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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.

1. 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, Vande Woestyne, De Smet, & Verstraete, 1994; Molly, Vande Woestyne, & Verstraete, 1993) and TIM (Minekus, Marteau, Havenaar, & Huis in't Veld, 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, Schilderink, Minekus, Havenaar, & Verwei, 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

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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 & 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 (L. 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 & Kamphues, 2018) or for the large intestine of pigs (Bindelle, Buldgen, Boudry, & Leterme, 2007), faecal 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, Bosch, Tagliapietra, Williams, & Verstegen, 2006), 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.

2. Material and methods

2.1. 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 29th of 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.

Table 1
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.

2.2. Culture media, pancreatic juice and bile

A culture medium (called lactation culture medium) was prepared drawing on the work of Molly *et al.* (Molly et al., 1994). The composition is shown in Table 1. 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.

2.3. 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 Fig. 1, 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

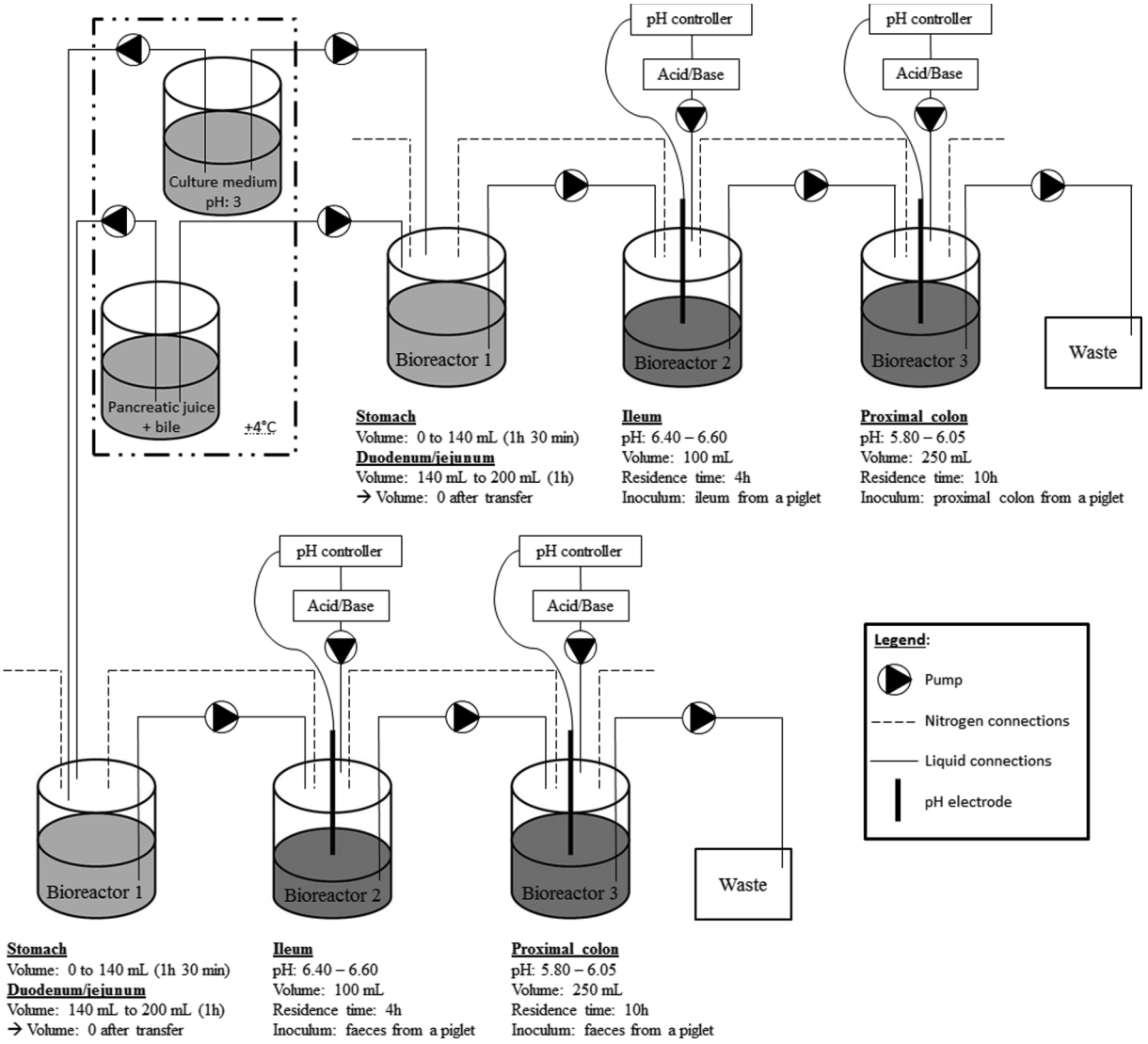

Fig. 1. Schematic representation of the baby-SPIME model used in the study (one run). The model consisted of three double-jacketed bioreactors (bioreactor 1: stomach/duodenum/jejunum, bioreactor 2: ileum and bioreactor 3: proximal colon). Three times a week, the culture medium and the pancreatic juice + 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.

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.

2.4. 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 17,000*g* 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.

2.5. 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.

3. Results

3.1. Microbiota of the samples

The microbiota composition of all the samples of the study is given in Fig. 2. The left side of Fig. 2 represents the ileal, colonic and fecal microbial composition of the inocula used to inoculate the baby-SPIME bioreactors; in the middle of the Fig. 2 are the results for ileum bioreactors (inoculated with ileum inocula or feces inocula); at the right side of the Fig. 2 are the results for colon bioreactors (inoculated with colon inocula or feces inocula).

3.1.1. 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 Fig. 2) and *Proteobacteria* (the *Gamma-Proteobacteria* class; purple in Fig. 2). 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.

3.1.2. 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%).

3.1.3. 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

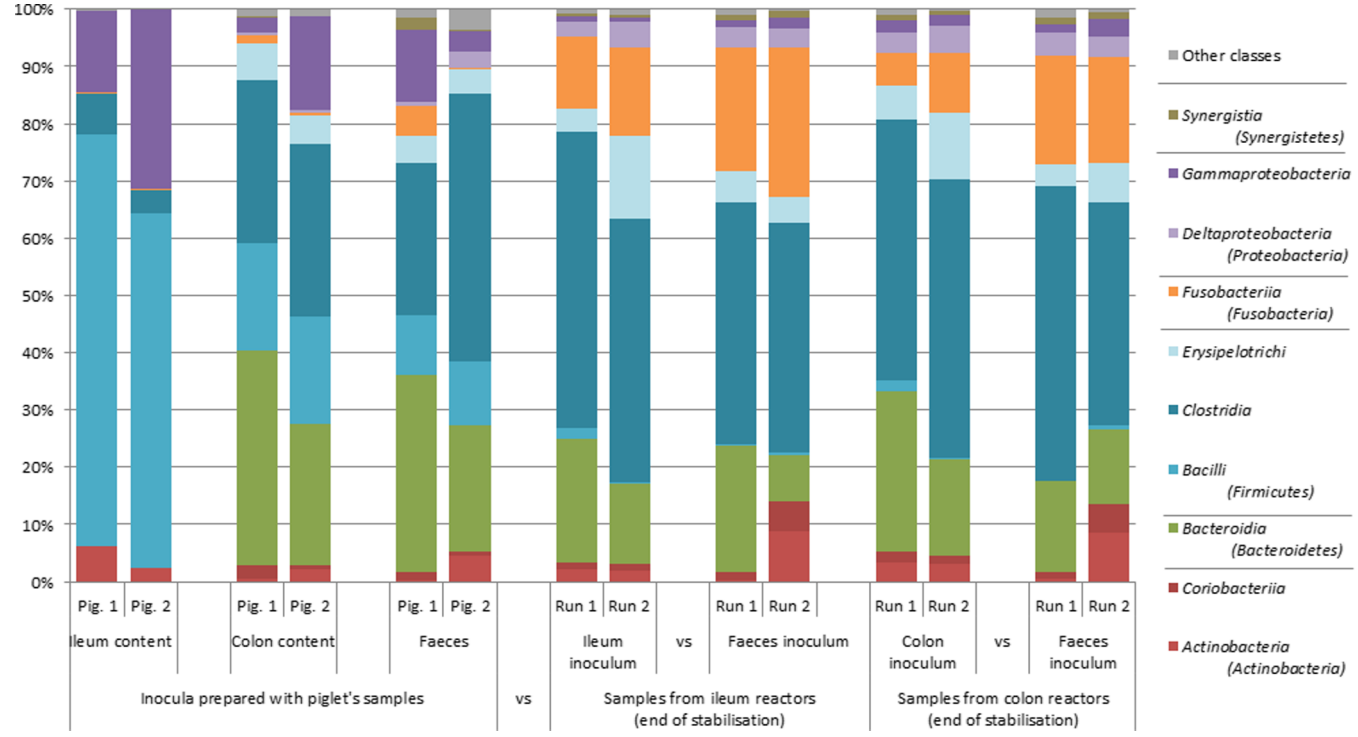

Fig. 2. Composition (in relative abundance, %) of the microbiota present in the inocula, in the ileum bioreactors and colon bioreactors. Classification is at the class level, with phylum between brackets. Samples coming from piglets (pig.) were used to prepare the inocula: pig. 1 for run 1, pig. 2 for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

Table 2
Relative abundances of bacteria (*in vivo vs in vitro* samples) following their use of oxygen.

	Run 1			Run 2			Bacteria (reference)
	Ileum content Piglet 1	Reactor content		Ileum content Piglet 2	Rector content		
		Inoc. feces	Inoc. ileum		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* (Goodfellow, Kämpfer, Busse, Trujillo, Suzuki, Ludwig, & Whitman, 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, Garrity, Jones, Krieg, Ludwig, Rainey, & Whitman, 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* (Goodfellow et al., 2012), *Enterobacteriaceae**(Garrity, 2005), *Lysinibacillus* (Ahmed, Yokota, Yamazoe, & Fujiwara, 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* (Goodfellow 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, Staley, Brown, Hedlund, Paster, Ward, & Whitman, 2010), *Bifidobacterium* (Goodfellow et al., 2012), *Bilophila* (Garrity, 2005), *Blautia* (C. Liu, Finegold, Song, & Lawson, 2008), *Dethiosulfovibrionaceae** (Bhandari & Gupta, 2012), *Lachnospiraceae*& ("Other" genus)* (De Vos et al., 2009), *Oscillospira* (Gophna, Konikoff, & Nielsen, 2017), *Prevotella* (Krieg et al., 2010), *Pyramidobacter* (Bhandari & Gupta, 2012), *Succiniclasticum* (De Vos et al., 2009), *Synergistaceae ("Other" genus)* (Bhandari & 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* (Goodfellow 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 & 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: biorector inoculated with feces inoculum; Inoc. ileum: bioreactor inoculated with ileum content 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”).

3.2. 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 2 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.

4. 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 & Kim, 2012). Among the *Firmicutes*, a high abundance of the *Bacilli* class is described, followed by *Clostridia* (De Rodas, Youmans, Danzeisen, Tran, & Johnson, 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 & 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, Brunelle, Trachsel, & Allen, 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, Yao, Perez-gutierrez, Smidt, & Zhu, 2008) – so important for health (Ferrando & 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, Bittinger, Esipova, Hou, Chau, Jiang, & Wu, 2018; Morris & Schmidt, 2013; Zheng, Kelly, & Colgan, 2015) and a radial (Albenberg et al., 2014; Morris & 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 & 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.* (Friedman et al., 2018): “The biomass of the oxygen-tolerant 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, Boudry, Portetelle, Théwis, & Bindelle, 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 semi-continuous model (Blake, Hillman, & Fenlon, 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, Zabielski, Hammon, & Metges, 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, Scorza, Sansonetti, & Tang, 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.

5. Conclusion

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 multi-compartment 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.

CRediT authorship contribution statement

S. Dufourny: Conceptualization, Methodology, Formal analysis, Investigation, Project administration, Writing - original draft. **N. Everaert:** Validation, Writing - original draft. **S. Lebrun:** Methodology, Resources. **M. Didelez:** Methodology, Resources. **J. Wavreille:** Resources. **E. Froidmont:** Writing-Review and Editing. **P. Rondia:** Project administration, Funding acquisition, Writing-Review and Editing. **V. Delcenserie:** Conceptualization, Methodology, Validation, Resources, Investigation, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

See Figs. 1 and 2.

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