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Evaluating the role of Chicory Flour in reducing the weaning stress in piglets

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Abstract

One of the main focuses in pig science is weaning as it is presumable the most sensitive period in a pig's life. At weaning, the maternal separation, the introduction to a new environment with new individuals and a new microbiota, and the switch from milk to solid feed all negatively impact an animal's well-being, which potentially results in a 'leaky gut' syndrome associated with diarrhea. Indeed, the structure itself of the intestine can be heavily impacted resulting in high permeability, allowing the uncontrolled passage of molecules, pathogens, and antigens to the intercellular space causing inflammation.

To answer this weaning stress associated problem, interventions on the microbiota may be taken by prebiotic supplementation with products containing inulin, cellulose, pectin, etc. An *in vivo* and an *in vitro* trial was organized to observe, measure and compare with already effective proven inulin, the effects of the chicory flour. Just like inulin powder, chicory flour has a high inulin content but also a non-negligible insoluble fiber fraction and other active compounds such as chlorogenic acids and sesquiterpene lactones which may play a role. Post weaning, the animals (21 days old) were divided in a control group, an inulin-supplemented group, and a chicory-supplemented group. The amount of administered prebiotics increased weekly (1.5g inulin/d on week 1, 2g inulin/d on week 2 and 2.5g inulin/d on week 3). In parallel, an *in vitro* digestion, following the INFOGEST protocol, and fermentation of piglet's feed with different proportions of the prebiotics were performed.

Inulin and chicory flour had no effect on pH, length of intestine or body weight evolution (p value > 0.05). Surprisingly, chicory reduced significantly the feed intake three weeks after weaning ($p<0.0001$). On day 29, chicory flour fed piglets showed significantly lower propionate levels ($p=0.0001$) and significantly higher acetate levels ($p=0.014$) as compared to inulin. Indeed, on D29, the chicory flour fed piglets showed significantly lower isobutyrate levels ($p=0.04$) and on D43, isovalerate levels were significantly lower ($p=0.0314$). The villus height was significantly higher in case of jejunum, ileum and duodenum of inulin fed piglets when compared to the chicory and control fed groups ($p<0.0001$).

In the case of inulin E, the digested inulin has shown the highest total cumulative gas production and also a higher time to half asymptote when compared to raw inulin fermentation ($p<0.05$). Only the feed with 2% of inulin had a higher total gas production when compared with Feed, F+ 0.5% and F+1% ($p<0.05$). The high proportions of lactate and acetate in the chicory fermentation, can be attributed to short chains of fructans. Gas production kinetics show that chicory has a higher time to half-asymptote than inulin and maximum production rate is lower than it is the case for inulin but it is reached faster. Chicory could thus be used as a prebiotic stimulating mainly in the upper GIT. Although equated for its inulin content, chicory was found to be less effective than inulin for its effect at *in vivo* as well as *in vitro* and this might be due to presence of other biomolecules in the chicory flour.

Key-words : inulin – chicory flour – infogest fermentation – SCFA – weaning – piglets

Résumé en français

Le sevrage est l'un des principaux centres d'intérêt de la science du porc, car il s'agit vraisemblablement de la période la plus sensible de la vie du porcelet. Au moment du sevrage, la séparation maternelle, l'introduction dans un nouvel environnement avec de nouveaux individus et un nouveau microbiote, ainsi que le passage du lait à l'alimentation solide, ont un impact négatif sur le bien-être de l'animal, qui se traduit par un "leaky gut" associées à la diarrhée. En effet, la structure même de l'intestin est impactée entraînant une forte perméabilité, permettant le passage incontrôlé de molécules, pathogènes, antigènes vers l'espace intercellulaire provoquant une inflammation.

Pour répondre à ce problème associé au stress du sevrage, on peut intervenir sur le microbiote par une supplémentation en prébiotiques tels que l'inuline, la cellulose, la pectine, etc. Un essai in vivo et un essai in vitro ont été organisés pour observer, mesurer et comparer les effets de la farine de chicorée avec ceux de l'inuline, dont l'efficacité a déjà été prouvée. Tout comme la poudre d'inuline, la farine de chicorée a une forte teneur en inuline mais aussi une fraction non négligeable de fibres insolubles et d'autres composés actifs comme les acides chlorogéniques et les lactones sesquiterpéniques qui peuvent jouer un rôle. Après le sevrage, les animaux (âgés de 21 jours) ont été divisés en un groupe témoin, un groupe supplémenté en inuline et un groupe supplémenté en chicorée. La quantité de prébiotiques administrés a augmenté chaque semaine (1,5 g d'inuline/j la première semaine, 2 g d'inuline/j la deuxième semaine et 2,5 g d'inuline/j la troisième semaine). En parallèle, une digestion in vitro, suivant le protocole INFOGEST, et une fermentation de l'aliment du porcelet avec différentes proportions de prébiotiques ont été réalisées.

L'inuline et la farine de chicorée n'ont eu aucun effet sur le pH, la longueur de l'intestin ou l'évolution du poids corporel (valeur $p > 0,05$). Etonnement, la chicorée a réduit de manière significative la prise alimentaire trois semaines après le sevrage ($p<0,0001$). Au jour 29, les porcelets nourris à la farine de chicorée ont montré des niveaux de propionate significativement plus bas ($p=0,0001$) et des niveaux d'acétate significativement plus élevés ($p=0,014$) par rapport à l'inuline. En effet, à J29, les porcelets nourris à la farine de chicorée ont montré des niveaux d'isobutyrate significativement plus bas ($p=0,04$) et à J43, les niveaux d'isovalérate sont significativement plus bas ($p=0,0314$). La hauteur des villosités était significativement plus élevée dans le jéjunum, l'iléon et le duodénum des porcelets nourris à l'inuline par rapport à la chicorée et au contrôle ($p<0,0001$).

Dans le cas de l'inuline E, l'inuline digérée a montré la production totale de gaz cumulée la plus élevée et également un temps plus élevé pour atteindre la demi-asymptote par rapport à la fermentation de l'inuline brute ($p<0,05$). Seul l'aliment contenant 2 % d'inuline a présenté une production totale de gaz plus élevée par rapport à l'aliment, F+ 0,5 % et F+1 % ($p<0,05$). Les proportions élevées de lactate et d'acétate dans la fermentation de la chicorée peuvent être attribuées aux chaînes courtes de fructanes. La cinétique de production de gaz montre que la chicorée a un temps de demi-asymptote plus élevé que l'inuline et que le taux de production maximum est plus faible que celui de l'inuline mais il est atteint plus rapidement. La chicorée pourrait donc être utilisée comme prébiotique stimulant principalement dans le tube digestif supérieur. Bien qu'elle soit équivalente pour sa teneur en inuline, la chicorée s'est avérée moins efficace que l'inuline pour son effet *in vivo* ainsi qu'*in vitro* et cela pourrait être dû à la présence d'autres biomolécules dans la farine de chicorée.

Mots clés : inuline - farine de chicorée - fermentation infogest - AGV - sevrage - porcelets

List of abbreviations

ADF : *Acid Detergent Fibre* (= cellulose et lignine)
ADFI: Average daily feed intake
ADG: Average daily gain
BCFA : Branched chain fatty acid
BW: Body weight
DP: Degree of polymerization
EHEC: Enterohaemorrhagic *E. coli*
FCR : Feed conversion ratio
FOS: Fructo-oligosaccharides
GIT: Gastrointestinal tract
GLP-1: Glucagon-like peptide-1
GPR : G protein-coupled receptors
HDAC: Histone deacetylase
HPSEC : High pressure size exclusion chromatography
IgA: Immunoglobulin A
JAMs: junctional adhesion molecules
MLN: Mesenteric lymph nodes
NDF : *Neutral Detergent Fibre* (= cellulose, hémicellulose et lignine)
PDI : Polydispersity Index
PP: Peyer’s patches
PWD: Post-weaning diarrhea
SCFA : Short chain fatty acid
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid
SSF: Simulated salivary fluid
T3SSS: Type III secretion system
TGF-β: Transforming growth factor-β
Tregs: Regulatory T cells
V:C ratio : villus height to crypt depth ratio

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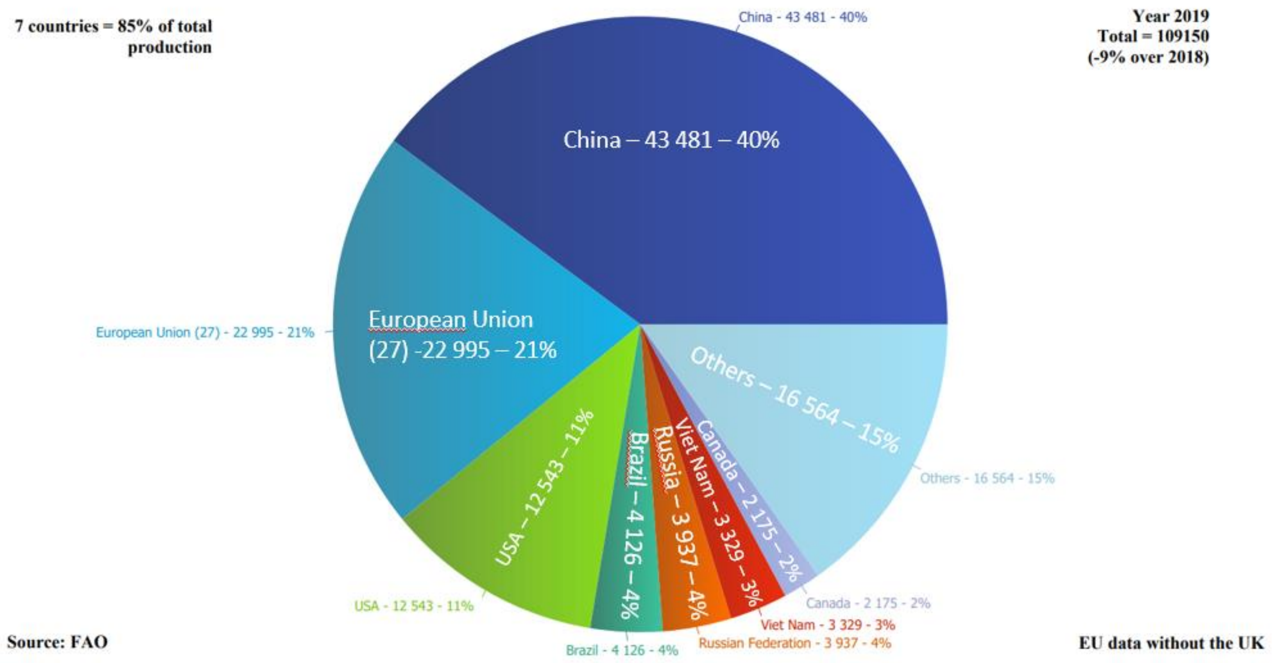
1. Introduction

1.1 Pig farming in the world

Pigs have major economic importance in the whole world. Depending on the country, consumption per capita can reach as high as 55.2kg as in Spain in 2019. In 2019, Spain was the biggest pork consumer per capita, followed by Poland with 54kg and Croatia with 53 kg (Pork Meat Consumption Per Capita, 2022). The European statistics center reports pig production and trade (Fig.1) (Pigmeat statistics, 2022). China is the world leader in pig meat production. Its production reached 43.481.000 T in 2019. However, per capita, pork consumption in 2017 in China was 38.4 kg only. The European Union follows with 22.995.000 T, equivalent to 21% of worldwide production. The USA is in third place with 12.543.000 T and 26.8kg of pork consumption per capita in 2017. While being the second worldwide pig meat producer, the EU is the top exporter of pig products (Augère-Granier, 2020). Pig trade takes place in different forms. Fresh or frozen meat, offals, fat, sausages, or sometimes even live animals are being sold and shipped internationally (Pigmeat statistics, 2022). In 2019 the EU exported 4.5 million T of pig products, and the trend is growing. The export grew 40% from 2017 to 2020. The main consumer of European pork is China (Augère-Granier, 2020).

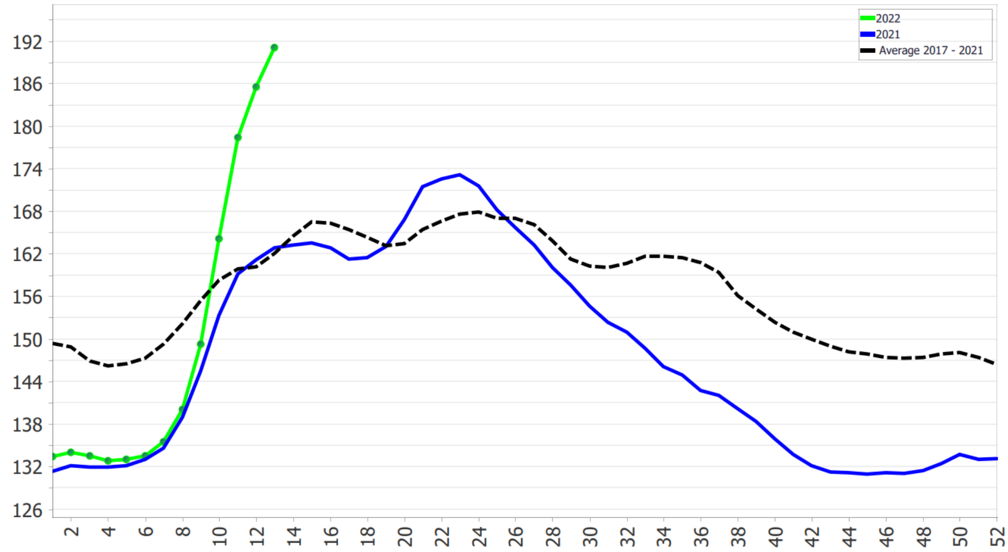


Source: FAO

EU data without the UK

Figure 1: World production of pig meat (in 1000 tonnes product weight) (year 2019) from the European statistic center

Pork prices are very volatile (Fig.2) and are influenced by factors such as product demand, production supply, feed prices or even diseases, etc. In the EU, prices range from 132€/100kg (beginning 2021) up to 192€/100kg (march 2022). The number of pig farms in Europe has decreased from 3.8 million down to 2.2 million between 2005 and 2013 (Pigmeat statistics, 2022). Yet, the average herd size has increased. This results in an increase in productivity per farm (Augère-Granier, 2020).



Source: MSs (Weekly communications under Reg. 2017/1185) **EU figures exclude UK (also for years prior to Brexit)**

Figure 2: Weekly evolution of the EU pig carcass prices (€/100kg) from the European statistic center

1.2 Pig farming in Belgium

Belgium is far from being the swine production leader. This title is shared between Germany and Spain. Indeed, in 2021, Belgium's production represented only 1.100.000 T while the production in Spain and Germany was almost 5 times bigger (5.194.000 T for Spain and 4.965.000 T in Germany) (EU-stat). Between 2005 and 2020, together with France, those 3 countries produced 51% of all pig meat in Europe (Fig.3).

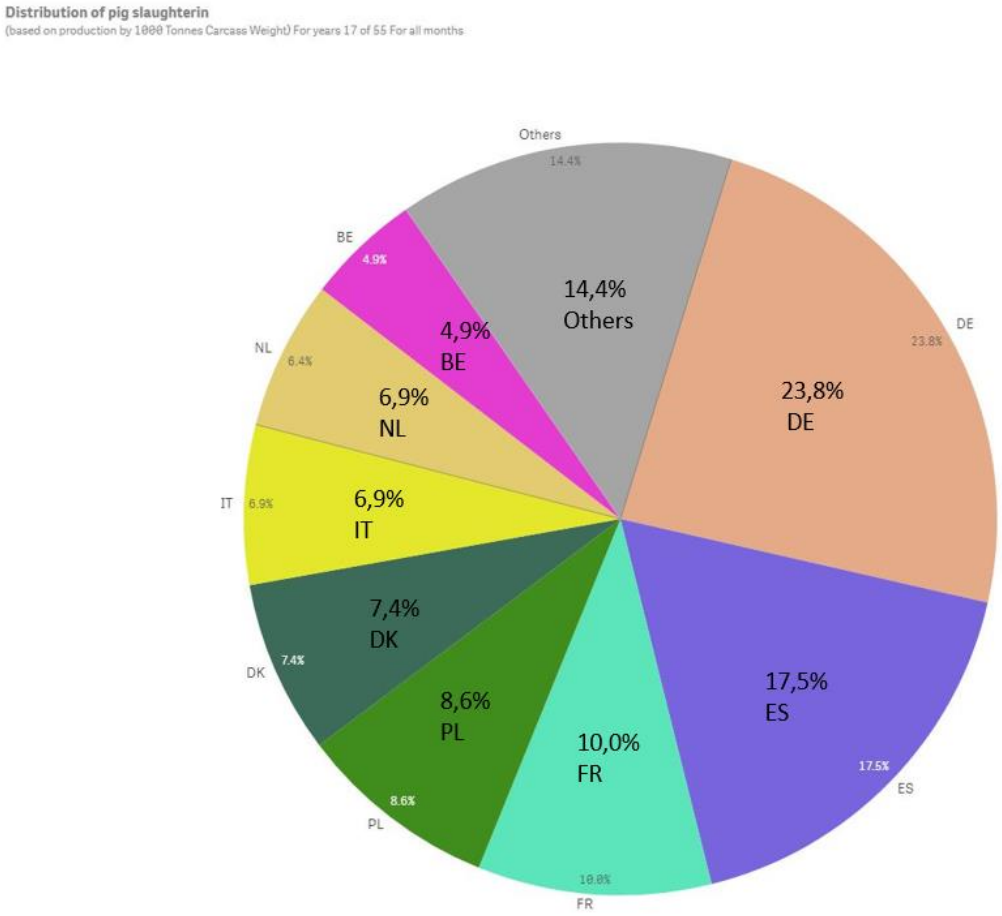


Figure 3 : Distribution of pig slaughtering in the European Union based on production by 1000 tonnes between 2005 and 2020 from the European statistic center

In 2020, pork was the main type of meat consumed in Belgium (51.8%), followed by poultry meat (18.7%) and beef (18.6%) (Supply balance sheets for meat, 2022) (table 1). The apparent meat consumption[1] grew up to 82 kg (all types combined) per capita in 2020. It gained 7 kg compared to the previous year (Table 1) that can be explained by an increase in net meat production, an increase in meat import (4.3%) and in meat export (3.7%) out of which pig meat illustrates a 5.7% increase in import and 7.1% increase of export (Supply balance sheets for meat, 2022).

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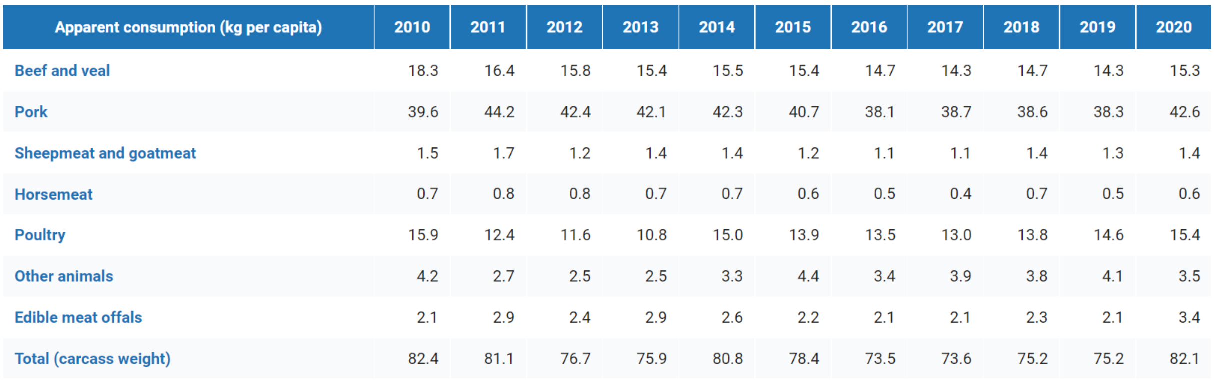
[1] The apparent meat consumption illustrates available meat on the market for one year related to the size of the population. It is not the actual meat consumption per capita.

Table 1 : Evolution of apparent meat consumption in carcass weight in Belgium (2010-2020)



Apparent consumption (kg per capita)	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Beef and veal	18.3	16.4	15.8	15.4	15.5	15.4	14.7	14.3	14.7	14.3	15.3
Pork	39.6	44.2	42.4	42.1	42.3	40.7	38.1	38.7	38.6	38.3	42.6
Sheepmeat and goatmeat	1.5	1.7	1.2	1.4	1.4	1.2	1.1	1.1	1.4	1.3	1.4
Horsemeat	0.7	0.8	0.8	0.7	0.7	0.6	0.5	0.4	0.7	0.5	0.6
Poultry	15.9	12.4	11.6	10.8	15.0	13.9	13.5	13.0	13.8	14.6	15.4
Other animals	4.2	2.7	2.5	2.5	3.3	4.4	3.4	3.9	3.8	4.1	3.5
Edible meat offals	2.1	2.9	2.4	2.9	2.6	2.2	2.1	2.1	2.3	2.1	3.4
Total (carcass weight)	82.4	81.1	76.7	75.9	80.8	78.4	73.5	73.6	75.2	75.2	82.1

1.3 Challenges in the industry

Environmental, animal welfare, and public health policies could strike heavily on animal farms. First, in 2019, agriculture represented 8% of total greenhouse gas emissions in Belgium. The main emissions account for methane and N_2O. Almost 20% of methane comes from faeces management, mainly from the swine industry (Émissions par gaz à effet de serre, 2022). As politics tend to strengthen laws aiming to reduce water and air pollution, the pig industry is running potentially high risk. Secondly, consumers are more concerned with animal welfare which results in lower meat consumption. To reassure the consumers, farmers are forced to provide better housing conditions for their animals resulting in higher production costs. Some veterinarian practices are more and more frowned upon, such as for instance male castration; however, they are a proven method to avoid boar taint. Analgesia and anesthesia while castrating and immunocastration are adequate operations but cost extra. Thirdly, feed prices are being very volatile because of recent events in the world such as for instance the war in Ukraine which strikes heavily on the industry. Lastly, antibiotics used to be an easy solution for animal health. At weaning, animals are put under high levels of stress while having weak immune defenses, making them prone to infections. Antibiotics used to be applied in a preventive matter. As antibiotic resistance rose, it put human health at risk which is not tolerated (Rhouma *et al.*, 2017). Antibiotic usage is now more and more regulated making it a less popular solution for post-weaning diarrhea. Same fate awaited ZnO which used to be applied in a similar way as antibiotics at weaning until June 2022 when it was prohibited in the European Union. Nevertheless, post-weaning diarrhea problems did not disappear and as antibiotic usage is less and less conceivable, the problem still needs to be faced. Promising research on pre- and probiotics can bring an answer to this problem.

To conclude, farmers need to face new policies while trying to stay competitive on the market and at the same time provide sufficient means for the growth of their animals. The numbers given for pig farming industry, the increase of pigs per farm as well as the many challenges mainly due to this intensive farming highlight the economic importance of pig industry and justify research on pigs. In the following chapters, the critical time around weaning where pigs face stress and diarrhea will be described in detail followed by pre- and probiotics supplementation to ease this issue.

1.4 Weaning

Weaning is a stressful event in the lives of piglets and is often associated with post-weaning diarrhea. From this period of life, they are moved away from their mothers, they are moved to a new environment with new individuals and are forced to switch from the milk diet to a solid one. All these factors contribute to high stress levels of the piglets, illustrated by high cortisol levels. Stress results in a decrease in pig's health, morbidity, loss of appetite or even aggressive behavior. The cortisol level is high which may cause intestinal inflammation resulting in an increased intestinal permeability, nutrient malabsorption and diarrhea ((Campbell, Crenshaw, & Polo, 2013); (Rhouma, Fairbrother, Beaudry, &

Let, 2017); (Madison & Kiecolt-Glaser, 2019)). Also, the sudden change of diet and the stress itself disturbs the microbiota equilibrium providing good colonizing opportunities for pathogenic microorganisms. At the same time, gut immunity is still unexperienced and unable to handle this situation properly. Enteric infections, the release of toxins and destruction of apical junctional complex occur often with diarrhea. *Escherichia coli* and *Salmonella typhimurium* are the 2 major pathogens in swine industry. Up to 50% of piglet deaths, yearly, is due by enterotoxigenic *E. coli* (ETEC) (Gresse, et al., 2017).

The reduction of appetite is associated with weaning. Indeed, solid foods (low in fat, high in carbohydrates) are less palatable than milk (high in fat, low in carbohydrate). This results in a lower metabolizable energy intake. On average, energy intake drops down to 60-70% of pre-weaning milk intake. A loss of weight is thus observed at this period of time. It ranges from 100 g up to 250 g but it is recovered after 2-4 days. Stress and low energy intake stimulate the liver for gluconeogenesis ((Le Dividich & Sève, 2000), (Thau, Gandhi, & Sharma, 2022)). Cortisol decreases glycogen synthesis in the liver, increases protein degradation in muscle cells to fuel the Krebs cycle but also increases lipolysis in adipose tissues to render glycerol which can be transformed into glucose (Thau, Gandhi, & Sharma, 2022). Also, a lower feed intake disturbs gut morphology and causes villi atrophy and hyperplasia of the crypts. Villus height is an indicator of intestinal integrity (Leblois, Early life programming of piglets' microbiota and gut health by maternal dietary fibre supplementation. Unpublished doctoral thesis, ULiège - Université de Liège. , 2018). In a study by Vente-Spreeuwenberg *et al.* (2003), it has been observed that villi height decreased for the first 3 days after weaning (394 µm -> 275µm) but afterwards, it increased. In 10 days, villi height did not recover its initial length and reached 324µm only. Also, villi height and crypt depth were not correlated with each other but villi height was indeed positively correlated with feed intake. As for crypt depth, there was no significant difference between the weaning day and 3 days after (166µm -> 183µm) but after 10 days, the depth increased (289µm) (Vente-Spreeuwenberg *et al.*, 2003).

Low feed intake after weaning resulted in lower villi heights indicating a poor intestinal integrity. To counter low feed intake, some tricks are applied to favor feed consumption like supplementation. Adding skim milk before and after weaning resulted in a higher consumption (257 >< 30 gDM/d) than those without supplementation (Le Dividich & Sève, 2000). The reduction of appetite reverberates on growth performances like average daily gain (ADG). Average daily gain plays a crucial role in the economic viability of pig farming as it illustrates growth performances. Indeed, the higher ADG, the shorter the growing time before the slaughtering weight is achieved. It is of interest to quickly achieve a high ADG after weaning as it will pave growth performances. Usually, it takes 2 weeks to reach the metabolic energy intake prior to weaning (Le Dividich & Sève, 2000). At the suckling period, this factor is influenced by the sex of the animal, its body weight, the blood glucose concentration and the size of the litter. Nuntapaitoon *et al.* (2018) found that at 7 days old, ADG was 163.7 g/d ± 67.32 but at 21 days, it was 216.8 g/d ± 63.05. Pre-weaning mortality could reach up to 12.6% (Nuntapaitoon, Muns, & Tummaruk, 2018). Yet, in Wang *et al.* (2020) experiment, at 49 days old, piglets weaned at 21 days old (6.48 kgBW ± 0.49) would present an ADG ranging from 155.55 g/d up to 218.89-235.32 g/d depending on the small intestine's length.

Weaning age has a strong influence on growth rate. Indeed, depending on the weaning age, initial weight (and thus also final weight) differed importantly in piglets weaned at 19 days (5.04 kg) and at 28d (7.26 kg) (Faccin, et al., 2020). In a study by Faccin *et al.* (2020), ADG (19d : 295g/d, 28d : 406g/d) as well as average daily feed intake (ADFI) (19d : 493 g/d, 28d : 661 g/d) increased with the weaning age. Removal rate of unfit animals decreased (19d : 8.01%, 28d : 1.65%) but the mortality rate remained similar (19 d : 0.71% , 28 d : 1.04%). Those factors contributed to significant differences at finishing stage. Indeed, the body weight at 136d was 114.99kg (19d) against 126.5kg (28d) and ADG at the final stage differed too (19d : 1.02kg/d, 28d : 1.07kg/d). They concluded that it is best to wean at 25d as the advantages are the greatest. Weaning after 25 days is still beneficial but the advantages are lower than at 25 days (Faccin, et al., 2020). Main *et al.* (2004) provided similar results with an improved ADG, but they also stated that mortality decreased. Indeed, ADG was 409 g/d ± 7 (weaning at 18 days) 482 g/d ± 13 (weaning at 18-19 days) and 474 g ± 7 (weaning at 21 days) 525 g/d ± 13 (weaning at 21-22 days) (Main). The mortality decreased linearly form 2.11% ± 0.76 (18 days), 1.56% ± 0.36 (18-19 days) down to 0.56% ± 0.76 (21 days), 1.30% ± 0.36 (21-22 days) (Main). In both studies, the initial weaning period affects growth performances.

The reduction of appetite at weaning leads to a caloric deficit and induces weakness in the animals making them more prone to infections. Yet, Rantzer, Crenshaw & Polo (1996) suggested that a restrictive diet during the first 3 to 8 days after weaning results in a lower diarrhea score and a lower haemolytic *Escherichia coli* infection (EHEC). The price to pay for a better animal health is high as weight gains are lower during this restricted diet (Rantzer, Svendsen, & Weström, 1996) and slaughtering is postponed by 6 to 10 days ((Campbell, Crenshaw, & Polo, 2013)) or even 10-28 days (Le Dividich & Sève, 2000). In normal feeding piglets, the diarrhea problem could partially be explained by vegetable allergens present in the feed. Amino acids like glycinin and β -conglicinin are allergenic proteins present in soybean meals. As a piglet's immune system is exposed for the first time to those molecules, its reaction can be excessive causing inflammation of the intestines resulting in the destruction of tight junctions which in turn could lead to nutrient malabsorption and diarrhea (Park, Lee, Cowieson, Pappenberger, & Woyengo, 2020). Reducing the exposure to allergens, reduces the pressure on the immune system as proved by Rantzer, Crenshaw & Weström (1996). Soya consumption causes weaned piglets to produce plasma IgG against those 2 amino acids (Bailey, et al., 2001).

Microbial infections can exacerbate even more the intestines resulting in post-weaning diarrhea like for example the proliferation of *E. coli* (Rhouma *et al.,* 2017). Indeed, dysbiosis in the microbiota of the intestines is another possibility that may result in a leaky gut (see chapter 1.5.2) leading to diarrhea. The disorder of the microbial population occurring at weaning disturbs protective properties of the microbiota and allows proliferation of pathogens resulting in inflammation and increased permeability. The intestine is no longer able to separate luminal content and peripheral tissues leading to an inflammatory response (Hiippala, et al., 2018). Gut maturation and activation of the immune system are occurring at the same time at weaning (Sureda, 2017). Reportedly in commercial farms, diarrhea occurs 40% during the first week after weaning, 69% during the second week and 50% during the third week by Vente-Spreeuwenberg, Verdonk & Vestegen (2003). Also, up to 17% of piglets die at weaning (Gresse, et al., 2017).

To conclude, good weaning practices are crucial to obtain good starting growth performances. At the same time piglets are confronted to stress, lack of appetite, allergens, microbiota disorders and immune deficiency, all having an effect on the intestines and leading to diarrhea. The following sections focus on a healthy and a leaky gut. Later, the usage of prebiotics as a solution to diarrhea would be explained and detailed, followed with the immune system as it is massively exposed at weaning.

1.5 Digestive tract

The digestive system is composed of the gastrointestinal tract and adjacent organs like the liver and the pancreas. The focus will be given to the small intestine as it lays at the hearth of the post-weaning diarrhea problem. First, a normal functioning intestine will be discussed followed by a comparison with the leaky gut. Later, as microbial fermentation takes place in the intestine, microbial structures before and after weaning will be discussed. A focus will be put on prebiotics and bacterial strains able to produce butyrate. Lastly, butyrate properties as it influences the immune system and the immune system itself will be explained.

1.5.1 Small intestine anatomy

The small intestine is composed of 3 parts; duodenum, jejunum and ileum and 4 concentric layers; mucosa, submucosa, muscularis externa, adventia (Kong, Zhang, & Zhang, 2018). There is also a mucin layer important for mucosal barrier as it prevents direct contact of the chyme with cells and provides a nutritive environment for commensal bacteria (Turner J. R., 2009). Structures like Peyers's patches (present in the ileum only), Lieberkühn crypts and villi are also found. Villi are absent in the caecum and the colon (Kong, Zhang, & Zhang, 2018). The mucosa layer of the intestine contains the epithelium, lamina muscularis and lamina propria. The epithelium is composed of different cells such as enterocytes (=IECs) [nutrients absorption], goblet cells [mucus secretion], paneth cells [antimicrobial factors secretion], enteroendocrine cells, stem cells, etc. (Fig. 4). Enterocytes are the lining cells of the epithelium. They contain brush-border enzymes allowing the breakdown of molecules into nutrients. Those nutrients are afterwards absorbed by those very same cells. Enterocytes are tightly connected together creating a concentration gradient between the intestine's lumen and the transcellular environment (Turner J. R., 2009). The gradient allows absorption of nutrients but other pathways are possible too such as passive permeability, transcellular route with or without the help of aqueous pores, active transport with a carrier and endocytosis (Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012).

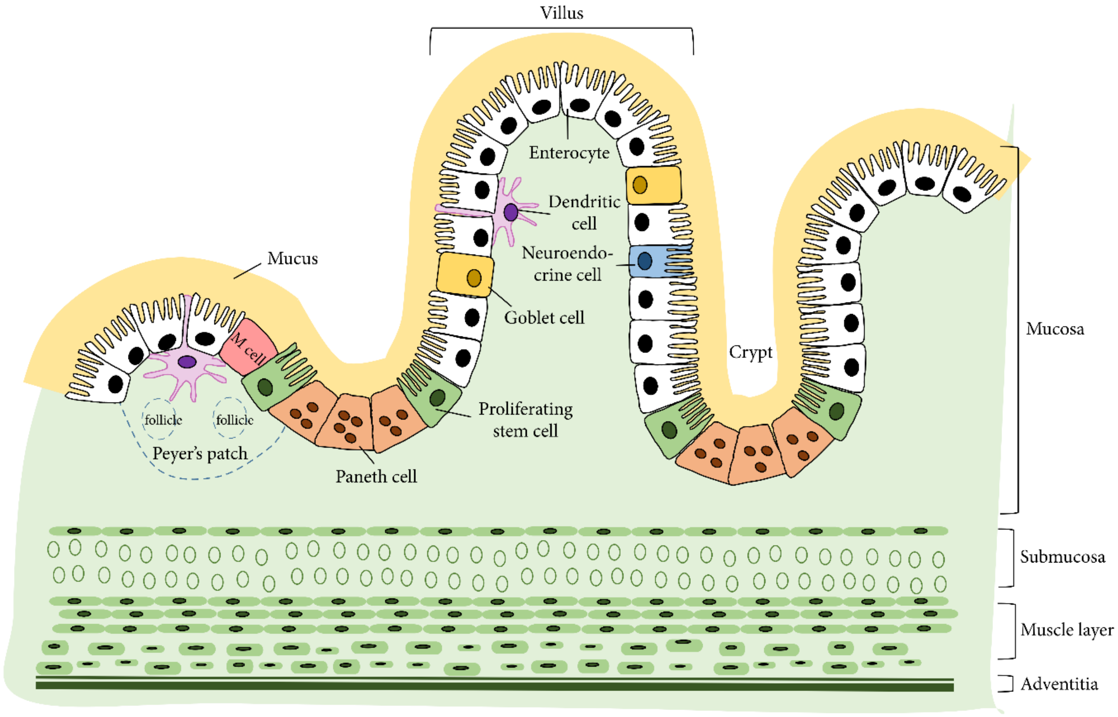


Figure 4 : Cross-sectional structure of small intestine from Kong, Zhang & Zhang. (2018)

The dense structure of the small intestine is made possible thanks to the apical junctional complex composed partially of tight junctions and subjacent adherens junctions (Fig.5). Both of which prevent free movement of microorganisms and particles across the membrane but allow water, ions and small particles to pass ((Kong, Zhang, & Zhang, 2018); (Otani & Furuse, 2020); (Sureda, 2017)). Indeed, intestines are a semi-permeable, dynamic and selective membrane. In fact, depending on their size and charge, some particles (up to 60 Å) can travel through pores of the apical junctional complex. Regarding the paracellular passage, claudins would sieve depending on the charge while junctional adhesion molecules (JAMs) depending on the size ((Turner J. R., 2009); (Otani & Furuse, 2020)). Depending on the position on the villi, pore size varies (Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012). A study by Fihn *et al.* (2000) showed that in rats particles as big as 4-5 Å at the tip, 10-15 Å (~3.5 kDa) at the base, at the crypt >20 Å can pass through ((Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012); (Fihn, Sjöqvist, & Jodal, 2000)).

The other constituent of the apical junctional complex, other than tight junctions, are the subjacent adherens junctions (Fig.5). Both complexes work together to make a tightly joint membrane. Desmosomes as well provide adhesion between cells (Turner J. R., 2009). Adherens junctions are composed of cadherin molecules (E-cadherin, beta-catenin, alpha-catenin) and actin. Desmosomes are composed of desmoglein, desmocollin, desmoplakin, and keratin filaments (Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012). Tight junctions on the other hand are made out of claudins, occludins, zona occludens (ZO) 1, 2 and 3, JAMs ((Turner J. R., 2009); (Otani & Furuse, 2020); (Campbell, Maiers, & DeMali, 2017)). The ZO proteins are scaffolding proteins able to form oligomers and interact with actin cytoskeleton, α-catenin, afadin, signaling proteins. They recrute claudins to drive tight junction assembly. Claudin protein family is comprised of 19 different proteins (Arrieta, Bistritz, & Meddings, 2006). Claudin-1, 3, 4, 5, and 8 strengthen the barrier, whereas claudin-2, 7, 10, and 12 weaken it (Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012). Zona occludens proteins bind claudins and actin filaments and allow control over permeability by contraction of actin fiber increasing intercellular space (Arrieta, Bistritz, & Meddings, 2006). This makes the junctional complex a dynamic one where space in between cells can vary.

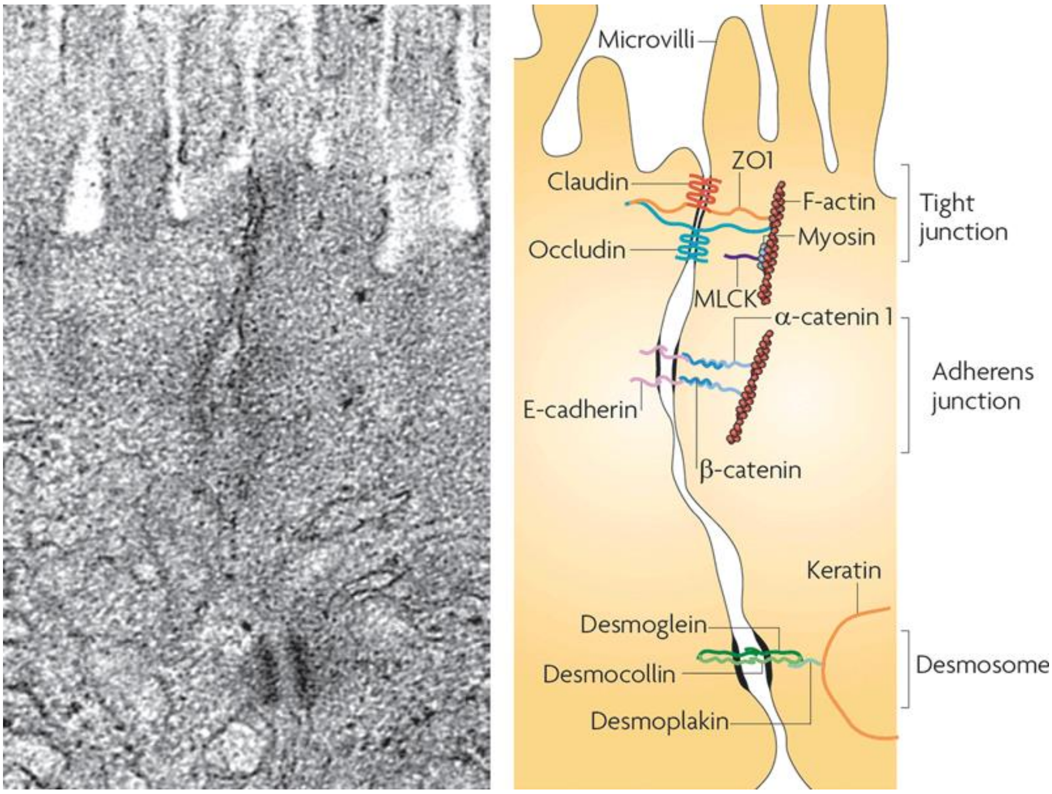


Figure 5: Tight junctions model from Turner J.R., 2009

1.5.2 Leaky gut

At weaning, piglets are exposed to many stressors which can have an effect on intestinal health. At weaning, a piglet’s intestinal immune system is exposed for the first time to feed antigens. The immune system is not yet able to distinguish and provide a suitable response to commensal bacteria, pathogens and antigens and this results in inflammation and destruction of cell structures. Disturbed intestines will cause digestive issues like nutrient malabsorption, increased intercellular space and diarrhea. Indeed, the intracellular space is high resulting in a so called ‘leaky gut’. In normal conditions, only water, ions and small molecules can take the paracellular pathway but under inflammatory circumstances, junctional complexes loosen and allow the uncontrolled passage of molecules (Sureda, 2017). Pathogens and particles take this pathway since intestinal membrane is easy to be breached and the result is infections and inflammation.

Pathogens can also actively increase intercellular space for colonization. Indeed, tight junction assembly/disassembly is regulated by internal or external signals affecting claudin expression resulting in a dynamic complex ((Arrieta, Bistritz, & Meddings, 2006); (Camilleri, Madsen, Spiller, Van Meerveld, & Verne, 2012)). When exposed to enteric bacteria, the small intestine will secrete zonulin as a defense mechanism to flush bacteria out by increasing intercellular space (Fasano, 2012). Zonulin levels can be used as an indicator of intestine permeability just like lactulose and mannose (see chapter 1.5.3 Permeability) and it is found to be positively correlated with urine lactulose/mannose levels in Fasano study (2012). Bacteria are able to loosen tight junctions on their own and actively increase permeability. For example, *Vibrio cholera* is able to take the zonulin pathway by stimulating protein kinase C resulting in actin contraction and thus in an increased intercellular space ((Arrieta, Bistritz, & Meddings, 2006); (Fasano, 2012)). The zonulin pathway is an example of permeability regulation by the zonulin protein.

Those attacks from the pathogens cause infections leading to inflammation of the intestine, weakness, slower growth and may lead to death. Enterohaemorrhagic *E. coli* (EHEC) bacteria are able to bind to host cells thanks to their type III secretion system (T3SSS) which is activated by succinate, a compound produced by commensal bacteria degrading mucus (Fig. 6) (Gresse, et al., 2017). Saccharolytic bacteria like Bacteroidales are able to digest mucus and release simple sugar molecules (fucose, galactose, sialic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose) that cross-fed other bacteria including pathogens (Bäumler). Fucose prevents EHEC gene expression for its metabolism and the expression of T3SS (Bäumler & Sperandio, 2016). Commensal bacteria like *Bacteroides thetaiotaomicron* can thus help pathogenic bacteria like EHEC infect the intestine. Succinase, another sugar produced by commensal bacteria, triggers the expression of mucinase in EHEC allowing it to pass through the mucus layer. Succinase is a compound produced by *B. thetaiotaomicron* for gluconeogenesis (producing lactate, glycerate too) in a poor nutrient environment. This metabolite production is sensed by transcriptomal regulator Cra by EHEC and upregulates its T3SS which is necessary to bind to enterocytes (Bäumler & Sperandio, 2016) (Fig. 6).

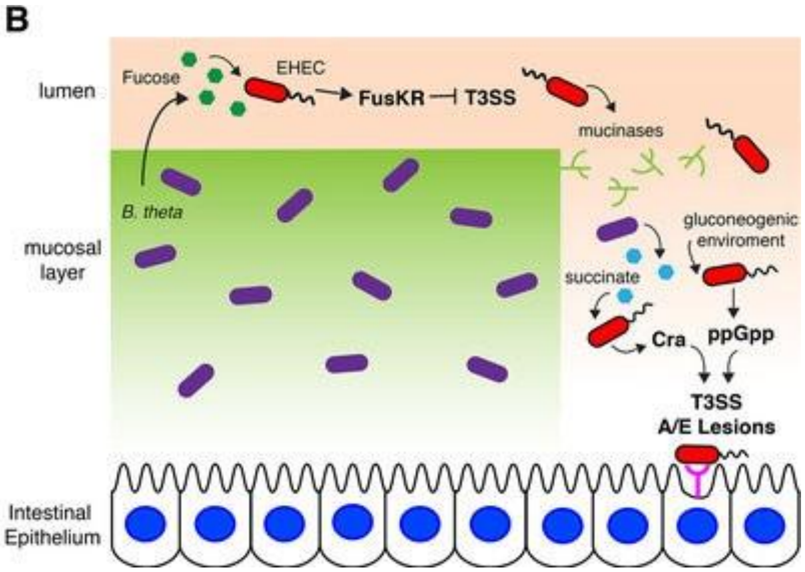


Figure 6: Virulence activation mechanism of E. coli regulated by succinate and fucose by De Nisco et al. (2018)

Morphology of the intestines takes a hit as height of villi and depth of the crypts are lowered too. In a study by Ji *et al.* (2019), piglets fed a diet containing Fusarium mycotoxins (deoxynivalenol and zearalenone) presented a down-regulation of claudin 3 protein, smaller villi height in duodenum, jejunum and ileum but also higher cell apoptosis. Toxins can accelerate cell apoptosis thus lowering membrane integrity. In this study, normal villi height can reach 360-390 µm while intestines exposed to toxins would lower them down to 313-335 µm and Claudin 3 number was lowered especially in the jejunum and ileum (Ji, Zhang, Zheng, & Yao, 2019). Toxins can disturb its distribution on the surface increasing permeability (Ji, Zhang, Zheng, & Yao, 2019).

To conclude, weaning is stressful which affects animal wellbeing. It results in diarrhea caused by pathogens, like enterotoxigenic or enterohaemorrhagic *E. coli or Salmonella typhimurium.* Indeed, the epithelial membrane is no more able to keep the intestinal content at bay. Physical and molecular functions as well as permeability are highly impacted.

1.5.3 Permeability

Permeability issues lay at the hearth of post weaning diarrhea issue. High permeability is illustrated by a reduced villi height, a higher cell apoptosis and bacterial infections. As the intestinal membrane does not prevent pathogen and toxin passage anymore, an inflammatory state sets in (Fig. 7). Permeability can be measured by different means like trans epithelial electrical resistance (TEER) or measuring the concentration of inert markers on both sides of the membranes. Those markers are not metabolized in the body and are thus secreted as they are. Small sugars can be used to determine intestinal permeability and intestinal absorption like mannitol (0.182 kDa), lactulose (0.36 kDa), xylose (0.15 kDa). Lactulose and mannitol are used together since lactulose is a disaccharide absorbed through the paracellular pathway in the crypts while mannitol is a monosaccharide sugar alcohol absorbed through the junctional complex or aqueous pores on the cells (Wijten, van der Meulen, & Verstegen, 2011). As explained earlier by Arrieta, Bistritz & Meddings (2006), pores allow the passage of big particules and makes the absorbtion of xylose, mannose, lactulose possible. Ratio of lactulose to mannitol is used to determine small intestinal permeability (Arrieta, Bistritz, & Meddings, 2006). A low ratio indicates good intestinal barrier with low paracellular passage. On the other hand, blood concentration can be influence by many premucosal factors (gastric emptying, intestinal transit time and bacterial degradation) and postmucosal factors (endogenous production, completeness of urinary collection and renal function) (Wijten, van der Meulen, & Verstegen, 2011).

In normal functioning intestine, enterocytes must create a barrier keeping the intestinal content away but it still needs to be able to absorb nutrients. Absorption is done thanks to transporters such a sodium dependent glucose transporter (SGLT1) and other sodium dependent transporter which actually activation leads to a natural widening of intercellular space. This increase in permeability allow the passage of particles of 2000 molecular weight up to 4000 molecular weight (Arrieta, Bistritz, & Meddings, 2006). This reaction is associated with phosphorylation of the myosin light chain kinase which is activated by a Ca^{2+} spike due to sodium concentration regulation. Normal functioning intestine can thus naturally allow the passage of some particles (Turner & Madara, 1995).

1.5.4 Microbiota

The microbiota refers to microorganisms living in the gastrointestinal tract. Bacteria, protozoa, fungi, archaea and viruses make up the microbiota. In young animals, the intestines are colonized by microorganisms encountered during environment exploration, skin microbiota from breastfeeding or even vaginal microbiota exposure during labor. The relationship between the animal and its microbiota is more than just commensal as the host has great interest to detain a “good” microbiota because it will provide protection against pathogen, provide nutritive values (like vitamins, SCFAs) or even stimulate immune system maturation. In return, the host provides the substrate on which microorganisms feed. In the healthy adult pig, gut microbiota is stable and dominated by anaerobic bacteria. *Lactobacilli*, member of the *Lactobacillaceae* family, and *Bifidobacteria*, member of the *Bifidobacteriaceae* family, are the most abundant bacteria in suckling piglets (Eberhard, et al., 2007). They are considered health promoting bacteria because they intervene in butyrate production.

1.5.4.1 Preweaning microbiota

Pre-weaning colon microbiota is mainly composed of phylla of *Firmicutes* (44.2 ± 2.9%), *Bacteroidetes* (39.6 ± 1.8%), *Proteobacteria* (6.9 ± 1.0%), *Fusobacteria* (5.7 ± 1.9%) and mainly composed of genus of *Prevotella*, *Lactobacillus* and *Bacteroides* (Lebois, et al., 2018). Besides, microorganisms modify their environment on their own to make it more suitable for themselves and other microbes. This is the case for *Streptococcus spp*. and *E. coli*, which are the first colonizers of neonate piglets and provide an oxygen-free environment favorable for later colonization of strict anaerobic bacteria like *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* ((Gresse, et al., 2017); (Konstantinov, et al., 2006)). Pre-weaning microbiota is mainly constituted of *Lactobacilli spp.* like *L. sobrius*, *L. reuteri*, *L. acidophilus* (*Firmicutes* phylum) and *Bifidobacteria spp*. (*Bacteroidetes* phyllum) (Sureda, 2017).

Gut microbiota composition is dynamic and can be influenced by the diet and the microorganisms from the surroundings. Petri *et al*. (2010) studied the evolution of the preweaned intestinal microbial population of piglets and found that for the first 0.5 days of life, the *Clostridiaceae* family (mainly *Clostridum perfringens*) was the most abundant one but it was quickly replaced by the Streptococcaceae family by the end of day 1. Also, the *Lactobacillaceae* abundance was slowly growing and by day 5, *Lactobacillaceae* were the most present ones (Petri, Hill, & Van Kessel, 2010). Over the next days, *Lactobacillaceae* dominated the intestine and reduced the relative importance of other bacterial families.

Thompson *et al.* (2008) worked on microbiota of early weaned piglets and found, no significant difference for the first 2 weeks in between piglets from the same pen and they actually shared 28.5% of microbial structures. Those piglets were separated from their mothers at 3 days and were fed a milk replacer solution. At 31 days of age, microbiota of piglets gained stability and similarity grew up to 62% with other piglets from the same pen.

1.5.4.2 Microbiota at weaning

The already settled microbiota structure occupies all available niches making it impossible for pathogens to colonize and thrive. With the change of the diet at weaning, a new type of element arrives to the intestine where the already settled microbiota is not adapted to ferment it, giving an opportunity for other strains of bacteria. In parallel, some pathogens can stimulate the immune system and induce inflammation in order to cause oxidative secretions, unfit for anaerobic bacteria, creating thus a niche for itself (Bäumler & Sperandio, 2016). One of the inflammation answers is antimicrobial secretions like NO but it is transformed into nitrate inside the lumen (Fig. 7) (Gresse, et al., 2017). It appears that *Enterobacteriaceae* such as *E.coli* or *S. typhimurium*, unlike *Clostridia* or *Bacteroidia,* are able to use this nitrate as energy source by nitrate reductase genes, giving them an advantage (Guevarra, et al., 2019). Inflammation affects also oxygen content in the lumen as more blood is drawn to the intestine favoring facultative anaerobes and reducing strict anaerobic bacteria diversity (Fig. 7) (Gresse, et al., 2017). At weaning, dysbiosis of the intestinal microbiota leads to the rise of gram negative bacteria population and pathogenic microorganisms resulting in post-weaning diarrhea (Sureda, 2017). The fecal microbiota in diarrheic piglets was associated with the increase in the relative abundance of families of *Prevotella*, *Sutterella*, *Campylobacter* and Fusobacteriaceae (Guevarra, et al., 2019). *Clostridium spp*., *Prevotella spp*. or facultative anaerobes such as *Proteobacteriaceae*, including *E. coli* thrive at the expense of *Lactobacillus* bacteria group and general microbe diversity ((Gresse, et al., 2017), (Konstantinov, et al., 2006)). In the study by Konstantinov *et al.* (2006), they found a significant difference for the microbial communities in the ileum and colon of weaned piglets between the 19th and the 23rd day while weaning was done at 21 days. Indeed, total count of *Lactobacilii* and particularly the population of *L. sobrius* and *L. reuterii* was lowered in the ileum of weaned piglets. Also, lactate concentrations were higher in the ileum of weaned piglets, yet no significant difference was observed for the total volatile fatty acids. Pathogens are the primary cause of diarrhea but the accumulation of lactate and succinate as well as an underdeveloped intestine and mucosal immune system also impact diarrhea (Konstantinov, et al., 2006).

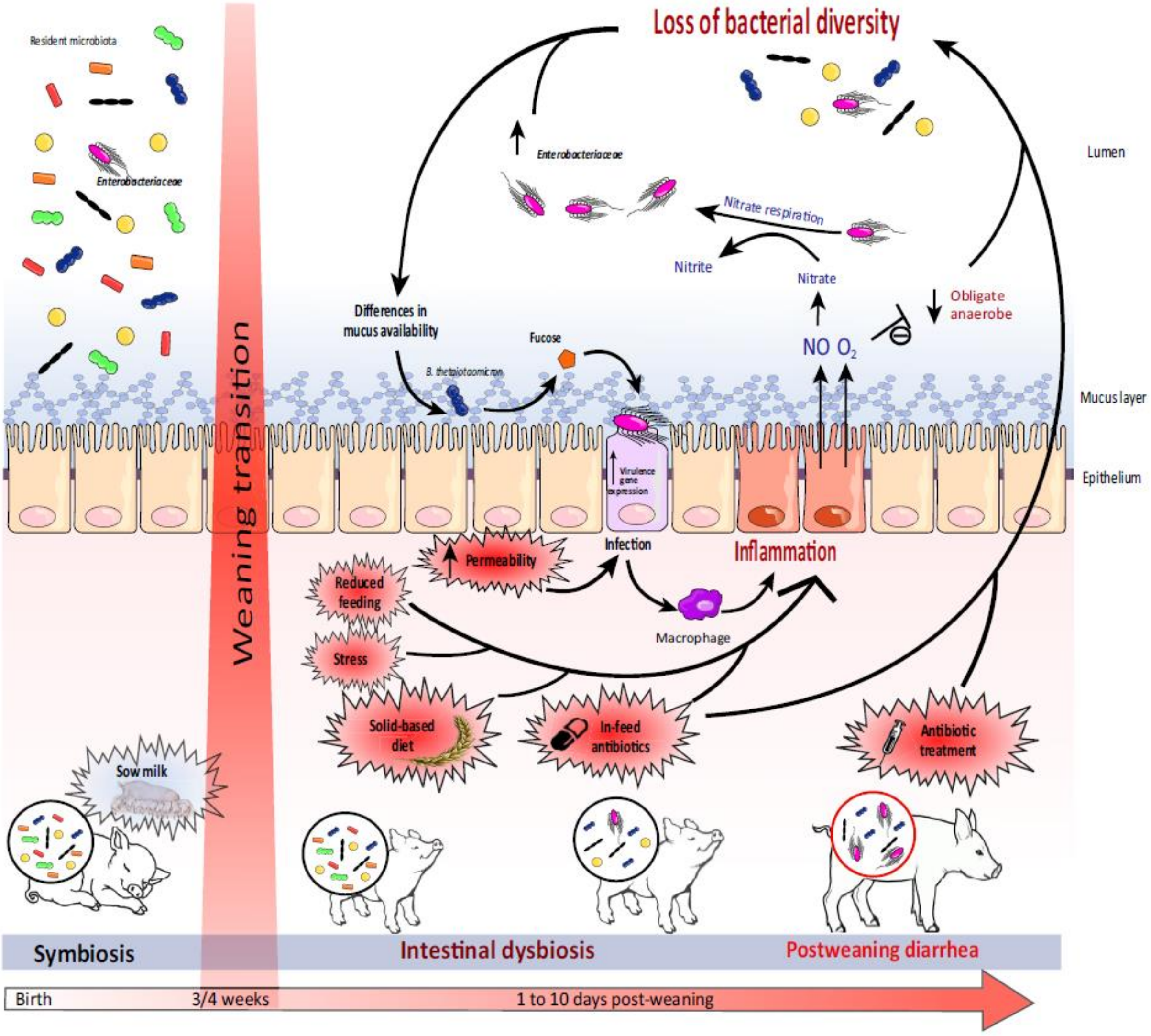


Figure 7 : Infection mechanism of E. coli from Gresse et al. (2017)

At weaning, stress and new feed particles arriving to the intestine alter the microbiota. Prebiotics may be given at this period to favor and maintain *Bifidobacteria* dominated flora as long as possible, preventing drastic changes. In a review by Flickinger *et al*. (2003), an experiment by Bunce *et al*. (1995) proved that piglets fed oligofructose and then infected with ETEC had better survival rates (mortality 100% -> 62.5%) and a higher *Bifidobacteria* content in fecal samples. Clostridia was not affected by oligofructose in fecal samples. Prebiotic supplementation with the diet can be used to shape the microbiota in order to provide health advantages. Prebiotics inclusion is mainly used to avoid drastic changes of the microbiota and to maintain *Bifidobacteria* domination as long as possible (Flickinger, Van Loo, & Fahey, 2003).

1.5.4.3. Prebiotics

Prebiotics are compounds undigested by the host in the higher part of the gastrointestinal tract. Host's enzymes may not be able to hydrolyze the bonds of the molecule or the particle is protected by indigestible compounds like for example resistant starch. Those particles continue their way through the gastrointestinal tract until they encounter microorganisms. Cellulose, hemicellulose, pectins, lignins, fructo-oligosaccharides, inulin or even chicory flour are examples of prebiotics. Prebiotics serve to feed and thereby stimulate the microbiota in order to obtain some health benefits for the host. For one, feeding beneficial or commensal microorganisms, makes it impossible for harmful pathogens to colonize the environment. This is obtained by increasing space and nutrient competition or by producing compounds lethal to those pathogens. Secondly, fermentation allows to obtain energetic benefits from indigestible particles as a form of SCFA that otherwise would simply be wasted. Lastly, some prebiotics may play a role in immunity, stimulating or toning down the immune response (Shoaib & et al, 2016).

Chicory (*Cichorium intybus*) root contains fructans, chlorogenic acids and sesquiterpene lactones which may bring health benefits. In Pouille *et al.* (2022), they found that 83% of benefits coming from chicory root consumption comes from the fructose type elements. Chlorogenic acids acts on insulin sensitivity in diabetic rats, has antioxidant properties *in vivo* and antimicrobial properties *in vitro* ((Pouille *et al.*, 2022), (Tajik, Tajik, Mack, & Enck, 2017)). Cells grown in a medium containing chlorogenic acid showed a dose-dependent secretion of GLP-1 (Domínguez Avila, García, Aguilar, & de la Rosa, 2017). Sesquiterpene lactones have antitumor, antimicrobial, antioxidant, hepatoprotective, antiprotozoal properties.

Inulin is a water-soluble fiber and can be used as a prebiotic (Shoaib & et al, 2016). It is a storage molecule in plants (Cherbut, 2002). Inulin is present in over 3.000 vegetables like chicory, dahlia, Jerusalem artichoke, asparagus, etc. (Shoaib & et al, 2016). Chicory root's dry matter is 70% composed out of inulin and can be grinded into a flour or be used to extract high-concentration inulin powder. Inulin is made up of one glucose unit and a chain of fructose linked together with β(2-1) bonds (Fig. 8) ((Shoaib & et al, 2016); (Bosscher, Van Loo, & Franck, 2006)). Beta bonds are not digestible by mammals (except for lactose). Microorganisms are able to break those bonds thanks to β-fructofuranosidase (Moens, Verce, & De Vuyst, 2017). Inulin is polymerized with an average degree of 12 but the chain's length may vary from 2 to 60 units ((Bosscher, Van Loo, & Franck, 2006); (Li, et al., 2018)). Fructo-oligosaccharides (FOS) groups molecules with a maximum degree of polymerization of 10. FOS are issued from chemical hydrolysis of inulin or synthesized by transfructolysation of sucrose by the glycosyl transferase enzyme ((Rossi, et al., 2005); (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002)).

Inulin's roles on the intestines are multiple. It affects trophicity and mucin expression (Cherbut, 2002). Prebiotics affect the morphology of the intestine resulting in an increased villi height : crypt depth ratio ((Uerlings, Schroyen, Bindelle, Bruggeman, & Everaert, 2021); (Verdonk, 2005)). Also, inulin stimulates *Bifidobacteria* and *Lactobacilli* (or more generally speaking lactic acid-producing bacteria) which prevent pathogenic microorganism's development by different means. Indeed, they secrete lactic acid as a by-product of inulin fermentation making the environment too acidic for pathogens (Konstantinov, et al., 2006). When the pH reaches 4.5-5.0, *E. coli* and *C. perfringens* levels drop to 0 while lactic acid-producing bacteria are not affected (Bosscher, Van Loo, & Franck, 2006). Inulin is fermented and by cross-feeding, different by-products are made such as SCFA (acetate, propionate, butyrate, valerate, formate), CO_2 and H_2 (Bosscher, Van Loo, & Franck, 2006). *Lactobacillus* strains are able to stimulate the proliferation of *Faecalibacterium prausnitzii* coinciding with high butyrate formation (Meimandipour, et al., 2010). SCFA are valorized by the animal as an energy source, supplying up to 70% energetic requirements of enterocytes, or for other properties like growth stimulation, retard cell atrophy, anti-inflammatory properties, etc. ((Rossi, et al., 2005); (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013)). Besides, as inulin is a water-soluble molecule and has gelling properties, it is useful for stool structure and frequency of defecation (Shoaib & et al, 2016).

In a study by Barszcz *et al*. (2020) where they tested different degrees of polymerization of inulin, intraepithelial lymphocyte counts and secretory IgA in the colon was not affected by Jerusalem artichoke tuber supplementation. It did however increase *Bifidobacterium* counts in the colon (Barszcz, Taciak, Tuśnio, Święch, & Skomiał, 2020). They determined that villus height in the duodenum decreased with inulin supplementation for the 2 types of inulin tested (10 DP and 23 DP) (510 → 375 or 368 μm) but in the jejunum the inulin with a degree of polymerization of 10 increased villus height when given at 1 or 2% (441 → 541µm). Villus height to crypt depth ratio increased in the same modality. Barszcz *et al.* (2020) concluded that the shorter ones are more active in the small intestine as they are faster and preferably fermented (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002). Stewart *et al.* (2008) found that short chains (FOS) have the highest fermentation rate at 0-4 hours while for long chains (inulin) it is between 12 and 24 hours. Besides, depending on the bacteria, some may favor shorter or longer chains for fermentation (Moens, Verce, & De Vuyst, 2017). Rossi's *et al.* (2005) confirmed that all *Bifidobacteria* strains are not equal for oligo fructose degradation. They said that not every *Bifidobacteria* is able to degrade inulin molecules but all are indeed able to degrade shorter chains like FOS. Inulin *in vitro* fermentation influenced the growth of *Clostridia, Lactobacilli,* coliforms and *Bacteroides* and led to a remarkable accumulation of butyric acid and lower amounts of acetic, lactic, and propionic acids. On the contrary, lactic and acetic acids were the major products of shorter chains of fructose fermentation ((Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002); (Rossi, et al., 2005)). These findings reflect the consequence of the different effects of FOS and inulin on microbiota composition and activity (Rossi, et al., 2005). In general, *Bifidobacteria* fermentation yields for every 2 mol of fructose, 2 mol of lactate and 3 mol of acetate (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002). In Perrin *et al.* (2002), long chains of fructose (70% DP >20 & 29% DP<20) by the fermentation of *B. angulatum* yield after 24 hours, high levels of lactate and acetate (3.51 ± 0.27 g/L and 4.03 ± 0.42 g/L respectively) comparing to the other 2 comparative strains (*B. longum* and *B. infantis*). *B. angulatum* with small chains (92% DP<20 & 5% DP>20) and average chain (60% DP<20 & 30% DP>20) gave the same acid production than the other 2 strains tested (*B.longum* and *B.infantis*) (6.95 ± 0.22g/L and 6.81 ± 0.42g/L from short chains and 5.07 ± 0.12 g/L and 4.66 ± 0.63 for average length chain for lactate and acetate respectively). By measuring residual oligofructose in the medium after fermentation, they concluded that *B. longum* and *B.infantis* do not ferment fructose chains >20 DP (residues 80-100%). On the opposite, *B. angulatum* used the shorter chain in a lesser extend (20% residues against <10% for the other 2 strains) than the larger one where 40% were fermented (60% residues). Bacterial multiplication and acid production is still higher with the smaller chains of fructose even for *B. angulutum.* Also, fermentation of long chain fructose starts only after a few hours as *Bifidobacteria* prefer shorter chains first. This could be applied to stimulate microbiota from distal zones of the gastrointestinal tract with the aim to shift from protein fermentation to carbohydrate fermentation. In a study conducted by Stewart *et al.* (2008), they concluded that raw FOS had a higher gas production rate between 0 and 4 hours (13.1 ± 0.1 µmol/mL/h vs 7.7 ± 0.5 µmol/mL/h) and that inulin had it greater between 12 and 24 hours (0.2 ± 0.3 µmol/mL/h vs -0.8 ± 0.08 µmol/mL/h for FOS).

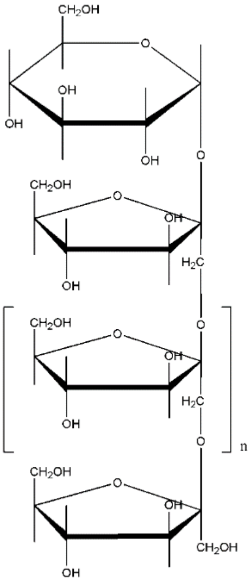
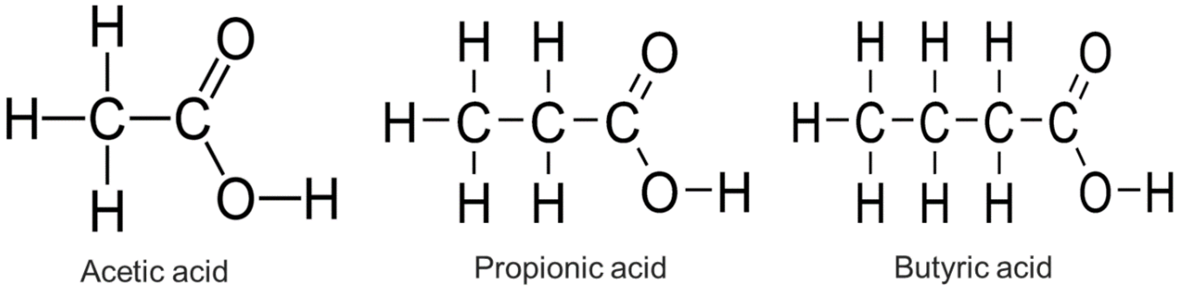


Figure 8 : Inulin structure where n is the number of fructose units in the chain

‘Commonly known’ inulin affects microbial diversity and increases the relative abundance of *Bifidobacterium* and *Lactobacillus* in colon and fecal samples resulting in a decrease of *Enterobacteriaceae* like *E. coli* and *Clostridium* like *Clostridium perfringens* populations ((Li, et al., 2018); (Paßlack, Vahjen, & Zentek, 2015); (Mair, Plitzner, Schedle, & Windisch, 2010)). Loh *et al.* (2006) confirmed that inulin affects the detection of *Bifidobacteria* in the colon of piglets fed with inulin (40% >< 13% of presence). Yet, a study conducted by Eberhard *et al.* (2007) did not find any significant influence of inulin on *Bifidobacterium* and *Lactobacillus* in the jejunum or caecum. (*Bifidobacterium* and *Lactobacillus* properties are explained in the next chapter.) On one hand, Mair *et al.* (2010) demonstrated a decline in enterococci population in the colon of piglets fed 0.4% inulin supplementation and an increase in aerobes in the jejunum. They observed lowering pH properties of inulin in the ileum but no significant difference of pH in the colon. Mair *et al.* (2010) confirmed Loh *et al. (2006)*’s conclusion on *Bifidobacteria* counts as 57.1% of piglets fed inulin had *Bifidobacteria* in their ileum against 19% in pigs with no inulin. On the other hand, Paßlack *et al.* (2015) found no influence of inulin on *Bifidobacteria* and thereby confirms Ebergard’s results. Inulin did increase the amount of *Enterococci* (aero-anaerobic gram + bacteria) in the faeces of a sow and lowered its pH. He also demonstrated a higher amount of *Clostridium leptum* (butyrate producing clostridium group) and *Enterococci* (lactic acid bacteria) in the caecum of piglets (Paßlack, Vahjen, & Zentek, 2015).

There seems to be no clear answer whether or not inulin acts on *Bifidobacterium* and *Lactobacillus* population but Moens *et al.* (2010) proposed a way of inulin utilization by *Lactobaccilli* and *Bifidobacteria* rendering butyrate with the help of other microorganisms confirming inulin properties of those 2 strains. Microbial fermentation of prebiotics results in the production of short-chain fatty acid like acetate, propionate or butyrate. The last one is peculiarly important as it has anti-inflammatory properties on the intestines. This explains thus the possible usage of prebiotics for the anti-inflammatory properties during weaning and justify its study.



Acetic acid Propionic acid Butyric acid

Figure 9: Chemical structure of acetic acid, propionic acid and butyric acid

1.5.4.4 Butyrate producing microbiota

The Clostridium clusters IV (=Clostridium leptum group) and XIVa (=Clostridium Coccoides group) are known as butyrate-producing species, (Hold *et al.* 2003; (Moens, Verce, & De Vuyst, 2017)). Those 2 groups make up 10-40% of all bacteria in human gut microbiota (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). The *Clostridium* cluster *XIVa* includes species belonging to the *Clostridium, Eubacterium, Ruminococcus, Coprococcus, Dorea, Lachnospira, Roseburia* and *Butyrivibrio* genera. *Clostridium* cluster *IV* is composed by the *Clostridium, Eubacterium, Ruminococcus* and *Anaerofilum* genera (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). Clostridia are generally strict anaerobic bacteria, gram-positive, spore forming. Most of the *Clostridia* group is commensal except for *C. perfringens, C. tetani* and *C.difficile* which are part of the Clostridium I and XI cluster.

Moens *et al.* (2017) worked on bacterial strains degrading inulin and producing butyrate and described a cross feeding pathway. First, inulin is degraded enzymatically by β-fructofuranosidase. Depending on the *Lactobaccilli* or *Bifidobacteria* bacteria strain used, free fructose, lactate, acetate or formate are produced. For example, *L. acidophilus* transforms short- and long-chain inulin into free fructose and then fully into lactate while *L. paracasei* and *B. longum* produce a mixture of lactate and acetate. *L. paracasei* gives more lactate than acetate and vice-versa for *B. longum*. Then, lactate or free fructose is transformed into butyrate. Lactate and acetate produced by members of the *Bifidobacteria* cross-feed butyrate-producing colon bacteria from the clostridium clusters IV (e.g., *Faecalibacterium prausnitzii* from *Ruminococcaceae*, cluster IV) and XIVa (e.g., *Anaerostipes caccae, Eubacterium hallii, Eubacterium rectale* and *Roseburia spp.*). Lactate can be transformed into butyrate but only in the presence of acetate (Moens, Verce, & De Vuyst, 2017). 2 molecules of acetyl coA are necessary for the formation of 1 molecule of butyrate (Fig. 19). Butyrate formation is mainly catalyzed by butyryl-CoA:acetate-CoA transferase but butyrate kinase can do so too (Duncan, Barcenilla, Stewart, Pryde, & Flint, 2002). The coenzyme A is transferred from the butyrate-coA molecules onto the acetate molecule providing a butyrate molecule. Butyrate is mostly produced from inulin fermentation while FOS fermentation gives more acetate and lactate. Butyrate production is not limited to *Clostridium* IV and XIV groups. There is a high abundance of bacteria able to produce butyrate in those groups but this capacity is wide-spread (Uerlings *et al.*, 2019).

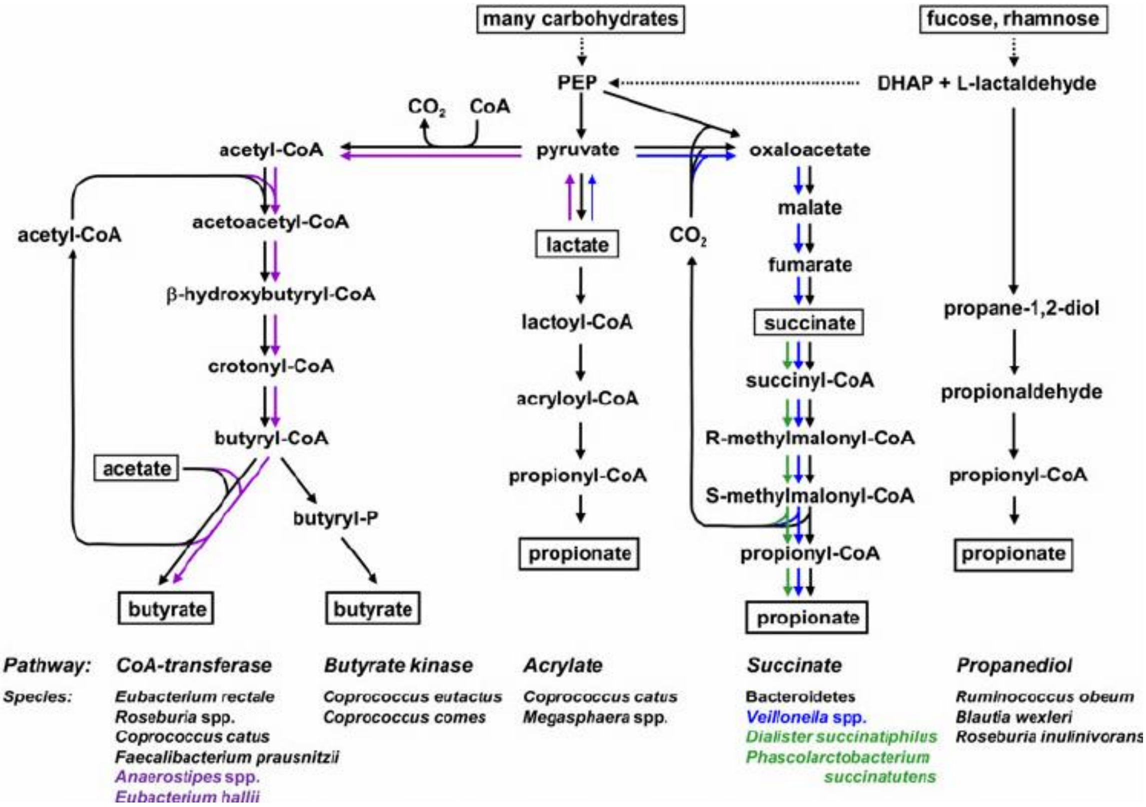


Figure 10 : Metabolic routes for butyrate and propionate formation by representative bacterial genera and species from the human colon by Flint et al. (2015).

Species shown in purple can utilise lactate to form butyrate; species shown in blue can utilise lactate, and those shown in green

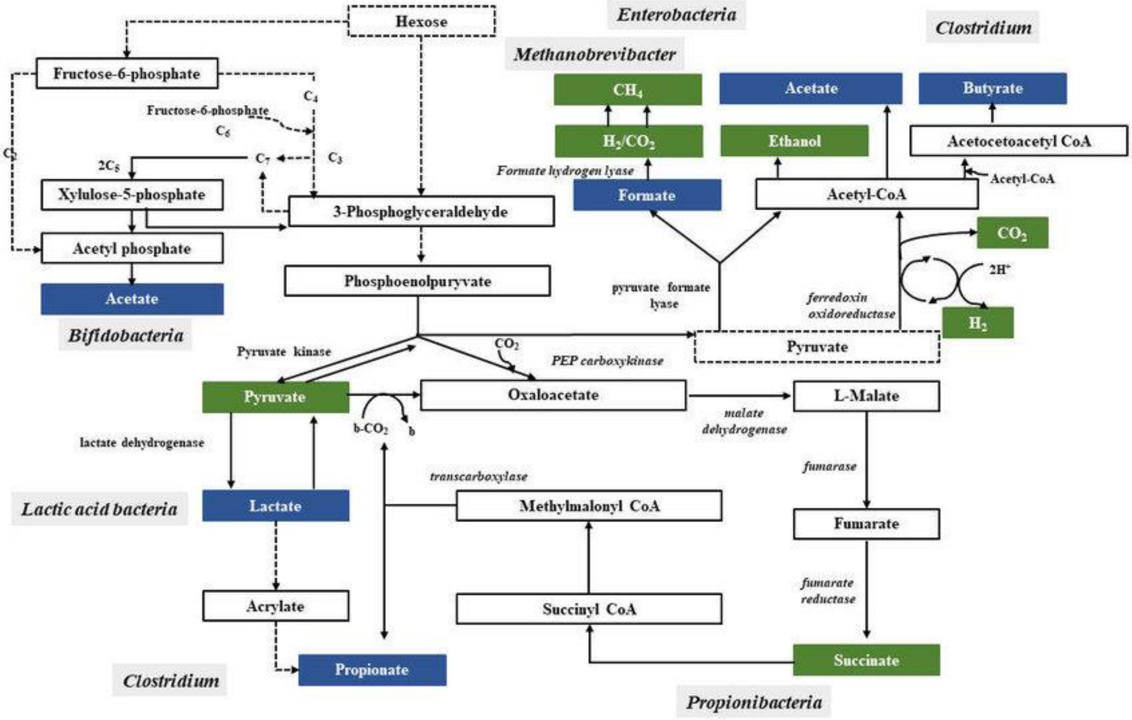


Figure 18 : Pathways leading to SCFAs and lactic acid production by intestinal bacteria by Markowiak-Kopeć & Śliżewska (2020)

1.5.4.5 SCFA

Prebiotics are fermented by microbes for their own energy metabolism. They produce SCFA as end-products such as acetate, propionate, butyrate, lactate and valerate (Fig. 9). CO_2 and H_2 are also produced. Hydrogen has a beneficial effect on epithelial cell proliferation as it is an anti-inflammatory, anti-oxidative and anti-apoptotic compound (Ji, Zhang, Zheng, & Yao, 2019). Undigested proteins will be degraded and branched amino-acids will be fermented resulting in branched-chain fatty acid production like valeric, isovaleric, isobutyric acid inducing an inflammatory compound formation. In a study by Uerlings *et al.* (2019) where inulin has been fermented *in vitro*, SCFA production has been measured at different time points. At 12 hours, inulin provided total SCFA 5.7 ± 0.1 mmol/g OM out of which 48.3 % ± 0.6 accounted for acetate, 40.6% ± 1.4 for propionate and 11.0% ± 0.9 for butyrate. Branched short chain fatty acid and lactate production were null at 12 hours and high at the 6 hours reaching 8.2% ± 4.5 for BCFA and 2.5 ± 0.1 mmol/g OM (Uerlings, et al., 2019).

In a study by Mair *et al.* (2010), inulin decreased acetate production (315.82 -> 296.51 mmol/kg DM) in the colon but increased lactic acid production (29.45 -> 63.89 mmol/kg DM). High lactate production indicated high lactic acid bacteria activity (LAB) but did not show any significant differences in bacterial structure. It also decreased total SCFA production in the colon (553.8 -> 521.17 mmol/kg DM P-value = 0.094). Mair *et al.* (2010) discovered high negative correlation for pH and lactic acid in ileum, for pH and propionate and butyrate in the colon.

A 48 hours fermentation of different substrates inoculated with bacteria from Clostridia IV and XIV was tested by Moens *et al* (2017). Pure fructose gave 12.5 ± 0.4 mM of butyrate. Lactate with acetate fermentation gave 37.2 ±0.1 mM butyrate and the fermentation of fructose and lactose in the presence of acetate gave the highest butyrate score of 77 ± 1.8 mM. Fermentation of pure lactate gave 0.0 ± 0.0 mM of butyrate. Moens *et al.* (2017) concluded that it was useful to have a bacterial strain able to degrade inulin into acetate as it is necessary for butyrate synthesis from lactate.

Propionate, just like butyrate, has health promoting properties like anti-inflammatory, anti-lipogenic, cholesterol–lowering, anti-carcinogenic actions. Propionate is produced following the succinate pathway (methylmalonyl-CoA by *Bacteroidetes*, *Negativicutes*), acrylate pathway (lactyol-CoA dehydratase by *Lachnosppiraceae*, *Negaticiutes*) or the propanediol pathway (propionaldehyde dehydrogenase by *Lachnospiraceae*) (Fig.10). *Lachnospiraceae* is part of clostridial cluster XIVa (Flint, Duncan, Scott, & Louis, 2015). Reichardt *et al.* (2014) worked on the different propionate pathways. They concluded that succinate pathway was the most abundant one while the acrylate one had a very limited distribution in the human gut microbiota. They found that the majority of bacteria that had genes for butyrate synthesis did not have genes for propionate synthesis and vice-versa. Thus, one bacteria will not be able to produce propionate and butyrate as major outputs of fermentation. Yet, some bacteria like *Clostridium catus* was able to transform lactate into propionate by the acrylate pathway but when fructose was present, mainly butyrate was produced. Same for *Roseburia inulinivorans*, it was producing propionate from fucose (propanediol pathway) but butyrate when exposed to glucose (Reichardt, et al., 2014).

In addition of being used for gluconeogenesis (propionate), directly as an energy source by the enterocyte (butyrate) or by peripheral tissues (acetate), SCFA can transfer messages by binding to free fatty acid receptors like G protein-coupled receptors Free fatty acid receptor (GPR) and stimulate hormone secretion. In fact, Propionate mainly stimulate the secretion of GIP, insulin, and amylin while butyrate stimulated the secretion of anorexigenic peptides like the gut hormone glucagon-like peptide-1 (GLP-1) or PYY ((Frost, et al., 2014); (Lin, et al., 2012)). Those hormones resulted in a significant lower feed intake and a lack of weight gain in mice. On the other hand, acetate, non-significantly, reduced by only 40% the expected weight gains in mice and increased, non-significantly, feed intake by 23% (Lin, et al., 2012). On the opposite, Frost *et al.* (2014) showed that acetate regulates body weight and the central nervous system. Indeed, a change in hypothalamic ACC and AMPK activities is observed with high acetate levels without changing the hormonal levels of GLP-1 and PYY. Acetate injections suppressed food intake for 2 hours and reduced it for up to 4 hours after the injection. In their case, acetate did not affect PYY and GLP-1 levels but sill showed anorexigenic properties (Frost, et al., 2014).

BCFA result from peptide degradation and amino acid fermentation. Valine is transformed into iso-butyrate, leucine into iso-valerate, isoleucine gives valine and 2-methyl-butyrate but other amino acids can be fermented into regular SCFA. Branched amino acids are first transaminated producing ketocompounds which are later transformed into acids. Indeed, leucine catabolism gives first α-ketoisocaproic acid which later on is converted into isovaleric acid (Thierry, Maillard, & Yvon, 2002). Protein degradation becomes gradually more important in the human distal colon part reaching 38% of total SCFA against only 17% in the proximal part ((Macfarlane, Gibson, Beatty, & Cummings, 1992); (Shim, Verdonck, Pellikaan, & Verstegen, 2007)).

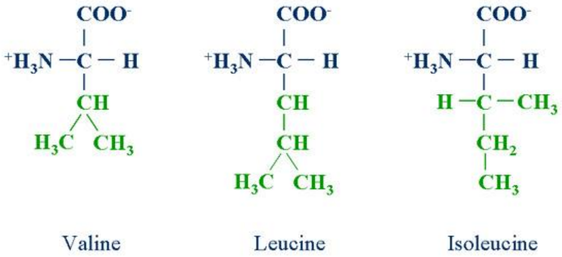


Figure 11: Molecular structure of branched amino acids

Butyrate

Butyrate can affect gene expression like for instance *NFKB* or *FOXP3*. Indeed, butyrate inhibits the activation of NFKB which is used for the expression of proinflammatory cytokines. This makes butyrate an anti-inflammatory compound (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). *FOXP3* is a gene necessary for regulatory T cells (Treg) which are able to produce anti-inflammatory interleukins. Butyrate promotes formation of regulatory T cells (Tregs). The mechanism behind is that butyrate lifts HDAC (an inhibitor of gene expression) of FoxP3 which is a protein useful for Tregs formation ((Cushing, Alvarado, & Ciorba, 2015); (Lin, et al., 2012)). Tregs are able to control T cell multiplication and cytokine production (Kondělková, et al., 2010). It is a mechanism for self-control of the immune system. Antigen-specific FoxP3+ Treg for non-pathogens are generated in mesenteric lymph nodes but are able to express CCR9 and integrin α4:β7 in order to fit in lamina propria. Tregs are induced by retinoic acid and transforming growth factor-β (TGF-β) produced by dendritic cells. Dendritic cells participate in anti-inflammation processes (Murphy & Weaver, 2017). *Clostridia* exerts a strong influence on the immune system. It can induce IgA-producing cells in the colon of mice and the development of T cell receptor by Il-6, IL-7 and TGF-β. *Clostridium* XIV and IV are strong inducers of colonic regulatory T cell accumulation since they secrete butyrate (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013). *Clostridium spp.* can affect the number and function of colonic Tregs, inducing naive $CD4^+$ T cells to differentiate into antigen-specific colonic Tregs that are able to enforce immune tolerance towards commensal bacteria. Increased production of IL-10 in Treg cells and decreased expression of NFKB lead to a consequent intestinal and systemic anti-inflammatory effect

Also, butyrate affects membrane permeability. Indeed, Mercado *et al.* (2013) reported that in cell culture, 1mM of butyrate induces a 12% increase in transepithelial resistance in LLC-PK1 cells exposed to it. This can be explained by a significant increase in tight junctions proteins : +37% claudin 1, +47% claudin 2, +96% claudin 3, +185% claudin 4, +299% claudin 5 (Mercado , et al., 2013).

1.6 *In-vitro* digestion, dialysis, fermentation

Animal experimenting brings ethical questions and so an ethical committee must examine the case. They apply the principles of humane experimental techniques proning the 3Rs standing for 'Replace, Reduce, Refine'. The first R stands for 'Replace' where animal experimenting, if possible, should be replace by a predictive model. Different tools and models exist such as *ex vivo* methods, cell culture, *in vitro,* etc. By deep understanding of all the processes taking place in the digestive tract, it is possible to recreate artificially all the steps required to mimic the digestive tract. Different protocols are available in the literature. However, there is still great potential for improvement, as not every animal alternative has been systematically compared with the *in vivo* results. Besides, *in vitro* models utilize exactly the same materials in between the laboratories reducing to minimum variability. By having a 'perfect' *in vitro* model, we would not only reduce experimental animals, we would be able to completely eliminate it. *In vitro* digestion allows to obtain digested samples without having to extract it from the animal. It is also quicker, less expensive and less laborious than animal experimenting. *In vitro* digestion also allows to collect feed digestibility data too.

The digestive tract can be summed up in 4 phases; the oral phase, the gastric phase and the intestinal phase (small and large intestine). All requiring different enzymes, pH values, buffers, salts and durations to follow as closely as possible the actual digestion *in vivo*. Many different protocols exist. In order to find a common ground in between laboratories around the word, the INFOGEST protocol was described by more than 200 scientists to consolidate conditions for a consensus on in-vitro digestion (Minekus, et al., 2014). The INFOGEST protocol details the conditions for the first 3 phases of in-vitro digestion (oral, gastric and intestinal). The small intestine is also the place of nutrient absorption which is not described in this protocol. Intestinal absorption can be mimicked artificially with the help of porous membranes where the diameter of the pore can be modulated. Indeed, depending on the size, the membranes allow the particles to seep out. When dialyzing digested feed containing soluble prebiotics, the aim is to eliminate small molecules and retains the fibers. Fiber molecules like inulin molecules are not meant to seep out through the membrane. They should continue their way to the next step where fermentation takes place.

The intestinal membrane is covered with mucin on which microbiota develop and feed. Mucins modify gas production kinetics and microbial population as it favors bacteria growing on mucus like *Lactobacillus sp*. (Uerlings, et al., 2019). Mucin carriers should be included to provide a better model. In a study by Tran *et al*. (2016), mucin carriers were tested in *in vitro* batch fermentation. The fermentation of inulin with mucin covered beads resulted in a higher gas production and an altered SCFA profile but *Lactobacillus spp.* and *Bifidobacterium spp.* populations decreased for inulin fermentation with mucin carriers. Also, the population of *Bacteroides spp.* increased. Same for *Firmicutes.* Acetate and butyrate production were positively but propionate production was negatively correlated with the type of substrate (cellulose or inulin) while bacterial communities were correlated with the presence of mucus. Bifidobacteria were considered acetate producing bacteria and Bacteroides, propionate producing one while Firmicutes oxydate acetate, producing methane. This resulted in low acetate levels (not a significant difference). *Enterobacteriaceae*, *Clostridiaceae* and Lactabacilli need mucin to proliferate. *Ruminococcus gnavus* (*Lachnospiraceae,* Firmicutes) uses mucin to produce propionate. Propionate production increased 6-8% with the introduction of mucins for inulin fermentation (Tran, et al., 2016). In Tran *et al*. (2016), gas kinetics parameters were significantly different for the ingredient, for the mucin (except for R_{max}). Maximum gas production was reached by inulin with mucins (297 mL/g), same for maximum rate production (57.5 mL/h/g). B was 5.63 h and T_{max}, 4.9 h. Also, propionate levels were higher in fermentation vials containing mucin carriers at 24 and 72 hours. For cellulose fermentation, acetate levels also increased and butyrate remained unchanged except at 24 hours where it slightly increased (6.8% + mucins >< 5.3% - mucins). BCFA was significantly decreased for cellulose and inulin fermentation with mucin carriers.

1.7 Immunity

Every day, animals are exposed to dangers like open-wounds, airborne diseases, allergies or even an attack from own microbiota in the intestine. Indeed, if the immune system is weakened, microorganisms can take over, multiply at the expense of the animal and produce toxins which will weaken it or even kill it. To avoid so, the intestine has different protective strategies. Firstly, apical junctional complex of the epithelial cell as well as the mucus layer provide a physical barrier. Secondly, active mechanisms of secretion or cell activation by the immune system are key for active adaptive healthy immunity. For example, the intestine can produce secretions like antimicrobial peptides and secretory immunoglobulin A (Hiippala, et al., 2018).

1.7.1 Mucosa associated lymphoid tissues structure

The mucosa associated lymphoid tissues (MALT) are the first line of defense against pathogens as it forms a barrier in contact with the exterior continuously exposing it to pathogens and foreign antigens (Fig. 12). Mechanical barriers like mucus and apical junctional complex are the first line of defense but can be breached, requiring an immune response (Shi, Shi, Duan, & Niu, 2017) MALT in the gastrointestinal tract is referred to as gut-associated lymphoid tissues (GALT) and include tissues like Peyer's patches, isolated lymphoid follicles (composed mainly of B cells), lymphocytes in between epithelial cells or in lamina propria ((Brandtzaeg, Kiyono, Pabst, & Russell, 2008); (Murphy & Weaver, 2017); (Watzl, Girrbach, & Roller, 2005)). In total, 60% of all lymphocytes are stored in the gut. Peyer's patches (PP) are a well-organized immune structure present in the small intestines composed of B-cells follicles with germinal center surrounded by T cells, a subepithelial dome, below an M cell, rich in dendritic cells, B cells and T cells. The M cell collects antigens from lumen by transcytosis and presents them via dendritic cells to lymphocytes hidden beneath it to induce the immune system. The neonatal Fc receptor (FcRn) can bind immunoglobulins or antigens creating a complex which in turn is presented to antigen presenting cells, dendric cells and T cells resulting in an immune response (Sureda, 2017). Macrophages and intraepithelial dendritic cells can also take antigens straight from the lumen. Isolated lymphoid follicles can be found in the small and the large intestine and they contain, just like Peyer's patches, M cells but are mainly composed of B cells unlike Peyer's patches (Murphy & Weaver, 2017). Both lymphoid organs are connected by lymphatics with mesenteric lymph nodes which intervene in the initiation and shaping of the immune response. T and B cells are primed in PP or mesenteric lymph nodes. Intraepithelial lymphocytes are mainly CD8 T cells while lamina propria has IgA producing plasma cells, CD4 and CD8 T cells (Murphy & Weaver, 2017).

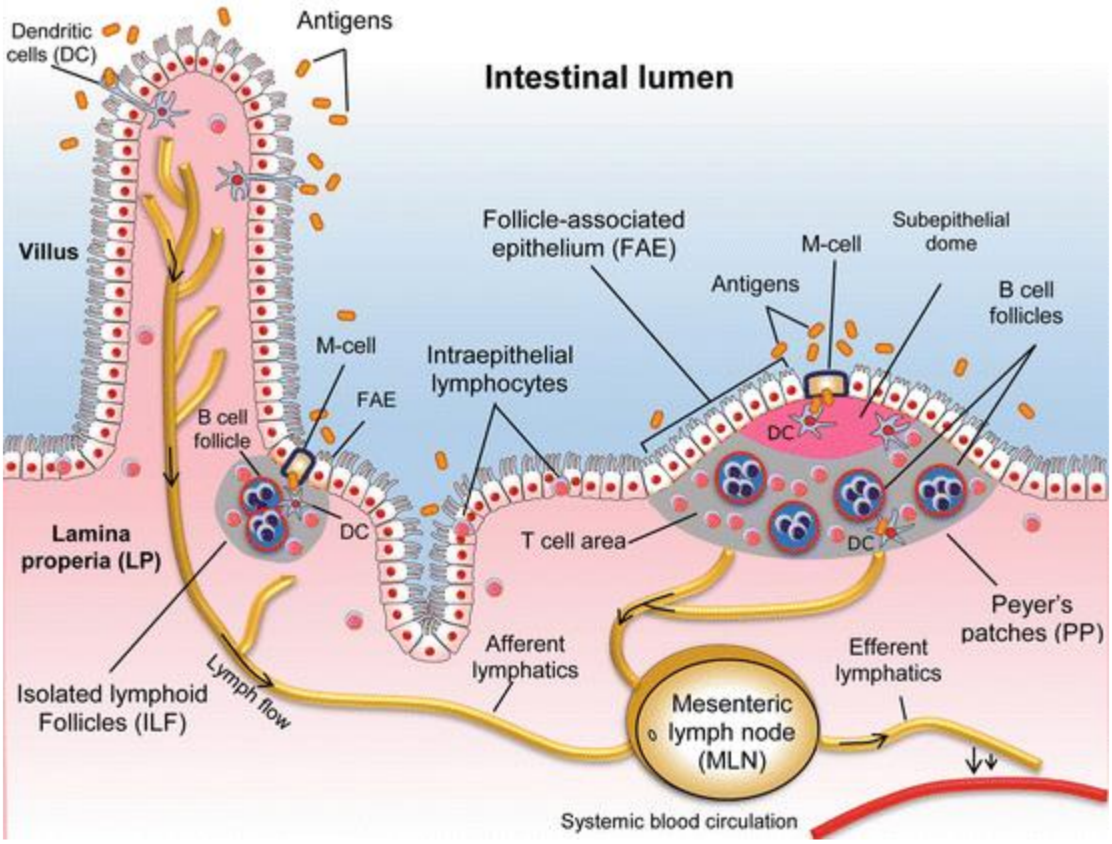


Figure 12 : Representation of gut-associated lymphoid tissues by Zgair et al. (2016)

1.7.2 IgA

At birth, mucosal immunity is barely present. GALT is continuously exposed to food antigens, commensal bacteria antigens and pathogenic antigens but it needs to distinguish them in order to provide a well-suited reaction. Indeed, an excessive reaction causing inflammation results in tissue damage like atrophy of villi, hypertrophy of crypts affecting nutrient digestion and absorption and a lower lifespan of cells which is the case at weaning (Bailey, et al., 2001). Excessive effector function leads to reactivity for food allergens but excessive regulatory function does not react to high commensal bacteria growth and leads to diarrhea. Cellular type responses are the most damaging while antibodies might be protective (Bailey, et al., 2001). Exposure to antigens, stimulates the immune system which develops into a mature type. Milk is the first type of feed received by neonates and provides passive immunity by IgA and TGF-β. Over time, those protective substances are depleted but the antigenic pressure from bacteria remains and is increased with the arrival of food requiring an active immune answer. The immune system needs to mature and gain experience.

Before weaning, the protection was ensured by milk IgA. It can bind toxins and pathogens inhibiting its entry in the epithelial cells but also neutralize particles that managed to pass through the membrane such as pathogens and toxins and neutralization of endosome content inside cells. Seventy-five percent of commensal bacteria are covered with IgA limiting its adherence and penetration. IgA concentration in fecal sample can be used to determine microbiota stability; high IgA levels can be found in stool samples of piglets of more than 30 days old, having a well-established microbiota (Thompson, Wang, & Holmes, 2008). In a study by Thompson *et al*. (2008), piglets were fed a soy/whey/casein (55:9:6) sow milk replacer from 3 till 36 days old. During the first days, they received their mother's colostrum. Piglets rely on maternal IgA from milk up to 10 days after colostrum intake, from 28 days onwards, it can be considered to be produced by the piglets itself (Thompson, Wang, & Holmes, 2008).

Regular milk contains IgA too and helps regulate commensal bacteria . IgA levels decline before the start of the own production leaving a gap where uncontrolled microbiota can develop (Fig. 13) (Thompson, Wang, & Holmes, 2008).

IgA is the major immunoglobulin produced by the intestines. IgA production begins with induction of class switching B cells in PP or mesenteric lymph nodes into IgA producing plasma cells. B cells class switching resulting in IgA plasma cells is initiated in PP or in mesenteric lymph node (MLN) and is induced by Il-6 and TGF-β (Leblois, Early life programming of piglets' microbiota and gut health by maternal dietary fibre supplementation. Unpublished doctoral thesis, ULiège - Université de Liège. , 2018). Activated cells will be able to take place in the lamina propria thanks to CCR9 and integrin α4:β7 which are homing molecules (Murphy & Weaver, 2017). Secreting plasma cells are located in lamina propria and in order to be secreted into the lumen, IgA need to bind the polymeric immunoglobulin receptor (pIgR) produced by immature epithelial cells at the base of villi crypts. Secretory immunoglobulin A (sIgA) stays in the mucus layer to ensure protection. By Camillerri *et al* (2012), enterocytes are also able to secrete IgA response (Abreu, 2010) and antimicrobial factors or cytokines too (Sureda, 2017).

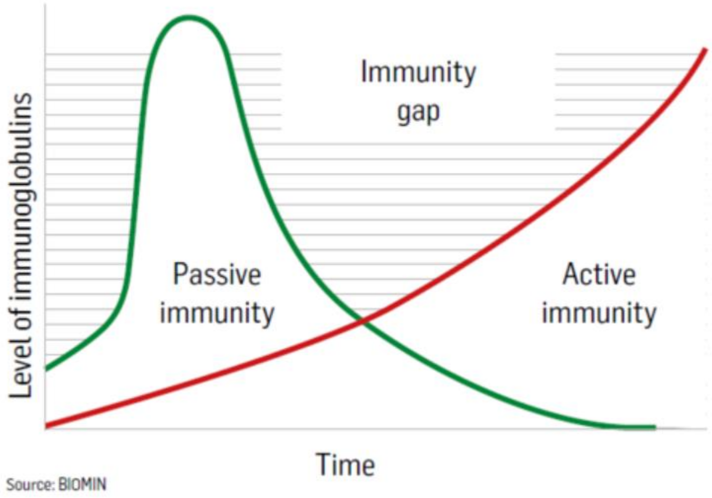


Figure 13: Immunoglobulins levels leaving an immunity gap

1.7.3 Lymphocyte regulation

Another way to ensure intestinal health are lymphocytes like regulatory T cells. Right below the epithelium are macrophages that produce IL-10 which limit inflammation and contributes to maintain antigen-specific tolerance necessary for FoxP3+ Treg. Under no inflammation or infection, antigen-specific FoxP3+ Treg for non-pathogens are generated in mesenteric lymph nodes and are able to express homing molecules (CCR9 and integrin α4:β7) to fit in between cells in lamina propria. Tregs are influenced by retinoic acid and transforming growth factor-β (TGF-β) produced by dendritic cells (Murphy & Weaver, 2017). Tregs are able to control T cell multiplication and cytokine production (Kondělková, et al., 2010). CD4 T-cells activation in the presence of inactive/latent TGF-β results in apoptosis but TGF-β latency is lost by mucosal inflammation and the active form prevents apoptosis of CD4 T-cells (Bailey, et al., 2001). In turn, clonal expansion follows with an increased production of IL-10, IL-4, TGF-β in order to limit immune response. Immunological tolerance is a form of regulation of the immune effector sites preventing inappropriate, damageable reactions.

2. Material and methods

2.1 Pig farming

In March 2022, 72 piglets (Landrace x Piétrain) were chosen from the CRAw facilities to hold the experiment. The animal experiment was approved by the ethical committee of the University of Liege (Ethical protocol 21-2385, Belgium) and all procedures were in compliance with Belgian and European regulations. The experiment started at weaning which was when the piglets reached 21 days of age and lasted for 3 weeks when piglets were 6 weeks old. In order to minimize variability, all piglets were castrated males with similar weight (5.48 ± 0.01 kg). The experiment was held in the Animal Experimentation and Production Center (CEPA) in Gembloux. The facilities provide 24 pens for the piglets (Fig. 18). Three piglets were thus distributed into each pen in a way that would give a similar inter-pen average weight (5.48 ± 0.5kg). Experimental modality repartition is explained in the paragraph 'Prebiotic application'. Each pen had a water tap and a feed dispenser that was daily cleaned, weighed and refilled in order to provide data for Average Daily Feed Intake (ADFI). Piglets were fed *ad libitum* a pig feed 'Baby-mix' mix by Quartes (Table 2). As requested by the ethical committee, temperature and humidity was checked daily. Each pen was equipped with a heating lamp to provide comfortable temperatures for the piglets. On the 2nd, 8th, 14th and 20th day, the piglets were weight individually to provide weight data for the Average Daily Gain (ADG) and to choose piglets to be sacrificed for the dissection.

Table 2 : Nutritional values and composition of Baby-rapid, piglet's feed, determined in the zootechnical laboratory

Nutritional values	
Dry matter	*87.11*
Protein	*21.6*
Fat	*4.5*
Ash	*Max 14*
NDF	*17.72*
ADF	*5.69*
Carbohydrate	*47.3*
Composition	
Barley (30.6%), soymeal (21.5%), corn (20.0%), wheat (16.3%), rice mill feed (3%), dried sugarbeet pulp (2.0%), spelt flour (2.0%), palm oil (1.3%), monocalcium phosphate (0.4%), sodium chloride (0.4%), soymeal (0.2%), calcium carbonate (0.1%)	

2.1.1 Prebiotic application

Twenty-four pens with 3 piglets each were allocated an experiment prebiotic modality. There were 3 tested prebiotic categories: inulin gavage, chicory flour gavage and isotonic solution gavage as control. The supplements were administrated daily by mouth force-feeding from day 22 until week 6. The allocation of different categories is illustrated in figure 18. Piglets were force-fed daily receiving each the same amount of prebiotics independently of their weight. Piglets received the prebiotics with the help of a force-feeding pistol (Fig. 14). Prebiotic gavage amount was increasing in a weekly manner where the first week (22 – 28 days old), piglets received 1.5 g/d of inulin, the second week (29 -35 days old) 2 g/d of inulin and the third week 2.5 g/d (36 - 43 days old). As inulin concentration in the Beneo - orafti 'pure inulin' product (OSCSJ8YHCOL) is 76.2 g/100g while in chicory flour from Waast Moulin (2000000006116) it is only 57 g/100g, solutions with different concentration had to be made in order to force-feed an equal volume to each piglet. The control group received an isotonic sucrose solution to mimic the gavage induced stress. The gavage volume increased as the weeks passed from 9mL to 12mL in the second week up to 15 mL the last week.

Table 3 : Nutrient composition and carbohydrate composition of the prebiotics, expressed per kgDM

Nutrients (g/kgDM)	**Inulin powder**	**Chicory Flour**
DM	940	937
Fat	1.3	6.8
Protein	38.5	43.2
Ash	max 90	max 90
Carbohydrate	900	430
NDF*	5.6	44.7
ADF	7.3	36.3
Inulin	762	571
CHO Composition (%)		
Fructose	8.57	10.90
Rhamnose	0	0.38
Arabinose	0.05	2.12
Xylose	0.08	0.46
Mannose	9.52	3.87
Glucose	15.82	10.5
Galactose	0.29	2.25

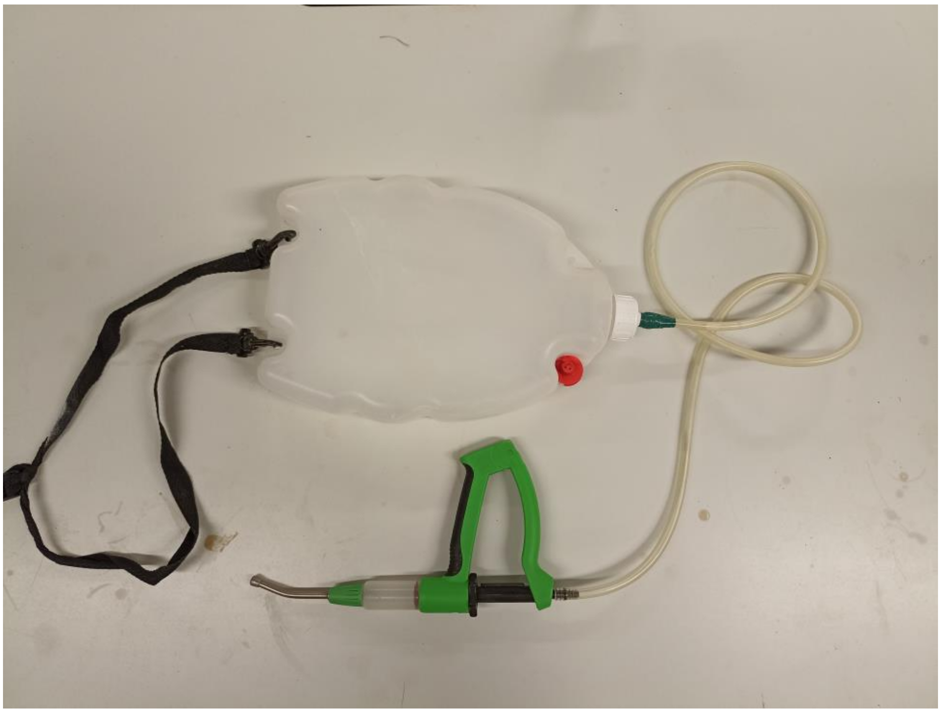


Figure 14 : Gavage pistol used for force feeding

2.1.2 Diarrhea score

In order to follow diarrheic evolution over the whole experiment, diarrhea score was taken daily in the morning before prebiotic gavage or feed weighing. The score has 4 levels to cover the whole diarrhea spectrum. Level 0 shows no diarrhea, firm stool. Level 1 shows soft stool in multiple pieces. Level 2 shows stool with no distinguishable single turd, just a load, and level 3 illustrates diarrhea, liquid stool. Examination was done always by the same person. After examination, all the pens were washed with a high-pressured water hose so that the next day evaluation would not be interfered by the previous day stools.

2.1.3 Dissections

Three dissections occurred during the experiment. The first one took place the first day of weaning with the beginning of the trial sacrificing 8 piglets aged 21 days. This was done still at CRAw, as to not cause any extra stress of transportation for the animals to be sacrificed. The second one took place on the 9th day of weaning, sacrificing 24 piglets of 29 days, 8 of each experimental group, and the last one on the 23rd day of weaning required 24 piglets of 43 days, again, 8 of each experimental group. The last dissection was on the 23rd day of the experiment marking its end. From the evening prior to the dissection, piglets fast overnight. For permeability measurements, a lactulose, mannose and xylose solution was applied exactly one hour before exsanguination (see permeability test).

Piglets were euthanized in an *Anoxia box* by nitrogen gas suffocation and sacrificed by exsanguination with the help of a scalpel cut on the jugular vein. The jejunum, ileum, caecum, colon tissues and contents were collected from each piglet. The gastrointestinal tract length and the pH of each compartment was measured. The small intestine parts were divided based on the length where the first 25% accounted for the duodenum, the next 50% were jejunum and the last 25% for ileum. Rectal content was also collected as well as liver and spleen sample tissues. Before storing, intestinal tissues were cleaned with phosphate buffer solution 1X (PBS) and cut into 2cm pieces to fit 2mL Eppendorf tubes. Tissues and content samples were snap frozen in liquid nitrogen and stored at -80°C for later use.

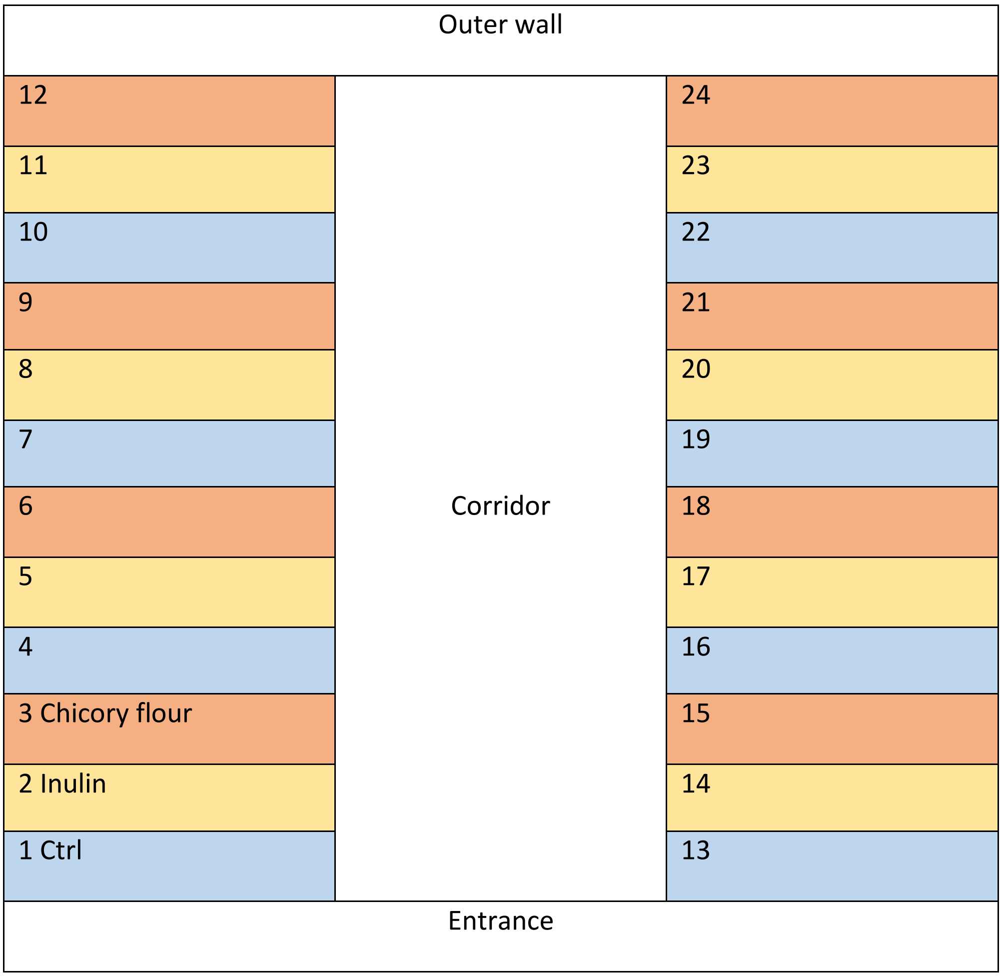
Duodenum, jejunum, ileum and colon tissues were also collected for structure analysis and thus 5cm long samples were put into formaldehyde. After 24 hours, those tissues moved from the initial formaldehyde solution into an ethanol one for long duration storage. In between each piglet, tools and surfaces were cleaned and sterilized.

2.1.4 Permeability test

In vivo intestinal permeability test was done using a sugar absorption test, by oral administration of a cocktail of marker probes, followed by blood sampling 1 hour after the administration. The markers (xylose, lactulose and mannitol) will be determined in the plasma by spectrophotometry and HPLC. In the evening prior to the dissection (12-15 hours), the feeders were emptied out for the overnight fast. On the dissection days, precisely one hour before exsanguination, the sugary solution of xylose (100 mg/kg BW), mannose (100 mg/kg BW) and lactulose (500 mg/kg BW) was mouth force-fed. One batch of solution was made the day of dissection and the volume given for each piglet was adjusted for its weight. The bleeding was then performed and blood from the jugular vein was collected. Blood samples were collected in coagulase coated blood tubes (18564 by Vacutest) that allow serum uptake by centrifugation at 2000 rpm for 10 minutes allowing the collection of supernatant in 2mL tubes to be frozen at -80°C until HPLC quantification. However, these analyses were not performed yet but they can give an idea of how permeable the intestines are in the different experimental modalities.

Table 22 : Trial modalities repartition

where blue is control, yellow is inulin gavage and orange is chicory flour



	Outer wall	
🟧 12	Corridor	🟧 24
🟨 11		🟨 23
🟦 10		🟦 22
🟧 9		🟧 21
🟨 8		🟨 20
🟦 7		🟦 19
🟧 6		🟧 18
🟨 5		🟨 17
🟦 4		🟦 16
🟧 3 Chicory flour		🟧 15
🟨 2 Inulin		🟨 14
🟦 1 Ctrl		🟦 13
	Entrance	

2.2 Histomorphology

Duodenum, jejunum, ileum and colon tissues were collected for structure analysis and so 5cm long samples were put into formaldehyde on the dissection day. They were first cleaned with PBS to remove fecal matter. After 24 hours, those tissues moved from formaldehyde solution into 70% ethanol solution for long duration storage. Those samples were sent to the GIGA Immunology laboratory (ULiège) for tissues treatment to obtain 5 µm thick slices on a microscope slides for tissues morphology observations. Tissues were first covered with paraffin before cutting with a microtome using blades Thermo MX35 Ultra (Thermo Fisher Scientific). Then they were treated with haematoxylin-eosin coloration. The observed parameters were villi height, crypt depth, and size of muscularis layer including lamina propria. The measurements were done with Toupview application and a Toupcam camera (UA510CA) mounted on top of a Olympus BX51 microscope.

2.3 Nucleic acid analysis

2.3.1 Microbial DNA extraction from content samples

Content samples of the colon and ileum were stored at -80°C after collection on the different dissection days. First, the frozen content samples were crushed using fire-sterilized lab pestle and mortar. To ensure that the samples did not melt, liquid nitrogen was used to cool down samples and tools. Once the samples were finely crushed, 0.4 mg were transferred into bead-beating tubes containing glass beans and the remaining crushed sample was put in a new 2mL tube for storage. DNA extraction was performed using the Qiagen QIAamp Powerfecal Pro DNA extraction kit (no. 51804). The protocol used was the one provided with the kit with slight adaptations to ensure good quality and quantity of extraction such as the increase of CD1 volume (Two time 640 µL with vortexing in between), the increase of CD2 (250µL) and the usage of a bead-beatter set at 6 m/s for 180s (with 5 minutes break after each 60s) . The kit contains Eppendorf tubes, bead-beating tubes, elution tubes and extraction solutions (CD1, CD2, CD3, CD5, CD6, EA). The quality of the extraction was assessed by Nanodrop (Thermo Fisher Scientific Nanodrop 2000, USA). CD2 contains inhibitor removal technology able to remove polysaccharides, proteins, and cell residues which may otherwise reduce extraction quality. CD3 is a high-concentration salt solution which is necessary to bind DNA to silica membrane. EA is a wash buffer solution able to remove proteins and other non-aqueous contaminants. CD5 is an ethanol-based wash solution able to remove residual salt, inhibitors and other contaminants without detaching the DNA from the silica membrane. CD6 is a solution used to detach DNA from the silica membrane.

Once the quality of the extraction is ensured, the samples ought to be multiplied with primers by PCR and analysed. However, these analyses were not performed yet but they can give an idea of microbial population structures.

2.3.2 Microbial DNA extraction from fermentation vials

DNA extraction of fermentation vials microbiota is identical for DNA extraction from fecal samples except the starting material is 1.7 mL of fermented liquid, instead 0.4 mg of crushed fecal sample, which is put in a bead-beating tube. Next, the tubes are centrifuged for 10 minutes at 13.000 rpm where all microbial cells and solid particles go to the bottom. Finally, the supernatant is eliminated. From here onwards, the sample follows the same steps as for DNA extraction from fecal matter (chapter 2.3.1).

2.3.3 Cellular RNA extraction from tissue samples

The RNA was extracted from colon and ileum tissues of the gastrointestinal tract to study the gene expression. RNA extraction was done using the Promega ReliaPrep RNA Tissue Miniprep System (no. Z6112). First, samples were crushed using fire-sterilized lab pestle and mortar and stored back in -80°C. For each sample, a lab spatula amount was taken up and mixed with a LBA + TG buffer allowing lysis and inactivation of nuclease from the sample. The Promega QuickProtocol provided with the kit was used. The kit contains Eppendorf tubes, elution tubes and purification solutions (RNA dilution buffer, isopropanol, RNA wash solutions, MnCl2, yellow core buffer, DNase I, column wash solution, nuclease-free water, 1-thioglycerol, $MnCl_2$, LBA buffer). The quality of the extraction was assessed by Nanodrop (Thermo Fisher Scientific Nanodrop 2000, USA). Pure RNA would present a A_{260}/A_{280} ratio of 2.0. Extracted RNA should present A_{260}/A_{230} ratio between 1.8-2.2.

2.4 *In vitro* digestion, dialysis and fermentation

Feed samples were digested using the INFOGEST protocol. Ten categories were chosen to do so with following afterwards *in vitro* dialysis and *in vitro* fermentation. *In vitro* digestion uses the same enzymes as an *in vivo* digestion in order to mimic as closely as possible the digestion in the piglets. In order to do so, the protocol of Minekus *et al.* (2014) (Fig. 15) was used which provides the information necessary to make buffer solutions (Table 6) and the volumes and concentrations of solutes to be used (Table 5). Dialysis was done based on lab protocol from the Zootechnical science unit, here at GxABT.

The 10 categories to be in-vitro digested were: pure Feed (Baby Rapid by Quartes lot : §ORDNR - §MENG_NR), pure inulin without equating, pure chicory flour equated with inulin powder inulin content, pure inulin powder equated with chicory flour inulin content, Feed + 0.5% inulin, Feed + 1% inulin, Feed +2% inulin, Feed + 0.5% chicory, Feed +1% chicory, Feed +2% chicory. Homogenizing substances with a < 2% concentration required a pre-mix. Also, in order to reduce size of feed particles, the feed samples were crushed with the help of an electric blender in order to increase contact surface of feed particles.

2.4.1 Digestion

A single digestive unit was a glass flask containing 2g (except for the 2 equating modalities) of feed to be digested. Each feed digestion modality had a repetition factor of 20. A water bath set at 37°C and 160 RPM is necessary to favor enzymatic reactions. The volumes added for 2g of sample were the ones in Table 5.

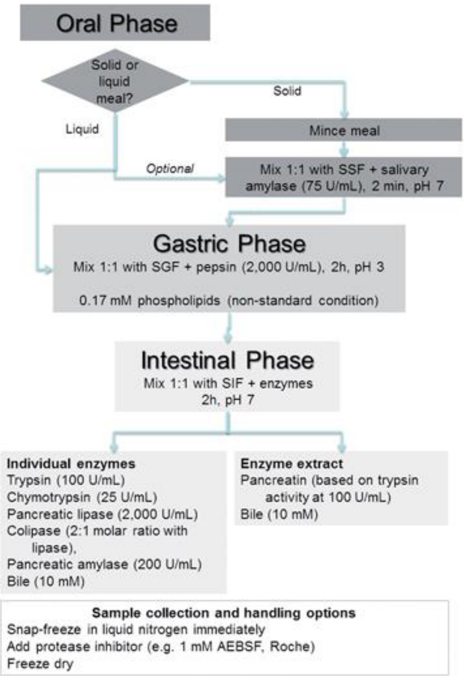


Figure 15: Overview and flow diagram of a simulated in vitro digestion method by Minekus et al. 2014

Firstly, a 2-minute-long oral mimicking phase with amylase was performed on the sample. This step imitates the saliva environment with simulated salivary fluid (SSF) buffer (pH 7) and amylase by Sigma-Aldrich (75 U/mL) (A1031). Next came, the acidic gastric phase for 2 hours where simulated gastric fluid (SGF) buffer (pH 3) is added with pepsin by Sigma-Aldrich (2000 U/mL) (P7000). The flasks were kept at 37°C in the water bath under gentle agitation. The last digestive step was the intestinal phase where the pH is neutral again and so simulated intestinal fluid (SIF) buffer (pH7) and pancreatin by Sigma-Aldrich (trypsin activity of 100 U/mL) (P7545) were added and kept at 37°C in the water bath under gentle agitation. Unlike the other enzymes, pancreatin was diluted in SIF buffer solution but likewise other enzymes, it was not centrifuged. Tips with a large opening were recommended to use. Between each step, the pH needed to be adjusted with NaOH 6M or HCl 6M solution.

Table 4 : Volumes of simulated fluids and enzymatic solutions added to 2 grams of sample to be digested based on INFOGEST protocol

	SSF	SGF	SIF
Simulated fluid	1.6 mL	3.2 mL	3.4 mL
$CaCl_2$	10 µL	2 µL	16 µL
Amylase	200 µL		
Lipase		200 µL	
Pepsin		200 µL	
Pancreatin			2 mL
Water	0.2 + 3 mL	400 µL	1 mL
Total volume added	4.8 mL	3.62 mL	4.5 mL

Table 5 : Stock solutions for the preparation of (1.25X) SSF, SGF, SIF.

The solutions are made for 400mL with actual intent to use as 500mL because of later dilution by enzymes, Ca^2 solution, etc. CaCl2(H2O)2 is added directly to the final mixture of fluid and food

Constituents	Stock concentration (g/L)	SSF	SGF	SIF
		Volume of stock (mL)	Volume of stock (mL)	Volume of stock (mL)
KCl	37.3	15.1	6.9	6.8
KH_2PO_4	68	3.7	0.9	0.8
$NaHCO_3$	84	6.8	25	42.5
NaCl	117	/	47.2	9.6
$MgCl_2(H_2O)_6$	30.5	0.5	0.1	1.1
$(NH_4)_2CO_3$	48	0.06	0.5	/
$CaCl_2(H_2O)_2$	44.1	1.5	0.15	0.6

2.4.2 Dialysis

The intestine is the place where digestion but also nutrient absorption takes place. To imitate nutrient absorption, porous membranes were used where small molecules can seep out. The remaining content inside the membrane was therefore similar to the remaining food chyme that would further be fermented.

Seventeen to eighteen cm long cellulose membrane tube segments (3.5kD) were cut and placed in water to facilitate their opening. Once hydrated, the membrane had to be closed at the bottom with a clip. The digesta was poured into the membrane tube and the flask was rinsed so every particle is collected. The remaining space in the tube was filled with water leaving a 3 cm gap for closure. Once fully sealed, the membrane was attached to a styrofoam floater and put in a 3L beaker with water and a magnetic stirrer to allow water movement around the membrane. Water was changed at 4, 18 and 21 hours. After soaking 24 hours in water, the samples were dialyzed and had to be freeze-dried by lyophilization. The content of the membrane was stored at -20°C. Once frozen, the boxes covered with a napkin were put in the lyophilizer (Delta 1-24 LSC by Christ) set at -50°C and at 0.647 mPa.

Membrane pore size trial

A trial with 2 different membranes was performed to optimize the pore size to mimic the best absorption. Membranes with 3.5kD and 6-8kD pore sizes were used for dialysis of digested inulin powder and chicory flour. 9 types of samples were collected; 3 types of substrates (feed, inulin powder, chicory flour) & 2 modalities (raw or digested and dialyzed) where 2 different membranes were used for the dialysis. As explained above, 17-18cm long membranes were used and water was changed after 4, 18 and 21 hours. After 24 hours of dialyzing, the contents were freeze-dried and analyzed for their inulin content. The expected result is that the small pore membrane reduces inulin losses seeping out but at the same time lets smaller molecules like simple sugars to seep out mimicking normal functioning intestine that would absorb simple sugars and leave fibers for later fermentation. Analyses on the contents were made by Chemistry Lab at GxABT following the method of Englyst and Cummings adapted by Aguedo as described by Uerlings *et al*., 2019. Fructan molecular weight distribution was characterized by 3 parameters; number-average molecular weight (M_n), weigh-average molecular weight (M_w) and PDI.

High pressure size exclusion chromatography (HPSEC) analysis on the digested samples done by Uerlings *et al.* (2019) provides reason to believe that 3.5 kD pore membrane would suit better to dialyze feeds containing inulin. In fact, they analyzed different substrates for its inulin content and size by HPSEC. Inulin powder number-average molecular weight was 2,87 kDa or 5.69 kD for weight-average molecular weight calculated by HPSEC. Chicory root powder was number-average molecular weight was 3.26 kDa or 7.02 kD for weight-average molecular weight calculated by HPSEC (Uerlings, et al., 2019). Thus, the molecular weight of the inulin fractions is variable. *In vivo*, inulin is absolutely not absorbed hence, it would be better to use membranes with pore sizes of 3.5kD as this would retain the maximum amount of inulin and only a minimalistic fraction would be lost. Therefore, the hypothesis is that the 3.5kD pore membrane would suit better for dialysis than the 7-8 kD pore membrane as it will prevent a high inulin loss.

2.4.3 Fermentation

The first fermentation was done on raw feed categories while the other fermentation was on 10 previously digested categories (see chapter 2.4.4). A control containing only mucins was also always applied. Each digested modality had a repetition factor of 4. As explained in the next paragraphs, after adding the substrate, the mucins carriers and the probiotics in the glass flasks, they were sealed off by a rubber stopper and crimp-caps. Next, the flasks were put in a water bath set at 39°C and speed 2 for rotations. After 2, 5, 8, 12, 16, 24 and 48 hours gas pressure measurements were taken with the help of a manometer (Tracker 200 id 221). Fluid collection was taken at 4, 8, 12, 24 and 48 hours, directly snap-frozen in liquid nitrogen and stored in a -80°C freezer. They were later used for HPLC and DNA extraction.

First, microbiota must be obtained from piglets so fecal samples were collected on their first day of weaning. Piglets were kept in farrowing units with access to feed and a space with a heat lamp. Fecal samples were collected by anal stimulation with a single use, sterile cotton swap into individual plastic bags. For transportation, fecal samples were put in a hermetic box with an anaerobic environment made possible thanks to ‘Microbiology Anaerocult C’ packets by Merck kGaA (1.16275.0001). Before freezing in liquid nitrogen, samples were homogenized in a hypoxia workstation and equally distributed in plastic bags. Later on, those fecal samples were used to make the inoculum suspension for the fermentation.

Next, mucin carriers need to be prepared. To do so, small plastic gears were covered with agarose by Sigma-Aldrich (A7002) and mucins by Sigma-Aldrich (M2378). Once boiled, agarose is cooled to solidify. It was used to stick mucins to the carriers. To do so 4 g of agarose and 700 µL of NaOH (7.5 M) was added to 400g of distilled water and warmed till boiling. When the solution started boiling, 20g of mucin were added. Then, the mixture was put in an oven at 75°C so the mixture stayed liquid while checking for any solid residues. Clumbs were broken down, if any were found. Next, the pH of this mixture was adjusted to 6.8 with 10M NaOH. The carriers can now dive in to be coated. Lastly, the coated carriers were collected on a petri dish without touching each other and were allowed to cool down to solidify in a fridge at 4°C. Three carriers were put in each fermentation flask. Their objective is to mimic the mucus coating the intestine.

Next, the menke's buffer solution was made out of 3 different solutions of salts (A, B and C) following the proportion from Table 7. Once all the solutes have been dissolved, the solution must be flushed with nitrogen gas to eliminate oxygen.

Table 6 : Menke's buffer solution components

Solutions	Components	Concentration	Mix for 1L buffer
Micro minerals Solution A	$CaCl_2.2H2O$ $MnCl_2.4H2O$ $CoCl_2.6H2O$ $FeCl_3.6H2O$ Distilled water	13.2mg 10g 1g 0.8g Fill up to 1L	1.2 mL
Micro buffer Solution B	$NaHCO_3$ NH_3Cl Distilled water	35g 2,7g Fill up to 1L	237 mL
Macro minerals Solution C	Na_2HPO4 KH_2PO4 $MgSO4.7H2O$ Distilled water	5,74g 6,2g 0,6g Fill up to 1L	237 mL
Distilled water			524.8 mL

Next, the probiotic suspension and the substrate had to be prepared in the glove box machine (Invivo, Led Techno, Heusden-Zolder, Belgium). The glove box machine replaces atmospheric gas by a nitrogen and 5% CO2 gas mixture. Each flask received 0.1g of prebiotic and 1mL of the inoculum suspension. The probiotic suspension was made as follows: first, the inoculum had to have a pH of 6.8. This should be achieved with 1M citric acid. Next, 200mL of buffer solution was added to the fecal matter and stomacher for 30 seconds at v1 speed (Awel Microbiology mixwel+). A sieve and a funnel was then used to transfer the fecal matter in the buffer bottle to obtain a 3% suspension. After that, the inoculum was ready to use and each flask must receive 1mL.

Each digested modality had a repetition factor of 4. The trial also included blanks containing only mucins. All the flasks were put in a water bath at 39°C and speed 2 for rotations. After 2, 5, 8, 12, 16, 24 and 48 hours gas pressure measurements were taken with the help of a manometer with a needle. Fluid collection followed that later will be used for HPLC and DNA extraction.

Gas kinetics

Pressure measures were taken by a manometer (Tracker 200 id 221) with a needle (dimensions: 0.6 x 25mm) on the 48 hours samples. After measuring the pressure, the pressure of all the vials was released with the help of a needle. The pressure measurements were not collected on each vials. A mathematical model provided by Groot *et al*. (1996) was used to plot a predictive production curve and to compare them between each other.

$$G = \frac{Axt^C}{t^C + B^C}$$

where G is gas accumulation to time; A (mL /g DM) is the maximum gas volume; B (h) is the time to half asymptote when G = A/2 and C is a constant determining the slope of the inflection point of the profile.

$$R\text{max} = \frac{AxB^C xCxTmax^{-C-1}}{(1 + B^C xTmax^{-C})^2}$$

R_{max} is the maximum rate of gas production (m L g^{-1} DM h^{-1})

$$Tmax = Bx(\frac{C-1}{C+1})^{1/C}$$

T_{max} is the time at which R_{max} is reached.

2.5 High performance liquid chromatography of short chain fatty acids

2.5.1 SCFA HPLC

Content samples of the different parts of the gastrointestinal tract were analyzed in the objective to measure SCFA content using High Performance Liquid Chromatography (HPLC) by the Waters E2695 Alliance HPLC machine equipped with a Aminex HPX-87H ion exclusion column (1250140). The samples were first centrifuged for 15 minutes at 13000 rpm. A volume of 1.6 mL of the supernatant was collected and acidified with H_2SO_4 (1M) until the phH was set between 2 and 3. Then, the samples were 2 times filtered (Chromafil AO-45/25 and Chromafil AO-20/25) to avoid clogging the chromatography column. Calibration solution of SCFA have to be prepared in order to ensure good quality of measurement (Table 8). With consecutive dilutions of this mother solution (25x, 50x, 100x, 200x, 400x), we obtain a precise scale of measurement. A flow rate of 0.6ml/min of H_2SO_4 (4M) was set in combined with a UV detector set at 210 nm allowing the quantification of acetate, propionate, butyrate, lactate, isobutyrate and branched short chain fatty acids.

The same principle as *in vivo* SCFA HPLC were applied for the fermentation sample of undigested feeds. Analyses were performed on the samples of 4, 8, 12 and 24 hours time point.

Table 7 : Compounds to disolve in 25 mL of milliQ water to obtain the mother solution to calibrate HPLC machine

Components	**Amount**
Lactic acid	1.3608 g
Acetic acid	1.2590 g
Propionic acid	1.2592 g
Iso-butyric acid	1.2350 g
Butyric acid	0.6238 g
Iso-valeric acid	0.6266 g
Valeric acid	0.6316 g

2.6 Statistical analysis

All collected data were analyzed using analysis of variance (ANOVA) on SAS Enterprise guide 7.1. Significant difference was considered by a probability level of <0.05. Tukey’s complement of analysis was applied to compare mean values of tested variables.

3. Results

3.1 Growing performances

During the whole experiment, feed consumption and the weight of the animals were measured regularly. Those data can be analyzed on their own for parameter like average daily gain (ADG) or average daily feed intake (ADFI) or combined for performance parameters like feed conversion ratio (FCR).

3.1.1 Weight evolution and average daily gain

Inulin and chicory flour have no effect on body weight evolution or the average daily gain (ADG) (P value > 0.05) although the amount of administered prebiotics increased weekly (1.5g inulin/d on week 1, 2g inulin/d on week 2 and 2.5g inulin/d on week 3) (Table 9). Although insignificant, the ADG for the control group was much lower than for the inulin and chicory flour group during the first week.

Table 8 : Average pen weights evolution throughout the duration of the experiment and Average Daily Gains per week

	Control	**Inulin**	**Chicory**	**p value**
Weight evolution (kg)				
D22	5.72 ± 0.02	5.77 ± 0.1	5.74 ± 0.04	0.8663
D28	5.75 ± 0.1	5.89 ± 0.05	5.86 ± 0.12	0.5961
D34	6.63 ± 0.16	6.54 ± 0.09	6.59 ± 0.13	0.9076
D40	7.91 ± 0.2	7.81 ± 0.14	7.55 ± 0.15	0.3756
ADG (g/d)				
D22-D28	4.24 ± 17.73	18.78 ± 16.72	19.68 ± 14.15	0.7559
D28-D34	147.04 ± 18.63	109.06 ± 17.67	120.62 ± 18.21	0.3366
D34-D40	212.94 ± 25.76	211.52 ± 16.48	160.67 ± 16.18	0.1333

Values are means ± SEM, n = 8 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the TUKEY test.

3.1.2 Average daily feed intake (ADFI)

Since multiple piglets were held in a single pen, it is not possible to know precisely individual feed intake. It is only possible to have an estimation by dividing total feed consumption by the number of animals per pen. By grouping feed intake data in a weekly matter, a significant feed intake difference (p<0.001) appears on the last week of the trial. Indeed, piglets fed chicory flour had an average daily feed intake of 364 ± 11 g/d while the control and inulin powder it was 440±13.3 g/d and 404±10.5 g/d respectively although the feed was given *ad libitum*. Also, although insignificant on week 2, lower feed intake was observed for inulin and chicory flour fed piglets (Table 10).

Table 9 : Average Daily Feed Intake (g/d) per week throughout the duration of the experiment

	Control	**Inulin**	**Chicory**	**p value**
D0-D22	Pre-Weaning Stage			
D23-D28	177 ± 12.2	168 ± 8.9	176 ± 11.6	0.8133
D29-D34	305 ± 12.9	288 ± 11	283 ± 9.98	0.3773
D34-D41	440 ± 13.3^a	404 ± 10.5^a	364 ± 11^b	**<0.0001**

Values are means ± SEM, n = 8 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the TUKEY test

3.1.3 Feed conversion ratio (FCR)

Feed conversion ratio was calculated with a sum of ingested feed over a period of time on the weight difference over the same period of time. Feed intake measures were obtained per pen while weight measurements were individual. A pen average weight was calculated and then, the pen-weight differences in between the days followed.

There is no significant difference between the experimental modality for feed conversion parameters for the 3 weeks (Table 11).

Table 10 : Feed Conversion ratio per week throughout the duration of the experiment

	Control	**Inulin**	**Chicory**	**p value**
D0-D22	Pre-Weaning Stage			
D28-D34	1.94 ± 0.24	3.59 ± 1.47	2.91 ± 0.98	0.5388
D34-D40	2.23 ±0.47	1.80 ± 0.10	2.41 ± 0.44	0.5030
D28-D40	1.88 ± 0.16	1.97 ± 0.10	2.18 ± 0.18	0.3615

Values are means ± SEM, n = 8 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the TUKEY test

3.1.4 Diarrhea score

Fecal consistency within each pen was visually assessed during the study. A 3 days average was calculated to describe the diarrhea score. No significant difference was observed in between the experimental modalities throughout the whole trial at three days interval (Table 12).

Table 11 : Average diarrhea score calculated per 3 days throughout the duration of the experiment

	Control	**Inulin**	**Chicory**
D23-D25	0.75 ± 0.225	0.75 ± 0.122	0.875 ± 0.218
D26-D28	1.21 ± 0.251	1.42 ± 0.186	1.5 ± 0.109
D29-D31	0.667 ± 0.154	1 ± 0.209	1 ± 0.275
D32-D34	1.42 ± 0.151	1.62 ± 0.231	1.46 ± 0.259
D35-D37	1.29 ± 0.278	1.29 ± 0.348	1.33 ± 0.167
D38-D40	0.958 ± 0.239	1.12 ± 0.235	0.75 ± 0.265

Diarrhoea score is measured on a scale from 0 to 3: (0) no diarrhoea; (1) slight; (2) middle; (3) acute. Values are means ± SEM, n = 8 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey test

3.2 pH and length of the gastrointestinal tract

The small intestine length is not significantly different between the modalities on the same dissection day but over time, as the piglets grew, their intestine grew too (Table 13). Prebiotics have influenced intestinal growth as inulin and chicory flour show a significantly different intestinal length which is not the case for the control modality (Table 14).

No changes in the pH of Cecum, Ileum and colon content was observed on either day (D29 or D43) among any group

Table 12 : Analysis of variance of length and pH values

Sampling Time	**Parameters**	**Control**	**Inulin**	**Chicory**	**p value**
Day 29	**Length**	8.19 ± 0.262	7.34 ± 0.271	7.56 ± 0.262	0.0884
	pH Caecum Content	5.97 ± 0.101	5.9 ± 0.112	6.16 ± 0.216	0.4702
	pH Ileum Content	6.7 ± 0.214	6.69 ± 0.349	7.31 ± 0.242	0.2087
	pH Colon content	6.63 ± 0.153	6.33 ± 0.162	6.47 ± 0.165	0.4234
Day 43	**Length**	8.77 ± 0.281	8.28 ± 0.332	8.97 ± 0.224	0.232
	pH Caecum content	6.51 ± 0.19	6.54 ± 0.105	6.6 ± 0.15	0.9078
	pH Ileum Content	7.25 ± 0.086	7.27 ± 0.099	7.37 ± 0.0769	0.5883
	pH Colon content	6.59 ± 0.122	6.63 ± 0.0894	6.68 ± 0.0747	0.7979

Values are means ± SEM, n = 8 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey test.

Table 13 : Analysis of variance of the prebiotic effect on intestinal growth over time

	Experimental modality	D29	D43	P value
D29 & D43	**Control**	8.19 ± 0.262	8.77 ± 0.281	0.1525
	Inulin	7.34 ± 0.271	8.28 ± 0.332	0.04603
	Chicory	7.56 ± 0.262	8.97 ± 0.224	0.0011

Analysis of variance of intestinal length growth for each experimental modality with a degree of certainty of 95%. Values are means ± SEM

3.3 *In vivo* short chain fatty acid production

The jejunum, ileum, colon and caecum contents collected on the 3 dissection days were analyzed for their SCFA profile.

3.3.1 Jejunum

There is no significant difference among the experimental modalities. Yet, a slight decreasing trend can be observed for the SCFA as it falls from 2.94± 0.4 mg/g on D21 to 0.883 ± 0.19 on D43. No significant differences were found for the SCFA profiles in between the experimental modalities. On the other hand, isobutyrate, butyrate, isovalerate and valerate were not at all detected on D21, D29 and D43 except isovalerate and valerate on D43 in the chicory group where a small fraction was detected (3.04 ± 3.04% and 1.41 ± 1.41% respectively). No significant difference was detected anyways. Lactate levels were 0.6 ± 0.08 mg/g on D21, 0.876 ± 0.25 mg/g on D29 and 0.525 ± 0.24 mg/g on D43 (Table 15).

3.3.2 Ileum

Total SCFA and lactate levels were not significantly different among the 3 experimental modalities. On day 29, chicory flour fed piglets showed a significantly lower propionate levels (p=0.0001) and significantly higher acetate levels (p=0.014) (Table 16). The presence of other SCFA was almost negligible throughout the experiment.

3.3.3 Colon

Total SCFA was 8.03 ± 1.06 mg/g on the D21 dissection and gradually rose for the next weeks to reach 10.7 ± 1.19 mg/g on D29 and 13.9 ± 1.36 mg/g on D43. Acetate and butyrate levels showed a positive growth trend throughout the experiment while the opposite trend applied for propionate levels. On the other hand, lactate levels remained low throughout the whole experiment.

BCFA levels are higher than in the upper GIT and reached 9.8 ± 1.458% on D21, 15.9 ± 2.77% on D29 and 26.9 ± 4.95% on D43. Indeed, on D29, the chicory flour fed piglets showed significantly lower isobutyrate levels (p=0.04) and on D43, isovalerate levels are significantly lower (p=0.0314). Valerate levels were not detectable prior to D43 (Table 17).

3.3.4 Caecum

There were no significant differences in between the experimental modalities for the SCFA. Isobutyrate was not detected on D29 and D43 yet a 3.11 ± 0.52 percentage was detected on D21. Butyrate levels increased on D43. Comparing to the upper GIT, BCFA are higher but no significant difference is observed among the experimental modalities (Table 18).

Table 14 : Chromatography of SCFA profile of the jejunum contents of piglets dissected on D21, D29 and D43

		Jejunum Experiment 1								
Days	**Treatment**	**Lactate (mg/g)**	**Total SCFAs (mg/g)**	**Acetate (%)**	**Propionate (%)**	**Isobutyrate (%)**	**Butyrate (%)**	**Isovalerate (%)**	**Valerate (%)**	**BCFA (%)**
D21	**Control**	0.6 ± 0.08	2.94 ± 0.4	27.79 ± 7.9	72.21 ± 7.9	0	0	0	0	0
D29	**Control**	0.876 ± 0.25	1.09 ± 0.22	24 ± 9.24	76 ± 9.24	0	0	0	0	0
	Inu	0.428 ± 0.06	0.811 ± 0.12	17.7 ± 9.14	69.8 ± 13.3	0	0	0	0	0
	Chico	0.579 ± 0.14	1.68 ± 0.6	27.1 ± 10.3	60.4 ± 12.9	0	0	0	0	0
	p value treatment	0.1883	0.2673	0.7816	0.6538	ND	ND	ND	ND	ND
D43	**Control**	0.525 ± 0.24	0.882 ± 0.19	21.5 ± 8.85	66 ± 12.6	0	0	0 ± 0	0 ± 0	0 ± 0
	Inu	0.513 ± 0.12	1.76 ± 0.49	40.2 ± 9.97	55.3 ± 10.2	0	0	3.04 ± 3.04	1.41 ± 1.41	4.45 ± 4.45
	Chico	0.27 ± 0.051	1.03 ± 0.12	42.8 ± 8.12	57.2 ± 8.12	0	0	0 ± 0	0 ± 0	0 ± 0
	p value treatment	0.4413	0.1262	0.2131	0.7453	ND	ND	0.384	0.3847	0.3847

Values are means ± SEM, n = 8 per treatment group. Means in a column without a common superscript letter differ ($P<0.05$) as analysed by one-way ANOVA and the Tukey test

Table 15 : Chromatography of SCFA profile of the ileum contents of piglets dissected on D21, D29 and D43

Ileum Experiment 1										
Days	**Treatment**	**Lactate (mg/g)**	**Total SCFAs (mg/g)**	**Acetate (%)**	**Propionate (%)**	**Isobutyrate (%)**	**Butyrate (%)**	**Isovalerate (%)**	**Valerate (%)**	**BCFA (%)**
D21	**Control**	0.89 ± 0.57	4.4 ± 2.1	16.89 ± 17.13	79.57 ± 15.40	0	0	3.52 ± 5.6	0	3.52 ± 5.6
D29	**Control**	2.24 ± 0.794	1.63 ± 0.426	33.7 ± 3.56^{b}	59.5 ± 2.66^{a}	0	0	6.72 ± 3.38	0	6.72 ± 3.38
	Inulin	1.23 ± 0.407	1.71 ± 0.24	38.5 ± 3.77^{b}	61.5 ± 3.77^{a}	0	0	0 ± 0	0	0 ± 0
	Chicory	0.819 ± 0.375	1.64 ± 0.55	59.9 ± 6.05^{a}	36.5 ± 4.39^{b}	0	0	3.63 ± 3.63	0	3.63 ± 3.63
	p value treatment	0.20	**0.990**	**0.014**	**0.0001**	ND	ND	0.273	ND	0.37
D43	**Control**	0.467 ± 0.087	1.72 ± 0.299	31.3 ± 8.16	66.8 ± 8.71	0	0 ± 0	1.89 ± 1.89	0 ± 0	1.89 ± 1.89
	Inulin	0.87 ± 0.67	1.18 ± 0.42	31.6 ± 11.9	38.7 ± 13.7	0	3.28 ± 3.28	0 ± 0	1.49 ± 1.49	1.49 ± 1.49
	Chicory	0.304 ± 0.0716	1.64 ± 0.331	49.3 ± 9.83	50.7 ± 9.83	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	p value treatment	0.5898	0.5222	0.3695	0.2141	ND	0.264	0.3847	ND	0.3847

Values are means ± SEM, n = 8 per treatment group. Means in a column without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey test

Table 16 : Chromatography of SCFA profile of the colon contents of piglets dissected on D21, D29 and D43

Colon Experiment 1

Days	Treatment	Lactate (mg/g)	Total SCFAs (mg/g)	Acetate (%)	Propionate (%)	Isobutyrate (%)	Butyrate (%)	Isovalerate (%)	Valerate (%)	BCFA (%)
D21	**Control**	0.38 ± 0.37	8.03 ± 1.06	20.55 ± 3.46	65.08 ± 5.34	2.95 ± 0.75	3.64 ± 1.32	6.77 ± 1.4	0	9.8 ± 1.458
D29	**Control**	1.46 ± 0.76	10.7 ± 1.19	30.3 ± 3.07	49.1 ± 3.43	1.62^{a} ± 0.562	4.82 ± 1.15	14.2 ± 2.46	0	15.9 ± 2.77
	Inulin	0.0481 ± 0.0481	12.4 ± 0.918	34.3 ± 2.88	42.2 ± 3.83	0.21^{b} ± 0.21	4.64 ± 0.571	18.7 ± 2.57	0	17.8 ± 3.3
	Chicory	0.417 ± 0.417	12.1 ± 1.92	32.5 ± 4.51	47.7 ± 3.96	0.351^{b} ± 0.351	6.09 ± 1.22	13.1 ± 3.03	0	11.7 ± 3.62
	p value treatment	**0.144**	**0.6486**	**0.7268**	**0.4063**	**0.04**	**0.56**	**0.3298**	**ND**	**0.4199**
D43	**Control**	0.0215 ± 0.0215	13.9 ± 1.36	32.2 ± 3.14	29.2 ± 2.61	0.875 ± 0.344	10.3 ± 1.22	25.6 ± 3.45^{ab}	1.84 ± 1.61	26.9 ± 4.95
	Inulin	0.017 ± 0.017	15.8 ± 0.848	28.8 ± 2.24	27.4 ± 1.77	0.59 ± 0.29	9.65 ± 0.868	33.6 ± 2.64^{a}	0 ± 0	34.2 ± 2.66
	Chicory	0.116 ± 0.0915	13.5 ± 1.63	34.2 ± 3.14	32.7 ± 2.72	1.3 ± 0.429	10.3 ± 0.9	19.3 ± 4.31^{b}	2.18 ± 1.43	22.8 ± 4.77
	p value treatment	**0.377**	**0.4525**	**0.419**	**0.3051**	**0.3861**	**0.8644**	**0.0314**	**0.4255**	**0.1835**

Values are means ± SEM, n = 8 per treatment group. Means in a column without a common superscript letter differ ($P<0.05$) as analysed by one-way ANOVA and the Tukey test

Table 17 : Chromatography of SCFA profile of the caecum contents of piglets dissected on D21, D29 and D43

		Caecum Experiment 1								
Days	**Treatment**	**Lactate (mg/g)**	**Total SCFAs (mg/g)**	**Acetate (%)**	**Propionate (%)**	**Isobutyrate (%)**	**Butyrate (%)**	**Isovalerate (%)**	**Valerate (%)**	**BCFA (%)**
D21	**Control**	3.38 ± 0.21	10.59 ± 0.92	31.11 ± 2.54	52.37 ± 4.28	3.11 ± 0.52	5.68 ± 1.24	6.64 ± 1.27	1.07 ± 3.04	10.84 ± 4.29
D29	**Control**	0.51 ± 0.282	5.60 ± 0.381	54.1 ± 4.45	25.2 ± 1.66	0	5.15 ± 1.16	9.94 ± 1.06	5.57 ± 3.64	15.50 ± 4.41
	Inu	0.04 ± 0.0.04	7.10 ± 0.845	48.92 ± 7.6	22.46 ± 4.04	0	4.74 ± 1.47	8.72 ± 2.21	1.65 ± 1.65	10.38 ± 1.87
	Chico	0.28 ± 0.244	6.89 ± 0.792	48.05 ± 7.81	25.59 ± 5.53	0	5.71 ± 2.3	7.24 ± 2.37	0.90 ± 0.441	8.14 ± 2.66
	p value treatment	0.32	0.287	0.7951	0.838	ND	0.9225	0.6316	0.3327	0.2633
D43	**Control**	0.49 ± 0.1	7.29 ± 0.674	48.74 ± 7.28	16.50 ± 3.11	0	8.47 ± 1.44	12.21 ± 1.92	1.57 ± 0.782	13.78 ± 2.31
	Inu	0.99 ± 0.218	9.14 ± 1.27	48.86 ± 4.25	28.15 ± 4.23	0	8.59 ± 0.965	12.07 ± 1.26	2.31 ± 1.34	14.38 ± 2.2
	Chico	0.70 ± 0.15	6.68 ± 0.623	54.84 ± 33.44	21.68 ± 2.98	0	9.9 ± 1.07	12.17 ± 0.841	1.38 ± 0.982	13.57 ± 1.3
	p value treatment	0.112	0.1608	0.6502	**0.0834**	ND	0.635	0.9973	0.8095	0.9559

Values are means ± SEM, n = 8 per treatment group. Means in a column without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey test

3.4 Fermentation gas kinetics

Two fermentations were performed. The first one was with raw substrates while the other one was with digested substrates.

3.4.1 Raw samples

Chicory flour had the lowest total gas production (P-value = 0.0074). Inulin (equated or not) had a significantly higher accumulated total gas production and was quickly fermented as the time to reach half asymptote was 5.08 ± 0.02h versus 6.19 ± 0.08 hours but also maximum rate of gas production was higher than for chicory flour (P-value = <0.0001). Strangely, the time to reach maximum gas production rate was similar for inulin W/E and chicory E but significantly higher than inulin E (Table 19).

Table 18 Gas fermentations parameters (A, B, R_{max}, T_{max}) modelled according to Groot et al (1996) of inulin and chicory flour in the presence of pre-weaned piglet faecal inoculum (n=4 fermentation vials)

Ingredients	**A (ml/g DM)**	**B (h)**	**R_{max} (mL g-1 DM h-1)**	**T_{max} (h)**
Inulin (W/E)	224^{a} ± 5.67	5.56^{b} ± 0.11	41.4^{a} ± 2.07	4.81^{a} ± 0.03
Inulin E	225^{a} ± 5.17	5.08^{b} ± 0.02	39.17^{a} ± 2.05	4.13^{b} ± 0.09
Chicory E	200^{b} ± 3.14	6.19^{a} ± 0.08	25.96^{b} ± 0.7	4.76^{a} ± 0.04
P value	0.0074	<0.0001	0.0003	<0.0001

A--Total gas produced; **B**-time to half asymptote; **R_{max}**--maximum rate of gas; **T_{max}**- time at which R_{max} is reached. Gas production parameters were recorded over 48 hours using a manometer. Values are means ± SEM, n = 4 per treatment group. Means in a column without a common superscript letter differ (*P*<0.05) as analyzed by one-way ANOVA and the TUKEY test.

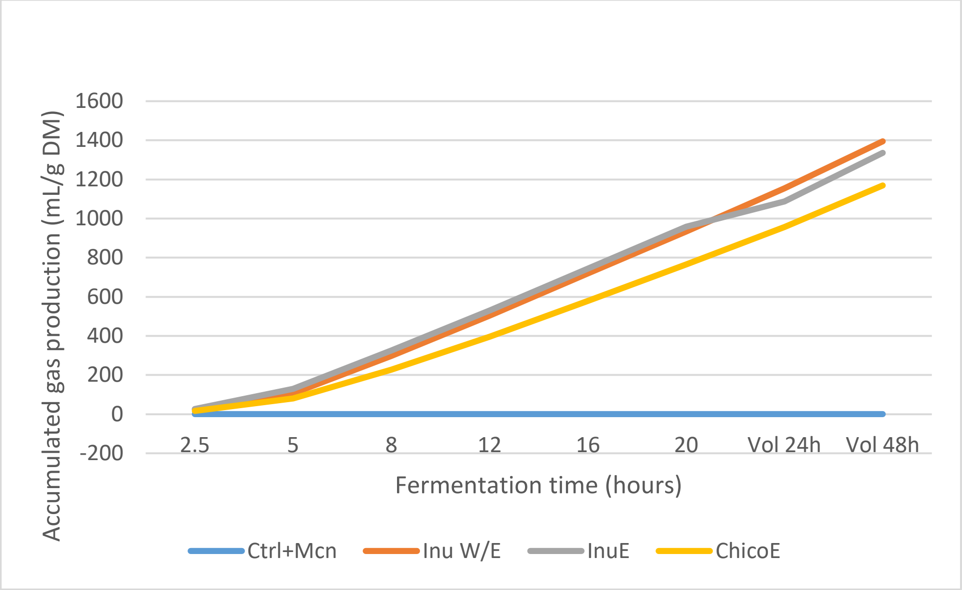


Figure 16 : Gas accumulation curves from the fermentation of raw chicory flour, inulin equated or not for the inulin content with chicory flour

3.4.2 Digested samples

First, a significant difference was observed for total gas production. Pure inulin had the highest value while the feed containing modalities had the lowest one ($p<0.001$). All the categories containing feed can be grouped together for having similar total gas production except for the feed with 2% of inulin. Indeed, the addition of 2% of inulin to feed gave higher total gas production levels compared to F, F+0.5% and F+1% ($p<0.001$). No similar observation can be made for the addition of chicory flour although inulin concentration was equated for inulin powder and chicory flour (Table 20).

R$_{max}$ and T $_{max}$ was reported to be double for inulin E and inulin W/E when compared to chicory E ($p<0.001$). At 2%, chicory had the lowest T$_{max}$, even lower than pure feed.

Table 19 : Gas fermentations parameters (A, B, R$_{max}$, T$_{max}$) modelled according to Groot et al (1996) of digested and dialysed inulin and chicory flour in the presence of pre-weaned piglet faecal inoculum (n=3 fermentation vials)

Ingredients	**A (ml/g DM)**	**B (h)**	**R$_{max}$ (mL g-1 DM h-1)**	**T$_{max}$ (h)**
Inu W/E	372 ± 11^{a}	6.6 ± 0.58	40 ± 7.1^{a}	4.1 ± 0.056^{ab}
InuE	314 ± 15^{a}	6.1 ± 0.26	40 ± 2.4^{a}	4.4 ± 0.32^{a}
ChicoE	219 ± 15^{bc}	7.6 ± 0.64	18 ± 2.2^{b}	2.4 ± 0.34^{cde}
F	172 ± 21^{c}	6.3 ± 0.45	17 ± 2^{b}	2.1 ± 0.38^{de}
F + Inu 0.5	171 ± 8.5^{c}	7.4 ± 0.52	14 ± 0.5^{b}	2.9 ± 0.29^{bde}
F + Chico 0.5	208 ± 12^{bc}	6.3 ± 0.43	20 ± 0.72^{b}	2 ± 0.25^{de}
F + Inu 1	170 ± 8.7^{c}	8.2 ± 0.68	13 ± 1.4^{b}	3.5 ± 0.26^{ad}
F + Chico 1	215 ± 15^{bc}	7.2 ± 0.54	18 ± 1^{b}	2.9 ± 0.28^{bde}
F + Inu 2	243 ± 6.6^{b}	6.4 ± 0.3	25 ± 2.2^{b}	3.6 ± 0.44^{abc}
F + Chico 2	228 ± 18^{bc}	6.3 ± 0.14	22 ± 2^{b}	2 ± 0.16^{e}
P value	<0.0001	NS	<0.0001	<0.0001

A--Total gas produced; **B**-time to half asymptote; **R$_{max}$**--maximum rate of gas; **T$_{max}$**- time at which R$_{max}$ is reached. Gas production parameters were recorded over 48 hours using a manometer. Values are means ± SEM, n = 4 per treatment group. Means in a row without a common superscript letter differ ($P<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

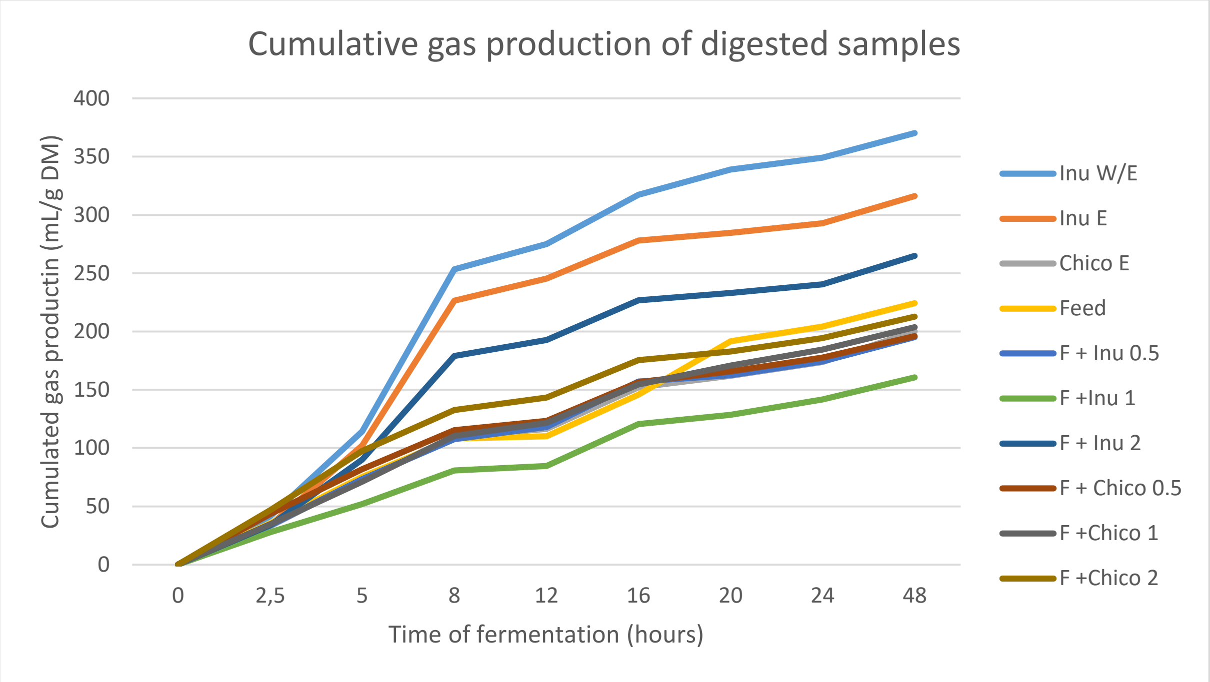


Figure 17 : Gas accumulation curves of the fermentation of digested and dialyzed samples of pure chicory flour, inulin equated or not for the inulin content with chicory flour and feed with proportions of the prebiotics

3.5 Prebiotic fermentation and Short chain fatty acid production

3.5.1 Raw samples

Total SCFA production was significantly different between the 3 categories at 12 hours and 24 hours. Inulin W/E showed a significant increase in total SCFA at 12 and 24 hours timepoint than the other 2 modalities. BCFA was higher for inulin fermentation at 4 hours but from 8 hours onwards, the values decreased significantly ($p<0.0001$). (Table 21).

At 4 hours, butyrate levels for chicory were significantly higher than the ones with inulin equated or not. As expected, the lactate levels decreased with time ($p<0.0001$) which was accompanied by increasing levels of butyrate ($p<0.0001$). Lactate production was high for the first 8 hours then it decreased. Indeed, at 4 hours, inulin W/E had the highest lactate production (2.39 ± 0.21 mmol/g) followed by inulin E (1.76 ± 0.07 mmol/g) and lastly chicory (1.3 ± 0.029 mmol/g). Then, at 8 hours, inulin and chicory equated for their inulin content showed similar lactate production (1.14 ± 0.1 mmol/g), which was lower than inulin W/E (2.33 ± 0.38). Acetate and lactate are the main produce of short chains fermentation ((Rossi, et al., 2005), (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002)). This suggests that chicory has a large proportion of short fructose chains.

Later on, inulin W/E had the greatest butyrate production at 8, 12 and 24 hours. The butyrate production was the highest for the 3 categories at the 12-hour time point but significantly different from each other (Table 21).

Acetate levels increased over time and inulin E and chicory E provided similar acetate levels throughout the duration of the fermentation except for the 8-hour time point. A reverse trend was observed in propionate levels as it goes from 4 to 24 hours. At 8 hours, inulin W/E had the lowest propionate production (45.05 ± 12.41%), chicory E had intermediate levels (54.27 ± 4.37%) and inulin E the highest (62.82 ± 1.45%) ($p<0.0001$).

Table 20 : Lactate, SCFA (mmolg-1) and molar ratio (%) after 4, 8, 12 & 24 hours of in vitro fermentation of the raw inulin and chicory flour

	Ingredients	**Lactate (mmol/g)**	**Total SCFAs (mmol/g)**	**Acetate (%)**	**Propionate (%)**	**Isobutyrate (%)**	**Butyrate (%)**	**Isovalerate (%)**	**Valerate (%)**	**BCFA (%)**
4	**Inulin W/E**	2.39^{a} ± 0.21	3.29 ± 0.53	34.85 ± 9.09	50.93 ± 7.37	0.27 ± 0.311	3.54^{b} ± 0.68	7.9^{a} ± 2.4	2.51^{a} ± 0.76	10.68^{a} ± 2.21
	Inulin E	1.76^{b} ± 0.07	3.74 ± 1.5	29.3 ± 10.65	59.78 ± 7.89	0.31 ± 0.329	2.32^{b} ± 1.71	6.92^{a} ± 4.16	1.37^{ab} ± 0.99	8.6^{a} ± 4.34
	Chicory E	1.3^{c} ± 0.029	3.23 ± 0.88	30.28 ± 8.9	59.45 ± 8.7	0.33 ± 0.13	9.27^{a} ± 0.74	0.67^{b} ± 0.28	0^{b} ± 0	1^{b} ± 0.34
8	**Inulin W/E**	2.33^{a} ± 0.38	6.06 ± 1.76	50.06^{a} ± 11.61	45.02^{b} ± 12.41	0.24^{a} ± 0.11	4.68^{a} ± 0.81	0	0	0.24^{a} ± 0.11
	Inulin E	1.43^{b} ± 0.1	6.66 ± 0.20	34.23^{b} ± 1.51	62.82^{a} ± 1.45	0.026^{b} ± 0.05	2.92^{b} ± 0.24	0	0	0.02^{b} ± 0.05
	Chicory E	1.12^{b} ± 0.07	6.38 ± 0.97	42.69^{ab} ± 4.41	54.27^{ab} ± 4.37	0.18^{a} ± 0.09	2.85^{b} ± 0.14	0	0	0.18^{a} ± 0.09
12	**Inulin W/E**	0.05 ± 0.01	6.66^{a} ± 1.021	56.02^{b} ± 4.57	29.08 ± 2.78	0	11.1^{a} ± 1.044	0	0	0
	Inulin E	0.04 ± 0.03	4.7^{b} ± 0.21	61.36^{a} ± 0.70	29.09 ± 0.99	0	9.55^{b} ± 0.052	0	0	0
	Chicory E	0.08 ± 0.01	5.69^{ab} ± 0.33	63.83^{a} ± 1.06	27.9 ± 0.62	1.086 ± 0.05	7.72^{c} ± 0.44	0	0	1.086 ± 0.05
24	**Inulin W/E**	0.13 ± 0.10	0.076^{a} ± 0.032	55.42^{b} ± 1.161	32.19 ± 1.03	0	11.3^{a} ± 0.59	0	0.72 ± 0.12	0.72 ± 0.12
	Inulin E	0.12 ± 0.05	0.025^{b} ± 0.007	58.84^{a} ± 0.25	32.27 ± 0.97	0	8.37^{b} ± 0.88	0	0.52 ± 0.14	0.52 ± 0.15
	Chicory E	0.126 ± 0.07	0.049^{ab} ± 0.02	60.57^{a} ± 1.19	31.49 ± 1.04	0	7.14^{b} ± 0.61	0	0.98 ± 0.3	0.98 ± 0.3
P-value ingredient		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.1483**	**<0.0001**	**0.0151**	**0.0004**	**0.0052**
P-value time		**<0.0001**	0.7078 (NS)	0.2336 (NS)	**0.007**	**0.0328**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P-value ingredient X time		**<0.0001**	0.0957 (NS)	**0.02**	**0.0386**	**0.3395**	**<0.0001**	**0.0326**	**<0.0001**	**0.0045**

These are net values for different times & ingredients, as those were corrected for control (mucin+ inoculum) fermentation products. Mean values (n=4 vials of fermentation) ± SEM. The unlike a, b, c letters for the same column and at one particular timepoint shows the significance different ($p<0.05$).

Inulin E & Chico E contains same quantity of inulin

Branch Chain Fatty Acids (BCFAs) = Isobutyrate + Isovalerate + Valerate

Total Short Chain Fatty Acids (SCFAs) = Acetic + Propionic + Isobutyrate + Butyrate + Isovalerate + Valerate

3.5.2 Digested samples

SCFA profile of digested samples was obtained on time and thus cannot be discussed.

3.6 Histomorphology

Villi height decreased post-weaning in the jejunum and ileum (Table 21) on D29 and D43. However, duodenal villi height was not affected. Piglets fed chicory flour has a significantly lower (p<0.0001) villi height at D29. On D43, they grew to similar levels as the control group ones. Inulin showed significantly higher villi height at all the time points and at all the locations. The villi heights of chicory fed piglets was no significantly different from the control ones expect on D29, in the jejunum, where it was slightly higher (305 ± 6µm) than the control group (246 ± 4µm) but lower than the inulin group (412 ± 5µm) (p<0.0001). On D29, in the duodenum, chicory group showed significantly lower villi height than the control group (251 ± 3µm vs 379 ± 3µm) (p<0.0001). As for the crypts depth, it deepened with time at all the parts. However, prebiotics influenced their evolution. On D29, in the jejunum and ileum, inulin increased crypts depth (p<0.0001) while chicory flour lowered it in the jejunum. On D43, prebiotics showed no significant difference with the control group. In the duodenum, inulin did not increase the depth on D29 and D43 but chicory lowered the depth on both days.

Width of the villi were also measured and there was no significant difference from the control ones for the duodenum at all time points and the ileum on D29. However, on D29 in the jejunum, chicory increased significantly the width (p<0.0001) but on D43, inulin increased it much more and chicory ones were the lowest. In the ileum, only on D43, there was a significant difference where chicory lowered villi width (p 0.0021).

In the ileum, the V:C ratio decreased over time but no significant difference can be seen on D29 unlike on D43 where inulin increased the ratio. In the jejunum, the prebiotics increased the ratio on D29 (p<0.0001) but on D43, chicory was no significantly different than the control group unlike the inulin group where it was significantly higher (p<0.0001).

Muscularis mucosae measured with the *tela submucosae* was not influence throughout the experiment at all the intestinal parts by the prebiotics. Except, punctually, on D29 in the duodenum, where inulin increased it significantly (p<0.0001). As for the *tunica muscularis*, it was not influenced at any time by the prebiotics in the jejunum. However, in the ileum and in the duodenum, there was a significant difference. In fact, in the duodenum, on D29, chicory fed piglets had intermediary tunica muscularis thickness comparing to the control and inulin group but on D43, the 2 prebiotic groups had similarly higher thickness (p 0.0002). While in the ileum, on D29, chicory lowered oppositely to inulin which increased the thickness (p<0.0001).

Table 21 : Histomorphology analysis of the duodenal, jejunal and ileal samples collected on the different dissection days

Intestine Part	**Days**	**Treatment**	**Villus Height (µm)**	**Villus Width (µm)**	**Crypt Depth (µm)**	**V:C ratio**	***Muscularis mucosae + Tela submucosae (µm)***	***Tunica muscularis (µm)***
Duodenum	**D21**	**Control**	290 ± 5	108 ± 2	258 ± 8	1.22 ± 0.04	46 ± 1	238 ± 3
	D29	**Control**	379 ± 3a	110 ± 1.69	265 ± 4a	1.46 ± 0.127a	44.7 ± 0.9^{b}	245 ± 3^{ab}
		Inulin	421 ± 3a	111 ± 1.23	276 ± 4.39a	1.56 ± 0.0267a	48.7 ± 0.8^{a}	237 ± 3^{b}
		Chicory	251 ± 3b	109 ± 1.4	249 ± 2b	1.02 ± 0.0179b	44.1 ± 0.7^{b}	249 ± 2^{a}
		p value	<0.0001	0.7424	<0.0001	<0.0001	0.0001	0.0035
	D43	**Control**	381 ± 7b	121 ± 2.18	306 ± 4.73a	1.28 ± 0.0337c	46.9 ± 1.1	247 ± 3^{b}
		Inulin	510 ± 6a	116 ± 1.62	307 ± 5.93a	1.71 ± 0.03a	47.4 ± 0.759	266 ± 4^{a}
		Chicory	381 ± 6b	117 ± 1.51	263 ± 4.01b	1.48 ± 0.0327b	47.8 ± 0.809	260 ± 3^{a}
		p value	<0.0001	0.1247	<0.0001	<0.0001	0.7768	0.0002
Jejunum	**D21**	**Control**	412 ± 11	111 ± 3	212 ± 6	2.08 ± 0.08	46 ± 1	235 ± 3
	D29	**Control**	246 ± 4c	108 ± 3b	230 ± 3b	1.09 ± 0.0233b	42 ± 1	239 ± 4
		Inulin	412 ± 5a	110 ± 3b	270 ± 5a	1.58 ± 0.0344a	43.3 ± 1	240 ± 3
		Chicory	305 ± 6b	122 ± 2a	208 ± 4c	1.51 ± 0.0405a	44.6 ± 1	230 ± 3
		p value	<0.0001	0.0172	<0.0001	<0.0001	0.0971	0.0794
	D43	**Control**	356 ± 11^{b}	129 ± 3^{ab}	267 ± 6	1.39 ± 0.0546^{b}	44.4 ± 1.01	260 ± 5
		Inulin	430 ± 6^{a}	134 ± 3^{a}	256 ± 5	1.73 ± 0.039^{a}	46.1 ± 1.27	264 ± 6
		Chicory	372 ± 9^{b}	121 ± 2^{b}	256 ± 5	1.52 ± 0.0472^{b}	47.3 ± 1.15	261 ± 4
		p value	<0.0001	0.0018	0.1925	<0.0001	0.2067	0.8493
Ileum	**D21**	**Control**	305 ± 7	105 ± 2	174 ± 5	1.89 ± 0.06	52.93 ± 2	225 ± 4
	D29	**Control**	252 ± 5^{b}	112 ± 2	215 ± 3^{b}	1.2 ± 0.0282	50 ± 1	260 ± 4^{b}
		Inulin	297 ± 5^{a}	114 ± 2	240 ± 3.^{a}	1.26 ± 0.0474	48.6 ± 2	318 ± 6^{a}
		Chicory	262 ± 7^{b}	110 ± 2	205 ± 4^{b}	1.32 ± 0.0427	46.8 ± 2	222 ± 6^{c}
		p value	<0.0001	0.4948	<0.0001	0.091	0.0957	<0.0001
	D43	**Control**	276 ± 5^{b}	129 ± 2^{a}	227 ± 5	1.28 ± 0.0423^{b}	44.4 ± 1	260 ± 5
		Inulin	385 ± 7^{a}	130 ± 2^{a}	232 ± 4	1.72 ± 0.0498^{a}	46.1 ± 1	264 ± 6
		Chicory	277 ± 6^{b}	121 ± 2^{b}	240 ± 3	1.17 ± 0.0255^{b}	47.3 ± 1	261 ± 4
		p value	<0.0001	0.0021	0.0746	<0.0001	0.1023	0.1276

Values are means ± SEM, n = 8 per treatment group. Means in a column without a common superscript letter differ (P<0.05) as analysed by one-way ANOVA and the Tukey test

4. Discussion

4.1 Growth performances

There are no significant differences in growth performances of piglets fed with inulin or chicory flour compared to the control piglets. The no effect was clearly evident although the prebiotic supplementation was increased in a weekly manner. The animals gained around 2 kg in around 20 days irrespective of the experimental modality. Average final weight of chicory flour fed piglets was slightly lower (7.55 ± 0.15 kg >< 7.91 ± 0.25kg) but it was not significant (P-value = 0.3756). As there are no weight differences, the same conclusions apply for ADG even though the analysis is not very conclusive because of the high standard error.

It is still worth checking if feed consumption differed. In fact, piglets could reach the same weight while consuming bigger amounts of feed, suggesting a lower energy yield from the digestion. In the first week, as expected, all piglets had a very low feed intake which may be explained by the loss of appetite due to the weaning process. In spite receiving the same equivalent amount of inulin and the same volume, there seems to be a significant difference in the last week (day 34-41) where piglets force fed chicory flour had a lower feed intake. The reduction in average daily feed intake for the chicory group can be attributed to the SCFA composition and the effect of polyphenols. According to studies, butyrate and propionate reduce appetite by increasing the synthesis of the gut hormone glucagon-like peptide-1 (GLP-1) or PYY (Lin, et al., 2012). Interestingly, *in vitro* fermentation of chicory flour of the present study showed, at 4 hours, a very high production of butyrate (9.27%) which was significantly higher than in the inulin (2.32%) and control (1.23%) group (Chapter 3.5.1). The *in vitro* SCFA result shows not significant difference in the composition of the propionate and acetate, hence only butyrate can be responsible for the lower feed intake in the Chicory group At the end of the present study, no weight differences were observed going against Lin *et al.* (2012) who observed significant weight differences in butyrate and propionate fed mice. Yet, chicory has other compounds like chlorogenic acids and polyphenols which may be also part of the explanation. Polyphenols can act on glucose levels by stimulating insulin and GLP-1 secretions (Domínguez Avila, García, Aguilar, & de la Rosa, 2017).

Furthermore, diarrhea score was also compared in-between the tested modalities throughout the duration of the experiment. The gastrointestinal health status is reflected in the diarrhea score and a higher score suggests a leaky gut which has a poor nutrient absorption. As seen in table 12, the diarrhea score did not differ significantly between the experimental modalities. Punctual occurrence of diarrhea, regardless of the experimental modality, do not reflect in a common trend. In fact, many factors contribute to the post-weaning diarrhea and a precise cause remains unknown. Indeed, ETEC proliferation, microbiota structure, livestock management, housing conditions, etc. are all mutually at the origin of PWD (Rhouma *et al.* (2017); (Gardner, Willeberg, & Mousing, 2002)). According to Madec *et al.* (1998), controlling the zootechnical conditions could serve as a sole basis for the avoidance of PWD and the emergence of diseases. Indeed, a low animal density, heating lamps for thermal comfort, high hygiene are important factors against PWD incidence (Madec, Bridoux, Bounaix, & Jestin, 1998). Additionally, the ethical committee put requirements on the housing conditions which, in fact, will influence the experiment. Weaning management tactics contributed to lower PWD incidence.

Feed conversion ratio is a parameter combining weight gain and feed consumption, giving one number to illustrate the growth performance. It can be influenced by the breed, sex, feed, health and the growing environment of the animal (He, et al., 2018). In the present study there seems to be no significant difference in between tested modalities. This result is counterintuitive as a lower feed intake is observed yet a similar weight is obtained for chicory fed piglets. Grela *et al.* (2014) verified the effects of inulin on growth performances of piglets and obtained positive results. By supplementing the diet with the equivalent of 20g of inulin/kg feed, they enhanced animal performances. In the present study, around 10 times less amounts of inulin were administered, as in most studies. The answer to prebiotic supplementation may be thus dose-dependent at a higher scale. Yet, the results can be highly variable throughout the literature (Flickinger, Van Loo, & Fahey, 2003).

The intestinal structure influences feed digestion and nutrient absorption. In a study by Wang *et al.* (2020) study, they concluded that the length of the intestine influences SCFA production in the small intestine as well as in a longer intestine, as more time is given for bacteria for fermentation. The SCFA production in the colon did not vary significantly (Wang, et al., 2020). They also confirmed that the length of the intestines is positively correlated with growth performances. In the present trial, the intestine length is not significantly different in between the 3 tested modalities but statistical analyses showed that there is an influence of prebiotics on intestinal growth as the intestinal length of the control piglets did not significantly increase over time unlike those of the prebiotic supplemented ones (Table 14). Therefore, the conclusion of Wang *et al*. (2020) 's conclusion that intestinal length reverberated on growth performances was not observed in the present study.

Intestinal structure informs about intestinal health status which is related to nutrient absorption. Including fructooligosaccharides at 0.4% of the diet was proved enough to have beneficial effects on intestinal structure (Csernus & Czeglédi, 2020). Yet, this is not always the case as explained by Csernus & Czeglédi where no effects of the inclusion of 0.5% of oligofructose were observed in the duodenum and in the ileum and the short-chain fructooligosaccharides did not increase ileal villi length.

As expected, weaning causes stress which damages intestinal structure, reducing villi height, deepening crypts and reducing nutrient absorption (Nabuurs, Hoogendoorn, Van Der Molen, & Van Osta, 1993). In Nabuurs *et al.* research *(1993)*, until 7 days after weaning, villus height was significantly lower in weaned piglets compared to unweaned ones yet, from the 11th day onwards, opposite trend appeared. Similarly, in the present study, jejunal and ileal villi is lowered 7 days after weaning but increases afterwards. There is a visible effect of the prebiotics on the histomorphological parameters as they increase it, especially the inulin, as can be observed with V:C ratio. The results in the present study go along what is present in the literature where the effects of prebiotics on growth performances have been proved ((Pierce K. , et al., 2006); (Eberhard, et al., 2007)). Also, an increased villus height in the small intestine could be caused by a higher production of SCFA (Rossi, et al., 2008). Acetate and butyrate could affect the renewal rate of the intestinal mucosa resulting in villi and crypt architecture modification (Cummings & Macfarnale, 1991). However, in two cases, duodenum on D29 and ileum on D43, chicory had lowering properties. On the other hand, chicory flour had a very limited effect and most of the time, did not differ significantly from the control group. However, chicory flour and inulin powder administration were equated for their inulin content. As the results show different effects for the 2 prebiotics, chicory flour must contain another active substance. Also, a lower feed intake, as it was the case for the chicory flour fed group, causes a decrease in villi height (Vente-Spreeuwenberg, Verdonk, & Verstegen, 2003).

pH measurement *in vitro* are not as illustrative as *in vivo* because buffers are used which prevent the evolution of pH, therefor the pH was measured at the time of slaughtering giving an idea of the pH differences in ileum, caecum and colon. Total SCFA was higher in the colon than in the caecum, yet it did not reverberate in the pH levels as the caecum was more acidic than the colon. Since microbial fermentation releases acids, it is expected that the pH drops. A higher microbial fermentation would be accompanied by a dropping pH levels (Deleu, Machiels, Raes, Verbeke, & Vermeire, 2021). Yet, data showed no significant difference between the 3 modalities similarly to Uerlings *et al.* (2019). In Flint *et al.* (2015), normal colon pH is between 5.5 and 7.5. First, the rate of fermentation would influence the acidification. Indeed, when a prebiotic is quickly fermented, it will be principally fermented in the upper GIT rather than in the lower GIT as it would be the case for inulin as suggested by (Uerlings, Schroyen, Bindelle, Bruggeman, & Everaert, 2021). In fact, pH levels are more variable in the ileum than in the colon in the present study. Also, the low inclusion levels of prebiotics in the present study may play a role for pH levels as it was already the case for FCR.

4.2 Short chain fatty acid production

As explained in the literature review, depending on the length of the fructose-oligosaccharide, different fermentation times and a different microbiota is necessary which on their own release a different SCFA profile. Shorter chains are preferable fermented over longer chains (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002). Inulin is a long molecule, requiring a long time for fermentation. It is reasonable to expect to see its influence further down the gastrointestinal tract rather than in the upper parts (Bossher *et al.*, 2006; (Barszcz, Taciak, Tuśnio, Święch, & Skomiał, 2020)). Also, fructose chains require β-fructofuranosidase which is not commonly present throughout the microbiota and depending on the position of the gastrointestinal tract different microbial structures can be encountered resulting in different fermentation profiles. It is worth mentioning that a bacterial strain that is able to produce acetate and butyrate is not able to produce propionate and vice-versa (Reichardt, et al., 2014).

In the jejunum, total SCFA levels reached around 1.09 ± 0.22 mg/g on day 29 corresponding to only acetate and propionate. The prebiotics are just beginning to be fermented as they encounter bacteria which can explain the low levels of SCFA. Further down the GIT, fermentation levels increase since the colon and the caecum hold a higher amount and diversity of microbiota which results in higher total SCFA production. Oppositely, lactate levels are higher in the upper GIT which is accordance with Brestenký *et al.* (2017), Franklin *et al.* (2002) and Meimandipour *et al.* (2011). This is independent of the experimental modalities as they all showed no significant difference. Also, Moens *et al.* (2017) and Belenger *et al.* (2007) explained that lactate can cross-feed bacteria and be used to synthesis acetate, propionate or butyrate. As butyrate levels are null in the upper GIT, it can be assumed that those bacteria are absent resulting in lactate accumulation. Opposite tendency can be observed in the colon and caecum where low levels of lactate but a slight increase in SCFA levels can be found in accordance with Meimandipour *et al.* (2010) and Belenguer *et al*. (2007).

Continuing on the differences between the higher and lower GIT, protein microbial degradation is greater in the lower GIT than it is in the upper GIT. BCFA levels issued from protein fermentation are not significantly different in between the experimental modalities on either day. In the lower GIT, the colon has significantly greater BCFA levels than the caecum which is in accordance with MacFarlane *et al.* (1992). In the present study, BCFA levels increased over time in the colon. Interestingly, a higher proportion of isobutyrate (p=0.04) and a lower level of isovalerate (p=0.03) were noted, respectively, on D29 and D43.

In accordance with Uerlings *et al*. (2020), no significant difference for total SCFA was observed in the distal small intestine. Yet, in the literature, different results can be obtained where inulin increased or decreased total SCFA levels or BCFA levels (Loh *et al*. 2006; Pierce *et al*. 2006; Eberhard *et al*. 2007; Halas *et al*. 2009). As already suggested previously, the reason for that could be the low inclusion of inulin. Indeed, in some cases, up to 10 times more inulin was administered

On D29, significantly higher levels of acetate (p=0.0014) and lower levels of propionate (p =0.0001) were found in the ileum of chicory fed piglets. No significant differences were observed on the other days nor in other parts of the intestine. Acetate and lactate are the main produce of short chains fermentation ((Rossi, et al., 2005); (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002)). It can thus be speculated that the chicory flour has more short chains of fructose.

Surprisingly, high acetate levels in the ileum were not reflected in the colon. If the bacteria responsible for acetate synthesis were already present in the jejunum, nothing should prevent them to colonize the colon and provide high production there too. The colon provides good fermentation conditions for bacteria and holds high levels of bacteria (Gaskins, 2001). The reason behind this may be that the competition is too high. The strain may go null on its own because of a too little competitiveness. It could be also that initial short chains providing high acetate levels have already been depleted. In the colon, total SCFA levels were around 10.7 ± 1.19 mg/g in D29 and 13.9 ± 1.36 mg/g on D43. Butyrate levels are now detectable (4.82 ± 1.15% and 10.3 ± 1.22%). This can be explained by a higher microbial presence and diversity in the colon which results in higher total SCFA production. By cross-feeding each other, bacteria can yield butyrate. In Rossi *et al*. (2005), inulin fermentation resulted in high levels of butyrate and low levels of acetate and propionate. Yet, it is not the present study in here and inulin supplementation did not differ significantly from the control. However, *in vitro* fermentation of chicory resulted in high levels of butyrate at the first 4 hours. *In vitro* fermentation, gave high levels of lactate, in the beginning compared to later fermentation. However, comparing to the other 2 modalities, the lactate level was the lowest for chicory but butyrate levels were the highest. This could be explained by a faster lactate metabolization into butyrate (Moens, Verce, & De Vuyst, 2017).

By comparing *in vivo* and *in vitro* fermentation, slight differences appear. Since, the fermentation substrate was the same in both cases and mucin carrier were used to closely mimic GIT, the difference could reside in the microbiota. Indeed, the inoculum used *in vitro* was collected on the first day of weaning. Later, piglets were introduced to a new environment which may contain different bacteria, not present in the inoculum collected earlier. Over time, new bacteria may have colonized the intestine and in the end modify fermentation profiles.

The high butyrate production in fructan rich products comparing to other prebiotics, i.e., chicory and inulin was reported in the past by Uerlings *et al* (2020) and therefore our study confirms it. ~~The total SCFA production is highly related to the inulin content and it is proved through this study.~~ The Chico E and Inu E when equated for the inulin content (Fructan) showed almost the same production of total SCFA. Interestingly a very high amount of butyrate was found in the chicory fermentation when compared to inulin and this might be due to specific substrate and microbiota composition but this needs to be confirmed with the microbiota analysis that is planned in the future.

Lactate, like SCFA, was the most abundant metabolite during the first 12 hours of the *in vitro* fermentation. The formation of lactate during *in vitro* fermentation is consistent with previous research by Uerlings *et al*., 2020. Lactate is a carbohydrate fermentation intermediary product that can be converted into acetate, propionate, and butyrate. This explains the decline in lactate and the significant increase in butyrate after 12 hours of fermentation. Butyrate may have also been formed from acetate because butyrate producing bacteria can be net acetate users (Macfarnale & Macfarnale, 2003). However, in our study, acetate was not found to be reduced after 12 hours of fermentation.

Also, in the first hours of the *in vitro* fermentation, BCFA were detectable. In the first 4 hours, inulin supplementation (equated or not) was associated with high levels of BCFA (10.68 ± 2.21%) which was not the case for chicory flour. Uerlings *et al*. (2019) found similar results at 6 hours. This could be explained by the fact that inulin is slowly degraded and shorter fructose chains are fermented first. Since they are not available, microbiota digest what is already available and thus turns to protein fermentation. This would momentarily increase BCFA proportions. Also, the inulin substrate contains 3.85% of protein while for chicory it is 4.32% (Table 3). Chicory must have shorter fructose chain and is thus easier fermentable as BCFA levels were lower for chicory than inulin. After another 4 hours, BCFA were <5% (while total SCFA increased to 6.66 ± 0.2 mmol/g) with, this time, inulin E having a lower BCFA proportion than the inulin W/E and chicory E. At 12 hours, the levels are low except for chicory and at 24 hours, it increases. The proportion is not significantly different between the 3 substrates but total SCFA production is indeed different for the 3 modalities. Chicory E has an intermediary production between the Inulin W/E and the inulin E one at 24 hours.

4.3 Gas kinetics

Chicory had a lower total cumulative gas production (A) and a lower rate of fermentation (R_{max}) than inulin powder for either raw or digested samples. As one of the modalities where inulin content for the inulin powder was equated to the one of chicory and the result was significantly different between the two, which means that the inulin content is not the only factor influencing fermentation. Uerlings *et al.* (2019), Pellikaan *et al.* (2007) and Shim *et al.* (2007) suggested that insoluble fiber content and low amounts of fructans with a high polymerization degree, as can be found in chicory flour, would cause a slower fermentation. Chicory flour reported higher NDF (4.4%) and ADF (3.63%) while inulin has lower proportions of NDF (0.56%) and ADF (0.73%) (Table 3). Tamaki *et al.* (2008) and Ramasamy *et al.* (2014), both reported that insoluble fibers are extensively fermented only after soluble fibers.

In the literature, in some cases, *in vitro* fermentation is done on raw or digested and dialyzed samples. In order to understand the differences, both were done in the present study. Digested chicory flour has its T_{max} lowered by half comparing to raw chicory (2.4 ± 0.34h vs 4.76 ± 0.04h) but there was no difference in the total gas production (200 ± 3.14mL/gDM for raw chicory and 219 ± 15 mL/g DM for digested chicory). The time to half asymptote was similar in between all digested samples but there was a significant difference in the raw samples where B was significantly higher for chicory E. The half time asymptote, although insignificantly, was higher for digested chicory sample with the T_{max} and R_{max} significantly lower than the raw chicory. This indicates the loss to a certain extent of smaller molecules (monosaccharides) during dialysis. Raw samples may contain, simple, smaller, easily fermentable sugars which may provide an initial boost for the microbiota. Thus, digestion and dialysis influences fermentation kinetics parameters.

In the case of inulin E, the digested inulin has shown the highest total cumulative gas production and also a higher time to half asymptote when compared to raw inulin fermentation ($p<0.05$). This can be due to high concentration of long chains of inulin in the digested and dialyzed form as the long chains take more time to be fermented. Short-chain fructans are rapidly fermentable, which would translate to fermentation occurring in the proximal colon. The long-chain fructans are more steadily fermented, which indicates that fermentation would occur over a longer portion of the colon (Stewart, Timm, & Slavin, 2008).

In digested samples, maximum production rate was significantly higher for inulin than chicory but it was reached slower than chicory. Chicory has a lower total cumulative gas (A) but it was quicker to reach T_{max} while inulin has a high fermentation capacity with a longer T_{max}. Earlier, SCFA profile of chicory fermentation suggested that it was fermented fast possibly because it has more short fructose chains than inulin powder resulting in visible higher acetate proportions (see chapter 3.3.2) ((Rossi, et al., 2005); (Perrin, Fougnies, Hill, Jacobs, & Schneider, 2002)).

In the present fermentation, the 3.5kD membranes were used while, on the contrary, Uerlings *et al*. (2019), digested and dialyzed the inulin using a 6-8kD size membrane. In result, the A value and R_{max} (A = 227 ± 4 ml/g , R_{max} = 20.3 ± 1.1 mL/h/g, T_{max} = 5.6 ± 0.2h) were quite lower in that study than what was found in the present study (A= 372 ± 11ml/g, R_{max} = 40 ± 7.1 mL/h/g, T_{max} = 4.1 ± 0.056 h). This clearly shows the importance of the membrane size to be used. Larger membranes would allow passage of certain fractions of fructans to pass and thus, causing a difference to mimic to the physiological condition. Surprisingly, in Tran *et al.* (2016), inulin fermentation with mucins reached 297 mL/g of total gas production and for maximum rate production 57.5 mL/h/g DM, with a B time of 5.63 h and a T_{max} of 4.9 h. In the present study, the values are slightly different for inulin; 314 ± 15 mL/g of total gas production, 40 ± 2.4 mL/h/g for maximum production rate, 6.1 ± 0.26h for B and 4.4 ± 0.32h for T_{max}. Although Tran *et al.* (2016) found to have similar results even without using a dialysis membrane however variation in the degree of polymerization of different fructans used in different studies can lead to different results.

Ramasamy *et al*. (2014) said that insoluble fibers are utilized only after 12 hours when all soluble fibers were already fermented. This could apply to the mixtures of feed with different proportions of prebiotics as feed contains cellulose. In our case, soluble fiber content differed in between all the modalities but half-time asymptote was not significantly different. Different fermentation duration could potentially be observed with a higher soluble fiber content.

Digested feed samples with all the different prebiotic combinations had all similar maximum production rates to one another and to the pure chicory modality. The B parameter was not significantly different neither between each modality meaning that the fermentation of all those categories was "over" around the same time. Time when this maximum production rate was reached, slightly differed but was considered between 2 and 3 hours. Inulin reached its maximum production rate after 4 hours only.

Increasing linearly inulin content did not increase linearly total gas production. Indeed, feed with 1% inulin had the lowest total gas production. The feed with 0.5% inulin similarly to the 1% inulin modality was significantly different from the 2% modality. Only the modality with 2% of inulin had a higher total gas production. Feed with 1% inulin had the lowest maximum production rate (13 ± 1.4 mL/h/g DM) but it was similar to the other modalities different than pure inulin. It reached its maximum production quickly, around the same time as pure inulin modalities just like feed with 2% inulin (3.6 ± 0.44h). Modalities containing chicory flour had a significantly lower T_{max} (2 ± 0.16h). Increasing the amount of chicory flour had no effect on gas production as it was similar to pure feed. Chicory did not influence T_{max} neither as it was similar to pure feed. Yet, Uerlings *et al*. (2019) concluded that the fermentation of different length chains had similar gas kinetics parameters and only the absolute amount of fructo-oligosaccharides will influence it. On the opposite, Bosscher, Van Loo & Franck stated that an *in vitro* fermentation of oligofructose is about 5 hours but may go up to 15 hours for long-chain fructans (Bosscher, Van Loo, & Franck, 2006). Obviously, this depends on the type of bacteria present in the inoculum and their abundance.

5. Personal contributions

During the previous six months, I have had the chance to work with Tushar Kulkarni (phD student) under the supervision of prof. Martine Schroyen. I am very grateful to them for welcoming me in the Animal production engineering and Nutrition team and for letting me contribute to the study.

In fact, in order to be allowed to participate in this study, I have had to take Felasa B classes which I successfully finished. This formation was both theoretical and practical and gave me good notions for animal experimenting. In fact, I applied what I learned with Vincent Servais (CRAw) and force fed the piglets. I also had to collect fecal samples for the *in vitro* fermentation which was a whole new experience. I had the responsibility to take care of all 72 piglets for almost 1 month and to take growth performances measurements. During the study, 3 dissections were held which gave me a first insight of time and general organization of a mammal dissection. Besides, I have also had the chance to go to Leuven to help with the dissection of chickens. This gave me an insight of the dissection of non-mammals and let me do some networking as it was done to help a fellow phD student.

Then, I learned to do INFOGEST *in vitro* digestion and *in vitro* fermentation. I realized how much preparations need to be done prior to the actual trial and let me gain first experience in this field. I would like to thank Sylvie Mabille and Ester Arévalo Sureda for teaching me how to and for helping during the fermentation procedure.

Later on, gas pressure and collected fluids from *in vitro* fermentation had to be analyzed. I learned how to obtain and utilize gas kinetics parameters following Groot *et al.* (1996) procedure. Then, I helped with DNA extraction from *in vivo* GIT contents and fermentation vials and with RNA extraction from tissues collected during *in vivo* sampling. This was very time consuming as all 2mL Eppendorf tubes contents had to be crushed manually. Also, histomorphology measurements had to be done to describe intestinal structure.

Besides, I did long sessions of literature review to get informed and find useful information to discuss my results.

Lastly, it happened that I had to briefly present this project on Terra innovation day. This was a useful exercise to practice presenting scientific research in public.

6. Conclusion

Prebiotics rich in fructans are meant to have bifidogenic properties and provide high butyrate production. Indeed, by favoring commensal bacteria in the intestine, pathogens struggle to colonize the environment and butyrate acts on the immune system where it stimulates regulatory T cells reducing its excessive reactions, thus protecting the intestine and *in fine* reduce occurrence of PWD.

The aim of this study was to compare, with inulin powder, the effect of chicory flour on PWD. The diarrhea score and the growth performance parameters like FCR and weight gains did not differ significantly. However, intestinal length growth was positively influenced by prebiotics and also, feed intake of the piglets fed chicory flour was lowered on the third week. This can be explained by polyphenols acting on GLP-1 levels and SCFA produced during fermentation.

Fructans are characterized by their degree of polymerization which can influence microbial fermentation and SCFA profile. Uerlings *et al* (2019) suggested that the length of the fructan chain does not influence gas kinetics parameters and that the absolute fructan content played on the rate of fermentation. However, *in vitro* fermentation of chicory flour mixed at different percentages with the feed, remained similar to pure feed fermentation. Linear dose dependence maybe can be observed at higher proportions. Also, outside of inulin, chicory flour is also composed of polyphenols and non-soluble fibers which may play a role in microbial fermentation. Soluble fiber would postpone the fermentation of non-soluble fibers extending total fermentation time. As this was not observed in the present study, this observation must apply to higher soluble fiber contents.

Then, SCFA chromatography of *in vivo* contents lets suggest that chicory has more short chains of fructose (<20DP) as, explained by Uerlings *et al.* (2019), it provided more acetate. Oppositely, gas parameters show that chicory was fermented for a longer period of time than inulin illustrated with higher B values and maximum production rate is lower than it is the case for inulin but it is reached faster. Chicory could thus be used a prebiotic stimulating mainly the upper GIT.

Chicory flour proved its role as a prebiotic stimulating gut health in post-weaned piglets but further studies on microbial structure, the degree of polymerization, SCFA profile from the fermentation of digested and dialyzed samples are necessary for a more conclusive opinion on this prebiotic.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Annexes

Average weights of piglets per pen on different measuring days, expressed in grams. For the first 3 measurements, there were 3 piglets per pen while on the last 2, there were only 2 piglets.

CATEGORY	LOGE	03/08/22	03/10/22	3/16/2022	3/22/2022	3/28/2022
CTRL 1	1	5493	5782,667	5971,333	6289	7951
INULIN 1	2	5453	5562	5846	6353	7199
CHICORY 1	3	5497	5489	5137	6068	6785
CTRL 2	4	5450	5651	5092	5663	6679
INULIN 2	5	5440	5790	5844	6532	8287
CHICORY 2	6	5486	5795	5859	6634	7693
CTRL 3	7	5520	5763	5840	6596	8149
INULIN 3	8	5510	6073	6157	6985	8294
CHICORY 3	9	5490	5874	6188	6800	8045
CTRL 4	10	5483	5763	5854	6751	7963
INULIN 4	11	5510	5684	5930	6592	7538
CHICORY 4	12	5520	5857	6038	6903	7316
CTRL 5	13	5460	5660	5777	7005	7523
INULIN 5	14	5353	6277	5782	6910	8296
CHICORY 5	15	5450	5638	5547	6299	7516
CTRL 6	16	5463	5813	5948	7024	7926
INULIN 6	17	5473	5288	5698	6497	7724
CHICORY 6	18	5526	5744	6117	6289	7308
CTRL 7	19	5490	5632	6050	7234	8888
INULIN 7	20	5483	5736	6054	6147	7502
CHICORY 7	21	5493	5792	6072	7272	8267
CTRL 8	22	5516	5712	5448	6476	8180
INULIN 8	23	5446	5778	5781	6310	7639
CHICORY 8	24	5493	5781	5956	6439	7487