乾燥食肉製品の品質および微生物学的特性に関する研究

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Studies on Quality and Microbiological Characteristics of Dry Meat Products

by

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Contents

General Introduction

Mongolia, which is situated in the Central Asia between Russia and China, has a population of 2.9 million people with an average density of 1.7 persons per square kilometer. However, more than half of the population lives in urban areas. About 700,000 people live in Ulaanbaatar, the capital city. The traditional nomadic or semi-nomadic life style is still practiced to about more than ten percent of the current population who live by herding livestock in the nation's countryside. Meat consumption has been very high in Mongolia especially during the last decade with per capita meat consumption increasing to an average of 100-120 kg /person per year. In developing country including Mongolia, the per capita consumption average is 31.1 kg/person per year. People in the world consume 42.1 kg/person per year (FAO, 2008). Total numbers of livestock reached 40.2 million in 2007. Traditionally, five types of livestock namely camels, cattle, horses, sheep and goats are found in Mongolia and graze mainly on natural pasture and their meat is produced for consumption. Meat is well known as an excellent source of protein and energy for our daily diets and after digestion, it provides excellent nutrients (Chang and Huang, 1991). Consumers are becoming more demanding about product quality (Steenkamp, 1990 ; Dalen, 1996), given the increasing importance to credence quality attributes in response to rising concerns on safety, health, convenience, locality, ethical factors, etc (Harrington, 1994; Wandel and Bugge, 1996 ; Anwander and Badertscher, 2001). These attributes mainly focus on the quality of the production process. In Mongolia, millions of livestock are slaughtered for food annually, more than half of them without the benefit of stunning and in poor hygienic condition. The propensity of meat and meat product quality depends on several factors including pre-slaughter events such as stress and post-slaughter events such as early postmortem

pH, carcass temperature, cold shortening, and techniques such as electrical stimulation (Bukley *et al.*, 1995). Furthermore, many factors on processing are also important for quality of products. Traditional dried meat products are major part of the daily diet in rural areas of Mongolia and fashionable food products in urban whose market has been remaining in a significant way. Drying has the advantage that the dried product can be preserved under ambient conditions and requires very little storage space, but the nutritional and sensory attributes of the product may be changed (Fellows, 2000) by lipid oxidation which is one of the major factors resulting in losses in fatty food quality by formation of products having negative effect on taste, aroma and nutritional value of the food, which are health hazard and associated with many types of biological damage in living tissues and increase risk of cardiovascular disease (Addis and Park, 1989; Chow, 1992) by potential formation of toxic compounds (Baron and Andersen, 2002). At the same time, during processing of meat product, microorganisms such as *Salmonella, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes,* as well as unwanted mould and yeast could contaminate the meat product causing a decrease in shelf-life of this product and serious public health concern.

In Mongolia, there are many types of meat products produced by different methods. These methods, although good, there is need for more scientific methods for upgrading quality and developing shelf-life of products. For example, fermented dry sausage would have good market value for relatively nomadic livestock countries such as Mongolia due to its capability of good storage at room temperature. In Mongolia, traditional cultures as well as starter culture are used in the dairy industry but meat products are still often not inoculated with starter cultures which depend on the indigenous flora, these harsh processes lead to significant losses in safety and quality of

products. Probably, technique based on meat starter cultures will be soon become a new method for meat processing when used in combination with antioxidants, such as flavonoids and other phenolic phytochemicals present in plants which are associated with reduced chronic disease risk (Singh and Marimuthu, 2005; Cieslik *et al.*, 2006) and are also safe for human consumption (Conner, 1993). This would be an attractive method, because it makes food more digestible and more palatable, and is considered as a harmless natural process by consumers.

Objectives

The overall objective was to improve quality and shelf life of dry meat products in Mongolia. The specific aims of the studies were to:

① determine the quality of traditional dried meats, produced in Mongolia

② try the processing of dry fermented sausages in Mongolia and evaluate their microbiological characteristics

③ examine the effect of various starter cultures in dry fermented sausage

④ determine the effect of food additives on gram negative bacteria

Chapter 1

Quality of dried meats from different livestock species

1.1. Introduction

In Mongolia, dried meat products derived from traditional simple processing continues to hold the large share in local meat production and consumption. This is because, dry meat products are important for nutrition, which essential for nomadic life styles in Mongolia. The dried meat products are commonly prepared by using beef, mutton, goat and camel meat. By the end of December each year, a large number of animals are slaughtered for preparation of dried meat products. Processing of dried meats is based on selection of the raw material, proper slice cutting and drying under natural temperatures and humidity during the coldest months of the year and long time storage in ambient temperature. Regarding long time freezing and storage, chemical and structural changes in muscle foods such as lipid oxidation have been reported (Stika *et al.*, 2007). Lipid oxidation is an important factor, which can result in quality losses in meat and meat products. It occurs during processing and storage of meat and meat products and this usually leads to the formation of numerous volatile compounds (Garcia *et al.*, 1991; Flores *et al.*, 1997), such as aldehydes that associated with warmed–over flavor (Tims and Watts, 1958; Pearson *et al.*, 1977). This was previously reported to correlate closely with other negative sensory alterations in meat product, and these included the formation of rancid odors and off-flavor development (Greene and Cumuze, 1981; Kanner, 1994). In addition, their marked relations to increase the risk of cardiovascular disease, diabetes, cancer and other pathologies in human (Guardiola *et*

al., 1996; Upston *et al.*,. 2002) were well noted.

In Mongolia, there is a wide range of meat products produced for domestic market. Nevertheless, it is difficult to assess the quality of products due to the lack of methodologically based research. There is no published scientific literature available about structural and chemical changes of traditional dried meat products; however, dried meat is normally assessed during long time of consumption.

1.2. Objectives

The objective of the current study was to examine the quality of 4 kind of dried meat through sensory quality parameters and lipid oxidation, and to provide useful product information to the consumer.

1.3. Materials and methods

1.3.1. Determination of consumer liking for type of dried meat

In this study, dried meat products were evaluated by randomly selecting 145 panelists who are citizens of Mongolia and familiar with dried meat products. The panelists were directly asked questions about dried meat products for overall, flavor and colour using 5 point scale without testing : 5- like very much, 4- like slightly, 3- neither like or dislike, 2- dislike slightly and 1- dislike very much. Consumer choice of dried meat from different meat types was also determined.

1.3.2. Samples of dried meat

Four kinds of different meat were purchased from meat market in Ulaanbaatar

city and sliced into small pieces. The pieces were placed on a string of bridge by suspension and dried in a dark room without temperature and humidity control for 3 months during winter and then kept until use.

1.3.3. Proximate analysis

The moisture, protein, fat and ash contents of 4 types dried meat were determined by using the Association of Official Analytical Chemists methods (AOAC).

Moisture content: A 2-3 g of sample was determined by using moisture analyzer (AND MX-50, Japan). Percentage of moisture was determined from the loss in weight.

Protein content: Crude protein was determined by Kjeldahl's method (AOAC-981.10), which was described as follows:

Digestion mixture preparation: The sample (1-1.5 g) was put in digestion flask (Kjeldahl flask) and then digested by heating (420 °C for 130 min) it in the presence of sulfuric acid and catalyst.

Dilution: After digestion, the flask was cooled and then digested solution was diluted with distilled water to 100 ml.

Distillation: Diluted solution, saturated NaOH and a little amount of distilled water were put into the distillation tube using a 10 ml pipette. Sulfuric acid and 2-3 drops of methylen blue-methyl red were added into the receiving flask. The sample was distilled until the solution in the receiving flask became 60 ml.

Titration: The distilled sample was titrated with NaOH solution using a buiret. The endpoint of titration is determined by the change in color, from purple to green.

The following equation can be used to determine the nitrogen concentration of a sample that weighs m grams using a xM NaOH solution for the titration.

$$\%N = \frac{X \text{ moles}}{1000cm^3} \times \frac{(Vs - Vb)cm^3}{mg} \times \frac{14\ g}{moles} \times 100$$

Where v_s and v_b are the titration volume of the sample and blank and 14 g is the molecular weight of nitrogen N. A blank sample is usually run at the same time as the material being analyzed to take into account any residual nitrogen. The nitrogen content is converted to a protein content using the appropriate conversion factor:

$$\% \text{ Protein} = F \times \%N$$

Lipid content: Lipid content in dried meat was determined according to Soxhlet extraction method (Association of Official Analytical Chemists1995). The thimble with the sample was placed in an extraction chamber, which is suspended above the flask containing solvent and below a condenser. The flask was heated and the solvent evaporated and moved up into the condenser where it is converted into liquid that trickles into the extraction chamber containing the sample. At the end of the extraction process (24-48h), the flask containing the solvent was removed, the solvent was evaporated and the mass of lipid remaining was measured (M_{lipid}). The percentage of lipid in the initial sample (M_{sample}) can then be calculated:

$$\% \text{ Lipid} = 100 \times (M_{lipid} / M_{sample})$$

Ash: Ash content was determined by samples in muffle furnace at 525 °C until constant weight. Percentage of ash was calculated in the sample.

1.3.4. Lipid peroxidation assay

Reagents:

Ten percent trichloroacetic acid (10% TCA): I weighed 50g trichloroacetic acid, dissolved it in about 400 ml distilled water and adjusted the volume up to 500 ml with distilled water.

0.02 M thiobarbituric acid (TBA): I dissolved 0.222 g TBA in approximately 200 ml distilled water and then adjusted the volume to 250 ml with distilled water. Protected the solution from light and stored in brown bottle.

Standard solution series: A mass of 0.0220 g was dissolved in 100 ml distilled water to prepare stock solution, which was determined to be 0.001 M tetraethoxypropene (TEP, Sigma Chem. Co., ST. Louis. Mo). Standards were prepared as the follows: 0.01, 0.025, 0.05, 0.075 and 0.1 ml of stock were separately added to tubes, which already contained 4.99, 4.975, 4.95, 4.925 and 4.9 ml of distilled water, respectively. A 5ml of distilled water, 10 ml of a 10% TCA and 5 ml 0.02 M TBA were added to the 4 ml of standard series separately before heating in water bath at 100°C for 35 min. Absorbance at 532 nm was recorded and signals of these standard solution series were used to establish a linear calibration line.

Sample preparation: Oxidative stability of dried meats was measured by Thiobarbituric acid reactive substance (TBARS) using the following procedure. Four grams of dried meat was placed in polyethylene stomather bag. Then 5ml of distilled water, 10 ml of a 10% aqueous solution of trichloroacetic acid, and 5 ml 0.02 M TBA was immediately added to stomacher bag. Samples were blended for 3 min in stomacher laboratory mixer (Exnizer 400, Organo, Japan). After centrifugation (3500 rpm for 10 min), supernatant was separated through a Toyo filter paper No.5C and then heated in boiling water bath for 35 min to complete the reaction. Intensity of pink colored complex was measured at 532 nm using spectrophotometer. The TBARS values were evaluated using a slope of TEP standard curve and expressed as mg TBARS per kg of dried meat.

1.3.5. Hexanal analysis

A 0.5 g of each dried meat was applied to a glass crimp top vial (10 ml, 24.5 mm

o.d., 50 mm height; Supelco Bellefonte, Pa., U.S.A.) and then sealed with both PTFE/ silocone septa (Supelco) and a silver aluminum seal (Supelco). The volatile compounds were extracted by using solid-phase micro extraction technique (SPME) with carboxen/polydimethylsiloxane (CAR/PDMS) as described by Watanabe et al (2008). The hexanal content was separated from volatile compounds in gas chromatograph (GC- MS-QP 2010), following the procedure described by Watanabe et al (2008). Hexanal was identified by comparing its retention time with that from standard compound.

1.3.6. Statictical analysis

Comparison of means among different kinds of dried meat products was performed by ANOVA. All statistical analyses were performed using SPSS 12 package (SPSS Inc., Chicago, USA, 2003)

1.4. Results and Discussion

This study describes the results of sensory quality on scaling method, chemical composition and lipid oxidation in 4 kinds of traditional dried meats. The scores of liking for flavor, colour and overall of each of dried meat are shown in Table 1.1. Dried meat from cattle had significantly ($p<0.05$) highest liking scores for flavor, colour and overall than all other dried meat used. In addition, goat liking scores was significantly ($p< 0.05$) higher than camel and mutton scores. Panelists scored dried meat from dislike very much to like very much, three characteristics of each dried meats had showed a similar scores that ranged from 4.5-4.8, 4.0-4.0, 3.3-3.5, 3.1-3.5, in beef, goat, mutton and camel dried meat, respectively. These results assume that important one or two

characteristic in 3 was not in selected dried meat. In Figure 1.1, percentages of consumer choice were 95.90%, 70.43%, 48.28% and 42.76% from 145 panelists for the cattle, goat, camel and sheep, respectively. Thus indicating that more than one kind of dried meat was consumed by one consumer. Dried meat made from beef and goat were highly consumed than other two. As discussed above, scores on flavor, colour and overall were different between dried meats with the highest scores found in dried beef, and an intermediate score found in dried goat meat. Extremely low scores were obtained in mutton and camel dried meat. A similar trend was found for percentage of consumer choice. The highest consumption in dried beef than in dried meat from small ruminants can be related to quantitative aspects of livestock production and amounts of meat from carcass weight. Meat production from camel is also high, however, camel numbers are the lowest in livestock numbers [Ministry of Food and Agriculture, 2007 (0.3 camels, 2.2 horses, 2.4 cattle, 17.0 sheep, 18.3 goats, a total of 40.2 millions)] and they are kept only in the south and west of Mongolia. Thus, beef is commonly used in traditional dried meat, therefore higher sensory scores in dried beef than in other types in present study may be dependent on consumer experience.

Table 1.2 shows the moisture, fat, protein and ash contents of different dried meat. Moisture contents of dried meat samples ranged from 9.57% to 14.60%. There were no significant differences in moisture values of different dried meat. The total fat content of dried goat meat was significantly higher ($p<0.05$) while protein and ash content were significantly lower ($p<0.05$) than other dried meats which showed no significantly differences. All dried meat had a high fat, protein and ash contents, compared with raw fresh meat.

When meat is sliced and aerobically stored for a long time, lipid oxidation in

products increased, reported in previous reports (Gray *et al.*, 1996). In the current study, four types of dried meat had higher TBARS values (Table 1.3) compared with fresh raw meat in the results reported by Descalzo *et al.* (2005), and also higher than the limited TBARS number reported by Pearson (1968), because processing and long-storage increase the lipid oxidation. In addition, traditionally, five types of livestock namely camels, cattle or yaks, horses, sheep and goats are found in Mongolia where they graze mainly on natural pasture and are not artificially fattened. Therefore, their meat is purely organic in nature and contain high contents of polyunsaturated fatty acid that is susceptible to oxidation (Kanner, 1994) in processed meat product (Drumn and Spainer, 1991) that causes the rapid development of meat rancidity and also affects colour, nutritional quality as well as texture (Kanner, 1994). Enser *et al.* (1998) concluded that polyunsaturated fatty acid content is higher in meat from pasture feeding than those from grain feeding. There were significant differences ($p<0.05$) in the values of TBARS while in those of hexanal no significant differences between dried meats (Table1.3). Standard deviation in results of hexanal in all dried meat was very high, it may be related to muscle type of animal, which is reflected in differences in chemical composition especially fatty acid composition. Hexanal is mainly associated with the development of lipid oxidation in meat products that contribute to the development of oxidative rancidity known as warmed – over flavor (Dupuy *et al.*, 1987). Among four types of dried meat, the highest TBARS value was in dried meat from camel, the lowest TBARS values were found in both beef and dried goat meat while lipid content was not significantly different except for dried goat meat. Discussion of the differences in dried meats between different species was difficult in this report. This is because TBARS value should be related to the concentration of polyunsaturated fatty acids, natural

antioxidants and enzymes (Gatellier *et al.*, 2004; Descalzo *et al.*, 2005), which depend on many factors such as gender, age, production region (Hoffman *et al.*, 2007) and diet (Warren *et al.*, 2008). In this study, observed TBARS values may be due to the difference in fatty acid composition of dried meat from different species. The percentage of PUFA and ratio of linoleic acid metabolites to linolenic acid metabolites (n-6/n-3) in camel meat is higher than those in other meat from domesticated ruminants as reported by Sinclair *et al* (1982) and Rawdah *et al* (1994). High levels of PUFA in meat products have previously been associated with high oxidative instability during storage (Ingene *et al.*, 1980) because their hydrolysis is higher than for monounsaturated and saturated fatty acids. Moreover, Ackman (1990) have shown the existence of positive correlation between deaths by coronary illnesses and high relation of n-6/n-3.

This study observed that dried meat products from different animal species were rich sources of lipid oxidation products. Chronic high ingestion of dried meat product can be a considerable risk to the public health, according to Kanner (2007), who suggested that accumulation of lipid peroxidation products in the body is known to present risks to human health. Lipid oxidation products, especially malondialdehyde (MDA) is one of the most abundant lipid peroxidation cytothoxins, and able to induce changes in blood low-density lipoproteins, resulting in the formation of atherosclerotic plaques and subsequently, of atherosclerosis and coronary artery disease (Pearson *et al.*, 1983) MDA is also mutagenic and carcinogenic (Basu and Marnett, 1983). According to the health statistics in 2004, cardiovascular diseases and cancer are number one and two, respectively leading causes of human deaths in Mongolia (Ministry of Health, National Center for Health Developmnt, 2004), which are probably associated with a diet low in

fruit and vegetable and diet rich in animal fat (Nutrition Research Center of the Public Health Institute, Ulaanbaatar, 2002). Consumers do not know about the problems that can emerge in the meat production, such as lipid oxidation products due to lack of information.

1.5. Conclusion

From the results it can be concluded that traditionally dried meat product from beef had highest panelists score, and lowest TBARS value compared to those from goat, sheep and camel. Nevertheless, need for extended shelf life and to protect the quality properties of four types of dried meats are urgently needed to augment the existing human health.

Table 1-1. Consumer liking for dried meat derived from various animals (Means ± ST for n=3)

	Flavor	Colour	Overall
Cattle	4.8 ± 0.6^{a}	4.5 ± 0.8^{a}	4.6 ± 0.5^{a}
Camel	3.4 ± 1.1^{c}	3.5 ± 1.1^{c}	3.1 ± 1.1^{c}
Sheep	3.5 ± 1.3^{c}	3.5 ± 1.2^{c}	3.4 ± 1.2^{c}
Goat	4.0 ± 1.2^{b}	4.0 ± 1.1^{b}	4.0 ± 1.0^{b}

a ,b, c Means in the same column with superscript letters in common are significantly different ($p < 0.05$)

5- like very much, 4- like slightly, 3- neither like or dislike, 2- dislike slightly, 1- dislike very much

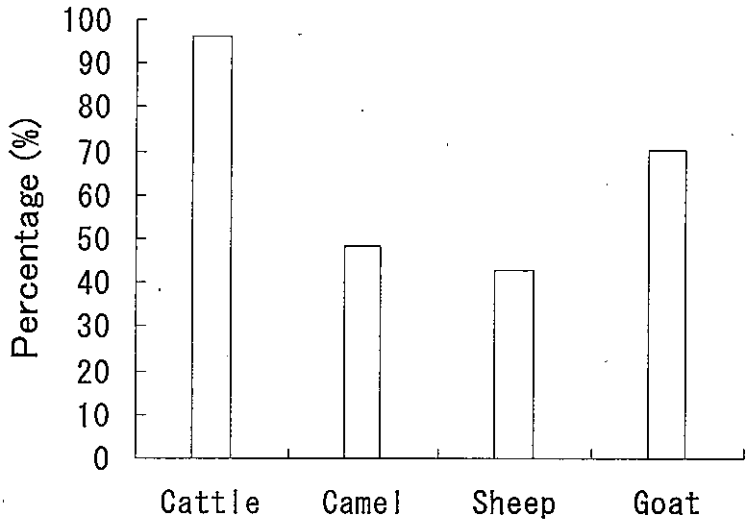

Fig 1-1. Consumer choice for 4 types of dried meat

Table 1-2. Chemical composition of dried meat from 4 species (%, means ± ST for n=3)

	Cattle	Camel	Sheep	Goat
Moisture	10.83 ± 2.27^{a}	14.60 ± 5.24^{a}	11.53 ± 4.12^{a}	9.57 ± 5.74^{a}
Protein	68.50 ± 8.35^{a}	70.83 ± 4.65^{a}	66.07 ± 5.60^{a}	52.07 ± 10.17^{b}
Lipid	18.23 ± 8.26^{b}	12.47 ± 10.02^{b}	19.00 ± 3.70^{b}	38.37 ± 10.77^{a}
Ash	3.43 ± 0.60^{a}	3.43 ± 0.25^{a}	3.47 ± 0.38^{a}	2.47 ± 0.61^{b}

a ,b Means in the same row with superscript letters in common are significantly different ($p < 0.05$)

Table 1-3. Lipid oxidation in dried meat derived from various animals. (Means ± ST for n=3)

Dried meat	TBARS value (mg/kg)	Hexanal value (intensity*)
Cattle	2.96 ± 0.65^{c}	83.2 ± 22.3^{a}
Camel	6.15 ± 0.66^{a}	79.3 ± 52.6^{a}
Sheep	4.44 ± 0.43^{b}	242.2 ± 84.1^{a}
Goat	2.59 ± 0.31^{c}	233.4 ± 194.6^{a}

a, b, c Means in the same column with superscript letters in common are significantly different ($p < 0.05$)

*Intensity expressed by peak area relative to that of 105.4 pmol of the 1, 2 dichlorobenzene internal standard

Chapter 2

Microbiological analysis of fermented sausages without starter culture

2.1. Introduction

Fermented dry sausages is defined as a mixture of comminuted fat and lean meat, salt, curing agent, sugar and spices, which is stuffed into casings, subjected to fermentation and then allowed to dry (Hugas and Monfort, 1997). Fermented meats are those that have been subjected to a combination of chemical curing, microbiological fermentation and drying, which then makes them safe to eat for several months after production .Most products are exposed to natural contamination of the raw materials that occurs during animal slaughtering and that increases during the manufacturing process. In meat products, this contaminating microflora colonises the processing unit's environment and the products in a continuous symbiotic exchange. Such microflora includes useful microorganisms for the fermentation and flavour of sausages, as well as lactic acid bacteria and sometimes even pathogenic bacteria. Many studies have reported microbiology of traditional fermented sausages in many parts of the world (Ming- Tsao Chen and Shiu-Lan Guo, 1992; Drosinos, 2005; Ferreira *et al.,* 2006). However, in Mongolia, there are no studies on dry fermented sausage because there is still no dry sausage.

2.2. Objectives

This study attempted to make fermented dry sausage with different meat in

Mongolia and to determine their microbiological characteristics.

2.3. Material and methods

2.3.1. Sources of materials

Meat and Fat: Five types of meat, which included beef, mutton, horse, goat and camel meat and caudal fat of sheep were collected from local meat market of Mongolia.

2.3.2 Making of sausages

Sausage mixture was prepared by mixing each meat with caudal fat in a proportion of 85/15. Other ingredients were added (w/w) as follows: 2% of salt, 1% of glucose, 0.6% of sugar, 0.2% of curing agent, 0.5% of black pepper, 0.5% of grain pepper, 0.3% of onion powder as well as 0.2% of garlic powder, and then stored at -20^{0}C until processing. Ingredients were mixed in bowl chopper and then stuffed in collagen casing. Sausages without starter culture were ripened at room temperature for 3 weeks.

2.3.3. Microbiological analysis

After aseptically removing and discarding outer casing of each sausage , 10 g of the edible part were aseptically taken and homogenized with 90 ml sterilized saline (0.85% NaCl) using physcotron NS－５１ (Microtec. Co., LTD., Japan), thus making a 1/8 dilution. Successive decimal dilutions were prepared by mixing 1 ml of the previous dilution with 9 ml sterile saline. A 1 ml volume of each dilution was inoculated in plates and then growth medium was added to them. Total aerobic microflora was enumerated in Standard Plate Count agar (SPC agar, Eiken chemical Co Ltd., Tokyo, Japan), after incubation at 37°C for 48 h; lactic acid bacteria in De Man, Rogosa, Sharpe agar (MRS agar, Oxoid, England), after incubation at 37°C for 72 h; Coliform group in Chromocult Coliform agar (Merk, Germany), after incubation at 37°C for 24 h; Staphylococci in

Mannital salt agar (Eiken, Japan), after incubation at 37°C for 48 h; Salmonella in DHL agar (Merck, Germany) after incubation at 37°C for 48 h. After the incubations, the plates with 30- 300 colonies were counted that expressed as $\log_{10}$ of CFU per gram.

2.3.4. Physico chemical analysis

The pH determination was performed using a pH meter (TOA pH METER HM-5S) and the measurements were performed in triplicate. Five grams of the sample was blended with 45 ml of distilled water and the sample homogenate was filtered through Toyo filter paper No.5A before taking a reading.

Dishes containing the samples (2 - 3 g) were placed in the oven and dried at 125°C for at least 24 hours by which time a constant weight should have been reached. The moisture was calculated by the following formula:

Moisture content (%) = weight of sample after drying / test sample weight x 100

Water activity (a_w) was determined using a hydrometer (Gunze Sangyo) at 20°C.

2.4. Statistical analysis

Comparison of means among different types of dry sausage was performed by ANOVA. All statistical analyses were performed using SPSS 12 package (SPSS Inc., Chicago, USA, 2003).

2.5. Results and discussion

A study on the absence and presence of the target food spoiling bacteria such as total aerobic bacteria, lactic acid bacteria, staphylococcus, salmonella and E.coli is required to evaluate microbial safety of fermented sausage produced in Mongolia. The data obtained from microbial analysis will be in the development of next trial for dry fermented sausage processing. Data of bacterial counts on plate count agar, MRS agar, Mannital salt agar, Chromocult coliform agar and DHL agar are shown in Table 2.1.

Counts of total aerobic bacteria and lactic acid bacteria were significantly higher while coliform bacteria were significantly lower in camel sausage than in other sausages. Lactic acid bacteria dominated in sausages except horse sausage in which staphylococci dominated. Coliform bacteria appeared in all sausages, ranging from 2.7 log CFU/g to 4.6 log CFU/g. Bacterial growths on DHL agar were loaded one time in triplicate microbiological analysis of fermented sausages made by beef and goat that had 4.6 log CFU/g and 2.5 log CFU/g, respectively. According to these results, undesirable bacteria may be appearing in raw sausages ripened without starter culture. This depends on many reasons. Used meats in this study were selected from meat market where meat produced by individual producers who may not be able to adequately control an environmental food safety hazard without cooperative effort. In addition, dry fermented sausages were processed in experimental room of University where poor hygiene and inadequate drinking water may have helped to increase the microbial load in sausages. Another reason might be due to lower lactic acid bacteria counts and higher pH values (Table 2.2) in sausages, therefore, their antimicrobial activity is too low to play a significant role as preservative as compared to other sausages as a previously reported (Benito *et al.*, 2007). Sausage made from beef showed significantly high counts of coliform bacteria as compared to other sausages. Cattle are a possible source of *Escherichia coli,* as reported by Chapman *et al.* (1993). As a consequence, food poisoning bacteria may be incorporated into meat products during manufacture.

In this study, low water activity and low moisture content viewed in Table 2.3 were evaluated and may have depended on lack of humidity in environment of ripening. The typical characteristics of naturally fermented sausages are influenced by extrinsic (temperature, relative humidity and air circulation) factors, as previously reported

(Dezacki, 1979; Dincer, 1982).

2.5. Conclusion

In conclusion, fermented sausage without starter culture made with meat from poor hygiene processing, and ripened in ambient temperature can give undesirable results on microbiological characteristics.

Table 2-1. Microbiological characteristics of 5 types of fermented sausage (mean $\log_{10}$ CFU g^{-1} ± SD for n=3)

	PCA	MRS agar	MSA	Chromocult agar	DHL agar
Cattle	5.3 ± 0.1 [b]	5.4 ± 0.3 [c]	4.1 ± 0.2 [b]	4.6 ± 0.2 [a]	3.5
Camel	7.2 ± 0.3 [a]	7.1 ± 0.3 [a]	4.2 ± 0.3 [b]	2.7 ± 0.7 [c]	ND
Horse	5.6 ± 0.3 [b]	5.2 ± 0.3 [c]	5.7 ± 0.3 [a]	3.8 ± 0.1 [ab]	ND
Sheep	5.6 ± 1.1 [b]	6.1 ± 0.4 [b]	4.4 ± 0.2 [b]	3.8 ± 0.5 [ab]	ND
Goat	6.5 ± 0.2 [a]	6.7 ± 0.2 [a]	4.6 ± 0.6 [b]	3.3 ± 0.3 [bc]	2.5

ND, none detected (< 30 CFU ml^{-1})

a ,b, c Means in the same column with superscript letters in common are significantly different ($p < 0.05$)

Table 2-2. pH of dry sausages made with different meat (Means ± ST for n=3)

Cattle	5.83 ± 0.07^{c}
Camel	5.53 ± 0.09^{d}
Horse	6.59 ± 0.03^{a}
Sheep	6.04 ± 0.05^{b}
Goat	5.75 ± 0.03^{c}

a ,b, c, d Means in the same column with superscript letters in common are significantly different ($p < 0.05$)

Table 2-3. Water activity and moisture of dry fermented sausages produced with different meat

	a_w	Moisture (%)
Cattle	0.792 ± 0.021	22.11 ± 1.80
Camel	0.747 ± 0.013	21.42 ± 1.29
Horse	0.762 ± 0.031	21.94 ± 2.74
Sheep	0.728 ± 0.009	19.72 ± 0.93
Goat	0.809 ± 0.003	27.40 ± 0.13

Values are expressed as mean ± SD for n=3

Chapter 3

Quality of fermented sausage with mould starter cultures

3.1. Introduction

The isolated indigenous microflora from traditional meat products are used as starter cultures with aim to reduce the ripening time as well as ensuring the quality and aroma of dry sausages. These microorganisms are well adapted in the meat environment and are capable of dominating the microflora of products. Nowadays, commercial starter cultures, which consist of one or several strains of lactic acid bacteria (LAB), Micrococcaceae, or a combination of both have been used in fermented meat products (Geisen *et al.*, 1992), to improve the quality and safety of the final product and the standardizes the production process (Hugas and Monfort, 1997; Lucke, 2000). In addition, mould starter culture is applied to sausage that improves safety and quality in products by preventing spontaneous colonization with unwanted mould, yeasts and bacteria (Lucke and Hechelmann, 1987), and delaying rancidity as well as stabilizing the colour through catalase activity, oxygen consumption and protection against light (Lucke and Hechelmann, 1987; Bruna *et al.*, 2001). However, their activity is not clearly studied in meat environment.

3.2. Objectives

In this study, different mould starter culture was used in sausages which contain SP 318 commercial starter culture and their quality characteristics were determined.

3.3. Materials and Methods

3.3.1. Sources of materials

Meat and Fat:

Ground pork meat and pork back fat were obtained from a meat packer 1 day after slaughter in a local area of Hokkaido, Japan, and stored at -30°C until use.

Commercial Starter cultures:

Commercial starter culture SP 318 (TEXEL, France) which contains *Lactobaccilus sakei, Pediococcus pentosaceus, Staphylococcus xylosus and Staphylococcus carnosus.*

Surface cultures are showed as follows:

PNT starter culture (TEXEL, France) containing *Penicillium nalgiovensis*

NEO starter culture (TEXEL, France) containing *Penicillium nalgiovensis* and *Penicillium candidum.*

LEM starter culture (TEXEL, France) containing *Penicillium nalgiovensis* and *Debaryomyces hansenii.*

3.3.2. Preparation of fermented sausage

Pork meat to pork back fat ratio of 85:15 was kept at -20^{0}C after mixing it with the following additives (% w/w): 2% of salt, 1% of glucose, 0.6% of sugar, 0.2% of curing agent, 0.5% of black pepper, 0.5% of grain pepper, 0.3% of onion powder and 0.2% of garlic powder. Thereafter, the product was thawed at 4°C before processing. The mixture was processed in mincer and inoculated with a starter culture of SP- 318 (0.1g/kg). Then, the mixtures were stuffed into natural casing (4.0 × 26 cm), and divided into four pieces, which were used to prepare four separate parts of fermented sausages that were manufactured in the following order: part1 (control) consisted of the initial mixture alone and part 2, 3 and 4 which were like part 1 but were superficially inoculated with starter culture of PNT, NEO and LEM, respectively, after fermentation

at 20° Cand 80% relative humidity for 3 days in ripening cabinet. After this, the temperature was decreased to 15°C from fourth day. Finally, the sausages were dried at 15°C, 65-75% relative humidity until the end of ripening, resulting in a total of 35 days of processing.

3.3.3. Physico- chemical analysis

The pH value, water activity and moisture content were determined using methods that were described in chapter 2.

NO_2 determination:

Reagents:

Sulfanilamide reagent: I dissolved 0.5 g of sulfanilamide ($NH_2C_6H_4SO_2NH_2$) in 20 ml of distilled water and 20 ml of hydrochloric acid. Thereafter, 40 ml of methanol was added to this solution, mixed thoroughly, and then stored in the brown bottle, in a cool place protected from light.

Naphthylethylenediamine reagent: Forty milliters of distilled water, 0.3 g of N-(1-naphthel) ethylenediamine ($C_{10}H_7NHCH_2$ CH_2NH_22HCl, Wako Pure. Chemical Industries.LTD, Japan), 1 ml of hydrochloric acid as well as 59 ml of methanol were mixed thoroughly and subsequantly kept in brown glass bottle in a cool place protected from light

Ten percent ammonium acetic acid: To prepare this solution, 100 g CH_3COONH_4 was dissolved in distilled water and the volume adjusted to1.000 ml and the pH to 9.0 with 0.1 M NaOH solution.

Standard solutions.

Dissolved 0.150 g $NaNO_2$ in about 500 ml distilled water and then adjusted the

volume to 1 litre with distilled water to have 100 ppm solution. The volumes 2, 5, 10, 15 and 20 ml were taken from this solution and then diluted to 100 ml to obtain 0.02, 0.05, 0.1and 0.2 ppm of extract, respectively. A 3 ml of aliquots were taken 4 times from each of above solutions and then dispensed in to each of the 4 tubes. Then, 0.3 ml of sulfanilamide reagent was added to 3 of the 4 tubes, and then 0.3 ml of naphthylethylenediamine reagent was added. To another one tube, 0.3 ml of distilled water was added, and after 20 min, the absorbance was measured at 540 nm using spectrophotometer (U best-50).

Determination of sample

Five grams of the sample and 30 ml of hot water were homogenized to get a homogeneous mixture, and then 5 ml of 0.5N NaOH as well as 5ml of 12% $ZnSO_4$ were added to the mixture, and kept at 80°C for 20 min. After cooling at room temperature, 5 ml of 10% CH_3COOH as well as distilled water were added to give a total volume of 100 ml. The entire mixture was transferred in to a centrifuge tube and centrifuged at 3,600 rpm for 25min. A 3 ml of sample filtrate was pipetted into each of the tubes and then 0.3 ml of sulphanlic acid reagent was added to these tubes and left to stand at room temperature for 5 min. A 0.3 ml of α-naphthylamine was then added, and allowed to stand for about 20 until the colour changes to pinkish – red. The optical densities were measured at a wavelength of 540 nm by a spectrophotometer (U best-50, Japan Spectroscopic Co. LTD), using distilled water blank.

Nitrite content was calculated from the calibration curve below.

NO_2 (ppm)= 1.05627026y-0.00869733 x n

y : The absorbance of sample (OD)

n : Dilution factor

Samples for determination of peptides and free amino acids were prepared according to the procedures described by Mikami *et al.* (1994).

Sausage homogenate was prepared by mixing a 10 g of sausage with 90 ml distillate water and then filtered to get the extract. A 2% trichloroacetic acid (TCA) solution was prepared by mixing 4 g of extract of sausage with the same weight of 4% TCA solution. It was incubated for 30 min at 37°C and centrifuged at 4500x g for 10 min. The supernatant was then filtered with Toyo filter paper No.5C. The filtrate was used for analysis of peptides and free amino acids. Peptide content was determined by the Lowry-Folin method (Lowry et al., 1951) using bovine serum albumin as a standard as follows:

Two point four milliliters of distilled water, 0.5 ml of 2% TCA- sausage extract and 1 ml of Lowry solution (2% Na_2CO_3, 0.1N NaOH; 2.7% $KNaC_4H_4O_6$ $4H_2O$) were pipetted into the distillation tubes using pipetman, and incubated in a water bath at 37°C for 15 min. The samples were taken, and 0.1 ml of 1N Folin was added and then warmed in a water bath again for 30min at 37°C. The absorbance of the solution was then measured at absorbance of 660 nm using an UV/VIS Spectrophotometer (Ubest-50, Japan Spectroscopic Co., LTD {JASCO}). The peptide content was calculated using standard formulas with bovine serum albumin as a standard.

Peptide content was calculated using the formula:

Peptide content = 0.09009 Y – 0.008243

Y: The absorbance of sample (OD, nm)

Free amino acid content was determined by using the OPA (o- Phthalaldehyde) reagent with amino acid analyzer (JASCO, Model 8000) using the lithium citrate buffer system.

Lithium citrate buffer system was prepared as follows:

1 st Buffer (0.15 N Li^+, pH 2.97)

2 nd Buffer (0.20 N Li^+, pH 3.28)

3 rd Buffer (0.30 N Li^+, pH 4.25)

4 th Buffer (0.90 N Li^+, pH 3.75)

5 th Buffer (1.23 N Li^+, pH 4.55)

6 th Buffer (0.3 N LiOH)

All Buffers were filtered using 0.45μm filter paper before analysis.

Preparation of OPA and HYPO buffers:

To prepare OPA buffer, 24.7g of H_3BO_3, 23g of KOH, 1.6g OPA and 10ml of methanol were dissolved in 980 ml of distilled water. The solution was filtered by 0.45μm filter paper, and 2.0ml of 2, 2'-Thiodiethanol as well as 4.0ml of 15% Brig-35 were added to the filtered solution.

To prepare HYPO buffer, 24.7g of H_3BO_3 and 23g of KOH, were dissolved in 980 ml of distilled water, and the resulting solution was filtered by 0.45μm filter paper. Both buffers were stored at 4^0C until using for the analysis.

Analysis: Free amino acid contents analysis was carried out using a fully automated amino acid analyzer (JASCO Model 8000 series) and was determined by the OPA reagent method. A 20 μl of 2% TCA – sausage extract was injected in a JASCO 851- AS. The separation of peaks was done in a JASCO 860 column Oven at 40°C. The amino acid standard was analyzed to monitor the retention time of peaks. The retention time of amino acid compounds were compared with those of the standard compounds for reliability of the amino acid compounds.

The calculation formula used in this procedure is presented below as,

Free amino acid content = Ax B (nmol) where

A is the molecular weight of amino acid, and

B is the detection content of each amino acid determined as the area of each amino acid of sausage divided by the area of each amino acid of standard.

3.3. 4. Determination of bacterial numbers

Sampling procedures and methods for determination of bacteria number were similar to those described in the previous experiment (see chapter 2).

3.3.5. Lipid oxidation assay

Samples

Lipid oxidative stability was determined in sausages mixtures divided into three batches each weighing 2 kg each. Commercial starter culture containing *Staphylococcus carnosus* and *Lactobacillus sakei* (F-SC-111 Bactoferm^{TM}, Chr.Hansen) was added in two parts simultaneously with one of them was superficially sprayed by mould starter culture(PNT starter culture, M-EK-6 Bactoferm^{TM}, Chr.Hansen). The third batch (control) consisted of the initial ingredients, seasoning and curing salts.

Reagents

To make acetic acid solution, 200 ml of acetic acid was dissolved in distilled water to get a final volume of 1000 ml and then the pH of this solution was adjusted to 3.5 with 10N NaOH. Next, TBA reagent was prepared by dissolving 0.8 g of 2-thiobarbituric acid in distilled water to get a final volume of 1000 ml. For BHT solution, 0.2 g of BHT was weighed and then dissolved in 25 ml of acetic acid. Additionally, SDS solution was prepared by dissolving 20.25 g of sodium dodecyl sulfate in approximately 200 ml distilled water and then the volume adjusted to 250 ml using distilled water. Finally, to prepare n- butanal: pyridine (15:1) solution, 450 ml of

n- butanol was mixed with 50 ml of pyridine.

Procedure

Five grams of the sample was added into a test tube. Thereafter, 1.5 ml of acetic acid solution, 1.5 ml of TBA reagent, 0.05 ml of 0.8% BHT solution, 0.2 ml of 8.1% SDS solution and 1 ml of distilled water were added to this sample. The test-tube was covered and stored at 5 °C for 60 min. After that, the sample was exposed at 90 °C for 60 min, and then cooled at room temperature. After cooling, 1 ml of distilled water and 5 ml of n- butanol:pyridine solution were added to cooled solution and the tube was then centrifuged at 3000 rpm for 10 min. The clear and colored supernatants were transferred into a curvette, and absorbance was measured at 535 nm using spectrophotometer (V-630 vio, Japan). The TBARS value was calculated based on the molar absorptivity of MDA (156,000 M^{-1} cm^{-1} at 535 nm) and the results were reported in mg MDA/kg of the sample.

3.3.6. Sensory evaluation

This was carried out at the end of ripening by a panel of 78 testers who related to students and lecturers at the Obihiro University. Each sausages were sliced to approximately 2 mm of thickness, and then assessed for colour, smell, sour, taste and overall according to the following 5 point hedonic scales (5 = excellent; 4 = good; 3 = acceptable; 2 = fair and 1 = unacceptable) .

3.4. Results

Growth of total aerobic microflora, lactic acid bacteria and coliform bacteria during the drying process is presented in Table 3.1. In all sausages, the microbiota and

lactic acid bacterial counts were similar during the ripening. Lactic acid bacterial counts were approximately 6.8 Log CFU/g, and increased rapidly during the first few days of ripening. They remained at the same number during fermentation period with values between 8.9 - 9.3 Log CFU/g at the end of the process. Undesirable bacteria were not detected in all sausages during ripening except for Coliform group, which was observed during the 2 weeks required for ripening to occur, with the values ranging from 6.3 Log CFU/g to 4.2 Log CFU/g. However, coliform group were absent from the 3 rd week to 5th week.

Results of pH in the sausages are shown in Table 3.2. The pH values of the sausages decreased in 7days from an initial value of 6.12 to between 4.46 and 4.55 but slightly increased to about 4.63 - 4.70 by the end of the process. Low pH led to the breakdown of nitrite resulting in nitrite residue of about 5.8 – 7.4 ppm at the end of ripening in all sausages (Table 3.3). Evaporated moisture and decreased water activity were also observed in sausages both with mould inoculated and without (Table 3.4, 3.5). Moisture content decreased throughout the ripening process form initial values of 62.01 % to 25.85 % – 28.28 %.Water contents in sausages at end of processing ranged from 0.798 to 0.808. The content of peptide in the sausages during processing is shown in Fig 3.1. The peptide content increased during the fermentation and drying process, it was slightly higher in control than in all sausages with mould.

The total free amino acid and each free amino acid are shown in Table 3.6. At the end of ripening, control had slightly higher content of free amino acid. Among individual free amino acids of all sausages, high level was shown by Glu and Lys while Pro and Arg were not detected at 5 weeks.

Table 3.7 shows the results of the descriptive sensory analysis. All sausages, including

control had good scores in all the evaluated properties.

The effect of starter cultures on the TBA values of the fermented sausage is shown in Table 3.8. The TBA values of the sausage with starter cultures slightly increased from 3.26 mg/kg to 3.40-3.70 mg/kg during the one week ripening, and then decreased to 1.28-1.57 mg/kg at end of ripening. For control, the TBA value initially increased from 2.84 mg/kg to 8.23 mg/kg at end value of ripening.

3.5. Discussion

Added SP-318 in sausage mixtures and inoculated 3 types of mould starter cultures on casing on the third day of fermentation were tested for their ability to accelerate the ripening process, and improve the safety of product through microbial, chemical and sensory characteristics. The results of the microbiological analysis showed that the lactic acid bacteria was the major microflora from the beginning of ripening, since the cell numbers of the total viable count and lactic acid bacteria count were similar in all samples as occurs in other fermented sausages (Bruna *et al.*, 2003) . This is because of the good adaptation of lactic acid bacteria to the meat environment, and their faster growth rates, which are displayed in fermentation with carbohydrate used in the processing of sausages. Fermentation is known to promote the growth of lactic acid bacteria (Demeyer *et al.*, 1986). In the present study, Coliform bacteria were observed from day 0 to the 2nd week, and then became negative on the 3rd week. Adding starter culture accelerated the formation of lactic acid during the processing of fermented sausages leading to drop in pH of the products thus inhibiting the growth of undesirable bacteria which could still be not completely killed during the 2 weeks of ripening. A wide variety of microrganisms such as *Salmonella* and *E.coli* has already been found in

fermented sausages (Ferreira et al., 2006). The pH values of the sausages rapidly decreased during the 7days but slightly increased by the end of the process. This result is in agreement with finding of other authors who reported that lactic acid bacteria are the major producers of lactic acid responsible for the decrease in pH and the increase in acidity during the fermentation (Benito *et al.,* 2007). Another reason is that accumulation of acid and decrease in pH during fermentation denatures proteins leading to an increase the release of amino acids. Kato *et al.* (1994) concluded that the protein degradation in the lactic fermented pork muscle was caused by proteases that originated from meats with the lactic acid fermentation acting as the enhancing effector. Likewise, Molly *et al.* (1997) suggested the responsibility of both, meat and bacterial proteinases, in the ripening and flavour generation in dry fermented sausages. Therefore, the increased in pH, may be together with decrease in the concentration of amino acids in sausages, which is responsible for the increase in ammonia concentration. Similar results have been reported by Dierick *et al.* (1974) and Huang and Lin (1992).

Peptide content and total free amino acid increased during the ripening in all samples but control was higher than mould inoculated sausages. This results indicated that amino acids could be transformed into amines, α -ketoacids or other amino acids via decarboxylation, deamination or transamination, respectively, by microorganisms growing on surface of product such as Brevibacterium linens and Pseudomonas, and Penicillium roqueforti (Hemme *et al.*, 1982). For the analysis of the individual amino acids, Gly and Lys concentrations increased but those of Arg and Pro decreased with the final product having no amino acid. This result suggested that Arg and Pro might be further decarboxylated into biogenic amines. Water content and water activity were decreased during ripening in all samples. The reason for higher moisture losses is most

probably due to the longer ripening period of dry sausages, in the present study (35 days) than that of in industrial scale sausages. Therefore, no marked differences were observed in moisture losses between the control and mould inoculated sausages, which are agreement with the pH values of the sausages. In this study, nitrite content greatly decreased in all samples. The decrease was related to high reactivity of nitrite (Cassens *et al.*, 1979), which converted to red nitrosylmyoglobin which is the normal pigment of uncooked cured sausage (Pegg and Shahidi, 1997).

Different starter cultures are known to result in different flavour compounds in dry sausage (Berdague *et al.*, 1993; Montel *et al.*, 1996). However, in present study, sensory scores of dry sausages fermented with superficially inoculated and non inoculated were almost identical. It is possible that due to their similar genetic background the SP-318 starter culture used in the present study did not result in different sensory profile. This is in consensus with the results of Meynier *et al.* (1999) who identified 80 aroma compounds from Milano salami from which over 60% of the compounds were identified to be of spice origin, while 19% came from lipid oxidation, 12% from amino acid catabolism and only 5% from fermentation processes.

The oxidative stability of fermented sausages during ripening process was evaluated by the TBA procedure. Results clearly demonstrate that starter culture including staphylococci had a major impact on the stability of products. Thus, the low TBARS values at the ripening time can be attributed to the barrier effect developed by the strains of staphylococci in starter culture. Staphylococci has catalase activity, thus prevented the lipid oxidation in products (Barriere *et al.*, 2001).

3.6. Conclusion

In conclusion, interior starter culture led to most of the chemical changes in all sausages compared with the superficial colonies of mould inoculated sausages. This is because mould starter cultures were only applied to the surface of the sausages so their action was limited. However, combined effects of both exterior and interior starter cultures could prevent the undesirable micro organisms and lipid oxidation products that contribute to no risk in sausages. This study reports that starter cultures used in sausages may be added to the formulation of fermented sausages, leading to a potentially safer product with no adverse effect on quality. Also, mould starter cultures inhibited growth of undesirable moulds on sausage casing indicating that these starter cultures could used to ensure food safety.

Table 3-1. Microbiological characteristics of dry fermented sausages with mould (mean $\log_{10}$ CFU g^{-1} ± SD for n=3)

		Day 0	Week 1	Week 2	Week 3	Week 4	Week 5
control	1	6.9±0.35	9.37±0.06	9.40±0.17	9.35±0.21	9.45±0.21	9.45±0.07
	2	6.77±0.38	9.33±0.06	9.37±0.06	9.35±0.21	9.45±0.07	9.30±0.14
	3	6.25±0.07	4.45±0.35	4.10±0.28	ND	ND	ND
PNT	1	6.90±0.35	9.27±0.12	9.43±0.06	9.30±0.14	9.30±0.14	9.25±0.07
	2	6.77±0.39	9.27±0.06	9.30±0.00	9.20±0.28	9.45±0.07	9.05±0.07
	3	6.25±0.07	5.15±1.34	4.00±0.00	ND	ND	ND
NEO	1	6.90±0.35	9.20±0.10	9.50±0.10	9.35+0.07	9.30+0.14	9.15±0.21
	2	6.77±0.40	9.13±0.15	9.37±0.15	9.35±0.07	9.25±0.07	8.90±0.28
	3	6.25±0.07	4.45±0.35	4.40±0.00	ND	ND	ND
LEM	1	6.90±0.35	9.17±0.23	9.53±0.12	9.45±0.07	9.35±0.07	9.40±0.28
	2	6.77±0.41	9.03±0.40	9.43±0.15	9.25±0.07	9.15±0.21	9.20±0.00
	3	6.25±0.07	5.10±0.28	4.20±0.00	ND	ND	ND

ND, none detected (< 30 CFU ml^{-1})

1: Total aerobic microflora

2: Lactic acid bacteria

3: Coliform group

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis,*

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii.*

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-2. pH of dry fermented sausages with mould (mean ± SD for n=3)

	Day 0	Week 1	Week 2	Week 3	Week 4	Week5
Control	6.12 ± 0.28	4.55 ± 0.06	4.57 ± 0.10	4.63 ± 0.17	4.65 ± 0.21	4.63 ± 0.14
PNT	6.12 ± 0.28	4.53 ± 0.08	4.59 ± 0.10	4.65 ± 0.13	4.65 ± 0.13	4.67 ± 0.10
NEO	6.12 ± 0.28	4.54 ± 0.10	4.63 ± 0.15	4.69 ± 0.19	4.60 ± 0.11	4.70 ± 0.15
LEM	6.12 ± 0.28	4.46 ± 0.05	4.57 ± 0.08	4.62 ± 0.07	4.61 ± 0.11	4.67 ± 0.09

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii*.

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-3. Nitrite content of dry fermented sausage with mould (ppm, mean ± SD for n=3)

	Day 0	Week 1	Week 2	Week 3	Week 4	Week 5
Control	145.7±2.9	16.0±0.4	10.0±2.0	9.9±1.7	9.7±2.6	6.5±2.8
PNT	145.7±2.9	16.4±3.8	12.3±2.8	10.9±4.0	6.3±0.7	5.8±2.8
NEO	145.7±2.9	16.0±0.9	11.8±3.0	11.5±0.8	7.9±1.0	7.2±2.5
LEM	145.7±2.9	16.9±2.2	10.7±2.3	10.6±2.6	8.1±1.3	7.4±2.2

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii.*

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-4. Moisture of dry sausages with mould (%, mean ± SD for n=3)

	Day 0	Week 1	Week 2	Week 3	Week 4	Week5
Control	62.01± 4.45	46.73± 3.55	39.12± 3.59	36.08± 3.93	30.37± 1.84	28.28± 3.25
PNT	62.01± 4.45	49.32± 5.95	38.58± 2.88	34.71± 2.02	27.92± 3.67	26.96± 1.74
NEO	62.01± 4.45	49.41± 6.37	38.86± 1.85	34.41± 2.65	30.23± 1.86	27.05± 1.93
LEM	62.01± 4.45	49.72± 3.25	41.88± 2.65	34.23± 1.64	29.89± 2.56	25.85± 0.84

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii.*

SP318*: P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-5. Water activity of fermented sausages at end of ripening

Control	0.808 ± 0.021
PNT	0.799 ± 0.022
NEO	0.798 ± 0.032
LEM	0.804 ± 0.023

Values are expressed as mean ± SD for n=3

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii.*

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

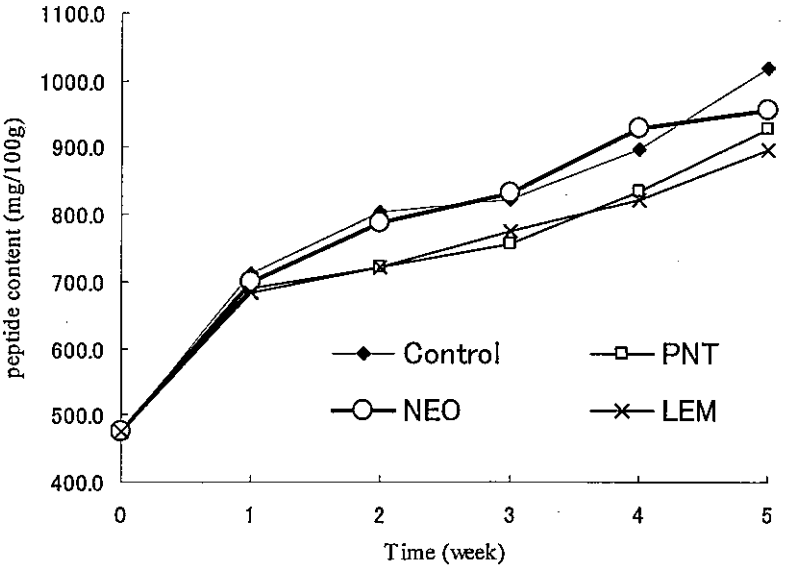

Fig 3.1 Peptide content of dry sausages with mould

Control: Starter culture SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii*.

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-6. Free amino acid of fermented sausages (mg/100g)

	Control	PNT	NEO	LEM
Asp	25.8	30.8	33.5	48.3
Thr	42.3	36.1	36.9	35.4
Ser	41.2	32.4	43.7	30.2
Asn	53.0	47.2	45.4	44.0
Glu	228.2	208.7	211.5	208.7
Pro	0.0	0.0	0.0	0.0
Gly	70.7	55.5	60.7	55.6
Ala	128.4	103.6	112.4	102.5
Val	64.5	60.1	58.2	62.0
Met	27.2	23.8	22.0	22.2
Ile	40.3	39.1	37.9	39.0
Leu	86.6	81.3	78.6	81.6
Tyr	39.5	38.0	36.6	37.8
Phe	50.5	48.6	45.8	47.9
Lys	124.8	120.6	118.2	123.4
His	30.5	29.0	28.3	29.1
Arg	0.0	0.0	0.0	0.0
Total	1053.2	954.4	969.3	967.4

Values are expressed as mean for n=3

Table 3-7. Sensory evaluation of fermented sausage with mould (mean ± SD for n=3)

	Colour	Smell	Sour	Taste	Overall
Control	3.57 ± 0.77	3.08 ± 0.90	3.55 ± 1.00	3.23 ± 0.90	3.27 ± 0.91
PNT	3.42 ± 0.77	2.97 ± 0.88	3.14 ± 0.82	3.11 ± 0.77	3.12 ± 0.70
NEO	3.30 ± 0.72	2.90 ± 0.87	3.10 ± 0.85	3.30 ± 0.83	3.20 ± 0.71
LEM	3.43 ± 0.74	3.08 ± 0.74	2.96 ± 0.95	3.16 ± 0.74	3.32 ± 0.66

Data are expressed as an average of 78 panelists and SD.

5: excellent, 4: very good, 3: good, 2: fair, 1: poor

Control: Starter culture—SP318, PNT1: SP318 + *Penicillium nalginovensis*,

NEO: SP318 + *P.nalginovensis* + *P.candidum*, LEM: SP318 + *P. nalginovensis* + *Debaryomyces hansenii.*

SP318: *P. pentosaceus* P208, *Lactobacillus. sake* L110, *Staphyloccus. carnosus* M72, *S. xylosus* M86.

Table 3-8. TBARS values of fermented sausages (mg/kg、 mean ± SD for n=3)

Ripening time	Control	Sausage with bacterial starter culture	Sausage with both starter cultures of bacterial and mould
Week 0	2.84 ± 0.62	3.26 ± 0.55	3.26 ± 0.55
Week 1	5.87 ± 0.92	3.40 ± 0.23	3.02 ± 0.34
Week 2	3.10 ± 0.25	1.50 ± 0.17	0.96 ± 0.29
Week 3	8.35 ± 0.68	1.40 ± 0.30	1.55 ± 0.19
Week 4	8.47 ± 0.33	1.33 ± 0.33	1.10 ± 0.18
Week 5	8.23 ± 0.77	1.28 ± 0.19	1.57 ± 0.27

Bacterial starter culture: *Staphylococcus carnosus* and *Lactobacillus sakei*

Mould starter culture: *Penicillium nalgiovensis*

Chapter 4

Effects of food additives on susceptibility of gram negative bacteria derived from fermented sausage

4.1. Introduction

Food additives are used extensively in fermented meat products and are involved in various reactions of meat environment which contributes to the development of taste, texture, consistency, or color. In addition, specific additives used in fermented products have shown important multiple effects for extending shelf life, which is determined by both microbiological (spoilage) and chemical (oxidation and physical) deterioration. Lactic acid bacteria (LAB) or their production and antioxidants can be given as examples.

Numerous studies have reported on the spoilage of meat and fermented meat products by pathogenic gram negative bacteria, including *Escherichia coli* (Glass. Kathleen *et al.*, 1992; Ferreira *et al.*, 2006) and *Salmonella* (Buchanan and Whiting, 1998; Escartin *et al.*, 1999), which produce toxins and cause several diseases in humans (Doyle and Schoeni, 1984; Padhye *et al.*, 1992; Centers for Disease Control, 1995; Pearse *et al.*, 2004). In addition, lipid oxidation in foods could also pose a human health risk (Pearson *et al.*, 1983; Kubow, 1992). To minimize those risks, desirable bacteria which produce antibacterial compounds and food additives are added to fermented meat products. There are many scientific reports about the positive effects of food additives such as antioxidants (Bozkurt and Erkmen, 2002; Ansorena and Astiasaran, 2004; Lee and Kunz, 2005; Bozkurt, 2006), curing agents (Noil *et al.*, 1990; Olesen *et al.*, 2004) and lactic

acid bacteria as starter culture (de Vuyst and Vandamme, 1994; Coffey *et al.*, 1998; Niku-Paavola *et al.*, 1999; Lucke, 2000; Kalalou *et al.*, 2004;) in fermented meat products. However, their correlation to such effects as well as the nature of their specific positive actions in the fermented products are not yet clear, especially against food pathogenic gram negative bacteria. Gram negative bacteria are highly resistant to foreign molecules such as antimicrobial compounds of LAB (Nikaido, 1989; Gao *et al.*, 1999) due to an effective barrier function of the outer membrane (Nikaido, 1994) which is absent in gram positive bacteria .

4.2. Objectives

The aim of this study was to establish a method for reducing the number of gram negative bacteria either through the use of food additives like lactic acid, BHA, BHA/BHT and curing agent alone or in combination with antimicrobial compounds produced by LAB.

4.3. Materials and Methods

Food additives: Synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydrooxytoluene (BHT) were purchased from MP Biomedicals (France) and Nacalai Tesque (Kyoto, Japan), respectively. Lactic acid was obtained from Kanto Chemical (Tokyo, Japan). Curing agent (a mixture containing 5% $NaNO_2$, 10% KNO_3 and 85% NaCl) was obtained from Chiyoda Industry Co. Ltd., Tokyo, Japan.

Experimental design: Sterile nutrient broths (Merck, Germany) were prepared with each of the following: 3 BHA concentrations (0.01%, 0.05%, 0.1%), BHA/BHT combination (0.01% BHA+0.01% BHT), 2 lactic acid concentrations (0.05%, 0.1%), curing agent (0.2%) or a negative control. Gram negative bacterial suspension was

prepared as described below and was inoculated in broth described above to a final concentration of 1% and then these broths were incubated at 30°C for 18 h. Thereafter, each of the culture broths was divided into three 9 ml portions and aliquots of 1 ml from each cell-free supernatant (CFS) prepared as described below were added to two portions and incubated for another 24 h period at 30°C, whilst the third portion served without CFS. The two portions with CFS were used to determine the combined effects of food additives and CFS at different pH on gram negative bacteria isolated from fermented sausage. After both of the incubation periods above, viable counts of gram negative bacteria were enumerated using the direct plating method on MacConkey agar. Furthermore, immediately after each of the two incubations, pH values were measured with a pH meter (HM -5S, TOA Electronics Ltd, Tokyo, Japan). These experiments were performed in triplicate.

Preparation of bacterial inoculum: Ten grams fermented sausage recovered at the end of processing was analyzed for the presence of gram negative bacteria. A homogenized sample (1:10, sample: sterile saline) was used to prepare serial dilutions ($10^{-1}-10^{-3}$) using sterile saline and an aliquot of 1 ml of each dilution was incubated with DHL agar (Merck, Germany). After aerobic incubation of plates for 24 h at 37°C, suspicious 9 colonies were picked from plate containing 2.9 x 10^2 colony counts and subcultured onto nutrient agar (Difco, U.S.A). Thereafter, cell morphology , gram reaction (Gram stain B& M, Merck, Germany), growth on MacConkey agar (Eiken, Japan) and gas production in BGLB broth (Eiken, Japan) were quantified in order to select gram negative bacteria. Bacteria which rod-shaped, gram negative, gas produced and pinkish in colour growth on MacConkey agar were stored in nutrient broth supplemented with 30% glycerol at -20°C until further analysis. Just before the experiment, frozen cultures

were activated in fresh nutrient broth (1%) at 37°C for 24 hours and stored at 4°C. To determine numbers of colony-forming units (cfu) diluted culture was plated on MacConkey agar and incubated at 37°C for 24 h. The refrigerated stationary-phase cells were diluted to a cell density of around 8 log cfu per ml and used as inoculum.

Preparation of crude cell-free supernatant (CFS) from lactic acid bacteria: The *Lactobacillus sakei* D-1001 from MMF-161 commercial starter culture (San-ei Sucrochemical Co.,Ltd, Japan) was cultivated in 100 ml of MRS broth at 30°C. After 72 h, CFS [which contained antimicrobial compounds from the lactic acid bacteria (Daeschel, 1989)] was obtained by centrifugation at 10,000 x*g* for 20 min at 4°C and then filtration through a 0.22 μm pore-size filter (Corning Incorporated Corning 431154, U.S.A.). To determine the effect of pH on the gram negative bacteria cultured with various additives in nutrient broth, we used CFS with 2 pH values. Non-adjusted pH of CFS was 4.0 and CFS adjusted to pH 6.0 by addition of 10N NaOH. Raising the pH was done to mitigate the antimicrobial effects of organic acid produced in the *Lactobacillus* culture.

Statistical analysis Enumerated populations of gram negative bacteria were transformed into $\log_{10}$ CFU per ml for data analysis. Analysis of variance (ANOVA) was applied to the data, followed by Turkey's test when significant differences of $p < 0.05$ and $p < 0.01$ were observed. SAS (Local, XP PRO) was used for the statistical analysis.

4.4. Results

pH The pH changes of broth cultures inoculated with gram negative bacteria and different food additives are shown in Table 5.1. After the initial 18 h incubation, pH

values of culture broth with BHA at different concentrations ranged from 6.19 ± 0.16 to 7.08 ± 0.03. For broths containing 0.05 % or 0.1% lactic acid or curing agent, pH values were 5.09 ± 0.01, 4.28 ± 0.02 and 6.43 ± 0.03, respectively. Compared to the control, significantly lower ($p < 0.05$) pH values were observed in these culture broths with lactic acid, while significantly higher ($p < 0.05$) pH values were observed in culture broth with 0.1% BHA. After additional 24 h incubation, pH changed depending on the presence of CFS at different pH. When compared to broth with or without CFS at pH 6.0, significant reduction ($p < 0.01$) in pH was observed after addition of CFS at pH 4.0. In all culture broths, decreased pH ranged from 4.13 ± 0.03 to 4.78 ± 0.03 after addition of CFS (pH 4.0). Culture broths with or without CFS at pH 6.0 in combination with BHA at various concentrations resulted in 5.80 ± 0.08 – 6.54 ± 0.02 and 6.28 ± 0 .29 – 7.12 ± 0.06 pH ranges respectively. The pH values of culture broth containing lactic acid at 0.05% concentration with and without CFS at pH 6.0 were 5.67 ± 0.06 and 6.07 ± 0.21 respectively, whilst culture broth containing lactic acid at 0.1% concentration with and without CFS were 4.60 ± 0.05 and 4.27 ± 0.03 respectively. Curing agent with or without CFS at pH 6.0 showed pH values of 6.37 ± 0.03 and 7.28 ± 0.09 respectively in culture broths.

Effect of different food additives in 18 h cultures: The populations of gram negative bacteria in nutrient broths containing synthetic antioxidant, lactic acid and curing salt are presented in Table 5.2. For treatment with 0.1% lactic acid, 0.01% or 0.05% BHA and 0.02% BHA/BHT, growth of gram negative bacteria was significantly ($p < 0.05$) reduced by approximately 4.8, 1, 1.3 and 1.5 log units respectively, as compared to the controls. The 0.1% BHA alone completely inhibited the growth of gram negative bacteria in 18 h culture broth at pH 7.08. Incubated culture broths with curing agent at

0.2% and lactic acid at 0.05% showed no significant differences in cell numbers, as compared to the control without additive.

Effect of CFS in 18+24 h culture: The effects of food additives on the susceptibility of gram negative bacteria to antimicrobial compounds from *Lactobacillus sakei* D-1001 were expressed as log-reduction of viable cell counts (Table 5.2). Analysis of this data revealed significant differences ($p < 0.01$) among 18+24 h cultures depending on addition of CFS at different pH. When CFS at pH 4.0 was added, a substantial population reduction was observed in all culture broths including control. No viable cells were detected following use of CFS at pH 4.0 in cultures with either 0.05% BHA or 0.02% BHA/BHT. Viable cell count was markedly reduced to 1.02 ± 0.03 log unit in cultures with 0.01% BHA in combination with CFS at pH 4.0. In contrast, viable counts of gram negative bacteria from 18h incubation were increased in 18+24 h culture broths regardless of presence or absence of the CFS at pH 6.0, except for culture with BHA at 0.05% and without CFS that slightly decreased.

The 0.1% lactic acid reduced the bacterial population to 3.77 ± 0.95 log units in 18 h culture broth and then the cell counts slightly increased to 4.37 ± 0.51 log units after the additional 24 h incubation period without CFS. For this additive, gram negative bacterial population was absent when CFS was added at either pH. On the other hand, when a lower concentration of lactic acid was used, CFS at pH 4.0 reduced cell counts from 8.23 ± 0.06 log unit to 4.0 ± 0.69 log units, a decrease of approximately 4.23 log units. However, the same additive with or without CFS at pH 6.0 showed an increase in cell counts. A similar trend was seen for cultures with curing agent.

4.5. Discussion

This study examined the effects of various food additives on gram negative bacteria in broth. Gram negative bacteria were isolated from fermented sausages made without starter culture. The fermented meat products might be spoiled by pathogens due to absence of a heating step which controls pathogen populations (Rodel, 1992; Gill and Landers, 2003). However, in many cases, spoilage bacteria can be reduced in numbers, either by inhibition or killed by various manufacturing methods such as lactic acid bacteria used in fermentation (Takeo *et al.*, 1994; Coffey *et al.*, 1998; Gonzalez and Diez, 2002; Kalalou *et al.*, 2004). In this study, *Lactobacillus sakei* was selected because is more competitive than other lactobacilli, is most commonly found in fermented sausages (Rantsiou and Cocolin, 2006), showing a shorter lag phase, a higher maximum growth rate, higher final cell density (Dossmann *et al.*, 1996) that contribute to hygienic quality of meat products by producing various antimicrobial compounds including organic acid, hydrogen peroxide, carbon dioxide, diacetyl and high molecular mass compounds like bacteriocins towards undesirable bacteria such as gram positive bacteria (Mataragas *et al.*, 2003). However, Tantillo *et al.* (2002) reported that gram negative bacteria were resistant to antimicrobial activity of *Lactobacillus sakei*. Lactic acid produced by lactic acid bacteria in sausage fermentation reduces pH to a point at which it has a preservative effect. pH values below 5.2 in fermented sausage are favorable for preservation and hygienic stability in fermented sausage (Leistner, 1995). Among all culture additives tested in this study, culture broths with 0.1% lactic acid showed the lowest pH. A pronounced drop in pH value was observed in all culture broths combined with added CFS at pH 4.0.

The increasing BHA concentration demonstrated a predictable trend in decreasing cell counts. BHA has antibacterial effects; however, the 0.1% concentration of BHA which is lethal and effective in total inhibition of gram negative bacterial cells is currently out of the legal regulation in food products (U.S. FDA: www.fda.gov). The combination of this synthetic antioxidant at various concentrations (0.01%-0.05%) and CFS at pH 4.0 has ability to suppress gram negative bacteria. In this study reduction of bacterial numbers was observed in combination of CFS at pH 4.0 with synthetic antioxidant even at the lowest concentration tested (0.01% BHA). It has previously been reported that inhibitory effect of synthetic antioxidant on pathogenic bacteria is due to its ability to disrupt cytoplasmic membranes of cells (Branen *et al.*, 1980; Degre and Sylvestre, 1983; Degre *et al.*, 1983). While there were no previous reports on the effects of a combination of synthetic antioxidant and CFS against gram negative bacteria.

Generally, lactic acid which is produced by lactic acid bacteria in fermented meat products, produces primarily antimicrobial effects against spoilage bacteria (Daeschel, 1989). In our study, the effects of 2 lactic acid concentrations alone or each concentration combined with CFS on gram negative bacteria in broth were tried. The 0.1% lactic acid alone markedly reduced gram negative bacteria in 18 h culture broth, but cells increased in 18+24 h culture broth. The 0.1% lactic acid concentration in combination with each CFS showed no viable cells and 0.05% lactic acid in combination with CFS at pH 4.0 reduced viable cell numbers. Furthermore, we have observed that control with CFS at pH 4.0 was also more efficient than control with or without CFS at pH 6.0. These results were confirmed with several reports published on inhibitory effects of lactic acid (Van Netten *et al.*, 1995; Pipek *et al.*, 2005) and that lactic acid increases the outer membrane permeability of gram negative bacteria

(Alakomi *et al.*, 2000).

Curing agents are commonly used in fermented sausage to aid in flavor development and to prevent lipid rancidity (Noil *et al.*, 1990; Olesen *et al.*, 2004). Curing agent concentration in this study was fixed at 0.2% because of results in our previous experiment (Mikami *et al.*, 2004). No antimicrobial effect was seen with 0.2% curing agent alone; this is similar to the control without additive. However, the combination of 0.2% curing agent and CFS at pH 4.0 seems to kill gram negative bacteria. Leroy *et al.* (1999) suggested that nitrite which is a component of curing agent, has a synergistic action with lactic acid that is commonly found in CFS (Lindgren and Dobrogosz, 1990). This explains our observation whereby gram negative bacteria was susceptible to curing agent when it is combined with CFS containing high concentration of lactic acid.

In the present study, all the investigated combinations at pH 4.78 ± 0.03 or below were effective in inhibition of gram negative bacteria derived from fermented sausage than when used as single components, except for 0.1% BHA which alone had inhibited all viable cells. This is in partial agreement with Leistner *et al.* (1995), who showed that two or more antimicrobial agents acting synergistically at suboptimal levels are more effective than each of them alone at the optimal level.

4.6. Conclusion

This study suggests that randomly used food additives could have an effect on increasing susceptibility of gram negative bacteria to antimicrobial compounds of *Lactobacillus sakei* D-1001, but only in low pH environments that depend on lactic acid concentration. In addition, the food additives used in this study at permitted concentrations by the Food and Drug Administration, in combination with antimicrobial compounds of LAB can be useful in devising control strategies for pathogenic gram

negative bacteria in acidified foods such as fermented meat products. Recently, naturally occurring antioxidants are being replaced by synthetic antioxidants because they exhibit similar antioxidant potency (Ramarathnam *et al.*, 1995; Zein, 2000; Bozkurt, 2006). Further studies are needed to investigate the efficiency of the combination of natural antioxidants and LAB compounds on gram negative bacteria.

Table 4.1. pH of the broth medium inoculated with gram negative bacteria and different additives

(mean ± SD for n=3)

Broth	18 h	18+24 h with CFS at pH 4.0	18+24 h with CFS at pH 6.0	18+24 h without CFS
control	6.28 ± 0.12^{b}	4.74 ± 0.05^{c}	6.20 ± 0.10^{b}	7.09 ± 0.26^{a}
0.01% BHA	6.19 ± 0.16^{b}	4.64 ± 0.04^{c}*	6.04 ± 0.08^{b}	6.70 ± 0.10^{a}*
0.02% BHA/BHT	6.20 ± 0.10^{b}	4.65 ± 0.03^{c}*	6.08 ± 0.04^{b}	6.78 ± 0.18^{a}
0.05% BHA	6.63 ± 0.56^{a}	4.60 ± 0.00^{b}*	5.80 ± 0.08^{a}*	6.28 ± 0.29^{a}*
0.1% BHA	7.08 ± 0.03^{a}*	4.59 ± 0.01^{c}*	6.54 ± 0.02^{b}*	7.12 ± 0.06^{a}
0.1% lactic acid	4.28 ± 0.02^{b}*	4.13 ± 0.03^{c}*	4.60 ± 0.05^{a}*	4.27 ± 0.03^{b}*
0.05% lactic acid	5.09 ± 0.01^{c}*	4.43 ± 0.03^{d}*	5.67 ± 0.06^{b}*	6.07 ± 0.21^{a}*
0.2% curing agent	6.43 ± 0.03^{b}	4.78 ± 0.03^{c}	6.37 ± 0.03^{b}*	7.28 ± 0.09^{a}*

* Means significantly different in same column with respect to the control ($p < 0.05$)

^{a,b,c,d} Means in the same row with superscript letters in common are significantly different ($p < 0.01$)

Table 4.2. Viable counts of gram negative bacteria in broth medium with different additives (mean $\log_{10}$ CFU $ml^{-1} \pm$ SD for n=3)

Broth	18 h	18+24 h with CFS at pH 4.0	18+24 h with CFS at pH 6.0	18+24 h without CFS
control	8.57 ± 0.15^{a}	6.77 ± 0.32^{b}	8.90 ± 0.10^{a}	9.07 ± 0.21^{a}
0.01% BHA	7.60 ± 0.30^{a} *	1.02 ± 0.03^{b}*	8.17 ± 0.21^{a}*	8.20 ± 0.36^{a}*
0.02% BHA/BHT	7.07 ± 0.32^{b} *	ND	8.20 ± 0.10^{a}*	8.10 ± 0.26^{a}*
0.05% BHA	7.23 ± 0.71^{ab}*	ND	7.80 ± 0.36^{a}*	6.37 ± 0.21^{b}*
0.1% BHA	ND	ND	ND	ND
0.1% lactic acid	3.77 ± 0.95^{a} *	ND	ND	4.37 ± 0.51^{a}*
0.05% lactic acid	8.23 ± 0.06^{a}	4.00 ± 0.69^{b}*	8.53 ± 0.12^{a}	8.73 ± 0.32^{a}
0.2% curing agent	8.67 ± 0.32^{a}	3.20 ± 1.57^{b}*	8.87 ± 0.25^{a}	9.10 ± 0.10^{a}

ND, none detected (< 30 CFU ml^{-1})

* Means significantly different in same column with respect to the control ($p < 0.05$)

[a,b] Means in the same row with different letters are significantly different ($p < 0.01$)

General discussion

This study was started with experiment on quality of Mongolian traditional dried meat because it has being kept long time for consumption. According to the results, traditional dried meats in Mongolia are characterized by an excess of lipid oxidation. Epidemiological research has demonstrated a relationship between this type of diet and emergence of a range of chronic diseases, including cardiovascular disease and different types of cancer (Kaeferstein and Clugston, 1995).

Epidemiological studies have shown that in countries with low total fat intake cardiovascular diseases are less prevalent (Connor and Connor, 1986). Reducing fat content in diet of Mongolia is very difficult because it is agricultural country and diet is based on animal production. Mongolian traditional dried beef has high energy content and considered to be the food of choice largely due to its nutritional value. Nevertheless, there is lipid oxidation and consumption of these products may be associated with the subsequent risk of cardiovascular disease and cancer in human. As a result of negligence, contamination can lead to suffering and loss of human health. So there is need to improve the product's quality and to modify processing and preservation techniques. In addition, dry products such as fermented sausage might be replaced by traditional dried meat because they exhibit similar preservation potency. Several authors have observed that during the ripening of dry fermented sausages lipid oxidation did not occur (Dominguez and Zumalacarregui, 1991; Gokalp, 1986; Nagy, Mihalyi and Incze, 1989). 1989). Reason for this was probably the antioxidative action of food additives such as spices and curing agent. It was also confirmed in the study on TBARS values of fermented sausages processed with starter culture including *Staphylococcus* which acts as a catalyst. Chapter 2 was aimed at trying the processing of fermented sausage, which

made without starter culture in Mongolia and studied their microbiological characteristics because microorganisms induce changes in flavour, nutritional quality, texture, safety and other characteristics, depending on their activity. According to these results, the source of different microorganisms in raw materials, which could contaminate the final products, appeared on MRS agar, Chromocult Coliform agar, Mannitol salt agar and DHL agar. In Mongolian domestic markets, it is difficult for consumers to follow food safety regulations or avoid the health hazards that compromise the safety and quality of food. According to official statistics of Mongolia, there are 243 and 1,383 case of salmonellosis and dysentery, respectively, in 1999 (National Statistical Office of Mongolia). To date, infectious diarrhea remains a major health problem during the summer months in Ulaanbaatar and a 34-bed unit at National Research Center for Infectious Diseases is dedicated to the care of patients with this disease.

Further experiment was needed to make the comparison with dry sausage made with different starter cultures and processed in good hygienic condition, according to Chapter 3. In this experiment, coliform bacteria appeared during 2 weeks when lactic acid bacteria dominated, and the pH rapidly decreased. In comparison to the above two studies on dry sausages, coliform bacteria could appear in the second week of processing of dry sausages with or without starter culture. However, 3rd week of processing of sausage with starter culture revealed no coliforms and it might be related to interactions of sausage formulations including food additives. This result was confirmed and explained by fourth experiment in Chapter 4, which determined the effects of food additives including antioxidant and antimicrobial on gram negative bacteria. Many studies have already been reported (Geisen *et al.*, 1992;Choi and Chin.,

2003; Mataragas *et al.*, 2003) on food additives, which have antimicrobial activity to gram positive bacteria in meat products while there is lack study on their combination to gram negative bacteria.

Conclusion

The present study has shown that traditional dried meat had high lipid oxidation products while in dry fermented sausage coliform bacteria appeared. Both poisoning of lipid oxidation products and microorganism on quality of dry meat products might be delayed if food additives are used, including antimicrobial and antioxidant substance.

General Summary

In order to improve quality and shelf life of dry meat products in Mongolia, the following four main objectives were achieved:

① To determine the quality of traditional dried meats produced of Mongolia

② To try the processing of dry fermented sausages in Mongolia and to evaluate their microbiological characteristics

③ To examine the effect of various starter cultures on dry fermented sausage

④ To determine the effect of food additives on gram negative bacteria

To achieve these objectives, the following four experiments were carried out:

1. Determination of quality of dried meat from different livestock species.

Questions were asked about preference for eating dried meats produced from raw meats of cattle, sheep, goat and camel. Evaluation of consumption liking by colour, flavor, overall desirability and percentage of consumer choice was carried out from 145 panelists who live in Ulaanbaatar. In results, low score in colour, flavor and overall desirability was shown in order for dried meats from cattle, goat, sheep and camel. According this result, consumer choice was high (90%) in dried meat from cattle, while the concentrate (50%) was low in dried meat from camel and sheep. Dried meat from cattle and goat had the highest TBARS value, followed by mutton and camel meat in that order. In conclusion, dried meat from cattle had the highest scores for the three characteristics and low value in lipid oxidation products.

2. Microbiological analysis of fermented sausages without starter culture.

In September 2006, caudal fat of sheep as well as different kinds of meat produced from cattle, sheep, horse, goat and camel were collected from meat market of Ulaanbaatar. Then sausage mixture was done by mixing each meat with caudal fat in the

proportion of 85/15. The other ingredients were added (w/w) as follows: 2% of salt, 1% of glucose, 0.6% of sugar, 0.2% of curing agent, 0.5% of black pepper, 0.5% of grain pepper, 0.3% of onion powder and 0.2% of garlic powder. Ingredients were mixed in bowl chopper and then stuffed in collagen casing. Sausages without starter culture were ripened at room temperature for 3 weeks. Five types of samples were tested for the presence of total aerobic bacteria, lactic acid bacteria, coliforms, staphylococci and salmonellae. Numbers of total aerobic bacteria and lactic acid bacteria were significantly higher in camel sausage than the others including beef sausage that showed low bacterial numbers. Total numbers of staphylococci in samples were $<10^4$ of numbers in horse sausage. There was bacterial growth on the DHL agar in one of the triplicate experiments. These sausages were made without starter culture and were ripened at low temperature with produced lack lactic acid. This corresponded with the high pH values of 5.83, 5.53, 6.59, 6.04 and 5.75 for sausages made from meat of cattle, camel, horse, sheep and goat, respectively.

3. Quality of fermented sausage with mould starter culture.

The role of bacteria adapted in meat products have been studied extensively. However, there are relative few studies on the role of mould adapted in such products. Therefore, in this experiment, dry fermented sausages were made by either spraying mould starter cultures to their surfaces or adding bacterial starter culture in formulation in order to study their characteristics. The present study showed that mould starter cultures did not alter the sensory characteristics of dry fermented sausages and both had high contents of peptide and free amino acid during ripening. Even though white mould grew on the surface of sausages, undesirable mould did not grow on their surfaces. During the ripening on the 1st week, lactic acid bacteria dominated and the pH rapidly

decreased to desirable value while coliforms grew until 3 week of ripening. It was concluded that low pH as well as another antimicrobial could inhibit the growth of undesirable bacteria. In addition, sausages fermented with starter culture had lower values of TBARS than naturally fermented sausage.

4. Effects of Food Additives on Susceptibility of Gram Negative Bacteria Derived from Fermented Sausage.

This study examined the effects of food additives on gram negative bacteria. Food additives used included synthetic antioxidants (butylated hydroxyanisole, BHA and butylated hydroxytoluene, BHT), curing agent and lactic acid with or without combination with cell free supernatant (CFS) containing antimicrobial compounds of *Lactobacillus sakei* D-1001. The gram negative bacteria was selected from fermented sausages and cultured with different food additives for 18 h in nutrient broth, and then another 24 h with CFS [pH adjusted to pH 6.0 to inactivate lactic acid, or pH 4.0 (not adjusted)]. BHA (0.1%) resulted in total viable cell inhibition following 18 h incubation. A reduction in cell growth was observed in culture broths with 0.1% lactic acid and synthetic antioxidants at different concentrations. Furthermore, greater susceptibility of gram negative bacteria could be obtained in 18+24 h cultures with combination of selected food additives and antimicrobial compounds of *Lactobacillus sakei* D-1001, in a low pH environment depending on lactic acid concentration.

In conclusion, traditional dried meat had high lipid oxidation products while in dry fermented sausage had coliform bacteria. The spoilage of dry meat products by lipid oxidation and microorganism might be delayed if antimicrobial agents and antioxidant substances are used as food additives.

要約

乾燥食肉製品の品質および微生物学的特性に関する研究

Studies on quality and microbiological characteristics of dry meat products

はじめに：モンゴルでは食肉および食肉加工製品の消費量が多い。しかし、これら食肉の生産や加工に関する衛生的・技術的な解決すべき多くの問題がある。特に、食肉加工製品に関する衛生あるいは健康危害に関する情報は少ない。こうした問題が放置されていることから、ひとたび食肉製品による汚染が発生すれば甚大な健康被害につながると想定されている。そこで食肉製品の品質改善が求められており、加工や保存、製造工程も修正されるべき点がある。モンゴルにおける食肉加工製品の良好な品質や十分な貯蔵期間を確保するために、以下に示す実験を行った。

実験 1：モンゴルにおける乾燥肉の嗜好性調査

モンゴルの伝統的乾燥肉；牛，ラクダ，羊および山羊の乾燥肉の嗜好性について聞き取り調査を行った．調査項目は，風味，色調，総合評価および食体験の有無で、任意に抽出したウランバートル市民 145 名から回答を得た．その結果、風味，色調、総合（全体的な受容性）とも項目間ではほぼ等しい評価を示す傾向で，ウシ，ヤギ，ヒツジ，ラクダの順に嗜好性が低くなることが判明した．この結果に対応して，食体験の有無もウシが約 90%と高く，ラクダおよびヒツジで 50%程度と低いことが明らかとなった．一般成分は、いずれの乾燥肉とも水分が少なくタンパク質、脂肪、灰分が多かった。ヤギの乾燥肉は他の三つの乾燥肉に比べ、タンパク質と灰分が有意に低かったが脂肪が有意に高かった。４種類の乾燥肉のうち、牛肉とヤギ肉の TBA 値が最も低かった。 すなわち、モンゴルにおいて最も好まれる乾燥肉は牛肉のもので、これは脂質の酸化が低

い製品であることが判明した。

実験 2：モンゴルで製造した醗酵ソーセージの細菌学的特長

2006 年 9 月、ウランバートル市において，モンゴルで一般的に消費されているウシ，ラクダ，ウマ，ヒツジおよびヤギ肉の挽肉を市場から購入し，これに羊脂 15%,食塩 2%，グルコース 1%，砂糖 0.6%，胡椒 0.8%，オニオン粉 0.2%，発色剤 0.2%を添加・混和し，コラーゲン人工ケーシングに充填後、室温で自然発酵させた．標準寒、MRS、MSA、DHL および Chromocult 天培地による細菌検査を行った。 自然発酵 3 週間目の製品の一般生菌数は、ラクダが最大で，ウシが最小である．この傾向は，乳酸菌数とも一致した．ブドウ球菌は、全ての試料で 10 の 4 乗であった．DHL 寒天による細菌は牛肉とヤギ肉で作られた発酵ソーセージの一部で陽性となった．これらが存在したのは，今回，スターターを使用しなかったため，十分な乳酸醗酵が生じていなかったのかもしれない．実際，この時点での製品のｐＨは，ウシ 5.83，ラクダ 5.53，ヒツジ 6.04 およびヤギ 5.75 と高かった．また，ウランバートル市の 10 月の平均気温が氷点下 1 度と低く初期の醗酵に問題があったのかもしれない．すなわち，衛生状態の良くない肉を使って、室温で熟成したスターターカルチャ-を添加しない自然発酵ソーセージでは、微生物学的に不適切な製品となると断定した。また、モンゴルで醗酵ソーセージを製造するにあたっては低温で比較的活性の強い乳酸菌の開発とその添加あるいは適した添加物の使用が望ましいと考えられた。

実験 3：カビスターターを用いた発酵ソーセージの特徴

発酵食肉食品の製造過程における細菌の役割については比較的よく研究されているが，カビに関する報告は少ない。そこで、今回、自然発酵の際に障害となる有害なカビの発生を防止する効果が期待されている市販のカビスターターを

表面に塗布し、内部には乳酸菌を主体とする市販スターターを添加した醗酵ソーセージを製造し、その特徴を調査した。

その結果、熟成中のペプチド、遊離アミノ酸量の増加、あるいは官能評価に対してカビスターターによる影響は少なかった。ソーセージの表面には、白カビが存在し、有害なカビは認められなかった。また、脂質酸化におけるスターターカルチャ - を用いたソーセージのＴＢＡＲＳ値は、スターターを使用しないものよりも低かった。熟成 1 週目で乳酸菌は優勢となり、ｐＨ は最終製品の値に近づいたが、大腸菌群は 3 週目まで生存した。このことは大腸菌群の死滅がｐＨの低下を主因とするだけではないことが示唆された。

実験 4 : 発酵ソーセージから得られたグラム陰性菌に対する食品添加物の影響について

モンゴルの食肉製品から分離したグラム陰性菌を指標菌として乳酸菌の効果を検討した。先ず乳酸菌の直接的な影響をプレート法で調査したが、明らかな効果は認められなかった。そこで、乳酸菌培養上澄といくつかの添加物との組み合わせによる効果を検討した。

その結果、乳酸、発色剤 、BHA 等を単独で加えた場合よりも、組み合わせて添加すると抑制効果が認められ、特に低いｐＨで顕著であることが判明した。

おわりに：これらにおいて、乾燥食肉製品であるモンゴルの伝統的な乾燥肉と乾燥発酵ソーセージについて細菌学的、化学的分析および消費者テストによって調べた。その結果、モンゴルの伝統的な乾燥肉は高い酸化脂質を含むことや、乾燥発酵ソーセージの熟成中に大腸菌群が出現することなどが明らかとなった。しかし、製品の品質を低下させる脂質酸化や有害微生物に関しては、抗菌物質や抗酸化剤を含む食品添加物が、適切に使われるならば改善される可能性が示唆された。また，モンゴルにおいては、醗酵ソーセージよりも乾燥肉が

一般的に普及し、牛肉の乾燥品が最も好まれているため、世界各国では一般的な豚肉よりも牛肉の醗酵ソーセージの開発が重要であると考えられた.

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