

***FAECALIBACTERIUM PRAUSNITZII*: ISOLATION, CHARACTERIZATION
AND EFFECTS ON DAIRY CALVES**

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FAECALIBACTERIUM PRAUSNITZII: ISOLATION, CHARACTERIZATION
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ABSTRACT

Replacement heifers are extremely important for the success of a dairy farm. Strategies to improve performance while maintaining welfare, health and expenses at optimal levels are the focus of many research groups. Results from a previous study showed that young calves with a higher relative abundance of *Faecalibacterium prausnitzii* had a better weight gain and decreased incidence of diarrhea during the preweaning period. Our hypothesis was that the administration of *F. prausnitzii* could improve gastrointestinal health and performance of preweaned heifers. The first objective of this thesis was to advance our knowledge on the anaerobic bacterium *F. prausnitzii* and its *in vitro* characteristics. The second objective was to test its effects *in vivo*, administering it to newborn calves.

In order to achieve our first objective, 203 isolates of *F. prausnitzii* were isolated from the feces of calves and piglets. Forty genetically distinct *F. prausnitzii* isolates were selected for further characterization. A large variability was observed among isolates for *in vitro* short chain fatty acid metabolism, growth, antibiotic resistance, and sensitivity to low pH and bile salts. Based on this data, 4 isolates with desirable characteristics were selected and used as part of a probiotic cocktail in the subsequent *in vivo* studies.

To accomplish our second objective two trials were completed. First a safety trial was conducted using 30 newborn bull calves. Since no adverse effects of the oral and rectal administration of *F. prausnitzii* to neonatal calves were observed, a large field trial was performed in a commercial farm. This last study demonstrated that the oral administration of *F. prausnitzii* reduced the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers.

The results presented in this thesis contribute to the knowledge about *F. prausnitzii* characteristics. Moreover, our studies demonstrated that *F. prausnitzii* administration to newborn calves is safe and proved the concept that this commensal bacterium is a promising probiotic for newborn calves. Further research is needed to evaluate *in vivo* mechanisms of action and interactions between this microbe, the gut microbiota and the host.

BIOGRAPHICAL SKETCH

Carla Foditsch, middle daughter of Klaus and Cibele, was born in Sao Paulo, Brazil, in 1986. Despite living in a big city, she always liked the country lifestyle. Her family has a ranch with a few cows and horses in a small town called Cunha, where she spent holidays and vacations. Cunha is beautiful, but it is located in a humble and remote place that limits dairy production.

Carla completed her veterinary degree at University of Sao Paulo in 2009. In 2010, she went to USA for an internship at the Rural Animal Health and Management department of the University of Illinois. It was a great challenge and she learned a lot from that experience. In 2011, her soon-to-be husband started his residency at the Cornell University in Ithaca, NY, where Carla moved right after she finished the internship in September 2011.

At Cornell University she met Dr. Rodrigo Bicalho, coincidentally Brazilian as well, and worked as a research assistant in his group until 2013, when she started her Masters of Science in the Department of Animal Science. She enjoyed working with dairy cows and calves at the farm and also working in the laboratory. She hopes that one day her research will help improve calf health and performance. Now she is looking forward to have some time with her loved ones in Brazil before starting to work again here in Ithaca.

This thesis is dedicated to my husband, Daniel, for all his support,
encouragement, patience and love.

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“None of us got to where we are alone. Whether the assistance we received was obvious or subtle, acknowledging someone's help is a big part of understanding the importance of saying thank you.”

Harvey Mackay

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TABLE OF CONTENTS

BIOGRAPHICAL SKETCH.....	III
ACKNOWLEDGMENTS.....	V
TABLE OF CONTENTS	VI
LIST OF FIGURES.....	VIII
LIST OF TABLES	IX
CHAPTER 1: INTRODUCTION.....	1
Highlights of the preweaned dairy heifer’s management practices.....	1
Gut microbiome.....	8
<i>Faecalibacterium prausnitzii</i>	12
Objectives	14
References	15
CHAPTER 2: ISOLATION AND CHARACTERIZATION OF <i>FAECALIBACTERIUM PRAUSNITZII</i> FROM CALVES AND PIGLETS*	26
Abstract.....	26
Introduction	27
Materials and Methods	29
Results	36
Discussion.....	48
References	51

CHAPTER 3: ORAL ADMINISTRATION OF <i>FAECALIBACTERIUM PRAUSNITZII</i> DECREASED THE INCIDENCE OF SEVERE DIARRHEA AND RELATED MORTALITY RATE AND INCREASED WEIGHT GAIN IN PREWEANED DAIRY HEIFERS.....	56
Introduction	57
Materials and Methods	59
Safety Trial	60
Randomized Field Trial.....	62
Results	70
Safety Trial	70
Randomized Field Trial.....	75
Discussion.....	85
References	91
CHAPTER 4: CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES...	97

LIST OF FIGURES

CHAPTER 2

Figure 2. 1: Growth of each isolate.	37
Figure 2. 2: Average difference of acetate and butyrate concentrations.	39
Figure 2. 3: Average difference of propionate and isobutyrate concentrations.	40
Figure 2. 4: Correlations between the average difference of SCFA concentrations and the growth performance.....	41
Figure 2. 5: Effect of pH on bacterial growth.	42
Figure 2. 6: Effect of bile salts on bacterial growth.	42
Figure 2. 7: Disk diffusion agar assay.....	44
Figure 2. 8: Phylogenetic tree.....	47

CHAPTER 3

Figure 3. 1: Distribution of scores of fecal consistency, dehydration, attitude and appetite during the 14 days of life of calves. Safety trial.	71
Figure 3. 2: Incidence of severe diarrhea from birth to weaning of dairy calves.....	76
Figure 3. 3: Effect of <i>F. prausnitzii</i> administration on mortality rate of preweaned dairy calves.	76
Figure 3. 4: Mean fecal consistency score (0-3) during the preweaning period.	77
Figure 3. 5: Operational taxonomic unit (OTU), Chao1 index and Shannon index....	79
Figure 3. 6: <i>Faecalibacterium</i> mean relative abundance.	80
Figure 3. 7: Heat map of the 30 most prevalent genera.	81
Figure 3. 8: Aggregate microbiome composition at the phylum level.....	82
Figure 3. 9: Relative abundance of Firmicutes and Bacteroidetes and their ratio.....	83
Figure 3. 10: Serum BHBA ($\mu\text{mol/l}$) concentration over the preweaning period.....	84

LIST OF TABLES

CHAPTER 2

Table 2.1: E-test. Resistance profile.....	45
Table 2.2: Multidrug resistance profile of <i>Faecalibacterium prausnitzii</i>	46
Table 3.1: Weight gain and average daily gain during the preweaning period.	78

CHAPTER 1: Introduction

The dairy industry has experienced significant changes in the last decades. Between 2001 and 2009, the number of farms decreased from 97,460 to 65,000, while the number of animals per farm increased (USDA, 2010b). The number of farms with 2,000 cattle or more increased from 12% to 30% between those years (USDA, 2010b). Genetic improvement along with better management practices are reflected by higher productivity per cow. Milk production has increased 15%, from 165,332 in 2001 to 189,320 million pounds in 2009, whereas the number of dairy cows has grown only 1%, from 9.10 to 9.20 million (USDA, 2010b).

Replacement heifers are extremely important for the success of a dairy farm. Strategies to improve animal performance while maintaining welfare, health, and expenses at optimal levels are the focus of many research groups. Preweaned calf health is an important challenge faced by dairy producers, with diarrhea being the main cause of morbidity during the preweaning period. The treatment of diarrhea with antibiotics, supportive care, and labor is expensive and new strategies to prevent and treat diarrhea will maximize productivity, animal welfare, and profitability.

Highlights of the preweaned dairy heifer's management practices

Newborn management

Post-natal calf care plays an important role in subsequent health. The major causes of newborn mortality include acidosis, parturient trauma, hypoglobulinemia,

congenital infections and deficiencies, and omphalophlebitis (Mee, 2008). From 1996 to 2004, the average mortality rate in the first 48h of life was 8%, being higher for calves born from nulliparous dams and for twins, as described in an observational study performed on Minnesota dairy herds (Silva del Rio et al., 2007).

Calves are born with an incomplete immune system due to the cotyledonary placenta and depend on the maternal colostrum to acquire immunoglobulins (Ig). The permeability of the mucosal epithelial cells to large molecules, including Ig, is optimal in the first 4 hours after birth and a linear decrease of absorption happens over the first 24h (Stott et al., 1979).

The quality and the volume of the colostrum produced by a cow depends on her breed, lactation number, dry period length, pre-parturient vaccination, nutrition, mastitis, and delayed colostrum collection (Godden, 2008). About 85 to 90% of the total Ig in colostrum is IgG (Larson et al., 1980); hence total Ig, often measured on dairies, is correlated with colostrum quality. Colostrum is also a source of energy, IgM, IgA, IgE, growth hormones, nonspecific antimicrobial factors, and cytokines for the newborn calf (McGuirk and Collins, 2004).

Failure of passive transfer (FPT) is a condition associated with increased morbidity and mortality of preweaned calves and occurs when the serum IgG concentration is less than 1000mg/dL between the 2nd and 7th days of life. It can occur due to several reasons, for example: if the calf does not receive colostrum, if colostrum is not administered in the first few hours of life, or if the colostrum's volume or quality is not adequate. The serum IgG concentration can be estimated using a refractometer; serum total protein of 5.0 g/dL is equivalent to 1000 mg/dL of

IgG (Calloway et al., 2002). In a study evaluating more than 2800 calves in Minnesota and Ontario, the incidence of FPT was 11% when the serum total protein cut-off point was 5.2 g/dL and 32% when the cut-off point was 5.7 g/dL (Windeyer et al., 2014). Failure of passive transfer was one of the risk factors for bovine respiratory disease (BRD) in this study, but it was a poor predictor of neonatal calf diarrhea. In general, the goal is to have more than 90% of the calves with adequate passive transfer. In summary, the administration of 10 to 12% of the calf's body weight (liters/kilograms) through esophageal feeding in the first hours after birth is recommended as general practice to increase the potential for passive transfer of immunity (Morin et al., 1997).

Umbilical antiseptics is controversial. The use of iodine 7% or chlorhexidine 0.5% was shown to decrease calf morbidity and mortality; however other studies say the opposite (Mee, 2008). Indeed, the umbilical cord should not be cut or ruptured prematurely. Maintenance of good hygiene in maternity pens, separation of different age groups, and adequate cleanliness and comfort of calves are strategies currently recommended to prevent future infections.

Housing and feeding systems

Among heifer-raising operations, 92.9 % used individual outside hutches to house preweaned calves in West region of US, while Eastern operations varied between individual outside hutches (30.6%), individual inside pens (30.6%), and groups housing (19.4%) (USDA, 2012). Differences between the two regions

primarily occur due to climate conditions. Some forms of individual housing prevent direct contact between calves, reducing the transmission of disease. Management of individually housed calves often requires more labor, and as a counterbalance, managers often place calves on a twice per day feeding program.

Group housing is becoming a more common practice as calves in these systems have been shown to have higher average daily gains and better social behaviors (Duve et al., 2012; Bernal-Rigoli et al., 2012; Cobb et al., 2014). Heifers that received *ad libitum* milk twice a day had a mean body weight 6.7 kg greater than heifers that received restricted volume of milk during the preweaning period (Uys et al., 2011). Cross-sucking behavior was increased for heifers fed restricted amount of milk in that study. Duve *et al.* 2012 showed that calves raised in groups increased play behavior and responded less to restrain. Group pens are equipped with automated systems that permit calves to consume milk *ad libitum*. Employees therefore spend less time feeding and cleaning, allowing for more time for calf care and other chores. Disadvantages of group housing are higher risk of disease transmission and the inability to observe individual feed consumption, which can be an indication of illness (Pereira et al., 2014).

The choice of milk fed to calves varies depending on availability, price, quality, and farmer preference. Pasteurized whole milk would be the best option from a nutritional perspective, but not economically, due to loss of saleable milk and labor and machine costs for pasteurization. According to the USDA, 86% of heifer-raising operations feed milk replacer to preweaned calves (USDA, 2012). Milk replacer has a lower chance of transmitting milk-borne disease or disease due to fecal and

environmental contamination, is easier to stock, and can be more economical than feeding milk. On the other hand, the weight gain during the preweaning period was higher for calves that had pasteurized nonsalable milk (26.7 ± 9.3 kg) compared to calves fed conventional milk replacer (20.1 ± 10.1 kg) (Godden et al., 2005). The same study reported lower morbidity (12.1 vs. 32.1%) and mortality rates (2.2 vs. 11.6%) for calves fed pasteurized waste milk compared to calves fed conventional milk replacer. Milk acidification is a common method of conservation of nonsalable milk because it inhibits bacterial growth. It is less expensive than pasteurization and commonly used in group housed calf facilities (Capel, 2013).

Diarrhea and pneumonia

The two major causes of morbidity and mortality during the preweaning period are pneumonia and diarrhea (Windeyer et al., 2014). The U.S. Department of Agriculture (USDA) surveyed morbidity and mortality rates in preweaned heifers in 2006 (USDA, 2010a). Digestive disorders (i.e. diarrhea and bloat) affected 23.9% of the population and were responsible for 56.5% of the deaths between birth and weaning. The overall mortality rate for that same period was 7.8% (USDA, 2010a). The high incidence of diarrhea is a persistent problem. Four years after the 2006 survey, the USDA reported that 25.3% of the preweaned calves had diarrhea (USDA, 2012). The overall mortality rate decreased to 4.2% and one third of the deaths were caused by digestive problems. The treatment of diarrhea with antibiotics, supportive

care and labor is expensive and new strategies to prevent diarrhea will maximize productivity, animal welfare, and profitability.

In the first two weeks of life neonatal calves are at risk for diarrhea caused by enterotoxigenic *E. coli*, Rotavirus, Coronavirus, *Salmonella spp.*, and *Cryptosporidium parvum* (McGuirk, 2008; Cho and Yoon, 2014; Meganck et al., 2014). Diarrhea can have different levels of severity, depending on pathogen and host. Some calves have only a gastrointestinal disorder characterized by liquid stools. Others can have systemic symptoms such as fever, loss of appetite, dehydration, and metabolic acidosis. Mild cases are often not treated, oral fluids are administered to calves with moderate dehydration, intravenous fluids are administered for severely dehydrated calves, and antibiotic therapy is recommended for calves showing systemic signs.

Second to diarrhea, respiratory diseases are a main cause of morbidity in preweaned dairy heifers. A 2010 survey indicated that 18.1% (± 5.9) of preweaned heifers were affected by pneumonia. Among weaned heifers, pneumonia was the main disease (11.2% ± 2.5) (USDA, 2012).

Pneumonia caused by Bovine Respiratory Disease (BRD) is normally accompanied by inflammation, consolidation, and can include abscessation and fibrosis of the lungs. Calves experiencing BRD may have fever, respiratory effort, nasal discharge, cough, depressed attitude and/or decreased appetite, (Guterbock, 2014). *Mannheimia haemolytica*, and *Histophilus somnus* can cause acute cases, while *Pasteurella multocida* and *Trupeerella pyogenes* (formely *Arcanobacterium pyogenes*) are associated with chronic pneumonia and can be induced by stressful events or acquired secondary to viral or *Mycoplasma spp.* infections (Rebhun, 1995).

According to the USDA survey cited above, of the preweaned calves affected with pneumonia 90.2% (\pm 6.5) are treated with antibiotics (USDA, 2012). The negative impact of BRD on growth, age at first calving, first lactation milk yield and survivability is not well defined yet and should be further characterized (Guterbock, 2014).

Antibiotic use in feed

Historically, sub-therapeutic doses of antibiotics have been used as growth promoters and disease prophylaxis in livestock. In 2010, more than 75% of heifer-raising operations used antibiotics in the feed (USDA, 2012). Ionophores, coccidiostats, and antibiotics act as growth promoters, inhibit coccidiosis, and prevent diarrhea and pneumonia, respectively. Due to concerns about antibiotic resistant microbes and the possible impact on human health, the use of antibiotics for growth promotion in food animals is being reconsidered in the United States and is already prohibited in Europe. Currently, antibiotics can only be used for therapeutic purposes; a licensed veterinarian is required to approve use of antibiotics in the feed of livestock animals, as enforced by the 2015 Veterinary Feed Directive ((FDA, 2015). The use of probiotics in food animals is a potential alternative to the use of antimicrobials in animal feed.

Records

Good record keeping of calves' weights, disease events, and treatments is a practice that should be applied at all dairy farms. The determination of morbidity and mortality rates, growth rates, stocking densities, seasonal patterns, can help in outbreak diagnosis, disease prevention, decision making, and problem solving. Good management of replacement heifers is the basis for the health and productivity of the whole herd.

Gut microbiome

The human gastrointestinal tract (GIT) is inhabited by 10^{14} microbe cells (100 trillion), 10 times the number of eukaryotic cells in the body (Luckey, 1972). The gut microbiota affects various aspects of postnatal life; it contributes to the development of the immune system (Round and Mazmanian, 2009; Peterson and Cardona, 2010) and controls energy balance by influencing energy expenditure and storage (Ridaura et al., 2013).

Commensal microbes are essential for the digestion of fiber, energy production and vitamin conversion. The fermentation of starches by commensal bacteria produces short chain fatty acids (SCFA): mainly butyrate, acetate and propionate (Cummings et al., 1987). The mucosal immune system allows the beneficial microbiota to colonize the gut and reacts against pathogenic bacteria. The SCFA play an important role in this immunological homeostasis, especially butyrate (Arpaia and Rudensky, 2014). A dysfunction of this homeostasis can result in Inflammatory Bowel Diseases (IBD;

Crohn's disease and ulcerative colitis), which correspond to chronic and progressive inflammatory disorders of the GIT (Kaser et al., 2010).

Short chain fatty acids limit inflammatory responses in the intestine by promoting regulatory T-cell generation and primarily butyrate inhibits histone deacetylase (HDAC), an enzyme active prior to DNA transcription, which inhibition has been studied as a treatment for cancer, anti-inflammatory diseases, psychiatric and neurologic diseases (Arpaia et al., 2013; Furusawa et al., 2013). Butyrate has several beneficial effects in the intestine, as reviewed by Guilloteau *et al.* 2010 and Ploger *et al.* 2012 (Guilloteau et al., 2010; Ploger et al., 2012). In summary, it is a major energy source to enterocytes; it stimulates cell proliferation, differentiation and maturation, and improves colonic barrier function. Another anti-inflammatory property of butyrate is the suppression of pro-inflammatory cytokine production by intestinal macrophages (Chang et al., 2014). In human patients with shigellosis, sodium butyrate enemas contributed to the reduction of inflammation in the large intestine epithelia (Raqib et al., 2012). This study reported a reduction of macrophages, white blood cells, and the pro-inflammatory cytokines, IL8 and IL1 β , in the feces of patients treated with sodium butyrate compared to patients that received placebo. Additionally, the expression of the anti-inflammatory cytokine LL-37 was induced by the treatment and its concentration was elevated in the feces of treated patients.

Butyrate has been proven to be beneficial to livestock as well; higher concentrations of butyrate in the rumen of growing steers were associated with better feed conversion efficiency (Guan et al., 2008). A positive effect on weight gain, health, and metabolic intermediates (plasma glucose, serum total protein, plasma

glucagon-like peptide-2) was observed when sodium butyrate was supplemented to calves in the milk replacer or starter mixture (Gorka et al., 2011a). Additionally, the same study showed that it indirectly stimulated rumen development. *Faecalibacterium prausnitzii* is a major butyrate producer.

Several studies described the relationship between the gut microbiome and energy balance. Together with host genotype and diet, the relative abundance and ratios between different bacterial taxa can influence weight gain. For example, a higher ratio of Firmicutes to Bacteroidetes has been correlated with obesity in mice and humans (Ley et al., 2005; Turnbaugh et al., 2006; Turnbaugh et al., 2009). Specifically, the level of *F. prausnitzii*, a Firmicutes, has been associated with weight gain of humans (Balamurugan et al., 2010) and calves (Oikonomou et al., 2013). The effect of the microbiota on body mass composition was demonstrated with germ-free mice (GF) colonized with uncultured fecal microbiota of four twins discordant for obesity (Ridaura et al., 2013). Fecal microbiota from obese donors caused a greater change in body composition and increase in fat mass compared to the microbiota of lean co-twin donors. When GF mice were cohoused with the obese mice, the obesity phenotype was transferred. However, when GF mice were cohoused with obese and lean mice, the lean microbiota was more prevalent and the adiposity phenotype was not developed. Obesity should be avoided in humans and animals since it can lead to several health disorders (i.e. diabetes mellitus and systemic hypertension). However, increased growth rate is desirable in young production animals.

Probiotics as defined by the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO) are “live micro-organisms

which, when administered in adequate amounts, confer a health benefit on the host” (Morelli and Capurso, 2012). Microbial mechanisms of action can be multifactorial. The micro-organism and its metabolites can act directly improving digestion and mucosal health; or indirectly, modulating the growth of other bacteria (Scott et al., 2015). Currently, examples of microbes considered as probiotics are *Lactobacilli*, *Bifidobacteria*, *Enterococci*, *E. coli* strain Nissle and the yeast *Saccharomyces boulardii* (Chen et al., 2014; Scott et al., 2015).

The effect of probiotics as a disease therapy has been extensively studied (Saggiaro, 2004; Sang et al., 2010; Hungin et al., 2013; Celiberto et al., 2015). In summary, probiotics prevent regression and reduce the intensity and duration of gastrointestinal inflammatory diseases. Hence, they can be used as adjunct therapies and to maintain clinical remission in patients with IBD.

Probiotics are a promising alternative to improve food animal productivity and health, and decrease the use of antimicrobials in animal feed. However, scientific evidence that specific microbes can be used to benefit animal health and performance is limited.

Faecalibacterium prausnitzii

F. prausnitzii (formally *Fusobacterium prausnitzii*) is a Gram-positive, anaerobic, rod-shaped, non-motile, non-spore-forming bacterium belonging to the phylum *Firmicutes* (Barcenilla et al., 2000; Duncan et al., 2002; Foditsch et al., 2014). It is part of the normal intestinal microbiota of many animal species and represents one of the most abundant bacteria encountered in the feces of healthy animals, such as humans (Suau et al., 2001; Hold et al., 2003; Walker et al., 2011), bovine (Oikonomou et al., 2013), swine (Haenen et al., 2013), mice (Nava and Stappenbeck, 2011), and poultry (Lund et al., 2010).

High levels of *F. prausnitzii* were associated with obesity in humans. Balamurugan *et al.* 2010 compared the fecal microbiota of obese and non-obese Indian children using real-time PCR and found significantly higher levels of *F. prausnitzii* in the feces of the obese (Balamurugan et al., 2010).

Low levels of *F. prausnitzii* have been associated with chronic inflammatory disorders of the GIT including Crohn's disease (Sokol et al., 2008; Wang et al., 2013) and ulcerative colitis (Machiels et al., 2013). In humans, lower proportion of *F. prausnitzii* in resected ileal mucosa of Crohn's patients was associated with post-surgery recurrence of the disease (Sokol et al., 2008). The anti-inflammatory and immunomodulatory capacities of *F. prausnitzii* and its supernatant have been demonstrated *in vitro* with cultured cells and *in vivo* with TNBS-induced colitis mice models (Sokol et al., 2008; Qiu et al., 2013; Miquel et al., 2015). Both bacterium and its supernatant induced the production of IL-10, an anti-inflammatory cytokine, while decreasing the secretion of the pro-inflammatory cytokines IFN- γ and IL-12 (Sokol et

al., 2008). The study also reported that *F. prausnitzii* metabolites inhibit the activation of NF-kB and the IL-8 secretion (Sokol et al., 2008). Additionally, a protective effect of *F. prausnitzii* on the intestinal barrier has been suggested (Martin et al., 2015; Miquel et al., 2015).

As mentioned previously, butyrate has several beneficial effects in the intestines, is a major energy source to enterocytes, stimulates cell proliferation, differentiation and maturation, and improves colonic barrier function (Guilloteau et al., 2010; Ploger et al., 2012). In ruminants, butyrate has been associated with rumen development, having a mitotic effect on ruminal papillae (Mentschel et al., 2001), gut maturation (Gorka et al., 2011b) and increased weight gain in calves (Gorka et al., 2011a). Considering this, the ability of *F. prausnitzii* to produce butyrate could contribute to positive affects observed during colonization of *F. prausnitzii* in the large intestines.

The anti-inflammatory properties of this commensal bacterium have previously been considered as dose-dependent, since a higher abundance of *F. prausnitzii* was correlated with lower disease activity markers in TNBS-induced colitis mice (Miquel et al., 2015). Additionally, the anti-inflammatory effects of the *F. prausnitzii* have been proven to be independent of the butyrate (Sokol et al., 2008).

A recent review emphasized the use *F. prausnitzii* as a “next generation probiotic” (Scott et al., 2015). Further studies are needed to approve the use of this beneficial microbe in human and animal health.

Objectives

Non-culture methods opened the horizons of microbiology and myths are being unveiled. The knowledge on bacterial communities from innumerable niches of the body and the environment advanced tremendously in the last decades with DNA sequencing techniques.

The fecal microbiota of calves during the preweaning period was characterized using a metagenomic approach in a previous study from our group (Oikonomou et al., 2013). This study provided evidence of the potential beneficial effects of *F. prausnitzii* in the GIT of neonatal calves. Higher prevalence of *F. prausnitzii* in the first week of life of Holstein calves was associated with improved weight gain and decrease in the incidence of diarrhea (Oikonomou et al., 2013).

Increased growth rate and low diarrhea incidence are major goals of preweaned heifer management. The preweaning period is critical for the performance of the heifers as lactating cows and for the profitability as well. As an example, preweaned dairy heifers are expected to yield more milk in their 1st lactation when they are purposely fed to exceed the nutrient requirements and have a greater average daily gain (ADG) (Soberon et al., 2012; Soberon and Van Amburgh, 2013).

The results described by Oikonomou *et al.* 2013 motivated us to focus our studies in this commensal bacterium and its potential use as a probiotic. The hypothesis was that the administration of *F. prausnitzii* could improve gastrointestinal health and performance of preweaned heifers. The first objective of this thesis was to advance our knowledge on the anaerobic bacterium *F. prausnitzii* and its *in vitro*

characteristics. The second objective was to test its effects *in vivo*, administering it to newborn calves. The long term goal is to develop a probiotic, depending on the results.

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CHAPTER 2: Isolation and characterization of *Faecalibacterium prausnitzii* from calves and piglets*

* Foditsch C, Santos TMA, Teixeira AGV, Pereira RVV, Dias JM, Gaeta N, Bicalho RC. (2014) Isolation and Characterization of *Faecalibacterium prausnitzii* from Calves and Piglets. PLoS ONE 9(12): e116465. doi:10.1371/journal.pone.0116465

Abstract

The goal of our study was to isolate and characterize *Faecalibacterium prausnitzii* from fecal samples of healthy calves and piglets, in order to develop a novel probiotic for livestock animals. We identified 203 isolates of *Faecalibacterium sp.*, which were clustered in 40 genetically distinct groups. One representative isolate from each cluster was selected for further characterization. The concentrations of the short chain fatty acids (SCFA) acetate, butyrate, propionate and isobutyrate in the culture media were measured by gas chromatography. We observed reduction in the concentration of acetate followed by concomitant increase in the concentration of butyrate, suggesting that the isolates were consuming acetate present in the media and producing butyrate. Butyrate production correlated positively with bacterial growth. Since butyrate has many benefits to the colonic epithelial cells, the selection of strains that produce higher amounts of butyrate is extremely important for the development of this potential probiotic.

The effect of pH and concentration of bile salts on bacterial growth was also evaluated in order to mimic the conditions encountered by *F. prausnitzii in vivo*. The

optimal pH for growth ranged between 5.5 and 6.7, while most isolates were inhibited by the lowest concentration of bile salts tested (0.1%). Antimicrobial resistance profile showed that most isolates of *Faecalibacterium sp.* were resistant against ciprofloxacin and sulfamethoxazole-trimethoprim. More than 50% of the isolates were resistant to tetracycline, amikacin, cefepime and ceftiofur. A total of 19 different combinations of multidrug resistance were observed among the isolates. Our results provide new insights into the cultural and physiological characteristics of *Faecalibacterium prausnitzii* illustrating large variability in SCFA production, *in vitro* growth, sensitivity to bile salts, and antibiotic resistance and suggesting that future probiotic candidates should be carefully studied before elected for *in vivo* studies.

Introduction

The gut microbiota plays a key role in shaping various aspects of postnatal life; it contributes to the development of the immune system (Round and Mazmanian, 2009; Peterson and Cardona, 2010) and controls energy balance by influencing energy expenditure and storage (Ridaura et al., 2013). Higher ratio of *Firmicutes* to *Bacteroidetes* in the gut has been associated with obesity in mice (Turnbaugh et al., 2006). Specifically, the level of *Faecalibacterium prausnitzii* has been shown to increase significantly in the microbiota isolated from fecal samples of obese children when compared to the microbiota from non-obese individuals (Balamurugan et al., 2010).

Low levels of *F. prausnitzii* have been associated with chronic inflammatory disorders of the gastrointestinal tract, including Crohn's disease (Wang et al., 2013)

and ulcerative colitis (Machiels et al., 2013). Recent research has shown that *F. prausnitzii* has anti-inflammatory and immunomodulatory capacities, which improved the 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice, partially due to secreted metabolites blocking NF- κ B activation and IL-8 production (Sokol et al., 2008; Qiu et al., 2013). Therefore, the use of *F. prausnitzii* as a probiotic might be a promising strategy for the treatment of Crohn's disease.

F. prausnitzii (formally *Fusobacterium prausnitzii*) is a Gram-positive, non-motile, non-spore-forming, butyrate producer bacterium belonging to the Firmicutes phylum (Duncan et al., 2002). It is part of the normal intestinal microbiota of many animal species and represents one of the most abundant bacteria encountered in the feces of healthy animals, such as humans (Suau et al., 2001; Hold et al., 2003; Walker et al., 2011), bovine (Oikonomou et al., 2013), swine (Haenen et al., 2013), mice (Nava and Stappenbeck, 2011), and poultry (Lund et al., 2010).

F. prausnitzii is a major butyrate producer (Barcenilla et al., 2000). Butyrate produces the highest energy value per mole compared to other short chain fatty acids (SCFA) (Maynard, 1979), it is an important energy source to the colonic epithelial cells, it has anti-inflammatory and epithelial barrier-preserving effects, and it regulates cell proliferation, differentiation, and apoptosis (Ploger et al., 2012). Butyrate has been proven to be beneficial to livestock as well; higher levels of butyrate in the rumen of growing steers were associated with better feed conversion efficiency (Guan et al., 2008). A positive effect on body weight gain, health, and metabolic intermediates (plasma glucose, serum total protein, plasma glucagon-like peptide-2) was observed when sodium butyrate was supplemented to calves in the milk replacer or starter

mixture (Gorka et al., 2011a). Additionally, the same study showed that it indirectly stimulated the rumen development. We previously characterized the fecal microbiota of calves during the pre-weaning period using a metagenomic approach and provided evidence of the potential beneficial effects of *F. prausnitzii* in the intestinal tract of neonatal calves (Oikonomou et al., 2013). Higher prevalence of *Faecalibacterium* sp. in the first week of life of Holstein calves was associated with improved weight gain and decrease in the incidence of diarrhea. The anti-inflammatory properties of *F. prausnitzii* and the production of butyrate are factors potentially contributing to the positive results observed in treated calves. Here, we characterize *Faecalibacterium prausnitzii* isolated from feces of calves and piglets to study cultural and physiological aspects of this commensal bacterium and to substantiate its use as a viable animal probiotic.

Materials and Methods

Ethics statement

Fecal samples were collected from calves that were housed on a large commercial dairy farm located near Ithaca NY and from piglets housed in Cornell University facilities. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University (Protocol number: 2012-0055). The fecal sample collections from the calves housed on a commercial dairy farm were authorized by the farm owner, who was aware of the procedure.

Bacterial strains and growth conditions

A reference strain of *F. prausnitzii* used as a control in our experiments was obtained from the DSMZ-German Collection of Microorganism and Cell Cultures (DSM17677, strain designation A2-165) (Barcenilla et al., 2000; Duncan et al., 2002). We developed a complex culture medium (herein referred to as VTR2RF) containing rumen fluid. The VTR2RF media used as transport, enrichment, and isolation media was composed of the anaerobic media Versa TREK REDOX 2 (Trek Diagnostic Systems, Cleveland-OH) supplemented with 30% filtered rumen fluid. Rumen fluid was collected from fistulated cows, centrifuged at $12,000 \times g$ for 30 min, the supernatant was filter-sterilized (Corning Incorporated Life Sciences, Tewksbury-MA) 3 times and stored at 4°C. VTR2RF agar was additionally supplemented with 0.5% (w/v) yeast extract (BD, Franklin Lakes, NJ), 5 mg/l (w/v) hemin (Sigma-Aldrich, St. Louis, MO), 1 mg/l (w/v) cellobiose (Sigma-Aldrich), 1 mg/ml (w/v) maltose (Sigma-Aldrich), and 0.5 mg/ml (w/v) L-cystein (Sigma-Aldrich) (Sokol et al., 2008).

Sample collection and isolation of anaerobic bacteria

Fecal samples were collected from 7-28 days-old healthy Holstein calves and from 10-30 days-old healthy piglets. The samples were gently collected from the rectum and immediately placed in a tube containing 12 ml of VTR2RF broth. The tubes were sealed and transported until further processing. The subsequent procedures were performed in an anaerobic chamber (BacBasic chamber, Sheldon Manufacturing, Inc., Cornelius,OR). All samples were serially diluted in Anaerobic Dilution Blank

(Anaerobe Systems, USA) and plated on VTR2RF agar. After 48h, about 10 typical colonies from each sample were selected and single-colony purified in VTR2RF agar. The isolates were stored at -80°C in VTR2RF broth containing 16% of glycerol.

DNA extraction, PCR and 16S rDNA sequencing

Genomic DNA was extracted from typical colonies using the InstaGene Matrix (Biorad, Hercules, CA) according to the manufacturer's instructions with some modifications. Briefly, one colony was inoculated in 200 µl of InstaGene Matrix, incubated at 56°C for 30 min, mixed using a vortex and incubated at 100°C for 8 min. After centrifugation at 13,400 × g for 1 min, the supernatant was collected and used for the PCR.

The DNA concentration was measured using a spectrophotometer (NanodropTM 1000 – NanoDrop Technologies, Rockland, DE) and, approximately, 350 ng of genomic DNA were used for PCR. The 16S rDNA gene was amplified by PCR using the universal eubacterial primers fD1 (forward primer) and rP2 (reverse primer) (Wood et al., 1998). The 100-µl reaction mix was composed of 50 µl of Green GoTaq Master Mix (2 × Green GoTaq Master consisting of Green GoTaq Reaction Buffer, 400 µM of each dNTP, and 3 mM MgCl₂; Promega, Madison, WI), 20 pmol of each primer, 350 ng of DNA, and nuclease-free water. Reaction conditions for the amplification were an initial cycle of 94°C for 5 min, 57°C for 2 min, and 72°C for 2 min, followed by 29 cycles of 94°C for 2 min, 57°C for 30 sec, and 72°C for 2 min, with a final cycle of 72°C for 10 min (Wood et al., 1998).

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD), according to the manufacturer's protocol, and sequenced at the Cornell University Life Sciences Core Laboratories Center. The sequences obtained were compared to sequences deposited in the Ribosomal Database Project Classifier (RDP – Center for Microbial Ecology, Michigan State University, East Lansing MI).

RAPD analysis

The genetic diversity of the isolates was assessed by random amplified polymorphic DNA PCR (RAPD-PCR) using the primer 1254 (Lopez-Siles et al., 2012). Amplification products were resolved by electrophoresis in a 2% (w/v) agarose gel and stained with 0.5 µg/ml of ethidium bromide (Wang et al., 1993). The RAPD-PCR profiles were visually compared and clustered in 40 genetically distinct groups of isolates. One representative isolate from each cluster was selected for further characterization, as described below.

Growth performance

The inoculum cultures were grown under the same conditions and standardized by optical density. Half microliter of each inoculum were inoculated in 40 ml of VTR2RF broth and incubated at 37°C for 48 h under anaerobic conditions. Two aliquots were aseptically removed from each bottle and the optical density (O.D.) of the culture sample was measured at $\lambda=600\text{nm}$ in a Synergy 2 Microplate Reader

(BioTek Instruments, Inc., Winooski, VT). The difference in average O.D. between 5 h and 48 h of incubation was used to calculate the growth performance. The procedure was repeated, at least, 2 times for each sample.

Short chain fatty acids metabolism

Faecalibacterium sp. isolates and the reference strain DSM 17677 were inoculated in 25 ml of VTR2RF broth and incubated at 37°C for 48 h under anaerobic conditions. The culture was centrifuged at 4000 × *g* for 10 min and the supernatant was collected. The concentration of acetate, butyrate, propionate and isobutyrate in the VTR2RF media before inoculation and in the supernatant of the culture was measured by gas chromatography. Samples were injected into a Perkin Elmer Autosystem XL Gas Chromatograph containing a Supelco packed column (Sigma-Aldrich, St. Louis, MO) and the analysis was performed according to the manufacturer's protocol (Supelco, 1998) at the Dairy One Cooperative, Ithaca, NY. The procedure was repeated, at least, 3 times for each sample.

Resistance to pH and bile salts

The pH of VTR2RF broth was adjusted to 6.7, 6.2, 5.5, 5.0, 4.5, 4.0 and 3.5 with HCl and the adjusted broth was inoculated with the *Faecalibacterium sp.* isolates or the reference strain DSM 17677. The O.D. of the cultures was measured at $\lambda=600\text{nm}$ in a Synergy 2 Microplate Reader (BioTek Instruments, Inc.) 0 h and 48 h after inoculation. Similarly, we evaluated bacterial growth in VTR2RF broth

supplemented with 0.1% (wt/vol), 0.25% (wt/vol), and 0.5% (wt/vol) bile salts (Sigma-Aldrich, St. Louis, MO) (Lopez-Siles et al., 2012).

Disk diffusion and Etest MIC

A preliminary susceptibility test was performed using the disk diffusion agar assay, adapted from the Performance Standards for Antimicrobial Disk Susceptibility Tests (CLSI, 2012). To standardize the inoculum density, the direct cell suspension was adjusted according to the 0.5 McFarland turbidity standard. The suspension was spread with a swab on a 150-mm VTR2RF agar plate and 12 antibiotic disks (ampicillin, ceftiofur, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, enrofloxacin, nalidixic acid, neomycin, streptomycin, sulfamethoxazole-trimethoprim and tetracycline) were deposited on the agar. The plates were also incubated at 37°C for 48 h under anaerobic conditions. The diameters of zones of complete inhibition were measured with calipers.

Additionally, the minimal inhibitory concentration (MIC) was estimated using the Etest (BioMérieux, Inc., Durham, NC), which consist of plastic strips coated with antimicrobials that create a concentration gradient as they diffuse into the agar (Rosenblatt and Gustafson, 1995). To standardize the inoculum density, the direct cell suspension was adjusted according to the 1.0 McFarland turbidity standard. The suspension was spread with a swab on two 150-mm VTR2RF agar plates and five Etest strips were added per plate, according to the manufacturer's instructions. The plates were incubated at 37°C for 48 h under anaerobic conditions. The intersection of

the ellipse of growth inhibition with the strip was considered as the MIC. The antibiotics tested were amikacin, ampicillin, cefepime, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, tetracycline and sulfamethoxazole-trimethoprim. For both the disk diffusion assay and the Etest, quality assurance was performed using *Bacteroides fragilis* ATCC 25285, as recommended by the CLSI (CLSI, 2004).

We estimated the resistance profile of the *Faecalibacterium sp.* isolates by comparing the MIC values with the standard values determined by the CLSI for *Bacteroides fragilis* ATCC 25285 (CLSI, 2004) and reported elsewhere (Wust and Wilkins, 1978; Chin and Neu, 1984). No published MIC breakpoint for amikacin and gentamicin were found in the literature, therefore the highest concentrations were considered as the MIC for these two aminoglycosides.

Phylogenetic analysis

The 16S rDNA sequences were aligned and manually trimmed using the cross-platform bioinformatics software *Geneious* 7.0.6 (Biomatters, Auckland, New Zealand). The sequences were compared with other sequences imported from the NCBI database. Sequences from bacteria belonging to other families of the *Clostridiales* order (*Ruminococcaceae*, *Syntrophomonadaceae*, *Peptostreptococcaceae*, *Clostridiaceae* and *Lachnospiraceae*) were included in the phylogenetic analysis. The sequences of our isolates have been deposited in GenBank (NCBI) under the accession numbers KJ957841 to KJ957877.

Statistical Analyses

Data were analyzed using JMP Pro (version 11, SAS Institute Inc., Cary, NC). Results are presented as mean and standard error of the mean, and as correlation coefficient (r) matrix for the multivariate analysis between the average difference of SCFA concentrations and the growth performance. Box and whiskers plots were generated using MedCalc Statistical Software version 13.1.2 (MedCalc Software, Ostend, Belgium).

Results

Faecalibacterium sp. isolation and identification

A total of 931 anaerobic bacteria were isolated from fecal samples collected from approximately 130 healthy Holstein heifer calves and 6 isolates were obtained from samples collected from 10 piglets. Based on the 16S rDNA sequences, 203 of these isolates were identified as *Faecalibacterium* sp., which were clustered in 40 genetically distinct groups according to the RAPD-PCR profiles. One representative isolate from each cluster was used for the subsequent analysis.

Growth performance

An increment in O.D. was observed for all isolates after 48 h of incubation (Figure 2.1). This increment was higher for the isolates that produced more butyrate suggesting that the growth rate is associated with higher butyrate production, as described further next.

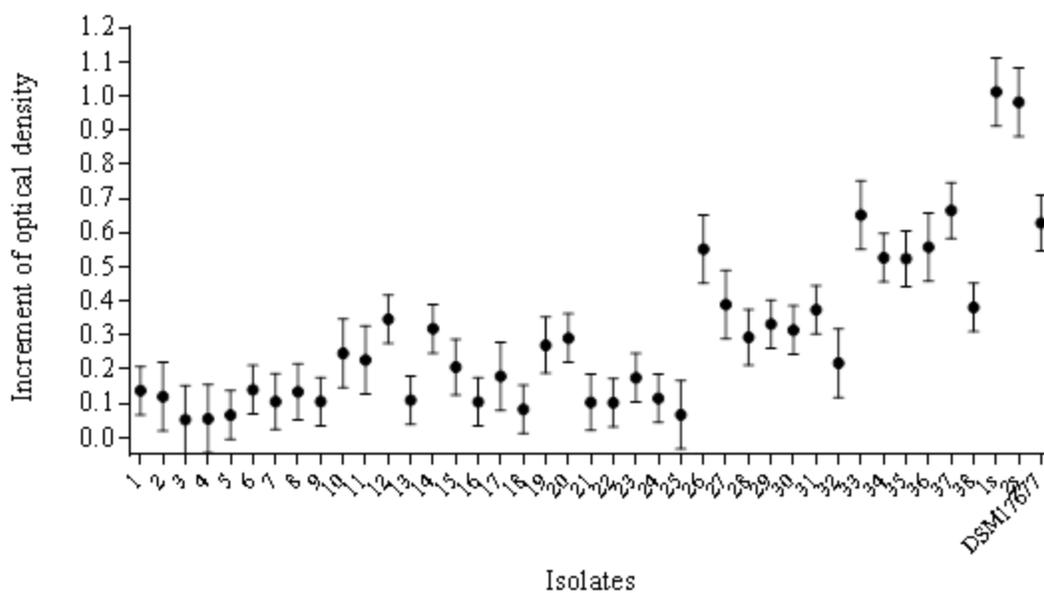


Figure 2.1: Growth of each isolate.

The growth of each of our 40 isolates and the DSM 17677 isolate, measured spectrophotometrically at hours 5, 17, 29, 41 and 48 using absorbance at the 600nm wavelength. The average optical density increment was calculated as the difference between 5 h and 48 h of incubation. Error bars indicate the standard error of the mean.

Short chain fatty acids metabolism

The average difference of the acetate and butyrate concentrations in the growth media before and after 48h of incubation for each *Faecalibacterium sp.* isolate is shown in Figure 2.2. The identification (bovine 1-38, swine 1S, 2S) of each *Faecalibacterium sp.* isolate described here was based on the butyrate production. After 48 h of incubation, there was an evident reduction in acetate concentration and concomitant increase in butyrate concentration. The average differences of propionate and isobutyrate concentrations in the medium before and after 48h of growth are shown in Figure 2.3. The concentration of propionate and isobutyrate decreased for most of the isolates; however, the differences in concentrations were not as conspicuous as the difference detected for acetate and butyrate. The scatterplot matrix (Figure 2.4) shows the correlations between the average difference of SCFA concentrations and the growth performance (fitted lines with confidence intervals are also shown). The correlation between the average difference of butyrate and the mean OD increment was $r=0.68$. There was a high negative correlation ($r= -0.87$) between the acetate and butyrate average concentrations, meaning that the high butyrate producers were consuming more acetate as well.

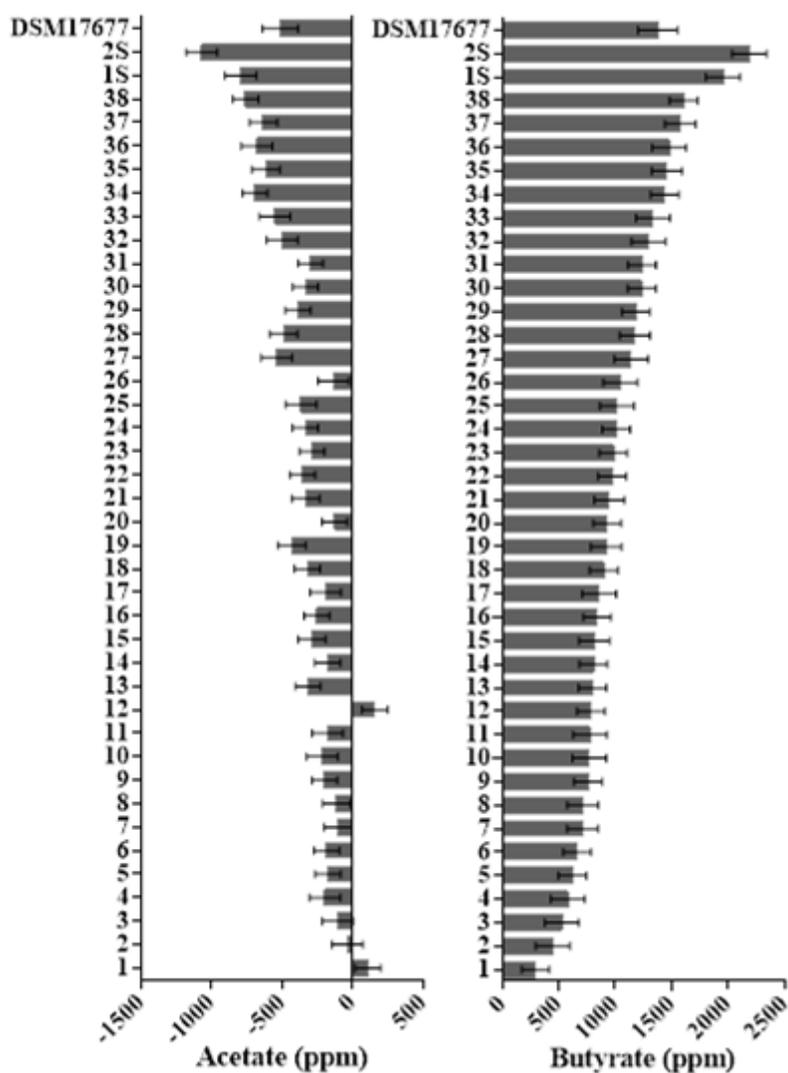


Figure 2.2: Average difference of acetate and butyrate concentrations.

Average difference of acetate and butyrate concentrations in the growth media after 48 h of incubation of each *F. prausnitzii* isolate. The bars represent the mean concentration in ppm. Error bars indicate the standard error of the mean. Based on the butyrate production, the *F. prausnitzii* isolates were entitled from 1-38 (bovine), and 1S and 2S (swine).

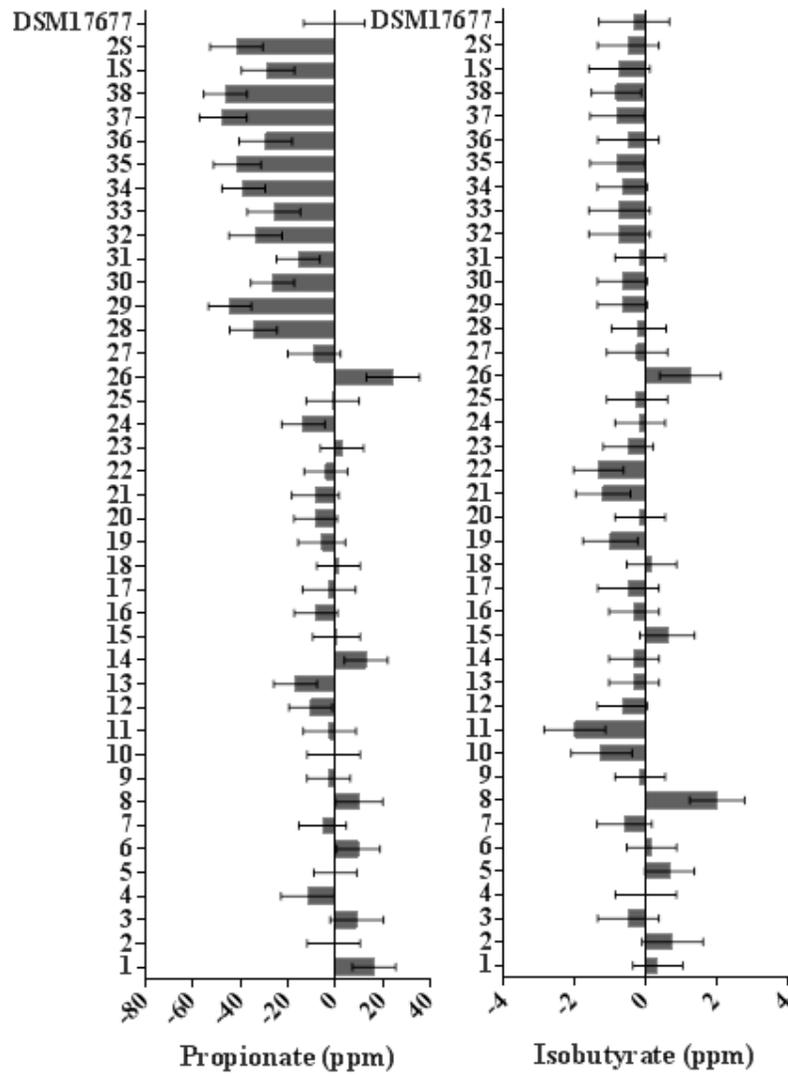


Figure 2.3: Average difference of propionate and isobutyrate concentrations.
 Average difference of propionate and isobutyrate concentrations in the growth media after 48 h of incubation of each *F. prausnitzii* isolate. The bars represent the mean concentration in ppm. Error bars indicate the standard error of the mean.

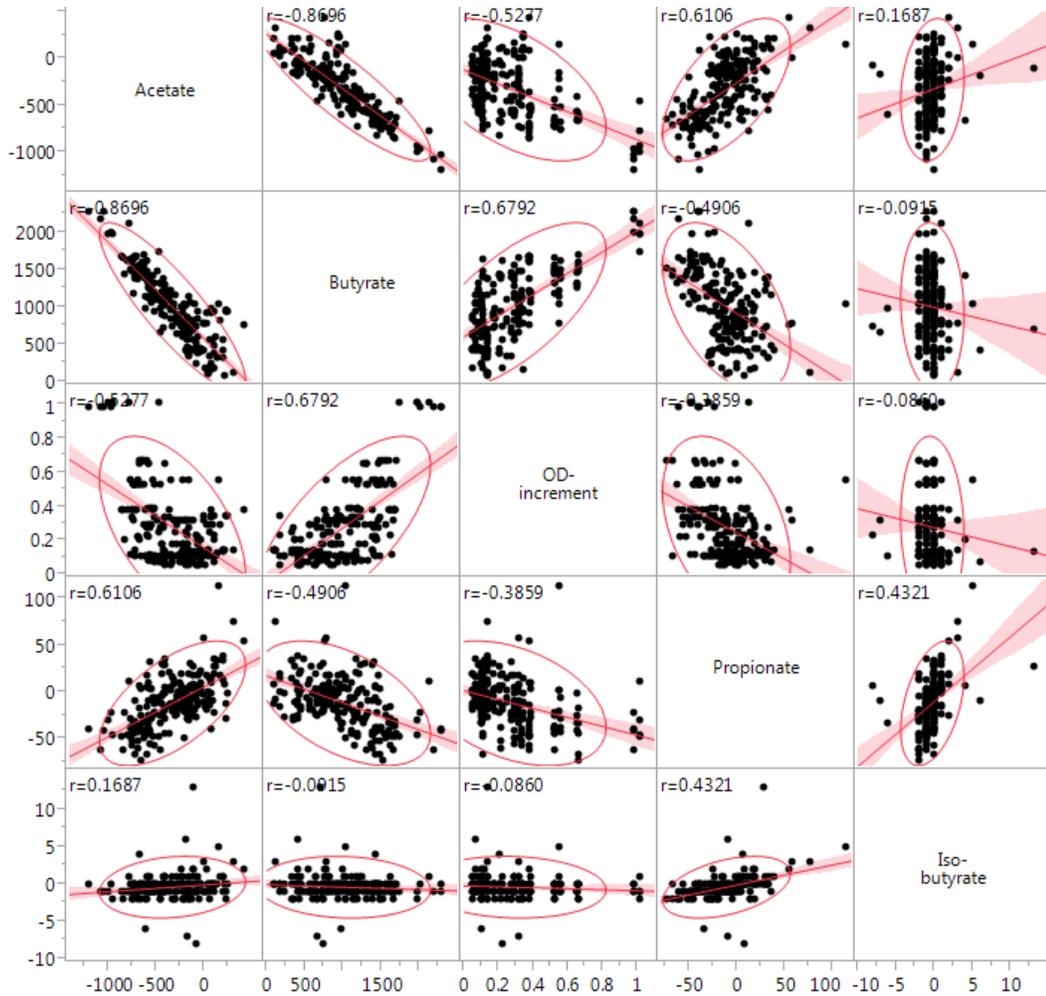


Figure 2.4: Correlations between the average difference of SCFA concentrations and the growth performance.

Correlations between the average difference of SCFA (acetate, butyrate, propionate and isobutyrate) concentrations and the growth performance of the *F. prausnitzii* isolates, represented by the increment of optical density. Correlation coefficients and fitted lines with confidence intervals are shown in each box.

Resistance to different pH and bile salts

Bacterial growth was observed in pH values between 5.0 and 6.7 (Figure 2.5).

No growth was observed in pH 4.5, 4.0, or 3.5. Bile salts inhibited growth of the

majority of the isolates (Figure 2.6) and only few isolates were able to grow in the presence of 0.1% (wt/vol) bile salt.

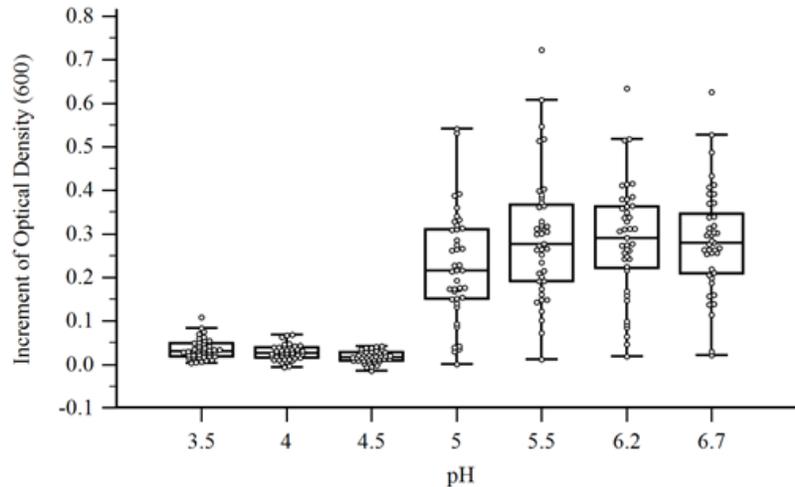


Figure 2.5: Effect of pH on bacterial growth.

The increment of optical density after 48h of incubation for each isolate in media containing different pH. Each box-and-whisker plot constitutes one pH value. The extent of the box encompasses the interquartile range of the optical density increment, whiskers extend to maximum and minimum values, and the line within each box represents the median. Outliers are represented as open black circles.

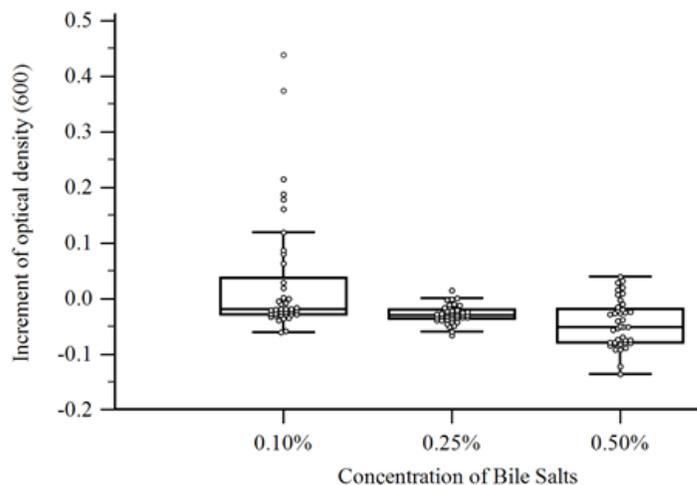


Figure 2.6: Effect of bile salts on bacterial growth.

The increment of optical density after 48 h of incubation for each isolate in media containing different bile salts concentrations are displayed. Box-and-whisker plots represent each bile salt concentration (0.1, 0.25 and 0.5%).

Disk diffusion and Etest MIC

Ciprofloxacin, enrofloxacin, nalidixic acid, neomycin and sulfamethoxazole-trimethoprim disks did not inhibit the growth of most isolates (Figure 2.7). Cefoxitin, ceftiofur and streptomycin had a small diameter of inhibition; the means and standard deviations were 1.33cm \pm 0.48, 0.85cm \pm 0.64, and 0.56cm \pm 0.63, respectively. The bovine isolates had a small inhibition zone for tetracycline (0.76cm \pm 0.49), which was different from the observed for the swine isolates and the DSM 17677 that ranged from 4.5 to 5.1cm. Larger inhibition zones were observed for most of the isolates in the presence of ampicillin (2.75cm \pm 0.61), ceftriaxone (2.11cm \pm 0.62) and chloramphenicol (3.98cm \pm 0.52).

According to the results obtained for the Etest (Table 2.1), all isolates were resistant to ciprofloxacin and sulfamethoxazole-trimethoprim; 82.8% were resistant to tetracycline; 55.2% were resistant to cefepime and cefoxitin; 34.5% were resistant to ceftriaxone; 27.6% were resistant to ampicillin; and none of the isolates was resistant to chloramphenicol. About 55.2% of the isolates grew in the highest concentration of amikacin tested and only 1 isolate was resistant to gentamicin.

The multidrug resistance profile of the *Faecalibacterium sp.* isolates is shown in Table 2.2. A total of 19 different combinations of antibiotic resistance were observed. The isolates were resistant to, at least, 2 antibiotics (1 isolate) and up to 8 antibiotics (2 isolates). All 29 isolates tested showed multiresistance to ciprofloxacin and sulfamethoxazole-trimethoprim.

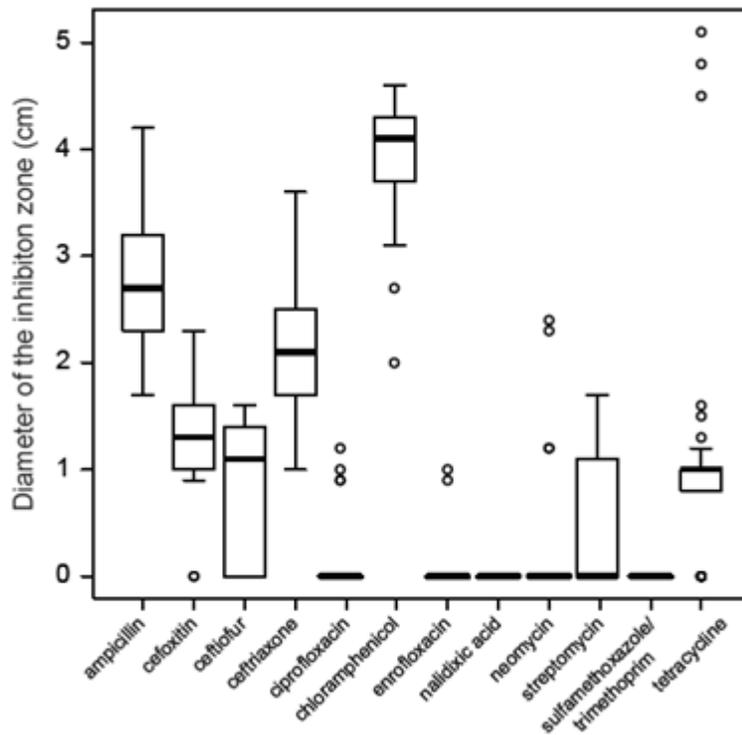


Figure 2.7: Disk diffusion agar assay.

Box-and-whisker plots depicting the diameter of the inhibition zone measured for each antimicrobial agent, as determined by the disk diffusion agar assay.

Table 2.1: E-test. Resistance profile.

Resistance profile of the *Faecalibacterium prausnitzii* isolated from feces of dairy calves and piglets, as determined by E-test (BioMérieux, Inc., Durham, NC). Distribution of the minimal inhibitory concentration (MIC) for the 29 isolates among the different antimicrobial concentrations (0.002 – 256 mg/μl). Shaded cells highlight the MIC range. Triple vertical bars mark the (approximate) breakpoint between sensitive and resistant. The resistance breakpoints are based on CLSI interpretative criteria for *Bacteroides fragilis* (ATCC 25285) (CLSI, 2012). No published MIC breakpoint for amikacin and gentamicin was found in the literature, therefore the highest concentration was considered as MIC.

Antimicrobial	Distribution of isolates (%)															Percentage of resistant isolates	MIC ₅₀	MIC ₉₀
	0.002 – 0.75	1 – 1.5	2 – 3	4	6	8	12	16	24	32	48	64	96	128	256			
Amikacin	-	-	-	-	3.4	-	3.4	3	7	6.9	10	6.9	3.4	-	55	55.2	256	256
Ampicillin	41.4	31	6.9	10	6.9	-	3.4	-	-	-	-	-	-	-	-	27.6	1	4
Cefepime	-	-	-	-	-	-	10	7	7	14	3.4	-	3.4	-	55	55.2	256	256
Cefoxitin	-	-	-	3.4	-	3.4	3.4	3	7	10	10	17	21	3.4	17	58.5	64	256
Ceftriaxone	-	-	-	3.4	3.4	17	17	7	7	10	-	6.9	10	3.4	14	34.5	24	256
Chloramphenicol	86.2	6.9	3.4	-	3.4	-	-	-	-	-	-	-	-	-	-	0	0.5	1
Ciprofloxacin	-	-	-	-	-	-	-	-	-	100	-	-	-	-	-	100	32	32
Gentamicin	3.4	6.9	21	31	21	10	-	-	-	3.4	-	-	-	-	3.4	3.4	4	8
Tetracycline	10.3	-	-	-	3.4	3.4	-	3	7	35	14	10	6.9	-	6.9	82.8	32	96
Sulfamethoxazole-trimethoprim	-	-	-	-	-	-	-	-	-	100	-	-	-	-	-	100	32	32

MIC₅₀, the MIC that inhibited at least 50% of the isolates; MIC₉₀, the MIC that inhibited at least 90% of the isolates.

Table 2.2: Multidrug resistance profile of *Faecalibacterium prausnitzii*.

Multidrug resistance profile of *Faecalibacterium prausnitzii* (n = 29 isolates) isolated from feces of dairy calves and piglets.

Resistance phenotype	Number of isolates
CIP-TRM	1
CIP-TRM-AMK	1
CIP-TRM-CEF	2
CIP-TRM-TET	1
CIP-TRM-AMK-TET	4
CIP-TRM-CEF-TET	2
CIP-TRM-CFX-TET	1
CIP-TRM-AMK-CEF-TET	1
CIP-TRM-AMK-CFX-TET	4
CIP-TRM-CEF-CET-TET	1
CIP-TRM-CFX-CET-TET	1
CIP-TRM-AMK-AMP-CEF-CFX	1
CIP-TRM-AMP-CEF-CET-TET	1
CIP-TRM-CEF-CET-CFX-TET	2
CIP-TRM-AMK-AMP-CEF-CET-TET	2
CIP-TRM-AMK-AMP-CEF-CFX-TET	1
CIP-TRM-AMK-AMP-CET-CFX-TET	1
CIP-TRM-AMK-AMP-CEF-CET-CFX-TET	1
CIP-TRM-AMK-AMP-CEF-CET-GET-TET	1

AMK, amikacin; AMP, ampicillin; CEF, cefepime; CET, ceftriaxone; CFX, ceftiofloxacin; CIP, ciprofloxacin; GET, gentamicin; TET, tetracycline; TRM, sulfamethoxazole-trimethoprim.

Phylogenetic analysis

A phylogenetic tree showing the relationships of 16S rDNA sequences from *Faecalibacterium* sp. with other bacteria belonging to the *Clostridiales* order and different families is shown in Figure 2.8. The sequences from our isolates were closely related with other 4 *Faecalibacterium prausnitzii* sequences (AJ270469, AJ270470, AJ413954, X85022). Since our *Faecalibacterium* sp. sequences had 100% match with

sequences from *Faecalibacterium prausnitzii*, and knowing that *prausnitzii* is the only specie described, we considered our isolates as *Faecalibacterium prausnitzii*.

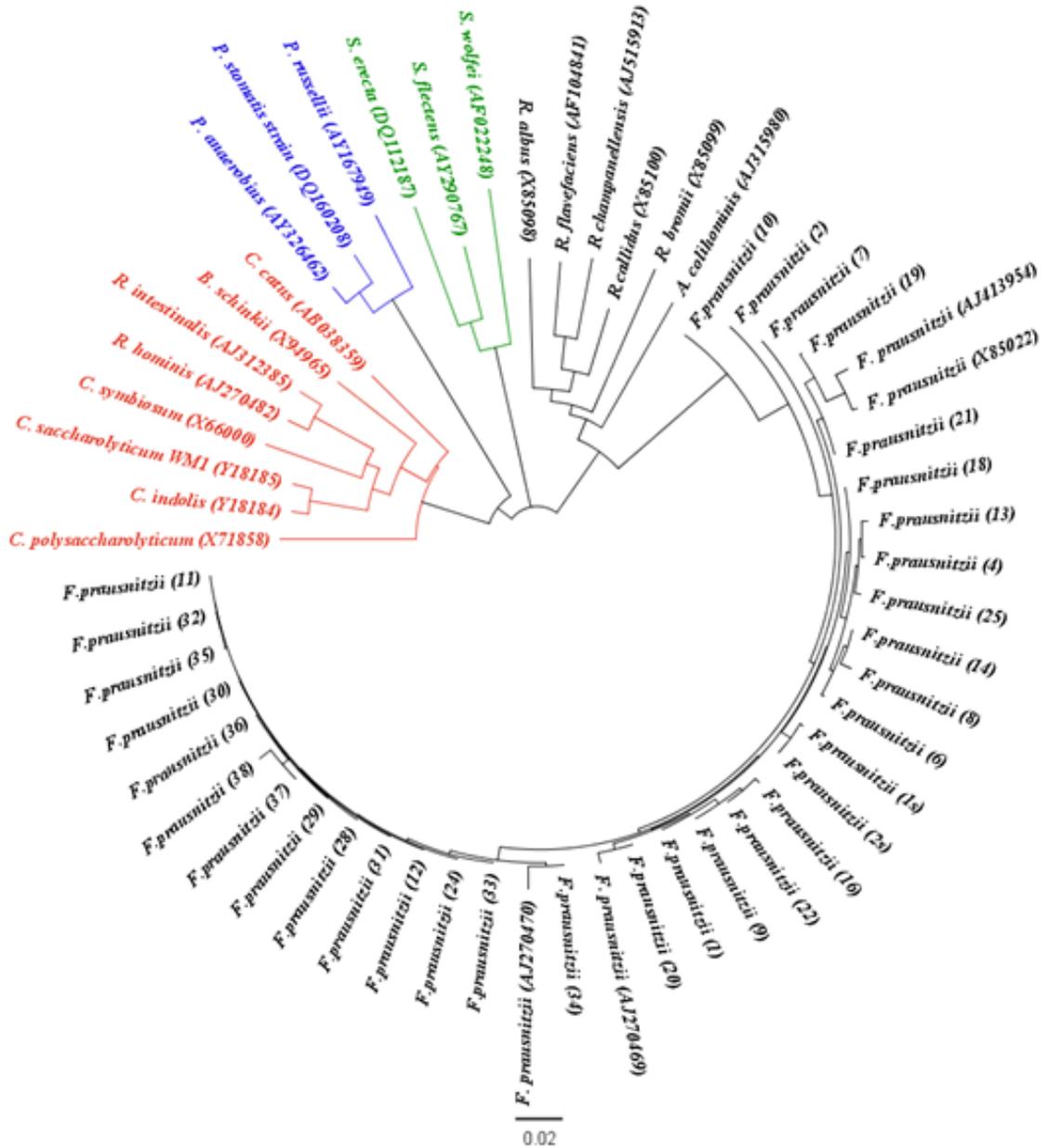


Figure 2.8: Phylogenetic tree.

Phylogenetic tree showing the relationships of 16S rDNA sequences of 33 *Faecalibacterium prausnitzii* isolates from our group with other bacteria of the belonging to the same order; *Ruminococcaceae* (black), *Syntrophomonadaceae* (green), *Peptostreptococcaceae* (blue), *Clostridiaceae* and *Lachnospiraceae* (red).

Discussion

Our study aim was the isolation and characterization of *Faecalibacterium prausnitzii* from feces of calves and piglets. *F. prausnitzii* has been the focus of many studies related to human health (Sokol et al., 2008; Balamurugan et al., 2010; Machiels et al., 2013), and the anti-inflammatory mechanisms (Qiu et al., 2013).

F. prausnitzii is extremely sensitive to oxygen and loses its viability if exposed to air for more than 2 minutes (Duncan et al., 2002). To survive in oxygenated niches within the human colon, *F. prausnitzii* uses an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen (Khan et al., 2012). The media used by our group as a transport and growth media has cysteine and yeast extract, which provides thiol and flavin, respectively. In addition, the ruminal fluid supplies acetate and other micronutrients required for growth.

The measurement of the SCFA metabolized by our isolates indicated that *F. prausnitzii* is an acetate consumer and butyrate producer, as previously described (Barcenilla et al., 2000; Duncan et al., 2004; Wrzosek et al., 2013). However, a substantial variability in the butyrate production was observed among the isolates. In general, isolates that were high butyrate producers also demonstrated high *in vitro* growth performance ($r = 0.68$). The two isolates from piglets showed the best growth performance and produced higher amounts of butyrate than the bovine and the DSM 17677 isolates.

The growth in adverse conditions, such as low pH or the presence of bile salts was evaluated in order to mimic the conditions encountered by *F. prausnitzii in vivo*. The low pH in the stomach or in the ruminant's abomasum could prevent or reduce the growth of

these bacteria. Our results are in agreement with previously reported pH values tolerated by *F. prausnitzii* (Lopez-Siles et al., 2012).

The fasting gastric pH observed in humans is about pH 1.7, which rose and peaked at pH 6.7 postprandially, then declined gradually back to the fasted state value in the next 2 hours (Dressman et al., 1990). The same study measured the pH in the duodenum, which varied between pH 5.4 and 6.1. The successful use of *F. prausnitzii* as probiotic will most likely depend on the administration method. According to the results obtained in our study, oral administration of *F. prausnitzii* would be recommended after feeding to avoid the low gastric pH, which could be deleterious to the bacterial cells. *F. prausnitzii* isolates were susceptible to the presence of bile salts *in vitro*. It has been reported that patients with hepatic and intestinal disorders have increased luminal concentration of bile salts (Duboc et al., 2013), which could explain the lower count of *F. prausnitzii* observed in those individuals.

On this study, all evaluated *F. prausnitzii* isolates were resistant to ciprofloxacin and sulfamethoxazole-trimethoprim. Additionally, most isolates were resistant to tetracycline, amikacin, cefepime and ceftazidime. To the best of our knowledge, this is the first study that evaluated antimicrobial susceptibility of *F. prausnitzii*. Resistance genes would allow the *F. prausnitzii* to survive and persist in the intestinal microbiota during antibiotic treatment; however, it could also act as reservoir of resistance genes and potentially transfer them to pathogenic bacterial species (van den Bogaard et al., 2002). It is our future interest to study the molecular mechanisms for antibiotic resistance in *F. prausnitzii* as well its potential role in resistance dissemination. Furthermore, *F.*

prausnitzii could be used as a model bacterium for studies of antibiotic resistance, since it is one of the most abundant intestinal microorganisms.

Aminoglycosides' entry in the bacterial cell is most efficiently achieved by energy obtained from electron transport using oxygen (or, alternatively, nitrate) as a terminal electron acceptor (Bryan et al., 1979). Therefore, anaerobes are less susceptible to aminoglycosides than aerobic bacteria due to impermeability (Magnet and Blanchard, 2005). High levels of resistance to aminoglycoside were expected due to the anaerobic nature of the isolates; however, only 55.2% and 3.4% of our *F. prausnitzii* isolates were resistant to the highest concentrations of amikacin and gentamicin, respectively.

About 83% of the isolates were resistant to tetracycline (MIC $\geq 16\mu\text{g/ml}$), while the MIC of the same antibiotic was considerably lower (MIC $< 0.2 \mu\text{g/ml}$) to the two swine and the human DSM 17677 isolates. The tetracycline resistance gene *tetW* is the most prevalent among anaerobic commensal gut bacteria and it has been found in the human fecal microbiota (Scott et al., 2000; Seville et al., 2009), pig feces (Kazimierczak et al., 2009) and bovine rumen (Barbosa et al., 1999). A possible tetracycline resistance gene could be present among isolates from calves. However, tetracycline is not an antibiotic routinely used by the farm where the calves' samples were collected. Resistance genes are present even in antibiotic-free animals and environmental sources such as water and soil are probably responsible for its dissemination (Chee-Sanford et al., 2001).

In summary, our results suggested that the isolates were consuming acetate present in the media and producing butyrate ($r = -0.87$) and that butyrate production correlated positively with bacterial growth ($r = 0.68$). The optimal pH for growth ranged

between 5.5 and 6.7, while most isolates were inhibited by of the lowest concentration of bile salts tested (0.1%). Antimicrobial resistance profile showed that most isolates of *F. prausnitzii* were resistant against ciprofloxacin and sulfamethoxazole-trimethoprim. More than 50% of the isolates were resistant to tetracycline, amikacin, cefepime and ceftiofur. A total of 19 different combinations of multidrug resistance were observed among the isolates. Our results provide new insights into the cultural and physiological characteristics of *Faecalibacterium prausnitzii* illustrating large variability in SCFA production, *in vitro* growth, sensitivity to bile salts, and antibiotic resistance and suggesting that future probiotic candidates should be carefully studied before elected for *in vivo* studies. The study of the use of the *Faecalibacterium prausnitzii* as a livestock probiotic is of our future interest, as well as the resistance genes and their potential transmission to relevant enteric pathogens such as *Salmonella* spp. and *E. coli*.

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CHAPTER 3: Oral administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers

Abstract

Probiotics are a promising alternative to improve food animal productivity and health. However, scientific evidence that specific microbes can be used to benefit animal health and performance is limited. The objective of this study was to evaluate the effects of administering a live culture of *F. prausnitzii* to newborn dairy calves on subsequent growth, health, and fecal microbiome. Initially, a safety trial was conducted using 30 newborn bull calves to assess potential adverse effects of the oral and rectal administration of *F. prausnitzii* to neonatal calves. No adverse reactions, such as increased body temperature, heart and respiratory rates, were observed after the administration of the treatments. All calves survived the experimental period, and there was no difference in fecal consistency score, attitude, appetite and dehydration between the treatment groups. The rectal route was not an efficient practice while the oral route ensures that the full dose is administered to the treated calves. Subsequently, a randomized field trial was completed in a commercial farm with preweaned calves. A total of 554 Holstein heifers were enrolled in one of two treatment groups: treated calves (FPTRT) and non-treated calves (control). Treated calves received two oral doses of *F. prausnitzii*, one at enrollment (1st week) and another one week later. The FPTRT group presented significantly lower incidence of severe diarrhea (3.1%) compared with the control group (6.8%). Treated calves also had lower mortality rate associated with severe

diarrhea (1.5%) compared to control calves (4.4%). Furthermore, FPTRT calves gained significantly more weight, 4.4 kg over the preweaning period, than control calves. The relative abundance of *F. prausnitzii* in the fecal microbiota was significantly higher in the 3rd and 5th weeks of life of FPTRT calves than of the control calves, as revealed by sequencing of the 16S rRNA gene. Our findings showed that oral administration of *F. prausnitzii* improves gastrointestinal health and growth of preweaned calves, supporting its use as a potential probiotic.

Introduction

Diarrhea is one of the major causes of morbidity in preweaned dairy heifers, resulting in significant economic losses and animal suffering. The U.S. Department of Agriculture (USDA) surveyed morbidity and mortality rates in preweaned heifers from heifer-raising operations in 2006 (USDA, 2010a). Digestive disorders (i.e. diarrhea and bloat) affected 23.9% of the population; the overall mortality rate was 7.8% and 56.5% of the deaths were caused by digestive problems, mainly diarrhea (USDA, 2010a). The high incidence of diarrhea is a persistent problem. Four years after the 2006 survey, the USDA reported that 25.3% of preweaned calves had diarrhea; the overall mortality rate decreased to 4.2% and digestive problems were responsible for one third of the deaths. (USDA, 2012). The treatment of diarrhea with antibiotics, supportive care and labor is expensive, thus development of new strategies to prevent diarrhea will maximize overall productivity, animal welfare and profitability.

Historically, sub-therapeutic doses of antibiotics have been used as growth promoters and disease prophylaxis in livestock animals. However, due to the emergence

of antibiotic resistant microbes, the use of antibiotics as growth promoters is now banned in the European Union and is limited in the United States. Effective in the spring of 2015, the Food and Drug Administration published new directives for the use of antimicrobials in the feed of livestock in the United States, creating a new category of products called veterinary feed directive drugs (VFD drugs). Under this directive, a VFD drug intended for use in or on animal feed must be used under the professional supervision of a licensed veterinarian (FDA, 2015). The use of probiotics is a potential alternative to the use of antimicrobials in livestock feed.

Faecalibacterium prausnitzii belongs to the phylum Firmicutes and is an obligate anaerobic, Gram-positive, rod-shaped, butyrate producing microorganism (Barcenilla et al., 2000; Foditsch et al., 2014) that is abundant in the feces of several animals (Suau et al., 2001; Hold et al., 2003; Lund et al., 2010; Walker et al., 2011; Nava and Stappenbeck, 2011; Oikonomou et al., 2013; Haenen et al., 2013). In humans, high levels of *F. prausnitzii* were associated with obesity (Balamurugan et al., 2010), while a low abundance of *F. prausnitzii* was linked to Inflammatory Bowel Disease (IBD), (i.e. Crohn's disease (Sokol et al., 2008; Wang et al., 2013) and ulcerative colitis) (Machiels et al., 2013). *F. prausnitzii* has anti-inflammatory properties, which have been demonstrated *in vitro* with cultured cells and *in vivo* with trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice models (Sokol et al., 2008; Qiu et al., 2013; Martin et al., 2015; Miquel et al., 2015). *F. prausnitzii* induces the production of the anti-inflammatory cytokine IL-10 and reduces the secretion of the pro-inflammatory cytokines IFN- γ and IL-12 (Miquel et al., 2015). Furthermore, *F. prausnitzii* and its supernatant decreased the severity of colitis in IBD mice models (Sokol et al., 2008; Qiu et al., 2013).

Additionally, the butyrate produced by *F. prausnitzii* is both an energy source to enterocytes and act as an anti-inflammatory agent (Segain et al., 2000).

In preweaned Holstein calves, higher relative abundance of *F. prausnitzii* in the first week of life was associated with enhanced weight gain and reduced incidence of diarrhea (Oikonomou et al., 2013). A recent study conducted by our research group isolated 203 *F. prausnitzii* isolates from the feces of calves and piglets (Foditsch et al., 2014). In that study, 40 genetically distinct *F. prausnitzii* isolates were selected for further characterization. A large variability was observed among isolates for *in vitro* short chain fatty acids (SCFA) metabolism, growth, antibiotic resistance, and sensitivity to low pH and bile salts. Based on this data, 4 isolates with desirable characteristics were selected and used as part of a probiotic cocktail in the *in vivo* studies described herein.

The first objective of the present study was to assess the safety of the oral and rectal administration of a live culture of *F. prausnitzii*. The second objective was to evaluate the effects of the oral administration of *F. prausnitzii* on preweaned calves' survivability, incidence of severe diarrhea, weight gain and fecal microbiome.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of The Animal Welfare Act of 1966 (AWA) (P.L. 89–544) and its amendments 1970 (P.L. 91–579); 1976 (P.L. 94–279), and 1985 (P.L. 99–198) which regulate transport, purchase, care, and treatment of animals used in research. The research protocol was reviewed and approved by the Institutional Animal Care and use Committee of Cornell University

(Protocol number: 2012-0055). The administration of *F. prausnitzii* culture to calves housed on the commercial dairy farm was authorized by the farm owner, who was aware of all experimental procedures.

Treatment preparation

Four *F. prausnitzii* isolates were selected from our culture collection based on greater capacity for *in vitro* butyrate production, growth and tolerance to low pH and bile salts as previously evaluated by our research group (Foditsch et al., 2014). The four isolates (ref. numbers 34, 35, 1S, and 2S; Foditsch *et al.* 2014) were cultured individually in a medium supplemented with 30% ruminal fluid as previously described (Foditsch et al., 2014). The average colony forming units (CFU) of each isolate was 1.43×10^7 CFU/mL. Equal volumes of the four cultures were mixed, frozen in 50 mL sterile disposable centrifuge tubes with 15% glycerol, and stored at -80°C . For quality assurance purposes, the CFU/mL was calculated at the time of administration; the average CFU was 1.34×10^7 CFU/mL, confirming that a live bacteria culture was administered to the calves. The placebo given to control calves in the safety trial contained the same growth medium without the bacterial culture.

Safety Trial

Animals and facilities

The safety trial was conducted from March to May of 2014 at the College of Veterinary Medicine, Cornell University. Thirty bull calves were obtained from a

commercial dairy farm that milked 2,800 Holstein cows near Ithaca, New York, USA. Immediately after birth, calves were removed from the maternity pens and were placed in dry sawdust bedded pens. Four liters of pooled, non-pasteurized colostrum from primiparous cows was administered to calves by esophageal feeder. Calves were transported from the farm to the College of Veterinary Medicine facility where they were housed individually in concrete stalls bedded with pine shavings. Calves were kept in the same stall during the 14 days of the research trial. Non-pasteurized whole milk was fed twice daily at approximately 10% of the body weight and water was available *ad libitum*.

Study design and data collection

A randomized clinical trial design was used. Thirty calves were randomly allocated into one of four treatment groups as follows: oral control (O-CONTROL, n = 5) calves received 80 mL of a placebo solution orally; oral treatment (O-FPTRT, n = 10) calves received 80 mL of live culture of *F. prausnitzii* orally; rectal control (R-CONTROL, n = 5) calves received 80 mL a placebo solution rectally; and rectal treatment (R-FPTRT, n = 10) calves received 80 mL of live culture of *F. prausnitzii* rectally. Control groups received a placebo containing the growth medium without the bacterial culture. Oral treatments were administered through an esophageal tube and rectal treatments were given with a 6 cm drench tube attached to a syringe. Treatments were administered on the second day of life in order to avoid interactions between colostrum's immune cells and the bacteria administered. Due to the *F. prausnitzii* sensitivity to low pHs (Foditsch et al., 2014), the treatments were administered 1 hour after milk feeding, when the abomasal pH increases approximately from 2 to 6 (Ahmed et

al., 2002). Calf health was assessed twice daily for the following parameters; fecal consistency (0 = well-formed; 1 = semi-formed; 2 = loose or watery feces not containing blood; and 3 = loose or watery feces containing blood), dehydration (0 = euhydrated; 1 = skin tented 2 to 6s; 2 = skin tented 6 to 10s; and 3 = skin tented \geq 10s), attitude (0 = alert; 1 = depressed; and 2 = non responsive) and appetite (0 = normal; 1 = consumed ½ bottle; 2 = consumed 1/4 bottle; and 3 = forced fed). The effect of treatment on fecal consistency, dehydration, attitude and appetite scores was assessed using ordinal logistic regression models fitted in JMP Pro 11 (SAS Institute Inc., NC, USA). The independent variables offered to the model were treatment group, age in days, and interaction terms between treatment and age.

Randomized Field Trial

Farm and management

The study was conducted from November 2014 to April 2015 at a commercial dairy farm. Immediately after birth, female calves were removed from the maternity pens, weighed, and placed in dry sawdust bedded pens. Four liters of pooled non-pasteurized colostrum from primiparous cows was administered to calves by esophageal tubing and calves had their umbilicus dipped in 7% iodine solution.

Newborn calves were transported twice daily from the maternity area to the calf barn. Calves were housed in a green-house barn divided into 30 identical pens with positive ventilation. Pens were separated by steel gates and calves were moved by birth order into each pen until maximum capacity was reached (20 calves/pen). Calves remained in the same pen until weaning.

Calves were fed *ad libitum* acidified non-saleable milk using a fully automated system with 6 nipples per pen. Acidification was performed in a sealed stainless-steel tank where cold milk (5°C) was mixed with organic acid under constant homogenization until a pH of 4.5 was reached. Acidified milk was directed to a smaller stainless-steel tank, warmed, and distributed to the pens. Acidified milk was offered to the calves from day 1 to 56 of life. All calves were weaned by reducing the daily milk availability starting on day 42 until complete absence of acidified milk at 57 days of life. Water and solid feed (calf starter mix) were offered *ad libitum* to all calves.

Health-related events (e.g. otitis, pneumonia and severe diarrhea) were recorded and treated as needed by farm employees. One dose of the macrolide antibiotic Zuprevo (Merck Animal Health, Summit, NJ) was given by the farm to all female calves at 8 to 14 days of age as a metaphylactic for bovine respiratory disease. All calves were disbudded by heat cauterization at approximately 4 weeks of age.

Study design and data collection

The treatment administered was a live microorganism and cross-contamination between calves in the same group was possible. Therefore, all calves in the same pen were enrolled in the same treatment group (oral treatment with *F. prausnitzii* (FPTRT) or control, at 5 ± 2 days of life). The first group was randomly selected, and the subsequent groups were alternated between control and FPTRT, resulting in the same number of calves for each treatment group per week.

The rumen microbiota gradually changes from aerobic to anaerobic during the calves' first weeks of life (Minato et al., 1992; Beharka et al., 1998; Jami et al., 2013),

therefore we chose to treat calves in the field trial with two 40ml doses of *Faecalibacterium sp.* culture, one dose at enrollment (1st week of life) and a second dose one week later, instead of only administering one 80ml dose on the second day of life, to increase the chances of its colonization in the large intestine. The control calves did not receive a placebo treatment or sodium bicarbonate.

In a group feeding system it was not possible to determine the time each calf was fed and to account for the increase of the abomasal pH, as in the safety trial. Additionally, the milk fed in the commercial farm was acidified and, as mentioned previously, *F. prausnitzii* is highly sensitive to low pHs. Therefore, we administered 130 mL of sodium bicarbonate (90 mg/mL) orally to FPTRT calves to buffer the low pH of the abomasum before administering the culture. Sodium bicarbonate at 0.6% was used previously to increase the pH of fermented waste milk to 6.0 in a study evaluating feeding value of fermented waste milk (Keith et al., 1983). In that study, calves received one of the four milk treatments (fresh milk, fresh waste milk, fermented waste milk, fermented waste milk with sodium bicarbonate) for 42 days and weight gain was not significantly different between groups. We estimated the dose of sodium bicarbonate considering the milk present in the abomasum and did not expect effects of the two administrations of bicarbonate, other than the neutralization of the abomasal pH prior to *F. prausnitzii* administration.

A total of 554 Holstein heifers were enrolled in the field trial, with 296 allocated to the control group and 258 to the FPTRT group. A subset of 35 calves/treatment was selected randomly for collection of fecal DNA through rectal swabs and evaluation of fecal microbiome. From these 70 calves, 45 calves (n=22, control; n=23, FPTRT) were

selected randomly for evaluation of serum β -hydroxybutyrate (BHBA) concentrations. Blood samples were collected from the jugular vein and fecal samples were collected using rectal swabs on the 1st (enrollment), 3rd, 5th and 7th weeks of life. Blood samples were centrifuged at 3000 x g for 10 minutes, after which serum was obtained. Serum and swabs were stored at -20°C until assayed. Fecal consistency scores were recorded weekly using a four level scoring system, as described in the safety trial. Calves were weighed using a Waypig 15 digital scale (Vittetoe Inc., Keota, IA, USA) at birth and again at weaning (56 ± 3 days of life; n=141 for the control group and n=146 for FPTRT group). Weight gain was calculated by subtracting the birth weight from the weight at weaning. The weight gain was divided by the age in days at the second weight (56 ± 3 days) to obtain the average daily gain (ADG). Due to equipment constraints, weights of a subset of calves (303) were obtained. Severe diarrhea and death events records were acquired from the farm's software (Dairy-Comp 305; Valley Ag Software, Tulare, CA, USA). Severe diarrhea was defined as dehydrated calves with loose or watery feces that were treated by the farm employees with oral electrolytes or intravenous fluids. Farm employees were blind to the treatment groups.

DNA extraction, amplification and purification

DNA of the fecal material from the 4 time points (1st, 3rd, 5th and 7th week of life) was extracted following the protocol previously used by Oikonomou *et al.* 2013. Briefly, each rectal swab was placed in 1.5 ml of nuclease-free water (Life Technologies, Grand Island, NY) and vortexed for at least two minutes. The swab was then removed and the sample centrifuged for 10 min at 13200g. The supernatant was discarded and the

remaining pellet was resuspended in 400 μ l of nuclease-free water. Isolation of microbial genomic DNA was performed by using a QIAamp DNA minikit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Besides the proteinase K and the Buffer AL, 40 μ l (10 mg/ml) of lysozyme (Sigma-Aldrich, St. Louis, MO) were added to the sample and the incubation at 56°C was extended for 12 h. The DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm.

The 16S rRNA gene was amplified by PCR from individual metagenomic DNA samples using barcoded primers. For amplification of the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene, primers 515F and 806R were used according to a previously described method optimized for the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) (Caporaso et al., 2012). The earth microbiome project (Gilbert et al., 2010) was used to select 280 different 12-bp error-correcting Golay barcodes for the 16S rRNA PCR, as previously described (Caporaso et al., 2012). The 5'-barcoded amplicons were generated in triplicate using 1 μ L DNA template, 2 X EconoTaq® Plus Green Master Mix (Lucigen®, Middleton, WI, USA), and 5 μ M of each primer. The PCR conditions for the 16S rRNA gene consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s, and a final elongation step of 72°C for 10 min. Blank controls, in which no DNA was added to the reaction, were performed for quality assurance. Replicate amplicons were pooled and visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/mL ethidium bromide. Amplicons were purified with a PCR DNA extraction kit (IBI

Scientific, Peosta, IA, USA) and the purified 16S rRNA amplicons were quantified using the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA, USA) and a Qubit fluorometer (Life Technologies).

Sequence library analysis and statistical analysis

Amplicon DNA aliquots were standardized to the same concentration and then pooled. Final equimolar libraries were sequenced using the MiSeq reagent kit v2 (300 cycles) on the Illumina MiSeq platform. The obtained 16S rRNA gene sequences were processed using the open source software pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0-dev (Caporaso et al., 2010). Sequences were filtered for quality using established guidelines (Bokulich et al., 2013). Sequences were binned into Operational Taxonomic Units (OTU) based on 97% identity using UCLUST (Edgar, 2010) against the Greengenes reference database (McDonald et al., 2012), May 2013 release. Low-abundance clusters were filtered and chimeric sequences were removed using USEARCH (Edgar, 2010). The classification of reads at multiple taxonomic levels (kingdom, phylum, class, order, family, and genus) used in the present study were obtained from the MiSeq Reporter and are based on the Greengenes database cited above.

Using the obtained OTU information, we evaluated each sample's richness using the Chao1 index, which is a nonparametric estimator of the minimum richness (number of OTU) and is based on the number of rare OTU (singletons and doublets) within samples. Microbiota diversity was measured using the Shannon index, which is a nonparametric diversity index that combines estimates of richness (the total number of OTU) and evenness (the relative abundance of OTU).

β-hydroxybutyrate analysis

β-hydroxybutyrate was measured for 180 serum samples. The Autokit Total Ketone Bodies (Wako Pure Chemical Industries Ltd., Richmond, VA, USA), a cyclic enzymatic method based on the oxidation of BHBA to acetoacetate by BHBA dehydrogenase, was chosen to measure serum BHBA due to its high sensitivity and high specificity.

Statistical analysis

Pearson chi-square test was used to compare the following categorical variables between treatment groups: parity of the dam (1, 2, 3), occurrence of twins (yes or no), and calving ease of the dam (assisted or non-assisted).

Kaplan-Meier survival analysis were performed using MedCalc Statistical Software version 13.1.2 (MedCalc Software, Ostend, Belgium) to compare the effect of oral *F. prausnitzii* administration on the incidence of severe diarrhea cases, on the mortality rate caused by severe diarrhea and on the overall mortality rate.

The effect of oral administration of *F. prausnitzii* on fecal consistency score was assessed using an ordinal logistic regression model fitted in JMP. The independent variables offered to the model were treatment group (control, FPTRT), week of life (1-8), and interaction term between treatment and week.

The effects of oral administration of *F. prausnitzii* on weight gain and ADG were evaluated by linear regression models fitted in JMP with calf as the experimental unit. Variables offered to the models included treatment (control and FPTRT), birth weight,

age at enrollment, age at weaning, parity of the dam (1, 2, 3), occurrence of twins, and calving ease of the dam (assisted or non-assisted). The interaction terms between treatment groups and all independent variables were evaluated in the model. Pen was fitted as a random effect. Manual backward variable elimination was undertaken considering main effects and interactions, which were retained in the model when $P \leq 0.05$.

Additionally, the relative abundance of *F. prausnitzii* in the 1st week of life of the subset of 70 calves was dichotomized in LowFP and HighFP. The mean relative abundance of *F. prausnitzii* and 95% confidence intervals were 0.42% (0.30 – 0.54) for the LowFP calves (n=20 control, n=18 FPTRT) and 17.99% (12.99 – 23.00) for the HighFP calves (n=15 control, n=17 FPTRT). ANOVA was used to evaluate the effect of the low and high abundance of *F. prausnitzii* in the first week of life on the weight gain of this subset of calves.

Faecalibacterium, Firmicutes and Bacteroidetes mean relative abundances, Firmicutes to Bacteroidetes ratio, and BHBA concentration were each compared using multiple linear mixed regression models in JMP. Variables offered to the models included treatment group, week of life, and the interaction terms between these two variables. Calf and pen were fitted as random effects. Number of OTU, Chao1 and Shannon indexes means was estimated using a similar linear mixed regression model accounting for the number of sequences to reduce bias.

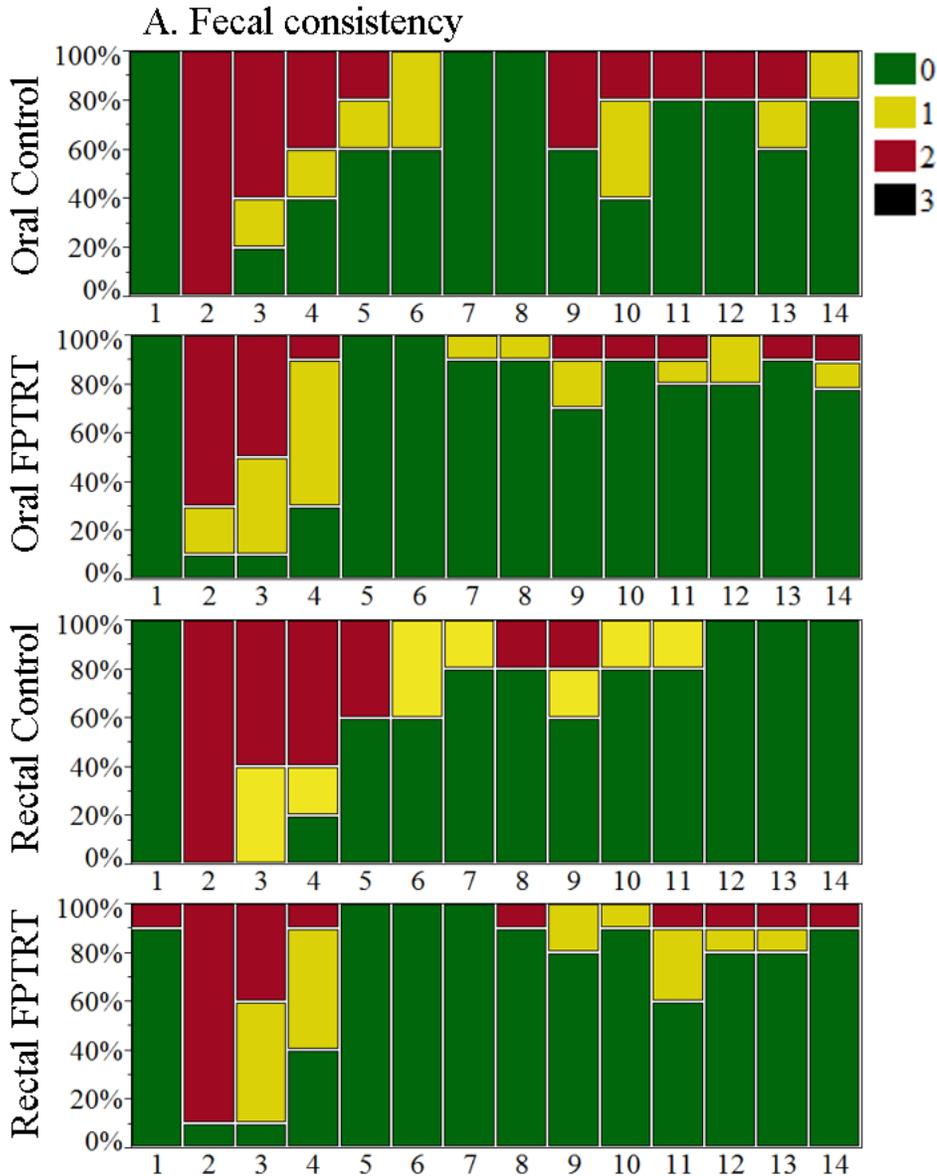
Results

Safety Trial

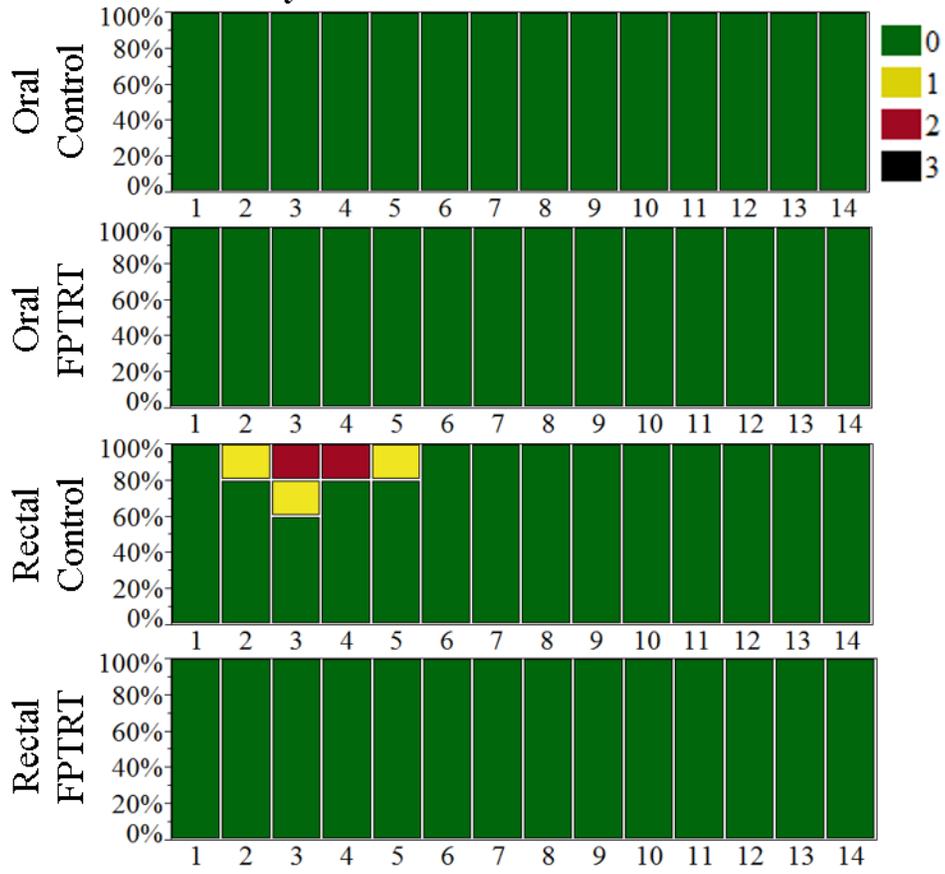
No adverse reactions, such as increased body temperature, heart and respiratory rates, were observed after the administration of the treatments and during the following days. All 30 bull calves survived the experimental period and there was no difference in fecal consistency score, attitude, appetite and dehydration between the 4 treatment groups ($P \geq 0.05$), as shown in Figure 3.1. We concluded that it was safe to administer *F. prausnitzii* culture to newborn calves. Although the rectal administration was a promising way of by-passing the low pH of the abomasum and the detrimental effect of bile salts, it was not an efficient practice. Most of the infused liquid was promptly excreted by the calf. Therefore, the oral route was selected for the field trial.

Figure 3. 1: Distribution of scores of fecal consistency, dehydration, attitude and appetite during the 14 days of life of calves. Safety trial.

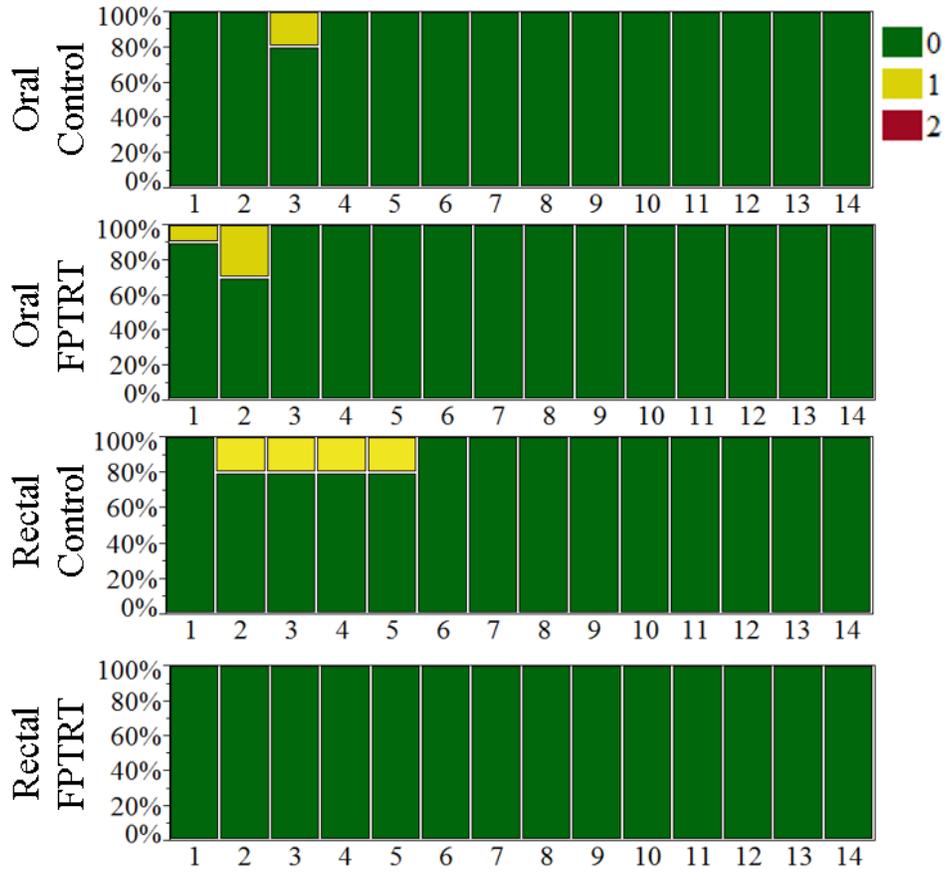
Distribution of scores of A) fecal consistency (0 = well-formed; 1 = semi-formed; 2 = loose or watery feces not containing blood; and 3 = loose or watery feces containing blood), B) dehydration (0 = euhydrated; 1 = skin tented 2 to 6s; 2 = skin tented 6 to 10s; and 3 = skin tented ≥ 10 s), C) attitude (0 = alert; 1 = depressed; and 2 = non responsive) and D) appetite (0 = normal; 1 = consumed $\frac{1}{2}$ bottle; 2 = consumed $\frac{1}{4}$ bottle; and 3 = forced fed) during the 14 days of life of the calves in the safety trial by treatment group.



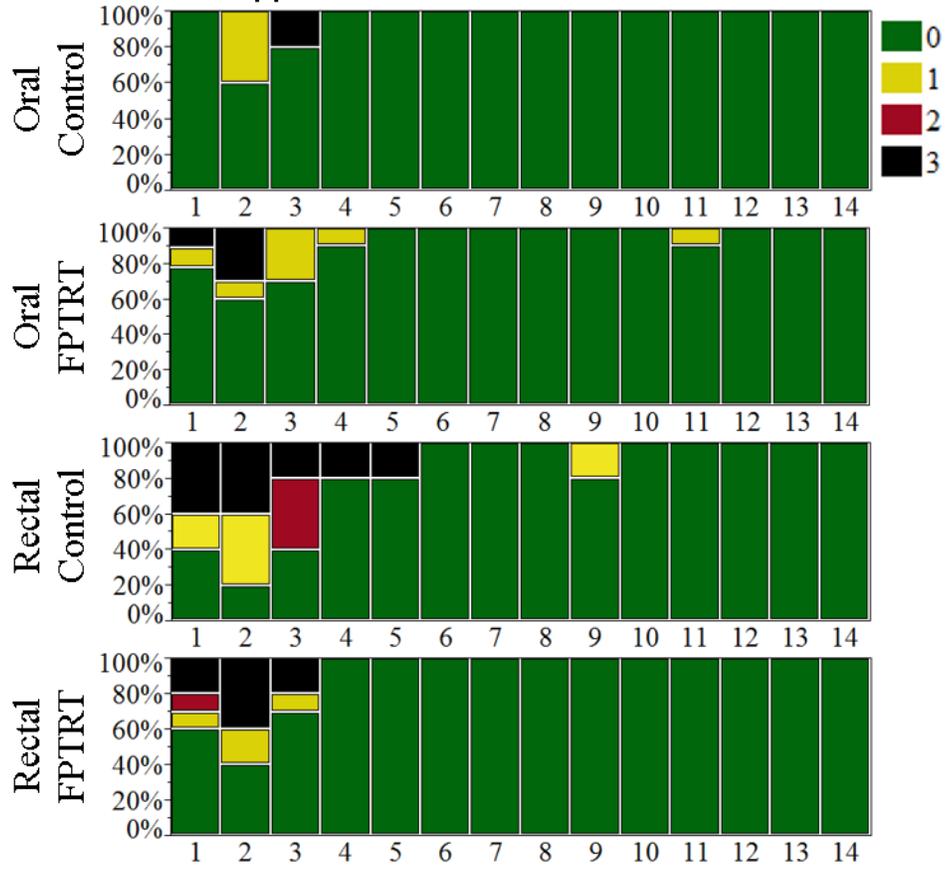
B. Dehydration



C. Attitude



D. Appetite



Randomized Field Trial

A total of 554 Holstein heifers were enrolled in this randomized field trial, 296 in the control group and 258 in the FPTRT group. A total of 22 were twins, 12 in the control group (4.10%) and 10 in the FPTRT group (3.89%; $P = 0.99$). Six control calves (2.05%) and 7 FPTRT calves (2.72%) were born with assistance ($P = 0.60$). The numbers of calves born from first lactation cows were 166 for control calves (56.66%) and 124 (48.25%) for FPTRT calves; from second lactation cows were 70 in the control group (23.89%) and 72 in the FPTRT (28.02%), and from third or more lactations cows were 57 in the control group (19.45%) and 61 in the FPTRT (23.74%), $P = 0.14$).

Calves that were treated with *F. prausnitzii* had significantly lower incidence of severe diarrhea over the preweaning period compared to the controls, 3.1% and 6.8%, respectively ($P = 0.05$), as depicted in Figure 3.2. Mortality rate associated with severe diarrhea was also significantly lower for FPTRT calves, 1.5%, compared to control calves, 4.4% ($P = 0.05$), and the overall mortality was numerically lower for the FPTRT group compared to the control, 3.9% and 6.1%, respectively ($P = 0.17$). Survival analysis graphs are presented in Figure 3.3.

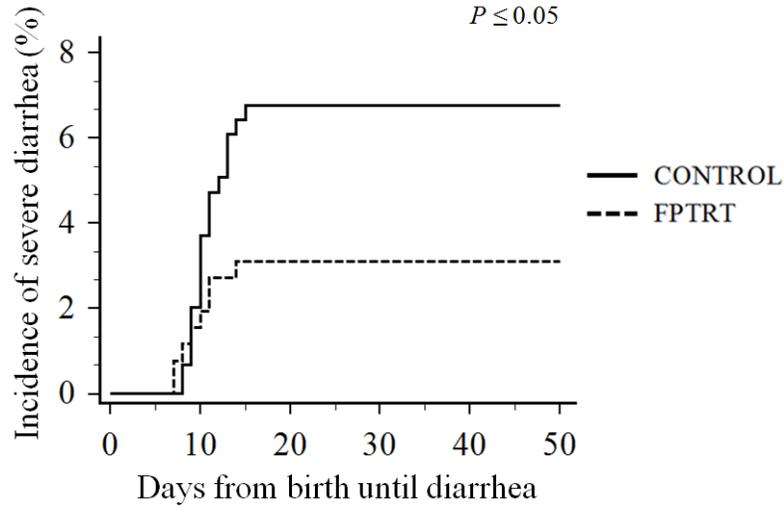


Figure 3. 2: Incidence of severe diarrhea cases from birth to weaning of dairy calves. Field trial.

Effect of *F. prausnitzii* administration on incidence of severe diarrhea according by treatment groups: FPTRT (N = 258) or control (N = 296). Severe diarrhea was defined as dehydrated calves with loose or watery feces that were treated by the farm employees with oral electrolytes or intravenous fluids.

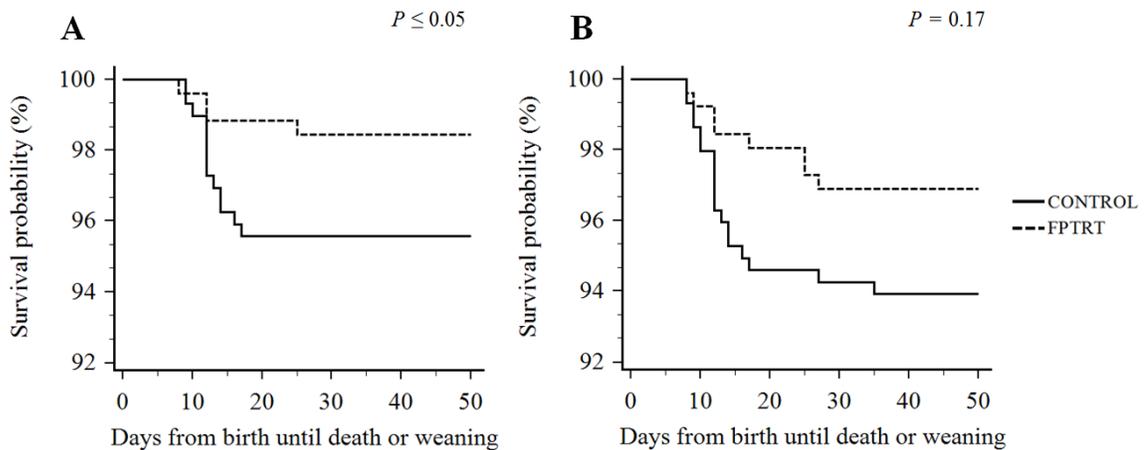


Figure 3. 3: Effect of *F. prausnitzii* administration on mortality rate of preweaned dairy calves. Field trial.

Kaplan-Meier survival analysis from birth to weaning of dairy calves by treatment groups: FPTRT (N = 258) or control (N = 296). A) Effect of *F. prausnitzii* administration on mortality rate related to severe diarrhea. B) Effect of *F. prausnitzii* administration on overall mortality.

The fecal consistency scores did not differ between treatments ($P = 0.38$). Figure 3.4 describes the percentage of each fecal consistency score according to study group during the 8 weeks of life of the treatment calves. Week of life had a significant effect on fecal consistency score ($P < 0.0001$). A higher incidence of diarrhea (score 2 and 3) was observed during the first 2 weeks of life.

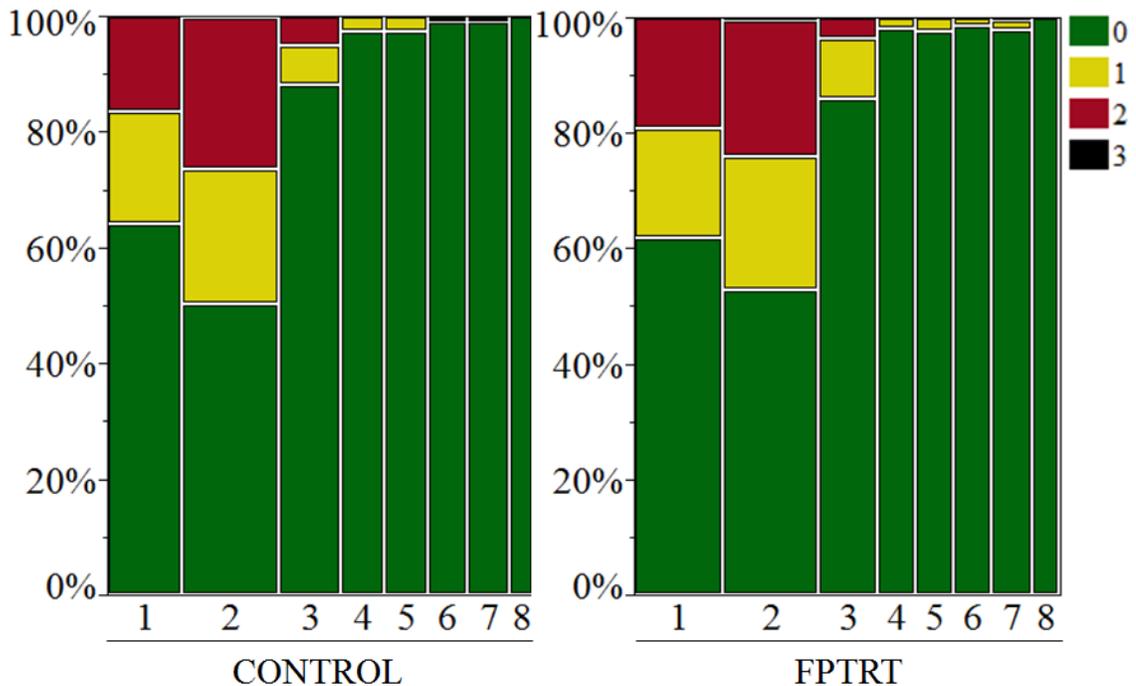


Figure 3. 4: Mean fecal consistency score (0-3) during the preweaning period. Field trial.

Dairy calves' fecal consistency score distribution during the 8 weeks of life by treatment groups: control and FPTRT. (0 = well-formed fecal samples; 1 = semi-formed fecal samples; 2 = loose or watery feces not containing blood; and 3 = loose or watery feces containing blood).

Calves in the FPTRT group gained significantly more weight than calves in the control group during the preweaning period ($P < 0.05$). Birth weight, weaning weight, weight gained during the preweaning period and ADG values are presented in Table 3.1 - A. When comparing calves with high or low prevalence of *F. prausnitzii* in the 1st week of life, the treatment had a significant effect on weight gain when administered to HighFP calves ($P < 0.05$), as shown in Table 3.1 - B.

Table 3.1: Weight gained and average daily gain during the preweaning period. Field trial.

A) Effect of oral administration of *F. prausnitzii* to newborn dairy calves weight gain during the preweaning period (N = 303). B) Effect of oral administration of *F. prausnitzii* to newborn dairy calves with high or low relative abundance of *F. prausnitzii* in the 1st week of life (LowFP and HighFP; N = 70).

A		<u>Control</u>		<u>FPTRT</u>		<i>P</i> -value
		Mean	SE	Mean	SE	
Birth weight, kg		36.0	0.32	36.8	0.27	0.08
Weaning weight, kg		75.9	1.08	80.2	1.05	<0.0001
Weight gain, kg		38.4	1.09	42.8	1.06	0.02
ADG, kg		0.68	0.02	0.77	0.02	0.01

B		<u>Control</u>		<u>FPTRT</u>		<i>P</i> -value
		Mean	SE	Mean	SE	
Weight gain, kg	LowFP	36.5	2.28	40.3	2.41	0.27
	HighFP	39.3	2.71	48.2	2.62	0.03
ADG, kg	LowFP	0.65	0.04	0.72	0.04	0.18
	HighFP	0.70	0.05	0.85	0.04	0.03

From the 280 rectal swabs collected, DNA was successfully extracted from 264 samples. Quality-filtered reads for 16S sequences yielded a total of 16,266,816 sequences with an average coverage of 61,617 sequences per sample. The mean number of sequences per sample and the 95% confidence interval were: 62,218 (60,209 - 64,227) for the control group's samples and 61,033 (59,055 - 63,012) for the FPTRT group's

samples. The effect of the interactions between the treatment groups and the week of life on the OTU, Chao 1 richness index and the Shannon diversity index are presented in Figure 3.5. There was a significant effect of week of life on the Chao ($P = 0.04$) and Shannon ($P < 0.0001$) indexes.

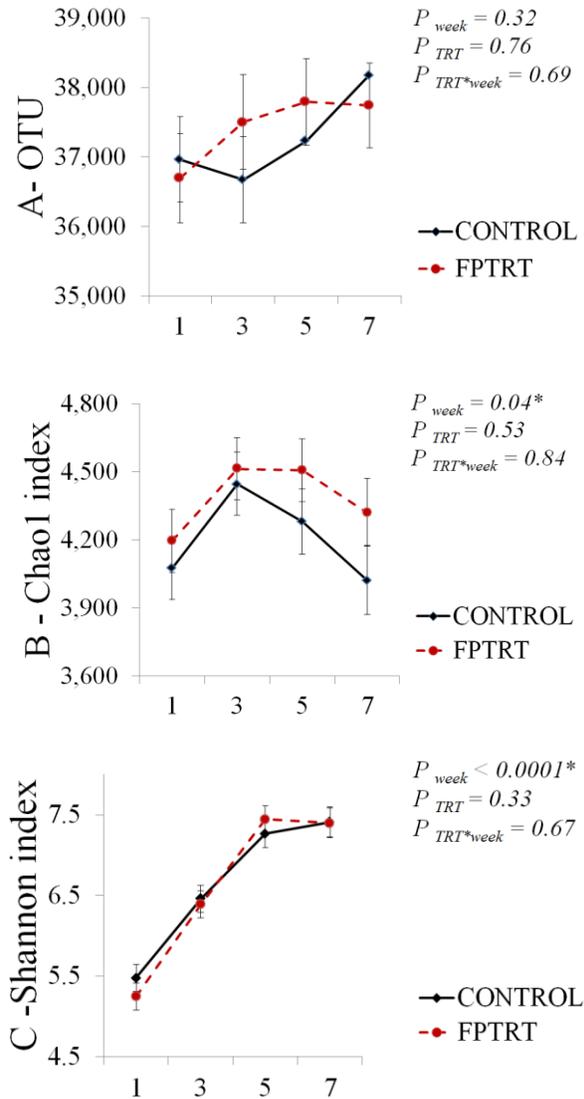


Figure 3. 5: Operational taxonomic unit (OTU), Chao1 index and Shannon index. Field trial.

OTU (A), Chao1 index (B) and Shannon index (C) according to treatment group. The error bars represent standard errors of the means.

The mean relative abundance of the genus *Faecalibacterium* was significantly higher in the FPTRT group in the 3rd and 5th weeks of life ($P < 0.05$) compared to the control group, as illustrated in Figure 3.6. Other bacterial genera were not significantly different between the study groups. The thirty most common genera for the two treatment groups are shown in a heat map (Figure 3.7). *Faecalibacterium* (mean 13.0%), *Bacteroides* (mean 12.2%), *Ruminococcus* (mean 10.8%), *Blautia* (mean 6.5%), and *Prevotella* (mean 5.6%) were the five most prevalent genera during the preweaning period. *Escherichia* was the 9th most prevalent genus (mean 3.3%), with an average prevalence of 10% in the first week of life and decreasing to less than 0.2% in the 7th week.

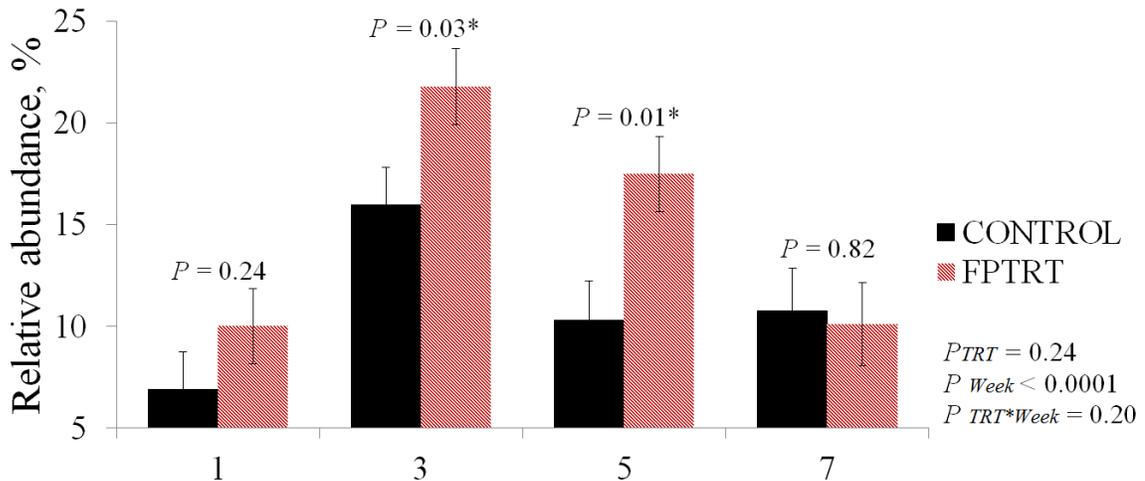


Figure 3. 6: *Faecalibacterium* mean relative abundance. Field trial.

Faecalibacterium mean relative abundance (Y axis, %) for each treatment group (control and FPTRT) over their 1st, 3rd, 5th and 7th week of life (X axis). The error bars represent the standard errors of the means.

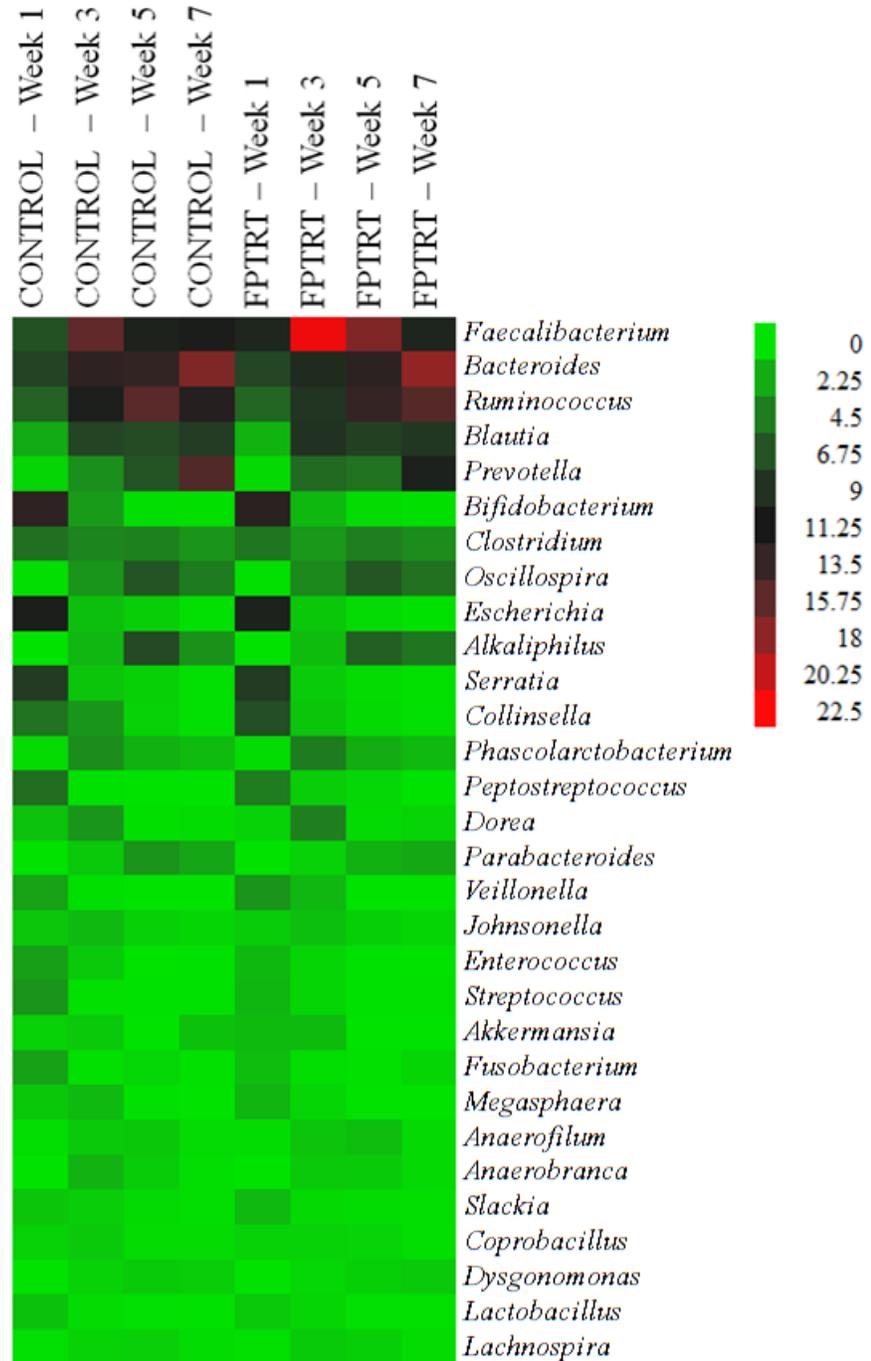


Figure 3. 7: Heat map illustrating the relative abundance of the 30 most prevalent genera. Field trial.

Heat map illustrating the relative abundance of the 30 most prevalent genera according to treatment group (control and FPTRT) over their 1st, 3rd, 5th and 7th week of life. The color and intensity of each square represent the value of the microbial relative abundance, as present in the legend.

The relative abundance of the ten most prevalent phyla present in the fecal samples is illustrated by treatment group in Figure 3.8. Firmicutes was the most abundant phylum (mean 61.0%), followed by Bacteroidetes (mean 20.7%), and Proteobacteria (mean 10.6%).

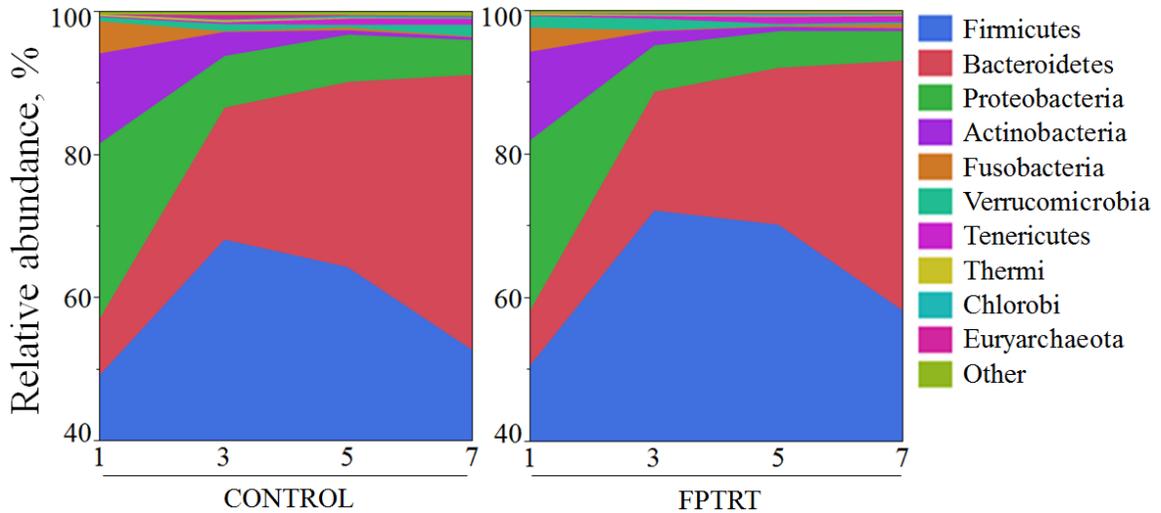


Figure 3. 8: Aggregate microbiome composition at the phylum level. Field trial.

Aggregate microbiome composition at the phylum level for 16S rRNA sequences according to treatment group (control and FPTRT) over their 1st, 3rd, 5th and 7th week of life. The y axis represents the mean relative abundance of OTUs for all samples evaluated within the specific week of life.

Firmicutes and Bacteroidetes mean relative abundances are presented in Figure 3.9-A and 3.9-B. The ratio of Firmicutes to Bacteroidetes is depicted in Figure 3.9-C. A higher ratio was observed in the 1st week and it decreased over time for both groups ($P < 0.0001$). Although the FPTRT group had numerically higher ratios than the control group over the time, the differences between the two treatment groups were not statistically significant.

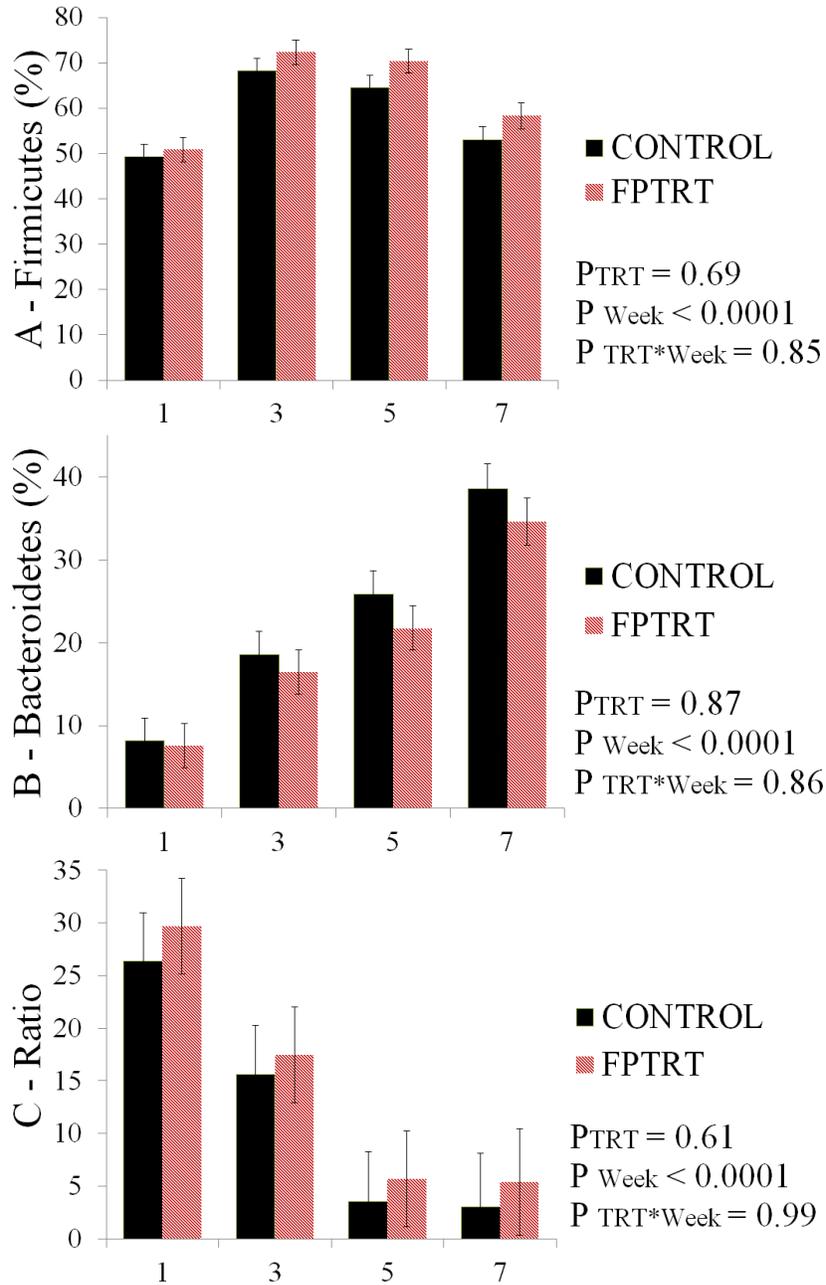


Figure 3. 9: Mean relative abundance of Firmicutes and Bacteroidetes and their ratio. Field trial.

Firmicutes mean relative abundance of control calves and FPTRT calves (A). Bacteroidetes mean relative abundance (B). Firmicutes to Bacteroidetes ratio (C). The X axis symbolizes the week of life. The Y axis represents the relative ratio (%) for A and B, and the ratio for graph C. The error bars represent standard errors of the means.

Serum BHBA concentration increased significantly over time ($P < 0.0001$) and was not affected by treatment ($P = 0.67$), as shown in Figure 3.10. Overall, the least square means ranged from 11.7 to 19.7 $\mu\text{mol/L}$.

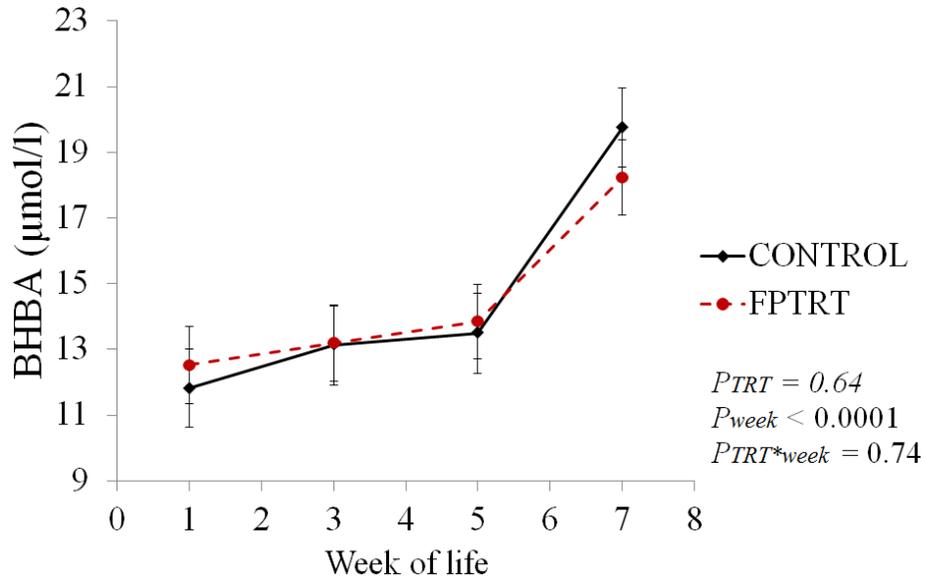


Figure 3. 10: Serum BHBA ($\mu\text{mol/l}$) concentration over the preweaning period. Field trial.

Serum BHBA ($\mu\text{mol/l}$) concentration over the preweaning period by treatment groups: FPTRT (dashed line; oral administration of *F. prausnitzii*) or control (solid line; no placebo). The error bars represent standard errors of the means.

Discussion

The use of *F. prausnitzii* as a probiotic treatment for newborn dairy calves was assessed. We confirmed its safety and efficacy and moreover determined that oral administration of *F. prausnitzii* live culture decreased the incidence of severe diarrhea and related mortality rate, while increasing weight gain in preweaned dairy heifers. This research demonstrates *F. prausnitzii* as a novel approach to enhance gastrointestinal health and weight gain in dairy calves.

The anti-inflammatory properties of *F. prausnitzii* and the production of butyrate are factors potentially contributing to the positive results observed in treated calves. The anti-inflammatory capacity of *F. prausnitzii* and its supernatant has previously been demonstrated both *in vitro* with cultured cells and *in vivo* using TNBS-induced colitis mice models (Sokol et al., 2008; Qiu et al., 2013; Miquel et al., 2015). Both bacterium and its supernatant induced the production of IL-10, an anti-inflammatory cytokine, while decreasing the secretion of IFN- γ and IL-12, which are pro-inflammatory cytokines (Sokol et al., 2008). This study also reported that *F. prausnitzii* metabolites inhibit the activation of NF-kB and the IL-8 secretion (Sokol et al., 2008). Additionally, a protective effect of *F. prausnitzii* on the intestinal barrier has been suggested (Martin et al., 2015; Miquel et al., 2015). We hypothesize that the administration of *F. prausnitzii* and its colonization in the lower GIT (cecum and colon), could attenuate the symptoms caused by infectious diarrhea in young calves or possibly prevent new infections from arising. Calf diarrhea plays a key role in the preweaned period and reducing scours incidence could positively affect animal health and welfare, as well as decrease labor, raising cost and antibiotic use.

As already stated, butyrate has several known beneficial effects in the intestines. It is a major energy source to colonocytes, it stimulates cell proliferation, differentiation and maturation, and improves colonic barrier function (Guilloteau et al., 2010; Ploger et al., 2012). In ruminants, butyrate has been associated with rumen development, having a mitotic effect on ruminal papillae (Mentschel et al., 2001), gut maturation (Gorka et al., 2011b) and increased weight gain in calves (Gorka et al., 2011a). Considering this, the ability of *F. prausnitzii* to produce butyrate could contribute to positive effects observed during colonization of *F. prausnitzii* in the large intestines.

Gastrointestinal epithelial cells use a large amount of the energy and nutrients provided by the diet and the microbial fermentation for absorption, transport of nutrients, and tissue maintenance (Baldwin et al., 2004). Dysbiosis and inflammation increase the energy expended by the enterocytes and also impair the intestinal barrier function, increasing the permeability to toxic substances (leaky gut) (Hollander, 1999; Bischoff et al., 2014; Geurts et al., 2014). Therefore, the enhanced growth observed in calves with higher *F. prausnitzii* prevalence could be partially explained by the reduced inflammation (decreased secretion of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines) and improved integrity of the intestinal barrier. Healthier calves could potentially have an increased appetite or have the same feed intake but extra energy available for weight gain due to less energy required for maintenance of a healthier gut.

A higher ratio of Firmicutes to Bacteroidetes has been correlated with obesity in mice and humans (Ley et al., 2005; Turnbaugh et al., 2006). Although this ratio was not significantly different between our study groups, it was higher for FPTRT calves compared to control calves. *F. prausnitzii* belongs to the phylum Firmicutes and has a

higher capacity for producing energy than other commensal bacteria. Balamurugan *et al.* 2009 compared the fecal microbiota of obese and non-obese Indian children using real-time PCR and found significantly higher levels of *F. prausnitzii* in the feces of the obese (Balamurugan *et al.*, 2010). Oikonomou *et al.* 2013 was the first to associate greater abundance of *F. prausnitzii* with higher weight gain and lower incidence of diarrhea in preweaned calves (Oikonomou *et al.*, 2013). Similarly, its relative abundance was increased in FPTRT calves, which gained more weight than controls. Obesity should be avoided in humans and animals, since it can lead to several health disorders (i.e. diabetes mellitus and systemic hypertension). However, increased growth rate is desirable in young production animals. As an example, preweaned dairy heifers are expected to yield more milk in their 1st lactation when they are purposely fed to exceed the nutrient requirements and have a greater ADG (Soberon *et al.*, 2012; Soberon and Van Amburgh, 2013). Hence, the administration of *F. prausnitzii* can be considered as a natural way to improve performance in preweaned heifers.

Calves that presented a high prevalence of *F. prausnitzii* on their 1st week of life (HighFP) and that received the additional *F. prausnitzii* culture gained significantly more weight compared to HighFP control calves. The *F. prausnitzii* isolates used for the probiotic cocktail were selected based on their high production of butyrate, better growth performance and resistance to low pH and bile salts. Treated calves were potentially colonized by more beneficial isolates present in the probiotic cocktail, irrespective of the higher relative abundance of the *F. prausnitzii* in their fecal microbiota. Another hypothesis is that HighFP have a gut environment more prone to the colonization of these new isolates administered.

The incidence of severe diarrhea was lower in the FPTRT group, as demonstrated through the survival analysis. Conversely, the mean fecal consistency score was not significantly different between FPTRT calves and control calves. Fecal consistency score alone cannot be a reliable indicator of disease, and diarrhea can be nutritional or infectious. We hypothesize that FPTRT calves were consuming a larger amount of milk, resulting in loose feces. This was similarly reported by Hill et al., 2010, in a study comparing different milk replacer programs. They observed greater average fecal consistency score from calves receiving a higher dry matter amount per day (Hill et al., 2010).

Short chain fatty acids, mainly butyrate, are converted to BHBA in the rumen epithelium and are metabolized in the liver (Bruss, 1997; Steele et al., 2011). We first speculated that calves in the FPTRT group would have higher prevalence of *F. prausnitzii* and hence have more butyrate, leading to greater serum BHBA concentration than control calves. However, if there was a higher production of butyrate by the *F. prausnitzii* in the FPTRT group, it would be in the large intestines (Malmuthuge et al., 2014), where most of the butyrate produced by the microbiota is consumed by the enterocytes and by other bacterial species. This might be one of the reasons that we did not detect differences in the serum BHBA concentration between the two groups. The rise on the BHBA concentration with age reported here is known to be correlated with the increase in starter intake and greater production of VFA by the rumen (Beharka et al., 1998; Galvao et al., 2005; Ślusarczyk et al., 2010).

A limited number of longitudinal studies have focused on weekly changes in fecal microbial diversity in calves during the preweaning period (Oikonomou et al., 2013;

Klein-Jobstl et al., 2014). In the calf field trial, Firmicutes was the most prevalent phylum, followed by Bacteroidetes and Proteobacteria. Our results are in agreement with Oikonomou *et al.* 2013 that studied weekly changes in the fecal microbiota of Holstein heifers. Klein-Jöbstl *et al.* 2014 collected fecal samples from 6 Simmental calves across 6 time points ranging from birth until 1 week after weaning (Klein-Jobstl et al., 2014). Calves were housed individually and were fed saleable pasteurized whole milk. They reported the same 3 phyla as the most prevalent, Firmicutes, Bacteroidetes and Proteobacteria, but in different ratios. Firmicutes was also the major phylum present in the in the fecal microbiota of 1 to 4 week old dairy calves fed non-pasteurized waste milk (Edrington et al., 2012). This cross-sectional study collected samples from 15 calves from each age group, which does not account for the individual variation over time. In addition, the increase in the Shannon index with age observed in the field trial is in agreement with the 3 studies cited above (Edrington et al., 2012; Oikonomou et al., 2013; Klein-Jobstl et al., 2014) and demonstrate that the calves' gut microbiota was increasing in diversity over time due to the consumption of solid feed.

The use of sterile cotton tipped swabs to collect fecal samples from the rectum requires less handling of animals and permits sampling of microbes encountered in the rectal mucosa and in the fecal material. A study by Malmuthuge *et al.* 2014 evaluated the taxonomic discrimination of commensal bacteria among the gastrointestinal tract (GIT) of preweaned calves by collecting tissue and content samples from the rumen, jejunum, ileum, cecum, and colon from 8 calves (Malmuthuge et al., 2014). The amplified 16S rRNA products were pooled by GIT segment and sequenced. They observed that *F. prausnitzii* was predominant in the mucosa-associated community from the cecum and

colon of preweaned calves' samples. Therefore, we considered the rectal swab as a better method for fecal sample collection, especially for targeting *F. prausnitzii*.

In conclusion, our studies demonstrated that *F. prausnitzii* administration to newborn calves is safe and proved the concept that this commensal bacterium is a promising probiotic for newborn calves. Further research is needed to evaluate *in vivo* mechanisms of action and interactions between this microbe, the gut microbiota and the host.

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CHAPTER 4: Conclusion and suggestions for future studies

The first objective of this thesis was to advance our knowledge on this anaerobic bacterium *F. prausnitzii* and its *in vitro* characteristics. The second objective was to test its effects *in vivo*, administering it to newborn calves. Both objectives were accomplished, generating useful scientific information. Despite this, additional research in this area is definitely needed. Further *in vitro* and *in vivo* studies are necessary to better understand the *F. prausnitzii* mechanisms of action and interactions between this microbe, the gut microbiota and the host.

The medium used in our studies to culture *F. prausnitzii* is very complex and expensive. The discovery of other alternatives to grow this very sensitive microorganism would facilitate its research. More *in vitro* studies evaluating the metabolites produced by *F. prausnitzii*, its action on intestinal cells, and its effects on growth of other commensal and pathogenic bacteria will be valuable. Studies in mice models might help in understanding how *F. prausnitzii* colonizes the gut of healthy animals.

The growth of the world population, the increase in income and urbanization are resulting in higher consumption of animal products. Livestock production systems have to be efficient to meet the high demand, while still maintaining the animal quality of life. Probiotics are a promising alternative to improve food animal productivity and health. The development of a probiotic of *F. prausnitzii* could benefit several species, including humans, domestic and production animals. More studies should be conducted in dairies with different management practices, morbidity and mortality

rates to evaluate the repeatability of our results. Studies with other animal species will be of significant value to science as well.

The protocol used in the field trial included sodium bicarbonate to buffer the abomasum pH, before administration of the *F. prausnitzii* liquid culture. Further studies are needed to discard the relevance of the sodium bicarbonate or the growth medium on the relative abundance of *F. prausnitzii* in the feces of preweaned calves. Different formulations should also be tested. Enteric protected capsules containing lyophilized *F. prausnitzii* would be ideal. It could facilitate the administration, and avoid the contact with oxygen and low pH.