Study of the microbial diversity of a panel of Belgian artisanal cheeses associated with challenge studies for *Listeria monocytogenes*.

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ABSTRACT

High throughput sequencing could become a powerful tool in food safety. This study was the first to investigate artisanal cheeses from Belgium (31 batches) using metagenetics, in relation to *Listeria monocytogenes* growth data acquired during a previous project. Five cheese types were considered, namely unripened acid-curd cheeses, smear- and mold-ripened soft cheeses, and Gouda-type and Saint-Paulin-type cheeses. Each batch was analyzed in triplicate the first and the last days of storage at 8 °C. Globally, 2,697 OTUs belonging to 277 genera and to 15 phyla were identified. *Lactococcus* was dominant in all types, but *Streptococcus* was co-dominant in smear-

ripened soft cheeses and Saint-Paulin-type cheeses. The dominant population was not always associated with added starter cultures. Bacterial richness and diversity were significantly higher in both types of soft cheeses than in other categories, including particular genera like *Prevotella*, *Faecalibacterium* and *Hafnia-Obesumbacterium* in mold-ripened cheeses and *Brevibacterium*, *Brachybacterium*, *Microbacterium*, *Bacteroides*, *Corynebacterium*, *Marinilactibacillus*, *Fusobacterium*, *Halomonas* and *Psychrobacter* in smear-ripened soft cheeses. A strong correlation was observed between no growth of *L. monocytogenes* in a smear-ripened cheese and the presence of an unknown *Fusobacterium* (relative abundance around 10%). This *in silico* correlation should be confirmed by further experiments *in vitro* and *in situ*.

Keywords

Metagenetics; cheese; bacteria; 16S rRNA gene; ecology; challenge studies.

1. Introduction

Cheese is one of the oldest dairy and fermented products, and was already produced 8,000 years ago in the Middle-East (Gobbetti et al., 2018b). Nowadays, more than 1,200 cheese varieties could be found worldwide, varying in terms of texture, aspect, aroma and flavor (Barthelemy and Sperat-Czar, 2001; Tilocca et al., 2020). Although some cheese varieties from France, Italy and Latin America have been extensively studied and registered as protected designation of origin (PDO), Belgian cheeses remain relatively unknown. However, cheese production is well established in Belgium, with more than 250 artisanal cheese producers and several famous industrial cheese factories (personal communication). Artisanal cheeses are essentially handmade in farms and using raw milk (Kamimura et al., 2020). Raw milk cheeses present more pronounced tastes and flavors than cheeses produced from heat treated milk (Yoon et al., 2016). In addition to sensorial and technological roles, microbiota of raw milk cheeses could play an antagonistic role against foodborne pathogens, including *Listeria monocytogenes* (Choi et al., 2020; Yoon et al., 2016). Cheese microbiota originates from two major sources, namely inoculated microorganisms and resident microbiota (Afshari et al., 2020). According to Dugat-Bony et al. (2016), inoculated microorganisms represent less than 50% of cheese microbiota, but this proportion is influenced by the type of cheese and the type of milk used for manufacture. The remaining part of the population is composed of the resident microbiota. The structure of the latter is influenced by a lot of factors, including raw milk microbiota (governed itself by farming practices), people working in the workshop, water- and airflows, production tools, surfaces, wooden shelves and natural ripening cellars (Irlinger et al., 2015).

Despite aforementioned advantages, raw milk cheeses have commonly been identified as potential vectors of *L. monocytogenes* (Gérard et al., 2018). As a consequence, several

63 listeriosis outbreaks associated with contaminated samples occurred worldwide
64 (Martinez-Rios and Dalgaard, 2018). During a previous project, challenge studies were
65 performed in order to determine the growth potential (δ) of *L. monocytogenes* (*i.e.* the
66 difference between final and initial levels of the pathogen during storage at 8 ± 1°C) in 32
67 Belgian artisanal cheeses (Gérard et al., 2020a). For some batches of soft and semi-hard
68 cheeses, an unexpected decrease of the levels of the pathogen during shelf-life was
69 observed. Physicochemical characteristics of the samples did not allow to explain this
70 inhibition.

71 A hypothesis was that resident microbiota of these cheeses acted as an inhibitor on
72 *L. monocytogenes*. For a long time, food microbiota has been exclusively studied using
73 classical culturing methods, missing the presence of all non-culturable microorganisms,
74 and underestimating its exceptional diversity (Afshari et al., 2020; Bozoudi et al., 2016).
75 The emergence of next generation sequencing (NGS) technologies allowed a huge
76 revolution in deciphering food microbiota, including cheese (Afshari et al., 2020).
77 Although NGS technologies were already used to characterize diverse food matrices,
78 their use in food safety remains an emerging trend (Weimer et al., 2016). The presence of
79 some particular bacterial species could be a clue to predict the ability of foodborne
80 pathogens, including *L.* monocytogenes, to grow or to be inhibited (Jagadeesan et al.,
81 2019).

82 Recently, various studies on the microbial diversity of multiple cheese varieties have
83 been conducted in diverse parts of the world, including Bola de Ocosingo (Mexico),
84 Cheddar (USA), Livanjski (Czech Republic), Mozzarella (Italy), Rushan (China) and
85 Serra da Canastra (Brazil) (Aldrete-Tapia et al., 2018; Choi et al., 2020; Kamimura et al.,
86 2020; Marino et al., 2019; Vladimir et al., 2020; Xue et al., 2018). To our knowledge, the
87 only Belgian cheese which has already been studied using metagenetics is Herve cheese,

which is the only Belgian cheese registered as PDO (Delcenserie et al., 2014). However, a lot of other products from Belgium deserve more attention.

The main aim of this study was to acquire an in depth knowledge of the microbiota of cheese varieties previously analyzed by challenge studies by Gérard et al. (2020a). For this purpose, the exact same batches as those used during challenge studies were considered. Potential correlations between the presence of bacterial taxa and δ of *L. monocytogenes* evaluated during these challenge studies were also explored, as a first approach.

2. Material and methods

2.1 Sampling and cheese definition

Based on previous knowledge acquired on Belgian artisanal cheeses (Gérard et al., 2020b), a classification into five major varieties was used during this study (see description in Table 1), based on manufacturing practices and final characteristics of the products, namely (a) unripened acid-curd cheeses (UACC), cheeses shaped or not, produced by lactic acidification and consumed without aging, *i.e.* fresh, (b) smear-ripened soft cheeses (SRSC), unpressed cheeses undergoing a short ripening period during which the product is regularly washed, resulting in a typical red to orange rind, (c) mold-ripened soft cheeses (MRSC), unpressed cheeses seeded with *Penicillium* spores resulting in a white rind, (d) Gouda-type semi-hard cheeses (GSHC), uncooked pressed cheeses of high weight (> 10 kg) and surrounded by an artificial coating and (e) Saint-Paulin-type semi-hard cheeses (SPSHC), uncooked pressed cheeses with a lower weight (typically 1.0-2.5 kg) and a natural crust. Both types of semi-hard cheeses have moisture on a fat-free basis (MFFB) higher than 54%. Hard cheeses (*i.e.* MFFB < 54) and blue-veined cheeses were not considered in this study, as these types are not common in Belgium. Cheeses

were considered as artisanal when they were transformed by hand directly in farms. Studied batches were distributed as follow: (a) 11 UACC, (b) 4 SRSC, (c) 4 MRSC, (d) 4 GSHC and (e) 8 SPSHC. All batches considered in the present paper are the same as those used in a previous study, published as Gérard et al. (2020a). Samples were collected from different farms, directly after production or after ripening, respectively for UACC and ripened cheeses, corresponding to day-0 in the following parts of this article. Each collected batch was composed of at least 12 cheese wheels.

2.2 Microbial challenge tests for L. monocytogenes

Gérard et al. (2020a) performed challenge studies for *L. monocytogenes* in cheese, in agreement with available guidelines and recommendations (EURL *Lm*, 2014; Federal Agency for the Safety of the Food Chain (FASFC), 2016). This part, as well as parts 2.3 to 2.5 are presented as a reminder of the methodology developed during the previous study of Gérard et al. (2020a). Among the 12 cheeses collected per batch, six were inoculated at a level of 100 cfu/g with a pool of three *L. monocytogenes* strains isolated from dairy products (12MOBO53LM, 12MOBO96LM and 12MOBO98LM) and provided by EURL *Lm*. Briefly, cryobeads containing each strain were suspended in 9 ml of brain heart infusion and stored at 37 °C for 18 h. These cultures were diluted 1:100 in brain heart infusion and stored for 7 days at 7 °C. Strains were then pooled in equivalent amounts. The six non-inoculated samples were used as control samples. The pathogen was inoculated in cheese cores using a syringe, except for SRSC and MRSC, for which the inoculum was divided between core and rind. For each batch, three controls and three inoculated cheeses were analyzed at day-0 (see section 2.3 and 2.4), while remaining cheeses were stored at 8 ± 1 °C until end of shelf-life. At this time point, the same analyses were performed. Shelf-life of 14 and 30 days was considered for UACC and ripened cheeses, respectively.

137 *2.3 Sample preparation*

138 Samples of 25 g of cheese, comprising both core and rind, were diluted 10-fold in
139 trisodium citrate (81 g of trisodium citrate + 4050 ml of purified water) and homogenized
140 using Stomacher 400 (Seward, Worthing, United Kingdom). Ten ml of this suspension
141 were kept at -80 °C until DNA extraction. The remaining volume was used for
142 microbiological enumerations.

143 *2.4 Microbiological enumerations*

144 *L. monocytogenes* was enumerated in samples at day-0 and end of shelf-life, using
145 RAPID'*L. mono* method, detailed by Gérard et al. (2020a). Total microbiota was
146 enumerated after pour-plate inoculation of 1 ml of cheese suspension with 15 ml of plate
147 count agar (Bio-Rad, Hercules, CA, USA), incubated at 22 °C for 72 h, as adapted from
148 ISO 4833-1:2013 method (International Organization for Standardization, 2013). LAB
149 counts were determined by pour-plate inoculation with 15 ml of De Man, Rogosa and
150 Sharpe agar (Tritium Microbiologie, Eindhoven, Netherlands), following the same
151 incubation scheme (International Organization for Standardization, 1998).

152 *2.5 δ calculation*

153 δ was calculated according to guidelines provided by EURL *Lm* (2014) and as described
154 by Gérard et al. (2020a), *i.e.*"as the difference between the median contamination at use-
155 by-date and the median contamination at day-0, expressed as $\log_{10}$ cfu/g".

156 *2.6 DNA Extraction*

157 For each batch, DNA was extracted from three samples at day-0 and three samples at the
158 end of shelf-life, using Fast DNA SPIN Kit with CLS-TC, from 200 µl of cheese
159 suspension (MP Biomedicals, Santa Ana, CA, USA). DNA concentration and quality

were checked using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracts were stored at -18 °C until use.

2.7 Libraries preparation and sequencing

Libraries were prepared under accreditation ISO 17025 by amplifying V1-V3 regions of the 16S rRNA bacterial gene. Sequences of forward and reverse primers, with overhand adapters, used during this study were 5'-GAGAGTTTGATYMTGGCTCAG-3' and 5'-ACCGCGGCTGCTGGCAC-3', respectively. Amplicons were purified using Agencourt AMPure XP bead kit (Beckman Coulter, Pasadena, CA, USA), indexed using Nextera XT index primers 1 and 2 (Illumina, San Diego, CA, USA), quantified by Quant-IT PicoGreen (Thermo Fisher Scientific, Waltham, MA, USA), and diluted to a concentration of 10 ng/µl. Each DNA sample was then quantified by qPCR with KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). Finally, samples were normalized, pooled and sequenced using Illumina MiSeq technology with v3 reagents (Illumina, San Diego, CA, USA), using paired end reads, by GIGA Genomics platform (Liège, Belgium). A co-sequencing of a mock community was conducted in order to assess error rate due to biases introduced during PCR and sequencing steps. Mock community was composed of a known proportion of *Carnobacterium maltaromaticum*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc carnosum*, *Pseudomonas aeruginosa* and *Streptococcus thermophilus*. For all sequencing runs, expected proportions of these bacteria were found. Negative controls were also used during DNA extraction and library preparation, and sequenced.

2.8 Bioinfiormatics

Sequence reads were processed using respectively Mothur v1.44.3 and VSearch for alignment, clustering and chimera detection (Rognes et al., 2016; Schloss et al., 2009).

Sequences were clustered into operational taxonomic units (OTUs) at 97% of identity. SILVA 138 database of full-length 16S rDNA gene sequences was used for alignments of unique sequences and taxonomical assignations (Quast et al., 2013). Finally, cleaned sequences were rarefied to 6,000 reads per sample. All sequence reads are publicly available on the website of National Center for Biotechnology Information (NCBI) under the Bioproject ID PRJNA672908.

2.9 Statistics

All statistical analyses were performed at the genus level, as identification at the species level based on short 16S rRNA gene sequences should only be considered carefully. Regarding α-diversity, ecological indicators, namely Goods's coverage, the number of genera, Chao1 estimator of richness, reciprocal Simpson diversity index and Simpson evenness were calculated using Mothur v1.44 (Schloss et al., 2009). For bacterial enumeration and α-diversity indicators, statistical differences between groups were identified by Kruskal-Wallis tests, using Minitab 17 (State College, PA, USA). Barplots were built using Microsoft Excel (Redmond, WA, USA), including only genera with relative abundance > 1% in at least one type of cheese at day-0 or end of shelf-life. Structure of the subdominant and minor communities, or β-diversity, was assessed using Yue and Clayton Theta dissimilarity matrices built using Mothur, taking into account the proportions of both shared and non-shared genera from the populations, and not comprising the dominant genera, *i.e. Lactococcus* and *Streptococcus* (Yue and Clayton, 2005). Non-metric multidimensional scaling (NMDS) was performed using Mothur, and considered as satisfying when stress value was < 0.20. Finally, plots were built using RStudio and R package ggplot2 (Wickham, 2016; RStudio Team, 2020). AMOVA were performed in order to reveal eventual significant population structure differences, using Mothur. For SHC and SRSC, in order to look for correlations between δ of

L. monocytogenes, calculated during challenge studies, and the presence of specific bacterial genera, canonical correspondence analyses were performed, using R package vegan (Oksanen et al., 2019). Observations were confirmed by building Spearman correlation matrices with R and FDR corrections for multitesting. Permutation tests were performed using R package wPerm (Weiss, 2015).

3. Results

3.1 Bacterial enumerations

Total microbiota at 22 °C and LAB at 22 °C were enumerated in all samples. Bacterial counts by type of cheese are summarized in Table 2 (averages ± standard deviations). In all types of cheese, level of total microbiota was comprised between 7.0 and 8.2 $\log_{10}$ cfu/g, on average, at both day-0 and end of shelf-life. Total and LAB counts were the lowest in GSHC at day-0. Both levels were significantly higher in UACC than in MRSC and GSHC. At end of shelf-life, levels did not differ significantly between types. A significant difference was observed between the levels of total microbiota in UACC at day-0 and end of shelf-life. The majority of the total microbiota was thus composed of LAB, with enumerations of at least 6.9 $\log_{10}$ cfu/g. At day-0, *L. monocytogenes* levels were always comprised between 1.48 and 2.71 $\log_{10}$ cfu/g. Globally, at end of shelf-life, final contamination was comprise between < 1 and > 7 $\log_{10}$ cfu g. A conclusion of challenge studies was that contamination systematically decreased during storage of UACC at 8±1 °C. Globally, both types of soft cheeses, *i.e.* SRSC and MRSC, allowed the growth of *L. monocytogenes*, but at different extents. Maximal levels reached in SRSC (around 4 $\log_{10}$ cfu/g) were lower than in MRSC (up to > 7 $\log_{10}$ cfu/g). An exception was observed for batch SRSC1, in which levels of the pathogen decreased during shelf-life. In GSHC and SPSHC, final levels were generally

lower than 3 $\log_{10}$ cfu/g, but huge inter-farms, inter-batches and intra-batch variability was observed.

3.2 α-diversity

α-diversity metrics, including number of observed genera, Chao1, reciprocal Simpson index and Simpson evenness, were used to assess community richness and diversity. Results are summarized in Table 3 for each type of cheese. For all samples at day-0 and end of shelf-life, Good's coverage was > 0.99, meaning that although the number of generated sequence reads (*i.e.* 6,000) was limited, this sampling effort allowed to produce an accurate caption of cheese microbial communities. For all types of cheese, no significant differences in bacterial richness and diversity were observed between samples at day-0. Regarding richness, at the end of shelf-life, the number of genera was significantly higher in soft cheeses (MRSC and SRSC), in comparison with all other types of cheese. Chao1 richness indicator confirmed this observation for SRSC at the end of shelf-life. Regarding diversity, reciprocal Simpson index enlightened the same conclusion. No significant differences were observed at day-0, regarding Simpson evenness but, at the end of shelf-life, significant differences were observed between soft cheeses and other types. Between day-0 and end of shelf-life, significant differences were observed for MRSC and SRSC regarding Simpson evenness.

3.3 Cheese microbiota

Challenge studies performed in accordance with EURL *Lm* (2014) guidelines require two sampling times, namely day-0 and end of shelf-life. Cheese microbiota was thus studied at these end points, in the exact same batches as in published paper of Gérard et al. (2020a). Overall, 1,107,561 reads were obtained after treatment of raw data in cheeses sampled at day-0 and end of shelf-life, and clustered into 2,697 OTUs, belonging to 277

genera and 15 phyla. Ninety-eight genera were common between samples from day-0 and end of shelf-life. One hundred and twenty-four and 55 unique genera were observed at day-0 and end of shelf-life, respectively. Only five phyla represented more than 1% of sequence reads in at least one type of cheese at day-0 or end of shelf-life, namely Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and Fusobacteria. Barplots of the bacterial genera in all types of cheese are presented in Fig. 1. For clarity and readability improvement, only genera with relative abundance > 1% in one type of cheese at day-0 and/or end of shelf-life were plotted. Supplementary files 1-5 show plots for individual samples.

3.3.1. Dominant microbiota

Bacteria from the genus *Lactococcus* were dominant in all types of cheese, at both day-0 and end of shelf-life. Most of these sequences corresponded to *Lactococcus lactis*, a major starter culture. A co-dominance of *Lactococcus* with *Streptococcus* (relative abundance > 25%) was observed in SRSC and SPSHC. A majority of *Streptococcus* sequences were linked to *S. thermophiles*.

Regarding UACC, GSHC and SPSHC, no other genera with relative abundance > 1% were observed. For the latter types of cheese, relative abundances of the dominant/co-dominant genera, *i.e. Lactococcus* and *Streptococcus*, were higher at end of shelf-life than at day-0. In SPSHC, cumulative proportion of both genera was 98.0 ± 3.5% and 99.0 ± 1.2% at day-0 and end of shelf-life, respectively. Nevertheless, 101 genera were observed in SPSHC at day-0, while only 40 were identified in GSHC (27 in common). At the end of shelf-life, only 38 genera were observed in each type of semi-hard cheese (19 in common). In contrast, relative abundances of *Lactococcus* and *Streptococcus* were lower at end of shelf-life than at day-0 in both types of soft cheeses.

3.3.2. NSLAB

Major NSLAB observed during this study included species from genera *Enterococcus*, *Lactobacillus* (including newly described genera *Companilactibacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Levilactobacillus* and *Ligilactobacillus*), *Lactococcus*, *Pediococcus*, *Streptococcus* and *Weissella*. Proportions of these genera were variable between cheese types, but often < 1% of relative abundance.

3.3.3. Other genera with relative abundance > 1%

Bifidobacterium, mainly *Bifidobacterium animalis* subsp. *lactis*, were observed at day-0, in all types of cheeses, but were not detected anymore at end of shelf-life.

Although *Lactococcus* and *Streptococcus* were (co-)dominant in SRSC and MRSC, additional genera with a relative abundance > 1% were observed in soft cheeses, including *Prevotella* (4.0 ± 13.7%; 1 cheese out of 4), *Faecalibacterium* (3.3 ± 9.9%, 1/4) and Lachnospiraceae family (1.0 ± 2.6%, 1/4) in MRSC, and *Brevibacterium* (11.3 ± 26.3%, 1/4), *Brachybacterium* (3.4 ± 7.7%, 2/4), *Microbacterium* (2.3 ± 5.8%, 2/4), *Bacteroides* (1.9 ± 6.3%, 2/4) and *Staphylococcus* (1.7 ± 5.2%, 3/4) in SRSC. In MRSC, *Prevotella*, *Faecalibacterium* and Lachnospiraceae were not observed at end of shelf-life samples. On the opposite, relative abundances of the genera *Hafnia-Obesumbacterium* (from 0.0 ± 0.1% to 15.5 ± 25.4%, 3/4) and *Enterococcus* (from undetected to 2.0 ± 4.1%, 3/4) were increased. In SRSC, *Bacteroides* was not detected anymore at the end of shelf-life, while RA of *Staphylococcus* fell to 0.2 ± 0.3%. *Corynebacterium* (2/4), *Marinilactibacillus* (4/4), *Fusobacterium* (1/4), *Halomonas* (1/4) and *Psychrobacter* (4/4) reached relative abundances > 1% at the end of shelf-life. In

addition to that, variability between some triplicates from a given batch was sometimes observed (see Supplementary files 1-6).

3.3.4. Foodborne pathogens

Regarding the detection of potential foodborne pathogens, metagenetics allowed to observe *L. monocytogenes*, *Escherichia coli* and *Staphylococcus* spp.

Using metagenetics based on V1-V3 regions of 16S rRNA gene sequencing and sampling effort of 6,000 sequences, *L. monocytogenes* was only detected in seven MRSC samples, at end of shelf-life. All types of cheese put together, eight OTUs associated to *Staphylococcus* were observed, including *Staphylococcus aureus* (10 reads) and *Staphylococcus equorum* (2,181 reads). *E. coli* was observed in 24 samples, mainly from UACC type, but generally at low levels (< 5 reads/sample).

3.3.5. Observation of unexpected bacterial genera

More surprising bacteria were also observed during this study. In three SRSC samples from the same factory, a huge proportion of an unknown species from the genus *Fusobacterium* has been observed, *i.e.* 12.18% of all sequence reads. Four OTUs from the genus *Ralstonia* were also observed in all types of cheese at day-0 and end of shelf-life, including *R. pickettii*.

3.4. β-diversity

Community structure, or β-diversity, was assessed not considering the two dominant bacterial genera, *i.e. Lactococcus* and *Streptococcus*, as their important weight in the analysis would have masked the potential differences between subdominant and minor communities. NMDS and AMOVA revealed an influence of the time of sampling on subdominant community structure in SRSC, MRSC, SPSHC and GSHC (Fig. 2C-F; all p-

values < 0.001). Subdominant community structure of UACC did not significantly vary during shelf-life (p-value = 0.160). Subdominant community structure was also compared between types of cheese. At day-0, few significant differences were observed, namely SPSHC *vs.* MRSC (p = 0.003) and SPSHC *vs.* UACC (p = 0.002). At end of shelf-life, subdominant community structure was more different between types of cheese, with all pairwise tests with p-values < 0.002, excepting for GSHC *vs.* SPSHC and GHSC *vs.* UACC, for which no significant differences were observed (Fig. 2A). Consequently, it appeared that the differentiation in cheese community structure occurred during storage at 8 °C.

3.5. Correlation between growth potential of L. monocytogenes and resident microbiota

Canonical correspondence analyses were performed in order to look for correlations between δ of *L. monocytogenes*, calculated from challenge studies (Gérard et al., 2020a), and the presence of specific genera identified using metagenetics. As a reminder, in this previous paper, it was reported that three batches of SRSC from a unique farm did not allow the growth of *L. monocytogenes*, with all δ comprised between -1.05 and -1.68 $\log_{10}$ cfu/g, from an initial contamination of approximatively 2 $\log_{10}$ cfu/g. A high inter-farm variability in δ values was also observed for both types of SHC. Canonical correspondence analysis triplots did not allow the identification of relevant correlations between δ of *L. monocytogenes* in SHC and the presence of particular bacterial genera. Canonical correspondence analysis triplot for SRSC was more interesting (Fig. 3). The three samples in which the pathogen was unable to grow (9-10-11) are clearly separated from other cheeses and located on the left part of the plot. Based on graphical representation, it seems that the inability of *L. monocytogenes* to grow in SRSC could be correlated to the dominance of *Lactococcus*. No growth of *L. monocytogenes* was also associated to the presence of the genera *Alkalibacterium*, *Arcobacter*,

Clostridiisalibacter, *Fusobacterium*, *Marinilactibacillus*, *Pseudoalteromonas*, *Psychrilyobacter* and *Staphylococcus*. Spearman correlation coefficients calculated with permutation tests confirmed that four of these genera were significantly correlated with the no growth of *L. monocytogenes*, namely *Lactococcus*, *Psychrilyobacter*, *Fusobacterium* and *Alkalibacterium*.

4. Discussion

4.1. Bacterial enumerations

Enumeration of total microbiota and LAB reached expected levels. Indeed, comparable values were reported by Delcenserie et al. (2014) and Kamimura et al. (2020) in Herve and Serra da Canastra, respectively. In cheese, LAB represent a majority of total microbiota. Most LAB generally come from starter cultures (SLAB), but non-starter LAB, known as NSLAB, are frequent (Choi et al., 2020). NSLAB are mainly facultative hetero-fermentative bacteria, including *Lacticaseibacillus* spp. (comprising species previously known as *Lactobacillus casei*, *Lactobacillus paracasei* or *Lactobacillus rhamnosus*) and *Lactiplantibacillus* spp., playing important roles in the development of cheese aromas and flavors (Choi et al., 2020; Zheng et al., 2020).

4.2. Cheese microbiota

4.2.1. Dominant microbiota

Lactococcus were dominant in all cheese types, but *Streptococcus* was co-dominant in SPSHC and SRSC. For the latter type of cheese, this observation was quite surprising. From Table 1, it can be seen that *S. thermophilus* was not used as starter culture during manufacture of SRSC, although it was the case during SPSHC production. From these facts, it should be said that dominant microbiota is not necessarily linked to selected

starter cultures. Regarding cheese dominant microbiota reported in the literature, Aldrete-Tapia et al. (2018) and Falardeau et al. (2019) observed the dominance of *S. thermophilus* in Bola de Ocosingo and Gruyere, respectively, while a dominance of *L. lactis* in Brie, Cheddar, cores of Epoisses, Herve, Jarlsberg and rinds of Saint-Marcellin was also reported (Delcenserie et al., 2014; Dugat-Bony et al., 2016; Falardeau et al., 2019). In Gouda cheese, Oh et al. (2016) reported only a really low relative abundance of the *Streptococcus* genus (< 0.1%). This is not in accordance with the present study, as the genus *Streptococcus* represented 2.0 ± 3.0% of the reads in GSHC at day-0 and end of shelf-life. Nevertheless, it can be observed that, from identical starter culture in GSHC and SPSHC, different bacterial profiles were obtained. A hypothesis to explain the dominance of *Streptococcus* in some samples could be the inhibitive effect of salt on the growth of *Lactococcus* (Ceugniez et al., 2017). Another one could be the influence of the temperature during cheese production, as *S. thermophilus* is a thermophilic LAB. Nevertheless, no (half-) cooked cheeses were included in this study. *Lactococcus* spp. and *Streptococcus* spp. are part of the dominant microbiota of raw milk (1-4 $\log_{10}$ cfu/g) and of the major commercial starters available for cheese production (Aldrete-Tapia et al., 2018; Tilocca et al., 2020). Kamimura et al. (2020) suggested that *Lactococcus* and *Streptococcus* are the most adapted genera regarding physicochemical conditions met during cheese production, ripening and storage. In Gruyere and Comte, a co-dominance of *Streptococcus* with *Lactobacillus* was already observed (Wei et al., 2016), but *Lactobacillus* was never in dominant position in our samples. During a study on Rushan cheese, Xue et al. (2018) identified *Acetobacter* and *Acinetobacter* as (co-)dominant genera but, in the present study, these genera were either not detected or had a really low relative abundance (< 0.1%), respectively. Another SLAB, *Leuconostoc*, mainly *Leuconostoc pseudomesenteroides*, was observed in all types of cheese at both sampling

400 points, but as a part of the subdominant population. It was also the case in Gouda cheese,
401 in which *Leuconostoc* represented around 1% of the sequences (Oh et al., 2016).
402 Although *Leuconostoc* is included in most commercial starters as citrate fermenter, it was
403 not used during manufacture of GSHC (Gobbetti et al., 2018a).

404 Regarding semi-hard cheeses, it was observed that bacterial richness was much lower in
405 GSHC (40 genera) than in SPSHC (101 genera). The coating around GSHC prevented the
406 development of surface microbiota, explaining these differences. Both types of semi-hard
407 cheese had a poorly diversified microbiota at the end of shelf-life, with only 38 observed
408 genera in total. In Edam, another semi-hard cheese similar to Gouda, genera *Acetobacter*,
409 *Alcaliphilus*, *Bacillus*, *Cellulomonas* and *Propionibacterium* were part of the
410 subdominant microbiota (Nalepa et al., 2020), but none of these taxa were observed in
411 SPSHC and GSHC from the present study.

412 4.2.2. NSLAB

413 Many genera of NSLAB were identified during this study. All these genera remained
414 subdominant or minor in our samples, but their presence in cheese was not surprising, as
415 NSLAB are part of natural raw milk microbiota. They have also been isolated from
416 cheese production environment (Choi et al., 2020).

417 4.2.3. Other genera with relative abundance > 1%

418 As detailed in part 3.3.3., *Bifidobacterium* were observed in all cheese types. Bacteria of
419 the latter genus are known for their probiotic properties (Demers-Mathieu et al., 2016).
420 Demers-Mathieu et al. (2016) mentioned that some *Bifidobacterium* species, including
421 *B. animalis* subsp. *lactis*, could survive in Cheddar up to several months of ageing and
422 storage. Delcenserie et al. (2013) discovered two *Bifidobacterium* species able to grow
423 during ripening of French cheeses, namely *B. crudilactis* and *B. mongoliense*, but the

latter species were not detected in our samples, and the genus was not identified anymore at end of shelf-life.

In SRSC and MRSC, subdominant microbiota was composed of several additional genera, at both day-0 and end of shelf-life, but differences were observed according to the cheese varieties. This inter-farm diversity is known as the *terroir* effect, and is a major characteristic of artisanal cheeses (Turbes et al., 2016). Nevertheless, this concept is questionable, as an opposed idea, observed by Wolfe et al. (2014), suggests that reproducible rind microbial communities could be found on cheese samples collected from various parts of the world. In other words, the impact of fermentation phenomena on cheese microbial composition could be greater than the geographical influence. Differences between cheeses within a given batch highlight the intrinsic variability of an artisanal production process, as well as the variability introduced by the sampling procedure. These variations could also be introduced by the sampling effort of 6,000 sequence reads per sample used in this work.

Most subdominant genera in SRSC and/or MRSC samples were already observed in cheese. *Brevibacterium* had an important relative abundance (> 10% at day-0 and end of shelf-life) in SRSC. Bacteria from these genera are rind colonizers, especially *Brevibacterium linens*, which is responsible for the red-orange color of SRSC rinds and was used as ripening starter in SRSC manufacture (Fox et al., 2017; Wei et al., 2016). *Staphylococcus* and *Micrococcus* also contribute to this aspect by producing pigments (Ceugniez et al., 2017). As already mentioned, *Staphylococcus* was observed in SRSC samples during this study, but it was not the case of *Micrococcus*. As alkalophiles, the presence of the genera *Corynebacterium* and *Brachybacterium* on the surface of washed rind cheeses is common, provided that this environment is de-acidified due to the metabolic activities of yeasts and moulds (Wei et al, 2016). In this study, relative

449 abundance of *Corynebacterium* was relatively low, especially at day-0 (0.1 ± 0.4% in
450 SPSHC and 0.2 ± 0.4% in SRSC), but was increased in SRSC at the end of shelf-life
451 (1.2 ± 2.2%). *Brachybacterium* were part of the subdominant population of SRSC, with
452 relative abundance of 3.4 ± 7.7% and 3.5 ± 7.5% at day-0 and end of shelf-life,
453 respectively. *Marinilactibacillus* (mainly *M. psychrotolerans*) and *Halomonas* are
454 halotolerant bacteria that were part of the subdominant microbiota of SRSC. They were
455 identified for the first time in seawater, and their presence in cheese can be attributed to
456 cross-contaminations during brining or salting (Yunita et al., 2018). *Halomonas* has often
457 been identified in short ripening cheeses, and could play important functions during
458 ripening (Quijada et al., 2018). *M. psychrotolerans* was already observed in Herve and
459 Munster, two red smear cheeses (Delcenserie et al., 2014; Dugat-Bony et al., 2016.
460 *Psychrobacter* was observed in all SRSC samples at the end of shelf-life. According to
461 Ceugniez et al. (2017), *Psychrobacter* is part of the raw milk microbiota, and its growth
462 is promoted in cheese, especially in case of cold ripening and during storage. Some
463 *Psychrobacter* species have also been isolated from seawater, and are thus halotolerant.
464 They could possibly been carried by brine and salt (Falardeau et al., 2019). Finally, the
465 presence of *Microbacterium* in various types of cheeses is well documented, originating
466 from raw milk and contributing to cheese flavor (Delcenserie et al., 2014; Irlinger et al.,
467 2015; Tilocca et al., 2020). *Bacteroides* are abundant in dairy farm environment, on teat
468 skin and in raw tank milk. Their presence in cheese has already been observed in multiple
469 varieties (Falardeau et al., 2019, Milani et al., 2019). These bacteria are part of the natural
470 human gut microbiota, and can be used as probiotics (Tan et al., 2019). Regarding
471 MRSC, the presence of *Faecalibacterium* is not a surprise, as this genus is commonly
472 found in raw milk (Savin et al., 2019). These strict anaerobes could find a suitable
473 environment in cheese cores (Fox et al., 2017). Quigley et al. (2012) observed for the first

474 time the presence of *Faecalibacterium* in cores of soft, semi-hard and hard cheese
475 samples. Interestingly, various species from this genus, including *F. prausnitzii*, are
476 known for their probiotic role (Savin et al., 2019). *Prevotella*, another genus including
477 strict anaerobes, was frequently observed in cheese since the emergence of NGS.
478 *Prevotella* were primarily identified in cow rumens, but were also observed in mouth,
479 nose and gut of cows (Fox et al., 2017). According to Frétin et al. (2018), individuals
480 from the family *Lachnospiraceae* are commonly found on the teat skin, as a result of
481 fecal contamination, provided that these bacteria are part of gut microbiota. Bacteria can
482 thus be transferred to raw milk during milking or to washing water during cleaning, and
483 be found in cheese. It was for instance the case in Parmesan (Milani et al., 2019). On the
484 opposite, Falardeau et al. (2019) observed *Lachnospiraceae* in dairy farms, milk and
485 cheese plants, but did not detect its presence in the final cheeses, including MRSC.
486 However, as DNA sequencing do not allow to distinguish dead and alive bacteria, it is
487 possible that all these anaerobes were not metabolically active anymore in cheese during
488 ripening and storage. *Hafnia alvei* is fecal and water contaminant which represented a
489 huge part of the subdominant microbiota in MRSC. This Gram-negative bacterium is
490 sometimes used as starter culture in MRSC and SRSC, as it influences cheese sensorial
491 properties by producing volatile sulfur compounds (Irlinger et al., 2015). To our
492 knowledge, *H. alvei* was not intentionally added in samples considered during this work.
493 A hypothesis to explain the peak in relative abundance of *H. alvei* in MRSC during
494 storage at 7 °C is that psychrotrophic Gram-negative bacteria are favored by these
495 conditions (Gobbetti et al., 2018b).

496 4.2.4. Foodborne pathogens

497 Three foodborne pathogens were identified using metagenetics, namely
498 *L. moncoytogenes*, *S. aureus* and *E. coli*. *L. monocytogenes* was only observed in seven

MRSC sample at end of shelf-life. During challenge studies performed by Gérard et al. (2020a), levels of the pathogen were the highest in concerned batches at end of shelf-life 6-7 $\log_{10}$ cfu/g), while level in other varieties was generally < 3 $\log_{10}$ cfu/g. Given the random sampling effort used in this study, *i.e.* 6,000 sequences/sample, and cheese total microbiota assessed by plate counts (*i.e.* 7-8 $\log_{10}$ cfu/g), it was expected that the sensitivity of metagenetics was not sufficient to detect *L. monocytogenes* in the latter samples, as it is also the case for many other minor microbial species. Indeed, the probability to randomly select sequences of minor bacteria is limited in contrast to sequences of sub-dominant or dominant microbiota. As expected, metagenetics is not the most adequate tool when looking for pathogens in food.

Regarding *Staphylococcus*, according to Gobbetti et al. (2018a), this genus is part of natural raw milk microbiota, but is also transmitted by cheesemakers' hands (Castellanos-Rozo et al., 2020). According to Irlinger et al. (2015), *Staphylococcus* spp. were identified on the rinds of nearly all cheese varieties, their halotolerance allowing them to find a suitable environment in and on cheese. The presence of *E. coli* in cheese is common (Lahou and Uyttendaele, 2017; Gérard et al., 2020a).

4.2.5. Observation of unexpected bacterial genera

As a reminder, *Fusobacterium* has been observed in three SRSC samples from a same batch, with relative abundance around 10%. The presence of *Fusobacterium* in cheese has already been reported by Delcenserie et al. (2014), but with a much lower relative abundance (2.54% and 4.39% in raw and pasteurized milk SRSC samples, respectively). To our knowledge, no other papers mentioned the presence of this genus in cheese. Interestingly, cheese samples from this farm were the only SRSC in which *L. monocytogenes* levels decreased during challenge studies (Gérard et al., 2020a). The

second unexpected genus observed in this study was *Ralstonia*. Species of this genus are known as plant pathogens, and can sometimes be found in raw milk (Salazar et al., 2018). However, *Ralstonia* are also known as potential contaminants from DNA extraction kits, reagents for PCR or water (Salter et al., 2014). Further investigations should be performed in order to confirm that these bacteria were metabolically active during cheese ripening and storage.

4.3. Correlation between growth potential of *L. monocytogenes* and resident microbiota

Canonical correspondence analysis did not identify correlations with the presence of particular genera and δ of *L. monocytogenes* in SPSHC. This variability could be explained by the bias introduced by the differential dispersion of *L. monocytogenes* into cheese following inoculation during challenge studies, as hypothesized by Gérard et al. (2020a). Another explanation could be differences in the composition of dominant microbiota at deeper taxonomic levels, *i.e.* species, subspecies or strains.

Canonical correspondence analysis performed for SRSC revealed more interesting results, with the three samples of interest (*i.e.* samples in which *L. monocytogenes* levels decreased during challenge studies performed by Gérard et al. (2020a)) clustered clearly apart from other batches. A first significant correlation was found with the presence of *Lactococcus* as only dominant genus. Although *Lactococcus* spp., including *L. lactis*, are known for their production of bacteriocins inhibiting the growth of *L. monocytogenes*, this correlation could doubtful as such, as *Lactococcus* was used as main starter during manufacture of all SRSC samples considered in this study. Nevertheless, inhibition of *L. monocytogenes* by *Lactococcus* spp. is often strain-dependent. Although some batches present similar levels of *Lactococcus* spp., the differential dominance of *Lactococcus* strains could be a clue to explain differences observed regarding δ of *L. monocytogenes*.

A strong correlation with the presence of *Fusobacterium* was reported by canonical correspondence analysis and Spearman correlation coefficients. As detailed in part 3.3.5., *Fusobacterium* represented 12.2 ± 3.0% of the sequences associated with the three samples not allowing the growth of the pathogen, and this genera was not observed in other samples. It seems that this genus represent the most interesting pathway to investigate, as its presence in cheese was only reported once, in 2014, in samples from the same producer, but with much lower levels. Other genera significantly correlated to the negative δ of *L. monocytogenes* were *Alkalibacterium* (29 reads), *Clostridiisalibacter* (26 reads) and *Psychrilyobacter* (27 reads). It was already reported that *Alkalibacterium kapii*, an alkalophilic bacteria, finding suitable environment on cheese surfaces, was able to inhibit the growth of *Listeria innocua* during Raclette cheese ripening (Roth et al., 2011). *Clostridiisalibacter* are halophilic bacteria which were already observed in SRSC (Delcenserie et al., 2014), but their ability to inhibit *L. monocytogenes* has never been investigated. *Psychrilyobacter* is a genus from the Fusobacteria phyla, which is commonly observed in marine environments. Its presence in cheese was never reported, although it was already observed in cheese production environment (Schön et al., 2016). All the latter genera represent thus interesting perspectives to investigate, in order to confirm their potential influence on the growth of *L. monocytogenes*.

5. Conclusion

Microbial populations of cheeses, especially subdominant and minor populations, are strongly influenced by many factors. Each paper on this topic identified novelties: new species, taxa observed in cheese for the first time, or at least unexpected relative abundance of known taxa. It was the case for Belgian samples investigated during this study. The major surprise was the identification of a high proportion (> 10%) of *Fusobacterium* in three SRSC samples from the same factory, which did not allow the

growth of *L. monocytogenes* during previously performed challenge studies. Otherwise, it was observed that the production technology has a strong influence on cheese subdominant microbiota, and that starter cultures did not always govern cheese microbial community structure. Regarding dominant microbiota, *Lactococcus* and/or *Streptococcus* were dominant in all cheese types, corresponding mainly to *L. lactis* and *S. thermophilus*. Nevertheless, strains could be different between cheese types or batches. A deeper knowledge could be acquired through analysis of oligotypes. Knowing with precisions strains met in each batch could allow to improve understanding of the results of challenge studies with *L. monocytogenes*, as production of bacteriocins or other antimicrobial compounds is strain dependent. Considering separately core and rind could also have been interesting. In addition to that, using NGS to study fungal communities of Belgian cheeses would represent an added-value. Correlations analyses were a first approach in order to draw hypotheses in order to explain the unexpected decrease of *L. monocytogenes* levels during storage of three SRSC samples from the same producer. Further studies should be performed in order to assess the real influence of the identified genera on the growth of the pathogen. It is also important to characterize in details the *Fusobacterium* spp., as observed species was not listed in databases. At least two species of this genus, *i.e. Fusobacterium nucleatum* and *Fusobacterium necrophorum*, are known as human pathogens. High relative abundance of *Fusobacterium gastrosuis* has also been associated to stomach ulceration in pigs. Food safety aspects associated to the presence of this unknown *Fusobacterium* should be investigated. Finally, it is now important to go beyond diversity studies, and metatranscriptomics could be a powerful tool in order to understand the role of bacterial taxa during cheese production and storage.

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801 **Table 1.** Characteristics of the five types of cheese considered during this study.

	UACC	**MRSC**	**SRSC**	**SPSHC**	**GSHC**
Starters	*Lactococcus lactis* subsp. *lactics* *Lactococcus lactis* subsp. *cremoris* *Leuconostoc* spp.	*Lactococcus lactis* subsp. *lactics* *Lactococcus lactis* subsp. *cremoris* *Leuconostoc* spp. *Penicillium candidum*	*Lactococcus lactis* subsp. *lactics* *Lactococcus lactis* subsp. *cremoris* *Leuconostoc* spp. *Brevibacterium linens*	*Lactococcus lactis* subsp. *lactics* *Lactococcus lactis* subsp. *cremoris* *Streptococcus thermophilus*	*Lactococcus lactis* subsp. *lactics* *Lactococcus lactis* subsp. *cremoris* *Streptococcus thermophilus*
Curd	Lactic	Enzymatic	Enzymatic	Enzymatic	Enzymatic
Type of milk	P: UACC9 R: UACC1-8 and UACC10-12	P: MRSC1 R: MRSC2-4	R	R	P: GSHC1-3 R: GSHC4
Maximal temperature during manufacture	Room temperature	< 40 °C	< 40 °C	< 40 °C	< 40 °C
Pressing	No	No	No	Yes	Yes
Specific ripening practices	/	Turning	Turning + Rind washing	Turning + Rind washing	Turning
Ripening duration (weeks)	/	2	3	4	8
Rind	No rind	White molds	Red/orange bacteria		Artificial coating
Weight (kg)	~0.25	0.25-0.30	0.30-0.50	1.00-2.50	> 10.00

Shelf-life (days)	14	30	30	30	30
pH (Gérard et al., 2020a)	~4.4	~5.8	~5.8	~5.8	~5.8
a_w (Gérard et al., 2020a)	~0.99	~0.97	~0.97	~0.96	~0.96
Dry matter (Gérard et al., 2020a)	~25%	~50%	~50%	~60%	~60%
Growth of *L. monocytogenes* (number of batches with growth/total number of batches; Gérard et al., 2020a)	0/11	4/4	3/4	4/8	3/4

802 Legend: P, pasteurized milk; R, raw milk.

803 **Table 2.** Total microbiota and LAB counts at day-0 and end of shelf-life
804 (averages ± standard deviations) expressed as $\log_{10}$ cfu/g.

Sampling times	Analyses	UACC	SRSC	MRSC	GSHC	SPSHC
Day-0	Total microbiota	8.216±0.381[a]	8.156±0.187[abc]	7.678±0.776[bc]	7.077±0.928[c]	8.014±0.408[ab]
	LAB	8.091±0.381[a]	7.930±0.266[abc]	7.263±0.804[bc]	7.012±0.682[c]	8.046±0.328[ab]
End of shelf-life	Total microbiota	7.661±0.639[a]	8.221±0.159[a]	7.714±0.702[a]	7.402±0.608[a]	7.926±0.314[a]
	LAB	7.934±0.501[a]	7.417±0.467[a]	7.642±0.793[a]	6.907±0.991[a]	7.823±0.361[a]

805 Legend : UACC, unripened acid-curd cheeses ; SRSC, smear-ripened soft cheeses ;
806 MRSC, mold-ripened soft cheeses ; GSHC, Gouda-type semi-hard cheeses ; SPSHC,
807 Saint-Paulin-type semi-hard cheeses ; all enumerations are expressed as $\log_{10}$ cfu/g; items
808 not sharing superscript letters are significantly different.

Table 3. α-diversity metrics by type of cheese.

Types of cheese	Good's coverage (%)		Number of genera			Chao1			Inverse Simpson			Simpson evenness		
	Day-0	End of shelf-life	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters
UACC	0.999±0.001	0.999±0.001	15.94±22.49^{a}	8.64±8.57^{b}	$H_{1,67}$=1.16 p=0.281	21.55±32.50^{a}	13.23±16.24^{b}	$H_{1,67}$=0.43 p=0.510	1.12±0.17^{b}	1.10±0.13^{b}	$H_{1,67}$=0.29 p=0.590	0.18±0.13^{a}	0.20±0.11^{a}	$H_{1,67}$=1.04 p=0.307
MRSC	0.999±0.001	0.999±0.000	26.92±34.46^{a}	18.00±6.30^{a}	$H_{1,24}$=3.41 p=0.065	34.90±42.30^{a}	22.50±8.49^{ab}	$H_{1,24}$=1.61 p=0.204	1.54±0.99^{ab}	1.51±0.74^{b}	$H_{1,24}$=1.92 p=0.166	0.12±0.07^{a}	0.08±0.02^{b}	$H_{1,24}$=2.08 p=0.149
SRSC	0.998±0.002	0.999±0.000	28.00±32.57^{a}	25.00±5.60^{a}	$H_{1,24}$=2.10 p=0.148	38.60±44.90^{a}	38.92±24.77^{a}	$H_{1,24}$=2.10 p=0.148	1.90±1.29^{a}	2.08±1.03^{a}	$H_{1,24}$=1.85 p=0.173	0.18±0.26^{a}	0.08±0.03^{b}	$H_{1,24}$=0.71 p=0.401
GSHC	0.999±0.000	0.999±0.001	7.08±4.19^{a}	8.92±7.05^{b}	$H_{1,24}$=0.16 p=0.686	10.29±7.35^{a}	13.53±14.36^{b}	$H_{1,24}$=0.04 p=0.840	1.07±0.08^{b}	1.20±0.30^{b}	$H_{1,24}$=0.12 p=0.729	0.20±0.12^{a}	0.19±0.10^{a}	$H_{1,24}$=0.21 0.64
SPSHC	0.999±0.000	0.999±0.000	10.64±10.58^{a}	7.29±3.32^{b}	$H_{1,49}$=0.14 p=0.711	17.00±20.60^{a}	9.01±5.30^{b}	$H_{1,49}$=0.26 p=0.610	1.24±0.26^{b}	1.25±0.27^{b}	$H_{1,49}$=0.05 p=0.826	0.21±0.14^{a}	0.20±0.08^{a}	$H_{1,49}$=0.41 p=0.522
Statistical letters			$H_{4.96}$=8.47	$H_{4.92}$=37.03		$H_{4.96}$=6.96	$H_{4.92}$=33.18		$H_{4,96}$=10.47	$H_{4.92}$=27.41		$H_{4,96}$=0,212	$H_{4.92}$=30.81	
p-value			0.076	***<0.001***		0.138	***<0.001***		***0.033***	***<0.001***		0.212	***<0.001***	

Legend : UACC, unripened acid-curd cheeses ; MRSC, mold-ripened soft cheeses ; SRSC, smear-ripened soft cheeses ; GSHC, Gouda-type semi-hard cheeses ; SPSHC, Saint-Paulin-type semi-hard cheeses ; within a column, values which do not shared superscript letters are statistically different; significant p-values are written in italic bold.

Table 4. Spearman correlation coefficient and significativity (p-values corrected for multitesting using FDR method) for the genera suspected to be correlated with no growth of *L. monocytogenes* from canonical correspondence analysis.

Genera	Spearman correlation coefficient	p-values
Lactococcus	-0.620	***0.002***
Psychrilyobacter	-0.511	***0.022***
Fusobacterium	-0.511	***0.024***
Alkalibacterium	-0.511	***0.024***
Clostridiisalibacter	-0.408	0.118
Staphylococcus	0.224	0.306
Pseudoalteromonas	-0.092	0.677
Arcobacter	-0.052	0.814
Marinilactibacillus	0.001	0.995

Legend : corrected p-values in italic bold are significant (*i.e.* < 0.050).

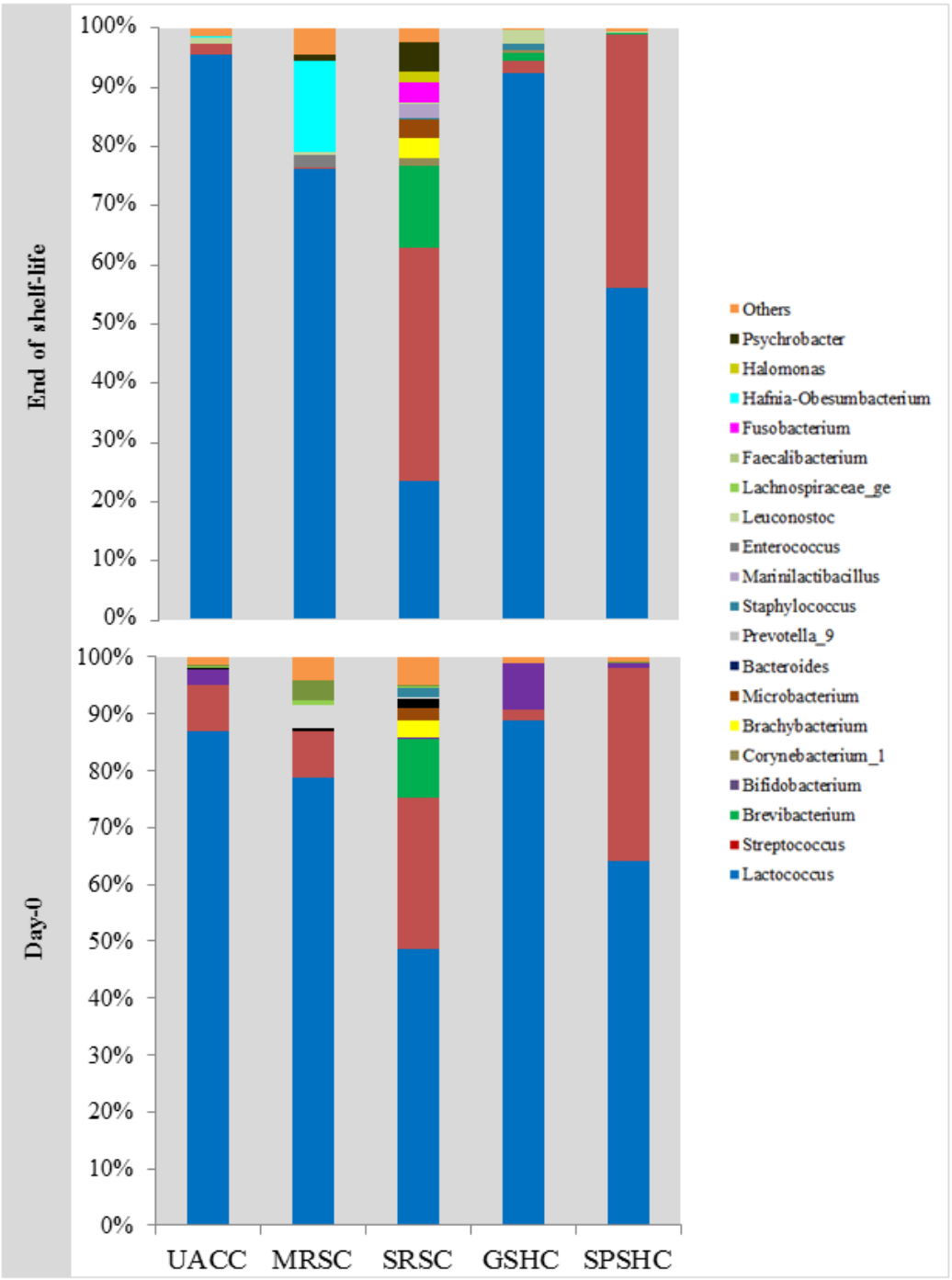

Fig. 1. Relative abundance of bacterial genera in all types of cheese at day-0 and end of shelf-life. Only genera with relative abundance > 1 % were plotted.

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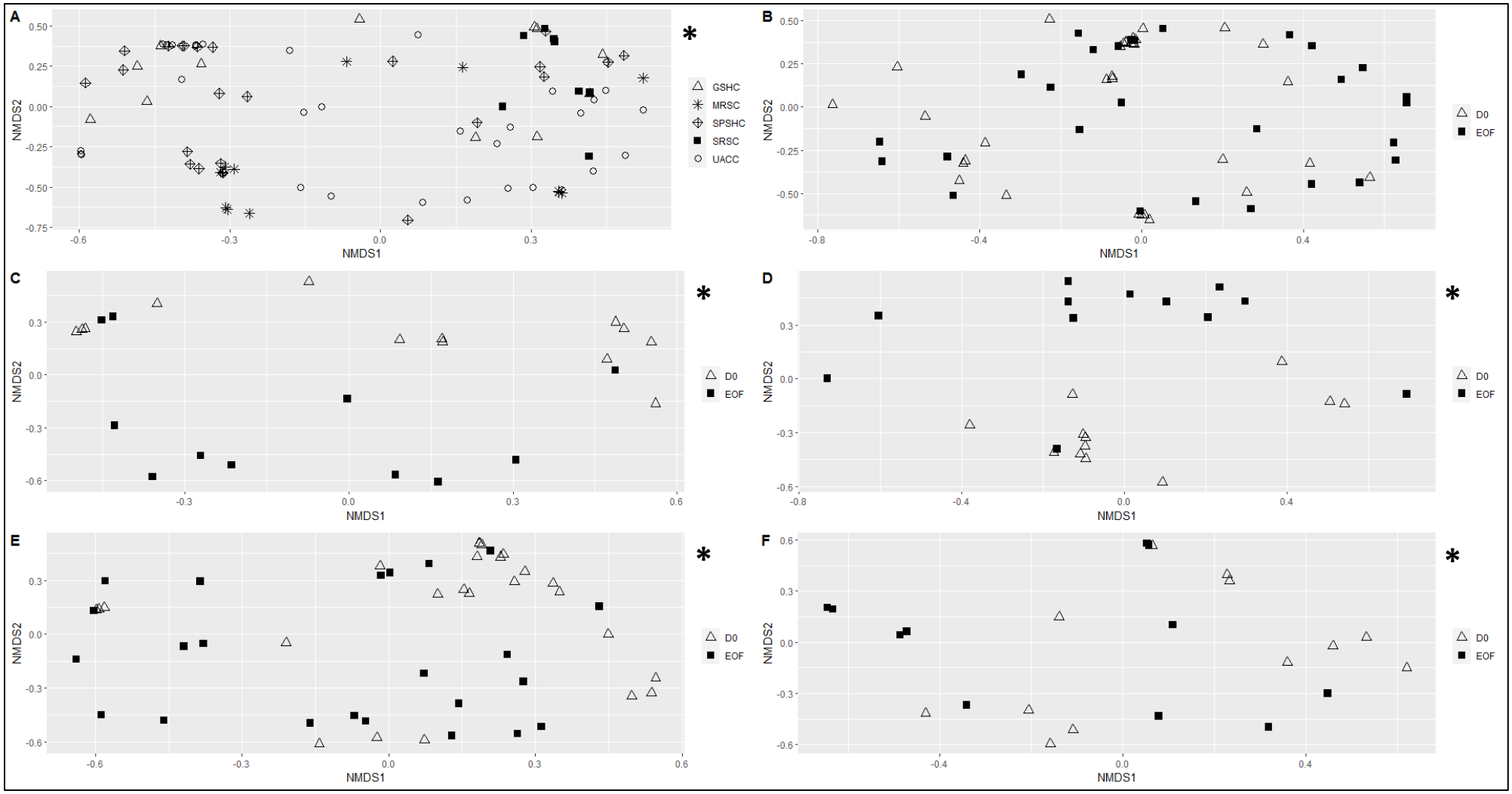


Fig. 2. NMDS highlighting differences in cheese subdominant community structure (Yue and Clayton theta dissimilarity matrix); A, all types of cheese at end of shelf-life; B, UACC; C, SRSC; D, MRSC; E, SPSHC; F, GSHC; D0, day-0; EOF, end of shelf-life; *, significant differences between groups (p-value < 0.050).

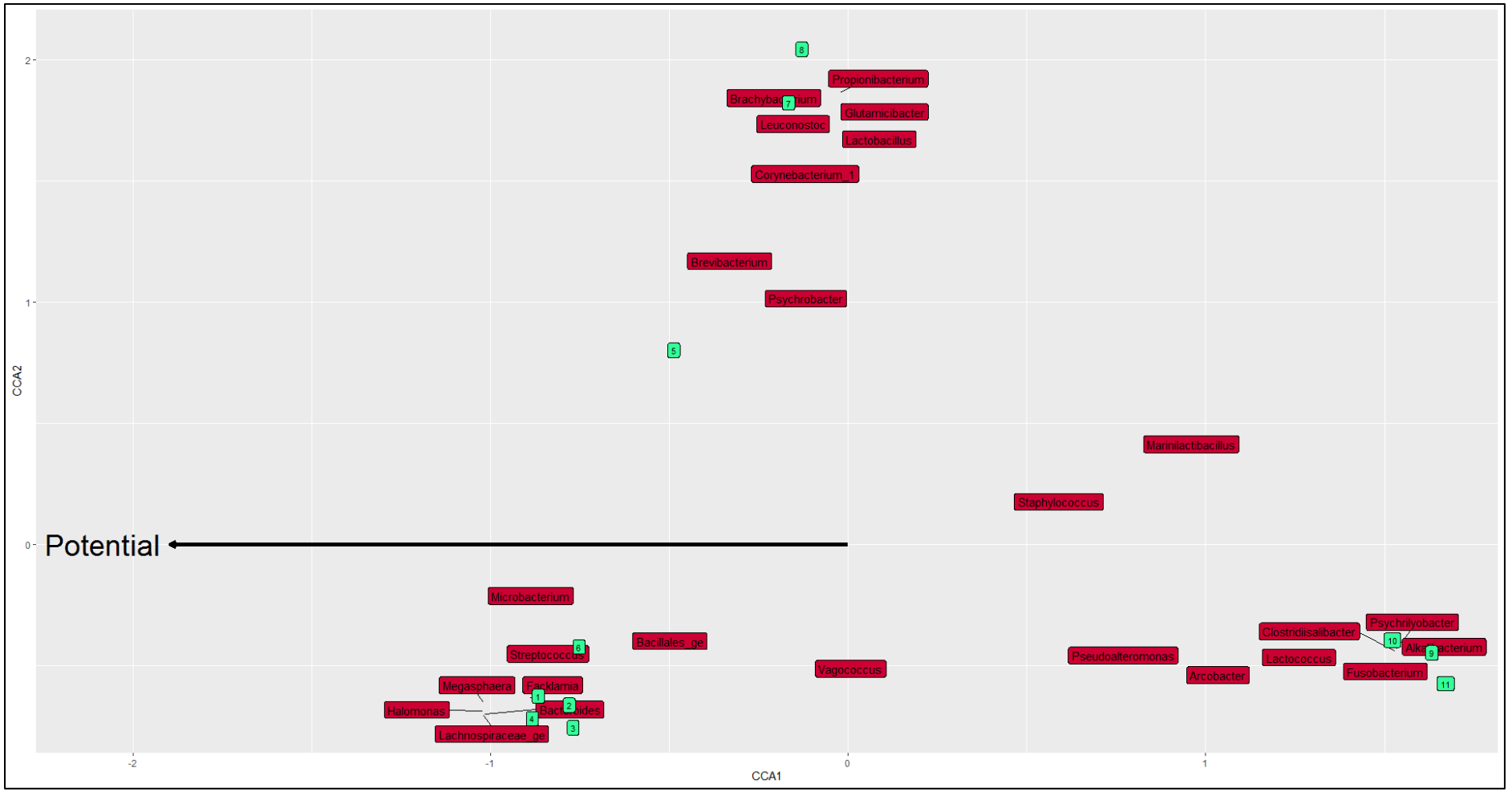
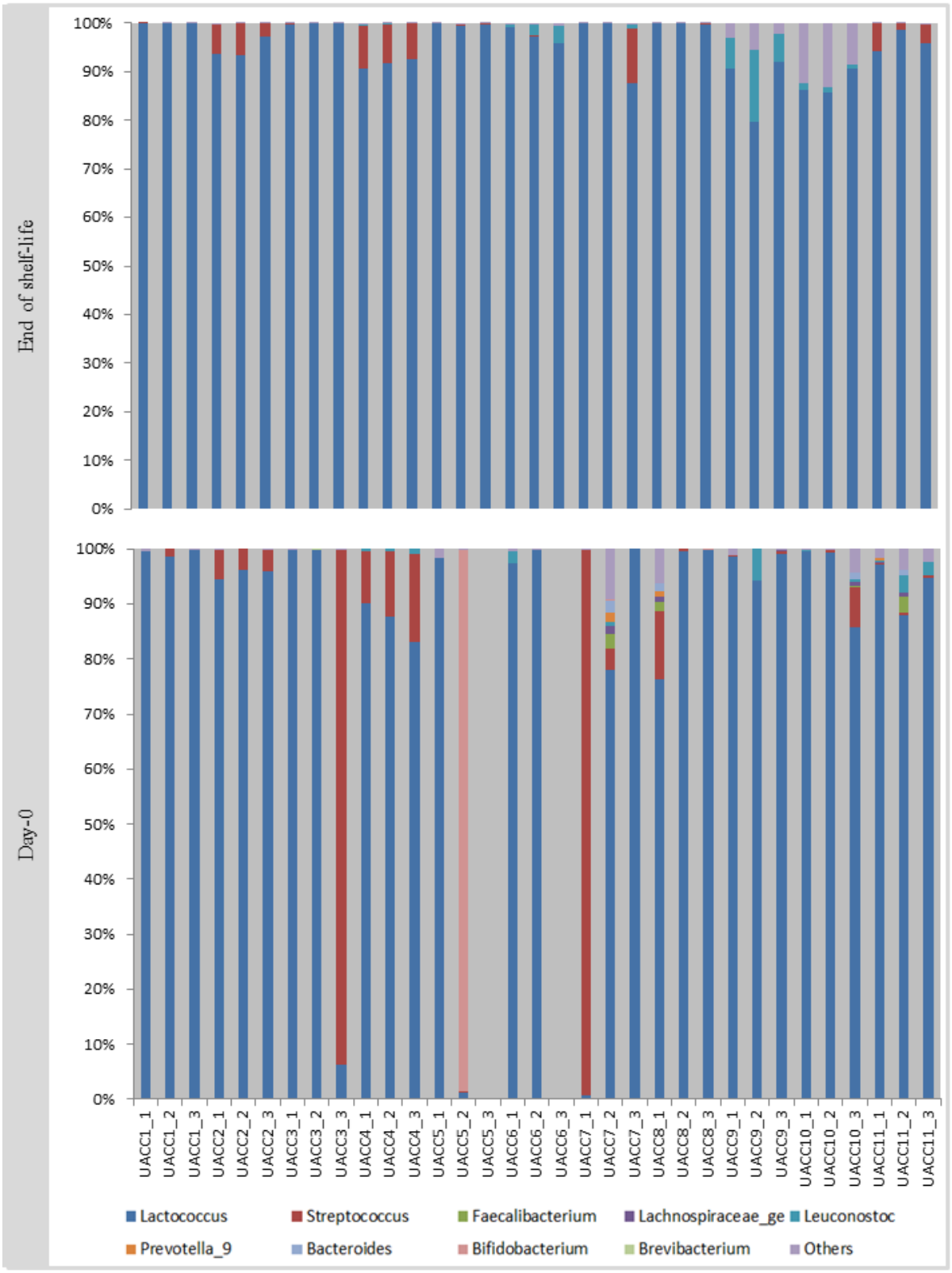
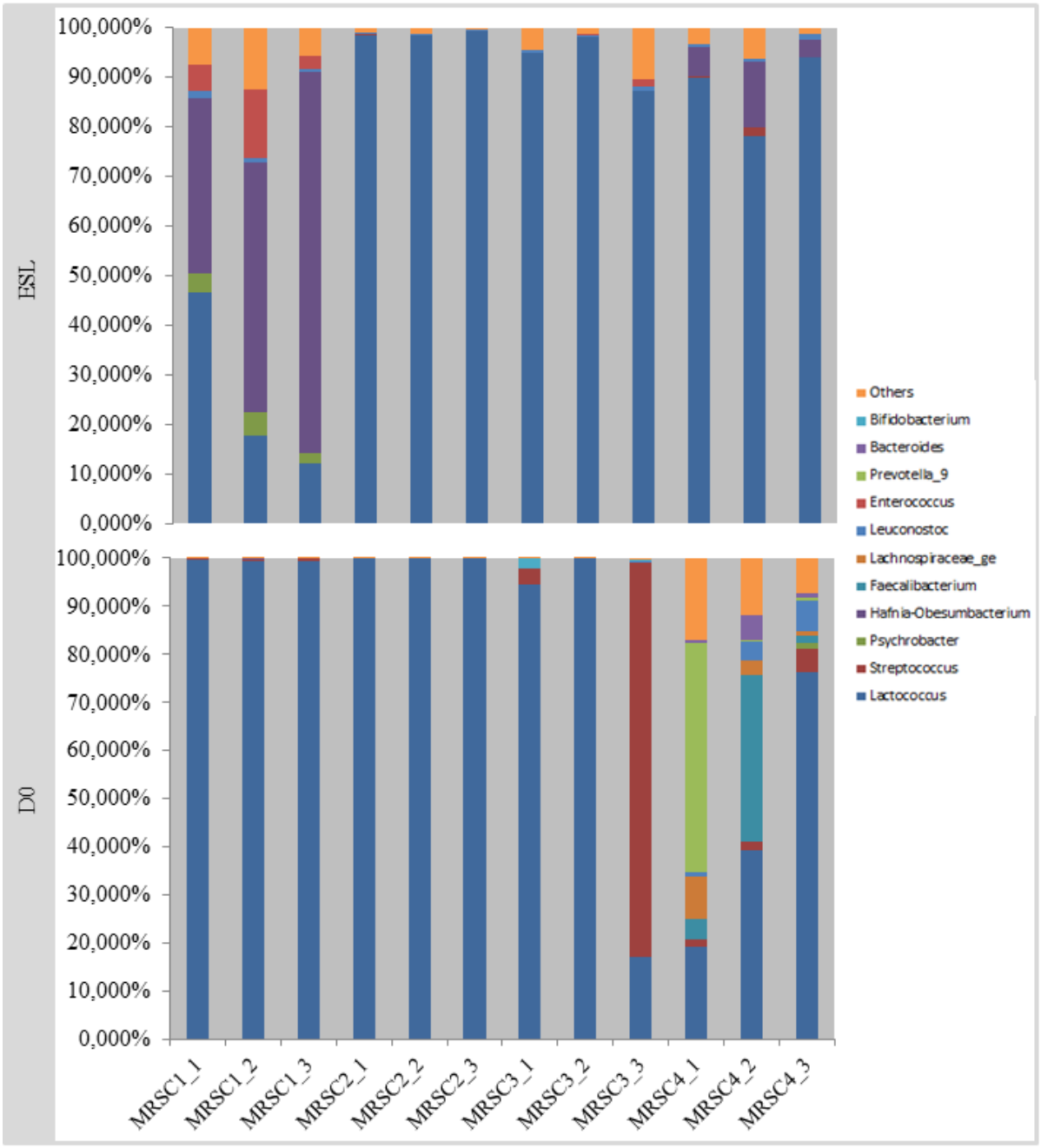


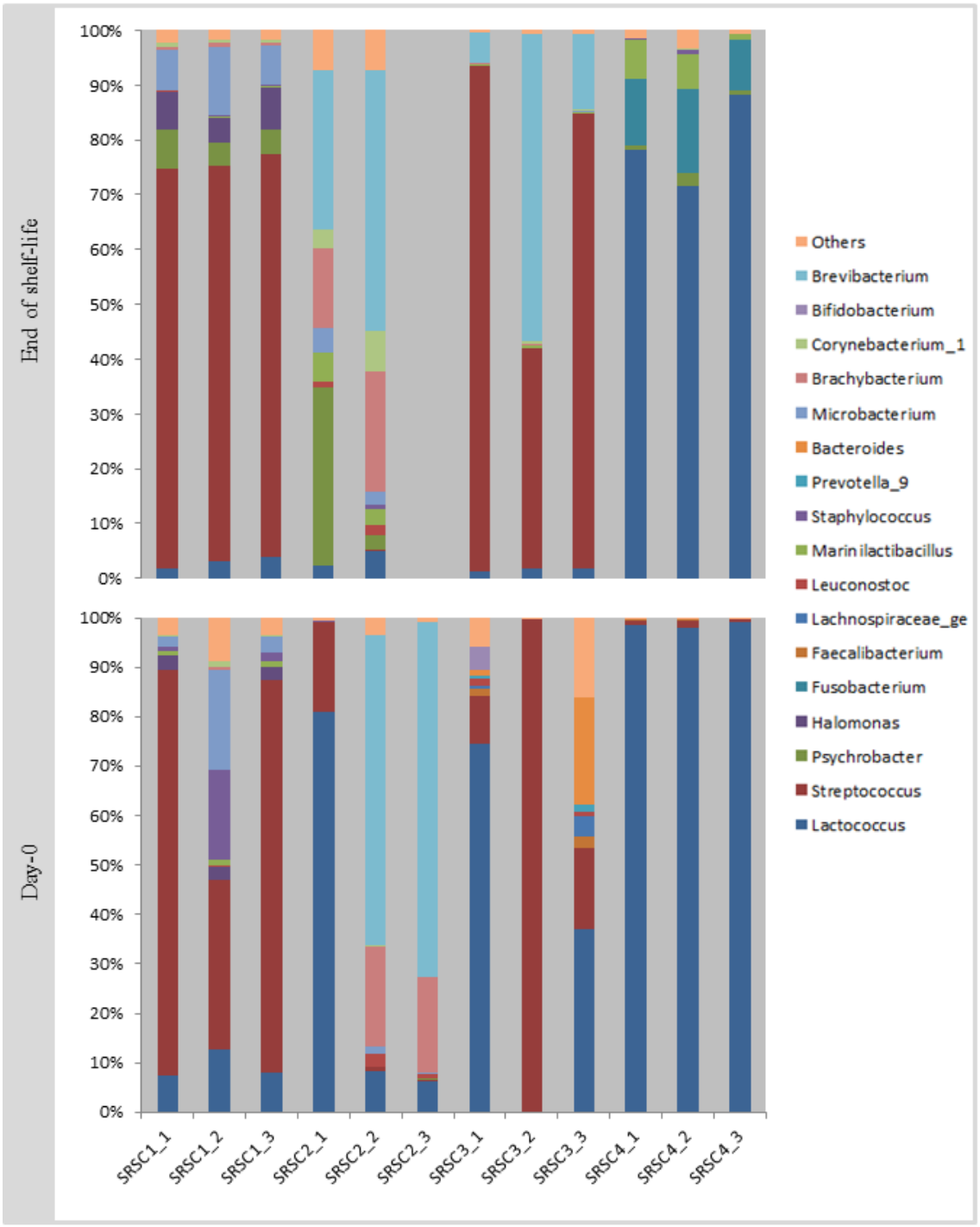
Fig. 3. Canonical correspondence analysis triplot for SRSC. Green labelled numbers correspond to cheese samples, red labels to bacterial genera and black arrow to positive δ of *L. monocytogenes*. Cheese samples not allowing the growth of *L. monocytogenes*, *i.e.* 9-10-11, are grouped on the right side of the figure.



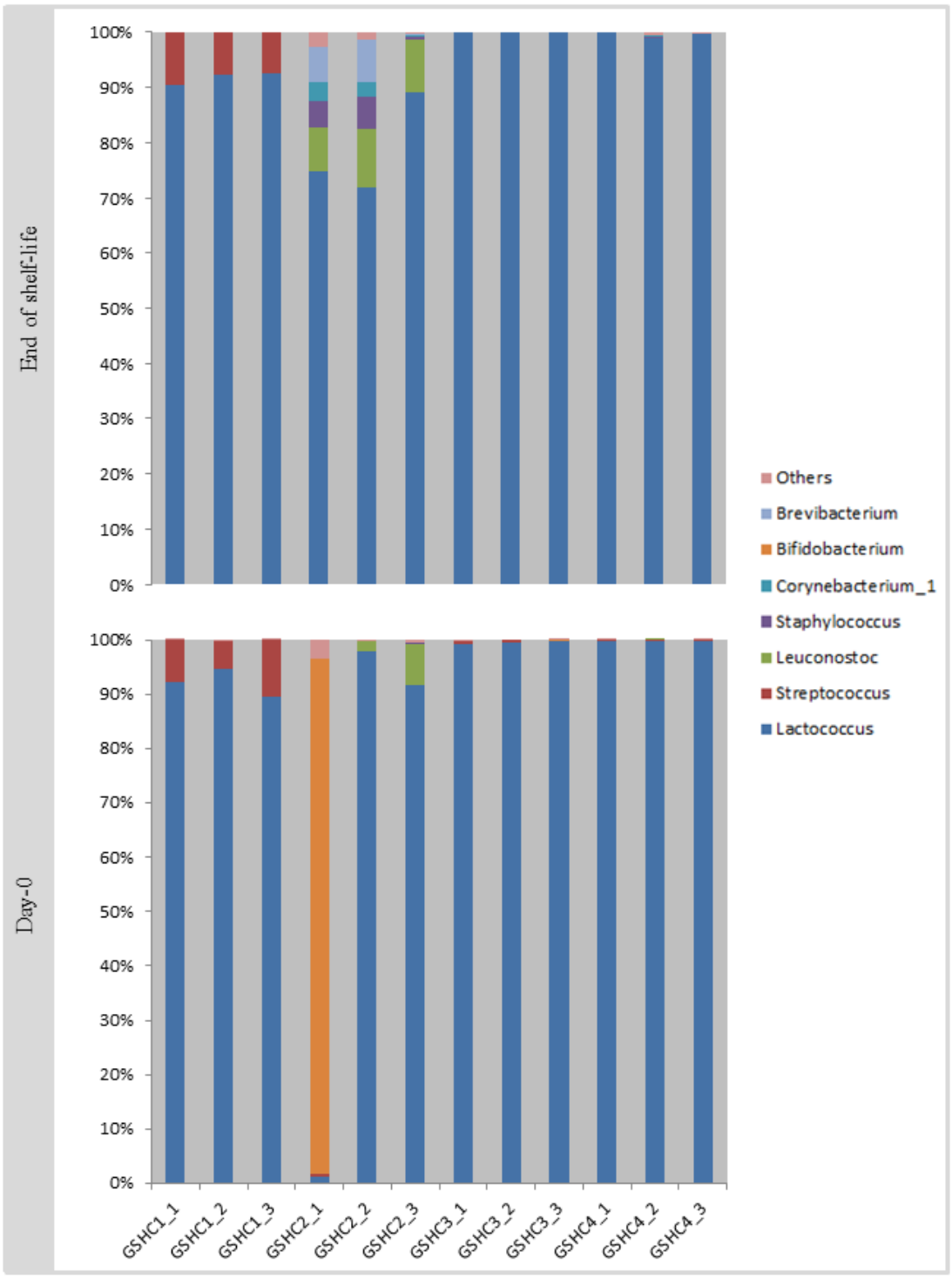
Supplementary material 1. Relative abundance of bacterial genera by UACC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.



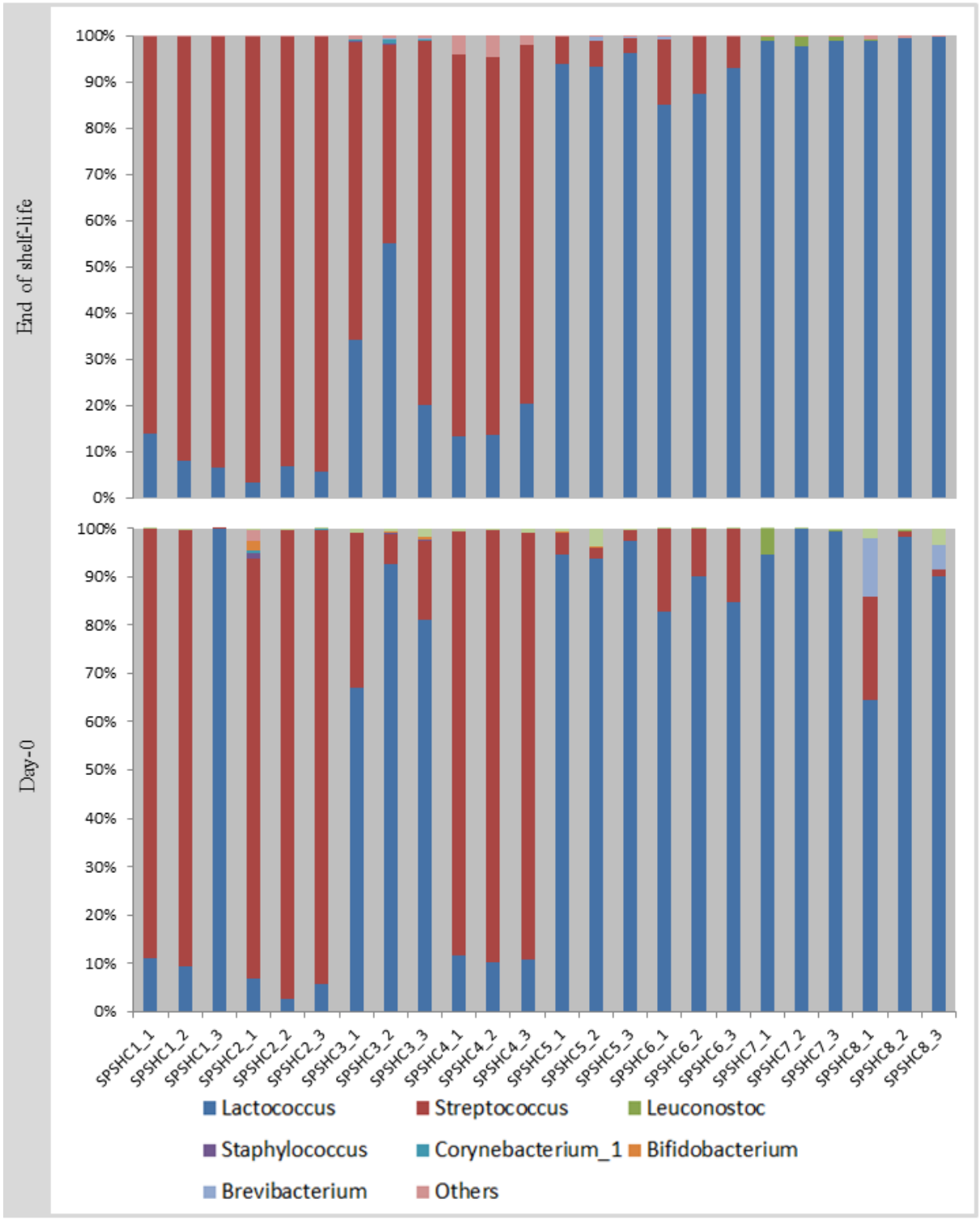
Supplementary material 2. Relative abundance of bacterial genera by MRSC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.



Supplementary material 3. Relative abundance of bacterial genera by SRSC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.



Supplementary material 4. Relative abundance of bacterial genera by GSHC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.



Supplementary material 5. Relative abundance of bacterial genera by SPSHC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.

Bacterial genera	UACC		MRSC		SRSC		GSHC		SPSHC	
	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life
Lactococcus	85.7±28.4	95.5±5.3	78.6±33.3	76.3±32.2	44.3±42.2	23.5±36.0	88.7±27.8	92.4±9.8	62.5±40.0	56.1±41.5
Streptococcus	8.8±23.8	1.9±3.2	7.9±23.4	0.2±0.5	28.7±37.0	39.5±39.7	2.1±3.6	2.0±3.7	35.5±40.4	42.9±41.3
Brevibacterium	/	/	/	/	11.3±26.3	13.8±20.9	/	1.2±2.8	0.1±0.5	0.1±0.2
Bifidobacterium	3.2±17.7	/	0.2±0.6	/	0.4±1.3	/	7.9±27.4	/	0.7±2.7	/
Corynebacterium	/	/	/	/	0.2±0.4	1.2±2.2	/	0.6±1.2	0.1±0.4	0.1±0.3
Brachybacterium	/	/	/	/	3.4±7.7	3.5±7.5	/	/	0.0±0.1	/
Microbacterium	/	/	/	/	2.3±5.8	3.1±4.3	/	/	/	/
Bacteroides	0.2±0.5	/	0.6±1.5	/	1.9±6.3	/	/	/	/	/
Prevotella	0.1±0.4	/	4.0±13.7	/	0.2±0.4	/	/	/	/	/
Staphylococcus	/	/	/	/	1.7±5.2	0.2±0.3	/	0.9±2.0	0.1±0.2	/
Marinilactibacillus	/	/	/	/	0.2±0.5	2.2±2.8	/	/	/	/
Enterococcus	/	/	/	2.0±4.1	/	/	/	/	/	/
Leuconostoc	0.5±1.2	1.2±2.9	1.0±2.1	0.6±0.4	0.5±0.8	0.3±0.6	0.8±2.3	2.3±4.3	0.2±1.1	0.2±0.5
Lachnospiraceae	0.1±0.3	/	1.1±2.6	/	0.4±1.2	/	/	/	/	/
Faecalibacterium	0.3±0.8	/	3.3±9.9	/	0.3±0.8	/	/	/	/	/
Fusobacterium	/	/	/	/	/	3.3±5.8	/	/	/	/
Hafnia-Obseumbacterium	/	/	0.0±0.1	15.5±25.4	/	/	/	/	/	/
Halomonas	/	/	/	/	0.7±1.2	1.8±3.1	/	/	/	/
Psychrobacter	/	/	0.1±0.3	0.9±1.7	/	5.0±9.4	/	/	/	/

Supplementary material 6. Average (± standard deviation) relative abundance (%) of main bacterial genera considered in this paper. UACC, unripened acid-curd cheeses; MRSC, mold-ripened soft cheeses; SRSC, smear-ripened soft cheeses; GSHC, Gouda-type semi-hard cheeses; SPSHC, Saint-Paulin-type semi-hard cheeses; D0, day-0; ESL, end of shelf-life; /, undetected genus.