Robustness and resilience of gut microbiota in ruminants in relation to feeding and nutritional management

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TANG THUY MINH

Graduate School of Environmental and Life Science

(Doctor's Course)

OKAYAMA UNIVERSITY

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DECLARATION

I hereby declare that my thesis/dissertation entitled:

"Robustness and resilience of gut microbiota in ruminants in relation to feeding and nutritional management"

has been composed by myself, that the work contained here in is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified.

Parts of this work have been published in Journal of Applied Microbiology and Biotechnology (2017).

Date

Signature

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CHAPTER 1
GENERAL INTRODUCTION

Gastrointestinal tract of animal is the natural habitat for a dense and dynamic microbial community. Ruminants and their diverse community of symbiotic microbiota is a typical example. This microbiota is in charge of digesting and fermenting plant materials into nutrient sources usable by the host. The ruminants acquire the fermentation products for body maintenance, growth, and milk production.

During early life, major changes take place in the composition of gut microbiota. The early and rapid colonization helps protect from pathogen invasion but the climax population establish for several years. In adults, the composition of this microbiota across the digestive tract is determined by a gradient of oxygen from food and water consumption and a gradient from tissue into the lumen. In summary, establishment of gut microbiota is a complex process which is influenced by microbes-host interactions and by several external and internal factors. The important extrinsic factors are bacteria of the environment, composition of maternal microbiota, diet, mode of delivery and medication, and the host-related factors like anatomical development of intestinal tract, peristalsis, bile acids, intestinal pH and immune systemic responses, microbial interactions and mucosal receptors.

Although several studies have been conducted for understanding the gut microbiota of ruminants, several questions remain. A substantial part of this resident microbiota is still to be discovered. Theoretically, this population has been contributed by digesta-associated and mucosa-associated microbiota. While mucosa-associated microbiota would be crucial for immunological priming, digesta-associated would be important for nutrient digestion. Rather than mucosa-associated microbiota, digesta-associated microbiota has intensively been studied targeting to rumen and feces microbiota, due to the easy and convenient sampling. Despite reports indicating that the core bacterial community is shared, other studies have observed high variation in the relative microbial abundance across samples. The divergence of abundance was suggested to be due to differences in genotype, aging, diet formulation, farm management, and geographical locations, food-borne microbiota. Similarly, differences were also observed in gut microbiota structure even when animals were kept under the same condition and showed the similar physiological profiles. In additon, recent studies have paid attention to individual intra-variation of gut microbiota in different segments across the gastrointestinal tract. Reports indicated that gut microbiota varied across the tract in abundance as well as in microbial composition. However, understanding about this fluctuation of gut microbiota is still limited.

Because microbial activity and metabolism in the gut is a key to secure health and productivity of ruminants, understanding robustness and resilience of the gut microbiota is of great importance. In this thesis, three experiments were carried out to examine variability, stability, and adaptability of gut microbiota in relation to feeding and nutritional management. Feces were used to determine the gut microbiota in heifer and dairy cow experiments, and digesta and mucosa samples collected from various gut segments were used in goat experiment. Silage, a rich source of lactic acid bacteria, was given to animals in the entire or a part of test period; hence, the effect of food-borne bacteria on the gut microbiota was also examined.

In the first experiment, fecal microbiota of seven Holstein calves were examined during growing stage, wherein weaning and commencement of silage feeding were practiced. The objective was to understand how gut microbial community changed over the time and if weaning and silage feeding had influence on gut microbiota during growing stage.

In the second experiment, microbiota of whole crop corn silage and feces of 18 silage-fed lactating Holstein cows were examined in Japan and China. The objective was to understand the variation and similarity of the fecal microbiota of dairy cows with regards to individuals, farms, and countries. And, we also examined if silage-associated microbiota affected the gut microbiota of lactating cows.

In the third experiment, gut microbiota was characterized across the gastrointestinal tract for both digesta and mucosa-associated microbiota using goat model. The *Lactobacillus* community was also examined to understand how this community inhabited from feed (silage) throughout different segments along the gastrointestinal tract.

CHAPTER 2
LITERATURE REVIEW

2.1. The role of gut bacteria

Gut bacteria have main functions including metabolic activities for rescue of energy and nutrients, trophic effects on intestinal epithelia, development of immune system and protection the host from invasion of foreign microbes (Guarner et al. 2003). Gut bacteria harbor different dominant bacterial phyla regarding host species. For human, the most popular phyla are *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*; for cattle, those are *Firmicutes*, *Bacteroides*, *Proteobacteria*, *Euryarchaeota* (Eckburg et al. 2005; Hogenova et al. 2011).

2.1.1. Metabolic function

The critical metabolic function of gut bacteria is related to the fermented process of non-digestible substances (exogenous substrates that are provided by food and endogenous substrates synthesized by host) and converting into chemical compounds, which subsequently are absorbed and digested by host animals (Guarner et al. 2003; Rambaud et al. 2006).

2.1.1.1. Carbohydrate metabolism

Non-digestible carbohydrates like resistant starch, cellulose, hemicelluloses, pectin, unabsorbed sugars and alcohols are converted into short chain fatty acids (mainly acetate, propionate and butyrate) and gases by the interaction of different kinds of bacteria. These fatty acids have an important contribution to host physiology. Butyrate which is the main source of energy for colonocytes is consumed by colonic epithelium while acetate and propionate play a role in modulation of glucose metabolism. These fermenting activities of bacteria are supposed to recover energy and nutrients for host as well as energy and nutrients supplying for their own growth and multiplication (Rambaud et al. 2006). Taking part in these processes, it's necessarily mentioned to amylolytic species (*Bacteroides*), cellulolytic species (*Ruminococcus*, *Clostridium*, *Eubacterium*, *Enterococcus* and *Propionibacterium*), xylanolytic species (*Clostridium*, *Butyrivibrio* and *Bacteroides*), pectinolytic species (*Bacteroides*, *Bifidiobacterium*, *Eubacterium* and *Clostridium*). In addition, many endogenous substrates that secreted by the host animal (mucopolysaccharides, mucins) were degraded by *Bacteroides*, *Bifidobacterium*, *Ruminococcus* based such enzymes as sialidase, specific α-glucosidase, β-D-galactosidase (Rambaud et al. 2006).

2.1.1.2. Protein metabolism

Food proteins are hydrolysed to peptides and amino acids by gut bacteria proteases consisting of serine-, cystine- and metallo-proteases. Abundance of bacterial species which has proteolytic activity belongs to genera *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium*, *Streptococcus*, and *Lactobacillus*. Especially in ruminant, hydrolysed amino acids are degraded further to create organic acids, ammonia and carbon dioxide. The rumen bacteria used the resulted ammonia and some small peptides, free amino acids for synthesis of microbial proteins. Once these organisms travel through to abomasum and intestine, then, microbial proteins are digested and absorbed. The formation of microbial protein implicated an important point that bacteria can synthesize essential and non – essential amino acids which adapt to the host's protein requirements (McDonald et al. 1995; Rambaud et al. 2006).

2.1.1.3. Vitamin synthesis

Gut bacteria also contribute partly to the vitamin synthesis. In ruminant, all the members of vitamin B complex and vitamin K are synthesized by rumen microorganisms. Vitamin B synthesis depends on the amounts of B vitamins supplied by feeds. This process enhances when feed-derived vitamin B is small, and conversely, decreases when vitamin B supply is relatively adequate. Therefore, in adult ruminant, these vitamins are independent of dietary source (McDonald et al. 1995).

2.1.2. Trophic function

2.1.2.1. Epithelial cell growth and differentiation

The role of gut bacteria on colonic physiology is based on their production of short chain fatty acids. A study of Alam et al. (1994) showed that the rate of crypt cells production decreased in colon of rats that were bred in germ-free environment than in conventional environment. It's implicated that commensal bacteria influence cell multiplication in the colon (Alam et al. 1994). The differentiation of epithelial cells is affected by resident bacterial interactions (Hopper et al. 2001; Guarner et al. 2003). Among fatty acids that stimulate cell proliferation and differentiation, butyrate inhibits cell proliferation but stimulates cell differentiation in neoplastic organ cell, and it also supports reversion from neoplastic to non-neoplastic phenotypes (Siavoshian et al. 2000; Guarner et al. 2003). Another study of Hooper et al. (1999) indicated that the *Bacteroides thetaiaotamicron* can secret a signal molecule stimulating the expression of fucose used as a nutrient resource by bacteria (Hooper et al. 1999; Callaway et al. 2012). The presence of *B. thetaiotamicron* is related to fucose expression among epithelial cells (Callaway et al. 2012).

2.1.2.2. Interactions between gut bacteria and host immunity

The communication between gut bacteria and host at mucosal interface plays a role in development of immune system. Microbial colonization is considered to affect the composition of gut-associated lymphoid tissue. Components of gut bacteria have an important role in the postnatal immune system development. In the early post natal period, a transient physiological inflammatory response with expansion of the mucosal-associated lymphatic tissue was induced by components of gut bacteria (Hrncir et al. 2008; Hogenova et al. 2011). Early exposure to microbes induces expansion and development of immune cells and tissues; and diversity of microorganisms partially influence subsequent ability of immune system to respond to allergens and infection (Bjorksten et al. 2001; Callaway et al. 2012). Reports showed that bacterial colonization of animal bred in a germ-free environment indicated an enhancement in the production of specific antibodies, a high density of lymphoid cells in gut mucosa and an increase in circulating concentration of immunoglobulin in the blood (Butler et al. 2000; Guarner et al. 2003; Tannock et al. 2001; Stepankova et al. 1998; Hogenova et al. 2011). Hrncir et al. (2008) reported that the development of regulatory T lymphocytes depends on the presence of the dietary bacterial components. Germ-free mice fed with small amount of lipopolysaccharide had fewer T lymphocytes (Hrncir et al. 2008). Interestingly, germ-free mice's bacterial colonization also stimulates the biochemical maturation of enterocytes, creating an alternation of brush-border enzymes close to those in conventional mice (Stepankova et al. 1998; Hogenova et al. 2011).

2.1.3. Protective function

Resident bacteria can be considered as an essential line of resistance to exogenous microbes' colonization, then, greatly relevant in prevention of pathogens. When the bacteria have an optimal composition, adherent non-pathogenic bacteria can compete for attachment sites in brush-border of intestinal cells in order to prevent attachment and invasion of the pathogenic microorganisms into epithelial cells and the circulation. As well, bacteria compete for nutrient availability and maintain their habitat by administering and consuming all resources. This symbiotic relationship between host-bacteria prevents undesirable overproduction of nutrient, which attract potentially pathogenic competitors. Finally, bacteria can inhibit the growth of their competitors by secreting antimicrobial substances – bacteriocins (Bernet et al. 1994; Hooper et al. 1999; Lievin et al. 2000; Guarner et al. 2003; Hogenova et al. 2011).

2.2. Temporal colonization of gut bacteria communities

During early life, major changes take place in the composition of gut bacteria. The fetal intestine is sterile and soaked in amniotic fluid. At birth, the intestine is still sterile. Following

delivery, multiple antigens challenge intestine of newborn. Within a few hours, bacteria appear in the feces. Facultative aerobes rapidly colonize the intestinal tract, gradually, consumption of oxygen leads the intestinal environment into a reduced one, allowing strict anaerobes to sequentially colonize it (Bezirtzoglou et al. 1997; Farano et al. 2003; Penders et al. 2006; Dominguez-Bello et al. 2011; Koenig et al. 2011; Callaway et al. 2012).

The climax intestinal bacterial composition is attained in successive stages. During passage through vagina new-born is exposed to maternal vaginal flora. According to studies, vaginal microbiota of mother and of the external ear canal of newborn showed the close qualitative and quantitative association; similar microbes were determined in 85% of mother–newborn pairs (Mandar et al. 1996; Farano et al. 2003). However, vaginal microbes usually do not settle in intestinal tract. The maternal intestinal flora is actually the source of bacteria for newborn. But in fact, some neonates acquire bacteria from environment rather than from mother (Farano et al. 2003).

The early and rapid colonization helps protect from pathogen invasion but the climax population establish for several years (Marques et al. 2010; Callaway et al. 2012). In adults, the composition of bacteria along digestive tract is determined by a gradient of oxygen from food and water consumption and a gradient from tissue into the lumen (Wilkinson et al. 2012; Callaway et al. 2012). In summary, establishment of gut bacteria is a complex process which is influenced by microbes-host interactions and by external and internal factors. The important extrinsic factors are bacteria of the environment, composition of maternal microbiota, diet, mode of delivery and medication, and the host-related factors like anatomical development of intestinal tract, peristalsis, bile acids, intestinal pH and immune systemic responses, microbial interactions and mucosal receptors (Mackie et al. 1999; Farano et al. 2003). The stability of intestinal microbiota changes throughout life (Spor et al. 2011; Claesson et al. 2011; Callaway et al. 2012).

Recently, several studies have been carried out to investigate the composition of bacterial communities in newborn calves and their changes with growth stages throughout the animal's life. Rumen and feces samples have been employed in this approaching (Jami et al. 2013; Li et al. 2012; Rey et al. 2013; Klein-Jobstl et al. 2014; Oikonomou et al. 2013).

In ruminal bacteria of calves, the dominant phyla found in all age groups were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Jami et al. 2013). And, Rey et al. (2013) reported that three phyla *Proteobacteria*, *Fusobacteria* and *Bacteroidetes* changed significantly over time. At 2 days of age, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were the dominant phyla and accounted for 70.4, 13.9 and 10.8% of total sequences, respectively (Fig. 2.1.). Jami et al. (2013) characterized that phylum *Firmicutes* in the samples taken from the 1–3-day-old-calves was abundant compared with the other groups, with the vast majority of the reads belonging to the genus *Streptococcus*.

This phylum decreased in the 2-month-old-calves and significantly increased again in the other older age groups. Phylum *Bacteroidetes* was significantly lower in samples taken from the 1–3-day-old-calves compared with all other groups, but became the most abundant phylum in samples from older animals. The genus composition of this phylum also changed considerably between the age groups: in the newborn animals (1- and 3-day-old) it was mainly composed of the genus *Bacteroides*, whereas in the older age groups, it was almost exclusively composed of *Prevotella*. It indicated that the changes occurred in rumen ecosystem after birth, reflected by a decline in aerobic and facultative anaerobic taxa and an increase in anaerobic ones (Jami et al. 2013). In a report of Rey et al. (2013), from 3 days of age, *Proteobacteria* relative abundance fell rapidly, exhibiting the lowest values between 3 and 15 days, while the *Bacteroidetes* phylum became dominant (56.3%). *Bacteroides* became prominent genus, others were *Prevotella*, *Actinobacillus*, *Fusobacterium*, and *Streptococcus* (Rey et al. 2013). Several specific functional bacterial species like *Ruminococcus flavefaciens*, *Ruminococcus albus* could already be found in the first days of life in the pre-functioning rumen. This proved that establishment in the rumen of bacterial species that are important for its proper function in adult animals begins on the first day of life, when animals are still being fed exclusively colostrum, before the intake of plant material (Jami et al. 2013). Ruminal function can be covered by different microbiota with quite different phylogenetic composition (Li et al. 2012). Hence, it is difficult to find relationships between bacterial community and ruminal functions or fermentation parameters (Rey et al. 2013).

When calves began to eat concentrate, a fall in *Bacteroides* abundance (from 16.9% to 7.1% after one day) and an increase in *Succinivibrio* (from 0.2% to 2.1%). *Succinivibrio* was found positively correlated with concentrate intake. As the concentrate intake progressively increased, several dominant genera declined or no longer detected, including *Granulicatella*, *Actinomyces*, *Bacteroides*, *Streptococcus*, *Gallibacterium*, *Fusobacterium* and *Pelistega*. The *Proteobacteria* phylum increased to 27.6%. *Prevotella* genus and *Coriobacterineae* family increased to achieve 41.5 and 2.9% relative abundances, respectively (Fig. 2.2). At the age of 1 month, calves ate more and more starter concentrate and started eating some hay leading to a significant increase in ruminal pH, cause disruption in the bacterial community: a selection phase of bacterial community would be set up with bacterial taxa that were more specific and adapted to these new substrates. Several genera belonging mainly to *Proteobacteria* and *Firmicutes* phyla, were no longer detected. At the same time, *Dialister* and *Syntrophococcus* increased to 1.2 and 0.6% relative abundances, respectively (Rey et al. 2013). However, in study of Jami et al. (2013), even though animals from the 6-month and 2-year-old groups received the same diet, their microbiota were still significantly different, which might indicate that at 6 months of age, the rumen microbiota undergoes

developmental changes that are independent of diet. Also, the study highlighted the mature rumen microbiota are more diverse, more specific, and more homogenous than that of primary microbiota which allows form more heterogeneity between different animals (Jami et al. 2013).

Phyla	Age (days)																			PRE	Age significance
	2	3	4	5	6	7	8	9	10	12	15	19	22	26	29	33	36	40	43	50	62	83	
Proteobacteria	70·4 ± 8·1		16·9 ± 3·1									27·6 ± 7·9											0·32	***
Bacteroidetes	13·9 ± 8·5		56·3 ± 5·8																				0·20	**
Firmicutes	13·9 ± 2·2																						-	NS
Actinobacteria	4·9 ± 1·4																						-	NS
Fusobacteria	4·7 ± 3·9											0·2 ± 0·4											0·23	**
Spirochaetes	0·4 ± 0·3																						-	NS
Fibrobacteres	0·3 ± 0·3																						-	NS

Phyla relative abundance	< 2 %	2 - 5 %	5 - 10 %	10 - 20 %	20 - 50 %	> 50 %
Composition of intake	colostrum	97% milk replacer to 5%				

Figure 2.1. Age classification of calf ruminal samples according to phyla relative abundance determined with TREE package of SYSTAT from birth to weaning, values expressed as mean percentages ± standard deviation of calf means. PRE indicates proportion of variability explained by division into age-classes. Significance of age in the repeated measurements analysis is referred as ***P ≤ 0.001, **P ≤ 0.01, NS for P > 0.05 (Rey et al. 2013).

In fecal microbiota of calves from birth until weaning, the predominant phyla were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. However, relative abundance was different across studies. Oikonomou et al. (2013) reported that *Firmicutes* was the major phylum in dairy calves, showing a prevalence that ranged from 63.84% to 81.90%, followed by *Bacteroidetes* (8.36% to 23.93%), *Proteobacteria* (3.72% to 9.75%), *Fusobacteria* (0.76% to 5.67%), and *Actinobacteria* (1.02% to 2.35%). Klein-Jobstl et al. (2014) characterized *Bacteroidetes* (69.3%), *Proteobacteria* (15.7%), and *Firmicutes* (14.8%), accounting for 99.8% of all reads in case of Simmental calves (Fig. 2.3). The diversity of fecal microbiota was found increased with advanced age and solid feed consumption (Oikonomou et al. 2013; Klein-Jobstl et al. 2014). In dairy calves, the abundance of *Firmicutes* increased from the first to the fourth week of life and then progressively decreased. A reverse pattern was observed for *Bacteroidetes* prevalence. It is possible that these changes reflect the gradual adaptation of the calf gastrointestinal tract first to milk consumption and later to consumption of solid feed. An observation made at the genus level supports this assumption. The prevalence of *Lactobacillus* spp. (known to be related to digestion of milk) reached a 14.74% maximum during the fourth week of calf life and then progressively decreased to reach 2.15% during the seventh week. The prevalence of *Bifidobacterium* spp. (also known to be related to digestion of milk) showed a similar pattern. It is known that the consumption of solid feed by dairy calves significantly increases after the fourth week of calf life (Oikonomou et al. 2013). But in case of Simmental calves, while *Bacteroidetes* decreased, *Firmicutes* increased over time. The overall

Firmicutes to Bacteroidetes ratio was 0.2 varying between 0.1 and 0.4 during the different stages of growing, with a tendency to increase with age. Genus *Bacteroides* is dominant from birth until calves were provided concentrate to diet.; and generally, this genus was decreasing overtime. The same trend of variation was found in *Lactobacillus* with a significant decrease over time (Klein-Jobstl et al. 2014).

Faecalibacterium prausnitzii is a butyrate-producing microorganism. Butyrate has the highest energy value per mole of the major rumen volatile fatty acids, is extensively metabolized by the rumen epithelium, and has mitogenic effects on the epithelium during development. Additionally, butyrate concentrations were found to be higher in the rumen fluid of steers that showed higher feed efficiency. Klein-Jobstl et al. (2014) reported that in Simmental calves, *Faecalibacterium* spp. decreased significantly over time. Oikonomou et al. (2013) indicated that *Faecalibacterium* spp. prevalence during the first week of calf life was found to be significantly associated with body weight gain during the pre-weaning period. Calves with higher abundance of *Faecalibacterium* spp. in the first week of life had showed the lower incidence of diarrhea (Oikonomou et al. 2013).

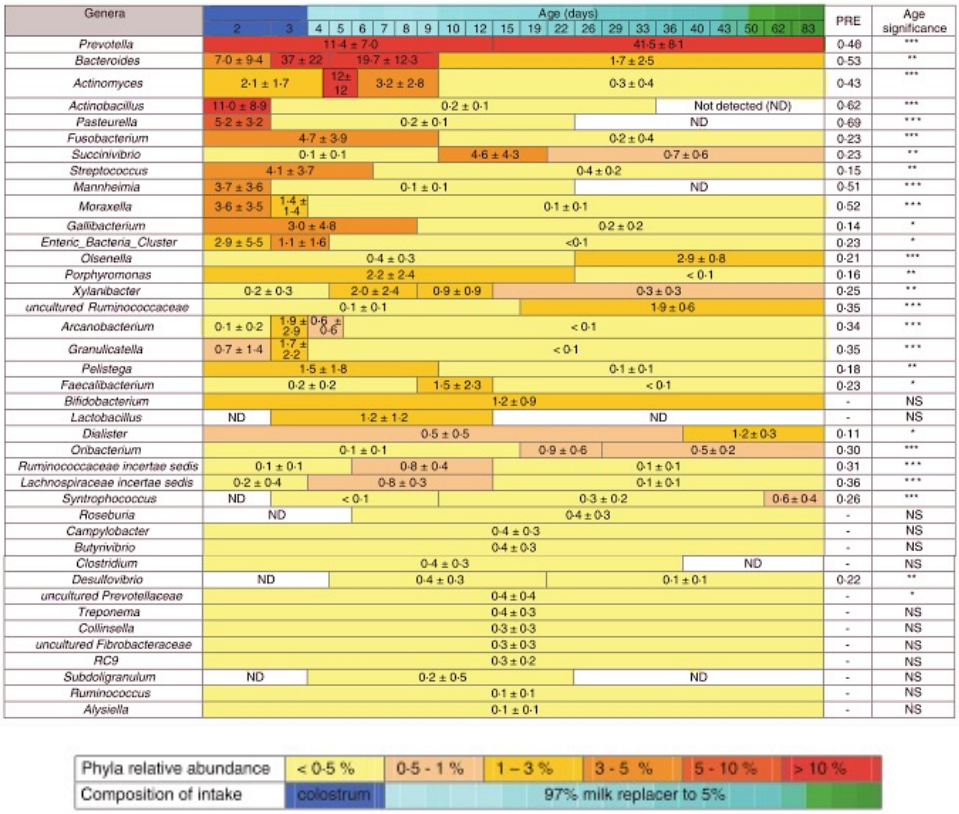

Figure 2.2 Age classification of calf ruminal samples according to genera relative abundance determined with TREE package of SYSTAT from birth to weaning, values expressed as mean percentages ± standard deviation of calf means. PRE indicates proportion of variability explained by division into age-classes. ND indicates not detected genera. Significance of age in the repeated measurements analysis is referred as ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05, NS for P> 0.05 (Rey et al. 2013).

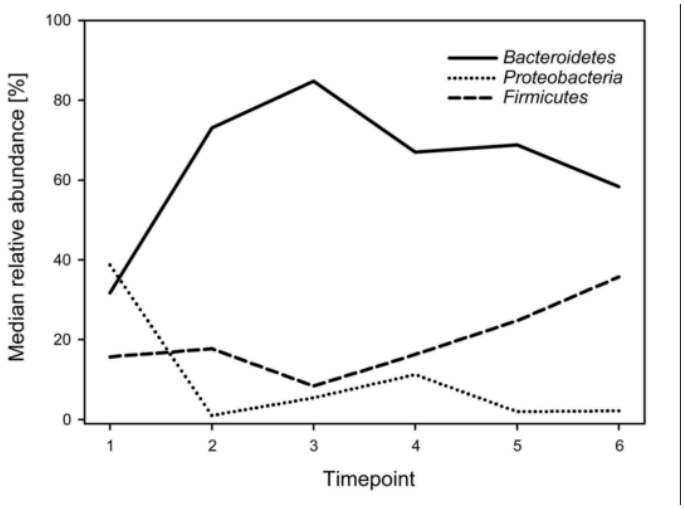

Figure 2.3. Median relative abundances of the three most abundant phyla during the six timepoints (12 hours, 2 weeks, 3 weeks, 6 weeks after calving, one week before and after weaning) (Klein-Jobstl et al. 2014).

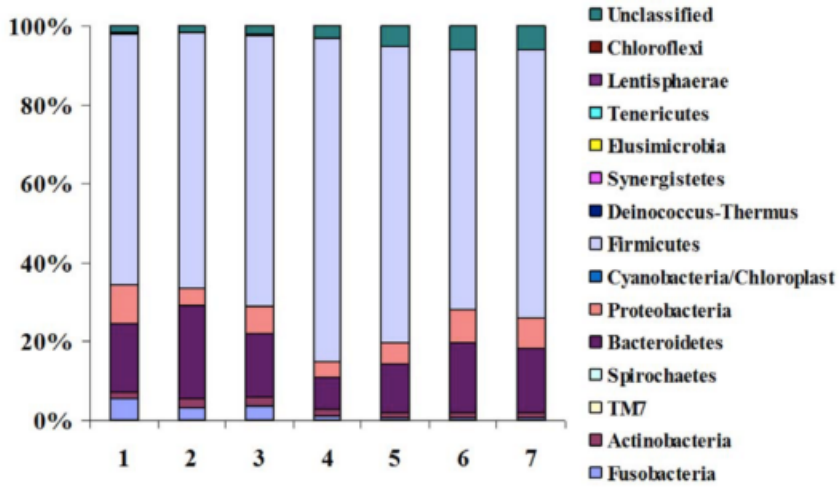

Figure 2.4. Aggregate microbiota composition at the phylum level by week of life (Oikonomou et al. 2013).

2.3. Characterizing microbiota across gastrointestinal tract of ruminants

The symbiosis between gastrointestinal bacterial communities and their mammalian host have been proved to have essential implications for overall animal health. The typical example of this relationship is exposed in the rumen of ruminants, where plant materials are digested in order to converse plant fibers into chemical compounds absorbed by the animal. Hence, the microbiota in bovine rumen have been examined extensively. In addition, fecal microbiota has been also studied due to the convenient sampling procedure. However, the role of microbiota in other segments such as small intestine and large intestine have received little consideration, and, limited understanding of characteristic microbiota in different parts along the gastrointestinal tract was achieved. Recently, there have been studies exploring the structure of microbiota across the

gastrointestinal tract of ruminant. These works have revealed there is a high intra-individual variation in the microbiota inhabiting within the different components of the gastrointestinal tract (de Oliveira et al. 2013; Mao et al. 2015; Zeng et al. 2017).

Genus	No. of reads	Relative abundance (%)
Bacteroides	144,517	57.00
Escherichia-Shigella	26,398	10.41
Faecalibacterium	12,097	4.77
Paraprevotella	10,969	4.33
Sutterella	7420	2.93
Rikenella	6666	2.63
Butyricicoccus	6519	2.57
Parabacteroides	4508	1.78
Lactobacillus	3683	1.45
Oscillibacter	3358	1.32
Paludibacter	2727	1.08
Pseudomonas	2615	1.03
Phocaeicola	2490	0.98
Lachnospiracea incertae sedis	2144	0.85
Alistipes	929	0.37
Clostridium XlVa	843	0.33
Clostridium XlVb	697	0.27
Klebsiella	641	0.25
Flavonifractor	585	0.23
Sporobacter	500	0.20
Butyricimonas	477	0.19
Ahrensia	457	0.18
Odoribacter	423	0.17
Acinetobacter	358	0.14
Barnesiella	302	0.12
Ethanoligenens	296	0.12
Butyrivibrio	293	0.12
Clostridium IV	283	0.11
Sum	243,195	95.92

Table 2.1. Relative abundance of the 28 most abundant genera (relative abundance > 0.1%) of Simmental calves from birth to weaning (Klein-Jobstl et al. 2014).

The symbiotic bacteria are traditionally including digesta-associated and mucosa-associated bacteria. These bacterial populations were supposed to interact with each other and contribute to host animal function.

Total bacterial populations in various regions were estimated with a real-time PCR analysis. Regional sites affected bacterial density of dairy cattle significantly. Higher digesta-associated bacterial numbers were observed in the colon and omasum, and higher mucosa-associated bacterial numbers were present in the rumen and omasum. In addition, mucosa-associated bacterial densities in the jejunum, ileum, cecum, colon and rectum were significantly lower than in their corresponding digesta. However, digesta-associated bacterial numbers in the duodenum were lower than mucosa-associated bacterial densities (Table 2.2) (Mao et al. 2015).

2.3.1. Microbial composition of digesta-associated population across gastrointestinal tract

The gastrointestinal tract of ruminant was differentiated into three regions, namely the forestomach (rumen, reticulum, omasum, and abomasum), small (duodenum, jejunum, and ileum) and large intestine (cecum, colon, and feces).

The diversity of microbiota was found higher in the stomach than in the small and large intestine. For instance, among different site along the gastrointestinal tract of dairy cow, rumen and abomasum harbored most of the phyla (19 phyla), while the lowest number was seen in cecum (12 phyla) (Mao et al. 2015). In dairy cow, within the gastrointestinal tract, the most abundant phyla were *Firmicutes* (64.81%), *Bacteroidetes* (15.06%), and *Proteobacteria* (13.29%). At genus level, *Prevotella* (5.27%), *Ruminococcus* (3.29%), *Acetitomaculum* (2.89%), *Butyrivibrio* (5.06%), as well as those unclassified derived from *Peptostreptococcaceae* (16.9%), *Ruminococcaceae* (8.69%), *Enterobacteriaceae* (11.1%), *Clostridiales* (2.46%), *Rikenellaceae* (3.64%) and *Bacteroidales* (4.03%) were predominant (Table 2.3) (Mao et al. 2015).

One report of Brazilian Nelore steer showed that the majority belonged to the phyla *Firmicutes* (41.22%), *Bacteroidetes* (33.51%), and *Proteobacteria* (12.15%). And, the most highly represented families included the *Ruminococcaceae* (11.14%), *Lachnospiraceae* (9.98%) and *Prevotellaceae* (8.67%) (de Oliveira et al. 2013). Zeng et al. (2017) indicated that in the gastrointestinal tract of Chinese Mongolian sheep harbored phyla *Firmicutes* (44.62%), *Bacteroidetes* (38.49%), *Proteobacteria* (4.11%), *Spirochaetes* (3.44%), and *Euryarchaeota* (1.78%); classes *Clostridia* (42.02%), *Bacteroidia* (37.30%), *Spirochaetes* (3.36%), Unclassified (2.80%), and *Fibrobacteria* (0.58%); 20 orders *Clostridiales* (42.01%), *Bacteroidales* (37.30%), *Spirochaetales* (3.26%), Unclassified (2.90%) and *Methanobacteriales* (1.83%); 38 families *Ruminococcaceae* (20.76%), *Prevotellaceae* (16.60%), *Lachnospiraceae* (8.37%), Unclassified (6.88%), and *Bacteroidaceae* (5.23%); 40 genera Unknown (20.76%), Unclassified (19.92%), *Prevotella* (15.56%), *Ruminococcus* (6.35%), and *Treponema* (3.26%) and 18 species Unknown (63.42%) Unclassified (21.70%), *Prevotella ruminicola* (5.45%), *Ruminococcus flavefaciens* (3.63%), and *Ruminobacter albus* (1.72%) (Zeng et al. 2017).

Table 2.2. Total bacterial density (numbers per gram wet weight) throughout the gastrointestinal tracts of dairy cattle*. Note. Means within the same column with different subscripts are significantly different from one another. *Copy number of 16S rRNA gene (copy.g^{-1}). ‡Sampling site effect on bacterial density throughout the GIT of dairy cattle. §Regional effect on bacterial density throughout the GIT of dairy calves (Mao et al. 2015).

Regions	Digesta	Mucosa tissue	*P* value‡
Rumen	$(7.79 \pm 1.52) \times 10^{10c}$	$(5.84 \pm 0.64) \times 10^{10a}$	0.575
Reticulum	$(7.50 \pm 1.1) \times 10^{10c}$	$(3.51 \pm 0.49) \times 10^{10ab}$	0.136
Omasum	$(6.81 \pm 0.43) \times 10^{11ab}$	$(6.51 \pm 0.95) \times 10^{10a}$	<0.001
Abomasum	$(3.05 \pm 1.32) \times 10^{10c}$	$(3.42 \pm 0.75) \times 10^{10b}$	0.645
Duodenum	$(1.14 \pm 0.20) \times 10^{9d}$	$(4.93 \pm 0.39) \times 10^{9b}$	0.002
Jejunum	$(4.36 \pm 1.47) \times 10^{10c}$	$(6.35 \pm \pm 0.46) \times 10^{9b}$	0.003
Ileum	$(4.81 \pm 1.33) \times 10^{10c}$	$(7.74 \pm 1.51) \times 10^{9b}$	<0.001
Cecum	$(2.13 \pm 0.33) \times 10^{11bc}$	$(5.48 \pm 0.29) \times 10^{9b}$	0.012
Colon	$(7.62 \pm 1.64) \times 10^{11a}$	$(4.95 \pm 0.42) \times 10^{9b}$	0.047
Rectum	$(7.35 \pm 2.13) \times 10^{10c}$	$(4.04 \pm 0.16) \times 10^{9b}$	<0.001
P value§	<0.001	<0.001	

Although *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were found represented within gastrointestinal tract of ruminant, they varied considerably among regions in abundance and in the number of genera composing them. In steer, *Bacteroidetes* was found predominantly from the reticulum to abomasum, whereas *Firmicutes* dominate within the small intestine (duodenum, jejunum and ileum) and within the large intestine (cecum, colon and feces) (de Oliveira et al. 2013). The gastrointestinal tract of sheep indicated *Firmicutes* was higher in the small intestine than in the stomach and large intestine, while *Bacteroidetes* showed the opposite trend of variation. In phylum *Firmicutes*, the two genera *Ruminococcus* and *Oscillospira* were the most abundant across the tract. While genus *Ruminococcus* showed a decrease trend in abundance from the stomach (15.45% of total bacterial population), small intestine (4.03%), to larger intestine (1.99%), genus *Oscillospira* indicated a gradual increase in abundance from 1.94% of total population in the stomach, 3.03% in small intestine to 4.42% in large intestine. Genus *Prevotella*, the most predominant genus within phylum *Bacteroidetes*, showed great variation between segments. This genus accounted for 34.88% (of total bacterial population) in the stomach, decreased into 7.78% in the small intestine, and lightly increased again in large intestine (13.42%). Genus *Bacteroides* was only detected in jejunum (9.27%), colon (10.63%), and feces (15.32%). The relative abundance of other genera [*Prevotella*], *Parabacteroides*, and *CF231* were found greater from the segment of ileum to feces, while they were not detected at remarkable level in other segments (Zeng et al. 2017).

In dairy cow, phylum *Firmicutes* dominated all bacterial communities along the tract except for in the duodenum, where *Proteobacteria* (53.9%) was predominant. *Bacteroidetes* was the second most prevalent in the forestomach, while *Proteobacteria* was the second most prevalent in

digesta samples of the jejunum, ileum, cecum and colon. *Firmicutes* was mainly composed of unclassified *Ruminococcaceae* (15.2%), unclassified *Christensenellaceae* (7.51%), and unclassified *Lachnospiraceae* (6.29%) in forestomach; unclassified *Lachnospiraceae* (6.06%), *Buryrivibrio* (8.19%), and *Ruminococcus* (4.41%) in small intestine; and, unclassified *Peptostreptococcaceae* (43.1%), *Turicibacter* (13.2%), and *Clostridium* (10.1%) in large intestine. Phylum *Bacteroidetes* was dominated by *Prevotella* (12.9%), unclassified *Rikenellaceae* (8.42%), and unclassified *Bacteroidales* (9.46%) in forestomach. High abundance of genus *Prevotella* in forestomach is thought to relate to its high genetic variability, which enables them to occupy various ecological niches within the rumen. However, the exact mechanism explaining the result that *Prevotella* was less abundant in small and large intestines is not clear yet. Phylum *Proteobacteria* was overwhelmed by unclassified *Enterobacteriaceae* in small intestine (27.3%), and large intestine (8.92%), but could not be detected at significant level in forestomach (Mao et al. 2015).

2.3.2. Microbial composition of mucosa-associated population across gastrointestinal tract

Gastrointestinal mucosa-associated microbiota could play important biological roles due to their close proximity to the animal host, but knowledge of their composition in cattle still is limited. Mao et al. (2015) indicated that significant differences in the diversity, composition, and structure of mucosa-associated bacteria communities among sections. *Firmicutes* (42.2%), *Bacteroidetes* (21%) and *Proteobacteria* (17.6%) were the most abundant phyla across the tract, and *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Tenericutes*, *Spirochaetae*, *Cyanobacteria* and *Lentisphaerae* were found in all samples. *Firmicutes* dominated all mucosa-associated bacterial communities along the tract except for in the duodenum, where *Proteobacteri*a (37.3%) was predominant. *Bacteroidetes* was the second most prevalent phyla in mucosal samples of the reticulum, omasum, abomasum, colon and rectum, whereas *Proteobacteria* was the second most prevalent in mucosal tissues of the rumen, jejunum and ileum. *Firmicutes* and *Spirochaetae* were the second most dominant phyla in mucosal samples of the duodenum and cecum, respectively. In *Firmicutes*, unclassified *Ruminoccocaceae* were found more abundant in forestomach (9.12%) and large intestine (16.1%) (Table 2.4). Unclassified *Peptostreptococcaceae* (6.32%), *Anaerovibrio* (4.05%) appeared predominant in large intestine. *Butyrivibirio* showed a higher abundance in rumen (12.1%) and reticulum (12.1%). As the mucosal butyrate producers release butyrate close to the epithelium, species of *Butyrivibrio* may enhance butyrate bioavailability for the host, which may be particularly useful in proliferating rumen and reticulum epithelium. The study highlighted the dominant presence of the aerobic bacterial genus *Acinetobacter* (12.1%), belonged to phylum *Proteobacteria*, in small intestine mucosa. This could be explained by the presence of oxygen at

the apical surface of the intestinal epithelial cells, representing a possible mechanism of exclusion of strictly anaerobic, extremely oxygen-sensitive microorganisms. In addition, the enrichment of genus *Treponema* (12.5%) (belonged to phylum *Spirochaetae*) in large intestine mucosa may have health implications. *Treponema* spp. has been reported that they were well adapted to oxidative stress. Additionally, *Treponema* spp. are believed to be associated with ulcerative mammary dermatitis and bovine digital dermatitis in cattle and contagious ovine digital dermatitis in. Thus, enhanced *Treponema* in large intestine mucosa could have deleterious effects on hindgut health (Mao et al. 2015).

When comparing between digesta and mucosa samples of each region along gastrointestinal tract, significant differences in composition and structure of bacterial communities were observed. Mao et al. (2015) showed that higher digesta-associated bacterial numbers were observed in the colon and omasum, and higher mucosa-associated bacterial numbers were present in the rumen and omasum. In addition, mucosa-associated bacterial densities in the jejunum, ileum, cecum, colon and rectum were significantly lower than in their corresponding digesta. However, digesta-associated bacterial numbers in the duodenum were lower than mucosa-associated bacterial densities.

At the phylum level, the proportion of *Firmicutes* in the digesta of the forestomach, jejunum and large intestine was significantly higher than in their corresponding mucosal tissues. The abundance of *Bacteroidetes* was in the digesta of the rumen and reticulum, while it was lower in the digesta of the duodenum, jejunum and large intestine when compared with their corresponding mucosal tissues. Mucosal tissues of the forestomach and rectum presented higher proportions of *Proteobacteria* than their corresponding digesta samples, while digesta of the duodenum showed a comparatively higher proportion of *Proteobacteria*. At genus level, higher proportions of the predominant *Prevotella*, unclassified *Ruminococcaceae*, unclassified *Enterobacteriaceae* and unclassified *Peptostreptococcaceae* in the digesta-associated microbiota and a larger percentage of *Butyrivibrio*, *Acinetobacter* and *Treponema* in the mucosa. Previous studies revealed that the genus *Prevotella*, unclassified *Ruminococcaceae* and unclassified *Peptostreptococcaceae* might play important roles in feed digestion and that members of the genera *Butyrivibrio*, *Acinetobacter* and *Treponema* were more involved in epithelium proliferation and diseases (Mao et al. 2015).

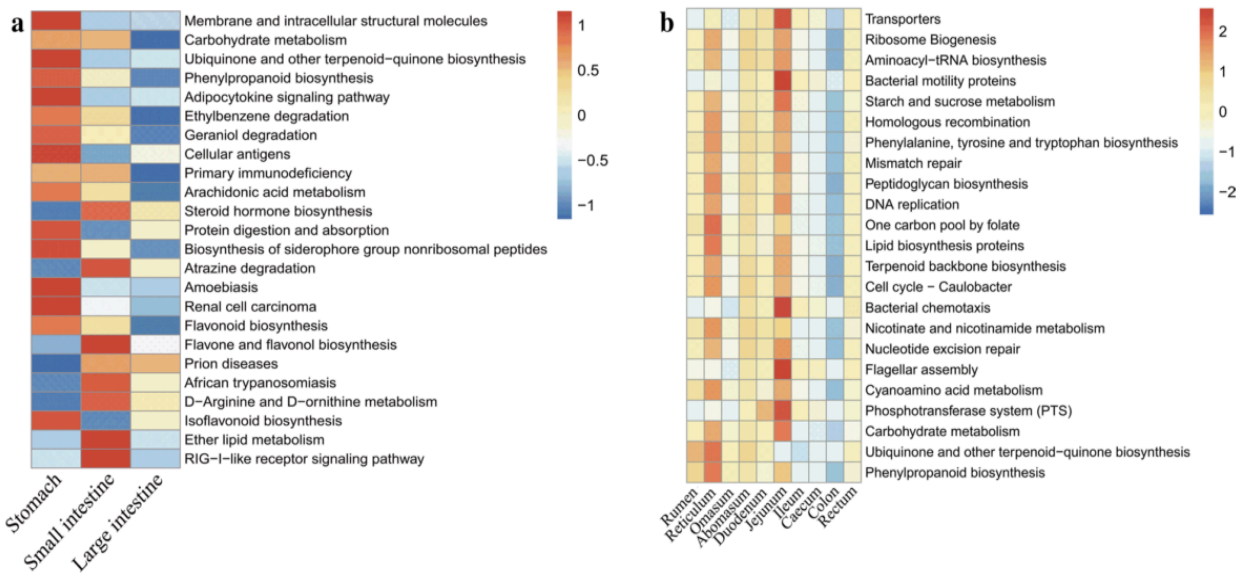

Figure 2.5. Predicted function of the gut micorbiota in the sheep of GIT. KEGG pathways were shown in two heatmaps. The bootstrap Mann–Whitney u-test was used to detect the gene distribution with cutoffs of $P < 0.05$, FDR <0.2, Mean counts >10,000 (a) and $P < 0.01$, FDR <0.1, Mean counts >10 (b) (Zeng et al. 2017).

2.3.3. Microbial composition of food-associated population in related to bacterial communities across gastrointestinal tract

There have been limited studies which involved in the relationship between food-associated and gut microbiota of cattle. de Oliveira et al. (2013) reported that corn and grass feed which provided to cattle was dominated by members of phylum *Proteobacteria*, and indicated no relation to gut microbiota of cattle across the gastrointestinal tract. According to the authors, this is not surprising given that the rumen, which is the first major organ that feed enters, is adept at selecting a very specific microbiota (Fig. 2.6). However, in human case, several studies have been carried out to shed light on the consequence of consumption of naturally fermented food. Lee et al. (1996) demonstrated that consumption of naturally fermented kimchi, which has been shown to have *Lactobacillaceae* and *Leuconostocaceae* as the dominant taxa (Jung et al. 2014), increased the population of *Lactobacillus* spp. and *Leuconostoc* spp. in human feces (Lee et al. 1996). Similarly, Han et al. (2015) found that intake of fermented kimchi raised up the populations of two phyla, *Proteobacteria* and *Actinobacteria*, and two genera *Bacteroides* spp. and *Prevotella* spp. in human feces (Han et al. 2015).

2.3.4. Prediction of molecular function of microbiota across gastrointestinal tract

The gastrointestinal microbiota presents many physiological functions that are lacking in the host, and therefore, they can be considered essential to cattle life. To gain insight into the

molecular functions of bacterial microbiota across cattle GITs, in many studies, PICRUSt was used to predict the metagenomic contribution of the communities observed. PICRUSt predicts metagenomic potential by imputing the available annotated genes within a known sequenced database, such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the Clusters of Orthologs Groups (COGs) catalogue, based on the presence/absence of OTUs in a 16S rRNA survey. With PICRUSt, one can calculate nearest sequenced taxon index (NSTI), which measures how closely related the average 16S rRNA sequence in an environmental sample is to an available sequenced genome. When this number is low, PICRUSt is likely to perform well in predicting the genomes of the organisms in an environmental sample.

Using PICRUSt as a predictive exploratory tool, Mao et al. (2015) inferred that 39 gene families were identified in the digesta and mucosa-associated microbiota samples. The results indicated that molecular function of digesta- and mucosa-associated microbiota were differentiated between forestomach and intestine (except for rectum samples). The majority of the genes belonged to membrane transport (17.8% indigesta-associated microbiota and 17.6% in mucosa-associated microbiota, respectively), carbohydrate metabolism (10.68% in digesta, 10.8% in mucosa), amino acid metabolism (8.36% in digesta, 9.13% in mucosa), replication and repair (7.70% in digesta, 7.62% in mucosa) and energy metabolism (4.69% in digesta, 4.84% in mucosa). The abundance of these genes is consistent with the general metabolic functions being essential for microbial survival. This study also revealed that significant differences in bacterial function among region across the gastrointestinal tract. For instance, in the digesta samples, the relative abundances of the genes involved in carbohydrate metabolism and replication and repair in the microbiota of forestomach samples were significantly higher than in the microbiota of the cecum and colon. In mucosal samples, there was a notable enrichment of genes related to amino acid metabolism in the duodenum when compared with those in the forestomach and large intestine. When comparing between digesta and mucosa, genes associated with amino acid metabolism were more enriched in the mucosa-associated microbiota than in their corresponding digesta. One possibility is that the mucosal tissue provides a decreased supply of carbohydrates and that bacteria may derive energy from amino acid fermentation, and the mucosal microbiota may be more necessary to amino acid degradation (Mao et al. 2015).

Zeng et al. (2017) also used PICRUSt to predict bacterial functional across gastrointestinal tract of sheep. Genes related to carbohydrate metabolism and bacterial flagellar assembly (“Carbohydrate metabolism”, “Peptidoglycan biosynthesis”, “Ethylbenzene degradation”, “Geraniol degradation”, “Primary immunodeficiency”, “Arachidonic acid metabolism”, “Biosynthesis of siderophore group nonribosomal peptides”, and “Flagellar assembly”) were more

abundant in the stomach and small intestine than in the large intestine. Genes primarily related to the metabolism of cofactors and vitamins ("Membrane and intracellular structural molecules", "Ubiquinone and other terpenoid-quinonebiosynthesis", and "Adipocytokine signaling pathway") were significantly abundant only in the stomach. And, two pathways ("Ether lipid metabolism" and "RIG-I-like recep- tor signaling pathway") were significantly abundant only in the small intestine (Fig. 2.5) (Zeng et al. 2017).

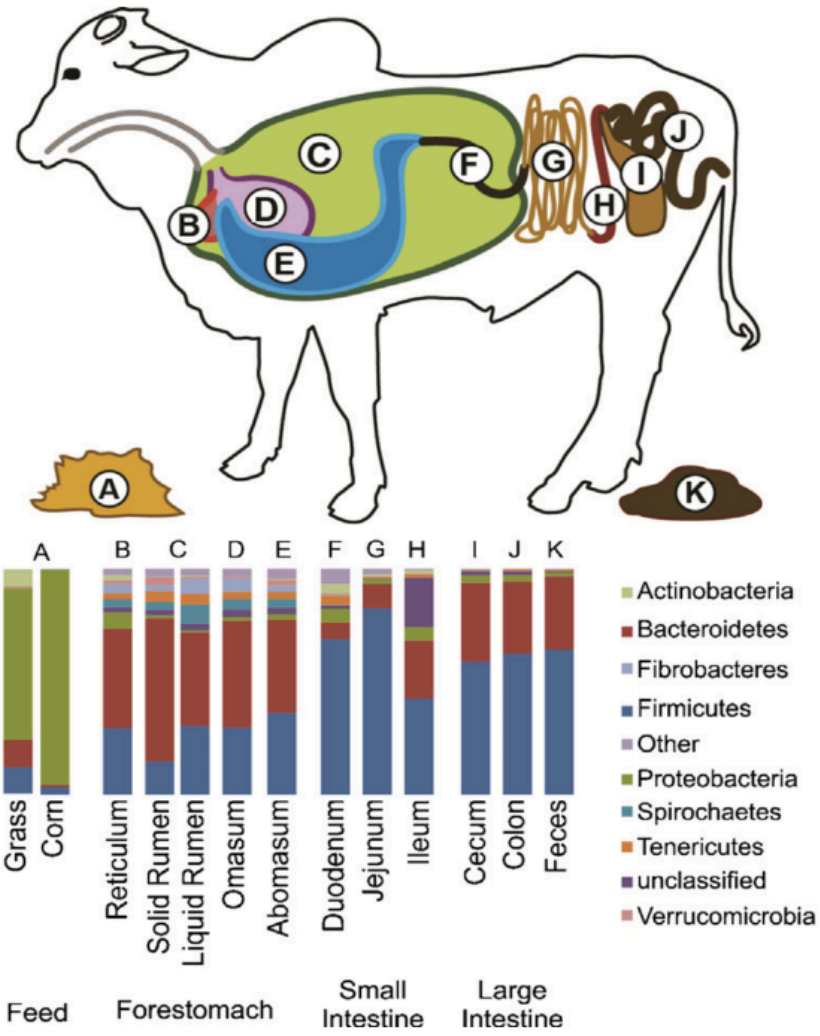

Figure 2.6. Distribution of 16S rRNA sequences from the feed and GIT of a Nelore steer. Depicted are sampling locations and the relative sequence abundances in phyla (de Oliveira et al. 2013).

Table 2.3. Comparison of the phyla in digesta samples across the gastrointestinal tract of dairy cattle (the multiple comparisons results were only presented for the phyla which average relative abundance ≥1% in at least one region). Mean in the same row with different superscripts represents a significant difference ($P < 0.05$) (Mao et al. 2015).

Phylum	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	SEM	P value	FDR*
Firmicutes	50.49^{ef}	53.86^{de}	50.96^{ef}	64.37^{cd}	38.28^{f}	68.52^{bc}	67.49^{bc}	79.15^{ab}	83.71^{a}	91.26^{a}	2.211	<0.001	<0.001
Bacteroidetes	42.39^{a}	38.98^{a}	40.59^{a}	19.66^{b}	1.78^{c}	1.66^{c}	1.31^{c}	0.41^{c}	0.99^{c}	2.87^{c}	2.353	<0.001	<0.001
Proteobacteria	1.15^{e}	1.12^{e}	1.21^{e}	3.48^{de}	53.87^{a}	17.49^{bc}	26.86^{b}	15.65^{bcd}	10.62^{cde}	1.4^{e}	2.223	<0.001	<0.001
Actinobacteria	1.27^{b}	1.23^{b}	1.13^{b}	5.18^{b}	4.29^{b}	9.62^{a}	3.12^{b}	3.97^{b}	3.32^{b}	2.96^{b}	0.402	<0.001	<0.001
Tenericutes	1.43^{b}	1.43^{b}	2.11^{a}	1.03^{b}	0.2^{c}	0.23^{c}	0.24^{c}	0.1^{c}	0.18^{c}	0.35^{c}	0.096	<0.001	<0.001
Spirochaetae	1.02^{b}	0.91^{bc}	0.59^{bcd}	2.03^{a}	0.06^{d}	0.07^{d}	0.04^{d}	0.08^{d}	0.31^{cd}	0.27^{cd}	0.089	<0.001	<0.001
Lentisphaerae	0.35^{bc}	0.3^{bc}	0.5^{b}	1.12^{a}	0.05^{bc}	0.14^{bc}	0.05^{bc}	0.01^{bc}	0.01^{bc}	0.01^{c}	0.053	<0.001	<0.001
Unclassified Bacteria	1.6b^{cde}	1.67^{abcd}	2.57^{a}	2.23^{ab}	1.23^{cdef}	1.97^{abc}	0.78^{def}	0.59^{f}	0.76^{def}	0.68^{ef}	0.105	<0.001	<0.001
Chloroflexi	0.13	0.1	0.06	0.05	0.03	0.02	0.02	0.005	0.004	0.003	0.009	0.004	0.005
Acidobacteria	<0.001	ND	ND	ND	ND	ND	ND	ND	ND	ND	<0.001	<0.001	<0.001
Armatimonadetes	0.01	0.01	0.02	0.02	0.002	0.001	ND	ND	0.003	ND	0.001	<0.001	<0.001
Chlamydiae	<0.001	ND	ND	ND	ND	ND	<0.001	ND	ND	ND	0.000	0.537	0.537
Chlorobi	ND	ND	ND	0.01	0.01	ND	ND	ND	ND	ND	0.001	0.524	0.537
Cyanobacteria	0.07	0.26	0.17	0.25	0.09	0.19	0.04	0.03	0.04	0.06	0.021	0.038	0.04
Deinococcus-Thermus	0.002	ND	<0.001	0.001	ND	ND	<0.001	ND	<0.00	ND	0.000	0.003	0.004
Elusimicrobia	0.02	0.01	0.04	0.09	0.003	0.003	0.001	ND	ND	<0.001	0.005	<0.001	<0.001
Fibrobacteres	0.02	0.08	0.02	0.35	0.01	0.01	0.002	ND	<0.001	0.003	0.019	<0.001	<0.001
Fusobacteria	0.02	0.02	0.01	0.06	0.06	0.06	0.02	0.005	0.005	0.01	0.006	0.017	0.021
Planctomycetes	ND	ND	<0.001	0.03	0.02	ND	ND	ND	ND	ND	0.003	0.526	0.537
Synergistetes	0.02	0.01	0.01	0.04	0.001	0.003	0.003	ND	ND	<0.001	0.002	<0.001	<0.001
Verrucomicrobia	0.02	0.01	<0.001	0.01	0.002	0.003	0.01	0.02	0.05	0.13	0.007	<0.001	<0.001

ND, not detected *FDR: False discovery rate.

Table 2.4. Comparison of the phyla in mucosal samples across the gastrointestinal tract of dairy cattle (the multiple comparisons results were only presented for the phyla which average relative abundance ≥1% in at least one region). Mean in the same row with different superscripts represents a significant difference ($P < 0.05$) (Mao et al. 2015).

Phylum	Rumen	Reticulum	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	SEM	P value	FDR*
Firmicutes	43.79^{ab}	43.01^{ab}	39.43^{ab}	27.4^{b}	34.21^{ab}	42.24^{ab}	52.15^{a}	48.29^{ab}	45.17^{ab}	46.57^{ab}	1.614	0.026	0.047
Bacteroidetes	21.98^{bc}	26.95^{ab}	39.08a	20.95^{bc}	10.33^{cd}	7.87^{d}	12.04^{cd}	18.16^{bcd}	21.75^{bc}	30.94^{ab}	1.438	<0.001	<0.001
Proteobacteria	22^{abc}	17.44^{bc}	10.88^{bc}	19.82^{abc}	37.26^{a}	27.84^{ab}	18.27^{bc}	7.92^{c}	8.48^{c}	5.69c	1.663	<0.001	<0.001
Actinobacteria	0.81^{c}	1.12^{c}	1.88^{c}	7.85^{ab}	10.94^{a}	12.62^{a}	10.59^{a}	3.09^{bc}	1.85^{c}	1.79c	0.686	<0.001	<0.001
Spirochaetae	2.49^{bc}	2.52^{bc}	1.13^{c}	1.27^{c}	0.31^{c}	0.63^{c}	0.39^{c}	18.75^{a}	13.85^{ab}	10.64^{abc}	1.105	<0.001	<0.001
Tenericutes	1.03^{b}	1.16^{b}	1.49^{b}	17.95^{a}	0.64^{b}	0.56^{b}	0.54^{b}	0.55^{b}	0.7^{b}	0.84^{b}	0.774	<0.001	<0.001
Unclassified Bacteria	6.59	5.93	4.23	2.2	4.85	6.03	4.75	2.27	6.53	1.6	0.533	0.262	0.317
Lentisphaerae	0.16^{b}	0.29^{b}	0.65^{b}	1.06^{a}	0.25^{b}	0.48b	0.47b	0.28b	0.22b	0.31b	0.047	<0.001	<0.001
Acidobacteria	ND	<0.001	ND	<0.001	0.05	0.17	0.01	0.001	0.02	0.005	0.015	0.250	0.317
Aquificae	ND	ND	ND	ND	ND	0.001	ND	ND	ND	ND	0.000	0.452	0.506
Armatimonadetes	0.01	0.002	0.01	0.01	0.03	ND	ND	<0.001	0.002	0.01	0.002	0.085	0.130
Chlamydiae	ND	ND	ND	ND	ND	<0.001	0.03	0.002	ND	ND	0.002	0.225	0.305
Chlorobi	ND	<0.001	ND	ND	0.01	0	0.01	0.01	ND	ND	0.001	0.635	0.635
Chloroflexi	0.02	0.03	0.03	0.03	0.06	0.22	0.07	0.01	0.02	0.004	0.018	0.225	0.305
Cyanobacteria	0.06	0.1	0.41	0.76	0.48	0.62	0.36	0.14	0.33	0.29	0.044	0.001	0.003
Deinococcus-Thermus	0.01	0.07	0.005	0.01	0.28	0.21	0.03	0.01	0.01	0.003	0.023	0.045	0.074
Elusimicrobia	0.08	0.13	0.13	0.18	0.03	0.07	0.1	0.02	0.02	0.02	0.010	<0.001	<0.001
Fibrobacteres	0.1	0.13	0.36	0.28	0.01	0.04	0.06	0.07	0.08	0.14	0.018	<0.001	<0.001
Fusobacteria	0.1	0.13	0.04	0.04	0.19	0.33	0.09	0.02	0.03	0.01	0.017	<0.001	<0.001
Gemmatimonadetes	ND	ND	ND	ND	ND	0.03	ND	ND	0.004	0.004	0.003	0.462	0.506
Planctomycetes	0.01	0.002	<0.001	ND	0.003	ND	ND	0.001	0.03	0.01	0.003	0.570	0.595
Synergistetes	0.77	0.97	0.21	0.18	0.05	0.01	0.01	0.01	0.01	0.02	0.046	<0.001	<0.001
Verrucomicrobia	0.01	0.01	0.03	0.01	0.03	0.03	0.04	0.39	0.9	1.13	0.092	0.016	0.031

ND, not detected; *FDR: False discovery rate

2.4. Factors influencing gut bacterial communities

2.4.1. Diet

Diet has been the most concerned parameter which drives the composition and metabolism of the colonic microbiota. Especially, the great impact of amount, type and balance of the main dietary macronutrients (carbohydrates, proteins and fats) is noticed (Scott et al. 2013).

2.4.1.1. Dietary carbohydrates

Cumming et al. (1991) reported that approximately 40 g of dietary carbohydrates per day have been escaped from digestion by host enzymes (Cummings et al. 1991; Scott et al. 2013). Many studies revealed that changing the amount and type of carbohydrate over periods (about four weeks) could create an extreme and rapid influence on the composition of the gut bacteria and its metabolites (Duncan et al. 2007; Russell et al. 2011; Walker et al. 2011; Scott et al. 2013), and dietary habit also makes an profound effect on bacterial composition. The studies of Qin et al. (2010) and of Yatsunenko et al. (2005) demonstrated that there has been a higher similarity between fecal bacteria of European and USA adults than that of South America and Malawi (Qui et al. 2010; Yatsunenko et al. 2012). The microbial communities of Malawians clusters were grouped together with those of Amerindian, but both were separated from USA adults' communities (Yatsunenko et al. 2012). It could be explained that Malawians and Amerindian groups owned a diet consisting of a large amount of plant-derived polysaccharides in comparison with a protein-rich diet of USA adults. Another study of De Filippo et al. (2010) showed that microbiota of the African children was dominated by *Bacteroidetes* (73%) compared to 27% *Bacteroidetes* and 51% *Firmicutes* in the EU children (De Filippo et al. 2010). The dominant *Bacteroidetes* species of African children, which are adept at fermenting xylans and other components of plant fiber, belonged to *Prevotella* and *Xylanibacter* genera which were absent in EU children (Hogenova et al. 2011).

Starch is a complex polysaccharide consisting of a mixture of amylose and amylopectin. The relative proportion of amylose and amylopectin also influences the bacterial composition to use different kinds of starch for growth (Scott et al. 2013). Mao et al. (2013) showed that when the proportion of dietary corn grain increased, the abundance of phylum *Actinobacteria* and *Planctomycetes* linearly decreased; phylum *Firmicutes* linearly increased; but there were no significant changes in the abundance of *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Proteobacteria* and *Tenericutes* (Mao et al. 2013). The abundance and digestibility of starch in grains created a shift in microbial community structure in grain-fed animals. An observation in cattle announced that across an increasing starch gradient, the relative abundance of phylum

Firmicutes decreased and phylum *Bacteroidetes* increased, and, the bacterial composition composing to these phyla also changed (Shanks et al. 2011).

2.4.1.2. Dietary proteins

The colon is an active site of protein which provides nitrogen for growth of saccharolytic bacteria and amino acids for fermentation by saccharolytic species. In human feces, *Bacteroides* species and *Clostridium perfringens*, propionibacteria, streptpcocci, bacilli, staphylococci are determined as the predominant proteolytic bacteria. Amongst fecal bacteria *Bacteroides* species has strong pepsidase activity. And, several bacterial groups can ferment amino acids and possess weak saccharolytic activity like peptococci, acidaminococci, veillonella, and some fusobacteria, eubacteria, clostridia (Macfarlane et al. 1997; Walker et al. 2005; Hamer et al. 2011; Scott et al. 2013).

Fermentation of protein results in the production of various potentially toxic products, such as amines and ammonia, phenols, indoles, and often accompanies with growth of potential pathogens. Excessive protein intake has been related to stimulate the growth of pathogenic species like *Clostridium perfringens*, and to decrease amount of beneficial bifidobacteria (Rist et al. 2013). Principally, dietary protein becomes favorable for bacterial fermentation when it escapes the digestion by host enzymes (Libao-Mercado et al. 2009; Rist et al. 2013). The source of protein may influence the microbial fermentation. For instance, highly digestible proteins like casein are digested by host enzymes, so that they are not available for microbial fermentation. In case of plant proteins, they are usually not completely digested and certainly become available for microbial fermentation especially at higher dietary protein levels (Pluske et al. 2002). One study showed that protein source (soybean meal, casein) has no influence on bifidiobacteria, lactobacilli and clostridia in ileal digesta of weaned piglets, but these bacterial groups were increased in piglets fed with soybean meal-based diet (Rist et al. 2013).

It has been suggested that increasing of carbohydrate supply can restrict proteolytic fermentation by enhancing bacterial proliferation, and then, utilization of peptides for bacterial proteosynthesis. Evidence from many studies indicated that a shift of carbohydrate:protein balance changes the profile of fermentation products, but changes in micobitota are still unclear (Bernalier-Donadille et al 2010; Scott et al. 2013).

2.4.1.3. Dietary fat

Zhang et al. (2012) have reported that the microbiota of mice fed with high-fat diet to induce obesity for 12 weeks and followed by a normal diet for 10 weeks has shown a different

microbial composition in obese group in high-fat diet but become indiscernible from control group after 10 weeks of normal diet treatment. Hence, the response of microbiota to the high-fat diet indicated that microbiota responds to the diet not to the obese phenotype (Zhang et al. 2012).

Data obtained from animal showed that the high-fat diet can force an increase in *Firmicutes* and decrease in *Bacteroidetes*. Gut flora can be considered as an essential target to manipulate obesity and related diseases (D'Aversa et al. 2013). A study of Mujico et al. (2013) indicated that high-fat diet administration induced an increase in body weight, all groups of *Firmicutes* (phylum *Firmicutes*, *Lactobacillus* group and *Clostridial* cluster XIVa); an decrease in *Bifidobacterium* spp. and the phylum *Bacteroidetes*. But for the high-fat diet administration supplemented with oleic acid-derived compound, the microbial changes were counteracted: a decrease in all groups of *Firmicutes* and an increase in *Bifidobacterium* spp. and the phylum *Bacteroidetes*, up to the level even higher than the control diet group. These data support a role for fatty acids related to obesity prevention (Mujico et al. 2013).

2.4.2. Antibiotics usage

Many antibiotics that have been used followed the idea of "detoxifying" the body by the rectal administration (Rambaud et al. 2006). They can reach the colon directly or after entering an enterohepatic cycle, and create an impact on the microbiota depending on class, spectrum or pharmacological properties. Hence, the adverse effects may happen: decreasing capacity of fermentation (increasing risk of diarrhea), decreasing in the barrier effect (increasing risk of the emergence of pathogens) and the occurrence of antibiotic resistance (Rambaud et al. 2006; Perez-Cobas et al. 2013). Perez-Cobas et al. (2013) reported that specific properties of antibiotics such as mode of action forced the selection of intestinal microbiota, and were responsible for the changes of bacterial composition during therapy. This study was carried out with 4 patients (A, B, C and D) who were treated different kinds of antibiotics owning bactericidal, antimicrobial and bacteriostatic effect. In patient A (treated with cell replication inhibitor antibiotic), both total (16S rRNA gene) and active (16S rRNA transcripts) microbiota indicated high dominance of the families *Lachnospiraceae* and *Ruminococcaceae* during treatment. Treatment of patient B (inhibitor of protein synthesis) resulted in high presence of *Enterobacteriaceae* and in active microbiota, there was an increase of *Bacteroides* genus after 5-day treatment. For patient C (cell envelop synthesis inhibitor antibiotic), *Oscillibacteriaceae*, *Ruminococcaceae*, *Rikenellaceae* and *Bacteroidaceae* were seen as the most abundant taxa. The first significant change in this case took place on day 6 with an increase

in *Parabacteroides*. And, on day 10 after treatment, *Enterobacteriaceae* and *Enterococcaceae* families increased. In patient D (cell envelop synthesis inhibitor antibiotic), *Enteriobacteraceae* and *Ruminococcaceae* were the most popular at the initial composition but, after that, were extremely affected by antibiotics due to an increase in resistant bacterial taxa of *Bacteroides* genus. After all, the bacterial composition and abundance of patients who suffered bactericidal antimicrobial agent (A, C and D) clustered together, and apart from those of patient who received bacteriostatic antibiotic (B) (Perez-Cobas et al. 2013). For the infant in the study of Penders et al. (2006), during the first 1 month of life, oral use of antibiotics resulted in a decrease of bifidobacteria and *Bacteroides fragilis* group species (Penders et al. 2006). A different study indicated that antibiotics created a suppression of all anaerobic bacteria, except for clostridia, and increased levels of *Klebsiella*, *Enterobacter*, *Citrobacter* and *Pseudomonas* spp. The action of antibiotics on bacterial communities is selective and its impacts can maintain even after the treatment has been terminated (Fanaro et al. 2003).

2.4.3. Prebiotics and probiotics

Prebiotics are defined as non-digestible food ingredients that are resistant to digestion and absorption, are fermented by cecal/colonic microbiota, and selectively stimulate growth and/or activity of bacteria that contribute to colonic and host health (Callaway et al. 2012). It's focused on oligosaccharides as health-promoting substrates, which can be food additives because of their low caloric value and ability of enhancing mineral absorption. Many oligosaccharides can modulate microbiota of large bowel by increasing bifidobacteria and lactobacilli populations and decreasing clostridia populations. There are three oligosaccharides classified as prebiotics: fructans, galactooligosaccharides and lactulose. Yusrizal et al. (2003) showed that supplementation of fructans created an improvement in weight gain, carcass weight and an increase in lactobacilli counts, concomitantly, a decrease in *Campylobacter* and *Salmonella* (Yusriszal et al. 2003; Gaggia et al. 2010). Another study reported the decrease of *Clostridium perfringens* number and bacterial endotoxin levels due to adding 0.5% of fructan-rich jerusalem artichokes syrup to drinking water in broilers (Kleessen et al. 2003; Gaggia et al. 2010).

Probiotics are defined as a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microbiota (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host (Schrezenmeir et al. 2001; Callaway et al. 2012).

The practice of supplementing probiotics and prebiotics to domestic animals during their growth becomes more widespread. It is possible that effectiveness of probiotics and prebiotics in some animal species may be an indirect consequence of speeding up the establishment of the dominant microbiota characteristic of the adult gastrointestinal tract. A study of Siew et al. (2013) showed that treatment of probiotics mix VSL#3 (contained 900 billion viable lyophilized bacteria consisting of four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve* and *B. infantis*), and one strain of *Streptococcus* (*Streptococcus salivarius* subsp. *thermophilus*)) demonstrated a significant reduction of *Bacteroides* and improved evenness of microbial profile (Ng et al. 2013). Younts-Dahl et al. (2004) reported a large-scale trial of steers fed with a standard steam-flaked corn-based finishing diet containing *L. acidophilus* NP51 demontrated a reduction of *E. coli* O157 fecal shedding by 57% (Younts-Dahl et al. 2004). Another field trial clearly showed that *E. coli* O157:H7 fecal shedding decreased (35%) in beef cattle, following daily administration of *L. acidophilus* NP51 (Peterson et al. 2007). Higgins et al. (2007) found that *Lactobacillus*-based probiotic cultures significantly reduced *Salmonella enteritidis* recovery in challenged neonatal broiler chicks but there were no relevant results towards *S. typhimurium* (Higgins et al. 2007; Higgins et al. 2008).

2.4.4. Host-related factors

In intestinal tract, the abiotic ecological factors controlling the microbiota are acidity, bile, pancreatic and intestinal secretions, mucus and defensins. Gastric acid secretion is the essential defense factor against colonization of the gastrointestinal tract by pathogens. Intestinal infection extremely increased in case of achlorhydria or administration of anti-secretory therapy because the number of bacteria needed for a trigger of infection is decreased (Marteau et al. 1997; Rambaud et al. 2006). Resistant ability to acid are greatly different among microorganisms, and some can survive through the stomach, which are selected as probiotics. Biliary acids and pancreatic juice also have affected bacterial membranes because of their antimicrobial properties (Drouault et al. 1999; Rambaud et al. 2006). The influence of mucus and defensins is still poorly understood. Mucus performs a physical barrier between lumen and epithelial cells of stomach, intestine and the colon. The secret of mucus, which is viscous, composed of mucoglycoprotein polymer, is decreased by fasting and total parenteral feeding. Bacterial flora can change its composition and properties by not only partial degradation but also influencing its synthesis. Mucus creates a physical barrier and also concentrates many

antimicrobial substances such as secretory immunoglobulin A, lactoferrin, lactoperoxidase and lysozyme. Besides, some of its sugars which can mimic bacterial receptors have the ability to bind various microorganisms. Defensins are antimicrobial peptides, acting by destroying the bacterial cell membrane, especially against *E. coli*, *Listeria monocytogenes*, *Salmonella* and *Candida albicans* (Ganz et al. 2003; Rambaud et al. 2006). However, their possible action on the saprophytic microbiota is not known.

Intestinal immune system has the function of controlling the pathogenic intestinal microbiota, indicated by the observation of disturbances in pathogenic flora in immune-deficient hosts. Hence, in case of IgA selective deficiency or common variable immunodeficiency, recurrent intestinal infection is increased. Depending on the severity of immune deficiency, the risk of bacterial colitis (*Campylobacter jejuni* and *coli*, *Salmonella*, *Clostridium difficile*), and then, cytomegalovirus infections, infections with different protozoa, mycobacteria or fungi is observed (Rambaud et al. 2006).

Differences in small intestine and colon's motility are responsible for the primary localization of microbiota. In adult animals, there is a gradient of oxygen from food and water consumption and from tissues into lumen that has effects on bacterial composition along digestive tract (Wilkinson et al. 2002; Callaway et al. 2012). There is an increase in total numbers of bacteria along the small intestinal tract, in the cecum, and in the proximal and distal large intestine resulting in falcutative microorganism being <0.01 – 1.0% of total population (Callaway et al. 2012).

A study of Khachatryan et al. (2008) showed the link between host genotype and the corresponding shifts in the gut microbiota. Mutations in a single host gene named MEFV (Mediterranean fever), which is involved in regulation of innate immunity, lead to specific restructuring of commensal gut microbiota. Analysis of gut bacterial diversity revealed highly specific, well-separated and distinct grouping, which depended on the allele carrier status of the host (Khachatryan et al. 2008). Guan et al. (2008) reported the important role of host genetics in rumen microbial structure. Analysis of detectable bacterial PCR-DGGE profiles showed that efficient steers (who have low residual feed intake) clustered together and were clearly separated from those obtained from inefficient steers (who have high residual feed intake), implicating that specific group may only inhabit in efficient steers. Likewise, the similarity of bacterial component in the rumen of efficient steers (91%) was more than those of inefficient steers (71%) (Guan et al. 2008). In another study, Li et al. (2016) examined the rumen microbiota of the parents and their offspring using the hybridization of sika deer and elk. The results indicated microbiota of the hybrids significantly differed from that of their parents,

suggesting the rumen microbiota were largely affected by host genetics, which may be related to the transmission of microbiota to the offspring during birth (Li et al. 2016).

2.4.5. Other factors

Penders et al. (2006) reported that delivery and birth characteristics have influence on infant's microbiota. Infant who was born through cesarean section had lower colonization rates and counts of bifidobacteria and *Bacteroides fragilis* group species, whereas prevalence and counts of *Clostridium difficile* and counts of *E. coli* were higher in comparison with vaginal delivery (Penders et al. 2006). However, the influence of delivery methods is less effective in developing countries because of some reports showed the same early colonization with enterobacteria in newborns delivered either vaginally or by caesarean section, in the hospital or at home (Adlerberth et al. 1991; Mackie et al. 1999; Fanaro et at. 2003). Preterm birth infants were colonized more often by *C. difficile* with higher counts compared with term infants. Formula – fed infants showed the colonization of *E. coli*, *C. difficile*, *B. fragilis* group and lactobacilli more than breastfed infants (Penders et al. 2006). *Staphylococci* have been found more frequently in breastfed infants, compared to bottled-fed infants (Fanaro et al. 2003). Another study of Harmsen et al. (2000) reported that in all breastfed infants the flora was dominated by bifidobacteria, whereas similar numbers of *Bacteroides* spp. and bifidiobacteria were found in most formula-fed infants (Harmsen et al. 2000; Fanaro et al. 2003). Interestingly, infants who have older siblings owned lower total bacterial counts per gram of feces than that of infants who had no sibling. Moreover, infants with older siblings indicated the greater proportion of bifidobacteria compared with infants without siblings (Penders et al. 2006).

Shanks et al. (2011) emphasized the role of management practices to community structures of fecal bacteria in cattle. Data suggested that animals subjected to similar management practices are more closely associated with one another than with animals from different groups. Respective key metabolic processes are most likely linked to particular management practices rather than to site-specific attributes, such as water source, elevation, humidity, or other factors closely associated with a particular geographic location (Shanks et al. 2011). The role of geography in shaping diversity of the gut microbiota was confirmed in case of house mice and human. Lewis et al. (2017) indicated that 8.4% of total variation of the fecal microbiota of breast-fed infants from Armenia and Georgia was contributed by the differences in location. Armenian infants had more *Enterobacteriaceae*, *Planococcaceae*, *Streptococcus*, *Enterococcus*, *Bacteriodes*, *Clostridium*, and *Staphylococcus* than those of Georgian infants (Lewis et al. 2017). In house mice, Linnenbrink et al. (2013) determined the

most significant contributing factor to microbial diversity between individuals to be geography, as measured by the distance between sampling sites. The authors showed that the variation of not only digesta-associated microbiota (11%) but also mucosa-associated microbiota (16%) was derived from geographic distance. The geography might be viewed as an approximation for the sum of environment effects such as local weather patterns, food intake, and behavior, culture in case of human. Therefore, the mechanism which drive the differences in the gut microbiota has not been identifiable yet (Linnenbrink et al. 2013).

CHAPTER 3
CHANGES IN GUT MICROBIOTA OF HOLSTEIN HEIFERS DURING GROWING STAGE

3.1. Abstract

Fecal microbiota of seven Holstein heifers was examined during growing stage, wherein weaning and commencement of silage feeding were practiced. Denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) were employed for microbiota analyses. Populations of total bacteria and *Bacteroides-Prevotella-Porphyromonas*, *Clostridium* XIVa, and *Clostridium* I&II groups were relatively stable in abundance during the growing stage, and those of *Lactobacillus* and *Bifidobacterium* groups decreased after weaning. Regarding bacterial composition, distinctive changes were shown after weaning in all bacterial taxa examined, whereas no changes were observed thereafter. The composition of *Lactobacillus* group changed over the growing stage, while maintaining high similarity between individual heifers. These findings indicated that weaning had a marked influence on the gut microbiota especially in the composition. Management during this transition period may be critical in shaping gut microbiota, whereas silage microbiota would have no effects.

3.2. Introduction

During early life, the primary colonization of bacteria is rapid but the climax microbiota population is gradually established over the time. Efficient growth of pre-weaned dairy calf is a precondition for the optimal performance in post-weaning period and further productivity (Soberon et al. 2012). The perception that calf microbiota is related to growth and health were indicated (Donovan et al. 2002), that inferred to the development of the immune system, the influence on host's physiology, including energy balance (Mazmanian et al. 2005; Backhed et al. 2004). A newborn calf is considered as a non-ruminant from a functional viewpoint. Calf relied almost on milk or milk replacer and a small amount of solid feed as a negligible level during the early weeks of life. Later, since weaning, the calf started increasing amount of solid feed, that supporting to the development of the forestomach, and leading to full function of a ruminant. These changes in anatomy and physiology of the gastrointestinal tract are accompanied with the succession of gastrointestinal microbiota. The transition of diet from milk to solid feed, for instance, roughage and concentrates, has also been demonstrated a substantial effect on the gastrointestinal microbiota (Callaway et al. 2010). Although several

studies had been conducted to clarify the establishment of gut microbiota in early life after calving, most of them focused on the very early time of colonization of gut microbiota in pre-weaned calves (Li et al. 2012; Oikonomou et al. 2013; Rey et al. 2013; Klein-Jobstl et al. 2014). Others focused on the development of gut microbiota in bovine from birth to adulthood by examining different groups of age (Jami et al. 2012). The sequential succession of gut microbiota during growing stage in dairy calves has not been elucidated. Hence, in this study, feces samples were repeatedly collected from seven Holstein heifers prior and posterior to weaning, and at the termination of growing stage to examine sequential changes taken place in gut microbiota during growing stage.

3.3. Materials and Methods

Feces sample collection and DNA extraction – Seven Holstein heifers were involved in this study. Feces samples were taken repeatedly three times during growing: prior and posterior to weaning, and at the termination of growing stage (3 weeks, 4 months, and 8 months after calving). At the third sampling time, calves had already started to take silages. Feces samples were kept on ice and transferred to laboratory, and further stored at -30^{o}C for downstream processing.

Bacterial DNA was extracted from 200 mg frozen feces sample using repeated bead beating plus column method (Yu and Morrison, 2004).

PCR amplification – Extracted DNA was subjected to PCR with universal primers including GC357f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACG GAGGCAGCAG-3') and 517r (5'-ATTACCGCGGCTGCTGG-3') for V3 region amplification. PCRs were also performed using the primer pairs of Bac303f (5'-GAAGGTC CCCCACATTG-3') and Bac708r (5'-CAATCGGAGTTCTTCGTG-3') for targeted *Bacteroides-Prevotella-Porphyromonas* community (Bartosch et al. 2004), Clost-f (5'-AAAGGRAGATTAATACCGCA TAA-3') and Clost-r (5'-TTCTTCCTAATCTCTACGCA-3') for targeted *Clostridium* cluster I&II community (Hung et al. 2008), Lab159f (5'-GGAAA CAGRTGCTAATACCG-3') and Lab617r (5'-CACCGCTACACATGGAG-3') for targeted *Lactobacillus* community (Heilig et al. 2002), and Bif 164f (5'-GGGTGGTAATGCCGGATG-3') and Bif 601r (5'-TAAGCGATGGACTTTCACACC-3') for targeted *Bifidobacterium* community (Bernhard et al. 2000). Nested PCRs were employed with universal primers on previous generated products from amplification of *Bacteroides*, *Clostridium* and *Lactobacillus* groups. PCR products were separated by electrophoresis on 2% agarose gel including ethidium bromide.

DGGE analysis – PCR products were separated by DGGE by using the DCode Universal Mutation Detection System (Bio-Rad Laboratory Inc.). Polyacrylamide gels consisted of 8% (v/v) polyacrylamide. Denaturing gradient (Urea and Formamide) of 25 - 50% was used for the separation of the generated. Electrophoresis was performed for 8 h at 150 V in TAE buffer at a constant temperature of 60°C. Gels were stained with SYBR Green, observed by UV transilluminator and photographed.

Species identification – PCR amplicons (with 357f (without GC clamp) and 517r primers) resulted from excised bands on DGGE gel were purified using FastGene® Gel/PCR Extraction Kits (Nippon Genetics Co., Ltd., Japan) according to the manufacturer's instructions, inserted in to pTAC–1 vector and cloned into Escherichia coli strain DH5α competent cells (DynaExpress TA Cloning Kit; BioDynamics Laboratory Inc., Tokyo, Japan). Positive white clones are randomly selected and confirmed for the presence of expected size inserts by PCR. Subsequently, positive colonies were sub-cultured into LB broth media. Extracted plasmids should be employed for a new PCR amplification followed by purification. To enhance the quality of sequencing, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instructions. DNA sequences were analyzed using an ABI PRISM 3130 sequencer (Applied Biosystems Inc., Foster City, Calif., U.S.A.). The Basic Local Alignment Search Tool (BLAST) program andGenBank databases were used to determine the closest relatives of partial 16S rRNA gene sequences. Unknown sequences that shared greater than 98% identity with a sequence in the BLAST database were considered as identified.

Quantitative PCR – A master mix for total quantification (357f (5'-ACGGGGGGCCTACGGA GGCAGCAG-3') and 517r (5'-ATTACCGCGGCTGCTGG-3')), *Bacteroides-Prevotella-Porphyromonas* population (BPP fwd (5'-GGTGTCGGCTTAAGTGCC AT-3') and BPP rev (5'-CGGA(C/T)GTAAGGGCCGTGC-3')), *Clostridium* cluster I&II population (Cl-perf fwd (5'-ATGCAAGTCGAGCGA(G/T)G-3') and Cl-perf rev (5'-TATGCGGTATTAATCT (C/T)CC TTT-3')), *Clostridum* cluster XIVa (Clost-Eub fwd (5′-CGGTACCTGACTAAGAA GC-3′) and Clost-Eub rev (5′-AGTTTYATTCTTGCGAACG-3′)), *Lactobacillus* population (Lact fwd (5'-AGCAGTAGGGAATCTTCCA-3') and Lact rev (5'-CACCGCTACACATGG AG-3')), *Bifidobacterium* population (Bifdo fwd (5′-TCGCGTCYGGTGTGAAAG-3′) and Bifdo rev (5′-CCACATCCAGCRTCCAC-3′)) (Rinttilä et al. 2004) was prepared for each well contained following reagents: 2 x KAPA SYBR FAST qPCR Master Mix Universal (Kapa Biosystem Inc.) that contained integrated antibody-mediated hot start, SYBR Green I fluorescent dye, $MgCl_2$, dNTPs, forward and reverse primers, and distilled water to a total of

23 µl per well. To these, 2 µl of each sample or standard was added, and the plates were briefly spun down and placed in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratory, Inc.) for analysis. Amplification programme involved an initial denaturation step at 95^{o}C for 5 min, followed by 35 cycles of denaturation at 95^{o}C for 15s, primer annealing at optimal temperatures for each target bacterial group for 20 s, extension at 72^{o}C for 30 s and an additional incubation step at 80-85^{o}C for 30s to collect the fluorescent data (Rinttilä et al. 2004).

Statistical analysis – Binary matrix resulted from DGGE banding patterns of *Lactobacillus* group was created to describe the presence or absence of individual band in all lanes. Resulted binary matrix was engaged to create Cluster Analysis to demonstrate similarity and differences between sampling times and individual calves, using Primer version 7 with Permanova + add-on software (Primer-E, Plymouth Marine Laboratory, and Plymouth, UK). Data for bacterial population were subjected to repeated measures of ANOVA and mean values were separated by Tukey's multiple range test. All data are presented as means with their standard deviation.

3.4. Results

3.4.1. Changes in bacterial population and structure during growing stage

Total bacterial population, the major bacterial groups (*Bacteroides-Prevotella-Porphyromonas; Clostridium XIVa*), pathogenic group (*Clostridium I&II*), and so-called beneficial groups (*Bifidobacterium*, *Lactobacillus*) were examined during growing stage of seven Holstein heifers (Table 3.1). In general, bacterial number was relatively stable during growing stage for total bacteria, *Bacteroides-Prevotella-Porphyromonas, Clostridium XIVa,* and *Clostridium I&II* population. These populations showed a light decrease after weaning and increase again in bacterial number after taking silage (Changes were not significant). Only *Lactobacillus* and *Bifidobacterium* population indicated a significant decrease in number after weaning (Table 3.1).

Cluster analysis results showed that similarity of total bacterial structure of Holstein heifers increased after weaning and no change was observed thereafter. Taking an insight into individual groups, the changing pattern was different between groups. *Bacteroides-Prevotella-Porphyromonas, Bifidobacterium* and *Clostridium I&II* groups formed two clear separated groups before and after weaning point. This indicated these bacterial structures were influenced by weaning than silage taking. *Lactobacillus* group showed significant changes in structure over the growing stage. This was demonstrated by three different groups at each sampling time, while maintaining synchronized composition between calves (Fig. 3.3).

Table 3.1. Quantification PCR results (log 16S rRNA gene copy number/g fresh matter) of total bacteria, *Bacteroides-Prevotella-Porphyromonas*, *Clostridium cluster XIVa, Clostridium I&II, Bifidobacterium,* and *Lactobacillus* population (Period 1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively).

	Total bacteria	***BPP***	***Clostridium* XIVa**	***Clostridium* I&II**	***Bifidobacterium***	***Lactobacillus***
Period 1	10.7 ± 0.66	10.1 ± 1.59	9.42 ± 1.18	6.19 ± 0.56	8.83 ± 0.62^{x}	6.28 ± 0.48^{x}
Period 2	10.6 ± 0.28	9.91 ± 0.34	8.96 ± 0.39	6.17 ± 0.30	7.85 ± 0.16^{y}	5.39 ± 0.25^{y}
Period 3	11.1 ± 0.17	10.5 ± 0.31	9.30 ± 0.24	6.02 ± 0.84	8.51 ± 1.11^{xy}	5.67 ± 0.20^{y}

Results are means ± standard deviation of seven samples in every sampling time. Samples that are labeled by different characters are considered statistically different.

DGGE profile and species identification of *Lactobacillus* community were represented in Figure 3.3. Banding pattern was different between periods. Composition of this community also changed during growing. Before weaning, *Lactobacillus* community was dominated by *L. delbrueckii* (band 1), *L crispatus* (band 3), *Faecalicoccus pleomorphus* (band 5), *L. gasseri* (band 6), *Streptococcus constellatus* (band 9), *L. amylolyticus* (band 10), *F. acidiformans* (band 11), *S. constellatus* (band 12), *L. johnsonii* (band 13, 14). After weaning, only few of these bacterial species were further maintained their presences, for instance, *L. gasseri* (band 6), *L. amylolyticus* (band 10), *L. johnsonii* (band 13). And, several species newly appeared instead, such as uncultured bacterium (band 2), *L. jonhsonii* (band 4), *L. pontis* (band 8). When animals took silages, *L. gasseri* (band 6) was gone, *L. pontis* (band 7) exclusively came to sight at this period in several heifeirs (Fig. 3.3).

Bifidobacterium community were also examined as Figure 3.4. The diversity of *Bifidobacterium* community was increased after weaning. Before weaning, this *Bifidobacterium* community was varied between calves, several species (band 2, band 6, *B. boum*-band 7, *B. pseudocatenulatum*-band 8, and band 9) was found in this period. After weaning, *Bifidobacterium* structure became similar between calves and there was no significant difference after calves consumed silage. *B. longum* (band 1), *B. pseudolongum* (band 4), and uncultured bacterium (band 5) were steadily present at period 2 and 3 in several calves (Fig. 3.4.).

3.4.2. Comparison between Holstein heifers and Japanese Black calves

The same examination was conducted for Japanese Black calves at the same periods during growing stage. The simple comparison was carried to understand the dynamic changes in gut microbiota regarding to breed. In general, similarity between cattle appeared to increase

after weaning period for both Japanese Black calves and Holstein heifers. While Holstein heifers revealed significant changes in composition before and after weaning, Japanese Black calves showed changes in bacterial structure at every single sampling time (Fig. 3.2).

The changing pattern of individual groups was different between breeds. In Japanese Black calves, *Bacteroides-Prevotella-Porphyromonas* and *Clostridium I&II* communities showed different banding pattern at every sampling time. Cluster analysis indicated that calf-to-calf variation of *Bacteroides-Prevotella-Porphyromo* composition became the lowest at the termination of growing period (period 3). However, in *Clostridium I&II*, the community showed the highest synchronization between individuals at 4 months (Fig. 3.2). The other periods inferred the differences in composition among individual calves. Interestingly, in both breeds, *Lactobacillus* community showed significant changes in diversity over the growing stage while maintaining synchronized composition between calves.

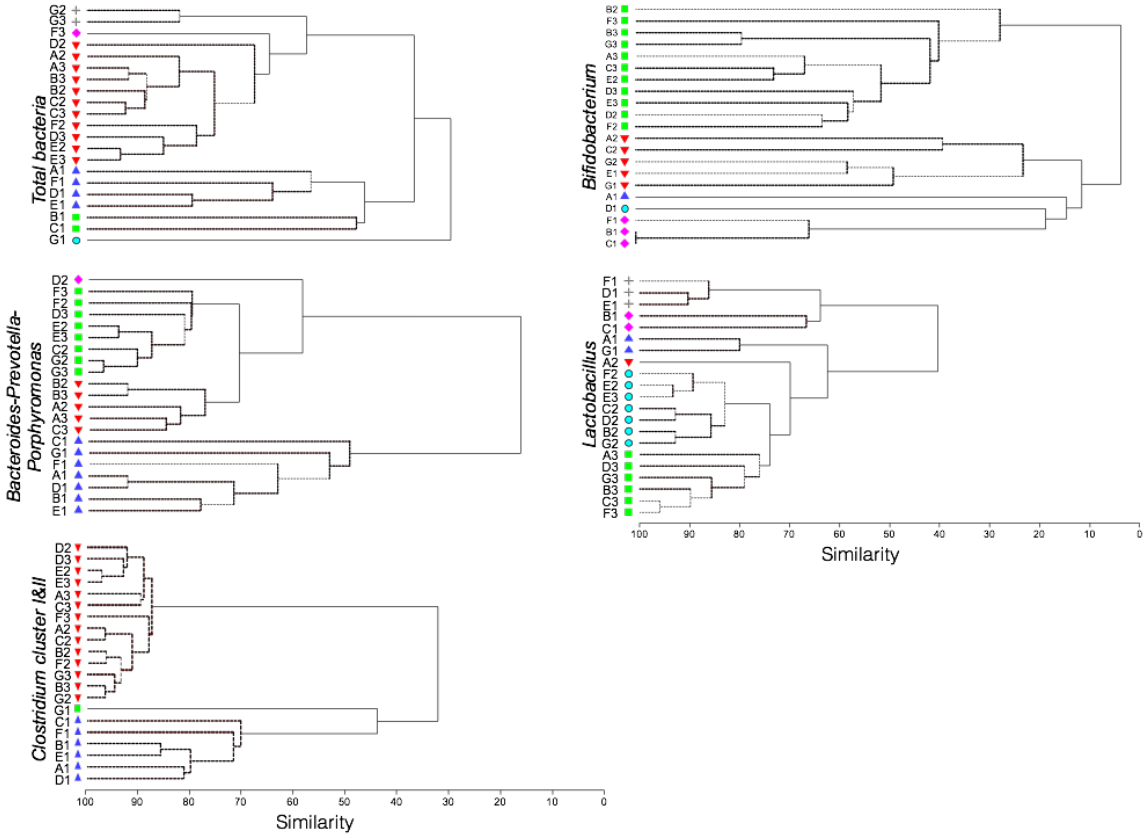

Figure 3.1. Cluster analysis results for *Lactobacillus, Bifidobacterium, Clostridium cluster I&II*, *Bacteroides-Prevotella-Porphyromonas*, and total bacterial community structure based on DGGE banding patterns of seven Holstein heifers (A, B, C, D, E, F, G, and H) during growing stage (1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively).

Japanese Black calves also disclosed changes taken place in bacterial population (Fig. 3.5). Total bacterial number tended to increase after weaning, whereas *Lactobacillus* population

decreased after weaning and reduced their size with advancement of age. Nevertheless, population size decreased in all cases at the last period. On the contrary, in Holstein heifers, bacterial population was relatively stable. Only *Lactobacillus* population showed the same trend of changing across periods (Fig. 3.5).

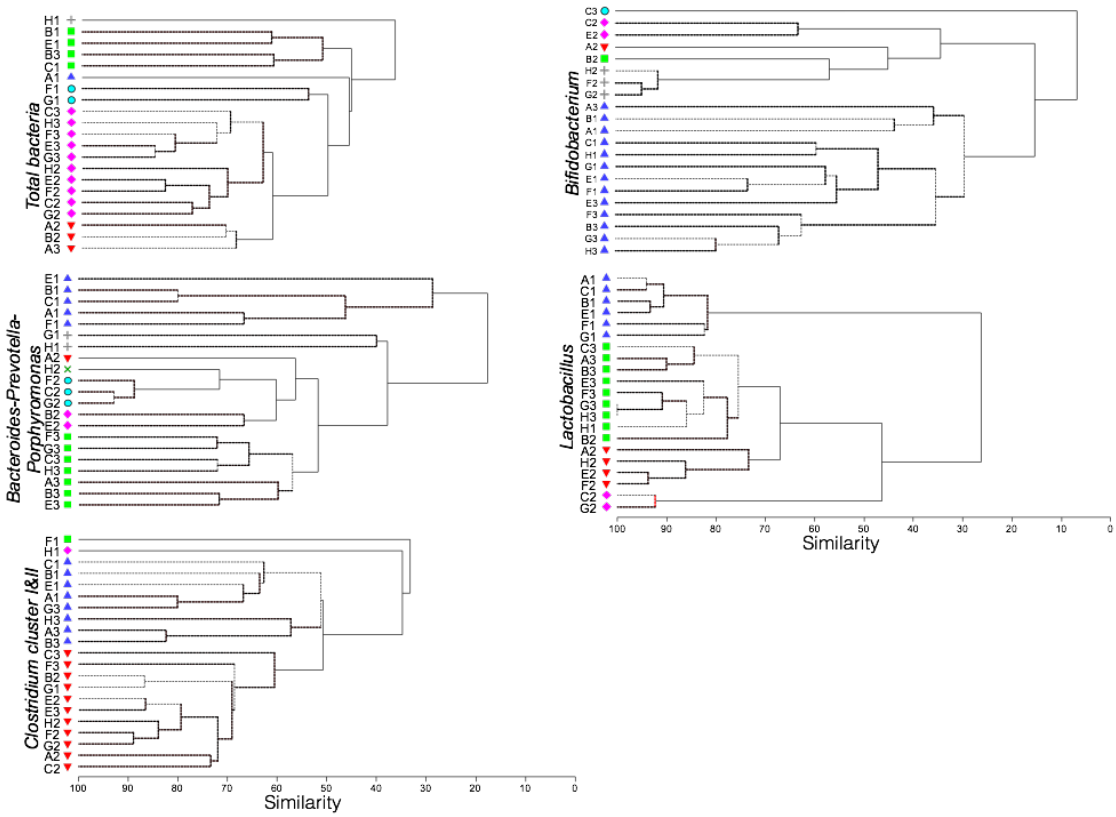

Figure 3.2. Cluster analysis results for *Lactobacillus, Bifidobacterium, Clostridium cluster I&II*, *Bacteroides-Prevotella-Porphyromonas*, and total bacterial community structure based on DGGE banding patterns of seven Japanese Black calves (A, B, C, D, E, F, G, and H) during growing stage (1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively).

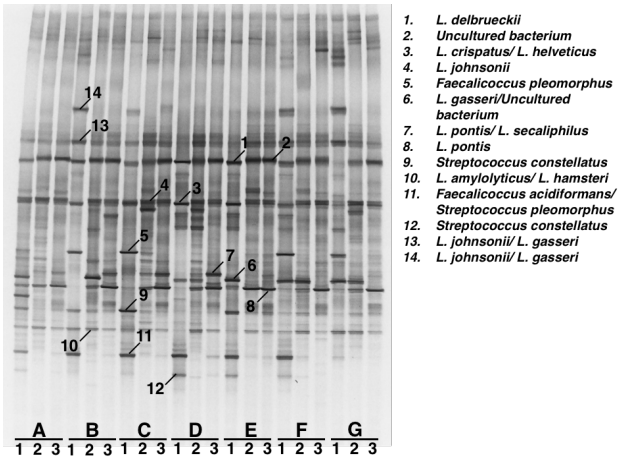

Figure 3.3. DGGE profile and species identification in *Lactobacillus* group of seven Holstein calves (A, B, C, D, E, F, and G) during growing stage (1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively).

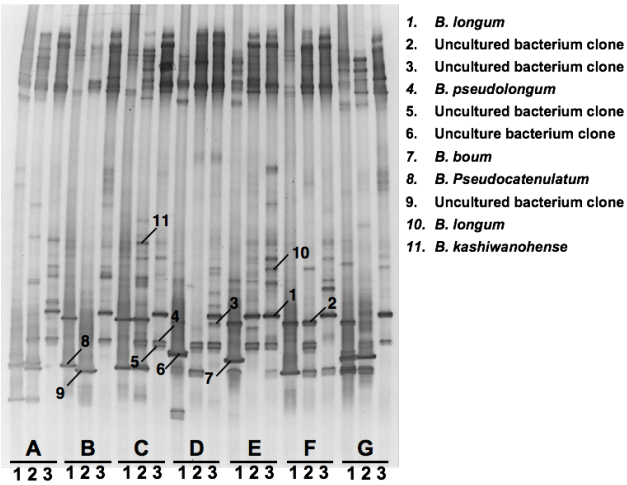

Figure 3.4. DGGE profile and species identification in *Bifidobacterium* group of seven Holstein calves (A, B, C, D, E, F, and G) during growing stage (1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively).

3.5. Discussion

In this study, fecal microbiota of seven young Holstein heifers was examined during growing stage, wherein weaning and commencement of silage feeding were employed. PCR-DGGE and qPCR were used for microbiota analyses. The changing pattern in gut microbiota of Holstein heifers during growing was different regarding to composition and abundance, and also with regard to bacterial groups. Weaning is the event that involving to the diet transition from liquid (milk) to solid feed, had a marked influence on the gut microbiota composition in our study.

Young ruminants at birth have an undeveloped rumen, until the system is fully matured they function as non-ruminant fed on milk-based diet that are not digested in the rumen but in the abomasum. The development of the rumen is an important physiological challenge for young ruminants, involves in the anatomical development, functional achievement and microbial colonization. In this study, total bacteria and *Bacteroides-Prevotella-Porphyromonas*, *Clostridium* XIVa, *Clostridium* I&II groups were stable in abundance over the time. Regarding to *Bacteroides-Prevotella-Porphyromonas* group, this group is the combination of the two most abundant genera (*Bacteroides* and *Preovotella*) of phylum *Bacteroidetes* in calves (Li et al. 2012; Rey et al. 2013). Even though the abundance of combination of these two genera was showed constant in Holstein heifers of our study, changing pattern of these individual genera was reported to be different by aging. In a report of Klein-Jobstl et al. (2014), during early development of Simmental calves, genus *Bacteroides* in feces

generally decreased in relative abundance over the time despite of its particularly high abundance before weaning (Klein-Jobstl et al. 2014). This was consistent to the findings in the rumen of developing calves (Li et al. 2012; Jami et al. 2013). The reason was supposed to the presence of *Bacteroides* in the vagina of healthy cows, and calves received *Bacteroides* by ingestion during passage through the birth canal (Klein-Jobstl et al. 2014). In adult cattle, relative abundance of *Bacteroides* was negatively associated with high fiber diet (Kim et al. 2014). Hence, the increase of fiber and decrease of milk consumption could explain for the decrease of *Bacteroides* with the advancement of age (Klein-Jobstl et al. 2014). In contrast to *Bacteroides*, genus *Prevotella* indicated an increase in abundance over the time (Jami et al. 2013). However, the concrete timing for *Prevotella* to take over the exclusive position of *Bacteroides* is yet to be known. Jami et al. (2013) reported the prevalence of *Prevotella* was seen from 2 months of age in rumen of Holstein calves, while Rey et al. (2013) detected the high abundance of this genus as early as 15 days of age (Jami et al. 2013; Rey et al. 2013). On the opposite side of other reports, Li et al. (2011) discovered the increase of *Bacteroides* and decrease of *Prevotella* from 14 days of age to 42 days of age of pre-ruminant calves (Li et al. 2011). After all, it is obvious to see the mutual compensation between these two genera in abundance in gut microbiota of cattle. This partly explained for the relatively stable in the combined abundance of these two genera in our study.

In the gastrointestinal tract, genus *Clostridium* make up a substantial part of the total bacteria in the gut microbiota. It is likely that genus *Clostridium* play a crucial role in gut homeostasis by interacting with the other resident microbe populations, but also by providing specific and essential functions, such as maintenance of overall gut function (Lopetuso et al. 2013). However, it has not been paid attention in several reports. In this study, we examined the two important groups of *Clostridia* in the gut, *Clostridium* XIVa and *Clostridium* I&II. Principally, the cluster *Clostridium* XIVa includes species belonging to the *Clostridium*, *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Dorea*, *Lachnospira*, *Roseburia* and *Butyrivibrio* genera. And, cluster *Clostridium* I&II (or *Clostridium perfringens* group) includes *Clostridium perfringens* and *Clostridium tetani*. While members of cluster *Clostridium* XIVa related to butyrate production that supporting to overall gut health, members of cluster *Clostridium* I&II possessed pathogenic species (Lopetuso et al. 2013). The stable abundance of genus *Clostridium* may implicate a certain balance status in gut microbiota promoting to a good condition of gut health of Holstein calves in our study.

Lactobacillus and *Bifidobacterium* was considered as beneficial bacterial groups in cattle (Callaway et al. 2012). The abundance of these two genera were found positively

correlated with the expression level of miRNAs, that relating to the gut development mechanism, for instance, the differentiation and proliferation of the cells of gastrointestinal tract (Liang et al. 2014). The gradual decrease in abundance of genera *Lactobacillus* and *Bifidobacterium* after weaning was consistent with previous reports (Oikonomou et al. 2013; Klein-Jobstl et al. 2014). Oikonomou et al. (2013) reported that the prevalence of *Lactobacillus* and *Bifidobacterium* reached the maximum during the first fourth week of calf life, and then progressively decreased over the time. The two genera were supposed to be related to digestion of milk, that explained for the low abundance after weaning (Oikonomou et al. 2013). Klein-Jobstl et al. (2014) showed that *Lactobacillus* had relatively low abundances after weaning. The early appearance of *Lactobacillus* might be due to milk feeding or the incorporation of these bacteria during passage of the vagina during natural birth (Dominguez-Bello et al. 2010; Klein-Jobstl et al. 2014).

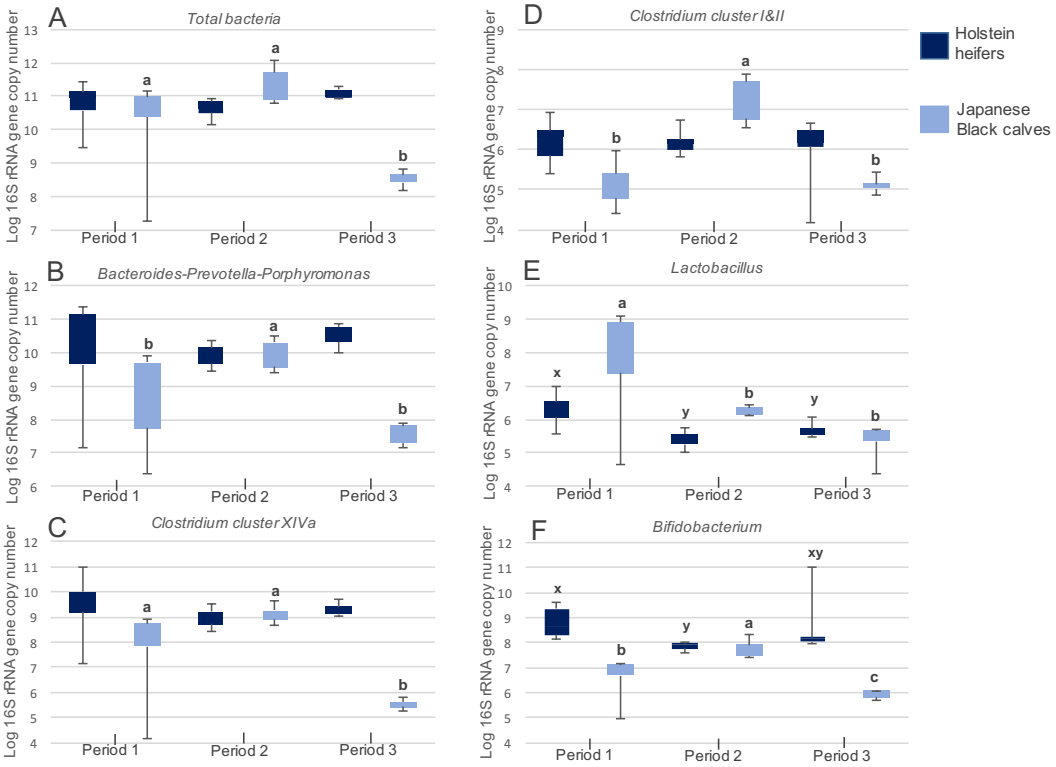

Figure 3.5. Comparison of total bacteria population (A), *Bacteroides-Prevotella-Porphyromonas* (B), *Clostridium cluster XIVa* (C), *Clostridium I&II* (D), *Lactobacillus* (E), *and Bifidobacterium* (F) population between Holstein heifers and Japanese Black calves during growing stage (Period 1, 2, and 3 - 3 weeks, 4 months and 8 months after calving, respectively). Boxes that are labeled by different characters are considered statistically different, for Japanese Black calves (a, b, and c) and for Holstein heifers (x, y).

In current study, the composition of total bacteria and several bacterial groups showed great differences before and after weaning but no change was observed thereafter. This finding indicated that weaning had a marked influence on gut microbiota (Fig. 3.1.). The similarity in composition between seven heifers increased with the advancement of age. Among bacterial groups examined, *Lactobacillus* showed higher similar between cows than other bacterial groups. The finding of our study were in agreement with the report of Jami et al. (2013). Report indicated that the diversity and within-group similarity increased with age, suggesting a more diverse but homogenous and specific mature community, compared with the more heterogenerous and less diverse primary community (Jami et al. 2013).

Holstein heifers and Japan Black calves examined in this study were raised with different dietary regime and in different farm environments. Shanks et al. (2011) showed that bovine management played an integral role in fecal microbial community structure than the site-specific attributes, such as water source, elevation, humidity and particular geographic location. This could account for the similarity of bacterial structure between calves kept under the same management, which was proven by cluster analysis (Fig 3.1&3.2). Likewise, Holstein heifers and Japanese Black calves evidently possessed their own pattern of changing in gut microbiota during growing. Combined analyses of DGGE and qPCR revealed that weaning period implicated greater on bacterial population than on bacterial composition in beef cattle, whereas contrary state was found in dairy cattle. *Lactobacillus* spp. community showed significant changes in composition over the growing stage regardless of breed, while maintaining the highest similarity between individual calves. The difference between two systems may also derived from the managing of young ruminants. In dairy farming, heifers are typically separated from their mother, on the contrary, in beef farming, the calves remain with their mothers until weaning. Abecia et al. (2014) implied that kid goats reared with mothers had greater rumen development than their twins that were fed on milk replacer and separated from adult animals (Abecia et al. 2014). This is consistent with report of De Paula Vieira et al. (2012) that calves grew up with the presence of older adult animals exhibited more frequent and longer visits to the feeder. That was hypothesized as a consequence of social learning (De Paula Vieira et al. 2012).

In conclusion, in Holstein heifers, populations of total bacteria and *Bacteroides-Prevotella-Porphyromonas*, *Clostridium* XIVa, and *Clostridium* I&II groups were relatively stable in abundance during the growing stage. *Lactobacillus* and *Bifidobacterium* groups decreased their abundance after weaning. Regarding bacterial composition, distinctive changes were shown after weaning in all bacterial taxa examined, whereas no changes were observed

thereafter. The composition of *Lactobacillus* group changed over the growing stage, while maintaining high similarity between individual heifers. These findings indicated that weaning had a marked influence on the gut microbiota especially in the composition. Management during this transition period may be critical in shaping gut microbiota, whereas silage microbiota would have no effects.

CHAPTER 4
VARIABILITY, STABILITY, AND RESILIENCE OF FECAL MICROBIOTA IN DAIRY COWS FED WHOLE CROP CORN SILAGE

4.1. Abstract

The microbiota of whole crop corn silage and feces of silage-fed dairy cows were examined. A total of 18 dairy cow feces were collected from six farms in Japan and China, and high-throughput Illumina sequencing of the V4 hypervariable region of 16S rRNA genes was performed. *Lactobacillaceae* were dominant in all silages, followed by *Acetobacteraceae*, *Bacillaceae*, and *Enterobacteriaceae*. In feces, the predominant families were *Ruminococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Rikenellaceae*, and *Paraprevotellaceae*. Therefore, *Lactobacillaceae* of corn silage appeared to be eliminated in the gastrointestinal tract. Although fecal microbiota composition was similar in most samples, relative abundances of several families, such as *Ruminococcaceae*, *Christensenellaceae*, *Turicibacteraceae*, and *Succinivibrionaceae* varied between farms and countries. In addition to the geographical location, differences in feeding management between total mixed ration feeding and separate feeding appeared to be involved in the variations. Moreover, a cow-to-cow variation for concentrate-associated families was demonstrated at the same farm; two cows showed high abundance of *Succinivibrionaceae* and *Prevotellaceae*, whereas another had a high abundance of *Porphyromonadaceae*. There was a negative correlation between forage-associated *Ruminococcaceae* and concentrate-associated *Succinivibrionaceae* and *Prevotellaceae* in 18 feces samples. *Succinivibrionaceae*, *Prevotellaceae*, *p-2534-18B5*, and *Spirochaetaceae* were regarded as highly variable taxa in this study. These findings help to improve our understanding of variation and similarity of the fecal microbiota of dairy cows with regard to individuals, farms, and countries. Microbiota of naturally fermented corn silage had no influence on the fecal microbiota of dairy cows.

4.2. Introduction

The relationship between gut microbiota and their host has been suggested to provide critical benefits to animals. Cattle and their diverse community of symbiotic microbiota is a typical example. These symbionts are in charge of digesting and fermenting plant materials into nutrient sources usable by the host. The cattle acquire the fermentation products for body maintenance, growth, and milk production (Jami and Mizrahi 2012a, b; Jewell et al. 2015). Although several studies have been conducted using next generation sequencing (NGS) for

understanding the gut microbiota of cattle, several questions remain (Menezes et al. 2011; Mao et al. 2015; Liu et al. 2016). Despite reports indicating that the core bacterial community is shared by all cattle; other studies have observed high variation in the relative microbial abundance across samples (Jami and Mizrahi 2012a, b; Jewell et al. 2015; Lima et al. 2015). The divergence of abundance was suggested to be due to differences in diet formulation, farm management, and geographical locations (Menezes et al. 2011; Shanks et al. 2011; Anderson et al. 2016; Khafipour et al. 2016). Similarly, differences were also observed in gut microbiota structure even when cows were supplied with the same diet and had similar milk yields and rumen fluid profiles (Welkie et al. 2009). There is therefore a need to fully understand the extent of variation between microbiota from individual cows under similar diet and management.

In dairy practice, silage feeding is a popular management method (Castillo et al. 2001; Broderick and Radloff 2004; Beauchemin and Wang 2005; Benchaar et al. 2007). Silage is a product of the ensiling process, in which lactic acid fermentation usually occurs. Therefore, it can be considered as a lactic acid bacteria (LAB)-rich nutrient source for dairy cows. Because dairy cows receive the silage microbiota steadily through daily meals, silage could confer probiosis especially when silage LAB have acceptable survivability and compatibility throughout the gastrointestinal tract. Although several studies have emphasized the effects of diet on shaping gut microbiota, whether food microbiota influence the gut microbiota has not been elucidated.

In the present study, we conducted a survey of the fecal microbiota of dairy cows fed corn silages in Japan and China. Corn silage has been reported to harbor a predominant proportion of *Lactobacillus* spp. (Ni et al. 2017), and NGS was utilized to get insight into the microbiota of silage and feces of silage-fed dairy cows. The objective was to improve our knowledge of variations in the fecal microbiota of dairy cows with regard to country, farm, and individual cows. We also examined whether silage microbiota influence gut microbiota.

4.3. Materials and methods

Sampling – A total of 18 feces samples were collected from six dairy farms located in Kumamoto Province (farms A, B, and C), Japan, and in Shan Xi Province (farm D), He Bei Province (farm E), and Beijing city (farm E), China. Holstein dairy cows were fed a diet containing whole-crop corn silage that was self-produced at each farm using a bunker silo without bacterial inoculants. In farms A, C, D, and E, feed was provided as a total mixed ration (TMR), and in farms B and F, forage and concentrate were provided separately (Table 4.1). The proportion of corn silage in the total diet varied from 0.20 to 0.35 on a DM basis. Silage

samples were collected by digging into the silo face to approximately a 0.2 m depth and manually removing approximately 500 g of silage. Five samples (2 outer samples from the top layer, 2 outer samples from the bottom layer, and 1 sample from the central part) were obtained from each bunker silo and were thoroughly mixed to prepare a representative sample. Approximately 500 g of silage and 1 g of feces were placed in a plastic bag and an Eppendorf tube, respectively, and kept on ice during transportation to the laboratory.

The DM content of silage was determined after oven drying at 60°C for 48 h. The pH value and fermentation products in water extracts were determined. Lactic acid, acetic acid, and ethanol contents were measured by ion-exclusion polymeric high-performance liquid chromatography with refractive index detection as previously described (Ni et al. 2017).

Illumina MiSeq sequencing – Silage samples were added to a 20× volume of sterilized phosphate-buffered saline (pH 7.4), and extraction was achieved through vigorous shaking for 10 min at ambient temperature. Bacterial pellets were obtained by centrifugation at 8000 × g for 15 min, then washed once with 1 mL of solution containing 0.05 M D-glucose, 0.025 M Tris-HCl (pH 8.0), and 0.01 M sodium EDTA (pH 8.0). After centrifugation at 15,000 × g for 2 min, bacterial cells were lysed with 180 μL of lysozyme solution (20 g/L lysozyme, 0.02 M Tris-HCl [pH 8.0], 0.002 M sodium EDTA [pH 8.0], 1.2 g/L Triton X-100) at 37°C for 1 h. Subsequent bacterial DNA purification was performed using a commercial kit (DNeasy Blood & Tissue Kit; Qiagen, Germantown, MD, USA) according to the manufacturer's recommendations. Bacterial DNA of the feces was purified using the mini DNeasy Stool Kit (Qiagen, Germantown, MD, USA).

The resulting DNA was subjected to 2-step polymerase chain reaction (PCR) procedures to generate amplicon libraries for NGS. In the first round PCR, primers targeting the V4 region of 16S rRNA genes (forward: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVG GGTWTCTAAT-3′; tail sequences are underlined) were used. The PCR protocol included initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and a final elongation at 72°C for 5 min. The PCR products were purified by electrophoretic separation on a 2.0% agarose gel using a Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan), and moved to a second round of PCR with adapter-attached primers. The PCR protocol included initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, elongation at 72°C for 30 s, and a

final elongation at 72°C for 5 min. PCR products were purified as described above for the first round products.

The purified amplicons were pair-end sequenced (2 × 250) on an Illumina MiSeq platform at the FASMAC Co., Ltd. (Kanagawa, Japan). Raw sequences were processed using the QIIME (version 1.9.0) microbial ecology pipeline. The 250 bp reads were truncated at any site receiving an average quality score < 20 over a 20 bp sliding window. Truncated reads that were shorter than 50 bp were discarded. Similarly, two nucleotide mismatches in primer matching were removed, sequences that overlapped more than 50 bp were assembled, and chimeric sequences were identified and removed. Only sequences of at least 130 bp long after quality filtering were grouped into operational taxonomic units (OTUs) using a 97% similarity threshold. Sequences were classified from the phylum to the genus level using the default settings of the Ribosomal Database Project classifier. The results of the sequence analysis are available in the DDBJ Sequence Read Archive under project identification number PRJDB5800.

Statistical analysis – Quantitative NGS data were subjected to one-way analysis of variance. Tukey's multiple range test was performed using the JMP software (version 11; SAS Institute, Tokyo, Japan) to examine the differences between farms and countries. Major families in both silage and feces samples were engaged to create a heat map using Primer version 7 with Permanova+ add-on software (Primer-E, Plymouth Marine Laboratory, Plymouth, UK). In addition, the data were subjected to the Shannon diversity index and coefficient of variation calculations.

4.4. Results

4.4.1. Silage fermentation and microbiota

Fermentation products of whole crop corn silage produced in bunker silos of Japan and China are presented in Table 4.1. The DM content of silage was observed different between Chinese (221 g/kg) and Japanese (299 g/kg) samples. Mean lactic acid, acetic acid, ethanol, and 1-propanol contents in Chinese silages were greater than those in Japanese silages. Although lactic acid was predominant in all silages, propionic acid was present in silages Cs and Ds and a small amount of butyric acid (<1 g/kg DM) was detected in silages Ds and Es. 1,2-propanediol was found in all silages except silage Cs.

The NGS results of the silage microbiota indicated huge taxonomic diversity including 29 phyla, 200 families, and 302 genera. Among them, 12 phyla, 50 families, and 30 genera were shared in all samples. At the family level, the number of taxa was 118, 96, 113, 141, 119,

and 91 for silage As, Bs, Cs, Ds, Es, and Fs, respectively (Table 4.2). *Lactobacillaceae* was found to be exclusively dominant, accounting for 75.2, 87.5, 45.7, 91.6, 73.6, and 82.2% of the total population (Table 4.3). Clusters based on the family level indicated three separate groups regardless of the country (Fig. 4.1). Silage Cs showed a distinctive bacterial structure with the lowest proportion of *Lactobacillaceae* (45.7%) among the silages. This was followed by the *Acetobacteraceae* (32.4%), *Bacillaceae* (4.63%), *Enterobacteriaceae* (3.44%), *Comamonadaceae* (3.32%), *Enterococcaceae* (2.65%), *Moraxellaceae* (2.29%), and *Planococcaceae* (1.53%) families, which were shown to be greater than those in other silages. The Bs, Es, and Fs silage groups were different from the As and Ds groups, because of the higher proportion of *Acetobacteraceae* representing 9.14, 20.6, and 16.8%, respectively, in the former. The Shannon index showed numerically greater value for silage Cs (1.581) than for other *Lactobacillaceae*-dominated silages.

4.4.2. Fecal microbiota

The heat map in Figure 1 shows 50 of the most abundant taxa at the family level. The fecal microbiota was shown to be unrelated to the silage microbiota by a distinct grouping pattern. The NGS demonstrated a total number of 35 phyla, 184 families, and 263 genera allocating for all feces samples. Among them, 9 phyla, 28 families, and 34 genera were consistently present in all feces samples and covered 98.9, 75.6, and 32.7% of the total bacterial populations. *Ruminococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Rikenellaceae*, and *Paraprevotellaceae* were the most abundant families, accounting for 22.8, 10.6, 7.17, 7.13, 5.94, and 5.59% of the total population on average across 18 dairy cows (Table 4.4). *Lactobacillaceae* contributed to only 0.04% of the total population in feces samples. The numbers of taxa (ranging from 71.0 to 83.3) were numerically lower, and the Shannon index values (ranging from 2.205 to 2.549) were numerically greater for feces than silages (Table 4.2).

4.4.3. Variations between cows, farms, and countries

The coefficient of variation (CV) was employed for taxa detected at >1% of the total population in order to understand the variation of fecal microbiota across 18 cows (Table 4.5). If the threshold was defined as >1.0, 4 families (*Succinivibrionaceae*, *Prevotellaceae*, *p-2534-18B5*, and *Spirochaetaceae*) and 3 genera (*Succinivibrio* spp., *Prevotella* spp., and *Treponema* spp.) were evaluated as highly variable taxa. If the threshold was lowered to >0.5, 4 families (*Veillonellaceae*, *RF16*, *S24-7*, and *Porphyromonadaceae*) and one genus (*Prevotella* spp. belonging to family *Paraprevotellaceae*) were added as moderately variable taxa.

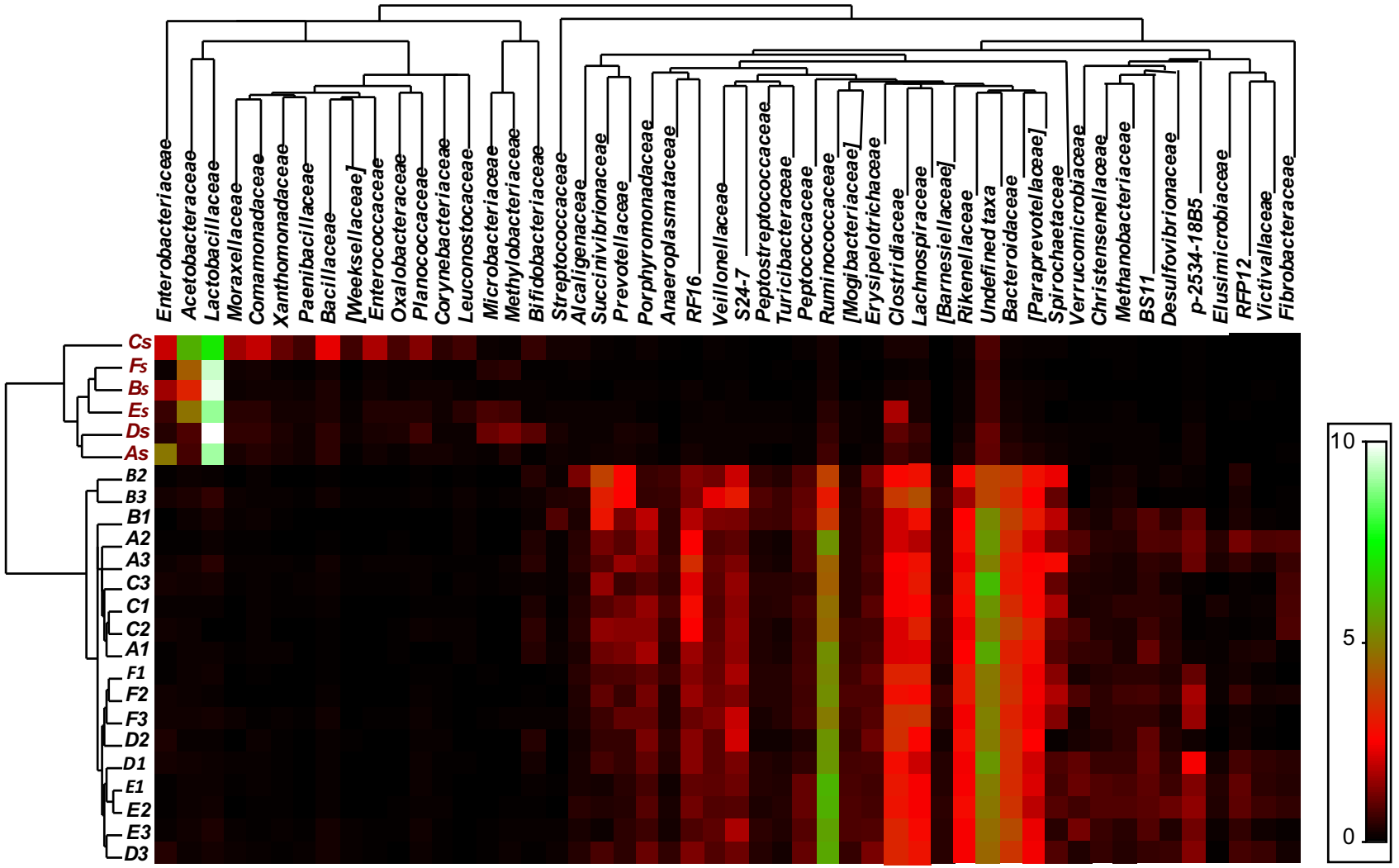

Figure 4.1. Heatmap representing the relative abundance of the major families (50 most abundant taxa) in silage and fecal microbiota. Clustering was performed using the Euclidean distance as a similarity metric. As, Bs, Cs, Ds, Es, and Fs indicate silage samples from farms A, B, C, D, E, and F, respectively. A-(1, 2, 3), B (1, 2, 3), C (1, 2, 3), D (1, 2, 3), E (1, 2, 3), and F (1, 2, 3) indicate feces samples from farms A, B, C, D, E, and F, respectively.

Three dairy farms (A, B, and C) in Japan were similar with regard to the number of taxa and Shannon index value for fecal microbiota at the family level (Table 4.2). However, the relative abundance of several families, such as *Ruminococcaceae*, *Peptococcaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, *Turicibacteraceae*, *Succinivibrionaceae*, and *Fibrobacteraceae* was statistically different between samples from the three farms (Table 4.4). In China, although the number of taxa was similar for samples from farms D, E, and F, the abundance of the bacteria varied, with respect to the families *Ruminococcaceae*, *Veillonellaceae*, *Mogibacteriaceae*, *Christensenellaceae*, *Turicibacteraceae*, *Succinivibrionaceae*, and *Spirochaetaceae*.

Firmicutes and *Bacteroidetes* were the first- and second-most predominant phyla in samples from both countries (Table 4.4). These two phyla contributed to 88.6 and 93.7% of total bacterial population in samples from Japan and China, respectively. The populations of *Ruminococcaceae*, *Clostridiaceae*, *Mogibacteriaceae*, and *Christensenellaceae* were higher, and those of *Paraprevotellaceae*, *RF16*, and *Prevotellaceae* were lower in samples from China than in those from Japan. The *Firmicutes* to *Bacteroidetes* ratio was, therefore, greater in Chinese samples (>1) than in Japanese samples (<1). Additionally, the unidentified family *p-2534-18B5*, *Succinivibrionaceae*, *Anaeroplasmataceae*, *Methanobacteriaceae*, and *Fibrobacteraceae* indicated statistical differences between the bacterial populations in samples from the two countries.

Cluster analysis revealed that, although differences between individual cows were observed, the composition of fecal microbiota was clearly different in the samples from the two countries. Moreover, two samples from farm B (B2 and B3) were grouped in a cluster separate from the cluster that included the other 16 feces samples. This grouping was determined based on the extremely high proportions of *Succinivibrionaceae* (9.83%), *Prevotellaceae* (4.41%), *Peptostreptococcaceae* (0.37%), and *Turicibacteraceae* (0.24%), and lower proportion of *Ruminococcaceae* (10.9%) in the B2 and B3 samples compared to those of other samples (Table 4.4). Therefore, cow-to-cow variation in farm B was greater than farm-to-farm and country-to-country variation.

Table 4.1. Feeding management, herd milk yield, diet formulation, and fermentation products of whole crop corn silage used in the six farms.

	Japan		China			
	A	B	C	D	E	F
Feeding management	Total mixed ration	Separate feeding	Total mixed ration	Total mixed ration	Total mixed ration	Separate feeding
Milk yield (g/day)	27	32	30	21	28	30
Diet formulation						
Corn silage	0.35	0.29	0.20	0.29	0.32	0.32
Other forages	0.16	0.21	0.39	0.11	0.18	0.16
Concentrates	0.49	0.50	0.41	0.38	0.50	0.52
Silage fermentation						
Dry matter (g/kg)	313	295	290	219	219	225
pH	3.98	3.94	3.88	3.92	3.51	3.52
Lactic acid (g/kg DM)	47.1	57.2	73.7	59.5	129	97.3
Acetic acid (g/kg DM)	33.8	46.7	29.2	51.1	45.4	26.6
Propionic acid (g/kg DM)	0.00	0.00	11.7	14.7	0.00	0.00
Butyric acid (g/kg DM)	0.00	0.00	0.00	0.62	0.74	0.00
Ethanol (g/kg DM)	3.94	10.2	9.78	15.1	34.2	19.7
1,2-Propanediol (g/kg DM)	25.2	42.9	0.00	5.50	25.1	13.3
1-Propanol (g/kg DM)	2.39	0.78	2.49	16.2	10.5	3.60

Table 4.2. Number of sequence reads, taxa, and Shannon diversity index values determined by next generation sequencing at the family level for the microbiota of whole crop corn silage and silage-fed dairy cows.

	Read	Taxa	Shannon
As	63574	118	0.781
Bs	71458	96	0.507
Cs	105573	113	1.581
Ds	76133	141	0.556
Es	76427	119	0.866
Fs	103149	91	0.537
A	69612 ± 14319	78.7 ± 29.8[a]	2.319 ± 0.147[a]
B	69232 ± 13384	71.0 ± 21.6[a]	2.549 ± 0.022[a]
C	64671 ± 6569	75.7 ± 22.9[a]	2.296 ± 0.100[a]
D	78106 ± 11620	83.3 ± 24.0[a]	2.226 ± 0.038[b]
E	65035 ± 11527	79.3 ± 21.4[a]	2.205 ± 0.021[b]
F	64308 ± 4394	71.0 ± 19.9[a]	2.319 ± 0.043[a]
Japan	67838 ± 10607	75.1 ± 21.9[a]	2.388 ± 0.150[a]
China	69150 ± 10817	77.9 ± 19.7[a]	2.250 ± 0.061[b]

4.5. Discussion

Several factors including diet formulation, management practice, and geographical location have been shown to influence the gut microbiota (Menezes et al. 2011; Shanks et al. 2011; Anderson et al. 2016; Khafipour et al. 2016). Regarding diet formulation, the forage-to-concentrate ratio is considered to have substantial effects on gut microbiota (Shanks et al. 2011; Khafipour et al. 2016). In the present study, dairy cows received diets with the forage-to-concentrate ratio varying from 62:38 to 52:48. The difference may be large enough to result in differences in fecal microbiota; however, farm-to-farm and country-to-country differences were observed even with similar forage-to-concentrate ratio. Because all cows were of the Holstein breed, farm-to-farm and country-to-country differences could, at least in part, be attributed to the differences in the geographical location.

Lactobacillaceae species accounted for the highest proportion of the total population of silage microbiota in all the six corn silages. At the family level, three separate groups were formed, and none of them were related to the country in which silage was produced. A group of silage Bs, Es, and Fs, another group of silage As and Ds, and silage Cs possessed their own patterns, and the divergence of *Acetobacteraceae* was the main cause for the group differentiation. *Acetobacter* spp. were the main contributor to the *Acetobacteraceae* family in this study. *Acetobacter* spp. are known to have strong crop specificity with respect to corn and cereal silages (Oude Elferink et al. 2001), and to have the ability to initiate aerobic deterioration

by oxidizing ethanol to acetic acid. Other typical spoilage-associated bacteria, e.g., *Bacillaceae* (*Bacillus* spp.) and *Enterobacteriaceae* (*Enterobacter* spp.), were also detected, and were especially high in silage Cs. However, all the silages examined were not obviously spoiled and were considered acceptable based on the pH and fermentation product content (Table 4.1). An exception of low abundance of *Lactobacillaceae* in silage Cs was similar to the finding of Kraut-Cohen et al. (2016), who observed a farm silage harboring <35% *Lactobacillaceae*, despite having pH and fermentation product content at acceptable levels. Among the six silage samples examined in the present study, only silage Cs had no detectable 1,2-propnediol, but the relevance to a large proportion of *Acetobacteraceae* was not known.

Table 4.3. Relative abundance of taxa detected at >0.1% of the total population in silage samples.

		As	Bs	Cs	Ds	Es	Fs
Phylum	*Firmicutes*	76.6	87.8	55.5	93.8	76.9	82.3
Family	*Lactobacillaceae*	75.2	87.5	45.7	91.6	73.6	82.2
Family	*Bacillaceae*	0.15	0.08	4.63	0.16	0.06	0.02
Family	*Clostridiaceae*	0.35	0.04	0.05	0.73	2.54	0.01
Family	*Enterococcaceae*	0.03	0.02	2.65	0.05	0.09	0.01
Family	*Planococcaceae*	0.04	0.02	1.53	0.31	0.09	0.01
Family	*Ruminococcaceae*	0.34	0.06	0.04	0.24	0.15	0.03
Family	*Lachnospiraceae*	0.16	0.05	0.05	0.28	0.05	0.01
Family	*Leuconostocaceae*	0.01	0.004	0.34	0.01	0.13	0.003
Family	*Paenibacillaceae*	0.02	0.01	0.35	0.03	0.03	0.001
Phylum	*Proteobacteria*	22.1	11.6	43.1	3.20	22.0	17.1
Family	*Acetobacteraceae*	0.42	9.14	32.4	0.51	20.6	16.8
Family	*Enterobacteriaceae*	21.1	2.28	3.44	0.12	0.26	0.01
Family	*Comamonadaceae*	0.10	0.03	3.32	0.20	0.15	0.04
Family	*Moraxellaceae*	0.05	0.01	2.29	0.20	0.13	0.02
Family	*Methylobacteriaceae*	0.08	0.01	0.01	1.36	0.35	0.16
Family	*Xanthomonadaceae*	0.05	0.02	0.86	0.07	0.04	0.01
Family	*Oxalobacteraceae*	0.01	0.01	0.45	0.07	0.09	0.01
Phylum	*Actinobacteria*	0.10	0.07	0.52	1.92	0.56	0.15
Family	*Microbacteriaceae*	0.01	0.003	0.02	0.97	0.44	0.11
Family	*Bifidobacteriaceae*	0.003	0.004	0.24	0.68	0.01	0.001
Phylum	*Bacteroidetes*	0.68	0.13	0.48	0.45	0.16	0.05

As-Fs indicate silages produced in bunker silos at farms A, B, C, D, E, and F, respectively.

The fact that *Lactobacillus* spp. dominate the microbiota of corn silage (Ni et al. 2017) was confirmed in this study. *Lactobacillaceae* ranged from 45.7% to 91.6% of the total bacterial population. However, in feces samples, *Lactobacillaceae* accounted for a very small proportion

(<0.1%), and appeared to be replaced by the predominance of *Ruminococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Rikenellaceae*, and *Paraprevotellaceae*. Although the decrease in *Lactobacillaceae* proportion could occur if silage microbiota was diluted in the rumen, the survival of *Lactobacillaceae* throughout the gastrointestinal tract of dairy cows was apparently not possible owing to the low compatibility. Indeed, cluster analysis indicated that silage microbiota was distinctively separated from fecal microbiota, and the grouping pattern of silage samples showed no relation to that of feces samples (Fig. 4.1). Similarly, although great variation in microbial diversity was observed in the six silage samples, especially for silage Cs with a low *Lactobacillaceae* and a high *Acetobacteraceae* proportion, no effects on the fecal microbiota of silage-fed dairy cows were observed. The number of detected taxa was higher in silage than in feces. Because of the unique dominance and more even distribution of *Lactobacillaceae*, however, the Shannon index value was lower for silage than feces.

The repeated microbiota consumption of silage in dairy cows is comparable to that of naturally fermented food in humans. Lee et al. (1996) demonstrated that consumption of naturally fermented kimchi, which has been shown to have *Lactobacillaceae* and *Leuconostocaceae* as the dominant taxa (Jung et al. 2014), increased the population of *Lactobacillus* spp. and *Leuconostoc* spp. in human feces. Similarly, Han et al. (2015) found that intake of fermented kimchi raised up the populations of two phyla, *Proteobacteria* and *Actinobacteria*, and two genera *Bacteroides* spp. and *Prevotella* spp. in human feces. Because only silage-fed dairy cows were examined in this study, the comparison between fermented and non-fermented crop feeding was not possible. Regardless, there were no distinctive differences in any taxa of fecal microbiota in response to ingestion of various silage microbiota, which were seprated into three separate clusters (Fig. 4.1). The low impact of diet microbiota on fecal microbiota of cattle was likely due to the polygastric digestive system compared to the monogastric system in humans.

Although several NGS studies on gut microbiota of dairy cows have been conducted, it is not easy to perform cross comparisons because of the differences in diet formulation, production level (lactating and non-lactating), examined gut segments (e.g., rumen and rectum), and targeted regions of 16S rRNA genes. Mao et al. (2015) studied the gut microbiota of lactating Holstein cows in China, but their reported proportion of *Firmicutes* and *Bacteroidetes* (91.3 and 2.87%, respectively) for rectum digesta (feces) was greatly different from that found in our study (46.1 and 45.1%, respectively). This discrepancy could be due to differences in targeted regions. Mao et al. (2015) analyzed V3/V4 regions whereas we examined the V4

region of 16S rRNA genes. Claesson et al. (2010) demonstrated that, regardless of pyrosequencing and Illumina platforms, the *Firmicutes*-to-*Bacteroidetes* ratio was much greater when the V3/V4 region was targeted than other regions such as V1/V2, V2/V3, V4/V5, V5/V6, and V7/V8. Similar to Mao et al. (2015), Derakhshani et al. (2016) reported the proportion of *Firmicutes* and *Bacteroidetes* at 80.3 and 11.1%, respectively, although they analyzed the same V4 region as targeted in this study. Since Derakhshani et al. (2016) examined the feces of Holstein bull calves, the difference in diet formulation may have affected the outcome of the microbiota analysis. Regarding the proportion of *Firmicutes* and *Bacteroidetes* for feces samples, our data was similar to that obtained for steers fed a late growing diet (moderate grain) containing 26% corn silage (Kim et al. 2014).

Among the families evaluated as highly variable taxa, *Succinivibrionaceae* and *Prevotellaceae* have been shown to correlate with grain feeding (Khafipour et al. 2016). Furthermore, *Spirochaetaceae* (mainly the genus *Treponema* spp.) has been shown to relate to the fiber degrading ability (De Filippo et al. 2010; Liu et al. 2016). At the phylum level, *Proteobacteria* (CV = 1.312) was the highest variance taxa in our study, which was in agreement with a study by Jami and Mizrahi (2012a) for rumen microbiota. Our finding that *Bacteroidetes* (CV = 0.121) was consistent was different from previously reported findings for cattle (Jami and Mizrahi 2012a) and human (Kurokawa et al. 2007; Arumugam et al. 2011). Variations in *Succinivibrionaceae* and *Prevotellaceae* were also seen between farms and countries. This difference could be seen from a high abundance of these two families (9.83% and 4.41% for *Succinivibrionaceae* and *Prevotellaceae*, respectively) for farm B (Table 4.4), especially with B2 and B3 cows. In B1 cow raised under the same management as B2 and B3, *Porphyromonadaceae*, which also showed a higher proportion under a high grain diet (Kim et al. 2014), was detected at a high abundance compared to *Succinivibrionaceae* and *Prevotellaceae*, indicating that gut microbiota may have adapted strategies under the same feeding regimen. Because the forage-to-concentrate ratio was similar between farms A and B, the high abundance of concentrate-associated taxa in farm B cows could not be attributed to diet formulation. Rather, the difference in feeding management, i.e., TMR and separate feeding, may have affected the abundance of forage- and concentrate-associated families. It has been demonstrated that, although cows may feed on diet of an intended formulation under TMR feeding, cows may consume a greater amount of concentrate than designed under separate feeding (Nocek et al. 1986). Maekawa et al. (2002) found that, even if the diet was provided at a 50:50 forage-to-concentrate ratio, the actual ratio consumed by cows was approximately 60:40 under separate feeding. In farm F cows that were also managed by separate feeding,

although the differences were small, the lowest proportion of forage-associated *Ruminococcaceae* and the highest proportion of concentrate-associated *Succinivibrionaceae* were observed, compared to other Chinese cows. Therefore, rather than the geographical location, feeding management could have led to the farm-to-farm differences in fecal microbiota in this study.

Table 4.4. Relative abundance of the taxa detected at >0.1% of the total population in feces samples from silage-fed dairy cows at 3 Japanese and 3 Chinese farms.

		Farm-to-Farm variation								**Country-to-Country variation**	
		Japan				China					
		A	B	C	SE	D	E	F	SE	Japan	China
Phylum	*Firmicutes*	41.2	37.2	40.6	2.63	51.9	55.1	50.3	1.53	39.7^{b}	52.4^{a}
Family	*Ruminococcaceae*	23.2^{a}	10.9^{b}	18.7^{ab}	1.85	28.2^{ab}	31.7^{a}	23.9^{b}	1.21	17.6^{b}	27.9^{a}
Family	*Clostridiaceae*	4.53	7.29	5.04	1.42	8.97	8.24	8.99	0.96	5.62^{b}	8.74^{a}
Family	*Lachnospiraceae*	4.83	9.76	7.73	1.73	5.74	5.71	9.03	1.07	7.44	6.83
Family	*Veillonellaceae*	0.73	2.37	0.63	0.66	0.63^{b}	0.69^{ab}	1.08^{a}	0.10	1.24	0.79
Family	*Erysipelotrichaceae*	0.47	0.71	0.47	0.15	0.92	0.45	0.64	0.15	0.55	0.67
Family	*Peptococcaceae*	0.47^{ab}	0.77^{a}	0.29^{b}	0.08	0.39	0.78	0.37	0.15	0.51	0.51
Family	*[Mogibacteriaceae]*	0.20	0.12	0.22	0.03	0.30^{b}	0.49^{a}	0.41^{ab}	0.04	0.18^{b}	0.40^{a}
Family	*Christensenellaceae*	0.14^{a}	0.03^{b}	0.08^{ab}	0.02	0.20^{ab}	0.46^{a}	0.15^{b}	0.06	0.09^{b}	0.27^{a}
Family	*Peptostreptococcaceae*	0.07^{b}	0.37^{a}	0.11^{b}	0.06	0.08	0.12	0.09	0.03	0.19	0.10
Family	*Turicibacteraceae*	0.04^{b}	0.24^{a}	0.10^{ab}	0.04	0.05^{b}	0.10^{a}	0.11^{a}	0.01	0.13	0.08
Phylum	*Bacteroidetes*	48.6	46.7	51.4	2.27	42.8	37.4	43.8	1.62	48.9^{a}	41.3^{b}
Family	*Bacteroidaceae*	9.06	11.7	10.4	1.05	10.8	11.8	10.1	1.94	10.4	10.9
Family	*Rikenellaceae*	5.84	4.79	6.25	0.90	5.82	5.83	7.13	0.68	5.63	6.26
Family	*[Paraprevotellaceae]*	5.45	6.96	7.15	0.82	4.87	4.06	5.04	0.46	6.52^{a}	4.65^{b}
Family	*RF16*	5.98	1.88	5.49	1.47	1.54	0.81	1.49	0.22	4.45^{a}	1.28^{b}
Family	*S24-7*	1.28	4.33	1.84	1.18	2.07	1.38	2.42	0.72	2.48	1.96
Family	*Prevotellaceae*	1.33	4.41	0.10	0.96	0.21	0.14	0.52	0.09	2.25^{a}	0.29^{b}
Family	*Porphyromonadaceae*	1.86	1.22	1.37	0.63	1.01	0.46	0.90	0.13	1.48	0.79
Family	*p-2534-18B5*	0.67	0.27	0.004	0.24	2.31	1.46	1.77	0.97	0.31^{b}	1.85^{a}
Family	*BS11*	0.56	0.20	0.22	0.16	0.65	0.67	0.27	0.13	0.33	0.53
Family	*[Barnesiellaceae]*	0.17	0.16	0.21	0.03	0.10	0.14	0.16	0.04	0.18	0.13
Phylum	*Proteobacteria*	1.78^{b}	10.7^{a}	2.06^{b}	1.12	0.90	1.06	0.92	0.31	4.84^{a}	0.96^{b}
Family	*Succinivibrionaceae*	0.95^{b}	9.83^{a}	1.45^{b}	0.95	0.35^{ab}	0.04^{b}	0.51^{a}	0.09	4.08^{a}	0.30^{b}
Family	*Desulfovibrionaceae*	0.26	0.06	0.09	0.08	0.19	0.46	0.11	0.16	0.14	0.25
Family	*Alcaligenaceae*	0.13	0.48	0.11	0.23	0.07	0.08	0.10	0.03	0.24	0.08
Phylum	*Tenericutes*	2.44	1.42	2.50	0.48	1.67	2.47	1.42	0.53	2.12	1.85
Family	*Anaeroplasmataceae*	0.22	0.28	0.30	0.05	0.16	0.04	0.16	0.04	0.27^{a}	0.12^{b}
Phylum	*Spirochaetes*	2.85	2.98	1.45	1.31	0.39^{b}	0.40^{b}	2.05^{a}	0.30	2.42	0.95
Family	*Spirochaetaceae*	2.74	2.97	1.45	1.33	0.36^{b}	0.39^{b}	1.94^{a}	0.25	2.39	0.89
Phylum	*Verrucomicrobia*	0.81	0.12	0.20	0.28	0.62^{ab}	1.48^{a}	0.31^{b}	0.26	0.37	0.80
Family	*Verrucomicrobiaceae*	0.32	0.04	0.19	0.10	0.32	0.85	0.20	0.22	0.18	0.46
Family	*RFP12*	0.47	0.08	0.01	0.20	0.28	0.58	0.11	0.14	0.18	0.32
Phylum	*Cyanobacteria*	0.43	0.12	0.17	0.20	0.29	0.54	0.05	0.20	0.24	0.29
Phylum	*Euryarchaeota*	0.09	0.11	0.11	0.04	0.22	0.39	0.27	0.08	0.10^{b}	0.29^{a}
Family	*Methanobacteriaceae*	0.08	0.10	0.10	0.04	0.19	0.38	0.24	0.08	0.09^{b}	0.27^{a}
Phylum	*Fibrobacteres*	0.28^{ab}	0.001^{b}	0.45^{a}	0.10	0.06	0.10	0.02	0.04	0.24^{a}	0.06^{b}
Family	*Fibrobacteraceae*	0.28^{ab}	0.001^{b}	0.45^{a}	0.10	0.06	0.10	0.02	0.04	0.24^{a}	0.06^{b}
Phylum	*Lentisphaerae*	0.21	0.004	0.01	0.09	0.17	0.16	0.02	0.07	0.07	0.12

A-F indicate feces samples collected from farms A, B, C, D, E, and F, respectively. Mean values with different superscript letters are significantly different ($p < 0.05$).

Table 4.5. Coefficient of variation calculated for the taxa detect at >0.1% of total population in feces samples collected from silage-fed dairy cows at 3 Japanese and 3 Chinese farms.

		SD	Mean	CV
Phylum	*Firmicutes*	7.519	46.0	0.163
Family	*Ruminococcaceae*	7.241	22.7	0.318
Genus	*Oscillospira*	0.380	1.98	0.192
Family	*Clostridiaceae*	2.549	7.18	0.355
Genus	*Clostridium*	2.453	6.72	0.365
Family	*Lachnospiraceae*	2.821	7.13	0.395
Family	*Veillonellaceae*	0.940	1.02	0.921
Phylum	*Bacteroidetes*	5.440	45.1	0.121
Family	*Bacteroidaceae*	1.672	10.6	0.157
Genus	*5-7N15*	1.990	7.74	0.257
Family	*Rikenellaceae*	1.358	5.94	0.229
Family	*[Paraprevotellaceae]*	1.507	5.59	0.270
Genus	*CF231*	1.345	3.87	0.348
Genus	*[Prevotella]*	0.693	1.20	0.576
Family	*RF16*	2.613	2.86	0.912
Family	*S24-7*	1.769	2.22	0.797
Family	*Prevotellaceae*	1.807	1.27	1.423
Genus	*Prevotella*	1.807	1.27	1.423
Family	*Porphyromonadaceae*	0.793	1.14	0.698
Family	*p-2534-18B5*	1.337	1.08	1.239
Phylum	*Proteobacteria*	3.804	2.90	1.312
Family	*Succinivibrionaceae*	3.682	2.19	1.682
Genus	*Succinivibrio*	2.735	1.44	1.900
Phylum	*Tenericutes*	0.897	1.99	0.452
Phylum	*Spirochaetes*	1.753	1.68	1.041
Family	*Spirochaetaceae*	1.748	1.64	1.066
Genus	*Treponema*	1.749	1.64	1.067

Ruminococcaceae and *Christensenellaceae* varied between farms as well as countries (Table 4.4). *Ruminococcaceae*, the greatest population in our data, is known as an active plant degrader, whereas the function of *Christensenellaceae* is still poorly understood. Goodrich et al. (2014) reported that this family is a heritable taxon in humans and is associated with a healthy state but not with the diet, and often co-occurs with *Methanobacteriaceae*. In our study, *Christensenellaceae* and *Methanobacteriaceae* accounted for similar relative abundances in samples from Japan and China.

Fiber-degrading *Ruminococcaceae* showed a positive correlation ($p < 0.01$) with *Christensenellaceae* ($r^2 = 0.638$), indicating a negative correlation with *Succinivibrionaceae* ($r^2 = 0.590$) and *Prevotellaceae* ($r^2 = 0.567$). *Ruminococcaceae* also showed positive correlations

with the other six families, i.e. *BS11* (r^2 = 0.381), *Verrucomicrobiaceae* (r^2 = 0.346), *Mogibacteriaceae* (r^2 = 0.540), RFP12 (r^2 = 0.327), *Desulfovibrionaceae* (r^2 = 0.312), and *Methanobacteriaceae* (r^2 = 0.358). Although the functions of these six families are yet unknown, these taxa could be involved in fiber-degrading activity. Meanwhile, although *Lachnospiraceae* is known as a fiber-degrading family, a negative correlation with *Ruminococcaceae* ($r^2 = 0.360$; $p < 0.01$) was observed in this study.

In conclusion, rather than diet (silage) microbiota, feeding management and geographical location may contribute greatly to shaping the gut microbiota of silage-fed dairy cows. Although variations between farms and countries were seen, neither highly variable nor stable families of microbiota in feces samples were affected by silage microbiota. An interesting cow-to-cow variation for concentrate-associated taxa was demonstrated in the same farm; two cows showed high abundance of *Succinivibrionaceae* and *Prevotellaceae*, whereas another cow had a high abundance of *Porphyromonadaceae*. Similarly, although diets with similar forage-to-concentrate were offered, cows under separate feeding had greater abundance of concentrate-associated families than those under TMR feeding. These findings help to envisage the effects of feeding management and diet formulation on gut microbiota of silage-fed dairy cows. Because assessment of rumen microbiota was not within the scope of this study, further investigation is necessary to warrant the relationship between diet microbiota, gut microbiota, and the health and productivity of dairy cows.

CHAPTER 5
MICROBIAL COMMUNITY IN THE GASTROINTESTINAL TRACT OF GOATS FED WHOLE CROP CORN SILAGE

5.1. Abstract

Gut microbial communities were examined across the gastrointestinal tract using goat model. All goats were fed corn silage as daily meal. Silage-associated *Lactobacillus* community had no influence on gut-associated *Lactobacillus* communities across the gastrointestinal tract of goats. In gut-associated samples, composition of mucosa-associated *Lactobacillus* community was more stable than the one of digesta samples, but relative abundance of this community was rather regionally fluctuated. Total microbial population and its component bacterial population (*Bacteroides-Prevotella-Porphyromonas*, *Enterobacteriaceae*, *Ruminococcus albus*, *Faecalibacterium prausnitzii*, *Fibrobacter succinogenes*) were varied in number across the tract for both digesta and mucosa samples. Throughout the tract, abomasum-associated microbiota indicated the greater differences in distribution between digesta and mucosa samples, while no difference was seen between digesta- and mucosa-associated small intestine microbiota. Proportion of *Bacteroides-Prevotella-Porphyromonas*, *F. succinogenes* and mucosa-associated *R. albus* showed significant variation across the tract, which all described their highest proportion in abomasum. Overall, this is the first study providing insight into the relation of silage-associated *Lactobacillus* community to the gut-associated ones throughout the gastrointestinal tract of goat. Digesta- and mucosa-associated microbiota were revealed intra-variation across different segments of the tract.

5.2. Introduction

Gastrointestinal tract of animal is the natural habitat for a dense and dynamic microbial community. This resident microbiota has been recognized to provide benefits to the animals, especially for ruminants with their symbiotic bacteria that fermenting plant materials into nutrient sources available for the host. But a substantial part of this resident microbiota is still to be discovered. Theoretically, this population has been contributed by three subpopulations, including digesta-associated, mucosa-associated, and food-associated microbiota (Cheng et al. 1979; Li et al. 2011). Comparing to mucosa- and food-associated population, digesta-associated population has intensively been studied targeting to rumen and feces microbiota, due to the easy and convenient sampling. However, recent studies have raised concern about intra-variation of gut microbiota in different segments across the gastrointestinal tract (de Oliveira et al. 2013).

It is indicated that gut microbiota varied across the tract in abundance as well as in microbial composition. Understanding about this fluctuation of gut microbiota is still limited (de Oliveira et al. 2013; Mao et al. 2015; Zeng et al. 2017). And, the contribution of mucosa-associated population to this dynamics is further restrictedly comprehended despite its crucial role to the host (Van den Abbeele et al. 2011).

Corn silage has been reported to harbor a rich source of *Lactobacillaceae*, which promoting to the ensiling process (Ni et al. 2017; Tang et al. 2017). The consumption of this silage-microbiota is considered to propose probiotic effect for the ruminants if these *Lactobacillaceae* could comply a satisfactory survivability throughout the gastrointestinal tract.

In this study, we examined gut microbial communities across the gastrointestinal tract using goat model. All goats were fed corn silage as daily meal. *Lactobacillus* community was examined to understand how this community inhabited throughout the gastrointestinal tract and if silage-associated *Lactobacillus* community had influence on gut-associated *Lactobacillus* communities. We also investigated the digesta- and mucosa- associated microbiota to satisfy our own knowledge about their variation in different segments across the gastrointestinal tract as well as the differences in distribution between these two populations.

5.3. Materials and methods

Animals and sample collection – This study used four female goats (body weight: 35.6 ± 3.3 kg). All goats were fed hay exclusively before experiment. The experimental diets were formulated to meet the energy requirement of the goats, containing 50% concentrates, 10% alfalfa hay, 40% whole crop corn silage on a DM basis. Goats were fed at 09:00 and 17:00 everyday during 14 days of the experimental period. Goats were kept in stalls and fed ad libitum to assure 10% orts, and they were given free access to fresh water during the trial.

Feces samples were collected at day 0 to compare feces microbiota before and after examination. On the day 14, the goats were sacrificed. Mucosa and digesta samples were collected from the rumen, abomasum, small intestine, cecum and rectum. Digesta samples of rumen, abomasum, cecum, and rectum were separately collected from each opened segment, and transferred to sterilized tube kept on ice. In case of small intestine, digesta sample was achieved by washing up the content of a 2-cm-in-length-small-intestine using sterilized saline water. The resulted liquid was collected into sterilized tube kept on ice. For mucosa samples, 2 cm^2-mucosa-tissue of each segment (except for rumen) was rinsed with sterilized saline water to eliminate contamination from the digesta. Sterilized glass slide was used to scrape the surface of mucosa tissue. The collected sample containing the mucosa-associated bacteria was washed

up by sterilized saline water and transferred into sterilized tube kept on ice. For rumen sample, after rinsing with sterilized saline water, the lining of the rumen wall harboring papillae was detached and directly transferred into sterilized tube. In addition, whole crop corn silage was collected. All of the resulted samples were then transferred to laboratory and stored at -30^{o}C prior to DNA extraction.

DNA extraction –All of the liquid samples were centrifuged shortly to achieve pellets. Bacterial DNA was extracted from achieved pellets (for liquid samples) and 200 mg of frozen samples (for digesta samples) using repeated bead beating plus column method (Yu and Morrison, 2004). DNA concentration of each sample was determined by Invitrogen Qubit 2.0 Fluorometer with Qubit® dsDNA BR Assay Kits (Life Technologies Ltd., Paisley, UK). DNA samples was stored at -30^{o}C for further examination.

PCR – DGGE analysis – Bacterial DNA was subject to PCR using primers Lab159f (5'-GGA AACAGRTGCTAATACCG-3') and Lab617r (5'-CACCGCTACACATGGAG-3') for targeted *Lactobacillus* community (Heilig et al. 2002). Nested PCR were employed with universal primers GC357f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCCTACGGAGGCAGCAG-3') and 517r (5'-ATTACCGCGGCTGCTGG-3'). Resulted PCR products were separated by DGGE by using the DCode Universal Mutation Detection System (Bio-Rad Laboratory Inc.). Polyacrylamide gels consisted of 8% (v/v) polyacrylamide. Denaturing gradient (Urea and Formamide) of 25 - 50% was used for the separation of the generated. Electrophoresis was performed for 8 h at 150 V in TAE buffer at a constant temperature of 60°C. Gels were stained with SYBR Green, observed by UV transilluminator and photographed.

Species identification – PCR amplicons (with 357f (without GC clamp) and 517r primers) resulted from excised bands on DGGE gel were purified using FastGene® Gel/PCR Extraction Kits (Nippon Genetics Co., Ltd., Japan) according to the manufacturer's instructions, inserted in to pTAC–1 vector and cloned into *Escherichia coli* strain DH5α competent cells (DynaExpress TA Cloning Kit; BioDynamics Laboratory Inc., Tokyo, Japan). Positive white clones are randomly selected and confirmed for the presence of expected size inserts by PCR. Subsequently, positive colonies were sub-cultured into LB broth media. Extracted plasmids should be employed for a new PCR amplification followed by purification. To enhance the quality of sequencing, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instructions. DNA sequences were analyzed using an ABI PRISM 3130 sequencer (Applied Biosystems Inc., Foster City, Calif., U.S.A.). The Basic Local Alignment Search Tool (BLAST) program andGenBank databases

were used to determine the closest relatives of partial 16S rRNA gene sequences. Unknown sequences that shared greater than 98% identity with a sequence in the BLAST database were considered as identified.

Quantitative PCR – A master mix for total quantification (357f (5'-ACGGGGGGCCT ACGGAGGCAGCAG-3') and 517r (5'-ATTACCGCGGCTGCTGG-3')), *Bacteroides-Prevotella-Porphyromonas* population (BPP fwd (5'-GGTGTCGGCTTAAGTGCCAT-3') and BPP rev (5'-CGGA(C/T)GTAAGGGCCGTGC-3')), *Clostridium* cluster I&II population (Cl-perf fwd (5'-ATGCAAGTCGAGCGA(G/T)G-3') and Cl-perf rev (5'-TATGCGGTAT TAATCT(C/T)CCTTT-3')), *Lactobacillus* population (Lact fwd (5'-AGCAGTAGGGAAT CTTCCA-3') and Lact rev (5'-CACCGCTACACATGGAG-3')), *Faecalibacterium prausnitzii* (Fprau-2 fwd (5'- CCCTTCAGTGCCGCAGT-3') and Fprau-2 rev (5'- GTCGCAGGATGTC AAGAC-3') ((Rinttilä et al. 2004), *Enterobacteriaceae* population (Eco1457 fwd (5'-CATTG ACGTTACCCGCAGAAGAAGC-3') and Eco1652 rev (5'-CTCTACGAGACTCAAGCTT GC-3')), *Ruminococcus albus* population (Ralb561 fwd (5'-CAGGTGTGAAATTTAGGG GC-3') and Ralb807 rev (5'-GTCAGTCCCCCCAC ACCTAG-3')) (Bartosh et al. 2004), *Fibrobacter succinogenes* (Fibsuc fwd (5′-GGTATGGGATGAGCTTGC-3′) and Fibsuc rev (5′-GCCTGCCCCTGAACTATC-3′)) (*Tajima et al. 2001)* was prepared for each well contained following reagents: 2 x KAPA SYBR FAST qPCR Master Mix Universal (Kapa Biosystem Inc.) that contained integrated antibody-mediated hot start, SYBR Green I fluorescent dye, $MgCl_2$, dNTPs, forward and reverse primers, and distilled water to a total of 23 µl per well. To these, 2 µl of each sample or standard was added, and the plates were briefly spun down and placed in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratory, Inc.) for analysis. Amplification programme involved an initial denaturation step at 95^{o}C for 5 min, followed by 35 cycles of denaturation at 95^{o}C for 15s, primer annealing at optimal temperatures for each target bacterial group for 20 s, extension at 72^{o}C for 30 s and an additional incubation step at 80-85^{o}C for 30s to collect the fluorescent data (Rinttilä et al. 2004).

Statistical analysis – Binary matrix resulted from DGGE banding patterns of *Lactobacillus* group was created to describe the presence or absence of individual band in all lanes. Resulted binary matrix was engaged to Principal Coordinates Analysis (PCO) to demonstrate similarity and differences between gut segments and mucosa vs. digesta samples using Primer version 7 with Permanova + add-on software (Primer-E, Plymouth Marine Laboratory, and Plymouth, UK). Quantitative PCR data were subjected to one-way analysis of variance. Tukey'smultiple range test was performed using the JMP software (version 11; SAS Institute, Tokyo, Japan) to examine the differences between segments and samples. test. All data are presented as means

with their pooled standard error.

Table 5.1. Proportion of *Lactobacillus* group, *R. albus*, *F. prausnitzii*, *Enterobacteriaceae*, *Bacteroides–Prevotella–Porphyromonas* group, *F. succinogenes* in digesta (D) and mucosa-adhered microbiota (M) of different sampling sites across the gastrointestinal tract of goat. Means with different subscripts are considered statistically different ($p<0.05$). [§]Sample type effect on bacterial population. [†]Regional effect on bacterial population.

	Regions	Rumen	Abomasum	Small Intestine	Cecum	Rectum	Feces 0 day	SE	P value[†]
Lactobacillus	D	0.0009	0.0038	21.2467	0.0014	0.0001	0.0012	8.66	
	M	0.0032	0.0113	0.0641	0.0230	0.0259	#	0.018	
P value[§]									
Ruminococcus albus	D	0.0085	0.0099	0.0139	0.0026	0.0034	0.0104	0.005	
	M	0.0037^{b}	0.0496^{a}	0.0065^{b}	0.0080^{b}	0.0358^{a}	#	0.006	*
P value[§]			*			*			
Faecalibacterium prausnitzii	D	0.1471	0.0941	0.2187	0.1847	0.2541	0.4929	0.093	
	M	0.5201	0.2526	0.5288	0.2326	0.2824	#	0.152	
P value[§]		*	*						
Enterobacteriaceae	D	0.0004	0.0008	0.0329	0.0968	0.4436	0.0549	0.101	
	M	0.0044	0.0137	0.0392	0.0435	0.0734	#	0.020	
P value[§]		*	*						
Baceteriode – Prevotella– Porphyromonas	D	8.2227^{ab}	19.264^{a}	0.0569^{b}	6.196^{ab}	7.295^{ab}	2.731^{b}	3.185	*
	M	5.1325^{b}	13.260^{a}	0.0654^{b}	1.5842^{b}	1.4479^{b}	#	1.614	*
P value[§]					*	*			
Fibrobacter succinogenes	D	1.0361^{ab}	1.4701^{a}	0.6704^{ab}	0.0442^{b}	0.0226^{b}	0.0225^{b}	0.249	*
	M	5.1972^{b}	26.461^{a}	0.5236^{b}	1.5863^{b}	4.5321^{b}	#	1.564	*
P value[§]		*	*		*	*			

5.4. Results

5.4.1. Habitation of *Lactobacillus* community from silage throughout the gastrointestinal tract of goat

The *Lactobacillus* community was detected in silage samples and in all segments of the gastrointestinal tract regardless of sample type. *Lactobacillus* population was found as high as 2.91×10^{10} copies/g on average in silage samples. But this population revealed a lower abundance in digesta- (1.36×10^{9} copies/g) and mucosa-associated (7.97×10^{7} copies/cm^2) samples. Mucosa-associated *Lactobacillus* population showed significant higher number in small intestine comparing to other segments, while digesta-associated *Lactobacillus* population was rather stable across the tract (Fig. 5.3C).

DGGE profile and species identification of *Lactobacillus* community revealed that the silage-associated *Lactobacillus* community was dominated by *L. acetotolerans*, *L. parafarraginis*, *L. buchneri*, and *L. kefiri*, while gut-associated *Lactobacillus* was belonged to

L. johnsonii, *L. helviticus*, *L. acetotolerans*, *L. buchneri*, *L. crispatus*, and many unidentified bacterium clone (Fig. 5.1).

PCoA profile indicated that silage-associated *Lactobacillus* community was different from gut-associated ones. Mucosa-associated samples were distributed closely into one group, indicating that the mucosa-associated *Lactobacillus* community was similar between different segments of the gastrointestinal tract. In general, digesta-associated *Lactobacillus* samples were not statistically similar to each other, but these samples were allocated unrelatedly from the mucosa-associated ones (except for small intestine samples). Feces samples collected at day-0 showed independent grouping pattern with other gut-associated samples collected at day-14 (Fig. 5.2).

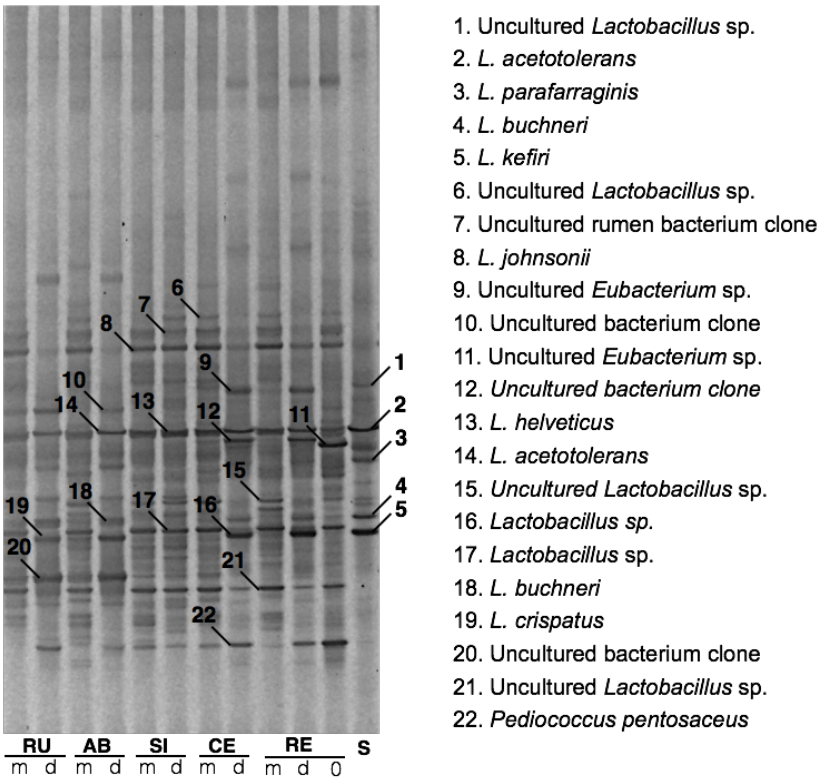

Figure 5.1. DGGE banding patterns and species identification of *Lactobacillus* group of silage sample (S) and digesta (d) and mucosa-associated samples (m) across gastrointestinal tract of goat including rectum (RE, RE-0: Feces sample collected before examining period), cecum (CE), small intestine (SI), abomasum (AB), rumen (RU).

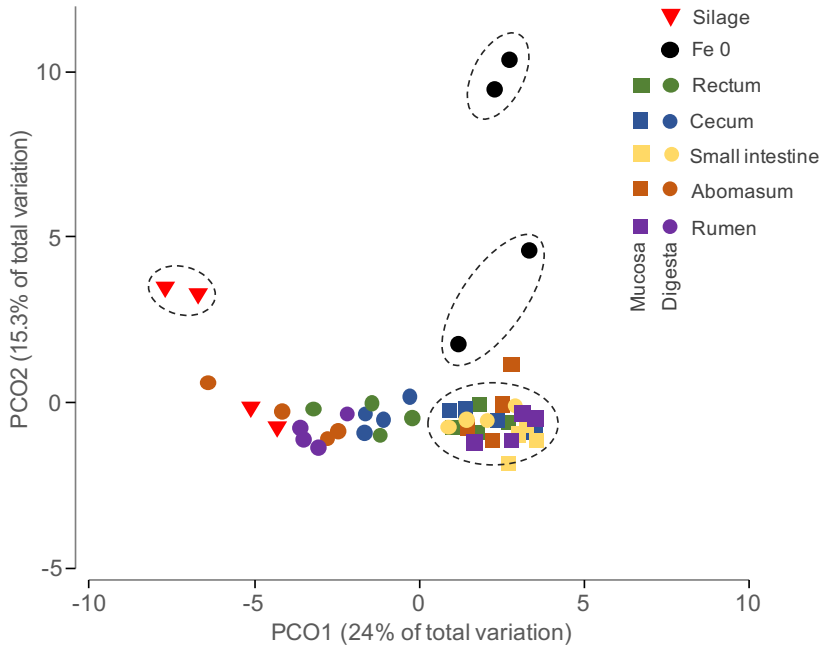

Figure 5.2. Principal Coordinate Analysis (PCoA) profile of *Lactobacillus* group based on DGGE banding pattern of digesta and mucosa-associated microbiota across the gastrointestinal tract of 4 goats and silage samples.

5.4.2. Microbial population changing across the gastrointestinal tract of goat

Total bacterial population showed different trends of distribution in digesta and mucosa samples. In digesta samples, total population appeared to be stable in different segments across the gastrointestinal tract, except for small intestine. Small intestine exposed the smallest bacterial population with two order lower in comparison to other segments. The opposite trend was observed in mucosa-associated samples. Small intestine samples indicated the most abundant population (Fig. 5.3A).

In digesta samples, population of component bacterial groups also appeared to be lowest in small intestine, except for *Lactobacillus* and *Enterobacteriaceae* group. *Bacteroides-Prevotella-Porphyromonas* group, *R. albus*, and *F. succinogenes* population showed their highest number in forestomach (rumen and abomasum). After a decline in number of small intestine, in large intestine (cecum and rectum) *Bacteroides-Prevotella-Porphyromonas* group and *R. albus* regained their number as great as their performance in forestomach, while *F. succinogenes* maintained the low number from small to large intestine. *Enterobacteriaceae* population was found smallest in abomasum and gradually increased their number to the largest population in rectum. *F. prausnitzii* showed a gradual decreased trend from rumen to small intestine and increased again to reach the highest population in rectum (Fig. 5.3).

In mucosa samples, across the gastrointestinal tract, the greatest number of several component bacterial groups was found in small intestine, such as *Lactobacillus*, *Enterobacteriaceae*, *R. albus*, and *F. prausnitzii*. The opposite pattern was observed for *Bacteroidetes-Prevotella-Porphyromonas* group and *F. succinogenes*, that showed smallest number in small intestine (Fig. 5.3).

5.4.3. Comparative evaluation of digesta- and mucosa-associated microbial population across the gastrointestinal tract of goat

The different sampling method was employed for digesta and mucosa samples, hence, the representing unit was also different. It's infeasible to make direct comparison between digesta (copies/g) and mucosa (copies/cm^2) samples for every segment across the gastrointestinal tract. Therefore, proportion of component bacterial group was calculated in order to earn a synchronized evaluation. The proportion of *Lactobacillus*, *Bacteroides-Prevotella-Porphyromonas*, *Enterobacteriaceace*, *R. albus*, *F. prausnitzii*, *F. succinogenes* of digesta and mucosa samples across the gastrointestinal tract were presented in the Table 1. *Lactobacillus*, *Enterobacteriaceae*, *F. prausnitzii* did not revealed any fluctuation in their proportion across the gastrointestinal tract regardless of sample type. *Bacteroides-Prevotella-Porphyromonas*, *F. succinogenes* and mucosa-associated *R. albus* showed significant variation across the tract, which all described their highest proportion in abomasum (Table 5.1).

When comparing between digest and their corresponding mucosa samples, abomasum was the segment that showed several differences between these two microbiota, referring to *R. albus*, *F. prausnitzii*, *F. succinogenes*, and *Enterobacteriaceae* population. Rumen indicated the greater proportion of mucosa-associated *F. prausnitzii*, *F. succinogenes*, and *Enterobacteriaceae* than those of digesta samples. Whereas, cecum and rectum revealed the greater proportion of digesta-associated *Bacteroides-Prevotella-Porphyromonas* and *F. succinogenes*. But *R. albus* exposed its higher proportion in mucosa-associated microbiota of rectum (Table 5.1).

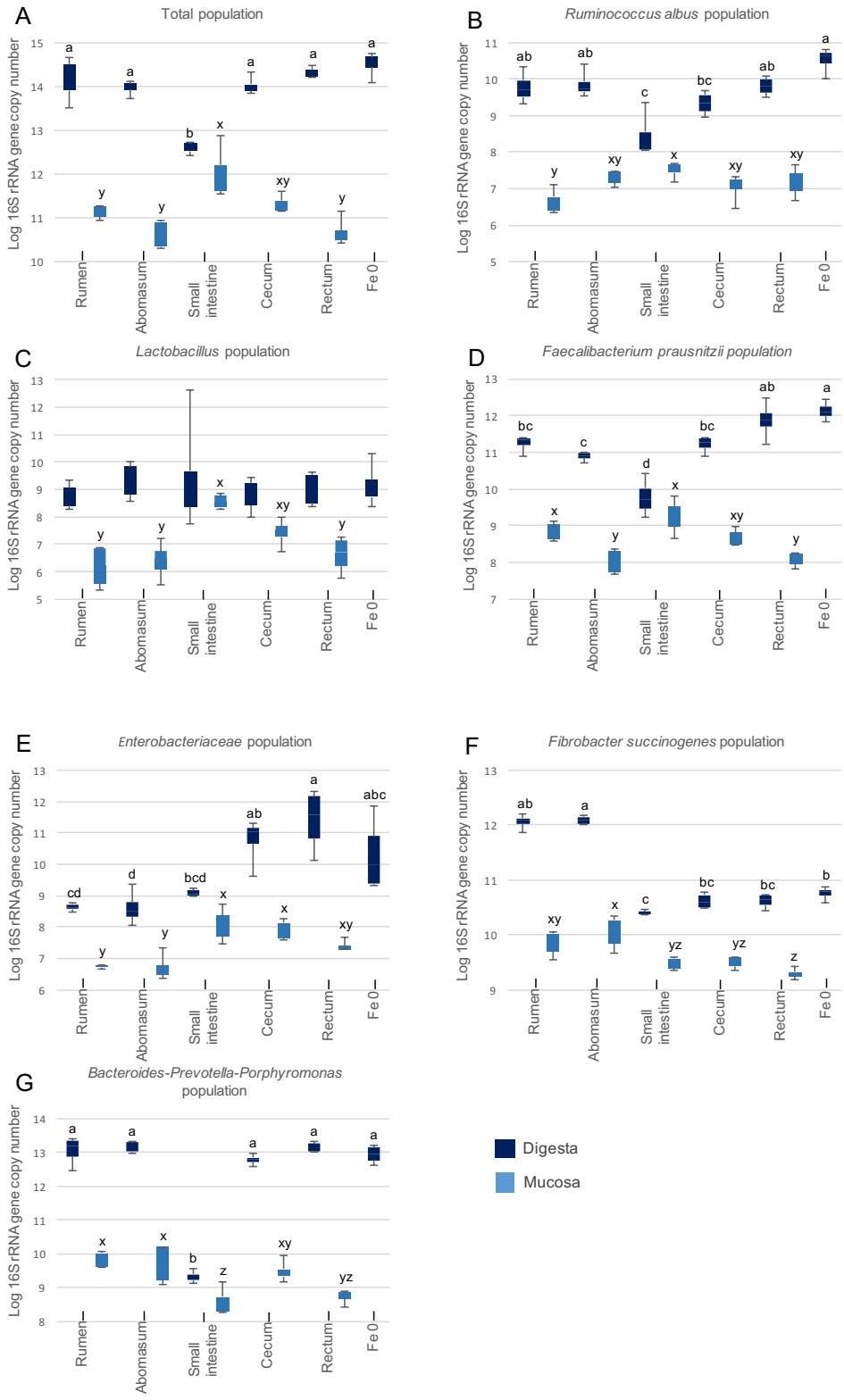

Figure 5.3. Relative abundance of total bacteria (A), *R. albus* (B), *Lactobacillus* group (C), *F. prausnitzii* (D), *Enterobacteriaceae* (E), *F. succinogenes* (F), *Bacteroides–Prevotella–Porphyromonas* group (G) population of digesta- (log 16S rRNA gene copies/g) and mucosa-associated (log 16S rRNA gene copies/cm^2) samples across the gastrointestinal tract of goat. Superscripts with different characters above their whiskers are considered statistically different (a, b, and c for digesta samples and x, y, and z for mucosa samples, $p<0.05$).

5.5. Discussion

Symbiotic gut microbiota colonizing the gastrointestinal of the animal have long been recognized to provide crucial benefits to the host. However, limited studies have been conducted to characterize the microbial community inhabiting in different segments across the gastrointestinal tract (de Oliveira et al. 2013; Gu et al. 2013; Mao et al. 2015; Zeng et al. 2017). In general, this symbiotic gut microbiota has been contributed by three subpopulations, including digesta-associated, mucosa-associated, and food-associated microbiota. These three microbial population has been supposed to interact with each other across the gastrointestinal tract (Cheng et al. 1979; Li et al. 2011). Hence, in the current study, microbiota of digesta-, mucosa- and feed-associated samples were examined using goat model for understanding about the dynamic and relation between these three microbiota across the gastrointestinal tract.

In silage, where lactic acid fermentation usually occurs, a rich source of lactic acid bacteria (LAB) could be found. The consumption of this rich LAB-silage-microbiota could supposed to a probiosis effect accumulation for the ruminants if these LAB could comply a satisfactory survivability and compatibility throughout the gastrointestinal tract. In this study, corn silage, which has been overwhelmed by *Lactobacillaceae* (Ni et al. 2017; Tang et al. 2017), was provided to goats. Hence, *Lactobacillus* community, the most important taxa contributing to the *Lactobacillaceae*, was examined for the structure and number in silage, in digesta and mucosa samples across the gastrointestinal tract. It is indicated that there had been variation in distribution of this community between different sampling sites and sampling types.

There were changes appeared in *Lactobacillus* group composition before and after examination based on PCoA profile. The 0-day-*Lactobacillus* community in feces was separated from the other 14-day-*Lactobacillus* communities (Fig. 5.2). However, all of the gut-associated samples showed a different pattern from silage-associated ones, that indicated silage-associated *Lactobacillus* community had no influence on gut-associated *Lactobacillus* communities across the gastrointestinal tract. Only *L. acetotolerans* was found in common between silage samples and all gut microbiota samples. Rather than silage-associated microbiota, changing in diet formulation from 100% fiber to 50% fiber and 50% concentrate was assumed to have effects on gut-associated microbiota.

Mucosa-associated *Lactobacillus* composition was more stable and differentiated from digesta-associated *Lactobacillus* community throughout the gastrointestinal tract, except for small intestine-associated samples that was similar between digesta and mucosa samples (Fig. 5.2). This finding is consistent with a study of Li et al. (2012) that suggested the tissue-associated bacteria may be less dynamic compared to the content-associated bacteria using

PCR-DGGE analysis (Li et al. 2012). However, although composition of mucosa-associated *Lactobacillus* community is stable across the gastrointestinal tract, relative abundance is rather regionally fluctuated (Fig. 5.3C). When comparing between digesta- and their corresponding mucosa-associated samples, proportion of mucosa-associated *Lactobacillus* was numerically higher than that of digesta-associated microbiota. Small intestine seemed to be the preferable habitat for *Lactobacillus*, which indicated the rather high proportion of this community comparing to other segments (difference is not significant).

Total bacterial quantification and several component bacterial populations were examined for all samples from digesta and mucosa samples across the gastrointestinal tract (Fig. 5.3). The lower number of small intestine-associated samples could be supposed to related to the different sample collecting comparing to other digesta samples. Small DNA yield and consequent small number of quantitative copies could be derived from the smaller amount of digesta samples from small intestine. The trend of variation in abundance of total population in digesta seemed to be contributed mainly by *Bacteroides-Prevotella-Porphyromonas* group, *R. albus*, and *F. prausnitzii* population (Fig. 5.3A, B, D, G). In our study, *Bacteroides* and *Prevotella*, the two main genera composing to phylum *Bacteroidetes*, showed their high population in the rumen and abumasum, that is consistent with other reports which indicated *Bacteroidetes* was the most abundant phylum in the forestomach of bovines (Mao et al. 2015; Peng et al. 2015). However, the increase of *Bacteroides* and *Prevotella* population in cecum and rectum of our study was opposite to finding of Mao et al. (2015), who implied that these genera would decline their abundance into neglectable level comparing to the one in forestomach of bovines, especially for digesta samples (Mao et al. 2015). The finding that number of dominant fibrolytic bacteria, *R. albus* and *F. succinogenes*, were consistently high in the rumen and abomasum, is in agreement with report of Zeng et al. (2015) in bovines. Zeng et al. (2017) using Illumina Miseq sequencing revealed that *Fibrobacter* was discovered only in the forestomach, and *Faecalibacterium* was determined only in large intestine (Zeng et al. 2017). Whereas, *F. succinogenes* and *F. prausnitzii* was detected at meaningful level in all segments of gastrointestinal tract of our goats (Fig. 5.3D, F). The different findings could be attributed to difference in techniques, among which qPCR has been considered more sensitive in discover the minor population than robust Miseq sequencing. The other possible reason could be explained by different host animals. In mucosa samples, except for *Bacteroides-Prevotella-Porphyromonas* and *F. succinogenes* population, small intestine showed a greater number of bacteria than other segments across the gastrointestinal tract. The cause of the increase in

bacterial density in the small intestine is not clear, and further studies are needed to clarify this concerning.

Principally, the distribution of bacteria in digesta and mucosa tissues is different. Digesta-associated bacteria are freely inhabiting in the lumen of gastrointestinal tract, while mucosa-associated bacteria are only adhered on the surface of the mucosa tissue. Despite the different distribution of these two microbiota, several reports had tried to equalize the source of sampling when comparing digesta- and mucosa-associated microbiota (Li et al. 2012; Mao et al. 2015). The equal volume or weight of sample could not provide a comparative evaluation for these microbial population. Hence, to reflect the actual density of the two microbiota, different units were used to represent data. The shortcoming is the difficulties of making cross comparison to other reports and also between digesta and mucosa samples in our own study. Proportion of several component bacterial populations were calculated in order to have a synchronized comparison (Table 5.1). There were several differences in microbial proportion between digesta and mucosa samples across the gastrointestinal tract. Abomasum was the segment that showed large variation between digesta and mucosa samples, while small intestine-associated digesta and mucosa were not differentiated in their microbial proportion (Table 5.1). *F. prausnitzii*, a well-known butyrate producer and a putative immune regulator at microbiota-host interface, was reported to be consistently and abundantly present in the mucosa-associated microbiota of the healthy object (Duncan et al. 2002; Soko et al. 2008; Willing et al. 2009; Van den Abbeele et al. 2011). The greater abundance of *F. prausnitzii* in mucosa-associated microbiota was confirmed in this study, significantly in rumen and abomasum (Table 5.1). The presence of *F. prausnitzii* in mucosa-associated microbiota supported to their ability of producing butyrate close to epithelium, enhancing the butyrate bioavailability for proliferating epithelium for the host animal (Van den Abbeele et al. 2011). Fibrolytic *F. succinogenes* and *R. albus* indicated their higher proportion of mucosa-associasted microbiota in several segments across the gastrointestinal tract. This finding was opposed to a study of Li et al (2012) that phyla *Fibrobacteres* was only detected in the bovine rumen digesta but not in rumen mucosa community (Li et al. 2012), while Malmuthuge et al. (2014) discorved Fibrobacter in both mucosa- and digesta-associated community of preweaned calves (Malmuthuge et al. 2014).

In conclusion, the study showed that silage-associated *Lactobacillus* community had no influence on gut-associated *Lactobacillus* communities across the gastrointestinal tract of goats. Composition of mucosa-associated *Lactobacillus* community is more stable than the one of digesta samples across the gastrointestinal tract, but relative abundance is rather regionally

fluctuated. Total microbial population and its component bacterial population were varied in number across the gastrointestinal tract of goats for both digesta and mucosa samples. Throughout the tract, abomasum-associated microbiota indicated the greater differences in bacterial composition between digesta and mucosa samples, while no difference was seen in digesta- and mucosa-associated small intestine microbiota.

CHAPTER 6
GENERAL CONCLUSION

The divergence of gut microbiota in ruminants is affected by many factors including genotype, aging, farm management, geographical locations, and diet formulation. Likewise, differences can be seen even when animals consume the same diet and show similar physiological profiles. Because microbial activity and metabolism in the gut is a key to secure health and productivity of ruminants, understanding robustness and resilience of the gut microbiota is of great importance. Therefore, in this thesis, three experiments were carried out to examine variability, stability, and adaptability of gut microbiota in relation to feeding and nutritional management.

In early life of Holstein heifers, weaning, with the transition of diet from milk to solid feed, showed a substantial effect on the gastrointestinal microbiota composition in all bacterial taxa examinned, whereas no changes were observed thereafter. The composition of *Lactobacillus* group changed over the growing stage, while maintaining high similarity between individual heifers. These findings indicated that weaning had a marked influence on the gut microbiota especially in the composition. Management during this transition period may be critical in shaping gut microbiota, whereas silage microbiota would have no effects.

In lactating cows, rather than diet (silage) microbiota, feeding management and geographical location may contribute greatly to shaping the gut microbiota of silage-fed dairy cows. Although fecal microbiota composition was similar in most samples, relative abundances of several families varied with regard to farms, countries and feeding managements. In addition, cow-to-cow variation for concentrate-associated families was also found in the same farm. *Lactobacillaceae* were dominant in all silages, while in feces, the predominant families were *Ruminococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, and *Rikenellaceae*. *Lactobacillaceae* of corn silage appeared to be eliminated in the gastrointestinal tract. These findings help to envisage the effects of feeding management and diet formulation on gut microbiota of silage-fed dairy cows.

Across the gastrointestinal tract of ruminants, regardless of digesta- and mucosa-associated samples, the bacterial populations varied across gut segments and between bacterial taxa. In digesta-associated samples, the populations of total bacteria. *Lactobacillus* spp. in silage had no influence on gut-associated *Lactobacillus* spp. Difference of *Lactobacillus* composition between digesta- and mucosa-associated samples was shown regardless of gut

segments. These results indicated dynamics of bacterial populations in the gastrointestinal tract and concrete differences between digesta- and mucosa-associated microbiota.

In general, diet formulation and feeding management showed substantial influence on shaping the gut microbiota in ruminants, whereas food-borne bacteria like silage lactic acid bacteria had little ability of modulation. Weaning had a great impact regardless of bacterial taxa and difference between total mixed ration feeding and separate feeding had effects on the proportions of concentrate- and fiber-associated bacteria. Variations with regard to farms, countries, and individual cows were also seen in relative abundances of several bacterial families. Differences between gut segments and between digesta- and mucosa-associated samples were distinctive. Because most of the findings to date are derived from rectum samples, there is still a large room for improvement on understanding the roles and functions of gut microbiota in ruminants.

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