Modulation of the gastrointestinal tract microbiota by two direct fed microbials and their efficacy as alternatives to antibiotic growth promoter use in calf management operations

Thèse

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Résumé

L'usage des produits microbiens administrés directement (aussi appelés probiotiques) gagne de l'intérêt comme alternative à l'utilisation des antibiotiques comme promoteurs de croissance dans les élevages. Cependant, très peu d'informations existent quant à l'influence des probiotiques sur la modulation du microbiote gastrointestinal et la réponse immunitaire innée chez le veau laitier. Les objectifs de cette thèse visaient à (1) Étudier l'effet de Lactobacillus acidophilus BT 1386 ou de Saccharomyces cerevisiae boulardii CNCM 1-1079 sur les constituants sanguins, biochimiques / chimiques du sang. (2) Déterminer les mécanismes potentiels d'une réponse immunitaire renforcée de Lactobacillus acidophilus BT 1386 et de Saccharomyces cerevisiae boulardii CNCM 1-1079. (3) Déterminer comment Lactobacillus acidophilus BT 1386 ou Saccharomyces cerevisiae boulardii CNCM 1-1079 modulent la composition de la communauté microbienne GIT de veau par séquençage de nouvelle génération de la région V3-V4 du gène ARNr 16S. (4) Comparer l'efficacité de ces deux DFM avec la tetracycline-néomycine, un promoteur de croissance antibiotique.

Quatre traitements ont été distribués aléatoirement à 48 veaux âgés de 2 à 7 jours (n=12). TÉMOIN : lactoremplaceur (LR) suivi d'une moulée de démarrage (MD); SCB) TÉMOIN + *Saccharomyces cereviseae* var. *boulardii* CNCM I-1079 [7,5 × 10^8 unités formatrices de colonie (CFU)/L de LR + 3 × 10^9 CFU/kg de MD]; LA) TÉMOIN + *Lactobacillus acidophilus BT 1386* (2,5 × 10^8 CFU/L de LR + 1 × 10^9 CFU/kg de MD); ATB) TÉMOIN + traitement antibiotique composé de chlortétracycline (528 mg/L de LR + 55 mg/kg de MD) et de néomycine (357 mg/L de LR). Les animaux ont été élevés selon les procédures d'élevage conventionnelles pendant les 96 jours de la période expérimentale. Des échantillons de sang ont été prélevés de la veine jugulaire à différents moments pendant les périodes de pré-sevrage (jours 1 à 42), de sevrage (jours 43 à 53) et de post-sevrage (jours 54 à 96). Aux jours 33 et 96 dans chacun des groupes, 4 veaux ont été euthanasiés afin de prélever des échantillons de tissus et de digesta.

Des SCB viables ont été retrouvées tout au long du tractus gastrointestinal, ainsi que dans les fèces des veaux en périodes pré- et post-sevrage. Autour du sevrage, les fèces du groupe SCB contenaient une population de lactobacilli plus importante que celles du groupe TÉMOIN. Au cours de la période pré-sevrage, la distribution des lactobacilli évoluait graduellement à travers les sections du tube digestif (colon > contenu iléal > rumen > muqueuse iléale). À l'exception du rumen, tous les autres compartiments présentaient une population de lactobacilli réduite en post- vs. en pré-sevrage. Comparativement aux groupes TÉMOIN et LA, la profondeur et la largeur des cryptes du colon des groupes SCB et ATB étaient réduites. Toujours comparativement aux groupes TÉMOIN et LA, le nombre de cellules caliciformes contenant des mucines neutres tendait à augmenter pour les groupes SCB et ATB, alors que le nombre de mucines acides augmentaient. Globalement, les traitements n'ont pas affecté les performances des animaux.

Pendant le sevrage, une amélioration de la stimulation oxydative et de la phagocytose, ainsi qu'une augmentation des concentrations des protéines de la phase aiguë, ont été observées chez les groupes SCB et LA. L'ajout de probiotiques à la diète du veau a eu moins d'impact sur la diversité bactérienne mais a tout de même modifié significativement l'abondance des différentes populations microbiennes, et ce plus particulièrement dans l'iléon. L'ajout de SCB ou de LA a réduit l'abondance de certains genres bactériens pathogènes, tels que *Streptococcus* et *Tyzzerella*_4, alors que cela a augmenté l'abondance de bactéries potentiellement bénéfiques pour l'hôte tel que celles appartenant au genre *Fibrobacter*. Par ailleurs, d'autres bactéries bénéfiques tel que *Rumminococcaceae* UCG 005 et *Olsenella* étaient aussi plus abondantes, mais seulement pour le traitement SCB. Les bactéries pathogènes *Peptoclostridium* et *Ruminococcus*_2 étaient respectivement moins abondantes lorsque les traitements SCB et LA étaient ajoutés à la ration. Les analyses de prédiction fonctionnelle ont montré qu'en plus des effets observés sur les voies métaboliques locales impliquées dans le cycle cellulaire, la sécrétion biliaire et les voies de signalisation de l'AMPc et du proteasome, l'ajout des deux formes de probiotiques a également affecté d'importantes voies impliquées au sein d'autres tissus comme la synthèse des hormones thyroïdiennes ou le fonctionnement des synapses dopaminergiques.

Cette étude suggère que les probiotiques, et plus particulièrement SCB, devraient être davantage considérés comme modulateur de la santé gastro-intestinale du veau laitier. Aussi, la supplémentation en SCB, en améliorant la réponse immunitaire innée, permettrait de stimuler le système immunitaire du veau avant l'infection, le préparant ainsi à mieux affronter les périodes plus sensibles comme celle du sevrage.

Le SCB et le LA ont modifié la composition en bactéries du GIT. Dans l'ensemble, cette étude a montré une démonstration remarquable de l'importance du DFM sur le microbiote de la TI. Cependant, il faut mieux comprendre les molécules et les mécanismes qui déterminent le rôle du microbiote, puis exploiter ces connaissances pour améliorer la santé et augmenter la production animale.

Abstract

There is interest in the use of direct-fed microbials (DFM) as substitutes for antibiotic growth promoters in farm animal production. However, little information exists on the effects of *Lactobacillus acidophilus BT 1386* (LA) and *Saccharomyces cereviseae* boulardii *CNCM I-1079* (SCB) on the modulation of the gastrointestinal tract (GIT) microbiota and innate immune responses in dairy calves. Therefore, the objectives of this thesis were to (1) investigate the effect of *Lactobacillus acidophilus BT 1386* or *Saccharomyces cerevisiae boulardii CNCM 1-1079* on blood cellular and biochemical/chemical constituents; (2) determine the potential mechanisms of enhanced immune response by *Lactobacillus acidophilus BT 1386* and *Saccharomyces cerevisiae boulardii CNCM 1-1079*; (3) determine how *Lactobacillus acidophilus BT 1386* or *Saccharomyces cerevisiae boulardii CNCM 1-1079* modulate calf GIT microbial community composition by next-generation sequencing of the V3-V4 region of the 16S rRNA gene and (4) compare the efficacy of these two DFM with tetracycline-neomycin, an antibiotic growth promoter.

Forty eight calves (2 to 7 days old) were randomly allocated to four treatments: 1) Control (CTRL) fed milk replacer (MR) and starter feed (SF); 2) CTRL supplemented with *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7.5×10^8 (CFU)/L MR + 3×10^9 CFU/kg SF); 3) CTRL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8 CFU/L MR + 1×10^9 CFU/kg SF); and 4) CTRL supplemented with antibiotics (ATB) chlortetracycline and neomycin (528 and 357 mg/L MR, respectively), and chlortetracycline (55 mg/kg SF). Animals were raised for 96 days following standard management procedures. Growth parameters (body weight and feed intake) of calves were recorded weekly. Four calves per treatment were euthanized on day 33 (pre-weaning) and an additional four calves per treatment on day 96 (post-weaning) to sample rumen and ileum tissues for real time quantitative polymerase chain reaction and colon for histomorphology. The ileum, colon and rumen were also analyzed for viability. Furthermore, samples of digesta (colon, ileum and rumen) and mucosa (colon and ileum) for bacterial characterization by sequencing the V3-V4 region of 16S rRNA gene. Weekly feces samples were collected for viability analysis. Blood samples were also collected for isolation of neutrophils and peripheral blood mononuclear cells for oxidative burst and phagocytosis analyses by flow cytometry. Serum measurements of acute phase proteins were done by ELISA.

Viable SCB were recovered throughout the GIT and in the feces pre- and post-weaning. The feces of SCB-treated calves showed a greater lactobacilli population compared with CTRL ($P < 0.01$) around weaning. In the pre-weaning period, the distribution of lactobacilli population differed along the digestive tract (colon > ileum content > rumen > ileum mucosa; $P < 0.001$). The lactobacilli population were significantly reduced in all compartments ($P = 0.02$) post-weaning compared to pre-weaning, except in the rumen. Crypts depth and width of the colon decreased ($P < 0.01$) whereas number of goblet cells containing neutral mucins tended to increase ($P = 0.058$) while acidic mucins increased ($P < 0.05$) in SCB- and ATB-treated calves compared with CTRL and

LA-treated calves. Overall, growth performances were not affected by treatment. There was improvement of both oxidative burst and phagocytosis by SCB and LA during weaning in calves. Similarly, the concentrations of acute phase proteins (C-reactive proteins and serum amyloid A proteins) were increased by SCB and LA during weaning.

The DFM had less impact on the bacteria diversity but had significant impact on the abundance of the bacteria community with most changes associated to treatments occurring in the ileum. SCB and LA reduced some pathogenic bacteria genera such as *Streptococcus, Tyzzerella*_4 and increased some potential beneficial bacteria such as *fibrobacter*. Meanwhile, *Rumminococcaceae* UCG 005 and *Olsenella,* also beneficial, were increased only by SCB treatment. The potential pathogenic bacterium, *Peptoclostridium,* was reduced by SCB only while LA reduced *Ruminococcus*_2. The functional prediction analyses indicated that besides affecting local pathways such as cell cycle, bile secretion, proteasome or cAMP signaling pathway, both DFM might also affect important pathways in other tissues such as thyroid hormone synthesis or Dopaminergic synapse in the brain.

Our results suggest that SCB is a modulator of gastrointestinal health and could prime the immune system prior to infection leading to an enhanced innate immune response in calves especially during periods of stress (e.g., weaning). Consequently, SCB might have the potential to strengthen calf immune system in the critical periods of disease susceptibility. Both SCB and LA changed the bacteria composition of the GIT. Overall, this study showed a remarkable demonstration of the importance of DFM on the GIT microbiota. However, what is needed is a complete and better understanding of the molecules and mechanisms driving the roles played by the microbiota and then to exploit this knowledge to improve health and increase animal production.

Table des matières

Résumé ii
Abstract iv
Table des matières vi
List of tables xi
List of figures xii
List of abbreviations xiv
Dédicaces xvi

Remerciements xvii
Avant-propos xix
Introduction 1
Chapitre 1 Literature review 3
1.1 Application of antibiotic growth promoters in farm animal production 3
1.2 Benefits of using of AGP in farm animal production 4
1.2.1 Enhance animal health status 4
1.2.2 Improve animal performance and production efficiency 4
1.2.3 Effects of antibiotic growth promoters on gastrointestinal microbiota 5
1.3 Problems associated with the use of antibiotic growth promoters 5
1.3.1 Development of resistance in animals and humans 5
1.3.2 Effect on human health 6
1.3.3 Potential public health hazard 7
1.3.4 Increase cost of production/loss in the production chain 7
1.3.5 Environmental contamination 8
1.4 Bans and anticipated bans 8
1.5 Alternatives to antibiotic growth promoters 9
1.5.1 Direct fed microbials 9
1.5.2 Prebiotics 10
1.5.3 In-feed enzymes 10
1.5.4 Phytogenic feed additives 10
1.5.5 Essential oils 11
1.5.6 Bacteriophages 11
1.5.7 Symbiotics 11

1.5.8 Organic and inorganic acids 11
1.5.9 Egg yolk and antibodies 12
1.5.10 Clay minerals 12
1.5.11 Rare earth elements 12
1.6 Application of direct fed microbials in animal production enterprises 12
1.6.1 Influence of DFM on animal performance 12
1.6.2 *Lactobacillus acidophilus* species as a direct fed microbial 19
1.6.3 *Saccharomyces cerevisiae* species as a direct fed microbial 19
1.6.4 Proposed mechanisms of action of direct fed microbials 20
1.6.4.1 Direct fed microbials and the innate immune system 22
1.6.4.2 Probable mechanisms of action of Saccharomyces cerevisiae and Lactobacillus acidophilus in the gastrointestinal tract 23
1.6.4.3 Direct fed microbials and the digestive system 24
1.6.4.4 Direct fed microbials and the gastrointestinal microbiota 25
1.7 Strategies for characterizing the gastrointestinal tract microbiota 25
1.7.1. The 16S rRNA gene sequencing for taxonomic profiling 27
1.7.2 Alternative marker genes 29
1.7.3 Limitations of the 16S rRNA 29
1.8 Direct fed microbials and animal (calf) production 29
1.9 Study hypotheses 30
1.10 Study objectives 30
1.11 References 30
Chapitre 2 Impact of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 on total lactobacilli population in the gastrointestinal tract and colon histomorphology of Holstein dairy calves 43
2.1 Résumé 44
2.2 Abstract 44
2.3 Keywords 45
2.4 Abbreviations 45
2.5 Introduction 46
2.6 Materials and methods 47
2.6.1 Calves, housing and experimental diets 47
2.6.2 Viability of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 in commercial products and feed 48
2.6.3 Sampling of feces and gastro intestinal tract content 49

2.6.4 Isolation of DNA and real-time qPCR quantification of total Lactobacilli in the gastro-intestinal tract and feces 49
2.6.5 Blood sampling and analysis ..... .50
2.6.6 Colon histomorphology 50
2.6.7 Quantification of goblet cells 50
2.6.8 Health status, medication and vaccination ..... .51
2.6.9 Feed intake and body weight measurements 51
2.6.10 Attitude and fecal scoring 51
2.6.11 Fecal sampling and detection of enterotoxigenic Escherichia coli O157:H7 51
2.6.12 Statistical analysis 52
2.7 Results 52
2.7.1 Viability of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 in commercial products, feeds, gastrointestinal tract and feces 52
2.7.2 Effect of treatment and weaning on total lactobacilli population in the gastrointestinal tract and in feces 53
2.7.3 Plasma glucose and β-hydroxybutyrate 54
2.7.4. Colon histomorphology 55
2.7.5 Quantification of neutral and acidic mucins 56
2.7.6 Calf health, feed intake, weight gain and feed efficiency 56
2.7.7 Presence of Escherichia coli O157:H7 in feces 58
2.8 Discussion 58
2.9 Conclusion ..... .60
2.10 Competing interest ..... .60
2.11 Acknowledgements ..... .60
2.12 References ..... .60
Chapitre 3 *Saccharomyces cerevisiae* boulardii CNCM 1-1079 and Lactobacillus acidophilus BT1386 influences innate immune response and serum levels of acute-phase proteins during weaning in Holstein calves ..... .64
3.1 Résumé ..... .65
3.2 Abstract ..... .65
3.3 Key words ..... .66
3.4 Abbreviations ..... .66
3.5 Introduction ..... .66
3.6 Materials and Methods ..... .67

3.6.1 Calves and experimental diets ..... .67
3.6.2 Blood sampling ..... .68
3.6.3 Isolation of peripheral blood mononuclear cells ..... .68
3.6.4 Culture of peripheral blood mononuclear cells and stimulation with lipopolysaccharide or pokeweed mitogen ..... .68
3.6.5 Quantification of interferon γ, interleukin 6 and tumor necrosis factor α in peripheral blood mononuclear cells culture supernatants ..... .69
3.6.6 Isolation of polymorphonuclear neutrophils ..... .69
3.6.7 Neutrophil oxidative burst assay ..... .69
3.6.8 Neutrophil phagocytosis assay ..... .70
3.6.9 Flow cytometric analysis for oxidative burst and phagocytosis ..... .70
3.6.10 Quantification of serum acute phase proteins ..... .71
3.6.11 Tissue sampling and RNA isolation ..... .71
3.6.12 Quantitative real time polymerase chain reaction ..... .71
3.7 Statistical analysis ..... .72
3.8 Results 74
3.8.1 Cytokine production in supernatant of peripheral blood mononuclear cells 74
3.8.2 Neutrophil oxidative burst and phagocytosis ..... .74
3.8.3 Concentrations of acute phase proteins ..... .78
3.8.4 Gene expression in the rumen and ileum ..... .79
3.9 Discussion ..... .80
3.10 Conclusion ..... .82
3.11 Acknowledgements ..... .82
3.12 References ..... .82
Chapitre 4 Direct-fed microbial supplementation influences the bacteria community composition of the gastrointestinal tract of pre- and post-weaned calves ..... .85
4.1 Résumé ..... .86
4.2 Abstract ..... .86
4.3 Key words 87
4.4 Introduction ..... .87
4.5 Results ..... .88
4.5.1 Data acquisition ..... .88
4.5.2 Bacterial diversity across treatments in GIT sites at pre-weaning (day 33) and post-weaning (day 96) ..... .89

4.5.3 Bacterial composition and differential abundance across treatments in GIT sites at pre-weaning and post-weaning periods 95
4.5.4 Predicted pathways of the relative changes due to treatments 113
4.6 Discussion 116
4.7 Materials and Methods 121
4.7.1 Animal treatments and samplings 121
4.7.2 DNA isolation and quantification 122
4.7.3 Amplification of bacterial ribosomal DNA and sequencing 122
4.7.4 Bioinformatics analysis 123
4.7.5 Assessment of diversity and statistical analysis 123
4.7.6 Bacterial Community Composition and differential relative abundance analyses 124
4.4.7 Functional prediction and differential analysis of predicted pathways 124
4.8 References 124
4.9 Acknowledgements 129
4.10 Author Contributions 129
4.11 Additional Information 130
4.12 Supplementary figure and tables 131
Conclusions 132
General conclusion 132
Implication 135
Limitations 135
Study perspectives 136
References 136

List of tables

Table 1.1. Antibiotics used for growth promotion and feed efficiency in food animals..... 4
Table 1.2. Beneficial properties described for some direct fed microbials..... 10
Table 1.3. Some direct-fed microbials (DFM) used in animal production and their effects..... 14
Table 1.4. Some experimental and computational methods available for studying the microbiome..... 26
Table 2.1. Chemical composition of milk replacer, starter feed and hay[a]..... . 48
Table 2.2. Effect of Lactobacillus acidophilus BT1386 (LA) and Saccharomyces cerevisiae boulardii CNCMI–1079 (SCB) on total lactobacilli (log10 CFU/g) in the gastrointestinal tract (GIT) of dairy calves receiving milk replacer (day 33, pre-weaning) or starter feed (day 96, post-weaning)……..53

Table 2.3. Mean differences between sites of the gastrointestinal tract (GIT) of total lactobacilli population (log10 CFU/g) in dairy calves supplemented with Lactobacillus acidophilus BT1386 and Saccharomyces cerevisiae boulardii CNCMI–1079 during pre-weaning (day 33) or post-weaning (day 96). ..... .54
Table 2.4. Effect of direct-fed microbials Lactobacillus acidophilus BT1386 and Saccharomyces cerevisiae boulardii CNCMI–1079 on total lactobacilli in feces (expressed as area under the curve) at different times relative to weaning[1]..... .54
Table 2.5. Mean plasma glucose and β-hydroxybutyrate during pre-weaning, weaning and post-weaning periods in dairy calves. ..... .55
Table 2.6. Effect of direct-fed microbials and antibiotics on colon histomorphology of Holstein calves..... 55
Table 2.7. Effect of direct-fed microbials and antibiotics on body weight, feed intake, feed efficiency and fecal scores of Holstein dairy calves. 57
Table 3.1. Primer sequences and concentration of genes quantified by real time PCR..... 73
Table 3.2. The effect of treatment on *ex vivo* production of cytokines in supernatant of peripheral blood mononuclear cells of calves..... 74
Table 3.3. The effect of treatment on acute phase proteins throughout the feeding trial..... .78
Table 4.1. Comparison of alpha diversity measures across treatments in gastrointestinal sites at pre- and post-weaning periods..... .91
Table 4.2. Significant differential abundant genera between control and SCB on day 33 (pre- weaning) and day 96 (post- weaning). 98
Table 4.3. Significant differential abundant genera between control and LA on day 33 (pre -weaning) and day 96 (post- weaning). ..... .101
Table 4.4. Significant differential abundant genera between control and ATB on day 33 (pre -weaning) and day 96 (post-weaning) periods ..... .103
Table 4.5. Highly significant differential abundant genera between SCB and ATB on day 33 (pre -weaning) and day 96 (post-weaning)[1]. ..... .108
Table 4.6. Highly significant differential abundant genera between LA and ATB on day 33 (pre- weaning) and day 96 (post- weaning)[1]. 111
Table 4.7. Predicted KEGG pathways significantly changed by treatments at each gastrointestinal site in the pre- and post-weaning periods. ..... .114

List of figures

Figure 1.1. Adapted from Suresh et al. 2017. The transfer of antibiotic resistance genes. 6
Figure 1.2. In ruminants, direct-fed microbials (DFM) may modulate ruminal fermentation, stimulate intestinal immune function, enhance nutrient absorption and competitively exclude select microbes from the small and large intestines. Additionally, some DFM may also remain viable after passage through the intestinal tract and excretion in feces which does not contaminate the environmental (Adapted from McAllister *et al*. 2011). 21
Figure 1.3. Proposed mechanisms of pathogen exclusion in the intestinal tract: (1) competion between the pathogen and DFM for nutrients; (2) direct antagonism through production of antimicrobials (bacteriocins); (3) competitive exclusion through occupation of binding sites; (4) enhanced gut health through restoration of epithelial intergrity; and (5) stimulation of immune response resulting in host – exclusion of the pathogens. Adapted from O'Toole and Cooney (2008) 23
Figure 1.4. Luminal conversion by intestinal microbes may play an important role in host–microbiota interactions. Orally consumed direct-fed microbial may be converted by intestinal microbes into bioactive compounds that could affect the health of the host and the intestinal microbiota. GABA = gamma-aminobutyric acid; SCFAs = short-chain fatty acids). Adapted from (Hemarajata & Versalovic 2013)..... 24
Figure 1.5. The 16S rRNA amplicon targeting the V3 –V4 region gene. The forward and reverse primers designed with overhang adapters are used to amplify the V3-V4 region of interest from genomicDNA.https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf..... 28
Figure 1.6. The variable (gray) and conserved regions (green) of the 16S rRNA gene (Source:https://biology.stackexchange.com/questions/54823/what-causes-the-variable-conserved-structure-in-the-16s-rrna-gene)..... 28
Figure 3.1. (a) Typical histograms showing fluorescent intensity and gating for quantification of oxidative burst. (i) Negative control: unstimulated polymophonuclear neutrophils (PMN; 0 nM PMA), (ii) PMN stimulated with phorbol myristate acetate (PMA;100 nM), and (iii) an example of gating for data acquisition using physical characteristics of granularity (side scatter) and size (forward scatter) of the PMN oxidative burst plot. Ten thousand cells were measured and acquired for data analysis. (b) Neutrophil oxidative burst capacity of calves on different treatments. CTRL = Control (milk replacer and starter feed from wk two); ATB = antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning, and at 3×10^9 CFU kg^{-1} starter feed after weaning); LA = CTRL + *Lactobacillus acidophilus* BT1386 (2.5×10^8 CFU L^{-1} milk replacer before weaning and at 1×10^9 CFU kg^{-1} starter feed after weaning). The mean fluorescence between SCB and CTRL was significant ($P < 0.05$) on d 53 (weaning), and on d 59 and d 87 (post-weaning). The mean fluorescence of LA increased significantly ($P < 0.05$) on d 53 as compared to CTRL. ..... …76
Figure 3.2. (a) Typical phagocytosis histograms showing (i) negative sample at 4 °C, (ii) positive sample at 37 °C, and (iii) an example of sample gating for data acquisition using physical characteristics of granularity (side scatter) and size (forward scatter) for the polymophonuclear neutrophil phagocytosis plot. (b) Neutrophil phagocytosis measured using flow cytometry with Alexa fluor as the fluorescent probe upon stimulation with lipopolysaccharide and pHrodo™ green fluorescent beads. CTRL = Control (milk replacer and starter feed from second wk); ATB = CTRL + antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning,

and at 3 × 10^9 CFU kg^{-1} starter feed after weaning); LA = CTRL + *Lactobacillus acidophilus* BT1386 (2.5 × 10^8 CFU L^{-1} milk replacer before weaning and at 1 × 10^9 CFU kg^{-1} starter feed after weaning). Mean phagocytic florescence increased significantly ($P < 0.05$) for calves on SCB and ATB treatments at d 47 as compared to CTRL. ..... …77
Figure 3.3. (a) Gene expression levels in the ileum on d 33 (gene expression data for d 33 was similar to d 96 so only d 33 data has been presented) (b) Gene expression levels in the rumen (d 33). Higher values, expressed as $\Delta\Delta C_t$, represent lower mRNA expression and vice versa. CTRL = Control fed milk replacer and starter feed from wk two; ATB = CTRL + antibiotics; chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning. SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM I-1079 at 7.5 × 10^8 CFU L^{-1} milk replacer before weaning and at 3 × 10^9 CFU kg^{-1} starter feed after weaning. LA = CTRL + *Lactobacillus acidophilus* BT1386 at 2.5 × 10^8 CFU L^{-1} milk replacer before weaning and at 1 × 10^9 CFU kg^{-1} starter feed after weaning..... 79
Figure 4.1. Distribution of 159 samples with complete 16S rRNA gene sequences of bacteria phylum and genera..... .89
Figure 4.2. Principal coordinate analysis (PCoA) plots for treatment effect on each site at pre- and post-weaning periods. (a) Principal coordinate analysis (PCoA) plots for treatment effect on Rumen digesta at pre-weaning and post-weaning. (b) Principal coordinate analysis (PCoA) plots for treatment effect on ileum mucosa at pre-weaning and post-weaning. (c) Principal coordinate analysis (PCoA) plots for treatment effect on ileum digesta at pre-weaning and post-weaning. (d) Principal coordinate analysis (PCoA) plots for treatment effect on colon digesta at pre-weaning and post-weaning. (e) Principal coordinate analysis (PCoA) plots for treatment effect on colon mucosa at pre-weaning and post-weaning. Distances between the samples are based on similarity in OTU composition (OTU similarity 97%). A greater distance implies lower similarity, whereas similar OTUs will cluster together. The clustering pattern of the bacterial communities were tested using PERMANOVA and ($P < 0.05$) were considered significant..... ..94
Figure 4.3. Stack bar charts of phylum level bacterial composition for the treatment effect on each site at pre- and post-weaning periods. CoM = colon mucosa, CoD = colon digesta, IM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta. 95
Figure 4.4. Stack bar charts of genus level bacterial composition for the treatment effect on each site at pre- and post-weaning periods. CoM = colon mucosa, CoD = colon digesta, IIM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta..... 96
Figure 4.5. The common and specific genera in the (a) pre-weaning and (b) post-weaning periods for the different treatments. 106
Figure S4.1. Stack bar charts of genus level bacterial composition for the treatment effect on each site at pre- and post-weaning periods. CoM = colon mucosa, CoD = colon digesta, IIM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta. 131

List of abbreviations

AB: Alcian blue
AGP: Antibiotic growth promoter
APP: Acute phase proteins;
ATB: Antibiotics
BHB: β-hydroxybutyrate
BP: Base pair
cAMP: Cyclic adenosine monophosphate
CFU: Colony forming units
CLDN: Claudin
CoM: Colon mucosa
CoD: Colon digesta
CRP: C-reactive proteins
CSS: Cumulative sum scaling
CTRL: Control
CV: Coefficient of variation
DA: Differential abundance
DNA: Deoxyribonucleic acid
DFM: Direct fed microbials
FC: Fold-change
GABA: Gamma-aminobutyric acid
GIT: Gastro intestinal tract
GOLD: Genome online data base
HBSS: Hank's balanced salt solution
HP: Haptoglobin
IFN-γ: Interferon gamma
IIM: Ileum mucosa
IID: Ileum digesta
IL-6: Interleukin 6
KEGG: Kyoto Encyclopedia of genes and genomes
LA: *Lactobacillus acidophilus* BT1386
LPS: Lipopolysaccharide
MALDI TOF: Matrix assisted laserdesortion ionization time of flight
MAPK: Mitogen-activated protein kinase
MR: Milk replacer
NFκB: Nuclear factor-kappaB
OTU: Operational taxonomic unit
PAS: Periodic acid Schiff
PBMC: Peripheral blood mononuclear cell
PCoA: Principal coordinate analysis
PCR: Polymerase chain reaction
PERMONOVA: Permutational multivariate analysis of variance
p.FDR: False discovery rate
PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PMA: Phorbol 12-myristate 13-acetate
PMN: Polymorphonuclear neutrophils
QIIME: Quantitative Insights Into Microbial Ecology
qPCR: quantitative real-time PCR
RNA: Ribonucleic acid

RuD: Rumen digesta
SAA2: Serum amyloid A2
SCB: *Saccharomyces cerevisiae* boulardii CNCM1-1079
SCFA: Short-chain fatty acids
SEM: Standard error of mean
SF: Starter feed
TLRs: Toll like receptors
TNFα: tumor necrosis factor alph
16S rRNA: 16S Ribosomal RNA Gene

This work is dedicated to my late father,
Fomenky Eric Njikem, who passed away in
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Avant-propos

This thesis is submitted to the "Faculté des études supérieures de l'Université Laval" to obtain the degree of Doctor of Philosophy of Science (Ph. D). The present thesis consists of a general introduction and five chapters. Chapter 1 is the literature review of the current state of the art on alternatives to antibiotics growth promoters and particularly the beneficial effects of direct fed microbials (DFM); *Saccharomyces cerevisiae* boulardii CNCMI 1079 (SCB) and *Lactobacillus acidophilus* BT1386 (LA) in animal farming and particularly in calf management. Chapters 2, 3 and 4 are presented in the form of manuscripts and describe three different sets of experiments and a general conclusion.

The objectives of the thesis were: (1) investigate the effect of *Lactobacillus acidophilus BT 1386* or *Saccharomyces cerevisiae boulardii CNCM 1-1079* on blood cellular and biochemical/chemical constituents. (2) Determine the potential mechanisms of enhanced immune response by *Lactobacillus acidophilus BT 1386* and *Saccharomyces cerevisiae boulardii CNCM 1-1079*. (3) Determine how *Lactobacillus acidophilus BT 1386* or *Saccharomyces cerevisiae boulardii CNCM 1-1079* modulate calf gastrointestinal tract (GIT) microbial community composition by next-generation sequencing of the V3-V4 region of the 16S rRNA gene. (4) Compare the efficacy of these two DFM with tetracycline-neomycin, an antibiotic growth promoter (ATB).

Three journal manuscripts have resulted from this research:

(1) **Fomenky B.E.**, Chiquette J., Bissonnette N., Talbot G., Chouinard P.Y. & Ibeagha-Awemu E.M. (2017) Impact of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 on total lactobacilli population in the gastrointestinal tract and colon histomorphology of Holstein dairy calves. *Animal Feed Science and Technology* **234**, 151-61.

(2) **Fomenky B.E.**, Chiquette J., Lessard M., Bissonnette N., Talbot G., Chouinard Y.P. & Ibeagha-Awemu E.M. (2018) Saccharomyces cerevisiae var. boulardii CNCM I-1079 and Lactobacillus acidophilus BT1386 influence innate immune response and serum levels of acute-phase proteins during weaning in Holstein calves. *Canadian Journal of Animal Science* **98**, 576-88.

(3) **Fomenky B.E.**, Do D.N., Talbot G., Chiquette J., Bissonnette N., Chouinard Y.P., Lessard M. & Ibeagha-Awemu E.M. (2018) Direct-fed microbial supplementation influences the bacteria community composition of the gastrointestinal tract of pre- and post-weaned calves. *Scientific Reports* **8**, 14147.

During her Ph.D., the candidate co-authored a book chapter and other journal publications:

Do D.N., Dudemaine P.-L., **Fomenky B.** & Ibeagha-Awemu E.M. (2017) Transcriptome Analysis of Non-Coding RNAs in Livestock Species: Elucidating the Ambiguity. In: *Applications of RNA-Seq and Omics Strategies - From*

Microorganisms to Human Health (eds. by Marchi FA, Cirillo PDR & Mateo EC), p. Ch. 05 (42 pages). InTech, Rijeka. A book Chapter

Ibeagha-Awemu E., Do D., Dudemaine P.-L., **Fomenky B.** & Bissonnette N. (2018) Integration of lncRNA and mRNA Transcriptome Analyses Reveals Genes and Pathways Potentially Involved in Calf Intestinal Growth and Development during the Early Weeks of Life. *Genes* **2018**, *9*(3), 142 (26 pages); doi:10.3390/genes9030142

D.N. Do, **B.E. Fomenky**, P-L. Dudemaine, N. Bissonnette & E.M. Ibeagha-Awemu (2018) Weighted gene co-expression network analysis reveals gene clusters and pathways related to rumen development in calves. Procceings of the World Congress on Genetics Applied to Livestock Production, February 11 to 16, 2018, Auckland, New Zealand: 11, 945 (5 pages).

Duy N. Do, Pier-Luc Dudemaine, **Bridget E. Fomenky**, Nathalie Bissonnette and Eveline M. Ibeagha-Awemu (2018) Integration of miRNA weighted gene co-expression network and miRNA-mRNA co-expression analyses for exploration of miRNA potential regulatory functions in calves' rumen development. Genomics. 2018 May 21. pii: S0888-7543(18)30125-3. doi: 10.1016/j.ygeno.2018.05.009

Duy N. Do Pier-Luc Dudemaine, **Bridget E. Fomenky** and Eveline M. Ibeagha-Awemu (2018) Integration of miRNA and mRNA co-expression reveals potential regulatory roles of miRNAs in calves' ileum before and after weaning Cells 2018 Sep 11;7(9). pii: E134. doi: 10.3390/cells7090134.

The candidate was awarded the Canadian Society of Animal Science travel award for her participation in the Canadian Society of Animal Science (CSAS)-Canadian Meat Science Association (CMSA) joint meeting in Ottawa in May of 2015.

Introduction

Over the years, intensive farming practises promoted the use of antibiotics at sub therapeutic levels to promote growth, feed efficiency, prevent diseases and increase productivity in farm animal production (Butaye *et al.* 2003; Suresh *et al.* 2017). Thus, the use of antibiotic growth promoters (AGP) has been very valuable in animal farming in past decades. However, AGP usage in farm animal production has led to the emergence, spread and transfer of antibiotic resistance genes in microbes found in animals, which are in turn transferable to humans (Lekshmi *et al.* 2017). A correlation was shown between AGP use and development of resistant commensal *Escherichia coli* in pigs, poultry and cattle in seven different countries (Chantziaras *et al.* 2014). Also, a recent worldwide survey identified four antibiotic resistant pathogens (*Staphylococcus aureus, Klebsiella pneumoniae,* non-typhoidal *Salmonella and Mycobacterium tuberculosis*) in food animals (Prestinaci *et al.* 2015). Additionally, beef, pork and poultry and even milk consumed by humans may contain small amounts of antibiotics residues (Kjeldgaard *et al.* 2012). The development of resistant pathogenic bacteria in food animals may eventually spread through the food chain (Ciara & Seamus 2008). The use of AGP have been shown to affect human health (Heuer *et al.* 2009), and could be a potential public health hazard (Grundmann *et al.* 2006). The pathogenic resistant bacteria may cause a loss in the production chain and also increase cost of production. In addition, antibiotic-related environmental pollution, now a worldwide concern, results from excreted feces and urine from farm animals often used as manure in agricultural farm lands (Lupo *et al.* 2012). Similarly, antibiotic-related environmental pollutants can also leach into water sources (Wooldridge 2012). Thus, there is public disquiet over antimicrobial resistance bacteria circulating among food-producing animals and the subsequent transmission to humans (Prestinaci *et al.* 2015). Consequently, the evaluation and development of effective alternatives to AGP that can enhance animal health, boost productivity as well as assure food safety are needed (Seal *et al.* 2013). Effective alternatives should be able to maintain current animal production levels without threatening public health (Millet & Maertens 2011). The European Union banned the use of antibiotics for growth promotion in 2006 (Regulation 1831/2003/EC). Public pressure may soon lead to similar bans in Canada. Therefore, there is urgent need for better alternatives to AGP for safe food production. Consequently, there is research and commercial interest in the development of natural feed additives like direct-fed microbials (DFM) for use in animal production.

Direct fed microbials are live microorganisms which, when administered in adequate amounts, confer health benefits to the host (Hill *et al.* 2014). For effective application of DFM in livestock production enterprises and to increase the usefulness of these additives, the mechanisms of action must be known. The identification of the most effective DFM that support optimal animal health and productivity will reduce the need for antibiotics use and the risk of transferring antibiotic resistant bacteria to the food chain (Guo *et al.* 2006).

Studies on the application of DFM in calves have shown enhanced inflammatory response for pathogen clearance in calves (Novak *et al*. 2012). Likewise, DFM can also improve intestinal microbial balance, promote intestinal digestion and increase animal growth performance (Sun *et al.* 2010), prevention of mycobacterium tuberculosis (Badiei *et al.* 2013) and reduction of fecal shedding of serotype O157:H7 (Schamberger *et al.* 2004). Direct fed microbial stimulates host's nonspecific resistance to microbial pathogens and thus may assist in the reduction of pathogens (Rahimi *et al.* 2011). Direct fed microbial have the potential to modify the composition of the gut microbiota and may contribute to optimizing beneficial functions (digestion, production of vitamin K, promotion and development of the immune system, and detoxification of harmful chemicals) of gut microbial communities resulting in improved gut health (Hemarajata & Versalovic 2013). The main driving force in shaping the gut microbiota during the lifetime of an animal is diet which interacts with gastrointestinal bacteria. (Thursby & Juge 2017). The early gut microbial composition and diversity have been linked to calf/host health (Oikonomou *et al.* 2013) (Hanning & Diaz-Sanchez 2015). The underlying mechanism of DFM functionality is often assumed to stem from their ability to impact the gut microbiota (Sanders 2016). Therefore, the study of microbial communities in the gastrointestinal tract will allow for more effective methods of improving animal health and productivity.

Chapitre 1 Literature review

1.1 Application of antibiotic growth promoters in farm animal production

About 70 years ago, it was discovered that antibiotics improved growth of farm animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues (Moore *et al.* 1946). Similarly, a small number of farmers in USA found that pigs fed penicillin fermented mixture grew faster (Wahlstrom *et al.* 1950; Hewes 1955). Subsequently, chlortetracycline, doxycycline and sulfonamides were used to promote the growth of calves, pigs and chicken (Cunha *et al.* 1951). The legal usage of antibiotics in feed has a history of over 60 years (Hao *et al.* 2014).

Antibiotic growth promoters (AGP) are antibiotics used at sub therapeutic levels continuously to improve growth, feed efficiency and to prevent diseases in agricultural animals (Nagaraja & Taylor, 1987; Barton, 2000). The bulk of antibiotic usage occurs in agricultural settings including use in farm animals (Landers *et al.* 2012). The use of AGP is the result of intensive farming attempting to increase quality and quantity of outputs on farms (Butaye *et al*. 2003).

The majority of antibiotic use is in agricultural settings (Landers *et al.* 2012) with most antibiotics (more than 40%) being added in animal feed at subtherapeutic levels for the improvement of animal production in the USA (Van Lunen 2003). Examples of commonly used AGP in livestock production enterprises are listed in (Table 1.1). Antibiotic growth promoters are administered to cattle for acceleration of weight gain (Shuford & Patel, 2005) and to control/prevent diseases (Phillips *et al*. 2004). In general, antibiotics including amoxicillin, penicillin, erythromycin, quinolones, gentamicin, novobiocin, tylosin, tilmicosin, and tetracycline are used extensively worldwide (Economou & Gousia 2015). For the beef industry, the use of antibiotics is mostly for the prevention of bovine pneumonia, diarrhea, and shipping fever commonly occurring in cattle (McEwen & Fedorka-Cray 2002) while in calves, prophylactic antibiotics are used to prevent opportunistic pathogens in the gut flora from thriving during stressful periods, to prevent and control intestinal infections, and to prevent diarrhea and pneumonia (Timmerman *et al*. 2005; McEwen & Fedorka-Cray 2002). Milk replacers for calves sometimes contain antimicrobials for disease prophylaxis (Thames *et al*. 2012; Kaneene *et al*. 2008). Additionally, some producers use feed containing low amounts of antibiotics all the time, seasonally or during outbreaks of diseases for weaned heifers aged below 12 months. Poor management practices like inadequate colostrum intake ultimately motivate the routine use of sub therapeutic and prophylactic antibiotics in feed and milk replacers (Raymond *et al.* 2006).

Table 1.1. Antibiotics used for growth promotion and feed efficiency in food animals

Group of Antibiotics	Antibiotics for use as growth promoters
Glycolipids	Bambermycin, avoparcin, ardacin
Streptogramins	Virginiamycin Avilamycin
Oligosaccharide	Gentamycin
Polypeptide	Bacitracin
Ionophore	Monensin, salinomycin
Macrolide	Tylosin, spiramycin, erythromycin
Tetracycline	Chlortetracycline, oxytetracycline
Quinoxalines	Carbadox, olaquidox
Elfamycin	Efrotomycin
Pleuromutilins	Tiamulin
beta Lactam	Penicillin

Adapted from Lekshmi *et al.* (2017)

1.2 Benefits of using of antibiotic growth promoters in farm animal production

1.2.1 Enhance animal health status

Antibiotic growth promoters' use has been linked to the reduction of opportunistic pathogens and subclinical infections (like respiratory diseases) in farm animals (Dibner & Richards, 2005). Also, antibiotics administered at low concentrations modulate enteric immune responses (Costa *et al*. 2011). Antibiotic growth promoters' use has been shown to reduce mortality and morbidity from clinical and subclinical infections in animals (Gersema & Helling, 1986). Similarly, AGP have been used for improvement of enteric health and prevention of nutrient degradation by the intestinal microflora (Hao *et al.* 2014). Generally, antibiotics work by limiting harmful microbes, reduce metabolic demands for the gastrointestinal system, stimulate the host immune system and hence draw nutrients for optimal performance (Nocek *et al*. 2011). Many AGP have made major contributions in the prevention and control of infectious diseases such as *Streptococcus pneumonia* in animals (Hoflack *et al.* 2001; Krausse & Schubert 2010).

1.2.2 Improve animal performance and production efficiency

Antibiotic growth promoters are used in farm animals for improving growth rate and feed conversion efficiency (feed/gain ratio), and in the prevention and control of diseases (Morris *et al.* 1990; Dibner & Richards 2005; Niewold 2007). Antibiotic growth promoters have been shown to improve performance of beef cattle on pasture in feedlots and to improve the average daily weight gain of farm animals (Goodrich *et al*. 1984). Antibiotic growth promoters have also been used to improve milk productivity in dairy cattle (Duffield & Bagg 2000). It is believed that the growth promoting effect of AGP is mediated through enhanced energy partitioning and control of gastro intestinal infections due to altered microbiota in the intestine (Backhed *et al.* 2005). Antibiotic growth

promoters alternate the normal intestinal microbial population of the animal resulting in more efficient digestion of feed and metabolic uptake of nutrients (Lin *et al*. 2013). The growth rate and feed conversion have been directly correlated with the ability to control *Clostridium perfringens*, the causative agent of clinical and subclinical necrotic enteritis of farm animals (Stutz & Lawton 1984).

1.2.3 Effects of antibiotic growth promoters on gastrointestinal microbiota

Antibiotics alter the community structure of the gut microbiome and have significant effects on the microbiota, consequently reducing colonization by microbes (Theriot & Young 2015).The use of antibiotics disturbs the ecology of the microbiota causing dysbiosis, hence the inability to perform vital functions such as nutrient supply, vitamin production, and protection from pathogens (Guarner & Malagelada 2003). Dysbiosis is linked with many health problems and is also associated with susceptibility to develop infectious diseases (Langdon *et al.* 2016). The growth-promoting effect of AGP is due to the interaction between the AGP and the gastrointestinal microbiota (Chapman & Johnson 2002). It has been noted that the use of AGP changes diversity and structure of microbial communities in the gastrointestinal tract of the animal (Lin 2011). The composition of the gastrointestinal microbiota has been shown to be affected by AGP; for example, lactobacilli populations were significantly reduced or increased in abundance in the intestine after AGP use (Dumonceaux *et al.* 2006; Wise & Siragusa 2007).

1.3 Problems associated with the use of antibiotic growth promoters

1.3.1 Development of resistance in animals and humans

Repeated use of AGP in farm animal production has led to the development of antimicrobial resistance in animals and subsequently humans (Szmolka & Nagy, 2013). Also, frequent antibiotics use on farms can produce resistant strains of microorganisms that may turn up in meat in the grocery stores (Mole, 2013). Antibiotic resistant bacteria have been found in a slaughter house in Poland (Wieczorek *et al*. 2013). There have been studies showing associations between the use of antibiotics and the occurrence of resistant enteric bacteria in cattle (Sato *et al*. 2005), swine (Mathew *et al*. 2005) and chicken (Takahashi *et al*. 2005). Pathogenic bacteria resistant to a number of antimicrobial agents were detected worldwide in the 1980s (Aarestrup *et al*. 2003). Generally, resistant bacteria remain in livestock and farm animal products during slaughter and processing, and then are passed along to humans who consume these products. The residual antibiotic in foods is thus improper for human consumption (Lindquist *et al*. 2014). Antibiotic resistant bacteria may be transmitted to the human population from farm animals through food, environment (water, air and soil) and by direct contact of the animals with humans (Marshall & Levy, 2011). Transmission of antimicrobial resistance to humans can be in the form of

either resistant pathogens or commensal organisms carrying transferable resistance genes (McEwen & Fedorka-Cray 2002; Mathur & Singh 2005).

Bacteria develops resistance either by exposure to low doses of antibiotics in their surrounding or is acquired from other bacteria through DNA transfer mechanisms (Lekshmi *et al.* 2017) (Figure 1.1). The spread of antibiotic resistance genes can be attributed to horizontal gene transfer. In addition, the clustering of several resistance genes on a single plasmid can lead to selection of multidrug resistant strains through a single horizontal transfer event (Barlow 2009). The mechanisms for horizontal gene transfer include transformation, transduction, and conjugation (von Wintersdorff *et al.* 2016).

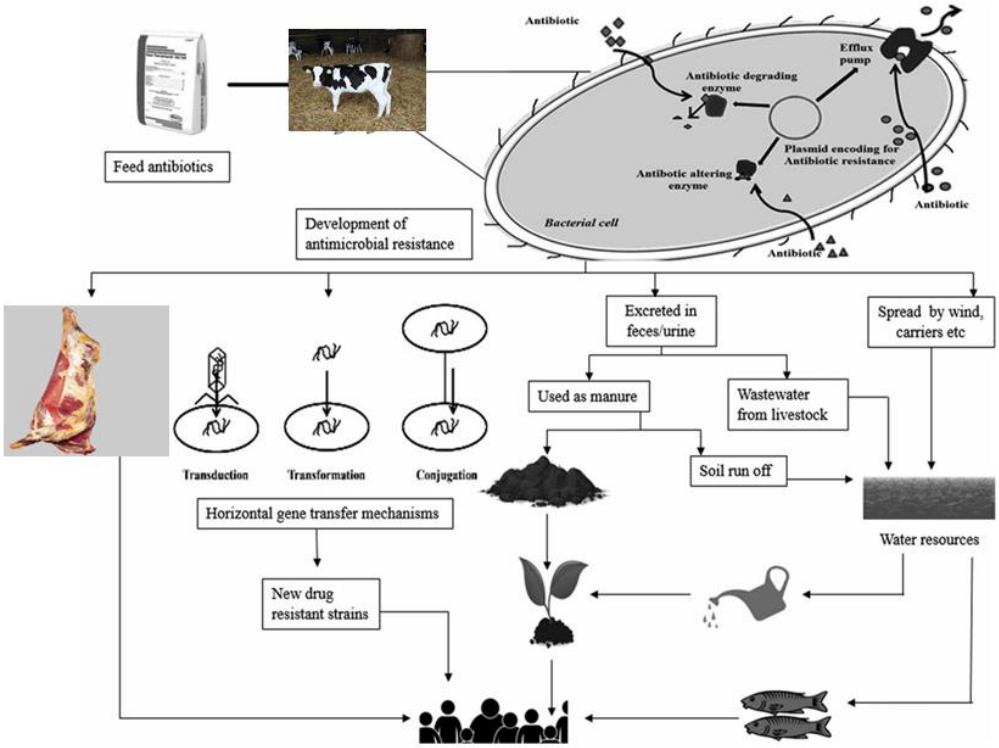

Figure 1.1. Adapted from Suresh et al. 2017. The transfer of antibiotic resistance genes.

1.3.2 Effect on human health

Human health can either be affected directly through residues of an antibiotic in meat and meat products (Kjeldgaard *et al.* 2012) indirectly, through the selection of antibiotic resistance determinants that may spread to a human pathogen (Marshall & Levy 2011b). The resistant pathogen can be acquired simply by ingesting them, and also contaminated meat and other cross-contaminated foods can cause millions of cases of gastrointestinal illnesses including salmonellosis and campylobacteriosis (Scallan *et al.* 2011). The transfer of resistant bacteria from food animals to humans is most evident in human bacterial pathogens that have food animal sources, such

as *Campylobacter*, which has reservoirs in chickens and turkeys, and *Salmonella*, which has reservoirs in cattle, chickens, pigs, and turkeys (Anderson *et al*. 2003). The multidrug resistant commensal *E. coli* is the most prevalent in the food animal industry, acting as reservoir for intra- and interspecific exchange through contaminated food to humans (Szmolka & Nagy, 2013).

1.3.3 Potential public health hazard

The presence of antibiotic-resistant bacteria in the food chain represents a growing public health problem because infections from resistant bacteria are increasingly difficult and expensive to treat. Its effects cause a high frequency of treatment failures and the duration of hospitalization is seen to increase (Roberts *et al*. 2009). Medical authorities are now challenged with infections with no available effective antibiotics to treat them since the causative bacteria has developed resistance (Barton & Hart 2001). According to the Center for Disease Control and Prevention (CDC), antimicrobial resistance cost an estimated $20 billion in excess health care expenses and $35 billion in other societal costs annually in the United States alone (CDC, 2011). Bengtsson & Greko (2014) refer to this situation as an economic disaster. The absence of alternatives to AGP may lead towards a post-antibiotic era, in which many common infections will be very difficult to cure and may even be life-threatening. For instance, in Canada, an *E. coli* 0157:H7 outbreak in beef products (traced back to XL Foods Inc.) in 2012 made 12 people sick (Larsen, 2013). Similarly, listeriosis outbreak in the summer/fall of 2008, linked to ready-to-eat meats produced at a Maple Leaf plant in Ontario, was a significant public health crisis that resulted in the deaths of 20 people across five Canadian provinces (Health Canada, 2009). In the United States alone, more than two million people are infected by antibiotic-resistant bacteria leading to the death of about 23,000 people annually (Ventola 2015).

1.3.4 Increase cost of production/loss in the production chain

Stakeholders in agriculture and agri-food sector may incur significant financial losses in terms of product recalls when health issues related to antibiotic residues in food products or antimicrobial resistance are detected. An example is the *E. coli* O157:H7 in beef products recall case of XL Foods Inc. in 2012. Contamination of food can happen at any stage of the production chain: raw materials used in animal nutrition, feed manufacturing, farm level, slaughter plant, meat processing, retail and preparation of meat at home. Pathogenic-resistant organisms growing in livestock can enter the food supply chain and could spread extensively in food products (Garofalo *et al.* 2007). With each product recall, cattle producers that sell to large processors also suffer. The cost of feeding market cattle can add up quickly; each day that cattle are held back from processing, there is additional cost (feeding and handling) to the producer. Furthermore, product recalls when there is contamination of the food chain may lead to closure of businesses.

1.3.5 Environmental contamination

Land application of animal manure represents a significant source of environmental exposure to these antibiotics (Topp *et al.* 2013). The fecal waste from thousands of animals reared under intensive conditions is often spread as fertilizer on pasture and crop lands. Similarly, frequently farm wastes containing bioactive veterinary drug residues and antimicrobial-resistant bacteria are disposed into bodies of water and can create reservoirs in the environment for antibiotic resistant bacteria (Vandendriessche *et al*. 2014). There is also the possibility of occupational hazards through exposure to aerosol and dust contaminated with antimicrobials in the environment. Residues from farm environments may contain antibiotics and antibiotic resistance genes that can contaminate natural environments. For example, vancomycin-resistant *Enterococcus* was found in meat and also in manure on farms where avoparacin was used as a growth promoter (Martinez, 2009). Also, it has been shown that all compounds including antibiotics after passing through wastewater treatment could be released directly into the environment (Kümmerer 2003). There is increasing evidence suggesting that Antibiotics resistance genes are ubiquitous in all natural environments (Berglund 2015). The source of these increasing resistant genes is most likely the routine discharge of antibiotics and resistance genes from wastewater or run-off from livestock facilities and agriculture (Berglund 2015).

1.4 Bans and anticipated bans

The use of AGP in farm animal production enterprises was banned in Denmark, Norway and Sweden in 1998–1999, 1995 and 1986, respectively (Grave *et al*. 2006). Beginning on January first, 2006, the European Union instituted a total ban on the use of AGP, although antibiotics use for therapeutic reasons is allowed. The Swedish and the Danish ban on the use of AGP has seen overall successes. Denmark introduced a monitoring system making it the first country to establish systematic and continuous monitoring of antimicrobial resistance in food animals and in humans. Similar programs have been implemented in other countries like Canada (The Canadian Integrated Program for Antimicrobial Resistance Surveillance, CIPARS) and the US (National Antimicrobial Resistance Monitoring System, NARMS). A major goal of the European ban on AGP was to reduce antibiotic resistance traits in the microbial flora of farm animals.

The Canadian Institute for Environmental Law and Policy (CIELAP) recommended that the usage of antibiotics as growth promoters in Canadian animal agriculture should be banned since 2009 (Hotlz, 2009). Human health was the main reason for these recommendations as a result of the emergence of strains of bacterial pathogens that are resistant to antibiotics. This recommendation was in light of both the European experience and other factors specific to Canadian situation. In order to execute a ban on the use of antibiotics for animal farming in Canada, there are about five major factors that need attention if CIELAP's recommendation is to be applied including (1) timing, (2) rationale and education, (3) cultural factors, (4) economics and international trade, and (5) an extended information/consultation process (Holtz, 2009). The lack of new

regulatory initiatives in Canada despite increasing concern about AGP for over a decade gives the matter urgency at this point to find alternatives. Thus, it is anticipated that an AGP ban will progressively be imposed in North America (Reti *et al*. 2013).

Presently, many countries including Canada, China, Australia, Brazil and Ukraine do not have any restrictions on AGP use and only have limited requirements to obtain veterinary prescriptions. These countries rely on voluntary actions to limit use for growth promotion, and Canada and Australia do report some limitations at the state or territory levels (Maron *et al.* 2013).

1.5 Alternatives to antibiotic growth promoters

In attempt to limit AGP use in animal farming, attention has been given to various alternatives. These alternatives could enhance animal health, boost productivity as well as assure food safety (Seal *et al.* 2013). Effective alternatives to AGP are therefore urgently needed to help maintain current animal production levels without threatening public health (Millet & Maertens, 2011). With the anticipated bans in Canada, the challenge and need to find alternative methods to control and prevent pathogenic bacteria spread in the food chain is increasing. Essential information on the impact of these alternatives on the host is also incomplete. There is thus the need to look for possible substitutes that could enhance the natural defense mechanisms of animals and reduce massive use of antibiotics. In this perspective, several products including direct fed microbials, prebiotics, in feed enzymes, phytogenic feed additives, bacteriophages, symbiotic etc are being developed as alternatives to AGP in livestock production.

1.5.1 Direct fed microbials

Direct fed microbials (DFM) are live microorganisms which, when administered in adequate amounts, confer health benefits to the host (Hill *et al.*2014). They are able to become part of the normal microbial flora of the intestine, survive the gastrointestinal passage, adhere and colonize the intestinal tract by competing with resident microbiota (Gaggia *et al*. 2010, Table 1.2). In addition, they are nontoxic and nonpathogenic to the host. Initially, DFM were used in young animals to promote gut health since they could accelerate the establishment of intestinal flora favoring feed digestion. Subsequently, it became possible for mixtures of DFM to be used for fiber digestion and prevention of ruminal acidosis in mature cattle. After this, second generation DFM have been produced from the knowledge of the effect of previously known DFM for the improvement of milk yield, growth and feed efficiency (McAllister *et al.* 2011). Some beneficial characteristics of DFM are shown in (Table 1.2).

Table 1.2. Beneficial properties described for some direct fed microbials

Host benefit	Microbial trait implication
Nontoxic and non-pathogenic	Normal inhabitants of the target species
Survival, colonisation	Competition with resistant microbiota
Pathogen burden reduction	Direct antagonism
Improved gut barrier function	Promote gut barrier integrity
Stimulation of immunity	Enhanced T-cell number and activity levels
Reducing of inflammation	Promote anti-inflammatory cytokine production
Production of antimicrobial substances	Antagonism towards pathogenic bacteria

Adapted from O'Toole & Cooney (2008)

1.5.2 Prebiotics

These are non-digestible food ingredients that beneficially affect host by selectively stimulating growth or activity of a restricted number of bacteria in the colon (Schrezenmeir & Vrese, 2001). Some prebiotics such as mannan-ologosaccharides have been shown to induce changes in gut morphology, lower pH of excreta and reduce bacterial challenge in the intestine of pigeons (Abd El-Khalek *et al.* 2012). Some common examples include inulin, fructooligosaccharide (FOS) and galactooligosaccharide.

1.5.3 In-feed enzymes

In -feed enzymes are commonly produced by bacteria (*Bacillus subtilis*), fungus (*Trichoderma reesei*, *Aspergillus niger*) or yeast (*Saccharomyces cerevisiae)* and are used in the feed industry. They help break down certain components of the feed like glucans, proteins and phytates that the animal may have problems digesting directly. In-feed enzymes are very effective at maximizing feed conversion efficiency and are mostly used in poultry production. Benefits of using in-feed enzymes include enhanced digestion and absorption of nutrients (Kiarie *et al*. 2013). Some examples of in feed enzymes include beta glucanase, xylanase, phytases, proteases, lipases and amylases.

1.5.4 Phytogenic feed additives

Phytogenic feed additives are plant-derived compounds incorporated into diets to improve the productivity of livestock through amelioration of feed properties, promotion of animal production performance, and improving the quality of derived foods. They include a wide variety of herbs, spices and plant extracts (Windisch *et al*. 2008; Wallace *et al*. 2010). They have beneficial effects such as improvement of host immunity, animal growth and production (Liu *et al*. 2012; Lee *et al.* 2011). Some examples include eugenol (a phenolic compound), *Yucca schidigera*, *Sanguinaria canadensis, and umbelliferal* (coriander and anise) and essential oils from thyme.

1.5.5 Essential oils

Essential oils are aromatic oily liquids obtained from plant materials and usually have the characteristic odor or flavor of the plant from which they are obtained (Stein & Kil 2006). These oils are generally recognized as safe by the Food and Drug Administration (FDA) and have been used as artificial flavourings and preservatives. Interest in using essential oils as replacement for antibiotics stems from results of *in vitro* studies showing that essential oils have antimicrobial activities against microflora commonly present in the gut (Michiels *et al.* 2009). The exact mode of action has not been established but the activity may be related to changes in lipid solubility at the surface of the bacteria (Michiels *et al.* 2009). Essential oils seem to control pathogenic bacteria with one trial showing positive results (Cho *et al.* 2006) while another study showed no beneficial effects (Huang *et al.* 2010).

1.5.6 Bacteriophages

Bacteriophages are bacterial viruses that are ubiquitous in the environment. For almost every bacterial species, there exists at least one bacteriophage that can specifically infect and ultimately destroy that particular bacterial group. Given these characteristics, bacteriophages have proven to be valuable in the control of foodborne pathogens. They have been used successfully in poultry (Atterbury *et al*. 2007) and some early work in cattle (Smith *et al*. 1987). Some examples include *Campylobacter* bacteriophage, *Escherichia coli* bacteriophage and *Listeria* bacteriophage.

1.5.7 Symbiotics

Symbiotics are mixtures of probiotics and prebiotics that may have synergetic effects (beneficial) on host (animal or man) through reduction of pathogenic bacteria population in the gastrointestinal tract (Kolida & Gibson, 2011). They have been used in farm animal production to improve health (Huyghebaert *et al*. 2011). Some examples include combinations of *Bifidobacteria* and fructooligosaccharides (FOS), *Lactobacillus* GG and inulins, and *Bifidobacteria* and *Lactobacilli* with FOS or inulin.

1.5.8 Organic and inorganic acids

Different combinations of organic and inorganic acids can be used in animal diets. Beneficial effects on growth performance and overall animal health have been shown when using acidic substances as feed additives (Quitmann *et al.* 2014). The effectiveness of feeding acids will vary with the type and also combinations of organic and inorganic acid, the animal's state and feed characteristics, in particular the diet's buffering capacity (de Lange *et al.* 2010). Organic acids have anti-microbial activity, can lower the pH of digesta in the stomach, and stimulate (pancreatic) enzyme production and activity in the small intestine (Quitman *et al.* 2014).

1.5.9 Egg yolk and antibodies

Egg yolk and antibodies also called immunoglobulin Y (IgY), appear to have considerable potential as alternatives to AGP to treat and prevent animal diseases (Kovacs-Nolan & Mine 2012). Egg yolk antibodies are produced as laying hens are injected with the organisms that cause specific diseases. The injection of these antigens induces an immune response in the hen which results in the production of antibodies. These antibodies are usually placed in the egg yolk. Booster inoculations are given to make sure there is a continuous transfer of antibodies from the hen to the egg yolk (Thacker 2013). These antibodies are then extracted from the egg yolk and processed. The antibodies could be administered in feed in several forms which include whole egg powder, whole yolk powder and water-soluble fraction powder (Xu *et al.* 2011).

1.5.10 Clay minerals

Naturally extracted clays (bentonites, zeolite, kaolin) and mixtures of various clays are added to animal diet and can bind and immobilize toxic materials in the gastrointestinal tract of animals (Thacker 2013). Clay minerals can bind aflatoxins, plant metabolites, heavy metals, and toxins. Clay minerals have been used extensively in swine diets for improving performance with diets containing mycotoxins (Schell *et al.* 1993). Clays have also been shown to prevent diarrhea in weaned pigs (Song *et al.* 2012).

1.5.11 Rare earth elements

Rare earth elements comprise the elements scandium, yttrium, lanthanum and lanthanoids (He & Rambeck 2000). The application of rare earth elements as feed additives for livestock has been practiced in China for decades (Panichev 2015). There is a report indicating significant improvements in performance using rare earth elements (He *et al.* 2001) though one report observed no change (Kraatz *et al.* 2006).

1.6 Application of direct fed microbials in animal production enterprises

1.6.1 Influence of direct fed microbials on animal performance

Direct fed microbials are used in cattle farming mainly to improve growth performance, milk production and feed conversion efficiency (LeJeune & Wetzel, 2007). Direct fed microbials have been used to possibly replace or reduce the need for antibiotics in neonatal and stressed calves (Krehbiel *et al.*2003). Direct fed microbials have been shown to improve the overall health of animals, improve the microbial balance in the gut, and to have antidiarrheal capacities (Timmerman *et al*. 2005; Donovan *et al*. 2002). Table 1.3 shows some DFM, their application and effects. Supplementing young calves' diets with DFM prevented the occurrence of possible imbalances in the normal microbiota in the intestinal tract and contributed to improved growth of young calves housed under stressful conditions by preventing diarrhea (Timmerman *et al*. 2005). Administration of DFM to

calves has consistently shown improved body weights, reduced incidence of diarrhea, reduced mortality rate and reduced need for antibiotic treatment against digestive and respiratory diseases (Agarwal *et al*. 2002; von Buenau *et al.* 2005; Galvao *et al*. 2005; Adams *et al*. 2008) (Table 1.3). Results from a meta-analysis showed that lactic acid bacteria have protective effects and reduced incidence of diarrhea in calves (Signorini *et al*. 2012). During the postpartum period, DFM supplementation increased dry matter intake, milk yield, and milk protein content of cows (Nocek *et al.* 2003).

Similarly, another meta-analysis by Desnoyers *et al*. (2009) showed that yeast supplementation in ruminants increased milk yield, rumen pH, rumen volatile fatty acids concentration, and organic matter digestibility as well as decreased rumen lactate concentration. Feeding yeast culture with grain improved health and minimized frequency of disease occurrence in dairy calves **(**Magalhaes *et al*. 2008**)** (Table 1.3). DFM are used sometimes in combination or as single strains. They have also been shown to act against zoonotic pathogens like *Escherichia coli* 0157:H7, *salmonella* sp and *staphylococcus aureus* (Tabe *et al*. *2008*). Lactobacillus species have shown immunomodulatory effects in humans and animal (Kim & Ji 2012) . Similarly, the yeast *Saccharomyces cerevisiae* has been observed to equally prevent the occurrence of many diseases in both animal and humans (Kelesidis & Pothoulakis 2012).

Table 1.3. Some direct-fed microbials (DFM) used in animal production and their effects

DFM	Company	Application and medium of delivery	Concentration administered	Effects studied	Results	References
Bacteria						
Bacillus subtilis	Blue Ribbon, Merrick's, Inc., Union Center, WI	Dairy calves Electrolytes scour treatment.	3 × 10^9 CFU/dose	Immune development	Enhanced inflammatory response for pathogen clearance	Novak *et al.* 2012
Bacillus subtilis natto Cultures	Prepared in the laboratory	Dairy calves In milk	1×10^{10} CFU	Rumen fermentation and development	Increased rate of rumen development	Sun *et al.*2011
Bacillus subtilis 166	Ivy Animal Health Overland Park, KS.	Feed lot cattle DFM premix added to the normal feed	6.4 x 10^8 CFU	Antimicrobial effect	No difference	Arthur *et al.* 2010
Propioibacterium jensenii 702	Denkavit.Pty Ltd. Melbourne, Victoria, Australia	Calves In Denkavit	4.78×10^{10} CFU	Digestive system	Successful gastrointestinal transit of bacterium Increase weight gain	Adams *et al.*2008
Prevotella bryantii 25A	Probios TC (Chr. Hansen, Milwaukee, WI	Dairy cows Administered directly with a syringe through the rumen cannula	2 ×10^{11} cells/dose	Efficacy as a probiotic & Role of probiotics in the regulation of rumen fermentation	Possible mitigation of inflammatory response from sub -acute acidosis	Chiquette *et al.* 2012)
Yeast						
Saccharomyces cerevisiae CNCM I-1077	Lallemand SAS, Toulouse, France	Dairy calves In the starter concentrate	1.5 × 10^6 CFU/g	Performance and rumen microbiota	Improvements in dry matter intake after weaning Increased presence of *R. albus*	Terré *et al.* 2015
Yeast Culture	A-Max, Vi-COR, Mason, IA	Dairy cattle	56 g/cow per day	Performance	Higher milk yield	Nocek *et al.*2011
Saccharomyces cerevisiae Yea-Sacc® 1026	Alltech Biotechnology	Dairy cattle	Ten grams	Milk production	Higher production of milk fat and milk protein but not yield	Kalmus *et al* 2009

	Center, Nicolasville YK, USA					
Yeast Culture	Diamond V XP Yeast Culture, Diamond V Mills Inc., Cedar Rapids, IA	Dairy calves Incorporated into grain	2% of DM	Performance, health, and immune-competence	Increase the number of phagocytized bacteria and killing of phagocytized bacteria	Magalhaes *et al*. 2008
Saccharomyces cerevisiae 1077 (SC) or *Saccharomyces boulardii* 1079 (SB)	Lallemand Animal Nutrition, Milwaukee, WI, USA	Holstein calves Diluted in 10 ml sterile water	2.0×10^{10} CFU/g 2.0×10^{10} CFU/g	Performance and rumen fermentation	SC modified ruminal fermentation dry matter intake was increased	Pinos-Rodríguez *et al.*2008
Saccharomyces cerevisiae	Lallemand Animal nutrition National Collection of Dairy Cultures, National Dairy Research Institute, India	Calves In milk	3 x 10(9) CFU/flask	Performance	Higher feed efficiency	Malik &Bandla2010
S. cerevisiae boulardii	Lallemand Animal Nutrition	Calves Top-dressed		Diarrhea days	Reduced diarrhea days	Galvao *et al.*2005
Saccharomyces cerevisiae Culture	Diamond V XP Yeast Culture; Diamond V Mills, Inc., Cedar Rapids, IA	Calves In grain	1, or 2% of dry matter	Effects on intake, growth, blood parameters, and rumen development	2% of the starter ration significantly increased starter and total dry matter intake, average daily gain	Lesmeister *et al,*2004
Cocktail *Lactobacillus acidophilu* (LA 51) & *P. freudenreichii* (PF 24)	Rosell Probiotics, Inc., Montreal, Quebec, Canada	Feedlot cattle	1×10^9 CFU of *L. acidophilus* 1×10^9 CFU	Fecal shedding of *Escherichia coli* O157:H7 and Salmonella	Reduced fecal shedding of E. coli O157:H7	Tabe *et al.*2008

2 strains of *Enterococcus faecium* (EF) & *Saccharomyces cerevisiae* (SC)	Probios TC (Chr. Hansen, Milwaukee, WI)	Dairy cows Administered directly with a syringe through the rumen cannula	5×10^9 cells/dose 2×10^9 cells/dose (SC)	Efficacy as a probiotic & Role of probiotics in the regulation of rumen fermentation	Possible mitigation of inflammatory response from sub -acute acidosis	Chiquette *et al.* 2012
Bacillus subtilis natto Na and N1 Culture	Culture	Calves Culture mixed with milk	1:1 vol/vol 1×10^{10} CFU	Pre-weaning Performance and immune function	Improved average daily gain. Advanced weaning age. Higher IgG, secrete more 1FN $_\gamma$ no difference in IgM and IgE levels	Sun *et al.*2010
Lactobacillus plantarum 220(LP)& Enterococcus faecium26(EF) & Clostridium butyricum Miyari (CBM)	Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan	Holstein calves Mixed in50ml of tap water	9×10^6 (CFU)/g (*EF)* (9×10^5 CFU/g) *CBM* (9×10^4 CFU/g)	Ruminal pH, Volatile fatty acids and bacterial flora	Improved mean ruminal pH in calves	Qadis *et al.* 2014
Protexin cocktail contains *Lactobacillus plantarum, Lactobacillus delbrueckii* ssp., *Bulgaricus, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Streptococcus salivarius,* ssp., *Thermophiles, Enterococcus faecium, Aspergillus oryzae, Candida pintolopesii*	Probiotics International Ltd., South Petherton, UK	Dairy calves	2 g of Protexin	Prevention of ileocecal infection	Decreased ileocecal infection by Mycobacterium tuberculosis	Badiei *et al.* 2013
Lactobacillus casei L. salivarius DSPV 315T Pediococcus acidilactici DSPV 006T	Cultured in the lab	Calves Administered with milk replacer	Daily dose of 10^9 CFU/kg body weight of each strain	Protection against Salmonella Dublin infection	Altered the response of animals against pathogen infection	Frizzo *et al.* 2012

Lactobacillus acidophilus NP51 and *Propionibacterium freudenreichii* NP24	Bovamine, Nutrition Physiology Corp., St. Cloud, MN	Dairy cattle Top dressed	4×10^9 CFU/head	Milk yield, efficiency of yield, and nutrient digestibility	Improved milk and protein yield, apparent digestibility of crude protein, neutral detergent fiber, and acid detergent fiber	Boyd *et al.* 2011
Combination of *Lactobacillus acidophilus* & *L. plantarum*	Bio Saver, Kemin Industries Inc. Des Moines, IA, USA	Newborn Calves In milk	(1.25 g/100 kg of milk).	Blood components immunoglobulin concentration	Increase in IgG concentration	Al-Saiady *et al*. 2010
L. acidophilus 27 sc culture	Propagated in the laboratories	Newborn Calves In milk	1.85×10^7 colony forming Unit/liter CFU l-1	Blood components immunoglobulin concentration		
Lactobacillus acidophilus (LA747) & *Propionibacteria freudenreichii* (PF24) acidophilus strain LA45	Nutrition Physiology Corp., Indianapolis, IN	Dairy cows mixing 45 g of finely ground corn with 5 g of DFM products	1×10^9 (CFU)/d LA747 2×10^9 CFU/d PF24 5×10^8 CFU/d of L. acidophilus strain LA45	Performance, nutrient digestibility, and rumen fermentation	DM intake did not differ, no differences in crude protein, or starch digestibility	Raeth-Knight *et al.* 2007
Lactobacillus cesei DSPV 318T & Lactobacillus salvirius DSPV315T		Calves		Performance	Stimulated earlier consumption of starter diet and earlier rumen development	Frizzo *et al*.2010
Bacillus subtilis natto Na and N1 Culture		Calves Culture mixed with milk	1:1vol/vol 1×10^{10} CFU	Pre-weaning Performance and immune function	Improved average daily gain. Advanced weaning age. Higher	Sun *et al*.2010

					IgG, secrete more 1FN $_{\gamma}$ no difference in IgM and IgE levels	
Lactobacilus rhamnous LHR19 & SP1 L.Plantarum LPAL & BG112 Bifido bacterium animalis lactis	Not mentioned	Horse 10ml of water or syrup	1×10^9 of each strain	Incidence of diarrhea and prevalence of fecal pathogens shedding	No benefit of administering DFM	Schoster *et al.* 2015

CFU-Colony forming units

1.6.2 *Lactobacillus acidophilus* species as a direct fed microbial

Most DFM used in cattle farming are lactic acid producing bacteria including *Lactobacillus acidophilus* (LA*) species (sp)*. *Lactobacillus acidophilus* is a homofermenter that ferments lactose efficiently to lactic acid as an end product (Cruywagen *et al.* 1996). *Lactobacillus acidophilus* is a short chain Gram positive bacterium with rod shape morphology and forms part of the normal intestinal microbiota of animals and humans (Anjum *et al.* 2013). Lactobacillus acidophilus has the potential to control pathogens (e.g. *Salmonella sp* and *Escherichia coli)* and alter or influence microbial communities by lactic acid antimicrobial activities (Servin, 2004). *Lactobacillus acidophilus* produces bacteriocins which have a broad spectrum of antagonistic effects against pathogens. Several bacteriocins of LA have been isolated and characterized. They are structurally similar, but their molecular weight varies as well as their spectrum of antimicrobial activities. These helps retain activities at a wide pH range along with strong inhibitory actions against pathogenic bacteria. In addition to bacteriocins, LA contains other polymeric substances such as exopolysaccharides which increase the colonization of probiotic bacteria by cell–cell interactions in the gastrointestinal tract (Kanmani *et al*. 2012). The surface properties of LA were recently screened and some isolates showed highly hydrophobic or auto agglutinating properties while some grew at very low pH and high bile concentrations (Maldonado *et al*. 2012). *Lactobacillus acidophilus* also produces a bio surfactant whose wide range of antimicrobial activities against bacterial pathogens as well as its anti-adhesive properties reduces the adhesion of pathogens onto gastric wall membranes (Kanmani *et al.* 2012). *Lactobacillus acidophilus* limits the activity of some pathogenic bacteria *in vitro* by producing hydrogen peroxide (Pridmore *et al*. 2008).

Application of two different probiotic preparations containing six *Lactobacillus sp.* of bovine and human origin were successful at reducing mortality rate, incidence of diarrhea and fecal coliforms counts in veal calves (Timmerman *et al*. 2005). Steers fed a standard steam-flaked corn-based finishing diet containing *L. acidophilus* NP51 showed a reduction of *E. coli* O157 fecal shedding by 57% (Younts-Dahl *et al.* 2004). Also, daily administrations of *L. acidophilus* NP51 for a period of two years reduced fecal shedding of *E. coli* 0157:H7 in beef cattle by 35% (Peterson *et al*. 2007, Tabe *et al*. 2008). Stress in neonates often leads to decreased population of *Lactobacilli* (Krehbiel *et al*. 2003) favoring the establishment of pathogenic bacteria such as *E. coli.etc.* Also, *Lactobacillus* can produce bile salt hydrolases, which are important to reduce lipid solubilization and absorption and even lower cholesterol levels (Ridlon *et al.* 2006).

1.6.3 *Saccharomyces cerevisiae* species as a direct fed microbial

Saccharomyces cerevisiae (SC) belongs to the group of simple eukaryotic cells (such as fungi and algae) and are part of the normal microbiota of ruminants making up <0.1% of ruminant microbiota (Czeruka *et al*. 2007). *Saccharomyces cerevisiae* has been used extensively as a DFM for ruminants including dairy cattle. Although yeast account for only a small proportion of the organism's microbiota, their cell size is ten times larger than that of bacteria and they can represent a significant stearic hindrance for bacteria pathogens (Czerucka *et al*. 2007). *Saccharomyces cerevisiae* survives transit through the gastrointestinal tract and inhibits the growth of a number of microbial pathogens.

Yeast is resistant to pH variations and is a good candidate as probiotics because while in the gastrointestinal tract, it is resistant to local stresses such as the presence of gastrointestinal enzymes, bile salts, and organic acids. There is first-hand evidence of its efficacy as an adjuvant agent for the treatment of diarrhea since it has been prescribed since the 20th century for diarrhea (Kelesidis & Pothoulakis, 2012). Auto aggregation and co-aggregation assays show that SC is able to co-aggregate with bacterial pathogens (Pizzolitto *et al*. 2012).

Saccharomyces cerevisiae cells have been alleged to eliminate traces of oxygen in the rumen, thereby helping oxygen sensitive bacteria to grow, as well as subsequent increase in viable anaerobic bacteria (Marden *et al*. 2008). Chiquette (2009) demonstrated that a combination of SC with *Enterococcus faecium* could effectively mitigate sub-acute acidosis symptoms in mid-lactation cows by increasing ruminal pH. The use of yeast as an additive has been associated with stabilization of ruminal pH and promotion of microbial growth (Bach *et al*. 2007). The addition of *SC* supplements may cause shifts in fibrolytic rumen bacterial populations, which could account for improvements in fiber digestibility (McAlister *et al*. 2011). *Saccharomyces cerevisiae boulardii* has been found to stimulate rumen microbial metabolism (Oeztuerk *et al.* 2005) while *SC* subspecies *boulardii* CNCM I-1079 has been shown to enhance immune response of stressed (from transportation) cattle (Keyser *et al.* 2007), decrease time spent in subacute rumen acidosis, decrease total volatile fatty acids concentration in the rumen (Thrune *et al.* 2009), and decrease ruminal lactate concentration (Guedes *et al.*2008). Recently, *SC* has been explored as modulators of rumen methanogenesis (Jeyanathan *et al*. 2014).

Incorporating yeast (SC) to calf diets reduced the incidence of diarrhea, health problems, elevated body temperature, antibiotic treatment and pre-weaning mortality rate, and improved fecal scores (Galvao *et al.* 2005; Magalhaes *et al.* 2008). It has been shown that metabolites present in yeast culture minimized the growth of enteric pathogens or reduced inflammatory response in the gut (Jensen *et al*. 2007).

1.6.4 Proposed mechanisms of action of direct fed microbials

Although studies on DFM as alternatives to AGP are encouraging in many experimental models, basic mechanisms cannot be defined clearly and are not well understood. Improving our understanding of the mode of action of DFM would increase our ability to select and use them appropriately in ruminant diets. A number of mechanisms by which DFM may maintain a healthy gut flora and animal performance have been proposed. Many studies have attempted to define potential mechanisms and possible ways by which DFM could modify digestion in a favourable way. These include: modification of ruminal acid production, promotion of establishment of required rumen microbial populations or improvement in ruminal fiber digestion (Figure 1.2, McAllister *et al*. 2011). Other proposed mechanisms include regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Figure 1. 2) and expression of bacteriocins (Mazmanian *et al*. 2008). The interference of DFM with the ability of pathogens to colonize and infect the mucosa is a known concept but the exact mechanism is still not known (Gill, 2003). Modification

of gut microbial population and alteration of rumen fermentation patterns have also been proposed for both bacterial and fungal DFM as a way to improve digestion (Brashears *et al*. 2003).

The production of bacteriocins is thought to be responsible for pathogen exclusion and it is one of the proposed mechanisms of action of DFM that is gaining wide acceptance. Bacteriocins are produced by both Gram-positive and Gram-negative bacteria and are made up of four different classes. Lantibiotics or class I bacteriocins are small heat-stable peptides (about 20 to 35 amino acids for the mature peptide). They act by forming pores in the cytoplasmic membrane of their target cell or by interfering with cell wall synthesis. Occasionally, they require the interaction of specific target or docking molecules for optimal activity. Class II bacteriocins are also small heat-stable peptides that, like the lantibiotics, are synthesized with a leader peptide. Bacterocins also act by forming pores in the cytoplasmic membrane of the target cell (Gillor *et al*. 2008). Class III bacteriocins are large proteins that possess bactericidal enzyme activities and class IV bacteriocins have lipid or carbohydrate moieties that are required for activity (Vermeiren *et al*. 2006). Some common examples of bacteriocins produced by known DFM that act against harmful bacteria include acidocin B (*LA*), acidophilucin A (L*A* LAPT 1060), mersacidin (*Bacillus subtillis*), plantaricin 423 (*L. plantarum* 423), plantaricin EF (*L. Plantarum* C11) and enterocin L50 (*E. faecium* L50).

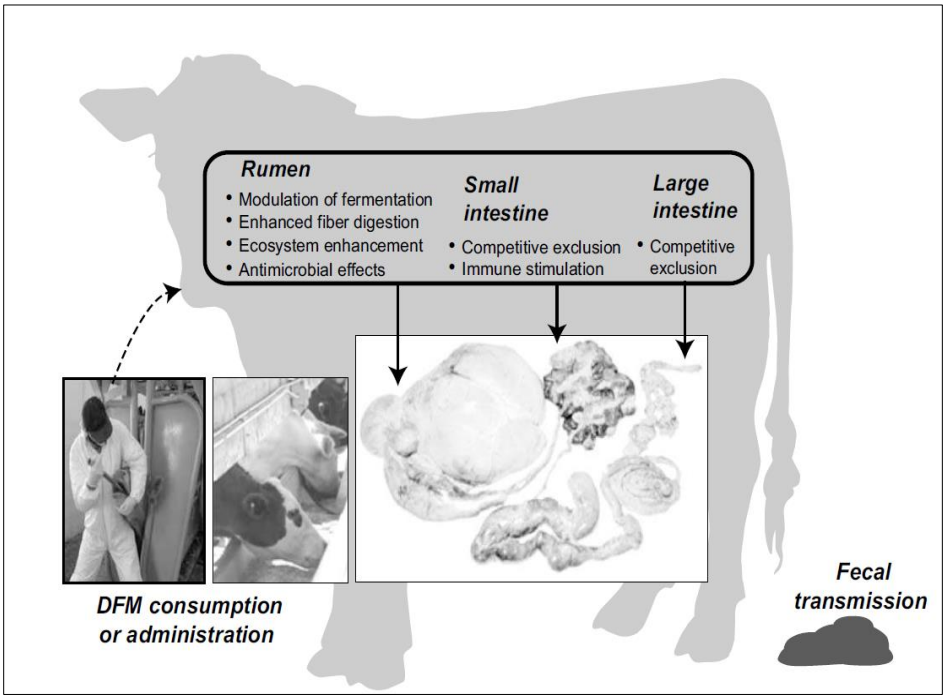

Figure 1.2. In ruminants, direct-fed microbials (DFM) may modulate ruminal fermentation, stimulate intestinal immune function, enhance nutrient absorption and competitively exclude select microbes from the small and large intestines. Additionally, some DFM may also remain viable after passage through the intestinal tract and excretion in feces which does not contaminate the environmental (Adapted from McAllister *et al*. 2011).

1.6.4.1 Direct fed microbials and the innate immune system

The innate immune system provides an immediate, but non-specific response which is very diverse and includes physical barriers, phagocytes complements and toll-like receptors (TLR) all serving to prevent infection, eliminate potential pathogens and initiate the inflammatory process (Mogensen 2009). The modulation of the innate immune system of cattle (calf) by DFM has not been well described. However, there are a few reports of stimulation of the innate immune system. A study on the administration of *Bacillus* based DFM to dairy calves enhanced inflammatory response for pathogen clearance (Novak *et al*. 2012).

Lactobacillus acidophilus isolates tested *in vitro* were capable of increasing oxidative burst and degranulation when compared with unstimulated controls in broilers (Farnell *et al.* 2006). Polymorpho neutrophils (PMN) are the first cells to arrive at the site of immune response (Oh *et al.* 2008). Polymorpho neutrophils generate reactive oxygen species (ROS) during phagocytosis particularly in response to pathogens. This functional response is known as oxidative burst and it is an essential player in innate immunity against invading microorganisms (Chen & Junger 2012). Phagocytosis was increased in groups on DFM-supplemented diets compared with those fed the control diet showing immunomodulatory effects of *Bacillus*-based DFM on innate immunity in broiler chickens (Waititu *et al*. 2014). The acute phase response is an intricate systemic early defence triggered by inflammation, infection and stress leading to increased hepatic synthesis of acute phase proteins (APP) (Cray *et al.* 2009). Some APP opsonize microorganisms and activate complement while others scavenge cellular remnants and free radicals, or neutralize proteolytic enzymes in order to directly protect the host (Petersen *et al.* 2004). Studies have also been done on the mediators of the acute phase response in feedlot steers in which DFM increased plasma concentrations of serum amyloid A (SAA), lipopolysaccharide binding protein (LBP), haptoglobin, and alpha1-acid glycoprotein (alpha1-AGP) indicating that DFM mediated the acute phase response (Emmanuel *et al*. 2007). A possible role of probiotics in mitigating the inflammatory response of dairy cows resulting from sub-acute acidosis has been proposed (Chiquette *et al.* 2012).

Metabolites present in yeast culture have been shown to induce inflammatory activity and altered chemokine receptor expression in human natural killer cells and B lymphocytes *in vitro* (Jensen *et al.* 2007). The detrimental activity of pathogens by yeast or SC is thought to be due to its ability to co-aggregate with bacterial pathogens (Pizzolitto *et al.* 2012). *Saccharomyces cerevisiae* boulardii CNCM I-1079 has been shown to enhance immune response of stressed cattle (Keyser *et al.* 2007). In addition, a *Bacillus*-based DFM was shown to enhance inflammatory response for more rapid pathogen clearance in calves (Novak *et al.* 2012). There are also indications that *Bacillus subtilis* are efficient immune modulators, initiating and improving immune functions in the presence of antigens in mice (Barnes *et al.* 2007). *Lactobacillus* species produces bio surfactant whose wide range of antimicrobial activities against bacterial pathogens as well as its anti-adherent properties reduces the adhesion of pathogens onto gastric wall membranes (Kanmani *et al.* 2013). *Lactobacillus acidophilus* limits the activity of some pathogenic bacteria *in vitro* by producing hydrogen peroxide (Pridmore *et al.* 2008). During the early period of life, there is inadequate immune development predisposing calves to increased susceptibility to infectious diseases during the weaning period (Baintner 2007).

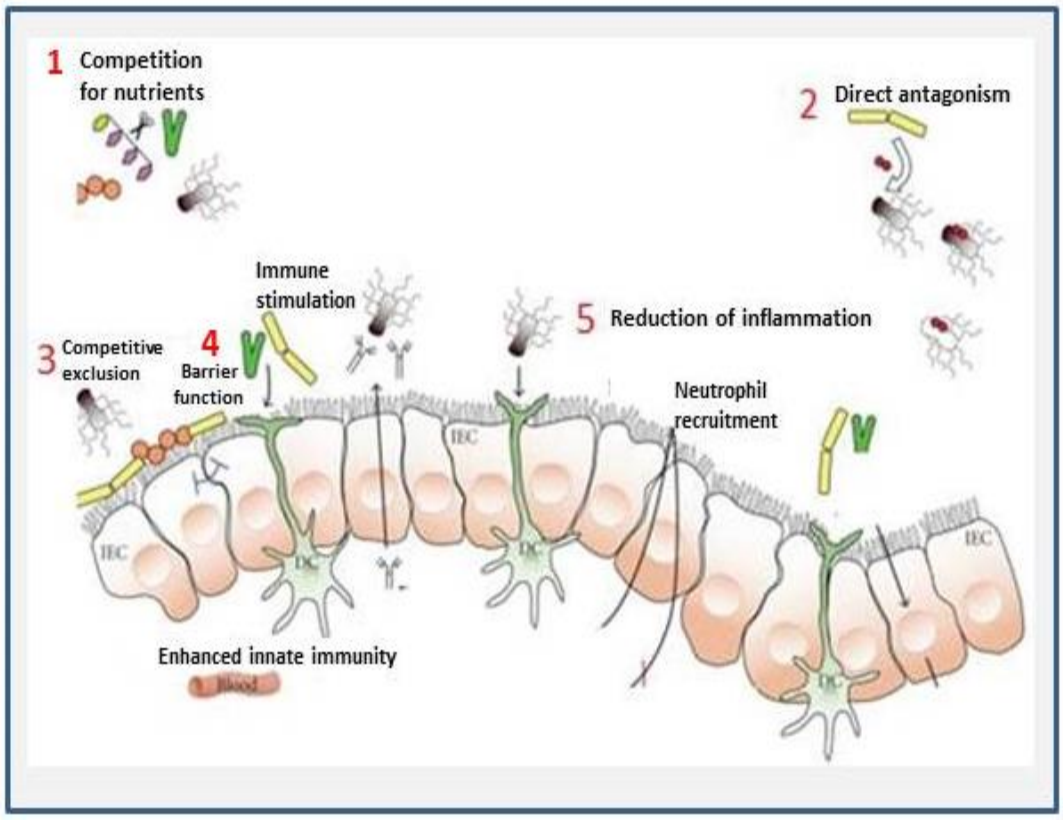

Figure 1.3. Proposed mechanisms of pathogen exclusion in the intestinal tract: (1) competition between the pathogen and direct fed microbial (DFM) for nutrients; (2) direct antagonism through production of antimicrobials (bacteriocins); (3) competitive exclusion through occupation of binding sites; (4) enhanced gut health through restoration of epithelial intergrity; and (5) stimulation of immune response resulting in host – exclusion of the pathogens. Adapted from O'Toole & Cooney (2008)

1.6.4.2 Probable mechanisms of action of Saccharomyces cerevisiae and Lactobacillus acidophilus in the gastrointestinal tract

One of the main mechanisms of DFM action is regulation of the immune system (Yan & Polk 2011). The stimulation, preparation and the functioning of the host immune system depends on the microbiota (Belkaid & Hand 2014). The luminal action of the GIT microbes plays key roles in the host –microbiota interactions. The DFM in the GIT consumed orally could convert nutrients into bioactive compounds (short chain fatty acid (SCFA), gamma-aminobutyric acid (GABA)) that in turn could have an effect on the GIT microbiota and hence the health of the, short-chain fatty acids animal (Figure 1.4). The luminal action can lead to antimicrobial activity which includes; inhibition of growth of bacteria and parasites, reduction of gut translocation of pathogens, neutralization of bacterial virulence factors and suppression of host cell adherence that interferes with bacterial colonization (Czerucka *et al.* 2007). There could be cross-talk with normal microbiota due to trophic action on the intestinal mucosa which decreases infected cells and stimulate the growth and differentiation of intestinal cells (Belkaid & Hand 2014). The microbiota could also restore fluid transport

pathways, stimulate protein and energy production, restore metabolic activities in colonic epithelial cells, secrete mitogenic factors that enhance cell restitution and enhance release of brush-border membrane enzymes in humans (Zanello *et al.* 2009; Canonici *et al.* 2011).The microbiota has also been seen to stimulate the production of glycoproteins in the brush border and production of intestinal polyamines (Kibe *et al.* 2014). Furthermore, the microbiota can restore normal levels of colonic SCFA (Morrison *et al.* 2016). It has been observed that bacteria play important roles in many metabolic pathways (e.g. secondary bile acids and SCFA pathways in humans) (Flint *et al.* 2012).

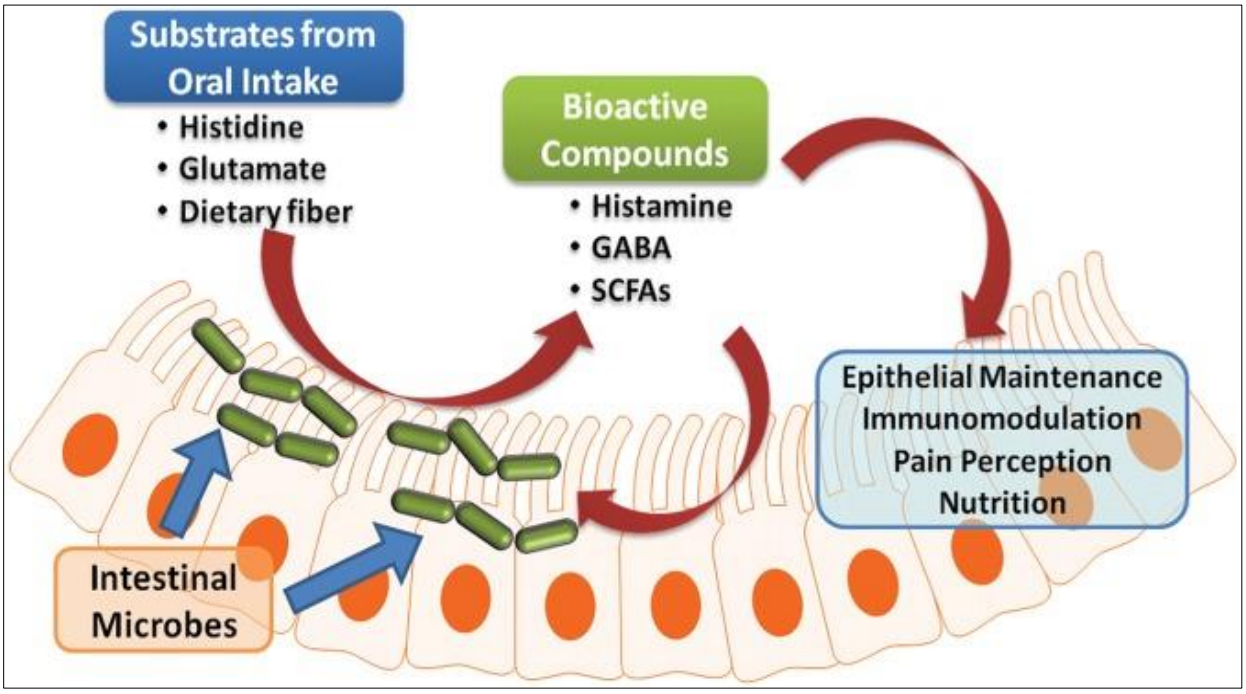

Figure 1.4. Luminal conversion by intestinal microbes may play an important role in host–microbiota interactions. Orally consumed direct-fed microbial may be converted by intestinal microbes into bioactive compounds that could affect the health of the host and the intestinal microbiota. GABA = gamma-aminobutyric acid; SCFA = short-chain fatty acids). Adapted from (Hemarajata & Versalovic 2013).

1.6.4.3 Direct fed microbials and the digestive system

Microbial populations in the digestive tract of animals influence nutritional processes and exert profound effects on the functional and morphological development of the gastrointestinal tract (Guarner, 2006). Changes in the diet of animals can lead to changes in microbiota composition and diversity (De Filippo *et al.* 2010; Maslowski & Mackay, 2011). Direct fed microbials are able to improve intestinal microbial balance; promote intestinal digestion thereby optimizing ruminal digestion. In the rumen, DFM modulate fermentation patterns and enhance fiber digestion; they can increase intestinal nutrient flow and absorption thereby improving diet digestibility (McAllister *et al.* 2011). Direct fed microbials are able to regulate the ruminant neonate microbial flora by promoting rumen development (Sun *et al*. 2011). Diect fed microbials ferment carbohydrates and produce SCFA which reduces intestinal pH and promote

the growth of intestinal cells and may affect cell differentiation, and improve digestion and absorption (Maslowski & Mackay 2011). Lactic acid bacteria inoculants applied at ensiling or added directly to the rumen fluid had the potential to increase the dry matter and neutral detergent fibre (Weinberg *et al*. 2007). Direct fed microbials stimulate intestinal metabolic activities such as increased production of vitamin B12, bacteriocins and propionic acid. A change in ruminal and ileal morphology of calves treated with DFM has been demonstrated. The average ileal height, crypt depth, total height (villus + crypt) and ruminal papillae width were greater in DFM (*L. acidophilus* and *P. freudenreichii*) treatment compared with controls in calves two weeks post weaning had more nutrient-absorptive area (Dick *et al*. 2013). Direct fed microbial (*Lactobacillus acidophilus* NP51 and *Propionibacterium freudenreichii* NP24) improved apparent digestibility of crude protein, neutral detergent fiber, and acid detergent fiber of lactating dairy cows (Boyd *et al*. 2011). The supplementation of yeast cell wall also increased villus height in the jejunum, width of villus in the ileum and number of goblet cells in villi of the jejunum and ileum (Muthusamy *et al.* 2011).

1.6.4.4 Direct fed microbials and the gastrointestinal microbiota

The term microbiome was first introduced by Joshua Lederberg as the 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally live together, determine health and disease of host (Lederberg & McCray 2001).The GIT microbiomes of farm animals are now well-known as important features of animal health, development and productivity (Celi *et al.* 2017). Early colonization of the gut is crucial for the functional, morphological and immunological development of the GIT (Hanning & Diaz-Sanchez 2015). Many factors impact the development of the gut and microbial colonization in calves during the early period of growth (Meale *et al.* 2016). Diet plays an essential role in providing the nutrients required by the host, but also by the microbiome. Some studies have shown that diet play pivotal roles on the species composition of the gastrointestinal microbiota (Callaway *et al.* 2010; Lai *et al.* 2016). The bacterial microbiota has established many mechanisms to impact the host, usually in a useful manner (Téllez *et al.* 2015). The developing GIT in calves offers unique possibilities for manipulating the intricate microbial communities (Yáñez-Ruiz *et al.* 2015). Feed additives like DFM can balance the the gutmicrobial population while improving the host immune system and conferring health benefits onto the host (Liao & Nyachoti 2017). The bacterial community composition and function in the GIT of calves is very important, since it can influence host health.

1.7 Strategies for characterizing the gastrointestinal tract microbiota

The approaches for characterising the GIT microbiota include: (1)16S rRNA gene sequencing or amplicon sequencing for taxonomic profiling, identification, classification and quantitation of bacteria within the gastrointestinal tract; (2) metagenomics for the identification and function of the different microbial communities which provides an opportunity to catalog the set of genes from the entire community (Qin *et al.* 2010). More so, the bacterial, archaea and fungi residing in the GIT can be studied (Gerritsen *et al.* 2011); and (3) metatranscriptomics which exploits RNA sequencing to determine which genes and pathways are being actively expressed within a microbial niche (Yeoman & White 2014). While metagenomics approaches provide information on microbial gene composition/functional

abundance, metatranscriptomics approaches provide details on the activities of the microbial community in terms of gene expression (Hugenholtz & Tyson 2008) (Table 1.4). Additionally, studies to understand the role of the gut microbes, the relationship and interactions between these microbes and the host need advanced "meta omics" approaches such as metaproteomics, and meta-metabolomic (Ross *et al.* 2012; Schloissnig *et al.* 2013) (Table 1.4).

Table 1.4. Some experimental and computational methods available for studying the microbiome

Approach	Definition	Molecules used	Level of information
Amplicon-based sequencing	Study of the taxonomy profiles by sequencing the targeted gene product	16S rRNA gene	Taxonomic composition, microbial abundance
Metagenome	Study of all genetic materials from a microbiome	Genomic DNA	Taxonomic and functional composition, gene abundance
Metatranscriptome	Study of all transcripts from a microbiome	RNA	Expression of genes
Metaproteome	Study of all proteins from a microbiome	protein	Protein profile, expression of proteins
Metametabolome	Study of all metabolites from a microbiome	Metabolites	Metabolite profile
Cultureomes	Study also minority populations and detects only viable bacteria	microbes	Replicate the natural environment
Stable isotope probing	Study DNA isolated from the target group of microorganisms can be characterized taxonomically and functionally by gene probing and sequence analysis	DNA	linking the identity of bacteria with their function in the environment

Adapted from Malmuthuge & Guan, 2016.

Using metaproteomics, it is possible to detect changes occurring even if there are no differences in microbiome profile (Franzosa *et al.* 2015). The detection of metabolites (metabolomics) in a microbial community is crucial as these molecules are important mediators of microbial interactions and microbial-host interactions (Franzosa *et al.* 2015).

The possibility of being capable of culturing distinct microbial populations (cultureomics) will certainly offer clues on potential methods to modify particular microbes in the microbiota community. Microbial culturomics (the culture of ''non-cultivable" species) is a recent concept based on the use of several culture conditions with identification by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) followed by the genome sequencing of the new species cultured (Lagier *et al.* 2015). Using culturomics, it is possible to identify minority populations, culture bacteria not restricted to eubacteria and allow the extensive categorization of new species (Greub 2012). The culturome method uses prolonged incubation and stringent anaerobic conditions, such as diffusion chambers simulating the natural environment of "uncultivable" microorganisms (Vartoukian *et al.* 2010). The stable-isotope probing is also a technique

that offers the possibility of identifying microorganisms actively involved in particular metabolic processes in conditions close to those occurring in situ (Hungate *et al.* 2015)

A combination of 16S amplicon-based approaches along with other omic approaches could improve our understanding of the GIT microbiota as well as its associations and the interaction with the host (calves). At the moment, there is not a single method that can be used to completely describe a microbial community both at the level of taxonomy and function. The combination of multiple techniques will offer the most complete and comprehensive description of the microbial community.

1.7.1. The 16S rRNA gene sequencing for taxonomic profiling

The 16S rRNA gene is used for the identification, classification and quantitation of bacteria within the gastrointestinal tract. The 16S rRNA gene is ubiquitous and is present in all bacteria, and it is the most conserved molecule generated from 30S rRNA precursor molecule (Rajendhran & Gunasekaran 2011). The highly conserved nature of the 16S rRNA gene makes possible the construction of universal primers that are able to amplify 16S rRNA genes from widely divergent bacteria (Figure 1.5). The 16S rRNA gene (approximately 1,500 base pair long) is part of the translation process making it easy to target a wide variety of bacteria (perfect universal target). The 16S rRNA contains nine variable regions interspersed between conserved regions (Figure 1.6). The hypervariable regions [(69-99 (V1), 137-242 (V2), 433-497 (V3), 576-682 (V4), 822-879 (V5), 986-1043 (V6), 1117-1173 (V7), 1243-1294 (V8) and 1435-1465 (V9)] of the 16S rRNA gene are used in differentiating bacterial species (Chakravorty *et al*. 2007). The conserved region makes universal amplification possible and variable region allows discrimination between specific bacteria.

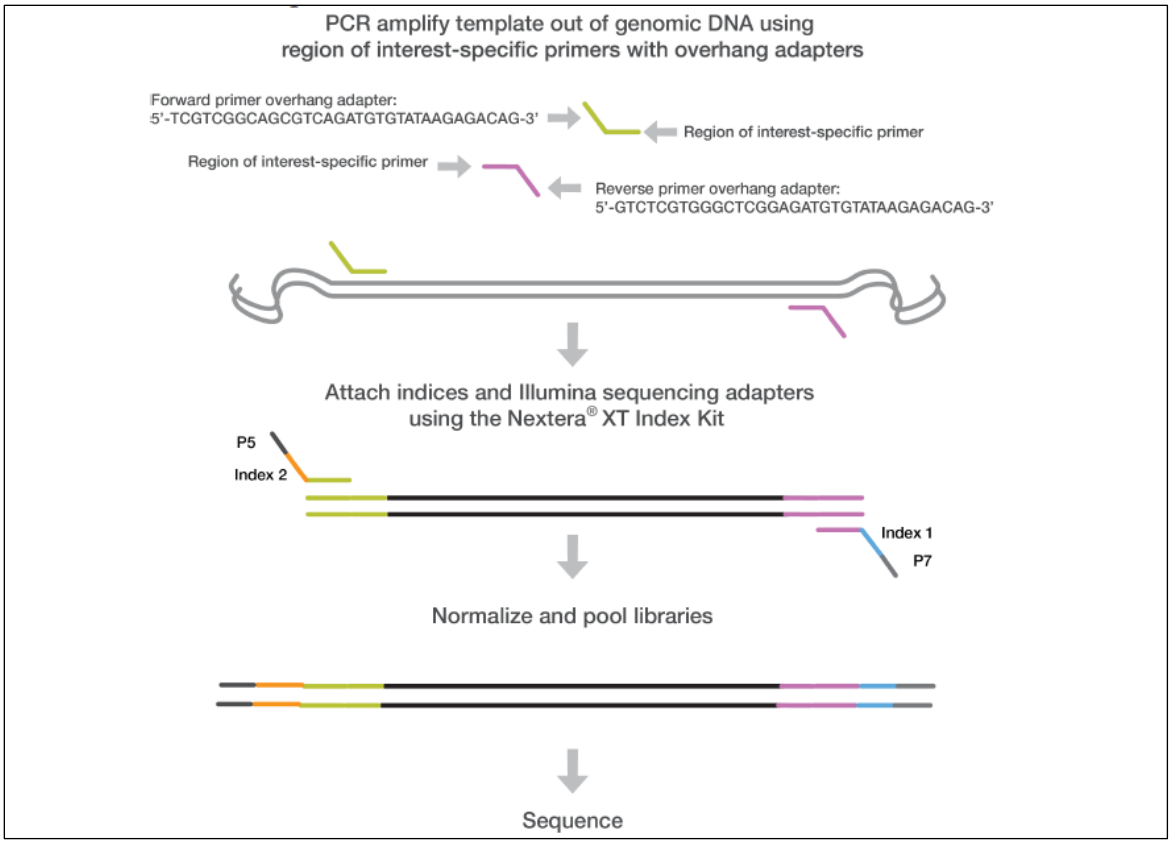

Figure 1.5. The 16S rRNA amplicon targeting the V3 –V4 region gene. The forward and reverse primers designed with overhang adapters are used to amplify the V3-V4 region of interest from genomicDNA.https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

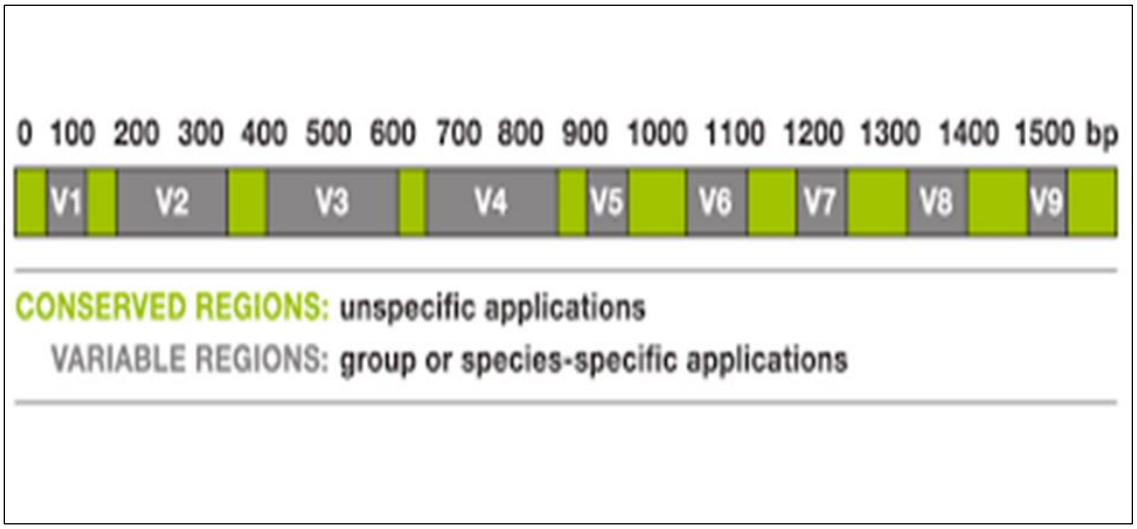

Figure 1.6. The variable (gray) and conserved regions (green) of the 16S rRNA gene (Source: https://biology.stackexchange.com/questions/54823/what-causes-the-variable-conserved-structure-in-the-16s-rrna-gene)

The 16S rRNA variable region used for bacteria characterization is an area of debate since the region of interest depends on experimental objectives, design, and sample type. Different 16S rRNA regions give different results with the differences arising from dissimilar regions chosen (Youssef *et al.* 2009). The combination of two or more variable regions together could offer sufficient sequence diversity to identify most bacterial species (Chakravorty *et al.* 2007). However, for example, the sequencing of the V3/V4 region revealed significant amplification bias compared to single regions (Claesson et al. 2010). In addition, error rates which are a bunch of unique reads and a ginormous distance matrix is problematic for analysing data (Kozich *et al.* 2013). Moreso, different sequencing platforms, sampling conditions will give different results (Harismendy *et al*. 2009).

1.7.2 Alternative marker genes

There exist a few other marker genes with single gene copies in the bacterial genome which could be used for taxonomic classification of bacteria. Examples include RNA polymerase beta subunit-encoding gene (rpoB), DNA gyrase (gyrB), cpn 60, GroEL chaperonin, and heat shock protein (dnaK) (Rajendhran & Gunasekaran 2011). However, the 16S rRNA gene still remains the main marker gene used in microbial studies conducted till date. The 16S rRNA is well established and sequencing costs are relatively cheaper compared to other sequencing methods.

1.7.3 Limitations of the 16S rRNA

One advantage of the 16S rRNA approach is that this only amplifies the specified region without host contamination and can have higher sensitivity due to target enrichment, and it is comparatively cheaper than other sequencing techniques (Poretsky *et al*. 2014). However, there are some drawbacks, including the fact that the target gene may not be truly universal; there could be primer bias and variant gene copy numbers (Poretsky *et al.* 2014). The bacterial species with higher variations in their genome can still be highly similar in their 16S rRNA gene sequences; hence there is difficulty in differentiating such species via 16S rRNA gene sequencing (Rajendhran & Gunasekaran 2011). Besides, if there is a horizontal gene transfer within the 16S rRNA gene, leading to mosaicism (presence of two or more populations of cells with different genotypes in one individual), it can also influence the taxonomic classification of bacteria (McDonald *et al*. 2005). More so, different primers are usually needed for archaea and eukaryotes (18S) and it is not possible to identify viruses.

1.8 Direct fed microbials and animal (calf) production

Effective DFM as alternatives to antibiotic additives like SCB and LA have the potential to effectively decrease sub-therapeutic and even therapeutic application of antibiotics in animal production. *Saccharomyces cerevisiae* and LA could enhance animal health at the farm level while ensuring safe and quality products for human consumption. Also, human health problems like development of antimicrobial resistance and environmental shedding of harmful bacteria will be reduced. The adoption of DFM as additives instead of AGP will play key roles in maintaining safe animal products. Also, further information will enable us find DFM that are more suited to particular production stages, whether

to help calves to establish a beneficial micro-floral population and control diarrhea or to help calves prevent infections during weaning hence minimising the use of antibiotics in calf management practises.

The present study provided an opportunity to understand the way DFM modulated calf GIT microbiota and enhanced host (calf) responses around birth and weaning.

1.9 Study hypotheses

1. The use of the DFM, *Lactobacillus acidophilus BT 1386 or Saccharomyces cerevisiae boulardii CNCM 1-1079* in calf feeding improves the digestive tract development, growth performance and health status of calves.
2. Feeding calves with *Lactobacillus acidophilus* BT 1386 or *Saccharomyces cerevisiae* boulardii CNCM 1-1079 modulates calf GIT microbiota leading to improved immune system development and the ability of calves to better resist infections and consequently reduced use of antibiotics in calf management operations

1.10 Study objectives

1. To investigate the effect of *Lactobacillus acidophilus* BT 1386 or *Saccharomyces cerevisiae* boulardii CNCM 1-1079 on blood cellular and biochemical/chemical constituents.
2. To determine the potential mechanisms of enhanced immune response by *Lactobacillus acidophilus* BT 1386 and *Saccharomyces cerevisiae* boulardii CNCM 1-1079.
3. To determine how *Lactobacillus acidophilus* BT 1386 or *Saccharomyces cerevisiae* boulardii CNCM 1-1079 modulate calf GIT microbial community composition by next-generation sequencing of the V3-V4 region of the 16S rRNA gene
4. To compare the efficacy of *Lactobacillus acidophilus* BT 1386 or *Saccharomyces cerevisiae* boulardii CNCM 1-1079 with tetracycline-neomycin, an antibiotic growth promoter

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Chapitre 2 Impact of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 on total lactobacilli population in the gastrointestinal tract and colon histomorphology of Holstein dairy calves

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2.1 Résumé

L'utilisation de produits microbiens à administration orale (PMAO) en remplacement des agents de croissance antibiotiques suscite de l'intérêt pour la production d'animaux d'élevage. Il existe toutefois peu d'information sur les effets des PMAO sur les veaux de race laitière. Cette étude visait à : 1) déterminer la viabilité des suppléments de PMAO dans l'alimentation et dans le tube digestif (TD) des veaux; 2) évaluer l'effet des PMAO sur la population de lactobacilles totaux dans le tube digestif et dans les excréments des veaux; et 3) examiner les effets des PMAO sur l'histomorphologie du côlon et le rendement global des veaux. Quarante-huit veaux (âgés de 2 à 7 jours) ont été soumis aléatoirement à l'un des quatre traitements suivants : 1) ration témoin (TEM) de lait de remplacement (LR), suivie d'un aliment de démarrage (AD); 2) TEM avec supplément de *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7,5 × 108 unités formant des colonies (UFC)/L LR + 3 × 109 UFC/kg AD); 3) TEM avec supplément de *Lactobacillus acidophilus* BT1386 (LA; 2,5 × 108 UFC/L LR + 1 × 109 UFC/kg AD); et 4) TEM avec antibiotiques (ATB) chlortétracycline et néomycine (528 et 357 mg/L LR, respectivement) et chlortétracycline (55 mg/kg AD). Quatre veaux par traitement ont été euthanasiés au jour 33 (présevrage) et quatre autres veaux par traitement ont été euthanasiés au jour 96 (post-sevrage) pour échantillonner différentes parties du TD. Les effets des traitements ont été analysés suivant un plan expérimental complètement aléatoire avec des mesures répétées et des ajustements de Tukey pour des niveaux multiples de comparaison. Les PMAO (SCB et LA) sont demeurés viables dans le produit commercial durant toute l'expérience. Le SCB viable a été récupéré dans tout le TD et dans les excréments des veaux avant et après le sevrage. Les PMAO n'ont pas eu d'effet sur la population de lactobacilles totaux dans le TD des veaux. Cependant, les excréments de veaux traités au SCB contenaient une plus grande population de lactobacilles comparativement aux TEM ($P < 0,01$) vers le sevrage. Durant la période précédant le sevrage, la distribution de la population de lactobacilles variait le long du TD (côlon>contenu de l'iléon>rumen>muqueuse de l'iléon; $P < 0,001$). La population de lactobacilles avait nettement diminué dans toutes les parties du TD ($P = 0,02$) après le sevrage comparativement à la période précédant le sevrage, sauf dans le rumen. La profondeur et la largeur des cryptes du côlon avaient diminué ($P < 0,01$), tandis que le nombre de cellules caliciformes contenant des mucines neutres avait généralement augmenté ($P = 0,058$) et que les mucines acides avaient augmenté ($P < 0,05$) chez les veaux traités au SCB et aux ATB, comparativement aux TEM et aux veaux traités au LA. Dans l'ensemble, les traitements n'ont pas eu d'incidence sur la performance de croissance. L'ingestion de SCB a stimulé la population de lactobacilles totaux vers le sevrage et a modifié la morphologie du côlon, ce qui pourrait avoir des effets bénéfiques durant la période de croissance précoce des veaux. Nos résultats donnent à penser que le SCB pourrait être utile comme modulateur de la santé gastro-intestinale des jeunes veaux de race laitière.

2.2 Abstract

There is interest in the use of direct-fed microbials (DFM) as substitutes for antibiotic growth promoters in farm animal production. However, little information exists on their effects in dairy calves. The aims of this study were to: 1)

determine the viability of supplemental DFM in feed and throughout the gastrointestinal tract (GIT) of calves; 2) evaluate the effect of DFM on total lactobacilli population in the digestive tract and in feces of calves; and 3) examine the influence of DFM on colon histomorphology and overall calf performance. Forty eight calves (2–7 days old) were randomly allocated to four treatments as follows: 1) Control (CTRL) fed milk replacer (MR) followed by starter feed (SF); 2) CTRL supplemented with Saccharomyces cerevisiae boulardii CNCMI-1079 (SCB; 7.5×10^8 colony forming units (CFU)/L MR + 3×10^9 CFU/kg SF); 3) CTRL supplemented with Lactobacillus acidophilus BT1386 (LA; 2.5 × 108 CFU/L MR + 1×10^9 CFU/kg SF); and 4) CTRL supplemented with antibiotics (ATB) chlortetracycline and neomycin (528 and 357 mg/L MR, respectively), and chlortetracycline (55 mg/kg SF). Four calves per treatment were euthanized on day 33 (pre-weaning) and an additional four calves per treatment on day 96 (post-weaning) to sample different sections of the GIT. The effects of treatments were analyzed following a completely randomized design with repeated measures and Tukey adjustments for multiple comparisons. The DFM (SCB and LA) remained viable in the commercial product throughout the period of the experiment. Viable SCB was recovered throughout the GIT and in the feces pre- and post-weaning. There was no effect of DFM on total lactobacilli population in the GIT of calves. However, feces of SCB-treated calves showed a greater lactobacilli population compared with CTRL ($P < 0.01$) around weaning. In the pre-weaning period, the distribution of lactobacilli population differed along the digestive tract (colon > ileum content > rumen > ileum mucosa; $P < 0.001$). The lactobacilli population was significantly reduced in all compartments ($P = 0.02$) post-weaning compared to pre-weaning, except in the rumen. Crypts depth and width of the colon decreased ($P < 0.01$) whereas number of goblet cells containing neutral mucins tended to increase ($P = 0.058$) while acidic mucins increased ($P < 0.05$) in SCB- and ATB-treated calves compared with CTRL and LA-treated calves. Overall, growth performances were not affected by treatment. Feeding SCB stimulated total lactobacilli population around weaning and altered colon morphology with potential beneficial effects during the early period of growth in calves. Our findings suggest that SCB would deserve more attention as a modulator of the gastrointestinal health in young dairy calves.

2.3 Keywords

Calves, Direct fed microbials, Histomorphology, Lactobacillus acidophilus, Saccharomyces cerevisiae boulardii, Viability

2.4 Abbreviations

AB: alcian blue**, ATB**: antibiotics, **BHB**: β-hydroxybutyrate, **CTRL**: control, **CFU:** colony forming units, **DFM**: direct fed microbials, **GIT:** gastro intestinal tract, **LA**: Lactobacillus acidophilus BT1386, **MR:** milk replacer, **PAS**: periodic acid Schiff**, SCB**: Saccharomyces cerevisiae boulardii CNCMI-1079**, SF**: starter feed

2.5 Introduction

There is increasing concern over the use of antibiotic growth promoters (AGP) in food-producing animals and the subsequent transmission of antibiotic-resistant bacteria to animals and humans (Sato et al., 2005; Szmolka and Nagy, 2013). Consequently, the European Union instituted a total ban on the use of AGPs in 2006 (Regulation 1831/2003/EC; European Union, 2003) and public pressure may soon lead to similar bans in North America. Already, several food retailers have made commitments to cease using meat from animals raised with AGPs in their products (PRNews, 2015; Wired, 2015). Possible substitutes which could enhance the natural defense mechanisms and reduce the use of AGP in animal production include direct-fed microbials (DFM) (Buntyn et al., 2016). Thus, there is growing research and commercial interest in the use of DFM as alternatives to AGP in farm animal production. Direct-fed microbials are a source of live, naturally occurring microorganisms that beneficially affect the host (Brashears et al., 2005).

Unlike antibiotics which have either bactericidal or bacteriostatic effects on bacteria, DFM induce alterations to the intestinal microbiome, enhance intestinal efficiency and modulate host immune response through indirect mechanisms (Irta, 2015; Lee et al., 2015; Buntyn et al., 2016). Studies on the application of DFM in dairy production have shown improved health status, and productivity in calves and dairy cows (Krehbiel et al., 2003; Gaggia et al., 2010; McAllister et al., 2011). The supplementation of DFM promoted intestinal digestion and improved intestinal microbial balance (Krehbiel et al., 2003), increased growth performance (Frizzo et al., 2010) and reduced mortality in calves (Donovan et al., 2002; Timmerman et al., 2005; Sun et al., 2010).

DFM have been shown to reduce the incidence of diarrhea and the need for antibiotic treatment against digestive and respiratory diseases, and improved overall health of calves (Adams et al., 2008; Agarwal et al., 2002; Galvao et al., 2005; von Buenau et al., 2005). Additionally, fecal shedding of Escherichia coli serotype O157:H7 was reduced in calves receiving DFM (Tabe et al., 2008). However, some studies did not associate beneficial effects with supplemental DFM. For example, Kim et al. (2011) reported no difference in feed intake, live weight or feed efficiency when neonatal calves received either control calf starter or starter supplemented with 0.2% hydrolysed yeast. Quigley et al. (1992) and Seymour et al. (1995) observed a decrease in dry matter intake with supplemental yeast culture while Magalhaes et al. (2008) and Huuskonen and Pesonen (2015) reported no effects of Saccharomyces cerevisiae on feed intake, feed efficiency or live weight gain in dairy calves.

In ruminants, the use of Saccharomyces cerevisiae as a DFM has been associated with promotion of rumen microbial growth (Bach et al., 2007), improved health, reduced frequency of diseases, abated use of antibiotics and reduced pre-weaning mortality rate of calves (Galvao et al., 2005; Magalhaes et al., 2008). Likewise, Lactobacillus acidophilus has been shown to provide protection against enteric infections and beneficial intestinal effects, by establishing desirable gut microflora in calves (Krehbiel et al., 2003). Results from a meta-analysis showed that lactic acid bacteria (such as Lactobacillus acidophilus) have protective effects against opportunistic pathogens at the intestinal level by maintaining a favorable microbial balance as well as reduced incidence of diarrhea in calves

(Signorini et al., 2012). These characteristics make both Saccharomyces cerevisiae and Lactobacillus acidophilus good candidates for inclusion in DFM products for livestock.

Gastrointestinal tract (GIT) development and microbial colonization during the early period of growth is controlled by endogenous factors, but exogenous variables including nutrition play key roles as well (Guilloteau et al., 2009; Malmuthuge et al., 2015). Lactobacilli, for example, constitute the predominant microbial population in the GIT of young ruminants fed milk only (Jiao et al., 2016). Furthermore, the overall lactobacilli population has been shown to promote health by modulating host defenses (Walter, 2008). In effect, suitable DFM should favor growth of the indigenous lactobacilli population inhabiting the GIT of calves.

Most information on the impact of DFM on intestinal morphology, modulation of the gut flora and tissue development has been gained from pigs (Baum et al., 2002; Sehm et al., 2007; Jiang et al., 2015) and poultry (Nava et al., 2005; Forder et al., 2007; Yang et al., 2009). Currently, little is known concerning the possible impact of supplemental DFM on colon histomorphology during the early period of calf's growth.

The objectives of this study were to: 1) determine the viability of supplemental DFM in feed and throughout the digestive tract of calves; 2) evaluate the effect of DFM on total lactobacilli population of calves throughout the digestive tract and in feces; and 3) examine the influence of DFM on colon histomorphology and overall calf performance.

2.6 Materials and methods

2.6.1 Calves, housing and experimental diets

Animal management and use procedures were according to guidelines by the Canadian Council on Animal Care (CCAC, 2009) and approved by the animal care and ethics committee of Agriculture and Agri-Food Canada (AAFC). Forty-eight (45 males and 3 females) Holstein calves, 2–7 days old were sourced from six commercial dairy herds in Quebec (Sherbrooke and surrounding communities) and from the dairy research farm of AAFC Sherbrooke Research and Development Centre. Calves received adequate colostrum (2 × 3 L) within 12 h after birth (Godden 2008). Animals were transported to the animal facility of the Sherbrooke Research and Development Centre, Agriculture and Agri-Food Canada. They were housed in individual pens (1.23 × 2.13 m) bedded with wood shavings and raised following standard management procedures for 14 weeks (experiment day 1–96). Animal environment was controlled and temperatures were between 15 and 25 °C. Calves were allowed an adaptation period of 3 days before the start of the experiment. Calves were randomly allocated to four treatment groups (n = 12/treatment): 1) the controls (CTRL) were bucket fed milk replacer (MR; Goliath XLR 27-16, La Coop, Montreal, QC, Canada) at 6 L/day (2 L three times a day) for the first 4 days, and at 9 L/day (4.5 L twice a day) from day 5 to the end of weaning (day 53). In addition, a starter feed (SF; Shur-Gain − Meunerie Sawyerville Inc., Cookshire-Eaton QC, Canada) about 2.5 kg/day was introduced gradually, starting on day 8 of the experiment; 2) the SCB group received the CTRL diet supplemented with Saccharomyces cerevisiae boulardii CNCMI-1079 (Levucell SB 20, Lallemand Animal Nutrition, Montreal, QC,

Canada) at 7.5 × 10^8 colony forming units (CFU)/L of MR before weaning and at 3 × 10^9 CFU/kg of SF after weaning (day 54–96); 3) the LA group received the CTRL diet supplemented with Lactobacillus acidophilus BT1386 (LA Micro-Cell FS, Lallemand Animal Nutrition) at 2.5 × 10^8 CFU/L of MR before weaning and at 1 × 10^9 CFU/kg of SF after weaning; and 4) the ATB group received the CTRL diet supplemented with chlortetracycline (528 mg/L) and neomycin at (357 mg/L) in MR before weaning and chlortetracycline at 55 mg/kg of SF after weaning (Vetoquinol Inc., Lavaltrie, QC, Canada). Weaning was gradually put in place beginning on day 43. The amount of MR was reduced by half after 3 consecutive days during the weaning period. Weaning was completed by day 53 when calves were eating at least 1 kg of SF for 3 consecutive days. The DFM (SCB and LA) and ATB were added and mixed directly into the MR. After weaning, the DFM (SCB and LA) and ATB were added in a premix and mixed thoroughly with the SF to ensure complete consumption, and fed with hay. Calves had ad libitum access to SF and water. The chemical composition of MR, SF and hay is shown in Table 2.1.

Table 2.1. Chemical composition of milk replacer, starter feed and hay[a].

Component[b]	Milk replacer	Starter feed	Hay
Dry matter, g/kg as fed	89.5	890	829
Organic matter	928	912	–
Crude protein	302	225	163
Lactose	439	–	–
Neutral detergent fiber[c]	–	135	687
Crude fat	179	45	–
Non fibre carbohydrates[d]	–	507	–
Calcium	9.0	11.0	–
Phosphorus	7.0	5.5	–
Metabolizable energy, MJ/kg of dry matter	12.43	10.25	–

[a] Chemical composition of milk replacer (Goliath XLR 27-16, La Coop, Montreal, QC, Canada) and starter feed (Shur-Gain – Meunerie Sawyerville Inc., Cookshire-Eaton, QC, Canada). Hay composition was obtained by infrared analysis by Agri-Analyse (Sherbrooke, QC, Canada).
[b] Expressed as g/kg dry matter, unless stated otherwise.
[c] NDFom excluding ash and including a heat induced amylase solution.
[d] Calculated as 100 − (Crude protein + Neutral detergent fiber + Crude fat + Ash).

2.6.2 Viability of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 in commercial products and feed

Samples of commercial products, MR and SF were collected weekly to assess the viability of the DFM (SCB and LA) prior to and after addition to calves' diet. Fifty g of the commercial product containing SCB or LA were diluted (1:5) in phosphate buffer saline. Similarly, SF (50 g) and MR (20 mL) were diluted 1:10 in peptone water (0.1%). Reconstituted substrates were homogenized 3 min in a high-speed blender followed by serial 10-fold dilutions in peptone water. The resulting homogenates (100 µL) were plated in triplicates on selective media Potato Dextrose Agar (BD Difco, Mississauga, ON, Canada) for the evaluation of SCB viability and Man Rogosa and Sharpe (BD Difco) agar

plates for the evaluation of LA viability. Plates of SCB and LA were incubated at 30 and 37 °C, respectively, in an anaerobic chamber for 48 h. The colony forming units (CFU) per gram of product were determined considering the dilution factor.

2.6.3 Sampling of feces and gastro intestinal tract content

Fresh feces (5 g) from all calves were collected on days 1, 8, 16, 23, 51, 54, 57 and 85 into 50-mL falcon tubes, mixed thoroughly with peptone water (45 mL) and placed on ice. The fecal samples were transported immediately to the laboratory, transferred into 20 mL scintillation vials and stored at −20 °C pending analysis.

Eight calves per treatment (4 on day 33) (pre-weaning) and 4 others on day 96 (post-weaning) were euthanized following standard procedures at a commercial abattoir. Ruminal content (60 mL) was collected, mixed by stirring in 2 L of saline and aliquoted in 120-mL collection cups (Medicus Health, Kentwood, MI, USA). Similarly, ileum and colon contents of approximately 20–25 mL were collected into 50-mL falcon tubes, kept on ice and transferred to the laboratory. Segments (10 cm) of the ileum were cut open longitudinally after removing its contents. The segments were further divided into two parts of 5 cm and rinsed three times with 25 mL saline in 50 mL falcon tubes. Ileal mucosal washings were obtained as previously described (Li et al., 2003). The resulting mucus washes, along with ruminal, ileum and colon contents (5 g or 5 mL) were diluted in 45 mL peptone water from which serial dilutions were made and plated as described above, for determination of DFM viability, or frozen at −80 °C for future real time quantitative PCR (qRT-PCR) for determination of total lactobacilli population.

2.6.4 Isolation of DNA and real-time qPCR quantification of total Lactobacilli in the gastro-intestinal tract and feces

Extractions of DNA from GIT samples (mucosal washing of the ileum and digesta from the rumen, ileum and colon), feces and LA commercial product were accomplished using the bead beating method with the ZR Fecal DNA MiniPrep kit (Zymo Research, Ivine, CA, USA) following manufacturer's instructions. The concentration and quality of DNA was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at −20 °C pending real time qPCR analysis. These procedures were only performed on calves receiving CTRL, LA and SCB treatments.

To quantify total lactobacilli, the following primers were used: F-lac 5'GCAGCAGTAGGGAATCTTCCA3' and R-lac 5'GCATTYCACCGCTACACATG3' (Castillo et al., 2006). The qPCR was performed on a Step One Plus Real-Time PCR System (Applied Biosystems). The qPCR mix was in a final volume of 10 µL and contained 5 µL Fast SYBR Green Master Mix (Applied Biosystems), 1 µL of each primer (300 nM), 1 ng DNA and RNase free water. A serial dilution was made of the DNA extracted from the commercial LA product (Micro-Cell FS) and used to generate a standard curve for total bacterial copy number evaluation. The thermal profile included an initial denaturation at 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s and finally,

a melt curve generated at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Quantification by real time qPCR for all samples was performed in triplicates.

2.6.5 Blood sampling and analysis

Blood samples were collected weekly; approximately 3 h after the morning meal, from the jugular vein of calves into EDTA coated vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After collection, samples were placed immediately on ice, transported to the laboratory and centrifuged at 1800 × g for 12 min at 4 °C. Aliquots of the resulting plasma were stored at −20 °C pending analysis of glucose and β-hydroxybutyrate (BHB).

The plasma concentrations of glucose and BHB were determined using glucose trinder assay kit (Sekisui Diagnostics P.E.I. Inc., Charlottetown, PE, Canada) and BHB reagent kit (Bio-Pacific Diagnostic Inc., Vancouver, BC, Canada), respectively, following manufacturers' protocols. The spectrophotometer (SpectramaxM2) was used for data acquisition and data were analyzed with SoftMax Pro software version 6.2, all from Molecular Devices Inc. (Molecular Devices Inc., Sunnyvale, CA, USA).

2.6.6 Colon histomorphology

Tissue samples from the mid part of the ascending colon (at 60% of the ascending length) were collected upon slaughtering on day 33 (pre-weaning) and day 96 (post-weaning) for histomorphology studies. Histomorphology of transverse colon sections were carried out as described previously (Baurhoo et al., 2011). Sections were then stained with haematoxylin and eosin for the measurements of crypt depth and width. Meanwhile, Alcian blue (AB), and periodic acid Schiff (PAS) stains were done for the quantification of goblet cells containing neutral and acidic mucins, respectively.

Digital photographs of the stained tissues were obtained using a slide scanner (NanoZoomer Digital Pathology, Hamamatsu Photonics, Boston, MA, USA). Morphometric measurements of crypt depth and basal width were performed using NDP.view 2.5 software (Hamamatsu Photonics). Crypt of Lieberkühn depth was taken as the distance from the crypt mouth to the base (lamina muscularis mucosae). The basal width was taken as the perpendicular distance from about 20% of the crypt length from the base. Crypt measurements were taken from 10 crypts in 8 different fields of each colon section. The crypt morphology was clearly distinguishable at 20 × magnification. Samples were measured in duplicates and the following specific criteria were implemented to ensure consistent measurements: use of complete crypts, and well oriented crypts which were representative of the rest of the tissue.

2.6.7 Quantification of goblet cells

Individual goblet cells can potentially produce different types of mucin concurrently; therefore quantification of specific goblet cells required two separate staining, AB for acid mucin and PAS for neutral mucins. Digital photographs of stained sections were similarly obtained using the digital slide scanner (NanoZoomer Digital Pathology, Hamamatsu Photonics) and the total numbers of AB positive cells were determined using image analysis with the NIH ImageJ

software (Schneider et al., 2012). The numbers of PAS-positive and AB-positive goblet cells were determined as the total occurrences of these respective cells per crypt outline.

2.6.8 Health status, medication and vaccination

Calves were monitored daily for health disorders. Rectal temperatures were taken from calves displaying clinical signs of disease such as diarrhea, coughing, increased respiratory frequency, depression and lack of appetite. An electrolyte solution (Calflyte II, Vetoquinol Inc., Lavaltrie, QC, Canada) was given in case of diarrhea outbreak until restoration of normal consistency of stool. Lactated Ringer Solution (Baxter Cooperation, Mississauga, ON, Canada) was administered if diarrhea persisted. Upon arrival at the research facility, animals received an intranasal vaccine (Inforce 3, Pfizer, Kirkland, QC, Canada) to protect against respiratory diseases (bovine rhinotracheitis, parainfluenza and respiratory syncytial viruses). The calves were vaccinated later on day 29 (Somnu-Star, Novartis, Mississauga, ON, Canada) to protect against pneumonia caused by Mannheimia (Pasteurella) haemolytica and Histophilus somni (haemophilus somnus).

2.6.9 Feed intake and body weight measurements

Intake of MR and SF (feed intake) was recorded daily. Body weight was recorded upon arrival for each calf, and then twice weekly for the first two weeks followed by once weekly until weaning. Calves were weighed twice weekly during the weaning process (day 43–53), and once weekly until the end of the experiment. Body weights were taken at the same time during the day, approximately 2 h after the morning meal. Feed efficiency for milk replacer and starter diet was generated from the ratio of daily feed intake (dry matter intake) and average daily gain.

2.6.10 Attitude and fecal scoring

Attitude and fecal consistency were recorded daily during morning feeding using a 1–4 scale (Magalhaes et al., 2008). Attitude scoring categories were as follows: 1) alert and responsive; 2) non-active; 3) depressed; and 4) moribund. Fecal consistency was scored as follows: 1) firm; 2) soft or of moderate consistency; 3) runny or mild diarrhea; and 4) watery and profuse diarrhea (Magalhaes et al., 2008). The presence of blood in the stool was also monitored. Average weekly attitude and fecal scores were generated from the daily records for individual calves.

2.6.11 Fecal sampling and detection of enterotoxigenic Escherichia coli O157:H7

Fecal samples were collected directly from the anus on days 1, 8, 51, and 85 into collection tubes and transferred to the laboratory. Samples (10 g) were weighed and mixed into 90 mL of trypticase soy broth (Becton Dickinson), ATB (0.5 mg of cefiximine; Lederle Laboratories, Pearl River, NY, USA), 10 mg of cefsuludin (Sigma aldrich, Winston Park, Oakville, ON, Canada), and 8 mg of vancomycin (Sigma Aldrich). The resulting mixture was thereafter homogenized in a high-speed blender for 5 min with a pulse after every minute. The homogenized mixture was incubated at 37 °C for 24 h in a rotating incubator followed by plating on cefiximine tellurite sorbitol MacConkey

agar (Thermo Scientific, Nepean, ON, Canada). Sorbitol negative bacteria such as Escherichia coli O157:H7 produce colorless colonies, whereas sorbitol positive bacteria produce pink colonies. A latex agglutination test was performed for the positive Escherichia coli O157:H7 colonies according to manufacturer's instructions.

2.6.12 Statistical analysis

Statistical analyses were performed using the MIXED procedure of SAS version 9.3software
(SAS Institute Inc., Cary, NC, USA). A completely randomized design with repeated measures was used for the analyses of the following variables: quantification of total lactobacilli, feed intake, weight gain, feed efficiency, attitude and fecal scores, plasma glucose and BHB concentrations, crypts depth and width, and neutral and acidic mucins. Total lactobacilli counts were submitted to a logarithmic transformation prior to analysis. The model included the fixed effects of treatment and day and their interaction, and random effect associated to calf. Multiple comparisons among means were carried out using a Tukey's adjustment. The effects were considered significant at $P < 0.05$. The statistical model used was as follows:

$$Yijk=\mu+\alpha i+dij+\tau k+(\alpha\tau)ik+eijk$$

Where:

Yijk = observation for animal j receiving treatment i at day k

μ = general mean

αi = fixed effect of treatment i

dij = random effect associated with animal j in treatment i

τk = fixed effect of day k

ατik = interaction between treatment i and day k

eijk = random error.

The effect of GIT sites was added to the model for the analysis of microbial populations.

2.7 Results

2.7.1 Viability of Saccharomyces cerevisiae boulardii CNCMI-1079 and Lactobacillus acidophilus BT1386 in commercial products, feeds, gastrointestinal tract and feces

Both SCB and LA remained viable throughout the study. In commercial products, mean counts were 1.8×10^{10} and 5.6×10^{9} CFU/g for SCB and LA, respectively. In MR, mean counts were 8.2×10^{8} and 7.9×10^{7} CFU/L for SCB and LA, respectively. In SF, mean counts were 4.5×10^{9} and 4.7×10^{7} CFU/g for SCB and LA, respectively.

Viable SCB (log10/mL or log10/g) were recovered from the rumen (4.89 ± 0.29 and 4.99 ± 0.17), ileum (4.69 ± 0.30 and 5.29 ± 0.17) and colon (5.33 ± 0.63 and 5.69 ± 0.32) sampled on day 33 (pre-weaning) and day 96 (post-weaning), respectively. Viable SCB were also recovered from feces of calves with a mean weekly viable count (log10/g) of 5.70 ± 0.29.

In the present study, it was not possible to specifically evaluate the viability of LA in the GIT and feces because it constitutes one of several lactobacillus species and strains indigenous to the GIT tract of calves. There was no growth medium that could specifically allow only the growth of LA on agar plates for its viability determination. Therefore, the viability of total lactobacilli was evaluated and reported.

2.7.2 Effect of treatment and weaning on total lactobacilli population in the gastrointestinal tract and in feces

There was no effect of treatment on the populations of total lactobacilli in the different segments of the GIT neither pre-weaning nor post-weaning (Table 2.2). Total lactobacilli were comprised of the indigenous lactobacilli naturally inhabiting the GIT of calves plus the LA supplied as a DFM. Total lactobacilli population in the ileum content ($P = 0.001$), ileum mucosa ($P = 0.023$) and colon ($P = 0.001$) was greater pre- as compared to post-weaning (Table 2.3). Pre-weaning, the greatest ($P < 0.001$) population was observed in the colon followed by the ileum content, the rumen and finally the ileum mucosa with very few lactobacilli (Table 2.3). Post-weaning, the population had drastically decreased, and the highest population was found in the colon and the least in the ileum mucosa ($P < 0.001$). An effect of treatment was detected on the population of lactobacilli quantified from calves' feces (Table 2.4). The addition of SCB stimulated total lactobacilli compared to CTRL and LA. These results were only significant when considering the period from the beginning of the study to the end of weaning.

Table 2.2. Effect of Lactobacillus acidophilus BT1386 (LA) and Saccharomyces cerevisiae boulardii CNCMI–1079 (SCB) on total lactobacilli (log10 CFU/g) in the gastrointestinal tract (GIT) of dairy calves receiving milk replacer (day 33, pre-weaning) or starter feed (day 96, post-weaning).

	Treatment [a]				
GIT site	CTRL	LA	SCB	SEM	P-value
Pre-weaning					
Rumen	2.43	2.13	2.24	0.20	0.57
Ileum content	3.87	3.42	3.48	0.33	0.61
Ileum mucosa	1.25	0.90	0.65	0.34	0.48
Colon	4.41	4.31	4.17	0.30	0.85
Post-weaning					
Rumen	1.98	2.18	1.80	0.27	0.62
Ileum content	2.14	2.03	1.66	0.26	0.43
Ileum mucosa	0.34	0.40	0.51	0.12	0.58
Colon	2.54	2.32	1.94	0.19	0.12

*These procedures were only performed on calves receiving CTRL, LA and SCB treatments.

[a]CTRL = Control fed milk replacer or starter feed; LA = CTRL + Lactobacillus acidophilus BT1386 at 2.5×10^8 CFU/L milk replacer before weaning and at 1×10^9 CFU/kg starter feed after weaning; SCB = CTRL + Saccharomyces cerevisiae boulardii CNCMI-1079 at 7.5×10^8 CFU/L milk replacer before weaning and at 3×10^9 CFU/kg starter feed after weaning.

Table 2.3. Mean differences between sites of the gastrointestinal tract (GIT) of total lactobacilli population (log10 CFU/g) in dairy calves supplemented with Lactobacillus acidophilus BT1386 and Saccharomyces cerevisiae boulardii CNCMI–1079 during pre-weaning (day 33) or post-weaning (day 96).

GIT site			P-value	
	Pre-weaning	Post-weaning	Time	Treatment × time
Rumen	2.27[c] ± 0.11	1.98[a] ± 0.16	0.161	0.50
Ileum content	3.59[b] ± 0.19	1.94[a] ± 0.15	0.001	0.76
Ileum mucosa	0.93[d] ± 0.20	0.42[b] ± 0.07	0.023	0.33
Colon	4.30[a],b ± 0.17	2.27[a] ± 0.11	0.001	0.77
P-value (GIT site)	P < 0.001	P < 0.001		

[a]Means with different superscripts are significantly different (P < 0.05).

[b]Means with different superscripts are significantly different (P < 0.05).

[c]Means with different superscripts are significantly different (P < 0.05).

Table 2.4. Effect of direct-fed microbials Lactobacillus acidophilus BT1386 and Saccharomyces cerevisiae boulardii CNCMI–1079 on total lactobacilli in feces (expressed as area under the curve) at different times relative to weaning[1].

	Treatment[2]				
	CTRL	LA	SCB	SEM	P-value
Total lactobacilli[3], cfu/g × d					
Days 1–24 (pre-weaning)	67.10	69.70	75.98	3.34	0.18
Days 1–54 (end of weaning)	114.57[b]	122.58[b]	141.21[a]	5.24	0.01
Days 1–96 (whole study)	131.99	144.17	164.71	11.57	0.15

[a]Means within a row with different superscripts differ (P = 0.01).

[b]Means within a row with different superscripts differ (P = 0.01).

[1]These procedures were only performed on calves receiving CTRL, LA and SCB treatments.

[2]CTRL = Control fed milk replacer or starter feed; LA = CTRL + Lactobacillus acidophilus BT1386 at 2.5 × 108 CFU/L milk replacer before weaning and at 1 × 109 CFU/kg starter feed after weaning; SCB = CTRL + Saccharomyces cerevisiae boulardii CNCMI-1079 at 7.5 × 108 CFU/L milk replacer before weaning and at 3 × 109 CFU/kg starter feed after weaning.

[3]Total lactobacilli are expressed as area under the curve; i.e., area generated by counts of lactobacilli/g feces x sampling days.

2.7.3 Plasma glucose and β-hydroxybutyrate

There was no effect of treatment on plasma glucose and BHB concentrations throughout the experimental period, as well as no interaction with time. However, the concentrations of plasma glucose decreased at weaning for all treatments compared to pre-weaning concentrations (P < 0.001) and these lower concentrations remained relatively stable post-weaning (Table 2.5). Meanwhile, the concentrations of BHB increased with age of calves (Table 2.5).

Table 2.5. Mean plasma glucose and β-hydroxybutyrate during pre-weaning, weaning and post-weaning periods in dairy calves.

Metabolite and time	Concentration, mmol/L	Effect of time, P-value
Glucose		
Pre-weaning	$6.56^{a} \pm 0.12$	0.001
Weaning	$5.54^{b} \pm 0.10$	
Post-weaning	$5.48^{b} \pm 0.10$	
β-Hydroxybutyrate		
Pre-weaning	$0.13^{c} \pm 0.01$	0.001
Weaning	$0.29^{b} \pm 0.02$	
Post-weaning	$0.59^{a} \pm 0.02$	

[a]Means within a column with different superscripts differ (P = 0.001) for each parameter.

[b]Means within a column with different superscripts differ (P = 0.001) for each parameter.

[c]Means within a column with different superscripts differ (P = 0.001) for each parameter.

2.7.4. Colon histomorphology

Colon crypt depths and widths were smaller (P < 0.01) in the SCB and ATB-treated calves as compared to the LA-treated or CTRL calves, both on day 33 (pre-weaning) and day 96 (post- weaning) (Table 2.6). There was no difference between pre- and post-weaning.

Table 2.6. Effect of direct-fed microbials and antibiotics on colon histomorphology of Holstein calves.

Measurement	*Treatment				P-value
	CTRL	LA	SCB	ATB	
Pre-weaning (day 33)					
Crypt depth (μm)	$474.64^{a}\pm8.24$	$436.03^{a}\pm31.20$	$361.37^{b}\pm6.62$	$341.36^{b}\pm10.00$	0.02
Crypt width (μm)	$92.63^{a}\pm1.94$	$85.91^{a}\pm2.85$	$73.86^{b}\pm1.36$	$71.12^{b}\pm1.17$	0.01
Number of goblet cells containing neutral mucins	$7.56^{b}\pm1.18$	$8.30^{b}\pm1.32$	$14.10^{a}\pm3.48$	$11.85^{a}\pm2.07$	0.06
Number of goblet cells containing acidic mucins	$68.00^{b}\pm7.05$	$78.00^{b}\pm.98$	$90.75^{a}\pm4.02$	$93.75^{a}\pm2.17$	0.04
Post −weaning (day 96)					
Crypt depth (μm)	$490.02^{a}\pm3.29$	$456.53^{a}\pm15.90$	$366.21^{b}\pm5.80$	$366.83^{b}\pm18.80$	0.01
Crypt width (μm)	$94.02^{a}\pm4.04$	$93.60^{a}\pm6.69$	$78.10^{b}\pm2.08$	$76.62^{b}\pm3.37$	0.03
Number of goblet cells containing neutral mucins	$6.23^{b}\pm0.84$	$6.90^{b}\pm0.29$	$12.40^{a}\pm1.57$	$8.32^{ab}\pm1.73$	0.06
Number of goblet cells containing acidic mucins	$72.00^{b}\pm4.56$	$80.00^{b}\pm5.46$	$97.00^{a}\pm8.66$	$100.50^{a}\pm2.90$	0.02

*CTRL = Control fed milk replacer or starter feed; LA = CTRL + Lactobacillus acidophilus BT1386 at 2.5 × 108 CFU/L milk replacer before weaning and at 1 × 109 CFU/kg starter feed after weaning; SCB = CTRL + Saccharomyces cerevisiae boulardii CNCMI-1079 at 7.5 × 108 CFU/L milk replacer before weaning and at 3 × 109 CFU/kg starter feed

after weaning; ATB = CTRL + antibiotics; chlortetracycline and neomycin at 528 and 357 mg/L milk replacer, respectively, before weaning and chlortetracycline at 55 mg/kg starter feed after weaning.
[a]For each period and measurement, row means with different superscripts differ significantly (P < 0.05) (n = 4).
[b]For each period and measurement, row means with different superscripts differ significantly (P < 0.05) (n = 4).

2.7.5 Quantification of neutral and acidic mucins

Goblet cells containing neutral mucins had a tendency to be greater (P = 0.06) in the SCB and ATB-treated calves compared to LA-treated and CTRL calves, both on day 33 (pre-weaning) and day 96 (post-weaning). Likewise, goblet cells containing acidic mucins were greater (P < 0.05) in the SCB- and ATB-treated calves on day 33 (pre-weaning; Table 2.6). After weaning, SCB- and ATB-treated calves continued to have a greater (P < 0.05) number of goblet cells containing acidic mucins compared to the other treatments (Table 2.6).

2.7.6 Calf health, feed intake, weight gain and feed efficiency

Calves were generally healthy, and there were no treatment differences observed for the various health conditions monitored. Rectal temperatures throughout the first 21 days of age did not differ (38.9 ± 1 °C) between groups.

There was no significant treatment effect on MR intake over the entire feeding trial. Starter feed intake increased linearly with age of calves for all treatments (Table 2.7). The ATB fed calves had a tendency to increase (P = 0.10) for the average daily gain on days 1–32 compared to CTRL (Table 2.7). There were no significant differences between treatments in SF intake or feed efficiency.

Attitude and fecal scores were unaffected by treatments (Table 2.7). The average attitude for all the animals was 1 (alert) throughout the entire project. The average fecal score of 2.03 on the first week decreased (P < 0.001) during week 2 of the experiment for all treatments. Fecal scores stabilised on the fifth week (1.03) until the end of the experiment. There were no differences in the occurrence of diarrhea between treatment groups.

Table 2.7. Effect of direct-fed microbials and antibiotics on body weight, feed intake, feed efficiency and fecal scores of Holstein dairy calves.

Parameter	[a]Treatment				SEM	P-value
	CTRL	ATB	SCB	LA		
Body weight, kg						
Pre-weaning (day 1)	48.08	46.77	47.41	46.39	2.49	0.96
Weaning (day 50)	87.02	88.97	85.55	84.53	3.78	0.84
Post weaning (day 88)	141.59	143.50	136.65	134.09	7.40	0.75
[b]Average daily gain, kg						
Pre-weaning (day 1–32)	0.70	0.82	0.72	0.70	0.08	0.10
Post – weaning (day 53–88)	1.43	1.45	1.36	1.34	0.15	084
[b]Average daily feed intake						
Milk replacer (day 1–32), L/day	6.88	6.98	6.94	6.83	0.39	0.98
Starter feed (day 53–88), kg/day	4.00	3.69	3.60	3.58	0.40	0.74
[c]Feed efficiency (kg dry matter/kg daily gain)						
Milk replacer, pre-weaning (day 1–32)	1.02	0.95	1.10	1.02	0.04	0.46
Starter feed, post-weaning (day 53–88)	2.66	2.56	2.58	2.65	0.09	0.89
[d]Fecal scores						
Pre-weaning (day 1–7)	1.99	1.99	1.97	2.19	0.18	0.78
Pre-weaning (day 8–14	1.77	2.02	1.83	2.16	0.16	0.29
Pre-weaning (day 15–21)	1.55	1.30	1.65	1.76	0.17	0.29
Pre-weaning (day 22–28)	1.20	1.14	1.19	1.37	0.13	0.53
Pre- weaning (day 29–35)	1.01	1.03	1.02	1.08	0.04	0.53
Pre- weaning (day 36–42)	1.02	1.01	1.01	1.03	0.12	0.72

[a]CTRL = Control fed milk replacer and starter feed; LA = control diet supplemented with Lactobacillus acidophilus BT1386 at 2.5 × 108 CFU/L milk replacer before weaning and at 1 × 109 CFU/kg starter feed after weaning; SCB = control diet supplemented with Saccharomyces cerevisiae boulardii CNCMI-1079 at 7.5 × 108 CFU/L milk replacer before weaning and at 3 × 109 CFU/kg starter feed after weaning. ATB = control diet supplemented with antibiotics; chlortetracycline and neomycin at 528 and 357 mg/L milk replacer, respectively, before weaning and chlortetracycline at 55 mg/kg starter feed after weaning.

[b]Values collected during the weaning period were too erratic to analyse since milk replacer intake was gradually reduced while started feed was gradually increased, which led to reduced feed intake in the early days of weaning.

[c]Feed efficiency was calculated for milk replacer and starter diet separately.

[d]Fecal scores categories were 1) firm; 2) soft or moderate consistency; 3) runny or mild diarrhea; 4) watery and profuse diarrhea.

2.7.7 Presence of Escherichia coli O157:H7 in feces

Escherichia coli O157:H7 was not present in the feces of calves throughout the experimental period. Observed colonies were between pink and white with very few samples having colourless colonies. The few colourless samples (positive) evaluated for latex agglutination showed no agglutination (negative) throughout the experimental period.

2.8 Discussion

The DFM SCB remained viable in the different sites of the GIT as well as in feces in the order of 10^5 CFU/g. Durand-Chaucheyras et al. (1998) also observed viable yeast counts (10^5 CFU/g) in feces of lambs 48 h following ingestion of the DFM yeast. There were more lactobacilli in feces of the SCB-treated calves implying that this yeast promoted the growth of this bacteria genus in the GIT. The main effect of SCB on total lactobacilli population occurred between day 24 and 54 (end of weaning). Our data is supported by Yoon and Stern (1996) who also observed that Saccharomyces cerevisiae stimulated the growth of lactate-utilizing bacteria in the rumen. Similarly, Saccharomyces cerevisiae bayanus EC-1118 enhanced the viability of Lactobacillus rhamnosus at 30 °C in fermented milk (Suharja et al., 2014). An explanation for this observation would be that Saccharomyces cerevisiae stimulated the growth of lactate producers, such as lactobacilli, which in turn provided the substrate for lactate-utilizing bacteria. An increased lactobacilli population is important for gastrointestinal health of host such as prevention of the growth of infective organisms (O'Toole and Cooney, 2008; Conlon and Bird, 2015). In the digestive tract, SCB can adhere to the intestinal cell and prevent translocation of pathogens (Martins et al., 2007). Direct-fed SCB, by promoting the growth of lactobacilli population in this study, actively increased beneficial microflora population that may function to prevent the adherence of potential pathogens (Nava et al., 2005).

Direct-fed SCB increased total lactobacilli population in feces around weaning but such effect was not observed along the digestive tract. This could be due to the fact that frequent (weekly) fecal samples were taken on the same calf as compared to GIT samples taken once per animal on day 33, and then on another set of animals on day 96. Total lactobacilli population in the GIT of calves post-weaning was reduced as compared to pre-weaning, as a consequence of diet change from MR to SF and hay after weaning. This observation is supported by a recent study by Jiao et al. (2016) on the ileal bacterial community composition in the early life of goats.

Direct-fed SCB altered colon morphology by reducing crypt depth and width during the early period of growth. Similarly, reduced colon crypt depth and width in calves fed Enterococcus faecium and lactulose has been reported (Fleige et al., 2007). Reduced colon crypt depth/width could reduce bacterial population in direct contact with the epithelial surface hence impeding translocation to the internal environment (Mello et al., 2012). There was increased mucin production in the SCB and ATB-treated calves compared with CTRL and LA. This effect is indicative of higher mucus production, and therefore of improved antibacterial properties (Deplancke and Gaskins, 2001; Machado-Neto et al., 2013). Direct-fed SCB altered the colon morphology and increased mucin production which is probably an

indication of early maturation in the group receiving this yeast (Sinha and Chakravorty, 1982; Nonose et al., 2009). Potential antimicrobial effects of SCB could therefore reduce the impacts of pathogens in the colonic flora.

Although SCB altered colon morphology (crypt depth and width, and mucin production), and increased the population of total lactobacilli in feces, it did not reflect on overall growth performance and feed intake of calves in this study. This was probably due to the small sample size, overall clean pen environment, and short duration of the experiment. In a randomized field trial with 554 Holstein calves, there was a significant increase in weight gain and low mortality rate under conditions of severe diarrhea during the pre-weaning period in calves treated with *Faecalibacterium prausnitzii* compared to control calves (Foditsch et al., 2015).

The effects of feeding Saccharomyces cerevisiae products on performance of calves have not been consistent. While some studies have revealed that inclusion of yeast products does not influence feed intake and body weight gain in the first days of life (Magalhaes et al., 2008; Kim et al., 2011), Galvao et al. (2005) reported increased grain intake, body weight gain, and blood glucose of calves after feeding live yeast incorporated into grain during the pre-weaning period, but not after weaning. The form of the yeast appears to play an important role in its effects on growth performance. Supplementation of live yeast improved feed efficiency of piglets on day 0 to 21 as compared to those fed heat-killed whole yeast (Jiang et al., 2015). There are several other factors such as strain, duration or frequency of administration, inclusion of mixed strains/species and dosage of administration that may influence the potential effects of DFM (Slattery et al., 2016; Buntyn et al., 2016). In addition, the format and characteristics of feed in which DFM cells are provided are critical to the determination of their effectiveness (Lee et al., 2015). The question remains therefore whether the lack of effect on growth performance of LA and SCB in the current study was due to insufficient supply (7.5×10^8 CFU/L of MR before weaning and 3×10^9 CFU/kg of SF after weaning for SCB, and 2.5×10^8 CFU/L of MR before weaning and 1×10^9 CFU/kg of SF after weaning for LA) or whether higher doses could have conferred more significant changes in the GIT and beneficial effects on growth performance.

Previous studies have indicated that both Lactobacillus acidophilus (Jenny et al., 1991; Cruywagen et al., 1996) and Saccharomyces cerevisiae (Galvao et al., 2005) can improve growth performance in calves. However, the optimum doses of LA and SCB required for increased growth performance effects have not been determined in dairy calves. A higher dose (3×10^9 CFU/kg dry matter) of Saccharomyces cerevisiae as well as mixed cultures were more effective in buffalo calves for improvement of growth parameters than lower supply (1×10^9 CFU/kg dry matter) and single strains (Malik and Bandla, 2010). In another study, there was no effect on performance using 1% yeast (dry matter basis), but 2% (dry matter basis) yeast (Saccharomyces cerevisiae) culture added to a texturized calf starter improved dry matter intake, body weight gain and feed efficiency compared to controls (Lesmeister et al., 2004).

Animals seem to respond better to DFM supplementation under stressful and unhygienic conditions (Frizzo et al., 2010; Krause et al., 2010) as compared to healthy, as well as non-stressed and highly sanitised conditions as in the present study. Greater body weight and increased SF intake was reported after feeding lactic acid bacterial inoculum of bovine origin composed of Lactobacillus casei DSPV 318T, *Lactobacillus salivarius* DSPV 315T and

Pediococcus acidilactici DSPV 006T to calves managed under stressful conditions of inadequate housing and unhygienic environment (Frizzo et al., 2010; Krause et al., 2010). Direct-fed microbials supplementation may have greater impact during disease or environmental challenges (Lessard et al., 2009; Manafi et al., 2016). Animals in this study were not challenged with a disease pathogen.

2.9 Conclusion

Feeding SCB stimulated total lactobacilli population around weaning and altered colon morphology with potential beneficial effects during the early period of growth. However, SCB had no effect on growth performance. Our data suggest that SCB would deserve more attention as a modulator of the gastrointestinal health in young dairy calves. An extensive study with more animals and longer duration could confirm the role of SCB on animal performance.

2.10 Competing interest

The authors have no competing interest to declare.

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Chapitre 3 *Saccharomyces cerevisiae* boulardii CNCM 1-1079 and Lactobacillus acidophilus BT1386 influences innate immune response and serum levels of acute-phase proteins during weaning in Holstein calves

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3.1 Résumé

Les buts de cette étude étaient d'évaluer les effets de Saccharomyces cerevisiae var. boulardii CNCM I-1079 (SCB) ou Lactobacillus acidophilus BT1386 (LA) sur (1) la réponse immunitaire innée, (2) les marqueurs de la réaction en phase aiguë et (3) l'expression de gènes immunitaires des tissus du rumen et de l'iléon des veaux holsteins. Quarante-huit veaux (~5 j d'âge) ont été alloués aléatoirement à quatre traitements comme suit : (1) témoin (CTRL — « control ») ayant reçu un remplacement de lait suivi d'une moulée de départ, (2) CTRL avec suppléments de SCB dans le lait et la moulée, (3) CTRL avec suppléments de LA dans le lait et la moulée et (4) CTRL avec suppléments d'antibiotiques (ATB; chlortétracycline et néomycine dans le lait et chlortétracycline dans la moulée). Le facteur de nécrose tumorale α (TNF-α — « tumour necrosis factor α ») a diminué (P < 0,05) au jour 66 (après sevrage) chez les veaux traités aux ATB. Il n'y avait pas d'effet de traitement sur la production des protéines interféron gamma (IFN-γ — « interferon γ ») et interleukine 6 (IL-6 — « interleukin 6 ») ni sur l'expression des gènes TLR4, TLR6, TLR9, TLR10, CLDN3, MUC1 et MUC 20. Les veaux ayant reçu les suppléments de SCB ou de LA avaient une plus grande (P < 0,05) stimulation oxydative au sevrage (jour 53) par rapport aux veaux CTRL. La stimulation oxyda- tive était aussi plus grande (P < 0,05) après le sevrage (jour 59 et jour 87) chez les veaux ayant reçu les suppléments de SCB. Les veaux ayant reçu des suppléments de SCB et de ATB avaient une plus grande (P < 0,05) activité dephagocytose pendant le sevrage (jour 47) par rapport aux veaux CTRL. La concentration de A2 amyloïde sérique (SAA2 — « serum amyloid A2 ») a augmenté (P < 0,05) chez les veaux ayant reçu des suppléments de SCB et LA (jour 53), tandis que la concentration de protéines C-réactives (CRP — « C-reactive proteins ») a augmenté (P < 0,05) chez les veaux ayant reçu les suppléments de SCB pendant le sevrage par rapport aux veaux CTRL. Nos résultats suggèrent que les suppléments de SCB pourraient améliorer la réponse immunitaire innée (stimulation oxydative et phagocytose) ainsi que les marqueurs de la réaction en phase aiguë (CRP et SAA2), surtout pendant des périodes critiques comme le sevrage.

3.2 Abstract

The aims of this study were to investigate the effect of *Saccharomyces cerevisiae boulardii* CNCM I-1079 (SCB) or *Lactobacillus acidophilus* BT1386 (LA) on 1) innate immune response, 2) markers of acute phase reaction and 3) immune gene expression of rumen and ileum tissues of Holstein calves. Forty-eight calves (~5 d old) were randomly allocated to four treatments as follows: (1) control (CTRL) fed milk replacer followed by starter feed, (2) CTRL supplemented with SCB in milk and feed, (3) CTRL supplemented with LA in milk and feed; and (4) CTRL supplemented with antibiotics (ATB; chlortetracycline and neomycin in milk, and chlortetracycline in feed). Tumour necrosis factor α decreased (*P*<0.05) on d 66 (post-weaning) for the ATB-treated calves. There were no treatment effects on production of IFNγ and IL-6 proteins and on expression of *TLR4, TLR6, TLR9, TLR10, CLDN3, MUC1* and *MUC 20* genes. Calves fed SCB or LA had a greater (*P*<0.05) oxidative burst at weaning (d 53) compared with CTRL. Oxidative burst was also greater (*P*<0.05) after weaning (d 59 and d 87) for SCB-fed calves. Calves fed SCB and ATB had higher (*P*<0.05)

phagocytosis activity during weaning (d 47) compared to CTRL. The concentration of SAA2 increased (P<0.05) in SCB- and LA-fed calves (d 53), while the concentration of CRP increased (P<0.05) in SCB-fed calves during weaning as compared to CTRL. Our results suggest that SCB could improve innate immune response (oxidative burst and phagocytosis) and markers of acute phase reaction (CRP and SAA2), especially during critical periods like weaning.

3.3 Key words

Acute phase proteins, calves, direct fed microbials, innate immune response, *Lactobacillus acidophilus* BT1386*, Saccharomyces cerevisiae boulardii* CNCM I-1079

3.4 Abbreviations

APP, acute phase proteins; **ATB**, antibiotics; **CFU**, colony forming units; **CRP**, C-reactive proteins; **CTRL**, control; **DFM**, direct fed microbials; **HBSS**, Hank's balanced salt solution; **HP**, haptoglobin; **IFN-γ**, interferon γ; **IL-6**, interleukin-6; **LA**, *Lactobacillus acidophilus* BT1386; **LPS**, lipopolysaccharide; **MR**, milk replacer ; **PBMC**, peripheral blood mononuclear cell; **PMA**, Phorbol 12-myristate 13-acetate; **PMN**, polymorphonuclear neutrophils; **SAA2**, serum amyloid A2; **SCB**, *Saccharomyces cerevisiae boulardii* CNCM I-1079; **SF**, starter feed; **TLR**, toll-like receptors; **TNF-α**, tumour necrosis factor α

3.5 Introduction

In attempt to limit the use of antibiotic growth promoters in animal farming, attention has been directed to the development of alternatives that can enhance animal health, boost productivity as well as assure food safety (Seal et al. 2013). Effective alternatives should be able to maintain current animal production levels without threatening public health (Millet and Maertens 2011). Feed additives for improving the natural host defense mechanisms and reducing the use of antibiotic growth promoters in animal production include direct fed microbials (DFM) such as *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Bacillus subtilis* (Buntyn et al. 2016; McAllister et al. 2011). Direct fed microbials are live microorganisms which, when administered in adequate amounts, confer health benefits to the host (FAO/WHO 2001).

Young calves are predisposed to enteric diseases which cause morbidity and mortality during the early period of growth (Lorenz et al. 2011). Therefore the prevention of these diseases using DFM like *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* is important to promote the health of calves (Uyeno et al. 2015). *Saccharomyces cerevisiae* has been reported to modulate immune gene expression as well as inhibit ETEC-mediated ERK1/2 and p38 signaling pathways in intestinal epithelial cells (Zanello et al. 2011). It was shown that lactobacilli activated dendritic cells by inducing the expression of Toll-like receptors (TLRs) (Mohamadzadeh and Klaenhammer 2008). Toll like receptors were also responsible for maintaining immune tolerance to resident commensal bacteria mounting immune responses against pathogens (Villena et al. 2014). The host (calves) are also protected from attack by potentially harmful microbial

microorganisms by the physical and chemical barriers created by the gastrointestinal epithelium (Servin 2004). Barrier function genes and antimicrobial peptides such as claudins, and mucins could also have an impact on innate immune response by alteration of the tight junction protein expression and contributing to impaired barrier function (Patel et al. 2012).

In calves, DFM such as *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* generally target the lower intestine and represent an interesting means to stabilize the gut microbiota and decrease the risk of pathogen colonization (Tlaskalová-Hogenová et al. 2011). Direct fed microbials are proposed to enhance intestinal health by preventing enteric pathogens from colonizing the intestine, increasing digestive capacity, lowering gut pH, and improving mucosal immunity (Uyeno et al. 2015). DFM also stimulate the development of a healthy microbiota predominated by beneficial bacteria (Guinane and Cotter 2013; Scott et al. 2015).

The beneficial impact of yeast or *Saccharomyces cerevisiae* on the detrimental activity of pathogens is thought to be due to its ability to co-aggregate with bacterial pathogens (Pizzolitto et al. 2012). *Saccharomyces cerevisiae* boulardii CNCM I-1079 (SCB) has been shown to enhance antibody response of stressed cattle (Keyser et al. 2007). A *Bacillus*-based DFM was shown to enhance inflammatory response for more rapid pathogen clearance in calves (Novak et al. 2012). There are also indications that *Bacillus subtilis* spores are efficient immune modulators, initiating and improving immune functions such as increased antibody and T cell responses to a co-administered soluble antigen in mice (Barnes et al. 2007). Lactobacillus species produces bio surfactants whose wide range of antimicrobial activities against bacterial pathogens as well as their anti-adherent properties impairs the adhesion of pathogens onto gastrointestinal wall membranes (Kanmani et al. 2013). *Lactobacillus acidophilus* limits the activity of some pathogenic bacteria in vitro by producing hydrogen peroxide (Pridmore et al. 2008).

The effect of *Saccharomyces cerevisiae boulardii* or *Lactobacillus acidophilus* on calf's ability to resist infections during the early period of growth has not been well studied. The aims of this study were to investigate the effects of SCB or *Lactobacillus acidophilus* BT1386 (LA) on (1) innate immune response, 2) markers of acute phase reaction and 3) some immune gene expression of rumen and ileum tissues of Holstein dairy calves.

3.6 Materials and Methods

3.6.1 Calves and experimental diets

Animal management and use procedures were according to the Canadian Council on Animal Care (CCAC 2009) and were approved by the Animal Care and Ethics Committee of Agriculture and Agri-Food Canada. Animal management procedures have been described in details previously (Fomenky et al. 2017). Briefly, 48 Holstein calves, 2 to 7 d old were randomly assigned to four treatment groups (n = 12 per treatment): (1) control treatment (CTRL)- fed milk replacer (Goliath XLR 27-16, La Coop, Montreal, QC, Canada) then starter feed (Calf Starter, Shur-Gain, St-Hyacinthe, QC, Canada) that was introduced gradually starting on day 8 of the experiment); (2) SCB treatment - fed CTRL diet supplemented with SCB (Levucell® SB, Lallemand Animal Nutrition, Montreal, QC, Canada; 7.5×10^8 CFU

L^{-1} milk replacer + 3 × 10^9 colony forming units (CFU) kg^{-1} starter feed after weaning); (3) LA treatment fed CTRL diet supplemented with LA (Micro-Cell® FS, Lallemand Animal Nutrition; 2.5 × 10^8 CFU L^{-1} milk replacer + 1 × 10^9 CFU kg^{-1} starter feed after weaning) and (4) antibiotic (ATB) treatment fed CTRL diet supplemented with chlortetracycline (528 mg L^{-1}) and neomycin at (357 mg L^{-1}) in milk replacer before weaning, and chlortetracycline at 55 mg kg^{-1} of starter feed after weaning (Vetoquinol Inc., Lavaltrie, QC, Canada). Calves were housed in individual pens, fed individually and water provided *ad libitum*. The animal trial was divided into three experimental periods d 1 to 42 (pre-weaning), d 43 to 53 (weaning) and d 54 to 96 (post-weaning).

3.6.2 Blood sampling

Blood (10 mL) was collected by jugular venipuncture into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing heparin for plasma separation at different times and transported to the laboratory immediately to perform assays described below. Blood was also collected into tubes at different times with no anticoagulant and transported to the laboratory for serum separation. The blood tubes for serum separation were left to stand for 1 h at room temperature and centrifuged at 850 × g for 15 min at 4 °C. The resulting serum was aliquoted and stored at -20 °C pending analysis.

3.6.3 Isolation of peripheral blood mononuclear cells

Whole blood samples collected on d 22 and 42 (pre-weaning), d 47 and 53 (weaning), and d 66 and 87 (post weaning) were used for the isolation of peripheral blood mononuclear cells (PBMC). The PBMC were isolated using ficoll density gradient. Briefly, blood samples were homogenized and centrifuged (1000 × g at 22 °C for 15 min). The buffy coat layer containing desired cells was harvested and diluted (1:15) in 1× Hank's Balanced Salt Solution (HBSS; Wisent Bio Products, St-Bruno, QC, Canada). The buffy coat mixture was gently layered on the surface of ficoll solution (12 mL; density 1.077 g mL^{-1}, Amersham Biosciences, Piscataway, NJ, USA) followed by centrifugation (750 × g) at 22 °C for 45 min. The supernatant was discarded and the white ring of cells on the top of ficoll (PBMC) was transferred into a 50-mL tube and washed in HBSS by centrifugation (500 × g at 22 °C for 8 min). The isolated PBMC were then suspended in red blood cell lysis buffer (2 mL; Sigma Aldrich, Oakville, ON, Canada) for 2 min to lyse any red blood cells carried over during the separation procedure. Subsequently, cells (PBMC) were washed (centrifuged at 300 × g at 22°C for 5 min) with 20 mL HBSS and counted using the Countess™ automated cell Counter (Invitrogen, Waltham, MA, USA). Cell viability was evaluated by the trypan blue dye exclusion method.

3.6.4 Culture of peripheral blood mononuclear cells and stimulation with lipopolysaccharide or pokeweed mitogen

Isolated PBMC were seeded in 6-well plates at a density of 1 × 10^7cells per well in complete culture medium (89% RPMI 1640 with phenol red and L-glutamine + 10% fetal bovine serum + 1% Penicillin-Streptomycin). Cells were stimulated (100 μL per well) with lipopolysaccharide (LPS; 0.5 μg mL^{-1}) for the quantification of tumour necrosis factor

α (TNF-α) or 100 µL per well of poke weed mitogen (PWM 0.5 µg mL^{-1}) for the quantification of interferon γ (IFN-γ) and interleukin-6 (IL-6). After incubation at 39 °C under 5% CO_2 for 72 h, the stimulated cells were transferred to 5-mL culture tubes, and centrifuged at 350 × g for 5 min at 22 °C. The supernatant was harvested and used for the quantification of the cytokines IFN-γ, TNF-α and IL-6 by ELISA as described below.

3.6.5 Quantification of interferon γ, interleukin 6 and tumor necrosis factor α in peripheral blood mononuclear cells culture supernatants

The PBMC supernatants were tested for the presence of IFN-γ and TNF-α by antigen-capture ELISA using the Bovine IFN-γ and TNF-α DuoSet ELISA (R&D systems Inc., McKinley Place, NE, USA), respectively according to manufacturer's protocols. Bovine IL-6 was assayed using the Bovine IL-6 ELISA Reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA) according to manufacturer's instructions. All samples including standards were tested in duplicates and the optical density values were read on a microplate spectrophotometer (Spectra MaxM2, Molecular Devices, CA, USA) at 450 nm wavelength using the SoftMax Pro® for data analysis. The intra- and inter-assay coefficients of variation were 5% or less. Results with coefficients of variations larger than 5% were repeated.

3.6.6 Isolation of polymorphonuclear neutrophils

Blood collected on d 15 and 42 (pre-weaning), d 47 and 53 (weaning), and d 59, 66 and 87 (post-weaning) were used for the isolation of PMN. The PMN were isolated immediately after collection, and used for oxidative burst and phagocytosis assay (Zhou et al. 2012). For the isolation of PMN, blood samples were homogenised and centrifuged as described above for the PBMC. After removal of the plasma and buffy coat layer, two third of the remaining red blood cell layer were equally discarded leaving one third (approximately 1.5 mL) of red blood cells. The remaining red blood cells were then lysed until all red cells were completely removed. To lyse the red blood cells, 8 mL red cell lysis buffer (8.3 g L^{-1} ammonium chloride in 0.01 M Tris-HCL buffer, adjusted to pH 7.5) were added to the tubes, mixed thoroughly and incubated for 2 min at room temperature followed by centrifugation at 1000 × g for 10 min. The supernatant was discarded followed by another lysing (6 mL red cell lysis buffer) and centrifugation at 500 × g for 7 min. After cell lysis was complete, cells were washed by adding 10 mL sterile HBSS (Wisent Inc., St-Bruno, QC, Canada) and centrifuged at 300 × g for 5 min. Another wash step was done at 200 g for 3 min to completely remove the red cells and recover the PMN. The PMN were suspended in HBSS and counted using the Countess automatic cell counter (Life Technologies Inc., Burlington, ON, Canada) then adjusted to the desired concentration of 5×10^6 cell mL^{-1}.

3.6.7 Neutrophil oxidative burst assay

Neutrophil oxidative burst was done as in (Rinaldi et al. 2007). The PMN (5×10^6 cells mL^{-1}) were pre-stimulated in a 15 mL conical tube with 1 µL LPS (1 µg mL^{-1}) from *E. coli* O111:B4 (Sigma-Aldrich, Oakville, ON, Canada) and incubated at 37ºC for 10 min under 5% CO_2. The pre-stimulated PMN were then distributed (100 µL per

well) into a 96-well plate on ice. Thereafter, a fluorescent probe, dihydrorhodamine 123 (10 µM; Life Technologies Inc.) was added (50 µL per well) to the pre-stimulated PMN and incubated at 37ºC for 15 min under 5% CO_2. Next, cells were stimulated with 100 nM Phorbol 12-myristate 13-acetate (PMA; 50 µL per well; Sigma-Aldrich) and incubated for 60 min at 37ºC under 5% CO_2. For the negative controls (non-stimulated cells), 8 mL HBSS was added and incubated. At the end of incubation, plates being protected from light were placed on ice for 5 min to stop the reaction. Plates were then centrifuged (500 × g at 4 °C for 5 min) and the supernatant removed. The PMN (5×10^6 cells mL^{-1}) were re-suspended in HBSS (200 µL) and transferred into flow cytometer tubes containing FACS fixing solution (200 µL; FACSFlow + 0.5% formaldehyde). The assessment of oxidative burst was measured by flow cytometry.

3.6.8 Neutrophil phagocytosis assay

The LPS pre-stimulated PMN (5×10^6 PMN mL^{-1}) were transferred (100 µL) into two separate 96-well plates; one at 37 °C and the other on ice (4 °C) at all times (negative control). The two plates were centrifuged (350 × g for 5 min at 4 °C) and the supernatant discarded. Then, phagocytosis beads, pHrodo™ *E. coli* bio particles® conjugate (100 µL; Life Technologies Inc.) sonicated 3 times for 20 s were added to the 96-well plate and incubated at 37 °C while HBSS (100 µL) was added to the negative control plate at 4 °C. Plates were incubated in darkness for 1 hr in the incubator at 37 °C and on ice respectively. At the end of the incubation period, the plate at 37 ºC was placed on ice for 5 min to stop the reaction. Plates were then centrifuged (500 × g for 5 min at 4 ºC) and the supernatant discarded. The PMN were re-suspended in 200 µL of cold bovine serum albumin solution (0.5%) and transferred to FACS tubes containing 200 µL of FACS fixing solution. The assessment of phagocytosis of the PMN was measured by flow cytometry.

3.6.9 Flow cytometric analysis for oxidative burst and phagocytosis

In PMN undergoing oxidative burst, the probe dihydrorhodamine 123 is oxidised to fluorescent rhodamine following activation. Reactive oxygen species are generated and the fluorescent rhodamine emits a signal at 480-nm wavelength. Ten thousand events (number of cells allowed to pass through the laser beam) were collected at the PMN gate. The fluorescent intensity was the main indicator to quantify oxidative burst and phagocytosis.

The phagocytic capacity was considered as the proportion of phagocytic cells that had ingested the phagocytized fluorescent pHrodo™ *E. coli* bio particles® (Life technologies Inc.) The phagocytic activity was determined as the mean fluorescent intensity, which equaled the mean number of bacteria phagocytized by the cells. The pHrodo™ *E. coli* bio particles® are coated with *E. coli* membrane.

All samples were measured in duplicates and the fluorescent intensity was measured for the stimulated cells compared with the non-stimulated controls. The background fluorescence (in the non-stimulated cells) was subtracted from total fluorescence to obtain values for each individual sample. The oxidative burst and phagocytosis fluorescence intensity of the PMN were acquired processed and analysed using BD FACS Canto II (BD Biosciences, Mississauga, ON, Canada) flow cytometer with BD FACS diva software version 8.0.

3.6.10 Quantification of serum acute phase proteins

Serum collected on d 15, 29 and 42 (pre-weaning), d 46, 49, 51 and 53 (weaning), and d 58 and 65 (post-weaning) were used for the measurement of acute phase proteins (APP). Three APP C-reactive protein (CRP), haptoglobin (HP) and serum amyloid A2 (SAA2) were analysed using the cow CRP, cow HP and cow SAA2 ELISA assay kits (Life Diagnostic Inc, West Chester, PA, USA), respectively, following manufacturer's instructions with slight modifications. All samples including standards were tested in duplicates, the optical density values was read on a microplate spectrophotometer (Spectra MaxM2, Molecular Devices, CA, USA) at 450-nm wavelength, and SoftMax Pro® was used for data analysis.

Preliminary trials indicated that the final dilution of the serum samples for CRP assay was optimal at 1:500. The intra- and inter-assay coefficient of variation was 3%. Optimal dilution for HP and SAA2 quantification was 1:100. The intra- and inter-assay coefficients of variation were below 5% for HP, and 10% for SAA2. Purified bovine CRP, SAA2 and HP standards included in the commercial kits were used to prepare standard curves used in the quantification of the proteins in samples.

3.6.11 Tissue sampling and RNA isolation

Four calves per treatment were humanely euthanized on d 33 (pre-weaning) and d 96 (post-weaning) following standard procedures at a commercial abattoir for collection of tissue samples from the rumen and ileum for RNA isolation. Day 33 was chosen to capture pre-weaning rumen development and d 96 to capture post-weaning rumen development. The tissues (rumen and ileum) were rinsed in phosphate buffered saline to remove digesta, cut into small fragments, snap frozen in liquid nitrogen and stored at -80°C pending RNA isolation.

Total RNA was purified using Qiazol lysis reagent and RNeasy kit (Qiagen Inc., Toronto, ON, Canada) following manufacturer's instructions. The RNA concentration and purity were measured with the Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies Inc., Wilmington, DE, USA) and quality assessed using Bio Analyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). All samples had an RNA integrity number value greater than eight.

3.6.12 Quantitative real time polymerase chain reaction

The expression levels of bovine TLRs (*TLR4*, *TLR6*, *TLR9* and *TLR10*), genes involved in the recognition of pathogen-associated molecular patterns by epithelial and immune cells, cell surface associated mucin (*MUC1* and *MUC20*) and tight junction (*CLDN3*) genes were analyzed by quantitative real time polymerase chain reaction. Reverse transcription was performed with the Superscript II Reverse Transcriptase (Life Technologies Inc.) using 1 µg of total RNA. Assays were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the amplification done using power SYBR green PCR master mix (Life Technologies Inc.). Primers (Table 3.1) were designed to span exon boundaries using Integrated DNA Technologies Real Time qPCR Assay tool (https://www.idtdna.com/scitools) or adapted from Charavaryamath et al. (2011). The forward and reverse primer concentrations were tested (150 to 900 nM) in various combinations to determine the optimal concentrations for the

assay. The best forward and reverse primer combinations yielding reproducible and earliest Ct values while retaining a sigmoidal curve were chosen as optimal (Table 3.1). The efficiencies of the primers ranged from 94 to 98%. The PCR reaction mix of 10 µL included 5 µL Power SYBR® Green PCR Master Mix (Life Technologies Inc.), 3 µL cDNA, and 0.1 U AmpErase Uracil N Glycosylase (Life Technologies Inc.) and 300 to 900 nM of each forward and reverse primer (Table 3.1). The thermal cycling conditions started with a Uracil N Glycosylase treatment at 25°C for 5 min followed by an initial denaturation/activation step at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s on a StepOnePlus Real-Time PCR System (Applied Biosystems). A melt curve was generated at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Two independent experiments were carried out and each data point was in triplicates. The relative quantification of gene expression was determined using the ΔΔCt method (Livak and Schmittgen 2001). The Normfinder analysis (Andersen et al. 2004) identified *RSP9* and *GAPDH* for rumen, and *UXT* and *GAPDH* for ileum as the best combination of reference genes under the experimental conditions out of three reference genes (*RPS9*, *GAPDH*, and *UXT)* tested.

3.7 Statistical analysis

Statistical analyses were performed using PROC Mixed of SAS version 9.3 software (SAS Institute Inc., Cary, NC). A completely randomized design with repeated measures was used for the analyses of cytokines (IFN-γ, IL-6, and TNF-α), APP (CRP, HP, and SAA2), oxidative burst, phagocytosis and immune genes (*TLR4, TLR6 TLR9, TLR10, MUC1*, *MUC20*, and CLDN3) expression levels. The model included the fixed effects of treatment, day and their interaction, and a random effect associated to calf. Multiple comparisons between means were carried out using Tukey's adjustment. The statistical model used was as follows:

$$Y_{ijk} = \mu + \alpha_i + d_{ij} + \tau_k + (\alpha\tau)_{ik} + e_{ijk}$$

Where: Y_{ijk} = observation for animal *j* receiving treatment *i* at day *k*; μ = general mean; α_i = fixed effect of treatment *i*; d_{ij} = random effect associated with animal *j* in treatment *i*; τ_k = fixed effect of day *k*; $\alpha\tau_{ik}$ = interaction between treatment *i* and day *k*; and e_{ijk} =random error.

Table 3.1. Primer sequences and concentration of genes quantified by real time PCR

Gene	Primer sequence	Primer concentration (nM)	Gene accession number	Size	Source
MUC1	MUC1_1594F: 5'CCTACCATCCTATGAGCGAGTA3	300	NM_174115.2	119	This study
	MUC1_1713R: 5'GGCTGCCAGGTTTGTATAAGA3'	300			
MUC20	MUC20_2080F: 5'CGGACAAGGTGGACACATATTA3'	300	XM_580797.8	166	This study
	MUC20_2226R: 5'GGGAGACTTGGACAGGAAAC3'	300			
CLDN3	CLDN3_362F: 5'CAGATGCAGTGCAAGGTGTA3'	300	NM_205801.2	96	This study
	CLDN3_429R: 5'TAGGATGGCGATGACGATGA3'	300			
GAPDH	GAPDH_276F: 5'TGGAAAGGCCATCACCATCT3'	900	NM_001034034.2	100	This study
	GAPDH_338R: 5'CCCACTTGATGTTGGCAG3'	300			
RSP9	RSP9_12F: 5'TTTCCAGAGCGTTGGCTTAG3'	300	NM_001101152.2	114	This study
	RSP9_126R: 5'GGACTTCTCGAAGGGTCTCC3'	900			
UXT	UXT_319F: 5'TGTGGCCCTTGGATATGGTT3'	900	NM_001037471.2	100	This study
	UXT_400R: 5'GGTTGTCGCTGAGCTCTGTG3'	300			
TLR4	Forward: 5'GGTTTCCACAAAAGCCGTAA3'	300	AY634630	137	Charavaryamath et al., 2011
	Reverse: 5'AGGACGATGAAGATGATGCC3'	300			
TLR6	Forward: 5'CGACATTGAAGGCACTGAAA3'	300	AY487803	148	Charavaryamath et al., 2011
	Reverse: 5'TCCTGAGGACAAAGCATGTG 3'	300			
TLR9	Forward: 5'CTCTCCTTGGACTGCTTTGG3'	300	AY859726	204	Charavaryamath et al., 2011
	Reverse: 5'CACTGCACTCTGCACCTTGT3'	300			
TLR10	Forward: 5'TCACCTGACATCTTTGCGAG3'	300	AY634632	187	Charavaryamath et al., 2011
	Reverse: 5'TCGGAATGGATTTCTTCCTG3'	300			

3.8 Results

3.8.1 Cytokine production in supernatant of peripheral blood mononuclear cells

TNF-α was significantly decreased ($P < 0.05$) in ATB-treated calves as compared to CTRL on d 66 (Table 3.2). There was no effect of treatment on IFN-γ and IL-6 concentrations throughout the study (Table 3.2).

Table 3.2. The effect of treatment on *ex vivo* production of cytokines in supernatant of peripheral blood mononuclear cells of calves.

Cytokine[1] (pg/mL)	Time (d)	Treatment[2]				SEM[3]	*P*-value
		CTRL	ATB	LA	SCB		
TNF-α							
	22	600	508	545	465	146	0.66
	42	451	346	342	307	68	0.37
	47	371	300	408	267	81	0.45
	53	224	143	225	155	54	0.17
	66	345[a]	111[b]	198[ab]	240[ab]	60	0.04
	87	189	113	126	158	49	0.37
IL-6							
	22	987	900	1064	1003	227	0.93
	42	1291	924	1302	1498	276	0.38
	47	925	642	1136	851	197	0.28
	53	1141	756	1219	1067	294	0.71
	66	859	683	1276	820	195	0.23
	87	932	803	1167	1046	236	0.70
IFN-γ							
	22	2.96	2.63	2.79	2.94	0.16	0.27
	42	2.81	2.60	2.74	2.93	0.17	0.57
	47	2.94	2.64	2.77	2.90	0.15	0.40
	53	2.72	2.86	3.00	2.96	0.21	0.78
	66	3.39	3.02	3.30	3.32	0.19	0.43
	87	2.71	2.42	2.35	2.66	0.24	0.54

[1]IFN-γ = interferon gamma; IL-6 = interleukin-6; TNF-α = tumour necrosis factor alpha.
[2]CTRL = Control (milk replacer and starter feed from week three); ATB = CTRL + antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning, and at 3×10^9 CFU kg^{-1} starter feed after weaning); LA = CTRL + *Lactobacillus acidophilus* BT1386 (2.5×10^8 CFU L^{-1} milk replacer before weaning and at 1×10^9 CFU kg^{-1} starter feed after weaning).
[3]SEM, standard error of the mean.
[a,b]Means within the same row with different lowercased letters differ significantly ($P < 0.05$).

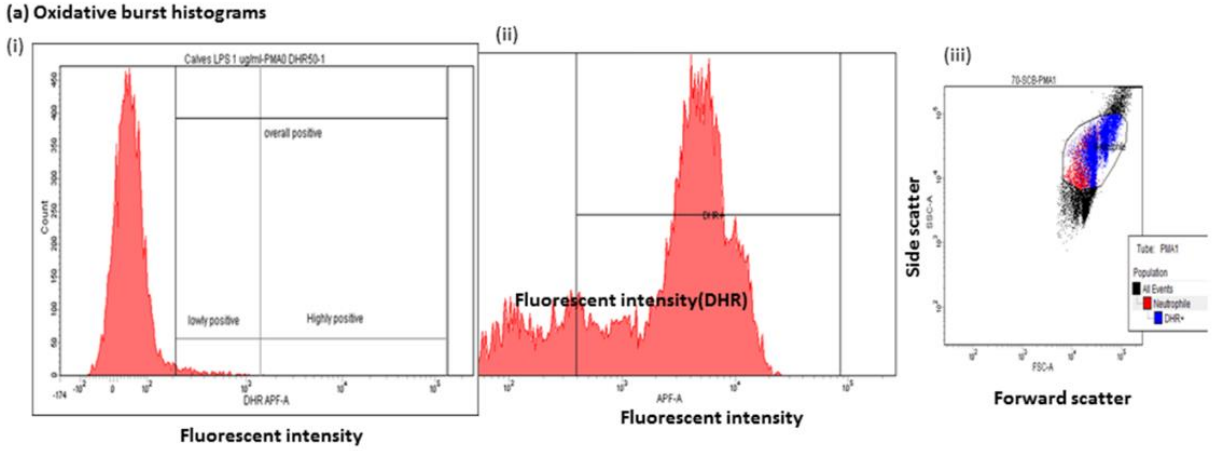
3.8.2 Neutrophil oxidative burst and phagocytosis

The histograms indicating the fluorescent intensity and gating for the quantification of oxidative burst capacity are shown in Fig. 3.1a. The mean fluorescence due to oxidative burst of the PMN of SCB-treated calves were generally higher (greater oxidative burst potential) throughout the study except on d 42 as compared to the other treatments (Fig.

3.1b). The oxidative burst of PMN was decreased during weaning (d 47) for all treatments with SCB showing a superior performance (numerically higher values) though not significant (Fig. 3.1b) as compared to the other treatments. The difference between SCB and CTRL (Fig. 3.1b) was significant ($P < 0.05$) on d 53 (weaning), and d 59 and d 87 (post-weaning). Similarly, mean fluorescence with LA increased significantly ($P < 0.05$) on d 53 as compared to CTRL.

The histograms showing the fluorescent intensity and gating for the quantification of phagocytosis are shown in Fig. 3.2a. The PMN from calves on SCB and ATB treatments showed increased ($P < 0.05$) phagocytic ability at weaning (d 47) as compared to CTRL (Fig. 3.2b). There were no significant differences between treatments in phagocytizing neutrophils on all the other days of sampling.

(a) Oxidative burst histograms

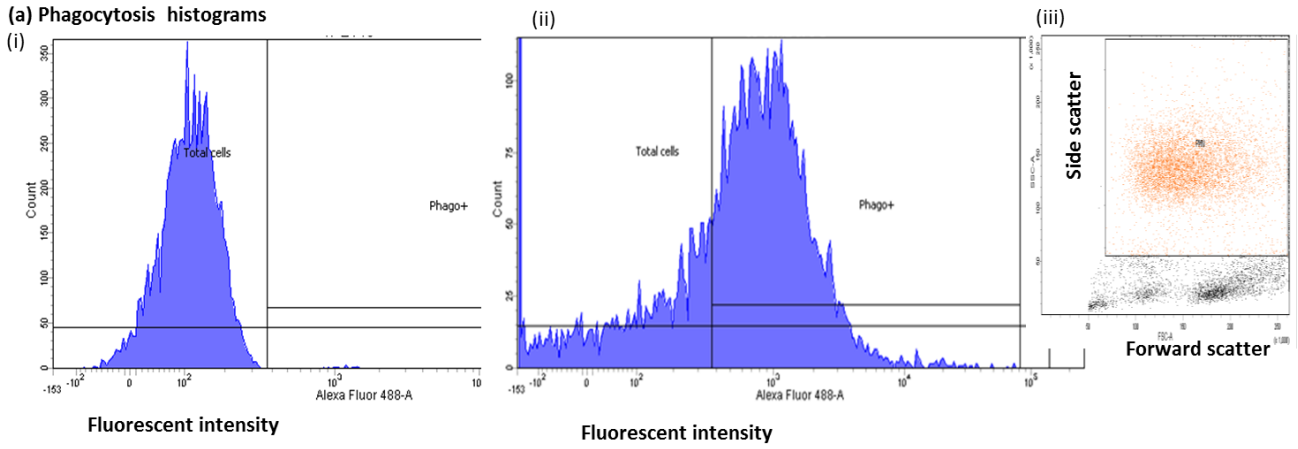


(b) Neutrophil oxidative burst capacity of calves on different treatments

Day	CTRL	SCB	ATB	LA	SEM	P-value
15	1922.50	3829.50	3066.75	2299.88	423.23	0.08
42	3203.50	2991.50	3818.25	2899.25	206.76	0.35
47	1200.00	2246.50	1076.00	1289.25	268.11	0.16
53	2344.75[a]	4042.25[b]	3180.75[a]	4229.25[b]	433.21	**0.01**
59	2620.75[a]	4397.00[b]	3463.25[a]	3207.75[a]	369.71	**0.02**
66	3792.75	6040.25	5624.25	4921.50	491.56	0.10
87	3068.75[a]	4199.00[b]	2295.37[a]	3479.38[a]	397.59	**0.02**

[a,b] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Figure 3.1. (a) Typical histograms showing fluorescent intensity and gating for quantification of oxidative burst. (i) Negative control: unstimulated polymophonuclear neutrophils (PMN; 0 nM PMA), (ii) PMN stimulated with phorbol myristate acetate (PMA;100 nM), and (iii) an example of gating for data acquisition using physical characteristics of granularity (side scatter) and size (forward scatter) of the PMN oxidative burst plot. Ten thousand cells were measured and acquired for data analysis. (b) Neutrophil oxidative burst capacity of calves on different treatments. CTRL = Control (milk replacer and starter feed from wk two); ATB = antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning, and at 3×10^9 CFU kg^{-1} starter feed after weaning); LA = CTRL + *Lactobacillus acidophilus* BT1386 (2.5×10^8 CFU L^{-1} milk replacer before weaning and at 1×10^9 CFU kg^{-1} starter feed after weaning). The mean fluorescence between SCB and CTRL was significant ($P < 0.05$) on d 53 (weaning), and on d 59 and d 87 (post-weaning). The mean fluorescence of LA increased significantly ($P < 0.05$) on d 53 as compared to CTRL.



(b) Neutrophil phagocytosis of calves on different treatments

Day	CTRL	ATB	LA	SCB	SEM	P-value
15	1199.25	1587.25	2058.50	2046.75	398.92	0.15
42	1526.00	2633.00	2931.00	3010.00	342.75	0.51
47	1925.00[a]	3119.50[b]	2691.00[a]	3140.50[b]	284.21	**0.02**
53	3438.23	4281.50	4848.25	4442.75	426.72	0.73
59	3256.75	3566.00	4399.25	4377.20	289.01	0.18
66	3116.50	3954.00	3954.75	4880.00	360.20	0.15
87	1139.00	1145.00	1123.00	1178.00	11.55	0.25

[a,b] Means within the same row with different superscripts differ significantly ($P < 0.05$).

Figure 3.2. (a) Typical phagocytosis histograms showing (i) negative sample at 4 °C, (ii) positive sample at 37 °C, and (iii) an example of sample gating for data acquisition using physical characteristics of granularity (side scatter) and size (forward scatter) for the polymophonuclear neutrophil phagocytosis plot. (b) Neutrophil phagocytosis measured using flow cytometry with Alexa fluor as the fluorescent probe upon stimulation with lipopolysaccharide and pHrodo™ green fluorescent beads. CTRL = Control (milk replacer and starter feed from second wk); ATB = CTRL + antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning, and at 3×10^9 CFU kg^{-1} starter feed after weaning); LA = CTRL + *Lactobacillus acidophilus* BT1386 (2.5×10^8 CFU L^{-1} milk replacer before weaning and at 1×10^9 CFU kg^{-1} starter feed after weaning). Mean phagocytic florescence increased significantly ($P < 0.05$) for calves on SCB and ATB treatments at d 47 as compared to CTRL.

3.8.3 Concentrations of acute phase proteins

Serum concentrations of CRP were higher throughout, but significantly ($P < 0.05$) on d 53 (weaning period) in SCB-treated calves when compared to CTRL, LA and ATB-treated calves (Table 3.3). Serum concentration of HP also increased for the SCB treated calves at weaning though not significantly (Table 3.3). Likewise, concentrations of SAA2 increased at weaning for SCB-treated (d 53; $P < 0.05$) and LA-treated (d 51; $P < 0.05$) calves when compared to CTRL (Table 3.3).

Table 3.3. The effect of treatment on acute phase proteins throughout the feeding trial

Day	[1]Treatments				[2]SEM	P-value
	CTRL	ATB	SCB	LA		
C-reactive protein (ng/mL)						
29	28622.00	21381.50	29231.50	26164.00	4336.50	0.44
42	26018.00	19104.00	32593.50	21636.50	6248.50	0.32
47	22833.50	20426.50	24514.00	22267.00	4333.50	0.86
51	19293.00	20830.50	22115.50	16682.50	4438.50	0.76
53	19332.00[a]	16290.50[a]	30605.50[b]	16922.50[a]	4609.00	0.02
58	19960.00	16660.50	22938.50	17795.00	4253.50	0.40
65	18595.00	19008.50	26346.50	20626.00	3832.50	0.30
Haptoglobin	(ng/mL)					
29	9400.42	7800.22	12200.29	12300.33	2033.50	0.30
42	11800.40	12300.29	12400.87	17300.69	3485.50	0.42
47	8400.49	10100.12	10800.82	11700.77	3375.40	0.73
49	10500.37	6600.16	11600.44	16500.95	4174.40	0.39
51	9600.54	8700.25	15300.94	10800.21	3440.00	0.39
53	11900.67	10000.39	18000.05	13000.09	3686.50	0.28
58	10300.23	9200.75	12000.68	10700.25	3427.40	0.91
65	9700.67	8300.10	11500.40	9100.59	3154.30	0.81
Serum amyloid protein (ng/mL)						
29	2200.89	2000.96	2600.45	2400.43	575.20	0.84
42	2300.16	1600.98	2200.45	1700.18	445.60	0.61
47	1500.36	1500.36	1700.26	2000.93	438.70	0.75
49	1200.06	1400.37	2800.32	2600.74	726.30	0.24
51	1300.43[a]	900.21[a]	1800.62[a]	2400.95[b]	359.50	0.02
53	1600.47[a]	1200.64[a]	2600.79[b]	1800.91[a]	477.10	0.05
58	1300.99	1200.05	1600.88	1700.74	411.70	0.66
65	1300.25	1200.24	1400.54	1600.87	409.80	0.81

[1]CTRL = Control (milk replacer and starter feed from week three); ATB = antibiotics (chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning); SCB = *Saccharomyces cerevisiae* boulardii CNCM1-1079 (7.5×10^8 CFU L^{-1} milk replacer before weaning, and at 3×10^9 CFU kg^{-1} starter feed after weaning); LA = *Lactobacillus acidophilus* BT1386 (2.5×10^8 CFU L^{-1} milk replacer before weaning and at 1×10^9 CFU kg^{-1} starter feed after weaning).
[2]SEM, standard error of mean.
[a,b]Means within the same row with different superscripts differ significantly ($P < 0.05$).

3.8.4 Gene expression in the rumen and ileum

The expression of studied genes (*TLR4, TLR6 TLR9, TLR10, CLDN3, MUC1*, and *MUC 20*) in the rumen and ileum were not significantly different between treatments (Fig. 3.3a and b).

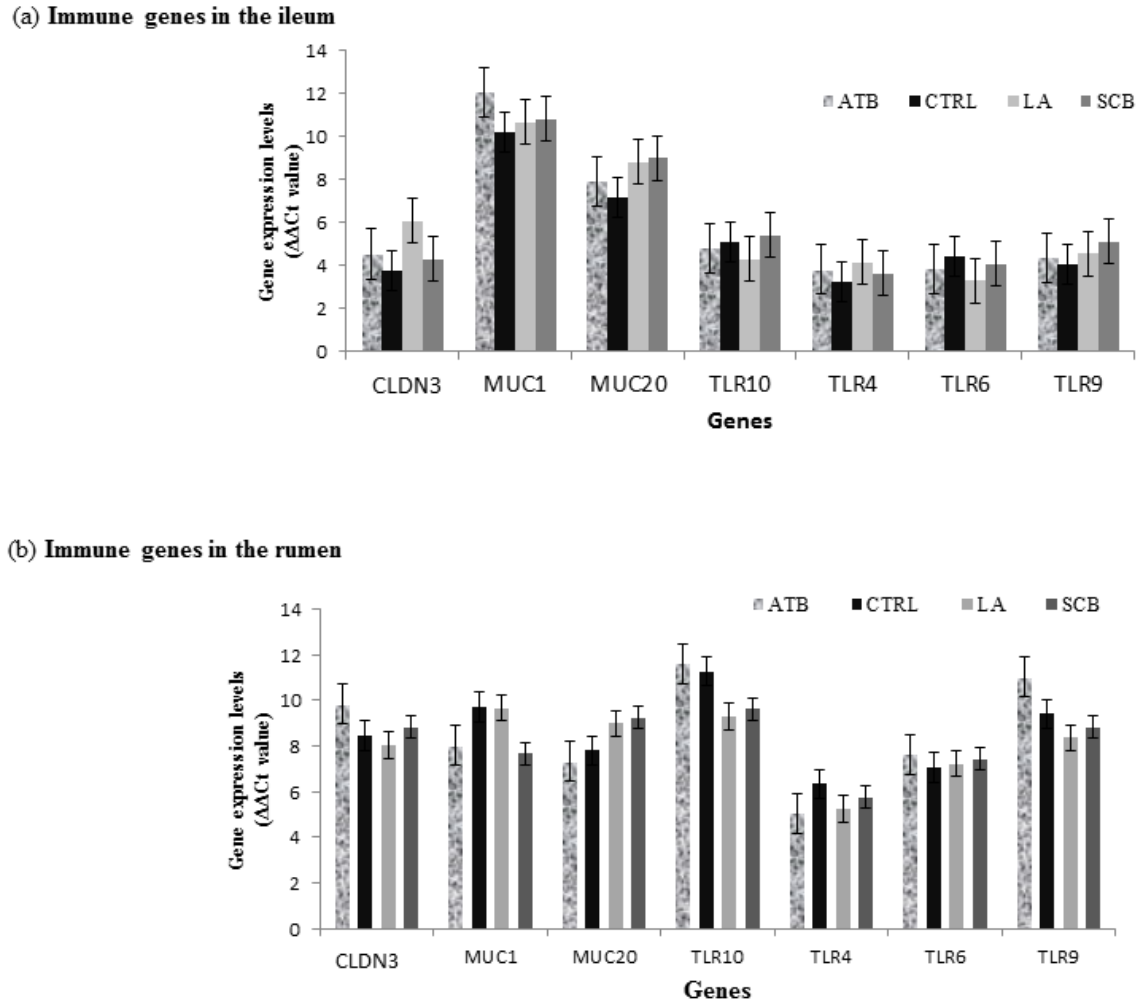


Figure 3.3. (a) Gene expression levels in the ileum on d 33 (gene expression data for d 33 was similar to d 96 so only d 33 data have been presented) (b) Gene expression levels in the rumen (d 33). Higher values, expressed as $\Delta\Delta C_t$, represent lower mRNA expression and vice versa. CTRL = Control fed milk replacer and starter feed from wk two; ATB = CTRL + antibiotics; chlortetracycline and neomycin at 528 and 357 mg L^{-1} milk replacer, respectively, before weaning and chlortetracycline at 55 mg kg^{-1} starter feed after weaning. SCB = CTRL + *Saccharomyces cerevisiae* boulardii CNCM I-1079 at 7.5 × 10^8 CFU L^{-1} milk replacer before weaning and at 3 × 10^9 CFU kg^{-1} starter feed after weaning. LA = CTRL + *Lactobacillus acidophilus* BT1386 at 2.5 × 10^8 CFU L^{-1} milk replacer before weaning and at 1 × 10^9 CFU kg^{-1} starter feed after weaning.

3.9 Discussion

In this study, SCB, LA and ATB showed innate immunomodulatory (oxidative burst and phagocytosis) effects in calves especially during the period of weaning. Since the immune system is immature in the early period of growth in calves, phagocytosis will be the main innate immune system used against pathogens to relieve infections (Kantari et al. 2008). Our results showed that SCB and LA could play a possible role in enhancing innate immune responses of calves during the critical period of stress due to weaning. Similar to our data, *Saccharomyces cerevisiae* boulardii and *Bacillus subtilis* have been reported to increase oxidative burst and phagocytosis in mice and broiler chickens (Lee et al. 2011; Rodrigues et al. 2000). Also, Ortuno et al. (2002) showed enhanced oxidative burst capacity and phagocytosis after oral administration of *Saccharomyces cerevisiae boulardii* to fish. The positive effects of *Saccharomyces cerevisiae* in enhancing the immune system and lessening negative effects related with stress and diseases in farm animals have been reviewed (Broadway et al. 2015). Thus, the ingestion of SCB can enhance systemic innate immunity and intestinal SCB could play a role in the activation of phagocytic cells like PMN (Clarke 2014). Phagocytosis is an innate immune defense mechanism, which is defined by the initiation of intricate signaling networks activated by contact with microorganisms (Batista et al. 2015). Phagocytic cells play a key role in the defense against infections, particularly bacterial infections. Phagocytosis and oxidative burst activity are two of the most essential and vital functions of the innate immune defense for the elimination of invading pathogens (Paape et al. 2003).

Our data showed that markers of the acute phase response were increased in SCB-treated (SAA2 and CRP) and LA-treated (SAA2) calves especially during the weaning period. Similar to our study, a combination of *Enterococcus faecium* and *Saccharomyces cerevisiae* have been reported to increase APP (SAA, lipopolysaccharide binding protein and HP) in feedlot steers (Emmanuel et al. 2007). The acute phase protein α (1)-acid glycoprotein (AGP) concentration increased in scouring calves given electrolyte containing *Bacillus subtilis* (Novak et al. 2012). The concentrations of APP in the blood are normally low but increase if there is an inflammatory response and translocation of bacteria in the bloodstream or during bacterial infection (Deignan et al. 2000); Petersen et al. 2004). The acute phase response is an intricate systemic early defence triggered by inflammation, infection and stress leading to increased hepatic synthesis of APP (Cray et al. 2009). Some APP opsonize microorganisms and activate complement while others scavenge cellular remnants and free radicals, or neutralize proteolytic enzymes in order to directly protect the host (Petersen et al. 2004). The APP have several roles, including the transport and recruitment of immune cells to sites of inflammation. Therefore, increased concentrations of CRP and SAA2 in SCB- and LA-treated calves likely participated directly or indirectly in the protection of calves, increased bacterial translocation and in priming the immune system. Moreover, translocation of yeast antigenic compounds such as α-d-glucan, β-d-glucan and mannan into the bloodstream (Czerucka et al. 2007; Emmanuel et al. 2007) of calves could have supported the increase of serum CRP and SAA2 in this study.

The expression of *TLRs* which are involved in recognition of pathogen-associated molecular patterns by epithelial and immune cells and also cell surface associated *MUC1* and *MUC20* and barrier function gene *CLDN3* in

the ileum and rumen were not changed by treatments. This is an indication that the DFM in this study used other mechanisms and other molecules involved in the enhancement of the innate immune system which probably stimulated acute phase reaction. The observation of no effect of treatment on TLR gene expression (*TLR4, 6, 9* and *10*) is in line with data reported by Trevisi et al. (2008) who did not find an effect of *Bifidobacterium animalis,* another DFM, on *TLR2* and *TLR4* gene expression in the jejunum of pigs two weeks after weaning. Contrary to our study, the supplementation of a combination of DFM *Saccharomyces cerevisiae* boulardi and *Bacillus subtilis* B10 led to an increase in *CLDN3* expression at the cell boundaries and prevented adverse pathogenic effects in chickens (Rajput et al. 2013). *Saccharomyces cerevisiae* was reported to increase the expression of *MUC1* (Zanello et al. 2011) and decrease the expression of *MUC20* (Zhou 2013) in porcine epithelial cells. These data suggest that the expression of these genes is not consistently downregulated or upregulated after feeding DFM. However, analysis of the transcription profile showed that the membrane associated mucins, *MUC1* and *MUC20,* were transcribed throughout the gastrointestinal tract (Hoorens et al. 2011).

Many mechanisms of action have been proposed for SCB when used as a DFM. These include intestinal microbial homeostasis regulation, interference with the ability of pathogens to colonize and infect the mucosa, local and systemic immune response modulation, stabilization of the gastrointestinal barrier function, induction of enzymatic activity favoring absorption and nutrition, and also inhibition of bacterial adhesion/translocation (Czerucka et al. 2007; Im and Pothoulakis 2010; Mazmanian et al. 2008; Ng et al. 2009; Pothoulakis 2009). A combination of these mechanisms might result in the reduction of pathogen's ability to adhere or colonize, therefore attenuating the immune response (Stier and Bischoff 2016). Although yeast account for only a small proportion (0.1%) of the ruminant microbiota, their cell size is ten times larger than that of bacteria and they can represent a significant steric hindrance for bacteria pathogens (Czerucka et al. 2007). We observed that SCB promoted the growth of total lactobacilli in the gastrointestinal tract (Fomenky et al. 2017) which might in turn produce bacteriocins/defensins that can kill certain gram-positive bacteria (Corr et al. 2007; Nes and Holo 2000). A major components of the yeast cell wall are polysaccharides such as α-d-glucan and β-d-glucan which interact directly with immune cells, and are also able to bind to bacteria to prevent attachment and colonization of pathogens (Broadway et al. 2015). The yeast cell wall polysaccharides are also able to block the fimbriae of pathogenic bacteria, and adsorb mycotoxins thus mediating their removal from the organism. Blocking of the receptors may prevent or eliminate infection (Kogan and Kocher 2007). The LA used in this study was also seen to modulate SAA2 (APP) and oxidative burst during weaning. Moreover, direct-fed LA had the potential to produce bacteriocins/defensins as a mechanism of enhancement.

Lactobacillus acidophilus BT1386 had less modulatory effects on the innate immune system and markers of acute phase reaction when compared to SCB. Direct-fed SCB has been shown to outperform other known probiotics, such as *Bifidobacterium sp.* and *Lactobacillus sp.* regarding immunomodulation (Martins et al. 2009). This is an indication that different mechanisms are involved in immune enhancement by different DFM and different DFM species work in different ways.

3.10 Conclusion

Feeding SCB enhanced the innate immune response (both oxidative burst and phagocytosis) and increased the concentrations of APP (CRP and SAA2) at weaning in calves. Similarly, LA enhanced SAA2 and oxidative burst at weaning. Our results suggest that SCB could stimulate acute phase response, and might serve to prime the immune system prior to infection leading to an enhanced innate immune response (oxidative burst capacity and phagocytosis) in calves especially at periods of stress (e.g., weaning). Therefore, SCB might have the potential to strengthen calf immune system in the critical periods of disease susceptibility. However, the expression of studied genes (*TLR4, TLR6 TLR9, TLR10, CLDN3, MUC1*, and *MUC 20*) in the rumen and ileum were not significantly different between treatments.

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Chapitre 4 Direct-fed microbial supplementation influences the bacteria community composition of the gastrointestinal tract of pre- and post-weaned calves

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4.1 Résumé

Cette étude visait à examiner les effets de l'ajout, à l'alimentation de veaux, de deux produits microbiens à administration orale (PMAO) (*Saccharomyces cerevisiae* boulardii CNCM I-1079 [SCB] et *Lactobacillus acidophilus* BT1386 [LA]) et d'un agent de croissance antibiotique (ATB). Trente-deux veaux de race laitière ont reçu une ration témoin (TEM) avec supplément de SCB, de LA ou d'ATB pendant 96 jours. Au jour 33 (présevrage, n = 16) et au jour 96 (post-sevrage, n = 16), on a prélevé du digesta du rumen, de l'iléon et du côlon ainsi que de la muqueuse de l'iléon et du côlon. La diversité et la composition bactériennes du tube digestif (TD) des veaux avant et après le sevrage ont été caractérisées au moyen du séquençage de la région V3-V4 du gène de l'ARN 16S des bactéries. Les PMAO ont eu un effet important sur la structure de la communauté bactérienne, la plupart des changements associés au traitement s'étant produits durant la période de présevrage et principalement dans l'iléon, mais les PMAO ont eu un effet moindre sur la diversité des bactéries. Le SCB et le LA ont entraîné une réduction notable des bactéries pathogènes potentielles appartenant aux genres *Streptococcus* et *Tyzzerella_4* (TFD ≤ 8,49E-06) et une augmentation des bactéries bénéfiques *Fibrobacter* (TFD ≤ 5,55E-04) par rapport à la ration témoin. D'autres bactéries bénéfiques potentielles, dont les bactéries *Rumminococcaceae UCG 005, Roseburia* et *Olsenella*, ont augmenté (TFD ≤ *1,30E-02*) uniquement avec le traitement SCB par rapport à la ration témoin. De plus, la bactérie pathogène *Peptoclostridium* a été réduite (TFD = 1,58E-02) par le SCB seulement, tandis que le LA a entraîné une diminution (TFD = 1,74E-05) du *Ruminococcus_2*. L'analyse de prédiction fonctionnelle semble indiquer que les deux PMAO ($p < 0,05$) ont eu une incidence sur des voies telles que le cycle cellulaire, la sécrétion de bile, le protéasome, la voie de signalisation cAMP, la voie de synthèse des hormones thyroïdiennes et la voie des synapses dopaminergiques. Comparativement aux PMAO, les ATB ont eu un effet similaire sur la diversité bactérienne dans tous les sites du TD, mais ont eu un effet plus important sur la composition bactérienne de l'iléon. De façon générale, cette étude fournit des éclaircissements sur les genres de bactéries qui sont touchés par les PMAO et sur les mécanismes possibles selon lesquels les PMAO agissent sur le microbiote du TD; l'étude pourrait donc faciliter la mise au point de PMAO en remplacement des ATB qui sont utilisés pour la gestion des veaux de race laitière.

4.2 Abstract

This study investigated the effect of supplementing the diet of calves with two direct fed microbials (DFMs) (*Saccharomyces cerevisiae*boulardii CNCM I-1079 (SCB) and *Lactobacillus acidophilus* BT1386 (LA)), and an antibiotic growth promoter (ATB). Thirty-two dairy calves were fed a control diet (CTL) supplemented with SCB, LA or ATB for 33 and 96 days. On day 33 (pre-weaning, n = 16) and day 96 (post-weaning, n = 16), digesta from the rumen, ileum, and colon, and mucosa from the ileum and colon were collected. The bacterial diversity and composition of the gastrointestinal tract (GIT) of pre- and post-weaned calves were characterized by sequencing the V3-V4 region of the bacterial 16S rRNA gene. The DFMs had significant impact on bacteria community structure with most changes associated with treatment occurring in the pre-weaning period and mostly in the ileum but less impact on bacteria

diversity. Both SCB and LA significantly reduced the potential pathogenic bacteria genera, *Streptococcus* and *Tyzzerella_4* (FDR ≤ 8.49E-06) and increased the beneficial bacteria, *Fibrobacter* (FDR ≤ 5.55E-04) compared to CTL. Other potential beneficial bacteria, including *Rumminococcaceae UCG 005, Roseburia* and *Olsenella*, were only increased (FDR ≤ *1.30E-02*) by SCB treatment compared to CTL. Furthermore, the pathogenic bacterium, *Peptoclostridium*, was reduced (FDR = 1.58E-02) by SCB only while LA reduced (FDR = 1.74E-05) *Ruminococcus_*2. Functional prediction analysis suggested that both DFMs impacted ($p < 0.05$) pathways such as cell cycle, bile secretion, proteasome, cAMP signaling pathway, thyroid hormone synthesis pathway and dopaminergic synapse pathway. Compared to the DFMs, ATB had similar impact on bacterial diversity in all GIT sites but greater impact on the bacterial composition of the ileum. Overall, this study provides an insight on the bacteria genera impacted by DFMs and the potential mechanisms by which DFMs affect the GIT microbiota and may therefore facilitate development of DFMs as alternatives to ATB use in dairy calf management.

4.3 Key words

Bacteria composition, colon, digesta, gastrointestinal tract, ileum, *Lactobacillus acidophilus,* mucosa, rumen, *Saccharomyces cerevisiae*, weaning

4.4 Introduction

The microbiota composition of the gastrointestinal tract (GIT) influences the health outcome of animals as well as their productivity[1,2]. The diversity and composition of the GIT microbiota can be influenced by many factors including age, diet, feeding method (management), and feed additives[3,4]. In particular, diet plays pivotal roles on the composition of the GIT microbiota[5,6,7]. Furthermore, diet and the weaning process affect the development of the GIT and microbial colonization in calves during the early period of growth[8,9]. Calf GIT is rapidly colonized by the maternal and environmental microorganisms during and after birth[4,10]. Consequently, exposure to beneficial microbes in the early period of growth will have relevant roles in health outcome[11]. It has been shown that diet and feeding management can be used to manipulate the rumen microbiota in ruminants with long lasting effects[12]. Likewise, microbial colonization and subsequent fermentation processes in the rumen during the early period of growth was influenced by feeding (natural or artificial) practice[13].

Direct fed microbials (DFMs) have been shown to provide health benefits to the host mainly by modulating the GIT microbiota in cattle or other ruminants, and humans[2,14,15]. By modifying the composition of the GIT microbiota, DFMs may contribute to optimize beneficial functions of GIT microbial communities such as digestion, production of vitamin K, promotion and development of the immune system, and detoxification of harmful chemicals resulting in improvement of GIT health[16]. While the diversity, composition, and complexity of calves GIT microbiota has been mostly derived from the analyses of fecal[17,18,19] and rumen microbiota[20,21], few studies have characterized the diversity and community composition in the different sections of the GIT of 5 years old cows and 10 months old sheep[22,23].

Manipulating the microbiota of the GIT through supplementation with DFMs is an attractive approach to improve and maintain animal health[24,25]. DFMs including *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* are naturally occurring microorganisms in the GIT[26,27]. Introducing *Saccharomyces cerevisiae* boulardii CNCM I-1079 (SCB) and *Lactobacillus acidophilus* BT1386 (LA) soon after birth could provide beneficial impact in the establishment of the GIT microbiota. An increase in the potentially beneficial phylum, Actinobacteria, and genera, Bifidobacterium and Collinsella, in the cecum and colon of yeast supplemented piglets[28] has been observed. Also, *Lactobacillus* spp. and *Bifidobacteruim* spp. were increased following treatment with several *Lactobacillus* species in a simulator of human intestinal microbial ecosystem[29]. Furthermore, SCB significantly improved the growth of total lactobacilli in the GIT especially around the weaning period and improved colon morphology[30]. Our hypothesis was that supplementation of calf's diet with SCB and LA will increase the colonisation and establishment of beneficial bacteria in the different GIT sites.

Therefore, the present study investigated the effect of feeding SCB and LA on the colonisation and development of the GIT microbiota, their effects on the composition of bacterial populations in different GIT sites and their potential mechanisms of action during the early period of calf's growth.

4.5 Results

4.5.1 Data acquisition

A total of 8,824,437 sequences of the 16S rRNA genes were generated from amplicon sequencing of 159 samples representing rumen (RuD), ileum (IlD) and colon (CoD) digesta and ileum (IlM) and colon (CoM) mucosa of 16 calves on day 33 (pre-weaning) and another 16 on day 96 (post-weaning) for a total of 4 calves per treatment (Control (CTL), SCB, LA, and an antibiotic growth promoter (ATB)). The mean number of sequences was 55,494.00 ± 1,969.00 per sample. A random sub-sample of sequences per sample were utilised for the normalisation of sequence numbers for other analyses. The sequencing depth was sufficient to cover each microbial community as shown on the rarefaction curves for each sample (Fig. S4.1). Overall, a total of 23 different phyla with 428 genera, 131 families, 81 order and 41 classes were detected (Fig. 4.**1**, Table **S4.1a–e**).

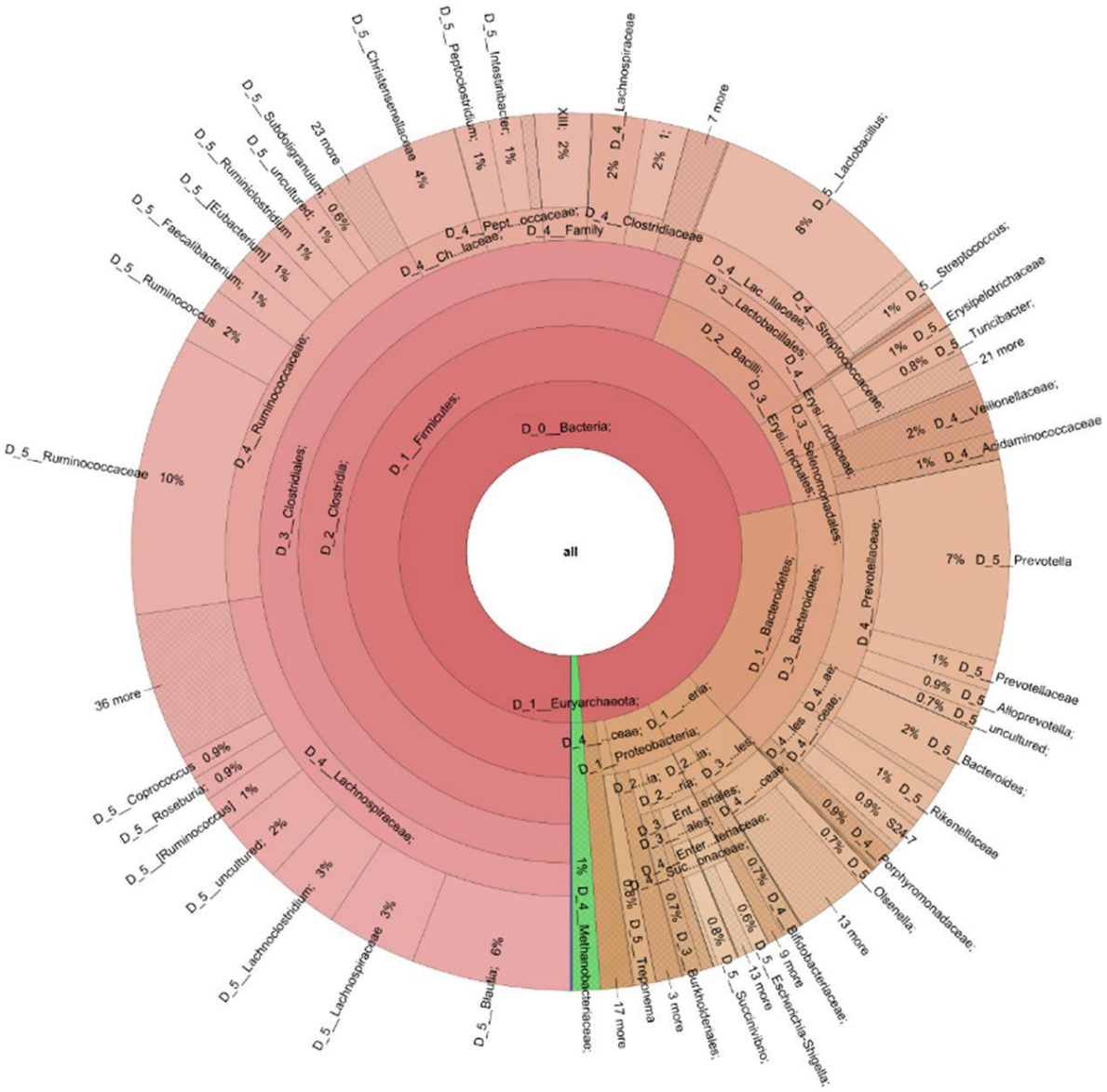

Figure 4.1. Distribution of 159 samples with complete 16S rRNA gene sequences of bacteria phylum and genera.

4.5.2 Bacterial diversity across treatments in GIT sites at pre-weaning (day 33) and post-weaning (day 96)

A pairwise comparison of treatments was done within each GIT site on day 33 (pre-weaning) and day 96 (post-weaning) separately. The results of alpha diversity indices are shown in Table 4.1. In the pre-weaning period, ATB had bacterial communities with a tendency for a greater Shannon diversity index ($p = 0.06$) compared to CTL in IIM (Table 4.1). On the contrary, animals supplemented with ATB had bacterial communities with lower ($p < 0.01$) Shannon diversity index compared to that of CTL in RuD (Table 4.1). Moreover, SCB treatment had greater ($p < 0.05$) Simpson diversity index compared to ATB in CoM and greater bacterial richness (Chao1, $p < 0.05$) compared to ATB

in CoD (Table 4.1). Meanwhile, LA had greater ($p < 0.01$) Shannon and Simpson diversity indices compared to ATB in CoM.

Table 4.1. Comparison of alpha diversity measures across treatments in gastrointestinal sites at pre- and post-weaning periods.

Gastrointestinal site	Alpha indices	Treatments[1]				P-value					
		CTRL	ATB	LA	SCB	ATB vs CTRL	ATB vs LA	ATB vs SCB	CTL vs LA	CTL vs SCB	LA vs SCB
Pre-weaning (day 33)											
Colon mucosa	Observed OTU	94.00	74.33	107.25	90.00	0.284	0.124	0.399	0.510	0.833	0.409
	Chao1	100.35	93.16	122.03	103.72	0.749	0.255	0.659	0.313	0.872	0.419
	Shannon	2.40	2.15	2.87	2.74	0.635	0.004	0.065	0.382	0.537	0.598
	Simpson	0.75	0.79	0.90	0.89	0.840	0.005	0.021	0.396	0.440	0.630
	InvSimpson	8.04	4.71	10.29	9.84	0.232	0.006	0.047	0.405	0.539	0.822
Colon digesta	Observed OTU	72.25	63.25	77.50	84.00	0.271	0.242	0.092	0.654	0.304	0.629
	Chao1	92.48	78.05	83.95	102.46	0.239	0.663	0.014	0.582	0.378	0.205
	Shannon	2.25	2.35	2.50	2.65	0.785	0.636	0.188	0.563	0.301	0.664
	Simpson	0.81	0.86	0.84	0.86	0.398	0.737	0.838	0.597	0.356	0.651
	InvSimpson	6.54	7.36	7.68	7.96	0.719	0.885	0.737	0.684	0.575	0.910
Ileum digesta	Observed OTU	61.00	71.75	70.25	53.25	0.484	0.920	0.212	0.462	0.445	0.132
	Chao1	76.86	91.30	89.90	70.55	0.382	0.938	0.242	0.419	0.660	0.264
	Shannon	1.25	1.05	1.44	1.42	0.739	0.512	0.516	0.681	0.688	0.978
	Simpson	0.50	0.43	0.55	0.58	0.755	0.621	0.516	0.821	0.663	0.819
	InvSimpson	2.34	2.74	2.72	3.07	0.741	0.992	0.814	0.663	0.500	0.771
Ileum mucosa	Observed OTU	67.50	105.50	84.50	103.75	0.064	0.280	0.940	0.326	0.146	0.416
	Chao1	82.90	113.53	91.46	107.25	0.077	0.172	0.764	0.604	0.294	0.480
	Shannon	1.19	2.51	1.84	1.71	0.057	0.398	0.246	0.413	0.449	0.867
	Simpson	0.45	0.78	0.60	0.55	0.092	0.411	0.264	0.532	0.637	0.853
	InvSimpson	2.34	8.13	5.63	2.99	0.216	0.614	0.261	0.339	0.578	0.432
Rumen digesta	Observed OTU	85.00	85.25	87.67	87.50	0.987	0.899	0.860	0.886	0.833	0.992
	Chao1	104.22	101.21	111.81	101.04	0.851	0.645	0.991	0.722	0.771	0.603
	Shannon	2.59	2.32	2.65	2.23	0.004	0.184	0.738	0.770	0.244	0.225
	Simpson	0.87	0.82	0.86	0.77	0.147	0.332	0.555	0.892	0.283	0.314
	InvSimpson	7.48	5.80	7.76	5.38	0.100	0.300	0.767	0.867	0.161	0.253

Gastrointestinal site	Alpha indices	Treatments[1]				P-value					
		CTRL	ATB	LA	SCB	ATB vs CTRL	ATB vs LA	ATB vs SCB	CTL vs LA	CTL vs SCB	LA vs SCB
Post –weaning (day 96)											
Colon mucosa	Observed OTU	115.25	112.25	96.25	99.75	0.664	0.215	0.463	0.167	0.384	0.852
	Chao1	125.32	118.77	105.10	112.82	0.500	0.343	0.675	0.202	0.419	0.662
	Shannon	2.83	3.19	2.92	2.67	0.191	0.104	0.104	0.724	0.627	0.378
	Simpson	0.84	0.90	0.88	0.79	0.266	0.442	0.122	0.390	0.497	0.168
	InvSimpson	8.35	10.71	9.04	6.54	0.482	0.362	0.213	0.829	0.650	0.420
Colon digesta	Observed OTU	111.25	84.25	104.50	111.50	0.019	0.046	0.017	0.494	0.980	0.473
	Chao1	122.37	98.08	112.91	123.02	0.069	0.164	0.064	0.366	0.957	0.339
	Shannon	3.04	2.57	3.25	2.99	0.102	0.041	0.132	0.150	0.756	0.089
	Simpson	0.89	0.77	0.93	0.85	0.103	0.053	0.205	0.005	0.247	0.049
	InvSimpson	9.25	5.81	15.09	7.68	0.231	0.022	0.531	0.001	0.418	0.013
Ileum digesta	Observed OTU	80.75	73.75	60.00	79.00	0.737	0.287	0.693	0.330	0.932	0.145
	Chao1	93.67	87.95	73.56	102.88	0.818	0.386	0.423	0.424	0.719	0.128
	Shannon	2.53	2.27	2.17	2.54	0.529	0.686	0.155	0.412	0.970	0.175
	Simpson	0.84	0.81	0.78	0.88	0.673	0.594	0.060	0.462	0.580	0.165
	InvSimpson	10.12	5.56	5.59	8.30	0.301	0.988	0.073	0.316	0.657	0.210
Ileum mucosa	Observed OTU	103.00	95.25	75.25	94.25	0.507	0.209	0.945	0.120	0.589	0.314
	Chao1	110.33	102.59	87.11	103.01	0.569	0.194	0.974	0.149	0.652	0.291
	Shannon	2.60	2.52	1.99	1.84	0.878	0.478	0.377	0.352	0.271	0.847
	Simpson	0.82	0.77	0.64	0.60	0.782	0.517	0.490	0.315	0.350	0.869
	InvSimpson	7.87	9.18	6.26	4.47	0.759	0.584	0.288	0.711	0.224	0.674
Rumen digesta	Observed OTU	84.50	83.75	89.00	94.50	0.938	0.611	0.169	0.681	0.253	0.537
	Chao1	96.30	105.88	96.18	114.13	0.408	0.446	0.410	0.992	0.103	0.146
	Shannon	2.50	2.55	2.55	2.82	0.804	0.971	0.181	0.815	0.184	0.158
	Simpson	0.81	0.82	0.83	0.88	0.733	0.916	0.235	0.614	0.051	0.236
	InvSimpson	5.42	6.54	6.43	9.95	0.483	0.952	0.253	0.406	0.138	0.227

[1]Treatments: CTRL: Control fed milk replacer followed by starter feed, ATB: CTRL supplemented with antibiotics (ATB) chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively), and chlortetracycline (55 mg/kg starter feed). LA: CTRL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8

CFU/L milk replacer + 1 × 10^9 CFU/kg starter feed) and SCB: CTRL supplemented with *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7.5 × 10^8 colony forming units (CFU)/L milk replacer + 3 × 10^9 CFU/kg starter feed).

In the post-weaning period, LA treatment had bacterial communities with greater ($p < 0.01$) Shannon, Simpson and InvSimpson diversity indices compared to ATB in CoD. SCB had bacterial communities with greater Simpson ($p < 0.05$) diversity index compared to CTL in RuD (Table 4.1).

For beta diversity, dissimilarities were mostly observed between periods, i.e. pre-weaning vs. post-weaning, as shown by the clustering pattern of the principal coordinate analysis (PCoA) plots at the different GIT sites (Fig. 4.2a–e). There was no dissimilarity ($p = 0.512$) in bacterial communities between treatments in RuD but a tendency ($p = 0.09$) was observed in IIM (Fig. 4.2a and c). However, there was a clear difference ($p < 0.01$) between all treatments in the pre-weaning period compared to the post-weaning period in IID (Fig. 4.2b), CoD (Fig. 4.2d) and CoM (Fig. 4.2e).

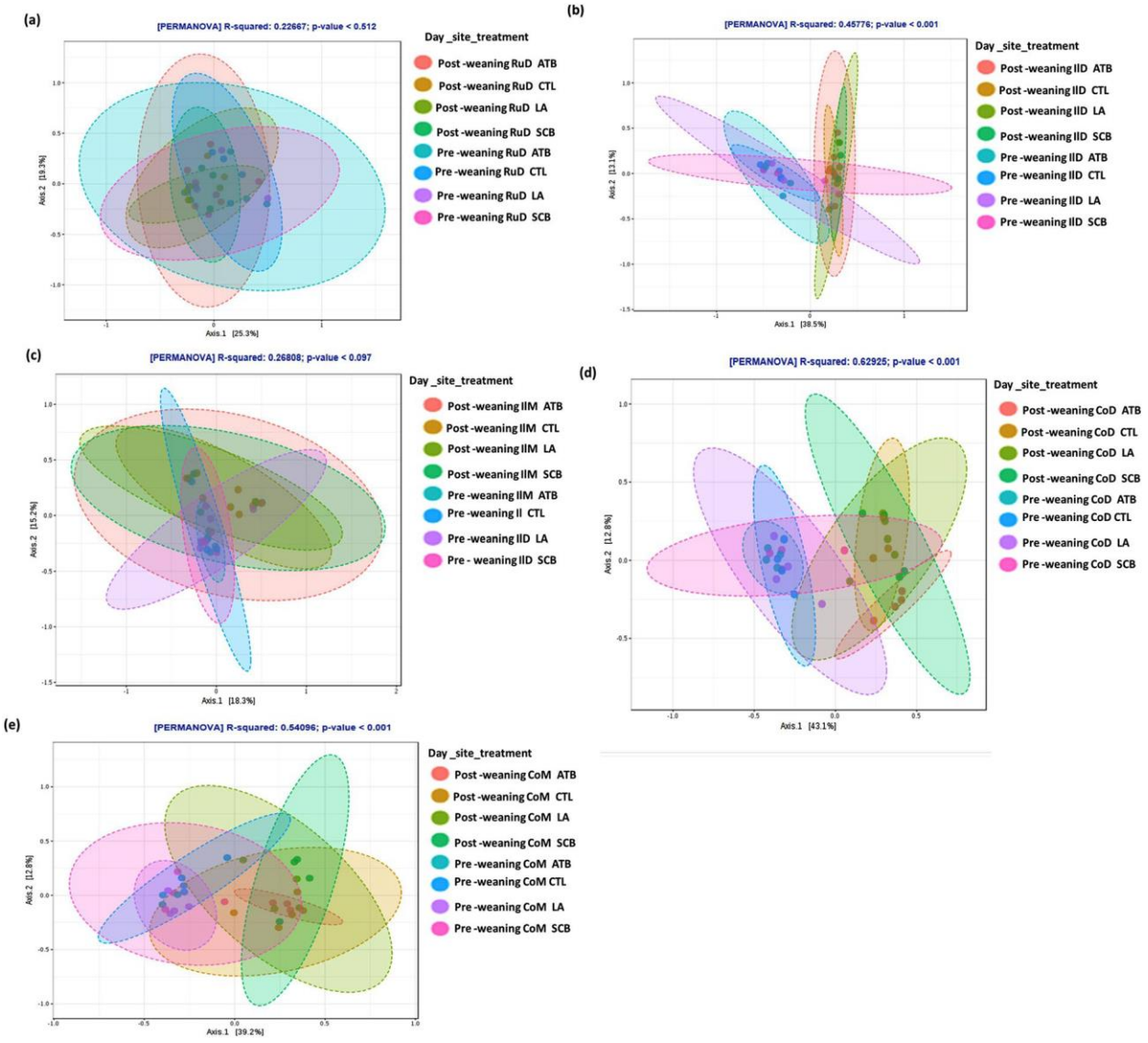

Figure 4.2. Principal coordinate analysis (PCoA) plots for treatment effect on each site at pre- and post-weaning periods. (a) Principal coordinate analysis (PCoA) plots for treatment effect on Rumen digesta at pre-weaning and post-weaning. (b) Principal coordinate analysis (PCoA) plots for treatment effect on ileum mucosa at pre-weaning and post-weaning. (c) Principal coordinate analysis (PCoA) plots for treatment effect on ileum digesta at pre-weaning and post-weaning. (d) Principal coordinate analysis (PCoA) plots for treatment effect on colon digesta at pre-weaning and post-

weaning. (e) Principal coordinate analysis (PCoA) plots for treatment effect on colon mucosa at pre-weaning and post-weaning. Distances between the samples are based on similarity in OTU composition (OTU similarity 97%). A greater distance implies lower similarity, whereas similar OTUs will cluster together. The clustering pattern of the bacterial communities were tested using PERMANOVA and ($P < 0.05$) were considered significant.

4.5.3 Bacterial composition and differential abundance across treatments in GIT sites at pre-weaning and post-weaning periods

The most abundant phyla in all treatments (SCB, LA, ATB and CTL) at all GIT sites were either Firmicutes or Bacteriodetes at both pre- and post-weaning periods. However, Proteobacteria was the most abundant (33.31%) phylum in IIM for calves fed LA in the pre-weaning period (Fig.4.3).

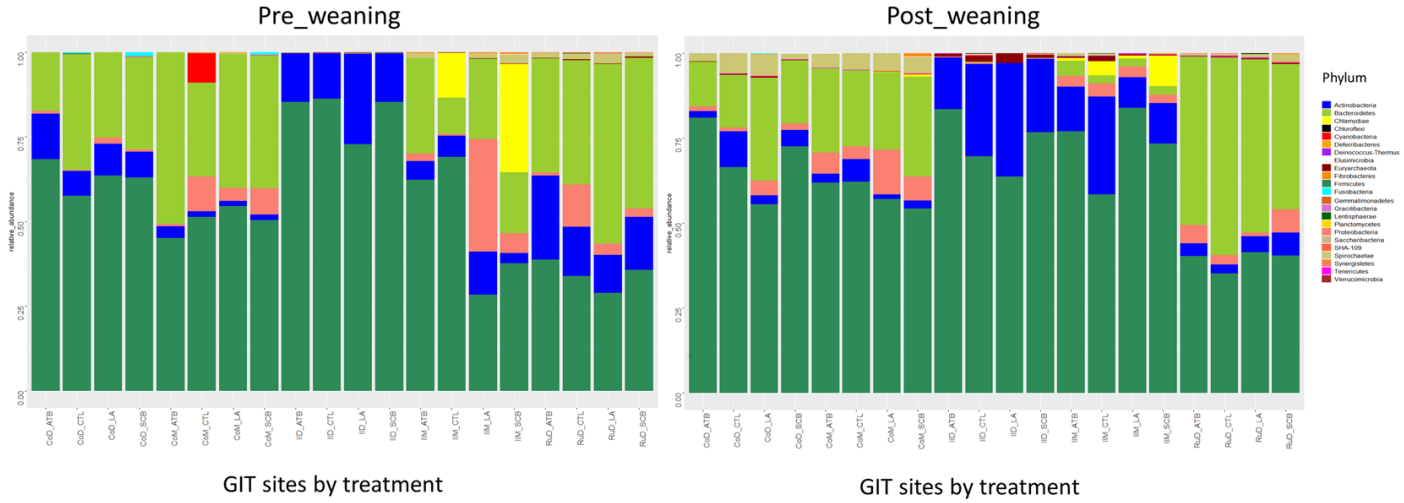

Figure 4.3. Stack bar charts of phylum level bacterial composition for the treatment effect on each site at pre- and post-weaning periods. CoM = colon mucosa, CoD = colon digesta, IM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta.

At the pre-weaning period, the most abundant genera for all treatments were *Blautia, Lactobacillus* and *Prevotella*_1 in CoD (17.1–21.9%), IID (43.1–66.7%) and RuD (19.5–40.7%), respectively (Table S4.2). While the most abundant genera were *Bacteriodetes* for ATB (22.5%) and LA (14.3%), *Streptococcus* for CTL (16.7%) and *Faecalibacteria* for SCB (13.2%) in CoM (Fig. 4.4).

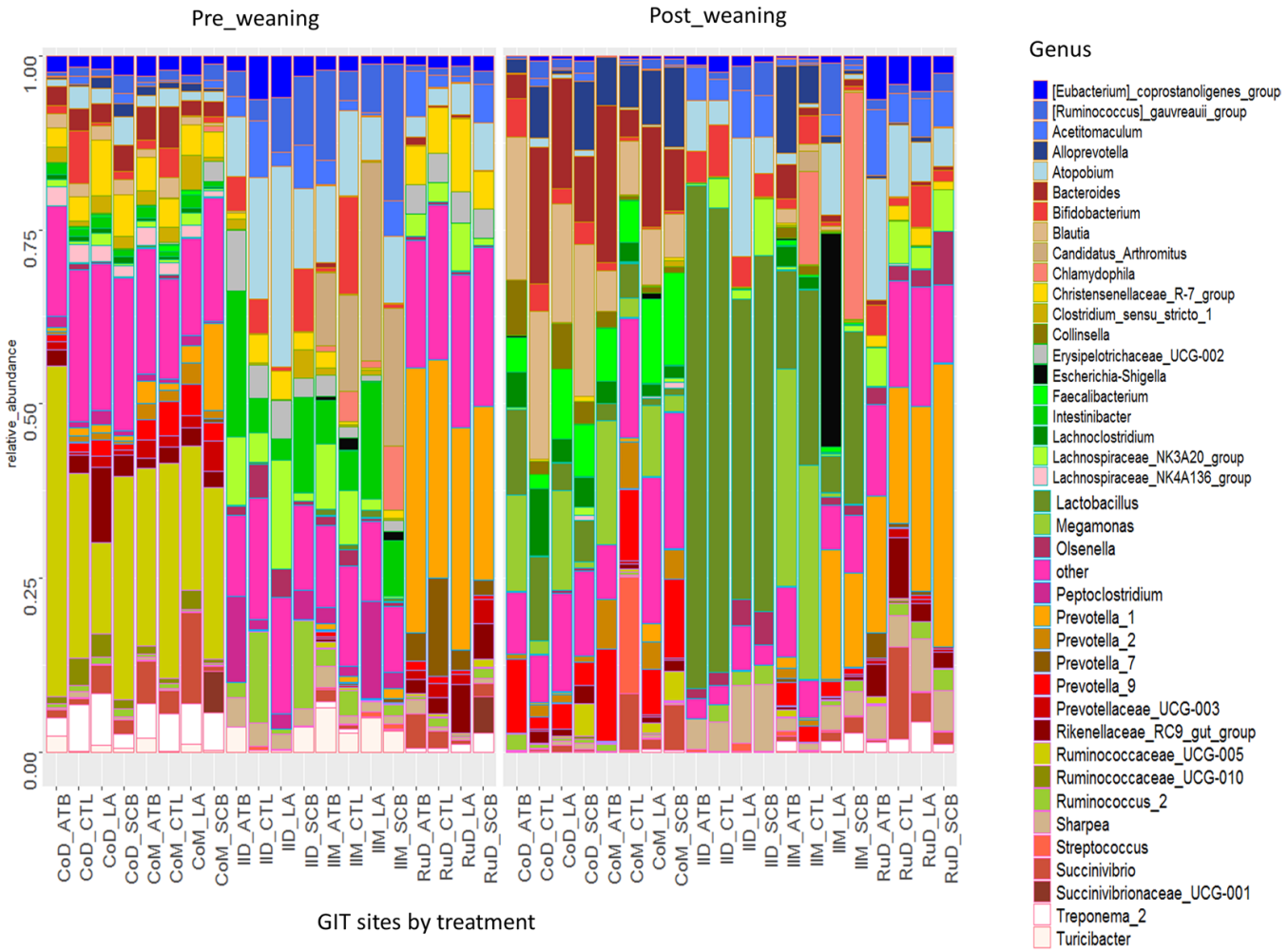

Figure 4.4. Stack bar charts of genus level bacterial composition for the treatment effect on each site at pre- and post-weaning periods. CoM = colon mucosa, CoD = colon digesta, IIM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta.

At the post-weaning period, *Ruminococcaceae*_UCG-005 was the most abundant genus in all treatments (13.2–47.5%) in CoD and CoM while *Atopobium* was the most abundant genus for both LA (28.8%) and CTL (17.5%) treatments and *Intestinibacter* for both ATB (20.9%) and SCB (13.6%) treatments in IID. *Candidatus_Arthromitus* was the most dominant genus for both LA (28.60%) and SCB (19.9%) treatments while *Bifidobacterium* was the most abundant genus for CTL (14%) and *Ruminococcus_gauvreauii_group* for ATB (12.9%) in IIM (Fig. 4.4). *Prevotella_1* was the most abundant genus for all treatments (24.9–38.1%) in RuD.

Significant differential abundant (DA) genera between treatments (SCB, LA and ATB) and CTL in the pre- and post-weaning periods are shown in Tables 4.2, 4.3 and 4.4, respectively. The numbers of DA genera and common genera between the three pairwise comparisons are also shown in Fig. 4.5 for pre- and post-weaning periods. At the pre-weaning period, SCB significantly reduced the abundance of *Streptococcus* (FDR = 8.49E-06) and *Prevotella_7* (FDR = 1.49E-02) in CoM but increased (FDR = 1.30E-02) the abundance

of *Ruminococcaceae_UCG-005* in CoD compared to CTL (Table 4.2). The SCB treatment also significantly changed the relative abundance of 42 and two genera in IIM and IID, respectively, but had no impact on the relative abundance of genera in RuD at the pre-weaning period. In IIM, the genera *Tyzzerella_4* (FDR = 4.27E-09) and *Ruminococcaceae_UCG-008* (FDR = 2.38E-04) had the highest log fold change reduction, while *Fibrobacter* (FDR = 5.5E-04) and *Roseburia* (FDR = 7.01E-04) had the highest log fold change increase by SCB compared to CTL. In IID, *Ruminiclostridium_5* and *Christensenellaceae_R-7* genera were two genera significantly reduced (FDR = 2.52E-02) by SCB compared to CTL in the pre-weaning period.

Table 4.2. Significant differential abundant genera between control and SCB on day 33 (pre- weaning) and day 96 (post- weaning).

Gastrointestinal site	Genus	Phylum	Base Mean	L2FC[1]	P-value	FDR[2]
Pre-weaning (day 33)						
Colon mucosa	*Prevotella_7*	*Bacteroidetes*	215.29	7.98	1.50E-04	1.49E-02
	Streptococcus	*Firmicutes*	649.99	10.13	4.29E-08	8.49E-06
Colon digesta	*Ruminococcaceae_UCG-005*	*Firmicutes*	827.05	−7.44	6.55E-05	1.30E-02
Ileum Mucosa	*Acidaminococcus*	*Firmicutes*	10.78	−6.50	7.81E-03	3.00E-02
	Bacteroides	*Bacteroidetes*	5956.66	5.23	1.61E-03	7.92E-03
	Bifidobacterium	*Actinobacteria*	5947.20	7.02	5.47E-05	7.01E-04
	Collinsella	*Actinobacteria*	3794.58	7.39	3.42E-05	5.55E-04
	Olsenella	*Actinobacteria*	1082.09	−5.16	6.18E-04	4.46E-03
	Desulfovibrio	*Proteobacteria*	105.72	−7.76	1.02E-04	1.06E-03
	Erysipelotrichaceae_UCG-001	*Firmicutes*	54.14	−6.10	8.42E-04	5.12E-03
	Erysipelatoclostridium	*Firmicutes*	70.82	6.67	1.05E-03	5.70E-03
	Erysipelotrichaceae_UCG-002	*Firmicutes*	208.05	6.39	1.63E-03	7.92E-03
	[Eubacterium]_nodatum_group	*Firmicutes*	134.12	−4.11	1.33E-03	6.94E-03
	Mogibacterium	*Firmicutes*	22.25	−5.22	2.53E-03	1.12E-02
	Fibrobacter	*Fibrobacteres*	61.34	−10.57	3.38E-05	5.55E-04
	Tyzzerella_4	*Firmicutes*	1532.14	14.77	2.92E-11	4.27E-09
	Lachnoclostridium	*Firmicutes*	5966.51	8.95	9.48E-09	6.92E-07
	Dorea	*Firmicutes*	160.16	8.90	2.54E-05	5.55E-04
	Roseburia	*Firmicutes*	521.27	−6.67	5.03E-05	7.01E-04
	Lachnospiraceae_NK3A20_group	*Firmicutes*	1293.21	−5.75	6.42E-04	4.46E-03
	Acetitomaculum	*Firmicutes*	2437.10	−5.41	1.01E-03	5.65E-03
	Howardella	*Firmicutes*	24.17	−5.11	2.07E-03	9.73E-03
	Blautia	*Firmicutes*	4016.00	4.58	2.69E-03	1.16E-02
	Lachnospiraceae_UCG-004	*Firmicutes*	106.46	5.05	1.17E-02	4.08E-02
	Peptoclostridium	*Firmicutes*	119.32	5.21	4.02E-03	1.58E-02

Gastrointestinal site	Genus	Phylum	Base Mean	L2FC[1]	P-value	FDR[2]
	Butyricimonas	*Bacteroidetes*	10.20	−7.99	3.08E-03	1.25E-02
	Prevotella_2	*Bacteroidetes*	2892.26	7.99	8.61E-06	3.14E-04
	Prevotella_1	*Bacteroidetes*	6592.34	−6.15	6.24E-05	7.01E-04
	Prevotellaceae_UCG-003	*Bacteroidetes*	276.10	−6.41	7.67E-04	5.09E-03
	Prevotellaceae_UCG-001	*Bacteroidetes*	222.20	−6.44	9.23E-04	5.39E-03
	Rikenellaceae_RC9_gut_group	*Bacteroidetes*	1036.14	−5.21	4.28E-04	3.68E-03
	Ruminococcaceae_UCG-008	*Firmicutes*	1016.82	9.13	4.89E-06	2.38E-04
	Pseudoflavonifractor	*Firmicutes*	135.88	8.84	2.92E-05	5.55E-04
	Ruminiclostridium_9	*Firmicutes*	362.16	7.40	2.46E-05	5.55E-04
	Ruminococcus_2	*Firmicutes*	656.41	−5.04	4.63E-04	3.75E-03
	Ruminococcus_1	*Firmicutes*	223.20	−6.04	8.17E-04	5.12E-03
	Ruminococcaceae_UCG-010	*Firmicutes*	105.32	−6.67	2.78E-03	1.16E-02
	[Eubacterium]_coprostanoligenes_group	*Firmicutes*	1381.11	−3.68	8.32E-03	3.11E-02
	Ruminococcaceae_UCG-014	*Firmicutes*	171.47	−3.91	9.17E-03	3.35E-02
	Ruminococcaceae_NK4A214_group	*Firmicutes*	234.29	−4.49	9.94E-03	3.54E-02
	Treponema_2	*Spirochaetae*	1088.68	−7.03	6.08E-05	7.01E-04
	Sphaerochaeta	*Spirochaetae*	32.86	−9.86	1.34E-04	1.31E-03
	Succinivibrio	*Proteobacteria*	2214.12	−6.38	4.23E-04	3.68E-03
	Veillonellaceae_UCG-001	*Firmicutes*	62.99	−9.46	5.44E-04	4.18E-03
	Megasphaera	*Firmicutes*	264.01	−6.21	2.47E-03	1.12E-02
Ileum digesta	*Christensenellaceae_R-7_group*	*Firmicutes*	896.60	5.89	2.54E-04	2.52E-02
	Ruminiclostridium_5	*Firmicutes*	144.45	6.65	1.45E-04	2.52E-02
Post weaning(day96)						
Rumen digesta	*Ruminococcaceae_UCG-008*	*Firmicutes*	1016.82	9.13	6.68E-05	1.32E-02
Ileum mucosa	*Prevotella_1*	*Bacteroidetes*	6592.35	−5.95	0.000266	2.24E-02
	Actinomyces	*Actinobacteria*	10.95	−8.02	0.000293	2.24E-02
	Streptococcus	*Firmicutes*	649.99	−6.72	0.000407	2.24E-02
	Rothia	*Actinobacteria*	13.17	−8.23	0.000452	2.24E-02

[1]L2FC: log2fold change: positive (+) value indicates a decrease in relative abundance in SCB compared to control while negative value (−) indicates an increase in relative abundance in SCB compared to control,
[2]FDR: P value corrected for False Discovery Rate:
SCB: *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7.5×10^8 colony forming units (CFU)/L milk replacer + 3×10^9 CFU/kg starter feed).

Table 4.3. Significant differential abundant genera between control and LA on day 33 (pre -weaning) and day 96 (post- weaning).

Gastrointestinal site	Genera	Phylum	Base Mean	L2FC[1]	P-value	FDR[2]
Pre-weaning (day 33)						
Colon mucosa	*Turicibacter*	*Firmicutes*	67.38	6.41	4.84E-04	3.19E-02
	Methylobacterium	*Proteobacteria*	84.95	8.82	2.37E-04	2.35E-02
	Streptococcus	*Firmicutes*	649.99	9.39	2.25E-07	4.45E-05
Ileum mucosa	*Phascolarctobacterium*	*Firmicutes*	1075.81	7.18	1.92E-04	2.33E-03
	Bacteroides	*Bacteroidetes*	5956.66	5.78	1.07E-03	1.04E-02
	Bifidobacterium	*Actinobacteria*	5947.2	6.71	2.82E-04	3.16E-03
	Collinsella	*Actinobacteria*	3794.58	9.5	9.73E-07	2.84E-05
	Erysipelatoclostridium	*Firmicutes*	70.82	11.12	1.29E-06	3.13E-05
	Fibrobacter	*Fibrobacteres*	61.34	−7.72	3.60E-03	3.09E-02
	Tyzzerella_4	*Firmicutes*	1532.14	14.25	4.13E-10	6.02E-08
	Lachnoclostridium	*Firmicutes*	5966.51	9.25	3.44E-08	1.67E-06
	Blautia	*Firmicutes*	4016	7.37	8.27E-06	1.51E-04
	Lachnospiraceae_UCG-004	*Firmicutes*	106.46	8.9	9.22E-05	1.50E-03
	Dorea	*Firmicutes*	160.16	8.44	1.41E-04	2.06E-03
	Intestinibacter	*Firmicutes*	189.47	6.16	2.35E-03	2.14E-02
	Prevotella_2	*Bacteroidetes*	2892.26	8.88	4.57E-06	9.53E-05
	Prevotella_9	*Bacteroidetes*	6462.15	6.66	1.80E-04	2.33E-03
	Ruminococcaceae_UCG-008	*Firmicutes*	1016.82	13.16	7.42E-09	5.42E-07
	Pseudoflavonifractor	*Firmicutes*	135.88	11.74	3.30E-07	1.21E-05
	Ruminiclostridium_9	*Firmicutes*	362.16	6.02	1.02E-03	1.04E-02
	Candidatus_Soleaferrea	*Firmicutes*	22.07	7.88	3.89E-03	3.16E-02
Post weaning (day 96)						
Ileum digesta	*Ruminococcus_2*	*Firmicutes*	656.41	7.45	3.95E-07	1.74E-05
	Lactobacillus	*Firmicutes*	19739.88	5.74	5.19E-04	8.93E-03
	Ruminiclostridium_9	*Firmicutes*	362.16	5.98	7.77E-04	8.93E-03
	Prevotella_1	*Bacteroidetes*	6592.35	5.75	8.12E-04	8.93E-03
	Acetitomaculum	*Firmicutes*	2437.1	5.15	2.13E-03	1.88E-02
	Ruminococcaceae_NK4A214_group	*Firmicutes*	234.29	4.48	3.36E-03	2.46E-02

[1]L2FC: log2fold change, positive (+) value indicates a decrease in relative abundance in LA compared to control while negative value (−) indicates increase in relative abundance in LA compared to control.
[2]FDR: p values corrected for False Discovery Rate.
LA: CTRL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8 CFU/L milk replacer.

Table 4.4. Significant differential abundant genera between control and ATB on day 33 (pre -weaning) and day 96 (post-weaning) periods

Gastrointestinal site	Genus	Phylum	Base mean	L2FC[1]	P-value	FDR[2]
Pre-weaning (day 33)						
Colon mucosa	*Streptococcus*	*Firmicutes*	649.99	8.06	3.01E-05	5.97E-03
Ileum digesta	*Actinomyces*	*Actinobacteria*	10.95	−5.69	4.04E-03	2.16E-02
	Bifidobacterium	*Actinobacteria*	5947.2	−5.76	8.48E-04	6.93E-03
	Olsenella	*Actinobacteria*	1082.09	−5.62	2.64E-05	4.59E-04
	Atopobium	*Actinobacteria*	1724.26	−3.36	1.24E-03	9.08E-03
	Collinsella	*Actinobacteria*	3794.58	−4.94	7.68E-03	3.56E-02
	Desulfovibrio	*Proteobacteria*	105.72	−12.62	4.34E-10	6.03E-08
	Erysipelotrichaceae_UCG-001	*Firmicutes*	54.14	−6.90	4.78E-06	1.59E-04
	Turicibacter	*Firmicutes*	67.38	−7.91	1.27E-04	1.96E-03
	Sharpea	*Firmicutes*	2807.21	−4.69	4.08E-04	4.05E-03
	[Eubacterium]_nodatum_group	*Firmicutes*	134.12	−3.80	1.53E-04	2.13E-03
	Mogibacterium	*Firmicutes*	22.25	−3.97	3.10E-03	1.79E-02
	Roseburia	*Firmicutes*	521.27	−7.21	2.20E-07	1.53E-05
	Syntrophococcus	*Firmicutes*	285.49	−6.50	5.42E-07	2.51E-05
	Blautia	*Firmicutes*	4016	−7.25	8.44E-06	1.96E-04
	Acetitomaculum	*Firmicutes*	2437.1	−5.21	9.25E-04	7.14E-03
	Howardella	*Firmicutes*	24.17	−3.53	7.96E-03	3.57E-02
	Lachnoclostridium	*Firmicutes*	5966.51	−3.99	9.61E-03	4.05E-02
	Methanosphaera	*Euryarchaeota*	24.49	−5.18	2.18E-04	2.76E-03
	Methylobacterium	*Proteobacteria*	84.95	−7.00	2.43E-03	1.52E-02
	Peptoclostridium	*Firmicutes*	119.32	−5.38	2.51E-03	1.52E-02
	Romboutsia	*Firmicutes*	10.11	−7.48	2.38E-03	1.52E-02
	Intestinibacter	*Firmicutes*	189.47	−5.03	4.23E-03	2.18E-02
	Prevotella_1	*Bacteroidetes*	6592.35	−5.11	2.13E-03	1.48E-02
	Rikenellaceae_RC9_gut_group	*Bacteroidetes*	1036.14	−4.54	7.55E-03	3.56E-02
	Ruminococcus_1	*Firmicutes*	223.2	−7.60	5.71E-06	1.59E-04
	Ruminococcaceae_NK4A214_group	*Firmicutes*	234.29	−5.26	2.75E-04	3.18E-03

	Ruminiclostridium	*Firmicutes*	6.81	−8.06	3.43E-04	3.67E-03
	[Eubacterium]_coprostanoligenes_group	*Firmicutes*	1381.11	−4.61	5.57E-04	5.16E-03
	Ruminococcaceae_UCG-002	*Firmicutes*	149.04	−6.54	6.98E-04	6.06E-03
	Anaerotruncus	*Firmicutes*	115.29	−5.59	6.06E-03	3.01E-02
	Ruminiclostridium_9	*Firmicutes*	362.16	4.97	8.34E-03	3.62E-02
	Treponema_2	*Spirochaetae*	1088.68	−5.80	3.69E-03	2.05E-02
	Cloacibacillus	*Synergistetes*	16.61	−7.12	1.14E-02	4.67E-02
	Megasphaera	*Firmicutes*	264.01	−8.18	2.45E-05	4.59E-04
Rumen digesta	*Phascolarctobacterium*	*Firmicutes*	1075.81	−5.82	3.84E-03	2.91E-02
	Bacteroides	*Bacteroidetes*	5956.66	−6.36	6.64E-04	1.88E-02
	Bifidobacterium	*Actinobacteria*	5947.2	−6.37	1.01E-03	1.88E-02
	Olsenella	*Actinobacteria*	1082.09	−4.82	1.49E-03	1.88E-02
	Atopobium	*Actinobacteria*	1724.26	−3.57	2.70E-03	2.75E-02
	Elusimicrobium	*Elusimicrobia*	4.43	−7.96	3.87E-03	2.91E-02
	Erysipelotrichaceae_UCG-001	*Firmicutes*	54.14	−4.51	6.93E-03	4.38E-02
	Mogibacterium	*Firmicutes*	22.25	−6.05	1.05E-04	7.67E-03
	[Eubacterium]_brachy_group	*Firmicutes*	19.74	−7.00	1.51E-03	1.88E-02
	[Eubacterium]_hallii_group	*Firmicutes*	28.17	−7.45	8.55E-04	1.88E-02
	Blautia	*Firmicutes*	4016	−5.89	1.55E-03	1.88E-02
	Syntrophococcus	*Firmicutes*	285.49	−4.71	1.35E-03	1.88E-02
	Lachnospiraceae_UCG-008	*Firmicutes*	12.39	−6.20	2.83E-03	2.75E-02
	Acetitomaculum	*Firmicutes*	2437.1	−5.11	3.99E-03	2.91E-02
	Lachnospiraceae_NK3A20_group	*Firmicutes*	1293.21	−5.43	3.20E-03	2.91E-02
	Methanosphaera	*Euryarchaeota*	24.49	−7.09	8.00E-06	1.17E-03
	Prevotella_2	*Bacteroidetes*	2892.26	−7.17	1.53E-03	1.88E-02
	Prevotella_9	*Bacteroidetes*	6462.15	−5.24	5.21E-03	3.45E-02
	Ruminococcaceae_UCG−013	*Firmicutes*	27.66	−8.91	2.77E-04	1.35E-02
	[Eubacterium]_coprostanoligenes_group	*Firmicutes*	1381.11	−5.19	6.23E-04	1.88E-02
	Ruminiclostridium	*Firmicutes*	6.81	−7.71	1.85E-03	2.08E-02
	Ruminococcaceae_UCG-002	*Firmicutes*	149.04	−5.60	4.56E-03	3.17E-02

	Streptococcus	*Firmicutes*	649.99	−5.44	7.20E-03	4.38E-02
	Ruminobacter	*Proteobacteria*	450.64	−7.89	3.45E-03	2.91E-02
Post- weaning (day 96)						
Ileum digesta	*Ruminococcus_2*	*Firmicutes*	656.41	5.11	1.99E-04	2.56E-02
	Ruminococcaceae_UCG-008	*Firmicutes*	1016.82	7.88	2.59E-04	2.56E-02
Ileum mucosa	*Dorea*	*Firmicutes*	160.16	−9.10	3.40E-05	2.74E-03
	Sutterella	*Proteobacteria*	99.95	−7.83	5.92E-05	2.74E-03
	Prevotellaceae_UCG-003	*Bacteroidetes*	276.1	−7.40	8.56E-05	2.74E-03
	Rikenellaceae_RC9_gut_group	*Bacteroidetes*	1036.14	−5.99	1.02E-04	2.74E-03
	Anaerovibrio	*Firmicutes*	241.51	−7.61	2.41E-04	5.15E-03
	Prevotella_1	*Bacteroidetes*	6592.35	−5.62	5.81E-04	1.04E-02
	Lachnoclostridium	*Firmicutes*	5966.51	−5.55	8.85E-04	1.35E-02
	Prevotella_9	*Bacteroidetes*	6462.15	−5.70	1.34E-03	1.79E-02
	Prevotella_2	*Bacteroidetes*	2892.26	−6.00	1.50E-03	1.79E-02
	Ruminococcaceae_UCG-005	*Firmicutes*	827.05	−5.30	2.70E-03	2.89E-02
	Treponema_2	*Spirochaetae*	1088.68	−5.32	3.52E-03	3.42E-02
	Ruminococcaceae_UCG-010	*Firmicutes*	105.32	−5.95	4.65E-03	4.15E-02
	Ruminococcaceae_UCG-009	*Firmicutes*	15.2	−7.25	5.71E-03	4.68E-02
	Succinivibrio	*Proteobacteria*	2214.12	−5.18	6.19E-03	4.68E-02
	Lachnospiraceae_NK4A136_group	*Firmicutes*	95.27	−5.57	6.56E-03	4.68E-02
	Prevotella_7	*Bacteroidetes*	215.29	5.9	7.29E-03	4.88E-02

[1]L2FC: log2fold change log 2-fold change, positive (+) value indicates a decrease in relative abundance in control compared to ATB while negative value (−) indicates increase in relative abundance in ATB compared to control.
[2]FDR: P value corrected for False Discovery Rate.
ATB: chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively), and chlortetracycline (55 mg/kg starter feed).

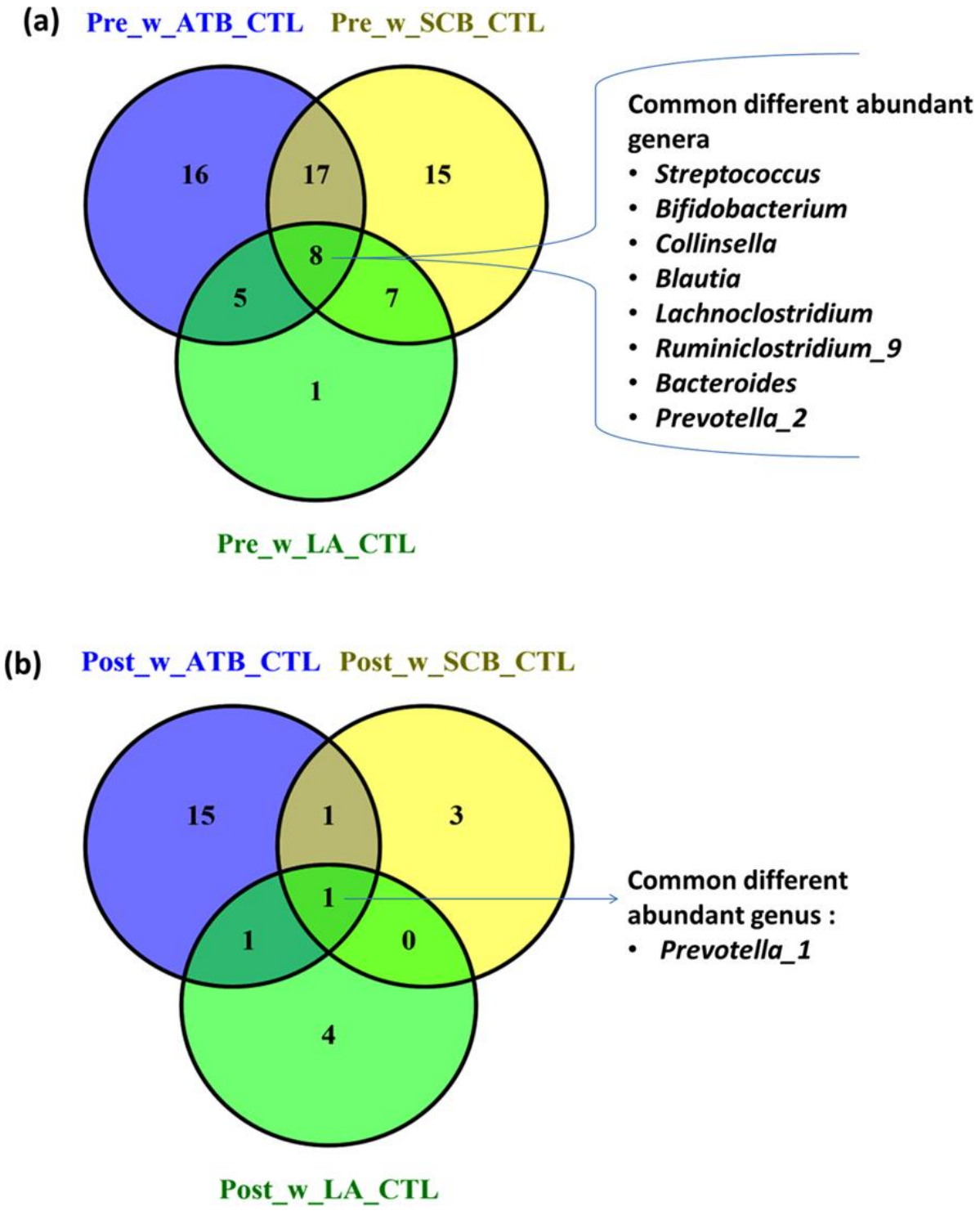

Figure 4.5. The common and specific genera in the (a) pre-weaning and (b) post-weaning periods for the different treatments.

In the post weaning period, SCB significantly reduced the abundance of *Ruminococcaceae_UCG-008* in RuD (FDR = 1.32E-02) but increased (FDR = 2.24E-02) the relative abundance of four genera (*Prevotella_1, Actinomycetes, Streptococcus* and *Rothia*) in IIM compared to CTL. Genera relative abundance in other sites was not affected by SCB in the post-weaning period (Table 4.2).

In the pre-weaning period, no genus was significantly affected by LA treatment in the RuD, IID and CoD compared to CTL, but three and 18 genera were significantly affected in CoM and IIM respectively. In IIM, *Tyzzerella_4, Ruminococcaceae_UCG-008* and *Lachnoclostridium* were the top three genera significantly reduced (FDR ≤ 1.67E-06) while *Fibrobacter* was significantly increased (FDR = 3.09E-02) by LA treatment compared to CTL (Table 4.3). In the post-weaning period, LA treatment impacted only the IID, by reducing (FDR ≤ 2.46E-02) the relative abundance of six genera (*Ruminococcus_2, Lactobacillus, Ruminiclostridium_9, Prevotella_1*, *Acetitomaculum and Ruminococcaceae_NKA214_group* (Table 4.3).

The ATB treatment had greater impact on genera relative abundance in IID and RuD at the pre-weaning period and in IIM at the post-weaning period (Table 4.4). The ATB treatment changed (FDR ≤ 9.08E-03) the relative abundance of 34 and 24 genera in IID and RuD in the pre-weaning period and 16 genera in IIM. *Streptococcus* was significantly reduced (FDR = 5.97E-03) by ATB treatment in CoM at the pre-weaning period. In the post-weaning period, *Dorea* (FDR = 2.74E-03) and *Anaerovibrio* (FDR = 5.15E-03) were significantly increased by ATB (Table 4.4). Comparisons between LA vs. ATB, SCB vs. ATB and SCB vs. LA are shown in Tables 4.5, 4.6 and S4.3. A total of 43 and 135 genera were significantly DA between LA vs. ATB (Table 4.5) and SCB vs. ATB (Table 4.6), respectively. Most DA genera for both pairwise comparisons were found in the pre-weaning period (40/43 for LA vs. ATB and 113/135 for SCB vs. ATB) as well as in the ileum (mucosa and digesta) (Tables 4.5 and 4.6). *Tyzzerella* 4 (FDR = 4.42E-11) and Ruminococcaceae_UCG-005 (FDR = 8.45E-07) were the most significant DA genera between SCB vs. ATB in the pre- and post-weaning period, respectively (Table 4.5). *Tyzzerella* 4 was also the most significant DA genus in the pre-weaning period when comparing LA vs. ATB (FDR = 7.91E-10) (Table 4.6).

Table 4.5. Highly significant differential abundant genera between SCB and ATB on day 33 (pre -weaning) and day 96 (post-weaning)[1].

Gastrointestinal site	Genus	Phylum	Base Mean	L2FC[2]	P-value	FDR[3]
Pre-weaning (day 33)						
Ileum mucosa	Tyzzerella [4]	Firmicutes	1532.14	16.85	3.32E-13	4.42E-11
	Lachnoclostridium	Firmicutes	5966.51	11.22	1.36E-11	9.04E-10
	Ruminococcaceae UCG-008	Firmicutes	1016.82	11.38	6.23E-08	2.56E-06
	Pseudoflavonifractor	Firmicutes	135.88	11.82	8.16E-08	2.56E-06
	Prevotella 2	Bacteroidetes	2892.26	10.14	9.63E-08	2.56E-06
	Ruminiclostridium 9	Firmicutes	362.16	9.75	1.38E-07	3.06E-06
	Collinsella	Actinobacteria	3794.58	9.19	1.20E-06	2.15E-05
	Bifidobacterium	Actinobacteria	5947.2	8.95	1.29E-06	2.15E-05
	Erysipelatoclostridium	Firmicutes	70.82	9.36	9.76E-06	1.44E-04
	Bacteroides	Bacteroidetes	5956.66	7.69	1.30E-05	1.73E-04
	Blautia	Firmicutes	4016	6.88	2.38E-05	2.87E-04
	Anaerotruncus	Firmicutes	115.29	6.98	7.22E-05	8.00E-04
	Subdoligranulum	Firmicutes	121.14	7.04	2.88E-04	2.83E-03
	Ruminococcus 1	Firmicutes	223.2	−6.80	3.18E-04	2.83E-03
	Lachnospiraceae FCS020 group	Firmicutes	7.11	7.67	3.19E-04	2.83E-03
	Lachnospiraceae UCG-004	Firmicutes	106.46	7.31	4.69E-04	3.90E-03
	Prevotella 1	Bacteroidetes	6592.34	−5.58	6.60E-04	5.16E-03
	Phascolarctobacterium	Firmicutes	1075.81	6.21	9.25E-04	6.84E-03
Ileum digesta	Desulfovibrio	Proteobacteria	105.72	10.35	1.47E-08	2.00E-06
	Ruminococcus 1	Firmicutes	223.2	8.65	2.70E-07	1.83E-05
	Lachnoclostridium	Firmicutes	5966.51	7.3	3.30E-06	1.50E-04
	Syntrophococcus	Firmicutes	285.49	5.74	8.76E-06	2.98E-04
	Methanosphaera	Euryarchaeota	24.49	5.85	3.00E-05	7.46E-04
	[Eubacterium] coprostanoligenes group	Firmicutes	1381.11	5.55	3.29E-05	7.46E-04
	Roseburia	Firmicutes	521.27	5.59	5.67E-05	1.06E-03
	Ruminococcaceae UCG-002	Firmicutes	149.04	8.32	6.23E-05	1.06E-03

	Intestinibacter	Firmicutes	189.47	7.55	7.65E-05	1.16E-03
	Streptococcus	Firmicutes	649.99	6.91	1.15E-04	1.57E-03
	Erysipelotrichaceae UCG-001	Firmicutes	54.14	5.61	1.45E-04	1.79E-03
	Atopobium	Actinobacteria	1724.26	3.83	2.38E-04	2.66E-03
	Methylobacterium	Proteobacteria	84.95	8.84	2.55E-04	2.66E-03
	Bifidobacterium	Actinobacteria	5947.2	6.24	3.01E-04	2.79E-03
	[Eubacterium] nodatum group	Firmicutes	134.12	3.62	3.08E-04	2.79E-03
	Ruminococcaceae NK4A214 group	Firmicutes	234.29	5.15	3.29E-04	2.80E-03
	Ruminococcaceae UCG-005	Firmicutes	827.05	6.72	5.24E-04	4.19E-03
	Turicibacter	Firmicutes	67.38	6.35	9.15E-04	6.91E-03
	Peptoclostridium	Firmicutes	119.32	5.81	1.08E-03	7.04E-03
	Lactobacillus	Firmicutes	19739.88	5.04	1.12E-03	7.04E-03
	Lachnospira	Firmicutes	13.66	8.73	1.15E-03	7.04E-03
	Christensenellaceae R-7 group	Firmicutes	896.6	5.28	1.15E-03	7.04E-03
	Sharpea	Firmicutes	2807.21	4.3	1.19E-03	7.04E-03
Rumen digesta	Prevotella 7	Bacteroidetes	215.29	9.8	3.16E-06	4.39E-04
	Ruminococcaceae UCG-005	Firmicutes	827.05	7.98	7.51E-06	5.22E-04
	Atopobium	Actinobacteria	1724.26	4.82	1.67E-05	7.72E-04
	Methanosphaera	Euryarchaeota	24.49	5.78	6.28E-05	2.14E-03
	Lachnospiraceae UCG-008	Firmicutes	12.39	7.98	8.59E-05	2.14E-03
	Streptococcus	Firmicutes	649.99	7.6	9.22E-05	2.14E-03
	[Eubacterium] coprostanoligenes group	Firmicutes	1381.11	5.51	1.15E-04	2.24E-03
	Ruminococcaceae NK4A214 group	Firmicutes	234.29	5.81	1.29E-04	2.24E-03
	Bifidobacterium	Actinobacteria	5947.2	6.72	2.49E-04	3.84E-03
	Lactobacillus	Firmicutes	19739.88	5.86	5.40E-04	7.33E-03
	Mogibacterium	Firmicutes	22.25	4.87	5.80E-04	7.33E-03
	Corynebacterium 1	Actinobacteria	33.17	8.08	6.50E-04	7.53E-03
Colon mucosa	Succinivibrio	Proteobacteria	2214.12	−8.17	1.84E-05	3.65E-03
	Tyzzerella 4	Firmicutes	1532.14	8.1	1.01E-04	9.98E-03
Colon digesta	Tyzzerella 4	Firmicutes	1532.14	8.08	2.59E-05	5.12E-03

Post-weaning (day 96)						
Ileum mucosa	Ruminococcaceae_UCG-005	Firmicutes	827.05	10.32	6.21E-09	8.45E-07
	Prevotellaceae_UCG-003	Bacteroidetes	276.1	9.3	8.50E-07	5.78E-05
	Dorea	Firmicutes	160.16	9.47	2.21E-06	8.60E-05
	Prevotella_7	Bacteroidetes	215.29	−9.86	2.53E-06	8.60E-05
	Anaerovibrio	Firmicutes	241.51	8.62	1.66E-05	3.96E-04
	Ruminococcaceae_UCG-010	Firmicutes	105.32	9.21	1.75E-05	3.96E-04
	Actinomyces	Actinobacteria	10.95	−8.84	3.36E-05	6.53E-04
	Phascolarctobacterium	Firmicutes	1075.81	7.9	5.58E-05	9.48E-04
	Lachnoclostridium	Firmicutes	5966.51	6.48	9.10E-05	1.37E-03
	Lachnospiraceae_NK4A136_group	Firmicutes	95.27	7.96	1.28E-04	1.74E-03
	Ruminiclostridium_9	Firmicutes	362.16	6.92	2.24E-04	2.68E-03
	Rothia	Actinobacteria	13.17	−8.46	2.36E-04	2.68E-03
	Prevotella_2	Bacteroidetes	2892.26	6.41	4.50E-04	4.71E-03
	Lachnospiraceae_UCG-005	Firmicutes	91.57	−8.64	8.16E-04	7.92E-03
	Faecalibacterium	Firmicutes	1438.15	5.82	1.06E-03	9.61E-03

[1]Results presented only for genera with FDR < 0.01; the complete results are presented in Table S4.3b.

ATB: chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively), and chlortetracycline (55 mg/kg starter feed), SCB: *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7.5×10^8 colony forming units (CFU)/L milk replacer + 3×10^9 CFU/kg starter feed).

[2]L2FC: log2fold change, positive (+) value indicates a decrease in relative abundance in SCB compared to ATB, negative value (−) indicates increase in relative abundance in SCB compared to ATB.

[3]FDR: P value corrected for False Discovery Rate.

Table 4.6. Highly significant differential abundant genera between LA and ATB on day 33 (pre- weaning) and day 96 (post- weaning)[1].

Gastrointestinal site	Genus	Phylum	Base Mean	L2FC[2]	P-value	FDR[3]
Pre-weaning (day 33)						
Ileum mucosa	Tyzzerella 4	Firmicutes	1532.14	16.34	5.69E-12	4.42E-11
	Lachnoclostridium	Firmicutes	5966.51	11.52	7.50E-11	9.04E-10
	Ruminococcaceae UCG-008	Firmicutes	1016.82	15.41	7.57E-11	2.56E-06
	Pseudoflavonifractor	Firmicutes	135.88	14.71	6.26E-10	2.56E-06
	Erysipelatoclostridium	Firmicutes	70.82	13.8	5.40E-09	2.56E-06
	Collinsella	Actinobacteria	3794.58	11.29	3.04E-08	3.06E-06
	Blautia	Firmicutes	4016	9.67	3.10E-08	2.15E-05
	Prevotella 2	Bacteroidetes	2892.26	11.03	5.94E-08	2.15E-05
	Lachnospiraceae FCS020 group	Firmicutes	7.11	7.93	1.81E-04	1.44E-04
	Candidatus Soleaferrea	Firmicutes	22.07	10.23	2.47E-04	1.73E-04
	Subdoligranulum	Firmicutes	121.14	7.2	5.03E-04	2.87E-04
	Ruminococcaceae UCG-005	Firmicutes	827.05	6.99	6.38E-04	8.00E-04
Ileum digesta	Desulfovibrio	Proteobacteria	105.72	11.33	4.72E-09	2.83E-03
	Lachnospiraceae UCG-004	Firmicutes	106.46	11.16	2.08E-06	2.83E-03
	Phascolarctobacterium	Firmicutes	1075.81	9.47	2.92E-06	2.83E-03
	Prevotella 9	Bacteroidetes	6462.15	8.57	5.05E-06	3.90E-03
	Bifidobacterium	Actinobacteria	5947.2	8.64	9.44E-06	5.16E-03
	Bacteroides	Bacteroidetes	5956.66	8.24	1.01E-05	6.84E-03
	Ruminiclostridium 9	Firmicutes	362.16	8.37	1.38E-05	2.00E-06
	Roseburia	Firmicutes	521.27	6.4	4.26E-06	1.83E-05
	Ruminococcus 1	Firmicutes	223.2	7.62	5.85E-06	1.50E-04
	Atopobium	Actinobacteria	1724.26	4.32	3.34E-05	2.98E-04
	Methanosphaera	Euryarchaeota	24.49	5.39	1.47E-04	7.46E-04
Post- weaning (day 96)						**7.46E-04**
Ileum digesta	Clostridium_sensu_stricto_1	Firmicutes	189.26	7.83	2.03E-05	1.06E-03

[1]The results presented for genera with FDR < 0.01; the complete results are presented in table S4.3c.

[2]L2FC: log2fold change: positive (+) value indicates a decrease in relative abundance in LA compared to ATB while negative value (−) indicates an increase in relative abundance in LA compared to ATB
LA: CTRL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8 CFU/L milk replacer + 1×10^9 CFU/kg starter feed)
ATB: chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively), and chlortetracycline (55 mg/kg starter feed)
[3]FDR: P value corrected for False Discovery Rate

Several genera were also found to be significantly DA between the two DFMs, and among them *Ruminobacter* (FDR = 1.72E-03) and *Lachnospiraceae*_UCG-008 (FDR = 3.71E-02) were the most significantly DA in pre- and post-weaning periods, respectively. *Ruminobacter, Moryella, Acetitomaculum* and *Prevotellaceae* UCG-001 were significantly reduced (FDR ≤ 7.96E-03) by SCB compared to LA (Table S4.3a).

4.5.4 Predicted pathways of the relative changes due to treatments

To investigate the potential molecular pathways by which the microbiota adapted to treatments, we performed metagenomics contribution of the communities observed and differential analyses of predicted pathways between control and treatments for each site in pre- and post-weaning periods using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A total of 6,205 KEGG orthologies (Table S4.4a) were predicted for all samples and assigned into 261 KEGG pathways (Table S4.4b). Metabolic pathway, biosynthesis of amino acids, ribosome, carbon metabolism and purine metabolism were the top 5 predicted pathways by relative abundance values for all GIT sites in both pre- and post-weaning periods (Table S4.4c). The ECM-receptor interaction and the AGE-RAGE signaling pathway in diabetic complications were only predicted for RuD, while Fc epsilon RI signaling pathway was uniquely predicted for IID (Table S4.4c). Several pathways such as endocrine resistance, spliceosome, rap1 signaling, gap junction, and cytosolic DNA-sensing pathway were also uniquely predicted for CoM (Table S4.4c). The changes in abundance values for predicted pathways varied between treatments, site and day.

At the pre-weaning period, the SCB treatment significantly ($p < 0.05$) influenced 6 pathways (cell cycle, EGFR tyrosine kinase inhibitor resistance, bile secretion, Fanconi anemia pathway, mRNA surveillance pathway and oxytocin signaling pathway) in IIM and 5 pathways (caffeine metabolism, cAMP signaling pathway, steroid biosynthesis, proteasome and dopaminergic synapse) in RuD but had no impact on other GIT sites (Table 4.7) compared to CTL treatment. The LA treatment significantly ($p < 0.05$) impacted 4 pathways (caffeine metabolism, cAMP signaling pathway, steroid biosynthesis, proteasome and dopaminergic synapse) in RuD only, compared to CTL. The ATB treatment had diverse effects including significant ($p < 0.05$) changes to steroid hormone biosynthesis pathway in CoM, bile secretion and caffeine metabolism in IIM and cAMP signaling pathway, steroid biosynthesis and proteasome pathways in RuD compared to CTL (Table 4.7).

Table 4.7. Predicted KEGG pathways significantly changed by treatments at each gastrointestinal site in the pre- and post-weaning periods.

Gastrointestinal site	Genus	Phylum
Pre-weaning (day 33)		
Colon mucosa	ATB	Steroid hormone biosynthesis
Ileum mucosa	ATB	Bile secretion
Rumen digesta	ATB	Caffeine metabolism
	ATB	cAMP signaling pathway
	ATB	Steroid biosynthesis
	ATB	Proteasome
	LA	Caffeine metabolism
	LA	cAMP signaling pathway
	LA	Steroid biosynthesis
	LA	Proteasome
	LA	Dopaminergic synapse
Ileum mucosa	SCB	Cell cycle
	SCB	EGFR tyrosine kinase inhibitor resistance
	SCB	Bile secretion
	SCB	Fanconi anemia pathway
	SCB	mRNA surveillance pathway
	SCB	Oxytocin signaling pathway
Rumen digesta	SCB	Caffeine metabolism
	SCB	cAMP signaling pathway
	SCB	Steroid biosynthesis
	SCB	Proteasome
	SCB	Dopaminergic synapse
Post-weaning (day 96)		
Colon mucosa	ATB	Caffeine metabolism
	ATB	Steroid biosynthesis
	ATB	cAMP signaling pathway
Ileum mucosa	ATB	RIG-I-like receptor signaling pathway
	ATB	D-Arginine and D-ornithine metabolism
	ATB	Butanoate metabolism
Ileum digesta	ATB	Thyroid hormone signaling pathway
	ATB	Ether lipid metabolism
Colon mucosa	LA	Caffeine metabolism
Ileum mucosa	LA	Cell cycle
	LA	EGFR tyrosine kinase inhibitor resistance
	LA	Oxytocin signaling pathway
	LA	mRNA surveillance pathway
	LA	Fanconi anemia pathway

Colon mucosa	SCB	Caffeine metabolism
	SCB	Dopaminergic synapse
	SCB	cAMP signaling pathway
	SCB	Serotonergic synapse
	SCB	Steroid biosynthesis
Ileum mucosa	SCB	RIG-I-like receptor signaling pathway
	SCB	Steroid biosynthesis
	SCB	Sphingolipid signaling pathway
	SCB	D-Arginine and D-ornithine metabolism
	SCB	Metabolism of xenobiotics by cytochrome P450
	SCB	Fructose and mannose metabolism
	SCB	Drug metabolism
Rumen digesta	SCB	Thyroid hormone signaling pathway
	SCB	Ether lipid metabolism
	SCB	Cell cycle
	SCB	Oxytocin signaling pathway
	SCB	EGFR tyrosine kinase inhibitor resistance
	SCB	mRNA surveillance pathway
	SCB	Ascorbate and aldarate metabolism
	SCB	Fanconi anemia pathway
	SCB	Riboflavin metabolism

[1]Treatment CTL: Control fed milk replacer followed by starter feed, ATB: CTL supplemented with antibiotics (ATB) chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively), and chlortetracycline (55 mg/kg starter feed). LA: CTL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8 CFU/L milk replacer + 1×10^9 CFU/kg starter feed) and SCB: CTL supplemented with *Saccharomyces cerevisiae* boulardii CNCMI-1079 (SCB; 7.5×10^8 colony forming units (CFU)/L milk replacer + 3×10^9 CFU/kg starter feed).
[2]L2FC: Log2fold change. Negative value indicate that treatment decreased the expression of pathway compared to control while positive value indicate that treatment increased the expression of pathway compared to control.
[3]FDR: False discovery rate corrected p-values.

At the post-weaning period, 5, 7 and 9 pathways were significantly ($p < 0.05$) changed by SCB compared to CTL in CoM, IIM and RuD, respectively (Table 4.7). The most significantly changed pathways by SCB during this period were caffeine metabolism ($p < 1.72E-05$), RIG-I-like receptor signaling pathway ($p < 5.57E-05$) and thyroid hormone signaling pathway ($p < 7.57E-07$) in CoM, IIM and RuD, respectively. Meanwhile, LA impacted the mucosa (IIM and CoM) only as it changed the abundance levels of caffeine metabolism ($p < 7.13E-04$) in CoM and of cell cycle ($p < 2.64E-04$) in IIM, EGFR tyrosine kinase inhibitor resistance, oxytocin signaling pathway, mRNA surveillance pathway and Fanconi anemia pathway ($p \leq 1.64E-03$) in IIM. The ATB treatment significantly changed ($p \leq 5.63E-04$) the abundance of thyroid hormone signaling pathway and ether lipid metabolism in IID, cAMP signaling pathway in CoM and RIG-I-like receptor signaling pathway, D-arginine and D-ornithine metabolism and Butanoate metabolism in IIM.

4.6 Discussion

Overall, the phylum Firmicutes was the most abundant in all GIT sites except the RuD where Bacteroidetes was the most dominant. Our results are supported by earlier reports of high relative abundance of Firmicutes in the GIT of pre-weaned Holstein calves or of Brazilian Nelore steer[31]. It is well documented that the bacterial community diversity pattern and composition differ across GIT sites[31,32]. In the current study, each GIT site was host to different bacteria community structures. In fact, we observed that CoM harboured a greater bacterial community diversity compared to other GIT sites. The colon is considered a fermentation tank for microbial fermentation of indigestible dietary substrates and the digesta is retained in the colon (large intestine) for a longer time compared to the small intestine (ileum), the colon being the hub of a more complex bacterial community[33]. In the colon, dietary fiber that escaped digestion in the upper digestive tract are broken down into short-chain fatty acids and, the increased availability of short chain fatty acids promotes the growth of some bacteria in the lower GIT sites. Therefore, the increased bacteria growth is expected to account for the richness of bacteria in the colon[34]. The IID had the lowest diversity compared to all other GIT sites. Peristaltic movements ensure a relatively short passage time through the ileum (3–5 h) by pushing the microbiota migration towards the large intestine, hence limited time for microorganisms to replicate and increase in numbers[35] in IID compared with other GIT sites investigated. Mucosa-associated microorganisms live in close contact with host cells; hence they execute different functions within the GIT compared to digesta microorganisms. This might account for the differences in diversity and composition of the ileum mucosa and digesta as seen in the current study.

As expected, alpha diversity measures were higher for post-weaning compared to pre-weaning. Likewise bacterial community composition was different in the post-weaning period as compared to the pre-weaning period in this study. In the early period of growth, the bacterial populations undergo dynamic changes in diversity and abundance as calf age[20]. Also, the bacterial communities in the GIT sites are significantly influenced by weaning[36]. The increased consumption of large amounts of solid feed and dietary shift from milk replacer with age has been given as the reason for age dependent increase in bacterial diversity[37]. The fermentation processes in the rumen is activated by the introduction of solid feed but there is a dramatic shift when milk is completely removed (weaning), greatly altering the composition of the ruminal and intestinal microbiomes8. The ruminal bacterial community is established before intake of solid food, but solid food arrival in turn shapes this community[38]. Dias *et al*.[39] indicated that diet and age concurrently drive changes in the structure and abundance of bacterial communities in the developing rumen in calves. The PCoA plots in this study clustered according to period (pre-weaning and post-weaning) which is in line with Wang *et al*.[23] who also indicated that bacteria communities clustered based on different age groups.

Previously, we recovered viable SCB and LA (total lactobacilli) throughout the GIT (rumen, ileum and colon) and feces of calves at the pre- and post-weaning periods[30,40]. Although growth performance (weight gain, feed intake and efficiency) was not affected by treatments[30], calves were generally healthy and the treatments (LA and SCB)

improved innate immune response (oxidative burst and phagocytosis) and markers of the acute phase reaction (CRP and SAA2), especially during weaning[40].

The current study indicated that DFMs had less impact on bacterial diversity but more impact on bacterial composition in the GIT sites in calves. The greater diversity of SCB or LA compared to ATB (Table 4.1) might be linked to the differences in the mechanisms of pathogen clearance by ATB in the GIT. ATB eliminates pathogen growth by direct killing including neighbouring commensals, and therefore completely changing the ecological niche[41]. The diversity of the GIT has been shown to decrease both by short-term and long-term usage of antibiotics[42,43]. Decreased diversity by the use of ATB resulted in dysbiosis of the GIT microbiota leading to undesired effects, such as antibiotic-associated diarrhea[44]. The effects of DFMs on bacterial composition of GIT microbiota was site specific. Interestingly, major changes associated with DFMs were mostly found in the ileum and rumen compared to the colon (Tables 4.2 and 4.3), while a higher impact was observed at the pre-weaning period compared to the post-weaning period. The DA communities were composed of bacteria genera with beneficial effects to the host. The genera were phylogenetically related, suggesting a high level of functional redundancy, which is often associated with stable microbial assemblages resistant to pathogens[45]. Changes in microbial community compositions have been attributed to diet[46]. Since LA and SCB treatments had different impacts, we will discuss the specific potential mechanisms for each DFM separately. For specific mechanisms, we will also focus our discussion on results reported at the genus level.

Perhaps, the most interesting results for SCB treatment was the significant reduction in the presence of *Tyzzerella_4* genus compared to control in IIM (Table 4.2).This genus belongs to *Lachnospiraceae* family and *Clostridia* class. Bacterial species of *Clostridia* class have the ability to form spores and some genera including *Tyzzerella_4* are linked to human diseases[47]. For instance, *Tyzzerella* and *Tyzzerella_*4 were associated to increased cardiovascular disease risk[47]. The SCB treatment reduced the presence of *Streptococcus* compared to control in CoM. The pathogenic *Streptococcus* genus is widely distributed on the mucosal surfaces of the animal GIT[48]. Therefore, it suggests that SCB was able to eliminate numerous pathogens in the colonic mucosa compared to CTL or the other treatments. The microbiota influences the immune system by obstructing invading pathogens and can also support the growth and production of immune cells[49,50]. The SCB treatment also reduced the abundance of *Peptoclostridium* (*Clostridium difficile*) in IIM a major pathogen linked with infectious diarrhea[51] (Table 4.2). In general, *Ruminococcaceae* are common digestive tract microbes that break down complex carbohydrates. SCB consumption positively influenced the establishment of *Ruminococcaceae* genera in the ileum of calves in this study. Brousseau *et al*.[52] also found *Ruminococcaceae* bacterial family in the colon of pigs fed SCB and suggested that SCB had the potential as feed additives to modulate bacterial populations associated with GIT health[52]. *Ruminococcaceae*, actively degrades plants; it has carbohydrate-active enzymes, sugar transport mechanisms, and metabolic pathways for the degradation of complex plant materials[41,53]. As a member of the *Ruminococcaceae* family, *Ruminococcus* is a mucin-degrader and this probably enhanced mucus production which could be the reason for improved inflammatory

responses in calves[54]. In a previous study, we also observed an increase in the concentration of markers associated with inflammatory response (acute phase proteins: CRP and SAA2) in calves fed LA or SCB[39]. Additionally, SCB also significantly increased the abundance of *Olsenella (Lactobacillus*reclassified as *Olsenella)* in IIM, a lactic acid bacterium that ferments carbohydrates to lactic acid[55]. This genus is bile-resistant and has the ability to utilise mucin[56]. Since *Olsenella* is a re-classification of lactobacillus species, its higher abundance supports our recent data in which we observed that SCB promoted the growth of total lactobacilli in the GIT of calves[33]. Surprisingly, the relative abundance of lactobacillus in LA treatment was similar to control in IIM at pre-weaning but decreased significantly ($p = 8.93E-03$) in IID at post-weaning as compared to control. One possible explanation for this observation is that LA was probably a substrate for some other beneficial bacteria which disallowed its increase in some GIT sites even after supplemental feeding of LA. It is known that the product of one microbe is usually the substrate for another[57].The genus Roseburia was also significantly ($p = 7.01E-04$) increased by SCB in IIM pre-weaning as compared to control. This is a commensal related genus producing short-chain fatty acids, particularly butyrate, which provides energy for cells in the GIT[58], affects motility, maintains immunity, and has anti-inflammatory properties[59,60]. *Roseburia* may affect various metabolic pathways and could also serve as biomarkers for beneficial flora in GIT health[60]. This genus metabolizes dietary components that stimulate their proliferation and metabolic activities[60]. In mice, it has been shown that an increase in the abundance of *Roseburia* is linked to reduction of glucose intolerance[61].

Many mechanisms of action of SCB have been directed against pathogenic microorganisms which include regulation of intestinal microbial homeostasis, interference with pathogens ability to colonize and infect the mucosa, modulation of local and systemic immune responses, and induction of enzymatic activity favoring absorption and nutrition. Consistent with the DA analyses, the major pathways changed by SCB treatment were in the IIM at the pre-weaning period. During this period, SCB significantly changed cell cycle, EGFR tyrosine kinase inhibitor resistance, bile secretion, Fanconi anemia pathway, mRNA surveillance pathway and oxytocin signaling pathway in IIM (Table 4.7). Since cell cycle and EGFR pathways are important for the regulation of cell proliferation, differentiation, growth, survival and motility, the SCB treatment might alter the bacterial abundance by influencing the genes or enzymes controlling these processes. Bile secretion pathway was also increased by SCB. This is a vital secretion essential for digestion and absorption of fats and fat-soluble vitamins in the small intestine[62]. In addition, bile is also an important route for elimination of excess cholesterol and many waste products, bilirubin, drugs and toxic compounds[63]. Bile acids appear to be a major regulator of the gut microbiota; and significant reduction in *Ruminococcaceae*[64] has been related to low bile acid levels in the intestine[65]. Bile acids have been shown to have direct and indirect (through FXR-induced antimicrobial peptides) antimicrobial effects on gut microbes[66].

Moreover, SCB treatment also altered the abundance of caffeine metabolism, cAMP signaling pathway, steroid biosynthesis, proteasome and dopaminergic synapse in the RuD. Steroid biosynthesis and proteasome are crucial pathways for lipid and protein metabolism while cAMP signaling pathway is important for second messengers signaling and have wide ranges of impact on cellular processes; therefore, it is not surprising that these pathways were

impacted by the SCB treatment. However, it is not clear how caffeine metabolism pathway is related to SCB treatment in RuD.

Overall, health benefits of DFMs interaction can be classified into three categories[67] as they can act directly within the GIT (level 1), they can also interact directly with the gastrointestinal mucus layer and epithelium (level 2) or they can have effects outside the GIT (level 3). The third level might reflect the effects of SCB on the dopaminergic synapse pathway. The SCB treatment might have impact on dopamine, an important and prototypical slow neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, motivation and reward, learning and memory, and endocrine regulation[68.] However, the exact mechanisms are not clear.

At the post-weaning period, SCB also had an effect on five different pathways (caffeine metabolism, dopaminergic synapse, cAMP signalling, serotonergic synapse and steroid biosynthesis) and among them serotonergic synapse was the only pathway not affected by SCB in the pre-weaning period. Notably, serotonin (5-Hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that plays important roles in physiological functions such as learning and memory, emotion, sleep, pain, motor function and endocrine secretion, as well as in pathological states including abnormal mood and cognition (http://www.genome.jp/kegg-bin/show_pathway?map=hsa04726&show_description=show). Interestingly, beside the effects on steroid metabolism, SCB increased the thyroid hormone signaling pathway ($p < 0.0001$) in RUD during the post-weaning period. Thyroid hormones are important regulators of growth, development and metabolism[69]; therefore it could be an important pathway involved in the SCB mechanism of action.

Generally, the LA treatment had less impact on the bacterial diversity (Table 4.1) but similar impact with SCB treatment on bacterial composition. At the pre-weaning period, LA also had greater impact on bacterial diversity in IIM compared to other GITs sites. Similar to SCB treatment, *Tyzzerella_4* was the most significant genus decreased (FDR = 6.02E-08) and *Fibrobacter* was the most significant genus increased (FDR = 3.09E-02) by LA treatment in IIM (Table 4.3). However, some genera were significantly (FDR ≤ 2.33E-03) changed only by LA treatment including *Phascolarctobacterium, Prevotella_9* and *Candidatus_Soleaferrea*. Little is known about the functions of *Phascolarctobacterium*, and *Candidatus_Soleaferrea* genera in calf's GIT but in human, *Phascolarctobacterium faecium* demonstrated a high colonization rate in the GIT[70]. In CoM, LA treatment also reduced *Turicibacter which* has been shown to possess putative immunomodulatory[71] and invasive properties and may cause subclinical infections in piglets[72].

In the post-weaning period, *Ruminococcus_2*, most significantly reduced by LA, has been shown to potentially associate with hyperinsulinaemia, intestinal permeability and hepatic inflammation in rats[73]. However, there is no information about the detrimental effects of this genus in calves.

In the pre-weaning period, the LA treatment had significant impact on KEGG pathways only in the RuD which is similar to the impact of SCB during this period. However, at the post-weaning period, LA did not have significant impact on these pathways in RuD, but significantly changed caffeine metabolism pathway in the CoM and five pathways

(cell cylce, EGFR tyrosine kinase inhibitor resistance, oxytocin signaling, mRNA surveillance and Fanconi anemia pathway) in IIM. Since these pathways were also significantly changed by SCB, we might assume similar potential mechanisms for SCB and LA in IIM.

The effects of antibiotics growth promoter on the bacteria community in the GIT system have been well documented. Several studies have shown that treatment with ATB altered the bacteria diversity[74,75] as well as the bacteria composition[75,76] in the GIT. The genera *Lactobacilli* and *C. perfringens* decreased in the ileum in broiler chickens fed low dose of avilamycin and salinomycin[77]. Meanwhile the abundance of lactobacilli particularly *L. gasseri*, was increased by tylosin in the ileum of pigs[78]. However, we observed less impact of ATB on the GIT bacteria community at the pre- and post-weaning periods in this study. ATB significantly changed the bacteria composition in IID and RuD only, at the pre-weaning period. Unlike SCB or LA, ATB had greatest impact on genera composition in the IID and RuD, since it significantly changed the abundance of 34 and 24 genera in these sites, respectively, at the pre-weaning period. *Desulfovibrio* and *Ruminiclostridium_9* were the most significantly decreased or increased genera, respectively, by ATB treatment in IID. Little is known about the roles of *Ruminiclostridium_9*in the GIT sites. Interestingly, no pathway was significantly changed by ATB treatment in IID and RuD at the pre-weaning period. Notably, ATB also reduced *Streptococcus* in the CoM and also significantly changed the abundance of steroid hormone biosynthesis pathways in the CoM at the pre-weaning period. In fact, *Streptococcus* was the top most DA general in all three treatments (LA, SCB and ATB) in the CoM. Some species of the *Streptococcus* genera are pathogenic such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*. However, *Streptococcus* was reduced in the treated samples with the largest reduction by SCB, followed by LA and ATB.

At the post-weaning period, *Sutterella* was DA by ATB. The genus *Sutterella* are commensals in the GIT with mild pro-inflammatory capacity in the human GIT[79].

At post-weaning, ATB impacted steroid biosynthesis pathway in the CoM but targeted three different pathways including RIG-I-like receptor signaling pathway, D-Arginine and D-ornithine metabolism and butanoate metabolism pathways in IIM. RIG-I-like receptor proteins including RIG-I, MDA5, and LGP2 are expressed in both immune and non-immune cells. Upon recognition of viral nucleic acids, RIG-I-like receptor proteins recruit specific intracellular adaptor proteins to initiate signaling pathways that lead to the synthesis of type I interferon and other inflammatory cytokines, which are important for eliminating viruses.

The results from direct comparison of DA genera between treatments confirmed that the GIT microbiota was more sensitive to treatments in the pre-weaning period compared to the post-weaning period since most genera were significantly DA in the pre-weaning period (Tables 4.5, 4.6 and S4.3). Moreover, fewer genera and sites were affected when comparing LA vs. ATB than the comparison between SCB vs. ATB. This suggests that there were more diverse impacts of SCB compared to other treatments. Notably, Tyzzerella_4 (potential pathogenic genera) was the most significant DA genera in the pre-weaning period in both comparisons (SCB vs. ATB and LA vs. ATB) (Tables 4.5 and 4.6) suggesting differences in mechanisms by which the antibiotics (ATB) and DFMs (SCB or LA) can

modulate pathogenic bacterial populations. Nevertheless, more studies are required to examine the distinct mechanisms by which DFMs impact the GIT of calves to enable development of effective DFMs.

The functional prediction analysis revealed more effects in the RuD contrary to data on diversity and abundance, which mostly influenced the ileum and colon. However, it is known that the level of abundance might not reflect the function of the bacteria and that roles played by the bacteria might be more important than abundance[80], thus our data should be interpreted with caution.

In summary, the current data showed that site and day had an effect on bacteria diversity. However, the effect of treatment on bacteria diversity was not significant for most sites even though an increase in diversity was observed in the colon. The bacterial composition of the GIT microbiota was altered due to supplementation with the two DFMs with most DA genera found in the ileum. Both DFM treatments reduced some pathogenic bacteria genera such as *Streptococcus or Tyzzerella_4*and increased the potential beneficial bacteria, *Fibrobacter*. Other potential beneficial bacteria including *Rumminococcaceae UCG 005, Roseburia* and *Olsenella* were increased by SCB treatment only. The functional prediction via pathways enrichment analyses indicated that besides affecting the local pathways such as cell cycle, bile secretion, proteasome or cAMP signaling pathway both DFMs also impacted other pathways such as thyroid hormone synthesis or dopaminergic synapse in the brain pathway. Moreover, these DFMs also shared some common mechanisms with ATB; however, they had more diverse target sites compared to the ATB which mainly targeted the colon microbiome. Although, this study indicates that DFM have site specific and age dependent effects on the calf gut microbiome, further system-omics related studies (meta-genomics, meta-transcriptomics, proteomics and metabolomics) are needed to better define the mechanisms related to these effects. Therefore, regional effects and age need to be taken into consideration when investigating the biological mechanisms by which DFMs affect the growth and development of calves at the early period of growth. Furthermore, the pre- and post-weaning samples were collected from different calves implying that some individual variation was expected to influence our results, thus our data should be cautiously interpreted.

4.7 Materials and Methods

4.7.1 Animal treatments and samplings

Animal management and use procedures were according to the Canadian Council on Animal Care[81] and were approved by the animal care and ethics committee of Agriculture and Agri-Food Canada. Animal management procedures have been described in details previously[30]. Briefly, thirty two calves (2–7 days old) were randomly allocated to four treatments as follows: (1) Control (CTRL)- calves bucket fed with milk replacer (Goliath XLR 27–16, La Coop, Montreal, QC, Canada) at 6 L/day (2 L three times a day) for the first 4 days, and at 9 L/day (4.5 L twice a day) from day 5 to the end of weaning (day 53))and starter feed (Shur-Gain—Meunerie Sawyerville Inc., Cookshire-Eaton QC, Canada) fed *ad libitum* from day 8 of the experiment; (2) CTRL supplemented with *Saccharomyces cerevisiae boulardii* CNCMI-1079 (SCB; 7.5×10^8 colony forming units (CFU)/L milk replacer + 3×10^9 CFU/kg starter

feed) (Levucell SB 20, Lallemand Animal Nutrition, Montreal, QC, Canada); (3) CTRL supplemented with *Lactobacillus acidophilus* BT1386 (LA; 2.5×10^8CFU/L milk replacer + 1×10^9 CFU/kg starter feed) (Micro-Cell FS, Lallemand Animal Nutrition) and (4) CTRL supplemented with antibiotics (ATB) chlortetracycline and neomycin (528 and 357 mg/L milk replacer, respectively) pre-weaning, and chlortetracycline (55 mg/kg starter feed) (Vetoquinol Inc., Lavaltrie, QC, Canada) post-weaning. Calves were housed in individual pens, fed individually and had *ad libitum* access to water. The animal trial lasted for 14 weeks (experiment day 1 to 96). Weaning was initiated on day 43 by reducing the quantity of milk replacer offered by half every day and it was completed on day 53 when animals were able to eat 1 kg of starter feed per day. Four calves per treatment were euthanized on day 33 (pre-weaning) and another set of four calves per treatment on day 96 (post-weaning) to collect digesta samples from the rumen, ileum and colon, and mucosal samples from the ileum and colon. The pre- and post-weaning samples were collected from different calves. Digesta samples were aseptically collected placed in sterile tubes followed by storage at −20 °C until DNA isolation. Mucosal scrapings from intestinal tissues (colon and ileum) were collected using the inoculum method as described previously[82] and stored at −80 °C until DNA isolation.

4.7.2 DNA isolation and quantification

Samples were thawed and kept on ice during the extraction process. The digesta were disrupted using a high-speed blender and mucosa samples as described above. DNA was isolated from the homogenate using the bead beating method with the ZR fecal DNA kit (Zymo Research Corp., Irvine, CA, USA) following manufacturer's instructions. The quantity and purity of isolated DNA was measured using spectrophotometry (Nano Drop Technologies, Wilmington, DE, USA) and diluted to a final concentration of 30 ng/µl.

4.7.3 Amplification of bacterial ribosomal DNA and sequencing

PCR primers targeting the 16S rRNA gene (V3–V4 region) were used to prepare amplicon libraries. Amplification of the 16S V3-V4 region was performed using sequence specific regions described previously[83] in a dual indexed PCR approach. Briefly, the following generic oligonucleotide sequences were used for amplification: Bakt_341F-long AATGATACGGCGACCACCGAGATCTACAC [index1] TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Bakt_805R-longCAAGCAGAAGACGGCATACGAGAT[index2] GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The PCR was carried out in a total volume of 50 µL that contains 1X Q5 buffer (NEB), 0.25 µM of each primer, 200 µM of each dNTPs, 1 U of Q5 High-Fidelity DNA polymerase and 1 µL of template cDNA. The PCR started with an initial denaturation at 98 °C for 30 s followed by 10 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 30 s, and 25 cycles of denaturation at 98 °C for 10 s, annealing at 65 °C for 10 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 2 min. The PCR reactions were purified using the Axygen PCR cleanup kit (Axygen). Quality of the purified PCR product was checked on a DNA7500 BioAnalyzer chip (Agilent) and quantified using a

Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). Barcoded Amplicons were pooled in equimolar concentrations and sequenced on the Illumina MiSeq (paired–end 300 bases with two index reads). Library preparation and sequencing was performed by L'Institut de Biologie Intégrative et des Systèmes (IBIS), de Université Laval, Quebec City, Canada.

4.7.4 Bioinformatics analysis

The downstream analysis of output fastq files was done using the pipeline of the open source software package QIIME[84]. Paired end reads were merged using FLASh[85]. Chimera detection was applied to the merged reads using Uchime[86]. The GOLD[87] database w as used for reference based detection. Taxomomic affiliation of the 16S data was studied using QIIME[84]. Demultiplexed and quality filtered sequences from pre-processing step were clustered into OTUs using VSEARCH[88]. An OTU (Operational Taxonomic Unit) was formed based on sequence identity with threshold defined at 0.97. After the clustering step, a representative sequence was picked for each OTU and a taxonomic identity was assigned to each representative sequence. The 16S database used was Greengenes while Uclust[86] was used for taxonomic assignment. Multiple alignments of the representative OTU sequences were generated with PyNAST[89], which aligns the sequences to 16S reference sequences. The relationship between sequences was studied by generating a phylogenetic tree with FastTree[90] followed by computing UniFrac distances. A rarefaction curve for each sample was plotted (observed OTUs metric) in order to estimate the depth of sequencing for each sample and to choose the rarefaction threshold for all samples. Results were generated after the cumulative sum scaling (CSS)[91] normalization method. The Amplicon-Seq pipeline provides taxonomic affiliation of data at different levels (Kingdom, Phylum, Class, Order, Family and Genus).

4.7.5 Assessment of diversity and statistical analysis

Samples were rarefied for alpha-diversity calculations and rarefaction curves generated (Fig. S4.1) in order to eliminate the bias caused by the different sample sizes[92]. The OTU table was rarefied across samples to the lowest sample depth using QIIME based on the Messene Twister pseudorandom number generator. Alpha diversity estimators including Chao1, observed OTUs, Shannon, Simpson and Inverted Simpson (Invsimpson) were calculated for the overall bacterial community using Phyloseq[93]. Mean alpha diversity estimates for each site, day, treatment and treatment by site by day were compared using the two-sided t-test in R program[94.]

The dataset was also subsampled to the minimum[95] to compare microbial composition between samples (β-diversity). Beta-diversity was measured by calculating the weighted and unweighted UniFrac distances[96] using Phyloseq default scripts. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots. Permutational multivariate analysis of variance (PERMONOVA[97]) was used to calculate P-values and to test differences of β-diversity among treatment groups for significance.

4.7.6 Bacterial Community Composition and differential relative abundance analyses

To investigate the relative abundance of the different genera, The MicrobiomeAnalyst[98] was used to obtain the most prevalent bacteria genera within each site.

To investigate the effect of treatment on the different genera, we did a pair wise comparison between each treatment and control, GIT site and day (33 and 96). Different abundance at genus level was compared between treatments and control as well as among treatments using the Wald Test method of DESeq2[99]. The samples with OTU total count <10,000 were removed. The normalization step was done for each pair of comparison separately[100] and taxa were considered significantly differentially abundant if p-corrected for false discovery rate (FDR) was <0.05. The FDR procedure is performed to reduce the type I error. In brief, this procedure includes the following steps: (1) uncorrected p-values are sorted in ascending order, (2) ranks to the p-values are assigned, (3) individual Benjamini-Hochberg critical p-values were calculated using the formula (i/m) q (i = the individual's p-value rank, m = total number of tests, q = the false discovery rate). In this analysis, a q-value (FDR) of ≤0.05 was considered significant

4.4.7 Functional prediction and differential analysis of predicted pathways

The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)[101] software was used for the prediction of functional genes of the classified members of the GIT microbiota resulting from reference-based OTU picking against Greengenes database. Predicted genes were then hierarchically clustered and categorized under Kyoto Encyclopedia of Genes and Genomes[102]orthologs (KOs). Predicted KOs was then converted into their associated pathways. The differential analyses of predicted pathways were done in DeSeq. 2 and only pathways predicted for at least 5 samples were used as input data. The pathways were considered significantly differentially predicted if p was <0.05. Since the enrichment relied on human data, we used a relaxed threshold (uncorrected p-values) to get a better overview of the impact of treatments on pathways.

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4.10 Author Contributions

EMI-A conceived and designed the experiments with inputs from G.T., J.C., N.B., M.L. and Y.P.C. B.E.F. performed the experiments; B.E.F. and D.N.D. analyzed the data; B.E.F., D.N.D., G.T. and EMI-A interpreted the data, B.E.F. drafted the manuscript with input from D.N.D. all authors revised and approved the final draft.

4.11 Additional Information

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4.12 Supplementary figure and tables

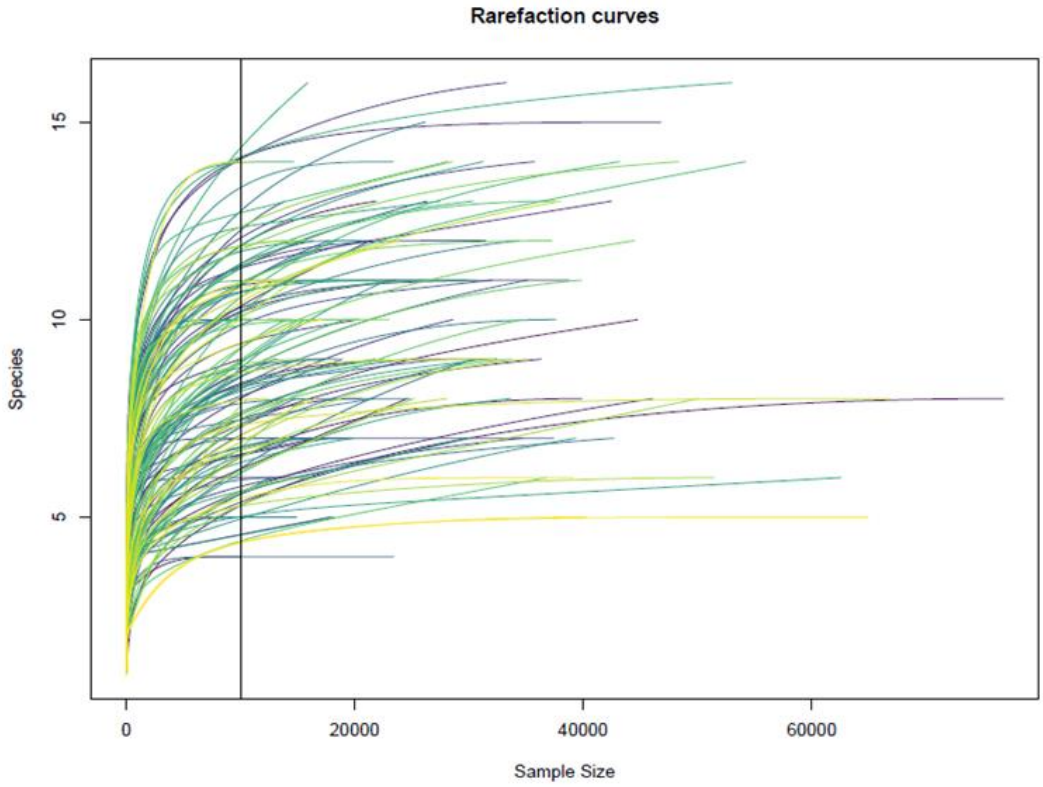

Figure S4.1. Rarefaction curves. Rarefaction curves (rarefied to the shallowest level for all our sequences and plots of estimation of observed OTU against sequencing efforts). The y-axis shows the number of OTUs detected, and the x-axis the number of taxa in the sequence subset analyzed

Table S4.1: The operational taxonomic units (OTU) present in the samples; from phylum to genus levels
Table S4.2: The dominant genera in the different gastrointestinal sites, Differential abundance at the phylum and genus level comparing sites, Top 20 genera differentially abundant between day 33(pre weaning) and day 96 (post weaning) periods
CoM = colon mucosa, CoD = colon digesta, IM = ileum mucosa, IID = ileum digesta, RuD = rumen digesta.
Table S4.3: The Differential abundant comparison between LA and SCB
Table S4.4: Kegg orthologies and pathways prediction for all samples

Supplementary tables S4.1, S4.2, and S4.4 are too large and are avilable at https://doi.org/10.1038/s41598-018-32375-5

Conclusions

General conclusion

The widespread use of AGP in food animal production has led to antimicrobial resistance in animals and subsequently humans (Marshall & Levy 2011). There have been incidences of contamination by antimicrobial resistance bacteria in the food production chain, and in animal products that have even led to deads (Founou *et al.* 2016) . Consequently, there is growing public disquiet over the use of AGP in food animal production and the associated health problems. Hence, there is need for alternatives with beneficial effects to maintain and improve animal health and productivity. The use of DFM like *Saccharomyces cerevisiae boulardii* (SCB) and *Lactobacillus acidophilus* BT1386 (LA) in animal production has beneficial effects.

The objectives of this thesis were to: (1) Investigate the effect ofLA or SCBon blood cellular and biochemical/chemical constituents. (2) Determine the potential mechanisms of enhanced immune response by LA and SCB. (3) Determine how LA or SCB modulate calf GIT microbial community composition by next-generation sequencing of the V3-V4 region of the 16S rRNA gene. (4) Compare the efficacy of these two DFM with tetracycline-neomycin, an antibiotic growth promoter.

The DFM (SCB and LA) remained viable in the commercial product throughout the period of the experiment (98 days). Viable SCB was recovered throughout the GIT and in the feces pre- and post-weaning (Fomenky *et al.* 2017). The properties of a DFM that determine its efficacy include surviving the passage to its target organ (colon), surviving the gastric acid and bile in the GIT (Kelesidis & Pothoulakis 2012). Similarly, other authors have also shown surviving passage of SCB to the colon in the GIT (Gorbach 2000; Graff *et al.* 2008).There was no effect of DFM on total lactobacilli population in the GIT tissues (sampled twice only) of calves. However, feces (collected at weekly intervals throughout the period of the experiment) of SCB-treated calves showed a greater lactobacilli population compared with CTL ($P < 0.01$) around weaning. In the pre-weaning period, the distribution of lactobacilli population differed along the digestive tract (colon>ileum content>rumen> ileum mucosa; $P < 0.001$). The lactobacilli population was significantly reduced in all compartments ($P = 0.02$) post-weaning compared to pre-weaning, except in the rumen. SCB improved the growth of total lactobacilli in the gut around weaning.

The environment for the growth of beneficial intestinal microbiota can be established by SCB and LA which creates an additional protection to the host mucus layer (Bischoff *et al.* 2014). Crypt depth and width of the colon decreased ($P < 0.01$) whereas the number of goblet cells containing neutral mucins tended to increase ($P=0.058$) while acidic mucins increased ($P < 0.05$) in SCB- and ATB-treated calves compared with CTL and LA-treated calves. The direct-fed yeast cells produced a prebiotic effect also via compounds such as oligosaccharides, amino acids, vitamins, and organic acids found within the yeast cells, which together can stimulate microbial communities to propagate in the GIT (Opsi *et al.* 2011). Currently, there are indications that

DFMs impact health and thus productivity in calf management by influencing the gastrointestinal microflora community in a beneficial way (Lee *et al.* 2010). Overall, growth performances were not affected by treatment. The DFM inclusion level has a significant effect on growth responses and even on the microflora composition (Mountzouris *et al.* 2010). The unimprovements in growth performance in calves in this study might be due to inadequate doses (inclusion levels) of the DFM used in the present study and the overall clean pen environment. It has been shown that there was improved growth performance after feeding lactic acid bacterial inoculum of bovine origin composed of Lactobacillus casei DSPV 318T, Lactobacillus salivarius DSPV 315T and Pediococcus acidilactici DSPV 006T to calves managed under stressful conditions of inadequate housing and unhygienic environment (Frizzo et al., 2010; Krause et al., 2010).

Tumour necrosis factor α decreased (P<0.05) on d 66 (post-weaning) for the ATB-treated calves. There were no treatment effects on production of interferon-γ and interleukin-6 proteins as well as on the expression of *TLR4, TLR6, TLR9, TLR10, CLDN3, MUC1* and *MUC 20* genes. Calves fed SCB or LA had a greater (P<0.05) oxidative burst at weaning (d 53) compared with CTL. Oxidative burst was also greater (P<0.05) after weaning (d 59 and d 87) for SCB-fed calves. Calves fed SCB and ATB had higher (P<0.05) phagocytosis activity during weaning (d 47) compared to CTRL. Similar to our study there was an increase in phagocytic activity of neutrophils when cells were incubated with pathogenic *E. coli*. (Magalhães et al.2008). The impact of SCB in preventing infectious pathogens is probably due to direct influence of *S. boulardii* on the modulation of the gut microbes (More & Swidsinski 2015). We showed that LA produces reactive oxygen species as measured by the oxidative burst capacity of neutrophils during weaning in our study (Fomenky *et al.* 2018). *Lactobacillus acidophilus* is known to limit the activity of some pathogenic bacteria by producing hydrogen peroxide (Pridmore *et al*. 2008). Lactobacillus species produces bio surfactants whose wide range of antimicrobial activities against bacterial pathogens as well as their anti-adherent properties impairs the adhesion of pathogens onto gastrointestinal wall membranes (Kanmani *et al.* 2013). In the yeast cell wall, there are many polysaccharides such as β-glucans and mannan-oligosaccharides identified as modulators of immunity (Jensen *et al.* 2008). The concentration of serum amyloid A (SAA2) increased (P<0.05) in SCB- and LA-fed calves (d 53), while the concentration of C-reactive protein (CRP) increased (P<0.05) in SCB-fed calves during weaning as compared to CTL. The beneficial effects of *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* has been more evident in calves under stressful conditions or exposed to pathogens (Alugongo *et al.* 2017). In this study, immune-enhancing effects were seen during weaning for both LA and SCB (Fomenky et al 2018).

The SCB and LA treatments had less impact on the bacteria diversity but had significant impact on the abundance of the bacteria community. However, most changes associated with treatment occured in the ileum. *Saccharomyces cerevisiae boulardii* has been shown to either reduce or increase the abundance of different bacteria genera with a dynamic shift in composition of the GIT (Gonçalves & Gallardo-Escárate 2017). The SCB and LA treatments significantly reduced some potential pathogenic bacteria genera (*Streptococcus* and

Tyzzerella_4) and increased potential beneficial bacteria (*Fibrobacter).* Other potential beneficial bacteria including *Rumminococcaceae UCG 005 Roseburia* and *Olsenella* were increased by SCB treatment only. Furthermore, the potential pathogenic bacterium *Peptoclostridium* was reduced by SCB only while LA reduced *Ruminococcus*_2. Direct fed microbials like LA and SCB are able to manipulate the intestinal microbial communities by suppressing the growth of pathogens and inducing the production of β-defensin and induce mucin production by the host (Hemarajata & Versalovic 2013**)**. It has been observed that LA can also influence the commensal microorganisms through the production of lactic acid and bacteriocins that inhibit the growth of pathogens and alter the ecological balance of the commensals in the GIT (Kailasapathy & Chin 2000).

The functional prediction analysis suggested that both DFM impacted pathways such as cell cycle, bile secretion, proteasome, cAMP signaling pathway thyroid hormone synthesis pathway and dopaminergic synapse pathway. The bacterial metabolites produced by the microbiota affect the functioning of the host, and the host–microbiome interactions involve the host detecting these bacteria metabolites (Kohl & Carey 2016**).** The main metabolite from bacteria known to have an influence on the functioning of the host are the short chain fatty acids; acetate, propionate and butyrate. However, other metabolites such as bile acids, amino acid derivatives and vitamins are also made by microorganisms in the GIT which all affect the functioning of the host (Kohl & Carey 2016**).** The bacterial metabolites also make available fuel acting as signaling molecules within the GIT and other sites in the host (Koh *et al.* 2016). Bile acids are also important signaling molecules that activate nuclear receptors and regulate their own synthesis and transport rates (Chiang 2013). Bile secretion is one of the major substrates for growth used by gut bacteria to produce short chain fatty acids (acetate, propionate, butyrate) accounting for a proportion (10%) of total caloric intake per day (Donohoe *et al.* 2011). Bile acids appear to be a major regulator of the gut microbiota; and bacterial dysbiosis characterized by significant reduction in *Ruminococcaceae* (Bajaj *et al.* 2014) has been related to low bile acid levels in the intestine (Kakiyama *et al.* 2013). Bile acids have been shown to have direct and indirect (through FXR-induced antimicrobial peptides) antimicrobial effects on gut microbes (Begley *et al.* 2005).The bacteria in the GIT seemed to interconnect with the gut–brain axis in this study. This might be through the production of neuroactive and neuroendocrine molecules such as serotonin, gamma-aminobutyric acid (GABA), histamine, noradrenaline and adrenaline (Bienenstock *et al.* 2010). In addition to our study, it has also been shown that consumption of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse (Bravo *et al.* 2011). From the GIT microbiota moving to the brain, there is possible production, expression and turnover of neurotransmitters such as GABA, serotonin and neurotrophic factors which modify enteric sensory neurons and bacterial metabolite activities thereby affecting the mucosal immune system (Carabotti *et al.* 2015). Meanwhile from the brain to the GIT microbiota there are alterations of the mucus and biofilms, mortality, intestinal permeability and alteration of the immune function (Carabotti *et al.* 2015). Compared to the DFM, ATB had similar impact on bacterial diversity in all GIT sites but had more impact on the bacteria composition in the ileum.

This study suggests that SCB would deserve more attention as a modulator of the gastrointestinal health in dairy calves. An extensive study with more animals and longer duration could confirm the role of SCB on animal performance. Feeding SCB enhanced the innate immune response (both oxidative burst and phagocytosis) and increased the concentrations of APP (CRP and SAA2) at weaning in calves. Similarly, LA enhanced SAA2 and oxidative burst at weaning. SCB could stimulate acute phase response, and might serve to prime the immune system prior to infection leading to an enhanced innate immune response (oxidative burst capacity and phagocytosis) in calves especially at periods of stress (e.g., weaning). Therefore, SCB might have the potential to strengthen calf immune system in the critical periods of disease susceptibility. Direct fed microbials have site specific and age dependent effects on the calf gut microbiota. Therefore, regional effects and age need to be taken into consideration when investigating the biological mechanisms by which DFM affect the growth and development of calves at the early period of growth.

The microbiome of the GIT is essential to animal nutrition and is important to animal health and development (Yeoman & White 2014).The study of microbiota-host relationships are paramount to improve our understanding of the effects of nutrition on animal health and performance. However, the key to microbiome studies will be to identify the key microbial elements in an ecological niche.

Implication

This study presents knowledge that can be used to improve health and increase animal production. The association of the microbiota and health and animal production has important consequences for the industry. This kind of study improves our understanding of the probiotic potential for altering intestinal health and opens the door to the study of microbiota interactions and certain nutritional therapies that could alter intestinal health and production performance.

The study has advanced knowledge of the biochemical mechanisms in the host (dairy calf) following supplemental feeding with probiotics. The process of immune defense and the maturation of the animal immunity, modulation of the microbiota and metabolism have been elucidated. An impressive range of areas of studies was implicated in this thesis and this subject is very important for the cattle industry.

Limitations

Our study demonstrated the importance of the microbiota in calf GIT. However, a complete and better understanding of the molecules and mechanisms driving the roles played by the microbiota is needed. Such information can be exploited to improve health and increase animal production. The complexity of the microbiome study will require an integrative multi'omics systems approach that will capture the microbial dynamics and host metrics on animal performance.

Study perspectives

In order to fully understand the importance of the gastrointestinal microbiome, an integrated approach encompassing metagenomics, metatranscriptomics, metaproteomics and metabolomics, etc. is necessary.

Metagenomics is the study of all microbes in a particular niche like the GIT. The 16sRNA studies could only allow the study of bacteria. We need to study the other microbes like protozoa, archea etc. to fully understand their roles. In addition, metagenomics will allow the study of the fuctions of the different microbes.

Metabolomics is the study of the metabolites in a specific ecological niche (e.g. GIT). All the microbes in the GIT produce metabolites therefore, it is important to study these products inorder to fully understand the functions of the different microbes in the GIT.

Metatranscriptomics is the study of the interactions between the different microbes in a specific ecological niche (e.g. GIT) and the host. Metatranscriptomics analysis will uncover the different pathways in which microbes interact with the host.

Metaproteomics is the study of all proteins from the microbiome the protein profile expression.

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