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Development of a modelling approach for characterization and prediction of bacterial spoilage microbiota dynamics in perishable foodstuffs

Développement d'une approche de modélisation pour la caractérisation et la prévision de la dynamique des microbiotes bactériens altérants dans les denrées alimentaires périssables

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“Happiness can be found in the darkest of times,
if one only remembers to turn on the light.”

- Albus P.W.B. Dumbledore,
Harry Potter and the Prisoner of Azkaban,
J.K. Rowling, 2004.

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Abstract

Food waste is currently a major problem since it is estimated that about one third of the food produced in the world is discarded before it is consumed. The reasons for these food losses and waste are varied and one cause is the bacterial spoilage, rendering foods unacceptable for consumption. The study of the dynamics of bacterial spoilage populations and the prediction of their dynamics would therefore be interesting to better understand and anticipate this phenomenon. This research focused on the study of predictive models for spoilage bacteria of fresh meat and meat products, considered as highly perishable foodstuffs. The two working matrices were pork minced meat and white pudding, considering variations in storage conditions (temperature and packaging).

The first chapter of this thesis provides a general overview of bacterial spoilage of meat and meat products, as well as factors that may promote or limit its development. The different techniques used in this study to characterize and modelize the dynamics of spoilage microbiota are also described.

This research was then divided into four main areas that are discussed in the other chapters: (1) describing the spoilage bacterial microbiota naturally present in the matrices studied; (2) characterizing the spoilage bacteria of interest for these products; (3) developing and validating predictive models with one or more bacteria; (4) and studying the metabolome of minced meat inoculated by spoilage microorganisms of interest.

These studies have demonstrated the interest of combining results from classical microbiology and 16S rDNA-based metagenetic to monitor and predict the dynamics of spoilage microbiota. For the white pudding, the bacteria of interest were *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Lactobacillus* spp. (*Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans*), *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pseudomonas psychrophila*, *Pseudomonas* sp., *Psychrobacter* spp. (*Psy. okhotskensis*, *Psy. urativorans*), *Raoultella terrigena* and *Serratia* sp. For minced pork samples they were *B. thermosphacta*, *Lb. algidus*, *Lc. piscium*, *Leuconostoc* spp. (*Ln. inhae*, *Ln. gelidum*), *Photobacterium* spp. (*Ph. kishitanii*, *Ph. phosphoreum*) and *Pseudomonas* spp. (*Ps. fragi*, *Ps. fluorescens*, *Ps. psychrophila*). The type of packaging and storage

temperature have a significant effect on the different dynamics, as well as the food companies and the production batches analyzed.

Some of these bacteria of interest were then inoculated on sterile and non-sterile matrices, stored at different temperatures and packaging. The growth parameters to each bacterium were collected: maximum growth rate, lag time, minimum and maximum bacterial populations, time to reach the stationary phase, time to reach the spoilage threshold, minimum growth temperature, etc. Packaging seems to have the greatest impact on the maximum growth rate, itself having the greatest influence on the microbiological shelf life of the foods studied.

Based on these data, good adjustments were obtained for the growth simulations, but overestimations were often observed. The same observations could be made by comparing the simulations performed on the white pudding with those available from software (ComBase and Sym'Previus). For minced pork, the data obtained allowed the development of three species interaction models based on the Lotka-Volterra (prey-predator model) and the modified Jameson models. The simulations obtained were validated by monitoring the spoilage microbiota of naturally contaminated pork minced meat matrices. The modified Jameson model obtained the best adjustments, although the prey-predator approach seems to be an interesting interaction model for complex microbiota. However, these proposals for models with three or more spoilage bacteria need to be validated by more experimental repetitions.

Finally, metabolomic analyses (^{1}H-NMR), in collaboration with CIRM-CHU, were performed in order to monitor the metabolites produced by inoculated bacteria in sterile minced pork samples. The dynamics of the metabolome for sterile non-inoculated matrices was also monitored. The different metabolomic patterns and metabolites produced were highlighted according to the inoculated bacteria and the food packaging. Moreover, the storage temperature seems to have the lowest impact on the metabolome.

Development of predictive models based on data obtained by multi-omics analyses, combined with classical microbiology, provide an interesting approach. Further research on the development of complex models integrating the dynamics of two or more spoilage bacteria, interacting with each other and with the natural microbiota of foodstuffs, will be also an important step for better understanding and anticipating the bacterial spoilage of perishable foodstuffs.

Résumé

Le gaspillage alimentaire est actuellement un problème majeur puisqu'il est estimé qu'environ un tiers de la nourriture produite dans le monde est jetée avant d'être consommée. Les raisons de ce gaspillage sont variées, et l'une des causes est l'altération bactérienne des denrées alimentaires, rendant celles-ci inacceptables à la consommation. L'étude de la dynamique des populations microbienne altérantes et la prédiction de leur évolution permettrait donc de mieux comprendre et d'anticiper ce phénomène. La présente recherche s'est intéressée à l'étude de modèles prédictifs dans le cadre de l'altération bactérienne de viandes fraîches et de produits à base de viande, considérés comme des matrices alimentaires très périssables. Les deux matrices de travail étudiées ont été la viande hachée de porc et le boudin blanc, en tenant compte des variations des conditions de stockage (température et conditionnement).

Le premier chapitre de cette thèse brosse un aperçu général concernant l'altération bactérienne des viandes et des produits à base de viande, ainsi que des facteurs pouvant favoriser ou limiter son développement. Les différentes techniques utilisées dans cette étude pour caractériser et modéliser les dynamiques des microbiotes altérantes sont également décrites.

Cette recherche a ensuite été divisée en quatre axes principaux qui sont abordés dans les autres chapitres : (1) la description du microbiote bactérien altérant naturellement présent dans les matrices étudiées ; (2) la caractérisation des bactéries altérantes d'intérêt pour ces produits ; (3) le développement et la validation de modèles prédictifs à une ou plusieurs bactéries ; (4) et l'étude du métabolome de la viande hachée inoculée par des bactéries altérantes d'intérêt.

Ces études ont démontré l'intérêt de combiner les résultats issus de la microbiologie classique et de la métagénétique basée sur l'ADNr 16S afin de suivre et de prédire la dynamique des microbiotes altérants. Pour le boudin blanc, les bactéries d'intérêt étaient *Brochothrix thermosphacta, Carnobacterium maltaromaticum*, *Lactobacillus* spp. (*Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans)*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pseudomonas psychrophila*, *Pseudomonas* sp., *Psychrobacter* spp. (*Psy. okhotskensis*, *Psy. urativorans), Raoultella terrigena* et *Serratia* sp. et, pour la viande hachée de porc, étaient *B. thermosphacta*, *Lb. algidus, Lc. piscium*,

Leuconostoc spp. (*Ln. inhae, Ln. gelidum)*, *Photobacterium* spp. (*Ph. kishitanii*, *Ph. phosphoreum)* et *Pseudomonas* spp. (*Ps. fragi*, *Ps. fluorescens*, *Ps. psychrophila*). Le type de conditionnement et la température de stockage ont eu un effet significatif sur les différentes dynamiques, ainsi que l'entreprise et le lot de production analysé.

Certaines de ces bactéries d'intérêt ont ensuite été inoculées sur des matrices alimentaires stériles et non stériles, stockées sous différentes températures et sous différents conditionnements. Les paramètres de croissance propres à chaque bactérie ont pu ainsi être collectés : taux de croissance maximal, temps de latence, populations bactériennes minimales et maximales, temps pour atteindre la phase stationnaire, temps pour atteindre le seuil d'altération, température minimale de croissance, etc. Le conditionnement semble avoir eu le plus d'impact sur le taux de croissance maximal, lui-même ayant eu le plus d'influence sur la durée de vie microbiologique des aliments étudiés.

De bons ajustements ont été obtenus pour les simulations de croissance réalisées sur base de ces données mais des surestimations ont souvent été observées. Ce même constat a pu être fait en comparant les simulations réalisées sur le boudin blanc à celles issues de logiciels de modélisation existants (ComBase et de Sym'Previus). Pour la viande hachée de porc, les données ainsi obtenues ont permis le développement de modèles d'interactions à trois espèces, basé sur les modèles de Lotka-Volterra (modèle proie-prédateur) et de Jameson modifié. Les simulations obtenues ont été validées par le suivi du microbiote altérant de matrices de viande hachée de porc naturellement contaminées. Le modèle modifié de Jameson a obtenu les meilleurs ajustements, même si l'approche proie-prédateur constitue un modèle d'interaction intéressant pour les microbiotes complexes. Toutefois, ces propositions de modèles à trois bactéries altérantes nécessitent d'être validées ultérieurement par plus de répétitions expérimentales.

Enfin, des analyses de métabolomique (RMN-^{1}H), en collaboration avec le CIRM-CHU, ont permis le suivi du métabolisme de certaines bactéries d'intérêts inoculées dans des échantillons de viande hachée de porc stériles, le suivi de l'évolution du métabolome de matrices stériles non inoculées ayant également été réalisée. Les différents patterns métaboliques et les métabolites produits ont pu être mis en évidence en fonction de la bactérie inoculée et du conditionnement. La température de stockage semble avoir eu le moins d'impact sur le métabolome.

Le développement de modèles prédictifs basés sur des données issues d'analyses multi-omiques, en association avec la microbiologie classique, est une approche intéressante. Des recherches ultérieures sur le développement de modèles complexes intégrant l'évolution de trois ou plus de trois bactéries altérantes, en interaction entre elles et avec le microbiote naturel des denrées alimentaires, constitueront également une démarche importante pour mieux comprendre et anticiper l'altération bactérienne des denrées alimentaires périssables.

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FIGURES.

EQUATIONS.

Abbreviation list

A.	
a_w	water activity
AHLs	acylated homoserine lactones
B.	
B.	*Brochothrix*
BAs	biogenic amines
C.	
°C	Celsius degree
C.	*Carnobacterium*
CFU	colony forming unit
E.	
EU	European union
F.	
FL	food losses
FLW	food losses and waste
FW	food wrap
G.	
g	gram
GDP	gross domestic product
H.	
h	hours
H.	*Hafnia*
^{1}H-NMR	proton nuclear magnetic resonance
K.	
kg	kilogram
L.	
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
L.	*Listeria*
Ln.	*Leuconostoc*
log	logarithm
M.	
MAP	modified atmosphere packaging
MAT	microbial aging testing
Mbp	millions of base pairs
MCP	maximum critical population
MCT	microbial challenge testing
min	minutes
MP	minced pork
MS	Mass spectrometry
N.	
NAFTA	North American Free Trade Agreement
NMR	nuclear magnetic resonance
O.	
OTU	operational taxonomic unit
P.	
Ph.	*Photobacterium*
Ps.	*Pseudomonas*
Psy.	*Psychrobacter*
Q.	
QS	quorum sensing
S.	
S.	*Serratia*
sec	seconds
Sh.	*Shewanella*
SSOs	specific spoilage organisms
T.	
TVB-N	total volatile basic nitrogen
U.	
USD	U.S. Dollars
V.	
VBNC	viable but non culturable
VOCs	volatile organic compounds
VP	vacuum packaging
W.	
W.	*Weissella*
WP	white pudding

Preface

In recent years, food losses and waste (FLW) become one of the major challenges for a sustainable food system. FLW can occur throughout the food chain, from production to household consumption, and represent an important economic, ecological, social and ethical challenge for our society. Globally, one third of food production is lost or wasted annually (Corrado *et al*., 2019; den Besten *et al*., 2017; Food and Agriculture Organization, 2011; Laso *et al*., 2018; Schumann and Schmid, 2018), representing around 1.3 billion tonnes of food products wasted every year in the world (Ishangulyyev *et al*., 2019; Pinter *et al*., 2014; Vanham *et al*., 2015). It is estimated that developed countries contribute to 56% of the world FLW (Ishangulyyev *et al*., 2019). In Belgium, the annual food loss (FL) is estimated around 3.6 million tonnes (De Boeck *et al*., 2017). These amounts are very important, especially when taken into account that malnutrition and hunger affect nearly a billion people worldwide (Food and Agriculture Organization, 2011; Laso *et al*., 2018). Moreover, FLW are a source of major environmental impacts (Henchion *et al*., 2014; Laso *et al*., 2018; Shafiee-Jood and Cai, 2016; Thornton, 2010; Xue *et al*., 2017), responsible for around 170 million tonnes of CO_2-equivalent of greenhouse gases productions in Europe (AFSCA, 2018; Parlement Européen Actualité, 2017). It is the equivalent of an economic cost of about 750 billion USD, which equals the GDP of Turkey (Xue *et al*., 2017). In European Union, this incurs a loss of 143 billion euros each year (Tonini *et al*., 2018). As a result, international institutions, as well in many European countries, want to increase efforts in better understanding this phenomenon (Corrado and Sala, 2018). In 2018, the European Parliament adopted new measures to reduce waste in the European Union by 50%. Since 2015, the Walloon Government adopted the REGAL action plan, which aims to reduce FLW by 30% between 2015 and 2025.

These FLW have many causes (De Boeck *et al*., 2017), as food safety issues or inadequate market systems (Muth *et al*., 2019), and also food spoilage (Ishangulyyev *et al*., 2019). The general requirements of food law (Regulation (EC) No 178/2002 of the European Parliament) indicated that food shall not be placed on the market if it is unsafe, but also that food shall be deemed to be unsafe if it is considered to be (i) injurious to health and/or (ii) unfit for human consumption for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay. To reduce the amount of FLW, prevention is very important (De Boeck *et al*., 2017). To perform this, the implementation of food safety (based on HACCP principles) and quality management systems for food producers to systematically control production processes is really important (den Besten *et al*.,

2017; Nychas *et al*., 2008). Predictive microbiology can also be used along with the HACCP system in order to prevent FLW. For perishable products, improving the performance of storage facilities, and so decrease food quality degradation, can be strategic for FLW reduction (Neff *et al*., 2017; Shafiee-Jood and Cai, 2016).

Chapter 1
Introduction

Chapter 1 involves a thorough review of the scientific literature.

This chapter is divided into six sections.

The first sections introduce the food losses and waste, and the food matrices studied in this research. They include a general overview of pork meat and meat products.

Others sections present bacterial spoilage, associated ecosystems and their metabolic pathways, as well as factors influencing the shelf life of these food products. The microbiological and metabolomic analysis used in this research, and also predictive microbiology, will also be included.

The last section is dedicated to the highlights concerning all points described above.

1.1. Food losses and waste

A worldwide major problem

As mentioned above, FLW are a worldwide major concern (Alexander *et al*., 2017; Betz *et al*., 2015; Corrado *et al*., 2019; Dal'Magro and Talamini, 2019; Muth *et al*., 2019; Salihoglu *et al*., 2018; Schmidt, 2019; Sheahan and Barrett, 2017). FLW are defined by the Food and Agriculture Organization (2011) as: "any food intended for human consumption which, at any stage of the food chain, is lost, discarded and/or degraded." But distinctions between the terms "food losses" (FL) and "food waste" are sometimes made in the literature (Ishangulyyev *et al*., 2019; Xue *et al*., 2017). Considering the definition of FLW established by the Committee on World Food Security (2014), FL can occur at any stage of the food chain before consumption (e.g. production, handling, storage and processing steps). Whatever the reason, FL correspond to a decrease in the quantity or quality of foodstuffs that were originally intended for human consumption. On the other hand, food waste can only occur at the distribution, marketing and consumption stages. Food waste corresponds to food that has been thrown away, spoiled for any reason or kept beyond its expiry date.

In Europe, FLW represent 20% of food production, with a mean of 88 billion tonnes of food wasted every year (Food and Agriculture Organization, 2011; Pagliaccia *et al*., 2016; Tonini *et al*., 2018; Zhang *et al*., 2018a) of which about 47 million tonnes are related to the consumer level (Food and Agriculture Organization, 2011). A European citizen discards on average 123 [55-190] kg of food every year (Barone *et al*., 2019; Philippidis *et al*., 2019; Vanham *et al*., 2015), and this amount reaches up to 345 kg for a resident of Belgium (**Figure 1**) (AFSCA, 2018; Food and Agriculture Organization, 2011). In the European Union, sectors contribute differently to FLW: 53% is from households, 19% from food processing, 12% from food services, 11% from primary production, and 5% from wholesale and retail sales (Parlement Européen Actualité, 2017; Schmidt, 2019). Most studies have identified that the highest proportion of wasted food types is the most perishable food items, like bakery products, fruits and vegetables. In the food supply chains of industrialized regions, meat and meat products represent 20% of FLW, that also mainly occurs at the consumption level (Food and Agriculture Organization, 2011). However, studies estimate that 30% (Scott and Andersson, 2015) to 80% (Vanham *et al*., 2015) of these losses are considered avoidable or possibly avoidable, representing edible food not consumed (Tonini *et al*., 2018).

For these reasons, reducing and preventing FLW are a major concern. To do this, it is important to better understand and predict FLW phenomena, including food spoilage.

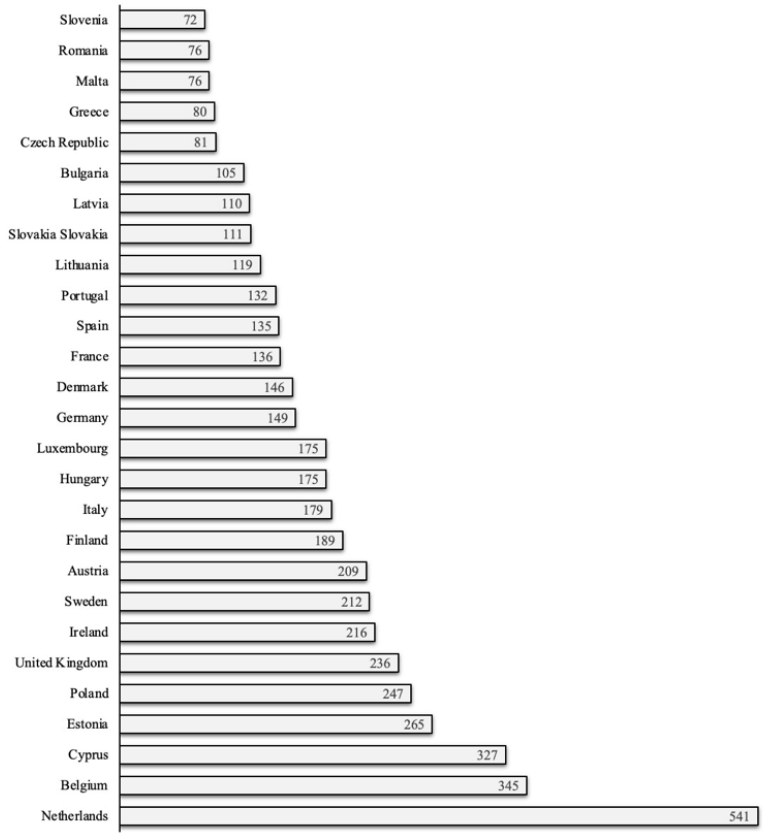


Figure 1. Food waste per country and *per capita* (expressed in kg) estimated in 2010 (AFSCA, 2018).

1.2. Food matrices studied in this research

Belgian minced pork and white pudding

Two types of pork meat matrix were selected for this research because they are considered as highly perishable foods, and therefore likely to be spoiled and thus could be lost or wasted (Doulgeraki *et al*., 2012; Ercolini *et al*., 2006): minced meat[1] and white pudding, a typical Belgian meat product[2]. Moreover, as discussed in the next section, pork meat and meat products account for a major part of Belgian consumption.

Minced pork (MP) samples were provided by local small and medium-sized Belgian manufacturers on the day of the production. According to the recipe, MP is composed of 100% minced pork (70% lean, 30% fat); no salt, spices, additives, eggs or sugar are added. On the day of the production, the water activity of this product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.05 (n = 12).

White pudding (WP) were also received from Belgian manufacturers the day following their production. According to the recipe, 10 kg of white pudding is composed of minced pork (8 kg), milk (2 L), salt (200 g), maize and wheat starches (130 g), pepper (25 g), spices (10 g), emulsifier (E450)[3] and taste enhancer (E621)[4], the rest de up with pork guts. The water activity of this product was 0.98 ± 0.02 and the pH value was 6.00 ± 0.20 (n = 4).

[1] According to Regulation (EC) No 853/2004 of the European parliament and of the council of 29 April 2004 laying down specific hygiene rules for food of animal origins, "minced meat" means boned meat that has been minced into fragments and contains less than 1% salt.

[2] According to Regulation (EC) No 853/2004 of the European parliament and of the council of 29 April 2004 laying down specific hygiene rules for food of animal origins, "meat products" means processed products resulting from the processing of meat or from the further processing of such processed products, so that the cut surface shows that the product no longer has the characteristics of fresh meat.

[3] Diphosphates (Regulation (EC) No 1333/2008 of the European parliament and of the council of 16 December 2008 on food additives).

[4] Monosodium glutamate (Regulation (EC) No 1333/2008 of the European parliament and of the council of 16 December 2008 on food additives).

1.3. Pork meat and meat products

You can eat all of the pig except the squeal

As described in the previous section, two types of Belgian pork meat matrices were selected in this research: minced pork and white pudding. Their physicochemical characteristics make them good candidates for studying bacterial spoilage mechanisms. Moreover, pork meat matrices are widely consumed throughout the world, and represent a large part of production and consumption in Belgium.

1.3.1. Worldwide meat production and consumption

Globally, meat production and consumption have a clear upward trend (**Figure 2**). In the past 50 years the consumption of poultry and pig meats has largely increased, whereas the total amount of beef consumed has been relatively stable.

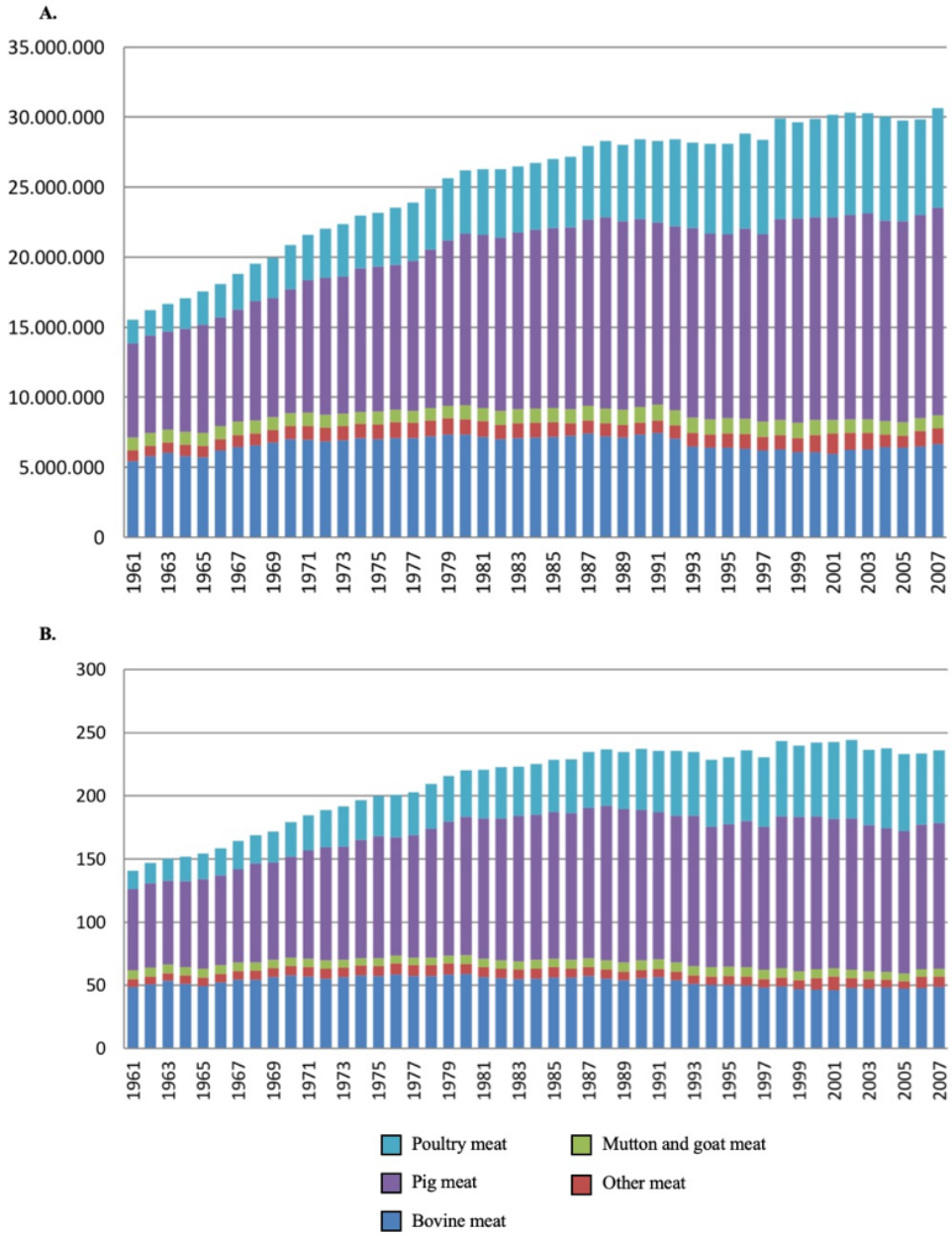


Figure 2. Meat consumption trends since 1961, represented by totals for group of eight European countries (Germany, France, Italy, Spain, United Kingdom, Netherlands, Hungary and Finland): (**A**) total meat (expressed in tons), and (**B**) *per capita* consumptions (Food and Agriculture Organization, 2019).

Considering pork meat, the main exporters are the EU, NAFTA and South America, and the main importers are China, Japan and Russia (**Figure 3**). In the United States and Canada, pork production continues to increase. In Brazil, production has decreased slightly due to economic difficulties, while Russia continues to develop its production and had begun exporting. In the EU, production is still at a high level, but may be very different between countries.

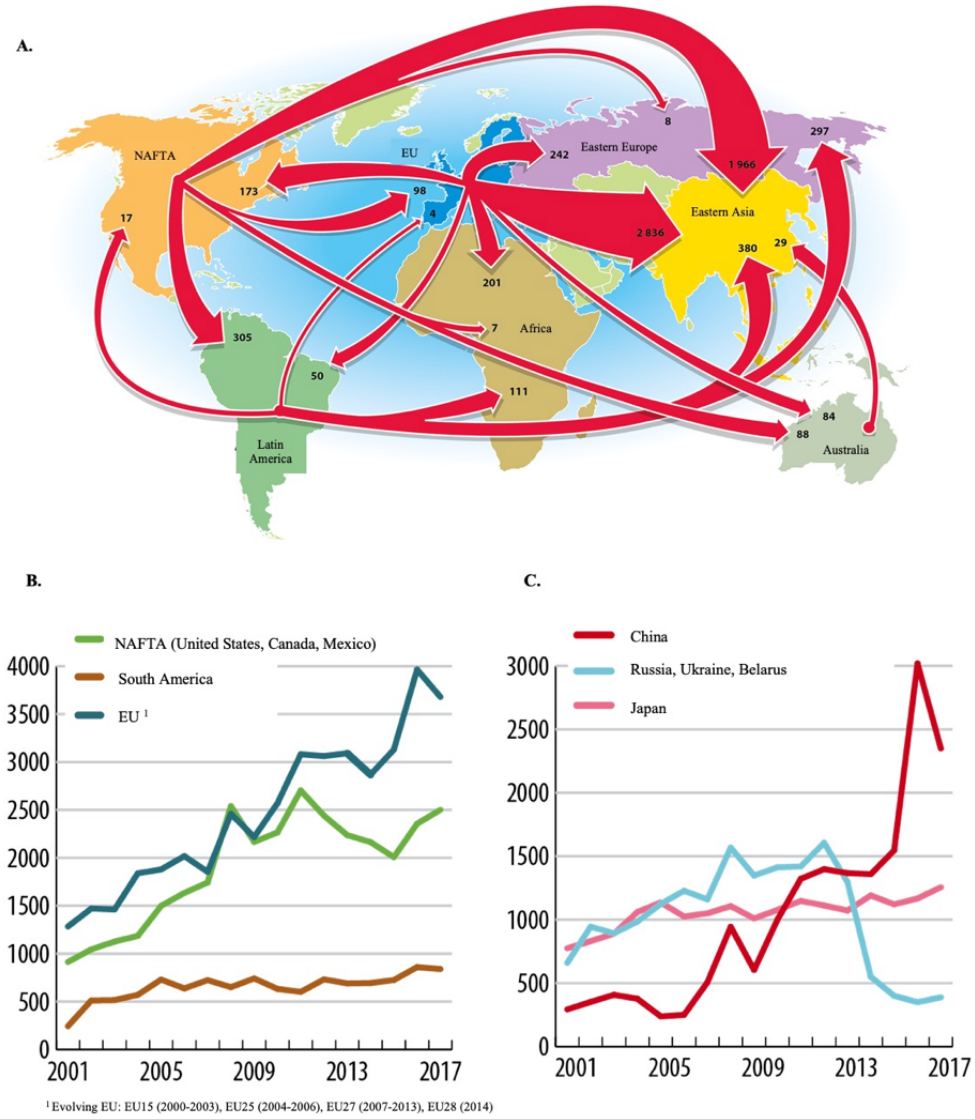


Figure 3. World production of pork meat and meat products (intra-EU and intra-NAFTA trade not included): (**A**) world flows in 2017 (expressed in thousands of tons), (**B**) exportation and (**C**) importation flows for the main countries concerned (expressed in thousands of tons) (IFIP, 2018).

Since 2005, pig production in the EU-28 has increased by 12% (expressed in tons), with alternating increases and decreases. In 2016, pig meat production was 23.9 million tonnes (**Table 1**). Since 2017 it has stabilized at 23.6 million tons (IFIP, 2018).

Table 1. Worldwide pig herd and pig production, expressed in million, in 2016 for the EU-28 (IFIP, 2018).

	Pig herd (per animal)	Pig production (tons)
Worldwide total	968.0	113.3
Asia	573.6	62.9
China	435.0	53.0
Vietnam	29.1	2.7
Philippines	12.2	1.5
Japan	9.3	1.3
Taiwan	5.4	0.8
South Korea	10.4	1.3
Europe	187.0	28.1
EU-28	147.2	23.9
Russia	21.9	2.9
Ukraine	6.8	0.8
North and Central America	96.2	14.6
USA	71.5	11.2
Canada	13.9	2.2
Mexico	10.7	1.2
South America	69.1	5.8
Brazil	39.2	3.7
Africa	36.6	5.8
Oceania	5.4	0.5
Australia	2.3	0.4

1.3.2. Belgian pork production and consumption

Until 2007, pig meat production in Belgium remained relatively stable (**Table 2**).

Table 2. Pig farms in the EU (expressed per 1000 livestock animals) (IFIP, 2018).

Countries	Years		Evolution between 2017 and 2007 (%)
	2017	2007	
Spain	29971	26061	+15.0
Germany	27578	27113	+1.7
France	13097	14969	-12.5
Denmark	12382	13170	-2.6
Netherlands	12296	11710	+5.0
Poland	11908	17621	-32.4
Italy	8571	9273	-7.6
Belgium	6108	6200	-1.5
United Kingdom	4713	4671	+0.9
Romania	4406	6565	+32.9
Hungary	2870	3871	-25.9
Austria	2820	3286	-14.2
Portugal	2165	1978	+9.5
Ireland	1616	1500	+7.7
Czech Republic	1532	2662	-42.5
Sweden	1382	1728	-20.0
Croatia	1121	1348	-16.9
Finland	1108	1427	-22.3
Greece	744	1038	-28.3
Slovakia	614	952	-35.5
Lithuania	612	923	-33.7
Bulgaria	593	889	-33.2
Cyprus	350	467	-25.1
Latvia	312	414	-22.6
Estonia	289	375	-22.8
Slovenia	257	543	-52.6
Luxembourg	91	86	+5.3
Malta	34	77	-55.8

The number of pig keepers has decreased steadily since the 1980s, both in Wallonia and in Flanders. But with a much higher labor/land ratio than in Wallonia, Flanders has developed horticulture and pig and poultry breeding. In 2007, Wallonia had 907 pig keepers (10,025 in 1980) compared to 6,069 in Flanders (31,753 in 1980). Currently, Wallonia holds only 5.5% of the national pig meat production capacity, compared to 94.5% for Flanders. Production is mainly found in Hainaut (39%), Liège (32%) and Namur (17%). This strong disparity between regions is reflected in the capacity for self-sufficiency through local production: Flanders has a self-sufficiency rate of around 340% and Wallonia only 25%. This means that about half of the pork produced in Belgium finds a commercial outlet in export markets (Apaq-W, 2018).

It should also be noted that pig meat remains the meat most consumed by Belgian citizens, representing 49% of all meat consumed in Belgium. Apparent consumption[5] was 50.2 kg in 2013 and 40.0 kg in 2017 (**Figure 4**) (Apaq-W, 2018; Meat Information Cell, 2016).

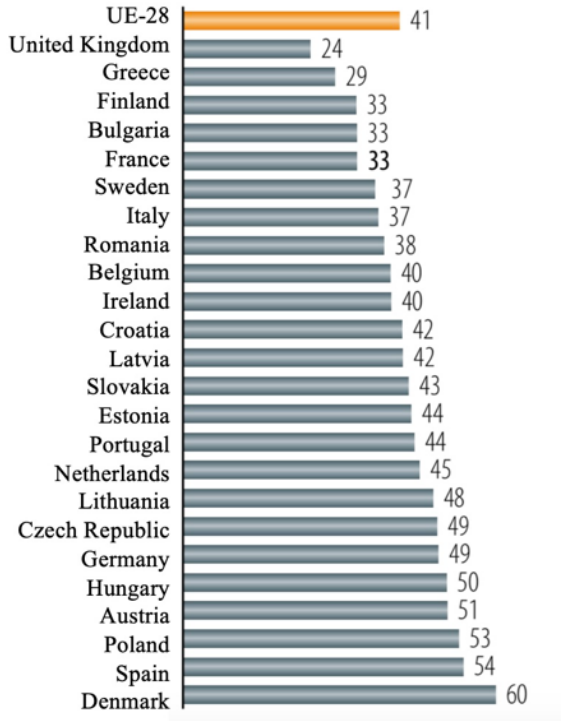


Figure 4. Pork meat consumption for European countries in 2017 (expressed in kg *per capita*) (IFIP, 2018).

1.3.3. Nutritional aspects of minced meat and white pudding

Pork meat and meat products have interesting nutritional values and are considered to be excellent source of nutritious proteins, essential amino acids, zinc, heme-iron and bioavailable vitamins, with a low carbohydrate content (Bohrer, 2017; Engstrom *et al*., 1997; Fernandez-Ginés *et al*., 2005; Lawrence, 2013; McAfee *et al*., 2010; O'Neil *et al*., 2012; Pellet and Young, 1990; Pereira and Vicente, 2013; Stabler and Allen, 2004; Web and O'Neill, 2008; Williams, 2007; Wood *et al*., 2008). Even though meat quality can be influenced by several factors, the protein content and vitamins and minerals available are generally consistent (Biesalski, 2005). The nutritional composition of the two matrices studied in this research is presented in **Table 3**.

[5] In the absence of actual consumption data, apparent consumption is calculated from the supply balances, by adding meat production and imports and subtracting meat exports, expressed per year and per *capita* (without distinction between pig meat, minced meat, meat preparations and meat products).

However, it is important to note that several studies have recently highlighted the adverse health effects associated with meat consumption (Alshahrani *et al*., 2019; Aune *et al*., 2008; Cross *et al*., 2007; Grosso *et al*., 2017; Micha *et al*., 2010; Wolk, 2017; Zelber-Sagi *et al*., 2018).

Table 3. Nutritional composition (amount per 100 g) of minced pork and white pudding (USDA, 2019; Ciqual, 2017).

	Pork meat (minced)	**White pudding**
Calories (kcal)	218.00	250.00
Fat (g)	16.00	20.30
Saturates (g)	5.10	10.10
Mono-unsaturated (g)	4.09	7.80
Polyunsaturated (g)	0.88	1.99
Cholesterol (mg)	68.00	70.10
Carbohydrate (g)	0.24	15.29
Protein (g)	17.99	7.14
Salt (g)	0.12	1.80
Vitamin B12 (µg)	0.73	0.30
Phosphorus (mg)	161.00	59.20
Iron (mg)	0.88	2.57
Zinc (mg)	1.91	0.76
Selenium (µg)	10.90	8.44

1.3.4. Microbial ecology of pork meat and meat products

The bacterial microbiota of foods is diverse and determined by the environment in which the living animals are raised. Carcass muscles can be considered as almost sterile. But contamination can occur during the evisceration and skinning stages, as well as during the cutting and/or processing stages by skin, respiratory and digestive tracts of animals, air, water, equipment surfaces, slaughterhouse environment, ingredients and cross-contaminated people, utensils and equipment (Brightwell *et al*., 2007; Gill and McGinnis, 2000; Gill and McGinnis, 2003; Mills *et al*., 2014; Nychas *et al*., 2008). Therefore, the bacterial species that are currently described in meat and meat products are frequently found in the soil, water and microbiota of animals. Total aerobic microbial load in pig carcasses generally ranges from 2.0 to 5.0 log CFU/cm^2 (Gill *et al*., 2000; Van Ba *et al*., 2019). Stellato *et al*. (2016) showed that the main microbiota of slaughterhouse samples include *Acinetobacter* spp., *Brochothrix* spp., *Pseudomonas* spp., *Psychrobacter* spp. and *Streptococcus* spp. Mills *et al*. (2018) also showed that lamb carcasses are contaminated by *Carnobacterium* spp., provided by the processing environment. Samapundo *et al*. (2019) have made the same observations for Belgian chicken cuts.

Pork meat is considered as highly perishable, with a high water content, a near-neutral pH and important nutrients available for bacterial growth, and thus for food spoilage (Doulgeraki *et al*., 2012; Ercolini *et al*., 2006; Gram *et al*., 2002; Liu *et al*., 2006). As discussed before, spoilage bacteria are responsible for FLW. However, these bacteria are less studied than pathogenic bacteria for the two selected matrices. Therefore, this research only focused on spoilage bacteria.

1.4. Meat spoilage

Spoiled or not spoiled: that's the question

Food spoilage is the result of many changes affecting the food and leading to the recognition of unacceptable sensory features by the consumer (Ercolini *et al*., 2006; Gram *et al*., 2002). Spoilage involves biological, physical and chemical activities that triggers product deterioration (Casaburi *et al*., 2015; Jääskeläinen *et al*., 2016; Mansur *et al*., 2019). The spoilage of meat and meat products can be caused in four ways: (i) microbiological growth (bacteria, yeast and mold); (ii) enzymatic activities (lipases and proteases); (iii) chemical reactions (browning and oxidation); and (iv) physical changes (e.g. by freezing, drying, high pressure, etc.) (Dave and Ghaly, 2011; Ercolini *et al*., 2006; Papuc *et al*., 2017; Schumann and Schmid, 2018). Food spoilage is mainly caused by growth of microorganisms, especially by bacteria (Del Blanco *et al*., 2017; Liu *et al*., 2006; Nychas *et al*., 2008; Stellato *et al*., 2017; Tsigarida *et al*., 2003; Zhang *et al*., 2019). Bacterial food spoilage can result in off-odor, color or texture defects due to the synthesis of molecules through various pathways (catabolism of proteins, carbohydrate and lipids present in meat), and thus renders products unsuitable for human consumption (Ercolini *et al*., 2006). As a nutrient-rich medium, with protein, lipids, minerals and vitamins, meat and meat products enables microorganisms to grow and consequently to express many metabolic functions (Casaburi *et al*., 2015; Iulietto *et al*., 2015).

It is really important to be able to correlate food spoilage with the bacterial communities of these products because organoleptic changes may vary according to the microbial association involved in this phenomenon (Ercolini *et al*., 2006). However, it is sometimes difficult to establish a direct link between a given deterioration phenomenon and one or more specific species. Moreover, not all the members of this microbiota contribute to food spoilage. Several studies have established that spoilage is caused only by a dominant fraction of the initial microbial association (Doulgeraki *et al*., 2012; Nychas *et al*., 2008), designated as Specific(/Ephemeral) Spoilage Organisms (S(E)SOs) (Benson *et al*., 2014; Chenoll *et al*., 2007; Gram *et al*., 2002; Huis in't Veld, 1996; Jorgensen *et al*., 2000; Koutsoumanis *et al*., 2008; Pennacchia *et al*., 2011; Zotta *et al*., 2019). First described by Mossel and Ingram (1955), SSOs can dominate the microbiota, reaching a high population density and thus producing several spoilage metabolites (Mansur *et al*., 2019; Wang *et al*., 2016b). Therefore, as discussed by De Filippis *et al*. (2013), the concept of a succession of spoilage-related microbial groups is very important, and many studies have been performed to investigate the dynamics and changes of the meat microbiota during storage.

1.4.1. Main spoilage effects

Spoilage effects are variable, from slime formation to color, odor, flavor or textural changes. These phenomena largely depend on the bacteria involved, influencing factors and available nutrients (Dainty *et al*., 1996; Iulietto *et al*., 2015; Nychas *et al*., 2008). Glucose is the first substrate preferentially metabolized by spoilage bacteria, especially by obligate aerobic species, such as *Pseudomonas* spp., and facultative anaerobic microorganisms (Koutsoumanis *et al*., 2008). When the diffusion gradient of glucose is not sufficient for spoilage communities, or when glucose reserves are depleted, lactate is the next energy source under aerobic and anaerobic conditions. Then, free amino acids, sugars and proteins are degraded, contributing to organoleptic changes through the release of volatile metabolites (Ercolini *et al*., 2006). Some of these spoilage effects and their synthetic mechanisms are developed in the subsections below and are summarized in **Table 4**.

1.4.1.1. Discoloration

The color of meat, especially red meat, is an important criterion of quality for consumers. Some bacterial species are well known to specifically affect the color by microbial discoloration and/or pigments production. Discoloration generally appears when the bacterial communities reach counts of between 7.5 and 8.0 log CFU/g (Iulietto *et al*., 2015).

Microbial discoloration occurs when the muscle pigment, myoglobin, is converted into green sulfmyoglobin by bacterial production of hydrogen sulfide. The production of hydrogen sulfide, derived from cysteine, is dependent on the availability of glucose and oxygen. Sulfmyoglobin seems not to be formed in anaerobic conditions (Borch *et al*., 1996).

Table 4. Example of some spoilage effects on meat and meat products.

General spoilage effect	Bacteria involved	Underlying mechanisms	References
Discoloration	*C. viridans*	Green discoloration in vacuum packaged by production of hydrogen peroxide.	Holley *et al*., 2002; Peirson *et al*., 2003b;
	Lactic acid bacteria (LAB)	Greening/graying by production of dihydrosulfide or hydrogen peroxide.	Borch *et al*., 1996; Peirson *et al*., 2003a;
	Lb. sakei, *H. alvei* and *Sh. putrefaciens*	Green sulfmyoglobin formation.	Borch *et al*., 1996; Vihavainen and Björkroth, 2007;
	Leuconostoc spp., *Ln. gelidum*	Greening by oxidizing nitrosomyochromogen.	Nychas *et al*., 2008; Vihavainen and Björkroth, 2007;
	Pseudomonas spp.	Production of a variety of pigments (blue, green, yellow, black and white). Yellow fluorescent pigments of *Ps. fluorescens* is produced by a siderophore for utilization of iron in meat. Blue-pigmentation, especially in dairy products and mozzarella cheese, due to a genetic cluster of all blue-pigmenting strains ("blue branch").	Andreani *et al*., 2014; Andreani *et al*., 2015; Cornelis, 2010; Martin *et al*., 2011; Nogarol *et al*., 2013;
	W. viridescens	Greening by oxidizing nitrosomyochromogen.	Duskova *et al*., 2013;
Off-odors and off-flavors	*B. thermosphacta*	Butter/rancid, sour/pungent and/or cheese/fermented off-odors.	Jaffrès *et al*., 2011; Joffraud *et al*., 2001; Stohr *et al*., 2001;
	LAB	Sour and acid aroma, produced in VP and MAP.	Borch *et al*., 1996; Casaburi *et al*., 2015; Jääskeläinen *et al*., 2016; Pin *et al*., 2012;
	Lactobacillus spp., *Enterobacteriaceae*, *B. thermosphacta*	Cheesy odor due to the production of acetoin, diacetyl and 3-methylbutanol (carbohydrate metabolism).	Borch *et al*., 1996; Casaburi *et al*., 2015;
	Ln. gasicomitatum	Buttery off-odor by production of diacetyl and acetoin.	Chaillou *et al*., 2005; Rimaux *et al*., 2011;
	Ph. phosphoreum	Fishy, urine and ammonia-like off-odors by trimethylamine production.	Dalgaard, 1995;
	Pseudomonas spp., *Enterobacteriaceae*	Sulfuric odor by the production of hydrogen sulfide and dimethyl sulfide (sulfur catabolism).	Borch *et al*., 1996; Casaburi *et al*., 2015;
	Ps. fragi	Rancid flavors by metabolisms of triglycerides.	Cleto *et al*., 2012;
Gas production	*B. thermosphacta*	Production of CO_2.	Koutsoumanis *et al*., 2008;
	Clostridium spp., *Enterobacteriaceae*,	Production of H_2 and CO_2 in VP beef.	Borch *et al*., 1996; Brightwell *et al*., 2007; Mills *et al*., 2015;
	LAB	CO_2 production reported in VP pork and beef.	Borch *et al*., 1996; Iulietto *et al*., 2015; Koutsoumanis *et al*., 2008; Yang *et al*., 2014;
Slime formation and textural changes	*Lactobacillus* spp., *Lb. sakei*; *Leuconostoc* spp.	Often observed in vacuum packed and cooked meat products by extracellular production (glucan production).	Iulietto *et al*., 2015; Notararigo *et al*., 2013; Nychas *et al*., 2008;
	Pseudomonas spp., *Enterococcus* spp., *Brochothrix* spp.,	Slime formation.	Nychas *et al*., 2008;
	W. viridescens	Greenish slime formation.	Björkroth and Korkeala, 1997; Duskova *et al*., 2013; Nychas *et al*., 2008

1.4.1.2. Off-odors and off-flavors

Off-odors and off-flavors are linked to the production of volatile organic compounds (VOCs), such as sulfur compounds, ketones, aldehydes, volatile fatty acids, esters, alcohols, ammonia and other metabolites, when free amino acids, fatty acids and sugars are used as source of energy for bacterial communities (Ercolini *et al*., 2006; Estevez *et al*., 2003; Lovestead and Bruno, 2010; Nychas *et al*., 2008; Rivas-Canedo *et al*., 2009). Some of these VOCs commonly identified during storage of meat and meat products are summarized in **Table 5** and can be associated to specific aroma (**Figure 5**). Unpleasant odors generally become perceptible when total bacterial count reaches 7.0-7.5 log CFU/g (Iulietto *et al*., 2015). However, the volatilome (the volatile fraction of the microbial catabolites) is a complex association of molecules that can be considered as pleasant or unpleasant by different people. The spoilage-associated microorganisms have already been reviewed for meat by Casaburi *et al*. (2015) and Mansur *et al*. (2019), and for MP by Del Blanco *et al*. (2017) and Zareian *et al*. (2018).

Table 5. Most commonly identified VOCs during storage of meat and meat products.

	Compound	Off-odor descriptor	Metabolic pathway	Responsible bacteria	Storage condition	References
Alcohols	3-Methyl-1-butanol 1-Octen-3-ol 2-Ethyl-1-hexanol Butanol Heptanol 1-Hexanol 3-Phenoxy-1-propanol 2,3-Butanediol Ethanol 2-Hexen-1ol 1-Octanol 2-Octen-1-ol 2-Nonen-1-ol 2-Methyl-1-dodecanol 2-Ethyl-1-decanol 2-Butoxy-ethanol 1-Heptenol-1-butoxy-2-propanol 1-Pentanol	Fermented, alcoholic, etherical Earthy Fruity Fruity Fruity Creamy/buttery, and fruity	Breakdown of proteins and amines acids, reduction of ketones, and aldehydes derived from lipid peroxidation	*B. thermosphacta*, *Carnobacterium* spp., *C. maltaromaticum*, *C. divergens*, *Lb. curvatus*, *Lb. sakei*, *Lc. lactis,* *Pseudomonas* spp., *Ps. fragi*, *Ps. fluorescens*, *S. proteamaculans,* *S. liquefaciens*,	Air, VP, MAP	Casaburi *et al*., 2011; Casaburi *et al*., 2014; Casaburi *et al*., 2015; Ercolini *et al*., 2009; Ercolini *et al*., 2010a; Faustman *et al*., 2010; Hernandez-Macedo, 2012; Jääskeläinen *et al*., 2013 ; Mansur *et al*., 2019; Smit *et al*., 2009 ; Tsigarida *et al*., 2003
Aldehydes	Hexanal Nonanal Benzaldehyde 3-methylbutanal 2-Methylbutanal Octanal Nonanal Heptanal 2-Methylpropanol Dimethyl disulfide Dimethyl trisulfide Methyl thioacetate Carbon disulfide	Acidic, fatty flavors, rancid aroma	Triglycerides hydrolysis, oxidation of unsaturated fatty acids, lipid autooxidation	*B. thermosphacta*, *Carnobacterium* spp., *C. maltaromaticum*, *Enterobacteriaceae*, *Ln. gasicomitatum*, *Pseudomonas* spp., *Ps. fragi*, *Ps. fluorescens*, *S. proteamaculans*,	Air and VP	Calkins and Hodgen, 2007; Casaburi *et al*., 2011; Casaburi *et al*., 2014; Ercolini *et al*., 2009; Ercolini *et al*., 2010a; Hernandez-Macedo, 2012; Jääskeläinen *et al*., 2013; Mansur *et al*., 2019

Table 5 (continued).

	Compound	Off-odor descriptor	Metabolic pathway	Responsible bacteria	Storage condition	References
Ketones	Acetoin Diacetyl 2-Butanone 3,3-dimethylbutan-2-one 3-Octanone 2-Heptanone 2-Nonanone 3-hydroxypentan-2one	Cheesy odor, buttery/creamy flavor Acetone-like ethereal, fruity	Lipolysis, microbial alkane degradation, dehydrogenation of secondary alcohols	*B. thermosphacta*, *Carnobacterium* spp., *C. maltaromaticum*, *Enterobacteriaceae*, *Leuconostoc* spp., *Ln. gasicomitatum*, *Pseudomonas* spp., *Ps. fragi*, *S. proteamaculans*	Air, VP, MAP	Casaburi *et al*., 2014; Casaburi *et al*., 2015; Del Blanco *et al*., 2017; Ercolini *et al*., 2009; Ercolini *et al*., 2010a; Jääskeläinen *et al*., 2013; Jääskeläinen *et al*., 2016; La Storia *et al*., 2012; Mansur *et al*., 2019 ; Pin *et al*., 2002; Tsigarida *et al*., 2003
Esters	Ethyl acetate Ethyl butanoate Ethyl-3-methylbutanoate Ethylheptanoate Ethyloctanoate Ethylhexanoate Ethyldecanoate Ethylidene diacetate	Fruity/ethereal off-flavor	Microbial esterase activity (esterification of alcohols and carbohydrates)	*B. thermosphacta*, *Carnobacterium* spp., *C. maltaromaticum*, Pseudomonas spp., Ps. *fragi*, *S. proteamaculans*	More frequently in aerobic conditions	Dainty and Mackey, 1992; Ercolini *et al*., 2009; Ercolini *et al*., 2010a; Mansur *et al*., 2019; Toldra, 1998; Tsigarida *et al*., 2003
Volatile fatty acids	Acetic acid Butanoic acid Formic acid Hexanoic acid 2-Methyl butanoic acid 3-Methyl butanoic acid	Acid/Roquefort cheese. Rancid/acid/cheesy/butter and fruity	Hydrolysis of triglycerides and phospholipids, amino acid degradation, or oxidation of ketones, esters and aldehydes	*B. thermosphacta*, *Carnobacterium* spp., *C. maltaromaticum*, *Lactobacillus* spp., *Lb. curvatus*, *Lb. sakei*, *Lc. piscium*, *Leuconostoc* spp., *Pseudomonas* spp.	Air, VP and MAP	Casaburi *et al*., 2011; Casaburi *et al*., 2014; Casaburi *et al*., 2015; Jääskeläinen *et al*., 2013; Ferrocino *et al*., 2013; Martin *et al*., 2007; Nychas *et al*., 2008; Tsigarida *et al*., 2003
Sulfur compounds	Dimethyl sulfide Dimethyl disulfide Dimethyl trisulfide Methyl thioacetate Carbon disulfide	Sulfurous/cooked onion	Degradation of sulfur-containing amino acids (methionine and cysteine)	*C. maltaromaticum*, *Pseudomonas* spp., *Ps. fragi*, *S. proteamaculans*	Air and VP	Ercolini *et al*., 2009; Ercolini *et al*., 2010a; Mansur *et al*., 2019; Nowak and Czyzowska, 2011; Tsigarida *et al*., 2003; Yvon and Rijnen, 2001

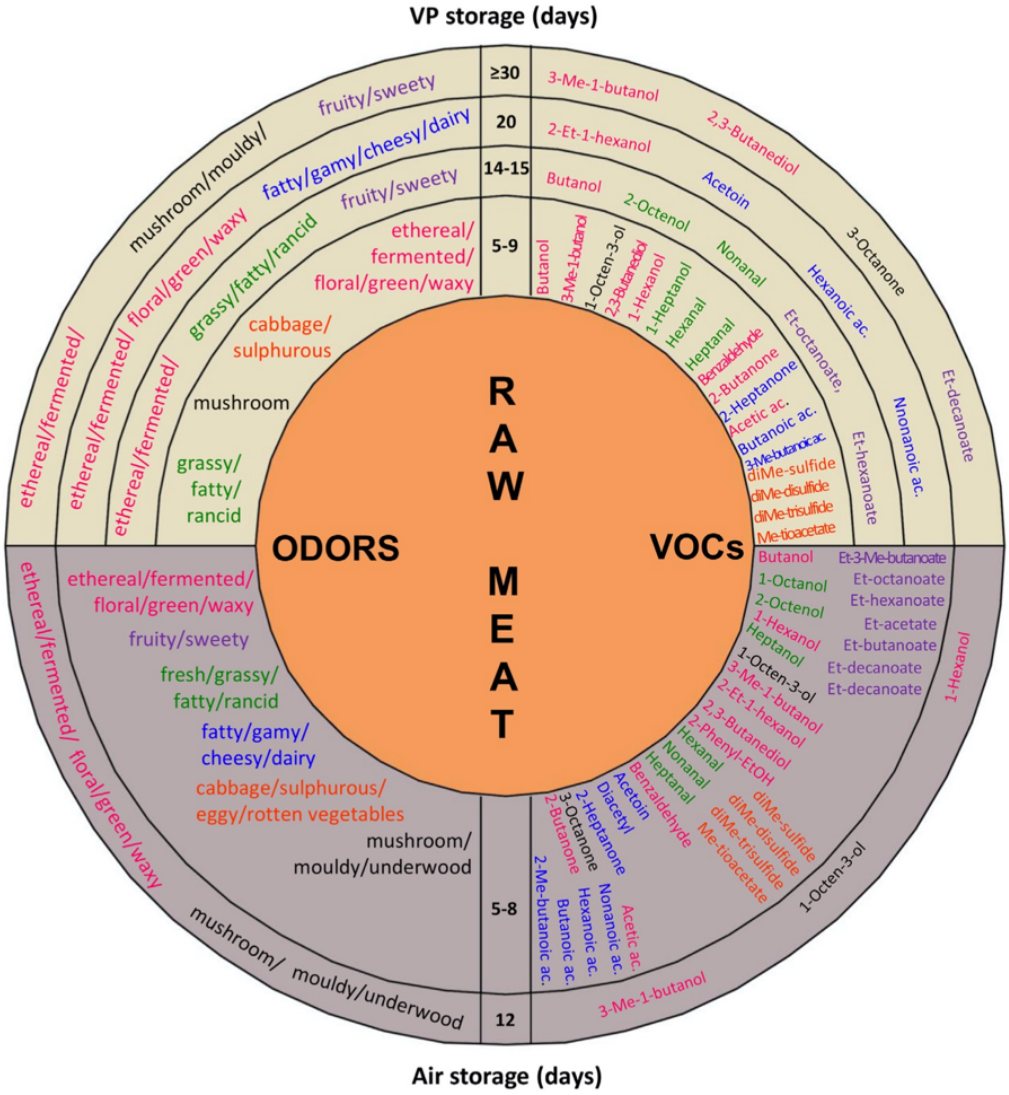


Figure 5. Schematic representation of meat spoilage aroma in meat during chill storage in air and in VP. Each odors descriptor, on the left side, are reported according to the corresponding VOCs, on the right side (Casaburi *et al*., 2015).

1.4.1.3. Gas production

Proteolysis caused by anaerobic microorganisms, especially in vacuum packaged (VP) meats, can lead to the accumulation of a large amount of gases, like hydrogen and carbon dioxide for example. *Clostridium* spp., in particular *Cl. estertheticum* and *Cl. gasigenes*, are often responsible for the "blown pack defect" due to a carbon dioxide production (Iulietto *et al*., 2015). But, depending on the nature of the gas that is produced and on the bacterial species responsible for it, this phenomenon may be associated with the production of off-odors.

1.4.1.4. Texture defects and slime formation

Consumers are also very sensitive to the visual aspect of meat and meat products. Slime production is generally associated with the bacterial polysaccharide polymers of lactic acid bacteria (LAB) group bacteria extending from the meat surface (Iulietto *et al*., 2015; Notararigo *et al*., 2013). This effect can be very important because it can lead to the production of biofilms that would modify interactions between resident species.

1.4.1.5. Biogenic amines production

The enzymatic decarboxylation of amino acids, or the transamination of aldehydes and ketones, by bacteria results in the formation and accumulation of biogenic amines (BAs) (Jastrzebska *et al*., 2016; Li *et al*., 2014). BAs are reported in various foods including meat, fish, cheese and wine (Kim *et al*., 2009; Stadnik and Dolatowski, 2010). They are nitrogenous compounds with an aromatic, aliphatic or heterocyclic structure (Papageorgiou *et al*., 2018). The most important BAs found in foods are b-phenylethylamine, cadaverine, histamine, putrescine, spermidine, spermine, tyramine and tryptamine (Cheng *et al*., 2016). They can have health implications, such as allergic reactions, but also contribute to spoilage due to their putrid aroma (Rodriguez *et al*., 2014; Stanborough *et al*., 2017). But since BA production is linked to bacterial activity, the level of BAs can therefore be reduced by measures such as approapriate packaging and storage temperature, for example (Doeun *et al*., 2017; Li *et al*., 2014; Nadon *et al*., 2001).

In spoiled pork meat, cadaverine, histamine, putrescine, tyramine, spermidine and spermine are the most frequently found BAs (Custodio *et al*., 2018; Del Rio *et al*., 2019; Min *et al*., 2004; Ngapo and Vachon, 2017; Papavergou *et al*., 2012). It has been reported that *Enterobacteriaceae*, LAB, *Pseudomonas* spp. and *Photobacterium* spp., especially *Ph. phosphoreum* and *Ph. iliopiscarium*, produce these BAs (Bover-Cid *et al*., 2003; Connil *et al*., 2002a; Connil *et al*., 2002b; Dalgaard *et al*., 2006; Hilgarth *et al*., 2018a; Jastrzebska *et al*., 2016; Kanki *et al*., 2007; Latorre-Moratala *et al*., 2012; Lavizzari *et al*., 2010; Remenant *et al*., 2015). Different strains of *B. thermosphacta* are also able to produce cadaverine, histamine, tyramine, tryptamine and putrescine (Casaburi *et al*., 2014; Nowak and Czyzowska, 2011; Paleologos *et al*., 2004; Stanborough *et al*., 2017). However, amino acid decarboxylase genes are not found in all strains of this species (Emborg *et al*., 2005; Stanborough *et al*., 2017). *C. divergens* is also described as a tyramine producer (Curiel *et al*., 2011).

On the other hand, several studies have highlighted the possibility of using BAs as indicators of meat quality during storage (Balamatsia *et al*., 2006; Hernandez-Jover *et al*., 1996; Kaniou *et al*., 2001; Lazaro *et al*., 2015; Rokka *et al*., 2004). Therefore, as proposed by Cheng *et al*. (2016), the sum of these compounds can be used as an index of pork meat quality and freshness. Li *et al*. (2014) also showed that putrescine and cadaverine can be used as spoilage indicators of chilled pork.

1.4.2. Influencing factors

Different spoilage-related species and strains can colonize the meat surface through adsorption and attachment by glycocalyx formation on the meat surface (Chung *et al*., 1989; Ercolini *et al*., 2006). These steps depend on intrinsic, extrinsic (**Table 6**) and implicit factors, as they can be used in hurdle technology to extend the shelf life of food products (Del Blanco *et al*., 2017; Jääskeläinen *et al*., 2016; Koutsoumanis and Nychas, 2000; Mansur *et al*., 2019; Nychas *et al*., 2008; Tsigarida *et al*., 2003; Zhao *et al*., 2015). Some intrinsic and extrinsic factors were considered in the experimental studies and predictive models of this research, and so are developed in the subsections below.

Table 6. Influencing factors on microbial growth.

Factors	Examples	References
Intrinsic	Type of animal (bovine, porcine), Physiological status of the animal at slaughter, Breed and feed diet, Age of animal at time of slaughter, Physical and chemical composition of the food matrices, Water activity, pH, Meat surface morphology, Nutrient availability, Initial microbiota, Presence and development of other bacteria, Bioprotective cultures, Natural antimicrobial substances, …	Argyri *et al*., 2015; Blixt and Borch, 2002; Casaburi *et al*., 2015; Dave and Ghaly, 2011; Del Blanco *et al*., 2017; Dolan *et al*., 2019; Doulgeraki *et al*., 2012; Ercolini *et al*., 2006; Iulietto *et al*., 2015; Kalschne *et al*., 2014; Koutsoumanis *et al*., 2006; Lambert *et al*., 1991; Mcdonald and Sun, 1999; Nychas *et al*., 2008
Extrinsic	Handling environment, Quality management system, Spread of contamination into slaughterhouses, Temperature, humidity and packaging system (gas composition and ratio) of storage, …	

1.4.2.1. Intrinsic factors

Meat composition is an important factor concerning the growth and survival of microorganisms. Meat and meat products are protein- and lipid-rich foods, with variable amounts of minerals and vitamins (Papuc *et al*., 2017).

The *postmortem* pH of meat is also an essential factor for microbial growth (Mills *et al*., 2014). pH is determined by the amount of lactic acid produced from glycogen during anaerobic glycolysis, reaching around 5.4-5.8 in muscle after slaughter. It is well known that a high pH favors bacterial growth (Aymerich *et al*., 2002; Iulietto *et al*., 2015; Wang *et al*., 2017). In contrast, *Enterobacteriaceae* and *B. thermosphacta* are inhibited by pH values lower than 5.8, while for *Shewanella putrefaciens* inhibition occurs at pH vaues < 6.0. But LAB growth seems not to be affected by pH (Mills *et al*., 2014).

Moreover, water activity (a_w), defined as the amount of water available for microbial growth, is also important for bacterial growth (Iulietto *et al*., 2015; Mills *et al*., 2014). Bacteria tend to grow at an a_w ranging from 0.75 to 1.00, whereas yeast and molds, more tolerant to higher osmotic pressures than bacteria, may grow slowly at an a_w of 0.62 (Aymerich *et al*., 2002; Gram *et al*., 2002; Iulietto *et al*., 2015).

1.4.2.2. Extrinsic factors

Temperature and packaging are among the most important parameters that determine the development of microbial communities, and hence are taken into account to extend the shelf life of meat products (**Table 7**) (Ahmed *et al*., 2017; Del Blanco *et al*., 2017; Fang *et al*., 2017; Ghaani *et al*., 2016; Holman *et al*., 2018; Jääskeläinen *et al*., 2016; Kerry *et al*., 2006; Liu *et al*., 2006; Mansur *et al*., 2019; McMillin, 2017; Pereira de Abreu *et al*., 2012; Pexara *et al*., 2002; Realini and Marcos, 2014). Spoilage microbiota in pork meat stored under different packaging and temperatures conditions have also been widely studied (Borch *et al*., 1996; Brightwell *et al*., 2009; Diez *et al*., 2008; Dougeraki *et al*., 2010; Fontana *et al*., 2006; Jeremiah *et al*., 1995; Jeremiah and Gibson, 1997; Jiang *et al*., 2010; Koutsoumanis *et al*., 2008; Labadie, 1999; Mano *et al*., 2000; Sakala *et al*., 2002; Sorheim *et al*., 1999).

Table 7. Spoilage microbiota associated to different packaging conditions.

Packaging conditions	Associated spoilage microbiota	References
Aerobic (air)	*Acinetobacter* spp., *Brochothrix* spp., *B. thermosphacta*, *Carnobacterium* spp., *Enterobacteriaceae*, *Flavobacterium* spp., LAB (*Lactobacillus* spp., *Leuconostoc* spp.), *Micrococcus* spp., *Moraxella* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Staphylococcus* spp.	Cantoni *et al*., 2000; Casaburi *et al*., 2015; Castellano *et al*., 2004; Doulgeraki *et al*., 2012; Ercolini *et al*., 2006; Gram *et al*., 2002; Iulietto *et al*., 2015; Jääskeläinen *et al*., 2016; Labadie, 1999; Mills *et al*., 2014; Nychas *et al*., 2008; Remenant *et al*., 2015; Rossaint *et al*., 2015; Russo *et al*., 2006; Tsigarida *et al*., 2000
MAP (not specified)	*B. thermosphacta*, *Carnobacterium* spp., *Enterobacteriaceae*, LAB (*Lactobacillus* spp., *Leuconostoc* spp.), *Pseudomonas* spp., *Serratia* spp.	Holman *et al*., 2018; Iulietto *et al*., 2015; Casaburi *et al*., 2015; Castellano *et al*., 2004; Labadie, 1999; Tsigarida *et al*., 2000; Cantoni *et al*., 2000; Casaburi *et al*., 2015; Ercolini *et al*., 2006; Remenant *et al*., 2015; Russo *et al*., 2006; Mills *et al*., 2014
MAP (< 50% CO_2, with O_2)	*B. thermosphacta*, LAB	Nychas *et al*., 2008
MAP (> 50% CO_2, with O_2)	*B. thermosphacta*, *Enterobacteriaceae*, LAB	Nychas *et al*., 2008
VP	*B. thermosphacta*, *Carnobacterium* spp., *Clostridium* spp., LAB (*Lactobacillus* spp., *Leuconostoc* spp.), *Pseudomonas* spp., *Sh. putrefaciens*	Cantoni *et al*., 2000; Casaburi *et al*., 2015; Castellano *et al*., 2004; Ercolini *et al*., 2006; Gram *et al*., 2002; Labadie, 1999; Mills *et al*., 2014; Nychas *et al*., 2008; Remenant *et al*., 2015; Russo *et al*., 2006; Tsigarida *et al*., 2000; Russo *et al*., 2006

Bacteria are classified into four categories according to their temperature range for growth: thermophilic, mesophilic, psychrophilic and psychrotrophs (**Table 8**). As mentioned in many studies, it

can be considered that most of the identified bacteria isolated from refrigerated meats are commonly psychrophilic, also known as cold-resistant bacteria. This concerns Gram-positive bacteria including *Arthrobacter*, *Bacillus*, *Brochothrix*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, *Microbacterium* and *Streptococcus*, and Gram-negative bacteria such as *Aeromonas*, *Enterobacteriaceae*, *Pseudomonas*, *Psychrobacter* and *Serratia* (Doulgeraki *et al*., 2012; Hantsiszacharov and Halpern, 2007; Iulietto *et al*., 2015; Moretro *et al*., 2016; Pennacchia *et al*., 2011; Rodrigues *et al*., 2009). At these low temperatures, psychrotolerant communities are responsible for off-odors, discoloration and slime production (Del Blanco *et al*., 2017; Gram *et al*., 2002; Wang *et al*., 2017; Zhang *et al*., 2019), due to the production of thermally stable lipases and proteases (Wei *et al*., 2019).

Table 8. Classification of spoilage bacteria according to their temperature ranges for growth. T_{min}, minimal temperature for growth; T_{opt}, optimal temperature for growth; T_{max}, maximal temperature for growth. Based on Bowman *et al*., 1997; Hilgarth *et al*., 2017; Russell *et al*., 1990; Zhang *et al*., 2015.

Type	T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)
thermophilic	30 to 40	55 to 60	60 to 90
Mesophilic	5 to 10	25 to 40	44 to 50
Psychrotroph	-2 to 5	20 to 35	30 to 40
Psychrophilic	-18 to -10	10 to 15	18 to 20

Food packaging, with several combinations of gaseous atmospheres, also has a significant impact on the spoilage microbiome (**Figure 6**) and so can be used as an effective method for extending the shelf life of meat (Blickstad and Molin, 1983; Borch, 1996; Dalcanton *et al*., 2013; Daniloski *et al*., 2019; Del Blanco *et al*., 2017; Kaur *et al*., 2017; Kiermeier *et al*., 2013; Lopusiewicz *et al*., 2018; Madival *et al*., 2009; Poyatos-Racionero *et al*., 2018; Rossaint *et al*., 2015; Sibande *et al*., 2016; Yost and Nattress, 2002). The characteristics of food packaging materials are also important to provide or not a barrier to the exchange of gases between the pack and the external atmosphere (Ercolini *et al*., 2006; Kurek *et al*., 2012; Toyonaga *et al*., 2016). Modified atmosphere packaging (MAP) and vacuum packaging (VP) are the most commonly used types of packaging (Jääskeläinen *et al*., 2016).

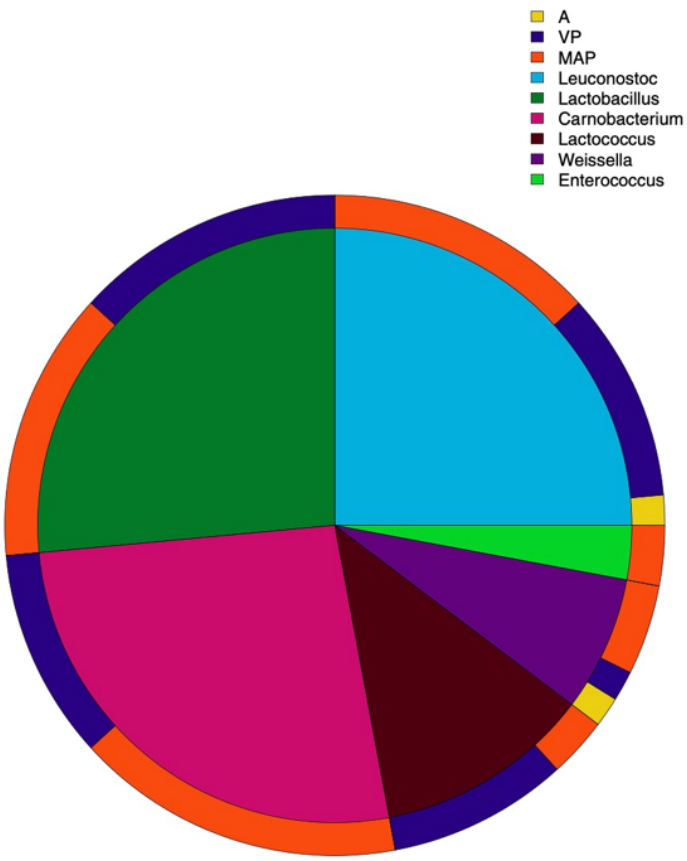


Figure 6. Occurrence of spoilage bacteria in meat according to the packaging storage condition (in air (A), in vacuum packaging (VP), in modified atmosphere packaging (MAP)) (Pothakos *et al*., 2015).

MAP consists of gas mixtures containing variable O_2 and CO_2 concentrations in order to inhibit the different spoilage-related bacteria, and is often associated with the use of low temperatures during storage (Ercolini *et al*., 2006). Gill and Tan (1979) reported greater inhibition of pseudomonads by carbon dioxide at 3°C rather than at 5°C. The lower inhibitory effect at a higher temperature is probably due to the limited formation of carbonic acid and dissociation to H^+ and HCO_3^-, by a lesser degree of dissolution in the aqueous phase of the products (Lambert *et al*., 1991). A high initial bacterial density can also result in an increased probability of more resistant microorganisms more resistant to the antibacterial effect of the gaseous atmosphere (Koutsoumanis *et al*., 2008). Carbon dioxide has a bacteriostatic effect for aerobic spoilage, thus allowing the growth of LAB (e.g. *Lactobacillus* spp. and *Leuconostoc* spp.) as spoilers (Arvanitoyannis and Stratakos, 2012; Devlieghere *et al*., 2001; Ercolini *et al*., 2006; Iulietto *et al*., 2015; Meredith *et al*., 2014; Yost and Nattress, 2002). Carbon dioxide concentrations up to 10% have been found to inhibit the growth of most *Pseudomonas* species (Rouger *et al*., 2018).

In meat stored under high oxygen and/or aerobically, aerobic or facultative aerobic Gram-negative bacteria can dominate the microbiota, especially *Pseudomonas* spp. (Doulgeraki *et al*., 2012; Koutsoumanis *et al*., 2006; Lambropoulu *et al*., 1996; Liu *et al*., 2006). However, facultative anaerobic

or anaerobic Gram-positive microbiota with less spoilage potential, such as *B. thermosphacta* and LAB (such as *Leuconostoc* and *Lactobacillus*), can occur under the association of low oxygen availability and carbon dioxide (Dolan *et al*., 2019; Ercolini *et al*., 2011; Gram *et al*., 2002; Jääskeläinen *et al*., 2016; Koutsoumanis *et al*., 2008; Jääskeläinen *et al*., 2016).

VP is also of interest to extend the shelf life of meat and meat products by preventing the growth of oxygen-requiring spoilage bacteria (Borch *et al*., 1996; Chen *et al*., 2012; Mansur *et al*., 2019). By respiratory activity, the residual oxygen at the meat surface-package interface is then converted to carbon dioxide (Mills *et al*., 2014). In addition, studies have demonstrated that *Lactobacillus* spp., *Pseudomonas* spp. and *Carnobacterium* spp. are the dominant bacteria in VP chilled pork (Jiang *et al*., 2010; Li *et al*., 2006; Zhao *et al*., 2015). Interestingly, *Enterobacteriaceae* are not always present as major spoilage bacteria in this kind of packaging. It has also been demonstrated that *Lactobacillus* may produce lactic acid in VP meat, which inhibits the growth of other families (Blixt and Borch, 2002). Wang *et al*. (2016a) have recently reported that 100% CO_2 packaging seems to be more effective against spoilage bacterial communities than VP.

1.4.2.3. Implicit and interaction factors

The factors described above can have synergistic or antagonistic effects on the spoilage microbiome and its dynamics. Moreover, spoilage microbiota can interact with each other (biotic interactions) and with the meat substrate (abiotic interactions) (Tsigarida *et al*., 2003; Zhang *et al*., 2015). These factors can be classified according to their beneficial or detrimental effects such as, for example, antagonism caused by the competition for iron, the change in profile of a bacterium by the nutrients supplied by another microorganism, bacterial communication, etc. (Gram *et al*., 2002). Some of these interactions will be presented in the discussion section, as they will be of great interest for the development of predictive models.

1.5. Main pork meat spoilage communities and their metabolic pathways

A synthetic review

The microbial ecology of meat and meat products (Borch *et al*., 1996; Chaillou *et al*., 2015; Drosinos and Board, 1995; Hilgarth *et al*., 2017; Jääskeläinen *et al*., 2016; Mansur *et al*., 2019; Miks-Krajnik *et al*., 2016; Nychas *et al*., 1991; Nychas *et al*., 2008; Stoops *et al*., 2015; Vester Laurtisen *et al*., 2019; Zhang *et al*., 2019), as well as that of pork meat (Benson *et al*., 2014; Del Blanco *et al*., 2017; Geeraerts *et al*., 2017; Koutsoumanis *et al*., 2008*;* Li *et al*., 2019a; Mann *et al*., 2016; Peruzy *et al*., 2019a; Zhao *et al*., 2015) is well described in the scientific literature. According to the storage conditions, pork meat communities can vary but *Acinetobacter* spp., *Aeromonas* spp., *Brochothrix* spp., *Clostridium* spp., cold-tolerant *Enterobaceriaceae*, *Enterococcus* spp., *Flavobacterium* spp., LAB (such as *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Carnobacterium* spp., etc.), *Micrococcus* spp., *Moraxella* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Rahnella* spp., *Staphylococcus* spp. and *Weissella* spp. are frequently described (Casaburi *et al*., 2015; De Filippis *et al*., 2013; Doulgeraki *et al*., 2012; Godziszewka *et al*., 2017; Koort *et al*., 2005 ; Liu *et al*., 2006 ; Pennacchia *et al*., 2009; Pennacchia *et al*., 2011; Pothakos *et al*., 2014; Stellato *et al*., 2016). Other recent scientific publications also described the microbial spoilage communities of pork meat and meat products by culture-dependent and -independent methods (**Table 9**). Spoilage generally occurs when SSOs grow to unacceptable levels, determined as between 7.0 log (Jääskeläinen *et al*., 2016; Koutsoumanis *et al*., 2008; Nychas *et al*., 2008; Pothakos *et al*., 2014; Reid *et al*., 2017; Spanu *et al*., 2018; Stoops *et al*., 2015; Zhao *et al*., 2015) and 8.0 log CFU/g (Chaillou *et al*., 2015; Fall *et al*., 2012; Nychas *et al*., 2008; Pothakos *et al*., 2014; Reid *et al*., 2017). Their sensory spoilage potential depends on their ability to produce metabolites such as aldehydes, ketones, esters, alcohols, organic acids, amines and sulphur compounds (Jääskeläinen *et al*., 2016).

A synthetic description of common spoilage bacteria described on pork meat and meat products, including their spoilage activity, is presented in the subsections below. Lastly, a short paragraph is also dedicated to spoilage by yeasts and molds.

Table 9. Some examples of recent scientific literature about the most potentially dominant spoilage bacteria represented in pork meat and meat products analyzed by culture-dependent and -independent techniques.

Samples	Packaging condition	Analysis	Dominant bacteria	References
Minced pork obtained from butchers' shops and supermarkets	Aerobic conditions, at 4 ± 2°C.	Microbial counts on plate count agar, cetrimide Fucidin cephaloridine agar (CFC), CFC pseudomonas supplement, streptomycin thallous acetate actidione agar (STAA) with STAA supplement.	*Pseudomonas* spp., *B. thermosphacta* and LAB.	Andritsos *et al*., 2012
Pork musculature scrapings	During veterinary inspection at slaughterhouse.	16S rRNA gene sequencing based on V1-V2 region.	*Aeromonas* spp., *Brochothrix* spp. and *Pseudomonas* spp.	Mann *et al*., 2016
Industrial pork raw sausages	Modified atmosphere (30% CO_2 – 70% O_2), at 7°C.	16S rRNA gene sequencing based on V3 region.	*Lactobacilliaceae* and *Listeriaceae*, mostly ascribed to *Lactobacillus* and *Brochothrix* genera, respectively.	Raimondi *et al*., 2018
Minced pork samples	Aerobic conditions, at 4°C.	16S rRNA gene sequencing based on V3-V4 region.	*Pseudomonas* spp. (*Ps. fragi*, *Ps. galenii*, *Ps. proteolytica*), *B. thermosphacta*, *Carnobacterium* spp., *Lc. lactis*, *Lb. sakei, Ln. gelidum and Ln. mesenteroides* are the most common genera. *Weissella* spp., *Debaryomyces* spp., *Escherichia coli*, *Rahnella* spp., *S. liquefaciens*, and *S. proteamaculans* are also detected.	Peruzy *et al*., 2019a
Fresh pork-pieces samples	Aerobic conditions, at 4°C.	16S rRNA gene sequencing based on V3-V4 region.	Dominant genera after 5 days of storage: *Pseudomonas, Photobacterium* and *Acinetobacter*.	Li *et al*., 2019a

1.5.1. Metabolic activity

In all packaging conditions, glucose, and other compounds from the glycolytic pathway, are the first substrates used by most spoilage bacteria described in pork meat (Casaburi *et al*., 2015; Del Blanco *et al*., 2017) and have been found to be the precursors of many off-odors compounds (VOCs) such as acetoin, diacetyl, acetic acid, iso-butyric acid, iso-valeric acid, 2-methylbutyric acid, 3-methylbutanol, 2 methylpropanol and ethanol (Nychas *et al*., 1998; Tsigarida *et al*., 2003). When glucose is depleted, spoilage bacteria produce undesirable VOCs by catabolism of other substrates such as lactate, gluconate, glucose-6-phosphate, pyruvate, propionate, formate, ethanol, acetate, amino acids, nucleotides and urea (**Table 10**) (Byun *et al*., 2003; Mills *et al*., 2014; Nychas *et al*., 2008). These compounds have been

associated with changes in pH and the production of volatile sulfur compounds during storage in aerobic conditions (Byun *et al*., 2003; Skandamis and Nychas, 2001). Lactate is also a source of energy for meat spoilage bacteria such as LAB, *B. thermosphacta* and *Pseudomonas* spp. (Nychas *et al*., 2008). It has been demonstrated that the concentration of glucose and lactate can affect the type and rate of spoilage, as they seem to be the principal precursors of the microbial metabolites that are responsible for off-odors (Casaburi *et al*., 2015).

Table 10. Substrates used by meat spoilage bacteria during growth under different packaging conditions. The number reported indicated the order of substrate utilization. A, *B. thermosphacta*; B, *Enterobacteriaceae*; C, LAB; D, *Pseudomonas* spp. According to studies of Casaburi *et al*. (2015) and Nychas *et al*. (2008).

Substrates	Aerobic				Anaerobic			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Glucose	1	1	1	1	1	1	1	1
Glucose-6-phosphate	2	2	2	2	2	2	2	2
Lactic acid	3	3		3				
Pyruvic acid				4				3
Gluconic acid				5				3
Gluconate-6-phosphate				6				3
Acetic acid								3
Amino acids	4	4		7		3	3	3
Ribose	5							
Glycerol	6							

Although many bacterial genome sequences are now available, few spoilage bacteria isolated from pork meat have been studied at a genomic level. Remenant *et al*. (2015) have summarized the available genome sequences of bacterial spoilers, and other publications have also described the genome sequence of meat and seafood spoilers (Chaillou *et al*., 2005; Illikoud *et al*., 2018; Mei *et al*., 2012; Paoli *et al*., 2017; Poirier *et al*., 2018a; Reichler *et al*., 2019; Singha *et al*., 2017; Stanborough *et al*., 2017; Yanzhen *et al*., 2016). Also, the Japanese Kyoto Encyclopedia of Genes and Genomes (KEGG) resource (https://www.kegg.jp/kegg/) procures various tools to investigate data issued from genomics and in particular from metabolomic pathways deduced from genome content (Liu *et al*., 2019a; Wu *et al*., 2017).

1.5.2. Common Gram-positive spoilage bacteria found in pork meat and meat products

Brochothrix spp. and LAB such as *Carnobacterium* spp., *Lactobacillus* spp. and *Leuconostoc* spp. are found in a variety of environmental habitats, including vegetable, meat, fish and milk products (Iskandar *et al*., 2017; Klaenhammer *et al*., 2005). They are important competitors in a variety of chilled meats stored under VP and MAP conditions (Ercolini *et al*., 2006; Lucquin *et al*., 2012; Vihavainen and Björkroth, 2009; Zotta *et al*., 2018). LAB have interesting processing characteristics (Fadda *et al*., 2010; Najjari *et al*., 2016; Stefanovic *et al*., 2017; Teusink and Molenaar,

2017) and health effects (Notararigo *et al*., 2013) and can be used as bioprotective microorganisms (Chaillou *et al*., 2014; Comi *et al*., 2015; Ghanbari *et al*., 2013; Siedler *et al*., 2019). The antagonistic and inhibitory properties of LAB for pathogenic and spoilage bacteria are due to different factors: (i) competition for nutrients, (ii) production of one or more antimicrobial peptides, such as bacteriocins, (iii) organic acid production, and (iv) production of hydrogen peroxide (Andreevskaya *et al*., 2018; Bartkiene *et al*., 2019; Ghanbari *et al*., 2013; Hilgarth *et al*., 2018b; Saraoui *et al*., 2016). Several antimicrobial compounds are produced by LAB against spoilage bacteria (**Figure 7**) but the most important are lactic and acetic acids which can act in different ways (**Figure 8**) (Siedler *et al*., 2019).

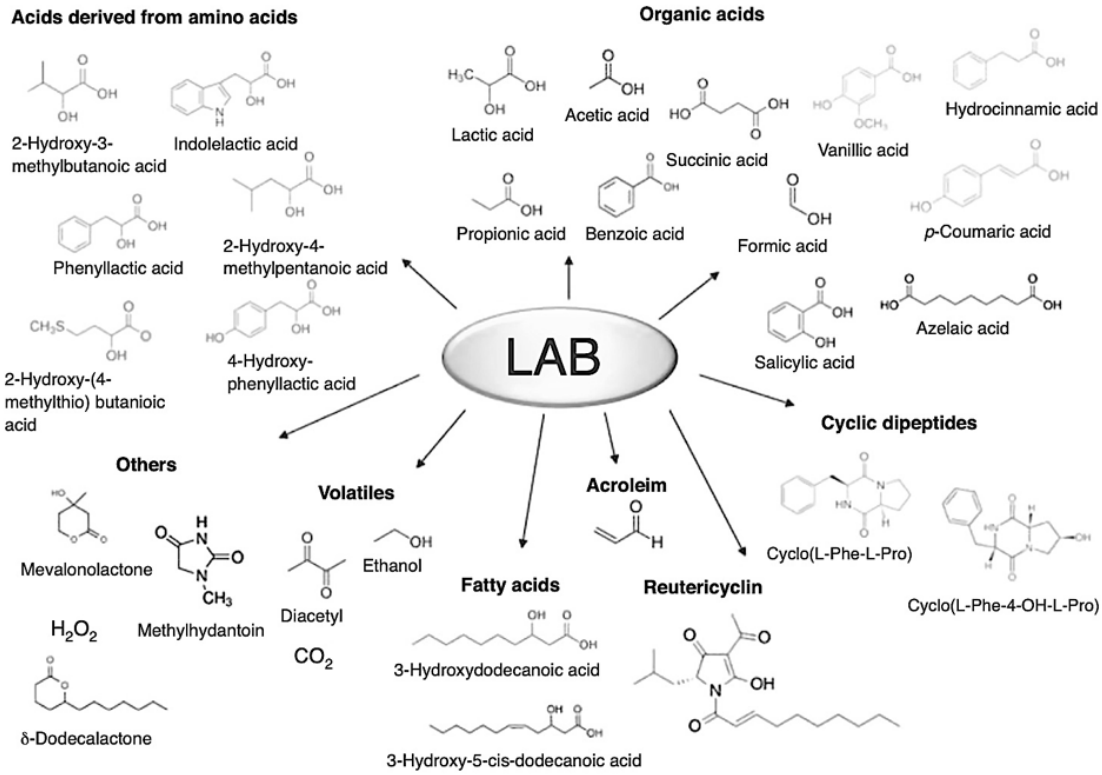


Figure 7. Antimicrobial compounds produced by LAB (Siedler *et al*., 2019).

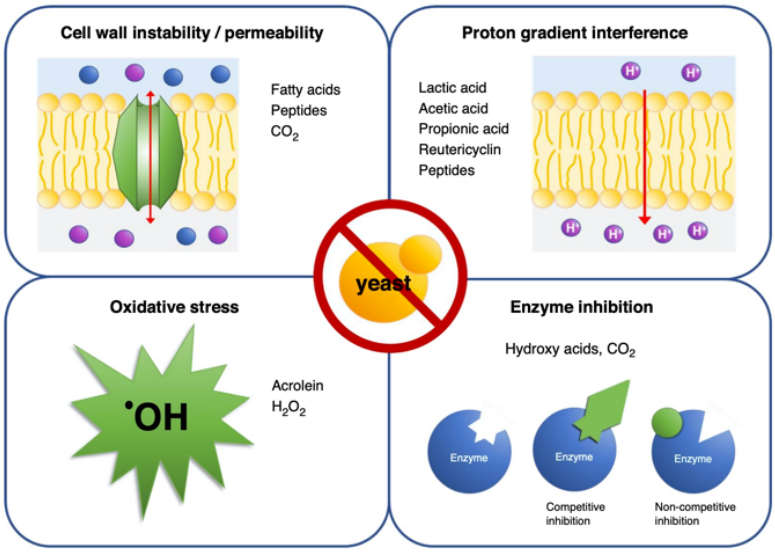


Figure 8. Some inhibitory mechanisms of antimicrobial compounds produced by LAB (Siedler *et al*., 2019).

However LAB are also linked to food spoilage (Hemme and Foucaud-Scheunemann, 2004; Iskandar *et al*., 2017; Iulietto *et al*., 2015; Klaenhammer *et al*., 2005), although the intra-species variation of LAB strains to cause spoilage has been recognized (Pothakos *et al*., 2015). LAB are oxygen-tolerant anaerobes which grow readily in the absence of oxygen and are not inhibited by carbon dioxide. Their spoilage effects generally occur at 8.0 log CFU/g (Mills *et al*., 2014). These microorganisms can be responsible for discoloration (Jääskeläinen *et al*., 2016; Vihavainen and Björkroth, 2007) or production of off-odor compounds (Casaburi *et al*., 2015). Lactate is the main end-product from glucose metabolism by LAB, in both in aerobic and anaerobic conditions (Del Blanco *et al*., 2017). Although some LAB can use amino acids to support their growth when the glucose concentration is low, the majority of these bacteria seem not to be able to use substrates other than lactate (Labadie, 1999; Nychas *et al*., 2008). According to their end-products of glucose fermentation LAB are classified as obligate homofermenters, producing only lactic acid, or as heterofermenters, producing a range of other end-products (Gänzle, 2015; Iulietto *et al*., 2015). Obligate and facultative heterofermentative LAB occur frequently in meat and meat products (Casaburi *et al*., 2015).

Lactobacillus spp., especially *Lb. sakei*, *Lb. curvatus*, *Lb. algidus*, *Lb. fuchuensis*, *Lb. graminis* and *Lb. oligofermentans*, are associated with the spoilage of meat (Audenaert *et al*., 2010; Jiang *et al*., 2010; Kato *et al*., 2000; Raimondi *et al*., 2018; Sanders *et al*., 2015; Stoops *et al*., 2015). With over 212 species described among LAB, *Lactobacillus* is a non-motile, facultative anaerobic, homo- or heterofermentative and catalase-negative bacterium unless heme is available (Abriouel *et al*., 2015; Chaillou *et al*., 2005).

The genus *Lactococcus* is also mainly encountered in chilled meat and seafood products, especially *Lc. lactis*, *Lc. piscium*, *Lc. plantarum* and *Lc. raffinolactis* (Pothakos *et al*., 2014; Rahkila *et*

al., 2012). Facultative anaerobic, psychrophilic, homofermentative, non-motile, catalase- and oxidase-negative microorganisms, *Lactococcus* spp. are able to growth under VP and MAP (Saraoui *et al*., 2016). Moreover, *Lc. piscium* is considered as potentially bioprotective for seafood products, limiting the growth of *B. thermosphacta* and *S. proteamaculans* (Marché *et al*., 2017).

Among species of the genus *Leuconostoc*, *Ln. gelidum*, *Ln. carnosum*, *Ln. mesenteroides* and *Ln. gasicomitatum* are the spoilers most frequently described in refrigerated meat (Jääskeläinen *et al*., 2013; Jääskeläinen *et al*., 2015; Johansson *et al*., 2011; Lyhs *et al*., 2004; Pothakos *et al*., 2015; Yang *et al*., 2009). *Leuconostoc* spp. are heterofermentative LAB, using lactose and citrate to produce acetate, ethanol, lactic acid and carbon dioxide (Cicotello *et al*., 2018). *Ln. gasicomitatum* can respire under high-oxygen MAP due to the production of cytochromes, thus increasing its growth rate and giving it a competitive advantage for growth (Jääskeläinen *et al*., 2016). Some strains of *Leuconostoc* spp. can also produce bacteriocins against pathogenic and spoilage bacteria (Hemme and Foucaud-Scheunemann, 2004).

Spoilers in various food matrices (Bohaychuk and Greer, 2003; Liang *et al*., 2012; Papadopoulu *et al*., 2012; Rattanasomboon *et al*., 1999; Russo *et al*., 2006; Samelis *et al*., 2000), *Brochothrix* spp. are the dominant microbiota in meat and seafood products stored under aerobic and anaerobic conditions (Illikoud *et al*., 2019a). Belonging to the family *Listeriaceae*, *B. thermosphacta* and *B. campestris* are the two main non-pathogenic species (Stanborough *et al*., 2017). *B. thermosphacta* is a psychrophilic, non-motile, halophilic, non-sporulating and rod-shaped bacterium. A facultative anaerobe, it is able to grow within a wide range of temperatures (Argyri *et al*., 2015; Odeyemi *et al*., 2018b; Pin *et al*., 2002). Production of off-odors is mainly due to the production of acetoin and diacetyl (Ercolini *et al*., 2006; Illikoud *et al*., 2018) and becomes perceptible at around 8.0 log CFU/g (Casaburi *et al*., 2014). Under high oxygen conditions, this bacterium can be considered as heterofermentative, with production of acetoin, while it shifts to homofermentative metabolism under low oxygen conditions and glucose availability, with the production of lactate (Casaburi *et al*., 2015; Labadie, 1999). In anaerobic conditions, *B. thermosphacta* preferentially uses glucose as substrate, while it consumes ribose, glycerol and amino acids in air packaging (Illikoud *et al*., 2019a; Nychas *et al*., 2008). However, studies concerning the proteolytic and lipolytic activities of *B. thermosphacta* are sometimes ambiguous (Braun and Sutherland, 2003). Labadie (1999) and Papadopoulu *et al*. (2019) reported non-proteolytic activity, while that is not the case for other strains. Indeed, despite their widespread occurrence within spoiling species, metabolic activity is not well known (Illikoud *et al*., 2018). Studies have recently shown that its spoilage ability is both strain- and environmental-dependent (Illikoud *et al*., 2019a), but also depends on the type of food matrix (Illikoud *et al*., 2019b).

Carnobacterium spp. are also frequently described in beef, poultry and pork meat (Pothakos *et al*., 2015). The genus *Carnobacterium* consists of 11 species, with *C. maltaromaticum* and *C. divergens* being the two most important (Afzal *et al*., 2010). They are non-motile LAB, found particularly in VP and MAP (Casaburi *et al*., 2015; Laursen *et al*., 2005; Vasilopoulos *et al*., 2008; Zhang *et al*., 2018c).

C. maltaromaticum strains present high inter-strains variability, but this bacterium is adapted to multiple and diverse environments, probably due to its larger genome size (3.7 Mbp) (Cailliez-Grimal *et al*., 2013; Leisner *et al*., 2007) and the presence of genes involved in the metabolism of branched-chain amino acids (Liu *et al*., 2008) and lactose (Iskandar *et al*., 2016). Moreover, *C. maltaromaticum* is able to grow at low temperature (below 0°C), high pH (superior to 9.5) and in the presence of up to 8% NaCl (Iskandar *et al*., 2017). Its spoilage effect on meat and meat products seems to be less than that of other spoilage bacteria (Zhang *et al*., 2018c). Indeed, *C. maltaromaticum* is described as a bioprotective species (Odeyemi *et al*., 2018b) due to its potential to produce bacteriocins against pathogenic bacteria, especially *Listeria monocytogenes* (Leisner *et al*., 2007), and spoilage bacteria (Spanu *et al*., 2018).

1.5.3. Common Gram-negative spoilage bacteria found in pork meat and meat products

Rarely reported in meat, *Photobacterium* spp. are well-known spoilage bacteria of seafood products in all packaging conditions (Fuertes-Perez *et al*., 2019; Remenant *et al*., 2015). However, some studies have found *Ph. phosphoreum* in pork and beef meats (Nieminen *et al*., 2016; Pennacchia *et al*., 2011; Stoops *et al*., 2015). Recently, Hilgarth *et al*. (2018a) showed that *Photobacterium* spp. are an underestimated spoiler of meat and Fuertes-Perez *et al*. (2019) have confirmed this observation. *Photobacterium* spp. are bioluminescent, psychrotolerant, motile and halophilic bacteria, with a high tolerance for CO_2 concentrations (Gram and Huss, 1996; Odeyemi *et al*., 2018b). *Ph. kishitanii* seems to be closely related to *Ph. phosphoreum* and *Ph. iliopiscarium* (Ast *et al*., 2007).

Moreover, *Pseudomonas fragi*, *Ps. fluorescens*, *Ps. lundesis* and *Ps. putida* are the most important taxa described in many foodstuffs (raw fish, ready-to-eat vegetables, dairy products) (Andreani *et al*., 2015; Caldera and Franzetti, 2014; Shen *et al*., 2015), as well as in meat and meat products stored in chilled and aerobic conditions (Casaburi *et al*., 2015; Del Blanco *et al*., 2017; Ercolini *et al*., 2010b; Iulietto *et al*., 2015; Jääskeläinen *et al*., 2016; Koutsoumanis *et al*., 2008; Labadie, 1999; Mohareb *et al*., 2015; Nychas *et al*., 2008; Remenant *et al*., 2015). The genus *Pseudomonas* includes obligatory aerobic rod-shaped, motile, mesophilic and psychrophilic, non-fermentative and oxidase positive species (Decimo *et al*., 2014; Odeyemi *et al*., 2018b; Peix *et al*., 2009; Wickramasinghe *et al*., 2019), with simple nutritional requirements (Anzai *et al*., 2000; Frapolli *et al*., 2007). The pseudomonads have a highly proteolytic activity, preferentially metabolizing glucose and nitrogenous compounds, such as amino acids (Ercolini *et al*., 2006; Iulietto *et al*., 2015; Wickramasinghe *et al*., 2019). However, because of their high iron requirement for growth, *Pseudomonas* spp. have a siderophore-mediated iron uptake system, but that is not the case for all strains. For example, *Ps. fragi* is considered to be a non-siderophore-producing species (Cornelis, 2010; Liu *et al*., 2017; Stanborough *et al*., 2018a). Spoilage activity is generally detected at a concentration of 7.0-8.0 log CFU/g, and is caused by the production of many VOCs responsible for off-odors (Casaburi *et al*., 2015; Del Blanco *et al*., 2017; Ghali *et al*., 2010; Iulietto *et al*., 2015; Nychas *et al*., 2008), and by discoloration produced

by colored or fluorescent pigments (Andreani *et al*., 2014). *Ps. fluorescens* and *Ps. putida* are pigmented species, which is not the case for *Ps. fragi* (Wickramasinghe *et al*., 2019). Since 2010, the "blue mozzarella cheese events" which occur in Europe have led to the investigation of the blue pigment phenotype of *Ps. fluorescens* (Andreani *et al*., 2015; Kumar *et al*., 2019). *Pseudomonas* spp. can also produce thermoresistant extracellular enzymes, such as proteases, lipases and lecithinases (Andreani *et al*., 2014; Arslan *et al*., 2011; De Jonghe *et al*., 2011; Dogan and Boot, 2003; Liu *et al*., 2007; Marchand *et al*., 2009; Mellor *et al*., 2011; Quigley *et al*., 2013; Stanborough *et al*., 2018b), as well as biofilms on food and equipment (Kerekes *et al*., 2013; Rossi *et al*., 2016; Wang *et al*., 2018b) by production of extracellular polysaccharides which give them an advantage over competitors (Hibbing *et al*., 2010; Nadell *et al*., 2016; Wickramasinghe *et al*., 2019). Nevertheless, Wang *et al*. (2018a, 2018c) showed that MAP seems to inhibits the spoilage activity of *Pseudomonas* spp. by decreasing metabolism and extracellular proteolytic activity. These observations were not supported by Hilgarth *et al*. (2019), who showed that *Pseudomonas* spp. appear to be well adapted to grow on meat stored under MAP conditions.

Moreover, *Shewanella* spp. is a genus closely related to *Pseudomonas* spp., which contributes significantly to bacterial food spoilage (Iulietto *et al*., 2015). *S. putrefaciens* is one of the most predominant spoilers in chilled-stored meat, especially under VP (Doulgeraki *et al*., 2012).

Psychrotolerant and halotolerant, *Psychrobacter* is described in seafood products, but also in fresh or salted meat (Moretro and Langsrud, 2017). It is a psychrophilic Gram-negative bacterium, non-motile, oxidase-positive, rod-shaped, aerobic and osmotolerant (Odeyemi *et al*., 2018b).

Finally, *Serratia* spp., *Enterobacter* spp., *Pantoea* spp., *Proteus* spp. and *Hafnia* spp. also often contribute to food spoilage (Ercolini *et al*., 2006). *Enterobacteriaceae* can metabolize amino acids to off-odor volatile compounds, such as foul-smelling diamines and sulfuric compounds (Remenant *et al*., 2015). *Enterobacteriaceae* are facultative anaerobes which metabolize glucose preferentially and then utilize amino acids for producing amines and dimethyl sulfide (Mills *et al*., 2014). Cold-tolerant *Enterobacteriaceae* such as *Hafnia alvei*, *S. liquefaciens* and *Enterobacter agglomerans* occur under aerobically conditions. But in terms of numbers they do not contribute significantly to the microbial associations for the spoilage of meat and meat products, and have been considered as indicators of food safety (Nychas *et al*., 2008).

1.5.4. Spoilage yeasts and molds

Filamentous fungi (commonly called "molds") and yeasts are responsible for about 5% of all FLW in developed countries (Snyder and Worobo, 2018; Snyder *et al*., 2019). However, they contribute only a small proportion of the natural microbiota of foods.

The dominant yeast species on meat include *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Rhodotorula* and *Saccharomyces* (Kabisch *et al*., 2016). Moreover, Nagy *et al*. (2014) and Kabisch *et al*. (2013) have recently isolated *Yarrowia* (*Y. porcina and Y. bubula*) and *Kazachstania psychrophila*,

respectively, from meats. For molds, it mainly concerns *Penicillium* and *Aspergillus*, especially in cured products (Bernardi *et al*., 2019; Perrone *et al*., 2019).

Mainly present in an air environment, they can disperse easily and contaminate the surfaces of meat and meat products (Dijksterhuis, 2019). Also, they are psychrotolerant and grow at low storage temperature (Kabisch *et al*., 2016), at water activities below 0.85 and under anaerobic conditions (Rico-Munoz *et al*., 2019). Food spoilage by yeasts and molds, occurring at 5.0-6.0 log CFU/g, is mainly due to the production of extracellular proteases and is characterized by discoloration, off-odors and off-flavors (dairy, buttery, fruity and putrid odors), swelling and slime formation (Hernandez *et al*., 2018).

1.6. Analytical tools

To a deeper understanding of bacterial food spoilage

To gain a deeper understanding of the dynamics of bacterial food spoilage, microbial challenge testing (MCT) and microbial aging testing (MAT) were applied in this research.

In MCT, products are inoculated by one or more bacteria at a certain level to observe their dynamics and/or their spoilage activity, for example. Products are either naturally contaminated or sterile. In MAT, naturally contaminated products are stored, and the naturally present microbiota is followed during shelf life. In both cases, food products are then stored under different storage conditions, in order to reflect or not the different storage conditions of commercialized products (Notermans and in't Veld, 1994; Rouger *et al*., 2017; Spanu *et al*., 2014). The specific storage lifetime applied in MCT and MAT corresponds to the period during which a product is expected to remain safe and at an appreciable quality, or for a longer period of time to observe the bacterial dynamics (Mills *et al*., 2014).

Different tools are available to monitor and predict the dynamics of microbial communities. In this research, classical plate counting, 16S rRNA gene sequencing, and proton nuclear magnetic resonance (^{1}H-NMR)-based metabolomic and predictive microbiology were used. In this section, a brief description of these techniques is presented.

1.6.1. Culture-dependent and -independent methods

Cultural methodologies using different culture media are mainly used to study bacterial ecosystems. However, some studies have shown that these techniques do not necessarily provide reliable information, due to the selectivity of the culture medium, incubation conditions and some viable but non culturable (VBNC) microorganisms (Duthoit *et al*., 2003; Justé *et al*., 2008). Therefore, molecular methods are interesting tools to analyze diversity within bacterial communities (Aguiar-Pulido *et al*., 2016; De Filippis *et al*., 2018; Franzosa *et al*., 2015; Hameed *et al*., 2018; Hugerth and Andersson, 2017; Jung *et al*., 2011; Menezes *et al*., 2020), alone or in association with other culture-dependent and -independent methods. Zhao *et al*. (2015) have already characterized bacterial and fungal changes by using 16S rDNA and 18S rDNA gene amplicon sequencing in combination to classical plate counts, in chilled VP pork during storage. Jaffrès *et al*. (2009) and Martin-Platero *et al*. (2008) also showed the interest of a polyphasic approach for other food matrices by combining cultural and non-cultural methods.

Not all culture-dependent and -independent methods are presented in this section: several review publications are available on the subject (Giraffa and Neviani, 2001; Hameed *et al*., 2018; Jany and Barbier, 2008; Justé *et al*., 2008; Vargas-Albores *et al*., 2018; Ziyaina *et al*., 2020). The research presented in this thesis only involved combining plate counts, 16s rRNA gene sequencing and PCR analyses. The use of culture-independent methods to characterize microbial communities has increased in recent years due to their practical benefits. As the isolation step of culture-dependent methods introduces biases because some species are unable to grow under the selected experimental conditions, culture-independent methods are based on the direct analysis of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), without any culturing step, to study the total diversity of food samples (Jany and Barbier, 2008; Zapka *et al*., 2017). Most of these methods use polymerase chain reaction (PCR) amplification of total DNA with universal (non-discriminative) primers to amplify all target sequences of a given population (Jany and Barbier, 2008; Justé *et al*., 2008; Su *et al*., 2012; Ziyaina *et al*., 2020). The increasing interest of culture-independent methods, namely those based on DNA sequencing, has been driven by advances in sequencing technologies and bioinformatic analysis tools, and also by a reduction in the cost of conducting such studies (Zapka *et al*., 2017). Methodologies based on detection and/or quantification of microbial DNA are characterized by faster analysis than conventional culture-based methods, higher sensitivity and specificity, and are able to provide reproductible results dealing with quantitative and qualitative compositions of a microbial community, even for the sub-dominant populations (Giraffa and Neviani, 2001; Postollec *et al*., 2011; Ziyaina *et al*., 2020). More particularly, real-time quantitative PCR (qPCR) is considered as a method of choice for the detection and quantification of microorganisms (Postollec *et al*., 2011).

Due to its high degree of conservation among bacteria and archaea domains, the 16S rRNA gene has been an excellent genetic marker for studying bacterial phylogeny and taxonomy (Cocolin *et al*., 2013; Vargas-Albores *et al*., 2018). One important advantage of the use of these two genes is that large sequence data-bases are available. On the other hand, the rRNA operon that codes for the 16S rRNA is frequently found as multi-copies, from 1 to 15 in bacteria (Cocolin *et al*., 2013; Vargas-Albores *et al*., 2018). These multi-copies often result in multi-signals, which complicate the analysis. The rpoB gene, encoding for the β-subunit of the RNA polymerase, has been proposed as an alternative (Cocolin *et al*., 2013). Moreover, PCR-based assays all suffer from amplification biases, as presentend in the review of Pollock *et al*. (2018). Indeed, any form of contamination of the sample, by even trace amounts of DNA, can produce misleading results (Ziyaina *et al*., 2020). Appropriate primer selection is also essential, but it must be noted that a perfect universal primer set does not exist for community profiling (Cocolin *et al*., 2013). Other biases could also come from inaccurate PCR amplification, such as preferential annealing to particular primer pairs or an incidence of chimeric PCR products with increasing numbers of PCR cycles, undetection of some genotypes due to low species abundance in the substrate, low species availability due to insufficient homogenization of the matrix, inadequate cell lysis that prevents release of nucleic acids, inhibition of PCR amplification, etc. (Jany and Barbier, 2008). Moreover, sampling

procedure and sample size rely on standard procedures in order to assess the microbial communities in a given environment and to compare different analyses. After sampling, sample treatment is the next crucial step and the choice for aerobic or anaerobic storage, washing, freezing or refrigeration procedures must be controlled (Justé *et al*., 2008). In addition, DNA-based techniques have sometimes been criticized because they do not distinguish living from dead cells. Some chemicals, such as propidium monoazide (PMA), could be used to differentiate between viable and non-viable organisms. Alternatively, RNA can be used as a target instead of DNA, in combination with reverse transcriptase PCR (RT-PCR). Since RNA is less stable than DNA, RNA will be degraded more quickly in dead organisms and could provide data on microbial activity by estimate transcript amounts (Justé *et al*., 2008; Postollec *et al*., 2011).

1.6.2. Metabolomic analysis

Other "omics" methods can be associated with these techniques in multi-omics approaches (Bergholz *et al*., 2014; Jääskeläinen *et al*., 2019; Kim *et al*., 2020) such as, for example, metabolomics (Remenant *et al*., 2015). Not all omics methods will be described in this section because only metabolomics was used in the present report.

Metabolomics is the qualitative and quantitative analysis of low molecular mass (< 1500 Da) metabolites (called the "metabolome") in complex biological samples (Verpoorte *et al*., 2008). First used in the fields of plant science and toxicology (Fiehn, 2002), metabolomics has recently emerged in food science ("*foodomics*") (Cevallos-Cevallos *et al*., 2009; Cifuentes, 2009; Marcone *et al*., 2013). This approach is very useful for addressing problems associated with food quality, authenticity (Lytou *et al*., 2019; Trivedi *et al*., 2016) and geographical origin assessments (Cubero-Leon *et al*., 2014; Locci *et al*., 2011), and also to describe metabolomic patterns linked to process and storage conditions (Consonni and Cagliani, 2010).

Nuclear magnetic resonance (NMR) is one of the most common approaches, with mass spectrometry (MS), used to analyze a metabolome because it is possible to obtain qualitative and quantitative information for a wide range of chemical metabolites (Trimigno *et al*., 2015). Moreover, this technique is available for solid and liquid samples, and is rapid, non-destructive and relatively affordable method (Mozzi *et al*., 2013). However, NMR has low sensitivity compared to MS (Rochfort, 2005).

Spectra are generally given by information of ^{1}H, ^{13}C and/or 2D-NMR analyses (Verpoorte *et al*., 2008), but high-resolution ^{1}H-NMR spectroscopy is mainly used (Mannina *et al*., 2011). Several studies have focused on metabolomic analysis by ^{1}H-NMR in foods (**Table 11**), but few of them have used this techniques in meat and meat products, especially in the context of food spoilage (Zhang *et al*., 2018d). In recent examples, Graham *et al*. (2010) have identified amino acid changes during different ageing periods of beef samples, Jung *et al*. (2010) have differentiated the geographical origin of beef samples,

Castejon *et al*. (2015) have analyzed the quality of beef meat exudate, Garcia-Garcia *et al*. (2018) have shown the interest of metabolomics to detect metabolites and compositional changes during different drying processes of fermented sausage and Yang *et al*. (2019) have studied the metabolomic profiles and taste of stewed pork hock in soy.

Table 11. Some example of studies that have used ^{1}H-NMR in foods.

Study objective	Food matrix	Reference
Metabolite changes during fermentation.	Traditional Korean salted seafood (*saeu-jeot*)	Jung *et al*. (2013)
Study of the ripening process.	Fiore Sardo cheese	Piras *et al*. (2013)
Evaluation of freshness.	Mussels	Aru *et al*. (2016)
Analyses of postharvest senescence.	Banana	Yuan *et al*. (2017)
Characterization of bacterial and yeast metabolome.	Fermentation starter (*Fen-Daqu*)	Li *et al*. (2018b)
Study of spoilage development during chilled storage.	Yellowfin tuna and salmon	Jääskeläinen *et al*. (2019)

Therefore, describing and characterizing the dynamics of bacterial spoilage in MP and WP by a multi-omics approach is an interesting subject, owing to the fact that these two matrices were never characterized with this method. In addition, predictive microbiology can be used to analyze the data obtained.

1.6.3. Predictive models

Predictive modeling, a sub-discipline of food microbiology, is also an interesting tool (Membré and Boué, 2018; Messens *et al*., 2018), especially associated with analytical methods (Cocolin *et al*., 2018; den Besten *et al*., 2018). Indeed, from available observations on the growth, survival or inactivation of bacteria, and depending on environmental factors, it is possible to predict the behaviour of the same microorganisms under various conditions (**Equation 1**).

$$y = f(x, \phi) \quad (1)$$

Where y is the output of the model, f is a mathematical function or equation, x are the input variables (also called factors), and ϕ are parameters estimated from the experimental data.

According to this, predictive microbiology assumes that the responses of bacterial populations to identical environmental conditions are reproducible, and so the dynamics of the microbial population always follows the same profile if the conditions are favorable to growth (**Figure 9**).

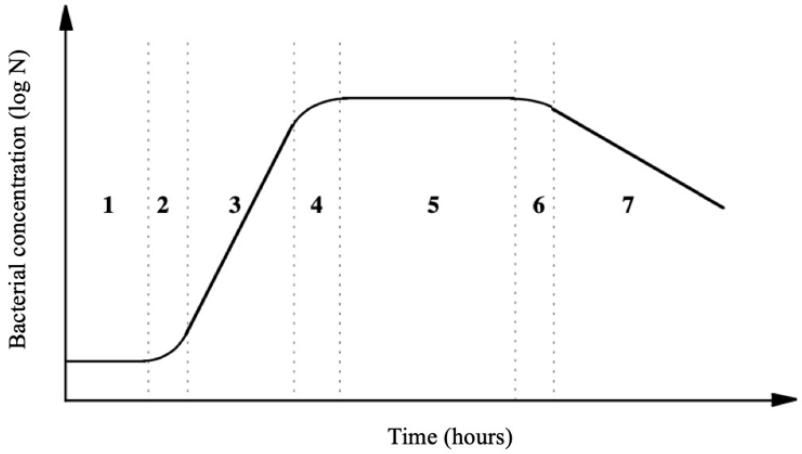


Figure 9. Growth curves of bacterial culture with its different phases: lag-time period (1), acceleration phase (2), exponential period (3), deceleration (4), stationary phase (5), decay phases (6, 7) (Buchanan, 1918).

Primary, secondary and tertiary models are then used to modelize the behavior of microorganisms (**Figure 10**).

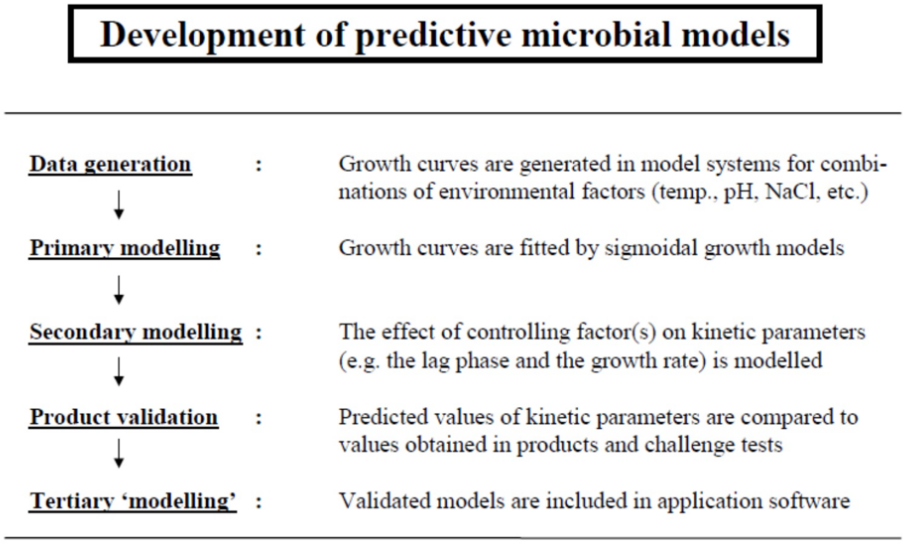


Figure 10. Steps for the development of predictive models (Fakruddin *et al*., 2011).

Primary models aim to reproduce the dynamics of the concentration of microorganisms over time in a given environment. The main growth parameters are then obtained by adjusting the bacterial curves with these models (**Figure 11**), in order to characterize each type of bacterial growth: lag phase (λ), maximal bacterial growth (μ_{max}), and initial and maximal bacterial concentration (N_0, N_{max}). It is assumed that 10 to 15 points are needed to better fit bacterial curves. As presented by Cornu *et al*. (2011) and Quinto *et al*. (2018), a modified generic primary growth model can be written as **Equation 2**, which combines different acceleration and deceleration phases.

$$\frac{1}{N(t)}\frac{dN(t)}{dt} = \frac{d(\ln(N(t)))}{dt} = \mu_{max}\ x\ \alpha(t)\ x\ f(t)\ (2)$$

Where $\frac{1}{N(t)}\frac{dN(t)}{dt}$ is the relative or instantaneous growth rate of the microorganism, N_t is the bacterial concentration at time t (log CFU/g), μ_{max} is the maximum growth rate (1/h), $\alpha(t)$ is an adjustment function, and $f(t)$ is an inhibition function, defined as **Equations 3** and **4**:

$$\alpha_t = \begin{cases} 0\ if\ t < LPD \\ 1\ if\ t \geq LPD \end{cases} (3)$$

$$f_t = \left(1 - \left(\frac{N_t}{N_{max}}\right)\right) (4)$$

Where LPD is the lag phase duration (hours), and N_{max} is the maximal population density (log CFU/g).

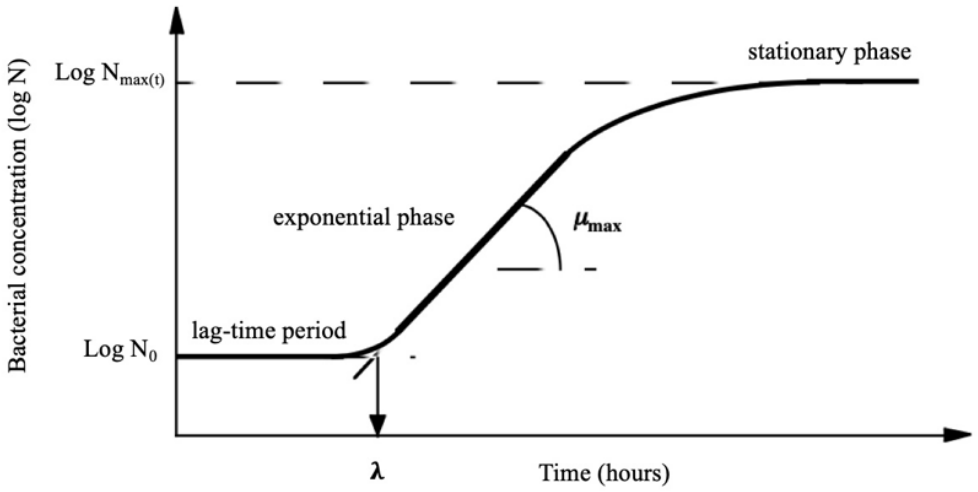


Figure 11. Growth parameters obtained from bacterial growth curves adjusted by primary models (Buchanan, 1918).

Among the primary models, the Baranyi and Roberts (1994) model is one of the most widely used and was applied in this study. This growth model is based on a differential equation (exponential phase) and completed by two adjustment functions (**Equation 5**). The first adjustment function describes the transition phase between the lag phase and the exponential phase. This function is represented by a parameter describing the physiological state of the bacteria, gradually increasing from 0 to 1. The second adjustment function describes the inhibition phase between the exponential phase and the stationary phase. It is based on the principle of a gradual decrease in nutrients. This inhibition function is based on the maximum concentration and a curvature parameter.

$$N_t = N_0 + \mu_{max} x\ A_t + ln\ [1 + \frac{exp(\mu_{max}\ x\ A_t) - 1}{exp(N_{max} - N_0)}]\ (5)$$

Where N_t the bacterial population at any time t (log CFU/g); N_{max} and N_0, the maximum and initial population level, respectively (log CFU/g); μ_{max}, the maximum specific growth rate (1/hour); and A_t, an adjustment function to define the LPD (**Equation 6**).

$$A_t = t + \frac{1}{\mu_{max}} x\, ln\left\{exp\left(-\mu_{max}\, x\, t\right) + exp(-h_0) - exp\left[\left(-\mu_{max}\, x\, t\right) - h_0\right]\right\} \quad (6)$$

Where h_0 is simply a transformation of the initial conditions.

Predictive microbiology models applied to model mixed cultures and/or microbial interactions in foods are also developed. The most two popular models for describing interactions between two bacterial populations are the Jameson-effect and the Lotka-Volterra models, which are used in this research.

In 1962, Jameson studied the competitive enrichment of *Salmonella* spp. and wrote: "*When two intestinal organisms, which do not mutually interact by colicines or bacteriophage, are inoculated together into a liquid medium, each organism normally follows at first a growth pattern similar to that which would have followed from a similar inoculum in the same medium in the absence of a competitor. Neither organism normally exhibits its awareness, to any appreciable degree, of the other's presence, until the bacterial density of one or other organism has risen to a level near to the molar concentration, when both organisms end their rapid multiplication*". In the late 1990s and early 2000s, there were numerous observations that many microbial interactions in foods are limited only to a reduction in the maximum population density, without any significant effect on the lag time or growth rate, and that the minority population decelerates when the majority or the total population count reaches its maximum level. On the basis of these observations, Ross *et al*. (2000) proposed to call this phenomenon the "Jameson effect". Then, Cornu (2001) incorporated the Jameson effect hypothesis into a growth model, assuming that the inhibition function is equal for all competitive microbial populations and would result from the competition for a common limiting resource. Thus, based on equation 2, an alternative deceleration function can be added for modeling the interaction of two bacterial species (**Equation 7**) (Cornu *et al*., 2011; Mejlholm and Dalgaard, 2007).

$$\begin{cases} \frac{1}{N_A(t)}\frac{dN_A(t)}{dt} = \mu_{max_{A(t)}}\, x\, \alpha_A(t)\, x\, \left(1 - \frac{N_{A(t)}}{N_{max_{A(t)}}}\right) x\, \left(1 - \frac{N_{B(t)}}{N_{max_{B(t)}}}\right) \\ \frac{1}{N_B(t)}\frac{dN_{Bt}}{dt} = \mu_{max_{B(t)}}\, x\, \alpha_B(t)\, x\, \left(1 - \frac{N_{B(t)}}{N_{max_{B(t)}}}\right) x\, \left(1 - \frac{N_{A(t)}}{N_{max_{A(t)}}}\right) \end{cases} \quad (7)$$

Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), N_{max} is the maximum population density (log CFU/g).

Moreover, in the 1920s, the Italian mathematician Vito Volterra proposed a differential equation model to describe the population dynamics of two interacting species and its "prey" (the Lotka-Volterra model) (**Equation 8**) (Chauvet *et al*., 2002).

$$\begin{cases} \frac{dx}{dt} = ax - bxy \\ \frac{dy}{dt} = -cy + dxy \end{cases} \quad (8)$$

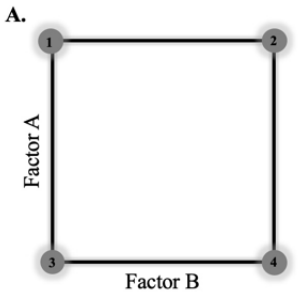
Where *y(t)* and *x(t)* are the predator and the prey populations, respectivelly, as functions of time, *a* is the natural growth rate of the prey in the absence of predators, *b* is the effet of predation on the prey, *c* is the natural death rate of the predator in the absence of prey, and *d* is the efficiency and propagation rate of the predator in the presence of prey.

Then, based on equation 2, Dens *et al.* (1999) and Vereecken *et al*. (2000) proposed to modify the deceleration function by including empirical parameters reflecting the degree of interaction between microbial species (F_{AB} and F_{BA}) for modelling the interaction of two bacterial species (**Equation 9**) (Cadavez *et al*., 2019; Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019; Liu *et al*., 2006).

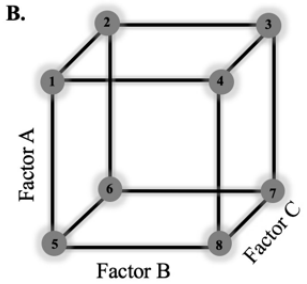
$$\begin{cases} \frac{1}{N_A(t)}\frac{dN_A(t)}{dt} = \mu_{max_{A(t)}}\, x\, \alpha_A(t)\, x\, \left(1 - \frac{N_A(t) + F_{AB}N_B(t)}{N_{\max A(t)}}\right) \\ \frac{1}{N_B(t)}\frac{dN_{Bt}}{dt} = \mu_{max_{B(t)}}\, x\, \alpha_B(t)\, x\, \left(1 - \frac{N_B(t) + F_{BA}\, N_A(t)}{N_{\max B(t)}}\right) \end{cases} \quad (9)$$

Where the parameters F_{AB} and F_{BA} are the coefficients of interaction measuring the effects of one species on the other.

Secondary models are then used to describe the influence of extrinsic (storage conditions) and intrinsic (food-specific characteristics) factors on primary growth parameters. These models can be single-factor, to study the influence of a factor on a variable, or multi-factorial, to study the influence of several factors on a variable. Complete factorial plans are preferred if factors have more than two levels (**Figure 12**). Moreover, environmental factors can be modelized according to two different approaches: (i) by a simultaneous approach (e.g. polynomial function), or (ii) by individual models, which are then combined to describe a global effect (e.g. gamma concept, square root or cardinal models).



Levels	Factors	
	A	B
1	+	-
2	+	+
3	-	-
4	-	+



Levels	Factors		
	A	B	C
1	+	-	-
2	+	-	+
3	+	+	+
4	+	+	-
5	-	-	-
6	-	-	+
7	-	+	+
8	-	+	-

Figure 12. Example of complete factorial plan for factors with two (A) and three (B) levels.

Among the empirical secondary models, reparametrized version of the square root models were used to assess the effects of temperature on the growth rates (**Equation 10**) (Ratkowsky *et al*., 1982). The growth rates were transformed by a square root to stabilize their variances. These models continue to be developed to obtain extensions.

$$\mu_{max} = \mu_{ref}\left(\frac{T-T_{min}}{T_{ref}-T_{min}}\right)^2 (10)$$

Where μ_{ref} is the reference growth rate obtained at T_{ref} = 20°C (1/hours), T is the temperature (°C) and T_{min} is the minimal temperature for growth (°C) found in the scientific literature for the studied bacterial species.

Validation is also a necessary step, since models must prove their predictive capabilities for complex foods.

Finally, tertiary models use expert systems and databases to establish relationships between primary and secondary models in an end-user interface (Psomas *et al*., 2011; Tamplin *et al*., 2018). As presented by Tenenhaus-Aziza and Ellouze (2015), several models are available. In this research, the ComBase predictive models software and Sym'Previus were used in experimental studies.

The ComBase predictive models software (https://www.combase.cc/) is a tertiary online model tool based on ComBase data to predict the growth or inactivation of microorganisms for studies of quantitative food microbiology. With its database completed by more than 50,000 records, ComBase provides predictive models to describe the dynamics (growth and inactivation) of several microorganisms in broth media or in different foods (Stavropoulou and Bezirtzoglou, 2019). The Predictor is based on the primary model of Baranyi and Roberts (1994) and a polynomial secondary model. On the other hand, Sym'Previus (https://symprevius.eu/fr) is a commercially available software that simulates the growth and inactivation of pathogens and spoilers in several food matrices in both static and dynamic conditions. Deterministic or probabilistic approaches can be used based on the software internal experimental data or on users' data (Tenenhaus-Aziza and Ellouze, 2015). Sym'Previus uses the model of Rosso *et al*. (1996) as the primary model and a cardinal type secondary model. However, although the number of models available is constantly increasing, only some of them take into account spoilage bacteria in meat and meat products. These kinds of models have been studied by da Silva *et al*. (2018), Dalcanton *et al*. (2013), Gospavic *et al*. (2008), Koutsoumanis (2009), Kreyenschmidt *et al*. (2010), Liu *et al*. (2006), Mataragas *et al*. (2006) and Zhang *et al*. (2011). Moreover, the majority of the models developed to predict the growth of SSOs in meat and meat products are based on the growth of two bacterial species in a food matrix (Giuffrida *et al*., 2009; Vereecken *et al*., 2000), most often to study the interaction between spoilage and pathogenic bacteria (Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019; Lebert *et al*., 2000; Mejlholm and Dalgaard, 2007; Pedrozo *et al*., 2019; Ye *et al*., 2014).

Therefore, it could be interesting to develop a model for two- or more- species, by considering the effect of multiple storage conditions and the natural food microbiota on the growth of specific spoilage bacteria. In the present manuscript, primary and secondary models for spoilage bacteria in WP and MP samples will be studied in order to explore predictive models with two and three spoilage species. In addition, validation steps are proposed for each of these experiments.

1.7. Highlights

- Food losses and waste are a worldwide major concern, representing annually 20% of the food production in Europe, which corresponds to around 345 kg of food wasted every year for a Belgian resident.
- These food losses and waste have many causes including bacterial food spoilage.
- Pork meat and meat products are widely produced and consumed, in Europe and in Belgium. Thus, two food matrices based on pork meat were selected for this research: minced meat and white pudding. These products are considered to be highly perishable foods, and therefore likely to be spoiled and thus thrown away.
- Bacterial food spoilage of minced pork has already been described, but that is not the case for white pudding. Moreover, bacterial food spoilage is a complex and not fully understood mechanism, largely depending on storage conditions, with several effects on food quality.
- Classical microbiology and metagenetics were mainly used to describe and follow the bacterial spoilage microbiota in meat and meat products. These methods could be used in a multi-omics approach, in association with metabolomics, in order to provide a more comprehensive analysis of the dynamics and metabolism in these products.
- Predictive microbiology is also an interesting tool to enhance the accuracy of growth predictions from microbial data obtained by culture-dependent and -independent methods. However, few models consider food spoilage bacteria, especially in models with interaction of two or more species.

Chapter 2
Objectives

The **general objective** of this work was to study and predict the dynamics of spoilage microbiota in minced pork and white pudding, according to different storage conditions.

The four principal topics of the present research with their **specific objectives** are presented in **Figure 13** and are described below.

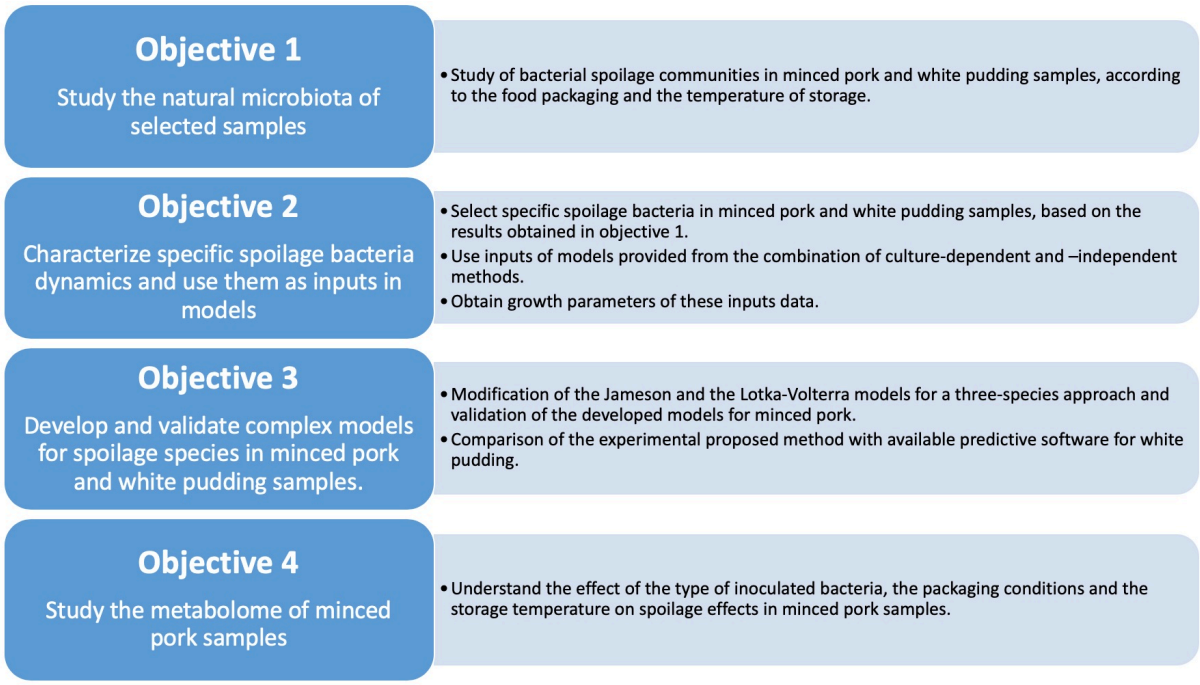


Figure 13. Summary of the objectives of this research work.

The first objective was to study the natural spoilage microbiota of minced pork and white pudding samples.

This topic aimed to describe the natural microbiota of samples according to the food packaging, as for food wrap and modified atmosphere, and to describe changes of microbial dynamics according to the temperature of storage, as isothermal and dynamics conditions.

The second objective was to characterize specific spoilage bacteria dynamics in selected food matrices, and use them as inputs in models.

Three specific objectives were performed in this topic.

The first was to select specific spoilage bacteria for the two food matrices, according to the previous objective.

The second specific objective was to use inputs of models provided from the combination of culture-dependent and culture-independent methods, in order to obtain estimate bacterial counts over time and storage, for mono-culture and co-culture experiments in samples.

And the last specific objective was to obtain growth parameters of these input data by fitting primary and secondary models (maximal bacterial growth, lag time, maximal and minimal bacterial populations, minimal to temperature for growth, time to reach the stationary phase and time to reach the spoilage-value level).

The third objectif of this research was to develop and validate complex models in <u>minced pork</u> and <u>white pudding</u> samples.

Predictive models are based on *B. thermosphacta*, *Pseudomonas* spp. (*Ps. fragi* and *Ps. fluorescens*) and *Ln. gelidum* for MP; and on *B. thermosphacta*, *Pseudomonas* sp. and *Psychrobacter* spp. for WP samples.

For minced pork, the specific objective was to develop a three species interaction model, based on Lotka-Volterra and modified Jameson effect models, for two packaging and three isothermal storage temperatures. And also to validate the new approach by naturally contaminated MP samples.

For white pudding, the specific objective was to develop a one-specie model with taking into account the natural microbiota of products, and to compare simulations with some available software;

The last objective was to study the metabolome in <u>minced pork</u> samples.

This topic aimed to understand the influence on spoilage effects in relation with the inoculated bacteria (*B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum*) in mono- and co-culture experiments, the packaging used, such as food wrap or modified atmosphere, and the temperature of storage, at 4°C, 8°C and 12°C.

Chapter 3
Experimental studies

Chapter 3 presents the experimental studies of this research for minced pork and white pudding samples. This chapter is divided into 6 scientific publications which correspond to the fourth objectives of this research (**Figure 14**).

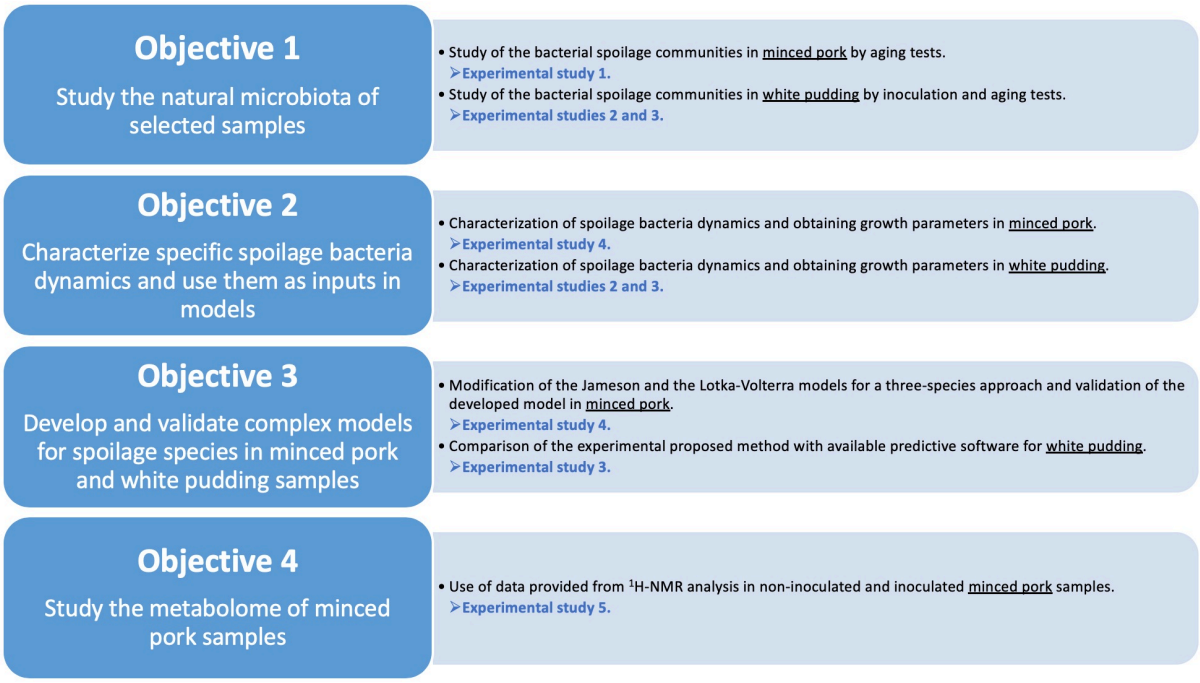


Figure 14. Summary of the objectives and the related experimental studies of this research work.

Experimental study 1 is dedicated to the description of natural microbiota in minced pork samples, and **studies 2 and 3** in white pudding (**Objective 1**).

Study 4 concerns the combination of culture-dependent and -independent methods in order to obtain bacterial dynamics and growth parameters in minced pork, and **experimental studies 2 and 3** in white pudding (**Objective 2**).

Moreover, **study 4** concerns the development of interaction models in minced pork samples, based on previous results described. A comparison between the experimental methods proposed with available predictive software, ComBase and Sym'Previus is also presented in **study 3** for white pudding (**Objective 3**).

Finally, **experimental study 5** is about study of the metabolome in minced pork samples, according to different storage conditions. This study is still ongoing, as not all the results have yet been reported by CIRM-CHU. A provisional and incomplete draft is therefore presented and will be completed as the work progresses (**Objective 4**).

Study 1

Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packed minced pork samples by 16S rDNA metagenetic analysis

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Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Abstract

⇒ **Objective 1.** Study the natural microbiota of minced pork

Although several studies have focused on the dynamics of bacterial food community, little is known about the variability of production batches and microbial changes that occur during storage. The aim of the study was to characterize the microbial spoilage community of MP samples, among different food production and storage, using both 16S rRNA gene sequencing and classical microbiology. Three batches of samples were obtained from four local Belgian facilities (A, B, C, D) and stored until shelf life under food wrap (FW) and MAP (CO_2 30%/O_2 70%), at constant and dynamic temperature. Analysis of 288 samples were performed by 16S rRNA gene sequencing in combination with counts of psychrophilic and LAB at 22°C. At the first day of storage, different psychrotolerant counts were observed between the four food companies (Kruskal-Wallis test, p-value<0.05). Results shown that lowest microbial counts were observed at the first day for industries D and A (4.2±0.4 and 5.6±0.1 log CFU/g, respectively), whereas industries B and C showed the highest results (7.5±0.4 and 7.2±0.4 log CFU/g). At the end of the shelf life, psychrophilic counts for all food companies was over 7.0 log CFU/g. With metagenetic, 48 operational taxonomic unit (OTU) were assigned. At the first day, the genus *Photobacterium* (86.7% and 19.9% for food industries A and C, respectively) and *Pseudomonas* (38.7% and 25.7% for food companies B and D, respectively) were dominant. During the storage, a total of 12 dominant genera (> 5% in relative abundance) were identified in MAP and 7 in FW. *Pseudomonas* was more present in FW and this genus was potentially replaced by *Brochothrix* in MAP (two-sided Welch's t-test, p-value<0.05). Also, a high Bray-Curtis dissimilarity in genus relative abundance was observed between food companies and batches. Although the bacteria consistently dominated the microbiota in our samples are known, results indicated that bacterial diversity needs to be addressed on the level of food companies, batches variation and food storage conditions. Present data illustrate that the combined approach provides complementary results on microbial dynamics in MP samples, considering batches and packaging variations.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Introduction

Meat and meat products are highly perishable, with colonization and development of a variety of microorganisms, especially bacteria. This is due to complex nutrient-rich environment with chemical and physical conditions favorable to bacterial development (Chaillou *et al*., 2015; Garnier *et al*., 2017; Nychas *et al*., 2008; Pennacchia *et al*., 2009). Moreover, minced meat can be contaminated by different types of microorganisms from several sources, such as raw materials, equipment, environment and handling involved in the production process. Abiotic factors (temperature, gaseous atmosphere, pH, NaCl levels, etc.) can also select certain bacteria (Mann *et al*., 2016; Rouger *et al*., 2018; Stellato *et al*., 2016). However, it is well known that richness and abundance of microbiota present in food products, and especially meats, play an important role in the microbial safety and the shelf life of the products (Pinu, 2016; Zhao *et al*., 2015). Microbial growth on meat to unacceptable levels and the various metabolic activities contribute to its deterioration by altering the structure, color and flavor of the meat (Mann *et al*., 2016). This leading to a reduction in food quality to the point of not being edible for human consumption (Holm *et al*., 2013; Silbande *et al*., 2016; Stellato *et al*., 2016), with alterations in the sensorial qualities of the product, particularly the aspect, with discoloration and gas production, and the presence of an off-odors and off-flavors (Stoops *et al*., 2015). Thus, food spoilage is problematic for two main reasons: first, it renders food unfit for human consumption and, secondly, it results in significant economic losses (Dalcanton *et al*., 2013; den Besten *et al*., 2017; Pinter *et al*., 2014).

As mentioned by Benson *et al*. (2014), the microbial population that colonizes and ultimately spoils minced pork is highly variable, depending on which groups of microbial taxa the product has been exposed to and perhaps even the order in which they are encountered. Using traditional cultivation methods, the microbial composition and diversity in fresh meat have been widely investigated (Zhao *et al*., 2015), but it is well known that traditional identification and culture-based methods for pathogens or food spoilage microbes are time-consuming (Pinu, 2016). Moreover, ecological studies at the genus-species level are required because the same storage conditions may affect differently the species in the same groups of bacteria (Pennacchia *et al*., 2011; Stoops *et al*., 2015), and because not all the members of this microbiota contribute to food spoilage. Several studies in meat microbiology have established that spoilage is caused only by a dominated fraction of the initial microbial association (Nychas *et al*., 2008). These spoilage microorganisms have been designated as Specific Spoilage Organisms (SSOs) (Benson *et al*., 2014; Zotta *et al*., 2019). Therefore, as discussed by De Filippis *et al*. (2013), the concept of succession of spoilage-related microbial groups is very important, and many studies have been

performed to investigate the dynamics and changes of the meat microbiota during storage.

Developed during the last decades, the next generation sequencing methodologies provide a powerful tool to study microbial community structure and composition shifts at different stages of ripening, allowing the detection of minor bacterial populations (Riquelme *et al*., 2015), at variable taxonomic depth (Chaillou *et al*., 2015; Parente *et al*., 2016; Pothakos *et al*., 2014). The introduction of molecular methods, especially culture-independent approaches, have contributed to the exploration of various food microbiota (Galimberti *et al*., 2015; Garofalo *et al*., 2017; Parlapani *et al*., 2018 ; Pinu, 2016), as for beverages (Elizaquivel *et al*., 2015), vegetables (Gu *et al*., 2018; Lee *et al*., 2017; Liu *et al*., 2019a), and for dairy (Ceugniez *et al*., 2017; Nalbantoglu *et al*., 2014; Porcellato *et al*., 2018; Riquelme *et al*., 2015), seafood (Li *et al*., 2018; Parlapani *et al*., 2018; Silbande *et al*., 2018), and meat products (Benson *et al*., 2014; Carrizosa *et al*., 2017; Cauchie *et al*., 2017; Cocolin *et al*., 2004; Delhalle *et al*., 2016; Greppi *et al*., 2015; Kaur *et al*., 2017; Korsak *et al*., 2017; Mann *et al*., 2016; Nieminen *et al*., 2012; Pennacchia *et al*., 2011; Peruzy *et al*., 2019a; Polka *et al*., 2015; Stoops *et al*., 2015; Vester Lauritzen *et al*., 2019 ; Zhao *et al*., 2015), in order to assess the microbial levels and diversity of food and food products (Lee *et al*., 2017; Nieminen *et al*., 2012; Pothakos *et al*., 2014 ; Rouger *et al*., 2018). The interest of this method to characterize the dominant spoilage bacteria in pork meat and meat products was also described (Andritsos *et al*., 2012; Li *et al*., 2019a; Mann *et al*., 2016; Peruzy *et al*., 2019a; Raimondi *et al*., 2018).

In this context, the aim of the present study was to assess the microbial spoilage community and dynamics of MP samples, among different conditions of production and food storage, using both 16S rRNA gene sequencing and classical microbiology.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Material and methods

2.1. Sampling

Fresh MP samples packed with a food wrap film were obtained from four local small and medium-sized Belgian manufacturers (food companies A, B, C and D) at the day of the production, corresponding to the day of slaughtering. Three batches for each manufacturer were used, with a one-week interval between sampling (**Figure 15**).

According to the recipe MP is composed of 100% minced pork (70% lean, 30% fat), no salt, no spices, no additives, no eggs and no sugar are added. At the day of the production, the water activity of this product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.05 (n = 12). pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Germany). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

MP samples were packed (100 g), in triplicate, in two different types of non-sterile packaging.

The first packaging concerns a tray (187x137x36, polyester 10 µm, homo-polymer polypropylene 50 µm, NutriPack, France) under MAP (CO_2 30% / O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France).

The second packaging concerns a tray (175x135x22, polystyrene) under FW packing using cling film (Clinofilm).

2.2. Food storage

According to the requirements for implementing microbiological tests of chilled perishable and highly perishable foodstuffs (AFNOR, NF V01-003, 2010), MP samples were stored during 3 days of shelf life under FW, and during 6 days under MAP, at constant and dynamic temperature: at (i) 2°C (± 1°C), (ii) 8°C (± 1°C), (iii) 12°C (± 1°C), and (iv) for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8°C ± 1°C), in climatic chambers (Sanyo MIR 254).

Samples were analyzed at the first day of inoculation (day 0) and at the last day of storage (day 3 in FW and day 6 in MAP, n=288).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

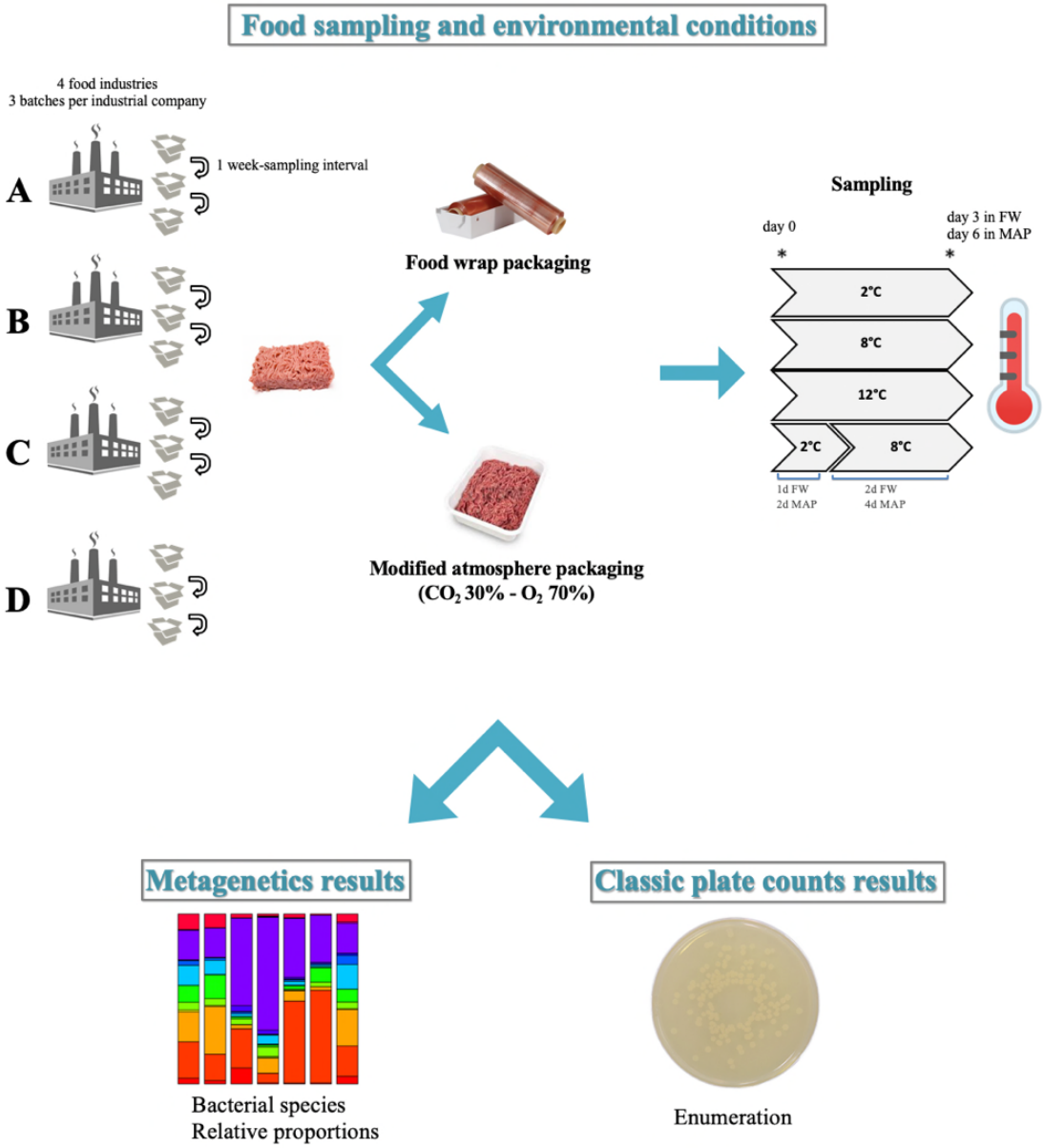

Figure 15. Schematic representation of the methods used.

2.3. Plate count enumeration

Twenty-five grams of product were randomly collected from the trays at the surface and at depth, without homogenization, and put into a Stomacher bag with a mesh screen liner (80 µm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10g/L peptone, 5g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (10 g/L peptone, 8.5 g/L sodium chloride, #CM0733,

Oxoid, Hampshire, England) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis (Spiral plater, DW Scientific, England). Total viable counts (TVC) for aerobic psychrophilic bacteria were performed on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France), and for LAB on de Man, Rogosa and Sharpe (MRS agar, #CM0361, Oxoid, Hampshire, England), after incubation at 22°C (Pothakos *et al*., 2014) for 72 h (model 1535 incubator, Shel Lab, Sheldon Manufacturing. Inc., USA).

2.4. DNA extraction and 16S rRNA gene sequencing

Bacterial DNA was extracted from each primary suspension, previously stored at – 80°C, using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV, Antwerp, Belgium) following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at – 20°C until used for 16S rRNA gene sequencing.

PCR-amplification of the V1-V3 region of the 16S rRNA gene library preparation were performed with the following primers (with Illumina overhand adapters), forward (5'-GAGAGTTTGATYMTGGCTCAG-3') and reverse (5'-ACCGCGGCTGCTGGCAC-3'). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 sec at 94°C), annealing (45 sec at 56°C) and extension (60 sec at 72°C), with a final elongation step (8 min at 72°C). These amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). After purification, PCR products were quantified using the Quanti-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, USA) and diluted to 10 ng/μL. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems, Wilmington, MA, USA) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina, San Diego, CA, USA).

2.5. Bioinformatics analysis

The 16S rRNA gene sequence reads were processed with MOTHUR (Schloss *et al*., 2009). The

quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS). The obtained read sets were compared to a reference data-set of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (http://www.arb-silva.de/, version v1.2.11) implemented in MOTHUR (Cauchie *et al*., 2017; Pothakos *et al*., 2014; Pruesse *et al*., 2012). The final reads were clustered into OTUs, using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA data-set 111, using a BLASTN algorithm. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (< 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Cauchie *et al*., 2017; Delcenserie *et al*., 2014).

2.6. 16S rRNA gene data analysis

A correcting factor for 16S rRNA copy numbers was applied for any taxon *i* (**Equation 11**) (Kembel *et al*., 2012; Louca *et al*., 2018).

$$A_i = {}^{N_k}/_{C_i} \quad (11)$$

Where A_i is the real abundance of 16S genes from the taxon in the sample, N_k is the number of reads for the taxon in the sample *k*, and C_i is determined by the genomic 16S copy number of that taxon. To obtain each gene copy number, Ribosomal RNA Database (rrnDB) (Stoddard *et al*., 2015) and EzBioCloud database (Yoon *et al*., 2017) were used.

Then, to compare the relative abundance of OTUs, the number of reads of each taxon were normalized (Nr_i) as described by Chaillou *et al*. (2015). Reads counts of each taxon *i* in the sample *k* were divided by a sample-specific scaling factor *(Si)* (**Equation 12**) (Fougy *et al*., 2016; Rouger *et al*., 2018):

$$Nr_i = {}^{A_i}/_{S_k} \quad (12)$$

Where A_i is the real abundance of 16S genes from that taxon obtained with a correcting factor for 16S rRNA copy numbers, S_k is the normalization factor associated with sample *k*.

The sample-specific scaling factor was calculated by (**Equation 13**):

$$S_k = {}^{T_k}/_{m_e} \quad (13)$$

Where S_k is the sample-specific scaling factor associated with sample *k*, T_k is the number of total reads in the sample *k*, m_e is the median value of total reads for all the samples of the dataset. Reads counts of

all samples were then transformed into a percentage of each OTU.

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de BioProject ID PRJNA551357. The raw data supporting the conclusions of this manuscript will be made available by EC to any qualified researcher.

2.7. Statistical analysis

2.7.1. Statistical analysis on microbiological results

Nonparametric statistical tests were used to compare the classical microbiology results between samples taken on the day of production and at the end of shelf life for a same temperature. With the help of R software (R Core Team, 2016), Kruskal-Wallis test was performed to make a comparison between the food industries on a certain day (i.e.; day 0 or day 3) (stats package, kruskal.test function). An Analysis of Covariance (ANCOVA) was also performed to evaluate the interactions between the storage conditions and the food origin on psychrophilic counts (FactoMineR package, AovSum function). All tests were considered as significant for a p-value < 0.05.

2.7.2. Statistical analysis on 16S rRNA gene results

Alpha diversity for each sample was evaluated by richness estimation (Chao1 estimator), microbial biodiversity (inverse of the Simpson index, coverage), and the population evenness (Simpson evenness) using MOTHUR (version 1.40.5) (http://www.mothur.org) (Riquelme *et al*., 2015; Zhao *et al*., 2015). Rarefaction curves were calculated for all samples to ensure that sequencing depth was sufficient: OTUs identified were plotted as a function of sequences obtained per sample. High diversity coverage was achieved with all curves reaching asymptotes from 3000 reads (**Figure 16**). Using Explicet, alpha and beta diversity indices were also calculated with bootstrapped sequencing data (http://www.explicet.org) (Mann *et al*., 2016; Robertson *et al*., 2013). Beta-diversity was assessed with Explicet using the Bray-Curtis index on a 0-1 scale. Using STAMP (v2+) software (http://www.kiwi.cs.dal.ca/Software/STAMP), a 2-sided Welch's *t*-test was performed on metagenetic results and confidence intervals were calculated according to the Newcombe-Wilson method. A Principal Component Analysis (PCoA) was also applied to classify and cluster samples according to the identified OTUs for the two packaging (Tukey-Kramer test in conjunction with an ANOVA) (Parlapani *et al*., 2018). The differences were considered significant for a corrected p-value less than 0.05 (Parks *et al*., 2014).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

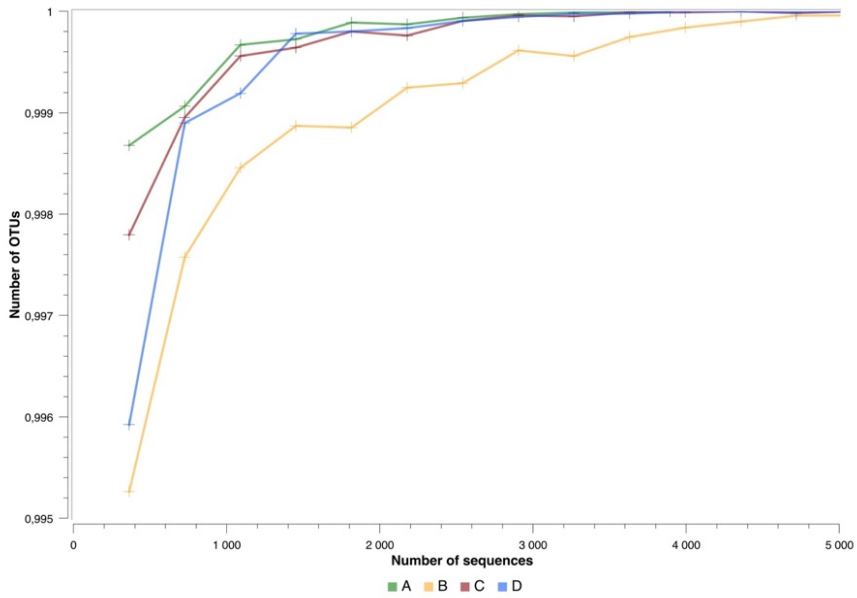

Figure 16. Rarefaction curves for all samples (food companies A, B, C and D), based on an OTU definition of 97% similarity (0.03 16S rRNA distance).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Results

3.1. Microbiological analysis

As expected, psychrophilic and lactic aerobic counts increased during the shelf life with increasing the temperature (**Tables 12 and 13**).

Compared to the TVC values, LAB counts showed highest results for food industries A and D.

At day 0, different microbiological counts were observed between food companies for TVC (Kruskal-Wallis test, H =9.43, p-value = 0.02) and for LAB (Kruskal-Wallis tests, H = 8.90, p-value = 0.04). The lowest psychrophilic populations were observed for food industries D (4.2 ± 0.4 log CFU/g) and A (5.6 ± 0.1 log CFU/g), whereas MP samples from B and C showed the highest results (7.5 ± 0.4 log CFU/g and 7.2 ± 0.4 log CFU/g, respectively).

At the end of the shelf life, the natural logarithm of the TVC for all food companies was over 7.0 log CFU/g. At this time, the Analysis of Covariance revealed also a significant effect of the food companies (p-value = 0.00000998) and the temperature of storage (p-value = 0.00000095) on microbial total counts. Psychrotolerant counts seemed also to be influenced by the interaction of the food industry and the temperature (p-value = 000442), but not by other interactions terms (p-value > 0.05).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Table 12. Results of psychrophilic aerobic counts in minced pork samples according to the origin, the food packaging and the temperature of storage. Values given as log CFU/g (mean ± SD, n=3) at 2°C, 8°C, 12°C and 2/8°C. ° significant Kruskal-Wallis value ($p<0.05$) with p-value between bracket; * significant Wilcoxon value ($p<0.05$).

Industries/packaging	Day 0	End of the shelf life			
		2°C	8°C	12°C	2/8°C
FW					
A	5.6 ± 0.1	6.5 ± 0.6	8.3 ± 0.4*	8.3 ± 0.5*	8.3 ± 0.3*
B	7.5 ± 0.4	7.5 ± 0.4	8.3 ± 0.0*	8.3 ± 0.2*	8.3 ± 0.9*
C	7.2 ± 0.4	7.3 ± 0.5	7.8 ± 0.0	7.8 ± 0.2	7.6 ± 1.3
D	4.2 ± 0.4	4.6 ± 0.2	7.2 ± 0.2*	8.3 ± 0.0*	6.6 ± 0.2*
Kruskal-Wallis test	9.43 (0.02)°	8.74 (0.03)°	9.02 (0.03)°	5.71 (0.13)	9.68 (0.02)°
MAP					
A	5.6 ± 0.1	6.5 ± 0.1*	7.9 ± 0.1*	8.3 ± 0.3*	7.9 ± 0.2*
B	7.5 ± 0.4	7.9 ± 0.1	8.3 ± 0.0*	8.3 ± 0.0*	8.3 ± 0.0*
C	7.2 ± 0.4	7.5 ± 0.2	7.6 ± 0.1	8.3 ± 0.1*	7.8 ± 0.6
D	4.2 ± 0.4	5.2 ± 0.3*	7.9 ± 0.1*	8.1 ± 0.1*	7.2 ± 0.1*
Kruskal-Wallis test	9.43 (0.02)°	10.39 (0.02)°	9.68 (0.02)°	3.45 (0.33)	8.94 (0.03)°

Table 13. Results of lactic aerobic counts in minced pork samples according to the origin, the food packaging and the temperature of storage. Values given as log CFU/g (mean ± SD, n=3) at 2°C, 8°C, 12°C and 2/8°C. ° significant Kruskal-Wallis value ($p<0.05$) with p-value between bracket; * significant t-student value ($p<0.05$).

Industries/packaging	Day 0	End of the shelf life			
		2°C	8°C	12°C	2/8°C
FW					
A	5.2 ± 0.2	6.4 ± 0.4	7.8 ± 0.1*	7.8 ± 0.2*	7.4 ± 0.2*
B	5.5 ± 0.6	5.5 ± 0.5	7.1 ± 0.3*	7.9 ± 0.2*	6.8 ± 0.4*
C	5.2 ± 0.7	6.7 ± 0.2*	7.4 ± 0.1*	7.6 ± 0.1*	7.0 ± 0.2*
D	3.5 ± 0.2	4.4 ± 0.3*	5.9 ± 0.4*	7.5 ± 0.1*	5.1 ± 0.3*
Kruskal-Wallis test	8.90 (0.04)°	9.15 (0.03)°	9.67 (0.02)°	7.62 (0.05)	8.44 (0.04)°
MAP					
A	5.2 ± 0.2	7.1 ± 0.2*	8.0 ± 0.18*	8.2 ± 0.09*	8.2 ± 0.09*
B	5.5 ± 0.6	6.6 ± 0.6*	7.8 ± 0.21*	7.7 ± 0.16*	7.8 ± 0.15*
C	5.2 ± 0.7	7.3 ± 0.2*	7.6 ± 0.06*	7.9 ± 0.09*	7.5 ± 0.07*
D	3.5 ± 0.2	5.2 ± 0.4*	7.5 ± 0.07*	7.8 ± 0.03*	6.8 ± 0.24*
Kruskal-Wallis test	8.90 (0.04)°	8.44 (0.04)°	9.05 (0.03)°	8.27 (0.04)°	9.45 (0.02)°

3.2. Carbon dioxide production

As shown in **Figure 17**, carbon dioxide values increased with highest temperatures, except for the food companies C and D which showed relatively stable measurements. Results at 2/8°C are not shown in this paper.

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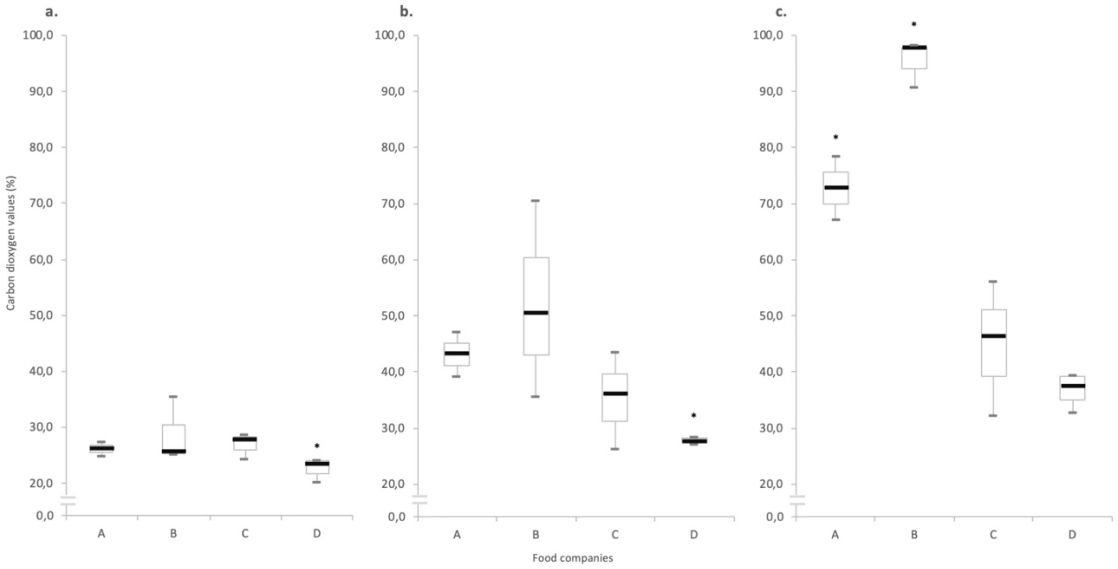

Figure 17. Box plots show the carbon dioxide measurements at the end of the shelf life, for the four food companies (A, B, C and D) at **(A)** 2°C, **(B)** 8°C, and **(C)** 12°C. The boxes represent the interquartile range between the first (Q1) and the third (Q3) quartiles; the vertical black line insides the box is the median obtained from the three batches analyzed by food industries; the two dotted line is the difference of 25% below the Q1 or above the Q3. The presence of stars indicated that samples deviated significantly from the carbon dioxide value at day 0 (30.0 ± 0.1%).

3.3. Alpha diversity of bacteria with 16S rDNA amplicon sequencing

Over 4,200 reads per sample were generated with 16S rDNA amplicon pyrosequencing. In total, 48 mains OTUs were assigned. The number of OTUs, the bacterial diversity, richness estimators and coverage are presented in Supplementary Material (**Tables 14, 15 and 16**). The highest number of identified species was encountered for the food industries C and D.

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Table 14. Alpha diversity from metagenetic analysis at day 0. Food companies (A, B, C, D), with three batches each (1, 2, 3).

Samples	No. of OTUs	Coverage (%)	Inv. Simpson	Chao richness	Simpson evenness
A1	8	99.98	1.33	8.00	0.17
A2	7	99.98	1.12	7.00	0.16
A3	6	99.98	1.25	6.00	0.21
B1	3	98.96	1.84	3.00	0.61
B2	5	98.86	1.30	5.00	0.26
B3	5	98.95	2.32	5.00	0.46
C1	8	97.80	2.18	8.50	0.27
C2	9	97.62	5.86	9.50	0.65
C3	4	98.98	1.29	4.00	0.32
D1	5	100.00	1.45	5.00	0.29
D2	11	94.06	4.80	26.00	0.44
D3	15	93.00	4.90	36.00	0.33

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Table 15. Alpha diversity from metagenetic analysis at the end of the shelf life in FW. Food companies (A, B, C, D), three batches each (1, 2, 3). At different temperature of storage: 2°C (a), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (b), 8°C (c), and 12°C (d).

Samples	No. of OTUs	Coverage (%)	Inv. Simpson	Chao richness	Simpson evenness
A1 a	8	100.00	3.18	8.00	0.40
A1 b	7	100.00	2.35	7.00	0.34
A1 c	8	100.00	1.98	8.00	0.25
A1 d	8	100.00	1.13	7.00	0.24
A2 a	7	99.95	1.23	7.50	0.18
A2 b	8	99.98	1.88	8.00	0.23
A2 c	7	100.00	2.33	7.00	0.33
A2 d	9	100.00	2.25	9.00	0.25
A3 a	5	99.98	1.05	5.00	0.21
A3 b	7	99.98	1.21	7.00	0.17
A3 c	6	100.00	1.36	6.00	0.23
A3 d	7	99.95	2.67	8.00	0.38
B1 a	3	98.95	2.06	3.00	0.69
B1 b	3	98.95	1.94	3.00	0.65
B1 c	4	97.98	2.01	5.00	0.50
B1 d	4	98.96	2.15	4.00	0.54
B2 a	3	100.00	2.68	3.00	0.89
B2 b	4	100.00	2.56	4.00	0.64
B2 c	7	97.78	3.00	8.00	0.43
B2 d	5	100.00	2.02	5.00	0.40
B3 a	3	100.00	1.26	3.00	0.42
B3 b	4	98.81	1.59	4.00	0.40
B3 c	6	97.80	1.85	6.50	0.31
B3 d	4	98.85	1.13	4.00	0.28
C1 a	3	98.96	1.14	3.00	0.38
C1 b	3	97.89	1.04	4.00	0.35
C1 c	3	97.94	1.04	4.00	0.35
C1 d	4	98.96	1.36	4.00	0.34
C2 a	7	95.60	1.50	13.00	0.21
C2 b	6	97.62	1.22	6.25	0.20
C2 c	5	96.47	1.13	6.50	0.23
C2 d	8	97.96	2.23	9.00	0.28
C3 a	3	97.85	1.04	4.00	0.35
C3 b	4	98.94	1.17	4.00	0.29
C3 c	4	97.87	1.14	5.00	0.29
C3 d	9	95.70	2.55	11.00	0.28
D1 a	5	97.85	1.38	6.00	0.28
D1 b	4	96.59	1.07	7.00	0.27
D1 c	5	97.80	1.29	5.50	0.26
D1 d	11	95.74	3.30	13.00	0.30
D2 a	4	97.85	2.00	5.00	0.50
D2 b	3	100.00	1.85	3.00	0.62
D2 c	4	100.00	1.59	4.00	0.40
D2 d	5	98.96	1.87	5.00	0.37
D3 a	7	95.79	2.59	13.00	0.37
D3 b	4	98.98	1.59	4.00	0.40
D3 c	4	100.00	1.59	4.00	0.40
D3 d	5	98.90	1.72	5.00	0.34

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Table 16. Alpha diversity from metagenetic analysis at the end of the shelf life in MAP. A, B, C and D, food companies; three batches each (1, 2, 3). At different temperature of storage: 2°C (a), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (b), 8°C (c), and 12°C (d).

Samples	No. of OTUs	Coverage (%)	Inv. Simpson	Chao richness	Simpson evenness
A1 a	8	100.00	2.52	8.00	0.32
A1 b	6	100.00	1.92	6.00	0.32
A1 c	9	100.00	1.74	9.00	0.19
A1 d	8	99.98	2.61	8.00	0.33
A2 a	8	100.00	1.25	8.00	0.16
A2 b	9	99.95	1.94	10.00	0.22
A2 c	8	100.00	2.55	8.00	0.32
A2 d	9	99.98	2.17	9.00	0.24
A3 a	7	100.00	1.40	7.00	0.20
A3 b	7	99.95	1.11	8.00	0.16
A3 c	7	100.00	1.09	7.00	0.16
A3 d	6	99.98	1.37	6.00	0.23
B1 a	4	97.85	1.42	5.00	0.35
B1 b	4	97.87	1.14	5.00	0.29
B1 c	7	97.98	1.27	7.25	0.18
B1 d	5	97.92	1.14	5.33	0.23
B2 a	6	98.91	2.89	6.00	0.48
B2 b	5	98.90	2.10	5.00	0.42
B2 c	5	100.00	2.56	5.00	0.51
B2 d	7	97.96	2.08	7.33	0.30
B3 a	5	98.98	2.53	5.00	0.51
B3 b	5	98.97	2.18	5.00	0.44
B3 c	6	96.74	2.70	9.00	0.45
B3 d	9	97.96	3.66	9.50	0.41
C1 a	4	98.95	1.11	4.00	0.28
C1 b	4	97.92	2.03	5.00	0.51
C1 c	4	98.96	1.59	4.00	0.40
C1 d	5	97.96	2.56	6.00	0.51
C2 a	5	98.96	3.22	5.00	0.64
C2 b	8	97.78	4.06	8.33	0.51
C2 c	5	98.95	1.36	5.00	0.27
C2 d	3	98.77	1.22	3.00	0.41
C3 a	2	98.96	1.02	2.00	0.51
C3 b	3	98.98	1.09	3.00	0.36
C3 c	3	98.97	1.11	3.00	0.37
C3 d	10	94.81	4.95	16.00	0.50
D1 a	4	100.00	1.41	4.00	0.35
D1 b	5	97.87	1.37	6.00	0.27
D1 c	6	96.91	1.71	9.00	0.28
D1 d	4	98.95	1.88	4.00	0.47
D2 a	7	97.94	2.44	8.00	0.35
D2 b	5	100.00	1.99	5.00	0.40
D2 c	5	98.96	1.83	5.00	0.37
D2 d	5	100.00	1.49	5.00	0.30
D3 a	4	98.90	1.17	4.00	0.29
D3 b	4	98.91	1.12	4.00	0.28
D3 c	4	98.99	1.43	4.00	0.36
D3 d	8	98.90	2.84	8.00	0.35

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3.4. Bacterial communities at the family and genus levels

The relative abundance results obtained by metagenetic analysis (expressed in %) in FW and MAP at family (**Figure 18**) and genus (**Figure 19**) levels (>5%) are represented in cumulated histograms for all samples. These data including the relative abundance of sequences are also summarized in Supplemental Material (**Tables 17, 18 and 19**). The taxa representing <5% in relative abundance were merged in the category of "Others". “Others” in FW are mainly composed by the genera *Bacillus*, *Carnobacterium*, *Enterococcus*, *Hafnia*, *Myroides*, *Rahnella*, *Staphylococcus*, *Serratia*, *Streptococcus*, *Weissella* and *Xanthomonas*. While it concerns *Bacillus*, *Carnobacterium*, *Enterococcus*, *Hafnia*, *Rahnella*, *Staphylococcus*, *Streptococcus* and *Xanthomonas* in MAP. Full data on taxa found in high (>5%) and low (<5%) frequencies will be made available by EC to any qualified researcher.

According to Figures 18 and 19, the food companies show a high variability in the distribution of read percentages at day 0. At this time, the genus *Photobacterium* is the most represented for A and C (86.7% and 19.9%, respectively), while it concerns the genus *Pseudomonas* for the industries B and D (38.7% and 25.7%, respectively).

At the end of the shelf life, a total of 12 genera were identified as dominant (taxa representing more than 5% in relative abundance) in MAP and only 7 genera in FW. These seven genera are all identical to those found in MAP.

For all samples, the percentage of “unassigned” reads was relatively low (7.1 ± 3.7).

Table 17. Distribution of metagenetic read percentages at genus level for each food companies, at day 0. At genus levels, the taxa representing <5% in relative abundance were merged in the category of “Others”.

Samples	*Aeromonas*	*Brochothrix*	*Carnobacterium*	*Chryseobacterium*	*Fusobacterium*	*Lactococcus*	*Leuconostoc*	*Photobacterium*	*Pseudomonas*	*Rhodococcus*	Others
A	0.00	1.35	0.00	0.00	0.00	0.00	0.00	86.77	0.00	0.00	11.88
B	0.00	5.20	0.00	0.00	0.00	0.00	0.00	0.00	38.71	0.00	56.09
C	0.00	1.76	10.08	0.00	0.00	9.50	6.85	19.98	0.00	0.00	51.84
D	6.45	6.03	0.00	8.25	13.47	0.00	0.00	0.00	25.67	11.27	28.86

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Table 18. Distribution of metagenetic read percentages at genus level during cold storage of MP in FW. At genus levels, the taxa representing <5% in relative abundance were merged in the category of "Others". A, B, C and D, food companies; three batches each (1, 2, 3). At different storage temperature: 2°C (a), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (b), 8°C (c), and 12°C (d).

Samples	*Acinetobacter*	*Brochothrix*	*Lactobacillus*	*Lactococcus*	*Leuconostoc*	*Photobacterium*	*Pseudomonas*	Others
A1 a	0.77	2.32	0.21	0.00	0.02	78.54	8.63	9.51
A1 b	5.56	15.66	0.15	0.00	0.08	40.25	32.24	6.06
A1 c	2.81	58.26	0.27	0.00	0.10	18.56	15.43	4.57
A1 d	5.43	63.23	0.30	0.33	0.12	1.92	22.30	6.37
A2 a	0.02	4.24	0.04	0.11	0.00	84.76	4.24	6.59
A2 b	0.11	8.39	0.07	0.18	0.07	66.04	8.39	16.76
A2 c	0.00	15.81	0.41	0.11	0.07	22.91	15.81	44.88
A2 d	0.05	20.33	0.17	0.07	0.07	17.67	20.33	41.30
A3 a	0.00	1.52	0.56	0.00	0.02	94.94	0.29	2.66
A3 b	0.00	5.97	1.81	0.18	0.02	87.33	0.71	3.99
A3 c	0.00	10.55	2.12	0.09	0.00	81.40	1.61	4.23
A3 d	0.00	16.79	0.29	0.23	0.03	36.72	40.32	5.62
B1 a	0.34	44.47	0.09	0.04	0.04	0.69	49.57	4.76
B1 b	0.31	58.73	0.13	0.20	0.33	1.40	34.99	3.90
B1 c	0.54	59.15	0.07	0.07	0.02	1.32	38.22	0.60
B1 d	0.62	45.72	0.18	0.13	0.18	2.02	47.33	3.82
B2 a	0.36	15.70	0.10	0.10	0.36	29.40	42.62	11.37
B2 b	3.59	22.00	0.25	0.22	0.05	15.03	52.21	6.65
B2 c	4.49	24.16	0.61	0.08	0.15	11.47	45.33	13.71
B2 d	1.81	58.23	0.02	0.15	0.05	23.54	3.23	12.96
B3 a	0.19	2.84	0.11	0.06	0.38	6.15	72.03	18.25
B3 b	0.49	3.30	0.21	0.15	0.83	15.47	65.25	14.30
B3 c	1.93	2.56	0.53	0.10	0.99	20.02	63.83	10.03
B3 d	1.40	2.19	0.24	0.02	0.39	1.77	81.54	12.44
C1 a	0.06	0.32	4.60	0.06	0.02	90.25	0.58	4.11
C1 b	0.04	0.42	0.94	0.15	0.06	92.66	0.69	5.04
C1 c	0.15	0.24	0.76	0.02	0.02	94.96	0.65	3.19
C1 d	0.02	1.16	82.22	0.07	7.11	6.13	0.31	3.00
C2 a	0.02	1.05	6.29	6.97	0.33	73.80	0.84	10.71
C2 b	0.18	1.63	2.49	1.16	0.51	76.34	1.70	16.00
C2 c	0.02	0.95	0.91	0.81	0.08	80.35	2.04	14.84
C2 d	0.03	1.06	5.26	2.86	0.31	63.43	17.52	9.53
C3 a	0.00	0.33	0.06	1.00	0.00	90.86	1.21	6.43
C3 b	0.00	0.62	0.27	3.74	0.00	86.66	1.98	6.73
C3 c	0.06	0.22	0.45	4.20	0.13	87.88	0.97	6.08
C3 d	0.12	1.07	0.92	3.73	0.12	32.10	49.12	12.82
D1 a	1.46	5.96	0.06	0.13	0.04	6.28	79.14	6.91
D1 b	0.13	1.43	0.02	0.11	0.04	0.77	85.27	12.22
D1 c	1.00	7.36	0.04	0.10	0.02	1.58	79.72	10.18
D1 d	1.01	37.49	3.17	2.24	3.05	6.81	36.19	10.05
D2 a	34.72	56.00	0.11	0.11	0.13	0.53	1.40	6.99
D2 b	0.11	2.01	0.06	0.30	0.37	60.81	27.75	8.59
D2 c	0.02	3.31	0.09	1.52	0.32	72.71	15.76	6.27
D2 d	0.02	1.69	0.10	15.93	0.80	9.05	68.10	4.30
D3 a	0.00	0.33	0.06	1.00	0.00	90.96	1.21	6.43
D3 b	0.00	0.62	0.27	3.74	0.00	86.66	1.98	6.73
D3 c	0.06	0.22	0.45	4.20	0.13	87.88	0.97	6.08
D3 d	0.12	1.07	0.92	3.73	0.12	32.10	49.12	12.82

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Table 19. Distribution of metagenetic read percentages at genus level during cold storage of MP in MAP. At genus levels, the taxa representing <5% in relative abundance were merged in the category of "Others". A, B, C and D, food companies; three batches each (1, 2, 3). At different storage temperature: 2°C (a), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (b), 8°C (c), and 12°C (d).

Samples	*Acinetobacter*	*Brochothrix*	*Enterobacter*	*Lactobacillus*	*Lactococcus*	*Leuconostoc*	*Myroides*	*Photobacterium*	*Pseudomonas*	*Serratia*	*Weissella*	Others
A1 a	0.21	43.75	0.00	4.93	0.05	1.44	0.00	40.09	3.95	0.00	0.00	5.25
A1 b	0.08	58.92	0.00	2.06	0.00	0.59	0.00	28.89	0.47	0.00	0.00	8.99
A1 c	2.30	21.05	0.00	0.97	0.13	0.56	0.00	66.71	0.35	0.00	0.00	7.74
A1 d	22.06	52.19	0.00	2.02	0.02	1.02	0.00	18.55	0.37	0.00	0.00	3.69
A2 a	0.00	3.23	0.00	1.74	0.31	0.38	0.00	85.12	0.33	0.00	4.16	4.64
A2 b	0.02	18.31	0.00	0.17	0.07	3.11	0.00	68.27	0.37	0.00	9.12	0.52
A2 c	0.00	12.38	0.00	0.43	0.06	12.95	0.00	55.43	0.23	0.00	12.78	5.53
A2 d	2.40	57.40	0.00	0.42	0.03	1.19	0.00	34.41	0.32	0.00	2.43	1.23
A3 a	0.00	3.23	0.00	11.52	0.27	1.63	0.00	77.79	0.16	0.00	0.00	5.23
A3 b	0.00	2.53	0.00	1.60	0.07	0.72	0.00	91.85	0.02	0.00	0.00	3.19
A3 c	0.00	0.87	0.00	2.79	0.09	0.26	0.00	93.48	0.07	0.00	0.00	2.37
A3 d	0.00	13.91	0.00	1.25	0.07	0.18	0.00	81.58	0.02	0.00	0.00	2.99
B1 a	0.04	77.10	0.00	0.00	0.10	0.08	0.00	0.72	14.18	0.02	0.00	6.77
B1 b	0.13	87.70	0.00	0.15	0.21	0.92	0.00	0.61	3.96	0.10	0.06	5.94
B1 c	1.56	87.60	0.00	0.65	0.26	1.62	0.00	3.03	1.64	0.73	0.00	2.83
B1 d	1.98	90.02	0.00	0.41	0.18	0.46	0.00	0.68	1.47	2.20	0.00	2.25
B2 a	2.63	44.51	0.00	0.60	0.37	0.35	28.26	1.96	13.29	0.22	0.00	7.01
B2 b	2.57	55.77	0.00	0.30	0.00	0.27	0.05	29.19	2.30	0.99	0.00	8.21
B2 c	26.03	52.63	0.00	0.48	0.03	0.08	0.14	12.51	1.89	1.80	0.00	3.31
B2 d	63.24	25.60	0.00	0.27	0.03	0.09	3.38	0.39	2.40	1.99	0.00	0.90
B3 a	0.03	4.67	0.00	39.74	0.00	46.66	0.00	0.60	5.21	0.00	0.00	2.52
B3 b	0.05	1.31	0.00	10.99	0.00	62.29	0.00	19.57	2.67	0.08	0.00	2.54
B3 c	32.16	1.45	0.00	1.18	0.05	11.56	0.00	44.57	0.93	0.35	0.00	7.38
B3 d	47.49	5.56	0.00	7.06	0.19	13.60	0.00	8.61	2.16	11.25	0.00	1.56
C1 a	0.04	77.10	0.00	0.00	0.10	0.08	0.00	0.72	14.18	0.02	0.00	6.77
C1 b	0.13	87.70	0.00	0.15	0.21	0.92	0.00	0.61	3.96	0.10	0.06	5.94
C1 c	1.56	87.60	0.00	0.65	0.26	1.62	0.00	3.03	1.64	0.73	0.00	2.83
C1 d	1.98	90.02	0.00	0.41	0.18	0.46	0.00	0.68	1.47	2.20	0.00	2.25
C2 a	0.06	0.21	0.37	15.56	29.44	42.07	0.00	8.25	0.00	0.03	0.00	2.59
C2 b	0.00	0.36	1.13	30.15	18.85	27.30	0.00	2.40	0.16	0.58	0.00	9.48
C2 c	0.03	0.15	0.55	8.96	2.32	2.11	0.00	80.62	0.03	0.10	0.00	4.35
C2 d	0.03	0.43	0.06	6.76	1.30	0.35	0.00	72.89	0.00	0.00	0.00	17.54
C3 a	0.00	0.09	0.00	0.42	0.94	0.02	0.00	95.03	0.00	0.00	0.00	3.50
C3 b	0.00	0.05	0.00	1.12	2.88	0.11	0.00	94.04	0.00	0.00	0.00	1.71
C3 c	0.00	0.15	0.13	0.69	3.58	0.08	0.00	91.61	0.05	0.00	0.00	3.41
C3 d	0.81	1.49	8.59	11.51	9.68	0.60	0.00	29.12	0.55	4.75	0.00	24.06
D1 a	0.40	11.96	0.00	0.15	0.30	0.02	0.00	1.66	80.04	0.30	0.00	3.16
D1 b	0.05	80.42	0.00	1.19	0.38	8.49	0.00	0.28	4.34	0.00	0.00	3.57
D1 c	0.05	71.83	0.00	0.19	2.99	0.99	0.00	19.27	1.11	0.00	0.00	2.87
D1 d	13.51	66.96	0.00	0.20	0.11	0.31	0.00	1.00	13.14	0.02	0.00	4.31
D2 a	0.20	7.77	0.00	8.68	8.14	60.50	0.00	0.25	0.84	0.00	9.77	2.08
D2 b	0.05	2.45	0.00	1.53	26.90	62.02	0.00	1.55	0.18	0.00	0.05	5.19
D2 c	0.00	8.29	0.00	0.26	69.19	16.21	0.00	1.96	0.14	0.00	0.00	3.33
D2 d	0.00	4.47	0.00	1.94	78.91	7.77	0.00	4.08	0.07	0.00	0.02	2.60
D3 a	0.13	84.17	0.00	3.04	0.09	2.65	0.00	0.24	1.43	0.02	0.00	7.85
D3 b	0.09	87.19	0.00	1.12	0.15	1.78	0.00	1.72	0.26	0.02	0.00	7.52
D3 c	0.15	81.60	0.13	0.40	2.82	1.06	0.00	12.88	0.08	0.03	0.00	0.54
D3 d	16.00	51.43	2.04	5.61	4.83	0.37	0.00	6.15	1.02	4.32	0.00	7.99

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

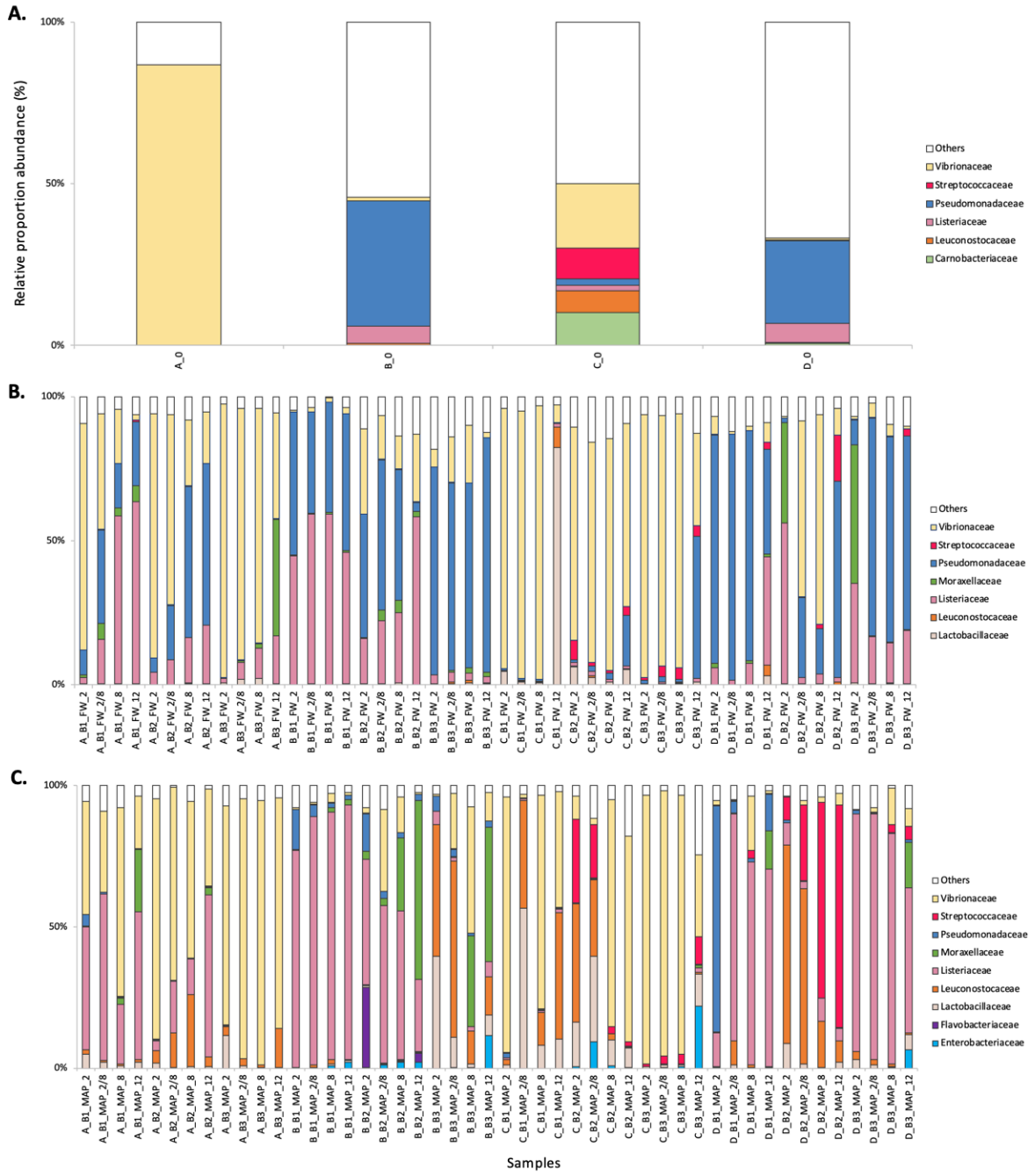

Figure 18. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetic at Family levels, during cold storage of minced pork in relation to the food packaging and the origin of samples (food companies and batches). **(A)** food samples analyzed at day 0 for the four companies (A, B, C and D), **(B)** storage in FW, **(C)** storage in MAP. At Family levels, the taxa representing <5% in relative abundance were merged in the category of "Others". Legend: batch 1 (B1), batch 2 (B2), batch 3 (B3), at 2°C (2), at 8°C (8), at 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

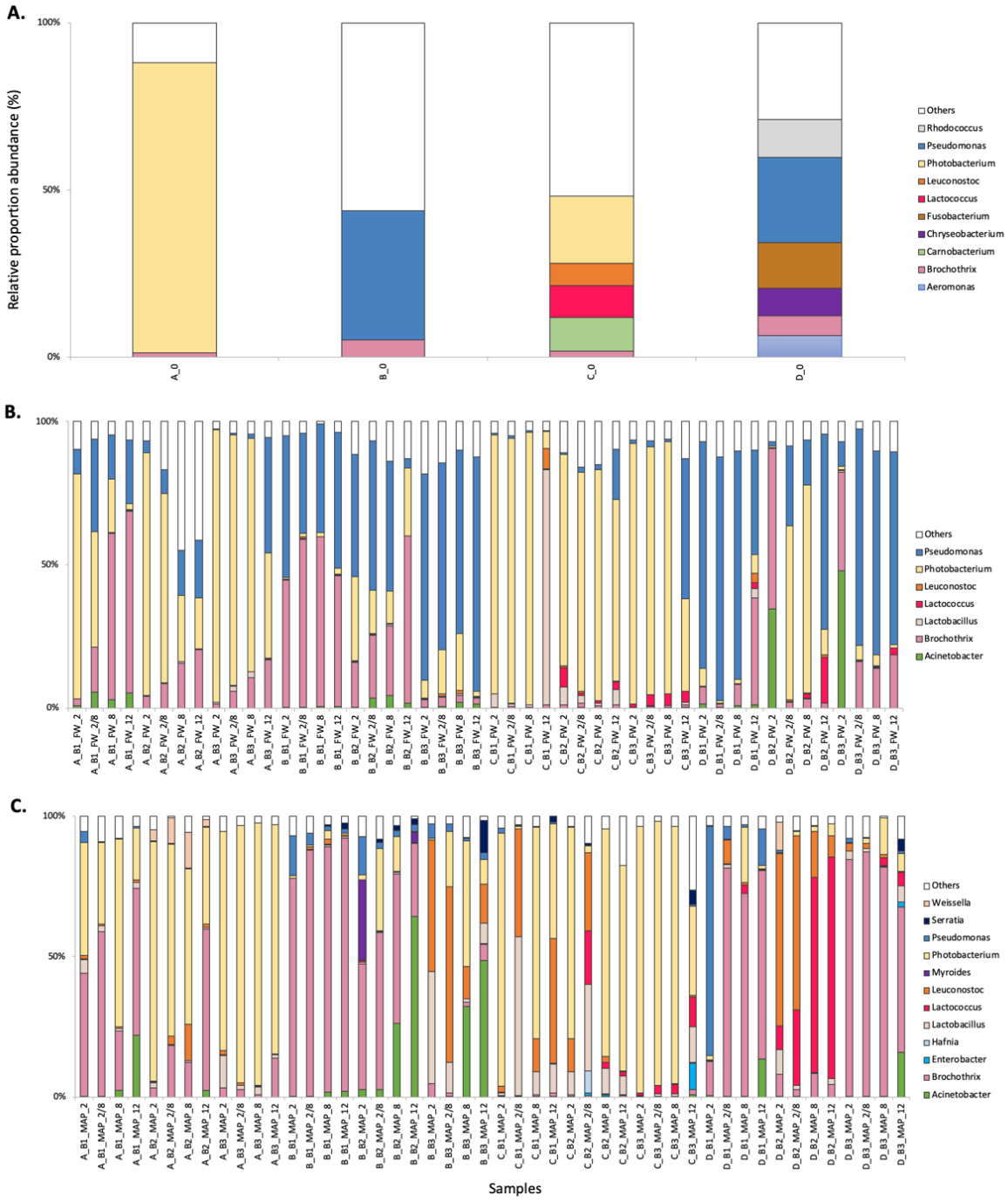

Figure 19. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial communities identified by metagenetic at genus levels, during cold storage of minced pork in relation to the food packaging and the origin of samples (food companies and batches). **(A)** food samples analyzed at day 0 for the four companies (A, B, C and D), **(B)** storage in FW, **(C)** storage in MAP. At genus levels, the taxa representing <5% in relative abundance were merged in the category of "Others". Legend: batch 1 (B1), batch 2 (B2), batch 3 (B3), at 2°C (2), at 8°C (8), at 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

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3.5. Effect of the food packaging on the bacterial communities

However, although dominant genera were identified across all samples, the two different types of packaging were characterized by different microbiota, with only some genera in common (**Figure 20**). At the end of the shelf life, *Pseudomonas* was more present in FW and this genus was potentially replaced by *Brochothrix* in MAP (Welch's t-test, p-value<0.05) (**Figure 21**).

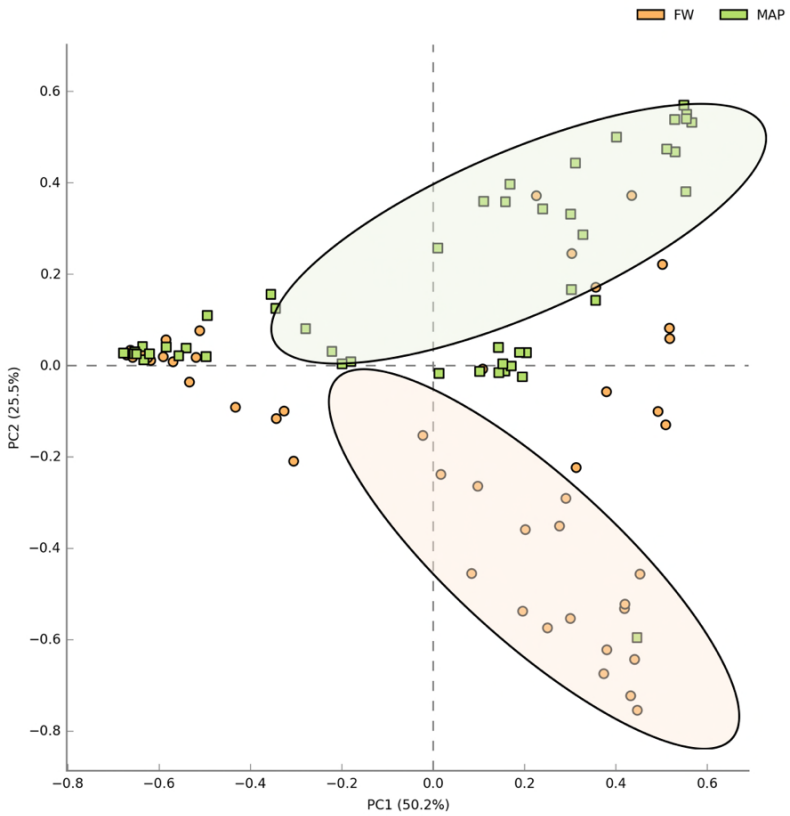

Figure 20. Principal component analysis for 16S rRNA gene sequence data in FW and MAP, among different origin (food companies and batches) and storage temperatures.

At this time, the major OTUs groups (**Figure 22**) are therefore different according to the food packaging: *B. thermosphacta*, *Lb. algidus*, *Ph. kishitanii*, *Ph. phosphoreum*, *Ps. psychrophila* and *Pseudomonas* sp. are dominant in FW. While it concerns *Acinetobacter* sp., *B. thermosphacta*, *Lb. algidus*, *Lc. piscium*, *Ln. inhae*, *Ln. gelidum*, *Leuconostoc* sp., *Ph. kishitanii*, *Ph. phosphoreum* and *Pseudomonas* sp. in MAP.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

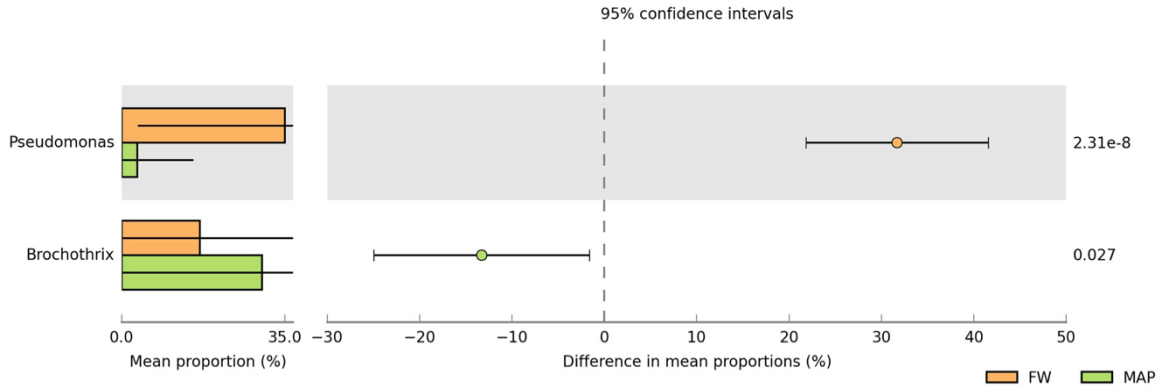

Figure 21. Extended bar plot showing the bacterial populations whose mean relative abundance differed between FW and MAP at genus scale. The relative abundance and the difference in mean proportions are illustrated for the statistically different taxa ($p < 0.05$).

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

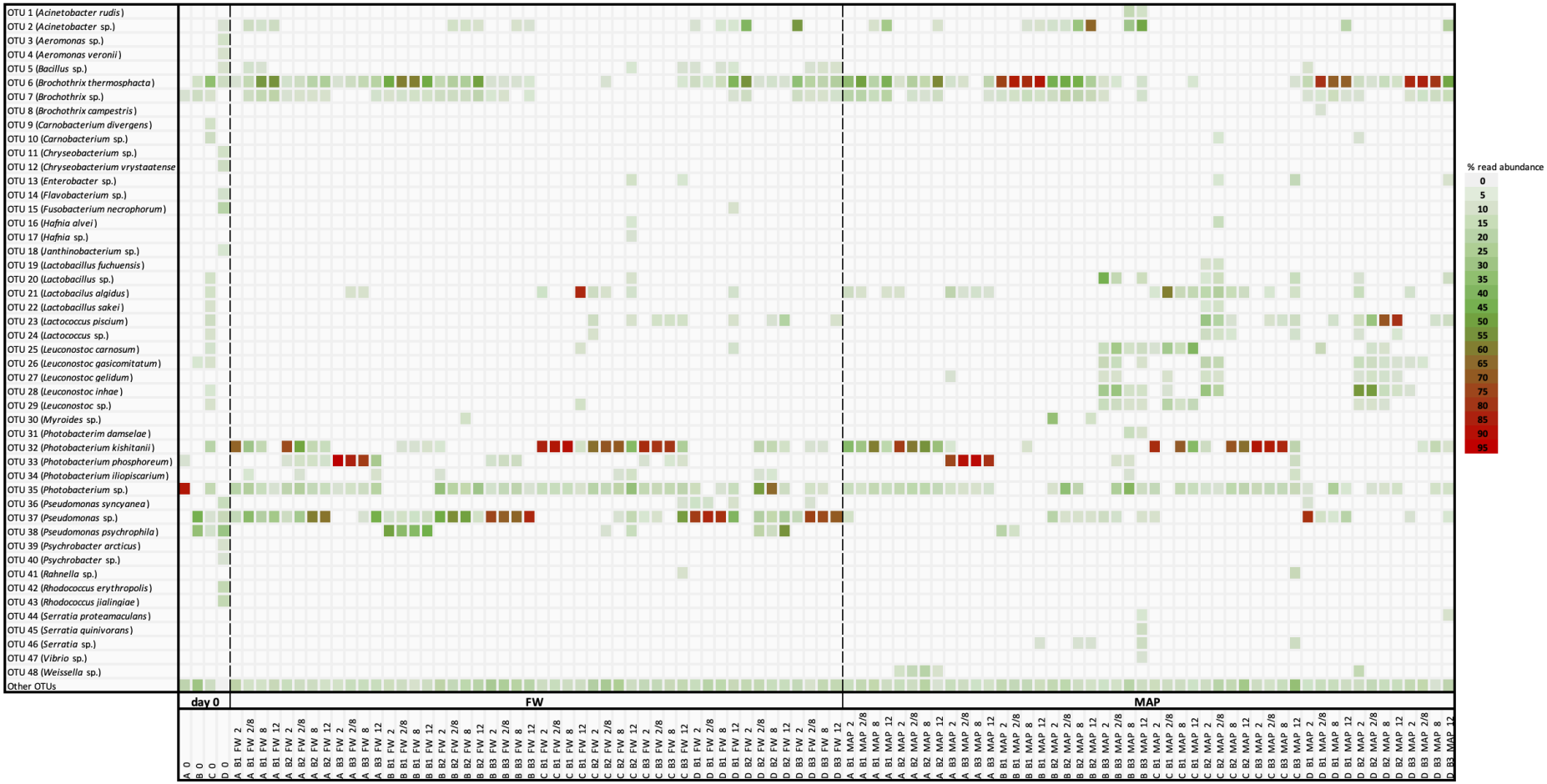

Figure 22. Heatmap of relative read abundance at species level for all samples (expressed in %) among the different storage conditions. Only the most abundant OTUs obtained in this study are specially indicated (>1%). Others OTUs are gathered in "Others OTUs". Legend: food companies (A, B, C and D), with three batches each (B1, B2, B3), analyzed at the first (0) and the last day of storage. Temperature of storage at 2°C (2), 8°C (8), 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

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3.6. Variability of the minced pork ecosystem between samples

Genus relative abundance shows a high Bray-Curtis dissimilarity during the storage, and between the food companies and batches (**Figure 23**).

At day 0, samples showed a high dissimilarity (>70%) with the metadata groupings at the end of the shelf life. At this time, the food company A seems not to shared OTUs in common with the three others food industries.

At the end of the shelf life, Bray-Curtis index seems indicating that a relative similarity exists for OTUs contained within food companies A and C, and within B and D. This index also indicates a relative similarity concerning the temperature of storage, except for the industry D.

A synthetic view about the Bray-Curtis index between samples according to the food origin and storage condition is summarized in **Table 20**.

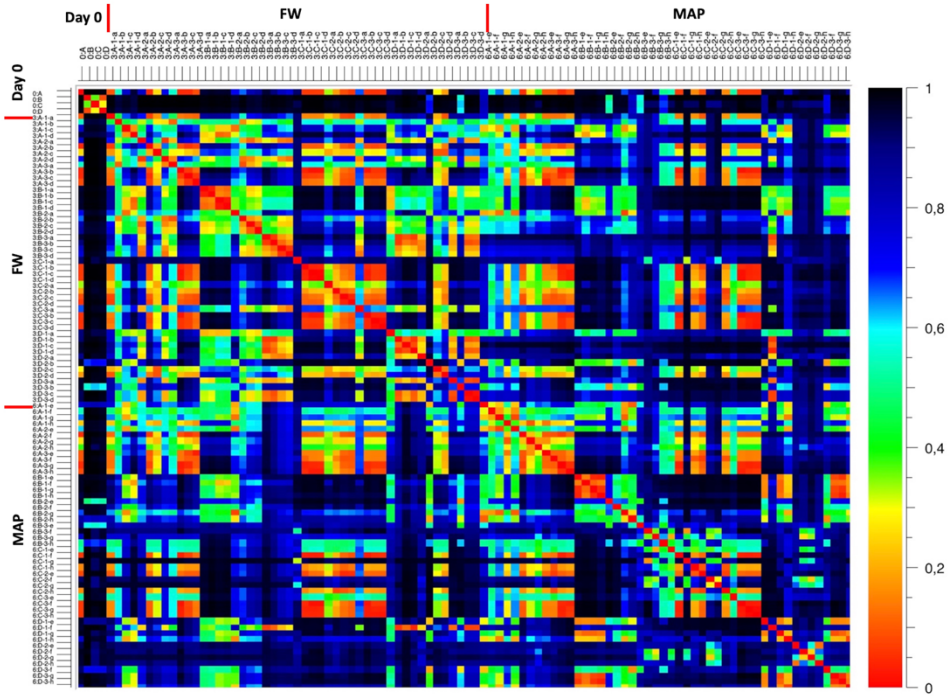

Figure 23. Global microbial dissimilarity obtained by metagenetic between samples for different conditions of storage. The heatmap shows the Bray-Curtis dissimilarity measure based on relative abundance of OTUS (genus scale). Values are given in dissimilarity counts (1= 100% dissimilar, 0=0% dissimilar). Legend: analysis at day 0 (0), at day 3 (3) and at day 6 (6); food companies (A, B, C and D); three batches each (1, 2, 3). Temperature of storage: 2°C in FW (a), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C in FW (b), 8°C in FW (c), 12°C in FW (d), 2°C in MAP (e), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C in MAP (f), 8°C in MAP (g), 12°C in MAP (h).

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Table 20. Dominant bacteria represented in MP samples according to storage conditions. At species level, the taxa representing < 20% in relative abundance were not considered as dominant in this table.

Food companies	First day of storage	Last day of storage		
		Batch	FW	MAP
A	*Photobacterium* sp., *Ph. phosphoreum*	1	*B. thermosphacta*, *Ph. kishitanii*, *Pseudomonas* sp.	*B. thermosphacta*, *Ph. kishitanii*
		2	*B. thermosphacta*, *Ph. kishitanii*, *Pseudomonas* sp.	*B. thermosphacta*, *Ph. kishitanii*, *Weissella* sp.
		3	*Ph. phosphoreum*, *Pseudomonas* sp.	*Ph. phosphoreum*
B	*Pseudomonas* sp., *Ps. psychrophila*	1	*B. thermosphacta*, *Ps. psychrophila*	*B. thermosphacta*, *Ps. psychrophila*
		2	*B. thermosphacta*, *Photobacterium* sp., *Pseudomonas* sp.	*Acinetobacter* sp., *B. thermosphacta*, *Photobacterium* sp.
		3	*Ph. kishitanii*, *Ph. phosphoreum*, *Pseudomonas* sp.	*Acinetobacter* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Ln. gelidum*, *Photobacterium* sp., *Ph. kishitanii*
C	*Photobacterium* sp., *Ph. kishitanii*	1	*Lb.algidus*, *Ph. kishitanii*	*Lb. algidus*, *Ln. carnosum*, *Ln. inhae*, *Ph. kishitanii*
		2	*Photobacterium* sp., *Ph. kishitanii*, *Pseudomonas* sp., *Ps. phychrophila*	*Lb. algidus*, *Lc. piscium*, *Ln. inhae*, *Ph. kishitanii*
		3	*Ph. kishitanii*, *Pseudomonas* sp.	*Ph. kishitanii*
D	*Pseudomonas* sp., *Ps. psychrophila*, *Ps. syncyanea*	1	*B. thermosphacta*, *Pseudomonas* sp.,	*B. thermosphacta*, *Photobacterium* sp., *Pseudomonas* sp.
		2	*Acinetobacter* sp., *B. thermosphacta*, *Photobacterium* sp., *Ps. psychrophila*	*B. thermosphacta*, *Lc. piscium*, *Ln. gelidum*, *Ln. inhae*
		3	*Acinetobacter* sp., *Brochothrix* sp., *B. thermosphacta*, *Pseudomonas* sp.,	*B. thermosphacta*, *Ph. kishitanii*

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Discussion

In this study, we investigated the microbial spoilage community and dynamics of MP samples, among different conditions of production and food storage, using both 16S rRNA gene sequencing and classical microbiology. Indeed, whereas the dynamics of the bacterial community of meat and meat products have been studied before, Stoops *et al*. (2015) reported that little is known about differences in microbial changes during storage, and among the variability between the production batches. Meat and meat products are highly perishable, with colonization and development of a great variety of microorganisms (Chaillou *et al*., 2015; Garnier *et al*., 2017; Nychas *et al*., 2008; Pennacchia *et al*., 2009; Stellato *et al*., 2016). The product composition (low/high pH, low/high concentration of glucose, water activity, …) and the storage conditions (temperature of storage and packaging conditions for example) may favor growth of microorganisms, that are responsible for the formation of spoilage (Argyri *et al*., 2015; Reid *et al*., 2017). This can lead to visible growth (slime, colonies), as textural changes, off-odors or off-flavors (Casaburi *et al*., 2014; Chaillou *et al*., 2015; Del Blanco *et al*., 2017; Stoops *et al*., 2015). In this context, minced meat is a potentially hazardous food product, vulnerable to bacterial spoilage, with a very short shelf life (Geeraerts *et al*., 2017) due to abundant and diverse substrates for bacterial growth and favorable growth conditions (Benson *et al*., 2014). In our study, the MP samples present a high water activity and a near-neutral pH which are in accordance with previous studies on this food matrix (Blixt and Borch, 2002; Andritsos *et al*., 2012).

The initial contamination of products, and also the initial level of LAB, is also a key factor that can influence the spoilage dynamics during storage (De Filippis *et al*., 2013). In our results, the microbial counts of the four manufacturers were quite different and psychrotolerant counts were higher for two food industries (**Tables 12 and 13**). High levels of initial contamination in MP samples were also observed by Peruzy *et al*. (2019a). This difference of the initial bacterial contamination is not in relation with the size of the company. These results can be explained by the fact that multiple sources of contamination can contribute to the initial composition of the meat microbiota (De Filippis *et al*., 2013), such as at the farm (hygiene practices, the conditions of animal transport, etc.) and at the slaughterhouse (automatic level of the process, cleaning practices, etc.). Initial carcass contamination can be also environmental, with contamination by tools, machines, and surfaces of slaughter equipment (Mann *et al*., 2016; Moretro and Langsrud, 2017). In addition, subsequent handling of meat in the operations of slicing, sectioning, portioning, and transferring in packages can determine further contamination in the handling points (Del Blanco *et al*., 2017).

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The bacterial count at the end of the shelf life was over 7.0 log CFU/g, indicating that meat had probably begun to be deteriorated and would not be suitable for human consumption (Zhao *et al*., 2015). Indeed, it is generally recognized that microbial spoilage of meat occurs when counts reach arbitrary level between 7.0 log CFU/g (Nychas *et al*., 2008; Pothakos *et al*., 2014; Reid *et al*., 2017; Spanu *et al*., 2018; Stoops *et al*., 2015) and 8.00 log CFU/g (Chaillou *et al*., 2015; Fall *et al*., 2012; Nychas *et al*., 2008; Pothakos *et al*., 2014; Reid *et al*., 2017). However, these values are only indicative and refer here to the total viable count. Food spoilage needs to be assessed to the genus-species level, because potentially protective bacteria can also occur in food products.

As discussed by Del Blanco *et al*. (2017), common approaches for delaying meat spoilage and improving meat shelf life are available, including good hygienic practices and all the storage conditions. Among these, low storage temperatures and adequate packaging are considered as the most important factors (Koutsoumanis *et al*., 2006; Andritsos *et al*., 2012; DeKaur *et al*., 2017). During the storage at 2°C, the arbitrary level of 7.0 log CFU/g was sometimes not reached. In addition, it can be observed that the microbial kinetics from 2°C to 8°C were quite similar to those at 8°C, as described by Cauchie *et al*. (2017).

In relation with the food packaging, the most common used in meat and meat products are VP and MAP (Caryé *et al*., 2005; Chaix *et al*., 2015a; Dalcanton *et al*., 2013; Koutsoumanis *et al*., 2008; Silbande *et al*., 2016). In this study, FW and MAP (30% CO_2 – 70% O_2) packaging are used. The composition of modified atmosphere systems can be an effective way to reduce the growth rate of spoilage aerobic organisms and modify the microbial ecology of the product. But their effectiveness strongly depends on the initial microbial contamination of raw materials, storage temperature, film permeability and the carbon dioxide concentration used (20-40% is commonly used to slow microbial growth) (Simpson and Carevic, 2004; Rotabakk *et al*., 2006; Stoops *et al*., 2015; Guillard *et al*., 2016; Saraiva *et al*., 2016; Couvert *et al*., 2017). The carbon dioxide concentration was here theoretically sufficient to limit the microbial growth. However, the higher percentage of oxygen can also enhance the growth of aerobic microbial communities in our samples. Moreover, some bacteria are able to grow in variable food packaging, as *Photobacterium* which is CO_2-tolerant (Dalgaard *et al*., 1995; Fuertes-Perez *et al*., 2019). Also, in accordance with Stoops *et al*. (2015), it can be observed a significant production of carbon dioxide. This production may be the reflect of the development of bacterial groups belonging to LAB, *Brochothrix* or *Enterobacteriaceae* (Caryé *et al*., 2005). As environment of slaughtering and processing steps (Stellato *et al*., 2016), packaging materials can also be a source of contamination because they are not sterile in this study. Further studies based on microbial contamination of food trays would also be interesting.

According to this, and based on the study by Stoops *et al*. (2015), viable counts are not suitable to characterize the microbial diversity of food products and to investigate thoroughly shifts in the

bacterial communities during storage. Indeed, culture-dependent techniques largely underestimated the species richness and abundance. For a more detailed characterization of microbial communities in samples, originating from different ecological niches, a sequence-based approach was used, allowing identification of OTUs at various taxonomic levels (species, genus or family levels) (Stoops *et al*., 2015). However, without extensive studies involving a large number of samples under different storage conditions it will not be possible to determine exactly the bacterial ecosystem and the role of individual spoilage species (Pennacchia *et al*., 2011; Rouger *et al*., 2018). According to this, we analyzed minced meat samples from four different food companies, with three different batches per industries. In addition to previous studies based on the microbial description of minced meat samples (Stoops *et al*., 2015, Peruzy *et al*., 2019a), our study aims to understand and monitor microbial dynamics and variability between food companies and food batches, according to different storage conditions.

In our results, the observed microbial diversity was relatively high, and the most abundant bacteria differ among samples. As observed by Stoops *et al*. (2015) in minced meat samples, an increase of microbial counts is coinciding with a decrease in bacterial diversity during storage. At the end of the storage period, the major genus taxa are represented by *Pseudomonas* in FW and *Brochothrix* in MAP. But it can also be observed a high diversity between food companies and batches (**Table 18**). Our results are in accordance with Peruzy *et al*. (2019a), which also observed a dominance of the genus *Pseudomonas*, *Brochothrix* and *Carnobacterium* in MP samples. Moreover, these results are not surprising because the microbial populations of refrigerated meat and pork-meat products are mainly composed by *Pseudomonas* spp., cold-tolerant *Enterobaceriaceae*, LAB (such as *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Carnobacterium* spp., etc.), *B. thermosphacta*, *Clostridium* spp. (Casaburi *et al*., 2014; Del Blanco *et al*., 2017; Geeraerts *et al*., 2017; Koort *et al*., 2005; Liu *et al*., 2006; Nychas *et al*., 2008; Pennacchia *et al*., 2009; Pennacchia *et al*., 2011; Stellato *et al*., 2016) and *Weissella* spp. (Pothakos *et al*., 2014; Stellato *et al*., 2016). Other genera isolated frequently from fresh pork meats are *Acinetobacter* spp., *Aeromonas* spp., *Enterococcus* spp. and *Moraxella* spp. (Mann *et al*., 2016; Zhao *et al*., 2015). However, these results are not completely in accordance with Stoops *et al*. (2015) because this study mentioned that *Lb. algidus* and *Leuconostoc* sp. became the dominant bacteria in minced meat samples stored at 5°C under MAP (66% O2, 25% CO2 and 9% N2). These differences can be explained by different meat compositions (beef in the study by Stoops *et al*. (2015) and pork in our study), the initial contamination of samples, and the gas mixture used.

The results also showed the interest of using culture-independent method to better understand the changes of food microbiota over time, and in each food companies, according to the storage conditions. Indeed, metagenetic approach produce a large amount of data in a very short time (Cocolin *et al*., 2018, den Besten *et al*., 2018), allowing to interpret and use these data to help agri-food companies in their decisions regarding food safety and quality decisions. Moreover, all the OTUs-species described

as potentially spoilers in our study are well described in the literature (**Table 21**), and in MP samples (Peruzy *et al*., 2019a; Stoops *et al*., 2015). The bacterial species present in our samples are also able to grow in meat matrices, and they are potentially responsible of spoilage effects, which can affect color, flavor, visual aspect, etc. (Pothakos *et al*., 2015). Sensory analyses would be interesting in this context, but were not performed in this study. Moreover, the enzymatic decarboxylation of amino acids, or the transamination of aldehydes and ketones, by bacteria results in the formation and accumulation of BAs (Jastrzebska *et al*., 2016). Biogenic amines (e.g.: b-phenylethylamine, cadaverine, histamine, putrescine, spermidine, spermine, tyramine and tryptamine) are reported in various foods including meat, fish, cheese and wine (Papageroegiou *et al*., 2018). They can have health implications, such as allergic reactions, but also contribute to spoilage due to their putrid aroma (Stanborough *et al*., 2017). Therefore, as proposed by Cheng *et al*. (2016), the sum of BAs could be used as an indicator of pork meat quality and freshness during storage. Li *et al*. (2014) also showed that some BAs could be used as spoilage indicators of chilled pork.

However, it is important to add that some bacteria can be considered as protective, such as some LAB. As mentioned by Singh *et al*. (2018), the presence of high LAB communities does not necessarily result in quality defect, and their intra-species variation to cause spoilage has already been recognized (Pothakos *et al*., 2015).

In the present study, we designed a method to collect MP samples in order to explore the bacterial communities and diversity among different food origin and storage conditions. Indeed, the modification of the composition of the spoilage microbiota during storage is an important factor in assessing food quality (Holm *et al*., 2013). Although the bacteria consistently dominated the microbiota of MP samples are known, results indicated that bacterial diversity needs to be addressed on the level of food companies and batches variations. As discussed by Rouger *et al*. (2017), it is important to overcome variability to better understand the factors underlying the diversity of spoilage bacterial communities, by (i) defining reproducible and reliable experimental conditions to lead to biological interpretation, or (ii) to multiplying sampling or experiments to obtain statistical significance of the results (Chaillou *et al*., 2015; Rouger *et al*., 2017). A seasonal effect on the microbial quality of minced meat has also been reported by Andritsos *et al*. (2012). In this paper, no conclusions about bacterial ecosystems for others food companies, or for different times of the year, should be dawn. Further data are so needed to determine diversity of spoilage microbiota in MP samples, according to others food industries, sampling periods and storage conditions. Also, a comparative evaluation of spoilage-related bacterial species and metabolic profiles, with growth parameters of these potentially spoilage bacteria in samples, will be studied in another study.

In conclusion, the combination of both culture-dependent and culture-independent analyses enabled us to explore the microbial communities of MP samples under different food origin and storage

conditions, as previously described by Stoops *et al*. (2015). In our study, microbial changes during storage were monitored, according to a sampling in four food companies and for several batches. In accordance with previous studies we found that *Pseudomonas* and *Brochothrix* dominate the community at the end of the shelf life in FW and MAP, respectively, together with *Photobacterium*. The major OTUs groups are also often associated with pork meat spoilage in the scientific literature. And these results are also in accordance with studies conducted on the microbiota of minced meat by Stoops *et al*. (2015) and Peruzy *et al*. (2019a). Psychrophilic spoilers dominated the microbiota of our samples, but each sample harbored a unique pork meat microbiota, depending on the manufacturing batch and the packaging used. The gas mixture and the temperature condition used in this study are probably the most important factors implied to the dynamics of the bacterial community. Further researches on the main contamination during slaughter production process, such as importance of processing environment, procedures and storage conditions, are desirable to provide a complete assessment of the microbiota of minced meat and to limit incidents of unexpected spoilage.

Study 1. Assessment of spoilage bacterial communities in food wrap and modified atmospheres-packaged minced pork samples by 16S rDNA metagenetic analysis.

Table 21. Examples of some microbial species occurring during chilled storage of meat and their potential spoilage effects.

Bacteria	Growth conditions	Spoilage effects	References
Actinetobacter spp.	Especially present in dairy and seafood products.	Low spoilage potential but can enhanced the growth of other spoilage bacteria by means of quorum sensing.	Ghasemi-Varnamkhasti *et al*., 2018; Hahne *et al*., 2019; Odeyemi *et al*., 2018a; Pinu, 2016.
Brochothrix spp.	In different gas composition, such as under air, modified atmosphere and vacuum packaging. More tolerant in oxygen-depleted and CO2-enriched environments.	Sour, acid and cheesy odor.	Del Blanco *et al*., 2017; Doulgeraki *et al*., 2012; Ercolini *et al*., 2011; Koutsoutamis *et al*., 2008; Nychas *et al*., 2008; Mann *et al*., 2016; Mansur *et al*., 2019; Reid *et al*., 2017; Zhao *et al*., 2015.
Carnobacterium spp.	In all types of packaging conditions. Predominance in low O_2 packaging.	Spoilage effect can vary, producing volatile molecules with low sensory impacts (fruity or fermented odors, …)	Casaburi *et al*., 2011; Doulgeraki *et al*., 2012 ; Pothakos *et al*., 2015.
Lactobacillus spp. (*Lb.* sakei, *Lb. fuchuensis*, *Lb. plantarum*, *Lb. curvatus*, *Lb. algidus*, *Lb. oligofermentans*, …)	In all types of packaging conditions. Predominance with high concentration of CO_2.	Severe acidification, emission of off-odor compounds and ropy slime. However, LAB may produce lactic acid, which inhibits the growth of other families of bacteria. And some species can produce bacteriocins.	Alvarez-Sieiro *et al*., 2016; Dalcanton *et al*., 2013; Doulgeraki *et al*., 2012; Fadda *et al*., 2010; Kato *et al*., 2000; Mann *et al*., 2016; Mansur *et al*., 2019; Nieminen *et al*., 2015; Pothakos *et al*., 2015; Stefanovic *et al*., 2017; Woraprayote *et al*., 2016; Zhao *et al*., 2015.
Lactococcus spp.	In various types of packaging.	Traditionally they have not been considered as spoilage microorganisms, but the spoilage potential of these bacteria is still scarcely known.	Dalcanton *et al*., 2013; Doulgeraki *et al*., 2012; Kato *et al*., 2000; Mann *et al*., 2016; Mansur *et al*., 2019; Pothakos *et al*., 2014; Rahkila *et al*., 2012; Zhao *et al*., 2015.
Leuconostoc spp. (*Ln. gelidum*, *Ln. carnosum*, *Ln. mesenteroides*, …)	Under aerobic, vacuum and modified atmosphere packaging. Predominance with high concentration of O_2.	Buttery aroma, formation of slime, blowing of packages, green discoloration.	Dalcanton *et al*., 2013; Doulgeraki *et al*., 2012; Kato *et al*., 2000; Mann *et al*., 2016; Mansur *et al*., 2019; Nieminen *et al*., 2015; Pothakos *et al*., 2015; Zhao *et al*., 2015.
Photobacterium spp.	Under air, vacuum and modified atmosphere packaging. More frequently present in seafood products.	Typically not associated with spoilage of meat. Responsible for reducing TMAO to TMA, off-odor (produce volatile organic compounds) and biogenic amine formation. The mechanism underlying spoilage has not been clarified.	Ast *et al*., 2007; Bjornsdottir-Butler *et al*., 2016; Fogarty *et al*., 2019; Jääskeläinena *et al*., 2019; Kuuliala *et al*., 2018; Li *et al*., 2019a; Moretro *et al*., 2016; Nieminen *et al*., 2016.
Pseudomonas spp.	In different gas composition, such as under air, modified atmosphere and vacuum packaging. Predominance under aerobic low temperature. Limitation in the bacterial microbiota by the presence of CO_2 and/or the limitation of O_2 in MAP.	Slime, discoloration, off-odor producing.	Andritsos *et al*., 2012; Del Blanco *et al*., 2017; Doulgeraki *et al*., 2012; Ercolini *et al*., 2011; Koutsoumanis *et al*., 2008; Liu *et al*., 2018; Nychas *et al*., 2008; Mann *et al*., 2016; Mansur *et al*., 2019; Reid *et al*., 2017; Spanu *et al*., 2018; Zhao *et al*., 2015.
Weissella spp.	Some can be found in salted and fermented foods. Present in vacuum packaging.	Greenish appearance. Can plays an important role in the fermentation process. Some species can produce bacteriocins.	Kariyawasam *et al*., 2019; Kim *et al*., 2017; Martins *et al*., 2016; Pothakos *et al*., 2015.

Study 2

The use of 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding

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Study 2. The use of 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding.

Study 2. The use of 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding.

Abstract

⇒ **Objective 1.** Study the natural microbiota of white pudding

⇒ **Objective 2.** Characterize specific spoilage bacteria dynamics and use them as inputs in models for white pudding

In order to control FLW, monitoring the microbial diversity of food products, during processing and storage is important, as studies have highlighted the metabolic activities of some microorganisms which can lead to spoilage. Knowledge of this diversity can be greatly improved by using a metagenetic approach based on high throughput 16S rRNA gene sequencing, which enables a much higher resolution than culture-based methods. Moreover, the Jameson effect, a phenomenon described by Jameson in 1962, is often used to classify bacterial strains within an ecosystem. According to this, we have studied the bacterial microbiota of Belgian WP during storage at different temperatures using culture-dependent and -independent methods. The product was inoculated with a mix of dominant strains previously isolated from this foodstuff at the end of its shelf life (*C. maltaromaticum*, *Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans*, *Lc. lactis*, *Ln. mesenteroides*, *R. terrigena* and *Serratia* sp.). Daily during 16 days, the absolute abundance of inoculated strain was monitored by combining total count on plate agar and metagenetic analysis. The results were confirmed by qPCR analysis. The growth of each species was modelled for each temperature conditions, representative of good or bad storage practices. These data allowed the bacterial strains subdivision into three classes based on criteria of growth parameters for the studied temperature: the "dominant", the "subdominant" and the "inhibited" bacterial species, according to their maximal concentration (N_{max}, log CFU/g), growth rate (μ_{max}, 1/h) and time to reach the stationary phase (*TRSP*, days). Thereby, depending on the storage conditions, these data have permitted to follow intrinsically the dyanmics of each strain on the bacterial ecosystem of Belgian WP. Interestingly, it has shown that the reliability of the Jameson effect can be discussed. For example, at 4°C when *Lc. lactis* and *Serratia* sp. stopped growth at day 12, at the same time *C. maltaromaticum* reached its maximal concentration and entered its stationary phase. In opposition to this, it can be noticed that in the same condition, the "sub-dominant" organisms continued their growth independently of the "dominant" species behavior. In this case, the Jameson effect was not illustrated. This pattern is described for all storage conditions with the same strain classifications. These results highlighted the importance of

combining metagenetic analysis and classical methods, with modeling, to offer a new tool for studying the dyanmics of microorganisms present in perishable food within different environmental conditions.

Study 2. The use of 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding.

Introduction

In past years, scientists who study the safety of highly perishable food products have focused their work on the detection and the control of pathogenic microorganisms. However, Food Law (Regulation (EC) N°178/2002) also integrates all products that are unfit for human consumption because of contamination, deterioration, decomposition or rotting into the definition of unsafe food. Around a third of all food produced for human consumption on Earth is lost or wasted. In Europe, the losses of initial meat production represent 20% and more than half of this occurs at animal production, slaughtering, processing and distribution steps (Food and Agriculture Organization, 2011; Kergourlay *et al*., 2015). These data highlight the importance of managing the microbiological quality of food products. Indeed, among the reasons for FLW, spoilage by bacteria that contaminate the food matrix and are able to develop during transformation steps and storage is a major issue (Lipinski *et al*., 2013; Remenant *et al*., 2015). For a clear and complete understanding of the mechanisms that lead to the spoilage of food products, classical microbiology is not sufficient. Fortunately, molecular technologies can elucidate the microbial communities, including the identification and quantification of culturable and non-culturable organisms, and can do so at a much higher resolution than was previously possible with culture-based methods (Kergourlay *et al*., 2015; Elizaquível *et al*., 2015). Many bacterial species putatively responsible for food spoilage have been reported, thanks to the development of high throughput sequencing methods, that allow for a more detailed and deeper description of bacterial species present in food (Benson *et al*., 2014; Chaillou *et al*., 2015; Delcenserie *et al*., 2014; Galimberti *et al*., 2015; Riquelme *et al*., 2015). These works are mainly limited to the description of the product's microbiota during its shelf life. However, spoilage is a complex process, resulting most often from incorrect storage temperatures and bacterial functions that are not fully understood. Spoilage is not only species and strain dependent, but also the result of interactions between strains. Few studies have described the dynamics of a whole microbiota in a food matrix with consideration of the storage parameters (Ercolini *et al*., 2011; Nieminen *et al*., 2012).

The present study proposes to follow the dynamics of the main bacterial species present in a famous Belgian meat product: the white pudding. For this, we inoculated a mix of strains previously isolated from aging tests on the same food matrix. The mix of inoculated strains has been studied in challenge tests at different storage temperatures, representative of good or bad practices. The growth of the added bacteria has been assessed daily at the same time by combining classical microbiology and

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16S rRNA metagenetic analysis (Esposito and Kirschberg, 2014) with the goal of obtaining quantitative results for each strain and to study their respective kinetics. Quantitative PCR (qPCR) analysis targeted on corresponding bacterial genera was used in order to validate the metagenetic approach.

There are two objectives in this study: the first is to reinforce the importance of combining classical microbiology and metagenetic analysis, with modeling, as a new tool to follow the dynamics of microorganisms present in perishable food within different environmental conditions. This approach can examine the potential for next-generation DNA sequencing methods to elucidate the detailed dynamics of microbial population during spoilage. To this end, a combination of metagenetic and traditional microbiological methods were used to quantify the microbiota of Belgian WP. The second is providing knowledge on the composition and dynamics of the emblematic bacterial species components of WP, and shown how it is affected by storage temperature.

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Material and methods

2.1. Food samples and selection of bacterial strains

The strains used in this study were previously isolated from Belgian WP at the end of their use-by date, by one Belgian manufacturer (five batches analyzed), after storage for a third of the storage period at 4°C and the remaining time at 8°C following the guidelines for implementing microbiological durability tests of chilled perishable and highly perishable foodstuffs (NF EN V01-003, 2010). The results of these first aging tests are not shown in this paper. Eight of the natural predominant strains isolated at the end of the shelf life, represented together more than 50% of the natural microbiota, were identified by sequencing of their 16S rRNA genes and used for the challenge-tests: *C. maltaromaticum*, *Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans*, *Lc.lactis*, *Ln. mesenteroides*, *R. terrigena* and *Serratia* sp. For this study, a short 16 days shelf life was evaluated for the Belgian WP.
Bacterial strains were stored at −80°C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, strains were transferred from the −80°C culture collection to Brain Heart Infusion (BHI) broth for *C. maltaromaticum*, *R. terrigena* and *Serratia sp.*, and de Man, Rogosa and Sharpe (MRS) broth for *Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans*, *Lc. lactis* and *Ln. mesenteroides* for 48 h at 22°C. The cultures were incubated overnight at 4 °C before inoculation.

2.2. Challenge tests

Thirty-three kilograms of white pudding (each 150 g) were received from a Belgian manufacturer the day following their production and stored at 4°C (composition: pork meat 64%, milk, bread, onions, salt and spices. No sugar was added). The natural microbiota was considered as insignificant because these products were inoculated by a concentrate mix of eight bacterial species who dominate the initial indigenous microbiota. The surface products were inoculated by soaking for 2 min in a bath of sterile water containing a mix of the eight bacterial strains at the same concentrations with the goal of reaching an approximatively global concentration of 3 log colony forming units (log CFU/g on the product), in duplicate (n = 192). Non-inoculated control samples were soaking for the same time in a bath of sterile water only, in duplicate (n = 24). After a drying step of 20 min at 10°C, WP were packed (300 g) in a tray (PP/EVOH/PP) under MAP (CO_2 30%/N_2 70%, Olympia V/G, Technovac, Italy) using packaging wrap (polyester 10 µm, homopolymer polypropylene 50 µm, NutriPack, France).

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According to the shelf life of the product, inoculated samples were stored at different temperatures, constant or dynamic: (i) for 16 days at 4°C (4°C), (ii) for 16 days at 8°C (8°C), (iii) for 16 days at 12°C (12°C), (iv) for 4 days at 4°C and for 12 days at 8°C (4–8°C), (v) for 4 days at 4°C, followed by a break of 4 h at 20°C than 12 days at 4 °C (4/20–4°C), (vi) 4 days at 4°C, followed by a break of 4 h at 20°C then 12 days at 8 °C (4/20–8°C). Control samples were only stored at the first day and at day 16.

2.3. Incubation and enumeration by conventional microbiological method

Each day during the 16-day storage period except on day 2, 25 g of product were put into a Stomacher bag with a mesh screen liner (80 μm pore size) (bioMérieux, Basingstoke, England, ref 80015) under aseptic conditions. Physiological water (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in peptone water (1 g/L peptone, 8.5 g/L sodium chloride) were prepared for microbiological analysis and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis in duplicate (Spiral plater, DW Scientific, England). A total count was made on Plate Count Agar (PCA) at 22°C for 48 h for the psychrophilic aerobic plate count, using the modified method specified by the International Organization for Standardization [ISO (2013, ISO 4833-2)]. Graphs were plotted with each of the day time points over the 16-day storage period (n = 192). Non-inoculated products were only analyzed at day 1 and day 16 (n = 24).

2.4. Total DNA extraction

Bacterial DNA was directly extracted from each primary suspension, which had been stored at −80°C, using the DNeasy Blood & Tissue DNA Extraction kit (Qiagen, Venlo, Netherlands), following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentrations and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). The quality and quantity of the products were confirmed by Picogreen double-stranded DNA (dsDNA) quantitation assay (Isogen, St-Pieters- Leeuw, Belgium). DNA samples were stored at −20°C until use for 16S rRNA gene pyrosequencing and qPCR analysis.

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2.5. Bacterial 16S rRNA gene amplification and barcoded pyrosequencing

16S rRNA PCR libraries targeting the V1–V3 hypervariable region were generated. Primers E9-29 and E514-430 (Brosius *et al*., 1981), specific for bacteria, were selected for their theoretical ability to generate the lowest amplification bias relative to amplification capability among the various bacterial phyla (Wang and Qian, 2009). The oligonucleotide design included 454 Life Sciences A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5′ end of each primer. The amplification mix contained 5 units (U) of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1× enzyme reaction buffer, 200 µM deoxynucleotide triphosphates (dNTPs) (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng of genomic DNA in a final volume of 100 µL. Thermocycling conditions consisted of a denaturation step of 15 min at 94°C, followed by 25 cycles of 40 s at 94°C, 40 s at 56°C, and 1 min at 72°C, with a final elongation step of 7 min at 72°C. These amplifications were performed on an EP Mastercycler Gradient System apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). The quality and quantity of the products were assessed by Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). Equal amounts of each of the PCR products were pooled and subsequently amplified by emulsion PCR. Pyrosequencing was performed with the Illumina sequencer (Illumina, Eindhoven, Netherlands) (2 × 300 bp). A mean 19,581 of reads per day were analyzed for all temperature conditions.

2.6. Bioinformatics and data analysis

The 16S rRNA gene sequence reads were processed with MOTHUR (Pothakos *et al*., 2014; Schloss *et al*., 2009). The quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS). The obtained reads sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pothakos *et al*., 2014). The final reads were clustered into OTUs using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA dataset 111 using a BLASTN algorithm (Delcenserie *et al*., 2014; Pothakos *et al*., 2014). For each OTU, a consensus detailed taxonomic identification was given

based upon the identity (<1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Delcenserie *et al.*, 2014; Pothakos *et al.*, 2014).

2.7. Estimate abundance results

The PCA results of the microbiota at 22°C (expressed in log CFU/g) and the relative proportions of strains given by metagenetic (expressed in %) were combined in order to obtain estimate counts for the strains (in log CFU/g). For this, relative abundance of bacteria obtained by metagenetic results were reported over the PCA real value (**Equation 14**). Indeed, thanks to the plate counts estimates, the proportions of the bacterial populations were transformed into concentrations. These results were used for statistical and graphical analysis.

$$C_{bacterial\ species} = (C_{total\ microbiota} \times P_{reads\ of\ bacterial\ species}) / 100 \quad (14)$$

Where $C_{bacterial\ species}$ is the estimated abundance concentration in the sample (log CFU/g), $C_{total\ microbiota}$ is the bacterial concentration per samples in the PCA analysis (log CFU/g), and $P_{reads\ of\ bacterial\ species}$ is the proportion of reads for the bacterial species per sample in the metagenetic analysis (expressed in % of the total number reads in the sample).

2.8. qPCR analysis

The primers described in **Table 22** were used for real-time PCR assay analysis using the Lightcycler 480 system (Roche, Basel, Switzerland). The real-time PCR reaction mixtures were combined in a 12 µL final volume containing 6 µL of LC 480 probe master mix (Roche, Basel, Switzerland), 2 µL of template DNA (at 5 ng/µL), 0.25 µL of primer pairs (10 µM each), 0.125 µL of Taqman probe (10 µM). The reaction conditions included the initiation step off 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The real-time system is supplied with the Lightcycler 480 Software version 1.5 using unique Roche algorithms for highly accurate and robust automated data analysis. Serial dilutions (10^6 to 1 copy numbers) of bacterial DNA were used for determining reference curves. The arithmetic mean of Cycle Threshold (CT) of the three repetitions was used in order to estimate the load of targeted bacterial populations present in the samples.

2.9. Statistical analysis

Using R software, the Analysis of Covariance (ANCOVA) test was used to evaluate if bacterial concentrations (log CFU/g) are equal across levels of a categorical independent variable (temperature conditions or microbial count method). With relation to temperature conditions, ANCOVA tests were

realized using the bacterial growth data (from day 1 to day 16) and the bacterial growth during the exponential phase data (from day 4 to day 8). All tests were considered as significant for a p-value < 0.05.

Table 22. Primers and probes designed for the qPCR tests allowing for the relative proportion of genera mainly present in WP to be estimated.

Target bacterial genus	Target gene	Primers	Sequence
Lactobacillus	Tuf	Lactobacillus-Tuf-F2	5'-GCYCACGTWGAATAYGAAAC-3'
		Lactobacillus-Tuf-R2	5'-CGDACTTCCATTTCAACYAAGTC-3'
		Lactobacillus-Tuf-FAM1	5'-TGTGGCATWGGRCCATCAGTTGC-3'
Lactococcus	rpoA	Lactococcus-RecA-F2	5'-GCCGAAATYGATGGYGAAAT-3'
		Lactococcus-RecA-R2	5'-CAACTTTTTCACGCAATTGGTTG-3'
		Lactococcus-RecA-FAM4	5'-TGATGTCWCAAGCYATGCGTAAAC-3'
Leuconostoc	Fus	Leuconostoc-Fus-F1	5'-TTCTTGTTCCATGAAATCCATTTG-3'
		Leuconostoc-Fus-R1	5'-GAATACCCACTAGAWCGTACAC-3'
		Leuconostoc-Fus-FAM1	5'-TGTGTTTCACCAATTTTGTGAATTTTACC-3'
Carnobacterium	rpoA	Carnobacterium-rpoA-F1	5'-ATTGGYGTATTACCAGTCGA-3'
		Carnobacterium-rpoA-R1	5'-AACCATCTGCCCATACATC-3'
		Carnobacterium-rpoA-FAM1	5'-CGATTTACACCCCAGTTAGTCGT-3'

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Results

3.1. Bacterial dynamics by classical microbiological analysis

Figure 24 shows the PCA results from inoculated Belgian WP at different temperatures. The bacterial population showed different dynamic changes depending on conditions of storage and stabilized between 8.5 and 9.2 log CFU/g. For the non-inoculated products, results were respectively inferior to 3 log CFU/g and the same as inoculated products at day 16.

As expected, the storage temperature had a strong impact on the bacterial dyanmics. A high storage temperature is correlated to a high growth rate during exponential phase and a stationary phase more rapidly reached. While the break at 20°C for 4 h doesn't seem to have a significant effect on the dyanmics of the culturable microbiota, the transition from 4°C to 8°C stimulated the growth of the microorganisms. It would be interesting to intrinsically study the effect of temperature conditions on the behavior of each strain inside the ecosystem.

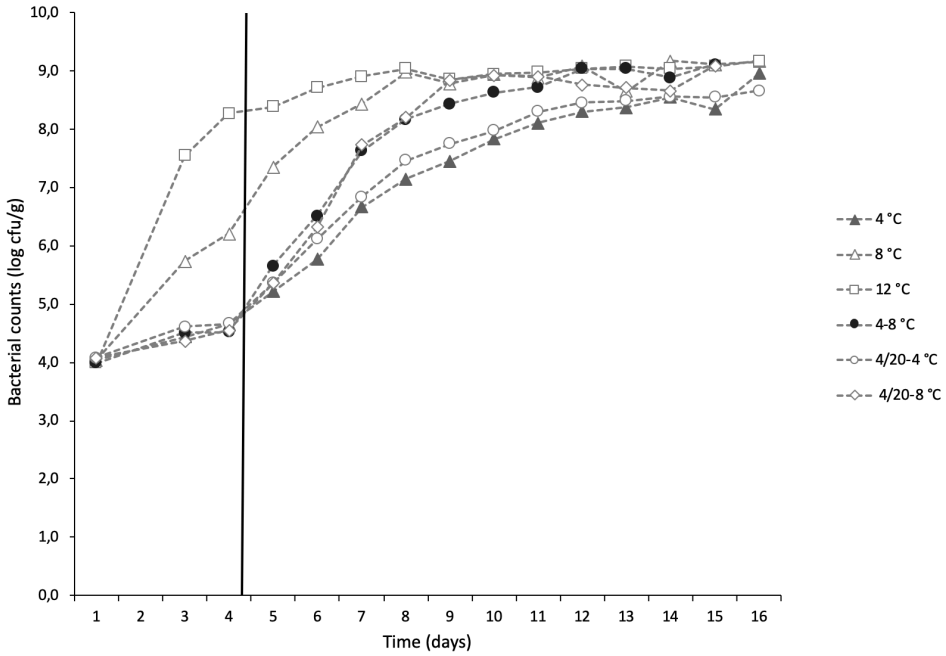

Figure 24. Enumeration of the total psychrophilic aerobic microorganisms from inoculated white pudding stored at different temperatures for 16 days. A solid line after the fourth day represents the transition from 4 °C to 8 °C for the 4-8 °C condition and the break at 20 °C for 4h for the 4-8 °C, 4/20-4 °C and 4/20-8 °C conditions.

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3.2. Relative abundance results obtained by metagenetic analysis

The distribution of read percentages for the eight major bacterial species for each sample (n = 768) in constant temperature shows that at day 7 the mix reach more than 70% of total reads in samples at 4°C. The same percentage is attained at day 3 both for 8°C and 12°C. The major bacterial species concerned *C. maltaromaticum* at 4°C and *Lc. lactis* at 12°C. All inoculated samples reached more than 90% at the end of shelf life product (**Table 23**) and the natural microbiota of the WP seems to become minor in contrast to the inoculated mix of the surface product. For the dynamic temperature condition, the same results were observed (**Table 24**).

In these two cases, some bacterial strains (*R. terrigena* and *Lb. oligofermentans*) were excluded from the results for better readability because they were often under the detection level for the metagenetic analysis (data not shown).

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Table 23. Distribution of reads percentages for the six major bacterial species inoculated for each samples conditions obtained by metagenetic analysis during shelf life product in constant temperature. Others strains represented the natural microbiota of WP; -, data under the detection limit.

Bacterial strains/temperature	**Days**														
	1	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
4°C															
C. maltaromaticum	1.1	3.2	4.5	11.5	25.8	70.5	86.8	96.7	96.4	93.9	90.7	76.7	65.8	50.4	42.1
Lc. lactis	0.3	0.2	0.1	0.4	1.2	0.2	0.2	-	-	-	-	0.1	-	-	-
Ln. mesenteroides	0.1	0.3	-	0.3	0.5	0.6	1.3	0.3	-	0.6	0.7	1.3	4.2	4.6	12.5
Lb. graminis	0.2	0.4	1.3	0.5	0.8	1.5	1.8	0.2	-	0.8	0.7	1.9	2.7	3.8	4.3
Serratia sp.	0.2	0.2	0.7	0.6	0.8	0.8	0.7	0.2	0.1	0.2	0.1	0.1	0.6	0.2	0.5
Lb. fuchuensis	0.2	0.4	0.6	0.6	1.5	1.9	3.1	0.4	1.1	2.8	4.2	13.6	18.1	35.8	32.3
Other strains	97.9	95.1	92.8	86.2	69.3	24.5	6.0	2.0	2.5	1.7	3.6	6.3	8.6	5.1	8.2
8°C															
C. maltaromaticum	1.1	33.8	60.4	69.8	75.1	48.8	43.0	18.7	8.4	14.0	7.0	6.7	8.2	8.3	3.4
Lb. fuchuensis	0.3	2.4	6.5	4.4	4.2	14.8	18.3	36.3	16.2	30.7	10.1	18.2	14.5	11.9	16.6
Lb. graminis	0.1	1.7	2.4	5.2	4.3	9.8	15.3	10.8	23.1	17.4	50.4	45.6	45.7	39.2	53.7
Ln. mesenteroides	0.2	2.3	5.9	3.5	2.6	4.3	3.0	3.1	0.5	1.7	2.4	3.0	2.7	4.1	2.3
Lc. lactis	0.2	1.8	3.1	4.3	4.3	5.0	0.7	0.2	0.7	1.0	0.6	0.9	0.9	0.7	1.2
Serratia sp.	0.2	1.2	2.6	5.4	3.8	8.0	8.5	19.1	26.1	23.0	15.7	14.9	15.7	26.2	12.4
Other strains	97.9	56.8	19.1	7.4	5.7	9.3	11.2	11.9	25.1	12.2	13.8	10.6	12.4	9.6	10.4
12°C															
C. maltaromaticum	1.1	8.0	2.7	0.8	0.7	0.7	0.8	1.1	0.8	0.8	1.0	0.4	0.6	0.5	0.6
Lb. fuchuensis	0.3	83.8	92.0	92.5	88.5	83.1	79.5	65.6	61.7	62.7	43.0	53.1	54.1	46.6	43.8
Lb. graminis	0.1	1.2	0.8	0.8	2.3	6.4	5.4	12.0	11.5	12.6	33.5	24.8	19.8	33.5	31.4
Ln. mesenteroides	0.2	1.3	0.4	0.9	0.8	1.2	0.6	1.1	-	2.0	0.9	1.4	1.8	1.4	1.6
Lc. lactis	0.2	1.7	0.6	0.1	0.5	0.2	0.2	0.1	0.1	0.1	0.2	0.3	0.2	-	0.1
Serratia sp.	0.2	0.7	0.3	0.4	2.9	4.5	3.7	9.5	14.2	17.1	10.6	13.6	14.7	11.7	12.7
Other strains	97.9	3.4	3.1	4.3	4.4	4.0	9.8	10.5	11.6	4.7	10.8	6.4	8.8	6.4	9.8

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Table 24. Distribution of reads percentages for the six major bacterial species inoculated for each samples conditions obtained by metagenetic analysis during shelf life product in dynamic temperature. Others strains represented the natural microbiota of WP; -, data under the detection limit.

Bacterial strains/temperature	**Days**													
	1	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
4-8°C														
C. maltaromaticum	1.1	8.7	2.6	50.4	49.8	89.5	86.3	72.0	48.5	27.0	14.6	15.2	7.3	11.9
Lc. lactis	0.3	20.2	10.8	4.1	16.0	0.3	0.4	0.4	1.0	2.6	2.3	2.7	2.4	3.6
Ln. mesenteroides	0.1	1.5	0.5	1.1	4.8	1.2	2.5	3.1	6.8	15.7	46.1	49.5	59.6	41.2
Lb. graminis	0.2	2.3	0.2	2.0	2.5	1.3	2.2	3.0	-	4.4	3.9	4.1	1.3	4.9
Serratia sp.	0.2	1.1	0.4	1.3	2.8	0.8	0.7	0.5	1.0	0.6	0.8	1.2	0.1	1.2
Lb. fuchuensis	0.2	0.9	0.3	1.7	7.3	2.7	4.7	11.3	26.4	36.9	23.4	17.7	11.3	27.0
Other strains	97.9	65.3	85.1	39.4	16.9	4.1	3.3	9.8	16.3	12.7	8.9	9.8	18.0	10.2
4/20-4°C														
C. maltaromaticum	1.1	3.9	2.8	32.7	29.8	69.8	92.4	80.5	92.7	83.7	68.5	57.9	35.1	29.1
Lc. lactis	0.3	1.0	2.3	16.9	19.9	-	-	-	-	0.1	0.1	-	0.1	0.1
Ln. mesenteroides	0.1	0.3	0.3	0.7	4.8	2.5	1.1	4.2	0.3	0.8	2.4	4.8	10.4	12.3
Lb. graminis	0.2	0.1	0.6	2.4	3.9	1.8	0.8	2.2	-	1.8	2.0	4.6	4.7	4.8
Serratia sp.	0.2	0.4	0.3	1.7	2.4	1.7	0.5	0.4	0.2	0.1	0.3	0.2	0.7	0.4
Lb. fuchuensis	0.2	0.2	0.3	1.4	5.6	3.9	2.2	8.8	2.9	5.2	15.1	25.0	37.7	39.6
Other strains	97.9	94.1	93.5	44.4	33.5	20.4	3.1	3.9	3.8	8.4	11.6	7.5	11.2	13.6
4/20-8°C														
C. maltaromaticum	1.1	2.3	3.0	35.5	38.4	88.0	89.9	56.6	36.7	24.2	12.0	13.0	12.6	9.2
Lc. lactis	0.3	1.2	0.8	24.4	21.3	0.8	0.2	2.5	9.1	7.4	12.3	11.6	21.1	8.2
Ln. mesenteroides	0.1	0.1	0.2	1.7	3.1	1.6	1.5	6.4	5.2	20.7	39.3	29.6	29.0	46.4
Lb. graminis	0.2	0.4	0.9	2.3	2.5	1.4	1.5	3.9	-	5.6	4.4	4.0	4.1	5.2
Serratia sp.	0.2	0.4	0.3	1.1	2.5	0.8	0.3	0.5	3.8	1.1	1.1	2.9	3.1	0.7
Lb. fuchuensis	0.é	0.2	0.6	1.6	4.2	1.6	2.3	20.6	29.7	30.3	16.2	25.1	20.7	17.5
Other strains	97.6	95.5	94.1	33.4	28.0	5.8	4.2	9.5	15.6	10.7	14.8	13.8	9.5	12.8

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3.3. Combining PCA results and relative abundance to obtain estimate counts

Table 25 shows growth parameters, for each strain, calculated from the combination of the PCA counts at 22°C and the relative proportions of strains given by metagenetic (estimate abundance results) for constant temperature conditions (at 4°C, 8°C and 12°C). Using R software these parameters were obtained by fitting to a primary model of bacterial curves according to the Baranyi equation (Delhalle *et al*., 2012; Ercolini *et al*., 2011; Zwietering *et al*., 1990). These parameters give the bacterial concentration at day 16 (N_{max}, log CFU/g), the maximal bacterial growth rate (μ_{max}, 1/h) and the time to reach the stationary phase (*TRSP*, days).

These results allowed the bacterial strain subdivision into three classes based on growth parameters for each temperature conditions studied. These three classes are respectively called “dominant”, “inhibited” and “subdominant” according to their growth parameters and their behavior observed inside the bacterial ecosystem.

The “dominant” bacterial species had three high growth parameters: they have the highest growth rate (μ_{max}), maximal concentration (N_{max}) between 8 and 9 log CFU/g, and rapidly reached the stationary phase during the shelf life of the product.

The “inhibited” bacterial species had a lesser or equal growth rate but they achieved an inferior N_{max} value, and stopped their growth at the same time as the “dominant” species.

The “subdominant” bacterial species are all other bacterial species that continued growth when the “dominant” organisms reached the stationary phase, which is the opposite to the “inhibited” bacteria, with a generally lesser growth rate than the “dominant” species. They reached the stationary phase lesser rapidly but they achieved a high maximal concentration.

According to the conditions of storage the bacterial ecosystem change: *C. maltaromaticum* is the “dominant” bacteria at 4°C and 8°C, while *Lc. lactis* dominates at 12°C. *Lc. lactis* is an “inhibited” and a “sub- dominant” bacterial species for conditions at 4°C and 8°C respectively. *Lb. graminis* is a “subdominant” bacterium at 4°C and an “inhibited” specie for the two other conditions. Interestingly, *Ln. mesenteroides* and *Lb. fuchuensis* were “subdominant” bacteria at all temperatures. On the other hand, *Serratia* sp. is an “inhibited” bacterium at all temperatures.

Table 26 shows the combination of the PCA counts of the microbiota at 22°C and the relative proportions of strains obtained by metagenetic (estimate abundance results) for storage conditions with changes of temperature (at 4–8°C, 4/20–4°C and 4/20–8°C). For these situations two parameters were studied: the time necessary to attain a 7 log CFU/g threshold of spoilage (days) and the statistical difference between conditions of storage by ANCOVA-tests based on the global growth and the growth rate during exponential phase.

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The results of the ANCOVA-tests show that strains have a better bacterial growth at 4–8°C than at 4°C, except for *C. maltaromaticum* that showed a statistically different growth rate only during the exponential phase. Consequently, all species reached the 7 log CFU/g threshold earlier at 4-8°C than at 4°C (**Table 26a**). For the break of 4 h at 20°C during storage this phenomenon was significantly weaker. Indeed, *Ln. mesenteroides* is the only species which showed a significant statistically effect in the two tested parameters of growth rate. The other strains have a better global growth at 4/20–4°C (except for *C. maltaromaticum*) and all species reached the 7 log CFU/g threshold earlier at 4/20–4°C than at 4°C (**Table 26b**). Results shows also that there were no significant statistical changes on the growth parameters between the break of 4 h at 20°C and the transition from 4°C to 8°C but *Lc. lactis* and *Serratia* sp. reached the 7 log CFU/g earlier (**Table 26c**).

Table 25. Growth parameters of bacterial strains in inoculated WP under constant storage conditions. N_{max}, bacterial concentration at day 16 (log CFU/g); *TRSP*, time to reach the stationary phase (days); μ_{max}, maximal bacterial growth rate (1/h). Bacterial strains were subdivided into three categorical classes as *D*, "dominant"; *S*, "subdominant"; and *I*, "inhibited".

Bacteria / temperature	N_{max}	*TRSP*	μ_{max}	Class
4°C				
C. maltaromaticum	8.6	12	0.07	*D*
Lb. fuchuensis	8.5	16	0.05	*S*
Lb. graminis	7.6	16	0.03	*S*
Ln. mesenteroides	8.1	16	0.03	*S*
Lc. lactis	4.9	12	0.05	*I*
Serratia sp.	6.7	12	0.04	*I*
8 °C				
C. maltaromaticum	8.1	8	0.10	*D*
Lc. lactis	8.4	10	0.09	*S*
Lb. fuchuensis	8.3	10	0.09	*S*
Ln. mesenteroides	8.9	10	0.10	*S*
Lb. graminis	7.6	8	0.08	*I*
Serratia sp.	6.7	8	0.10	*I*
12 °C				
Lc. Lactis	8.9	4	0.25	*D*
Lb. fuchuensis	8.3	11	0.14	*S*
Ln. mesenteroides	8.7	11	0.10	*S*
C. maltaromaticum	7.0	4	0.10	*I*
Lb. graminis	7.4	4	0.11	*I*
Serratia sp.	6.0	4	0.12	*I*

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Table 26. Comparison of bacterial strains in inoculated WP subject to storage conditions with changes of temperature, according to the time taken to reach a 7.0 log CFU/g threshold and ANCOVA-test based on the global growth and the growth rate during exponential phase. (a) 4°C vs. 4-8°C; (b) 4°C vs. 4/20-4°C; (c) 4-8°C vs. 4/20-8°C. -, data out of range; >, superior value, ϕ, no significant statistical difference; * significant statistical difference (p-value < 0.05); ** high significant statistical difference (p-value < 0.01); *** highly significant statistical difference (p-value < 0.001).

Bacterial strains	7.0 log CFU/g threshold (days)		ANCOVA-test	
	4°C	4-8°C	Global growth	Growth rate during exponential phase
a.				
C. maltaromaticum	8	7	ϕ	4-8 > 4**
Lc. lactis	-	11	4-8 > 4***	4-8 > 4**
Lb. fuchuensis	12	8	4-8 > 4**	4-8 > 4**
Lb. graminis	14	9	4-8 > 4**	4-8 > 4**
Ln. mesenteroides	14	9	4-8 > 4***	4-8 > 4***
Serratia sp.	-	12	4-8 > 4**	4-8 > 4**
b.				
C. maltaromaticum	8	7	ϕ	ϕ
Lc. lactis	-	-	4/20-4 > 4*	-
Lb. fuchuensis	12	11	4/20-4 > 4*	ϕ
Lb. graminis	14	12	4/20-4 > 4*	ϕ
Ln. mesenteroides	14	12	4/20-4 > 4**	4/20-4 > 4*
Serratia sp.	-	-	4/20-4 > 4*	ϕ
c.				
C. maltaromaticum	7	7	ϕ	ϕ
Lc. lactis	11	9	ϕ	ϕ
Lb. fuchuensis	8	8	ϕ	ϕ
Lb. graminis	9	9	ϕ	ϕ
Ln. mesenteroides	9	9	ϕ	ϕ
Serratia sp.	12	11	ϕ	ϕ

3.4. Comparison with qPCR results

Figure 25 shows the qPCR counts for four genera at 4 (Fig. 25A), 8 (Fig. 25B) and 12°C (Fig. 25C). The comparison between metagenetic results and the LAB genus specific qPCR are summarized in **Table 27**. On average, the population overestimation was equal to 1.1 log CFU/g in qPCR test at 4°C for *Lactobacillus* and *Leuconostoc*. Indeed, bacterial curves are convergent except for *Lactobacillus* and *Leuconostoc* at 4°C.

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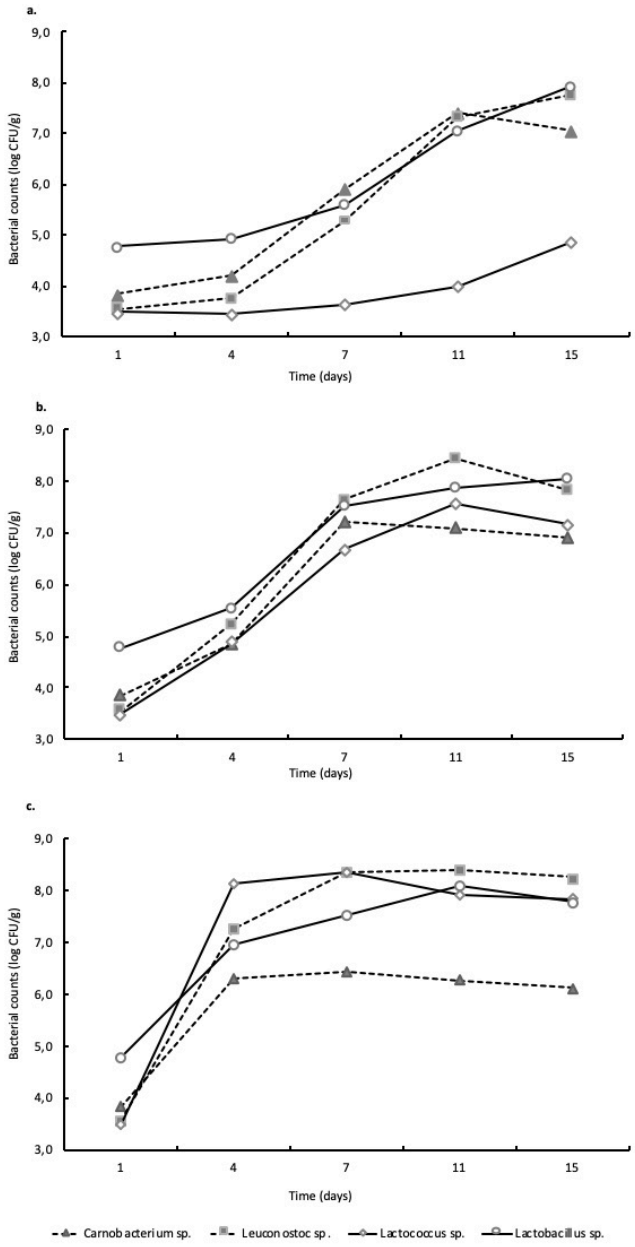

Figure 25. qPCR counts from inoculated white pudding stored at 4°C (**A**), 8°C (**B**) and 12°C (**C**).

Table 27. Comparison between qPCR and estimate abundance results (log CFU/g) for days 1, 4, 7, 11 and 15 with ANCOVA-test. Estimate abundance results: obtained by combination of the PCA results counts at 22°C and the relative proportions of strains given by metagenetic. >, superior value; ϕ, no significant statistical difference; * significant statistical difference (p-value < 0.05); ** very significant statistical difference (p-value < 0.01).

	Carnobacterium	***Lactobacillus***	***Lactococcus***	***Leuconostoc***
4°C	ϕ	qPCR > Meta**	ϕ	qPCR > Meta*
8°C	ϕ	ϕ	ϕ	ϕ
12°C	ϕ	ϕ	ϕ	ϕ

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Discussion

Based on the primary results given on total count on plate agar, the influence of temperature on the development of a whole ecosystem on Belgian WP was observed. The power of metagenetic analysis, when added to these basics results, has allowed us to closely follow the dynamics of each strain inoculated on the product during its shelf life. In addition, the data have been validated by a qPCR analysis where no significant differences were seen for the quantification of the genera studied except for *Lactobacillus* and *Leuconostoc* at 4°C. These small differences at the beginning of the shelf life can be explained by the detection of DNA from dead bacteria naturally present in large quantities on the raw meat and resulting from microbial destruction during the manufacturing process. This means that the qPCR analysis has detected some DNA fragments from dead organisms that haven't evidently grown on plate agar, leading to a weak overestimation of the qPCR results at the beginning of the experiment. This phenomenon is lesser in metagenetic analysis because of the high variability of strains presents in the product at the beginning of the experiment. Later during the challenge-test, this difference between the two techniques becomes negligible. Indeed, gradually throughout the experiment, the *Lactobacillus* and *Leuconostoc* species become a part of the dominant microbiota that leads to a dilution effect of the dead bacterial DNA by the living bacteria's DNA.

The large amount of data provided by the combination of the culture-dependent and culture-independent techniques has given useful information about the growth of each strain during challenge tests. Metagenetic analysis also allows for the assessment of the dynamics of bacterial species within a food matrix. It permitted classification of bacterial strains into different categories according to their behavior in the ecosystem. The so-called “dominant” bacterial species rapidly reached the stationary phase at a concentration of between 8 and 9 log CFU/g while at the same time the “inhibited” strains stopped their growth at a lower concentration. This phenomenon was described by Jameson in 1962 and recently reviewed by other scientists as follows: “the minority population decelerates when the majority or the total population count reaches its maximum” (Ross *et al*., 2000; Mellefont *et al*., 2008; Irlinger and Mounier, 2009; Cornu *et al*., 2011). This Jameson effect was clearly observed in our study, for example at 4°C (Table 25), when *Lc. lactis* and *Serratia* sp. stopped growth at day 12, at the same time *C. maltaromaticum* reached its maximal concentration and entered its stationary phase. In opposition to this, it can be noticed that in the same condition, some “sub-dominant” organisms continued their growth independently of the “dominant” species behavior. In this case, the Jameson effect was not illustrated. This pattern is described for all storage conditions with the same strain classifications (**Table 25**). This

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phenomenon was also observed by others scientists and they proposed that the growth of the minority population is only partly inhibited after the majority population has reached its stationary phase (Gnanou Besse *et al*., 2006; Cornu *et al*., 2011). This can be explained by the fact that the minority population is only partly affected by the limiting resource and/or inhibiting waste product that led it to stop growing (Gnanou Besse *et al*., 2006; Cornu *et al*., 2011).

According to this, the bacterial strain subdivision based on growth parameters can be represented as (**Table 28**):

If ($Nmax_{bacterial\ strain} > Nmax_{others}$) & ($\mu max_{bacterial\ strain} > \mu max_{others}$) & ($TRSP_{bacterial\ strain} < TRSP_{others}$) = *"dominant" bacterial species.*

If ($Nmax_{bacterial\ strain} \cong Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} > TRSP_{others}$) = *"subdominant" bacterial species.*

If ($Nmax_{bacterial\ strain} < Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} = TRSP_{others}$) = *"inhibited" bacterial species.*

Where N_{max} (bacterial concentration at day 16, log CFU/g), μ_{max} (maximal bacterial growth rate, 1/h) and *TRSP* (time to reach the stationary phase, days) are growth parameters.

Table 28. Bacterial strain subdivision based on growth parameters in three categorical classes: *D* ("dominant"), *S* ("subdominant"), *I* ("inhibited"). $\mu_{max,}$ maximal bacterial growth rate (1/h); N_{max}, bacterial concentration at day 16 (log CFU/g); *TRSP*, time to reach the stationary phase (days).

Class	μ_{max}	N_{max}	*TRSP*	Growth parameters
D	The highest	Maximal value. Between 8 and 9 log CFU/g	Rapidly reached	If ($Nmax_{bacterial\ strain} > Nmax_{others}$) & ($\mu max_{bacterial\ strain} > \mu max_{others}$) & ($TRSP_{bacterial\ strain} < TRSP_{others}$)
S	Generally lesser	High value	Continue to growth when the D organisms reached the stationary phase	If ($Nmax_{bacterial\ strain} \cong Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} > TRSP_{others}$)
I	Lesser or equal	Inferior value	The same as the D organisms	If ($Nmax_{bacterial\ strain} < Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} = TRSP_{others}$)

Regarding the "inhibited" strains, hypotheses can be made about inconvenient growth temperatures (T_{min}) and/or a microbial competition with the rest of the ecosystem. The composition of WP seems not to have an effect on strain competiveness. According to scientific literature, *Serratia* sp. has a minimum growth temperature (T_{min}) of 0°C (Labadie, 1999) and would normally grow at 4°C. But at this temperature, it is classified as an "inhibited" strain. Therefore, the inhibition of the bacterial growth of *Serratia* sp. is probably due to an ecosystem effect. Concerning *Lc.lactis*, one study set its T_{min} at 10°C (Labadie, 1999), while in our experiment a normal growth pattern was observed at 8°C, allowing its classification in the "sub-dominant" group. In this case, the inhibited development of this organism at 4°C is probably due to an ecosystem effect coupled with a temperature effect. Indeed, when

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Lc. lactis grew at 12°C, it became the dominant microbiota and was more competitive than *C. maltaromaticum* which seems to be more adapted to lower temperatures (T_{min} of *C. maltaromaticum* = 0°C, (Casaburi *et al*., 2011)). For *Ln. mesenteroides* and *Lb. fuchuensis*, for which T_{min} are respectively 4°C (Osmanagaoglu and Kiran, 2011) and 2°C (Zwietering *et al*., 1993), they stayed "subdominant" for all the temperature conditions tested. The rest of the ecosystem probably does not affect their growth, insofar as they never gained predominance but they were not inhibited either.

The results of the challenge tests with temperature breaks or changes are consistent with our previous observations. Indeed, *C. maltaromaticum* seems to be more adapted to low temperatures. To this end, this bacterium didn't take a great benefit in its growth when the storage temperature moved to 20°C for 4 h (**Table 26**). In contrast, the growth parameters of *Ln. mesenteroides* rose during the transition from 4°C to 8°C or with the break of 4 h at 20°C. This is consistent with the fact that its optimal growth temperature (T_{opt}) is between 20°C and 35°C (Zwietering *et al*., 1993, Jin *et al*., 2012). *Lc. lactis* also has a T_{opt} around 25°C but any improvement of its growth parameters was not observed. The hypothesis is made that the break time of 4 h was too short to see a significant effect. In conclusion, a break of 4 h at 20°C is prejudicial only if the storage temperature (4°C) is respected during the entire life of the product. Moreover, it is commonly admitted that the customer's fridge is rarely at 4°C (Lagendijk *et al*., 2010). By taking account of this fact, the lack of respect for good temperature storage (8°C instead of 4°C), particularly in customer's fridges, is more prejudicial than a break of the cold chain for up to 4 h. However, an indication about the true temperature in the product during the 4 h of breaking time at 20°C would be necessary before making this conclusion. Indeed, the internal temperature of WP samples may stay colder than 20°C, due to the thickness of this product, explaining the apparent absence of effect or a weak effect.

In the future, it will be interesting to explore the interactions in the WP ecosystem more deeply. Further studies will focus on the comprehension of the mechanisms that force the "inhibited" strains to stop their growth in the early stage of the shelf life of the product. Indeed, it is commonly accepted that the self-limiting growth process in microbial ecosystem is supposed to be due to (i) the exhaustion of one of the essential nutrients, (ii) the accumulation of metabolic waste products which inhibit growth, and/or (iii) the lowering of pH due to acid production (Cornu *et al*., 2011). According to the data already obtained, we could suppose that competition for space or nutrient has an effect. The action of a bacteriocin is also not excluded and could for example explain the lack of development of some strains inoculated into the product: *Lb. oligofermentans* and *R. terrigena*. It would also be interesting to know the spoilage or biopreservative potential of all the strains inoculated in the Belgian WP in this study. Another challenge will be in differentiating the nature of the ecosystem interactions: strain dependent or species dependent. Finally, this supply of new information will be a good start for future experiments

when it is considered that the natural contamination of a food product is more complex that an inoculation of eight bacterial strains from different species.

Our applications of the 16S rRNA gene-based amplicon sequencing has now extended our view of the dynamic behavior of complex microbial populations in Belgian WP, revealing the quantitative displacement of taxa that occur during microbial successions. By integrating metagenetic with traditional microbiological analysis we have now extended this view of a highly quantitative characterization of dynamic changes that occur during refrigerated storage. In addition to the predictive microbiology, these data also permit to classify the population dynamics into three major classes, based on growth parameters.

In conclusions, metagenetic analysis offers a new tool for identifying microorganisms present in perishable foods and for studying their dynamics within different environmental conditions. The information that can be obtained provides a clear picture of the microbial community. Microbiological ecology studies have shown that the microbiota of food is much more diverse than the cultivable group of bacteria studied by the use of culture media. The use of these new technologies will open a new era for modeling and predictive microbiology. In this study, these results provide valuable information for discussing about the theory of the Jameson effect. In addition, it will help food business operators to have a better view of the quality of their product by differentiating between the spoilage or bioprotective microbiota. Moreover, it will provide knowledge on the composition and dynamics of WP and shown how it is affected by storage temperature. Indeed, many food manufacturers, government agencies, retailers, distribution quality laboratories and researchers use classical culture media without being able to precisely identify the bacterial communities present within the food. In the future, new gold standards for food quality will need to be developed in order to allow the use of metagenetic as a complementary technique for characterizing the bacterial microbiota of products and its use should be considered as a technique for quality control, for accurately determining the length of shelf life and for developing new food products and/or new storage advices.

Study 3

Bacterial spoilage communities in Belgian food-wrap white pudding: 16S rDNA gene sequencing combining with modelling approach without interaction under isothermal conditions

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Version submitted in Food Research International (under review)

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Abstract

⇒ **Objective 1.** Study the natural microbiota of white pudding

⇒ **Objective 2.** Characterize specific spoilage bacteria dynamics and use them as inputs in models for white pudding

The aim of this study was to characterize the microbial spoilage community of Belgian WP samples using both culture-dependent and -independent methods in order to gain knowledge on spoilage bacteria. Growth models without interaction for three dominant bacteria of these samples were also performed under isothermal conditions. The ecology of the naturally contaminated WP was studied by classic microbiological plate counting and 16S rDNA amplicon sequencing at several periods during air storage at constant temperatures. At the first day, results showed a high variability and the genus *Pseudomonas* was the most represented. During the storage, other genera such as *Brochothrix* and *Psychrobacter* became dominant. To simulate microbial behavior over the storage, microbiological plate counts were then coupled to the metagenetics results in order to obtain bacterial estimations. Based on these results, the growth parameters were estimated and used to predict the microbial behavior of *B. thermosphacta*, *Pseudomonas* spp. and *Psychrobacter* spp. Three different tools were used to perform this: ComBase, Sym'Previus and the growth Baranyi function. All models showed relatively good statistical fits, but an overestimation was mainly observed. These results are in accordance with some publications in the scientific literature, but further researches are needed to better compare the three approaches. Moreover, compared to culture-based methods on selective media and previous culture-independent techniques, metagenetic analysis combined with predictive models and classical microbiology give interesting information to predict the dynamics of spoilage bacteria. Despite of the limited number of experimental datasets, the growth parameters and simulations presented in this study are interesting for the example shown, by adding to existing database for spoilage bacteria, and could be applied to others foods and food processes. Nevertheless, extensive surveys involving a large number of samples and validation data-set are required to provide a proof of concept, and to better predict the dynamics of individual microbial species in the spoilage of meat and meat products.

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Introduction

Emerging as an active area of research in the 1980s, predictive microbiology has become a useful tool in microbial shelf life prediction, quality control and risk assessment (Den Besten *et al*., 2017; Huang, 2014; Tenenhaus-Aziza and Ellouze, 2015). Predictive microbiology is a research discipline of food microbiology that uses mathematical models to describe the dynamics (growth and survival) of the populations of microorganisms undergoing complex physical, chemical and biological changes in the environment during processing, transportation, distribution and storage of foods (Fakruddin *et al*., 2012; Huang, 2014; Pla *et al*., 2015). Various models are available to predict the effects of temperature, pH, a_w, organic acids, modified atmosphere and other factors on microbial growth (Li *et al*., 2017; Martinez-Rios *et al*., 2016).

In predictive models, the change in microbial numbers is typically segmented into kinetic parameters, including lag time, growth rate (or inactivation rate), and maximum population density (Garre *et al*., 2017; Tamplin, 2018; Tarlak *et al*., 2018). After generating primary curves over a range of environmental conditions relevant to how the model will be applied, kinetic parameters are translated into 'secondary' models that describe changes in parameters as a function of the environment (e.g. the change in growth rate as a function of food storage temperature) (Baranyi and da Silva, 2017; Psomas *et al*., 2011). Then, based on the secondary and primary models, a 'tertiary' model is produced, which becomes the interface between the model and the end-user, in which environmental values are entered that result in estimations of microbial growth. Examples of tertiary model interfaces include Excel spreadsheets such as the American Meat Institute's process lethality calculator (http://www.amif.org/), and stand-alone software, such as ComBase Predictor (http://browser.combase.cc/ComBase_Predictor.aspx?model1⁄41), the USDA Pathogen Modeling Program (https://pmp.errc.ars. usda.gov/PMPOnline.aspx) (Membré and Lambert, 2008; Tamplin, 2018), and Sym'Previus (htpps://symprevius.eu/fr) (Tenenhaus-Aziza and Ellouze, 2015). More than 700 predictive models have been reported with potential applications for food. These are found in publications, in stand-alone software, and online. They include models based on microbial growth/inactivation in bacteriological media and in specific food matrices (Tamplin, 2018). However, the majority of models have been developed for pathogens, while very few ones describe the responses of spoilage bacteria (Alfaro *et al*., 2013; Membré and Lambert, 2008). Moreover, only a few studies have described the growth of a whole microbiota in a food matrix with consideration of various storage parameters and of species and/or strain dependent interactions (Ercolini *et al*., 2011; Nieminen *et al*.,

2012; Pothakos *et al*., 2015; Rouger *et al*., 2018). Even if some models consider the competition between pathogens and spoilers, most common predictive microbiology models have been generated to study one bacterial specie at various times and temperatures during the storage of foods (Ye *et al*., 2014). Furthermore, most of the data used were derived from conventional microbiological methods, which have practical limits compared to modern non-culturable methods giving a more realistic picture of microbial community dynamics in food products (Ye *et al*., 2014).

Indeed, the microbial diversity in meat and meat products have been widely investigated using traditional cultivation methods (Zhao *et al*., 2015), but it is well known that traditional identification and culture-based methods are not sufficient. In the last decades, the introduction of molecular methods, mainly culture-independent approaches, have contributed to the exploration of food microbiota and to a better description of bacterial species present in food (Benson *et al*., 2014; Chaillou *et al*., 2015; Galimberti *et al*., 2015; Riquelme *et al*., 2015). Among the culture-independent techniques, 16S rDNA amplicon sequencing has emerged as a powerful tool for studying the bacterial composition of various ecosystems (Elizaquivel *et al*., 2015; Parente *et al*., 2016). Nevertheless, only few studies have considered whole microbiota in a food matrix with consideration of various storage parameters and of species dependent interactions (Ercolini *et al*., 2011; Nieminen *et al*., 2012; Pothakos *et al*., 2014; Rouger *et al*., 2018).

The objective of this study was to thus provide a description and a prediction of the dynamics of bacterial spoilage communities in a typical Belgian meat product by combining metagenetics and classic microbiology results with predictive models.

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Material and methods

2.1. White pudding samples

Fresh WP packed under air with a food wrap film was obtained from a local Belgian manufacturer. According to the recipe, ten kg of white pudding is composed of pork minced meat (8 kg), milk (2 L), salt (200 g), maize and wheat starches (130 g), pepper (25 g), spices (10 g) and filled in pork guts.

The water activity of this product was 0.98 ± 0.02 and the pH value was 6.00 ± 0.20 (n = 4). pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Germany). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

2.2. Durability studies

Durability studies were performed according to the requirements for implementing microbiological tests of chilled perishable and highly perishable foodstuffs (AFNOR, 2010). Naturally contaminated WP samples were put (300 g), in triplicate, in a tray (175x135x22mm) under FW (cling film). The packaging film used is a common food film with a high permeability for the atmospheric gases.

WP samples were stored during 12 days of shelf life at isothermal temperature of 4°C (± 1°C), 8°C (± 1°C) and 12°C (± 1°C) in climactic chambers (Sanyo MIR 254).

At each day of the trials, total viable counts (TVC) and 16S rDNA amplicon sequencing were carried out on all the samples.

2.3. Plate count enumeration

Twenty-five grams of product were put into a Stomacher bag with a mesh screen liner (80 μm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10 g/L peptone, 5 g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were

homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (1.0 g/L peptone, 8.5 g/L sodium chloride, #CM0733, Oxoid, Hampshire, England) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis in triplicate (Spiral plater, DW Scientific, England). Total viable counts (TVC) for the aerobic psychrophilic microbiota were enumerated on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France) after 72 h at 22°C (model 1535 incubator, Shel Lab, Sheldon Manufacturing. Inc., USA).

2.4. Total DNA extraction

Bacterial DNA was extracted from each primary suspension, previously stored at – 80°C, using the DNeasy Blood & Tissue DNA Extraction kit (Qiagen, Venlo, The Netherlands), following the manufacturer's recommendations. The DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at – 20°C until used for 16S rRNA amplicon pyrosequencing.

2.5. Bacterial 16S rRNA gene amplification and barcoded pyrosequencing

16S rRNA PCR libraries targeting the V1-V3 hypervariable region were generated. Primers E9-29 and E514-430 (Brosius *et al.*, 1981), specific for bacteria, were selected for their theoretical ability to generate the lowest amplification bias relative to amplification capability among the various bacterial phyla (Wang and Qian, 2009). The oligonucleotide design included 454 Life Sciences A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs), fused to the 5' end of each primer. The amplification mix contained 5 units (U) of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1 x enzyme reaction buffer, 200 µM deoxynucleotide triphosphates (dNTPs) (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng of genomic DNA in a final volume of 100 µL. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 sec at 94°C), annealing (45 s at 56°C) and extension (60 s at 72°C), with a final elongation step (8 min at 72°C). The amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). The quality and quantity of the products were confirmed by Picogreen double-stranded DNA (dsDNA) quantitation assay (Isogen, St-Pieters-Leeuw,

Belgium). Equal amounts of each of the PCR products were pooled and subsequently amplified by emulsion PCR. Sequencing was performed using the Roche GS-Junior Genome Sequencer instrument (Roche) (2 x 300 bp).

2.6. Bioinformatics analysis

The 16S rRNA gene sequence reads were processed with MOTHUR (Schloss *et al*., 2009). The quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS).

The obtained read sets were compared to a reference data-set of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (http://www.arb-silva.de/, version 1.15, 2015) implemented in MOTHUR (Pothakos *et al*., 2014; Pruesse *et al*., 2007). The final reads were clustered into OTUs, using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA data-set 111, using a BLASTN algorithm. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (< 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Delcenserie *et* al., 2014; Pothakos *et al*., 2014).

2.7. 16S rRNA gene analysis

Alpha diversity was evaluated by richness estimation (Chao1 estimator), microbial biodiversity (inverse of the Simpson index, coverage), and the population evenness (Simpson evenness) using MOTHUR (version 1.40.5) (http://www.mothur.org) (Riquelme *et al*., 2015; Zhao *et al*., 2015). Rarefaction curves were calculated for all samples to ensure that sequencing depth was sufficient, OTUs identified were plotted as a function of sequences obtained per sample. High diversity coverage was achieved with all curves reaching asymptotes from 1000 reads (**Figure 26**).

Alpha and beta diversity indices were also calculated with bootstrapped sequencing data using Explicet (http://www.explicet.org) (Mann *et al*., 2016; Robertson *et al*., 2013). Using Explicet, bacterial population dissimilarity was assessed using the Bray-Curtis index on a 0-1 scale.

A 2-sided Welch's t-test was performed on metagenetics results and confidence intervals were calculated according to the Newcombe-Wilson method using STAMP (v2+) software

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(http://www.kiwi.cs.dal.ca/Software/STAMP). A Tukey-Kramer test in conjunction with an ANOVA was also applied for Principal Component Analysis (PCoA) in order to classify cluster samples according to the identified OTUs for the three temperatures. The differences were considered significant for a corrected p-value lesser than 0.05 (Parks *et al.*, 2014).

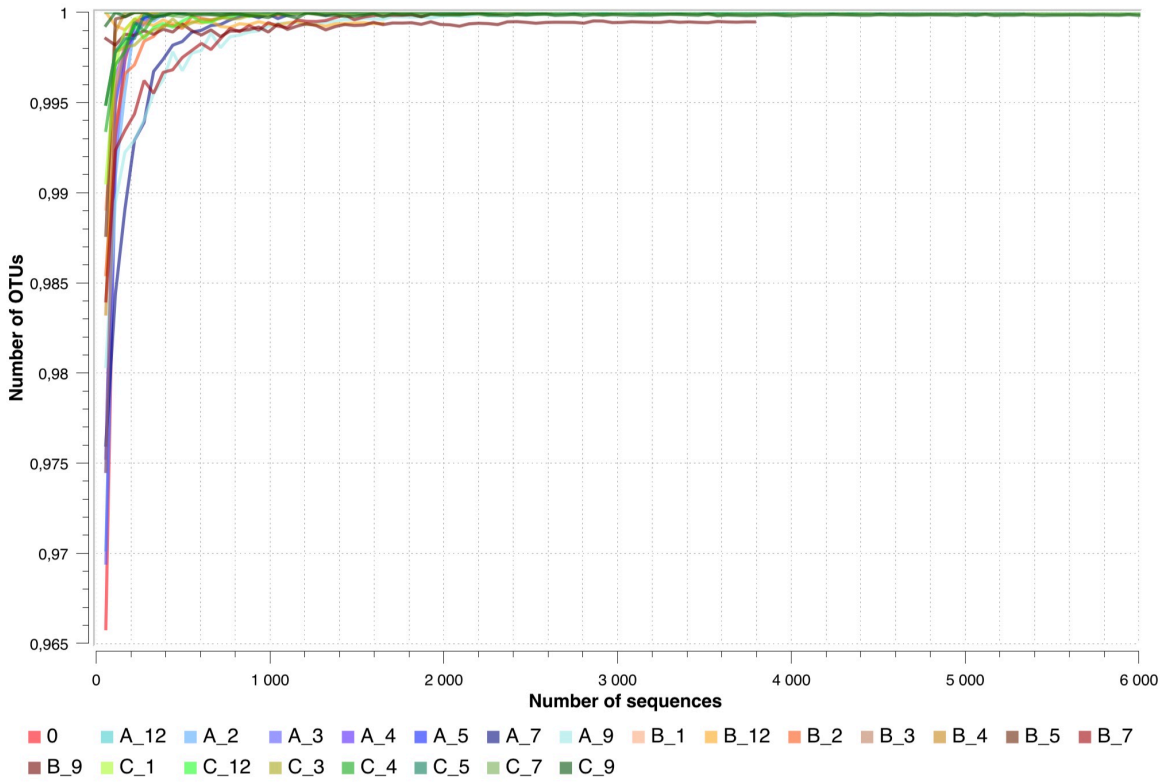

Figure 26. Rarefaction curves for samples daily analyzed at 4°C (A), 8°C (B) and 12°C (C), based on an OTU definition of 97% similarity (0.03 16S rRNA).

2.8. Bacterial estimations over storage

As described by Kembel *et al.* (2012) and Louca *et al.* (2018), correcting factors for 16S rRNA copy numbers were applied. To obtain each gene copy number, Ribosomal RNA Database (rrnDB) (Stoddard *et al.*, 2015) and EzBioCloud database (Yoon *et al.*, 2017) were used. Reads counts of all samples were then transformed into a percentage of each OTU. The percentage of each OTU was finally converted as a proportion of the total viable count, obtained by classical microbiological analysis, in order to estimate counts for each species (in $\log_{10}$ CFU/g, and expressed as mean ± standard deviation (SD)), as described by Cauchie *et al.* (2017) and Vandeputte *et al.* (2017).

Nonparametric statistical tests were used to compare the classical microbiology results from samples taken for two different storage temperatures. Analysis of covariance (ANCOVA) test was also performed to compare bacterial dynamics at 4, 8 and 12°C. All tests were considered as significant for

a p-value < 0.05.

2.9. Bacterial growth parameters

Based on previous results obtained, dominant bacteria were selected in WP samples and only these bacteria were then modelized. The primary growth model of Baranyi and Roberts (1994) was used to fit the data and estimate the growth parameters: lag phase duration (*LPD*), initial bacterial concentration (N_0), maximal bacterial concentration (N_{max}) and maximal growth rate (μ_{max}). A reparametrized version of the square root secondary model (Ratkowsky *et al*., 1982) was then used to assess the effects of temperature on the growth rates. All fittings were performed using the nlsMicrobio package (Baty and Delignette-Muller, 2013) from the open source R software (R Core Team, 2019). Extracts of the code in R for primary and secondary fittings are given in Supplemental Material.

2.10. Growth predictions and validation

Simulations were run at 4°C, 8°C and 12°C for each dominant bacterium, using three independent software programs: the Baranyi function in R software, ComBase Predictor and Sym'Previus. To run the simulations, information about the initial microorganism load (N_0), the maximum microorganisms load (N_{max}), the lag phase duration (*LPD*), the temperature and the physico-chemical characteristics (pH, a_w) were set to the observed values during the durability studies and introduced in the different software. Extracts of the code in R for predictive simulations are given in **R-commands 1**. The ANCOVA test was also used to compare validation data-set and each of the simulation results. All tests were considered as significant for a p-value < 0.05.

R-commands 1.

```
# Packages used #
require (nlsMicrobio)
require (lattice)
require (deSolve)
require (growthrates)
# Primary growth fitting #
baranyi
data<-data.frame(t=c(0,24,48,72,96,168,288),LOG10N=c(1.49,1.55,1.94,2.45,3.83,6.37,9.16))
preview(formula=baranyi,data=data,start=list(lag=48,mumax=0.07,LOG10N0=1.49,LOG10Nmax=9.16))
primary<-
nls(formula=baranyi,data=BT4data,start=list(lag=48,mumax=0.07,LOG10N0=1.49,LOG10Nmax=9.16))
# Secondary model fitting #
sqrt<-as.formula("sqrtmumax~sqrt((T>Tmin)*muref*((T-Tmin)/(20-Tmin))^2)")
secondary<-data.frame(T=c(4,8,12),sqrtmumax=sqrt(c(0.07,0.10,0.24)))
preview(formula=sqrt,data=secondary,start=list(Tmin=-3.36,muref=0.53))
# Growth prediction by Baranyi function #
baranyi
time<-c(0,48,96,120,216,288)
```

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```
y<-grow_baranyi(time,c(y0=1.49,mumax=0.05,K=8.51,h0=6.24))
y
```

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Results

3.1. Classical microbiology

Plate counts according to the temperature of storage are shown in **Figure 27**. The bacterial growth kinetics showed different dynamic changes depending on storage temperature: a high storage temperature is correlated to a faster bacterial growth during exponential phase and a shorter lag-time. At day 0, the initial bacterial concentration was around 2.90 ± 0.60 log CFU/g. Then, the bacterial population showed different dynamic changes depending on temperature, and stabilized between 8.00 and 9.00 log CFU/g. Significant differences ($p < 0.05$, ANCOVA test) were observed between the bacterial curves at 4°C and 8°C (p-value = 0.0318), and between those at 4°C and 12°C (p-value = 0.0009). No statistical differences were observed between the bacterial growth curves at 8°C and 12°C (p-value = 0.4569).

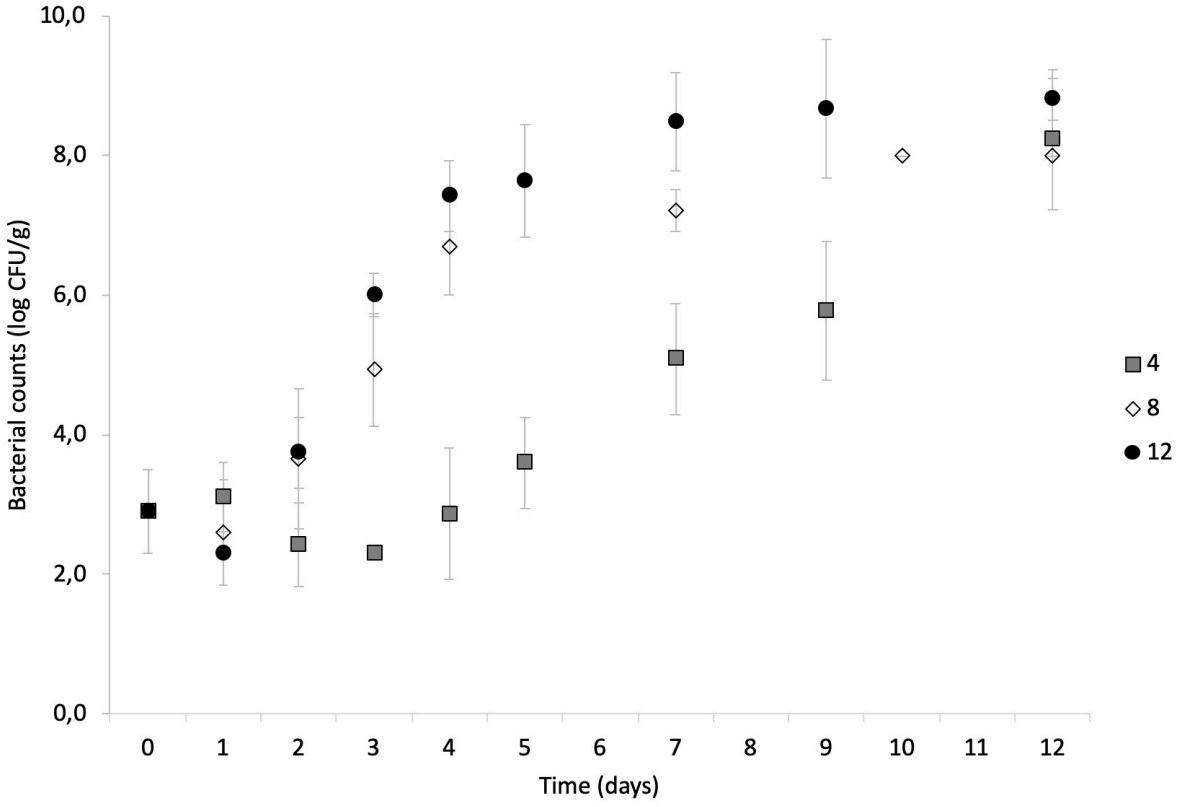

Figure 27. Plate counts for white pudding stored in food wrap packaging at 4°C (squares), 8°C (diamonds) and 12°C (triangles).

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3.2. Bacterial identification with 16S rDNA amplicon sequencing

Over 5,300 reads were generated per sample with pyrosequencing. In total, 14 main OTUs were assigned. The number of OTUs, the bacterial diversity, richness estimators and coverage are presented in Supplemental Material (**Table 29**). The highest number of identified species was encountered at 4°C, with a brutal decrease observed at 12°C.

Table 29. Alpha diversity from metagenetic analysis. For three temperature of storage at (A) 4°C, (B) 8°C, and (C) 12°C. Analysis were performed at different day of storage (day 0, 1, 2, 3, 4, 5, 7, 9 and 12).

Samples	No. Of OTUs	Coverage (%)	Inv. Simpson	Chao richness	Simpson evenness
d0	8	100.00	2.07	8	0.26
A-1	8	100.00	2.14	8	0.27
A-2	8	100.00	2.04	8	0.25
A-3	8	100.00	2.03	8	0.25
A-4	8	100.00	2.41	8	0.30
A-5	8	100.00	2.80	8	0.35
A-7	8	100.00	2.05	8	0.26
A-9	8	100.00	2.19	8	0.27
A-12	2	100.00	1.88	2	0.94
B-1	7	100.00	2.18	7	0.31
B-2	7	100.00	2.12	7	0.30
B-3	7	100.00	2.12	7	0.30
B-4	7	100.00	2.70	7	0.39
B-5	7	100.00	3.99	7	0.57
B-7	7	100.00	1.27	7	0.18
B-9	5	99.95	1.71	6	0.34
B-12	3	99.94	1.96	3	0.65
C-1	4	100.00	1.95	4	0.49
C-2	4	100.00	1.91	4	0.48
C-3	3	100.00	2.01	3	0.67
C-4	3	100.00	2.04	3	0.68
C-5	3	100.00	2.00	3	0.67
C-7	4	99.99	2.52	4	0.63
C-9	3	99.98	2.00	3	0.67
C-12	3	100.00	2.00	3	0.67

The relative abundance results obtained by metagenetic analysis (expressed in %) at Family (**Figure 28**) and Genus (**Figure 29**) levels (>5%) are represented in cumulated histograms for all samples. These data including the relative abundance of sequences are also summarized in Supplemental Material (**Table 30**). The taxa representing <5% in relative abundance were merged in the category of "Others". “Others” are mainly composed by the genera *Acinetobacter*, *Bacillus*, *Enterococcus*, *Gibbsiella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, *Pantoae* and *Pseudoalteromonas*. For all samples, the percentage of “unassigned” reads was relatively low (4.3 ± 2.8). Full data on taxa found in

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high (>5%) and low (<5%) frequencies will be made available by the authors to any qualified researcher. The observed microbial diversity was relatively high at the first day of storage, with a dominance of *Pseudomonas* (>50%). During storage, an increase of microbial counts is coinciding with a decrease in bacterial diversity: it concerns *Brochothrix* (61.3%, 41.7% and 50.5% at 4°C, 8°C and 12°C, respectively) and *Psychrobacter* (37.0%, 56.2% and 42.2%, respectively).

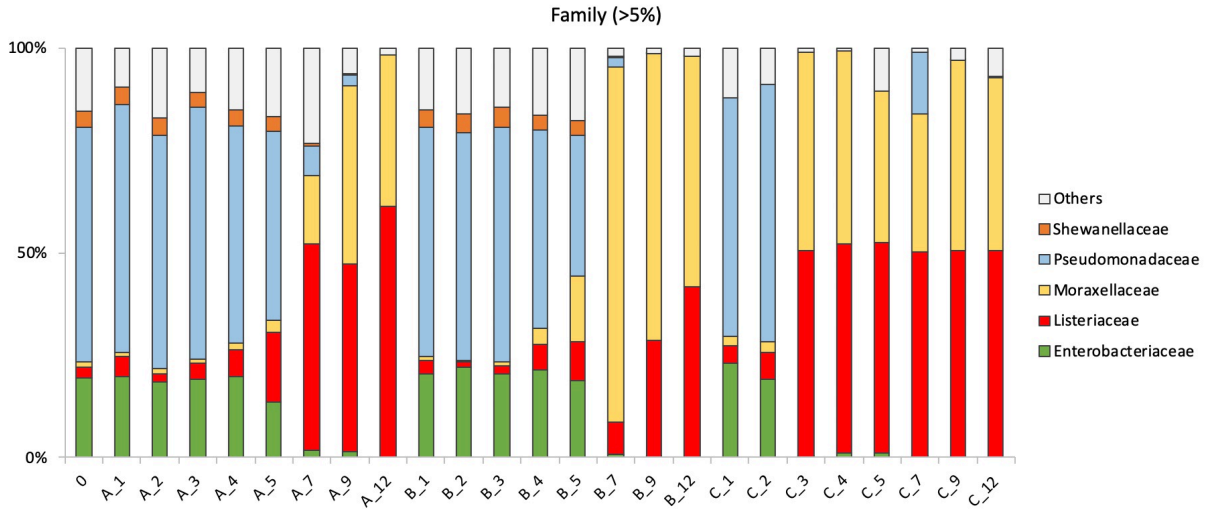

Figure 28. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at Family levels, during cold storage of white pudding samples (at 4°C (A), at 8°C (B) and at 12°C (C)). The taxa representing <1% in relative abundance of sequences are all gathered in the group "Others species".

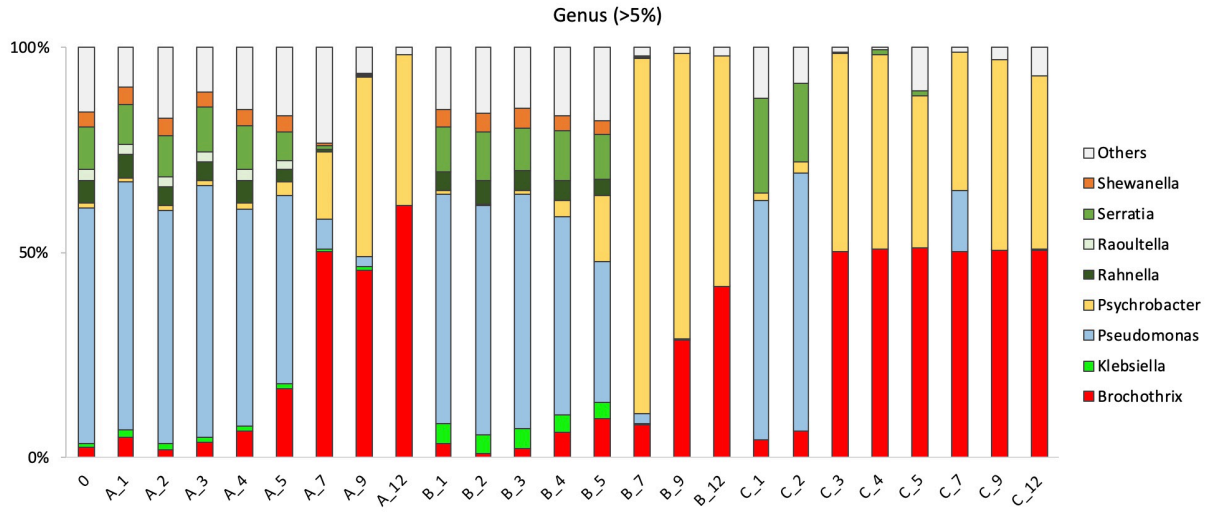

Figure 29. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at Genus levels, during cold storage of white pudding samples (at 4°C (A), at 8°C (B) and at 12°C (C)). The taxa representing <1% in relative abundance of sequences are all

gathered in the group "Others species".

Table 30. Distribution of metagenetic read percentages at Genus level during storage at three isothermal temperature. At Genus levels, the taxa representing <5% in relative abundance were merged in the category of "Others"; -*, data under the detection limit.

Bacterial genera / temperature	**Days**								
	0	1	2	3	4	5	7	9	12
4°C									
Brochothrix	2.40	4.95	1.96	3.84	6.50	16.77	50.32	45.76	61.34
Klebsiella	1.13	1.86	1.45	1.23	1.12	1.31	0.56	0.70	-*
Pseudomonas	57.35	60.41	56.78	61.41	52.81	45.94	7.16	2.70	-*
Psychrobacter	1.35	1.03	1.35	1.06	1.61	3.14	16.51	43.51	37.00
Rahnella	5.28	5.57	4.41	4.58	5.62	3.16	0.54	0.34	-*
Raoultella	2.78	2.47	2.60	2.37	2.64	2.21	0.17	0.05	-*
Serratia	10.34	9.90	10.08	10.87	10.55	6.96	0.69	0.30	-*
Shewanella	3.78	4.33	4.27	3.76	3.96	3.72	0.59	0.25	-*
Others	15.59	9.48	17.10	10.87	15.19	16.77	23.47	6.40	1.66
8°C									
Brochothrix	2.40	3.34	1.11	2.09	6.12	9.39	7.84	28.73	41.71
Klebsiella	1.13	4.87	4.50	4.96	4.41	4.11	0.30	0.01	-*
Pseudomonas	57.35	55.88	55.74	57.08	48.30	34.43	2.42	0.12	0.03
Psychrobacter	1.35	0.94	0.29	1.00	3.85	16.02	86.65	69.68	56.21
Rahnella	5.28	4.78	6.01	4.94	4.99	3.85	0.11	-*	-*
Raoultella	2.78	-*	-*	-*	-*	-*	-*	-*	-*
Serratia	10.34	10.71	11.68	10.39	12.11	10.86	0.30	-*	-*
Shewanella	3.78	4.32	4.54	4.85	3.75	3.37	0.30	0.01	-*
Others	15.59	15.16	16.12	14.70	16.49	17.96	2.08	1.44	2.05
12°C									
Brochothrix	2.40	4.34	6.52	50.24	50.84	51.28	50.20	50.57	50.50
Klebsiella	1.13	-*	-*	-*	-*	-*	-*	-*	-*
Pseudomonas	57.35	58.30	62.98	-*	-*	-*	14.92	0.01	0.30
Psychrobacter	1.35	1.98	2.54	48.21	47.34	36.99	33.63	46.47	42.21
Rahnella	5.28	-*	-*	-*	-*	-*	-*	-*	-*
Raoultella	2.78	-*	-*	-*	-*	-*	-*	-*	-*
Serratia	10.34	23.11	19.12	0.30	1.16	1.24	0.01	-*	-*
Shewanella	3.78	-*	-*	-*	-*	-*	-*	-*	-*
Others	15.59	12.27	8.84	1.25	0.66	10.49	1.25	2.95	6.99

At species level (**Figure 30**), the major OTUs concerns *Pseudomonas* sp. and *Ps. psychrophila* at the first day. While it concerns *B. thermosphacta*, *Psy. okhotskensis* and *Psy. urativorans* at the end of the shelf life.

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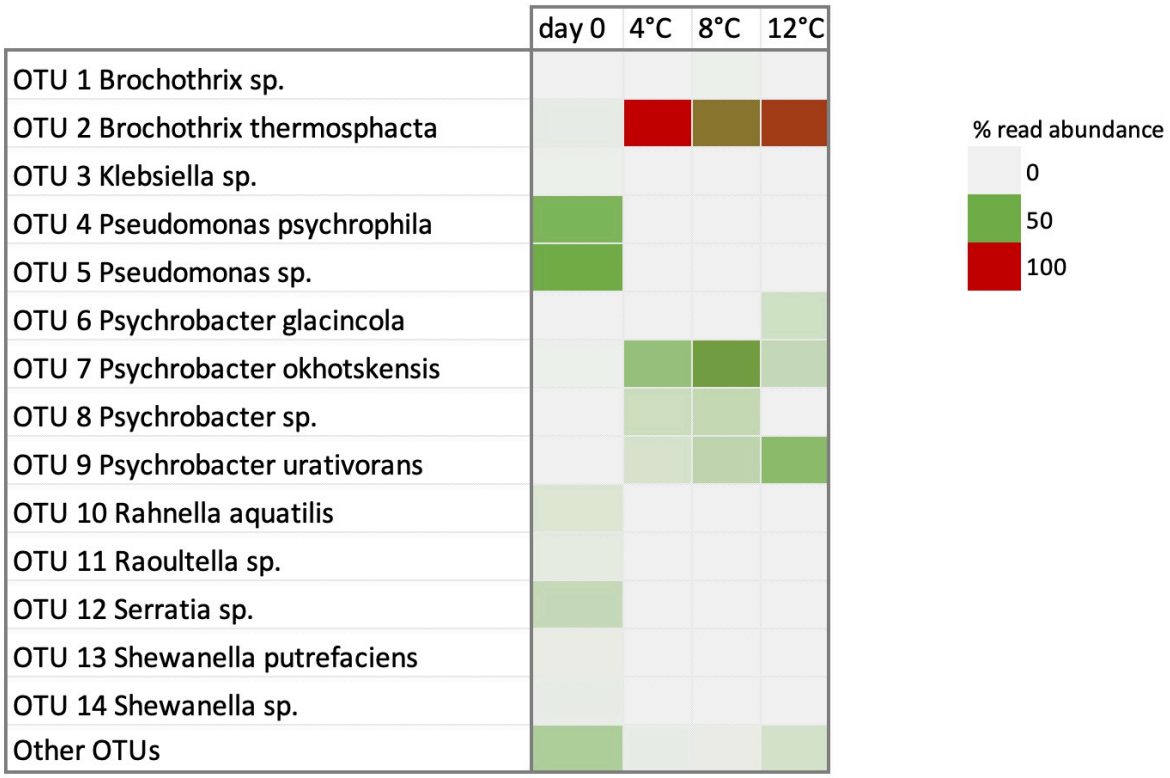

Figure 30. Heatmap of relative read abundance at species level for all samples (expressed in %) among different storage temperature (at 4°C, 8°C and 12°C). Only the most abundant OTUs obtained in this study are specially indicated (>1%). All others OTUs are gathered in "Other OTUs".

3.3. Comparison between the food storage conditions

The metagenetic dissimilarity and cluster results obtained for the different storage temperature are showed in **Figures 31** and **32**. The best similarity is observed between OTUs at 4°C and 8°C. During the storage period an increase in the dissimilarity was also observed (**Figure 33**).

Study 3. Bacterial spoilage communities in Belgian food-wrap white pudding: 16S rDNA gene sequencing combining with modelling approach without interaction under isothermal conditions.

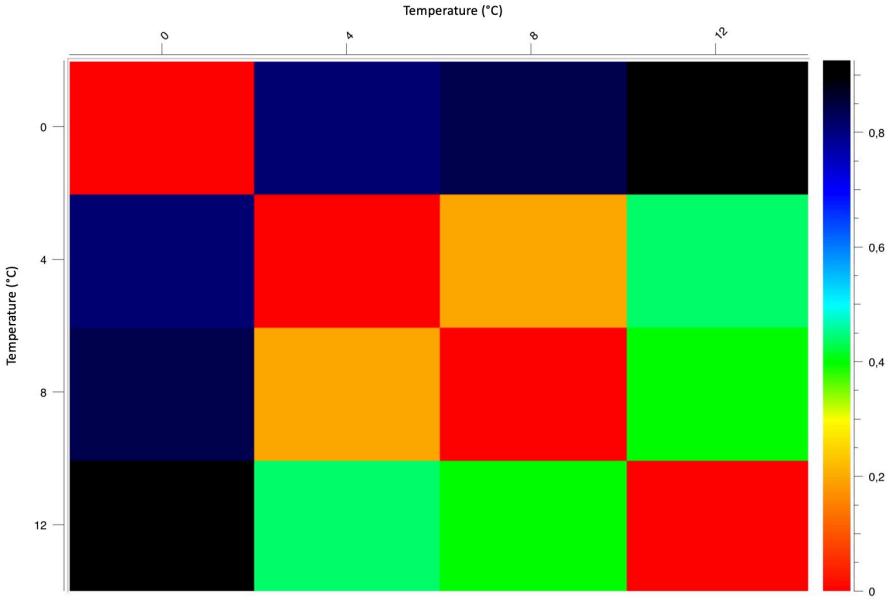

Figure 31. Microbial community dissimilarity between samples at the first and the last day of storage (at 4°C (4), 8°C (8) and 12°C (12)). The heatmap shows the Bray-Curtis dissimilarity measure based on relative abundance of OTUS (genus scale). Values are given in dissimilarity counts (1= 100% dissimilar, 0=0% dissimilar).

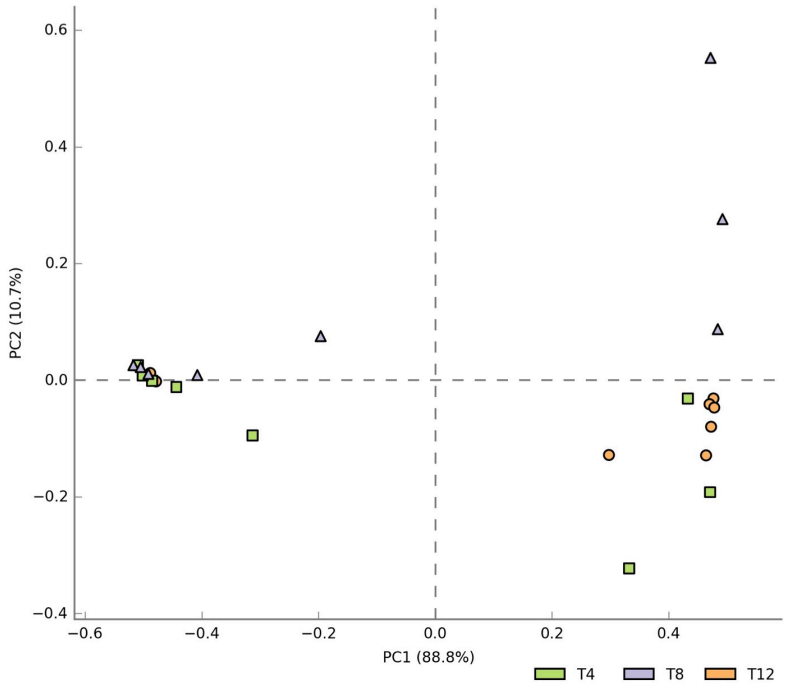

Figure 32. Principal component analysis for 16S rRNA gene sequence data for isothermal conditions (T) at 4, 8, and 12°C.

Study 3. Bacterial spoilage communities in Belgian food-wrap white pudding: 16S rDNA gene sequencing combining with modelling approach without interaction under isothermal conditions.

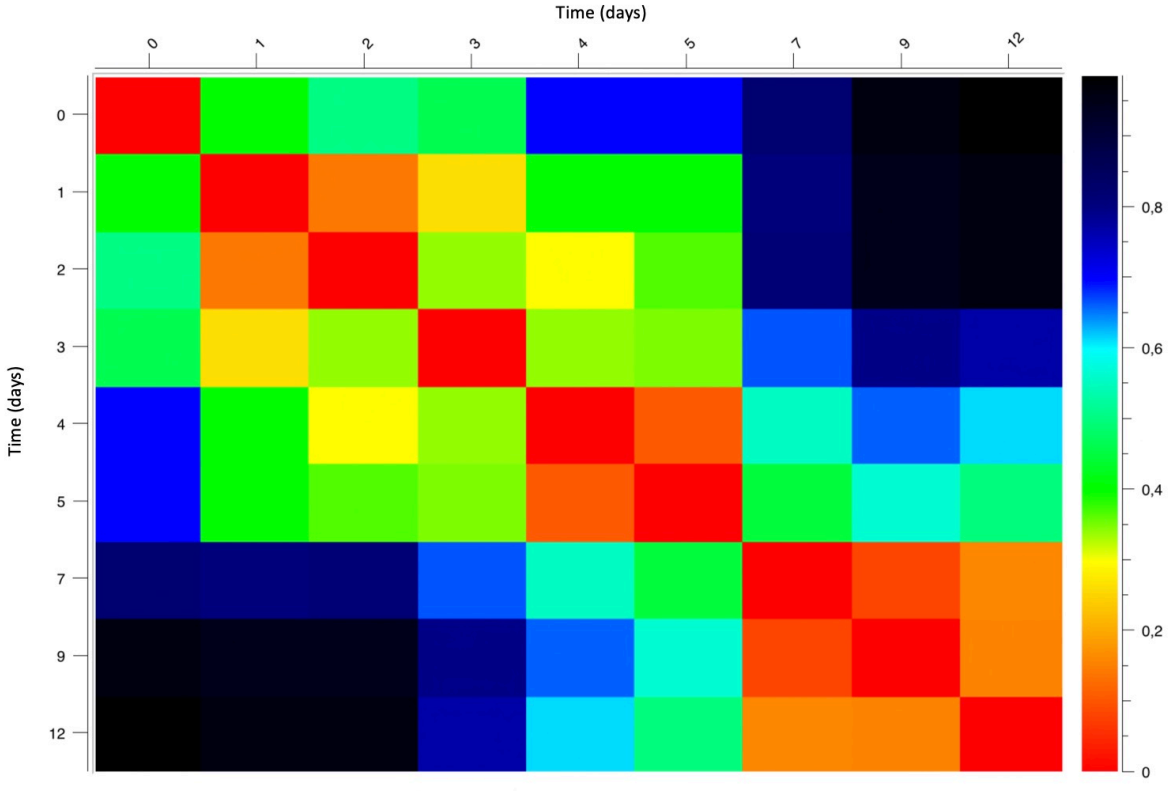

Figure 33. Microbial community dissimilarity between days of samples analysis (at day 0, 1, 2, 3, 4, 5, 7, 9 and 12). The heatmap shows the Bray-Curtis dissimilarity measure based on relative abundance of OTUS (genus scale). Values are given in dissimilarity counts (1 = 100% dissimilar, 0 = 0% dissimilar).

3.4. Growth parameters

For the rest of the study, *B. thermosphacta*, *Pseudomonas* sp. and *Psychrobacter* spp. (*Psy. urativorans* and *Psy. okhotskensis*) were, therefore, selected as the main spoilers in WP samples as they are the dominant population during shelf life in this product, and because these bacteria are well described in the literature and in predictive model's database.

The duplicates from the durability study were used for growth predictions. On the other hand, the third data-set was used to compare predictions and observations for validation purposes.

As a next step, the metagenetics data were thus combined with the plate counts results (**Table 31**) in order to obtain primary and secondary growth parameters (**Table 32**).

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Table 31. Bacterial concentrations (log CFU/g) used in the models for *B. thermosphacta*, *Pseudomonas* sp. and *Psychrobacter* spp. at 4°C, 8°C and 12°C; -[a], no analysis performed.

Conditions	Time (hours)												
	0	**24**	**48**	**72**	**96**	**120**	**144**	**168**	**192**	**216**	**240**	**264**	**288**
						B. thermosphacta							
4°C	1.49	-[a]	1.55	1.94	2.45	3.83	-[a]	-[a]	-[a]	6.37	-[a]	-[a]	9.16
8°C	1.49	-[a]	-[a]	1.94	3.22	4.89	-[a]	6.82	-[a]	7.90	-[a]	-[a]	8.51
12°C	1.49	1.84	3.43	6.90	-[a]	8.27	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	8.83
						Pseudomonas sp.							
4°C	2.53	-[a]	2.53	-[a]	2.83	3.75	-[a]	4.54	-[a]	4.80	-[a]	-[a]	-[a]
8°C	2.53	2.69	-[a]	-[a]	3.73	-[a]	-[a]	-[a]	-[a]	5.06	-[a]	-[a]	5.01
12°C	2.53	2.77	3.99	4.52	6.55	6.93	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
						Psychrobacter spp. (*Psy. urativorans* and *Psy. okhotskensis*)							
4°C	1.22	-[a]	1.44	-[a]	-[a]	2.97	-[a]	4.78	-[a]	6.04	-[a]	-[a]	8.43
8°C	1.22	-[a]	-[a]	-[a]	-[a]	4.80	-[a]	6.86	-[a]	7.59	-[a]	-[a]	8.38
12°C	1.22	-[a]	-[a]	5.92	6.78	7.91	-[a]	8.61	-[a]	9.15	-[a]	-[a]	9.10

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Table 32. Estimation of the primary and secondary parameters at 4, 8 and 12°C. [a] bacterial concentration at day 0, [b] bacterial concentration at the end of the shelf life product, [c] lag phase duration, [d] maximal bacterial growth rate (1/h), [e] bacterial growth rate of reference (1/h) obtained using a reparametrized version of the square root secondary model, [f] minimal temperature for growth (°C), coefficient of determination (r^2) and confidence interval of 95% (in square brackets).

Primary parameters of growth	***B. thermosphacta***	***Pseudomonas* sp.**	***Psychrobacter* spp.**
4°C			
N_0 (log CFU/g)[a]	1.49 [1.39-1.59]	2.53 [2.44-2.62]	1.22 [1.21-1.23]
N_{max} (log CFU/g)[b]	9.16 [9.16-9.16]	4.80 [4.80-4.80]	8.53 [8.53-8.53]
LPD (h)[c]	48 [48-50]	66 [65-67]	50 [50-50]
μ_{max} (1/h)[d]	0.07 [0.07-0.07]	0.05 [0.05-0.05]	0.07 [0.07-0.07]
r^2	0.99	0.98	0.98
8°C			
N_0 (log CFU/g)	1.49 [1.39-1.59]	2.53 [2.44-2.62]	1.22 [1.21-1.23]
N_{max} (log CFU/g)	8.51 [8.51-8.51]	5.01 [5.01-5.70]	8.38 [8.38-8.38]
LPD (h)	48 [48-50]	40 [40-40]	40 [40-40]
μ_{max} (1/h)	0.10 [0.10-0.10]	0.07 [0.03-0.07]	0.10 [0.10-0.10]
r^2	0.99	0.98	0.98
12°C			
N_0 (log CFU/g)	1.49 [1.39-1.59]	2.53 [2.44-2.62]	1.22 [1.21-1.23]
N_{max} (log CFU/g)	8.83 [8.83-8.83]	6.93 [6.93-6.93]	9.10 [9.10-9.10]
LPD (h)	24 [24-24]	20 [20-20]	10 [10-10]
μ_{max} (1/h)	0.24 [0.24-0.24]	0.11 [0.11-0.11]	0.15 [0.15-0.15]
r^2	0.99	0.99	0.99
Secondary parameters of growth			
μ_{ref}[e]	0.53 [0.53–0.53]	0.25 [0.21-0.25]	0.29 [0.29-0.29]
r^2	0.99	0.99	0.99
T_{min}[f]	-3.36 (33)	-5.00 (58, 59)	-10.00 (4)

3.5. Growth models and validation step

Table 33 gives an overview of the simulation hypothesis used for the three software. Growth predictions for each bacterium are also presented in **Tables 34, 35** and **36**. For the 3 bacteria concerned (*B. thermosphacta*, *Pseudomonas* sp. and *Psychrobacter* spp.), the comparison between the observations from the validation data-set given by the three software programs for is presented in **Figure 34**. *Psychrobacter* spp. could not be modeled in ComBase because the database did not include this bacterium.

A relatively good agreement was obtained between the observations from the validation data-set and simulations, especially for *Pseudomonas* sp. Moreover, the predictions of Sym'Previus and

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Baranyi's model seems often quite similar. But overestimations were frequently observed for *B. thermosphacta* and *Psychrobacter* spp., at 8°C and 4°C, respectively. On the maximum population density, ComBase also showed higher values (approximatively 8.00 log CFU/g) compared to the other two software programs.

Table 33. Simulation hypothesis used for the three predictive software programs. -[a], not considered in the model; -[b], calculated by polynomial model of the tool; -[c], calculated by model of the tool for a reference at 8°C; -[d], model based on the reparametrized version of the square root secondary model; -[e], bacteria not available in the ComBase Predictor database.

Predictive software	**Temp. (°C)**	**pH**	**a_w**	***N_0*** **(log CFU/g)**	***N_{max}*** **(log CFU/g)**	***LPD*** **(h)**	***μ_{max}*** **(1/h)**	***Phys. state***
				B. thermosphacta				
ComBase	4, 8, 12	6.00	0.98	1.49	-[a]	-[a]	-[b]	5.75×10^{-7}
Sym'Previus	4, 8, 12	6.00	0.98	1.49	8.51	48[c]	0.13[c]	-[a]
R	4, 8, 12	-[a]	-[a]	1.49	8.51	48	0.05,0.13,0.23[d]	-[a]
				Pseudomonas sp.				
ComBase	4, 8, 12	6.00	0.98	2.53	-[a]	-[a]	-[b]	1.58×10^{-3}
Sym'Previus	4, 8, 12	6.00	0.98	2.53	5.01	40[c]	0.07[c]	-[a]
R	4, 8, 12	-[a]	-[a]	2.53	5.01	40	0.03,0.07,0.12[d]	-[a]
				Psychrobacter spp.				
ComBase	-[e]	-[e]	-[e]	-[e]	-[e]	-[e]	-[e]	-[e]
Sym'Previus	4, 8, 12	6.00	0.98	1.22	8.38	40[c]	0.09[c]	-[a]
R	4, 8, 12	-[a]	-[a]	1.22	8.38	40	0.05,0.09,0.13[d]	-[a]

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Table 34. Simulations of bacterial growth using Sym'Previus, ComBase and the Baranyi function for *B. thermosphacta*. These results were compared with validation data-set; -*, significant statistical difference by ANCOVA-test (p-value < 0.05) between the validation data-set and each of the predictive simulations; -[a], no analysis performed.

Parameters (Temperature / hours)		Simulations of growth			Validation data-set
		ComBase	Sym'Previus	Baranyi function	
4°C	0	1.52	1.49	1.49	1.49
	24	-[a]	-[a]	-[a]	-[a]
	48	1.52	2.01	1.51	1.82
	72	-[a]	-[a]	-[a]	-[a]
	96	1.53	5.25	1.76	2.67
	120	1.65	6.77	2.33	3.85
	144	-[a]	-[a]	-[a]	-[a]
	168	-[a]	-[a]	-[a]	-[a]
	192	-[a]	-[a]	-[a]	-[a]
	216	5.65	8.42	8.71	6.30
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	7.75	8.45	8.49	8.11
8°C	0	1.52	1.49*	1.49*	1.49
	24	-[a]	-[a]	-[a]	-[a]
	48	-[a]	-[a]	-[a]	-[a]
	72	1.81	8.30*	7.09*	1.81
	96	3.57	8.44*	8.43*	3.56
	120	5.63	8.47*	8.50*	4.86
	144	-[a]	-[a]	-[a]	-[a]
	168	7.80	8.48*	8.50*	7.68
	192	-[a]	-[a]	-[a]	-[a]
	216	7.83	8.50*	8.51*	6.46
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	7.83	8.50*	8.51*	8.53
12°C	0	1.52	1.49	1.49	1.49
	24	1.52	4.34	2.03	2.05
	48	1.87	8.40	8.19	4.63
	72	4.78	8.49	8.50	6.86
	96	-[a]	-[a]	-[a]	-[a]
	120	7.82	8.50	8.51	7.50
	144	-[a]	-[a]	-[a]	-[a]
	168	-[a]	-[a]	-[a]	-[a]
	192	-[a]	-[a]	-[a]	-[a]
	216	-[a]	-[a]	-[a]	-[a]
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	7.83	8.50	8.51	9.34

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Table 35. Simulations of bacteria growth using Sym'Previus, ComBase and the Baranyi function for *Pseudomonas* sp.. These results were compared with validation data-set; -*, significant statistical difference by ANCOVA-test (p-value < 0.05) between the validation data-set and each of the predictive simulations; -[a], no analysis performed.

Parameters (Temperature / hours)		Simulations of growth			Validation data-set
		ComBase	Sym'Previus	Baranyi function	
4°C	0	2.52	2.53	2.53	2.53
	24	-[a]	-[a]	-[a]	-[a]
	48	2.54	2.65	2.75	2.81
	72	-[a]	-[a]	-[a]	-[a]
	96	2.81	3.92	3.37	3.27
	120	3.27	4.51	3.82	3.57
	144	-[a]	-[a]	-[a]	-[a]
	168	4.59	4.90	4.57	4.69
	192	-[a]	-[a]	-[a]	-[a]
	216	5.97	4.96	4.89	5.20
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	-[a]	-[a]	-[a]	-[a]
8°C	0	2.52*	2.53	2.53	2.53
	24	-[a]	-[a]	-[a]	-[a]
	48	-[a]	-[a]	-[a]	-[a]
	72	-[a]	-[a]	-[a]	-[a]
	96	4.29*	4.96	4.91	3.95
	120	-[a]	-[a]	-[a]	-[a]
	144	-[a]	-[a]	-[a]	-[a]
	168	-[a]	-[a]	-[a]	-[a]
	192	-[a]	-[a]	-[a]	-[a]
	216	8.23*	4.99	5.00	4.36
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	8.26*	5.01	5.01	5.31
12°C	0	2.52	2.53	2.53	2.53
	24	2.56	3.47	3.37	2.67
	48	3.35	4.88	4.77	4.14
	72	5.07	4.99	4.99	4.68
	96	6.81	5.01	5.00	6.00
	120	8.03	5.01	5.01	6.37
	144	-[a]	-[a]	-[a]	-[a]
	168	-[a]	-[a]	-[a]	-[a]
	192	-[a]	-[a]	-[a]	-[a]
	216	-[a]	-[a]	-[a]	-[a]
	240	-[a]	-[a]	-[a]	-[a]
	264	-[a]	-[a]	-[a]	-[a]
	288	-[a]	-[a]	-[a]	-[a]

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Table 36. Simulations of bacterial growth using Sym'Previus, ComBase and the Baranyi function for *Psychrobacter* spp.. These results were compared with validation data-set; -*, significant statistical difference by ANCOVA-test (p-value < 0.05) between the validation data-set and each of the predictive simulations; -a, bacteria not available in the ComBase Predictor database; -b, no analysis performed.

Parameters (Temperature / hours)		Simulations of growth			Validation data-set
		ComBase	Sym'Previus	R software	
4°C	0	-a	1.22*	1.22*	1.22
	24	-b	-b	-b	-b
	48	-a	1.83*	1.49*	1.75
	72	-b	-b	-b	-b
	96	-b	-b	-b	-b
	120	-a	5.04*	5.62*	2.49
	144	-b	-b	-b	-b
	168	-a	7.19*	7.99*	4.92
	192	-b	-b	-b	-b
	216	-a	8.28*	8.34*	5.79
	240	-b	-b	-b	-b
	264	-b	-b	-b	-b
	288	-a	8.37*	8.37*	7.35
8°C	0	-a	1.22	1.22	1.22
	24	-b	-b	-b	-b
	48	-b	-b	-b	-b
	72	-b	-b	-b	-b
	96	-b	-b	-b	-b
	120	-a	8.35	8.34	5.04
	144	-b	-b	-b	-b
	168	-a	8.37	8.37	7.18
	192	-b	-b	-b	-b
	216	-a	8.37	8.37	7.57
	240	-b	-b	-b	-b
	264	-b	-b	-b	-b
	288	-a	8.37	8.38	8.39
12°C	0	-a	1.22	1.22	1.22
	24	-b	-b	-b	-b
	48	-b	-b	-b	-b
	72	-a	8.34	8.22	5.77
	96	-a	8.38	8.37	5.72
	120	-a	8.38	8.37	8.07
	144	-b	-b	-b	-b
	168	-a	8.38	8.38	8.62
	192	-b	-b	-b	-b
	216	-a	8.38	8.38	9.11
	240	-b	-b	-b	-b
	264	-b	-b	-b	-b
	288	-a	8.38	8.38	9.50

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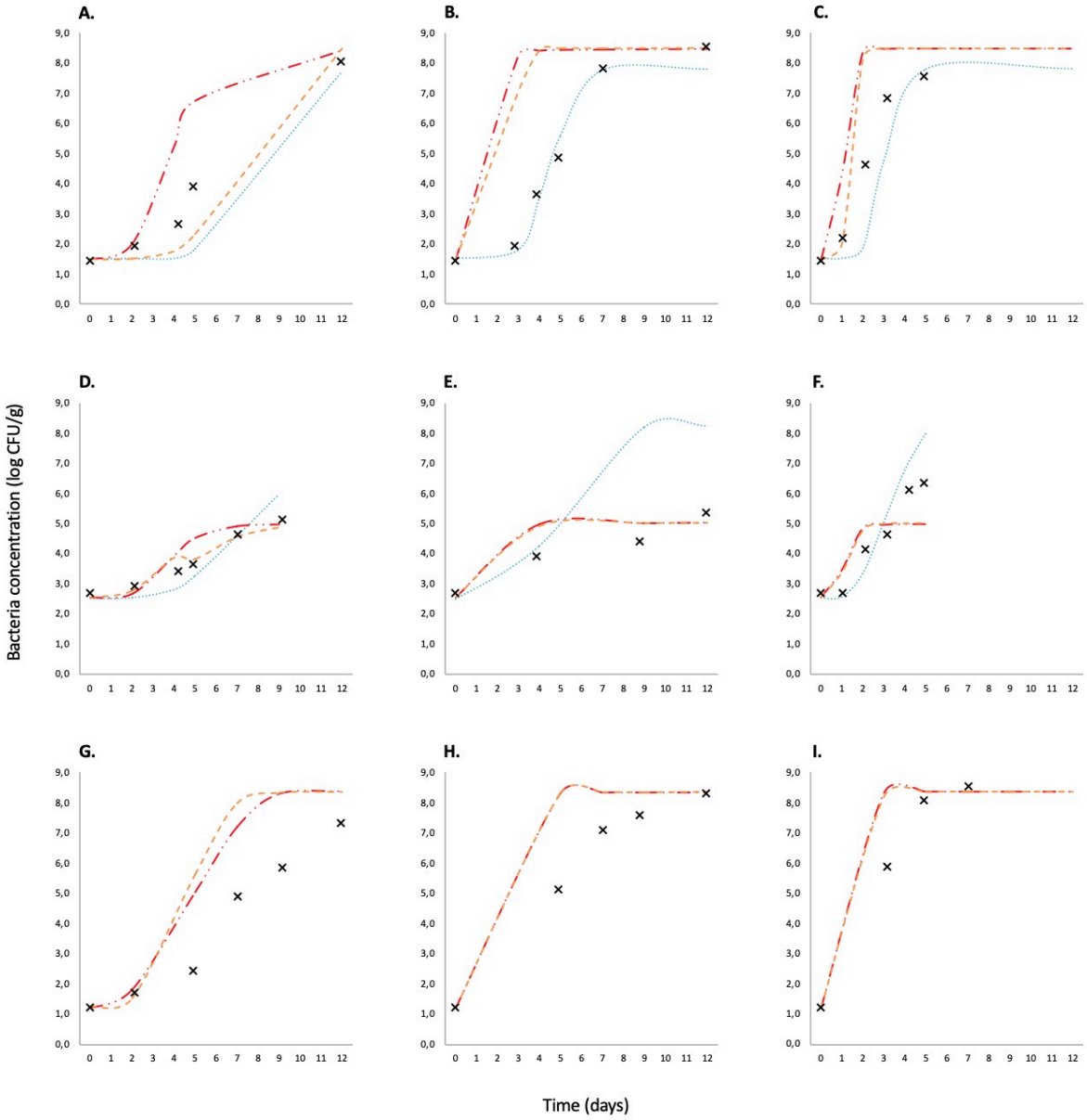

Figure 34. Observed (x) and predicted results (lines) for *B. thermosphacta* at 4 (A), 8 (B) and 12°C (C), for *Pseudomonas* sp. at 4 (D), 8 (E) and 12°C (F), and for *Psychrobacter* spp. at 4 (G), 8 (H) and 12°C (I). The predicted data obtained by the three software are indicted by lines: ComBase (blue, dotted line), Sym'Previus (red, dashed line) and the Baranyi function (green, broken line).

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Discussion

This experiment aims at following the bacterial growth in Belgian WP stored at isothermal conditions under food-wrap packaging.

At the end of the storage period, the bacterial counts were over 7.0 log CFU/g, indicating, given the nature of the dominant bacterial populations, that the product had probably begun to be spoiled and would be no longer suitable for human consumption. Indeed, it is generally recognized that microbial spoilage of meat occurs when counts reach arbitrary level between 7.0 log CFU/g (Reid *et al*., 2017; Spanu *et al*., 2018; Stoops *et al*., 2015; Zhao *et al*., 2015) and 8.0 log CFU/g (Chaillou *et al*., 2015; Fall *et al*., 2012; Nychas *et al*., 2008; Pothakos *et al*., 2014). Although culturable method is widely used to follow the microbial growth in a food matrix, these results are not informative enough alone to describe the dynamics of the bacterial communities. Indeed, the microbial diversity in terms of species richness and abundance is grossly underestimated by culture-dependent techniques (Stoops *et al*., 2015). Moreover, the problem with studies of the microbiota associated with food spoilage is that many articles focused only on one bacterial specie and did not consider the whole bacterial microbiota and their dynamics (Rouger *et al*., 2017). According to this, a sequence-based approach at the genus-species level can be used as a supplementary technique to elucidate the detailed dynamics of the microbial population during storage, because different species in the same groups of bacteria may be differently affected by the same storage conditions. In this study, the metagenetics data were combined with the plate counts results, regarding the detected major population (*B. thermosphacta*, *Psychrobacter* sp. and *Pseudomonas* sp.) whose is able to cultivate in these conditions. Moreover, association of relative abundance results with cell counts is well described in microbiome analysis (Amend *et al*., 2010; Guo *et al*., 2019). This method was also described by Vandeputte *et al*. (2017), who associated flow cytometric enumeration of microbial cells with sequencing data (relative microbiome profiles, RMP) in order to obtain quantitative microbiome profiles (QMP). In this study, QMP allow to investigate microbiota variations and to reduce the microbial abundance of the microbiome associated with Crohn's disease. Comparatively with the relative microbiome profiles (RMP), this method enables quantitative assessment of microbiota variations, even if all of the current biases of this approach were not addressed. Moreover, Vandeputte *et al*. (2017) also showed that quantitative qPCR (qPCR) and flow cytometric enumeration of microbial cells yield comparable abundance profiles. This point is important as we validated this method by a qPCR approach in another experimental study (Cauchie *et al*., 2017). It could be so interesting to validate the proposed method by other qPCR or flow cytometric enumerations.

However, the inability of differentiation between viable and non-viable cells by culture-independent DNA-based methods remains an important drawback, which could result in a significant over-estimation of viable species (Scariot *et al*., 2018; Tantikachornkiat *et al*., 2016). Systematic biases of processing and analysis steps in culture-dependent and -independent methods must also be considered (Louca *et al*., 2018; Pollock *et al*., 2018; Salter *et al*., 2014).

During storage, the major genus in WP samples are represented by *Brochothrix*, *Pseudomonas* and *Psychrobacter*. These results are not surprising because the microbial populations of refrigerated meat and pork-meat products are mainly composed by *Pseudomonas* spp., cold-tolerant *Enterobaceriaceae*, LAB (such as *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Carnobacterium* spp., etc.), *B. thermosphacta*, *Clostridium* spp. (Del Blanco *et al*., 2017; Geeraerts *et al*., 2017; Zhao *et al*., 2015), *Psychrobacter* spp. (Casaburi *et al*., 2015; Ferrocino *et al*., 2017; Zhang *et al*., 2018b) and *Weissella* spp. (Stellato *et al*., 2016). Other genera frequently isolated from fresh pork meats are *Acinetobacter* spp., *Aeromonas* spp., *Enterococcus* spp. and *Moraxella* spp. (Mann *et* al., 2016; Zhao *et al*., 2015). Their abundance and contribution to spoilage are largely influenced by the storage conditions (Stoops *et al*., 2015), like temperature and oxygen availability (Crotta *et al*., 2016), because not all the members of this microbiota contribute to spoilage.

For the modeling part of the study, only *Brochothrix*, *Pseudomonas* and *Psychrobacter* were used in fitting models. These bacteria represented the dominant spoilage bacteria of Belgian WP samples according to time and storage temperature. These bacteria are also known to be able to grow in different gas compositions (Ercolini *et al*., 2011; Mann *et al*., 2016; Mansur *et al*., 2019; Nychas *et al*., 2008; Reid *et al*., 2017) and are psychrophilic (Leroi *et al*., 2012; Martinez-Rios *et al*., 2016). Under aerobic low temperature conditions, the spoilage consortium of bacteria is usually dominated by *Pseudomonas* spp., which triggers slime, discoloration and off-odor producing (Liu *et al*., 2018; Reid *et al*., 2017; Spanu *et al*., 2018). *Brochothrix* spp. (especially *B. thermosphacta*) seems to be also the most abundant bacteria in minced meat (Del Blanco *et al*., 2017; Koutsoumanis *et al*., 2008), leading to spoilage of meat by a sour, acid and cheesy odor (Fall *et al*., 2012). Moreover, *Psychrobacter* is a food spoilage bacterium, especially in fermented meat under refrigerated conditions, associated with various spoilage compounds (Ferrocino *et al*., 2017; Zhang *et al*., 2018b).

In this study, the growth of each bacteria was assessed daily at the same time, by combining classical microbiology and 16S rDNA amplicon sequencing, with the aim to explore the microbial community and to obtain quantitative results (Fougy *et al*., 2016). However, without extensive surveys involving a large number of samples it will not be possible to characterize the food spoilage microbiota and to study their dynamics (Pennacchia *et al*., 2011). In further studies, it would be thus interesting to study the microbiota diversity by multiply sampling or experiments with the metagenetic approach, by tests in different storage conditions (temperature, packaging, …), and by evaluating the interactions

between samples during food production, because they can all influence the shelf life of the product (Rouger *et al*., 2017). Models should also include all other bacteria present in our samples. In this paper, no conclusions about subdominant bacteria or bacterial ecosystems for other storage conditions should be dawn. Further data are so needed to determine and follow spoilage bacterial communities in Belgian WP samples, but the method used could be applied for others food and food process.

In addition to predictive microbiology, these data permitted us to simulate the growth of each dominant bacterium. Even if none of the predictive simulations gave an identical microbial curve to the validation kinetics obtained by the aging-test, good statistical fits were obtained, showing that the approach combining the metagenetics and simulations based on an accurate database is promising. It should nevertheless be noticed that an overestimation was sometimes observed. In a worst-case assumption, this overestimation could be beneficial to decision makers in a spoilage risk analysis (Crotta *et al*., 2016; Membré and Lambert, 2008). Moreover, these results are in accordance with the study of Nyhan *et al*. (2018) where the calculated growth rates of *Listeria* spp. were underestimated with ComBase Predictor. In contrast, Leroi *et al*. (2012) demonstrated an overestimation of the maximum growth rate for *B. thermosphacta* in cooked peeled shrimp with the same software. As reported in the literature, the best performance factors were mainly observed with R and Sym'Previus (Valerio *et al*., 2015). Some hypotheses can explain these differences beside the fact that prediction errors can originate from biological and environmental variability, and the inaccuracy of the mathematical models and assumptions used (Baranyi and da Silva, 2017). ComBase model doesn't allow to modulate the N_{max} to observed lower values, in contrast with the two others. Food matrix and intrinsic parameters may have an effect on the bacterial growth. But only ComBase and Sym'Previus software applications consider the intrinsic parameters of Belgian WP, such as pH and water activity. That can maybe explain why an overestimation is mainly observed. It is possible to add intrinsic parameters in the Baranyi function, but more cardinal values about the spoilage microorganisms are needed (T_{min}, T_{max}, T_{opt}, $a_{w(min)}$, $a_{w(opt)}$, $a_{w(max)}$, pH_{min}, pH_{opt}, pH_{max}, …). Moreover, growth predictions did not take into account the effect of competitive microbiota present in the product and only focused on three targeted species. But, as mentioned in the results section, other bacterial species were present. Significant statistical differences between simulations and validation data-set are probably due to an interaction between bacterial species, compared to the behavior of each individual species in the same food matrix. On this basis, it would be worth including microbial interactions between microorganisms to better predict microbial growth or inactivation in WP, and in complex food ecosystems (Tenenhaus-Aziza and Ellouze, 2015). In addition, the three dominant selected bacteria were in an internal database of ComBase but it was not possible to select a precise bacterial species or a type of food matrix. And it is well known that predictions may be influenced by the condition of storage and/or the selected bacterial species. Thus, it could be interesting to explore the use of metagenetics analysis coupled to predictive models for other food storage

conditions (like packaging, temperature, etc.) and for several sampling, to give a better proof of concept on the interest to combine these methods. Indeed, data collection in this study is not sufficient enough. But the aim was to give a first exploration case in this specific context.

Finally, the third data-set used as a validation data derive from single repetition. This may also explain the differences between predicted and observed values. Further studies are needed to determine variability between predictive models, according to several validation data-set.

Based on all this, this study allows to describe and to follow the spoilage bacterial dynamics of Belgian WP samples. Moreover, *B. thermosphacta*, *Pseudomonas* sp. and *Psychrobacter* spp. were used as models in three predictive software, showing an interesting example in this case. But further studies are needed to demonstrate the interest of combining classical microbiology with metagenetics, and to associate these results with predictive modeling. Moreover, further researches on the bacterial communities' dynamics for meat and meat products in different conditions and for multiple samples are desirable to provide more complete predictions, and also to better compare the predictive models available.

Compared to culture-based methods on selective media and previous culture-independent techniques, metagenetic analysis combined with classical microbiology and predictive models gave interesting information. Microbial ecology studies have shown that the microbiota of food is much more diverse than that reported by the use of culture media. In this study, microbial changes during storage at three isothermal conditions in FW were monitored. In accordance with previous studies we found that *Pseudomonas*, *Brochothrix* and *Psychrobacter* dominate the community during storage. The growth parameters and models presented in this study are interesting for the example shown, by adding to existing database for spoilage bacteria, and could be applied to other foods and food processes. However, further researches on the bacterial communities' dynamics for Belgian WP, during storage in different storage conditions and for multiple samples, are important to provide a more complete evaluation of the microbial spoilage of WP, and to then use these results in predictive models. Extensive surveys involving a large number of samples and validation data-set are also required to provide a proof of concept, and to better predict the growth of microbial species in the spoilage of meat and meat products.

Study 4

Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples

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Abstract

⇒ **Objective 2.** Characterize specific spoilage bacteria dynamics and use them as inputs in models for minced pork

⇒ **Objective 3.** Develop and validate complex models for spoilage species in minced pork

The aim of this study was to obtain the growth parameters of specific spoilage microorganisms previously isolated in MP samples, and to develop a three spoilage species interaction model under different storage conditions. Naturally contaminated samples were used to validate this approach by considering the effect of the food microbiota. Three groups of bacteria were inoculated on irradiated samples, in mono- and in co-culture experiments (n = 1152): *B. thermosphacta*, *Ln. gelidum* and *Pseudomonas* spp. (*Ps. fluorescens* and *Ps. fragi*). Samples were stored in two food packaging (FW and MAP (CO_2 30% / O_2 70%)) at three isothermal conditions (4°C, 8°C and 12°C). Analysis were carried out by using both 16S rRNA gene amplicon sequencing and classical microbiology in order to estimate bacterial counts during the storage period. Growth parameters were obtained by fitting primary (Baranyi) and secondary (square root) models. The food packaging shows the highest impact on bacterial growth rates, which in turn have the strongest influence on the shelf life of food products. Based on these results, a three spoilage species interaction model was developed by using the modified Jameson-effect model and the Lotka-Volterra (prey-predator) model. The modified Jameson-effect model showed slightly better performances, with 40–86% out of the observed counts falling into the Acceptable Simulation Zone. It only concerns 14-48% for the prey-predator approach. These results can be explained by the fact that the dynamics of experimental and validation datasets seems to follow a Jameson behavior. On the other hand, the Lotka-Volterra model is based on complex interaction factors, which are included in highly variable intervals. More datasets are probably needed to obtained reliable factors, and so better model fittings, especially for three or more spoilage species interaction models. Further studies are also needed to better understand the interaction of spoilage bacteria between them and in the presence of natural microbiota.

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Introduction

During production and distribution steps, spoilage of meat and meat products may occur, rendering them unacceptable for human food consumption. Spoilage is mainly caused by microbial growth, which triggers alterations in the sensorial qualities of the product, with off-odor and off-flavor, discoloration, texture changes, etc. (Cauchie *et al*., 2017; Dalcanton *et al*., 2013; Den Besten *et al*., 2017; Kreyenschmidt *et al*., 2010; Pinter *et al*., 2014; Torngren *et al*., 2018). It is well known that the initial bacterial counts on meat and meat products is highly variable (Benson *et al*., 2014), but several studies have established that only a dominant fraction of the microbiota, designated as SSOs, contributes to spoilage (Benson *et al*., 2014; Kreyenschmidt *et al*., 2010; Nychas *et al*., 2008; Pennacchia *et al*., 2011; Zotta *et al*., 2019). In this context, predictive microbiology can be a helpful tool because the prediction of microbial growth, especially SSOs, enables food industries to optimize their production and storage managements, and thus reduce their economic losses (Fakruddin *et al*., 2012; Kreyenschmidt *et al*., 2010; Li *et al*., 2017; Tamplin, 2018).

During the last years, several models have been developed to predict the growth of SSOs in meat and meat products (Dalcanton *et al*., 2013; Koutsoumanis, 2009; Kreyenschmidt *et al*., 2010; Liu *et al*., 2006; Mataragas *et al*., 2006; Mejlholm and Dalgaard, 2013). But the majority of the developed models are based on the growth of two bacterial species in a food matrix (Giuffrida *et al*., 2007; Vereecken *et al*., 2000), most often to study the interaction between spoilage and pathogenic bacteria (Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019; Giuffrida *et al*., 2009; Lebert *et al*., 2000; Mejlholm and Dalgaard, 2007; Pedrozo *et al*., 2019; Ye *et al*., 2014). Moreover, these models often describe the growth of the SSOs depending on the storage temperature (Antunes-Rohling *et al*., 2019; Dominguez and Schaffner, 2007; Gospavic *et al*., 2008; Kreyenschmidt *et al*., 2010; Longhi *et al*., 2013; Psomas *et al*., 2011) or the packaging conditions (Chaix *et al*., 2015b; Couvert *et al*., 2019; Devlieghere *et al*., 1999; Guillard *et al*., 2016; Kapetanakou *et al*., 2019), but do not always consider the interaction of these storage conditions for the growth of spoilage bacteria (Augustin and Carlier, 2000; Correia Peres Costa *et al*., 2019; Dalcanton *et al*., 2018; Kakagianni *et al*., 2018; Le Marc *et al*., 2002; Nyhan *et al*., 2018; Pinon *et al*., 2004; Rosso *et al*., 1995).

As mentioned by Correia Peres Costa *et al*., 2019: “interaction models are usually intended to quantify how much the growth of one population is reduced by the growth of other populations”. In this context two model approaches are generally used to describe the microbial interaction: (i) those based on the modified Jameson-effect phenomenon (Cauchie *et al*., 2017; Cornu *et al*., 2011; Correia Peres

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Costa *et al*., 2019; Jameson, 1962; Ye *et al*., 2014), and (ii) those based on the predator-prey models (Lotka-Volterra equation) (Berlow et al., 2004; Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019; Dens *et al*., 1999; Giuffrida *et al*., 2007; Mounier *et al*., 2008; Powell *et al*., 2004; Ye *et al*., 2014).

As described by Cornu *et al*. (2011), the Jameson-effect model assumes that: "(i) many microbial interactions in foods limit the maximum population density, without any significant effect on the lag time, and (ii) the growth of the minority population is only partly inhibited after the majority population count has reached its stationary phase (maximum critical population, *MCP*, expressed in log CFU/g)". The modified Jameson-effect model makes the hypothesis that there is one single inhibition function for both populations, hence both populations are similarly inhibited by the same limiting resource, the same waste products and/or by change in pH (Cornu *et al*., 2011). Recently, Quinto *et al*. (2018) have developed a three-strains model based on the modified Jameson-effect equation for inoculated spoilage and pathogenic bacteria in a reconstituted sterile skimmed milk. This study considers the effect of two bacteria, *Ps. fluorescens* and *L. innocua*, on the bacterial growth of *L. monocytogenes*. But the effect of the natural food microbiota on the growth of specific spoilage bacteria need to be studied (Rouger *et al*., 2017) in order to predict bacterial growth resulting from several interactions between three or more spoilage species (Ye *et al*., 2014). This approach needs to be studied.

The Lotka-Volterra model can be considered as a prey-predator model that includes competition for a common substrate (Cornu *et al*., 2011). As cited by Chauvet *et al*. (2002), the Lotka-Volterra model for a three species food chain approach can be considered as: "the lowest-level prey *x* is preyed upon by a mid-level species *y*, which, in turn, is preyed upon by a top-level predator *z*". However, this hypothesis cannot always be applied in food matrix. Indeed, the growth of a bacterium (B_A) presents simultaneously with other bacteria in a food matrix (B_B and B_C) can be affected by three different ways: (i) B_A growth with a reduced growth rate after that B_B and B_C reach their maximal population densities (N_{max}, expressed in log CFU/g), (ii) B_A stops growing when B_B and B_C reach their N_{max}, and (iii) B_A declines when B_B and B_C reach their N_{max} (Cauchie *et al*., 2017; Correia Peres Costa *et al*., 2019). It could be so interesting to develop a Lotka-Volterra model for a three species approach, by considering the effect of the natural food microbiota for the growth of specific spoilage bacteria. Also, this approach is, to the best knowledge of the authors, not available in the literature.

Based on these, the objectives of the present study were (i) to obtain the growth parameters of three specific spoilage microorganisms previously isolated in MP samples, according to different storage conditions, (ii) to develop a three spoilage species interaction model based on available models, under FW and MAP, at isothermal conditions, and (iii) to validate this approach with naturally contaminated food samples stored under different storage conditions.

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Material and methods

2.1. Sampling

Fresh MP samples were obtained from a local Belgian manufacturer at the day of the production, corresponding to the day of slaughtering. MP samples were packed by the manufacturer in a polypropylene tray under cling film (high film permeability).

According to the recipe MP is composed of 100% pork mince (70% lean, 30% fat), no salt, no spices, no additives, no eggs and no sugar are added.

At the day of the production, the water activity of the product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.05 (n = 12). pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

Food samples were then stored at -20°C and irradiated by gamma irradiation (17.5 ± 0.4 kGy) at the same temperature (Sterigenics, Fleurus, Belgium) to limit the adverse effects of irradiation at this dose (Kim *et al*., 2002; Ham *et al*., 2017; Wang *et al*., 2018d).

2.2. Bacterial strains

As described in the study of Cauchie *et al*. (2019), three specific spoilage microorganisms were previously isolated from different batches of naturally contaminated Belgian MP samples at the end of their use-by date. Samples were stored under two packaging (under air and MAP - 30% CO_2 - 70% O_2) and 3 temperature conditions (4°C, 8°C and 12°C). These predominant strains, represented more than 50% of the natural microbiota, were identified by 16S rRNA sequencing and used for experiments: *B. thermosphacta* (MM008), *Ln. gelidum* (MM045) *Pseudomonas* spp. (*Ps. fluorescens* MM026 and *Ps. fragi* MM014). *Ps. fluorescens* and *Ps. fragi* were used together because experiments were carried out in an exploratory approach to the proposed method, thus wishing to consider a wide diversity of *Pseudomonas* species most frequently found in MP.

B. thermosphacta MM008, *Ln. gelidum* (MM045), *Ps. fragi* MM014 and *Ps. fluorescens* MM026 were stored at -80°C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, strains were transferred from the -80°C culture collection to Brain Heart Infusion (BHI) broth for 48 h at 22°C. The bacterial suspensions were incubated overnight at 4°C before inoculation at stationary

phase (7.00 log CFU/mL).

2.3. Inoculation experiments

The three selected bacteria suspensions were inoculated on irradiated MP samples (1% v/w), in triplicate, for mono-culture and co-culture experiments with the objective to reach an average concentration of 3.0 log colony forming units (log CFU/g on the product).

Mono-culture experiments were performed by inoculation of individual bacterial strains: *B. thermosphacta* MM008, *Pseudomonas* spp. (*Ps. fluorescens* MM026, *Ps. fragi* MM014, 1:1 ratio), and *Ln. gelidum* MM045.

Co-culture experiments were performed by inoculation of a mix containing *B. thermosphacta* MM008, Pseudomonas spp. (*Ps. fragi* MM0014 and *Ps. fluorescens* MM0026, 1:1 ratio) and *Ln. gelidum* MM045 (1:1:1 ratio).

Non-inoculated control samples were homogenized, in triplicate, by adding the same quantity of sterile water only.

After inoculation, MP samples were mixed in a Kenwood mixer for 2 min in speed 2 (Kenwood, Mechelen, Belgium).

Inoculated and non-inoculated MP samples were then packed (50 g) in two different type of non-sterile packaging. The first packaging was a high barrier tray (187x137x36, polyester 10 µm, homo-polymer polypropylene 50 µm, NutriPack, France) under MAP (CO_2 30% / O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France). The second packaging concerns a weak barrier tray (175x135x22, polystyrene) under FW using cling film (Clinofilm).

In this study, MP samples were stored during a 13-days shelf life at isothermal temperature: (i) 4°C (± 1°C), (ii) 8°C (± 1°C), (iii) and 12°C (± 1°C), in climatic chambers (Sanyo MIR 254) (288 samples for 4 experiments, n = 1152 samples) (**Figure 35**). A storage time of 13 days was defined in this study in order to obtain a sufficient number of points for modeling, allowing us to predict all the growth phases.

The codes used for each experiment, depending on the inoculated bacteria and storage conditions, are listed in **Table 37**.

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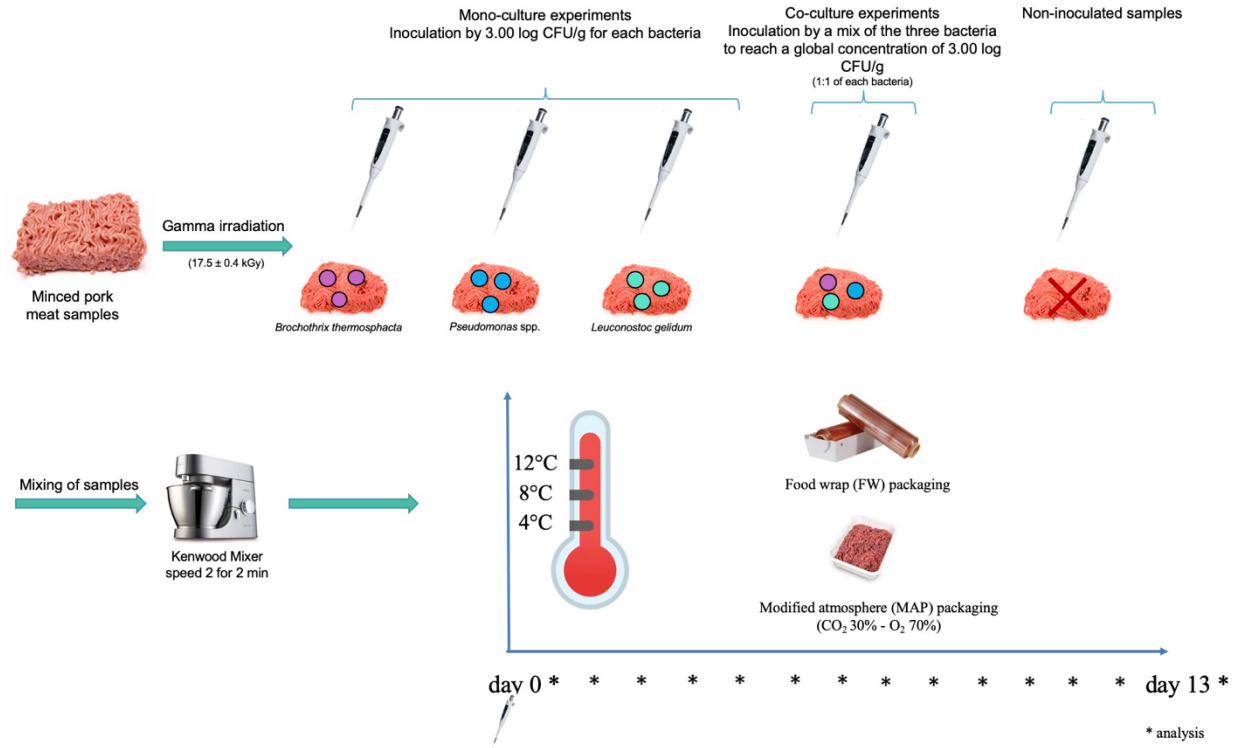

Figure 35. Inoculation experiments performed in food samples.

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Table 37. List of the codes used for the experiments, depending on the inoculated bacteria and storage conditions. FW, food wrap packaging; MAP, modified atmosphere packaging (CO_2 30% / O_2 70% ± 0.1%); mono, mono-culture experiments; co, co-culture experiments with by individually tracking the inoculated bacteria by metagenetic analysis (*B. thermosphacta*, $_{co(A)}$; *Pseudomonas* spp., $_{co(B)}$; *Ln. gelidum*, $_{co(C)}$).

Experiments	Food packaging	Temperature (°C)	Bacterial species	Codes
Mono-culture	FW	4	*B. thermosphacta*	A_{mono}
	FW	8		B_{mono}
	FW	12		C_{mono}
	MAP	4		D_{mono}
	MAP	8		E_{mono}
	MAP	12		F_{mono}
Mono-culture	FW	4	*Pseudomonas* spp.	G_{mono}
	FW	8		H_{mono}
	FW	12		I_{mono}
	MAP	4		J_{mono}
	MAP	8		K_{mono}
	MAP	12		L_{mono}
Mono-culture	FW	4	*Ln. gelidum*	M_{mono}
	FW	8		N_{mono}
	FW	12		O_{mono}
	MAP	4		P_{mono}
	MAP	8		Q_{mono}
	MAP	12		R_{mono}
Co-culture	FW	4	*B. thermosphacta*	$A_{co(A)}$
			Pseudomonas spp.	$A_{co(B)}$
			Ln. gelidum	$A_{co(C)}$
	FW	8	*B. thermosphacta*	$B_{co(A)}$
			Pseudomonas spp.	$B_{co(B)}$
			Ln. gelidum	$B_{co(C)}$
	FW	12	*B. thermosphacta*	$C_{co(A)}$
			Pseudomonas spp.	$C_{co(B)}$
			Ln. gelidum	$C_{co(C)}$
	MAP	4	*B. thermosphacta*	$D_{co(A)}$
			Pseudomonas spp.	$D_{co(B)}$
			Ln. gelidum	$D_{co(C)}$
	MAP	8	*B. thermosphacta*	$E_{co(A)}$
			Pseudomonas spp.	$E_{co(B)}$
			Ln. gelidum	$E_{co(C)}$
	MAP	12	*B. thermosphacta*	$F_{co(A)}$
			Pseudomonas spp.	$F_{co(B)}$
			Ln. gelidum	$F_{co(C)}$

2.4. pH and gas composition measurements

At the first and the last day of storage, pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne).

Oxygen and carbon oxygen concentrations of samples stored in MAP were monitored daily (CheckMate 3, Dansesor, France).

Nonparametric statistical tests were used to compared the pH values and the gas measurements between samples. All tests were considered as significant for a p-value < 0.05.

2.5. Plate count enumeration

Twenty-five grams of product were put into a Stomacher bag with a mesh screen liner (80 µm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10g/L peptone, 5g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (1.0 g/L peptone, 8.5 g/L sodium chloride, #CM0733, Oxoid, Hampshire, England) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis (Spiral plater, DW Scientific, England).

Total viable counts (TVC) for the aerobic psychrophilic microbiota were enumerated on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France) after 72 h at 22°C (model 1535 incubator, Shel Lab, Sheldon Manufacturing. Inc., USA).

Plate counts were performed for mono- and co-culture experiments, and transformed in decimal logarithmic values. Samples for both experiments were enumerated at the first day of inoculation (day 0) and daily until the last day of storage (day 13). None specific agar media were used in co-culture experiments to separately enumerate the three inoculated species. Non-inoculated control samples were analyzed at day 0 and at day 13.

Using R software (R Core Team, 2019), a covariance analysis (ANCOVA) was performed to evaluate the effect of the storage conditions on plate counts (FactoMineR package, Le *et al*., 2008). All tests were considered as significant for a p-value < 0.05.

2.6. 16S rDNA amplicon sequecing

A 16S rDNA metagenetic approach was used for mono- and co-culture experiments.

In mono-culture experiments, metagenetic analysis were performed at the first day of inoculation (day 0) and at the last day of storage (day 13) for samples stored at 4°C.

In co-culture experiments, samples were analyzed at day 0 and daily until day 13. The results were then correlated with plate counts in order to obtain estimate bacterial abundance over storage (see section 2.6.3.).

No 16S rDNA metagenetic analysis were performed for non-inoculated control samples.

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2.6.1. DNA extraction and 16S rDNA amplicon sequencing

Bacterial DNA was extracted from each primary suspension, previously stored at – 80°C, using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV, Antwerp, Belgium) following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at – 20°C until used for 16S rDNA amplicon sequencing.

PCR-amplification of the V1-V3 region of the 16S rDNA library preparation were performed with the following primers (with Illumina overhand adapters), forward (5'-GAGAGTTTGATYMTGGCTCAG-3') and reverse (5'-ACCGCGGCTGCTGGCAC-3'). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 sec at 94°C), annealing (45 sec at 56°C) and extension (60 sec at 72°C), with a final elongation step (8 min at 72°C). These amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). After purification, PCR products were quantified using the Quanti-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, USA) and diluted to 10 ng/μL. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems, Wilmington, MA, USA) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina, San Diego, CA, USA).

2.6.2. Bioinformatics analysis

The 16S rRNA gene sequence reads were processed with MOTHUR. The quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS). The obtained read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (http://www.arb-silva.de/, version v1.2.11) implemented in MOTHUR. The final reads were clustered into operational taxonomic units (OTUs), using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was

attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cutoff. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA dataset 111, using a BLASTN algorithm. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (< 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not).

2.6.3. 16S rRNA gene analysis and bacterial abundance

A correcting factor for 16S rDNA gene copy numbers was applied for any taxon *i* (see **Equation 11**). To obtain each gene copy number, Ribosomal RNA Database (rrnDB) (Stoddard *et al*., 2015) and EzBioCloud database (Yoon *et al*., 2017) were used.

Then, to compare the relative abundance of OTUs, the number of reads of each taxon were normalized as described by Chaillou *et al*. (2015). Reads counts of each taxon *i* in the sample *k* were divided by a sample-specific scaling factor *(Si)* (see **Equations 12** and **13**) (Fougy *et al*., 2016; Rouger *et al*., 2018). Reads counts of all samples were then transformed into a percentage of each OTU.

For co-culture experiments, the percentage of each OUT was finally converted as a proportion of the total viable count, obtained by classical microbiological analysis, in order to estimate counts for each species (in $\log_{10}$ CFU/g, and expressed as mean ± standard deviation (SD)) (see **Equation 14**), as described by Cauchie *et al*. (2017). All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de BioProject ID PRJNA590608. The raw data supporting the conclusions of this manuscript will be made available by EC to any qualified researcher.

2.7. Approach used to develop the interaction model

As proposed by Correia Peres Costa *et al*. (2019), a step-wise approach (**Figure 36**) was followed to develop interaction models simulating the growth of specific spoilage microorganisms.

Firstly, primary and secondary models were performed on mono-culture experiments to obtain the kinetic parameters (section 2.7.1.): lag phase duration (*LPD*, hours), maximum specific growth rate (μ_{max}, 1/hours), initial and maximal population densities (N_0 and N_{max}, respectively, log CFU/g), theoretical minimal temperature of growth (T_{min}, °C), growth rate obtained at the reference temperature of 20°C (μ_{ref}, 1/hours), and minimal shelf life (*MSL*). The *MSL* is the time for the plate counts reaching approximatively 7.0 log CFU/g (expressed as Spoilage value according to the scientific literature, S_{val}).

Secondly, the same approach was applied for co-culture experiments in order to obtain the growth parameters (section 2.7.1.), and to compare them with those on mono-culture experiments

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(section 2.7.2.). The Pearson's correlation coefficient was also used to choose the highest influencing growth parameters on the microbial shelf life of MP samples (section 2.7.2.).

Thirdly, all of these results were used to estimate competitions parameters in interaction models for a three species approach, based on the modified Jameson-effect model and Lotka-Volterra model (section 2.7.3.).

Finally, validation of growth and interaction parameters obtained by the three species models were performed with naturally contaminated MP samples stored under different conditions (section 2.7.4.).

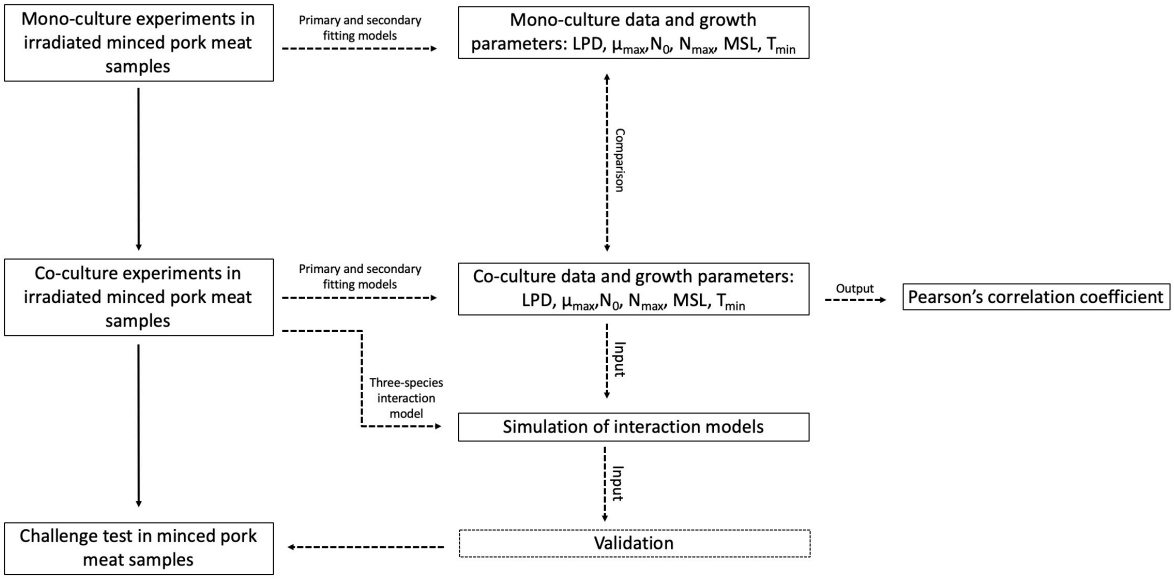

Figure 36. Schematic overview of the step-wise method used for the development of a three spoilage species interaction model.

2.7.1. Primary and secondary model for the fitting of experimental data

The primary model of Baranyi and Roberts (1994) (see **Equations 5** and **6**) was fitted to the experiment dataset obtained for mono- and co-culture experiments. Experimental dataset is obtained by plate counts in mono-culture, and by estimate abundance based on metagenetic results in co-culture. All the data from the three replicates were modeled. All fittings were performed using the nlsMicrobio package (function: baranyi, Baty and Delignette-Muller, 2013) from the open source R software (R Core Team, 2019).

The adequacy of the primary models to describe the experimental data was observed by using the root mean square error of the residuals (*RrMSE*, standard deviation of the residuals) (**Equation 15**) and the coefficient of multiple determination (R^2, the fraction of the square of the deviations of the

observed values about their mean explained by the equation fitted to the experimental data) (**Equation 16**).

$$RrMSE = \sqrt{\frac{RSS}{DF}} = \frac{\sum_{i=1}^{n}(x_i^0 - x_i^f)^2}{n-s} \quad (15)$$

Where *RSS*, the residual sum of square; *DF*, the degrees of freedom; *n*, the number of data points; *s*, the number of parameters of the model; x_i^0, the observed values; and x_i^f, the fitted values.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(observed_i - predicted_i)^2}{\sum_{i=1}^{n}(observed_i - mean)^2} \quad (16)$$

Where *n*, the total number of data points; *mean*, the average value from all observed values.

A reparametrized version of the square root secondary model (Ratkowsky *et al*., 1982) (see **Equation 10**) was then used in R (R Core Team, 2019) to assess the effects of temperature on the growth rates. T_{min} value, the minimal temperature for growth (°C), were found in the scientific literature for the studied bacterial species: - 3.36°C for *B. thermosphacta* (Leroi *et al*., 2012); -5.00°C for *Pseudomonas* spp. (Rashid *et al*., 2001); and +1.00°C for *Ln. gelidum* (Kim *et al*., 2000). For comparison, T_{min} values were also estimated by the Rosso primary model (Rosso *et al*., 1995) and the square root model (Ratkowsky *et al*., 1983).

For secondary models, the coefficient of multiple determination (R^2) and the goodness of fit (*GoF*, root meat square error of the model, analogous to the accuracy factor*)* were used (**Equation 17**).

$$GoF = \frac{\sum_{i=1}^{n}(x_i^0 - x_i^f)^2}{n} \quad (17)$$

Extracts of the code in R for primary and secondary fittings are given in **R-commands 2**.

R-commands 2. Data fitting by primary and secondary models.

```
require(nlsMicrobio)
require(lattice)
require(deSolve)
require(growthrates)
baranyi
LOG10N ~ LOG10Nmax + log10((-1 + exp(mumax * lag) + exp(mumax *
   t))/(exp(mumax * t) - 1 + exp(mumax * lag) * 10^(LOG10Nmax -
   LOG10N0)))
environment: namespace:nlsMicrobio
data<-data.frame(t=c(0,24,48,72,96,120,168,216,288),LOG10N=c(1.49,1.55,1.94,2.45,3.83,6.37,9.16))
preview(formula=baranyi,data=data,start=list(lag=48,mumax=0.07,LOG10N0=1.49,LOG10Nmax=9.16))
primary<-
nls(formula=baranyi,data=BT4data,start=list(lag=48,mumax=0.07,LOG10N0=1.49,LOG10Nmax=9.16))
sqrt<-as.formula("sqrtmumax~sqrt((T>Tmin)*muref*((T-Tmin)/(20-Tmin))^2)")
secondary<-data.frame(T=c(4,8,12),sqrtmumax=sqrt(c(0.07,0.10,0.24)))
preview(formula=sqrt,data=secondary,start=list(Tmin=-3.36,muref=0.53))
time<-c(0,48,96,120,216,288)
y<-grow_baranyi(time,c(y0=1.49,mumax=0.05,K=8.51,h0=6.24))
```

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2.7.2. Correlations between growth parameters

An analysis of covariance (ANCOVA) was performed to evaluate if the maximal bacterial growth rates (μ_{max}) were significantly different between the two food packaging. All tests were considered as significant for a p-value < 0.05. Extracts of the code in R for ANCOVA analysis are given in **R-commands 3**.

Using R software (R Core Team, 2019), correlations between the minimal shelf life (*MSL*) and the growth parameters (μ_{max}, *LPD*, N_0, N_{max}) were obtained by the Pearson's correlation coefficient (r) in mono-culture and co-culture experiments (Liu *et al*., 2006; Miks-Krajnik *et al*., 2016). High correlations were considered when $|r| > 0.7000$ (Miks-Krajnik *et al*., 2016). The best influencing growth parameter on the microbial shelf life was chosen according to the Pearson's correlations coefficient.

Then, a reduction ratio (α) was calculated to quantify the interaction effect on μ_{max} by inoculated bacteria in co-culture experiments (**Equation 18**) (Correia Peres Costa *et al*., 2019).

$$\alpha = 1 - \frac{(p_{co})}{(p_{mono})} \quad (18)$$

Where α is the reduction ratio; p_{co} and p_{mono} are the growth parameters obtained in co-culture and mono-culture experiments, respectively.

R-commands 3. Analysis of covariance (ANCOVA) for bacterial growth parameters.

```
require(FactoMineR)
require(readr)
ancova<- read_delim("~/Desktop/Data.txt", "\t", escape_double = FALSE, trim_ws = TRUE)
test.ancova<-
AovSum(counts~packaging*temperature+time+packaging:time+packaging:temperature+temperature:time,data=
ancova)
test.ancova$Ftest
```

2.7.3. Modelling microbial interactions for *Brochothrix thermosphacta*, *Pseudomonas* spp., and *Leuconostoc gelidum*

Two well-known interactions models for two species were modified to predict the simultaneous growth of the three-inoculated spoilage bacteria in irradiated MP samples: the modified Jameson-effect model and the Lotka-Volterra model (Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019).

In the modified Jameson-effect model, the deceleration function can be replaced by **Equation 19** (Cadavez *et al*., 2019; Cornu *et al*., 2011; Quinto *et al*., 2018; Mejlholm and Dalgaard, 2007).

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$$\begin{cases} f_A(t) = \left(1 - \frac{N_A(t)}{N_{max_{A(t)}}}\right)\left(1 - \frac{N_B(t)}{N_{max_{B(t)}}}\right) \\ f_B(t) = \left(1 - \frac{N_A(t)}{N_{MCP_{A(t)}}}\right)\left(1 - \frac{N_B(t)}{N_{max_B(t)}}\right) \; if \; N_A(t) \geq N_{MCP_{A(t)}} \\ f_B(t) = 0 \; if \; N_A(t) \geq N_{MCP_{A(t)}} \end{cases} \quad (19)$$

Where N_t is the bacterial concentration at time t (log CFU/g), $N_{max(t)}$ is the maximal population density (log CFU/g), $N_{MCP(t)}$ is maximum critical population (log CFU/g) that the bacterium should be reached to inhibit the growth of the other populations. *MCP* is inferior to its own maximum population density (N_{max}) (Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019).

Using R software (R Core Team, 2019), the modified Jameson-effect model (**Equation 19**) was applied on mono-culture experiment data with the functions of Baranyi, Buchanan and without-lag (package nlsMicrobio, Baty and Delignette-Muller, 2013). The function without lag shown the best fitting in all cases (**Table 38**). This model was then selected in the rest of the study, by using the growth parameters obtained on co-culture experiments. Extracts of the code in R for the modified Jameson-effect models for two species are given in **R-commands 4**.

Table 38. Goodness-of-fit indexes for the two species modified Jameson-effect model on mono-culture experiment data, by using Baranyi, Buchanan and without-lag functions. *RrMSE*, the root mean square error of the residuals mean sum of square; -[a], no bacterial fitting obtained.

Two species Model	*RrMSE*		
	Baranyi	**Buchanan**	**without-lag**
A_{mono}-G_{mono}	0.3528	- [a]	0.5787
A_{mono}-M_{mono}	- [a]	- [a]	0.6879
G_{mono}-M_{mono}	- [a]	- [a]	0.3640
B_{mono}-H_{mono}	0.3870	- [a]	0.8679
B_{mono}-N_{mono}	0.1284	- [a]	0.7059
H_{mono}-N_{mono}	0.1821	0.1830	0.3422
C_{mono}-I_{mono}	0.2486	- [a]	0.7820
C_{mono}-O_{mono}	0.5000	- [a]	0.7681
I_{mono}-O_{mono}	- [a]	- [a]	0.4453
D_{mono}-J_{mono}	- [a]	- [a]	0.4690
D_{mono}-P_{mono}	- [a]	- [a]	0.4743
J_{mono}-P_{mono}	- [a]	- [a]	0.2591
Q_{mono}-E_{mono}	0.1668	- [a]	0.3046
Q_{mono}-K_{mono}	0.6390	- [a]	0.6867
E_{mono}-K_{mono}	0.6390	- [a]	0.8276
F_{mono}-L_{mono}	0.2665	- [a]	0.6745
F_{mono}-R_{mono}	0.3011	- [a]	0.3502
L_{mono}-R_{mono}	- [a]	- [a]	1.0511

R-commands 4. Modified Jameson-effect model without lag phase used for competition of two bacterial species.

```
library(nlsMicrobio)
t<-c(0,24,48,72,168,192,240,312)
data.specie1<-c(3.84,3.08,3.76,4.54,7.74,7.63,7.68,7.90)
rep.specie1<-rep(1,8)
```

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```
specie1<-data.frame(t,rep.specie1,data.specie1)
data.specie2<-c(3.15,3.43,4.52,5.64,9.45,9.51,9.90,10.21)
rep.specie2<-rep(2,8)
specie2<-data.frame(t,rep.specie2,data.specie2)
names(specie1)<-c("t","flora","LOG10N")
names(specie2)<-c("t","flora","LOG10N")
rbind(specie1,specie2)
specie.1.vs.2<-rbind(specie1,specie2)
summary(specie.1.vs.2)
modified.jameson.baranyi.two.bacteria<-formula(LOG10N ~ (flora == 1) * ((t <= tmcp) * (LOG10N0_1 + mumax_1 * t/log(10) + log10(exp(-mumax_1 * t) * (1 - exp(-mumax_1 * lag_1)) + exp(-mumax_1 * lag_1))) + (t > tmcp) * (LOG10N0_1 + mumax_1 * tmcp /log(10) + log10(exp( mumax_1 * tmcp) * (1 - exp(-mumax_1 * lag_1)) + exp(-mumax_1 * lag_1)))) + (flora == 2) * ((t <= tmcp) * (LOG10N0_2 + mumax_2 * t/log(10) + log10(exp(-mumax_2 * t) * (1 - exp(-mumax_2 * lag_2)) + exp(-mumax_2 * lag_2))) + (t > tmcp) * (LOG10N0_2 + mumax_2 * tmcp /log(10) + log10(exp(-mumax_2 * tmcp) * (1 - exp(-mumax_2 * lag_2)) + exp(-mumax_2 * lag_2)))))
modified.jameson.buchanan.two.bacteria<-formula(LOG10N ~ (flora == 1) * ((t <= lag_1) * LOG10N0_1 + ((t > lag_1) & (t < tmcp)) * (LOG10N0_1 + mumax_1/log(10) * (t - lag_1)) + (t >= tmcp) * (LOG10N0_1 + mumax_1/log(10) * (tmcp - lag_1))) + (flora == 2) * ((t <= lag_2) * LOG10N0_2 + ((t > lag_2) & (t < tmcp)) * (LOG10N0_2 + mumax_2/log(10) * (t - lag_2)) + (t >= tmcp) * (LOG10N0_2 + mumax_2/log(10) * (tmcp - lag_2))))
modified.jameson.without.lag.two.bacteria<-formula(LOG10N ~ (flora == 1) * ((t < tmcp) * (LOG10N0_1 + mumax_1/log(10) * t) + (t >= tmcp) * (LOG10N0_1 + mumax_1/log(10) * tmcp)) + (flora == 2) * ((t < tmcp) * (LOG10N0_2 + mumax_2/log(10) * t) + (t >= tmcp) * (LOG10N0_2 + mumax_2/log(10) * tmcp)))
specie.1.vs.2.without.lag<-nls(modified.jameson.without.lag.two.bacteria,
specie.1.vs.2,list(mumax_1=0.07,LOG10N0_1=3.84,tmcp=310,mumax_2=0.08,LOG10N0_2=3.15))
specie.1.vs.2.without.lag
summary(specie.1.vs.2.without.lag)
twocolors <- c("red","blue")
npoints <- 100
seq.t <- seq(0,max(specie.1.vs.2$t),length.out=npoints)
prednls3.1 <- predict(specie.1.vs.2.without.lag,data.frame(t=seq.t,flora=rep(1,npoints)))
prednls3.2 <- predict(specie.1.vs.2.without.lag,data.frame(t=seq.t,flora=rep(2,npoints)))
plot(specie.1.vs.2$t, specie.1.vs.2$LOG10N,col=twocolors[specie.1.vs.2$flora],xlab="t",ylab="LOG10N")
lines(seq.t,prednls3.1,col=twocolors[1])
lines(seq.t,prednls3.2,col=twocolors[2])
```

For a three species mixed culture model, Quinto *et al.* (2018) recently proposed a modification of the logistic deceleration model (**Equation 20**).

$$f(t) = \left(1 - \frac{N_A(t) + N_B(t) + N_C(t)}{N_{\max tot}}\right) \quad (20)$$

Where $N_A(t)$, $N_B(t)$, and $N_C(t)$ are the cell concentration of microorganism *A*, *B*, or *C* in co-culture at time *t*; $N_{max\ tot}$ is the maximal total population density (including all species present) and consequently the overall carrying capacity of the system from the three species co-cultured.

However, this study only considers the effect of *Ps. fluorescens* and *L. innocua* on the bacterial growth of *L. monocytogenes*. In our study, the aim of co-culture experiments was to consider the global effect of three inoculated bacterial species and the bacterial interaction on each other. According to this, the modified Jameson-effect model was re-defined for a three species model that was used in this study (**Equation 21**).

$$\frac{1}{N_{tot}(t)} \frac{dN_{tot(t)}}{dt} = \mu_{max_{(Bm,Ps,Lg)(t)}} x\ \alpha_{(Bm,Ps,Lg)}(t)\ x \left(1 - \frac{N_{Bm(t)} + N_{Ps(t)} + N_{Lg(t)}}{N_{MCP}(t)}\right) \quad (21)$$

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Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), $\alpha(t)$ is an adjustment function, N_{MCP} is the maximum critical population of each bacterium (log CFU/g). Extracts of the code in R for the three species modified Jameson-effect models are given in **R-commands 5**.

Supplementary R-commands 5. Modified Jameson-effect model without lag phase used for competition of three bacterial species.

```
library(nlsMicrobio)
t<-c(0,24,72,144,312)
A<-c(2.71,2.67,2.97,3.83,3.83)
fA<-rep(1,5)
metaA<-data.frame(t,fA,A)
B<-c(3.07,3.13,4.24,4.14,4.76)
fB<-rep(2,5)
metaB<-data.frame(t,fB,B)
C<-c(3.00,3.04,4.31,6.81,8.36)
fC<-rep(3,5)
metaC<-data.frame(t,fC,C)
names(metaA)<-c("t","flora","LOG10N")
names(metaB)<-c("t","flora","LOG10N")
names(metaC)<-c("t","flora","LOG10N")
rbind(metaA,metaB,metaC)
ABC<-rbind(metaA,metaB,metaC)
ABC
summary(ABC)
modified.jameson.without.lag.three.species<-formula(LOG10N ~ (flora == 1) * ((t < tmcp) * (LOG10N0_1 + mumax_1/log(10) *  t) + (t >= tmcp) * (LOG10N0_1 + mumax_1/log(10) * tmcp)) + (flora == 2) * ((t < tmcp) * (LOG10N0_2 + mumax_2/log(10) * t) + (t >= tmcp) * (LOG10N0_2 + mumax_2/log(10) * tmcp)) + (flora == 3) * ((t < tmcp) * (LOG10N0_3 + mumax_3/log(10) * t) + (t >= tmcp) * (LOG10N0_3 + mumax_3/log(10) * tmcp)))
modified.jameson.without.lag.ABC<-
nls(modified.jameson.without.lag.three.species,ABC,list(mumax_1=0.02,LOG10N0_1=2.71,tmcp=144,mumax_2=0.06,LOG10N0_2=3.07,mumax_3=0.01,LOG10N0_3=3.00)
overview(modified.jameson.without.lag.ABC)
predict(modified.jameson.without.lag.ABC)
threecolors <- c("red","blue","pink")
npoints <- 100
seq.t <- seq(0,max(ABC$t),length.out=npoints)
prednls3.1 <-predict(modified.jameson.without.lag.ABC,data.frame(t=seq.t,flora=rep(1,npoints)))
prednls3.2 <- predict(modified.jameson.without.lag.ABC,data.frame(t=seq.t,flora=rep(2,npoints)))
prednls3.3 <- predict(modified.jameson.without.lag.ABC,data.frame(t=seq.t,flora=rep(3,npoints)))
plot(ABC$t,ABC$LOG10N,col=threecolors[ABC$flora],xlab="t",ylab="LOG10N")
lines(seq.t,prednls3.1,col=threecolors[1])
lines(seq.t,prednls3.2,col=threecolors[2])
lines(seq.t,prednls3.3,col=threecolors[3])
```

In the two species model based on the Lotka-Volterra equation, the deceleration function includes empirical parameters reflecting the degree of interaction between microbial species (F_{AB} and F_{BA}) (see **Equation 9**) (Cadavez *et al*., 2019; Cornu *et al*., 2011; Correia Peres Costa *et al*., 2019; Liu *et al*., 2006). Using R software (R Core Team, 2019), the Lotka-Volterra model was also re-defined for a three species interaction model, represented by **Equation 22**.

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$$\begin{cases} \frac{1}{N_A(t)}\frac{dN_{A(t)}}{dt} = \mu_{max_{A(t)}} x\ \alpha_A(t)\ x\left(1 - \frac{N_{A(t0)} + (F_{ABC}\ x\ F_{ACB\ x}\ N_{BC\ (t0)})}{N_{max_{A(t)}}}\right) \\ \frac{1}{N_B(t)}\frac{dN_{B(t)}}{dt} = \mu_{max_{B(t)}} x\ \alpha_B(t)\ x\left(1 - \frac{N_{B(t0)} + (F_{BAC}\ x\ F_{BCA\ x}\ N_{AC\ (t0)})}{N_{max_{B(t)}}}\right) \\ \frac{1}{N_C(t)}\frac{dN_{C(t)}}{dt} = \mu_{max_{C(t)}} x\ \alpha_C(t)\ x\left(1 - \frac{N_{C(t0)} + (F_{CAB}\ x\ F_{CBA\ x}\ N_{AB\ (t0)})}{N_{max_{C(t)}}}\right) \end{cases} \quad (22)$$

Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), $\alpha(t)$ is an adjustment function, $F_{A,B,C}$ are the coefficient of interaction measuring the effects of one species on the others, N_{max} is the maximum population density (log CFU/g). Extracts of the code in R for the three species Lotka-Volterra models are given in **R-commands 6**.

R-commands 6. Lotka-Volterra model used for competition of three bacterial species.

```
library(deSolve)
library(nlsMicrobio)
library(car)
LVmodel.three<-function(Time,State,Pars){
  with(as.list(c(State,Pars)),{
    dx=mumax1*(1-((x+alpha*delta*3)/Nmax1))
    dy=mumax2*(1-((y+beta*epsilon*3)/Nmax2))
    dz=mumax2*(1-((z+gamma*zeta*3)/Nmax3))
    return(list(c(dx,dy,dz)))
  })
}
Pars<-c(mumax1=0.13,mumax2=0.32,mumax3=0.15,alpha=0.3833,beta=-0.3382,gamma=0.2456,delta=-
0.2456,epsilon=0.1546,zeta=-0.1345,Nmax1=8.83,Nmax2=8.87,Nmax3=6.78)
State<-c(x=3.84,y=4.00,z=3.85)
Time<-seq(0,13,by=1)
three.bacteria.LVmod<-as.data.frame(ode(func=LVmodel.three,y=State,parms=Pars,time=Time))
three.bacteria.LVmod
summary(three.bacteria.LVmod)
matplot(three.bacteria.LVmod[,-1],type="l",xlab="time",ylab="population")
```

Comparison of the two models was assessed by root mean square error (*RMSE*) and coefficient of determination (R^2) (Correia Peres Costa *et al*., 2019), as previously described in the section above (sections 2.7.1.).

2.7.4. Model validation

Validation of the developed three species interaction models was performed using a new dataset of experimental data.

Fresh MP samples were obtained from a local Belgian manufacturer at the day of the production, corresponding to the day of slaughtering. MP samples were packed by the manufacturer in a polypropylene tray under cling film. Samples have the same composition as described above.

Samples were not irradiated and not inoculated in order to follow the dynamics of the natural

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food microbiota. MP samples were also packed (50 g) in two different packaging, in triplicate.

The first packaging was a tray (187x137x36, polyester 10 µm, homo-polymer polypropylene 50 µm, NutriPack, France) under MAP (CO_2 30% / O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France). The second packaging consisted in a tray (175x135x22, polystyrene) under FW using cling film (Clinofilm).

In this study, MP samples were stored during a 13 days shelf life at isothermal temperature: (i) 4°C (± 1°C), (ii) 8°C (± 1°C), (iii) and 12°C (± 1°C), in climatic chambers (Sanyo MIR 254).

Samples (n = 288) were then analyzed at the first day of inoculation (day 0) and daily until the last day of storage (day 13). Analyses were performed by classical plate counts and 16S rDNA metagenetics, as methods previously described in the sections above (sections 2.6. and 2.7.), in order to estimate bacterial counts over the storage.

The performance of the developed interaction models was evaluated by the acceptable simulation zone (*ASZ*) approach. Model performance is considered acceptable when at least 70% of the observed log counts values are within the *ASZ*, defined as ± 0.5 log-units from the simulated concentration in log units (Correia Peres Costa *et al*., 2019).

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Results

3.1. 16S rDNA metagenetic results

Despite of the inability of differentiation between viable and non-viable cells by the culture-independent DNA-based methods used, high level (>95%) of relative abundance for each inoculated bacterium was observed for mono-culture experiments (**Figure 37**).

The relative abundance results for co-culture experiments (expressed in %) at genus levels (>1%) are represented in cumulated histograms for all samples in FW (**Figure 38**) and MAP (**Figure 39**). These data including the relative abundance of sequences are also summarized in Supplemental Material (**Table 39**).

The taxa representing <1% in relative abundance were merged in the category of "Others". “Others” are mainly composed by the genera *Aeromonas*, *Arthrobacter*, *Bacteroides*, *Carnobacterium*, *Chryseobacterium*, *Enterococcus*, *Flavobacterium*, *Kurthia*, *Lactobacillus*, *Lactococcus*, *Mannheimia*, *Massilia*, *Micrococcus*, *Moraxella*, *Myroides*, *Ottowia*, *Peptococcus*, *Photobacterium*, *Porphyromonas*, *Propionibacterium*, *Rothia*, *Serratia* and *Staphylococcus*. Full data on taxa found in high (>1%) and low (<1%) frequencies will be made available by EC to any qualified researcher.

At day 0, small differences between the distribution of read percentages for the three inoculated bacteria are observed (11.8%, 27.4% and 23.3% for *Brochothrix*, *Pseudomonas* and *Leuconostoc*, respectively).

At day 3 in FW, *Brochothrix* became under the detection limit. At this same time, *Pseudomonas* became the most represented genus (>90%), and remained during the 13 days of storage.

In MAP, *Leuconostoc* and *Pseudomonas* were equally distributed during the first days of storage, but *Leuconostoc* became the most represented genus (>90%) after 3 days and until the end of storage.

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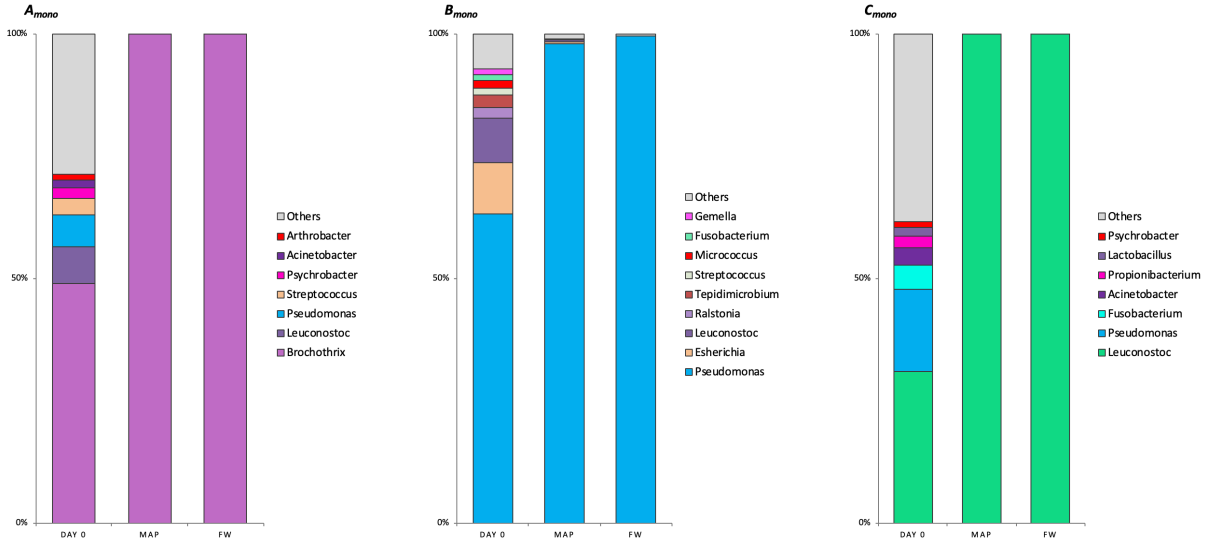

Figure 37. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at genus levels in mono-culture experiment, at day 0 and 13, at 4°C: (A) for *B. thermosphacta*, (B) for *Pseudomonas* spp., and (C) for *Ln. gelidum*. At genus levels, the taxa representing <1% in relative abundance were merged in the category of "Others".

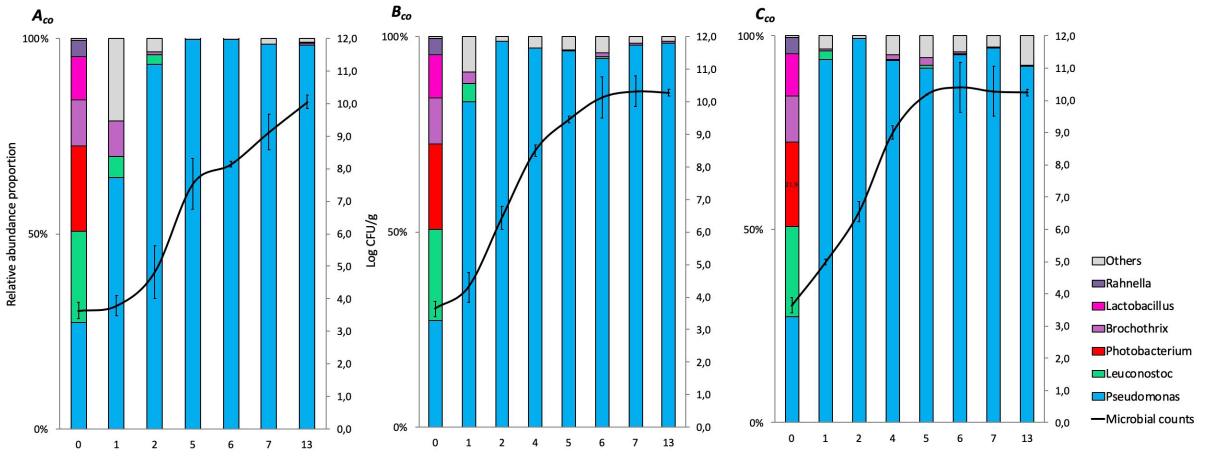

Figure 38. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at genus levels in co-culture experiment during storage in FW (***A*****$_{co}$**, at 4°C; ***B*****$_{co}$**, at 8°C; ***C*****$_{co}$**, at 12°C). At genus levels, the taxa representing <1% in relative abundance were merged in the category of "Others". The solid represents the plate counts (means and standard deviation of the three replicates).

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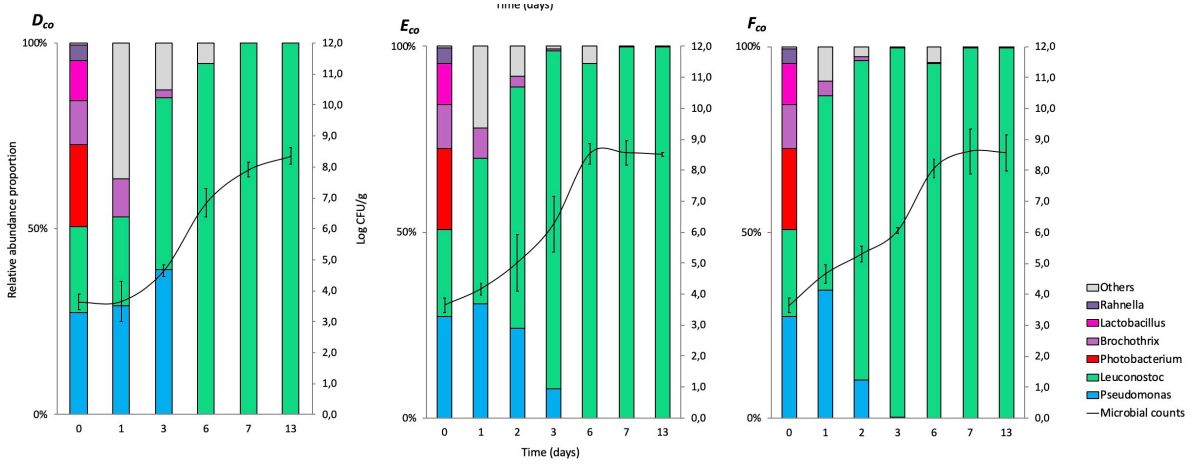

Figure 39. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at genus levels in co-culture experiment during storage in MAP (***D*****$_{co}$**, at 4°C; ***E*****$_{co}$**, at 8°C; ***F*****$_{co}$**, at 12°C). At genus levels, the taxa representing <1% in relative abundance were merged in the category of "Others". The solid represents the plate counts (means and standard deviation of the three replicates).

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Table 39. Distribution of metagenetics reads percentages at genus level for co-culture experiments. At genus levels, the taxa representing <1% in relative abundance were merged in the category of "Others"; -*, data under the detection limit; -[a], no analysis performed this day.

Genus	Days								
	0	1	2	3	4	5	6	7	13
					FW				
4°C									
Brochothrix	11.80	9.10	0.75	-[a]	-[a]	-*	-*	-*	0.52
Lactobacillus	10.96	-*	-*	-[a]	-[a]	-*	-*	-*	0.10
Leuconostoc	23.29	5.30	2.35	-[a]	-[a]	0.10	0.10	0.05	0.07
Photobacterium	21.92	-*	-*	-[a]	-[a]	-*	-*	-*	-*
Pseudomonas	27.40	64.40	93.44	-[a]	-[a]	99.80	99.80	98.51	98.25
Rahnella	4.11	-*	-*	-[a]	-[a]	-*	-*	-*	-*
Others	0.53	21.20	3.45	-[a]	-[a]	0.10	0.10	1.42	1.06
8°C									
Brochothrix	11.80	2.90	-*	-[a]	-*	-*	1.00	0.43	0.52
Lactobacillus	10.96	-*	-*	-[a]	-*	-*	-*	-*	0.10
Leuconostoc	23.29	4.80	0.07	-[a]	-*	0.20	0.30	0.08	0.07
Photobacterium	21.92	-*	-*	-[a]	-*	-*	-*	-*	-*
Pseudomonas	27.40	83.30	98.80	-[a]	97.10	96.50	94.50	97.85	98.25
Rahnella	4.11	-*	-*	-[a]	-*	-*	-*	-*	-*
Others	0.53	9.00	1.12	-[a]	2.90	3.30	4.20	1.65	1.06
12°C									
Brochothrix	11.80	0.40	-*	-[a]	1.40	1.90	0.60	0.20	0.10
Lactobacillus	10.96	-*	-*	-[a]	-*	-*	-*	0.03	-*
Leuconostoc	23.29	2.20	0.03	-[a]	0.20	0.70	0.10	0.07	0.07
Photobacterium	21.92	-*	-*	-[a]	-*	-*	-*	-*	0.07
Pseudomonas	27.40	93.90	99.41	-[a]	93.60	91.70	95.20	96.91	92.06
Rahnella	4.11	-*	-*	-[a]	-*	-*	-*	-*	-*
Others	0.53	3.50	0.55	-[a]	4.80	5.70	4.10	2.79	7.68
					MAP				
4°C									
Brochothrix	11.80	10.10	-[a]	2.09	-[a]	-[a]	0.10	-*	-*
Lactobacillus	10.96	-*	-[a]	0.00	-[a]	-[a]	-*	0.03	-*
Leuconostoc	23.29	23.90	-[a]	46.23	-[a]	-[a]	94.20	99.74	99.92
Photobacterium	21.92	-*	-[a]	0.00	-[a]	-[a]	-*	-*	-*
Pseudomonas	27.40	29.40	-[a]	39.0	-[a]	-[a]	0.20	0.23	0.03
Rahnella	4.11	-*	-[a]	0.00	-[a]	-[a]	-*	-*	-*
Others	0.53	36.60	-[a]	12.67	-[a]	-[a]	5.50	-*	0.05
8°C									
Brochothrix	11.80	8.10	2.80	0.50	-[a]	-[a]	-*	-*	0.03
Lactobacillus	10.96	-*	-*	-*	-[a]	-[a]	-*	-*	-*
Leuconostoc	23.29	39.10	65.0	90.89	-[a]	-[a]	95.40	99.94	99.92
Photobacterium	21.92	-*	-*	-*	-[a]	-[a]	-*	0.03	-*
Pseudomonas	27.40	30.80	24.20	7.95	-[a]	-[a]	-*	0.03	0.03
Rahnella	4.11	-*	-*	-*	-[a]	-[a]	-*	-*	-*
Others	0.53	22.00	8.00	0.76	-[a]	-[a]	4.60	-*	0.03
12°C									
Brochothrix	11.80	3.90	1.00	0	-[a]	-[a]	0.10	0.08	0.21
Lactobacillus	10.96	-*	-*	-*	-[a]	-[a]	-*	0.05	0.03
Leuconostoc	23.29	52.40	86.10	99.41	-[a]	-[a]	95.50	99.63	99.61
Photobacterium	21.92	-*	-*	-*	-[a]	-[a]	-*	-*	-*
Pseudomonas	27.40	34.40	10.20	0.20	-[a]	-[a]	-*	0.10	0.03
Rahnella	4.11	-*	-*	-*	-[a]	-[a]	-*	-*	-*
Others	0.53	9.30	2.70	0.39	-[a]	-[a]	4.40	0.10	0.13

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

3.2. Plate counts and estimated abundance

In mono-culture experiments, plate counts for *B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum* increased during the shelf life with increasing the temperature (**Tables 40**).

At the end of the shelf life, the bacterial count was higher than 7.0 log CFU/g, except for some samples stored in MAP. During the storage, a high growth rate and a more rapidly reached stationary phase were also correlated to FW and the highest storage temperatures.

No bacterial growth was observed on PCA for the control samples (limit detection < 3.0 log CFU/g) (data not shown in this paper).

For co-culture experiments, the metagenetic data were combined with the plate counts results in order to obtain estimated bacterial counts (**Table 41**).

As previously observed, estimate counts increased during the shelf life with increasing the temperature. At the end of the shelf life, the bacterial count was over 7.0 log CFU/g, except for *B. thermosphacta* and *Pseudomonas* spp. stored in MAP. During the storage, the same growth profiles as mono-culture experiments were observed.

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 40. Microbiological counts (log CFU/g) for mono-culture expriments in minced pork samples stored during 13-days shelf life, at constant temperature, in FW and MAP (CO_2 30% / O_2 70% ± 0.1%). See Table 37 for list of the codes used. Mean values with standard deviations of the three repliacates; -[a], no analysis performed for the day.

Codes	Days													
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A_{mono}	3.84±0.03	3.08±0.10	3.76±0.07	4.54±0.12	- [a]	- [a]	7.24±0.11	7.74±0.17	7.63±0.10	8.17±0.33	7.68±0.15	- [a]	- [a]	7.90±0.15
B_{mono}	3.84±0.03	6.76±0.04	7.49±0.11	8.25±0.07	8.51±0.10	8.58±0.06	8.85±0.02	8.77±0.15	9.05±0.03	8.79±0.21	- [a]	- [a]	- [a]	9.00±0.01
C_{mono}	3.84±0.03	7.68±0.08	8.29±0.13	8.66±0.04	8.99±0.09	9.01±0.23	9.11±0.10	8.81±0.28	9.03±0.03	8.91±0.16	- [a]	- [a]	- [a]	9.27±0.08
D_{mono}	3.84±0.03	- [a]	- [a]	2.17±0.30	- [a]	- [a]	4.11±0.01	4.01±0.14	4.35±0.03	5.24±0.05	4.99±0.12	- [a]	- [a]	5.43±0.06
E_{mono}	3.84±0.03	- [a]	5.88±0.10	6.11±0.11	7.11±0.02	7.86±0.10	8.21±0.04	8.43±0.11	8.43±0.16	8.41±0.10	8.38±0.16	- [a]	7.86±0.07	8.76±0.03
F_{mono}	3.84±0.03	- [a]	7.10±0.04	7.76±0.23	8.35±0.04	8.58±0.06	8.40±0.12	8.44±0.07	8.32±0.03	9.16±0.08	8.67±0.40	- [a]	8.83±0.02	8.71±0.06
G_{mono}	3.15±0.59	3.43±0.11	4.52±0.23	5.64±0.19	- [a]	- [a]	- [a]	9.45±0.13	9.51±0.07	- [a]	9.90±0.29	- [a]	- [a]	10.21±0.03
H_{mono}	3.15±0.59	3.86±0.17	5.36±0.03	7.69±0.17	9.04±0.05	9.67±0.03	- [a]	9.62±0.15	10.34±0.24	10.39±0.40	10.11±0.28	- [a]	- [a]	10.15±0.17
I_{mono}	3.15±0.59	4.93±0.15	- [a]	9.81±0.04	9.85±0.29	9.95±0.34	10.15±0.82	10.26±0.08	10.14±0.10	- [a]	9.87±0.19	- [a]	- [a]	9.80±0.42
J_{mono}	3.15±0.59	- [a]	3.48±0.06	- [a]	- [a]	3.90±0.11	4.87±0.34	4.55±0.12	- [a]	- [a]	- [a]	- [a]	4.73±0.01	4.90±0.01
K_{mono}	3.15±0.59	3.52±0.01	4.16±0.05	- [a]	- [a]	5.41±0.08	6.33±0.07	6.52±0.14	- [a]	6.59±0.17	- [a]	- [a]	7.83±0.13	8.37±0.08
L_{mono}	3.15±0.59	4.47±0.07	6.08±0.03	- [a]	- [a]	- [a]	9.42±0.28	9.58±0.23	- [a]	9.80±0.41	- [a]	- [a]	9.87±0.06	9.85±0.14
M_{mono}	4.00±0.02	4.07±0.01	4.38±0.01	4.61±0.12	- [a]	- [a]	6.17±0.05	- [a]	- [a]	- [a]	8.62±0.09	- [a]	- [a]	8.42±0.06
N_{mono}	4.00±0.02	4.58±0.08	5.84±0.02	- [a]	7.57±0.10	- [a]	8.61±0.13	- [a]	8.73±0.07	- [a]	8.84±0.09	- [a]	- [a]	8.77±0.30
O_{mono}	4.00±0.02	5.38±0.01	6.84±0.13	8.35±0.09	7.56±0.01	- [a]	8.64±0.13	- [a]	- [a]	- [a]	8.82±0.23	- [a]	- [a]	8.62±0.18
P_{mono}	4.00±0.02	4.18±0.09	- [a]	- [a]	6.31±0.17	- [a]	6.84±0.06	7.85±0.01	- [a]	7.78±0.21	- [a]	- [a]	8.00±0.10	8.39±0.12
Q_{mono}	4.00±0.02	4.75±0.03	- [a]	- [a]	8.06±0.01	- [a]	8.38±0.05	8.49±0.16	- [a]	8.85±0.01	- [a]	- [a]	- [a]	8.75±0.19
R_{mono}	4.00±0.02	8.32±0.15	7.28±0.01	- [a]	8.35±0.06	- [a]	8.36±0.09	8.64±0.10	- [a]	8.89±0.07	- [a]	- [a]	- [a]	8.87±0.11

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Table 41. Estimate bacterial counts for co-culture experiment. See Table 37 for list of the codes used. Mean values with standard deviations of the three replicates; -[a], no analysis performed for the day.

Code	Time (days)								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**13**
$A_{co(A)}$	2.71±0.24	2.75±0.31	2.71±0.81	- [a]	- [a]	- [a]	- [a]	- [a]	7.77±0.20
$A_{co(B)}$	3.07±0.24	3.60±0.31	4.80±0.81	- [a]	- [a]	7.54±0.77	8.14±0.08	9.12±0.53	10.04±0.20
$A_{co(C)}$	3.00±0.24	2.52±0.31	3.20±0.81	- [a]	- [a]	4.54±0.77	5.14±0.08	5.79±0.53	6.92±0.20
$B_{co(A)}$	2.71±0.24	2.26±0.31	- [a]	- [a]	- [a]	- [a]	7.13±0.53	7.68±0.20	8.00±0.10
$B_{co(B)}$	3.07±0.24	4.23±0.46	6.43±0.34	- [a]	8.49±0.18	9.43±0.10	10.11±0.64	10.31±0.47	10.27±0.10
$B_{co(C)}$	3.00±0.24	2.48±0.31	1.70±0.81	- [a]	- [a]	5.44±0.08	6.61±0.08	6.93±0.20	7.15±0.10
$C_{co(A)}$	2.71±0.24	2.58±0.09	- [a]	- [a]	7.15±0.20	8.46±0.02	8.18±0.77	7.58±0.78	7.24±0.10
$C_{co(B)}$	3.07±0.24	4.95±0.09	6.55±0.30	- [a]	8.97±0.20	10.14±0.02	10.38±0.77	10.26±0.78	10.21±0.10
$C_{co(C)}$	3.00±0.24	3.32±0.09	3.04±0.30	- [a]	6.30±0.20	8.02±0.02	7.41±0.77	7.10±0.78	7.06±0.10
$D_{co(A)}$	2.71±0.24	2.67±0.64	- [a]	2.97±0.19	- [a]	- [a]	3.83±0.46	- [a]	3.83±0.46
$D_{co(B)}$	3.07±0.24	3.13±0.64	- [a]	4.24±0.19	- [a]	- [a]	4.14±0.46	5.28±0.23	4.76±0.28
$D_{co(C)}$	3.00±0.24	3.04±0.64	- [a]	4.31±0.19	- [a]	- [a]	6.81±0.46	7.91±0.23	8.36±0.28
$E_{co(A)}$	2.71±0.24	3.07±0.19	3.46±0.90	3.95±0.90	- [a]	- [a]	- [a]	- [a]	4.94±0.07
$E_{co(B)}$	3.07±0.24	3.65±0.19	4.39±0.90	5.15±0.90	- [a]	- [a]	- [a]	5.00±0.39	4.94±0.07
$E_{co(C)}$	3.00±0.24	3.76±0.19	4.82±0.90	6.21±0.90	- [a]	- [a]	8.51±0.33	8.56±0.39	8.50±0.07
$F_{co(A)}$	2.71±0.24	3.25±0.30	3.30±0.25	- [a]	- [a]	- [a]	5.05±0.30	5.51±0.72	5.88±0.58
$F_{co(B)}$	3.07±0.24	4.20±0.30	4.31±0.25	3.34±0.10	- [a]	- [a]	- [a]	5.63±0.72	4.98±0.58
$F_{co(C)}$	3.00±0.24	4.38±0.30	5.24±0.25	6.05±0.10	- [a]	- [a]	8.03±0.30	8.61±0.72	8.57±0.58

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3.3. pH and gas measurements

A significant increase of pH is observed for MP samples inoculated by *Pseudomonas* spp. (7.54 ± 0.76, n = 5, p-value = 0.01) compared to the control samples (5.79 ± 0.05, n = 10).

In co-culture experiments, pH values at the end of the shelf life were not different to control samples (5.87 ± 0.02, n = 5) (**Figure 40**).

A relatively stable concentration of carbon dioxide was observed in MAP at the end of the shelf life. Except for MP samples inoculated with *Pseudomonas* spp., which reached a higher significant carbon dioxide value (100.0 ± 0.1 %) at 12 °C (**Figure 41**).

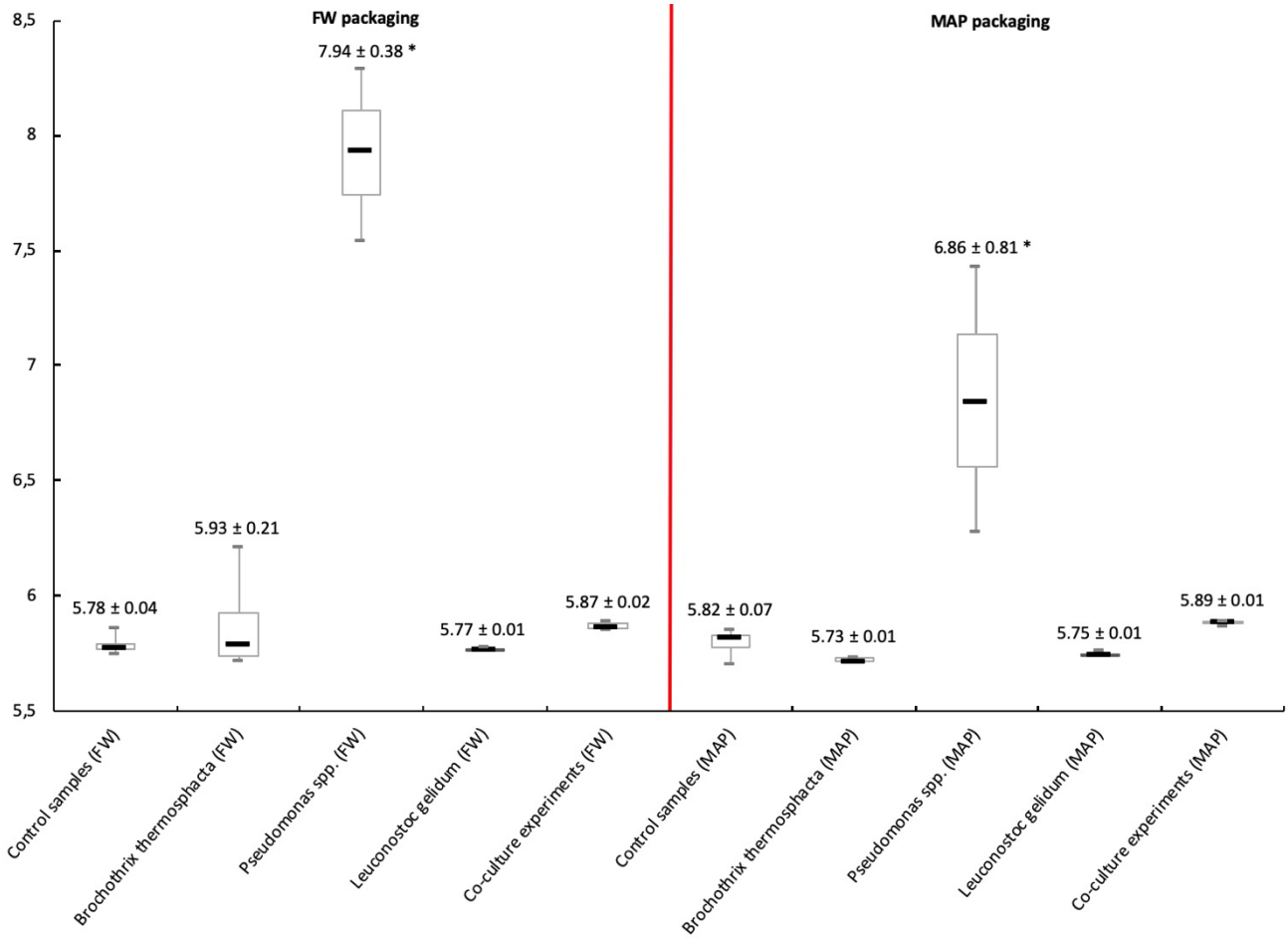

Figure 40. Comparison of pH values for control samples with mono- and co-culture experiments, at day 13 for all packaging conditions. * significant statistical difference (p-value < 0.05).

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

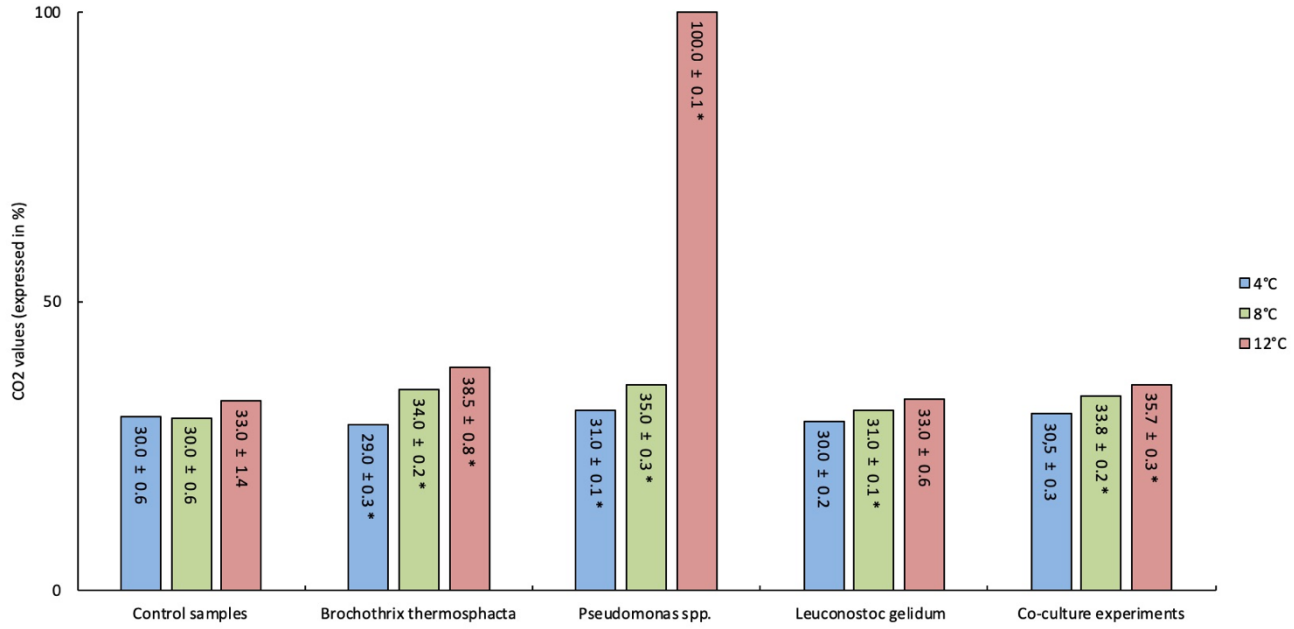

Figure 41. Comparison of carbon dioxide measurements for control samples with mono- and co-culture experiments, at day 13, in MAP. * significant statistical difference (p-value < 0.05).

3.4. Microbial growth parameters

Results of the primary and secondary model fittings for mono- and co-culture experiments are shown in **Tables 42** and **43**. Growth parameters from mono-culture experiments are based on plate counts, and those from co-culture experiments are based on estimate abundance (obtained by the association of metagenetic and plate counts results).

Good fit indexes were obtained in all cases (**Tables 44** and **45**).

Growth parameters showed different dynamic changes depending on storage temperature: a high storage temperature is correlated to a high growth rate during exponential phase and a lower lag-time. These growth parameters are also higher in FW than in MAP.

The *MSL* value is more rapidly reached in FW, except for *Ln. gelidum*.

Moreover, the S_{val} was never reached in MAP for MP samples inoculated by *Pseudomonas* spp. and *B. thermosphacta* during the 13-days shelf life at 4°C.

Based on these results, the dynamics of μ_{max} between a large range of temperature (from -6°C to +25°C) in FW and MAP was performed for mono- and co-culture experiments (**Figure 42**).

It can be clearly observed that *Ln. gelidum* had a highest growth rate in MAP, while it concerns *B. thermosphacta* in FW in mono-culture experiments. *B. thermosphacta* had the lowest one in co-culture experiments.

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 42. Observed kinetic parameters of mono- and co-culture experiments, calculated by Baranyi equation without interactions. See Table 37 for list of the codes used. Mean values with standard deviation (SD represent three samples per experiment) or with the 95% confidence intervals (lower limit and upper limit); μ_{max} , maximal specific growth rate (1/h); *LPD,* lag phase duration (h); N_0, initial bacterial concentration (log CFU/g); N_{max}, maximum bacterial concentration (log CFU/g); *RSS,* Residual Sum of Square of the model; S_{val}, spoilage values of 7.00 log CFU/g (Y (yes) or N(not) if this value is reached during the 13-days shelf life); *MSL,* predictions of the minimal shelf life for the product (days).

	μ_{max}	*LPD*	N_0	N_{max}	*RSS*	S_{val}	*MSL*
A_{mono}	0.09 [0.09-0.08]	51 [53-51]	3.84±0.03	7.90±0.15	0.000442	Y	5.7 [5.8-5.6]
B_{mono}	0.21 [0.21-0.19]	0 [0-0]	3.84±0.03	8.79±0.21	0.000255	Y	1.5 [1.5-1.4]
C_{mono}	0.39 [0.39-0.35]	0 [0-0]	3.84±0.03	9.11±0.10	0.000558	Y	0.8 [0.8-0.8]
D_{mono}	0.03 [0.03-0.03]	20 [20-17]	3.84±0.03	4.99±0.12	0.005700	N	15.3 [15.8-14.7]
E_{mono}	0.07 [0.07-0.07]	0 [0-0]	3.84±0.03	8.43±0.16	0.005700	Y	3.8 [3.9-3.7]
F_{mono}	0.13 [0.13-0.12]	0 [0-0]	3.84±0.03	8.83±0.16	0.005260	Y	1.9 [1.9-1.4]
G_{mono}	0.06 [0.06-0.06]	24 [24-24]	3.15±0.59	9.90±0.29	0.010900	Y	4.5 [4.6-4.2]
H_{mono}	0.13 [0.13-0.13]	10 [10-10]	3.15±0.59	10.15±0.17	0.010900	Y	2.7 [2.8-2.6]
I_{mono}	0.23 [0.23-0.23]	0 [0-0]	3.15±0.59	9.95±0.34	0.010900	Y	1.8 [1.9-1.7]
J_{mono}	0.04 [0.04-0.04]	48 [48-48]	3.15±0.59	4.90±0.01	0.001210	N	21.8 [22.6-20.9]
K_{mono}	0.08 [0.08-0.08]	27 [27-27]	3.15±0.59	8.37±0.08	0.001210	Y	9.0 [9.2-8.8]
L_{mono}	0.13 [0.13-0.13]	0 [0-0]	3.15±0.59	9.87±0.06	0.001210	Y	3.5 [3.6-3.3]
M_{mono}	0.01 [0.01-0.01]	48 [48-48]	4.00±0.02	8.42±0.06	0.017900	Y	7.1 [7.2-7.0]
N_{mono}	0.07 [0.08-0.07]	10 [12-10]	4.00±0.02	8.77±0.30	0.023000	Y	3.4 [3.4-3.3]
O_{mono}	0.18 [0.19-0.18]	0 [0-0]	4.00±0.02	8.64±0.13	0.017900	Y	2.5 [2.5-2.4]
P_{mono}	0.02 [0.02-0.02]	17 [19-15]	4.00±0.02	8.00±0.10	0.025600	Y	6.2 [6.4-5.5]
Q_{mono}	0.13 [0.13-0.13]	0 [0-0]	4.00±0.02	8.75±0.19	0.023700	Y	3.0 [3.0-2.3]
R_{mono}	0.32 [0.33-0.32]	0 [0-0]	4.00±0.02	8.87±0.11	0.025600	Y	1.2 [1.2-1.1]
$A_{co(A)}$	0.03 [0.03-0.03]	36 [36-36]	2.71±0.24	7.77±0.20	0.000490	Y	11.2 [11.6-10.6]
$A_{co(B)}$	0.05 [0.06-0.05]	12 [12-12]	3.07±0.24	10.04±0.20	0.098240	Y	5.4 [6.1-4.8]
$A_{co(C)}$	0.01 [0.01-0.01]	24 [30-24]	3.00±0.24	6.92±0.20	0.002650	Y	11.6 [12.3-10.6]
$B_{co(A)}$	0.07 [0.08-0.07]	12 [12-12]	2.71±0.24	8.00±0.10	0.014000	Y	7.8 [8.3-7.3]
$B_{co(B)}$	0.11 [0.12-0.11]	0 [0-0]	3.07±0.24	10.27±0.20	0.472000	Y	3.8 [4.2-3.5]
$B_{co(C)}$	0.05 [0.05-0.05]	24 [24-24]	3.00±0.24	7.15±0.10	0.016460	Y	8.5 [8.8-8.2]
$C_{co(A)}$	0.13 [0.15-0.12]	0 [0-0]	2.71±0.24	7.58±0.92	0.117000	Y	6.0 [6.4-5.6]
$C_{co(B)}$	0.19 [0.20-0.19]	0 [0-0]	3.07±0.24	10.26±0.78	0.472000	Y	3.5 [3.9-3.3]
$C_{co(C)}$	0.12 [0.13-0.11]	0 [0-0]	3.00±0.24	7.10±0.90	0.000840	Y	6.6 [7.1-6.1]
$D_{co(A)}$	0.02 [0.02-0.01]	46 [59-10]	2.71±0.24	3.83±0.46	0.000150	N	21.0 [20.5-16.8]
$D_{co(B)}$	0.06 [0.06-0.03]	48 [48-48]	3.07±0.24	4.76±0.75	0.135300	N	17.2 [17.4-16.9]
$D_{co(C)}$	0.01 [0.02-0.01]	12 [12-12]	3.00±0.24	8.36±0.28	0.046870	Y	7.6 [8.2-7.0]
$E_{co(A)}$	0.04 [0.06-0.03]	16 [16-16]	2.71±0.24	4.94±0.07	0.005560	N	23.1 [24.0-15.6]
$E_{co(B)}$	0.12 [0.12-0.07]	16 [16-16]	3.07±0.24	5.00±0.40	0.059240	N	14.4 [21.2-8.5]
$E_{co(C)}$	0.08 [0.08-0.07]	6 [6-6]	3.00±0.24	8.50±0.45	0.076910	Y	5.9 [6.6-5.1]
$F_{co(A)}$	0.07 [0.10-0.06]	0 [0-0]	2.71±0.24	5.88±0.01	0.006320	N	14.0 [16.7-11.8]
$F_{co(B)}$	0.20 [0.21-0.12]	0 [0-0]	3.07±0.24	5.00±0.56	0.015400	N	14.0 [17.5-11.3]
$F_{co(C)}$	0.20 [0.20-0.16]	0 [0-0]	3.00±0.24	8.57±0.73	0.030760	Y	5.9 [6.6-5.2]

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 43. Estimation of the secondary parameters obtained by the square root model without interactions. Mean values with the 95% confidence intervals (lower limit and upper limit); -[a], not calculated in the model; T_{min}, minimal temperature for growth (°C) provided from scientific litterature; *Adjusted* T_{min}, minimal temperature for growth (°C) provided from adjustment by the Rosso model (°C); μ_{ref}, bacterial growth rate at the reference (1/h) obtained using a reparametrized version of the square root secondary model; *RSS*, Residual Sum of Square for the μ_{ref} value.

Mono-culture experiments		T_{min}	*Adjusted* T_{min}	μ_{ref}	***RSS***
FW	*B. thermosphacta*	-3.36	-3.36	0.99 [0.99-0.89]	0.000668
FW	*Pseudomonas* spp.	-5.00	-5.02	0.42 [0.42-0.42]	0.001070
FW	*Ln. gelidum*	+1.00	+1.40	0.39 [0.41-0.39]	0.004580
MAP	*B. thermosphacta*	-3.36	-3.36	0.33 [0.33-0.32]	0.000003
MAP	*Pseudomonas* spp.	-5.00	-5.02	0.24 [0.24-0.24]	0.000323
MAP	*Ln. gelidum*	+1.00	+1.40	0.71 [0.73-0.71]	0.000033
Co-culture experiments					
FW	*B. thermosphacta*	-3.36	- [a]	0.30 [0.35-0.28]	0.000193
FW	*Pseudomonas* spp.	-5.02	- [a]	0.42 [0.44-0.42]	0.000190
FW	*Ln. gelidum*	+1.40	- [a]	0.35 [0.40-0.34]	0.000008
MAP	*B. thermosphacta*	-3.36	- [a]	0.17 [0.24-0.13]	0.000092
MAP	*Pseudomonas* spp.	-5.02	- [a]	0.43 [0.46-0.27]	0.023100
MAP	*Ln. gelidum*	+1.40	- [a]	0.59 [0.61-0.49]	0.000750

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 44. Goodness-of-fit indexes used in primary models for fitting the experimental data in mono- and co-culture experiments. *RrMSE*, the root mean square error of the residuals (standard deviation of the residuals); R^2, the coefficient of multiple determination (the fraction of the square of the deviations of the observed values about their mean explained by the equation fitted to the experimental data).

Models	*RrMSE*	R^2
A_{mono}	0.0070	0.9996
B_{mono}	0.0053	0.9997
C_{mono}	0.0079	0.9994
D_{mono}	0.0252	0.9943
E_{mono}	0.0252	0.9943
F_{mono}	0.0242	0.9947
G_{mono}	0.0348	0.9891
H_{mono}	0.0348	0.9891
I_{mono}	0.0348	0.9891
J_{mono}	0.0116	0.9988
K_{mono}	0.0116	0.9988
L_{mono}	0.0116	0.9988
M_{mono}	0.0446	0.9821
N_{mono}	0.0506	0.9770
O_{mono}	0.0446	0.9821
P_{mono}	0.0533	0.9744
Q_{mono}	0.0513	0.9763
R_{mono}	0.0533	0.9744
$A_{co(A)}$	0.0074	0.9995
$A_{co(B)}$	0.1045	0.9018
$A_{co(C)}$	0.0172	0.9974
$B_{co(A)}$	0.0394	0.9860
$B_{co(B)}$	0.2290	0.4280
$B_{co(C)}$	0.0428	0.9835
$C_{co(A)}$	0.1140	0.8830
$C_{co(B)}$	0.2290	0.5280
$C_{co(C)}$	0.0097	0.9992
$D_{co(A)}$	0.0041	0.9999
$D_{co(B)}$	0.1226	0.8647
$D_{co(C)}$	0.0722	0.9531
$E_{co(A)}$	0.0249	0.9944
$E_{co(B)}$	0.0811	0.9408
$E_{co(C)}$	0.0924	0.9231
$F_{co(A)}$	0.0265	0.9937
$F_{co(B)}$	0.0414	0.9846
$F_{co(C)}$	0.0585	0.9692

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 45. Goodness-of-fit indexes used in secondary models for fitting the experimental data in mono- and co-culture experiments. *GoF*, the goodness of fit (root meat square error of the model, analogous to the accuracy factor*)*; R^2 , the coefficient of multiple determination (the fraction of the square of the deviations of the observed values about their mean explained by the equation fitted to the experimental data).

Mono-culture experiments		***GoF***	***R²***
FW	*B. thermosphacta*	0.0183	0.9993
FW	*Pseudomonas* spp.	0.0231	0.9989
FW	*L. gelidum*	0.0479	0.9954
MAP	*B. thermosphacta*	0.0012	1.0000
MAP	*Pseudomonas* spp.	0.0127	0.9997
MAP	*L. gelidum*	0.0041	0.9989
Co-culture experiments			
FW	*B. thermosphacta*	0.0098	0.9998
FW	*Pseudomonas* spp.	0.0097	0.9998
FW	*L. gelidum*	0.0020	1.0000
MAP	*B. thermosphacta*	0.0068	0.9999
MAP	*Pseudomonas* spp.	0.1075	0.9769
MAP	*L. gelidum*	0.0194	0.9993

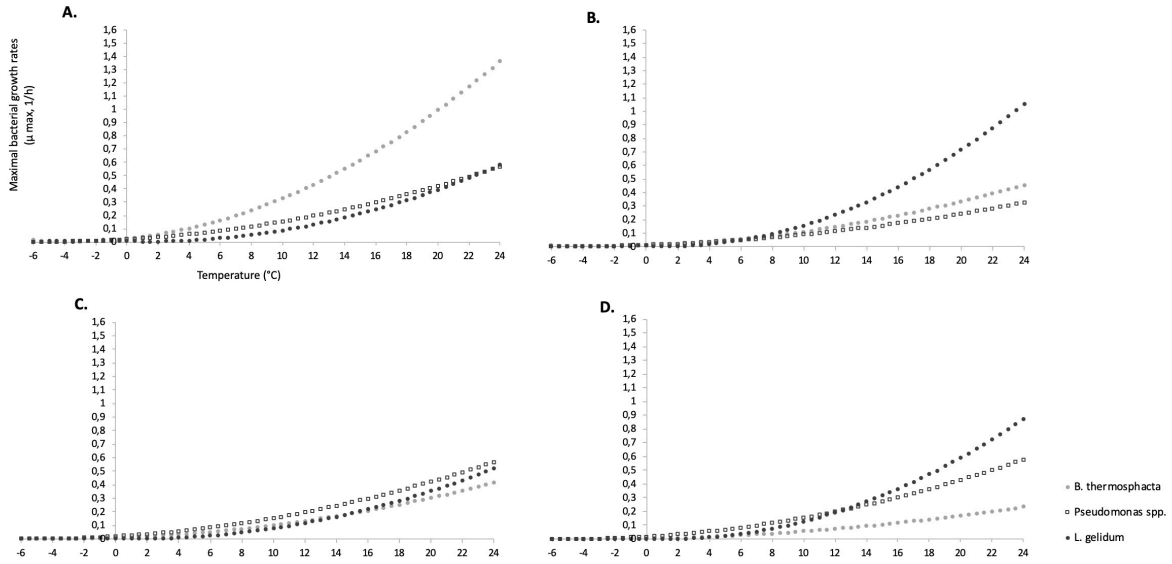

Figure 42. Dynamics of μ_{max} between a large range of temperature (from -6°C to +25°C) for mono-culture experiments in FW (**A**) and MAP (**B**), and for co-culture experiments in FW (**C**) and MAP (**D**).

3.5. Correlations between growth parameters obtained in mono- and co-culture experiments

Correlations between growth parameters of *B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum* for mono-culture and co-culture experiments are presented in **Table 46**.

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It can be observed that the maximum specific growth rate (μ_{max}) of microorganisms was negatively correlated with microbial shelf life. The correlation was higher in mono-culture (-0.8660 to -0.9572) than in co-culture experiments (-0.0339 to -0.9160).

Lag phase duration (*LPD*) of all microorganisms showed good correlation. High correlations of μ_{max} and *LPD* were observed in FW for co-culture experiments.

N_0 showed little correlations than the two others parameters, except for mono-culture of *Pseudomonas* spp. stored in FW.

Moreover, no obvious correlation has been shown between N_{max} with shelf life for co-cultures experiments.

In conclusion, the results showed in our study that the microbial shelf life of MP samples is mainly correlated with μ_{max} and *LPD* than by N_{max} and N_0. Even if the correlations are lower for experiments carried out in co-culture under MAP.

It was also showed that μ_{max} seems to be mainly influenced by the food packaging (**Table 47**), and by the interaction of the storage conditions applied in this study (packaging and temperature). These results were confirmed by the study of the reduction ratio α (**Figure 43**). *B. thermosphacta* and *Ln. gelidum* presented a higher reduction in FW. But an increase was observed for *Pseudomonas* spp. in MAP. Indeed, μ_{max} of *Pseudomonas* spp. was 0.04, 0.08 and 0.13, at 4°C, 8°C and 12°C, respectively, in mono-culture experiments. While the parameter was gradually increasing to 0.06 (α = -50.0%), 0.12 (α = -50.0%) and 0.20 (α = -53.8%), at 4°C, 8°C and 12°C, respectively, in co-culture experiments. However, N_{max} values of this bacterium was lesser in co-culture than in mono-culture experiments.

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Table 46. Correlations between growth parameters and the minimal shelf life (*MSL*) for mono-culture and co-culture experiments. N_0, the initial bacterial population (log CFU/g); N_{max}, the maximal bacterial population (log CFU/g); *LPD*, the lag phase duration (h); μ_{max} (the maximum specific growth rate (1/h).

Bacterial species/packaging	Growth parameters	Mono-culture experiments Pearsons-correlations (*r*)	CI	p-value	Co-culture experiments Pearsons-correlations (*r*)	CI	p-value
FW							
B.thermosphacta	μ_{max}	-0.8660	-0.9715;-0.4771	0.0025	-0.9144	-0.9821;-0.6376	0.0005
	LPD	0.9920	0.9608;0.9983	1.52^{-07}	0.9839	0.9227;0.9967	1.71^{-06}
	N_0	0.0188	-0.6534;0.6745	0.9617	0.1763	-0.5524;0.7523	0.6500
	N_{max}	-0.9553	-0.9908;-0.7965	5.94^{-05}	0.2151	-0.5238;0.7693	0.5783
Pseudomonas spp.	μ_{max}	-0.9548	-0.9907;-0.7945	6.17^{-05}	-0.7774	-0.9507;-0.2344	0.0136
	LPD	0.9905	0.9542;0.9980	2.63^{-07}	0.9013	0.5911;0.9792	0.0008
	N_0	0.9903	-0.6048;0.7160	0.7999	0.3903	-0.3696;0.8373	0.2990
	N_{max}	-0.0675	-0.7002;0.6245	0.8629	0.0278	-0.6482;0.6783	0.9434
Ln. gelidum	μ_{max}	-0.8784	-0.9742;-0.5144	0.0018	-0.9160	-0.9824;-0.6434	0.0005
	LPD	0.9989	0.9948;0.9997	1.23^{-10}	0.8251	0.3563;0.9620	0.0061
	N_0	0.0271	-0.6486;0.6790	0.9448	0.2163	-0.5228;0.7698	0.5760
	N_{max}	-0.5478	-0.8886;0.1828	0.1268	-0.0568	-0.6947;0.6311	0.8846
MAP							
B.thermosphacta	μ_{max}	-0.8819	-0.9750;-0.5258	0.0016	-0.2501	-0.7839;0.4965	0.5164
	LPD	0.9881	0.9424;0.9975	5.95^{-07}	0.5490	-0.1811;0.8890	0.1257
	N_0	0.0411	-0.6405;0.6864	0.9164	0.5858	-0.1281;0.8998	0.0973
	N_{max}	-0.9925	-0.9984;-0.9637	1.15^{-07}	-0.4274	-0.8502;0.3304	0.2511
Pseudomonas spp.	μ_{max}	-0.9572	-0.9912;-0.8047	5.09^{-05}	-0.0339	-0.6827;0.6446	0.9308
	LPD	0.9549	0.7951;0.9907	6.10^{-05}	0.3844	-0.3755;0.8352	0.3070
	N_0	0.0425	-0.6396;0.6872	0.9134	0.7422	0.1540;0.9420	0.2202
	N_{max}	-0.9977	-0.9995;-0.9890	1.66^{-09}	0.2979	-0.4565;0.8031	0.4362
Ln. gelidum	μ_{max}	-0.9283	-0.9851;-0.6891	0.0003	-0.5587	-0.8919;0.1675	0.1178
	LPD	0.9424	0.7438;0.9881	0.0001	0.7049	0.0768;0.9325	0.0339
	N_0	0.1130	-0.5958;0.7228	0.7722	0.5667	-0.1561;0.8942	0.1116
	N_{max}	-0.8983	-0.9786;-0.5806	0.0009	0.3732	-0.3867;0.8313	0.3225

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 47. Effect of food storage conditions on the maximal bacterial growth rates (μ_{max}, 1/h) for mono- and co-cultures experiments (analysis of covariance, ANCOVA). [a], interaction effect of packaging and temperature on bacterial growth rates; [*], significant statistical effect ($p<0.05$).

Experiments	Effects		
	Packaging	Temperature	Packaging*temperature[a]
Mono-culture			
B. thermosphacta	0.0113*	0.0003*	0.0001*
Pseudomonas spp.	0.4133	0.7389	0.0050*
Ln. gelidum	0.1655	0.0015*	0.4331
Co-culture			
B. thermosphacta	0.0280*	0.8072	0.0016*
Pseudomonas spp.	0.3063	0.3564	0.8114
Ln. gelidum	0.1030	0.1691	0.8728

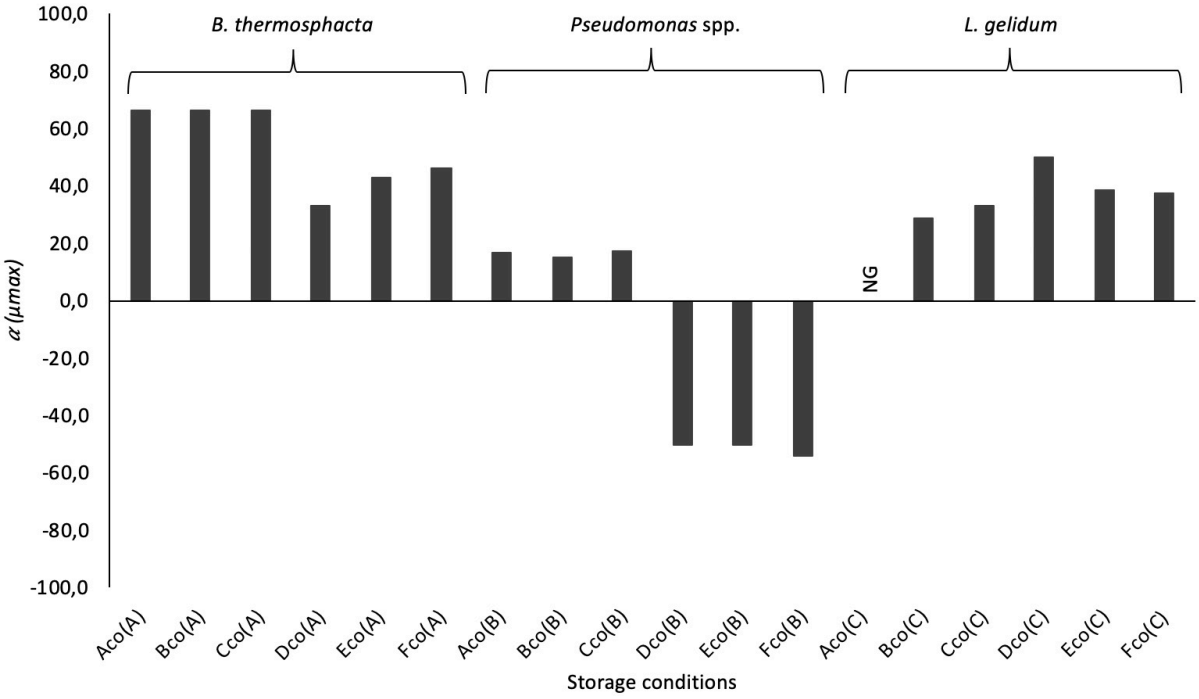

Figure 43. Reduction ratio (α), in %, of the parameters μ_{max} for *B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum* in co-culture experiments at different storage conditions (see Table 37 for legend). The negative bars represents an increase in co-culture for the specific parameters. No growth of bacteria (NG) was only observed for *Ln. gelidum* in MAP at 4°C.

3.6. Three species interaction models and validation step

Estimated growth parameters and goodness-of-fit indexes for the two developed interaction model are available in **Table 48**.

The Lotka-Volterra model showed lower *RrMSE* values but the interaction factors are

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

sometimes included in high intervals.

Simulations provided by the predictive models based on the modified Jameson-effect model and the Lotka-Volterra equations, are represented in **Figures 44** and **45.**

The modified Jameson-effect model showed the best model performance (*ASZ*), with a mean of 63 ± 23%, while the Lotka-Volterra model showed lesser percentages (31 ± 17% (n = 18)). Eight simulated models based on the equation of the modified Jameson-effect model can be considered as acceptable, because at least 70% of the observed log counts values are within the *ASZ*.

Table 48. Estimated growth parameters of the three species modified Jameson-effect and Lotka-Volterra models, with goodness-of-fit indexes. See Table 37 for the list of codes used. Mean values with the 95% confidence intervals (lower limit and upper limit); *RrMSE*, the root mean square error of the residuals; μ_{max}, the maximum growth rate (1/h); F_{ABC}, F_{ACB}, F_{BAC}, F_{BCA}, F_{CAB}, F_{CBA}, the coefficient of interaction measuring the effects of one species on the others ($_A$, *B. thermosphacta*; $_B$, *Pseudomonas* spp.; $_C$, *Ln. gelidum*; respectively).

	Modified Jameson-effect model		**Lotka-Volterra model**						
	RrMSE	μ_{max}	***RrMSE***	F_{ABC}	F_{ACB}	F_{BAC}	F_{BCA}	F_{CAB}	F_{CBA}
$A_{co(A)}$	0.261	0.047 [0.019;0.076]	0.154	-0.90 [-5.41;-0.19]	-1.10 [-5.13;-0.18]	2.20 [0.92;2.81]	0.45 [0.35;1.08]	0.50 [0.19;1.82]	1.99 [0.54;5.00]
$A_{co(B)}$	0.273	0.065 [0.031;0.097]	0.171	-0.90 [-5.41;-0.19]	-1.10 [-5.13;-0.18]	2.20 [0.92;2.81]	0.45 [0.35;1.08]	0.50 [0.19;1.82]	1.99 [0.54;5.00]
$A_{co(C)}$	0.284	0.039 [0.013;0.065]	0.199	-0.90 [-5.41;-0.19]	-1.10 [-5.13;-0.18]	2.20 [0.92;2.81]	0.45 [0.35;1.08]	0.50 [0.19;1.82]	1.99 [0.54;5.00]
$B_{co(A)}$	0.372	0.230 [0.019;0.380]	0.113	0.05 [-0.02;0.09]	6.02 [3.53;6.55]	0.90 [0.85;0.99]	1.08 [0.67;1.11]	-5.51 [-5.73;-0.27]	-0.04 [-0.05;-0.03]
$B_{co(B)}$	0.273	0.317 [0.031;0.485]	0.365	0.05 [-0.02;0.09]	6.02 [3.53;6.55]	0.90 [0.85;0.99]	1.08 [0.67;1.11]	-5.51 [-5.73;-0.27]	-0.04 [-0.05;-0.03]
$B_{co(C)}$	0.284	0.184 [0.015;0.327]	0.108	0.05 [-0.02;0.09]	6.02 [3.53;6.55]	0.90 [0.85;0.99]	1.08 [0.67;1.11]	-5.51 [-5.73;-0.27]	-0.04 [-0.05;-0.03]
$C_{co(A)}$	0.224	0.111 [0.082;0.140]	0.216	0.11 [0.04;0.17]	0.38 [0.17;0.50]	0.62 [0.61;0.63]	1.15 [1.06;1.21]	0.78 [0.60;1.06]	0.12 [0.12;0.15]
$C_{co(B)}$	0.248	0.136 [0.105;0.169]	0.294	0.11 [0.04;0.17]	0.38 [0.17;0.50]	0.62 [0.61;0.63]	1.15 [1.06;1.21]	0.78 [0.60;1.06]	0.12 [0.12;0.15]
$C_{co(C)}$	0.250	0.090 [0.062;0.116]	0.186	0.11 [0.04;0.17]	0.38 [0.17;0.50]	0.62 [0.61;0.63]	1.15 [1.06;1.21]	0.78 [0.60;1.06]	0.12 [0.12;0.15]
$D_{co(A)}$	0.187	0.015 [0.004;0.028]	0.056	-0.06 [-0.14;0.15]	-11.08 [-11.08;-3.72]	2.21 [1.80;2.21]	0.45 [0.45;0.48]	-5.05 [-5.05;0.50]	0.13 [-0.32;0.37]
$D_{co(B)}$	0.186	0.018 [0.004;0.033]	0.205	-0.06 [-0.14;0.15]	-11.08 [-11.08;-3.72]	2.21 [1.80;2.21]	0.45 [0.45;0.48]	-5.05 [-5.05;0.50]	0.13 [-0.32;0.37]
$D_{co(C)}$	0.223	0.064 [0.004;0.084]	0.083	-0.06 [-0.14;0.15]	-11.08 [-11.08;-3.72]	2.21 [1.80;2.21]	0.45 [0.45;0.48]	-5.05 [-5.05;0.50]	0.13 [-0.32;0.37]
$E_{co(A)}$	0.187	0.044 [0.023;0.095]	0.050	0.26 [-0.24;0.26]	3.08 [-3.96;3.08]	4.40 [1.31;4.40]	0.14 [0.11;0.75]	-0.28 [-0.28;3.01]	-0.74 [-0.74;0.32]
$E_{co(B)}$	0.228	0.039 [0.014;0.096]	0.094	0.26 [-0.24;0.26]	3.08 [-3.96;3.08]	4.40 [1.31;4.40]	0.14 [0.11;0.75]	-0.28 [-0.28;3.01]	-0.74 [-0.74;0.32]
$E_{co(C)}$	0.186	0.110 [0.055;0.184]	0.119	0.26 [-0.24;0.26]	3.08 [-3.96;3.08]	4.40 [1.31;4.40]	0.14 [0.11;0.75]	-0.28 [-0.28;3.01]	-0.74 [-0.74;0.32]
$F_{co(A)}$	0.192	0.056 [0.015;0.095]	0.203	-0.15 [-0.19;0.02]	-0.11 [-0.20;0.01]	0.66 [0.40;0.83]	0.47 [0.43;0.48]	0.63 [0.60;0.63]	1.19 [1.19;1.27]
$F_{co(B)}$	0.228	0.035 [0.010;0.096]	0.189	-0.15 [-0.19;0.02]	-0.11 [-0.20;0.01]	0.66 [0.40;0.83]	0.47 [0.43;0.48]	0.63 [0.60;0.63]	1.19 [1.19;1.27]
$F_{co(C)}$	0.186	0.100 [0.046;0.184]	0.221	-0.15 [-0.19;0.02]	-0.11 [-0.20;0.01]	0.66 [0.40;0.83]	0.47 [0.43;0.48]	0.63 [0.60;0.63]	1.19 [1.19;1.27]

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

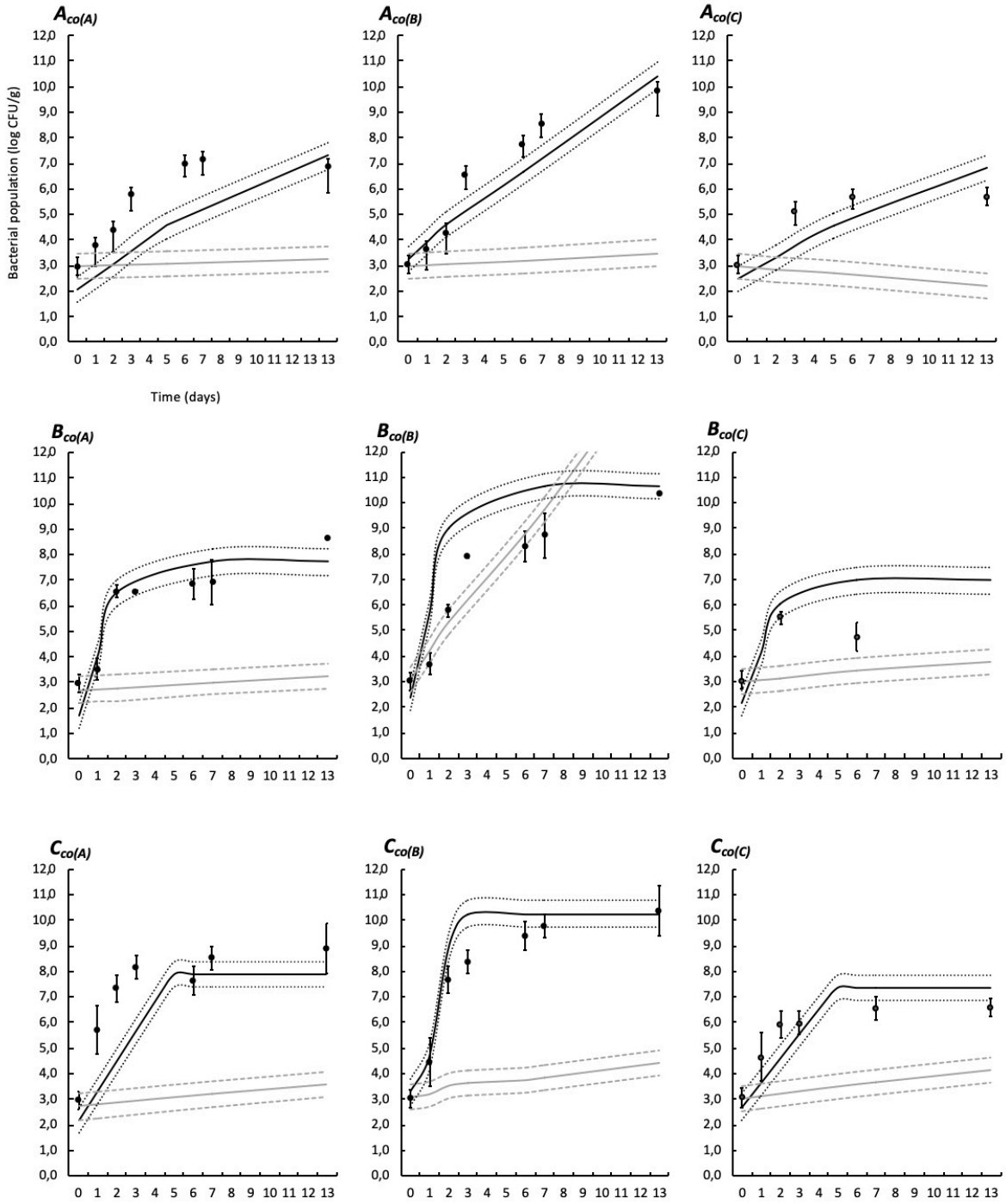

Figure 44. Experimental observed data (validation dataset, means and standard deviation of the three replicates) and simulations provided by the predictive models based on the modified Jameson-effect equation and on the Lotka-Volterra equation in food wrap. See Table 37 for list of the codes used. Black solid lines represent the Jameson-effect model, grey solid lines represent the Lotka-Volterra model. Dashed and dotted lines represent the acceptable simulation zone (ASZ) used to compare observations versus predictions of the interaction models.

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

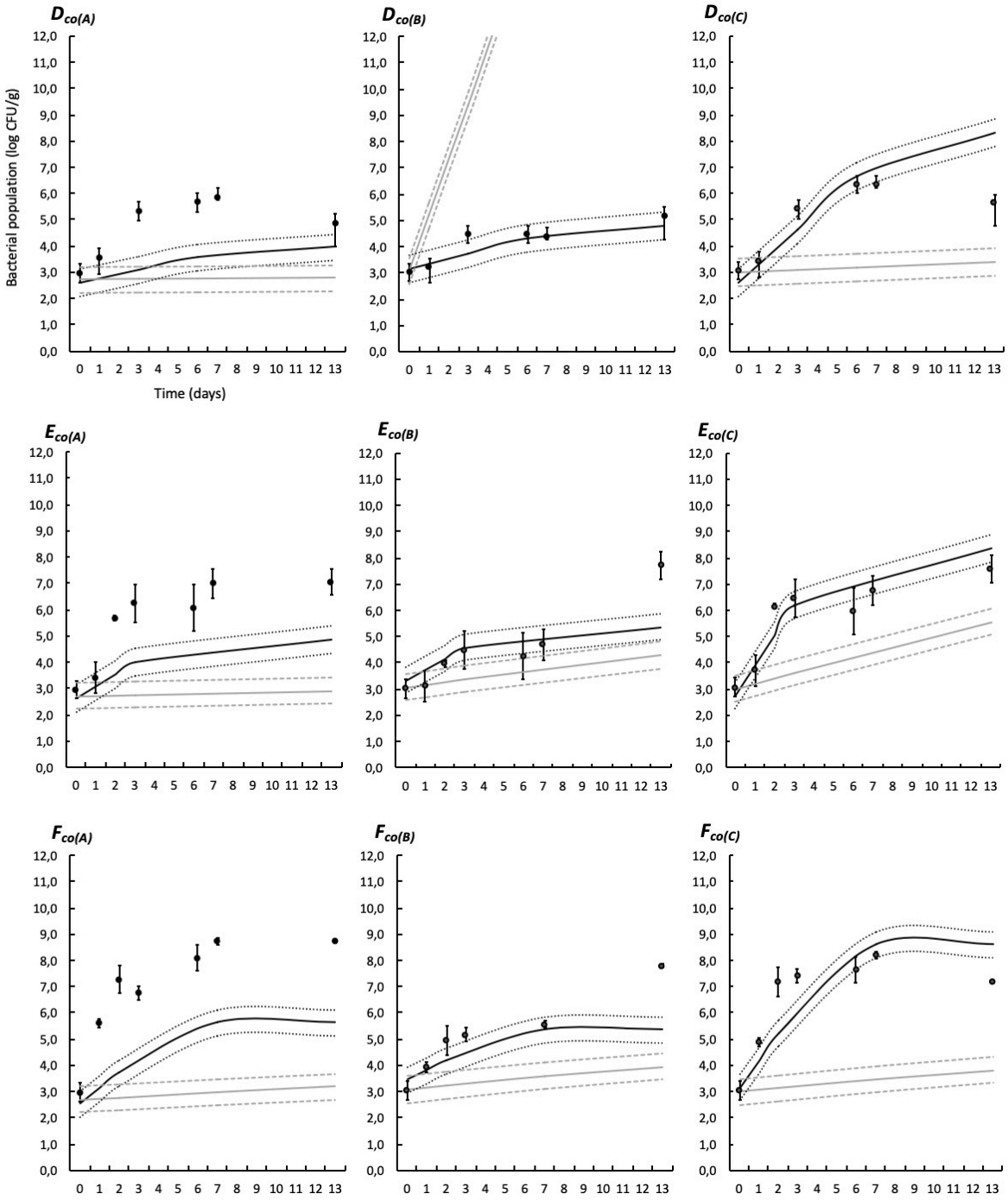

Figure 45. Experimental observed data (validation dataset, means and standard deviation of the three replicates) and simulations provided by the predictive models based on the modified Jameson-effect equation and on the Lotka-Volterra equation in modified atmosphere packaging. See Table 37 for list of the codes used. Black solid lines represent the Jameson-effect model, grey solid lines represent the Lotka-Volterra model. Dashed and dotted lines represent the acceptable simulation zone (ASZ) used to compare observations versus predictions of the interaction models.

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

3.7. Validation dataset

As previously described, plate counts in validation dataset increased during the shelf life with increasing the temperature (**Figures 46** and **47**).

At the end of the shelf life, the natural logarithm of the bacterial count was over 7.0 log CFU/g.

During the storage, a high growth rate and a more rapidly reached stationary phase are also correlated to FW and the highest storage temperatures.

No bacterial growth was observed on PCA for the control samples (limit detection < 3.00 log CFU/g) (data not shown in this paper).

The relative abundance results obtained by metagenetic analysis (expressed in %) at species levels (>1%) are represented in cumulated histograms for validation dataset in Supplemental Material for FW (**Table 49**) and MAP (**Table 50**). The metagenetic data were then combined with the plate counts results in order to obtain estimated bacterial counts (**Table 51**).

At day 0, the distribution of read percentages shows high values (> 90%) of *Photobacterium* spp., *Ph. kishitanii* and *Ph. illiopiscarium*.

In FW, *Pseudomonas* spp. reached higher values at day 3, and became the most represented bacteria until the end of the shelf life (>90%). *B. thermosphacta* reached lesser values, with 3.22% at the end of the shelf life. *Ln. gelidum* was always under the detection limit. These results are in accordance with those obtained in co-culture experiments.

In MAP, *Photobacterium* spp. was the most represented genus (>90%) during storage. However, low levels of *B. thermosphacta* and *Ln. gelidum* were observed at 8°C and 12°C. *Pseudomonas spp.* was always under the detection limit. These results are different from those obtained in co-culture experiments.

Moreover, pH value of the validation dataset at the end of the shelf life was statistically different to control samples (7.06 ± 0.80, $n = 7$, p-value = 0.01).

At the same time, the concentration of carbon dioxide also showed higher values than control samples (35.5 ± 1.64, 56.7 ± 2.17, and 96.7 ± 5.57, at 4°C, 8°C and 12°C, respectively).

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

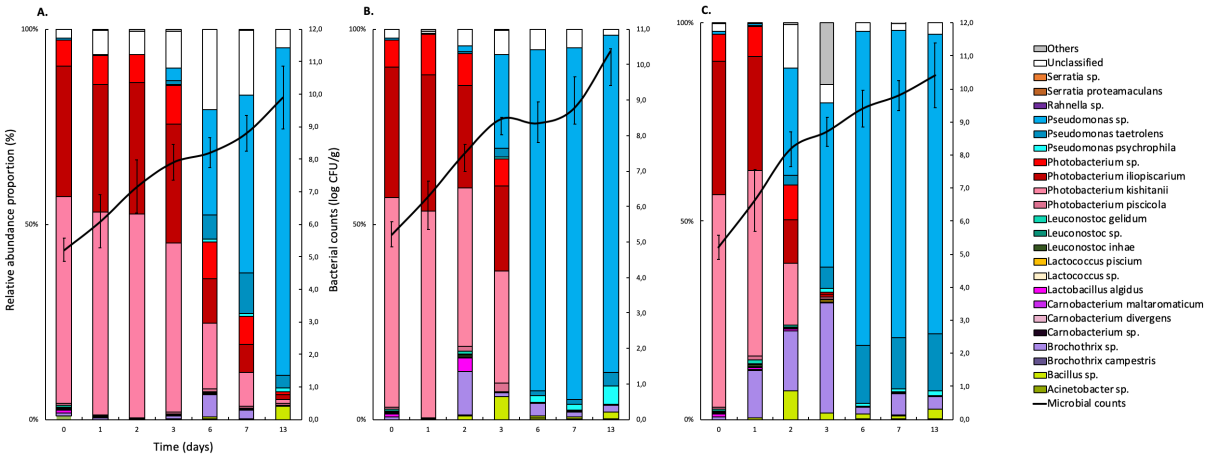

Figure 46. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at species levels in validation dataset during storage in FW (**A**, at 4°C; **B**, at 8°C; **C**, at 12°C) At species levels, the taxa representing <1% in relative abundance were merged in the category of "Others". The solid represents the plate counts (means and standard deviation of the three replicates).

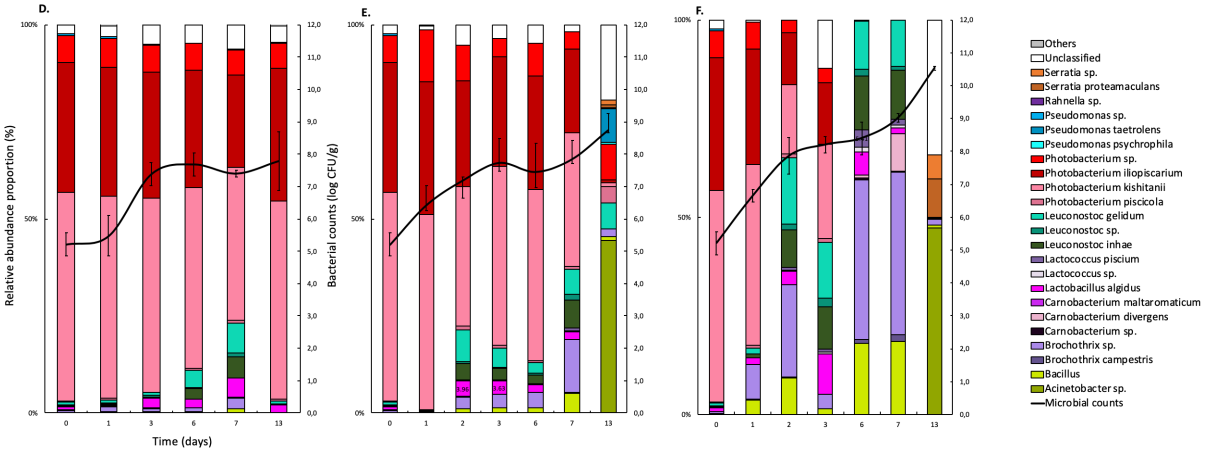

Figure 47. Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at species levels in validation dataset during storage in MAP (**D**, at 4°C; **E**, at 8°C; **F**, at 12°C). At species levels, the taxa representing <1% in relative abundance were merged in the category of "Others". The solid represents the plate counts (means and standard deviation of the three replicates).

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 49. Distribution of metagenetic reads percentages at species level for validation dataset, during storage of MP samples in FW. At species levels, the taxa representing <1% in relative abundance were merged in the category of "Others"; Temp., temperature (°C); -*, data under the detection limit.

Temp.	Days	*Acinetobacter* sp.	*Bacillus* sp.	*B. campestris*	*Brochothrix* sp.	*Carnobacterium* sp.	*C. divergens*	*C. maltaromaticum*	*Lb. algidus*	*Lactococcus* sp.	*Lc. piscium*	*Ln. inhae*	*Leuconostos* sp.	*Ln. gelidum*	*Ph. kishitanii*	*Ph. illiopiscarium*	*Photobacterium* sp.	*Ph. piscicola*	*Ps. psychrophila*	*Ps. taetrolens*	*Pseudomonas* sp.	*Rahnella* sp.	*S. proteamaculans*	*Serratia* sp.	Unclassified	Others
4°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	- *	- *	0.45	- *	- *	0.03	0.05	0.03	0.08	- *	- *	0.19	0.19	52.23	32.68	7.24	- *	0.11	0.24	- *	- *	- *	6.10	0.39
	2	- *	- *	- *	0.17	- *	0.03	- *	- *	- *	- *	- *	- *	0.03	0.11	52.41	33.46	7.19	- *	- *	0.14	- *	- *	- *	5.84	0.63
	3	- *	0.09	- *	0.69	0.03	0.03	- *	0.21	0.03	0.03	0.03	0.03	0.15	0.51	43.46	30.32	9.94	0.15	1.02	3.41	- *	- *	- *	9.30	0.58
	6	0.03	0.52	- *	5.80	- *	- *	- *	0.17	0.06	0.09	0.09	- *	0.29	0.73	16.80	11.37	9.40	0.73	6.24	27.13	- *	- *	- *	20.57	0.00
	7	- *	0.25	- *	2.13	0.03	- *	- *	0.09	0.06	0.09	- *	- *	0.09	0.68	8.66	7.21	7.15	0.62	10.45	45.65	- *	- *	- *	16.50	0.33
	13	- *	3.35	- *	0.09	- *	- *	- *	0.15	- *	- *	0.03	- *	- *	0.53	1.06	1.12	0.88	0.94	3.20	83.92	- *	- *	- *	4.61	0.15
8°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	0.06	- *	0.17	- *	- *	- *	- *	- *	- *	- *	- *	0.06	0.14	52.98	34.93	10.37	- *	0.03	0.22	- *	- *	- *	0.47	0.58
	2	- *	0.90	0.13	11.16	0.10	0.07	- *	3.27	0.07	0.33	0.40	0.23	0.80	1.30	40.58	26.09	8.28	0.03	0.43	1.50	- *	- *	- *	4.24	0.07
	3	- *	5.77	- *	1.17	- *	- *	- *	0.07	- *	- *	0.15	- *	- *	2.04	28.76	21.75	7.15	0.29	2.34	23.87	- *	- *	- *	6.20	0.44
	6	0.37	0.55	0.05	3.14	0.02	0.05	- *	- *	- *	0.02	0.02	- *	0.02	- *	0.12	0.02	0.02	1.62	1.14	87.41	- *	- *	- *	5.35	0.05
	7	0.09	0.42	0.02	1.39	- *	0.02	- *	0.02	- *	- *	- *	- *	- *	- *	0.14	0.12	0.02	1.53	1.20	90.35	- *	- *	- *	4.65	0.02
	13	0.03	1.75	- *	1.78	- *	- *	- *	- *	- *	- *	- *	- *	- *	0.03	0.19	0.11	0.03	4.71	3.39	86.48	- *	- *	- *	1.51	0.00
12°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	0.32	0.10	11.98	- *	0.13	- *	0.64	- *	0.19	0.54	0.19	0.89	0.96	46.75	28.86	7.56	- *	0.26	0.41	- *	- *	- *	0.11	0.13
	2	- *	7.15	- *	15.04	- *	0.14	- *	0.53	- *	0.11	0.21	0.14	0.42	- *	15.68	11.00	8.70	- *	2.37	27.03	- *	- *	- *	10.90	0.57
	3	- *	1.50	- *	27.86	0.03	- *	- *	0.25	- *	0.37	0.09	0.06	0.12	- *	0.56	0.78	0.34	0.99	5.40	41.53	- *	- *	- *	4.65	15.46
	6	0.24	1.16	0.06	1.66	0.03	- *	0.06	- *	- *	- *	- *	- *	- *	- *	- *	- *	- *	0.74	14.60	79.32	- *	- *	- *	2.14	0.00
	7	0.12	0.84	0.09	5.45	- *	0.09	0.12	0.03	- *	- *	0.03	0.06	- *	- *	- *	0.03	- *	0.93	12.90	77.34	- *	- *	- *	1.89	0.09
	13	0.19	2.34	0.06	3.22	- *	- *	- *	- *	- *	- *	- *	- *	0.03	- *	- *	- *	- *	1.20	14.54	75.37	- *	0.06	- *	2.94	0.00

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 50. Distribution of metagenetic reads percentages at species level for validation dataset, during storage of MP samples in MAP. At species levels, the taxa representing <1% in relative abundance were merged in the category of "Others"; Temp., temperature (°C); -*, data under the detection limit; -[a], no analysis performed this day.

Temp.	Days	*Acinetobacter* sp.	*Bacillus* sp.	*B. campestris*	*Brochothrix* sp.	*Carnobacterium* sp.	*C. divergens*	*C. maltaromaticum*	*Lb. algidus*	*Lactococcus* sp.	*Lc. piscium*	*Ln. inhae*	*Leuconostoc* sp.	*Ln. gelidum*	*Ph. kishitanii*	*Ph. illiopiscarium*	*Photobacterium* sp.	*Ph. piscicola*	*Ps. psychrophila*	*Ps. taetrolens*	*Pseudomonas* sp.	*Rahnella* sp.	*S. proteamaculans*	*Serratia* sp.	Unclassified	Others
4°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	- *	- *	1.23	- *	- *	0.14	0.36	0.06	0.03	0.22	0.14	0.78	0.42	52.17	33.45	7.41	- *	0.11	0.45	- *	- *	- *	2.80	0.24
	2	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]	- [a]
	3	- *	- *	- *	0.92	- *	- *	0.03	2.49	0.03	0.09	0.31	0.31	0.74	0.22	50.15	32.39	7.13	- *	0.03	0.09	- *	- *	- *	4.90	0.17
	6	0.03	- *	- *	0.97	- *	- *	- *	2.13	0.03	- *	2.76	0.21	4.43	0.67	46.77	30.34	6.86	- *	- *	0.06	- *	- *	- *	4.52	0.21
	7	- *	- *	- *	2.88	0.16	0.06	- *	4.85	0.13	0.09	5.42	1.06	7.74	0.75	39.74	23.99	6.61	- *	- *	0.09	- *	- *	- *	6.36	0.06
	13	- *	0.03	- *	0.12	- *	- *	- *	2.06	- *	- *	0.23	- *	0.70	0.46	51.06	34.16	6.32	- *	- *	0.23	- *				
8°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	- *	- *	0.10	- *	- *	- *	0.12	0.02	0.15	0.10	0.07	0.12	0.07	50.38	34.10	13.36	- *	- *	0.05	- *	- *	- *	0.94	0.40
	2	- *	- *	0.03	2.97	0.03	- *	0.06	3.96	0.06	0.21	4.24	0.51	8.50	0.76	36.47	27.48	9.35	- *	0.03	0.03	- *	- *	- *	5.29	0.00
	3	- *	- *	0.06	3.34	- *	- *	- *	3.63	- *	0.23	2.95	0.34	5.26	0.51	46.69	28.73	4.76	- *	- *	0.06	- *	- *	- *	3.57	0.00
	6	- *	- *	- *	3.95	- *	- *	- *	2.03	0.06	0.09	2.34	0.49	2.82	0.55	44.57	29.63	8.56	- *	- *	0.06	- *	- *	- *	4.85	0.00
	7	- *	0.07	0.35	14.16	0.03	- *	0.07	2.09	0.21	0.83	7.62	1.39	6.92	0.83	36.24	22.47	4.83	- *	0.07	- *	- *	- *	- *	1.81	0.00
	13	44.73	0.09	- *	2.07	- *	- *	- *	- *	- *	- *	0.09	- *	6.80	4.01	1.17	0.81	9.18	0.36	8.87	0.32	- *	0.59	1.31	19.62	0.00
12°C	0	- *	- *	- *	0.56	- *	0.03	- *	0.86	0.18	0.12	0.21	0.12	0.59	0.35	53.75	33.54	6.80	0.03	0.06	0.56	- *	- *	- *	2.10	0.15
	1	- *	0.03	0.16	9.23	- *	- *	- *	1.65	- *	0.16	0.82	0.19	1.46	0.73	47.57	30.19	7.07	- *	0.06	0.13	- *	- *	- *	0.54	0.00
	2	- *	- *	0.39	25.64	0.06	0.03	- *	3.80	0.33	0.56	10.58	1.69	18.54	0.98	19.32	14.56	3.36	- *	0.03	0.09	- *	- *	- *	- *	0.03
	3	- *	- *	0.06	3.59	0.03	0.03	- *	10.47	0.30	0.79	10.90	2.37	14.31	0.88	24.26	15.80	3.71	- *	- *	0.09	- *	- *	- *	12.36	0.03
	6	- *	- *	1.19	49.13	0.60	1.06	- *	7.26	1.36	5.41	16.53	2.09	15.03	0.20	0.08	0.03	0.03	- *	- *	- *	- *	- *	- *	- *	0.00
	7	- *	- *	2.04	50.50	0.47	11.52	- *	1.74	0.97	1.80	15.35	1.20	14.22	- *	0.07	0.07	0.03	- *	- *	0.03	- *	- *	- *	- *	- *
	13	47.53	- *	0.04	1.53	- *	- *	- *	- *	- *	- *	0.27	- *0.04	- *	- *	- *	- *	- *	- *	0.04	0.13	- *	9.84	6.06	34.50	0.00

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Table 51. Estimate bacterial counts calculated for validation dataset. Mean values with lower and upper confidence intervals; -*, data under the detection limit.

Conditions/ Bacteria	Time (days) 0	1	2	3	6	7	13
FW 4°C				- [a]			
B.thermosphacta	2.96 [3.31-2.60]	3.75 [4.10-2.94]	4.37 [4.72-3.55]	5.74 [6.09-5.18]	6.96 [7.31-6.51]	7.13 [7.48-6.58]	6.84 [7.19-5.88]
Pseudomonas spp.	3.02 [3.37-2.66]	3.63 [3.98-2.83]	4.29 [4.64-3.47]	6.57 [6.92-6.01]	7.73 [8.08-7.28]	8.55 [8.90-8.00]	9.84 [10.19-8.88]
L. gelidum	3.06 [3.41-2.71]	- *	- *	5.16 [5.51-4.60]	5.66 [6.01-5.21]	- *	5.68 [6.03-5.33]
FW 8°C							
B.thermosphacta	2.96 [3.31-2.60]	3.51 [3.93-3.09]	6.55 [6.80-6.31]	6.54 [6.57-6.51]	6.85 [7.44-6.26]	6.92 [7.80-6.04]	8.64 [8.71-8.57]
Pseudomonas spp.	3.02 [3.37-2.66]	3.68 [4.10-3.26]	5.80 [6.04-5.56]	7.90 [7.93-7.87]	8.31 [8.90-7.72]	8.74 [9.62-7.86]	10.37 [10.44-10.30]
L. gelidum	3.06 [3.41-2.71]	- *	5.52 [5.76-5.28]	- *	4.75 [5.34-4.16]	- *	- *
FW 12°C							
B.thermosphacta	2.96 [3.31-2.60]	5.69 [6.63-4.75]	7.36 [7.88-6.83]	8.14 [8.59-7.69]	7.62 [8.17-7.07]	8.54 [8.99-8.09]	8.91 [9.89-7.94]
Pseudomonas spp.	3.02 [3.37-2.66]	4.44 [5.38-3.50]	7.65 [8.17-7.12]	8.38 [8.83-7.93]	9.38 [9.93-8.83]	9.76 [10.21-9.31]	10.36 [11.34-9.38]
L. gelidum	3.06 [3.41-2.71]	4.65 [5.59-3.71]	5.93 [6.46-5.41]	5.97 [6.42-5.52]	- *	6.57 [7.02-6.12]	6.59 [6.94-6.24]
MAP 4°C							
B.thermosphacta	2.96 [3.31-2.60]	3.56 [3.91-2.94]	- *	5.34 [5.69-4.99]	5.67 [6.02-5.32]	5.86 [6.21-5.76]	4.85 [5.20-3.96]
Pseudomonas spp.	3.02 [3.37-2.66]	3.22 [3.57-2.60]	- *	4.47 [4.82-4.11]	4.47 [4.82-4.12]	4.37 [4.72-4.27]	5.15 [5.50-4.26]
L. gelidum	3.06 [3.41-2.71]	3.34 [3.79-2.81]	- *	5.40 [5.75-5.04]	6.35 [6.70-6.00]	6.34 [6.69-6.24]	5.63 [5.98-4.74]
MAP 8°C							
B.thermosphacta	2.96 [3.31-2.60]	3.42 [4.02-2.82]	5.66 [5.76-5.56]	6.26 [6.99-5.52]	6.05 6.94-5.16]	6.99 [7.57-6.41]	7.05 [7.56-6.54]
Pseudomonas spp.	3.02 [3.37-2.66]	3.12 [3.52-2.52]	3.97 [4.07-3.87]	4.49 [5.22-3.75]	4.24 [5.13-3.35]	4.68 [5.26-4.10]	7.72 [8.23-7.21]
L. gelidum	3.06 [3.41-2.71]	3.72 [4.32-3.12]	6.14 [6.24-6.04]	6.47 [7.21-5.74]	5.97 [6.86-5.08]	6.76 [7.34-6.18]	7.57 [8.08-7.06]
MAP 12°C							
B.thermosphacta	2.96 [3.31-2.60]	5.62 [5.80-5.44]	7.27 [7.81-6.73]	6.75 [7.01-6.49]	8.09 [8.59-7.59]	8.73 [8.87-8.59]	8.71 [8.77-8.65]
Pseudomonas spp.	3.02 [3.37-2.66]	3.93 [4.11-3.75]	4.94 [5.48-4.40]	5.16 [5.42-4.90]	- *	5.55 [5.69-5.41]	7.78 [7.84-7.72]
L. gelidum	3.06 [3.41-2.71]	4.87 [5.05-4.69]	7.17 [7.71-6.63]	7.42 [7.68-7.16]	7.63 [8.13-7.13]	8.21 [8.35-8.07]	7.18 [7.24-7.12]

Study 4. Modelling the growth and interaction between *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum* in minced pork samples.

Discussion

The present study aimed to obtain the growth parameters of three specific spoilage microorganisms previously isolated in MP samples, and to develop a three spoilage species interaction model under different storage conditions. *B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum* were previously isolated as predominant strains (>50% reads) from different batches of Belgian MP samples at the end of their use-by-date (Cauchie *et al*., 2019). Considered as the main representative spoilage species in meat and meat products (Andritsos *et al*., 2012; Casaburi *et al*., 2014; De Filippis *et al*., 2013; Del Blanco *et al*., 2017; Geeraerts *et al*., 2017; Koort *et al*., 2005; Li *et al*., 2019a; Liu *et al*., 2006; Mann *et al*., 2016; Mansur *et al*., 2019; Nychas *et al*., 2008; Pennacchia *et al*., 2009; Pennacchia *et al*., 2011; Peruzy *et al*., 2019a; Raimondi *et al*., 2018; Stellato *et al*., 2016; Stoops *et al*., 2015; Zhao *et al*., 2015), these bacteria were inoculated on irradiated MP samples, in mono- and in co-culture experiments.

However, the selection of dominant and non-dominant species in inoculation experiments could have been more interesting in order to better represent the natural contamination of MP, and thus to better model the impact of sub-dominant microbiota. Indeed, others taxa were also present in MP samples but in lesser abundance, even if they are considered as dominant taxa in several studies: *Photobacterium* spp. (Ast *et al*., 2007; Bjornsdottir-Butler *et al*., 2016; Fogarty *et al*., 2019; Jääskeläinen *et al*., 2019; Kuuliala *et al*., 2018; Moretro *et al*., 2016; Nieminen *et al*., 2016) and *Lactobacillus* spp. (especially *Lb. algidus*) (Alvarez-Sieiro *et al*., 2016; Dalcanton *et al*., 2013; Doulgeraki *et al*., 2012; Fadda *et al*., 2010; Kato *et al*., 2000; Nieminen *et al*., 2015; Pothakos *et al*., 2015; Stefanovic *et al*., 2017; Woraprayote *et al*., 2016). According to this, they were not included in models of this study, as all others non-dominant microbiota. Moreover, *Ps. fluorescens* and *Ps. fragi* were used together in experiments. The objective of this study was to offer an exploratory approach to the proposed method by following the common genus formed by the two species mentioned. So, it would have been interesting to inoculate MP samples with both species in different batches, as behavior of these species is different according to the storage conditions.

The inputs of models were provided from culture-dependent and culture-independent analysis performed on inoculation experiments. The association of both techniques allow us to obtain estimate abundance during storage in co-culture experiments. Although we acknowledge that the plate count method is not able to assess all the microbial populations in presence, the combination of these two methods was previously validated by a quantitative PCR (qPCR) approach (Cauchie *et al*., 2017). This approach was also used in others studies (Chaillou *et al*., 2015; Delhalle *et al*., 2016). Fougy *et al*. (2016)

also showed that this conversion can be used to obtain an extrapolated estimation of the bacterial concentration, and may be used in food industries. But comparison of these results with counts on selective media would also be interesting to study in the future. Moreover, even if this method overestimates the bacterial concentration, it could be beneficial in a worst-case risk assumption for food industries (Crotta *et al*., 2016; Membré and Boué, 2018).

In this study, models show relatively good fitting indexes (*RrMSE* and R^2). Good performances (*ASZ*) in the three species interaction approach were also obtained, especially with the modified Jameson-effect model.

The growth parameters of the three specific spoilage microorganisms were obtained for mono- and co-culture experiments by fittings primary and secondary models (**Tables 42** and **43**). The food packaging shows the highest impact on bacterial growth rates (μ_{max}), which in turn have the strongest influence on the shelf life of food products (Simpson and Carevic, 2004; Stoops *et al*., 2015; Guillard *et al*., 2016; Saraiva *et al*., 2016; Couvert *et al*., 2017). In accordance with Liu *et al*. (2006), N_0 showed a little correlation with the microbial shelf life in mono- and co-culture experiments, indicated that the storage outcome of food seems to be not completely determined by the initial microbial counts. Moreover, no obvious correlation has been shown between N_{max} and shelf life in co-cultures experiments. This can be explained by the fact that meat shelf life is determined primarily by the metabolic patterns of the spoilage microbiota, rather than by total counts of bacteria (Liu *et al*., 2006). However, it can be observed that the parameters obtained in single culture were quite different from those in co-culture, especially for *Pseudomonas* spp. and *B. thermosphacta*. In FW, *B. thermosphacta* grew faster on mono-culture, but this behavior was not detected in co-culture. On the opposite, *Pseudomonas* spp. became the dominant bacteria in FW in the presence of the two others microorganisms. These differences between mono- and co-culture inoculations have already been observed by Hibbing *et al*. (2010) and Quinto *et al*. (2018).

On the other hand, observations in co-culture experiments showed that the suppression of the two other bacteria occurred when the dominant one reached its *MCP*. This result reveals a potential Jameson effect between populations, rather than a prey-predator trend. According to these, differences between mono- and co-cultures experiments could maybe be explained by two hypotheses: (i) a non-specific interaction involving the Jameson effect, where growth inhibition is the result from a depletion in nutrient bioavailability and toxicity increase when the dominant bacteria reaches N_{MCP}; and (ii) a specific interaction due to the modification of the food matrix where bacteria are growing (i.e. catabolism of carbon sources, the production of by products such as carbon dioxide and acids, …) (Bruce *et al*., 2017; Correia Peres Costa *et al*., 2019; Kumariya *et al*., 2019; Quinto *et al*., 2018). Nadell *et al*. (2016) have mentioned that *Ps. fluorescens* can produces extracellular matrix materials to give them an advantage over competitors. Quorum sensing (QS) could also be related to this inhibition by the

dominant bacteria, by exchanging information to synchronize bacterial behavior in mixed-culture (Dubey and Ben-Yehuda, 2011; Ng and Basster, 2009; Quinto *et al*., 2018).

The development of a three spoilage species interaction model was then performed using two models: the modified Jameson-effect and the Lotka-Volterra (**Figures 44** and **45**). The modified Jameson-effect model showed slightly better fits than the Lotka-Volterra equation, with 40 – 86% out of the observed counts falling into the *ASZ*, indicating a satisfactory model performance. It only concerns 14-48% for the prey-predator approach. These results can be explained by the fact that the dynamics of experimental and validation datasets seems to follow a Jameson behavior, because the minority bacteria decelerate when the majority one reaches the *MCP* (Cornu *et al*. (2011). Moreover, the modified Jameson-effect equation is considering growth parameters (μ_{max}, t_{MCP} and N_0) for modeling (Equation 21). These parameters are obtained by primary and secondary fittings, and are relatively reliable in our study due to the numbers of samples analyzed. On the other hand, the Lotka-Volterra model is based on complex interaction factors (Equation 22) which are obtained by linear regression. Due to the high variability of interactions that can be simulated, particularly in three or more species models, these interaction factors must necessarily be as accurate as possible. In this study, interaction factors are included in highly variable intervals (**Table 48**), with some variations observed according to the temperature (Correia Peres Costa *et al*., 2019; Mejlholm and Dalgaard, 2015; Moller *et al*., 2013). More datasets are probably needed to obtained reliable factors. Also, the Lotka-Volterra model could be modified for a more realistic approach by considering the effect of other influencing factors (e.g. environmental conditions such as several storage and packaging conditions, bacteriocin production, etc.) (Baka *et al*., 2014; Powell *et al*., 2004).

More inoculation experiments are so needed to develop better predictive models, especially for a three or more spoilage species interaction approach. And also, to better understand the dynamics of spoilage bacteria towards each other and in the presence of natural microbiota. As mentioned by Quinto *et al*. (2018): "it is well known that a spoilage microorganism can either stimulate, inhibit or have no effect on the growth of the pathogenic species". So, it could be interesting to study interactions between spoilage microorganisms, with production of metabolites or other substances as interaction factors. It would also be interesting to investigate co-culture experiments with two species. Moreover, metabolites production by each of the inoculated bacteria, as inputs interacting models, will be studied in another scientific publication.

Finally, naturally contaminated samples were used to validate the developed models by considering the effect of the food microbiota. Differences with co-culture experiments were obtained: a predominance of *Photobacterium* spp. (>90% of reads) was observed in MAP (**Figure 47**). It could be interesting to take also into account this bacterium for modeling interactions. The addition of this bacterium could possibly improve the reliability of predictions, particularly for the Lotka-Volterra

model. Moreover, *Photobacterium* spp. is not well recovered on PCA at 22°C (Dalgaard *et al*., 1997; Hilgarth *et al*., 2018c). According to this, improving cultivation methods for this bacterium is important to obtain more reliable results. Further studies are so needed to develop more realistic interacting predictive models, especially in a three or more spoilage species interaction approach, and to develop new food preservation process.

In conclusion, new omics technologies, such as metagenetics and metabolomics, are important to characterize and to follow the dynamics of bacterial microbiota and metabolites in complex food matrices. New generations of predictive models will probably need to be developed, by considering the results provided by these techniques. These models will provide a better understanding of the interactions between microorganisms and food, and microorganisms between them.

Study 5

A NMR-based metabolomics study of minced pork samples inoculated with *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum*

Version in progress (to be submitted in Food Microbiology)

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Abstract

⇒ **Objective 4.** Study the metabolome of minced pork samples

The aim of this study was to assess bacterial meat spoilage through the dynamics of microbial counts and changes in the metabolic profile of MP samples using NMR-based metabolomics. Three dominant bacterial isolates were inoculated in irradiated samples in mono-culture experiments: *B. thermosphacta*, *Pseudomonas* spp. (*Ps. fluorescens* and *Ps. fragi*) and *Ln. gelidum*. Samples were stored under food packaging at constant temperature during 13 days. For all conditions, irradiated non-inoculated samples were also stored. Analysis were carried out by using microbial counts and ^{1}H-NMR. The multivariate analysis (OPLS-DA) shows a clear discrimination between: (i) the non-inoculated product at day 0 and 13, (ii) the inoculated and non-inoculated samples, (iii) the type of strain, and (iv) the packaging conditions. It can be observed that the type of strain inoculated has a higher impact on the metabolome than that of the packaging conditions. Some metabolites are also significantly increased: acetate, cadaverine, glutamate and succinate in samples inoculated with *Pseudomonas* spp., and acetate, acetoin and isobutyrate in samples inoculated with *B. thermosphacta*. The results for *Ln. gelidum* have not yet been reported. Exploration of the correlations of NMR-based metabolomics results with others microbial parameters suggested that their use might be a useful tool to provide information on minced pork spoilage.

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Introduction

Food spoilage is a major problem for the food industry and consumers because it renders products unacceptable for consumption and consequently leads to significant food waste and economic losses (Garnier *et al*., 2017). In Europe, the losses of initial meat production represent 20% and more than half of this occurs at animal production, slaughtering, processing and distribution steps (Food and Agriculture Organization, 2011; Kergourlay *et al*. 2015). Foof waste which is a major issue, is mainly due to contamination and development in a food matrix of spoilage bacteria during the transformation and storage steps (Lipsinki *et al*., 2013; Remenant *et al*., 2015). It is well established that spoilage of meat is the result of decomposition and formation of metabolites caused by the growth and enzymatic activity of microorganisms (Argyri *et al*., 2015), highlighting the importance of managing the quality of food products.

Food quality is commonly described using sensory evaluation, microbial inspection, biochemical methods, and proteome analysis. New "omics" approaches and more specifically metabolomics, which deals with the study of the metabolite's profiles of samples, are relatively new investigation tools in food science ("foodomics") (Aru *et al*., 2016; Cevallos-Cevallos *et al*., 2009). Metabolomics was initially applied to fields of plant science and toxicology (Fiehn, 2002) and has gained popularity in the last 10 years, as shown by the recent surge in the number of metabolomic studies in foods to obtain metabolite profiling of salmon, meat, honey, milk, olive oil, wine, tea and others plants (Ibanez *et al*., 2013; Jung *et al*., 2010; Mannina *et al*., 2012; Piras *et al*., 2013; Ritota *et al*., 2012; Singh *et al*., 2017; Trimigno *et al*., 2015). NMR spectroscopy and more specifically proton NMR is, together with MS, the most widely used analytical platform for metabolomic analysis (Marcone *et al*., 2013; Pinu, 2016; Sundekilde *et al*., 2013).

Several studies showed the interest of metabolomics for the study of food quality (Castro-Puyana and Herrero, 2013; Tarachawin *et al*., 2007; Zanardi *et al*., 2015) with assessment of food spoilage (Duarte *et al*., 2006; Pinter *et al*., 2014), geographical origin (Boffo *et al*., 2012; Brescia *et al*., 2003; Cubero-Leon *et al*., 2014; Jung *et al*., 2010; Mannina *et al*., 2001; Schievano *et al*., 2008; Shintu and Caldarelli, 2006), authenticity assessment (Aru *et al*., 2016; Jiang and Bratcher, 2016; Charlton *et al*., 2002; Chen *et al*., 2016; Chen *et al*., 2017; Mazzei and Piccolo, 2012), and metabolomic profiles linking to process and storage conditions (Consonni and Cagliani, 2008; Piras *et al*., 2013). However, among all these studies only a few have been performed on beef meat (Castejon *et al*., 2015; Jung *et al*., 2010; Zanardi *et al*., 2015) and none of them are concerning pork meat.

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Because meat has a high water content and an abundance of nutrients available for bacteria, that renders is one of the most perishable foods (Ercolini *et al*., 2011), no metabolite profiles from inoculated meat by putatively spoilage microorganisms have been reported to date. The main reason is due to the dynamic and complex nature of this system and to large gaps of knowledge on the interactions between microbiota, food structure and sensorial aspect (flavor, aroma, texture, color) (Piras *et al*., 2013). However, the putative interest for the association between microbial development and chemical changes occurring during the storage of meat is recognized as a potential means of revealing indicators of meat quality or freshness (Castejon *et al*., 2015; Ercolini *et al*., 2011; Jung *et al*., 2010).

Moreover, it is expected that the combination of metabolomic data with other complementary approaches (classical microbiology and quality parameters) can give the opportunity to gain deeper insights into, and have a better comprehension of the spoilage mechanisms (Consonni and Cagliani, 2008; Mannina *et al*., 2012). It has proved to be an attractive technique offering in a single experiment an overview of a wide range of low molecular-mass compounds (<1500 Da) present in a matrix by detecting all ^{1}H containing metabolites with concentrations above level of ten micromolar (Dufour *et al*., 2015; Shumilina *et al*., 2016; Verpoorte *et al*., 2008; Yuan *et al*., 2017).

According to these, the objectives of the present study were (i) to explore the dynamics of metabolomic profiles by ^{1}H-NMR in MP samples, during storage under different packaging and temperature conditions, and (ii) to associate putative metabolites with the dynamics of each inoculated spoilage bacterial strains.

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Material and methods

2.1. Bacterial strains and sampling

As described in the study of Cauchie *et al*. (2019), three specific spoilage microorganisms were previously isolated from different batches of naturally contaminated Belgian MP samples at the end of their use-by date. Samples were stored under 2 packaging (under air and MAP (30% CO_2 - 70% O_2)) and 3 temperature conditions (4°C, 8°C and 12°C). These predominant strains, represented more than 50% of the natural microbiota, were identified by 16S rRNA sequencing and used for experiments: *B. thermosphacta* (MM008), *Ln. gelidum* (MM045) and *Pseudomonas* spp. (*Ps. fluorescens* MM026 and *Ps. fragi* MM014). *Ps. fluorescens* and *Ps. fragi* were used in a mix because they correspond to the most frequently spoilage species in MP. Bacterial strains were stored at −80 °C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, strains were transferred from the −80 °C culture collection to Brain Heart Infusion (BHI) broth for 48 h at 22 °C. The cultures were incubated overnight at 4 °C before inoculation.

Fresh MP samples were obtained from a local Belgian manufacturer at the day of the production, corresponding to the day of slaughtering. MP samples were packed by the manufacturer in a polypropylene tray under cling film. According to the recipe MP is composed of 100% minced pork (70% lean, 30% fat), no salt, no spices, no additives, no eggs and no sugar were added. At the day of the production, the water activity of the product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.02 (n = 12). pH of the homogenized samples (5 g in 45 ml KCl) was measured with a pH meter (Knick 765 Calimatic, Germany). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

Food samples were then irradiated by gamma irradiation at 17.5 ± 0.4 kGy (Sterigenics, Fleurus, Belgium) and were stored until used at -20 °C.

2.2. Inoculation experiments

The three selected bacteria were inoculated on irradiated MP samples (1% v/w), in triplicate, with the objective to reach an average concentration of 3.0 log colony forming units (log CFU/g on the product). Experiments were performed by inoculation of individual bacterial strains (mono-culture experiments): *B. thermosphacta* MM008, *Pseudomonas* spp. (*Ps. fluorescens* MM026, *Ps. fragi*

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MM014 and MM015), and *Ln. gelidum* MM045 (n = 465 samples). Non-inoculated control samples were homogenized, in triplicate, by adding the same quantity of sterile water only (n = 36 samples).

After inoculation, MP samples were mixed in a Kenwood mixer for 2 min in speed 2 (Kenwood, Mechelen, Belgium).

Inoculated and non-inoculated MP samples were then packed (50 g) in two different type of non-sterile packaging. The first packaging was a tray (187x137x36, polyester 10 µm, homo-polymer polypropylene 50 µm, NutriPack, France) under MAP (CO_2 30% / O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France). The second packaging concerns a tray (175x135x22, polystyrene) under FW using cling film (Clinofilm).

In this study, MP samples were stored during 13 days at isothermal temperature: (i) 4°C (± 1°C), (ii) 8°C (± 1°C) and (iii) 12°C (± 1°C), in climatic chambers (Sanyo MIR 254).

The codes used for each experiment, depending on the inoculated bacteria and storage conditions, are listed in **Table 52**.

Table 52. List of the codes used for the experiments, depending on the inoculated bacteria and storage conditions. FW, food wrap packaging; MAP, modified atmosphere packaging (CO_2 30% / O_2 70% ± 0.1%).

Bacterial species	Food packaging	Temperature (°C)	Codes
B. thermosphacta	FW	4	A
	FW	8	B
	FW	12	C
	MAP	4	D
	MAP	8	E
	MAP	12	F
Pseudomonas spp.	FW	4	G
	FW	8	H
	FW	12	I
	MAP	4	J
	MAP	8	K
	MAP	12	L
Ln. gelidum	FW	4	M
	FW	8	N
	FW	12	O
	MAP	4	P
	MAP	8	Q
	MAP	12	R

Moreover, irradiated MP samples were also inoculated by a mix containing the same bacterial strains (1:1:1 ratio) (co-culture experiments). These co-culture experiments were stored under the same storage conditions as mono-culture experiments.

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2.3. pH and gas composition measurements

At the first and the last day of storage, pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Germany).

Oxygen and carbon dioxide concentrations of samples stored in MAP were monitored daily (CheckMate 3, Dansensor, France).

Nonparametric statistical tests were used to compare the pH values and the gas measurements between samples. All tests were considered as significant for a p-value <0.05.

2.4. Plate count enumeration

Twenty-five grams of product were put into a Stomacher bag with a mesh screen liner (80 µm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10 g/L peptone, 5 g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (1.0 g/L peptone 8.5 g/L sodium chloride, #CM0733, Oxoid, Hampshire, England) were prepared for microbiological analysis and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis in duplicate (Spiral plater, DW Scientific, England). Total viable count (TVC) for the psychrophilic microbiota were enumerated on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France) after 72 h at 22°C (model 1535 incubator, Shel Lab, Sheldon Manufacturing Inc., USA). Enumerations were performed for mono- and co-culture experiments, and transformed in decimal logarithmic values. Samples from both experiments were analyzed at the first day of inoculation (day 0) and daily until the last day of storage (day 13). Non-inoculated samples were only analyzed at day 0 and at day 13.

2.5. Fittings of bacterial curves

Growth curves of each bacteria were fitted by primary model of Baranyi and Roberts (1994) to estimate growth parameters: initial bacterial concentration (N_0), maximal bacterial concentration (N_{max}), lag-time duration (*lag*) and maximal growth rates (μ_{max}). All fittings were performed on triplicate data sets, using the nlsMicrobio package (Baty and Delignette-Muller, 2013) from the open source R software (R Core Team, 2019).

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2.6. Sample preparation for NMR analysis

^{1}H-NMR analysis of MP samples was realized at day 0 and day 13, in five repetitions, for inoculated and non-inoculated samples. A volume of 500 µl deuterated phosphate butter (DPB, pH 7.4) was added to 100 mg of each meat samples. The mixture was homogenized with a vortex during 1 min and then centrifuged 2x30 sec at 5.000 rpm. Supplemental 300 µl of D_2O were added and the mixture was homogenized with a vortex 1 min and then left at room temperature for 15 min. A final centrifugation step during 15 min at 13.000 rpm was performed and the supernatants (650 µl) were supplemented with 100 µl of DPB, 100 µl of a 5 mM solution of maleic acid and 5 µl of a 10 mg/ml trimethylsilyl-3-propionic-d4 (TMSP) solution and transferred to a 5-mm NMR tube for the analysis by NMR. Maleic acid was used as internal standard for quantification and TMSP for the zero calibration.

2.7. NMR measurements

All samples were recorded at 298 Kelvin on a Bruker Advance spectrometer operating at 500 MHz for the proton signal acquisition. The instrument was equipped with a 5 mm TCI cryoprobe with a Z-gradient. ^{1}H-NMR spectra of the samples were acquired using a CPMG relaxation-editing sequence with presaturation. The CPMG experiment used a RD-90-(t-180-t)-n-sequence with a relaxation delay (RD) of 2 s, a spin echo delay (t) of 400 ms and the number of loops (n) equal to 80. The water suppression pulse was placed during the relaxation delay (RD). The number of transients was typically 32 and a number of 4 dummy scans was chosen. The data were processed with the Bruker Topspin 3.2 software with a standard parameter set. Phase and baseline corrections were performed manually over the entire range of the spectra and the δ scale was calibrated to 0 ppm using the internal standard TMSP. In order to prevent any bias related to the day of acquisition on the equipment, meat extracts were randomly analyzed in single blind: at the time of sample preparation and NMR acquisition, the analyst did not know which number matched a particular meat sample.

2.8. Multivariate analysis for NMR spectra

For statistical analysis, optimized ^{1}H-NMR spectra were automatically baseline-corrected and reduced to ASCII files using AMIX software (version 3.9.14; Bruker). The spectral intensities were normalized to total intensities and reduced to integrated regions of equal width (0.04 ppm) corresponding to the 0.5–10.00 ppm region. Because of the residual signals of water and maleic acid, regions between 4.7 and 5 ppm (water signal) and 5.6–6.2 ppm (maleic acid signal) were removed before analysis. The reduced and normalized NMR spectral data were imported into SIMCA (version

13.0.3, Umetrics AB, Umea Sweden). Pareto scaling was applied to bucket tables and discriminant analysis (DA) such as PCA (Principal Component Analysis), PLS-DA (Partial Least Squares Discriminant Analysis), OPLS-DA (orthogonal partial least squares discriminant analysis) and PLS (Partial Least Square) regression were performed. SIMCA was used to generate all PCA, PLS, PLS-DA, and OPLS-DA models and plots. PCA was only used to detect possible outliers and determine intrinsic clusters within the dataset, while PLS-DA maximized the separation and OPLS-DA facilitated the graphic visualization of differences and similarities between groups. The quality of OPLS-DA models was determined by the goodness of fit (R^2) and the predictability was calculated on the basis of the fraction correctly predicted in one-seventh cross-validation (Q^2).

2.9. Metabolite identification

Spectra for each minced meat sample was acquired by ^{1}H-NMR. Based on 2D experiments, previous research and websites, such as FooDB version 1.0 (http://foodb.ca), water-soluble metabolites were detected and annotated. From PLS-DA loading plots, metabolites with higher loadings were identified. Signals with values of Variable Importance in Projection (VIP) higher than 1 were considered as significant, and further validated using t-test with Metaboanalyst (http://www.metaboanalyst.ca). Metabolite identification was then performed using the open-access database NMR suite 8.1 (Chenomx inc., Edmonton, Canada), the free web-based tool HMDB (http://www.hmdb.ca) and tables. Each metabolite identified was finally confirmed by performing peak correlation plots from 2D-NMR spectra (COSY and HSQC).

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Results

3.1. Microbial counts

As expected, the storage temperature and the packaging conditions have a strong impact on the bacterial dynamics (**Table 53**). During storage, a more rapidly reached stationary phase is correlated to the FW packaging and the highest temperature.

At the end of the shelf life, the natural logarithm of bacterial counts was over the spoilage threshold of 7.00 log CFU/g, except for some samples stored in MAP at low temperature.

No bacterial growth was observed on PCA for the control samples (limit detection < 3.00 log CFU/g) (data not shown).

3.2. pH and gas measurements

A significant increase of pH was observed for MP samples inoculated by *Pseudomonas* spp. (7.54 ± 0.76, $n = 5$, p-value = 0.01) compared to non-inoculated samples (5.79 ± 0.05, $n = 10$). For the two others inoculated bacteria, no statistical differences was observed when comparing to control samples.

Moreover, a relatively stable composition of carbon dioxide in MAP was observed at the end of the shelf life for all inoculated samples [30.0 – 38.5 %], except for MP samples inoculated with *Pseudomonas* spp. that reached a higher amount at 12°C (100.0 ± 0.1 %).

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Table 53. Microbiological counts (log CFU/g) for mono-culture expriments in minced pork samples stored during 13-days shelf life, at constant temperature, in FW and MAP (CO_2 30% / O_2 70% ± 0.1%). See Table 52 for list of the codes used. Mean values with standard deviations of the three repliacates; -[a], no analysis performed for the day.

Codes	Days													
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A	3.84±0.03	3.08±0.10	3.76±0.07	4.54±0.12	- [a]	- [a]	7.24±0.11	7.74±0.17	7.63±0.10	8.17±0.33	7.68±0.15	- [a]	- [a]	7.90±0.15
B	3.84±0.03	6.76±0.04	7.49±0.11	8.25±0.07	8.51±0.10	8.58±0.06	8.85±0.02	8.77±0.15	9.05±0.03	8.79±0.21	- [a]	- [a]	- [a]	9.00±0.01
C	3.84±0.03	7.68±0.08	8.29±0.13	8.66±0.04	8.99±0.09	9.01±0.23	9.11±0.10	8.81±0.28	9.03±0.03	8.91±0.16	- [a]	- [a]	- [a]	9.27±0.08
D	3.84±0.03	- [a]	- [a]	2.17±0.30	- [a]	- [a]	4.11±0.01	4.01±0.14	4.35±0.03	5.24±0.05	4.99±0.12	- [a]	- [a]	5.43±0.06
E	3.84±0.03	- [a]	5.88±0.10	6.11±0.11	7.11±0.02	7.86±0.10	8.21±0.04	8.43±0.11	8.43±0.16	8.41±0.10	8.38±0.16	- [a]	7.86±0.07	8.76±0.03
F	3.84±0.03	- [a]	7.10±0.04	7.76±0.23	8.35±0.04	8.58±0.06	8.40±0.12	8.44±0.07	8.32±0.03	9.16±0.08	8.67±0.40	- [a]	8.83±0.02	8.71±0.06
G	3.15±0.59	3.43±0.11	4.52±0.23	5.64±0.19	- [a]	- [a]	- [a]	9.45±0.13	9.51±0.07	- [a]	9.90±0.29	- [a]	- [a]	10.21±0.03
H	3.15±0.59	3.86±0.17	5.36±0.03	7.69±0.17	9.04±0.05	9.67±0.03	- [a]	9.62±0.15	10.34±0.24	10.39±0.40	10.11±0.28	- [a]	- [a]	10.15±0.17
I	3.15±0.59	4.93±0.15	- [a]	9.81±0.04	9.85±0.29	9.95±0.34	10.15±0.82	10.26±0.08	10.14±0.10	- [a]	9.87±0.19	- [a]	- [a]	9.80±0.42
J	3.15±0.59	- [a]	3.48±0.06	- [a]	- [a]	3.90±0.11	4.87±0.34	4.55±0.12	- [a]	- [a]	- [a]	- [a]	4.73±0.01	4.90±0.01
K	3.15±0.59	3.52±0.01	4.16±0.05	- [a]	- [a]	5.41±0.08	6.33±0.07	6.52±0.14	- [a]	6.59±0.17	- [a]	- [a]	7.83±0.13	8.37±0.08
L	3.15±0.59	4.47±0.07	6.08±0.03	- [a]	- [a]	- [a]	9.42±0.28	9.58±0.23	- [a]	9.80±0.41	- [a]	- [a]	9.87±0.06	9.85±0.14
M	4.00±0.02	4.07±0.01	4.38±0.01	4.61±0.12	- [a]	- [a]	6.17±0.05	- [a]	- [a]	- [a]	8.62±0.09	- [a]	- [a]	8.42±0.06
N	4.00±0.02	4.58±0.08	5.84±0.02	- [a]	7.57±0.10	- [a]	8.61±0.13	- [a]	8.73±0.07	- [a]	8.84±0.09	- [a]	- [a]	8.77±0.30
O	4.00±0.02	5.38±0.01	6.84±0.13	8.35±0.09	7.56±0.01	- [a]	8.64±0.13	- [a]	- [a]	- [a]	8.82±0.23	- [a]	- [a]	8.62±0.18
P	4.00±0.02	4.18±0.09	- [a]	- [a]	6.31±0.17	- [a]	6.84±0.06	7.85±0.01	- [a]	7.78±0.21	- [a]	- [a]	8.00±0.10	8.39±0.12
Q	4.00±0.02	4.75±0.03	- [a]	- [a]	8.06±0.01	- [a]	8.38±0.05	8.49±0.16	- [a]	8.85±0.01	- [a]	- [a]	- [a]	8.75±0.19
R	4.00±0.02	8.32±0.15	7.28±0.01	- [a]	8.35±0.06	- [a]	8.36±0.09	8.64±0.10	- [a]	8.89±0.07	- [a]	- [a]	- [a]	8.87±0.11

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1.3. Microbial growth parameters

Results of the primary model fittings are shown in **Table 54**. Good fit indexes were obtained in all cases.

Bacterial growth parameters showed different dynamic changes depending on storage temperature and packaging condition. Indeed, the storage temperature had a strong impact on the maximal growth rate and lag-time: a high storage temperature is correlated to a high growth rate during exponential phase and a lower lag-time duration. The maximal bacterial growth is also higher in the FW packaging.

Table 54. Observed kinetic parameters calculated by Baranyi equation. See Table 52 for list of the codes used. Mean values with standard deviation (SD represent three samples per experiment) or with the 95% confidence intervals (lower limit and upper limit); μ_{max} , maximal specific growth rate (1/h); *LPD,* lag phase duration (h); N_0, initial bacterial concentration (log CFU/g); N_{max}, maximum bacterial concentration (log CFU/g); *RSS,* Residual Sum of Square of the model.

	μ_{max}	*LPD*	N_0	N_{max}	*RSS*
A	0.09 [0.09-0.08]	51 [53-51]	3.84±0.03	7.90±0.15	0.000442
B	0.21 [0.21-0.19]	0 [0-0]	3.84±0.03	8.79±0.21	0.000255
C	0.39 [0.39-0.35]	0 [0-0]	3.84±0.03	9.11±0.10	0.000558
D	0.03 [0.03-0.03]	20 [20-17]	3.84±0.03	4.99±0.12	0.005700
E	0.07 [0.07-0.07]	0 [0-0]	3.84±0.03	8.43±0.16	0.005700
F	0.13 [0.13-0.12]	0 [0-0]	3.84±0.03	8.83±0.16	0.005260
G	0.06 [0.06-0.06]	24 [24-24]	3.15±0.59	9.90±0.29	0.010900
H	0.13 [0.13-0.13]	10 [10-10]	3.15±0.59	10.15±0.17	0.010900
I	0.23 [0.23-0.23]	0 [0-0]	3.15±0.59	9.95±0.34	0.010900
J	0.04 [0.04-0.04]	48 [48-48]	3.15±0.59	4.90±0.01	0.001210
K	0.08 [0.08-0.08]	27 [27-27]	3.15±0.59	8.37±0.08	0.001210
L	0.13 [0.13-0.13]	0 [0-0]	3.15±0.59	9.87±0.06	0.001210
M	0.01 [0.01-0.01]	48 [48-48]	4.00±0.02	8.42±0.06	0.017900
N	0.07 [0.08-0.07]	10 [12-10]	4.00±0.02	8.77±0.30	0.023000
O	0.18 [0.19-0.18]	0 [0-0]	4.00±0.02	8.64±0.13	0.017900
P	0.02 [0.02-0.02]	17 [19-15]	4.00±0.02	8.00±0.10	0.025600
Q	0.13 [0.13-0.13]	0 [0-0]	4.00±0.02	8.75±0.19	0.023700
R	0.32 [0.33-0.32]	0 [0-0]	4.00±0.02	8.87±0.11	0.025600

3.4. NMR metabolomic profiling of the meat ageing-process

In order to assess the dynamics of the metabolomic profile of minced meat samples during the storage period, OPLS-DA was performed on the NMR spectra between non-inoculated MP samples at day 0 and 13 (**Figure 48**). Score-plot analysis reveals an excellent separation between samples (model values of R^2=0.925, Q2=0.903, 4 principal components). VIP analysis revealed 32 features with

values ≥1 potentially significant. Among those features, 12 metabolites were identified with accuracy. Threonine, lactate, creatine and formic acid decreased during the storage; while capric acid, isoleucine, leucine, valine, alanine, acetate, adipate and inosine increased.

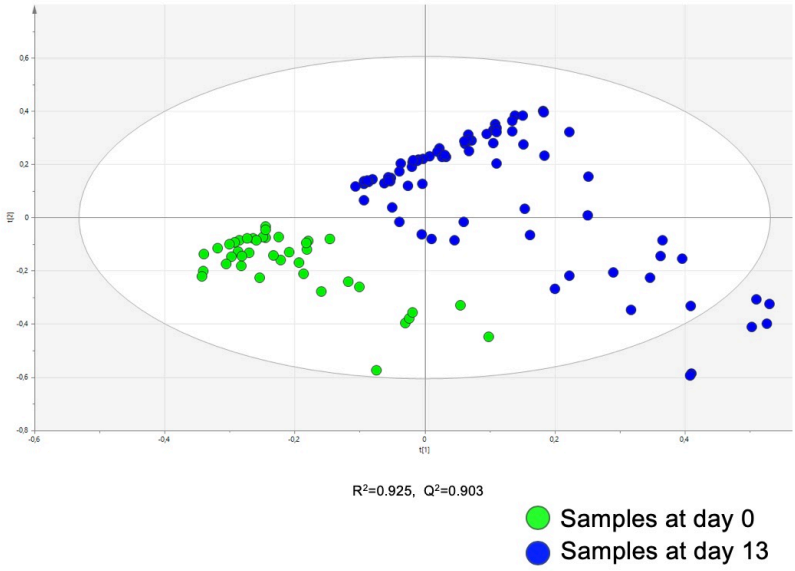

Figure 48. OPLS-DA based on NMR spectra between non-inoculated samples analyzed at day 0 and 13.

3.5. Comparison of inoculated and non-inoculated samples on the metabolic profiles

OPLS-DA was then carried out on all spectra between inoculated (all inoculated conditions combined) and non-inoculated MP samples at day 13 (**Figure 49**). Score-plot analysis also reveals excellent separation between samples (R2=0.858, Q2=0.838).

Non-inoculated group appeared homogeneous, indicating that packaging and storage temperature have a limited impact on their metabolomic profile. However, an important intra-variability within inoculated samples was observed, indicating a likely effect of the storage parameters. Indeed, inoculated samples seemed to be separated into three distinct clusters. The first sub-group is located farthest from the non-inoculated samples on the score-plot and is composed of MP samples inoculated with *Pseudomonas* spp. (stored in FW at 4, 8, and 12°C and in MAP at 12°C). The second intermediate subgroup contains MP samples inoculated with *B. thermosphacta* (stored in FW at 4, 8, and 12°C and in MAP at 12°C). The last subgroup, closest to the non-inoculated group, is composed of samples inoculated with *Ln. gelidum* (all packaging and temperatures), and those with *Pseudomonas* spp. and *B. thermosphacta* kept in MAP at 4 and 8°C. Thus, *Pseudomonas* spp. seem to have a more important effect on the meat pork minced meat metabolic profiles, followed by *B. thermosphacta*, while *Ln. gelidum* seems to exert little modification on meat samples.

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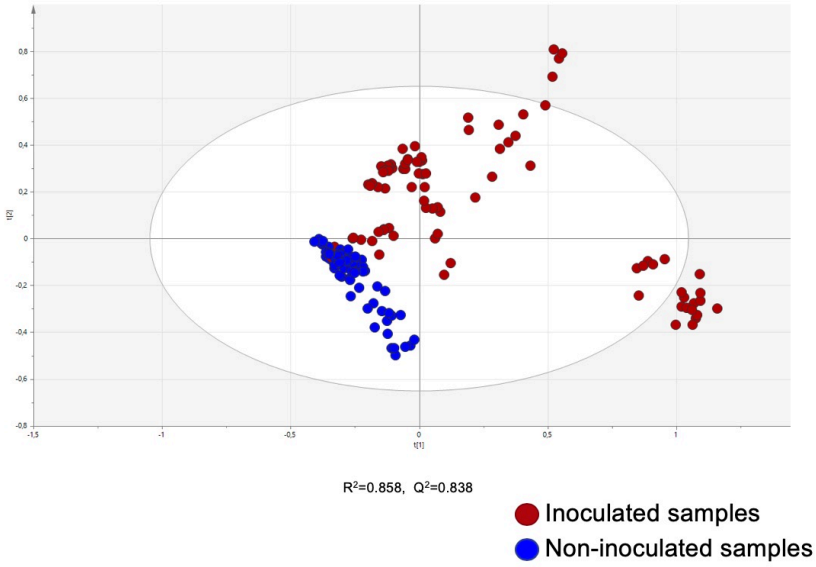

Figure 49. OPLS-DA between all non-inoculated and inoculated samples at day 13.

3.6. Influencing factors on the metabolomic profiles of inoculated minced pork samples

Influencing factors are likely to impact the metabolomic profiles of inoculated MP samples: the inoculated bacterial species, the food packaging and the temperature of storage (**Figure 50**). These results are still ongoing, as not all scare plots and metabolites have yet been reported by CIRM-CHU.

Score plots highlighted a significant discrimination between inoculation experiments with specific bacterial specie (*B. thermosphacta*, *Pseudomonas* spp. and *Ln. gelidum*) at day 13 (Figure 50A). These results also highlighted a significant effect of food packaging, revealing a clear discrimination between samples stored in FW and in MAP (Figure 50B), while the temperature of storage seems to have a little effect on metabolome profile (Figure 50C).

Significant increase or decrease of metabolites responsible for the separation between non-inoculated samples and each inoculated bacteria are described in **Table 55** (quantity not yet available). At day 13, some metabolites increased in each inoculated samples. It mainly concerns acetate, cadaverine, glutamate and succinate for *Pseudomonas* spp., and acetate, acetoin and isobutyrate for *B. thermosphacta*. The results for *Ln. gelidum* have not yet been reported.

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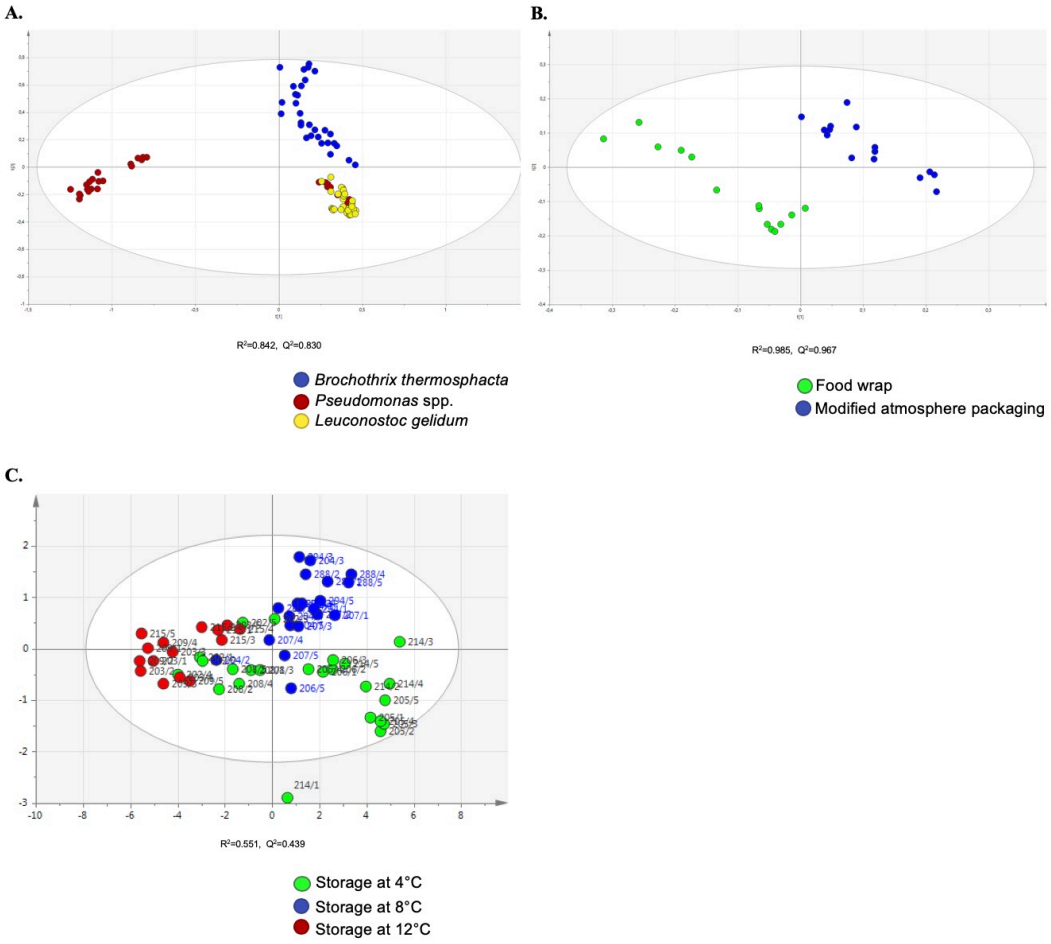

Figure 50. Metabolomic patterns between inoculated samples at day 13 and according to the inoculated bacteria (**A**), the packaging condition (**B**) and the temperature of storage (**C**).

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Table 55. Metabolites profiles at day 13 between inoculated and non-inoculated samples stored at 4, 8 and 12°C, in both FW and MAP. Metabolites increased (↑), decreased (↓) or not modified (-).

Identified metabolite	Inoculated bacteria	Inoculated *vs* non-inoculated samples stored at 4°C	Inoculated *vs* non-inoculated samples stored at 8°C	Inoculated *vs* non-inoculated samples stored at 12°C
Acetate	*B. thermosphacta*	↑	↑	↑
	Pseudomonas spp.	↑	↑	↑
Acetoin	*B. thermosphacta*	↑	↑	↑
	Pseudomonas spp.	-	-	-
Alanine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↓	↓	↓
Aminopentanoate	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	-	-	↑
Anserine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↓	↓	↓
Cadaverine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↑	↑	↑
Creatine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↓	↓	↓
Glucose	*B. thermosphacta*	↓	↓	↓
	Pseudomonas spp.	↓	↓	↓
Glutamate	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↑	↑	↑
Glutamine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↓	↓	↓
Leucine/ isoleucine	*B. thermosphacta*	↓	↓	-
	Pseudomonas spp.	-	↑	↑
Isobutyrate	*B. thermosphacta*	↑	↑	↑
	Pseudomonas spp.	-	-	-
Lactic acid	*B. thermosphacta*	-	↓	↓
	Pseudomonas spp.	↓	↓	↓
Succinate	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	↑	↑	↑
Tyrosine	*B. thermosphacta*	-	-	-
	Pseudomonas spp.	-	-	↑

A preliminary analysis of NMR pattern for comparing mono- and co-culture experiments is available but full results are not yet available (**Figure 51**). It appears that a clear distinction can be made between each mono-culture experiments, as described above. And that co-culture experiments seems to correspond to mono-cultures experiments for MP samples inoculated by *Pseudomonas* spp. and *Ln. gelidum*. These results are in accordance with those obtained in study 4, where *Pseudomonas* spp. become the dominant specie in FW, and *Ln. gelidum* in MAP for co-culture experiments. A detailed comparison between each inoculation experiments shows clearly these results (**Figure 52**). But more precise results concerning the comparison of mono- and co-culture experiments are still being studied.

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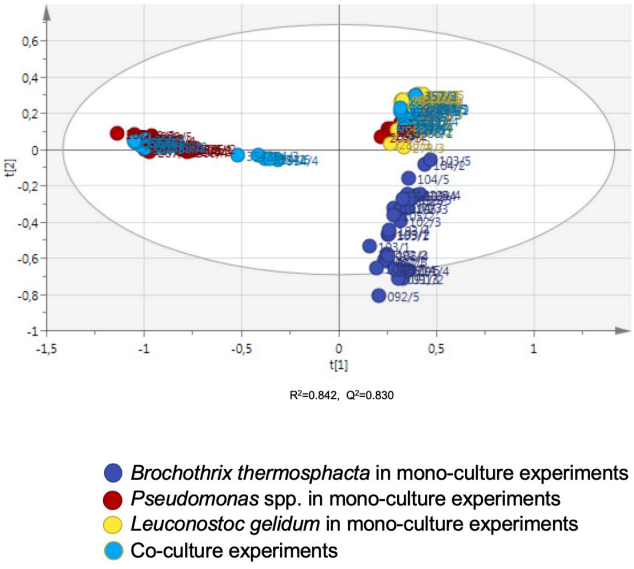

Figure 51. Comparison of NMR spectra between all samples in mono-culture and co-culture experiments.

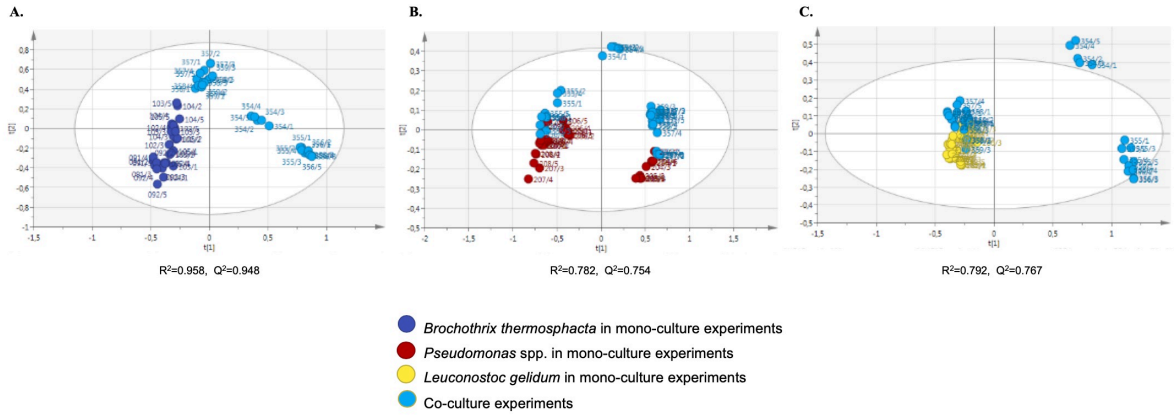

Figure 52. Comparison of each NMR spectrum between co-culture and mono-culture experiments, for *B. thermosphacta* (**A**), *Pseudomonas* spp. (**B**) and *Ln. gelidum* (**C**).

Study 5. A NMR-based metabolomics study of minced pork samples inoculated with *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum.*

Discussion

Thanks to NMR-based metabolomics, the exploration of the correlations of metabolites with other parameters, such as microbiological counts, pH changes and gas composition, is feasible in order to provide information on minced pork spoilage. Spoilage occurs when the formation of off-flavors, off-odors, discoloration, slime, or any other changes in physical appearance or chemical characteristics make the food unacceptable for human consumption (Ercolini *et al.*, 2011). Indeed, the qualitative and quantitative analyses of metabolic compounds present, as a consequence of microbial activity, in a more integrated holistic approach, regardless of storage conditions (e.g. temperature, type of packaging) (Argyri *et al.*, 2015). In MAP, all bacteria reached stationary phase. Moreover, a high growth rate and a more rapidly reached stationary phase were correlated with the highest storage temperatures. In FW, *Pseudomonas* spp. reached the highest microbiological counts at the end of storage. These results are of interest because this bacterium is considered as specific spoilage organism in meat products, responsible for unpleasant odors and flavors (Pothakos *et al.*, 2015; Saraoui *et al.*, 2017).

Although most spoilage bacteria are proteolytic, they initially grow by utilizing the most readily available carbohydrates and nonprotein nitrogen. Glucose, lactic acid, and certain amino acids, followed by water-soluble proteins, are the precursors of metabolites that are responsible for meat spoilage. Moreover, concentrations of the precursors can influence the rate and extent of spoilage. It is the accumulation of microbial metabolites, such as aldehydes, ketones, esters, alcohols, organic acids, amines, and sulphur compounds, that triggers the meat spoilage (Ercolini *et al.*, 2011). The determination of metabolites reveals interesting relationships between nutrient consumption and possible variations in metabolic pathways (Castro-Puyana and Herrero, 2013). Analysis of NMR patterns reveals a clear discrimination between all tested conditions in this study: (i) the non-inoculated products analyzed at day 0 and 13, showing the natural dynamics of irradiated meat according to the storage conditions; (ii) the inoculated and non-inoculated samples at day 13; (iii) the inoculated bacterial species on the samples; and finally (iv) the packaging conditions, between FW and MAP. The storage temperature seems also to have a significant effect but with a lesser impact than the other parameters.

For non-inoculated samples, a decrease of threonine, lactate, creatine and formic acid amounts, and an increase of capric acid, isoleucine, leucine, valine, alanine, acetate, adipate and inosine amounts was observed during storage. These results are partially in accordance with Ercolini *et al.* (2011), who showed a decrease of lactate and creatine in beef chops stored at 4°C under bacteriocin-activated antimicrobial packaging.

Study 5. A NMR-based metabolomics study of minced pork samples inoculated with *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Leuconostoc gelidum.*

Samples inoculated with *Pseudomonas* spp. exhibit the most important differences from the non-inoculated samples, while those inoculated with *Ln. gelidum* is the worst model. Samples spiked with *Pseudomonas* spp. also showed the highest pH and carbon dioxide in MAP compared to non-inoculated samples. The change in pH of food products is usually a good index for quality assessment, commonly related to the accumulation of lactic acid produced in anoxic condition (Aru *et al.*, 2016). We also observed some metabolites with amount increased in all inoculated samples. Those are acetate, cadaverine, glutamate and succinate in samples with *Pseudomonas* spp., and acetate, acetoin and isobutyrate in the presence of *B. thermosphacta*. The results for *Ln. gelidum* have not yet been fully analyzed. All metabolites were assigned into Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/kegg/). Mapping results of KEGG pathway help to explain the different increases and decreases of metabolites for *B. thermosphacta* and *Pseudomonas* spp. (*Ps. fragi* and *Ps. fluorescens*). However, a further study on this subject must be carried out once the complete results have been obtained. Moreover, these results are in accordance with scientific studies, especially those of Zareian *et al*. (2018) who evaluated VOCs in raw pork samples stored at 4°C under MAP, which is the same as the one used in experiments in MP samples (70% O_2 – 30% CO_2). These results, along with those of Zareian *et al*. (2018), could offer important insights for potential future implementation of specific sensors for shelf life of MP. According to others studies, acetate seems to be mainly produced by *Pseudomonas* spp. (*Ps. fragi* and *Ps. taetrolens*) in meat and meat products and be associated with off-odors (Stanborough *et al*., 2018b). Based on this, Mansur *et al.*, 2019 have proposed to use acetate, as well as acetoin, as spoilage indicators for chilled-beef stored under air and VP. Argyri *et al*. (2015) had already proposed to use acetoin as a spoilage indicator for ground beef. Acetoin is mainly produced under aerobic conditions by LAB and *B. thermosphacta*, and is associated with a sour-sweet odor (Ercolini *et al*., 2011; Panseri *et al*., 2018; Raimondi *et al*., 2019; Russo *et al*., 2006). Illikoud *et al*. (2019a) showed that strains of *B. campestris* and *B. thermosphacta* produced acetoin and diacetyl in beef minced meat and peeled shrimp juices. This was also observed by Stanborough *et al*. (2017) who analyzed the genome of *B. thermosphacta*, and by Casaburi *et al*. (2014) who monitored activities of strains of *B. thermosphacta in vitro* and in beef meat. However, acetoin production seems to be highly variable between strains (Illikoud *et al*., 2019b). Moreover, cadaverine, with putrescine and phenylethylamine, are the predominant BAs found in pork meat samples (Bartkiene *et al*., 2019). Many bacteria, belonging to the *Enterobacteriaceae* family, are able to produce cadaverine because they can produce ornithine and lysine decarboxylase enzymes (Chaidoutis *et al*., 2019). *B. thermosphacta* does not appear to be a cadaverine-producing bacterium but, instead, can produce histamine (Casaburi *et al*., 2014).

According to this, metabolites and their pathways could give important information about the spoilage capacity of inoculated bacteria, and particularly for *Pseudomonas* spp. which seems to be of

interest in our product. More complete genomic analysis, coupled with transcriptome and volatilome analyses, are necessary to better understand spoilage mechanisms. Moreover, preliminary analysis of NMR pattern for comparing mono- and co-culture experiments show interesting results, in accordance with those obtained in experimental study 4. Further analyses are in progress and migh enable to understand the underlying mechanisms of growth and interaction. These mechanisms are sometimes very complex and still little studied, especially for spoilage bacteria. Cross-feeding (also called "public goods") is a good example of complex interactions that are not yet fully understood (D'Souza *et al*., 2018; Smith *et al*., 2019). This phenomenon has already been described for LAB (Seth and Taga, 2014), pathogenic bacteria (Ziesack *et al*., 2019), especially *E. coli* (Dal Co *et al*., 2019; Pande *et al*., 2014; Pande *et al*., 2015), and in the gut microbiota (Henriques *et al*., 2019; Turroni *et al*., 2018), although none related to food spoilage bacteria. It could therefore be interesting to study these phenomena in future research, as using them in modeling to better understand and predict food spoilage. NMR analysis on naturally contaminated MP samples, in order to compare the metabolomic profiles with the results of inoculation experiments, are also important for validation purpose. This was not performed in this study and should therefore be studied in further studies.

In conclusion, these results support the use of NMR-based metabolomics as an easy valuable tool to provide information on minced pork spoilage and to follow intrinsically the dynamics of the metabolomic pattern linked to a specific bacterium in complex bacterial ecosystems. These data also suggest that NMR-based metabolomics is an efficient method to distinguish fingerprinting differences between samples, and to distinguish metabolites as putative biomarkers of spoilage products.

Highlights

Highlights.

Highlights.

Some highlights of the six experimental studies described previsouly are presented in this subsection.

- Investigation of the microbial spoilage communities and their dynamics using both 16S rRNA gene sequencing and classical microbiology offers a more detailed characterization of bacterial microbiota in samples. The approach of combining these data in predictive models seems also promising.
- Without extensive studies involving a large number of samples under different food companies, batches production and storage conditions, it will not be possible to determine exactly the variability of bacterial ecosystems, and thus the role of individual spoilage species.
- Food spoilage needs to be assessed to species levels, because potentially protective bacteria, such as some lactic acid bacteria, can also occur in food products.
- Sensory analyses would be interesting to assess food spoilage. The sum of biogenic amines could also be used as quality indicator of meat freshness during storage.
- Low storage temperatures and adequate packaging are considered as the most important factors for improving meat shelf life by delaying meat spoilage.
- Predictive models based on one bacterial species show relatively good adjustments although not always, in particular for complex prediction with three species. More datasets are probably needed to obtain reliable factors that could be used in predictive models.
- It is also important to consider all intrinsic and extrinsic factors, and microbial interactions between different types of microorganisms and within the food matrix, in order to obtain more accurate models.
- Investigation of metabolite production by ^{1}H-NMR-based metabolomics is an interesting tool, providing usefull information for monitoring the shelf life of perishable products.
- Metabolomic patterns and interaction of each bacteria (e.g. cross-feeding) could also be used as input in modeling to better understand and predict food spoilage.
- Validation is an essential step: more experimental data, derived from multiple repetitions, are needed to validate the developed models.

Chapter 4
General discussion and perspectives

Chapter 4 involves a general discussion and perspectives of this research.

Three sections are presented and are dedicated to the objectives of this thesis:

(1) Investigation of the natural spoilage microbiota, and characterization of the dynamics of specific related bacteria, in minced pork and white pudding samples (**Objectives 1 and 2**).

(2) Development of complex species-growth models in minced pork and white pudding samples (**Objective 3**).

(3) Study of the metabolome in minced pork samples (**Objective 4**).

4.1. Investigation of microbial spoilage communities

The aim of this topic was to describe the natural spoilage microbiota of samples and their dynamics depending on food packaging and storage temperature. Characterization of specific related spoilage bacteria in these selected food matrices was also performed, and these data were used as inputs for predictive models in order to obtain growth parameters.

4.1.1. Describing the natural spoilage microbiota of minced pork and white pudding samples

Experimental studies showed that the combination of plate counts and 16S rRNA gene sequencing enables monitoring of the dynamics of microbial food spoilage communities. The advantages of these techniques have already been demonstrated in several studies, but none of them had been interested in their association or in the use of the data obtained as inputs for predictive models.

Although we acknowledge that the plate count method is not able to completely assess all the microbial populations present, the combination of these two methods was validated by a quantitative PCR (qPCR) approach in study 2. Fougy *et al*. (2016) showed that this conversion can be used to obtain an extrapolated estimation of the bacterial concentration. Association of relative abundance results with cell counts is also described for microbiome analysis (Amend *et al*., 2010; Guo *et al*., 2019). And Vandeputte *et al*. (2017) associated flow cytometric enumeration of microbial cells with sequencing data in order to obtain quantitative microbiome profiles. However, the inability of differentiation between viable and non-viable cells by culture-independent DNA-based methods in experimental studies remains an important drawback, which could result in significant overestimation of viable species (Scariot *et al*., 2018; Tantikachornkiat *et al*., 2016). Systematic biases of processing and analysis steps in culture-dependent and -independent methods must also be considered in further studies (Louca *et al*., 2018; Pollock *et al*., 2018; Salter *et al*., 2014). Despite this, models based on these results in experimental studies show relatively good fitting indices. In this context, further studies and repetitions are needed to demonstrate the interest of combining culture-dependent and -independent methods to explore bacterial variability between food samples, depending on their origin, production environment and storage conditions, and then to use these data as inputs for predictive models.

Moreover, it could be interesting to compare other associated methods, such as flow cytometric enumerations, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Rahi and Vaishampayan, 2020; Seuylemezian *et al*., 2018) or *gyrB* sequencing with the results obtained. Peruzy *et al*. (2019b) have shown that 16S rRNA sequencing does not always detect some bacteria, demonstrating the necessity to combine new analysis techniques such as MALDI-TOF MS. A comparison between amplicon sequencing based on 16S rDNA (V3-V4) and on *gyrB*, an alternative genetic marker, has also been carried out for different types of meat and seafood products by Poirier *et al*. (2018b). This study showed better accuracy for bacterial richness by using *gyrB* sequencing. It could also be interesting to compare intra-species diversity results obtained from *gyrB* and 16S rDNA (V1-V3) amplicon sequencing for the studied samples.

Experimental studies also showed that *Pseudomonas* and *Brochothrix* dominate the community of MP samples at the end of the shelf life in FW and MAP, respectively, together with *Photobacterium*, whereas the major genus taxa are represented by *Brochothrix*, *Pseudomonas* and *Psychrobacter* in WP. These results can be explained by the fact that multiple sources of contamination can contribute to the initial composition of the meat microbiota. Further researches on the main contamination routes during the slaughtering, cutting and processing processes, such as the processing environment, procedures and storage conditions, are thus desirable to provide a complete assessment of the microbiome of minced meat and white pudding, and so to limit incidents of unexpected spoilage.

4.1.2. Characterization of specific related spoilage microorganisms in studied samples

Specific spoilage bacteria were selected for the two studied matrices.

- For white pudding samples:
  - *B. thermosphacta*, C. *maltaromaticum*, *Lb. fuchuensis*, *Lb. graminis*, *Ln. mesenteroides*, *Lc. lactis*, *Pseudomonas* sp., *Psychrobacter* spp. (*Psy. okhotskensis* and *Psy. urativorans*) and *Serratia* sp..
- For minced pork samples:
  - *B. thermosphacta*, *Pseudomonas* spp. (*Ps. fragi* and *Ps. fluorescens*) and *Ln. gelidum*.

The growth parameters of these selected bacteria (maximal bacterial growth, lag phase duration, maximal and minimal bacterial populations, minimal temperature for growth, time to reach the stationary phase and time to reach the spoilage value level) were obtained by fitting primary and secondary models for naturally contaminated or inoculated samples.

These results are very important for creating a comprehensive database of spoilage microorganisms, but accurate cardinal values are needed to improve the robustness of growth prediction.

Currently, these parameters are mainly studied for pathogenic bacteria (Martinez-Rios et al., 2019) and for spoilage bacteria in culture medium. An approach in food would therefore be very interesting and could be complementary to the other databases available, such as ComBase and Sym'Previus.

The general perspective of this thesis is therefore to create a comprehensive database for the growth of spoilage microorganisms in food, in connection with existing data or databases (Microbial Responses Viewer, ComBase, Sym'Previus), and thus to develop a web interface useful to researchers or agro-industries.

4.2. Development of complex predictive models

The aim of this topic was to develop complex models for spoilage species in MP and WP samples, and to validate and compare these approaches.

4.2.1. Development of spoilage species growth models

Predictive models without interactions were based on naturally contaminated WP samples. Good adjustments were obtained for the growth simulations in WP, even if overestimations were often observed. By comparing these simulations with those available from other software, an overestimation was mainly observed with Sym'Previus, while an underestimation was more frequently observed during the lag-phase period with ComBase.

For minced pork, the data obtained obtained enabled the development of a three species interaction model based on the Lotka-Volterra (prey-predator model) and the modified Jameson models. The best adjusments were obtained with the modified Jameson model, although the prey-predator approach seems to be an interesting interaction model for complex microbiota.

As we know, there is a lack of information and of predictive models for spoilage bacteria (Alfaro *et al*., 2013). Further studies and repetitions are thus needed to develop more realistic interacting predictive models, especially in approaches considering the interaction of two or more spoilage species interaction approach, and to develop new food preservation processes.

The Jameson model seems to be more appropriate for describing the dynamics of spoilage organisms for the MP matrices studied. Indeed, this phenomenon was observed in MP samples where the minority bacteria decelerate when the majority one reaches the *MCP*. It was also the case in WP where bacterial strains were classified into three categories according to their behavior in the ecosystem: "dominant", "subdominant" or "inhibited".

On the other hand, the Lotka-Volterra model should be used in further studies with more accurate interaction factors, and also for obtaining more datasets such as for the growth rates and bacterial concentrations of each taxon (den Besten *et al*., 2018). Indeed, more datasets are probably

needed to obtain reliable factors. Thank to this, the Lotka-Volterra model could be modified to make a more realistic model by considering the effect of environmental conditions and interactions, such as bacteriocin production, QS, etc.

Knowledge of bacterial interactions is crucial for understanding the dynamics of bacterial communities and global metabolic activities, and also for developing a new modeling approach (Zhang *et al*., 2015). The interactions between microorganisms may be classified on the basis of their effects as being detrimental or beneficial : (i) antagonism, due to a change in environmental conditions; (ii) QS, by bacterial communication and/or (iii) metabiosis, by interdependent ways between microorganisms (Gram *et al*., 2002; Hibbing *et al*., 2010).

Antagonism is mainly caused by the competition for nutrients and for iron, by bacterial siderophore production, by bacteriocin production and/or by lowering of pH. LAB are particularly able to cause a decrease of pH and to produce bacteriocins. Some Gram-negative bacteria may also produce NH_3 and trimethyl-amine, which are toxic to other bacteria and sometimes to the producing organism itself. *Pseudomonas* spp., in particular the fluorescent group, can produce a wide range of antibacterial and antifungal compounds such as antibiotics and cyanide. These bacteria are also competitors for iron through the production of siderophores, biofilms and QS molecules. As discussed by Tsigarida *et al*. (2003), this bacterial group can also inhibit the growth of *S. putrefaciens* by producing siderophores.

Quorum sensing can also be related in mixed culture to synchronized bacterial behavior. The dominant bacteria inhibit other microorganisms by exchanging information (low molecular weight signaling molecules) (Diggle *et al*., 2007). Indeed, when a microorganism reaches its maximum population density, the concentration of the signaling molecules produced also reaches its maximum, indicating that the second species of the mixed culture should complete its growth because the maximum total population density of the culture (or carrying capacity of the system) has been reached. It is now in evidence that QS regulates food spoilage by cell-to-cell communication. Nychas *et al*. (2009) found that the growth rate of *Ps. fluorescens* and *S. marcescens* are increased by QS compounds extracted from meat. Russo *et al*. (2006) also found that the growth of *B. thermosphacta* decreased in the presence of LAB. Moreover, Gram-negative bacteria have the capability to coordinate the expression of certain phenotypic traits (e.g. hydrolytic enzymes) through bacterial communication via N-acyl homoserine lactones (AHLs) (Whitehead *et al*., 2001). *Serratia* spp. and *Hafnia alvei* have been identified as the dominating AHL producers in VP meats. AHLs are also produced by *Pseudomonas* spp. and some strains of *Photobacterium* spp. (Gram *et al*., 2002), even though Bruhn *et al*. (2004) mentioned that *Pseudomonas* isolates do not produce a detectable amount of AHLs. These signaling molecules become detectable when *Enterobacteriaceae* reached around 5.0 to 7.0 log CFU/g (Lopes Martins *et al*., 2018), and at around 8.0 to 9.0 log CFU/g for *Pseudomonadaceae* (Ammor *et al*., 2008). However, the role of AHLs in the spoilage of food remains unclear. Studying AHLs might offer further insights into spoilage metabolisms and related VOC production. Several proteolytic and lipolytic activities also appear to be

regulated by QS for a variety of Gram-positive and -negative bacteria (Ammor *et al*., 2008). It could be interesting to better understand these mechanisms, and also to design an approach for specific blocking of these communication systems, hence reducing or preventing spoilage reactions and extending shelf life (Nychas *et al*., 2009). As discussed by Tiwari *et al*. (2016), natural or synthetic QS inhibitors or QS quenching compounds can be used as novel biopreservatives against food spoilage bacteria, by disturbing microbial cell-to-cell communication. However, this approach is difficult due to the complex nature of food and multiple communications between bacteria.

On the other hand, metabiosis occurs due to a change in the spoilage profile of an organism by the supply of nutrients from another microorganism, creating a favorable environment. Several studies have shown that despite the inhibitory activity of *Pseudomonas* spp., their presence may enhance the growth of some microorganisms. Synergistic interactions were also observed by Lapointe *et al*. (2019) in tri-species biofilms (*Ps. fluorescens*, *Lb. plantarum* and *Ln. pseudomesenteroides*). In addition, cross-feeding interactions may contribute to the creation of an environment favorable for bacterial populations through complementary metabolic pathways, as a result of adaptation to an environmental change (Dal Co *et al*., 2019; Smith *et al*., 2019). Considered as cooperative social behavior, cross-feeding enables intra- and inter-species exchanges of several metabolites (D'Souza *et al*., 2018; Henriques *et al*., 2019; Turroni *et al*., 2018). These "public goods", which benefit not only the producer but also other cells in the neighboring group or population, are released into the extracellular environment by secretion, diffusion or connections between cells (e.g. bacterial nanotubes) (D'Souza *et al*., 2018; Pande *et al*., 2015; Ziesack *et al*., 2019). The meta-analysis of D'Souza *et al*. (2018) revealed that cross-feeding of metabolites is a common phenomenon between bacterial species, but also between bacteria of other kingdoms. The study of these behaviors could therefore be of interest, particularly concerning metabolic pathways potentially involved in spoilage.

In terms of modeling approaches, changes in model inputs are thus expected: building predictive models of interactions will increase our knowledge of spoilage bacteria in meat and meat products, and consequently will reduce uncertainty. This progress will benefit the whole community of safety assessors and research scientists from academia, regulatory agencies and industries (den Besten *et al*., 2018). Andreevskaya *et al*. (2018) have studied interactions between *Ln. gelidum*, *Lc. piscium* and *Lb. oligofermentans* in individual, pairwise and triple cultures. It was concluded that interactions between members of LAB communities are not well known. Moreover, all influencing factors need to be included in models, such as temperature, packaging, food composition and characteristics, as well as the main source of variability (Couvert *et al*., 2010). Currently, only some predictive models based on spoilage bacteria have included several factors (Kapetanakou *et al*., 2019).

A one-step model may also be an interesting approach, providing better fit to the data and minimizing errors. This approach involves directly forming a single global model by fitting the raw data to the primary and secondary models in a single step (Jewell, 2012). Akkermans *et al*. (2018), Huang

(2015), Jewell (2012) and Li *et al*. (2019b) have demonstrated that the one-step method gives a better fit to the data and yields more accurate estimations and precise calculations of the model, with less prediction uncertainty, than the two-step method. Moreover, the one-step method allows a more robust model on smaller data sets. Currently, this approach has not been well tested and has only been studied for pathogenic bacteria (Hwang and Huang, 2019).

Another future area of interest is to explore the use of antimicrobial agents in meat and meat products in order to preserve food quality and to extend shelf life (Singh, 2018). Numerous compounds (e.g. nisin, chitosan, lysozyme, essential oils, L-carnitine, conjugated linoleic acid, carboxylic acids and plant extracts) have been employed as potential preservatives to prolong the shelf life of meat products, but little is known about the changes that may occur in the fingerprint of VOCs and related potential characteristic biomarkers.

4.2.2. Validation step

The predictive simulations obtained were validated by monitoring the spoilage microbiota of naturally contaminated MP matrices. But differences with experiments were observed: *Photobacterium* was a predominant taxon in MP samples in MAP, while *Psychrobacter* spp. was predominant in WP samples. Even though preliminary studies of the most representative taxa were performed for these experiments, it could be interesting to take into account these bacteria for modelized interactions. These differences may thus explain the reliability of predictions, especially for the Lotka-Volterra model.

These proposals for models, with one or more spoilage bacteria, need to be validated by more experimental repetitions. Indeed, models based on background food microorganisms are less common and future predictive models are likely to include the behavior of the whole ecosystem (den Besten *et al*., 2018).

The general perspective of this thesis is therefore to develop new predictive models, by combining accurate data of bacterial kinetics, interactions and other growth mechanisms, in order to better predict spoilage bacteria dynamics and gene expression in meat and meat products. And thus offer more reliable and accurate modeling tools to scientists and agri-food stakeholders.

4.3. Metabolomic approach for bacterial food spoilage

The aim of this topic was to understand the influence of spoilage bacteria and storage conditions on the metabolome of MP samples.

4.3.1. Study of the spoilage metabolome of inoculated minced pork samples

Our results demonstrated that the use of ^{1}H-NMR-based metabolomics in study 6 is an interesting non-targeted approach, identifying several metabolites in inoculated and non-inoculated MP samples.

Other studies have shown that Raman microspectroscopy is also a fast, robust and non-destructive method for detection of spoilage bacteria. Klein *et al*. (2019) have shown the interest of this technique for the detection of seven spoilage microorganisms in agar medium, with an error rate of 3.5%. This technique seems to be promising and has the potential to be used for rapid differentiation of microorganisms and to determine microbial contamination in food safety issues. Jaafreh *et al*. (2018) used a portable fiber-optic Raman spectrometer, in conjunction with chemometric analysis, in order to rapidly detect and predict poultry spoilage. They concluded that this technique is a reliable and fast method for evaluation of poultry freshness during storage. But further investigations are required to use this method for determining the real shelf life of food products. It may therefore be interesting to study it, in association with ^{1}H-NMR, and to compare the two approaches.

In our study, the multivariate analysis (PLS-DA) showed a clear discrimination between samples: (i) the non-inoculated products analyzed at day 0 and 13, (ii) the inoculated and non-inoculated samples at day 13, (iii) the type of inoculated bacteria, and (iv) the packaging conditions. It could be observed that the inoculated bacteria have a more important impact on the metabolome than the packaging conditions. Some metabolites are also significantly increased: mainly acetate, cadaverine, glutamate and succinate for *Pseudomonas* spp., and acetate, acetoin and isobutyrate for *B. thermosphacta*. The results for *Ln. gelidum* are not yet available. Mapping results of KEGG pathway could help to explain the different increases and decreases of metabolites for *B. thermosphacta* and

Pseudomonas spp., as well as the available scientific studies on the metabolism of spoilage bacteria. Therefore, a more detailed discussion on this subject should be conducted once the full results have been obtained. Exploration of correlations of NMR-based metabolomic results with other microbial parameters also needs to be developed, as they may suggest their use as a possible tool to provide information on MP spoilage.

It is also important to increase our understanding of food spoilage mechanisms and metabolisms in order to better detect and ultimately prevent this phenomenon. In all studies, the bacterial count at the end of the shelf life was over 7.0 log CFU/g, indicating that MP and WP had probably begun to deteriorate and would not be suitable for human consumption. As reviewed by Wang *et al*. (2016b) other microbial VOCs could be studied as indicators of microbial spoilage in meat and meat products. Indeed, the sum of BAs could be used as spoilage indicators (Vieira *et al*., 2019), such as total volatile basic nitrogen (TVB-N) (e.g. ammonia, dimethylamine and trimethylamine). Moreover, sensory analyses would be interesting in this context, but were not performed in these studies. In association with predictive models, new detection systems, such as electronic noses, biosensors and conjugated polymer nanocomposite-based chemical sensors, are also promising candidates for the detection and monitoring of food spoilage (Ghasemi-Varnamkhasti *et al*., 2018; Pavase *et al*., 2018; Tamplin, 2018). Indeed, the VOC release profiles provided useful information that could be used for developing specific sensors to monitor shelf life (Zareian *et al*., 2018). At the moment, few sensors have the capabilities to detect food spoilage (Dudnyk *et al*., 2018) and they are mainly studied for pathogenic bacteria (Tait *et al*., 2014). Nevertheless, color indicators for monitoring the freshness of skinless chicken breast were proposed by Ruckson *et al*. (2014).

Study of the production of bacteriocins, bacteriophages, endolysins and BAs is also important to enhance our comprehension of food spoilage.

The use of bacteriocins is of great interest, as they may be added as biopreservatives to improve the microbial stability and safety of food products (Juturu and Wu, 2018). Several studies have been performed on bacteriocins to improve the safety of foods (Deegan *et al*., 2006), but little is known about how they could improve food quality. All genera of LAB are able to produce a broad spectrum of bacteriocins (Tumbarski *et al*., 2018) and by 2005, 185 bacteriocins produced by LAB had been identified (Woraprayote *et al*., 2016). Nisin, the best known LAB bacteriocin, is produced by *Lc. lactis* subsp. *lactis* and has a large antimicrobial spectrum, against Gram-positive and -negative bacteria (Ahmad *et al*., 2017). Although the effect of nisin on the total spoilage microbiota is well established, limited information is available about its effect on some species or strains of spoilage bacteria, and how bacteriocin can influence the spoilage dynamics during meat storage (Doulgeraki *et al*., 2012). Indeed, nisin reduces viable counts of *E. coli*, *S. aureus* and slime-producing bacteria in a meat model system (Garriga *et al*., 2002). Synergistic activity of nisin and lysozyme against Gram-

positive bacteria has also been observed, including activity against spoilage lactobacilli and *S. aureus* (Chung and Hancock, 2000; Nattress and Baker, 2003). *Carnobacterium* species, especially *C. divergens* and *C. maltaromaticum*, may also produce bacteriocins which are effective towards LAB, *Enterococcus* and *L. monocytogenes*. It has been demonstrated that strains of the same *Carnobacterium* species may produce different bacteriocins, and that the production rate for the same bacteriocin differs among strains. Thus, variations in the antibacterial spectra can be expected within the same species (Doulgeraki *et al*., 2012). *Ps. fluorescens* seems also to be able to produce bacteriocins but inhibition occurs less frequently between coexisting isolates (Bruce *et al*., 2017). Moreover, caution should be taken when using bacteriocins due to the resistance of pathogenic and spoilage bacteria to bacteriocins that have been mentioned above (Kumariya *et al*., 2019).

Moreover, bacteriophages and endolysins have a promising role in food processing, preservation and safety. Bacteriophages (or phages) have been recognized for their great effectiveness as antimicrobial candidates because of their ubiquity, self-replication capacity, low inherent toxicity and their high specificity against a target host or host range (Yezhi *et al*., 2016). The potential of bacteriophages and phage-encoded lytic enzymes to control specific pathogens in foods has been investigated intensively in recent years, as for example for the biocontrol of *Salmonella* spp., *Campylobacter* spp., *Listeria* spp. and *E. coli* (Greer, 2005; Hagens and Loessner, 2007). In recent years, several delivery systems using phages in food packaging have been developed but most of them are mainly directed mainly to controlling the growth of pathogenic species in foods products (Colom *et al*., 2015; Korehei and Kadla, 2014; Vonasek *et al*., 2014). Concerning spoilage bacteria, it has been shown that *Brochothrix* phage A3 is able to limit off-odor formation and increase the storage life of pork adipose tissue (Greer and Dilts, 2002). More recently, Alves *et al*. (2019) used bacteriophages loaded on sodium alginate-based films to prevent microbial poultry spoilage caused by *Ps. fluorescens*. These authors showed that a decrease in phage viability was detected after 8 weeks under refrigerated conditions, and that phages containing films applied on commercial poultry fillets were able to control bacterial growth for a period of up to 5 days. On the other hand, the control of environmental biofilms produced by *Ps. fluorescens* was investigated by Sillankorva *et al*. (2008), but phage behavior on biofilms is still poorly studied.

Because of the specific action of endolysins, they also offer an interesting option for the biological control of unwanted bacteria without having any effect on the natural microbiota (Loessner, 2005). But only a few studies have tested their antimicrobial effect against spoilage microbiota. Zinoviadou *et al*. (2010) showed the interest of using ε-polylysine against the spoilage microbiota of fresh beef in order to reduce the growth of LAB and pseudomonads. The direct application of purified bacteriophage endolysins to foods or raw products was also studied by Kilcher *et al*. (2010) for the biocontrol of *B. thermosphacta*. These concepts should be further explored in order to prevent spoilage phenomenon, but also to minimize pathogenic bacterial levels as well.

Moreover, knowledge of the regulatory mechanisms of bacterial food spoilage is still very limited. Transcriptomics could be interesting to fully understand how spoilage microorganisms regulate their metabolic pathways under specific conditions. But these studies are mainly focused on pathogenic bacteria (Lamas *et al*., 2019), and only few transcriptome analyses concerns spoilage bacteria. Wang *et al*. (2018a) and Liu *et al*. (2017) have shown the response of *Pseudomonas* species under stress conditions. Höll *et al*. (2019) have also used metatranscriptomics for predicting metabolite production by *Photobacterium* spp. in poultry meat stored in MAP. Recently, Liu *et al.* (2019b) have demonstrated that RpoS, an alternative sigma factor induced in stationary growth phase and under stress conditions, plays an important role in modulating the spoilage activity of *Ps. fluorescens* by regulating resistance to different stress conditions, extracellular AHL levels, extracellular proteases, biofilm formation and TVB-N production. It could be also interesting to provide insights into the regulatory mechanisms of *Pseudomonas* species and those of other food spoilage bacteria.

4.3.2. Association of metabolomics with predictive microbiology

Metabolite production by each of the inoculated bacteria, as inputs in interaction models, should be studied. Metagenomics, metatranscriptomics, metaproteomics and metabolomics have the potential to produce a large amount of data in a very short time, by integrating multi-omics data in microbiological risk assessment (Cocolin *et al*., 2018; Mu *et al*., 2020). Integrative studies have the power to provide a new approach for the investigation of microbial communities, enabling data integration, which can be used to better understand the interactions between community members (Aguiar-Pulido *et al*., 2016). Approaching the food ecosystem from different methods enables a "holistic" representation of which microorganisms are present, how they behave, how they interact and which are the phenotypic manifestations in this complex arena. However, multi-omics data also require careful data integration strategies. A further goal of data integration could also be the construction of predictive models (Franzosa *et al*., 2015).

The expected outcome may have an invaluable impact on food safety, in order to reduce the risk associated with foodborne pathogens, but also to better control spoilage processes. However, before this becomes reality, a number of hurdles have to be overcome. More specifically we have to learn how to translate molecular events into practical applications, which will give the food industry a concrete solution to make food products more safe and stable.

The general perspective of this thesis is therefore to study in more detail the metabolism related to spoilage bacteria in meat and meat products; to integrate these data into an "omics" approach, which could thus contribute to a better understanding and an anticipation of bacterial food spoilage; and also to improve predictive models in order to offer better tools to assist risk management.

Conclusions

In conclusion, this research proposed the study and the prediction of the dynamics of specific spoilage bacteria in minced pork and white pudding samples, depending on different storage conditions.

The results obtained and the proposed approaches could be further studied for extending shelf life and so preventing food losses and waste. Indeed, food spoilage must be addressed at the species levels and also be related to food companies, production batches and storage conditions. Further experimental studies are therefore needed to better characterize the kinetics and the behavior of these bacteria. Further researches and more experimental repetitions are also needed to better understand and predict food spoilage of meat and meat products, but also for all perishable foods.

Predictive models can provide valuable assistance in this area, but more integrated models are needed to obtain reliable predictions that could be used in practical applications. Several methods are now available to monitor and understand spoilage microbiota, which could be used in multi-omics approaches. This concept is essential to provide more accurate data and new perspectives for modeling.

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Annexes

Annexe 1

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The use of 16S rRNA gene metagenetic monitoring of refrigerated food products for understanding the kinetics of microbial subpopulations at different storage temperatures: the example of white pudding

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ABSTRACT

In order to control food losses and wastage, monitoring the microbial diversity of food products, during processing and storage is important, as studies have highlighted the metabolic activities of some microorganisms which can lead to spoilage. Knowledge of this diversity can be greatly improved by using a metagenetic approach based on high throughput 16S rRNA gene sequencing, which enables a much higher resolution than culture-based methods. Moreover, the Jameson effect, a phenomenon described by Jameson in 1962, is often used to classify bacterial strains within an ecosystem. According to this, we have studied the bacterial microbiota of Belgian white pudding during storage at different temperatures using culture-dependent and independent methods. The product was inoculated with a mix of dominant strains previously isolated from this foodstuff at the end of its shelf life (*Carnobacterium maltaromaticum*, *Lactobacillus fuchuensis*, *Lactobacillus graminis*, *Lactobacillus oligofermentans*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Raoultella terrigena* and *Serratia* sp.). Daily during 16 days, the absolute abundance of inoculated strain was monitored by combining total count on plate agar and metagenetic analysis. The results were confirmed by qPCR analysis. The growth of each species was modelled for each temperature conditions, representative of good or bad storage practices. These data allowed the bacterial strains subdivision into three classes based on criteria of growth parameters for the studied temperature: the "dominant", the "subdominant" and the "inhibited" bacterial species, according to their maximal concentration (Nmax, log CFU/g), growth rate (μmax, 1/h) and time to reach the stationary phase (TRSP, days). Thereby, depending on the storage conditions, these data have permitted to follow intrinsically the evolution of each strain on the bacterial ecosystem of Belgian white pudding. Interestingly, it has shown that the reliability of the Jameson effect can be discussed. For example, at 4 °C when *Lactococcus lactis* and *Serratia* sp. stopped growth at day 12, at the same time *Carnobacterium maltaromaticum* reached its maximal concentration and entered its stationary phase. In opposition to this, it can be noticed that in the same condition, the "sub-dominant" organisms continued their growth independently of the "dominant" species behaviour. In this case, the Jameson effect was not illustrated. This pattern is described for all storage conditions with the same strain classifications. These results highlighted the importance of combining metagenetic analysis and classical methods, with modelling, to offer a new tool for studying the evolution of microorganisms present in perishable food within different environmental conditions.

1. Introduction

In past years, scientists who study the safety of highly perishable food products have focussed their work on the detection and the control of pathogenic microorganisms. However, Food Law (Regulation (EC) N°178/2002) also integrates all products that are unfit for human consumption because of contamination, deterioration, decomposition or rotting into the definition of unsafe food. Around a third of all food produced for human consumption on Earth is lost or wasted. In Europe, the losses of initial meat production represent 20% and more than half of this occurs at animal production, slaughtering, processing and distribution steps (Food and Agriculture Organization, 2011; Kergourlay et al., 2015). These data highlight the importance of managing the microbiological quality of food products. Indeed, among the reasons for food

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loss and waste, spoilage by bacteria that contaminate the food matrix and are able to develop during transformation steps and storage is a major issue (Lipinski et al., 2013; Remenant et al., 2015).

For a clear and complete understanding of the mechanisms that lead to the spoilage of food products, classical microbiology is not sufficient. Fortunately, molecular technologies can elucidate the microbial communities, including the identification and quantification of culturable and non-culturable organisms, and can do so at a much higher resolution than was previously possible with culture-based methods (Kergourlay et al., 2015; Elizaquível et al., 2015). Many bacterial species putatively responsible for food spoilage have been reported, thanks to the development of high throughput sequencing methods, that allow for a more detailed and deeper description of bacterial species present in food (Benson et al., 2014; Chaillou et al., 2014; Delcenserie et al., 2014; Galimberti et al., 2015; Riquelme et al., 2015). These works are mainly limited to the description of the product's microbiota during its shelf life. However, spoilage is a complex process, resulting most often from incorrect storage temperatures and bacterial functions that are not fully understood. Spoilage is not only species and strain dependent, but also the result of interactions between strains. Few studies have described the evolution of a whole microbiota in a food matrix with consideration of the storage parameters (Ercolini et al., 2011; Nieminen et al., 2012).

The present study proposes to follow the evolution of the main bacterial species present in a famous Belgian meat product: the white pudding. For this, we inoculated a mix of strains previously isolated from aging tests on the same food matrix. The mix of inoculated strains has been studied in challenge tests at different storage temperatures, representative of good or bad practices. The growth of the added bacteria has been assessed daily at the same time by combining classical microbiology and 16S rRNA metagenetic analysis (Esposito and Kirschberg, 2014) with the goal of obtaining quantitative results for each strain and to study their respective kinetics. Quantitative PCR (qPCR) analysis targeted on corresponding bacterial genera was used in order to validate the metagenetic approach.

There are two objectives in this study: the first is to reinforce the importance of combining classical microbiology and metagenetics analysis, with modelling, as a new tool to follow the evolution of microorganisms present in perishable food within different environmental conditions. This approach can examine the potential for next-generation DNA sequencing methods to elucidate the detailed dynamics of microbial population during spoilage. To this end, a combination of metagenetics and traditional microbiological methods were used to quantify the microbiota of Belgian white pudding. The second is providing knowledge on the composition and dynamics of the emblematic bacterial species components of white pudding, and shown how it is affected by storage temperature.

2. Material and methods

2.1. Food samples and selection of bacterial strains

The strains used in this study were previously isolated from Belgian white puddings at the end of their use-by date, by one Belgian manufacturer (five batches analysed), after storage for a third of the storage period at 4 °C and the remaining time at 8 °C following the guidelines for implementing microbiological durability tests of chilled perishable and highly perishable foodstuffs (NF EN V01-003, 2010). The results of these first aging tests are not shown in this paper. Eight of the natural predominant strains isolated at the end of the shelf life, represented together more than 50% of the natural microbiota, were identified by sequencing of their 16S rRNA genes and used for the challenge-tests: *Carnobacterium maltaromaticum*, *Lactobacillus fuchuensis*, *Lactobacillus graminis*, *Lactobacillus oligofermentans*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Raoultella terrigena* and *Serratia* sp. For this study, a short 16 days shelf life was evaluated for the Belgian white pudding.

Bacterial strains were stored at −80 °C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, strains were transferred from the −80 °C culture collection to Brain Heart Infusion (BHI) broth for *C. maltaromaticum*, *R. terrigena* and *Serratia* sp., and de Man, Rogosa and Sharpe (MRS) broth for *Lb. fuchuensis*, *Lb. graminis*, *Lb. oligofermentans*, *Lc. lactis* and *Ln. mesenteroides* for 48 h at 22 °C. The cultures were incubated overnight at 4 °C before inoculation.

2.2. Challenge tests

Thirty-three kilograms of white puddings (each 150 g) were received from a Belgian manufacturer the day following their production and stored at 4 °C (composition: pork meat 64%, milk, bread, onions, salt and spices. No sugar was added). The natural microbiota was considered as insignificant because these products were inoculated by a concentrate mix of eight bacterial species who dominate the initial indigenous microbiota. The surface products were inoculated by soaking for 2 min in a bath of sterile water containing a mix of the eight bacterial strains at the same concentrations with the goal of reaching an approximatively global concentration of 3 log colony forming units (log CFU/g on the product), in duplicate (n = 192). Non-inoculated control samples were soaking for the same time in a bath of sterile water only, in duplicate (n = 24). After a drying step of 20 min at 10 °C, white puddings were packed (300 g) in a tray (PP/EVOH/PP) under modified atmosphere (CO_2 30%/N_2 70%, Olympia V/G, Technovac, Italy) using packaging wrap (polyester 10 μm, homopolymer polypropylene 50 μm, NutriPack, France). According to the shelf life of the product, inoculated samples were stored at different temperatures, constant or dynamic: (*i*) for 16 days at 4 °C (4 °C), (*ii*) for 16 days at 8 °C (8 °C), (*iii*) for 16 days at 12 °C (12 °C), (*iv*) for 4 days at 4 °C and for 12 days at 8 °C (4–8 °C), (*v*) for 4 days at 4 °C, followed by a break of 4 h at 20 °C than 12 days at 4 °C (4/20–4 °C), (*vi*) 4 days at 4 °C, followed by a break of 4 h at 20 °C then 12 days at 8 °C (4/20–8 °C). Control samples were only stored at the first day and at day 16.

2.3. Incubation and enumeration by conventional microbiological method

Each day during the 16-day storage period except on day 2, 25 g of product were put into a Stomacher bag with a mesh screen liner (80 μm pore size) (bioMérieux, Basingstoke, England, ref 80015) under aseptic conditions. Physiological water (225 mL) was automatically added to each bag (Dilumat, Biomerieux, Belgium) and the samples were homogenised for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in peptone water (1 g/L peptone, 8.5 g/L sodium chloride) were prepared for microbiological analysis and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis in duplicate (Spiral plater, DW Scientific, England). A total count was made on Plate Count Agar (PCA) at 22 °C for 48 h for the psychrotrophic aerobic plate count, using the modified method specified by the International Organization for Standardization [ISO (2013, ISO 4833-2)]. Graphs were plotted with each of the day time points over the 16-day storage period (n = 192). Non-inoculated products were only analysed at day 1 and day 16 (n = 24).

2.4. Total DNA extraction

Bacterial DNA was directly extracted from each primary suspension, which had been stored at −80 °C, using the DNeasy Blood & Tissue DNA Extraction kit (Qiagen, Venlo, Netherlands), following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentrations and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). The quality and quantity of the products were confirmed by Picogreen

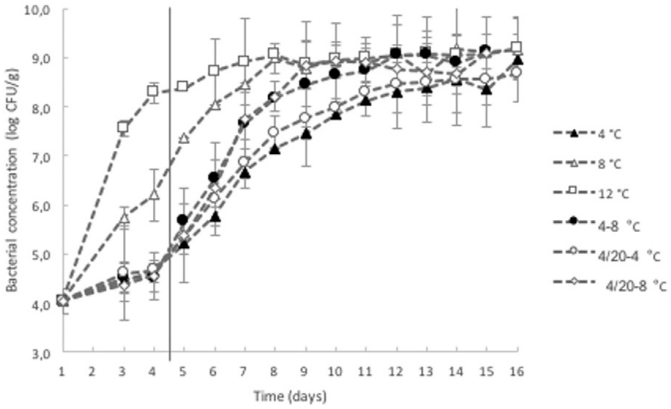

Fig. 1. Enumeration of the total psychrotrophic aerobic microorganisms from inoculated white puddings stored at different temperatures for 16 days. A solid line after the fourth day represents the transition from 4 °C to 8 °C for the 4–8 °C condition and the break at 20 °C for 4 h for the 4–8 °C, 4/20–4 °C and 4/20–8 °C conditions. The average of the replicate plating is plotted, with the standard deviation indicated by the whiskers.

double-stranded DNA (dsDNA) quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at −20 °C until use for 16S rRNA amplicon pyrosequencing and qPCR analysis.

2.5. Bacterial 16S rRNA gene amplification and barcoded pyrosequencing

16S rRNA PCR libraries targeting the V1–V3 hypervariable region were generated. Primers E9-29 and E514-430 (Brosius et al., 1981), specific for bacteria, were selected for their theoretical ability to generate the lowest amplification bias relative to amplification capability among the various bacterial phyla (Wang and Qian, 2009). The oligonucleotide design included 454 Life Sciences A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5′ end of each primer. The amplification mix contained 5 units (U) of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1× enzyme reaction buffer, 200 μM deoxynucleotide triphosphates (dNTPs) (Eurogentec, Liège, Belgium), 0.2 μM of each primer and 100 ng of genomic DNA in a final volume of 100 μL. Thermocycling conditions consisted of a denaturation step of 15 min at 94 °C, followed by 25 cycles of 40 s at 94 °C, 40 s at 56 °C, and 1 min at 72 °C, with a final elongation step of 7 min at 72 °C. These amplifications were performed on an EP Mastercycler Gradient System apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). The quality and quantity of the products were assessed by Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). Equal amounts of each of the PCR products were pooled and subsequently amplified by emulsion PCR. Pyrosequencing was performed with the Illumina sequencer (Illumina, Eindhoven, Netherlands) (2 × 300 bp). A mean 19,581 of reads per day were analysed for all temperature conditions.

2.6. Bioinformatics and data analysis

The 16S rRNA gene sequence reads were processed with MOTHUR (Pothakos et al., 2014; Schloss et al., 2009). The quality of all sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute, http://microbiomeutil.sourceforge.net/#A_CS). The obtained reads sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pothakos et al., 2014). The final reads were clustered into operational taxonomic units (OTUs) using the nearest neighbour algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU

Table 1
Primers and probes designed for the qPCR tests.

Target bacterial genus	Target gene	Primers	Sequence
Lactobacillus	Tuf	Lactobacillus-Tuf-F2	5′-GCYCACGTWGAATAYGAAAC-3′
		Lactobacillus-Tuf-R2	5′-CGDACTTCCATTTCAACYAAGTC-3′
		Lactobacillus-Tuf-FAM1	5′-TGTGGCATWGGRCCATCAGTTGC-3′
Lactococcus	RecA	Lactococcus-RecA-F2	5′-GCCGAAATYGATGGYGAAAT-3′
		Lactococcus-RecA-R2	5′-CAACTTTTTCACGCAATTGGTTG-3′
		Lactococcus-RecA-FAM4	5′-TGATGTCWCAAGCYATGCGTAAAC-3′
Leuconostoc	Fus	Leuconostoc-Fus-F1	5′-TTCTTGTTCCATGAAATCCATTTG-3′
		Leuconostoc-Fus-R1	5′-GAATACCCACTAGAWCGTACAC-3′
		Leuconostoc-Fus-FAM1	5′-TGTGTTTCACCAATTTTGTGAATTTTACC-3′
Carnobacterium	rpoA	Carnobacterium-rpoA-F1	5′-ATTGGYGTATTACCAGTCGA-3′
		Carnobacterium-rpoA-R1	5′-AACCATCTGCCCATACATC-3′
		Carnobacterium-rpoA-FAM1	5′-CGATTTACACCCCAGTTAGTCGT-3′

Primers and probes designed for the qPCR tests allowing for the relative proportion of genera mainly present in Belgian white pudding to be estimated.

Table 2
Metagenetics results for constant temperature condition.

	Days														
Bacterial strains/temperature	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16
4 °C															
C. maltaromaticum	1.1	3.2	4.5	11.5	25.8	70.5	86.8	96.7	96.4	93.9	90.7	76.7	65.8	50.4	42.1
Lc. lactis	0.3	0.2	0.1	0.4	1.2	0.2	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Ln. mesenteroides	0.1	0.3	0.0	0.3	0.5	0.6	1.3	0.3	0.0	0.6	0.7	1.3	4.2	4.6	12.5
Lb. graminis	0.2	0.4	1.3	0.5	0.8	1.5	1.8	0.2	0.0	0.8	0.7	1.9	2.7	3.8	4.3
Serratia sp.	0.2	0.2	0.7	0.6	0.8	0.8	0.7	0.2	0.1	0.2	0.1	0.1	0.6	0.2	0.5
Lb. fuchuensis	0.2	0.4	0.6	0.6	1.5	1.9	3.1	0.4	1.1	2.8	4.2	13.6	18.1	35.8	32.3
Other strains	97.9	95.1	92.8	86.2	69.3	24.5	6.0	2.0	2.5	1.7	3.6	6.3	8.6	5.1	8.2
8 °C															
C. maltaromaticum	1.1	33.8	60.4	69.8	75.1	48.8	43.0	18.7	8.4	14.0	7.0	6.7	8.2	8.3	3.4
Lb. fuchuensis	0.3	2.4	6.5	4.4	4.2	14.8	18.3	36.3	16.2	30.7	10.1	18.2	14.5	11.9	16.6
Lb. graminis	0.1	1.7	2.4	5.2	4.3	9.8	15.3	10.8	23.1	17.4	50.4	45.6	45.7	39.2	53.7
Ln. mesenteroides	0.2	2.3	5.9	3.5	2.6	4.3	3.0	3.1	0.5	1.7	2.4	3.0	2.7	4.1	2.3
Lc. Lactis	0.2	1.8	3.1	4.3	4.3	5.0	0.7	0.2	0.7	1.0	0.6	0.9	0.9	0.7	1.2
Serratia sp.	0.2	1.2	2.6	5.4	3.8	8.0	8.5	19.1	26.1	23.0	15.7	14.9	15.7	26.2	12.4
Other strains	97.9	56.8	19.1	7.4	5.7	9.3	11.2	11.9	25.1	12.2	13.8	10.6	12.4	9.6	10.4
12 °C															
C. maltaromaticum	1.1	8.0	2.7	0.8	0.7	0.7	0.8	1.1	0.8	0.8	1.0	0.4	0.6	0.5	0.6
Lb. fuchuensis	0.3	83.8	92.0	92.5	88.5	83.1	79.5	65.6	61.7	62.7	43.0	53.1	54.1	46.6	43.8
Lb. graminis	0.1	1.2	0.8	0.8	2.3	6.4	5.4	12.0	11.5	12.6	33.5	24.8	19.8	33.5	31.4
Ln. mesenteroides	0.2	1.3	0.4	0.9	0.8	1.2	0.6	1.1	0.0	2.0	0.9	1.4	1.8	1.4	1.6
Lc. Lactis	0.2	1.7	0.6	0.1	0.5	0.2	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.0	0.1
Serratia sp.	0.2	0.7	0.3	0.4	2.9	4.5	3.7	9.5	14.2	17.1	10.6	13.6	14.7	11.7	12.7
Other strains	97.9	3.4	3.1	4.3	4.4	4.0	9.8	10.5	11.6	4.7	10.8	6.4	8.8	6.4	9.8

Distribution of reads percentages for the six major bacterial species inoculated for each samples conditions obtained by metagenetics analysis during shelf life product in constant temperature. Others strains represented the natural microbiota of white pudding.

were compared to the SILVA dataset 111 using a BLASTN algorithm (Delcenserie et al., 2014; Pothakos et al., 2014). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (<1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Delcenserie et al., 2014; Pothakos et al., 2014).

2.7. Estimate abundance results

The PCA results of the microflora at 22 °C (Fig. 1, expressed in log CFU/g) and the relative proportions of strains given by metagenetics (Tables 2 and 3, expressed in %) were combined in order to obtain estimate counts for the strains (in log CFU/g). For this, relative abundance of

Table 3
Metagenetics results for dynamic temperature condition.

	Days													
Bacterial strains/temperature	1	3	4	5	6	7	8	9	10	11	12	13	14	15
4–8 °C														
C. maltaromaticum	1.1	8.7	2.6	50.4	49.8	89.5	86.3	72.0	48.5	27.0	14.6	15.2	7.3	11.9
Lc. lactis	0.3	20.2	10.8	4.1	16.0	0.3	0.4	0.4	1.0	2.6	2.3	2.7	2.4	3.6
Ln. mesenteroides	0.1	1.5	0.5	1.1	4.8	1.2	2.5	3.1	6.8	15.7	46.1	49.4	59.6	41.2
Lb. graminis	0.2	2.3	0.2	2.0	2.5	1.3	2.2	3.0	0.0	4.4	3.9	4.1	1.3	4.9
Serratia sp.	0.2	1.1	0.4	1.3	2.8	0.8	0.7	0.5	1.0	0.6	0.8	1.2	0.1	1.2
Lb. fuchuensis	0.2	0.9	0.3	1.7	7.3	2.7	4.7	11.3	26.4	36.9	23.4	17.7	11.3	27.0
Other strains	97.9	65.3	85.1	39.4	16.9	4.1	3.3	9.8	16.3	12.7	8.9	9.8	18.0	10.2
4/20–4 °C														
C. maltaromaticum	1.1	3.9	2.8	32.7	29.8	69.8	92.4	80.5	92.7	83.7	68.5	57.9	35.1	29.1
Lc. lactis	0.3	1.0	2.3	16.9	19.9	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.1
Ln. mesenteroides	0.1	0.3	0.3	0.7	4.8	2.5	1.1	4.2	0.3	0.8	2.4	4.8	10.4	12.3
Lb. graminis	0.2	0.1	0.6	2.4	3.9	1.8	0.8	2.2	0.0	1.8	2.0	4.6	4.7	4.8
Serratia sp.	0.2	0.4	0.3	1.7	2.4	1.7	0.5	0.4	0.2	0.1	0/3	0.2	0.7	0.4
Lb. fuchuensis	0.2	0.2	0.3	1.4	5.6	3.9	2.2	8.8	2.9	5.2	15.1	25.0	37.7	39.6
Other strains	97.9	94.1	93.5	44.4	33.5	20.4	3.1	3.9	3.8	8.4	11.6	7.5	11.2	13.6
4/20–8 °C														
C. maltaromaticum	1.1	2.3	3.0	35.5	38.4	88.0	89.9	56.6	36.7	24.2	12.0	13.0	12.6	9.2
Lc. lactis	0.3	1.2	0.8	24.4	21.3	0.8	0.2	2.5	9.1	7.4	12.3	11.6	21.1	8.2
Ln. mesenteroides	0.1	0.1	0.2	1.7	3.1	1.6	1.5	6.4	5.2	20.7	39.3	29.6	29.0	46.4
Lb. graminis	0.2	0.4	0.9	2.3	2.5	1.4	1.5	3.9	0.0	5.6	4.4	4.0	4.1	5.2
Serratia sp.	0.2	0.4	0.3	1.1	2.5	0.8	0.3	0.5	3.8	1.1	1.1	2.9	3.1	0.7
Lb. fuchuensis	0.2	0.2	0.6	1.6	4.2	1.6	2.3	20.6	29.7	30.3	16.2	25.1	20.7	17.5
Other strains	97.6	95.5	94.1	33.4	28.0	5.8	4.2	9.5	15.6	10.7	14.8	13.8	9.5	12.8

Distribution of reads percentages for the six major bacterial species inoculated for each samples conditions obtained by metagenetics analysis during shelf life product in dynamic temperature. Others strains represented the natural microbiota of white pudding.

bacteria obtained by metagenetic results were reported over the PCA real value (Eq. 1). Indeed, thanks to the plate counts estimates, the proportions of the bacterial populations were transformed into concentrations. These results were used for statistical and graphical analysis.

$$C_{bacterial\ strain} = (C_{total\ flora} \times N_{reads\ of\ bacterial\ strain})/100 \quad \text{Eq. 1}$$

Where $C_{bacterial\ strain}$ is the estimated abundance concentration in the sample (log CFU/g).

$C_{total\ flora}$ is the bacterial concentration per samples in the PCA analysis (log CFU/g).

$N_{reads\ of\ bacterial\ strain}$ is the number of reads for the bacterial strain per sample in the metagenetic analysis (expressed in % of the total number reads in the sample).

2.8. qPCR analysis

The primers described in Table 1 were used for real-time PCR assay analysis using the Lightcycler 480 system (Roche, Basel, Switzerland). The real-time PCR reaction mixtures were combined in a 12 μL final volume containing 6 μL of LC480 probe master mix (Roche, Basel, Switzerland), 2 μL of template DNA (at 5 ng/μL), 0.25 μL of primer pairs (10 μM each), 0.125 μL of Taqman probe (10 μM). The reaction conditions included the initiation step off 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The real-time system is supplied with the Lightcycler 480 Software version 1.5 using unique Roche algorithms for highly accurate and robust automated data analysis. Serial dilutions (10^6 to 1 copy numbers) of bacterial DNA were used for determining reference curves. The arithmetic mean of Cycle Threshold (CT) of the three repetitions was used in order to estimate the load of targeted bacterial populations present in the samples.

2.9. Statistical analysis

Using R software, the Analysis of Covariance (ANCOVA) test was used to evaluate if bacterial concentrations (log CFU/g) are equal across levels of a categorical independent variable (temperature conditions or microbial count method). With relation to temperature conditions, ANCOVA tests were realised using the bacterial growth data (from day 1 to day 16) and the bacterial growth during the exponential phase data (from day 4 to day 8). All tests were considered as significant for a p-value of <0.05.

3. Results

3.1. Bacterial evolution by classical microbiological analysis

Fig. 1 shows the PCA results from inoculated Belgian white puddings at different temperatures. The bacterial population showed different dynamic changes depending on conditions of storage and stabilised between 8.5 and 9.2 log CFU/g. For the non-inoculated products, results were respectively inferior to 3 log CFU/g and the same as inoculated products at day 16.

As expected, the storage temperature had a strong impact on the bacterial evolutions. A high storage temperature is correlated to a high growth rate during exponential phase and a stationary phase more rapidly reached. While the break at 20 °C for 4 h doesn't seem to have a significant effect on the evolution of the culturable microflora, the transition from 4 °C to 8 °C stimulated the growth of the microorganisms. It would be interesting to intrinsically study the effect of temperature conditions on the behaviour of each strain inside the ecosystem.

3.2. Relative abundance results obtained by metagenetics analysis

The distribution of reads percentages for the eight major bacterial species for each samples ($n = 768$) in constant temperature shows that at day 7 the mix reach more than 70% of total reads in samples for 4 °C. The same percentage is attained at day 3 both for 8 °C and 12 °C. The major bacterial species concerned *C. maltaromaticum* at 4 °C and *Lc. lactis* at 12 °C. All inoculated samples reached more than 90% at the end of shelf-life product (Table 2) and the natural microbiota of the white pudding seems to become minor in contrast to the inoculated

Table 4
Growth parameters of bacterial strains in inoculated white puddings under constant storage conditions.

	Nmax	TRSP	μmax	Class
4 °C				
C. maltaromaticum	8.6	12	0.07	D
Lb. fuchuensis	8.5	16	0.05	S
Lb. graminis	7.6	16	0.03	S
Ln. mesenteroides	8.1	16	0.03	S
Lc. Lactis	4.9	12	0.05	I
Serratia sp.	6.7	12	0.04	I
8 °C				
C. maltaromaticum	8.1	8	0.10	D
Lc. Lactis	8.4	10	0.09	S
Lb. fuchuensis	8.3	10	0.09	S
Ln. mesenteroides	8.9	10	0.10	S
Lb. graminis	7.6	8	0.08	I
Serratia sp.	6.7	8	0.10	I
12 °C				
Lc. Lactis	8.9	4	0.25	D
Lb. fuchuensis	8.3	11	0.14	S
Ln. mesenteroides	8.7	11	0.10	S
C. maltaromaticum	7.0	4	0.10	I
Lb. graminis	7.4	4	0.11	I
Serratia sp.	6.0	4	0.12	I

Bacterial concentration at day 16 (Nmax, log CFU/g), time to reach the stationary phase (TRSP, days) and maximal bacterial growth rate (μmax, 1/h). Bacterial strains were subdivided into three categorical classes: D ("dominant"), S ("subdominant"), I ("inhibited").

Table 5
Comparison of bacterial strains in inoculated white puddings subject to storage conditions with changes of temperature, according to the time taken to reach a 7 log CFU/g threshold and ANCOVA-test based on the global growth and the growth rate during exponential phase.

Bacterial strains	7 log CFU/g threshold (days)		ANCOVA-test	
	4 °C	4–8 °C	Global growth	Growth rate during exponential phase
a.				
C. maltaromaticum	8	7	Ø	4–8 > 4**
Lc. lactis	–	11	4–8 > 4***	4–8 > 4**
Lb. fuchuensis	12	8	4–8 > 4**	4–8 > 4**
Lb. graminis	14	9	4–8 > 4**	4–8 > 4**
Ln. mesenteroides	14	9	4–8 > 4***	4–8 > 4***
Serratia sp.	–	12	4–8 > 4**	4–8 > 4**
b.				
C. maltaromaticum	8	7	Ø	Ø
Lc. lactis	–	–	4/20–4 > 4*	–
Lb. fuchuensis	12	11	4/20–4 > 4*	Ø
Lb. graminis	14	12	4/20–4 > 4*	Ø
Ln. mesenteroides	14	12	4/20–4 > 4**	4/20–4 > 4*
Serratia sp.	–	–	4/20–4 > 4*	Ø
c.				
C. maltaromaticum	7	7	Ø	Ø
Lc. lactis	11	9	Ø	Ø
Lb. fuchuensis	8	8	Ø	Ø
Lb. graminis	9	9	Ø	Ø
Ln. mesenteroides	9	9	Ø	Ø
Serratia sp.	12	11	Ø	Ø

(a) 4 °C vs. 4–8 °C, (b) 4 °C vs. 4/20–4 °C, (c) 4–8 °C vs. 4/20–8 °C.
– data out of range, > superior value, Ø no significant statistical difference.
* Significant statistical difference, p-value < 0.05.
** High significant statistical difference, p-value < 0.01.
*** Highly significant statistical difference, p-value < 0.001.

mix of the surface product. For the dynamic temperature condition, the same results were observed (Table 3).

In these two cases, some bacterial strains (*R. terrigena* and *Lb. oligofermentans*) were excluded from the results for better readability because they were often under the detection level for the metagenetic analysis (data not shown).

3.3. Combining PCA results and relative abundance to obtain estimate counts

Table 4 shows growth parameters, for each strain, calculated from the combination of the PCA counts at 22 °C and the relative proportions of strains given by metagenetics (estimate abundance results) for constant temperature conditions (at 4 °C, 8 °C and 12 °C). Using R software these parameters were obtained by fitting to a primary model of bacterial curves according to the Baranyi equation (Delhalle et al., 2012; Ercolini et al., 2011; Zwietering et al., 1990). These parameters give the bacterial concentration at day 16 (Nmax, log CFU/g), the maximal bacterial growth rate (μmax, 1/h) and the time to reach the stationary phase (TRSP, days).

These results allowed the bacterial strain subdivision into three classes based on growth parameters for each temperature conditions studied. These three classes are respectively called “dominant”, “inhibited”

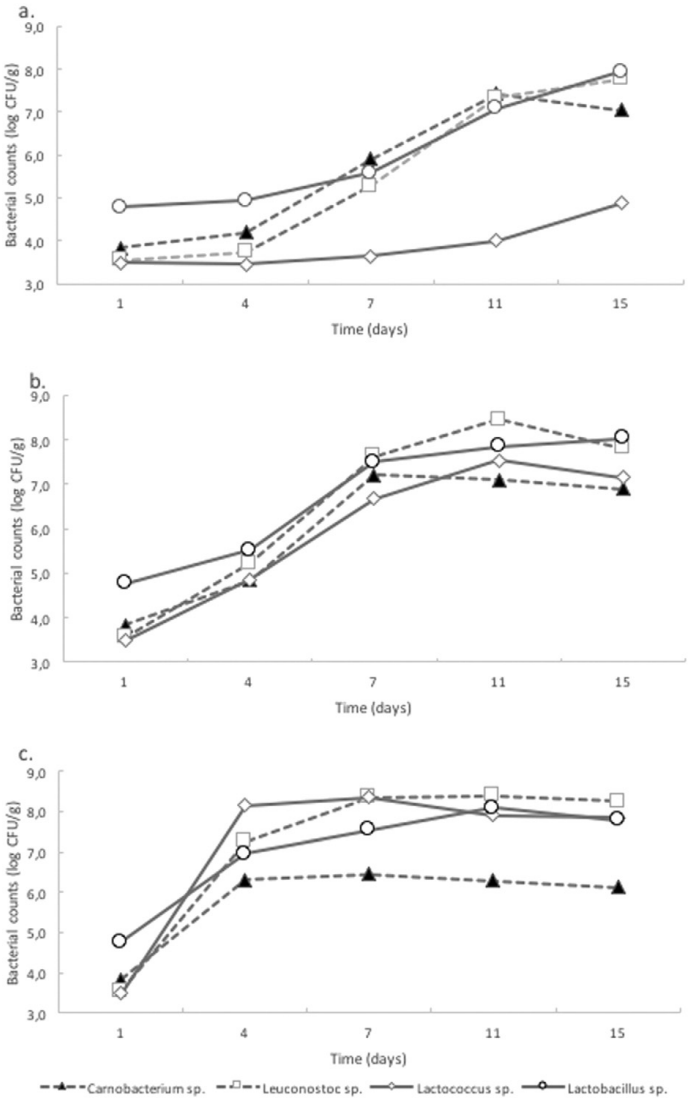

Fig. 2. qPCR counts from inoculated white puddings stored at 4 °C (a), 8 °C (b) and 12 °C (c).

and "subdominant" according to their growth parameters and their behaviour observed inside the bacterial ecosystem:

The "dominant" bacterial species had three high growth parameters: they have the highest growth rate (μmax), maximal concentration (Nmax) between 8 and 9 log CFU/g, and rapidly reached the stationary phase during the shelf life of the product.

The "inhibited" bacterial species had a lesser or equal growth rate but they achieved an inferior Nmax value, and stopped their growth at the same time as the "dominant" species.

The "subdominant" bacterial species are all other bacterial species that continued growth when the "dominant" organisms reached the stationary phase, which is the opposite to the "inhibited" bacteria, with a generally lesser growth rate than the "dominant" species. They reached the stationary phase lesser rapidly but they achieved a high maximal concentration.

According to the conditions of storage the bacterial ecosystem change: *C. maltaromaticum* is the "dominant" bacteria at 4 °C and 8 °C, while *Lc. lactis* dominates at 12 °C. *Lc. lactis* is an "inhibited" and a "subdominant" bacterial species for conditions at 4 °C and 8 °C respectively. *Lb. graminis* is a "subdominant" bacteria at 4 °C and an "inhibited" species for the two other conditions. Interestingly, *Ln. mesenteroides* and *Lb. fuchuensis* were "subdominant" bacteria at all temperatures. On the other hand, *Serratia* sp. is an "inhibited" bacterium at all temperatures.

Table 5 shows the combination of the PCA counts of the microflora at 22 °C and the relative proportions of strains obtained by metagenetics (estimate abundance results) for storage conditions with changes of temperature (at 4–8 °C, 4/20–4 °C and 4/20–8 °C). For these situations two parameters were studied: the time necessary to attain a 7 log CFU/g threshold of spoilage (days) and the statistical difference between conditions of storage by ANCOVA-tests based on the global growth and the growth rate during exponential phase.

The results of the ANCOVA-tests show that strains have a better bacterial growth at 4–8 °C than at 4 °C, except for *C. maltaromaticum* that showed a statistically different growth rate only during the exponential phase. Consequently, all species reached the 7 log CFU/g threshold earlier at 4–8 °C than at 4 °C (Table 5(a)). For the break of 4 h at 20 °C during storage this phenomenon was significantly weaker. Indeed, *Ln. mesenteroides* is the only species which showed a significant statistically effect in the two tested parameters of growth rate. The other strains have a better global growth at 4/20–4 °C (except for *C. maltaromaticum*) and all species reached the 7 log CFU/g threshold earlier at 4/20–4 °C than at 4 °C (Table 5(b)). Results shows also that there were no significant statistical changes on the growth parameters between the break of 4 h at 20 °C and the transition from 4 °C to 8 °C but *Lc. lactis* and *Serratia* sp. reached the 7 log CFU/g earlier (Table 5(c)).

3.4. Comparison with qPCR results

Fig. 2 shows the qPCR counts for four genus at 4 °C (Fig. 2a), 8 °C (Fig. 2b) and 12 °C (Fig. 2c). The comparison between metagenetics results and the LAB genus specific qPCR are summarised in Table 6. On average, the population overestimation was equal to 1.1 log CFU/g in qPCR test at 4 °C for *Lactobacillus* and *Leuconostoc*. Indeed, bacterial curves are convergent except for *Lactobacillus* and *Leuconostoc* at 4 °C.

Table 6
Comparison between qPCR and estimate abundance results (log CFU g^{-1}) for days 1, 4, 7, 11 and 15 with ANCOVA-test.

	Carnobacterium	*Lactobacillus*	*Lactococcus*	*Leuconostoc*
4 °C	Ø	qPCR > Meta**	Ø	qPCR > Meta*
8 °C	Ø	Ø	Ø	Ø
12 °C	Ø	Ø	Ø	Ø

Estimate abundance results: obtained by combination of the PCA counts at 22 °C and the relative proportions of strains given by metagenetics.
> superior value, Ø no significant statistical difference.
* Significant statistical difference, p-value <0.05.
** Very significant statistical difference, p-value <0.01.

4. Discussion

Based on the primary results given on total count on plate agar, the influence of temperature on the development of a whole ecosystem on Belgian white puddings was observed. The power of metagenetic analysis, when added to these basics results, has allowed us to closely follow the evolution of each strain inoculated on the product during its shelf life. In addition, the data have been validated by a qPCR analysis where no significant differences were seen for the quantification of the genera studied except for *Lactobacillus* and *Leuconostoc* at 4 °C. These small differences at the beginning of the shelf life can be explained by the detection of DNA from dead bacteria naturally present in large quantities on the raw meat and resulting from microbial destruction during the manufacturing process. This means that the qPCR analysis has detected some DNA fragments from dead organisms that haven't evidently grown on plate agar, leading to a weak overestimation of the qPCR results at the beginning of the experiment. This phenomenon is lesser in metagenetics analysis because of the high variability of strains presents in the product at the beginning of the experiment. Later during the challenge-test, this difference between the two techniques becomes negligible. Indeed, gradually throughout the experiment, the *Lactobacillus* and *Leuconostoc* species become a part of the dominant microflora that leads to a dilution effect of the dead bacterial DNA by the living bacteria's DNA.

The large amount of data provided by the combination of the culture-dependent and culture-independent techniques has given useful information about the growth of each strain during challenge tests. Metagenetic analysis also allows for the assessment of the dynamics of bacterial species within a food matrix. It permitted classification of bacterial strains into different categories according to their behaviour in the ecosystem. The so-called "dominant" bacterial species rapidly reached the stationary phase at a concentration of between 8 and 9 log CFU/g while at the same time the "inhibited" strains stopped their growth at a lower concentration. This phenomenon was described by Jameson in 1962 and recently reviewed by other scientists as follows: "the minority population decelerates when the majority or the total population count reaches its maximum" (Ross et al., 2000; Mellefont et al., 2008; Irlinger and Mounier, 2009; Cornu et al., 2011). This Jameson effect was clearly observed in our study, for example at 4 °C (Table 2), when *Lc. lactis* and *Serratia* sp. stopped growth at day 12, at the same time *C. maltaromaticum* reached its maximal concentration and entered its stationary phase. In opposition to this, it can be noticed that in the same condition, the "sub-dominant" organisms continued their growth independently of the "dominant" species behaviour. In this case, the Jameson effect was not illustrated. This pattern is described for all storage conditions with the same strain classifications (Table 2). This phenomenon was also observed by others scientists and they proposed that the growth of the minority population is only partly inhibited after the majority population has reached its stationary phase (Gnanou Besse et al., 2006; Cornu et al., 2011). This can be explained by the fact that the minority population is only partly affected by the limiting resource and/or inhibiting waste product that led it to stop growing (Gnanou Besse et al., 2006; Cornu et al., 2011).

According to this, the bacterial strain subdivision based on growth parameters can be represented as (Table 7):

$$\text{If } (Nmax_{\text{bacterial strain}} > Nmax_{\text{others}}) \& (\mu max_{\text{bacterial strain}} > \mu max_{\text{others}}) \& (TRSP_{\text{bacterial strain}} < TRSP_{\text{others}}) = \text{"dominant" bacterial species.}$$

$$\text{If } (Nmax_{\text{bacterial strain}} \cong Nmax_{\text{others}}) \& (\mu max_{\text{bacterial strain}} \leq \mu max_{\text{others}}) \& (TRSP_{\text{bacterial strain}} > TRSP_{\text{others}}) = \text{"subdominant" bacterial species.}$$

Table 7
Bacterial strain subdivision based on growth parameters in three categorical classes: D ("dominant"), S ("subdominant"), I ("inhibited").

Class	μmax	Nmax	TRSP	Growth parameters
D	The highest	Maximal value. Between 8 and 9 log CFU g^{-1}	Rapidly reached	If ($Nmax_{bacterial\ strain} > Nmax_{others}$) & ($\mu max_{bacterial\ strain} > \mu max_{others}$) & ($TRSP_{bacterial\ strain} < TRSP_{others}$)
S	Generally lesser	High value	Continue to growth when the D organisms reached the stationary phase	If ($Nmax_{bacterial\ strain} \cong Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} > TRSP_{others}$)
I	Lesser or equal	Inferior value	The same as the D organisms	If ($Nmax_{bacterial\ strain} < Nmax_{others}$) & ($\mu max_{bacterial\ strain} \leq \mu max_{others}$) & ($TRSP_{bacterial\ strain} = TRSP_{others}$)

Maximal bacterial growth rate (μmax, 1/h), bacterial concentration at day 16 (Nmax, log CFU/g) and time to reach the stationary phase (TRSP, days).

If $(Nmax_{bacterial\ strain} < Nmax_{others}) \& (\mu max_{bacterial\ strain} \leq \mu max_{others})$ $\& (TRSP_{bacterial\ strain} = TRSP_{others}) =$ "inhibited" bacterial species.

Where Nmax (bacterial concentration at day 16, log CFU/g), μmax (maximal bacterial growth rate, 1/h) and TRSP (time to reach the stationary phase, days) are growth parameters.

Regarding the "inhibited" strains, hypotheses can be made about inconvenient growth temperatures (T_{min}) and/or a microbial competition with the rest of the ecosystem. The composition of white pudding seems not to have an effect on strains competiveness. According to scientific literature, *Serratia* sp. has a minimum growth temperature (T_{min}) of 0 °C (Labadie, 1999) and would normally grow at 4 °C. But at this temperature, it is classified as an "inhibited" strain. Therefore, the inhibition of the bacterial growth of *Serratia* sp. is probably due to an ecosystem effect. Concerning *Lactococcus lactis*, one study set its T_{min} at 10 °C (Labadie, 1999), while in our experiment a normal growth pattern was observed at 8 °C, allowing its classification in the "sub-dominant" group. In this case, the inhibited development of this organism at 4 °C is probably due to an ecosystem effect coupled with a temperature effect. Indeed, when *Lc. lactis* grew at 12 °C, it became the dominant flora and was more competitive than *C. maltaromaticum* which seems to be more adapted to lower temperatures (T_{min} of *C. maltaromaticum* = 0 °C, (Casaburi et al., 2011)). For *Ln. mesenteroides* and *Lb. fuchensis*, for which T_{min} are respectively 4 °C (Osmanagaoglu and Kiran, 2011) and 2 °C (Zwietering et al., 1993), they stayed "subdominant" for all the temperature conditions tested. The rest of the ecosystem probably does not affect their growth, insofar as they never gained predominance but they were not inhibited either.

The results of the challenge tests with temperature breaks or changes are consistent with our previous observations. Indeed, *C. maltaromaticum* seems to be more adapted to low temperatures. To this end, this bacterium didn't take a great benefit in its growth when the storage temperature moved to 20 °C for 4 h (Table 3). In contrast, the growth parameters of *Ln. mesenteroides* rose during the transition from 4 °C to 8 °C or with the break of 4 h at 20 °C. This is consistent with the fact that its optimal growth temperature (T_{opt}) is between 20 °C and 35 °C (Zwietering et al., 1993, Jin et al., 2012). *Lc. lactis* also has a T_{opt} around 25 °C but any improvement of its growth parameters was not observed. The hypothesis is made that the break time of 4 h was too short to see a significant effect. In conclusion, a break of 4 h at 20 °C is prejudicial only if the storage temperature (4 °C) is respected during the entire life of the product. Moreover it is commonly admitted that the customer's fridge is rarely at 4 °C (Lagendijk et al., 2010). By taking account of this fact, the lack of respect for good temperature storage (8 °C instead of 4 °C), particularly in customer's fridges, is more prejudicial than a break of the cold chain for up to 4 h. However, an indication about the true temperature in the product during the 4 h of breaking time at 20 °C would be necessary before making this conclusion. Indeed, the internal temperature of white pudding samples may stay colder than 20 °C, due to the thickness of this product, explaining the apparent absence of effect or a weak effect.

In the future, it will be interesting to explore the interactions in the white pudding ecosystem more deeply. Further studies will focus on the comprehension of the mechanisms that force the "inhibited" strains to stop their growth in the early stage of the shelf life of the product. Indeed, it is commonly accepted that the self-limiting growth process in microbial ecosystem is supposed to be due to (i) the exhaustion of one of the essential nutrients, (ii) the accumulation of metabolic waste products which inhibit growth, and/or (iii) the lowering of pH due to acid production (Cornu et al., 2011). According to the data already obtained, we could suppose that competition for space or nutrient has an effect. The action of a bacteriocin is also not excluded and could for example explain the lack of development of some strains inoculated into the product: *Lb. oligofermentans* and *R. terrigena*. It would also be interesting to know the spoilage or biopreservative potential of all the strains inoculated in the Belgian white pudding in this study. Another challenge will be in differentiating the nature of the ecosystem interactions: strain dependent or species dependent. Finally, this supply of new information will be a good start for future experiments when it is considered that the natural contamination of a food product is more complex that an inoculation of eight bacterial strains from different species.

Our applications of the 16S rRNA gene-based pyrosequencing has now extended our view of the dynamic behaviour of complex microbial populations in Belgian white pudding, revealing the quantitative displacement of taxa that occur during microbial successions. By integrating metagenetics with traditional microbiological analysis we have now extended this view of a highly quantitative characterization of dynamic changes that occur during refrigerated storage. In addition to the predictive microbiology, these data also permit to classify the population dynamics into three major classes, based on growth parameters.

5. Conclusions

Metagenetic analysis offers a new tool for identifying microorganisms present in perishable foods and for studying their evolution within different environmental conditions. The information that can be obtained provides a clear picture of the microbial community. Microbiological ecology studies have shown that the microbiota of food is much more diverse than the cultivable group of bacteria studied by the use of culture media. The use of these new technologies will open a new era for modelling and predictive microbiology. In this study, these results provide valuable informations for discussing about the theory of the Jameson effect. In addition, it will help food business operators to have a better view of the quality of their product by differentiating between the spoilage or bioprotective microflora. Moreover, it will provide knowledge on the composition and dynamics of white pudding and shown how it is affected by storage temperature. Indeed, many food manufacturers, government agencies, retailers, distribution quality laboratories and researchers use classical culture media without being able to precisely identify the bacterial communities present within the food. In the future, new gold standards for food quality will need to be developed in order to allow the use of metagenetics as a complementary

technique for characterizing the bacterial flora of products and its use should be considered as a technique for quality control, for accurately determining the length of shelf life and for developing new food products and/or new storage advices.

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Annexe 2

frontiers in Microbiology

ORIGINAL RESEARCH
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Assessment of Spoilage Bacterial Communities in Food Wrap and Modified Atmospheres-Packed Minced Pork Meat Samples by 16S rDNA Metagenetic Analysis

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Although several studies have focused on the dynamics of bacterial food community, little is known about the variability of batch production and microbial changes that occur during storage. The aim of the study was to characterize the microbial spoilage community of minced pork meat samples, among different food production and storage, using both 16S rRNA gene sequencing and classical microbiology. Three batches of samples were obtained from four local Belgian facilities (A–D) and stored until shelf life under food wrap (FW) and modified atmosphere packaging (MAP, CO_2 30%/O_2 70%), at constant and dynamic temperature. Analysis of 288 samples were performed by 16S rRNA gene sequencing in combination with counts of psychrotrophic and lactic acid bacteria at 22°C. At the first day of storage, different psychrotrophic counts were observed between the four food companies (Kruskal-Wallist test, p-value < 0.05). Results shown that lowest microbial counts were observed at the first day for industries D and A (4.2 ± 0.4 and 5.6 ± 0.1 log CFU/g, respectively), whereas industries B and C showed the highest results (7.5 ± 0.4 and 7.2 ± 0.4 log CFU/g). At the end of the shelf life, psychrotrophic counts for all food companies was over 7.0 log CFU/g. With metagenetics, 48 OTUs were assigned. At the first day, the genus *Photobacterium* (86.7 and 19.9% for food industries A and C, respectively) and *Pseudomonas* (38.7 and 25.7% for food companies B and D, respectively) were dominant. During the storage, a total of 12 dominant genera (>5% in relative abundance) were identified in MAP and 7 in FW. *Pseudomonas* was more present in FW and this genus was potentially replaced by *Brochothrix* in MAP (two-sided Welch's t-test, p-value < 0.05). Also, a high Bray-Curtis dissimilarity in genus relative abundance was observed between food companies and batches. Although the bacteria consistently dominated the microbiota in

our samples are known, results indicated that bacterial diversity needs to be addressed on the level of food companies, batches variation and food storage conditions. Present data illustrate that the combined approach provides complementary results on microbial dynamics in minced pork meat samples, considering batches and packaging variations.

Keywords: minced meat, metagenetics, spoilage bacteria, modified atmosphere packaging, food wrap packaging

INTRODUCTION

Meat and meat products are highly perishable, with colonization and development of a variety of microorganisms, especially bacteria. This is due to complex nutrient-rich environment with chemical and physical conditions favorable to bacterial development (Nychas et al., 2008; Pennacchia et al., 2009; Chaillou et al., 2015; Garnier et al., 2017). Moreover, minced meat can be contaminated by different types of microorganisms from several sources, such as raw materials, equipment, environment and handling involved in the production process. Abiotic factors (temperature, gaseous atmosphere, pH, NaCl levels, etc.) can also select certain bacteria (Mann et al., 2016; Stellato et al., 2016; Rouger et al., 2018). However, it is well known that richness and abundance of microbiota present in food products, and especially meats, play an important role in the microbial safety and the shelf life of the products (Zhao et al., 2015; Pinu, 2016). Microbial growth on meat to unacceptable levels and the various metabolic activities contribute to its deterioration by altering the structure, color and flavor of the meat (Mann et al., 2016). This leading to a reduction in food quality to the point of not being edible for human consumption (Holm et al., 2013; Silbande et al., 2016; Stellato et al., 2016), with alterations in the sensorial qualities of the product, particularly the aspect, with discoloration and gas production, and the presence of an off-odors and off-flavors (Stoops et al., 2015). Thus, food spoilage is problematic for two main reasons: first, it renders food unfit for human consumption and, secondly, it results in significant economic losses (Dalcanton et al., 2013; Pinter et al., 2014; Den Besten et al., 2017).

As mentioned by Benson et al. (2014), the microbial population that colonizes and ultimately spoils minced pork meat is highly variable, depending on which groups of microbial taxa the product has been exposed to and perhaps even the order in which they are encountered. Using traditional cultivation methods, the microbial composition and diversity in fresh meat have been widely investigated (Zhao et al., 2015), but it is well known that traditional identification and culture-based methods for pathogens or food spoilage microbes are time-consuming (Pinu, 2016). Moreover, ecological studies at the genus-species level are required because the same storage conditions may affect differently the species in the same groups of bacteria (Pennacchia et al., 2011; Stoops et al., 2015), and because not all the members of this microbiota contribute to food spoilage. Several studies in meat microbiology have established that spoilage is caused only by a dominated fraction of the initial microbial association (Nychas et al., 2008). These spoilage microorganisms have been designated as Ephemeral/Specific Spoilage Organisms (E(S)SOs) (Benson et al., 2014; Zotta et al., 2019). Therefore, as discussed by De Filippis et al. (2013), the concept of succession of spoilage-related microbial groups is very important, and many studies have been performed to investigate the dynamics and changes of the meat microbiota during storage.

Developed during the last decades, the next generation sequencing methodologies provide a powerful tool to study microbial community structure and composition shifts at different stages of ripening, allowing the detection of minor bacterial populations (Riquelme et al., 2015), at variable taxonomic depth (Pothakos et al., 2014; Chaillou et al., 2015; Parente et al., 2016). The introduction of molecular methods, especially culture-independent approaches, have contributed to the exploration of various food microbiota (Galimberti et al., 2015; Pinu, 2016; Garofalo et al., 2017; Parlapani et al., 2018), as for beverages (Elizaquivel et al., 2015), vegetables (Lee et al., 2017; Gu et al., 2018; Liu et al., 2019), and for dairy (Nalbantoglu et al., 2014; Riquelme et al., 2015; Ceugniez et al., 2017; Porcellato et al., 2018), seafood (Li et al., 2018; Parlapani et al., 2018; Silbande et al., 2018), and meat products (Cocolin et al., 2004; Pennacchia et al., 2011; Nieminen et al., 2012; Benson et al., 2014; Greppi et al., 2015; Polka et al., 2015; Stoops et al., 2015; Zhao et al., 2015; Delhalle et al., 2016; Mann et al., 2016; Carrizosa et al., 2017; Cauchie et al., 2017; Kaur et al., 2017; Korsak et al., 2017; Peruzy et al., 2019; Vester Lauritsen et al., 2019), in order to assess the microbial levels and diversity of food and food products (Nieminen et al., 2012; Pothakos et al., 2014; Lee et al., 2017; Rouger et al., 2018). The interest of this method to characterize the dominant spoilage bacteria in pork meat and meat products was also described (Andritsos et al., 2012; Mann et al., 2016; Raimondi et al., 2018; Li et al., 2019; Peruzy et al., 2019).

In this context, the aim of the present study was to assess the microbial spoilage community and dynamics of minced pork meat samples, among different conditions of production and food storage, using both 16S rRNA gene sequencing and classical microbiology.

MATERIALS AND METHODS

Sampling

Fresh minced pork meat (MPM) samples packed with a food wrap film were obtained from four local small and medium-sized Belgian manufacturers (food companies A, B, C, and D) at the day of the production, corresponding to the day of slaughtering. Three batches for each manufacturer were used, with a 1-week interval between sampling (**Supplementary Figure S1**).

According to the recipe MPM is composed of 100% minced pork meat (70% lean, 30% fat), no salt, no spices, no additives,

no eggs and no sugar are added. At the day of the production, the water activity of this product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.05 (n = 12). pH of the homogenized samples (5 g in 45 ml of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

Minced pork meat samples were packed (100 g), in triplicate, in two different types of non-sterile packaging.

The first packaging concerns a tray (187 × 137 × 36, polyester 10 μm, homo-polymer polypropylene 50 μm, NutriPack, France) under modified atmosphere (MAP, CO_2 30%/O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France).

The second packaging concerns a tray (175 × 135 × 22, polystyrene) under food wrap packing (FW) using cling film (Clinofilm).

Food Storage

According to the requirements for implementing microbiological tests of chilled perishable and highly perishable foodstuffs (AFNOR, 2010, NF V01-003), MPM samples were stored during 3 days of shelf life under FW, and during 6 days under MAP packaging, at constant and dynamic temperature: at (i) 2°C (± 1°C), (ii) 8°C (± 1°C), (iii) 12°C (±1°C), and (iv) for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8°C ± 1°C), in climatic chambers (Sanyo MIR 254).

Samples were analyzed at the first day of inoculation (day 0) and at the last day of storage (day 3 in FW and day 6 in MAP, n = 288) (**Supplementary Figure S1**).

Plate Count Enumeration

Twenty-five grams of product were randomly collected from the trays at the surface and at depth, without homogenization, and put into a Stomacher bag with a mesh screen liner (80 μm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10 g/L peptone, 5 g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (10 g/L peptone, 8.5 g/L sodium chloride, #CM0733, Oxoid, Hampshire, England) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis (Spiral plater, DW Scientific, England). Total viable counts (TVC) for the aerobic psychrotrophic flora were performed on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France), and for the lactic acid bacteria (LAB) on de Man, Rogosa and Sharpe (MRS agar, #CM0361, Oxoid, Hampshire, England), after incubation at 22°C (Pothakos et al., 2014) for 72 h (model 1535 incubator, Shel Lab, Sheldon Manufacturing Inc., United States).

DNA Extraction and 16S rDNA Amplicon Sequencing

Bacterial DNA was extracted from each primary suspension, previously stored at −80°C, using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV, Antwerp, Belgium) following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, Sint-Pieters-Leeuw, Belgium). DNA samples were stored at −20°C until used for 16S rDNA amplicon sequencing.

PCR-amplification of the V1-V3 region of the 16S rDNA library preparation were performed with the following primers (with Illumina overhand adapters), forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGGCAC-3′). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, United States) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 s at 94°C), annealing (45 s at 56°C) and extension (60 s at 72°C), with a final elongation step (8 min at 72°C). These amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). After purification, PCR products were quantified using the Quanti-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, United States) and diluted to 10 ng/μL. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems, Wilmington, MA, United States) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina, San Diego, CA, United States).

Bioinformatics Analysis

The 16S rRNA gene sequence reads were processed with MOTHUR (Schloss et al., 2009). The quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute)[1]. The obtained read sets were compared to a reference data-set of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences (version v1.2.11)[2] implemented in MOTHUR (Pruesse et al., 2012; Pothakos et al., 2014; Cauchie et al., 2017). The final reads were clustered into operational taxonomic units (OTUs), using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut off. A taxonomic identity was attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cut off. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique

[1]http://microbiomeutil.sourceforge.net/#A_CS

[2]http://www.arb-silva.de/

sequences for each OTU were compared to the SILVA data-set 111, using a BLASTN algorithm. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (<1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not) (Delcenserie et al., 2014; Cauchie et al., 2017).

16S rDNA Data Analysis

A correcting factor for 16S rDNA gene copy numbers was applied for any taxon *i* (Eq. 1) (Kembel et al., 2012; Louca et al., 2018).

$$A_i = {}^{N_k}/_{C_i} \quad \text{(Eq. 1)}$$

Where A_i is the real abundance of 16S genes from the taxon in the sample, N_k is the number of reads for the taxon in the sample *k*, and C_i is determined by the genomic 16S copy number of that taxon. To obtain each gene copy number, Ribosomal RNA Database (rrnDB) (Stoddard et al., 2015) and EzBioCloud database (Yoon et al., 2017) were used.

Then, to compare the relative abundance of OTUs, the number of reads of each taxon were normalized (Nr_i) as described by Chaillou et al. (2015). Reads counts of each taxon *i* in the sample *k* were divided by a sample-specific scaling factor *(Si)* (Eq. 2) (Fougy et al., 2016; Rouger et al., 2018):

$$Nr_i = {}^{A_i}/_{S_k} \quad \text{(Eq. 2)}$$

Where A_i is the real abundance of 16S genes from that taxon obtained with a correcting factor for 16S rDNA gene copy numbers, S_k is the normalization factor associated with sample *k*.

The sample-specific scaling factor was calculated by (Eq. 3):

$$S_k = {}^{T_k}/_{m_e} \quad \text{(Eq. 3)}$$

Where S_k is the sample-specific scaling factor associated with sample *k*, T_k is the number of total reads in the sample *k*, m_e is the median value of total reads for all the samples of the dataset. Reads counts of all samples were then transformed into a percentage of each OTUs.

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de BioProject ID PRJNA551357. The raw data supporting the conclusions of this manuscript will be made available by EC to any qualified researcher.

Statistical Analysis

Statistical Analysis on Microbiological Results

Non-parametric statistical tests were used to compare the classical microbiology result between samples taken on the day of production and at the end of shelf life for a same temperature. With the help of R software (R Core Team, 2016), Kruskal-Wallis test was performed to make a comparison between the food industries on a certain day (i.e., day 0 or day 3) (stats package, kruskal.test function). An Analysis of Covariance (ANCOVA) was also performed to evaluate the interactions between the storage conditions and the food origin on psychrotrophic counts (FactoMineR package, AovSum function). All tests were considered as significant for a *p*-value of < 0.05.

Statistical Analysis on 16S rDNA Results

Alpha diversity for each sample was evaluated by richness estimation (Chao1 estimator), microbial biodiversity (inverse of the Simpson index, coverage), and the population evenness (Simpson evenness) using MOTHUR (version 1.40.5)[3] (Riquelme et al., 2015; Zhao et al., 2015). Rarefaction curves were calculated for all samples to ensure that sequencing depth was sufficient: OTUs identified were plotted as a function of sequences obtained per sample. High diversity coverage was achieved with all curves reaching asymptotes from 3000 reads (**Supplementary Figure S2**). Using Explicet, alpha and beta diversity indices were also calculated with bootstrapped sequencing data[4] (Robertson et al., 2013; Mann et al., 2016).

Beta-diversity was assessed with Explicet using the Bray-Curtis index on a 0-1 scale.

Using STAMP (v2+) software[5], a 2-sided Welch's *t*-test was performed on metagenetic results and confidence intervals were calculated according to the Newcombe-Wilson method. A Principal Component Analysis (PCoA) was also applied to classify and cluster samples according to the identified OTUs for the two packaging (Tukey-Kramer test in conjunction with an ANOVA) (Parlapani et al., 2018). The differences were considered significant for a corrected *p*-value of less than 0.05 (Parks et al., 2014).

RESULTS

Microbiological Analysis

As expected, psychrotrophic and lactic aerobic counts increased during the shelf life with increasing the temperature (**Tables 1**, **2**).

Compared to the TVC values, LAB counts showed highest results for food industries A and D.

At day 0, different microbiological counts were observed between food companies for TVC (Kruskal-Wallis test, $H = 9.43$, p-value = 0.02) and for LAB (Kruskal-Wallis tests, $H = 8.90$, p-value = 0.04). The lowest psychrotrophic populations were observed for food industries D (4.2 ± 0.4 log CFU/g) and A (5.6 ± 0.1 log CFU/g), whereas minced pork meat samples from B to C showed the highest results (7.5 ± 0.4 and 7.2 ± 0.4 log CFU/g, respectively).

At the end of the shelf life, the natural logarithm of the TVC for all food companies was over 7.0 log CFU/g. At this time, the Analysis of Covariance revealed also a significant effect of the food companies (p-value = 0.00000998) and the temperature of storage (p-value = 0.00000095) on microbial total counts. Psychrotrophic counts seems also to be influenced by the interaction of the food industry and the temperature

[3]http://www.mothur.org

[4]http://www.explicet.org

[5]https://www.mybiosoftware.com/stamp-2-0-0-analyze-metagenomic-profiles.html

TABLE 1 | Results of psychrotrophic aerobic counts in minced pork meat samples according to the origin, the food packaging and the temperature of storage.

Industries/packaging	Day 0	End of the shelf life (day 3)			
		2°C	8°C	12°C	2/8°C
FW					
A	5.6 ± 0.1	6.5 ± 0.6	8.3 ± 0.4*	8.3 ± 0.5*	8.3 ± 0.3*
B	7.5 ± 0.4	7.5 ± 0.4	8.3 ± 0.0*	8.3 ± 0.2*	8.3 ± 0.9*
C	7.2 ± 0.4	7.3 ± 0.5	7.8 ± 0.0	7.8 ± 0.2	7.6 ± 1.3
D	4.2 ± 0.4	4.6 ± 0.2	7.2 ± 0.2*	8.3 ± 0.0*	6.6 ± 0.2*
Kruskal-Wallis test	9.43 (0.02)°	8.74 (0.03)°	9.02 (0.03)°	5.71 (0.13)	9.68 (0.02)°
MAP					
A	5.6 ± 0.1	6.5 ± 0.1*	7.9 ± 0.1*	8.3 ± 0.3*	7.9 ± 0.2*
B	7.5 ± 0.4	7.9 ± 0.1	8.3 ± 0.0*	8.3 ± 0.0*	8.3 ± 0.0*
C	7.2 ± 0.4	7.5 ± 0.2	7.6 ± 0.1	8.3 ± 0.1*	7.8 ± 0.6
D	4.2 ± 0.4	5.2 ± 0.3*	7.9 ± 0.1*	8.1 ± 0.1*	7.2 ± 0.1*
Kruskal-Wallis test	9.43 (0.02)°	10.39 (0.02)°	9.68 (0.02)°	3.45 (0.33)	8.94 (0.03)°

*Values given as log CFU/g (mean ± SD, n = 3) at 2, 8, 12, and 2/8°C. FW (food wrap packaging), MAP (modified atmosphere packaging),° significant Kruskal-Wallis value (p < 0.05) with p-value between bracket, *significant Wilcoxon value (p < 0.05).*

TABLE 2 | Results of lactic aerobic counts in minced pork meat samples according to the origin, the food packaging and the temperature of storage.

Industries/packaging	Day 0	End of the shelf life (day 6)			
		2°C	8°C	12°C	2/8°C
FW					
A	5.2 ± 0.2	6.4 ± 0.4	7.8 ± 0.1*	7.8 ± 0.2*	7.4 ± 0.2*
B	5.5 ± 0.6	5.5 ± 0.5	7.1 ± 0.3*	7.9 ± 0.2*	6.8 ± 0.4*
C	5.2 ± 0.7	6.7 ± 0.2*	7.4 ± 0.1*	7.6 ± 0.1*	7.0 ± 0.2*
D	3.5 ± 0.2	4.4 ± 0.3*	5.9 ± 0.4*	7.5 ± 0.1*	5.1 ± 0.3*
Kruskal-Wallis test	8.90 (0.04)°	9.15 (0.03)°	9.67 (0.02)°	7.62 (0.05)	8.44 (0.04)°
MAP					
A	5.2 ± 0.2	7.1 ± 0.2*	8.0 ± 0.18*	8.2 ± 0.09*	8.2 ± 0.09*
B	5.5 ± 0.6	6.6 ± 0.6*	7.8 ± 0.21*	7.7 ± 0.16*	7.8 ± 0.15*
C	5.2 ± 0.7	7.3 ± 0.2*	7.6 ± 0.06*	7.9 ± 0.09*	7.5 ± 0.07*
D	3.5 ± 0.2	5.2 ± 0.4*	7.5 ± 0.07*	7.8 ± 0.03*	6.8 ± 0.24*
Kruskal-Wallis test	8.90 (0.04)°	8.44 (0.04)°	9.05 (0.03)°	8.27 (0.04)°	9.45 (0.02)°

*Values given as log CFU/g (mean ± SD, n = 3) at 2, 8, 12, and 2/8°C. FW (food wrap packaging), MAP (modified atmosphere packaging).° Significant Kruskal-Wallis value (p < 0.05) with p-value between bracket, *significant t-student value (p < 0.05).*

(*p*-value = 000442), but not by others interactions terms (*p*-value > 0.05).

Carbon Dioxygen Production

As shown in **Figure 1**, carbon dioxygen values increased with highest temperatures, except for the food companies C and D which shown relatively stable measurements. Results at 2/8°C are not shown in this paper.

Alpha Diversity of Bacteria With 16S rDNA Amplicon Sequencing

Over 4,200 reads per sample were generated with pyrosequencing. In total, 48 mains OTUs were assigned. The number of OTUs, the bacterial diversity, richness estimators and coverage are presented in **Supplementary Tables S1–S3**). The highest number of identified species was encountered for the food industries C and D.

Bacterial Communities at the Family and Genus Levels

The relative abundance results obtained by metagenetics analysis (expressed in%) in FW and MAP packaging at Family (**Figure 2**) and Genus (**Figure 3**) levels (>5%) are represented in cumulated histograms for all samples. These data including the relative abundance of sequences are also summarized in **Supplementary Tables S4–S6**). The taxa representing <5% in relative abundance were merged in the category of "Others." "Others" in FW are mainly composed by the genera *Bacillus*, *Carnobacterium*, *Enterococcus*, *Hafnia*, *Myroides*, *Rahnella*, *Staphylococcus*, *Serratia*, *Streptococcus*, *Weissella* and *Xanthomonas* in FW. While it concerns *Bacillus*, *Carnobacterium*, *Enterococcus*, *Hafnia*,

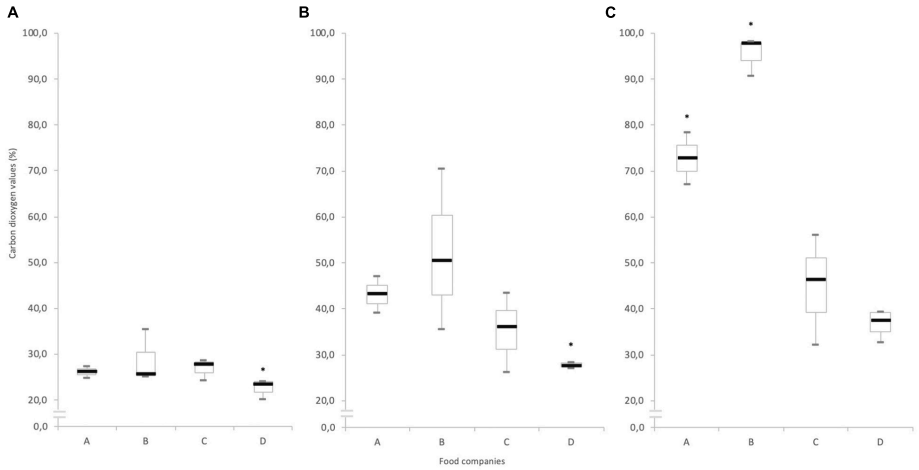

FIGURE 1 | Box plots show the carbon dioxide measurements at the end of the shelf life, for the four food companies (A–D) at **(A)** 2°C, **(B)** 8°C, and **(C)** 12°C. The boxes represent the interquartile range between the first (Q1) and the third (Q3) quartiles; the vertical black line insides the box is the median obtained from the three batches analyzed by food industries; the two dotted line is the difference of 25% below the Q1 or above the Q3. The presence of stars indicated that samples deviated significantly from the carbon dioxide value at day 0 (30.0 ± 0.1%).

Rahnella, *Staphylococcus*, *Streptococcus* and *Xanthomonas* in MAP. Full data on taxa found in high (>5%) and low (<5%) frequencies will be made available by EC to any qualified researcher.

According to **Figures 2**, **3**, the food companies show a high variability in the distribution of read percentages at day 0. At this time, the genus *Photobacterium* is the most represented for A and C (86.7 and 19.9%, respectively), while it concerns the genus *Pseudomonas* for the industries B and D (38.7 and 25.7%, respectively).

At the end of the shelf life, a total of 12 genera were identified as dominant (taxa representing more than 5% in relative abundance) in MAP and only seven genera in FW. These seven genera are all identical to those found in MAP.

For all samples, the percentage of "unassigned" reads was relatively low (7.1 ± 3.7).

Effect of the Food Packaging on the Bacterial Communities

However, although dominant genera were identified across all samples, the two different types of packaging were characterized by different microbiota, with only some genera in common (**Supplementary Figure S3**). At the end of the shelf life, *Pseudomonas* was more present in FW and this genus was potentially replaced by *Brochothrix* in the MAP packaging (Welch's *t*-test, *p*-value < 0.05) (**Figure 4**).

At this time, the major OTUs groups (**Figure 5**) are therefore different according to the food packaging: *Brochothrix thermosphacta*, *Lactobacillus algidus*, *Photobacterium kishitanii*, *Photobacterium phosphoreum*, *Pseudomonas psychrophila*, and *Pseudomonas* sp. are dominant in FW. While it concerns *Acinetobacter* sp., *Brochothrix thermosphacta*, *Lactobacillus algidus*, *Lactococcus piscium*, *Leuconostoc inhae*, *Leuconostoc gelidum*, *Leuconostoc* sp., *Photobacterium kishitanii*, *Photobacterium phosphoreum*, and *Pseudomonas* sp. in MAP.

Variability of the Minced Pork Meat Ecosystem Between Samples

Genus relative abundance shows a high Bray-Curtis dissimilarity during the storage, and between the food companies and batches (**Figure 6**).

At day 0, samples showed a high dissimilarity (>70%) with the metadata groupings at the end of the shelf life. At this time, the food company A seems not to shared OTUs in common with the three others food industries.

At the end of the shelf life, Bray-Curtis index seems indicating that a relative similarity exists for OTUs contained within food companies A and C, and within B and D. This index also indicates a relative similarity concerning the temperature of storage, except for the industry D.

A synthetic view about the Bray-Curtis index between samples according to the food origin and storage condition is summarized in **Table 3**.

DISCUSSION

In this study, we investigated the microbial spoilage community and dynamics of minced pork meat samples, among different

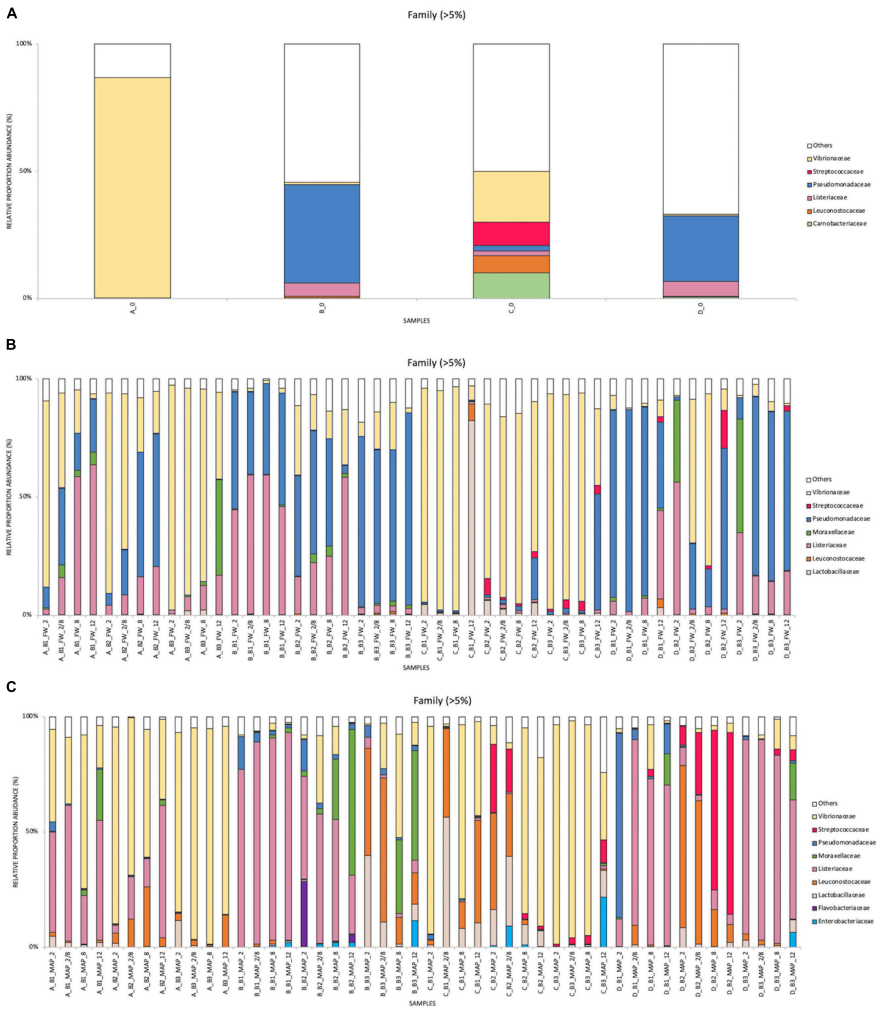

FIGURE 2 | Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at Family levels, during cold storage of minced pork meat in relation to the food packaging and the origin of samples (food companies and batches). **(A)** food samples analyzed at day 0 for the four companies (A–D), **(B)** storage in FW (food wrap) packaging, **(C)** storage in MAP (modified atmosphere) packaging. At Family levels, the taxa representing <5% in relative abundance were merged in the category of "Others". Legend: batch 1 (B1), batch 2 (B2), batch 3 (B3), at 2°C (2), at 8°C (8), at 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

conditions of production and food storage, using both 16S rRNA gene sequencing and classical microbiology. Indeed, whereas the dynamics of the bacterial community of meat and meat products have been studied before, Stoops et al. (2015) reported that little is known about differences in microbial changes during storage, and among the variability of batches production. Meat and meat products are highly perishable, with colonization and development of a great variety of microorganisms (Nychas et al., 2008; Pennacchia et al., 2009; Chaillou et al., 2015; Stellato et al., 2016; Garnier et al., 2017). The product composition (low/high pH, low/high concentration of glucose, water activity, . . .) and the storage conditions (temperature of storage and packaging

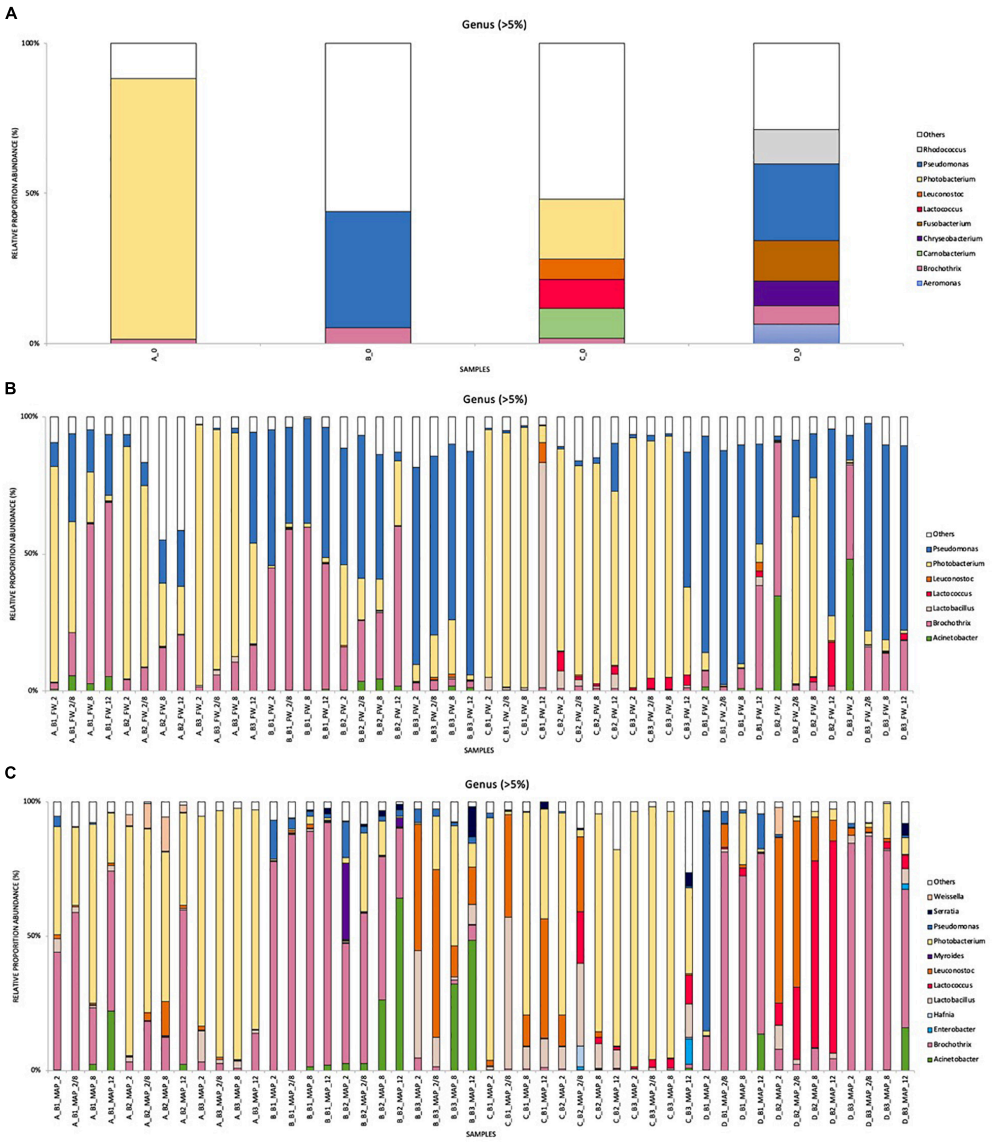

FIGURE 3 | Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at Genus levels, during cold storage of minced pork meat in relation to the food packaging and the origin of samples (food companies and batches). **(A)** food samples analyzed at day 0 for the four companies (A–D), **(B)** storage in FW (food wrap) packaging, **(C)** storage in MAP (modified atmosphere) packaging. At Genus levels, the taxa representing <5% in relative abundance were merged in the category of "Others". These data including the relative abundance of sequences are also summarized in **Supplementary Tables S4–S6**. Legend: batch 1 (B1), batch 2 (B2), batch 3 (B3), at 2°C (2), at 8°C (8), at 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

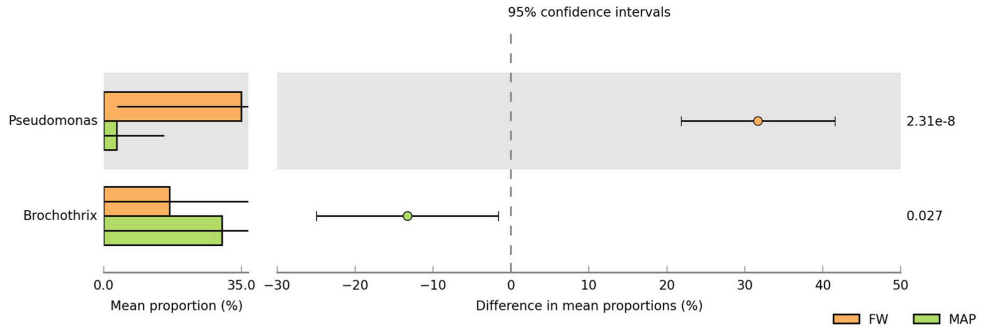

FIGURE 4 | Extended bar plot showing the bacterial populations whose mean relative abundance differed between food wrap (FW) and modified atmosphere (MAP) packaging at genus scale. The relative abundance and the difference in mean proportions are illustrated for the statistically different taxa ($p < 0.05$).

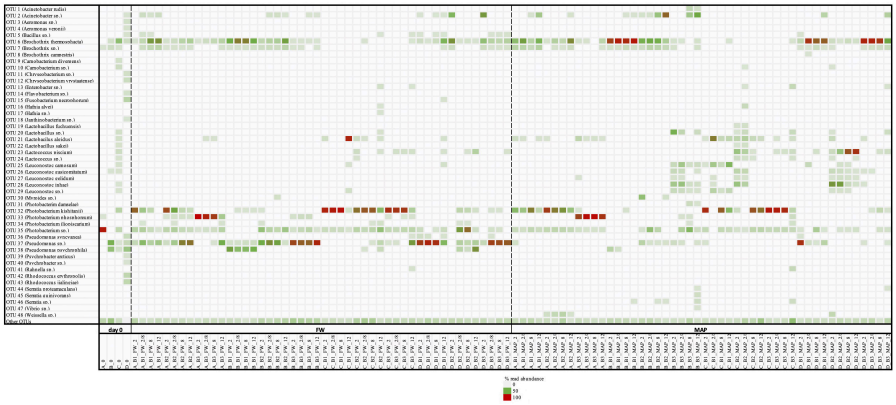

FIGURE 5 | Heatmap of relative read abundance at species level for all samples (expressed in %) among the different storage conditions. Only the most abundant OTUs obtained in this study are specially indicated (> 1%). Others OTUs are gathered in "Others OTUs." Legend: food companies (A–D), with three batches each (B1, B2, B3), analyzed at the first (0) and the last day of storage, in food wrap (FW) and modified atmosphere (MAP) packaging. Temperature of storage: 2°C (2), 8°C (8), 12°C (12), and for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C (2/8).

conditions for example) may favor growth of microorganisms, that are responsible for the formation of spoilage (Argyri et al., 2015; Reid et al., 2017). This can lead to visible growth (slime, colonies), as textural changes, off-odors or off-flavors (Casaburi et al., 2014; Chaillou et al., 2015; Stoops et al., 2015; Del Blanco et al., 2017). In this context, minced meat is a potentially hazardous food product, vulnerable to bacterial spoilage, with a very short shelf life (Geeraerts et al., 2017) due to abundant and diverse substrate for bacterial growth and favorable growth conditions (Benson et al., 2014). In our study, the minced pork meat samples present a high water activity and a near-neutral pH which are in accordance with previous studies on this food matrix (Blixt and Borch, 2002; Andritsos et al., 2012).

The initial contamination of products, and also the initial level of lactic acid bacteria, is also a key factor that can influence the spoilage dynamic during storage (De Filippis et al., 2013). In our results, the microbial counts of the four manufacturers were quite different and psychrotrophic counts were higher for two food industries (**Tables 1**, **2**). High levels of initial contamination in minced pork meat samples were also observed by Peruzy et al. (2019). This difference of the initial bacterial contamination is not in relation with the size of the company. These results can be explained by the fact that multiple sources of contamination can contribute to the initial composition of the meat microbiota (De Filippis et al., 2013), such as at the farm (hygiene practices, the conditions of animal transport, etc.) and at the

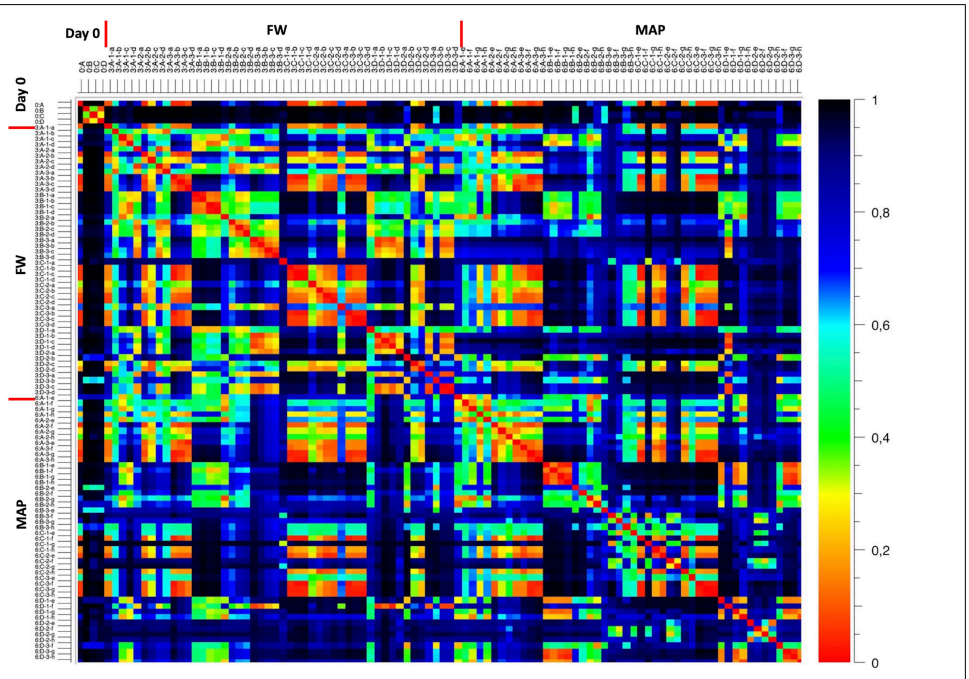

FIGURE 6 | Global microbial dissimilarity obtained by metagenetics between samples for different conditions of storage. The heatmap shows the Bray-Curtis dissimilarity measure based on relative abundance of OTUS (genus scale). Values are given in dissimilarity counts (1 = 100% dissimilar, 0 = 0% dissimilar). Legend: analysis at day 0 (0), at day 3 (3) and at day 6 (6); food companies (A–D); three batches each (1, 2, 3); in food wrap (FW) and in modified atmosphere (MAP) packaging. Temperature of storage: 2°C in FW (A), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C in FW (B), 8°C in FW (C), 12°C in FW (D), 2°C in MAP (E), for a third of the shelf life at 2°C and for the rest of the shelf life at 8°C in MAP (F), 8°C in MAP (G), 12°C in MAP (H).

slaughterhouse (automatic level of the process, cleaning practices, etc.). Initial carcass contamination can be also environmental, with contamination by tools, machines, and surfaces of slaughter equipment (Mann et al., 2016; Moretro et al., 2016). In addition, subsequent handling of meat in the operations of slicing, sectioning, portioning, and transferring in packages can determine further contamination in the handling points (Del Blanco et al., 2017).

The bacterial count at the end of the shelf life was over 7.0 log CFU/g, indicating that meat had probably begun to be deteriorated and would not be suitable for human consumption (Zhao et al., 2015). Indeed, it is generally recognized that microbial spoilage of meat occurs when counts reach arbitrary level between 7.0 log CFU/g (Nychas et al., 2008; Pothakos et al., 2014; Stoops et al., 2015; Reid et al., 2017; Spanu et al., 2018) and 8.00 log CFU/g (Nychas et al., 2008; Fall et al., 2012; Pothakos et al., 2014; Chaillou et al., 2015; Reid et al., 2017). However, these values are only indicative and refer here to the total viable count. Food spoilage needs to be assessed to the genus-species level, because potentially protective bacteria can also occur in food products.

As discussed by Del Blanco et al. (2017), common approaches for delaying meat spoilage and improving meat shelf life are available, including good hygienic practices and all the storage conditions. Among these, low storage temperatures and adequate packaging are considered as the most important factors (Koutsoumanis et al., 2006; Andritsos et al., 2012; Kaur et al., 2017). During the storage at 2°C, the arbitrary level of 7.0 log CFU/g was sometimes not reached. In addition, it can be observed that the microbial kinetics from 2 to 8°C were quite similar to those at 8°C, as described by Cauchie et al. (2017).

In relation with the food packaging, the most common used in meat and meat products are vacuum packaging and modified atmosphere packaging (MAP) (Caryé et al., 2005; Koutsoumanis et al., 2008; Dalcanton et al., 2013; Chaix et al., 2015; Silbande et al., 2016). In this study, a food wrap (FW) and a MAP (30% CO_2 – 70% O_2) packaging are used. The composition of modified atmosphere systems can be an effective way to reduce the growth rate of spoilage aerobic organisms and modify the microbial ecology of the product. But their effectiveness strongly depends on the initial microbial contamination of raw materials, storage temperature, film permeability and the

TABLE 3 | Dominant bacteria represented in minced pork meat samples according to storage conditions.

Food companies	First day of storage	Last day of storage		
		Batch	FW	MAP
A	*Photobacterium* sp., *Ph. phosphoreum*	1	*Brochothrix thermosphacta, Photobacterium kishitanii, Pseudomonas* sp.	*B. thermosphacta, Ph. kishitanii*
		2	*B. thermosphacta, Ph. kishitanii, Pseudomonas* sp.	*B. thermosphacta, Ph. kishitanii, Weissella* sp.
		3	*Ph. phosphoreum, Pseudomonas* sp.	*Ph. phosphoreum*
B	*Pseudomonas* sp., *Ps. psychrophila*	1	*B. thermosphacta, Ps. psychrophila*	*B. thermosphacta, Ps. psychrophila*
		2	*B. thermosphacta, Photobacterium* sp., *Pseudomonas* sp.	*Acinetobacter* sp., *B. thermosphacta, Photobacterium* sp.
		3	*Ph. kishitanii, Ph. phosphoreum, Pseudomonas* sp.	*Acinetobacter* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Ln. gelidum, Photobacterium* sp., *Ph. kishitanii*
C	*Photobacterium* sp., *Ph. kishitanii*	1	*Lactobacillus algidus, Ph. kishitanii*	*Lb. algidus, Ln. carnosum, Ln. inhae, Ph. kishitanii*
		2	*Photobacterium* sp., *Ph. kishitanii, Pseudomonas* sp., *Ps. phychrophila*	*Lb. algidus, Lactococcus piscium, Ln. inhae, Ph. kishitanii*
		3	*Ph. kishitanii, Pseudomonas* sp.	*Ph. kishitanii*
D	*Pseudomonas* sp., *Ps. psychrophila*, *Ps. syncyanea*	1	*B. thermosphacta, Pseudomonas* sp.,	*B. thermosphacta, Photobacterium* sp., *Pseudomonas* sp.
		2	*Acinetobacter* sp., *B. thermosphacta, Photobacterium* sp., *Ps. psychrophila*	*B. thermosphacta, Lc. piscium, Ln. gelidum, Ln. inhae*
		3	*Acinetobacter* sp., *Brochothrix* sp., *B. thermosphacta, Pseudomonas* sp.,	*B. thermosphacta, Ph. kishitanii*

At species level, the taxa representing <20% in relative abundance were not considered as dominant in this table. FW (food wrap packaging), MAP (modified atmosphere packaging).

carbon dioxide concentration used (20–40% is commonly used to suppress microbial growth) (Simpson and Carevic, 2004; Rotabakk et al., 2006; Stoops et al., 2015; Guillard et al., 2016; Saraiva et al., 2016; Couvert et al., 2017). The carbon dioxide concentration was here theoretically sufficient to limit the microbial growth. However, the higher percentage of oxygen can also enhance the growth of aerobic microbial communities in our samples. Moreover, some bacteria are able to grow in variable food packaging, as *Photobacterium* which is CO_2-tolerant (Dalgaard, 1995; Fuertez-Perez et al., 2019). Also, in accordance with Stoops et al. (2015), it can be observed a significant production of carbon dioxide. This production may

be the reflect of the development of bacterial groups belonging to lactic acid bacteria, *Brochothrix* or *Enterobacteriaceae* (Caryé et al., 2005). As environment of slaughtering and processing steps (Stellato et al., 2016), packaging materials can also be a source of contamination because they are not sterile in study. Further studies based on microbial contamination of food trays would also be interesting.

According to this, and based on the study by Stoops et al. (2015), viable counts are not suitable to characterize the microbial diversity of food products and to investigate thoroughly shifts in the bacterial communities during storage. Indeed, culture-dependent techniques largely underestimated the species richness and abundance. For a more detailed characterization of microbial communities in samples, originating from different

TABLE 4 | Examples of some microbial species occurring during chilled storage of meat and their potential spoilage effects.

Bacteria	Growth conditions	Spoilage effects	References
Actinetobacter spp.	Especially present in dairy and seafood products.	Low spoilage potential but can enhanced the growth of other spoilage bacteria by means of quorum sensing.	Pinu, 2016; Ghasemi-Varnamkhasti et al., 2018; Odeyemi et al., 2018; Hahne et al., 2019
Brochothrix spp.	In different gas composition, such as under air, modified atmosphere and vacuum-packaging. More tolerant in oxygen-depleted and CO2-enriched environments.	Sour, acid and cheesy odor.	Koutsoumanis et al., 2008; Nychas et al., 2008; Ercolini et al., 2011; Doulgeraki et al., 2012; Zhao et al., 2015; Mann et al., 2016; Del Blanco et al., 2017; Reid et al., 2017; Mansur et al., 2019
Carnobacterium spp.	In all types of packaging conditions. Predominance in low O_2 packaging.	Spoilage effect can vary, producing volatile molecules with low sensory impacts (fruity or fermented odors, . . .)	Casaburi et al., 2011; Doulgeraki et al., 2012; Pothakos et al., 2015
Lactobacillus spp. (*Lb.* sakei, *Lb. fuchuensis*, *Lb. plantarum*, *Lb. curvatus*, *Lb. algidus*, *Lb. oligofermentans*, . . .)	In all types of packaging conditions. Predominance with high concentration of CO_2.	Severe acidification, emission of off-odor compounds and ropy slime. However, lactic acid bacteria may produce lactic acid, which inhibits the growth of other families of bacteria. And some species can produce bacteriocins.	Kato et al., 2000; Fadda et al., 2010; Doulgeraki et al., 2012; Dalcanton et al., 2013; Nieminen et al., 2015; Pothakos et al., 2015; Zhao et al., 2015; Alvarez-Sieiro et al., 2016; Mann et al., 2016; Woraprayote et al., 2016; Stefanovic et al., 2017; Mansur et al., 2019
Lactococcus spp.	In various types of packaging.	Traditionally they have not been considered as spoilage microorganisms, but the spoilage potential of these bacteria is still scarcely known.	Kato et al., 2000; Doulgeraki et al., 2012; Rahkila et al., 2012; Dalcanton et al., 2013; Pothakos et al., 2014; Zhao et al., 2015; Mann et al., 2016; Mansur et al., 2019
Leuconostoc spp. (*Ln. gelidum*, *Ln. carnosum*, *Ln. mesenteroides*, . . .)	Under aerobic, vacuum and modified atmosphere packaging. Predominance with high concentration of O_2.	Buttery aroma, formation of slime, blowing of packages, green discoloration.	Kato et al., 2000; Doulgeraki et al., 2012; Dalcanton et al., 2013; Nieminen et al., 2015; Pothakos et al., 2015; Zhao et al., 2015; Mann et al., 2016; Mansur et al., 2019
Photobacterium spp.	Under air, vacuum and modified atmosphere packaging. More frequently present in seafood products.	Typically not associated with spoilage of meat. Responsible for reducing TMAO to TMA, off-odor (produce volatile organic compounds) and biogenic amine formation. The mechanism underlying spoilage has not been clarified.	Nieminen et al., 2016; Li et al., 2019
Pseudomonas spp.	In different gas composition, such as under air, modified atmosphere and vacuum-packaging. Predominance under aerobic low temperature. Limitation in the bacterial flora by the presence of CO_2 and/or the limitation of O_2 in MAP packaging.	Slime, discoloration, off-odor producing.	Koutsoumanis et al., 2008; Nychas et al., 2008; Ercolini et al., 2011; Andritsos et al., 2012; Doulgeraki et al., 2012; Zhao et al., 2015; Mann et al., 2016; Del Blanco et al., 2017; Reid et al., 2017; Liu et al., 2018; Spanu et al., 2018; Mansur et al., 2019
Weissella spp.	Some can be found in salted and fermented foods. Present in vacuum packaging.	Greenish appearance. Can plays an important role in the fermentation process. Some species can produce bacteriocins.	Pothakos et al., 2015; Martins et al., 2016; Kim et al., 2017; Kariyawasam et al., 2019

ecological niches, a sequence-based approach was used, allowing identification of OTUs at various taxonomic levels (species, genus or family levels) (Stoops et al., 2015). However, without extensive studies involving a large number of samples under different storage conditions it will not be possible to determine exactly the bacterial ecosystem and the role of individual spoilage species (Pennacchia et al., 2011; Rouger et al., 2018). According to this, we analyzed minced meat samples from four different food companies, with three different batches per industries. In addition to previous studies based on the microbial description of minced meat samples (Stoops et al., 2015; Peruzy et al., 2019), our study aims to understand and monitor microbial dynamics and variability between food companies and food batches, according to different storage conditions.

In our results, the observed microbial diversity was relatively high, and the most abundant bacteria differ among samples. As observed by Stoops et al. (2015) in minced meat samples, an increase of microbial counts is coinciding with a decrease in bacterial diversity during storage. At the end of the storage period, the major genus taxa are represented by *Pseudomonas* in FW and *Brochothrix* in MAP. But it can also be observed a high diversity between food companies and batches (**Table 3**). Our results are in accordance with Peruzy et al. (2019), which also observed a dominance of the genus *Pseudomonas*, *Brochothrix*, and *Carnobacterium* in minced pork meat samples. Moreover, these results are not surprising because the microbial populations of refrigerated meat and pork-meat products are mainly composed by *Pseudomonas* spp., cold tolerant *Enterobaceriaceae*, lactic acid bacteria (such as *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Carnobacterium* spp., etc.), *Brochothrix thermosphacta*, *Clostridium* spp. (Koort et al., 2005; Liu et al., 2006; Nychas et al., 2008; Pennacchia et al., 2009, 2011; Casaburi et al., 2014; Stellato et al., 2016; Del Blanco et al., 2017; Geeraerts et al., 2017) and *Weissella* spp. (Pothakos et al., 2014; Stellato et al., 2016). Other genera isolated frequently from fresh pork meats are *Acinetobacter* spp., *Aeromonas* spp., *Enterococcus* spp., and *Moraxella* spp. (Zhao et al., 2015; Mann et al., 2016). However, these results are not completely in accordance with Stoops et al. (2015) because this study mentioned that *Lactobacillus algidus* and *Leuconostoc* sp. became the dominant bacteria in minced meat samples stored at 5°C under modified atmosphere (66% O2, 25% CO2, and 9% N2). These differences can be explained by different meat compositions (beef in the study by Stoops et al. (2015) and pork in our study), the initial contamination of samples, and the gas mixture used.

The results also showed the interest of using culture-independent method to better understand the changes of food microbiota over time, and in each food companies, according to the storage conditions. Indeed, metagenetics approach produce a large amount of data in a very short time (Cocolin et al., 2018; Den Besten et al., 2018), allowing to interpret and use these data to help agri-food companies in their decisions regarding food safety and quality decisions. Moreover, all the OTUs-species described as potentially spoilers in our study are well described in the literature (**Table 4**), and in minced pork meat samples (Stoops et al., 2015; Peruzy et al., 2019). The bacterial species present in our samples are also able to grow in meat matrices, and they are potentially responsible of spoilage effects, which can affect color, flavor, visual aspect, etc. (Pothakos et al., 2015). Sensory analyses would be interesting in this context, but were not performed in this study. Moreover, the enzymatic decarboxylation of amino acids, or the transamination of aldehydes and ketones, by bacteria results in the formation and accumulation of biogenic amines (BAs) (Jastrzębska et al., 2016). Biogenic amines (e.g., b-phenylethylamine, cadaverine, histamine, putrescrine, spermidine, spermine, tyramine and tryptamine) are reported in various foods including meat, fish, cheese, and wine (Papageorgiou et al., 2018). They can have health implications, such as allergic reactions, but also contribute to spoilage due to their putrid aroma (Stanborough et al., 2017). Therefore, as proposed by Cheng et al. (2016), the sum of BAs could be used as an indicator of pork meat quality and freshness during storage. Li et al. (2014) also showed that some BAs could be used as spoilage indicators of chilled pork.

However, it is important to add that some bacteria can be considered as protective, such as some lactic acid bacteria. As mentioned by Singh (2018), the presence of high LAB communities does not necessarily result in quality defect, and their intra-species variation to cause spoilage has already been recognized (Pothakos et al., 2015).

In the present study, we designed a method to collect MPM samples in order to explore the bacterial communities and diversity among different food origin and storage conditions. Indeed, the modification of the composition of the spoilage flora during storage is an important factor in assessing food quality (Holm et al., 2013). Although the bacteria consistently dominated the microbiota of MPM samples are known, results indicated that bacterial diversity needs to be addressed on the level of food companies and batches variations. As discussed by Rouger et al. (2017), it is important to overcome variability to better understand the factors underlying the diversity of spoilage bacterial communities, by (i) defining reproducible and reliable experimental conditions to lead to biological interpretation, or (ii) to multiplying sampling or experiments to obtain statistical significance of the results (Chaillou et al., 2015; Rouger et al., 2017). A seasonal effect on the microbial quality of minced meat has also been reported by Andritsos et al. (2012). In this paper, no conclusions about bacterial ecosystems for others food companies, or for different times of the year, should be dawn. Further data are so needed to determine diversity of spoilage microbiota in minced pork meat samples, according to others food industries, sampling periods and storage conditions. Also, a comparative evaluation of spoilage-related bacterial species and metabolic profiles, with growth parameters of these potentially spoilage bacteria in samples, will be studied in another study.

In conclusion, the combination of both culture-dependent and culture-independent analyses enabled us to explore the microbial communities of minced pork meat samples under different food origin and storage conditions, as previously described by Stoops et al. (2015). In our study, microbial changes during storage were monitored, according to a sampling in four food companies and for several batches. In accordance with previous

studies we found that *Pseudomonas* and *Brochothrix* dominate the community at the end of the shelf life in FW and MAP, respectively, together with *Photobacterium*. The major OTUs groups are also often associated with pork meat spoilage in the scientific literature. And these results are also in accordance with studies conducted on the microbiota of minced meat by Stoops et al. (2015) and Peruzy et al. (2019). Psychrophilic spoilers dominated the microbiota of our samples, but each sample harbored a unique pork meat microbiota, depending on the manufacturing batch and the packaging used. The gas mixture and the temperature condition used in this study are probably the most important factors implied to the dynamics of the bacterial community. Further researches on the main contamination during slaughter production process, such as importance of processing environment, procedures and storage conditions, are desirable to provide a complete assessment of the microbiome of minced meat and to limit incidents of unexpected spoilage.

DATA AVAILABILITY STATEMENT

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under the BioProject ID PRJNA551357.

AUTHOR CONTRIBUTIONS

EC did the experiments, interpreted the results and wrote the manuscript. LD performed the experiments, supervised analyses and revised the manuscript. BT, PF, FF, GB, and GD were involved in the design of the study and provided help for interpretation of the results. AT and SB participated to the experiments. NK participated to the design of the study, interpretation of the results and writing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03074/full#supplementary-material

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Conflict of Interest: PF and SB (Quality Partner sa, Liège, Belgium) were employed by the Department of Food Sciences (Faculty of Veterinary Medicine, University of Liège, Liège, Belgium) to perform 16S rRNA gene amplicon sequencing.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annexe 3

frontiers in Microbiology

ORIGINAL RESEARCH
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Modeling the Growth and Interaction Between *Brochothrix thermosphacta*, *Pseudomonas* spp., and *Leuconostoc gelidum* in Minced Pork Samples

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The aim of this study was to obtain the growth parameters of specific spoilage micro-organisms previously isolated in minced pork (MP) samples and to develop a three-spoilage species interaction model under different storage conditions. Naturally contaminated samples were used to validate this approach by considering the effect of the food microbiota. Three groups of bacteria were inoculated on irradiated samples, in mono- and in co-culture experiments (n = 1152): *Brochothrix thermosphacta*, *Leuconostoc gelidum*, and *Pseudomonas* spp. (*Pseudomonas fluorescens* and *Pseudomonas fragi*). Samples were stored in two food packaging [food wrap and modified atmosphere packaging (CO_2 30%/O_2 70%)] at three isothermal conditions (4, 8, and 12°C). Analysis was carried out by using both 16S rRNA gene amplicon sequencing and classical microbiology in order to estimate bacterial counts during the storage period. Growth parameters were obtained by fitting primary (Baranyi) and secondary (square root) models. The food packaging shows the highest impact on bacterial growth rates, which in turn have the strongest influence on the shelf life of food products. Based on these results, a three-spoilage species interaction model was developed by using the modified Jameson-effect model and the Lotka Volterra (prey–predator) model. The modified Jameson-effect model showed slightly better performances, with 40–86% out of the observed counts falling into the Acceptable Simulation Zone (ASZ). It only concerns 14–48% for the prey–predator approach. These results can be explained by the fact that the dynamics of experimental and validation datasets seems to follow a Jameson behavior. On the other hand, the Lotka Volterra model is based on complex interaction factors, which are included in highly variable intervals. More datasets are probably needed to obtained reliable factors, and so better model fittings, especially for three- or more-spoilage species interaction models. Further studies are also needed to better understand the interaction of spoilage bacteria between them and in the presence of natural microbiota.

Keywords: predictive microbiology, growth parameters, interaction models, *Brochothrix thermosphacta*, *Pseudomonas* spp., *Leuconostoc gelidum*, Jameson-effect model, Lotka Volterra model

INTRODUCTION

During production and distribution steps, spoilage of meat and meat products may occur, rendering them unacceptable for human food consumption. Spoilage is mainly caused by microbial growth, which triggers alterations in the sensorial qualities of the product, with off-odor and off-flavor, discoloration, texture changes, etc. (Kreyenschmidt et al., 2010; Dalcanton et al., 2013; Pinter et al., 2014; Cauchie et al., 2017; Den Besten et al., 2017; Torngren et al., 2018). It is well known that the initial bacterial counts on meat and meat products is highly variable (Benson et al., 2014), but several studies have established that only a dominant fraction of the microbiota, designated as specific spoilage organisms (SSOs), contributes to spoilage (Nychas et al., 2008; Kreyenschmidt et al., 2010; Pennacchia et al., 2011; Benson et al., 2014; Zotta et al., 2019). In this context, predictive microbiology can be a helpful tool because the prediction of microbial growth, especially SSOs, enables food industries to optimize their production and storage managements, and thus reduce their economic losses (Kreyenschmidt et al., 2010; Fakruddin et al., 2012; Li et al., 2017; Tamplin, 2018).

During the last years, several models have been developed to predict the growth of SSOs in meat and meat products (Liu et al., 2006; Mataragas et al., 2006; Koutsoumanis, 2009; Kreyenschmidt et al., 2010; Dalcanton et al., 2013; Mejlholm and Dalgaard, 2013). But the majority of the developed models are based on the growth of two bacterial species in a food matrix (Vereecken et al., 2000; Giuffrida et al., 2007), most often to study the interaction between spoilage and pathogenic bacteria (Lebert et al., 2000; Mejlholm and Dalgaard, 2007; Giuffrida et al., 2009; Cornu et al., 2011; Ye et al., 2014; Correia Peres Costa et al., 2019; Pedrozo et al., 2019). Moreover, these models often describe the growth of the SSOs depending on the storage temperature (Dominguez and Schaffner, 2007; Gospavic et al., 2008; Kreyenschmidt et al., 2010; Psomas et al., 2011; Longhi et al., 2013; Antunes-Rohling et al., 2019) or the packaging conditions (Devlieghere et al., 1999; Chaix et al., 2015; Guillard et al., 2016; Couvert et al., 2019; Kapetanakou et al., 2019), but do not always consider the interaction of these storage conditions for the growth of spoilage bacteria (Rosso et al., 1995; Augustin and Carlier, 2000; Le Marc et al., 2002; Pinon et al., 2004; Dalcanton et al., 2018; Kakagianni et al., 2018; Nyhan et al., 2018; Correia Peres Costa et al., 2019).

As mentioned by Correia Peres Costa et al. (2019): "interaction models are usually intended to quantify how much the growth of one population is reduced by the growth of other populations." In this context, two model approaches are generally used to describe the microbial interaction: (i) those based on the modified Jameson-effect phenomenon (Jameson, 1962; Cornu et al., 2011; Ye et al., 2014; Cauchie et al., 2017; Correia Peres Costa et al., 2019), and (ii) those based on the predator-prey models (Lotka Volterra equation) (Dens et al., 1999; Berlow et al., 2004; Powell et al., 2004; Giuffrida et al., 2007; Mounier et al., 2008; Cornu et al., 2011; Ye et al., 2014; Correia Peres Costa et al., 2019).

As described by Cornu et al. (2011), the Jameson-effect model assumes that: "(i) many microbial interactions in foods limit the maximum population density, without any significant effect on the lag time, and (ii) the growth of the minority population is only partly inhibited after the majority population count has reached its stationary phase [maximum critical population, *MCP*, expressed in log colony forming units (CFU)/g]." The modified Jameson-effect model makes the hypothesis that there is one single inhibition function for both populations; hence, both populations are similarly inhibited by the same limiting resource, the same waste products, and/or by change in pH (Cornu et al., 2011). Recently, Quinto et al. (2018) have developed a three-strain model based on the modified Jameson-effect equation for inoculated spoilage and pathogenic bacteria in a reconstituted sterile skimmed milk. This study considers the effect of two bacteria, *Pseudomonas fluorescens* and *Listeria innocua*, on the bacterial growth of *Listeria monocytogenes*. But the effect of the natural food microbiota on the growth of specific spoilage bacteria needs to be studied (Rouger et al., 2017) in order to predict bacterial growth resulting from several interactions between three or more spoilage species (Ye et al., 2014). This approach needs to be studied.

The Lotka Volterra model can be considered as a prey-predator model that includes competition for a common substrate (Cornu et al., 2011). As cited by Chauvet et al. (2002), the Lotka Volterra model for a three-species food chain approach can be considered as: "the lowest-level prey x is preyed upon by a mid-level species y, which, in turn, is preyed upon by a top-level predator z." However, this hypothesis cannot always be applied in food matrix. Indeed, the growth of a bacterium (B_A) presents simultaneously with other bacteria in a food matrix (B_B and B_C) can be affected by three different ways: (i) B_A growth with a reduced growth rate after that B_B and B_C reach their maximal population densities (N_{max}, expressed in log CFU/g), (ii) B_A stops growing when B_B and B_C reach their N_{max}, and (iii) B_A declines when B_B and B_C reach their N_{max} (Cauchie et al., 2017; Correia Peres Costa et al., 2019). It could be so interesting to develop a Lotka Volterra model for a three-species approach, by considering the effect of the natural food microbiota for the growth of specific spoilage bacteria. Also, this approach is, to the best knowledge of the authors, not available in the literature.

Based on these, the objectives of the present study were (i) to obtain the growth parameters of three specific spoilage micro-organisms previously isolated in minced pork (MP) samples, according to different storage conditions, (ii) to develop a three-spoilage species interaction model based on available models, under food wrap and modified atmosphere packaging, at isothermal conditions, and (iii) to validate this approach with naturally contaminated food samples stored under different storage conditions.

MATERIALS AND METHODS

Sampling

Fresh MP samples were obtained from a local Belgian manufacturer at the day of the production, corresponding to the day of slaughtering. MP samples were packed by the manufacturer in a polypropylene tray under cling film (high film permeability).

According to the recipe, MP is composed of 100% pork mince (70% lean, 30% fat), no salt, no spices, no additives, no eggs, and no sugar are added.

At the day of the production, the water activity of the product was 0.98 ± 0.02 and the pH value was 5.80 ± 0.05 (n = 12). pH of the homogenized samples (5 g in 45 mL of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne). The water activity was measured for homogenized samples on the basis of the relative humidity measurement of the air balance in the micro enclosure at 25 ± 0.4°C (Thermoconstanter TH200, Novasina, Switzerland).

Food samples were then stored at −20°C and irradiated by gamma irradiation (17.5 ± 0.4 kGy) at the same temperature (Sterigenics, Fleurus, Belgium) to limit the adverse effects of irradiation at this dose (Kim et al., 2002; Ham et al., 2017; Wang et al., 2018).

Bacterial Strains

As described in the study of Cauchie et al. (2019), three specific spoilage micro-organisms were previously isolated from different batches of naturally contaminated Belgian MP samples at the end of their use-by date. Samples were stored under two packaging (under air and modified atmosphere—30% CO_2–70% O_2) and three temperature conditions (4, 8, and 12°C). These predominant strains, represented more than 50% of the natural microbiota, were identified by 16S rRNA sequencing and used for experiments: *Brochothrix thermosphacta* (MM008), *Leuconostoc gelidum* (MM045) *Pseudomonas* spp. (*P. fluorescens* MM026 and *Pseudomonas fragi* MM014). *P. fluorescens* and *P. fragi* were used together because experiments were carried out in an exploratory approach to the proposed method, thus wishing to consider a wide diversity of *Pseudomonas* species most frequently found in MP.

Brochothrix thermosphacta MM008, *L. gelidum* (MM045), *P. fragi* MM014, and *P. fluorescens* MM026 were stored at −80°C in nutrient broth with 30% glycerol as a cryoprotective agent. Before use, strains were transferred from the −80°C culture collection to Brain Heart Infusion (BHI) broth for 48 h at 22°C. The bacterial suspensions were incubated overnight at 4°C before inoculation at stationary phase (7.00 log CFU/mL).

Inoculation Experiments

The three selected bacteria suspensions were inoculated on irradiated MP samples (1% v/w), in triplicate, for mono-culture and co-culture experiments with the objective to reach an average concentration of 3.0 log CFU/g (on the product).

Mono-culture experiments were performed by inoculation of individual bacterial strains: *B. thermosphacta* MM008, *Pseudomonas* spp. (*P. fluorescens* MM026, *P. fragi* MM014, 1:1 ratio), and *L. gelidum* MM045.

Co-culture experiments were performed by inoculation of a mix containing *B. thermosphacta* MM008, Pseudomonas spp. (*P. fragi* MM0014 and *P. fluorescens* MM0026, 1:1 ratio), and *L. gelidum* MM045 (1:1:1 ratio).

Non-inoculated control samples were homogenized, in triplicate, by adding the same quantity of sterile water only.

After inoculation, MP samples were mixed in a Kenwood mixer for 2 min in speed 2 (Kenwood, Mechelen, Belgium).

Inoculated and non-inoculated MP samples were then packed (50 g) in two different types of non-sterile packaging. The first packaging was a high barrier tray (187 × 137 × 36, polyester 10 μm, homo-polymer polypropylene 50 μm, NutriPack, France) under modified atmosphere (MAP, CO_2 30%/O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France). The second packaging concerns a weak barrier tray (175 × 135 × 22, polystyrene) under food wrap packing (FW) using cling film (Clinofilm).

In this study, MP samples were stored during a 13-days shelf life at isothermal temperature: (i) 4°C (±1°C), (ii) 8°C (±1°C), (iii) and 12°C (±1°C), in climatic chambers (Sanyo MIR 254) (288 samples for four experiments, n = 1152 samples) (**Supplementary Figure S1**). A storage time of 13 days was defined in this study in order to obtain a sufficient number of points for modeling, allowing us to predict all the growth phases.

The codes used for each experiment, depending on the inoculated bacteria and storage conditions, are listed in **Table 1**.

pH and Gas Composition Measurements

At the first and the last day of storage, pH of the homogenized samples (5 g in 45 mL of KCl) was measured with a pH meter (Knick 765 Calimatic, Allemagne).

Oxygen and carbon oxygen concentrations of samples stored in modified atmosphere packaging were monitored daily (CheckMate 3, Dansesor, France).

Non-parametric statistical tests were used to compare the pH values and the gas measurements between samples. All tests were considered as significant for a p-value < 0.05.

Plate Count Enumeration

Twenty-five grams of product were put into a Stomacher bag with a mesh screen liner (80 μm pore size) (Biomérieux, Basingstoke, England, ref 80015) under aseptic conditions. Buffered peptone water (BPW, 10 g/L peptone, 5 g/L sodium chloride, #3564684, Bio-Rad, Marnes-la-Coquette, France) (225 mL) was automatically added to each bag (Dilumat, Biomérieux, Belgium) and the samples were homogenized for 2 min in a Stomacher (Bagmixer, Interscience, France). From this primary suspension, decimal dilutions in maximum recovery diluent (1.0 g/L peptone, 8.5 g/L sodium chloride, #CM0733, Oxoid, Hampshire, England) were prepared for microbiological analysis, and 0.1 mL aliquots of the appropriate dilutions were plated onto media for each analysis (Spiral plater, DW Scientific, England).

Total viable counts (TVCs) for the aerobic psychrophilic microbiota were enumerated on plate count agar (PCA agar, #3544475, Bio-Rad, Marnes-la-Coquette, France) after 72 h at 22°C (model 1535 incubator, Shel Lab, Sheldon Manufacturing, Inc., United States).

Plate counts were performed for mono- and co-culture experiments, and transformed in decimal logarithmic values. Samples for both experiments were enumerated at the first day of inoculation (day 0) and daily until the last day of storage (day 13). None specific agar media were used in co-culture experiments to separately enumerate the three inoculated species. Non-inoculated control samples were analyzed at day 0 and at day 13.

TABLE 1 | List of the codes used for the experiments, depending on the inoculated bacteria and storage conditions.

Experiments	Food packaging	Temperature (°C)	Bacterial species	Codes
Mono-culture	FW	4	*B. thermosphacta*	A_{mono}
	FW	8		B_{mono}
	FW	12		C_{mono}
	MAP	4		D_{mono}
	MAP	8		E_{mono}
	MAP	12		F_{mono}
Mono-culture	FW	4	*Pseudomonas* spp.	G_{mono}
	FW	8		H_{mono}
	FW	12		I_{mono}
	MAP	4		J_{mono}
	MAP	8		K_{mono}
	MAP	12		L_{mono}
Mono-culture	FW	4	*L. gelidum*	M_{mono}
	FW	8		N_{mono}
	FW	12		O_{mono}
	MAP	4		P_{mono}
	MAP	8		Q_{mono}
	MAP	12		R_{mono}
Co-culture	FW	4	*B. thermosphacta*	$A_{co(A)}$
			Pseudomonas spp.	$A_{co(B)}$
			L. gelidum	$A_{co(C)}$
	FW	8	*B. thermosphacta*	$B_{co(A)}$
			Pseudomonas spp.	$B_{co(B)}$
			L. gelidum	$B_{co(C)}$
	FW	12	*B. thermosphacta*	$C_{co(A)}$
			Pseudomonas spp.	$C_{co(B)}$
			L. gelidum	$C_{co(C)}$
	MAP	4	*B. thermosphacta*	$D_{co(A)}$
			Pseudomonas spp.	$D_{co(B)}$
			L. gelidum	$D_{co(C)}$
	MAP	8	*B. thermosphacta*	$E_{co(A)}$
			Pseudomonas spp.	$E_{co(B)}$
			L. gelidum	$E_{co(C)}$
	MAP	12	*B. thermosphacta*	$F_{co(A)}$
			Pseudomonas spp.	$F_{co(B)}$
			L. gelidum	$F_{co(C)}$

FW, food wrap; MAP, modified atmosphere packaging (CO_2 30%/O_2 70% ± 0.1%); mono, mono-culture experiments; co, co-culture experiments with by individually tracking the inoculated bacteria by metagenetic analysis [B. thermosphacta, $_{co(A)}$; Pseudomonas spp., $_{co(B)}$; L. gelidum, $_{co(C)}$].

Using R software (R Core Team, 2019), an analysis of covariance (ANCOVA) was performed to evaluate the effect of the storage conditions on plate counts (FactoMineR package, Le et al., 2008). All tests were considered as significant for a p-value < 0.05.

16S rDNA Metagenetic Approach

A 16S rDNA metagenetic approach was used for mono- and co-culture experiments.

In mono-culture experiments, metagenetic analysis were performed at the first day of inoculation (day 0) and at the last day of storage (day 13) for samples stored at 4°C.

In co-culture experiments, samples were analyzed at day 0 and daily until day 13. The results were then correlated with plate counts in order to obtain estimate bacterial abundance over storage (see section "16S rDNA Data Analysis and Bacterial Abundance").

No 16S rDNA metagenetic analysis was performed for non-inoculated control samples.

DNA Extraction and 16S rDNA Amplicon Sequencing

Bacterial DNA was extracted from each primary suspension, previously stored at −80°C, using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV, Antwerp, Belgium) following the manufacturer's recommendations. The resulting DNA extracts were eluted in DNAse/RNAse free water and their concentration and purity were evaluated by means of optical density using the NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at −20°C until used for 16S rDNA amplicon sequencing.

PCR-amplification of the V1–V3 region of the 16S rDNA library preparation was performed with the following primers (with Illumina overhand adapters), forward (5′-GAGAGTTTGA TYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGG CAC-3′). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, United States) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. Thermocycling conditions consisted of a denaturation step of 4 min at 94°C, followed by 25 cycles of denaturation (15 s at 94°C), annealing (45 s at 56°C), and extension (60 s at 72°C), with a final elongation step (8 min at 72°C). These amplifications were performed on an EP Mastercycler Gradient System device (Eppendorf, Hamburg, Germany). The PCR products of approximately 650 nucleotides were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, Netherlands). After purification, PCR products were quantified using the Quanti-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, United States) and diluted to 10 ng/μL. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR® FAST quantitative PCR (qPCR) Kit (KapaBiosystems, Wilmington, MA, United States) before normalization, pooling, and sequencing on a MiSeq sequencer using V3 reagents (Illumina, San Diego, CA, United States).

Bioinformatics Analysis

The 16S rRNA gene sequence reads were processed with MOTHUR. The quality of all sequence reads was denoised using the Pyronoise algorithm implemented in MOTHUR. The sequences were checked for the presence of chimeric amplification using ChimeraSlayer (developed by the Broad Institute[1]). The obtained read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database of full-length rRNA gene sequences[2]

[1]http://microbiomeutil.sourceforge.net/#A_CS
[2]http://www.arb-silva.de/

(version v1.2.11) implemented in MOTHUR. The final reads were clustered into operational taxonomic units (OTUs), using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was attributed to each OTU by comparison to the SILVA database, using an 80% homogeneity cutoff. As MOTHUR is not dedicated to the taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared to the SILVA dataset 111, using a BLASTN algorithm. For each OTU, a consensus detailed taxonomic identification was given based upon the identity (<1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not).

16S rDNA Data Analysis and Bacterial Abundance

A correcting factor for 16S rDNA gene copy numbers was applied for any taxon *i* (Eq. 1).

$$A_i = N_k / C_i \quad (1)$$

Where A_i is the real abundance of 16S genes from the taxon in the sample, N_k is the number of reads for the taxon in the sample *k*, and C_i is determined by the genomic 16S copy number of that taxon. To obtain each gene copy number, Ribosomal RNA Database (rrnDB) (Stoddard et al., 2015) and EzBioCloud database (Yoon et al., 2017) were used.

Then, to compare the relative abundance of OTUs, the number of reads of each taxon was normalized as described by Chaillou et al. (2015). Reads counts of each taxon *i* in the sample *k* were divided by a sample-specific scaling factor (*Si*) (Eq. 2) (Fougy et al., 2016; Rouger et al., 2018):

$$Nr_i = A_i / S_k \quad (2)$$

Where Nr_i is the normalized number of reads for the taxon in the sample, A_i is the real abundance of 16S rRNA genes from that taxon obtained with a correcting factor for 16S rRNA gene copy numbers, and S_k is the normalization factor associated with sample *k*.

The sample-specific scaling factor was calculated by (Eq. 3):

$$S_k = T_k / m_e \quad (3)$$

Where S_k is the sample-specific scaling factor associated with sample *k*, T_k is the number of total reads in the sample *k*, and m_e is the median value of total reads for all the samples of the dataset. Reads counts of all samples were then transformed into a percentage of each OTU.

For co-culture experiments, the percentage of each OTUs was finally converted as a proportion of the TVC, obtained by classical microbiological analysis, in order to estimate counts for each species [in $\log_{10}$ CFU/g, and expressed as mean ± standard deviation (SD)] (Eq. 4), as described by Cauchie et al. (2017).

$$C_{bacterial\ species} = (C_{total\ microbiota} \times P_{reads\ of\ bacterial\ species})/100 \quad (4)$$

Where $C_{bacterial\ species}$ is the estimated abundance concentration in the sample (log CFU/g), $C_{total\ microbiota}$ is the bacterial concentration per samples in the PCA analysis (log CFU/g), and $P_{reads\ of\ bacterial\ species}$ is the proportion of reads for the bacterial species per sample in the metagenetic analysis (expressed in% of the total number reads in the sample).

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de BioProject ID PRJNA590608. The raw data supporting the conclusions of this article will be made available by EC to any qualified researcher.

Approach Used to Develop the Interaction Model

As proposed by Correia Peres Costa et al. (2019), a step-wise approach (**Figure 1**) was followed to develop interaction models simulating the growth of specific spoilage micro-organisms.

First, primary and secondary models were performed on mono-culture experiments to obtain the kinetic parameters (section "Primary and Secondary Model for the Fitting of Experimental Data"): lag phase duration (*LPD*, hours), maximum specific growth rate (μ_{max}, 1/hours), initial and maximal population densities (N_0 and N_{max}, respectively, log CFU/g), theoretical minimal temperature of growth (T_{min},°C), growth rate obtained at the reference temperature of 20°C (μ_{ref}, 1/hours), and minimal shelf life (*MSL*). The *MSL* is the time for the plate counts reaching approximatively 7.0 log CFU/g (expressed as Spoilage value according to the scientific literature, S_{val}).

Second, the same approach was applied for co-culture experiments in order to obtain the growth parameters (section "Primary and Secondary Model for the Fitting of Experimental Data"), and to compare them with those on mono-culture experiments (section "Correlations Between Growth Parameters"). The Pearson's correlation coefficient was also used to choose the highest influencing growth parameters on the microbial shelf life of MP samples (section "Correlations Between Growth Parameters").

Third, all of these results were used to estimate competitions parameters in interaction models for a three-species approach, based on the modified Jameson-effect model and Lotka Volterra model (section "Modeling Microbial Interactions for *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum*").

Finally, validation of growth and interaction parameters obtained by the three-species models was performed with naturally contaminated MP samples stored under different conditions (section "Model Validation").

Primary and Secondary Model for the Fitting of Experimental Data

The primary model of Baranyi and Roberts (1994) (Eq. 5) was fitted to the experiment dataset obtained for mono- and co-culture experiments. Experimental dataset is obtained by plate counts in mono-culture, and by estimate abundance based on metagenetic results in co-culture. All the data from the three replicates were modeled.

Based on primary fitting, the growth kinetic parameters were obtained.

$$N_t = N_0 + \mu_{max} \times A_t + \ln\left[1 + \frac{\exp(\mu_{max} \times A_t) - 1}{\exp(N_{max} - N_0)}\right] \quad (5)$$

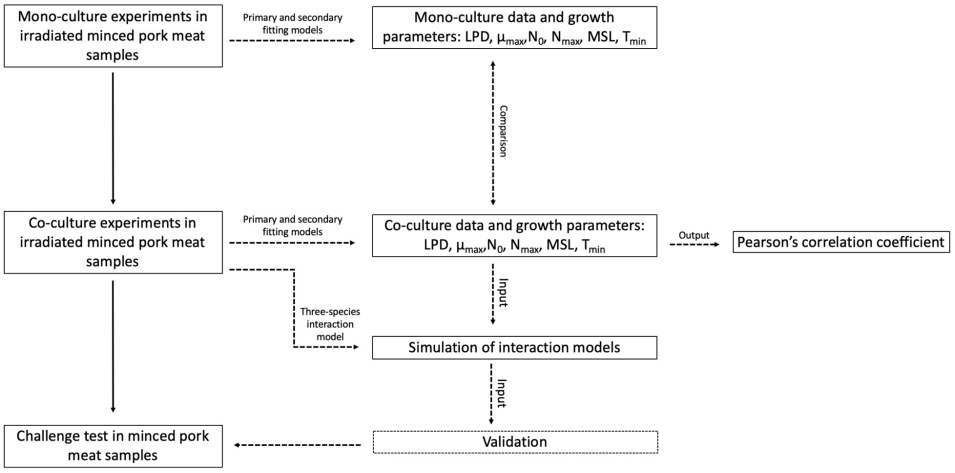

FIGURE 1 | Schematic overview of the step-wise method used for the development of a three-spoilage species interaction model.

Where N_t the bacterial population at any time t (log CFU/g); N_{max} and N_0, the maximum and initial population level, respectively (log CFU/g); μ_{max}, the maximum specific growth rate (1/hour); and A_t, an adjustment function to define the *LPD* (Eq. 6).

$$A_t = t + \frac{1}{\mu_{\max}} \times \ln\{\exp(-\mu_{\max} \times t) + \exp(-h_0) - \exp[(-\mu_{\max} \times t) - h_0]\} \quad (6)$$

Where h_0 is simply a transformation of the initial conditions.

All fittings were performed using the nlsMicrobio package (function: baranyi, Baty and Delignette-Muller, 2013) from the open source R software (R Core Team, 2019).

The adequacy of the primary models to describe the experimental data was observed by using the root-mean-square error of the residuals (*RrMSE*, SD of the residuals) (Eq. 7) and the coefficient of multiple determination (R^2, the fraction of the square of the deviations of the observed values about their mean explained by the equation fitted to the experimental data) (Eq. 8).

$$RrMSE = \sqrt{\frac{RSS}{DF}} = \frac{\sum_{i=1}^{n}(x_i^0 - x_i^f)^2}{n-s} \quad (7)$$

Where *RSS*, the residual sum of square; *DF*, the degrees of freedom; n, the number of data points; s, the number of parameters of the model; ${x_i}^0$, the observed values; and ${x_i}^f$, the fitted values.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(observed_i - predicted_i)^2}{\sum_{i=1}^{n}(observed_i - mean)^2} \quad (8)$$

Where n, the total number of data points; *mean*, the average value from all observed values.

A reparameterized version of the square root secondary model (Ratkowsky et al., 1982) (Eq. 9) was then used in R (R Core Team, 2019) to assess the effects of temperature on the growth rates.

$$\mu_{\max} = \mu_{\text{ref}}\left(\frac{T - T_{\min}}{T_{\text{ref}} - T_{\min}}\right)^2 \quad (9)$$

Where μ_{ref} is the reference growth rate obtained at T_{ref} = 20°C (1/hours), T is the temperature (°C), and T_{min} is the minimal temperature for growth (°C) found in the scientific literature for the studied bacterial species: −3.36°C for *B. thermosphacta* (Leroi et al., 2012); −5.00°C for *Pseudomonas* spp. (Rashid et al., 2001); and + 1.00°C for *L. gelidum* (Kim et al., 2000).

For comparison, T_{min} values were also estimated by the Rosso primary model (Rosso et al., 1995) and the square root model (Ratkowsky et al., 1983) (Eq. 10).

$$\sqrt{\mu_{max}} = btimes(T - T_{min}) \quad (10)$$

Where μ_{max} is the maximal growth rate (1/hours), b is a constant parameter obtained by linear regression, T is the temperature (°C), and T_{min} is the minimal temperature for growth (°C).

For secondary models, the coefficient of multiple determination (R^2) and the goodness of fit (*GoF*, root-meat-square error of the model, analogous to the accuracy factor) were used (Eq. 11).

$$GoF = \frac{\sum_{i=1}^{n}(x_i^0 - x_i^f)^2}{n} \quad (11)$$

Extracts of the code in R for primary and secondary fittings are given in **Supplementary Material** (R-commands 1).

Correlations Between Growth Parameters

An analysis of covariance was performed to evaluate if the maximal bacterial growth rates (μ_{max}) were significantly different between the two food packaging. All tests were considered as significant for a p-value of < 0.05. Extracts of the code in R for ANCOVA analysis are given in **Supplementary Material** (R-commands 2).

Using R software (R Core Team, 2019), correlations between the minimal shelf life (*MSL*) and the growth parameters (μ_{max}, *LPD*, N_0, N_{max}) were obtained by the Pearson's correlation coefficient (r) in mono-culture and co-culture experiments (Liu et al., 2006; Miks-Krajnik et al., 2016). High correlations were considered when $|r| > 0.7000$ (Miks-Krajnik et al., 2016). The best influencing growth parameter on the microbial shelf life was chosen according to the Pearson's correlations coefficient.

Then, a reduction ratio (α) was calculated to quantify the interaction effect on μ_{max} by inoculated bacteria in co-culture experiments (Eq. 12) (Correia Peres Costa et al., 2019).

$$\alpha = 1 - \frac{(p_{co})}{(p_{mono})} \tag{12}$$

Where α is the reduction ratio; p_{co} and p_{mono} are the growth parameters obtained in co-culture and mono-culture experiments, respectively.

Modeling Microbial Interactions for *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum*

Two well-known interactions models for two-species were modified to predict the simultaneous growth of the three-inoculated spoilage bacteria in irradiated MP samples: the modified Jameson-effect model and the Lotka Volterra model (Cornu et al., 2011; Correia Peres Costa et al., 2019).

As presented by Cornu et al. (2011) and Quinto et al. (2018), a modified generic primary growth model can be written as Eq. 13.

$$\frac{1}{N(t)}\frac{dN(t)}{dt} = \frac{d(\ln(N(t)))}{dt} = \mu_{max} \times \alpha(t) \times f(t) \tag{13}$$

Where $\frac{1}{N(t)}\frac{dN(t)}{dt}$ is the relative or instantaneous growth rate of the microorganism, N_t is the bacterial concentration at time t (log CFU/g), μ_{max} is the maximum growth rate (1/h), $\alpha(t)$ is an adjustment function, and $f(t)$ is an inhibition function, defined as Eqs 14 and 15:

$$\alpha_t = \begin{cases} 0 \text{ if } t < LPD \\ 1 \text{ if } t \geq LPD \end{cases} \tag{14}$$

$$f_t = \left(1 - \left(\frac{N_t}{N_{max}}\right)\right) \tag{15}$$

Where *LPD* is the lag phase duration (hours) and N_{max} is the maximal population density (log CFU/g).

Based on Eq. 13, an alternative deceleration function can be added for modeling the interaction of two bacterial species (Jameson-effect model) (Eq. 16) (Mejlholm and Dalgaard, 2007; Cornu et al., 2011).

$$\begin{cases} \frac{1}{N_A(t)}\frac{dN_A(t)}{dt} = \mu_{max\,A(t)} \times \alpha_A(t) \times \left(1 - \frac{N_{A(t)}}{N_{max\,A(t)}}\right) \times \left(1 - \frac{N_{B(t)}}{N_{max\,B(t)}}\right) \\ \frac{1}{N_B(t)}\frac{dN_{Bt}}{dt} = \mu_{max\,B(t)} \times \alpha_B(t) \times \left(1 - \frac{N_{B(t)}}{N_{max\,B(t)}}\right) \times \left(1 - \frac{N_{A(t)}}{N_{max\,A(t)}}\right) \end{cases} \tag{16}$$

Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), and N_{max} is the maximum population density (log CFU/g).

In the modified Jameson-effect model, the deceleration function can be replaced by Eq. 17 (Mejlholm and Dalgaard, 2007; Cornu et al., 2011; Quinto et al., 2018; Cadavez et al., 2019).

$$\begin{cases} f_A(t) = \left(1 - \frac{N_A(t)}{N_{max_{A(t)}}}\right)\left(1 - \frac{N_B(t)}{N_{max_{B(t)}}}\right) \\ f_B(t) = \left(1 - \frac{N_A(t)}{N_{MCP_{A(t)}}}\right)\left(1 - \frac{N_B(t)}{N_{max_B(t)}}\right) \text{ if } N_A(t) \geq N_{MCP_{A(t)}} \\ f_B(t) = 0 \text{ if } N_A(t) \geq N_{MCP_{A(t)}} \end{cases} \tag{17}$$

Where N_t is the bacterial concentration at time t (log CFU/g), $N_{max(t)}$ is the maximal population density (log CFU/g), and $N_{MCP(t)}$ is maximum critical population (log CFU/g) that the bacterium should be reached to inhibit the growth of the other populations. *MCP* is inferior to its own maximum population density (N_{max}) (Cornu et al., 2011; Correia Peres Costa et al., 2019).

Using R software (R Core Team, 2019), the modified Jameson-effect model (Eq. 17) was applied on mono-culture experiment data with the functions of Baranyi, Buchanan and without-lag (package nlsMicrobio, Baty and Delignette-Muller, 2013). The function without lag shown the best fitting in all cases (**Supplementary Table S1**). This model was then selected in the rest of the study, by using the growth parameters obtained on co-culture experiments. Extracts of the code in R for the modified Jameson-effect models for two species are given in **Supplementary Material** (R-commands 3).

For a three-species mixed culture model, Quinto et al. (2018) recently proposed a modification of the logistic deceleration model (Eq. 18).

$$f(t) = \left(1 - \frac{N_A(t) + N_B(t) + N_C(t)}{N_{\max tot}}\right) \tag{18}$$

Where $N_A(t)$, $N_B(t)$, and $N_C(t)$ are the cell concentration of microorganism *A*, *B*, or *C* in co-culture at time t; N_{maxtot} is the maximal total population density (including all species present) and consequently the overall carrying capacity of the system from the three-species co-cultured.

However, this study only considers the effect of *P. fluorescens* and *L. innocua* on the bacterial growth of *L. monocytogenes*.

In our study, the aim of co-culture experiments was to consider the global effect of three inoculated bacterial species and the bacterial interaction on each other.

According to this, the modified Jameson-effect model was re-defined for a three-species model that was used in this study (Eq. 19).

$$\frac{1}{N_{tot}(t)}\frac{dN_{tot(t)}}{dt} = \mu_{max(Bm,Ps,Lg)(t)} \times \alpha_{(Bm,Ps,Lg)}(t) \times \left(1 - \frac{N_{Bm(t)} + N_{Ps(t)} + N_{Lg(t)}}{N_{MCP}(t)}\right) \quad (19)$$

Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), $\alpha(t)$ is an adjustment function, and N_{MCP} is the maximum critical population of each bacterium (log CFU/g).

Extracts of the code in R for the three-species modified Jameson-effect models are given in **Supplementary Material** (R-commands 4).

In the two-species model based on the Lotka Volterra equation, the deceleration function can be replaced by Eq. 20 (Cornu et al., 2011), which includes empirical parameters reflecting the degree of interaction between microbial species (F_{AB} and F_{BA}) (Liu et al., 2006; Cornu et al., 2011; Cadavez et al., 2019; Correia Peres Costa et al., 2019).

$$\begin{cases} f_A(t) = \left(1 - \dfrac{N_A(t) + F_{AB}N_B(t)}{N_{\max A(t)}}\right) \\ f_B(t) = \left(1 - \dfrac{N_B(t) + F_{BA}N_A(t)}{N_{\max B}(t)}\right) \end{cases} \quad (20)$$

Where the parameters F_{AB} and F_{BA} are the coefficients of interaction measuring the effects of one species on the other.

Using R software (R Core Team, 2019), the Lotka Volterra model (Eq. 20) was also re-defined for a three-species interaction model, represented by Eq. 21.

$$\begin{cases} \dfrac{1}{N_A(t)}\dfrac{dN_{A(t)}}{dt} = \mu_{max\,A(t)} \times \alpha_A(t) \times \left(1 - \dfrac{N_{A(t0)} + (F_{ABC} \times F_{ACB} \times N_{BC(t0)})}{N_{max\,A(t)}}\right) \\ \dfrac{1}{N_B(t)}\dfrac{dN_{B(t)}}{dt} = \mu_{max\,B(t)} \times \alpha_B(t) \times \left(1 - \dfrac{N_{B(t0)} + (F_{BAC} \times F_{BCA} \times N_{AC(t0)})}{N_{max\,B(t)}}\right) \\ \dfrac{1}{N_C(t)}\dfrac{dN_{C(t)}}{dt} = \mu_{max\,C(t)} \times \alpha_C(t) \times \left(1 - \dfrac{N_{C(t0)} + (F_{CAB} \times F_{CBA} \times N_{AB(t0)})}{N_{max\,C(t)}}\right) \end{cases} \quad (21)$$

Where N is the cell concentration (log CFU/g) at time t (h), μ_{max} is the maximum specific growth rate (1/h), $\alpha(t)$ is an adjustment function, $F_{A,B,C}$ are the coefficient of interaction measuring the effects of one species on the others, and N_{max} is the maximum population density (log CFU/g).

Extracts of the code in R for the three-species Lotka Volterra models are given in **Supplementary Material** (R-commands 5).

Comparison of the two models was assessed by root-mean-square error (*RMSE*) and coefficient of determination (R^2) (Correia Peres Costa et al., 2019), as previously described in the section above (Section 2.7.1.).

Model Validation

Validation of the developed three-species interaction models was performed using a new dataset of experimental data.

Fresh MP samples were obtained from a local Belgian manufacturer at the day of the production, corresponding to the day of slaughtering. MP samples were packed by the manufacturer in a polypropylene tray under cling film. Samples have the same composition as described above.

Samples were not irradiated and not inoculated in order to follow the dynamics of the natural food microbiota. MP samples were also packed (50 g) in two different packaging, in triplicate.

The first packaging was a tray (187 × 137 × 36, polyester 10 µm, homo-polymer polypropylene 50 µm, NutriPack, France) under modified atmosphere (MAP, CO_2 30%/O_2 70% ± 0.1%) (Olympia V/G, Technovac, Italy) using packaging wrap (PP/EVOH/PP) with random gas measurements (CheckMate 3, Dansensor, France). The second packaging consisted in a tray (175 × 135 × 22, polystyrene) under FW using cling film (Clinofilm).

In this study, MP samples were stored during a 13 days shelf life at isothermal temperature: (i) 4°C (±1°C), (ii) 8°C (±1°C), (iii) and 12°C (±1°C), in climatic chambers (Sanyo MIR 254).

Samples (n = 288) were then analyzed at the first day of inoculation (day 0) and daily until the last day of storage (day 13). Analyses were performed by classical plate counts and 16S rDNA metagenetics, as methods previously described in the sections above (sections "16S rDNA Metagenetic Approach" and "Approach Used to Develop the Interaction Model"), in order to estimate bacterial counts over the storage.

The performance of the developed interaction models was evaluated by the acceptable simulation zone (*ASZ*) approach. Model performance is considered acceptable when at least 70% of the observed log counts values are within the *ASZ*, defined as ± 0.5 log-units from the simulated concentration in log units (Correia Peres Costa et al., 2019).

RESULTS

16S rDNA Metagenetic Results

Despite of the inability of differentiation between viable and non-viable cells by the culture-independent DNA-based methods used, high level (>95%) of relative abundance for each inoculated bacterium was observed for mono-culture experiments (**Supplementary Figure S2**).

The relative abundance results for co-culture experiments (expressed in%) at genus levels (>1%) are represented in cumulated histograms for all samples in FW (**Figure 2**) and MAP (**Figure 3**). These data including the relative abundance of sequences are also summarized in **Supplementary Table S2**.

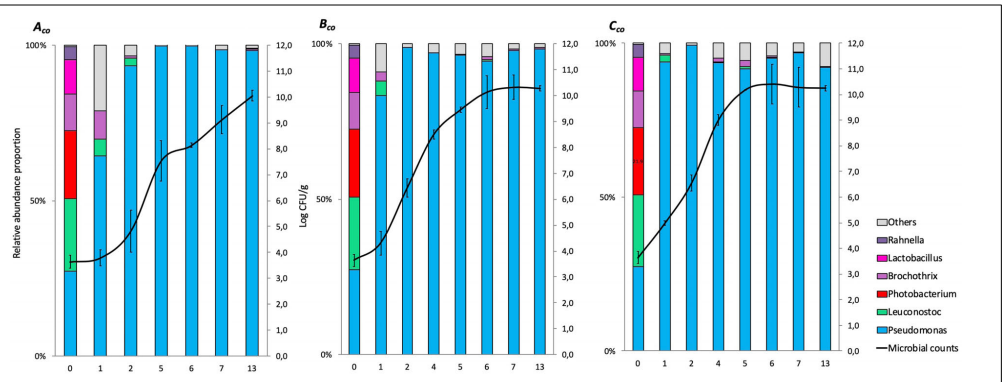

FIGURE 2 | Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at genus levels in co-culture experiment during storage in food wrap ($\boldsymbol{A}_{co}$, at 4°C; $\boldsymbol{B}_{co}$, at 8°C; $\boldsymbol{C}_{co}$, at 12°C). At genus levels, the taxa representing < 1% in relative abundance were merged in the category of "Others." The solid represents the plate counts (means and standard deviation of the three replicates).

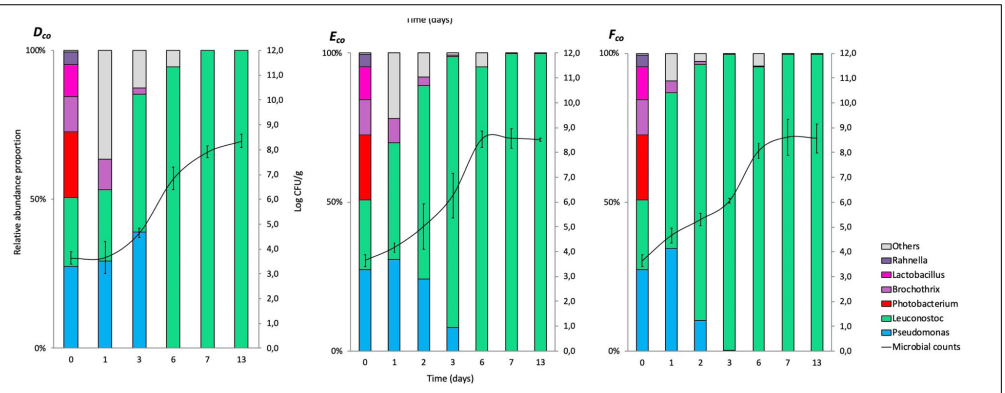

FIGURE 3 | Cumulated histograms of the relative abundance (%) of taxa and the dynamics of the bacterial community identified by metagenetics at genus levels in co-culture experiment during storage in modified atmosphere packaging ($\boldsymbol{D}_{co}$, at 4°C; $\boldsymbol{E}_{co}$, at 8°C; $\boldsymbol{F}_{co}$, at 12°C). At genus levels, the taxa representing < 1% in relative abundance were merged in the category of "Others." The solid represents the plate counts (means and standard deviation of the three replicates).

The taxa representing < 1% in relative abundance were merged in the category of "Others." "Others" are mainly composed by the genera *Aeromonas*, *Arthrobacter*, *Bacteroides*, *Carnobacterium*, *Chryseobacterium*, *Enterococcus*, *Flavobacterium*, *Kurthia*, *Lactobacillus*, *Lactococcus*, *Mannheimia*, *Massilia*, *Micrococcus*, *Moraxella*, *Myroides*, *Ottowia*, *Peptococcus*, *Photobacterium*, *Porphyromonas*, *Propionibacterium*, *Rothia*, *Serratia*, and *Staphylococcus*. Full data on taxa found in high (>1%) and low (<1%) frequencies will be made available by EC to any qualified researcher.

At day 0, small differences between the distribution of read percentages for the three inoculated bacteria are observed (11.8, 27.4, and 23.3% for *Brochothrix*, *Pseudomonas*, and *Leuconostoc*, respectively).

At day 3 in FW, *Brochothrix* became under the detection limit. At this same time, *Pseudomonas* became the most represented genus (>90%), and remained during the 13 days of storage.

In MAP, *Leuconostoc* and *Pseudomonas* were equally distributed during the first days of storage, but *Leuconostoc* became the most represented genus (>90%) after 3 days and until the end of storage.

Plate Counts and Estimated Abundance

In mono-culture experiments, plate counts for *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum* increased during the shelf life with increasing the temperature (**Table 2**).

At the end of the shelf life, the bacterial count was higher than 7.0 log CFU/g, except for some samples stored in MAP.

TABLE 2 | Microbiological counts (log CFU/g) for mono-culture experiments in minced pork samples stored during 13-days shelf life, at constant temperature, in food wrap (FW) and modified atmosphere packaging (MAP, CO_2 30%/O_2 70% ± 0.1%).

	Days													
Codes	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A_{mono}	3.84 ± 0.03	3.08 ± 0.10	3.76 ± 0.07	4.54 ± 0.12	–[a]	–[a]	7.24 ± 0.11	7.74 ± 0.17	7.63 ± 0.10	8.17 ± 0.33	7.68 ± 0.15	–[a]	–[a]	7.90 ± 0.15
B_{mono}	3.84 ± 0.03	6.76 ± 0.04	7.49 ± 0.11	8.25 ± 0.07	8.51 ± 0.10	8.58 ± 0.06	8.85 ± 0.02	8.77 ± 0.15	9.05 ± 0.03	8.79 ± 0.21	–[a]	–[a]	–[a]	9.00 ± 0.01
C_{mono}	3.84 ± 0.03	7.68 ± 0.08	8.29 ± 0.13	8.66 ± 0.04	8.99 ± 0.09	9.01 ± 0.23	9.11 ± 0.10	8.81 ± 0.28	9.03 ± 0.03	8.91 ± 0.16	–[a]	–[a]	–[a]	9.27 ± 0.08
D_{mono}	3.84 ± 0.03	–[a]	–[a]	2.17 ± 0.30	–[a]	–[a]	4.11 ± 0.01	4.01 ± 0.14	4.35 ± 0.03	5.24 ± 0.05	4.99 ± 0.12	–[a]	–[a]	5.43 ± 0.06
E_{mono}	3.84 ± 0.03	–[a]	5.88 ± 0.10	6.11 ± 0.11	7.11 ± 0.02	7.86 ± 0.10	8.21 ± 0.04	8.43 ± 0.11	8.43 ± 0.16	8.41 ± 0.10	8.38 ± 0.16	–[a]	7.86 ± 0.07	8.76 ± 0.03
F_{mono}	3.84 ± 0.03	–[a]	7.10 ± 0.04	7.76 ± 0.23	8.35 ± 0.04	8.58 ± 0.06	8.40 ± 0.12	8.44 ± 0.07	8.32 ± 0.03	9.16 ± 0.08	8.67 ± 0.40	–[a]	8.83 ± 0.02	8.71 ± 0.06
G_{mono}	3.15 ± 0.59	3.43 ± 0.11	4.52 ± 0.23	5.64 ± 0.19	–[a]	–[a]	–[a]	9.45 ± 0.13	9.51 ± 0.07	–[a]	9.90 ± 0.29	–[a]	–[a]	10.21 ± 0.03
H_{mono}	3.15 ± 0.59	3.86 ± 0.17	5.36 ± 0.03	7.69 ± 0.17	9.04 ± 0.05	9.67 ± 0.03	–[a]	9.62 ± 0.15	10.34 ± 0.24	10.39 ± 0.40	10.11 ± 0.28	–[a]	–[a]	10.15 ± 0.17
I_{mono}	3.15 ± 0.59	4.93 ± 0.15	–[a]	9.81 ± 0.04	9.85 ± 0.29	9.95 ± 0.34	10.15 ± 0.82	10.26 ± 0.08	10.14 ± 0.10	–[a]	9.87 ± 0.19	–[a]	–[a]	9.80 ± 0.42
J_{mono}	3.15 ± 0.59	–[a]	3.48 ± 0.06	–[a]	–[a]	3.90 ± 0.11	4.87 ± 0.34	4.55 ± 0.12	–[a]	–[a]	–[a]	–[a]	4.73 ± 0.01	4.90 ± 0.01
K_{mono}	3.15 ± 0.59	3.52 ± 0.01	4.16 ± 0.05	–[a]	–[a]	5.41 ± 0.08	6.33 ± 0.07	6.52 ± 0.14	–[a]	6.59 ± 0.17	–[a]	–[a]	7.83 ± 0.13	8.37 ± 0.08
L_{mono}	3.15 ± 0.59	4.47 ± 0.07	6.08 ± 0.03	–[a]	–[a]	–[a]	9.42 ± 0.28	9.58 ± 0.23	–[a]	9.80 ± 0.41	–[a]	–[a]	9.87 ± 0.06	9.85 ± 0.14
M_{mono}	4.00 ± 0.02	4.07 ± 0.01	4.38 ± 0.01	4.61 ± 0.12	–[a]	–[a]	6.17 ± 0.05	–[a]	–[a]	–[a]	8.62 ± 0.09	–[a]	–[a]	8.42 ± 0.06
N_{mono}	4.00 ± 0.02	4.58 ± 0.08	5.84 ± 0.02	–[a]	7.57 ± 0.10	–[a]	8.61 ± 0.13	–[a]	8.73 ± 0.07	–[a]	8.84 ± 0.09	–[a]	–[a]	8.77 ± 0.30
O_{mono}	4.00 ± 0.02	5.38 ± 0.01	6.84 ± 0.13	8.35 ± 0.09	7.56 ± 0.01	–[a]	8.64 ± 0.13	–[a]	–[a]	–[a]	8.82 ± 0.23	–[a]	–[a]	8.62 ± 0.18
P_{mono}	4.00 ± 0.02	4.18 ± 0.09	–[a]	–[a]	6.31 ± 0.17	–[a]	6.84 ± 0.06	7.85 ± 0.01	–[a]	7.78 ± 0.21	–[a]	–[a]	8.00 ± 0.10	8.39 ± 0.12
Q_{mono}	4.00 ± 0.02	4.75 ± 0.03	–[a]	–[a]	8.06 ± 0.01	–[a]	8.38 ± 0.05	8.49 ± 0.16	–[a]	8.85 ± 0.01	–[a]	–[a]	–[a]	8.75 ± 0.19
R_{mono}	4.00 ± 0.02	8.32 ± 0.15	7.28 ± 0.01	–[a]	8.35 ± 0.06	–[a]	8.36 ± 0.09	8.64 ± 0.10	–[a]	8.89 ± 0.07	–[a]	–[a]	–[a]	8.87 ± 0.11

*See **Table 1** for list of the codes used. Mean values with standard deviations of the three replicates. –[a] no analysis performed for the day.*

TABLE 3 | Estimate bacterial counts for co-culture experiment.

	Time (days)								
Code	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**13**
$A_{co(A)}$	2.71 ± 0.24	2.75 ± 0.31	2.71 ± 0.81	–[a]	–[a]	–[a]	–[a]	–[a]	7.77 ± 0.20
$A_{co(B)}$	3.07 ± 0.24	3.60 ± 0.31	4.80 ± 0.81	–[a]	–[a]	7.54 ± 0.77	8.14 ± 0.08	9.12 ± 0.53	10.04 ± 0.20
$A_{co(C)}$	3.00 ± 0.24	2.52 ± 0.31	3.20 ± 0.81	–[a]	–[a]	4.54 ± 0.77	5.14 ± 0.08	5.79 ± 0.53	6.92 ± 0.20
$B_{co(A)}$	2.71 ± 0.24	2.26 ± 0.31	–[a]	–[a]	–[a]	–[a]	7.13 ± 0.53	7.68 ± 0.20	8.00 ± 0.10
$B_{co(B)}$	3.07 ± 0.24	4.23 ± 0.46	6.43 ± 0.34	–[a]	8.49 ± 0.18	9.43 ± 0.10	10.11 ± 0.64	10.31 ± 0.47	10.27 ± 0.10
$B_{co(C)}$	3.00 ± 0.24	2.48 ± 0.31	1.70 ± 0.81	–[a]	–[a]	5.44 ± 0.08	6.61 ± 0.08	6.93 ± 0.20	7.15 ± 0.10
$C_{co(A)}$	2.71 ± 0.24	2.58 ± 0.09	–[a]	–[a]	7.15 ± 0.20	8.46 ± 0.02	8.18 ± 0.77	7.58 ± 0.78	7.24 ± 0.10
$C_{co(B)}$	3.07 ± 0.24	4.95 ± 0.09	6.55 ± 0.30	–[a]	8.97 ± 0.20	10.14 ± 0.02	10.38 ± 0.77	10.26 ± 0.78	10.21 ± 0.10
$C_{co(C)}$	3.00 ± 0.24	3.32 ± 0.09	3.04 ± 0.30	–[a]	6.30 ± 0.20	8.02 ± 0.02	7.41 ± 0.77	7.10 ± 0.78	7.06 ± 0.10
$D_{co(A)}$	2.71 ± 0.24	2.67 ± 0.64	–[a]	2.97 ± 0.19	–[a]	–[a]	3.83 ± 0.46	–[a]	3.83 ± 0.46
$D_{co(B)}$	3.07 ± 0.24	3.13 ± 0.64	–[a]	4.24 ± 0.19	–[a]	–[a]	4.14 ± 0.46	5.28 ± 0.23	4.76 ± 0.28
$D_{co(C)}$	3.00 ± 0.24	3.04 ± 0.64	–[a]	4.31 ± 0.19	–[a]	–[a]	6.81 ± 0.46	7.91 ± 0.23	8.36 ± 0.28
$E_{co(A)}$	2.71 ± 0.24	3.07 ± 0.19	3.46 ± 0.90	3.95 ± 0.90	–[a]	–[a]	–[a]	–[a]	4.94 ± 0.07
$E_{co(B)}$	3.07 ± 0.24	3.65 ± 0.19	4.39 ± 0.90	5.15 ± 0.90	–[a]	–[a]	–[a]	5.00 ± 0.39	4.94 ± 0.07
$E_{co(C)}$	3.00 ± 0.24	3.76 ± 0.19	4.82 ± 0.90	6.21 ± 0.90	–[a]	–[a]	8.51 ± 0.33	8.56 ± 0.39	8.50 ± 0.07
$F_{co(A)}$	2.71 ± 0.24	3.25 ± 0.30	3.30 ± 0.25	–[a]	–[a]	–[a]	5.05 ± 0.30	5.51 ± 0.72	5.88 ± 0.58
$F_{co(B)}$	3.07 ± 0.24	4.20 ± 0.30	4.31 ± 0.25	3.34 ± 0.10	–[a]	–[a]	–[a]	5.63 ± 0.72	4.98 ± 0.58
$F_{co(C)}$	3.00 ± 0.24	4.38 ± 0.30	5.24 ± 0.25	6.05 ± 0.10	–[a]	–[a]	8.03 ± 0.30	8.61 ± 0.72	8.57 ± 0.58

*See **Table 1** for list of the codes used. Mean values with standard deviations of the three replicates. FW, food wrap; MAP, modified atmosphere packaging (CO_2 30%/O_2 70% ± 0.1%), –[a] no analysis performed for the day.*

During the storage, a high growth rate and a more rapidly reached stationary phase were also correlated to FW and the highest storage temperatures.

No bacterial growth was observed on PCA for the control samples (limit detection < 3.0 log CFU/g) (data not shown in this paper).

For co-culture experiments, the metagenetic data were combined with the plate counts results in order to obtain estimated bacterial counts (**Table 3**).

As previously observed, estimate counts increased during the shelf life with increasing the temperature. At the end of the shelf life, the bacterial count was over 7.0 log CFU/g, except for *B. thermosphacta* and *Pseudomonas* spp. stored in MAP. During the storage, the same growth profiles as mono-culture experiments were observed.

pH and Gas Measurements

A significant increase of pH is observed for MP samples inoculated by *Pseudomonas* spp. (7.54 ± 0.76, n = 5, p-value = 0.01) compared to the control samples (5.79 ± 0.05, n = 10).

In co-culture experiments, pH values at the end of the shelf life were not different to control samples (5.87 ± 0.02, n = 5) (**Supplementary Figure S3**).

A relatively stable concentration of carbon dioxide was observed in MAP at the end of the shelf life. Except for MP samples inoculated with *Pseudomonas* spp., which reached a higher significant carbon dioxide value (100.0 ± 0.1%) at 12°C (**Supplementary Figure S4**).

Microbial Growth Parameters

Results of the primary and secondary model fittings for mono- and co-culture experiments are shown in **Tables 4**, **5**. Growth parameters from mono-culture experiments are based on plate counts, and those from co-culture experiments are based on estimate abundance (obtained by the association of metagenetic and plate counts results).

Good fit indexes were obtained in all cases (**Supplementary Tables S3, S4**).

Growth parameters showed different dynamic changes depending on storage temperature: a high storage temperature is correlated to a high growth rate during exponential phase and a lower lag-time. These growth parameters are also higher in FW than in MAP.

The *MSL* value is more rapidly reached in FW, except for *L. gelidum*.

Moreover, the S_{val} was never reached in MAP for MP samples inoculated by *Pseudomonas* spp. and *B. thermosphacta* during the 13-days shelf-life at 4°C.

Based on these results, the evolution of μ_{max} between a large range of temperature (from −6 to +25°C) in FW and MAP was performed for mono- and co-culture experiments (**Figure 4**).

It can be clearly observed that *L. gelidum* had a highest growth rate in MAP, while it concerns *B. thermosphacta* in FW in mono-culture experiments. *B. thermosphacta* had the lowest one in co-culture experiments.

Correlations Between Growth Parameters Obtained in Mono- and Co-culture Experiments

Correlations between growth parameters of *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum* for mono-culture and co-culture experiments are presented in **Table 6**.

It can be observed that the maximum specific growth rate (μ_{max}) of micro-organisms was negatively correlated with

TABLE 4 | Observed kinetic parameters of mono- and co-culture experiments, calculated by Baranyi equation without interactions.

	μ_{max}	*LPD*	N_0	N_{max}	*RSS*	S_{val}	*MSL*
A_{mono}	0.09 [0.09–0.08]	51 [53–51]	3.84 ± 0.03	7.90 ± 0.15	0.000442	Y	5.7 [5.8–5.6]
B_{mono}	0.21 [0.21–0.19]	0 [0–0]	3.84 ± 0.03	8.79 ± 0.21	0.000255	Y	1.5 [1.5–1.4]
C_{mono}	0.39 [0.39–0.35]	0 [0–0]	3.84 ± 0.03	9.11 ± 0.10	0.000558	Y	0.8 [0.8–0.8]
D_{mono}	0.03 [0.03–0.03]	20 [20–17]	3.84 ± 0.03	4.99 ± 0.12	0.005700	N	15.3 [15.8–14.7]
E_{mono}	0.07 [0.07–0.07]	0 [0–0]	3.84 ± 0.03	8.43 ± 0.16	0.005700	Y	3.8 [3.9–3.7]
F_{mono}	0.13 [0.13–0.12]	0 [0–0]	3.84 ± 0.03	8.83 ± 0.16	0.005260	Y	1.9 [1.9–1.4]
G_{mono}	0.06 [0.06–0.06]	24 [24–24]	3.15 ± 0.59	9.90 ± 0.29	0.010900	Y	4.5 [4.6–4.2]
H_{mono}	0.13 [0.13–0.13]	10 [10–10]	3.15 ± 0.59	10.15 ± 0.17	0.010900	Y	2.7 [2.8–2.6]
I_{mono}	0.23 [0.23–0.23]	0 [0–0]	3.15 ± 0.59	9.95 ± 0.34	0.010900	Y	1.8 [1.9–1.7]
J_{mono}	0.04 [0.04–0.04]	48 [48–48]	3.15 ± 0.59	4.90 ± 0.01	0.001210	N	21.8 [22.6–20.9]
K_{mono}	0.08 [0.08–0.08]	27 [27–27]	3.15 ± 0.59	8.37 ± 0.08	0.001210	Y	9.0 [9.2–8.8]
L_{mono}	0.13 [0.13–0.13]	0 [0–0]	3.15 ± 0.59	9.87 ± 0.06	0.001210	Y	3.5 [3.6–3.3]
M_{mono}	0.01 [0.01–0.01]	48 [48–48]	4.00 ± 0.02	8.42 ± 0.06	0.017900	Y	7.1 [7.2–7.0]
N_{mono}	0.07 [0.08–0.07]	10 [12–10]	4.00 ± 0.02	8.77 ± 0.30	0.023000	Y	3.4 [3.4–3.3]
O_{mono}	0.18 [0.19–0.18]	0 [0–0]	4.00 ± 0.02	8.64 ± 0.13	0.017900	Y	2.5 [2.5–2.4]
P_{mono}	0.02 [0.02–0.02]	17 [19–15]	4.00 ± 0.02	8.00 ± 0.10	0.025600	Y	6.2 [6.4–5.5]
Q_{mono}	0.13 [0.13–0.13]	0 [0–0]	4.00 ± 0.02	8.75 ± 0.19	0.023700	Y	3.0 [3.0–2.3]
R_{mono}	0.32 [0.33–0.32]	0 [0–0]	4.00 ± 0.02	8.87 ± 0.11	0.025600	Y	1.2 [1.2–1.1]
$A_{co(A)}$	0.03 [0.03–0.03]	36 [36–36]	2.71 ± 0.24	7.77 ± 0.20	0.000490	Y	11.2 [11.6–10.6]
$A_{co(B)}$	0.05 [0.06–0.05]	12 [12–12]	3.07 ± 0.24	10.04 ± 0.20	0.098240	Y	5.4 [6.1–4.8]
$A_{co(C)}$	0.01 [0.01–0.01]	24 [30–24]	3.00 ± 0.24	6.92 ± 0.20	0.002650	N	11.6 [12.3–10.6]
$B_{co(A)}$	0.07 [0.08–0.07]	12 [12–12]	2.71 ± 0.24	8.00 ± 0.10	0.014000	Y	7.8 [8.3–7.3]
$B_{co(B)}$	0.11 [0.12–0.11]	0 [0–0]	3.07 ± 0.24	10.27 ± 0.20	0.472000	Y	3.8 [4.2–3.5]
$B_{co(C)}$	0.05 [0.05–0.05]	24 [24–24]	3.00 ± 0.24	7.15 ± 0.10	0.016460	Y	8.5 [8.8–8.2]
$C_{co(A)}$	0.13 [0.15–0.12]	0 [0–0]	2.71 ± 0.24	7.58 ± 0.92	0.117000	Y	6.0 [6.4–5.6]
$C_{co(B)}$	0.19 [0.20–0.19]	0 [0–0]	3.07 ± 0.24	10.26 ± 0.78	0.472000	Y	3.5 [3.9–3.3]
$C_{co(C)}$	0.12 [0.13–0.11]	0 [0–0]	3.00 ± 0.24	7.10 ± 0.90	0.000840	Y	6.6 [7.1–6.1]
$D_{co(A)}$	0.02 [0.02–0.01]	46 [59–10]	2.71 ± 0.24	3.83 ± 0.46	0.000150	N	21.0 [20.5–16.8]
$D_{co(B)}$	0.06 [0.06–0.03]	48 [48–48]	3.07 ± 0.24	4.76 ± 0.75	0.135300	N	17.2 [17.4–16.9]
$D_{co(C)}$	0.01 [0.02–0.01]	12 [12–12]	3.00 ± 0.24	8.36 ± 0.28	0.046870	Y	7.6 [8.2–7.0]
$E_{co(A)}$	0.04 [0.06–0.03]	16 [16–16]	2.71 ± 0.24	4.94 ± 0.07	0.005560	N	23.1 [24.0–15.6]
$E_{co(B)}$	0.12 [0.12–0.07]	16 [16–16]	3.07 ± 0.24	5.00 ± 0.40	0.059240	N	14.4 [21.2–8.5]
$E_{co(C)}$	0.08 [0.08–0.07]	6 [6–6]	3.00 ± 0.24	8.50 ± 0.45	0.076910	Y	5.9 [6.6–5.1]
$F_{co(A)}$	0.07 [0.10–0.06]	0 [0–0]	2.71 ± 0.24	5.88 ± 0.01	0.006320	N	14.0 [16.7–11.8]
$F_{co(B)}$	0.20 [0.21–0.12]	0 [0–0]	3.07 ± 0.24	5.00 ± 0.56	0.015400	N	14.0 [17.5–11.3]
$F_{co(C)}$	0.20 [0.20–0.16]	0 [0–0]	3.00 ± 0.24	8.57 ± 0.73	0.030760	Y	5.9 [6.6–5.2]

*See **Table 1** for list of the codes used. Mean values with standard deviation (SD represent three samples per experiment) or with the 95% confidence intervals (lower limit and upper limit); μ_{max}, maximal specific growth rate (1/h); LPD, lag phase duration (h); N_0, initial bacterial concentration (log CFU/g); N_{max}, maximum bacterial concentration (log CFU/g); RSS, residual sum of square of the model; S_{val}, spoilage values of 7.00 log CFU/g [Y (yes) or N(not) if this value is reached during the 13-days shelf life]; MSL, predictions of the minimal shelf life for the product (days).*

microbial shelf life. The correlation was higher in mono-culture (−0.8660 to −0.9572) than in co-culture experiments (−0.0339 to −0.9160).

Lag phase duration (*LPD*) of all micro-organisms showed good correlation. High correlations of μ_{max} and *LPD* were observed in FW for co-culture experiments.

N_0 showed little correlations than the two others parameters, except for mono-culture of *Pseudomonas* spp. stored in FW.

Moreover, no obvious correlation has been shown between N_{max} with shelf life for co-cultures experiments.

In conclusion, the results showed in our study that the microbial shelf life of MP samples is mainly correlated with μ_{max} and *LPD* than by N_{max} and N_0. Even if the correlations are lower for experiments carried out in co-culture under MAP.

It was also showed that μ_{max} seems to be mainly influenced by the food packaging (**Table 7**), and by the interaction of the storage conditions applied in this study (packaging and temperature). These results were confirmed by the study of the reduction ratio α (**Figure 5**). *B. thermosphacta* and *L. gelidum* presented a higher reduction in FW. But an increase was observed for *Pseudomonas* spp. in MAP. Indeed, μ_{max} of *Pseudomonas* spp. was 0.04, 0.08, and 0.13, at 4, 8, and 12°C, respectively, in mono-culture experiments. While the parameter was gradually increasing to 0.06 (α = −50.0%), 0.12 (α = −50.0%), and 0.20 (α = −53.8%), at

TABLE 5 | Estimation of the secondary parameters obtained by the square root model without interactions.

Mono-culture experiments		T_{min}	Adjusted T_{min}	μ_{ref}	RSS
FW	*B. thermosphacta*	−3.36	−3.36	0.99 [0.99–0.89]	0.000668
FW	*Pseudomonas* spp.	−5.00	−5.02	0.42 [0.42–0.42]	0.001070
FW	*Ln. gelidum*	+1.00	+1.40	0.39 [0.41–0.39]	0.004580
MAP	*B. thermosphacta*	−3.36	−3.36	0.33 [0.33–0.32]	0.000003
MAP	*Pseudomonas* spp.	−5.00	−5.02	0.24 [0.24–0.24]	0.000323
MAP	*Ln. gelidum*	+1.00	+1.40	0.71 [0.73–0.71]	0.000033
Co-culture experiments					
FW	*B. thermosphacta*	−3.36	–[a]	0.30 [0.35–0.28]	0.000193
FW	*Pseudomonas* spp.	−5.02	–[a]	0.42 [0.44–0.42]	0.000190
FW	*Ln. gelidum*	+1.40	–[a]	0.35 [0.40–0.34]	0.000008
MAP	*B. thermosphacta*	−3.36	–[a]	0.17 [0.24–0.13]	0.000092
MAP	*Pseudomonas* spp.	−5.02	–[a]	0.43 [0.46–0.27]	0.023100
MAP	*Ln. gelidum*	+1.40	–[a]	0.59 [0.61–0.49]	0.000750

Mean values with the 95% confidence intervals (lower limit and upper limit). FW, food wrap; MAP, modified atmosphere packaging (CO_2 30%/O_2 70% ± 0.1%); –[a] not calculated in the model; T_{min}, minimal temperature for growth (°C) provided from scientific literature; Adjusted T_{min}, minimal temperature for growth (°C) provided from adjustment by the Rosso model (°C); μ_{ref}, bacterial growth rate at the reference (1/h) obtained using a reparameterized version of the square root secondary model; RSS, residual sum of square for the μ_{ref} value.

4, 8, and 12°C, respectively, in co-culture experiments. However, N_{max} values of this bacterium were lesser in co-culture than in mono-culture experiments.

Three-Species Interaction Models and Validation Step

Estimated growth parameters and goodness-of-fit indexes for the two developed interaction models are available in **Table 8**.

The Lotka Volterra model showed lower *RrMSE* values but the interaction factors are sometimes included in high intervals.

Simulations provided by the predictive models based on the modified Jameson-effect model and the Lotka Volterra equations are represented in **Figures 6**, **7**.

The modified Jameson-effect model showed the best model performance (*ASZ*), with a mean of 63 ± 23%, while the Lotka Volterra model showed lesser percentages [31 ± 17% (n = 18)]. Eight simulated models based on the equation of the modified Jameson-effect model can be considered as acceptable, because at least 70% of the observed log counts values are within the *ASZ*.

Validation Dataset

As previously described, plate counts in validation dataset increased during the shelf life with increasing the temperature (**Supplementary Figures S5, S6**).

At the end of the shelf life, the natural logarithm of the bacterial count was over 7.0 log CFU/g.

During the storage, a high growth rate and a more rapidly reached stationary phase are also correlated to FW and the highest storage temperatures.

No bacterial growth was observed on PCA for the control samples (limit detection < 3.00 log CFU/g) (data not shown in this paper).

The relative abundance results obtained by metagenetic analysis (expressed in%) at species levels (>1%) are represented in cumulated histograms for validation dataset in **Supplementary Material** for FW (**Supplementary Table S5**) and MAP (**Supplementary Table S6**). The metagenetic data were then combined with the plate counts results in order to obtain estimated bacterial counts (**Supplementary Table S7**).

At day 0, the distribution of read percentages shows high values (>90%) of *Photobacterium* spp., *Photobacterium kishitanii* and *Photobacterium illiopiscarium*.

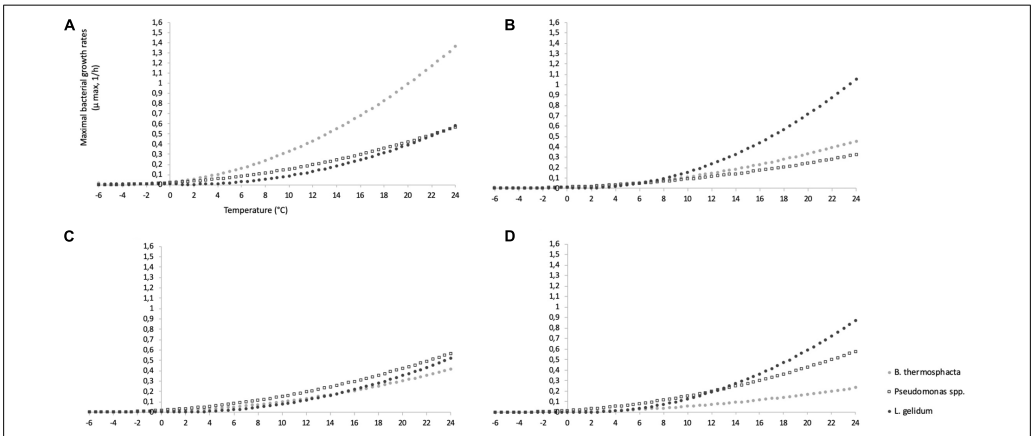

FIGURE 4 | Evolution of μ_{max} between a large range of temperature (from −6 to + 25°C) for mono-culture experiments in FW **(A)** and MAP **(B)**, and for co-culture experiments in FW **(C)** and MAP **(D)**.

TABLE 6 | Correlations between growth parameters and the minimal shelf life (*MSL*) for mono-culture and co-culture experiments.

		Mono-culture experiments			Co-culture experiments		
Bacterial species/ packaging	**Growth parameters**	**Pearsons-correlations (*r*)**	**CI**	***p*-value**	**Pearsons-correlations (*r*)**	**CI**	***p*-value**
FW							
B. thermosphacta	μ_{max}	−0.8660	−0.9715; −0.4771	0.0025	−0.9144	−0.9821; −0.6376	0.0005
	LPD	0.9920	0.9608; 0.9983	1.52^{-07}	0.9839	0.9227; 0.9967	1.71^{-06}
	N_0	0.0188	−0.6534; 0.6745	0.9617	0.1763	−0.5524; 0.7523	0.6500
	N_{max}	−0.9553	−0.9908; −0.7965	5.94^{-05}	0.2151	−0.5238; 0.7693	0.5783
Pseudomonas spp.	μ_{max}	−0.9548	−0.9907; −0.7945	6.17^{-05}	−0.7774	−0.9507; −0.2344	0.0136
	LPD	0.9905	0.9542; 0.9980	2.63^{-07}	0.9013	0.5911; 0.9792	0.0008
	N_0	0.9903	−0.6048; 0.7160	0.7999	0.3903	−0.3696; 0.8373	0.2990
	N_{max}	−0.0675	−0.7002; 0.6245	0.8629	0.0278	−0.6482; 0.6783	0.9434
L. gelidum	μ_{max}	−0.8784	−0.9742; −0.5144	0.0018	−0.9160	−0.9824; −0.6434	0.0005
	LPD	0.9989	0.9948; 0.9997	1.23^{-10}	0.8251	0.3563; 0.9620	0.0061
	N_0	0.0271	−0.6486; 0.6790	0.9448	0.2163	−0.5228; 0.7698	0.5760
	N_{max}	−0.5478	−0.8886; 0.1828	0.1268	−0.0568	−0.6947; 0.6311	0.8846
MAP							
B. thermosphacta	μ_{max}	−0.8819	−0.9750; −0.5258	0.0016	−0.2501	−0.7839; 0.4965	0.5164
	LPD	0.9881	0.9424; 0.9975	5.95^{-07}	0.5490	−0.1811; 0.8890	0.1257
	N_0	0.0411	−0.6405; 0.6864	0.9164	0.5858	−0.1281; 0.8998	0.0973
	N_{max}	−0.9925	−0.9984; −0.9637	1.15^{-07}	−0.4274	−0.8502; 0.3304	0.2511
Pseudomonas spp.	μ_{max}	−0.9572	−0.9912; −0.8047	5.09^{-05}	−0.0339	−0.6827; 0.6446	0.9308
	LPD	0.9549	0.7951; 0.9907	6.10^{-05}	0.3844	−0.3755; 0.8352	0.3070
	N_0	0.0425	−0.6396; 0.6872	0.9134	0.7422	0.1540; 0.9420	0.2202
	N_{max}	−0.9977	−0.9995; −0.9890	1.66^{-09}	0.2979	−0.4565; 0.8031	0.4362
L. gelidum	μ_{max}	−0.9283	−0.9851; −0.6891	0.0003	−0.5587	−0.8919; 0.1675	0.1178
	LPD	0.9424	0.7438; 0.9881	0.0001	0.7049	0.0768; 0.9325	0.0339
	N_0	0.1130	−0.5958; 0.7228	0.7722	0.5667	−0.1561; 0.8942	0.1116
	N_{max}	−0.8983	−0.9786; −0.5806	0.0009	0.3732	−0.3867; 0.8313	0.3225

N_0, the initial bacterial population (log CFU/g); N_{max}, the maximal bacterial population (log CFU/g); LPD, the lag phase duration (h), μ_{max} (the maximum specific growth rate (1/h).

In FW, *Pseudomonas* spp. reached higher values at day 3, and became the most represented bacteria until the end of the shelf-life (>90%). *B. thermosphacta* reached lesser values, with 3.22% at the end of the shelf-life. *L. gelidum* was always under the detection limit. These results are in accordance with those obtained in co-culture experiments.

In MAP, *Photobacterium* spp. was the most represented genus (>90%) during storage. However, low levels of *B. thermosphacta* and *L. gelidum* were observed at 8 and 12°C. *Pseudomonas* spp. was always under the detection limit. These results are different from those obtained in co-culture experiments.

Moreover, pH value of the validation dataset at the end of the shelf-life was statistically different to control samples (7.06 ± 0.80, $n = 7$, p-value = 0.01).

At the same time, the concentration of carbon dioxide also showed higher values than control samples (35.5 ± 1.64, 56.7 ± 2.17, and 96.7 ± 5.57, at 4, 8, and 12°C, respectively).

TABLE 7 | Effect of food storage conditions on the maximal bacterial growth rates (μ_{max}, 1/h) for mono- and co-cultures experiments (analysis of covariance, ANCOVA).

	Effects		
Experiments	**Packaging**	**Temperature**	**Packaging * temperature[a]**
Mono-culture			
B. thermosphacta	0.0113*	0.0003*	0.0001*
Pseudomonas spp.	0.4133	0.7389	0.0050*
L. gelidum	0.1655	0.0015*	0.4331
Co-culture			
B. thermosphacta	0.0280*	0.8072	0.0016*
Pseudomonas spp.	0.3063	0.3564	0.8114
L. gelidum	0.1030	0.1691	0.8728

*[a]Interaction effect of packaging and temperature on bacterial growth rates; *significant statistical effect ($p < 0.05$).*

DISCUSSION

The present study aimed to obtain the growth parameters of three specific spoilage micro-organisms previously isolated in MP samples, and to develop a three-spoilage species interaction model under different storage conditions. *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum* were previously isolated as

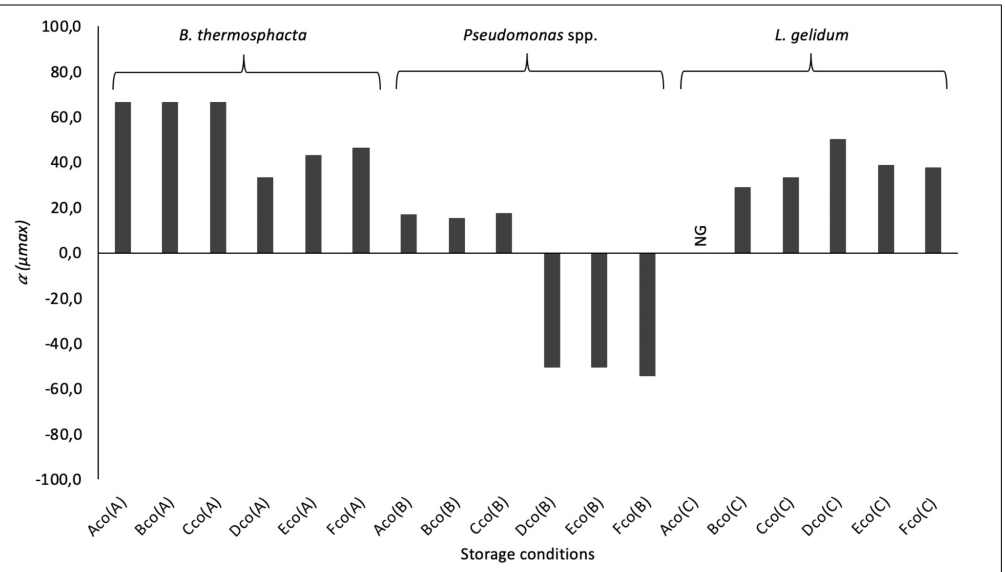

FIGURE 5 | Reduction ratio (α), in%, of the parameters μ_{max} for *B. thermosphacta*, *Pseudomonas* spp., and *L. gelidum* in co-culture experiments at different storage conditions (see **Table 1** for legend). The negative bars represent an increase in co-culture for the specific parameters. No growth of bacteria (NG) was only observed for *Ln. gelidum* in MAP at 4°C.

predominant strains (>50% reads) from different batches of Belgian MP samples at the end of their use-by-date (Cauchie et al., 2019). Considered as the main representative spoilage species in meat and meat products (Koort et al., 2005; Liu et al., 2006; Nychas et al., 2008; Pennacchia et al., 2009, 2011; Andritsos et al., 2012; De Filippis et al., 2013; Casaburi et al., 2014; Stoops et al., 2015; Zhao et al., 2015; Mann et al., 2016; Stellato et al., 2016; Del Blanco et al., 2017; Geeraerts et al., 2017; Raimondi et al., 2018; Li et al., 2019; Mansur et al., 2019; Peruzy et al., 2019), these bacteria were inoculated on irradiated MP samples, in mono- and in co-culture experiments.

However, the selection of dominant and non-dominant species in inoculation experiments could have been more interesting in order to better represent the natural contamination of MP, and thus to better model the impact of sub-dominant microbiota. Indeed, others taxa were also present in MP samples but in lesser abundance, even if they are considered as dominant taxa in several studies: *Photobacterium* spp. (Ast et al., 2007; Bjornsdottir-Butler et al., 2016; Moretro et al., 2016; Nieminen et al., 2016; Kuuliala et al., 2018; Fogarty et al., 2019; Jääskeläinen et al., 2019) and *Lactobacillus* spp. (especially *Lactobacillus algidus*) (Kato et al., 2000; Fadda et al., 2010; Doulgeraki et al., 2012; Dalcanton et al., 2013; Nieminen et al., 2015; Pothakos et al., 2015; Alvarez-Sieiro et al., 2016; Woraprayote et al., 2016; Stefanovic et al., 2017). According to this, they were not included in models of this study, as all others non-dominant microbiota. Moreover, *P. fluorescens* and *P. fragi* were used together in experiments. The objective of this study was to offer an exploratory approach to the proposed method by following the common genus formed by the two species mentioned. So, it would have been interesting to inoculate MP samples with both species in different batches, as behavior of these species is different according to the storage conditions.

The inputs of models were provided from culture-dependent and culture-independent analysis performed on inoculation experiments. The association of both techniques allows us to obtain estimate abundance during storage in co-culture experiments. Although we acknowledge that the plate count method is not able to assess all the microbial populations in presence, the combination of these two methods was previously validated by a qPCR approach (Cauchie et al., 2017). This approach was also used in others studies (Chaillou et al., 2015; Delhalle et al., 2016). Fougy et al. (2016) also showed that this conversion can be used to obtain an extrapolated estimation of the bacterial concentration, and may be used in food industries. But comparison of these results with counts on selective media would also be interesting to study in the future. Moreover, even if this method overestimates the bacterial concentration, it could be beneficial in a worst-case risk assumption for food industries (Crotta et al., 2016; Membré and Boué, 2018).

In this study, models show relatively good fitting indexes (*RrMSE* and R^2). Good performances (*ASZ*) in the three-species interaction approach were also obtained, especially with the modified Jameson-effect model.

TABLE 8 | Estimated growth parameters of the three-species modified Jameson-effect and Lotka Volterra models, with goodness-of-fit indexes.

	Modified Jameson-effect model		Lotka Volterra model						
	RrMSE	μ_{max}	***RrMSE***	F_{ABC}	F_{ACB}	F_{BAC}	F_{BCA}	F_{CAB}	F_{CBA}
$A_{co(A)}$	0.261	0.047 [0.019; 0.076]	0.154	−0.90 [−5.41; −0.19]	−1.10 [−5.13; −0.18]	2.20 [0.92; 2.81]	0.45 [0.35; 1.08]	0.50 [0.19; 1.82]	1.99 [0.54; 5.00]
$A_{co(B)}$	0.273	0.065 [0.031; 0.097]	0.171	−0.90 [−5.41; −0.19]	−1.10 [−5.13; −0.18]	2.20 [0.92; 2.81]	0.45 [0.35; 1.08]	0.50 [0.19; 1.82]	1.99 [0.54; 5.00]
$A_{co(C)}$	0.284	0.039 [0.013; 0.065]	0.199	−0.90 [−5.41; −0.19]	−1.10 [−5.13; −0.18]	2.20 [0.92; 2.81]	0.45 [0.35; 1.08]	0.50 [0.19; 1.82]	1.99 [0.54; 5.00]
$B_{co(A)}$	0.372	0.230 [0.019; 0.380]	0.113	0.05 [−0.02; 0.09]	6.02 [3.53; 6.55]	0.90 [0.85; 0.99]	1.08 [0.67; 1.11]	−5.51 [−5.73; −0.27]	−0.04 [−0.05; −0.03]
$B_{co(B)}$	0.273	0.317 [0.031; 0.485]	0.365	0.05 [−0.02; 0.09]	6.02 [3.53; 6.55]	0.90 [0.85; 0.99]	1.08 [0.67; 1.11]	−5.51 [−5.73; −0.27]	−0.04 [−0.05; −0.03]
$B_{co(C)}$	0.284	0.184 [0.015; 0.327]	0.108	0.05 [−0.02; 0.09]	6.02 [3.53; 6.55]	0.90 [0.85; 0.99]	1.08 [0.67; 1.11]	−5.51 [−5.73; −0.27]	−0.04 [−0.05; −0.03]
$C_{co(A)}$	0.224	0.111 [0.082; 0.140]	0.216	0.11 [0.04; 0.17]	0.38 [0.17; 0.50]	0.62 [0.61; 0.63]	1.15 [1.06; 1.21]	0.78 [0.60; 1.06]	0.12 [0.12; 0.15]
$C_{co(B)}$	0.248	0.136 [0.105; 0.169]	0.294	0.11 [0.04; 0.17]	0.38 [0.17; 0.50]	0.62 [0.61; 0.63]	1.15 [1.06; 1.21]	0.78 [0.60; 1.06]	0.12 [0.12; 0.15]
$C_{co(C)}$	0.250	0.090 [0.062; 0.116]	0.186	0.11 [0.04; 0.17]	0.38 [0.17; 0.50]	0.62 [0.61; 0.63]	1.15 [1.06; 1.21]	0.78 [0.60; 1.06]	0.12 [0.12; 0.15]
$D_{co(A)}$	0.187	0.015 [0.004; 0.028]	0.056	−0.06 [−0.14; 0.15]	−11.08 [−11.08; −3.72]	2.21 [1.80; 2.21]	0.45 [0.45; 0.48]	−5.05 [−5.05; 0.50]	0.13 [−0.32; 0.37]
$D_{co(B)}$	0.186	0.018 [0.004; 0.033]	0.205	−0.06 [−0.14; 0.15]	−11.08 [−11.08; −3.72]	2.21 [1.80; 2.21]	0.45 [0.45; 0.48]	−5.05 [−5.05; 0.50]	0.13 [−0.32; 0.37]
$D_{co(C)}$	0.223	0.064 [0.004; 0.084]	0.083	−0.06 [−0.14; 0.15]	−11.08 [−11.08; −3.72]	2.21 [1.80; 2.21]	0.45 [0.45; 0.48]	−5.05 [−5.05; 0.50]	0.13 [−0.32; 0.37]
$E_{co(A)}$	0.187	0.044 [0.023; 0.095]	0.050	0.26 [−0.24; 0.26]	3.08 [−3.96; 3.08]	4.40 [1.31; 4.40]	0.14 [0.11; 0.75]	−0.28 [−0.28; 3.01]	−0.74 [−0.74; 0.32]
$E_{co(B)}$	0.228	0.039 [0.014; 0.096]	0.094	0.26 [−0.24; 0.26]	3.08 [−3.96; 3.08]	4.40 [1.31; 4.40]	0.14 [0.11; 0.75]	−0.28 [−0.28; 3.01]	−0.74 [−0.74; 0.32]
$E_{co(C)}$	0.186	0.110 [0.055; 0.184]	0.119	0.26 [−0.24; 0.26]	3.08 [−3.96; 3.08]	4.40 [1.31; 4.40]	0.14 [0.11; 0.75]	−0.28 [−0.28; 3.01]	−0.74 [−0.74; 0.32]
$F_{co(A)}$	0.192	0.056 [0.015; 0.095]	0.203	−0.15 [−0.19; 0.02]	−0.11 [−0.20; 0.01]	0.66 [0.40; 0.83]	0.47 [0.43; 0.48]	0.63 [0.60; 0.63]	1.19 [1.19; 1.27]
$F_{co(B)}$	0.228	0.035 [0.010; 0.096]	0.189	−0.15 [−0.19; 0.02]	−0.11 [−0.20; 0.01]	0.66 [0.40; 0.83]	0.47 [0.43; 0.48]	0.63 [0.60; 0.63]	1.19 [1.19; 1.27]
$F_{co(C)}$	0.186	0.100 [0.046; 0.184]	0.221	−0.15 [−0.19; 0.02]	−0.11 [−0.20; 0.01]	0.66 [0.40; 0.83]	0.47 [0.43; 0.48]	0.63 [0.60; 0.63]	1.19 [1.19; 1.27]

*See **Table 1** for the list of codes used. Mean values with the 95% confidence intervals (lower limit and upper limit). RrMSE, the root-mean-square error of the residuals; μ_{max}, the maximum growth rate (1/h); F_{ABC}, F_{ACB}, F_{BAC}, F_{BCA}, F_{CAB}, F_{CBA}, the coefficient of interaction measuring the effects of one species on the others ($_A$, B. thermosphacta; $_B$, Pseudomonas spp.; $_C$, L. gelidum; respectively).*

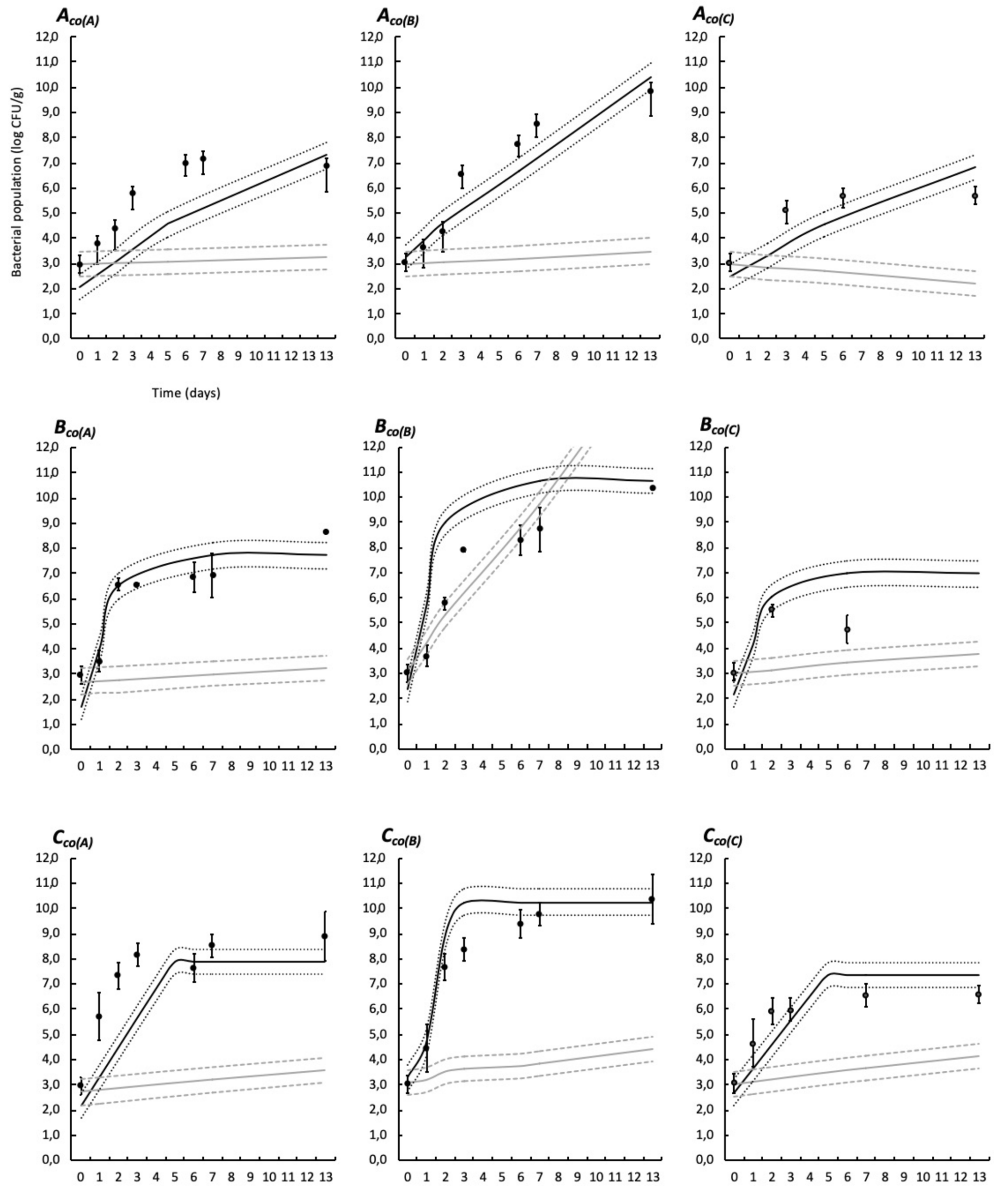

FIGURE 6 | Experimental observed data (validation dataset, means, and standard deviation of the three replicates) and simulations provided by the predictive models based on the modified Jameson-effect equation and on the Lotka Volterra equation in food wrap. See **Table 1** for list of the codes used. Black solid lines represent the Jameson-effect model and gray solid lines represent the Lotka Volterra model. Dashed and dotted lines represent the acceptable simulation zone (ASZ) used to compare observations versus predictions of the interaction models.

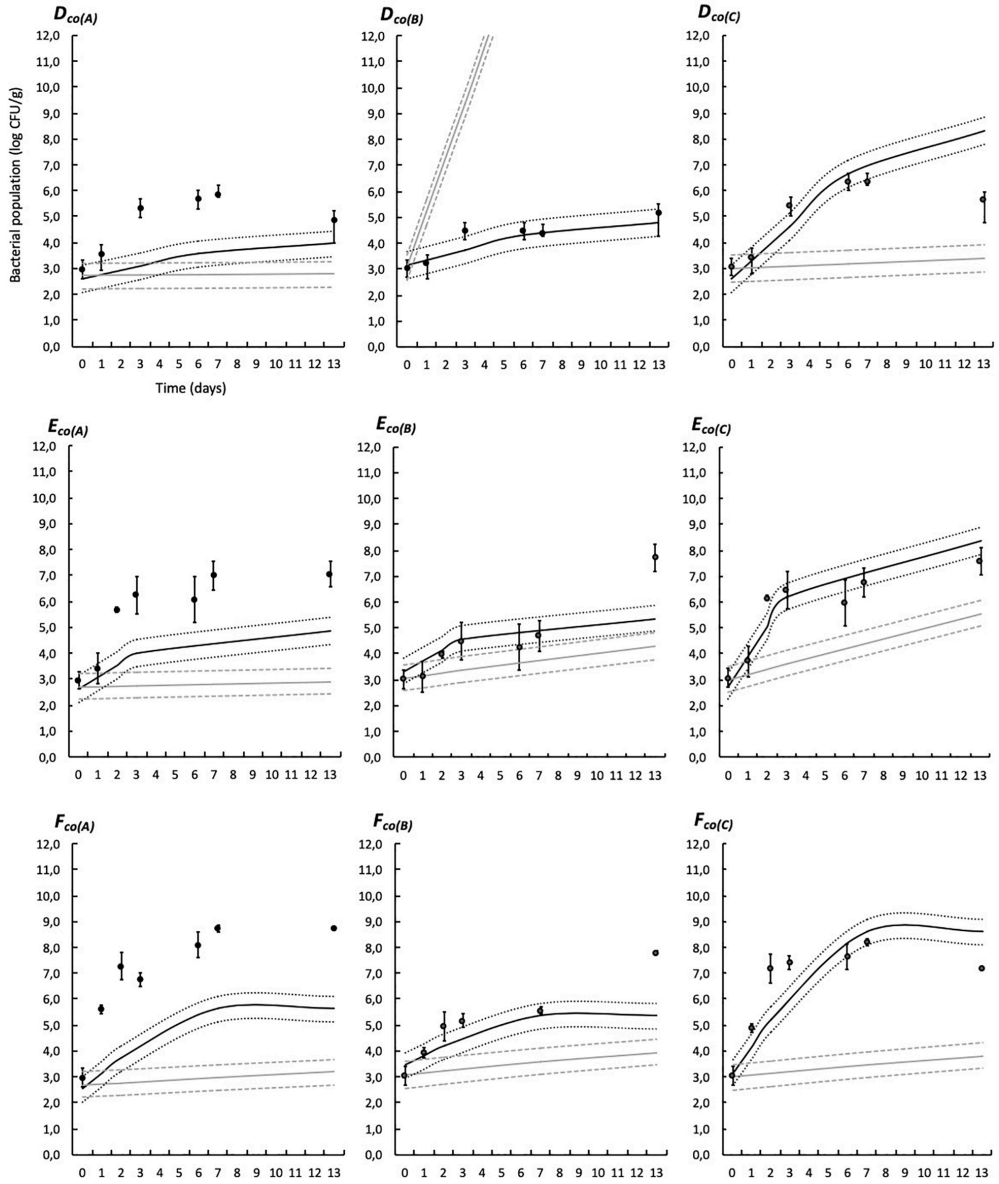

FIGURE 7 | Experimental observed data (validation dataset, means, and standard deviation of the three replicates) and simulations provided by the predictive models based on the modified Jameson-effect equation and on the Lotka Volterra equation in modified atmosphere packaging. See **Table 1** for list of the codes used. Black solid lines represent the Jameson-effect model and gray solid lines represent the Lotka Volterra model. Dashed and dotted lines represent the acceptable simulation zone (ASZ) used to compare observations versus predictions of the interaction models.

The growth parameters of the three specific spoilage micro-organisms were obtained for mono- and co-culture experiments by fittings primary and secondary models (**Tables 4**, **5**). The food packaging shows the highest impact on bacterial growth rates (μ_{max}), which in turn have the strongest influence on the shelf life of food products (Simpson and Carevic, 2004; Stoops et al., 2015; Guillard et al., 2016; Saraiva et al., 2016; Couvert et al., 2017). In accordance with Liu et al. (2006), N_0 showed a little correlation with the microbial shelf life in mono- and co-culture experiments, indicated that the storage outcome of food seems to be not completely determined by the initial microbial counts. Moreover, no obvious correlation has been shown between N_{max} and shelf life in co-cultures experiments. This can be explained by the fact that meat shelf life is determined primarily by the metabolic patterns of the spoilage microbiota, rather than by total counts of bacteria (Liu et al., 2006). However, it can be observed that the parameters obtained in single culture were quite different from those in co-culture, especially for *Pseudomonas* spp. and *B. thermosphacta*. In FW, *B. thermosphacta* grew faster on mono-culture, but this behavior was not detected in co-culture. On the opposite, *Pseudomonas* spp. became the dominant bacteria in FW in the presence of the two others micro-organisms. These differences between mono- and co-culture inoculations have already been observed by Hibbing et al. (2010) and Quinto et al. (2018).

On the other hand, observations in co-culture experiments showed that the suppression of the two other bacteria occurred when the dominant one reached its *MCP*. This result reveals a potential Jameson effect between populations, rather than a prey-predator trend. According to these, differences between mono- and co-cultures experiments could maybe be explained by two hypotheses: (i) a non-specific interaction involving the Jameson effect, where growth inhibition is the result from a depletion in nutrient bioavailability and toxicity increase when the dominant bacteria reaches N_{MCP}; and (ii) a specific interaction due to the modification of the food matrix where bacteria are growing (i.e., catabolism of carbon sources, the production of by products such as carbon dioxide and acids, . . .) (Bruce et al., 2017; Quinto et al., 2018; Correia Peres Costa et al., 2019; Kumariya et al., 2019). Nadell et al. (2016) have mentioned that *P. fluorescens* can produces extracellular matrix materials to give them an advantage over competitors. Quorum sensing could also be related to this inhibition by the dominant bacteria, by exchanging information to synchronize bacterial behavior in mixed-culture (Ng and Bassler, 2009; Dubey and Ben-Yehuda, 2011; Quinto et al., 2018).

The development of a three-spoilage species interaction model was then performed using two models: the modified Jameson-effect and the Lotka Volterra (**Figures 6**, **7**). The modified Jameson-effect model showed slightly better fits than the Lotka Volterra equation, with 40–86% out of the observed counts falling into the *ASZ*, indicating a satisfactory model performance. It only concerns 14–48% for the prey-predator approach. These results can be explained by the fact that the dynamics of experimental and validation datasets seems to follow a Jameson behavior, because the minority bacteria decelerate when the majority one reaches the *MCP* (Cornu et al., 2011). Moreover, the modified Jameson-effect equation is considering growth parameters (μ_{max}, t_{MCP}, and N_0) for modeling (Eq. 19). These parameters are obtained by primary and secondary fittings, and are relatively reliable in our study due to the numbers of samples analyzed. On the other hand, the Lotka Volterra model is based on complex interaction factors (Eq. 21) which are obtained by linear regression. Due to the high variability of interactions that can be simulated, particularly in three or more species models, these interaction factors must necessarily be as accurate as possible. In this study, interaction factors are included in highly variable intervals (**Table 8**), with some variations observed according to the temperature (Moller et al., 2013; Mejlholm and Dalgaard, 2015; Correia Peres Costa et al., 2019). More datasets are probably needed to obtained reliable factors. Also, the Lotka Volterra model could be modified for a more realistic approach by considering the effect of other influencing factors (e.g., environmental conditions such as several storage and packaging conditions, bacteriocin production, etc.) (Powell et al., 2004; Baka et al., 2014).

More inoculation experiments are so needed to develop better predictive models, especially for a three- or more-spoilage species interaction approach. And also, to better understand the dynamics of spoilage bacteria toward each other and in the presence of natural microbiota. As mentioned by Quinto et al. (2018): "it is well known that a spoilage microorganism can either stimulate, inhibit, or have no effect on the growth of the pathogenic species." So, it could be interesting to study interactions between spoilage microorganisms, with production of metabolites or other substances as interaction factors. It would also be interesting to investigate co-culture experiments with two species. Moreover, metabolites production by each of the inoculated bacteria, as inputs interacting models, will be studied in another scientific publication.

Finally, naturally contaminated samples were used to validate the developed models by considering the effect of the food microbiota. Differences with co-culture experiments were obtained: a predominance of *Photobacterium* spp. (>90% of reads) was observed in MAP (**Supplementary Figure S5**). It could be interesting to take also into account this bacterium for modeling interactions. The addition of this bacterium could possibly improve the reliability of predictions, particularly for the Lotka Volterra model. Moreover, *Photobacterium* spp. is not well recovered on PCA at 22°C (Dalgaard et al., 1997; Hilgarth et al., 2018). According to this, improving cultivation methods for this bacterium is important to obtain more reliable results. Further studies are so needed to develop more realistic interacting predictive models, especially in a three- or more-spoilage species interaction approach, and to develop new food preservation process.

CONCLUSION

New omics technologies, such as metagenetics and metabolomics, are important to characterize and to follow the dynamics of bacterial microbiota and metabolites in complex food matrices. New generations of predictive models will probably need to be developed, by considering the results provided by these

techniques. These models will provide a better understanding of the interactions between microorganisms and food, and microorganisms between them.

DATA AVAILABILITY STATEMENT

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de BioProject ID PRJNA590608.

AUTHOR CONTRIBUTIONS

EC did the experiments, interpreted the results, and wrote the manuscript. LD and GB performed the experiments, supervised analyses, and revisited the manuscript. BT, PF, FF, and GD were involved in the design of the study and provide help for interpretation of the results. AT and SB participated to the experiments. NK participated to the interpretation of the results and revisited of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00639/full#supplementary-material

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Conflict of Interest: SB and PF was employed by Quality Partner sa.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

List of publications

The full list of publications and communications of Emilie Cauchie can be accessed at:
https://orbi.uliege.be/browse?type=author&value=Cauchie,%20Emilie%20p083529

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“Everything that has a beginning, has an end.”

- Agent Smith,

Matrix,

Andy and Lary Wachowski, 1999