Assessment of milk and gut microbiota of lactating Jersey cows in relation to milk quality and animal health management

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NGUYEN DANG QUI

Graduate School of Environmental and Life Science

(Doctor's Course)

OKAYAMA UNIVERSITY

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DECLARATION

I hereby declare that my dissertation entitled:

"Assessment of milk and gut microbiota of lactating Jersey cows in relation to milk quality and animal health management."

has been composed by myself, that the work contained herein 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 Animal Science.

Date

14th July 2020

Signature

Nguyen Dang Qui

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CHAPTER 1

GENERAL INTRODUCTION

Bovine milk is an ideal environment for the growth of many microorganisms due to its high nutritional content. A lot of bacterial species, including both desirable and undesirable ones, are detected in raw milk, and thus the milk microbiota may affect cow's health, milk quality and safety of dairy products. Several psychrotrophic bacteria in milk, e.g. *Pseudomonas* spp., *Bacillus* spp., and *Acinetobacter* spp., have ability to grow at low temperatures and become a major cause of milk spoilage, persisting and proliferating during cold storage, producing heat-resistant proteases and lipases which lower nutritional value and alter organoleptic properties of the milk. Besides, the presence of some pathogenic bacteria in raw milk such as *Escherichia coli, Staphylococcus aureus*, and *Streptococcus agalactiae*, will cause a significant increase in somatic cell count (SCC) and increase the occurrence of mastitis. Mastitis lowers productivity and increases farm costs, including veterinary diagnostics, medicines, and even risks leading to elimination of the diseased cows. Understanding microbiota of raw milk is crucial to be able to improve milk quality and prevent mastitis.

Recent studies have revealed that gut microbiota may directly or indirectly contribute to milk productivity, milk quality and cow's health. Genetic and functional aspects of the rumen microbiota are believed to be associated with feed efficiency of dairy cattle, highlighting that manipulation of both compositional and functional outcomes of the rumen microbiota is vital for managing cattle and improving feed efficiency, which subsequently will affect milk productivity. Among the complex rumen microbes, bacteria are the major microbial group contributing to the production of volatile fatty acids (VFAs) and microbial proteins that are utilized by dairy cows for milk production, and the shifts in ruminal VFAs and microbial proteins can directly affect milk yield and milk protein content. Some previous studies indicated that dietary β-carotene supplementation may enhance rumen function, i.e. rumen bacteria's growth and cellulose digestion.

However, the information on the effect of supplemental β-carotene on composition of rumen microbiota as well as hindgut microbiota of dairy cow is scared.

Beside Holsteins, Jersey cow is the second largest dairy breed. Because Jerseys tend to be more efficient and typically have fewer reproductive challenges than Holsteins, reconsideration of purebred Jerseys and a crossbreed between Holsteins and Jerseys has been discussed recently. One strong feature of Jersey products is their yellowness due to high amount of β-carotene in milk. Jersey cows have ability to absorb more β-carotene than Holstein, and the conversion ratio from dietary β-carotene to retinol is different between two breeds, which subsequently affects β-carotene and retinol concentration in milk. The use of β-carotene as a supplement for dairy cows has been applied for a long time, since β-carotene is a precursor of vitamin A and is also an antioxidant, which can directly enhance cow's immunity. Nevertheless, there have been few reports documenting concentrations of fat-soluble micronutrients; i.e. vitamin A, vitamin E and β-carotene in blood and milk of Jersey cows; and milk microbiota; gut microbiota compositions as well as plasma metabolites concentration during β-carotene supplementation. Understanding the milk microbiota, gut microbiota compositions, fat-soluble micronutrients, and metabolic profiles while supplementing β-carotene is important to improve milk quality and cow's health.

In this dissertation, three experiments were carried out to examine the variability and stability of milk microbiota, gut microbiota, and blood metabolites concentrations in relation to seasonal changes and feeding management. Individual milk samples were used to examine the milk microbiota, feces and rumen fluid were used to examine gut microbiota, blood plasma was used to examine metabolic profiles of Jersey cows.

In the first experiment, milk, feces, and blood samples of lactating Jersey cows were collected from two farms and in cool and hot seasons. The objectives were to characterize the milk microbiota of Jersey cows, to examine differences in the milk and fecal microbiota between farms and seasons, and to determine if the metabolic profile of the cows can be associated with a variation in the milk microbiota.

In the second experiment, milk, rumen fluid, feces and blood were collected from 10 healthy lactating Jersey cows supplemented with synthetic β-carotene for one month. The objectives were to examine the effect of β-carotene supplementation 1) on plasma metabolites concentration, 2) on

fat-soluble micronutrients in blood and milk, and 3) on ruminal fermentation, rumen microbiota and hindgut microbiota of lactating Jersey cows.

In the third experiment, milk microbiota, milk components, and fat-soluble micronutrients composition of ten lactating Jersey cows supplemented with β-carotene were examined. The objectives were 1) examine the effect of β-carotene supplementation on milk yield and milk composition, 2) to characterize the indigenous microbiota composition of Jersey milk, 3) to examine whether β-carotene supplementation affect milk microbiota, and 4) to identify the relationships between milk microbial composition and milk components.

CHAPTER 2

LITERATURE REVIEWS

2.1. The association of milk composition with milk quality

Today, not only the nutritional value of milk but also other physiological properties of milk components have attracted interest. Bovine milk contains approximately 87% water, 4.6% lactose, 3.4% protein, 4.2% fat, 0.8% minerals and 0.1% vitamins (Ma & Lindmark, 2008). Milk composition has a dynamic nature, and the composition varies with breed, stage of lactation, season, age, nutrition, energy balance and health status of the udder.

The dairy breeds have the most significant effect on composition of milk. The most commonly-found-breeds, i.e., Friesian, Jersey, Guernsey, Ayrshire, Brown Swiss, and Holstein, have fairly similar lactose levels, but milk fat and protein vary considerably. These differences are partly genetic in origin and partly the results of environmental and physiological factors. Within a herd of cows of a single breed, there are considerable variations in milk composition between individual cows. For example, the milk fat content in Jerseys can range from 4% to 7%, with an average of about 5.0% (Wiley & Sons, 2002).

The milk fat consists mainly of triacylglycerols, approximately 95% of the lipid fraction. Triacylglycerols are composed of fatty acids of different length (C4 – C24) and saturation. Each triacylglycerol molecule is built with a fatty acid combination giving the molecule liquid form at body temperature. Other milk lipids are diacylglycerol (about 2% of the lipid fraction), cholesterol (less than 0.5%), phospholipids (about 1%) and free fatty acids (FFA) (about 0.1%). In addition, there are trace amounts of ether lipids, hydrocarbons, fat-soluble vitamins, flavor compounds and compounds introduced by the feed (Ma & Lindmark, 2008).

The opinions on the consumption of milk and its products have been changing since the moment of stating the presence of biologically active, health promoting substances in the milk. Fatty acids (FAs) in milk fat are considered to be important nutritional components of the diets of a substantial part of the human population. According to scientific knowledge, they can also affect human health. In the case of dairy farming, the FA profile is also seen as an important factor in the technological quality of raw milk. Therefore, the FA profile has the potential to significantly

contribute to the production of dairy products with higher added value. It is equally important to study the health and technological impacts of FAs.

Efforts to carry out practical improvements of milk FA profiles to benefit consumers are usually driven by two reasons: (1) from a nutritional point of view, a lower proportion of saturated FAs (SFA) and a higher proportion of unsaturated FAs (UFA), especially polyunsaturated FAs (PUFA) n-3, is desirable; and (2) from a usability point of view, higher proportions of UFA are preferred (i.e., easier spreadability of butter is desirable for consumers). However, there are also problems associated with having high UFA content in milk fat, including its lower stability and the accompanying phenomena such as oxidation and possible sensory changes.

Some factors affecting the FA profile of milk (such as altitude, breed, lactation order (parity), lactation stage (days in milk), and diet) have been described previously; nevertheless, these factors continue to be studied because of their wide-range of variation and their large number of possible mutually combined effects. In some studies using multifactorial datasets, the main factors affecting milk FA composition were feeding ration, herd, cow´s individuality, and lactation stage; whereas, breed and parity showed only small effects. Although animal factors evidently affect the FA profile of milk fat, the main factors are related to dairy cow nutrition. A number of papers showing specific nutritional effects of cow diet on milk FA profiles have been published. These results regularly show that increasing the proportion of fresh (pasture) or preserved forage (generally fiber) as compared to grain concentrates and increasing the proportion of oilseeds in feed concentrates as compared to non-oleaginous seeds in dairy cow feeding rations improves the milk FA profile by increasing UFA and rumenic acid (RA; C18:2 c9, t11; isomer of conjugated linoleic acid (CLA)) content in milk fat (Hanuš, Samkov, & Kˇ, 2018).

Saturated fatty acids

More than half of the milk fatty acids are saturated. The specific health effects of individual fatty acids have been extensively studied. Butyric acid (C4:0) is a well-known modulator of gene function, and may also play a role in cancer prevention. Caprylic and capric acids (C8:0 and C10:0) may have antiviral activities, and caprylic acid has been reported to delay tumor growth. Lauric acid (C12:0) may have antiviral and antibacterial functions, and might act as an anti caries and anti-plaque agent. Interestingly, *Helicobacter pylori* can in fact be killed by this fatty acid. Another

interesting observation is that capric and lauric acid are reported to inhibit COX-I and COX-II. Stearic acid (C18:0) does not seem to increase serum cholesterol concentration, and is not atherogenic. It would appear, accordingly, that some of the saturated fatty acids in milk have neutral or even positive effects on health. In contrast to this, the saturated fatty acids lauric-, myristic-(C14:0) and palmitic (C16:0) acid have low-density lipoprotein (LDL)- and high-density lipoprotein- (HDL) cholesterol-increasing properties. High intake of these acids raises blood cholesterol levels, and diets rich in saturated fat have been regarded to contribute to development of heart diseases, weight gain and obesity. Association between consumption of milk and milk products and serum total cholesterol, LDL cholesterol and HDL cholesterol has been reported. High cholesterol levels are a risk factor for coronary heart disease (CHD), with LDL cholesterol and a high ratio between LDL and HDL cholesterol enhancing the risk of CHD. Several intervention studies have shown that diets containing low-fat dairy products have been associated with favorable changes in serum cholesterol. However, milk fat consumption has been shown to have less pronounced effects on serum lipids than could be expected from the fat content.

Unsaturated fatty acids

Oleic acid (C18:1 cis-9) is the single unsaturated fatty acid with the highest concentration in milk (about 25 % oleic acid). Accordingly, milk and milk products contribute substantially to the dietary intake of oleic acid in many countries. Oleic acid is considered to be favorable for health, as diets with high amounts of monounsaturated fatty acid will lower both plasma cholesterol, LDL-cholesterol and triacylglycerol concentrations, and replacement of saturated fatty acids with cis-unsaturated fatty acids reduces risk for coronary artery disease. Several studies also indicate a cancer protective effect of oleic acid, but the data are not fully convincing.

Fatty acids are the main building material of cell membranes. The unsaturated fatty acids are reactive as they may give oxidative stress with free radicals and secondary peroxidation products (different aldehydes such as malonedialdehyde and 4-hydroxynonenale) that may be harmful to proteins and DNA in the cells. This may contribute to cancer and to mitochondrial aging processes caused by mutations in mitochondrial DNA. The enzyme lechitin/cholesterol acyl transferace (LCAT), having an important role in reverse cholesterol transport, is sensitive to oxidative stress and it is also inhibited by minimally oxidized LDL. Oleic acid is more stable to oxidation than the

omega-3 and omega-6 fatty acids, and it can partly replace these fatty acids in both triacylglycerols and membrane lipids.

The main PUFA in milk are linoleic- (C18:2 omega-6) and alpha-linolenic (C18:3 omega-3) acid. These fatty acids may be converted to fatty acids with 20 carbon atoms, i.e. arachidonic acid (C20:4 omega-6) and eicosapentaenoic acid, (EPA) (C20:5 omega-3), and further converted to eicosanoids; metabolically very active compounds with local functions. Eicosanoids derived from linoleic acid, via arachidonic acid, may enhance blood platelet aggregation and thereby increase the coronary risk, in contrary to eicosanoids produced form the long omega-3 fatty acids. EPA has the ability to partially block the conversion of the omega-6 fatty acids to harmful eicosanoids, thereby reducing the cardiovascular risk and inhibiting tumor genesis. PUFA may also affect signal transduction and gene expression. It is conceivable therefore that the type of fatty acid in the membrane governs several metabolic functions.

In milk the ratio between omega-6 and omega-3- fatty acids is low and favorable compared to most other nonmarine products. This ratio is greatly influenced by the feeding regime. A favorable meal should be rich in oleic acid and have a low ratio between omega-6 fatty acids and omega-3 fatty acids, perhaps near 1–2:1. Indeed, milk fat fits into this description probably better than any other food item.

Conjugated linoleic acid (CLA)

Bovine milk, milk products and bovine meat are the main dietary sources of the cis9, trans 11 isomer of conjugated linoleic acid (c9, t11-CLA). In most cases this isomer is the most abundant CLA-isomer in bovine milk.

Minor amounts of other geometrical and positional isomers of CLA also occur in milk (such as the t7, c9 and t10, c12-CLA), with different biological effects. Milk content of c9, t11-CLA vary considerably but may constitute about 0,6 % of the fat fraction.

The health effects of CLA have been discussed. CLA is considered as fatness-preventing, anti-atherosclerotic, anti-cancerogenic and immunity-stimulating factor. Administration of c9, t11-CLA has shown to modulate plasma lipid concentration in both human and animal models. Some studies but not all have shown that addition of CLA isomer mixtures (c9, t11 and t10, c12)

to a diet affects plasma lipids. Studies have shown that especially c9, t11-CLA can improve plasma cholesterol status. In a study with healthy men, Tricon et al. found a significant reduction in plasma total cholesterol concentration by c9, t11-CLA. The results concerning the effects of CLA on serum triglycerides are controversial. Tricon et al. observed a decrease in serum triglycerides by c9, t11-CLA compared to t10, c12-CLA in humans, and Roche et al. found serum triglycerides and unesterified FA to be decreased by c9, t11-CLA in ob/ob-mice.

In experimental animals, CLA has been shown to have anticarcinogenic effects. Prospective data from a Swedish study suggest that high intakes of high-fat dairy foods and CLA may reduce the risk of colorectal cancer. The knowledge of CLA's effects in metabolism and the reported anti-proliferative and pro-apoptotic effect of CLA on various types of cancer cells makes CLA to an interesting, and possible therapeutic agent in nutritional cancer therapy. The mechanisms by which CLA might affect metabolism are many. It is suggested that CLA competes with arachidonic acid in the cyclooxygenase reaction, resulting in reduced concentration of prostaglandins and thromboxane in the 2-series. CLA may suppress the gene expression of cyclooxygenase and reduce the release of pro-inflammatory cytokines such as TNF alpha and interleukins in animals. CLA also activates the PPARs transcription factors, and CLA may reduce the initial step in NF-kappa B activation and thereby reduce cytokines, adhesions molecules and other stress-induced molecules.

Changes in the CLA level in the milk fat may occur due to the following reasons: addition of oily plants to a ration, manipulation of rumen fermentation, and direct addition of CLA to the feed. Numerous studies, including Brzóska (1998), Focant et al. (1998), Kelly et al. (1998), Dhiman et al. (1999), Pisulewski et al. (1999), Timothy et al. (2000), and Solomon et al. (2000), concerned modification of fatty acid composition via feeding system. The CLA values, as obtained in the studies of Dhiman et al. (1999), amounted to: 0.34 g/100 g of fat in the control group, 0.69 g for the groups fed the rations containing the addition of soy seeds, and 0.6 g for the groups fed the diet with cotton seed addition. In the studies of Chouinard et al. (1999), the following values of CLA were obtained: 0.43 g/100g of fat for the control group and from 0.95 to 1.52 g for the cows receiving CLA in the ration. The extremely favorable effect of pasture feeding on the level of unsaturated fatty acids, especially of CLA, was found. Kelly et al. (1998) obtained the mean

content of CLA in the milk of cows fed the total mixed ration (TMR) diet on the level of 0.45 g/100 g of fat (Na, Karaszewska, & Zdziarski, 2003).

Trans vaccenic acid (VA)

The main trans 18:1 isomer in milk fat is vaccenic acid, (c18:1, t11, VA), but trans double bonds in position 4 to 16 is also observed in low concentrations in milk fat. The amount of VA in milk fat may vary; constituting 1.7%, or 4–6 % of the total fatty acid content. Typically, the concentration of VA may be about 2–4% when the cows are on fresh pasture and about 1–2 % on indoor feeding. Normally, naturally increase in c9, t11-CLA in milk also results in increased concentration of VA.

VA has a double role in metabolism as it is both a trans fatty acid and a precursor for c9, t11-CLA. Vaccenic acid can be converted to c9, t11-CLA in rodents, pigs and humans.

Trans fatty acids have been shown to increase blood lipids. Industrially produced trans-fatty acids are shown to increase the risk of coronary heart disease as they have adverse influence on the ratio of LDL on HDL, and on Lp. It has been questioned if VA has these same adverse effects. In one study with hamster, Meijer et al. found that VA was more detrimental to cardiovascular risk than elaidic acid (18:1, t9) due to a more increasing effect on LDL/HDL cholesterol ratio. Furthermore, Clifton et al. showed that VA was an independent predictor of a first myocardial infarction. In contrast to this, it has been shown by Willett et al. that trans-fatty acids from animals did not give an increased risk for CHD. As recently demonstrated by Tricon et al, a combination of naturally increased concentration of VA and c9, t11-CLA in milk fat did not result in detrimental effects on most cardiovascular disease risk parameters. However, it remains to clarify if VA has unhealthy effects on blood lipids.

The dietetic and health promoting values of milk fat are determined not only by fatty acids, but also by the content of the present vitamins and carotene. β-carotene together with vitamin A (retinol) and vitamin E (α-tocopherol) are essential for quality and nutritional value of milk and dairy products.

Vitamin A has many functions in the body. In addition to helping the eyes adjust to light changes, vitamin A plays an important role in bone growth, tooth development, reproduction, cell

division, gene expression, and regulation of the immune system. The skin, eyes, and mucous membranes of the mouth, nose, throat and lungs depend on vitamin A to remain moist. Vitamin A is also an important antioxidant that may play a role in the prevention of certain cancers.

β-Carotene is the main safe dietary source of vitamin A, essential for normal growth and development, immune system function, healthy skin and epithelia and vision. β-Carotene has antioxidant properties that can help neutralize free radicals – reactive oxygen molecules potentially damaging lipids in cell membranes, proteins and DNA. All these changes and a high oxidative stress may lead to the development of cardiovascular disease, chronic inflammation and cancer. In vitro studies indicate that β-carotene can also inhibit the oxidation of fats under certain conditions.

Vitamin E benefits the body by acting as an antioxidant, and protecting vitamins A and C, red blood cells, and essential fatty acids from destruction. Research from decades ago suggested that taking antioxidant supplements, vitamin E in particular, might help prevent heart disease and cancer. However, newer findings indicate that people who take antioxidant and vitamin E supplements are not better protected against heart disease and cancer than non-supplement users. Many studies show a link between regularly eating an antioxidant rich diet full of fruits and vegetables, and a lower risk for heart disease, cancer, Alzheimer's Disease, and several other diseases. Essentially, research indicates that to receive the full benefits of antioxidants and phytonutrients in the diet, one should consume these compounds in the form of fruits, vegetables, nuts, seeds, and milk, not as supplements.

2.2. The association of milk microbiota with milk quality and animal health

Bovine milk is an ideal environment for the growth of many microorganisms due to its high nutritional content. A lot of bacterial species, including both desirable and undesirable ones, are detected in raw milk, and thus the milk microbiota may affect cow's health, milk quality and safety of dairy products. Milk in healthy udder cells is thought to be sterile (Tolle, 1980) but thereafter becomes colonized by bacteria from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil and other environments. In general, there are three basic sources of microbial contamination of milk: (1) from within the udder, (2) from the exterior of the teats and udder, and (3) from the milk handling and storage equipment (Wiley & Sons, 2002).

2.2.1. The association of milk microbiota with milk quality

The microbial composition in raw milk has a significant influence on shelf life, organoleptic quality, spoilage and yields of the raw milk, processed milk as well as on the other dairy products (Samaržija, Zamberlin, & Pogačić, 2012). Hence, it is important to assess the composition of the microbiota in raw milk and its impacts to the quality of dairy products (Addis et al., 2016). Being a rich and nutritious fluid, milk supports the growth of many microorganisms. In addition to their contribution to milk fermentation by transforming lactose in lactate, they can bring about a variety of attributes that impact the sensory and textural characteristics of the dairy products derived from milk. Furthermore, contamination with, and subsequent growth of potentially pathogenic bacteria (or with toxins produced by them) in milk can have implications for human health and are therefore relevant issues to consider.

Raw milk can be contaminated with psychrotrophic bacteria from a variety of sources including air, water, soil, and milking equipment (Vacheyrou et al., 2011). Psychrotrophic bacteria are ubiquitous organisms that have the ability to grow at 7 °C or below, regardless of their optimal growth temperature (Sørhaug and Stepaniak, 1997). Their optimal metabolic activity is expressed at temperatures between 20 and 30 °C. However, they can grow and multiply at low temperatures through an enrichment of polyunsaturated fatty acid in their membrane lipids. These bacteria usually account for less than 10% of the total microflora of raw milk, but invariably become predominant during the prolonged storage and transportation of raw milk at low temperatures (Sørhaug and Stepaniak, 1997; Yuan et al., 2018).

Psychrotrophic bacteria isolated from cooled milk belong to Gram-negative and Gram-positive genera and are taxonomically classified into seven classes. *Gammaprotobacteria*, *Bacilli* and *Actinobacteria* are the dominant classes containing between 19 and 21 species, while *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* are the four less significant classes (HantsisZacharov and Halpern, 2007; Samaržija et al., 2012). At genus level, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Stenotrophomosnas*, *Flavobacterium*, *Chryseobacterium*, and *Serratia* are the most frequently isolated from raw milk (Vithanage et al., 2016; Yuan et al., 2018). Among them, *Bacillus*, *Stenotrophomonas*, *Acinetobacter*, and *Pseudomonas* are

considered potentially pathogenic bacteria. These bacterial species are associated with infections in humans and animals, particularly in cases of immune-repression, and they show pronounced resistance to antibiotics (Foght et al., 1996; Svensson et al., 2006.; Munsch-Alatossava and Alatossava, 2005; Beena et al., 2011; Samaržija et al., 2012).

Pseudomonas in milk

The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria. Owing to the fact that the nutritional requirements of *Pseudomonas* spp. are very simple, representatives of the genus have been detected in virtually all natural habitats (e.g., soil, house dust, fresh water and clouds), and have also been isolated from clinical instruments, aseptic solutions, cosmetics and medical products (Franzetti and Scarpellini, 2007). As such, it is not surprising that members of the genus *Pseudomonas* have long been recognized as the predominant group of psychrotrophic bacteria recovered from spoiled refrigerated milk (Chen et al., 2003). Among the pseudomonads, *P. fluorescens* is generally considered to be the principal spoilage agent of pasteurized milk (Mcphee and Griffiths, 2011). The majority of these bacteria (58-91 %) have the ability to show distinct enzymatic extracellular proteolytic, lipolytic and phospholipolytic activity (Wang and Jayarao, 1999; Wiedmann et al.,2000).

The efficient cold adaption of the psychrotrophic pseudomonads is believed to be linked to the possession of elevated levels (between 59 to 72%) of unsaturated lipids in their cell membranes that impart the ability to efficiently maintain membrane functionality (specifically solute transport and the secretion of extracellular enzymes) at refrigeration temperatures (Fonseca et al., 2011; Jay, 2005). Furthermore, these species are able to proliferate in milk, an environment where the concentration of free iron is low, due to the production of the diffusible fluorescent pigment pyoverdine, which acts as a siderophore, allowing the bacteria to effectively sequester iron from lactoferrin (Mcphee and Griffiths, 2011).

De Jonghe et al. (2011) examined the growth of psychrotrophic pseudomonads in raw milk under conditions that simulated prolonged storage (4 days on the farm, 8 hours in transport and 24 hours of storage at the dairy plant) at suboptimal (6 °C) and optimal (4 °C) storage temperatures. The numbers of *Pseudomonas* were similar during the first 72 h of storage at either temperature.

However, by the end of the experiment, a striking difference of 2 log cfu/mL was reported between the optimal and suboptimal storage conditions. Moreover, *Pseudomonas* counts reached the same levels as the total aerobic plate counts by the end of the experiment (10^6 and 10^8 cfu/mL for optimally and sub-optimally cooled milk, respectively). Unfortunately, direct comparisons between the work of De Jonghe et al. (2011) and similar studies (Martin et al., 2011) are difficult to perform due to methodological differences and the specific strains investigated. In this context, the importance of the choice of strain was highlighted in the work of Jaspe et al. (1995), which demonstrated that *Pseudomonas* spp. isolated from milk that had been stored at 7 °C for three days grew ten times faster at 7 °C, had 1000-fold more proteolytic activity, and were 280-fold more lipolytic than *Pseudomonas* spp. isolated from freshly drawn milk.

Phenotypic analysis of microorganisms isolated from raw milk by Mcphee and Griffiths (2011) demonstrated that *P. fluorescens biovar I* (32.1% of isolates), *P. fragi* (29.6%), *P. lundensis* (19.8%), and *P. fluorescens biovar III* (17.3%) were the most commonly isolated species, while Marchand et al. (2009) demonstrated that *P. lundensis* and *P. fragi* were the predominant milk spoilers in Belgian raw milk samples.

Similarly, He et al. (2009) found that pseudomonads predominated in cold stored pasteurized milk at 10 and 5 days before expiration as well as on the expiration day, although they also detected significant numbers of *Streptococcus* spp. and *Buttiauxella* spp. in all samples. Pseudomonads also predominated in the microbiota cultured from the crevices of cleaned devices sampled at a milk processing plant, demonstrating their potential roles as post collection contaminants (Cleto et al., 2012). The ability of this group of microbes to resist cleaning is linked to the fact that many species are effective biofilm producers (Bai and Rai, 2011; Simões et al., 2008). The complex and multilayered structures of biofilms allow the bacterial communities to live in a sessile and protected environment. Yet, when population densities in the biofilms become high, bacteria are released into the environment, providing a continuous source of planktonic bacteria capable of replication within milk (Oliveira, Favarin, Luchese, & Mcintosh, 2015)**.**

The Gram-positive psychrotrophic bacteria isolated from raw milk include following genera: *Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus, Arthrobacter, Streptococcus, Staphylococcus* and *Lactobacillus*. With the exception of *Arthrobacter* and

Lactobacillus, the other genera of that group belong to the thermo-resistant psychrotrophic bacteria (Washam et al., 1977).

Bacillus spp. are today considered the main microbial causes for the spoilage of milk and milk products, and the main reason for significant economic losses in the dairy industry (Meer et al., 1991; Brown, 2000). Wong et al. (1988) confirmed the presence of *B. cereus* in 52 % of ice cream samples, 29 % powdered milk samples, 17 % of fermented milk samples and 2 % of pasteurized milk samples. According to the study by Griffiths and Phillips (1990), about 50 % of the *Bacillus* spp. strains isolated from milk are even capable of growing at a temperature of 2 °C. According to their physiological characteristics, they belong to the mesophilic and thermophilic psychrotrophic strains (S ørhaung and Stepaniak, 1997; Kumarsan et al., 2007). *Bacillus* spp. are a very heterogeneous group of bacteria characterized by different nutritional requirements, the ability to grow in a wide range of temperatures and pH values and show different resistance to osmotic pressure. Due to the different physiological properties they express, the standardization of procedures to isolate these bacteria from milk and dairy products, as well as to define the conditions for their inactivation are made more difficult (McGuiggan et al., 1994; Francis et al., 1998).

Among the bacteria belonging to genus *Bacillus*, from raw, heat treated milk and dairy products *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. circulans* are the most commonly isolated species. The spores of these thermoresistant psychrotrophic aerobic or facultative anaerobic bacteria are activated immediately after the heat treatment of milk by forming vegetative forms. In relation to *Pseudomonas* spp., the vegetative cells of *Bacillus* spp. have a greater capacity to form broad spectrum of thermostable extracellular and intracellular hydrolytic enzymes (Chen et al., 2003; 2004). In 40-84 % of cases, *Bacillus* spp. (with the predominance of the species *B. cereus*) isolated from milk expresses both proteolytic and lipolytic activities, and approximately in 80 % of cases also a phospholipolytic activity (Muir, 1996; Matta and Punj, 1999). Furthermore, certain species of the genus Bacillus can produce more than one type of proteinases simultaneously. However, according to their hydrolytic characteristics these enzymes are comparable to the hydrolytic enzymes formed by *Pseudomonas fluorescens*.

In addition to hydrolytic thermostable enzymes, *Bacillus* species such as *B. cereus*, *B. licheniformis* and *B. subtilis* are able to form different types of toxins which are implicated in food

borne diseases (Salkinoja-Salonen et al., 1999; Svensson et al., 2006). It is particularly interesting that *Bacillus cereus*, which is a very common contaminant of milk and dairy products, can produce several different enterotoxins that are responsible for human infections or intoxication. For example, haemolytic BL (HBL), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK) are associated with gastrointestinal diseases and/or other systematic infections in humans. These enterotoxins are released in the small intestine after consumption of the contaminated product. However, the emetic enterotoxins such as the cereulides that *B. cereus* secretes directly into food are responsible for intoxication that is manifested with the appearance of nausea and vomiting within 1-6 hours of consumption of the product (Senesi and Ghelardi, 2010).

Beside the ability to grow at low temperatures, a variety of psychrotrophic bacterial species (primarily represented by pseudomonads) found in raw milk produce heat-stable proteases and lipases, generally during the late log or early stationary growth phases when the cell density is high. Many of the produced enzymes retain significant activity after pasteurization (72-75 °C/15-20 s) and even UHT treatment (130-150 °C/2-4 s), and may subsequently degrade proteins and fats present in the processed products (De Jonghe et al., 2011; Oliveira et al., 2015).

Heat resistant extracellular proteinases and lipases produced by a broad spectrum of psychrotrophic bacteria at low temperatures contribute to the spoilage of milk and dairy products. These enzymes can survive all successive processing conditions and remain active in processed dairy products. For example, the decimal reduction time (D-value) of proteases produced by *Pseudomonas* spp. was 416.67 min at 75 °C and 250 min at 85 °C, indicating that these enzymes are difficult to inactivate by normal thermal processing techniques (Machado et al., 2016). Consequently, these heat-stable enzymes may lead to unacceptable biochemical changes, a decrease in nutritional value, and reduced shelf-life of dairy products. Lipases catalyze the hydrolysis of triglycerides which cause rancid, butyric, or soapy flavors and also may lead to a reduction in milk foaming properties. Proteases hydrolyze casein fractions and produce defects described as bitter off-flavors and result in age gelation. Phospholipases degrade the integrity of the milk fat globule membrane, facilitating more lipolysis by milk's natural lipases. β-Galactosidases catalyze the hydrolysis of β-1,4- galactosidic bonds in lactose of milk. Therefore, spoilage caused by psychrotrophic bacteria and their enzymes is a major concern in the dairy industry (Yuan et al., 2018).

A reduction in cheese yield and tainting are the two most frequently reported negative effects in cheese production that are attributed to psychrotrophic-derived enzymes (Mcphee and Griffiths, 2011). Less frequently reported effects include the alteration of starter activity and/or growth rates and rennet coagulation times. Reduced yields in cheese production occur mainly because soluble casein degradation products (peptides and amino acids) may be lost into the whey instead of forming part of the curd (Mcphee and Griffiths, 2011). The tainting problems are due to the action of proteases, which generate bitter flavors, and lipases, which hydrolyze milk fat yielding free fatty acids (FFAS) and generate strong flavors that in the majority of cases are considered undesirable (Deeth, 2006; Mankai et al., 2012). 'Age gelation' of UHT milk is an irreversible phenomenon characterized by a change in the physical state that is manifested by a rise in viscosity of more than 10 mPa.S (at 20 °C), followed by the formation of a gel and loss of fluidity (Datta and Deeth, 2001). According to Sørhaug and Stepaniak (1997), a psychrotrophic population of 5.5 log cfu/mL in raw milk causes UHT milk gelation after 20 weeks of storage, while populations between 6.9 and 7.2 logs will cause the same effect between 2 and 10 weeks (Oliveira et al., 2015).

2.2.2. The association of milk microbiota with mastitis

Inflammation of the mammary gland, or mastitis, is a highly prevalent disease of dairy cows, caused by intramammary infection (IMI) derived from bacteria. Mastitis is the most important disease for the dairy industry worldwide, causing economic losses due to reduced milk production, reduced milk quality, discarded milk, lower probability of conception, premature culling, drugs and treatment cost. The decrease in milk production per cow resulting from mastitis is estimated approximately 15% of the milk production. (Addis et al., 2016). Mastitic cows lower the milk quality through the increase of somatic cell counts and decrease of milk compositions. In addition, mastitic quarters have a higher bacterial load than healthy quarters (Taponen et al., 2019).

Many bacteria in milk are known to be related with mastitis. *Staphylococcus aureus* and *Streptococcus agalactiae* are regarded as the most common contagious pathogens, and coagulase-negative *Staphylococci*, *Escherichia coli*, *Streptococcus uberis*, and *Streptococcus dysagalactiae* are regarded as the most common environmental pathogens (Bradley, 2002).

There has been extensive research of possible mechanisms adopted by mastitis-causing pathogens to avoid removal by regular milking and to evade the immune system. In summary, research has demonstrated the ability of *Str. uberis* to resist phagocytosis (Thomas et al., 1994) and intracellular killing by leukocytes (Leigh et al., 1990). Adherence is not important in the early stages of pathogenesis of *Str. uberis* though the ability of certain strains to adhere both to extra-cellular matrix (Lammers et al., 2001) and to bovine mammary epithelial cells in the presence of fibronectin (Almeida et al., 1999) may be important in the subsequent development of persistent infection.

In contrast to *Str. uberis* the mechanisms of persistence of *E. coli* in the bovine mammary gland are less well understood. Serum resistance of *E. coli* has previously been associated with organism virulence in the bovine mammary environment and serum resistant strains have been shown experimentally to be capable of surviving for protracted lengths of time in the mammary gland. As early as 1979 researchers demonstrated experimentally the ability of *E. coli* to cause persistent infection in the mammary gland in the absence of a significant immune response and demonstrated the presence of viable bacteria within neutrophils (Hill et al., 1979). Survival of bacteria in neutrophils alone is unlikely to be the only mechanism of persistence especially considering the short half-life of neutrophils in milk, which would necessitate repeated invasion of fresh neutrophils by the bacteria and hence their exposure to the immune system. More recent research has demonstrated the ability of *E. coli* to adhere to mammary cells, mediated both with and without the presence of fibronectin (Lammers et al., 2001). Researchers in both the Netherlands and the UK have demonstrated an increase in the ability of 'recurrent' strains of *E. coli* to adhere to and invade the Mac-T tissue culture adapted bovine mammary epithelial cell line. Further research has demonstrated that certain *E. coli* strains were as adherent as *Str. dysgalactiae* and more adherent than *Str. uberis*, though less adherent than *S. aureus* (Dopfer et al., 2001).This same research investigated the mechanisms by which two *E. coli* strains invaded epithelial cells, but although demonstrating the role of cytoskeletal elements in the invasion process, it failed to detect genes encoding proteins characteristic of EPEC strains of E. coli. The authors hypothesized the existence of some cytoskeletal mediated uptake reliant on the presence and action of certain phosphokinases by an unknown mechanism (Dopfer et al., 2001).

E. coli is recognized as a highly adaptive organism existing as both a commensal and as a pathogen; its ability to acquire exogenous DNA, and hence virulence genes, is well established (Dozois & Curtiss, 1999). This process could play a role in the emergence of udder adapted strains. Many different subsets of *E. coli* have also been demonstrated, such as enterohaemorrhagic, enteroinvasive and enteropathogenic, and it is not unreasonable to expect that another such subset more adapted to the mammary environment may already be present but be, as yet, unidentified (Bradley, 2002).

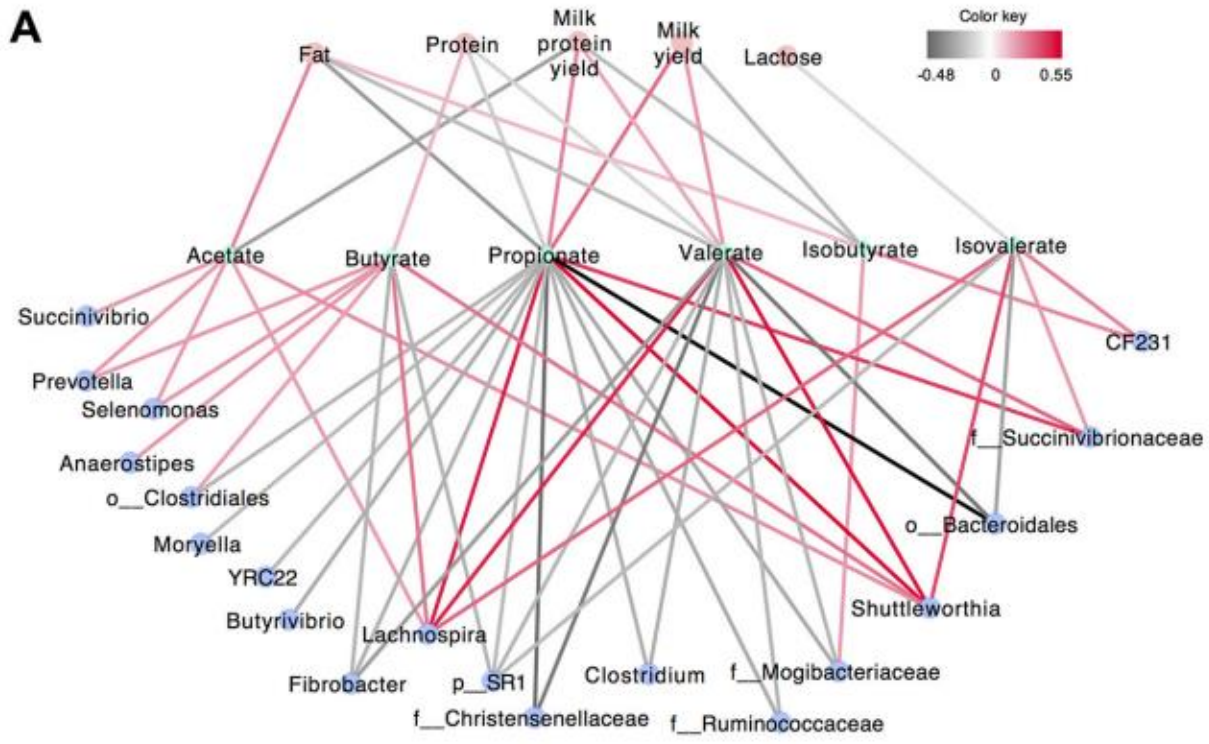
2.3. The association of gut microbiota with milk quality and animal health

2.3.1. The roles of rumen microbiota

The bovine rumen houses a complex microbiota that is responsible for cattle's ability to convert indigestible plant mass into energy for its growth and milk production. With the advent of high-throughput sequencing technologies in recent years, a large amount of data on the composition and function of the rumen microbiota has been generated (Hara, Neves, & Song, 2020). The microbial population in the rumen is diverse, including bacteria, archaea, protozoa, and fungi, 95% of which are bacteria. In a recent survey of cultured rumen bacteria from culture collections, scientific literature, and public databases, cultured rumen bacteria were noted in 88 existing known genera belonging to 9 phyla, with Firmicutes (45 genera), Proteobacteria (20 genera), Actinobacteria (11 genera), and Bacteroidetes (6 genera) representing most of the genera (Firkins & Yu, 2015).

Rumen bacteria are the major microbial group contributing to the production of volatile fatty acids (VFAs) and microbial proteins that are utilized by dairy cows for the majority of their energy and protein requirements. It has been reported that the amount of ruminal VFAs and microbial proteins derived from microbial fermentation are key factors that directly affect milk performance, i.e., milk production and milk fat and protein synthesis (Seymour et al., 2005). Xue et al. (2018) recently revealed the relationship between ruminal VFAs and lactation traits and the rumen microbiota. Analysis of Spearman correlations between lactation traits and rumen fermentation measurements from this study showed that several VFAs were significantly associated in a moderate way with milk yield and milk composition, and these VFAs were also correlated with

the relative abundances of specific bacterial genera. Specifically, positive correlations were found between milk yield and proportions of propionate and valerate. These two VFAs were positively correlated with relative abundances of the genera *Lachnospira* and *Shuttleworthia* as well as with an unclassified genus from the family *Succinivibrionaceae*. Milk protein content was positively correlated with butyrate proportion, and butyrate was positively correlated with the relative abundances of the genera *Prevotella*, *Selenomonas*, *Anaerostipes*, *Lachnospira*, and *Shuttleworthia* as well as with an unclassified genus that belongs to the order *Clostridiales*. Positive correlations were found between milk fat content and acetate proportion, while acetate concentration was positively correlated with the genera *Succinivibrio*, *Prevotella*, *Selenomonas*, *Lachnospira*, *Shuttleworthia* and an unclassified genus from the family *Succinivibrionaceae*. Positive correlations were also detected between milk fat content and iso-butyrate proportion, and iso-butyrate was positively correlated with relative abundances of the genus *CF231* and an unclassified genus from the family *Mogibacteriaceae*.



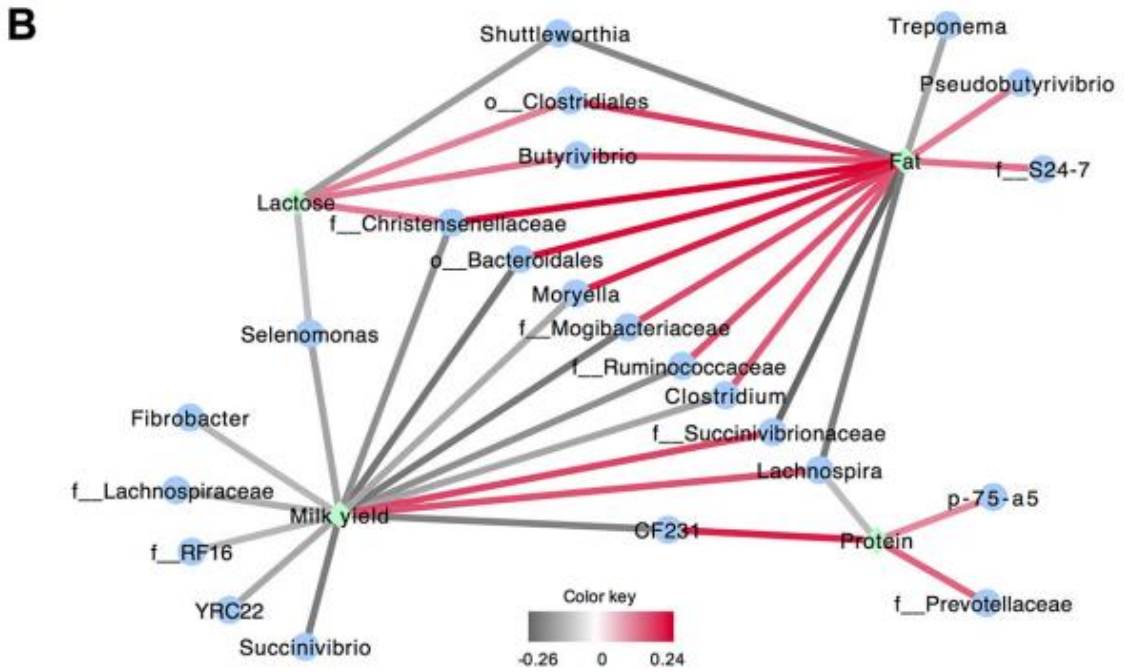


Figure 2.1. Correlation networks showing associations between lactation performance, rumen fermentation products, and bacterial genera (with relative abundance of > 0.1% in at least 60% of all the cows). (A) The correlation network of lactation performance parameters, rumen fermentation parameters, and rumen bacterial genera. Only significant (P < 0.05) correlations were chosen to be displayed in the network. (B) The correlation network of lactation performance and rumen bacterial genera. Only strong (correlation coefficient R > 0.2 or <-0.2) and significant (P < 0.05) correlations were chosen to be displayed in the network. The edge width and color (red, positive; black, negative) are proportional to the correlation strength.

Differences in rumen microbial compositions have been linked to feed efficiency in dairy cattle. Recent studies have revealed that the rumen microbiome may directly or indirectly contribute to milking traits. Specific bacterial taxa are associated with milk yield and milk composition (Jami et al., 2014; Jewell et al., 2015). For example, Jami et al. (2014) provided evidence that various physiological processes in the host animal are correlated with specific rumen bacteria. Their study demonstrated for the first time that the ratio of Firmicutes-to-Bacteroidetes

is positively correlated with milk-fat yield (Bainbridge, 2016)**.** Higher percentages of Firmicutes compensated for the lower abundances of Bacteroidetes. A decreased abundance of Bacteroidetes in comparison to Firmicutes was resulted in increased milk-fat percentages. Similar results were found by Bainbridge et al. (2016), where moderate correlations were found between milk yield, protein percentage, fat yield and bacterial communities. Xue et al. (2018) further examined the relationship between the rumen bacteriome and lactation performance. Milk yield possessed the most complex relationships with bacterial genera, including two positive relationships and 13 negative relationships. Among them, the two positive correlations between milk yield and the relative abundance of the genus *Lachnospira* and that of an unclassified genus belonging to the *Succinivibrionaceae* family were consistent with the positive relationships between the relative abundances of these bacterial taxa and proportions of propionate and valerate. Milk fat was positively correlated with relative abundances of nine genera, including *Butyrivibrio*, *Pseudobutyrivibrio*, *Clostridium*, and *Moryella* and unclassified genera belonging to the families *Christensenellaceae*, *Mogibacteriaceae*, *Ruminococcaceae*, *Clostridiales*, and S24-7, and the order *Bacteroidales*. Milk fat was negatively correlated with the relative abundances of four genera, including *Lachnospira*, *Shuttleworthia*, and *Treponema* and an unclassified genus belonging to the *Succinivibrionaceae*. The positive relationship between milk fat and the relative abundance of an unclassified genus belonging to *Mogibacteriaceae* was consistent with the positive relationship between this taxon and iso-butyrate. Milk protein was positively correlated with the relative abundances of three genera, including CF231 and p-75-a5 and an unclassified genus belonging to the family *Prevotellaceae* and was negatively correlated with the relative abundance of *Lachnospira*. These studies indicate the potential role of the rumen microbiota in modulating milk performance and milk quality.

2.3.2. The roles of hindgut microbiota

Hindgut is defined as the large intestine, which consists of the cecum, colon, and rectum. The role of hindgut fermentation and its microbiota in ruminant nutrition and health has received little research attention in recent decades. Although the contribution of the hindgut to total-tract nutrient digestion is substantially less than the contribution from the rumen, hindgut fermentation and its microbiota affect animal production and health (Gressley, Hall, & Armentano, 2011).

The wet digesta in the hindgut of the cow accounts for approximately 2% of body weight compared with approximately 14% of the rumen's volume of a cow (Dado and Allen, 1995). The differences in volume between hindgut and rumen indicate that the hindgut has about 14% of the capacity for fermentation of the rumen. However, particle retention time in the hindgut is considerably less than that in the rumen, i.e., 13h vs. 30h, which may reduce the extent of substrate fermentation in the hindgut versus the rumen.

Similar to rumen, bacteria reside in the hindgut also possess cellulase, protease, deaminase, and urease activities, and products of fermentation include VFA, NH_3, and microbial cells. Bacterial populations are very dense, at 10^{10} to 10^{12} cfu/ml, with greater than 95% anaerobes. The hindgut, similar to rumen, contains distinct populations of bacteria including luminal bacteria and epithelium-associated bacteria; however, mucus-associated bacteria are an additional population in the hindgut (Hume, 1997).

There is increasing evidence that the hindgut and its resident microbiota also make important contributions to cattle health and production. However, in contrast to the rumen, the fundamental roles of the hindgut microbiota and its contribution to ruminant health and production are poorly understood. Microbial fermentation in the hindgut may be responsible for up to 30% of cellulose and hemicellulose degradation in ruminants, though smaller figures have also been proposed. Lower dietary energy production in the hindgut compartments is likely due to a combination of factors, including reduced retention time of digesta in the hindgut compartments versus in the rumen, as well as the fact that substrates entering the cecum and colon already have been partially digested by enzymes in the rumen (microbial) and small intestine (host and microbial). However, dietary energy derived from the hindgut is likely an important contributor to energy availability in cattle throughout all stages of production, and hindgut fermentation could be of elevated importance to the calf during the first days and weeks of life, before the rumen becomes fully developed.

The hindgut microbiota diverges in composition according to intestinal segments, likely reflecting differences in physical, chemical, and biological conditions in each compartment. The cecum and colon, with the functions of protein and carbohydrate digestion and absorption, are dominated by Firmicutes and Bacteroidetes taxa. Augmenting the hypothesized importance of the hindgut microbes to animal performance, several taxa in large intestine have been related to feed

efficiency status, with abundances of *Butyrivibrio*, *Pseudobutyrivibrio*, *Prevotella*, *Anaeroplasma*, *Paludibacter*, *Faecalibacterium*, and *Succinivibrio*, reported as being divergent across feed efficiency phenotypes. These findings indicate that the microbial communities of the hindgut may indeed be closely related to cattle production efficiency.

Unlike in the rumen, where there remains incongruence over the presence of any robust host immune mechanisms that propagate gut health, the lower-gut regions (including small intestine and large intestine) are highly active in terms of immune function, with the mucosal immune system comprising physical (mucosal/epithelial layers) and chemical (antimicrobial peptides, secretory IgA) barriers, as well as pattern-recognition receptors (for example toll-like receptors, TLRs) and containing a wide array of immune cells that contribute to host defense. As such, with the lower-gut regions known to be vital to immune system development in monogastric animals, there is also increasing evidence that the microbial communities of the lower gut contribute to immune system establishment and homeostasis in beef cattle that directly impact animal gut health in addition to their roles in feed digestion and energy production. In this regard, starter feeding as part of normal early-life calf management influenced both bacterial diversity and the expression of genes (TLR10 and TLR2) related to the effectiveness of the host mucosal immune response in the lower gut. In a follow-up study, total counts of mucosa-associated and luminal bacteria in the small intestine of pre-weaned dairy calves were closely correlated with the expression of genes encoding host immune response, while the same authors also showed that interaction between the commensal gut microbes and expression of specific host microRNAs may contribute to immune system development in the neonatal calf gut. A recent study of functional metagenomic profiles derived from the ileal tissue of *Lactobacillus*-dominant calves showed elevated expression of genes involved in "leukocyte and lymphocyte chemotaxis" and the "cytokine/chemokine-mediated signaling pathway". Taken together, these observations suggest the importance of lower-gut microbiota in immune system development in dairy calves, which may lay the foundation for improving the health of neonatal calves through nutritional manipulation strategies. This is supported by the close relationship between microbial perturbation or dysbiosis in the gut and ruminant health. One example is the onset of hindgut acidosis, which occurs when rapidly digestible carbohydrates overflow to the hindgut for fermentation. The accumulation of acidic fermentation products, such as short-chain fatty acids, is suspected to decrease the luminal pH,

leading to changes in microbial composition and damage to the gut epithelium, with detrimental effects on animal productivity and health. While clear relationships between the ruminal microorganisms and acidosis have been demonstrated, relationships between hindgut acidosis and the changes of lower-gut microbiota in the ruminant remain poorly understood. Evaluating this relationship in future studies may pave the way for manipulation of lower-gut communities as an avenue to improve intestinal health in cattle (Hara et al., 2020).

2.4. The use of β-carotene in animal nutrition

Retinoids are not synthesized by the body (in humans or cattle) and are therefore called essential micronutrients as they are required in small amounts from external sources such as the diet. Retinoids are available in food, firstly; via provitamin A or carotenoids in vegetables and leaves, and secondly as preformed vitamin A obtained from animal sources such as meat and milk product, i.e. the vitamin has already been previously obtained from vegetables and leaves consumed by the animal. Provitamin A is the name given to over 600 carotenoids of which β-carotene is one; Figure 2.2 shows the pathway in which β-carotene is transformed to vitamin A. This pathway illustrates the central cleavage of β-carotene to form two molecules of retinal or one molecule of β-apo-carotenal plus one molecule of β-ionone, both being intermediate substrates. Retinal is an intermediate in synthesis of retinoic acid and undergoes reductase to form retinol. Retinol has no biological function. It is dehydrolized to form retinyl esters creating a less toxic form of the vitamin molecule for storage. Retinyl esters are the storage unit of vitamin A and can be hydrolyzed to form retinol (Packer et al., 2004).

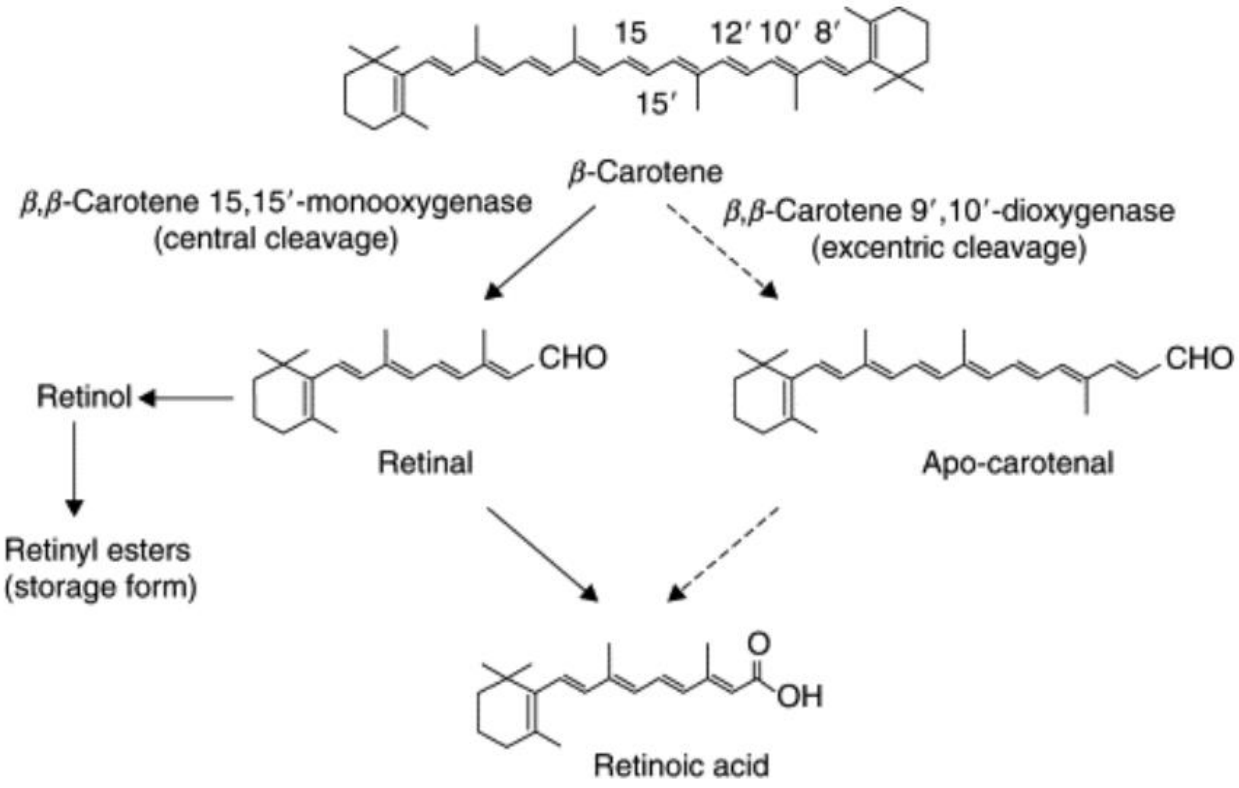


Figure 2.2. The formation of retinoic acid from β-carotene

Retinol, by definition, is true vitamin A, which in the body is converted to retinyl ester for storage, i.e., it is the storage form of the retinol molecule. Retinol itself has no biological purpose but is oxidized to retinal, a molecule important in vision (Packer et al., 2004). Vitamin A is virtually colorless, is soluble in fat and is a long-chain, unsaturated alcohol possessing five double bonds. The most common form of vitamin A found in animal tissues is all-trans-vitamin A, but changes within the molecule catalyzed by moisture, heat or light result in the formation of cis-forms which greatly reduce the effectiveness of vitamin A. Vitamin A is required for a number of physiological processes, including; good vision, resistance to infectious diseases, correct functioning of epithelial cells, healthy and correct bone growth and reproduction.

2.4.1. Chemical structure of β-carotene

Carotenoids are evident as the pigments in plants as the orange-yellow and green color of leaves. There are over 600 types of carotenoids, but so far as we know only 60 have a function in plants and animals. Within the plant, carotenoids aid in photosynthesis and are usually found in high concentrations within the grana of chloroplasts, they are divided into two groups namely the Carotenes mostly seen as orange pigments that contain no oxygen and the xanthophylls or yellow pigments which contain oxygen. β-Carotene ($C_{40}H_{56}$) also known as; carotene, β-carotene, provitamin A, provitamin A1 and carotene Type I, is the most occurring form of carotene and of all the carotenes has the highest activity related to vitamin A. Its molecular structure is shown in Figure 2.3.

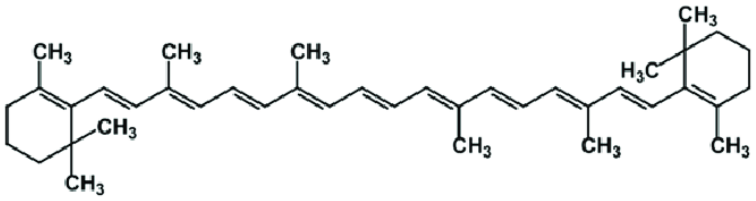


Figure 2.3. Molecular structure of β-carotene

2.4.2. Functions of β-carotene

The main function of β-carotene is that of vitamin A precursor, being converted within the gut or surrounding tissues. Reports have also shown that carotenoids have antioxidant properties; they are able to deactivate the effects of chemicals such as oxygen and free radicals, preventing the effects of potentially harmful processes such as lipid peroxidation (Olson, 1996, McDowell, 2000). Interest in β-carotene as an antioxidant peaked when it was reported that vitamins had antioxidant effects which were believed to reduce the effect of free radicals in tissues.

Trials have shown that supplementing dairy cows with β-carotene at 300 mg/day during the dry period reduced the occurrence of mastitis. Interestingly, in the same trial mastitis was not reduced for animals only receiving vitamin A supplementation (Chew and Johnston, 1985). The function of β-carotene as an antioxidant is further confirmed by an in vitro study by Schweigert (2003) showing the prevention of cross linking (damaging effects of free radicals) in the presence

of β-carotene, with another study showing a reduction of vitamin E but not β-carotene during elevated levels of free radicals, suggesting that free radicals denatured vitamin E but not β-carotene (Schweigert, 2003).

With regard to immune function, there is much evidence pointing to the ability of β-carotene to aid in uplifting the functions of the immune system. β-Carotene has been revealed to aid in the response of lymphocytes, natural killer cells and macrophages. With respect to health and specifically udder health, β-carotene supplementation has been shown to have a stabilizing effect on polymorphonuclear neutrophils (PMNs) which make up the primary defense against bacteria in the udder. This is evident in dose-response trials where the incidence of intra-mammary infections and mastitis was reduced by the supplementation of β-carotene but not of vitamin A (McDowell, 2000). It has also been proven that animals experiencing a vitamin A deficiency have a reduced mobility of natural killer cells, a reduction in the production of antibodies, a reduction in lymphocyte response and a higher inclination towards infection. This shows that vitamin A aids in regulating the immune response whereas β-carotene has an antioxidant role in blood and milk and aids in improving the efficiency of polymorphonuclear neutrophils against *Staphylococcus aureus* (McDowell, 2000).

A number of papers have reported the positive effect of β-carotene on reproduction. β-Carotene does this by affecting structures of reproduction such as follicles and the corpus luteum (Schweigert, 2006). β-Carotene has also been associated with increasing reproductive efficiency by increasing conception and reducing cysts. In the corpus luteum (CL), β-carotene shows a higher concentration than in any other part of the cow. It has been suggested that it has a highly specific role other than that of vitamin A precursor (Buiter, 1998). When vitamin A was greatly reduced during winter feeding, β-carotene was shown to have a positive effect on luteal progesterone (McDowell, 2000). β-Carotene has also been reported to have a specific function in the CL (Buiter, 1998). Arikan and Rodway (2000) reported that progesterone synthesis is at its highest when β-carotene plasma concentrations are at their lowest. This was observed in one experiment on heifers reported by Lotthammer (1978). The heifers in the third trial had received no previous β-carotene versus heifers in trials 1 and 2. Comparisons of the CL with those of the previous trials showed clearly that CL growth in the control group was regressed and did not reach the same mature size when compared to that of the supplemented group (Lotthammer, 1978). On the contrary, in some

other studies, it was found that β-carotene had no effect on fertility (Akordor et al., 1986), or incidence of mastitis (Oldham et al., 1991). These responses, post β-carotene supplementation, have been suggested to be due to amount of β-carotene fed, time of supplementation, β-carotene status at initiation of trial, environmental effects and effects of other ingredients in the diet (Herdt and Seymour, 2006).

2.4.3. Absorption of β-carotene

The process of digestion by pepsin in the stomach and proteolytic enzymes in the small intestine, break down animal feeds and liberate vitamin A and carotenoids from proteins. Bile salts within the duodenum are then able to break up large fatty masses of carotenoids and retinyl esters into smaller parts so that enzymes can digest them easily (McDowell, 2000). For vitamin A and carotenoids to be absorbed in the intestine they must become soluble in a mixed-micelle solution. In order for retinyl esters to be absorbed into the mucosal cell they must first be hydrolyzed to retinol within the intestine wall. Retinol is then re-esterified in the mucosal cell by long chain fatty acids and incorporated as carotenoids into chylomicra which are transported to the general circulation by way of the lymphatic system. Chylomicrons are lipoprotein particles composed of triglycerides, phospholipids, cholesterol and proteins. Their function is to assist in transportation of hydrophobic substances such as lipids, obtained from the diet, to other parts of the body through the water-based system of blood. The liver takes up the retinyl esters and stores them within parenchymal cells, the retinal binds to a transport protein called retinol-binding protein (RBP) thereby making retinol mobile. Retinol-binding protein in turn binds to a larger molecule called transthyretin (TTR) in the blood, making it more resistant to glomerular filtration and renal catabolism. In essence as retinol movement is regulated by the procedures that control the synthesis and secretion of RBP by the liver, RBP is solely accountable for the mobilization of retinol from the liver to the vitamin target sites. The outcome of carotenoids is largely not understood as, unlike retinol, carotenoids are not stored in the liver. In the general circulation, carotenoids are connected to low density lipoproteins, but the chain of events from their removal from chylomicra, until they join up with these lipoproteins, are unknown.

In review of a number of studies, regarding vitamins and health, it is clear that the role of vitamin A is well understood in its effect on disease control as opposed to β-carotene's role in the same area. It is clear that both vitamin A and β-carotene aid in combating infections by aiding the mechanisms of host defense, even though many papers discuss this topic, there are only a few that discuss the effects of vitamins on defense mechanisms in domestic animals (Chew, 1987).

The process whereby vitamin A is synthesized from β-carotene is thought to mostly occur within the intestinal mucosa; however, this process can also take place in the liver or other organs (McGinnis,1988) and in cattle may occur within the corpora lutea (Sklan, 1983). In order for this conversion to take place, the carotene must possess one β-ionone ring in the presence of β-carotene -15,15-dioxygenase and retinaldehyde reductase. The function of -15,15-dioxygenase is to initiate the process of β-carotene cleavage at the double bond in the center of the molecule. This produces two molecules of retinol. Retinaldehyde reductase changes retinal to retinol by the process of reduction (Wolf, 1995). Vitamin A and β-carotene are absorbed in the intestine, the absorption of vitamin A is about 80 to 90% and β-carotene is about 50 to 60%. In the lymph system, vitamin A is conveyed via a carrier in the form of a low-density lipoprotein which takes it to the liver. The main factor effecting the secretion of RBP from the liver is the vitamin A status, as well as oestrogen, protein and zinc. Therefore, in the case of vitamin A deficiency, secretion of RBP from the liver is blocked, increasing the RBP content of the liver and reducing that in blood plasma (McDowell, 2000).

There is evidence that β-carotene is transmitted from plasma to follicular fluid by passive transfer. Only lipoproteins of high density can pass through the blood-follicle barrier due to the structure of the molecular sieve and their low molecular weight. There are two possible routes of entry for vitamin A found in follicular fluid. Firstly by crossing the blood-follicular barrier by way of transportation via carrier proteins, transfer rate by this method is influenced only by molecule charge and size, therefore implying a constant transfer rate, secondly β-carotene is absorbed by granulose cells, converted to vitamin A and transferred to the follicular fluid via carrier proteins (Schweigert, 2006).

2.4.4. Factors affecting absorption of β-carotene

2.4.4.1. Animal effect

With regards to animal effect, vitamins require transportation through the digestive tract, thus availability is directly proportional to transportation through the rumen and absorption in the intestine. Vitamins are released in the rumen by the action of digestion, a prerequisite to absorption, therefore the efficiency of this release is primarily important to vitamin bioavailability but this may vary with plant type. The losses of vitamin A in the rumen that have been reported are large, approximately 40-60% but no exact values are available. Vitamin A is denatured in the rumen by chemical reactions, and rumen bacteria cause; oxidation, degradation and engulfment. It would seem that the absorption of vitamin A through the intestinal wall has not been demonstrated to date, possibly as the size of the vitamin A molecule is so large. β-Carotene losses in the rumen range from 3 to 32% with an average of 20%, probably due to hydrogenation; the losses of vitamin A are hypothesized to undergo reductive degradation (Potkanski et al., 1974). It could therefore be concluded that due to the broad range of feeds given and the influence of rumen fermentation the nutrient profile found in the rumen is variable and the chemical structure of vitamins undergo substantial modification (Williams et al., 1998).

2.4.4.2. Breed effect

Different breeds were found to have varying abilities to convert carotene to vitamin A. In order of efficiency: Holstein, Ayrshire, Jersey and Guernsey. No significant ($P < 0.05$) difference was found between Holstein and Ayrshire. The different breeds had similar responses to β-carotene and vitamin A intake but the range of plasma β-carotene and vitamin A fluctuation varied between breeds, with vitamin A to a much lower degree than β-carotene. Vitamin A fluctuations did not follow that of the β-carotene levels but seemed to have a lag effect (Sutton et al, 1945).

2.4.5. β-Carotene and animal performance

The role of vitamin A and β-carotene in prevention of animal diseases is well documented. Vitamin A is necessary for all cellular division and differentiation (Herdt and Stowe, 1991), and plays a key role in inhibition of keratinization. Deficiency of vitamin A culminates with

hyperkeratinization of the secretory epithelium, increasing the susceptibility to diseases (Reddy and Frey, 1990).

β-Carotene, a precursor of vitamin A, functions as an antioxidant reducing superoxide formation within the phagocyte (Sordillo et al., 1997) and can directly enhance immunity with reproductive and mammary benefits (Chew, 1993). Vitamin A is also related to immunity and mastitis. It plays an important role in maintaining epithelial tissue health and preserving the integrity of the mucosal surface (Sordillo et al., 1997); which may contribute in preventing the entrance of mastitis causing pathogens into the mammary gland.

2.4.5.1. Milk production

Arechiga et al. (1998) conducted three experiments on two farms in the South and North of Florida to test the effects of β-carotene supplementation on dairy cattle during heat stress. Animals were split into two groups, namely; the control group receiving no supplemental β-carotene and the supplemental group receiving 400 mg of β-carotene daily. Animals were supplemented from 15 days postpartum for 2 months and blood plasma was analyzed for β-carotene, retinyl palmitate, retinol, α-tocopherol and progesterone. Overall milk yield for supplemented cows showed an increased in all 3 experiments as a result of supplemental β-carotene with increases of 11%, 6% and 7% in order. Experiment 1 ($P < 0.05$) and experiment 3 ($P < 0.01$) showed significant differences for predicted milk yield at 305 days of lactation. Folman et al. (1987) reported that only cows in lactation 4 and higher had an increased milk yield whereas Akordor et al. (1986) reported no effect of β-carotene supplementation on milk production. Kawashima et al. (2009) found no differences for 305-day milk yield. Rakes et al. (1985) tested the effect of β-carotene supplementation on different diets in lactating cows with regards to milk yield but no differences were found between treatment groups for milk yield.

In a study to evaluate the effect of β-carotene on milk yield (de Ondarza et al., 2009), 515 cows were used and treatment animals received 425 mg of β-carotene daily. However, β-carotene supplementation had no effect on milk yield. De Ondarza and Engstrom (2009) investigated the effect of supplementing β-carotene (425 mg/cow/d), on lactating Holstein cows with low concentrations of β-carotene in serum (< 3 μg/mL) and adequate supplementation of vitamin A

(8400 I.U/kg) to examine any effects on milk yield. Animals were supplemented for 120 days and milk production was measured. Supplementation had no effect on 3.5% FCM but there was a tendency ($P < 0.01$) for cows in early lactation and lactation 3 and higher to produce more milk. Folman et al. (1987) reported that younger animals in a supplemented group receiving β-carotene (500 mg/d) during both the dry and lactation periods were shown to have a higher FCM yield than similar animals in the control group only receiving 69 mg of retinyl acetate per cow/day. From the literature cited, it is clear that responses to β-carotene supplementation on milk yield is variable and is affected by various factors as discussed in the previous sections of this chapter.

2.4.5.2. Milk composition

De Ondarza and Engstrom (2009) reported that β-carotene had no effect on overall milk fat (kg/d), milk true protein % or milk true protein production; however there was a tendency ($P < 0.01$) for early lactation cows in lactation 3 or higher to produce more milk fat (kg/d). Supplementation of β-carotene resulted in an average increase in Milk Urea Nitrogen (MUN) (mg/dL) by 2%, affecting cows in second lactation and those less than 100 DIM. This is, however, biologically insignificant. In another experiment by de Ondarza et al. (2009) the percentage of milk fat was 3.25% and 3.18% for the supplemented and control group respectively. However, milk fat production (kg/day) was unaffected by β-carotene supplementation but data did show trends ($P < 0.01$) of milk fat increase in favor of the β-carotene supplemented group. β-Carotene supplementation had no effect on milk true protein percentage and production. Kawashima et al. (2009) found no differences for the effect of supplemental β-carotene on milk composition as did Rakes et al. (1985) who tested the effect of β-carotene supplementation on milk fat percentage and found no differences between treatment groups. As is the case with milk production, β-carotene supplementation effects on milk composition are variable but are mostly not affected.

2.4.5.3. Somatic cell count

In an experiment by Bindas et al. (1984), 78 cattle were assigned to either a β-carotene supplemented group receiving 600 mg of β-carotene daily, or a control group receiving no supplemental β-carotene from 30 to 60 DIM. The supplemented group experienced maximum

concentrations of 2.45 µg/ml β-carotene at about week 10 and the control group of 1.50 µg/ml by about week 7. The supplemented group was found to have a lower comparative somatic cell count (SCC) but this was not significant. Rakes et al. (1985) tested the effect of β-carotene supplementation on different diets in lactating cows with regards to SCC and found that SCC was lower for Lucerne-fed cows than maize fed cows, this was also true for β-carotene supplemented cows as compared to control or un-supplemented cows but this was not a significant difference. De Ondarza and Engstrom (2009) found β-carotene supplementation had no effect on the SCC as did de Ondarza et al. (2009). Oldham et al. (1991) reported that neither β-carotene nor vitamin A supplementation had an effect on reducing the SCC. In general, β-carotene supplementation does not seem to affect the SCC of milk, based on the results of the studies mentioned above.

2.4.5.4. Growth

It was suggested that β-carotene may have a general metabolic effect on ruminant performance. Folman et al. (1987) found β-carotene supplementation to have a positive effect on growth, this was again confirmed when Folman et al. (1979) established that a β-carotene supplemented group experienced a higher growth rate and average daily gain than that of the control group, but this increase in body weight was only observed during the last 3 months of the trial when animals were put in the same yard, suggesting that the supplemented group had a higher DMI. A contradicting article stated that β-carotene supplementation had no effect on the average live weight or change of live weight during the trial duration (Ducker et al., 1984).

2.4.5.5. β-Carotene in milk

Breed type was found to have an effect of secretion of β-carotene levels and amount in milk but was found to be of less importance when compared to the effect of diet on the amount of β-carotene in milk (Nozière et al., 2006). It has been reported that ruminants are poor absorbers of β-carotene; this was confirmed in an experiment by Ascarelli et al. (1985) who found only a few milligrams of β-carotene daily in milk of an un-supplemented herd. A general increase of vitamin A in milk was seen, as cows progressed in lactation, directly related to the high intake of these vitamins in the diet. This can be associated with the low rate of uptake of β-carotene from the

blood, as these molecules are generally associated with high density proteins for transportation in ruminants, whereas molecules associated with low density lipoproteins are easily absorbed. However, in a trial by Nozière et al. (2006) plasma concentrations of β-carotene accounted for only 20% of variation in milk levels. β-Carotene levels in colostrum are high as close-up animals absorb β-carotene to supply to calves at birth, but levels drop as milk returns to normal (Bindas et al., 1984).

2.4.6. β-Carotene and animal health

2.4.6.1. β-Carotene levels in cattle

Katsoulos et al. (2005) reported that β-carotene and vitamin E were lowest at calving compared to any other time, in a trial initiated at 30 days prepartum and continuing up to 10 months postpartum. β-Carotene concentrations during the first month in lactation were lower than plasma concentrations during the dry period. Average plasma concentrations of β-carotene were significantly higher in group A, which consisted of animals 4 years of age or younger, compared to group B, consisting of animals older than 4 years. This trial confirmed changes in β-carotene from the dry period to the end of lactation, with age playing a major role in levels of fat-soluble vitamins in plasma. The rapid decrease of β-carotene plasma concentration at calving was due to lactation inception, as β-carotene is excreted in © University of Pretoria 29 colostrum and lost through oxidation and a decrease in DMI at this time. Vitamin A, however, was found to experience no change.

2.4.6.2. Udder health and mastitis

Compromised udder health, which is directly related to mastitis, is one of the most economically important conditions in dairy herds. It is the single highest cause of premature culling. Farmers constantly underestimate the true cost of mastitis. The associated financial losses include; cost of medication, discarded milk not fit for human consumption, veterinarian consultation, labor, reduced milk yield, premature culling, expense of replacement animals, cost of feed for animals not producing and reduced price per liter for lower quality milk related to increased SCC. Mastitis management, however, remains the best means of control (Halasal et al., 2007).

Chew et al. (1982) found that a deficiency of vitamin A and β-carotene may be linked to udder infections in cows. Proposed explanations for this include: a weakening of the udder lining due to reduced keratin secretion, resulting in a successful attack by organisms causing mastitis; reduced transportation of immunoglobulins and leukocytes to the infected area and the rate of transfer of β-carotene and vitamin A from plasma to milk or differences in the conversion of β-carotene to vitamin A which takes place in the intestine. Chew et al. (1982) further stated that cattle with reduced concentrations of plasma β-carotene and vitamin A scored higher on the California Mastitis Test. Dahlquist and Chew (1985) reported that supplemental β-carotene reduced the incidence of new udder infections acquired during the dry period. Chew and Johnston (1985) reported that supplemental β-carotene reduced the SCC count in cattle during the lactation period. Wang et al. (1988) found that β-carotene supplementation had a positive effect on reducing mastitis whereas Kawashima et al. (2009) and Oldham et al. (1991) did not. Similar to many other production measures, the effect of β-carotene supplementation on the incidence of mastitis is variable and inconclusive.

2.4.6.3. Immune function

Kawashima et al. (2009) showed that reduced plasma concentrations of β-carotene in anovulatory cattle may correlate to inadequate support of the immune function. In review of a number of studies regarding vitamins and health, it is clear that the role of vitamin A is well understood in its effect on disease control as opposed to β-carotene's role in the same area. It is clear that both vitamin A and β-carotene aid in combating infections by aiding the mechanisms of host defense. Although many papers discuss this topic, there are only a few papers that discuss the effects of vitamins on defense mechanisms in domestic animals. Chew (1987) reported that vitamins may help in aiding mammary health. Chew (1993) stated that β-carotene may also function as an antioxidant. This antioxidant function is further confirmed by Rapoport et al. (1998) and Weiss (1998). Van den Berg et al. (2000) reported the antioxidant nature of carotenoids in ruminant diets and stated that this function aids in cell communication and immune function by shielding cells from free radical attacks.

Michal et al. (1994) reported that β-carotene obtained from the diet was able to boost host defense mechanisms by enhancing lymphocyte and phagocyte function, reducing the occurrence

of several reproductive disorders. There are many reports of positive effects on human health by β-carotene supplementation, including prevention of certain cancers, improved immune function, tumor restraint, aid in reduction of coronary heart disease, cataract suppression and the reduction of deterioration related to aging (Umeno et al., 2005).

2.4.6.4. Calf health

Kaewlamun et al. (2011) investigated the responses to dietary β-carotene supplementation of dairy cows during the dry period. The supplemented group received 1 g of β-carotene daily. Results displayed an increase in plasma β-carotene status and an increase in β-carotene content of colostrum in the supplemented group (3.10 ± 0.23 mg/L) versus the control group (1.44 ± 0.24 mg/L). Results reported from a number of studies show that concentrations in colostrum can range widely for β-carotene (17.8 to 342.9 μg/dL) and vitamin A (32.9 to 450.0 μg/dL). These variations have been linked to the following factors; individual effect, breed effect, effect of lactation number, effect of diet given during the dry period and incidence of mastitis as well as the decline of both of these vitamins supplied in milk over time after calving (Kume and Tanabe, 1993). Parrish et al. (1953) reported that absorption by calves during the first week of life of vitamin A and β-carotene was 81-95% and only 38-65%, respectively. In a study conducted by Nonnecke et al. (1999), 3 groups of Jersey bull calves received colostrum for the first week of life and milk replacer for 7 weeks thereafter. The first group, the control group, received no supplementation of vitamin A. The second group was supplemented with 32 000 I.U vitamin A and the third group was supplemented with β-carotene equivalent to 20 000 I.U. vitamin A/day. It was found that supplemented vitamin A had an influence on the composition of mononuclear leukocyte population (MNL) or white blood cell population in calves, increasing the growth of leukocytes associated with the recognition and response to antigens. Supplemental β-carotene had no effect on the MNL population. Vitamin A supplied in the diet to calves may help in improving the rate of development of the calf's immune system, furthermore the vitamin A supplied to calves in milk replacer can change the bioavailability of vitamin E and the composition of the peripheral blood MNL, improving calf health.

Lotthammer (1978) reported low incidence of diarrhea in calves when their dams were supplemented with β-carotene. Calves with β-carotene-unsupplemented dams were shown to have

much lower concentration of gamma-globulins and vitamin A versus those with supplemented dams, prior to both groups receiving colostrum. Kume and Tanabe (1993) agreed that the vitamin status of calves relies on both the amount of vitamin in colostrum, as well as that absorbed via the placenta during late pregnancy and that this is more important for vitamin A than β-carotene, as β-carotene is available in milk at higher levels than vitamin A. Optimal β-carotene concentrations have also been shown to improve calf health status by reducing the magnitude and incidence of diarrhea and pneumonia (Byers et al., 1956). Quigley et al. (1995) and Quigley and Drewry (1998) confirmed that colostrum passively transfers immunoglobulins, other proteins and nutrients to the calf which supports the immune system and results in the reduction and extent of scouring. It is important that feed is of high quality prepartum, as this affects the vitamin status of calves via placental transfer and colostrum, consequently calves fed low vitamin colostrum require supplementation to maintain optimal health. Kume and Toharma (2001) stated that vitamin A and β-carotene deserved further investigation in improving calf health due to their findings.

2.5. Reconsidering of Jersey cows for milk production

The Holstein and Jersey dairy breeds have emerged to be the top two dairy breeds in the world in popularity. The popularity is primarily due to the two breeds having the ability to convert large quantities of forage-based diets into milk and milk components. According to New Zealand dairy statistics (2016-2017), Jersey milks have 5.6% of fat, 4.21% of protein, 9.91 % solids not fat on average while Holstein milks have lower value with 4.48% of fat, 3.76% of protein, and 8.24% of solids not fat. With the trend toward milk component pricing, the shift has been away from fluid milk toward milk solids. With the higher percent components in Jersey milk, producers feel the Jersey cow is more suited for today's marketplace.

Holsteins have been the major breed in dairy farming because of their overwhelming productivity compared with other breeds, including Jerseys. The high milk yield of Holsteins, however, often forces the cows to encounter a serious negative energy balance when they enter lactation after parturition. The state of negative energy balance may increase the number of services per pregnancy and extend the time of days open, accounting in part, for the decline in reproductive ability (Brown et al., 2012).

A comparison study by Washburn was carried out in 2002 (Washburn et al., 2002), who compared reproduction, mastitis, and body condition of seasonally calved Holstein and Jersey cows and concluded that Breed differences were significant, as Jersey cows had less clinical mastitis, higher BCS, higher insemination rates, higher conception rates, and lower culling rates than Holsteins. In particular, Jerseys had higher conception rates (59.6 vs. 49.5 ± 3.3%) and higher percentages of cows pregnant in 75 d (78.1 vs. 57.9 ± 3.9%) than Holsteins. Jerseys had half as many clinical cases of mastitis per cow as Holsteins. Jerseys had higher condition scores and lower body weights than Holsteins.

Jersey cow is the second largest dairy breed. Because Jerseys tend to be more efficient, typically have fewer reproductive challenges, have less environmental impact, have lower culling rate, more sustainable, and have higher heat tolerant ability than Holsteins; reconsideration of purebred Jerseys and a crossbreed between Holsteins and Jerseys has been discussed recently.

2.5.1. Feed efficiency

A Jersey cow is very efficient at converting dry matter into milk. Most studies in New Zealand and internationally indicate the feed conversion efficiency of Jersey (g MS/kg DM) is superior to Friesians in the order of 9-13% and as high as 18.7%. Differences are even greater when expressed as g MS/kg BW – in the order of 20-30% more MS/kg BW produced by Jerseys compared with Friesians, due to a combination of the increased feed conversion efficiency (FCE) and the fact that Jerseys eat more per kg liveweight than Friesians. These findings are found for both total mixed ration and pasture-based diets but, on average, are greater for pasture-based systems. For both g MS/kg DM and kg DM/kg BW, Jersey x Holstein crossbreeds are generally intermediate between parent breeds with a small but significant heterosis component.

2.5.2. Reproduction

A number of studies have compared Jerseys and Holsteins using reproductive measures. One of the comprehensive studies was conducted in North Carolina (Fonseca et al., 1983). The Holstein cows averaged 109.2 days open versus 94.8 days open for the Jerseys. Table 2.1 showed some

comparisons about reproductive measures of Jersey and Holstein, in which Jerseys have shorter days from parturition, shorter interval to cervix involution, shorter interval to uterine involution, shorter interval from parturition to 1st detected estrus, higher in percentage detected in estrus and higher first service conception rate.

Table 2.1. Means for reproductive measures of Jerseys and Holsteins (Fonseca, 1983).

Traits	Holsteins	Jerseys
Days from parturition to 1st ovulation	20.8	20
Interval to cervix involution (days)	23.7	20.9
Interval to uterine involution (days)	23.8	21.7
Interval from parturition to 1st detected estrus	66.9	37.2
Percentage detected in estrus (overall)	43	73
1st service conception rate, percent	49	72

A possible factor influencing reproductive performance is calf size. A research in North Carolina determined Jerseys produce female calves equal to 5.5 - 5.6 % of dam's weight, while Holsteins produce female calves equal to 5.8 - 6.5 % of dam's weight. Research results indicate Jerseys are more efficient in reproductive performance than Holsteins.

2.5.3. Environmental impact

Jerseys are kinder on the environment. Compare with Holstein, Jerseys require less feed for maintenance, which means less production of greenhouse gasses and urinary nitrogen (Garrick, Holmes, Blair, & Spelman, 2000**,** Sneddon & Baudracco, 2011). If a Holstein herd is replaced by a Jersey herd of similar genetic merit and at numbers to produce the same amount of milk solids (MS), the maintenance requirement of the Jersey herd will be 5.5 - 8 % less than the Holstein herd. These differences will be reflected in reduced greenhouse gas (GHG) and N leaching outputs. In essence, without impacting significantly on MS production, a farmer with a herd containing a high proportion of Holstein genetics can reduce both the GHG emissions and N leaching figure if these animals are replaced with cows of predominantly Jersey genetics.

2.5.4. Culling rate

Jerseys have a number of advantages in lowering the culling rate in the dairy population. Studies consistently demonstrate that Jerseys develop less clinical mastitis (Jury et al., 2005**,** Taylor et al., 2007) and lameness (Taylor, et al., 2013) than Holsteins and J x HF. In one large New Zealand study, the seasonal cumulative incidence of clinical mastitis was; Friesians, 15.8%, J x HF, 12.4%, Jersey, 7.6% (Taylor et al., 2007). Mastitis and lameness combined account for almost 75% of total disease problems on farm in New Zealand and are a significant cause of cows being culled. Fewer replacements in Jerseys also reduces rearing costs, thus improving the profitability of the whole farm system.

Numerous studies also confirm that Jerseys have a higher rate of cycling prior to mating than other breeds which results in less hormonal intervention and/or improved in calf rates (Mccarthy et al., 2012).

2.5.5. Milking frequency

Milking once a day (OAD) compared to twice a day (TAD) is gaining popularity. In 2016, 9% of herds were milked OAD for the entire season and 47% of herds milked OAD as part herds or part seasons (making a success of full-season once-a-day milking). OAD milking saves energy, needs less water for milk cooling and shed washing and requires less labor.

Like twice-a-day milking systems, the Jersey in an OAD system is more efficient than both Holsteins and J x HF cows when expressed as g MS/kg LW. When farmed together on OAD, Jerseys produced 9% and 6% more milk solids per kg liveweight than Holsteins and J x HF respectively over the first 150 days of lactation. Crossbred and Jersey cows were less affected than Holstein cows by OAD milking with a reduction in milk yield traits of ≤ 19.0%, while in Holstein cows the reduction ranged between 19% – 25%. (Lembeye et al., 2014).

Jersey breed is the most adaptable to an OAD milking system due to its more concentrated milk. This means that the negative impact of OAD on milk solids production is less, and farmer opinion is that udder breakdown is reduced compared with those breeds that produce less concentrated milk, like J x HF and HF cows. OAD systems are more sustainable from energy,

water and labor use perspective. This sustainability is maximized if the herd is Jersey as there is reduced culling because of udder breakdown and mastitis (Dalley & Clark, 2005), and therefore fewer replacements required.

2.5.6. Heat tolerant ability

Whether measured as ambient temperature or calculated as the temperature-humidity index (THI), Jersey cows tolerate the heat much better than Holsteins. As ambient temperatures increase, Holstein cow rectal temperature, heart rate, respiratory rate and milk solids production changes at a lower temperature than Jersey (Bryant, 2006)**,**.

Smith (2013) compared the effects of heat stress on milk yield, milk components and somatic cell score in Holstein and Jersey cows revealed that Jersey cows appeared to be more heat tolerant than Holstein cows. More specifically, Holstein milk yield decreased during heat stress, whereas Jersey milk yield increased. Milk fat percentage for Holstein and Jersey cows declined during heat stress. Holstein fat-corrected milk yield decreased during heat stress, whereas Jersey fat-corrected milk yield during heat stress did not differ from that during non-heat stress. During heat stress (HS), somatic cell score increased in milk from Holstein and Jersey cows compared with non-heat stress. In the second analysis, HS was categorized as mild, moderate, or severe. The corresponding temperature-humidity index (THI) values were THI ≥72 but <79, THI ≥79 but <90, and THI ≥90. Holstein milk yield declined during moderate and severe heat stress, whereas Jersey milk yield declined during severe heat stress. Holstein milk fat percentage was less during moderate and severe heat stress compared with milk fat percentage during mild HS. Jersey milk fat percentage did not differ with regard to heat stress category (Smith et al., 2013).

Another study by Bryant (2006) reported that Jerseys can tolerate an average THI of 75 over a 24-hour period (or 25.5^0C at 70% humidity). In contrast, the Holsteins level of tolerance is lower at a THI of 68 (or 21^0C at 70% humidity. For every unit of THI above this maximum tolerable level, there is a production loss of 10g MS/day (Bryant, 2006). As our climate warms, the relative heat tolerance of Jerseys will become a significant benefit of the breed.

Because of the merits mentioned above, i.e., more efficient, fewer reproductive challenges, less environmental impact, lower culling rate, more sustainable, and higher heat tolerant ability than Holsteins; reconsideration of purebred Jerseys and a crossbreed between Holsteins and Jerseys should be considered. On the other hand; more studies on rumen microbiota, milk microbiota, and fecal microbiota should be carried out on purebred Jerseys and their crossbreeds to maximize milk productivity, milk quality, and minimize greenhouse gas emission.

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CHAPTER 3

EXAMINATION OF MILK MICROBIOTA, FECAL MICROBIOTA, AND BLOOD METABOLITES OF JERSEY COWS IN COOL AND HOT SEASONS

3.1. INTRODUCTION

Milk is an ideal environment for the growth of many microorganisms due to its high nutritional content. A lot of species, including both desirable and undesirable ones, are detected in raw milk, and thus the milk microbiota may affect the quality and safety of dairy products (Quigley et al., 2013). Some bacteria in milk, including members of *Lactococcus* spp., *Lactobacillus* spp., *Streptococcus* spp., *Propionibacterium* spp., and *Leuconostoc* spp. can exert a beneficial role in altering the taste, appearance, and texture of milk and dairy products (Quigley et al., 2013). Meanwhile, several psychrotrophic bacteria, e.g. *Pseudomonas* spp., *Bacillus* spp., and *Acinetobacter* spp., have the ability to grow at low temperatures and become a major cause of milk spoilage, persisting and proliferating during cold storage and producing proteases and lipases (Meer et al., 1991; Vithanage et al., 2016). Proteases may reduce the nutrient and economic value of milk by the hydrolysis of casein during milk processing, and lipases can convert lipids into free fatty acids, resulting in unexpected flavor and altering organoleptic properties (Hantsis-Zacharov & Halpern, 2007).

Purebred Holsteins have been the major breed in dairy farming because of their overwhelming productivity compared with other breeds. The high milk yield of Holsteins, however, often forces the cows to encounter a serious negative energy balance when they enter lactation after parturition. The state of negative energy balance may increase the number of services per pregnancy and extend the time of days open, accounting in part, for the decline in reproductive ability (Brown et al., 2012). Milk yield of Jersey cows is ≈30% lower and the negative energy balance after parturition may be less critical compared to that of Holstein cows. The number of services per pregnancy and the time of days open can remain at acceptable levels; hence, reconsideration of purebred Jerseys and a crossbreed between Holsteins and Jerseys has been discussed (Kristensen et al., 2015).

High protein and fat content are attractive features of Jersey milk, which contains 20–30% higher levels compared to Holstein milk, and the amount of cheese produced per kg of milk is >20% greater when Jersey milk is used (Alstrup et al., 2015). A difference in the milk composition between breeds may provoke differences in the milk microbiota. However, information on the microbiota of Jersey milk is limited compared to that of Holstein milk. The microbiota of Holstein milk has been shown to vary by season and farm management (Li et al. 2018; Wu et al., 2019; Nguyen, Wu, & Nishino, 2019); therefore, studies on the microbiota of Jersey milk should be conducted while examining season-to-season and farm-to-farm differences. Likewise, although the microbiota of bulk tank milk may be of greater importance for milk processing in the industry (Skeie, Hàland, Thorsen, Narvhus, & Porcellato, 2019), understanding the composition of the microbiota of individual cow milk can also be of value for herd management.

In this study, we visited two Jersey farms in cool (November 2017, (Nov)) and hot (July 2018, (Jul)) seasons and collected samples of individual cow milk, blood, and feces, and that of bulk tank milk. The microbiota of the milk and feces were analyzed using 16S rRNA gene amplicon sequencing, and metabolic profiles of the cows were evaluated using blood metabolite levels. The objectives of this study were to characterize the milk microbiota of Jersey cows, to examine differences in the milk and fecal microbiota between farms and seasons, and to determine if the metabolic profile of the cows can be associated with a variation in the milk microbiota.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection

Feces and milk samples were collected in cool (Nov) and hot (Jul) seasons. Two farms (F1 and F2) were located in the Hiruzen region, Okayama prefecture, and both used a tie-stall housing and pipeline milking system. Daily minimum and maximum temperatures on the day of sampling were 1.7 and 12.3 °C for Nov 2017 and 19.9 and 30.9 °C for Jul 2018, respectively. The herd size of the farms ranged between 30–40 animals. Both farms fed the cows a diet containing timothy silage, alfalfa hay, cracked corn, and rolled barley as the main ingredients; however, the forage to concentrate ratio was higher in F1 (60:40) than in F2 (45:55). Milk, blood, and feces were collected individually from eight cows with no systemic signs of production diseases, such as mastitis,

metritis, lameness, ketosis, and hypocalcemia. The cows were selected at random during the November 2017 sampling, and the same cows were chosen if they kept producing milk during the July 2018 sampling. Eventually, half of the cows were replaced between the Nov and Jul samplings. Individual milk samples were collected by a sampling device attached to the milking parlor, first at 18:00 and the next at 6:00 the next morning, before being pooled together as a composite milk sample. Samples of bulk tank milk were collected from each farm in the morning. Feces were collected from the rectum and blood was collected from the caudal vein. The sampling was performed between 10:00–11:00 at F1 and between 13:00–14:00 at F2. All samples were kept on ice during transportation and subsequently frozen at -30 °C until further analysis. All procedures and protocols for animal experiments were approved by the Animal Care and Use Committee, Okayama University (OKU-2016290), Japan.

3.2.2. Blood metabolites and milk components analysis

A total of 32 plasma samples were analyzed for non-esterified fatty acids (NEFA), glucose (Glu), total cholesterol (T-Cho), albumin (Alb), urea nitrogen (UN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) using commercially available kits (Fujifilm Wako Pure Chemicals Co., Tokyo, Japan). Plasma haptoglobin (Hp) was examined using a bovine Hp ELISA kit (Life Diagnostics, Inc., West Chester). High performance liquid chromatography determination of β–carotene, retinol, and α–tocopherol levels was performed according to the procedures of Talwar, Ha, Cooney, Brownlee & O'Reilly (1998). The protein, fat, and solids-not-fat (SNF) contents of the milk were determined using a CombiFoss FT+ analyzer (Foss Allé, Hillerød, Denmark). Somatic cell count (SCC) was estimated by N-acetyl -β-D-glucosaminidase (NAGase) activity assay in milk (Wu et al. 2019).

3.2.3. Bacterial DNA extraction

To collect bacterial DNA from samples, 1 mL of milk was centrifuged at 15,000 rpm for 30 min at 4 °C. Fat and supernatants were removed and the pellets were re-suspended in 500 μL solution I containing 0.05 M D-glucose, 0.025 M Tris-HCl (pH 8.0), and 0.01 M sodium EDTA (pH 8.0). The mixture was then centrifuged again for 2 min at 15,000 rpm and the supernatant was

removed. Bacterial pellets were re-suspended in 180 μL lysis buffer including 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 1.2% Triton X-100, 20 mg lysozyme, and distilled water. After incubation at 37 °C for 1 h, proteinase K was added to remove the proteins. The mixtures were used to isolate genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Bacterial DNA of fecal samples was extracted from 0.2 g wet weight and purified using the mini DNeasy Stool Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions.

3.2.4. Quantification of total bacteria

The total bacteria population in milk and feces was determined using quantitative real-time PCR targeting the V3 region of the 16S rRNA gene (Wu et al., 2019). Each PCR tube contained 2 μL DNA template, 1 μL forward primer 357f (5'-ACGGGGGGCCTACGGAGGCAGCAG-3'), 1 μL reverse primer 517r (5'-ATTACCGCGGCTGCTGG-3'), 12.5 μL KAPA SYBR FAST Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), 0.5 μL bovine serum albumin, and 8 μL distilled water, in a final reaction volume of 25 μL. The qPCR was performed on a Mini Opticon real-time PCR system (Bio-Rad Laboratories Inc., Tokyo, Japan). The amplification program involved an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s and an additional incubation step at 80 °C for 30 s. A standard curve was established using 1010 to 103 copies of the 16S rRNA gene/μL isolated from Escherichia coli plasmid DNA (JCM 1649).

3.2.5. Illumina MiSeq sequencing

The bacterial DNA of milk samples was subjected to two-step PCR procedures to generate an amplicon library for MiSeq sequencing (Nguyen, Wu, & Nishino, 2019). The first round PCR was employed using primers targeting the V4 region of the 16S rRNA gene (forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3′; tail sequences are underlined). The PCR protocol included an initial denaturation at 94 °C for 2

min, followed by 35 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. For fecal samples, similar cycling parameters were used except 25 cycles were used instead of 35 cycles. The PCR products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and subsequently used as DNA templates for second round PCR with adapter-attached primers. The second round PCR protocol included an 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. After the purification process described above, the purified DNA was pair-end sequenced (2 × 250 bp) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

The archived raw sequences were processed using quantitative insights into microbial ecology (QIIME, version 1.9.1) software. The raw sequences were filtered to remove reads at any sites receiving a quality score lower than 25 and a length shorter than 135 bp. Sequences that overlapped more than 60 bp with a maximum 20% difference were joined paired ends, followed by the identification and removal of chimeric sequences. Only the remaining high quality sequences were grouped into operational taxonomic units (OTUs) with a 97% similarity threshold. Bacterial clustering was analyzed from the phylum to genus level.

3.2.6. Statistical analysis

All data were statistically analyzed using JMP software (version 11, SAS Institute, Tokyo, Japan). The statistical significance of the differences was determined by non-parametric Wilcoxon test. Principle coordinate analysis (PCoA) was performed using Primer version 7 with Permanova+ add-on software (Primer-E, Plymouth Marine Laboratory, Plymouth, UK).

3.3. RESULTS

3.3.1. Milk production, milk components, and blood metabolite levels

No differences were observed in days in milk (123–198 days), parity (2.75–4.88), milk yield (20.2–22.2 kg/day/cow), and milk composition (3.80–4.04% protein, 4.66–5.08% fat, 9.27–9.47%

SNF content, and 2.72–3.74 × 10^5 cells/mL SCC) between the farms and seasons (Table 1). The levels of UN, T-Cho, and Glu were higher for F1 than F2 cows, and AST was greater for F2 than F1 cows. Season-to-season differences were found for Alb, Glu, AST, and ALT (Nov < Jul) and β–carotene and retinol (Nov > Jul).

Table 3.1. Days in milk, milk yield, milk composition, and plasma metabolite concentrations of Jersey cows examined at two farms in cool and hot seasons.

	Farm 1		Farm 2			Wilcoxon test	
Farm	Nov	Jul	Nov	Jul	SE	Farm	Season
Days in milk	153	123	146	198	40.6	NS	NS
Parity	4.88	3.13	3.13	2.75	0.82	NS	NS
Milk yield (kg/d)	20.2	23.4	22.2	21.0	2.13	NS	NS
Milk composition							
Protein (%)	4.04	3.80	3.82	4.03	0.13	NS	NS
Fat (%)	4.82	4.66	4.69	5.08	0.22	NS	NS
Solid-not-fat (%)	9.40	9.35	9.27	9.47	0.13	NS	NS
SCC (x10^5 cells/mL)	3.74	3.40	3.40	2.72	0.52	NS	NS
Plasma metabolites							
Albumin (g/dL)	3.64	4.06	3.95	4.24	0.10	NS	**
Urea nitrogen (mg/dL)	18.1	21.1	15.5	15.1	1.10	**	NS
Total cholesterol (mg/dL)	173	199	149	143	10.31	**	NS
NEFA (mEq/L)	0.07	0.22	0.05	0.13	0.04	NS	**
Glucose (mg/dL)	62.7	63.3	59.3	54.8	1.99	*	NS
AST (units/L)	20.9	23.9	27.6	35.4	2.23	**	*
ALT (units/L)	3.05	7.05	3.98	8.62	0.66	NS	**
β-carotene (μg/mL)	13.8	2.82	11.9	0.35	1.58	NS	**
Vitamin A (μg/mL)	1.97	0.12	6.47	0.11	0.37	NS	**
Vitamin E (μg/mL)	1.40	1.76	3.08	1.45	0.38	NS	NS
Haptoglobin (μg/L)	106	108	118	92.4	8.11	NS	NS

SCC; somatic cell count, NEFA; non-esterified fatty acid, AST; aspartate aminotransferase, ALT; alanine aminotransferase. P-value is calculated by Wilcoxon test.

3.3.2. Milk microbiota

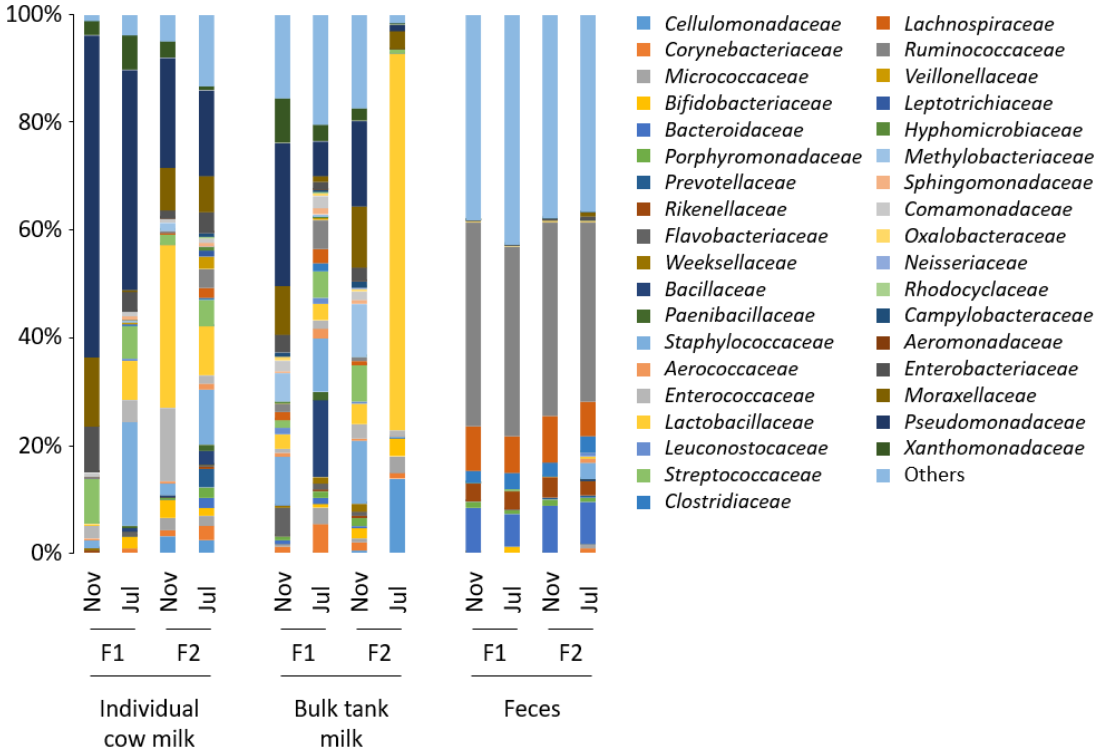

Figure 3.1. Relative abundance of individual milk, bulk tank milk, and fecal microbiota of Jersey cows examined at two farms in cool and hot seasons.

The MiSeq sequencing resulted in non-chimeric sequence reads with an average of 41,657 and 44,536 for milk and feces samples, respectively. No differences were found in alpha diversity indices (OTU number, Chao 1, phylogenetic diversity whole tree, and Shannon) of the milk microbiota between seasons (Figure 3.1; Table 3.2). Proteobacteria (30.7–85.3%) and Firmicutes (13.4–55.1%) were two prevalent phyla regardless of the farm and season. At the family level, the prevalent taxa were *Pseudomonadaceae* (16.1–59.7%), *Lactobacillaceae* (0.55–30.0%), *Staphylococcaceae* (1.84–19.4%), *Moraxellaceae* (0.24–12.9%), and *Enterococcaceae* (1.41–13.9%). For Nov samples, the five most abundant taxa were *Pseudomonadaceae*, *Moraxellaceae*, *Enterobacteriaceae*, *Streptococcaceae*, and *Xanthomonadaeae* for F1 cows, and *Lactobacillaceae*,

Pseudomonadaceae, *Enterococcaceae*, *Moraxellaceae*, and *Bifidobacteriaceae* for F2 cows. For Jul samples, four of the five most abundant taxa, i.e. *Pseudomonadaceae*, *Staphylococcaceae*, *Lactobacillaceae*, and *Streptococcaceae*, were common for F1 and F2 cows. Among these prevalent families, the abundance of *Staphylococcaceae* showed a season-to-season difference (Nov < Jul), and those of *Pseudomonadaceae*, *Streptococcaceae*, *Enterobacteriaceae*, and *Xanthomonadaceae* (F1 > F2), and *Lactobacillaceae* and *Bifidobacteriaceae* (F1 < F2) demonstrated farm-to-farm differences.

Prevalent families of bulk tank milk microbiota appeared to be different compared with those of individually collected milk (Figure 3.1; Table 3.3). For example, the five most abundant families of Nov samples were *Pseudomonadaceae*, *Staphylococcaceae*, *Moraxellaceae*, *Xanthomonadaeae*, and *Methylobacteriaceae* for the F1 bulk tank milk, however, *Staphylococcaceae* and *Methylobacteriaceae* were not found in the five most abundant families of individually collected F1 milk. Likewise, *Staphylococcaceae* and *Streptococcaceae* were found in the five most abundant families of the F2 bulk tank milk but they were not observed in those of individually collected F2 milk. There were large differences of bulk tank milk microbiota between Nov and Jul samples regardless of the farm. F1 and F2 bulk tank milk had *Bacillaceae* and *Lactobacillaceae*, respectively, as the most prevalent families, which were not found in the five abundant taxa in Nov samples. Regarding the F2 bulk tank milk, none of the three most abundant taxa of the Jul samples, i.e. *Lactobacillaceae*, *Cellulomonadaceae*, and *Bifidobacteriaceae* were found in common with the Nov samples.

Table 3.2. Total population, diversity indices, and relative abundance of milk microbiota of Jersey cows examined at two farms in cool and hot seasons.

	Farm 1		Farm 2			Wilcoxon test	
	Nov	Jul	Nov	Jul	SE	Farm	Season
Total bacterial population (log10 copies/mL)	7.07	6.71	6.59	6.84	0.29	NS	NS
Observed OTU	657	797	934	1160	106	**	NS
Chao 1	1288	1514	1626	1958	179	*	NS
PD whole tree	27.9	35.7	46.5	54.4	4.77	**	NS
Shannon	3.83	4.28	5.11	5.86	0.37	**	NS

Actinobacteria	0.18	11.2	3.00	8.96	1.28	**	NS
Actinomycetaceae	0.00	0.03	0.04	0.43	0.10	**	*
Cellulomonadaceae	0.02	0.02	2.90	2.40	0.91	**	NS
Corynebacteriaceae	0.06	0.58	1.17	2.43	0.35	**	*
Gordoniaceae	0.00	0.00	0.01	0.18	0.09	NS	NS
Microbacteriaceae	0.04	0.04	0.30	0.15	0.07	**	NS
Micrococcaceae	0.03	0.08	2.47	2.10	0.68	**	NS
Bifidobacteriaceae	0.02	2.25	3.20	1.25	0.71	*	NS
Bacteroidates	0.88	1.09	2.09	10.4	2.46	NS	NS
Bacteroidaceae	0.03	0.05	0.05	1.97	0.31	NS	*
Porphyromonadaceae	0.04	0.04	0.33	1.98	0.70	**	NS
Prevotellaceae	0.00	0.01	0.02	3.28	1.56	**	*
Rikenellaceae	0.01	0.02	0.01	0.33	0.10	NS	**
S24-7	0.00	0.01	0.00	0.66	0.13	*	**
Paraprevotellaceae	0.00	0.00	0.00	0.40	0.12	**	*
Cytophagaceae	0.00	0.28	0.04	0.08	0.11	NS	NS
Flavobacteriaceae	0.10	0.60	0.21	0.60	0.29	NS	NS
Weeksellaceae	0.31	0.16	0.07	0.13	0.11	NS	NS
Sphingobacteriaceae	0.35	0.85	0.25	0.03	0.33	NS	**
Firmicutes	13.4	55.1	39.9	42.7	7.78	*	NS
Bacillaceae	0.01	0.82	0.03	2.65	0.54	NS	**
Paenibacillaceae	0.00	0.12	0.00	0.83	0.33	NS	**
Planococcaceae	0.03	0.27	0.20	0.17	0.12	NS	*
Staphylococcaceae	1.84	19.4	2.41	10.3	6.53	NS	*
Aerococcaceae	0.05	0.07	0.34	1.20	0.46	NS	NS
Enterococcaceae	2.24	4.11	13.9	1.41	2.39	NS	*
Lactobacillaceae	0.55	7.39	30.0	9.20	5.15	*	NS
Streptococcaceae	8.36	6.20	1.99	4.64	1.48	**	NS
Turicibacteraceae	0.00	0.05	0.03	0.40	0.07	NS	**
Clostridiaceae	0.01	0.05	0.08	0.60	0.10	**	*
Lachnospiraceae	0.06	0.10	0.18	1.65	0.21	**	*

Ruminococcaceae	0.07	0.26	0.26	3.70	1.01	*	**
Veillonellaceae	0.00	0.28	0.02	2.14	1.01	NS	**
Erysipelotrichaceae	0.01	0.04	0.05	0.63	0.13	**	**
Fusobacteria	0.00	0.02	0.08	1.84	0.62	**	**
Fusobacteriaceae	0.00	0.03	0.02	0.76	0.28	NS	*
Leptotrichiaceae	0.00	0.05	0.00	1.09	0.34	*	**
Proteobacteria	85.3	30.7	53.7	31.9	8.01	**	*
Caulobacteraceae	0.12	0.05	0.53	0.05	0.14	NS	**
Brucellaceae	0.13	0.26	0.15	0.24	0.13	NS	*
Hyphomicrobiaceae	0.00	0.05	0.12	0.70	0.34	NS	NS
Methylobacteriaceae	0.20	0.04	1.46	0.12	0.31	NS	**
Phyllobacteriaceae	0.00	0.01	0.01	0.38	0.19	NS	NS
Acetobacteraceae	0.00	0.00	0.51	0.01	0.06	**	*
Sphingomonadaceae	0.01	0.92	0.13	0.84	0.50	NS	NS
Comamonadaceae	0.68	0.65	0.59	0.45	0.30	NS	NS
Rhodocyclaceae	0.00	0.00	0.03	0.25	0.08	*	NS
Campylobacteraceae	0.03	0.00	0.19	0.76	0.36	**	**
Enterobacteriaceae	8.62	3.69	1.52	3.76	2.20	**	NS
Pasteurellaceae	0.00	0.01	0.00	0.32	0.14	NS	**
Moraxellaceae	12.9	0.24	7.72	6.81	3.22	NS	**
Pseudomonadaceae	59.7	40.9	20.6	16.1	7.39	**	NS
Xanthomonadaceae	2.73	6.48	2.96	0.49	1.20	*	NS
Spirochaetes	0.00	0.00	0.01	0.21	0.07	**	*
Spirochaetaceae	0.00	0.01	0.00	0.21	0.07	*	*
Verrucomicrobia	0.00	0.03	0.05	0.36	0.07	NS	**
Verrucomicrobiaceae	0.00	0.05	0.03	0.36	0.07	NS	**
Thermi	0.00	0.01	0.20	0.01	0.10	NS	NS
Deinococcaceae	0.00	0.01	0.20	0.01	0.10	NS	NS

Phyla and families having a relative abundance of > 1% in at least one sample are indicated.

Table 3.3. Relative abundance of bulk tank milk microbiota of Jersey cows examined at two farms in cool and hot seasons.

	Farm 1		Farm 2	
	Nov	Jul	Nov	Jul
Total bacterial population (log10 copies/mL)	7.41	7.30	7.12	7.57
SCC (x10^5 cells/mL)	3.35	3.35	2.92	3.30
Actinobacteria	4.03	9.71	7.17	21.9
Cellulomonadaceae	0.01	0.04	0.27	13.7
Corynebacteriaceae	0.96	5.15	1.54	1.13
Microbacteriaceae	0.66	0.59	2.15	0.10
Micrococcaceae	0.41	3.07	0.72	2.94
Promicromonosporaceae	1.49	0.00	0.00	0.00
Bifidobacteriaceae	0.29	0.65	2.19	3.54
Bacteroidetes	9.00	7.28	7.16	0.03
Bacteroidaceae	0.47	1.33	0.07	0.00
Porphyromonadaceae	0.96	1.07	1.74	0.01
Flavobacteriaceae	5.16	1.35	0.99	0.00
Weeksellaceae	0.46	1.05	1.49	0.01
Sphingobacteriaceae	1.14	0.06	2.35	0.00
Firmicutes	20.7	54.2	28.7	72.8
Bacillaceae	0.01	14.2	0.01	0.00
Paenibacillaceae	0.00	1.53	0.00	0.00
Staphylococcaceae	9.18	9.96	11.8	0.05
Aerococcaceae	0.61	1.96	0.37	0.00
Enterococcaceae	0.74	1.54	2.45	1.29
Lactobacillaceae	2.60	2.76	3.71	70.1
Leuconostocaceae	1.26	1.11	0.48	0.00
Streptococcaceae	1.26	5.21	6.80	0.56

Turicibacteraceae	0.07	1.15	0.00	0.00
Clostridiaceae	0.29	1.43	0.15	0.01
Lachnospiraceae	1.28	2.47	0.59	0.01
Ruminococcaceae	1.57	5.56	0.78	0.03
Proteobacteria	60.2	20.5	50.4	5.09
Caulobacteraceae	1.26	0.14	3.18	0.00
Methylobacteriaceae	5.29	0.35	9.99	0.00
Rhizobiaceae	1.93	1.90	0.00	0.00
Sphingomonadaceae	0.65	1.25	0.59	0.00
Comamonadaceae	1.70	2.34	1.66	0.00
Campylobacteraceae	0.80	0.31	1.01	0.00
Enterobacteriaceae	3.61	1.77	2.58	0.01
Moraxellaceae	9.12	1.13	11.4	3.43
Pseudomonadaceae	26.3	6.29	16.0	1.04
Xanthomonadaceae	8.17	2.96	2.28	0.61
Verrucomicrobia	0.05	1.04	0.00	0.00
Verrucomicrobiaceae	0.05	1.04	0.00	0.00

SCC; somatic cell count. Phyla and families having a relative abundance of > 1% in at least one sample are indicated.

3.3.3. Fecal microbiota

Differences between farms and seasons were observed for the Chao 1 index; the diversity of feces of F2 cows was higher than the diversity of feces of F1 cows, and Nov samples had a greater diversity than Jul samples (Figure 3.1; Table 3.4). Four predominant phyla in the feces were Firmicutes (64.1–65.9%), Bacteroidetes (22.1–26.6%), Actinobacteria (1.09–2.69%), and Proteobacteria (0.29–1.74%) (Table 3). No difference in their abundance was observed regardless of the farm and season, except that the abundance of Proteobacteria was greater in Jul than Nov. At the family level, the five most abundant taxa were *Ruminococcaceae* (33.3–37.7%),

Bacteroidaceae (6.06–8.48%), *Lachnospiraceae* (6.46–8.70%), *Rikenellaceae* (2.95–3.58%), and *Clostridiaceae* (2.22–3.32%). These prevalent families were stable between farms and between seasons, whereas season-to-season variations were seen for *Lachnospiraceae* (Nov > Jul) and *Clostridiaceae* (Nov < Jul).

Table 3.4. Total population, diversity indices, and relative abundance of fecal microbiota of Jersey cows examined at two farms in cool and hot seasons.

	Farm 1		Farm 2			Wilcoxon test	
	Nov	Jul	Nov	Jul	SE	Farm	Season
Total bacterial population (log10 copies/mL)	9.61	9.51	9.54	9.60	0.14	NS	NS
OTU number	2590	2463	2587	2528	83.3	NS	NS
Chao 1	4448	4137	4855	4574	160	*	*
PD whole tree	99.8	94.4	99.7	98.3	2.46	NS	NS
Shannon	8.76	8.83	8.67	8.56	0.12	NS	NS
Actinobacteria	1.09	1.49	2.69	2.36	1.14	NS	NS
Methanobacteriaceae	1.05	1.32	1.61	0.73	0.22	NS	NS
Corynebacteriaceae	0.00	0.00	0.02	0.74	0.37	**	*
Micrococcaceae	0.00	0.00	0.00	0.79	0.40	NS	NS
Bifidobacteriaceae	0.03	0.17	1.06	0.07	0.49	NS	NS
Bacteroidetes	25.7	26.6	25.2	22.1	1.60	NS	NS
Bacteroidaceae	8.32	8.48	6.06	7.83	1.01	NS	NS
Porphyromonadaceae	0.99	1.32	0.75	0.8	0.10	NS	**
RF16	0.83	1.2	1.15	0.59	0.17	NS	NS
Rikenellaceae	3.25	3.58	3.17	2.95	0.30	NS	NS
S24-7	0.81	1.06	1.68	1.07	0.19	NS	NS
Paraprevotellaceae	1.02	1.54	1.24	0.78	0.13	NS	NS
p-2534-18B5	0.55	0.11	0.31	0.12	0.13	*	NS
Firmicutes	65.9	64.1	64.8	65.8	1.35	NS	NS
Bacillaceae	0.04	0.01	0.02	0.13	0.06	NS	NS
Planococcaceae	0.00	0.00	0.00	0.34	0.17	NS	NS
Staphylococcaceae	0.01	0.01	0.03	2.98	1.49	NS	*

Aerococcaceae	0.00	0.00	0.01	1.03	0.51	NS	NS
Leuconostocaceae	0.00	0.00	0.02	0.72	0.36	NS	**
Turicibacteraceae	0.27	0.27	0.89	0.84	0.24	NS	*
Clostridiaceae	2.22	2.88	3.32	2.97	0.37	NS	*
Lachnospiraceae	8.24	8.7	6.46	6.5	0.57	NS	**
Peptostreptococcaceae	0.21	0.27	0.91	0.84	0.25	NS	**
Ruminococcaceae	37.7	35.8	35.4	33.3	2.07	NS	NS
Mogibacteriaceae	1.38	1.22	1.66	1.47	0.17	NS	NS
Erysipelotrichaceae	1.79	1.72	1.74	1.52	0.17	NS	NS
Proteobacteria	0.29	1.09	0.15	1.72	0.56	NS	*
Succinivibrionaceae	0.25	1.00	0.08	0.15	0.21	NS	**
Enterobacteriaceae	0.01	0.06	0.01	0.66	0.29	NS	NS
Moraxellaceae	0.00	0.00	0.01	0.59	0.29	NS	**
Spirochaetes	0.78	0.63	0.68	0.42	0.12	NS	NS
Spirochaetaceae	0.78	0.63	0.68	0.42	0.12	NS	NS
Verrucomicrobia	0.49	0.42	0.34	1.28	0.18	NS	NS
Verrucomicrobiaceae	0.49	0.42	0.34	1.28	0.18	NS	NS

Phyla and families having a relative abundance of > 1% in at least one sample are indicated.

3.3.4. Relationships between milk and fecal microbiota

The relationship between the milk and fecal microbiota, as analyzed by PCoA, showed a clear separation between milk and feces regardless of the farm and season (Figure 3.2). The fecal microbiota was closely grouped regardless of the farm and season, with the exception of one sample from the F2 cows that had relatively higher abundance of *Staphylococcaceae* and lower abundance of *Ruminococcaceae*, which was grouped with milk samples. The fecal microbiota featured typical gut microbiota including the five most abundant taxa, i.e. *Ruminococcaceae*, *Bacterioidaceae*, *Lachnospiraceae*, *Rikenellaceae*, and *Clostridiaceae*. The milk microbiota appeared to be separated by the farm, but several samples were grouped together across the two farms. The majority of the F1 milk microbiota was grouped together regardless of the season, and featured *Enterobacteriaceae*, *Pseudomonadaceae*, and *Streptococcaceae*. The F2 milk microbiota

appeared to be separated into three groups; one group of Nov samples featured *Xanthomonadaceae* and *Enterococcaceae*, and another group of Nov samples featured *Lactobacillaceae*, *Bifidobacteriaceae*, and *Cellulomonadaceae*. Regardless, the PCoA demonstrated that the fecal microbiota was unrelated to the milk microbiota.

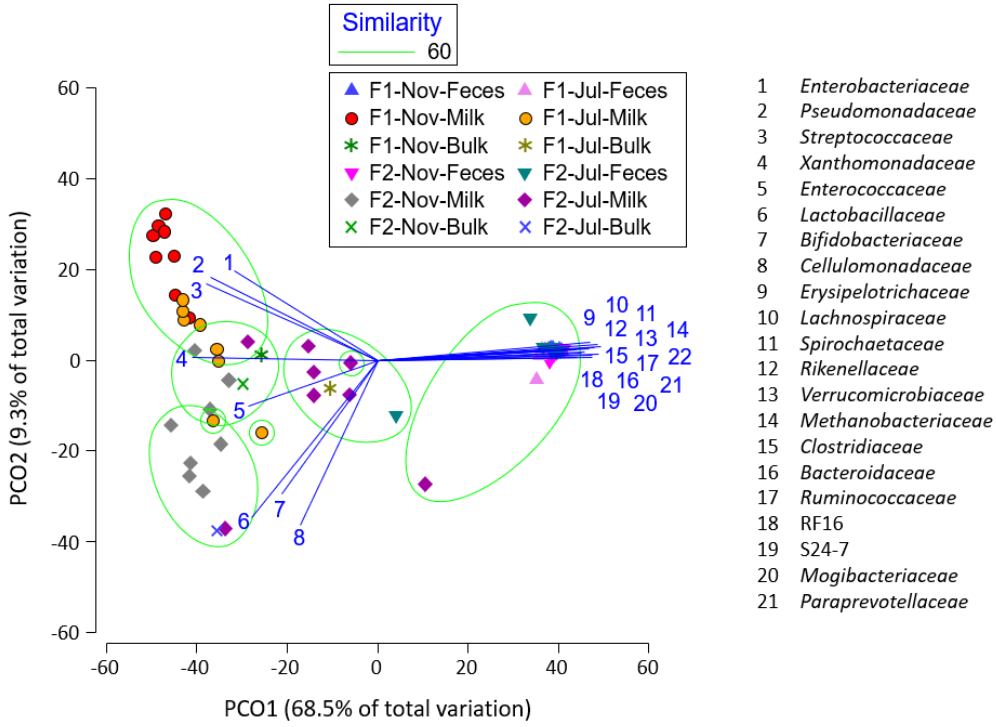

Figure 3.2. Principle Coordinate Analysis (PCoA) shows the relationship between the milk and fecal microbiota.

3.4. DISCUSSION

One cow exhibited an extremely long (> 500 days) lactation period at the time of sampling, and thus the average of the days in milk was substantially long (197 days) for F2 Jul samples. Data for the milk yield (≈20 kg/day/cow) and milk composition (≈3.7% protein and ≈4.8% fat content) were normal for Jersey milk. Although higher levels of SCC (2.72–3.74 × 105 cells/mL) in milk suggested that several cows may have been infected with mastitis pathogens, no cows were

considered to be having clinical or sub-clinical mastitis at the time of sampling. Further, no significant relationship was seen between SCC and blood metabolite levels based on the Pearson correlation coefficient.

Distinctive farm-to-farm and season-to-season differences were seen in blood metabolite levels, whereas there were few differences in milk yield and composition. The fact that the levels of T-Cho and Glu were higher while AST levels were lower for F1 than F2 cows indicated that an acceptable milk production with sufficient energy status and ameliorated liver damage in cows could be achieved by a high forage to concentrate ratio in the feed. Likewise, the lower levels of UN for F2 than F1 cows suggested that the greater amount of concentrate provided to F2 cows could help improve protein utilization. Because the levels of Alb and NEFA were higher and those of β-carotene and retinol were lower in Jul than Nov, cows may have used more energy and antioxidants to accommodate the hot environment. Although the blood T-Cho levels are known to correlate with milk yield (Kayano & Kida, 2015), a relationship was not observed in this study.

In contrast to the differences observed in blood metabolite levels, prevalent families of the fecal microbiota were stable regardless of the farm and season. It has been demonstrated that high concentrate feeding could increase concentrate-associated taxa, e.g. *Ruminococcaceae* and *Lachnospiraceae*, and decrease forage-associated taxa, e.g. *Prevotellaceae* (Khafipour et al., 2016). However, Zhang et al. (2018) reported that, when diets differing in forage to concentrate ratio (80:20, 60:40, 40:60, and 20:80) were given, significant changes in fecal microbiota were seen between 80:20 and 20:80, between 80:20 and 40:60, and between 40:60 and 20:80 diets; hence, differences between the diets at the farms F1 (60:40) and F2 (45:55) were probably not sufficient to result in any differences in the fecal microbiota. Although a season-to-season difference was observed for minor taxa, i.e. *Corynebacteriaceae*, *Staphylococcaceae*, *Clostridiaceae*, and *Lachnospiraceae*, our previous survey of fecal microbiota of Holstein cows did not find seasonal changes in those taxa (Nguyen, Wu, & Nishino, 2019). In this study, a greater abundance of *Clostridiaceae* and a lower abundance of *Lachnospiraceae* in Jul than Nov were recorded in both the F1 and F2 farms. *Lachnospiraceae* and *Ruminococcaceae* are regarded as families associated with forage feeding; hence, the cows may have consumed less amounts of fodder in the hot season. However, relative abundance of *Succinivibrionaceae*, a family associated with grain and fermentable starch feeding (Khafipour et al., 2016), also appeared to be lower in Jul.

Differences of the milk microbiota between farms were clear for the relative abundance of *Pseudomonadaceae*, *Enterobacteriaceae*, and *Streptococcaceae* (F1 > F2) and those of *Lactobacillaceae*, *Bifidobacteriaceae*, *Cellulomonadaceae*, and *Micrococcaceae* (F1 < F2), suggesting that milk microbiota could vary according to farm management and environment. Sources of contamination could be seen throughout the farms, including bedding, teat surfaces, milking parlors, feed, water, air-borne dust, and others (Quigley et al., 2013). Distinctive differences due to season were also found for *Staphylococcaceae* (Jul > Nov) and *Moraxellaceae* (Jul < Nov); hence, milk microbiota may change depending on the season. These results agree with the findings of Metzger et al. (2018), who demonstrated seasonal variation of the abundance of *Staphylococcus* spp. (*Staphylococcaceae*), *Acinetobacter* spp. (*Moraxellaceae*), and *Aerococcus* spp. (*Aerococcaceae*). Likewise, Li et al. (2016) reported that the abundance of *Pseudomonas* spp. (*Pseudomonadaceae*), *Propionibacterium* spp. (*Propionibacteriaceae*), and *Flavobacterium* spp. (*Flavobacteriaceae*) was negatively correlated with temperature, and that of *Bacillus* spp. (*Bacillaceae*), *Lactobacillus* spp. (*Lactobacillaceae*), and *Bifidobacterium* spp. (*Bifidobacteriaceae*) was positively correlated with temperature. Indeed, *Pseudomonadaceae* was the most prevalent in Nov samples regardless of the farm, and *Bacillaceae* (F1) and *Lactobacillaceae* (F2) were the most prevalent in Jul samples. Psychrophilic *Pseudomonas* spp. in milk can exhibit higher growth in winter than in summer (Marchand et al., 2009); hence, milk spoilage caused by *Pseudomonas* spp. would become a problem during winter. Regardless, the abundance of *Pseudomonadaceae* did not indicate clear seasonal variation in this study.

Two F1 cows and one F2 cow showed quite high (>40%) abundance of *Staphylococcaceae* in summer, raising the mean values for the herds to high levels. Nevertheless, the SCC (1.97-3.65 X 105 cells/mL) of the milk was not extremely high and the symptoms of clinical and sub-clinical mastitis were not observed in the cows. When Pearson correlation coefficient was calculated to examine the linear relationship between SCC and relative abundance of milk microbiota (51 families that had a relative abundance of > 1% in at least one sample), only *Weeksellaceae* ($r = 0.479$, $P < 0.01$) and *Comamonadaceae* ($r = 0.372$, $P < 0.05$) showed significant relationship. Typical mastitis pathogens belong to *Staphylococcaceae* ($r = 0.017$), *Streptococcaceae* ($r = 0.045$), and *Enterobacteriaceae* ($r = 0.034$) families, while fecal microbiota predominantly belong to *Ruminococcaceae* ($r = -0.142$), *Bacteroidaceae* ($r = -0.196$), and *Lachnospiraceae* ($r = -0.212$)

families; thus, mastitis pathogens and fecal microbiota were shown to be unrelated. Neither *Weeksellaceae* nor *Comamonadaceae* are regarded as pathogens associated with mastitis; hence, contamination of single bacterial spp. may not be sufficient to account for variation in the SCC of clinically normal cows.

It is difficult to assert that the microbiota data of this study was representative of Jersey cow milk, because milk microbiota can be altered by environmental factors including farm management and season. However, the finding that the relative abundance of Pseudomonadaceae was high compared to that reported for Holstein milk (Wu et al., 2019; Nguyen, Wu, & Nishino, 2019) was noteworthy. Pseudomonas spp. are known to have a high proteolytic activity; hence, a high abundance of this spoilage-associated species may be related to higher protein content in Jersey milk (≈3.7%) than in Holstein milk (≈3.0%). High fat content is also a distinctive property of Jersey milk, and thus the abundance of *Moraxellaceae*, a family that lipolytic *Acinetobacter* spp. belong to, was expected to be high. However, the abundance of *Moraxellaceae* recorded in this study (0.24–12.9%) appeared to be the same as those reported for Holstein milk (Wu et al., 2019; Nguyen, Wu, & Nishino, 2019). *Acinetobacter* spp. are regarded as psychrophilic, and thus a higher abundance of *Moraxellaceae* in Nov than Jul milk samples appeared reasonable.

Although statistical analysis comparing the microbiota of bulk tank milk with individual cow milk was not appropriate because of the small number of bulk tank milk samples, the finding that the abundance of *Pseudomonadaceae* was numerically lower in bulk tank milk compared with individually collected milk was interesting. *Pseudomonas* spp. are psychrophilic but the growth seemed to be inhibited by low temperature storage in the bulk tank. Vithanage et al. (2016) reported that *Pseudomonas* (*Pseudomonadaceae*), *Bacillus* (*Bacillaceae*), *Microbacterium* (*Microbacteriaceae*), *Lactococcus* (*Streptococcaceae*), *Acinetobacter* (*Moraxellaceae*), and *Hafnia* (*Enterobacteriaceae*) are frequently isolated from bulk tank milk. Many of their findings obtained by plate-culture were in agreement with our results obtained by 16S rRNA gene amplicon sequencing, except the markedly high abundance of *Lactobacillaceae* in Jul samples of F2 bulk tank milk. The abundance of *Lactobacillaceae* in individual cow milk averaged 5.78%, but this taxon surprisingly increased to 70.1% in bulk tank milk. This numerical increase of *Lactobacillaceae* from individual cow milk to bulk tank milk might be due to an unusual management of bulk tank milk when the Jul samples were collected.

The PCoA demonstrated that microbiota of individual cow milk was separated from that of bulk tank milk. The milk microbiota of F1, which featured *Enterobacteriaceae*, *Pseudomonadaceae*, and *Streptococcaceae*, was grouped together and thus appeared unaffected by the season. The milk microbiota of F2 was separated into three groups; two groups were Nov samples and the other was Jul samples, indicating that the milk microbiota of F2 varied within the herd and between the seasons. In a study examining milk microbiota of Holstein cows managed under a free stall housing system, a season-to-season difference was apparent, but differences within the farm were not observed (Nguyen, Wu, & Nishino, 2019). Although it was consistently observed that the microbiota of milk and feces were unrelated in clinically normal cows, further studies need to be carried out to clarify the factors affecting the microbiota of individual milk and bulk tank milk under a tie stall housing system.

3.5. CONCLUSION

In the milk microbiota of Jersey cows, *Pseudomonadaceae* and *Moraxellaceae* were found at a relatively high abundance, and *Lactobacillaceae* could occasionally become prevalent. The abundance of *Staphylococcaceae* may increase in the hot season. The microbiota of bulk tank milk appeared to be different from that of individual cow milk, but the growth of psychrophilic *Pseudomonadaceae* may effectively be suppressed by the low temperature storage in the bulk tank. PCoA indicated that milk microbiota was unrelated to fecal microbiota. The finding that the relative abundance of *Pseudomonadaceae* and *Moraxellaceae* appeared to be higher compared to those reported for Holstein milk suggested that the high protein and fat content of the Jersey milk may result in a large abundance of proteolytic and lipolytic taxa in the milk microbiota.

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CHAPTER 4

EFFECT OF SUPPLEMENTAL β-CAROTENE ON MILK PRODUCTION, MILK QUALITY AND HEALTH OF LACTATING JERSEY COW

4.1. INTRODUCTION

Due to the increase in demands for high-quality milk, many researchers have devoted studies on improving the value of milk by manipulating the composition and fat-soluble micronutrients in milk and dairy products by feed-management practices (Martin et al., 2004). β-carotene together with vitamin A (retinol) and vitamin E (α-tocopherol) are essential for quality and nutritional value of milk and dairy products. Since the β-carotene content in feedstuff is limited, adding commercial products is effective. Previous studies indicated that responses to β-carotene supplementation were inconsistent. Some studies found supplemental β-carotene to have a positive effect on milk yield and digestive function. Heat-stressed cows supplemented with 400 mg β-carotene increased milk yield by 11% (Ondarza, Wilson, & Engstrom, 2009). Oldham et al. (1991) supplemented cows with 300 mg β-carotene and increased milk yield by 6.4%, with this difference approaching significance. Rakes et al (1985) supplemented cows with 300 mg β-carotene and numerically lowered SCC content of milk without significantly improving milk production. Wang et al. (1988) found that cows supplemented with 300 mg β-carotene required fewer clinical mastitis treatments. Chew (1981) demonstrated that β-carotene and retinol concentration in plasma was associated with severity of mastitis. Oldham et al. (1991), however, reported no reduction in the incidence of mastitis.

Beside its role as a precursor of vitamin A, β-carotene is also a natural antioxidant and can directly enhance immunity by protecting cells against free radical attack, enhance host defense mechanisms by lymphocyte and phagocyte functions (Boon P Chew, 1992). For instance, Holstein cows supplemented with 300 to 600 mg/d of β- carotene from week 4 prepartum through week 4 postpartum showed increased mitogen-induced (concanavalin A, phytohemagglutinin, and pokeweed mitogen) lymphocyte proliferation (Heinnan et al., 1990) during the peripartum period. Cows supplemented with 120,000 IU/d of vitamin A did not show a similar response profile. Likewise, blood lymphocytes isolated from Holstein cows during the peripartum period and incubated with 1×10^{-9} M β-carotene had higher lymphocyte proliferation induced by concanavalin

A than did unsupplemented cultures; retinol had no effect on lymphocyte proliferation, whereas retinoic acid was suppressive (Daniel et al., 1991). The stimulatory effects of β-carotene (1×10^{-8} to 1×10^{-6} M) on bovine lymphocytes in vitro have similarly been demonstrated in non-lactating, primiparous Holsteins (Daniel et al., 1986). That group of studies suggested a specific effect of β-carotene. In addition to modulating lymphocyte function, β-carotene also modulates other host defense cells. In the presence of 10^{-8} to 10^{-7} M β-carotene in vitro, bovine blood and mammary polymorphonuclear leukocytes (PMN) isolated from cows during the peripartum period showed enhanced ability to kill *Staphylococcus aureus* (Daniel et al., 1991b). Similarly, blood PMN isolated from Holstein cows fed 300 to 600 mg/d of β-carotene from week 4 prepartum through week 4 postpartum showed higher killing ability against bacteria during the peripartum period (Michal et al., 1990). The increased bacterial killing could be accounted for partly by increased myeloperoxidase activity in the PMN and seems to be unrelated to changes in the production of superoxides (Michal et al., 1990).

Supplemental β-carotene, in addition, may enhance rumen function. In vitro growth of rumen bacteria and cellulose digestion has been increased with the addition of β-carotene in the presence of safflower oil (Hino, Andoh, & Ohgi, 1993). Other dietary antioxidants have increased fiber digestion when fed with diets containing high levels of unsaturated fat in continuous culture (Vázquez-Añón & Jenkins, 2007). Perhaps β-carotene may perform a positive role as an antioxidant in the rumen. However, the information on the effect of supplemental β-carotene on ruminal microbiota as well as hindgut microbiota of dairy cow is scared.

Jersey cows have ability to absorb more β-carotene than Holstein, and the conversion ratio from β-carotene to retinol is different between two breeds, which in turn affects β-carotene and retinol concentration in milk. Nevertheless, there have been few reports documenting vitamin A, vitamin E and β-carotene concentrations in blood and milk of Jersey cows, as well as plasma metabolites concentration, during β-carotene supplementation and termination.

The objectives of this study were to examine the effect of β-carotene supplementation 1) on milk yield and milk composition of lactating Jersey cows, 2) on ruminal fermentation, rumen microbiota and hindgut microbiota, 3) on plasma metabolites concentration, and 4) on fat-soluble micronutrients in blood and milk of lactating Jersey cows.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design

The experiment was conducted on Chukoku Shikoku college of dairy farming in Hiruzen area, Okayama prefecture, Japan, on 10 healthy multiparous lactating Jersey cows. The cows were housed in a free-stall barn and fed total mixed ration (TMR) before and during experiment period. The study was 2 months in length, beginning on 1st November 2019, and ending on 26th December 2019. In the first month, the cows were supplied with 1000 mg β-carotene/cow/d (10 g Rovimix β-carotene containing 10% β-carotene; DSM Nutrition Japan K.K., Tokyo, Japan) by mixing with TMR daily, followed by 1 month without supplying β-carotene supplement.

4.2.2. Sample collection

Milk, blood, and rumen fluid were collected 3 times from 10 cows, i.e. before (0M), after 1 month (1M+) of the β-carotene supplementation, and after 1 month (1M-) of β-carotene termination. For milk component analysis, individual milk samples were collected by a sampling device attached to the milking parlor, first collection was at 18:00 and second collection was at 6:00 the next morning, before being pooled together as a composite milk sample. For fat-soluble micronutrients analysis, milk samples were collected manually and evenly from four udders and pooled together as a composite milk sample. Blood were sampled through jugular vein and kept in a 5 mL heparin-containing tube, rumen fluid was collected using flexible stainless spring tube (Lumenar stomach evacuator outfit, Fujihira Industry Co. Ltd., Tokyo, Japan), and feces was collected through rectum. All samples were kept dark and cold during transportation and subsequently frozen at -30 °C until further analysis. All procedures and protocols for animal experiments were approved by the Animal Care and Use Committee, Okayama University, Japan.

4.2.3. Blood metabolites and milk components analysis

A total of 30 blood samples were centrifuged 3000 rpm in 15 min and plasma were collected and analyzed for non-esterified fatty acids (NEFA), total cholesterol (T-Cho), albumin (Alb),

blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Calcium (Ca), and Phosphorus (P) using commercially available kits (Fujifilm Wako Pure Chemicals Co., Tokyo, Japan).

Milk composition including protein, fat, and solids-not-fat (SNF) were determined using a CombiFoss FT+ analyzer (Foss Allé, Hillerød, Denmark). Direct microscopic method was used (Paape at al. (2001)) for somatic cell count (SCC) enumeration.

4.2.4. Fat-soluble micronutrients extraction and determination

High performance liquid chromatography (HPLC) were used to determine retinol, α–tocopherol, and β–carotene levels in plasma and milk following the procedures of Talwar et al., (1998). Briefly, 100 µl of internal standard retinol acetate 10 µmol/l were added to 400 µl plasma or milk samples, and the retinol, α–tocopherol, and β–carotene were extracted with 1 ml of hexane. The samples were vortex mixed and centrifuged at 2500 rpm/min for 20 min. Part (0.6 ml) of the hexane layer was collected and evaporated to dryness under a stream of nitrogen at 40^{o}C. The residue was reconstituted in 100 µl of mobile phase, and filtered through a 0.02 polytetrafloroethylene membrane, followed by injection of 20 µl aliquot into Inertsil ODS-80A (C 18) column, 150 x 4.6 mm (Shimadzu Co., Kyoto, Japan). The isocratic mobile phase for chromatography was methanol:acetonitrile:tetrahydrofuran (75:20:5, v/v). Mobile phase was filtered through a 0.2 µm PTFE membrane filter and pumped at a flow rate of 0.6 ml/min. The absorbance at 325 nm, 290 nm, and 450 nm for retinol, α–tocopherol, and β–carotene, respectively, was detected using a LC-10AT HPLC system (Shimadzu Co., Kyoto, Japan) fitted with a CR-6A data processor (Shimadzu Co., Kyoto, Japan) and a SPD-10A variable wavelength detector (Shimadzu Co., Kyoto, Japan). The peaks of retinol, α–tocopherol, and β–carotene were identified and quantified by comparing with external and internal standards, which had retention time of 4.6, 9.6, and 26.05 min, respectively.

4.2.5. Volatile fatty acids measurements

Regarding volatile fatty acids (VFAs) determination, 100 µl 10% Trichloroacetic acid was added into 100 µl filtered rumen fluid, mixed well and kept overnight at 4^{o}C before centrifuging (8000 rpm for 10 min at 4^{o}C). The supernatant was used to measure the VFAs concentration by a capillary column gas chromatograph (Cottyn & Boucque, 1968).

4.2.6. Bacterial DNA extraction

Bacterial DNAs from rumen fluid and feces samples were extracted and purified following repeated bead beating plus column method of Yu & Morrison (2004). Briefly, 0.2 mL of the rumen fluid and 0.2 g wet weight of feces samples were lysed with 1 mL of lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)]. Cell lysis was achieved by bead beating in the presence of 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). After bead beating, most of the impurities and the SDS are removed by precipitation with 200 µL 10 M ammonium acetate, and then the nucleic acids are recovered by precipitation with 700 µL isopropanol. Genomic DNA can then be purified via sequential digestions with 2 µL DNase-free RNase and 15 µL proteinase K, followed by the use of QIAamp columns from DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

The bacterial DNAs of rumen fluid and feces samples were subjected to two-step PCR procedures to generate an amplicon library for MiSeq sequencing (Nguyen, Wu, & Nishino, 2019). The first round PCR was employed using primers targeting the V4 region of the 16S rRNA gene (forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3′; tail sequences are underlined). The PCR protocol included an initial denaturation at 94 °C for 2 min, followed by 30 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 using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and subsequently used as DNA templates for second round PCR with adapter-attached primers. The

second round PCR protocol included an 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. After the purification process described above, the purified DNA was pair-end sequenced (2 × 250 bp) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

The archived raw sequences were processed using quantitative insights into microbial ecology (QIIME 2, version 2019.10) software. The raw sequences were denoised via DADA2 and low-quality sequences were truncated. Sequences that overlapped more than 60 bp were merged, followed by the identification and removal of chimeric sequences. Only high-quality sequences remained were grouped into operational taxonomic units (OTUs) with a 97% similarity threshold. Bacterial clustering was analyzed from the phylum to family level.

4.2.7. Statistical analysis

Statistical analysis was performed using JMP software (version 13, SAS Institute, Tokyo, Japan). The data was subjected to one-way analysis of variance, and the means were compared using Tukey HSD method. Spearman correlation analysis were calculated using JMP software and network analysis were performed using Cytoscape software (version 3.8.0).

4.3. RESULTS AND DISCUSSION

4.3.1. Effect of supplemental β-carotene on milk yield and milk composition

Table 4.1. Milk yield and milk compositions of Jersey cows before (0M), after 1 month of β-carotene supplementation (1M+), and after 1 month of β-carotene termination (1M-).

	0M	1M+	1M-	SE
Milk yield (kg/d)	28.61^{a}	24.47^{ab}	20.33^{b}	1.56
Milk composition				
Protein (%)	3.88^{b}	4.25^{a}	4.53^{a}	0.65
Fat (%)	5.05^{b}	4.86^{b}	5.86^{a}	0.73
Solid-not-fat (%)	9.38^{b}	9.76^{a}	9.90^{a}	0.98
SCC (log10 cell/mL)	5.68^{a}	4.66^{b}	4.78^{b}	0.71

a, b – values in the rows with different letters differs significantly ($P < 0.05$)

Data for the milk yield (20-28 kg/day/cow) and milk composition (3.8-4.5% protein, 4.8-5.8% fat content, and 9.3-9.9% solid-not-fat) were normal for Jersey cows. After 1 month of β-carotene supplementation, milk yield gradually decreased and significant difference was observed after 1 month of β-carotene depletion. Clearly, β-carotene supplementation did not affect milk yield, and the decrease of milk yield during 2 months of experiment was also observed for the whole herd and in other farms which didn't use β-carotene supplement. Fat, protein, and SNF, on the other hand, showed increased trend, regardless of β-carotene supplementation or termination, indicating that dietary β-carotene is not the determining factor of milk components. The increase of fat, protein and SNF probably because of the decrease of milk yield production, which made the milk more condense. Somatic cell counts noticeably decreased when cows were fed with β-carotene (5.68 vs. 4.66 log10 cells/mL) and remained at lower level at 1M- than before supplementation (0M) ($P < 0.05$).

Though milk yield was numerically lower at 1M+, there was no significant difference in milk yield and milk fat percentage ($P > 0.05$) before and after 1 month of β-carotene supplementation, which was consistent with Rakes (1985), Wang (1988), Akordor (1986), and Bindas (1984). Milk protein (3.88–4.25%) and solid-not-fat (9.38–9.76%) significantly increased after 1 month feeding β-carotene ($P < 0.05$), probably due to the numerical decrease in milk yield. Probably, the decrease in milk yield and increase in milk protein and milk fat was due to seasonal change, which were consistently observed from whole-herd-data and other farms' data (unpublished). Somatic cell counts noticeably decreased when cows were fed with β-carotene for a month (5.68 vs. 4.66 log10 cells/mL), and slightly increased (4.66 vs 4.78 log10 cells/mL) after one month of depletion. Hence, β-carotene might decrease SCC.

4.3.2. Effect of supplemental β-carotene on plasma metabolites concentration

Table 4.2. Plasma metabolites concentration of Jersey cows before (0M), after 1 month (1M+) of β-carotene supplementation, and after 1 month (1M-) of β-carotene termination.

Plasma metabolites	0M	1M+	1M-	SE
Albumin (g/dL)	3.70	3.79	3.86	0.11
Urea nitrogen (mg/dL)	9.02^{b}	11.02^{a}	9.80^{ab}	0.39
Total cholesterol (mg/dL)	245.2^{a}	213.5^{ab}	187.5^{b}	16.13
NEFA (mEq/L)	398.9	290.0	276.7	37.41
AST (units/L)	56.81	62.59	56.88	3.64
ALT (units/L)	11.48	13.03	12.82	1.24
Ca (mg/dL)	6.23	6.29	5.77	0.27
P (mg/dL)	4.79	5.19	5.80	0.32

a, b – values in the rows with different letters differs significantly ($P < 0.05$)

No difference was observed in albumin, NEFA, AST, ALT, Ca, and P concentration during 2 months of β-carotene supplementation and termination period ($P > 0.05$). Significant increase was seen in blood urea nitrogen concentration after 1 month (1M+) of β-carotene supplementation and decreased again after 1 month of β-carotene termination. Nevertheless, total cholesterol showed a linear decrease from 245.2 (0M) to 213.5 (1M+), and significantly differed at 1M- with average concentration of 187.5 mM ($P < 0.05$).

The increase in plasma urea nitrogen indicated a decreased utilization of rumen ammonia for microbial protein synthesis. However, it cannot be excluded that increased plasma urea nitrogen resulted from higher degradation of dietary N. In this respect, supplemental β-carotene might increase feed consumption of the cows, which in turn increased dietary N intake, as the cows were fed ad libitum during experimental period. Though level of plasma urea nitrogen increased during 1 month of the supplementation, its level was acceptably low, and those of total cholesterol was sufficiently high.

4.3.3. Effect of supplemental β-carotene on fat-soluble vitamin concentration in plasma and milk

Table 4.3. Retinol, α-tocopherol, and β-carotene concentration in plasma and milk of Jersey cows before (0M), after 1 month (1M+) of β-carotene supplementation, and after 1 month (1M-) of β-carotene termination.

	0M	1M+	1M-	SE
Plasma				
Retinol (µg/mL)	2.40	3.05	2.39	0.42
α-tocopherol (µg/mL)	2.50[a]	1.89[ab]	1.43[b]	0.31
β-carotene (µg/mL)	8.59[b]	15.83[a]	10.83[ab]	1.89
Milk				
Retinol (µg/mL)	0.08[b]	0.20[a]	0.21[a]	0.01
α-tocopherol (µg/mL)	8.11[b]	13.14[ab]	18.00[a]	2.47
β-carotene (µg/mL)	1.01[c]	3.82[a]	2.98[b]	0.22

a, b, c – values in the rows with different letters differs significantly ($P < 0.05$)

Table 4.3 shows no difference in plasma retinol, plasma α-tocopherol, and milk α-tocopherol after 1 month of β-carotene supplementation ($P > 0.05$). Predictively, plasma β-carotene, milk β-carotene and milk retinol significantly increased after 1 month of supplementation ($P < 0.05$). One month after β-carotene termination (1M-), plasma β-carotene returned to normal level ($P > 0.05$) (10.83 µg/mL vs 8.59 µg/mL at 1M- and 0M, respectively) whereas milk retinol (0.21 µg/mL vs 0.08 µg/mL) and milk β-carotene (2.98 vs 1.01 µg/mL) were maintained at higher level than before supplementation (0M) ($P < 0.05$).

In cattle, many studies have been focused on the role of β-carotene in reproduction, and the suggestion that it may be essential for normal reproduction in cattle is still a matter of debate. Moreover, studying the absorption and metabolism of β-carotene in cattle is particularly relevant because β-carotene is the main source of vitamin A in milk.

In this study, plasma retinol concentration was quite stable, which was consistent with other studies (Fuquay, 2011). An increase in dietary β-carotene supply has been shown to lead to higher plasma concentrations of β-carotene in lactating cows but not of circulating retinol, because retinoic acid is a potent regulator of gene expression, its concentration has to be regulated precisely in circulating blood to avoid any deleterious effect due to a high concentration of vitamin A (Fuquay, 2011). Although plasma α-tocopherol and milk α-tocopherol showed no significant change ($P > 0.05$), supplemental β-carotene numerically increased milk α-tocopherol and decreased plasma α-tocopherol, indicating that β-carotene supplementation seemed to facilitate the α-tocopherol diffusion from plasma to milk. The interaction between β-carotene and α-tocopherol within the dairy cattle's body remains unclear and needs further studies to elucidate. In this

experiment, milk retinol, but not plasma retinol, and milk β-carotene were maintained at high level even after 1 month of termination suggesting that β-carotene might be stored and converted to retinol in the mammary gland.

4.3.4. Effect of supplemental β-carotene on rumen fermentation, rumen fluid microbiota and fecal microbiota composition

Table 4.4. Volatile fatty acids (VFAs) concentration in the rumen fluid of Jersey cows before (0M), after 1 month (1M+) of β-carotene supplementation, and after 1 month (1M-) of β-carotene termination.

VFAs (mM)	0M	1M+	1M-	SE
Acetate	53.73^{b}	63.96^{ab}	75.11^{a}	3.87
Propionate	15.60^{b}	16.71^{ab}	20.43^{a}	1.34
Iso-butyrate	1.31^{a}	0.81^{b}	0.85^{ab}	0.13
Butyrate	9.62^{b}	10.43^{b}	13.77^{a}	0.94
Total VFAs	80.26^{b}	91.91^{ab}	110.17^{a}	6.07
C2:C3 ratio	3.52	3.91	3.72	0.13

a, b – values in the rows with different letters differs significantly ($P < 0.05$)

Table 4.4 showed the variation of volatile fatty acids concentration following the β-carotene supplementation and termination. It seemed that β-carotene didn't have significant effect on rumen fermentation. Comparing with before supplementation (0M), the content of acetate, propionate, butyrate, total VFAs and acetate:propionate ratio showed no significant difference after 1 month of supplementation (1M+) ($P > 0.05$). Only iso-butyrate significantly decreased from 1.31 mM to 0.81 mM at 0M and 1M+, respectively. After 1 month of β-carotene termination; acetate, propionate, butyrate, and total VFAs concentration kept increasing and significantly higher ($P < 0.05$) than before supplementation (75.11 mM vs 53.73 mM, 20.43 mM vs 15.6 mM, 13.77 mM vs 9.62 mM, and 110.2 mM vs 80.26 mM at 1M- and 0M, respectively).

There are few reports about the effect of supplemental β-carotene on rumen fermentation. Hino, Andoh, & Ohgi (1993) reported that β-carotene plus α-tocopherol enhanced bacterial cell yield and cellulose digestion by the increased cellulolytic bacteria in the presence of safflower oil, caprate, stearate, or linoleate in *in vitro* incubation. Our results were inconsistent with Yan, Sun, & Zhao (2007) who concluded that β-carotene supplementation increased the acetic acid concentrations *in vitro*, but did not influence total VFA production.

Table 4.5. Ruminal microbiota of Jersey cows before (0M), after 1 month (1M+) of the β-carotene supplementation, and after 1 month (1M-) of β-carotene termination

Family	β-Carotene supplementation			
	0M	1M+	1M-	SE
Euryarchaeota	0.51	0.59	0.67	0.13
Methanobacteriaceae	0.51	0.59	0.67	0.13
Actinobacteria	0.16	0.16	0.18	0.10
Bifidobacteriaceae	0.16	0.16	0.18	0.10
Bacteroidetes	53.77^{b}	58.84^{ab}	62.36^{a}	1.45
BS11	0.26	0.41	0.53	0.10
Prevotellaceae	32.68^{ab}	31.19^{b}	38.19^{a}	1.94
RF16	1.31	2.13	1.38	0.24
S24-7	2.62	3.39	2.80	0.28
[Paraprevotellaceae]	3.69^{b}	4.98^{a}	4.81^{a}	0.28
Fibrobacteres	0.48^{ab}	0.72^{a}	0.27^{b}	0.10
Fibrobacteraceae	0.48^{ab}	0.72^{a}	0.27^{b}	0.10
Firmicutes	35.14^{a}	29.23^{b}	29.41^{b}	1.35
Leuconostocaceae	0.65^{a}	0.00^{b}	0.00^{b}	0.13
Clostridiaceae	1.64	1.21	1.35	0.13
Lachnospiraceae	6.46^{a}	4.97^{b}	4.47^{b}	0.35
Ruminococcaceae	12.01	10.35	10.70	1.09
Veillonellaceae	2.15	2.59	2.58	0.36
[Mogibacteriaceae]	0.76	0.64	0.63	0.10
Erysipelotrichaceae	1.11	1.20	1.26	0.05
Proteobacteria	3.71	3.21	0.76	0.83
Succinivibrionaceae	2.90	2.52	0.60	0.81
Spirochaetes	0.81	0.87	1.28	0.16
Spirochaetaceae	0.81	0.87	1.28	0.16
Tenericutes	3.10	3.25	2.69	0.27
Anaeroplasmataceae	0.24^{b}	0.58^{a}	0.55^{a}	0.08
Mycoplasmataceae	0.15	0.31	0.42	0.08
Verrucomicrobia	0.42	0.45	0.33	0.09
RFP12	0.42	0.45	0.33	0.09

Other	1.89[b]	2.69[a]	2.04[ab]	0.22

a, b – values in the rows with different letters differs significantly (P < 0.05)

The effect of supplemental β-carotene on rumen microbiota was not clear (Table 4.5). After 1 month of supplementation (1M+), many phyla, i.e., Bacteroidetes, Actinobacteria, Euryarchaeota, Fibrobacteres, Proteobacteria, and Tenericutes showed no significant difference (P > 0.05). Only Firmicutes taxon showed significant decrease (P < 0.05).

At family level, the majority of taxa were unchanged, i.e., *Prevotellaceae*, *Ruminococcaceae*, *Veillonellaceae*, *Succinivibrionaceae*, *S24-7*, and *RF16*, except for *Paraprevotellaceae* and *Anaeroplasmataceae*, which significantly increased during β-carotene supplementation period. *Lachnospiraceae*, on the other hand, significantly decreased after 1 month of supplementation, and *Leuconostocaceae* was completely removed when cows fed β-carotene for 1 month (P < 0.05).

Table 4.6. Fecal microbiota of Jersey cows before (0M), after 1 month (1M+) of the β-carotene supplementation, and after 1 month (1M-) of β-carotene termination

Family	β-Carotene supplementation			
	0M	1M+	1M-	SE
Euryarchaeota	2.67[a]	1.60[b]	1.52[b]	0.20
Methanobacteriaceae	2.67[a]	1.60[b]	1.52[b]	0.20
Actinobacteria	1.07	0.31	0.38	0.27
Bifidobacteriaceae	1.07	0.31	0.38	0.27
Bacteroidetes	21.65[a]	20.55[a]	13.95[b]	1.65
Bacteroidaceae	5.56[a]	3.99[ab]	2.05[b]	0.71
Porphyromonadaceae	0.32[b]	0.64[a]	0.33[b]	0.08
Prevotellaceae	2.17	0.67	0.62	0.54
RF16	0.43[b]	1.07[a]	0.69[ab]	0.12
Rikenellaceae	1.15[b]	2.38[a]	1.45[b]	0.23
S24-7	3.87	2.79	2.67	0.43
[Paraprevotellaceae]	2.14	2.52	1.30	0.35
Firmicutes	69.16[b]	70.47[b]	77.95[a]	1.72
Turicibacteraceae	3.39	2.13	3.87	0.49
Clostridiaceae	3.51[a]	2.20[b]	2.73[ab]	0.34
Lachnospiraceae	12.68	13.00	16.32	1.27
Peptostreptococcaceae	3.37[a]	1.92[b]	2.55[ab]	0.35
Ruminococcaceae	31.04[b]	34.82[a]	37.33[a]	0.94
Veillonellaceae	0.61[a]	0.32[ab]	0.24[b]	0.09

[Mogibacteriaceae]	1.41	1.45	1.16	0.10
Erysipelotrichaceae	2.72	3.38	3.23	0.24
Proteobacteria	1.45	1.07	1.52	0.69
Alcaligenaceae	0.01	0.59	1.09	0.65
Succinivibrionaceae	1.44^{a}	0.48^{b}	0.44^{b}	0.21
Other	4.00^{b}	6.00^{a}	4.67^{ab}	0.38

a, b– values in the rows with different letters differs significantly ($P < 0.05$)

At phylum level, no difference was found in the fecal microbiota after 1 month of β-carotene feeding except for the decrease of *Euryarchaeota* taxon ($P < 0.05$). At family level, fecal microbiota showed significant variation after 1 month of β-carotene supplementation. High dominance taxa, i.e., *Ruminococcaceae*, *Rikenellaceae*, *RF16*, and *Porphyromonadaceae* showed significant increase in relative abundance meanwhile *Methanobacteriaceae*, *Clostridiaceae*, and *Succinivibrionaceae* showed significant decrease ($P < 0.05$) after 1-month-supplementation period.

4.3.5. Relationship between blood metabolites, VFAs, rumen microbiota and fecal microbiota

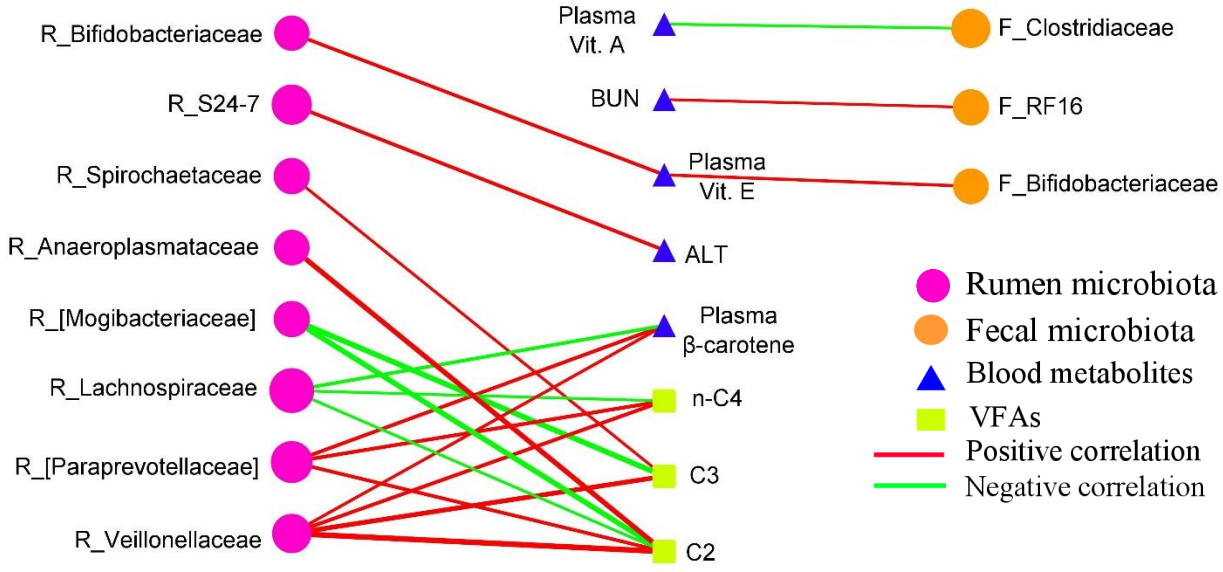

Figure 4.1. Correlation between blood metabolites, VFAs, rumen microbiota and fecal microbiota. Only strong (correlation coefficient R > 0.6 or <-0.6) and significant (P < 0.01) correlations were chosen to be displayed in the network. The edge width and color (red, positive;

green, negative) are proportional to the correlation strength. The circle sizes are proportional to the relative abundance of bacterial families. Circle color indicates microbiota types (purple, rumen microbiota; orange, fecal microbiota). Blue triangle indicates blood metabolites, green rectangle indicates volatile fatty acids.

Figure 4.1 showed the correlation between blood metabolites, VFAs, rumen microbiota and fecal microbiota. Many families in rumen had correlations with VFAs and blood metabolites. For example, *Veillonellaceae*, *Paraprevotellaceae*, *Anaeroplasmataceae*, and *Spirochaetaceae* have positive correlation with VFAs whereas *Lachnospiraceae*, *Mogibacteriaceae* have negative correlation with VFAs. Plasma β-carotene was positively correlated with *Veilonellaceae*, *Paraprevotellaceae* and negative with *Lachnospiraceae*. ALT had positive correlation with S24-7 and plasma vitamin E had positive correlation with *Bifidobacteriaceae*.

As per fecal microbiota and blood metabolites relationship, some bacterial families in feces were correlated with blood metabolites, i.e., *Bifidobacteriaceae* was positively correlated with plasma vitamin E, *RF-16* was positively correlated with BUN, and *Clostridiaceae* was negatively correlated with plasma vitamin A.

4.4. CONCLUSION

The supplementation of 1000 mg β-carotene for 1 month did not show clear effect on milk yield and milk composition but significantly decrease SCC. Plasma NEFA, Albumin, AST, ALT, Ca, and P concentrations were not affected by supplemental β-carotene while urea nitrogen was increased and total cholesterol was decreased. Supplemental β-carotene apparently increased plasma β-carotene, milk β-carotene and milk retinol concentration, which increased the nutritional value of milk.

Regarding rumen fermentation, β-carotene supplementation did not have significant effect on major VFAs, i.e., acetate, propionate, butyrate, total VFAs and acetate:propionate ratio. As per rumen and hindgut microbiota, supplemental β-carotene did not have significant effect on rumen microbiota composition but had more effect on hindgut microbiota. β-Carotene supplementation alleviated relative abundance of *Methanobacteriaceae*, *Clostridiaceae*, and *Succinivibrionaceae*

taxa while increased those of *Ruminococcaceae*, *Rikenellaceae*, *RF16,* and *Porphyromonadaceae* in the hindgut. Further studies are needed to elucidate the affecting mechanism of β-carotene on hindgut microbiota and its possible effect on animal health.

Many positive and negative correlation between rumen microbiota with VFAs and blood metabolites were revealed, which suggest the potentials to improve milk yield and animal health by manipulation of these rumen bacteria.

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CHAPTER 5

CHARACTERIZATION OF INDIGENOUS MICROBIOTA COMPOSITION OF JERSEY MILK AND ITS RELATIONSHIP WITH MILK COMPONENTS DURING β-CAROTENE SUPPLEMENTATION PERIOD

5.1. INTRODUCTION

Indigenous milk microbiota composition can affect milk quality and safety of dairy products, which are still produced using unpasteurized milk, such as artisan cheeses. In addition, indigenous milk microbiota is also important in shaping the gut microbiota of calves at the early growth stage. The ability to characterize the indigenous milk microbiota and control their microbial composition is critical in monitoring milk quality and shaping gut microbiota of the calves.

Beside Holsteins, Jersey cows are the second largest dairy breed. Because Jerseys tend to be more efficient and typically have fewer reproductive challenges than Holsteins, reconsideration of purebred Jerseys and a crossbreed between Holsteins and Jerseys has been discussed recently. Though Jerseys produce less milk than Holsteins, they have less incident of mastitis and other transition-related disease, and their milk have higher nutrients contents, i.e. fat, protein, and solid not fat (SNF), than Holsteins.

The breed difference has been shown to alter milk microbiota composition, even when they were raised on the same farm and under the same conditions (Cremonesi et al., 2018). Cremonesi investigated milk microbiota diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows and demonstrated that milk microbial populations were very different in the two breeds along all the timepoints within the transition period. More specifically, the milk microbiota of the two breeds was characterized by significant differences in the average abundance of *Streptococcaceae* (HF 29.3%, REN 74.1%) and *Lactobacillaceae* (HF 6.9%, REN 14.0%). Significant differences were observed also for *Ruminococcaceae*, *Bradyrhizobiaceae*, *Aerococcaceae* and *Staphylococcaceae*, which were found almost exclusively in HF milk.

The differences in milk nutrient composition between breeds, i.e., Holstein and Jersey, may suggest the differences in milk microbiota. One study on human have revealed that nutrient components in human milk (Kumar et al., 2016) have been recently substantiated as factors that may influence its microbiota composition (Padilha et al., 2019). Boix-amorós et al., (2016), who investigated relationship between milk microbiota, bacterial load, macronutrients, and human cells during lactation, identified some positive and negative relationships between some milk components and specific bacterial genera. For instance, the amount of proteins were positively correlated with the proportion of *Bacillus*, *Peptoniphilus*, and *Anaerococcus* in the samples, whereas lactose levels were negatively correlated with *Enterobacter* and *Actinomyces*, indicating potential prebiotic and antagonistic effects for bacterial growth. In the case of fat, whose content in milk is known to increase through breastfeeding, it was negatively correlated with the proportion of *Staphylococcus*, which suggested high fat content in milk could potentially be protective of mastitis risk. However, the information on the relationship of milk microbiota with milk nutrient components in dairy cows has not been well established.

Diets are believed to be one of the efficient ways to modulate the milk composition, and subsequently, milk microbiota of dairy cows. Zhang et al. (2014) was the first who reported the impacts of diet on changes in the composition of milk microbiota of Holstein cows by using next generation sequencing. β-carotene, in addition, is widely used as a dietary supplement for dairy cows to improve milk yield (Ondarza et al., 2009), lower SCC (Rakes et al., 1985), and reduce mastitis incident (Wang et al., 1988). Beside its role as a precursor of vitamin A, β-carotene is also a natural antioxidant which can directly enhance immunity and host defense mechanisms (Boon P Chew, 1992). Nevertheless, the question is still open on whether β-carotene supplementation affect milk microbiota and milk composition.

The objectives of this study were to characterize the indigenous microbiota composition of Jersey milk, to identify the relationships between milk microbiota composition and milk components, and to examine whether β-carotene supplementation affect milk microbiota and milk components.

5.2. MATERIALS AND METHODS

5.2.1. Experimental design

The experiment was conducted on Chukoku Shikoku college of dairy farming in Hiruzen area, Okayama prefecture, Japan. A total of 90 lactating Jersey cows were housed in a free-stall barn and fed total mixed ration (TMR) before and during experiment period. The study was 2 months in length, beginning on 1st November 2019, and ending on 26th December 2019. In the first month, the cows were supplied with 1000 mg β-carotene/cow/d (10 g ROVIMIX® β-carotene containing 10% β-carotene; DSM Nutrition Japan K.K., Tokyo, Japan) by mixing with TMR daily, followed by 1 month without supplying β-carotene supplement.

5.2.2. Sample collection

During two-month period, milk samples were collected 3 times from 10 randomly selected cows, i.e. before (0M), after 1 month (1M+) of the β-carotene supplementation, and after 1 month (1M-) of β-carotene termination. For milk component analysis, individual milk samples were collected at 0M, 1M+, and 1M- by a sampling device attached to the milking parlor, first collection was at 18:00 and second collection was at 6:00 the next morning, before being pooled together as a composite milk sample. For milk microbiota analysis, individual milk samples were aseptically collected by hands from 5:00 to 6:00 every sampling day. The udder and teat surfaces were cleaned and disinfected with iodine and ethanol-soaked-cotton, and 3-4 streams of foremilk were discarded before collecting the milk samples. Each individual milk sample was collected manually from 4 quarters, then mixed together to make a composite milk sample. These milk samples were also used for fat-soluble micronutrients analysis. Right after the collection, all milk samples were dipped in liquid nitrogen and kept dark during transportation and subsequently frozen at -30 °C until further analysis. All procedures and protocols for animal experiments were approved by the Animal Care and Use Committee, Okayama University, Japan.

5.2.3. Milk components and milk fatty acids compositions analysis

Milk components including protein, fat, and solids-not-fat (SNF) were determined using a CombiFoss FT+ analyzer (Foss Allé, Hillerød, Denmark). Direct microscopic method was used (Paape at al. (2001)) for somatic cell count (SCC) enumeration.

Milk fat was extracted by the standard procedure of Hara and Radin (1978) using the solvent mixture of chloroform and methanol in a ratio of 2 : 1. The FAs were converted into methyl esters via trans-esterification with a solution of boron trifluoride in methanol (Rule, 1997). Fatty acid composition was determined using a Shimadzu GC2010 Plus gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a BD-23 column (30 m length, D=0.25 mm, Agilent Technology Inc, USA). The chromatographic conditions were as follows: carrier gas, helium; injection volume, 1 µL; injector temperature, 200 ^{0}C, detector temperature, 250 ^{0}C; oven temperature program, initial 140 ^{0}C, then increased at 4 ^{0}C min−1 to 240 ^{0}C and keep for 15 min. Quantification was determined through area normalization, with an external standard mixture of fatty acid methyl esters (Sigma-Aldrich, Steinheim, Germany). Fatty acid composition was calculated as the percentage of each individual fatty acid relative to the total fatty acids.

5.2.4. Fat-soluble micronutrients extraction and determination

High performance liquid chromatography (HPLC) were used to determine retinol, α–tocopherol, and β–carotene levels in milk following the procedures of Talwar et al., (1998). Briefly, 100 µl of internal standard retinol acetate 10 µmol/l were added to 400 µl of milk samples, and the retinol, α–tocopherol, and β–carotene were extracted with 1 ml of hexane. The samples were vortex mixed and centrifuged at 2500 rpm/min for 20 min. Part (0.6 ml) of the hexane layer was collected and evaporated to dryness under a stream of nitrogen at 40^{o}C. The residue was reconstituted in 100 µl of mobile phase, and filtered through a 0.02 polytetrafloroethylene membrane, followed by injection of 20 µl aliquot into Inertsil ODS-80A (C 18) column, 150 x 4.6 mm (Shimadzu Co., Kyoto, Japan). The isocratic mobile phase for chromatography was methanol:acetonitrile:tetrahydrofuran (75:20:5, v/v). Mobile phase was filtered through a 0.2 µm PTFE membrane filter and pumped at a flow rate of 0.6 ml/min. The absorbance at 325 nm, 290 nm, and 450 nm for retinol, α–tocopherol, and β–carotene, respectively, was detected using a LC-

10AT HPLC system (Shimadzu Co., Kyoto, Japan) fitted with a CR-6A data processor (Shimadzu Co., Kyoto, Japan) and a SPD-10A variable wavelength detector (Shimadzu Co., Kyoto, Japan). The peaks of retinol, α–tocopherol, and β–carotene were identified and quantified by comparing with external and internal standards, which had retention time of 4.6, 9.6, and 26.05 min, respectively.

5.2.5. Bacterial DNA extraction

Bacterial DNAs from milk samples were extracted and purified following repeated bead beating plus column method of Yu & Morrison (2004). Briefly, bacterial DNA from 1.5 mL of milk sample was lysed with 1 mL of lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS)]. Cell lysis was achieved by bead beating in the presence of 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). After bead beating, most of the impurities and the SDS are removed by precipitation with 200 µL 10 M ammonium acetate, and then the nucleic acids are recovered by precipitation with 700 µL isopropanol. Genomic DNA can then be purified via sequential digestions with 2 µL DNase-free RNase and 15 µL proteinase K, followed by the use of QIAamp columns from DNeasy Stool Mini Kit (Qiagen, Germantown, MD, USA).

The bacterial DNAs of milk samples were subjected to two-step PCR procedures to generate an amplicon library for MiSeq sequencing (Nguyen, Wu, & Nishino, 2019). The first round PCR was employed using primers targeting the V4 region of the 16S rRNA gene (forward: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3′; reverse: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3′; tail sequences are underlined). The PCR protocol included an initial denaturation at 94 °C for 2 min, followed by 35 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 using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and subsequently used as DNA templates for second round PCR with adapter-attached primers. The second round PCR protocol included an 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. After the purification process described above, the purified DNA was pair-end sequenced (2 × 250 bp) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

The archived raw sequences were processed using quantitative insights into microbial ecology (QIIME2, version 2019.10) software. The raw sequences were denoised via DADA2 and low-quality sequences were truncated. Sequences that overlapped more than 60 bp were merged, followed by the identification and removal of chimeric sequences. Only high-quality sequences remained were grouped into operational taxonomic units (OTUs) with a 97% similarity threshold. Bacterial clustering was analyzed from the phylum to family level.

5.2.6. Statistical analysis

Statistical analysis was performed using JMP software (version 13, SAS Institute, Tokyo, Japan). The data was subjected to one-way analysis of variance, and the means were compared using Tukey HSD method. Spearman correlation analysis were also carried out using JMP software and network analysis were performed using Cytoscape software (version 3.8.0).

5.3. RESULTS AND DISCUSSION

5.3.1. Effect of supplemental β-carotene on milk yield and milk composition

Table 5.1. Milk yield and milk compositions of Jersey cows before (0M), after 1 month of β-carotene supplementation (1M+), and after 1 month of β-carotene depletion (1M-).

	0M	1M+	1M-	SE
Milk yield (kg/d)	28.61^{a}	24.47^{ab}	20.33^{b}	1.56
Milk composition				
Protein (%)	3.88^{b}	4.25^{a}	4.53^{a}	0.65
Fat (%)	5.05^{b}	4.86^{b}	5.86^{a}	0.73
Solid-not-fat (%)	9.38^{b}	9.76^{a}	9.90^{a}	0.98
SCC (log10 cell/mL)	5.68^{a}	4.66^{b}	4.78^{b}	0.71

a, b – values in the rows with different letters differs significantly ($P < 0.05$)

Data for the milk yield (20-28 kg/day/cow) and milk composition (3.8-4.5% protein, 4.8-5.8% fat content, and 9.3-9.9% solid-not-fat) were normal for Jersey cows. After 1 month of β-carotene supplementation, milk yield gradually decreased and significant difference was observed after 1 month of β-carotene depletion. Clearly, β-carotene supplementation did not affect milk yield, and the decrease of milk yield during 2 months of experiment was also observed for the whole herd and in other farms, which probably due to seasonal effect. Fat, protein, and SNF, on the other hand, showed increased trend, regardless of β-carotene supplementation or termination, indicating that dietary β-carotene is not the determining factor of milk components. The increase of fat, protein and SNF probably because of the decrease of milk yield production, which made the milk more condense. Somatic cell counts noticeably decreased when cows were fed with β-carotene (5.68 vs. 4.66 log10 cells/mL).

Though milk yield was numerically lower, there was no significant difference in milk yield and milk fat percentage ($P > 0.05$) before and after 1 month of β-carotene supplementation, which was consistent with Rakes (1985), Wang (1988), Akordor (1986), and Bindas (1984). Milk protein (3.88–4.25%) and solid-not-fat (9.38–9.76%) significantly increased after 1 month feeding β-carotene ($P < 0.05$), probably due to the numerical decrease in milk yield. The decrease in milk yield and increase in milk protein and milk fat probably due to seasonal change, which were consistently observed from whole-herd-data and other farms' data (unpublished). Somatic cell counts noticeably decreased when cows were fed with β-carotene for a month (5.68 vs. 4.66 log10 cells/mL), and slightly increased (4.66 vs 4.78 log10 cells/mL) after one month of depletion. Hence, β-carotene might decrease the number of SCC.

5.3.2. Concentration of fat-soluble micronutrients in plasma and milk

Table 5.2. Retinol, α-tocopherol, and β-carotene concentration in plasma and milk of Jersey cows before (0M), after 1 month (1M+) of the β-carotene supplementation, and after 1 month (1M-) of β-carotene termination.

	0M	1M+	1M-	SE
Plasma				

Retinol (µg/mL)	2.40	3.05	2.39	0.42
α-tocopherol (µg/mL)	2.50^{a}	1.89^{ab}	1.43^{b}	0.27
β-carotene (µg/mL)	8.59^{b}	15.83^{a}	10.83^{ab}	1.65
Milk				
Retinol (µg/mL)	0.08^{b}	0.20^{a}	0.21^{a}	0.01
α-tocopherol (µg/mL)	8.11^{b}	13.14^{ab}	18.00^{a}	2.60
β-carotene (µg/mL)	1.01^{c}	3.82^{a}	2.98^{b}	0.20

a, b – values in the rows with different letters differs significantly ($P < 0.05$)

Table 5.2 shows no significant difference in plasma retinol, plasma α-tocopherol, and milk α-tocopherol between 0M, and 1M+ ($P > 0.05$). Predictively, plasma β-carotene, milk β-carotene and milk retinol significantly higher after 1 month of supplementation ($P < 0.05$). After 1 month (1M-) of β-carotene termination, plasma β-carotene returned to normal level ($P > 0.05$) with 10.83 µg/mL at 1M-; whereas milk retinol and milk β-carotene were maintained at high level than before supplementation (0M) ($P < 0.05$) with 2.5 and 2.6 times higher, respectively.

In this study, plasma retinol concentration was quite stable, which was consistent with other studies (Fuquay, 2011). An increase in dietary β-carotene supply has been shown to elevate plasma concentrations of β-carotene in lactating cows but not of circulating retinol, because retinoic acid is a potent regulator of gene expression, its concentration has to be regulated precisely in circulating blood to avoid any deleterious effect due to a high concentration of vitamin A (Fuquay, 2011). Although plasma α-tocopherol and milk α-tocopherol showed no significant change ($P > 0.05$), supplemental β-carotene numerically increased milk α-tocopherol and decreased plasma α-tocopherol (Table 5.2), indicating that β-carotene supplementation seemed to facilitate the α-tocopherol diffusion from plasma to milk. The interaction between β-carotene and α-tocopherol within the dairy cattle's body remains unclear and needs further studies to elucidate. In this experiment, milk retinol, but not plasma retinol, and milk β-carotene were maintained at high level even after 1 month of termination suggesting that β-carotene might be stored and converted to retinol in the mammary gland (Fuquay, 2011).

5.3.3. Indigenous microbial composition of Jersey milk

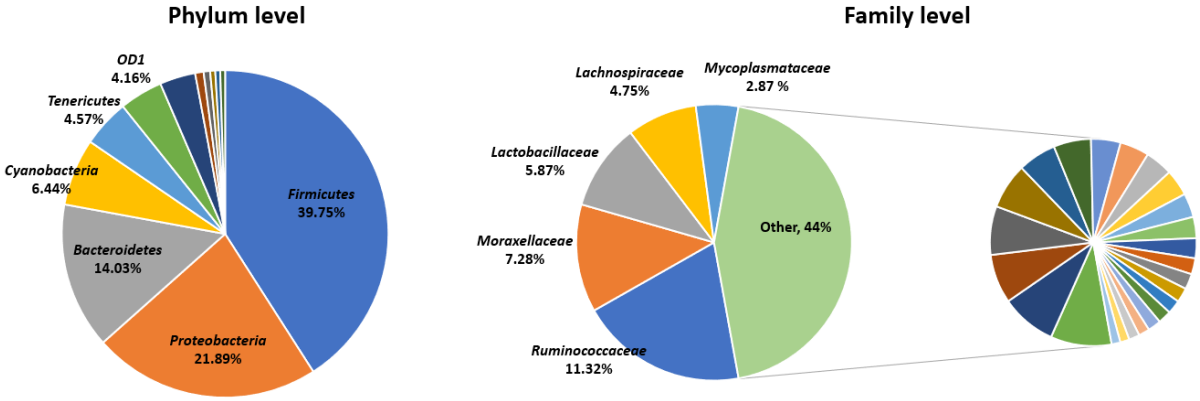

Figure 5.1. Relative abundance of major microbial taxa in Jersey milk at phylum and family level. The data was averaged from 30 milk samples collected from 10 healthy Jersey cows in 3 times collection.

At phylum level, 4 out of 15 phyla were abundant in Jersey milk, accounted for 82.11% in relative abundance, i.e., 39.75% was *Firmicutes*, 21.89% was *Proteobacteria*, 14.03% was *Bacteroidetes*, and 6.44% was *Cyanobacteria*. At family level, 5 out of 46 families were predominant, i.e., 11.32 % was *Ruminococcaceae*, 7.28% was *Moraxellaceace*, 5.87% was *Lactobacillaceae*, 4.74% was *Lachnospiraceae*, and 2.87% was *Mycoplasmataceae* (Figure 5.1). The remaining 44% belonged to 24 other families, demonstrating the high diversity of bacterial community in Jersey milk.

5.3.4. Effect of supplemental β-carotene on milk microbiota

Table 5.3. Relative abundance of milk microbiota of Jersey cows at phylum and family level. Families having a relative abundance of > 1% in at least one sample are indicated. 0M, 1M+, and 1M- indicate the time before β-carotene supplementation (0M), after 1 month of supplementation (1M+), and after 1 month of β-carotene termination (1M-), respectively.

Phylum | Family	β-Carotene supplementation			
	0M	1M+	1M-	SE

Euryarchaeota	0.16[b]	0.69[a]	0.35[b]	0.09
Methanobacteriaceae	0.16[b]	0.69[a]	0.35[b]	0.09
Actinobacteria	1.77[a]	0.39[b]	0.53[b]	0.11
Corynebacteriaceae	1.77[a]	0.39[b]	0.53[b]	0.11
Bacteroidetes	7.82[b]	13.67[a]	9.50[b]	0.74
Bacteroidaceae	1.34	1.27	0.71	0.20
Porphyromonadaceae	0.71	0.60	0.49	0.07
Prevotellaceae	1.45[b]	2.51[a]	2.30[ab]	0.25
Rikenellaceae	0.50[ab]	0.85[a]	0.44[b]	0.11
S24-7	0.89[b]	1.72[a]	1.05[b]	0.15
[Paraprevotellaceae]	0.71	1.09	0.93	0.14
Firmicutes	38.73	36.08	41.26	2.07
Bacillaceae	2.36[a]	0.82[b]	0.69[b]	0.12
Paenibacillaceae	0.83[a]	0.09[b]	0.11[b]	0.09
Staphylococcaceae	3.79[a]	1.37[b]	1.77[b]	0.55
Aerococcaceae	3.24[a]	0.96[b]	0.91[b]	0.39
Lactobacillaceae	9.41[a]	2.67[b]	7.25[ab]	1.63
Streptococcaceae	1.01[a]	0.19[b]	0.42[b]	0.08
Turicibacteraceae	0.50	1.14	0.88	0.21
Clostridiaceae	0.84	1.28	1.15	0.20
Lachnospiraceae	3.76	4.99	5.40	0.64
Peptostreptococcaceae	0.87	0.92	0.82	0.15
Ruminococcaceae	7.30[b]	14.50[a]	12.11[ab]	1.41
Veillonellaceae	0.40	0.29	0.26	0.06
[Mogibacteriaceae]	0.42	0.71	0.51	0.08
Erysipelotrichaceae	0.97[b]	1.54[b]	3.90[a]	0.56
Proteobacteria	9.78[b]	12.69[ab]	15.34[a]	1.22
Comamonadaceae	1.70[a]	1.23[ab]	1.12[b]	0.14
Succinivibrionaceae	0.52	0.38	0.36	0.11
Enterobacteriaceae	0.77	0.60	0.74	0.12
Moraxellaceae	3.62[b]	7.92[a]	11.18[a]	1.15
Pseudomonadaceae	0.98[a]	0.31[b]	0.27[b]	0.06
Tenericutes	3.87[b]	5.08[a]	4.00[ab]	0.34
Mycoplasmataceae	2.95	2.80	2.52	0.23
Unclassified	37.88[a]	31.40[ab]	29.03[b]	1.95

At phylum level, the effect of supplemental β-carotene on milk microbiota was not clear (Table 5.3). Major phyla showed no significant difference during 2-month experiment period, including Firmicutes and Proteobacteria; meanwhile many others showed significant changes. Bacteroidetes, Tenericutes, and Euyarchaeota significantly increased after 1 month of β-carotene

supplementation (1M+) and decreased after 1 month of β-carotene termination (1M-). Actinobacteria; on the other hand; showed significant decrease at 1M+ and remained at low abundance at 1M- (Table 5.3).

At family level, different taxa showed distinct responses to β-carotene supplementation. Some families showed no significant changes in relative abundance by β-carotene supplementation ($P > 0.05$), i.e., *Lachnospiraceae, Mycoplasmataceae, Bacteroidaceae, Porphyromonadaceae, Paraprevotellaceae, Turicibacteraceae,* and *Clostridiaceae*. Relative abundance of *Ruminococcaceae, Moraxellaceae, Prevotellaceae,* S24-7 and *Methanobacteriaceae* significantly increased after 1 month of feeding β-carotene (1M+). At 1M-, that of *Moraxellaceae* kept increasing while *Ruminococcaceae, Prevotellaceae,* S24-7 and *Methanobacteriaceae* showed no difference compared with before β-carotene supplementation (0M). On the contrary, *Bacillaceae, Paenibacillaceae, Staphylococcaceae, Aerococcaceae*, *Lactobacillaceae,* and *Streptococcaceae* decreased significantly at 1M+ and remained at low abundance at 1M- except for *Lactobacillaceae*, which increased again after stopping β-carotene supplementation (Table 5.3).

5.3.5. Relationship between milk microbiota and milk components

Relationships between milk microbiota and milk components, including fat, protein, SNF, retinol (vitamin A), α-tocopherol (vitamin E), and β-carotene were revealed. Figure 5.2 indicated that milk microbiota showed sophisticated interaction with milk components. Milk fat showed positive correlation with *Streptococcaceae* and negative correlation with *Pseudomonadaceae*. Protein showed positive correlation with *Lachnospiraceae* and negative correlation with *Prevotellaceae*. Milk urea nitrogen was positively correlated with *Streptococcaceae* and negatively correlated with *Methanobacteriaceae*. Somatic cell count was positively correlated with *Staphylococcaceae* and *Bacteroidaceae*. Fat-soluble micronutrients had a lot of relationship with milk microbiota; in which milk β-carotene had positive correlation with 8 families and negative correlation with 9 families. Milk vitamin A had positive correlation with 6 families and negative correlation with 9 families. Vitamin E had only 1 positive correlation and 3 negative correlation with milk microbiota. Noticeably, many negative correlations between fat-soluble micronutrients and mastitis-related bacteria and spoilage-related bacteria were observed. For

instant, both milk β-carotene and milk vitamin A were negatively correlated with mastitis-related bacteria, i.e., *Staphylococcaceae*, *Streptococcaceae*, and *Corynebacteriaceae*; and were also negatively correlated with spoilage-related bacteria, i.e., *Pseudomonadaceae* and *Bacillaceae*. Milk vitamin E was negatively correlated with *Corynebacteriaceae* and *Pseudomonadaceae*. This suggested that fat-soluble micronutrients, i.e., vitamin A, vitamin E, and β-carotene can potentially suppress the growth of mastitis-related bacteria and spoilage-related bacteria.

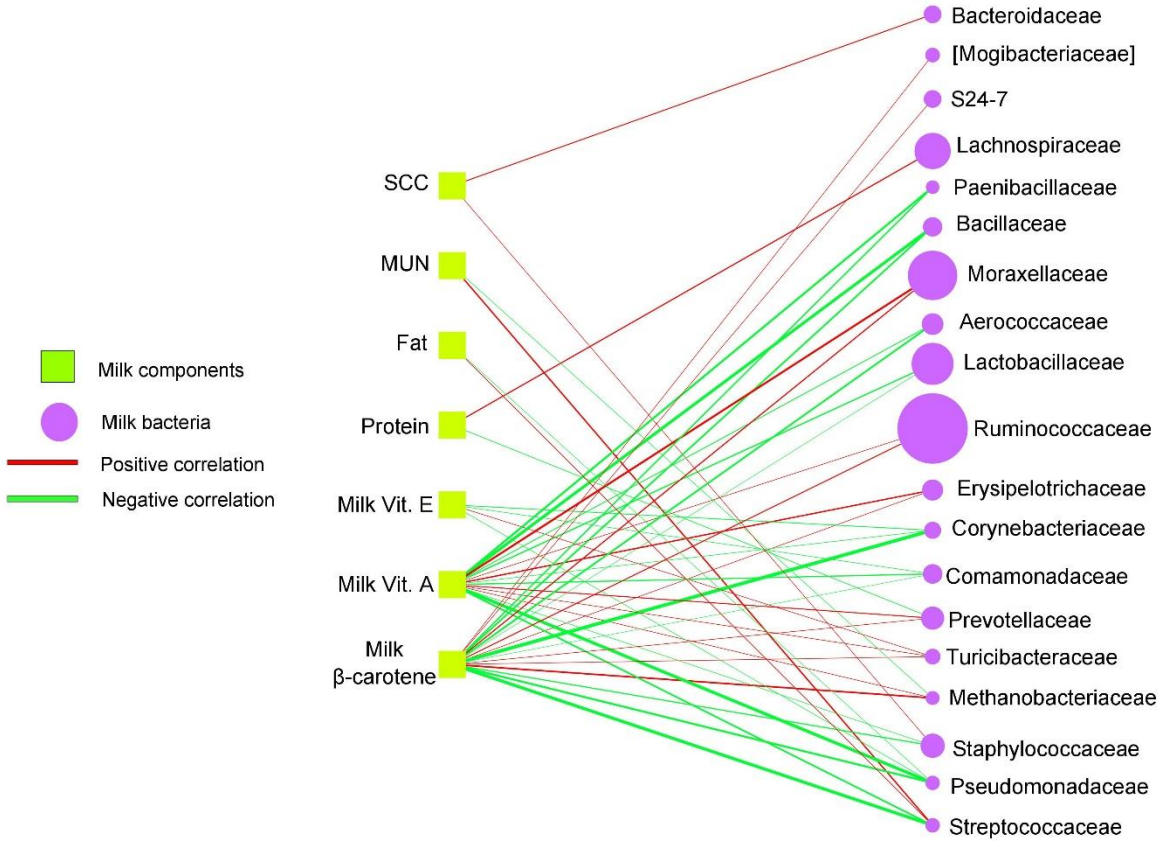

Figure 5.2. Correlation analysis showing the relationship between milk microbiota and milk components. Only strong (correlation coefficient R > 0.6 or <-0.6) and significant (P < 0.01) correlations were chosen to be displayed in the network. The edge width and color (red, positive; green, negative) are proportional to the correlation strength. The circle sizes are proportional to the relative abundance of bacterial families. Green square indicates milk compositions. Purple circle indicates milk microbiota.

5.4. CONCLUSION

β-carotene supplementation did not significantly affect milk yield and milk components but significantly increased milk β-carotene, milk retinol and numerically increased milk α-tocopherol even after 1 month of β-carotene termination.

Ruminococcaceae, *Moraxellaceace*, *Lactobacillaceae*, *Lachnospiraceae* and *Mycoplasmataceae* were predominant families of Jersey milk.

Different taxa showed distinct responses to β-carotene supplementation. β-carotene supplementation increased relative abundance of *Ruminococcaceae*, *Moraxellaceae*, *Prevotellaceae*, S24-7, and *Methanobacteriaceae* while decreased that of *Lactobacillaceae*, *Staphylococcaceae*, *Aerococcaceae*, *Bacillaceae*, *Corynebacteriaceae*, *Streptococcaceae*, *Pseudomonadaceae*, and *Paenibacillaceae*.

Milk microbiota showed sophisticated interaction with milk components. Milk fat showed positive correlation with *Staphylococcaceae* and *Bacteroidaceae*. Milk protein showed positive correlation with *Lachnospiraceae* and negative correlation with *Prevotellaceae*. Milk urea nitrogen was positively correlated with *Streptococcaceae* and negatively correlated with *Methanobacteriaceae*. Somatic cell count was positively correlated with *Staphylococcaceae* and *Bacteroidaceae*.

Many negative correlations between fat-soluble micronutrients and mastitis-related bacteria and spoilage-related bacteria were observed suggesting that fat-soluble micronutrients, i.e., vitamin A, vitamin E, and β-carotene can potentially suppress the growth of mastitis-related bacteria and spoilage-related bacteria.

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CHAPTER 6

GENERAL CONCLUSION

The variation of microbial composition in milk is affected by many factors including seasons, genotype, and feed ingredients. Because the management of microbial composition in milk is the key to secure udder health, high-quality and safety of milk and dairy products, understanding the variation of milk microbiota in relation to season, genotype, and feeding is of great importance. In addition, gut microbiota may directly or indirectly contribute to milk productivity, milk quality and cow's health. Therefore, three experiments were presented in this dissertation to examine the variability and stability of milk microbiota and gut microbiota of Jersey cows in relation to seasonal changes and feeding management.

In the first experiment, relative abundance of milk microbiota showed significant difference between two farms and two seasons, though milk yield, milk composition and fecal microbiota were similar. In addition, fecal microbiota was unrelated with milk microbiota regardless of the farm and seasons. This suggests seasons have substantial effect on shaping milk microbiota.

In the second experiment, the use of dietary β-carotene supplementation increased blood urea nitrogen and decreased total cholesterol. Regarding rumen fermentation, β-carotene supplementation did not have significant effect on major VFAs, i.e., acetate, propionate, butyrate, total VFAs and acetate:propionate ratio. As per rumen and hindgut microbiota, supplemental β-carotene did not have significant effect on rumen microbiota composition but had more effect on hindgut microbiota. β-Carotene supplementation alleviated relative abundance of *Methanobacteriaceae*, *Clostridiaceae*, and *Succinivibrionaceae* taxa while increased those of *Ruminococcaceae*, *Rikenellaceae*, RF16, and *Porphyromonadaceae* in the hindgut. Further studies are needed to elucidate the affecting mechanism of β-carotene on hindgut microbiota and its possible effect on animal health.

In the third experiment, β-carotene supplementation did not affect milk yield and milk composition. Nutritional value of the milk was also increased with supplemental β-carotene by increasing milk β-carotene, milk retinol, and milk α-tocopherol concentration. Jersey milk

microbiota was revealed with *Ruminococcaceae*, *Moraxellaceace*, *Lactobacillaceae*, *Lachnospiraceae* and *Mycoplasmataceae* were predominant families. On the other hand, dietary β-carotene supplementation had substantial effect on milk microbiota composition. β-carotene supplementation increased relative abundance of *Ruminococcaceae*, *Moraxellaceae*, *Prevotellaceae*, *S24-7*, and *Methanobacteriaceae* while decreased that of *Lactobacillaceae*, *Staphylococcaceae*, *Aerococcaceae*, *Bacillaceae*, *Corynebacteriaceae*, *Streptococcaceae*, *Pseudomonadaceae*, and *Paenibacillaceae* taxa. The sophisticated interaction between milk microbiota and milk components was revealed. Milk fat showed positive correlation with *Staphylococcaceae* and *Bacteroidaceae*. Milk protein showed positive correlation with *Lachnospiraceae* and negative correlation with *Prevotellaceae*. Milk urea nitrogen was positively correlated with *Streptococcaceae* and negatively correlated with *Methanobacteriaceae*. Somatic cell count was positively correlated with *Staphylococcaceae* and *Bacteroidaceae*.

The findings indicated that dietary β-carotene supplementation may not only increase fat-soluble micronutrients in milk but also modulate the milk microbiota composition. Many negative correlations between fat-soluble micronutrients and mastitis-related bacteria and spoilage-related bacteria were observed suggesting that fat-soluble micronutrients, i.e., vitamin A, vitamin E, and β-carotene can potentially suppress the growth of mastitis-related bacteria and spoilage-related bacteria.