Study of foodborne disease: Hygienic status and prevalence of food-borne pathogens in domestic natural cheeses produced in Hokkaido, Japan

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Contents

Abbreviations

A	AIEC	adherent invasive *Escherichia coli*
B	BAM	bacteriological analytical manual
	BHI	brain heart infusion
	BPW	buffered peptone water
	BTS	bacterial test standard
C	CDC	Center for Disease Control and Prevention
D	DDW	double distilled water
	DHL	deoxycholate hydrogen sulfide lactose
E	EAEC	enteroaggregative *Escherichia coli*
	EHEC	enterohemorrhagic *Escherichia coli*
	EIEC	enteroinvasive *Escherichia coli*
	ELISA	enzyme-linked immunosorbent assay
	EMB	eosin methylene blue
	EPEC	enteropathogenic *Escherichia coli*
	ETEC	enterotoxigenic *Escherichia coli*
F	FDA	Food and Drug Administration
	FSIS	food safety and inspection service
H	HACCP	hazard analysis critical control point
	HC	hemorrhagic colitis
	HCCA	α-cyano-4-hydroxycinnamic acid
	HUS	hemolytic uremic syndrome
I	ISO	international standards organization
L	LEB	*Listeria* enrichment broth
M	MALDI	matrix-assisted laser desorption ionization
	MHLW	Ministry of Health, Labor and Welfare
P	PALCAM	polymyxin-acriflavin-lithium chloride-ceftazidime-esculin-mannitol

	PCR	polymerase chain reaction
R	RV broth	Rappaport-Vassiliadis broth
S	SEA	staphylococcal enterotoxin A
	SEB	staphylococcal enterotoxin B
	SEC	staphylococcal enterotoxin C
	SED	staphylococcal enterotoxin D
	SEE	staphylococcal enterotoxin E
	SEG	staphylococcal enterotoxin G
	SEH	staphylococcal enterotoxin H
	SEI	staphylococcal enterotoxin I
	SPC	standard plate count
	STEC	Shiga toxin-producing *Escherichia coli*
U	USDA	United States Department of Agriculture

General introduction

1. Growing cheese consumption in Japan

Natural cheese is a dairy food made from milk, and it is one of the oldest and most traditional human foods consumed worldwide. Perhaps, accidental milk curdling might have been the reason for the beginning of cheese making practice and nowadays; it is one of the most diverse and most commonly consumed foods around the world [48]. According to the international dairy food association, cheese is also a major manufactured dairy product, and it has significant importance to the dairy industry [72]. Consumption of cheese, however, varies from country to country and from region to region in different parts of the world. For instance, Western nations have more custom of cheese consumption than others, and they are leading the first lines of global cheese consumers list [45, 48]. In Japan, however, consumption of cheese is a relatively recent practice and more public awareness and wider cheese consumption habit begun after the Second World War [17]. Since the flavor of the fermented milk product was not popular among Japanese consumers, consumption of natural cheese did not popularize in earlier times and processed cheese gained more acceptance than the natural cheese. Besides, the absence of cooling facilities in earlier times also has played its part to limit the growth of natural cheese consumption in Japan [45].

However, following the tremendous economic development of Japan and exposure of the Japanese public to the western food cultures, consumption of natural cheese has started to increase from time to time [17, 101]. Consequently, the per capita cheese consumption at present is estimated to be about 2 kg per year [17, 45] and some informal estimates also show it as 2.4 kg per year. Even though this quantity is much lower as compared to the consumption in other developed countries, it indicates the presence of a continuous increase in the natural cheese consumption in Japan [17, 101]. Moreover, a further increase of the consumption is predicted as more public is becoming consumers

of foods that contain cheese as its ingredient. The estimates of the gross average annual consumption in Japan shows that 261000, 284000, and 301000 metric tons of natural cheese is consumed during 2010, 2011, and 2012 respectively [101]. As a response to the increase in the consumption, there is also the increase in domestic production and import from different countries. For instance, during the consecutive years of 2010, 2011, and 2012, estimates show that the country produced an estimated 48000, 49000, and 50000 metric tons of natural cheese. Among these, the majority of the product comes from Hokkaido [101]. In Hokkaido, eastern sub-region of Tokachi is the primary place where the vast majority of the dairy farms and cheese makers operate.

2. Hokkaido as a major supplier of domestic natural cheese

Hokkaido is Japan's major agrarian region that supplies a vast array of agricultural products including crop, livestock, and fishery products [53]. Among these, the natural cheese produced and supplied from this region is estimated to amount 90% of the domestic natural cheese production. Import dominates Japanese cheese market where the country supplies about 80% of the cheese consumed in Japan through import. The major suppliers of the imported cheeses are Australia and New Zealand where about 75% of the product comes from these countries [17]. Meanwhile, the remaining portion of the imported cheese comes from other countries including Europe and the US. However, 20% of the natural cheese consumed in the country is produced domestically, and production of domestic natural cheese is increasing from time to time [17, 45, 101]. From the total small scale and large scale cheese making factories in Japan, 54% of them are based in Hokkaido and 33% of the factories are located in Eastern part of the region. Eastern Hokkaido is famous for dairy production. Natural cheeses from this sub-region are more popular and highly preferred by most of the domestic natural cheese consumers in the country.

3. Cheese making process and the importance of production hygiene

Natural Cheese is made by curdling of raw or pasteurized milk, and it has several steps that start from selecting and standardizing milk until obtaining the finished product. Among others, milk

selection, standardization, coagulation, whey removal, molding, salting and maturation are some of the steps commonly followed in the cheese-making processes [48]. Each step requires implementation of careful cheese-making practice to avoid contamination and ensure the safety of the product. The occurrence of contamination could affect the quality of cheese and make the product unfit for public consumption due to the possible health risk to consumers. Contaminated raw milk could be one of the potential sources of natural cheese contamination [3, 76]. To eliminate such contamination, therefore, cheese making is mostly based on pasteurized milk. As pasteurization is expected to eliminate foodborne pathogens, it is a prerequisite to using pasteurized milk for cheese making in Japan.

Though pasteurization is expected to eliminate foodborne pathogens, failure in its process may lead to the spoilage of milk and then to the finished products including natural cheese. Once contaminants get access to the natural cheese, their growth can be influenced by several factors. Among others, storage at room temperature, improper packaging, and high moisture content of the product could favor the growth of pathogens and may lead to the health risk of the consumers. A report of microbial risk assessment showed that extra hard and hard type of natural cheeses made from raw milk had very unlikely results to pose a risk to consumers due to foodborne pathogens. However, molded raw milk cheddar, feta and camembert cheeses had a higher likelihood of public health risk due to the growth and survival of pathogenic *E. coli* [3]. Thus, paying due care for the cheese making steps and avoiding post processing contamination could help to prevent the occurence of pathogens and resulting health risks to the consumers.

4. Foodborne pathogens associated with natural cheese consumption

Natural cheese is the concentrated form of milk comprising of nutrients that can supply dietary requirements of humans and support the growth and survival of microorganisms. Therefore, it contains natural microflora of the cheese such as lactic acid bacteria, manually added starter and non-starter cultures. Moreover, adulterated cheese may also contain contaminants including foodborne pathogens. Among the pathogens, *Listeria monocytogenes, Salmonella,* pathogenic

Escherichia coli and *Staphylococcus aureus* are more often associated with cheese related foodborne disease outbreaks worldwide [3, 131]. In fact, the outbreak caused by these pathogens due to the consumption of natural cheese is not common in Japan. However, there is gap of information on the prevalence of these pathogens on domestic natural cheese produced in Japan. Since the foodborne outbreaks of these pathogens associated with the consumption of cheese are reported in other countries, it is necessary to assess and know their prevalence in natural cheeses produced in Hokkaido, Japan. An earlier report from Hokkaido showed the outbreak of *L. monocytogenes* caused by the consumption of contaminated cheese and that was the only report on foodborne listeriosis outbreak in Japan so far [90]. Moreover, another study also showed the prevalence of *S. aureus* in the cheese from this region and some of the isolates were positive to enterotoxin genes such as *seg* and *sei* [56]. Despite the presence of such few reports, however, there is a lack of information on the prevalence of these pathogens in domestic natural cheese. Therefore, this dissertation has the general objective of assessing hygienic status and prevalence of foodborne pathogens in natural cheese produced in Hokkaido. Thus, in this section I briefly describe these four major foodborne pathogens based on their characters, epidemiology, source and route of transmission, clinical significance, isolation and identification, and prevention and control.

Listeria monocytogenes

Characteristics

Listeria spp. are short, Gram-positive, rod-shaped, and psychrotrophic bacteria with rounded ends. The cells can be found singly or in short chains and sometimes in long filaments. They are catalase positive, oxidase negative, and facultative anaerobic microorganisms [84, 85]. *L. monocytogenes* is motile at the temperatures between 20ºC and 25ºC with peritrichous flagella showing characteristic tumbling movement. However, it is not motile at other growth temperature conditions including the optimum temperature of 37ºC. Among its main features, *L. monocytogenes* grows in many foods including those treated with high salt or food grade acids. As the organism is cold tolerant, it can also grow in those foods stored at refrigerated temperature conditions and poses

a health risk to the consumers. Its ability to endure harsh conditions allows the pathogen to grow and reach a high number of cells in the foods stored in refrigerators as storage time extends. *L. monocytogenes* is a zoonotic pathogen that cause different infections including mild gastroenteritis up to fatal septicemia, encephalitis, abortion or stillbirth [84, 85].

There are six species of *Listeria* classically known that include *L. monocytogenes, L. ivanovii, L. welshimeri, L. grayi, L. innocula* and *L. seligeri.* Among these, *L. monocytogenes* is pathogenic both for humans and animals. *L. ivanovi*, however, is pathogenic for animals, and rarely associated with the human infection while other species are mostly nonpathogenic. Apart from the previously known six species, four novel species of *Listeria* are reported recently. These include *L. rocourtiae*, *L. marthii*, *L. fleischmannii,* and *L. weihenstephanensis* making the number of *Listeria* species be ten [47, 79, 84, 85].

Epidemiology

L. monocytogenes is widely distributed in nature, and it is a zoonotic pathogen both for animals and human. Human listeriosis has a high fatality rate of up to 30% in the risk group such as pregnant women, infant, elderly and immunocompromised people. According to the report from the center for disease control and prevention (CDC), *L. monocytogenes* is one of the major foodborne pathogens. It results in an estimated 1591 illnesses, 1455 hospitalizations, and 255 deaths in the US per year [117]. A surveillance result from France also showed that an annual incidence of 3.4 listeriosis cases occurred per 1000000 inhabitants during 2001 – 2003 [46]. Another study from Germany indicated that an estimated 0.62 cases per 100000 inhabitants occur per year, and that report also showed increasing trend of listeriosis than the previous years [75]. The national survey result of Japan also showed an estimated incidence of listeriosis as 0.65 cases per million inhabitants that are lower than the cases in other developed countries [102]. Other reports from different regions of the world also indicate the distribution of *L. monocytogenes* all over the world [11, 24].

Source and route of transmission

L. monocytogenes is a ubiquitous microorganism that exist in a broad range of the environment. Among others, it can be isolated from soil, water, plants, decaying vegetation, slaughterhouse wastes, sewage, food processing environments, and others [116]. Therefore, it can get access to foods of different origin including dairy products, meat products, vegetables, bakery products, sea foods and ready-to-eat foods from various sources. Human listeriosis is believed to transmit mainly through consumption of contaminated foods [15, 116]. Since *L. monocytogenes* is ubiquitous in the environment, it can contaminate cheese at different stages of the cheese-making process. A study conducted by Brito and colleagues indicated the storage coolers as the principal source of contamination for cheeses produced from pasteurized milk [12]. Moreover, Kousta and colleagues [76], also reviewed that milk as raw material and cheese making environment can be the primary sources of cheese contamination.

Clinical significance

Listeriosis is a foodborne disease that affect a particular group of people designated as risk group due to their weakened immunity. These include elderly people, newborn infants, pregnant women or immunocompromised individuals. Listeriosis has the clinical signs that involve febrile gastrointestinal illnesses with mild and flue like symptoms. The disease may also develop into more invasive forms of listeriosis including meningitis, meningoencephalitis and septicemia in the risk groups [2, 15, 122]. The pathogen requires a long incubation period of about 30 days where the range varies between 3 and 70 days [2, 122]. Healthy individuals with normal immunocompetence may not develop symptoms, and if they develop, it appear in a mild flu-like symptoms or mild enteric form of illness. On the risk groups, however, the disease is manifested in more systemic form, and it has a high fatality rate of about 30% [122].

Isolation and identification

Several methods have been developed and used to isolate and identify *L. monocytogenes*, in various specimens including food samples. Conventional bacteriological methods of International Standards Organization (ISO) and Food and Drug Administration (FDA), are some of the most commonly used methods to inspect this pathogen. Moreover, methods of the Food Safety Inspection Service (USDA-FSIS), and International Dairy Federation (IDF) are also commonly used for the detection of *L. monocytogenes* from different food samples [33]. Most of these methods involve two steps that include pre-enrichment and enrichment processes in selective culture media followed by biochemical identification of suspected *L. monocytogenes* colonies. Since *Listeria* spp. are capable of hydrolyzing esculin into esculetin, the culture methods comprise esculin and ferric ion as important components to indicate the presence of esculin hydrolyzing bacteria. Then esculin reacts with ferric ion resulting in the formation of blackening complex in the media, and this indicates the occurrence of esculin hydrolyzing bacteria in the target sample.

The enrichment methods also contain selective agents that support the growth of *L. monocytogenes* from a given sample while inhibiting other microorganisms. Among these, lithium chloride is used for amplification of the pathogen in the presence of gram-negative bacteria while nalidixic acid is inhibitory for gram-negative bacteria through interference with DNA gyrase. Moreover, acriflavine, polymyxin B, and ceftazidime are used for inhibition of Gram-positive cocci and to prevent the growth of gram-negative rods that are background microflora of food and environmental samples [33].

Since the start of cold enrichment techniques, a variety of detection methods have been used for isolation and identification of *L. monocytogenes.* However, none of the methods is best for the selective isolation and identification of the pathogen. Thus, efforts for improving the existing methods are part of the continuous processes of scientific community's efforts. Among the current enrichment methods, UVM broth is recommended approach by USDA-FSIS for selective isolation of *L. monocytogenes* from meat, egg, and poultry samples. It contains per liter of proteose peptone (5 g), tryptone (5 g), lab-lamco powder (5 g), yeast extract (5 g) and sodium chloride (20 g). Moreover, it contains $Ha_2HPO_4.2H_2O$ (12 g), KH_2PO_4 (1.35 g), esculin (1 g), nalidixic acid (20 mg) and acriflavine HCl (12 mg). The medium is also called *Listeria* enrichment broth I (LEB I) and

similar media with the modification of its nalidixic acid content to (25 mg/l) is known as LEB II. Frazer broth is another enrichment media commonly used for isolation of *L. monocytogenes.* This broth is also a modification of LEB II, and it contains 3 g/l of lithium chloride and the components in the LEB II [33].

Moreover, selective agar media are also used for differential identification of the pathogen. Among these polymyxin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol (PALCAM) agar and Oxford agar are most commonly used solid media. More recently, chromogenic agar media have also been developed taking advantage of the specific enzyme activity that exist in *L. monocytogenes* and *L. ivanovii*, but not in other *Listeria* spp. Even though the culture methods are efficient to discriminate *Listeria monocytogenes* selectively, food-processing industries increasingly prefer to use more rapid quality control tests. These methods include immunosorbent assay (ELISA) and polymerase chain reaction (PCR) – based techniques. Such methods could deliver results within a short time than culture methods and allow batches of foods for quick release following completion of the tests [33, 43].

Prevention and control

Since *L. monocytogenes* is a ubiquitous microbe in the environment including food processing setups, it is hard to prevent contamination completely. However, implementation of appropriate hygiene and sanitation steps with good manufacturing practices and hazard analysis critical control point (HACCP) system can help for prevention and control of this pathogen. As *L. monocytogenes* is susceptible to cooking temperature, heat treatment and prevention of recontamination are also useful options to prevent the risk of listeriosis due to the consumption of contaminated foods [55]. Furthermore, adjusting acidification in the early stages of the cheesemaking process is indicated as a useful option to prevent the development of *L. monocytogenes* effectively with low-level contamination in raw milk cheese [95].

Different countries set the standard limit as control option so that foods that contain this pathogen below the specified limit could be considered as safe for public consumption. The limits

may vary based on actual conditions of each country. However, all the existing regulations require either the absence or lower than 100 cfu/g of *L. monocytogenes* in cheese and other ready-to-eat food samples [32, 59, 77]. For instance, USA has a strict rule of zero tolerance policy where detection of *L. monocytogenes* in cheese or any food sample may make the food subject to seizure [77]. According to the regulation of European Union, soft cheese should be free from *L. monocytogenes* per 25 g of the sample while less than 100 cfu/g of the other type cheese sample is acceptable [32].

Salmonella

Characteristics

Salmonellae are facultative anaerobic, gram-negative, small rod-shaped bacteria. They are the member of the *Enterobacteriaceae* family. Except *Salmonella* Pullorum and *Salmonella* Gallinarum, *Salmonellae* are motile with peritrichous flagella. They are catalase positive, oxidase negative and reduce nitrates to nitrites. *Salmonella* produces hydrogen sulfide, decarboxylate lysine, and they are negative to indole and urease [8]. *Salmonellae* are also mesophilic bacteria that have a broad range of growth temperature extending between 5ºC and 46ºC where optimum growth temperature is around 37ºC. However, they are killed by pasteurization temperature and time. *Salmonellae* also have a broad range of pH between four and nine while optimum pH being around neutrality. *Salmonella* do not multiply at an A_w of 0.94, especially in combination with a pH of 5.5 and below, but the cells survive in frozen and dried states for a long time. They can also proliferate in many foods without affecting the acceptance qualities [60]

The genus *Salmonella* has two species namely *S. enterica* and *S. bongori. S. enterica* is also subdivided into six subspecies based on their specific biochemical and genomic characteristics. As differentiation tool, Roman numbers and specific names associated with the geographic location are used to designate these six subspecies. Accordingly *S. enterica* subsp. *enterica* is assigned by the Roman number (I); and likewise *S. enterica* subsp. *salamae* (II); *S. enterica* subsp. *arizonae* (IIIa); *S.*

enterica subsp. *diarizonae* (IIIb); *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* is designated by the Roman number (VI) [60]. Among these, *S. enterica* subsp. *enterica* causes the majority of foodborne diseases.

Epidemiology

Salmonella is a zoonotic pathogen that causes foodborne bacterial disease worldwide. According to the estimates, it causes 93.8 million cases of foodborne illnesses around the globe leading to 155,000 deaths each year [89]. Another estimate also showed that *Salmonella* causes about 1 million illness per year in the United States alone which result in 19336 hospitalizations and 378 deaths [117]. A food poisoning statistics report of the Ministry of Health Labor and Welfare of Japan also showed that *Salmonella* caused an estimated 1518 illnesses in 67 incidents occurred in various parts of the country in 2009 [93]. The annual burden report of different regions of the world showed that *Salmonella* is estimated to cause about 56300 illnesses in North Africa and Middle East. Moreover, it causes 2.5 million illnesses in Africa, 53.6 million in Asia-Pacific and Oceania, 29.8 million in South and South East Asia, 5.1 million in Europe and 2.2 million in the Americas [89].

Source and route of transmission

Salmonella is transmitted through fecal-oral route mainly by the means of eating contaminated food. Consumption of contaminated non-animal food products, contaminated water, foods of animal origin or contact with reservoir animals could also be some of the possible routes of *Salmonella* transmission [89]. Moreover, mass production and distribution of the food products disseminate pathogens rapidly to the communities. Farm animals are the primary reservoir for non-typhoid *Salmonella* spp. Sanchez-Vargas and colleagues reviewed that *Salmonella* naturally occurs in poultry, sheep, goats, pigs, reptiles, amphibians, pet rodents, dogs, cats, and in a variety of wild animals [115]. Thus, consumption of foods contaminated with a fecal matter of these animals also could result in transmission of *Salmonella*. Furthermore, infections of *Salmonella* associated with pet transmission also reported to affect infants and lead to invasive disease and severe complications

[115]. Thus, though the majority of human *Salmonella* infections are foodborne, salmonellosis can also be acquired through, contact with infected animals, direct human-to-human transmission, or contact with a variety of reservoir animals [16, 115, 123].

Clinical significance

Salmonella causes foodborne salmonellosis that vary from mild and self-limiting gastroenteritis to sometimes systemic infections such as bacteremia. It has the incubation period of 7 to 72 hr after ingestion of the contaminated food. The symptoms include fever, nausea, abdominal cramping, and non-bloody diarrhea that may tend to decrease within few hr or days. In some cases, septicemia could result as a complication of gastroenteritis leading to fatal cases in immunocompromised patients. Prolonged septicemic infections may also cause localized tissue and organ infections, especially in those previously damaged or diseased individuals. Although their severity and duration may depend on the type and amount of pathogens ingested and on the susceptibility of the host, symptoms may last up to 2 to 7 days [31, 124].

Isolation and identification

Regardless of the contamination level, the presence of *Salmonella* in a given food is considered as a significant risk due to its low infective dose. Therefore, isolation and identification of *Salmonella* is conducted routinely following conventional culture techniques based on the distinct features of this pathogen [61]. Since, food samples might have passed through different treatment methods such as cold storage or heat treatment, the cells of the pathogen could appear in the injured state. This condition may also result in reduced recovery rate of the *Salmonella* cells by culture method and could lead to false negative results that in its turn could pose the potential health risk to the consumers. For this reason, isolation methods usually involve pre-enrichment steps to allow the recovery of injured cells. The pre-enrichment step of food sample uses non-selective media such as buffered peptone water. However, a selective Rappaport Vassiliadis (RV) – broth is used for the enrichment step that help the selective isolation of the *Salmonella*. Then the culture from RV broth

is streaked on the selective differential agar media. RV broth contains malachite green and magnesium chloride that inhibits other bacteria and allows the growth of *Salmonella*. Among others, Desoxycholate agar, *Salmonella-Shigella* agar, modified brilliant green agar, xylose lysine Desoxycholate agar and brilliant green MacConkey agar represent selective agar media used for isolation of *Salmonella*. Moreover, Bismuth sulfite agar, Mannitol lysine crystal violet brilliant green agar and chromogenic agar can also be used for selective isolation of *Salmonella* from various samples.

Salmonella can be further identified by using biochemical tests. Among others, gram stains and responses to various sugar fermentation tests are commonly used methods for identification of these pathogens. Moreover, ELISA and PCR-based methods are also available that has high efficiency for discrimination and getting the result with a shorter time than the culture based methods [111].

Prevention and control

Keeping proper sanitation measures, safe and hygienic food handling practice, and enhancing public awareness through appropriate education are some of the essential steps for the prevention of foodborne salmonellosis. As *Salmonella* is widely present in the gastrointestinal tract of food animals, it can be shed in feces that can be the source of food contamination. Hence, prevention measures for such cases should be focused on implementing proper hygienic measures and to avoid fecal contamination of foods. Among others, foods of animal origin such as eggs, poultry, and beef should be cooked properly to prevent *Salmonella* infection. Milk and dairy products should also be heat treated properly including pasteurization and appropriate cooking. Moreover, it is important to keep the hygiene of food making environment such as kitchen surfaces and utensils to prevent cross contamination of other food items due to the faulty practice of hygiene during household food making. Furthermore, it is important to avoid contact with gastrointestinal fluid with the carcass in the slaughter houses. Avoiding the contact of infants to pet animals is also suggested as these animals are reservoirs for *Salmonella*. However, in the case of an adult, to wash

hands properly following the contacts of pets could be sufficient to prevent the risk of salmonellosis [115].

Pathogenic *Escherichia coli*

Characteristics

E. coli is a gram-negative, facultative anaerobic, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. These bacteria are commensal microorganisms that exist in the lower intestinal tract of human hosts and other animals in a beneficial mutualistic relationship. As components of natural microflora in the human intestinal tract and other warm-blooded animals, *E. coli* can be detected in big number from the fecal samples. They are considered as harmless and commonly used as indicators of fecal contamination in foods. Also, if their number is high in food samples, it may suggest a possible presence of the pathogenic group of *E. coli* or other enteric pathogens in foods. Although many strains are known to be harmless inhabitants of the gastrointestinal system, some of the *E. coli* strains may be pathogenic and cause disease in humans. These pathogenic *E. coli* are characterized by their serogroup, virulence genes, and production of toxins and associated disease symptoms. Accordingly, there are classes of *E. coli* pathotypes known to cause foodborne gastrointestinal disease in humans. These include enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC). Moreover, enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and adherent invasive *E. coli* (AIEC) are among the known pathotypes of pathogenic *E. coli* [28, 37, 72].

Epidemiology

Among pathogenic *E. coli*, more focus of research is given for STEC specifically for EHEC, and more data is available for this group than other pathotypes. The pathotypes of pathogenic *E. coli*, however, include STEC O157, non-O157 STEC, ETEC and other diarrheagenic *E. coli* than STEC and ETEC [117]. Therefore, apart from STEC O157, other groups such as O26, O45, O103, O111,

O121, and O145 may also cause severe infections that could result in hemorrhagic colitis and hemolytic uremic syndrome. Diseases related to the pathogenic *E. coli* are widely distributed all over the world. A report of CDC shows an estimated 205781 illnesses, 2429 hospitalizations and 20 deaths occur in the US per year due to pathogenic *E. coli* infections [117]. A review of Croxen and colleagues [28] also showed that the prevalence of EPEC goes beyond developed countries. Though it is associated with infant diarrhea in developing countries, the problem remains in developed countries too. In the US, however, the rate related to this group of pathogens is reducing. Nevertheless, among all pathogenic *E. coli*, EHEC continues as a concern of foodborne public health issue worldwide [28, 37]. The report by Scallan and colleagues showed that EHEC alone results in an estimated 112572 illness and 271 hospitalizations in the US annually [117]. The CDC estimate also shows that the rate of EHEC incidence between 2005 and 2010 was 0.97 per 100000 inhabitants for O157 while the estimate for non-O157 was 1.10 per 100000 people. The hospitalization rate for the same period, however, was 43% for O157 while it was 18% for non-O157 groups. The fatality rate also was twice high in the case of O157 while it was relatively little in the case of non-O157 *E. coli.* The incidence of O157, however, dropped by 42% in 2011 than the earlier years in the US. In Canada, Australia, and Europe the incidence reports show higher figures than those reported by CDC in the US [28].

Source and route of transmission

The main route of transmission is a fecal-oral route through consumption of food contaminated with the pathogen. Ruminants are the major reservoir of STEC, and consumption of the food contaminated with cattle manure is the primary source for STEC transmission [28, 37]. Food contamination with cattle manure can occur through different routes and lead to the incidence of foodborne illnesses on the consumers. Direct contact with reservoir animals may also result in human infection in the absence of appropriate hygiene. Moreover, the human infection could also occur by direct contact with infected individuals especially in the places such as care centers [28].

Thus washing hand after the contact with animals or infected individuals should be a regular practice before handling foods.

Clinical significance

Pathogenic *E. coli* cause a various range of clinical syndromes that involve bloody or watery diarrhea based on the pathotypes. Among these, infection of STEC can range from mild watery diarrhea to bloody diarrhea (hemorrhagic colitis) and may result in the development of the hemolytic uremic syndrome (HUS) [28, 37]. The incubation period before the onset of the symptoms could be about three days. Despite the variation in severity, both O157 and non-O157 STEC can cause similar symptoms including bloody diarrhea and HUS on children, elderly or immunocompromised people. On the other hand, EPEC causes infectious diarrhea accompanied by fever, vomiting and dehydration in children under two years of age. It has a rapid onset of three-hour incubation time on human volunteers after ingesting wild-type bacteria. Other clinical features of this group include the intolerance for the cow's milk and failure to respond oral rehydration therapy. Clinical manifestation of EIEC is similar with that of *Shigella* that include bacillary dysentery and watery diarrhea containing blood and mucus [28].

Isolation and identification

Pathogenic *E. coli* comprises several pathotypes and STEC is one of the pathotypes that have more than 400 serotypes. However, only a few of these serotypes are known to be pathogenic for humans [28, 37]. There are standard isolation and identification methods including FDA's procedures indicated in bacteriological analytical manual and USDA/FSIS methods among others. The isolation methods of the pathogenic *E. coli* are also variable that include culture techniques and immunological or molecular-based identification methods. Since food samples might initially contain low numbers of STEC together with a high level of competing microflora, selective enrichment step might be required for the isolation of STEC [128]. Such enrichment step from inspection of processed foods could allow the recovery of injured or stressed bacterial cells due to

harsh manufacturing processes [28, 51]. Different enrichment protocols may possibly use varying basal broths, while diversity of selective agents are also added and used with various incubation times and temperature combinations.

Prevention and control

Sanitizing and keeping good hygienic measure is the best way to preventing contamination of food with pathogenic *E. coli.* Since *E. coli* are highly sensitive to heat, proper temperature treatment including pasteurization or cooking foods can also help considerably to prevent infection by these pathogens. Similarly, proper refrigeration and prevention of cross-contamination are among the useful measure to control pathogenic *E. coli* in ready-to-eat food including cheese. Moreover, keeping the appropriate hygienic condition of food making environment is also an important practice to prevent the contamination. Furthermore, personal hygiene and proper storage of foods in refrigeration with the prevention of cross-contamination are essential measures to prevent and control pathogenic *E. coli* in foods.

Staphylococcus aureus

Characteristics

Staphylococcus aureus is a gram-positive coccus that has characteristic cell arrangement of the grape-like structure. The cells appear singly, in paired cocci, or in clustered structures. It is a non-motile and non-spore forming bacteria that is facultative anaerobic [86]. *S. aureus* ferment mannitol, produce coagulase and catalase, and also produce thermostable nuclease (TNase). It grows in a wide temperature range that vary between 7ºC and 46ºC while it optimally grows in the mesophilic temperatures between 30ºC and 37ºC. *S. aureus* also grows in relatively small A_w (0.86) and low pH (4.8). Moreover, it can also grow in high salt and sugar concentrations of up to 15%, and in anaerobic conditions [67, 86, 112]. Therefore, due to their ability to grow under adverse conditions, *S. aureus* can multiply in many foods including natural cheeses. However, they are weak competitors to the native flora that found in foods, and the presence of active starter culture inhibits

their growth [112]. *S. aureus* exists in the skin, hair, and mucosal surfaces of healthy humans and animals. Moreover, anterior nares of the healthy people could be commonly inhabited by *S. aureus* [4, 86]. Therefore, most of the staphylococcal foodborne diseases are thought to result from consumption of contaminated foods because of the unhygienic practices by the food handlers. Some of the *S. aureus* strains are known to produce heat-stable enterotoxins. Therefore, once *S. aureus* gets access to foods, it can grow and produce a staphylococcal enterotoxin that could cause food poisoning.

Epidemiology

S. aureus causes foodborne bacterial intoxication worldwide. The data from the Japanese Ministry of Health, Labor, and Welfare shows that *S. aureus* is also one of the common pathogens causing foodborne bacterial diseases in Japan [93]. It caused a total of 2525 staphylococcal food poisoning outbreaks that resulted in 59, 964 illnesses, and three deaths during 20 years period of 1980 – 1999 in Japan [121]. Asao and colleagues [5] also reported an extensive outbreak of the staphylococcal foodborne outbreak in Kansai area of Japan. That outbreak was caused following the consumption of dairy products contaminated with the staphylococcal enterotoxins and it affected 13420 people in the year 2000. Moreover, *S. aureus* is also the second most important foodborne pathogen in France next to *Salmonella.* According to the report, 86 confirmed, and 173 suspected foodborne outbreaks were caused in France due to *S. aureus* among a total of 1787 foodborne illnesses reported during 2001 – 2003 [70]. *S. aureus* is also one of the causative agents of foodborne diseases in the US where according to CDC estimate, it result in 241148 illness, 1064 hospitalizations and six deaths in each year [117]. As it is part of the natural flora of humans and commensal organism in healthy individuals, the scope of foodborne diseases caused by this pathogen could be worldwide and possibly underreported.

Source and route of transmission

S. aureus is a commensal bacterium that inhabits the skin and anterior nares of humans. Among healthy people about 30% of the population is considered as permanent carriers [74, 86]. Moreover, *S. aureus* also could exist in the skin and hair of humans and domestic animals [4, 86]. Therefore, food handlers are considered as the primary source of food contamination by *S. aureus*. Especially, for the heat treated foods, contamination due to the faulty handling is the main route for the transmission of the pathogen to foods. Though it is poorly competent, *S. aureus* can grow in wide varieties of foods once it enter to the food. Among others, milk and its products, cream filled pastries and cheeses are some of the commonly incriminated foods for staphylococcal food poisoning. Moreover, meat and meat products, poultry and egg products, salads, and bakery products, cooked meals, and sandwich fillings are associated with staphylococcal food poisoning [4, 86, 112].

Clinical significance

Consumption of one or more staphylococcal enterotoxins pre-produced in the food may result in the staphylococcal food poisoning. Staphylococcal foodborne illnesses have a rapid onset that normally develop within 30 min to 8 hr following ingestion of the contaminated food. The symptoms include nausea, vomiting, and abdominal cramps and with or without diarrhea [4, 86]. The symptoms may also include headaches, cold sweats and rapid pulse, transient changes in blood pressure, prostration and dehydration depending on the amount of toxin ingested. Moreover, the amount of enterotoxin that cause the illness can vary depending on the susceptibility and immune condition of the individuals. However, 100 – 200 ng of the toxin is thought to be enough to cause symptoms [112]. A report of major Japanese outbreak estimated that about 20 – 100 ng of the enterotoxin caused symptoms on the consumers [5]. Despite its high incidence and economic pressure on the patients, staphylococcal foodborne illness is self-limiting, and recovery from it takes one to two days. However, in rare cases it may result in complication or hospitalization [67]. Staphylococcal food poisoning is one of the top most causes of foodborne bacterial illnesses in

Japan. However, the death rate related to staphylococcal food poisoning is low or, no death recorded from it in recent years [73, 93].

Isolation and identification

S. aureus is isolated from food samples and other specimens following one of the standard methods such as ISO method and FDA methods. Enrichment in selective agar media is used for isolation of the pathogen and coagulase tests, thermo nuclease analysis, and other biochemical tests are used for further identification [14, 68, 108]. Standard methods are based on the specific growth requirement of the bacteria and Baird-Parker Agar and Mannitol Salt Agar with egg yolk are most commonly used agar media for isolation of *S. aureus.* Baird Parker Agar contains Tryptone and Beef Extract as main carbon and nitrogen source. Yeast extract is another component used as an essential nutrient supplement and as a primary source of vitamins and growth stimulant for *S. aureus.* Also, the growth of the bacteria is stimulated by the presence of glycine and sodium pyruvate. Moreover, glycine, lithium chloride and potassium tellurite act as a selective agent, and egg yolk is the substrate used to detect lecithinase or lipase activity produced by *S. aureus*. Mannitol Salt Agar is another media used for selective isolation of *S. aureus.* In this medium, most other bacteria are selectively inhibited by the presence of high sodium chloride concentration. *S. aureus* ferments mannitol and formation of acid due to this turns the media into yellow. Most of the other coagulase-negative staphylococci do not ferment mannitol and grow as red colonies. Phenol red is the pH indicator that is converted to yellow when the fermentation of mannitol occurs reducing the pH of the medium to lower than 6.8. In the absence of mannitol fermentation, however, the medium remains red. Mannitol Salt Agar also contain peptone and beef extract that supply the nutrient requirement of the bacteria including carbon, nitrogen and other essential growth factors such as trace nutrients.

Following the isolation of *S. aureus* on selective media, identification is achieved through respective biochemical analysis unique to this pathogen that include colony morphology and gram staining. Moreover, coagulase test, latex agglutination test, DNase and thermo nuclease test, and

tests based on commercial biochemical kits are used for identification. Furthermore, PCR-based tests of molecular methods targeting specific genetic markers of *S. aureus* can be used for identification [14]. Among others, coagulase test is most commonly used for identification of *S. aureus* in routine laboratory activities [68]. There are two types of coagulase tests used for screening and identifying *S. aureus*. These include slide coagulase test (SCT) and tube coagulase test (TCT). Bound coagulase produced by the *S. aureus* also known as a clumping factor can be identified by using slide coagulase test. The presence of bound coagulase on the cell wall of the bacteria will cause the cells to clump or stick together forming a kind of clot. As a result, every cell of *S. aureus* stick to each other showing the resultant clumping of cells [68, 69]. On the other hand, tube coagulase test is used to identify the presence of free extracellular coagulase. Free coagulase is the enzyme released by the cell during its growth in the plasma. The enzyme forms coagulation of plasma due to its effect on blood clotting process by cleaving soluble fibrinogen into fibrin resulting in a gel-like clot in the samples positive for coagulase test [69]. These tests are used as the standard method for routine screening and identification of *S. aureus*.

Virulence factors

S. aureus has diverse virulence factors that include adhesions and extracellular proteins that enable them to colonize and cause various kind of illnesses [105]. Among others, staphylococcal enterotoxins are the most important virulence factors related to the food poisoning illnesses. Classically five staphylococcal enterotoxins (SEA, SEB, SEC, SED, and SEE) are known for their involvement in staphylococcal food poisoning incidences. However, at present there are about 22 staphylococcal enterotoxins identified among which some of them are known to be emetic and recognized for their involvement in foodborne outbreaks [4, 52, 63]. Consequently, those toxins having emetic activities are designated as staphylococcal enterotoxins. However, the other group either those lacking emetic activity or those that are not yet examined for such activity are designated as staphylococcal enterotoxin like super-antigens [4, 81].

Prevention and control

Since *S. aureus* can contaminate foods mainly due to faulty hygienic practice, improving overall production hygiene is the most important way to prevent contamination. Moreover, since the pathogen is highly susceptible to heat treatment, appropriate temperature treatment including pasteurization or cooking is a highly useful preventive measure. Furthermore, proper personal hygiene that involve washing hands and equipment properly, wearing masks, wearing hair nets, using separate equipment among others are useful for the prevention of contamination [67]. Additionally, storing natural cheese at their specific temperatures or in the temperature lower than 5ºC may help to prevent growth of the pathogen and possible toxin production as *S. aureus* does not grow below 5ºC [67, 112].

5. Microbial standards of natural cheese

Different countries set the microbial standards to ensure food safety and strengthen prevention and control measure of foodborne diseases. These rules set the limits that indicate a given food below the specified limit of microorganisms could be safe for public consumption. According to the European Commission (EC) standard, natural cheese should be negative for *Salmonella* and pathogenic *E. coli*. Due to their low infective doses, any detection of these pathogens in foods is considered as a threat to public health. The EC standard also describes the limit of *L. monocytogenes* in natural cheese that it should be absent or lower than 100 cfu/g in the natural cheese at the removal from the processing. Moreover, *S. aureus* count also should be below 1000 cfu/g at the time of removal from processing establishment for the cheese made from heat treated or pasteurized milk while it shall not exceed 10000 cfu/ml for raw milk cheese [32, 35]. According to these criteria, the coliform count also should not exceed 10^5 cfu/g in the natural cheese sample [32]. Meanwhile, the regulation initiated by USDA in 1987 states that ready-to-eat foods should be free from *L. monocytogenes*, and that guideline is applicable in the US for all ready-to-eat

foods [77]. The FDA regulation also require food products to be free from *Salmonella* and pathogenic *E. coli* [38]. The USDHHS also recommend coliform count in grade 'A' milk and its product should not exceed 10 cfu/g [39]. A standard of the UK also has a similar limit, and it states that *S. aureus* and *L. monocytogenes* in natural cheese shall not exceed 100 cfu/g. Moreover, natural cheese shall be negative for *Salmonella* and pathogenic *E. coli* while the coliform count in it shall not exceed 10000 cfu/g [107]. In Japan, the ministry of health, labor and welfare require the absence of *L. monocytogenes* in natural cheese [62]. Moreover, the guideline of Hokkaido regional accreditation body requires that natural cheese should not contain coliform per gram of sample while it should also be negative to *L. monocytogenes* per 25 g sample.

6. Aims of the present study

Although the regulatory bodies related to food safety and public health require cheese to be negative to coliform and *L. monocytogenes*; there is no microbial standard for domestic natural cheese in Japan. The recent amendment made on the "Ministerial Ordinance Concerning Compositional Standards Etc. for Milk and Milk Products", emphases on the prevention of *S. aureus* through pasteurization of milk and maintaining storage below 10ºC [92]. Though contaminated milk could be one of the sources for cheese contamination, pasteurized milk cheese and heat treated products can also get contaminated through improper sanitation and faulty post-processing handling. Among others, *L. monocytogenes, S. aureus, Salmonella,* and Pathogenic *E. coli* are also associated with cheese related foodborne disease outbreaks [3, 131]. As cheese production and consumption is growing in Japan from time to time [45, 101], therefore, it is important to have microbiological standards for domestic natural cheeses based on actual conditions of the country. Having such standards, could help producers to make better quality cheese and to ensure public health safety from foodborne diseases that might arise following improper hygiene.

Apart from the gap in the microbial standard itself, there is a shortage of suitable data to use as baseline information for suggesting the limits of possible microbiological standards for domestic natural cheese. Moreover, there is a lack of the precise surveillance system to monitor the

prevalence of foodborne pathogens in natural cheese. Furthermore, the information on the prevalence of these pathogens in domestic natural cheese is scarce. Therefore, this work was planned to study the hygienic status, microbial quality and prevalence of food-borne pathogens in domestic natural cheese and generate information that can be used as input for further monitoring system development. Hence, this dissertation has three specific objectives. The first one is to assess hygienic status and microbial quality of the domestic natural cheese produced in Hokkaido and the first chapter deals with this study. The second objective was to investigate the prevalence of food-borne pathogens in domestic natural cheese produced in Hokkaido, and this is dealt in the second chapter. The third objective was to characterize virulence factors and antibiotic resistance of the pathogens identified during the screening, and that one is discussed in the third chapter. The information obtained through this work shows the hygienic status of domestic natural cheese. Moreover, it could also serve as a baseline information for further works and provide useful input for the preparation of microbial standard for the natural cheeses produced in Japan.

Chapter I

Assessment of hygienic status and microbial quality of domestic natural cheese produced in Hokkaido, Japan

1. Introduction

Food hygiene includes all conditions and measures necessary to ensure the safety and suitability of food at all stages from production up to consumption [26]. The hygiene assessment is, therefore, essential means used to measure sanitation and wholesomeness of the food products. The evaluation of hygiene is conducted by measuring various indicators such as bacterial counts including coliform, total bacteria and spore formers count among others. Since Cheese is a fermented dairy product, and starter and/or non-starter microbes might also be added to it, its standard plate content could be high. However, the counts of coliform, are expected to be small or absent in the product made in good hygienic condition. In Japan, using pasteurized milk for making dairy products is a requirement [62, 92]. If raw milk is used for cheese making, the product should be processed in equivalent temperature of 63°C for 30 minutes. It is for that reason that Hokkaido's regional accreditation body demand natural cheese to be negative for coliform counts per gram of sample. Since coliforms are highly susceptible to heat treatment, their occurrence in cheese made from pasteurized or heat treated milk could indicate post-pasteurization contamination or failure in the process of heat treatment including pasteurization.

Moreover, total aerobic bacteria, and spore-forming bacteria are important contaminants in the dairy industry that indicate the hygiene of milk and dairy products. Since spore formers are ubiquitous microorganisms, they can be isolated from soil, the dust of the barn, manure, a fecal matter of the dairy animals, feeds and the environmental samples. They contaminate raw milk from either source and, therefore, raw milk is the most common source of product contamination by these group of microorganisms [80]. As spores cannot be destroyed by heat treatment or pasteurization temperature, once they contaminate the raw milk, they may lead to spoilage of the products. *Bacillus* spp. are the most common aerobic spore-forming contaminants in the dairy industry. Among others, *B. licheniformis, B. subtilis,* and *B. cereus* are some of the most frequently identified spore formers prevalent in dairy products [65, 80]. Moreover, reports also showed a prevalence of coliform and foodborne pathogens in natural cheese made from either from raw or pasteurized milk [44, 83, 131]. Therefore, it is important to keep good manufacturing practices in the cheese making

process to prevent product contamination and to ensure the safety of consumers. Indeed, the incidence of foodborne disease related to the consumption of natural cheese is rare in Japan. That could be related to required use of pasteurized milk for cheese making and implementation of sound production hygiene.

Also, consumption of natural cheese is becoming common, and it is increasing year after year in the diets of Japanese people [17, 18, 45, 101]. Though the majority of cheese consumed in the country is imported, there is also a substantial increase in domestic production and consumption while Hokkaido produces the biggest amount (90%) of indigenous cheese products. Despite the high level of production hygiene and superior quality of dairy products enjoyed in the country, little information is available on the hygienic status and microbial quality of domestic natural cheese produced in Hokkaido. Therefore, the purpose of this study was to assess the hygienic status and microbial quality of the natural cheese produced in Hokkaido. Bacterial counts such as coliform count, spore-formers, and standard plate counts were employed to evaluate the hygienic status and microbial quality of the domestic natural cheese. The results showed the commendable hygienic state of the natural cheese produced in Hokkaido and suggested the need to maintain proper hygienic status while striving for further improvement.

2. Materials and methods

Sampling site and sample collection

A total of 200 natural cheese samples made from cows' milk were purchased during December 2012 to January 2014 from local stores, farm dairy shops and annual natural cheese contest events held in Obihiro city. The samples include soft (100), semi-hard (48) and hard (52) types of natural cheeses produced in the farm dairies. Following the purchase, samples were transported to the Food Microbiology and Immunology Laboratory of Obihiro University of Agriculture and Veterinary Medicine in the cold condition using Ice boxes. Upon the delivery to the laboratory, appropriate pieces of information were recorded including cheese type, producing companies, expiry dates, package types used and the storage temperatures of natural cheeses. Then samples were stored at refrigeration temperature of 4ºC and analysis was carried out within the shelf life of the samples.

Standard plate count

A standard plate count (SPC) was conducted following the methods mentioned in Bacteriological Analytical Manual (BAM) of the FDA [91]. I also used the method of Ministry of Health Labor and Welfare (MHLW) in this study. Briefly, 25 g of cheese was weighed aseptically into filter stomacher bag (Filter bag type P, GSI Creos Corporation, Tokyo, Japan). Then 225 ml buffered peptone water (BPW, OXOID, Basingstoke, UK) was added to the bag. Then, the samples were homogenized in the pulsed stomaching mixer (AES Laboratoire, Combourg, France) for 30 sec. twice. Following that, 10 ml of the sample homogenates were taken into sterile test tubes and serially diluted ten times in buffered peptone water until desired level. Next, 1 ml of diluted sample homogenates were pipetted in duplicate to the sterile plates labeled appropriately. Then about 20 ml of molten standard plate count agar (OXOID) was poured followed by mixing gently. The plates were then allowed for few minutes until they set and then they were incubated at 37ºC for 48 hr. Consequently, bacterial counts were conducted using pen type colony counter.

Spore formers count

To count spore formers, 25 g of the samples were aseptically removed from the original samples to filter stomacher bag and homogenized in 225 ml of BPW for 30 sec. twice in pulsed stomaching mixer (AES Laboratoire). Then, 10 ml of the sample homogenates were pipetted and subjected to the heat treatment of 80ºC for 10 minutes in a revolving water bath to inactivate vegetative cells. Then the sample homogenates were kept at room temperature for a while and cooled. Next, 10 ml of the sample homogenates were pipetted and serially diluted ten times in buffered peptone water. Then 1 ml of the diluted samples were pipetted to the appropriately labeled sterile petri-dishes in duplicate. Subsequently about 20 ml of the molten standard plate count agar (OXOID) was pour plated and mixed gently. Then, following the incubation of the plates at 37ºC for 48 hr, colonies were counted using pen type colony counter.

Identification of spore formers

A small portion of distinct colonies from spore former bacteria were picked, streaked on Brain Heart Infusion (BHI) agar plates (BD - Becton, Dickinson, and Company) and incubated overnight at 37ºC. Single colonies of the overnight culture grown on BHI agar plates were then picked and smeared on the 96 well stainless steel target plate (Bruker Daltonik, Germany) using a disposable loop. The samples were then allowed to dry at room temperature and overlaid with 1μL of α-cyano-4-hydroxycinnamic acid. Then, the samples were again allowed to dry at room temperature. Next the plate was subjected to Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-TOF MS) machine (autoflex-04S, Bruker Daltonik). Finally, the profile spectra of mass spectrometry were analyzed using MALDI Biotyper 2.0 software (Bruker Daltonik) according to the reference database.

Coliform count and confirmation

A 25 g of samples were aseptically removed from the original packages and homogenized with 225 ml of buffered peptone water using pulsed stomacher (AES Laboratoire). Then 10 ml of the sample homogenates were pipetted into a sterile test tube followed by ten times serial dilution in buffered peptone water up to the desired level. One milliliter of the diluted samples then pipetted into the sterile petri dish in duplicate. Next, 20 ml of the molten Desoxycholate agar (MERCK, Germany) was poured on plates, mixed gently, and then the plates were allowed to set. Then, about 4 ml of molten Desoxycholate agar was overlaid, and the plates were further allowed to set and incubated at 35ºC for 24 hr. Finally, the colonies were counted using pen type colony counter. Then at least two to five representative colonies were picked and streaked onto Eosin Methylene Blue (EMB) agar plates (Eiken Chemical, Tokyo, Japan) followed by incubation at 37ºC for 24 hr. Colonies with different color appearance such as dark pink or greenish colonies with metallic shine were considered as confirmation for the presence of coliforms [78].

3. Result

SPC

A total of 200 natural cheese samples were inspected for SPC. Among them, 93% of the samples had minimum and maximum bacterial counts of 10 cfu/g and 1.3 x 10^9 cfu/g, respectively. Moreover, most of the samples (75.5%) had counts ranging between 10^5 cfu/g and 10^9 cfu/g (Fig. 1.1.). The result shows the expected limit for fermented foods including natural cheese. Seven percent of the total samples, however, had SPC below the detection limit (<10 cfu/g). Meanwhile, 90% of the 100 soft cheese samples inspected had minimum and maximum SPC of 10 cfu/g and 7.4 x 10^8 cfu/g, respectively. However, 10% of the soft cheese samples had counts below the detection limit. Among the samples with detectable numbers, 14% of them had the results ranging from 10 cfu/g to 6.6 x 10^4 cfu/g. The other 76% of the samples, however, had the SPC values ranging between 1.2 x 10^5 cfu/g and 7.4 x 10^8 cfu/g, respectively. The minimum and maximum SPC for 94% (n=48) of semi-hard types of natural cheese samples were 25 cfu/g and 3.4 x 10^8 cfu/g, respectively. The other 6% of the samples under this category, however, had counts lower than the detection limit. Among the samples with detectable counts, most of them (88%) had counts ranging from 1.6 x 10^5 cfu/g to 3.4 x 10^8 cfu/g. On the other hand, the remaining 6% of the samples had counts 3.1 x 10^4 cfu/g or lower. Among the hard type of natural cheese samples (n= 52), 99.5% of them had the minimum and maximum SPC of 55 cfu/g and 1.3 x 10^9 cfu/g, respectively. On the other hand, the remaining 0.5% of the samples, had counts below the detection limit. Moreover, among the samples with SPC in this category, 17% of the samples had bacterial counts equal to or lower than 9.1 x 10^4 cfu/g. The remaining 82.5% of the samples, however, had counts ranging between 1.0 x 10^5 cfu/g and 1.3 x 10^9 cfu/g, respectively.

Spore formers count and identification

A total of 200 samples were inspected for spore former counts. Among these, 41% of the samples had counts below the detection limit (10 cfu/g) (Fig. 1.2). The overall spore former count

result showed that the minimum and the maximum spore former counts were 10 cfu/g and 5.2 x 10^5 cfu/g, respectively. Among the total samples inspected for spore forming bacteria (n=200), 77.5% of them had counts equal or below 9.1 x 10^2 cfu/g. However, the remaining 22.5% of the samples had counts varying between 1.0 x 10^3 cfu/g and 5.2 x 10^5 cfu/g.

When spore former counts evaluated among the cheese types, 40% of the soft cheeses samples had counts below the detection limit (10 cfu/g). The other 60% of the soft cheese samples had minimum and maximum counts of 10 cfu/g and 5.2 x 10^5 cfu/g, respectively. Among these, 37% of the samples had counts ranging between 10 cfu/g and 5.2 x 10^2 cfu/g. The remaining 23% of the samples, however, had minimum and maximum spore former counts of 1.0 x 10^3 cfu/g and 5.2 x 10^5 cfu/g, respectively. The semi-hard cheese also had 46% of the samples with the spore former counts of lower than the detection limit. On the other hand, the other 54% of the samples in this category had counts varying between 10 cfu/g and 1.8 x 10^5 cfu/g. Among these samples, 29% of them had the counts varying between 10 cfu/g and 80 cfu/g. However, the minimum and maximum counts for the remaining 17% of the samples were 1.2 x 10^2 cfu/g and 1.8 x 10^5 cfu/g, respectively. On the other hand, the hard type of the natural cheese had the spore former counts of lower than the detection limit for 40% samples. The 60% of hard cheese samples, however, had counts varying between 10 cfu/g and 2.6 x 10^5 cfu/g. Among these, 38% of the samples had counts varying between 10 and 9.1 x 10^2 cfu/g. The remaining 22% of the samples, however, had a minimum and maximum spore former counts of 1.2 x 10^3 cfu/g and 2.6 x 10^5 cfu/g, respectively.

Spore former identification was conducted for the isolates from the second sampling as these samples had relatively higher counts than those in the first sampling. We used MALDI-TOF MS method for the identification. Distinct colonies of the suspected spore formers were collected from a total of 15 samples and used for this inspection. Single colonies of the presumptive spore former bacteria from samples with higher counts were used for identification procedures. The result showed that predominant spore-former bacteria identified in this study were *Bacillus licheniformis* and *Paenibacillus pubuli* with the score values varying between 1.71 and 1.99 that indicate the genus level identification of the bacteria.

Coliform count and confirmation

Among a total of 200 samples inspected, 78% of them had coliform counts below the detection limit (Fig.1.3). However, 22% of the samples had coliform counts varying between 10 cfu/g and 6.3 x 10^7 cfu/g. Among these, 8% of them had counts equal to or more than 1.1 x 10^5 cfu/g. Two samples among these had the highest counts of 1.5 x 10^7 cfu/g and 6.3 x 10^7 cfu/g. The remaining 14% of the samples, however, had the counts of coliform below 10^5 cfu/g.

When evaluated based on the different cheese type, 71% of the 100 soft cheese samples had counts below the detection limit. The result of 29% samples, however, showed counts ranging between 10 cfu/g and 1.5 x 10^7 cfu/g. Among these, 13% of the samples had counts varying between 1.1 x 10^5 cfu/g and 1.5 x 10^7 cfu/g. Another 16% of these samples had counts ranging from 10 cfu/g up to 2.9 x 10^4 cfu/g. From a total of 48 semi-hard natural cheese samples, 83% of them had counts below the detection limit. The remaining 17% of them, however, had counts varying between 75 cfu/g and 6.3 x 10^7 cfu/g. Among these 6% of the samples have counts varying between 3.0 x 10^5 and 6.3 x 10^7 cfu/g. However, 11% of the semi-hard cheese samples had counts of 6.6 x 10^4 cfu/g or lower. Similarly from the 52 samples of the hard type of natural cheese, 87% of them had the counts below the detection limit. On the other hand, the remaining 13% samples had counts ranging between 10 cfu/g and 4.6 x 10^4 cfu/g.

Following the growth of coliform on Desoxycholate agar, at least five representative colonies were picked and streaked on EMB agar. The growth of dark pink or greenish colonies with metallic shine were considered as confirmation of coliform contamination.

Coliform positivity and package type

A total of 200 natural cheese samples were evaluated for their package types and coliform positivity. The package types include vacuum-sealed plastic packages (122 samples) and primary paper wrapping with a different combination of secondary packages (22 samples). Moreover, samples with a primarily plastic film wrapping with various combinations of secondary packages

(25 samples) as well as those with the package type of paper wrapping alone that involve 20 samples were also evaluated. The remaining 11 samples were either packed in combination with aluminum foil or plastic can and can only and all the samples were investigated for their condition of coliform contamination (Table 1.2).

Amongst all the package types, the samples packed with paper wrapping alone had the highest proportion (55%) of coliform positivity. The natural cheese samples that had primary paper wrapping with various combination of secondary packages also had 32% of coliform-positive samples. Moreover, though they are in small number, the samples packed in primary aluminum foil wrapping followed by different secondary packages had 29% of the coliform-positive samples of their group. On the other hand, only 16% of the 122 plastic vacuum sealed samples had while 21% of the plastic film wrapped samples also contain coliform. However, no coliform-positive samples were found among those packed in the plastic containers and can (Table 1.2).

4. Discussion

The purpose of this study was to assess the hygienic status and microbial quality of domestic natural cheese produced in Hokkaido. The coliform count is considered as a principal indicator of the dairy production hygiene and standard plate count, and spore-former counts were also used as important components of the hygiene indicators. The results showed that 78% of the 200 samples were negative for coliform counts indicating the higher level of hygienic status of the domestic natural cheese. Moreover, although 22% of the samples (n=200) were positive for coliform counts 14% of them had counts lower than 10^5 cfu/g. However, the remaining 8% of the samples had minimum and maximum counts of 1.0 x 10^5 cfu/g and 6.3 x 10^7 cfu/g, respectively. According to the European Commission standards, the natural cheese with coliform counts of over 10^5 cfu/g has unsatisfactory quality [32, 35]. However, 92% of the domestic natural cheese samples assessed in this study qualify the good quality based on the European standard. Different studies also showed that some of the samples positive to coliform had unsatisfactory quality, and the highest counts among those reach up to 10^7 cfu/g [71, 131]. In Japan, milk used for cheese making is required to be pasteurized [62, 92]. In the case of using raw milk, the product should be processed at the equivalent temperature of 63ºC for 30 min. Thus, as coliforms are highly susceptible to heat treatment, their occurrence in natural cheese made from pasteurized milk is not expected. The presence of coliform in 22% of the samples with a higher number of counts in some specimens (8%), indicates the possibility of contamination after cheese making. Since coliforms are indicators of production hygiene, their occurrence in the high number shows the presence of either low sanitation, fecal contamination or improper storage after cheese making [71].

Moreover, the high coliform count may also suggest the possibility of product contamination with other enteric pathogens that can affect the health of consumers. Among others, enteric pathogens such as *Salmonella* and pathogenic *E. coli* could transmit through fecal contamination of foods. Thus, the occurrence of coliform in a high number could suggest the possibility of the cheese contamination with these or other enteric pathogens. Therefore, different countries set standards to monitor the level of coliform in foods including cheese though the

acceptable limits may vary depending on the regulations of each country. Among these, the US Ordinance for grade "A" milk, requires that coliform should not exceed 10 cfu/ml in the sample of the grade "A" milk or its products [39]. Similarly, the guideline of the Hokkaido Regional Accreditation Body requires natural cheese should be negative to coliform count per gram of sample. On the other hand, the standard of EC for milk and dairy products requires that coliform count in the natural cheese should not exceed 10^5 cfu/g [32]. Based on these regulations, 78% of the samples in this study meet the requirement of Hokkaido regional accreditation body and US standard for grade "A" milk. Moreover, 92% of the samples also qualify the requirements of European standard suggesting that the domestic natural cheese produced in Hokkaido has fine hygienic status. However, the further effort might be needed to produce the natural cheese with more hygiene and ensure the safety of the consumers.

Among the cheese types, the soft cheese had the highest coliform positivity (29%) followed by semi-hard (17%) and hard (13%) types of natural cheeses. Amongst the factors, high moisture content and relatively low acidity might have favored the growth of coliform in soft cheese than in semi-hard or hard type of the natural cheeses. Earlier studies reported that maturation of raw milk cheese results in the reduction of coliform counts. Such decrease was thought to happen due to the decline of moisture contents and increased activity of natural flora of lactic acid bacteria [19, 76, 100, 131]. Moreover, package type might have played an important role in the prevention of contamination. The result of this study showed that samples wrapped with the paper had the highest coliform positivity (55%) than others (Table 1.2). Moreover, the majority of these samples were the soft type of natural cheeses. Next to these, primary paper wrapped samples with other secondary packages had higher coliform-positive samples (32%) than the samples with other packages. These results show that samples with paper wrapped packages are more susceptible to coliform contamination than samples with other package types. Microbiological quality of natural cheese can be influenced by several factors including equipment and environmental hygiene during production. Moreover, the use of good quality milk, proper packaging and handling, as well as appropriate storage conditions, are essential components to maintaining good hygienic quality of the natural cheese. This study, however, didn't determine, whether the highest coliform prevalence in paper-

wrapped cheese was due to pre-packaging or post-packaging contamination. However, the results clearly indicate that appropriate packaging such as plastic packaging and the combination of primary and secondary packaging could reduce the risk of coliform contamination. Therefore, choosing an appropriate packaging suitable for the product quality is a simple and practical measure that producers could follow since proper package could help to reduce the risk of unnecessary contamination and ensure the food safety.

Spore former bacteria are ubiquitous microorganisms and important in the dairy food hygiene. Once the spores get access to milk, they can endure pasteurization temperature and may cause spoilage on the processed product [40, 65]. Spore former bacteria could contaminate dairy products from various sources including water, cheese clothes, knives, packing materials and others. Moreover, raw milk as raw material is thought be the primary source of dairy product contamination with spore formers [120]. Spore former could cause not only spoilage of milk products but also some of them such as *Bacillus cereus* could cause food poisoning, and they are a public health concern. Among the total of 200 samples inspected in the present study, 59% of them were positive to the spore former counts. However, most of the samples (77.5%, n=200) had counts equal to or lower than 9.1 x 10^2 cfu/g. The counts for the remaining 22.5% of the samples ranged between 1.0 x 10^3 and 5.2 x 10^5 cfu/g. The result also shows that *Bacillus licheniformis* and *Paenibacillus pubuli* were the dominant spore-formers identified using MALDI-TOF MS technique. *Bacillus licheniformis* is commonly isolated from raw milk samples and considered as harmless [94, 119, 125]. On the other hand, some reports show that *Bacillus licheniformis* can cause delayed fermentation in yogurt making [125]; produce heat-stable toxins and cytotoxic activity to cells [82, 94, 114]. Moreover, it was also reported that *Bacillus licheniformis* may cause mastitis in dairy animals [99]. Furthermore, *Paenibacillus* spp. is also psychrotolerant spore forming bacteria that also has the spores with thermotolerant properties [110]. They are widely distributed in soil and farm environment and can be isolated from raw milk as well as pasteurized milk samples and cause spoilage of processed dairy products [27, 110, 118]. They emerged as a challenge to the dairy industry and responsible for the considerable spoilage of pasteurized milk and related products. In this study, we did not conduct the experiment on toxin production by the spore former isolates.

However, reports indicate the possible risks due to the contamination of these bacteria that may result not only in product spoilage but also in food poisoning incidences [82, 94, 114]. Due to its acidity and occurrence of competitive microflora such as lactic acid bacteria, *B. cereus* may not survive in relatively inhospitable cheese environment [113] and the threat spore formers pose due to cheese consumption could be minimal. However, the study also indicated that despite its numbers decreased, the vegetative cells of *B. cereus* survived cheese ripening period suggesting potential threat [97]. Therefore, it is important to devise a mechanism to prevent or reduce contamination of the product by *Bacillus* spp. to ensure the safety of the product and to avoid possible food poisoning incidences.

SPC shows the growth of all viable aerobic mesophilic bacteria that can grow on plate count agar. Since natural cheese is a fermented dairy product, it can have high SPC. However, the occurrence of both SPC and coliform counts in large number could indicate poor microbial quality and low hygienic status of a given food product. In this study, 8% of the samples had high coliform counts that fall under unsatisfactory quality based on European Standard [32]. These samples also had high SPC where the highest being 7.4×10^8 cfu/g. The rate of high bacterial counts for both coliform and SPC may indicate the possibility of failure in either the pasteurization or heating process during cheese making. Moreover, inappropriate storage of the product after processing could favor the growth of contaminants and increase the number of these bacteria rendering the cheese to lose its quality.

In conclusion, most of the samples (78%) inspected in this study were negative to coliform count. Moreover, the SPC and spore formers' counts are mostly in the acceptable range showing that the hygienic status and microbial quality of natural cheese produced in Hokkaido is in fine condition. However, the occurrence of coliform in 22% of the samples indicates suboptimal hygienic status based on the Ministerial ordinance and requirements of Hokkaido Regional Accreditation Body. The result suggests the importance for the further improvement of the existing good production hygiene to supply wholesome product and ensure the safety of consumers.

Summary

Food hygiene includes the application of all necessary measures to ensure safety and suitability of food for public consumption. Therefore, hygiene is monitored by different ways and coliform count is important means used in the dairy industry. Though they are not pathogenic, coliform indicate the hygiene of food and may also suggest the possibility of other enteric pathogen contamination based on their level. In Japan, using pasteurized milk for cheese making is a prerequisite as it helps to eliminate pathogens and contaminants including coliform. However, failure in the pasteurization process or post-processing contamination may render cheese for inferior hygienic quality and pose a concern for the safety of the product. To assess the hygienic status and microbial quality of domestic natural cheese, a total of 200 natural cheese samples collected and analyzed from different parts of Hokkaido. Standard plate count, coliform count, and spore formers' counts were conducted following standard methods. Matrix-assisted laser desorption ionization time of flight mass spectrometer method was also employed to identify spore formers. The result showed that the SPC in 93% samples varied between 10 cfu/g and 1.3×10^9 cfu/g where overall counts are within the expected limit for the fermented dairy products. Similarly, 59% of the samples had spore former counts varying between 10 cfu/g and 5.2×10^5 cfu/g while the remaining 41% had counts below the detection limit. Among the spore former isolates, MALDI-TOF MS result showed that *Bacillus licheniformis* and *Paenibacillus pubuli* were predominant. The coliform count also revealed that 78% of the samples were negative to the coliform count indicating the good hygienic quality of the domestic natural cheese. However, 22% of the samples had coliform counts varying between 10 cfu/g and 6.3×10^7 cfu/g. Since it is a pre-requisite to use pasteurized milk for cheese making in Japan and as coliform is highly susceptible to heat treatment, their occurrence in cheese is not expected. Thus, the result suggests possible contamination of the product after cheese making due to faulty handling or failure in the heating process. Finally, the overall hygienic condition of the domestic natural cheese is in its fine status. However, more effort might be needed to produce a natural cheese with greater hygiene that complies Ministerial Ordinance and the requirements of Hokkaido Regional Accreditation Body and to ensure the safety of consumers.

Table1.1. The number of natural cheese samples used in this study.

Location[a]	Number of samples	Cheese type		
		Soft	Semi-hard	Hard
Central	31	17	8	6
North	8	4	0	4
East	161	79	40	42
Total	200	100	48	52

[a] Locations where natural cheese samples produced in different parts of Hokkaido.

Table 1.2. Coliform positivity of natural cheeses in different package types.

Cheese type	Proportion of coliform-positive samples (%)							Total (%)
	AF+[a]	Pa[b]	Pa+[c]	PVS[d]	PFW[e]	PC[f]	Can	
Soft	1/5	9/14	7/22	10/40	2/9	0/9	0/1	29/100 (29)
Semi-hard	1/2	1/3	0	6/38	0/5	0	0	8/48 (17)
Hard	0	1/3	0	3/39	3/10	0	0	7/52 (13)
Total	2/7 (29)	11/20 (55)	7/22 (32)	19/117 (16)	5/24 (21)	0/9 (0)	0/1 (0)	44/200 (22)

[a] Primary packaging: aluminum foil wrap, secondary: none, plastic bag or wooden box.

[b] Paper wrapping only.

[c] Primary packaging: paper, secondary: carbon box, wooden box or plastic bag.

[d] Plastic vacuum seal.

[e] Plastic film wrapping.

[f] Plastic container.

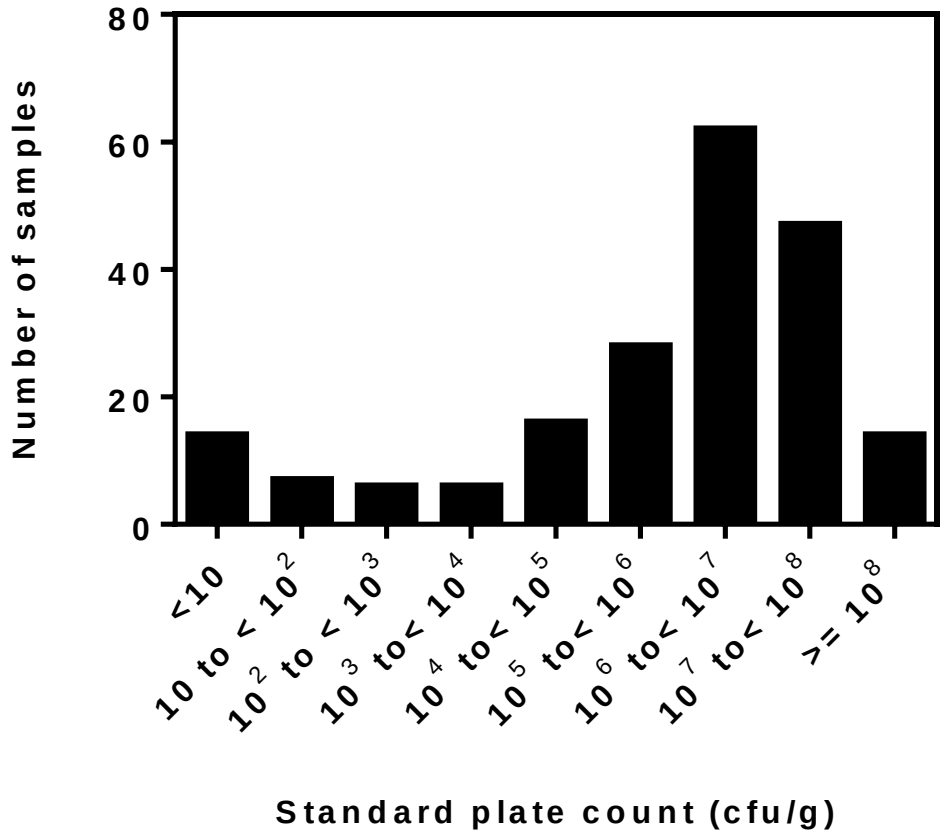


Fig. 1.1. SPC result. A total of 200 samples inspected for SPC.

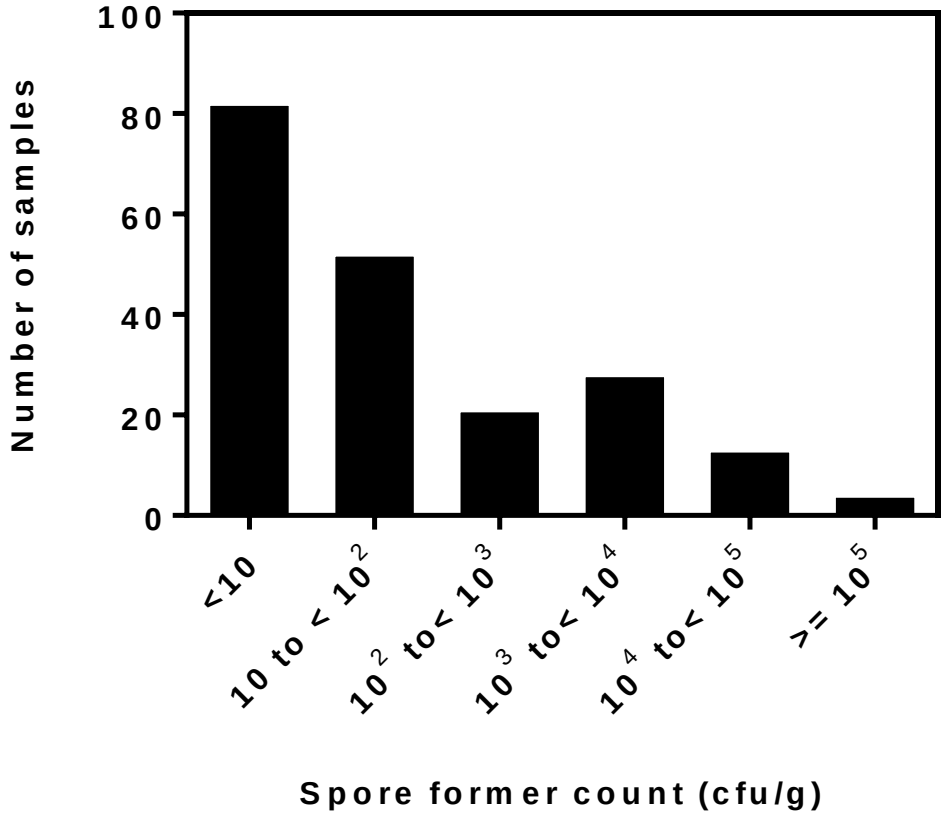


Fig 1.2. Spore former count result. A total of 200 samples inspected for spore formers counts.

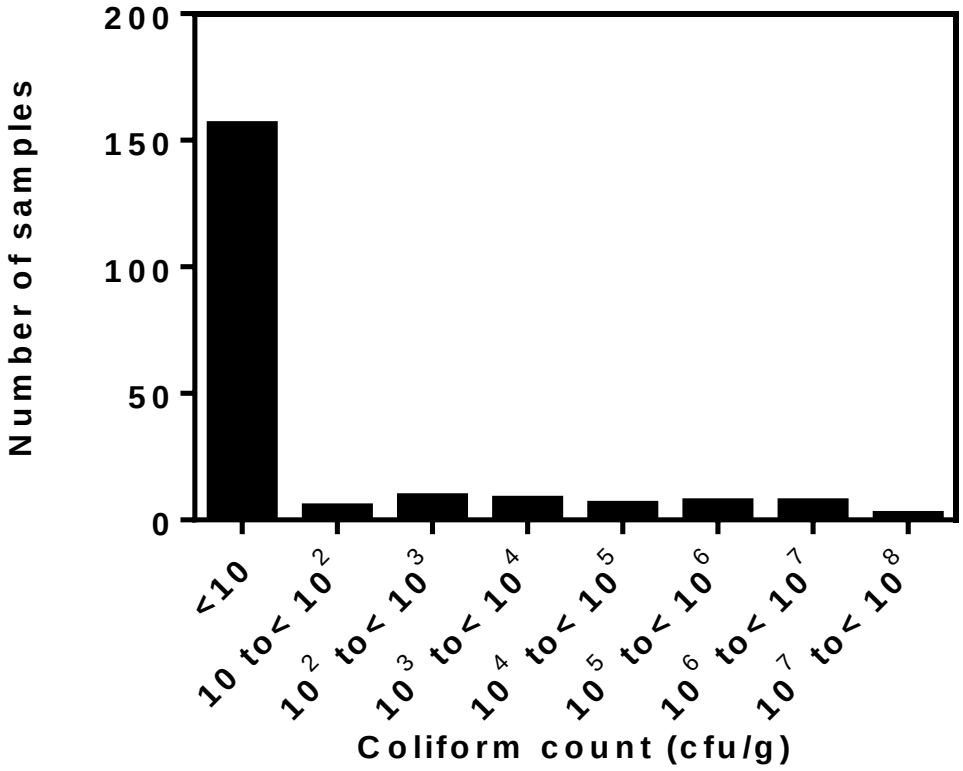


Fig. 1.3. Coliform count result. A total of 200 samples inspected for coliform counts.

Chapter II

Prevalence of Foodborne Pathogens in Domestic Natural Cheese Produced in Hokkaido, Japan

1. Introduction

Natural Cheese is a fermented dairy product made by curdling of milk from dairy animals with rennet or action of lactic acid bacteria. It is produced at various levels such as at household level, small-scale farm dairies and big cheese-making industries. Farm Dairies produce natural cheese using either pasteurized milk, sub pasteurization temperature treated milk or raw milk. Cheese contamination could occur when raw milk is used for cheese making. Once the contaminants or pathogens get access to the cheese, they may survive, proliferate, and cause a health hazard to the consumers. However, pasteurization eliminates pathogenic organisms from the milk and reduces the potential risk of foodborne illnesses due to the contaminated products. Moreover, aging of raw milk cheese for 60 days at the temperature of not less than 1.7ºC is also acceptable practice in several countries to eliminate foodborne pathogens [13, 32, 44].

Foodborne disease related to the consumption of natural cheese is not frequent in Japan. Among others, implementation of the good hygienic practice in the production process and a low level of cheese consumption as compared to other developed countries might have contributed to the absence of such incidences. However, production and consumption of natural cheese is increasing in Japan from time to time [17, 45, 101]. This increase might also contribute to the increase in sporadic outbreaks related to the consumption of natural cheese. Moreover, natural cheese related foodborne outbreak caused by *Listeria monocytogenes* was reported in Japan recently despite the implementation of good hygienic practices in production setups [90]. On the other hand, the prevalence of cheese related foodborne outbreaks are more frequently reported in different countries of the world [1, 22, 34, 54, 88, 104, 126]. Among others, *L. monocytogenes,* pathogenic *E. coli, Salmonella* spp., and *S. aureus* are more commonly detected pathogens from the outbreaks caused by consumption of contaminated cheeses [13, 76, 131].

L. monocytogenes is a ubiquitous foodborne pathogen. Reports show that outbreaks and sporadic cases of listeriosis occurred as a result of consuming milk or dairy products in different countries including US and Europe in the past decades [13, 76, 122]. Previous domestic studies in

Japan conducted from 1992 to 1994 reported the incidence of *L. monocytogenes* contamination in raw milk [133]. However, a foodborne listeriosis outbreak was reported in 2005 that was occurred in 2001 due to consumption of contaminated natural cheese [90]. Besides *L. monocytogenes*, pathogenic *E. coli* also causes concern for public health due to the consumption of contaminated cheese. Among pathogenic *E. coli*, Shiga toxin-producing *E. coli* (STEC) is the most important pathogen. It causes disease outbreaks that are characterized by enteric symptoms and diarrhea that could result in hemorrhagic colitis (HC) and lethal HUS [28, 37]. *Salmonella* are also another important foodborne pathogens that cause major illnesses and death worldwide [89, 117] and it causes an estimated 39085 illnesses per year in Japan alone [96]. However, information on its association with domestic natural cheese is scarce. Staphylococcal food poisoning related to the consumption of dairy products was reported in Japan previously [5]. However, only a limited information is available related to the prevalence of *S. aureus* in domestic natural cheese and the potential threat it poses. Therefore, this study was conducted to investigate the prevalence of *Salmonella* spp., pathogenic *E. coli*, *L. monocytogenes*, and *S. aureus* in domestic natural cheese produced in Hokkaido.

2. Materials and methods

Sample collection

A total of 200 natural cheese samples made from cows' milk were randomly purchased from different stores in Hokkaido during December 2012 to January 2014. The samples include soft (n=100), semi-hard (n=48) and hard type (n=52) of natural cheeses packed with various packages. Following the purchase, samples were kept in the ice box and transported to Food Microbiology and Immunology Laboratory of the Obihiro University of Agriculture and Veterinary Medicine. Upon delivery in the lab, cheese type, producing companies, and expiry dates were recorded. Then the samples were stored in the refrigerator (4ºC) until inspected within their shelf lives.

Sample preparation

The sample homogenates were prepared by aseptically removing 25 g from the original packages into sterile filter-stomaching bags (Filter bag type P). A 225 ml of appropriate pre-enrichment media were also added to the stomaching bags containing the samples. The samples were then homogeneously mixed in a pulsed stomacher (AES Laboratoire) twice for 30 sec. The sample homogenates were then subjected to the inspection of *L. monocytogenes*, *Salmonella*, *S. aureus* and pathogenic *E. coli* following respective standard protocols.

Inspection of *Listeria monocytogenes*

Detection of *L. monocytogenes* was conducted as described in International Organization for Standardization [43]. Briefly, 25 g of samples were pre-enriched in 225 ml of half Frazer broth (OXOID) and incubated for 24 hr at 30ºC. From the pre-enriched sample, 0.1 ml of culture was enriched in 10 ml of Fraser broth and incubated at 35ºC for 48 h. Then, a loop full of the cultures were streaked on PALCAM agar (PALCAM agar base, OXOID) and incubated at 37ºC. The agar plates were subsequently examined for bacterial growth after 24 and 48 hr of incubation. From

plates showing bacterial growth, five typical colonies (gray green colonies with black precipitate) were picked up and streaked on BHI agar (BBL™ Brain Heart Infusion). Then the plates were incubated at 37ºC for 24 h. Then, further identification was conducted using MALDI-TOF MS method.

For identification of presumptive *L. monocytogenes* isolates, MALDI-TOF MS analysis was carried out following appropriate directions. Briefly, bacterial cells of a single colony grown on BHI agar plates were transferred in duplicate to a stainless steel target plate of 96 well (Bruker Daltonik GmbH, Germany) using a disposable loop. Bruker bacterial test standard (BTS) (1 µl) was transferred to the target MALDI plate and used to calibrate the spectrometer following the direction of the manufacturer. The targets with sample and BTS were then overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA). The overlaid sample on the target plate was then allowed to dry at room temperature. The plate was then subjected to MALDI-TOF Mass Spectrometer machine (BRUKER DALTONICS®, autoflex®-04S). Then the obtained profile spectra were analyzed using MALDI Biotyper 2.0 software, according to the reference database.

Inspection of *Salmonella* spp.

Isolation and detection of *Salmonella* spp. were carried out following the procedures indicated in FDA's Bacteriological Analytical Manual Online. Briefly, 25 g of samples were pre-enriched in 225 ml of buffered peptone water (MERCK) and incubated at 35ºC for 18 hr. Next, 0.1 ml of each sample homogenate was enriched into 10 ml of Rappaport-Vassiliadis (RV) broth (OXOID). Then the enriched culture was incubated at 42ºC for 18 hr. Next a loop full of RV culture was then streaked on Deoxycholate Hydrogen sulfide Lactose (DHL) agar (DHL; Eiken Chemical, Tokyo, Japan) and CHROMagar™ *Salmonella* and incubated at 37ºC for 24 hr. Five presumptive colonies were collected and streaked on BHI agar for further identification using MALDI-TOF MS method as indicated in the earlier part. For the detection of *Shigella* spp., *Shigella* broth base (OXOID), and SS agar (OXOID) were used following the directions of the manufacturers.

Inspection of pathogenic *Escherichia coli*

The pathotypes of *E. coli* assessed is this study include STEC, EIEC, ETEC, and EAEC. The screening was performed by real-time PCR based on their associated genetic markers. Briefly 25 g of samples were pre-enriched in 225 ml of mEC broth with novobiocin (MERCK). After 24 hr incubation at 37ºC, DNA was extracted from 2 ml of pre-enriched broth. For the DNA extraction, PrepMan® Ultra Sample Preparation Kit (Applied Biosystems, Foster City, CA, USA) was used. Real-Time PCR analysis was performed using QuickPrimer kit (*stx1, stx2, ipaH, LT, EASTI,* and *STI*) (Takara Bio, Shiga, Japan).

Inspection of *Staphylococcus aureus*

A 25 g of original samples were aseptically removed and added to sterile stomacher bag (Filter bag type P) followed by the addition of 225 ml of peptone water. The samples were then homogenized in a stomacher (AES Laboratoire) by agitating for 30 sec. twice. Then 10 ml of the sample homogenates were pipetted into the sterile test tube, and ten times serial dilution was conducted in sterile peptone water. From this, 0.1 ml of the sample homogenates were pipetted into Mannitol Salt Agar with Egg Yolk in duplicate and spread plated. After 48 hr incubation at 35ºC, the creamy yellowish colonies turning the agar media into yellow were counted as suspected *S. aureus* colonies. From these five representative colonies were picked and streaked on Nutrient agar (Eiken Chemical) and incubated at 35ºC for 24 hr. Then few colonies were picked and mixed with 0.5 ml of plasma and incubated for three hours at 37ºC to check for coagulation. Any coagulation was considered as a positive indication for coagulase-positive *S. aureus.* The test tubes were further incubated at room temperature for 24 hr and checked for the occurrence of coagulase activity [69].

3. Result

Listeria monocytogenes

A total of 126 samples were screened for the detection of *L monocytogenes* following the standard method of ISO 11290 – 1. Despite the occurrence of suspected colonies to be *Listeria* spp. from eight samples, the result shows that none of the samples inspected were positive for the pathogen (Table 2.1). Moreover, the analysis of MALDI-TOF MS for the suspected samples showed that the isolates of those suspected colonies were neither *L monocytogenes* nor other *Listeria* spp. However, the isolates were identified as either *Staphylococcus* spp. or *Bacillus* spp. Among them, *S. xylosus*, *B. licheniformis*, and *B. pumilus* were the most commonly identified bacteria.

***Salmonella* spp.**

Inspection for *Salmonella* spp. was conducted on 126 samples of natural cheese following standard methods of FDA described in BAM. None of the samples found positive for this pathogen. However, presumptive colonies grew on some plates of chromogenic agar that had a similar morphology to *Salmonella* spp. Hence, further investigation was carried out using MALDI-TOF MS method and the result showed that all of the samples were negative for *Salmonella* spp. (Table 2.1). The MALDI-TOF MS result for these isolates showed that the detected colonies as *Serratia marcescens*, *Enterobacter asbureae*, *Hafnia alvei*, *Klebsiella pneumonia* ssp. *pneumonia* or *Pseudomonas stutzeri*.

Pathogenic *Escherichia coli*

A total of 120 samples were inspected for pathogenic *E. coli* including STEC, EIEC, and ETEC. However, 79 samples were inspected for the detection of EAEC. Following the enrichment of the sample in mEC broth with novobiocin, DNA extraction was conducted, and Real-Time PCR analysis was done as aforementioned. The result indicated that none of the samples contain

pathogenic *E. coli* (Table 2.2). However, one of the samples was found to be positive for *ipaH* gene suggesting the possible contamination with EIEC or *Shigella* spp. Thus, attempts were made to recover the bacteria from the sample using the culture method on specific media for *Shigella*, but neither of these bacteria was detected.

Staphylococcus aureus

A total of 74 samples were analyzed for the detection of *S. aureus*. The analysis was made using culture method on Mannitol salt agar with egg yolk (Eiken Chemicals), and 50% of the samples showed no growth of colonies. Another 50% of the samples, however, had the growth of *Staphylococcus* spp. with the lowest and highest counts of 50 cfu/g and 6.3×10^8 cfu/g, respectively. Also, 76% of the samples including the negative ones had *Staphylococcus* spp. counts of lower than 10^5 cfu/g while the counts of the remaining 24% exceed 10^5 cfu/g. Among those with higher *Staphylococcus* spp. growth, 14 samples (19%) had counts varying between 1.08×10^5 and 4.75×10^6 cfu/g while another 4 samples (5%) had the counts varying between 4.8×10^7 cfu/g and 6.3×10^8 cfu/g (Table 2.3). Moreover, among these, three samples (4%) were identified to contain coagulase positive staphylococci, which are considered as *S. aureus*. The *S. aureus* counts of these three samples were 2.7×10^4 cfu/g, 5.95×10^4 cfu/g and 7.5×10^4 cfu/g respectively. Among these samples, two of them were collected from the same producer but they were different batches of the products.

The isolates screened were capable of fermenting mannitol and, as a result, the color of the media changes to yellow. Moreover, they showed egg yolk activity, and they were capable of growing at high salt concentration. Furthermore, they are coagulase positive and gram-positive cocci in very similar appearance to the *S. aureus* strain used as a positive control. Therefore, the isolates were considered as *S. aureus* isolates and kept for further inspection.

4. Discussion

This study investigated the prevalence of foodborne pathogens in domestic natural cheese produced in Hokkaido. Standard methods of FDA and MHLWF with MALDI-TOF MS and real-time PCR procedures were employed for the detection and identification of the four principal foodborne pathogens. These include *L. monocytogenes, Salmonella* spp., pathogenic *E. coli*, and *S. aureus*. These bacteria are the most commonly reported pathogens in recent cheese related foodborne outbreaks [1, 22, 34, 54, 88, 104, 126]. In Japan, foodborne outbreaks related to the consumption of natural cheese were not reported until the one that occurred in 2001 in Hokkaido and caused by *L. monocytogenes* [90]. That was the only outbreak of foodborne listeriosis in Japan so far. There is, however, concern about the safety of the domestic natural cheese while information on the prevalence of these pathogens is limited. The results of this study, however, shows that *L. monocytogenes* and *Salmonella* were not detected in 126 samples inspected while coagulase-positive *S. aureus* was detected in 3 out of the 74 samples. Moreover, only one sample had *ipaH* gene suggesting possible contamination of the sample by either EIEC or *Shigella* spp. However, none of these pathogens was recovered using culture based technique where the result shows that neither of these pathogens is prevalent in the natural cheese sample. The results demonstrate that the foodborne pathogenic bacteria associated with cheese related outbreaks are not prevalent in domestic natural cheese indicating good microbiological quality and safety of the products.

L. monocytogenes is characterized by its high fatality rate in risk groups. Therefore, strict rules are followed to prevent it in the food samples that demand either absence or below 100 cfu/g [32, 59, 77]. The result of the present study shows that none of the 126 samples analyzed were positive for *L. monocytogenes* indicating domestic natural cheese qualifies the requirements of the MHLW for this pathogen. Since using pasteurized milk for cheese making is a prerequisite in Japan [62, 92], the absence of the pathogen in the product is expected. Among others, implementation of good manufacturing practices at all stages of the process, might have contributed to the absence of pathogens in domestic natural cheese. The result indicates proper level of safety and hygienic status of domestic natural cheese produced in Hokkaido. The guideline of the Ministry of Health, Labor

and Welfare and Hokkaido's Regional Accreditation Body require the absence of *L. monocytogenes* in natural cheese. Similar results were also reported from other countries. A recent report from US showed the absence of *L. monocytogenes* in a nationwide survey of that inspected 41 raw milk cheese samples [13]. Similarly, *L. monocytogenes* was not detected in 50 raw milk cheese samples inspected in Italy [44]. Another report [58] also showed that *L. monocytogenes* were not detected in caprine milk cheese while 1.4% of the cheese from bovine milk was positive for the pathogen in Norway. Other researchers [83] also reported that 2% of the samples analyzed for national cheese surveillance had *L. monocytogenes* in the greater amount than European standard while 98% of the samples complies the standard. The survey conducted on Scottish artisanal cheese, however, showed that 86% of the samples were positive for one or more of the foodborne pathogens including *L. monocytogenes* among 28 samples inspected [131].

Salmonella is one of the leading causes of foodborne diseases worldwide. It results in 1.4 million illnesses and 400 deaths in the US per year while 39085 incidences were reported to occur because of this pathogen annually in Japan [96, 129]. Although most of its incidences are related to poultry products and meats, illnesses of *Salmonella* spp. also were associated with consumption of contaminated dairy products. Among these, several sporadic outbreaks related to the consumption of cheese were reported in different countries [1, 22, 34, 104, 126]. In the present survey, however, *Salmonella* was not detected in 126 domestic natural cheese samples inspected. The absence of *Salmonella* indicates that the domestic natural cheese produced in Hokkaido is safe, and it has good hygienic as well as microbiological safety status. Similar results were reported by various researchers from different countries [13, 44, 83]. Use of pasteurized milk for cheese making, the presence of good manufacturing practice and implementation of proper production hygiene could be some of the possible reasons for the absence of this pathogen in domestic natural cheese.

In the meantime, pathogenic *E. coli* including STEC, EIEC, EPEC, and ETEC were not found in 120 samples inspected. However, one of the samples (0.8%) were found to be positive for the *ipaH* gene suggesting possible contamination at some point in the production process. Since the *ipaH* gene is a multi-copy that exist in either EIEC or *Shigella* spp., [7], the occurrence this gene in the natural cheese indicates the incidence of contamination by either of these pathogens. The

occurrence of EIEC or *Shigella spp.* in natural cheese is risky for the health of consumers. Due to the high invasiveness and a small infective dose of these pathogens, their presence in natural cheese could lead to the health risk of consumers. However, the culture method could not recover any of these pathogens. Therefore, the result suggests that none of these pathogens are prevalent in domestic natural cheese produced in Hokkaido. An earlier report showed outbreak of *S. sonnei* that affected over 200 people in Spain and it was related to regionally manufactured fresh pasteurized milk cheese [42]. Epidemiological investigation of that study suggested that an infected employee at the cheese factory might have been the source of contamination.

On the other hand, 50% of the 74 natural cheese samples inspected were positive for *Staphylococcus* spp. Among the total samples, 76% of them had *Staphylococcus* spp. counts of lower than 10^5 cfu/g. Therefore, these samples qualify the right microbial quality level based on European Recommendation 2005/2073/EC on microbiological criteria for raw and thermized milk cheese [44]. From the remaining 24% of the samples, 19% of them could be classified as poor quality while the other 5% are categorized as bad quality based on the same European criteria for raw milk cheese. Even though the foodborne disease incidence due to *Staphylococcus* spp. other than *S. aureus* is uncommon, studies show the properties of enterotoxin production by coagulase-negative *Staphylococcus* spp. [41, 127]. Moreover, another study also indicated foodborne disease outbreak incidence related to the coagulase negative *Staphylococcus* spp. and prevention of these organisms is highly important [20]. As they are part of the normal flora of the humans, mostly the incidence of *Staphylococcus* spp. in the cheese is believed to come from improper handling after cheese making. Therefore, avoiding direct contact with the product and using hygienic measures can help to prevent contamination.

Moreover, coagulase-positive *S. aureus* was detected in 4% of the samples. The counts of *S. aureus* in these three samples were 2.7×10^4 cfu/g, 5.95×10^4 cfu/g, and 7.5×10^4 cfu/g. These samples were categorized under unsatisfactory quality based on a European standard for pasteurized milk cheese that requires 10^4 cfu/g or lower for acceptable quality [32, 35]. Giammanco and colleagues also reported that 50% of the 50 raw milk cheese samples were positive for *Staphylococcus* spp. and 6% of them had coagulase-positive *S. aureus* with a higher amount than

European standard limits [44]. Earlier study conducted in Hokkaido also showed that 38 out of 535 natural cheese samples (7%) were positive for *S. aureus.* Among them, some of the isolates harbor enterotoxin genes of *seg* and *sei* [56]. In another study conducted by Brooks and colleagues, only 3 out of the 41 samples (7.3%) were positive for *S. aureus.* One of these samples also had counts higher than the requirements of European standard [13]. Moreover, another study [83] reported that 2% of the natural cheese samples from raw and pasteurized milk had *S. aureus* in a higher amount than the requirements of the European standard. The result of the present study also shows the large number of positive samples for *Staphylococcus* spp. indicating the occurrence of contamination possibly due to mishandling. Moreover, a high count of *S. aureus* in 4% of the samples also indicates the presence of potential risk to the health of consumers and the need for improving production hygiene.

Different studies conducted on the four pathogens in natural cheeses showed varying results from place to place. Among these, a study carried out in the US described that none of the pathogens mentioned earlier were prevalent in 41 samples [13]. In another survey conducted in the UK, *Salmonella* was not detected from 1819 raw or thermized milk cheese and 2618 pasteurized milk cheese samples. However, that study reported that 2% of the samples had unsatisfactory quality because of the presence of *S. aureus*, *E. coli*, and *L. monocytogenes* in a greater amount than the European recommendation [83]. A study conducted in Italy also showed that no *L. monocytogenes* were detected in 50 samples inspected. However *S. aureus*, *E. coli*, *Staphylococcaceae*, and *Enterobacteriaceae* were detected in 6%, 44%, 42% and 50% of the samples respectively. The results were at a higher level than European standard, and the quality of that particular cheese was unsatisfactory [44]. In Spain, a study reported that 2.4% of the raw milk cheese samples were positive for STEC [21]. Though cheese related foodborne disease outbreaks attributed to the pathogens mentioned above occur on a sporadic basis in other countries, it is not common in Japan. Moreover, the result of the present study also shows the absence of these foodborne pathogens in domestic natural cheese produced in Hokkaido. This result indicates that the natural cheese produced in Hokkaido is safe and it is in relatively better hygienic status than those produced in other countries.

Among others, the practice of good production hygiene and a low level of natural cheese consumption might have contributed to the low incidence of cheese related outbreaks in Japan. The average cheese consumption of the Japanese people at present is about 2 kg per year per person that is about 10 times lower than those in European countries. Reports, however, predict that the market for domestically produced natural cheese will continue to grow from year to year, and that could result in the increase of production [17, 45, 101]. Thus, the increase in consumption and production may influence the risk of foodborne illness in the future. Therefore, conducting a similar survey on small and large scale on the periodic basis is important to ensure the food safety of domestic natural cheese. Such study could help to identify potential risks and to prevent possible incidence of foodborne disease outbreaks that may result in health risk of the consumers

In conclusion, the current result indicates that the microbiological quality and hygienic status of natural cheese produced in Hokkaido is mostly in the fine and satisfactory status. Among others, the required use of pasteurized milk for the production of dairy products including natural cheese might have contributed to being in such a status. Moreover, implementation of good manufacturing and hygienic practices across all production stages from farm to fork might have contributed to the absence of these foodborne pathogens. However, the prevalence of *Staphylococcus* spp. in a large number of samples and occurrence of coagulase-positive *S. aureus* in few samples indicates a potential risk to the consumers and suggest the need for maintaining improved production hygiene.

5. Summary

Cheese is considered as one of the safest foods, and it is widely consumed all over the world. However, concerns have aroused about its safety following reports of sporadic outbreaks related to its consumption. Natural cheese could be contaminated with foodborne pathogens when raw milk is used for cheese making or post cheese making contamination is incriminated. Though using pasteurized milk for cheese making is a prerequisite in Japan, an outbreak of *L. monocytogenes* has been reported previously in Hokkaido leaving concerns among consumers. Moreover, Shiga toxin-producing *E. coli, Salmonella,* and *S. aureus* are implicated in foodborne outbreaks related to the consumption of cheese in other countries. This study was, therefore, conducted to investigate the prevalence of *Salmonella* spp., pathogenic *E. coli, L. monocytogenes*, and *S. aureus* in domestic natural cheese. A total of 200 natural cheese samples made from cows' milk were randomly purchased from different stores in Hokkaido and used for this study. Standard methods of ISO, FDA, and MHLW with PCR and MALDI-TOF MS were used for detection and identification of the pathogens. The results showed that none of the samples inspected contain *Salmonella* and *L. monocytogenes*. One of the 120 samples (0.8%) had an *ipaH* gene that indicate the possible contamination by Enteroinvasive *E. coli* or *Shigella* as this gene is a multi-copy that exclusively exist in these pathogens. However, neither of the pathogens were recovered by the culturing technique suggesting that none of them are prevalent in the domestic natural cheese. Though 50% of the samples were positive for *Staphylococcus* spp., only three out of the 74 samples found to contain coagulase-positive *S. aureus.* The result demonstrates that the natural cheese made in Hokkaido is negative for common foodborne pathogens related to foodborne outbreaks and has a lower rate of *S. aureus* incidence. The occurrence of *S. aureus* in 4% of the samples, however, indicate potential risk to the consumers due to possible food poisoning. The result also suggests the importance of maintaining the existing good hygienic practices to prevent contamination with the pathogens and ensure consumers' safety.

Table 2.1. Pathogenic bacteria detection from cheese samples.

Pathogens inspected	Total samples tested	Number of positive samples
L. monocytogenes	126	0
Salmonella spp.	126	0
S. aureus	74	3

All of the samples analyzed were negative for *L. monocytogenes* and *Salmonella*, and only 3 samples out of 74 appear positive for *S. aureus.*

Table 2.2. Real-time PCR analysis result showing the prevalence of pathogenic *E. coli* in natural cheese samples.

Pathogenic *E. coli*	Target virulence genes	Total number of samples tested	Number of positive samples
STEC	*stx1*	120	0
	stx2	120	0
EIEC[a]	*ipaH*	120	1
ETEC	LT	120	0
	STI	120	0
EAEC	EAST1	79	0

[a] Only One sample had positive amplification for EIEC, but the bacteria were not detected by culture method.

Table 2.3. Prevalence of *Staphylococcus* spp. in different types of domestic natural cheese produced in Hokkaido.

Staphylococcus spp. count (cfu/g)	Cheese types			Total (%)
	Soft (n=34)	Semi-hard (n=15)	Hard (n=25)	
$<10^{1}$ [a]	24	3	10	37 (50)
$10^{1} – 10^{2}$	0	1	0	1
$10^{2} – 10^{3}$	2	1	0	3
$10^{3} – 10^{4}$	0	1	2	3
$10^{4} – 10^{5}$	3	4	5	12
$10^{5} – 10^{6}$	0	3	3	6
$10^{6} – 10^{7}$	4	2	2	8
$10^{7} – 10^{8}$	0	0	1	1
$10^{8} – 10^{9}$	1	0	2	3
Total[b]	10	12	15	37 (50)
Grand total	34	15	25	74 (100)

[a] Negative samples with no *Staphylococcus* spp. count.

[b] Positive samples with counts of $10^{1} – 10^{9}$ cfu/g.

Chapter III

Characterization of Enterotoxins and Antibiotic Resistance of Staphylococcus aureus Isolates from Natural Cheese in Hokkaido, Japan

1. Introduction

The staphylococcal foodborne disease is one of the most common forms of bacterial foodborne intoxication worldwide [67, 70, 121, 132]. It is caused when a food contaminated with *S. aureus* and containing one or more types of pre-produced staphylococcal enterotoxins is ingested. Classical staphylococcal enterotoxins (A-E) such as SEA, SEB, SEC, SEC, SED and SEE are responsible for most food poisoning incidences. More recently, newly identified enterotoxins including staphylococcal enterotoxin H (SEH) are also involved in foodborne disease incidences [66] while another study also reported the involvement of SEG and SEI in the staphylococcal foodborne outbreaks [64]. The disease of staphylococcal food poisoning is characterized by rapid onset after the ingestion of contaminated food [67, 70]. Its onset may depend on the immune status of the consumers and amount and type of the enterotoxin ingested. Symptoms of the staphylococcal foodborne disease include nausea, vomiting, and abdominal cramping with or without diarrhea. Though the illness is self-limiting in most cases, it may be severe and require hospitalization depending on the immune status of the patients and amount and type of the enterotoxins ingested [67]. In Japan, staphylococcal food poisoning is one of the major public health concerns attributed to the consumption of different contaminated foods [121]. For instance, consumption of contaminated crisps at university festival resulted in a staphylococcal outbreak in Nagoya [73]. Moreover, consumption of contaminated dairy products was implicated in a staphylococcal foodborne disease that caused a major outbreak in Kansai area [5].

Staphylococcal enterotoxins are 22 – 30 kDa small molecular weight proteins that show an emetic response in the patients and they are members of superantigens [4, 6, 52, 86]. There are five classical staphylococcal enterotoxins known to cause foodborne illnesses. These include enterotoxin A, B, C (C1, C2, C3), D, and E. Among these, enterotoxin A is the most predominant toxin responsible for the staphylococcal foodborne disease [23, 63, 132]. Following the use of more recent analytical techniques including PCR and complete genome sequence analysis, currently about 22 enterotoxins are identified [4, 63]. Among them, some are known to be emetic and considered as enterotoxins (SEs). Others on the other hand either lack emetic activity or not yet studied for their

emetic activity and designated as staphylococcal enterotoxin-like (SEl) superantigens [4, 52, 81]. Staphylococcal enterotoxins are produced during the growth of *S. aureus* in contaminated foods or growth media [29, 30, 103]. Once the toxins are produced and released, they cannot be destroyed by pasteurization temperatures and retain their biological activity [4, 5]. Moreover, they are also not affected by freezing, change in pH and action of proteolytic enzymes such as pepsin [4, 86, 112]. Thus, even though the pasteurization temperature kills the bacteria, the toxins may remain in foods and lead to the onset of food poisoning illnesses.

Even though sporadic cases of cheese related staphylococcal foodborne outbreaks reported elsewhere [36, 64, 70], there is no report on staphylococcal foodborne outbreak related to the consumption of natural cheese in Japan. However, an earlier study conducted in Hokkaido showed the occurrence of *S. aureus* in domestic natural cheese and some isolates harbor enterotoxin genes *seg*, and *sei* [56]. Besides enterotoxin production, *S. aureus* also pose challenges due to its antibiotic resistance characteristics and little is known about this property from cheese isolates in Japan. Therefore, the present study was conducted to characterize the enterotoxin genes profile, and antibiotic resistance properties of the *S. aureus* isolates obtained from domestic natural cheese.

2. Materials and methods

Bacterial strains and growth condition

S. aureus strain (clinical strain, laboratory stock) was used as a positive control for staphylococcal enterotoxin genes *sea* and *seh*. An overnight culture of the strain prepared in BHI broth was spread plated on BHI agar and incubated at 37ºC overnight. Similarly, a total of eight test strains of *S. aureus* isolates obtained from domestic natural cheese were grown on BHI agar overnight. Single colonies of the overnight cultures were aseptically picked and used for DNA extraction. The DNA extraction was done following the manufacturer's instructions of PrepMan Ultra sample preparation kit (Applied Biosystems).

Enterotoxin detection

Detection of staphylococcal enterotoxins was conducted following single PCR procedures as shown in Blaiotta *et al.* [10]. Eight sets of primers were used to detect the enterotoxin genes of *sea, seb, sec, sed, see, seg, sei* and *seh* (Table 3.1). Thus, eight separate PCR experiments were carried out for each isolate to screen for their enterotoxin genes. The PCR analysis was conducted in a total volume of 25 µl reaction mixture. The mixture contain 3 µl of template DNA (30–50 ng), 2.5 µl of 10x EX Taq DNA polymerase buffer (Takara Bio, Tokyo, Japan), 2.5 µl of 25 mmol/l $MgCl_2$, and 2.5 µl of dNTP mix from 2.5 mmol/l stock solution. In addition, 0.1 µl of each primer from 10 pmol/l stock solution and 0.2 µl of EX Taq DNA polymerase (stock solution 5 U/µl) (Takara Bio, Tokyo, Japan) were used per reaction mixture. Moreover, double distilled water (DDW) was used as a negative control for PCR amplification while template DNA from the clinical strain was used as a positive control for *sea* and *seh* enterotoxin genes.

The PCR protocol was set as 3 min at 95ºC for the initial denaturing step; 30 cycles of 3 sec. at 95ºC denaturing step and 59ºC for 75 sec. the annealing-extension step. The final extension step was done at 72ºC for 3 min after 30 cycles. Five µl of the PCR products were used for agarose gel

electrophoresis in 1 x TAE buffer (2%, w/v) at 100 V for 30 min. A 100 bp DNA Ladder marker (Takara Bio, Japan) was used to determine the molecular mass of the PCR products.

Antibiotic resistance test

The antibiotic resistance test was conducted using the eight S. aureus isolates obtained from natural cheese samples produced in Hokkaido. A disc diffusion method was used to investigate the antibiotic resistance properties of the isolates. Overnight cultures of the isolates were prepared on BHI agar plates (BD). Single colonies of the overnight cultures 35ºC were then picked up and suspended in sterile saline (0.85% NaCl). The cell suspensions of each culture were adjusted to the McFarland's 0.5 standard and then plated in duplicate on Muller Hinton Agar plates (MERCK). Within 15 minutes of plating, three to four discs were applied to each plate to investigate the antibiotic resistance of the isolates. The antibiotics discs used to assess the resistance of *S. aureus* strains include penicillin, oxacillin, amikacin, tobramycin, minocycline, imipenem, and vancomycin. The discs were gently pressed down to ensure complete contact with the agar surface of the plates. Moreover, they were placed in the way that the discs may not be closer than 24 mm from the center to each other to avoid the possibility of overlapping in the clear zone formation. Within 15 minutes of disc application, the plates were incubated at 35ºC. After 20 hr of incubation, records were taken in mm by measuring the diameters of clear zones. Result interpretations were done as resistant, intermediate or susceptible following standard guidelines [25].

3. Results

Enterotoxin detection

Eight isolates of *S. aureus* were subjected to the separate experiments to detect eight staphylococcal enterotoxin genes. The target enterotoxin genes include *sea, seb, sec, sed, see, seg, seh* and *sei,* and PCR analysis was conducted to identify the prevalence of these enterotoxin genes in the isolates. The results showed that one of the eight isolates (12.5%) had a positive result for enterotoxin genes of the *sea* and *seh* (Fig. 3.1). The other seven isolates, however, did not show positivity for any of the enterotoxin genes analyzed.

Antibiotic resistance

Among all the isolates screened for the antibiotics resistance, none of them was resistant to oxacillin (Table 3.2 and 3.3). Moreover, all of the isolates tested were susceptible to tobramycin, minocycline, imipenem and vancomycin (Table 3.2). Furthermore, the result also showed that 12.5% of the isolates were susceptible to all the antibiotics tested including penicillin (Table 3.2). On the other hand, the results showed that a total of 62.5% of the isolates were resistant to penicillin. Also, 12.5% isolates had an intermediate response to amikacin (Table 3.2 and 3.3).

4. Discussion

In this study, the enterotoxin genes and antibiotic resistance of *S. aureus* isolates obtained from natural cheeses produced in Hokkaido were characterized. The results showed that 12.5% of the *S. aureus* isolates harbor genes for SEA (*sea*) and SEH (*seh*). Similar, result was reported elsewhere from earlier study showing that enterotoxin A was detected from 12% of the *S. aureus* isolates obtained from cheese [9]. Other reports from different parts of the world also showed that SEA was the most prevalent enterotoxin among *S. aureus* isolates than the other enterotoxins [70, 98, 105, 109]. Moreover, among the 22 staphylococcal enterotoxins identified so far, most of the staphylococcal food poisoning is caused by classical enterotoxins particularly SEA [5, 63, 70]. For instance, a study conducted in the United Kingdom showed that SEA caused 79% of staphylococcal foodborne outbreaks in the country [130]. Another study also reported that SEA was responsible for repeated food poisoning outbreaks in France [70]. Furthermore, SEA was also known as responsible enterotoxins in several staphylococcal food poisoning outbreaks in the US [67]. Meanwhile, 90% of the isolates from food poisoning outbreak were reported to harbor this enterotoxin in a study conducted in the South Korea [23]. SEA is also the most common cause of foodborne outbreaks in Japan [121]. Among the staphylococcal food poisoning outbreaks reported in recent years in this country, the extensive outbreak that occurred in Kansai area was the major one. It was caused due to the consumption of contaminated dairy products, and it has affected 13420 people. Asao and colleagues reported that the causative agent for that outbreak was SEA [5]. A More recent outbreak that occurred due to the consumption of contaminated crisp at annual student festival of Nagoya University was also caused by SEA and SEC [73].

Moreover, among the newly identified enterotoxins, SEH had emetic activity in the animal model [4, 70]. Moreover, this enterotoxin was also known to cause food poisoning outbreak [66]. Another report [57] also showed that SEH was one of the responsible causative agents for the extensive staphylococcal outbreaks that occurred in Japan. Cha and colleagues in the study conducted in the Korea also reported that more than half of the *S. aureus* isolates investigated for

enterotoxins had *seh* in combination with *sea* [23]. A previous study conducted in Hokkaido on cheese showed that no staphylococcal enterotoxins were detected in 20 *S. aureus* isolates. However, the same study described that 13 of the 38 isolates investigated using the molecular method had enterotoxin genes such as *sea*, *seb*, *sec*, *seg* and *sei* [56]. According to the report of that study, however, no enterotoxins were detected by TRANSIA PLATE staphylococcal enterotoxin detection method. Apart from the detection of enterotoxin genes such as *sea,* and *seh*, I did not conduct an evaluation of enterotoxin production by the isolates. However, once the isolates are known to harbor the enterotoxin gene, they could have a high likelihood of enterotoxin production [9, 30, 70, 98, 105]. Therefore, the occurrence these genes in isolates from domestic natural cheeses may indicate the potential risk of staphylococcal food poisoning and the need for maintaining proper hygienic measures to prevent staphylococcal contamination.

Antibiotic resistance of *S. aureus* strains has become a challenging characteristic of these pathogens. Since *S. aureus* is the pathogen associated with both community-based and hospital-related infection, the increase in its antibiotic resistance is a threat to public health. Its resistance to several drug leaves the limited choice for the treatment and control of this pathogen. After the introduction of penicillin as a therapeutic option in 1940, the level of mortality related to *S. aureus* has dropped dramatically [87]. However, not after a long time had the resistant strains noticed in the hospitals and soon spread widely. At present penicillin resistance is considered as one of the most common features of *S. aureus* strains. Besides to penicillin, *S. aureus* has also developed resistance to other beta-lactam drugs making its control difficult. According to the result of this study, 62.5% of isolates were resistant to penicillin. From an earlier study, similar results were reported [70] that shows 57.6% of the isolates analyzed were resistant to penicillin. Other researchers [50] also reported that 53.8% of the isolates were resistant to this antibiotic. Another report also indicated more resistance in 96.3% of milk and dairy product isolates in Turkey [49]. Meanwhile, a separate study [132] also showed that 96.2% of the isolates from foodborne outbreaks in China were resistant to penicillin.

Moreover, 12.5% of the isolates had an intermediate response for amikacin. On the other hand, none of the strains was resistant to oxacillin and vancomycin. Oxacillin resistance is related with the resistance to methicillin, and high oxacillin resistance was reported among some food isolates [106]. Staphylococcal foodborne diseases can be caused by either methicillin-resistant staphylococci or methicillin-susceptible ones as long as they contain enterotoxins responsible for food poisoning. Moreover, antimicrobial resistance could be transferred among microorganisms through foods due to either the occurrence of antimicrobial residues or ingestion of resistant strains of the food microflora [105, 106]. Apart from beta-lactam antibiotics such as penicillin and methicillin, the multi-drug resistance of *S. aureus* is becoming a concern nowadays. Since vancomycin is used as an alternative treatment for systemic infection of methicillin-resistant strains, some strains resistant to vancomycin has started to emerge and pose further concern [106]. In this study, however, none of the strains isolated from natural cheese was multidrug resistant, and no vancomycin resistance was observed.

In conclusion, this study reports the prevalence of staphylococcal enterotoxin genes such as *sea* and *seh* in *S. aureus* strains isolated from domestic natural cheese produced in Hokkaido. *S. aureus* is susceptible to heat treatment, and pasteurization temperature can kill it. However, the staphylococcal enterotoxins are heat stable and can maintain its biological activities even after cooking or heat treatment. Thus, their occurrence in foods could pose potential threats to the health of consumers as it may result in foodborne disease incidences. Therefore, implementation of the proper hygienic measure is highly essential to prevent contamination of natural cheese with the pathogens. Moreover, keeping the natural cheese at appropriate storage conditions indicated on their labels or at refrigeration temperature may prevent the growth of the organisms and reduce the related risk of enterotoxin production.

5. Summary

Staphylococcal foodborne disease is one of the most common bacterial foodborne intoxication worldwide. It is caused by ingestion of contaminated foods with preformed enterotoxins. *S. aureus* can get access to food due to improper hygiene and faulty storage that may favor the growth of the pathogen in foods and allow the production of the enterotoxins. This study was conducted to characterize staphylococcal enterotoxins in the *S. aureus* isolates and their antibiotic resistance properties. I used a conventional PCR procedures and disc diffusion methods for the enterotoxin detection and antibiotic resistance experiments. I also used respective specific primers for enterotoxin genes of *sea, seb, sec, sed, see, seg, seh* and *sei* for the detection of enterotoxins. A clinical strain of *S. aureus* was used as a positive control for the enterotoxin genes of the *sea* and *seh* while DDW was used as negative control for the PCR amplification. Overnight cultures of positive control and test strains were prepared in BHI plates and incubated at 35ºC. Single colonies of the respective test strains were picked, and DNA extraction was conducted following Prep Man Ultra sample preparation kit. The appropriate concentration of the template was used for PCR analysis in 25 µl reaction mixture. Antibiotic resistance tests were also conducted using discs of penicillin, oxacillin, amikacin, tobramycin, minocycline, imipenem, and vancomycin. From all the strains inspected, enterotoxin genes of *sea* and *seh* were detected in 12.5% of the isolates inspected. Moreover, 65.5% of the isolates were resistant to penicillin while 12.5% were susceptible for all of the antibiotics evaluated. The presence of staphylococcal enterotoxins *sea* and *seh* may represent the potential concern of food poisoning. Appropriate hygienic measures and proper storage temperature should be followed to prevent the *S. aureus* contamination and incidence of public health hazard due to staphylococcal food poisoning.

Table 3.1. Primer sequences used for the detection of staphylococcal enterotoxin genes in the isolates obtained from natural cheese.

Primer	Nucleotide sequence (3'- 5')	Amplicon size (bp)	Target gene
SEA F	cctttggaaacggttaaaacg	127	*sea*
SEA R	tctgaaccttcccatcaaaaac		
SEB F	tcgcatcaaactgacaaacg	477	*seb*
SEB R	gcaggtactctataagtgcctgc		
SEC F	ctcaagaactagacataaaagctagg	271	*sec*
SEC R	tcaaaatcggattaacattatcc		
SED F	ctagtttggtaatatctcctttaaacg	319	*sed*
SED R	ttaatgctatatcttatagggtaaacatc		
SEE F	cagtacctatagataaagttaaaacaagc	656	*see*
SEE R	taacttaccgtcgacccttc		
SEG F	aattatgtgaatgctcaacccgatc	642	*seg*
SEG R	aaacttatatggaacaaaaggtactagttc		
SEH F	caatcacatcatatgcgaaagcag	375	*seh*
SEH R	catctacccaaacattagcacc		
SEI F	ctcaaggtgatattggtgtagg	577	*sei*
SEI R	aaaaaacttacaggcagtccatctc		

Table 3.2. Antibiotic resistance of the isolates studied using disc diffusion method.

Antibiotics	*S. aureus* isolates							
	SA 5.2	SA 5.3	SA 5.5	SA 37.1	SA 37.3	SA 37.4	SA 37.5	SA 51.1
Penicillin	S	S	R	S	R	R	R	R
Oxacillin	S	S	S	S	S	S	S	S
Amikacin	S	I	S	S	S	S	S	S
Tobramycin	S	S	S	S	S	S	S	S
Minocycline	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S	S

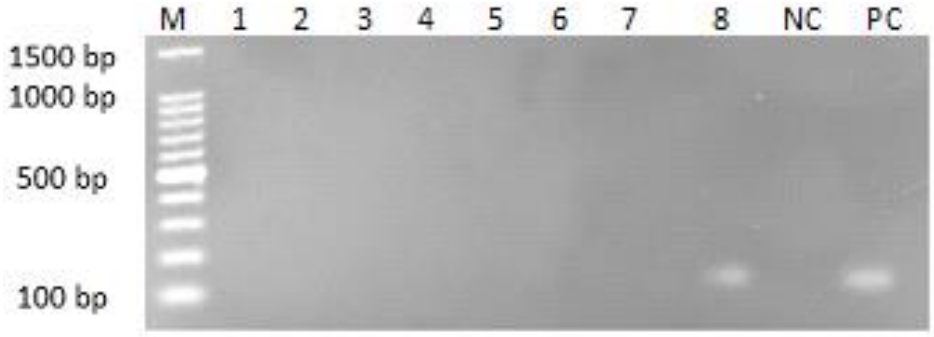
R, resistant; I, intermediate; S, susceptible.

Table 3. 3. Percentages of antibiotics resistant isolates

Antibiotics	Percent of isolates		
	Resistant	Intermediate	Susceptible
Penicillin	62.5	0	37.5
Oxacillin	0	0	100
Amikacin	0	12.5	87.5
Others[a]	0	0	100

[a] Others include tobramycin, minocycline, imipenem and vancomycin.

(A) *sea*



(B) *seh*

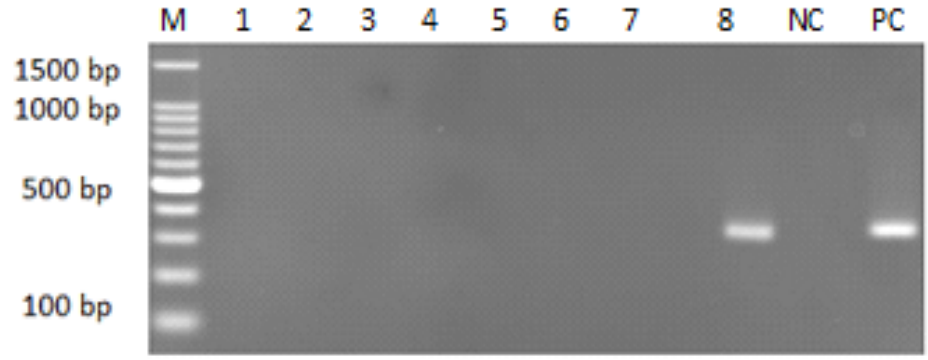


Fig. 3.1. PCR detection of staphylococcal enterotoxin genes in *S. aureus* isolates. *S. aureus* isolates were PCR-analyzed for staphylococcal enterotoxin genes, *sea* (A) and *seh* (B). Lane 1, SA 5.2; lane 2, SA 5.3; lane 3, SA 5.5; lane 4, SA 37.1; lane 5, SA 37.3; lane 6, SA 37.4; lane 7, SA 37.5; lane 8, SA 51; NC, negative control; PC, positive control; M, DNA size marker.

General discussion

Production and consumption of natural cheese are growing from time to time in Japan, and Hokkaido is the major agrarian region where several agricultural products are produced including domestic natural cheese [17, 45, 101]. Natural cheese is produced by curdling of raw or pasteurized milk from healthy dairy animals including cow, buffalo, sheep, and goat. Cows' milk is most commonly used for cheese making, and natural cheese is one of the most commonly consumed foods of animal origin. The milk used for cheese making is allowed to develop acidity either by lactic acid bacteria fermentation, the action of manually added starter culture, rennet or food grade organic and inorganic acids to facilitate the curdling process. Several steps are followed in cheese-making including whey removal, molding, packing and storage among others while each step require appropriate care to prevent contamination and resultant food spoilage.

In Japan, consumption of cheese is becoming popular as a western type of foods that use cheese as an ingredient are becoming more common. Once the natural cheese was not popular and had a little acceptability among the most Japanese populace. After the World War II, however, there has been tremendous economic development in the country and increased exposure of the Japanese public to the western food cultures [18]. Especially the movement of Japanese people to overseas and that of the western public to Japan has increased westernization process contributing to the growth of cheese popularity in Japanese foods. Following such movements, it became common to find western style food shops such as French and Italian restaurants in the major cities of the country. Such restaurants are known to use cheese as the essential recipe of their foods. Moreover, through time, the establishment of such businesses expanded all over the country contributing to the increase of cheese consumption in the wider scale. Furthermore, cheese makers and importers associations played a great role to promote consumption of natural cheese and conduct regional and national cheese contests that had great roles in increasing consumption of cheese in the country.

Though it is low as compared to other developed countries, the current Japanese per capita consumption of cheese is increasing and currently estimated to be about 2 kg [45]. Moreover, wide varieties of natural cheeses are readily available in most of the local stores all over the country. Therefore, Japanese public enjoys a variety of cheese based on their preferences. Furthermore, production and consumption of natural cheese keeps increasing in the past decades and at present domestic production accounts for about 20% of the overall cheese consumed in the country.

Hokkaido is the major agrarian region of Japan that produce a vast array of agricultural products, seafood, and fresh dairy products including natural cheese [53]. This region alone supplies 50% of the nation's milk and 90% of the domestically produced natural cheese. Being the nation's highest producer, Hokkaido takes an important place to supply the country with a high quality and microbiologically safe agricultural product. In this regard, the region pays due attention to the quality and safety of agricultural products in general and dairy products in particular including natural cheese. For this reason, Hokkaido Regional Accreditation Body has set a guideline for the hygienic production of domestic natural cheese. According to this guideline, natural cheese should be negative for *L. monocytogenes* per 25 gram of sample, and it should be negative for coliform per gram of sample. This guideline is the first of its kind in the country as far as natural cheese is concerned, and no other microbiological standard related to the natural cheese is available. As production and consumption of domestic natural cheese are increasing from time to time, it is important to have a microbiological standard to encourage producers for better quality and prevent the health of consumers. Moreover, the continued increase in production and consumption of natural cheese in the country suggests the need for closer monitoring and further improving production hygiene to ensure the food safety.

Indeed, foodborne disease outbreaks related to the consumption of natural cheese is not common in Japan. The absence of such cases could be related to high level of hygienic practice in the country's food production sector. Moreover, the low level of cheese consumption in the country, as compared to the other developed countries, could have played its role. In European and North American countries, however, foodborne disease outbreaks related to the consumption of natural

cheese are more frequent than in Japan. Among others, *L. monocytogenes, Salmonella*, pathogenic *E. coli*, and *S. aureus* are frequently associated with foodborne disease outbreaks in relation to the consumption of natural cheese [1, 22, 34, 76, 104, 126, 131]. An earlier report in Japan also showed the occurrence of *L. monocytogenes* outbreak in Hokkaido. It was related to the consumption of natural cheese, and it was the only reported outbreak of foodborne listeriosis in the country [90]. Moreover another report indicated the prevalence of *S. aureus* in natural cheese where some of the isolates are known to be Enterotoxigenic [56].

However, except those few reports on *L. monocytogenes* outbreak and prevalence of *S. aureus*, no comprehensive information is available about the hygienic status and microbial quality of domestic natural cheese produced in Hokkaido. Moreover, there is a lack of surveillance system required to obtain up-to-date information. On the other hand, there is a continued increase in production and consumption of domestic natural cheese in Japan. Thus, the information on the hygienic and microbiological quality of domestic natural cheese also need to be available and updated regularly to prevent any potential foodborne disease incidences and ensure public health safety. Therefore, the purpose of this study was to assess the hygienic status and prevalence of foodborne pathogens in domestic natural cheese produced in Hokkaido. Specifically I assessed the hygienic status of the domestic natural cheeses focusing on the coliform, spore-forming bacteria, and standard plate count. As indicator organisms, these are used to monitor overall hygiene of dairy foods and potential implication for the occurrence of the foodborne pathogen. Moreover, I investigated the prevalence of foodborne pathogens such as *L. monocytogenes, Salmonella,* pathogenic *E. coli*, and *S. aureus* following standard methodologies to investigate potential hazards on the health of consumers. Furthermore, Characterization of *S. aureus* isolates was also carried out in terms of their virulence characters specifically detection of enterotoxin genes and antibiotic resistance.

The results demonstrated that the hygienic status of domestic natural cheese is in its fine condition. More than 78% of the samples inspected appear negative for the coliform count, and none of the samples contains *L. monocytogenes, Salmonella*, and pathogenic *E. coli*. The result

shows that the domestic natural cheese produced in Hokkaido is in its proper hygienic and safety status for public consumption. However, there are samples that were positive coliform counts, and fail to comply with the requirements of regional accreditation body indicating the need for improvement of production hygiene. Moreover, some of the samples contain *S. aureus* and among the isolates few of them were Enterotoxigenic. Specifically genes for enterotoxin A (*sea*) and enterotoxin H (*seh*) were detected from those isolates. Both of these enterotoxins has been reported as the causative agents of foodborne outbreaks in different countries and Japan as well [5, 57, 66]. The occurrence of such pathogen in natural cheese could indicate the presence potential public health risk due to consumption of contaminated natural cheese and suggests the need of further improvement on the existing best production practices.

General summary

Natural Cheese is a fermented dairy food consumed all over the world including Japan. As it is a concentrated milk product, it is not only highly nutritious food for humans but also it can allow the growth of microorganisms. Lactic acid bacteria and other starters are used in the fermentation of the cheese making process and categorized under the useful group of microorganisms. However, in the absence of careful implementation of hygienic production foodborne pathogens may get access and render the product unsafe and unfit for public consumption. In this dissertation, I analyzed a total of 200 samples of domestic natural cheese to evaluate its hygienic status and prevalence of foodborne pathogens. In the first chapter I investigated the hygienic status of domestic natural cheese with particular focus on the coliform count, spore formers' count, and standard plate count. These counts are classical methods that are used to estimate the hygienic status of dairy products. I used standard bacteriological analytical methods for the bacterial count and matrix-assisted laser desorption ionization time of flight mass spectrometer method to identify spore formers. The result showed that the standard plate count varied between 10 cfu/g and 1.3 x 10^9 cfu/g where overall counts are within the expected limit for the fermented dairy products. Similarly, 59% of the samples had spore former counts varying between 10 cfu/g and 5.2 x 10^5 cfu/g while the remaining 41% had counts below the detection limit. Among the spore former isolates, MALDI-TOF MS result showed that *Bacillus licheniformis* and *Paenibacillus pubuli* were predominant. The coliform count also revealed that 78% of the samples were negative for coliform indicating the good hygienic quality of the domestic natural cheese. However, 22% of the samples had coliform counts varying between an estimated 10 cfu/g and 6.3 x 10^7 cfu/g. The occurrence of coliform in cheese indicate contamination after cheese making due to faulty handling or failure in the heating process. However, the overall hygienic condition of the domestic natural cheese is at its virtuous status. However, more effort might be needed to produce more hygienic products that

comply the requirements of Hokkaido regional accreditation body and to ensure the safety of consumers.

Foodborne disease outbreak related to the consumption of natural cheese is not common in Japan. However, *L. monocytogenes, Salmonella*, pathogenic *E. coli* and *S. aureus* are frequently associated with cheese related foodborne outbreaks in other countries. However, the information about the prevalence of these pathogens in Japanese domestic natural cheese is scarce. Moreover, the earlier report showed the occurrence *L. monocytogenes* outbreak related to the consumption of contaminated cheese in Hokkaido. Therefore, in the second chapter I investigated the prevalence of these four foodborne pathogens in domestic natural cheese produced in Hokkaido. The contamination of natural cheese could occur in any of the cheese making stage including milk selection, standardization, and the addition of starter, molding, aging and storage among others. Indeed, it is a prerequisite to use pasteurized milk for cheese making in Japan. However, reports from other countries showed that both pasteurized, and raw milk cheeses are related to the foodborne disease outbreaks use to these pathogens. Therefore, a total of 200 natural cheese samples made in Hokkaido were randomly purchased from different stores and used to assess the prevalence of these pathogens in the domestic natural cheese. Among these, 126 samples were used to inspect *Salmonella* and *Listeria monocytogenes* while 120 of them were also used to investigate pathogenic *E. coli.* Moreover, another 74 samples were used to assess *S. aureus* in domestic natural cheeses produced in Hokkaido. Standard methods of ISO, FDA, and MHLW, coupled with PCR and MALDI-TOF MS were used for detection and identification of the pathogens. The results showed that none of the samples inspected contain *Salmonella* and *L. monocytogenes*. Moreover, only one of the 120 samples (0.8%) had an *ipaH* gene that indicate the possible contamination by Enteroinvasive *E. coli* or *Shigella*, yet the culturing technique recovered neither of these pathogens. Therefore, no pathogenic *E. coli* was detected in domestic natural cheese. Among the 74 samples analyzed for *S. aureus*, however, 50% of the samples contain *Staphylococcus* spp. and 4% of them found to contain *S. aureus*. The result from the second chapter demonstrates domestic natural cheese is negative for common foodborne pathogens related to foodborne outbreaks and has a lower rate of

S. aureus incidence. The absence of the pathogens implies that there are commendable production hygiene and minimal potential risk of the foodborne outbreak related to the consumption of natural cheese. However, despite it is low, the occurrence of *S. aureus* in 4% samples indicate potential public health risk due to the consumption of contaminated natural cheese. Thus, the result suggests the need to improve the existing best hygienic practice to prevent contamination of the product with the pathogens and ensure consumers' safety.

In the third chapter, I assessed the enterotoxin gene profiles of *S. aureus* isolates obtained from the natural cheese samples. *S. aureus* is a commensal microorganism that resides in anterior nares and skin of healthy humans and animals. Its occurrence in heat treated foods is associated with the cross-contamination by food handlers. *S. aureus* produces enterotoxins in foods that cause food poisoning that is the most common form of bacterial foodborne intoxication. The disease is caused by ingestion of contaminated foods with preformed staphylococcal enterotoxins. At present, about 22 staphylococcal enterotoxins are identified yet only a few of them are known to cause foodborne diseases. Among SEA is the most important and widely distributed among the *S. aureus* isolates. The study of the third chapter was conducted to characterize staphylococcal enterotoxins in the *S. aureus* isolates and the antibiotic resistance properties of the isolates. I used conventional PCR steps for the detection of staphylococcal enterotoxin genes and disc diffusion methods for antibiotic resistance experiments. Specific primers for enterotoxin genes of *sea, seb, sec, sed, see, seg, seh,* and *sei* were used to identify the presence of these enterotoxin genes. A clinical strain of *S. aureus* was used as positive control for the enterotoxin genes of the *sea* and *seh* while DDW was used as a negative control for the PCR amplification. Overnight cultures of positive control and test strains were prepared in BHI plates and incubated at 35ºC. Single colonies of the respective test and control strains were picked, and DNA extraction was conducted following Prep Man Ultra sample preparation kit. The appropriate concentration of the template was used for PCR analysis in 25 µl reaction mixture. Antibiotic resistance tests were also conducted using discs of penicillin, oxacillin, amikacin, tobramycin, minocycline, imipenem, and vancomycin. From all the strains inspected, enterotoxin genes of *sea* and *seh* were detected in 12.5% of the isolates inspected. Moreover, 87.5%

of the isolates were resistant for one or more of the antibiotics tested while 12.5% were susceptible for all. Penicillin resistance was the most commonly detected in 62.5% of the isolates as expected. The presence of staphylococcal enterotoxins *sea* and *seh* among the isolated of the domestic natural cheese may represent a potential public health risk due to food poisoning. Therefore, use of appropriate hygienic measures and proper storage temperature could help to prevent the incidence of staphylococcal food poisoning and related public health hazard.

In conclusion, this study shows that the hygienic status and microbial quality of the domestic natural cheese is fine. The absence of coliform in over 78% of the samples inspected shows a high level of production hygiene practiced by the cheese makers in the region. Moreover, foodborne pathogens most commonly associated with outbreaks related to the consumption of contaminated cheese are not prevalent in the domestic natural cheese. This result shows that natural cheeses produced in Hokkaido complies the requirements to supply wholesome and safe dairy products based on the existing standards. However, the occurrence of coliform in 22% of the samples and *S. aureus* in 4% of the samples with some enterotoxigenic strains indicate the need for periodic monitoring and further improvement of the existing best production hygiene.

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Japanese Summary