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Development of a gastrointestinal model to study the interest of apple pomace during weaning of piglets

Développement d'un modèle gastro-intestinal en vue d'étudier l'intérêt du marc de pomme durant le sevrage des porcelets

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Abbreviations

ADF	Acid detergent fiber
ADG	Average daily gain
ADL	Acid detergent lignin
AP	Apple pomace
ARCOL	Artificial colon model
Avg	Average
Baby-SPIME	Baby Simulator of Pig intestinal Microbial Ecosystem
C2	Acetic acid
C3	Propionic acid
(i)C4	(iso)Butyric acid
(i)C5	(iso)Valeric acid
C6	Hexanoic acid
CEPA	Centre d'Expérimentation en Productions Animales
CFU	Colony-forming unit
CHO	Carbohydrates
CRA-W	Centre wallon de Recherches agronomiques
DM	Dry matter
DP	Degree of polymerization
EFE	Energetic feed efficiency
ETEC	Enterotoxigenic E. coli
FCR	Feed conversion ratio
FDR	False discovery rate
GC	Gaz chromatography
GIT	Gastrointestinal tract
HF	Human feces
HMWDF	High molar weight dietary fiber
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC-UV	High performance liquid chromatography – ultraviolet detector
INRA-AFZ	Institut National de la Recherche Agronomique - Association Française de Zootechnie
IS	Internal standard
KW	Kruskal-Wallis
LAP	Medium with apple pomace
LOQ	Limit of quantification
LMWDF	Low molar weight dietary fiber
Max	Maximum
Min	Minimum
MJ	Méga joule
N_2	Nitrogen
NAP	Medium without apple pomace
Nb	Number of values
NDF	Neutral detergent fiber
NE	Net energy
NF	Norme française
NGP	Next-generation probiotics
ns	Not significant
NSP	Non-starch polysaccharides
OTU	Operational taxonomic unit
PD	Phylogenetic diversity
PW AP	Apple pomace culture medium (control medium +AP)

PWD	Post-weaning diarrhea
QC	Quality control
(R)RT	(Relative) retention time
SCFA	Short-chain fatty acid
SD	Standard deviation
SH	Scientific hypotheses
SHIME	Simulator of Human intestinal Microbial Ecosystem
TFI	Total feed intake
TIM	TNO gastrointestinal model
TO	Technical objective
VFAs	Volatile fatty acids
VL/CD	Villus length/crypts depth
ZnO	Zinc oxide

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Summary–Résumé

Summary

In the field of piglet nutrition, researches in recent decades have focused on nutritional alternatives to antibiotics or zinc oxide that prevent intestinal concerns during weaning–a critical production step associated with loss of performance and intestinal dysbiosis. In this context, the use of in vitro model is of great interest to study fermentation potential of feed ingredients or additives. The aim of the thesis was to develop an in vitro gastrointestinal model of the piglet able to reproduce weaning, in order to test apple pomace (AP) as a post-weaning feed ingredient. AP is known to be rich in dietary fiber, polyphenols and triterpenes, which could assume a positive effect on the production of short chain fatty acids and microbiota. Additionally, an in vivo study using AP in a post-weaning piglets' diet was provided to evaluate the effects–assuming positive–on performance, intestinal architecture and microbiota as well as to generate data for further discussion on the model, in vitro observations and the interest of AP around weaning.

To obtain the piglet model, the dynamic and multi-compartments SHIME (Simulator of Human intestinal Microbial Ecosystem) device has been identified as an adequate model to transpose to the piglet–baby-SPIME (baby Simulator of Pig Intestinal Microbial Ecosystem). It had to include 1°) an ileum bioreactor inoculated to be able to study the fermentation process in this compartment in addition to the large intestine and 2°) a weaning step in the chronology of each run to obtain a shift in the microbiota profile as observed in vivo at weaning. Regarding the microbiota of the baby-SPIME, the main phyla present in the model were Firmicutes, Bacteroidetes and Proteobacteria although Bacteroidetes decreased after inoculation ($p = 0.043$ in ileum, $p = 0.021$ in colon) and Delta-Proteobacteria tended to be favored ($p = 0.083$ in ileum, $p = 0.043$ in colon) over Gamma-Proteobacteria. The designed model led to under-estimation of Bacilli–in particular *Lactobacillus sp*. in the ileum and a weak representation of Bacteroidia in the proximal colon. However, *Mitsuokella* and *Prevotella* were part of the main genera of the model along with *Bifidobacterium*, *Fusobacterium*, *Megasphaera* and *Bacteroides*. As a result of weaning, two major changes–that can occur in vivo–were detected in the system: firstly, Firmicutes diminished when Bacteroidetes increased, especially in the proximal colon; secondly, *Bacteroides* decreased and *Prevotella* increased (mean value for four runs). However, microbiota profile and SCFA in the ileum bioreactor did not conform to the expectations. It acted more like a pre-colon and had to be improved.

Despite the limitations of the model, AP was tested in the baby-SPIME (0.65/16.85 g/L of medium). The results showed that AP led to bacterial groups and metabolic pathways favoring a higher molar proportion of propionate and a lower one of butyrate in the acetate: propionate: butyrate profile. Apple pomace tended to decrease the relative abundance of Bifidobacteriaceae ($p = 0.067$) in the ileum bioreactor and increased that of Prevotellaceae ($p = 0.036$) in the colon bioreactor when observing results in predominant bacteria. Apple pomace, however, led to an increase, in average values, of the relative

abundances of bacteria belonging to Bacteroidetes phylum or even *Akkermansia sp*. belonging to the phylum Verrucomicrobia and related to the production of propionate.

In vivo, AP was used at two levels of incorporation into post-weaning diets. Apple pomace was evaluated in weaning diets for a period of 5 weeks from weaning of piglets at 28 days onwards. No effects of AP were observed on the production of SCFA in feces or in caecum. However, significant effects were observed on microbiota in terms of richness or groups of bacteria, depending on the samples and the level of incorporation of AP in the diet, in addition to other parameters. The AP diets were beneficial to average daily gain calculated at week 3 ($p = 0.038$) and to some parameters of intestinal architecture on day 35 post-weaning. A supplementation level of 4% AP was beneficial for feed conversion ratio ($p = 0.002$) and energetic feed efficiency ($p = 0.004$) on day 35 post-weaning. Apple pomace tended to alter feces consistency (softer to liquid, $p = 0.096$) and increased counts of excreted pathogens ($p = 0.072$). Four percent AP influenced microbiota richness and bacteria profile as observed for the phylum Bacteroidetes or the class Clostridia. In this way, Bacteroidetes appeared as a point of convergence between in vitro and in vivo data. To conclude the experiment, a level of 4% AP in diet appeared to be an interesting weaning strategy which should be evaluated in a large cohort to finely assess the risk of dysbiosis due to excretion of pathogens at the beginning of the post-weaning period.

The thesis allowed the development of a new model suitable to study microbiota of piglets during weaning (baby-SPIME) and highlighted possible future research questions on the evolution of the baby-SPIME model (e.g. to mimic passive absorption of some components with the dialysis system of the SHIME, to use reagents from the porcine species such as porcine bile, to use mucin-beads and to combine a quantitative approach to the compositional data of the microbiota) that could probably improve the fermentation occurring in the ileum bioreactor. With the in vivo experiment, a concept that emerged was the beneficial effect of AP on the critical step of piglet weaning probably due to its monosaccharides, fiber, and polyphenols content by improving the energetic absorption—in the small intestine—and by modifying the gut microbiome. This concept showed the importance of combining in vitro and in vivo studies. It also showed the interest of ongoing research experiments to elucidate the mechanisms of action of AP on metabolism.

Résumé

Dans le domaine de la nutrition porcine, les recherches de ces dernières décennies ont largement porté sur les alternatives nutritionnelles aux antibiotiques ou à l'oxyde de zinc pour assurer la santé intestinale au moment du sevrage – une étape critique de la production qui peut mener à des pertes de performance et à une dysbiose intestinale. Dans ce contexte, l'utilisation d'un modèle in vitro est d'un grand intérêt pour étudier le potentiel de fermentation d'une matière première ou d'un additif. L'objectif de cette thèse consistait à développer un modèle gastro-intestinal du porcelet incluant une étape de sevrage pour tester le marc de pomme afin de l'intégrer dans des aliments de post-sevrage. Le marc de pomme est connu pour être riche en fibres alimentaires, en polyphénols et en triterpènes, ce qui pourrait avoir un effet positif sur la production d'acides gras à chaîne courte et le microbiote. En complément, une expérimentation sur porcelets a été prévue pour évaluer l'effet du marc de pomme sur les performances, l'architecture intestinale et le microbiote et également obtenir des données permettant d'approfondir la discussion sur le modèle, les résultats in vitro et l'intérêt du marc de pomme au sevrage.

Pour obtenir le modèle porcelet, le modèle dynamique et multi-compartiments SHIME (Simulator of Human intestinal Microbial Ecosystem) a été identifié comme un modèle adéquat à adapter pour le porcelet – baby-SPIME (baby Simulator of Pig Intestinal Microbial Ecosystem). Il doit inclure 1°) un bioréacteur iléon inoculé afin de modéliser l'étude des fermentations qui se produisent en fin d'intestin grêle et 2°) une étape de sevrage intégrée dans la chronologie de chaque expérimentation afin de créer un changement du microbiote proche de celui rencontré in vivo. Concernant le microbiote du baby-SPIME, les principaux phyla présents dans le modèle étaient les Firmicutes, Bacteroidetes et Proteobacteria bien que le phyla des Bacteroidetes diminuait après inoculation ($p = 0.043$ dans l'iléon, $p = 0.021$ dans le côlon) et que le phyla Delta-Proteobacteria était favorisé – ou tendait à l'être – ($p = 0.083$ dans l'iléon, $p = 0.043$ dans le côlon) par rapport aux Gamma-Proteobacteria. Le modèle ainsi établi amenait une sous-estimation des bactéries de la classe Bacilli – en particulier *Lactobacillus sp*. dans l'iléon et à une plus faible représentation des Bacteroidia dans le côlon proximal. Cependant, les bactéries des genres *Mitsuokella* et *Prevotella* faisaient partie des principaux genres bactériens retrouvés dans le modèle avec *Bifidobacterium*, *Fusobacterium*, *Megasphaera* et *Bacteroides*. Résultant de l'étape de sevrage en bioréacteurs, deux évolutions majeures – pouvant se produire in vivo – ont été observées in vitro : premièrement, l'abondance de Firmicutes diminuait alors que celle de Bacteroidetes augmentait, particulièrement dans le côlon proximal ; deuxièmement, *Bacteroides* diminuait et *Prevotella* augmentait (en valeur moyenne sur 4 expérimentations distinctes). Cependant, le profil du microbiote et les acides gras à chaines courtes dans le bioréacteur iléon ne s'est pas révélé conforme à ce qui était attendu. Il s'est avéré agir plutôt à l'image d'un pré-côlon.

Malgré la limitation observée du modèle, le marc de pomme a été testé dans le baby-SPIME. Les résultats ont montré que le marc de pomme a favorisé des bactéries et des voies métaboliques liées

au propionate en induisant une augmentation du ratio molaire de cet acide gras volatil tout en induisant une diminution de celui du butyrate dans l'équilibre acétate: propionate: butyrate. Le marc de pomme tendait à réduire l'abondance relative des Bifidobacteriaceae ($p = 0.067$) dans le bioréacteur iléon et il a augmenté celle des Prevotellaceae ($p = 0.036$) dans le bioréacteur du côlon proximal lorsque sont observées les bactéries dominantes dans le système. Le marc de pomme a par contre amené à une augmentation, en valeur moyenne, des bactéries appartenant au phylum Bacteroidetes et aussi à *Akkermansia sp*. du phylum Verrucomicrobia, toutes deux productrices de propionate.

In vivo, le marc de pomme a été ajouté à deux niveaux d'incorporation dans un aliment post-sevrage. Le marc de pomme a été évalué dès le sevrage de porcelets de 28 jours d'âge, et distribué pendant une période de 5 semaines. Aucun effet du marc de pomme n'a été observé sur la production des acides gras volatils dans les fèces ou dans le caecum. Cependant, des effets significatifs ont été observés sur le microbiote (richesse et diversité des groupes bactériens, avec effet dépendant du type d'échantillon et du contenu d'incorporation du marc de pomme) et sur d'autres paramètres. L'expérience a mené à observer que les régimes marc de pomme favorisaient le gain quotidien moyen calculé à la semaine 3 de post-sevrage ($p = 0,038$) et certains paramètres de l'architecture intestinale au 35ème jour post-sevrage. Le régime à 4% de marc de pomme a été bénéfique sur le taux de conversion alimentaire ($p = 0,002$) et l'efficacité énergétique ($p = 0,004$) au jour 35 après le sevrage. Le marc de pomme a eu tendance à diminuer la consistance des matières fécales (plus molles à liquides, $p = 0,096$) et à augmenter le nombre d'agents pathogènes excrétés ($p = 0,072$). L'aliment à 4% de marc de pomme a influencé la richesse du microbiote et le profil bactérien comme observé pour le phylum Bacteroidetes ou la classe Clostridia. En ce sens, Bacteroidetes est apparu comme un point de convergence entre les données in vitro et in vivo. Pour conclure l'expérimentation, le régime 4% marc de pomme apparaît comme une stratégie de sevrage intéressante qui doit être évaluée sur une large cohorte pour évaluer plus finement le risque de dysbiose dû à l'excrétion d'agents pathogènes au début de la période post-sevrage.

La thèse a permis le développement d'un modèle gastrointestinal permettant l'étude du microbiote du porcelet au moment du sevrage (baby-SPIME) et a mis en lumière les possibles futures recherches à mener pour faire évoluer le modèle baby-SPIME (par exemple en reproduisant l'absorption passive d'une partie des ingrédients à l'aide d'un système de dialyse combiné au SHIME, en utilisant des réactifs de l'espèce porcine tels que la bile porcine, en utilisant des perles de mucine et en combinant une approche quantitative aux données de composition du microbiote) qui pourraient probablement améliorer les fermentations du bioréacteur iléon. Avec l'expérimentation in vivo, un concept qui a émergé était l'effet bénéfique du marc de pomme pour la période critique du sevrage probablement grâce à son contenu en monosaccharides, fibres et polyphénols en améliorant l'absorption d'énergie – dans l'intestin grêle – et en modifiant le microbiome intestinal. Ce concept a montré l'importance de combiner des études in vitro et in vivo. Il a également montré l'intérêt de poursuivre les recherches sur le marc de pomme pour élucider les mécanismes d'action en jeu sur le métabolisme.

Introduction

At the beginning of the thesis–in 2015–pig production was one of the fastest growing animal productions in the world. Global demand for pork was steadily increasing for several decades and it was the most widely consumed meat in the world. The increase in herds was mainly observed in Asia. It was slower or even stable in North America and Europe. It was accelerating in Africa (FAO, 2016). Since then, the African Swine Fever outbreak and the COVID-19 crisis have severely disrupted pork production, international trade of this meat and its consumption. However, the outlook for 2030 points to an increase in global production and consumption of meat for which a third of the global increase is expected to come from pork, that should be the second largest source of meat products in the world (FAO, 2021).

Factors that will support production are herd expansion (especially in China and in the Americas) and increased productivity per animal (FAO 2021). One axe on which to act to improve productivity is the gastrointestinal disorders. Indeed, the intestinal tract diseases are amongst the most important economic problems affecting pig production, with the recurring endemic enteric diseases having the greatest long-term impact on health and animal productivity (Thomson and friendship, 2019). Post-weaning diarrhea is one of them–with neonatal diarrhea, proliferative enteropathy and swine dysentery; it has a cost for producers and it represents an opportunity to improve health of piglets and so animal welfare and producer's income (Sjölund et al., 2014).

Weaning–in commercial production–is often at the origin of post-weaning diarrhea. Indeed, weaning induces physiological changes that can lead to the proliferation of enteropathogenic bacteria resulting in serious post-weaning enteropathies such as colibacillosis (Luppi et al., 2016; Thomson and Friendship, 2019). Antibiotics have long been used as a means of prevention, followed up by zinc oxide. Due to human health concern (increasing antibiotic resistance) and environmental concern, alternatives are more and more used for the control of post-weaning diarrhea (Rhouma et al., 2017). This is described in **PART 1** of the introduction.

Amongst the multiple alternative strategies, some are related to feed. A possible feeding strategy–subject of the thesis–is the use of apple pomace. Indeed, this by-product–described in **PART 2** of the introduction–is known for its antioxidative, anti-inflammatory, antiproliferative, antibacterial and antiviral effects (Waldbauer et al., 2017). Those are relevant properties in the context of weaning disorders. However, in Wallonia, this by-product is generally spread over the fields or used for the production of biogas but no use aims at a health-oriented added value (data not shown in the thesis). By the work of the thesis, a possible way of orienting the co-product is being considered for a local level use.

Finally, to test a feeding strategy, in vitro gastrointestinal models are useful tools as alternative to (or before) animal experimentation. They are described in **PART 3** of the introduction with a focus on swine models–a model adapted to weaning conditions being required to test apple pomace.

PART 1. Weaning of Piglets

1.1 Weaning process and impact

Weaning of piglets is the process ensuring the transition from an animal greatly dependent on sow milk to a self-reliance eating animal.

In the wild, as described in Pluske et al. (2003), weaning is an extended process that takes several months–following the individual–during which the piglet has time to evolve on behavioral, nutritional and immunological aspects. The decrease of milk production engages the first foraging behaviors of the piglets that progressively get into a larger social group with a minimum of aggression. The milk–20% of dry matter highly digestible due to its lactose, protein and fat content–is progressively replaced by solid feed–15 to 30% dry matter; leading time to the immature gastrointestinal tract of piglet to adapt physiologically and microbiologically to a new regimen. The health status of the youth animal is influenced by the appropriate evolution of all these factors.

In rearing conditions, weaning is generally an abrupt and premature event –3 to 5 weeks after parturition in the current production methods–consisting to separate the piglet from the mother or to stop artificial milk feeding (Jean-Blain, 2002). Weaning in breeding conditions leads to multiple external factors of stress: change in diet (from a 20% dry matter milk feed to an 80-90% dry matter plant-based diet), in physical and social environments including the maternal separation and the disruption of the established social group (Weary et al., 2008). In few hours, piglet has to adapt to a novel environment including unfamiliar feeding and drinking equipments, unfamiliar piglets, with no more stimulus from sow to start feeding, and potential lack of thermal comfort (Pluske et al., 2003). These stressors alter intestinal structure and functions, nervous and hormonal pathways and immune system that may have an impact on short and long-term performances and health status of the piglet (Campbell et al., 2013; Niewold, 2015).

Abrupt weaning results in reduced piglets' feed intake with subsequent damages on gut health and functions (Jayaraman and Nyachoti, 2017). Due to this nutritional deficit or anorexia period–depending on the time required by the piglets to get used to the novel environment and regain homeostasis–a depression in growth (very low or negative growth)–is observed. Modifications on the intestinal architecture and functions happen, i.e., decrease of villi height, increase of crypts depth and intestinal cell mitosis, the reduction of the brush border enzyme activity and the reduction of the absorptive capacity of nutrients and electrolytes (Pluske et al., 2003). Additionally, impacts on the gut barrier function, on piglets' immunity and on microbiota are also described. An inflammatory response of the intestinal mucosa is observed and makes animal sensitive to antigens, toxins and translocation of bacteria (Gresse et al., 2017; Jayaraman and Nyachoti, 2017; J. Lallès et al., 2007). As a consequence of these disturbances, weaning generates post-weaning symptoms in piglets, including post-weaning diarrhea (van Beers-Schreurs et al., 1992).

The post-weaning diarrhea (PWD) is described as a multifactorial endemic disease–including multiple infectious and non-infectious causes (AMCRA, 2018). Virus (as coronavirus or rotavirus), bacteria (*Escherichia coli*, *Clostridium perfringens* type A or *Salmonella*), parasite or feed are at the origin of most of the digestive affections. While some diseases from viral origin require only symptomatic treatment, bacterial causes requires antibiotic (colistin or gentamycin treatment for weaning enteritis) (Coudert, 2018).

Amongst the common infectious agents of PWD are enterotoxigenic *E. coli* (ETEC) pathotype, more precisely the F4 and F18 attachment factors (Luppi et al., 2016). Fairbrother et al. (2005) reported the pathogenesis of *E. coli* PWD. When ingested, *E. coli* colonizes the mucus layer of the small intestine or get attached to receptors of epithelium. They massively proliferate to represent in the order of 10^9 CFU/g of tissue and the degree of proliferation determines disease infectious status. For a long time, antibiotics were largely used to promote growth of piglets and prevent disease. But the problematic of bacteria resistance to antibiotics in animal and human health issues, combined to observed aggravation of post-weaning syndromes, highlighted the global issue to find alternative strategies to antimicrobials (Fairbrother et al., 2005; Heo et al., 2013; Rhouma et al., 2017). Since January 1, 2006, in Europe, antibiotics for non-therapeutic purposes are banned of the animal nutrition (European Parliament and Council of the European Union, 2003). In Belgium, two major evolution of the legislation contributed to reducing the quantity of antibiotics used in animal production (Agence Fédérale des médicaments et des produits de santé, 2017; SPF Santé publique Sécurité de la Chaîne alimentaire et Environnement, 2016). As an immediate and effective alternative to antibiotics for piglets at weaning, zinc oxide (ZnO) was largely used to prevent PWD. Following unclear mechanisms, it reduces diarrhea and mortality and improves piglets' growth (Fairbrother et al., 2005). But alternative strategies had also to be found (Heo et al., 2013; Satessa et al., 2020). Indeed, due to the impacts of zinc on the environment, in particular the leaching and run-off of zinc to surface water, the use of ZnO is being phased out in Europe (end of authorisation period scheduled for July 27, 2026; European Commission, 2016).

To reduce the risk of PWD, a combination of preventive measures has to be reasoned on a case-by-case basis, depending on the farm situation (AMCRA, 2018; Fairbrother et al., 2005). Due to the fact that weaning is a stressful event leading to reduction of the feed intake with increased microbiological risk, it seems appropriate to reduce as much as possible stress, to stimulate the feed intake and manage the microbiological risk to ensure gut health and performance (Heo et al., 2013; Jayaraman and Nyachoti, 2017). This can be done through appropriate husbandry practices including feeding and nutrition strategies, animal welfare, biosecurity practices and prevention of disease (Jayaraman and Nyachoti, 2017). For the rearing practices implemented before weaning, the piglet gastrointestinal tract has to be progressively prepared to deal with diet evolution. It is done by offering creep feed (Jean-Blain, 2002). Although this does not guarantee that piglets experienced with it (Pluske et al., 2003), it appears however as an advantageous practice to maintain growth performances and gut health when well offered to piglets (Jayaraman and Nyachoti, 2017), i.e., creep feed regularly refreshed and at

disposal of piglets in multiple areas of the case. The vaccination of piglets before weaning against ETEC is also an encouraging strategy (Fairbrother et al., 2005; Rhouma et al., 2017). For the rearing practices implemented at weaning, the age of piglet must be a good compromise between the physiological adaptation of the young animal and the constraints of animal production desired by the breeder (Jean-Blain, 2002). Four parameters guide it: the piglet aptitude, the sow reproductive performances, the farming organization and the legislation (IFIP - Institut du porc, 2013); this last one requiring a minimal age of 28 days–or 21 days if specialised housings exist for piglets–in order to ensure animal welfare (Council of the European Union, 2009). For the rearing practices implemented after weaning, the preventive measures includes to maintain piglets in the suitable thermal zone, avoiding drafts when ventilating and favouring *ad libitum* feeding strategy (Rhouma et al., 2017) with an appropriate access to water in quantity and quality (Pluske et al., 2003). Increasing feeder space to reduce social stress and to improve feeding behaviour in group is also advised (Jayaraman and Nyachoti, 2017).

The feeding strategy at weaning appears as one of the most important weaning parameters to control with precision. Supplying a continuous flow of nutrients to the intestine allows to maintain the villous architecture while reducing enteric disorders (Pluske et al., 2003). To manage the nutritional aspect of weaning, producers are advised to furnish during a short time interval a weaning diet–1st age diet containing raw materials as fat or lactoserum–to ensure the transition followed by a second diet–2nd age diet–less expensive (IFIP - Institut du porc, 2013). As an example of what producers are advised, 1st age diet has to furnish 10 to 11 MJ NE/kg feed and 1.3 g of digestible lysine/MJ NE and not too much crude protein. The requirements for the 2nd age diet are 9.0 to 10.5 MJ NE/kg feed and 1.15 to 1.20 g digestible lysine/MJ NE; at this stage, piglets regulate their ingestion and producers/nutritionists can vary the raw materials to reduce cost. Following piglets and management practices, a third diet can be supplied during the post-weaning with the constraint to provide 1.0 g digestible lysine/MJ NE to piglets. To manage the risk of enteric disorders, different nonantibiotic alternatives have been identified and tested with more or less efficacy: organic acids (well recognized as alternative), specific amino acids, spray-dried animal plasma protein (well recognized alternative), dietary protein source and level, antimicrobial peptides, medium-chain triglycerides/fatty acids, specific egg yolk antibodies, bacteriophages, prebiotics, probiotics, synbiotics, exogenous enzymes, milk products, clay minerals, medicinal plants and essential oils (Gresse et al., 2017; Heo et al., 2013; Niewold, 2015; Rhouma et al., 2017). Prebiotics and probiotics are considered by some authors as major alternatives options due to their impacts on gut health and microbiota (Gresse et al., 2017; J.-P. Lallès et al., 2007).

1.2 Impact of weaning on the digestive system

The digestive system of the pig – monogastric omnivorous mammal has a dominant nutritional role. In addition, the GIT plays a protective role for the organism (intestinal barrier and immune defence) and interacts with the host's endocrine system (Evans et al., 2013).

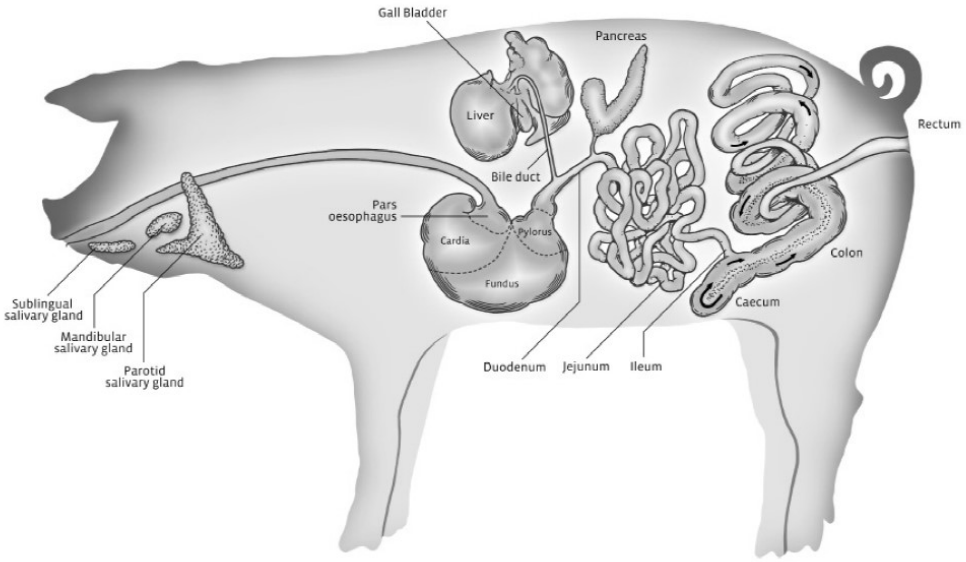

Figure 1: Illustration of the porcine digestive system (Lærke and Hedemann, 2012).

The first site of feed digestion after the mouth is the stomach–9 liters, 30% of the GIT volume of an adult pig. It includes three regions with specific pH and enzymatic .conditions (Leborgne et al., 2012). The first–the cardiac gland region–has a higher pH due to the saliva and the secretions of the cardia glands. They provide suitable conditions for activity of the salivary amylase and to bacterial fermentation on carbohydrates (CHO). Mucus is secreted to protect the epithelium from the acidity. The second and third regions–the fundus and the pylorus gland regions–have lower pH although pH increases near the end of the stomach due to reflux from small intestine (Snoeck et al., 2004a). The mucosa secretes gastric juice, containing chymosin, hydrochloric acid and pepsinogen. Mucus is also produced to protect the pylorus epithelium from acidity. Low pH ensures the conversion of pepsinogen into pepsin that allows first hydrolysis of protein to take place (Heo et al., 2013; Snoeck et al., 2004a). Moreover, low pH has a beneficial health effect due to its bactericidal action on many pathogens as *E. coli* (Heo et al., 2013). In the stomach, microbiota is scarce (10^4 – 10^5 bacteria/g of content) and is mainly considered as a deglutition one (Fonty and Chaucheyras-Durand, 2007).

The second site of digestion is the small intestine–18 to 20 meters, 10 liters, 33% of the GIT volume of an adult pig. The small intestine plays an essential role in chemical digestion and absorption. The chemical digestion occurs through the enzymes produced by the intestine itself but also through products coming from the liver and the pancreas. Indeed, the glands of the mucosa produce substances

containing several enzymes (lipase, peptidases, dipeptidases, maltase, isomaltase, saccharase and lactase) and mucus; the liver produce bile that permits to neutralize the acidity of the chyme and makes easier the digestion of lipids emulsified by bile salts; pancreatic juice contains lipase, amylase, trypsin, chymotrypsin and peptidases (Leborgne et al., 2012). The small intestine includes three successive sections: duodenum, jejunum and ileum (Leborgne et al., 2012). The duodenum–1/16 of the small intestine (Snoeck et al., 2004a)–is the zone receiving alkaline pancreatic juice and bile. The jejunum–11/16 of the small intestine–is the site major of nutrients absorption (Crevieu-Gabriel, 1999). The ileum–¼ of the small intestine–is the site where enzymatic digestion, due to glands secretions, progressively finishes and is the site of the first intestinal fermentation for highly fermentescible CHO (Williams et al., 2005) as starch, some soluble β-glucans, oligosaccharides and short chain inulin (Molist et al., 2014). The architecture of the small intestinal mucosa is crucial for the proper functioning of the digestive process. The mucosa forms villi at the base of which open crypts (Figure 2). Villi are finger-like protrusions whose enterocytes show a brush border surface, increasing the exchange surface of the epithelium (Hérin et al., 2015). Crypts are tubular invaginations of the surface epithelium. They contain epithelial stem cells required for repopulation of the epithelial cells of the villi. Other crypt cells ensure also the secretion of fluids and electrolytes–essential for nutrient digestion and absorption (Heo et al., 2013). The chyme progress through the small intestine rapidly and regularly, and influence the abundance of the microbiota (from 10^4 - 10^5/g of content in duodenum to 10^7 - 10^9/g of content in ileum; Fonty and Chaucheyras-Durand, 2007). The pH significantly increases from the beginning to the end of the small intestine (Snoeck et al., 2004a).

The third site of digestion is the large intestine. Seat of the absorption of water, minerals and products of fermentation, the large intestine includes the caecum, the colon and the rectum. The caecum–2 liters, 7% of the GIT volume–is a cylindroid pouch at the junction of the ileum and the colon and is carried by this one. The pH is lower than those of the ileum. The colon–4 to 5 meters, 9 liters, 30% of the GIT volume–includes two parts, an ascending voluminous part (helicoidal colon) followed by a smaller transversal part that ends in the rectum. The pH is the same than in the caecum. In the large intestine, the intestinal enzymatic activity is over; the glands of the mucosa produce only mucus (Leborgne et al., 2012). The microbiota is abundant since the caecum. It ensures a saccharolytic activity considered as beneficial for health. Indeed, it uses the wall carbohydrates from plant feed and residual starch as substrates, which leads to the production of short-chain fatty acids (SCFA) as acetate, propionate and butyrate–an energy source for the pig (Fonty and Chaucheyras-Durand, 2007; Leborgne et al., 2012; Molist et al., 2014). The microbiota ensures also a proteolytic activity by the fermentation of residual nitrogenous and endogenous materials providers of certain fatty acids–particularly branch-chain fatty acids (BCFA) as isobutyrate, valerate and isovalerate but also phenols, indoles, amines and ammonia–metabolites potentially toxic to the host (Fonty and Chaucheyras-Durand, 2007; Jha and Berrocoso, 2016). Finally, the bacteria also produce vitamin B but in an amount insufficient to cover the needs of the animal (Fonty and Chaucheyras-Durand, 2007; Leborgne et al., 2012).

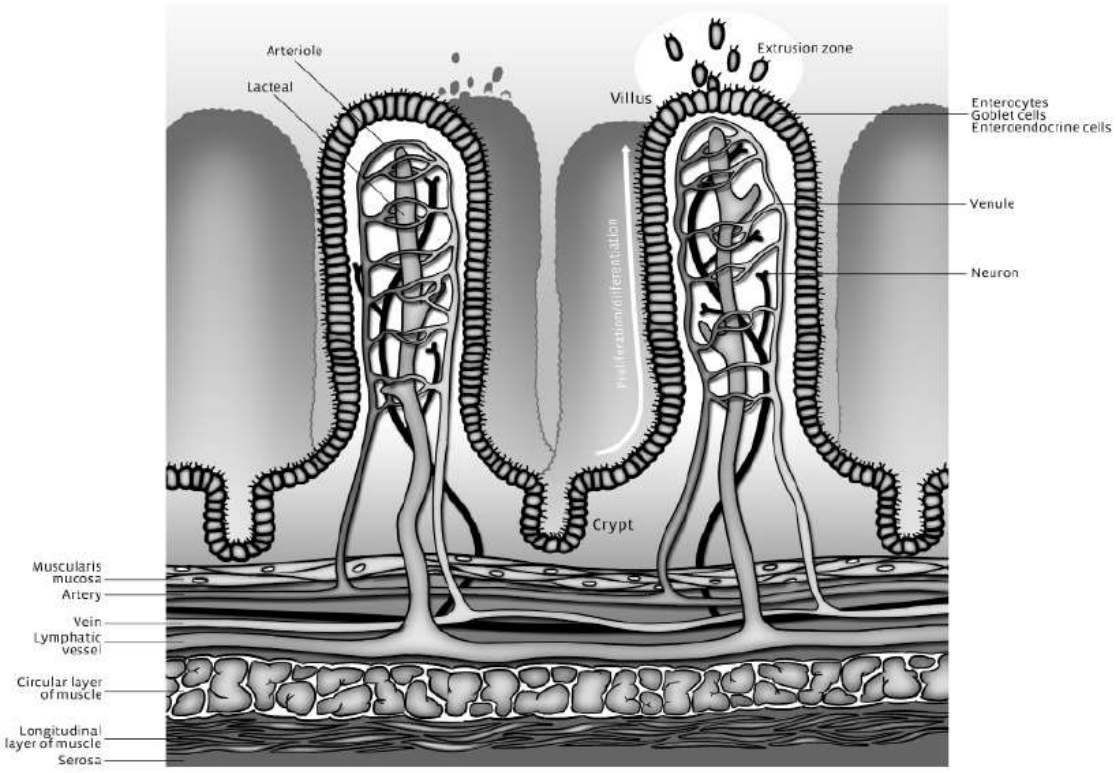

Figure 2: illustration of the small intestinal wall (Lærke and Hedemann, 2012).

Weaning leads to structural and functional changes of the digestive system. Following the works of Pluske et al. (2003), Montagne et al. (2006) and Heo et al. (2013), the immature intestinal structure declines and moves on to a mature intestinal structure including parameters adapted to the new diet. Indeed, before weaning, the sow's milk–rich in simple molecules–is rapidly digested. The proximal parts of the small intestine are particularly active for this digestion. At weaning, the GIT has to digest a diet containing complex proteins and carbohydrates with various antinutritional factors. It requires more sophisticated digestion mechanisms for which the distal parts of the GIT, since the ileum, are more active. The regional distribution of monosaccharide transporters evolves consequently all along the small intestine with weaning (Figure 3).

With an abrupt weaning, the evolution of the intestinal structure occurs also abruptly. As a whole, in few days, the digestive system has to growth intensely to maintain or increase its digestive and absorptive functions. The relative masses (g/kg bodyweight) of the stomach and the large intestine increase while that of the small intestine decreases; the relative mass of the liver and pancreas increases in relation with the evolution of the metabolic and biosynthetic function activities. In a particular way, the decline of the immature structure–that culminates 3 to 5 days post-weaning–primarily concerns the proximal small intestine with the atrophy of the villi (phase 1) and is followed by the increase of the crypts depth that initiates the maturation phase of the digestive system during which enzymes and regionalized nutrient transporter capacities evolves (phase 2). The effect of weaning on the gastric and small intestinal enzymes' activity is equivocal in the literature, the discrepancies between studies being

probably due to experimental variations (Heo et al., 2013). The enzymatic adaptation is not immediate and occurs from the second day post-weaning to the four or five days post-weaning; the activity of lactase decreases while the activity of pepsin, amylase, maltase and saccharase increases (Leborgne et al., 2012). In addition to these changes, weaning also influences the transit time. Indeed, in the immediate post-weaning, the transit time is lengthened in all compartments of the GIT–a phenomenon called intestinal stasis that is thus coupled with a poor digestion of feed (Molist et al., 2014)–and it takes up to three weeks not to be longer affected by weaning; transit time is then regularised with feed being more retained in the stomach and the colon (Snoeck et al., 2004b). The pH also evolves; it decreases in the first half of the small intestine and in the caecum within two weeks after weaning (Snoeck et al., 2004a).

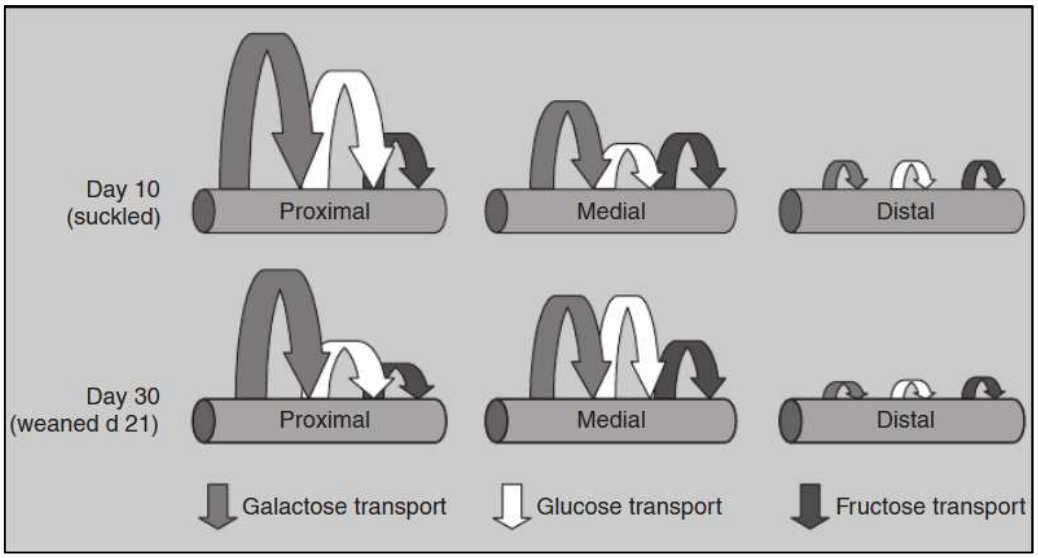

Figure 3: Regional and temporal distribution of monosaccharide transporters of the small intestine (Pluske et al., 2003).

As a consequence of the structural weaning perturbations, the net absorption of fluid and electrolytes is reduced; the absorption of nutrients is compromised. And it could contribute to high incidences of osmotic diarrhea (Heo et al., 2013). As a consequence of the functional weaning perturbations, the large intestine is potentially overload with readily fermentable nutrients–starch becoming a main fermentative substrate for the microbiota instead of the non-starch polysaccharides for which the fermentative process is reduced (Bach Knudsen et al., 2012). As a consequence of the transit time and pH perturbations in the immediate post-weaning, susceptibility of piglet to pathogens increases (Heo et al., 2013).

1.3 Impact of weaning on the fermentative process

1.3.1 Intestinal microbiota

Microbiota is defined by Marchesi and Ravel (2015) as "The assemblage of microorganisms present in a defined environment." Considering the gastrointestinal tract of an individual, the microbiota can be approached as a succession in space and times of bacterial groups that has a role in nutrition and health of their host (Flint et al., 2012) and that impacts production parameters such as body weight and meat quality of animal production (Knecht et al., 2020). The bacterial population of the pig's colon is estimated to 10^{10} - 10^{11} bacteria per gram of digesta (Guevarra et al., 2019). Each microorganism occupies its proper niche, in which ecological and nutritional conditions are favorable to its multiplication. And this organization would be all the more strict in the segments of the digestive tract where the residence times are longer (Fonty and Chaucheyras-Durand, 2007).

A large amount of bacterial genera are common between humans and most animals but quantity and proportion of bacteria are well species-specific and varies depending on age, physiological conditions encountered by bacteria, host's digestive secretions, diet composition and gut site (Montagne et al., 2003). Globally, it can be considered that the gastrointestinal and fecal microbiota of the pig is mainly composed of members from the Firmicutes, Bacteroidetes and Proteobacteria phyla with Firmicutes being dominant all along the tract (Gresse et al., 2019). The microbiota includes also members from Actinobacteria, Spirochetes, Synergistetes and Fusobacteria phyla (De Rodas et al., 2018; Gresse et al., 2019; Liu et al., 2019; Yang et al., 2019), as well as Acidobacteria (Zhao et al., 2015) or Verrucomicrobia (Slifierz et al., 2015). In their meta-analysis of the swine microbiota, Holman et al. (2017) listed the 20 most abundant genera found in their selected studies: *Prevotella*, *Bacteroides*, *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Treponema*, *Helicobacter*, *Succinivibrio*, *Alloprevotella*, RC9 gut group, *Blautia*, *Streptococcus*, *Escherichia-Shigella*, *Turicibacter*, *Faecalibacterium*, *Sarcina*, *Megasphaera*, *Phascolarctobacterium*, *Parabacteroides* and *Pseudobutyrivibrio*. The first 4 genera are common with the first reference gene catalogue of Estelle et al. (2018) that found in addition *Eubacterium* as major bacterial genus of the pig's gut microbiome. To describe more precisely the microbiota of pig, this requires to consider its variation in space–from stomach to faeces–and in time–across ages.

Regarding the evolution in space, it can be considered that, longitudinally in the tract, a facultative anaerobic–even aerobic–population localized in the small intestine evolves to an obligate anaerobic population more and more close to that found in faeces (Bach Knudsen et al., 2012; Zhao et al., 2015). In parallel, the α-diversity of the microbiota increased with the progression toward the intestine (Gresse et al., 2019; Li et al., 2019). Moreover, an evolution occurs also radially in the intestinal segment due to the well different environment provided by the mucosa, mucus layer and lumen (Bach Knudsen et al., 2012; Fonty and Chaucheyras-Durand, 2007; Montagne et al., 2003). Authors that studied the microbiota of pig all along the gastrointestinal tract observed that each segment has its own

bacterial particularities (De Rodas et al., 2018; Gresse et al., 2019; Holman et al., 2017) but 3 distinct profiles emerge. They correspond to the anatomic zone of the tract: duodenum-jejunum, ileum and large intestine (Liu et al., 2019). In the digesta of preweaned piglet, the duodenum-jejunum part mainly includes Firmicutes but Actinobacteria and Proteobacteria are also found (Gresse et al., 2019). This is in agreement with the observation of De Rodas et al. (2018) specifying that–in Firmicutes–Bacilli is more abundant that Clostridia. *Lactobacillaceae*, *Pasteurellaceae*, *Peptostreptococaceae* and *Streptococcaceae* are the main families found in duodenum-jejunum (Gresse et al., 2019). De Rodas et al. (2018) also observed some Bacteroidetes in this segment of the tract. It is confirmed in the work of Liu et al. (2019) in which *Lactobacillus* and *Bacteroides* were the most abundant genera found.

In ileum, Firmicutes is dominant with Proteobacteria which are also well established (Gresse et al., 2019; Li et al., 2019). In addition to the families described in duodenum-jejunum, Enterobacteriaceae is also found (Gresse et al., 2019). This converges with the observations of De Rodas et al. (2018) who also show a dominance of Bacilli in this part of the tract. However, the works of Liu et al. (2019) diverge. Indeed, the authors mainly observed *Fusobacterium* and *Escherichia* in this part of the GIT. It is suggested that *Fusobacterium* act as pathobiont in young pigs, wich means that its normal evolution tends to decrease in abundance excepted in case of trouble where *Fusobacterium* can proliferate (Chen et al., 2017). The difference between studies may depend on the health status of the animals. It may also depend on another factor such as the breed of pig–Meishan being known to harbour more *Fusobacterium* (Guevarra et al., 2019). *Lactobacillus*, *Actinobacillus*, *Terrisporobacter*, *Streptococcus* and *Clostridium sensu stricto* 1 are described as the dominant genera in ileum (Li et al., 2019).

In the large intestine, Firmicutes is present with Bacteroidetes; Proteobacteria and Epsilonbacteraeota are also present but mainly in the mucosa (Gresse et al., 2019). Clostridia is more abundant than Bacilli (De Rodas et al., 2018). The main families observed belong to Ruminococcaceae, Lachnospiraceae, Prevotellaceae and Bacteroidaceae (Gresse et al., 2019). Fusobacteriaceae and Lactobacillaceae seem also abundant in other studies (Li et al., 2019). *Prevotella* appears as the major genus in this part of the tract (Holman et al., 2017; Liu et al., 2019). *Lactobacillus*–although less abundant than in ileum–remains important in colon as well as other genera such as *Alloprevotella*, *Fusobacterium* or *Ruminococcus* 2 (Li et al., 2019). From the study of Gresse et al. (2019), the most prevalent genera found all along the GIT are *Lactobacillus*, *Actinobacillus*, *Romboutsia*, *Escherichia-Shigella*, *Terrisporobacter* and *Campylobacter*; this last one regarding only some individuals. When considering mucosal *vs*. digesta samples, Proteobacteria and Epsilonbacteroaeta are better represented in the mucus (Gresse et al., 2019). Several taxa are of significantly higher relative abundance in the mucosa, including *Anaerovibrio*, *Bacteroides*, *Desulfovibrio*, *Helicobacter*, *Oscillospira*, *Phascolarctobacterium*, and *Prevotella* (De Rodas et al., 2018). An observation shared by all the authors is the highest interindividual variability of the microbiota in the small intestine compared to a more diverse but more stable microbiota in the large intestine (De Rodas et al., 2018; Gresse et al., 2019; Liu et al., 2019).

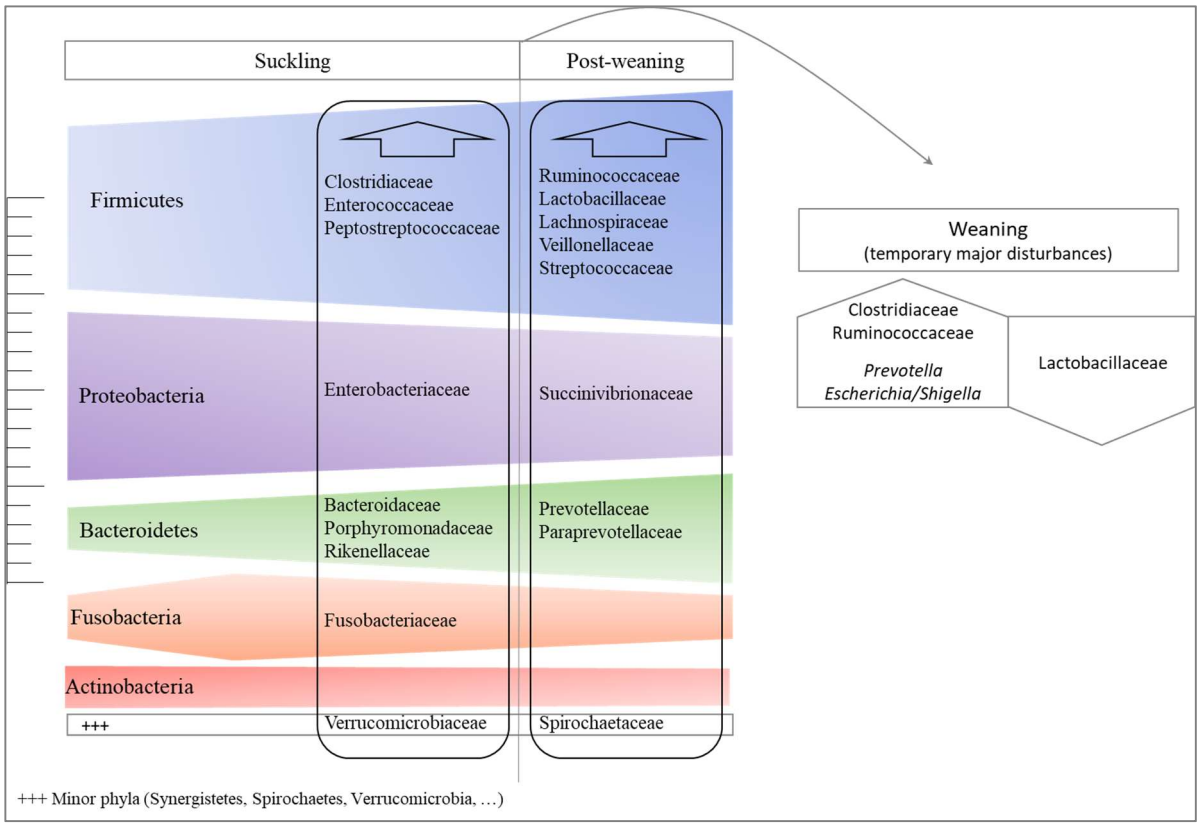

Figure 4: Evolution of the microbiota in faeces from birth to the post-weaning period (adapted from Guevarra et al., 2019; Slifierz et al., 2015)

Regarding the evolution over time (Figure 4), at the beginning of its life, the piglet is the host of a population of facultative aerobic or anaerobic bacteria which evolves into adulthood due to dietary and environmental changes. The microbiota becomes more diverse and abundant changing from bacteria favoured by a milk substrate to bacteria favoured by a vegetal substrate (Knecht et al., 2020; Niu et al., 2015; Pajarillo et al., 2014). Slifierz et al. (2015) studied the fecal microbiota of piglet from birth to 49 days of age. They concluded that the microbiota of piglet underwent an important evolution during these 7 weeks. Before weaning, Jurburg and Bossers (2021) identified 4 stages of bacterial development (stage 1: day 1, stage 2: days 2 to 4, stage 3: days 5 to 21, stage 4: days 28 to 35; no weaning during the experiment) with characteristic modifications between stages. At stage 1, Firmicutes is one of the two dominant phyla encountered. Its dominant *Clostridium sensu stricto* and *Streptococcus* population lets progressively place to a more diverse profile of Firmicutes–including *Lactobacillus* and *Faecalibacterium* in higher relative abundances–at the last stage. Lachnospiraceae and several members of Ruminococcaceae gradually increase in abundance. Proteobacteria, the second dominant phylum at stage 1 (predominance of *Escherichia-Shigella*) partially decreased with age in favour of Bacteroidetes for which Rikenellaceae and Muribaculaceae dominate. *Fusobacterium* decreases with age after a transient increase observed in the second stage.

Weaning is an event leading to a shift of the microbiota; a bacterial transition takes place with the new diet (Kim & Isaacson, 2015; Konstantinov et al., 2004). It can lead to an abrupt loss of richness

and a perturbation of the balance in the microbial communities (Yang et al., 2019). It is on the fourth day that the effects seems most marked (Tao et al., 2015). Five to ten days are required to restore stability amongst the microbiota and to lead to a suitable activity for the new complex diet in presence (Bach Knudsen et al., 2012; Chen et al., 2017; Estellé et al., 2016). In the immediate weaning (Figure 4), it can be considered that the major changes concern the increase of Clostridiaceae, Ruminococcaceae and a dramatical decrease of Lactobacillaceae. Moreover, *Prevotella* increases and *Escherichia-Shigella* is observed in relative high abundance compared to those observed before or afterweaning (De Rodas et al., 2018). After these major disturbances of the microbiota, the evolutions observed at the end of the suckling period continues post-weaning till stabilisation. Firmicutes increase when Fusobacteria and Proteobacteria decrease (Chen et al., 2017; Yang et al., 2019). Actinobacteria decrease (Yang et al., 2019) or remain stable in abundance (Chen et al., 2017). Bacteroidetes increased (Chen et al., 2017; Guevarra et al., 2018), with some authors observing even an inversion of the post-weaning Firmicutes: Bacteroidetes ratio in faeces (Pajarillo et al., 2014). In the GIT, it is noticed that some authors also observe in some studies a dominance of Bacteroidetes on Firmicutes at least in the caecum (Knecht et al., 2020) but others don't (De Rodas et al., 2018). At the family level, the major evolutions of Firmicutes concern the decrease of Clostridiaceae (Karasova et al., 2021; Luise et al., 2021) in parallel with the increase of Lachnospiraceae (Luise et al., 2021), Lactobacillaceae (Guevarra et al., 2018; Karasova et al., 2021) and Veillonellaceae (Guevarra et al., 2018; Karasova et al., 2021). Ruminococcaceae increase (Guevarra et al., 2018; Karasova et al., 2021) or remain stable (Luise et al., 2021). Regarding Bacteroidetes, Prevotellaceae increase when Bacteroidaceae decrease (Guevarra et al., 2019; Karasova et al., 2021; Luise et al., 2021). Enterobacteriaceae (Guevarra et al., 2019; Karasova et al., 2021; Luise et al., 2021) as well as Fusobacteriaceae (Karasova et al., 2021) continue their gradual decrease observed even before weaning. When it comes to the genus level of the classification, a greater disparity exists in the observations made between studies. Consensus seems to exist for some bacteria, such as a postweaning increase of *Prevotella* (De Rodas et al., 2018; Guevarra et al., 2018; Han et al., 2017; Luise et al., 2021; Mach et al., 2015), *Blautia* (Chen et al., 2017; De Rodas et al., 2018; Luise et al., 2021), *Roseburia* (Chen et al., 2017; De Rodas et al., 2018; Luise et al., 2021) and *Feacalibacterium* (De Rodas et al., 2018; Luise et al., 2021; Yang et al., 2019) or the decrease of *Escherichia-Shigella* (Chen et al., 2017; Mach et al., 2015; Yang et al., 2019). But this is not the case for some others bacteria such as *Lactobacillus* that dominate before weaning in some studies (Chen et al., 2017; Mach et al., 2015; Yang et al., 2019) or after weaning in others (Guevarra et al., 2018; Han et al., 2017). It may be noticed that some factors–as the animal itself–can influence the profile of the microbiota. As a first example, during suckling, the Yorkshire breed exhibits more *Bacteroides* while the Meishan breed exhibits more Fusobacteriaceae and Erysipelotrichaceae (Guevarra et al., 2019). As a second example, post-weaning, *Bacteroides* is the third more abundant genus in lighter piglets whereas it is Faecalibacterium in heavier piglets (Yang et al., 2019). This makes difficult to map the profiles in terms of bacterial genera before and after weaning. Stabilized, the microbiota of a piglet could however belong to 1 of the identified

enterotypes. In 2016, Estellé et al. (2016) has identified two probable enterotypes : A (microbiota rich in Ruminococcus and Treponema) or B (microbiota rich in Prevotella and Mitsuokella). More recently, Luise et al. (2021) instead identified 4 enterotypes: Prevotella, Faecalibacterium, Roseburia and Lachnospira, which may correspond to a gradual maturation shift of the microbiota from pre- to post-weaning.

The susceptibility of piglets to PWD may be indicated by its early bacterial gut composition (Guevarra et al., 2019). Different studies highlight certain microbial characteristics such as the reflect of a predisposition to diarrhea. Karasova et al. (2021) identified a higher abundance of Actinobacteria before weaning as an indicator of such predisposition while, surprisingly, *Chlamydia* and *Helicobacter* were indicators of more resistant piglets. For Yang et al. (2019), an altered relationship between *Prevotella* and *Escherichia* during preweaning could be the main cause of post-weaning diarrhea. Indeed, in their experiment, healthy piglets harboured a higher relative abundance of *Escherichia* and a lower abundance of *Prevotella* during the whole period with the sow. Conversely, piglets harbouring a higher relative abundance of *Prevotella* compared to *Escherichia* during the same period were those belonging to the diarrheic category. Related to the beginning of PWD, a reduced number of *Bacteroides* jointly with a reduced numbers of *Ruminococcus*, *Bulleidia* and *Treponema* is observed (Yang et al., 2019). After weaning, a microbiota harbouring more Clostridiales, Deltaproteobacteria, Selenomodales, *Fusobacterium*, *Akkermansia* and *Anaerovibrio* seems linked to diarrheic piglets while a microbiota harbouring more *Prevotella* and *Faecalibacterium* seems linked to healthy piglets (Karasova et al., 2021); *Faecalibacterium* also being identified as being more abundant in heavier piglets than lighter piglets by Yang et al. (2019). At the family level, the higher relative abundances of Prevotellaceae, Lachnospiraceae, Ruminococcaceae and Lactobacillaceae appear as post-weaning indicators of healthy piglets (Guevarra, 2019).

1.3.2 Products of fermentation

Bacteria of the pig gastrointestinal tract are in touch with materials that were not digested by host secretions. It occurs mainly in the distal part of the tract from the ileum to the rectum, being dominant in the large intestine (Jha and Berrocoso, 2016). These materials are endogenous or exogenous and they mainly consists on dietary fiber and proteins not digested in the small intestine (Fonty and Chaucheyras-Durand, 2007). The products of fermentations are predominantly short chain fatty acids (SCFA), but also lactate, succinate, H_2O_2, various gazes (CO_2, H_2 and CH_4) and bacterial cell biomass (Montagne et al., 2003).

SCFA are saturated aliphatic organic acids ranging from 1 to 6 carbons. The three most important–in terms of quantity–are acetate (2 carbons), propionate (3 carbons) and butyrate (4 carbons) which constitute 90% - 95% of the SCFA found in the colon and faeces in an approximate molar ratio of 60:20:20 (den Besten et al., 2013; Ríos-covián et al., 2016). In less quantity are found formate

(1 carbon), valerate (5 carbons) and caproate (6 carbons). Are also found branched chain fatty acids for 5% of the total produced SCFA; consisting on isobutyrate, isovalerate and 2-methyl butyrate (Ríos-covián et al., 2016). SCFA concentrations evolve all along the fermentative intestinal tract, showing a balance between what is produced and what is absorbed (Fonty and Chaucheyras-Durand, 2007; Macfarlane and Macfarlane, 2003). So, the concentration of SCFA in the faeces does not reflect their production in the intestine (den Besten et al., 2013) because they are rapidly and massively (95% to 99% of total SCFA) used or absorbed from the gut lumen (Montagne et al., 2003). Indeed, they are directly taken up by the intestinal cells–for the benefit of the cells themselves as energy sources–or they pass into the blood to go to certain organs in which they are used as a substrate or as a signal molecule (den Besten et al., 2013). Ultimately, the excess amounts of SCFA circulating in the blood are metabolized by the liver in order to avoid toxic systemic concentrations.

SCFA are more and more considered as key mediators linking nutrition, intestinal microbiota, physiology and pathology (Ríos-covián et al., 2016). They indeed are identified as having a role directly on the microbiota (against pathogenic bacteria due to modification of the luminal pH or due to cross-feeding interactions between bacteria), in the gut barrier function, glucid and lipid metabolisms, on the immune system and inflammatory response or on the appetite regulation (Morrison and Preston, 2016; Ríos-covián et al., 2016). More precisely, acetate from protective *Bifidobacteria*–in relation with consumption of fructose in the distal colon–would play a role of protection against enteropathogenic infection at least in mice models (Fukuda et al., 2011). Acetate is a source of energy used by the muscles, the brain (Fonty and Chaucheyras-Durand, 2007) and the liver and is incorporated in the lipid biosynthesis (den Besten et al., 2013; Ríos-covián et al., 2016). Propionate is a precursor in gluconeogenesis in the intestine and in the liver (den Besten et al., 2013). It plays a role in the activation of hormonal and nervous systems (Koh et al., 2016). Butyrate is used as an energy source mainly by colonocytes and increases mucin production–favorable to reduction of bacterial adhesion (Ríos-covián et al., 2016). It plays also a role in intestinal gluconeogenesis (De Vadder et al., 2014) as well as in the activation of hormonal and nervous systems (Koh et al., 2016).

SCFA production–types and levels–depends on factors linked with the host and the diet. Depending on the substrates available–carbohydrates, proteins or molecules as nitrate and sulfate (these last two molecules favoring production of acetate instead of butyrate for example), the microbial composition and the transit time, different metabolic pathways can be taken (Figure 5), leading to different SCFA profile (Macfarlane and Macfarlane, 2003).

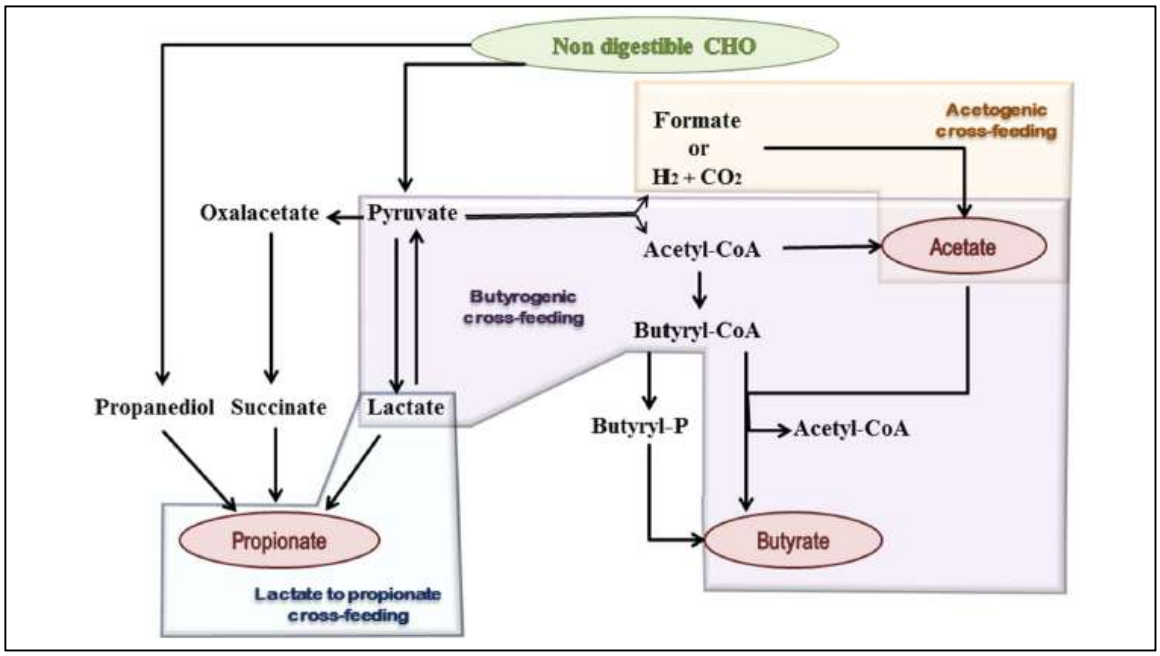

Figure 5: Schematic representation of microbial metabolic pathways and cross-feeding mechanisms, contributing to SCFA formation in the human gut according to Ríos-covián et al. (2016).

Like microbiota, concentrations and proportions of SCFA in the intestine of piglets shows a large interindividual variability and differences between the different anatomical regions are observed (Gresse et al., 2019). Before weaning, the total concentration in SCFA increases from small intestine to caecum (Franklin et al., 2002). The caecum shows the highest production of SCFA of the large intestine, followed by the proximal colon; The distal colon and rectum are equivalent in terms of total SCFA concentrations (Gresse et al., 2019). The concentration of the three major SCFA–acetate, propionate and butyrate–depends on the location in the intestine. All the three are lower in the small intestine (39.9 mM) compared to the large intestine (109.7 mM in caecum) (Franklin et al., 2002). Acetate is significantly higher in the caecum while propionate and butyrate are significantly higher in the proximal than distal colon (Gresse et al., 2019). In terms of proportion, depending on the authors, about 80% - 90% of the SCFA in the ileum are composed of acetate (Franklin et al., 2002; Mathew et al., 1996). It decreases to about 60% - 65% in the large intestine (Franklin et al., 2002; Gresse et al., 2019). Ratios 65:25:10 can be observed in the colon for acetate, propionate and butyrate respectively (Freire et al., 2000; Shim et al., 2005).

With weaning, it is expected that concentration and profile of SCFA evolves due to the presence of various dietary fiber in the solid feed, but also due–in the immediate post-weaning–to the presence of starch in the large intestine (K. E. Bach Knudsen et al., 2012; Laree et al., 2007). Indeed, weaning showed significant effects on SCFA concentrations (Franklin et al., 2002) with diet exerting an important effect (Awati et al., 2006b). Acetate, butyrate, iso-valerate and total SCFA decrease post-weaning in the small intestine while L- and D-lactate increase (Mathew et al., 1996). In the large

intestine, decreases of individual or total SCFA concentrations are more or less significantly observed in the immediate post-weaning period (Nakatani et al., 2018; Zhou et al., 2019) but re-increases are observed in the late post-weaning period (Zhou et al., 2019). In faeces, butyrate and acetate were observed to increase post-weaning probably due to the increase of some bacterial genera such as *Faecalibacterium*, *Blautia*, *Roseburia* and *Prevotella* (Meng et al., 2020).

PART 2. Apple pomace

Apple pomace is described in the filipodia database–the online encyclopaedia of animal feeds– as "the solid residue that remains after milling and pressing of apples for cider, apple juice or puree production […] and contains peel, flesh, stem, core, seeds and juice residues". From a nutritional aspect (Figure 6), AP is poor in protein and rich in carbohydrates–sugars and fiber (Heuzé et al., 2018). The dietary fiber concept has long been discussed until 2009 when the CODEX Alimentarius commission published a harmonized definition that includes all carbohydrates that are neither digested nor absorbed in the small intestine and which have a degree of polymerization of 10 or more monomeric units. A certain flexibility lies in the possibility to include carbohydrates from 3 to 9 monomeric units, which EFSA and the FDA have adopted. The fibers therefore include in one hand non-starch polysaccharides such as cellulose, hemicelluloses, pectins, resistant starch, indigestible oligosaccharides (inulin, oligofructose) and, in the other hand, lignins (Gill et al., 2021; Jones, 2014).

Main analysis	Unit	Avg	SD	Min	Max	Nb
Dry matter	% as fed	91.2	1.3	87.8	94.7	224
Crude protein	% DM	8.0	2.3	3.6	11.4	119
Crude fibre	% DM	36.0	6.8	18.1	49.6	224
NDF	% DM	65.1	7.4	48.2	74.6	70
ADF	% DM	57.7	8.0	36.7	67.1	70
Lignin	% DM	26.8	3.3	16.2	35.0	172
Cell walls, water insoluble	% DM	72.8				1
Ether extract	% DM	2.8	0.9	1.2	4.7	50
Ash	% DM	2.1	1.0	0.8	5.0	67
Starch (polarimetry)	% DM	8.1	6.2	0.9	18.4	7
Total sugars	% DM	5.5	1.8	2.6	13.9	111
Fructose	% DM	14.5		14.5	14.5	2
Gross energy	MJ/kg DM	19.4				*

Minerals	Unit	Avg	SD	Min	Max	Nb
Calcium	g/kg DM	0.6	0.2	0.2	1.1	25
Phosphorus	g/kg DM	1.4	0.3	1.0	2.0	27
Potassium	g/kg DM	1.9	0.7	1.2	3.4	18
Sodium	g/kg DM	2.7	5.3	0.1	15.0	20
Manganese	mg/kg DM	7		6	9	2
Zinc	mg/kg DM	4		3	5	2
Copper	mg/kg DM	5		5	5	2
Iron	mg/kg DM	72	19	60	100	4

Figure 6: Nutritional table for dehydrated apple pomace (Feedipedia, 2020). Avg: average or predicted value; SD: standard deviation; Min: minimum value; Max: maximum value; Nb: number of values (samples) used; * indicates that the average value was obtained by an equation.

Carbohydrates are the main source of energy of piglet diet and are digested in the different segment of the GIT following the organisation of the molecules itself or between them (Bach Knudsen et al., 2012). To clarify what includes carbohydrates and their becoming in the digestive tract, it is interesting to consider the classification of carbohydrates (Figure 7). Indeed, they can be classified either biochemically or nutritionally (Bach Knudsen et al., 2012; Siret, 2002).The biochemical classification is based on their molecular size–or degree of polymerization (DP)–of carbohydrates. Monosaccharides–DP 1–are basic molecules. Disaccharides–DP 2–consist of two linked basic molecules. Oligosaccharides have DP ranging from 3 to 9. Polysaccharides include molecules whose DP ≥ 10, being classified as starch components or non-starch polysaccharides (NSP). The nutritional classification of the carbohydrates is based on the endogenous capacity to digest them. They are classified into two categories. The first is the digestible carbohydrates (components directly assimilable by the organism–as oses or derivatives of oses …–or assimilable after degradation by endogenous digestive enzymes–as diholosides, starch or glycogen …). The second category is the non-digestible carbohydrates. It includes components which are not totally hydrolyzed by endogenous digestive enzymes. These carbohydrates–oligosaccharides, resistant starch and NSP–constitute an important part of the dietary fiber (Siret, 2002) and serves as main substrates for bacterial fermentation in the distal part of the gut; having an impact–positive or negative–on gut health because influencing the microbiota and the production of end-products of fermentation (Montagne et al., 2003).

Category	DP	Type of component	Endogenous enzymes	Prebiotic CHO
Monosaccharides	1	Glucose		–
		Fructose		–
Disaccharides	2	Sucrose	+	–
		Lactose	+	–
Oligosaccharides	3	Raffinose	–	+/–
	4	Stachyose	–	+/–
	5	Verbascose	–	+/–
	3–9	Fructo-oligosaccharides	–	+++
		Xylo-oligosaccharides	–	+
		Trans-galactooligosaccharides	–	+
Polysaccharides				
A. Starch	≥10	Rapidly digestible	+	–
		Slowly digestible	+	–
		Resistant (RS)	+	++
B. Non-starch (NSP)	≥10			
Cell wall NSP		Cellulose	–	–
		β-Glucan	–	+/–
		Arabinoxylans	–	+/–
		Xyloglucans	–	–
		Rhamnogalacturans	–	–
		Galactans	–	–
Non-cell wall NSP		Fructans/inulin	–	+++
		Mannans	–	–
		Guar gum	–	–
		Pectins	–	–

DP, degree of polymerization; CHO, carbohydrates; RS, resistant starch; NSP, non-starch polysaccharides.

Figure 7: Classification of some carbohydrates (Bach Knudsen et al., 2012).

The effect of dietary fiber on animal nutrition can be positive or negative. Amongst the most important factors influencing the fermentability of dietary fiber–and thus their effect–are the source of fiber itself and its solubility, degree of lignification, processing, level of inclusion in the diet, intestinal

transit time, age and weight of animal and the microbial composition of the host (Montagne et al., 2003). The solubility of the NSP fraction is a property of the dietary fiber that is noted in the literature as nutritionally of high importance (Bach Knudsen et al., 2012). I.ndeed, solubility influences the physicochemical properties of the digesta–the viscosity and hydration of which interfering with the gastric emptying rate and the transit time–as well as the digestive and the fermentative process (contact between enzymes and substrate, absorption of nutrients, creation of microenvironment favorable or not to proliferation of pathogenic bacteria). So soluble dietary fiber has long been considered as having negative impact (Bach Knudsen et al., 2012; Molist et al., 2014; Montagne et al., 2003). But soluble fiber has also showed having a positive impact on the fermentative process in the large intestine (Zacharias et al., 2004). Actually, the effect of soluble dietary fiber seems largely to depend on their primary structure and source, on their molecular weight and on the concentration of polymer (Bach Knudsen et al., 2012); for example low-methyl esterified pectin showing different impact on digestion/fermentation than high-methyl esterified pectin (Tian et al., 2017). Soluble fiber is considered as more easily and rapidly fermented that insoluble fiber–for which fermentation occurs more distally in the GIT (Montagne et al., 2003). Soluble fiber impacts the kinetics of digestion of all components of the diet. For example, it delays the digestion of starch and proteins that become fermentative substrate in the large intestine or it modulates the microbiota to favour degradation of dietary fiber instead of protein at the end of colon, beneficially for colon health (Tian et al., 2017). Recently, Larsen et al. (2019) confirmed the link between structural features of pectin and positive modulation of the gut microbiota. They identified in vitro at least five factors including the degree of esterification of the polygalacturonic acid–monomer constituting pectin–as the most important parameter. Soluble and insoluble fiber are both present in plants in a ratio that varies with the type and stage of maturity of the plants (Montagne et al., 2003). For piglets at weaning, it seems advised to favour the inclusion of moderate amounts of insoluble fiber just after weaning and to incorporate progressively higher amounts of soluble and fermentable fiber sources later in order to avoid overload of fermentable substrate in the immature GIT of piglets.

Apple pomace is one of the main commercial sources of pectin, providing high-methoxyl pectin or low-methoxyl pectin–obtained by deesterification of high-methoxyl pectin (Thakur et al., 1997). Apple pomace is well known as source of dietary fiber but also others biomolecules beneficial for health such as phenolic and terpenic compounds (Boyer and Liu, 2004; Grigoras et al., 2013; Kołodziejczyk et al., 2007; Lu and Foo, 1997) with beneficial effect of each molecule that appears more effective when they are combined (Aprikian et al., 2003). Apple Pomace can represent a raw material of great interest for different applications such as production of lactic acid (Gullón et al., 2007), as well as being a source of dietary fibre or polyphenolic components (Kołodziejczyk et al., 2007; Rana et al., 2015), and as a possible functional food for the agri-food sector (Reis et al., 2014). Apple Pomace seems to have positive health effects in rats when used as a feed ingredient–improving the antioxidant status of the animals, by increasing the antioxidant capacity of the lipid fraction of serum, reducing the blood glucose level and

increasing the fermentation process in the distal part of the gastrointestinal tract (Juśkiewicz et al., 2012). Incorporated at 3.5%, beneficial effects were also observed in weaned piglets on the gut morphology (Sehm et al., 2007) or on other parameters such as intestinal bacteria, blood parameters or gene expression of immunological markers (Sehm et al., 2011).

Although beneficial effects may thus be expected, it must be kept in mind that the composition of AP can largely vary in space and time. For example, the apple cultivars used for the juice extraction can influence the physico-chemical properties and antioxidant activities of the residual AP (Andre et al., 2012; Sato et al., 2010). The use of enzymes for extraction of juice (Thakur et al., 1997) or blanching and drying process employed on apple pomace (Heras-Ramírez et al., 2011) have an impact on its composition. So, the potential variability in the composition of the matrix encourages continuing research on this healthy raw material for its introduction in the feed chain at a large scale and shows the importance, for feed producers, of properly characterizing the matrix before using it.

PART 3. In vitro models

As an alternative to in vivo (human, animal) studies–that can ethically, technically or economically be difficult to carry out–, in vitro digestion and/or fermentation models were progressively developed since the 1990s (Nissen et al., 2020; Sensoy, 2021). Although « No in vitro technique could ever surpass or even equal the results that can be obtained from a living animal, most particularly in terms of assessing its health and well-being » (Williams et al., 2005), models are recognised as useful tools–by authorities–in terms of animal nutrition (Rychen et al., 2018). It is however essential to know the features, the strengths and the limits of each model to choose the most suitable for the study to be carried out (Nissen et al., 2020). Indeed, each model integrates in a more or less specialized way the mechanical, digestive or fermentation aspects of the digestion process.

Amongst the fermentation models that have gradually been developed, two major systems exist: the static models *vs* the dynamic models (Dupont, 2016; Fonty and Chaucheyras-Durand, 2007).

Static models–or batch models–are closed fermenters in which microorganisms can grow after inoculation. The device may be basic (a simple flask inoculated with a defined group of bacteria) or more complex (pH and temperature-controlled reactor inoculated with fecal suspension) and recreate the physicochemical conditions–usually through fixed parameters–of the targeted intestinal compartment. These models are inexpensive, easy to set up and to use, fast and accessible. It is a good screening technique that permits the study of metabolites profiles with, however, the limitations to be oversimplified and limited to short-term tests (Dupont, 2016; Moon et al., 2016; Nissen et al., 2020). Due to the plethora of batch models available in the literature, making comparisons between studies difficult, a consensual protocol was developed (Brodkorb et al., 2019; Minekus et al., 2014).

Dynamic models include the (semi-)continuous flow of fresh medium in the device with fermentative juice that is regularly removed from the system, better simulating the physiological reality. It permits to study more complex ecosystems, over a prolonged time (several tens of days) in one (monocompartmental system) or more compartments (multicompartmental system)–thus extending the study of the microbiota to different niches in a single equipment for these last ones. These dynamic models allow a high reproducibility between studies. However, they can present–following the model–the limitations of being time and efforts consuming. They can also be less accessible and bulky (Dupont, 2016; Moon et al., 2016; Nissen et al., 2020; Williams et al., 2005). A study comparing continuous–multicompartmental versus continuous–single colon model (Firrman et al., 2021) showed that alpha-diversity of the microbiota was comparable between both models but a marked difference was observed regarding abundance of some taxa such as Fusobacteriaceae or Bacteroidaceae. In addition, the single vessel community produced significantly less SCFA compared to multicompartmental model. Those data provide insight on possible applications and bias of a simplified versus more complex model.

The main fermentation models described in the literature are:

- (M-)SHIME (Molly et al., 1994)

SHIME (Simulator of Human Intestinal Microbial Ecosystem, ProDigest, Gent, Belgium) system is a computer-controlled system of the gastrointestinal tract through five double-jacketed glass units (two units dedicated to stomach and small intestine and three units dedicated to large intestine). The system ensures a movement through magnetic stirrers. Passive absorption mechanisms can be mimicked using a dialysis membrane. Anaerobiosis of the system is maintained using nitrogen. The culture medium is mixed in the system with pancreatic juice and bile solutions prepared for the run. The microbial communities colonizing the system are colon region specific, with the proximal regions containing saccharolytic microbes (e.g., *Bacteroides* spp. and *Eubacterium* spp.) while the distal regions contain mucin degrading bacteria (e.g., *Akkermansia* spp.) A shift is observed between this human in vitro model and in vivo experiments which results in an increased ratio of Bacteroidetes/Firmicutes in vitro with an enrichment of propionate producing bacteria (*Clostridium* cluster IX) compared to butyrate producing bacteria (*Clostridium* cluster IV and XIV). M-SHIME is an improvement of the SHIME system initially described. It includes mucin beads in the colon units serving as mucin-covered microcosms (Van den Abbeele et al., 2012). This allows to maintain a microbiota associated to mucin layer such as *Lactobacillus mucosae* and *Lactobacillus rhamnosus* GG. This is closer to the microbial communities observed in vivo and it allows more specific study of the mucosal microbiota.

- TIM-2 (Minekus et al., 1999)

TIM-2 (TNO, Zeist, The Netherlands) is a computer-controlled system mimicking the large intestine through four glass units. The system ensures a peristaltic movement. Absorption is done through hollow-fibers membranes. Anaerobiosis of the system is maintained using nitrogen. Due to the fact that no digestion process is included, the system requires the use of a special medium–the simulated ileal efflux medium (Nissen et al., 2020). TIM-2 can be coupled with TIM-1–the dynamic multi-compartmental digestive system simulating conditions in the stomach and small intestine–or tiny-TIM (the simplified Tim- model) although they do not run jointly (Sensoy, 2021). As other models, this one is also under constant optimization (Dupont et al., 2018). This model is at the origin of the TIMpediatric model that is able to simulate the dynamic conditions in the lumen of the upper gastrointestinal tract for three paediatric age groups: neonates, infants and toddlers (Havenaar et al., 2013; Roussel et al., 2016). It is also at the origin of the model that reproduces the specific digestive conditions of the elderly (Denis et al., 2016). The model can also be used to mimic the gastrointestinal conditions and colon microbiota observed for pigs (Avantaggiato et al., 2007; Martinez et al., 2013) and dogs (Smeets-Peeters et al., 1999). TIM-2 systems has been validated with pooled or individual fecal samples (Aguirre et al., 2014b), from healthy volunteers (Tabernero et al., 2011), from obese donors (Aguirre et al., 2014a), or patients with intestinal disorders (Rose et al., 2010). The method of preparation of a human inoculum has been the topic of a separate work to frame the use of the model (Aguirre et al., 2015).

- A dynamic in vitro model for the human ileum (Stolaki et al., 2019)

Based on the concept of TIM models, this human ileum model is composed of two linked glass units with inner flexible walls. It ensures a computer-controlled peristaltic movement. It includes a dialysis system of hollow fibers. The system is flushed with nitrogen gas. The culture medium is based on the medium described by Gibson et al. (1988), adapted from the TIM experience, and it simulates material passing the ileo-caecal valve. The pH–7.2–is set to agree with a postpandrial pH found in vivo. Different inocula were tested and faeces appeared an adequate inoculum for subsequent studies to stabilise faster the microbiota. Acetate, propionate and butyrate are present in the model in a ratio 6:3:1.

- (M-)ARCOL (Blanquet-Diot et al., 2012; Cordonnier et al., 2015)

ARCOL (Artificial colon; University of Auvergne, Clermont-Ferrand, France) is a mono-compartmental colonic model operating in semi-continuous conditions. The system (Applikon, Delft, The Netherlands) ensures a movement of the fermentation liquid by stirring and a continuous homogenisation of the atmosphere. After initial flushing with nitrogen, the fermentative process allows the maintenance of anaerobic conditions in the bioreactor. This is the first model allowing to maintain of anaerobiosis using the metabolic activity of the microbiota. Passive absorption is performed through hollow-fibers membranes. The culture medium reproduces ileal effluent. M-ARCOL is an adaptation of ARCOL, by using mucin beads (Deschamps et al., 2020). Up to date, ARCOL has been validated to reproduce the colonic microbiota of humans, preruminant calves, pigs and piglets.

- Mini-Bio

Mini-Bio (Applikon, Delft, The Netherlands) is described by Nissen et al. (2020) as a flexible system operating as batch or dynamic model generating high speed and massive data. The software can manage up to 32 bioreactors with a minimum capacity of 50 mL. MICODE (multi-unit in vitro colon gut model), obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, The Netherlands) and controlled by Lucullus PIMS software (Applikon Biotechnology) has been described recently (Nissen et al., 2020). The model was able to maintain a low Firmicutes to Bacteroidetes ratio, confirming the capacity of the model to simulate a healthy in vivo microbiota for a limited period of time (24h).

- PolyFermS (Zihler Berner et al., 2013)

PolyFermS is a continuous upper colonic model including 3 to 5 bioreactors (1 inoculum including fecal beads, 1 control and 1 to 3 test reactors) assembled in parallel. The system ensures a movement through stirrers and it does not include an absorption membrane. The anaerobiosis is maintained via CO_2 flushing. A medium mimicking intestinal chyme is prepared adapted from Macfarlane (Macfarlane et al., 1998). This model allows to improve the maintain of species diversity and abundance during long-term experiments through a specific process of immobilisation. This process consists on trapping the fecal microbiota via micro-encapsulation technique. Each of the five reactors of the model are inoculated with independent fecal inoculum, and the model can keep a stable microbial

community for 38 days (Nissen et al., 2020; Zihler Berner et al., 2013). PolyFermS is time and effort consuming, but stable over time and comparable to in vivo results.

- SIMGI (Verhoeckx et al., 2015)

SIMGI (CSIC-UAM, Madrid, Spain) is an automated gastrointestinal system including 5 compartments (stomach, small intestine and three colonic regions), with the digestive compartments and the fermentative compartments operating jointly or independently. Particularities of this model lies in the stomach. Indeed, the system ensures a peristaltic movement in this compartment and its pH acidification follows curves as encountered in vivo. The system is continuously flushed with nitrogen. The medium can be prepared based on Macfarlane's works (Macfarlane et al., 1998). The experimental period can last up to 6 days only. Regarding intestinal microbiota, the model is less close to in vivo experiments compared to other models (Nissen et al., 2020).

- CoMiniGut (Wiese et al., 2018)

CoMiniGut (University of Copenhagen, Frederiksberg, Denmark) is an in vitro colon model working by multiple of five parallel stirred reactor units of 5 mL contained in a composite climate box with controlled temperature. The anaerobiosis is maintained through nitrogen flushing and by using anaerogen sachets. The pH evolves until 24h–the endpoint–to simulate the ascending, transverse and descending colon conditions. The model allows to test a high number of experimental conditions in small volumes which is really useful for investigation of rare and expensive compounds and/or to increase statistical power of the experiments.

- TSI (Cieplak et al., 2018)

TSI (The Smallest Intestine) is partially adapted from the CoMiniGut model (cabinet, temperature and pH control). It simulates the stomach and the small intestine process including absorption via a dialysis cassette. It includes five parallel stirred batch-like reactors of 12 mL. The anaerobiosis is maintained via nitrogen and anaerobic sachets. Two fluids are used-one gastric and one intestinal, based on Minekus studies (Minekus et al., 2014). The pH and time conditions applied to the reactors evolves progressively to follow the simulation of duodenum, jejunum and ileum. A consortium of ileal bacteria is added in the reactors during this last part of the cycle. The model serves as screening tool. The model is less realistic and reproducible than other models, due to less strict environmental settings, but present the advantages to be logistically flexible and operator friendly (Nissen et al., 2020).

Fermentation models dedicated to swine:

- Porcine ileum (Blake et al., 2003) and colon model (Khaddour et al., 1998) adapted from the Hillman's works (1990s)

This mono-compartmental model has a 500 mL working volume reactor. It is stirred to allow a basal anaerobic niche region in the lower part that includes glass beads. In the upper part of the reactor, a low level of aeration is performed. The reactor does not include any absorption device. Media are prepared following the Macfarlane's works (Macfarlane et al., 1989b, 1989a). The porcine ileum is usually used to test antimicrobials or diet on microbiota. Using Vancomycin as example, data showed to be reproducible over time, describing that antibiotics influence the ileal bacteria (Blake et al., 2003). For the colon, the model is recognised by authors as appropriate for mid colon research considering the bacterial limitations (lowest *Bifidobacterium* representation and highest coliform proportion). The system requires low maintenance and minimal technical input.

- Cositec

Cositec is a semi-continuous fermentation model for the distal part of the intestine of pig (Breves et al., 2000; von Heimendahl et al., 2010). The anaerobic reactor contains 2 nylon bags. To begin the run, one bag is used to inoculate and the second-one is used to supply freeze-dried caecal particles. Then, the two bags are alternatively, every 24h, replaced to supply fresh-dried particles. An electromotor ensures the movement in the reactor. The model was used to test the effect of live yeast on the fermentation process of finishing pig. The effect of live yeast was visible with the largest dose of yeast. The shift in the microbiota was particularly initiated from the bacteria collected from the wash of the feed bag instead of the liquid associated bacteria (Pinloche et al., 2012).

- PolyFermS (Tanner et al., 2014a)

PolyFermS was adapted for the swine proximal colon by using swine fecal beads in the inoculum reactor and by adaptation of the fermentation conditions. The model, that stably reproduces the swine microbiota and metabolic activity for at least 54 days, allows the simultaneous investigation and direct comparison of different treatments on the same porcine gut microbiota. In particular, it can be used to study nutritional effects of pro- and prebiotics. It was studied for *Bifidobacterium thermophilum* RBL67 and prebiotics (Fibrulose F97, Vivinal GOS 90 and Bio-Mos) (MOS) on inhibition of *Salmonella Typhimurium* N-15 colonization (Tanner et al., 2014b).

- (M-)Pigut-IVM (Fleury et al., 2017; Gresse et al., 2021)

Pigut-IVM is a continuous piglet colonic model. The system (Global Process Concept, Périgny, France) ensures a movement of the fermentation liquid by stirring and a continuous homogenisation of the atmosphere for which anaerobiosis is maintained via the fermentation gazes (initial sparging with nitrogen gas). Absorption is performed using hollow-fibers membranes. The culture medium reproduces ileal effluent. M-Pigut-IVM is an adaptation of Pigut-IVM, by using mucin beads compartment and

mimicking the feed deprivation of weaning. The system (Applikon biotechnology, delft, the Netherlands) includes a bag filled with CO_2 to avoid any air contamination during the deprivation. A nutritive medium, mimicking ileal chyme, is prepared considering the digestibility indices of the compounds for piglets. Pigut-IVM was used for testing the probiotic *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 while applying an antibiotic disturbance with colistin. The model can be used for the screening of drugs or new dietary compounds (pre- and probiotics) (Fleury et al., 2017).

Over time, improvements have been made to batch and dynamic systems by for example adding a mucous environment (mucin beads) in the reactors in order to avoid the washout of the inoculum and to offer a niche for the mucous biofilm (Fleury et al., 2017; Moon et al., 2016; Tran et al., 2016; Van den Abbeele et al., 2012). Another major improvement was to combine a cell line (e.g. Caco-2) or *ex vivo* intestinal segment to the fermentation model in order to gain information about the host response (Marzorati et al., 2014; Moon et al., 2016; Nissen et al., 2020), gradually joining more complex models particularly dedicated to the study of the host-microbiota interaction as are the organoïds or the gut-on-a-chip model (Aguilar-Rojas et al., 2020). More recently, the improvements aimed to reduce the dimensions of the models–going through miniaturisation (Cryptobiotix, 2021)–while increasing the robustness of the results by increasing the number of simultaneous tests and reducing resources (Cieplak et al., 2018; Wiese et al., 2018). As perspectives, major evolution of the models proposed in the literature lies in the establishment of a set of standardized digestive and fermentative methods for food carbohydrates research and the introduction of modern bioinformatic and artificial intelligence technology to the systems (Ji et al., 2021).

Objective

The aim of the thesis was to develop an in vitro gastrointestinal model of the piglet including weaning in order to test AP–a product obtained after milling and pressing of apples–as a dietary supplement to reduce the challenges at weaning. Indeed, due to the increased interest of piglets' health at weaning and the importance of in vitro models as screening tools before performing animal experiments, a model dedicated to piglets at weaning appeared important to develop. Once equipped with this model, the fermentative digestion of AP could be studied to evaluate its interest since it was hypothesized that AP–due to its chemical composition–influenced the intestinal microbiota and the products of its fermentation in such a way to reduce intestinal weaning disorders. Additionally, an in vivo study using AP in a post-weaning piglets' diet to evaluate the effects on performance, intestinal architecture and microbiota would be of high interest to be carried out in order to evaluate the model, the in vitro observations and the interest of AP around weaning and for post-weaning.

In order to reach the aim of the thesis in terms of in vitro model, the SHIME® model–Simulator of Human intestinal Microbial Ecosystem–was chosen to benefit from its specificities, namely a dynamic and multi-compartment tool. The SHIME had to be modified into a baby-SPIME model (Simulator of Pig Intestinal Microbial Ecosystem, dedicated to the luminal microbiota of piglet) with a special focus on mimicking in vitro weaning at 28 days of age and including ileal plus colonic microbiota. Once wthe baby-SPIME was developed, AP could be included in the culture medium used to test its effect on short chain fatty acids (SCFA)–the products of fermentation–and on bacterial composition studied through next generation sequencing data. Then, data obtained through the in vivo experiment could be used to support additional discussion about the benefit of AP around weaning and for post-weaning. Prior to these in vitro/in vivo research studies, an analytical study had first to be done. The analytical study concerned the quantification of the SCFA. Indeed, the chosen model generates a large number of samples for which the SCFA content is used to evaluate the good evolution of the fermentation process.

The different studies of the thesis are illustrated in Figure 8. They are divided into 3 parts. First, the preparatory work part, includes the study 1 that aimed to develop a quantitative method of SCFA from C2 to C6 including isobutyric and isovaleric acids in samples provided by an experimental run of the adapted SHIME model (Technical objective–TO–1). Second, the in vitro model part, includes two studies that aimed the development (study 2) and some ways of improvement (study 3) of the model (Technical objective - TO 2). This part includes also the in vitro test of AP (study 4) to evaluate its effect on SCFA and microbiota (scientific hypothesis–SH–1). Third–the in vivo part–includes the animal experimentation study that aimed to evaluate the effects of dried AP incorporated at two levels into a post-weaning diet (a positive control level set at 4% and a lower intermediate level set at 2%)–on growth performance, on intestinal morphology and on the microbiota of piglets from weaning at 28 days old over a 5-week period (study 5, SH 2).

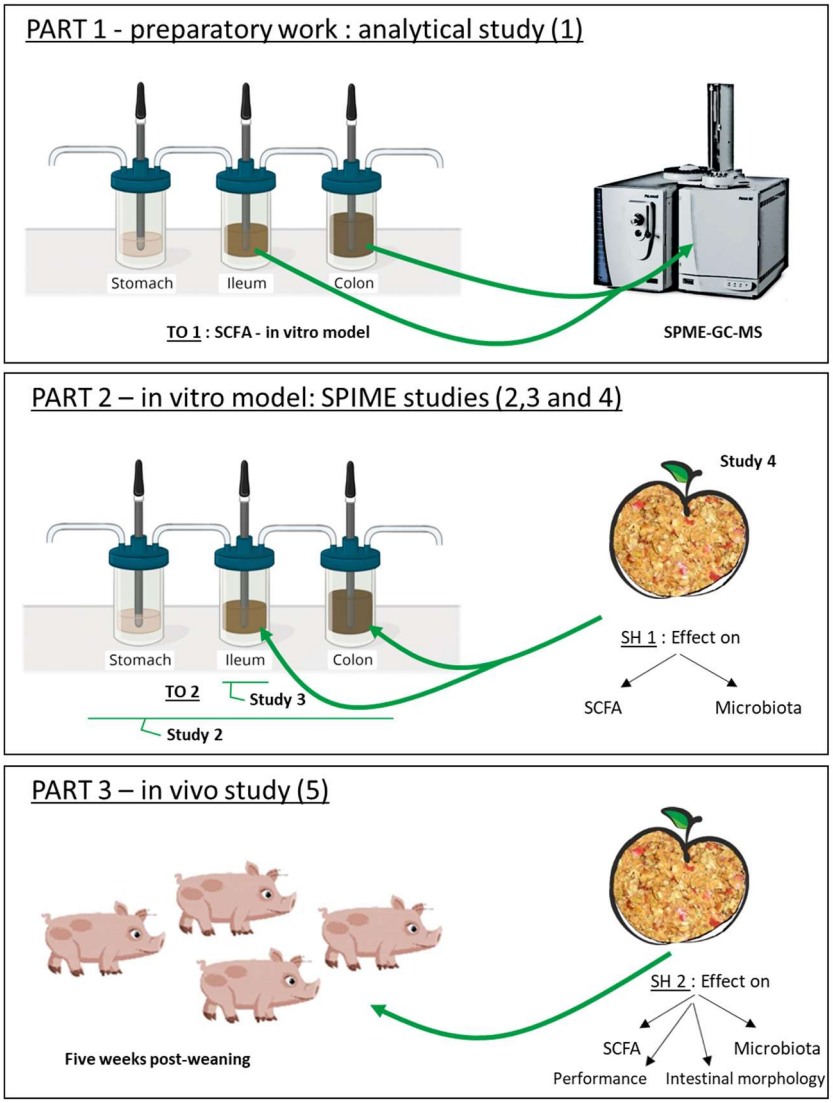

Figure 8: Illustration of the objective of the thesis through the technical objectives (TO) and scientific hypotheses (SH).

Experimental Section

PART 1: Preparatory Work– Analytical Study

The use of the SHIME model generates a large number of samples for which the SCFA content has to be quantified to evaluate, amongst other parameters, the good evolution of the fermentation process. In the literature, different methods are described to quantify the SCFA from faeces amongst which gas chromatography appears to be more commonly used, followed by high performance liquid chromatography, nuclear magnetic resonance and capillary electrophoresis (Primec et al., 2017). Large variations between/or in each technique exist in terms of sample preparation, accuracy or sensitivity of the methods. At least between the two first more used methods, gas chromatography is considered as the most reliable considering parameters such as limit of quantification, precision, accuracy, sample preparation, and volume of the sample required for measurement (Chatterjee et al., 2018). When choosing a proper method for fecal SCFA quantification, the recommendation that is done to researchers is to consider 3 important parameters. First, the instrumentation available in the laboratory. Second, time consumption. Indeed, all additional steps that prolonged the time for analysis (pretreatment procedures, …) increases the risk of losing SCFA and increases cost due to consumable consumption. In this view, SPME is considered as a modern technique reducing excessive use of solvent and sample dilution. Third, the cost for analysis, overall when a large number of samples are expected (Primec et al., 2017). SPME-GC-MS method appeared as the most appropriate for the thesis: available at the laboratory, reducing the pre-analysis treatment of the samples that are, in addition, produced in large numbers with baby-SPIME. It was the chosen method that is scientifically considered as acceptable for fecal samples. In the framework of this thesis, samples coming from a first adaptation of the SHIME model were used to develop the method. Those results are presented in Study 1.

Study 1 -

Development of an analytical method to detect short-chain fatty acids by SPME-GC–MS in samples coming from an in vitro gastrointestinal model

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Abstract

Short chain fatty acids (SCFA) are end-products of intestinal bacterial fermentation. The concentrations of fermentation metabolites are closely related to the microbial activity that occurs in various digestive compartments. The fermentation products may vary qualitatively and quantitatively, especially within the colon. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME), an in vitro dynamic and multicompartment model of the human intestinal tract, can be adapted to mimic the piglet gastrointestinal tract. In this context, a quantitative method, based on solid phase microextraction gas chromatography coupled to mass spectrometry (SPME–GC–MS), was developed for the determination of seven short chain fatty acids, *i.e.* acetic, propionic, butyric, isobutyric, valeric, isovaleric and hexanoic acids, in samples coming from this experimental in vitro gastrointestinal model. The advantage of the SPME-GC–MS technique is that the seven compounds could be determined in a single run, after a simple and rapid sample treatment, without any other extraction than the automatic SPME. The developed method was validated in accordance to the European and US FDA guidelines and showed good specificity/selectivity. In addition, limits of detection and quantification ranged from 8 to 72 mg L^{-1} and from 16 to 144 mg L^{-1}, respectively. Two internal quality control samples spiked at different concentrations were analyzed to assess the trueness of the developed method, which ranged between 97.7 and 122.4% of the expected value, for the seven compounds analyzed. The method was successfully applied to twenty samples coming from a gastrointestinal model, with different inocula. The developed method might be used as a general method for measuring SCFA in biological samples.

Introduction

Short chain fatty acids (SCFA) are end-products of fermentation of carbohydrates and proteins, from endogenous or diet origin, by the intestinal bacteria (Fonty and Chaucheyras-Durand, 2007). They consist of saturated aliphatic organic acids from 2 to 6 carbons, also known as volatile fatty acids (VFA): acetic (C2), propionic (C3), butyric (C4), isobutyric (iC4), valeric (C5), isovaleric (iC5) and hexanoic (C6) acids, with C2, C3 and C4 representing > 95% of the SCFA encountered in colon or faeces (den Besten et al., 2013).

The amount and types of SCFA produced in a healthy individual depend largely on the substrate availability, the transit time and the bacterial composition of the microbiota (Macfarlane and Macfarlane, 2003). In the pig gastrointestinal tract, microorganisms are in contact with exogenous or undigested substrate from the distal ileum to the rectum (Jha and Berrocoso, 2016; Williams et al., 2005). It is expected that the total concentration in SCFA and the different proportion between each of the acids vary all along the intestinal tract of a weaned piglet (Awati et al., 2006b). Intestinal SCFA content has to be considered as a balance between the produced and absorbed SCFA (Macfarlane and Macfarlane, 2003). As an example of in vivo profile for weaned piglet, the ratios 65:25:10 are usually observed in the colon for C2, C3 and C4, respectively (Freire et al., 2000; Shim et al., 2005). From an "in vitro colon" point of view, the ratio can be slightly different (60:20:20%) with a total concentration in SCFA, higher in vitro than in vivo (Fleury et al., 2017).

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME), a dynamic in vitro and multicompartment gastrointestinal model, is a well-established validated human model (Van den Abbeele et al., 2012, 2010; Venema and van den Abbeele, 2013). Its adaptation to other animal gastrointestinal environments such as piglet microbial environment is possible through the adjustment of new parameters that can be validated. Indeed, the pH of the different compartments can be adapted, in addition to the diet, the temperature and the intestinal microbiota that is inoculated into the system. The microbial composition and the metabolic activity of such model have to be monitored to ensure its stabilization before starting any trial, because two weeks of stabilization are necessary to reach the stabilization of the microbiota (Van den Abbeele et al., 2010). As the metabolic activity of microbiota from the gut can be measured *via* the SCFA content, the development of an adapted method measuring produced SCFA in bioreactors is crucial to monitoring the validation of gastrointestinal models for new animal species.

The two in vitro pig models described in the literature, namely PolyFermS (Tanner et al., 2014a) and PigutIVM (Fleury et al., 2017), refer to HPLC-UV as a method to measure the concentrations of SCFA. In this work, the development of a chromatographic method to quantify SCFA is described. This method is using a step of solid phase microextraction (SPME) to trap the compounds on a fiber before separation and detection of the volatile compounds by gas chromatography coupled to mass spectrometry (GC–MS).

The developed SPME-GC–MS method has been used to evaluate the concentration of SCFA from C2 to C6, including isobutyric and isovaleric acids, in samples provided by an experimental run of an adapted SHIME model. The developed method used 2-methylvaleric acid as internal standard (Bianchi et al., 2011).

Material and methods

Chemical reagents. Acetic acid (99.9% purity), propionic acid (99.8% purity), isobutyric acid (99.7% purity), butyric acid (99.6% purity), isovaleric acid (99.8% purity), valeric acid (99.8% purity), hexanoic acid (99.6% purity) and 2-methylvaleric (99.9% purity) were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). Water was of Chromanorm quality and provided by VWR International (West Chester, Pennsylvania, USA). Sulfuric acid (95–97% purity) was obtained from Merck (Darmstadt, Germany).

Standard solutions. Seven individual stock solutions (1–4.5 mg mL^{-1}) were prepared by dissolving each SCFA standard in HPLC grade water. A pool containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and hexanoic acids at a concentration of 0.720, 0.405, 0.080, 0.350, 0.070, 0.240 and 0.160 mg mL^{-1}, respectively, was prepared by diluting a specific volume of each of the stock solutions with HPLC grade water in a 20 mL volumetric flask.

A 0.2 mg mL^{-1} solution of 2-methylvaleric acid was prepared in water to be used as internal standard (IS). All standard solutions were stored in glass vials for maximum 6 months at +4 °C.

A 0.9M H_2SO_4 solution was prepared by diluting H_2SO_4 in water.

Culture medium. The medium used to feed the gastrointestinal system, called "feed medium", was prepared according to Molly et al. (1993) to make it closer to piglet feed: mucin (6.0 g L^{-1}), proteose-peptone (1.0 g L^{-1}), potato starch (1.0 g L^{-1}), L-cysteine hydrochloride (0.2 g L^{-1}), all from Prodigest (Gent, Belgium), and a post-weaning diet (8.0 g L^{-1}). This diet (ABZDiervoeding, Nijkerk, The Netherlands) contained mainly barley (30%), soy (29%), wheat (14%), maize (5%) and oat flakes (5%) as raw materials and was grinded to particles of 250 μm.

The culture medium was prepared in 5 L bottles and then autoclaved during 35 min at 121 °C. Each bottle was then agitated and let stand for 10 min before pumping 4 L of the supernatant. They were stored in a fridge and the pH was adjusted at 3.0 before being provided to the first vessel. Pancreatic juice was prepared in 2 L bottles. It contained sodium hydrogen carbonate (2.5 g L^{-1}, VWR Chemicals, Radnol, Pennsylvania, USA) and pancreatin (0.9 g L^{-1}, ProDigest) (personal communication of ProDigest). Oxgall (4.0 g L^{-1}, ProDigest) was added.

Samples used for the validation of the method. Since no Certified Reference Material of SCFA is available in biological samples, two in-house Quality Controls (QC) samples of "feed medium" fortified with standard solutions of SCFA at two different levels were used to assess the performance of the developed method.

QC1 contained concentrations of 360 mg L^{-1} of acetic acid, 202.5 mg L^{-1} of propionic acid, 40 mg L^{-1} of isobutyric acid, 175 mg L^{-1} of butyric acid, 35 mg L^{-1} of isovaleric acid, 120 mg L^{-1} of

valeric acid, 80 mg L^{-1} of hexanoic acid. QC2 was containing concentrations of SCFA five-time higher than QC1.

The two QCs were analyzed ten times each on different days.

Sample preparation. Twenty-five microliters of intestinal content were pipetted into a 20 mL glass vial. Forty microliters of internal standard at a concentration of 0.2 mg mL^{-1}, 15 µL of 0.9M sulfuric acid and 920 µL of feed medium were added. The vial was vortexed and placed on the autosampler of the SPME-GC–MS until analysis.

SPME-GC–MS parameters. The method used to analyze SCFA was adapted from Bianchi et al. (2011). SCFA were extracted with a SPME fiber on a Triplus RSH Autosampler (Thermo Fisher Scientific), separated on a Focus GC gas chromatograph (Thermo Fisher Scientific) using a Supelcowax-10 column (30m×0.25 mm, 0.2 µm) (Supelco, Bellefonte, PA, USA) and analyzed with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific).

SPME conditions were as follows: DVB/CAR/PDMS fiber (Supelco), agitation temperature 60 °C with an extraction time of 20 min. Temperature and time of desorption had been preliminarily evaluated and were set at 250 °C and 5 min, respectively. Fiber conditioning postinjection was performed at 270 °C during 10 min.

The GC conditions were: inlet: 250 °C; splitless injection; helium as the carrier gas at constant pressure of 250 kPa; temperature program: 50 °C for 5.5 min, followed by an increase of 75 °C min^{-1} to 170 °C for 2 min, then 10 °C min^{-1} to 200 °C for 3 min; total run time was 15.10 min.

The peaks were identified by comparing their mass spectrum and retention times with those of the corresponding standards. The MS conditions were: transfer line: 230 °C; ion source: 220 °C; collision energy: 35 eV; positive ionization mode. The SCFA were detected using full scan mode in a mass range between 40 and 150 Da in a single segment window. In each chromatographic run, different ions were monitored for each SCFA analyzed, which allowed to perform detection and quantitative analysis (Table 1).

Table 1: Retention times (RT, minutes) and mass of ions followed in MS for each compound analyzed (m/z).

	Compounds		RT (min)	Ions (*m*/*z*)
1	C2	Acetic acid	8.43	43+60
2	C3	Propionic acid	8.78	56+57
3	iC4	Isobutyric acid	9.11	41+43
4	C4	Butyric acid	9.21	73+55
5	iC5	Isovaleric acid	9.44	87+69
6	C5	Valeric acid	9.87	60+42
7	IS	2-Me-valeric acid	10.05	74+87
8	C6	Hexanoic acid	10.66	60+73

IS: Internal standard.

Calibration curves. Seven samples of feed medium spiked with the internal standard at a concentration of 0.2 mg mL^{-1} and with concentrations ranging from 144 to 7200 mg L^{-1} for acetic acid, 81 to 4050 mg L^{-1} for propionic acid, 16 to 800 mg L^{-1} for isobutyric acid, 70 to 3500 mg L^{-1} for butyric acid, 14 to 700 mg L^{-1} for isovaleric acid, 48 to 2400 mg L^{-1} for valeric acid and 32 to 1600 mg L^{-1} for hexanoic acid were extracted simultaneously with the samples. The concentration range of the calibration curve was chosen to cover the range of concentrations usually observed for each SCFA analyzed in biological samples coming from the gastrointestinal model. These seven samples were used to construct the calibration curves: the response (ratio between each SCFA and the internal standard peak areas) was plotted versus standard concentrations. Calibration points were injected before each series of samples and the extract spiked at a concentration corresponding to the central point of the calibration curve was injected one more time after all the samples.

The choice of the best regression model was studied using the statistic F-test, which is also known as Mandel fitting test (Mandel, 1968). A quadratic regression was used, and no "fit weighting" was applied. The correlation coefficients R^2 associated with those curves were higher than 0.99. It was also established that only one point of the curve can deviate from the curve by > 20% of the corresponding calculated value.

Confirmation criteria. SCFA were considered as positively identified in samples if the ratio between the chromatographic retention time of the analyte and that of the corresponding IS, *i.e.* the relative retention time (RRT) of the analyte, corresponded to that of the average retention time of the calibration solutions within a ± 0.5% tolerance and the peak area ratio of the two transitions of the native analytes corresponded to that of the averaged transition ratio of the calibration solutions within the tolerances set by the Commission Decision 2002/657/EC (European Commission 2002/657/EC, 2002).

Evaluation of SCFA in samples coming from an in vitro piglet gastrointestinal model:

Samples. Samples were obtained during an experimental run of a modified SHIME model (Van den Abbeele et al., 2010). It consists in a dynamic multi-compartment in vitro model mimicking a part of the gastrointestinal tract of a piglet. It begins with a first bioreactor mimicking stomach, duodenum/jejunum, a second bioreactor for ileum and finally, a third bioreactor for the proximal colon. The faeces and intestinal content used to inoculate the system were obtained from a healthy weaned piglet of 35 days old. After inoculation of the system, a stabilization period of two weeks had been applied.

Sample collection. Twenty samples of the different liquids of fermentation (2 mL) were collected during the two weeks of stabilization. Two samples were obtained during the first phase of stabilization of the microbiota (week 1) and three samples were taken at the end of the stabilization period of the microbiota (week 2).

Just after their collection, samples were centrifuged 2 min at 17,000g (Micro Star 17, VWR). Aliquots and supernatants were separated. Supernatants were filtered (0.45 μm, Whatman). All extra samples, aliquots and supernatants were immediately stored at −20 °C until SPME-GC–MS.

The intervention on the piglet donor was approved by the ethical committee of the University of Liège (Belgium)–file no 1824 and was in compliance with European (European Parliament and Council of the European Union, 2010) and Belgian (SPF Santé publique Sécurité de la Chaîne alimentaire et Environnement, 2013) regulations governing the protection of animals used for scientific purposes.

Statistics. Minitab 17 software (Minitab Inc., USA) was used for statistical analysis. The normality of data and equality of variances were evaluated. A paired sample t-test was performed. Conditions were considered as statistically significant with $p < 0.05$.

Results and Discussion

Choice of the analytical technique. Historically analyzed by distillation or using a colorimetric method developed by Montgomery et al. (1962) in environmental aqueous samples, SCFA are determined nowadays in various samples mainly by HPLC-UV, GC-FID, titrimetric or spectrofluorimetric methods (Chatterjee et al., 2018). Those techniques show variations between each other (sample preparation, accuracy, sensitivity …). However, according to Chatterjee et al. (2018), the determination of SCFAs via GC is the most reliable amongst the aforementioned methods with regard to LOQ, precision, accuracy, sample preparation, and volume of the sample required for measurement.

Nevertheless, it is also well known that the sensitivity and accuracy of the SPME-based methods are quite influenced by the "matrix effect" (Deng et al., 2004; Rincon et al., 2014). What is more, SPME is based on an equilibrium partition which means that, after a sufficient time, equilibrium is established between the solid phase formed by the fiber and the gas or liquid phase, leading to a non-exhaustive extraction. In order to counterbalance these weaknesses, an internal standard (2-methylvaleric acid) was added in every sample analyzed, matrix-matched calibration curves were used and a two different Quality Control samples were analyzed with every series of analysis. In addition, compared to other chromatography methods, the SPME system maintains a cleaner chromatographic column. Also, the sample is not directly injected in the machine compared to liquid injection; therefore, the fiber stays clean and not immersed in the sample. The fiber enables a specific extraction compared to headspace injection because the volatiles are trapped on the SPME fiber during the agitation extraction step. Another advantage of the SPME-GC–MS technique is that the seven compounds could be determined in a single run, after a simple and rapid sample treatment, without any other extraction than the automatic SPME.

SPME optimization. The DVB/CAR/PDMS fiber type was chosen because, as specified by the supplier, it is suitable for the analysis of flavor compounds (volatiles and semi-volatiles, C3-C20) with low molecular weight (MW 40-275). Using standard solutions, the suitability of the fiber was confirmed by a good separation of the eight SCFA (data not shown).

In order to optimize the extraction temperature, different extraction temperatures applied to the incubation oven (40, 50, 60, 70, 75, 80, 90 and 100 °C) were tested three times for each volatile fatty acid including the internal standard with an extraction time of 15 min. Figure 9A was then constructed using on the y-axis the average of the chromatographic peak areas and on the x-axis the extracted temperature tested. The results observed in Figure 9A show an optimum extraction temperature of 60 °C for iC5, 70 °C for C2, C3, C4 and IS, 75 °C for C5, 80 °C for C6, while for iC4 this optimum is at about 50 °C. Since the IS and the SCFA presented different extraction behaviors depending on the temperature, the temperature of 60 °C was chosen as a compromise for the 8 fatty acids to be analyzed. Consequently, this extraction temperature will be used to test the extraction time. To optimize this

parameter, the tests were carried out with different extraction times (5, 15, 25, 35, 45 and 60 min) at a temperature of 60 °C. Figure 9B shows that the optimum extraction time for C5 and C6 is 60 min, but a too long extraction time seems to be not in favor of iC4 and iC5. It is therefore again necessary to make a compromise between the optimum values observed for each of the 8 AGV, while keeping in mind that the whole analysis should not take too much time. The optimum extraction time chosen for all compounds is 20 min.

In conclusion, the parameters selected are an extraction temperature of 60 °C and an extraction time of 20 min. Even if the equilibrium state is not the same for every SCFA at 60 °C, the use of a calibration curve extracted in the same conditions of time and temperature as a real sample enables to have reliable results.

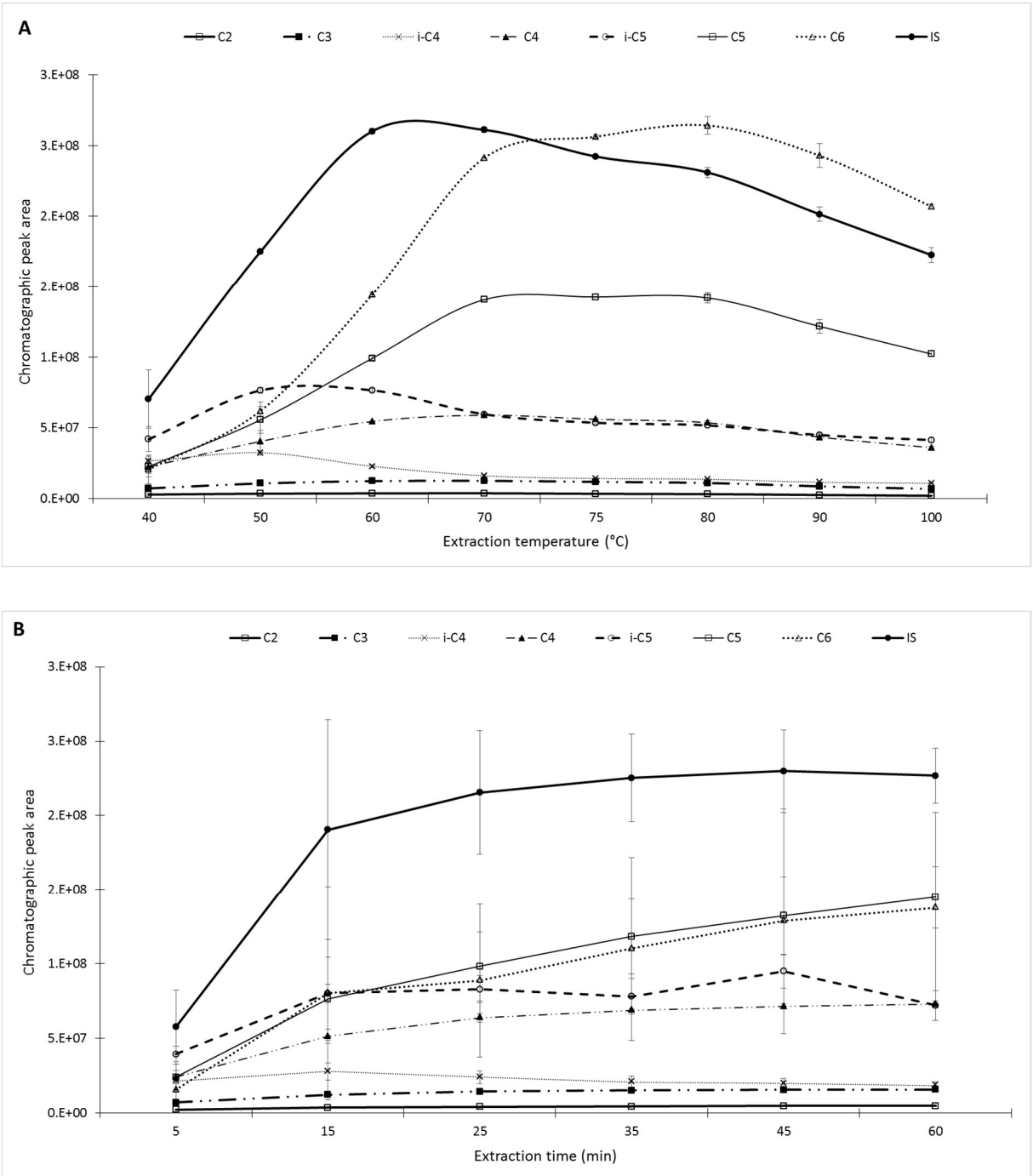

Figure 9 : Chromatographic peak areas versus the extracted temperature tested (°C) (A) or versus the extraction time (min) (B) for acetic (C2), propionic (C3), isobutyric (iC4), butyric (C4), isovaleric (iC5), valeric (C5), hexanoic (C6) acids and 2-methyl-valeric acid, used as internal standard.

Performances of the SPME-GC–MS analytical method. The developed method to measure fatty acids fulfils the criteria in accordance to European and US FDA guidelines (European Commission 2002/657/EC, 2002; US Food and Drug Administration, 2018). In the absence of specific guidelines for SCFA analysis, we used those texts, which provide guidelines to evaluate the performance of the screening and confirmatory methods used for organic residues and contaminants analysis: selectivity, specificity, recovery, reproducibility. Table 2 presents the validation parameters of the SPME-GC‑MS method for the quantification of 7 SCFA in feed medium.

Selectivity and specificity. The absence of significant peaks was shown in the blank feed medium (Figure 10 A) and the presence of quantifiable peaks was seen in the QCs (feed medium spiked with 7 SCFA at 2 different levels of concentration, Figure 10 B). When a peak was detected in the blanks, it was shown that the relative retention times and/or the transition ratios (ratio between the peak area when following one m/z ratio and that when following another m/z ration for an analyte) did not correspond to those of the SCFA analyzed here. For the QCs, it was also shown that the variations of relative retention times and of transition ratios corresponded to that of the calibration solution at a tolerance of ± 0.5% for the RRTs, and ± 10% for the transition ratios of ions with relative intensities higher than 50% of the base peak (European Commission 2002/657/EC, 2002).

As it can be seen on Figure 10B, a good separation was achieved for the eight compounds in a sample of feed medium spiked with the internal standard at a concentration of 0.2 mg mL^{-1} and with 2880 mg L^{-1} of acetic acid, 1620 mg L^{-1} of propionic acid, 320 mg L^{-1} of isobutyric acid, 1400 mg mL^{-1} of butyric acid, 280 mg L^{-1} for isovaleric acid, 960 mg L^{-1} of valeric acid and 640 mg L^{-1} for hexanoic acid. Some tailing can be observed for the chromatographic peaks, with acetic acid peak (8.43 min) showing more tailing than the other SCFA due to its high volatility. The peaks were identified by comparing their mass spectrum and retention times with those of the corresponding standards and by quantifying them with specific m/z ratios as shown in Table 1.

Calibration curves. For calibration curves, the quadratic regression provided a good curve fitting, *i.e.* with low residue values and correlation coefficients R^2 associated with those curves higher than 0.99 for the 7 SCFA (Table 2). The minimum value fixed for that parameter was 0.98. It was also established that only one point of the curve can deviate from the curve by > 20% of the corresponding calculated value.

Table 2: Validation parameters of the SPME-GC-MS method for the quantification of 7 SCFA in feed medium. The two values for reproducibility and recovery are respective to the two mean introduced concentration values (*n*=10).

SCFA	Targeted spiking levels ($mg*L^{-1}$)	Mean introduced concentrations ($mg*L^{-1}$)	LOD ($mg*L^{-1}$)	LOQ ($mg*L^{-1}$)	Reproducibility (CV, %)	Recovery (%)	Correlation (R^2)	Calibration curve equations
C2	360.0 – 1800.0	405.3 – 1793.1	72.0	144.0	15.1 – 6.3	112.6 – 99.6	0.9959	$y=0.056+1.264E-04*x+4.017E-09*x^2$
C3	202.5 – 1012.5	213.2 – 1020.6	40.5	81.0	5.7 – 5.7	105.3 – 100.8	0.9966	$y=0.002+2.673E-05*x+1.008E-09*x^2$
iC4	40.0 – 200.0	43.5 – 195.4	8.0	16.0	14.9 – 5.7	108.9 – 97.7	0.9970	$y=0.003+4.366E-04*x-3.391E-07*x^2$
C4	175.0 – 875.0	189.7 – 870.6	35.0	70.0	7.5 – 4.7	108.4 – 99.5	0.9972	$y=0.036+2.164E-04*x-1.276E-08*x^2$
iC5	35.0 – 175.0	42.8 – 184.1	7.0	14.0	15.3 – 6.0	122.4 – 105.2	0.9972	$y=0.001+1.554E-04*x-1.171E-07*x^2$
C5	120.0 – 600.0	129.4 – 595.8	24.0	48.0	6.6 – 3.8	107.9 – 99.3	0.9986	$y=0.020+1.732E-03*x+6.453E-08*x^2$
C6	80.0 – 400.0	89.0 – 392.9	16.0	32.0	6.2 – 3.5	111.3 – 98.2	0.9985	$y=0.029+1.489E-03*x+4.108E-07*x^2$

C2: acetic acid; C3: propionic acid; iC4: isobutyric acid; C4: butyric acid; iC5: isovaleric acid; C5: valeric acid; C6: hexanoic acid.

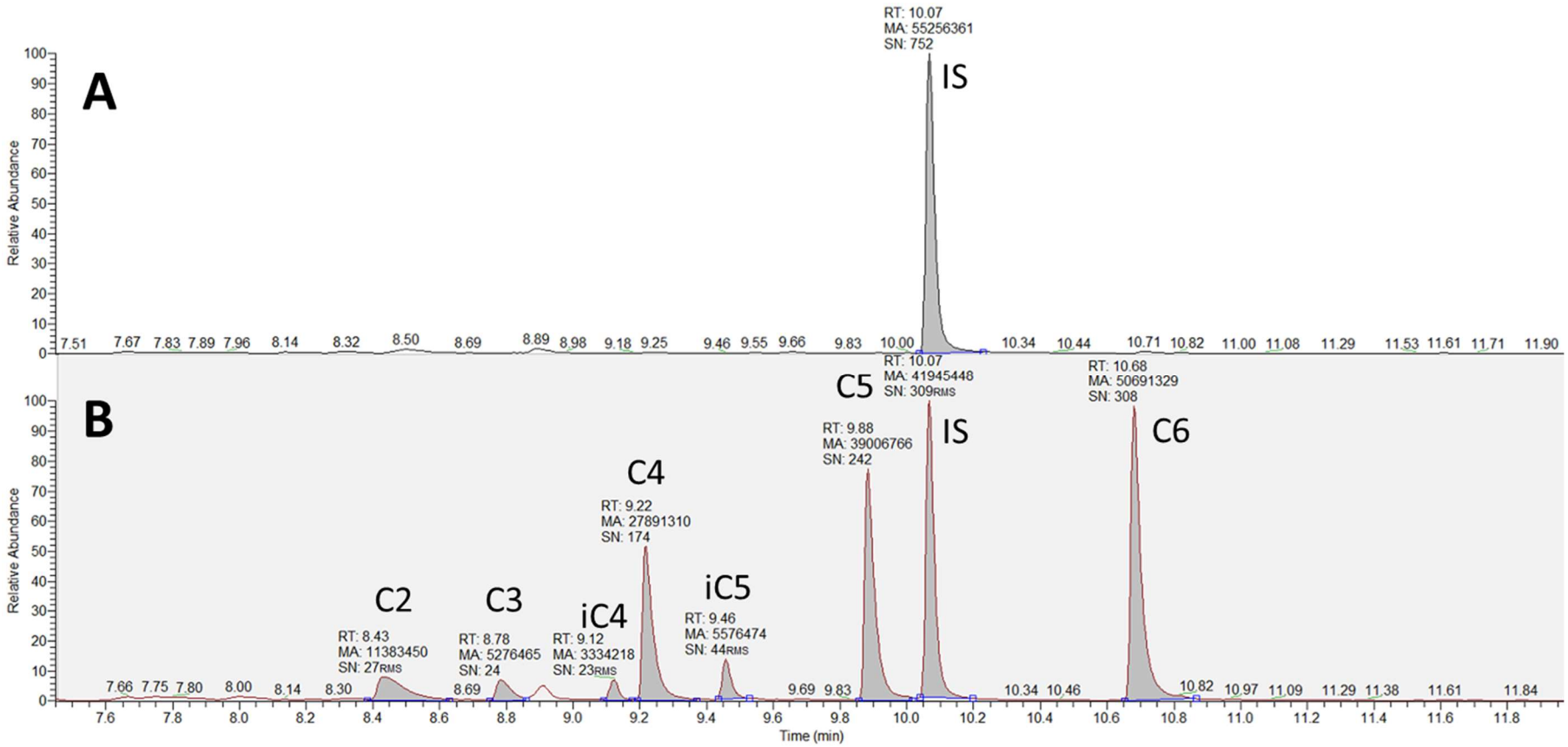

Figure 10: Chromatogram of a sample of feed medium only spiked with the internal standard at a concentration of 0.2 mg mL−1 (A) and of a sample of feed medium spiked with the internal standard at a concentration of 0.2 mg mL−1and with 2880 mg L−1 of acetic acid, 1620 mg L^{-1} of propionic acid, 320 mg L^{-1} of isobutyric acid, 1400 mg L^{-1} of butyric acid, 280 mg L^{-1} for isovaleric acid, 960 mg L^{-1} of valeric acid and 640 mg L^{-1} for hexanoic acid (B). C2: acetic acid; C3: propionic acid; iC4: isobutyric acid; C4: butyric acid; iC5: isovaleric acid; C5: valeric acid; IS (internal standard) consists on 2-methyl-valeric acid; C6: hexanoic acid.

Limit of quantification and limit of detection. The limit of quantification (LOQ) was fixed as the content of SCFA corresponding to the first point of the calibration curve (after checking that the signal to noise ratio was higher than 10 at that level). The LOQ were 144, 81, 16, 70, 14, 48 and 32 mg L^{-1}, for C2, C3, iC4, C4, iC5, C5 and C6, respectively. The limit of detection (LOD) was set at LOQ/2, after checking that the signal to noise ratio was higher than 3 at that level (Table 2).

Recovery and intermediate precision. Two QC samples were analyzed ten times each on different days to assess the performance of the developed method. QC1 contained concentrations of 360 mg L^{-1} of acetic acid, 202.5 mg L^{-1} of propionic acid, 40 mg L^{-1} of isobutyric acid, 175 mg L^{-1} of butyric acid, 35 mg L^{-1} of isovaleric acid, 120 mg L^{-1} of valeric acid, 80 mg L^{-1} of hexanoic acid. QC2 was containing concentrations of SCFA five-time higher than QC1. The estimated recovery and intermediate precision, corresponding to the inter-day variation of the results, are presented in Table 2. The intermediate precision is expressed as the relative standard deviation (RSD). The Commission Decision 2002/657/EC specifies that, for mass fractions higher than 1 mg kg^{-1}, the RSD must be below 16%. As shown in Table 2, this was the case for the seven SCFA, in the two QCs analyzed.

Regarding the recovery, the Commission Decision 2002/657/EC specifies that, for mass fractions higher than 10 μg kg^{-1}, the recovery should be included in a range between −20% and +10% of the certified concentration. For QC1, the recovery is a bit exceeding the upper limit of the permitted range for C2, iC5 and C6 with 112.6, 122.4 and 111.3%, respectively. For QC2, the measured recovery is included in the permitted range for the 7 SCFA.

Comparison of the developed method with published methods. A summary of the published methods developed for the detection of SCFA in human or animal intestinal content or faeces by SPME-GC–MS is presented in Table 3, including the comparison with the method proposed in this study. Medium (290 μL or 250 mg) to high amounts (3 g) of samples of human faeces are analyzed, with or without the use of a simulator of gastrointestinal tract. In our study, the only one working with piglet faeces, a small quantity (25 μL) of sample is used in the simulator of gastrointestinal tract.

When comparing the sample preparation of the different methods, only four methods (Mills et al., 1999; Di Cagno et al., 2011; Saa et al., 2014 and our method) propose a very quick and simple sample preparation, without any vial transfer. According to Table 3, the SPME extraction time can vary from 20 to 120 min.

Table 3: Comparison of the method described in this manuscript with published methods developed for the detection of SCFA in human or animal intestinal content or faeces by SPME-GC-MS.

Reference	SCFA analyzed	Sample type	Sample quantity	Sample preparation	Validation of the method	Internal standard	SPME extraction time / temperature
Zamora-Gasga et al. (2015)	C2, C3, C4	HF used in an SGT	Not mentioned	Centrifugation / vial transfer of supernatant	Not mentioned	Not mentioned	120 min / 45 °C
Luzardo-Ocampo et al. (2018)	C2, C3, C4	HF used in a SGT	Not mentioned	Centrifugation/ vial transfer of supernatant	Not mentioned	Not mentioned	120 min / 45 °C
Saa et al. (2014)	C2, C3, 2-methyl-C4, 3-methyl-C4, C4, 4-methyl-C5, C5, C6	HF	3 g	Addition of IS	Not mentioned	4-methyl-2-pentanol	40 min / 45 °C
Tamargo et al. (2018)	C2, C3, C4	HF used in a SGT	290 µl	Addition of IS, H_2SO_4 / vial transfer	Not mentioned	2-methylvaleric acid	25 min / 40 °C
Mills et al. (1999)	formic, C2, C3, iC4, 2-methyl-C4, C4, iC5, C5, iC6, C6	HF	1 g	Addition of NaCl, water, IS / Fiber loaded with derivatizing agent	yes	Deuterated formic, acetic and propionic acids	30 min / 50 °C
Bianchi et al. (2011)	C2, C3, iC4, C4, iC5, C5	HF	290 µl	Addition of IS and H_2SO_4 / vial transfer	yes	2-methylvaleric acid	20 min / 60 °C
Fiori et al. (2018)	C2, C3, C4, C5	HF	250 mg	Addition of $HClO_4$ / vial	yes	Deuterated-C4	30 min / 70 °C
Herrera-Cazares et al. (2019)	C2, C3, C4	HF used in a SGT	3 g	Centrifugation / vial transfer of supernatant	Not mentioned	Not mentioned	120 min / 45 °C
Di Cagno et al. (2011)	Total SCFA	HF	3 g	Addition of IS	Not mentioned	4-methyl-2-pentanol	40 min / 45 °C
Cueva et al. (2015)	C2, C3, 2-methyl-C3, C4, 2-methyl-C4, C5, C6, C8, C10	HF used in a SGT	290 µl	Addition of IS and H_2SO_4 / vial transfer	Not mentioned	2-methylvaleric acid	25 min / 40 °C
Gil-Sanchez et al. (2018)	C2, C3, C4	HF used in a SGT	290 µl	Addition of IS and H_2SO_4 / vial transfer	Not mentioned	2-methylvaleric acid	25 min / 40 °C
This study	C2, C3, iC4, C4, iC5, C5, C6	Piglet faeces used in a SGT	25 µl	Addition of IS, H_2SO_4, feed medium	yes	2-methylvaleric acid	20 min / 60 °C

HF: human faeces; SGT stands for “simulator of gastrointestinal tract”.

In our study, as well as in only 3 other studies out of 12 of the methods referenced in Table 3, a validation of the method used to detect SCFA was performed. The performances of the developed analytical method were similar to those published by Bianchi et al. (2011), Fiori et al. (2018) and Mills et al. (1999), with good precision, line arity and precision. Concerning the sensitivity of the method, the range of concentration evaluated in this work was not chosen to be the lowest as possible but to cover the range of concentrations usually observed for each SCFA analyzed in biological samples coming from the gastrointestinal model.

Evaluation of SCFA in samples coming from an in vitro piglet gastrointestinal model. The concentrations of SCFA (mg L^{-1}) contained in each sample coming from the experimental run on modified SHIME model inoculated either with fecal material or intestinal content are shown in Table 4.

During the stabilization phase of fecal material inoculation, the average total SCFA concentration was always lower in bioreactor mimicking ileum, with an average of 3242 ± 108 mg L^{-1}, than in bioreactor mimicking proximal colon where the average total SCFA content was 3759 ± 201 mg L^{-1}. The same observation was made when the model was inoculated with intestinal content, where the average total SCFA concentration in the bioreactor mimicking ileum was of 3159 ± 42 mg L^{-1} while it was 3461 ± 246 mg L^{-1} in the bioreactor mimicking proximal colon. Anyway, those differences were not statistically significant, with $p = 0.069$ for fecal material inoculation and $p = 0.195$ for intestinal content inoculation, respectively. When comparing the total SCFA content from ileum and proximal colon, whatever the inoculum chosen, the difference was statistically significant with $p = 0.012$.

Regarding the stabilization phases, the SCFA concentrations measured were not used for any further calculation but were only used to assess the stability of the system.

In addition to the total SCFA concentrations of the samples, the importance of each acid in the profile, in terms of concentration, must be considered. Acetic acid presented the highest concentrations compared to any other SCFA. This is consistent with the literature and the published in vitro models (Bianchi et al., 2011; Fehlbaum et al., 2016; Fleury et al., 2017). This compound presented a great variability of concentration between samples included in a same group. For acetic acid, standard deviations were higher in colon samples than in ileum samples. This was also observed for all the others except isobutyric, isovaleric acids. Regarding hexanoic acid, this was observed only for the samples obtained when the system was inoculated with real intestinal content rather than faeces.

Table 4: SPME-GC-MS measurement of SCFA concentration (mg L-1) in samples from the experimental run on modified SHIME model inoculated either with fecal material or intestinal content.

Vessel		Phase	Sampling week	SCFA concentration (mg L^{-1})								
				C2	C3	iC4	C4	iC5	C5	C6	TOTAL SCFA	Average total SCFA ± SD
Inoculum Fecal Material	Ileum	Before stabilization	1	2182	730	49	506	85	214	<LOQ	3765	3691 ± 106
				1722	690	52	789	80	283	<LOQ	3616	
		End of stabilization	2	1341	751	51	765	74	384	<LOQ	3366	3242 ± 108
				1257	679	56	784	81	333	<LOQ	3190	
				1199	702	51	770	69	344	34	3169	
	Proximal colon	Before stabilization	1	2027	667	66	597	99	274	<LOQ	3729	4120 ± 552
				2356	837	55	816	93	353	<LOQ	4510	
		End of stabilization	2	1434	827	45	774	83	524	<LOQ	3688	3759 ± 201
				1801	816	52	780	78	459	<LOQ	3986	
				1567	747	50	718	77	444	<LOQ	3603	
Inoculum Intestinal content	Ileum	Before stabilization	1	1179	353	54	250	120	56	<LOQ	2011	2108 ± 137
				1278	213	60	466	101	87	<LOQ	2205	
		End of stabilization	2	1758	264	47	795	96	181	61	3202	3159 ± 42
				1689	222	50	848	102	177	71	3158	
				1650	271	44	815	96	173	69	3117	
	Proximal colon	Before stabilization	1	1470	564	60	562	118	215	<LOQ	2990	3445 ± 644
				2248	581	51	737	101	182	<LOQ	3900	
		End of stabilization	2	1752	455	46	753	88	215	125	3435	3461 ± 246
				1567	392	51	647	84	237	249	3228	
				1950	392	54	656	81	238	348	3719	
		LOQ		144	81	16	70	14	48	32		

LOQ: Limit of Quantification

Valeric acid was the fourth more present SCFA in the analyzed samples, following acetic, propionic and butyric acids. Then, after valeric acid, isovaleric and isobutyric acids were the two last SCFA that were present. As seen in vivo, these SCFA were expected to be present as minor components of the profile, in this order of importance (Kraler et al., 2015). Regarding hexanoic acid, its concentration in the samples was most of the time below the limit of quantification of 32 mg L^{-1}. However, the samples obtained from real intestinal content inoculum appeared more appropriate to measure hexanoic acid concentration in the model with values ranging from 125 to 348 mg L^{-1}.

To compare the range of values obtained with other in vitro models mentioned in the literature, acid concentrations of samples collected after the stabilization period were converted in mM, leading to concentrations of total SCFA fluctuating between 43 and 56 mM. This model generated lower SCFA concentrations than PolyFermS (Tanner et al., 2014a) or PigutIVM (Fleury et al., 2017) (> 150 mM) but the composition of the nutritional medium added to the model can probably explain this difference.

Indeed, the medium used in the modified SHIME system contained maximum 16.2 g L^{-1} of nutrients while the medium from the PigutIVM contained > 35 g L^{-1} of carbohydrates and proteins.

Conclusions

Regarding the SCFA analyzed, the performance of the method assessed with both QC samples showed good specificity/selectivity and suitable limits of detection and quantification. Moreover, the measured concentrations were in accordance to what is found in the literature, also indicating that the developed method is suitable for compounds quantification in samples coming from in vitro gastrointestinal models. The average total level of SCFA measured at the end of the stabilization phase was always lower in ileum bioreactor than in proximal colon bioreactor. In addition to the total SCFA concentrations of the samples, the importance of each acid, in terms of profile and concentration, was assessed and values were in accordance with expected values.

In conclusion, a specific SPME-GC–MS method for the analysis of seven short chain fatty acids has been developed. The seven compounds could be determined in a single run, after a simple and fast sample treatment, without any other extraction than the automatic SPME. The method was applied with success to different kinds of samples obtained from a gastrointestinal model mimicking piglet intestinal tract. The developed method might be used as a general method for measuring SCFA in biological samples.

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PART 2: In Vitro Model–SPIME studies

The increased interest of piglets' health at weaning in a mondial One Health context, combined with more environmentally friendly production methods, set the framework of this research. This aimed to study a particular weaning strategy that would reduce intestinal disorders due to the physiological effects of the feeding transition. In the literature, different models and methods dedicated to such studies of intestinal disorders are described. Those methods allow the study of intestinal markers such as SCFA, microbiota or inflammatory markers (Niewold, 2015) before resorting to or to replace animal experiments. At disposal in the laboratory was the SHIME model, a multicompartment dynamic system that could be adapted to particular study. In the case of this thesis, the model was adapted according to the piglet physiology parameters, to allow the study of ileal and colonic fermentation process through SCFA and bacterial profile analysis. Moreover, the model was adapted to add a weaning step. The work led to the baby-SPIME model (Simulator of Pig Intestinal Microbial Ecosystem dedicated to the luminal microbiota of piglet with a special focus on mimicking in vitro weaning at 28 days of age). The development of the model is described in **Study 2**.

To optimize the microbiota in the baby-SPIME bioreactors, the assumption was made that inoculation with real intestinal content instead of feces could be beneficial to maintain bacteria present in the ileum or the proximal colon that could have disappeared from the feces. Therefore, **Study 3** aimed to compare the microbiota in the ileum and colon of the baby-SPIME inoculated with real intestinal content vs feces to assess the added-value of using real intestinal content. Oxygenation of the ileum compartment was considered as well.

Next, the aim of **Study 4** was to evaluate the effect of AP on the short chain fatty acids (SCFA) production and on the microbiota of piglet after weaning by using the baby-SPIME model as a proof of concept.

Study 2 -

Baby-SPIME: A dynamic in vitro piglet model mimicking gut microbiota during the weaning process

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Abstract

The study aimed to adapt the SHIME® model, developed to simulate human digestion and fermentation, to a baby-SPIME (baby Simulator of Pig Intestinal Microbial Ecosystem). What is a unique feature of this model is its twofold objective of introducing an ileal microbial community and mimicking a dietary weaning transition. This model should then be ideally suited to test a dietary weaning strategy of piglets in vitro. Regarding the microbiota, the main phyla making up the model were Firmicutes, Bacteroidetes and Proteobacteria although Bacteroidetes decreased after inoculation ($p = 0.043$ in ileum, $p = 0.021$ in colon) and Delta-Proteobacteria were favoured ($p = 0.083$ in ileum, $p = 0.043$ in colon) compared to Gamma-Proteobacteria. The designed model led to a low representation of Bacilli–especially *Lactobacillus sp*. in the ileum and a weak representation of Bacteroidia in the proximal colon. However, *Mitsuokella* and *Prevotella* were part of the major genera of the model along with *Bifidobacterium*, *Fusobacterium*, *Megasphaera* and *Bacteroides*. As a result of weaning, two major changes–normally occurring in vivo–were detected in the system: firstly, Firmicutes diminished when Bacteroidetes increased particularly in the proximal colon; secondly, *Bacteroides* decreased and *Prevotella* increased (mean value for four runs). In terms of metabolite production, while a ratio acetate: propionate: butyrate of 60:26:14 was obtained in post-weaned colon, the expected inversion of the ratio propionate: butyrate in the post-weaned ileum was unfortunately not observed. To conclude, the so-called baby-SPIME model meets expectations regarding the resident microbiota of the proximal colon bioreactor and the metabolites produced thereof. In terms of the evolution of major groups of bacteria, the in vitro weaning process appeared to be successful. However, higher concentration of butyric acid would have been expected in ileum part of newly weaned piglets, as observed in vivo. The microbiota in the ileum bioreactor seemed in fact to act like a pre-colon. This suggests that microbial profile in ileum bioreactor had to be improved.

Introduction

The use of in vitro models in animal experimentation has become an interesting alternative which no longer needs to be demonstrated, as seen in pig production where several of these models have been thoroughly developed. These are particularly well adapted to research that focuses on the fermentability of feed ingredients (Williams et al., 2005) or on the impact of drugs on piglet gut microbiota in the veterinary field (Fleury et al., 2017) for example.

Two major types of systems exist: batch models as the gas production technique described in the works of Bindelle et al. (2007) and (semi-)continuous models asOsitec (von Heimendahl et al., 2010); or PolyFermS (Tanner et al., 2014a); or PigutIVM (Fleury et al., 2017). They are mimicking the colon of adult pigs except for PigutIVM that is mimicking the colon of piglets.

Currently, none dynamic and multi-compartment model includes an ileum fermentation portion. Yet, the ileum plays an important role in porcine digestion. Indeed, the first–and potentially extensive–fermentation of the rapidly fermentable carbohydrates occurs in this part of the gastrointestinal tract (GIT) (Williams et al., 2005) while the digestion of plant polysaccharides occurs in the colon, leading to a clear differentiation of the microbial composition in these two digestive compartments (Crespo-Piazuelo et al., 2018). In this way, bacteria belonging to the *Lactobacillus* and *Clostridium* genera are observed in the small intestine while bacteria belonging to the *Prevotella* genus or to the Ruminococcaceae family are found in the colon (Crespo-Piazuelo et al., 2018), suggesting upgrading an in vitro model with an ileal simulation as an interesting possibility.

Moreover, none of these in vitro models has been used to study the transition from a lactation diet to a post-weaning diet. Weaning is a critical period for piglets as the relatively stable microbial population undergoes a huge modification after the introduction of solid food (Kim and Isaacson, 2015; Konstantinov et al., 2004). Significant compositional and functional differences have been reported in the microbiome of piglets as a result of this stressful event (Guevarra et al., 2018). And the effects of weaning seem to be higher in the ileum compared to the colon (Tao et al., 2015). These observations encourage to attempt to reproduce a weaning in bioreactors, including an ileum bioreactor.

Amongst the dynamic and multi-compartments models that are developed, the SHIME® (Simulator of Human Intestinal Microbial Ecosystem) model (Van den Abbeele et al., 2010) can display the functions of both ileum (*i.e.* abiotic factors) and colon (*i.e.* abiotic factors and gut microbes) simultaneously. It consists of an in vitro dynamic, multi-compartment gastrointestinal model, which includes a stomach, a small intestine, and three consecutive colon compartments (ascending/transverse/descending colon) in its classic set-up that can be modified according to the research question. After being inoculated with human fecal microbiota, the 3 parts of the colon offer a suitable environment for a reproducible microbial colonization of bioreactors by human microbial communities (Van den Abbeele et al., 2010).

The aim of the present study was to modify the SHIME into a baby-SPIME model (Simulator of Pig Intestinal Microbial Ecosystem, dedicated to the luminal microbiota of piglet) with a special focus on mimicking in vitro weaning at 28 days of age and introducing an ileal microbiota. This model should make feasible the in vitro testing of dietary weaning strategies for piglets.

Material and methods

Equipment. A SHIME® equipment (ProDigest Bvba, Gent, Belgium) as described by Van den Abbeele et al. (2010), was used to build the baby-SPIME model. Briefly, the SHIME system consists of a cabinet equipped with 24 peristaltic pumps and 6 double-jacketed bioreactors linked to a hot-water bath. All units are connected to a computer designed to standardize the different parameters of the system (temperature, pH, transfer time). The pumps provide the transfer of culture media, pancreatic juice, bile, acid (HCl 0.5 M), base (NaOH 0.5 M) and all the fermentation liquids from one bioreactor to another during a complete run. Manual quality controls are regularly performed to check the parameters and samples are taken 3 times a week at fixed intervals (days and times).

Regarding the baby-SPIME model, the cabinet was divided into two independent units containing three bioreactors as illustrated in Figure 11. Bioreactor 1, not inoculated, simulated the stomach and duodenum/jejunum digestion. Bioreactors 2 and 3, inoculated with piglets' faeces, simulated the functions of an ileum and a proximal colon, respectively. The feeding cycle was scheduled three times a day based on a total retention time of 14 h. During each cycle, culture media (140 mL), maintained at 4 °C, flowed into bioreactor 1 for 1 h 30 min. Then, pancreatic juice + bile (60 mL), also maintained at 4 °C, was added to the same bioreactor for 1 hr, after which the content of bioreactors 1, 2 and 3 was made to flow simultaneously into bioreactors 2, 3 and a waste, respectively. The flowing rates served two purposes: empty bioreactor 1 (from 200 mL to 0 mL); and obtain a residence time of 4 h and 10 h in bioreactors 2 (constant volume of 100 mL) and 3 (constant volume of 250 mL), respectively. For the ileum bioreactor, the minimal volume required was used to take into account the emptying of the small intestine that happens in vivo, while maintaining a good fermentation process in bioreactor. For the colon bioreactor, the volume used in the SHIME model was maintained for the development of the baby-SPIME model. The anaerobic condition of all bioreactors was maintained by flushing with nitrogen (N_2) once a day for 10 min. Additionally, they were continuously stirred (300 rpm) and kept at 39.5 °C. The pH of bioreactors 2 and 3 was continuously monitored by pH controllers maintaining pH ranges of [6.40–6.60] in bioreactor 2 (ileum) and [5.80–6.05] in bioreactor 3 (proximal colon) by using NaOH (0.5 M) or HCl (0.5 M). Four runs were managed (4 different donors). Every run lasted 4 weeks: 2 weeks for the stabilization of the microbiota in the lactation phase, a weaning procedure (replacement of the culture medium), and a 2-week post-weaning phase.

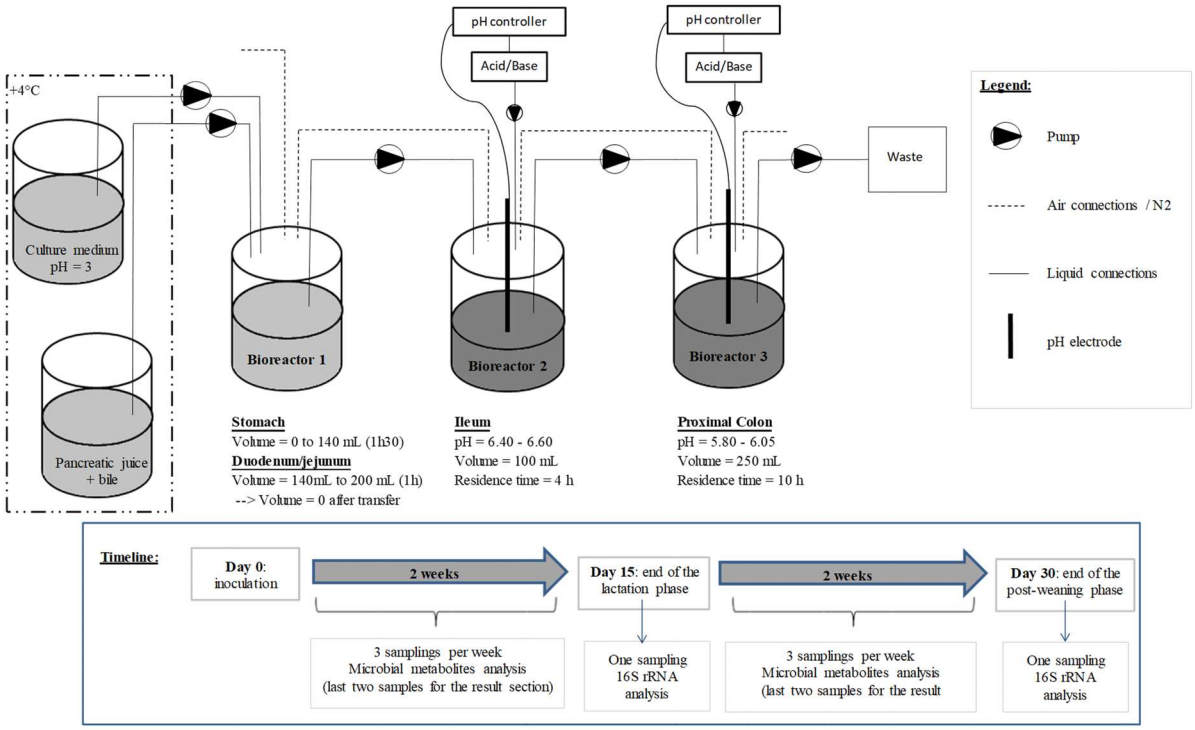

Figure 11: Schematic representation of the baby-SPIME model. It consists of three double-jacketed bioreactors (bioreactor 1: stomach/duodenum/jejunum, bioreactor 2: ileum and bioreactor 3: proximal colon). Three times a day, the culture medium entered bioreactor 1 (stomach digestion) through liquid connections controlled by pumps. Then pancreatic juice and bile entered bioreactor 1 (duodenum/jejunum digestion) following instructions given in the figure. The liquid was then made to flow simultaneously toward the ileum and proximal colon until reaching a waste. The system was flushed once a day with nitrogen (N_2) through the air connection system. The bioreactors were constantly stirred and kept at 39.5 °C. Throughout the run, ileum and colon pH were checked and adjusted to fixed pH ranges.

Inocula and culture media. The intervention on piglets was approved by the ethical committee of the University of Liège (ULiège, Liège, Belgium)–file n°1823 and it was in compliance with European (Directive 2010/63/EU) and Belgian (Royal Decree of the 29 May 2013) regulations governing the protection of animals used for scientific purposes.

The faeces of four [Piétrain×Landrace] suckling piglets (27 days old) free of antibiotics were used to prepare the inocula for the study. The four samples were taken at the same farm (Walloon Agricultural Research Centre, CRA-W, Gembloux, Belgium) with several weeks between the sampling. During transportation the faeces were kept in ice under anaerobic conditions. A single donor was used to prepare the inoculum for a run and a single inoculum prepared for both ileum and colon bioreactors of the same run. The inoculum was obtained by adding faeces to an anaerobic phosphate buffer solution (pH 7.0; 1:5, weight: volume) and homogenizing for 10 min. The filtrate was injected simultaneously in the ileum bioreactor (5 mL) and the proximal colon bioreactor (12.5 mL). Before inoculation, these two bioreactors were filled with non-acidified lactation culture medium (100 mL for ileum bioreactor and 250 mL for colon bioreactor) and the pH was automatically adjusted in each bioreactor according to its required range.

For each run, two different culture media (lactation and post-weaning media) were prepared drawing on the work of Molly et al. (1993). Their composition is shown in Table 5. The culture media were prepared in 5 L bottles and autoclaved during 35 min at 121 °C. The post-weaning medium required a special preparation in order to avoid the clogging of feeding tubes: after heating, the medium was homogenized and allowed to sediment for 10 min before pumping 4 L of the supernatant as a base. Separately, 1L of the same medium was prepared, autoclaved and added to the base medium ensuring that every fraction of the various fibres contained in the post-weaning diet was transferred. The media were stored at 4 °C and the pH was adjusted to 3.0 before using in the first bioreactor simulating the gastric conditions.

Pancreatic juice was prepared in 2 L bottles with autoclaved water. It contained (personal communication of ProDigest) sodium hydrogencarbonate (2.5 g/L, VWR Chemicals, Radnol, Pennsylvania, USA) and pancreatin (0.9 g/L, ProDigest). Bile (Oxgall, 4.0 g/L, ProDigest) was added.

Table 5: Composition of the culture media.

Ingredients	Lactation culture medium	Post-weaning culture medium
Mucin (Sigma-Aldrich, St-Louis, Missouri, USA)	6.0 g/L	6.0 g/L
Proteose-Peptone n°3 (BD Bacto Biosciences, Franklin Lakes, New-Jersey, USA)	1.0 g/L	1.0 g/L
Potato starch (Sigma-Aldrich, St-Louis, Missouri, USA)	1.0 g/L	1.0 g/L
L-Cysteine hydrochloride (Merck, Darmstadt, Germany)	0.2 g/L	0.2 g/L
Nuklospray Yoghurt[1] (Dumoulin, Andenne, Belgium)	8.0 g/L	0.0 g/L
Post-weaning diet for piglets[2] (ABZDiervoeding, Nijkerk, The Netherlands)	0.0 g/L	8.0 g/L

g/L: grams per litre. [1] Commercial complementary milk replacer feed for piglets containing, among others, whey powder, vegetable oils and wheat flour. [2] Grinded to particles of 250 µm Composition: Barley (30.00%), Wheat (14.41%), Maize (5.00%), Oat flakes (5.00%), Toasted soybeans (15.00%), Soya meal (13.87%), Potato protein (2.00%), Bread flour (5.00%), Soya oil (0.36%), Fat filled whey powder (4.67%), Chalk (1.05%), Monocalciumphosphate (1.01%), Salt (0.54%), Methionine (0.16%), L-lysine HCL (0.47%), L-threonine (0.11%), Lysine/tryptophan mix (0.02%), Flavoring (0.20%), Vitamins (0.40%), Start/BL.15CU (premix containing Cu, Fe, Zn, Mn, Se, I and vitamins A, B2, B3, B5, D3, E, K3; 0.40%), Phytase (0.33%).

Sample collection. Before adding the culture medium, a 9 mL sample was taken from the ileum and proximal colon bioreactors 3 times a week at fixed intervals of days and times from the beginning to the end of the run in order to standardize the sampling all along the run. Each collected sample was subdivided as follows: 2 mL for microbial metabolites analysis; 1 mL for high throughput sequencing analysis; and the remaining 6 mL for extra potential analyses. Samples for microbial metabolites analysis and high throughput sequencing analysis were centrifuged for 2 min at 17,000g to recover the supernatant of the first one and the pellet of the second one, respectively. Supernatants dedicated to

metabolites analysis were filtered (0.45 µm). Samples were stored at −20 °C until analyses were performed.

All samples were analysed for microbial metabolites because the concentration of the metabolites detected in the samples was used to monitor the system, ensuring that the microbiota was well stabilised for the last day of the lactation phase (after the two first weeks of the run) and for the last day of the post-weaning phase (the last day of the 4 weeks that ran the run).

Samples used for the microbial metabolites analysis: the two last samples of the lactation phase and the two last samples of the post-weaning phase were used to calculate a mean value in metabolites for the lactation and the post-weaning phases of a run. The data shown in the manuscript are the averages obtained from the four runs.

Samples used for the high throughput sequencing analysis: the last sample of the lactation phase and the post-weaning phase, for the four runs, were used to obtain the microbiota results.

Short-chain fatty acids of ileal and colon effluents (by SPME-GC–MS). Samples were analysed for their short-chain fatty acids (SCFA) content. The analysed compounds were acetic (C2), propionic (C3), isobutyric (iC4), butyric (C4), isovaleric (iC5), valeric (C5) and hexanoic acids (C6).

The SPME-GC–MS method developed for the management of the baby-SPIME is described by Douny et al. (2019). Briefly, 25 µL of baby-SPIME samples were pipetted into a 20 mL glass vial. Forty microlitres of internal standard (2-methylvaleric acid) at a concentration of 0.2 mg/mL, 15 µL of 0.9M sulfuric acid and 920 µL of culture medium were then added. For this purpose, a lactation culture medium and a post-weaning culture medium were used to analyse the samples taken during the lactation and post-weaning phases, respectively. The mixture was vortexed and placed on the autosampler of the SPME-GC–MS system until an analysis could be performed. SCFA were extracted with a SPME fibre, separated on a Focus GC gas chromatograph (Thermo Fisher Scientific) using a Supelcowax-10 column (30m×0.25 mm, 0.2 µm) (Supelco, Bellefonte, PA, USA) and analysed with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific). The agitation temperature was set at 60 °C and the extraction time at 20 min.

The results given by SPME-GC–MS, in mg/L of sample, were converted into mmol/L (±SEM). Ratios C2:C3:C4 were then calculated.

16S rRNA gene sequencing. DNA extraction and sequencing of all the samples were performed by DNA Vision (Gosselies, Belgium) following their internal quality SOP. DNA was extracted from frozen pellets with the DNeasy Blood & Tissue kit according to the manufacturer's instructions Qiagen (Qiagen Benelux B.V., Venlo, The Netherlands). DNA was quantified and qualitatively assessed on a NanoDrop 2000 from Thermo Scientific™ and by PicoGreenVICTOR X3 (PerkinElmer) using the Quant-it PicoGreen dsDNA Assay kit from Invitrogen. The 16S targeted region V3-V4 was amplified by PCR, purified and tagged. Libraries were indexed using the NEXTERA XT

Index kit V2 from Illumina. The high throughput sequencing was carried out on Illumina Miseq in paired-end sequencing (2×250 bp) by targeting an average of 10,000 reads per sample. Finally, the bioinformatic analysis was executed with the QIIME (Quantitative Insights Into Microbial Ecology) software, version 1.9.0 with "Greengenes 13_8" as database and recommended parameters to use QIIME scripts. The OTU (Operational Taxonomic Unit) table was generated based on a 97% sequence similarity of the sequencing reads to cluster OTUs. Only samples presenting more than 5,000 reads were used for taxonomic analysis. Similarly, samples with the same normalized number of reads were used for the beta diversity analysis.

The results were expressed in relative abundance–a percentage of the total bacteria (±SEM).

Statistical analysis. A paired t-test was applied to the short-chain fatty acids results (sum of SCFA, C2, C3, iC4, C4, C5, C6) first to compare ileum vs colon samples in each phase and then to compare lactation vs post-weaning phase samples in ileum and colon bioreactors.

Iso-valeric acid data did not follow a normal distribution; a nonparametric Kruskal-Wallis test was used following the same comparison modalities.

For the 16S rRNA gene sequencing results, alpha diversity statistical analysis was based on a non-parametric t-test (Monte Carlo permutations to calculate p-value) comparing groups of samples two by two. Beta diversity statistical analysis was done at different levels of the taxonomy classification to detect differences in read abundances between groups of samples. The non-parametric Kruskal-Wallis test used for this purpose gave a p-value (K-W p-value) that was subsequently adjusted using the Benjamini-Hochberg FDR procedure for multiple comparisons (FDR p-value).

A p-value between 0.01 and 0.05, or equal to 0.05, was considered statistically significant. A p-value between 0.05 and 0.1, or equal to 0.1, was considered a trend. Otherwise, a p-value higher than 0.1 was considered not significant (ns).

Results

Alpha-diversity of the microbial ecosystem. The results of the alpha-diversity (Shannon and observed OTU) are given in Table 6. No statistical difference was evident except between the inocula and the lactation phase samples. The Chao 1 index of the proximal colon samples showed an increasing statistical trend (344 lactation phase *vs* 446 post-weaning phase).

Table 6 : Alpha-diversity results.

Index	Inocula	Lactation phase			Post-weaning phase			Effect of weaning
		Ileum	Prox. colon	p	Ileum	Prox. colon	p	p
Shannon	6.66[a]	4.52[b]	4.58[b]	0.01	4.91	4.92	ns	ns
Observed OTU	783[c]	252[d]	239[d]	0.03	281	288	ns	ns
Chao 1	1196[e]	393[f]	344[f]	0,02	427	446	ns	Ileum: ns Colon: 0.05

Analysed samples are the last sample of each phase (samples taken 2 weeks after the inoculation for the lactation phase, samples taken 2 weeks after the weaning transition for the post-weaning phase), n=4 (4 runs).
p means p-value, ns means not significant, prox. means proximal, n=4.
a to f: values with different exponents within a row are statistically different.

Taxonomy of the microbial ecosystem at the end of the lactation phase:

Phyla–classes–families. At the phyla level of the bacterial taxonomy, Firmicutes, Bacteroidetes and Proteobacteria were dominant in the inocula samples (90.9% ± 2.4). At the end of the lactation phase, the sum of these 3 phyla reached 71.7% ± 4.5 in the ileum and 76.3% ± 3.6 in the proximal colon (Supplemental material), with Bacteroidetes (class of Bacteroidia) being significantly diminished in both bioreactors (from 32.6% ± 1.8 in inocula to 13.0% ± 3.2, p = 0.043, in the ileum and 12.8% ± 1.1, p = 0.021, in the proximal colon). In this phylum, [Paraprevotellaceae] family was less abundant in the ileum bioreactor than in the proximal colon bioreactor (respectively 0.0% ± 0.0 and 0.1% ± 0.0, p = 0.043). The Muribaculaceae (S24-7) family tended to be less abundant in the ileum bioreactor than in the proximal colon bioreactor (respectively 0.2% ± 0.1 and 0.7% ± 0.2, p = 0.083). Following stabilization, the Fusobacteria phylum was less abundant in the inocula samples (1.3% ± 1.1) than in the ileum (14.4% ± 5.8, p = 0.043) or the proximal colon (13.6% ± 4.4, p = 0.043) bioreactors. Actinobacteria tended to be less abundant in the inocula (1.7% ± 0.9) compared to the ileum (10.5% ± 3.2, p = 0.083) or the colon (8.5% ± 2.7, p = 0.083). In this phylum, the Nocardiaceae family tended to be less abundant in the ileum bioreactor than in the proximal colon bioreactor (respectively 0.0% ± 0.0 and 0.1% ± 0.0, p = 0.083).

Regarding the Proteobacteria phylum, two classes were significantly present in inocula and bioreactors, namely Gamma-Proteobacteria and Delta-Proteobacteria (Figure 12). Gamma-Proteobacteria were present in inocula (4.4% ± 2.7), ileum (1.2% ± 0.4) and proximal colon (1.6 ± 0.4).

It included the Enterobacteriaceae family–among them the well-known *Escherichia* genus. The abundance of this family in inocula equaled 3.8% ± 2.2 of the total sample bacteria. It reached 1.1% ± 0.3 in the ileum and 1.5% ± 0.5 in the proximal colon. Delta-Proteobacteria was represented mainly by Desulfovibrionaceae that included *Desulfovibrio* and *Bilophila* genera. Delta-Proteobacteria tended to be more abundant both in the ileum (2.8% ± 0.4, p = 0.083) and in the colon (3.3% ± 0.4, p = 0.043) of the baby-SPIME model, as compared to inocula (1.2% ± 0.6).

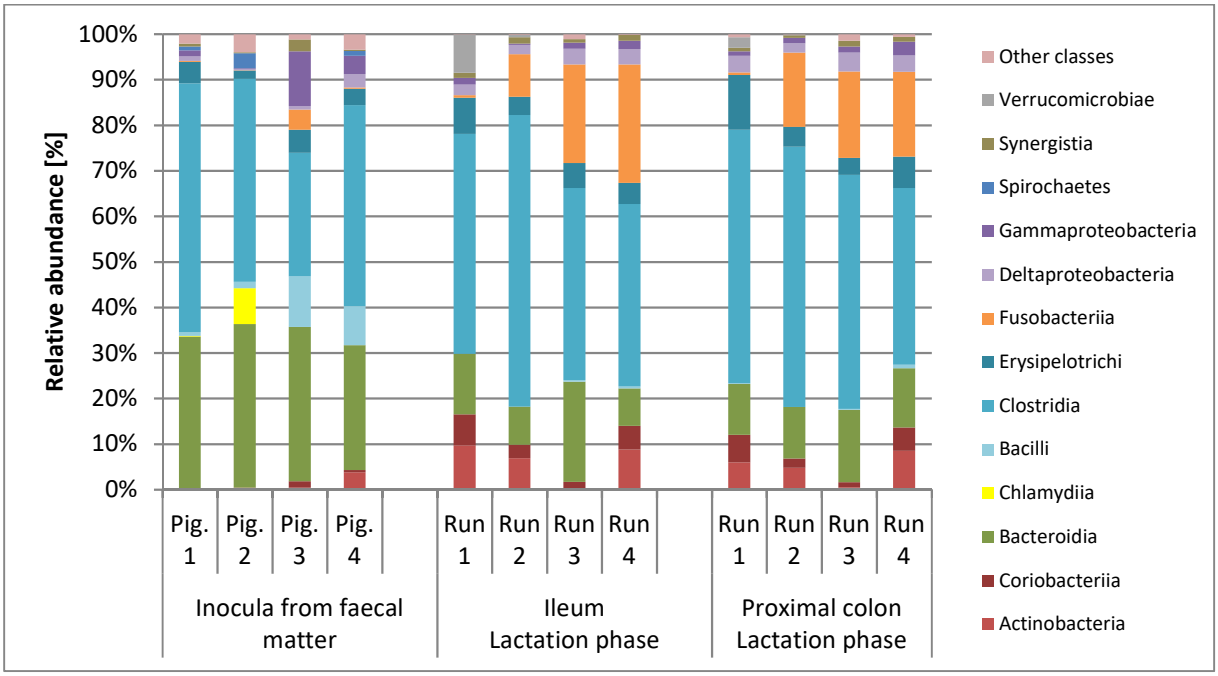

Figure 12: Class composition of microbiota in the inocula (prepared with faeces of 4 piglets (pig)) and in the corresponding samples of the lactation phase from ileum and proximal colon bioreactors of the baby-SPIME model. Samples were taken the last day of each phase (2 weeks after inoculation for lactation phase and 2 weeks after the weaning transition). Classes are grouped by phyla: Actinobacteria (red), Bacteroidetes (green), Chlamydiae (yellow), Firmicutes (blue), Fusobacteria (orange), Proteobacteria (mauve), Spirochaetes (blue), Synergistetes (brown) and Verrucomicrobia (grey).

Included in the phyla Firmicutes, the class Bacilli showed decreasing relative abundance from inocula (5.5% ± 2.6) to the ileum (0.1% ± 0.1, p = 0.021) and the proximal colon (0.2% ± 0.2, p = 0.021). Regarding specifically–within this class–the family Lactobacillaceae, this was abundant in the inocula via *Lactobacillus sp.* (4.5% ± 2.0) but it was detected–using 16S rRNA analysis–at a maximum level of 0.1% in the ileum and the colon during the third run (data not shown).

Verrucomicrobia, present in the model principally through *Akkermansia* sp., showed no statistical difference between inocula and bioreactors. The ileum of the first run accommodated this phylum particularly well compared to other ileum (8.4% in the first run *vs* 0.2% on average for the others runs) or colon samples (2.3% *vs* 0.1%, data not shown).

Genera. Forty-nine genera (data not shown) with a relative abundance higher than 0.1% were identified in the ileum or colon. These genera represented more than 99% of the total bacteria of the model but only 64.0% of those found in the inocula. As shown in Figure 13, these included *Mitsuokella*, *Fusobacterium*, *Prevotella*, *Megasphaera*, *Bifidobacterium*, *Sharpea* or *Bacteroides*.

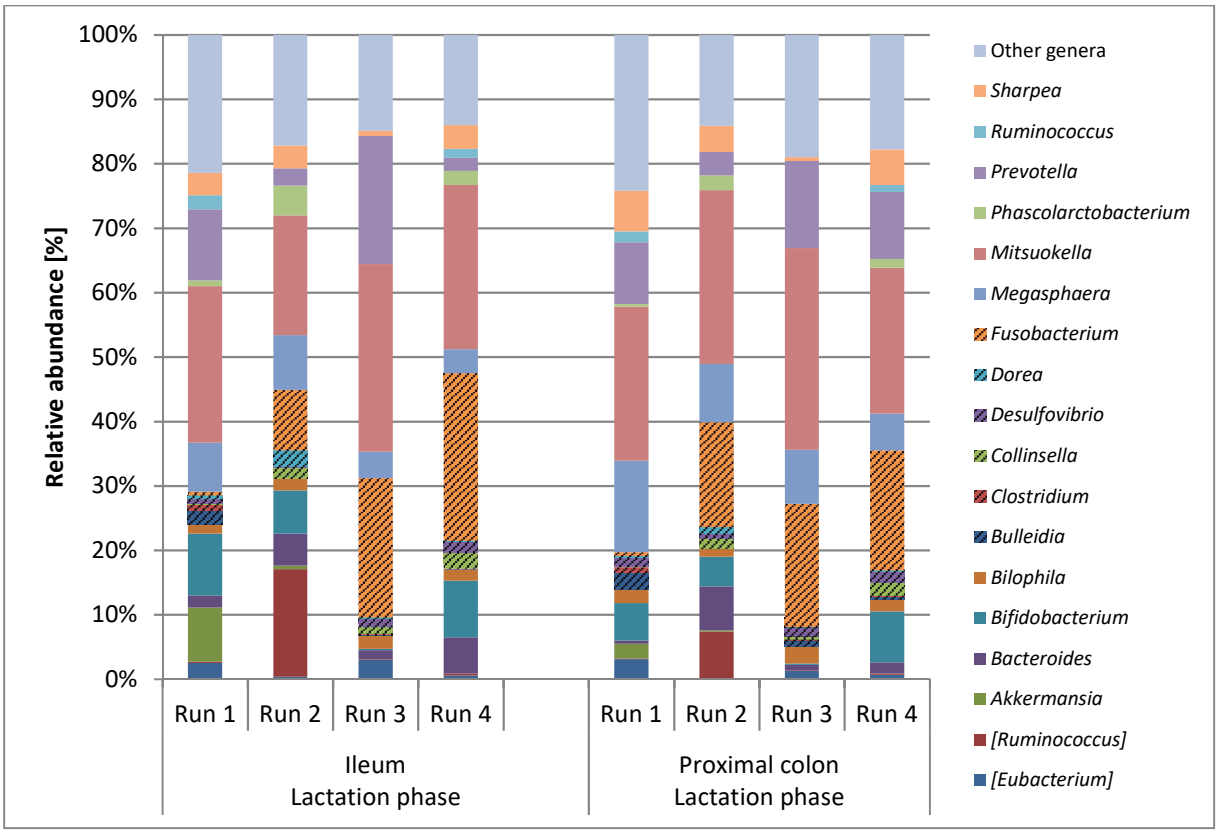

Figure 13: Genera composition of microbiota in the ileum and proximal colon at the end of the lactation phase (samples taken 2 weeks after inoculation).

Comparison between ileum and proximal colon. While no statistical differences in microbial composition were observed at the phylum level, at the family level of the classification, some differences could be detected. However, these differences were not observed in the case of abundant families but among sparser families: [Paraprevotellaceae], Nocardiaceae and Muribaculaceae (S24–7); the latter belonging to a family with a high relative abundance in the inocula (6.5 ± 2.3).

Taxonomy of the microbial ecosystem at the end of the post-weaning phase. Regarding the phyla found at the end of the post-weaning phase of the proximal colon (Figure 14), the relative abundances of Bacteroidetes increased while the relative abundances of Firmicutes and Proteobacteria diminished, except for the second run.

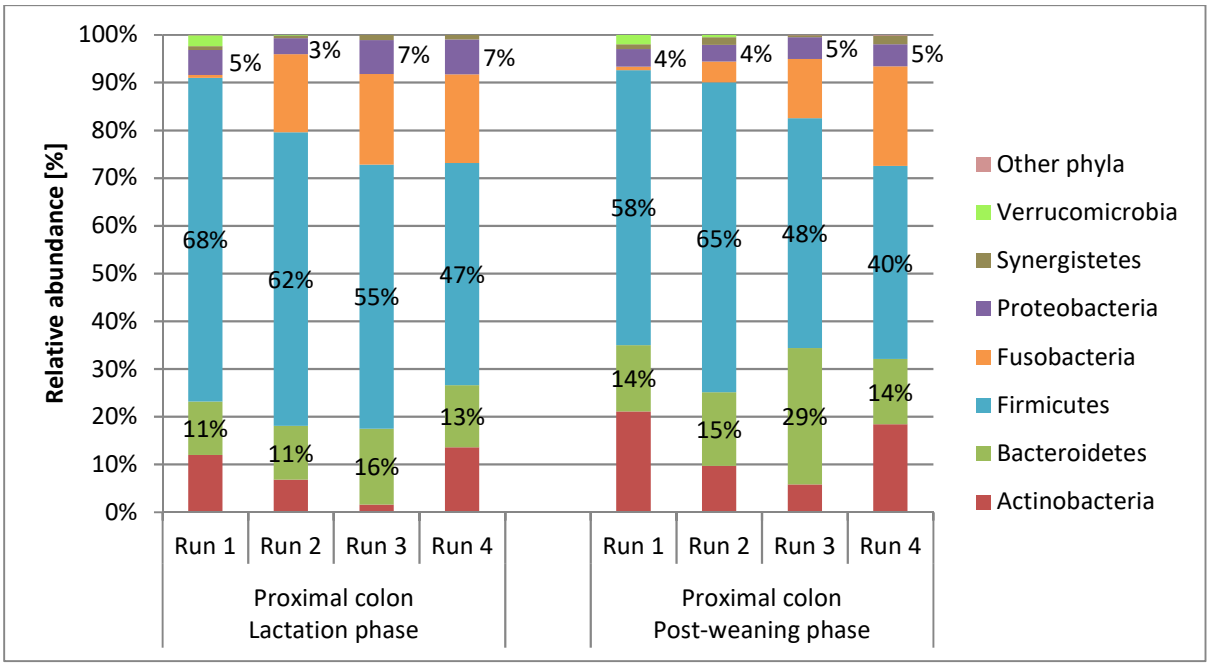

Figure 14: Phyla composition of microbiota in the proximal colon at the end of the lactation phase (samples taken 2 weeks after inoculation) and at the end of the post-weaning phase (samples taken 2 weeks after weaning transition).

At the class level (Supplemental material), statistical differences associated with weaning were detected in the ileum and proximal colon. Regarding the ileum, Erysipelotrichi (Firmicutes) increased in relative abundance (+5.0%, p = 0.043) and Actinobacteria (Actinobacteria) tended to increase (+6.5%, p = 0.083). In the proximal colon, Gamma-Proteobacteria (Proteobacteria) decreased (−0.9%, p = 0.021).

At the family level, statistical differences appeared at the end of weaning in the ileum and proximal colon. Regarding the former, Erysipelotrichaceae (Firmicutes) increased (+5.0%, p = 0.043) and Bifidobacteriaceae (Actinobacteria) tended to increase (+6.3%, p = 0.083) when Veillonellaceae (Firmicutes) tended to decrease (−6.8%, p = 0.083). In terms of the colon, [Paraprevotellaceae] (Bacteroidetes) increased (+0.1%, p = 0.021) while Enterobacteriaceae (Proteobacteria) and Veillonellaceae (Firmicutes) decreased or tended to decrease (−0.9%, p = 0.021 and −9.6%, p = 0.083, respectively).

Metabolites results. The SCFA profile of each sample was observed in order to confirm the stabilization of the system at the end of the lactation phase and at the end of the post-weaning phase in order to exploit the last 2 samples of each phase. Results of the metabolites through the SCFA are given in Table 7.

During the lactation phase, the total SCFA concentration tended to be higher (p = 0.098) in the ileum (67.3 ± 2.4 mM) compared to that of in the proximal colon (62.9 ± 3.3 mM). A trend value (p = 0.053) was observed on acetic acid (33.5 ± 2.7mM in ileum vs 30.1 ± 1.8mM in proximal colon).

The weaning in the ileum and the proximal colon was statistically perceptible on isovaleric acid concentrations (p = 0.021) seen by a drop from 2.1 ± 1.5mM in the ileum and 1.9 ± 1.0mM in the proximal colon, respectively, to 1.0 ± 0.2mM in each of the two bioreactors.

In the post-weaning samples, butyric acid concentration was significantly higher in the ileum (8.2 ± 0.8 mM) than in the proximal colon (7.8 ± 1.0 mM; p = 0.026).

Regarding the general profile of SCFA, acetic acid showed the highest concentration values followed by propionic, butyric, valeric and hexanoic acids. Isobutyric and isovaleric acid concentrations were weak and close to the hexanoic acid value.

In terms of proportions between acetic, propionic and butyric acids (C2: C3: C4), weaning tended to increase the proportion of acetic acid in the ileum (57:28:15 for lactation phase vs 60:26:14 for post-weaning phase, p = 0.096 for C2) and the proximal colon (56:29:15 *vs* 60:26:14, respectively, p = 0.060 for C2).

Table 7: Short-chain fatty acids (SCFA) contained in baby-SPIME samples.

SCFA	Lactation phase			Post-weaning phase			Effect of weaning
	Ileum	Proximal colon	p	Ileum	Proximal colon	p	p
Total SCFA	67.3 ± 2.4	62.9 ± 3.3	0.098	66.3 ± 6.5	61.4 ± 9.3	ns	ns
Acetic acid	33.5 ± 2.7	30.1 ± 1.8	0.053	35.4 ± 3.9	32.2 ± 5.9	ns	ns
Propionic acid	16.5 ± 2.2	15.5 ± 2.0	ns	15.2 ± 2.5	14.0 ± 3.2	ns	ns
Iso-butyric acid	0.6 ± 0.2	0.6 ± 0.1	ns	0.5 ± 0.1	0.5 ± 0.1	ns	ns
Butyric acid	8.5 ± 0.3	8.3 ± 0.9	ns	8.2 ± 0.8	7.8 ± 1.0	0.026	ns
Iso-valeric acid	2.1 ± 1.5	1.9 ± 1.0	ns	1.0 ± 0.2	1.0 ± 0.2	ns	On ileum: p = 0.021; On colon: p = 0.021
Valeric acid	5.0 ± 0.7	5.0 ± 0.7	ns	4.9 ± 1.2	4.6 ± 1.3	ns	ns
Hexanoic acid	1.1 ± 0.4	1.5 ± 0.4	ns	1.1 ± 0.4	1.3 ± 0.3	ns	ns

Concentrations in mmol/L of sample ± SEM, n=4, p means p-value, ns means not significant.

Discussion

The SHIME®–an in vitro dynamic multi-compartment model dedicated to human researches (Van den Abbeele et al., 2010)–is particularly well suited to be adapted to an in vitro piglet model ensuring the presence of an ileum and allowing a weaning process to take place in bioreactors. These two particularities constitute novelties in comparison with existing models (PolyFermS and PigutIVM). Compared to the human model, different parameters had to be adapted according to the piglet physiology (*i.e.*, volume, transit time, concentration of digestive secretions and culture media …) with special attention to pH due to its potential effect on microbiota (Ilhan et al., 2017). Indeed, the constraint of maintaining equal parameters in the system in order to study the effect of the culture media on microbiota make it mandatory to fix the pH ranges for the lactation as well as post-weaning phases. A pH of 6.5 was chosen for the ileum, and a pH of 5.9 for the proximal colon (Snoeck et al., 2004a). Define parameters for the retention time was also not easy in these particular conditions. They were set considering the constraints of an in vitro digestion model regarding the data from the literature (Davis et al., 2001; Snoeck et al., 2004b).

The bioreactors were inoculated with a preparation that contained the faeces of a suckling piglet. Faeces from 4 piglets were used to study and establish the present model. Each donor, originating in the same farm but different litters (time and space), allowed starting an individual run. This represented a constraint due to variations that occur in the microbiome between animals of the same age and involving variations in the microbial ecosystem of baby-SPIME. But it has been shown that same age groups share more similarities than animals of different ages (Isaacson and Kim, 2012). This feature was a deliberate choice as it offered the opportunity to study metabolic and microbial activities linked to a specific individual in the future. This can be considered as an advantage of the baby-SPIME model as compared against PigutIVM, *i.e.* the in vitro piglet model validated using a pool of faeces from 8 weaned piglets.

Introducing an ileum in an in vitro dynamic model. Based on the microbiota observed in the ileum and colon of the model, it can be concluded that Firmicutes, Bacteroidetes and, to a lesser extent, Proteobacteria were the main phyla present, in agreement with in vivo observations (De Rodas et al., 2018; Kim and Isaacson, 2015; Niu et al., 2015; Pajarillo et al., 2014). Indeed, according to the literature, Firmicutes and Bacteroidetes can make up 90% of the total bacteria, a fact confirmed by the in vitro model developed by Fleury et al. (2017). The baby-SPIME did not reach this level mainly due to the presence of Fusobacteria.

Recent work on piglet intestinal microbiota (De Rodas et al., 2018) revealed that Firmicutes are represented by Clostridia and Bacilli, the former found in high relative abundance in the colon and the latter in high relative abundance in the ileum. Moreover, in this study Bacteroidetes, through Bacteroidia, were also an important class but less dominant in the ileum than in the colon. Baby-SPIME led to similar observations, but two major discrepancies were also observed. Firstly, the relative

abundance of Bacteroidia in the colon of baby-SPIME was weak compared to the results observed in vivo in the colon by the team of De Rodas et al. (2018)–closer to 13% rather than 35%. Li et al. (2019) also observed more Bacteroidetes in the colon. The explanation of the lack of Bacteroidetes in the proximal colon bioreactors can probably be found in the use of Oxgall bile (Begley et al., 2005; Islam et al., 2011) without a dialysis module. Secondly, the relative abundance of Bacilli was also weak in vitro, and this point affected particularly the ileum profile where the relative abundance of Bacilli reached a value of only 0.1% in the baby-SPIME. Regarding the particularities of Bacteroidia and Bacilli relative abundances, the ileum and the proximal colon of baby-SPIME were quite comparable in terms of microbial populations, and quite similar to the profiles expected in the colon of piglets. Despite everything, the model seems to accurately reflect well expectations set forth in the literature, such as for example, the higher presence of *E. coli* found in the ileum *vs* that found in the colon (Zhao et al., 2015) as we observed with our qPCR results (data not shown). The ileum bioreactor seemed so to play the role of a pre-colon, suggesting the necessity to improve its microbial composition.

In light of the weakness of some bacterial population belonging to Bacilli class, including populations such as *Lactobacillus spp*., in ileum compared to in vivo studies (Pieper et al., 2008), the culture conditions could be improved. These culture conditions seemed suitable to ensure the presence of hardly cultivable bacteria, such as *Akkermansia muciniphila* (van der Ark et al., 2018) at least during one run. But they did not seem suitable to sufficiently promote the growth of bacteria of great interest such as *Lactobacillus spp*. in any run. As proposed by several authors (Fleury et al., 2017; Tran et al., 2016; Van den Abbeele et al., 2012), adding a solid substrate of mucin to replicate the intestinal mucin coat could help to improve the current model. Indeed, from mucosa to the lumen, each microorganism populates its niche helped by favourable ecological and food conditions, especially in a segment where retention time is high (Fonty and Chaucheyras-Durand, 2007). In all likelihood, the solid mucin substrate could give a more accurate representation of the in vivo process of development of *Lactobacillus spp*. (Van den Abbeele et al., 2012). It would also contribute to better differentiate the ileum from the colon. Similarly, as seen in the work of (Tran et al., 2016), the solid mucin environment would probably allow counterbalancing the proportions of Gamma-Proteobacteria and Delta-Proteobacteria in order to yield an even more realistic model. In addition, the new balance between bacterial populations achieved with a solid mucin environment would probably contribute to improve the relative abundance of Bacteroidia in the colon of baby-SPIME.

Regarding Actinobacteria, Fusobacteria and–to a lesser extent–Verrucomicrobia, they were more abundant in the baby-SPIME model than in vivo. Their relative abundances were variable from one run to another. Therefore, in future runs, it will be difficult to predict the profile they will develop in the bioreactors after the stabilization of the microbiota. Indeed, relative abundance by the end of the stabilization period could be low or high and could be assimilated to individual variations of the system. Fusobacteria, present through Fusobacterium, particularly held our attention. They are common in the gastrointestinal tract of human and animals (De Witte et al., 2017b; Krieg et al., 2010) and can be more

abundant in the microbiome profile of captive animals rather than in wild breed due to the composition of their feed (Wang et al., 2016). Perhaps the culture medium can partially explain the overabundance of this bacterium in the model.

To discuss the results observed at a lower level of the classification, Mach et al. (2015) hypothesized the existence of two clusters in piglet's microbiota in vivo: Ruminococcaceae on the one hand and *Prevotella* on the other. After weaning, the cluster Ruminococcaceae is enriched with *Treponema* while the *Prevotella* cluster is enriched with *Mitsuokella* (Mach et al., 2015). Interestingly, in the baby-SPIME model, the microbiota seemed to evolve from the Ruminococcaceae cluster in the inoculum to the *Prevotella* cluster for lactation and weaning stages. Indeed, the inoculum is enriched in Ruminococcaceae while lactation and post-weaning phases are enriched with *Mitsuokella* and *Prevotella*, two important genera of the model with *Fusobacterium*, *Bifidobacterium Megasphaera* and *Bacteroides* among others. The Ruminococcaceae cluster is more adapted to lactation periods because it includes bacteria capable of digesting free milk oligosaccharides. At the opposite end, the *Prevotella* cluster, since it is better adapted to the degradation of complex dietary polysaccharides, appears to derive more advantages from post-weaning diets. Perhaps cluster evolution could originate from the ability of *Prevotella* to degrade the glycoprotein of mucin in a mucin- enriched environment (Pajarillo et al., 2014 quoting both Wright et al., 2000 and Rho et al., 2005). The culture media of the baby-SPIME model being very rich in mucin (6.0 g/L on a total of 16.2 g/L of ingredients), could explain the evolution of the system toward the *Mitsuokella*/*Prevotella* cluster.

Finally, another prospect in the use of the model consists to systematically perform qPCR analyses on specific bacterium as *E. coli* because the only 16S rRNA data barcoding appeared not to be sufficient to quantify or to follow bacteria present in low proportions.

Ensuring a weaning stage in bioreactors. Weaning in bioreactors generally causes Firmicutes to decrease when Bacteroidetes increase, without leading to an inversion of the ratio as observed in vivo (Pajarillo et al., 2014). This is mainly seen in colon bioreactors and is contingent on the ability of Bacteroidetes to degrade complex carbohydrates (Thomas et al., 2011; Wang et al., 2016). But one of the 4 runs did not confirm this (both Firmicutes and Bacteroidetes increased) and the reasons remain unclear since the quality control mechanisms of the system appear to be working properly. Gamma-Proteobacteria decreased from the steady-state lactation to the steady-state post-weaning phases, as seen in vivo across ages (De Rodas et al., 2018).

Moreover, another important bacterial modification in agreement with in vivo weaning trials consists of a shift, in Bacteroidetes phyla, between *Bacteroides*–that decreased–and *Prevotella*–that increased (Pajarillo et al., 2014). This is explained in the literature by the favourable substrate for each of these bacteria: mono- and oligosaccharides contained in milk products in the case of *Bacteroides* and hemicellulose in the case of *Prevotella* (Kim and Isaacson, 2015 citing both Hayashi et al., 2007 and Lamendella et al., 2011). Recent in vivo work confirmed the significant increase of *Prevotella* as well

as *Lactobacillus* following weaning (Guevarra et al., 2018). In the baby-SPIME model, the average relative abundance of *Bacteroides* was lower in the postweaned ileum but statistically confirmed through *Bacteroides fragilis* and *Bacteroides uniformis* (data not shown). The average relative abundance of *Prevotella* was higher in the post-weaned proximal colon although this was not statistically confirmed.

Interestingly, the differences between the lactation and the post-weaning microbial profiles were rather limited. This could probably be explained by the composition of the culture medium. Indeed, the lactation culture medium provided to the baby-SPIME contains a complementary milk replacer feed with wheat flour and others typical postweaned raw materials. The provision of Nuklospray to bacteria during the lactation phase probably led to a first shift of the microbial populations and could explain why the shift of the microbiota after in vitro weaning is less remarkable compared to the in vivo observation (Kim and Isaacson, 2015; Slifierz et al., 2015). It would be of great interest to substitute part of this milk replacer feed in the medium by a milk powder free of wheat and other typical post-weaned raw materials. It would allow verifying the hypothesis of observing a stronger shift of the microbiota through this strategy.

The microbial discrepancy observed in the ileum bioreactors during the lactation phase can probably help to explain the odd SCFA profile in the ileum of post-weaning baby-SPIME. Indeed, during the different runs, SCFA were produced in the bioreactors. They were monitored to determine the evolution of their profiles during trials. The profile of the different SCFA (concentration of C2 > C3 > C4 > C5 > C6, concentration of iC5 > iC4 and close to the concentration of C6) and the ratios between acetic, propionic and butyric acids were in concordance with the literature when comparing the proximal colon of baby-SPIME with both in vitro models (PolyFermS and PigutIVM) or with in vivo data (Awati et al., 2006b; Kraler et al., 2015). The concentration of total SCFA was lower in baby-SPIME than in PigutIVM but this can be explained by the composition of the culture media (16.2 g/L for all ingredients for the baby-SPIME culture medium vs 35 g/L of carbohydrates and proteins for the culture medium of PigutIVM). However, in the ileum, higher concentration of butyric acid *vs* propionic acid would have been expected in newly weaned piglets, as observed in in vivo studies (Awati et al., 2006b; Kraler et al., 2015). This confirms that microbial profile in ileum bioreactor had to be improved even for the post-weaning phase. Three parameters that have to be investigated concern the richness of the medium in simple carbohydrates (Poeker et al., 2019), the anaerobic condition of the system (Zhao et al., 2015) and the type of inoculum–real ileal content instead of fecal matter (Awati et al., 2006a). Improve these parameters of the baby-SPIME model will also improve the dynamic evolution of *Lactobacillus* spp. in the bioreactor, so important to help piglets at weaning (Guevarra et al., 2018). Moreover, *Lactobacillus*–well known to metabolize highly fermentable carbohydrates–seems also to play a crucial role for the utilisation of complex carbohydrates (Guevarra et al., 2018) increasing the interest of promoting its growth in the bioreactors.

Conclusions

The purpose of the study was to adapt the SHIME® model, developed for human research, to a baby-SPIME model (Simulator of Pig Intestinal Microbial Ecosystem, dedicated to the luminal microbiota of piglet) including an inoculated ileum in addition to the colon, and a weaning transition in bioreactors. This adaptation would allow the study of weaning dietary strategies. The baby-SPIME model thus developed appears to meet expectations for the proximal colon in terms of microbial profile and the production of short-chain fatty acids. In vitro weaning seems to be successful regarding firstly the evolution of Firmicutes and Bacteroidetes in the proximal colon, and secondly the evolution of *Bacteroides* and *Prevotella*. As regards the ileum bioreactor, an obstacle has yet to be overcome especially in terms of improving the relative abundance of the class Bacilli in the model. Testing an inoculation with intestinal content instead of faeces, adding a solid mucin-environment and/or using milk powder for suckling piglets have all been considered possible improvements to the model.

Annex: additional description of data not shown

Material and methods: complementary qPCR analysis

DNA was extracted from samples obtained from the first two runs of the study. This was done in order to confirm the presence of two important bacteria: *Lactobacillus* sp. and *Escherichia coli*. The DNeasy Blood & Tissue extraction kit (Qiagen Benelux B.V., Venlo, The Netherlands) was used following the manufacturer's instructions. Samples were quantified and tested for purity using a NanoDrop 2000 (Thermo Scientific, USA) and integrity was tested on a 1% agarose gel. The PCR mixture contained: 2 µL of isolated DNA (100 ng), 10 µL of SYBR Premix EX TaqTM II, 0.4 µL of ROX Reference Dey II (50 ×, Tli RNase H plus, Takara, Japan), 1 µL of each primer (Table A1) and 5.6 µL of nuclease-free water. The PCR reaction programme was applied in an ABI StepOnePlus (Applied Bio systems, USA) system with the following programme: 30s heating at 95 °C, followed by 40 cycles of denaturation (5s at 95°C), annealing (30s at 60 °C) and extension (45s at 72 °C). A melting curve analysis was performed to check the specificity of the primers and a standard curve was determined to ensure amplification efficiencies between 90% and 110% (Table A1).
The $2^{-\Delta\Delta Ct}$ method (Barszcz et al., 2016) was used to analyse the selected bacteria, where a pooled sample created by mixing all the DNA samples was used as the calibrator sample (Bustin et al., 2009).

Table A1: qPCR: primers and efficiency

Bacteria	5'-primer-3'	Efficiency (%)
Total bacteria	F- CGTGCCAGCCGCGGTAATACG	101
	R- GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	
Lactobacillus	F- CATCCAGTGCAAACCTAAGAG	101
	R- GATCCGCTTGCCTTCGCA	
Escherichia coli	F- GGGAGTAAAGTTAATACCTTTGCTC	101
	R-TTCCCGAAGGCACATTCT	

Results: qPCR analysis

The results of the qPCR analysis, presented in Table A2, confirmed the presence of *Escherichia coli* in the model. For *Lactobacillus sp.*, qPCR analysis confirmed the presence of the bacterium in inoculum and its absence in the ileum and colon samples for piglet 1 and piglet 2.

Table A2: qPCR results: relative amounts [arbitrary units] of samples coming from the two first runs of the baby-SPIME model. The $2^{-\Delta\Delta Ct}$ method (Barszcz, Taciak, and Skomiał 2016) was used to analyse the selected bacteria compared to the total bacteria

		Lactobacillus	*Escherichia coli*
1st piglet	Inoculum	0.04	48.16
	Lactating ileum	0.00	0.21
	Lactating colon	0.00	0.18
	Post-weaned ileum	0.00	0.81
	Post-weaned colon	0.00	0.11
2nd piglet	Inoculum	0.31	7.09
	Lactating ileum	0.00	1.11
	Lactating colon	0.00	0.46
	Post-weaned ileum	0.00	0.81
	Post-weaned colon	0.00	0.52

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Availability of data and materials

Raw sequences can be found on the EMBL Nucleotide Sequence Database (ENA–European Nucleotide Archive) under the project accession number PRJEB30341.

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Supplemental material

Additional file - Table 1: Composition of the microbiota (family level) in baby-SPIME (Simulator of Pig Intestinal Microbial Ecosystem)

Phyla (kingdom)				Samples at the end of the lactation phase								Samples at the end of the post-weaning phase					
Classes	Families	Inocula		Ileum (IL)			Proximal Colon (PC)					Ileum			Proximal Colon		
		RA (%)	SEM	RA (%)	SEM	p/FDR: inoc-IL	RA (%)	SEM	p/FDR: inoc-PC	p/FDR: IL-PC		RA (%)	SEM	p/FDR: wean	RA (%)	SEM	p/FDR: IL-PC	p/FDR: wean
Euryarchaeota (Archaea)		**0.3**	**0.2**	**0.0**	**0.0**		**0.0**	**0.0**	*/T			**0.1**	**0.1**		**0.0**	**0.0**		
Methanobacteria	*Methanobacteriaceae*	0.3	0.2	0.0	0.0		0.0	0.0	*/ns			0.1	0.1		0.0	0.0		
Thermoplasmata	*[Methanomassiliicoc.]*	0.1	0.0	0.0	0.0		0.0	0.0				0.0	0.0		0.0	0.0		
Actinobacteria (Bacteria)		**1.7**	**0.9**	**10.5**	**3.2**	T/ns	**8.5**	**2.7**	T/ns			**17.9**	**3.9**		**13.7**	**3.6**		
Actinobacteria	*Corynebacteriaceae*	1.0	0.8	0.0	0.0	*/T	0.2	0.1				0.1	0.0		0.1	0.1		
	Dietziaceae	0.1	0.1	0.0	0.0	*/ns	0.0	0.0	*/ns			0.0	0.0		0.0	0.0		
	Micrococcaceae	0.1	0.1	0.0	0.0	*/T	0.0	0.0	*/T			0.0	0.0		0.0	0.0		
	Nocardiaceae	0.0	0.0	0.0	0.0	*/T	0.1	0.0	*/T	T/ns		0.1	0.1		0.1	0.1		
	Bifidobacteriaceae	0.0	0.0	6.3	2.1	*/T	4.6	1.6	*/T			12.6	2.3	T/ns	10.2	2.8		
Coriobacteriia	*Coriobacteriaceae*	0.5	0.3	4.1	1.2	*/T	3.6	1.2	*/ns			5.1	1.8		3.4	0.8		
Bacteroidetes (Bacteria)		**32.6**	**1.8**	**13.0**	**3.2**	*/T	**12.9**	**1.1**	*/T			**12.1**	**2.7**		**17.9**	**3.6**		
Bacteroidia	"undefined"	7.5	1.8	0.0	0.0	*/T	0.0	0.0	*/T			0.0	0.0		0.1	0.1		
	[Barnesiellaceae]	0.2	0.2	0.0	0.0		0.0	0.0				0.0	0.0		0.0	0.0		
	[Odoribacteraceae]	1.2	0.8	0.0	0.0	*/T	0.0	0.0	*/T			0.0	0.0		0.0	0.0		
	[Paraprevotellaceae]	2.1	0.7	0.0	0.0	*/T	0.1	0.0	*/T	*/ns		0.1	0.0		0.2	0.0	*/ns	*/ns
	Bacteroidaceae	5.0	2.2	3.5	1.1		2.5	1.5				1.8	0.7		2.7	0.9		
	p-2534-18B5	5.0	2.0	0.0	0.0	T/ns	0.0	0.0	*/ns			0.0	0.0		0.0	0.0		
	Porphyromonadaceae	1.3	0.4	0.4	0.1	*/ns	0.4	0.1	*/T			0.8	0.4		1.1	0.5		
	Prevotellaceae	3.0	1.4	8.9	4.2		9.3	2.1	*/ns			9.5	2.9		13.2	3.4		
	RF16	0.9	0.8	0.0	0.0	*/ns	0.0	0.0	*/ns			0.0	0.0		0.0	0.0		
	Rikenellaceae	0.2	0.1	0.0	0.0		0.0	0.0	*/ns			0.0	0.0		0.0	0.0		
	Muribaculaceae (S24-7)	6.5	2.3	0.2	0.1	*/T	0.7	0.2	*/T	T/ns		0.1	0.0		0.6	0.3	*/ns	
Chlamydiae (Bacteria)		**2.0**	**2.0**	**0.0**	**0.0**		**0.0**	**0.0**				**0.0**	**0.0**		**0.0**	**0.0**		
Chlamydiia	*Chlamydiaceae*	2.0	2.0	0.0	0.0		0.0	0.0				0.0	0.0		0.0	0.0		
Cyanobacteria (Bacteria)		**0.1**	**0.1**	**0.0**	**0.0**	*/*	**0.0**	**0.0**	*/T			**0.0**	**0.0**		**0.0**	**0.0**		
4C0d-2	"undefined"	0.1	0.1	0.0	0.0	*/T	0.0	0.0	*/ns			0.0	0.0		0.0	0.0		

Phyla (kingdom)				Samples at the end of the lactation phase						Samples at the end of the post-weaning phase					
Classes	Families	Inocula		Ileum (IL)			Proximal Colon (PC)			Ileum			Proximal Colon		
		RA (%)	SEM	RA (%)	SEM	p/FDR: inoc-IL	RA (%)	SEM	p/FDR: inoc-PC	p/FDR: IL-PC	RA (%)	SEM	p/FDR: wean	RA (%)	SEM	p/FDR: IL-PC	p/FDR: wean
Deferribacteres (Bacteria)		**0.1**	**0.0**	**0.0**	**0.0**	*/T	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
Deferribacteres	*Deferribacteraceae*	0.1	0.0	0.0	0.0	*/ns	0.0	0.0	*/ns		0.0	0.0		0.0	0.0		
Firmicutes (Bacteria)		**51.9**	**3.87**	**54.3**	**5.1**		**57.7**	**4.6**			**53.7**	**3.9**		**52.8**	**5.4**		
Bacilli	*Planococcaceae*	0.0	0.0	0.1	0.1		0.2	0.2	*/ns		0.1	0.1		0.1	0.1		
	Enterococcaceae	0.8	0.7	0.0	0.0		0.0	0.0			0.0	0.0		0.0	0.0		
	Lactobacillaceae	4.5	2.0	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Streptococcaceae	0.2	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Turicibacteraceae	0.1	0.0	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
Clostridia	"undefined"	7.5	3.4	1.0	0.3		1.0	0.5	T/ns		1.6	0.5		1.2	0.1		
	[Mogibacteriaceae]	0.4	0.1	0.4	0.1		0.5	0.1			0.5	0.2		0.4	0.1		
	Christensenellaceae	4.0	2.3	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Clostridiaceae	2.3	0.3	0.4	0.2	*/T	0.3	0.2	*/T		0.6	0.2		0.5	0.2		
	Dehalobacteriaceae	0.2	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Eubacteriaceae	0.0	0.0	0.0	0.0		0.1	0.0			0.0	0.0		0.0	0.0		
	Lachnospiraceae	5.4	2.2	10.2	5.7		5.6	2.5			9.4	1.0		8.5	2.9		
	Peptostreptococcaceae	0.1	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Ruminococcaceae	19.6	2.4	1.3	0.4	*/T	1.0	0.4	*/T		2.7	1.6		1.7	0.7		
	Veillonellaceae	3.1	1.4	35.2	1.1	*/T	42.4	3.4	*/T		28.4	3.9	T/ns	32.9	3.0		T/ns
Erysipelotrichi	*Erysipelotrichaceae*	3.8	0.7	5.5	0.9		6.7	1.9			10.5	1.4	*/ns	7.6	1.2		
Fusobacteria (Bacteria)		**1.3**	**1.1**	**14.4**	**5.8**	*/T	**13.6**	**4.4**	*/T		**9.0**	**2.9**		**9.6**	**4.5**		
Fusobacteriia	*Fusobacteriaceae*	1.3	1.1	14.4	5.8	*/ns	13.6	4.4	*/ns		9.0	2.9		9.6	4.5		
Lentisphaerae (Bacteria)		**0.1**	**0.1**	**0.0**	**0.0**	*/*	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
[Lentisphaeria]	*R4-45B*	0.1	0.0	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
Planctomycetes (Bacteria)		**0.4**	**0.3**	**0.0**	**0.0**	*/T	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
Planctomycetia	*Pirellulaceae*	0.4	0.3	0.0	0.0	*/ns	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
Proteobacteria (Bacteria)		**6.3**	**2.8**	**4.4**	**0.8**		**5.8**	**0.9**			**3.6**	**0.4**		**4.1**	**0.3**		
Alphaproteob.	"undefined"	0.1	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Brucellaceae	0.0	0.0	0.1	0.1		0.2	0.1	T/ns		0.1	0.0		0.1	0.0		
	Rhizobiaceae	0.0	0.0	0.1	0.1		0.1	0.1			0.0	0.0		0.1	0.1		

Phyla (kingdom)				Samples at the end of the lactation phase							Samples at the end of the post-weaning phase						
Classes	Families	Inocula		Ileum (IL)			Proximal Colon (PC)				Ileum			Proximal Colon			
		RA (%)	SEM	RA (%)	SEM	p/FDR: inoc-IL	RA (%)	SEM	p/FDR: inoc-PC	p/FDR: IL-PC	RA (%)	SEM	p/FDR: wean	RA (%)	SEM	p/FDR: IL-PC	p/FDR: wean
Betaproteob.	*Alcaligenaceae*	0.1	0.1	0.1	0.1		0.1	0.1			0.1	0.1		0.1	0.1		
	Comamonadaceae	0.3	0.2	0.1	0.1		0.2	0.1			0.2	0.1		0.3	0.1		
Deltaproteob.	*Desulfovibrionaceae*	1.1	0.4	2.8	0.4	T/ns	3.4	0.4	*/ns		2.6	0.1		2.8	0.1		
	"unclassified"	0.2	0.2	0.0	0.0	*/ns	0.0	0.0	*/ns		0.0	0.0		0.0	0.0		
Epsilonproteob.	*Campylobacteraceae*	0.3	0.1	0.0	0.0	*/T	0.1	0.1			0.1	0.1		0.2	0.2		
Gammaproteob.	*Succinivibrionaceae*	0.5	0.5	0.0	0.0		0.0	0.0			0.0	0.0		0.0	0.0		
	Enterobacteriaceae	3.8	2.2	1.1	0.3		1.5	0.5			0.4	0.1		0.5	0.1		*/ns
	Pasteurellaceae	0.1	0.1	0.0	0.0	*/T	0.0	0.0	T/ns		0.0	0.0		0.0	0.0		
	Pseudomonadaceae	0.0	0.0	0.0	0.0		0.0	0.0			0.1	0.1		0.0	0.0		
	Xanthomonadaceae	0.0	0.0	0.1	0.1		0.1	0.1			0.1	0.1		0.1	0.1		
Spirochaetes (Bacteria)		**1.4**	**0.7**	**0.0**	**0.0**	*/*	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
Spirochaetes	*Sphaerochaetaceae*	0.6	0.3	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	Spirochaetaceae	0.8	0.5	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
Synergistetes (Bacteria)		**0.9**	**0.6**	**1.2**	**0.1**		**0.9**	**0.2**			**1.9**	**0.6**		**1.2**	**0.3**		
Synergistia	*Dethiosulfovibrionaceae*	0.7	0.6	0.5	0.2		0.4	0.2			1.1	0.4		0.8	0.3		
	Synergistaceae	0.2	0.1	0.7	0.2	*/ns	0.5	0.2	T/ns		0.8	0.2		0.4	0.1		
Tenericutes (Bacteria)		**0.3**	**0.2**	**0.0**	**0.0**	*/*	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
Mollicutes	"undefined"	0.2	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
RF3	"undefined"	0.2	0.1	0.0	0.0	*/ns	0.0	0.0	*/ns		0.0	0.0		0.0	0.0		
TM7 (Bacteria)		**0.2**	**0.1**	**0.0**	**0.0**	*/*	**0.0**	**0.0**	*/T		**0.0**	**0.0**		**0.0**	**0.0**		
TM7-3	"undefined"	0.1	0.0	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
	F16	0.2	0.1	0.0	0.0	*/T	0.0	0.0	*/T		0.0	0.0		0.0	0.0		
Verrucomicrobia (Bacteria)		**0.2**	**0.2**	**2.3**	**2.1**		**0.6**	**0.6**			**1.7**	**1.4**		**0.6**	**0.5**		
Verruco-5	*RFP12*	0.2	0.1	0.0	0.0	*/ns	0.0	0.0	*/ns		0.0	0.0		0.0	0.0		
Verrucomicrobiae	*Verrucomicrobiaceae*	0.0	0.0	2.2	2.1		0.6	0.6			1.7	1.4		0.6	0.5		

Families with a relative abundance (RA) of minimum 0.1% in at least one of the samples were included in the table. Results in RA expressed a percentage (%) of the total bacteria. p/FDR were obtained by the Kruskal-Wallis test (p) that was adjusted using the Benjamini Hochberg FDR procedure for multiple comparisons (FDR). A p-value between 0.01 and (or equal to) 0.05 was considered statistically significant (symbolized by a star *). A p-value between 0.05 and (or equal to) 0.1 was considered a trend (T). Otherwise, p-value was considered as not significant (ns). A blank p/FDR means ns/ns.

Study 3 -

Oxygen as a key parameter in in vitro dynamic and multi-compartment models to improve microbiome studies of the small intestine?

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Abstract

In vitro digestion and fermentation models are frequently used for human and animal research purposes. Different dynamic and multi-compartment models exist, but none have been validated with representative microbiota in the distal parts of the small intestine. We recently developed a dynamic and multi-compartment piglet model introducing microbiota in an ileum bioreactor. However, it presented discrepancies compared to in vivo data. Recommendations are available to standardize studies in this field. They target the digestion model but include elements of a fermentation model. But no recommendation is given concerning control of the atmosphere. The gastrointestinal tract is generally associated with anaerobiosis to conduct a good fermentation process. In this study, we attempted to improve the ileal microbiota of the piglet model by testing inoculation: real intestinal content *vs* feces; the latter being generally used for ethical and economical aspects. Results showed a positive effect of using real intestinal content. Fusobacteriia were less abundant in the model, *Bacteroidia* were better maintained in the colon. But for the ileum, results showed that anoxic conditions in the ileum bioreactor conditioned the microbial profile probably more than the type of inoculum itself, leading to the general conclusion that in vitro dynamic and multi-compartment models probably have to get oxygenated to improve microbiome studies of the small intestine.

Introduction

Several in vitro digestion and fermentation models exist for human and animal research purposes. They can either be mono-compartmental or multi-compartmental systems; they can concern biochemical and mechanical aspects (digestion) or the microbial aspect (fermentation) (Dupont et al., 2018). Two major dynamic and multi-compartmental models were initially developed and validated–commonly called SHIME (Molly et al., 1994, 1993) and TIM (Minekus et al., 1995)–although alternative systems exist (Guerra et al., 2016). And both were progressively improved (Minekus et al., 1999; Van den Abbeele et al., 2012; Zeijdner et al., 2015), going on to develop for example a specific module to study interactions between bacteria and their host for SHIME (Marzorati et al., 2014). Recommendations are described in the literature regarding standardization of methods and comparison of results for in vitro digestion models (Minekus et al., 2014), but no appropriate recommendation is given about the atmosphere composition for fermentation systems. The TIM system controls the anaerobiosis of the colonic compartment by flushing with nitrogen (Minekus et al., 1999). The SHIME system initially ensured anaerobiosis with a 84%: 8%: 8% N_2–CO_2–H_2 atmosphere (Molly et al., 1993) but evolved towards flushing the headspace with nitrogen only (Van den Abbeele et al., 2010). Other in vitro models ensure anaerobiosis through the gaseous atmosphere generated by microbial activity, as in the ARCOL system (Dupont et al., 2018) or in the PigutIVM (Fleury et al., 2017).

Despite the diversity of existing in vitro models, it seems that to our knowledge a full dynamic and multi-compartment in vitro system that includes representative microbiota in the distal parts of the small intestine does not yet exist (Dupont et al., 2018; Guerra et al., 2012; Venema and van den Abbeele, 2013). Recently, a dynamic in vitro piglet model including an ileum, the baby-SPIME (baby Simulator of Pig Intestinal Microbial Ecosystem), has been developed (Dufourny et al., 2019). This last one–an adaptation of the SHIME® model–consists of three successive bioreactors (stomach, ileum and proximal colon) for which the ileum and proximal colon have been inoculated to mimic the microbiota of those compartments. This model is classically inoculated with feces based on the hypothesis that microbiota is able to differentiate itself following the physiological constraints that are applied to the system, as described previously (Liu et al., 2018; Molly et al., 1994; Van den Abbeele et al., 2010). Moreover, in the past decades, experiments were performed to confirm the interest to inoculate in vitro fermentation systems with feces. For the cecum of monogastric animal (Youssef and Kamphues, 2018) or for the large intestine of pigs (Bindelle et al., 2007), fecal inocula gave for example similar fractional rates of degradation or final gas production than intestinal inocula. However, in the case of ileum for piglets, the use of real intestinal content seems advised to study the potential of feed ingredients (Awati et al., 2006a), which is the main objective of the baby-SPIME. Yet, the latter presents a lack of Bacilli in the ileum together with a lack of Bacteroidia in the colon (Dufourny et al., 2019). To optimize the microbiota in the baby-SPIME bioreactors, the assumption was made that inoculation with real intestinal content instead of feces could be beneficial to maintaining bacteria present in the ileum or the proximal colon

that could have disappeared from the feces. The aim of the study was to compare the microbiota in the ileum and colon of the baby-SPIME inoculated with real intestinal content *vs* feces to assess the added-value of using real intestinal content. It was done in the light of modern techniques of gut microbiota analysis.

Material and methods

Inocula. The intervention on piglets was approved by the ethical committee of the University of Liège (ULiège, Liège, Belgium)–file n°1823. The intervention was in compliance with European (Directive 2010/63/EU) and Belgian (Royal Decree of the 29 May 2013) regulations governing the protection of animals used for scientific purposes.

The ileal and colon content as well as the feces of two [Piétrain × Landrace] suckling piglets of the Walloon Agricultural Research Centre (CRA-W, Gembloux, Belgium) were used to prepare the inocula of the study. Twenty-seven-day old piglets free of antibiotic treatment were selected, with several weeks between the sampling. Feces were sampled directly from the piglets and kept in ice under anaerobic conditions. Piglets were then euthanized to remove the intestinal content. Ileum content was sampled in the last quarter of the small intestine near the ileo-cecal junction. Colon content was sampled in the proximal colon one meter just after the ileo-cecal junction. The intestinal contents from the ileum and colon were also kept in ice under anaerobic conditions during transportation until the preparation of the inocula. Samples were not frozen. The procedure took 3 h.

A single donor was used to prepare the inocula of a SHIME® (ProDigest Bvba, Gent, Belgium). Two successive runs of a SHIME® were managed. For each run, one inoculum was prepared using the sample coming from ileal content to inoculate an ileum bioreactor. One inoculum was prepared using the sample coming from proximal colon content to inoculate a colon bioreactor. The last inoculum was prepared with the feces to inoculate both an ileum and a colon bioreactor.

Inocula were prepared by adding either intestinal content or feces to an anaerobic phosphate buffer solution (pH 7.0; 1:5, weight: volume) and homogenizing for 10 min. After a macroscopic filtration using stomacher bags, the filtrate was injected simultaneously in the ileum bioreactor (5 mL) and in the proximal colon bioreactor (12.5 mL). Before inoculation, these two bioreactors were filled with non-acidified culture medium (100 mL for the ileum bioreactor and 250 mL for the colon bioreactor) and the pH was automatically adjusted in each bioreactor according to its required range.

Culture media, pancreatic juice and bile. A culture medium (called lactation culture medium) was prepared drawing on the work of Molly et al. (1994). The composition is shown in Table 8. It was prepared in 5 L bottles and autoclaved for 35 min at 121 °C. Bottles were stored at 4 °C and the pH was adjusted to 3.0 before using in the first bioreactor.

Pancreatic juice was prepared in 2 L bottles. It contained (personal communication of ProDigest) sodium hydrogen carbonate (2.5 g/L, VWR Chemicals, Radnol, Pennsylvania, USA) and pancreatin (0.9 g/L, ProDigest) in autoclaved water. Bile (Oxgall, 4.0 g/L, ProDigest) was added.

Table 8: Composition of the culture medium.

Ingredients	Lactation culture medium
Mucin (Sigma-Aldrich, St-Louis, Missouri, USA)	6.0 g/L
Proteose-Peptone n°3 (BD Bacto Biosciences, Franklin Lakes, New-Jersey, USA)	1.0 g/L
Potato starch (Sigma-Aldrich, St-Louis, Missouri, USA)	1.0 g/L
L-Cysteine hydrochloride (Merck, Darmstadt, Germany)	0.2 g/L
Nuklospray Yogurt[1] (Dumoulin, Andenne, Belgium)	8.0 g/L

g/L: grams per liter. [1] Commercial complementary milk replacer feed for piglets containing, among others, whey powder, vegetable oils and wheat flour.

Equipment. SHIME® equipment (ProDigest Bvba, Gent, Belgium) was used for this study. The classic set-up was modified following the baby-SPIME model described in the works of (Dufourny et al., 2019). Briefly, the cabinet was divided into two independent units each containing three double-jacketed bioreactors linked to a hot-water bath. As illustrated in Figure 15, bioreactor 1–not inoculated–simulated the stomach and duodenum/jejunum digestion; bioreactors 2 and 3–inoculated–simulated the functions of an ileum and a proximal colon respectively. The first half cabinet was dedicated to the inocula prepared with real intestinal content and the second half cabinet for inocula prepared with feces. All components were connected to a computer designed to standardize the different parameters of the system (temperature, pH, transfer time). The pumps provided the transfer of the culture media, pancreatic juice, bile, acid (HCl 0.5 M), base (NaOH 0.5 M) and all the fermentation liquids from one bioreactor to another during a complete run. Manual quality controls were regularly performed to check the parameters and samples were taken three times a week at fixed intervals (days and times).The feeding cycles were scheduled three times a day based on a total retention time of 14 h. During each cycle, culture media (140 mL, flow rate: 4.67 mL/min), maintained at 4 °C, flowed into bioreactor 1 for 1 h 30 min. Then, pancreatic juice/oxgall (60 mL, flow rate: 4.00 mL/min), also maintained at 4 °C, was added to the same bioreactor for 1 h; pH in bioreactor 1 was considered being at 6.8. After this time, and simultaneously, the content of bioreactors 1, 2 and 3 was programmed to flow into bioreactors 2, 3 and a waste, respectively. The flow rate of 3.50 mL/min was calculated to serve two purposes: to empty bioreactor 1 (200 mL to 0 mL); and to obtain a residence time of 14 h (4 h in the ileum bioreactors–constant volume of 100 mL–and 10 h in the colon bioreactors–constant volume of 250 mL). The anaerobic condition of all bioreactors was maintained by flushing with nitrogen (N_2) once a day for 10 min. They were continuously stirred (300 rpm) and kept at 39.5 °C. The pH of bioreactors 2 and 3 was continuously monitored by pH controllers maintaining pH ranges of [6.40‑6.60] in bioreactor 2 (ileum) and [5.80‑6.05] in bioreactor 3 (proximal colon) by using NaOH (0.5 M) or HCl (0.5 M). Two runs were managed (two different donors). Every run lasted 2 weeks for stabilization of the microbiota into a simulated lactation phase.

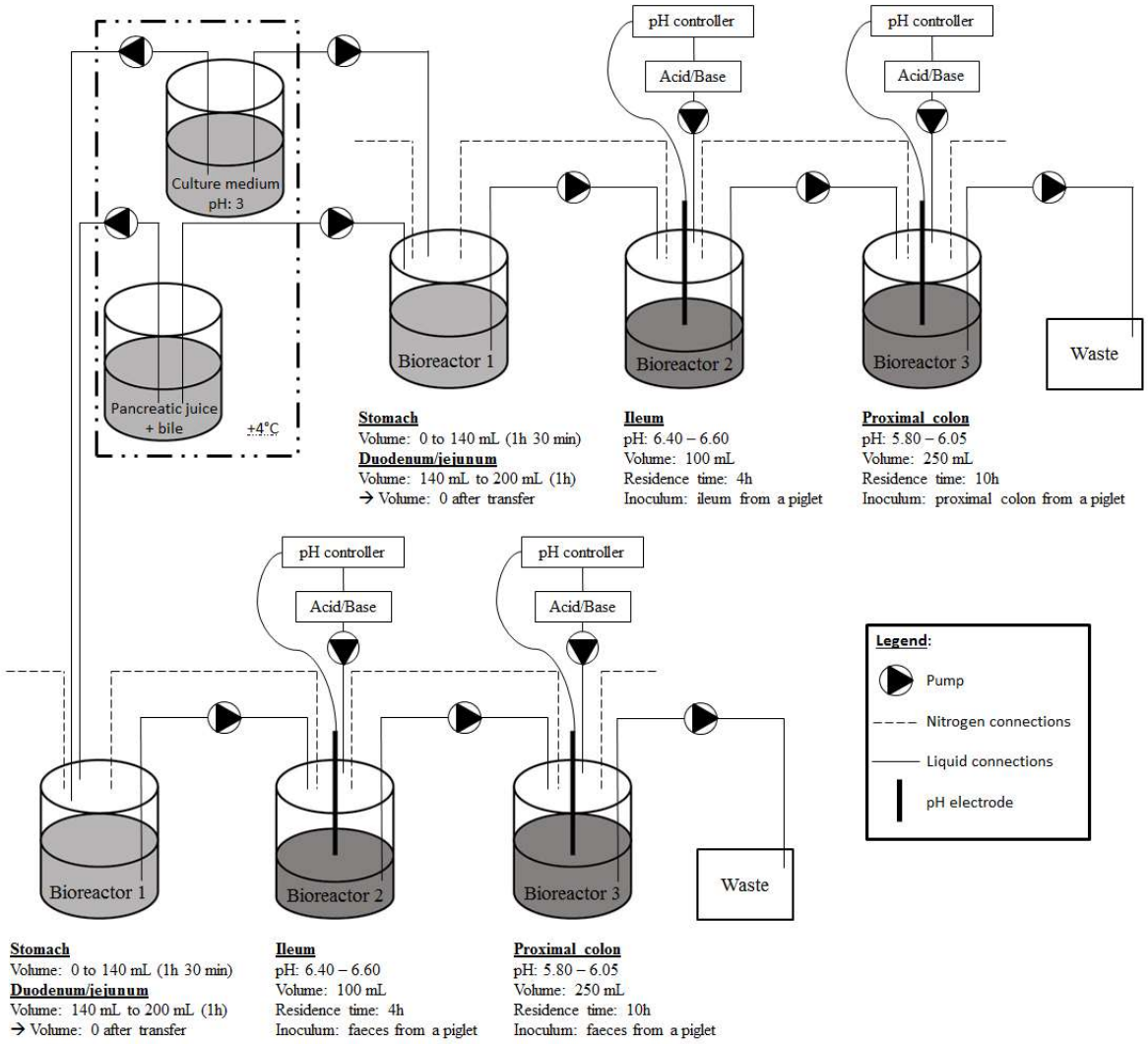

Figure 15: Schematic representation of the baby-SPIME model used in the study (one run). The model consisted of three double-jacketed bioreactors (bioreactor 1: stomach/duodenum/jejunum, bioreactor 2: ileum and bioreactor 3: proximal colon). Three times a week, the culture medium and the pancreatic juice+bile entered bioreactor 1, one after the other, through liquid connections controlled by pumps. This was done following the instructions given in the figure. Then liquid formed by medium/pancreatic juice/bile was made to flow simultaneously towards the ileum and proximal colon until reaching a biological container following the instructions of the figure. The system was flushed once a day with nitrogen (N_2) through the air connection system. The bioreactors were constantly stirred and kept at 39.5 °C. Ileum and colon pH were continuously checked and adjusted to the fixed pH ranges. Inocula were prepared with real intestinal content, or feces of a single piglet (one run). They were introduced in the corresponding bioreactor, real intestinal content in parallel with feces.

Sample collection. A 9 mL sample–for each sampling time point–was taken 3 times a week at fixed intervals of days and times (before adding the culture medium) from the ileum and proximal colon bioreactors. It was done from the beginning to the end of the run in order to standardize the sampling all along the run. Each collected sample was subdivided as follows: 2 mL for microbial metabolites analysis and one mL for high throughput sequencing analysis. The samples were centrifuged for 2 min at 17,000g to collect the pellet and immediately stored at −20 °C before performing analysis. All samples were

analyzed for microbial metabolites in the supernatant because the concentration of the metabolites (short chain fatty acids) detected in the samples was used to monitor the system, ensuring that the microbiota was well stabilized for the last day of the lactation phase. The last sample of the lactation phase was used for high throughput sequencing analysis.

16S rRNA gene sequencing. DNA extraction and sequencing of all the samples were performed by DNA Vision (Gosselies, Belgium) following their internal Standard Operating Procedure. DNA was extracted from frozen pellets with the DNeasy Blood & Tissue kit according to the Qiagen manufacturer's instructions (Qiagen Benelux B.V., Venlo, The Netherlands). The DNA was quantified and qualitatively assessed on a NanoDrop 2000 from Thermo Scientific™ and by PicoGreenVICTOR X3 (PerkinElmer) using the Quant-it PicoGreen dsDNA Assay kit from Invitrogen. The 16S targeted region V3-V4 was amplified by PCR, purified and tagged. Libraries were indexed using the NEXTERA XT Index kit V2 from Illumina. The high throughput sequencing was carried out on Illumina Miseq in paired-end sequencing (2x250bp) by targeting an average of 10,000 reads per sample. Finally, the bioinformatic analysis was executed with the QIIME (Quantitative Insights Into Microbial Ecology) software, version 1.9.0 with "Greengenes 13_8" as database and recommended parameters to use QIIME scripts. The OTU (Operational Taxonomic Unit) table was generated based on a 97% sequence similarity of the sequencing reads to cluster OTUs. Only samples presenting more than 5,000 reads were used for taxonomic analysis. Similarly, samples with the same normalized number of reads were used for the Beta diversity analysis.

Results

Microbiota of the samples. The microbiota composition of all the samples of the study is given in Figure 16. The left side of Figure 16 represents the ileal, colonic and fecal microbial composition of the inocula used to inoculate the baby-SPIME bioreactors; in the middle of Figure 16 are the results for ileum bioreactors (inoculated with ileum inocula or feces inocula); at the right side of Figure 16 are the results for colon bioreactors (inoculated with colon inocula or feces inocula).

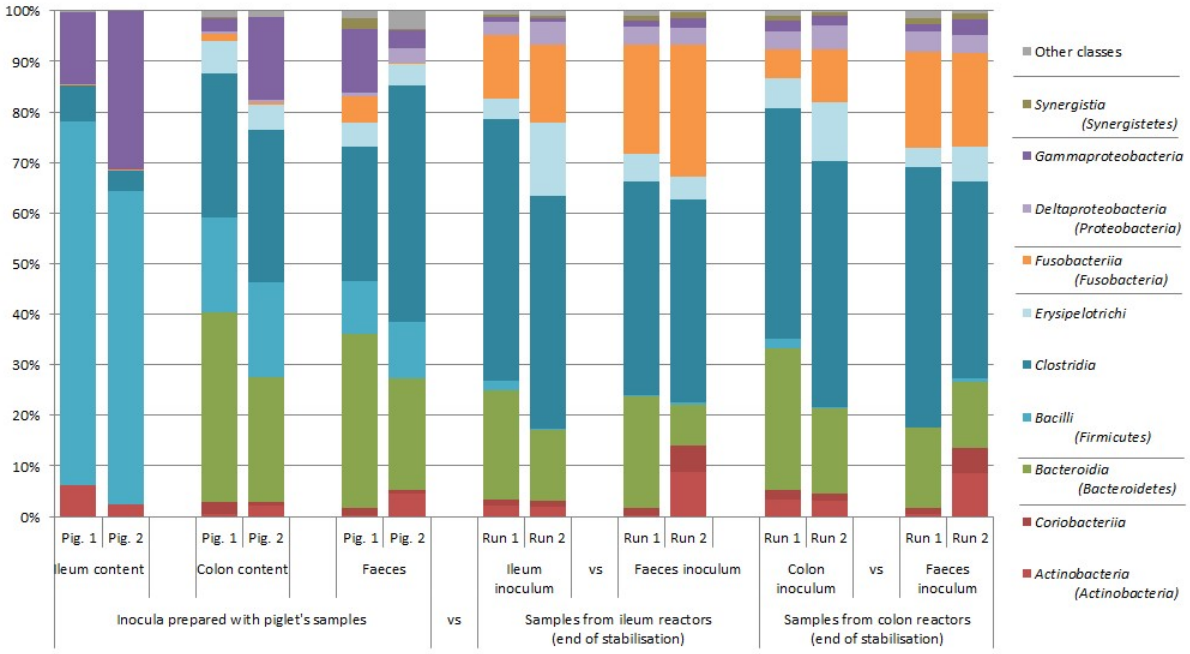

Figure 16: Composition (in relative abundance, %) of the microbiota present in the inocula, in the ileum bioreactors and colon bioreactors. Classification is at the class level, with phylum between brackets. Samples coming from piglets (pig.) were used to prepare the inocula: pig. 1 for run 1, pig. 2 for run 2.

Microbiota present in the inocula (piglet's samples). Ileum microbiota was characterized by high relative abundances of Firmicutes (especially the Bacilli class; blue colors in Figure 16) and Proteobacteria (the Gamma-Proteobacteria class; purple in Figure 16). Together, they represented more than 90% of the samples. *Lactobacillus sp.* and *Streptococcus sp.* were the two dominant genera of the Bacilli class in the samples (data not shown; 47.1% and 16.9% respectively in the ileum samples from piglet 1; 49.4% and 9.0% in the samples from piglet 2).

Proximal colon microbiota was much more similar to feces than ileum microbiota, containing at least a quarter of Bacteroidetes from Bacteroidia class.

Feces microbiota contained the highest levels of relative abundance of bacteria from the Clostridia, Bacteroidia and Bacilli classes.

Microbiota present in the ileum bioreactors. After two weeks of stabilization of the baby-SPIME, the observed profile of bacteria present in the ileum bioreactors was closer to colon content or feces of piglet's samples than piglet's ileum content. This was observed independently of ileal or fecal inoculum. This was mainly due to the presence of Bacteroidia. In addition, Gamma-Proteobacteria was less represented in bioreactors while Fusobacteriia was more represented.

Several differences in microbiota between bioreactors inoculated with ileal or fecal inoculum were observed during the 2 runs. Especially, bioreactors inoculated with ileum inoculum presented more Firmicutes than bioreactors inoculated with feces inoculum (mean=59.2% *vs* 46.5% including 1.0% of Bacilli *vs* 0.3%) and less Fusobacteriia (14.0% *vs* 23.9%) as well as Proteobacteria (mean=4.7% *vs* 5.6% with Gamma-Proteobacteria: 0.7% *vs* 1.6%).

Microbiota present in the proximal colon bioreactors. After two weeks of stabilization of the microbiota in the baby- SPIME, colon bioreactors were deficient in Bacteroidia and Bacilli, and enriched in Fusobacteriia and Clostridia compared to in vivo colonic samples.

Several differences in microbiota between bioreactors inoculated with colon or fecal inoculum were observed. Especially, bioreactors inoculated with colon inoculum presented lower abundance of Fusobacteriia (mean=8.1% inoculum colon *vs* 18.8% inoculum feces) and higher abundance of Bacteroidia (mean=22.5% inoculum colon *vs* 14.5% inoculum feces). Especially for this one, bacteria from *Prevotella sp*. were more abundant in bioreactors inoculated with colon inoculum instead of feces (piglet 1: 21.9% in "inocula colon content", 23.9% in bioreactor "colon inoculum" *vs* 13.5% in bioreactor "feces inoculum"; piglet 2: 13.1% in "inocula colon content", 15.2% in bioreactor "colon inoculum" *vs* 10.4% in bioreactor "feces inoculum").

Oxygen tolerance of the microbiota from the ileum inocula and ileum bioreactors. The bacteria present in the ileum or in the different ileal bioreactors were graded in Table 9 considering a gradient in their tolerance of oxygen. Interestingly, in ileal content of piglet, more than 90% of the bacteria, in relative abundance, were classified in the categories of obligate aerobe to facultative anaerobe, including the microaerophilic, nanaerobic or aerotolerant bacteria. It was in contrast to what was observed in the bioreactors at the end of the stabilization period. More than 90% of these bacteria were classified in the categories of anaerobe to obligate anaerobe.

Table 9: Relative abundances of bacteria (in vivo *vs* in vitro samples) following their use of oxygen.

	Run 1			Run 2			Bacteria (reference)
	Ileum content	Reactor content		Ileum content	Reactor content		
	Piglet 1	Inoc. feces	Inoc. ileum	Piglet 2	Inoc. feces	Inoc. ileum	
From obligate aerobe…							
(Obligate) aerobe	0.7%	1.4%	1.3%	0.1%	0.0%	0.0%	*Achromobacter* (Garrity, 2005), *Agrobacterium* (Garrity, 2005), Alcaligenaceae* (Garrity, 2005), *Comamonas* (Garrity, 2005), *Dietzia* (Whitman et al., 2012), *Ochrobactrum* (Garrity, 2005), *Oligella* (Garrity, 2005), *Pseudomonadacea** (Garrity, 2005), *Psychrobacter* (Garrity, 2005), *Stenotrophomonas* (Garrity, 2005)
Strictly or facultative aerobe	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%	Planococcaceae *("Other" genus)* (De Vos et al., 2009), *Sporosarcina* (De Vos et al., 2009)
Aerobe or facultative anaerobe	20.0%	0.9%	0.1%	33.3%	1.2%	0.2%	*Actinobacillus* (Garrity, 2005), *Corynebacterium* (Whitman et al., 2012), Enterobacteriaceae* (Garrity, 2005), *Lysinibacillus* (Ahmed et al., 2007), Pasteurellaceae *("Other" genus)* (Garrity, 2005)
Nanaerobe, Microaerophilic, Aerotolerant	0.1%	0.2%	0.0%	0.0%	0.6%	2.2%	*S24-7** (Ormerod et al., 2016), *Sutterella* (Garrity, 2005), *Trichococcus* (De Vos et al., 2009)
Facultative anaerobe	71.3%	0.1%	1.9%	61.7%	0.9%	0.4%	*Aerococcacea** (De Vos et al., 2009), *Aerococcus* (De Vos et al., 2009), *Citrobacter* (Garrity, 2005), *Enterococcus* (De Vos et al., 2009), *Facklamia* (De Vos et al., 2009), *Jeotgalicoccus* (De Vos et al., 2009), *Klebsiella* (Garrity, 2005), *Lactobacillus* (De Vos et al., 2009), *Streptococcus* (De Vos et al., 2009), *Weissella* (De Vos et al., 2009)
Subtotal:	**92.3%**	**2.6%**	**3.3%**	**95.1%**	**2.7%**	**2.8%**	
… To obligate anaerobe							
Strictly or facultative anaerobe	0.0%	0.5%	1.2%	0.2%	2.7%	0.9%	Coriobacteriaceae (Whitman et al., 2012), Peptostreptococcaceae* (De Vos et al., 2009)
Anaerobe	3.1%	31.9%	34.9%	0.2%	24.4%	21.3%	*Acidaminococcus* (De Vos et al., 2009), *Bacteroides* (Krieg et al., 2010), *Bifidobacterium* (Whitman et al., 2012), *Bilophila* (Garrity, 2005), *Blautia* (Liu et al., 2008), Dethiosulfovibrionaceae* (Bhandari and Gupta, 2012), Lachnospiraceae**& "Other" genu)* (De Vos et al., 2009), *Oscillospira* (Gophna et al., 2017), *Prevotella* (Krieg et al., 2010), *Pyramidobacter* (Bhandari and Gupta, 2012), *Succiniclasticum* (De Vos et al., 2009), *Synergistaceae ("Other" genus)* (Bhandari and Gupta, 2012), *Turicibacter* (De Vos et al., 2009), *Veillonella* (De Vos et al., 2009)
Strictly/Obligate anaerobe	1.7%	63.5%	58.0%	0.7%	68.5%	70.5%	*Anaerococcus* (De Vos et al., 2009), *Anaerovibrio* (De Vos et al., 2009), *Bulleidia* (De Vos et al., 2009), *Catenibacterium* (De Vos et al., 2009), *Clostridium* (De Vos et al., 2009), *Collinsella* (Whitman et al., 2012), *Desulfovibrio* (Garrity, 2005), *Dorea* (De Vos et al., 2009), *[Eubacterium]* (De Maesschalck et al., 2014), *Fusobacterium* (Krieg et al., 2010), *Megasphaera* (De Vos et al., 2009), *Mitsuokella* (De Vos et al., 2009), *Parabacteroides* (Sakamoto and Benno, 2006), *Phascolarctobacterium* (De Vos et al., 2009), *Roseburia* (De Vos et al., 2009), Ruminococcaceae* (De Vos et al., 2009), *Ruminococcus* (De Vos et al., 2009), *Sharpea* (Morita et al., 2008), *Succinivibrio* (Garrity, 2005)
Subtotal:	**4.8%**	**95.9%**	**94.1%**	**1.1%**	**95.6%**	**92.7%**	
Not defined	**2.9%**	**1.5%**	**2.6%**	**3.8%**	**1.7%**	**4.5%**	

* : undefined genus; Inoc. feces: bioreactor inoculated with feces inoculum; Inoc. ileum: bioreactor inoculated with ileum content inoculum.

Discussion

The microbial composition of the inocula prepared with piglet's samples was in accordance with the literature. For the ileum inocula, a dominance of Firmicutes and Proteobacteria was observed, with this last one being in the expected range − 5% to 40% of total microbiota (Isaacson and Kim, 2012). Among the Firmicutes, a high abundance of the Bacilli class is described, followed by Clostridia (De Rodas et al., 2018), as observed here. For the colon inocula, Firmicutes and Bacteroidetes were dominant phyla of bacteria in the samples. According to previous studies, in terms of classes of bacteria, the trio Clostridia (Firmicutes), Bacteroidia (Bacteroidetes) and Bacilli (Firmicutes) was well dominant (De Rodas et al., 2018). For the feces inocula, samples were rich in Firmicutes and Bacteroidetes (Isaacson and Kim, 2012) and the profile of feces microbiota was also more similar to the microbiota of the colon compared to the one of the ileum (Zhao et al., 2015).

In ileum bioreactors, after 2 weeks of stabilization, there were reduced relative abundances of Bacilli and Gamma-Proteobacteria and there was an increase of Clostridia, Fusobacteriia and Bacteroidia, compared to the ileum inoculum. In the colon bioreactors, there was a reduced relative abundance of Bacilli, Gamma-Proteobacteria and Bacteroidia and there was an increase of Clostridia and Fusobacteriia compared to the colon inoculum. When inoculating bioreactors with intestinal inocula instead of feces inocula, it was hypothesized that favorable differences would be highlighted, improving the model. Fusobacteriia were less abundant in the bioreactors at the end of the stabilization period when the inoculum was prepared with intestinal content; it was a first positive observation because the relative abundance of Fusobacteriia in baby-SPIME model–as described in Dufourny et al. (2019)–was too high compared to in vivo data. In addition, more Bacteroidia were maintained in the colon bioreactors and that constituted another positive observation because the relative abundance of Bacteroidia in the colon of baby-SPIME model was weaker taking into account the relative abundance expected in the intestine of piglets. *Prevotella sp*., as an important representative of the Bacteroidia phylum in swine (Holman et al., 2017), presented improved relative abundances with intestinal colon content inoculum. However, Bacteroidia were also maintained with high relative abundance in the ileum bioreactors. This observation was not expected because Bacteroidia were not detected by high throughput sequencing in the ileum inoculum and so they should not grow to that extent in the bioreactors. For Bacilli, these were not abundant enough especially in the ileum; there was a slight increase effect on the average value for the two runs (from less than 0.5% in fecal inoculum to 1.0% in intestinal inoculum). But it didn't live up to our expectations. Finally, regarding Gamma-Proteobacteria, these seemed to be better maintained with a fecal inoculum for these two runs.

In view of the limiting impact of the kind of inoculum on the microbial profiles in the bioreactors, a reflection was made about the bacterial environment in the system. Firstly, *Lactobacillus sp*.–so important in the ileum (Pieper et al., 2008) and probably in feed strategy (Guevarra et al., 2018)–

was not as much present as expected. Secondly, *Streptococcus sp*. (Su et al., 2008)–so important for health (Ferrando and Schultsz, 2016)–was also scarce in the ileum bioreactors; leading to the situation that these two facultative anaerobes were barely present and they did not sufficiently contribute to the ecosystem that was established in vitro. All the bacteria present in the ileum inocula and in the bioreactors dedicated to ileum were then classified into different categories based on the need or not of oxygen, following a gradient from obligate aerobiosis to obligate anaerobiosis. The gap between the high relative abundance of bacteria able to use oxygen in piglet's ileum inocula (more than 90% of the inocula) that was not able to set up in ileal bioreactors (less than 3.5%) became evident. In addition, it also became evident that the presence of colonic bacteria such as *Mitsuokella sp*. or *Ruminococcus sp*. in ileum bioreactors was probably due to the lack of facultative aerobic/anaerobic bacteria. By flushing bioreactors with nitrogen and by adding reducing agent in culture media, sufficiently anaerobiosic conditions for the in vitro gut microbiota was assured; the opposite could have been a criticism of the model (Van den Abbeele et al., 2010). But it probably disturbed the introduction of an ileum microbiota in the dedicated bioreactor. Indeed, the microbiota of pigs evolves from the mouth to the end of the colon with dominant aerobes or facultative anaerobes in the small intestine *vs* anaerobes in the colon (Zhao et al., 2015). Evidence piles up in the literature to demonstrate a gradient in oxygen in the gut from a longitudinal (Friedman et al., 2018; Morris and Schmidt, 2013; Zheng et al., 2015) and a radial (Albenberg et al., 2014; Morris and Schmidt, 2013; Zheng et al., 2015) point of view. It is known that the intestinal tract of mammals presents a microoxic zone along the mucosa and that its impact on bacteria was underestimated (Morris and Schmidt, 2013). The richness in oxygen of the proximal small intestine may be explained by multiple factors (vascularisation of the tissue, presence of villosities, liquid chile and pancreatico-biliary secretions) (Friedman et al., 2018). This level would deplete until the cecum due to the consumption of oxygen by aerotolerant bacteria (Albenberg et al., 2014) and by oxidative processes (e.g. lipid oxidation) (Friedman et al., 2018). The growth of the bacteria in their respective niches and their interactions would be explained by this oxygen availability in the ileum compared to more anoxic conditions in the colon (Crespo-Piazuelo et al., 2018). To quote Friedman et al. (2018): "The biomass of the oxygentolerant bacteria in luminal contents determines the level of oxygen in the intestinal luminal environment". Baby-SPIME did probably not offer sufficient microoxic conditions in the bioreactors. This parameter had probably a significant impact on the microbial profile in the bioreactors. Surprisingly, the effect could be more significant than the type of inoculum itself.

Avoiding the incorporation of a reducing agent in the medium is probably a prerequisite for the improvement of the baby-SPIME model, as seen in a batch model (Poelaert et al., 2012). The microbiota in the ileum bioreactor at the end of the stabilization period will probably benefit from the less anoxic conditions offered by the modified culture medium. However, there is a risk that other bacteria such as *Desulfovibrio sp*. that require a reducing agent in the media for its growth (Garrity, 2005) would be penalized. An improvement for the ileum microbiota can induce degradation for the colon microbiota and it should be evaluated before any protocol modification.

A second way for improvement certainly lies in management of the baby-SPIME atmosphere. The nitrogen-flushing actions that are applied to maintain anaerobic gastro-intestinal conditions could be adapted taking into account the need of oxygen in the ileum bioreactor and the know-how of the semi-continuous model of Blake et al. (2003).

In light of the present results and discussion, oxygen seems to play a key role in the ileum bioreactor although other parameters indubitably come under consideration to explain the weakness of the in vitro ileal microbiota (e.g. the content of simple carbohydrates of the culture medium; Lee et al., 2011; Poeker et al., 2019). But it now appears essential to maintain microaerophilic conditions in the ileum of the porcine in vitro dynamic and multi-compartment model–so called baby-SPIME. More generally, considering that this oxygen-modulated microbiota profile is found not only in pigs (Hillman, 1998) but also in other animals such as the mouse (Gu et al., 2013) and considering that the pig is also studied for human issues (Freeman et al., 2012; Guilloteau et al., 2010); while keeping in mind that the major pathogens of human intestine are facultative anaerobes for which the oxygen seems to play a key role in their virulence (Marteyn et al., 2011), this reflection on the need of oxygen in the ileal bioreactor may probably also be applied for human and other animal in vitro dynamic and multi-compartment models using an inoculated small intestine bioreactor.

Conclusions

The aim of the study was to determine the added-value of inoculating an in vitro multi-compartment gastro-intestinal piglet model with intestinal content instead of feces. Results showed positive aspects in terms of Fusobacteriia abundances in the ileum and colon bioreactors, as well as Bacteroidetes in the colon bioreactor. Results were more reserved for Proteobacteria and Bacilli abundances in the ileum bioreactor. In addition, our results also showed that anoxic conditions in the ileum bioreactor influenced the microbial profile more than the type of inoculum itself, leading to the conclusion that in vitro dynamic and multicompartment models including an ileal microbiota probably need to get oxygenated to improve microbiome studies of the small intestine.

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Data availability

Raw sequences can be found on the EMBL Nucleotide Sequence Database (ENA–European Nucleotide Archive) under the project accession number PRJEB34273.

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Study 4 -

Apple pomace modulates the microbiota and increases the propionate ratio in an in vitro piglet gastrointestinal model

Fermentation 8 (2022) 408

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Abstract

Apple pomace (AP) contains biomolecules that induce changes in intestinal fermentations of monogastric with positive expected health effects. The weaning of piglets can induce economic losses due to intestinal disturbances; new weaning strategies are, thus, welcome. The purpose of this study was to test the effect of AP on fermentation products by using baby-SPIME, an in vitro multicompartment model dedicated to piglet weaning. A comparison was done on short chain fatty acid (SCFA) ratio and the microbiota induced in bioreactors between a control culture medium vs. an AP culture medium. The results of 2 preliminary runs showed that AP medium increased the molar ratio of propionate ($p = 0.021$) and decreased the molar ratio of butyrate ($p = 0.009$). Moreover, this medium increased the cumulative relative abundance of *Prevotella* sp. and *Akkermansia* sp. in bioreactors. AP could promote an ecosystem enriched with bacteria known as next-generation probiotics (NGP)—likely influencing the energy metabolism of piglets by their fermentation metabolites. AP could be used as a dietary strategy to influence bacterial changes in the intestine by stimulating the growth of bacteria identified as NGP.

Introduction

Apple and its by-product apple pomace (AP)—obtained after extraction of juice—have long been studied because of their components of high interest such as dietary fibers, phenolic and terpenic compounds (Boyer and Liu, 2004; Grigoras et al., 2013; Lu and Foo, 1997; Reis et al., 2014; Waldbauer et al., 2017). At present, scientists are trying to find the best ways of finding value for AP, given how promising this co-product appears in the context of a circular economy (Awasthi et al., 2021; Okoro et al., 2021). In the food and feed sectors, AP remains poorly used (Lyu et al., 2020); however, this could change with -omic technological advances (Sabater et al., 2021). AP is already known to influence the intestinal fermentation of monogastrics. In rats, this ingredient increased intestinal fermentation and resulted in positive antioxidant health effects as well as the reduction of blood glucose levels (Juśkiewicz et al., 2012). In piglets, it increased the number of total colonic bacteria as well as the number of *Lactobacilli* in feces, and these effects were also observed in the gut morphology, blood parameters and gene expression of immunological markers (Sehm et al., 2011, 2007). AP has also been tested in an in vitro batch model inoculated with feces from pre-weaned piglets (Uerlings et al., 2020). Results suggested that the AP matrix offers a certain fermentative potential, but, in a panel of by-products, AP was not classed at the top of the ranking possibilities.

However, oligosaccharides derived from apple pectin have also been shown to have strong bacteriostatic effect against *Escherichia coli* and *Staphylococcus aureus* (Martinov et al., 2017). Biomolecules present in the apple skins—triterpenic or polyphenolic compounds such as ursolic acid and phloridzin—are known to be effective against pathogens (Waldbauer et al., 2017). This is of particular interest for piglets at weaning, considering that weaning induces multiple stressors (Pluske et al., 2003; Weary et al., 2008) that lead, among others, to immediate intestinal and immune disorders and may impact longterm performance and health (Campbell et al., 2013; Niewold, 2015). For the swine industry, there is an economic impact associated to post-weaning diarrhea (PWD)—commonly associated with enterotoxigenic *Escherichia coli* infection (Rhouma et al., 2017). Prebiotics and probiotics are major strategies against PWD (Gresse et al., 2017).

For the above-mentioned reasons and due to the nature of its components, AP appears as an interesting matrix to study in a multi-compartments gastrointestinal model in an attempt to decipher the matrix–microbiota interaction. The baby-SPIME (Simulator of Pig Intestinal Microbial Ecosystem) model was previously developed to study in vitro dietary weaning strategies for piglets (Dufourny et al., 2019). This model was used to study the effects of AP on short chain fatty acid (SCFA) production and on the microbiota of piglets after weaning.

Material and methods

Equipment, inocula, culture media and sample collection. The baby-SPIME model, requiring the use of the SHIME® equipment (ProDigest Bvba, Gent, Belgium), was used for the experiment (Dufourny et al., 2019). One-half cabinet of 3 bioreactors (stomach, inoculated pre-colon and inoculated colon) was used as control and one-half cabinet was dedicated to test AP (Figure 17). The timeline for 1 run included 2 weeks of the lactation phase, a weaning step (modification of the culture medium) and 2 weeks of the post-weaning phase. Two runs were managed with two different donors.

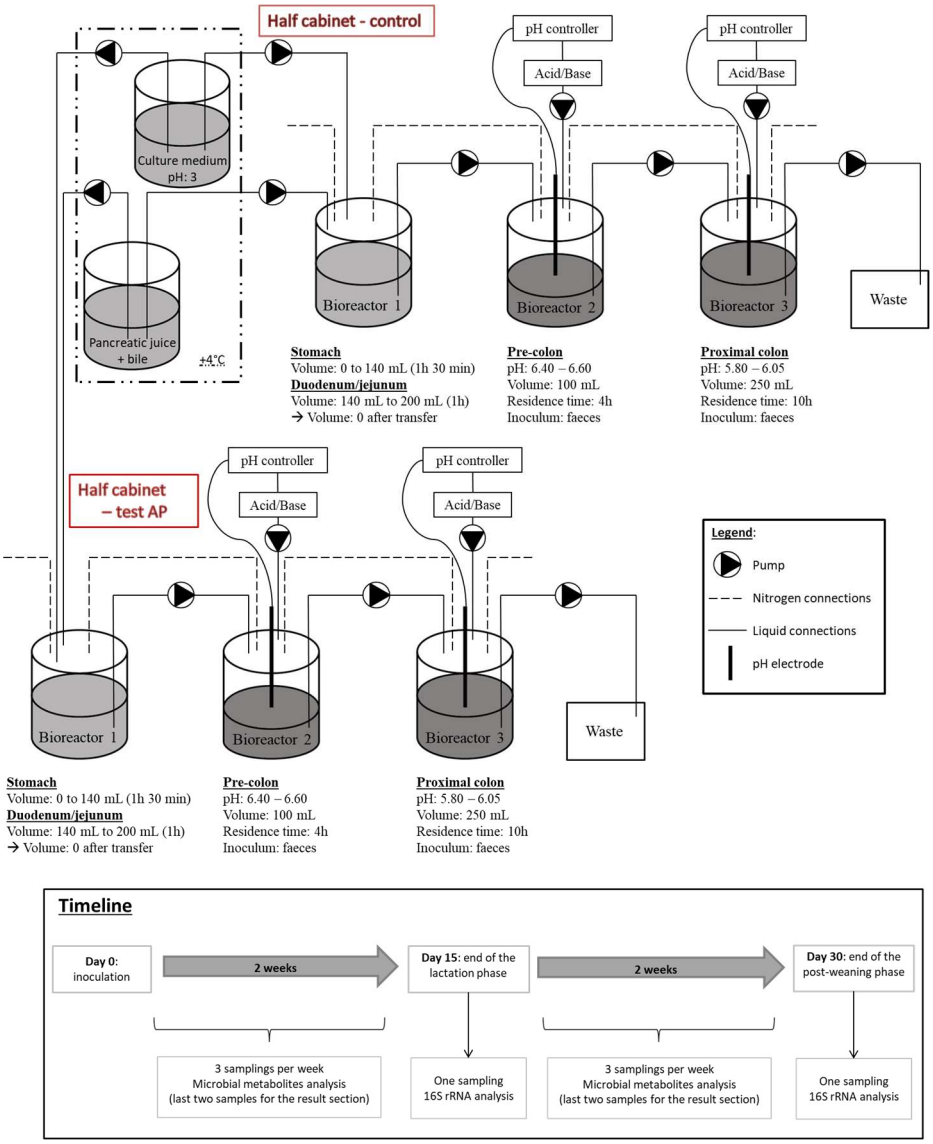

Figure 17: Baby-SPIME model: a SHIME equipment divided into two half cabinets of three double-jacketed bioreactors receiving the culture media three times a day. Bioreactor 1 with medium (stomach digestion) received pancreatic juice and bile (duodenum/jejunum digestion) following instructions given in the figure. Then the liquid flowed simultaneously toward the pre-colon and proximal colon until a waste container. Bioreactors 2 and 3 were inoculated with feces from one piglet–two different piglets for the two runs. The timeline of the run is described.

The donors were two [Piétrain×Landrace] suckling piglets (27 days old) free of antibiotics. Faeces were used to prepare the inocula for the study as previously described (Dufourny et al., 2019). The two samples were taken at the same farm, at the same time, in two different litters. They were kept on ice under anaerobic conditions during transportation. A single donor was used to prepare the inoculum for a run. A single inoculum was prepared for both pre-colon and colon bioreactors for each run.

Three different culture media were prepared (Table 10): 1° lactation culture medium, 2° post-weaning culture medium and 3° post-weaning culture medium with AP (Extr'Apple SAS, Pleudihen-sur-Rance, France). Culture media and pancreatic juice were prepared as previously described (Dufourny et al., 2019).

Samples from pre-colon and proximal colon bioreactors were taken 3 times a week at fixed intervals of days and times for microbial metabolites analysis (2 mL) and/or 16SrRNA sequencing (1 mL) as presented in the timeline of Figure 17. Samples were centrifuged (2 min at 17,000g). For the microbial metabolite analysis, the supernatants were collected and filtered (0.45 µm). For the sequencing, the pellets were collected. Supernatants and pellets were stored at −20 °C until analyses.

Table 10 : Composition of the culture media.

Ingredients	Lactation CM	PW Control CM	PW AP CM
Mucin (Sigma-Aldrich, St-Louis, MO, USA)	6.0 g/L	6.0 g/L	6.0 g/L
Proteose-Peptone n°3 (BD Bacto Biosciences, Franklin Lakes, NJ, USA)	1.0 g/L	1.0 g/L	1.0 g/L
Potato starch (Sigma-Aldrich, St-Louis, MO, USA)	1.0 g/L	1.0 g/L	1.0 g/L
L-Cysteine hydrochloride (Merck, Darmstadt, Germany)	0.2 g/L	0.2 g/L	0.2 g/L
Nuklospray Yoghurt[1] (Dumoulin, Andenne, Belgium)	8.0 g/L	0.0 g/L	0.0 g/L
Post-weaning diet for piglets[2] (ABZDiervoeding, Nijkerk, The Netherlands)	0.0 g/L	8.0 g/L	8.0 g/L
Apple pomace (Extr'Apple SAS, Pleudihen-sur-Rance, France)	0.0 g/L	0.0 g/L	0.65 g/L

g/L: grams per litre, CM: culture medium, AP: apple pomace, PW: post-weaning. [1] Commercial complementary milk replacer feed for piglets containing, among others, whey powder, vegetable oils and wheat flour. [2] Grinded to particles of 250 µm Composition: Barley (30.00%), Wheat (14.41%), Maize (5.00%), Oat flakes (5.00%), Toasted soybeans (15.00%), Soya meal (13.87%), Potato protein (2.00%), Bread flour (5.00%), Soya oil (0.36%), Fat filled whey powder (4.67%), Chalk (1.05%), Monocalciumphosphate (1.01%), Salt (0.54%), Methionine (0.16%), L-lysine HCL (0.47%), L-threonine (0.11%), Lysine/tryptophan mix (0.02%), Flavoring (0.20%), Vitamins (0.40%), Start/BL.15CU (premix containing Cu, Fe, Zn, Mn, Se, I and vitamins A, B2, B3, B5, D3, E, K3; 0.40%), Phytase (0.33%).

Microbial metabolites. Samples were analyzed for their SCFA content as previously described (Douny et al., 2019) by SPME-GC-MS. The protocol included a step of SCFA extraction with an SPME fiber, a step of separation on a Supelcowax-10 column (30 m x 0.25 mm, 0.2 µm; Supelco, Bellefonte, PA, USA) on a Focus GC gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) and a step of analysis with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific,Waltham, MA,

USA). The extraction temperature and time were 60°C and 20 min. The results (mg/L) were converted into mmol/L. Ratios C2:C3:C4 were then calculated.

16S rRNA gene sequencing. DNA extraction and sequencing (DNA Vision, Gosselies, Belgium) was done using the DNeasy Blood & Tissue kit (Qiagen Benelux B.V., Venlo, The Netherlands), a NanoDrop 2000 (Thermo Scientific™,Waltham, MA, USA) and PicoGreenVICTOR X3 (PerkinElmer, Waltham, MA, USA) for the quantitative and qualitative assessments of the DNA, using the Quant-it PicoGreen dsDNA Assay kit from Invitrogen. The V3–V4 region was amplified by PCR, purified and tagged. The NEXTERA XT Index kit V2 from Illumina was used to index libraries. The high throughput sequencing was carried out on Illumina Miseq in paired-end sequencing (2 _ 250 bp) by targeting an average of 10,000 reads per sample. QIIME2 (Bolyen et al., 2019) was used for the bioinformatics analysis. Demultiplexed paired-end sequencing reads were denoised with DADA2 (Callahan et al., 2016) to generate amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using the q2-feature-classifier plugin (Bokulich et al., 2018) together with the Greengenes 13_8 database (Caporaso et al., 2010).

Statistics. A two-way analysis of variance (GLM procedure of Minitab 18, Minitab Inc., State College, Pennsylvania, PA, USA)—including the bioreactor and the culture medium as fixed factors—was also applied to the microbial metabolite results to main relative abundance results of the microbiota. The ANCOM method (Mandal et al., 2015) was used to identify ASVs that were differentially abundant across sample groups at the genus taxonomic rank.

A p-value ≤ 0.05 was significant. A p-value between 0.05 and 0.1 or equal to 0.1 was a trend. A p-value > 0.1 was not significant (ns).

Results

SCFA results. SCFA and molar ratios C2:C3:C4 for pre-colon and proximal colon effluents are given in Table 11. The SCFA results between PW control and PW AP were not statistically different either in the pre-colon or in the proximal colon. However, statistical differences were observed in molar ratios C2:C3:C4. Apple pomace led to an increase in propionate proportion and a decrease in butyrate.

Table 11: SCFA results–and molar ratio C2, C3 and C4–for ileal and colon effluents of baby-SPIME (control *vs* AP).

SCFA	Pre-colon bioreactors		Proximal colon bioreactors		p-value		
	Control (*n*=2)	AP (*n*=2)	Control (*n*=2)	AP (*n*=2)	Bioreactor (B)	Medium (M)	B*M
C2	2019	2146	2157	1685	ns	ns	ns
C3	1126	1352	1200	1165	ns	ns	ns
iC4	38.1	44.5	36.1	44.6	ns	ns	ns
C4	692	579	714	556	ns	ns	ns
iC5	103	98	111	92	ns	ns	ns
C5	556	531	591	494	ns	ns	ns
C6	110	137	166	118	ns	ns	ns
Sum	4644	4887	4975	4155	ns	ns	ns
Ratio C2 (%)	59.3	58.8	59.6	56.1	ns	ns	ns
Ratio C3 (%)	26.9	30.4	27.0	31.4	ns	0.021	ns
Ratio C4 (%)	13.8	10.8	13.5	12.6	ns	0.009	ns

AP: apple pomace, SCFA: short chain fatty acids, C2: acetic acid, C3: propionic acid, iC4: Isobutyric acid, C4: butyric acid, iC5: isovaleric acid, C5: valeric acid and C6: hexanoic acid.

16S rRNA gene sequencing. The alpha-diversity parameters—Shannon observed features, phylogenetic diversity (Figure 18) and evenness—were not influenced by the use of AP.

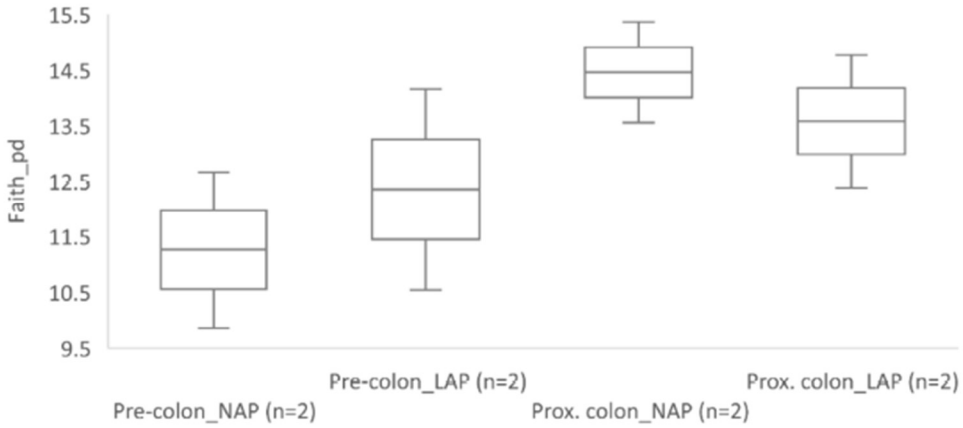

Figure 18 : Faith's phylogenetic diversity observed in the pre-colon and proximal colon bioreactors of baby-SPIME when using AP (LAP) or not (NAP) for two donor piglets.

Results of the taxonomic composition analysis are given in Figure 19 at the phylum level. Compared to the control condition (NAP), the use of apple pomace (LAP) tended to decrease the proportions of Firmicutes and Actinobacteria and to increase those of Bacteroidetes (except in the

pre-colon of piglet 1) and Verrucomicrobia. The only representative of Verrucomicrobia in bioreactors belonged to the *Akkermansia* genus.

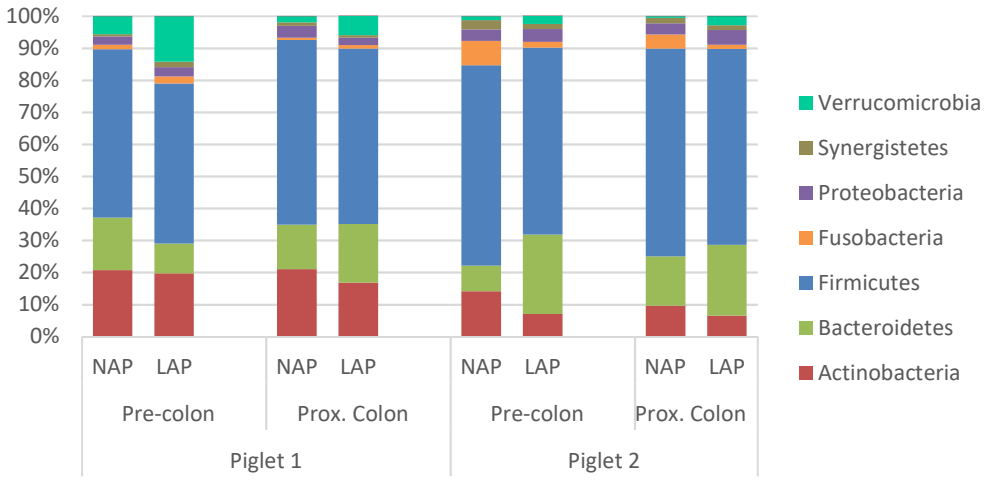

Figure 19: Relative abundances of the phyla observed in the pre-colon and proximal colon bioreactors of baby-SPIME when using AP (LAP) or not (NAP) for two donor piglets.

Relative abundances of the genera observed in the pre-colon and proximal colon bioreactors are presented in Supplementary Material—Figure S1. The ANCOM analysis showed that the *Catenibacterium* genus displayed significantly different abundances across NAP and LAP sample groups (respectively 0.0% vs. 3.6%). The ANOVA analysis showed that the cumulative relative abundance of *Akkermansia* sp. and *Prevotella* sp. Was significantly different across NAP and LAP sample groups (respectively 11.5% vs. 21.2%, p = 0.022). Differences in these microbial genus levels are presented in Figure 20.

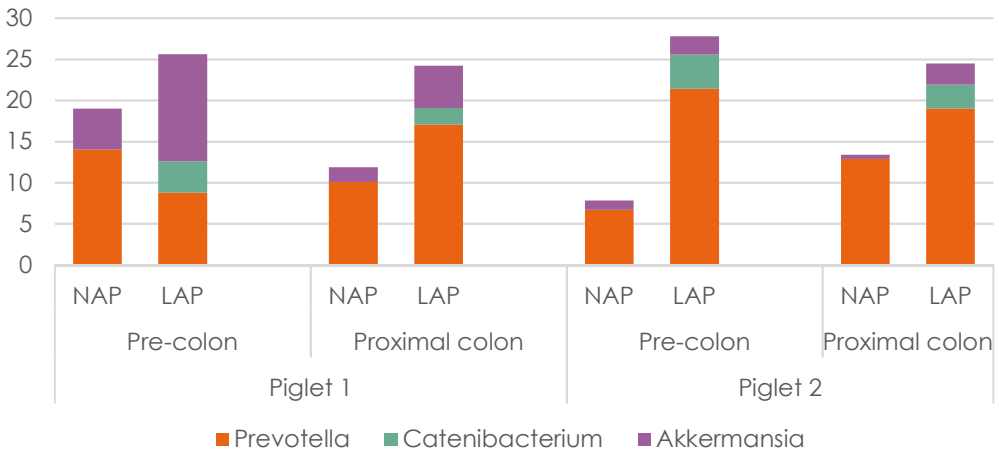

Figure 20 : Relative abundances [%] of three genera observed in the pre-colon and proximal colon bioreactors of baby-SPIME showing significant differences when using AP (LAP) or not (NAP) for two donor piglets.

Discussion

When setting-up a fermentation model, the question arises as to whether or not to pool the feces of individuals. Considering the work of Aguirre and colleagues (Aguirre et al., 2014b), the preparation of a fecal sample pool is suitable when comparing different experimental conditions such as changes in carbohydrate substrate. The results of their study argue in favor of a pool to reduce the effects of inter-individual variability. However, the aim of the present experiment targeted post-weaning diarrhea, for which some piglets' microbiota can be more predisposed than others (Karasova et al., 2021). It is interesting to note the individual particularities, keeping in mind the limitation of the statistics.

AP modified the molar ratios of C2:C3:C4 and the microbiota composition in pre-colon and proximal colon bioreactors. In particular, the proportion of propionate increased when that of butyrate decreased. Although not significant, this molar ratio evolution seemed to originate from a decrease in acetate. Acetate is known to be a common co-substrate for the CoA-transferase route—one of the two metabolic pathways with kinase leading to propionate and butyrate production (Louis and Flint, 2017). As a substrate for the bacterial fermentation and cross-feeding interactions, AP seemed thus to increase this CoA-transferase route in the in vitro piglet model in favor of propionate.

The relative abundance of Actinobacteria and Firmicutes decrease when Bacteroidetes and/or Verrucomicrobia numerically increased in the bioreactors. Observing this dynamic between propionate and the above-mentioned bacteria—independently from AP—is in accordance with the literature. Indeed, in the different microbial metabolic pathways leading to SCFA production in a human study, Bacteroidetes and Verrucomicrobia are known to be users of the succinate pathway leading to propionate production (Louis and Flint, 2017). In pigs, when propionate was directly infused in the caecum, authors showed a decrease of butyrate in the colon, an increase of Bacteroidetes and a decrease of Firmicutes (Zhang et al., 2018).

The effect observed of AP on propionate is also consistent with some observations of studies using rats (Skinner et al., 2018). Concerning a certain fraction of AP, this fraction is included in the diet supplementation of fiber-rich colloid juices from AP in the study of Sembries and colleagues (Sembries et al., 2003). The microflora fermentation increased in the caecum—the main site of intestinal fermentation in rats—leading to significantly higher SCFA, acetate and propionate yields. This fraction seems to be also present in the unprocessed AP used in the study of Juskiewicz and colleagues (Juśkiewicz et al., 2012) observing the results of acetic, propionic and butyric acid concentrations in digesta. This fraction seemed to be active in the baby-SPIME model. Moreover, in a previous in vivo trial on piglets, with the same AP used, an effect on Bacteroidetes was observed with 4% inclusion of AP in a post-weaning diet (Dufourny et al., 2021). In this trial, Bacteroidetes appeared as the second more abundant phylum in the feces of the 4%AP piglets instead of the third most abundant for 0%AP and 2%AP piglets on the 8th post-weaning day. The results showed that baby-SPIME highlighted an

effect of AP on propionate and propionate-producing bacteria, although more runs are required to consolidate the statistics of the study.

Propionate is known to act on the nervous and immune systems (Li et al., 2017)—for example, by increasing the gene expression of NF-κB and IL-18 in pigs (Zhang et al., 2018) or by acting on the regulation of Treg cells, which have immunosuppressive activity and participate in the regulation of intestinal inflammation (Chénard et al., 2020). AP appears in the literature as a matrix with positive immune effect for piglets through the regulation of inflammatory gene expression, e.g., NF-κB, cyclin D1 or caspase 3 (Sehm et al., 2006).

Propionate is also known to act on energy metabolism. Indeed, in humans, propionate exerts beneficial health effects by showing cholesterol-lowering and anti-lipogenic effects and by promoting satiety in the individual (Hosseini et al., 2011; Louis and Flint, 2017). Propionate interacts with gluconeogenesis in the intestine and the liver (De Vadder et al., 2014; Koh et al., 2016). As a particular effect of propionate on the gastrointestinal tract, the fatty acid increases the activity of the glucose-6-phosphatase in the jejunum (De Vadder et al., 2014)—an enzyme that ends the glucogenolysis cycle by releasing glucose. AP appears well in the literature as a matrix with positive effect on metabolic disorders for humans (Skinner et al., 2018). Thus, the results suggest an effect of AP on the regulation of energy metabolism in piglets.

By acting on the immune system and the energy metabolism through SCFA, AP could interact in two important mechanisms of piglets at weaning. The fight against pathogens is also of crucial importance at weaning. In addition, the results suggest that AP promoted the growth of bacteria considered as next-generation probiotics (NGP)—anaerobic gut commensal bacteria suppressing mucosal inflammation (Luo et al., 2021)—such as *Prevotella* sp. or *Akkermansia* sp. (Barbosa et al., 2022; Cani and de Vos, 2017; Chang et al., 2019; El Hage et al., 2020; Langella et al., 2019) that appear of interest for the “health” bio-industry. For example, Karasova and colleagues (Karasova et al., 2021) identified *Prevotella* spp. as an indicator of resistance to post-weaning diarrhea for piglets. A link has also been established in the literature between diet polyphenols (AP contains some), *Akkermansia muciniphila* and intestinal health in terms of barrier integrity, immune response and resident intestinal microbiota in humans (Kumari et al., 2021; Roopchand et al., 2015). For piglets, *Akkermansia muciniphila* appears more disturbing. While Karasova and colleagues (Karasova et al., 2021) observed an increased abundance of this bacteria in diarrheic piglets, Luo and colleagues (Luo et al., 2021) published the promising NGP potential of *Akkermansia muciniphila* through their in vitro intestinal porcine enterocytes model. NGP are also of interest for swine production. Indeed, adding substrates in the feed that will promote the growth of specific symbiotic bacteria will benefit the gut health of pigs (Luo et al., 2022). Introducing dietary components enhancing the growth of specific bacteria is a pathway that can lead to positive changes in the microbiota for better health of the host (Wallenborn and Vonaesch, 2022). Adequate substrate is furnished to the NGPs to promote growth in their niche (Kumari et al., 2021), ensuring an appropriate colonization resistance in the intestine of the host through these

bacteria interacting with the host immune system. Considering the concept “nourish the NGPs”, AP appears in this study as an interesting dietary component to achieve this purpose.

To conclude, the preliminary results from the test of dried AP in an in vitro multicompartment piglet model tended to show that AP could promote an ecosystem enriched in Bacteroidetes and/or Verrucomicrobia with a likely effect on the energy metabolism of the host by their fermentation metabolites. The AP component could be used as a dietary strategy to influence bacterial changes in the intestine by stimulating the growth of bacteria potentially considered as NGP.

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Acknowledgments

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Data Availability Statement

The datasets analyzed for this study can be found in the ENA database (https://www.ebi.ac.uk/ena/browser/home, 10 March 2021) under the accession number PRJEB43583.

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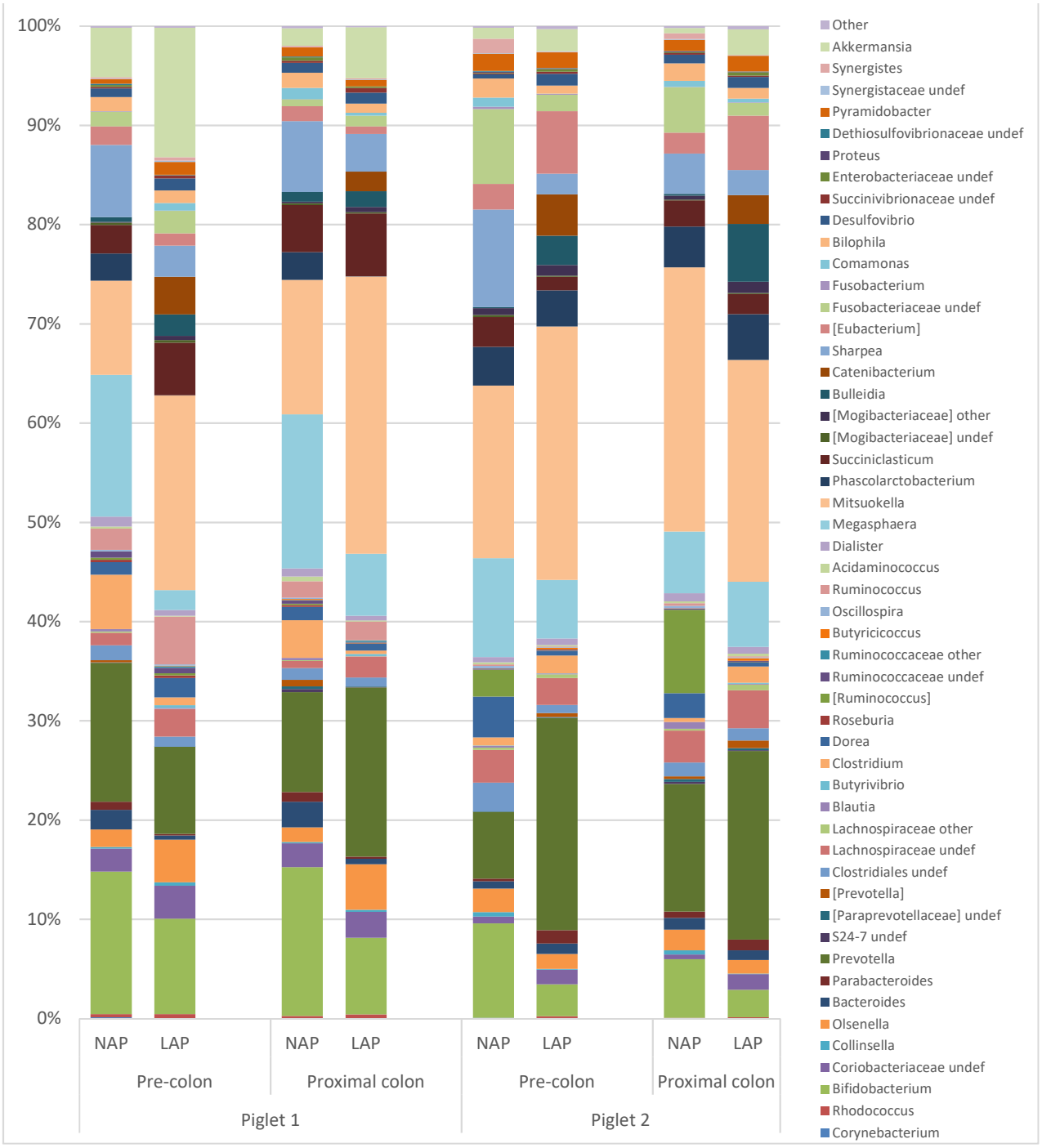
Supplementary material – Figure S1



Supplementary Material - Figure S1. Relative abundances of the genera observed in the pre-colon and proximal colon bioreactors of baby-SPIME when using AP (LAP) or not (NAP) for two donor piglets.

PART 3: In Vivo Study

Apple pomace is a by-product that presents interesting properties for food and feed sectors. In the literature, it was yet tested in diet for piglets–with positive impact reported. Due to the fact that the chemical composition of AP varies depending on variety of apple or on process applied on apples, additional researches are of interest. Moreover, studying the by-product from a whole microbiota perspective remains little explored in the literature. Here was the opportunity to carry out a piglet's experimentation which makes possible to increment data on the rate of incorporation of the by-product and also to ensure an assessment of the matrix at different time points of post-weaning, particularly on the microbiota. Moreover, the in vivo experiment can be performed with the same matrix used in vitro; the results obtained through the experiment serving to reinforce the discussion of the in vitro observations. Therefore, **Study 5** aimed to evaluate the effects of dried AP incorporated at two levels into a post-weaning diet (a positive control level set at 4% and a lower intermediate level set at 2%) – on growth performance, on intestinal morphology and on the microbiota of piglets from weaning at 28 days old over a 5-week period and globally discuss about AP as a weaning strategy.

Study 5 -

Apple Pomace and Performance, Intestinal Morphology and Microbiota of Weaned Piglets—A Weaning Strategy for Gut Health?

Microorganisms 9 (2021) 572

Sandrine Dufourny, Nadine Antoine, Elena Pitchugina, Véronique Delcenserie, Stéphane Godbout, Caroline Douny, Marie-Louise Scippo, Eric Froidmont, Pierre Rondia, José Wavreille, Nadia Everaert

Abstract

Apple pomace (AP) is known to be rich in biomolecules beneficial for health and it may advantageously be used to overcome the critical step of piglets' weaning. The study aimed to determine the effect of two levels of incorporation of AP on the performance, intestinal morphology and microbiota of weaned piglets and investigate this feed ingredient as a weaning strategy. An experiment was performed with 42 piglets from weaning (28 days old) over a five-week period, including three iso-energetic and iso-nitrogenous diets (0%, 2%, and 4% dried AP diets) with seven pen-repetitions per diet (two pigs per pen). AP diets were beneficial for the average daily gain calculated on week 3 ($p = 0.038$) and some parameters of the intestinal architecture on the 35 post-weaning day. The 4% AP diet was beneficial for the feed conversion ratio ($p = 0.002$) and the energetic feed efficiency ($p = 0.004$) on the 35 post-weaning day. AP tended to influence the consistency of feces (softer to liquid, $p = 0.096$) and increased the counts of excreted pathogens ($p = 0.072$). Four percent AP influenced the richness of the microbiota and the bacteria profile as observed for the phylum Bacteroidetes or the class Clostridia. The 4% AP diet appeared as an interesting weaning strategy that should be evaluated in a large cohort.

Introduction

Apple is a fruit rich in phytochemicals linked with good health indicators for humans (e.g. decreased risk of cancer or cardiovascular disease, positively associated with general pulmonary health); in particular its content in both dietary fiber and phenolic compounds may partially contribute to this beneficial effect (Boyer and Liu, 2004). Similarly, the product derived from apple juice extraction process, apple pomace (AP) is known to contain biomolecules beneficial for health such as phenolic and terpenic compounds (Grigoras et al., 2013; Lu and Foo, 1997). AP can represent a raw material of great interest for different applications such as the production of lactic acid (Gullón et al., 2007), as well as being a source of dietary fiber or polyphenolic components (Kołodziejczyk et al., 2007; Rana et al., 2015), and as a possible functional food for the agri-food sector (Reis et al., 2014). AP seems to have positive health effects in rats when used as a feed ingredient. Indeed, it improved the antioxidant status of the animals–by increasing the activity of superoxide dismutase in a hemolysate of erythrocytes and by increasing the antioxidant capacity of the lipid fraction of serum. It also reduced the blood glucose level of the rats and increased the fermentation process in the distal part of their gastrointestinal tract (Juśkiewicz et al., 2012). Incorporated at 3.5% in a weaner diet for piglet, beneficial effects were observed on the gut morphology of the animals (Sehm et al., 2007) or on other parameters such as intestinal bacteria, blood parameters or gene expression of immunological markers (Sehm et al., 2011).

Although beneficial effects may thus be expected, it must be kept in mind that the composition of AP can largely vary in space and time. For example, the apple cultivars used for the juice extraction can influence the physico-chemical properties and antioxidant activities of the residual AP (Andre et al., 2012; Sato et al., 2010). The use of enzymes for extraction of juice (Thakur et al., 1997) or the blanching and drying process employed on AP (Heras-Ramírez et al., 2011) have an impact on its composition. So, the potential variability in the composition of the matrix encourages continuing research on this healthy raw material for its introduction in the feed chain at a large scale and shows the importance, for feed producers, of properly characterizing the matrix before using it.

Weaning is a critical step in pig production. Indeed, weaning is generally an abrupt and precocious event–3 to 5 weeks after parturition in the current production methods–and leads to multiple external factors of stress: change in the diet from a 20% dry matter milk feed to an 80-90% dry matter plant-based diet, change in physical and social environments including the maternal separation and the disruption of the established social group (Pluske et al., 2003; Weary et al., 2008). In a few hours, the piglet has to adapt to a novel environment including unfamiliar feeding and drinking equipment, unfamiliar piglets, with no more stimulus from the sow to start feeding, and potential lack of thermal comfort (Pluske et al., 2003). All these stressors induce a reduction of piglets' feed intake and multiple biological stresses related to the intestinal structure and functions, to the nervous and hormonal pathways and to the immune system that may have an impact on short and long-term performance and health status of the piglet (Campbell et al., 2013; Jayaraman and Nyachoti, 2017; Niewold, 2015).

In a few days after weaning, the young intestinal structure has to adapt to the new diet. Modifications of the intestinal architecture and functions relate to the decrease of villi height, the increase of crypts depth and intestinal cell mitosis, the reduction of brush border enzyme activity and the reduction of the absorptive capacity of nutrients and electrolytes (Pluske et al., 2003). In addition to these physiological disturbances, impacts on the gut barrier function, the piglets' immunity and the microbiota are also described. It induces an inflammatory response of the intestinal mucosa and makes the animal sensitive to antigens, toxins and translocation of bacteria (Gresse et al., 2017; Jayaraman and Nyachoti, 2017; J. Lallès et al., 2007). As a consequence of all these disturbances, weaning generates post-weaning symptoms in piglets, including post-weaning diarrhea often induced by the enterotoxigenic *Escherichia coli* (van Beers-Schreurs et al., 1992).

Weaning requires nutritional management (J.-P. Lallès et al., 2007) in order to prevent or counteract the negative effects on growth performance and intestinal disorders (Heo et al., 2013) and to ensure lifelong gut health for pigs (Moeser et al., 2017). AP at an incorporation dose of 3.5% is a promising matrix (Sehm et al., 2011, 2007). However, technical and economic constraints linked with the production of dried AP, may wonder whether a lower dose of AP also results in beneficial effects in weaned piglets. Consequently, the aim of the study was to evaluate the effects of dried AP–incorporated at two levels into a post-weaning diet (a positive control level set at 4% and a lower intermediate level set at 2%)–on growth performance, on intestinal morphology and on the microbiota–of the faeces during the post-weaning as well as that of the caecum at the end of the post-weaning period–and globally discuss about AP as a weaning strategy. AP diets, particularly the higher dose, showed positive effects on some performance indicators explained partially by the intestinal architecture. The faeces of the 4%AP piglets tended to be softer, to count more excreted pathogens and to have an enriched microbiota. In the caecum on the 35 post-weaning day, the microbiota of 0% AP and 4% AP piglets were rather similar. Considering globally the results of the experiment, the 4% AP diet appeared as an interesting weaning strategy that merits to be evaluated in a large cohort to evaluate finely the risk of dysbiosis due to the excretion of pathogens in the beginning of the post-weaning period.

Material and methods

Experimental design:

Animals, diets and housing

The intervention on piglets was approved by the ethical committee of the University of Liège (ULiège, Liège, Belgium)—file n°1823. The intervention was in compliance with European (Directive 2010/63/EU) and Belgian (Royal Decree of the 29 May 2013) regulations governing the protection of animals used for scientific purposes.

Forty-two piglets [Pietrain x Landrace] from the Walloon Agricultural Research Centre (CRA-W, Gembloux, Belgium)—21 females and 21 castrated males, free of antibiotics—were weaned at 28 days of age and transported to the Animal Production Centre in Gembloux (CEPA-University of Liège, Gembloux, Belgium) for 35 days of post-weaning rearing.

During the lactation period, piglets received a creep feed (meal dry feed) for 10 days before weaning. The creep feed was composed of 50% milk powder and 50% of the control diet (SCAR, Herve, Belgium). During the post-weaning period, piglets received one of the three diets (meal dry feed) formulated for the experiment (control diet with no AP—0% AP-, experimental diet containing 2% dried AP—2% AP-, experimental diet containing 4% dried AP—4% AP-; Table 12; SCAR, Herve, Belgium). Diets were formulated to be iso-energetic (9.6 MJ EN/kg) and iso-nitrogenous (17.5% CP), having in mind the recommendations of the NRC. To formulate the experimental AP diets, a portion of wheat was removed from the 0% AP diet that was substituted by AP and—in a lesser extent—by soybean meal and soya oil.

Table 12: Composition of the post-weaning diets in %.

Ingredients	0% AP	2% AP	4% AP
Wheat	36.89	34.16	31.45
Barley	20.00	20.00	20.00
Soybean meal	16.40	16.83	17.28
Maize	15.00	15.00	15.00
Premix10916 (Inve Belgie, Dendermonde, Belgium)	7.50	7.50	7.50
Sugar beet pulp	2.50	2.50	2.50
AP	0.00	2.00	4.00
Soya oil	1.45	1.72	1.97
L-threonine	0.115	0.120	0.120
Monocalcium phosphate	0.100	0.100	0.100
Tryptophan	0.035	0.035	0.040
DL-Methionine	0.015	0.025	0.030
Rovimix® E50	0.005	0.005	0.005

0% AP, control diet, 2% AP, 2% apple pomace diet, 4% AP, 4% apple pomace diet. Rovimix® is a commercial source of vitamin E used in animal nutrition.

For the post-weaning housing, piglets were assigned in pair (1 female and 1 male) to seven boxes (1.5 m2, polymer grating) for each of the three diets following a randomized complete

block design. The boxes of the piglets were gathered in 7 blocks (including each the 3 diets), considering a potential thermic gradient in the housing.

Piglets were fed ad libitum and the enrichment of the boxes was done with steel chains. All boxes were washed with water every day after scoring the piglet's fecal consistency.

Sampling of Feces

A sample of feces was collected directly from the rectum of each male two times, i.e., on day 8 and day 28, during the post-weaning period. A part of the samples of day 8 was directly used fresh to detect excreted pathogens. All the samples (day 8 and day 28) were stored at −80 °C, until DNA extraction.

Sampling of Intestinal Tissues and Contents

On the last day of the experimental period–day 35–the male piglets were euthanized by isoflurane inhalation followed by bleeding. Their gastrointestinal tract was removed and each intestinal segment was isolated. The chyme and the mucosal layer–collected to be representative of a part of the ileum (from 1 m to 50 cm before the ileo-cecal junction), caecum and proximal colon (collected from 30 cm after the ileo-cecal junction) were immediately snap-frozen in liquid nitrogen and maintained at −80 °C until DNA extraction. Five cm of tissue samples from the duodenum (from 25 cm after the stomach), jejunum (from 2 m after the stomach) and ileum were collected, rinsed with a saline solution and fixed in 10% formol until morphological analyses.

Experimental Measurements

Chemical Analyses

AP was chemically analyzed for the mono-, and disaccharides, fiber, and polyphenols profile by the Biomass and Green Technologies laboratory (University of Liège, Gembloux, Belgium) using gas chromatography (GC) after derivatization (Aguedo et al., 2013) and high-performance anion exchange chromatography (HPAEC-PAD) (Uerlings et al., 2020). Rhamnose, arabinose, xylose, mannose, galactose, and glucose were determined after aqueous extraction by GC including derivatization step. Saccharose and fructose were determined in the aqueous extract by HPAEC-PAD. Arabino-, xylo- and galacto-oligosaccharides were determined by GC after acid hydrolysis and derivatization. The solvent used was dichloromethane. Glucuronic acid, galacturonic acid and constituting sugars were determined by HPAEC-PAD and GC after hydrolysis. Total polyphenols were determined, after extraction of the phenolic compounds with a polar solvent, by spectrophotometry after oxidation of the phenolic compounds by the Folin–Ciocalteu reagent (Dedrie et al., 2015).

Diets were chemically analyzed by CRA-W (Gembloux, Belgium) for their content in humidity, crude protein, crude ash, reducing sugars, total sugars, starch, cellulose and crude fat following Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of

sampling and analysis for the official control of feed. The NDF, ADF, ADL content were analyzed following Standard NFV18-122 August 1997 for animal feedstuffs—Determination of sequential cell-wall—Method by treatment with neutral and acid detergent and sulfuric acid.

AP and diets were chemically analyzed by Upscience (Saint-Nolff, France) for their insoluble high molar weight dietary fiber (HMWDF), soluble HMWDF and soluble low molar weight dietary fiber (LMWDF) following AOAC 991.43 method.

Zootechnical Performance

Piglets were weighed (Giropes G1308, Pesage Warnier, Hannut, Belgium) on a weekly basis, with the initial weight being the weaning weight. The average daily gain (ADG) was calculated per box, on a weekly and cumulative basis (from initial weighting to specific date of weighting), with the final ADG being those calculated using the initial weight and final weight of piglets. The total feed intake (TFI) and the feed conversion ratio (FCR) were calculated at the end of the experiment per box. The energetic feed efficiency (EFE) was calculated as the ratio between the total net energy of the diet ingested per box and the total weight gain of the piglets of the box. The net energy of the diet was calculated with EvaPig® software (v 1.3.1.7, INRA–AFZ–Ajinomoto Eurolysine S.A.S., Paris, France) using the results of the feed chemical analyses.

Scoring of Piglet's Fecal Consistency

The consistency of the piglet's feces was visually evaluated on a daily basis per box from the second day of weaning until the 15 post-weaning day. Two scores were attributed per box (minimum and maximum scores). The rating scale included 6 ranks (Oliviero et al., 2009) from score 0 to score 5. The score 0 was an absence of feces. The score 1 was multiple free pellets of feces. The score 2 was aggregated pellets shaping the feces. The score 3 was firm feces, shaped as a cylinder). The score 4 was soft feces, not shaped as a cylinder. The score 5 was liquid feces.

Excreted Pathogens

The presence/abundance of pathogens that are known to be involved in the diarrhea of piglets was investigated on the 8 post-weaning day through the use of the Rainbow Piglet ScoursTM Bio K 351 (Bio-X Diagnostics S.A., Rochefort, Belgium)—detecting Rotavirus, *E. coli* F4, F5, F18, F41 attachment factors, *Clostridium difficile*, *Clostridium perfringens* and Cryptosporidium—following the manufacturer's instructions.

Intestinal Morphology

The morphological measurements of the intestinal segments were determined following a protocol previously described (Laitat et al., 2015). The segments, fixed in 10% formol, were dehydrated and embedded in paraffin wax. For each intestinal segment, four sections of 5 µm were

obtained and stained with Alcian blue for mucous detection following routine methods. The sections obtained were scanned, digitized using an imaging system for virtual microscopy (Dotslide, Olympus, Belgium) and analyzed with a Java image morphometric processing program (Image J software, National Institute of Health, Bethesda, Maryland, USA). Villus length and crypt depth were measured in order to obtain a total of 20 measurements of crypt and villus per section (80 per pig and per intestinal segment).

Short Chain Fatty Acids (SCFA)

The SCFA analyzed were acetic (C2), propionic (C3), isobutyric (iC4), butyric (C4), isovaleric (iC5), valeric (C5) and hexanoic acids (C6). The SCFA content of the feces and chyme from the caecum was measured by SPME-GC-MS following a protocol previously described (Douny et al., 2019). Between 20 and 25 mg of samples were introduced into a 20 mL glass vial. Forty µl of internal standard (2-methylvaleric acid) at a concentration of 0.2 mg/mL, 15 µl of 0.9 M sulfuric acid, and 920 µL of water were then added. The mixture was vortexed and placed on the autosampler of the SPME-GC-MS system until analysis. SCFA were extracted with a SPME fiber, separated on a Focus GC gas chromatograph (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a Supelcowax-10 column (30 m × 0.25 mm, 0.2 µm) (Supelco, Bellefonte, PA, USA) and analyzed with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The agitation temperature was set at 60 °C and the extraction time at 20 min.

Composition and Richness of the Microbiota

The composition and richness of the microbiota were analyzed on all fecal and cecal samples. DNA extraction and sequencing of all the samples were performed by DNA Vision (Gosselies, Belgium) following their internal quality SOP. DNA was extracted from frozen pellets with the DNeasy Blood & Tissue kit according to the instructions of the manufacturer Qiagen (Qiagen Benelux B.V., Venlo, The Netherlands). DNA was quantified and qualitatively assessed on a NanoDrop 2000 from Thermo Scientific™ and by PicoGreen VICTOR X3 (PerkinElmer) using the Quant-it PicoGreen dsDNA Assay kit from Invitrogen. The 16S targeted region V3-V4 was amplified by PCR, purified and tagged. Libraries were indexed using the NEXTERA XT Index kit V2 from Illumina. The high throughput sequencing was carried out on Illumina Miseq in paired-end sequencing (2 × 250 bp) by targeting an average of 10,000 reads per sample. Finally, the bioinformatic analysis was executed with the QIIME (Quantitative Insights Into Microbial Ecology) software, version 1.9.0 with “Greengenes 13_8” as database and recommended parameters to use QIIME scripts. The OTU (Operational Taxonomic Unit) table was generated based on a 97% sequence similarity of the sequencing reads to cluster OTUs. Only samples presenting more than 5,000 reads were used for taxonomic analysis. Similarly, samples with the same normalized number of reads were used for the beta diversity (OTU) analysis for which the results were expressed in relative abundance—a percentage of the total bacteria.

Beta diversity (group comparisons)—representing comparison of microbial communities based on their composition—generated a matrix where dissimilarities/distances were calculated between every pair of group samples and that was visualized with Principal Coordinates Analysis. The group comparison analysis was performed for the Weighted Unifrac distance—based on the abundance of observed organisms.

The parameters of alpha-diversity that were analyzed are Chao1, observed operational taxonomic unit, phylogenetic diversity whole tree and Shannon index.

Statistical Analyses

Parametric Tests

The conditions for applying the analysis of variance on the quantitative data of the experiment have been verified. The assumptions of the data normality and the equality of variances according to the treatment were confirmed.

Zootechnical performance measured at the end of the post-weaning phase, calculated as mean values for the global post-weaning period (final weight, final ADG, TFI, FCR, and EFE) and intestinal morphology measurements were analyzed using a two-way analysis of variance with “diet” as fixed factor and “block” as random factor (GLM procedure, SAS v9.4, SAS Inst. Inc., Cary, NC, USA). The Student–Newman and Keuls test was used to structure the averages. SCFA data from chyme samples were analyzed following the same procedure using Minitab software (Minitab 18, Minitab Inc., State College, PA, USA).

Zootechnical performance measured during the post-weaning period (ADG calculated per box, on a weekly and cumulative basis) and SCFA data from fecal samples were analyzed as repeated measures following a split-plot analysis of variance (Dagnelie, 2012) with “diet” as fixed factor, “piglet” as random factor and “week” as fixed factor (Minitab 18, Minitab Inc., State College, PA, USA). Moreover, as a complement of information, analyses of variance were also performed separately on the different dates of the weekly ADG.

A p-value lower or equal to 0.05 was considered statistically significant. A p-value between 0.05 and 0.1, or equal to 0.1, was considered a trend. Otherwise, a p-value higher than 0.1 was considered not significant (ns).

Non-Parametric Tests

The qualitative data (pathogens in feces and fecal consistency scores) were analyzed using non-parametric tests for which categories were defined in order to comply with the test application conditions.

The qualitative data obtained from the detection of pathogens by Rainbow kit were analyzed using a non-parametric chi-square test of association (Minitab 18, Minitab Inc., State College, PA, USA) ranking the diet factor into two categories (no AP in the diet and AP in the diet) and the

results into two categories (negative: no pathogen detected or $<10^6$ CFU/g; positive: pathogen detected or $>10^6$ CFU/g).

The qualitative data obtained from the piglet's fecal consistency were analyzed using a non-parametric Cochran test (Minitab 18, Minitab Inc., State College, PA, USA), ranking the diet factor into two categories (no AP in the diet and AP in the diet) and the results of the maximum scores into two categories (category A included the scores 0 to 3; category B included the scores 4 and 5 that was considered as more diarrheic).

The statistical analysis for the alpha-diversity data of the microbiota was based on a non-parametric t-test (Monte Carlo permutations to calculate p-value) comparing groups of samples two by two. The analysis for the beta-diversity data (OTU) was done at different levels of the taxonomy classification to detect differences in read abundances between groups of samples. The non-parametric Kruskal–Wallis test was used for this purpose (KW p-value) and it was subsequently adjusted using the Benjamini–Hochberg false discovery rate procedure for multiple comparisons (FDR p-value). The analysis for the beta-diversity data (group comparison, weighted Unifrac distance) was done through the Adonis statistical test.

A p-value lower or equal to 0.05 was considered statistically significant. A p-value between 0.05 and 0.1, or equal to 0.1, was considered a trend. Otherwise, a p-value higher than 0.1 was considered not significant (ns).

Results

Feed chemical analyses.

Results of the chemical analyses are given in Table 13 for the AP and in Table 14 for the diets.

Table 13: Characterization of the apple pomace used in the study (mono-, and disaccharides, fiber and polyphenols).

Chemical Component	AP
Rhamnose	<0.1 g/100 g DM
Arabinose	<0.1 g/100 g DM
Xylose	<0.1 g/100 g DM
Mannose	<0.1 g/100 g DM
Glucose	3.4 g/100 g DM
Galactose	2.1 g/100 g DM
Fructose	13.5 g/100 g DM
Saccharose	2.9 g/100 g DM
Arabino-oligosaccharide	0.5 g/100 g DM
Xylo-oligosaccharide	0.3 g/100 g DM
Galacto-oligosaccharide	0.2 g/100 g DM
Galacturonic acid	<0.1 g/100 g DM
Glucuronic acid	<0.1 g/100 g DM
Total polyphenols (Folin-Ciocalteu)	26.7 mg gallic acid/g DM
Quercetin	23.1 µg/g DM
Phloretin	2.8 µg/g DM
Insoluble HMWDF	45.2 g/100 g DM
Soluble HMWDF	12.6 g/100 g DM
Soluble LMWDF	0.5 g/100 g DM
Soluble DF/insoluble DF	0.29

AP, apple pomace; DM, dry matter; HMWDF, high molar weight dietary fiber; LMWDF, low molar weight dietary fiber.

Table 14: Chemical analyses of the diets.

Chemical Component	0% AP	2% AP	4% AP
Dry Matter (%)	88.0	87.3	88.2
Crude protein (% DM)	18.1	18.7	18.6
Crude ash (% DM)	6.13	5.51	5.45
Reducing sugars (% DM)	1.43	1.76	2.47
Total sugars (% DM)	6.40	7.17	7.93
Starch (% DM)	44.10	43.41	42.23
NDF (% DM)	13.49	13.20	13.20
ADF (% DM)	6.49	6.56	6.68
ADL (% DM)	1.21	1.15	1.38
Cellulose (% DM)	5.13	5.12	4.93
Crude Fat (% DM)	3.34	3.55	3.50
Insoluble HMWDF (% DM)	17.3	17.4	16.7
Soluble HMWDF (% DM)	4.7	4.9	5.1
Soluble LMWDF (% DM)	3.3	3.0	3.1
Soluble DF/insoluble DF	0.46	0.45	0.49

ADF, acid detergent fiber; ADL, acid detergent lignin; DM, dry matter; HMWDF, high molar weight dietary fiber; LMWDF, low molar weight dietary fiber; NDF, neutral detergent fiber; 0% AP, control diet, 2% AP, 2% apple pomace diet, 4% AP, 4% apple pomace diet.

Zootechnical Performance

Piglets from two boxes were excluded from the experimental set-up due to health issues in the first days of the experiment. The first piglet—0% AP box—was excluded due to the infection of a hoof of the female piglet. The second piglet—4% AP box was excluded due to a paw problem of the male piglet.

For the performance at the end of the post-weaning period, final weight, final ADG and TFI were not statistically different between diets (Table 15). FCR, as well as EFE of piglets that had received the 4% AP diet were significantly lower compared to piglets of the 0% AP and 2% AP diets.

Table 15: Zootechnical performance of piglets receiving control or experimental diets during 35 days of the post-weaning rearing period.

Zootechnical Parameter	0% AP	2% AP	4% AP	SEM	*p*-Value Diet	*p*-Value Block
Initial weight (kg)	8.4	8.4	8.4	0.1	ns	ns
Final weight (kg)	21.0	22.6	23.1	0.4	ns	ns
Final ADG (kg/d)	0.361	0.406	0.421	0.012	ns	ns
TFI (kg DM)	19.8	21.7	20.5	0.5	ns	ns
FCR	1.79 [a]	1.75 [a]	1.59 [b]	0.03	0.002	ns
EFE (MJ NE/kg gain)	16.9 [a]	16.8 [a]	15.2 [b]	0.2	0.004	ns

[a, b] values assigned different letter within a row are statistically different; 0% AP, control diet (n = 6); 2% AP, diet containing 2% dried apple pomace (n = 7); 4% AP, diet containing 4% dried apple pomace (n = 6); ADG, average daily gain; DM, dry matter; EFE, energetic feed efficiency; FCR, feed conversion ratio; NE, net energy; ns, not significant; TFI, total feed intake.

For the weekly ADG measured during the post-weaning period, the statistical analyses using repeated measures did not differ between diets. However, analyses of variance performed on ADG week

by week (Table 16) revealed that ADG in week 3 was higher for 2% AP and 4% AP diet than that of 0% AP diet. ADG in week 2 and week 4 showed a trend to be different.

Table 16: Average daily weight gain (ADG) of piglets receiving control or experimental diets week by week.

ADG	0% AP	2% AP	4% AP	SEM	*p*-Value Diet	*p*-Value Block
ADG week 1 (d0–d + 7)	0.118	0.134	0.143	0.013	ns	ns
ADG week 2 (d + 7–d + 14)	0.304	0.261	0.350	0.020	0.083	ns
ADG week 3 (d + 14–d + 21)	0.367 [b]	0.459 [a]	0.458 [a]	0.017	0.038	ns
ADG week 4 (d + 21–d + 28)	0.489	0.563	0.537	0.018	0.085	ns
ADG week 5 (d + 28–d + 35)	0.530	0.613	0.614	0.020	ns	ns

[a, b] values assigned different letter within a row are statistically different; 0% AP, control diet (n = 6); 2% AP, diet containing 2% dried apple pomace (n = 7); 4% AP, diet containing 4% dried apple pomace (n = 6); ADG, average daily gain; d, day with d0 as the first day of the experiment.

Scoring of Piglet's Fecal Consistency and Excreted Pathogens

The results obtained from the observation of the feces (maximum score in each box) during the first two weeks of the post-weaning period showed a trend (p = 0.096) for more softer to liquid feces with AP diets than with the 0% AP diet (Table 17, left side). The results obtained through the use of the Rainbow kits showed a trend for more pathogens with diets containing AP than with the 0% AP diet (Table 17, right side, p = 0.072).

Table 17: Contingency tables for the scoring of fecal consistency during the first 14 days post-weaning period and detection of the excreted pathogens in feces on the 8 post-weaning day.

Diet	Statistical Parameter	Fecal Consistency Scoring [1]			Pathogens in Feces [2]		
		A	B	Total	Negative	Positive	Total
Apple pomace	Count	51	144	195	84	20	104
	Expected count	57.47	137.53		87.58	16.42	
	Adjusted Residuals	−1.809	1.809		−1.713	1.713	
No apple pomace	Count	33	57	90	44	4	48
	Expected count	26.53	63.47		40.42	7.58	
	Adjusted Residuals	1.809	−1.809		1.713	−1.713	
Total	Count	84	201	285	128	24	152
p-value			0.096			0.072	

[1] Contingency table from a Cochran test done on the maximum scores of fecal consistency. Feces were observed per box on a daily basis from the second day of weaning until the 15 post-weaning day. Consistency A included the results from no feces to normal feces (scores 0 to 3), consistency B included soft to liquid feces. [2] Contingency table from a Chi-Square test of Association counting the presence (positive test or $>10^6$ CFU) or absence (negative test or $<10^6$ CFU) for Rotavirus, *E. Coli* F4, F5, F18, F41 attachment factors, *Clostridium difficile*, *Clostridium perfringens*, and Cryptosporidium. Fecal samples were taken on the 8 post-weaning day.

Intestinal Morphology

The results of the morphological measurements performed on the three small intestinal segments showed a significant effect of the diet on the duodenal villus length and on the ileal ratio villus length/crypts depth (Table 18). Piglets receiving the 4% AP diet presented higher duodenal villus length than piglets from the 0% AP diet; with intermediate values for the 2% AP piglets. In the same way, piglets receiving the 4% AP diet had a higher ileal ratio villus length/crypt depth than piglets with the 0% AP diet (with the difference between 4% AP and 0% AP mainly due to the higher villus length of 4% AP in mean value). No effect of the diet was observed on jejunum measurements although numerical differences between the mean values in jejunum were similar to those observed in the duodenum.

Table 18: Results of the morphological measurement (villus and crypts) on the three upper intestinal segments on the 35 post-weaning day.

Intestinal Parameter	0% AP	2% AP	4% AP	SEM	*p*-Value Diet	*p*-Value Block
Duodenum	(*n* = 6)	(*n* = 7)	(*n* = 6)			
Villus length (µm)	320 [b]	381 [ab]	429 [a]	16.4	0.018	ns
Crypts depth (µm)	436	471	448	10.5	ns	ns
VL/CD	0.74	0.81	0.96	0.039	ns	ns
Jejunum	(*n* = 6)	(*n* = 6)	(*n* = 5)			
Villus length (µm)	354	366	420	20.5	ns	ns
Crypts depth (µm)	314	325	324	8.9	ns	ns
VL/CD	1.14	1.16	1.31	0.07	ns	ns
Ileum	(*n* = 6)	(*n* = 7)	(*n* = 6)			
Villus length (µm)	240	273	315	13.4	ns	ns
Crypts depth (µm)	255	270	242	7.9	ns	ns
VL/CD	0.94 [b]	1.03 [ab]	1.33 [a]	0.07	0.039	ns

[a, b] values assigned a different letter within a row are statistically different; 0% AP, control diet; 2% AP, diet containing 2% dried apple pomace; 4% AP, diet containing 4% dried apple pomace; VL/CD, ratio villus length/crypts depth.

Short Chain Fatty Acids (SCFA)

No SCFA differences between groups were observed in the samples of feces on the 8 and 28 post-weaning days (calculated as g/kg feces or as mmol/kg feces) and in the caecum samples (calculated as g/kg cecal content or as mmol/kg cecal content) (data not shown).

Richness and Composition of the Microbiota

Fecal Microbiota Composition on Day 8 Post-Weaning

The Shannon index tended (p = 0.094) to be higher in the feces of 4% AP piglets (5.7) than in the feces of 0% AP piglets (5.0), while the Shannon index of the 2% AP piglet's feces was 5.4. Other indices (Chao1, observed operational taxonomic unit, phylogenetic diversity whole tree) were not different. The weighted Unifrac distance tended to be different between groups (p = 0.052, Appendix A).

At the phylum level of classification (Figure 21), for the 0%AP and 2% AP piglets, the phyla showing the higher relative abundances—by decreasing order—were Firmicutes, Actinobacteria and Bacteroidetes (containing Bacteroidia as unique class in all the samples of the study). For the 4% AP piglets, it was Firmicutes, then Bacteroidetes that was more abundant than Actinobacteria. For all piglets, the phylum Proteobacteria was scarce (0.0% for 0% AP, 0.1% for 2% AP, and 0.5% for 4% AP).

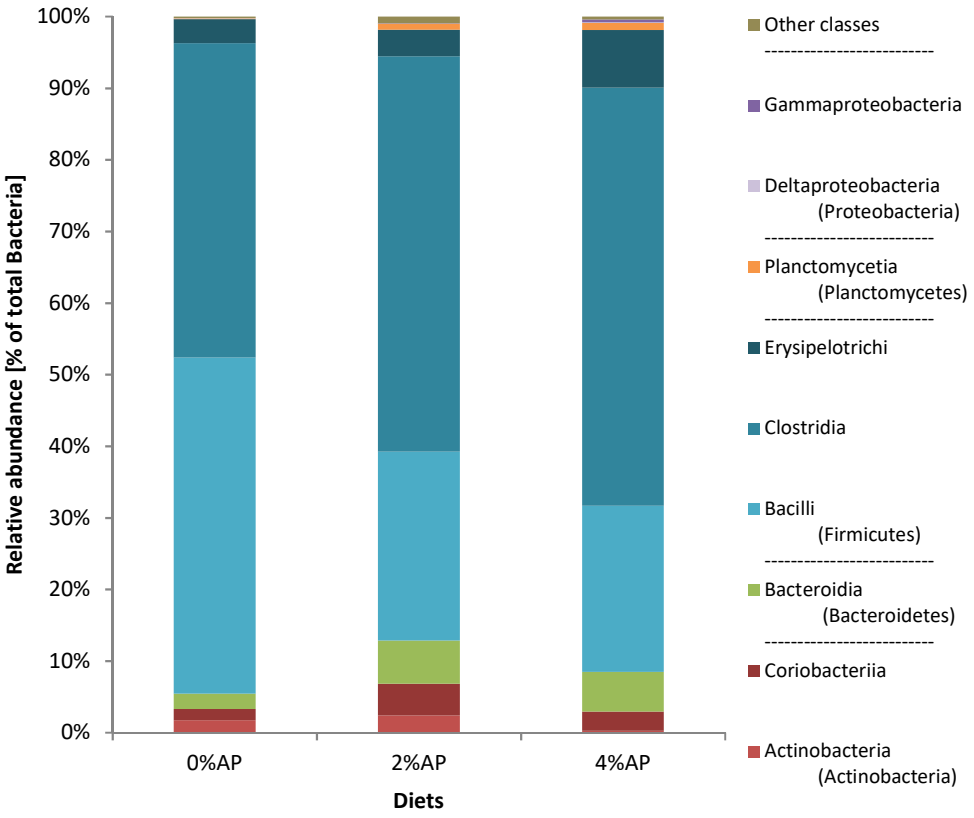


Figure 21 : Composition (phyla and classes) of the microbiota in feces of piglets on the 8 post-weaning day. Diet 0% AP, control diet (*n* = 5), 2% AP, diet containing 2% dried apple pomace (*n* = 7), 4% AP, diet containing 4% dried apple pomace (*n* = 6).

The class Clostridia was twice as abundant in comparison to Bacilli in AP diets while they were equal in the diet without AP (Figure 21).

Coriobacteriaceae, Lactobacillaceae and Enterobacteriaceae were bacterial families influenced by the diet as well as *Dorea*, *Slackia*, *Ruminococcus*, and *Catenibacterium* when considering the genus level of the classification (Appendix B and Supplementary file—Figure S1).

Fecal Microbiota Composition on Day 28 Post-Weaning

The indexes describing the α-diversity did not differ between the diets. The weighted Unifrac distance tended to be different between groups (p = 0.069, Appendix A).

As observed on the 8 post-weaning day, the phylum Firmicutes showed the highest relative abundance in the fecal microbiota of piglets on the 28 post-weaning day (Figure 22). It was then followed for the three diets by the phylum Bacteroidetes and then Actinobacteria. The relative abundance of Proteobacteria was below 1% (0.2% for 0% AP, 0.1% for 2% AP, and 0.2% for 4% AP; p = not significant).

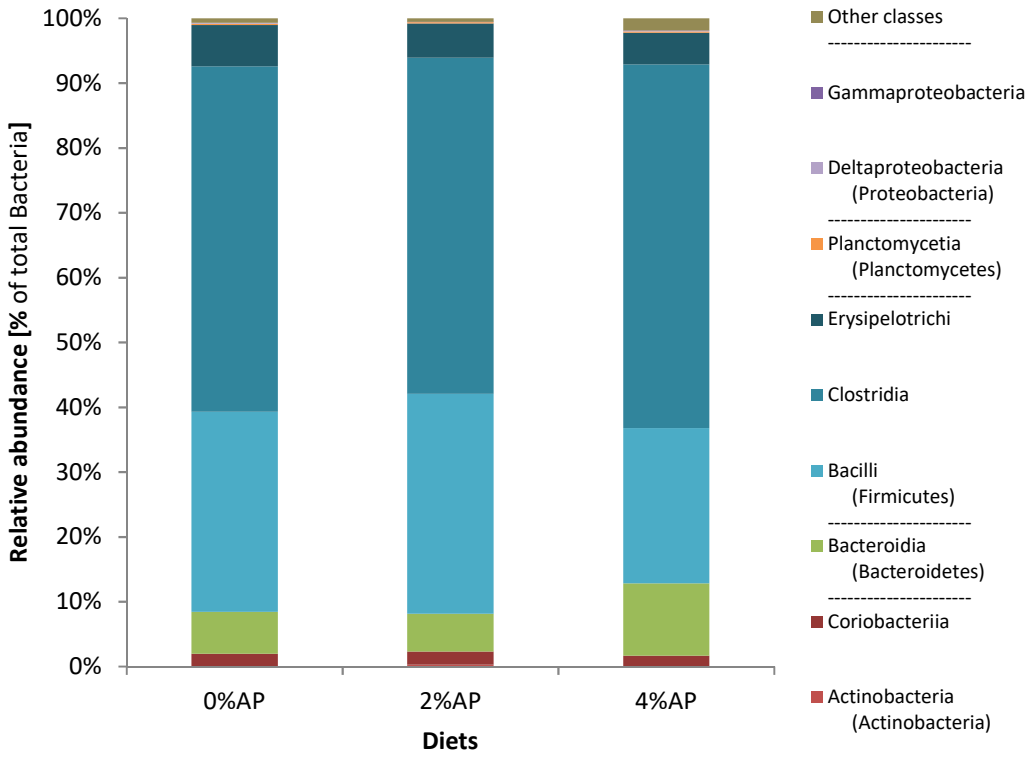


Figure 22 : Composition (phyla and classes) of the microbiota in feces of piglets on the 28 post-weaning day. Diet 0% AP, control diet (*n* = 6), 2% AP, diet containing 2% dried apple pomace (*n* = 7), 4% AP, diet containing 4% dried apple pomace (*n* = 6).

The class Clostridia was the first class of bacteria (>50% relative abundance) for AP diets and it became also the first class for the 0% AP diet at this timepoint (Figure 22).

Amongst the more abundant families—cited by decreasing order—were Lactobacillaceae, Lachnospiraceae, and Ruminococcaceae for the 0% AP and 2% AP piglets. It was Lactobacillaceae, Ruminococcaceae, and Lachnospiraceae for the 4% AP diet (Table 19). Between these three families, Ruminococcaceae showed a significantly higher relative abundance for the 4% AP diet than for the 2% AP diet (p = 0.047) and a trend to be more abundant than for the 0% AP diet (p = 0.078). Clostridiaceae showed also a significantly higher relative abundance for the 4% AP diet than for the 2% AP diet (p = 0.028); 0% AP was intermediate and not statistically different from 2% AP and 4% AP. Veillonellaceae showed the lowest abundance for the 4% AP diet than for the 0% AP and 2% AP diet (p = 0.009).

Table 19: Relative abundances (% of total bacteria) of families and species of the microbiota in feces of piglets on the 28 post-weaning day.

Phylum-Class Family—Genus (If app.)—Species (If app.)	0%AP	2%AP	4%AP	SEM	*p*-Value KW/FDR
Actinobacteria–Actinobacteria-					
Bifidobacteriaceae	0.0	0.3	0.0	0.06	ns/ns
Actinobacteria–Coriobacteriia-					
Coriobacteriaceae	1.9	1.9	1.8	0.21	ns/ns
Bacteroidetes–Bacteroidia-					
Bacteroidales (undef. fam.)	0.1	0.2	0.3	0.06	ns/ns
Prevotellaceae	2.2	3.2	1.4	0.31	ns/ns
Muribaculaceae (formerly called S24-7)	4.0	2.5	8.7	1.46	ns/ns
(**Paraprevotellaceae**)	0.1	0.1	0.0	0.01	ns/ns
Cyanobacteria–4C0d-2					
YS2 (undef. fam.)	0.1	0.2	0.2	0.04	ns/ns
Firmicutes–Bacilli-					
Enterococcaceae	0.1	0.0	0.0	0.03	ns/ns
Lactobacillaceae	25.3	31.3	23.2	2.91	ns/ns
Streptococcaceae	6.1	3.0	1.8	0.84	ns/ns
Firmicutes–Clostridia-					
Clostridiales (Other fam.)	0.1	0.1	0.2	0.03	ns/ns
Clostridiales (undef. fam.)	3.7	2.7	5.5	0.44	0.099/ns
Christensenellaceae	1.5	0.9	3.1	0.61	ns/ns
Clostridiaceae	3.5 ^{ab}	2.3 ^{b}	5.9 ^{a}	0.64	0.028/ns
Clostridiaceae -SMB53 (undef. sp)	0.6 ^{ab}	0.4 ^{b}	1.1 ^{a}	0.14	0.036/ns
Clostridiaceae (undefined genus)	2.8 ^{ab}	1.6 ^{b}	4.6 ^{a}	0.54	0.019/ns
Dehalobacteriaceae	0.1	0.0	0.1	0.02	ns/ns
Eubacteriaceae	0.0	0.2	0.0	0.05	ns/ns
Lachnospiraceae	16.3	19.8	14.1	1.50	ns/ns
Lachnospiraceae -*Blautia* (other sp.)	0.1 ^{a}	0.0 ^{b}	0.0 ^{b}	0.02	0.024/ns
Lachnospiraceae -*Dorea* (other sp.)	0.8 ^{a}	0.6 ^{a}	0.2 ^{b}	0.12	0.024/ns
Lachnospiraceae -*Lachnospira* (undef. sp)	0.4 ^{ab}	0.4 ^{a}	0.1 ^{b}	0.06	0.036/ns
Peptococcaceae	0.2	0.1	0.2	0.02	ns/ns
Peptostreptococcaceae	0.1	0.1	0.1	0.02	ns/ns
Ruminococcaceae	13.6 ^{ab}	12.9 ^{b}	19.8 ^{a}	1.27	0.047/ns
Veillonellaceae	12.2 ^{a}	11.4 ^{a}	4.8 ^{b}	1.12	0.009/ns
Veillonellaceae -*Dialister* (undef. sp)	2.3 ^{a}	2.1 ^{ab}	0.5 ^{b}	0.32	0.016/ns
Veillonellaceae -*Megasphaera* (undef. sp)	5.7 ^{a}	6.6 ^{a}	2.8 ^{b}	0.59	0.015/ns
Veillonellaceae -*Mitsuokella* (undef. sp)	2.4 ^{a}	1.1 ^{b}	0.6 ^{b}	0.29	0.023/ns
Veillonellaceae -*Mitsuokella multacida*	0.1	0.5	0.1	0.09	0.063/ns
Veillonellaceae -(undef. genus)	0.5	0.3	0.1	0.08	0.064/ns
[**Mogibacteriaceae**]	1.4	1.2	1.6	0.16	ns/ns
Firmicutes–Erysipelotrichi-					
Erysipelotrichaceae	6.6	5.0	5.3	0.62	ns/ns
Erysipelotrichaceae -[*Eubacterium*] *cylindroides*	0.7	0.3	0.6	0.09	0.092/ns
Erysipelotrichaceae -*Bulleidia* p-1630-c5	1.3	0.9	0.6	0.01	0.085/ns
Erysipelotrichaceae -L7A_E11 (undef. sp)	0.0	0.0	0.1	0.03	0.060/ns
Erysipelotrichaceae -(undef. genus)	0.1	0.1	0.2	0.02	0.085/ns
Planctomycetes–Planctomycetia-					
Pirellulaceae	0.2	0.2	0.2	0.08	ns/ns
Proteobacteria–Deltaproteobacteria-					
Desulfovibrionaceae	0.1	0.0	0.1	0.02	0.071/ns
-*Desulfovibrio* (undef. sp)	0.1	0.0	0.1		0.100/ns
Proteobacteria–Gammaproteobacteria-					
Succinivibrionaceae	0.0	0.1	0.0	0.01	ns/ns
TM7–TM7-3-					
F16	0.0	0.1	0.1	0.03	ns/ns
Tenericutes–Mollicutes-					
RF39 (undef. fam.)	0.1	0.1	0.1	0.01	ns/ns
WPS-2—(undefined class)-					
(undef. fam.)	0.0	0.0	0.6	0.18	0.065/ns

Families showing a relative abundance > 0.1% in at least one diet were included. Only species showing a statistical trend or significance were included; relative abundance in % of the total bacteria. $^{a, b}$ values assigned a different letter within a row are statistically different; 0%AP, control diet (n = 6); 2%AP, diet containing 2% dried apple pomace (n = 7); 4%AP, diet containing 4% dried apple pomace (n = 6); if app., if applicable; KW/FDR, Kruskal–Wallis/Benjamini–Hochberg false discovery rate; ns = not significant; undef. fam., undefined family; undef. sp, undefined species.

At the genus/species levels of the classification (Table 19, Supplementary file—Figure S2), two bacteria from the Clostridiaceae family were significantly more abundant in the feces of the 4% AP piglets (SMB 53_undefined species and Clostridiaceae_undefined genus) compared to the 2% AP piglets, with intermediate values for the 0% AP group. In contrast, *Blautia*_other sp., *Dorea*_other sp.—both from Lachnospiraceae family—and undefined species of *Dialister*, *Megasphaera*, and *Mitsuokella*—these three bacteria from the Veillonellaceae family—were more abundant in the 0% AP group compared to the 4% AP; results for the 2% AP diet was statistically not different from 0% AP or 4% AP following the bacteria as seen in Table 19.

Cecal Microbiota Composition on Day 35 Post-Weaning

The indices describing the α-diversity of the cecal microbiota of the chyme (Chao 1, number of operational taxonomic unit and phylogenetic diversity) revealed an increased richness for the 4% AP piglets compared to the 2% AP piglets (p-value significant for the three parameters), the indices for 0% AP were intermediate (Table 20). The weighted Unifrac distance was not different between groups (p = ns, Appendix A).The profile of the microbiota present in the chyme of the caecum (data not shown) was similar between diets except for *Coprococcus* ("undefined species") that was more abundant in the 0% AP piglets (1.3%, p = 0.014) than in the 2% AP piglets (0.6%), while the abundance for the 4% AP piglets was intermediate (1.1%). A trend was also observed for Muribaculaceae (0.1% for 0% AP, 0.1% for 2% AP, 0.2% for 4% AP; p = 0.078) and *Lachnospira* ("undefined species"; 0.1% for 0% AP, 0.4% for 2% AP, 0.5% for 4% AP; p = 0.069).

Table 20: Alpha-diversity indexes of the microbiota from the chyme and the mucosa of the caecum on the 35 post-weaning day.

Index	0% AP	2% AP	4% AP	*p*-Value KW
Chyme				
Chao 1	1103 [ab]	990 [b]	1189 [a]	0.015
OTU	707 [ab]	633 [b]	790 [a]	0.024
PD Whole Tree	40.0 [ab]	36.1 [b]	44.3 [a]	0.030
Shannon	5.4	5.3	5.7	ns
Mucosa				
Chao 1	771 [ab]	668 [b]	849 [a]	0.045
OTU	491	423	527	0.084
PD Whole Tree	34.1	30.2	36.4	ns
Shannon	6.0	5.5	6.1	ns

[a, b] values assigned a different letter within a row are statistically different; 0% AP, control diet; 2% AP, diet containing 2% dried apple pomace; 4% AP, diet containing 4% dried apple pomace; KW, Kruskal–Wallis; ns, not significant; OTU, operational taxonomic unit; PD, phylogenetic diversity.

The indices describing the α-diversity of the cecal microbiota of the mucosa were less influenced by the diet than chyme samples. Chao 1 was the only index of the α-diversity of the microbiota to be statistically higher for 4% AP piglets compared to 2% AP piglets, while 0% AP had an intermediate value (Table 20). The number of operational taxonomic units tended to be more substantial for 4% AP piglets. The Weighted Unifrac distance tended to be different between groups ($p = 0.054$, Appendix A). From a bacterial point of view (Table 21), more differences were visible between diets compared to the chyme results. Firmicutes were more abundant for 2% AP piglets ($p = 0.045$) than for 0% AP and 4% AP piglets; Bacteroidetes were more abundant for 0% AP and 4% AP piglets ($p = 0.049$) than for 2% AP piglets. The third dominant phylum—Proteobacteria—tended ($p = 0.053$) to be more abundant in 0% AP piglets (5.6%) and less abundant in 2% AP piglets (1.3%). Statistical differences or trends were observed for families Muribaculaceae, Pasteurellaceae, Peptostreptococcaceae and Prevotellaceae as well as for species *Acidaminococcus* "undefined species", *Actinobacillus* "other species", *Campylobacter* ("undefined species"), *Coprococcus* "undefined species", *Megamonas* "undefined species", *Mitsuokella multacida*, *Oscillospira* "undefined species" and *Prevotella* "undefined species".

Table 21: Relative abundances (% of total bacteria) of the microbiota from the mucosa of the caecum in piglets on the 35 post-weaning day.

Phylum-Class: Family–Genus–Species	0% AP	2% AP	4% AP	SEM	*p*-Value KW/FDR
Bacteroidetes	**14.6 [a]**	**6.5 [b]**	**13.6 [a]**	**1.27**	**0.049/ns**
Bacteroidia:	**14.6 [a]**	**6.5 [b]**	**13.6 [a]**	**1.27**	**0.049/ns**
Prevotellaceae	11.9	5.2	10.5	1.08	0.060/ns
Prevotellaceae–*Prevotella* (undef. sp.)	3.0 [a]	1.0 [b]	3.6 [a]	0.36	0.010/ns
Muribaculaceae (formerly called s24-7)	0.2 [ab]	0.1 [b]	0.3 [a]	0.03	0.037/ns
Firmicutes	**78.3 [b]**	**90.1 [a]**	**82.4 [b]**	**1.68**	**0.045/ns**
Clostridia:	**46.5**	**49.4**	**54.5**	**2.02**	**ns/ns**
Lachnospiraceae–*Coprococcus* (undef. sp.)	0.7	0.5	0.9	0.07	0.073/ns
Peptostreptococcaceae	0.1	0.1	0.1	0.01	0.074/ns
Ruminococcaceae–*Oscillospira* (undef. sp.)	0.2 [a]	0.1 [b]	0.3 [a]	0.03	0.037/ns
Veillonellaceae–*Acidaminococcus* (undef. sp.)	0.1	0.2	0.3	0.04	0.088/ns
Veillonellaceae–*Megamonas* (undef. sp.)	0.4	3.5	0.1	0.97	0.070/ns
Veillonellaceae–*Mitsuokella multacida*	0.0	0.5	0.1	0.10	0.094/ns
Proteobacteria	**5.6**	**1.3**	**3.0**	**0.63**	**0.053/ns**
Epsilonproteobacteria:	**3.2**	**0.9**	**1.4**	**0.39**	**ns/ns**
Campylobacteraceae–*Campylobacter* (undef. sp.)	2.5	0.5	0.9	0.34	0.083/ns
Gammaproteobacteria:	**2.1**	**0.3**	**1.4**	**0.33**	**ns/ns**
Pasteurellaceae	0.2	0.0	0.0	0.03	0.057/ns
Pasteurellaceae–*Actinobacillus* (Other sp.)	0.1 [a]	0.0 [b]	0.0 [a]	0.02	0.030/ns

[a, b] values assigned a different letter within a row are statistically different; 0% AP, control diet (n = 6); 2% AP, diet containing 2% dried apple pomace (n = 7); 4% AP, diet containing 4% dried apple pomace (n = 6); KW/FDR, Kruskal–Wallis/Benjamini–Hochberg false discovery rate; ns, not significant; OTU, operational taxonomic unit; PD, phylogenetic diversity; undef. sp., undefined species. Are shown in the table the bacteria with statistical differences.

Discussion

Zootechnical Performance and Intestinal Morphology

During the post-weaning period, AP improved the ADG of piglets on week 3. At the end of the post-weaning period, only piglets that had received the 4% AP diet showed a lower FCR and EFE compared to 0% AP piglets, which may partially be explained by the improved intestinal morphology, i.e., a higher villus length in the duodenum and a higher VL/CD in the ileum compared to the 0% AP piglets. Indeed, the villus length/crypt depth ratio is a good indicator for estimating the likely digestive capacity of the small intestine (Montagne et al., 2003). These observations are in line with those of Sehm and colleagues (Sehm et al., 2007) who showed a beneficial effect of 3.5% of dry AP on the villus height at certain time points of the post-weaning period. It could be hypothesized that this improved small intestinal morphology may be explained by the higher level of reducing sugars in the AP diets (mainly in the 4% AP diet), particularly due to fructose from AP. Indeed, AP is rich in fructose and it was demonstrated—in humans—that fructose is primarily metabolized in the small intestine (Jang et al., 2018), where it is an interesting source of energy for the enterocytes. As the zootechnical performance at the end of post-weaning for the 2% AP piglets were similar to those of 0% AP piglets and as the intestinal morphology results were intermediate and statistically not different from those of 0% AP and 4% AP piglets, reducing the level of incorporation of AP in the diet seems not appropriate concerning zootechnical performance mainly due to a stunted growth at the beginning of the post-weaning period.

Scoring of Piglet's Fecal Consistency and Excreted Pathogens

On the eighth post-weaning day, more pathogens tended to be detected in the feces of piglets receiving AP (through the Rainbow kit), together with the presence of numerically softer/liquid feces during the first two post-weaning weeks. It is known that healthy individuals can live in equilibrium with pathogens without clinical symptoms as long as there is equilibrium between the host and its entire microbiota (Delarras, 2014). On the one hand, the softer and liquid feces observed could be due to the pathogens (Montagne et al., 2003), which can represent a threat for the piglets' health, leading to a potential use of antibiotics. On the other hand, we cannot exclude the hypothesis that the softer feces can be due to the fiber composition of the AP diets. Indeed, the water holding capacity of some fiber (Brambillasca et al., 2015) and the possible abrasive effect of large/coarse fiber (McRorie and McKeown, 2017) may affect the intestinal mucosa and the consistency of the feces. This invites further unravelling the reasons why softer feces are observed with AP to confirm or refute the risk of an increasing use of antibiotics with AP, which can compromise the use of this ingredient as a weaning strategy. Under the good sanitary conditions offered during the experiment, pathogens did not appear as a threat and were not the cause of poor performance. However, it would be of great interest to test AP in large cohort to assess the risk of dysbiosis with a 4% AP diet and confirm or refute the ability of AP to maintain homeostasis or the absence of clinical symptoms.

Microbiota and SCFA in Feces and Cecum

Although some bacterial indicators on the 8 post-weaning day may appear less favourable with AP (e.g., Enterobacteriaceae more abundant in 2% AP feces than 0% AP feces or Lactobacillaceae less abundant with AP), several indicators point to a positive effect of a 4% AP addition in the post-weaning diet.

Firstly, the richness of the microbiota in the feces seems better for the 4% AP diet for this critical period (eighth post-weaning day), which is an important factor for health (Dominguez Bello et al., 2018), increasing ecological stability and resilience after a stress-related disturbance (Hemarajata and Versalovic, 2012). Furthermore, this higher α-diversity for the 4% AP group was clearly observed in the chyme and on the mucosa of the caecum on the 35 post-weaning day.

Secondly, the second most abundant phylum in the feces for the 4% AP piglets on the eighth post-weaning day was Bacteroidetes which can also be considered as positive, seen their potential for microbial enrichment (Thomas et al., 2011) or metabolism modulation for human health (Rios-Covian et al., 2017). In line with this high relative abundance of Bacteroidetes in feces of piglets from the 4% AP diet on the 8 post-weaning day—reflecting the fermentation process at the end of the large intestine—the Bacteroidetes were also highly present in the cecal mucosa on the 35 post-weaning day—as a result of the fermentation process at the beginning of the large intestine. It should however be noted that in the cecal mucosa on the 35 post-weaning day, the relative abundance of the Bacteroidetes were also high in the 0% group, as compared to the 2% AP group, but the Bacteroidetes were the third phylum in feces of 0% AP and 2% AP piglets, on the 8 post-weaning day. However, caution is required when interpreting the results for Bacteroidetes because this phylum also includes bacteria that can become problematic for health, as seen with *Prevotella copri*, acting in a beneficial or detrimental manner depending on the context (Ley, 2016; Wexler, 2007).

Thirdly, the higher abundance of Clostridia in the feces of the AP group on the eighth post-weaning day further supports the beneficial effect of AP, seen their importance for the maintenance of immune and gut homeostasis (Lopetuso et al., 2013; Spees et al., 2013).

Fourthly, the higher relative abundance of the Ruminococcaceae in the feces of 4% AP piglets compared to those of 0% AP and 2% AP piglets on the 28 post-weaning day, is in line with a possible improved resistance/tolerance to pathogens (Schroyen et al., 2016). In the cecal mucosa again, both the 0% and 4% AP groups had the highest relative abundance of Ruminococcaceae compared to the 2% AP group.

Lastly, although only a trend, the higher relative abundance of Proteobacteria in the mucosa of the caecum of 0% AP piglets on the 35 post-weaning day together with the lowest relative abundance of Firmicutes—compared to the AP supplemented piglets—may indicate towards a state of microbial dysbiosis, as also observed in young layer chicks (Mon et al., 2015).

It is worthy to note that the AP treatment seemed to affect more the composition of the microbiota of the mucosa than of the chyme and the composition of the microbiota in the 2% AP group did overall seem to differ more from the 0% and 4% AP groups.

Remarkably, no differences in SCFA between the different groups were observed in the feces on the 8 and 28 post-weaning days or in the caecum on the 35 post-weaning day. It is unclear why the differences in the diversity and composition of the microbiota seemed not to be reflected in changes in the SCFA.

AP as a Weaning Strategy for Gut Health

Taking together the data on performance, intestinal morphology and microbiota, the 4% AP diet showed potential to use as a feed ingredient for the post-weaning period in the experimental conditions applied, while the 2% AP diet seemed less interesting at the beginning of the post-weaning. It is uncertain which component of the AP was beneficial for the intestinal morphology and modulated the microbiota. AP used in the experiment was fairly rich in fructose, leading to more reducing sugars in the AP diets. As fructose may give energy to the small intestine (Jang et al., 2018) during the feed transition and was combined with an extra oil in AP diets, it may have affected the gut morphology. In this way, using a 4% AP diet during the post-weaning influenced positively the digestive process in the small intestine. In addition, the literature shows that the profile in dietary fiber of AP offers healthy components for the large intestine (Boyer and Liu, 2004), amongst which pectin is known to exhibit positive effects on gut health parameters such as immunomodulation (Popov and Ovodov, 2013) or microbiota modulation (Larsen et al., 2019), due to its impact on the digestive and fermentative processes (Tian et al., 2017). However, the content of glucuronic acid and galacturonic acid in AP was rather low, which leaves the question if the pectins (<1 g/100 g DM) or the oligosaccharides (1 g/100 g DM of arabino-, xylo-, and galacto-oligosaccharides)—oligosaccharides that may also influence the immune response (Bland et al., 2004)—played a prebiotic effect (Al-Tamimi et al., 2005; Maki et al., 2012; Olano-Martin et al., 2002). Other important bioactive components of AP are the polyphenols; the AP used in the experiment seemed well endowed with it compared to cultivars quantified in the literature—twice (Li et al., 2020) to five times more (Sato et al., 2010) than values found in the literature. In their review on polyphenols—gut microbiota and health-, Espin and colleagues (Espín et al., 2017) stated the emerging concept of 3P for gut health (probiotic, prebiotic and polyphenols) that promotes polyphenols to the same biological level of prebiotics. They explained the two-way interaction linking polyphenols and guts microbiota. Polyphenols shape the microbiota—enhancing the presence and abundance of bacteria beneficial for health—and microbiota catabolizes the polyphenols into metabolites often more active and absorbed by the colon than native forms. In a rat experiment on apple pectin and a polyphenol rich fraction of apple extract, Aprikian and colleagues (Aprikian et al., 2003) concluded that their combination is more effective than their separate supplementation. We support this hypothesis that AP exerts beneficial effects for health by its global matrix effect (involving fructose,

oligosaccharides and other prebiotic components, and polyphenols) influencing in its entirety the digestive and the fermentative process in piglets.

The "gut health" principle consists of the equilibrium between the diet, the host (epithelium, mucus layer and gut-associated lymphoid tissue) and its microbiota (commensal bacteria and transient bacteria, including pathogens) (Montagne et al., 2003). The AP diets still need to be evaluated from a "host response" point of view to fully appreciate the effect of such a diet at weaning. Indeed, many mechanisms related to the barrier properties of the gastrointestinal tract are to be explored, such as permeability of the epithelium or interleukins and growth factors secretion (Moeser et al., 2017). From the bacterial equilibrium observed in the study, referring to the work of Spees and colleagues (Spees et al., 2013), a beneficial effect of the 4% AP diet can be expected on some immune factors. Moreover, Sehm and colleagues (Sehm et al., 2007) showed a beneficial effect of AP on gut-associated lymphoid tissue by reducing its activity.

Emerging Concept from the Results

The nutrients in AP may give a beneficial effect on the critical step of piglet's weaning due to its monosaccharides, fiber, and polyphenols content by improving the energetic absorption—in the small intestine—and by modifying the gut microbiome.

At weaning, the digestive system of the piglet undergoes structural (intestinal architecture) and functional (enzymatic baggage) changes. The infant intestinal structure is replaced by a mature intestinal structure that is adapted to the new diet (Heo et al., 2013; Pluske et al., 2003). We hypothesized that during this transition, the monosaccharides profile of AP maintained an energetic absorption—despite the global energetic deficiency status of piglet—by compensating the loss of the highly digestible carbohydrates of the milk.

Moreover, due to the weaning perturbations, the large intestine is potentially overloaded with readily fermentable nutrients—starch becoming a main fermentative substrate for the microbiota instead of the non-starch polysaccharides (Bach Knudsen et al., 2012). By incorporating AP, the level of sugars in the diet progressively increased contrarily to the level of starch that decreased and AP appeared beneficial in this way, enabling a good absorption of monosaccharides in the small intestine.

As a last identified consequence of adding AP, the dietary fiber and polyphenolic compounds (Grigoras et al., 2013; Lu and Foo, 1997)—known to interact with the microbiota and to have beneficial health effect (Espín et al., 2017)—modulated the intestinal ecosystem so that the new balance resulted in healthy piglets, at least in this small study.

Conclusions

AP, at a level of 4% of incorporation, had positive effects on piglet's performance, intestinal morphology and microbiota during the post-weaning period. A lower level of inclusion of AP—set at 2%—did not appear sufficient to induce these changes. AP constitutes a matrix of high interest for the feed sector due to its composition in dietary fiber (including oligosaccharides), biomolecules beneficial for health (as polyphenols), and probably also through its reduced sugars profile (fructose content). The results suggest that AP could be used as a suitable weaning strategy for gut health although the impact of the 4% AP diet on the gut epithelium and immune system need yet to be investigated. AP needs also to be investigated in large cohort to better evaluate the bacterial load of 4% AP diet in the context of the reduction of antibiotics in animal production and the phase-out of zinc oxide in weaner diets.

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The authors thank Martine Laitat (FARAH, ULiège) for her help in feces scoring, Richard Agneessens (CRA-W) for the feed chemical analyses, DNA Vision and SCAR for the collaboration, Maxence Didelez (CRA-W) for the experimental phase, the "pig team" of CRA-W and all collaborators (CRA-W and Gbx-ABT) for the help the last day of the experiment.

Data Availability Statement

The datasets analyzed for this study can be found in the ENA database (https://www.ebi.ac.uk/ena/browser/home, 30 June 2020) under the accession number PRJEB38962.

Appendix A

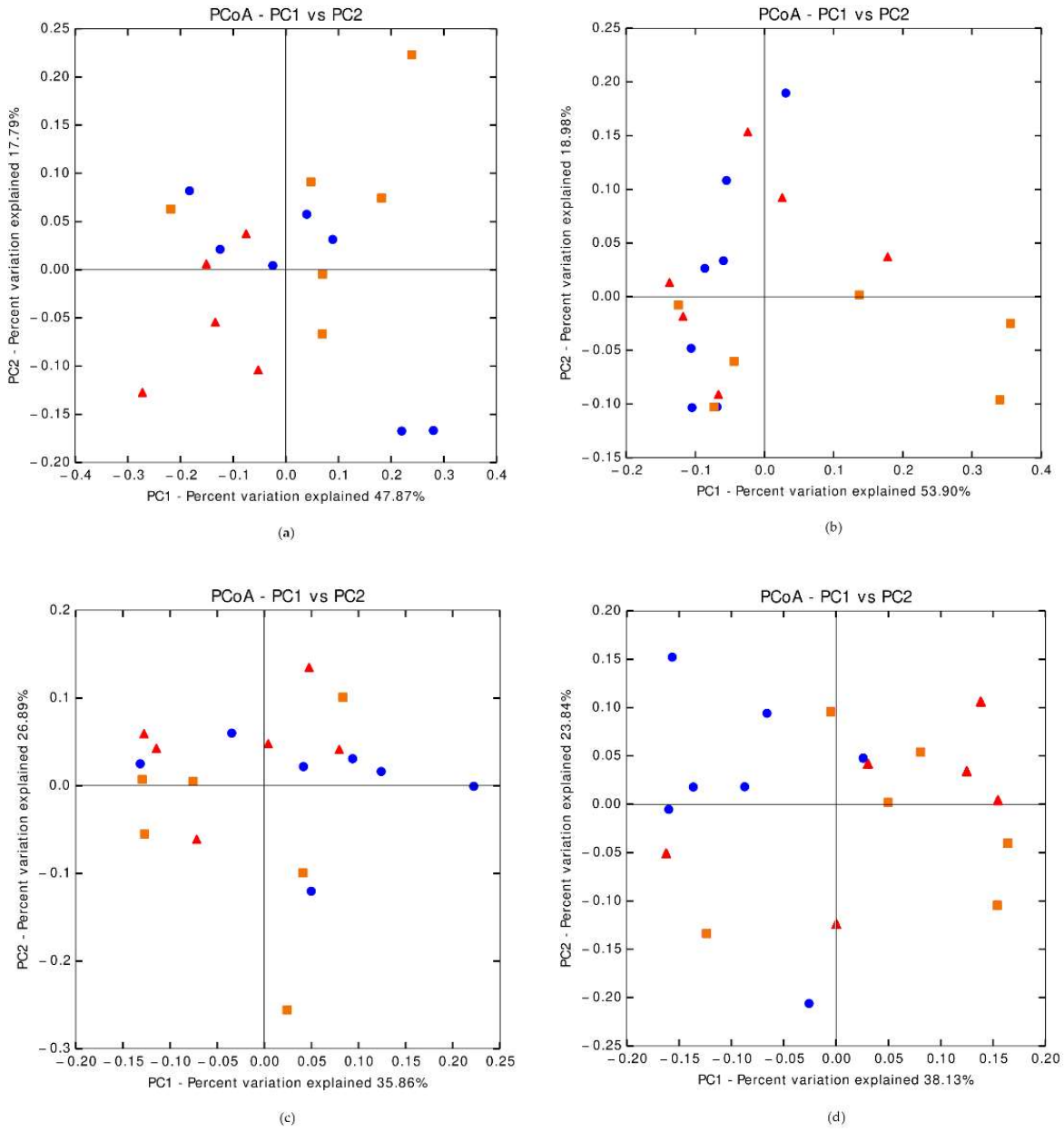

Figure A1. Principal Coordinate Analysis of beta diversity. (a) Weighted Unifrac distance for fecal samples on the 8 post-weaning day (p = 0.052); (b) Weighted Unifrac distance for fecal samples on the 28 post-weaning day (p = 0.069); (c) Weighted Unifrac distance for chyme samples from caecum on the 35 post-weaning day (p = 0.244); (d) Weighted Unifrac distance for mucosal samples form caecum on 35 post-weaning day (p = 0.054); triangle = 0%AP diet, circle = 2%AP diet, square = 4%AP diet.

Appendix B

Table A1. Relative abundance of family and genus of microbiota feces on day 8 post-weaning influenced by the diets.

Bacteria	0% AP	2% AP	4% AP	*p*-Value KW/FDR
Feces on day 8 post-weaning (family)				
Coriobacteriaceae	1.6%	4.5%	2.5%	0.080/ns
Lactobacillaceae	45.5%	25.5%	23.1%	0.053/ns
Enterobacteriaceae	0.0%	0.1%	0.4%	0.020/ns
Feces on day 8 post-weaning (genus)				
Dorea	1.3% [a]	0.2% [b]	0.7% [ab]	0.024/ns
Slackia	0.0%	0.1%	0.0%	0.039 [1]/ns
Ruminococcus	1.4%	0.6%	1.0%	0.049 [1]/ns
Catenibacterium	0.5%	0.7%	4.3%	0.037 [1]/ns

[a, b] values assigned a different letter within a row are statistically different; 0% AP, control diet (n = 5); 2% AP, diet containing 2% dried apple pomace (n = 7); 4% AP, diet containing 4% dried apple pomace (n = 6); KW/FDR, Kruskal-Wallis/Benjamini-Hochberg false discovery rate; ns, not significant. [1] the result of the multiple comparisons Kruskal-Wallis test performed was not significant.

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Supplemental material

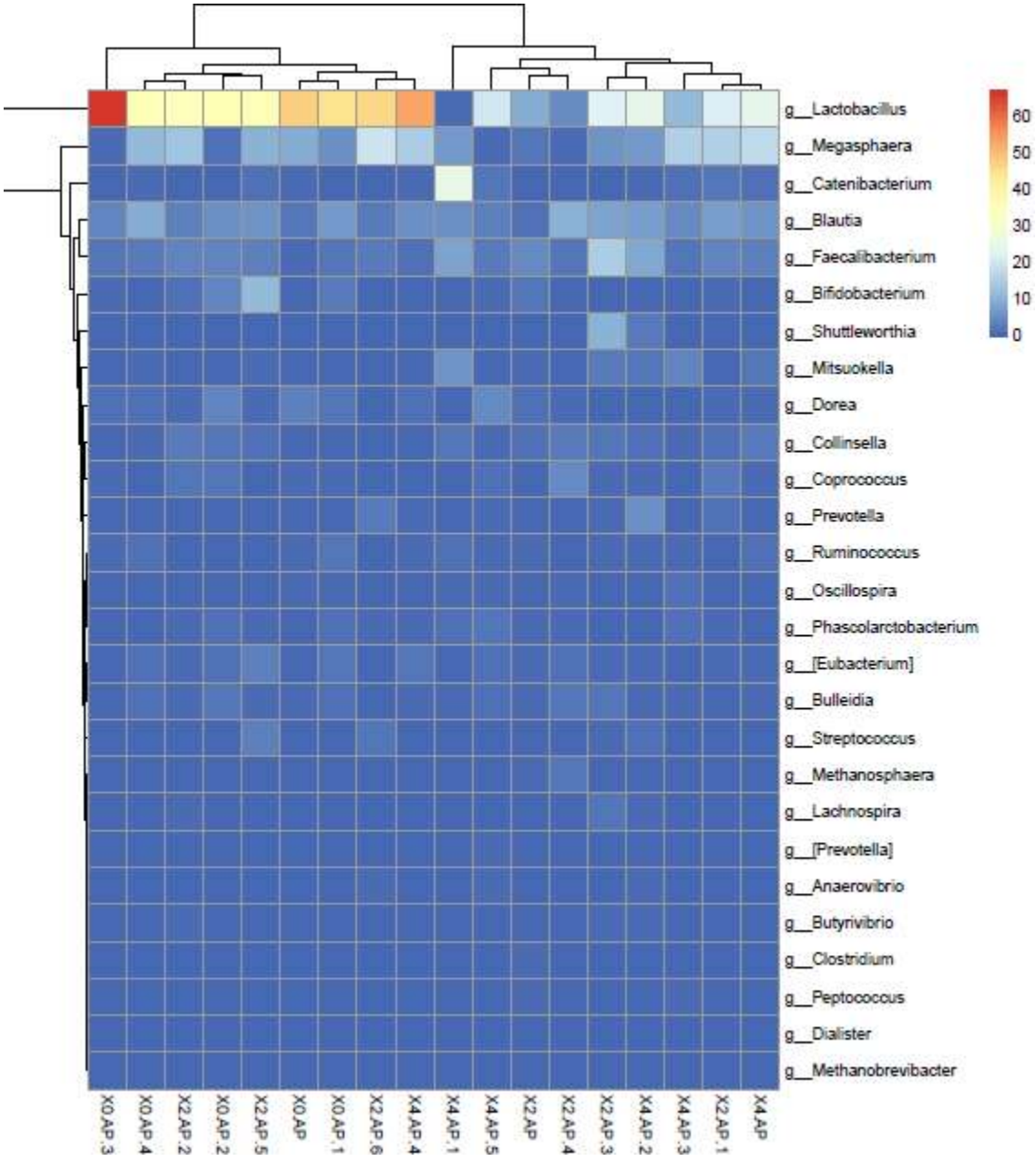

Figure S1: Genus abundance Heat Map for faeces on the 8 post-weaning day. Genera with at least 0.5% of relative abundance in one sample for each diet were used. X0.AP: 0%AP diet; X2.AP: 2%AP diet; X4.AP: 4%AP diet.

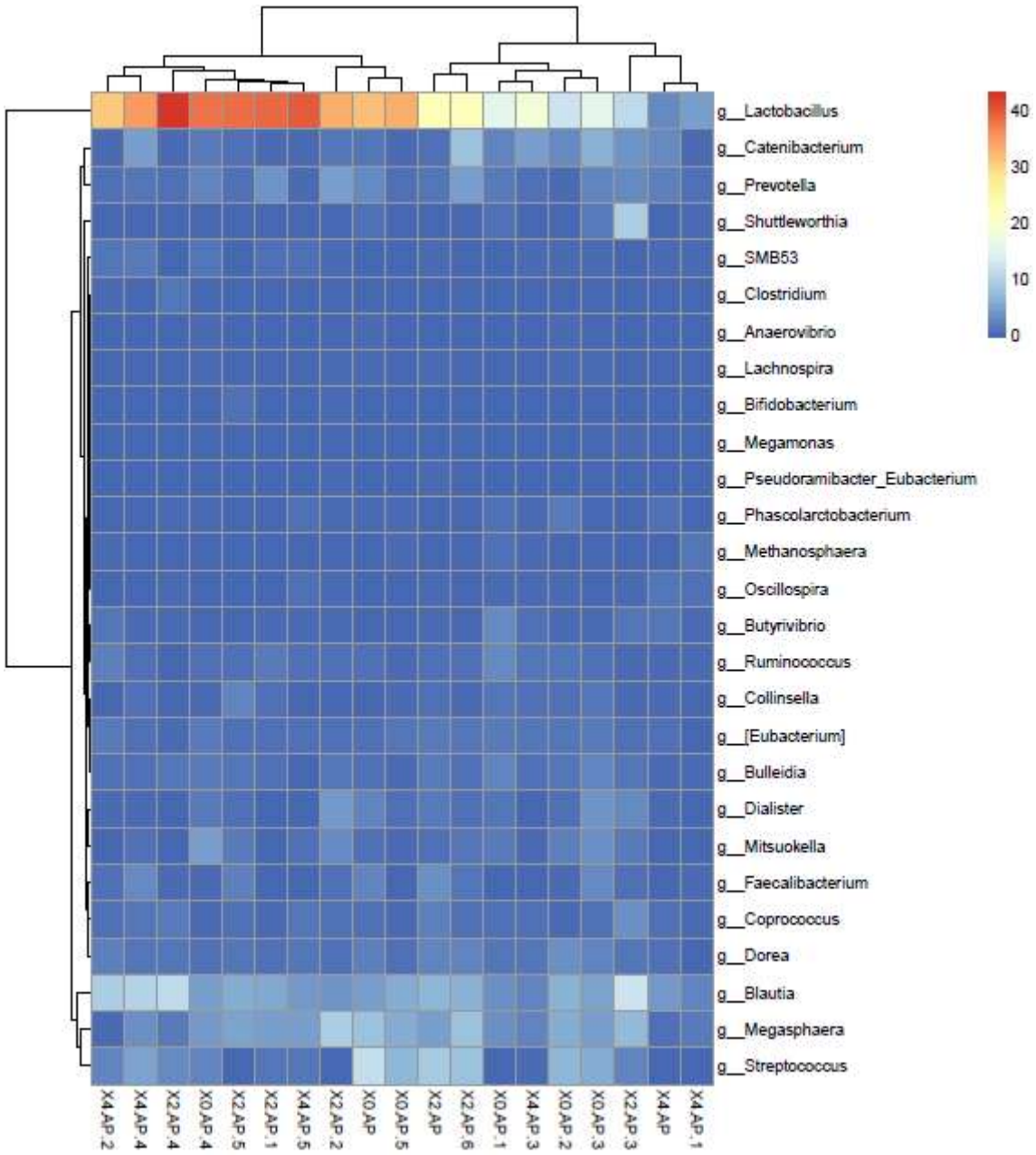

Figure S2: Genus abundance Heat Map for faeces on the 28 post-weaning day. Genera with at least 0.5% of relative abundance in one sample for each diet were used. X0.AP: 0%AP diet; X2.AP: 2%AP diet; X4.AP: 4%AP diet

Discussion–Perspectives

Overview

The aim of the thesis was to develop an in vitro gastrointestinal model of the piglet including weaning in order to test the interest of AP as a feed ingredient around weaning. The underlying hypothesis was an effect of AP–due to its chemical composition–on the intestinal microbiota and the products of its fermentation in such a way as to reduce intestinal weaning disorders. In addition, AP has been used in an animal experiment to obtain in vivo data. Prior to this work, a technical constraint had to be lifted, namely to measure the SCFA in the samples collected. The main results–achievement of technical objectives (TO) and scientific hypotheses (SH)–are illustrated in Figure 23. In summary, the method to quantify SCFA from samples was developed considering the constraint of the laboratory. SCFA in samples coming from an experimental run of the SHIME were successfully quantified using an SPME-GC-MS method (study 1). The baby-SPIME model was developed (study 2). From that study, we can conclude that the fermentation process in the ileum bioreactor needs to be improved but the hypothesis that the type of inoculum was the main factor to be modified could not be validated by the obtained results (study 3). Apple pomace was however tested and an effect of the matrix was observed both on SCFA and microbiota profiles. In vivo, the use of AP didn't have an effect on SCFA but had on the microbiota. Moreover, effects were also observed on performance and intestinal morphology, confirming beneficial effect of AP for the post-weaning period. These results will be discussed below.

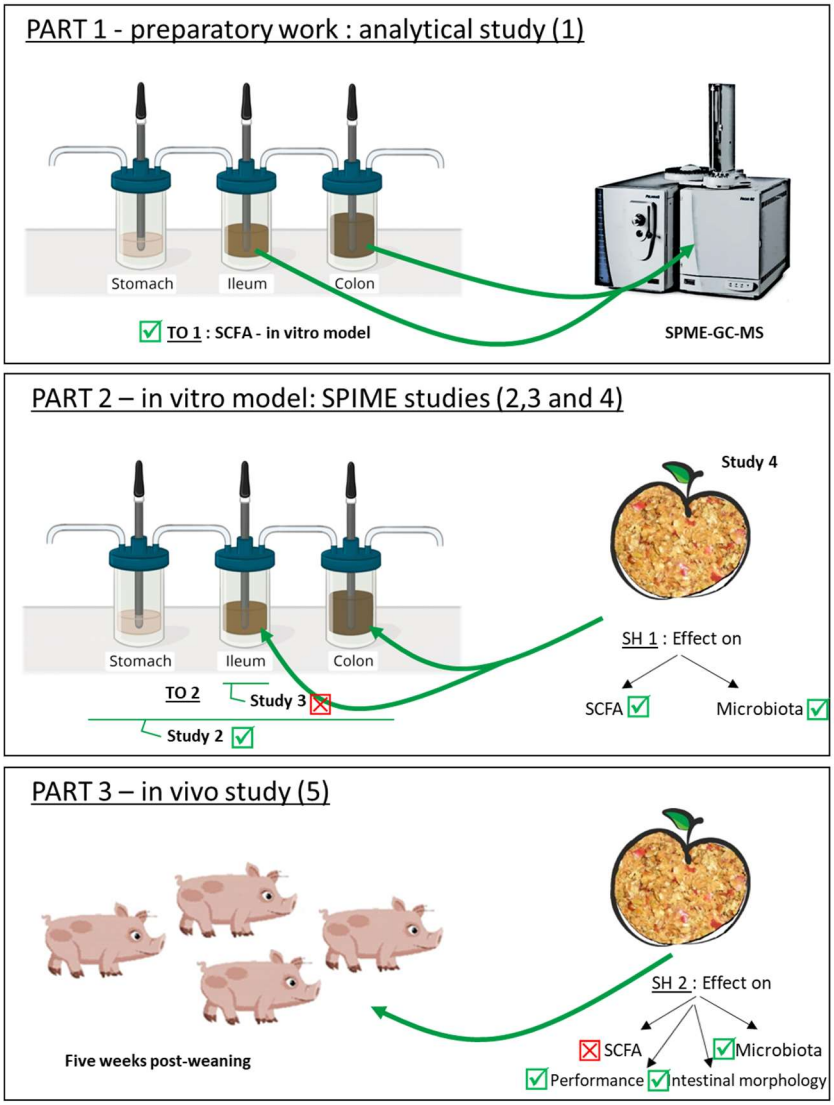

Figure 23: Illustration of the main results obtained during the thesis (TO: technical objective, SH: scientific hypothesis, SCFA: short chain fatty acids).

Effects of AP in vitro and in vivo around weaning and for post-weaning

- **In vitro**

The use of AP in bioreactors had an impact on the production of SCFA and on the microbiota. The ratio of propionate increased in the ileal bioreactor while that of butyrate decreased. And the culture of bacteria from the phyla Bacteroidetes and Verrucomicrobia (through *Akkermansia muciniphila*) was favored over those of Bifidobacteriaceae (*Bifidobacterium*) and Firmicutes. We know from the literature that propionate and butyrate are fermentation products from distinct subsets of gut bacteria, unlike acetate which is a fermentation product of most gut anaerobes (Louis and Flint, 2017). Propionate is well an end product of the metabolic pathways involving Bacteroidetes and *Akkermansia muciniphila* (Reichardt et al., 2014). Zhang et al. (2018) showed that an infusion of propionate in the caecum of piglet led to a decrease in the production of butyrate in the colon and that the composition of the colonic

microbiota was modified–Bacteroidetes increased and Firmicutes decreased; which is consistent with our in vitro observations. In mice, observations on the microbiota–similar to those made in the baby-SPIME with AP–were done with Concord grape polyphenols. *Akkermansia muciniphila* increased and the ratio of Firmicutes to Bacteroidetes decreased, resulting in lower intestinal and systemic inflammation (Roopchand et al., 2015). It shows that many mechanisms of action of AP still have to be elucidated despite its known antioxidative, anti-inflammatory, antibacterial and antiviral activities (Lu and Foo, 2000; Skinner et al., 2018; Waldbauer et al., 2017).

In a panel of fructan- and pectin- rich by-products, AP did not appear in other in vitro studies as a promising ingredient for its fermentative capacity for health purposes (Uerlings et al., 2019). In our study, however, AP showed that when added to a "control" diet, the propionate pathway seemed to be favored during the fermentative process. Propionate is known to be an efficient gluconeogenic substrate in the gut and liver (Koh et al., 2016) and known to impact glucose-6-phosphatase activity in the jejunum (De Vadder et al., 2014), an enzyme that ends the glycogenolysis cycle by releasing glucose (Raisonnier, 2003). Moreover, propionate acts on the nervous and immune systems (Li et al., 2017)–by for example increasing the expression of inflammation-related genes NF-κB and IL-18 (Zhang et al., 2018) or by acting on the regulation of Treg cells that have an immunosuppressive activity and play a key role in the regulation of intestinal inflammation (Chénard et al., 2020). In addition, in vitro AP seemed to favor bacteria from Bacteroidetes phylum and *Akkermansia muciniphila*. Some of these bacteria are known to maintain intestinal immune homeostasis in human gut health (Maier et al., 2015). Thus, AP may have an impact on the energetic metabolism and on the immune system of piglets–two fundamental issues at weaning. However, in a study of Karasova et al. (2021), a microbiota enriched with *Akkermansia* amongst other bacteria appeared to be linked to diarrheic post-weaning piglets while a microbiota enriched in *Prevotella* appeared to be linked to healthy piglets. In vitro, an increase in both is observed in the study. A significant effect is observed for Prevotellaceae, known to be a post-weaning indicator of healthy piglets (Guevarra et al., 2019).

A particular mode of fermentation of AP to SCFA was observed in the baby-SPIME for the ileum and colon bioreactors. Indeed, we observed a decrease in the concentration of SCFA from the first to the second when testing AP in bioreactors compared to the control diet, suggesting that AP could be more advantageous at the beginning of the fermentation process regarding the SCFA production and be involved in the production of other metabolites later in the fermentation process. Apple pomace was used without the dialysis module or without being dialised before entering the system. Probably this step may improve the fermentation process. But an in-depth-study remains interesting to investigate the polyphenols bioaccessibility of AP in the ileum and the colon bioreactors as done in other in vitro model as TIM (Etcheverry et al., 2012). Indeed, in vitro, it was demonstrated that polyphenols can have significant effects on the abundance of bacteria communities (Grant et al., 2020). In vivo, polyphenols are known to reach the colon almost unaltered where they are catabolized by the gut microbiota to bioactive metabolites (Espín et al., 2017). Polyphenols can regulate gut microbial ecology, making it

more conducive to health by increasing the abundances of probiotics (Ma et al., 2020). Confirming through a new experimental study the possibility of evaluating polyphenols in the colon bioreactors of the baby-SPIME would be an additional argument in favor of the model and it would be of great interest to improve knowledges about by-products fermentation such as AP.

- **In vivo**

The use of AP in weaning diets (2% and 4% dried AP) led to the observation of multiple parameters concerning performance, intestinal architecture and microbiota (in faeces 8 and 28 days post-weaning and from the intestine, 35 days post-weaning).

Apple pomace diets were beneficial for the average daily gain calculated at week 3 and the gut architecture in duodenum and ileum at day 35 post-weaning. A diet including 4% AP was beneficial for the FCR and the EFE at day 35 post-weaning.

Faeces were scored up to day 15 post-weaning during which AP piglets showed a trend towards softer to watery faeces. They showed a trend to shed more pathogens on day 8 post-weaning. The diet including 4% AP influenced the richness of the microbiota and the bacterial profile. On day 8 post-weaning in faeces, the second most abundant phylum for 4% AP piglets was Bacteroidetes instead of Actinobacteria for 0% AP and 2% AP piglets.

At day 35 post-weaning, the effect of AP on microbiota was particularly observed in the caecum but it is possible that due to the reduced sample size for this intestinal portion (n=4, data not shown) no statistical significance was observed for the colon. Moreover, in the caecum, the chyme microbiota was quite similar for the different diets. The most contrasting effect was observed between the 2% AP diet and the 4% AP diet for mucosal samples. Indeed, 2% AP piglets showed the lowest microbial diversity indices while 4% AP piglets showed the highest; those of 0% AP piglets being intermediate and not different. And the composition of the microbiota in the 2% AP group did overall seem to differ more from the 0% and 4% AP groups.

Regarding Bacteroidetes, 4% and 0% group showed higher relative abundance compared to 2% AP. But a strong difference between the 4% and 0% groups lied in the trend to observe more Proteobacteria in 0% AP piglets in the caecum. Moreover, the mucosa of the proximal colon (n = 4, data not shown) of 0% AP piglets contained 9.9% of Epsilonproteobacteria, a class of Proteobacteria that has to be carefully examined for health human issue (Cornelius et al., 2012). Epsilonproteobacteria includes *Campylobacter* and *Helicobacter*, both associated with human and/or animal digestive pathologies (Bohr et al., 2002; De Witte et al., 2017a; Haesebrouck et al., 2009; Lee et al., 2016; Pointon, 1989). This class of bacteria was less abundant in 2% AP piglets (7.4 %, n=6) and 4% AP piglets (4.8 %, n=4).

Surprisingly, no effects of AP were observed on SCFA between groups in faecal or intestinal samples.

- **In vitro *vs* in vivo**

Williams et al. (2019) explains in their review on dietary fiber that the best approach to obtain a comprehensive overview of the mechanisms and effects of dietary components on health would be to

integrate findings from epidemiology, in vivo, in vitro, and clinical studies. In the present work, we had the opportunity to discuss in vitro and in vivo data together and to try to better understand the impact of AP on piglets.

Among the specific points that seemed important to discuss, the first concerns SCFA production. The proportion of propionate increased in vitro with AP while no effects were observed in vivo in the caecum. Hosseini et al. (2011) suggests that propionate production can be fully appreciated in vitro because of the absence of intestinal absorption. Also an accumulation of the metabolites is expected in the bioreactor (Venema and van den Abbeele, 2013). Other analysis could be considered in vivo, for example the study of the activity of glucose-6-phosphatase in the small intestine, to establish the link with an expected effect of propionate on the intestine. We hypothesized that the content in fructose of AP may have an impact on the improved performance (FCR and EFE), which is a source of energy for the small intestine. The in vitro results, through the effect of AP on the level of propionate, tended to show that other mechanisms of energy metabolism of piglets could also be involved to valorize the feed more efficiently.

Secondly, the phylum Bacteroidetes seems to play a key role in the fermentation process of AP. In vitro, a significant effect was demonstrated through the increase of Prevotellaceae (otherwise, non-significant increases were observed). In vivo, Bacteroidetes also seemed to play a particular role as seen yet in faeces of 4% AP piglets on the 8 post-weaning day.

Thirdly, the effect of AP Ruminococcaceae family in particular was contradictory by observing in vitro *vs* in vivo results. While the abundance increased in the 4%AP group, in vitro AP led to a decrease. Ruminococcaceae being a post-weaning indicator of healthy piglets (Guevarra et al., 2019), this divergence between both models is problematic.

Fourthly, the kinetics of fermentation of AP in baby-SPIME showed the interest to investigate the fermentation of AP in vitro and in vivo in depth to better assess the kinetics of fermentation of polyphenols.

Finally, in vivo data showed that 4% AP piglets carried more pathogens in faeces on day 8 post-weaning. Should this be viewed as a threat? In the in vivo experiment, the microbiota of the piglets tended to more richness; performances were not affected and no antibiotic treatments were necessary. In an experimental human challenge model, enterotoxigenic *E. Coli* infection significantly induces intestinal and systemic inflammation in mild to severe diarrhea subjects as well as in asymptomatic subjects (Brubaker et al., 2021). Testing a mixture of plant polyphenols on weaned piglets, Jiang et al. (2014) showed that supplementation may improve the antioxidant capacities of piglets challenged with *Escherichia coli*. Different mechanisms probably occurred in vivo to explain the “non-negative” effect observed on 4% AP piglets. They could be related to the polyphenol content of AP or with such other components as triterpens (data not shown) that are known for their positive effects on health (Andre et al., 2012). In vitro data suggested a possible way of action through propionate. An experiment on large cohort is now required to confirm or refute health problem at weaning.

Baby-SPIME model

In vitro models are useful tools that evolve over time. Scientists are constantly improving the tools. In the animal feed sector, different models can be used with their own advantages and limitations. As part of the thesis, the opportunity to use a dynamic multi-compartment model arose to investigate research questions related to intestinal disorders in piglets around weaning. By choosing this model, the study was able to focus on the fermentative process of a piglet microbiota and thus attempt to include a microbial transition as observed during weaning in vivo when plant ingredients are included in the feed of piglets. Due to the fact that diet is known to influence the gut microbiota in its composition and function as well as the production of microbial-associated small molecules and metabolites (Li et al., 2017), the model was seen as an important tool to study both the microbiota and its metabolites. On the one hand, the gut microbiota has emerged as an important immunologic and metabolic organ influencing animal health and disease (Aluthge et al., 2019). On the other hand, bacterial metabolites, in particular SCFA, that are produced are important energy and signaling molecules for the host (Koh et al., 2016). So, the use of the model can give information about groups of bacteria favored by a diet and it can give indications on the quantity and quality of the metabolites produced. However, a model remains a model with all its limitations and possible biases.

Among the biases identified in baby-SPIME model was an under-representation of Bacilli within Firmicutes, an under-representation of Gamma-Proteobacteria in favor of Delta-Proteobacteria within Proteobacteria. Moreover, no Epsilon-Proteobacteria were observed. Conversely, Fusobacteria were overrepresented. In post-weaned bioreactors, Firmicutes decreased whereas they should rather have increase. Conversely, Actinobacteria and Fusobacteria should have decreased (Chen et al., 2017; Yang et al., 2019). During the development of the baby-SPIME, the physiological parameters applied in each bioreactor were set within an acceptable range before and after weaning. This made it possible to modify only one parameter in the study, in this case, the diet. This approach was justified to avoid variations related to the influence of secondary parameters on the experimental results. It is a choice but it is also a limit because, in vivo, parameters–likely to affect the microbiota such as pH or transit time-evolve (Heo et al., 2013; Snoeck et al., 2004a) and it appears as a source of bias. Moreover, other important factors can be cited as limitations and have now to be improved. First of all, integrate complex nutrients in culture media in the absence of a dialysis membrane induced an accumulation of metabolites in the medium that reached the ileum and colon bioreactors. Most models described in the literature includes this absorption step. Models–that do not have one–eventually overcome it because this limitation can, for example, induce an overestimation of some components such as some phenolic compounds (Wojtunik-Kulesza et al., 2020), 5 to 10% of the overall ingested polyphenols being yet absorbed in the small intestine (Ray and Mukherjee, 2021). The molecules that are normally absorbed are unavailable as substrate for the microbiota in vivo while they serve as cross-feeding substrate in bioreactors. Although dialysis membrane seems still to be oversimplified (Wojtunik-Kulesza et al., 2020), the baby-SPIME model could be improved by using the dialysis system to mimic passive

absorption and so better simulate the nutrient uptake that occurs in the small intestine. Another way to proceed could be to calculate the exact unabsorbed portion of the food/feed to be administered in the model as it is the case with the media of the classic SHIME system. Indeed, the media used in baby-SPIME were prepared to maximize the presence of components that are not absorbed by the intestine (50%). A part of the complex piglet diet was however maintained to keep all the complex nutrients of the real diet available (50%). Some particular compounds could be assessed separately, leading to recalculate the composition of the medium. Secondly, oxgall was used as source of bile. Indeed, it is more relevant to use additives from the studied species if available on the market. In this case, in the study of Hagi et al. (2020), oxgall had an effect on bacteria of the microbiota such as *Akkermansia muciniphila*–present in the model–while porcine bile had none. The Baby-SPIME model could be improved in the future by using additive related to pig as porcine bile. Thirdly, the baby-SPIME was used without mucin-beads that permits to include a mucous environment. In the literature, the in vitro models evolved progressively to include this environment. It was indeed done for M-SHIME, M-ARCOL or M-Pigut-IVM. It has to be noticed that mucin beads were used during the early stage of development of baby-SPIME but the solution to enrich the medium with mucin was finally preferred, leading to the presence of mucin-related bacteria as *Akkermansia sp*. Fourthly, microbial analysis was performed using high-throughput sequencing. This method gives relative but not quantitative data regarding the microbiota. This makes it difficult to detect groups actually influenced by an experimental treatment. A quantitative analysis carried out in parallel improves the analysis of the results and removes interpretation biases. The methods proposed to determine the bacterial load of a fecal-type sample are qPCR and flow cytometry, two methods which have their own advantages and disadvantages and which therefore do not lead to a consensus on the preferred method (Galazzo et al., 2020; Jian et al., 2021). The baby-SPIME model should evolve to the analysis of the microbiota towards a Quantitative Microbiome Profiling approach as explained by Vandeputte et al. (2017). These are some parameters on which to work now to improve the model and make it more compliant.

When setting up the model, one of the most important points that seemed to need improvement was the fermentation process of the ileum bioreactor. This statement was based on the observations of microbiota that was too anoxic to be representative of the ileal compartment. In addition, the bacterial load seemed too important to be representative of ileum and was more related to a pre-colon. Additional analysis of the total bacterial load of ileum and colon could have given additional arguments to discuss it. It was partially done through a bacterial count in MRS medium in samples coming from two runs in the early development of the model. Although MRS appears as a selective medium, results gave information on the difference between ileum and colon in vitro samples. The counts observed in the in vitro ileum samples (log 6–log 7 cfu/mL) were higher than those observed in the in vitro colon samples (log 4–log 6 cfu/mL). The opposite was rather expected, the microbial load of the large intestine being higher than the load in the small intestine. The SCFA observations converged also for such conclusion. While the sum of C2, C3 and C4 in ileum should be lower than in large intestine–a factor close to 3

(Franklin et al., 2002)–, in baby-SPIME the difference was not so conspicuous (less than 10% of difference on a 66 mM basis) and the proportion of acetate was too low in ileum bioreactor when considering the proportion of 80 to 90% found in vivo (Franklin et al., 2002; Mathew et al., 1996). However, it has to be noticed that the recent dynamic model for the human ileum also encountered difficulties concerning the SCFA concentrations (Stolaki et al., 2019). The authors referred to the particular dynamic of the diet in this compartment that functions with peak loads of substrates.

It was hypothesized that the inoculation of the bioreactors with intestinal content would improve the fermentation process. However, the results showed that this approach was unsuccessful. As mentioned earlier, another approach to consider is the improvement of the feed administered in the model. Indeed, even if half or the part of the feed was optimized for the use in the SHIME model, the other part, specific to piglet, must be improved. When setting up the model, there was a desire to add a representative portion of each of the fibers usually present in piglet feed. However, despite a maximum reduction in the quantities administered, the proportions of some were probably still too high. This was especially true in the case of AP. One way to improve the model on this point–as discussed above–could be to subject this portion of food to dialysis in order to keep only the portion actually reaching the colon.

In vitro, when observing the pH evolution between the ileum to the colon bioreactor, it decreased to mimic the in vivo evolution. In vivo, the pH decreased from ileum to caecum and then re-increased from caecum to rectum (Snoeck et al., 2004a). Due to the in vitro pH control, the fermentation that occurred in the ileum bioreactor had to be necessarily more representative of what is happening near the ileocecal junction–and not representative of the caecum where the pH is lower than ileum and colon. However, a parallel can be made between the observations in vitro in the ileum and colon bioreactors in baby-SPIME when using AP–ingredient known to have an effect on propionate in rats (Skinner et al., 2018)–and the results observed by Zhang et al. (2018) in the colon of pigs when propionate was directly infused in caecum. In presence of fermentable components as such present in AP, SCFA were more produced in the "ileum" bioreactor–with a higher proportion of acetate–when in vivo this observation is done in the caecum (Gresse et al., 2019). Being able to reproduce in bioreactors, bacterial dynamics such as those observed in vivo in the caecum seems of great interest when we see in vivo the difference observed with 2% and 4% AP in this compartment of the large intestine. Without drawing the conclusion that the baby-SPIME would reproduce the fermentation of the caecum, it seems important to explore these aspects after having improved the model.

To conclude, the developed baby-SPIME model fulfills its function to test AP and discuss the ingredient as a weaning strategy. The In vitro results showed an effect of AP on the production of propionate, the ratio of which increased, as well as on propionate-producing bacteria, such as certain Bacteroidetes or *Akkermansia*. In vivo data did not highlight an effect of AP on SCFA. However, the results showed a particular effect of AP on Bacteroidetes in fecal samples on day 8 post-weaning. Indeed, it was the second most abundant phylum for 4%AP piglets while it was at the third position for 0%AP and 2%AP piglets. Moreover, 4% AP improved piglet performance, gut architecture and

microbiota. Regarding the in vitro model, the results however showed certain limitations in terms of microbiota and SCFA, in particular in the ileum bioreactor which acted as a pre-colon bioreactor. Different solutions have been suggested to improve the baby-SPIME model: the use of a dialysis system, the use of bile salts and pancreatic enzymes of porcine origin and mucin beads. Moreover, a quantitative approach for microbial profiling should be combined with next-generation sequencing data. The ileum bioreactor would likely benefit from these improvements.

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