THE EFFECT OF WEANING FOOD SUBSTRATE ON SEGMENTED FILAMENTOUS BACTERIA IN INFANT SMALL INTESTINAL IMMUNE BARRIER MATURATION

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Nutrition

at Massey University, Palmerston North, New Zealand.

(School of Food and Advanced Technology)

LINDA AKOTH AKINYI OEMCKE

2021

To my loving husband Jamie,

my mother Risper, brother Peter, sister Winnie

&

my late father Dr John Ogutu Onunga

Abstract

Appropriate and complete maturation of the gastrointestinal tract (GIT) barriers is crucial as it contributes to overall health and wellness. Maturation of the small intestinal immunological barrier has gained interest due to GIT-associated disorders such as inflammatory bowel disease thought to be caused by improper maturation.

The transition from milk-based feeding to complementary feeding in healthy infants at weaning introduces new antigens and microbes into the GIT. These changes induce immune cell production and is thought to be the final stages in the maturation process of the immunological barrier. It is during this maturation process at weaning that segmented filamentous bacteria (SFB) are thought to play a role. These Gram-positive, obligate anaerobic, spore-forming commensals have been observed in the faeces of 6–25-month-old infants and the terminal ileum of weanling rodents. The abundance of ileal and faecal SFB increases at weaning, peaks then decreases post-weaning and remains at that lower plateau throughout life. The transient abundance change of SFB has also been correlated with immune markers, immunoglobulin A (IgA) concentration in faeces and interleukin 17 (IL-17) concentration in blood plasma. The reported correlation between SFB, IgA and IL-17, and the timing at which the abundance of SFB changes at weaning, suggests that weaning foods might have an influence on changes in SFB abundance and hence on the immunological barrier. The published SFB genome identified carbohydrate metabolic and transport genes and a published study also reported an influence of complex substrates from the diet on SFB abundance. These findings suggested that a diet supplemented with complex carbohydrates may enhance the ileal SFB abundance, which had not been investigated at weaning previously.

The aim therefore of this thesis was to investigate whether the complex carbohydrate inulin would influence the ileal and faecal abundances of SFB at weaning and would modulate the concentration of the GIT immune markers, IgA in faeces, and IL-17 in plasma. The hypothesis was that a weaning diet enriched with inulin would increase the peak abundance of SFB in the terminal ileum which would then enhance GIT immune barrier maturation.

Initial method development showed that the temporal profile of SFB colonisation in the ileal tissue and contents of weanling rats was similar to those published for mice and infants of corresponding weaning age. Additionally, and for the first time, whole tissue homogenisation was favoured over ileal mucosal scraping as the ideal collection technique due to lower

variability in whole ileal tissue data. These methods were implemented in a final study where inulin was selected because it is commonly found in weaning foods such as fruit and vegetables and is also routinely added to bovine-based milk formulas to supplement the deficit of oligosaccharides (compared to human milk). Results revealed that inulin did not influence the peak abundance of SFB, regardless of inulin dosing (0%, 2.5%, 5%, 10%) or sample type (ileal tissue, ileal contents, faeces), three days post-weaning in 24-day-old Sprague-Dawley rats. There were no differences of inulin dosing on ileal and faecal SFB abundances, blood plasma IL-17 and faecal IgA concentrations, nor between male and female rats.

This outcome from the inulin intervention suggests that SFB may not utilise inulin directly or a longer period of adaptation to the inulin-supplemented diet might be required to assess if there is a long-term effect on ileal SFB abundance. The findings do not rule out other complex carbohydrates with potential influence on ileal SFB abundance. Further investigation would entail determining any interactive effects among inulin-supplemented diet, SFB abundance, and immune markers at broader time-points beyond the expected peak of SFB abundance post-weaning. In addition, analysis could be carried out to determine the abundance of other microbes relative to the predicted pre- and post-weaning SFB abundance changes.

Further investigations will advance our understanding on the ability of specific food substrates to manipulate SFB and important members of the GIT microbiota and in turn support the development of high-value foods for overall health benefits in infants.

Acknowledgements

I would like to express my sincere gratitude to my supervisors, Professor Warren McNabb, Professor Nicole Roy, Dr Rachel Anderson and Dr Jasna Rakonjac for their support during my PhD journey. Their constructive feedback throughout has challenged me to become more confident as a budding scientist. Thank you for the helpful advice and discussions which also encouraged me to think broadly but also stay on track and not lose sight of the end goal. A special thank you to Rachel who has always been there for me from our very first meeting when she picked me up at the airport. Even with the number of responsibilities you have on your plate, you have always made a concerted effort to make sure I was making progress. You recognised the growth in my abilities and courage throughout this journey and I will forever be grateful. Thank you to Jasna; despite the fact that you were added to my supervisory panel a year into my studies, you constantly provided positive feedback and great ideas for the following steps in my project. You also have always had encouraging words to keep me going especially when I wanted to give up. Thank you to the School of Food and Advanced Technology at Massey as well as AgResearch Grasslands, Te Ohu Rangahau Kai and the Riddet Institute for providing the facilities to conduct the laboratory work and complete this project. This PhD fellowship was funded by the Tertiary Education Commision via the Riddet Institute Centre of Research Excellence (CoRE).

I would like to acknowledge the animal technical support and training provided by Hailey Gillespie, Susan Doohan, Dr Hilary Dewhurst, Melanie Van Gendt, Charlotte Hurst, Dr Eva Maier, Trent Olson, Ben Harvie (AgResearch Grasslands, Palmerston North), Robert Smith and Ric Broadhurst (AgResearch Ruakura, Hamilton, NZ) during the animal study planning and experimental phase of my project. Thank you to Research Diets Inc., New Jersey, for the assistance with customizing the diets used in the animal studies. Thank you to Dr Mark McCann (AgResearch Grasslands, Te Ohu Rangahau Kai) for my initial laboratory training and troubleshooting whenever problems arose in the lab. Thank you also to Dr Eric Altermann for the instruction and constructive feedback on the SFB genome included in my literature review which also resulted in a published peer-reviewed article. I would also like to thank Denise Martin, Terri Palmer and Ansley Te Hiwi for a lot of the administrative work that went into many aspects of my project and my time as a student.

Cheers to my colleagues that have been on the PhD journey with me. It was helpful to have others that identified with the highs, lows and ordinary moments of life as a student. Even after

repeated declarations of loathing science, I can safely say I still enjoy it and have a newfound appreciation for it.

A very special thanks to my family my mother Risper, my siblings Peter & Winnie and my extended family who have encouraged me all this while, more so mother dearest who, for some deluded reason, thinks I am working on a cure for cancer even though I've explained to her so many times that I am not. Thank you also to my wonderful husband Jamie for the constant love and encouragement especially during the moments I was tempted to throw in the towel. You are a real one. Also, your wife is back to normal. I dedicate this to my late father, Dr John Ogutu Onunga, who always reminded my siblings and I that we could do and be whatever we wanted, if we worked for it. He himself was an example of that. *Erokamano.*

Most importantly, I would like to thank God without whom I would not have come this far. I know I do not deserve it, but for some reason You saw it fit to have me in this place and time for this task. *Erokamano Nyasaye.*

Ukitaka kuenda haraka enda pekee yako, ukitaka kuenda mbali, endeni pamoja.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BALB/c	Bagg Albino c
C57BL/6	C57 black 6
COGs	Cluster of orthologous groups
CRISPR	Clustered regularly interspaced short palindromic repeats
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FliG	Flagellar motor switch protein
FliM	Flagellar motor switch protein
FliN	Flagellar motor switch protein
GF	Germ- free
GHS	General health score
GIT	Gastrointestinal tract
IgA	Immunoglobulin A
IL-17	Interleukin 17
NOD	Non-obese diabetic
OadA	decarboxylate alpha
OD	Optical density

PCR	polymerase chain reaction
PK	Pyruvate kinase
PND	Post-natal day
qPCR	Real-time polymerase chain reaction
SCFA	Short chain fatty acid
SCID	Severe combined immunodeficiency
SEM	Standard error mean
SFB	Segmented filamentous bacteria
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
SWR/J	Swiss
Th17	T helper 17

CHAPTER 1: INTRODUCTION AND THESIS OUTLINE

1.1. Research background and rationale of the thesis

The intestinal barrier is made up of four main components i.e., the immunological, physical, chemical, and microbiological barriers. The part of the intestinal barrier bordering the lumen, the microbiological barrier, is where the majority of the intestinal microbes reside. The microbiological barrier influences epithelial survival, proliferation and metabolism, proliferation [1]. The microbes that reside here prevent the attachment of pathogens by competing for adherence to epithelial spaces, producing antimicrobial compounds, stimulating increased production of mucin, and secreting chemicals that allow microbes to communicate and increase their numbers thereby supressing pathogens [1]. Other functions of the intestinal microbiota include nutrient acquisition and energy regulation [2]. Most of the microbes found in this barrier are commensals as they require nutrients for their growth and maintenance from the GIT.

The chemical barrier consists of two mucus layers that cover the intestinal epithelium. The main components of the mucus layers are heavily glycosylated proteins secreted as mucins. The mucus lubricates the epithelium to minimise sheer stress on the physical barrier, and also acts as a barrier against unwanted substances [3]. The inner mucus layer contains fewer intestinal microbes compared to the outer mucus layer. Antimicrobial peptides found within the inner mucus layer help prevent contact between bacteria and the epithelial layer [3]. There are however microbes which reside in the inner mucus layer attached to the epithelial cells and they are believed to have beneficial effects on immunity and barrier function for GIT development and maintenance.

The physical barrier is located below the chemical barrier and is a layer of columnar epithelial cells organised into crypts and villi in the small intestine [4, 5]. This barrier is selectively permeable, preventing the passage of foreign antigens, microorganisms, and harmful

intraluminal substances [6]. It also selectively allows the transport and absorption of electrolytes, water, and essential dietary nutrients into the bloodstream [5, 7-10]. The intestinal epithelium has structural integrity and cell activity, provided by protein complexes, required to perform specific functions such as allowing the passage of nutrients, fluids, electrolytes, small macromolecules, but also preventing the passage of larger molecules [11]. These protein complexes connect and interconnect epithelial cell membranes to the basement membrane [11].

The immunological barrier is the innermost layer found within the lamina propria, where immune cells are located. The defence provided by the immune cells maintains an immune-supressed state to avoid reactions to dietary antigens and microbiota [12], though non-pathogenic bacteria still induce mild immune reactions contributing to normal low-level inflammation [13].

The development and maturation of the small intestinal barriers begins *in utero* and involve a number of processes. The processes during development include the formation of enterocytes, formation of a crypt-villus architecture [14], detection and closure of tight junctions of the apical junctional complex [15], maturation of the absorptive and secretory capabilities of the mucosa [16], expression of mucin [17], production of defensins and lysozyme [18, 19] and recruitment of epithelial lymphocytes [20].

Appropriate and complete maturation of the small intestinal barrier is crucial as it contributes to overall gastrointestinal tract (GIT) health and wellness. Maturation of the immunological barrier in particular has gained interest due to disorders associated with improper or incomplete maturation of this barrier such as inflammatory bowel diseases like Crohn's disease and ulcerative colitis [21]. Infant immunological barrier maturation is important as during complementary feeding from about 6 to 24 months of age, the infant GIT becomes a conduit for solid foods which contain new antigens and microbes that may trigger immune responses.

The low-level responses by the GIT immune system, in healthy infants, do not cause any detrimental effects. This induction of immune cell production is thus thought to be the final stage in the maturation process of the infant immunological barrier. It is during that complementary feeding stage that the abundance of a group of microbes increases peaks and plateaus over time [22-24].

These bacteria known as segmented filamentous bacteria (SFB) are Gram-positive, obligate anaerobic, spore-forming commensals and have been observed in the terminal ileum of vertebrates during early life [22-29]. The origin of SFB is still unknown though their reported life cycle suggests that SFB may be transferred from the mother to the child when faecal spores seed the oral cavity of infants during vaginal birth, or SFB spores could be passed from the mother to the embryo via the placenta in infants born via Caesarean-section. Therefore, during postnatal development, pre-weaning, SFB are thought to be present in the terminal ileum as they have been isolated from the faeces of infants under 6 to 25 months of age [24]. These microbes have also been isolated from the terminal ileum and faeces of young mice [22], chickens [24], rats [23, 28] and piglets [27].

As the infant diet transitions from exclusively milk to one complemented with solids at weaning, the abundance of SFB changes. As observed in several studies, the abundance of ileal and faecal SFB increases during weaning to a peak point, then decreases and plateaus [22-24]. During complementary feeding, new substrates become available to the intestinal microbial population causing their abundance to shift from one that is exclusively milk-based to one that resembles that of an adult [30]. The absorptive function of the small intestine also matures during weaning [31] and seems to coincide with these changes in SFB abundance and other microbes in the ileum. Thus the change in the abundance of SFB at weaning suggests an effect on SFB by novel substrates which are not present in breast milk and formula and which dairy alone cannot cause [32].

The dietary changes at weaning also coincide with changes in the immune system responses to antigens and microbes present in the food. Studies have reported a correlation observed between SFB and immune markers, immunoglobulin A (IgA) [22, 33-38] and interleukin 17 (IL-17) [39-41]. IgA is an antimicrobial which inhibits pathogen attachment to the mucosa which inhibits SFB attachment to the ileal mucosa [22]. This observation was supported by an aberrant expansion of SFB reported in healthy germ-free (GF) mice with an IgA-deficient gut [33]. SFB also induce T helper 17 (Th17) cells to produce IL-17 in healthy rodents [39-41] indicating a positive correlation. The production of IgA and IL-17 at the weaning and post-weaning stages suggests a mature immune system and barrier. Additionally, the correlation observed between SFB and both immune markers IgA and IL-17 implies that SFB may have a role in the maturation process of the immune barrier.

The timing of SFB abundance changes around weaning [22-24] suggests that the inclusion of complementary foods might be a way to alter their abundance and hence effects on the epithelial and immune cells in the ileum. SFB are proposed to obtain nutrients from the ileal lumen [32, 42-46], and directly from the host as they cannot successfully survive outside the environment of the ileum [47]. SFB may have evolved to attach to the ileal mucosa and during complementary feeding potentially derive nutrients in a cross-feeding manner with other bacteria residing in the inner mucus layer of the chemical barrier. If this is the case, then substrates from the diet might modulate SFB abundance at weaning, which had not previously been investigated. Therefore, an approach would be to provide substrates to enhance their abundance and perhaps functionality though only at weaning and immediately post-weaning to avoid any risks posed by a sustained increase in the abundance of SFB. Moreover, as SFB have been associated with some immune-related and inflammatory bowel diseases later in life [48-52], this created the necessity to maintain an appropriate bacterial load of SFB to avoid possible activation of any putative pathogenic genes that these microbes may harbour.

Therefore, the hypothesis was that *a weaning diet enriched with a food substrate would increase the abundance of SFB in the terminal ileum of weanling mice, which would then influence the concentration of GIT immune markers*. This hypothesis could be tested by feeding weanling mice a diet enriched with a selected substrate and then quantify the ileal and faecal abundance of SFB and the concentration of faecal IgA and plasma IL-17.

To test the overall hypothesis, the initial step was to determine the substrates SFB may utilise. A literature search resulting in a literature review (Chapter 2) summaries published studies which investigated the effect of food substrates on the abundance of SFB in healthy adult rodents. These studies indicated that a balanced diet, compared to a milk-only diet, influenced the abundance of SFB. Whole genome sequencing resulted in the published rat and mouse SFB genomes which predicted the presence of a high abundance of cluster of orthologous groups (COGs) for carbohydrates. The presence of multiple carbohydrate metabolic pathways and the evidence that a balanced diet enhances SFB abundance suggested that SFB might benefit from more complex carbohydrates compared to the disaccharides found in milk.

These findings led to the search for a substrate characterised as a complex carbohydrate, is considered safe for infant consumption, and can be included in the complementary diet. The substrate selected was inulin. This soluble fibre is considered safe for infant consumption as it is routinely added to bovine-based milk formulas which have fewer milk oligosaccharides compared to human breast milk. There are also reported benefits of inulin which include reduced incidences of flatulence and bloating [53] and enhanced abundance of beneficial bacteria [54] and short-chain fatty acids (SCFA) (acetate, propionate and butyrate) production [55, 56]. In breastfed infants, SCFA reportedly create an acidic environment that prevents the growth of pathogenic bacteria [57] and are thought to be involved in immune system activation [58, 59].

The overall goal of this PhD project was thus *to increase the abundance of SFB in the terminal ileum of weanling mice at weaning with a solid diet enriched with inulin and observe changes in the concentration of the GIT immune markers.* The results would inform whether the weaning diet indeed influences the maturation of the GIT immune barrier in infancy.

Deciphering how SFB assist in this process may contribute to the current knowledge of GIT development and eventually promote life-long health. Future research will also further our understanding of the ability of specific food substrates to manipulate important members of the GIT microbiota in infancy and, in turn, support the development of high-value early-life foods.

1.2. Specific objectives and hypotheses

It was necessary to establish a working model to quantify the abundance of SFB in the ileum and identify the immune markers correlated with SFB in rodents. Therefore, the first objective was to quantify SFB abundance in the ileum and faeces (Chapter 3) from an already established mouse model [22].

Hypothesis: Changes in SFB abundance in ileum tissue, ileum contents and faeces in mice, before and immediately after weaning, would show a temporal profile similar to what has been reported in other mouse studies and infants of a similar life stage.

Swiss (SWR/J), Bagg Albino c (BALB/c) and C57 black 6 (C57BL/6) mice were supplied by the AgResearch Small Animal Facility. Faecal screening was used to select the strain which harboured SFB. The selected mouse strain was then used for determining the abundance of SFB in ileal tissue, contents, and faecal samples pre- and post-weaning. The faecal screening, mouse experimental design and sample collection were carried out by Dr Eva Maier, Kelly Dunstan, Charlotte Hurst, Jason Peters (AgResearch Grasslands) and Ric Broadhurst, Robert Smith, Dr Matthew Barnett (AgResearch Ruakura). It was important to determine when SFB was most abundant and the most appropriate time-points for quantification. The abundance of SFB would also be correlated with the concentration of immune markers, IL-17 and IgA which have been reported in the literature [34-37, 39-41, 60]. The results from that study indicated that further method development was required.

The second objective was to establish a weanling rat model for SFB quantification, which had not previously been reported in the literature (Chapter 4). The study also compared whole tissue homogenisation and mucosal scraping to determine the best method for collecting ileal tissue samples for SFB quantification [23].

Hypothesis: Homogenisation of whole ileal tissue from weanling rats allows more accurate quantification of SFB compared to mucosal scraping in determining the abundance of SFB over time.

Following the selection of a food substrate from the literature search and the necessary developments of Chapters 3 and 4, the overall hypothesis could then be tested (Chapter 5). Finally, the fourth objective sought to explore whether the selected food substrate (inulin) and what dose would increase or decrease the abundance of SFB in weanling rats.

Hypothesis: Inulin, a prebiotic fibre, would increase the abundance of SFB in the ileum and faeces in a dose-dependent manner in weaned rat pups.

An overview of the structure of this thesis is shown in Figure 1.1.

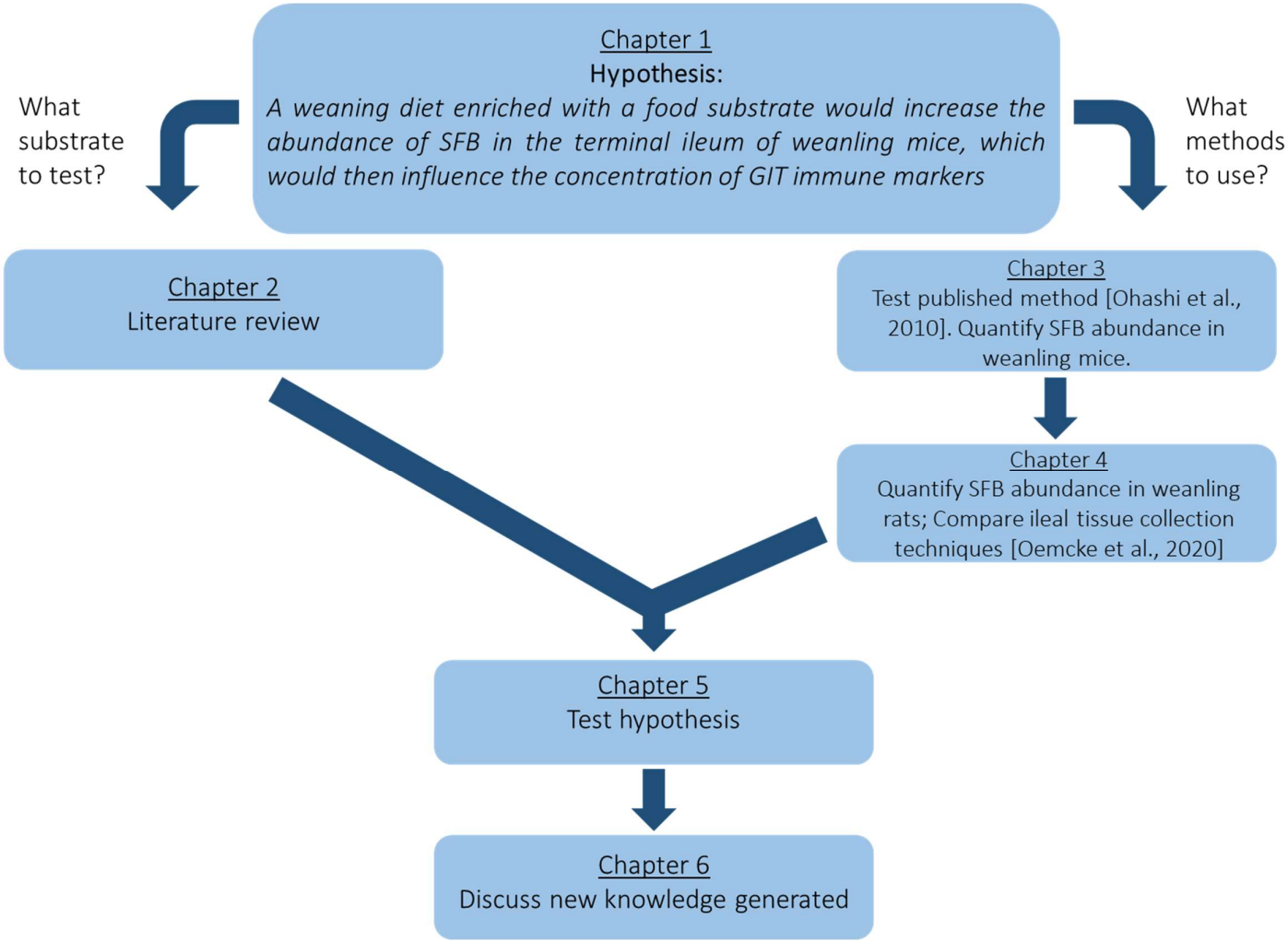

Figure 1.1. *Overview of the structure of the PhD thesis.*

1.3. Thesis structure

This Chapter is followed by the literature review (Chapter 2), which provides an overview of SFB, identifying these microbes in invertebrates and vertebrates. The review describes studies carried out on SFB and their perceived role in the GIT. It reports the temporal profile observed when the abundance of SFB changes at weaning and the reported correlation between SFB presence and GIT immune factors. These suggest that SFB may play a role in the maturation of the small intestine immunological barrier in infancy. As the temporal profile of commensal SFB changes in abundance at weaning, the weaning diet is suggested to influence these microbes. The literature review highlights this knowledge gap to determine what food nutrients if any, modulate the abundance of SFB and, in turn, the infant immunological barrier.

Chapter 3 reports the study which attempted to test the published mouse study to quantify SFB abundance pre- and post-weaning. A temporal profile of SFB in weanling mice was determined, including the correlation between SFB and faecal IgA and plasma IL-17. Chapter 4 highlights the comparison between two techniques previously used to collect ileum tissue for SFB quantification, whole tissue homogenisation and mucosal scraping. This study attempted to optimise the ileum tissue collection technique and the animal model used in determining the temporal profile of SFB abundance over time. The correlation between SFB and IgA and IL-17 was also determined in weanling rats. The abundance of SFB was also compared between sexes suggesting a possible influence of sex hormones on SFB, which was not previously done. The change in SFB abundance at weaning suggested an influence of diet on these microbes. The observed abundance changes following the weaning diet prompted the investigation (Chapter 5) on whether inulin would affect the abundance of SFB and, consequently, the concentration of faecal IgA and plasma IL-17 in pre- and post-weaning rats. Chapter 6 finally discusses the new knowledge generated, conclusion and areas of future research.

CHAPTER 2: REVIEW OF LITERATURE

This Chapter was published as a review in the journal *Frontiers in Nutrition*: Oemcke LA, Anderson RC, Altermann E, Roy NC and McNabb WC (2021) The Role of Segmented Filamentous Bacteria in Immune Barrier Maturation of the Small Intestine at Weaning. Frontiers in Nutrition: p. 950. (doi: 10.3389/fnut.2021.759137).

The content of this Chapter has been modified to meet the requirements for the thesis

2.1. Abstract

The microbiological, physical, chemical, and immunological barriers of the GIT begin developing *in utero* and finish maturing postnatally. Maturation of these barriers is essential for the proper functioning of the GIT. Maturation, particularly of the immunological barrier, involves bacteria. SFB which are anaerobic, spore-forming commensals have been linked to immune activation. The presence of, and changes in SFB abundance have been positively correlated to immune markers (cytokines and immunoglobulins) in the mouse ileum and stool samples, pre- and post-weaning. The abundance of SFB in infant stool increases from 6 months, peaks around 12 months and plateaus 25 months post-weaning. Changes in SFB abundance at these times correlate positively with the production of IL-17 and IgA, indicating their involvement in immune function and maturation. Additionally, the peak in SFB abundance when the milk-only diet is complemented by solid foods hints at a diet effect. SFB genome analysis revealed genes coding for enzymes involved in metabolic pathways for survival, growth, and development, host mucosal attachment and substrate acquisition. This narrative review discusses the current knowledge of SFB and their suggested effects on the small intestine immune system. Referencing the published genomes of rat and mouse SFB, the use of food substrates to modulate SFB abundance is proposed while considering their effects on other microbes. Changes in the immune response caused by the interaction of food substrate with SFB may provide insight into their role in infant immunological barrier maturation.

2.2.Introduction

It is widely accepted that, amongst other factors, the microbial consortia in all regions of the body contribute to maintaining homeostasis and good health [61-64]. These positive effects, specifically of the GIT microbes, created an interest in how the microbiota interacts with the host cells. Most of the knowledge from bacteria in the upper and lower GIT originated from studies carried out with faecal samples [65-67]. Faeces have been used to represent microbes of the lower GIT mostly. Some studies have also used faeces as a proxy of the upper GIT [68, 69] as the human small intestine is more difficult to access even with the use of invasive techniques [70, 71]. Nevertheless, some microbes present in high abundance in the small intestine have caught scientists' attention, as emerging evidence points to their significant involvement with the immune system [34, 43, 45, 72, 73].

SFB were discovered attaching to the ileal mucosa in invertebrates [74] and healthy vertebrates [22-24, 27, 28, 45]. These Gram-positive, anaerobic, spore-forming, commensal microbes have been classified within the Firmicutes phylum and are related to *Clostridia*. SFB possess a unique holdfast structure which facilitates their attachment to the ileal mucosa. During the lifecycle of SFB, the segmented filaments divide and elongate, forming viable intracellular offspring or spores in adverse conditions [72]. The closely reported phylogenetic relationship of SFB with *Clostridia* sparked an interest in their function because they were seen to occupy the distal ileum [22-24, 27, 28, 45]. In this region, SFB mainly attach to the ileal epithelium overlying the Peyer's patches where naïve T cells undergo antigen-driven activation and expansion to yield T helper cells [45, 75]. Studies reported positive correlations of SFB abundance with the concentration of IL-17 in plasma [39, 41, 76-78] and IgA in faeces [22, 33]. The concentration of these immune markers were also correlated with SFB abundance in the ileal contents of 4-6 week old mice [22, 35, 36], however these influences were not

exclusive to SFB [65, 79-81]. Interestingly, these effects occurred without signs of systemic inflammation in the host [39, 41, 76-78].

Additionally, the presence of SFB is variable; their abundance in the ileum of infants increases at weaning from 6 months, peaks around 12 months and plateaus until 25 months post-weaning [24, 82]. A similar pattern has been reported in BALB/c mice [22], institute of cancer research (ICR) albino mice [24] and Sprague-Dawley rats [23] where SFB abundance increased at weaning from 20 days, peaked around 24-28 days and plateaued until 50 days post-weaning. This timing of abundance change corresponds with the infant transitioning from a milk-based diet to one with solid foods. The increased dietary complexity is known to contribute to the immune system maturation progression and drives colonisation by a different and more diverse microbiota in the GIT [82-84] possibly including SFB. Figure 2.1 details the abundance of SFB as suggested to be involved in infant GIT immune maturation.

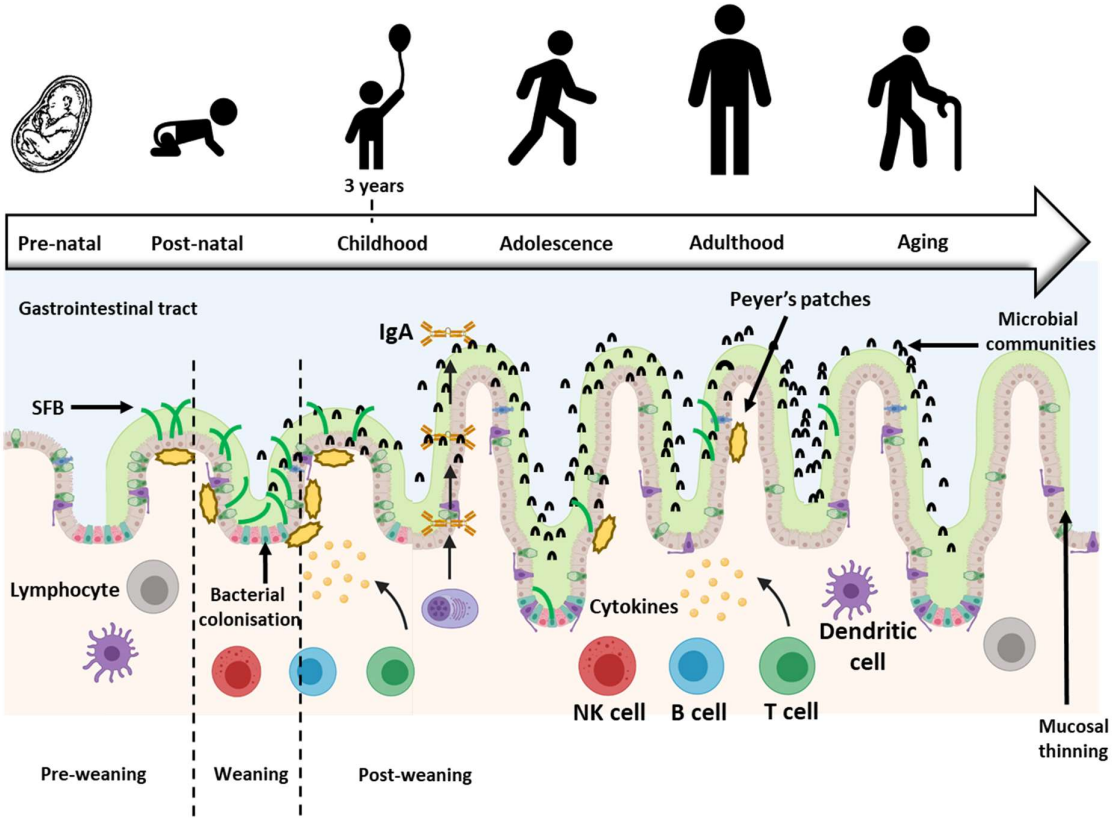

Figure 2.1. *Development of the gastrointestinal tract (GIT) throughout life. The GIT barriers continue developing and maturing postnatally until about three years of age when the resident microbiota resembles that of an adult. During this time, SFB abundance changes, first increasing and then decreasing at weaning, but SFB abundance remains low into adulthood. (Figure adapted from Jašarević, et al. [85]; Created with BioRender.com and PowerPoint). SFB: Segmented filamentous bacteria; IgA: Immunoglobulin A.*

The reports of positive correlation of plasma IL-17 and faecal IgA production with abundance changes of SFB in ileal contents or stools at weaning created an interest in studying these microbes and their influence on the infant GIT immunological barrier development. *In vitro* studies were carried out initially and proved challenging as SFB did not grow outside the ileum [47, 86]. SFB are reportedly anaerobic yet attach to epithelial cells that require oxygen. The attempts to further investigate the effects of SFB on the immune system *in vitro* became a significant technical challenge due to the poor viability of these microbes. SFB were therefore studied using animal models [22, 23, 47, 86] and ileal or stool samples from human participants [24, 26, 70].

Ohashi, et al. [22] investigated IgA concentration and SFB abundance from ileal contents in weanling mice. Their study reported that SFB abundance exhibited a temporal profile increasing post-weaning immediately, peaked, then decreased and plateaued off. Additionally, the concentration of IgA showed a negative association with SFB abundance. A study also reported that food substrates from a balanced diet compared to a milk powder diet increased the abundance of SFB in the ileum of 4 and 8-week-old mice [32]. Consideration should be given to study the potential of various food substrates to increase the abundance of ileal SFB and related changes in markers of immune maturation during the weaning transition.

Further to that, analysis of the SFB genome revealed genes encoding for enzymes involved in metabolic pathways which these microbes utilise for survival, growth, and development [87, 88]. Data sets of enzymes in the glycolytic and pentose phosphate pathways of SFB predict the use of some of the by-products in metabolic pathways for synthesising amino acids, vitamins, and cofactors within the genome [87, 88]. The genome of SFB is smaller (~1.57 Mb) compared to that of their relatives *Clostridium* (3.97 Mb). The genome reportedly lacks genes for biosynthesis of most amino acids. Amino acid transporters and permeases found in the genome

imply SFB uptake amino acids possibly from dietary sources and host protein degradation by proteases secreted by SFB. The absence of these important genes could explain their commensal nature [87] and possibly reflect the size of the genome. Attachment of SFB to the ileal mucosa and abundance change following the introduction of solid foods suggest the genome may not support all functions required to be free-living. However, genome size does not determine whether a microbe can be free-living or not.

This narrative review analyses the suggested role of SFB in infant immunological barrier maturation and whether modulation of SFB abundance could positively impact immune maturation of the small intestine for later health benefits. Referencing the published genomes of rodent SFB, the use of food substrates to potentially modulate SFB abundance is proposed while also considering the effects on the immune system and other GIT microbes.

2.3.Development and maturation of the small intestinal barrier

The small intestinal barrier comprises the microbiological and chemical barriers located in the lumen as well as the physical and immunological barriers in the mucosa. The microbiological barrier, located above the mucus layers, houses most of the microbes. Some microbes produce antimicrobial peptides that inhibit pathogen attachment by limiting their growth while other microbes are involved in nutrient acquisition and energy regulation [1, 2, 89, 90]. Below the microbiological barrier lies the chemical barrier that consists of an outer, less viscous mucus layer followed by the inner mucus layer containing fewer microbes. The inner mucus layer contains free-floating microbes and those, such as SFB, which attach to the epithelium.

Below the chemical barrier is the physical barrier known as the epithelium. It comprises columnar epithelial cells organised into crypts and villi [4]. At the base of the epithelial crypts, Paneth cells secrete antimicrobial peptides, including α-defensins, lysozyme and phospholipase, which prevent the growth of pathogenic microbes [3]. In the physical barrier, protein complexes provide structural integrity and act as channels that allow or prevent the passage of substances contributing to this barrier's selective permeability [11]. The immunological barrier is the innermost layer where the immune system provides defence against pathogens and antigens and exists in an immune-suppressed state maintaining homeostasis even with dietary antigens and microbiota that pass through the physical barrier [12].

Of interest is the immunological barrier where immune cells begin appearing at approximately 6 months of gestation. Recruitment of epithelial lymphocytes in the GIT begins signalling the functioning of an immature immunological barrier [20]. Naïve T cells undergo activation and expansion in the Peyer’s patches yielding T helper cells which are later stimulated to produce cytokines involved in immune responses [75]. Naïve B cells also begin differentiating into

plasma cells that produce immunoglobulins which recognise and bind to pathogenic bacterial or viral antigens and assist in their destruction [91]. Fully developed during gestation, the immune system matures post-birth possibly along with the microbiological barrier. Maturation of the immunological barrier is thought to occur at weaning (about 6 months of age) when foods containing antigens and microbes are introduced and may trigger immune responses in the infant GIT. It is during complementary feeding, when solid foods are introduced into the infant's milk-only diet, that the abundance of SFB reportedly increases, peaks, then plateaus. The studies which reported a positive correlation of SFB abundance with IL-17 [39, 41, 60] and IgA [22] production, led to the suggestion that these microbes may play a role in GIT immune system maturation.

2.4.Background on segmented filamentous bacteria

Reported initially over 150 years ago, SFB were observed in the ileum of invertebrates [74], firmly attaching to the epithelial lining [25]. SFB were initially given the provisional name *Candidatus arthromitus* [92]. However, the first filamentous bacteria isolated from arthropods, though morphologically similar to those isolated from vertebrates, were shown to belong to the *Lachnospiraceae*, a family within the order C*lostridiales*, after analysis of 16S rRNA gene sequences [93]. *Candidatus arthromitus* showed an apparent absence of SFB-like 16S rRNA gene sequences present in vertebrate SFB. It may have been an indicator of a different species of SFB within the same genera inhabiting invertebrates and vertebrates. Therefore, another taxonomic classification was proposed and accepted [94] to name the species isolated from vertebrates. It was named *Candidatus savagella* [95], which is within the family *Savagellaceae* [96], a credit to Dwayne C. Savage, the American gut microbiologist who first observed and described them in the ileum of rodents [25]. Rods and filaments of SFB were identified by light microscopy and fluorescent *in situ* hybridisation [24, 97] and later PCR methods were used to detect SFB in the ileum of mice and rats [22, 23].

SFB are reported to replicate in the ileum through a life cycle deduced by electron microscopy in rodents (Figure 2.2A) [98]. SFB exist in two forms, a vegetative segment containing a holdfast structure that allows them to anchor to the host epithelial cells, and as spores which are intracellular offspring encapsulated during adverse conditions. These morphologically distinct intrasegmental bodies indicate that SFB exist in vegetative and dormant states [99, 100]. The 'holdfast' structure on vegetative segments allows SFB to anchor onto ileal epithelial cells and lengthen, forming primary and secondary segments [99, 100]. These segments double in length and form filaments in which a mother cell forms, engulfing the subsequent daughter cell, which then divides to form two viable intracellular offspring [99, 100]. The offspring then exit the filament and later attach to the host epithelium or form spores under stressful conditions

[99, 101, 102], which may then be transferred to another host of the same species [103]. This observation was confirmed by an *in vitro* investigation in SFB gene diversity and host-specificity of four flagellin genes in mice and rats which revealed two relatively conserved and two variable genes and confirmed the preferential attachment of SFB to the epithelial mucosa of their host [103].

The origin of SFB in the GIT is uncertain mainly due to the inability to isolate microbes from a developing foetus. Presence of SFB in the placenta or meconium is yet to be elucidated, and it is unclear when and how they colonise the ileal mucosa. The presence of SFB in dormant stages suggests that their offspring may be transferred via maternal faeces to seed the infant during vaginal birth. The infant may also ingest SFB which can form spores to protect their offspring from the harsh environment of the upper GIT prior to arriving and attaching to the ileal mucosa. Additionally, SFB spores could also be passed from the mother to the embryo via the placenta in infants born via Caesarean-section (Figure 2.2B). These theories may explain the origin of SFB in infants, though they have not been demonstrated experimentally. Other potential sources of SFB including food or the environment (soils, plants, water bodies) have yet to be investigated as the GIT has so far remained the main focus of SFB studies. As maturation continues in infancy, SFB abundance then increases, peaks then decreases and plateaus at weaning [22, 23, 78]. Reportedly present in the GIT throughout an individual's lifetime, the abundance of SFB is thought to decrease post-weaning possibly due to completed maturation of the GIT immunological barrier. The decreased abundance might also be caused by competitive inhibition by later-arriving microbes whose growth may be encouraged by the new weaning diet.

The relative abundance of genes predicted to encode cell cycle control functions, envelope biogenesis, and trafficking was higher in the mouse SFB genome than in other clostridia. They may reflect the complex cell differentiation processes during this lifecycle [104]. Four

predicted N-acetylmuramoyl-L-alanine amidases unique to SFB (cell wall hydrolases, PF01510, PF05105, PF01520) are also hypothesised as necessary in forming the different cell morphotypes and may be responsible for releasing 'holdfasts' and spores from the filaments [105].

In postnatal life, SFB are present in the ileum of healthy weanling rodents [22]. They have also been detected in infant and adult humans' faeces, though collecting and analysing ileal samples are preferred [24]. However, collecting ileal samples in conscious subjects requires invasive diagnostic tools such as scopes [106]. Performing these manipulations in infants for routine SFB study is challenging due to the ethical restrictions of researching this age group. Therefore, animal models are currently the most appropriate way to study SFB.

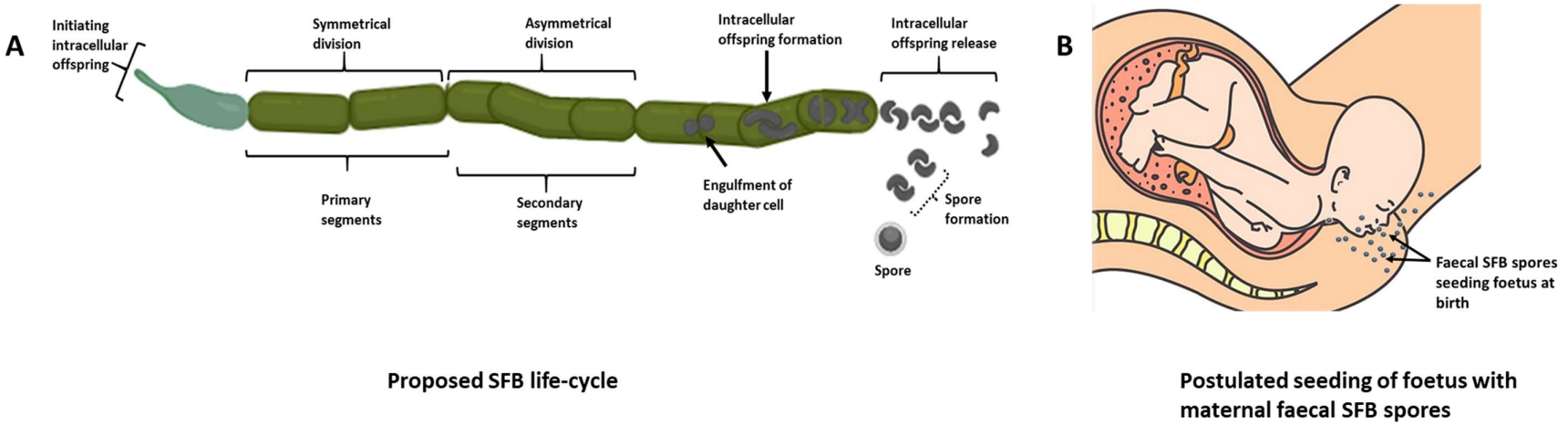

Figure 2.2. The proposed life cycle of SFB

(A) *The 'holdfast' structure on vegetative segments allows SFB to anchor onto host epithelial cells and lengthen, forming primary and secondary segments. These lengthen to form a filaments or mother cell which engulfs the subsequent daughter cell. The daughter cell divides to form two viable intracellular offspring which exit the filament and attach to the host epithelium or form spores, which are transferred to another host of the same species.* ***(B)*** *SFB faecal spores may seed the oral cavity during vaginal birth, or SFB spores could be passed from the mother to the embryo via the placenta in infants born via Caesarean-section. (Figure 2.2A adapted from Schnupf, et al. [47]; Created with BioRender.com) (Figure 2.2B with permission from EPI-NO. Source: https://www.epino.de/en/birth-preparation.html).*

2.4.1. SFB in humans

The discovery and characterisation of SFB in the ileum of healthy rodents drew interest due to their reported influence on markers of the GIT immune system. Later detection of SFB in the faeces of healthy infants and adults suggests that SFB form part of the normal GIT microbiota and, like rodents, may also influence the GIT immune system. Knowledge of the role of SFB in humans is still scarce, and a time-course study elucidating the temporal profile of SFB at the crucial weaning stage in infants has yet to be attempted.

A comparative 16S rRNA gene analysis of SFB in healthy human faeces, mice ilea and chicken ilea indicated similarities in the abundance change of these microbes at similar stages of development [24]. The similar findings of SFB abundance in human faeces and mice and chicken ilea implied that ileal SFB in humans exhibit a temporal profile of abundance change pre- and post-weaning, and then persist into adulthood and old age as part of the normal microbiota. SFB have also been detected in ileostomy samples of adult patients with ulcerative colitis [50], though the implications of disease-cause by SFB have not been verified. Other than indicating the presence of SFB in the human GIT, these studies provide limited evidence of the interaction of these microbes with the immune system. Current knowledge of SFB interactions in humans comes from studies carried out on animal models. However, these observations might not necessarily translate to humans due to differences in the GIT microbiota profiles between species.

2.4.2. SFB and the immune system

The discovery of SFB in the ileum of vertebrates prompted investigations of their role in the GIT. Studies on how SFB interact with the immune system indicate that SFB do not appear to cause ill-effects in healthy vertebrates [39, 41].

2.4.2.1.Immunoglobulin A and Interleukin 17

Studies on SFB have investigated their relationship with IgA, the most abundant immunoglobulin occurring in the body. IgA acts to block excessive bacterial adherence or translocation, mediates the neutralisation of toxins and viruses, and removes unwanted macromolecular structures on the epithelium in the GIT [107]. IgA in infancy reportedly originates from breast milk contributing to high levels found in the GIT lumen during the first month which gradually decrease until 5 months of age [108], then remain relatively low and stable until 24 months of age [109]. Postnatal microbial colonisation and maturation of the GIT stimulate host production of IgA, and the luminal levels slowly increase [110].

The presence of IgA in the GIT is important, especially in infants below 6 months of age with an immature immune system. Interactions between SFB and IgA drew attention following reports of SFB inducing IgA production in Swiss [34, 35], BALB/c [35, 37] and C3H/HeN [36] adult mice mono-associated with SFB. These findings suggest that SFB stimulated the germinal centres in the Peyer's patches [76]. Further investigation in rodents showed that IgA production increased with increasing SFB abundance and continued increasing as SFB abundance decreased from about 4 weeks postnatally [22, 35, 36]. A report on the aberrant expansion of mainly SFB and other anaerobes in the absence of hypermutated IgA in adult C57BL/6 mice [33] points at the function of IgA in regulating the bacterial composition of the GIT. IgA

production is, however, not exclusively linked to SFB. Other commensals, such as Gram-negative *Morganella morganii* [36] and Gram-positive probiotic *Bifidobacteria* [65, 80, 81], induce the production of IgA. B cells which are the origin of IgA may thus be stimulated by microbial colonisation, including SFB, resulting in increased IgA levels [36]. The reported correlation of IgA with SFB [34-37] may be evidence of IgA maintaining a homeostatic balance within the microbiota and remains a point of interest in the suggested role of SFB in postnatal immunological barrier maturation.

The effect of SFB on various immunoglobulins was compared to determine a suitable immune marker to correlate with the abundance of SFB. The concentrations of IgA, IgG, and IgM from small intestinal secretions of 6–8-week-old male and female Swiss mice were determined by an enzyme-linked immunosorbent assay (ELISA). The concentrations in the SFB-positive and SFB-free specific-pathogen-free (SPF) mice were compared against one another. The results showed a significant increase in the concentration of IgA, compared to IgG and IgM, in the SFB-positive mice, similar to what is observed in mice after weaning [34]. Another study also reported that SFB promoted the development of both intraepithelial lymphocytes and IgA-producing cells in the small intestine of male BALB/c SFB-mice [38].

Many reports on SFB have focused on their ability to stimulate the production of the pro-inflammatory cytokine IL-17 furthering the interest in their influence on GIT immunity [39-41, 60]. IL-17 is essential for host defence against infection by invading pathogens at mucosal surfaces [111, 112]. When faecal microbes, without SFB, from Jackson C57BL/6J mice, were introduced into GF mice, Th17 cells were not induced until SFB was added [39]. Mice lacking SFB in their microbiota had fewer Th17 cells in the ileum than mice with a normal SFB population. SFB also specifically induced Th17 cells in the small intestinal lamina propria when introduced into GF Swiss-Webster mice [39]. The production of Th17 cells demonstrated

maturation of the immunological barrier after SFB were introduced into GF mice. Non-colonised control GF mice were also observed to have no Th17 cells, implying an immature immunological barrier [34, 39].

Reports of the influence of SFB on ileal IL-17 production [39, 40, 60], including the reported mechanisms by which SFB achieve this [113, 114], suggest a positive correlation between both. Immunisation of adult mice with SFB flagellins (FliC3) resulted in higher upregulation of small intestine epithelial cell factors controlling the differentiation of Th17 (Duox2, Duoxa2, Nos2) and also promoting the production of IL-17 [114]. Further exploration of IL-17 production by SFB in adult mice has revealed that SFB and ileal epithelial cells communicate by generating endocytic vesicles at the interface of SFB-epithelial cell synapses. The interaction of SFB with the epithelial cells triggers the formation of endocytic vesicles through clathrin-independent and dynamin-dependent endocytosis. These vesicles contain an SFB cell wall-associated protein (P3340), an immunodominant T cell antigen for generating mucosal Th17 cells [113]. The vesicles are released into the ileal epithelial cells, and P3340 induce activation of lamina propria antigen-specific Th17 cells, and subsequently, IL-17 is produced [115]. Thus, these observations indicate that SFB flagellins are involved in upregulating ileal epithelial cell genes, which in turn induce IL-17 production.

Like IgA, Th17 cell production is also not exclusive to SFB [39]. This was demonstrated when C57BL/6 GF mice mono-associated with SFB induced Th17 cells to a lesser extent than GF mice colonised by SFB and a more complex microbiota (SFB and 8 defined commensals) in the small intestine [39, 116]. This reported interaction between SFB and other microbes in IL-17 production highlights the synergy among commensals and their influence on the immune system, including Th17 cells. Alternatively, differentiation of Th17 cells was induced in Taconic B6 and Jackson B6 mice treated with antibiotics and then exposed to normal SPF

bacteria [60]. Upon further investigation, members of the *Bacteroidetes* phylum were reported to be involved indicating that SFB may not be the only microbes capable of inducing IL-17 production.

Overall, the literature reports that SFB-upregulated epithelial cell factors are involved in IL-17 production in adult mice. These observations imply the requirement of a mature GIT in investigating the role of SFB in postnatal immunological barrier maturation.

2.4.2.2.Immune-mediated disease

Research into SFB has highlighted their association with both disease cause [49-52] and protection [48, 117]. The findings suggest that having an appropriate number of SFB maintains a delicate balance between these microbes and the host immune system.

The effort to decipher the role of SFB arose from studies which investigated the involvement of SFB in several functional GIT and autoimmune diseases. Studies with severe combined immunodeficiency (SCID) adult mice colonised with only SPF bacteria, only SFB, or a combination of both reportedly developed clinical signs of colitis [52], while GF SCID mice or SCID mice inoculated with SPF microbiota exhibited no signs of colitis. The development of colitis in the mice suggested that dendritic cells were activated by their colonisation of the ileum [118]. SFB were also detected in ileal mucosa samples of both ulcerative colitis adult patients and healthy controls, indicating the presence of these microbes in both groups might not be linked to the disease [50]. It was hypothesised that the ulcerative colitis patients' samples might have exhibited a higher SFB load compared to the healthy controls [50]. It is plausible that higher numbers of SFB induced the production of IL-17 to unhealthy levels [49, 119] though this was not measured. A higher abundance of SFB was also reported in faecal samples

of adult patients with diarrhoea-associated irritable bowel syndrome than those with constipation-associated irritable bowel syndrome [51]. Autoimmune diseases have also been associated with SFB where the Th17 cell population in arthritis and multiple sclerosis GF adult mouse models inoculated with SFB provoked an onset of the diseases [49, 120]. These reports highlight the complexities of the suggested effects of SFB in disease and cannot be restricted to a single type of pathology, nor infer causality.

Reports from the sequenced rat and mouse SFB genomes revealed that SFB lack the genes encoding for known toxins and virulence factors present in pathogenic *Clostridia* [106]. This observation and the lack of apparent inflammatory reactions where SFB colonise the ileum suggest that SFB may stimulate IL-17 production without pathological consequences. Phylogenetic analysis of 16S rRNA indicates that SFB and pathogenic *Clostridia* such as *Clostridium. tetani, C. perfringens* and *C. fallax* share a common ancestor, albeit distantly (Figure 2.3). Therefore, it is also likely that SFB may have unique genes that are absent in pathogenic *Clostridia,* and which may be activated under immune pathologic events or when the numbers of SFB cross a threshold. Sequencing of SFB isolates from stool samples of healthy adults [24] and those with ulcerative colitis [50] would be required to identify genomic differences between SFB genome-types.

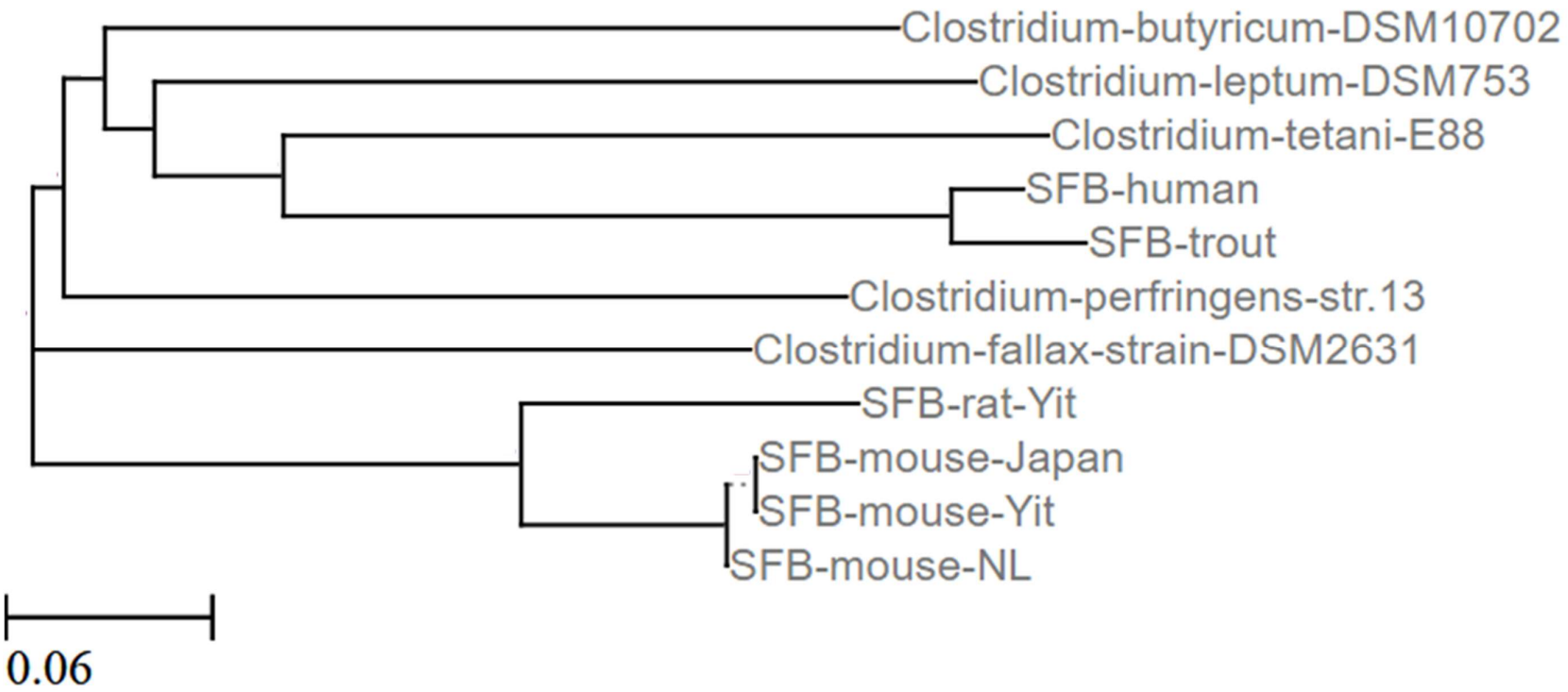

Figure 2.3. Phylogenetic tree relating SFB to Clostridium species. *This phylogenetic tree shows the relationship between complete (SFB mouse-Yit, SFB-rat-Yit, SFB-mouse-Japan, SFB-mouse-NL) and partial (SFB-human, SFB-trout) SFB genomes and some pathogenic (C. perfringens, C. fallax, C. tetani) and non-pathogenic (C. butyricum, C. leptum) Clostridium species published in the NCBI database (Created using the EMBL-EMI Multiple Sequence Alignment Clustal Omega tool and ETE toolkit-Phylogenetic Tree viewer).*

Comparison of sequences among SFB filaments isolated from SFB-monocolonised mice along with published SFB genome sequences revealed the presence of single nucleotide polymorphisms (SNPs) [104]. In nature, SNPs occur frequently and, in some cases, cause silent point mutations with no effect, but they can also cause nonsense mutations which affect the functionality of a gene. Pamp, et al. [104] combined reads, *de novo*, from five individual SFB filaments (SFB-1 to SFB-5) from faecal material of SFB-monocolonised mice to form the co-assembly "SFB-co". A second co-assembly, "SFB-mouse-SU", which was closely related to their SFB was also assembled using the published SFB mouse genome [SFB-mouse-Yit (AP012209)] [87]. Some of the loci exhibiting SNPs in the individual SFB filaments included oxaloacetate decarboxylate alpha (OadA), pyruvate kinase (PK) and flagellar motor switch protein (FliN). A conserved lysine residue in OadA, which generates pyruvate from oxaloacetate, was substituted by threonine. A similar mutation in *Vibrio cholerae* renders OadA ineffective. For PK which catalyses phosphoenolpyruvate to pyruvate, a valine residue was changed to alanine. The SNP in FliN, which together with FliG and FliM forms the switch complex that controls the direction of flagella rotation, resulted in a predicted threonine to alanine change [87]. These mutations of FliN in bacteria have reported failure in flagella export and rotation [121, 122]. The polymorphisms observed among the five SFB filaments are suggested to be minor variants which coexist within a population of SFB in an animal colony. Multiple genome sequence comparisons of other SFB genomes revealed chromosomal features whereby the highest variability include CRISPR-arrays, phage-related genes and hypothetical proteins [104] which indicate heterogeneity and evolution of SFB lineages within colonies of similar species.

Finotti, et al. [50] also sequenced an SFB PCR product from colorectal biopsy samples of 35 to 70-year-old males and females with ulcerative colitis. The sequence was compared to whole-genome sequences of SFB from healthy mouse, rat, turkey, and a partial human SFB sequence

in the NCBI Reference Sequence Database (Table 1). Their results highlighted nucleotide changes in the ulcerative colitis SFB coding sequences at positions 64, 68, 81 and 85 reflecting amino acid differences from aromatic to branched-chain amino acids, negatively charged to uncharged, aromatic to aliphatic and hydrophobic to positively charged, respectively. The sequenced colorectal SFB genes were representative of SFB from the ileum, but without further information, it is unclear whether ileal and colorectal SFB are similar or not. The lack of complete SFB sequences from healthy humans in the database creates a challenge in making informed comparisons between SFB isolated from healthy versus diseased adults.

Table 2.1. *Nucleotide changes observed in human SFB PCR product sequence from the terminal ileum of 35 to 70-year-old males and females with ulcerative colitis [50] compared to codons from the SFB genes of healthy rat and mice. The nucleotide positions correspond with the nucleotide bases in bold in the amino acid codons. Letters in parentheses are the single-letter abbreviations of the amino acids.*

NUCLEOTIDE POSITION	**64**	**68**	**81**	**85**
RAT	A**T**C	G**AT**	**A**TA	C**A**T
	Isoleucine (I)	Aspartate (D)	Tyrosine (Y)	Methionine (M)
MOUSE	T**T**C	G**A**T	**A**TA	C**C**T
	Phenylalanine (F)	Aspartate (D)	Tyrosine (Y)	Leucine (L)
HUMAN (ULCERATIVE COLITIS PATIENTS)	A**T**T	G**G**A	**G**CC	A**A**A
	Isoleucine (I)	Glycine (G)	Alanine (A)	Lysine (K)

In contrast, SFB have also been associated with disease protection against both type 1 diabetes [48] and rotavirus infection [117] in adult mice. Non-obese diabetic (NOD) adult mice inoculated with SFB were reported to have high levels of IL-17-expressing $CD4^+$ cells when compared to SFB-negative NOD mice. The SFB-positive NOD mice also did not develop diabetes though levels of insulitis, a marker for type 1 diabetes, were similar to those in SFB-negative NOD mice. This result implies that SFB colonisation may not block the trigger of diabetes but might modulate the progression of the disease [48].

In addition, the comparison of two different SFB strains administered to GF Rag1-knockout adult mice, which lack mature B or T cells, showed reduced rotavirus infectivity. The mechanism by which this effect happened was independent of Th17 cells because SFB administration promoted enterocyte proliferation, migration, and luminal shedding of rotavirus-infected cells. The observations hinted that SFB may prevent infection by hindering the rotavirus from utilising a surface component to bind to the ileal mucosa. The protective effect against rotavirus infection was, however, conferred more strongly by one of the two SFB strains administered to rotavirus-susceptible Rag1-knockout adult mice, highlighting a potential role of strain-specific phenotypes [117]. These reports indicate synergies possibly among SFB strains, along with other microbes, cell signalling receptors and immune system mediators.

The presence of SFB in healthy [24] and diseased [50] adults indicates these microbes may have persisted from childhood. Based on the abundance of SFB relative to the absence of or incidence of disease, perhaps an appropriate and critical number of SFB may confer beneficial effects, and beyond this threshold, there might be detrimental effects. Whether the effects of SFB are specific to an age group or whether they continue to influence the GIT immune system

later in life are unclear and remain as points of interest in the interaction of SFB in health and disease.

2.5.Impact of early-life nutrition on ileal SFB abundance

The timing of SFB abundance changes around weaning [22-24] suggests that the inclusion of complementary foods might be a way to alter their abundance and hence effects on the host epithelial and immune cells in the ileum. The absorptive function of the small intestine matures during weaning in infants [31] and seems to coincide with these changes in SFB (and other microbes) abundance in the ileum. SFB are proposed to obtain nutrients from the ileal lumen [32, 39, 42-46] and directly from the host, though this has not been confirmed. SFB may have evolved to attach to the ileal mucosa and derive nutrients in a cross-feeding manner with other bacteria residing in the inner mucus layer of the chemical barrier.

The abundance of SFB might increase with the increasingly diverse diet that infants consume from weaning, which might have subsequent effects on the immunological barrier maturation. However, the risks that an increase in SFB abundance may pose cannot be ignored. As discussed in Section 2.4.2.2, these microbes may be associated with some diseases [48-52]. However, observations from healthy SFB-mono-associated mice [39, 60], as well as healthy humans (1 day-old to 72 years old), have shown that SFB do not predispose them to disease, which suggests that changes in the profile of SFB abundance can occur without adverse pathological effects. These reports strongly hint at the necessity of an appropriate bacterial load of SFB to avoid possible activation of putative pathogenic genes that these microbes may harbour. SFB are thought to influence the production of immune cells naturally, and this process may exacerbate immune-mediated diseases and explain the association with disease. Immune-mediated diseases may also likely have a reverse effect and trigger opportunistic

pathogenicity in SFB, though this is yet to be investigated. The effects of a transient increase in SFB abundance cannot be inferred from studies with SFB-mono-colonised mice. The cause of SFB abundance decrease post-weaning is still unknown, though it is believed to be due to the completion of immune barrier maturation which coincides with increased diet diversity. This is thought to occur when solid foods induce differences in the mucosa-associated microbiota via gene regulation of lipid and carbohydrate metabolism, influencing energy balance and body weight, inflammatory response increase and altering endocrine functions [123-125]. SFB also coexist with other microbes in the community and attempts to alter the abundance of SFB, even for a limited amount of time, may impact other members. It is therefore unclear how these potential interactions might affect the immune system and overall health.

2.6. Substrate selection

The analysis of the rat and mouse SFB genomes is necessary to infer the functions of predicted genes and thereby predict the utilisation of substrates by SFB. The lack of certain metabolic genes in the SFB genome and evidence that SFB reside in the nutrient-rich environment of the ileum possibly explains its reduced genome size (~1.57 Mb) compared to their relatives *Clostridium* (3.97 Mb). Results from the published rat and mouse SFB genomes revealed the presence of carbohydrate, amino acid, proteins, vitamins and mineral permeases, import/export transporters and ABC-type transporters [87]. Continuous reduction within the SFB genome over time may have contributed to their commensalism as they are presently thought to acquire nutrients directly from the host.

Compared with selected clostridia, annotation of the SFB genomes indicated the absence of gene products involved in amino acid synthesis. Alternatively, the presence of genes coding

for amino acid transporters and permeases implies the requirement of essential amino acids that may be acquired via absorption [64, 87, 88]. Additionally, only a small fraction of enzymes required for synthesising amino acids and cofactors have been predicted [87, 88]. An analysis of the SFB genome has also indicated the presence of an N-acetylglucosaminidase family protein. Analysis of the draft genome of human SFB from ileostomy, revealed the presence of one tentatively extracellular N-acetylglucosaminidase, nine glycoside hydrolases representing 6 different families, as well as several cell surface-bound and extracellular proteases [126]. These enzymes together might hydrolyse nutrients within the ileum whose metabolic products are then imported into the SFB cell reflecting the reported commensal nature of these microbes. Analysis of the human SFB draft genome indicated a lack of tricarboxylic acid cycle enzymes, similar to rat and mouse SFB. There were also no proteins identified that could be assumed to take part in the electron transport chain, suggesting a fermentative lifestyle. An interesting observation was that human SFB contains genes for biotin synthesis (BIOA, B, D, F, W, and X), which are lacking in rat and mouse SFB. This observation may demonstrate physiological or dietary differences of the hosts, or host microbial community, as some GIT microbes can synthesise biotin while others cannot.

Analysis of the SFB genome also revealed the presence of enzymes predicted to be involved in the glycolytic and pentose phosphate pathways. A complete set of genes encoding for enzymes for the glycolytic pathway was revealed. For the pentose phosphate pathway, enzymes for the oxidative phase were not predicted, though at least two catalases and one peroxiredoxin were detected which may contribute to the tolerance of SFB to the microaerobic environment of the small intestinal lumen [87]. The predicted presence of these glycolytic and pentose phosphate enzymes suggests that carbohydrate macromolecules are transported into the cell and utilised by SFB as the main energy source. Another possibility is that SFB may possess

extracellular hydrolases which allow them to metabolise host carbohydrates though the evidence of genes responsible for this has not been reported.

The published SFB genome [87] predicted permeases and import/export-type transporters for sugars which reflect the metabolism and transport of carbohydrates. The sugars transported include mannose a component of the glycolytic pathway which is used in the cell wall structure and malate – a source of carbon used in the tricarboxylic acid cycle and ribose – essential for synthesising adenosine triphosphate. SFB are also thought to uptake cellobiose, ascorbate and fructose whose metabolism results in glyceraldehyde-3-phosphate of the glycolytic pathway. Moreover, the preferred niche of SFB is the ileum, where the brush border is located [87]. This microvilli-covered surface on the ileal epithelium contains enzymes which degrade disaccharides into simple sugars that are then absorbed into the bloodstream [127].

At weaning when the abundance of SFB changes, the addition of complex carbohydrates into the diet is thought to encourage their growth [22, 23] and that of other microbes within the ileum [128-131]. The infant GIT, pre-weaning, is mainly colonised by members from the phyla *Actinobacteria*, *Proteobacteria*, *Bacteriodetes*, and *Firmicutes* [132]. In exclusively breastfed infants, the GIT is dominated by *Bifidobacteria spp (Actinobacteria)* [133] which are associated with the production of SCFAs and vitamins, modulation of the immune system and prevention of GIT disorders [133, 134]. The production of SCFAs by *Bifidobacteria spp* suggests their involvement in carbohydrate fermentation. These carbohydrates which include non-digestible oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin are reportedly selective in increasing bifidobacterial numbers and decreasing *Enterococci* and *Escherichia* [128, 129].

Throughout all weaning stages, infants are able to ferment simple carbohydrates. Early at weaning though, infants develop the ability to ferment oligosaccharides, and at the later stages

of weaning are able to ferment complex carbohydrates [135]. Therefore, during complementary feeding, when complex food substrates are introduced into the infant's GIT, the abundance of *Firmicutes* which includes *Clostridia spp*, in which SFB are classified, also increases [136, 137].

The recommended weaning diet includes protein, fats, minerals, vitamins, and carbohydrate prebiotics [138] to keep up with infant nutritional requirements during growth and development. The carbohydrate prebiotics in the complementary diet feed GIT microbiota which benefit the infant [133, 134]. As the relative abundance of microbial genera such as *Bifidobacteria* increases [139], the abundance of *Clostridia spp* [136, 137] and *Streptococcus spp* and *Veillonella spp* also increase in the ileum [64]. At this time, *Streptococcus spp* are reportedly involved in simple carbohydrate fermentation and yield lactate as the main fermentation end-product which is a source of carbon for *Veillonella spp* [140]. Based on predictions from the SFB genome, SFB may also contribute to the production of lactate [87], which in a cross-feeding relationship like *Streptococcus spp*, may provide carbon for *Veillonella spp*. *Bifidobacteria spp* also contribute in SCFA-production as they increase at weaning [141] and may be involved in the suggested cross-feeding relationship with SFB.

Carbohydrates from the mainly milk-based diet pre-weaning may be an initial source of nutrients for SFB, though not adequate to modulate their abundance. This was concluded from a report of whole-milk-based diets containing dairy carbohydrates such as lactose and milk oligosaccharides that did not increase ileal SFB abundance in mice at weaning [32]. Mice that were fed a purified whole milk powder diet had lower SFB abundance in the ileum than those on a balanced milk-based diet. The basal diet comprised skim milk powder, Lucerne (alfalfa) meal, native corn starch, soybean oil, ground barley, fish meal, soybean protein concentrate, wheat middlings, corn protein concentrate, molasses, vitamin premix, mineral premix, calcium carbonate, and sodium chloride. These results indicate that the purified diet lacked more

complex nutrients essential for optimum SFB growth [32]. The introduction of carbohydrate prebiotics when the abundance of SFB is reported to change suggests these complex substrates may modulate the abundance of these microbes. SFB genome analysis has shown a higher abundance of COGs for carbohydrate metabolism and transport in SFB compared to other clostridial species [87]. SFB may utilise the metabolites from microbial fermentation and may also uptake some remnant products of digestion.

Investigating the suggested influence of complex carbohydrates on SFB abundance would involve enriching a weaning diet with a carbohydrate substrate typically present in the infant diet. One of these carbohydrates include inulin which is present in certain fruits and vegetables [142] given as weaning foods to infants. Inulin belongs to a class of soluble dietary fibres known as fructans and occurs naturally as a reserve carbohydrate in plants [143]. It is digested by residential bacteria and encourages the growth of SCFA-producing microbiota [144, 145]. Its unique chemical structure is made up of compounds with low chemical reactivity and resistant to digestion by the human GIT [146]. Inulin is routinely added to infant formulas [147] mainly manufactured from bovine milk which, compared to human breastmilk [148], has a lower concentration of milk oligosaccharides [149]. The complex molecular structures of these milk oligosaccharides are digested by residential bacteria which is key for developing a diverse and balanced microbial community in the infant GIT [150].

Inulin has also been reported to have several health benefits. These include reduced incidences of flatulence and bloating [53] and enhanced abundance of beneficial bacteria. Inulin promotes the production of SCFAs (acetate, propionate and butyrate) in the colon which creates an acidic environment that prevents the growth of pathogenic bacteria SCFAs are also thought to be involved in immune system activation [57]. Inulin has also been reported to improve epithelial integrity and barrier function and increase the expression of tight junctions (claudin-2 and occludin) mainly in the colon [151]. The reported production of acetate, via the glycolytic

pathway within the SFB genome, which is then converted to ethanol suggests that SFB may contribute some modulatory effects through SCFA-production. Additionally, the introduction of inulin (present in weaning foods) mainly occurs at the same time SFB is reported to increase in abundance. If this is the case, supplementation of the diet with inulin might possibly influence the abundance of SFB.

Inulin has not previously been reported in SFB studies and can be easily incorporated in the diet in a dose-dependent manner to investigate the effects on SFB abundance. SFB are suggested to utilise nutrients in the ileum resulting from the consumption of a balanced diet, though the mechanism they use is still unclear. However, the reported presence of import and export transporters in the cell wall of SFB filaments suggests this is how they uptake metabolic products [87]. This process of nutrient import and export in SFB filaments may provide energy for the reported life cycle. The presence of orthologous flagellar genes in SFB implies similarities in bacterial morphology determined by the arrangement of flagellin in the filament and attachment and nutrient absorption mechanisms to *Clostridium spp* [152]. Previously, the sequenced rat SFB genome revealed about 164 coding sequences as conserved hypothetical proteins or did not match any in the public database [87]. Recent annotation however has revealed 84 and 14 hypothetical proteins in the rat and mouse SFB genomes respectively. Majority of these genes are predicted to be involved in protein and carbohydrate metabolism related to cellular maintenance and make it difficult to predict whether nutrients can influence SFB abundance and mode of action on the infant immunological barrier.

2.7.Future perspectives

Initial research on SFB led to assumptions that these microbes could be studied in the laboratory using the common microbiological techniques. However, the difficulty in culturing

them in the laboratory required alternatives to decipher the role of SFB in the ileum. Thus far SFB seem to be the only group of anaerobes which require mucosal epithelial attachment to the ileum for survival. Their pattern of abundance change pre- and post-weaning in the ileum is also unique to them. Their preferred location of attachment in the ileum over Peyer's patches also reiterates the importance of understanding the function of SFB. This niche of SFB in the ileum may position them to uptake nutrients from digestion, allowing them to influence the immune system. Whether SFB directly benefit the immune barrier or whether the immune system of the GIT triggers SFB to influence immunity in a feedback loop or enhance the action of other microbes remains unknown.

The reported influence of SFB on the immune system encouraged research on the effects of SFB presence or absence in immune-mediated disease. Perhaps in some cases, SFB contribute to the progression of the disease, though the reported absence of clostridial virulence genes imply that SFB may indeed offer protection from disease. Thus far, the results from these studies are varied and inconclusive, reflecting the complex association between SFB and the immune system.

The lack of knowledge of the complex relationship between SFB and the immune system is an important factor in dietary intervention studies. The proposal to enrich diets with substrates to manipulate the abundance of SFB is feasible compared to delivering them as a supplement [47]. SFB require an attachment to the ileal mucosa in order to induce the GIT immune system [115]. Hypotheses surrounding diet and SFB have been proposed [32], though knowledge about which dietary substrates affect SFB abundance is unclear. A prime candidate is carbohydrates [64, 87]. Regardless of the impact on SFB abundance around weaning, observing the effects of a diet on the transient change in SFB abundance in infancy may give some clues on the role of SFB in immune barrier maturation.

Further investigation could lead to identifying how SFB interact with other microbes in the ileum. One group has suggested exploring the targeted use of metagenomic alteration of the gut microbiome by *In situ* Conjugation (MAGIC) [153]. They propose modifying SFB by harnessing naturally occurring horizontal gene transfer activity using an *Escherichia coli* strain as a donor to deliver engineered DNA. They reported achieving transient expression of the engineered DNA in the microbiome. It is unknown, however, whether SFB are naturally competent. Thus, it cannot be assumed that SFB would express the engineered DNA. Knowledge from this and more work on SFB may contribute to deducing the mechanisms by which these commensals uptake, utilise nutrients, and survive in the ileum. Understanding the function of SFB in the ileum may give more insight into the interaction of microbes with food substrates on the immune system.

2.8. Research questions

The following research questions arose from knowledge gaps in the literature on SFB function:

1. Does early-life nutrition (weaning food) influence the population of SFB in the terminal ileum?
2. What specific food substrate (in the weaning diet) influences the abundance of SFB? Would this, in turn, influence select GIT immune markers?
3. Can these food substrates be added to and used to improve milk formulas as well as weaning foods?
4. Will an increase in SFB abundance lead to improved infant GIT immune barrier maturation?
5. What are the mechanisms by which nutrition influence SFB?

This PhD project sought to answer questions 1 and 2 as the work was feasible within the time limitation. The overall hypothesis of this project was that *a solid weaning diet enriched with inulin with a particular food substrate would increase the abundance of SFB in the ileal tissue, contents and faeces of weanling Sprague-Dawley rats which would then enhance GIT immune barrier maturation*.

Quantification of SFB abundance from a previously established weanling mouse model was carried out to ensure the mouse strains in AgResearch contained ileal and faecal SFB. The study also set out to determine the most appropriate time-points for quantifying SFB in the weanling mouse. The ileal and faecal abundances of SFB was correlated with the concentration of immune markers; plasma IL-17 and faecal IgA.

A weanling rat model for SFB quantification was established for the first time which could provide enough samples for analysis to compare ileal tissue collection techniques; whole tissue homogenisation and mucosal scraping. This was to determine the best method to collect ileal tissue samples for SFB quantification.

In the following study, inulin was then added to enrich a solid weaning diet, and then the ileal abundance of SFB was quantified. The differences in the abundance of SFB amongst the groups fed diets enriched with the food substrate were then compared. Questions 3 to 5 are directions based on the results from the studies in this project.

CHAPTER 3: THE ABUNDANCE OF SEGMENTED FILAMENTOUS BACTERIA IN THE ILEUM AND FAECES OF WEANLING MICE

3.1. Abstract

The proper development of the physical, chemical, microbiological, and immunological barriers of the GIT is important for health and wellbeing. The maturation, particularly of the small intestine immunological barrier, is thought to involve SFB which attach to the mucosa. The abundance of SFB in ileal tissue, contents and faeces has been positively correlated with the plasma and faecal concentrations of IL-17 and IgA. Studies in mice and infants reported that the abundance of SFB increased and decreased post-weaning though it was unclear whether the change was influenced by weaning.

It was initially important to ensure the strains of mice in this study harboured SFB in the ileum as the presence of SFB may vary among strains as well as animal facilities. The faeces of Swiss (SWR/J), BALB/c and C57BL/6 mice were screened to confirm the presence of SFB and to select the mouse strain for the study. Changes in ileal and faecal SFB abundance in Swiss (SWR/J) mice were hypothesised to follow a similar pattern post-weaning as reported in infants and BALB/c mice of a similar stage. The profile of SFB colonisation was characterised, immediately pre- and post-weaning, in the ileal tissue, contents and faeces and its association with two immune markers of GIT maturation, plasma IL-17 and faecal IgA. The abundance of SFB between males and females was also compared.

Whole ileal tissue and contents were collected from 20, 21, 26, 31, 36 and 41-day old mice. The abundance of SFB was quantified in distal ileal tissue and luminal contents, and faeces by qPCR using SFB-specific primers. Concentrations of IL-17 and IgA in plasma and faeces, respectively, were determined by antibody-specific ELISAs.

Significant differences in SFB abundance in ileal luminal contents were observed; abundance peaked at day 26 and remained similar at other time-points. The SFB abundance in ileal tissue and faeces did not change across the time-points. This observation contradicts published

findings and may have been due to high variability in the data from this study caused by inadequate ileal tissue and faecal sample sizes. There were no differences observed in SFB abundance between male and female mice in the ileal tissue ($p=0.734$) and faeces ($p=0.075$). There were significant differences in the abundance of SFB in the ileal luminal contents ($p=0.004$) over time, though no differences between male and female mice. There were significant differences in plasma IL-17 concentration between days, with the concentration at 31, 36, 41 days being significantly higher than that at 20, 21 and 26 days. There were no differences in faecal IgA concentration, unlike in reports in mice and infants. There was large variability of SFB abundance in the ileal tissue and faeces and of IgA in faeces between individual mice. The variability may have been caused by the small size of the weanling mice which produced very little tissue samples for analysis. This implied that using a larger animal model to provide adequate samples for analysis might give more reliable results and increase the statistical power, thus overcoming the limitations of this study.

3.2. Introduction

The epithelium of the GIT is a semi-permeable membrane instrumental in filtering the components that pass into the circulatory system [6, 7] and thus important for health and wellbeing. Nutrients and water are absorbed and transported across the epithelium, whilst harmful substances and pathogens have limited transport across a healthy epithelium.

During the development of the human epithelial barrier of the GIT *in utero*, the columnar-shaped enterocytes appear at 8 weeks [14]. By 10 weeks, tight junctions, which hold the enterocytes together, are detected, making the barrier semi-permeable [15]. By 12 weeks, the crypt-villus architecture, where cells involved in host defence and signalling reside, is formed [14]. At 24 weeks, the intraepithelial lymphocytes are recruited to promote immune barrier function [20]. Secretion of mucin from goblet cells is detected as early as 6.5 weeks, though the patterns of expression undergo maturation throughout gestation [17]. Following birth, maturation of the GIT includes deepening of the crypts, microbial colonisation of the epithelial mucosa and recruitment of immune cells from the immunological barrier [130, 154, 155]. It is uncertain whether the microbes colonise the GIT during gestation as it is believed to be sterile at birth [156, 157].

At weaning and during GIT maturation in humans, solid foods complement the main milk diet [158]. This dietary transition provides a more diverse range of substrates to the resident microbes for growth and metabolism [82, 84] and creates new immune challenges for the maturing infant [159]. This transition creates a significant shift in the microbiota composition until it stabilises around 3 years of age to a profile resembling that of an adult [130].

The segmented filamentous bacteria are one of the members of the microbiota that were initially detected in the ileal tissue of mice and rats over two weeks old [160]. They have since been observed in other mammals [45, 46], including infants and older adults [24]. The origin

of SFB, however, is yet to be determined. Its life cycle [25] suggests that offspring may be seeded with vegetative segments during the birthing process. Mechanistic studies have shown that SFB attach to the terminal ileal mucosa overlying the Peyer's patches [22, 160], where naïve T cells undergo antigen-driven activation and expansion into T helper cells [75]. Further investigation showed that the abundance of SFB in faecal samples had been correlated positively with the presence of faecal IgA [22] and plasma IL-17 [39-41, 49, 60]. These associations suggest that these microbes may be involved in the maturation process of the immune barrier at infancy [39, 60, 76].

Ohashi, et al. [22] reported a temporal change in SFB abundance in the ileal tissue of mice, pre- and post-weaning. They observed SFB abundance changes from pre-weaning postnatal day (PND) 18 through to PND 105 [22], with the greatest changes observed mainly at weaning (PND 21), peaking at PND 28 and the decreasing in abundance towards PND 49 until PND 105. It remained unclear whether the transition to a more diverse diet at weaning caused or was related to the changes in SFB abundance.

In vitro studies have proven challenging as SFB prefer an environment with low to no oxygen and yet need to attach to epithelial cells that require oxygen. Additionally, ethical challenges associated with obtaining ileal samples from infants to decipher the role of SFB have resulted in requiring alternative models. Therefore, animals remain the primary experimental model to study SFB and its role in the immune barrier in infants during this dietary transition. Most SFB studies have been carried out using mice [22, 24, 25, 87, 102, 117].

Hypothesis and aims

The hypothesis of the research described in this thesis Chapter was that changes in SFB abundance before and immediately after weaning in ileal tissue, contents and faeces in weanling mice would show a temporal profile similar to what has been reported in other mouse studies and infants of a similar stage.

The most common mouse strains used in SFB studies are C57BL/6 mice, Swiss mice, and BALB/c mice [29, 34, 41, 43, 48, 115, 117, 161, 162]. The colonisation pattern of SFB possibly varies between mice breeds and facilities. Therefore, the first aim was to determine whether SFB were present in the Swiss (SWR/J), BALB/c and C57BL/6 mouse strains available at the AgResearch Small Animal Colony in Ruakura Research Centre. Faecal SFB DNA was amplified by polymerase chain reaction (PCR) using SFB-specific primers and visualised via gel electrophoresis.

The second aim was to determine when SFB were most abundant and the most appropriate time-points for quantifying SFB in weanling mice. The abundance of SFB was quantified from the ileal tissue and ileal luminal contents and faeces of SWR/J mice at days 20, 21, 26, 31, 36 and 41 post-birth. DNA from the samples was extracted, and SFB abundance determined qPCR using SFB-specific primers. Immune markers (plasma IL-17, faecal IgA) were also analysed [22, 39]. The concentrations of IL-17 in blood plasma and IgA in the ileal luminal contents were analysed by enzyme-linked immunosorbent assay (ELISA). The anticipated outcome would contribute to future experiments investigating the reported influence of SFB on the maturation of the infant immunological small intestinal barrier.

3.3.Materials and methods

3.3.1. Faecal screening of mouse strains for SFB[1]

The experimental procedures were carried out with the approval of the AgResearch Limited Grasslands Animal Ethics committee (Animal Ethics Application No: 14041) following the recommendations of the New Zealand Animal Welfare Act 1999.

Therefore, to ensure the mice used in the study harboured SFB, faecal screening was performed on SWR/J, BALB/c and C57BL/6 mouse strains regularly bred at the AgResearch Small Animal Colony in Ruakura (Hamilton, NZ).

For faecal screening, DNA was extracted from a minimum of 200 mg of faeces using the NucleoSpin® Soil extraction kit (Macherey-Nagel) according to the manufacturer's instructions. The samples were vigorously agitated to release the intracellular contents, spores, and SFB DNA. The concentration and purity of DNA from ileal tissue, ileal luminal contents and faeces were measured using a Nanodrop® ND-1000 Spectrophotometer (Analytical Technologies, Thermo Fisher Scientific, Massachusetts, USA). An absorbance 260/280 ratio of 1.8 was considered acceptable for pure DNA. The samples met the required DNA quality criteria for PCR.

An optimised protocol based on the procedure used in the Franklin Laboratory in St. Louis, Missouri, USA, was utilised for the PCR analysis. DNA samples from faeces were then quantified. The SFB synthetic product was used as a positive control, and nuclease-free water was used as a negative control. The SFB primers, confirmed by 16S sequencing, used were forward primer SFB 779F 5'- TGT GGG TTG TGA ATA ACA AT -3', reverse primer SFB

[1] The faecal screening, mouse experimental design and sample collection were carried out by Dr Eva Maier, Kelly Dunstan, Charlotte Hurst, Jason Peters (AgResearch Grasslands) and Ric Broadhurst, Robert Smith, Dr Matthew Barnett (AgResearch Ruakura) prior to the PhD student's start date in 2017.

1008R 5'- GCG AGC TTC CCT CAT TAC AAG G -3' (Integrated DNA Technologies, Singapore). The PCR parameters in the Biometra TAdvanced thermal cycler (Labgene Scientific SA, Switzerland) included a 1 cycle of the Lysis Stage at 95°C for 3 minutes, 40 cycles of the PCR Stage; denaturation at 95°C for 15 seconds; annealing at 64°C for 30 seconds; extension at 72°C for 30 seconds, and 1 cycle of the Hold Stage at 16°C. The PCR products were then visualised by performing gel electrophoresis. The PCR product samples were run on a 0.8% agarose gel which was run at 80V for 75 minutes. The SFB bands on the gels were then visualised under ultraviolet light.

3.3.2. Mouse experimental design

Conventionally raised SWR/J mouse pups (male and female) were used in this study. Fifty pups were randomised into six time-points: 20, 21, 26, 31, 36 and 41 days after birth. The first five time-points had eight pups each, and the last time-point had ten pups. The pups included 24 females and 26 males which were distributed across the six time-points: females (PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=2, PND41 n=6) and males (PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=6, PND41 n=4)). A power analysis (80% power) of the experimental design determined between 8-12 pups would be randomised into six time-points each. Power calculations were carried out based on a simple 2-group comparison with data from the study of Ohashi, et al. [22]. From that study, a standard deviation of 300 units/g of tissue and a minimum difference of interest of 600 units/g of tissue were calculated to achieve the desired level of significance of 0.05.

The mice were mated at AgResearch Ruakura and housed in a room where the temperature was maintained at 21°C. They were fed a regular rodent diet (Meat Free Rat and Mouse Diet, Specialty Feeds, Australia) and had access to water *ad libitum* until the dams gave birth.

Samples were collected from pre-weaned pups at 20 days of age. A second sampling time-point at 21 days was completed at Ruakura to avoid the stress associated with weaning and transportation to a different site. The remaining mouse pups were weaned at 21 days and transported to AgResearch Grasslands (Palmerston North, NZ) at 23 days. At Grasslands, the pups were also housed in a room maintained at 21°C. They were fed a regular rodent diet (Meat Free Rat and Mouse Diet, Specialty Feeds, Australia) and had access to water *ad libitum*.

The pups were randomised into time-point groups, labelled on their tails using non-toxic marker pens, whilst remaining in their birth litters throughout the experiment. This procedure was to avoid interaction between animals from different litters, which would influence the microbiota of the pups. All pups were checked daily, weighed once a week, and fresh food and water topped up as required. Their General Health Score (GHS), which ranges from 5 (healthy) to 1 (requires euthanisation), was checked daily. Any mice with a GHS of 3 or 4 were to be closely monitored, and if their condition did not improve, the mice would have been humanely euthanised. All mice had a GHS of 5 throughout the study.

3.3.3. Sample collection

During the sampling days, the pups were humanely euthanised by asphyxiation with carbon dioxide in an individual cage and cervical dislocation. Blood samples were taken by cardiac puncture with a needle coated with ethylenediaminetetraacetic acid (EDTA) (Invitrogen; Thermo Fisher Scientific, Massachusetts, USA). The blood samples were centrifuged at 2,000 x g for 10 minutes at 4°C, and the supernatant (blood plasma) was pipetted into cryotubes and snap-frozen in liquid nitrogen and stored at -80°C for later analysis. Faecal samples were collected directly from the rectum post-mortem and snap-frozen in liquid nitrogen. The distal ileal tissue and luminal contents were collected by cutting before the start of the caecum and

were collected into cryotubes and snap-frozen in liquid nitrogen. All samples were stored at -80°C for later genomic DNA extraction.

3.3.4. Sample preparation and genomic DNA extraction[2]

Genomic DNA was extracted from the ileal tissue using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Shanghai, China) according to the manufacturer's instructions. Before DNA extraction, the tissue was weighed and then disrupted using a tissue homogeniser (Omni International TH, Georgia, USA) in the extraction buffer. The suggested minimum weight for tissue to be used for DNA extraction is 20 mg [163]. However, the weights of the tissue collected varied between 4-15 mg. The homogeniser probe was cleaned with two different solutions of 70% ethanol, 100% ethanol and distilled water (milliQ H_2O) between each sample to prevent cross-contamination.

DNA was extracted from ileal luminal contents and faeces using the NucleoSpin® Soil extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The suggested minimum weight for faeces and ileal luminal contents is 200 mg though the weight of the faecal samples collected varied (24-172 mg). The weight of the ileal luminal contents was much lower with a smaller variation (4-5 mg). These samples were vigorously agitated to release the intracellular contents, spores, and SFB DNA.

The concentration and purity of DNA from ileal tissue, ileal luminal contents and faeces were measured using a Nanodrop® ND-1000 Spectrophotometer (Analytical Technologies, Thermo Fisher Scientific, Massachusetts, USA). An absorbance 260/280 ratio of 1.8 was considered

[2] Experimental work was continued by the PhD student.

acceptable for pure DNA. All samples met the required DNA quality criteria for quantification by qPCR.

3.3.5. Quantitative polymerase chain reaction

An optimised protocol based on the procedure used in the Franklin Laboratory in Columbia, Missouri, USA, was utilised for the qPCR analysis. A standard curve was made using an SFB synthetic product (Integrated DNA technologies, Singapore). DNA samples from ileal tissue, ileal luminal contents, and faeces were then quantified. The SFB synthetic product was used as a positive control, and nuclease-free water was used as a negative control. The SFB primers, confirmed by 16S sequencing, used were forward primer SFB 779F 5'- TGT GGG TTG TGA ATA ACA AT -3', reverse primer SFB 1008R 5'- GCG AGC TTC CCT CAT TAC AAG G -3' (Integrated DNA technologies, Singapore). The conditions in the Quantstudio 3D Digital PCR thermal cycler (Applied Biosystems™; Thermo Fisher Scientific, Massachusetts, USA) included a Hold Stage at 95°C in 3 minutes, the PCR Stage; denaturation at 95°C for 3 minutes; annealing at 64°C for 30 seconds; extension at 72°C for 30 seconds. A melting-curve analysis using SYBR green was performed to determine the specificity of the PCR by slowly heating the mixtures from 55–95°C for 1 second, 60°C for 30 seconds and 95°C for 1 second.

3.3.6. Enzyme-linked immunosorbent assay of immune biomarkers

The concentrations of IL-17 in plasma and IgA in faeces were analysed using an antibody-specific Mouse IL-17 ELISA Kit® and Mouse IgA ELISA Kit® (Cusabio Biotech Co. Ltd, Wuhan, China) according to the manufacturer's instructions. A FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, California, USA) was used to determine the optical

density (OD). The OD was measured at 450 nm, which were subtracted from 540 nm to determine the final OD readings. Standard curves, used to change the raw data OD readings to concentrations, were created for IL-17 and IgA using CurveExpert Professional (v 2.7.0) by plotting the mean absorbance for each standard against the concentration. Linear and polynomial regressions were generated by plotting the mean absorbance for each standard against the concentration of plasma IL-17 and faecal IgA, respectively. The limit of detection for plasma IL-17 and faecal IgA was 0.02 ng/mL and 0.82 ng/mL, respectively.

3.3.7. Statistical analysis

A one-way analysis of variance (ANOVA) was performed in Minitab® 18 Statistical Software (State College, Pennsylvania) to determine if there were any significant differences in the abundance of SFB and concentration of IL-17 and IgA between 18-41 days pre- and post-weaning. The Shapiro-Wilk test was used to verify that the data were normally distributed, and that homogeneity of variance was met. The data were log-transformed to meet the requirements of normal distribution and homogeneity of variance. The transformed data were normally distributed. A two-way ANOVA was also performed to determine whether the interaction between sex and age influenced SFB abundance over time. Where there was statistical significance, a post-hoc Tukey's test was performed to show where the differences lay. Differences were considered statistically significant when the probability value was inferior to 0.05 ($p<0.05$).

3.4. Results

3.4.1. Faecal screening of mouse strains for SFB

Gel electrophoresis of faecal DNA-PCR products from SWR/J, BALB/c and C57BL/6 mouse strains indicated that all three strains harboured SFB. However, in the SWR/J mice, SFB appeared to be more abundant (Figure 3.1). The SWR/J mouse strain was also readily available from the breeding colony and thus was chosen for the main study.

3.4.2. Quantification of SFB

The qPCR analysis was performed in ileal tissue, ileal luminal contents, and faeces to determine the abundance of SFB in pre- and post-weaning mice. No significant differences in SFB abundance in the ileal tissue ($p=0.734$) were observed over time (Figure 3.2). There was more variability in SFB abundance in the ileal tissue ($p=0.734$) and faeces ($p=0.075$) compared to SFB abundance in the ileal luminal contents ($p=0.004$). Significant differences in the abundance of SFB in the ileal luminal contents ($p=0.004$) were also noted over time (Figure 3.3). SFB abundance increased from weaning, peaked at 26 days after birth and then decreased and plateaued. A post-hoc Tukey's test indicated that SFB abundance was higher at 26 days post-birth compared to 20, 21 and 41 days. There were no significant differences ($p=0.075$) in SFB abundance in the faeces over time (Figure 3.4).

Differences in SFB abundance in the ileal tissue, ileal contents and faeces were also compared between sexes. A two-way ANOVA showed no significant differences in SFB abundance between the pre- and post-weaning male and female pups (Figures 3.5, 3.6, 3.7).

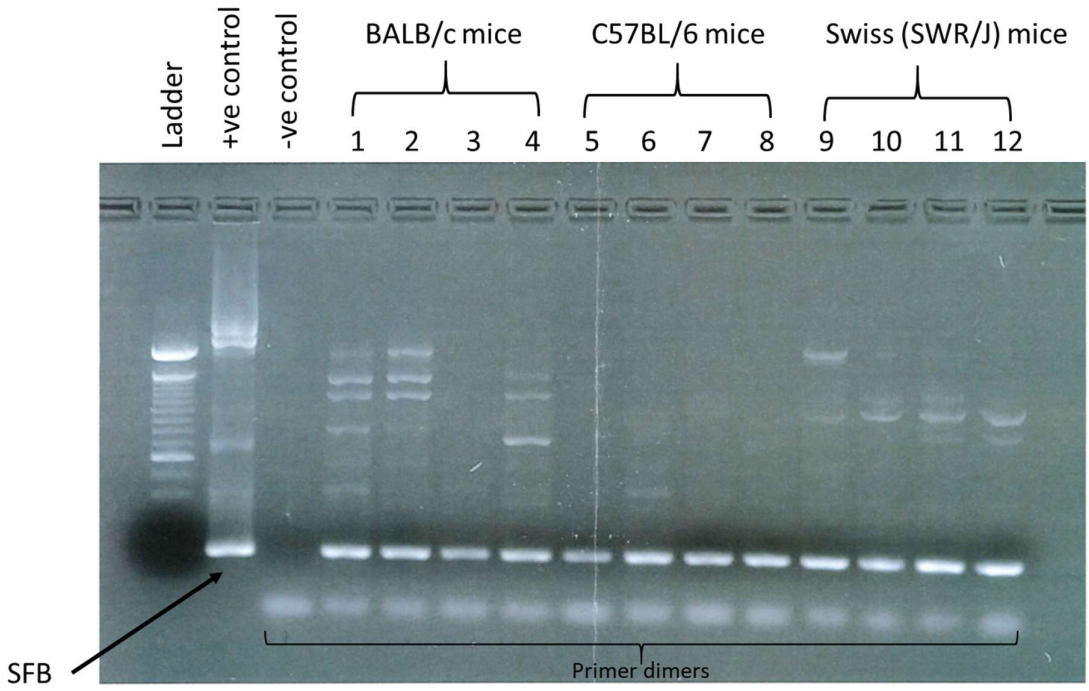

Figure 3.1. *Gel electrophoresis of PCR products indicating the presence of SFB in the faeces of BALB/c, C57BL/6 and Swiss (SWR/J) mice (n=3 each). A synthetic SFB product was used as the positive control and nuclease-free water as the negative control. SFB was present in all mouse strains, with the bands being brighter in the Swiss mice.*

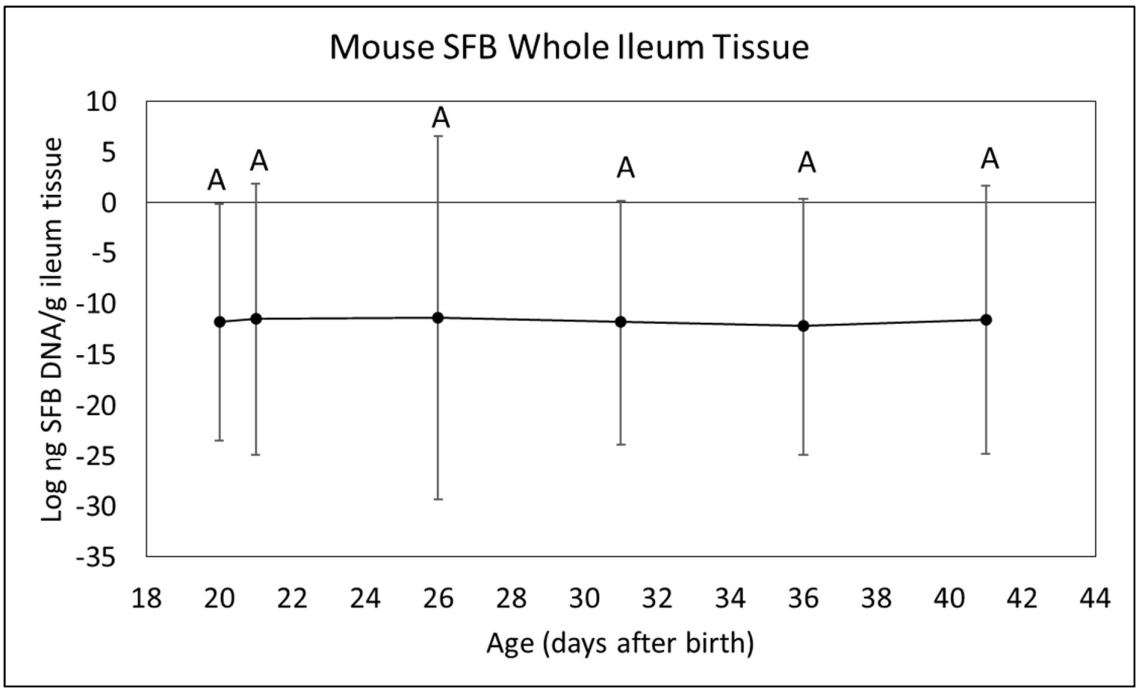

Figure 3.2. *The abundance of SFB in the whole distal ileal tissue of conventionally reared Swiss (SWR/J) male and female mouse pups. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=8 pups (18-36 days) and n=10 pups (41 days). The bars represent the standard error mean (SEM). There were no statistical differences in SFB abundance among the time-points (p=0.734). Values without common letters differ significantly (p<0.05).*

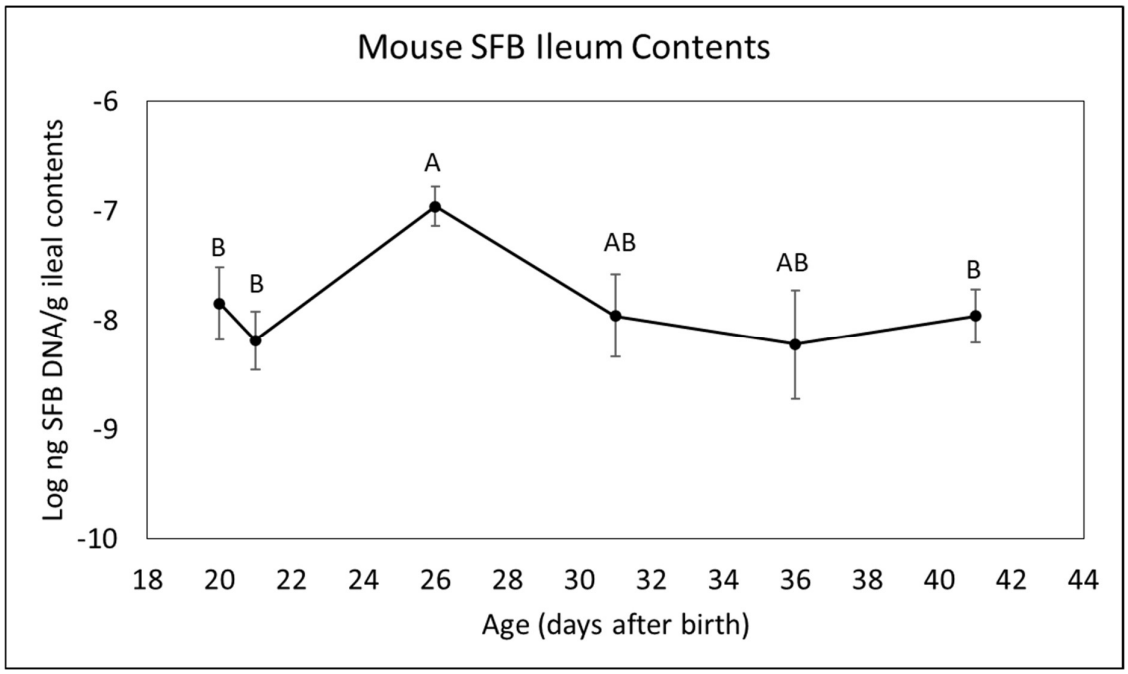

Figure 3.3. *The abundance of SFB from distal ileal luminal contents of conventionally reared Swiss (SWR/J) male and female mouse pups. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=8 pups (18-36 days) and n=10 pups (41 days). The bars represent the standard error mean (SEM). There were significant differences among the time-points, and a post-hoc Tukey's test indicated that SFB abundance was higher at 26 days compared to 20, 21 and 41 days (p=0.004). Values without common letters differ significantly.*

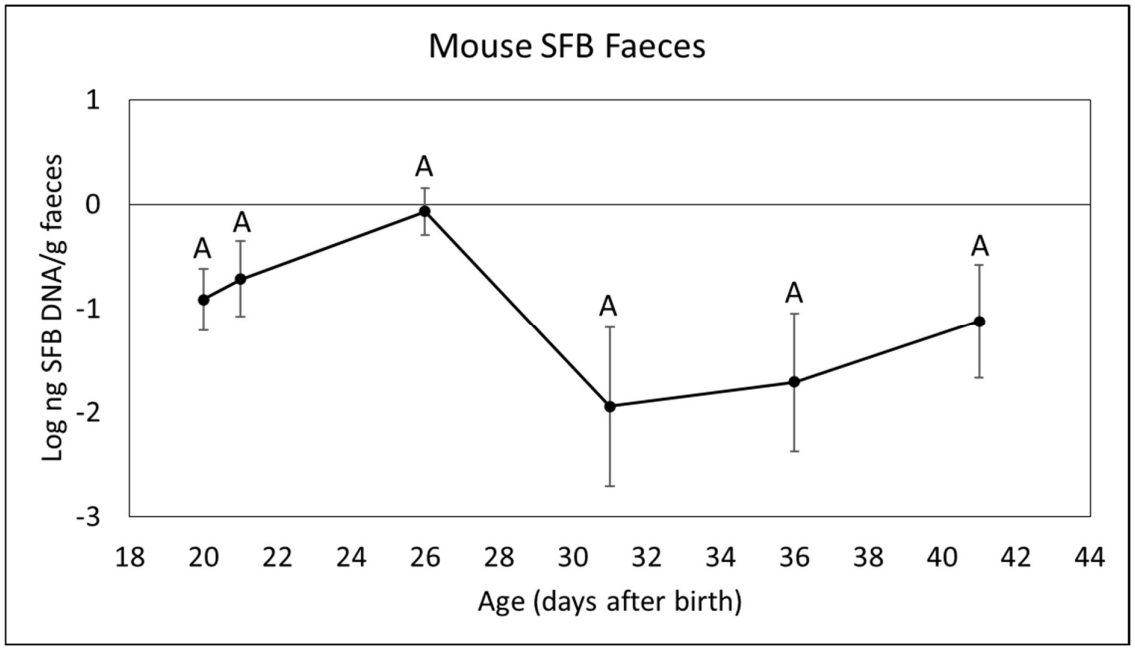

Figure 3.4. *The abundance of SFB from faeces of conventionally reared Swiss (SWR/J) male and female mouse pups. Samples were collected directly from the terminal colon and rectum. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=8 pups (18-36 days) and n=10 pups (41 days). The bars represent the standard error mean (SEM). There were no differences among the time-points (p=0.075). Values without common letters differ significantly.*

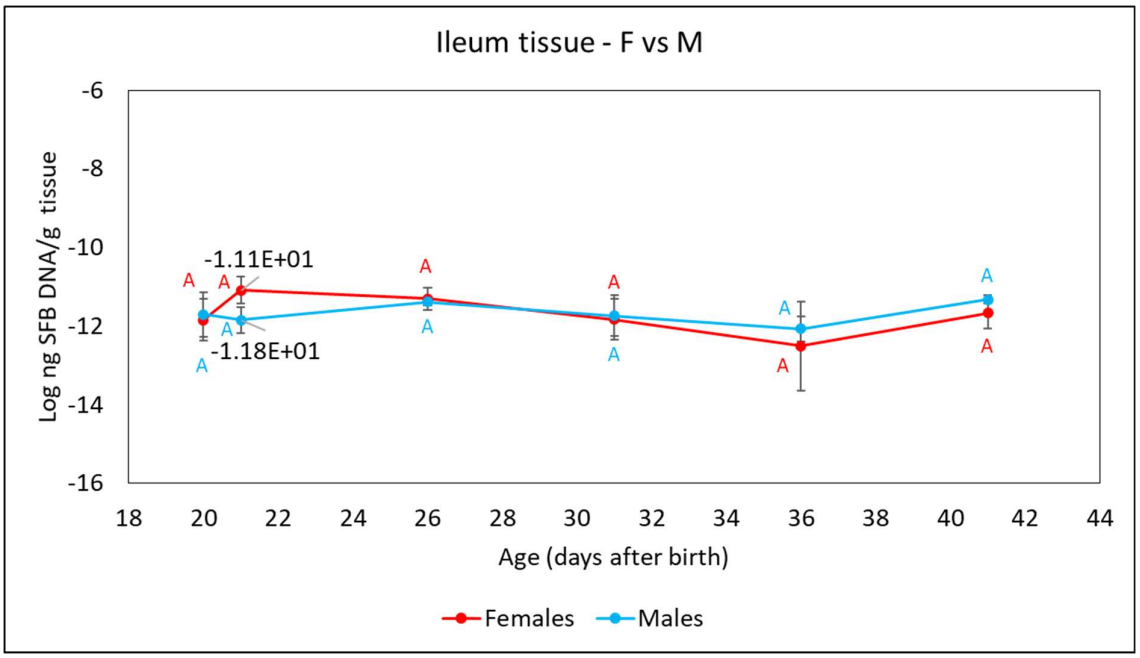

Figure 3.5. *Comparison of the abundance of SFB in the ileal tissue between female (F) and male (M) Swiss (SWR/J) mouse pups. The rat pups were weaned on day 21. Data are plotted on a logarithm ten scale and are shown as the mean values of females (n=24; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=2, PND41 n=6) versus males (n=26; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=6, PND41 n=4). The bars represent the standard error mean (SEM). There were no significant differences between sex and time-points (time-points p=0.397, sex p=0.905, time-points vs sex p=0.791).*

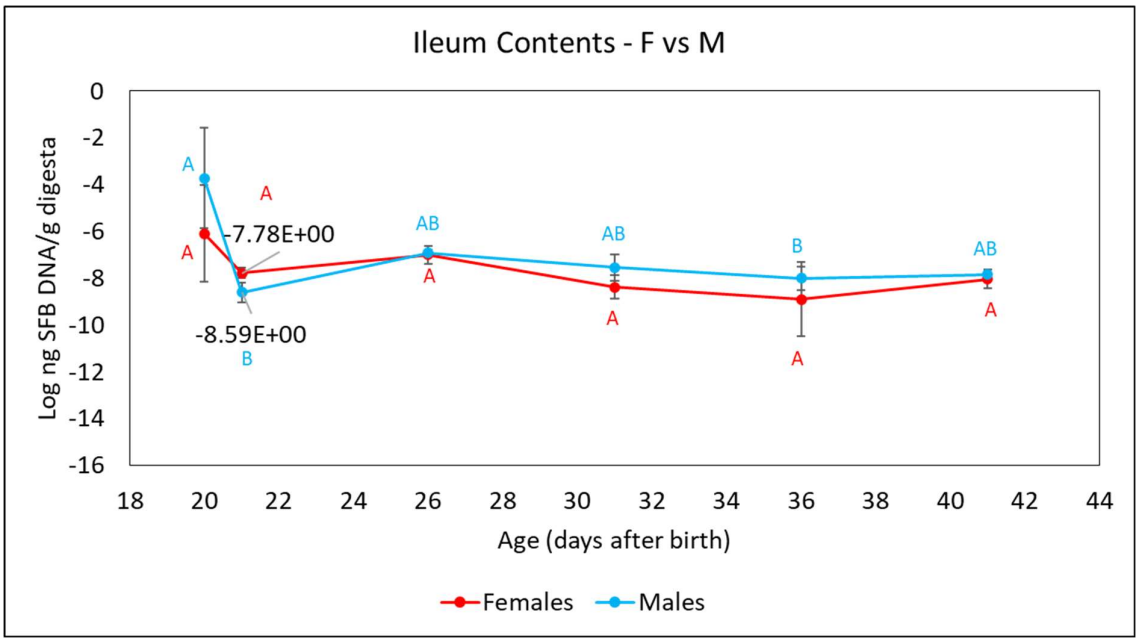

Figure 3.6. *Comparison of the abundance of SFB in the ileal luminal contents between female (F) and male (M) Swiss (SWR/J) mouse pups. The rat pups were weaned on day 21. Data are plotted on a logarithm ten scale and are shown as the mean values of females (n=24; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=2, PND41 n=6) versus males (n=26; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=6, PND41 n=4). The bars represent the standard error mean (SEM). There were no significant differences between sex and time-points (time-points p=0.007, sex p=0.284, time-points vs sex p=0.669).*

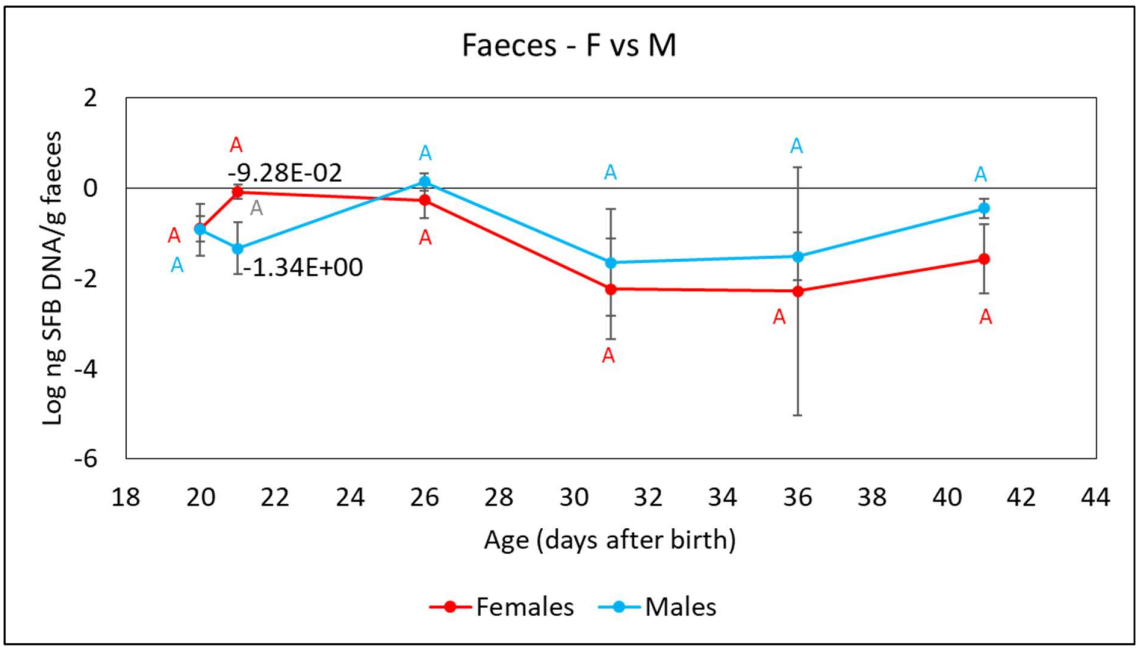

Figure 3.7. *Comparison of the abundance of SFB in the faeces between female (F) and male (M) Swiss (SWR/J) mouse pups. The rat pups were weaned on day 21. Data are plotted on a logarithm ten scale and are shown as the mean values of females (n=24; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=2, PND41 n=6) versus males (n=26; PND20 n=4, PND21 n=4, PND26 n=4, PND31 n=4, PND36 n=6, PND41 n=4). The bars represent the standard error mean (SEM). There were no significant differences between sex and time-points (time p=0.149, sex p=0.542, time vs sex p=0.673)*

3.4.3. Quantification of plasma IL-17 and faecal IgA concentrations

Antibody-specific ELISAs were performed to determine the concentration of IL-17 in the plasma and IgA in the faeces. The data were plotted on a logarithm 10 scale and shown as the mean values of pups in each time-point. A one-way ANOVA showed significant differences in the concentration of plasma IL-17 concentration over time ($p<0.05$) (Figure 3.8), and a post-hoc Tukey's test indicated that the concentration at 31, 36 and 41 days after birth was significantly higher than at 20 days. There were also differences between 21, 26 and 31 days, with the concentration at 31 days after birth being significantly higher. The concentrations of IgA in the faeces remained similar over time ($p=0.231$) (Figure 3.9).

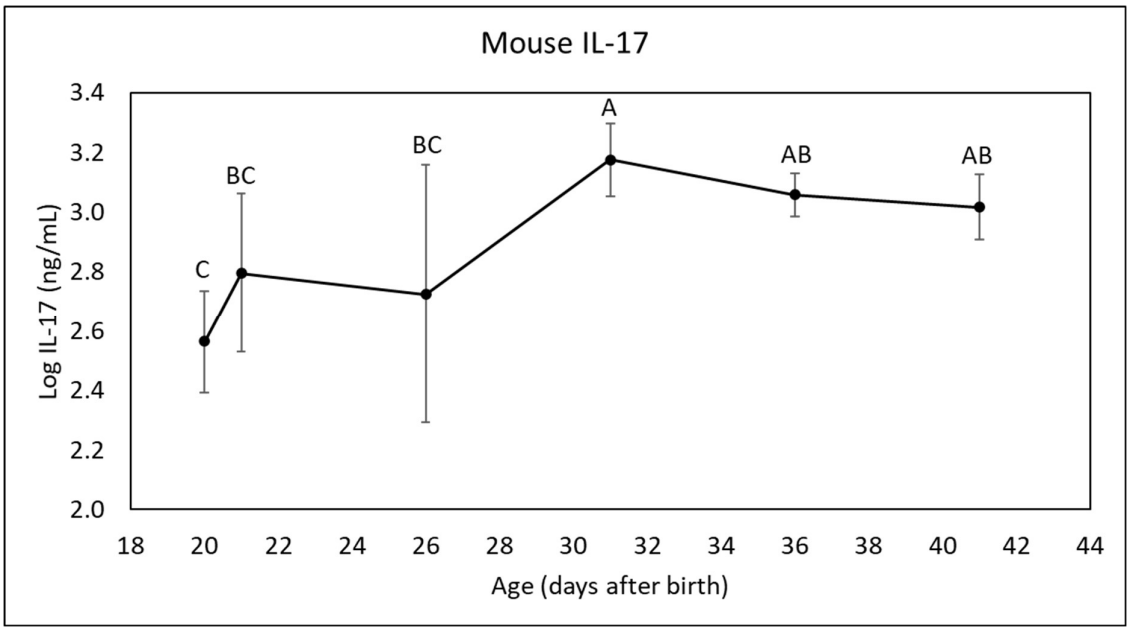

Figure 3.8. *The concentration of IL-17 in the blood plasma of conventionally reared Swiss (SWR/J) male and female mouse pups. Samples were collected by cardiac puncture. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=8 pups (18-36 days) and n=10 pups (41 days). The bars represent the standard deviation (SD). There were significant differences among the time-points, and a post-hoc Tukey's test showed that IL-17 concentration at 31, 36 and 41 days was significantly higher than at 20 days ($p<0.05$). Values without common letters differ significantly.*

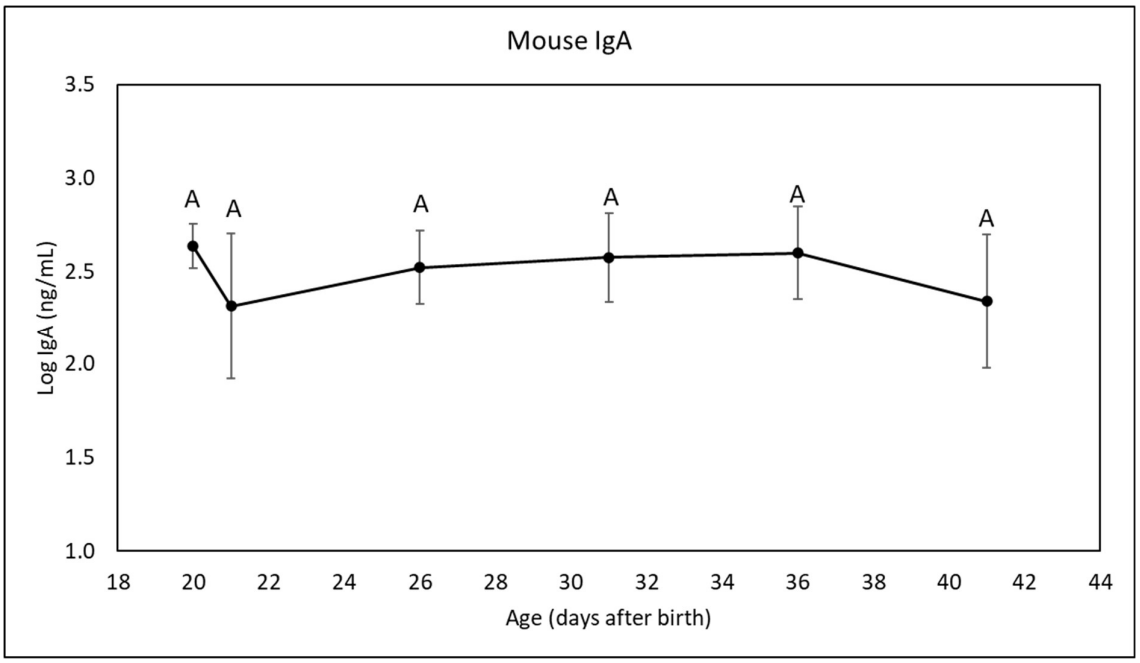

Figure 3.9. *The concentration of IgA in the faeces of conventionally reared Swiss (SWR/J) male and female mouse pups. Samples were collected directly from the rectum. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=8 pups (18-36 days) and n=10 pups (41 days). The bars represent the standard deviation (SD). There were no statistical differences in IgA concentration among the time-points (p=0.231). Values without common letters differ significantly.*

3.5. Discussion

The findings from this study partially supported the hypothesis that changes in SFB abundance before and immediately after weaning in ileal tissue, contents and faeces in weanling mice would show a temporal profile similar to what has been reported. The results showed changes in the abundance of SFB in ileal contents but not in ileal tissue and faeces. The mouse strains housed at the AgResearch Small Animal Colony in Ruakura were initially screened to ensure they harboured SFB. Results from PCR and gel electrophoresis confirmed the presence of SFB in the faeces of all mouse strains. This result suggested that the abundance of SFB in the SWR/J mice was higher than in the BALB/c and C57BL/6 mice. Of the three strains, SWR/J mice were readily available and were thus selected for this study.

The abundance of SFB in the ileal luminal contents of the SWR/J mice pups increased from 21 days, peaked at 26 days, then decreased and plateaued. The results reported here showed that the temporal profile of SFB in the ileal luminal contents of mice pre- and post-weaning was similar to what has been previously reported [22, 24]. The significance observed in the temporal profile of SFB abundance in the ileal contents may have been due to the presence of vegetative or dormant SFB segments [99]. It is plausible that the SFB segments may have detached from the ileal mucosa or transferred from other sections of the small intestine to the ileum.

The recommended weight for ileal luminal contents for genomic DNA extraction is 200 mg. However, the available samples were only between 4-6 mg, and this limited sample size may have contributed to the variation in reported SFB abundance over time. Similarly, the variable weights of the samples might explain the lack of differences in SFB abundance over time in the faeces. An average of 200 mg is recommended for genomic DNA extraction, though in this study, the weights of faecal samples collected varied between 24-172 mg and may have

influenced SFB abundance. The lack of significance in the distal ileal tissue, compared to what has been previously reported [22], may have also been due to the small and variable amounts of tissue available from the pre-weaned 20-day-old mice. The suggested minimum weight for tissue to be used for DNA extraction is 20 mg. The weights of distal ileal tissue available were between 4-15 mg. However, the concentration of SFB detected in the ileal tissue was 0.67-fold lower than the ileal luminal contents. The differences in the weights of biological materials (ileal tissue and faeces) possibly increased variability in the data, thereby decreasing the accuracy of the overall results.

The techniques used in sample processing may have also contributed to variation in the data. Ohashi, et al. [22] reported that the temporal profile of SFB abundance in weanling mice to be higher in the distal section compared to the proximal and medial sections of the ileal tissue. In comparison, the results here showed no differences in SFB abundance in the distal ileal tissue. These results call into question the methodology used for tissue sample collection. Whole tissue homogenisation used in this study was similar to that used by Godon, et al. [164] and Ohashi, et al. [22] to collect and prepare the ileal tissue for SFB quantification. Another technique, ileal mucosal scraping, has also been used to collect tissue samples containing attached SFB [43]. It is unclear which of the two techniques is better for ileal tissue collection to quantify the abundance of SFB, which requires further investigation.

Another factor reported to influence microbial abundance is sex differences. Studies have reported that the abundance of microbial groups varies in different strains of male and female pubescent and adult mice [165, 166]. This observation suggests an interaction between genotype and sex. Compared to pubescent and adult mice, the levels of sex hormones in weanling mice are much lower, and the result may be a smaller influence on the GIT microbiota. This indicates that other differences between sexes in early life might instead influence the composition of the microbiota in the GIT. The effects may be caused by sexual

dimorphism, whereby differences in characteristics beyond sexual organs are exhibited. These include differences between the immune system, age, diet, body mass index, species, and strain [165-170]. SFB abundance may be influenced by these factors and reflects the importance of considering their influence (including sex) on microbiota in studies with animals at the pre-puberty stage. In this study however, there were no differences in SFB abundance in the ileal tissue, contents and faeces between sexes, pre- and post-weaning.

There were differences in the plasma concentration of IL-17, increasing from day 20 to 21, 26 and 31, then plateauing off at day 36 until 41. This pattern is similar to the temporal profile of SFB abundance observed in the ileal luminal contents in this study and agrees with reports of the positive correlation between IL-17 concentration in plasma and SFB presence in the ileum [39-41]. In this study, the concentration of faecal IgA remained similar over time which is contrary to what Ohashi, et al. [22] observed. They reported that faecal IgA concentrations were low immediately after being weaned at 20 days and then increased late post-weaning at 28-105 days [22, 37]. The lack of differences could be attributed to an immature immune system in the mouse pups as the time frame of the study was shorter and the mice were younger compared to the results from the previous study. Alternatively, sufficient sample quantity is needed to measure IgA concentration accurately.

This study provides inconsistent findings compared to published studies. Technical limitations must be addressed to assess the temporal profile of SFB abundance more accurately over closer time-points pre- and post-weaning in males and females. The limitations of this study could be overcome by using an animal model that would provide enough sample for the accurate quantification of SFB in the ileum (contents and tissues) and faeces at the pre- and post-weaning age. It would also be important to choose a better sample type and method for collecting and preparing ileal tissue for SFB quantification as these steps influence the variability of results.

In conclusion, the results showed that the temporal profile of SFB colonisation in the ileal luminal contents (but not in ileal tissue and faeces) of weanling mice was similar to what has been published for mice and human infants of a similar developmental stage. However, the variable temporal profiles of the abundance of SFB in the ileal tissue and faeces and IgA in faeces suggest further improvement of the experimental protocols (higher sample quantity, optimal extraction) to overcome the limitations in the current study.

CHAPTER 4: WHOLE TISSUE HOMOGENISATION PREFERABLE TO MUCOSAL SCRAPING IN DETERMINING THE TEMPORAL PROFILE OF SEGMENTED FILAMENTOUS BACTERIA IN THE ILEUM OF WEANLING RATS

This Chapter was published as an original research article in the journal *Access Microbiology*: Oemcke, L. A., Anderson, R. C., Rakonjac, J., McNabb, W. C., & Roy, N. C. Whole tissue homogenization preferable to mucosal scraping in determining the temporal profile of segmented filamentous bacteria in the ileum of weanling rats. *Access Microbiology*, p. 000218 (2021).

The content of this Chapter has been modified to meet the requirements for the thesis.

4.1. Abstract

The study of SFB in weanling mice in Chapter 3 has shown a positive association between ileal SFB abundance and plasma IL-17, but not with faecal IgA. The results suggested that the size of the mouse ileum was a limitation for analytical accuracy especially at the weaning stage. The weanling mice produced very small tissue sample sizes that may have contributed to large variations in the resulting SFB abundance data. A rat model was thus proposed as a suitable alternative as the first observation of SFB presence was reported in rats, though most studies use mice. Additionally, weanling rats would provide sufficiently large samples for multiple biological analyses.

Changes in SFB abundance over time in rats were hypothesised to follow a similar pattern as reported in mice and infants. The profile of SFB colonisation was characterised in the ileal tissue, its contents, and its association with two immune markers of GIT maturation, IL-17, and IgA. Two published techniques for collecting ileal tissue were also compared to determine which would yield data on SFB abundance with the least variability.

Whole ileal tissue and ileal mucosal scrapings were collected from 20 to 32-day old Sprague-Dawley rats. SFB abundance was quantified from proximal, middle, and distal ileal tissues and contents and faeces by qPCR using SFB-specific primers. Concentrations of plasma IL-17 and faecal IgA were determined by antibody-specific ELISAs.

Significant differences in SFB abundance were observed from the whole and scraped tissues peaking at day 22. The variability in whole ileal data was less, favouring it as a better collection technique. A similar pattern of SFB abundance was observed in ileal contents and faeces peaking on day 24, suggesting faeces can be used as a proxy for ileal SFB abundance. SFB abundance at day 26 was higher in females compared to males across all samples. There were

significant differences in faecal IgA concentration only between days 20, 30 and 32 and none in plasma IL-17 concentration, which was not similar to reports in mice and infants.

4.2. Introduction

SFB were initially observed by microscope attached to the ileal mucosa of rats [160], though most published studies on SFB have been performed in mice [22, 32, 41, 43, 60, 76, 87, 103]. Mice have been the most frequently used animal model to study SFB pre- and post-weaning, though this is not without challenges; for example, obtaining enough ileal tissue and content samples for accurate SFB quantification and conducting multiple analyses to characterise its effects on the host.

Two methods for obtaining ileal samples for SFB quantification have been described in the published literature [43, 164]. Ericsson, et al. [43] attempted to create pure inocula of SFB by developing a cost-effective method of isolating SFB from complex microbes in the ileum. Using an aseptic technique, they rinsed the distal ileum of 2-3-day-old SFB-positive BALB/c mice. The ileal tissue was cut longitudinally, exposing the mucosa, which was then scraped with a sterile scalpel blade. The scrapes contained epithelial cells, and mucosa-associated bacteria (including SFB) were then transferred into sterile media. Ohashi, et al. [22] used the grinding method [164] to collect ileal tissue along with mucosa-associated bacteria. This method involves grinding whole tissue with a pestle and mortar on ice then transferring the ground up tissue into a buffer for DNA extraction.

Hypothesis and aims

The hypothesis of the research described in this thesis Chapter was that changes in SFB abundance in ileal tissue and contents over time in rats would have a similar pattern to what has been reported in mice and infants.

The first aim was to determine the temporal profile of SFB abundance in pre- and post-weaning rats. A weanling rat model was used to evaluate the profile of SFB colonisation and some markers of immune maturation of the ileum pre- and post-weaning. Samples from pre- and post-weaning mice are very small, which results in increased variability in the data, as reported in Chapter 3. The larger strain of rat, Sprague-Dawley, was selected as SFB were initially observed microscopically attached to the ileal mucosa [160].

The second aim was to determine which technique between whole tissue homogenisation and mucosal scraping would yield optimum results and with less variability. Two techniques used in published studies for collecting ileal tissue samples for SFB quantification, ileal mucosal scraping [43] and whole tissue grinding or homogenisation [164], were compared to determine whether one technique would yield better results with reduced variability.

Following the use of these two techniques, the abundance of SFB was quantified by quantitative polymerase chain reaction (qPCR). Immune markers suggested to correlate with the presence of SFB in the ileum were also analysed [22, 39]. IL-17 from blood plasma and IgA from the ileal contents were analysed by ELISAs.

4.3.Materials and methods

4.3.1. Rat experiment

The study was approved by the AgResearch Limited Grasslands Animal Ethics Committee (Animal Ethics Application No: 14485) under the recommendations of the New Zealand Animal Welfare Act 1999.

Conventionally raised Sprague-Dawley rat pups (male and female) were used. Eight pregnant Sprague-Dawley dams were obtained from AgResearch Ruakura (Hamilton, NZ) and transported to AgResearch Grasslands (Palmerston North, NZ) by air at 15 days of gestation. They were maintained at 21°C and provided with a commercial rodent diet (Meat Free Rat and Mouse Diet, Specialty Feeds, Australia) and water *ad libitum*. The dams were individually caged under dark and light cycles. Five litters were birthed on the first day, two litters on the second day and one litter on the third day. All dams and pups were maintained at 21°C. The pups were weaned on day 21, and the dams were removed from the birth cages.

A power analysis (80% power) of the experimental design determined between 8-12 pups would be randomised into seven time-points each (total of between 56-84 pups). Power calculations were carried out based on a simple 2-group comparison with data from the study of Ohashi, et al. [22]. From that study, a standard deviation of 852 units/g of tissue and a minimum difference of interest of 1300 units/g of tissue were calculated to achieve the desired level of significance of 0.05. That number of pups was necessary to compare the two tissue collection techniques. Ninety-two pups (57 females and 35 males) were evenly distributed across time-points. The pups were randomised into seven time-points: 20, 22, 24, 26, 28, 30 and 32 days after birth (n=13 for day 20, n=14 for others). They were identified by ear-punching at day 19 to meet the welfare norms for this procedure. The pups remained in their birth litters throughout the experiment to avoid interaction among animals from different litters,

which would influence the GIT microbiota of the pups. All dams and pups were checked daily, weighed once a week, and fresh food and water topped up as required. Their General Health Score (GHS), which ranges from 5 (healthy) to 1 (requires euthanisation), was checked daily, and the rats with a GHS of 3 or 4 were closely monitored. If their condition deteriorated, they were euthanised by intraperitoneal injection with pentobarbital. All rats had a GHS of 5 throughout the study.

4.3.2. Sample collection

At each sampling time point, the pups were euthanised by asphyxiation with carbon dioxide in an individual cage followed by cervical dislocation. Blood was drawn by cardiac puncture into a needle coated with ethylenediaminetetraacetic acid (EDTA) (Invitrogen; Thermo Fisher Scientific, Massachusetts, USA). The blood samples were centrifuged at 2,000 x g for 10 minutes at 4°C, and the supernatant (plasma) was pipetted into cryotubes and snap-frozen in liquid nitrogen before storage at -80°C for later analysis. Faecal samples were collected from the terminal colon or rectum post-mortem and snap-frozen in liquid nitrogen and stored at -80°C for DNA extraction. The distal portion of the ileum adjacent to the caecum was cut into three sections of 3 cm each. The ileal contents from each section were collected separately. The ileal tissue sections were then cut open longitudinally and down the middle into two pieces. The top sections of the tissue were collected as whole tissue. The bottom sections were each scraped three times with a plastic tissue scraper in the same direction using a similar amount of force to decrease variability in the sample weights. The scrapings and whole tissues were collected into cryotubes and snap-frozen in liquid nitrogen and stored at -80°C for DNA extraction (Figure 4.1).

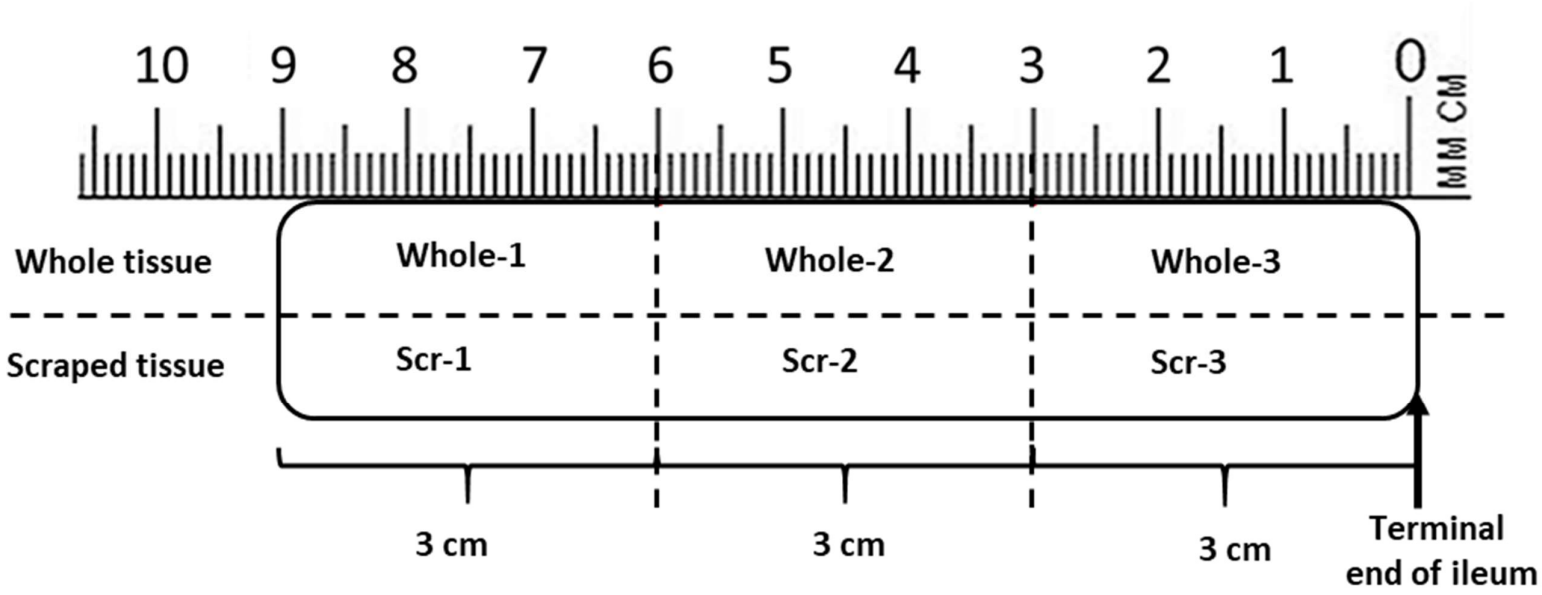

Figure 4.1. *Schematic of how the ileal tissue was cut into three sections of 3 cm each. The ileal sections were measured from the caecum, which borders the terminal end of the ileum. The sections were cut into 3 cm each, cut open and cut down the middle longitudinally. The top sections were collected as whole tissue samples for homogenisation. The bottom sections were scraped. Both whole and scraped tissue were used for genomic DNA extraction. 1-proximal section, 2-middle section, 3-terminal section.*

4.3.3. Sample processing

The methods for sample preparation and genomic DNA extraction, qPCR and ELISA were described in Chapter 3 in Sections 3.3.4, 3.3.5, 3.3.6. In the ELISA for this study, the limit of detection for plasma IL-17 and faecal IgA was 0.05 ng/mL and 0.88 ng/mL, respectively.

4.3.4. Statistical analysis

A one-way ANOVA was performed in Minitab® 18 to determine if there were any significant differences in the abundance of SFB and concentration of plasma IL-17 and faecal IgA over time between days 20-32 pre- and post-weaning. A two-way ANOVA was also performed to determine whether the interaction between sex and age influenced SFB abundance over time. The Ryan-Joiner's (like Shapiro-Wilk) test and Levene's test were used to verify that the data were normally distributed, and that homogeneity of variance was met. The data were log-transformed to meet the requirements of normal distribution and homogeneity of variance. The transformed data were normally distributed. A one-way ANOVA was performed using the logarithm-transformed data. Where there was statistical significance, a post-hoc Tukey's test was performed to show where the differences lay. Differences were considered statistically significant when the probability value was inferior to 0.05 ($p<0.05$). An analysis of covariance (ANCOVA) was performed to determine whether the sex of the rats influenced their weight gain with minimal effects from age. Weight gain was the response, sex was the explanatory factor, and age was the covariate (control variable).

4.4. Results

4.4.1. Quantification of SFB

The initial data of the abundance of SFB were skewed. Therefore, to meet conditions of normality, the data were transformed and plotted on a logarithm ten scale. The transformed data were normally distributed. All figures with untransformed data are included in Appendix I (Figure 4.7a-f, Figure 4.8a-d, Figure 4.9, Figure 4.10, Figure 4.11).

The abundance of SFB increased from weaning, peaked at day 22, and then plateaued (Figure 4.2a-f). Significant differences were observed in the temporal abundance of SFB in the proximal ($p<0.05$) (Figure 4.2a) and distal ($p<0.05$) (Figure 4.2c) sections of the whole ileal tissue from day 20, peaking at day 22 then decreasing from day 26, but not in the middle section ($p=0.086$) (Figure 4.2b). For the scraped ileal tissue, significant differences were observed in the temporal abundance of SFB in the middle ($p=0.008$) (Figure 4.2e) and distal ($p=0.003$) (Figure 4.2f) sections from day 20, peaking at day 22 then decreasing from day 26, but not in the proximal section ($p=0.066$) (Figure 4.2d). In all cases, SFB abundance peaked at day 22 and then plateaued. There was less variability in SFB abundance in the whole ileal tissue (Figure 4.2a-c, average SEM: 2.71E-01) compared to that from the ileal scrapes (Figure 4.2d-f, average SEM: 3.18E-01). There were significant differences in the abundance of SFB in the proximal ($p<0.05$) (Figure 4.3a) and middle ($p<0.05$) (Figure 4.3b) sections of ileal contents over time between postnatal days 20 and postnatal days 22, 24, 26, 28 and 32. There were no significant differences in SFB abundance in ileal contents from the distal (Figure 4.3c) section between day 22 and 32.

There were significant differences in SFB abundance over time in the faeces ($p<0.05$) (Figure 4.3d), increasing from day 20, peaking at day 24 then decreasing from day 28. The faecal SFB

abundance peaked later at day 24 compared to the ileal tissue at day 22 though there were no significant differences.

Differences in SFB abundance in all sample types measured were also compared between sexes. There was an unexpected difference in SFB abundance at day 26 between male and female rat pups which was not observed in the mouse pups (Chapter 3). SFB abundance in males sharply decreased, while in females, there was a gradual decrease until the plateau was reached at day 28 (Figure 4.4). The male pups also gained weight quicker than females (Table 4.1). There were significant differences in weight gain between males and females when age was used as a covariate ($p<0.05$).

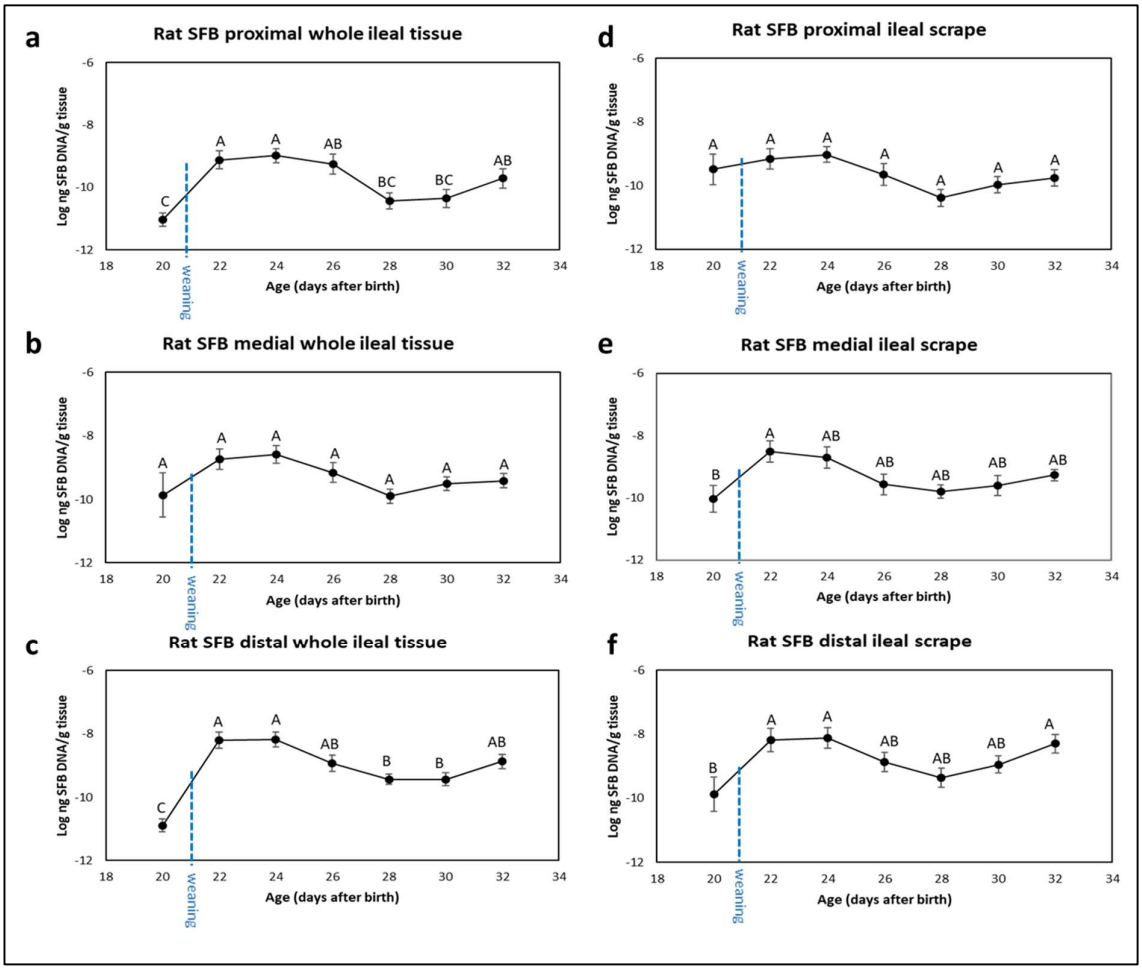

Figure 4.2. *Abundance of SFB from whole ileal tissue (a, b, c) and ileal mucosal scrapes (d, e, f) of conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle, and distal ileum. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=14 pups (20 days) and n=13 pups (22-32 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

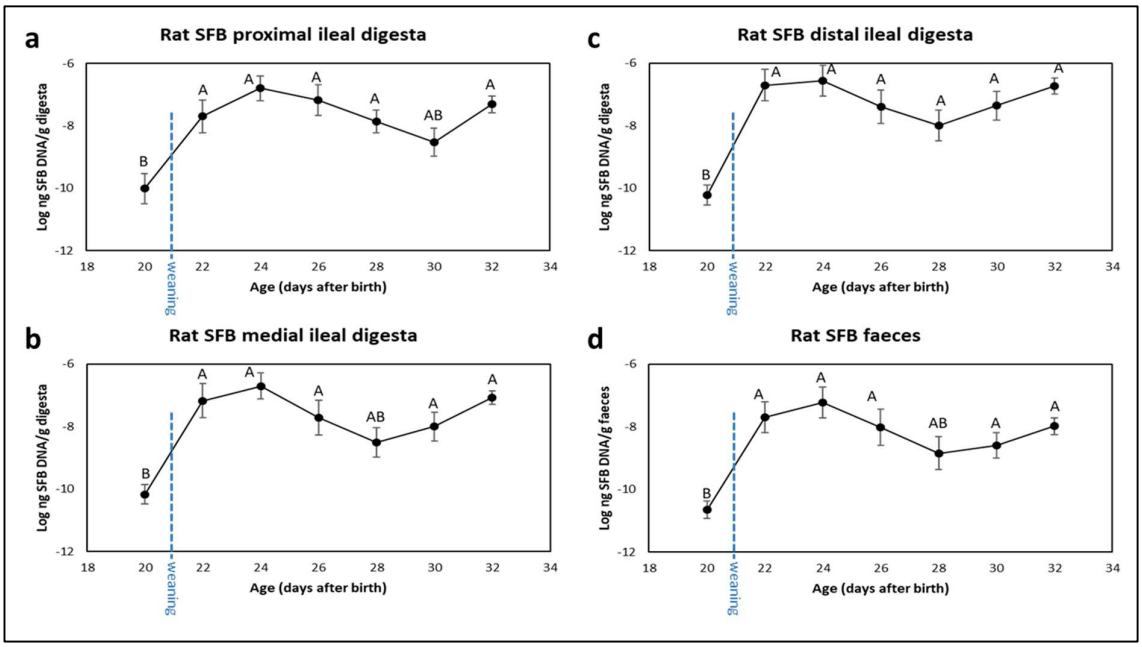

Figure 4.3. *Abundance of SFB from ileal contents (a, b, c) and faeces (d) of conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle, and distal ileum. Data are plotted on a logarithm 10 scale and are shown as the mean values of n=14 pups (20 days) and n=13 pups (22-32 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

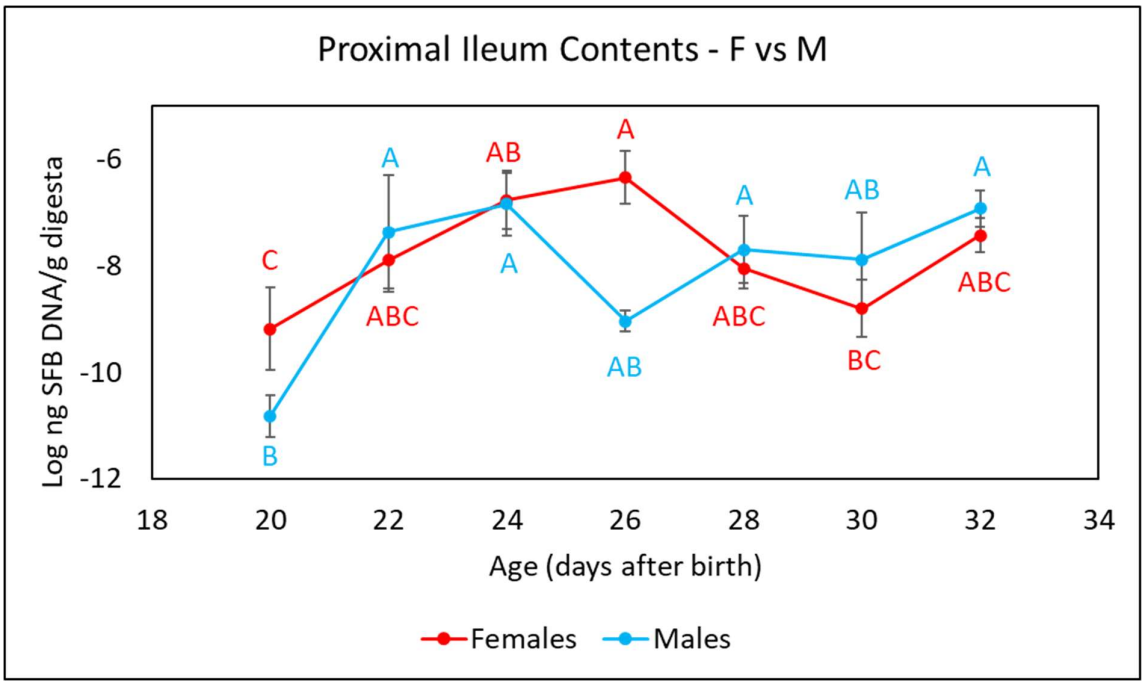

Figure 4.4. *Comparison of the abundance of SFB in the proximal ileal content samples between female (F) and male (M) Sprague-Dawley rat pups. The rat pups were weaned on day 21. Data are plotted on a logarithm ten scale and are shown as the mean values of females (n=57) versus males (n=35). Values without common letters differ significantly, $p < 0.05$.*

Table 4.1. Mean ± SEM weight gain in grams (g) of pre- and post-weaning male and female Sprague-Dawley rats. The values represent weight gained between the initial weigh-ins (at postnatal days 15, 17, 18) and before sample collection from pups at all time-points. Compared to females, males gained more weight, and male pups were the heaviest at terminal sample collection. An ANOVA and post-hoc Tukey's test showed significant differences in average weight gain in males among time-points 1-2, 4-6 and 7. There were significant differences in average weight gain in females at all time-points; $p < 0.05$.

Time-point	Males	Females
1	7.43 ± 0.98^{d}	7.00 ± 0.61^{g}
2	16.92 ± 1.74^{cd}	15.76 ± 1.08^{f}
3	25.80 ± 2.61^{c}	26.74 ± 1.60^{e}
4	44.48 ± 2.41^{b}	39.44 ± 1.08^{d}
5	56.20 ± 3.56^{b}	50.85 ± 2.86^{c}
6	53.23 ± 4.54^{b}	60.27 ± 2.58^{b}
7	92.93 ± 11.52^{a}	74.56 ± 1.72^{a}

4.4.2. Quantification of plasma IL-17 and faecal IgA concentrations

There were significant differences in the concentration of IgA in faeces ($p<0.05$), which decreased from day 24 to 32 (Figure 4.5). A Tukey's post-hoc test showed that there were significant differences in the faecal IgA concentration between days 20, 24 and days 30, 32. There were no significant changes in the concentration of IL-17 in plasma over time ($p=0.730$) (Figure 4.6).

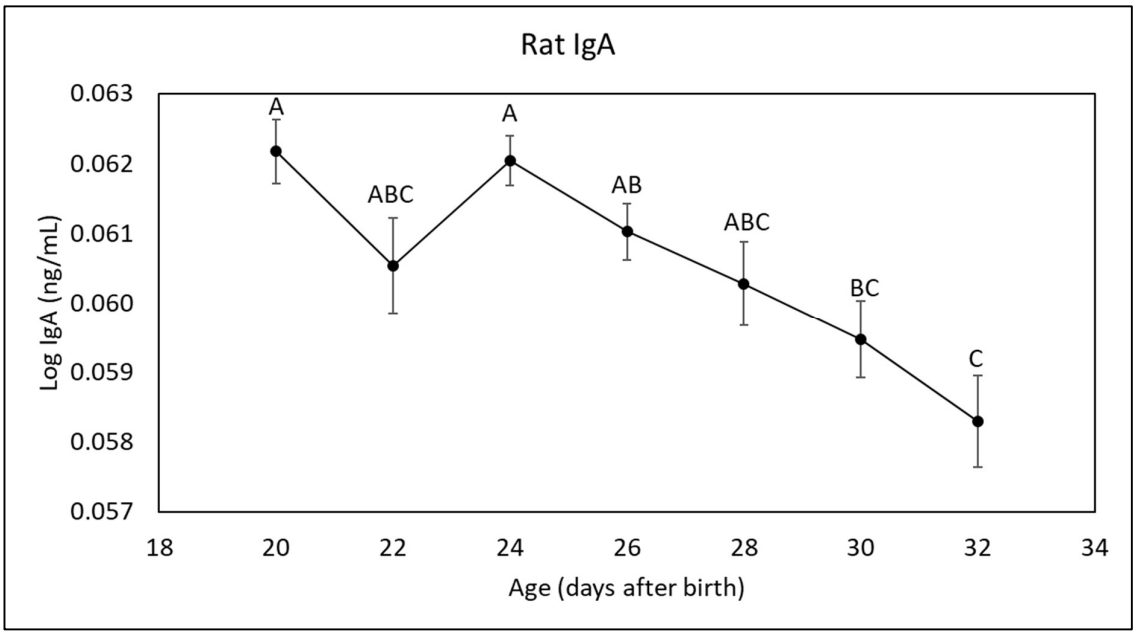

Figure 4.5. *Concentration of IgA in faeces of conventionally reared male and female Sprague-Dawley rat pups. The rat pups were weaned on day 21. The limit of detection was 0.88 ng/mL. Data are plotted on a logarithm ten scale are shown as the mean values of n=13 pups (22-32 days) and n=14 pups (20 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

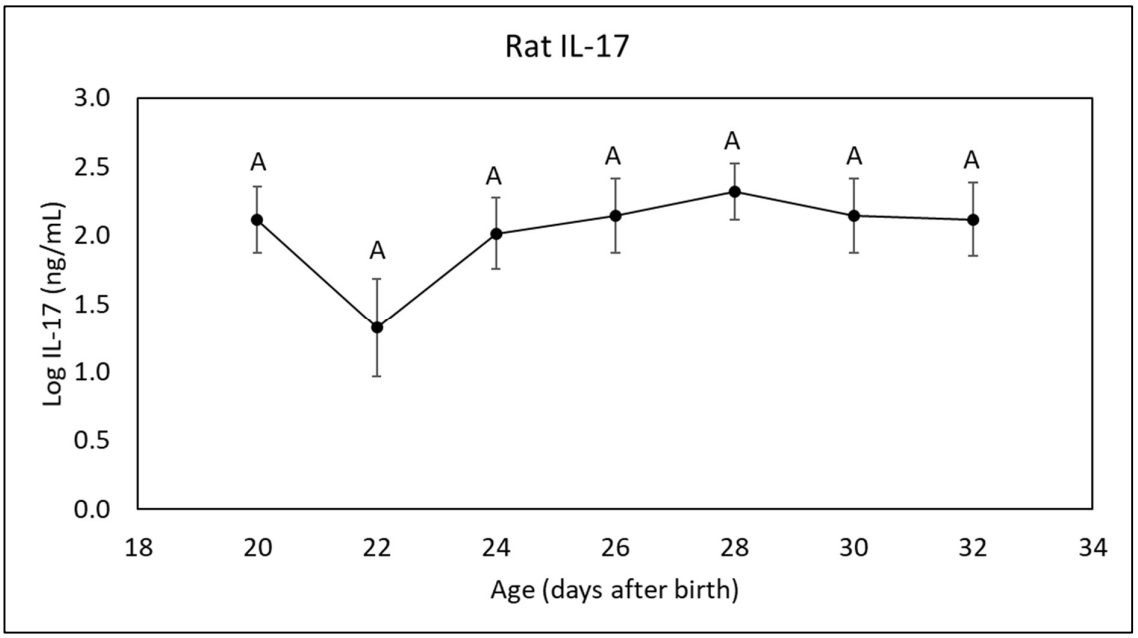

Figure 4.6. *Concentration of IL-17 in plasma of conventionally reared male and female Sprague-Dawley rat pups. The rat pups were weaned on day 21. The limit of detection was 0.05 ng/mL. Data are plotted on a logarithm ten scale are shown as the mean values of n=13 pups (22-32 days) and n=14 pups (20 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

4.5. Discussion

This study shows that the abundance of SFB in ileal tissue and contents of rats measured pre- and post-weaning was similar to the temporal profiles published in mice and infants of corresponding age [22, 24]. Here, the abundance of SFB increased post-weaning, peaked at days 22 and 24 and decreased until a plateau was reached at day 26 until the last measurement on day 32. Other studies suggest that the abundance of SFB remains relatively constant after weaning throughout life [24, 78].

The results also showed that of the two tissue collection techniques, whole tissue homogenisation gave less variable tissue weights compared with mucosal scraping. SFB abundance data was similar to what has been reported [22]. Interestingly, the SFB data from homogenisation was less variable compared to the scraped data. High variability in the scraped data was likely partly due to scraped tissue sticking to the collection tools, thus decreasing the amount of tissue available. The fragility of the ileum at that age also made scraping of the tissue challenging, resulting in variable weights. Whole tissue homogenisation was, therefore, a simpler method of collecting ileal tissue samples from rats for analysis pre- and post-weaning.

The pattern of SFB abundance in the ileal tissue and contents over time was also reflected in the faeces, suggesting faeces could be used as a proxy for SFB abundance in the ileum. The faecal SFB abundance peaked later at day 24 compared to the ileal tissue at day 22; abundance in the ileum would, therefore, be assumed to peak earlier. This non-invasive approach of using faeces aligns with the Three Rs of animal research that include replacement, reduction and refinement, which utilise alternatives to terminal animal sampling, reduce the number of animals used and follow procedures that minimise pain and stress [171]. This alternative to terminal sampling to obtain ileal tissue content could be applied in time-series and intervention

studies attempting to understand influences on SFB abundance caused by diet, disease, medication or ageing [50].

The significant difference in the interaction of sex with age indicates that SFB abundance in the proximal ileal contents over time is different in males and females. It suggests that this interaction may be present in the middle and distal ileal contents though a study with more statistical power would be required to make this conclusion. The abundance of SFB was unexpectedly different between females and males on day 26 only. This result may be a random difference in the rats sampled on that day. The observation also suggests that sex-based hormonal differences might influence SFB abundance. It may result from physiological differences in GIT development between sexes [172] as the male pups gained weight quicker. Before puberty, oestradiol concentrations peak at day 15 in both sexes and remain low until day 39, while testosterone concentrations in both sexes remain low between days 1 and 19 then decrease between days 20 and 30 [173, 174]. Rats reach puberty between days 30 and 42 in females and days 42 and 55 in males [175]. Sex hormones were not measured here, so it is unclear whether a change in their concentrations leading up to puberty might have influenced SFB abundance in the samples analysed. The abundance of SFB between sexes was not reported in a similar study which involved six male and nine female mouse pups [22]. Thus far, the study here is the only one that has reported this finding which highlights the importance of considering gut microbiota changes between sexes.

In this study, there was no difference in the plasma concentration of IL-17 from days 22 to 32. However, the results from Chapter 3 in mice showed differences in plasma concentration of IL-17 between days 18 and 41; the concentration peaked at day 32. These results also contrast with literature reports of a positive association between ileal SFB abundance and plasma IL-17 production in adult mice two to three weeks following SFB colonisation [39, 41, 72].

Several plasma samples across all time-points measured here had IL-17 concentrations lower than the calculated detection threshold, increasing the variability in the data. At day 22, most of the rats had zero readings, and no measurements were done after day 32.

The faecal concentration of IgA was variable from days 20 to 28, then decreased until day 32. This result disagrees with other studies where faecal IgA concentrations reportedly increased only late post-weaning [22, 37]. Other studies showed that oral inoculation of SFB in older conventional and germ-free mice initially stimulates the production of IgA [37], with IgA later inhibiting SFB colonisation [22, 29]. It is plausible that breastmilk IgA may have contributed to the high IgA concentrations found in the faecal samples at day 20, as observed in the caecal contents of pre-weaned mice [22]. Other microbes may be involved, as they change in abundance around weaning and are also known to stimulate the production and secretion of IgA by immune cells [36, 79, 80]. The numerical drop of faecal IgA concentration at day 22 may have resulted from weaning on day 21, then the increase at day 24 may have resulted from increased luminal IgA. A broader age range, with a later developmental stage, may give a clearer picture of how IgA concentration changes [22].

In conclusion, the result showed that the temporal profile of SFB colonisation in the ileal tissue and contents of weanling rats was similar to those published for mice and infants of corresponding age. Lower variability in whole ileal tissue data favours it as a preferred tissue collection technique. The data also show that faeces can also be used as a proxy for SFB abundance in the ileum. The immune markers did not give similar results to those reported in mice and infants, making it difficult to conclude on the influence of SFB on plasma IL-17 and faecal IgA. A future study would involve 16S rRNA sequencing of purified DNA samples, and with knowledge from the published SFB genome compare changes in the temporal profile of SFB abundance over time with other GIT microbiota pre- and post-weaning.

CHAPTER 5: COMPARING EFFECTS OF INULIN DOSES ON THE TEMPORAL PROFILE OF ILEAL AND FAECAL SFB ABUNDANCE AND ASSOCIATED IMMUNE MARKERS IN WEANLING RATS

5.1. Abstract

The study in this Chapter sought to investigate the suggested influence of diet on SFB abundance. The reported changes in the temporal profile of SFB at weaning when the GIT immune system is thought to mature points to an influence by the weaning diet on SFB abundance. To determine metabolism within SFB, they were examined to determine similarities to members of the *Clostridium* genus. Comparative genome analysis of SFB with pathogenic and non-pathogenic clostridial species reported the presence of COGs and pathways for carbohydrate metabolism in SFB. These clostridial species; *Clostridium tetani E88, C. perfringens strain 13 and C. stricklandii DSM 519* did not contain the reported carbohydrate COGs. This, and their preferred attachment to the distal ileum, suggest SFB may use carbohydrates or metabolites of carbohydrate fermentation from their niche.

The dietary carbohydrate selected, inulin, was based on oligosaccharides being routinely added to commercial infant formula and weaning diets. Inulin is known to promote the growth of beneficial commensals in the GIT and reportedly improve GIT function. These include reduced incidences of flatulence and bloating. Inulin reportedly promotes production of SCFAs which help prevent the growth of pathogenic bacteria and enhance abundance of beneficial bacteria. The SCFAs also reportedly activate the immune system, improve epithelial integrity and barrier function, and increase expression of tight junctions.

Inulin (at 2.5%, 5%, 10%) was thus hypothesised to increase the peak abundance of SFB in male and female weanling rats three days after weaning (day 24). The effect of inulin dosing (at 0%, 2.5%, 5%, 10%) on the profile of SFB colonisation was characterised at day 24 post-weaning compared to day 20 pre-weaning in the ileal tissue, contents, and faeces. Associations between SFB abundance and two immune markers of GIT maturation (plasma IL-17 and faecal IgA) were observed.

Inulin did not influence the abundance of SFB, regardless of sample type, in post-weaning rats. There were no differences observed in SFB abundance between male and female rats, regardless of inulin dosing or sample type. There were no differences of inulin dosing on blood plasma IL-17 and faecal IgA concentration, nor between male and female rats.

The results indicate that inulin fed in a dose-dependent manner to weaned Sprague-Dawley rats did not influence the peak abundance of SFB or the plasma concentration of IL-17 and faecal concentration of IgA. SFB may not utilise inulin directly but possibly the products of its fermentation in a cross-feeding manner with other bacteria in the microbial community, however this did not affect any parameters measured in this study.

5.2. Introduction

During postnatal development, pre-weaning, breastfed full-term infants receive required nutrition from their mother's milk. Where this is impossible, infant formulas that are made from ruminant (mostly bovine) milk or alternative formulas (soy or specialised) are used. Compared to human breastmilk, bovine milk has a higher fat, protein and mineral content, and a lower oligosaccharide content [176]. Bovine milk is skimmed and diluted, then vegetable oils, vitamins, minerals, and prebiotics are added to resemble the composition of human breastmilk [176]. Prebiotics added to infant formula include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose and acidic oligosaccharides [177].

As infants mature, their nutritional requirements become greater than what breastmilk can provide, creating the necessity for a complementary solid diet. The recommended weaning diet includes protein, fats, minerals, vitamins, digestible carbohydrates, and prebiotic carbohydrates [178]. As these new substrates become available to the intestinal microbial population, a shift is caused in microbial abundance from one that is exclusively milk-based and begins to resemble that of an adult [30]. As discussed in Chapter 2, the changes in microbial abundance at weaning include also SFB [22, 23] suggesting an effect by substrates other than those present only in milk [32].

Initial studies investigating the effect of food substrates on the abundance of SFB in healthy adult rodents reported that a balanced diet (basal diet comprised skim milk powder, Lucerne (alfalfa) meal, native corn starch, soybean oil, ground barley, fish meal, soybean protein concentrate, wheat middlings, corn protein concentrate, molasses, vitamin premix, mineral premix, calcium carbonate, and sodium chloride), compared to a purified whole milk powder diet, influenced the abundance of ileal SFB [32]. Later 16S rRNA analysis which resulted in the published rat and mouse SFB genomes predicted the presence of a high abundance of COGs

for carbohydrate metabolism and transport compared to other clostridial species; *Clostridium tetani E88, C. perfringens strain 13 and C. stricklandii DSM 519* [87]. The predicted genes from this analysis include those involved in metabolism of mannose – a component of the glycolytic pathway – which is used in the cell wall structure and malate – a source of carbon and ribose – which is used in cellular respiration. Cellobiose, ascorbate and fructose were also predicted to be metabolised resulting in glyceraldehyde-3-phosphate of the glycolytic pathway [87]. The preferred niche of SFB in the ileum (brush border) [87] supports these predictions, as this microvilli-covered surface on the ileal epithelium contains enzymes which degrade disaccharides into simple sugars which are absorbed into the bloodstream [127]. The presence of simple sugars in this region along with genes encoding for permeases and import/export-type transporters for sugars detected in the SFB genome suggests SFB may utilise simple carbohydrates for routine growth and maintenance [87]. Results from the previously described studies have suggested that SFB might benefit from more complex carbohydrates compared to only simple ones such as those found in milk [32]. Analysis of the SFB genome indicated a lack of tricarboxylic acid cycle enzymes [87]. A complete set of genes encoding for enzymes in the glycolytic pathway was identified [87]. In addition, there were no proteins identified that could be assumed to take part in the electron transport chain, suggesting a fermentative lifestyle [87].

Prior to weaning, in exclusively breastfed infants, *Bifidobacteria spp* dominate the GIT [133] and are associated with the production of SCFAs and vitamins, modulation of the immune system and prevention of GIT disorders [133, 134]. The role of *Bifidobacteria spp* in fermenting non-digestible oligosaccharides such as FOS, GOS and inulin result in their increased abundance [128, 129]. As the abundance of *Bifidobacteria spp* increases [139], the abundance of *Clostridia spp* [136, 137] and *Streptococcus spp* and *Veillonella spp* also increase in the ileum [64]. *Streptococcus spp* yield lactate from simple carbohydrate fermentation,

which is a source of carbon for *Veillonella spp* [140]. Since SFB are predicted to produce lactate [87], they may also be a source of carbon for *Veillonella spp* in a cross-feeding relationship. The production of SCFAs by *Bifidobacteria spp* at weaning [141] suggests they may be involved in the suggested cross-feeding relationship with SFB. This likely relationship might be important to consider for dietary modulation of the ileal SFB abundance around weaning.

Inulin is a carbohydrate substrate that is typically present in certain fruits and vegetables [142] given as weaning foods to infants, and is also typically added to bovine-milk formula [177]. Inulin belongs to a class of soluble dietary fibres known as fructans and occurs naturally as a reserve carbohydrate in plants [143]. Its unique chemical structure made up of compounds with low chemical reactivity results in it resisting small intestinal digestion [146]. Inulin is digested by bacteria in the ileum and (mostly) colon where it encourages the growth of the bacteria known to produce SCFAs [144, 145], increase the ratio of beneficial Firmicutes to Bacteroidetes at the phylum level and decrease colonic epithelial permeability in non-obese diabetic mice [151].

Inulin is also considered safe for infant consumption [179]. Studies in infants formula supplemented with inulin reported a significant increase of beneficial faecal bacteria and decrease in coliform bacteria as well as softer faeces and increased faecal weights which imply improved bile production and bacterial fermentation [179, 180]. Additional studies on inulin-supplemented diets reported decreased flatulence and bloating in healthy adults fed 5-10 g inulin [53], enhanced abundance of beneficial bacteria in 10% inulin-fed eight-week old piglets [54], and SCFA-production in stool samples of an *in vitro* continuous adult fermentation system [55, 56] suggests that pre-weaned and weaned infants benefit from these properties of inulin. Additionally, the acidic environment created by the increased SCFA-production not only inhibits the growth of pathogenic bacteria [57], but also increases the expression of tight

and adherens junctions genes [181] and also activates the immature infant immune system [58, 59].

To illustrate the protective effects of inulin on the GIT, a study compared effects of dietary inulin only, a dietary inulin with *Trichuris suis* infection and *Trichuris suis* infection only in weaned 8-week-old pigs [54]. The 16S rRNA amplicon sequencing showed that diet supplemented with 10% (w/w) long-chain purified chicory inulin fed to 8-week-old weaned pigs for 6 weeks increased abundance of beneficial bacteria throughout all segments of the gut [9]. Compared with the control, the inulin-fed pigs had a higher relative abundance of Actinobacteria, Bacteroidetes and Proteobacteria, and a lower relative abundance of Firmicutes in the ileum [9]. The inulin-fed pigs exhibited higher relative abundances of *Bifidobacteriaceae* (Actinobacteria), *Prevotellaceae* (Bacteroidetes), *Gammaproteobacteria* and *Deltaproteobateria* (Proteobacteria) and higher *Ruminococcaceae* and other members within *Clostridiales* (Firmicutes) [54]. Taxonomic classification following Illumina sequencing placed SFB within the order *Clostridiales* [182], and SFB are further classified within the family *Savagellaceae* [96]. The increased abundance of members within *Clostridiales* in the study by Stolzenbach, et al. [54] may include SFB though the effect of inulin specifically on these microbes has not been investigated.

Hypothesis and aims

The hypothesis of the research described in this Chapter was that feeding a diet enriched in inulin would increase the peak abundance of ileal and faecal SFB and the concentration of the immune markers of GIT maturation in a dose-dependent manner in weanling male and female Sprague-Dawley rats.

The aim of this study was to determine if inulin, and at what dose, would increase the peak abundance of ileal and faecal SFB in weanling rats. The rat diets were formulated with different

amounts of inulin (0%, 2.5%, 5% and 10%). These amounts of inulin were based off study by Young, et al. [183] where they fed weanling rats similar doses of resistant starch which had no negative effects on the weanling rats and influenced the host gut microbiota. Immune markers (IL-17 from blood plasma and IgA from faecal samples) suggested to correlate with the presence of SFB in the ileum were also analysed [22, 39] by antibody-specific ELISAs.

5.3. Methods

5.3.1. Rat experiment

The study was approved by the AgResearch Grasslands Animal Ethics committee (Animal Ethics Application No: 14992) under the recommendations of the NZ Animal Welfare Act 1999. Nine pregnant conventional Sprague-Dawley dams were obtained from AgResearch Ruakura (Hamilton, NZ) and transported to AgResearch Grasslands (Palmerston North, NZ) by road at 15 days of gestation. The dams were individually caged under dark and light cycles in a room at 21°C and provided with a casein-based open standard rodent diet from Research Diets Inc. (New Jersey, USA) and water *ad libitum*. This open standard rodent diet contained 0% inulin and was used as the control diet.

One litter was birthed on the first day, four litters on the second day, two litters on the third day and two litters on the fourth day. After birth, all dams and pups were maintained at 21°C, and the dams continued to receive the open standard rodent diet (control) and water *ad libitum* during the suckling period. All dams and pups were checked daily, weighed once a week, and fresh food and water topped up as required. Their General Health Score (GHS), which ranged from 5 (healthy) to 1 (requires euthanisation), was checked daily, and the rats with a GHS of 3 or 4 were closely monitored. If their condition did not improve, they were immediately humanely euthanised. A total of 106 pups were born. This number of pups, which was in excess

of what was required for this study, was planned for in the event there was a loss of pups. Any loss would therefore not affect the study design. Three pups overall were humanely euthanised at two days postnatally to reduce the number of pups per dam, and this is part of the small animal standard operating procedures at AgResearch. This practice ensures there is not an excess of pups per dam, which may lead to some pups not receiving enough nourishment and may affect growth and development and possibly GIT microbial abundance. A few days later, 35 pups were rejected by their mothers (GHS of 1) and were humanely euthanised. Two pups were missing and presumed to have been eaten by their mother, and one pup was found dead. The remaining 65 rat pups had a GHS of 5 and were used for the study.

A power analysis (80% power) of the experimental design determined between 8 pups would be randomised into the control group and each inulin-dose group for a total of 40 pups. Power calculations were carried out based on a simple 2-group comparison with data from the study in Chapter 4. From that study, a standard deviation of 1.07 units/g of tissue and a minimum difference of interest of 1.7 units/g of tissue were calculated to achieve the desired level of significance of 0.05. Sixty-five pups (33 females and 32 males) were randomised into two time-points: 20 and 24 days after birth. The pups were identified by ear-punching at day 14 to meet the welfare norms for this procedure. The control group consisted of pre-weaned 20-day old pups, which remained in their birth litters until sample collection. Thirteen pups from the control group were selected at random and euthanised at day 20 for sample collection. The remaining 52 pups were separated into the four inulin dose groups at weaning on day 21 in different cages, and samples were collected on day 24. The pups received their inulin-supplemented diets over a period of 4 days (Table 5.1). Interactions between pups from different litters were minimised. The treatment diets included modified open standard diets with 2.5% inulin, 5% inulin, and 10% inulin but no dextrose (Table 5.2).

Table 5.1. Study design of male (M) and female (F) Sprague-Dawley rats fed diets with different inulin concentrations (0%, 2.5%, 5% and 10%). Samples were collected at day 20. The remaining pups were weaned at day 21 and fed one of the diets and euthanised for sampling at day 24.

Diet (% inulin)	***N* *(M:F)***	**Age of rat pups (postnatal days)**		
		Sampling 1	Weaning diet	Sampling 2
Control (0%)	13 (6:7)	20	-	-
Control (0%)	10 (4:6)	-	21	24
Treatment (2.5%)	14 (7:7)	-	21	24
Treatment (5%)	14 (6:8)	-	21	24
Treatment (10%)	14 (9:5)	-	21	24

Table 5.2. Open Standard Diet (OSD) with 15kcal% Fat, 50 g Cellulose and modifications to inulin content. The casein-based OSD for weanling rats was modified to include 0% (blue), 2.5% (yellow), 5% (green) and 10% (red) inulin. The diet contained protein, carbohydrates, and fats for the pregnant and breastfeeding rats and later developing rat pups at the required minimum guidelines. The modified diets were identified by different coloured dyes.

Product #	**D11112242**		**New Formula 1**		**New Formula 2**		**New Formula 3**		**New Formula 4**	
	Open Standard Diet		Modified OSD		Modified OSD		Modified OSD		Modified OSD	
	50 g Cellulose		0% Inulin, Pellets		2.5% Inulin, Pellets		5% Inulin, Pellets		10% Inulin, Pellets	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	20	20	20	20	19	20	19	20	18	20
Carbohydrate	64	65	64	65	65	65	65	65	66	65
Fat	7	15	7	15	7	15	7	15	6	15
Total		100		100		100		100		100
kcal/gm	3.96		3.96		3.90		3.84		3.72	

Ingredient	**Gm**	**Kcal**	**Gm**	**Kcal**	**Gm**	**kcal**	**Gm**	**Kcal**	**gm**	**kcal**
Casein	200	800	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12	3	12
Corn Starch	525.5	2102	525.5	2102	515.5	2062	505.5	2022	484.2	1936.8
Maltodextrin 10	125	500	125	500	125	500	125	500	125	500
Dextrose	0	0	0	0	0	0	0	0	0	0
Cellulose, BW200	50	0	50	0	50	0	50	0	50	0
Inulin	**0**	**0**	**0**	**0**	**26.5**	**39.75**	**53**	**79.5**	**110**	**165**
Soybean Oil	70	630	70	630	70	630	70	630	70	630
Lard	0	0	0	0	0	0	0	0	0	0
Mineral Mix S10026	10	0	10	0	10	0	10	0	10	0

Dicalcium Phosphate	13	0	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0	2	0
Blue Dye, FD&C #1	0	0	0.05	0	0	0	0.025	0	0	0
Yellow Dye, FD&C #5	0	0	0	0	0.05	0	0.025	0	0	0
Red Dye, FD&C #40	0	0	0	0	0	0	0	0	0.05	0
Total	**1030.50**	**4084**	**1030.55**	**4084**	**1047.05**	**4084**	**1063.53**	**4084**	**1099.20**	**4084**

5.3.2. Sample processing

The methods for sample collection, sample preparation and genomic DNA extraction, qPCR and ELISA were described in Chapter 3 in Sections 3.3.3, 3.3.4, 3.3.5, 3.3.6. In the ELISA for this study, the limits of detection for plasma IL-17 and faecal IgA were 0.72 ng/mL and 1.03 ng/mL, respectively.

5.3.3. Statistical analysis

A Friedman test was performed in Minitab® 18 to determine if male and female rat pups fed diets supplemented with inulin in a dose-dependent manner resulted in any significant differences in the peak abundance of SFB, the concentration of IL-17 in plasma and IgA in faeces at 24 postnatal days. This nonparametric test was used as the data were not normally distributed, and the influence of two factors (age and inulin dose) on SFB abundance were compared. The data met the required four assumptions: the dependent variable should be measured at a continuous level, the independent variable should consist of two or more independent groups, there should be no relationship between the observations in each group or between the groups themselves, the distributions in each group should have the same variability.

The abundance of SFB and concentration of IL-17 and IgA were measured at a continuous level. Four inulin doses were representing different diet groups, the observations made in each inulin dose group were independent of each other, and the distributions in each inulin dose group had similar variability. A probability value inferior to 0.05 ($p<0.05$) indicated differences in the median number among groups.

5.4. Results

5.4.1. Quantification of SFB

The initial data on the abundance of SFB were skewed. Therefore, to meet the required assumptions (normality and homogeneity of variance) for the statistical test, the data were transformed and plotted on a logarithm ten scale. The transformed data were normally distributed. All figures with untransformed data are included in Appendix II (Figure 5.11 to Figure 5.20).

The Friedman test showed that there were no significant differences in SFB abundance in the ileum tissue ($p=0.267$) (Figure 5.1), ileum contents ($p=0.355$) (Figure 5.3) and faeces ($p=0.308$) (Figure 5.5) among the weanling rats fed inulin supplemented diets, regardless of the dose of inulin. The abundance of SFB in the ileum tissue of the 20-day-old rats, 2.5% and 10% inulin-fed rats seemed lower compared to the abundance of the 0% (24 days) and 5% inulin-fed rats (Figure 5.1). The abundance of SFB in the ileum contents of the 0%, 2.5%, 5% and 10% inulin-fed rats at 24-days seemed lower compared to the abundance of the 20-day-old rats (Figure 5.3). The abundance of SFB in the faeces of the 5% and 10% inulin-fed rats at 24-days seemed lower compared to the abundance of the 20-day-old rats as well as the 0% and 2.5% inulin-fed rats (Figure 5.5).

There were no significant differences in SFB abundance between males and females (Figures 5.2, 5.4, 5.6). A visual comparison between sexes showed that the 20-day-old female rats had a lower abundance of SFB. The 2.5% and 10% inulin-fed male rats exhibited a lower abundance of SFB. Female rats fed a diet containing 0% of inulin (24 days) exhibited a lower abundance of SFB and male rats fed 10% inulin exhibited a lower abundance of SFB in the ileum tissue (Figure 5.2). The male rats fed 2.5% and 5% inulin exhibited a lower abundance of SFB. Female rats fed 0% and 10% inulin at 24 days exhibited a lower abundance of SFB in

the ileum contents (Figure 5.4). The male rats fed 5% and 10% inulin exhibited a lower abundance of SFB in the faeces (Figure 5.6).

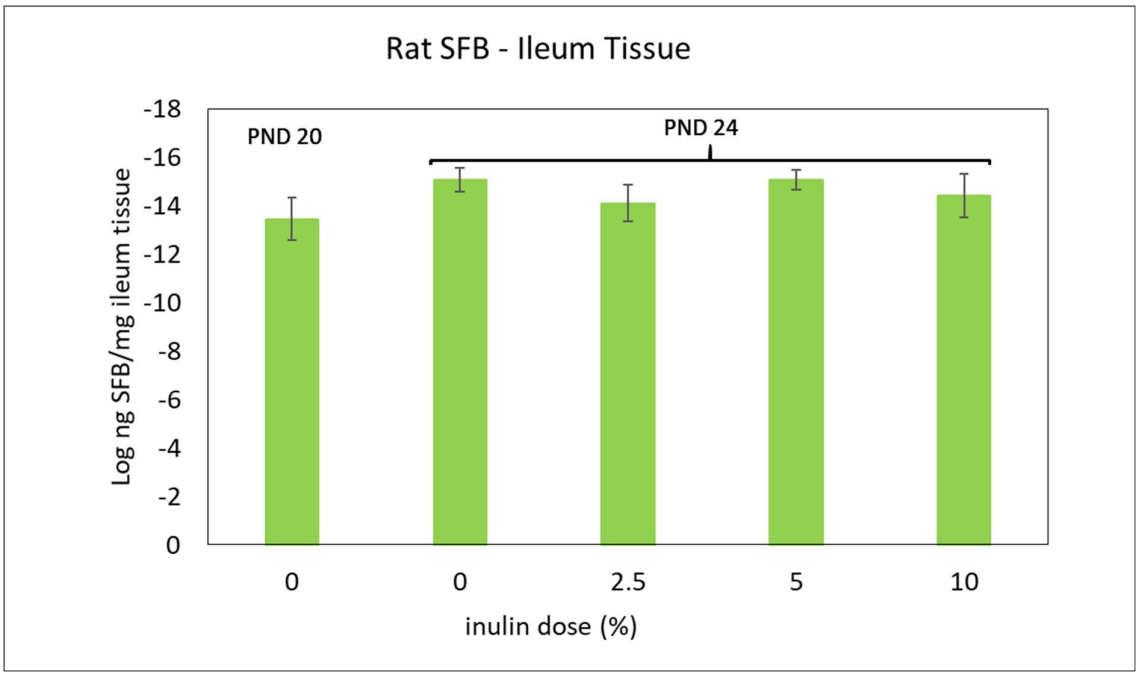

Figure 5.1. *Abundance of SFB from whole ileum tissue of conventionally reared Sprague-Dawley rat pups fed 0%, 2.5%, 5% and 10% inulin diets. Samples were collected from the distal ileum. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses at 24 days (p=0.267).*

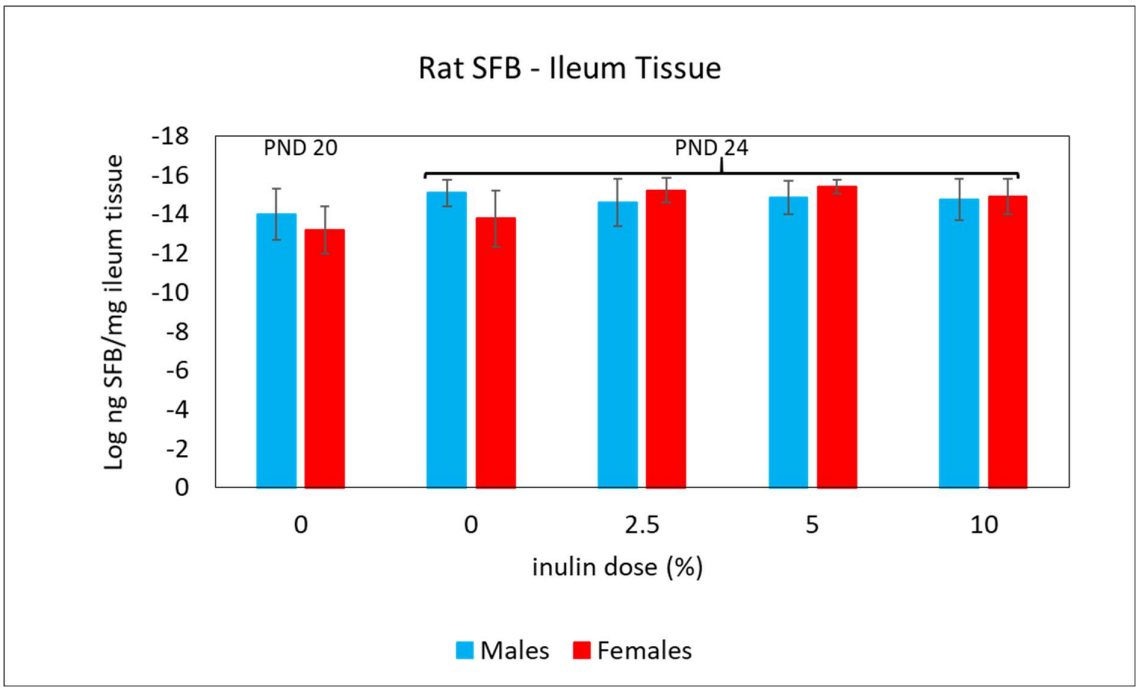

Figure 5.2. *Abundance of SFB from whole ileum tissue of conventionally reared male and female Sprague-Dawley rat pups fed 0%, 2.5%, 5% and 10% inulin diets. Samples were collected from the distal ileum. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 24 days (p=0.267).*

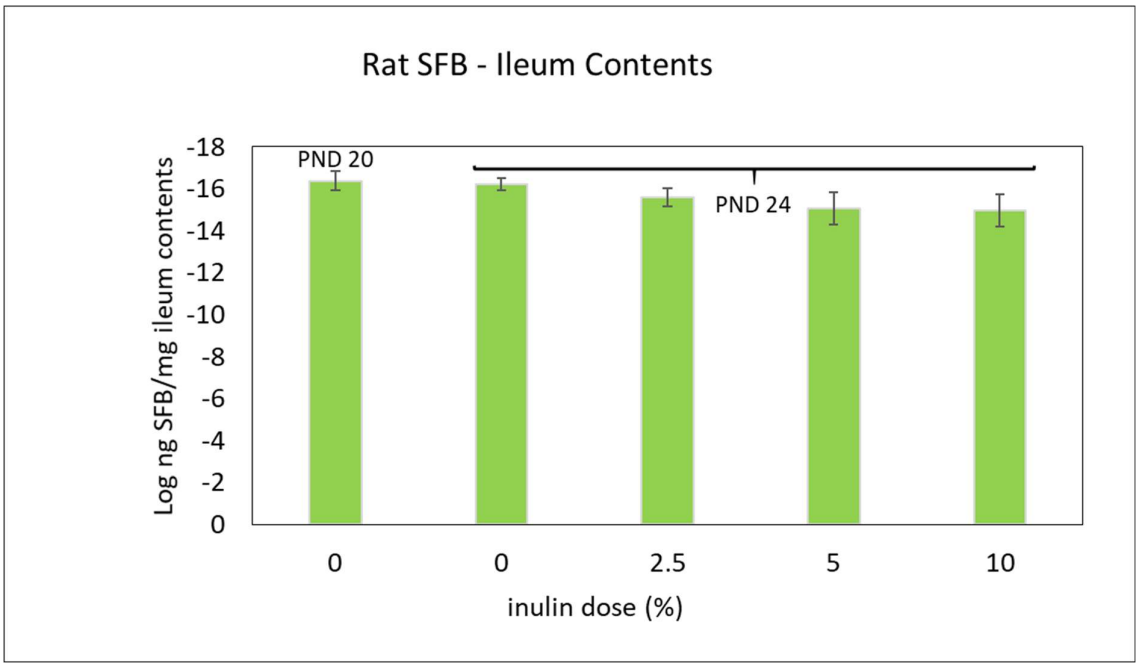

Figure 5.3. *Abundance of SFB from ileum contents of conventionally reared Sprague-Dawley rat pups. Samples were collected from the distal ileum. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses at 24 days (p=0.355).*

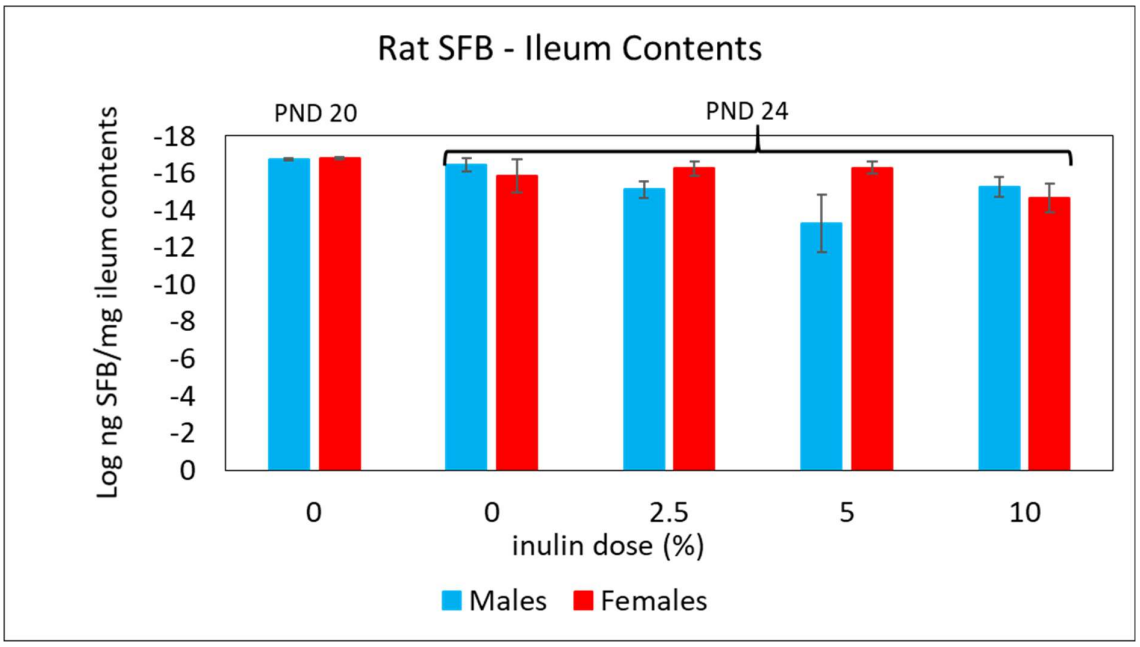

Figure 5.4. *Abundance of SFB from ileum contents of conventionally reared male and female Sprague-Dawley rat pups. Samples were collected from the distal ileum. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 24 days (p=0.355).*

Figure 5.5. *Abundance of SFB from faeces of conventionally reared Sprague-Dawley rat pups. Samples were collected from the distal colon. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses at 24 days (p=0.308).*

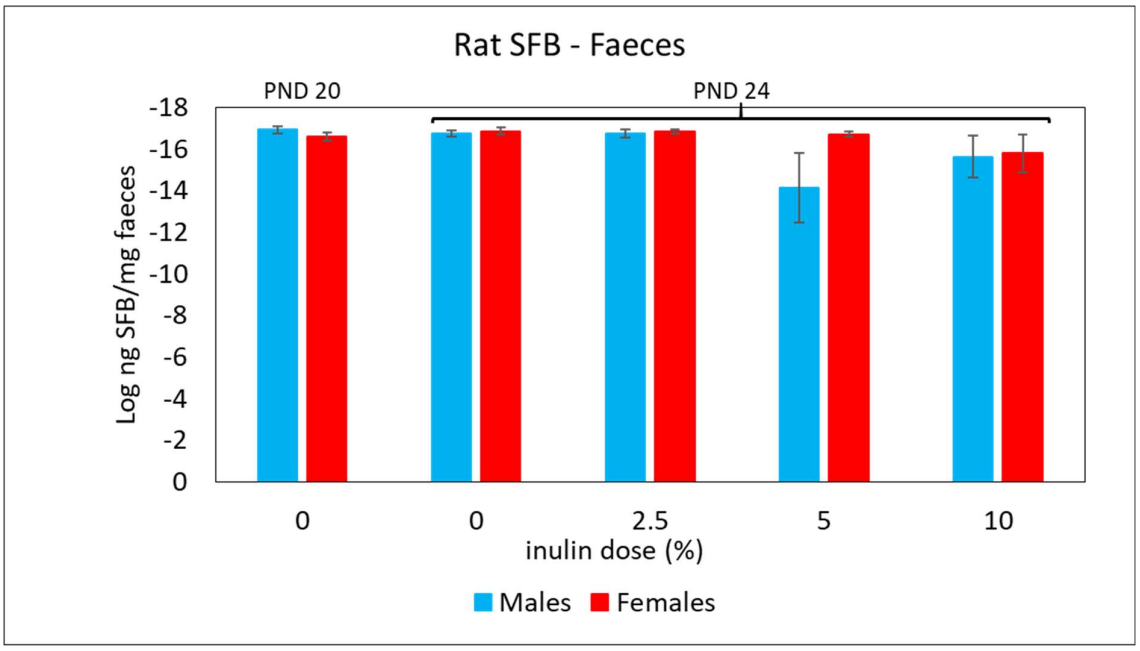

Figure 5.6. *Abundance of SFB from faeces of conventionally reared male and female Sprague-Dawley rat pups. Samples were collected from the distal colon. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 24 days (p=0.308).*

5.4.2. Quantification of plasma IL-17 and faecal IgA concentrations

The Friedman test showed that there were no significant differences in the concentrations of faecal IgA (p=0.592) (Figure 5.7) and blood plasma IL-17 (p=0.155) (Figure 5.9) among the weanling rats fed inulin-supplemented diets, regardless of the inulin dose. Only one rat in the 20-day-old (0% inulin) group had a concentration value of faecal IgA above the calculated limit of detection (1.03 ng/mL) resulting in the variable data point (Figure 5.7). The lower IL-17 concentration data at day 20 and 24 (0% inulin) compared to those at day 24 (2.5%, 5%, 10% inulin) was also the result of one rat in the day 20 and 24 (0% inulin) group with values above the calculated limit of detection (0.72 ng/mL) (Figure 5.9).

There were also no significant differences in the faecal IgA and plasma IL-17 concentrations between males and females (Figures 5.8, 5.10). The variability in the 20-day-old (0% inulin) group was caused by a female rat which exhibited a higher IgA concentration than the others (Figure 5.8). The lower IL-17 concentration in the sex comparison data at days 20 and 24 was a result of only two female rats in each group exhibiting a concentration value above the calculated limit of detection (1.03 ng/mL) (Figure 5.10). The higher faecal IgA and plasma IL-17 concentrations were all from different female rats.

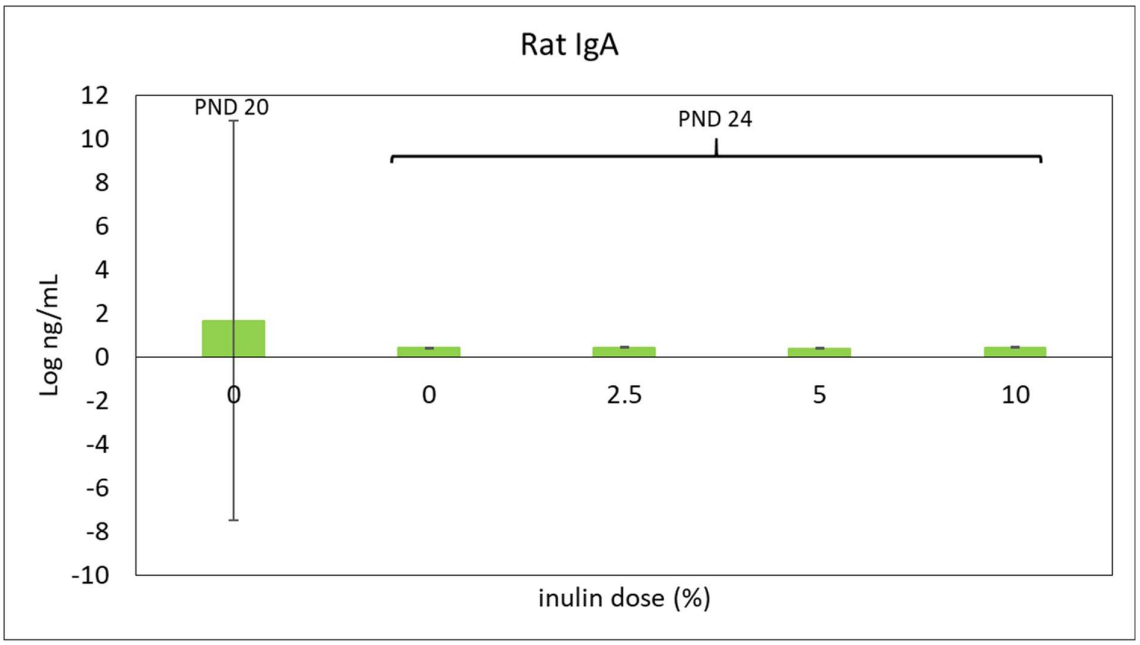

Figure 5.7. *Concentration of IgA in faeces of conventionally reared Sprague-Dawley rat pups. The limit of detection for faecal IgA was 1.03 ng/mL (Log value: 0.013 ng/mL). Data are plotted on a logarithm ten scale and are shown as the mean values of n=13 pups (0% inulin; 20 days), n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IgA concentration between the inulin doses at 20 and 24 days (p=0.592).*

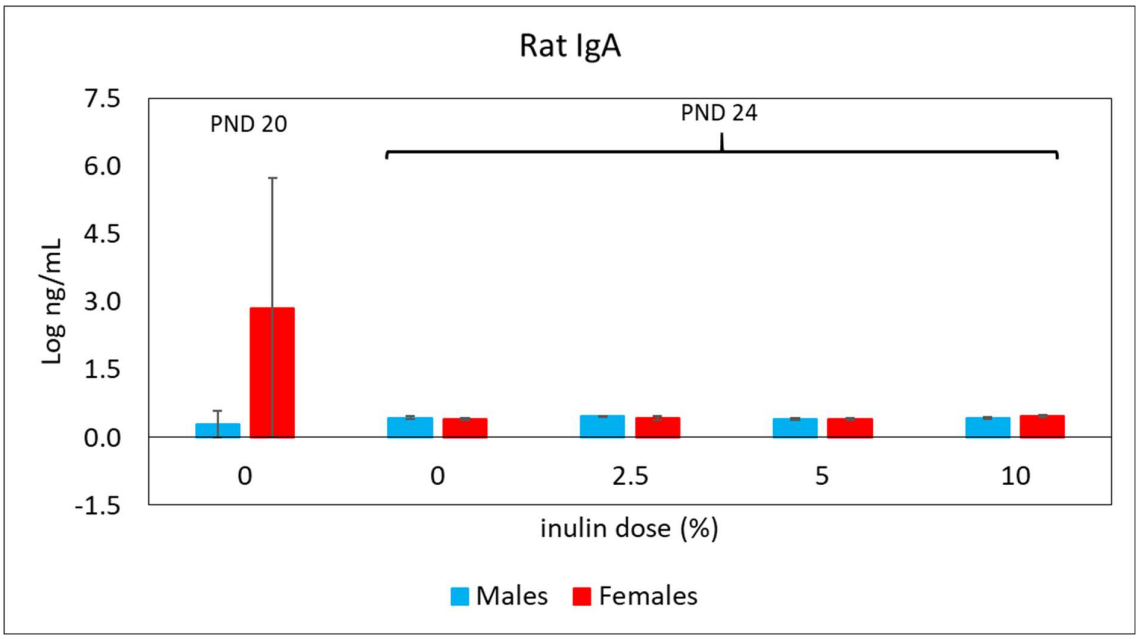

Figure 5.8. *Concentration of IgA in faeces of conventionally reared male and female Sprague-Dawley rat pups. The limit of detection for faecal IgA was 1.03 ng/mL. Data are plotted on a logarithm ten scale and are shown as the mean values of n=13 pups (0% inulin; 20 days), n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IgA concentration between males and females fed different inulin doses at 20 and 24 days (p=0.592).*

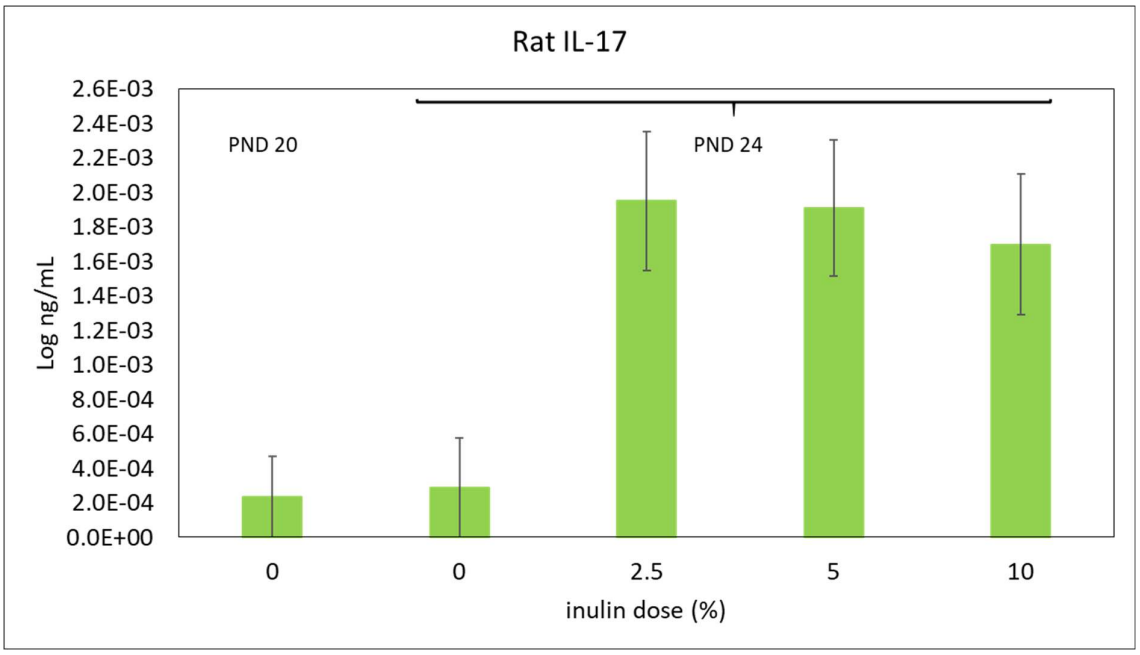

Figure 5.9. *Concentration of IL-17 in plasma of conventionally reared Sprague-Dawley rat pups. The limit of detection for plasma IL-17 was 0.72 ng/mL (Log value: -0.14 ng/mL). Data are plotted on a logarithm ten scale and are shown as the mean values of n=13 pups (0% inulin; 20 days) n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IL-17 concentration between the inulin doses at 20 and 24 days (p=0.155).*

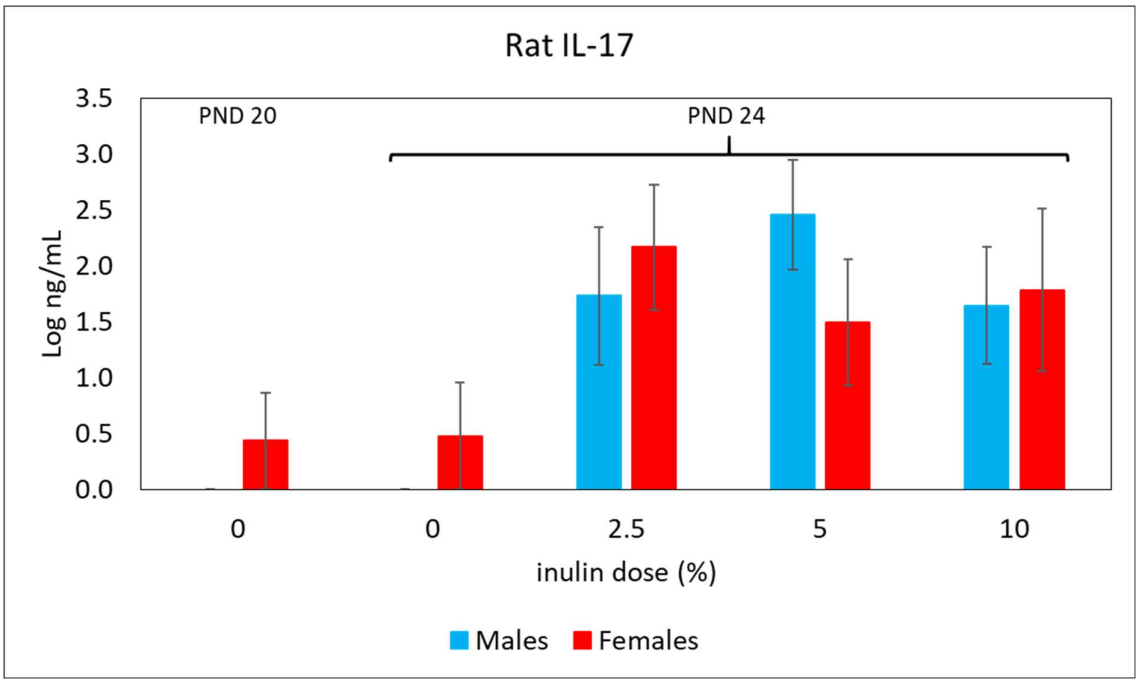

Figure 5.10. *Concentration of IL-17 in plasma of conventionally reared male and female Sprague-Dawley rat pups. The limit of detection for plasma IL-17 was 0.72 ng/mL. Data are plotted on a logarithm ten scale and are shown as the mean values of n=13 pups (0% inulin; 20 days) n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IL-17 concentration between males and females fed different inulin doses at 20 and 24 days (p=0.155).*

5.5. Discussion

This study shows that the abundance of SFB in weanling Sprague-Dawley rats, regardless of sampling type or their sex, was not influenced by inulin added to a regular rodent diet. This finding did not support the study's hypothesis that inulin would increase the peak abundance of SFB reported to be a few days after weaning (day 22 postnatally). In this study, the abundance of SFB was determined on postnatal days 20 and 24, based on the previous studies published in the literature [22, 23] and in Chapter 4. In these studies, SFB abundance was lowest at 20 days (pre-weaning) and was highest between 22 and 24 days postnatally.

Results in the control group (24-day-old rats, 0% inulin) in this study were as expected when compared to previous reports [22, 23]. The abundance of SFB was higher in the 24-day-old 0% inulin-fed rats (though not significant) compared to the pre-weaned 20-day-old rats. The lack of differences in SFB abundance between day 20 and the expected peak at day 24 in response to inulin-supplemented diets may reflect the short-term nature of the study. However, long-term effects of inulin on the abundance of SFB in the ileum remain to be determined. There may be other types of prebiotics or carbohydrates which could modulate the ileal SFB abundance. The numerous genes coding for carbohydrate metabolic pathways reported in the rat and mouse SFB genomes [87] suggest that SFB would be responsive to an increased availability of carbohydrates in the ileal lumen. Comparing genes from the three published SFB genomes [87, 126] indicated a high abundance of COGs for carbohydrate metabolism and transport, giving insight into which nutrient requirements and therefore dietary substrates might be effective. A longer-term study would be the next step in order to observe what influence, if any, inulin may have on the abundance of SFB and how it correlates with IgA and IL-17 concentration over time.

The lack of effect of inulin-enriched diets for plasma IL-17 and faecal IgA, with the majority of the data being below detection limit, was not surprising given the lack of effect of inulin-enriched diets on SFB abundance. Additionally, the concentration of faecal IgA in the 20-day-old pre-weaned rats was not significant compared to the 24-day-old 0% inulin-fed rats. The concentration of plasma IL-17 in the 20-day-old pre-weaned rats was lower compared to the 24-day-old 0% inulin-fed rats, also similar to the rats in the study reported in Chapter 3. The lower concentration of IL-17 in the 20-day-old rats was similar to results in Chapter 3 and the reported positive association with SFB abundance [39]. For the 24-day-old 0% inulin-fed rats, the concentrations of IL-17 in all but one rat were below the limit of detection (0.72 ng/mL) and may have caused the lower overall concentration of IL-17 in the control group.

Decreased levels of IL-17 have been reported in healthy female non-obese diabetic mice fed an inulin-supplemented diet from weaning up to 24 weeks [151] and healthy mouse pups exposed to inulin perinatally up to 2 weeks, by the dams being fed and inulin-enriched diet [184]. These observations may indicate a protective modulatory effect of inulin on pro-inflammatory cytokines in autoimmune disease and possibly an immature immune system, respectively. The disease state of the mice may have also stimulated the production of other tolerance immune markers, possibly masking the effects on IL-17 production. Among other immune markers, serum IgA levels were elevated in healthy dams and pups exposed both perinatally and postnatally to diets supplemented with inulin-containing prebiotic mixes [184, 185]. Elevated serum IgA levels in the mouse pups suggest an influence of prebiotics on an immature immune system [184, 185]. Prebiotics reportedly stimulate the activity of lactic acid bacteria such as *Lactobacillus* [186], and the utilisation of prebiotics by lactobacilli possibly stimulated IgA production. It is unclear whether a similar interaction would be observed with SFB. Further investigation of the relationships between SFB abundance and immune markers with broader time-points are required. Equally, understanding the associations between SFB

abundance and other microbe communities might give clues. However, given the levels of IgA and IL-17 concentration below the limit of detection, it is unclear whether inulin did have an effect on the concentration of these immune markers.

This study utilised both male and female rats, but sex did not have any influence on either SFB abundance at day 20 or day 24 postnatally. In Chapter 4, the results indicated a difference in SFB abundance between males and females in the proximal ileum contents at day 26 only. In this study, there were also no differences in the concentration of plasma IL-17 and faecal IgA at 20 and 24 days between both male and female rat pups. Studies on SFB and the immune system reported positive correlations with the concentration of IL-17 in plasma of adult mice [39, 41] and IgA in faeces of weaned mice (7-15 weeks) [22, 37] respectively. This was supported by results from Chapter 4 which indicated the faecal IgA concentration decreased from 24 until 32 days postnatally.

This study had limitations including some brought upon by the Corona virus disease 2019 (COVID19) lockdown such as older dams leading to lower breeding rate and road transportation in between facilities. This study was limited to two sample collection days; pre-weaning day 20 and post-weaning day 24 specifically to determine if inulin and at what dose would influence peak SFB abundance at day 24. It was thus impossible to determine the longer-term effects of the inulin-enriched diets on SFB abundance and the concentrations of plasma IL-17 and faecal IgA. Factors may also have triggered stress responses in the dams and pups and contributed to the loss of pups in the study. These include the age of the dams, mode of transportation of dams, different staff handling the rats and increased noise levels in the AgResearch Grasslands animal facility. The dams used for the study were much older (7-8 months old) than the recommended breeding age (3-4 months of age) and had never produced litters. These older dams were less accustomed to being handled and were prone to stress from

pregnancy and birthing, decreasing breeding success [187]. The restrictions at the time limited air travel meaning the rats could only be transported by road, which being longer may have stressed the dams. Transportation from AgResearch Ruakura to AgResearch Grasslands Small Animal Facilities was necessary because breeding at Grasslands was not practical due to the numbers required and biosecurity.

Other limiting factors, not related to COVID19, related to staff issues regarding animal handling. At AgResearch Grasslands, the dams were handled by different staff prior to and after birthing. These observations together with adaptations to the facility and staff may have stressed these older dams. These factors possibly contributed to the 40% of pups being rejected by the dam post-birth. however, the loss of pups did not affect the design of this study as this was mitigated by originally planning for pup numbers in excess of what was required. Regarding the success of future animal studies, it will be important for the animals to be handled by a single or a limited number of animal staff in order to reduce or prevent stress induced in the rodents prior to and post-birthing. Additionally, housing the animals in a quiet area may also allow them to acclimatize better to a different animal facility if transportation is absolutely necessary.

In conclusion, the results indicate that inulin-enriched diets (2.5%, 5% and 10%) fed to male and female Sprague-Dawley weanling rats between weaning at day 21 and day 24 did not influence the abundance of SFB in ileal tissue, ileal contents and faeces and the concentration of IL-17 in plasma and IgA in faeces. Both immune markers have concentrations in most pups below detection limits. This outcome suggests that SFB may not utilise inulin directly or a longer period of adaptation to the inulin-supplemented diet might be required to assess if there is a long-term effect on ileal SFB abundance. The published SFB genomes indicate that SFB reportedly metabolise pyruvate to produce acetate as well as lactate [87] which is a fermentation metabolic product of some *Streptococcus spp* [64]. Interestingly, early-life GOS

intervention in neonatal pigs (8 and 21 days) resulted in the increased ileal content abundance of *Firmicutes*, in which SFB are classified, and decreased abundance of *Proteobacteria*, which include mainly pathogens. Additionally, there was an increased concentration of SCFAs and lactate in the ileal contents [188].

The findings do not rule out other prebiotics with potential influence on ileal SFB abundance. Further investigation would entail determining any interactive effects among inulin-supplemented diet, SFB abundance, and immune markers at broader time-points beyond the expected peak of SFB abundance post-weaning. In addition, analysis could be carried out to determine the abundance of other microbes relative to the predicted pre- and post-weaning SFB abundance changes.

CHAPTER 6: OVERALL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

6.1. Discussion

The research carried out for this PhD dissertation investigated the role of a food substrate in the weaning diet on modulating the abundance of SFB in the ileum, which is in turn thought to influence maturation of the GIT immune barrier in infancy (Figure 6.1).

Originally observed in invertebrates [74, 93] and later in vertebrates [25], SFB were identified attaching to the epithelial mucosa of the ileum particularly where the Peyer's patches are located. This observation piqued an interest as these microbes, which are members within *Clostridiales*, did not induce inflammatory responses in the GIT, which is common in pathogenic *Clostridia*. Sequencing of the rat and mouse SFB genomes later revealed that SFB lack toxin and virulence genes which are present in pathogenic *Clostridia* [106].

Studies in adult animal models indicated that the presence of SFB was positively correlated with higher concentrations of plasma IL-17 [39-41, 60], whereas a lower abundance or absence of SFB was associated with higher concentrations of faecal IgA [22, 33, 35, 36]. Investigations on early detection of SFB in the ileum and/or faeces revealed their presence in infants [24] and weanling rodents [22] and a temporal profile where their abundance increases at weaning, peaks and decreases then plateaus post-weaning [22, 23]. SFB were thus hypothesised to originate from spores found in maternal faeces that may seed the infant during vaginal birth or be passed from the mother to the embryo via the placenta in infants born via Caesarean-section. Interestingly, the abundance of SFB was reported to remain at a plateau throughout an individual's lifetime without detrimental effects [24].

The initial reported temporal profile of SFB abundance indicated a negative association with the concentration of faecal IgA post-weaning [22]. Results from the Study reported in Chapter 3 indicated a positive association between the temporal profile of ileal SFB abundance and plasma IL-17, suggesting that SFB may be involved in inducing the production of IL-17 as in

previous reports [39, 41, 115]. The association between SFB and faecal IgA and plasma IL-17 implied that SFB may be involved in the maturation of the GIT immune barrier in infancy. Other microbes have been reported to induce the production of IL-17 and IgA. Of note however, was the continuous increase in IgA production as SFB abundance increased and then decreased and plateaued from about 4 weeks postnatally in weanling mice [22, 35, 36], and the aberrant expansion of mainly SFB and other anaerobes in the absence of hypermutated IgA in adult C57BL/6 mice [33].

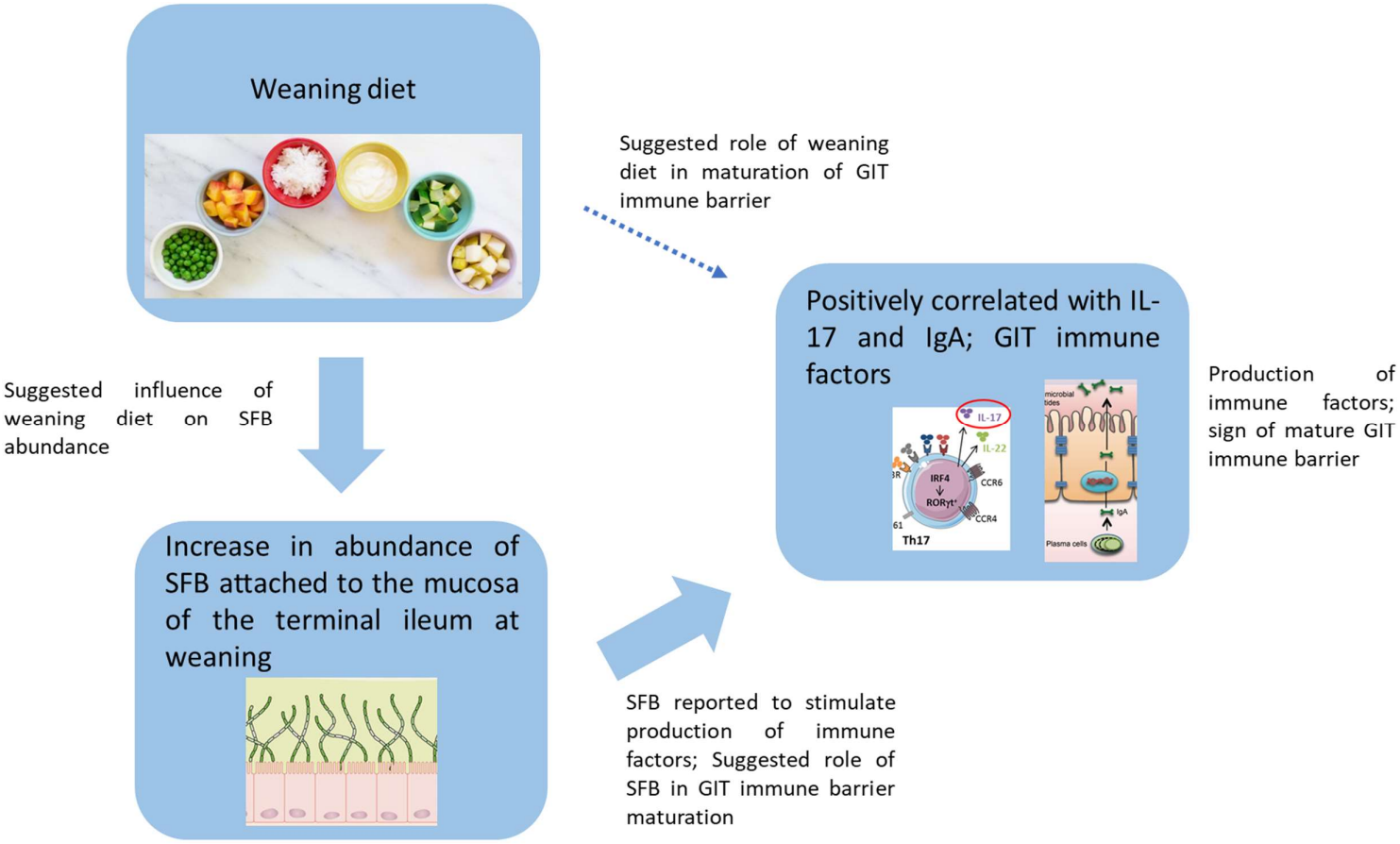

Figure 6.1. Schematic overview of the suggested role of SFB in the maturation of the infant GIT immune barrier and how the weaning diet may influence this role.

It was also further reported that mice lacking SFB in their microbiota had fewer Th17 cells in the ileum than mice with a normal SFB population and that Th17 cells were induced in the small intestinal lamina propria only after SFB were introduced [39].

Additionally, the observed change in abundance of SFB at weaning [22, 23] suggested that the complementary diet may have an influence on the function of these microbes. Complementing the exclusively-milk diet with solid foods at weaning introduces complex substrates containing novel antigens and microbes associated with food which might stimulate the infant GIT immune system. This aspect was a knowledge gap highlighted in the overview of existing literature.

This PhD project therefore sought to answer two questions that arose from the highlighted knowledge gaps:

1. Does early-life nutrition (weaning food) influence the abundance of SFB in the terminal ileum?
2. What specific food substrate \in the weaning diet influences the abundance of SFB? Would this in turn have an influence on selected GIT immune markers?

This led to the overall hypothesis that *a weaning diet enriched with a particular food substrate would increase the abundance of SFB in the terminal ileum which would then enhance markers of the GIT immune barrier maturation*.

Specific objectives were then carried out in order to test the hypothesis:

a) Conducting a literature search which resulted in a literature review (Chapter 2) that highlighted the published rat and mouse SFB genomes which predicted substrates that these commensal microbes may utilise. This review allowed for a food substrate inulin to be selected that SFB were thought to utilise.

b) Adapting a weanling mouse model for the quantification of SFB abundance, which had been reported in the literature [22] (Chapter 3). It was important to determine the most appropriate time-points for quantifying SFB in the weanling mouse model. The ileal abundance of SFB was also correlated with the concentration of one of the identified immune markers; plasma IL-17.

c) Establishing a weanling rat model for SFB quantification which had not been reported (Chapter 4). The study also compared between whole tissue homogenisation and mucosal scraping to determine the best method to collect ileal tissue samples for SFB quantification [23].

d) Exploring whether the selected food substrate inulin, and what dose, would increase or decrease the peak abundance of SFB in weanling rats. Following the selection of a food substrate, inulin, from the literature search and the necessary developments of the experimental methods, the hypothesis could then be tested (Chapter 5).

The first question inquired whether early-life nutrition (weaning food) had any influence on the abundance of SFB in the ileal contents or attached to the terminal ileum.

Prior to answering this question, the study carried out in mice (Chapter 3) attempted to replicate the observation by Ohashi, et al. [22] in their observation of the temporal profile of ileal SFB abundance in weanling mice. They reported the abundance of SFB changed from pre-weaning at day 18 until day 105 (3.5 months) [22]. The greatest changes in SFB abundance occurred primarily at weaning (day 21), peaked at day 28 and then decreased towards day 49 until day 105. Their observation indicated that changes in the abundance of SFB occurred in the window immediately pre- and post-weaning.

The first limitation of the previous study was that it was carried out in one strain of mice (BALB/c). It was unclear whether other mouse strains harboured SFB. It was important

therefore to repeat the study to ensure it was reproducible in other strains of weanling mice. The second limitation was the temporal profile and changes in SFB abundance were observed over a period of more than three months (18, 22, 28, 49, 105 days) postnatally. Given the broad age range, it was unclear when exactly the abundance of SFB increased and decreased between the postnatal days. Therefore, it was important to approximate the time-points when changes in SFB abundance occurred which led to the question that the weaning diet might influence the abundance of these microbes in the ileum.

This study reported in Chapter 3 also correlated the presence of SFB in the contents of the terminal ileum with the concentration of plasma IL-17. There were differences in the abundance of SFB in the ileum contents pre- and post-weaning. However, the SFB abundance in ileal tissue and faeces did not change over time. That observation contradicted the published findings [22] and may have been due to high variability in the data compared to published results caused by inadequate ileum tissue and faecal sample sizes. There were also no differences observed in SFB abundance between male and female mice, regardless of sample type.

The significant differences in plasma IL-17 concentration pre- and post-weaning confirmed the positive association reported between SFB and IL-17 production in other studies [39-41, 60]. There were however no differences in faecal IgA concentration over time, which was unexpected as previously a decrease in SFB abundance was negatively associated with an increase in luminal IgA concentration post-weaning [22, 33].

A major limitation of the study in Chapter 3 was the higher variability in the SFB and IgA data caused by inadequate ileum tissue, ileum contents and faecal sample sizes available for analysis from weanling mice. The suggestion was that the large differences in the weights of the samples possibly increased variability in the data, thereby decreasing the accuracy of the results. It is

possible that the type of mouse strain [189] used also contributed to the results. The study reported in Chapter 3 used a Swiss mouse strain whereas Ohashi, et al. [22] used the BALB/c strain. Differences in the genetic make-up may cause varied physiological development and hormonal responses and may have varying effects on GIT and immune interactions [189]. Another limitation was the variability of the results reported in Chapter 3 which may have been caused by the ileal tissue collection method. Of the two tissue collection methods that have been used to isolate SFB from the ileal mucosa, it was important to determine the best technique in order to decrease variability in SFB abundance data.

To overcome these limitations, a rodent model was selected that would provide enough samples for the accurate quantification of SFB in the ileum (tissue and contents) and faeces at the pre- and post-weaning age. Additionally, choosing a better method for collecting and preparing ileal tissue for SFB quantification was important as these steps influence the variability of results. The research in Chapter 4 compared whole tissue homogenisation and ileal mucosal scraping, two techniques used for collecting tissue samples for SFB quantification. It also sought to determine whether the weanling rat could provide enough samples for optimum SFB quantification.

The following method development study (Chapter 4) compared whole tissue homogenisation and ileal mucosal scraping. These two techniques, which had previously only been utilised in mouse SFB studies, were used in Sprague-Dawley rats in this study. The results indicated that whole tissue homogenisation [164] was preferrable to mucosal scraping [43] for collecting ileal tissue samples for SFB quantification [23]. The weanling rat was selected as the animal model as it could provide adequate samples for optimum SFB quantification.

For the first time, the abundance of ileal and faecal SFB between males and female rats was compared which revealed an unexpected difference at a single time-point. SFB abundance in

males sharply decreased while in females, there was a gradual decrease until the plateau was reached. That observation, however, was only different in the proximal ileal contents and may have been spurious. This was still an important observation, as prior to this study, there were no data comparing the abundance of SFB between sexes.

Notably, this study also utilised rats as the animal model for SFB, which had never been previously reported. SFB were initially observed naturally-occurring in the ileum of Sprague-Dawley rats [160] but no temporal abundance of SFB was measured in this study and other studies with weanling rats. Most published studies on SFB, particularly focused on immune system effects, have been performed on mice [22, 32, 41, 43, 60, 76, 87, 103]. This may be due to their small size and low maintenance costs [190]. Additionally, most animal studies use mice due to analytical resources being readily available for mice [190]. Only one other group determined the abundance of SFB in weanling mice, though their sample size for each of the five time-points was only five mice [22]. This aspect was improved on in Chapter 4, which included seven time-points with an average of thirteen rat pups each, ensuring decreased variability in the results. Noting that SFB appear about three weeks postnatally [22], mice at that life stage proved to be too small to provide enough samples for analysis (Chapter 3). The results from the rat study reported in Chapter 4 indicated that rats are as acceptable as mice are for use in SFB studies, though particularly for SFB studies at the pre- and post-weaning stage. The SFB abundance profile in weanling mice has also been demonstrated in weanling rats. Weanling rats are also larger than weanling mice and thus have more tissue for analyses which would decrease variability in the results.

The main limitation in the Chapter 4 study may have been the shorter time frame of the study. Compared to the mice in Chapter 3 which ranged from 20 to 41 days old, the rats ranged from 20 to 32 days old. The shorter time frame was used as the major changes in the abundance of

SFB have been observed to occur immediately pre- and post-weaning [22, 23]. A study with a time-frame similar to the one in Chapter 3 could allow for further physiological changes in the weanling rats. These important changes influence the profile of SFB abundance and the interaction between sex and age on SFB abundance. In this study, there were significant differences in faecal IgA concentration over time but none in plasma IL-17 concentration. The results from IL-17 contrasted those from Chapter 3 and those reported in the literature [39, 41, 72]. It would also be important to measure the concentrations of plasma IL-17 and faecal IgA perhaps for an extra week after 32 postnatal days. These immune factors may not necessarily change immediately with SFB abundance, but possibly at a later postnatal age [22] or in the adult stage [39, 41, 72].

The method developed in Chapter 4, along with the knowledge gained from the literature review in Chapter 2, was then used to test the project hypothesis of the study described in Chapter 5. The second question in this PhD thesis dissertation inquired whether inulin added to the weaning diet would influence the abundance of SFB. Changes in the temporal profile of SFB were reported immediately pre- and post-weaning [22, 23] suggesting an influence by the weaning food, introduced to complement the exclusively milk diet. As SFB are already present in the ileum pre-weaning, it is possible that the weaning diet provides novel substrates utilised by the resident microbiota including SFB, influencing ileal abundance. The concentration of IL-17 in plasma and IgA in faeces were also measured to determine whether the inulin-supplemented diet would in turn have an influence on these immune markers.

An analysis of the published rat and mouse SFB genomes [87] indicated the presence of multiple clusters of orthologous groups and pathways for carbohydrate metabolism. This and their preferred attachment in the distal ileum suggest SFB may acquire simple carbohydrates or metabolites of carbohydrate fermentation from their niche. Published studies have reported the presence of indigestible oligosaccharides (naturally present in human breastmilk), such as

inulin, which are added to bovine-based infant formulas to supplement the low levels of oligosaccharides [177].

Among dietary fibre, inulin is also a soluble fibre that is considered safe for infant consumption. Studies in infants fed formula supplemented with inulin reported a significant increase of beneficial faecal bacteria and decrease in coliform bacteria as well as softer faeces and increased faecal weights which imply improved bile production and bacterial fermentation [179, 180]. Additional studies on inulin-supplemented diets reported decreased flatulence and bloating in healthy adults fed 5-10 g inulin daily for five days [53], enhanced abundance of beneficial bacteria in 10% inulin-fed eight-week old piglets [54], and short chain fatty acid (SCFA) production in stool samples of an *in vitro* continuous adult fermentation system [55, 56] suggests that pre-weaned and weaned infants benefit from these properties of inulin. Additionally, the acidic environment created by the increased SCFA-production not only inhibits the growth of pathogenic bacteria [57] but also reportedly activates the immature infant immune system [58, 59]. Feeding 8-week old piglets an exclusive weaning diet modified with 10% inulin resulted in an increased relative abundance of members within *Clostridiales* in which SFB are classified [54]. Furthermore, MALDI-TOF mass spectrometry analysis of the ileal mucosa of 10-day old piglets revealed that mixed feeding including a milk-based diet and a solid diet supplemented with 3% inulin induced the up-regulation of transcriptional, translational, folding and posttranslational modification proteins that may stimulate epithelial cell proliferation [191]. This would be beneficial in pre-weaned infants as well as mice and rats during rapid growth and development of the GIT and it would also provide a medium on which SFB can attach via their holdfast structure [99].

In Chapter 5, inulin was used to determine whether diets enriched with inulin at doses ranging from 2.5% to 10% would influence the abundance of SFB in ileum (tissue, contents) and faecal samples at 24 days. However, there were no differences observed in peak abundance of SFB,

blood plasma IL-17 and faecal IgA concentration between male and female rats, regardless of inulin dosing or sample type. Inulin may not have any effect on the abundance of SFB in ileum (tissue, contents) and faecal samples. Klaasen, et al. [32] reported similar results, though their study investigated the effects of cellulose and pectin on SFB abundance in mice that were 28-56 days old, past the weaning age. In Chapter 5, only SFB abundance from the lowest (day 20) and highest (day 24) were measured based on results obtained from Chapter 4. It is unlikely that inulin resisted the increase in SFB abundance, as the SFB abundance from ileum tissue, contents and faeces was well within the upper and lower limits of the standard curve.

The reason for selecting inulin doses ranging from 2.5%-10% was based on the following studies in which infants and piglets of a similar life stage. The inulin supplemented diets of a similar dosage that were fed to the infants and piglets did not have negative effects on the overall health. The studies also reported benefits to the gut microbiota from the inulin-supplemented diets.

Seven-month-old infants fed 1.25 g/day of inulin were reported to have increased relative abundance of faecal *Bifidobacterium spp*. as well as reduced abundance of Gram-positive cocci and coliform bacteria compared to infants fed 0.75 g/day or 1 g/day of inulin [180]. In that study, inulin consumption at 1 g/day and 1.25 g/day resulted in decreased faecal pH and changes in faecal weight, faecal texture, and colour, implying improved bile production and bacterial fermentation [180]. That study concluded that inulin consumption in formula-fed infants post-weaning positively affected the microbial composition of faeces and faecal properties [180]. Healthy 12-week-old infants fed an average 1.5 g/day of inulin were reported to have increased relative abundances of faecal *Bifidobacterium* and *Lactobacillus*, without affecting the number of Bacteroides or the total anaerobic count. The faeces of the infants also tended to become softer and the amount of faeces produced increased [179].

In another study, 16S rRNA amplicon sequencing of ileal tissue from piglets fed 10% w/w inulin reported higher relative abundances of *Bifidobacteriaceae* (Actinobacteria), *Prevotellaceae* (Bacteroidetes), *Gammaproteobacteria* and *Deltaproteobateria* (Proteobacteria) and higher abundances *Ruminococcaceae* and members within *Clostridiales* (Firmicutes) [54]. These observations confirmed that members within *Clostridiales* are influenced by inulin supplementation in piglets.

As SFB are classified within *Clostridiales,* the study in Chapter 5 investigated whether SFB are influenced by inulin, which had never been investigated. Despite a lack of change in the abundance of SFB, it is possible that their activity may have increased, and they may ferment inulin or uptake metabolites produced by fermentation of inulin from other microbes in the ileal community. The higher relative abundance of other bacterial species with inulin supplementation suggests the ability to ferment inulin or utilise fermentation products pre- and post-weaning [54].

Some of the limitations of the study in Chapter 5 include those brought upon by the Corona virus disease 2019 (COVID19) lockdown. Rats were maintained in the AgResearch small animal facility in Ruakura (Hamilton, NZ) prior to the lockdown in New Zealand. The study occurred after the restrictions were lifted which meant the rats by then were older than the recommended breeding age. Additionally, restrictions at the time limited air travel meaning the rats could only be transported by road, which was longer. The older age of the dams and the mode of transportation were potential sources of stress, although no stress-related readouts of the rats were measured. These potential stress factors have potentially contributed to a reduction of rat pup numbers. The loss of pups did not affect the study design as this was mitigated by planning for pup numbers in excess of what was required.

The third question was related to the possible use of a selected food substrate, with positive effects on SFB abundance, in improving milk formulas. Although the results in this study indicated no effects by inulin on SFB abundance, this prebiotic is an important polysaccharide routinely added to commercial milk formulas [147]. It was important to select a food substrate approved for consumption by pre- and post-weaned infants as that is the targeted age group. Therefore, any future work on investigating the effects of diet on SFB in infants would require the use of approved food substrates due to the strict guidelines of studying this age group.

Overall, the limitations from this PhD project and the current study of SFB include:

Focus on one microbe (SFB) – The maturation of the infant GIT immunological barrier may rely on more than one group of microbes. This may be due to complex interactions, such as cross-feeding among the microbial groups of the ileum as well as influences from the weaning diet. The value in investigating these suggested mechanisms may expand the current knowledge on how SFB may influence the GIT immunological barrier maturation.

Focus on one carbohydrate source (inulin) – The weaning diet consists of a complex mix of substrates which might work together to modulate SFB abundance and in turn the immune system. Therefore, investigating the influence of a single food substrate on SFB abundance and the immune system might lack that complexity necessary to look at the mechanisms of action among food and microbes and the immune system. Even though it is easier to investigate the effects of a single factor, it is important to carry out these investigations within the greater context of the diet, GIT microbiota and immune system as a whole. That would likely allow for more informative conclusions on how diet and SFB as well as other microbes modulate immunological barrier maturation.

A limited range of indicators – This can be mitigated by investigating other immune markers that have been associated with SFB abundance pre- and post-weaning. Additionally,

determining gene expression of the immune markers may better indicate whether SFB abundance induced their production or not.

Use of a rodent model – Rodents are less similar to humans and therefore the effects observed from rodent studies may not translate in clinical studies. Therefore, to have more informative observations, dietary, time-course studies could be conducted with infants where the abundance of SFB can be quantified from faeces which are a proxy for SFB abundance in the ileum.

The limited study timeframe (24 days) – In the present study, the effect of inulin was investigated on the peak abundance of SFB which was 24 days post-birth. This time-point was compared only with SFB abundance at day 20 (pre-weaning). This limited timeframe did not allow for further observations on any effects of inulin on SFB abundance outside those dates. Therefore, future dietary intervention studies will require multiple time-points in order to provide conclusive results on whether inulin or other food substrates modulate SFB abundance and thus the GIT immunological barrier at weaning.

6.2. Conclusion

The overall goal of this thesis was to investigate the suggested influence of the weaning diet on the ileal abundance of SFB and if any effects modulated the GIT immune system. In conclusion, inulin added to a solid diet had no modulatory effects on the abundance of ileal SFB pre- and post-weaning.

It is possible that metabolites from the fermentation of inulin by other microbes in the ileum are utilised by SFB in a cross-feeding manner. The carbohydrate pathways predicted in the SFB genome hint that carbohydrates may mainly be metabolised by SFB for bacterial growth and maintenance. It is also likely that the presence of multiple carbohydrate metabolic pathways may be due to metabolic redundancy [192] which prevents the disruption of key metabolic processes.

The window of marked increase and eventual decrease of SFB abundance may simply be a response to more complex substrates present in the weaning diet. The lack of consistent differences in the associations between ileal and faecal SFB, plasma IL-17 and faecal IgA hint that the effects on the immune system may occur later than the period of observation in this research. Another biomarker of the immune system has been investigated such as IL-23. IL-23 has pro-inflammatory properties [193, 194] and enhances the expansion of Th17 cells and further production of IL-17 [195]. IL-23 is also secreted by activated macrophages and dendritic cells in the GIT mucosa [196]. Antigen-presenting cells reportedly produce IL-23 upon recognising SFB that are not adhered to the ileal mucosa [197]. Research has also shown that IL-23 can enhance Th17 cell expansion with the help of IL-6 [198] which functions in autoimmunity. These cytokines, which are produced by CD4 T cells that have undergone clonal expansion, can be easily quantified by ELISA. The concentration of IL-23 and IL-6 would then be related with that of IL-17 to infer the effect of SFB on the immune system.

The finding from this thesis also highlighted the influence of sex on SFB abundance which is an important biological factor that affects the microbiota composition in the GIT. Sex should be considered in future investigations on SFB since the effects on the GIT immune barrier will likely differ between males and females during maturation in infancy. This observation has been demonstrated in early life where males reportedly exhibit higher monocyte and basophil counts [199], higher natural killer cell frequencies [200] and have greater pro-inflammatory responses than females [201].

Despite the inconclusive result in relation to the effect of inulin on SFB abundance, new knowledge was developed during the course of this research. The new knowledge included the establishment of rats as an appropriate model for SFB studies especially at the weaning stage. A suitable technique for collecting ileal tissue samples for SFB quantification was also determined. The effects of sex differences on the abundance of SFB were also compared and is a factor that should not be ignored in future studies. This model for SFB studies is now available to test other substrates that may affect SFB abundance at the weaning stage.

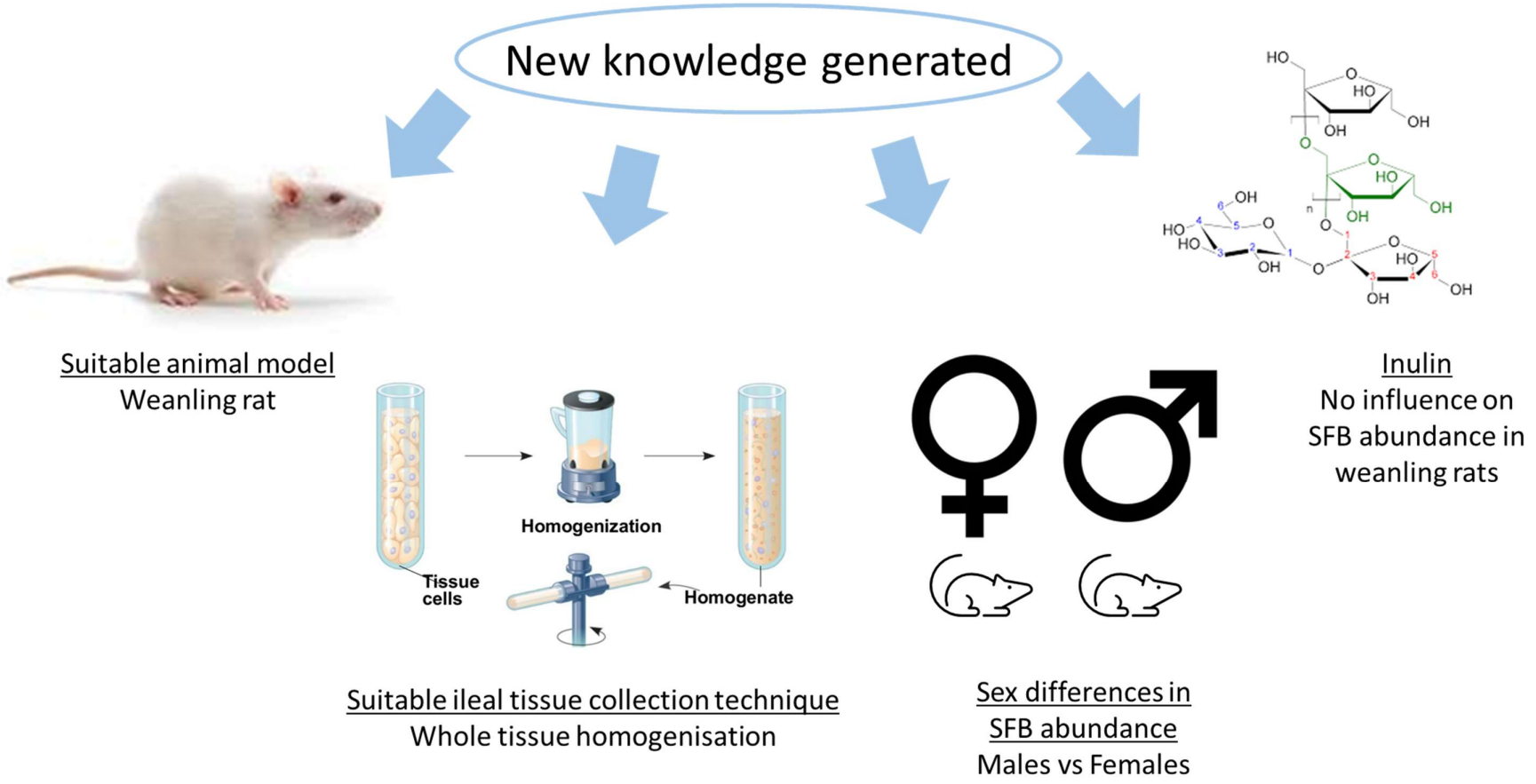

Figure 6.2. *The new knowledge contributed by this PhD project.*

6.3. Future directions

Given that the studies detailed in the PhD thesis dissertation have indicated a lack of influence of inulin on the temporal profile of SFB abundance at weaning, further investigations into the food substrates involved are necessary. Any future work on investigating the effects of diet on SFB in infants would also require the use of approved food substrates due to the strict guidelines of studying this age group. Furthermore, careful modulation of SFB abundance at weaning is recommended as well as avoiding a sustained increase in abundance which at a later age post-weaning could have detrimental effects on the immune system.

Further work would also include investigating the relationship between SFB and other distal ileum-resident microbes. Sequencing techniques such as 16S rRNA analysis utilising the genomic DNA samples from the rat studies in this project (Chapter 4 and 5) may be beneficial. The analysis would determine the relative abundances of microbial groups based on their taxonomic classifications in the terminal ileum. Comparing the abundances of the microbial groups with that of SFB over time may give an idea of their possible interactions when the GIT immune barrier is suggested to mature.

Finally, further investigations of the type of food substrates and/or weaning diets that might influence SFB abundance and modulate GIT immune barrier maturation are essential. Deciphering the perceived role of SFB in this process may contribute to the current knowledge of GIT development and eventually promote life-long health. The proposed research will also further our understanding on the ability of specific food substrates to manipulate important members of the GIT microbiota and in turn support the development of high-value foods.

References

1. Neish, A.S., *Microbes in Gastrointestinal Health and Disease.* Gastroenterology, 2009. **136**(1): p. 65-80.
2. Palmer, C., E.M. Bik, D.B. DiGiulio, D.A. Relman, and P.O. Brown, *Development of the human infant intestinal microbiota.* PLoS biology, 2007. **5**(7): p. e177.
3. Deplancke, B. and H.R. Gaskins, *Microbial modulation of innate defense: goblet cells and the intestinal mucus layer–.* The American journal of clinical nutrition, 2001. **73**(6): p. 1131S-1141S.
4. Van Der Flier, L.G. and H. Clevers, *Stem cells, self-renewal, and differentiation in the intestinal epithelium*, in *Annual Review of Physiology*. 2009. p. 241-260.
5. Groschwitz, K.R. and S.P. Hogan, *Intestinal barrier function: Molecular regulation and disease pathogenesis.* Journal of Allergy and Clinical Immunology, 2009. **124**(1): p. 3-20.
6. Podolsky, D.K., *V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1999. **277**(3): p. G495-G499.
7. Kunzelmann, K. and M. Mall, *Electrolyte transport in the mammalian colon: Mechanisms and implications for disease.* Physiological Reviews, 2002. **82**(1): p. 245-289.
8. Bröer, S., *Amino acid transport across mammalian intestinal and renal epithelia.* Physiological Reviews, 2008. **88**(1): p. 249-286.
9. Ferraris, R.P., *Dietary and developmental regulation of intestinal sugar transport.* Biochemical Journal, 2001. **360**(2): p. 265-276.
10. Anderson, R., J. Dalziel, P. Gopal, S. Bassett, A. Ellis, and N. Roy, *The role of intestinal barrier function in early life in the development of colitis*, in *Colitis*. 2012, InTech.
11. Salvo-Romero, E., C. Alonso-Cotoner, C. Pardo-Camacho, M. Casado-Bedmar, and M. Vicario, *The intestinal barrier function and its involvement in digestive disease.* Revista Espanola de Enfermedades Digestivas, 2015. **107**(11): p. 686-696.
12. Rautava, S. and W.A. Walker, *Immunophysiology and Nutrition of the Gut.* Nutrition in pediatrics: basic science and clinical applications, 2008: p. 251-260.
13. Bibiloni, R. and E.J. Schiffrin, *Intestinal host-microbe interactions under physiological and pathological conditions.* International journal of inflammation, 2010. **2010**.
14. Van Elburg, R.M., W.P.F. Fetter, C.M. Bunkers, and H.S.A. Heymans, *Intestinal permeability in relation to birth weight and gestational and postnatal age.* Archives of Disease in Childhood: Fetal and Neonatal Edition, 2003. **88**(1): p. F52-F55.
15. Patel, R.M., L.S. Myers, A.R. Kurundkar, A. Maheshwari, A. Nusrat, and P.W. Lin, *Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function.* American Journal of Pathology, 2012. **180**(2): p. 626-635.
16. Lebenthal, A. and E. Lebenthal, *The ontogeny of the small intestinal epithelium.* Journal of Parenteral and Enteral Nutrition, 1999. **23**(5_suppl): p. S3-S6.
17. Buisine, M., L. Devisme, T. Savidge, C. Gespach, B. Gosselin, N. Porchet, and J. Aubert, *Mucin gene expression in human embryonic and fetal intestine.* Gut, 1998. **43**(4): p. 519-524.
18. Mallow, E.B., A. Harris, N. Salzman, J.P. Russell, R.J. DeBerardinis, E. Ruchelli, and C.L. Bevins, *Human enteric defensins: Gene structure and developmental expression.* Journal of Biological Chemistry, 1996. **271**(8): p. 4038-4045.
19. Rumbo, M. and E.J. Schiffrin, *Ontogeny of intestinal epithelium immune functions: Developmental and environmental regulation.* Cellular and Molecular Life Sciences, 2005. **62**(12): p. 1288-1296.

20. Weitkamp, J.-H., M.J. Rosen, Z. Zhao, T. Koyama, D. Geem, T.L. Denning, M.T. Rock, D.J. Moore, M.D. Halpern, and P. Matta, *Small intestinal intraepithelial TCRγδ+ T lymphocytes are present in the premature intestine but selectively reduced in surgical necrotizing enterocolitis.* PloS one, 2014. **9**(6): p. e99042.
21. Glocker, E. and B. Grimbacher, *Inflammatory bowel disease: is it a primary immunodeficiency?* Cellular and Molecular Life Sciences, 2012. **69**(1): p. 41-48.
22. Ohashi, Y., M. Hiraguchi, C. Sunaba, C. Tanaka, T. Fujisawa, and K. Ushida, *Colonization of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice.* Anaerobe, 2010. **16**(5): p. 543-546.
23. Oemcke, L.A., R.C. Anderson, J. Rakonjac, W.C. McNabb, and N.C. Roy, *Whole tissue homogenization preferable to mucosal scraping in determining the temporal profile of segmented filamentous bacteria in the ileum of weanling rats.* Access Microbiology, 2021: p. 000218.
24. Yin, Y., Y. Wang, L. Zhu, W. Liu, N. Liao, M. Jiang, B. Zhu, H.D. Yu, C. Xiang, and X. Wang, *Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens.* ISME Journal, 2013. **7**(3): p. 615-621.
25. Davis, C.P. and D.C. Savage, *Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract.* Infection and Immunity, 1974. **10**(4): p. 948-956.
26. Bo, C., L. Li, Y. Elitsur, and H.D. Yu, *Presence of segmented filamentous bacteria in human children and its potential role in the modulation of human gut immunity.* Frontiers in microbiology, 2018. **9**: p. 1403.
27. Tajima, K., H. Ohmori, M. Tohno, H. Ohtsu, T. Tsukahara, and R. Aminov, *Segmented filamentous bacteria are a major group in terminal ileum of piglets.* Anaerobe, 2013. **23**: p. 109-111.
28. Garland, C.D., A. Lee, and M.R. Dickson, *Segmented filamentous bacteria in the rodent small intestine: Their colonization of growing animals and possible role in host resistance toSalmonella.* Microbial ecology, 1982. **8**(2): p. 181-190.
29. Jiang, H.-Q., N.A. Bos, and J.J. Cebra, *Timing, localization, and persistence of colonization by segmented filamentous bacteria in the neonatal mouse gut depend on immune status of mothers and pups.* Infection and Immunity, 2001. **69**(6): p. 3611-3617.
30. Yatsunenko, T., F.E. Rey, M.J. Manary, I. Trehan, M.G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R.N. Baldassano, and A.P. Anokhin, *Human gut microbiome viewed across age and geography.* nature, 2012. **486**(7402): p. 222-227.
31. Parrett, A. and C. Edwards, *In vitro fermentation of carbohydrate by breast fed and formula fed infants.* Archives of disease in childhood, 1997. **76**(3): p. 249-253.
32. Klaasen, H., J. Koopman, M. Van den Brink, P. Scholten, and A. Beynen, *Influence of macronutrients on segmented filamentous bacteria in the small intestine of mice.* Microbial Ecology in Health and Disease, 1991. **4**(1): p. 47-51.
33. Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan, *Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut.* Proceedings of the National Academy of Sciences, 2004. **101**(7): p. 1981-1986.
34. Klaasen, H., P. Van der Heijden, W. Stok, F. Poelma, J. Koopman, M. Van den Brink, M. Bakker, W. Eling, and A. Beynen, *Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice.* Infection and immunity, 1993. **61**(1): p. 303-306.
35. Snel, J., M. Bakker, and P. Heidt, *Quantification of antigen-specific immunoglobulin A after oral booster immunization with ovalbumin in mice mono-associated with*

segmented filamentous bacteria or Clostridiuminnocuum. Immunology letters, 1997. **58**(1): p. 25-28.
36. Talham, G.L., H.-Q. Jiang, N.A. Bos, and J.J. Cebra, *Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system.* Infection and immunity, 1999. **67**(4): p. 1992-2000.
37. Umesaki, Y., Y. Okada, S. Matsumoto, A. Imaoka, and H. Setoyama, *Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse.* Microbiology and immunology, 1995. **39**(8): p. 555-562.
38. Umesaki, Y., H. Setoyama, S. Matsumoto, A. Imaoka, and K. Itoh, *Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system.* Infection and Immunity, 1999. **67**(7): p. 3504-3511.
39. Ivanov, I.I., K. Atarashi, N. Manel, E.L. Brodie, T. Shima, U. Karaoz, D. Wei, K.C. Goldfarb, C.A. Santee, and S.V. Lynch, *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-498.
40. Farkas, A.M., C. Panea, Y. Goto, G. Nakato, M. Galan-Diez, S. Narushima, K. Honda, and I.I. Ivanov, *Induction of Th17 cells by segmented filamentous bacteria in the murine intestine.* Journal of immunological methods, 2015. **421**: p. 104-111.
41. Goto, Y., C. Panea, G. Nakato, A. Cebula, C. Lee, M.G. Diez, T.M. Laufer, L. Ignatowicz, and I.I. Ivanov, *Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation.* Immunity, 2014. **40**(4): p. 594-607.
42. Ericsson, A.C., C.E. Hagan, D.J. Davis, and C.L. Franklin, *Segmented filamentous bacteria: commensal microbes with potential effects on research.* Comparative medicine, 2014. **64**(2): p. 90-98.
43. Ericsson, A.C., G. Turner, L. Montoya, A. Wolfe, S. Meeker, C. Hsu, L. Maggio-Price, and C.L. Franklin, *Isolation of segmented filamentous bacteria from complex gut microbiota.* BioTechniques, 2015. **59**(2): p. 94-98.
44. Ivanov, I.I. and D.R. Littman, *Segmented filamentous bacteria take the stage.* Mucosal immunology, 2010. **3**(3): p. 209.
45. Klaasen, H.L.B.M., J.P. Koopman, F.G.J. Poelma, and A.C. Beynen, *Intestinal, segmented, filamentous bacteria.* FEMS Microbiology Letters, 1992. **88**(3-4): p. 165-179.
46. Klaasen, H.L.B.M., J.P. Koopman, M.E. Van Den Brink, M.H. Bakker, F.G.J. Poelma, and A.C. Beynen, *Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species.* Laboratory Animals, 1993. **27**(2): p. 141-150.
47. Schnupf, P., V. Gaboriau-Routhiau, M. Gros, R. Friedman, M. Moya-Nilges, G. Nigro, N. Cerf-Bensussan, and P.J. Sansonetti, *Growth and host interaction of mouse segmented filamentous bacteria in vitro.* Nature, 2015. **520**: p. 99.
48. Kriegel, M.A., E. Sefik, J.A. Hill, H.-J. Wu, C. Benoist, and D. Mathis, *Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice.* Proceedings of the National Academy of Sciences, 2011. **108**(28): p. 11548-11553.
49. Wu, H.-J., I.I. Ivanov, J. Darce, K. Hattori, T. Shima, Y. Umesaki, D.R. Littman, C. Benoist, and D. Mathis, *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-827.
50. Finotti, A., J. Gasparello, I. Lampronti, L.C. Cosenza, G. Maconi, V. Matarese, V. Gentili, D. Di Luca, R. Gambari, and M. Caselli, *PCR detection of segmented*

filamentous bacteria in the terminal ileum of patients with ulcerative colitis. BMJ Open Gastroenterology, 2017. **4**(1).

51. Shukla, R., U. Ghoshal, T.N. Dhole, and U.C. Ghoshal, *Fecal microbiota in patients with irritable bowel syndrome compared with healthy controls using real-time polymerase chain reaction: an evidence of dysbiosis.* Digestive diseases and sciences, 2015. **60**(10): p. 2953-2962.
52. Stepankova, R., F. Powrie, O. Kofronova, H. Kozakova, T. Hudcovic, T. Hrncir, H. Uhlig, S. Read, Z. Rehakova, and O. Benada, *Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells.* Inflammatory bowel diseases, 2007. **13**(10): p. 1202-1211.
53. Bonnema, A.L., L.W. Kolberg, W. Thomas, and J.L. Slavin, *Gastrointestinal tolerance of chicory inulin products.* Journal of the American Dietetic Association, 2010. **110**(6): p. 865-868.
54. Stolzenbach, S., L.J. Myhill, L.O.B. Andersen, L. Krych, H. Mejer, A.R. Williams, P. Nejsum, C.R. Stensvold, D.S. Nielsen, and S.M. Thamsborg, *Dietary inulin and Trichuris suis infection promote beneficial bacteria throughout the porcine gut.* Frontiers in microbiology, 2020. **11**: p. 312.
55. Patterson, J.K., K. Yasuda, R.M. Welch, D.D. Miller, and X.G. Lei, *Supplemental dietary inulin of variable chain lengths alters intestinal bacterial populations in young pigs.* The Journal of nutrition, 2010. **140**(12): p. 2158-2161.
56. Poeker, S.A., A. Geirnaert, L. Berchtold, A. Greppi, L. Krych, R.E. Steinert, T. de Wouters, and C. Lacroix, *Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS).* Scientific reports, 2018. **8**(1): p. 1-12.
57. Morrow, A.L., G.M. Ruiz-Palacios, X. Jiang, and D.S. Newburg, *Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea.* The Journal of nutrition, 2005. **135**(5): p. 1304-1307.
58. Brown, A.J., S.M. Goldsworthy, A.A. Barnes, M.M. Eilert, L. Tcheang, D. Daniels, A.I. Muir, M.J. Wigglesworth, I. Kinghorn, and N.J. Fraser, *The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids.* Journal of Biological Chemistry, 2003. **278**(13): p. 11312-11319.
59. Le Poul, E., C. Loison, S. Struyf, J.-Y. Springael, V. Lannoy, M.-E. Decobecq, S. Brezillon, V. Dupriez, G. Vassart, and J. Van Damme, *Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation.* Journal of Biological Chemistry, 2003. **278**(28): p. 25481-25489.
60. Ivanov, I.I., R. de Llanos Frutos, N. Manel, K. Yoshinaga, D.B. Rifkin, R.B. Sartor, B.B. Finlay, and D.R. Littman, *Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.* Cell host & microbe, 2008. **4**(4): p. 337-349.
61. Aas JA, P.B., Stokes LN, Olsen I, Dewhirst FE., *Defining the normal bacterial flora of the oral cavity.* Journal of Clinical Microbiology, 2005. **43**(11): p. 5721-5732.
62. Adlerberth, I., D.P. Strachan, P.M. Matricardi, S. Ahrné, L. Orfei, N. Åberg, M.R. Perkin, S. Tripodi, B. Hesselmar, R. Saalman, A.R. Coates, C.L. Bonanno, V. Panetta, and A.E. Wold, *Gut microbiota and development of atopic eczema in 3 European birth cohorts.* Journal of Allergy and Clinical Immunology, 2007. **120**(2): p. 343-350.
63. Allegretti JR, H.M., *Restoring the gut microbiome for the treatment of inflammatory bowel diseases.* World Journal of Gastroenterology, 2014. **20**(13).

64. Zoetendal, E.G., J. Raes, B. Van Den Bogert, M. Arumugam, C.C. Booijink, F.J. Troost, P. Bork, M. Wels, W.M. De Vos, and M. Kleerebezem, *The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates.* ISME Journal, 2012. **6**(7): p. 1415-1426.
65. Bevilacqua, L., M. Ovidi, E. Di Mattia, L.D. Trovatelli, and F. Canganella, *Screening of Bifidobacterium strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens.* Microbiological research, 2003. **158**(2): p. 179.
66. Fagerhol, M.K., *Calprotectin, a faecal marker of organic gastrointestinal abnormality.* The Lancet, 2000. **356**(9244): p. 1783-1784.
67. Vásquez, A., S. Ahrné, B. Jeppsson, and G. Molin, *Oral administration of Lactobacillus and Bifidobacterium strains of intestinal and vaginal origin to healthy human females: Re-isolation from faeces and vagina.* Microbial Ecology in Health and Disease, 2011. **17**(1).
68. Yan, W., C. Sun, J. Zheng, C. Wen, C. Ji, D. Zhang, Y. Chen, Z. Hou, and N. Yang, *Efficacy of fecal sampling as a gut proxy in the study of chicken gut microbiota.* Frontiers in microbiology, 2019. **10**: p. 2126.
69. Guo, M., M. Miao, Y. Wang, M. Duan, F. Yang, Y. Chen, W. Yuan, and H. Zheng, *Developmental differences in the intestinal microbiota of Chinese 1-year-old infants and 4-year-old children.* Scientific reports, 2020. **10**(1): p. 1-9.
70. Jonsson, H., *Segmented filamentous bacteria in human ileostomy samples after high-fiber intake.* FEMS Microbiology Letters, 2013. **342**(1): p. 24-29.
71. Van den Bogert, B., O. Erkus, J. Boekhorst, M.d. Goffau, E.J. Smid, E.G. Zoetendal, and M. Kleerebezem, *Diversity of human small intestinal Streptococcus and Veillonella populations.* FEMS Microbiology Ecology, 2013. **85**(2): p. 376-388.
72. Schnupf, P., V. Gaboriau-Routhiau, and N. Cerf-Bensussan, *Host interactions with Segmented Filamentous Bacteria: An unusual trade-off that drives the post-natal maturation of the gut immune system.* Seminars in Immunology, 2013. **25**(5): p. 342-351.
73. Schnupf, P., V. Gaboriau-Routhiau, P.J. Sansonetti, and N. Cerf-Bensussan, *Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world.* Current Opinion in Microbiology, 2017. **35**: p. 100-109.
74. Leidy, J., *On the existence of entophyta in healthy animals, as a natural condition.* Proceedings of the Academy of Natural Sciences of Philadelphia, 1849. **4**: p. 225-233.
75. Agace, W.W., *T-cell recruitment to the intestinal mucosa.* Trends in immunology, 2008. **29**(11): p. 514-522.
76. Lécuyer, E., S. Rakotobe, H. Lengliné-Garnier, C. Lebreton, M. Picard, C. Juste, R. Fritzen, G. Eberl, K.D. McCoy, and A.J. Macpherson, *Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses.* Immunity, 2014. **40**(4): p. 608-620.
77. Yi, J., J. Jung, D. Han, C. Surh, and Y. Lee, *Segmented Filamentous Bacteria Induce Divergent Populations of Antigen-Specific CD4 T Cells in the Small Intestine.* Molecules and cells, 2019. **42**(3): p. 228.
78. Chen, B., W. Liu, S. Bie, K. Peng, X. Shu, W. Gu, X. Wang, C. Xiang, and M. Jiang, *Adherent Bacteria in the Terminal Ileum of Children Correlates with Increased Th17 Cell Activation and Luminal Secretory IgA.* Available at SSRN 3397178, 2019.
79. Wilmore, J.R., B.T. Gaudette, D.G. Atria, T. Hashemi, D.D. Jones, C.A. Gardner, S.D. Cole, A.M. Misic, D.P. Beiting, and D. Allman, *Commensal microbes induce serum IgA responses that protect against polymicrobial sepsis.* Cell host & microbe, 2018. **23**(3): p. 302-311.

80. Cheikhyoussef, A., N. Pogori, H. Chen, F. Tian, W. Chen, J. Tang, and H. Zhang, *Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances (BLIS) produced by Bifidobacterium infantis BCRC 14602.* Food Control, 2009. **20**(6): p. 553-559.
81. Collado, M., A. Gonzalez, R. Gonzalez, M. Hernandez, M. Ferrus, and Y. Sanz, *Antimicrobial peptides are among the antagonistic metabolites produced by Bifidobacterium against Helicobacter pylori.* International journal of antimicrobial agents, 2005. **25**(5): p. 385-391.
82. Thompson, A.L., A. Monteagudo-Mera, M.B. Cadenas, M.L. Lampl, and M.A. Azcarate-Peril, *Milk-and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome.* Frontiers in cellular and infection microbiology, 2015. **5**: p. 3.
83. Lopetuso, L.R., F. Scaldaferri, V. Petito, and A. Gasbarrini, *Commensal Clostridia: leading players in the maintenance of gut homeostasis.* Gut pathogens, 2013. **5**(1): p. 23.
84. Amarri, S., F. Benatti, M. Callegari, Y. Shahkhalili, F. Chauffard, F. Rochat, K. Acheson, C. Hager, J. Benyacoub, and E. Galli, *Changes of gut microbiota and immune markers during the complementary feeding period in healthy breast-fed infants.* Journal of pediatric gastroenterology and nutrition, 2006. **42**(5): p. 488-495.
85. Jašarević, E., K.E. Morrison, and T.L. Bale, *Sex differences in the gut microbiome–brain axis across the lifespan.* Phil. Trans. R. Soc. B, 2016. **371**(1688): p. 20150122.
86. Gerard, E.P., FR), David, Bikard (Paris, FR), Pamela, Schnupf (Paris, FR), Nadine, Cerf Bensussan (Paris, FR), Valerie, Gaboriau-routhiau (Clamart, FR), Philippe, Sansonetti (Paris, FR), *Method of culturing segmented filamentous bacteria in vitro.*, E.P. Office, Editor. 2017, Institut Pasteur (Paris, FR),Fondation Imagine (Paris, FR),Assistance Publique - Hopitaux de Paris (Paris, FR),Universite Paris Descartes (Paris, FR),Institut National de la Sante et de la Recherche Medicale (INSERM) (Paris, FR).
87. Prakash, T., K. Oshima, H. Morita, S. Fukuda, A. Imaoka, N. Kumar, V.K. Sharma, S.W. Kim, M. Takahashi, N. Saitou, T.D. Taylor, H. Ohno, Y. Umesaki, and M. Hattori, *Complete genome sequences of rat and mouse segmented filamentous bacteria, a potent inducer of Th17 cell differentiation.* Cell Host and Microbe, 2011. **10**(3): p. 273-284.
88. Sczesnak, A., N. Segata, X. Qin, D. Gevers, J.F. Petrosino, C. Huttenhower, D.R. Littman, and I.I. Ivanov, *The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment.* Cell host & microbe, 2011. **10**(3): p. 260-272.
89. Zhang, L., A. Rozek, and R.E. Hancock, *Interaction of cationic antimicrobial peptides with model membranes.* Journal of Biological Chemistry, 2001. **276**(38): p. 35714-35722.
90. Zhang, L.-j. and R.L. Gallo, *Antimicrobial peptides.* Current Biology, 2016. **26**(1): p. R14-R19.
91. Litman, G.W., J.P. Rast, M.J. Shamblott, R.N. Haire, M. Hulst, W. Roess, R.T. Litman, K.R. Hinds-Frey, A. Zilch, and C.T. Amemiya, *Phylogenetic diversification of immunoglobulin genes and the antibody repertoire.* Molecular biology and evolution, 1993. **10**(1): p. 60-72.
92. Snel, J., P. Heinen, H. Blok, R. Carman, A. Duncan, P. Allen, and M. Collins, *Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus".* International Journal of Systematic and Evolutionary Microbiology, 1995. **45**(4): p. 780-782.

93. Thompson, C.L., R. Vier, A. Mikaelyan, T. Wienemann, and A. Brune, *'Candidatus Arthromitus' revised: segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae.* Environmental microbiology, 2012. **14**(6): p. 1454-1465.
94. McFall-Ngai, M., M.G. Hadfield, T.C. Bosch, H.V. Carey, T. Domazet-Lošo, A.E. Douglas, N. Dubilier, G. Eberl, T. Fukami, and S.F. Gilbert, *Animals in a bacterial world, a new imperative for the life sciences.* Proceedings of the National Academy of Sciences, 2013. **110**(9): p. 3229-3236.
95. Thompson, C.L., A. Mikaelyan, and A. Brune, *Immune-modulating gut symbionts are not "candidatus Arthromitus".* Mucosal Immunology, 2013. **6**(1): p. 200-201.
96. The University of Queensland, A. *Genome Taxonomy Database*. 2021; Available from: https://gtdb.ecogenomic.org/genomes?gid=GCF_000225365.1.
97. Child, M.W., A. Kennedy, A.W. Walker, B. Bahrami, S. Macfarlane, and G.T. Macfarlane, *Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques.* FEMS Microbiology Ecology, 2006. **55**(2): p. 299-310.
98. Ferguson, D.J.P. and A. Birch-Andersen, *Electron Microscopy of a Filamentous, Segmented Bacterium Attached to the Small Intestine of Mice from a Laboratory Animal Colony in Denmark.* Acta Pathologica Microbiologica Scandinavica Section B Microbiology, 1979. **87 B**(1-6): p. 247-252.
99. Chase, D.G. and S.L. Erlandsen, *Evidence for a complex life cycle and endospore formation in the attached, filamentous, segmented bacterium from murine ileum.* Journal of Bacteriology, 1976. **127**(1): p. 572-583.
100. Kuwahara, T., Y. Ogura, K. Oshima, K. Kurokawa, T. Ooka, H. Hirakawa, T. Itoh, H. Nakayama-Imaohji, M. Ichimura, K. Itoh, C. Ishifune, Y. Maekawa, K. Yasutomo, M. Hattori, and T. Hayashi, *The lifestyle of the segmented filamentous bacterium: A non-culturable gut-associated immunostimulating microbe inferred by whole-genome sequencing.* DNA Research, 2011. **18**(4): p. 291-303.
101. Snellen, J.E. and D.C. Savage, *Freeze-fracture study of the filamentous, segmented microorganism attached to the murine small bowel.* Journal of Bacteriology, 1978. **134**(3): p. 1099-1107.
102. Davis, C.P. and D.C. Savage, *Effect of penicillin on the succession, attachment, and morphology of segmented, filamentous microbes in the murine small bowel.* Infection and Immunity, 1976. **13**(1): p. 180-188.
103. Chen, H., Y. Yin, Y. Wang, X. Wang, and C. Xiang, *Host specificity of Segmented Filamentous Bacteria (SFB) flagellins affects their interaction patterns with mouse ileal mucosal proteins.* Applied and Environmental Microbiology, 2017.
104. Pamp, S.J., E.D. Harrington, S.R. Quake, D.A. Relman, and P.C. Blainey, *Single-cell sequencing provides clues about the host interactions of segmented filamentous bacteria (SFB).* Genome research, 2012. **22**(6): p. 1107-1119.
105. Pasztor, L., A.-K. Ziebandt, M. Nega, M. Schlag, S. Haase, M. Franz-Wachtel, J. Madlung, A. Nordheim, D.E. Heinrichs, and F. Götz, *Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins.* Journal of Biological Chemistry, 2010. **285**(47): p. 36794-36803.
106. Caselli, M., F. Cassol, V. Gentili, and D. Di Luca, *Genome sequences of segmented filamentous bacteria in animals: implications for human research.* Gut Microbes, 2012. **3**(5): p. 401-405.
107. Macpherson, A.J., M.B. Geuking, E. Slack, S. Hapfelmeier, and K.D. McCoy, *The habitat, double life, citizenship, and forgetfulness of IgA.* Immunological Reviews, 2012. **245**(1): p. 132-146.

108. Maruyama, K., M. Hida, T. Kohgo, and Y. Fukunaga, *Changes in salivary and fecal secretory IgA in infants under different feeding regimens.* Pediatrics International, 2009. **51**(3): p. 342-345.
109. Dion, C., P. Montagne, M.C. Bene, and G. Faure, *Measurement of faecal immunoglobulin a levels in young children.* Journal of clinical laboratory analysis, 2004. **18**(3): p. 195-199.
110. Lindner, C., B. Wahl, L. Föhse, S. Suerbaum, A.J. Macpherson, I. Prinz, and O. Pabst, *Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine.* Journal of experimental medicine, 2012. **209**(2): p. 365-377.
111. Aujla, S.J., P.J. Dubin, and J.K. Kolls. *Th17 cells and mucosal host defense*. in *Seminars in immunology*. 2007. Elsevier.
112. Bettelli, E., M. Oukka, and V.K. Kuchroo, *T H-17 cells in the circle of immunity and autoimmunity.* Nature immunology, 2007. **8**(4): p. 345.
113. Yang, Y., M.B. Torchinsky, M. Gobert, H. Xiong, M. Xu, J.L. Linehan, F. Alonzo, C. Ng, A. Chen, and X. Lin, *Focused specificity of intestinal T H 17 cells towards commensal bacterial antigens.* Nature, 2014. **510**(7503): p. 152.
114. Wang, Y., C. Xiang, H. Chen, Y. Yin, X. Chen, Y. Zhao, Y. Wu, and Y. Li, *Induction of intestinal Th17 cells by flagellins from segmented filamentous bacteria.* Frontiers in immunology, 2019. **10**: p. 2750.
115. Ladinsky, M.S., L.P. Araujo, X. Zhang, J. Veltri, M. Galan-Diez, S. Soualhi, C. Lee, K. Irie, E.Y. Pinker, and S. Narushima, *Endocytosis of commensal antigens by intestinal epithelial cells regulates mucosal T cell homeostasis.* Science, 2019. **363**(6431): p. eaat4042.
116. Geuking, M.B., J. Cahenzli, M.A. Lawson, D.C. Ng, E. Slack, S. Hapfelmeier, K.D. McCoy, and A.J. Macpherson, *Intestinal bacterial colonization induces mutualistic regulatory T cell responses.* Immunity, 2011. **34**(5): p. 794-806.
117. Shi, Z., J. Zou, Z. Zhang, X. Zhao, J. Noriega, B. Zhang, C. Zhao, H. Ingle, K. Bittinger, and L.M. Mattei, *Segmented Filamentous Bacteria Prevent and Cure Rotavirus Infection.* Cell, 2019. **179**(3): p. 644-658.
118. Uhlig, H.H. and F. Powrie, *Dendritic cells and the intestinal bacterial flora: a role for localized mucosal immune responses.* Journal of Clinical Investigation, 2003. **112**(5): p. 648.
119. Teng, F., C.N. Klinger, K.M. Felix, C.P. Bradley, E. Wu, N.L. Tran, Y. Umesaki, and H.-J.J. Wu, *Gut microbiota drive autoimmune arthritis by promoting differentiation and migration of Peyer's patch T follicular helper cells.* Immunity, 2016. **44**(4): p. 875-888.
120. Lee, Y.K., J.S. Menezes, Y. Umesaki, and S.K. Mazmanian, *Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis.* Proceedings of the National Academy of Sciences, 2011. **108**(Supplement 1): p. 4615-4622.
121. Irikura, V., M. Kihara, S. Yamaguchi, H. Sockett, and R. Macnab, *Salmonella typhimurium fliG and fliN mutations causing defects in assembly, rotation, and switching of the flagellar motor.* Journal of Bacteriology, 1993. **175**(3): p. 802-810.
122. Brown, P.N., M.A. Mathews, L.A. Joss, C.P. Hill, and D.F. Blair, *Crystal structure of the flagellar rotor protein FliN from Thermotoga maritima.* Journal of Bacteriology, 2005. **187**(8): p. 2890-2902.
123. Gill, S.R., M. Pop, R.T. DeBoy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett, and K.E. Nelson, *Metagenomic analysis of the human distal gut microbiome.* science, 2006. **312**(5778): p. 1355-1359.

124. Greiner, T. and F. Bäckhed, *Effects of the gut microbiota on obesity and glucose homeostasis.* Trends in Endocrinology & Metabolism, 2011. **22**(4): p. 117-123.
125. Nauta, A.J., K. Ben Amor, J. Knol, J. Garssen, and E. Van der Beek, *Relevance of pre- and postnatal nutrition to development and interplay between the microbiota and metabolic and immune systems.* The American journal of clinical nutrition, 2013. **98**(2): p. 586S-593S.
126. Jonsson, H., L.W. Hugerth, J. Sundh, E. Lundin, and A.F. Andersson, *Genome sequence of segmented filamentous bacteria present in the human intestine.* Communications biology, 2020. **3**(1): p. 1-9.
127. Wang, X. and R. Andersson, *Intestinal brush border membrane function.* Scandinavian journal of gastroenterology, 1994. **29**(4): p. 289-299.
128. Kapiki, A., C. Costalos, C. Oikonomidou, A. Triantafyllidou, E. Loukatou, and V. Pertrohilou, *The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants.* Early human development, 2007. **83**(5): p. 335-339.
129. Veereman-Wauters, G., S. Staelens, H. Van de Broek, K. Plaskie, F. Wesling, L. Roger, A. McCartney, and P. Assam, *Physiological and bifidogenic effects of prebiotic supplements in infant formulae.* Journal of pediatric gastroenterology and nutrition, 2011. **52**(6): p. 763-771.
130. Bäckhed, F., J. Roswall, Y. Peng, Q. Feng, H. Jia, P. Kovatcheva-Datchary, Y. Li, Y. Xia, H. Xie, H. Zhong, M.T. Khan, J. Zhang, J. Li, L. Xiao, J. Al-Aama, D. Zhang, Y.S. Lee, D. Kotowska, C. Colding, V. Tremaroli, Y. Yin, S. Bergman, X. Xu, L. Madsen, K. Kristiansen, J. Dahlgren, and W. Jun, *Dynamics and stabilization of the human gut microbiome during the first year of life.* Cell Host and Microbe, 2015. **17**(5): p. 690-703.
131. Koenig, J.E., A. Spor, N. Scalfone, A.D. Fricker, J. Stombaugh, R. Knight, L.T. Angenent, and R.E. Ley, *Succession of microbial consortia in the developing infant gut microbiome.* Proceedings of the National Academy of Sciences, 2011. **108**(Supplement 1): p. 4578-4585.
132. Fan, W., G. Huo, X. Li, L. Yang, and C. Duan, *Impact of diet in shaping gut microbiota revealed by a comparative study in infants during the first six months of life.* Journal of Microbiology and Biotechnology, 2014. **24**(2): p. 133-143.
133. Roger, L.C., A. Costabile, D.T. Holland, L. Hoyles, and A.L. McCartney, *Examination of faecal Bifidobacterium populations in breast-and formula-fed infants during the first 18 months of life.* Microbiology, 2010. **156**(11): p. 3329-3341.
134. O'callaghan, A. and D. Van Sinderen, *Bifidobacteria and their role as members of the human gut microbiota.* Frontiers in microbiology, 2016. **7**: p. 925.
135. Parrett, A.M., C.A. Edwards, and E. Lokerse, *Colonic fermentation capacity in vitro: development during weaning in breast-fed infants is slower for complex carbohydrates than for sugars.* The American journal of clinical nutrition, 1997. **65**(4): p. 927-933.
136. Semova, I., J.D. Carten, J. Stombaugh, L.C. Mackey, R. Knight, S.A. Farber, and J.F. Rawls, *Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish.* Cell host & microbe, 2012. **12**(3): p. 277-288.
137. Drago, L., M. Toscano, V. Rodighiero, E. De Vecchi, and G. Mogna, *Cultivable and pyrosequenced fecal microflora in centenarians and young subjects.* Journal of clinical gastroenterology, 2012. **46**: p. S81-S84.
138. Agostoni, C., I. Axelsson, O. Goulet, B. Koletzko, K.F. Michaelsen, J.W. Puntis, J. Rigo, R. Shamir, H. Szajewska, and D. Turck, *Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition.* Journal of pediatric gastroenterology and nutrition, 2004. **39**(5): p. 465-473.

139. Wall, R., S.G. Hussey, C.A. Ryan, M. O'neill, G. Fitzgerald, C. Stanton, and R.P. Ross, *Presence of two Lactobacillus and Bifidobacterium probiotic strains in the neonatal ileum.* The ISME journal, 2008. **2**(1): p. 83-91.
140. Ng, S.K. and I.R. Hamilton, *Lactate metabolism by Veillonella parvula.* Journal of bacteriology, 1971. **105**(3): p. 999-1005.
141. Wang, Y., E.K. Mortimer, K.G. Katundu, N. Kalanga, L.E. Leong, G.L. Gopalsamy, C.T. Christophersen, A.C. Richard, A. Shivasami, and G.C. Abell, *The capacity of the fecal microbiota from Malawian infants to ferment resistant starch.* Frontiers in microbiology, 2019. **10**: p. 1459.
142. Van Loo, J., P. Coussement, L. De Leenheer, H. Hoebregs, and G. Smits, *On the presence of inulin and oligofructose as natural ingredients in the western diet.* Critical Reviews in Food Science & Nutrition, 1995. **35**(6): p. 525-552.
143. Vijn, I. and S. Smeekens, *Fructan: more than a reserve carbohydrate?* Plant physiology, 1999. **120**(2): p. 351-360.
144. Hoffman, J.D., L.M. Yanckello, G. Chlipala, T.C. Hammond, S.D. McCulloch, I. Parikh, S. Sun, J.M. Morganti, S.J. Green, and A.-L. Lin, *Dietary inulin alters the gut microbiome, enhances systemic metabolism and reduces neuroinflammation in an APOE4 mouse model.* PloS one, 2019. **14**(8): p. e0221828.
145. Kleessen, B., L. Hartmann, and M. Blaut, *Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora.* British Journal of Nutrition, 2001. **86**(2): p. 291-300.
146. Mensink, M.A., H.W. Frijlink, K. van der Voort Maarschalk, and W.L. Hinrichs, *Inulin, a flexible oligosaccharide I: Review of its physicochemical characteristics.* Carbohydrate polymers, 2015. **130**: p. 405-419.
147. Oswari, H., A.D. Widodo, F. Handayani, M. Juffrie, T. Sundjaya, J. Bindels, and B. Hegar, *Dosage-Related Prebiotic Effects of Inulin in Formula-Fed Infants.* Pediatric gastroenterology, hepatology & nutrition, 2019. **22**(1): p. 63-71.
148. Gura, T., *Nature's first functional food.* Science, 2014. **345**(6198): p. 747-749.
149. Tao, N., E. DePeters, J. German, R. Grimm, and C.B. Lebrilla, *Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry.* Journal of dairy science, 2009. **92**(7): p. 2991-3001.
150. Walker, W.A., *Initial intestinal colonization in the human infant and immune homeostasis.* Annals of Nutrition and Metabolism, 2013. **63**(Suppl. 2): p. 8-15.
151. Chen, K., H. Chen, M.M. Faas, B.J. de Haan, J. Li, P. Xiao, H. Zhang, J. Diana, P. de Vos, and J. Sun, *Specific inulin-type fructan fibers protect against autoimmune diabetes by modulating gut immunity, barrier function, and microbiota homeostasis.* Molecular nutrition & food research, 2017. **61**(8): p. 1601006.
152. Vonderviszt, F. and K. Namba, *Structure, function and assembly of flagellar axial proteins.* Fibrous Proteins, 2008: p. 58-76.
153. Ronda, C., S.P. Chen, V. Cabral, S.J. Yaung, and H.H. Wang, *Metagenomic engineering of the mammalian gut microbiome in situ.* Nature methods, 2019. **16**(2): p. 167.
154. Hu, J., Y. Nomura, A. Bashir, H. Fernandez-Hernandez, S. Itzkowitz, Z. Pei, J. Stone, H. Loudon, and I. Peter, *Diversified microbiota of meconium is affected by maternal diabetes status.* PLoS ONE, 2013. **8**(11).
155. Walker, R.W., J.C. Clemente, I. Peter, and R.J.F. Loos, *The prenatal gut microbiome: Are we colonized with bacteria in utero?* Pediatric Obesity, 2017.
156. Escherich, T., *The intestinal bacteria of the neonate and breast-fed infant.* Reviews of infectious diseases, 1989. **11**(2): p. 352-356.

157. Funkhouser, L.J. and S.R. Bordenstein, *Mom Knows Best: The Universality of Maternal Microbial Transmission.* PLoS Biology, 2013. **11**(8).
158. Vail, B., P. Prentice, D.B. Dunger, I.A. Hughes, C.L. Acerini, and K.K. Ong, *Age at weaning and infant growth: primary analysis and systematic review.* The Journal of pediatrics, 2015. **167**(2): p. 317-324. e1.
159. Rowland, I., G. Gibson, A. Heinken, K. Scott, J. Swann, I. Thiele, and K. Tuohy, *Gut microbiota functions: metabolism of nutrients and other food components.* European journal of nutrition, 2018. **57**(1): p. 1-24.
160. Savage, D.C., *Localization of certain indigenous microorganisms on the ileal villi of rats.* Journal of Bacteriology, 1969. **97**(3): p. 1505.
161. Caselli, M., J. Holton, P. Boldrini, D. Vaira, and G. Calò, *Morphology of segmented filamentous bacteria and their patterns of contact with the follicle-associated epithelium of the mouse terminal ileum: implications for the relationship with the immune system.* Gut microbes, 2010. **1**(6): p. 367-372.
162. Fuentes, S., M. Egert, M. Jimenez-Valera, M. Monteoliva-Sanchez, A. Ruiz-Bravo, and H. Smidt, *A strain of Lactobacillus plantarum affects segmented filamentous bacteria in the intestine of immunosuppressed mice.* FEMS microbiology ecology, 2008. **63**(1): p. 65-72.
163. *AllPrep DNA/RNA/Protein Mini Handbook*. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/multianalyte-and-virus/allprep-dnarnaprotein-mini-kit/.
164. Godon, J.J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta, *Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis.* Applied and Environmental Microbiology, 1997. **63**(7): p. 2802-2813.
165. Org, E., M. Mehrabian, B.W. Parks, P. Shipkova, X. Liu, T.A. Drake, and A.J. Lusis, *Sex differences and hormonal effects on gut microbiota composition in mice.* Gut microbes, 2016. **7**(4): p. 313-322.
166. Elderman, M., F. Hugenholtz, C. Belzer, M. Boekschoten, A. van Beek, B. de Haan, H. Savelkoul, P. de Vos, and M. Faas, *Sex and strain dependent differences in mucosal immunology and microbiota composition in mice.* Biology of sex differences, 2018. **9**(1): p. 26.
167. Dominianni, C., R. Sinha, J.J. Goedert, Z. Pei, L. Yang, R.B. Hayes, and J. Ahn, *Sex, body mass index, and dietary fiber intake influence the human gut microbiome.* PloS one, 2015. **10**(4): p. e0124599.
168. Bolnick, D.I., L.K. Snowberg, P.E. Hirsch, C.L. Lauber, E. Org, B. Parks, A.J. Lusis, R. Knight, J.G. Caporaso, and R. Svanbäck, *Individual diet has sex-dependent effects on vertebrate gut microbiota.* Nature communications, 2014. **5**: p. 4500.
169. Yurkovetskiy, L., M. Burrows, A.A. Khan, L. Graham, P. Volchkov, L. Becker, D. Antonopoulos, Y. Umesaki, and A.V. Chervonsky, *Gender bias in autoimmunity is influenced by microbiota.* Immunity, 2013. **39**(2): p. 400-412.
170. Takagi, T., Y. Naito, R. Inoue, S. Kashiwagi, K. Uchiyama, K. Mizushima, S. Tsuchiya, O. Dohi, N. Yoshida, and K. Kamada, *Differences in gut microbiota associated with age, sex, and stool consistency in healthy Japanese subjects.* Journal of gastroenterology, 2019. **54**(1): p. 53-63.
171. Fenwick, N., G. Griffin, and C. Gauthier, *The welfare of animals used in science: How the "Three Rs" ethic guides improvements.* The Canadian Veterinary Journal, 2009. **50**(5): p. 523.
172. Afonso-Pereira, F., L. Dou, S.J. Trenfield, C.M. Madla, S. Murdan, J. Sousa, F. Veiga, and A.W. Basit, *Sex differences in the gastrointestinal tract of rats and the implications*

for oral drug delivery. European Journal of Pharmaceutical Sciences, 2018. **115**: p. 339-344.
173. Walker, D.M., D. Kirson, L.F. Perez, and A.C. Gore, *Molecular profiling of postnatal development of the hypothalamus in female and male rats.* Biology of reproduction, 2012. **87**(6): p. 129, 1-12.
174. Döhler, K. and W. Wuttke, *Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats.* Endocrinology, 1975. **97**(4): p. 898-907.
175. Ojeda, S., W. Andrews, J. Advis, and S.S. White, *Recent advances in the endocrinology of puberty.* Endocrine reviews, 1980. **1**(3): p. 228-257.
176. Koletzko, B., S. Baker, G. Cleghorn, U.F. Neto, S. Gopalan, O. Hernell, Q.S. Hock, P. Jirapinyo, B. Lonnerdal, P. Pencharz, H. Pzyrembel, J. Ramirez-Mayans, R. Shamir, D. Turck, Y. Yamashiro, and D. Zong-Yi, *Global standard for the composition of infant formula: Recommendations of an ESPGHAN coordinated international expert group.* Journal of Pediatric Gastroenterology and Nutrition, 2005. **41**(5): p. 584-599.
177. Boehm, G.n. and G. Moro, *Structural and functional aspects of prebiotics used in infant nutrition.* The Journal of nutrition, 2008. **138**(9): p. 1818S-1828S.
178. Fewtrell, M., J. Bronsky, C. Campoy, M. Domellöf, N. Embleton, N.F. Mis, I. Hojsak, J.M. Hulst, F. Indrio, and A. Lapillonne, *Complementary feeding: a position paper by the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Committee on Nutrition.* Journal of pediatric gastroenterology and nutrition, 2017. **64**(1): p. 119-132.
179. Sook-He, K., D. Lee, and D. Meyer, *Supplementation of infant formula with native inulin has a prebiotic effect in formula-fed babies.* Asia Pacific journal of clinical nutrition, 2007. **16**(1): p. 172.
180. Yap, W.K., S. Mohamed, M.H. Jamal, M. Diederick, and Y.A. Manap, *Changes in infants faecal characteristics and microbiota by inulin supplementation.* Journal of clinical biochemistry and nutrition, 2008. **43**(3): p. 159-166.
181. Uerlings, J., M. Schroyen, E. Willems, S. Tanghe, G. Bruggeman, J. Bindelle, and N. Everaert, *Differential effects of inulin or its fermentation metabolites on gut barrier and immune function of porcine intestinal epithelial cells.* Journal of Functional Foods, 2020. **67**: p. 103855.
182. Bolotin, A., T. De Wouters, P. Schnupf, C. Bouchier, V. Loux, M. Rhimi, A. Jamet, R. Dervyn, S. Boudebbouze, and H.M. Blottière, *Genome sequence of "Candidatus Arthromitus" sp. strain SFB-mouse-NL, a commensal bacterium with a key role in postnatal maturation of gut immune functions.* Genome announcements, 2014. **2**(4).
183. Young, W., N.C. Roy, J. Lee, B. Lawley, D. Otter, G. Henderson, and G.W. Tannock, *Bowel microbiota moderate host physiological responses to dietary konjac in weanling rats.* The Journal of nutrition, 2013. **143**(7): p. 1052-1060.
184. Gourbeyre, P., N. Desbuards, G. Grémy, S. Le Gall, M. Champ, S. Denery-Papini, and M. Bodinier, *Exposure to a galactooligosaccharides/inulin prebiotic mix at different developmental time points differentially modulates immune responses in mice.* Journal of agricultural and food chemistry, 2012. **60**(48): p. 11942-11951.
185. Gourbeyre, P., N. Desbuards, G. Grémy, O. Tranquet, M. Champ, S. Denery-Papini, and M. Bodinier, *Perinatal and postweaning exposure to galactooligosaccharides/inulin prebiotics induced biomarkers linked to tolerance mechanism in a mouse model of strong allergic sensitization.* Journal of agricultural and food chemistry, 2013. **61**(26): p. 6311-6320.

186. Gourbeyre, P., S. Denery, and M. Bodinier, *Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions.* Journal of leukocyte biology, 2011. **89**(5): p. 685-695.
187. Canadian Council on Animal Care, C., *Guide to the Care and Use of Experimental Animals.* 1984. **1 (2nd ed)**.
188. Tian, S., J. Wang, H. Yu, J. Wang, and W. Zhu, *Changes in ileal microbial composition and microbial metabolism by an early-life galacto-oligosaccharides intervention in a neonatal porcine model.* Nutrients, 2019. **11**(8): p. 1753.
189. Simon, M.M., S. Greenaway, J.K. White, H. Fuchs, V. Gailus-Durner, S. Wells, T. Sorg, K. Wong, E. Bedu, E.J. Cartwright, R. Dacquin, S. Djebali, J. Estabel, J. Graw, N.J. Ingham, I.J. Jackson, A. Lengeling, S. Mandillo, J. Marvel, H. Meziane, F. Preitner, O. Puk, M. Roux, D.J. Adams, S. Atkins, A. Ayadi, L. Becker, A. Blake, D. Brooker, H. Cater, M.-F. Champy, R. Combe, P. Danecek, A. di Fenza, H. Gates, A.-K. Gerdin, E. Golini, J.M. Hancock, W. Hans, S.M. Hölter, T. Hough, P. Jurdic, T.M. Keane, H. Morgan, W. Müller, F. Neff, G. Nicholson, B. Pasche, L.-A. Roberson, J. Rozman, M. Sanderson, L. Santos, M. Selloum, C. Shannon, A. Southwell, G.P. Tocchini-Valentini, V.E. Vancollie, H. Westerberg, W. Wurst, M. Zi, B. Yalcin, R. Ramirez-Solis, K.P. Steel, A.-M. Mallon, M.H. de Angelis, Y. Herault, and S.D.M. Brown, *A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains.* Genome Biology, 2013. **14**(7): p. R82.
190. Bryda, E.C., *The Mighty Mouse: the impact of rodents on advances in biomedical research.* Missouri medicine, 2013. **110**(3): p. 207.
191. Herosimczyk, A., A. Lepczyński, M. Ożgo, M. Barszcz, M. Marynowska, A. Tuśnio, M. Taciak, A. Markulen, and J. Skomiał, *Proteome changes in ileal mucosa of young pigs resulting from different levels of native chicory inulin in the diet.* Journal of Animal and Feed Sciences, 2018. **27**(3): p. 229-237.
192. Sambamoorthy, G. and K. Raman, *Understanding the evolution of functional redundancy in metabolic networks.* Bioinformatics, 2018. **34**(17): p. i981-i987.
193. Oppmann, B., R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, and K. Singh, *Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12.* Immunity, 2000. **13**(5): p. 715-725.
194. Kopp, T., P. Lenz, C. Bello-Fernandez, R.A. Kastelein, T.S. Kupper, and G. Stingl, *IL-23 production by cosecretion of endogenous p19 and transgenic p40 in keratin 14/p40 transgenic mice: evidence for enhanced cutaneous immunity.* The Journal of Immunology, 2003. **170**(11): p. 5438-5444.
195. Aggarwal, S., N. Ghilardi, M.-H. Xie, F.J. de Sauvage, and A.L. Gurney, *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17.* Journal of Biological Chemistry, 2003. **278**(3): p. 1910-1914.
196. McKenzie, B.S., R.A. Kastelein, and D.J. Cua, *Understanding the IL-23–IL-17 immune pathway.* Trends in immunology, 2006. **27**(1): p. 17-23.
197. Sano, T., W. Huang, J.A. Hall, Y. Yang, A. Chen, S.J. Gavzy, J.-Y. Lee, J.W. Ziel, E.R. Miraldi, and A.I. Domingos, *An IL-23R/IL-22 circuit regulates epithelial serum amyloid A to promote local effector Th17 responses.* Cell, 2015. **163**(2): p. 381-393.
198. Ghoreschi, K., A. Laurence, X.-P. Yang, C.M. Tato, M.J. McGeachy, J.E. Konkel, H.L. Ramos, L. Wei, T.S. Davidson, and N. Bouladoux, *Generation of pathogenic TH 17 cells in the absence of TGF-β signalling.* Nature, 2010. **467**(7318): p. 967-971.
199. Bellamy, G., R. Hinchliffe, K. Crawshaw, A. Finn, and F. Bell, *Total and differential leucocyte counts in infants at 2, 5 and 13 months of age.* Clinical & Laboratory Haematology, 2000. **22**(2): p. 81-87.

200. Lee, B.W., H.K. Yap, F.T. Chew, T.C. Quah, K. Prabhakaran, G.S. Chan, S.C. Wong, and C.C. Seah, *Age-and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: From birth to adulthood.* Cytometry: The Journal of the International Society for Analytical Cytology, 1996. **26**(1): p. 8-15.
201. Casimir, G.J., F. Heldenbergh, L. Hanssens, S. Mulier, C. Heinrichs, N. Lefevre, J. Désir, F. Corazza, and J. Duchateau, *Gender differences and inflammation: an in vitro model of blood cells stimulation in prepubescent children.* Journal of inflammation, 2010. **7**(1): p. 1-7.

Appendix I

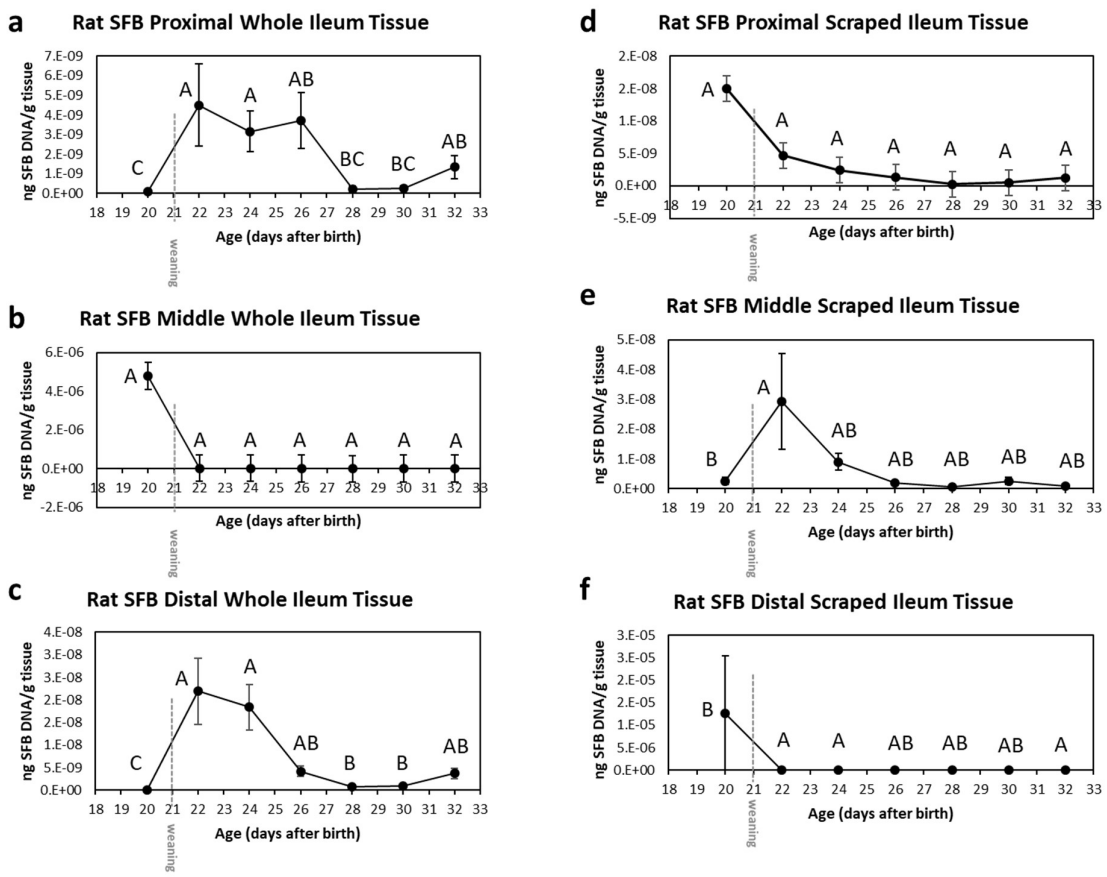

Figure 4.7. *Abundance of SFB in the whole ileal tissue (a, b, c) and ileal mucosal scrapes (d, e, f) of conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle, and distal ileum. Data are untransformed and are shown as the mean values of n=14 pups (20 days postnatally) and n=13 pups (22-32 days postnatally). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

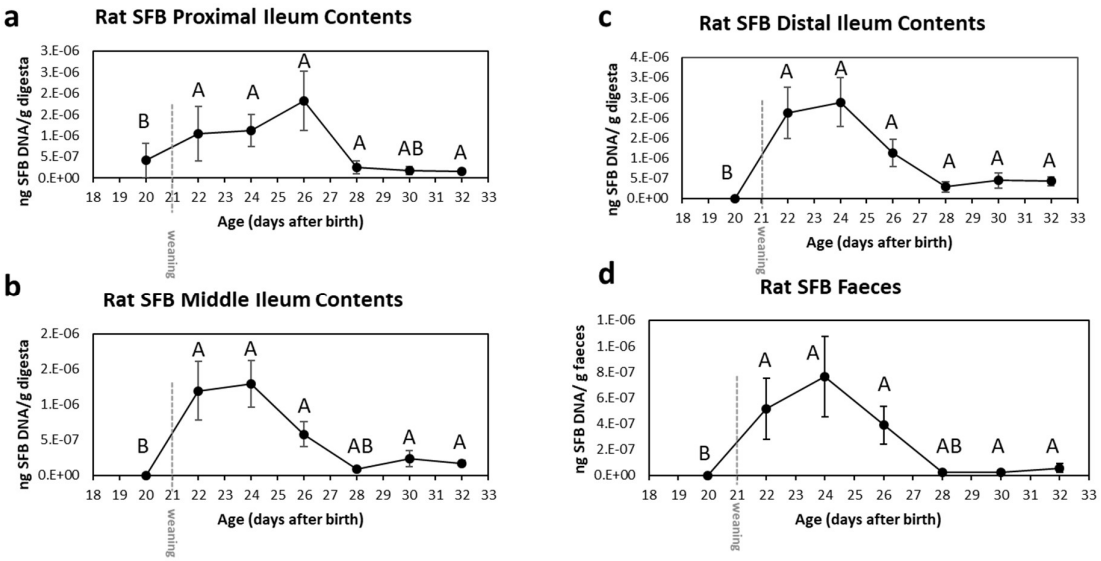

Figure 4.8. *The abundance of SFB in the ileal content (a, b, c) and faecal (d) samples collected from conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle, and distal ileum. Data are untransformed and are shown as the mean values of n=14 pups (20 days postnatally) and n=13 pups (22-32 days postnatally). The bars represent the standard error mean (SEM). Values without common letters differ significantly, $p < 0.05$.*

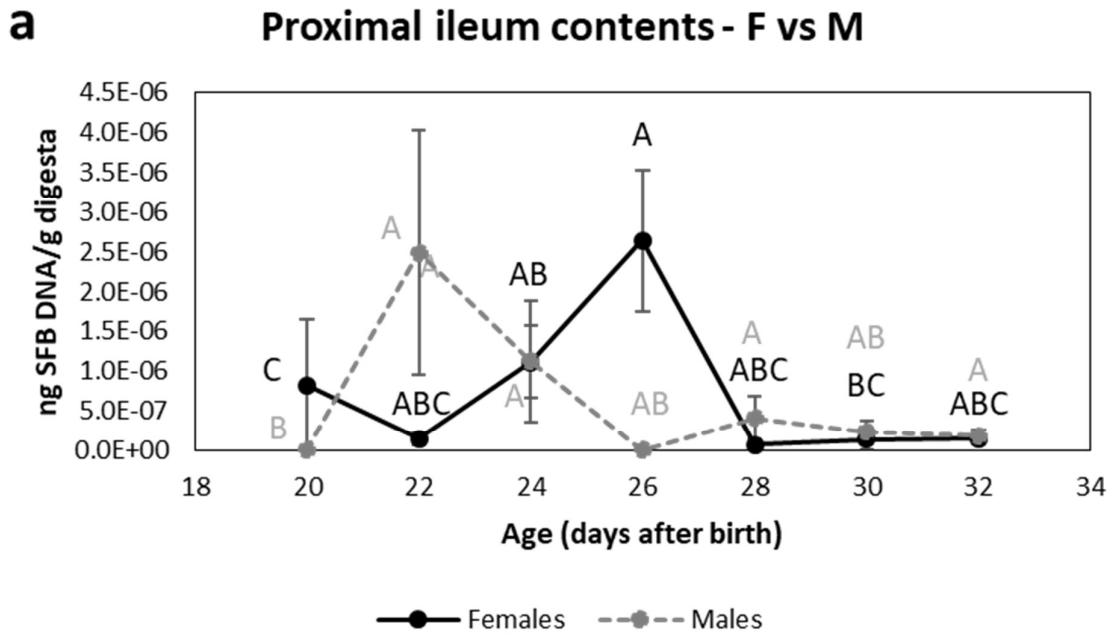

Figure 4.9. *Comparison of the abundance of SFB in the proximal ileal content samples between female and male Sprague-Dawley rat pups. The rat pups were weaned on day 21. Data are untransformed and are shown as the mean values of females (n=57) versus males (n=35). Values without common letters differ significantly, $p < 0.05$.*

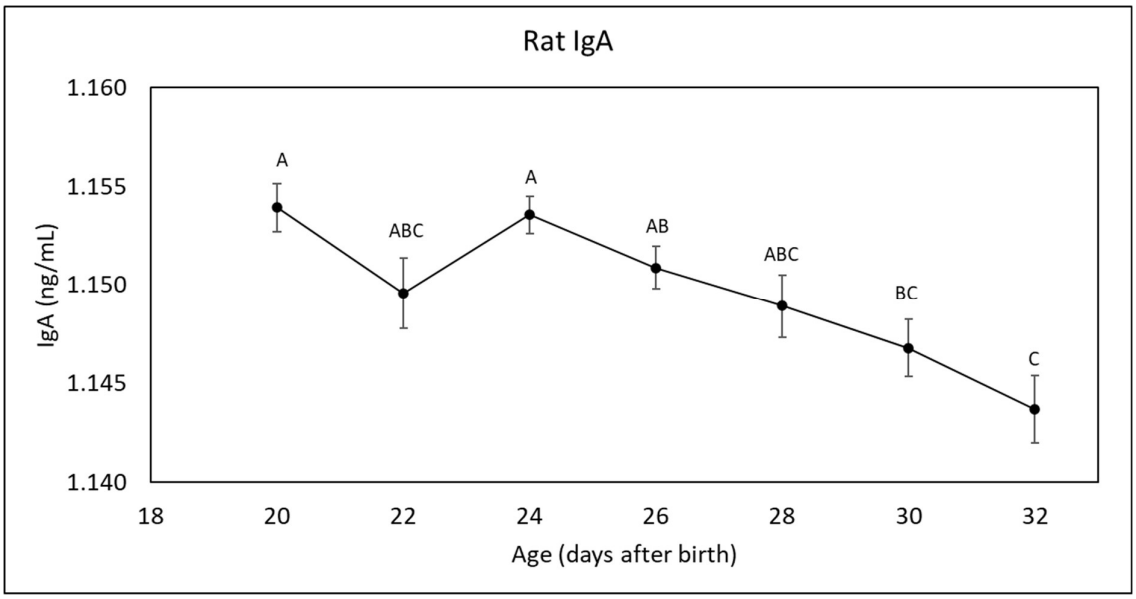

Figure 4.10. *Concentration of IgA in faeces of conventionally reared male and female Sprague-Dawley rat pups. The rat pups were weaned on day 21. Data are untransformed and are shown as the mean values of n=13 pups (22-32 days) and n=14 pups (20 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly,* $p < 0.05$.

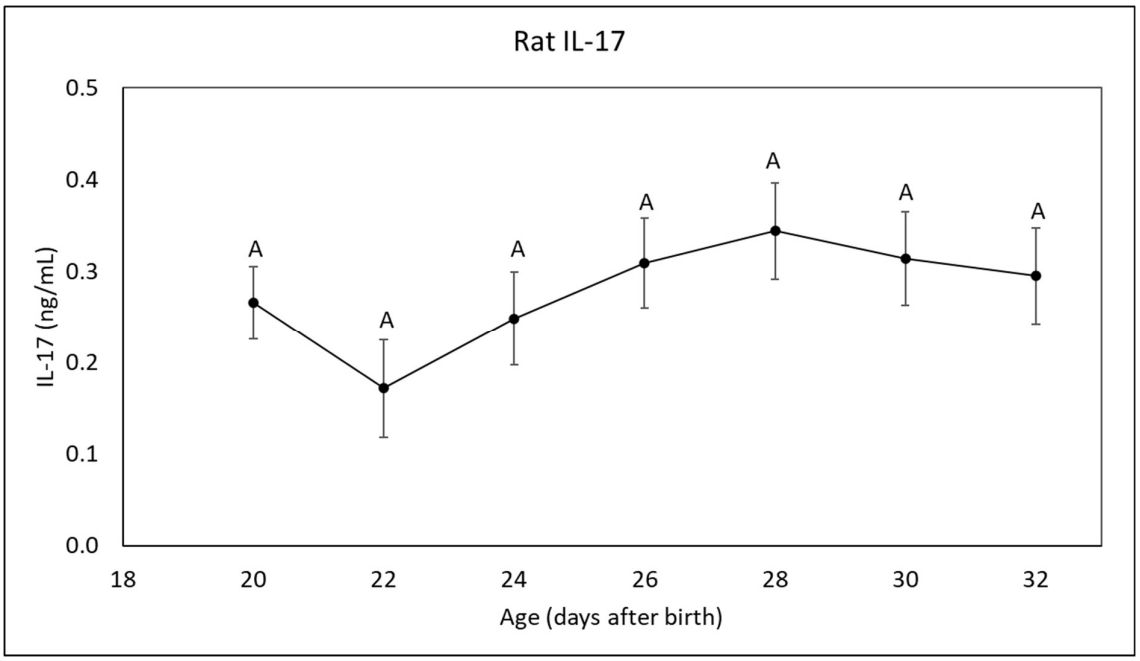

Figure 4.11. *Concentration of IL-17in plasma of conventionally reared male and female Sprague-Dawley rat pups. The rat pups were weaned on day 21. Data are untransformed and are shown as the mean values of n=13 pups (22-32 days) and n=14 pups (20 days). The bars represent the standard error mean (SEM). Values without common letters differ significantly,* $p < 0.05$.

Appendix II

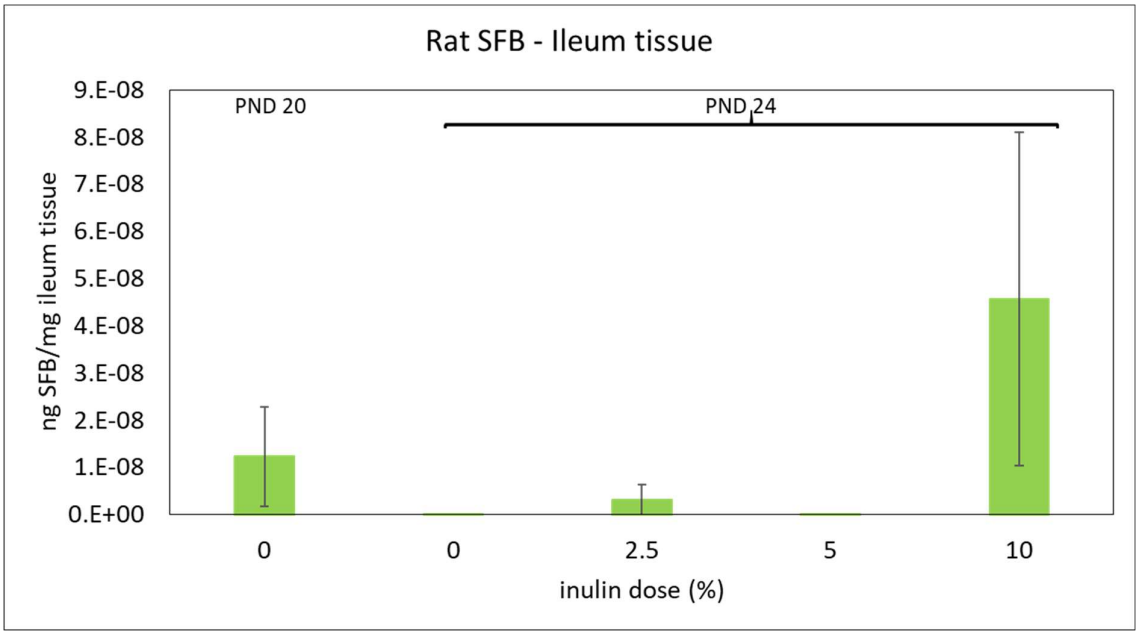

Figure 5.11. *Abundance of SFB from whole ileum tissue of conventionally reared Sprague-Dawley rat pups fed 0% 2.5%, 5% and 10% inulin diets. Samples were collected from the distal ileum. Data are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses at 20 and 24 days (H(4)=3.16; p=0.531).*

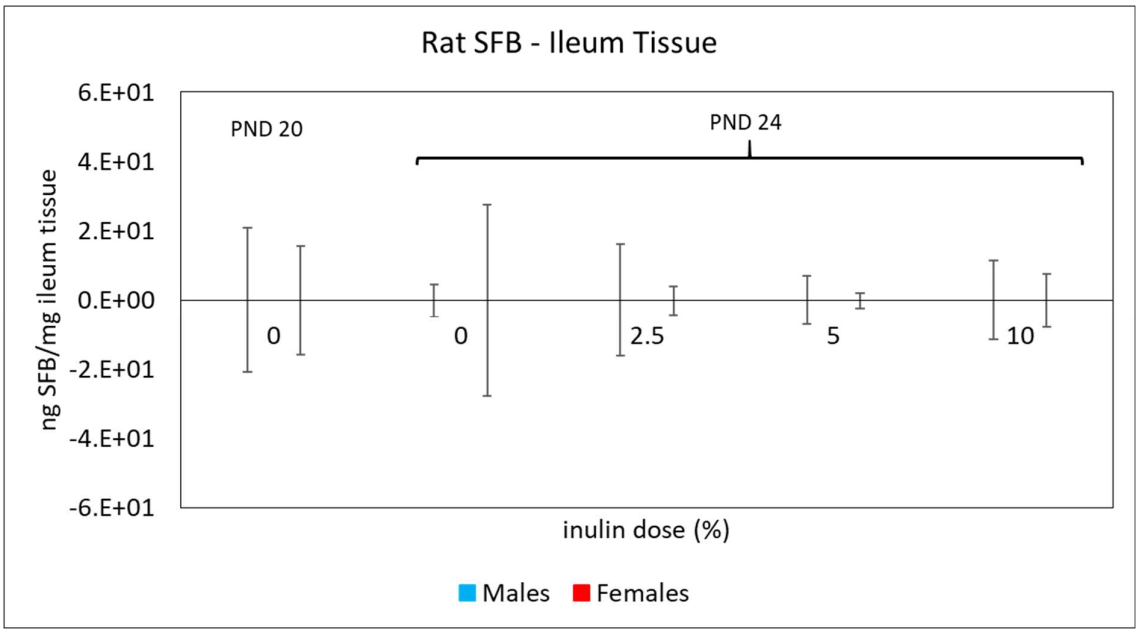

Figure 5.12. *Abundance of SFB from whole ileum tissue of conventionally reared male and female Sprague-Dawley rat pups fed 0%, 2.5%, 5% and 10% inulin diets. Samples were collected from the distal ileum. Data are plotted on a logarithm 10 scale and are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 20 and 24 days (p=0.267).*

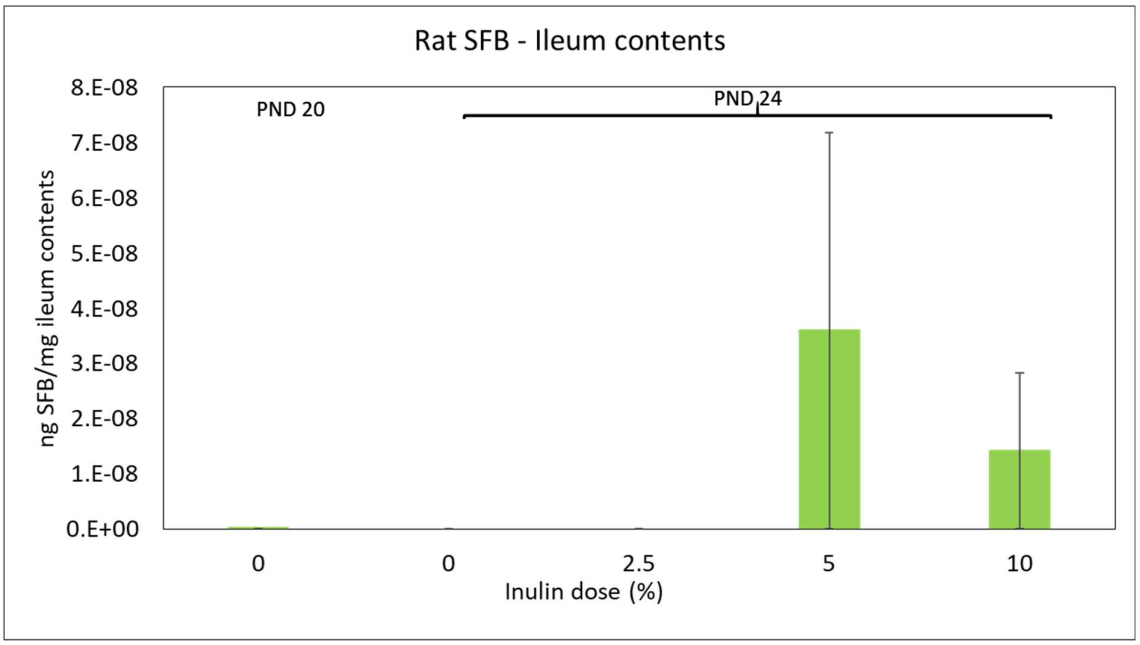

Figure 5.13. *Abundance of SFB from ileum contents of conventionally reared Sprague-Dawley rat pups. Samples were collected from the distal ileum. Data are shown as mean values of n=12 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses (H(4)=2.55; p=0.637).*

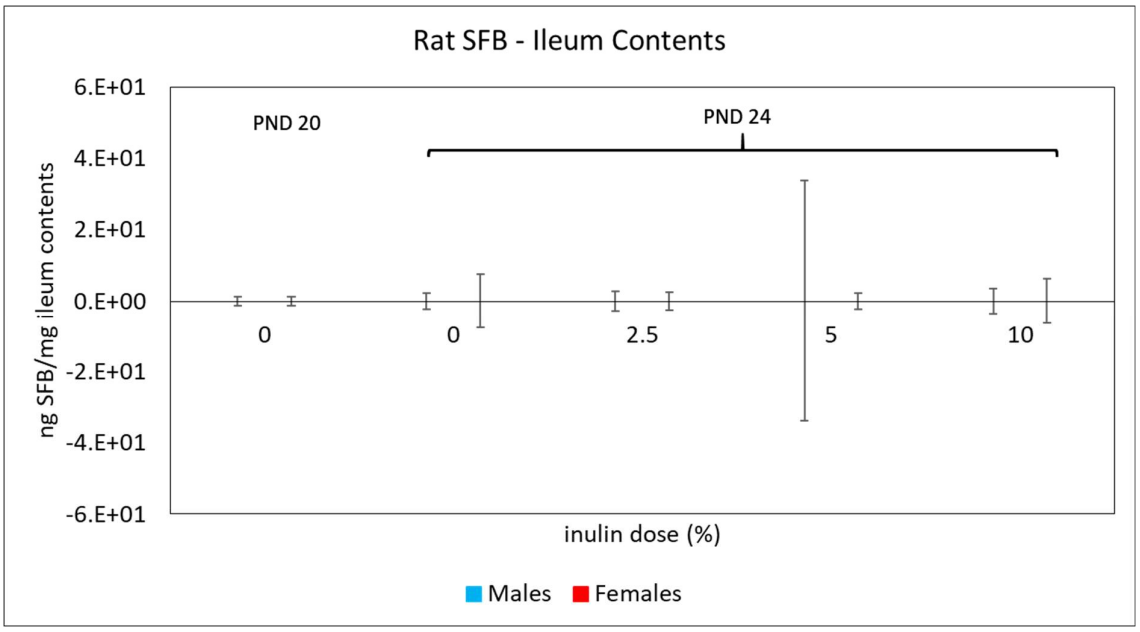

Figure 5.14. *Abundance of SFB from ileum contents of conventionally reared male and female Sprague-Dawley rat pups. Samples were collected from the distal ileum. Data are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 20 and 24 days (p=0.355).*

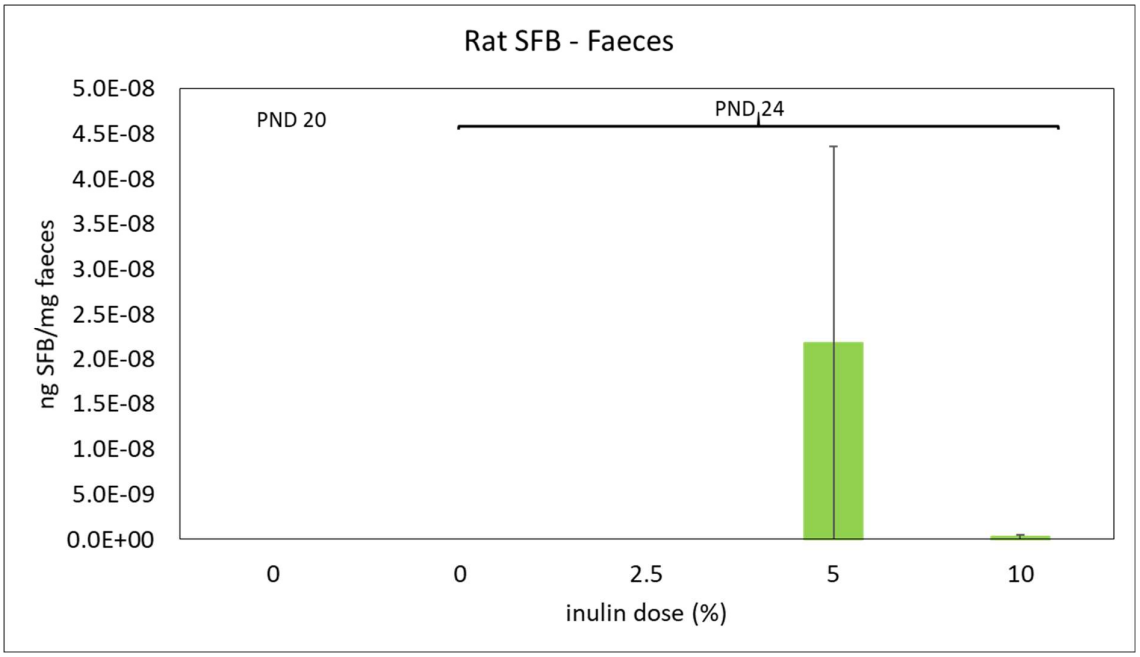

Figure 5.15. *Abundance of SFB from faeces of conventionally reared Sprague-Dawley rat pups. Samples were collected from the distal colon. Data are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between the inulin doses (H(4)=4.04; p=0.401).*

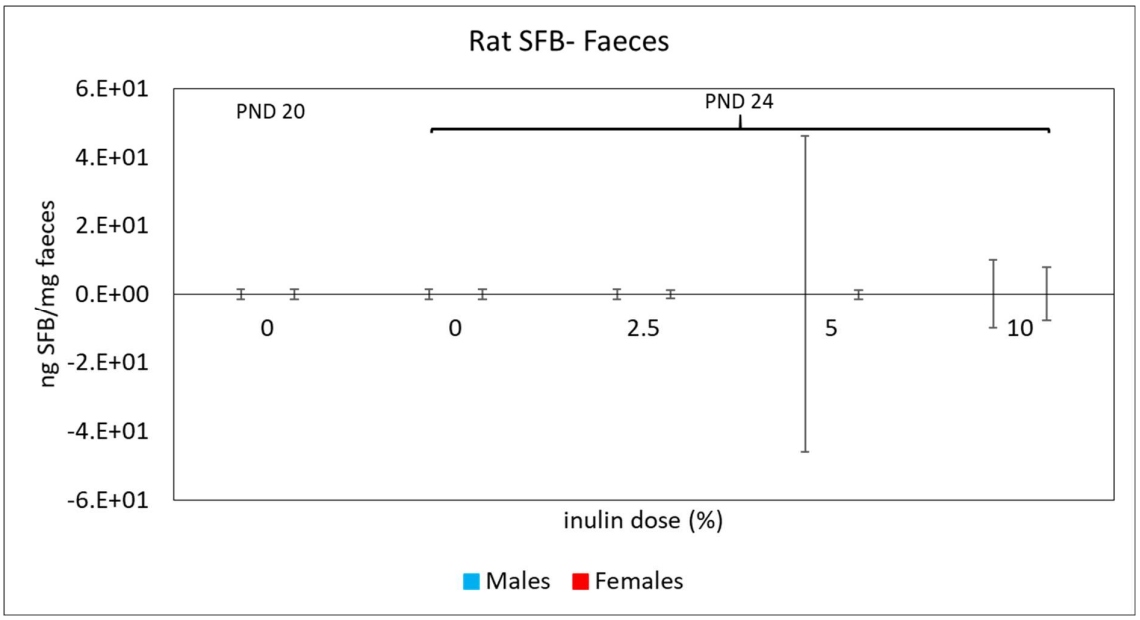

Figure 5.16. *Abundance of SFB from faeces of conventionally reared male and female Sprague-Dawley rat pups. Samples were collected from the distal colon. Data are shown as mean values of n=13 pups (0%; 20 days), n=10 pups (0%; 24 days), n=14 pups (2.5%, 5%, 10%; 24 days). The bars represent the standard error mean (SEM). There were no differences in SFB abundance between males and females fed different inulin doses at 20 and 24 days (p=0.308).*

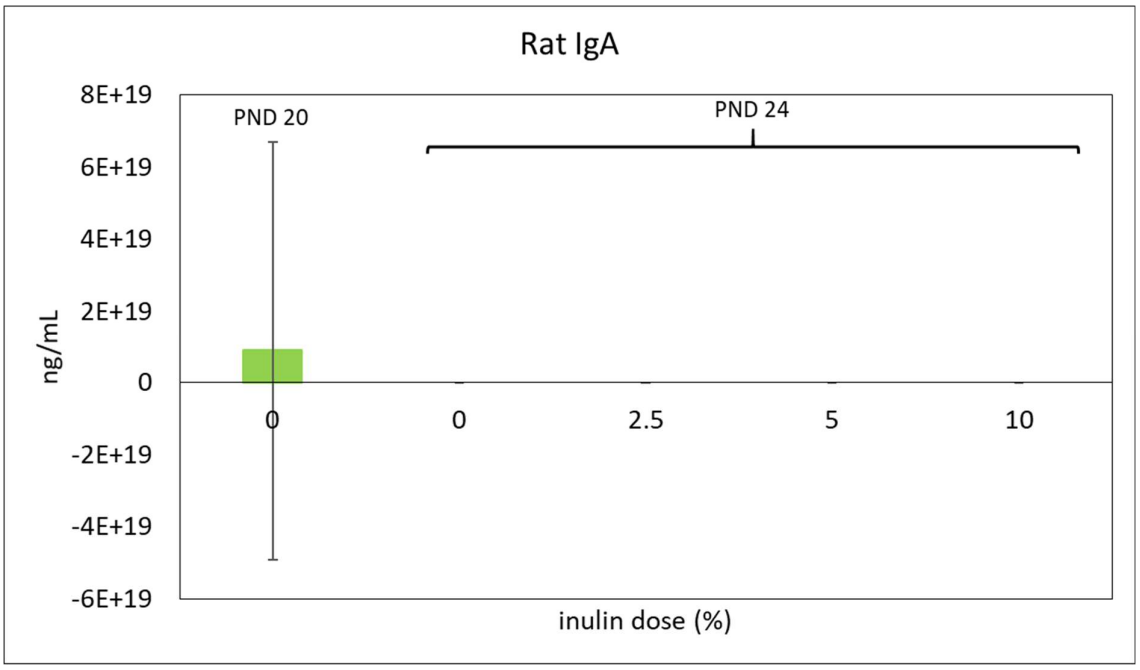

Figure 5.17. *Concentration of IgA in faeces of conventionally reared Sprague-Dawley rat pups. The limit of detection for faecal IgA was 1.03 ng/mL. Data are shown as the mean values of n=13 pups (0% inulin; 20 days), n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). The concentration of IgA in the 20-day-old rats fed 0% inulin diet was significantly lower compared to that of the 24-day-old rats fed 0%, 2.5%, 5% and 10% inulin diets (H(4)=18.30; p=0.001).*

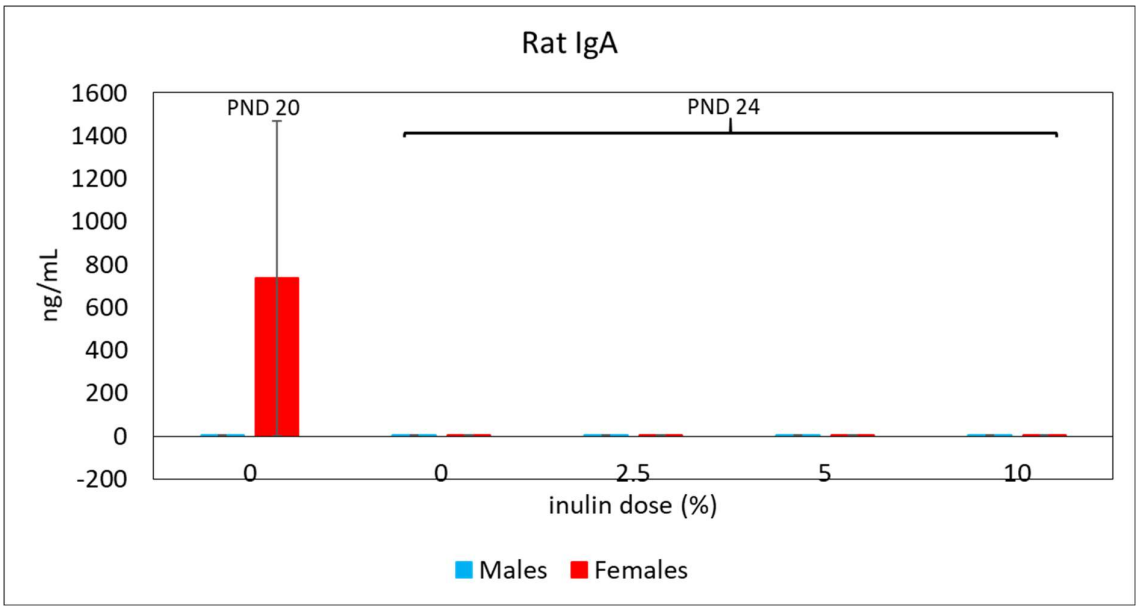

Figure 5.18. *Concentration of IgA in faeces of conventionally reared male and female Sprague-Dawley rat pups. The limit of detection for faecal IgA was 1.03 ng/mL. Data are shown as the mean values of n=13 pups (0% inulin; 20 days), n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IgA concentration between males and females fed different inulin doses at 20 and 24 days (p=0.592).*

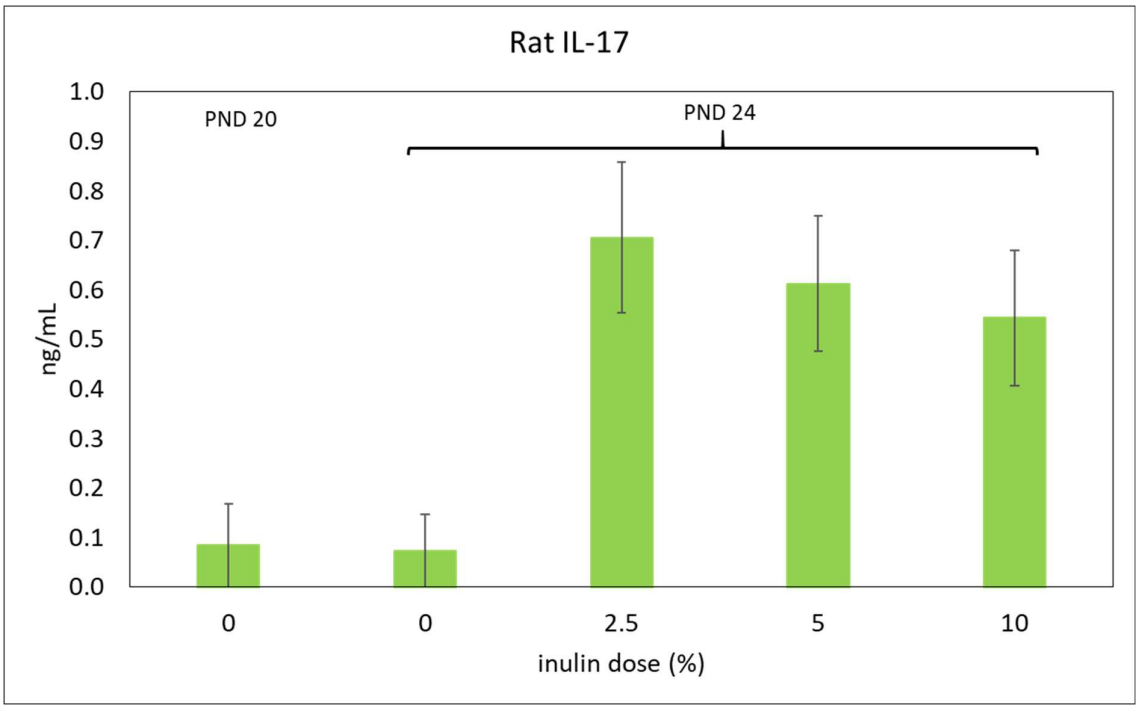

Figure 5.19. *Concentration of IL-17 in plasma of conventionally reared Sprague-Dawley rat pups. The limit of detection for plasma IL-17 was 0.72 ng/mL. Data are shown as the mean values of n=13 pups (0% inulin; 20 days), n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). The concentrations of IL-17 in the 20 and 24-day-old rats fed the 0% inulin diet were significantly lower compared to those of the 24-day-old rats on the 2.5%, 5% and 10% inulin diets (H(4)=16.95; p=0.002).*

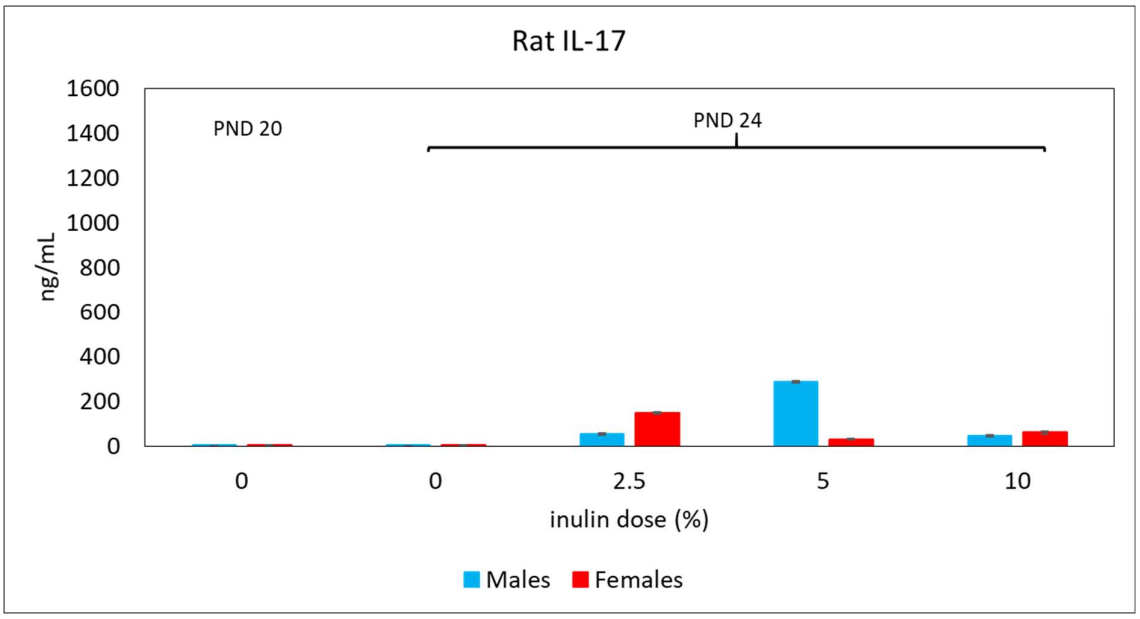

Figure 5.20. *Concentration of IL-17 in plasma of conventionally reared male and female Sprague-Dawley rat pups. The limit of detection for plasma IL-17 was 0.72 ng/mL. Data are shown as the mean values of n=13 pups (0% inulin; 20 days) n=10 pups (0% inulin; 24 days), n=14 pups (2.5%, 5%, 10% inulin; 24 days). The bars represent the standard error mean (SEM). There were no differences in IL-17 concentration between males and females fed different inulin doses at 20 and 24 days (p=0.155).*

Appendix IIIa

Animal Ethics Application No. 14041

AE Application 14041 ~ (Status=CLOSED)(Applicant=MAIERE) Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning

Group	Line	Question	Answer
		ASSOCIATED Documents	
		AE Modification 2259 ~ CLOSED	(AE APPLICATION 14041) Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning
		AE Stats 7457 ~ ACCEPTED	(AE APPLICATION 14041) Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning
		AE Report A 14041 ~ ACCEPTED	(AE APPLICATION 14041) Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning
		AE Report B 14041 ~ ACCEPTED	(AE APPLICATION 14041) Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning
		ADMINISTRATIVE Details	
0	1	**Title**	Determination of the abundance of segmented filamentous bacteria in ileal and faecal samples of mice pre- and post-weaning
0	3	**Applicant**	Eva Maier
0	4	**Institution**	AgResearch Limited
0	5	**Business Address**	AgResearch Grasslands Tennent Drive Palmerston North 4442
0	6	**Phone**	06 351 8326
0	7	**Mobile phone**	0211572041
0	8	**Email**	eva.maier@agresearch.co.nz
0	9	**Contact details**	
0	10	**Name of person responsible for the animals (MUST be named on and sign the personnel page)**	Eva Maier
0	11	**Phone**	06 351 8326
0	12	**Mobile phone**	021157204
0	13	**Email**	eva.maier@agresearch.co.nz
0	14	**Person responsible for entry of Trial Drug and animal manipulation data into Animal Tools database. (AgResearch requirement only ~ MUST be named on and sign the personnel page). If no AgR facilities are being used please enter Not Applicable**	Not Applicable
0	15	**Program Manager (MUST be named on and sign the personnel page)**	Nicole Roy/ Rachel Anderson
0	16	**Biometrician (MUST be named on and sign the personnel page - or hard [paper] copy if no computer access)**	Catherine Lloyd-West
0	17	**Facility Manager (MUST be named on and sign the personnel page - or hard [paper] copy if no computer access)**	Dan Robinson, Ric Broadhurst
0	18	**Is this a new experiment?**	Yes
0	19	**Project Dates ~ PLEASE enter all dates in the form requested [dd/mm/yy] AND ensure that the start date you enter occurs after the next meeting of your committee**	
0	20	**Start Date (dd/mm/yy)**	06/03/2017
0	21	**Finish Date (dd/mm/yy)**	27/03/2017

0	23	**Are there FOOD SAFETY issues under the Animal Products Act, or Regulatory approval associated with this project? If you are using Laboratory Rodents and/or other**	No
		species that are never used for human consumption or pet food, or if the animals in the project will be safe to enter the food chain, please type the word NO and then proceed to the next question. However, if some or all of the animals must be excluded from entering the food chain because of drugs they will be given or because of Regulatory Approval requirements, please type YES and then explain why. Animals that may not enter the food chain must be PINK TAGGED.	
0	24	**Māori Consultation. If there is any uncertainty, it is the applicant's responsibility to approach local Māori representatives for clarification.**	
0	25	**Do any aspects of this project require consultation with Māori?**	No
0	27	**Are you requesting URGENT consideration? Please only tick YES if the AEC needs to consider this Application before the next scheduled meeting.**	No
0	29	**Database Administration**	
0	30	**From the Dropdown, AgResearch users please indicate the name of your Section; Parented users please select EXTERNAL USER**	Food and Bio-based Products
		AEC JURISDICTION	
1	1	**Which committee are you applying to? (Use Dropdown box)**	Grasslands
1	2	**Some aspects of this work will also be undertaken under the jurisdiction of the AgResearch AEC at (Use Dropdown box)**	Ruakura
1	5	**Are staff, animals, or facilities of other organisation(s) involved in this project?**	No
1	7	**Does the Ethics committee of that institution(s) have a copy of this application?**	No
1	8	**Has this application, a similar or largely similar application been submitted to another Ethics Committee on a previous occasion?**	No
		REGULATORY APPROVAL	
2	1	**Any regulatory approval must be obtained before submission and proof that all necessary approval(s) have been granted must be attached**	
2	2	**Are approvals required from an outside body(ies)? Please answer either YES or NO**	No
2	3	**If any aspects of this proposal require approval from a regulatory body(s) please select from list below**	
		LAY SUMMARY	
4	1	**Confidentiality**	
4	2	**Is information in this application commercially sensitive?**	No
4	4	**PROJECT SUMMARY ~ Please use language that lay people can understand. The answers on each**	

		line must contain fewer than 7800 characters (including spaces). The size is not checked until you save and when the limit is exceeded the current input is rejected. Enter your information in small inputs and save regularly. If your answer becomes too large you will be prompted to insert an extra line to save your current input	
4	5	**Objectives**	The objective of this study is to determine the abundance of segmented filamentous bacteria (SFB) in ileal and faecal samples of mice from 18 till 43 days after birth (shortly before weaning until 3 weeks post-weaning). This will enable us to determine the best time point in which to collect SFB from weanling mice for future in vitro co-culture experiments investigating how SFB influence the maturation of the intestinal barrier.
4	6	**Introduction**	Our intestinal barrier is instrumental in deciding which components to let into our bodies and which to refuse admission. Dysfunction of this barrier, commonly referred to as 'leaky gut', is linked to inflammatory and autoimmune diseases, not only in the intestines, but throughout the body. Therefore, it is essential that we unravel how healthy intestinal barrier function develops during infancy as a step towards reducing the occurrence of these illnesses. Segmented filamentous bacteria (SFB), obligate anaerobic (oxygen intolerant) intestinal bacteria that firmly attach to intestinal epithelial cells, have been shown to be crucial for the maturation of the intestinal barrier during infancy. However, the mechanism by which this occurs is still unknown, mostly because until recently, the culture of SFB in the laboratory has been impossible. Now methods have been developed that allow the isolation of SFB from a complex microbiota of mice [1]. However, this method results in the isolation of SFB attached to ileal cells which are cultured under microaerophilic conditions (low oxygen concentration) which is suboptimal for both the obligate anaerobic SFB and the oxygen-requiring mouse intestinal epithelial cells. In our future research we will use the novel apical anaerobic co- culture model developed at AgResearch [2], which separates aerobic and anaerobic compartments, to co-culture live SFB with oxygen-requiring mouse intestinal epithelial cells to study their interactions. In mice SFB colonise at weaning and disappear once maturation is complete. The colonisation pattern of SFB can vary between mice breeds and facilities, therefore our first aim is to determine whether the mice colonies bred at the AgResearch Small Animal Colony (Ruakura) are colonised by SFB and the time point when SFB is most abundant in mice after weaning. This study is required to determine the most appropriate time point for the isolation of SFB of the mouse microbiota for future in vitro experiments.
4	7	**Additional Introduction**	References: 1. Ericsson AC, Turner G, Montoya L, Wolfe A, Meeker S, Hsu C, Maggio-Price L & Franklin CL (2015). Isolation of segmented filamentous bacteria from complex gut microbiota. BioTechniques, 59:94-9 2. Ulluwishewa, D. et al. Live *Faecalibacterium prausnitzii* in an apical anaerobic model of the intestinal epithelial barrier. Cell. Microbiol. 17, 226-240, (2015
4	8	**Methods ~ (If you have a Table to present, please append it to the Application as a PDF)**	The mouse strain used for this experiment will be determined based on the faecal screening of several mouse strains of the AgResearch Small Animal Colony (Ruakura) for the presence of SFB. 48 weanling male and female mice of the chosen strain will be obtained from the AgResearch Small Animal Colony (Ruakura). The mice will be transported from Ruakura in appropriate rodent transport containers and following IATA guidelines. A New Zealand courier company will be used in order to assist with tracking the shipment and ensuring animals are cared for throughout the trip. A time course study will be performed to determine the abundance of SFB in ileal scrape and faeces samples. The time points will be 18, 23, 28, 33, 38 and 43 days after birth. At each time point ileal scrape and faeces samples of 8 mice will be collected. Since the first time point will be pre-weaning the sampling will be performed at the AgResearch Small Animal Colony (Ruakura). The mice will be weaned at 21 days after birth. The second time point (23 days) will also be performed in Ruakura to avoid the mice being stressed by both weaning and transport. The mice will then be brought into AgResearch Grasslands Ulyatt-Reid Facility Small Animal Facility. The mice will be maintained under PC2 containment and conventional conditions at a constant temperature of 20 degrees Celsius with a 12h light/dark cycle. They will be allowed ad libitum access to normal rodent diet. Drinking water will be provided ad libitum and replaced as required. Procedure for faecal samples: The bacterial DNA will be extracted from the faecal samples and the abundance of SFB in the faecal samples will be determined by real-time PCR. Procedure for ileal scrape samples: Following euthanasia, the terminal ileum will be removed, flushed with sterile PBS, opened length wise and the inside of the ileum will be carefully scraped. The tissue samples will be used to extract bacterial DNA and the abundance of SFB attached to the ileal tissue samples will be determined by real-time PCR.

			Procedure for biomarkers: In addition to the determination of SFB abundance over time, the concentration of immunoglobulin A (IgA) in the digesta and the concentration of interleukin 17 (IL-17) in the blood will be determined by ELISA as parameters that correlate with the SFB colonisation.
4	9	**Design ~ (If you have a complicated diagram to present, please append it to the Application as a PDF)**	Only untreated healthy mice will be used for this experiment. All mice will be allowed ad libitum access to normal rodent diet. Drinking water will be provided ad libitum and replaced as required. Ileal scrape and faecal samples will be taken 18, 23, 28, 33, 38 and 43 days after birth and 8 mice will be sampled per time point.
4	10	**Timetable of events ~ (If you have a Table or complicated diagram to present, please append it to the Application as a PDF)**	Ruakura: Day 0 (18 days after birth, pre-weaning): Collecting faecal samples and ileal scrape of 8 mice. Ruakura: Day 3: Weaning (21 days after birth). Ruakura: Day 5 (post-weaning, 23 days after birth): Collecting faecal samples and ileal scrape of 8 mice. Between day 5 and 10: Mice will be transported from the AgResearch Small Animal Colony (Ruakura) to the AgResearch Grasslands Small Animal Facility. Grasslands: Day 10 (28 days after birth): Collecting faecal samples and ileal scrape of 8 mice. Grasslands: Day 15 (33 days after birth): Collecting faecal samples and ileal scrape of 8 mice. Grasslands: Day 20 (38 days after birth): Collecting faecal samples and ileal scrape of 8 mice. Grasslands: Day 25 (43 days after birth): Collecting faecal samples and ileal scrape of 8 mice.
4	11	**Expected outcomes**	This mouse experiment will allow us to determine whether the chosen mouse strain is colonised by SFB and if so at what time point after weaning SFB colonise the mice. Based on this information we will determine the time range in which to sample mice to collect tissue for SFB culturing in our future experiments.
4	12	**Contingency Plans**	As only healthy mice fed a standard diet will be used, we do not anticipate there will be any problems arising during this experiment.
4	13	**SYNOPSIS ~ for evaluation of the Ethical Cost/Benefit (Note that from 2009 the Grading system has changed. The grades now become A, B, C, D and E. If you have any doubts check the MAF guidelines link in the HELP File.)**	
4	14	**Briefly summarise the number of groups of animals, number of animals per group, manipulations (and Grading) to be done on animals in each group, drugs/chemicals to be administered to each group and the main benefit expected from this work.**	Only untreated, healthy mice will be used for this experiment. From the total of 48 mice, samples will be taken over 25 days at 6 time points (18, 23, 28, 33, 38 and 43 days after birth) and per time point faecal samples and ileal scrape will be taken from 8 mice. Expected benefit from this work: This study will allow us to determine the best time point to harvest SFB from a complex mouse microbiota. This will enable us to isolate SFB for future in vitro co-culture experiments investigating how SFB influence the maturation of the intestinal barrier.
		PROJECT TYPE	
5	2	**This application is a (Use Dropdown box)**	New application
5	3	**If this is a renewal of a previous approval (minimal changes to previously approved proposal) please complete the following - otherwise you must complete a full application**	
		SCIENCE Justification	
6	1	**Immediate Goal of this project:**	The goal of this study is to determine the abundance of SFB in ileal and faecal samples of mice from 18 till 43 days after birth (shortly before weaning until 3 weeks post- weaning).
6	2	**Purpose of your experimental programme:**	The purpose is to be able to determine whether the mouse colonies at the AgResearch Small Animal Colony (Ruakura) naturally harbour SFB. Through a preliminary screening of faecal samples from several mouse strains the most appropriate strain will be chosen for this experiment in order to determine the abundance of SFB in ileal scrape and faecal
			samples of mice from 18 till 43 days after birth (shortly before weaning until 3 weeks post-weaning). This experiment is necessary to determine the best time point for SFB isolation to harvest SFB for future in vitro co-culture experiments using the apical anaerobic co-culture model.

6	3	**Longer term benefits of the research programme:**	The longer term benefit will be knowledge on how SFB influence intestinal function, particularly the maturation of the intestinal barrier, an important parameter for life-long health.
6	4	**Has this application been reviewed by your peers**	Yes
6	5	**If YES, please detail how the project has been peer reviewed**	Peer reviewed by Dr Rachel Anderson and Prof Nicole Roy.
6	6	**Are you aware of similar/previous work in this field either in NZ or overseas?**	Yes
6	7	**If YES, please detail how similar/previous work in this field either in NZ or overseas relates to your proposal:**	Several studies have determined the abundance of SFB in various species, such as humans, mice, and chickens (e.g., Yin et al., 2013, Jonsson et al. 2013, Ohashi et al., 2010). Furthermore, Ericsson et al. (2015) have isolated SFB from a complex mouse microbiota. However, the colonisation pattern of SFB can vary between mice breeds and facilities, therefore the study proposed here is required in order to determine whether the mice colonies bred at the AgResearch Small Animal Colony (Ruakura) are colonised by SFB and the time point when SFB is most abundant in mice after weaning.
		ANIMAL USE Justification	
7	1	**Total number of animals being used**	48
7	2	**What is the highest MAF grading of manipulations that will apply to this project (use dropdown) - The grades must reflect the summed impacts of both the initial state of the animal and the induced effect of the experimental procedure, not the induced effect alone**	B (LITTLE IMPACT)
7	3	**How many animals will be in this grade?**	48
7	4	**How did you choose which species/breed/strain of animal to use for this project?**	We will choose the strain of mice used for this project based on the prior screening of faecal samples for the abundance of SFB. Once we have the results of that preliminary screen, we will inform the ethics committee of the breed we have selected. We have chosen to use male and female mice for this study since the data used for the power analysis was also from male and female mice.
7	5	**Why is it necessary to use sentient animals to achieve the goal(s) of this work?**	The abundance of SFB in ileal and faecal samples in mice can only be determined using animals.
7	6	**[REDUCTION] How have you reduced the number of animals you propose to use to the minimum compatible with achieving the purpose of the work?**	By doing a power analysis.
7	7	**[REFINEMENT] In what ways have you sought to minimise the noxiousness of the procedures you propose to use?**	The mice are not treated in any way and the healthy mice will be humanely euthanised by C02 asphyxiation and cervical dislocation before ileal samples are collected.
7	8	**[REPLACEMENT] What alternatives to using animals have been considered and why have they been rejected?**	We are not able to do this in humans so require an animal model.
		PUBLICATION	
8	1	**How will the results of this work be published or disseminated**	Published in a peer-reviewed international scientific journal.
		BIOMETRIC EVALUATION	
9	1	**Was there a Power Based assessment of the adequacy of sample size(s)**	Yes
9	2	**Main Hypothesis of interest**	SFB colonisation in the ileum reaches a maximum shortly after weaning and then levels reduce over time.
9	3	**Main variable of interest**	Abundance of SFB
9	4	**Experimental design**	Ileal scrape and faecal samples will be taken 18, 23, 28, 33, 38 and 43 days after birth and 8 mice will be sampled per time point.
9	5	**Biometricians comments**	A power analysis has been carried out based on data provided in relevant literature. The differences in SFB levels over the time period are expected to be large compared to the observed variability, so the number of mice proposed is sufficient to give 80% power to detect significant differences of 2 standard deviations. The levels and variability of IL-17 are unknown, so this is a screening study for that aspect of the trial.
9	6	**Data used in Power analysis**	Data from the publication: Ohashi, Y. et al. Colonisation of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. Anaerobe 16, 543-546, (2010).

9	7	**Standard deviation (SD) of the main variable**	300 units/g tissue
9	8	**Where was the SD obtained from**	Data from the publication: Ohashi, Y. et al. Colonisation of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. Anaerobe 16, 543-546, (2010).
9	9	**Minimum true difference of interest**	600 units/g tissue
9	10	**Desired power of the experiment**	80%
9	11	**Desired level of significance**	0.05
9	12	**Resulting sample size**	8
		ANIMAL INFORMATION	
10	1	**Animal Details (Use Dropdown boxes)**	
10	2	**Species ~ Breed or strain**	Mice ~ Strain to be chosen based on preliminary screening of faecal samples for SFB
10	3	**Common name ~ Gender**	Strain to be chosen based on preliminary screening of faecal samples for SFB ~ Mixed sex
10	4	**Reproductive status ~ Age**	Normal and Non-pregnant ~ Pre- and post-weaning
10	5	**Number being used ~ Health status**	48 ~ Healthy
10	6	**Additional Animal Details (Use Dropdown boxes)**	
10	11	**Additional Animal Details (Use Dropdown boxes)**	
10	16	**Additional Animal Details (Use Dropdown boxes)**	
10	22	**Additional Animal Details (Use Dropdown boxes)**	
10	27	**Additional Animal Details (Use Dropdown boxes)**	
10	32	**Previous use of animals**	
10	33	**Have any of the animals been used in previous experiments (i.e., with a different Project number)**	No
		ANIMAL WELFARE	
11	1	**Please detail the animal husbandry and welfare principles that will be applied throughout the project**	
11	2	**In preparation for the experiment**	Animals will be obtained from AgResearch Small Animal Colony (Ruakura).
11	3	**While in the experiment**	The mice will be housed 4 mice per cage under conventional conditions. Feed and water will be supplied ad libitum.
11	4	**After recovery/use (feeding, post-operative care, analgesia, antibiotics, etc.)**	N/A
11	5	**At the end of the experiment**	Animals will be humanely euthanised by C02 asphyxiation and cervical dislocation before samples are collected for analysis.
11	6	**Please detail any other animal welfare considerations (e.g., transport)**	All mice will be checked daily, weighed once a week, and fresh food and water topped up as required. General Health Score (GHS) will be checked daily. GHS ranges from 5 (healthy) to 1 (almost dead). A mouse with a GHS score of 3 or 4 will be closely monitored and if their condition does not improve, they will be humanely euthanised. Once a GHS of 2 is attained mice will be euthanised within the day. A mouse with a GHS of 1 will be immediately euthanised, as will one that has a weight loss greater than 10% from previous weighing. The mice will be transported from Ruakura in appropriate rodent transport containers and following IATA guidelines. A New Zealand courier company will be used in order to assist
			with tracking the shipment and ensuring animals are cared for throughout the trip.
11	7	**Monitoring - any animals that die during the experiment from causes not immediately obvious from clinical signs MUST BE POST- MORTEMED.**	SPECIES
11	8	**Please detail how the Facility, Animal welfare, Operators etc: of this project will be monitored**	All mice will be monitored daily, weighed once a week, and fed twice weekly. Fresh water bottles will be provided as required (normal water).
11	9	**Please indicate the signs/behaviours you will monitor**	REPRODUCTIVE
11	10	**Body weight ~ Frequency**	Yes ~ 1 x weekly

11	11	**Weight loss ~ Frequency**	Yes ~ 1 x weekly
11	12	**Water intake ~ Frequency**	Yes ~ 1 x weekly
11	13	**Food intake ~ Frequency**	Yes ~ 1 x weekly
11	14	**Posture ~ Frequency**	Yes ~ 1 x weekly
11	15	**Gait/movement ~ Frequency**	Yes ~ 1 x weekly
11	16	**Coat condition ~ Frequency**	Yes ~ 1 x weekly
11	17	**Vocalisation ~ Frequency**	No
11	18	**Respiration ~ Frequency**	No
11	19	**Faecal consistency ~ Frequency**	Yes ~ 1 x weekly
11	20	**Vaccination site reaction ~ Frequency**	No
11	21	**Fistula cleanliness ~ Frequency**	No
11	22	**Fly strike ~ Frequency**	No
11	23	**Parasites ~ Frequency**	No
11	24	**Haemorrhage ~ Frequency**	No
11	25	**Oedema/swelling ~ Frequency**	No
11	26	**Infection ~ Frequency**	No
11	27	**Self-mutilation ~ Frequency**	No
11	28	**CNS signs ~ Frequency**	No
11	30	**If you detect adverse effects, how will they be managed? - any animals that die during the experiment from causes not immediately obvious from clinical signs MUST BE POST-MORTEMED.**	Mice will be checked daily for specific health parameters. Any animal that dies during the experiment of causes not immediately obvious will be post-mortemed.
11	31	**Disposal of animals - How will the animals be disposed of? (Use Dropdown box)**	
11	32		Euthanased
11	33	**If sold or retained, where will the animals be located to (Facility, location, conditions etc)**	N/A
11	34	**If euthanised indicate method of euthanasia or slaughter**	
11	35	**Electric stun and exsanguinate ~ captive bolt/exsanguinate**	No ~ No
11	36	**Anaesthetics overdose ~ Cervical dislocation**	No ~ Yes
11	37	**CO2 chamber ~ Guillotine**	Yes ~ No
11	39	**If euthanised or slaughtered, please supply details (Facility, location, conditions etc)**	AgResearch Ulyatt Reid small animal unit, CO2 asphyxiation and cervical dislocation
11	40	**Who will perform the procedure (Detail experience and SOP numbers)**	Euthanasia will be performed by trained personnel (GM SOP 01 09)
11	41	**How will carcasses be disposed**	incineration
		of?	
11	42	**Treatment endpoints: Provide details of endpoints that will result in cessation of treatments**	
11	43	**Not applicable**	No
11	44	**Loss of weight ~ Details**	Yes ~ If a mouse has weight loss 10% or more over 7 days the mouse will be euthanised
11	45	**Intervention trigger (e.g.) ~ Details**	No ~ N/A
11	46	**Tumour size ~ Details**	No ~ N/A
11	47	**Irritation by implant/device ~ Details**	No ~ N/A
11	48	**Metabolic upset ~ Details**	No ~ N/A
11	49	**Death ~ Details**	No ~ N/A

11	50	**Euthanasia of moribund animals ~ Details**	No ~ N/A
11	51	**Euthanasia on clinical threshold ~ Details**	No ~ N/A
11	52	**Other ~ Details**	No ~ N/A
11	60	**CONTINGENCY PLANS ~ How do you plan to deal with an emergency/unforeseen circumstances that may affect the welfare of the animals.**	
11	61	**Have you made any contingency plans?**	All staff responsible for animals will be contactable after hours. The animals will be checked at least once a day for GHS. If any animals become sick (a GHS of 3 or lower) they will be checked at least twice daily or immediately euthanised (as outlined above).
11	62	**Please detail how you will deal with unexpected events, or alternatively why you think such plans are unnecessary.**	In the case of unexpected events that compromise the health and welfare of the mice, the experiment will be terminated, and mice will be euthanised.
		NON-SURGICAL Manipulation	
12	1	**SAMPLING**	
12	2	**Tissue collection ~ Blood collection**	Yes ~ No
12	6	**If there is an SOP approved by your AEC for this manipulation, please cut the name and number from the appropriate file found on the PAR~SOP page and paste in the space below. If no approved SOP is available, please describe the methodology.**	All tissue and blood sampling will occur following humane euthanasia of animals by CO2 asphyxiation and cervical dislocation (GM SOP01 09).
12	7	**DRUG administration**	
12	8	**Intramuscular ~ Intracardiac**	No ~ No
12	9	**Subcutaneous ~ Per os by mouth**	No ~ No
12	10	**Intraperitoneal ~ Drug in water bottle**	No ~ No
12	11	**Intravenous ~ Other**	No ~ No
12	14	**BEHAVIOUR and HANDLING**	Yes ~ 3 x weekly
12	15	**Observation only ~ Enforced activity**	Yes ~ No
12	16	**Physical restraint ~ Nutritional regime**	Yes ~ No
12	17	**Other**	No
12	20	**EXPOSURE to**	No
12	21	**Parasite ~ Micro-organism**	No ~ No
12	22	**Off-licence drug ~ Off-licence chemical**	No ~ No
12	23	**Biological product ~ Radiation**	No ~ No
12	24	**Electrical stimulation ~ Abnormal environment**	No ~ No
12	25	**Other**	No
12	28	**WILD ANIMALS**	No
12	29	**Handling ~ Capture**	No ~ No
12	30	**Marking, Tagging or Branding ~ Transmitter attachment or implantation**	No ~ No
12	32	**If other use of WILD ANIMALS, please describe**	
12	35	**Have you made contingency plans for major risk factors**	No
		SURGICAL Manipulation	
13	1	**Animals will survive surgery ~ Animals will NOT survive surgery**	No ~ No
13	2	**Manipulations for which SOPs have been approved**	

13	4	**For all manipulations where an SOP has not been approved, please enter the following information:**	
13	5	**ASEPTIC TECHNIQUE**	
13	7	**ANAESTHESIA and ANALGESIA**	
13	8	**Will the animals receive pre- emptive analgesia**	No
13	11	**Depth of Anaesthesia - Please indicate how this will be tested**	
13	12	**Thermal methods ~ Electrical methods**	No ~ No
13	13	**Mechanical methods ~ Chemical methods**	No ~ No
13	14	**Metabolic methods ~ Local inflammatory reactions**	No ~ No
13	15	**Deep pain reflex response**	No
13	16	**MONITORING**	
13	19	**Will you be using standard monitoring sheets**	No
13	22	**POST-OPERATIVE support**	
13	23	**Not applicable, animals do not recover**	No
13	24	**Conservation of body heat ~ Monitoring sheets**	No ~ No
13	25	**Fluid administration ~ Recovery in individual cages**	No ~ No
13	26	**Administration of analgesics ~ Other**	No ~ No
13	28	**ENDPOINTS**	
		DRUGS and CHEMICALS	
14	1	**List all drugs and Chemicals to be administered to animals during this project below._____ PLEASE NOTE: You are required to complete a separate IDAO for EACH DRUG/CHEMICAL that is to be used in this project**	
14	3	**If you are using drugs that do not have a COVERSHEET, please go to HELP on this page and use the link to download the template (second**	
		line below the green box). Complete one sheet for each drug and/or chemical. Send the completed form electronically to Grant Shackell. These will be made available in the PAR~SOP function for future reference.	
14	4	**Have you had to make up new PAR COVERSHEET(s) for this project**	No
14	6	**UNREGISTERED Drugs or Chemicals**	
14	20	**Will ANY drugs used in this project (other than OTC Products) be administered by ANY PERSON who is NOT a registered Veterinarian [Use Dropdown] _____This question is an internal trigger for IDAO creation, names are not required here.**	NO
		TISSUE COLLECTION	
21	1	**If you are requesting permission to collect tissue following euthanasia, and you are not manipulating the animals before they are euthanised you must complete this page**	

21	2	**If this is NOT an application to collect animal tissue only, you must complete a full application**	
21	3	**Species (Use Dropdown box)**	Mice
21	5	**How many animals of each species will be used?**	48
21	6	**What tissue(s) are to be collected?**	Ileum, digesta, blood
21	7	**If the animals are being euthanised only for the purpose of tissue collection, please indicate what method of euthanasia will be used**	CO2 and cervical dislocation
		SOURCE(s) and TRIAL SITE(s)	
97	1	**This section allows statistics to be gathered for MAF and Animal use information**	
97	2	**Please ensure you have selected the correct trial site as information is transferred to the Animal Tools database. Please note that you may select more than one trail site where relevant.**	
97	3	**MICE**	Source = AGR Ruakura Small Animal Unit Trial Site = AGR Grasslands Animal Facility (Ulyatt/Reid) Number = 48
		98. NOTES ~ Read only	
98	1	**Status Change**	(maiere 05/12/2016) SUBMIT
98	2	**Committee Decision**	(15/12/2016 RESUBMITcosgroveg) Hi Eva, 1. Any comment from the biometrician for section 9.5? 2. The AEC recommend mice have a one week stand down after transport to new facility. That is difficult to provide given the timeline of this experiment. Could the stress associated with weaning, followed closely by transport affect colonisation and abundance of SFB? Can the trial be completed in a single location to avoid the transport near weaning, or at least a further time point sampling be completed at Ruakura prior to transport, to increase the interval between weaning and transport? 3. Which day of study are they weaned? 4. Sections 9.9/9.7. What are the units of main variable? For questions 1, 3 and 4, edit the application, as required. For question respond 3 respond via Applicant Note, or in the application, as appropriate.
98	3	**Applicant Note**	(16/12/2016 maiere) To the animal ethics committee:
			Thanks a lot for your comments. I have addressed them as followed: Comment 1: The biometrician (Catherine Lloyd-West) has added a comment in section 9.5. Comment 2: In order to avoid the stress associated with weaning, followed closely by transport, we decided to have another sampling time point be completed at Ruakura prior to transport to increase the interval between weaning and transport, as suggested by the comments of the committee. To complete the study completely in Ruakura is not feasible for us because in the future we need to be able to isolate SFB at the determined maximum SFB level in Palmerston North to be able to use the bacteria in subsequent in vitro studies. I have updated the application form where appropriate to include this change. Comment 3: The mice are weaned after 21 days of age which would be Day 3 of the study. Comment 4: I have included the units in Sections 9.9/9.7.
98	4	**Status Change**	(maiere 16/12/2016) SUBMIT

98	5	**Applicant Note**	(21/12/2016 cosgroveg) Hi Gerald, For future studies there will not be repeated sampling that precludes the stand-down period, since we will only sample the mice at the time point with the maximum SFB levels as determined in this study. Based on previous studies the maximum levels of SFB occur around 4 weeks after birth, so 1 week after weaning. However, this could vary between breeds/facilities, so the purpose of this study is to determine the time point for maximum levels of SFB in the mouse colony we readily have access to (Ruakura). In future studies the mice will be transported to Palmerston North at the time point of maximum SFB colonisation (estimated 1 week post weaning) and the samples taken immediately on arrival for culturing. Kind regards, Eva _____ From: Cosgrove, Gerald Sent: Friday, 16 December 2016 16:34 To: Maier, Eva Subject: RE: AE application Hi Eva, Could you please clarify your comment about the sampling. You refer to future studies and the need to isolate SFB at the maximum levels, for in vitro use in PN. Can we assume that for those studies there won't be the same need for transferring mice soon after weaning and the repeated sampling that precludes the stand-down period? The AEC want to know if this trial design is one-off or setting a precedent in terms of this arrangement. Gerald
98	6	**Committee Decision**	(21/12/2016 APPROVEDcosgroveg)
98	7	**Committee Note**	(27/02/2017 kirkm) Hi Eva • Section 4.8 Confirm what strain of mice has been selected based on pre-trial faecal screening. • For future trials if the best time of sampling is around the weaning stage consider including a group of non-transport animals to compare with the transport group to identify if any stress related difference are related to transport. Michelle
98	8	**Applicant Note**	(01/03/2017 maiere) Hailey Gillespie (Animal Facility Manager) will help with euthanizing the mice on the first sampling day in Palmerston North (12 March).
98	9	**Committee Note**	(02/03/2017 kirkm) Answer to Committee Note 98.7 • Section 4.8 Confirm what strain of mice has been selected based on pre-trial faecal screening. * From MOD 2259, Section 1.12 The SWISS mouse strain has been selected based on pre-trial faecal screening.
98	10	**Applicant Note**	(11/03/2017 maiere) Charlotte O'Neill (Small Animal Technician) will help with euthanizing the mice and sampling.
98	11	**Committee Decision**	(22/02/2018 ACCEPTEDkirkm) This project finished in 2017 and associated reports have been accepted. This application will now be closed. Michelle
98	12	**Committee Decision**	(22/02/2018 CLOSEDkirkm) This project finished in 2017 and associated reports have been accepted. This application will now be closed. Michelle
		PERSONNEL and Sign off	
99	1	**Committee**	GRASSLANDS
99	99	**ANDERSONRC ~ approved ~ Job (Science Team Leader - Food Functionality - Model Systems) Location (Grasslands; Alan Johns Building. Tues-8-4.30pm. Mon-Fri-8- 2.30pm)**	Project Leader
99	99	**BROADHURSTR ~ approved ~ Job (Casual Senior Scientist Small Animal Colony) Location (Ruakura; Small Animals, An Phys)**	Facility manager
99	99	**DUNSTANK ~ approved ~ Job (Laboratory Manager) Location (Grasslands; Alan Johns - Rm F28)**	Animal husbandry
99	99	**GillespieH ~ Job (Animal Facility Manager) Location (Grasslands; Lab Admin Building)**	Animal Facility Manager - returned from Leave. Added for viewing purposes
99	99	**LLOYDWESTC ~ approved ~ Job (Statistician) Location (Grasslands; LC Rm 27 - East Wing)**	Statistician

99	99	**MAIERE ~ approved ~ Job (Lab Manager) Location (Grasslands; Alan Johns G27)**	Animal Husbandry
99	99	**ONEILLC ~ Job (Technician, Food Nutrition & Health) Location (Grasslands; Lab Admin Building #41 Rm A-03.)**	Assistant animal technician
99	99	**PETERSJ ~ approved ~ Job (Research Associate) Location (Grasslands; Alan Johns building, Rm G25)**	Sampling
99	99	**ROBINSONDA ~ approved ~ Job (Animal Technician) Location (Grasslands; Lab Admin Rm A-03)**	Acting animal facility manager
99	99	**ROYN ~ approved ~ Job (Science Team Leader Food Nutrition & Health) Location (Grasslands; Alan John Building, Level 2)**	Team Leader
99	99	**SHERIFFG ~ approved ~ Job (Senior Technician) Location (Ruakura; Small Animal Colony First Aid)**	Animal technician
99	99	**SMITHBG ~ approved ~ Job (Research Associate) Location (Ruakura; Small Animals, An Phys, Fire Warden, First Aid)**	Animal technician
99	99	**SMITHF ~ approved ~ Job (Research Technician) Location (Grasslands; Animal Admin Building)**	Animal technician

Appendix IIIb

Animal Ethics Application No. 14485

AE Application 14485 ~ (Status=APPROVED) (Applicant=OgutuL) Determination of the abundance of segmented filamentous bacteria in ileal samples of rats pre- and post-weaning

Group	Line	Question	Answer
		ASSOCIATED Documents	
		Attached pdf 9134	Additional Introduction
		Attached pdf 9153	Updated-Experimental Methods and Design
		0. ADMINISTRATIVE Details	
0	1	**Title**	Determination of the abundance of segmented filamentous bacteria in ileal samples of rats pre- and post-weaning
0	3	**Applicant**	Linda Ogutu
0	4	**Institution**	AgResearch Limited
0	5	**Business Address**	AgResearch Grasslands Tennent Drive Palmerston North 4442
0	6	**Phone**	06 351 8372
0	7	**Mobile phone**	0211775545
0	8	**Email**	linda.ogutu@agresearch.co.nz
0	9	**Contact details**	
0	10	**Name of person responsible for the animals during manipulations (MUST be named on and sign the personnel page). If more than one facility is being used, all persons responsible for animals must be named and sign this Application.**	Linda Ogutu
0	11	**Phone**	06 351 8372
0	12	**Mobile phone**	0211775545
0	13	**Email**	linda.ogutu@agresearch.co.nz
0	14	**Person responsible for entry of Trial Drug and animal manipulation data into Animal Tools database. (AgResearch requirement only ~ MUST be named on and sign the personnel page). If no AgR facilities are being used please enter Not Applicable**	Not Applicable
0	15	**Program Manager (MUST be named on and sign the personnel page)**	Nicole Roy/Rachel Anderson
0	16	**Biometrician (MUST be named on and sign the personnel page - or hard [paper] copy if no computer access)**	Peter Johnstone
0	17	**Facility Manager - If more than one facility is being used, all Managers must be named and sign this Application. (MUST be named on and sign the personnel page - or hard [paper] copy if no computer access)**	Hailey Gillespie, Ric Broadhurst
0	18	**Is this a new experiment?**	Yes
0	19	**Project Dates ~ PLEASE enter all dates in the form requested [dd/mm/yy] AND ensure that the start date you enter occurs after the next meeting of your committee. If you do not expect to renew this Application, please do not enter a Renewal Date**	
0	20	**Start Date (dd/mm/yy)**	01/08/2018

0	21	**Finish Date (dd/mm/yy)**	30/10/2018
0	23	**Are there FOOD SAFETY issues under the Animal Products Act, or Regulatory approval, associated with this project (PLEASE NOTE: The FOOD CHAIN includes food for either HUMAN CONSUMPTION or PET FOOD)? If you are using Laboratory Rodents and/or other species that are never used for human consumption or pet food, or if the animals in the project will be safe to enter the food chain, please type the word NO and then proceed to the next question. However, if some or all of the animals must be excluded from EVER entering the food chain because of the manipulations, drugs they will be given or because of Regulatory Approval requirements, please type YES and then explain why. AgResearch animals that may not enter the food chain must be PINK TAGGED.**	No
0	24	**Māori Consultation. If there is any uncertainty, it is the applicant's responsibility to approach local Māori representatives for clarification.**	
0	25	**Do any aspects of this project require consultation with Māori?**	No
0	27	**Are you requesting URGENT consideration? Please only tick YES if the AEC needs to consider this Application before the next scheduled meeting.**	No
0	29	**Database Administration**	
0	30	**From the Dropdown, AgResearch users please indicate the name of your Science Group and Team; Parented users please select EXTERNAL USER**	Food and Bio-based Products (Food Nutrition & Health)
		1. AEC JURISDICTION	
1	1	**Which committee are you applying to? (Use Dropdown box)**	Grasslands
1	2	**Some aspects of this work will also be undertaken under the jurisdiction of the AgResearch AEC at (Use Dropdown box)**	Ruakura
1	5	**Are staff, animals, or facilities of another organisation(s) which has an Animal Ethics Committee involved in this project?**	No
1	7	**Does the Ethics committee of that institution(s) have a copy of this application?**	No
1	8	**Has this application, a similar or largely similar application been submitted to another Ethics Committee on a previous occasion?**	No
		2. REGULATORY APPROVAL	
2	1	**Any regulatory approval must be obtained before submission and proof that all necessary approval(s) have been granted must be attached**	
2	2	**Are approvals required from an outside body(ies)? Please answer either YES or NO**	No
2	3	**If any aspects of this proposal require approval from a regulatory body(s) please select from list below**	

		4. LAY SUMMARY	
4	1	**Confidentiality**	
4	2	**Is information in this Application and any Reports associated with it commercially sensitive?**	No
4	4	**PROJECT SUMMARY ~ Please use language that lay people can understand. The answers on each line must contain fewer than 7800 characters (including spaces). The size is not checked until you save and when the limit is exceeded the current input is rejected. Enter your information in small inputs and save regularly. If your answer becomes too large you will be prompted to insert an extra line to save your current input**	
4	5	**Objectives**	The objective of this study is to determine the abundance of SFB in ileal tissue and faecal samples of rat pups pre- and post-weaning. This will be done by developing the best technique for extracting segmented filamentous bacteria (SFB) from the ileal tissue of rat pups from 20 until 32 days after birth. Day 20 will be the pre-weaning age and the rat pups will be weaned on day 21. This is based on observations from the results of Ohashi et al., 2010 where there was a very low population of SFB in the ileum of mouse pups from their pre-weaning age of 18 days. SFB abundance was observed and quantified from day 20 onwards. Results from the March 2017 trial also supported this as the abundance of SFB was low at the pre-weaning age of day 20 and gradually increased from weaning (day 21) and peaked post-weaning (day 26). As mice and rats are similar, the assumption is that the time-course of SFB abundance observed in mice will be similar to that in rats. According to the current literature, there are no studies of SFB time-courses that have been carried out in rats. Additionally, the rat pups at Ruakura are typically weaned 21 days after birth. The information on SFB prevalence at different time-points will be useful in designing future studies, where early-life diet will be used to attempt to stimulate the abundance of SFB in the ileum, which has shown to influence the maturation of the infant intestinal barrier. Currently there are no known negatives of either naturally high or artificially stimulated higher levels of SFB in rodents or humans as this has not been explored.
4	6	**Introduction**	The intestinal barrier in the gastrointestinal tract (GIT) is a semi-permeable membrane which allows some components through, whilst preventing unwanted components from passing into our bodies [1,2]. Leaky gut is the term given for the dysfunction of this barrier, and it has been linked to autoimmune and inflammatory disorders both in the GIT and the entire body [3]. SFB are Gram-positive, obligate anaerobic, commensals that have been shown to play a role in the maturation of the intestinal barrier during infancy. The mechanism by which SFB works to do this remains unknown as this bacterium has not been successfully cultured in vitro. Mice are commonly used in studies of SFB. This commensal has been previously isolated in the terminal ileum of rats [4], and a few techniques can be employed to isolate SFB from these rodents. One method involves performing ileal mucosal scrapes [5]. Grinding or homogenisation of ileal tissue is another method that has been employed for isolation of SFB from the terminal ileum [6]. The results between these two methods are variable, and the best isolation technique may be influenced by the morphology and attachment of these bacteria to the ileal tissue. Therefore, determining the best technique to isolate SFB will be beneficial to determine the difference in SFB abundance in the ileum between time-points pre- and post- weaning. This will be used for future studies where diet will be used to attempt to stimulate an increase in the abundance of SFB in the ileum, which has been shown to influence the maturation of the intestinal barrier.

4	7	**Methods ~ (If you have a Table to present, please append it to the Application as a PDF)**	The rat strain used for this experiment will be Sprague-Dawley. This rat strain was used in a former study by Davis and Savage [7,8] where they found naturally- occurring SFB in the terminal ileum. Rats were chosen for this study as they are larger than mice and more samples will be available for analysis. The time course study used will include pups aged: 20, 22, 24, 26, 28, 30, and 32 days after birth. These will correspond to time-points 1, 2, 3, 4, 5, 6, and 7 the days pups will be euthanised for sample collection. The number of pups required in each time-point will be at least 8. After weaning, the pups will be kept in their birth litters post-weaning throughout the study. This is to ensure the GIT microbiota is not influenced in any way by co-housing with pups from other litters. One dam per time- point i.e., 7 dams for 7 time-points was agreed on to produce enough pups for each time-point. If 12 pups for each of the 7 litters are available, they will be randomised evenly between groups. However, the pup litter sizes can vary between 2-17 pups, therefore it was suggested that 1 extra dam be included resulting in a total of 8 dams. Eight dams will therefore be used to produce the required pup litters for the study. Rats produce on average 11-13 pups per litter, therefore the total animals (mixed gender) born is likely to be between 88-104 pups i.e., 8 dams producing between 11- 13 pups. For this study, 56 pups will be required, and extra pups born will be randomised across the time-points to increase sample sizes. A greater sample size will reduce variability in the SFB abundance data. For litter sizes over 12 pups, excess pups will be euthanised to avoid cross-fostering due to a possible maternal influence on the pups' gut microbiomes. Also, 12 pups per dam will be sufficient as each pup will be able to suckle. More than 12 pups per dam may mean smaller pup sizes as not all pups will be able to suckle at the same time. Rats will be mated at the AgResearch Small Animal Colony in Ruakura, and the dams will be transported to Grasslands after 13-16 days which is optimal time to allow for a pregnancy diagnosis. The dams will be brought into the AgResearch Grasslands Ulyatt-Reid Small Animal Facility. They will be maintained under physical containment level 2 (PC2) and conventional conditions at a constant temperature of 20 degrees Celsius with a 12 hr light/dark cycle. They will be allowed ad libitum access to a normal rodent diet and water until the rat pups are born. The water will be replaced as required. The normal rodent diet is a meat-free rat and mouse diet containing wheat, barley, lupins, soya meal, fish meal, mixed vegetable oils, canola oil, salt, calcium carbonate, dicalcium phosphate, magnesium oxide, and a vitamin and trace mineral premix. Once the pups are born, they will be maintained in the same cages with their mothers under PC2 containment and conventional conditions at a constant temperature of 20 degrees Celsius with a 12 hr light/dark cycle. The pups will suckle, and the mothers will be allowed ad libitum access to a normal rodent diet and water. The pre-weaning day will be at 20 days after birth, which will be time-point 1 for euthanasia and ileal sample collection. The pups will then be weaned at day 21. The succeeding days after birth i.e., 22, 24 26, 28, 30 and 32 will be time-points 2, 3, 4, 5, 6, and 7 respectively. The pups will remain in their birth litters post-weaning and will be randomised into sampling time-points. Birthing is expected over a 2-3 day period, the expected span for 8 dams, so sampling will be done based on pup weaning time. At 19-21 days old, all pups will be weaned. Pup allocation to time-points is appended in a PDF. This sample collection method is ideal as we assume diet change (breastmilk pre-weaning and rodent feed & water post-weaning) has an effect on SFB abundance in the terminal ileum, not age. Sampling will be simplified and done every other day for 2 weeks.
4	8	**Design ~ (If you have a complicated diagram to present, please append it to the Application as a PDF)**	Experimental design appended as a PDF.
4	9	**Timetable of events ~ (If you have a Table or complicated diagram to present, please append it to the Application as a PDF)**	Ruakura: Day 0-Rat pairing and mating Transportation of pregnant rats (dams) from Ruakura to Grasslands: Day 13-16 after mating Grasslands: Maintain dams until birth of pups Grasslands: Day 0 - birth of pups Grasslands: Maintain pups until weaning time Grasslands: Day 20 after birth: Pre-weaning; collecting samples of more than 8 pups if available Grasslands: Day 22 after birth: Post-weaning; collecting samples of more than 8 pups if available Grasslands: Day 24 after birth: Collecting samples of more than 8 pups if available Grasslands: Day 26 after birth: Collecting samples of more than 8 pups if available Grasslands: Day 28 after birth: Collecting samples of more than 8 pups if available Grasslands: Day 30 after birth: Collecting samples of more than 8 pups if available Grasslands: Day 32 after birth: Collecting samples of more than 8 pups if available
4	10	**Expected outcomes**	This study will allow us to determine the best technique to use for extracting SFB from the ileal tissue of rat pups, in order to determine the abundance of SFB in ileal tissue and faecal samples of rat pups pre- and post-weaning. The best technique will be used in future studies where diet will be used to attempt to stimulate an increase in the abundance of SFB in the ileum of rat pups.

4	11	**Contingency Plans**	A concern in this study is losses due to stress of transport and change in location in dams. Precautions will be taken to standardise housing, bedding, feed, and cleaning protocols between Ruakura and Grasslands to minimise the changes to these dams. Animal welfare guidelines require transportation of pregnant dams to be completed by day 17 of pregnancy. Only healthy rats fed a standard rodent diet will be used in this study.
4	12	**SYNOPSIS ~ for evaluation of the Ethical Cost/Benefit. The grades are A, B, C, D and E. If you have any doubts check the MPI guidelines link in the HELP File.)**	
4	13	**Briefly summarise the number of of animals, number of animals manipulations (and Grading) to be done on animals in each group, drugs/chemicals to be administered to each group and the main benefit expected from this work.**	One hundred and twelve untreated, healthy rats will be used for this experiment. Dams produce on average 11-13 pups, therefore for the study, 8 dams and up to 104 pups will be maintained at PC2 conditions. Samples will be collected from 56 rat pups over a 12 day period at 7 time-points (20, 22, 24, 26, 28, 30, and 32 days after birth). Each time-point will consist of 8 to 12 **groups per group,** pups. The samples to be collected include ileal tissue for homogenisation, ileal scrape samples, ileal digesta, faecal samples, and blood plasma. Extra pups born will be randomised across the time-points to increase sample sizes. A greater sample size will reduce variability in the SFB abundance data. This study will allow us to determine the best technique to use for extracting SFB from the ileal tissue of rat pups, in order to determine the abundance of SFB in ileal tissue and faecal samples pre- and post-weaning. The best SFB extraction technique will be beneficial for future studies where early life nutrition will be used to attempt to stimulate an increase in the abundance of SFB in the ileum of rat pups.
		5. PROJECT TYPE	
5	2	**This application is a (Use Dropdown box)**	New application
5	3	**If this Application is a renewal of a previous approval, please complete this page before moving to section 6.**	
		6. SCIENCE Justification	
6	1	**Immediate Goal of this project:**	The goal of this study is to determine the best technique to use for extracting SFB from the ileal tissue of rat pups from 20 until 32 days after birth i.e., pre-weaning and 2 weeks post-weaning.
6	2	**Purpose of your experimental programme:**	The purpose is to determine whether ileal mucosal scraping or ileal tissue homogenisation is the better technique for isolating SFB from the ileum of rat pups.
6	3	**Longer term benefits of the research programme:**	This will be beneficial in the long-term as the better technique will be routinely used for isolating SFB from the ileum of rat pups in successive experiments.
6	4	**Has this application been reviewed by your peers**	Yes
6	5	**If YES, please detail how the project has been peer reviewed**	Peer reviewed by Prof Nicole Roy and Dr Rachel Anderson as well technical staff involved in the study.
6	6	**Are you aware of similar/previous work in this field either in NZ or overseas?**	Yes
6	7	**If YES, please detail how similar/previous work in this field either in NZ or overseas relates to your proposal:**	A number of studies (e.g., Ohashi et al., 2010; Ericsson et al., 2015) have been previously carried out where SFB was isolated from the ileum of mice using ileal mucosal scraping. An animal study was also carried out in March 2017 at Grasslands where ileal tissue homogenisation was used to isolate SFB from the terminal ileum of mice. SFB has been isolated using both techniques. However, it is unclear which technique results in a greater abundance of SFB isolation.
		7. ANIMAL USE Justification	
7	1	**Total number of animals being used**	112
7	2	**What is the highest MPI grading of manipulations that will apply to this project (use dropdown) - The grades must reflect the summed impacts of both the initial state of the animal and the induced effect of the experimental procedure, not the induced effect alone**	C (MODERATE IMPACT)
7	3	**How many animals will be in this grade?**	112

7	4	**How did you choose which species/breed/strain of animal to use for this project?**	We chose the Sprague-Dawley rat strain to use for this project as SFB was initially isolated from this strain. Rat pups are also larger animals compared to mouse pups' therefore the sample sizes will be larger. In a previous animal study where SFB was isolated from mouse pups, the tissue samples collected from some animals were too small to be analysed which may have contributed to the variability in the data from the analyses.
7	5	**Why is it necessary to use sentient animals to achieve the goal(s) of this work?**	The abundance of SFB in ileal tissue and faecal samples in rats can only be determined using animals.
7	6	**[REDUCTION] How have you reduced the number of animals you propose to use to the minimum compatible with achieving the purpose of the work?**	By doing a power analysis.
7	7	**[REFINEMENT] In what ways have you sought to minimise the noxiousness of the procedures you propose to use?**	The rats are not treated in any way and the healthy rat pups will be humanely euthanised by C02 asphyxiation and cervical dislocation using GMSOP 01 09 before ileal samples are collected by trained technicians. Extra pups born in excess of 12 per dam will be humanely euthanised 2 days post- birth by the AgResearch veterinarian using GMSOP 0413.
7	8	**[REPLACEMENT] What alternatives to using animals havebeen considered and why have they been rejected?**	We are not able to do this in humans, therefore an animal model is required.
		8. PUBLICATION	
8	1	**How will the results of this work be published or disseminated**	The results will be published in a peer-reviewed international scientific journal.
		9. BIOMETRIC EVALUATION	
9	1	**Was there a Power Based assessment of the adequacy of sample size(s)**	Yes
9	2	**Main Hypothesis of interest**	Whole ileal tissue homogenisation may be suitable for isolating SFB to allow for accurate observation in the changes in SFB abundance over time pre- and post- weaning.
9	3	**Main variable of interest**	Abundance of SFB over time pre- and post-weaning.
9	4	**Experimental design**	It was initially decided that 7 dams will be required to produce a little more than the 56 rat pups required for this experiment. Dams commonly produce litters of between 11-13 pups on average but vary between 2-17 pups per litter. Therefore, an extra dam was included to ensure sufficient pups available for the study. Therefore, 8 dams will be used to produce between 88-104 pups for the study. Ileal tissue samples from mucosal scraping and for homogenisation will be taken 20, 22, 24, 26, 28, 30 and 32 days after birth and at least 8 rat pups will be sampled per time-point.
9	5	**Biometricians comments**	To compare the 2 techniques at each time-point, we will collect tissue from 8-12 pups at each time-point, and tissue from each pup will be used for both techniques. It would be better to use 12 pups, but the experiment is still worth doing if we are unfortunate and get only 8.
9	6	**Data used in Power analysis**	Data from: Ohashi, Y. et al. Colonisation of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. Anaerobe 16, 543-546, (2010).
9	7	**Standard deviation (SD) of the main variable**	852 units/g of tissue
9	8	**Where was the SD obtained from**	Data from: Ohashi, Y. et al. Colonisation of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. Anaerobe 16, 543-546, (2010).
9	9	**Minimum true difference of interest**	1300 units/g of tissue
9	10	**Desired power of the experiment**	80% to 90%
9	11	**Desired level of significance**	0.05
9	12	**Resulting sample size**	8 to 12
		10. ANIMAL INFORMATION	
10	1	**Animal Details (Use Dropdown boxes)**	
10	2	**Species ~ Breed or strain**	Rats ~ Sprague-Dawley

10	3	**Common name ~ Gender**	~ Female
10	4	**Reproductive status ~ Age**	Pregnant
10	5	**Number being used ~ Health status**	8 ~ Healthy
10	6	**Additional Animal Details (Use Dropdown boxes)**	
10	7	**Additional Species ~ Breed or strain**	Rats ~ Sprague-Dawley
10	8	**Additional Common name ~ Gender**	~ Mixed sex
10	9	**Additional Reproductive status ~ Age**	Normal and Non-pregnant ~ Pre- and post-weaning
10	10	**Additional Number being used ~ Health status**	104 ~ Healthy
10	11	**1 Additional Animal Details (Use Dropdown boxes)**	
10	16	**2 Additional Animal Details (Use Dropdown boxes)**	
10	22	**3 Additional Animal Details (Use Dropdown boxes)**	
10	27	**4 Additional Animal Details (Use Dropdown boxes)**	
10	32	**Previous use of animals**	
10	33	**Have any of the animals been used in previous experiments (i.e., with a different Project number)**	No
		11. ANIMAL WELFARE	
11	1	**Please detail the animal husbandry and welfare principles that will be applied throughout the project**	
11	2	**In preparation for the experiment**	Charley O'Neill, the animal technician at Grasslands has received training from Ric Broadhurst and Robert Smith at Ruakura on caring for and birthing the dams. They will both continue to offer ongoing support once the dams are transported to Grasslands and throughout the animal study. Chrissie Butts at Plant& Food Research was also contacted by email to discuss how to care for breeding animals, though did not respond. Eight pregnant rats (dams) will be obtained from AgResearch Small Animal Colony (Ruakura) and transported to AgResearch Ulyatt-Reid Animal Facility (Grasslands). The dams will be maintained under PC2 conditions until the rat pups are born. Dams produce an average of 11-13 pups each, therefore the birth of 104 pups will be planned for. For this study, only 56 pups are required i.e., 8 pups each in 7 time- points. However, more than 56 pups may be used if there are more pups born. Extra samples collected will reduce the variability of the SFB data during analysis. The rat pups to be used in the experiment will be left with their mothers to suckle pre-weaning. They will then be separated from their mothers post-weaning but kept in their birth litters, and then randomised into the 7 time-points for the experiment. Extra pups (excess of 12 pups per dam) born will not be cross-fostered as is commonly done but will be euthanized 2 days post-birth using GMSOP 04 13. Given that the pups will be 2 days old, we will follow sodium pentobarbitone euthanasia with decapitation to confirm death. At this age, the pups are regarded as neonates and unsuitable for CO2. The aim is to avoid cross-fostering which may impact the abundance of SFB in the gut of the pups due to a possible maternal influence on the gut microbiome. For the extra pups born i.e., more than 56, some may be randomised into the 7 time- point groups for extra sample collection. More samples will reduce variability in the data analysis as advised by the Biometrician.
11	3	**While in the experiment**	The 8 dams will be housed individually for whelping (birthing) under PC2 conditions with rodent feed and water being supplied ad libitum. The rat pups will be housed with their mothers pre-weaning as they suckle. At weaning time, they will be separated from their mothers and housed in their litter groups under PC2 conditions with rodent feed and water being supplied ad libitum.
11	4	**After recovery/use (feeding, post- operative care, analgesia, antibiotics,** N/A **etc.)**	

11	5	**At the end of the experiment**	The rat pups will be euthanised humanely by CO2 asphyxiation and cervical dislocation prior to tissue sample collection for analysis. The extra pups born will be randomised into the time-point groups for extra sample collection. Dependent on how may pups are born, a few animals may be used at the Ulyatt-Reid Animal Facility or for training purposes under Susan Doohan, the Veterinarian and Animal Welfare Officer. The dams cannot be returned to Ruakura as once they leave the PC2 facility, they cannot be reintroduced into that facility. After the birth of pups, they will be used for training purposes at the Ulyatt-Reid Animal Facility. Alicia Barnett has expressed interest in obtaining rat tissues. This will be coordinated by Hailey Gillespie and Susan Doohan.
11	6	**Please detail any other animal welfare considerations (e.g., transport)**	Dams will be transported from Ruakura in appropriate rodent transport containers and following IATA guidelines. A New Zealand courier company will be used in order to assist with tracking the shipment and ensuring animals are cared for throughout the trip. All dams and rat pups after birth will be checked daily, weighed once a week, and fresh food and water topped up as required. General Health Score (GHS) will be checked daily. GHS ranges from 5 (healthy) to 1 (almost dead). A rat with a GHS score of 3 or 4 will be closely monitored and if their condition does not improve they will be humanely euthanised. Once a GHS of 2 is attained, rats will be monitored hourly or euthanised immediately depending on their condition. A rat with a GHS of 1 will be immediately euthanised, as will one that has a weight loss greater than 10% from previous weighing. For this trial only healthy animals will be used, therefore proactive euthanisation is preferred. There would be no reason to delay euthanisation since few animals would recover, and if they did it may be with intervention anyway.
11	7	**Monitoring - any animals that die during the experiment from causes not immediately obvious from clinical signs MUST BE POST-MORTEMED and the AEC notified immediately**	
11	8	**Please detail how the Facility, Animal welfare, Operators etc: of this project will be monitored**	All rats (dams and pups) will be monitored daily, weighed once a week, and fed twice weekly. The 8 dams will be housed in individual cages. Their weights and body conditions will be easily compared. Fresh (normal) water and rodent feed will be provided as required. The rat pups will be retained in their birth litters post-weaning and will be individually identified with different non-toxic marker pen colours in order to compare their weights and body conditions. Fresh (normal) water and rodent feed will be provided as required. Susan Doohan, the Veterinarian and Animal Welfare Officer will monitor visit to access the grading of manipulations.
11	9	**Please indicate the signs/behaviours you will monitor and how frequently they will be monitored**	
11	10	**Body weight ~ Frequency**	Yes ~ 1 x weekly
11	11	**Weight loss ~ Frequency**	Yes ~ 1 x weekly
11	12	**Water intake ~ Frequency**	Yes ~ 1 x weekly
11	13	**Food intake ~ Frequency**	Yes ~ 1 x weekly
11	14	**Posture ~ Frequency**	Yes ~ 1 x daily
11	15	**Gait/movement ~ Frequency**	Yes ~ 1 x daily
11	16	**Coat condition ~ Frequency**	Yes ~ 1 x daily
11	17	**Vocalisation ~ Frequency**	No
11	18	**Respiration ~ Frequency**	No
11	19	**Faecal consistency ~ Frequency**	Yes ~ 1 x daily
11	20	**Vaccination site reaction ~ Frequency**	No
11	21	**Fistula cleanliness ~ Frequency**	No
11	22	**Fly strike ~ Frequency**	No
11	23	**Parasites ~ Frequency**	No
11	24	**Haemorrhage ~ Frequency**	No
11	25	**Oedema/swelling ~ Frequency**	No

11	26	**Infection ~ Frequency**	No
11	27	**Self-mutilation ~ Frequency**	No
11	28	**CNS signs ~ Frequency**	No
11	30	**If you detect adverse effects, how will they be managed? - any animals that die during the experiment from causes not immediately obvious from clinical signs MUST BE POST- MORTEMED.**	Rats will be checked daily for specific health parameters. Any animal that dies during the experiment of causes not immediately obvious will be post-mortemed.
11	31	**Disposal of animals - How will the animals be disposed of? (Use Dropdown box)**	Euthanased
11	32	**Disposal other**	Dams will be euthanised or transferred to another trial if possible
11	34	**If animals are to be euthanised indicate method of euthanasia or slaughter**	
11	35	**Electric stun and exsanguinate ~ captive bolt/exsanguinate**	No ~ No
11	36	**Anaesthetics overdose ~ Cervical dislocation**	Yes ~ Yes
11	37	**CO2 chamber ~ Guillotine**	Yes ~ No
11	39	**If animals are to be euthanised or slaughtered, please supply details (Facility, location, conditions etc)**	AgResearch Ulyatt Reid small animal unit; Grasslands; CO2 asphyxiation and cervical dislocation for animals in study. For pups born in excess of 12 per dam; day 2 post-birth euthanisation with sodium pentobarbitone and decapitation following GMSOP 04 13.
11	40	**Who will perform the procedure (Detail experience and SOP numbers)**	Euthanasia of pups 2 days post-birth and of dams will be performed by AgResearch Veterinarian, Susan Doohan following GM SOP 04 13. Euthanasia of pups during the sampling days will be performed by trained technicians following GM SOP 01 09.
11	41	**How will carcasses be disposed of?**	Incineration
11	42	**Treatment endpoints: Provide details of endpoints that will result in cessation of treatments**	
11	43	**Not applicable**	No
11	44	**Loss of weight ~ Details**	Yes ~ If a rat has weight loss of 10% or more over 7 days the rat will be euthanised
11	45	**Intervention trigger (e.g.) ~ Details**	No
11	46	**Tumour size ~ Details**	No
11	47	**Irritation by implant/device ~ Details**	No
11	48	**Metabolic upset ~ Details**	No
11	49	**Death ~ Details**	No
11	50	**Euthanasia of moribund animals ~ Details**	No
11	51	**Euthanasia on clinical threshold ~ Details**	No
11	52	**Other ~ Details**	No
11	60	**CONTINGENCY PLANS ~ How do you plan to deal with an emergency/unforeseen circumstances that may affect the welfare of the animals.**	
11	61	**Have you made any contingency plans?**	All staff responsible for animals will be contactable after hours. The animals will be checked at least once a day for GHS. If any animals become sick (a GHS of 3 or lower) they will be checked at least twice daily or immediately euthanised (as outlined above).
11	62	**Please detail how you will deal with unexpected events, or alternatively why you think such plans are unnecessary.**	in the case of unexpected events that compromise the health and welfare of the rats, the experiment will be terminated and rats will be euthanised.
11	65	**ADVERSE EVENTS**	
11	70	**Any adverse events that impinge on animal welfare are to be reported to the AEC immediately.**	

		12. NON-SURGICAL Manipulation	
12	1	**SAMPLING**	
12	2	**If you are collecting samples, CHOOSE SAMPLE TYPE FROM DROPDOWN below**	Tissue sample
12	6	**If there is an SOP approved by your AEC for the SAMPLING manipulation (s) please cut the name and number from the appropriate file found on the RVM/SOP page and paste in the space below. If no approved SOP is available, please describe the methodology.**	All tissue and blood sampling will occur following humane euthanasia of animals by CO2 asphyxiation and cervical dislocation (GMSOP 01 09).
12	7	**DRUG administration**	
12	8	**Intramuscular ~ Intracardiac**	No ~ No
12	9	**Subcutaneous ~ Per os by mouth**	No ~ No
12	10	**Intraperitoneal ~ Drug in water bottle**	Yes ~ No
12	11	**Intravenous ~ Other**	No ~ No
12	13	**If there is an SOP approved by your AEC for this manipulation please cut the name and number from the appropriate files found on the RVM/SOP page and paste in the space below. If no approved SOP is available please describe the methodology.**	for pups born in excess of 12, they will be euthanised on day 2 post-birth following GMSOP 04 13. Faeces will be collected from the rat pups' ante-mortem and post-mortem. Ante-mortem, the pups will be isolated into 500 mL pottles for a maximum of 15 minutes for faecal collection. Post-mortem, the faeces will be collected directly from the lower colon of the gastrointestinal tract.
12	14	**BEHAVIOUR and HANDLING**	
12	15	**Observation only ~ Enforced activity**	Yes ~ No
12	16	**Physical restraint ~ Nutritional regime**	Yes ~ No
12	17	**Other**	No
12	20	**EXPOSURE to**	
12	21	**Parasite ~ Micro-organism**	No ~ No
12	22	**Off-licence drug ~ Off-licence chemical**	No ~ No
12	23	**Biological product ~ Radiation**	No ~ No
12	24	**Electrical stimulation ~ Abnormal environment**	No ~ No
12	25	**Other**	No
12	28	**WILD (Non Domesticated) ANIMALS**	
12	29	**Handling ~ Capture**	No ~ No
12	30	**Marking, Tagging or Branding ~ Transmitter attachment or implantation**	No ~ No
12	32	**If other use of WILD (Non Domesticated) ANIMALS please describe**	
		13. SURGICAL Manipulation	
13	1	**Animals will survive surgery ~ Animals will NOT survive surgery**	No ~ No
13	2	**Manipulations for which SOPs have been approved**	
13	4	**For all manipulations where an SOP has not been approved, please enter the following information:**	
13	5	**ASEPTIC TECHNIQUE**	
13	7	**ANAESTHESIA and ANALGESIA**	
13	8	**Will the animals receive pre-emptive analgesia**	No

13	11	**Depth of Anaesthesia - Please indicate how this will be tested**	
13	12	**Thermal methods ~Electrical methods**	No ~ No
13	13	**Mechanical methods ~ Chemical methods**	No ~ No
13	14	**Metabolic methods ~ Local inflammatory reactions**	No ~ No
13	15	**Deep pain reflex response**	No
13	16	**MONITORING**	
13	19	**Will you be using standard monitoring sheets**	No
13	22	**POST-OPERATIVE support**	
13	23	**Not applicable, animals do not recover**	No
13	24	**Conservation of body heat ~ Monitoring sheets**	No ~ No
13	25	**Fluid administration ~ Recovery in individual cages**	No ~ No
13	26	**Administration of analgesics ~ Other**	No ~ No
13	28	**ENDPOINTS**	
		14. DRUGS and CHEMICALS	
14	1	**List all drugs and Chemicals to be administered to animals during this project below._____ PLEASE NOTE: You are required to complete a separate IDAO for EACH DRUG/CHEMICAL that is to be used in this project and is not an OTC compound or being administered by a registered veterinarian.**	
14	2	**If the drugs have been ACCEPTED by the AEC in terms of label information as appropriate for the routine use you intend, please cut the name of the drug and its coversheet number from the appropriate file(s) found on the RVM/SOP page and paste in the space below. IF NO DRUGS ARE TO BE ADMINISTERED, PLEASE DO NOT WRITE IN THIS SPACE**	Sodium Pentobarbitone 300. No IDAO is required as drug will be administered by the AgResearch veterinarian.
14	3	**If you are using drugs that do not have a COVERSHEET, please go to HELP on this page and use the link to download the template (second line below the green box). Complete one sheet for each drug and/or chemical. Send the completed form electronically to Mariette Komene. These will be made available in the RVM/SOP function for future reference.**	
14	4	**Have you had to make up new RVM COVERSHEET(s) for this project**	No
14	6	**UNREGISTERED Drugs or Chemicals**	
14	20	**Will ANY drugs used in this project (other than OTC Products) be administered by ANY PERSON who is NOT a registered Veterinarian (please answer either YES or NO) _____This question is an internal trigger for IDAO creation, names are not required here.**	No
		21. TISSUE COLLECTION	

21	1	**If you are requesting permission to collect tissue following euthanasia, even if you are not manipulating the animals before they are euthanised you must complete this page**	
21	2	**If this is an application primarily to collect animal tissue only, you must complete a full application**	
21	3	**Species (Use Dropdown box)**	Rats
21	5	**How many animals of each species will be used?**	8 dams - for birthing pups 104 pups - for tissue collection
21	6	**What tissue(s) are to be collected?**	ileum, digesta, blood
21	7	**If the animals are being euthanised for the purpose of tissue collection, please indicate what method of euthanasia will be used**	CO2 asphyxiation and cervical dislocation
21	8	**Have any other staff who might be interested in other tissues been advised?**	No
		97. SOURCE(s) and TRIAL SITE(s)	
97	1	**This section allows statistics to be gathered for MPI (Animal use information) and data transfer into Animal Tools for AgR animals.**	
97	2	**Please ensure you have selected the correct Trial Site as information is transferred to the Animal Tools database. Please note that you may select more than one Trial Site where relevant depending on the fate of the animals. If the endpoint is returning/transporting animals to a AgR Farm, ensure you add this as an additional Trial Site.**	
97	3	**RATS**	Source = AGR Ruakura Small Animal Unit Trial Site = AGR Grasslands Animal Facility (Ulyatt/Reid) Number = 112
		98. NOTES ~ Read only	
98	1	**Status Change**	(ogutul 09/07/2018) SUBMIT

98	2	**Committee Decision**	(26/07/2018 RESUBMITcosgroveg) Hi Linda, Below are some questions and comments that have arisen from recent AEC discussion. 1. Expand the objective to reflect that more than just the best technique (for tissue preparation) is being assessed in this application, given that the experimental design and protocol is also new, and information on prevalence at different time points will be used to design future studies. 2. Add further science information to section 4.5 (objective). Weaning effect or age effect? Would a pre-weaning time point (Day 15 and/or 18) be beneficial? Rats usually start to self-wean and eat hard food from 15 days. To keep the number of samplings the same could you reduce a later time point? Check literature and comment. 3. Future objectives include 'stimulate the abundance of SFB'. Are there any known negatives of either naturally high, or artificially stimulated higher levels of SFB in rodents or in humans? 4. As Grasslands has not done breeding of rodents recently contact Chrissie Butts at Plant & Food Research to discuss how to care for breeding animals. It is recommended not to handle/count the pups on Day 0 as this may unsettle the dam. Leave until Day 2 if possible, with visibly small, weak, or discoloured/isolated pups selected preferentially for removal. 5. Recommended to increase grading of manipulations to "C" due to possible adverse effects from manipulation of litter size (handling at a vulnerable time period). 6. Susan to complete pentobarb euthanasia and monitor visit to access the grading of manipulations. 7. Confirm fate of dams. Can they be re-used for other trials? Check within team and with Hailey for possible uses. Contact Susan or myself if you require clarification on these questions. Gerald
98	3	**Status Change**	(ogutul 03/08/2018) SUBMIT
98	4	**Committee Decision**	(09/08/2018 APPROVEDcosgroveg)
		99. PERSONNEL and Sign off	
99	1	**Committee**	GRASSLANDS

99	1	**APPLICANT: All personnel who will manage the animals or perform manipulations on them, The Programme leader, Facility manager Biometrician and the person responsible for entering info on the animal database must sign this application.** **PLEASE Describe clearly what each person's role in the project is.** **All personnel who will perform manipulations on the specified animals must:** **A - Have been trained and be competent in the manipulations specified (unless this is a specified training protocol)** **B - Be conversant with the AgResearch Code of Ethical Conduct.** **TICKING THE APPROVE BOX IMPLIES THAT YOU HAVE:** **A - read and understood this application** **B - agreed to comply with all statutory requirements regarding the use of animals in experiments** **C - agreed to comply with all statutory requirements regarding the use of any Restricted Veterinary Medicines specified for use in this project.** **Any person whose name is NOT selected from a dropdown list may not be recognised by the auto email function in the database.**	
99	99	**ANDERSONRC ~ approved ~ Job (Senior Research Scientist) Location (Grasslands; Alan Johns Building. Tues-8-4.30pm. Mon-Fri-8-2.30pm)**	Project Leader, Senior Research Scientist
99	99	**BOWDENJ ~ Job (Assistant Animal Technician) Location (Grasslands;)**	Assistant animal technician
99	99	**BROADHURSTR ~ approved ~ Job (Senior Scientist Small Animal Colony) Location (Ruakura; Small Animals, An Phys)**	Facility Manager, Ruakura Senior Scientist Small Animal Colony
99	99	**DEWHURSTH ~ approved ~ Job (Technician) Location (Grasslands; Alan Johns F27 & Animal Facility Buildings.)**	Tissue sampling
99	99	**DOOHANS ~ approved ~ Job (Veterinarian and Animal Welfare Officer) Location (Grasslands; Animal Facilities - Ulyatt-Reid Facility)**	Day 0 pup Euthanasia
99	99	**DUNSTANK ~ approved ~ Job (Research Technician) Location (Grasslands; Alan Johns - Rm F18)**	Animal Technician
99	99	**GILLESPIEH ~ approved ~ Job (Animal Facility Manager) Location (Grasslands; Lab Admin Building)**	Grasslands Animal Facility Manager
99	99	**JOHNSTONEP ~ approved ~ Job (Senior Statistician) Location (Invermay; Room 10 Cullen building)**	Statistician

99	99	**OGUTUL ~ approved ~ Job (PhD Student) Location(Grasslands; PhD Student Room)**	PhD student
99	99	**ONEILLC ~ approved ~ Job (Small Animal Technician) Location (Grasslands; Lab Admin Building #41 Rm A-03.)**	Euthanasia for trial and day to day care
99	99	**ROYN ~ approved ~ Job (Science Team Leader Food Nutrition & Health) Location (Grasslands; Alan John Building, Level 2)**	Team Leader
99	99	**SMITHBG ~ approved ~ Job (Research Associate) Location (Ruakura; Small Animals, An Phys, Fire Warden, First Aid)**	Animal Technician. Ruakura
99	99	**VANKEULENM ~ approved ~ Job (Technician) Location (Grasslands;)**	Tissue sampling

Appendix IIIc

Animal Ethics Application No. 14992

AE Application 14992 ~ (Status=CLOSED) (Applicant=OemckeL) Investigating the dosage of inulin in a rodent diet suitable for influencing segmented filamentous bacteria abundancein the ileum of post-weaning Sprague-Dawley rats

Group	Line	Question	Answer
		ASSOCIATED Documents	
		ADVERSE EVENT 196 ~ ACCEPTED	(AE APPLICATION 14992) Investigating the dosage of inulin in a rodent diet suitablefor influencing segmented filamentous bacteria abundance in the ileum of post- weaning Sprague-Dawley rats
		AE Stats 8243 ~ ACCEPTED	(AE APPLICATION 14992) Investigating the dosage of inulin in a rodent diet suitablefor influencing segmented filamentous bacteria abundance in the ileum of post- weaning Sprague-Dawley rats
		Attached pdf 11227	Inulin dosage study-Experimental design
		AE Report A 14992 ~ ACCEPTED	(AE APPLICATION 14992) Investigating the dosage of inulin in a rodent diet suitablefor influencing segmented filamentous bacteria abundance in the ileum of post- weaning Sprague-Dawley rats
		AE Report B 14992 ~ ACCEPTED	(AE APPLICATION 14992) Investigating the dosage of inulin in a rodent diet suitablefor influencing segmented filamentous bacteria abundance in the ileum of post- weaning Sprague-Dawley rats
		0. ADMINISTRATIVE DETAILS (Please include Species and Model in your Title)	
0	1	**Title**	Investigating the dosage of inulin in a rodent diet suitable for influencing segmented filamentous bacteria abundance in the ileum of post-weaning Sprague-Dawley rats
0	3	**Applicant**	Linda Oemcke
0	4	**Institution**	AgResearch Limited
0	5	**Business Address**	AgResearch Grasslands Tennent Drive Palmerston North 4442
0	6	**Phone**	06 351 8372
0	7	**Mobile phone**	0212775545
0	8	**Email**	Linda.Oemcke@agresearch.co.nz
0	9	**Contact details**	
0	10	**Person responsible for animals during manipulations (MUST be named on and sign the personnel page). If more than one facility is being used, all persons responsiblefor animals must be named and signthis Application.**	Linda Oemcke
0	11	**Phone**	06 351 8372
0	12	**Mobile phone**	0211775545
0	13	**Email**	Linda.Oemcke@agresearch.co.nz
0	14	**Person responsible for entry of Trial Drug and animal manipulation data into the Animal Use database. (AgResearch requirement only ~ MUST be named on and sign the personnel page). If no AgResearch facilities are being used please enterNot Applicable**	Not Applicable
0	15	**Program Manager / Principal Investigator (MUST be named on and sign the personnel page)**	Rachel Anderson
0	16	**Biometrician (MUST be named on and sign the personnel page)**	Peter Green

0	17	**Facility Manager - If more than one facility is being used, all Managers must be named and sign this Application. (MUST be named on andsign the personnel page)**	Hailey Gillespie
0	19	**Project Dates ~ PLEASE enter all dates in the form requested [dd/mm/yy] AND ensure that the startdate you enter occurs after the next meeting of your committee. If you donot expect to renew this Application,please do not enter a Renewal Date**	
0	20	**Start Date (dd/mm/yy)**	15/06/2020
0	21	**Finish Date (dd/mm/yy)**	02/08/2020
0	22	**Reminder Date (dd/mm/yy) Enter a date to receive an email reminder thatthe Approval Period is due to end (Recommend 2 Months).**	
0	23	**Are there FOOD SAFETY issues under the Animal Products Act, or Regulatory approval, associated withthis project (PLEASE NOTE: The FOOD CHAIN includes food for eitherHUMAN CONSUMPTION or PET FOOD)? If you are using Laboratory Rodents and/or other species that are never used for human consumption or pet food, or if the animals in the project will be safe to enter the food chain, please type the word NO and then proceed to the next question. However, if some or all of the animals must be excluded from EVER entering the food chain because of the manipulations, drugs they will be given or because of Regulatory Approval requirements, please type YES and then explain why. AgResearch animals that must not enter the food chain must be PINK TAGGED.**	No
0	24	**Māori Consultation. If there is any uncertainty, it is the applicant's responsibility to approach local Māori representatives for clarification.**	
0	25	**Does this project require consultationwith Māori?**	No
0	26	**If yes, please indicate what aspects are of interest to Māori. If necessary,evidence of approval from iwi or hapumust be appended as a pdf**	
0	27	**Are you requesting URGENT consideration? Only tick YES if the AEC needs to consider this Application before the next scheduled meeting.**	No
0	28	**If YES, please state why the Application is urgent**	
0	29	**Database Administration**	
0	30	**From the Dropdown, AgResearch users select your Science Group andTeam; Parented users select EXTERNAL USER**	Food and Bio-based Products (Food Nutrition & Health)
		1. AEC JURISDICTION	
1	1	**Which committee are you applyingto? (Use Dropdown box)**	Grasslands
1	2	**Some aspects of this work will alsobe undertaken under the jurisdictionof the AgResearch AEC at (Use Dropdown box)**	Ruakura

1	3	**Some aspects of this work will alsobe undertaken under the jurisdictionof the AgResearch AEC at (Use Dropdown box)**	
1	5	**Are staff, animals or facilities of another organisation(s) which has anAnimal Ethics Committee involved inthis project?**	No
1	6	**If YES Name other organisation(s) involved in this project**	
1	7	**Does the Ethics committee of that organisation(s) have a copy of this application?**	No
1	8	**Has this application, or a similar application been submitted to another Ethics Committee on a previous occasion?**	No
1	9	**If YES, please provide the name ofthe committee that the applicationwas previously submitted to and details of the submission**	
		2. REGULATORY APPROVAL	
2	1	**Any regulatory approval must be obtained before submission and proof that all necessary approval(s)have been granted must be attached**	
2	2	**Are approvals required from an outside body (ies? Please answer either YES or NO**	NO
2	3	**If any aspects of this proposal require approval from a regulatory body(s) please select from list below**	
2	4	**Approval is required from (Use Dropdown box)**	
2	5	**Approval authority ~ Please supply approval number, and note that a pdfof approval has been attached**	
2	6	**Approval is also required from (Use Dropdown box)**	
2	7	**Approval authority ~ Please supply approval number, and note that a pdfof approval has been attached**	
2	8	**Approval is also required from (Use Dropdown box)**	
2	9	**Approval authority ~ Please supply approval number, and note that a pdfof approval has been attached**	
2	10	**If Other please specify ~ Please supply approval number, and notethat a pdf of approval has been attached**	
		3. CONFIDENTIALITY	
3	1	**Is information in this Application andany Reports associated with it CONFIDENTIAL?**	
3	2	**If YES, which aspects are CONFIDENTIAL?**	
		4. LAY SUMMARY	
4	1	**NOTE (Please use lay language.Each line must be less than 7800characters - Save regularly)**	

4	5	**Objectives**	The objective of this study is to investigate the dose of inulin in a rodent diet suitablefor influencing the abundance of SFB in ileal tissue, ileal contents, and faeces of pre-[PND 20] and post-weaning [PND 24] Sprague-Dawley rats. PND 20 which is the pre-weaning age group will be the baseline for SFB abundanceand weaning will occur at PND 21. The pups at PND 20 and 24 will be fed diets withdifferent inulin doses and SFB abundance will be quantified to determine which dosehas a greater effect. PND 24 was chosen as it is the age when SFB abundance peaked in the 2018 rat study. This is a preliminary study to help decide on the best dose of inulin andexperimental design for the main time course study.
4	6	**Introduction**	The intestinal barrier in the gastrointestinal tract (GIT) is a semi-permeable membrane which allows some components through, whilst preventing unwanted components from passing into our bodies [1, 2]. Development of this barrier in humans begins in utero with the appearance of enterocytes at 8 weeks gestation anddetection of tight junctions at 10 weeks. At 12 weeks the crypt-villus architecture (where cells involved in host defence and signalling reside) is formed, at 24 weeks intestinal epithelial lymphocytes are recruited then at 40 weeks goblet cells begin to express mucin. At birth, not all structures of the GIT are fully formed therefore maturation continues until weaning when solid foods are introduced to complementthe milk-based diet. At weaning, segmented filamentous bacteria (SFB) reportedly increase in abundance suggesting the role of diet in influencing the abundance of these microbes. SFB are Gram-positive, obligate anaerobic, commensals that adhere to the mucosaof the ileum and are reported to play a role in the maturation of the intestinal immunological barrier during infancy [3-5]. The mechanisms by which SFB function remain unknown as unsuccessful in vitro culture has prevented in-depth investigations. This has limited studies on SFB to in vivo work in rodents particularly mice and rats. Results from the AE 14485 study confirmed the rat as a suitable model for SFB studies and whole tissue homogenisation as the preferred method ofcollecting ileum tissue samples for SFB quantification. The suggested role of the weaning diet on influencing SFB abundance led to this method development study which would segue into further investigations on the impact of an enriched diet onSFB abundance in the ileum pre- and post-weaning. Of interest would therefore be to investigate what types of substrates would influencechanges in the abundance of SFB and if these changes modulate the GIT immune system in weanling rats. A look at the SFB genome shows multiple metabolic pathways for carbohydrate metabolism, and this assisted in deciding what substrate to use to enrich a regularrodent diet with [6]. The use of enriching rodent diet using inulin and other fibres indicated its safety for rodent consumption [7, 8]. Inulin is a prebiotic, a soluble dietary fibre, which cannot be digested by humans but is digested by probiotics thereby encouraging growth of gut microbiota. Also, the addition of inulin to infant formulas [9] indicated its safety for consumption in that age group which is ultimatelythe target. The goal was then to create a rat diet that emulated a weaning diet in infants. Firstly, it was important to confirm whether the natural composition of rat milk contains inulin or not. This allows for a baseline (control) from which a rat diet can then be formulated to contain the desired amount of inulin. As inulin is not present in rat breastmilk [10, 11], the absence of inulin in the rat diet would also not have negative effects on the pups. Previously, a combination of galactooligosaccharides with inulin (GOS/inulin) and given as a supplement reportedly increased bacterial translocation in artificially reared new-born rats [12]. Despite this observation, there have been no adverse effects associated with supplementation of infant formulas with GOS/inulin infull and pre-term infants as inulin is also not found in human breastmilk [13]. Additionally, when germ-free rats colonised by human faecal bacteria were fed inulinand fructooligosaccharides there was an increase in numbers of bacterial members from clostridial cluster XIVa group in the colon [7]. Taxonomical classification suggests SFB to be part of clostridial cluster XIVa, though the effect of inulin specifically on SFB abundance has not yet been reported. These observations contributed to inulin being selected as the substrate for rodent diet enrichment.

4	7	**Methods ~ (If you have a Table, append it to the Application as a PDF)**	The rat strain for this experiment will be Sprague-Dawley. This strain was used in a former study by Davis and Savage [6] where they found naturally occurring SFB in the terminal ileum. A 2018 rat study also confirmed the presence of SFB in this strainover time pre- and post-weaning. The study will include male and female pups aged 20 and 24 days after birth. Therewill be only 2 time-points i.e., PND 20 the pre-weaned pups and baseline for SFB abundance and PND 24 as SFB abundance peaked in the 2018 rat study. It is therefore important to compare baseline SFB abundance and peak SFB abundancewith different inulin doses to observe which dose produces the greatest effect. Terminal sampling will occur at PND 20 for the collection of ileum samples and faeces. Caecum samples and colon samples will also be collected to investigate any influence of SFB abundance in these regions of the gut. Blood plasma and faeces willalso be collected for interleukin 17 and immunoglobulin A analyses respectively which have been correlated with SFB presence in the ileum. Rats will be mated at the AgResearch Small Animal Colony in Ruakura, and the dams will be transported to Grasslands after 13-16 days which is optimal time to allow for a pregnancy diagnosis. The dams will be brought into the Ulyatt-Reid SmallAnimal Facility. They will be maintained under physical containment level 2 (PC2) and conventional conditions at a constant temperature of 20 degrees Celsius with a 12-hr light/dark cycle. They will be allowed ad libitum access to the control diet (0% inulin) which is suitable for pregnant/lactating rats and water until the pups are born.The water will be replaced as required. The control diet is one formulated by Research Diets Inc. The dams will be fed the control diet containing 0% inulin whilst the pups will be fed a diet pertaining to their inulin dose group. Once the pups are born, they will be maintained in the same cages with their mothersunder PC2 containment and conventional conditions at a constant temperature of 20 degrees Celsius with a 12-hr light/dark cycle. The pups will suckle, and the mothers will be allowed ad libitum access to their control diet and water. One group of PND 20 pups will be euthanised for sample collection. The remainingpups will be weaned at PND 21 and separated into different cages for each inulindose (0%, 2.5%, 5%, 10%). The pups will remain in their birth litters post-weaning and will be randomised between the 2 sampling days. Birthing is expected over a 3-day period, the expected span for 9 dams, so samplingwill be done based on pup weaning time. At 19-21 days old, all pups will be weaned.This collection method is ideal as we assume diet change (breastmilk pre-weaningand rodent feed & water post-weaning) influences SFB abundance, not age. The mating of rats will be staggered to allow for sample collection over 3 or 4 days avoiding having too many animals in one day.
4	8	**Design ~ (If you have a diagram, append it to the Application as a PDF)**	Experimental design appended as a PDF.
4	9	**Timetable of events ~ (If you have a Table or diagram, append it to the Application as a PDF)**	Ruakura: Day 0 - Rat pairing and mating Transportation of dams from Ruakura to Grasslands: Day 13-16 after mating Grasslands: Maintain dams until birth of pups Grasslands: Day 0 - birth of pups Grasslands: Maintain pups until weaning time Grasslands: Day 20 after birth: Pre-weaning; collecting samples of 16 pups or more Grasslands: Day 21 after birth: Weaning of remaining pups Grasslands: Day 24 after birth: Collecting samples of 64 pups or more
4	10	**Expected outcomes**	This study will allow us to determine the dose of inulin required to influence SFB abundance in the ileum, caecum, colon, and faeces. The chosen dose of inulin willthen be used in the following experiment where the influence on SFB abundance over a longer period will be investigated.
4	12	**SUMMARY OF ANIMAL MANIPULATIONS**	
4	13	**Describe the groups of animals, number of animals per group, manipulations, Grading (A, B, C, D or E)of animals in each group, drugs/compounds to be administeredto each group and the benefits expected from this work.**	For PND 20 (pre-weaning - baseline) there will be an average of 8 pups for each sex.For PND 24 with 4 inulin doses for both sexes there will be 64 pups. Therefore, to produce enough pups of both sexes, 9 dams will be required. With dams producingan average of 12 pups per litter 108 pups may be produced. One hundred and eight untreated, healthy rat pups of mixed sex will be used for this experiment. The samples to be collected include ileum, caecum, colon, and faecal samples. This studywill allow us to determine the appropriate dose of inulin required to cause an observable change in SFB abundance.
		5. PROJECT TYPE	
5	2	**This application is a (Use Dropdown box)**	New application
5	3	**If this application is a renewal of a previous approval, please complete this page before moving to section 6.**	
5	4	**Previous number(s):**	
5	5	**Previous title:**	

5	6	**Lay summary of results from previous approval:**	
5	7	**Significance of these results:**	
5	8	**Reasons for continuing:**	
		6. SCIENCE Justification	
6	1	**Immediate Goal of this project:**	The goal of this study is to determine which dose of inulin i.e., 0%, 2.5%, 5% and 10%in a rodent diet has any influence on the abundance of SFB in ileum tissue, contents, and faeces in post-weaning rats. SFB abundance change will be observed on PND 24.
6	2	**Purpose of your experimental programme:**	The purpose is to determine which dose of inulin would be sufficient to influence SFB abundance in post-weaning rats. The chosen dose of inulin will then be used in AE 14984 to investigate the timings ofSFB abundance change as well as the size of the changes.
6	3	**Longer term benefits of the research programme:**	If there are differences in SFB abundance caused by inulin, that will be used in a larger rat study to investigate the size of SFB abundance changes and the timing ofthose changes post-weaning. Results from this study may then contribute to the design of weaning diets to improveinfant gut development for lifelong health.
6	4	**Has this application been reviewed byyour peers**	Yes
6	5	**If YES, please detail how the project has been peer reviewed**	Peer reviewed by Dr Rachel Anderson as well as technical staff involved in the study.
6	6	**Are you aware of similar/previouswork in this field either in NZ or overseas?**	Yes
6	7	**If YES, please detail how similar/previous work in this field either in NZ or overseas relates to your proposal:**	A number of studies (e.g., Ohashi et al., 2010; Ericsson et al., 2015) have been previously carried out where SFB was isolated from the ileum of mice using ileal mucosal scraping. A 2017 mouse SFB study at Grasslands confirmed the observations made by Ohashi et al., 2010. The previous rat SFB study (AE 14485) also confirmed the presence of SFB in Sprague-Dawley rat pups over time. The changes in SFB abundance were reported after the pups were weaned suggesting the role diet may play in influencing SFB abundance. If diet indeed plays a role, theremay be a substrate(s) that affects the abundance, and it is unclear which one it is. Still, with all the SFB studies carried out there have been no studies on the role ofdiet on SFB abundance.
		7. ANIMAL USE Justification	
7	1	**Total number of animals being used**	117
7	2	**What is the highest MPI grading of manipulations that will apply to this project (use dropdown) - The grades must reflect the summed impacts of both the initial state of the animal andthe induced effect of the experimental procedure, not the induced effect alone**	C (MODERATE IMPACT)
7	3	**How many animals will be in this grade?**	108
7	4	**How did you choose which species/breed/strain of animal to usefor this project?**	We chose the Sprague-Dawley rat strain to use as SFB is confirmed as beingpresent pre- and post-weaning.
7	5	**Why is it necessary to use sentient animals to achieve the goal(s) of this work?**	The abundance of SFB in ileum tissue, ileum contents, caecum tissue, caecum contents, colon tissue, colon contents and faeces in rats can only be determinedusing animals.
7	6	**[REDUCTION] Describe how you reduced the number of animals proposed to the minimum number that can achieve the aims of the study?**	By doing a power analysis.
7	7	**[REFINEMENT] Describe the aspectsof the study you have put in place to minimise the impact of the manipulations you have proposed?**	The rats are not treated with chemical reagents. They will be fed multiple diets (0%,2.5%, 5% and 10% inulin). The healthy rat pups will be humanely euthanised by CO2asphyxiation and cervical dislocation using GMSOP 01 09 before ileal and faecal samples are collected by trained technicians.

7	8	**[REPLACEMENT] Describe the ways you could replace the use of animalswhile achieving the aims of the study? Why is avoiding the use of animals not possible?**	It is not ethical to take ileal samples from human infants therefore we need to userodents to model this.
7	9	**Are there likely to be any animals specifically bred or acquired for this study and euthanised without being manipulated, i.e., SURPLUS? If yes, provide details (Rodents supplied from RUAKURA SAC will have the surplus numbers collected by the SAC)**	No. All animals will be manipulated. The dams will be transported from Ruakura to Grasslands have their litter sizes altered (day 2 pup euthanasia) to prevent overcrowding during breastfeeding. Additionally, any surplus pups after day 2 euthanasia will be randomised amongst the 4 inulin treatment groups.
		8. PUBLICATION	
8	1	**How will the results of this work be published or disseminated**	The results will be used in the following work which will involve investigating the influence of an inulin-enriched diet (with the appropriately chosen dose) on the abundance of SFB over time. The results will also be included in my PhD thesis.
		9. BIOMETRIC EVALUATION	
9	1	**Was there a Power Based assessment of the adequacy of sample size(s)**	Yes
9	2	**Main Hypothesis of interest**	A diet enriched with 5% inulin will lead to an increase in SFB abundance in pre- andpost-weaning Sprague-Dawley rats.
9	3	**Main variable of interest**	Effect of 0%, 2.5%, 5% and 10% inulin on the abundance of SFB post-weaning (PND24).
9	4	**Experimental design**	For this study, animals of mixed sex will be used, and SFB abundance compared between both sexes. For PND 20 (pre-weaning - baseline) there will be an average of8 pups for each sex. For PND 24 with 4 inulin doses for both sexes there will be 64 pups. In order to produce enough pups of both sexes, 9 dams will be required. Withdams producing an average of 12 pups, the total number of pups may be 108 pups. The samples to be collected on PND 20 and 24 include ileum, caecum, colon, andfaecal samples.
9	5	**Biometricians comments**	When data from this study is available, the power analysis for AE 14984 will be checked/updated.
9	6	**Data used in Power analysis**	Data from: 2018 Rat SFB study. Determination of the abundance of segmented filamentousbacteria in ileal samples of pre- and post-weaning Sprague-Dawley rats.
9	7	**Standard deviation (SD) of the main variable**	1.07
9	8	**Where was the SD obtained from**	Data from: 2018 Rat SFB study. Determination of the abundance of segmented filamentousbacteria in ileal samples of pre- and post-weaning Sprague-Dawley rats.
9	9	**Minimum true difference of interest**	1.7
9	10	**Desired power of the experiment**	80%
9	11	**Desired level of significance**	0.05
9	12	**Resulting sample size**	8
9	13	**If a power analysis has not been performed, please explain why here**	
		10. ANIMAL INFORMATION	
10	1	**Animal Details (Use Dropdown boxes)**	
10	2	**Species ~ Breed or strain**	Rats ~ Sprague-Dawley
10	3	**Common name ~ Gender**	~ Female
10	4	**Reproductive status ~ Age**	Pregnant
10	5	**Number being used ~ Health status**	9 ~ Healthy
10	6	**Additional Animal Details (Use Dropdown boxes)**	
10	7	**Additional Species ~ Breed or strain**	Rats ~ Sprague-Dawley
10	8	**Additional Common name ~ Gender**	~ Mixed sex
10	9	**Additional Reproductive status ~ Age**	Normal and Non-pregnant ~ Pre- and post-weaning

10	10	**Additional Number being used ~ Health status**	108 ~ Healthy
10	11	**1 Additional Animal Details (Use Dropdown boxes)**	
10	12	**1 Additional Species ~ Breed or strain**	
10	13	**1 Additional Common name ~ Gender**	
10	14	**1 Additional Reproductive status ~Age**	
10	15	**1 Additional Number being used ~ Health status**	
10	16	**2 Additional Animal Details (Use Dropdown boxes)**	
10	17	**2 Additional Species ~ Breed or strain**	
10	18	**2 Additional Common name ~ Gender**	
10	19	**2 Additional Reproductive status ~Age**	
10	20	**2 Additional Number being used ~ Health status**	
10	22	**3 Additional Animal Details (Use Dropdown boxes)**	
10	23	**3 Additional Species ~ Breed or strain**	
10	24	**3 Additional Common name ~ Gender**	
10	25	**3 Additional Reproductive status ~Age**	
10	26	**3 Additional Number being used ~ Health status**	
10	27	**4 Additional Animal Details (Use Dropdown boxes)**	
10	28	**4 Additional Species ~ Breed or strain**	
10	29	**4 Additional Common name ~ Gender**	
10	30	**4 Additional Reproductive status ~Age**	
10	31	**4 Additional Number being used ~ Health status**	
10	32	**Previous use of animals**	
10	33	**Have any of the animals been used in previous experiments (i.e., with a different AE Application number)**	No
10	34	**If YES What were the animals usedfor previously?**	
10	35	**When and where did the previous use occur?**	
10	36	**What were the effects of the previoususe?**	
10	37	**Please present information to justifyre-using these animals**	
10	38	**SURPLUS Animal Details, as described in 7.9 (Use Dropdown boxes)**	
10	39	**Species ~ Breed or strain**	
10	40	**Common name ~ Gender**	~ Female
10	41	**Reproductive status ~ .Age**	
10	42	**Number being used ~ Health status**	
		11. ANIMAL WELFARE	

11	1	**Please detail the animal husbandryand welfare principles that will be applied:**	
11	2	**In preparation for the experiment**	Nine dams will be obtained from AgResearch Small Animal Colony (Ruakura) and transported to AgResearch Ulyatt-Reid Animal Facility (Grasslands). Prior to and after mating the rats will be fed a control diet (0% inulin) which is fed as a normal dietin rodent breeding facilities. This is to ensure the gut microbiota of the dams is not affected by differences in diets between housing facilities. The dams will be maintained under PC2 conditions until the rat pups are born. Dams produce on average 12 pups each, therefore, to produce enough pups withrelatively equal numbers of each sex, 9 dams will be required which may produce108 pups. The pups will be left with their mothers to suckle pre-weaning. The first group of pupsat PND 20 will the euthanised for sample collection. The pups will then be separated from their mothers at weaning on PND 21 but kept in their birth litters. Extra pups (excess of 12 pups per dam) born will not be cross-fostered as is commonly done but will be euthanised 2 days post-birth using GMSOP 04 13. Giventhat the pups will be 2 days old, we will follow sodium pentobarbitone euthanasia withdecapitation to confirm death. At this age, the pups are regarded as neonates and unsuitable for CO2. The aim is to avoid cross-fostering which may impact the abundance of SFB in the gut of the pups due to a possible maternal influence on the gut microbiome. For extra pups born, some may be randomised into the time-point groups for extrasample collection. More samples will reduce variability in the data analysis.
11	3	**While in the experiment**	The dams will be housed individually for whelping (birthing) under PC2 conditionswith rodent feed and water being supplied ad libitum. The rat pups will be housed with their mothers pre-weaning as they suckle. At weaning time, they will be separated from their mothers and housed in their litter groups under PC2 conditions. They will be divided into different cages based on thedose of inulin they are to receive through their diet.
11	5	**At the end of the experiment**	The rat pups will be euthanised humanely by CO2 asphyxiation and cervical dislocation prior to tissue sample collection for analysis. The dams cannot be returned to Ruakura as once they leave the PC2 facility, they cannot be reintroduced into that facility. After the weaning of pups, the dams may beused for other training purposes at the Ulyatt-Reid Animal Facility under appropriateethics approval only otherwise they will be euthanised with CO2. Jason Peters has expressed interest in obtaining rat tissues. This will be coordinated by Hailey Gillespie and Susan Doohan.
11	6	**Please detail any other animal welfare considerations (e.g., transport)**	Dams will be transported from Ruakura in appropriate rodent transport containers and following IATA guidelines. A New Zealand courier company will be used in orderto assist with tracking the shipment and ensuring animals are cared for throughout the trip. All dams and rat pups after birth will be checked daily, weighed once a week, andfresh food and water topped up as required. General Health Score (GHS) will be checked daily. GHS ranges from 5 (healthy) to 1 (almost dead). A rat with a GHS score of 3 or 4 will be closely monitored and if their condition does not improve they will be humanely euthanised. Once a GHS of 2 is attained, rats willbe monitored hourly or euthanised immediately depending on their condition. A ratwith a GHS of 1 will be immediately euthanised, as will one that has a weight loss greater than 10% from previous weighing. For this trial only healthy animals will be used, therefore proactive euthanisation is preferred. There would be no reason to delay euthanisation since few animals wouldrecover, and if they did it may be with intervention anyway.
11	7	**Monitoring - any animals that die during the experiment from causes not immediately obvious from clinicalsigns MUST BE POST-MORTEMED and the AEC notified immediately**	
11	8	**Please describe how animal welfarewill be monitored**	All rats (dams and pups) will be monitored daily, weighed once a week, and fed twice weekly. The dams will be housed in individual cages. Their weights and body conditions will be easily compared. Fresh (normal) water and a control diet (0%

			inulin) rodent feed will be provided as required. The rat pups will be maintained in their birth litters post-weaning and will be individually identified by ear-punching according to GMSOP 08_17 which is reportedto be safe for pups younger than PND 21. The ear punching GMSOP 08_17 statesthat it can be done after 2 weeks of age, as this is when the pinna is completely separated from the head. Marking will be done in order to compare their weights andbody conditions. Fresh (normal) water and rodent feed will be provided as required. Susan Doohan, the Veterinarian and Animal Welfare Officer will monitor visits toassess the grading of manipulations.
11	9	**Indicate which of the following youwill monitor and how frequently. Ifyou are using a monitoring sheet attach it as a pdf.**	
11	10	**Body weight ~ Frequency**	Yes ~ 1 x weekly
11	11	**Weight loss ~ Frequency**	Yes ~ 1 x weekly
11	12	**Water intake ~ Frequency**	Yes ~ 1 x weekly
11	13	**Food intake ~ Frequency**	Yes ~ 1 x weekly
11	14	**Posture ~ Frequency**	Yes ~ 1 x weekly
11	15	**Gait/movement ~ Frequency**	Yes ~ 1 x weekly
11	16	**Coat condition ~ Frequency**	Yes ~ 1 x weekly
11	17	**Vocalisation ~ Frequency**	No
11	18	**Respiration ~ Frequency**	No
11	19	**Faecal consistency ~ Frequency**	Yes ~ 1 x weekly
11	20	**Vaccination site reaction ~ Frequency**	No
11	21	**Fistula cleanliness ~ Frequency**	No
11	22	**Fly strike ~ Frequency**	No
11	23	**Parasites ~ Frequency**	No
11	24	**Haemorrhage ~ Frequency**	No
11	25	**Oedema/swelling ~ Frequency**	No
11	26	**Infection ~ Frequency**	No
11	27	**Self-mutilation ~ Frequency**	No
11	28	**CNS signs ~ Frequency**	No
11	29	**Other**	
11	30	**How will you manage adverse effects? - NOTE: any animals that dieduring the study from causes not immediately obvious from clinical signs MUST BE POST-MORTEMED.**	Rats will be checked daily for specific health parameters. Any animal that dies duringthe experiment of causes not immediately obvious will be post-mortemed.
11	31	**DISPOSAL OFANIMALS - How will the animals be disposed of? (Use Dropdown box)**	Euthanased
11	32	**Disposal other**	Dams will be euthanised or used for training or transferred to another study if possible.
11	33	**If sold or retained, where will the animals be moved to (Facility, location, conditions etc)**	
11	34	**If animals will be euthanised what method will be used**	
11	35	**Electric stun and exsanguinate ~ captive bolt/exsanguinate**	No ~ No
11	36	**Anaesthetics overdose ~ Cervical dislocation**	Yes ~ Yes
11	37	**CO2 chamber ~ Guillotine**	Yes ~ No
11	38	**Other**	
11	39	**Facility, location, and conditions of euthanasia**	AgResearch Ulyatt-Reid Small Animal Unit; Grasslands; CO2 asphyxiation and cervical dislocation for animals in study.

11	40	**Who will carry out the euthanasia? (Detail experience and SOP numbers)**	Euthanasia of pups during the sampling days will be performed by trained technicians following GM SOP 01 09.
11	41	**How will carcasses be disposed?**	Incineration
11	42	**STUDY ENDPOINTS: These are planned endpoints that if reached will result in cessation of treatments to an animal and/or removal from the study**	
11	43	**Endpoints are not applicable for this study (MUST be a very good reason)**	No
11	44	**Loss of weight ~ Details**	Yes ~ If a rat has weight loss of 10% or more over 7 days the rat will be euthanised
11	45	**Intervention trigger (e.g.) ~ Details**	No
11	46	**Tumour size ~ Details**	No
11	47	**Irritation by implant/device ~ Details**	No
11	48	**Metabolic upset ~ Details**	No
11	49	**Death ~ Details**	No
11	51	**Euthanasia on clinical threshold ~ Details**	No
11	52	**Other ~ Details**	No
11	60	**CONTINGENCY PLANS ~ How do you plan to deal with an emergency/unexpected circumstances that may affect the welfare of the animals.**	
11	61	**Have you made any contingency plans, and if not, why?**	Yes.
11	62	**Please detail your contingency plans,**	All staff responsible for animals will be contactable after hours. The animals will be checked at least once a day for GHS. If any animals become sick (a GHS of 3 orlower) they will be monitored at least 3 times daily or immediately euthanised (asoutlined above). In the case of unexpected events that compromise the health and welfare of the rats,the experiment will be terminated, and rats will be euthanised.
11	65	**ADVERSE OR UNEXPECTED EVENTS THAT COULD AFFECT ANIMAL WELFARE MUST BE REPORTED TO THE AEC IMMEDIATELY**	
		12. NON-SURGICAL MANIPULATION	
12	1	**SAMPLING**	
12	2	**If you are collecting samples, CHOOSE SAMPLE TYPE FROM DROPDOWN below**	Tissue sample
12	6	**If there is an SOP approved by your AEC for the SAMPLING manipulation (s) please cut the name and number from the appropriate file found on the RVM/SOP page and paste in the space below. If no approved SOP is available, please describe the methodology.**	All tissue and blood sampling will occur following humane euthanasia of animals byCO2 asphyxiation and cervical dislocation (GM SOP 01 09).
12	7	**DRUG administration**	
12	8	**Intramuscular ~ Intracardiac**	No ~ No
12	9	**Subcutaneous ~ Per os by mouth**	No ~ No
12	10	**Intraperitoneal ~ Drug in water bottle**	No ~ No
12	11	**Intravenous ~ Other**	No ~ No
12	12	**If Other DRUG administration method, please list**	

12	13	**If there is an SOP approved by your AEC for this manipulation, please cutthe name and number from the appropriate file found on the RVM/SOP page and paste in the space below. If no approved SOP isavailable, please describe the methodology.**	
12	14	**BEHAVIOUR and HANDLING**	
12	15	**Observation only ~ Enforced activity**	Yes ~ No
12	16	**Physical restraint ~ Nutritional regime**	Yes ~ Yes
12	17	**Other**	No
12	18	**If Other BEHAVIOUR and HANDLING manipulation, please list**	
12	19	**If there is an SOP approved by your AEC for the BEHAVIOUR and HANDLING manipulation(s) please cut the name and number from the appropriate file found on the RVM/SOP page and paste in the space below. If no approved SOP isavailable, please describe the methodology.**	All the dams will be fed a control diet (0% inulin). This will prevent any effects of andiet enriched on the gut microbiota of pups born. The pups will be separated into cages where different each group will be fed a diet enriched with different doses of inulin.
12	20	**EXPOSURE to**	
12	21	**Parasite ~ Micro-organism**	No ~ No
12	22	**Off-licence drug ~ Off-licence compound**	No ~ No
12	23	**Biological product ~ Radiation**	No ~ No
12	24	**Electrical stimulation ~ Abnormal environment**	No ~ No
12	25	**Other**	No
12	26	**If Other EXPOSURE, please list**	
12	27	**If there is an SOP approved by your AEC for the EXPOSURE manipulation(s) please cut the nameand number from the appropriate filefound on the RVM/SOP page and paste in the space below. If no approved SOP is available, please describe the methodology.**	
12	28	**WILD (Non Domesticated) ANIMALS**	
12	29	**Handling ~ Capture**	No ~ No
12	30	**Marking, Tagging or Branding ~ Transmitter attachment or implantation**	No ~ No
12	31	**If there is an SOP approved by your AEC for this manipulation, please cutthe name and number from the appropriate file found on the RVM/SOP page and paste in the space below. If no approved SOP isavailable, please describe the methodology.**	
12	32	**If other use of WILD (Non Domesticated) ANIMALS, pleasedescribe**	
12	33	**If you are performing manipulationsnot listed above, please detail**	
12	34	**If there is an SOP approved by your AEC for the WILD ANIMAL manipulation(s) please cut the nameand number from the appropriate filefound on the RVM/SOP page and paste in the space below. If no approved SOP is available, please describe the methodology.**	

12	35	**Have you made contingency plans for major risk factors to the WILD ANIMALS**	
12	36	**If yes detail your contingency plans**	
		13. SURGICAL MANIPULATION	
13	1	**Animals will survive surgery ~ Animals will NOT survive surgery**	
13	2	**Manipulations with APPROVED SOPs**	
13	3	**TITLE and NUMBER of the SOP. If no approved SOP is available, please describe the methodology.**	
13	4	**Manipulations without an approvedan SOP - Provide the following information:**	
13	5	**ASEPTIC TECHNIQUE**	
13	6	**Details of aseptic techniques to beused, including preparation**	
13	7	**ANAESTHESIA and ANALGESIA**	
13	8	**Will animals receive pre-emptive analgesia?**	
13	9	**If not, why will pre-emptive analgesianot be administered?**	
13	10	**Detail how the effectiveness of analgesia will be tested if given**	
13	11	**Depth of Anaesthesia - Please indicate how this will be monitored**	
13	12	**Eye Position ~ Body Temperature**	
13	13	**Heart Rate ~ Respiratory Rate**	
13	14	**Blood Pressure ~ Superficial/DeepPain Reflex**	
13	15	**Other method, please state:**	
13	17	**Duration of anaesthetic monitoring period**	
13	18	**Any further parameters to be monitored?**	
13	19	**Will you be using an anaesthesia monitoring sheet?**	
13	20	**Who will perform the monitoring?**	
13	21	**If not using a monitoring sheet, please state why:**	
13	22	**POST-OPERATIVE CARE**	
13	23	**Not applicable, animals do not recover**	
13	24	**Conservation of body heat ~ Specific posture for recovery**	
13	25	**Fluid administration ~ Recovery in individual cages**	
13	26	**Administration of analgesics ~ Other**	
13	27	**Please detail any other POST-OPERATIVE support and monitoring**	
13	28	**ENDPOINTS**	
13	29	**The endpoint of these manipulationsis (Use Dropdown box)**	
13	30	**If Euthanasia upon clinical thresholdor Other, please specify**	
		14. DRUGS and COMPOUNDS	

14	1	**List all drugs and Compounds to be administered to animals during this project below. PLEASE NOTE: You are required to complete a separate IDAO for each DRUG that is to be used in this project that is not an OTCcompound or being administered by a registered veterinarian.**	
14	2	**If the drugs have been ACCEPTED bythe AEC in terms of label informationas appropriate for the routine use youintend, please cut the name of the drug and its coversheet number fromthe appropriate file(s) found on the RVM/SOP page and paste in the space below. IF NO DRUGS ARE TO BE ADMINISTERED, PLEASE DO NOTWRITE IN THIS SPACE**	Sodium Pentobarbitone 300. No IDAO is required as drug will be administered by the AgResearch veterinarian.
14	3	**If you are using drugs that do not have a COVERSHEET, please go to HELP on this page and use the link todownload the template (second line below the green box). Complete one sheet for each drug and/or chemical.Send the completed form electronically to Jim Webster. These will be made available in the RVM/SOP function for future reference.**	
14	4	**Have you had to make up new RVM COVERSHEET(s) for this project**	No
14	5	**If YES, please name the drugs you have created the new RVM COVERSHEET(s) for**	
14	6	**UNREGISTERED Drugs or Compounds**	
14	7	**Name of Drug or Compound**	
14	8	**Composition**	
14	9	**Route of Administration**	
14	20	**Will ANY drugs used in this project (other than OTC Products) be administered by ANY PERSON who is NOT a registered Veterinarian (please No answer either YES or NO) _____This question is an internal trigger for IDAO creation, names are not required here.**	
		21. TISSUE COLLECTION	
21	1	**If animals are being killed specificallyto allow tissues to be collected a full APPLICATION is required and numbers reported in a STATISTICS Form as Killed to use body or tissues**	
21	5	**Animals are being Killed for Tissue Collection**	Yes
21	6	**What tissue(s) are to be collected?**	ileum tissue, ileum contents, caecum tissue, caecum contents, colon tissue, coloncontents, blood, faeces
21	10	**If the animals will be manipulated prior to collection of the tissue please detail the manipulations**	The animals will be fed a diet containing different doses of inulin. Prior to tissue collection the animals will be euthanised by CO2 asphyxiation and cervical dislocation.
		97. SOURCE(s) and TRIAL SITE(s)	
97	1	**This section allows statistics to be gathered for MPI (Animal use information) and data transfer into Animal Tools for AgR animals.**	

97	2	**Please ensure you have selected the correct Trial Site as information is transferred to the Animal Tools database. Please note that you may select more than one Trial Site where relevant depending on the fate of the animals. If the endpoint is returning/transporting animals to a AgR Farm, ensure you add this as an additional Trial Site.**	
97	3	**RATS**	Source = AGR Ruakura Small Animal Unit Trial Site = AGR Grasslands Animal Facility (Ulyatt/Reid) Number = 117
		98. NOTES ~ Read only	
98	1	**Applicant Note**	(05/03/2020 ogutul) Currently, there are only 2 personnel (Linda Ogutu and CharlotteO'Neill) included for the sample collection days. Personnel modification for sample collection will be done once new technical staff are hired.
98	2	**Status Change**	(ogutul 09/03/2020) SUBMIT
98	3	**Committee Decision**	(19/03/2020 RESUBMITkirkm) Hi Linda Discussed by GAEC prior to approval update: 1. Check application for errors in animal numbers, litter size, gender etc. Seem to bedifferent numbers in sections. 2. Consider reducing to 9 dams, this should give 80 pups. It is expected that 12 pupsis the average. The dams are not surplus animals as they are manipulated. 3. Confirm there is enough technical trained staff on site to complete this trial with thecurrent changes in location, positions, and staff. Include a detailed list of staff names, experience, and manipulations once this is known. Discuss any staff training requirements with Susan Doohan. Michelle
98	4	**Applicant Note**	(20/03/2020 ogutul) The process of finalising the hiring of two new staff with small animal experience is currently ongoing. They will be assisting with: 1. Day to day care of the dams (after they arrive from Ruakura) and pups that are eventually born. 2. Ear punching to mark the pups for the study. 3. Euthanasia (CO_2 asphyxiation and cardiac puncture) of pups during sample collection on pups aged 20 and 24 days old.
98	5	**Status Change**	(ogutul 20/03/2020) SUBMIT
98	6	**Applicant Note**	(20/03/2020 ogutul) The process of finalising the hiring of two new staff with small animal experience is currently ongoing. They will be assisting with: 1. Day to day care of the dams (after they arrive from Ruakura) and pups that are eventually born. 2. Ear punching to mark the pups for the study. 3. Euthanasia (CO_2 asphyxiation and cardiac puncture) of pups during the sample collection days.
98	7	**Applicant Note**	(02/04/2020 ogutul) Implications of delaying the start of the second trial AE 14984?The current lock-down (due to covid-19) will delay all animal trials (including pilot trialAE 14992) as we are currently unsure when we will return to campus. Will the pilot AE 14992 trial be completed, results analysed, and report A be completed prior to starting mating animals for this trial? This is the ideal case scenario and will depend on when the pilot trial AE 14992 will be carried out andanalysis completed in order to start trial AE 14984.
98	8	**Committee Decision**	(11/05/2020 RESUBMITkirkm) Hi LindaThanks for the updates late March. We understand COVID-19 may have impacted research priorities, timing, and staff availability, so please revisit the application, and let the committee know what is expected to occur with the trial and the animals. In addition to previously requested updates, minor alterations such as timings only may be updated via applicant note, major changes should be submitted via a modification. AEC approval will need to begiven before starting this trial. Michelle

98	9	**Applicant Note**	(21/05/2020 ogutul) Timings for inulin dose comparison rat study: Start date - 15 June 2020 (Breeding of rats in Ruakura) Finish date - 2 August 2020 (Last day of sample collection. Dependent on birth datesof rat pups)
98	10	**Applicant Note**	(26/05/2020 ogutul) In case the alert level due to Covid-19 goes back up to level 3 or 4 and animal studies are stalled for a longer time, I plan to collect more samples as a back up for my PhD project. That way there will be other tissue options for analysis. The extra samples (tissue and contents): Duodenum Jejunum Stomach Spleen Liver Thyroid gland Brain
98	11	**Applicant Note**	(26/05/2020 ogutul) In case the alert level due to Covid-19 goes back up to level 3 or 4 and animal studies are stalled for a longer time, I plan to collect more samples as a back up for my PhD project. That way there will be other tissue options for analysis. The extra samples (tissue and contents): Duodenum Jejunum Stomach Spleen Liver Thyroid gland Brain
98	12	**Status Change**	(ogutul 26/05/2020) SUBMIT
98	13	**Committee Note**	(09/06/2020 kirkm) Hi Linda The GAEC has given Pro Tem approval for breeding to commence.The committee will review the updates next week. With the recent facility staff changes is there any new staff needing to be named i.e.,Ben Harvie named as Facility Small Animal Tech. Michelle
98	14	**Committee Decision**	(09/06/2020 RESUBMITkirkm) Hi Linda Resubmitted to allow Ben Harvie as the Facility Small Animal Tech to sign. Michelle
98	15	**Administrator Note**	(17/06/2020 bormanv) Linda's maiden name Ogutu has been updated to her marriedname Oemcke
98	16	**Status Change**	(oemckel 17/06/2020) SUBMIT
98	17	**Committee Decision**	(19/06/2020 APPROVEDkirkm) Hi Linda Approved. Please complete reporting for this pilot study on the dosage, prior to starting the follow on trial AE 14984. Michelle
98	18	**Personnel Note**	(24/06/2020 oemckel) Faye Benjamin and Anna Shortall are additional personnel asweekend support for daily care of the animals.
98	19	**Committee Decision**	(24/06/2020 RESUBMITkirkm) Hi Linda Resubmitted to allow for new personnel to sign electronically. Faye Benjamin and Anna Shortall are additional personnel as weekend support for daily care of the animals. Michelle Michelle
98	20	**Applicant Note**	(29/06/2020 oemckel) Dams will now be transported from Ruakura in appropriate rodent transport containers, by direct route, in a private vehicle by an AgResearch employee, with care taken to ensure appropriate restraint and temperature during thejourney.
98	21	**Status Change**	(oemckel 29/06/2020) SUBMIT

98	22	**Committee Note**	(30/06/2020 cosgroveg) Hi Linda, Thanks for this confirmation on mode of transport from Ruakura to Grasslands. We understand Bobby will prepare the rats and load for transport and discuss as necessary with Nic. (this applies also to mice for Natalie Parlane). Ben and Hailey willbe at Grasslands to receive them. Gerald
98	23	**Committee Decision**	(02/07/2020 APPROVEDkirkm) Hi Linda Thanks for updates in staff. Changed back to approved. Michelle
98	24	**Coordinator's note**	(23/12/2020 bormanv) This application has been transferred over from GRASSLANDS to RUAKURA committee due to the closure of the GRASSLANDScommittee as of 8/12/2020. No action is required by the applicant to this note. All future reports will need to be submitted to the RUAKURA committee.
98	25	**Committee Decision**	(18/02/2021 CLOSEDwebsterj)
		99. PERSONNEL SIGNATURES	
99	1	**Committee**	RUAKURA
99	1	**APPLICANT: All personnel who will manage the animals or perform manipulations on them, The Programme leader, Facility manager Biometrician and the person responsible for entering info on the animal database must sign this application.** **PLEASE Describe clearly what each person's role in the project is.** **All personnel who will perform manipulations on the specified animals must:** **A - Have been trained and be competent in the manipulations specified (unless this is a specified training protocol)** **B - Be conversant with the AgResearch Code of Ethical Conduct.** **TICKING THE APPROVE BOX** **IMPLIES THAT YOU HAVE:** **A - read and understood this application** **B - agreed to comply with all statutory requirements regarding theuse of animals in experiments** **C - agreed to comply with all statutory requirements regarding theuse of any Restricted Veterinary Medicines specified for use in this project.** **Any person whose name is NOT selected from a dropdown list may not be recognised by the auto emailfunction in the database.**	
99	99	**ANDERSONRC ~ approved ~ Job (Science Team Leader - Food Functionality - Model Systems) Location (Grasslands; Alan Johns Building. Tues-8-4.30pm. Mon-Fri-8-2.30pm)**	Project Leader
99	99	**BENJAMINF ~ approved ~ Job (Animal Facility Technician) Location (Grasslands;)**	Weekend support for daily care of animals

99	99	**DOOHANS ~ approved ~ Job (Veterinarian and Animal Welfare Officer) Location (Grasslands; Animal Facilities - Ulyatt-Reid Facility)**	Day 2 pup Euthanasia
99	99	**GILLESPIEH ~ approved ~ Job (Animal Facility Manager) Location (Grasslands; Lab Admin Building)**	Grasslands Animal Facility Manager
99	99	**GREENP ~ approved ~ Job (Statistician) Location (Grasslands; Lionel Corkill East)**	Biometrician
99	99	**HARVIEB ~ approved ~ Job (Small Animal Technician) Location (Grasslands;)**	Day to day maintenance of rats
99	99	**OEMCKEL ~ approved ~ Job (PhD Student) Location (Grasslands;)**	PhD student
99	99	**OLSONT ~ approved ~ Job (Senior Technician) Location (Grasslands;)**	Euthanasia/sample collection
99	99	**ONEILLC ~ approved ~ Job (Technician, Food Nutrition & Health) Location (Grasslands; Lab Admin Building #41 Rm A-03.)**	Day to day care/euthanasia/sampling/ear punching
99	99	**SHORTALLA ~ Job (Assistant Animal Technician) Location (Grasslands;)**	May provide weekend support for daily care of animals alongside another technician
99	99	**SMITHBG ~ approved ~ Job (Research Associate) Location (Ruakura; Small Animals, An Phys, Fire Warden, First Aid)**	Animal Technician, Ruakura

Appendix IV

Sampling schedules

Sampling schedule (T294) - Mouse study_March 2017

				Litter born 14/02			
Day	Date	Day (#)	manipulation	Age of mice (days after birth)	Sampling location	Mice (#) before/after sampling	Personnel
Monday	6/03/2017	0	Faecal samples of all mice (50), Pre-weaning: Sampling of 8 mice, Weaning of the rest	20	Ruakura	50/42	Eva, Ric, Matt
Tuesday	7/03/2017	1	Sampling of 8 mice+ faecal samples from all mice (42)	21	Ruakura	42/34	Eva, Ric (Matt?)
Wednesday	8/03/2017	2		22		34	
Thursday	9/03/2017	3	Transport to Palmerston North	23		34	Pick up by facility staff
Friday	10/03/2017	4		24		34	
Saturday	11/03/2017	5		25		34	
Sunday	12/03/2017	6	Sampling of 8 mice+ faecal samples from all mice (34)	26	Palmerston North	34/26	Eva, Alicia, Hailey
Monday	13/03/2017	7		27		26	
Tuesday	14/03/2017	8		28		26	
Wednesday	15/03/2017	9		29		26	
Thursday	16/03/2017	10		30		26	
Friday	17/03/2017	11	Sampling of 8 mice+ faecal samples from all mice (26)	31	Palmerston North	26/18	Eva, Alicia, Kelly D.
Saturday	18/03/2017	12		32		18	
Sunday	19/03/2017	13		33		18	
Monday	20/03/2017	14		34		18	
Tuesday	21/03/2017	15		35		18	
Wednesday	22/03/2017	16	Sampling of 8 mice+ faecal samples from all mice (18)	36	Palmerston North	18,10	Eva, Alicia, Kelly D.
Thursday	23/03/2017	17		37		10	
Friday	24/03/2017	18		38		10	
Saturday	25/03/2017	19		39		10	
Sunday	26/03/2017	20		40		10	
Monday	27/03/2017	21	Sampling of 10 mice+ faecal samples from all mice (10)	41	Palmerston North	10/0	Eva, Alicia, Kelly D.

NOTE! Cage 1 has 2 ♂s of cage 4 → 1 with Yellow sampled 07/03 (Black, Green, Yellow), 1 left (Black, Green, Red)
Cage 4 has 1 ♀ of Cage 1 → sampled 07/03

Mouse #	Cage #	Colour	Strain	Born on	Sex	weaning (days after birth)	timepoint	Euthanised on
1	2 each from cage 1-4		Swiss	14/02/2017	female	20	1 (pre-weaning)	6/03/2017
2			Swiss	14/02/2017	~~female~~ male	20	1 (pre-weaning)	6/03/2017
3			Swiss	14/02/2017	female	20	1 (pre-weaning)	6/03/2017
4			Swiss	14/02/2017	~~female~~ male	20	1 (pre-weaning)	6/03/2017
5			Swiss	14/02/2017	~~male~~ female	20	1 (pre-weaning)	6/03/2017
6			Swiss	14/02/2017	male	20	1 (pre-weaning)	6/03/2017
7			Swiss	14/02/2017	~~male~~ female	20	1 (pre-weaning)	6/03/2017
8			Swiss	14/02/2017	male	20	1 (pre-weaning)	6/03/2017
9	5	BG	Swiss	14/02/2017	~~female~~ male	20	2 (post-weaning)	7/03/2017
10	5	WG	Swiss	14/02/2017	female	20	2 (post-weaning)	7/03/2017
11	4	WY	Swiss	14/02/2017	female	20	2 (post-weaning)	7/03/2017
12	4	BY	Swiss	14/02/2017	~~female~~ male	20	2 (post-weaning)	7/03/2017
13	1	WY	Swiss	14/02/2017	~~male~~ female	20	2 (post-weaning)	7/03/2017
14	1	BY	Swiss	14/02/2017	male	20	2 (post-weaning)	7/03/2017
15	2	WY	Swiss	14/02/2017	~~male~~ female	20	2 (post-weaning)	7/03/2017
16	2	BY	Swiss	14/02/2017	male	20	2 (post-weaning)	7/03/2017
17	1	BGR	Swiss	14/02/2017	~~female~~ male	20	3 (post-weaning)	12/03/2017
18	1	WR	Swiss	14/02/2017	female	20	3 (post-weaning)	12/03/2017
19	2	BR	Swiss	14/02/2017	~~female~~ male	20	3 (post-weaning)	12/03/2017
20	2	WR	Swiss	14/02/2017	female	20	3 (post-weaning)	12/03/2017
21	3	BY	Swiss	14/02/2017	male	20	3 (post-weaning)	12/03/2017
22	3	WY	Swiss	14/02/2017	~~male~~ female	20	3 (post-weaning)	12/03/2017
23	5	BY	Swiss	14/02/2017	male	20	3 (post-weaning)	12/03/2017
24	5	WY	Swiss	14/02/2017	~~male~~ female	20	3 (post-weaning)	12/03/2017
25	1	BO	Swiss	14/02/2017	~~female~~ male	20	4 (post-weaning)	17/03/2017
26	1	WO	Swiss	14/02/2017	female	20	4 (post-weaning)	17/03/2017
27	3	BR	Swiss	14/02/2017	~~female~~ male	20	4 (post-weaning)	17/03/2017
28	3	WR	Swiss	14/02/2017	female	20	4 (post-weaning)	17/03/2017
29	4	WR	Swiss	14/02/2017	~~male~~ female	20	4 (post-weaning)	17/03/2017
30	4	BR	Swiss	14/02/2017	male	20	4 (post-weaning)	17/03/2017
31	5	WR	Swiss	14/02/2017	~~male~~ Female	20	4 (post-weaning)	17/03/2017
32	5	BR	Swiss	14/02/2017	male	20	4 (post-weaning)	17/03/2017
33	2	BO	Swiss	14/02/2017	~~female~~ male	20	5 (post-weaning)	22/03/2017
34	2	WO	Swiss	14/02/2017	female	20	5 (post-weaning)	22/03/2017
35	3	BO	Swiss	14/02/2017	~~female~~ male	20	5 (post-weaning)	22/03/2017
36	3	WO	Swiss	14/02/2017	female	20	5 (post-weaning)	22/03/2017
37	4	BO	Swiss	14/02/2017	~~male~~ male	20	5 (post-weaning)	22/03/2017
38	4	WO	Swiss	14/02/2017	male	20	5 (post-weaning)	22/03/2017
39	5	BO	Swiss	14/02/2017	~~male~~ male	20	5 (post-weaning)	22/03/2017
40	5	WO	Swiss	14/02/2017	male	20	5 (post-weaning)	22/03/2017
41	1	BB	Swiss	14/02/2017	~~female~~ ~~male~~ male	20	6 (post-weaning)	27/03/2017
42	1	WB	Swiss	14/02/2017	female	20	6 (post-weaning)	27/03/2017
43	2	BB	Swiss	14/02/2017	~~female~~ ~~male~~ female	20	6 (post-weaning)	27/03/2017
44	2	WB	Swiss	14/02/2017	female	20	6 (post-weaning)	27/03/2017
45	3	BB	Swiss	14/02/2017	male	20	6 (post-weaning)	27/03/2017
46	3	WB	Swiss	14/02/2017	~~male~~ female	20	6 (post-weaning)	27/03/2017
47	4	BB	Swiss	14/02/2017	male	20	6 (post-weaning)	27/03/2017
48	4	WB	Swiss	14/02/2017	~~male~~ female	20	6 (post-weaning)	27/03/2017
49	5	BB	Swiss	14/02/2017	male	20	6 (post-weaning)	27/03/2017
50	5	WB	Swiss	14/02/2017	~~male~~ female	20	6 (post-weaning)	27/03/2017

B = Black head = male
W = nothing on head = female

Colours: Blue, Orange, Red, Yellow, Green

Cages: 5
each cage: 4 or 5 ♂s : Blue, Orange, Red, Yellow, Green
4 or 5 ♀s : " " " " "

Sampling schedule (T321) - Rat study_ September 2018

Sampling day	D.O.B.	Age of pups	Date		D.O.B.	Age of pups	Date		D.O.B.	Age of pups	Date		Pups being sampled
	9/09/2018	0	9/09/2018	Sunday			9/09/2018	Sunday			9/09/2018	Sunday	
		1	10/09/2018	Monday	10/09/2018	0	10/09/2018	Monday			10/09/2018	Monday	
		2	11/09/2018	Tuesday		1	11/09/2018	Tuesday			11/09/2018	Tuesday	
		3	12/09/2018	Wednesday		2	12/09/2018	Wednesday	12/09/2018	0	12/09/2018	Wednesday	
		4	13/09/2018	Thursday		3	13/09/2018	Thursday		1	13/09/2018	Thursday	
		5	14/09/2018	Friday		4	14/09/2018	Friday		2	14/09/2018	Friday	
		6	15/09/2018	Saturday		5	15/09/2018	Saturday		3	15/09/2018	Saturday	
		7	16/09/2018	Sunday		6	16/09/2018	Sunday		4	16/09/2018	Sunday	
		8	17/09/2018	Monday		7	17/09/2018	Monday		5	17/09/2018	Monday	
		9	18/09/2018	Tuesday		8	18/09/2018	Tuesday		6	18/09/2018	Tuesday	
		10	19/09/2018	Wednesday		9	19/09/2018	Wednesday		7	19/09/2018	Wednesday	
		11	20/09/2018	Thursday		10	20/09/2018	Thursday		8	20/09/2018	Thursday	
		12	21/09/2018	Friday		11	21/09/2018	Friday		9	21/09/2018	Friday	
		13	22/09/2018	Saturday		12	22/09/2018	Saturday		10	22/09/2018	Saturday	
		14	23/09/2018	Sunday		13	23/09/2018	Sunday		11	23/09/2018	Sunday	
		15	24/09/2018	Monday		14	24/09/2018	Monday		12	24/09/2018	Monday	
		16	25/09/2018	Tuesday		15	25/09/2018	Tuesday		13	25/09/2018	Tuesday	
		17	26/09/2018	Wednesday		16	26/09/2018	Wednesday		14	26/09/2018	Wednesday	
		18	27/09/2018	Thursday		17	27/09/2018	Thursday		15	27/09/2018	Thursday	
		19	28/09/2018	Friday		18	28/09/2018	Friday		16	28/09/2018	Friday	
	1st	20	29/09/2018	Saturday		19	29/09/2018	Saturday		17	29/09/2018	Saturday	14
		21	30/09/2018	Sunday		20	30/09/2018	Sunday		18	30/09/2018	Sunday	
	2nd	22	1/10/2018	Monday		21	1/10/2018	Monday		19	1/10/2018	Monday	13
		23	2/10/2018	Tuesday		22	2/10/2018	Tuesday		20	2/10/2018	Tuesday	
	3rd	24	3/10/2018	Wednesday		23	3/10/2018	Wednesday		21	3/10/2018	Wednesday	13
		25	4/10/2018	Thursday		24	4/10/2018	Thursday		22	4/10/2018	Thursday	
	4th	26	5/10/2018	Friday		25	5/10/2018	Friday		23	5/10/2018	Friday	13
		27	6/10/2018	Saturday		26	6/10/2018	Saturday		24	6/10/2018	Saturday	
	5th	28	7/10/2018	Sunday		27	7/10/2018	Sunday		25	7/10/2018	Sunday	13
		29	8/10/2018	Monday		28	8/10/2018	Monday		26	8/10/2018	Monday	
	6th	30	9/10/2018	Tuesday		29	9/10/2018	Tuesday		27	9/10/2018	Tuesday	13
		31	10/10/2018	Wednesday		30	10/10/2018	Wednesday		28	10/10/2018	Wednesday	
	7th	32	11/10/2018	Thursday		31	11/10/2018	Thursday		29	11/10/2018	Thursday	11
	8th									31	13/10/2018	Saturday	2

KEY

- Sampling days (weighing pups prior)
- D.O.B.
- Weighing pups
- Weaning age
- Completed

AE # 14485: Determination of the abundance of SFB in ileal samples of rats pre- and post-weaning

	Litter #	Date born	# Born	Date if any euthanised (and why)	New Total	Time-points	Date sexed	# Males	# Females	Notes
Dams	1	10/09/2018	10	N/A	**10**	**14**	13-Sep	6	4	
	2	10/09/2018	12	N/A	**12**	**13**	13-Sep	5	7	
	3	9/09/2018	12	N/A	**12**	**13**	13-Sep	3	9	
	4	9/09/2018	14	11/9/2018 - 2 pups in excess	**12**	**13**	13-Sep	5	7	
	5	9/09/2018	10	N/A	**10**	**13**	13-Sep	5	5	
	6	9/09/2018	14	11/9/2018 - 2 pups in excess	**12**	**13**	13-Sep	1	11	
	7	12/09/2018	15	14/9/2018 - 3 pups in excess	**12**	**13**	17-Sep	5	7	13/9 a.m. - Dam quite stressed out. Susan to give health check in afternoon pm, looking OK. Lots of pups.
	8	9/09/2018	12	N/A	**12**		13-Sep	4	8	
			99		92					

Total animals - 100; 92 pups and 8 dams

Day		12 Saturday	14 Monday	13 Wednesday	12 Friday	13 Sunday	13 Tuesday	13 Thursday	2 Saturday
Date		29/09/2018	1/10/2018	3/10/2018	5/10/2018	7/10/2018	9/10/2018	11/10/2018	13/10/2018
Number of pups being sampled	**Time-points**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**7**
	Age (days after birth)	20	22	24	26	28	30	32	
1	**Randomisation of pups**	3.1	3.2	3.3	3.4	3.5	3.6	3.7	
2		3.8	3.9	3.10	3.11	3.12	4.1	4.2	
3		4.3	4.4	4.5	4.6	4.7	4.8	4.9	
4		4.10	4.11	4.12	5.1	5.2	5.3	5.4	
5		5.5	5.6	5.7	5.8	5.9	5.10	6.1	
6		6.2	6.3	6.4	6.5	6.6	6.7	6.8	
7		6.9	6.10	6.11	6.12	8.1	8.2	8.3	
8		8.4	8.5	8.6	8.7	8.8	8.9	8.10	
9		8.11	8.12	1.1	1.2	1.3	1.4	1.5	
10		1.6	1.7	1.8	1.9	1.10	2.1	2.2	
11		2.3	2.4	2.5	2.6	2.7	2.8	2.9	
12		2.10	2.11	2.12	7.1	7.2	7.3	7.4	7.7
13			7.5	7.6		7.8	7.9	7.10	7.12
14			7.11						

The randomisation of pups was done by allocating numbers to the pups based on the litters/dams they came from.
There were 8 dams (pregnant rats) that had litters of rat pups. There are currently a total of 92 pups for the rat study.
All pups will be used in the rat study. All pups have been randomised into the 7 time-points.
The first number represents the dam/litter the pup is from.
The second number represents the number of the pup in the litter.
i.e. 7.4 = litter #7, pup #4; 8.12 = litter #8, pup #12
The total number of pups per litter are as shown above in the '**New Total**' column.

Time-point 1 = 12 pups, Time-points 2&3 = 14 pups, Time-points 4-7 = 13 pups.
Pups from litter 7 were born later and will therefore be moved up one time-point.
i.e. 7.5 and 7.11 will be time-point 1 pups, 7.6 and 7.12 will be time-point 2 pups etc

This is based on the number of pups that were born, and as indicated on the animal ethics application, extra pups would be added to the different time-points in order to decrease variation in the SFB abundance data.
Markings' on the right of the table represent the markings that will be used to identify the pups.
Marking the pups is useful as they will be kept in their birth litters even during the sampling days.
This is to ensure that the gut microbiota populations are not influenced by placing pups from different litters together.
The type of markers used will be non-toxic Stoetling markers which are routinely used in the Small Animal Facility at Ruakura.

Estimated sampling time for each pup = **15 minutes**
Average total sampling time each day = **3.5/4 hours**

Markers available
on back

on back

on back

1st day

Saturday (29/09/2018) **12 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary
ileal tissue collection	Melanie
post-mort faeces-DNA	Charley Linda

2nd day

Monday (1/10/2018) **14 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

3rd day

Wednesday (3/10/2018) **14 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

4th day

Friday (5/10/2018) **13 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

5th day

Sunday (7/10/2018) **13 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

6th day

Tuesday (9/10/2018) **13 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

7th day

Thursday (11/10/2018) **13 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary & Charley
ileal tissue collection	Melanie
post-mort faeces-DNA	Linda

8th day

Saturday (13/10/2018) **2 pups**

Task	Staff
Euthanasia Cardiac puncture Plasma ante-mort faeces-IgA	Hilary
ileal tissue collection	Linda
post-mort faeces-DNA	

Note:
The tasks can be switched around.
More people will be needed for collecting the ileal tissue.
ONLY ileal tissue will be collected, as in the AE 14485 application.

Sampling schedule (T351) - Rat Inulin study_August 2020

	D.O.B	Age of pups	Date	Day	D.O.B	Age of pups	Date	Day	D.O.B	Age of pups	Date	Day	D.O.B	Age of pups	Date	Day
	8/07/2020	0	8/07/2020	Wednesday			8/07/2020	Wednesday			8/07/2020	Wednesday			8/07/2020	Wednesday
	1 litter	1	9/07/2020	Thursday	9/07/2020	0	9/07/2020	Thursday			9/07/2020	Thursday			9/07/2020	Thursday
		2	10/07/2020	Friday	4 litters	1	10/07/2020	Friday	10/07/2020	0	10/07/2020	Friday			10/07/2020	Friday
		3	11/07/2020	Saturday		2	11/07/2020	Saturday	2 litters	1	11/07/2020	Saturday			11/07/2020	Saturday
		4	12/07/2020	Sunday		3	12/07/2020	Sunday		2	12/07/2020	Sunday	12/07/2020	0	12/07/2020	Sunday
		5	13/07/2020	Monday		4	13/07/2020	Monday		3	13/07/2020	Monday	1 litter	1	13/07/2020	Monday
		6	14/07/2020	Tuesday		5	14/07/2020	Tuesday		4	14/07/2020	Tuesday		2	14/07/2020	Tuesday
		7	15/07/2020	Wednesday		6	15/07/2020	Wednesday		5	15/07/2020	Wednesday		3	15/07/2020	Wednesday
		8	16/07/2020	Thursday		7	16/07/2020	Thursday		6	16/07/2020	Thursday		4	16/07/2020	Thursday
		9	17/07/2020	Friday		8	17/07/2020	Friday		7	17/07/2020	Friday		5	17/07/2020	Friday
		10	18/07/2020	Saturday		9	18/07/2020	Saturday		8	18/07/2020	Saturday		6	18/07/2020	Saturday
		11	19/07/2020	Sunday		10	19/07/2020	Sunday		9	19/07/2020	Sunday		7	19/07/2020	Sunday
		12	20/07/2020	Monday		11	20/07/2020	Monday		10	20/07/2020	Monday		8	20/07/2020	Monday
		13	21/07/2020	Tuesday		12	21/07/2020	Tuesday		11	21/07/2020	Tuesday		9	21/07/2020	Tuesday
Ear-punching		14	22/07/2020	Wednesday		13	22/07/2020	Wednesday		12	22/07/2020	Wednesday		10	22/07/2020	Wednesday
		15	23/07/2020	Thursday		14	23/07/2020	Thursday		13	23/07/2020	Thursday		11	23/07/2020	Thursday
		16	24/07/2020	Friday		15	24/07/2020	Friday		14	24/07/2020	Friday		12	24/07/2020	Friday
		17	25/07/2020	Saturday		16	25/07/2020	Saturday		15	25/07/2020	Saturday		13	25/07/2020	Saturday
		18	26/07/2020	Sunday		17	26/07/2020	Sunday		16	26/07/2020	Sunday		14	26/07/2020	Sunday
		19	27/07/2020	Monday		18	27/07/2020	Monday		17	27/07/2020	Monday		15	27/07/2020	Monday
		20	28/07/2020	Tuesday		19	28/07/2020	Tuesday		18	28/07/2020	Tuesday		16	28/07/2020	Tuesday
		21	29/07/2020	Wednesday		20	29/07/2020	Wednesday		19	29/07/2020	Wednesday		17	29/07/2020	Wednesday
		22	30/07/2020	Thursday		21	30/07/2020	Thursday		20	30/07/2020	Thursday		18	30/07/2020	Thursday
		23	31/07/2020	Friday		22	31/07/2020	Friday		21	31/07/2020	Friday		19	31/07/2020	Friday
		24	1/08/2020	Saturday		23	1/08/2020	Saturday		22	1/08/2020	Saturday		20	1/08/2020	Saturday
		25				24	2/08/2020	Sunday		23	2/08/2020	Sunday		21	2/08/2020	Sunday
		26				25				24	3/08/2020	Monday		22	3/08/2020	Monday
		27				26				25				23	4/08/2020	Tuesday
		28				27				26				24	5/08/2020	Wednesday

PND 20	13 pups	0% inulin
PND 24	13 pups	0% inulin
	14 pups	2.5% inulin
	14 pups	5% inulin
	14 pups	10% inulin

KEY

D.O.B.

Weighing pups

Weaning day

Sampling days

	Litter 2	Sex	ear-mark	weight (gm)	Litter 3	Sex	ear-mark	weight (gm)	Litter 4	Sex	ear-mark	weight (gm)	Litter 5
1	2.1	M	none	24.8	3.1	F	none	26.7	4.1	M	none	11.2	5.1
2	2.2	F	RF	26.8	3.2	F	RF	29.3					5.2
3	2.3	M	RM	27.0	3.3	F	RM	31.6					5.3
4	2.4	M	RB	27.5	3.4	M	RB	29.8					5.4
5	2.5	F	LF	28.2	3.5	F	LF	27.3					5.5
6	2.6	F	LM	22.6	3.6	M	LM	29.9					5.6
7	2.7	M	LB	24.5	3.7	M	LB	32.4					5.7
8	2.8	F	BF	26.6	3.8	M	BF	27.8					5.8
9	2.9	M	BM	26.7	3.9	F	BM	28.2					5.9
10	2.10	F	BB	27.5	3.10	F	BB	28.8					5.10
11	2.11	M	RF-LB	26.0	3.11	M	RF-LB	33					5.11
12	2.12	F	LF-RB	26.6	3.12	M	RB-LM	29					5.12
	Males	6			Males	6			Males	1			Males
	Females	6			Females	6			Females	0			Females

Males	32
Females	33
Total	**65**

Sex	ear-mark	weight (gm)	Litter 6	Sex	ear-mark	weight (gm)	Litter 7	Sex	ear-mark	weight (gm)	Litter 8	Sex	ear-mark
M	none	23.7	6.1	F	none	33.5	7.1	M	none	30.7	8.1	M	none
F	RF	22.7	6.2	F	RF	31.2	7.2	F	RF	29.8	8.2	M	RF
M	RM	26.2	6.3	M	RM	29.8	7.3	F	RM	26.8			
F	RB	24.9	6.4	F	RB	33	7.4	M	RB	28.3			
F	LF	26.3	6.5	F	LF	29.3	7.5	F	LF	28.2			
M	LM	25.6					7.6	F	LM	28.7			
M	LB	26.5					7.7	F	LB	26.4			
F	BF	23.1					7.8	M	BF	30.1			
M	BM	25.2					7.9	F	BM	28.2			
M	BB	26.7					7.10	F	BB	25.0			
F	RF-LB	23.1											
M	LF-RB	25.3											
7			Males	1			Males	3			Males	2	
5			Females	4			Females	7			Females	0	

weight (gm)	Litter 9	Sex	ear-mark	weight (mg)
16.6	9.1	M	none	29.4
20.7	9.2	F	RF	31.7
	9.3	F	RM	27.0
	9.4	M	RB	25.1
	9.5	M	LF	28.4
	9.6	F	LM	29.0
	9.7	M	LB	27.4
	9.8	F	BF	30.0
	9.9	F	BM	28.3
	9.10	M	BB	27.3
	9.11	M	RF-LB	29.7
	Males	6		
	Females	5		

	Inulin		0%				2.5%				5%				10%			
		sampling	Cage 1	Sex	Ear-punch		sampling	Cage 8	Sex	Ear-punch	sampling	Cage 12	Sex	Ear-punch		sampling	Cage 17	Sex	Ear-punch
PND	20	30/07/2020	2.1	M	none			2.3	M	RM	24 3/08/2020	2.8	F	BF			2.10	F	BB
			2.2	F	RF			2.4	M	RB		2.9	M	BM	24	3/08/2020	2.11	M	RF-LB
	24	31/07/2020	3.1	F	none	24	3/08/2020	2.5	F	LF							2.12	F	LF-RB
			3.2	F	RF			2.6	F	LM									
								2.7	M	LB									
		sampling	Cage 2	Sex	Ear-punch		sampling	Cage 9	Sex	Ear-punch	sampling	Cage 13	Sex	Ear-punch		sampling	Cage 18	Sex	Ear-punch
PND	20	28/07/2020	3.3	F	RM			3.5	F	LF	24 31/07/2020	3.8	M	BF			3.10	F	BB
			3.4	M	RB	24	31/07/2020	3.6	M	LM		3.9	F	BM	24	31/07/2020	3.11	M	RF-LB
			6.1	F	none			3.7	M	LB							3.12	M	LF-RB
	24	31/07/2020	6.2	F	RF														
			6.3	M	RM														
		sampling	Cage 3	Sex	Ear-punch		sampling	Cage 10	Sex	Ear-punch	sampling	Cage 14	Sex	Ear-punch		sampling	Cage 19	Sex	Ear-punch
PND	20	28/07/2020	4.1	M	none			5.5	F	LF	24 4/08/2020	5.8	F	BF			5.10	M	BB
						24	4/08/2020	5.6	M	LM		5.9	M	BM	24	4/08/2020	5.11	F	RF-LB
	24	4/08/2020	5.2	F	RF			5.7	M	LB							5.12	M	LF-RB
		sampling	Cage 4	Sex	Ear-punch		sampling	Cage 11	Sex	Ear-punch	sampling	Cage 15	Sex	Ear-punch		sampling	Cage 20	Sex	Ear-punch
PND	20	31/07/2020	5.3	M	RM			7.5	F	LF		7.8	M	BF	24	31/07/2020	9.10	M	BB
			5.4	F	RB	24	3/08/2020	7.6	F	LM	24 3/08/2020	7.9	F	BM			9.11	M	RF-LB
	24	31/07/2020	9.3	F	RM			7.7	F	LB		7.10	F	BB					
			9.4	M	RB														
		sampling	Cage 5	Sex	Ear-punch						sampling	Cage 16	Sex	Ear-punch		sampling	Cage 21	Sex	Ear-punch
PND	20	28/07/2020	6.4	F	RB		Males	7				9.5	M	LF	24	3/08/2020	7.3	F	RM
			6.5	F	LF		Females	7				9.6	F	LM			7.4	M	RB
											24 31/07/2020	9.7	M	LB					
												9.8	F	BF		sampling	Cage 22	Sex	Ear-punch
		sampling	Cage 6	Sex	Ear-punch							9.9	F	BM	24	4/08/2020	5.1	M	none
PND	20	30/07/2020	7.1	M	none														
			7.2	F	RF							Males	6				Males	9	
	24	31/07/2020	8.1	M	none							Females	8				Females	5	
			8.2	M	RF														
		sampling	Cage 7		Ear-punch														
PND	20	28/07/2020	9.1	M	none														
			9.2	F	RF														

KEY

1.1	Litter/dam no.1 and pup no. 1
3.12	Litter/dam no. 3 and pup no. 12

Ear-punch	Abbrev.
none	none
R-front	RF
R-middle	RM
R-back	RB
L-front	LF
L-middle	LM
L-back	LB
Both-front	BF
Both-middl	BM
Both-back	BB
R-front,L-ba	RF-LB
L-front,R-ba	LF-RB

Notes:

Inulin dose 0% contains more pups as this includes pre-weaned pups and pups on the 0% inulin diet.
There will be 36 cages in total to house the pups.
Pups will be housed with their litter-mates only to prevent gut microbiota profiles from being influenced by members of other litters.
The smallest litter in a cage post-weaning will consist of 2 pups.
The largest litter in a cage post-weaning will consist of 5 pups.
The pups will be identified by ear-punching following the patterns described in the KEY above.

PND 20	**13 pups**		PND 24	**14 pups**		PND 24	**14 pups**		PND 24	**14 pups**		Total	**65 pups**	
PND 24	**10 pups**													
				Sampling date	**Pup #s**	**Age**								
	Males	10		28/07/2020	7 pups	PND20								
	Females	13		30/07/2020	4 pups	PND20								
				31/07/2020	26 pups	PND20,24	PND20-2 pups;PND24-24 pups							
				3/08/2020	18 pups	PND24								
	Rats pups already sampled.			4/08/2020	10 pups	PND24								

Appendix V

frontiers in Nutrition

REVIEW
published: 18 November 2021
doi: 10.3389/fnut.2021.759137

The Role of Segmented Filamentous Bacteria in Immune Barrier Maturation of the Small Intestine at Weaning

Linda A. Oemcke[1,2,3], *Rachel C. Anderson*[1,3*], *Eric Altermann*[1,4,5], *Nicole C. Roy*[1,5,6] *and Warren C. McNabb*[1,5]

[1] *Riddet Institute, Massey University, Palmerston North, New Zealand,* [2] *School of Food and Advanced Technology, Massey University, Palmerston North, New Zealand,* [3] *Smart Foods Innovation Centre of Excellence, AgResearch, Palmerston North, New Zealand,* [4] *Consumer Interface Innovation Centre of Excellence, AgResearch, Palmerston North, New Zealand,* [5] *High-Value Nutrition National Science Challenge, Auckland, New Zealand,* [6] *Department of Human Nutrition, University of Otago, Dunedin, New Zealand*

OPEN ACCESS

Edited by:
Clara G. De Los Reyes-Gavilan, Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Aaron Conrad Ericsson, University of Missouri, United States
Ravinder K. Nagpal, Florida State University, United States

***Correspondence:**
Rachel C. Anderson
Rachel.Anderson@agresearch.co.nz

Specialty section:
This article was submitted to Nutrition and Microbes, a section of the journal Frontiers in Nutrition

Received: *15 August 2021*
Accepted: *25 October 2021*
Published: *18 November 2021*

Citation:
Oemcke LA, Anderson RC, Altermann E, Roy NC and McNabb WC (2021) The Role of Segmented Filamentous Bacteria in Immune Barrier Maturation of the Small Intestine at Weaning. Front. Nutr. 8:759137. doi: 10.3389/fnut.2021.759137

The microbiological, physical, chemical, and immunological barriers of the gastrointestinal tract (GIT) begin developing *in utero* and finish maturing postnatally. Maturation of these barriers is essential for the proper functioning of the GIT. Maturation, particularly of the immunological barrier, involves stimulation by bacteria. Segmented filamentous bacteria (SFB) which are anaerobic, spore-forming commensals have been linked to immune activation. The presence and changes in SFB abundance have been positively correlated to immune markers (cytokines and immunoglobulins) in the rat ileum and stool samples, pre- and post-weaning. The abundance of SFB in infant stool increases from 6 months, peaks around 12 months and plateaus 25 months post-weaning. Changes in SFB abundance at these times correlate positively and negatively with the production of interleukin 17 (IL 17) and immunoglobulin A (IgA), respectively, indicating involvement in immune function and maturation. Additionally, the peak in SFB abundance when a human milk diet was complemented by solid foods hints at a diet effect. SFB genome analysis revealed enzymes involved in metabolic pathways for survival, growth and development, host mucosal attachment and substrate acquisition. This narrative review discusses the current knowledge of SFB and their suggested effects on the small intestine immune system. Referencing the published genomes of rat and mouse SFB, the use of food substrates to modulate SFB abundance is proposed while considering their effects on other microbes. Changes in the immune response caused by the interaction of food substrate with SFB may provide insight into their role in infant immunological barrier maturation.

Keywords: gastrointestinal tract, segmented filamentous bacteria, interleukin 17, immunoglobulin A, immunological barrier, food substrate, weaning diet

INTRODUCTION

It is widely accepted that, amongst other factors, the microbial consortia in all regions of the body contribute to maintaining homeostasis and good health (1–4). These positive effects, specifically of the gastrointestinal tract (GIT) microbes, created an interest in how the microbiota interacts with host cells. Most of the knowledge regarding upper and lower GIT microbiomes comes from research using fecal samples, and the knowledge of upper GIT communities comes from research using samples collected from those sites (5–7). Feces have been used to represent microbes of the lower GIT. Some studies have also used feces as a proxy of the upper GIT (8, 9) as the human small intestine is more difficult to access even with the use of invasive techniques (10, 11). Nevertheless, some microbes present in high abundance in the small intestine have caught scientists' attention, as emerging evidence in their involvement with the immune system points to their significance (12–16).

Segmented filamentous bacteria (SFB) were discovered attaching to the ileal mucosa in invertebrates (17) and healthy vertebrates (12–16, 18). These Gram-positive, anaerobic, spore-forming, commensal microbes have been classified within the Firmicutes phylum and are within the *Clostridiaceae* family. SFB possess a unique holdfast structure which facilitates their attachment to the ileal mucosa. During the lifecycle of SFB, the segmented filaments divide and elongate, forming viable intracellular offspring or spores in adverse conditions (15). The classification of SFB sparked an interest in their function as they were seen to occupy the distal ileum (19, 20). In this region, SFB attach to the ileal epithelium overlying the Peyer's patches where naïve T cells undergo antigen-driven activation and expansion to yield T helper cells (14, 19). Studies reported a positive correlation of SFB abundance with the concentration of interleukin 17 (IL-17) in plasma (20–24) and a negative correlation of immunoglobulin A (IgA) in feces and ileum contents of 4–6 week old mice (25–27), though this is not exclusive to SFB (5, 28–30). Interestingly, these effects occur without signs of systemic inflammation in the host (20–24).

Additionally, the presence of SFB is variable; their abundance in the ileum of infants increases at weaning from 6 months, peaks around 12 months and plateaus until 25 months post-weaning (24, 31, 32). A similar pattern has been reported in BALB/c mice (25), ICR (Institute of Cancer Research) mice (32) and Sprague-Dawley rats (18) where SFB abundance increased at weaning from 20 days, peaked around 24–28 days and plateaued until 50 days post-weaning. This timing of abundance change corresponds with the infant transitioning from a milk-based diet to one which increasingly includes solid foods. The increased dietary complexity is known to contribute to the immune system maturation progression and drives colonization by a different and more diverse microbiota in the GIT (31, 33, 34) possibly including SFB. **Figure 1** details the abundance of SFB as suggested to be involved in infant GIT immune maturation.

The reports of positive or negative correlation of IL-17 and IgA production with abundance changes of SFB in ileal contents or stools at weaning created an interest in studying these microbes and their influence on the infant GIT immunological barrier development. *In vitro* studies were carried out initially and proved challenging as SFB did not grow outside the ileum (36, 37). SFB are reportedly anaerobic yet attach to epithelial cells that require oxygen. The attempts to further investigate the effects of SFB on the immune system *in vitro* became a significant technical challenge due to the poor viability of these microbes. SFB were therefore studied using animal models (18, 25, 36, 37) and stool samples from human participants (10, 32, 38).

Ohashi et al. (25) investigated IgA concentration and SFB abundance from ileal contents in weanling mice. Their study reported that SFB abundance exhibited a temporal profile; it increased post-weaning immediately, peaked, then decreased and plateaued. Additionally, the concentration of IgA showed a negative correlation with SFB abundance. A study also reported that the abundance of SFB was higher in the ileum of 4 and 8-week-old mice fed a composite diet based on natural ingredients when compared to mice fed a solely milk powder diet. However, when purified diets containing the macronutrients from the composite diet were tested, no fat, carbohydrate or fiber fraction appeared to influence SFB abundance. Whilst it was not possible to identify a single nutritional factor responsible for SFB abundance, it was clear that composition of complex diets was at least one factor responsible for SFB abundance (39). Consideration should be given to the potential of various food substrates to increase the abundance of ileal SFB and related changes in markers of immune maturation during the weaning transition.

Further to that, analysis of the SFB genome revealed enzymes involved in metabolic pathways which these microbes utilize for survival, growth, and development (4, 40, 41). Data sets of enzymes in the glycolytic and pentose phosphate pathways of SFB predict the use of some of the by-products in metabolic pathways for synthesizing amino acids, vitamins, and cofactors within the genome (40, 41). The genome of SFB is smaller (∼1.57 Mb) compared to that of their relatives *Clostridium* (3.97 Mb). The genome reportedly lacks genes for biosynthesis of most amino acids. Amino acid transporters and permeases found in the genome imply SFB uptake amino acids possibly from dietary sources and host protein degradation by proteases secreted by SFB. The absence of these important genes could explain their commensal nature (40) and possibly reflect the size of the genome. Attachment of SFB to the ileal mucosa and abundance change following the introduction of solid foods suggest the genome may not support all functions required to be free-living. However, genome size does not determine whether a microbe can be free-living or not.

This narrative review analyzes the suggested role of SFB in infant immunological barrier maturation and whether modulation of SFB abundance could positively impact immune maturation of the small intestine for later health benefits. Referencing the published genomes of rodent SFB, the use of food substrates to potentially modulate SFB abundance is proposed while also considering the effects on the immune system and other GIT microbes.

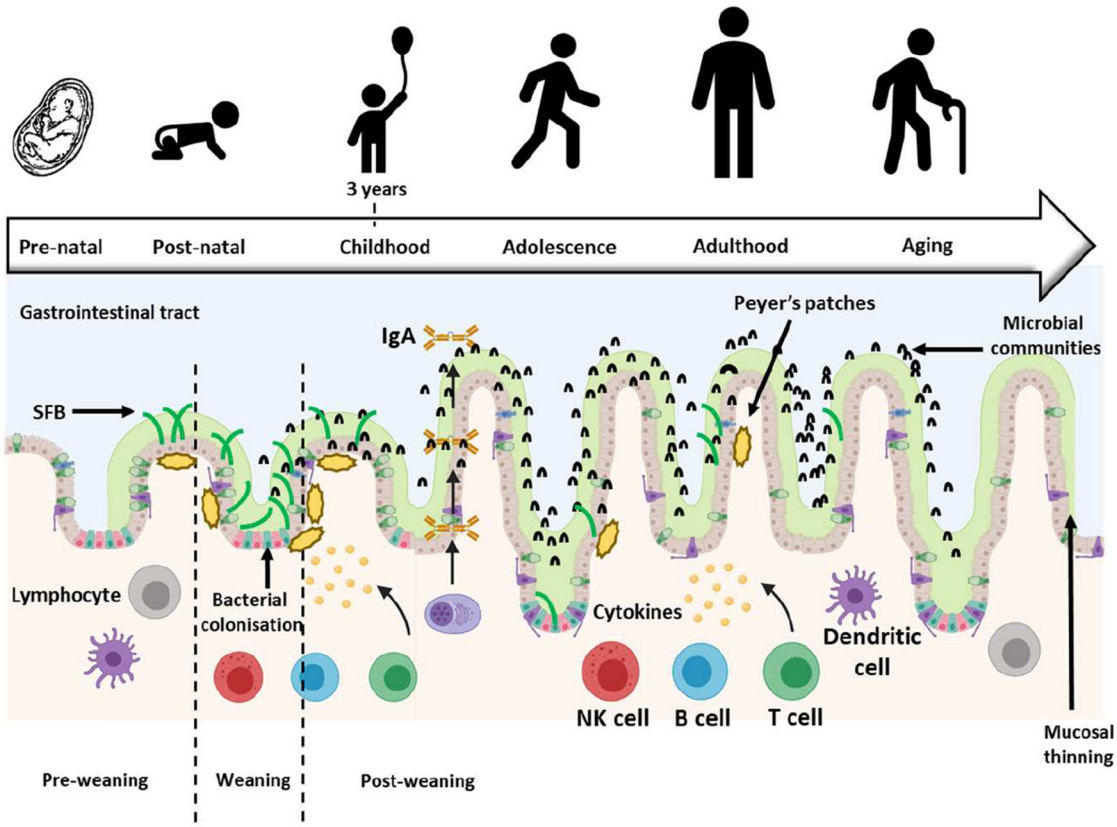

FIGURE 1 | Development of the gastrointestinal tract (GIT) throughout life. The GIT barriers continue developing and maturing postnatally until about 3 years of age when the resident microbiota resembles that of an adult. During this time, SFB abundance changes, first increasing and then decreasing at weaning, but SFB abundance remains low into adulthood [Figure adapted from Jašarević et al. (35); Created with BioRender.com and PowerPoint]. SFB, Segmented filamentous bacteria; IgA, Immunoglobulin A.

DEVELOPMENT AND MATURATION OF THE SMALL INTESTINAL BARRIER

The small intestinal barrier comprises the microbiological and chemical barriers, as well as the physical and immunological barriers in the mucosa. The microbiological barrier, located above the mucus layers, houses most of the microbes. Some microbes produce antimicrobial peptides that inhibit pathogen attachment by limiting their growth while other microbes are involved in nutrient acquisition and energy regulation (42–45). Below the microbiological barrier lies the chemical barrier that consists of an outer, less viscous mucus layer followed by the inner mucus layer containing fewer microbes. The inner mucus layer contains free-floating microbes and those, such as SFB, which attach to the epithelium.

Below the chemical barrier is the physical barrier known as the epithelium. It comprises columnar epithelial cells organized into crypts and villi (46). At the base of the epithelial crypts, Paneth cells secrete antimicrobial peptides, including defensins, lysozyme and phospholipase, which prevent the growth of pathogenic microbes (47). In the physical barrier, protein complexes provide structural integrity and act as channels that allow or prevent the passage of substances contributing to this barrier's selective permeability (48). The immunological barrier is the innermost layer where the immune system provides defense against pathogens and antigens and exists in an immune-suppressed state maintaining homeostasis even with dietary antigens and microbiota that pass through the physical barrier (49).

Of interest is the immunological barrier where immune cells begin appearing at ∼6 months of gestation. Recruitment of epithelial lymphocytes in the GIT begins signaling the functioning of an immature immunological barrier (50). Naïve T cells undergo activation and expansion in the Peyer's patches yielding T helper cells which are later stimulated to produce cytokines involved in immune responses (19). Naïve B cells also begin differentiating into plasma cells that produce immunoglobulins which recognize and bind to pathogenic bacterial or viral antigens and assist in their destruction (51). Fully developed during gestation, the immune system matures post-birth possibly along with the microbiological barrier. Maturation of the immunological barrier is thought to occur at

weaning (about 6 months of age) when foods containing antigens and microbes are introduced and may trigger immune responses in the infant GIT. It is during complementary feeding, when solid foods are introduced into the infant's milk-only diet, that the abundance of SFB reportedly increases, peaks, then plateaus. The studies which reported a positive and negative correlation of SFB abundance with IL-17 (20, 21, 52, 53). SFB also reportedly initially induce the production of IgA suggesting a positive correlation (54). However, the continued increase in luminal IgA concentration reportedly results in decreased SFB abundance in a somewhat self-regulating system whereby IgA restrains and possibly prevents an overgrowth of SFB in the ileum. This implies a negative correlation of IgA concentration with SFB abundance. These reported correlations led to the suggestion that SFB may play a role in influencing GIT immune system maturation.

The timing of SFB abundance changes around weaning (18, 25, 32) suggests that the inclusion of complementary foods might be a way to alter their abundance and hence effects on the epithelial and immune cells in the ileum. SFB are proposed to obtain nutrients from the ileal lumen (12, 14, 39, 55–57), and directly from the host as they cannot successfully survive outside the environment of the ileum (36). SFB may have evolved to attach to the ileal mucosa and during complementary feeding potentially derive nutrients in a cross-feeding manner with other bacteria residing in the inner mucus layer of the chemical barrier. If this is the case, then substrates from the diet might modulate SFB abundance at weaning, which had not previously been investigated. Therefore, an approach would be to provide substrates to enhance their abundance and perhaps functionality though only at weaning and immediately post-weaning to avoid any risks posed by a sustained increase in the abundance of SFB.

BACKGROUND ON SEGMENTED FILAMENTOUS BACTERIA

Reported initially over 150 years ago, SFB were observed in the ileum of invertebrates (17), firmly attaching to the epithelial lining (58). SFB were initially given the provisional name *Candidatus Arthromitus* (59). However, the first filamentous bacteria isolated from arthropods, though morphologically similar to those isolated from vertebrates, were shown to belong to the *Lachnospiraceae*, a family within the order *Clostridiales*, after analysis of 16S rRNA gene sequences (58). *Arthromitus* showed an apparent absence of SFB-like 16S rRNA gene sequences present in vertebrate SFB. This suggested different strains of SFB inhabiting invertebrates and vertebrates. Therefore, another taxonomic classification was proposed and accepted (60) to name the species isolated from vertebrates. It was named *Candidatus Savagella* (61) and provisionally classified under Savagellaceae, a credit to Dwayne C. Savage, the American gut microbiologist who first observed and described them in the ileum of rodents (62). Rods and filaments of SFB were identified by light microscopy and fluorescent *in situ* hybridization (32, 63) and later PCR methods were used to detect SFB in the ileum of rats (64). SFB are reported to replicate in the ileum through a life cycle deduced by electron microscopy in rodents (**Figure 2A**) (73). SFB exist in two forms, a vegetative segment containing a holdfast structure that allows them to anchor to the host epithelial cells, and as spores which are intracellular offspring encapsulated during adverse conditions. These morphologically distinct intrasegmental bodies indicate that SFB exist in vegetative and dormant states (67). Once the SFB intracellular offspring are released, they are transferred to another host of the same species (70). This observation was

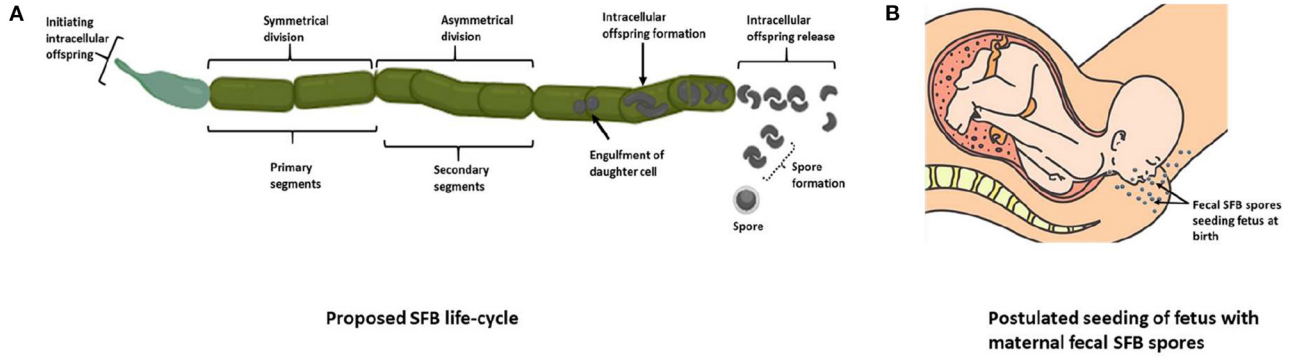

FIGURE 2 | The proposed life cycle of SFB. **(A)** The "holdfast" structure on vegetative segments allows SFB to anchor onto host epithelial cells and lengthen, forming primary and secondary segments (65). These segments double in length and form filaments in which a mother cell forms, engulfing the subsequent daughter cell, which then divides to form two viable intracellular offspring (66). The offspring then exit the filament and later attach to the host epithelium or form spores under stressful conditions (67–69), which may then be transferred to another host of the same species (70). **(B)** SFB fecal spores may seed the oral cavity during vaginal birth, or SFB spores could be passed from the mother to the embryo via the placenta in infants born *via* Cesarean-section. These theories may explain the origin of SFB in infants, though they have not been demonstrated experimentally. The relative abundance of genes predicted to encode cell cycle control functions, envelope biogenesis, and trafficking was higher in the mouse SFB genome than in other clostridia. They may reflect the complex cell differentiation processes during this life cycle (71). Four predicted N-acetylmuramoyl-L-alanine amidases unique to SFB (cell wall hydrolases, PF01510, PF05105, and PF01520) are also hypothesized as necessary in forming the different cell morphotypes and may be responsible for releasing "holdfasts" and spores from the filaments (72) [**(A)** adapted from Schnupf et al. (36); Created with BioRender.com] [**(B)** with permission from EPI-NO. Source: https://www.epino.de/en/birth-preparation.html]. SFB, Segmented filamentous bacteria.

confirmed by an *in vitro* investigation in SFB gene diversity and host-specificity of four flagellin genes in mice and rats which revealed two relatively conserved and two-variable genes and confirmed the preferential attachment of SFB to the epithelial mucosa of their host (70).

The origin of SFB in the GIT is thought to be via vertical transmission, as with other GIT microbes, from the mother to the fetus. The presence of SFB in dormant stages suggests that their spores found in maternal feces may seed the infant during vaginal birth (**Figure 2B**). In the case of infants born *via* Cesarean-section, it is unclear how SFB are present in the infant GIT. SFB spores could also be passed from the mother to the embryo via the placenta and may be the origin of SFB in infants born *via* Cesarean-section if SFB are even present in these infants. This theory, however, has no reported evidence.

In postnatal life, SFB are present in the ileum of healthy weanling rodents (25). They have also been detected in infant, and adult humans' feces though collecting and analyzing ileal samples are preferred (32). However, collecting ileal samples in conscious subjects requires invasive diagnostic tools such as scopes (74). Performing these manipulations in infants for routine SFB study is challenging due to the ethical restrictions of researching this age group. Therefore, animal models are currently the most appropriate way to study SFB.

SFB in Humans

The discovery and characterization of SFB in the ileum of healthy rodents drew interest due to their reported influence on markers of the GIT immune system. Later detection of SFB in the feces of healthy infants and adults suggests that SFB form part of the normal GIT microbiota and, like rodents, may also influence the GIT immune system. Knowledge of the role of SFB in humans is still scarce, and a time-course study elucidating the temporal profile of SFB at the crucial weaning stage in infants has yet to be attempted.

A comparative 16S rRNA gene analysis of SFB in healthy humans feces, mice ilea and chicken ilea indicated similarities in the abundance change of these microbes at similar stages of development (32). The similar findings of SFB abundance in human feces and mice and chicken ilea implied that ileal SFB in humans exhibit a temporal profile of abundance change pre- and post-weaning, and then persist into adulthood and old age as part of the normal microbiota. SFB have also been detected in ileostomy samples of adult patients with ulcerative colitis (64), though the implications of disease-cause by SFB have not been verified. Other than indicating the presence of SFB in the human GIT, these studies provide limited evidence of the interaction of these microbes with the immune system. Current knowledge comes from studies carried out using animal models. However, these observations might not necessarily translate to humans due to differences in the GIT microbiota and immune cell profiles between them.

SFB and the Immune System

The discovery of SFB in the ileum of vertebrates prompted investigations of their role in the GIT. Studies on how SFB interact with the immune system indicate that SFB do not appear to cause ill-effects in healthy vertebrates.

Immunoglobulin A and Interleukin 17

Studies on SFB have investigated their relationship with IgA, the most abundant immunoglobulin occurring in the body. IgA acts to block excessive bacterial adherence or translocation, mediates the neutralization of toxins and viruses, and removes unwanted macromolecular structures on the epithelium in the GIT (75). IgA in infancy reportedly originates from breast milk contributing to high levels found in the GIT lumen during the 1st month which gradually decrease until 5 months of age (76), then remain relatively low and stable until 24 months of age (77). Postnatal microbial colonization and maturation of the GIT stimulate host production of IgA, and the luminal levels slowly increase (78).

The presence of IgA in the GIT is important, especially in infants below 6 months of age with an immature immune system. Interactions between SFB and IgA drew attention following reports of SFB inducing IgA production in Swiss (13, 26), BALB/c (26, 79), and C3H/HeN (27) adult mice mono-associated with SFB. These findings suggest that SFB stimulated the germinal centers in the Peyer's patches (22). Further investigation in rodents showed that IgA production increased with increasing SFB abundance and continued increasing as SFB abundance decreased from about 4 weeks postnatally (25–27). A report on the aberrant expansion of mainly SFB and other anaerobes in the absence of hypermutated IgA in adult C57BL/6 mice (54) points at the function of IgA in regulating the bacterial composition of the GIT. The observation from this study suggests that IgA restrains growth of SFB. Therefore, though studies report an initial positive correlation between SFB and IgA, a further increase in IgA concentration results in decreasing SFB abundance, a negative correlation. IgA concentrations increase or decrease is, however, not exclusively linked to SFB. Other commensals, such as Gram-negative *Morganella morganii* (27) and Gram-positive probiotic *Bifidobacteria* (5, 29, 30), induce the production of IgA. B cells which are the origin of IgA may thus be stimulated by microbial colonization, including SFB, resulting in increased IgA levels (27). The reported correlation of IgA with SFB (13, 26, 27, 79) may be evidence of IgA maintaining a homeostatic balance within the microbiota and remains a point of interest in the suggested role of SFB in postnatal immunological barrier maturation.

Many reports on SFB have focused on their ability to stimulate the production of the pro-inflammatory cytokine IL-17 furthering the interest in their influence on GIT immunity (20, 21, 52, 53). IL-17 is essential for host defense against infection by invading pathogens at mucosal surfaces (80, 81). When fecal microbes, without SFB, from Jackson C57BL/6J mice, were introduced into germ-free (GF) mice, Th17 cells were not induced until SFB was added (21). Mice lacking SFB in their microbiota had fewer Th17 cells in the ileum than mice with a normal SFB population. SFB also specifically induced Th17 cells in the small intestinal lamina propria when introduced into GF Swiss-Webster mice (21). The production of Th17 cells demonstrated maturation of the immunological barrier after SFB

were introduced into GF mice. Non-colonized control GF mice were also observed to have no Th17 cells, implying an immature immunological barrier (13, 21).

Reports of the influence of SFB on ileal IL-17 production (21, 52, 53), including the reported mechanisms by which SFB achieve this (82, 83), suggest a positive correlation between both. Immunization of adult mice with SFB flagellins (FliC3) resulted in higher upregulation of small intestine epithelial cell factors controlling the differentiation of Th17 (Duox2, Duoxa2, and Nos2) and also promoting the production of IL-17 (83). Further exploration of IL-17 production by SFB in adult mice has revealed that SFB and host ileal epithelial cells communicate by generating endocytic vesicles at the interface of SFB-epithelial cell synapses. The interaction of SFB with the epithelial cells triggers the formation of endocytic vesicles through clathrin-independent and dynamin-dependent endocytosis. These vesicles contain an SFB cell wall-associated protein (P3340), an immunodominant T cell antigen for generating mucosal Th17 cells (82). The vesicles are released into the host epithelial cells, and P3340 induce activation of lamina propria antigen-specific Th17 cells, and subsequently, IL-17 is produced (84). Thus, these observations indicate that SFB flagellins are involved in upregulating ileal epithelial cell genes, which in turn induce IL-17 production.

Like IgA, Th17 cell production is also not exclusive to SFB (21). This was demonstrated when C57BL/6 GF mice mono-associated with SFB induced Th17 cells to a lesser extent than GF mice colonized by SFB and a more complex microbiota (SFB and eight defined commensals) in the small intestine (21, 85). This reported interaction between SFB and other microbes in IL-17 production highlights the synergy among commensals and their influence on the immune system, including Th17 cells. Alternatively, differentiation of Th17 cells was induced in Taconic B6 and Jackson B6 mice treated with antibiotics and then exposed to normal specific-pathogen-free bacteria (52). Upon further investigation, members of the *Bacteroidetes* phylum were reported to be involved indicating that SFB may not be the only microbes capable of inducing IL-17 production.

Overall, the literature reports that SFB-upregulated epithelial cell factors are involved in IL-17 production in adult mice. These observations in adult mice imply the presence of a mature GIT is required and this needs to be considered when investigating the role of SFB in immunological barrier maturation.

Immune-Mediated Disease

Research into SFB has highlighted their association with both disease cause (64, 86–88) and protection (89, 90). The findings hint at the abundance of SFB, maintaining a delicate balance between these microbes and the host immune system.

The effort to decipher the role of SFB arose from studies which investigated the involvement of SFB in several functional GIT and autoimmune diseases. Studies with immunodeficient adult mice colonized with only specific-pathogen-free bacteria, only SFB, or a combination of both reportedly developed clinical signs of colitis (87), suggesting that dendritic cells were activated by their colonization of the ileum (91). SFB were also detected in ileal mucosa samples of adult patients with ulcerative colitis. However, their presence might not be linked to the disease (64), and it is hypothesized that the patients' samples might have simply exhibited a higher SFB load (64). It is plausible that higher numbers of SFB induced the production of IL-17 to abnormal levels (86, 92) though this was not measured. A higher abundance of SFB was also reported in fecal samples of adult patients with diarrhea-associated irritable bowel syndrome than those with constipation-associated irritable bowel syndrome (88). Autoimmune diseases have also been associated with SFB where the Th17 cell population in GF adult mouse models of human arthritis and multiple sclerosis inoculated with SFB provoked an onset of the diseases (86, 93). These reports highlight the complexities of the suggested effects of SFB in disease and cannot be restricted to a single type of pathology, nor infer causality.

Mining of sequenced and annotated rat and mouse SFB genomes revealed that SFB lack the genes encoding for known toxins and virulence factors present in pathogenic *Clostridia* (74). This observation and the absence of apparent inflammatory reactions where SFB colonize the ileum suggest that SFB may stimulate IL-17 production without pathological consequences. Phylogenetic analysis of whole SFB genomes indicates that SFB and pathogenic *Clostridia* such as *C. tetani, C. perfringens,* and *C. fallax* share a common ancestor though distantly (**Figure 3**). Sequencing of SFB isolates from stool samples of healthy adults (32) and those with ulcerative colitis (64) would be required to identify genomic differences between SFB genome-types.

Comparison of genome sequences among SFB filaments isolated from SFB-monocolonized mice along with published SFB genome sequences revealed the presence of single nucleotide polymorphisms (SNPs) (71). SNPs occur frequently and, in some cases, cause missense mutations with no effect, but they can also cause non-sense mutations which affect gene functionality and alter phenotypes. Pamp et al. (71) combined reads, *de novo*, from five individual SFB filaments (SFB-1 to SFB-5) from SFB-monocolonized mice to form the genomic co-assembly "SFB-co." A second co-assembly, "SFB-mouse-SU," which was closely related to their SFB was also assembled using the published SFB mouse genome [SFB-mouse-Yit (AP012209)] (40). Some of the loci exhibiting SNPs in the individual SFB filaments included genes encoding for oxaloacetate decarboxylate alpha (OadA), pyruvate kinase (PK) and flagellar motor switch protein (FliN). A conserved lysine residue in OadA, which generates pyruvate from oxaloacetate, was substituted by threonine. A similar mutation in *Vibrio cholerae* renders OadA ineffective. For PK which catalyzes phosphoenolpyruvate to pyruvate, a valine residue was changed to alanine. The SNP in FliN, which together with FliG and FliM forms the switch complex that controls the direction of flagella rotation, resulted in a predicted threonine to alanine change (40). These mutations of FliN in bacteria are reported to result in failure in flagella export and rotation (94, 95). The polymorphisms observed among the five SFB filaments are likely minor variants which coexist within a population of SFB in an animal colony. Multiple genome sequence comparisons of other SFB genomes revealed chromosomal features whereby the highest variability include CRISPR-arrays, phage-related genes and hypothetical proteins (71) which indicate heterogeneity and evolution of SFB lineages within colonies of similar species.

Finotti et al. (64) also sequenced SFB PCR amplicons from colorectal biopsy samples of 35–70 year-old males and females with ulcerative colitis. The sequences were compared to whole-genome sequences of SFB from healthy mouse, rat, turkey, and a partial human SFB sequence in the NCBI Reference Sequence Database (**Table 1**). Results highlighted nucleotide changes in the ulcerative colitis SFB sequences at positions 64, 68, 81, and 85 reflecting amino acid differences from aromatic to branch-chain, negatively charged to uncharged, aromatic to aliphatic and hydrophobic to positively charged, respectively. The sequenced colorectal SFB genes were representative of SFB from the ileum, but without further information, it is unclear whether ileal and colorectal SFB are genetically similar or not. The lack of complete SFB sequences from healthy humans in the database creates a challenge in making informed comparisons between SFB isolated from healthy vs. diseased adults.

SFB have been associated with disease protection against type 1 diabetes (89) and rotavirus infection (90) in adult mice. Non-obese diabetic (NOD) adult mice inoculated with SFB were reported to have high levels of IL-17-expressing $CD4^+$ cells when compared to SFB-negative NOD mice. The SFB-positive NOD mice also did not develop diabetes though levels of insulitis, a marker for type 1 diabetes, were similar to those in SFB-negative NOD mice. This result implies that SFB colonization may not block the trigger of diabetes but might modulate the progression of the disease (89). Also, the comparison of two different SFB strains administered to GF Rag1-knockout (lack mature B or T cells) adult mice showed reduced rotavirus infectivity. The mechanism by which this effect happened was independent of Th17 cells as SFB administration promoted enterocyte proliferation, migration, and luminal shedding of rotavirus-infected cells. The observations hinted that SFB may prevent infection by hindering rotavirus from utilizing a surface component to bind to the ileal epithelial mucosa. The protective effect against rotavirus infection was, however, conferred more strongly by one of the two SFB strains administered to rotavirus-susceptible Rag1-knockout adult mice, highlighting a potential role of strain-specific phenotypes (90). These reports indicate synergies possibly among SFB strains, along with other microbes, cell signaling receptors and immune system mediators. IgA production was not assessed in the mice as a previous study by Corthesy et al. (96) indicated that IgA contribute to rotavirus protection via intracellular neutralization and not *via* immune exclusion. Immune exclusion involves SIgA preventing pathogens and antigens from gaining access to the intestinal epithelium. This suggests the decreased ability of SIgA to protect the mice from RV infection.

The presence of SFB in healthy (32) and diseased (64) adults indicates these microbes may have persisted from childhood. Based on the abundance of SFB relative to the absence of or incidence of disease, perhaps an appropriate and critical number of SFB may confer beneficial effects, and beyond this threshold, there might be detrimental effects. Whether the effects of SFB are

TABLE 1 | Nucleotide changes observed in human SFB gene from the terminal ileum of 35–70-year-old males and females with ulcerative colitis (64) compared to codons from the SFB genes of healthy rat and mice.

Nucleotide position	64	68	81	85
Rat	A**T**C Isoleucine (I)	G**AT** Aspartate (D)	**A**TA Tyrosine (Y)	C**A**T Methionine (M)
Mouse	T**T**C Phenylalanine (F)	G**A**T Aspartate (D)	**A**TA Tyrosine (Y)	C**C**T Leucine (L)
Human (ulcerative colitis patients)	A**T**T Isoleucine (I)	G**G**A Glycine (G)	**G**CC Alanine (A)	A**A**A Lysine (K)

The nucleotide positions correspond with the nucleotide bases in bold in the amino acid codons. Letters in parentheses are the single-letter abbreviations of the amino acids.

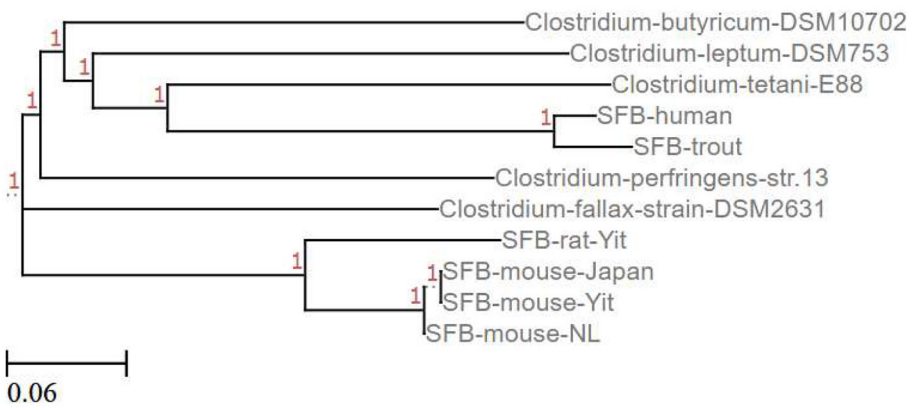

FIGURE 3 | Phylogenetic tree relating SFB to Clostridium species. This phylogenetic tree shows the relationship between complete (SFB mouse-Yit, SFB-rat-Yit, SFB-mouse-Japan, and SFB-mouse-NL) and partial (SFB-human and SFB-trout) SFB genomes and some pathogenic (C. perfringens, C. fallax, and C. tetani) and non-pathogenic (C. butyricum and C. leptum) Clostridium species published in the NCBI database (Created using the EMBL-EMI Multiple Sequence Alignment Clustal Omega tool and ETE toolkit-Phylogenetic Tree viewer).

specific to an age group or whether they continue to influence the GIT immune system later in life are unclear and remain as points of interest in the interaction of SFB in health and disease.

Impact of Early-Life Nutrition on Ileal SFB Abundance

The timing of SFB abundance change at weaning (18, 25, 32) suggests that the inclusion of complementary foods may alter their abundance as well as their effects on the host epithelial and immune cells in the ileum. The absorptive function of the small intestine matures during weaning in infants (97) and seems to coincide with these changes in SFB (and other microbes) abundance in the ileum. SFB are proposed to obtain nutrients from the ileal lumen (12, 14, 21, 39, 55–57), and directly from the host. SFB may have evolved to attach to the ileal mucosa and derive nutrients in a cross-feeding manner with other bacteria residing in the inner mucus layer of the chemical barrier.

The abundance of SFB might increase with the increasingly diverse diet that infants consume from weaning, which might have subsequent effects on the immunological barrier maturation. However, the risks that an increase in SFB abundance may pose cannot be ignored. As discussed in section Immune-Mediated Disease, these microbes may be associated with some diseases (64, 86–89). However, observations from healthy SFB-mono-associated mice (21, 52), as well as healthy humans (1 day-old to 72 years old), have shown that SFB do not predispose them to disease, which suggests that changes in the profile of SFB abundance can occur without adverse pathological effects. These reports hint at the necessity of an appropriate bacterial load of SFB to avoid possible activation of putative pathogenic genes that these microbes may harbor.

SFB are thought to influence the production of immune cells, and this process may exacerbate immune-mediated diseases and explain the association with disease. Immune-mediated diseases may also likely have a reverse effect and trigger opportunistic pathogenicity in SFB, though this is yet to be investigated. The effects of a transient increase in SFB abundance cannot be inferred from studies with SFB-mono-colonized mice. The cause of the SFB abundance decrease post-weaning is still unknown. One suggestion is that the abundance of other anaerobic microbiota may rapidly increase and compete with SFB resulting in decreased SFB abundance. If SFB are indeed involved in immune barrier maturation, the decrease in SFB abundance may be due to the completion of immune barrier maturation which coincides with increased diet diversity (98–100). However, this still remains unclear. SFB also coexist with other microbes in the community and attempts to alter the abundance, even for a limited amount of time, may impact other members. It is therefore unclear how these potential interactions might affect the immune system and overall health.

SFB Genes and Substrates

The analysis of the SFB genomes is necessary to infer the functions of predicted gene products and thereby model the utilization of substrates by SFB. Compared with selected *Clostridia*, annotation of SFB genomes indicated the absence of gene products involved in amino acid synthesis. Alternatively, the presence of genes coding for amino acid transporters and permeases implies a requirement for essential amino acids (4, 40, 41). *In silico* analyses of adult mouse and rat fecal SFB genomes revealed the presence of enzymes predicted to be involved in the glycolytic and pentose phosphate pathways. For the pentose phosphate pathway, enzymes for the oxidative phase were not predicted, though at least two catalases and one peroxiredoxin were detected which may contribute to the tolerance of SFB to the microaerobic environment of the small intestinal lumen (40). The predicted presence of these glycolytic and pentose phosphate enzymes suggests that carbohydrate macromolecules may be transported into the cell and utilized by SFB as an energy source.

Additionally, only a small fraction of enzymes required for synthesizing amino acids and co-factors have been predicted (40, 41). The lack of certain metabolic genes in the SFB genome and evidence that SFB reside in the nutrient-rich environment of the ileum may be indicative of ongoing reductive genome evolution and explain the reduced genome size (∼1.57 Mb) compared to closely related *Clostrida* (3.97 Mb). Results from the published rat and mouse SFB genomes revealed the presence of carbohydrate, amino acid, protein, vitamin and mineral permeases, import/export transporters and ABC-type transporters (40). Continuous evolution within the SFB genome may have contributed to their commensalism as they are presently thought to acquire essential nutrients directly from the host.

The published rat and mouse SFB genomes (40) revealed predicted genes which reflect the transport and metabolism of carbohydrates. These include mannose, a component of the glycolytic pathway which is employed in the cell wall structure and malate–a source of carbon and ribose–used in cellular respiration. SFB are also thought to uptake cellobiose, ascorbate and fructose whose metabolism results in glyceraldehyde-3-phosphate of the glycolytic pathway. Moreover, the preferred niche of SFB is the ileum, where the brush border is located (40). This microvilli-covered surface on the ileal epithelium contains enzymes which degrade disaccharides into simple sugars that are then absorbed into the bloodstream (101). The presence of simple sugars in this region along with permeases and import/export-type transporters for sugars detected in the SFB genome points toward SFB being able to thrive on simple sugars (40).

An analysis of the SFB rat genome also indicated the presence of an N-acetylglucosaminidase family protein. N-acetylglucosaminidases are essential enzymes involved in the hydrolysis of complex oligosaccharides and their presence in the genome could point toward the ability of SFB to metabolize these kinds of carbohydrates. Analysis of the draft genome of human SFB from ileostomy patients, revealed the presence of one tentative extracellular N-acetylglucosaminidase, nine glycoside hydrolases representing six different families, as well as several cell surface-bound and extracellular proteases (102). Analysis of the human SFB draft genome indicated a lack of tricarboxylic acid cycle enzymes, similar to rat and mouse SFB. There were also no proteins identified that could be assumed to take part in the electron transport chain, confirming a fermentative lifestyle. An interesting observation was that human SFB contains genes for biotin synthesis (*bioA, B, D, F, W*, and *X*), which are lacking in rat

and mouse SFB. This observation may demonstrate physiological or dietary differences of the hosts, or host microbial community, as some gut microbes can synthesize biotin while others cannot.

Zoetendal et al. (4) reasoned that the microbes from the *Clostridium* class XIVa, could utilize simple carbohydrate fermentation products from anaerobes such as some *Streptococcus spp* in a cross-feeding manner to produce butyrate (**Figure 4**). However, SFB reportedly metabolize pyruvate to produce acetate or ethanol but not butyrate evidenced by the lack of uptake transporters, though they reportedly metabolize pyruvate into lactate (40) like some *Streptococcus spp* (4).

Although diets post-weaning can contain low levels of simple sugars, whole-milk-based diets pre-weaning contain dairy carbohydrates such as lactose and oligosaccharides, but these diets did not increase ileal SFB abundance in mice at weaning, indicating that these metabolic pathways probably don't play a dominant role in SFB (39). Earlier work showed that mice fed a purified whole milk powder diet had lower SFB abundance in the ileum than those on a balanced composite diet. The composite diet comprised skim milk powder, Lucerne (alfalfa) meal, native corn starch, soybean oil, ground barley, fish meal, soybean protein concentrate, wheat middling's, corn protein concentrate, molasses, vitamin premix, mineral premix, calcium carbonate, and sodium chloride. These results suggest that the whole milk powder diet lacked additional nutrient(s) essential for optimum SFB growth. It is also possible that the reduced abundance of SFB in the mice receiving purified whole milk powder may have been an indirect effect, whereby some other microbe on which SFB rely are the primary effect (39).

Enriching a weaning diet would involve using a carbohydrate substrate which is typically added to the infant diet such as inulin. Inulin belongs to a class of soluble dietary fibers known as fructans and occurs naturally as a reserve carbohydrate in plants (103). It is digested by probiotics and encourages the growth of short-chain fatty acid (SCFA) producing microbiota (104, 105). Its unique chemical structure is made up of compounds with low chemical reactivity and resistant to digestion by the human GIT (106). Inulin is routinely added to infant formulas (107) mainly manufactured from bovine milk which, compared to human breastmilk (108), has a lower concentration of milk oligosaccharides (109). The complex molecular structures of these milk oligosaccharides are digested by probiotic bacteria which is key for developing a diverse and balanced microbial community in the infant GIT (110).

Inulin has also been reported to have several health benefits. These include reduced incidences of flatulence and bloating (111) and enhanced abundance of beneficial bacteria. Inulin promotes the production of SCFAs (acetate, propionate and butyrate) which creates an acidic environment that prevents the growth of pathogenic bacteria. SCFAs are also thought to be involved in immune system activation (112). Inulin has also been reported to improve epithelial integrity and barrier function and increase the expression of TJs (claudin-2 and occludin) (113). The reported production of acetate by SFB via the glycolytic pathway which is then converted to ethanol suggests that SFB may contribute some modulatory effects through SCFA production.

As an infant matures, a weaning diet is provided to complement breastmilk or formula to keep up with

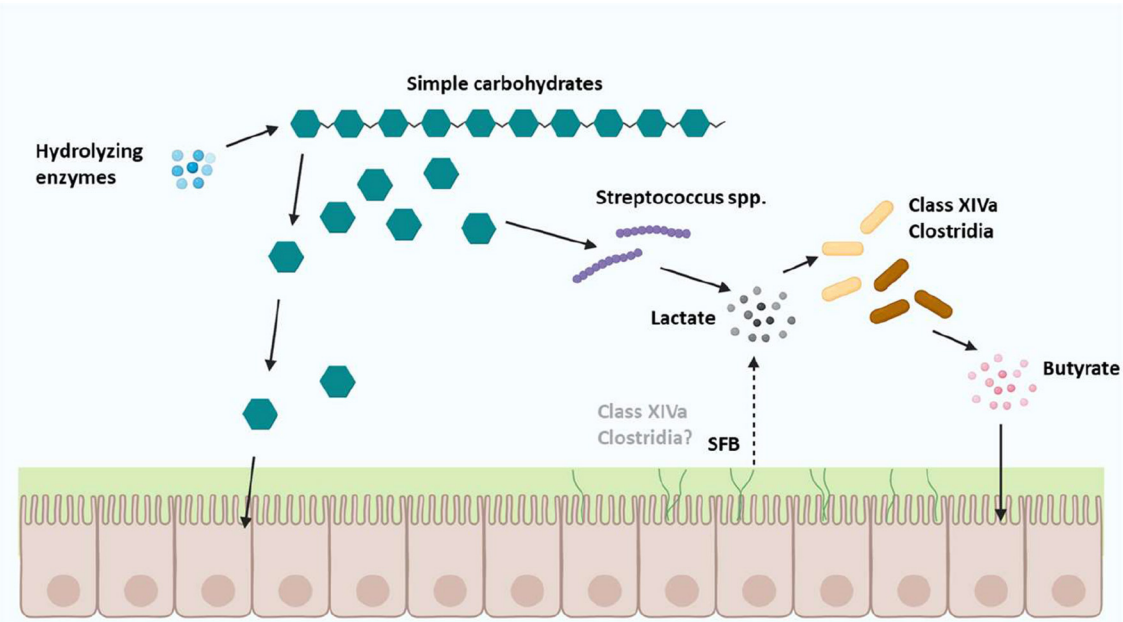

FIGURE 4 | Cross-feeding of carbohydrate substrates between Clostridium class XIVa microbes and other Firmicutes. Cross-feeding with microbes from Clostridium class XIVa, and the Firmicutes phylum (42) might include SFB which are proposed to be a separate genus of Clostridia, based on a small number of SFB orthologs identified in host-associated Clostridia [Figure adapted with permission from Zoetendal et al. (4); Created with BioRender.com].

the nutritional requirements during development. The recommended weaning diet includes protein, fats, minerals, vitamins, and carbohydrate prebiotics, including inulin (114). Inulin has not previously been reported in SFB studies and can be easily incorporated in the diet in a dose-dependent manner to investigate the effects on SFB abundance. A mixture of inulin and fructooligosaccharides was tested in GF rats colonized by human fecal bacteria. The rats exhibited an increase in numbers of bacterial members from *Clostridium* class XIVa in the colon (105) but the effects of inulin specifically on SFB has not yet been investigated.

SFB are suggested to utilize nutrients in the ileum resulting from the consumption of a balanced diet, though the mechanism they use is still unclear. However, the reported presence of import and export transporters in the cell membrane of SFB filaments suggests this is how they uptake metabolic products (40). This process of nutrient import in SFB filaments may provide energy sources for the reported life cycle. The presence of orthologous flagellar genes in SFB implies similarities in bacterial flagellar morphology to Clostridium species and may include the arrangement of flagellin in the filament and attachment and nutrient uptake mechanisms (115).

Future Perspectives

The difficulty in culturing SFB in the laboratory using the common microbiological techniques required alternatives to decipher the role of SFB in the ileum. SFB are unique as they require mucosal epithelial attachment to the ileum for survival. Their pattern of abundance change pre- and post-weaning in the ileum, as currently known, is also unique to them. Their preferred location of attachment in the ileum over Peyer's patches reiterates the importance of understanding the function of SFB. This niche of SFB in the ileum may position them to receive nutrients from digestion and influence the immune system. Whether SFB directly benefit the host GIT immune barrier or whether the host immune system triggers SFB to influence immunity in a feedback loop or enhance the action of other microbes remains unknown.

The reported influence of SFB on the immune system encouraged research on the effects of SFB presence or absence in immune-mediated disease. Perhaps in some cases, SFB contribute to the progression of the disease, though the reported absence of clostridial virulence genes imply that SFB may indeed offer protection from disease. Thus, far the results from these studies are varied and inconclusive, reflecting the complex association between SFB and the immune system.

The lack of knowledge of the complex relationship between SFB and the immune system is an important factor in dietary intervention studies. The proposal to enrich diets with substrates to manipulate the abundance of SFB is feasible compared to delivering SFB as a supplement (36). Hypotheses surrounding diet and SFB have been proposed (39), though there is a lack of knowledge about which dietary substrates affect SFB abundance. A prime candidate is carbohydrates (4, 40). Regardless of the impact on SFB abundance around weaning, observing the effects of a diet enriched in carbohydrate on the transient change in SFB abundance in infancy may give some clues on the role of SFB in immune barrier maturation.

Further investigation could lead to identifying how SFB interact with other microbes in the ileum. One group has suggested exploring the targeted use of metagenomic alteration of the gut microbiome by *in situ* conjugation (MAGIC) (116). They propose modifying SFB by harnessing naturally occurring horizontal gene transfer activity using an *Escherichia coli* strain as a donor to deliver engineered DNA. They reported achieving transient expression of the engineered DNA in the microbiome. It is unknown, however, whether SFB are naturally competent. Knowledge from this and more work on SFB may contribute to deducing the mechanisms by which these commensals uptake, utilize nutrients, and survive in the ileum. Understanding the function of SFB in the ileum may give more insight into the interaction of microbes with nutrition on the immune system.

The scarce knowledge on SFB is unsurprising, considering most studies have focused mainly on the effects of SFB presence and absence on the GIT immune system. Therefore, this new avenue of utilizing diet to decipher any influence on SFB could increase the understanding of the suggested role of these unique commensals in small intestine immune barrier maturation.

AUTHOR CONTRIBUTIONS

LO, RA, NR, and WM contributed to the conception of the review. The draft manuscript was prepared by LO. EA and NR contributed to the genomics portion of the review. All authors contributed to the preparation of the manuscript.

FUNDING

LO was supported by a PhD Fellowship from the Riddet Institute Centre of Research Excellence, funded by the Tertiary Education Commission.

ACKNOWLEDGMENTS

Appreciation is given to AgResearch Grasslands and the Riddet Institute for providing the required facilities for the work on this manuscript.

REFERENCES

1. Aas JA, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* (2005) 43:5721–32. doi: 10.1128/JCM.43.11.5721-5732.2005
2. Adlerberth I, Strachan DP, Matricardi PM, Ahrné S, Orfei L, Åberg N, Perkin MR, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol.* (2007) 120:343–50. doi: 10.1016/j.jaci.2007.05.018
3. Allegretti JR Hamilton HJ. Restoring the gut microbiome for the treatment of inflammatory bowel diseases. *World J Gastroenterol.* (2014) 20:3468. doi: 10.3748/wjg.v20.i13.3468
4. Zoetendal EG, Raes J, Van Den Bogert B, Arumugam M, Booijink CC, Troost FJ, et al. The human small intestinal microbiota is driven by rapid

uptake and conversion of simple carbohydrates. *ISME J.* (2012) 6:1415–26. doi: 10.1038/ismej.2011.212
5. Bevilacqua L, Ovidi M, Di Mattia E, Trovatelli LD, Canganella F. Screening of Bifidobacterium strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens. *Microbiol Res.* (2003) 158:179. doi: 10.1078/0944-5013-00192
6. Fagerhol MK. Calprotectin a faecal marker of organic gastrointestinal abnormality. *Lancet.* (2000) 356:1783–4. doi: 10.1016/S0140-6736(00)03224-4
7. Vásquez A, Ahrné S, Jeppsson B, Molin G. Oral administration of *Lactobacillus* and *Bifidobacterium* strains of intestinal and vaginal origin to healthy human females: Re-isolation from faeces and vagina. *Microb Ecol Health Dis.* (2011) 17:7811. doi: 10.3402/mehd.v17i1.7811
8. Yan W, Sun C, Zheng J, Wen C, Ji C, Zhang D, et al. Efficacy of fecal sampling as a gut proxy in the study of chicken gut microbiota. *Front Microbiol.* (2019) 10:2126. doi: 10.3389/fmicb.2019.02126
9. Guo M, Miao M, Wang Y, Duan M, Yang F, Chen Y, et al. Developmental differences in the intestinal microbiota of Chinese 1-year-old infants and 4-year-old children. *Sci Rep.* (2020) 10:1–9. doi: 10.1038/s41598-020-76591-4
10. Jonsson H. Segmented filamentous bacteria in human ileostomy samples after high-fiber intake. *FEMS Microbiol Lett.* (2013) 342:24–9. doi: 10.1111/1574-6968.12103
11. Van den Bogert B, Erkus O, Boekhorst J, Goffau Md, Smid EJ, Zoetendal EG, et al. Diversity of human small intestinal *Streptococcus* and *Veillonella* populations. *FEMS Microbiol Ecol.* (2013) 85:376–88. doi: 10.1111/1574-6941.12127
12. Ericsson AC, Turner G, Montoya L, Wolfe A, Meeker S, Hsu C, et al. Isolation of segmented filamentous bacteria from complex gut microbiota. *BioTechniques.* (2015) 59:94–8. doi: 10.2144/000114319
13. Klaasen H, Van der Heijden P, Stok W, Poelma F, Koopman J, Van den Brink M, et al. Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. *Infect Immunity.* (1993) 61:303–6. doi: 10.1128/iai.61.1.303-306.1993
14. Klaasen HLBM, Koopman JP, Poelma FGJ, Beynen AC. Intestinal, segmented, filamentous bacteria. *FEMS Microbiol Lett.* (1992) 88:165–79. doi: 10.1111/j.1574-6968.1992.tb04986.x
15. Schnupf P, Gaboriau-Routhiau V, Cerf-Bensussan N. Host interactions with Segmented Filamentous Bacteria: An unusual trade-off that drives the post-natal maturation of the gut immune system. *Seminars Immunol.* (2013) 25:342–51. doi: 10.1016/j.smim.2013.09.001
16. Schnupf P, Gaboriau-Routhiau V, Sansonetti PJ, Cerf-Bensussan N. Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world. *Curr Opin Microbiol.* (2017) 35:100–9. doi: 10.1016/j.mib.2017.03.004
17. Leidy J. On the existence of entophyta in healthy animals, as a natural condition. *Proc Acad Nat Sci Philadelphia.* (1849) 4:225–33.
18. Oemcke LA, Anderson RC, Rakonjac J, McNabb WC, Roy NC. Whole tissue homogenization preferable to mucosal scraping in determining the temporal profile of segmented filamentous bacteria in the ileum of weanling rats. *Access Microbiol.* (2021) 3:000218. doi: 10.1099/acmi.0.000218
19. Agace WW. T-cell recruitment to the intestinal mucosa. *Trends Immunol.* (2008) 29:514–22. doi: 10.1016/j.it.2008.08.003
20. Goto Y, Panea C, Nakato G, Cebula A, Lee C, Diez MG, et al. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity.* (2014) 40:594–607. doi: 10.1016/j.immuni.2014.03.005
21. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell.* (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
22. Lécuyer E, Rakotobe S, Lengliné-Garnier H, Lebreton C, Picard M, Juste C, et al. Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses. *Immunity.* (2014) 40:608–20. doi: 10.1016/j.immuni.2014.03.009
23. Yi J, Jung J, Han D, Surh C, Lee Y. Segmented filamentous bacteria induce divergent populations of antigen-specific CD4 T cells in the small intestine. *Mol Cells.* (2019) 42:228. doi: 10.14348/molcells.2018.0424
24. Chen B, Liu W, Bie S, Peng K, Shu X, Gu W, et al. Adherent bacteria in the terminal ileum of children correlates with increased Th17 cell activation and luminal secretory IgA. *Front Pharmacol.* (2019) 11:588560. doi: 10.2139/ssrn.3397178
25. Ohashi Y, Hiraguchi M, Sunaba C, Tanaka C, Fujisawa T, Ushida K. Colonization of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. *Anaerobe.* (2010) 16:543–6. doi: 10.1016/j.anaerobe.2010.07.006
26. Snel J, Bakker M, Heidt P. Quantification of antigen-specific immunoglobulin A after oral booster immunization with ovalbumin in mice mono-associated with segmented filamentous bacteria or Clostridiuminnocuum. *Immunol Lett.* (1997) 58:25–28. doi: 10.1016/S0165-2478(97)02715-6
27. Talham GL, Jiang H-Q, Bos NA, Cebra JJ. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immunity.* (1999) 67:1992–2000. doi: 10.1128/IAI.67.4.1992-2000.1999
28. Wilmore JR, Gaudette BT, Atria DG, Hashemi T, Jones DD, Gardner CA, et al. Commensal microbes induce serum IgA responses that protect against polymicrobial sepsis. *Cell Host Microbe.* (2018) 23:302–11. doi: 10.1016/j.chom.2018.01.005
29. Cheikhyoussef A, Pogori N, Chen H, Tian F, Chen W, Tang J, et al. Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances (BLIS) produced by Bifidobacterium infantis BCRC 14602. *Food Control.* (2009) 20:553–9. doi: 10.1016/j.foodcont.2008.08.003
30. Collado M, Gonzalez A, Gonzalez R, Hernandez M, Ferrus M, Sanz Y. Antimicrobial peptides are among the antagonistic metabolites produced by Bifidobacterium against Helicobacter pylori. *Int J Antimicrob Agents.* (2005) 25:385–91. doi: 10.1016/j.ijantimicag.2005.01.017
31. Thompson AL, Monteagudo-Mera A, Cadenas MB, Lampl ML, Azcarate-Peril MA. Milk-and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome. *Front Cell Infect Microbiol.* (2015) 5:3. doi: 10.3389/fcimb.2015.00003
32. Yin Y, Wang Y, Zhu L, Liu W, Liao N, Jiang M, et al. Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens. *ISME J.* (2013) 7:615–21. doi: 10.1038/ismej.2012.128
33. Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathogens.* (2013) 5:23. doi: 10.1186/1757-4749-5-23
34. Amarri S, Benatti F, Callegari M, Shahkhalili Y, Chauffard F, Rochat F, et al. Changes of gut microbiota and immune markers during the complementary feeding period in healthy breast-fed infants. *J Pediatric Gastroenterol Nutr.* (2006) 42:488–95. doi: 10.1097/01.mpg.0000221907.14523.6d
35. Jašarević E, Morrison KE, Bale TL. Sex differences in the gut microbiome-brain axis across the lifespan. *Phil Trans R Soc B.* (2016) 371:20150122. doi: 10.1098/rstb.2015.0122
36. Schnupf P, Gaboriau-Routhiau V, Gros M, Friedman R, Moya-Nilges M, Nigro G, et al. Growth and host interaction of mouse segmented filamentous bacteria *in vitro*. *Nature.* (2015) 520:99. doi: 10.1038/nature14027
37. Gerard EP. *Method of Culturing Segmented Filamentous Bacteria in vitro. Office EP, Editor*. Institut Pasteur (Paris, FR),Fondation Imagine (Paris, FR), Assistance Publique - Hopitaux de Paris (Paris, FR), Universite Paris Descartes (Paris, FR), Institut National de la Sante et de la Recherche Medicale (INSERM) (Paris, FR) (2017).
38. Bo C, Li L, Elitsur Y, Yu HD. Presence of segmented filamentous bacteria in human children and its potential role in the modulation of human gut immunity. *Front Microbiol.* (2018) 9:1403. doi: 10.3389/fmicb.2018.01403
39. Klaasen H, Koopman J, Van den Brink M, Scholten P, Beynen A. Influence of macronutrients on segmented filamentous bacteria in the small intestine of mice. *Microb Ecol Health Dis.* (1991) 4:47–51. doi: 10.3109/08910609109140263
40. Prakash T, Oshima K, Morita H, Fukuda S, Imaoka A, Kumar N, et al. Complete genome sequences of rat and mouse segmented filamentous bacteria, a potent inducer of Th17 cell differentiation. *Cell Host Microbe.* (2011) 10:273–84. doi: 10.1016/j.chom.2011.08.007
41. Sczesnak A, Segata N, Qin X, Gevers D, Petrosino JF, Huttenhower C, et al. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. *Cell Host Microbe.* (2011) 10:260–72. doi: 10.1016/j.chom.2011.08.005

42. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. *PLoS Biol.* (2007) 5:e177. doi: 10.1371/journal.pbio.0050177
43. Zhang L, Rozek A, Hancock RE. Interaction of cationic antimicrobial peptides with model membranes. *J Biol Chem.* (2001) 276:35714–22. doi: 10.1074/jbc.M104925200
44. Zhang, LJ, Gallo RL. Antimicrobial peptides. *Curr Biol.* (2016) 26:R14–9. doi: 10.1016/j.cub.2015.11.017
45. Neish AS. Microbes in gastrointestinal health and disease. *Gastroenterology.* (2009) 136:65–80. doi: 10.1053/j.gastro.2008.10.080
46. Van Der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Ann Rev Physiol.* (2009) 71:241–60. doi: 10.1146/annurev.physiol.010908.163145
47. Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am J Clin Nutr.* (2001) 73:1131S−41. doi: 10.1093/ajcn/73.6.1131S
48. Salvo-Romero E, Alonso-Cotoner C, Pardo-Camacho C, Casado-Bedmar M, Vicario M. The intestinal barrier function and its involvement in digestive disease. *Rev Espanola Enfermedades Digestivas.* (2015) 107:686–96. doi: 10.17235/reed.2015.3846/2015
49. Rautava S, Walker WA. Immunophysiology and nutrition of the gut. *Nutr Pediatr.* (2008) 2008:251–60.
50. Weitkamp JH, Rosen MJ, Zhao Z, Koyama T, Geem D, Denning TL, et al. Small intestinal intraepithelial TCRγδ+ T lymphocytes are present in the premature intestine but selectively reduced in surgical necrotizing enterocolitis. *PLoS ONE.* (2014) 9:e99042. doi: 10.1371/journal.pone.0099042
51. Weber MS, Prod'Homme T, Patarroyo JC, Molnarfi N, Karnezis T, Lehmann-Horn K, et al. B-cell activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity. *Ann Neurol.* (2010) 68:369–83. doi: 10.1002/ana.22081
52. Ivanov II, de Llanos Frutos R, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe.* (2008) 4:337–49. doi: 10.1016/j.chom.2008.09.009
53. Farkas AM, Panea C, Goto Y, Nakato G, Galan-Diez M, Narushima S, et al. Induction of Th17 cells by segmented filamentous bacteria in the murine intestine. *J Immunol Methods.* (2015) 421:104–11. doi: 10.1016/j.jim.2015.03.020
54. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci USA.* (2004) 101:1981–6. doi: 10.1073/pnas.0307317101
55. Ericsson AC, Hagan CE, Davis DJ, Franklin CL. Segmented filamentous bacteria: commensal microbes with potential effects on research. *Compar Med.* (2014) 64:90–8.
56. Ivanov II, Littman DR. Segmented filamentous bacteria take the stage. *Mucosal Immunol.* (2010) 3:209. doi: 10.1038/mi.2010.3
57. Klaasen HLBM, Koopman JP, Van Den Brink ME, Bakker MH, Poelma FGJ, Beynen AC. Intestinal, segmented, filamentous bacteria in a wide range of vertebrate species. *Lab Animals.* (1993) 27:141–50. doi: 10.1258/002367793780810441
58. Thompson CL, Vier R, Mikaelyan A, Wienemann T, Brune A. 'Candidatus Arthromitus' revised: Segmented filamentous bacteria in arthropod guts are members of *Lachnospiraceae*. *Environ Microbiol.* (2012) 14:1454–65. doi: 10.1111/j.1462-2920.2012.02731.x
59. Snel J, Heinen P, Blok H, Carman R, Duncan A, Allen P, et al. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". *Int J Syst Evolut Microbiol.* (1995) 45:780–2. doi: 10.1099/00207713-45-4-780
60. McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Lošo T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci USA.* (2013) 110:3229–36. doi: 10.1073/pnas.1218525110
61. Thompson CL, Mikaelyan A, Brune A. Immune-modulating gut symbionts are not "candidatus Arthromitus." *Mucosal Immunol.* (2013) 6:200–1. doi: 10.1038/mi.2012.91
62. Davis CP, Savage DC. Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract. *Infect Immunity.* (1974) 10:948–56. doi: 10.1128/iai.10.4.948-956.1974
63. Child MW, Kennedy A, Walker AW, Bahrami B, Macfarlane S, Macfarlane GT. Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques. *FEMS Microbiol Ecol.* (2006) 55:299–310. doi: 10.1111/j.1574-6941.2005.00016.x
64. Finotti A, Gasparello J, Lampronti I, Cosenza LC, Maconi G, Matarese V, et al. PCR detection of segmented filamentous bacteria in the terminal ileum of patients with ulcerative colitis. *BMJ Open Gastroenterol.* (2017) 4:172. doi: 10.1136/bmjgast-2017-000172
65. Kuwahara T, Ogura Y, Oshima K, Kurokawa K, Ooka T, Hirakawa H, et al. The lifestyle of the segmented filamentous bacterium: A non-culturable gut-associated immunostimulating microbe inferred by whole-genome sequencing. *DNA Res.* (2011) 18:291–303. doi: 10.1093/dnares/dsr022
66. Angert ER. Alternatives to binary fission in bacteria. *Nat Rev Microbiol.* (2005) 3:214–24. doi: 10.1038/nrmicro1096
67. Chase DG, Erlandsen SL. Evidence for a complex life cycle and endospore formation in the attached, filamentous, segmented bacterium from murine ileum. *J Bacteriol.* (1976) 127:572–83. doi: 10.1128/jb.127.1.572-583.1976
68. Snellen JE, Savage DC. Freeze-fracture study of the filamentous, segmented microorganism attached to the murine small bowel. *J Bacteriol.* (1978) 134:1099–107. doi: 10.1128/jb.134.3.1099-1107.1978
69. Davis CP, Savage DC. Effect of penicillin on the succession, attachment, and morphology of segmented, filamentous microbes in the murine small bowel. *Infect Immunity.* (1976) 13:180–8. doi: 10.1128/iai.13.1.180-188.1976
70. Chen H, Yin Y, Wang Y, Wang X, Xiang C. Host specificity of Segmented Filamentous Bacteria (SFB) flagellins affects their interaction patterns with mouse ileal mucosal proteins. *Appl Environ Microbiol.* (2017) 83:e01061-17. doi: 10.1128/AEM.01061-17
71. Pamp SJ, Harrington ED, Quake SR, Relman DA, Blainey PC. Single-cell sequencing provides clues about the host interactions of segmented filamentous bacteria (SFB). *Genome Res.* (2012) 22:1107–19. doi: 10.1101/gr.131482.111
72. Pasztor L, Ziebandt A-K, Nega M, Schlag M, Haase S, Franz-Wachtel M, et al. Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. *J Biol Chem.* (2010) 285:36794–803. doi: 10.1074/jbc.M110.167312
73. Ferguson DJP, Birch-Andersen A. Electron microscopy of a filamentous, segmented bacterium attached to the small intestine of mice from a laboratory animal colony in Denmark. *Acta Pathol Microbiol Scand Section B Microbiol.* (1979) 87:247–52. doi: 10.1111/j.1699-0463.1979.tb02434.x
74. Caselli M, Cassol F, Gentili V, Di Luca D. Genome sequences of segmented filamentous bacteria in animals: implications for human research. *Gut Microbes.* (2012) 3:401–5. doi: 10.4161/gmic.20736
75. Macpherson AJ, Geuking MB, Slack E, Hapfelmeier S, McCoy KD. The habitat, double life, citizenship, and forgetfulness of IgA. *Immunol Rev.* (2012) 245:132–46. doi: 10.1111/j.1600-065X.2011.01072.x
76. Maruyama K, Hida M, Kohgo T, Fukunaga Y. Changes in salivary and fecal secretory IgA in infants under different feeding regimens. *Pediatr Int.* (2009) 51:342–5. doi: 10.1111/j.1442-200X.2008.02748.x
77. Dion C, Montagne P, Bene MC, Faure G. Measurement of faecal immunoglobulin a levels in young children. *J Clin Lab Analysis.* (2004) 18:195–9. doi: 10.1002/jcla.20022
78. Lindner C, Wahl B, Föhse L, Suerbaum S, Macpherson AJ, Prinz I, et al. Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine. *J Experi Med.* (2012) 209:365–77. doi: 10.1084/jem.20111980
79. Umesaki Y, Okada Y, Matsumoto S, Imaoka A, Setoyama H. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol.* (1995) 39:555–62. doi: 10.1111/j.1348-0421.1995.tb02242.x
80. Aujla SJ, Dubin PJ, Kolls JK. *Th17 Cells and Mucosal Host Defense Seminars in Immunology.* Pittsburgh, PA: Elsevier (2007). doi: 10.1016/j.smim.2007.10.009
81. Bettelli E, Oukka M, Kuchroo VK. T H-17 cells in the circle of immunity and autoimmunity. *Nat Immunol.* (2007) 8:345. doi: 10.1038/ni0407-345

82. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, et al. Focused specificity of intestinal T H 17 cells towards commensal bacterial antigens. *Nature.* (2014) 510:152. doi: 10.1038/nature13279
83. Wang Y, Xiang C, Chen H, Yin Y, Chen X, Zhao Y, et al. Induction of intestinal Th17 cells by flagellins from segmented filamentous bacteria. *Front Immunol.* (2019) 10:2750. doi: 10.3389/fimmu.2019.02750
84. Ladinsky MS, Araujo LP, Zhang X, Veltri J, Galan-Diez M, Soualhi S, et al. Endocytosis of commensal antigens by intestinal epithelial cells regulates mucosal T cell homeostasis. *Science.* (2019) 363:eaat4042. doi: 10.1126/science.aat4042
85. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity.* (2011) 34:794–806. doi: 10.1016/j.immuni.2011.03.021
86. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity.* (2010) 32:815–27. doi: 10.1016/j.immuni.2010.06.001
87. Stepankova R, Powrie F, Kofronova O, Kozakova H, Hudcovic T, Hrncir T, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. *Inflamm Bowel Dis.* (2007) 13:1202–11. doi: 10.1002/ibd.20221
88. Shukla R, Ghoshal U, Dhole TN, Ghoshal UC. Fecal microbiota in patients with irritable bowel syndrome compared with healthy controls using real-time polymerase chain reaction: an evidence of dysbiosis. *Digestive Dis Sci.* (2015) 60:2953–62. doi: 10.1007/s10620-015-3607-y
89. Kriegel MA, Sefik E, Hill JA, Wu H-J, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci.* (2011) 108:11548–53. doi: 10.1073/pnas.1108924108
90. Shi Z, Zou J, Zhang Z, Zhao X, Noriega J, Zhang B, et al. Segmented filamentous bacteria prevent and cure rotavirus infection. *Cell.* (2019) 179:644–58. doi: 10.1016/j.cell.2019.09.028
91. Uhlig HH, Powrie F. Dendritic cells and the intestinal bacterial flora: a role for localized mucosal immune responses. *J Clin Investig.* (2003) 112:648. doi: 10.1172/JCI19545
92. Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, et al. Gut microbiota drive autoimmune arthritis by promoting differentiation and migration of Peyer's patch T follicular helper cells. *Immunity.* (2016) 44:875–88. doi: 10.1016/j.immuni.2016.03.013
93. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA.* (2011) 108:4615–22. doi: 10.1073/pnas.1000082107
94. Irikura V, Kihara M, Yamaguchi S, Sockett H, Macnab R. Salmonella typhimurium fliG and fliN mutations causing defects in assembly, rotation, and switching of the flagellar motor. *J Bacteriol.* (1993) 175:802–10. doi: 10.1128/jb.175.3.802-810.1993
95. Brown PN, Mathews MA, Joss LA, Hill CP, Blair DF. Crystal structure of the flagellar rotor protein FliN from Thermotoga maritima. *J Bacteriol.* (2005) 187:2890–902. doi: 10.1128/JB.187.8.2890-2902.2005
96. Corthésy B, Benureau Y, Perrier C, Fourgeux C, Parez N, Greenberg H, et al. Rotavirus anti-VP6 secretory immunoglobulin a contributes to protection via intracellular neutralization but not via immune exclusion. *J Virol.* (2006) 80:10692–9. doi: 10.1128/JVI.00927-06
97. Parrett A, Edwards C. *In vitro* fermentation of carbohydrate by breast fed and formula fed infants. *Arch Dis Childhood.* (1997) 76:249–53. doi: 10.1136/adc.76.3.249
98. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science.* (2006) 312:1355–9. doi: 10.1126/science.1124234
99. Greiner T, Bäckhed F. Effects of the gut microbiota on obesity and glucose homeostasis. *Trends Endocrinol Metab.* (2011) 22:117–23. doi: 10.1016/j.tem.2011.01.002
100. Nauta AJ, Ben Amor K, Knol J, Garssen J, Van der Beek E. Relevance of pre-and postnatal nutrition to development and interplay between the microbiota and metabolic and immune systems. *Am J Clin Nutr.* (2013) 98:586S−93. doi: 10.3945/ajcn.112.039644
101. Wang X, Andersson R. Intestinal brush border membrane function. *Scand J Gastroenterol.* (1994) 29:289–99. doi: 10.3109/00365529409094838
102. Jonsson H, Hugerth LW, Sundh J, Lundin E, Andersson AF. Genome sequence of segmented filamentous bacteria present in the human intestine. *Commun Biol.* (2020) 3:1–9. doi: 10.1038/s42003-020-01214-7
103. Vijn I, Smeekens S. Fructan: more than a reserve carbohydrate? *Plant Physiol.* (1999) 120:351–60. doi: 10.1104/pp.120.2.351
104. Hoffman JD, Yanckello LM, Chlipala G, Hammond TC, McCulloch SD, Parikh I, et al. Dietary inulin alters the gut microbiome, enhances systemic metabolism and reduces neuroinflammation in an APOE4 mouse model. *PLoS ONE.* (2019) 14:e0221828. doi: 10.1371/journal.pone.0221828
105. Kleessen B, Hartmann L, Blaut M. Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora. *Br J Nutr.* (2001) 86:291–300. doi: 10.1079/BJN2001403
106. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WL. Inulin, a flexible oligosaccharide I: Review of its physicochemical characteristics. *Carbohyd Polym.* (2015) 130:405–19. doi: 10.1016/j.carbpol.2015.05.026
107. Oswari H, Widodo AD, Handayani F, Juffrie M, Sundjaya T, Bindels J, et al. Dosage-related prebiotic effects of inulin in formula-fed infants. *Pediatric Gastroenterol Hepatol Nutri.* (2019) 22:63–71. doi: 10.5223/pghn.2019.22.1.63
108. Gura T. Nature's first functional food. *Science.* (2014) 345:747–9. doi: 10.1126/science.345.6198.747
109. Tao N, DePeters E, German J, Grimm R, Lebrilla CB. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J Dairy Sci.* (2009) 92:2991–3001. doi: 10.3168/jds.2008-1642
110. Walker WA. Initial intestinal colonization in the human infant and immune homeostasis. *Ann Nutr Metab.* (2013) 63(Suppl. 2):8–15. doi: 10.1159/000354907
111. Bonnema AL, Kolberg LW, Thomas W, Slavin JL. Gastrointestinal tolerance of chicory inulin products. *J Am Dietetic Assoc.* (2010) 110:865–8. doi: 10.1016/j.jada.2010.03.025
112. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr.* (2005) 135:1304–7. doi: 10.1093/jn/135.5.1304
113. Chen K, Chen H, Faas MM, de Haan BJ, Li J, Xiao P, et al. Specific inulin-type fructan fibers protect against autoimmune diabetes by modulating gut immunity, barrier function, microbiota homeostasis. *Mol Nutr Food Res.* (2017) 61:1601006. doi: 10.1002/mnfr.201601006
114. Agostoni C, Axelsson I, Goulet O, Koletzko B, Michaelsen KF, Puntis JW, et al. Prebiotic oligosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr.* (2004) 39:465–73. doi: 10.1097/00005176-200411000-00003
115. Vonderviszt F, Namba K. Structure, function and assembly of flagellar axial proteins. *Fibrous Proteins.* (2008) 2008:58–76.
116. Ronda C, Chen SP, Cabral V, Yaung SJ, Wang HH. Metagenomic engineering of the mammalian gut microbiome *in situ*. *Nat Methods.* (2019) 16:167. doi: 10.1038/s41592-018-0301-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

ACCESS MICROBIOLOGY

RESEARCH ARTICLE

Oemcke *et al.*, *Access Microbiology*

DOI 10.1099/acmi.0.000218

MICROBIOLOGY SOCIETY

OPEN ACCESS

Whole tissue homogenization preferable to mucosal scraping in determining the temporal profile of segmented filamentous bacteria in the ileum of weanling rats

Linda A. Oemcke[1,2,3,*], Rachel C. Anderson[1,3], Jasna Rakonjac[4], Warren C. McNabb[1,5] and Nicole C. Roy[1,5,6,7]

Abstract

Segmented filamentous bacteria (SFB) are thought to play a role in small intestine immunological maturation. Studies in weanling mice have shown a positive correlation between ileal SFB abundance and plasma and faecal interleukin 17 (IL-17) and immunoglobulin A (IgA) concentrations. Although the first observation of SFB presence was reported in rats, most studies use mice. The size of the mouse ileum is a limitation whereas the rat could be a suitable alternative for sufficient samples. Changes in SFB abundance over time in rats were hypothesized to follow the pattern reported in mice and infants. We characterized the profile of SFB colonization in the ileum tissue and contents and its correlation with two immune markers of gastrointestinal tract (GIT) maturation. We also compared two published ileum collection techniques to determine which yields data on SFB abundance with least variability. Whole ileal tissue and ileal mucosal scrapings were collected from 20- to 32-day-old Sprague-Dawley rats. SFB abundance was quantified from proximal, middle and distal ileal tissues, contents and faeces by quantitative PCR using SFB-specific primers. Antibody-specific ELISAs were used to determine IL-17 and IgA concentrations. Significant differences in SFB abundance were observed from whole and scraped tissues peaking at day 22. Variability in whole ileum data was less, favouring it as a better collection technique. A similar pattern of SFB abundance was observed in ileum contents and faeces peaking at day 24, suggesting faeces can be a proxy for ileal SFB abundance. SFB abundance at day 26 was higher in females than males across all samples. There were significant differences in IgA concentration between days 20, 30 and 32 and none in IL-17 concentration, which was different from reports in mice and infants.

INTRODUCTION

The identification of segmented filamentous bacteria (SFB) naturally occurring in invertebrates [1] and vertebrates [2–5] over 150 years ago [1] created an interest in these microbes. They are Gram-positive, spore-forming, facultative anaerobic commensals, which attach selectively to the mucosa of the terminal ileum [6–8] overlying Peyer's patches, where naïve T cells undergo antigen-driven activation and expansion to yield T helper cells [9, 10]. The current literature reports that SFB mainly reside in the ileum [6, 11, 12] rather than the large intestine with the absence of Peyer's patches in the large intestine [13] possibly contributing to this. Additionally, the selective colonization of the ileum mucosa by SFB might be explained by their ability to survive the microaerophilic conditions present in the ileum [14, 15]. The investigation into their effects during postnatal development has given insight into a symbiotic relationship with the host, primarily due to their suggested link with markers of immune maturation of the small intestine [16–20].

Commensal and pathogenic bacteria are known to modulate the abundance and activity of many immune markers of the maturation of the GIT. Both beneficial and detrimental effects on the host have been reported [21–24], and this is also the case for SFB. For instance, in adulthood, the abundance of

Received 30 September 2020; Accepted 04 March 2021; Published 23 March 2021

Author affiliations: [1]Riddet Institute, Massey University, Palmerston North, New Zealand; [2]School of Food and Advanced Technology, Massey University, Palmerston North, New Zealand; [3]AgResearch, Grasslands Research Centre, Palmerston North, New Zealand; [4]School of Fundamental Sciences, Massey University, Palmerston North, New Zealand; [5]High-Value Nutrition National Science Challenge, Auckland, New Zealand; [6]Department of Human Nutrition, University of Otago, Dunedin, New Zealand; [7]Liggins Institute, University of Auckland, Auckland, New Zealand.

***Correspondence:** Linda A. Oemcke, Linda.Oemcke.1@uni.massey.ac.nz

Keywords: segmented filamentous bacteria; ileum; whole tissue homogenization; mucosal scraping; interleukin 17; immunoglobulin A.

Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; GIT, gastrointestinal tract; IgA, immunoglobulin A; IL-17, interleukin 17; OD, optical density; PND, post-natal day; qPCR, quantitative polymerase chain reaction; SEM, standard error mean; SFB, segmented filamentous bacteria.

Four supplementary figures are available with the online version of this article.

SFB in stool samples has been positively associated with autoimmune and inflammatory bowel disease [11, 25, 26]. Studies have reported SFB to induce the production of the pro-inflammatory cytokine interleukin 17 (IL-17) in plasma [16–19, 27], and the antibody immunoglobulin A (IgA) in ileal contents and faeces of 4–6-week-old mice [20, 28, 29]. They also reportedly cause a weak inflammatory response in adult mice and children between 6 months and 15 years of age [16–19, 27]. The dampened response might be due to the lack of clostridial virulence-related genes [30] or absence of gene-encoding sortase, the enzyme that mediates anchoring of most cell-wall proteins in some pathogenic Gram-positive bacteria [31], from the SFB genome. Further to that, benefits of SFB have been suggested, including type 1 diabetes protection in adult non-obese diabetic mice involving IL-17 [32] and prevention of rotavirus infection in adult mice though independent of immune cell involvement [33].

The SFB were initially observed by microscope in the ileum tissue of rats [34], though most published studies on SFB have been performed in mice [2, 17, 18, 20, 30, 35–37]. It is unclear whether SFB in rodents are detectable prenatally up to 2 weeks postnatally. SFB appear about 3 weeks postnatally in mice and rats [20], increase at weaning [38], peak then plateau about 4 weeks later [20, 27, 39], highlighting their transient colonization profile. SFB may, therefore, provide benefits primarily around weaning, likely preventing overstimulation of the immune system later in life.

There are also technical limitations in the quantification of SFB. *In vitro* studies have been challenging as these microbes thrive in environments of low to no oxygen concentrations while requiring an attachment to epithelial cells that need oxygen [14, 40]. Human infant studies rely on faecal samples as a proxy of ileal abundance, as there are ethical challenges associated with obtaining ileal samples. Mice have been the most frequently used animal model to study SFB pre- and post-weaning, though this is not without challenges; for example, obtaining enough ileal tissue and content samples for accurate SFB quantification and conducting multiple analyses to characterise its effects on the host.

Two methods for obtaining ileal samples for SFB quantification have been described in the published literature [2, 20, 41]. Ericsson *et al.* [2] attempted to create pure inocula of SFB by developing a cost-effective method of isolating SFB from complex microbes in the ileum. Using an aseptic technique, they rinsed the distal ileum of 2–3-day-old SFB-positive Balb/c mice. The ileal tissue was cut longitudinally exposing the mucosa, which was then scraped with a sterile scalpel blade. The scrapes contained epithelial cells, and mucosa-associated bacteria (including SFB) were then transferred into sterile media. Ohashi *et al.* [20] used a grinding method [41] to collect ileum tissue along with mucosa-associated bacteria. This method involves grinding whole tissue with a pestle and mortar on ice then transferring the ground up tissue into a buffer for DNA extraction.

Therefore, the study hypothesis was that changes in SFB abundance in ileal tissue and contents over time in rats would have a similar pattern to that reported in mice and infants. A weanling rat model was used to evaluate the profile of SFB colonization and some markers of immune maturation of the ileum pre- and post-weaning. Two techniques used in published studies for collecting ileum tissue samples for SFB quantification, ileal mucosal scraping [2] and whole tissue grinding or homogenization [41], were compared to determine whether one technique would yield better results with reduced variability. Following the use of these two techniques, the abundance of SFB was quantified by quantitative PCR (qPCR), and IL-17 and IgA concentrations by ELISA.

METHODS

Rat experiment

The study was approved by the AgResearch Limited Grasslands Animal Ethics Committee (Animal Ethics Application No: 14485) under the recommendations of the New Zealand Animal Welfare Act 1999.

Conventionally raised Sprague-Dawley rat pups (male and female) were used. Eight pregnant Sprague-Dawley dams were obtained from AgResearch Ruakura (Hamilton, NZ) and transported to AgResearch Grasslands (Palmerston North, NZ) at 15 days of gestation. They were maintained at 21 °C and provided with a commercial rodent diet (Meat Free Rat and Mouse Diet, Specialty Feeds, Australia) and water *ad libitum*. The dams were individually caged under dark and light cycles. Five litters were birthed on the first day, two litters on the second day and one litter on the third day. All dams and pups were maintained at 21 °C. The pups were weaned on day 21, and the dams were removed from the birth cages.

Ninety-two pups (57 females and 35 males) were evenly distributed across time-points. The pups were randomized into seven time-points: 20, 22, 24, 26, 28, 30 and 32 days after birth (n=13 for day 20, n=14 for others). They were identified by ear-punching at day 19 to meet the welfare norms for this procedure. The pups remained in their birth litters throughout the experiment to avoid interaction among animals from different litters which would influence the gut microbiota of the pups. All dams and pups were checked daily, weighed once a week, and fresh food and water topped up as required. Their General Health Score (GHS), which ranges from 5 (healthy) to 1 (requires euthanization) was checked daily, and the rats with a GHS of 3 or 4 were closely monitored. If their condition deteriorated, they were euthanized by intraperitoneal injection with pentobarbitol. All rats had a GHS of 5 throughout the study.

Sample collection

At each sampling time point, the pups were euthanized by asphyxiation with carbon dioxide in an individual cage followed by cervical dislocation. Blood was drawn by cardiac puncture into a needle coated with Ethylenediaminetetraacetic acid (EDTA) (Invitrogen; Thermo Fisher Scientific). The blood samples were centrifuged at 2000 ***g*** for 10 min at 4 °C, and the supernatant (plasma) was pipetted into

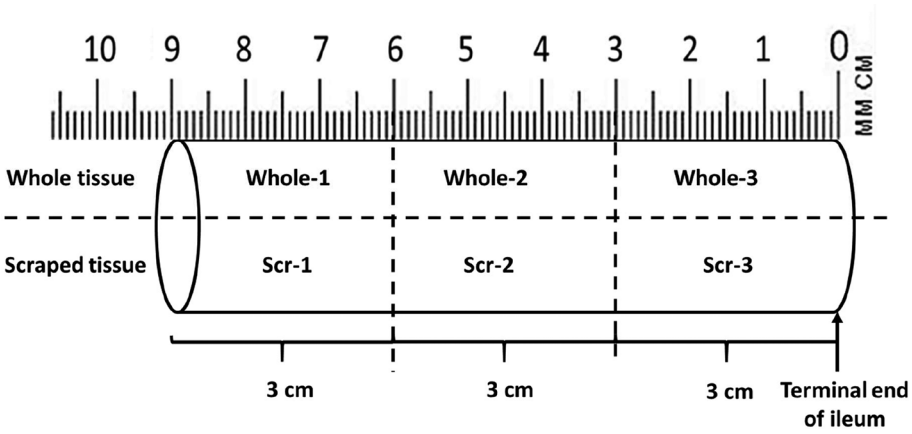

Fig. 1. Schematic of how the ileum tissue was cut into three sections of 3 cm each. The ileum sections were measured up from the caecum, which borders the terminal end of the ileum. The sections were cut into 3 cm each, cut open and down the middle longitudinally. The top sections were collected as whole tissue samples. The bottom sections were scraped and used. Both whole and scraped tissue were homogenized before genomic DNA extraction. 1-proximal section, 2-middle section, 3-terminal section.

cryotubes and snap-frozen in liquid nitrogen before storage at −80 °C for later analysis. Faecal samples were collected from the terminal colon or rectum post-mortem and snap-frozen in liquid nitrogen and stored at −80 °C for DNA extraction. The distal portion of the ileum adjacent to the caecum was cut into three sections of 3 cm each. The ileal contents from each section were collected separately. The ileal tissue sections were then cut open longitudinally and down the middle into two pieces. The top sections of the tissue were collected as whole tissue. The bottom sections were each scraped three times with a plastic tissue scraper in the same direction using a similar amount of force to decrease variability in the sample weights (Fig. 1). The scrapings and whole tissues were collected into cryotubes and snap-frozen in liquid nitrogen and stored at −80 °C for DNA extraction.

Sample preparation and genomic DNA extraction

Genomic DNA was extracted from the whole and scraped ileum tissue samples using the Qiagen AllPrep DNA/RNA/Protein Mini Kit according to the manufacturer's instructions. Before DNA extraction, both tissue samples were disrupted using a tissue homogeniser (Omni International TH, Georgia, USA). The homogenizer probe was cleaned with two different solutions of 70% ethanol, 100% ethanol and distilled water between each sample to prevent cross-contamination. DNA was extracted from ileal contents and faeces using the NucleoSpin Soil extraction kit (Macherey-Nagel) according to the manufacturer's instructions. These samples were vigorously agitated in a bead beater to release the DNA from the SFB intracellular contents and spores. The concentration and purity of DNA from ileum tissue, ileum contents and faeces were measured using a Nanodrop ND-1000 Spectrophotometer (Analytical Technologies, Thermo Fisher Scientific, MA, USA). A ratio of 1.8 was considered acceptable for pure DNA. The DNA samples were then ready for quantification by qPCR.

QuantitativePCR to quantify SFB

An optimized protocol based on the one from the Franklin Laboratory in St. Louis, Missouri, USA (personal communication) was utilized for the qPCR analysis. A standard curve was generated using a recombinant plasmid DNA containing the SFB 16S rRNA gene fragment (Integrated DNA technologies). The master mix consisted of 5 µl of KAPA SYBR Green I, 0.2 µl of Rox low reference dye (Sigma-Aldrich), 0.2 µl each of forward and reverse primers (Integrated DNA Technologies), and 3.4 µl of nuclease-free water (Invitrogen). To this mix, 1 µl of the recombinant plasmid DNA (positive control), 1 µl of nuclease-free water (negative control) or 1 µl of sample DNA (ileum tissue, contents, or faecal) were added for a total of 10 µl in each reaction well on a 96-well plate. The technical replicates used were four each. The SFB primers were forward primer SFB 779F 5′- TGT GGG TTG TGA ATA ACA AT −3′, reverse primer SFB 1008R 5′- GCG AGC TTC CCT CAT TAC AAG G −3′ [42] (Integrated DNA technologies). The conditions in the Quantstudio 3D Digital PCR thermal cycler (Applied Biosystems; Thermo Fisher Scientific, MA, USA) included a hold stage at 95 °C for 3 min, the PCR Stage; 40 cycles of denaturation at 95 °C for 3 min; annealing at 64 °C for 30 s; extension at 72 °C for 30 s. A melting-curve analysis using SYBR green was performed to determine the specificity of the PCR by slowly heating the mixtures from 55–95 °C for 1 s, 60 °C for 30 s and 95 °C for 1 s. Duplicate negative controls were run to assess the specificity and to rule out contamination. Data were analysed, and the relative quantification (fold) of SFB DNA was performed using the ΔΔCt method.

ELISA of immune markers

The concentrations of IL-17 in plasma and IgA in faeces from male and female rats were analysed using an antibody-specific Rat IL-17 ELISA Kit and Rat IgA ELISA Kit (Cusabio Biotech) according to the manufacturer's instructions. A FlexStation

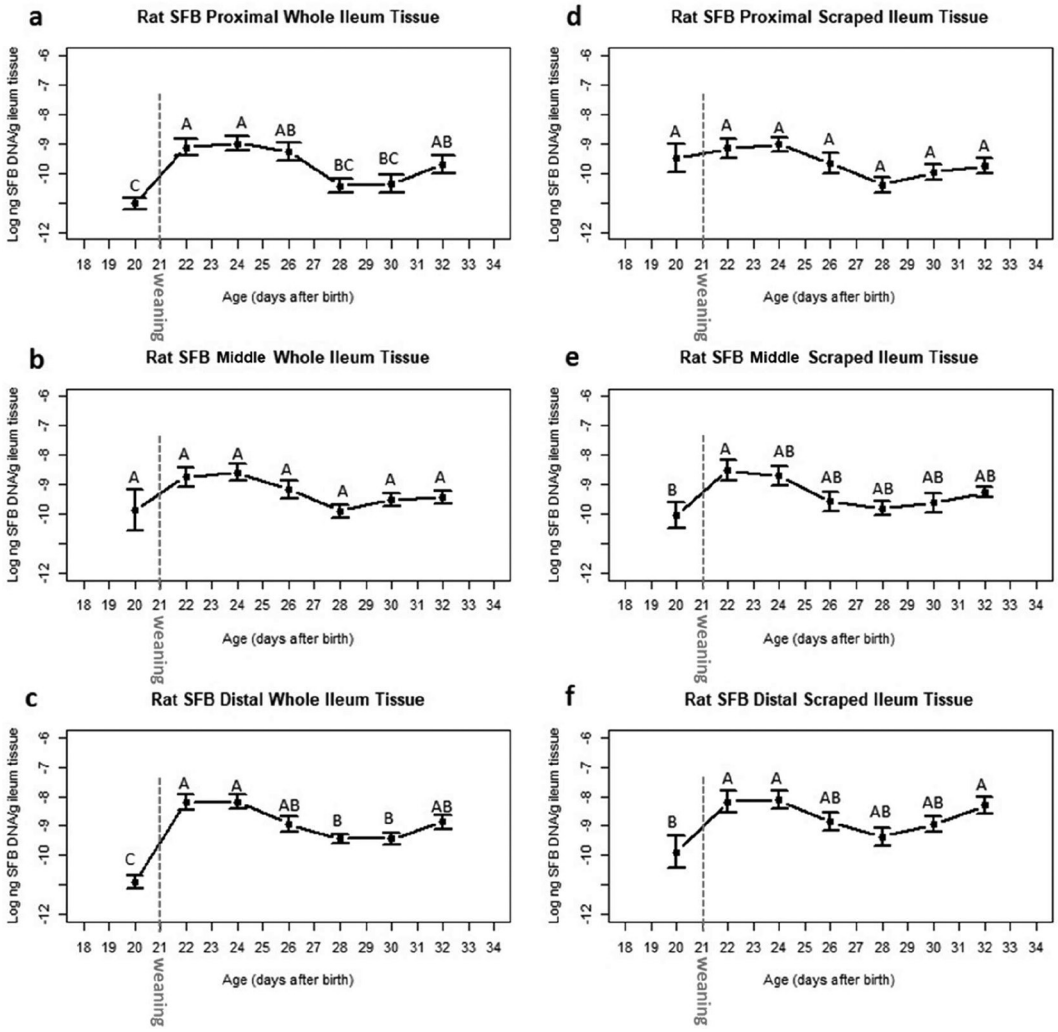

Fig. 2. Abundance of SFB in the whole ileal tissue (a–c) and ileal mucosal scrapes (d–f) of conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle and distal ileum. Data are plotted on a logarithm ten scale and are shown as the mean values of n=14 pups (20 days postnatally) and n=13 pups (22–32 days postnatally). The bars represent the SEM. Values without common letters differ significantly P <0.05.

3 Multi-Mode Microplate Reader (Molecular Devices, CA, USA) was used to determine the optical density (OD). The OD was measured at 450 nm, which was subtracted from 540 nm to determine the final OD readings. Standard curves, used to change the raw data OD readings to concentrations, were created for IL-17 and IgA using Genstat (18th Edition). Linear and polynomial regressions were generated by plotting the mean absorbance for each standard against the concentration of plasma IL-17 and faecal IgA, respectively. The limit of detection for plasma IL-17 and faecal IgA was 0.05 ng ml^{-1} and 0.88 ng ml^{-1}, respectively.

Statistical analysis

A one-way ANOVA was performed in Minitab 18 to determine if there were any significant differences in the abundance of SFB and concentration of IL-17 and IgA over time between days 20–32 pre- and post-weaning. A two-way ANOVA was also performed to determine whether the interaction between sex and age influenced SFB abundance over time. The Ryan-Joiner's (like Shapiro–Wilk) test and Levene's test were used to verify that the data were normally distributed, and that homogeneity of variance was met. The data were log-transformed to meet the requirements of normal distribution and homogeneity of variance. The one-way ANOVA was performed using the logarithm-transformed data. Where there was statistical significance, a post-hoc Tukey's test was performed to show where the differences lay. Differences were considered statistically significant when the probability value was inferior to 0.05. An analysis of covariance (ANCOVA) was performed to determine whether the sex of the rats influenced their weight gain with minimal effects from age. Weight gain was the response, sex was the explanatory factor, and age was the covariate (control variable).

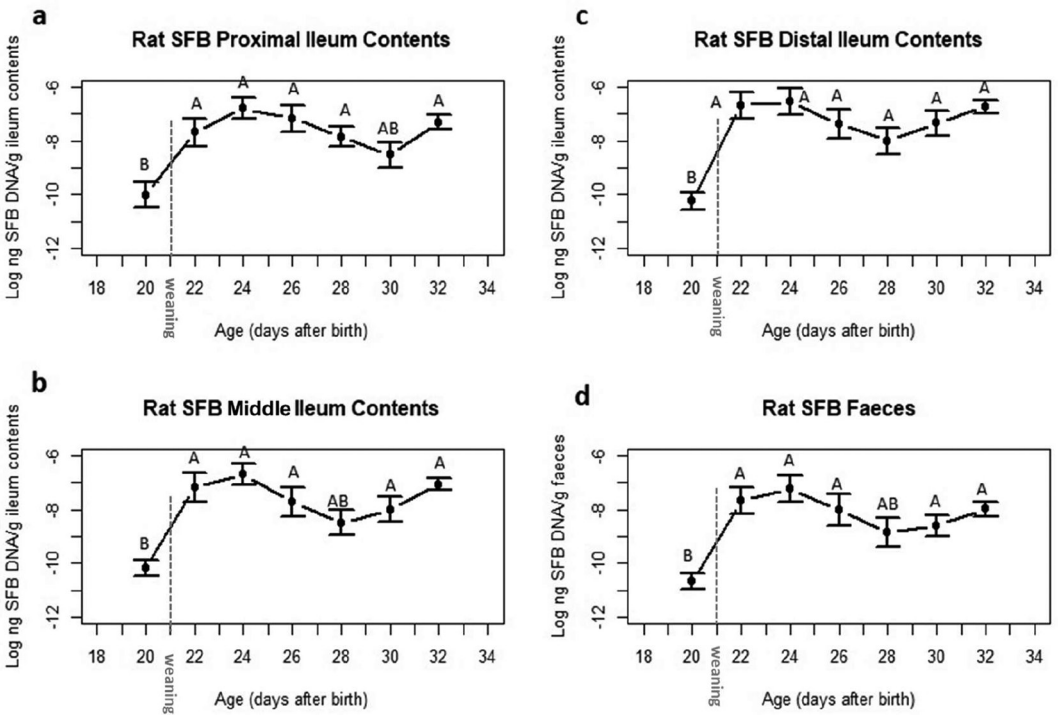

Fig. 3. The abundance of SFB in the ileal content (a–c) and faecal (d) samples collected from conventionally reared Sprague-Dawley rat pups. Samples were collected from the proximal, middle and distal ileum. Data are plotted on a logarithm ten scale and are shown as the mean values of *n*=14 pups (20 days postnatally) and *n*=13 pups (22–32 days postnatally). The bars represent the SEM. Values without common letters differ significantly *P* <0.05.

RESULTS

Quantification of SFB

The initial data of the abundance of SFB were skewed, therefore, to meet conditions of normality the data were transformed and plotted on a logarithm ten scale. All figures with untransformed data are included in the Supplementary Material, available in the online version of this article. The abundance of SFB increased from weaning, peaked at PND 22, and then plateaued (Figs 2a–f and S1a–f). Significant differences were observed in the temporal abundance of SFB in the proximal (Figs 2a and S1a) and distal (Figs 2c and S1c) sections of the whole ileum tissue from day 20, peaking at day 22 then decreasing from day 26, but not in the middle

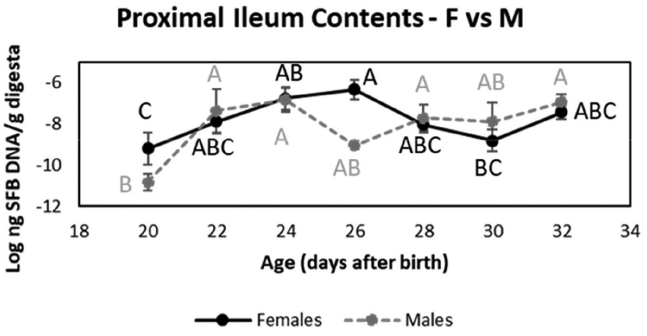

Fig. 4. Comparison of the abundance of SFB in the proximal ileal content samples between female and male Sprague-Dawley rat pups. The rat pups were weaned on day 21. Data are plotted on a logarithm ten scale and are shown as the mean values of females (*n*=57) versus males (*n*=35). The bars represent the SEM. Values without common letters differ significantly *P*<0.05.

Table 1. Mean±SEM wt gain in grams (g) of pre- and post-weaning male and female Sprague-Dawley rats. The values represent weight gained between the initial weigh-ins (at postnatal days 15, 17, 18) and before sample collection from pups at all time-points. Compared to females, males gained more weight, and male pups were the heaviest at terminal sample collection. An ANOVA and post-hoc Tukey's test showed there were significant differences in average weight gain in males among time-points 1–2, 4–6 and 7. There were significant differences in average weight gain in females at all time-points; *P* <0.05

Time-point	Males	Females
1	7.43±0.98^{d}	7.00 ± 0.61^{g}
2	16.92 ± 1.74^{cd}	15.76 ± 1.08^{f}
3	25.80 ± 2.61^{c}	26.74±1.60^{e}
4	44.48±2.41^{b}	39.44±1.08^{d}
5	56.20±3.56^{b}	50.85 ± 2.86^{c}
6	53.23±4.54^{b}	60.27±2.58^{b}
7	92.93±11.52^{a}	74.56±1.72^{a}

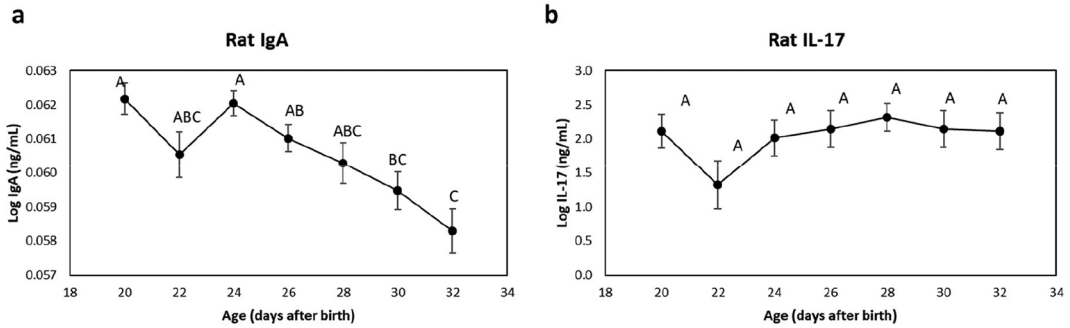

Fig. 5. Concentration of IgA (a) in faeces and IL-17 (b) in plasma of conventionally reared male and female Sprague-Dawley rat pups. The limit of detection for faecal IgA was 0.88 ng ml^{-1}, and plasma IL-17 was 0.05 ng ml^{-1}, respectively. Data are plotted on a *logarithm ten* scale and are shown as the mean values of *n*=13 pups (22–32 days) and *n*=14 pups (20 days). The bars represent the SEM. Values without common letters differ significantly P <0.05.

(Figs 2b and S1b) section. For the scraped ileum tissue, significant differences were observed in the temporal abundance of SFB in the middle (Figs 2e and S1e) and distal (Figs 2f and S1f) sections from day 20, peaking at day 22 then decreasing from day 26, but not in the proximal (Figs 2d and S1d) section. In all cases, SFB abundance peaked at day 22 and then plateaued. There was less variability in SFB abundance in the proximal, middle and distal whole ileum tissue (Fig. 2a–c; average SEM*: 2.71E-01;* Fig. S1a–c; average SEM: *1.84E-07*) compared to that from the proximal, middle, and distal ileal scrapes (Fig. 2d–f; average SEM: *3.18E-01;* Fig. S1d–f; average SEM: *6.10E-07*). There were significant differences in the abundance of SFB in the proximal (Figs 3a and S2a) and middle (Figs 3b and S2b) section of ileal contents over time between postnatal days 20 and postnatal days 22, 24, 26, 28 and 32. There were no significant differences in SFB abundance in ileal contents from the distal (Figs 3c and S2c) section between day 22 and 32.

There were significant differences in SFB abundance over time in the faeces (Figs 3d and S2d) increasing from day 20, peaking at day 24, then decreasing from day 28. The faecal SFB abundance peaked later at day 24 compared to the ileum tissue at day 22 though there were no significant differences.

There were significant differences in the interaction between sex and age on SFB abundance in the proximal ileum contents only. There was an unexpected difference in SFB abundance at day 26 between male and female pups. SFB abundance in males sharply decreased while in females, there was a gradual decrease until the plateau was reached at day 28 (Figs 4 and S3). The male pups also gained weight quicker than females (Table 1). There were significant differences in weight gain between males and females when age was used as a covariate.

Analysis of IgA and IL-17

The IgA and IL-17 data were transformed and plotted on a logarithm ten scale to meet conditions of normality. All figures with untransformed data are included in the Supplementary Material. There were significant differences in the concentration of IgA in faeces (Figs 5a and S4a), which decreased postnatally from day 24 to 32. Tukey's post-hoc test showed that there were differences in the faecal IgA concentration between days 20, 24 and days 30, 32. There were no significant changes in the concentration of IL-17 in plasma over time (Figs 5b and S4b).

DISCUSSION

This study shows that the abundance of SFB in ileum tissue and content of rats measured pre- and post-weaning was similar to the temporal profiles published in mice and infants of corresponding age [20, 39]. Here, the abundance of SFB increased post-weaning, peaked at days 22 and 24 and decreased until a plateau was reached at day 26 until the last measurement on day 32. Other studies suggest that the abundance of SFB remains relatively constant after weaning throughout life [27, 39].

The results also showed that of the two tissue collection techniques, whole tissue homogenization gave less variable tissue weights compared with mucosal scraping. SFB abundance data was similar to what has been reported [20]. Interestingly, the SFB data from homogenization was less variable compared to the scraped data. High variability in the scraped data was likely partly due to scraped tissue sticking to the collection tools, thus decreasing the amount of tissue available. The fragility of the ileum at that age also made scraping of the tissue challenging, resulting in variable weights. Whole tissue homogenisation was, therefore, a simpler method of collecting ileum tissue samples from rats for analysis pre- and post-weaning.

The pattern of SFB abundance in the ileum tissue and contents overtime was also reflected in the faeces, suggesting faeces could be used as a proxy for SFB abundance in the ileum. The faecal SFB abundance peaked later at day 24 compared to the ileum tissue at day 22; abundance in the ileum would, therefore, be assumed to peak earlier. This non-invasive approach of using faeces aligns with the three Rs of animal research that include replacement, reduction and refinement, which utilize alternatives to terminal animal sampling, reduce the

number of animals used and follow procedures that minimize pain and stress [43]. This alternative to terminal sampling to obtain ileal tissue content could be applied in time-series and intervention studies attempting to understand influences on SFB abundance caused by diet, disease, medication or ageing [11].

The significant difference in the interaction of sex with age indicates that SFB abundance in the proximal ileum contents over time is different in males and females. It suggests that this interaction may be present in the middle and distal ileum contents though a study with more statistical power would be required to make this conclusion. The abundance of SFB was unexpectedly different between females and males on day 26 only. This result may be a random difference in the rats sampled on that day. The observation also suggests that sex-based hormonal differences might influence SFB abundance. It may result from physiological differences in GIT development between sexes [44] as the male pups gained weight quicker. Before puberty, oestradiol concentrations peak at day 15 in both sexes and remain low until day 39, while testosterone concentrations in both sexes remain low between days 1 and 19 then decrease between days 20 and 30 [45, 46]. Rats reach puberty between days 30 and 42 in females and days 42 and 55 in males [47]. Sex hormones were not measured here, so it is unclear whether a change in their concentrations leading up to puberty might have influenced SFB abundance in the samples analysed. The abundance of SFB between sexes was not reported in a similar study, which involved six male and nine female mouse pups [20]. Thus far, our study is the only one that has reported this finding, which highlights the importance of considering gut microbiota changes between sexes.

In this study, there was no difference in the plasma concentration of IL-17 from days 22 to 32. However, our previous (unpublished) results in mice showed differences in plasma concentration of IL-17 between days 18 and 41; the concentration peaked at day 32. These results also contrast with literature reports of a positive correlation between ileal SFB abundance and plasma IL-17 production in adult mice 2 to 3 weeks following SFB colonization [4, 16, 17]. Several plasma samples across all time-points measured here had IL-17 concentrations lower than the calculated detection threshold, increasing the variability in the data. At day 22, most of the rats had zero-readings, and no measurements were done after day 32.

The faecal concentration of IgA was variable from days 20 to 28 then decreased until day 32. This result is in disagreement with other studies where faecal IgA concentrations reportedly increased only late post-weaning [20, 48]. Other studies showed that oral inoculation of SFB in older conventional and germ-free mice initially stimulates the production of IgA [48], with IgA later inhibiting SFB colonization [20, 49]. It is plausible that breastmilk IgA may have contributed to the high IgA concentrations found in the faecal samples at day 20, as observed in caecal contents of pre-weaned mice [20]. Other microbes may be involved, as they change in abundance around weaning, and are also known to stimulate production and secretion of IgA by immune cells [21, 23, 29]. The numerical drop of faecal IgA concentration at day 22 may have resulted from weaning on day 21, then the increase at day 24 may have resulted from increased luminal IgA. A broader age range, with a later developmental stage, may give a clearer picture of how IgA concentration changes [20] as the duration of this study was short.

In conclusion, the results showed that the temporal profile of SFB colonization in the ileum tissue and content of weanling rats was similar to those published for mice and infants of corresponding age. Lower variability in whole ileal tissue data favours it as a preferred tissue collection technique. The data also show that faeces can also be used as a proxy for SFB abundance in the ileum. The immune markers did not give similar results to those reported in mice and infants making it difficult to conclude on the influence of SFB on IL-17 and IgA. This indicates the importance of investigations on the temporal profile of SFB and their effects on IL-17 and IgA, particularly at weaning. The results may determine whether the studies are reproducible and contribute to current knowledge of SFB. The study model and results may also help guide the design of further studies investigating the perceived role of SFB on small intestine immunological barrier maturation.

Funding information
Linda A. Oemcke was supported by a PhD Fellowship from the Riddet Institute Centre of Research Excellence, funded by the Tertiary Education Commission. The research was funded by the same grant.

Acknowledgements
Appreciation is given to AgResearch Grasslands, and Ruakura staff for providing the required facilities and materials for the work carried out. Thanks to the staff who provided technical assistance with the animal study; Charlotte Hurst, Hilary Dewhurst and Melanie Van Gendt and the AgResearch animal welfare officer; Susan Doohan. Thanks to Peter Green for assistance with statistics.

Author contributions
The experimental design was devised by R.C.A. and L.A.O. Animal work was undertaken by L.A.O. with assistance from C.H., H.D. and M.V.G., Laboratory work was undertaken by L.A.O. Statistical analyses were conducted by L.A.O. with assistance from P.G. All authors contributed to the preparation of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was approved by the AgResearch Limited Grasslands Animal Ethics Committee (Animal Ethics Application No: 14485) under the recommendations of the New Zealand Animal Welfare Act 1999.

References

1. Leidy J. On the existence of entophyta in healthy animals, as a natural condition. *Proc Acad Nat Sci Philadelphia* 1849;4:225–233.
2. Ericsson AC, Turner G, Montoya L, Wolfe A, Meeker S *et al*. Isolation of segmented filamentous bacteria from complex gut microbiota. *Biotechniques* 2015;59:94–98.
3. Klaasen H, Van der Heijden PJ, Stok W, Poelma FG, Koopman JP *et al*. Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. *Infect Immun* 1993;61:303–306.
4. Schnupf P, Gaboriau-Routhiau V, Cerf-Bensussan N. Host interactions with segmented filamentous bacteria: an unusual trade-off

that drives the post-natal maturation of the gut immune system. *Semin Immunol* 2013;25:342–351.

5. Schnupf P, Gaboriau-Routhiau V, Sansonetti PJ, Cerf-Bensussan N. Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world. *Curr Opin Microbiol* 2017;35:100–109.
6. Ericsson AC, Hagan CE, Davis DJ, Franklin CL. Segmented filamentous bacteria: commensal microbes with potential effects on research. *Comp Med* 2014;64:90–98.
7. Liao N, Yin Y, Sun G, Xiang C, Liu D *et al*. Colonization and distribution of segmented filamentous bacteria (SFB) in chicken gastrointestinal tract and their relationship with host immunity. *FEMS Microbiol Ecol* 2012;81:395–406.
8. Bohórquez DV, Bohórquez NE, Ferket PR. Ultrastructural development of the small intestinal mucosa in the embryo and turkey poult: a light and electron microscopy study. *Poult Sci* 2011;90:842–855.
9. Agace WW. T-cell recruitment to the intestinal mucosa. *Trends Immunol* 2008;29:514–522.
10. Klaasen H, Koopman JP, Poelma FGJ, Intestinal BAC. Segmented, filamentous bacteria. *FEMS Microbiology Letters* 1992;88:165–179.
11. Finotti A, Gasparello J, Lampronti I, Cosenza LC, Maconi G *et al*. PCR detection of segmented filamentous bacteria in the terminal ileum of patients with ulcerative colitis. *BMJ Open Gastroenterol* 2017;4:e000172.
12. Caselli M, Holton J, Boldrini P, Vaira D, Calò G. Morphology of segmented filamentous bacteria and their patterns of contact with the follicle-associated epithelium of the mouse terminal ileum: implications for the relationship with the immune system. *Gut Microbes* 2010;1:367–372.
13. Jinturkar KA, Rathi MN, Misra A. Gene delivery using physical methods. *Challenges in Delivery of Therapeutic Genomics and Proteomics*. Elsevier; 2011. pp. 83–126.
14. Schnupf P, Gaboriau-Routhiau V, Gros M, Friedman R, Moya-Nilges M. Growth and host interaction of mouse segmented filamentous bacteria in vitro. *Nature* 2015;520:99–103.
15. Friedman ES, Bittinger K, Esipova TV, Hou L, Chau L *et al*. Microbes vs. chemistry in the origin of the anaerobic gut lumen. *Proc Natl Acad Sci U S A* 2018;115:4170–4175.
16. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T *et al*. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009;139:485–498.
17. Goto Y, Panea C, Nakato G, Cebula A, Lee C *et al*. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity* 2014;40:594–607.
18. Lécuyer E, Rakotobe S, Lengliné-Garnier H, Lebreton C, Picard M *et al*. Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses. *Immunity* 2014;40:608–620.
19. Yi J, Jung J, Han D, Surh CD, Lee YJ. Segmented filamentous bacteria induce divergent populations of antigen-specific CD4 T cells in the small intestine. *Mol Cells* 2019;42:228.
20. Ohashi Y, Hiraguchi M, Sunaba C, Tanaka C, Fujisawa T *et al*. Colonization of segmented filamentous bacteria and its interaction with the luminal IgA level in conventional mice. *Anaerobe* 2010;16:543–546.
21. Wilmore JR, Gaudette BT, Gomez Atria D, Hashemi T, Jones DD *et al*. Commensal microbes induce serum IgA responses that protect against polymicrobial sepsis. *Cell Host Microbe* 2018;23:302–311.
22. Bevilacqua L, Ovidi M, Di Mattia E, Trovatelli LD, Canganella F. Screening of Bifidobacterium strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens. *Microbiol Res* 2003;158:179–185.
23. Cheikhyoussef A, Pogori N, Chen H, Tian F, Chen W *et al*. Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances (BLIS) produced by Bifidobacterium infantis BCRC 14602. *Food Control* 2009;20:553–559.
24. Collado MC, Gonzalez A, Gonzalez R, Hernandez M, Ferrus MA *et al*. Antimicrobial peptides are among the antagonistic metabolites produced by Bifidobacterium against *Helicobacter pylori*. *Int J Antimicrob Agents* 2005;25:385–391.
25. Wu H-J, Ivanov II, Darce J, Hattori K, Shima T *et al*. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 2010;32:815–827.
26. Calasan J, Waldschmitt N, Ahmed MA, Metwaly A, Sarnaghi SK *et al*. Tu1858–segmented filamentous bacteria induce alternative Th17 differentiation and ileo-colonic crohn's disease-like inflammation. *Gastroenterology* 2019;156:S-1149.
27. Chen B, Liu W, Bie S, Peng K, Shu X *et al*. Adherent bacteria in the terminal ileum of children correlates with increased Th17 cell activation and luminal secretory IgA. *SSRN Journal* 2019;3397178.
28. Snel J, Bakker MH, Heidt PJ. Quantification of antigen-specific immunoglobulin A after oral booster immunization with ovalbumin in mice mono-associated with segmented filamentous bacteria or *Clostridium innocuum*. *Immunol Lett* 1997;58:25–28.
29. Talham GL, Jiang HQ, Bos NA, Cebra JJ. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun* 1999;67:1992–2000.
30. Prakash T, Oshima K, Morita H, Fukuda S, Imaoka A *et al*. Complete genome sequences of rat and mouse segmented filamentous bacteria, a potent inducer of Th17 cell differentiation. *Cell Host Microbe* 2011;10:273–284.
31. Jonsson H, Hugerth LW, Sundh J, Lundin E, Andersson AF. Genome sequence of segmented filamentous bacteria present in the human intestine. *Communications Biology* 2020;3:1–9.
32. Kriegel MA, Sefik E, Hill JA, Wu H-J, Benoist C *et al*. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 2011;108:11548–11553.
33. Shi Z, Zou J, Zhang Z, Zhao X, Noriega J *et al*. Segmented filamentous bacteria prevent and cure rotavirus infection. *Cell* 2019;179:644–658.
34. Savage DC. Localization of certain Indigenous microorganisms on the ileal villi of rats. *J Bacteriol* 1969;97:1505–1506.
35. Ivanov II, Frutos RdeL, Manel N, Yoshinaga K, Rifkin DB *et al*. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 2008;4:337–349.
36. Chen H, Yin Y, Wang Y, Wang X, Xiang C. Host specificity of segmented filamentous bacteria (SFB) flagellins affects their interaction patterns with mouse ileal mucosal proteins. *Appl Environ Microbiol* 2017.
37. Klaasen HLBM, Koopman JP, Van Den Brink ME, Scholten PM, Beynen AC. Influence of macronutrients on segmented filamentous bacteria in the small intestine of mice. *Microb Ecol Health Dis* 1991;4:47–51.
38. Thompson AL, Monteagudo-Mera A, Cadenas MB, Lampl ML, Azcarate-Peril MA. Milk- and solid-feeding practices and daycare attendance are associated with differences in bacterial diversity, predominant communities, and metabolic and immune function of the infant gut microbiome. *Front Cell Infect Microbiol* 2015;5:3.
39. Yin Y, Wang Y, Zhu L, Liu W, Liao N *et al*. Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens. *Isme J* 2013;7:615–621.
40. Eberl G, Bikard D, Schnupf P, Cerf Bensussan N, Gaboriau-Routhiau V *et al*. Method of culturing segmented filamentous bacteria *in vitro*. Patent No. EP15830866. Munich, Germany: European Patent Office; 2017.
41. Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol* 1997;63:2802–2813.
42. Snel J, Heinen PP, Blok HJ, Carman RJ, Duncan AJ *et al*. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of *"Candidatus Arthromitus"*. *Int J Syst Bacteriol* 1995;45:780–782.

43. Fenwick N, Griffin G, Gauthier C. The welfare of animals used in science: how the "Three Rs" ethic guides improvements. *Can Vet J* 2009;50:523.
44. Afonso-Pereira F, Dou L, Trenfield SJ, Madla CM, Murdan S *et al*. Sex differences in the gastrointestinal tract of rats and the implications for oral drug delivery. *Eur J Pharm Sci* 2018;115:339–344.
45. Walker DM, Kirson D, Perez LF, Gore AC. Molecular profiling of postnatal development of the hypothalamus in female and male Rats1. *Biol Reprod* 2012;87:121–112.
46. Döhler KD, Wuttke W. Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. *Endocrinology* 1975;97:898–907.
47. Ojeda SR, Andrews WW, Advis JP, White SS. Recent advances in the endocrinology of puberty. *Endocr Rev* 1980;1:228–257.
48. Umesaki Y, Okada Y, Matsumoto S, Imaoka A, Setoyama H. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol* 1995;39:555–562.
49. Jiang HQ, Bos NA, Cebra JJ. Timing, localization, and persistence of colonization by segmented filamentous bacteria in the neonatal mouse gut depend on immune status of mothers and pups. *Infect Immun* 2001;69:3611–3617.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Linda Oemcke
Name/title of Primary Supervisor:	Warren McNabb
In which chapter is the manuscript /published work:	Chapter 2

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Oemcke LA, Anderson RC, Altermann E, Roy NC and McNabb WC (2021) The Role of Segmented Filamentous Bacteria in Immune Barrier Maturation of the Small Intestine at Weaning. Frontiers in Nutrition: p. 950. (doi: 10.3389/fnut.2021.759137)

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate:

- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Linda Oemcke Digitally signed by Linda Oemcke Date: 2021.12.02 16:51:30 +13'00'
Date:	02-Dec-2021
Primary Supervisor's Signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2021.12.03 09:10:25 +13'00'
Date:	3-Dec-2021

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Linda Oemcke
Name/title of Primary Supervisor:	Warren McNabb
In which chapter is the manuscript /published work:	Chapter 4

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Oemcke, L. A., Anderson, R. C., Rakonjac, J., McNabb, W. C., & Roy, N. C. Whole tissue homogenization preferable to mucosal scraping in determining the temporal profile of segmented filamentous bacteria in the ileum of weanling rats. Access Microbiology, p. 000218 (2021).

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate:

- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Linda Oemcke Digitally signed by Linda Oemcke Date: 2021.07.30 08:27:17 +12'00'
Date:	30-Jul-2021
Primary Supervisor's Signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2021.08.02 14:34:12 +12'00'
Date:	2-Aug-2021

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.