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

Sandrine Dufourny[a,*], Nadia Everaert[b], Sarah Lebrun[c], Caroline Douny[d], Marie-Louise Scippo[d], Bing Li[b], Bernard Taminiau[e], Massimo Marzorati[f,g], José Wavreille[h], Eric Froidmont[a], Pierre Rondia[a], Véronique Delcenserie[c]

[a] *Animal Nutrition and Sustainability Unit, Production and Sectors Department, Walloon Agricultural Research Centre, Gembloux, Belgium*
[b] *Precision Livestock and Nutrition laboratory, AgroBioChem Department, TERRA Teaching and Research Centre, University of Liège, Gembloux, Belgium*
[c] *Food Quality Management, Food Science Department, FARAH, University of Liège, Liège, Belgium*
[d] *Laboratory of Food Analysis, Food Sciences Department, FARAH, University of Liège, Liège, Belgium*
[e] *Food Microbiology, Food Science Department, FARAH, University of Liège, Liège, Belgium*
[f] *ProDigest Bvba, Gent, Belgium*
[g] *CMET, University of Ghent, Gent, Belgium*
[h] *Animal Breeding, Quality Production and Welfare Unit, Production and Sectors Department, Walloon Agricultural Research Centre, Gembloux, Belgium*

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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 constitutes 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 the dietary weaning strategies 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.

1. 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

* Corresponding author at: Walloon Agricultural Research Centre, Rue de Liroux, 8 B-5030, Gembloux, Belgium.
E-mail addresses: s.dufourny@cra.wallonie.be (S. Dufourny), nadia.everaert@uliege.be (N. Everaert), sarah.lebrun@uliege.be (S. Lebrun), cdouny@uliege.be (C. Douny), mlscippo@uliege.be (M.-L. Scippo), bli@student.uliege.be (B. Li), bernard.taminiau@uliege.be (B. Taminiau), Massimo.Marzorati@prodigest.eu (M. Marzorati), j.wavreille@cra.wallonie.be (J. Wavreille), e.froidmont@cra.wallonie.be (E. Froidmont), p.rondia@cra.wallonie.be (P. Rondia), veronique.delcenserie@uliege.be (V. Delcenserie).

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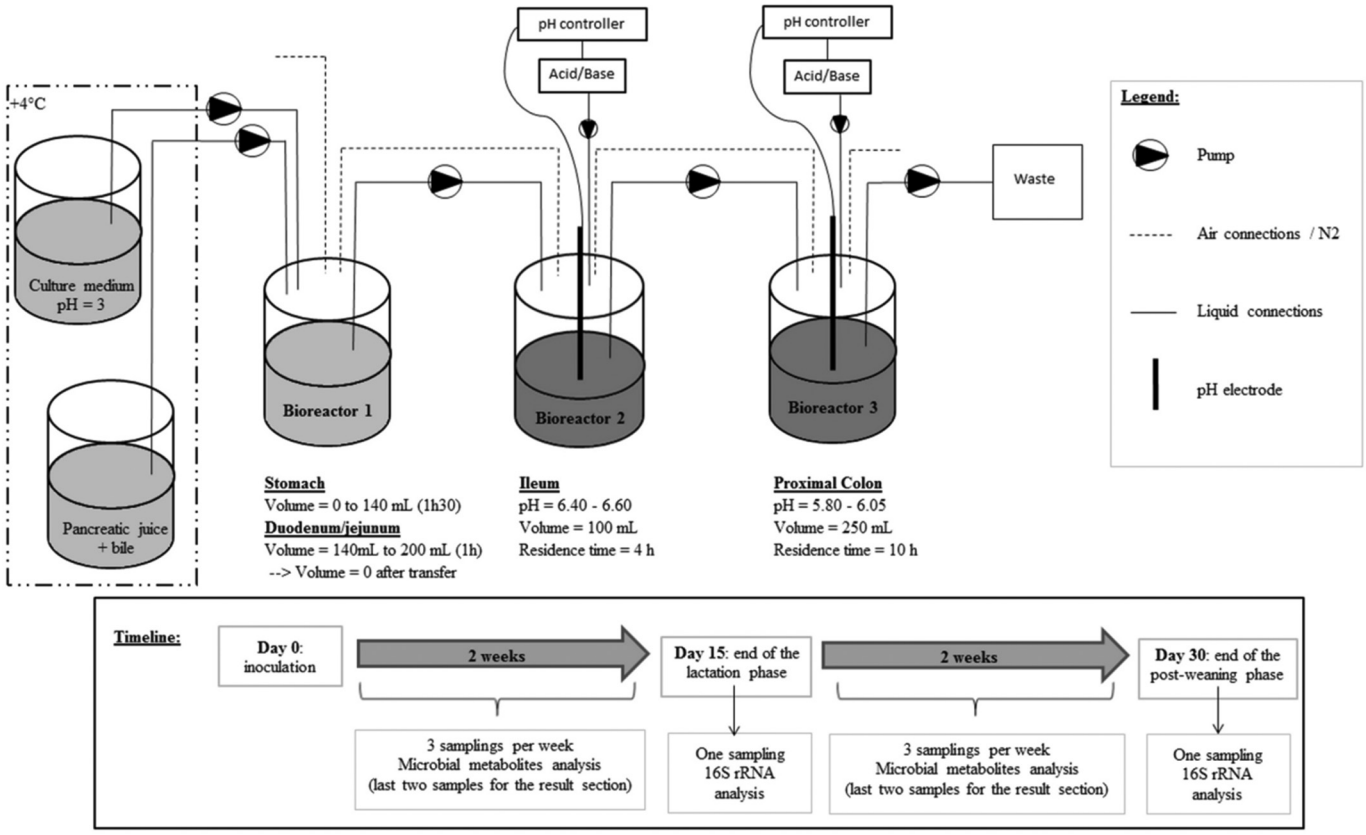

Fig. 1. Schematic representation of the baby-SPIME model. It consists of three double-jacketed bioreactors (bioreactor 1: stomach/duodenum/jejunum, bioreactor 2: ileum and bioreactor 3: proximal colon). Three times a day, the culture medium entered bioreactor 1 (stomach digestion) through liquid connections 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.

(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 as Positec (von Heimendahl et al., 2010); or PolyFermS (Tanner et al., 2014); 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.

Among 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 faecal 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.

2. Material and methods

2.1. Equipment

A SHIME® equipment (ProDigest Bvba, Gent, Belgium) as described by Van den Abbeele et al. (2010) (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 Fig. 1. 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.

2.2. 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 was in compliance with European (Directive 2010/63/EU) and Belgian (Royal Decree of the 29th of 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 1. 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.

2.3. 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 17,000*g* 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.

2.4. 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. (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 0.9 M 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 (30 m × 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.

Table 1
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
Protéose-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[a] (Dumoulin, Andenne, Belgium)	8.0 g/L	0.0 g/L
Post-weaning diet for piglets[b] (ABZDiervoeding, Nijkerk, The Netherlands)	0.0 g/L	8.0 g/L

g/L: grams per litre.

[a] Commercial complementary milk replacer feed for piglets containing, among others, whey powder, vegetable oils and wheat flour.

[b] 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%).

Table 2
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.
a to f: values with different exponents within a row are statistically different.

2.5. 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 5000 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).

2.6. 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 non-parametric 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).

3. Results

3.1. Alpha-diversity of the microbial ecosystem

The results of the alpha-diversity (Shannon and observed OTU) are given in Table 2. 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).

3.2. Taxonomy of the microbial ecosystem at the end of the lactation phase

3.2.1. 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 (Additional file – Table 1), with *Bacteroidetes* (class of *Bacteroidia)* being significantly diminished in both bioreactors (from 32.6% ± 1.8 in inocula to 13.0% ± 3.2, $p = .043$, in the ileum and 12.8% ± 1.1, $p = .021$, 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 = .043). The *Muribaculaceae* (S24-7) family tended to

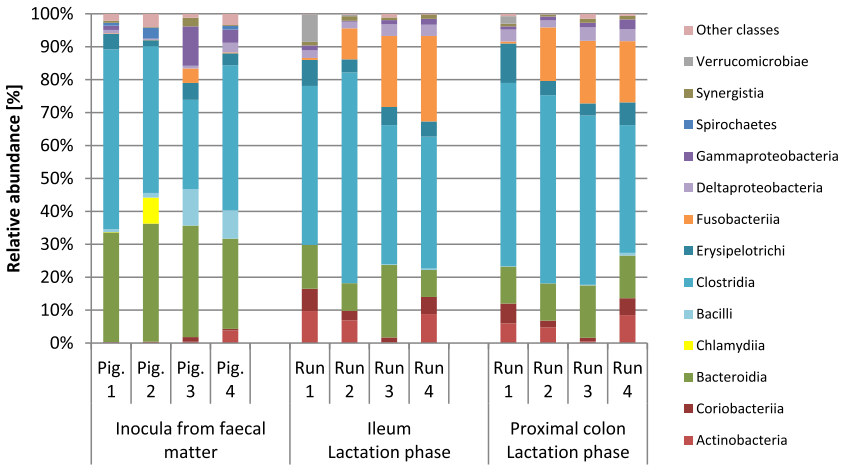

Fig. 2. Class composition of microbiota in the inocula (prepared with faeces of 4 piglets (pig)) and in the corresponding samples of the lactation phase from ileum and proximal colon bioreactors of the baby-SPIME model. Samples were taken the last day of each phase (2 weeks after inoculation for lactation 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)*.

be less abundant in the ileum bioreactor than in the proximal colon bioreactor (respectively 0.2% ± 0.1 and 0.7% ± 0.2, $p = .083$). 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 = .043) or the proximal colon (13.6% ± 4.4, p = .043) bioreactors. *Actinobacteria* tended to be less abundant in the inocula (1.7% ± 0.9) compared to the ileum (10.5% ± 3.2, p = .083) or the colon (8.5% ± 2.7, p = .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 = .083).

Regarding the *Proteobacteria* phylum, two classes were significantly present in inocula and bioreactors, namely *Gamma-Proteobacteria* and *Delta-Proteobacteria* (Fig. 2). *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 equalled 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 = .083) and in the colon (3.3% ± 0.4, $p = .043$) of the baby-SPIME model, as compared to inocula (1.2% ± 0.6).

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, $p = .021$) and the proximal colon (0.2% ± 0.2, p = .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).

3.2.2. 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 Fig. 3, these included *Mitsuokella*, *Fusobacterium*, *Prevotella, Megasphaera*, *Bifidobacterium*, *Sharpea* or *Bacteroides*.

3.2.3. 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).

3.3. 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 (Fig. 4), the relative abundances of *Bacteroidetes* increased while the relative abundances of *Firmicutes* and *Proteobacteria* diminished, except for the second run.

At the class level (Additional file – Table 1), statistical differences associated with weaning were detected in the ileum and proximal colon. Regarding the ileum, *Erysipelotrichi* (*Firmicutes*) increased in relative abundance (+5.0%, $p = .043$) and *Actinobacteria* (*Actinobacteria*) tended to increase (+6.5%, $p = .083$). In the proximal colon, *Gamma-Proteobacteria* (*Proteobacteria)* decreased (−0.9%, $p = .021$).

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 = .043) and *Bifidobacteriaceae* (*Actinobacteria*) tended to increase (+6.3%, p = .083) when *Veillonellaceae* (*Firmicutes*) tended to decrease (−6.8%, p = .083). In terms of the colon, *[Paraprevotellaceae]* (*Bacteroidetes*) increased (+0.1%, p = .021) while *Enterobacteriaceae* (*Proteobacteria*) and *Veillonellaceae* (*Firmicutes*) decreased or tended to decrease (−0.9%, p = .021 and −9.6%, p = .083, respectively).

3.4. 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 3.

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 ± 2.7 mM in ileum *vs* 30.1 ± 1.8 mM 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 ± 1.5 mM in the ileum and 1.9 ± 1.0 mM in the

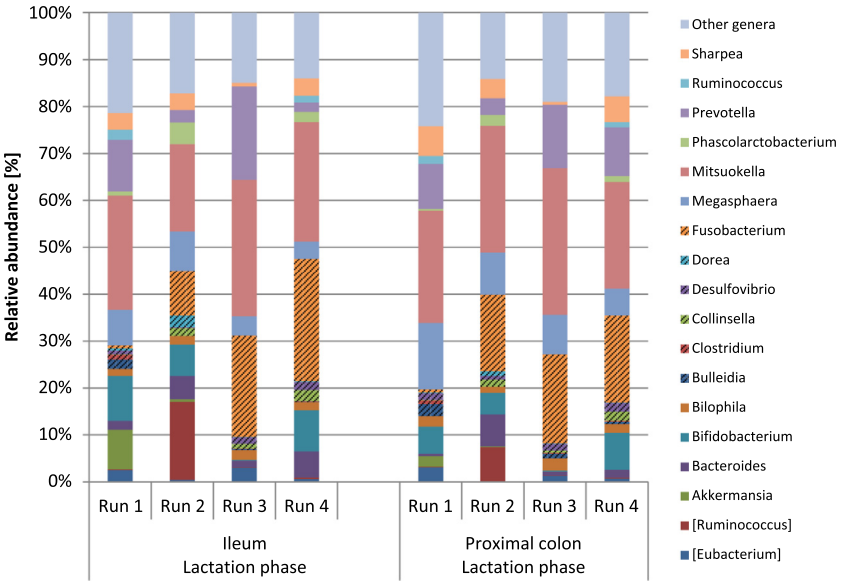

Fig. 3. Genera composition of microbiota in the ileum and proximal colon at the end of the lactation phase (samples taken 2 weeks after inoculation)

proximal colon, respectively, to 1.0 ± 0.2 mM 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; $p = .026$).

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, $p = .096$ for C2) and the proximal colon (56: 29: 15 *vs* 60: 26: 14, respectively, $p = .060$ for C2).

4. 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

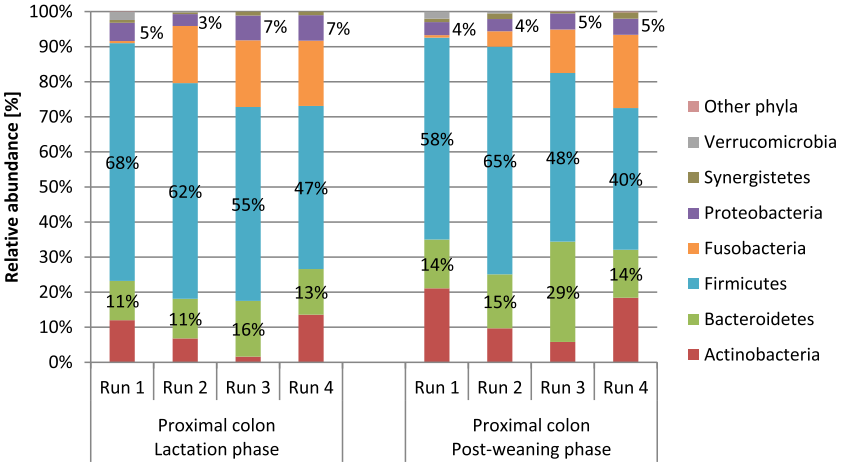

Fig. 4. Phyla composition of microbiota in the proximal colon at the end of the lactation phase (samples taken 2 weeks after inoculation) and at the end of the post-weaning phase (samples taken 2 weeks after weaning transition).

Table 3
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
Isobutyric 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
Isovaleric 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.

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.

4.1. 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. (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. (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 high 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. (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., 2017; 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.

4.2. 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 post-weaned 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 post-weaned 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 faecal 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.

5. 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.

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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Declaration of Competing Interest

MM was employed by company ProDigest Bvba, Gent, Belgium. All other authors declare no competing interests.

Authors' contributions

SD, NE, PR, VD: contributed to all steps of the work; SL: design and operation of baby-SPIME; CD and MLS: short-chain fatty acids analyses; BL: qPCR analyses (data not shown but discussed); BT: additional 16S rRNA gene sequencing analyses; MM: design of baby-SPIME; JW: inoculation of baby-SPIME; EF: discussion of results. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105735.

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