Master's Thesis of Science in Agriculture

Screening and Validation of Flavor-forming Starter Bacteria for Korean Gouda-type Cheese Model

풍미 생산 스타터 박테리아의 선발 및 한국형 고다 타입 치즈 모델에서의 유효성 검증

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Screening and Validation of Flavor-forming Starter Bacteria for Korean Gouda-type Cheese Model

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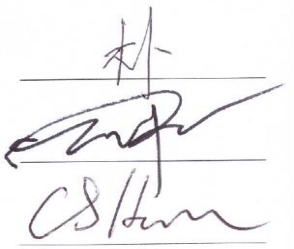

Abstract

Lactic acid bacteria (LAB) have an important role in dairy fermentations, notably as cheese starter cultures. They cause not only rapid acidification and coagulant of milk through the production of lactic acid, but also flavor formation. Cheese flavor results from a complex combination of flavor compounds. The flavor compounds in cheeses are derived from milk composition which is mainly composed of lactose, milk casein, and milk fat. During cheese production and ripening period, various enzymes from milk, rennet, starter cultures, and non-starter LABs are involved in the flavor formation pathways; glycolysis, proteolysis, and lipolysis. Among these three pathways, starter LABs are especially related into amino acids degradation, presumably the origin of major flavor compounds. Therefore, specific key enzymes (AdhE; Bifunctional Alcohol/Aldehyde dehydrogenase, BcaT; Branched chain aminotransferase, AraT; Aromatic aminotransferase, EstA; Esterase, Adh; Alcohol dehydrogenase, CitP; Citrate permease, KdcA; Keto acid decarboxylase) appear to be major criteria for the selection of flavor-forming starter bacteria. By using the multiplex PCR assay for detection of genes for those enzymes, 44 of flavor-forming LAB strains were screened. Among them, the most putative strains which have a possibility to flavor formation in cheese were selected. They were four of *Lactococcus lactis* subsp. *lactis*

strains and one of *Lactococcus lactis* subsp. *cremoris* strain. Each of five strains were further characterized and assessed for industrial use. The combinations of starter bacteria were validated in miniature Gouda-type cheese model. Flavor compounds of tested miniature cheeses were analyzed and profiled by using the Hercules Ⅱ electronic nose (Alpha M.O.S., France). Compared to industrial cheese starter (CHN-11, Chr.hansen, Denmark), selected flavor-forming starter bacteria showed more variety flavor profile. Methylbutanone and 1-propanol, 2-methyl- was only found in test groups. Among the test groups, flavor profile of Test 1 evolved most at the last day of ripening.

Keyword : Lactic acid bacteria/ Starter culture/ Flavor/ Gouda cheese

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Chapter 1. Introduction

1.1. Study background

Milk and dairy products, including cheeses are important components of the diet and can play an essential role in meeting nutritional requirements. Although South Korea has low per capita natural cheese consumption compared to other countries such as Greece, France, Germany and Italy where consume more than 20 kg of cheese per capita (International Dairy Federation, 2010), it has been grown up to 2.1 kg per capita (Figure 1). The constant increase in cheese consumption is due to the adoption of Western life-style. This phenomenon is positive to promote domestic dairy industry. As the domestic cheese market is growing, cheese consumer and producer focus on new type of cheese which have interesting characteristics such as flavor.

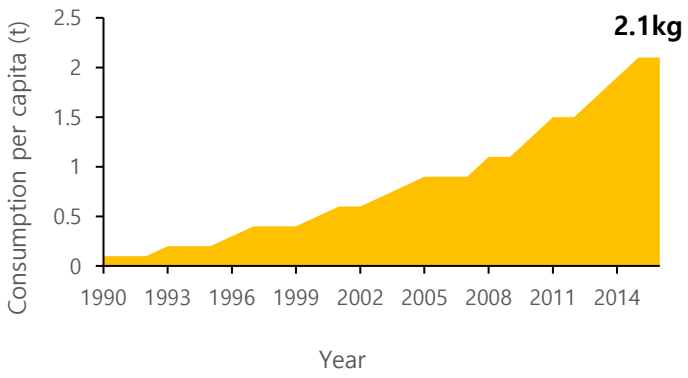

Figure 1. Natural cheese consumption per capita in South Korea (Korea Dairy Committee, 2017).

1.2. Purpose of research

Starter culture used in manufacturing cheeses such as Gouda, Edam, and Cheddar usually consisting of mesophilic LAB, mainly *Lactococcus lactis* spp. Important characteristics of starter cultures related to cheese making are acidification activity, proteolytic activity and flavor production. Flavor is one of the most important attributes of cheese, therefore it has received much attention. Cheese flavor development is a very complex process, originating from a combination of microbiological, biochemical and technological aspects. Starter cultures play a key role in the flavor development during ripening of cheese (Urbach, 1993, Broome and Limsowtin, 1998). For the new flavor of cheeses, new starter LAB strains can be applicated. These new strains are so-called 'wild strains' were isolated from different dairy source and other non-dairy sources such as fermented foods (Klijn *et al.*, 1995, Soda *et al.*, 2003, Kuda *et al.*, 2016). In some of previous studies, wild strains have the ability to produce flavor distinctly different from those produced by industrial starter cultures (Buckenhüskes, 1993, H.E. Ayad *et al.*, 2000, Ayad *et al.*, 2003, Smit *et al.*, 2005a, Ayad, 2009). Wild starter strains may have a good potential for developing improved cheese flavor. For our knowledge, the present is the first study to screen the novel flavor-forming cheese starter strains originated from Gangwon (Korea) and validated their flavor-forming ability in Gouda-type cheese model.

Chapter 2. Review of Literature

2.1. Cheese

Cheese is the generic name for a group of fermented milk-based food products produced throughout the world in a great diversity of flavors, textures, and forms. It is commonly believed that cheese evolved in the Fertile Crescent between the Tigris and Euphrates rivers, in Iraq, some 8,000 years ago, during the so-called Agricultural Revolution, when certain plants and animals were domesticated as sources of food (Fox *et al.*, 2000). Although humankind didn't know the concept of microorganism related to cheese in the very first time, they soon recognized the nutritive value of milk and used milk for produce of wide range of fermented dairy products. In 1665, Robert Hooke, the scientist of the microscope development, recorded very first note of observation of microbes associated with cheese. From recognition of the role of microorganisms in cheesemaking, scientific understanding of the identities and roles of microorganisms have been inquired (Donnelly, 2014).

2.1.1. Gouda-type cheese

Gouda cheese has its origin in the Netherlands owing its name to a village bearing the same name. Gouda cheese is a more generic name for the large group of rennet coagulated semi-hard cheeses often referred to as Dutch-type. In general, they are produced from partially skimmed or whole bovine milk and have a yellowish color with wheel-shaped body (Victor R. Preedy, 2013).

According to the Korea Custom Service Report in 2014, Gouda cheese rapidly increased imports weight than Cheddar cheese (Figure 2). Report data is reflected the interest of Gouda cheese in Korea.

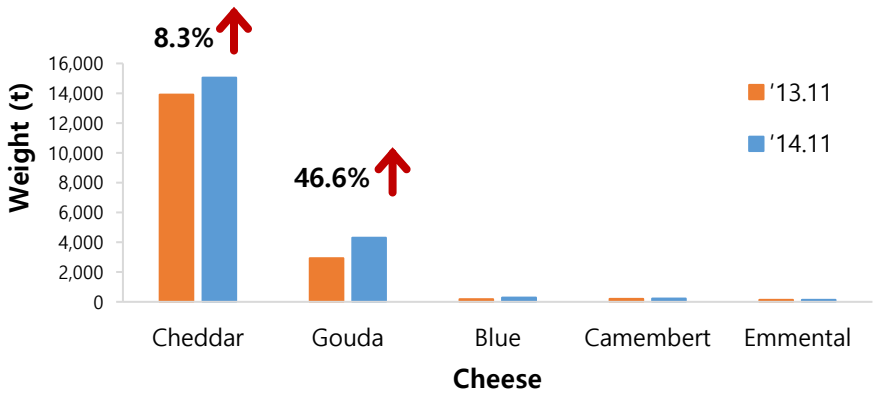

Figure 2. Accumulated imports of natural cheese in 13' and 14' (Korea Custom Service Report, 2014).

2.2. Cheese starter culture

2.2.1. Starter lactic acid bacteria

A starter culture can be defined as a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process. The group of lactic acid bacteria (LAB) occupies a central role in these processes, and has a long and safe history of application and consumption in the production of fermented foods and beverages (Holzapfel and Wood, 1995, Leroy and De Vuyst, 2004).

In the manufacture of most cheeses, carefully selected strains of different species of LAB are added to the milk shortly before renneting (Fox *et al.*, 2000). Starter strains have an important role during the cheese ripening due to (1) the production of lactic acid, which cause the curd formation, acts as a preservative and contributes to the acids flavor of the cheese, (2) metabolism of citrates, which is regarded as essential compounds for flavor production, (3) metabolism of the milk protein, especially in amino acid breakdown, (4) some contribution to the breakdown of the diglycerides formed from the milk triglycerides by lipase (Hassan *et al.*, 2013).

Starter cultures are commonly divided into mesophilic cultures (with an optimum temperature of about 30 ℃) and thermophilic cultures (with an optimum temperature of about 42 ℃). The main mesophilic

culture is *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* spp. is also found. The thermophilic cultures almost always consist of two organisms, *Streptococcus thermophilus* and either *Lactobacillus helveticus*, *Lb. delbrueckii* subsp. *lactis*, or *Lb. delbrueckii* susp. *bulgaricus*.

2.2.2. Gouda-type cheese starter

Gouda-type cheeses are made of the mesophilic cheese culture which contains mainly *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*. In addition, *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and/or *leuconostoc* spp. Commonly the combination of these starter bacteria is called DL-cultures as D from *streptococcus diacetilactis*, the old name for *Lc. lactis* subsp. biovar *diacetylactis* and L from the first letter of *Leuconostoc* (Fox *et al.*, 2000). *Lc lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are principal acid producers, while *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and Leuconostoc spp. are the secondary microflora (Ayad, 2001), which ferment citrate with the production of CO_2, diacetyl, and acetate.

2.3. Cheese flavor

The flavor compounds in cheese originate from degradation of the major milk components; lactose, citrate, milk proteins (namely called casein), and milk fat during ripening period which, can be a few weeks to more than 2 years long (Urbach, 1995, Singh *et al.*, 2003).

Table 1. Flavor compounds generated from the 3 principal milk constituents during ripening of cheese (Singh *et al.*, 2003).

Lactose & Citrate	Casein	Milk fat
Lactate	Peptides	Fatty acids
Pyruvate	Amino acids	Keto acids
CO_2	Acetic acid	Methyl ketones
Diacetyl	Ammonia	Lactones
Acetoin	Pyruvate	
2,3−butandiol	Aldehydes	
Acetaldehyde	Alcohols	
Acetic acid	Carboxylic acid	
Ethanol	Sulfur compounds	

2.3.1. Glycolysis of lactose and citrate

Lactose is converted into lactic acid during the cheese ripening accordance with growth of starter LAB. Lactic acid causes a decrease pH in cheese. The *Lactococcus* strains only produce lactic acid as long as they are growing and lactose is available, whereas *Leuconostoc* strains produce acetic acid and ethanol, beside lactic acid (Ardö and Varming, 2010). These acid productions contribute to a fresh acidic flavor of the cheeses. The lactic acid produced will only be further

metabolized into various flavor compounds by non-starter LAB originated in milk or adjunct starter cultures, finally such as ethyl esters were formed in long time ripened semi-hard and hard cheeses.

The strains of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. of the DL-starter convert the citric acid in the cheese into acetic acid, diacetyl, acetoin, 2,3-butanediol and CO_2. CO_2 is responsible for eye formation in Gouda-type cheese, while diacetyl and acetate are important in flavor formation (Victor R. Preedy, 2013).

2.3.2. Proteolysis of milk casein

The starter Lactococcus strains have a well characterized cell envelope-associated protease (CEP) that is also named PrtP or lactocepin (Tan *et al.*, 1993). It hydrolyses β-casein and α_{s1}-casein in milk. However, chymosin and plasmin have mainly responsible for peptide production (Exterkate *et al.*, 1997). Peptides that are released from casein may have a role in bitter or umami flavor in cheese depending on their amino acid sequences (Ardö and Varming, 2010). However, only minor flavor differences have been observed in cheese trials conducted with LAB with altered peptidase activities, although significant changes in the amino acid pool were observed, indicating that the conversion of amino acid to flavor compounds is the rate-limiting step in flavor formation from proteins (Steele *et al.*,

2013).

From casein metabolism, LAB release amino acids and use them in their protein synthesis, cell metabolism and as energy sources (Ardö and Varming, 2010). The first step of amino acid catabolism is transamination activity, which is bottleneck for flavor formation route. The transamination step initiate to transform an amino acid into its corresponding α-keto acid by moving the amino group to α-keto acid acceptor. All aminotransferases described so far in LAB use α-ketoglutarate and produce glutamate (Yvon and Rijnen, 2001, Ardö, 2006). The amount of α-ketoglutarate available is a limiting factor for the flavor compounds formation in cheese (Yvon *et al.*, 1997). The glutamic acid produced by the aminotransferase activities may be regenerated into α-ketoglutarate by the bacterial enzyme glutamate dehydrogenases (GDH) that have been found in some strains of *Lactococcus lactis* starter (Tanous *et al.*, 2002). The α-keto acids are intermediate compounds in the flavor formation as they can be metabolized through a range of enzymatic reactions to provide various flavor compounds such as aldehydes, alcohols, carboxylic acids and esters.

2.3.3. Lipolysis of milk fat

Various flavor compounds are also derived from milk fat. Lipases and esterases of LAB seemed to be the main lipolytic factors in Cheddar and Dutch-type cheese made from pasteurized milk. The esterolytic/lipolytic enzymes in starter *Lactococcus* and *Leuconostoc* can hydrolyze a range of esters of free fatty acids (FFAs), tri-, di-, and monoglycerides in cheese (Collins *et al.*, 2003). Despite the presence of these enzymes, LAB, especially *Lactococcus* and *Lactobacillus* spp. are generally considered to be weakly lipolytic in comparison to species such as *Pseudomonas*, *Acinetobacter* and *Flavobacterium* (Holland and Coolbear, 1996, Liu *et al.*, 2001). However, because of their presence in cheese at high numbers over an extended ripening period, LAB are considered likely to be responsible for the liberation of significant levels of FFA. Only limited amounts of fat derived compounds should be present in many of the semi-hard cheese varieties, and they cause off flavors or sometimes fruity and sharp flavor. However, key flavor compounds in cheese varieties generally produced by mould from raw milk (Ardö and Varming, 2010).

2.3.4. Gouda-type cheese flavor

While a sharp, nutty flavor is typical of aged Cheddar from some parts of the world, a similar profile in Gouda cheeses is totally unacceptable (Weimer, 2007). Instead, Gouda-type cheese have malty, cabbage, garlic, and sulphurous flavors. Although key flavor components in Gouda-type cheese are derived mainly from amino acids, there exist another flavor compounds such as diacetyl, ketones, aldehydes, and fatty acids (Table 2). However, extensive lipolysis is considered undesirable in Gouda cheeses because high levels of fatty acids lead to rancidity (Hassan *et al.*, 2013).

Table 2. Key flavor compounds in Gouda cheese (modified from Singh, T., *et al.* 2003, Yvon, M. 2006, and Hassan *et al.* 2013).

Associated flavor compounds			Flavor note
Amino acid	Leucine	3-mehylbutanal	malty, cheese, dark chocolate
		3-methylbutanol	malty, alcoholic, fruity, grainy
	Isoleucine	2-methylpropanol	malty, alcoholic
	Methionine	Methanethiol	cooked cabbage, garilic, onion, sulphurous
		Dimethyldisulfide (DMDS)	cabbage, onion, garlic, ripened-cheese
		Dimethyltrisulfide (DMTS)	cabbage, Garlic, putrid, sulfurous
		Dimethylsulfide (DMS)	cabbage, garlic, sulphurous
Sugar	Lactose, Citrate	Diacetyl (2,3-butanedione)	buttery, nuts
Fat		Butyric acid	sweaty, cheesy, fecal
		Butanone	etheric
		Hexanal	green
		Pentanal	pungent, almond-like

Chapter 3. Material and Methods

3.1. Selection of the flavor-forming starter bacteria

3.1.1. Sample collection

All samples were collected from Gangwon-do, South Korea. 20 samples of raw milk, 33 samples of Kimchi, 15 samples of Jeotgal, 11 samples of Makgeolli, and 1 sample of Cheonggukjang were used for this study (Table 3). The collected samples were transferred to laboratory within 6 h, and then immediately the portion of sample was distributed in 50 ㎖ cornical tube and stored at 4 ℃ refrigerator before the lactic acid bacteria isolation.

Table 3. Origin of the samples used for LAB isolation in this study.

Sample	Gangwon-do, South Korea				
	Pyeongchang	Hoengseong	Gangneung	Other district	No.
Raw milk	10	10	·	·	20
Kimchi	27	·	6	·	33
Jeotgal	9	·	6	·	15
Makgeolli	·	2	·	9	11
Cheonggukjang	·	·	1	·	1
Total					80

3.1.2. Isolation of lactic acid bacteria

Colleted samples were serially diluted 10-fold using 0.85 % NaCl solution (Sigma-Aldrich, USA) and homogenized. Serial dilutions were plated on de Man, Rogosa and Sharp (MRS) (Difco, USA) agar for Lactobacilli isolation, M17 (Difco, USA) supplemented with 0.5 % lactose (GM17) (Difco, USA) agar for Lactococci isolation. Inoculated plates were incubated at 30 ℃ under aerobic condition for 48 h. After incubation, colonies of different morphologies were selected and inoculated same media. Isolates incubated for 24 h at arranged condition then streaked on matched agar media and incubated again. To confirm the isolates were LAB, plate count agar containing bromocresol purple (BCP) (Eiken chemical Co., Ltd, Japan) method was used. Only the yellow-color colonies on BCP agar were selected. Subsequently, gram staining (Becton, Dickinson and Company, USA) and testing for the absence of catalase were done. Catalase negative and also gram positive isolates were selected and stored in 20 % glycerol (Sigma-Aldrich, USA) at -80 ℃ (Figure 3).

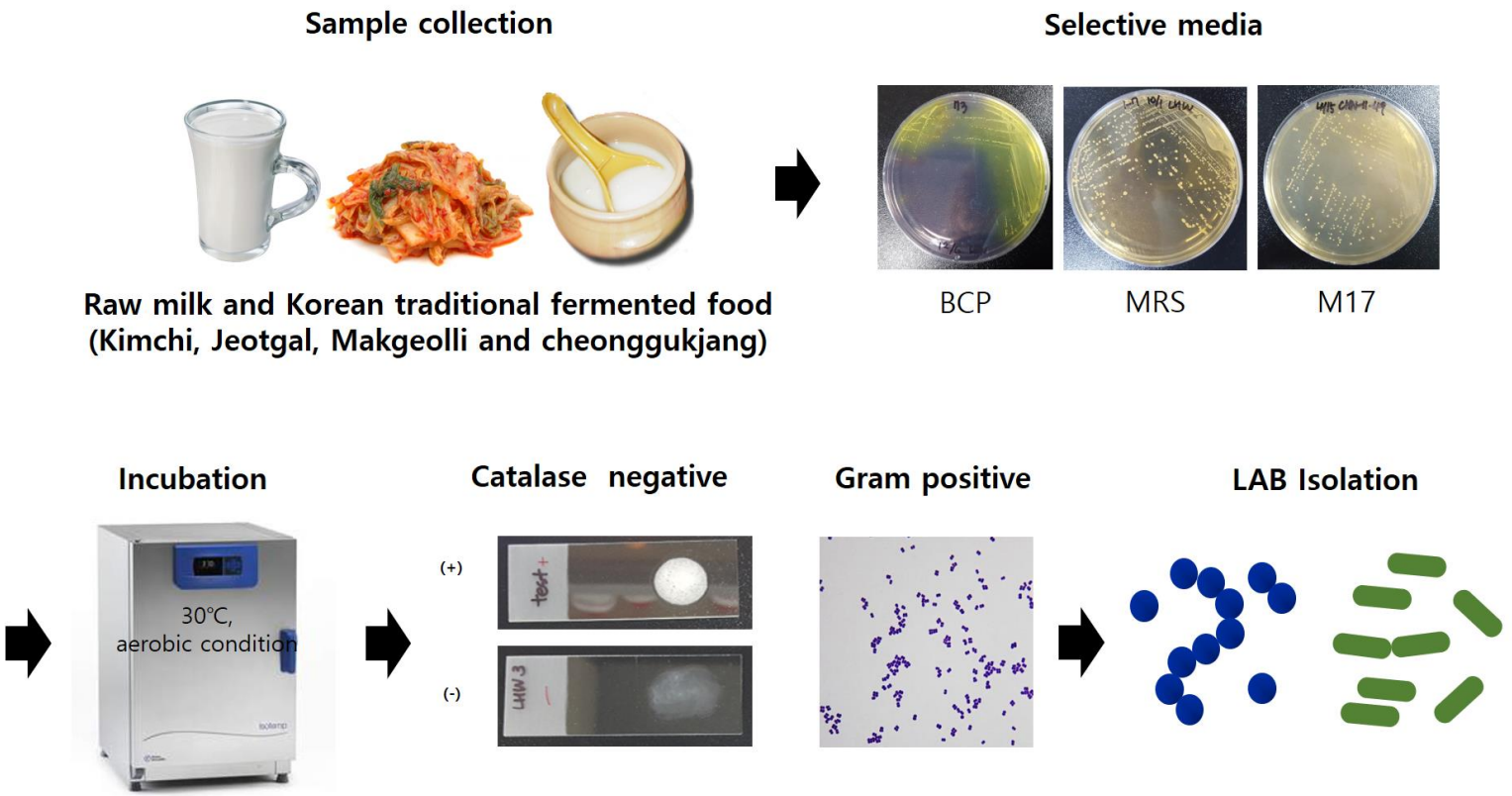

Figure 3 . Scheme for isolation of lactic acid bacteria.

3.1.3. Acidification and coagulant activity

The acidification activity was measured by using ORION STAR A211 pH-meter (Thermo Scientific, USA) after 6 h and 48 h incubation at 30 ℃ in sterilized 10 % skimmed milk (Oxoid, UK). The coagulant activity was evaluated by the appearance of visual coagulum of 10 % skimmed milk in the test tube, after leaning it manually (H.E. Ayad *et al.*, 2000, Randazzo *et al.*, 2007).

3.1.4. Proteolytic and lipolytic activity

Proteolytic and lipolytic properties of each isolates were assessed on two differential media, skim milk agar and tributyrin agar, respectively. Skim milk agar were made as follows: 25 g of skim milk (Sigma-Aldrich, USA) powder was reconstituted with 250 ㎖ of distilled water. The mixture was stirred thoroughly and autoclaved at 115 ℃ for 20 min. Likewise, 500 ㎖ of 2.5 % agar (Difco, USA) solution was sterilized (Pailin *et al.*, 2001). Skim milk and agar solution were mixed and then distributed into plates. Tributyrin agar (Sigma-Aldrich, USA) were made according to the manual (Van Hoorde *et al.*, 2010).

3.1.5. Multiplex PCR

The multiplex PCR assay for detection of genes for enzymes that involved in flavor-forming pathway include *adhE*; Bifunctional Alcohol/Aldehyde dehydrogenase, *bcaT*; Branched chain aminotransferase, *araT*; Aromatic aminotransferase, *estA*; Esterase, *adh*; Alcohol dehydrogenase, *citP*; Citrate permease, *kdcA*; Keto acid decarboxylase. Because of PCR products size, these target enzymes were grouped into three (Table 4). Primers were designed manually and were commercially obtained (Bioneer, South Korea). For performing multiplex PCR, streaking plate of each isolates were prepared. Amplification was performed on a PCR system in 20 ㎕ reaction mixture consisting of *i*-Taq™ 2X PCR Master mix Solution (iNtRON, South Korea), 10 pmole of the relevant forward and reverse primers and one colony of each streaking plate. The PCR program of the first and second group was as follows, respectively: 30 cycles of 30 sec at 95 ℃, 20 sec at 43 ℃, 2.2 min 72 ℃, and 30 cycles of 30 sec at 95 ℃, 20 sec at 41 ℃, 2.2 min at 72 ℃. The third group was amplified by touchdown PCR (Figure 4). The annealing temperature of the earliest steps of touchdown PCR was programmed to decrease in increments (-1 ℃) for every subsequent set of cycles. The first sequence amplified was further amplified by subsequent 25 cycles. The amplified products of each groups were run on 1.5 % agarose in

1x TAE buffer at 70 v for 40 min in the presence of 100 bp marker (Bioneer, South Korea) and visualized by staining with loading dye (Bioneer, South Korea).

Table 4. Primers used in this study.

Group	Target gene*	Primer	Sequences (5'-3')	T_M (℃)	GC (%)	Amplicon (bp)	Reference
1	*adhE*	AAdh_F	CAAGTCGATACTATTGTC	48.0	38	2158	In this study
		AAdh_R	TTTATGAGCAAGTGAGTG	49.9	38		
	bcaT	BcAT_F	TACCTTTTCGTTATATCGC	49.8	36	884	In this study
		BcAT_R	TCCTACTTCTGTTTCTGAA	49.9	36		
	araT	ArAT_F	GTCATTATACTGGAATGG	47.7	38	750	In this study
		ArAT_R	CAAGGTCTGACATCTTTT	49.0	38		
	estA	Est_F	GGGATGAATCGAAAAGTT	48.8	38	617	In this study
		Est_R	CCTAATTTTTTCAACTCGG	48.0	36		
2	*adh*	Adh_F	TGTTGAAAAGGAACTTCG	48.7	38	880	In this study
		Adh_R	AATTGGTTTTACCTTACCC	48.3	36		
	citP	P4(S)	GGAGTTGGTGCTGGTATTGTG	60.2	52	616	Klijn *et al.*, 1995
		P5(A)	CCAACCCTGCTGTAATAGCAG	59.7	52		
3	*kdcA*	3fw	GTATACAGTAGGACATTACC	51.1	40	1630	Yvon, M., 2006
		1633Rev	GCTCAGCAAATAATTTACCC	51.7	40		

* *adhE*, bifunctional aldehyde/alcohol dehydrogenase; *bcaT*, branched-chain aminostransferase; *araT*, aromatic aminotransferase; *estA*, esterase A; *adh*, alcohol dehydrogenase; *citP*, citrate permease; *kdcA*, keto acid decarboxylase.

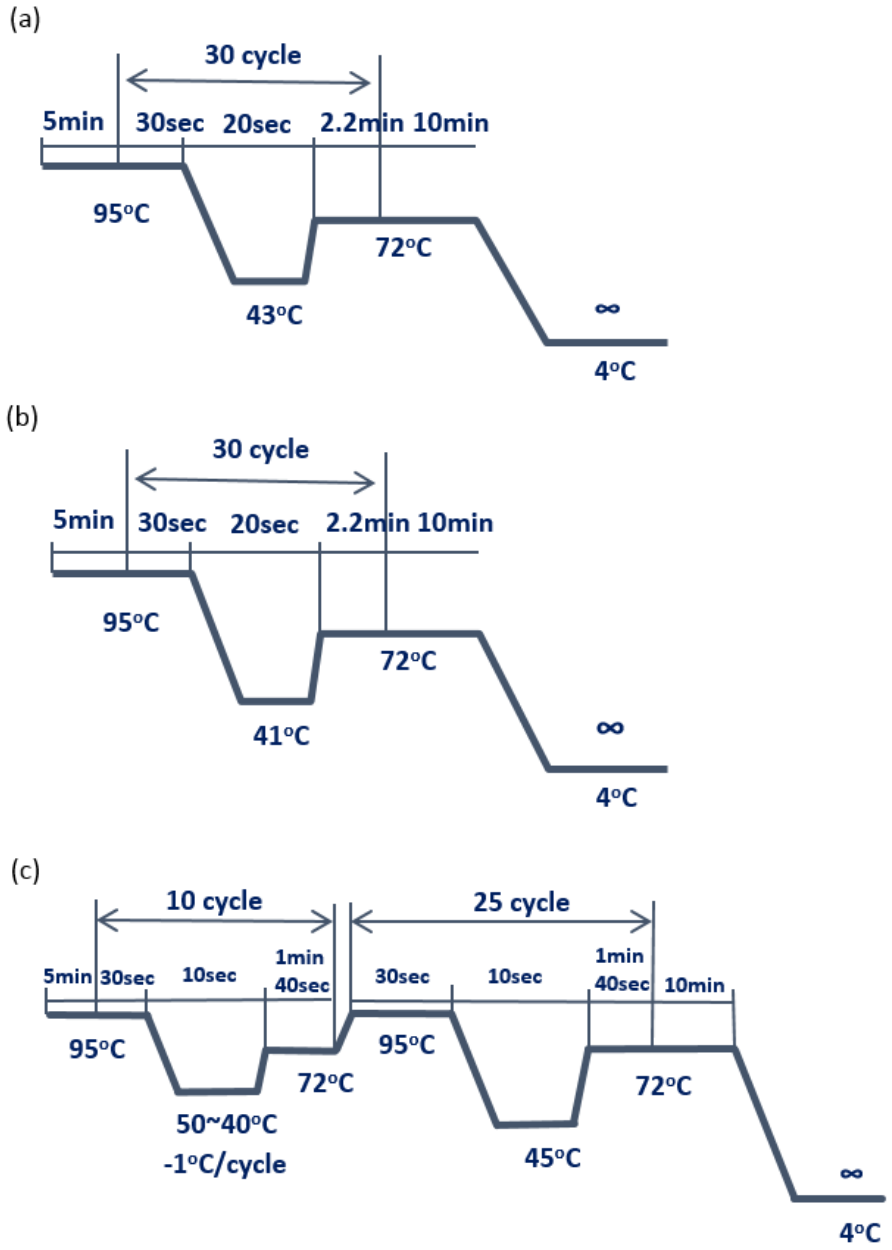

Figure 4. PCR procedure of the group 1 (a), group 2 (b), and group 3 (c).

3.1.6. Enzymatic assay

Each strain was inoculated at 1 % (v/v) in 10 ㎖ of media and incubated under 30℃, aerobic condition for 18h. Cells were harvested by centrifugation (15,000 rpm, 5 min, 4 ℃), washed twice with phosphate-buffered saline (Mediatech, Inc., USA), and added to microtubes, which contained 2.0 g of Zirconia/silica beads (0.1 mm dia., BioSpec Products, USA). Each cell-free extracts (CFEs) were obtained by follow as: two cycle of 30 sec disruption at the highest speed of the disruptor (Mini-Beadbeater-16, Biospec Products) and 1 min cooling on ice. After disruption, CFEs were cooled down immediately by using ice. A CFE consisted of the cell lysate centrifuged (15,000 rpm, 10min, 4 ℃) in order to separate the beads, cells, and cell debris. The CFEs were stored at -20 ℃ until used (Peralta *et al.*, 2016).

The GDH activities of the CFEs were analyzed by using commercial colorimetric glutamic acid assay (R-biopharma, Germany). By the GDH, oxidative deamination of Glu produce reduced cofactor (NADH). In the presence of diaphorase, the NADH formed converts iodonitrotetrazolium (INT) to produce colorimetric Formazan (Figure 5). The assay was performed by using 96 well plates (SPL Life Sciences Co., Ltd., South Korea). The reaction mixture containing 80 ㎕ of distilled water, 80 ㎕ of potassium phosphate/triethanolamine buffer (pH 8.6), 40 ㎕ of 100 mM of L-Glutamic acid (Sigma-Aldrich,

USA), 40 ㎕ of INT, 40 ㎕ of NAD · diaphorase (600 ㎕ in total). Except L-Glutamic acids, all provided in the kit. After the addition of 60 ㎕ of CFE to the reaction mixture, immediately, mixture was distributed 200 ㎕ to each of 3 wells and incubated for 3 h at 30 ℃ and measured the absorbance at 492 nm (Kieronczyk *et al.*, 2003).

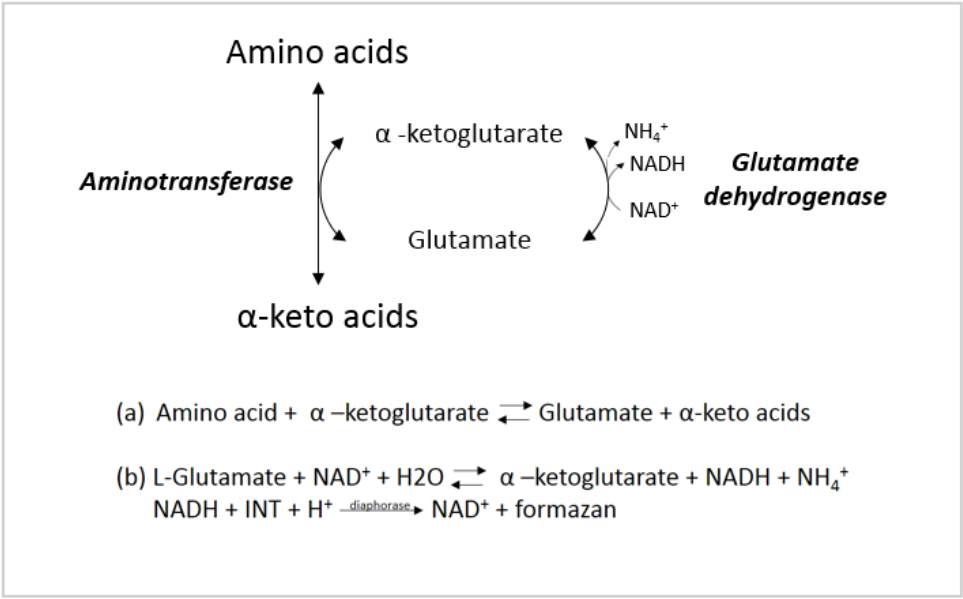

Figure 5. Aminotransferase (AT) reaction (a) and Glutamate assay reaction (b) mechanisms.

3.2. Characterization of the selected starter bacteria

3.2.1. 16s rRNA sequence analysis

The isolated strains were identified using the 16s rRNA sequence analysis. The 16s rRNA gene of the strains was amplified by PCR using universal primer set 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The PCR was performed using thermal cycler according to the following protocol: Initial DNA denaturation, 3 min at 95 ℃; 20 cycles of denaturation for 30 sec at 95 ℃, annealing for 30 sec at 55 ℃, and extension for 1 min at 72 ℃; and final elongation for 5 min at 72 ℃. The 16s rRNA gene was sequenced at Macrogen corporation (South Korea) and then compared to those in the GenBank database using the BLAST algorithm (National centre for Biotechnology Information, USA).

3.2.2. API test

API 50 CH and API ZYM (BioMérieux, France) were used for the carbohydrate fermentation and enzymatic profiling, respectively. The API tests were performed according to the manufacturer's instructions. Briefly, strains were harvested in the late exponential phase and inoculated into API 50 CH test strips, and incubated at 30 ℃ for 48 h. Cell concentration was adjusted to 0.1 at O.D. 600 nm (SPECTROstar Nano, BMG LABTECH, Germany) and inoculated into API ZYM test strips. API ZYM result recorded after 4 h of incubation at 30 ℃.

3.2.3. Growth curve

Growth curve of the selected strains were cultivated at 30 ℃ with 1 % (v/v) inoculation in M17 (Difco, USA) broth supplemented 0.5 % Lactose (Difco, USA) and 0.05 % Glucose (Sigma-Aldrich, USA) (GM17). Growth of bacterial concentrations were determined by measuring optical density (O.D.) at 600 nm (SPECTROstar Nano, BMG LABTECH, Germany).

3.2.4. Autolysis ability

According to the method of Soda *et al.* (2003), the rate of cell autolysis was measured. Briefly, cells of different cultures were harvested by centrifugation (8,000 rpm, 15 min, 4 ℃) (1248R, Labogene, South Korea) and washed twice by 0.85% NaCl (Sigma-Aldrich, USA). The obtained pellet was then resuspended in 0.01 M phosphate buffer (pH 5.5) (Mediatech, Inc., USA) containing 0.5 M sodium chloride (Sigma-Aldrich, USA). The cell suspension was initially adjusted at an optical density of 0.9 to 1.0 at 600 nm by spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany). The percentage decrease in optical density at 600 nm after incubation for 6, 24, and 48 h at 12 ℃ was calculated.

3.2.5. Stability to freeze-drying process

The grown cultures were subcultured twice and inoculated (1 %, v/v) in 500 ㎖ of GM17 broth. The culture were incubated over night at 30 ℃ at 100 rpm. Grown cells were harvested by centrifugation at 4000 rpm for 20 min at 4 ℃. The cell pellets were suspended in 10 ㎖ of sterile freeze-drying medium composed of skim milk 10 % (Oxoid, UK), lactose 3 % (Difco, USA), yeast extract 0.1 % (Difco, USA), then stored overnight in deep-freezer (IE2386D, Thermo Fisher Scientific, USA) at -80 ℃, and finally dried in a freeze dryer (MCFD8518, IlShinBioBase Co. Ltd., South Korea) at -80 ℃ for 24h. The freeze-dried powder of each strain was weighted and stored at -20 ℃. The viability of strains was evaluated 1 month following freeze-drying, as follow: 0.1 g of each freeze-dried strain was resuspended in 1 ㎖ of skim milk (10 %, w/v) for 30 min, plated in duplicate onto GM17 agar and incubated at optimal conditions (Carafa *et al.*, 2016).

3.3. Validation of the selected strains in cheese model

3.3.1. Manufacture of miniature cheese model

According to the method of Hynes *et al.* (2000), the miniature model cheeses were manufactured. In order to obatain an acceptable standard, the miniature cheeses were made from whole, homogenized and pasteurized milk. The milk was analyzed by Milkoscan FT2 (Foss Analytical A/S, Denmark) (Table 5). Six miniature cheeses, two control groups and four test groups, were made of different starter culture. The starter strains were inoculated into sterile 10 % skim milk. After 18 h of incubation at 30 ℃, each cultures was mixed as described below (Table 6). Liquid rennet extract of bovine origin (STD PLUS 290, Chr.Hansen, Denmark) was used after filtering (pore size, 0.2 ㎛, Minisart® Syringe Filter, Satorius AG, Germany) to avoid contamination. The cheeses were produced in sterile 500 ㎖ centrifuge bottles. 400 ㎖ of milk (30 ℃) was filled into bottles and inoculated with the starter (2 %, v/v) plus 80 ㎕ of rennet. The bottles were covered immediately and inverted by manually three times, after which they were kept in a water bath at 30 ℃. Coagulation time was controlled by leaning the bottles gently to test adhesion of casein to their sides. After coagulation, the bottles were maintained in a water bath for a while. The coagulum was cut with sterile disposable steel tools. The mixture of whey – curd particles was agitated for 20 min in

a mechanical stirrer by inversion of the bottles (10 rpm). About 40 % (160 ㎖) of the whey was discarded and replaced by an equal volume of sterile water at 30 ℃. Curd washing was completed by stirring for 10 min as described above.

The bottles were centrifuged at 320 g for 10 min at room temperature in order to remove most of the aqueous phase. After that, curd was transferred to sterile cylindrical 150㎖ recipients, and re-centrifuged at 1400 g for 1 h. The whey was discarded and the curd was inverted in the same container. A final centrifugation was carried out as above for 30 min. After centrifugation, miniature cheeses were obtained. The miniature cheeses were kept in their sterile containers and salted by pouring 32 ㎖ of sterile saturated brine (330 g/L of NaCl, pH adjusted to 5.4) at 10 ℃ into the same containers. After 5 min, the brine was removed, and the cheeses were stored to ripening for 7 days at 30 ℃ and 30 days at 10 ℃ (Figure 6).

Table 5. Composition of raw milk analyzed by Milkoscan FT2.

Composition	Contents (%)
Milk fat	3.69
Milk protein	2.99
Lactose	4.58
Solids non fat (SNF)	8.61
Total solid (TS)	12.20

Table 6. Combinations of starter strains used for miniature cheese control and test groups and their inoculum ratios.

Group		Combination of strains	Ratio
Control	1	*L. lactis* subsp. *cremoris* CHN-11-3, *L. lactis* subsp. *lactis* IL1403	7:3
	2	*L. lactis* subsp. *cremoris* CHN-11-3, *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* biovar *diacetylactis* CHN-11-1, *L. pseudomesenteroides* CHN-11-48	7:1:1:1
Test	1	*L. lactis* subsp. *cremoris* C46, *L. lactis* subsp. *lactis* L5	7:3
	2	*L. lactis* subsp. *cremoris* C46, *L. lactis* subsp. *lactis* L56	7:3
	3	*L. lactis* subsp. *cremoris* C46, *L. lactis* subsp. *lactis* L23	7:3
	4	*L. lactis* subsp. *cremoris* C46, *L. lactis* subsp. *lactis* L48	7:3

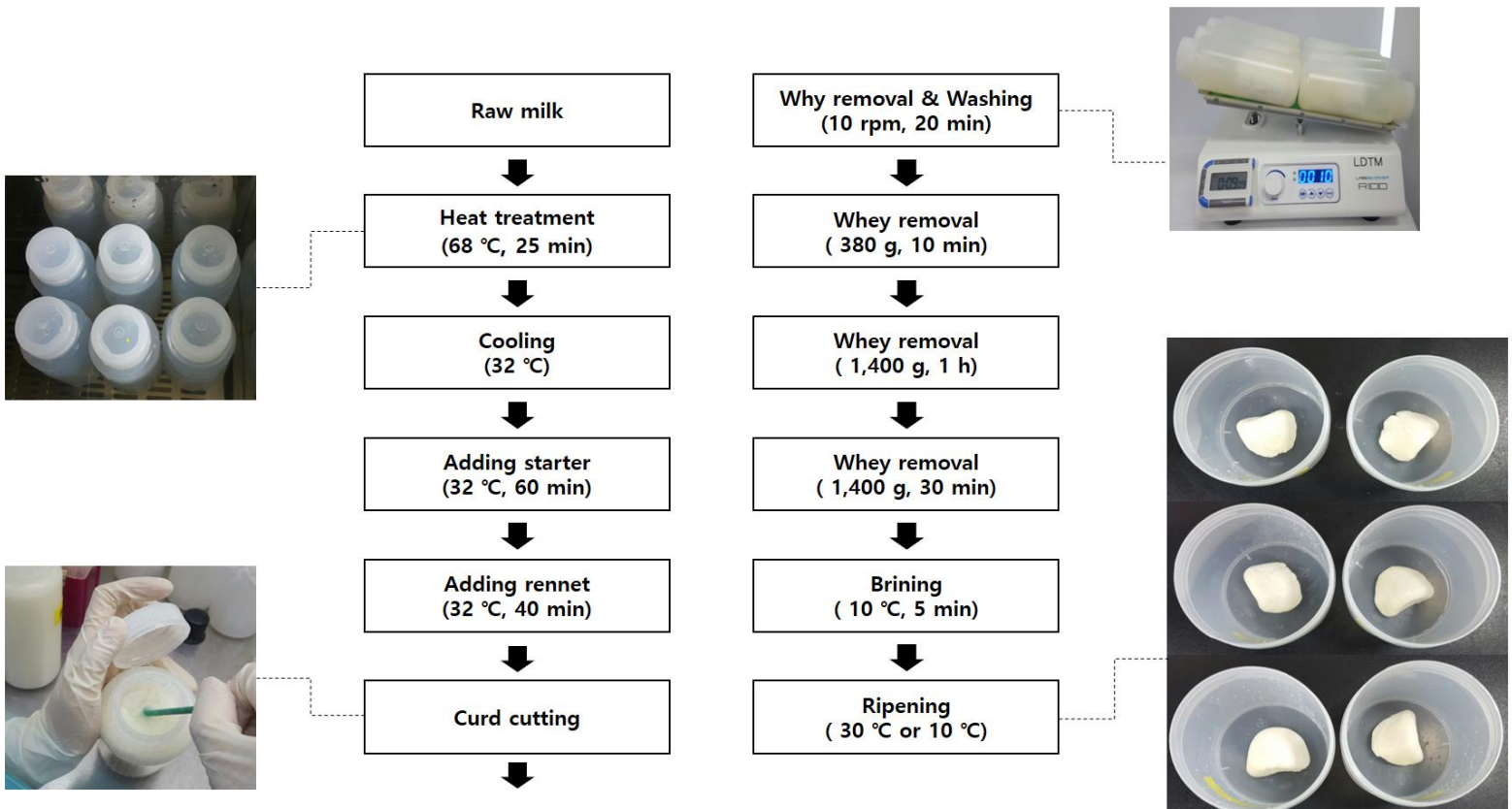

Figure 6. Procedure of miniature Gouda-type cheese model manufacturing.

3.3.2. Electronic nose analysis

The apparatus, Heracles Ⅱ Analyzer / GC type Electronic Nose produced by Alpha M.O.S (France), consists of a dual-column flash gas-chromatograph, coupled with a Combi PAL Auto-Sampler System (CTC Analytics AG, Switzerland) which is useful for processing multiple samples in batches. The analysis was performed according to the manual of Alpha M.O.S. Gas samples from the headspace after pre-incubation 20 min at 70 ℃ are taken from each 10 ㎖ vial containing 1 g of cheese and delivered by the auto-sampler to an inlets injector in which compounds flash evaporate. After passing through a pre-concentration TENAX adsorbent trap, the volatiles carried by the hydrogen are sent, equally, to two capillary chromatographic columns. The two columns have different polarities (one apolar, GC#: DB-5 and the other one with medium polarity, GC#2: DB-WAX), so that various volatile compounds are separated on both of them in the same time and detected by flame ionization detectors (FID) working in parallel. The analytical conditions for an automatic gas injection with a 2.5 ㎖ HS syringe from the head-space are as follows: quantity of sample: 1 g in a 10 ㎖ vial, sample incubation 20 min at 70 ℃ (agitation speed 500 rpm, agitation on 5 s, agitation off 2 s, flushing time 90 s), syringe temperature 80 ℃, fill speed 500 ㎕/s, injection volume 5,000 ㎕, injection speed 125 ㎕/s, injection temperature 200 ℃, injection pressure 10 kPa, injection time

45 s, trap temperature 50 ℃, trap pre-heating temperature 35 ℃, column temperature program 50 ℃ (initial, hold time 2 s) up to 250 ℃ (21 s) with an increment of 1 ℃/s to 80 ℃ and an increment of 3 ℃ /s to 250 ℃, detector temperature 260 ℃. The two chromatograms recorded are analyzed by specialized software AroChemBase (Alpha M.O.S, France) for sensory descriptions and Discriminant function analysis (DFA). DFA take the original sets of data and identify some linear combinations of the initial variables, combinations called discriminant factors which allow a simpler representation of the initial data while preserving as much as possible the information contained by it.

Table 7. Instrument and working conditions for identification of flavor compounds in miniature Gouda-type cheeses by electronic nose.

Instrument	Heracles Ⅱ Electronic Nose (Alpha M.O.S, France)
Column	DB-5 (30 m x 0.25 mm i.d., film thickness = 1.0 ㎛) DB-WAX (30 m x 0.32 mm i.d., film thickness = 0.25 ㎛)
Carrier gas	Hydrogen gas
Oven temp.	50 ℃ (2 s) → 1 ℃/s → 80 ℃ → 3 ℃/s → 250 ℃ (21 s)
Injector temp.	200 ℃
Detector temp.	260 ℃
Detector	Flame ionization detectors (FID)

Chapter 4. Results

4.1. Selection of the flavor-forming starter bacteria

4.1.1. Isolation of lactic acid bacteria

80 samples were collected from Gangwon region, South Korea. From the sample collection, total 615 strains were isolated (Table 7).

Table 8. The number of isolates obtained from sample collection.

Samples	No. of samples	No. of isolates
Raw milk	20	306
Kimchi	33	181
Jeotgal	15	91
Makgeolli	11	31
Cheonggukjang	1	6
Total	80	615

4.1.2. Acidification and coagulant activity

One of the main roles of starter bacteria in cheese manufacture is to provide rapid acidification. Table 8 shows the acid production after 6 and 48 h of groups of strains were considered. Among 615 isolates, 164 isolates show the coagulant positive activity (Figure 7).

Table 9. Acidification ability (after 6 and 48 h) of 164 isolates.

⊿pH		No. of isolates		
		Class Ⅰ[a] (⊿pH < 1.5)	Class Ⅱ[b] (1.5 ≤ ⊿pH < 2.0)	Class Ⅲ[c] (⊿pH≥ 2.0)
⊿pH 6 h	Control	4	0	0
	Isolates	163	1	0
⊿pH 48 h	Control	2	2	0
	Isolates	24	62	78

[a] Class Ⅰ: Low acidifying isolates, causing a pH decrease lower than 1.5 pH units.
[b] Class Ⅱ: The group of medium acidifying activity, showing a pH drop of between 1.5 and 2.0 units.
[c] Class Ⅲ: highly acidifying isolates showing a pH decrease of over 2 pH units.

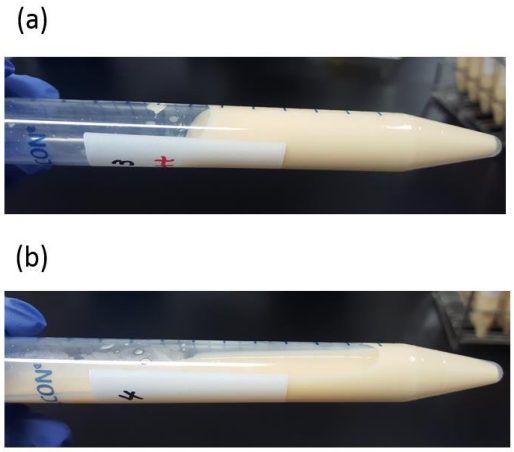

Figure 7. Coagulant positive (a) and negative (b) activity in 10 % skim milk.

4.1.3. Assessment of proteolysis and lipolysis

There were no significant strains that have proteolytic and lipolytic activity on skim milk agar or tributyrin agar at 30 ℃ (Figure 8).

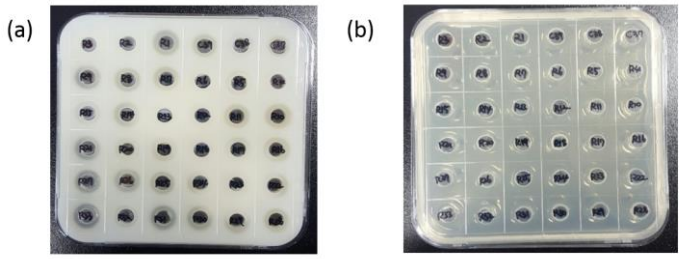

Figure 8. Proteolytic activity (a) and lipolytic activity (b) by agar well diffusion.

4.1.4. Detection of enzymes involved in the flavor-forming pathways

Multiplex PCR assay was rapid and reasonable method for screening of strains with specific key enzymes involved in flavor formation pathway. Examples of PCR product visualization are shown in Figure 9 and summarized in Table 8. As expected, control *L. lactis* subsp. *lactis* biovar. *diacetylactis* CHN-11-1 encoded all genes and *L. lactis* subsp. *lactis* IL1403 encoded all genes except *citP*. Whereas, *L. lactis* subsp. *cremoris* CHN-11-3 encoded only *bcaT* and *L. pseudomesenteroides* CHN-11-48 encoded none of the target genes. In the tested 164 isolates, 44 isolates express at least three of target genes. There were no isolates which possess *citP* and only three isolates possess *kdcA*.

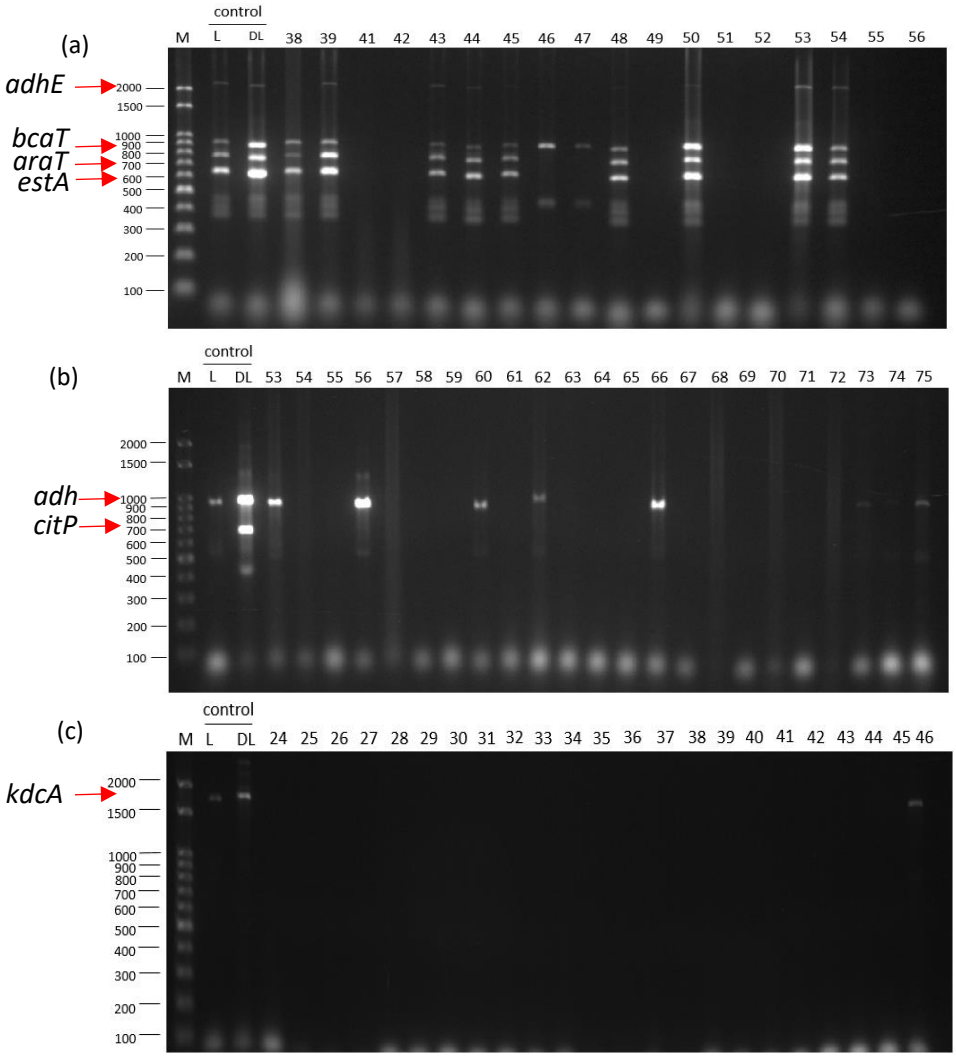

Figure 9. Agarose gel electrophoretic analysis of multiplex PCR of 164 isolates with the *adhE, bcaT, araT, estA* (a), *adh, citP* (b), and *kdcA* (c). M: 100bp ladder, L: *Lactococcus lactis* subsp. *lactis* IL1403, DL: *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CHN-11-1.

Table 10. The summary of application of the multiplex PCR to detect enzymes involved in the flavor-forming pathways.

	Target gene [a,b]						
	adhE	*bcaT*	*araT*	*estA*	*adh*	*citP*	*kdcA*
Control							
*L.*subsp. *lactis* IL1403	+	+	+	+	+	−	+
L. lactis subsp. *cremoris* CHN-11-3	−	+	−	−	−	−	−
L. lactis subsp. *lactis* biovar *diacetylactis* CHN-11-1	+	+	+	+	+	+	+
L. pseudomesenteroides CHN-11-48	−	−	−	−	−	−	−
Strains							
L. lactis subsp. *lactis* L5	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L9	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L19	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L20	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L21	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L23	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L29	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L39	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L43	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L44	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L45	+	+	+	+	+	−	−
L. lactis subsp. *cremoris* C46	−	+	−	−	−	−	+
L. lactis subsp. *lactis* L48	+	+	+	+	−	−	+
L. lactis subsp. *lactis* L54	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L30	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L53	+	+	+	+	+	−	−
L. lactis subsp. *lactis* L56	+	+	+	+	+	−	+
L. lactis subsp. *lactis* L66	+	+	+	+	+	−	−

[a] *adhE*, bifunctional aldehyde/alcohol dehydrogenase; *bcaT*, branched-chain aminostransferase; *araT*, aromatic aminotransferase; *estA*, esterase A; *adh*, alcohol dehydrogenase; *citP*, citrate permease; *kdcA*, keto acid decarboxylase.
[b] + , gene present; − , gene absent.

4.1.5. Glutamate dehydrogenase activity assessment

Different levels of NAD-dependent GDH were detected in the strains assayed (Figure. 10). The 16 strains of *L. lactics* strains among the 44 strains had the significant levels of GDH activity compared to CHN-11-1. The GDH levels in the other strains were not significant.

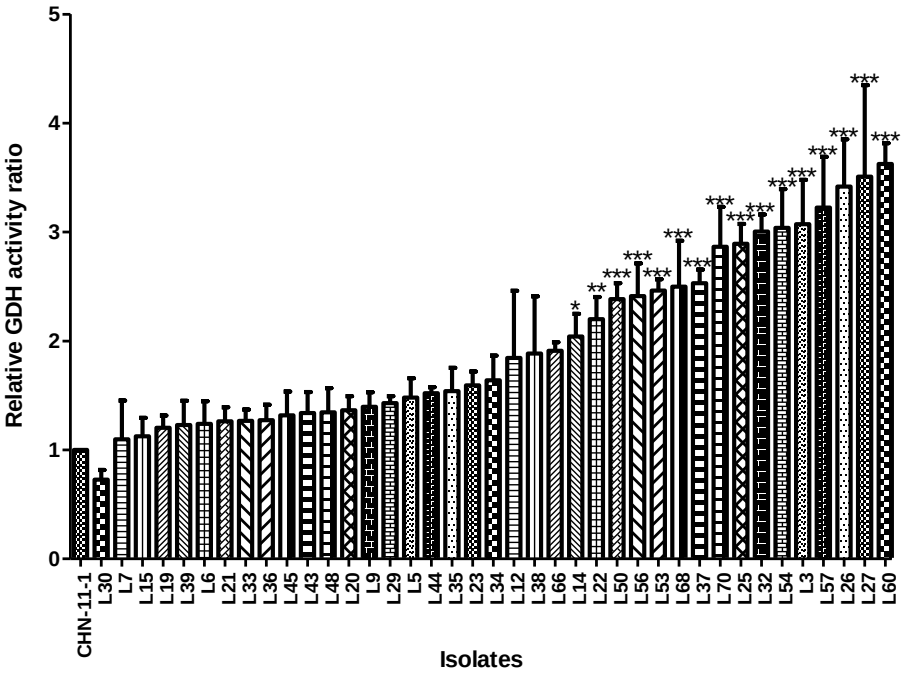

Figure 10. The relative GDH activity of isolates was measured as a ratio compared with CHN-11-1. *P* value were analyzed by one-way analysis of variance (ANOVA) ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$). Error bars indicate standard deviations of triplicate determinations. CHN-11-1: *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* CHN-11-1.

4.2. Characterization of the selected starter bacteria

4.2.1. Identification of the selected strains

Selected 44 isolates were identified by using 16s rRNA gene BLAST (> 99 %). One was identified as *Lactococcus lactis* subsp. *cremoris* strain and two were identified as *Lactobacillus casei* strains. The rest of the isolates were *Lactococcus lactis* subsp. *lactis* strains.

4.2.2. Biochemical property

The selected *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains showed identical patterns of fermentation compared to control *L. lactis* subsp. *lactis* IL1403 (IL1403) and *L. lactis* subsp. *cremoris* CHN-11-3 (CHN-11-3), respectively (Figure 11). Especially, *L. lactis* subsp. *lactis* L5 (L5) and *L. lactis* subsp. *lactis* L23 (L23) strains showed strong carbohydrates fermentation activity. Also, the selected strains L5 were showed slightly strong on enzyme activity.

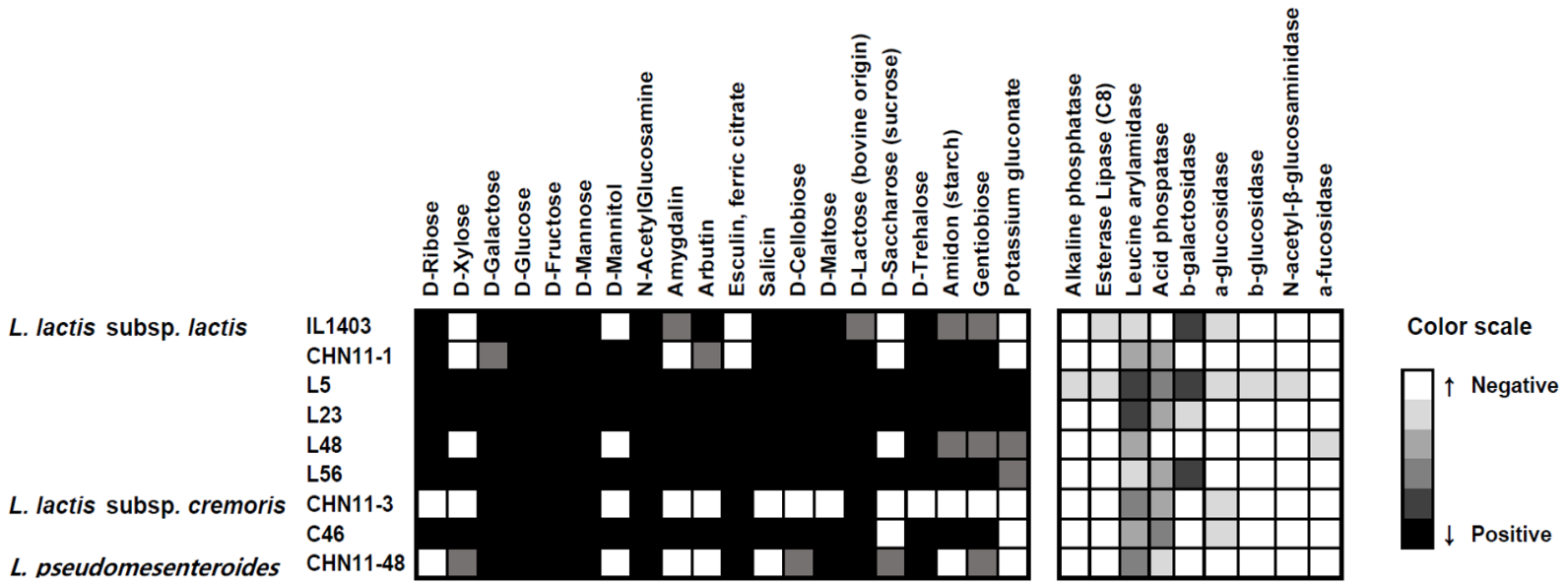

Figure 11. Carbohydrates fermentation and enzymatic patterns of selected *L. lactics* subsp. *lactis* strains and *L. lactics* subsp. *cremoris* strain were assessed by API 50 CHL and API ZYM kit, respectively. The biochemical properties are shown in a black–white color scale, where black represents positive activity and white represents negative activity.

4.2.3. Growth curve

Although all *L. lactis* strains showed rapid growth in initial incubation time, the growth curve patterns were strain specific. *L. lactis* subsp. *lactis* strains slightly rapid increase than *L. lactis* subsp. *cremoris* strain, however, the maximum growth of *L. lactis* subsp. *cremoris* strain was higher than *L. lactis* subsp. *lactis* strains. *L. pseudomesenteroides* strain showed low growth activity in entire incubation time (Figure 12).

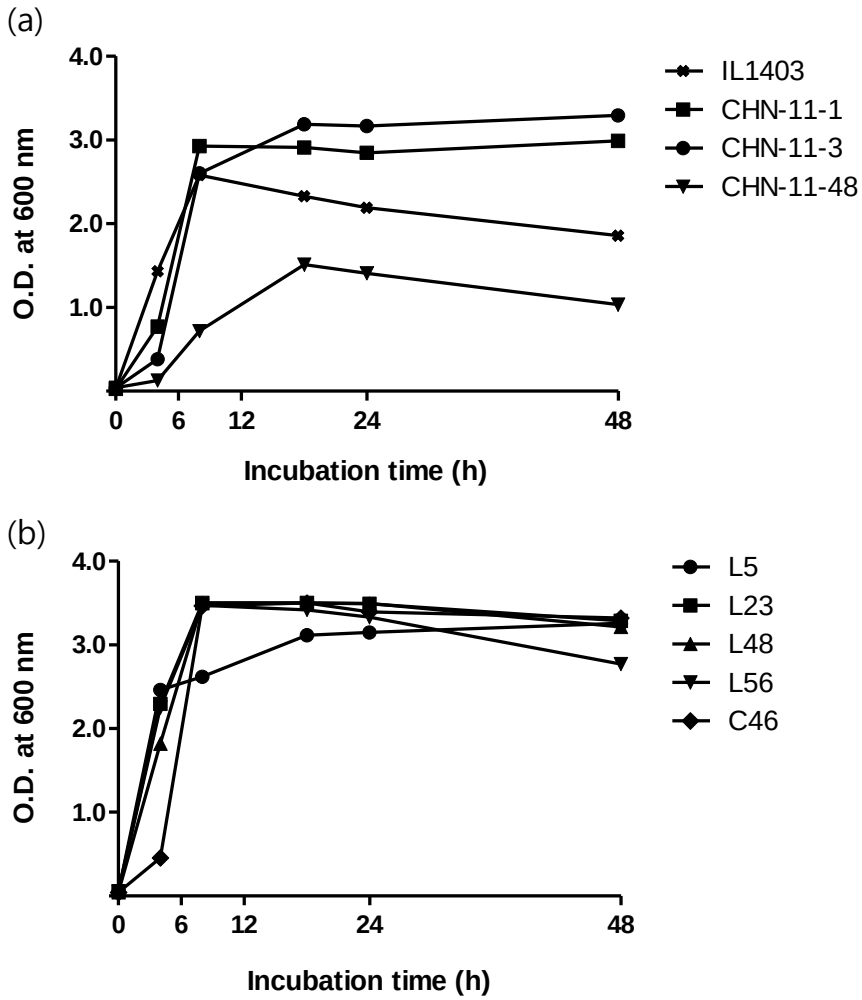

Figure 12. The growth pattern of control cheese starter strains and the combination of selected strains in GM17 media at 30 ℃. Control 4 strains (a), the selected strains; L5, L23, L48, L56, and C46 (b).

4.2.4. Assessment of autolysis ability

Compared to control IL1403, selected *L. lactis* subsp. *lactis* strains have high autolysis activity except *L. lactis* subsp. *lactis* L48 (L48). Selected *L. lactis* subsp. *cremoris* C46 (C46) also have high autolysis activity compared to control CHN-11-3 (Figure 13).

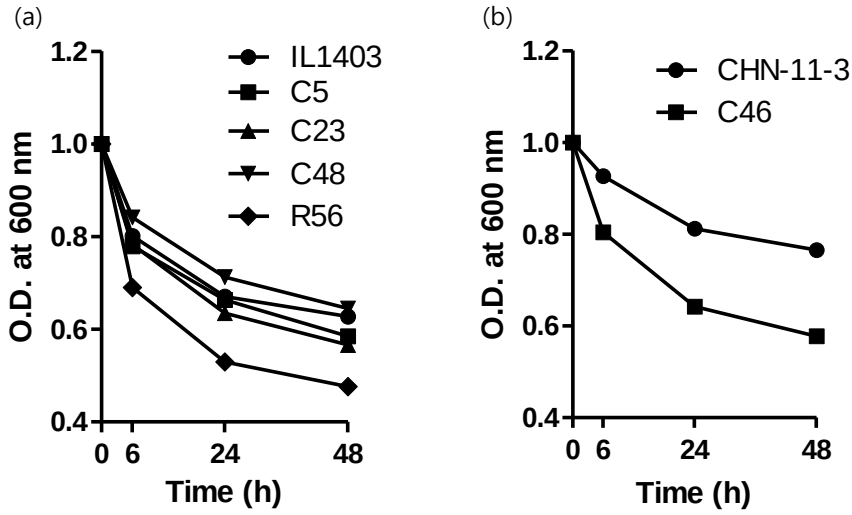

Figure 13. Autolysis activity of isolated *L. lactis* subsp. *lactis* strains (a) and *L. lactis* subsp. *cremoris* strains (b).

4.2.5. Stability to freeze-drying process

The stability of control 4 strains and selected 5 strains to freeze-drying process were assessed (Table 11). Control strains decreased 1 or 2 Log CFU/g in the viable cell counts after freeze-drying, however, the selected 5 strains maintained their viable cell counts or decreased only 1 Log CFU/g. Selected strains were more stable to freeze-drying process compared to control strains.

Table 11. The viable cell counts of the control strains and selected strains are reported before and after the freeze-drying process.

	Before freeze-drying (Log CFU/g)	After freeze-drying (Log CFU/g)
Control		
IL1403	11.64±0.00	10.43±0.09
CHN-11-1	10.51±0.21	8.83±0.13
CHN-11-3	10.72±0.11	8.91±0.01
CHN-11-48	10.81±0.19	9.96±0.17
Strains		
L5	10.47±0.05	10.73±0.04
L23	10.80±0.17	10.25±0.07
L48	11.55±0.02	10.49±0.08
L56	11.33±0.01	10.38±0.09
C46	11.64±0.05	10.43±0.22

4.3. Validation of the selected strains in cheese model

4.3.1. Analysis of miniature cheese model

Two of control miniature cheeses and four of test miniature cheeses were manufactured and ripened at 10 ℃ or 30 ℃. Total 12 of miniature cheeses were weighted and yield of each cheeses was calculated (Table 12). The average weight and yield of all miniature cheeses was 49.32 g and 12.33 %, respectively.

Acidification of each cheeses during the production and ripening period was showed in Figure 14. pH in 10 ℃ ripened miniature cheeses continuously decreased until 21 days, however, pH slightly increased at 28 days, except Con.4 and Test 4. pH in 10 ℃ ripened miniature cheeses rapid decreased during first 24 h and then increased.

Table 12. Weight (g) and yield (%) of model cheese immediately after manufacture.

	10℃ ripening		30℃ ripening	
	weight (g)	yield (%, w/v)	weight (g)	yield (%, w/v)
Con.2	50.83	12.71	47.68	11.92
Con.4	45.26	11.32	45.87	11.47
Test 1	60.33	15.08	43.12	10.78
Test 2	65.15	16.29	41.63	10.41
Test 3	51.92	12.98	47.68	11.92
Test 4	51.50	12.88	40.82	10.21

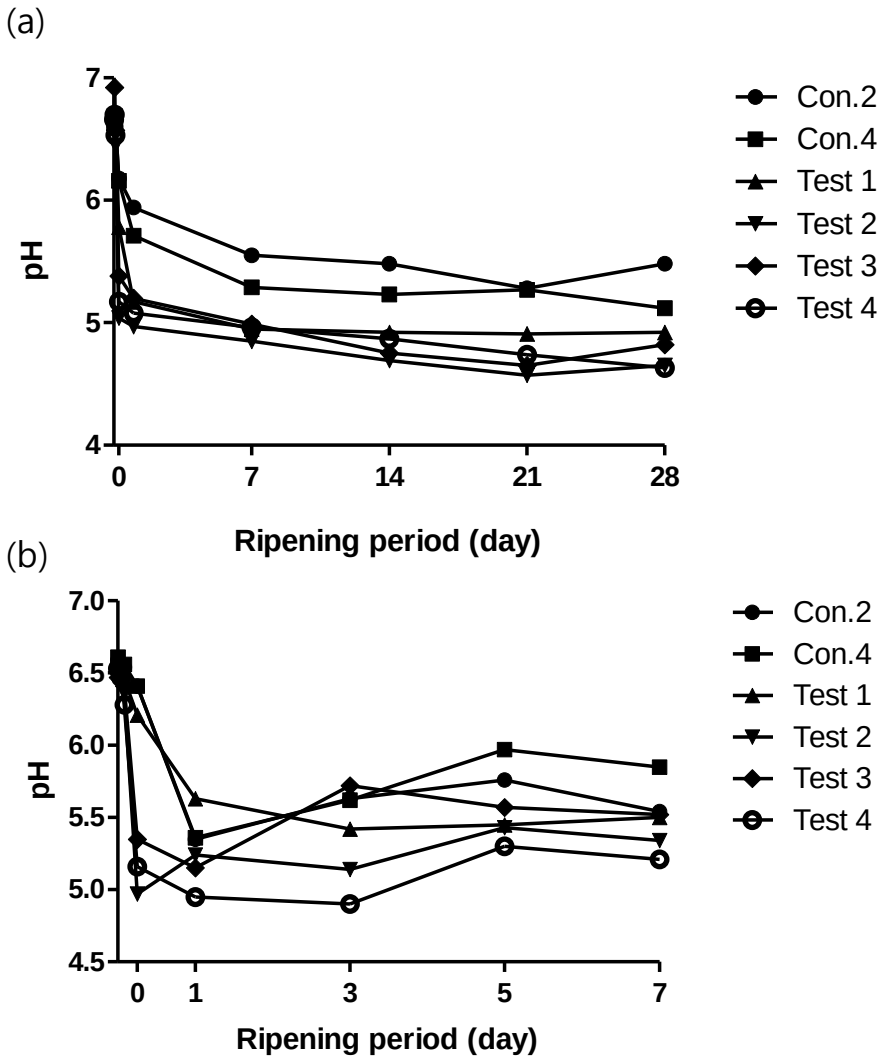

Figure 14. Change in pH during ripening at 10 ℃ (a) and 30 ℃ (b).

4.3.2. Flavor compounds analysis

4.3.2.1. Discriminant Function Analysis (DFA)

Figure 15 shows the DFA of the raw data obtained with the electronic nose. In the DFA of 10 ℃ ripened miniature cheeses, it was possible to discriminate between control groups and test groups by using the first three discriminant factors (DF1; 21.300 %, DF2; 12.599 %, and DF3; 11.061 %). It was also possible to discriminate the ripening periods by using the first three discriminant factors (DF1; 48.481 %, DF2; 32.726 %, and DF3; 9.149 %). In the DFA of 30 ℃ ripened miniature cheeses, each clusters of 0, 3, 7 days were clearly classified (Hong *et al.*, 2012).

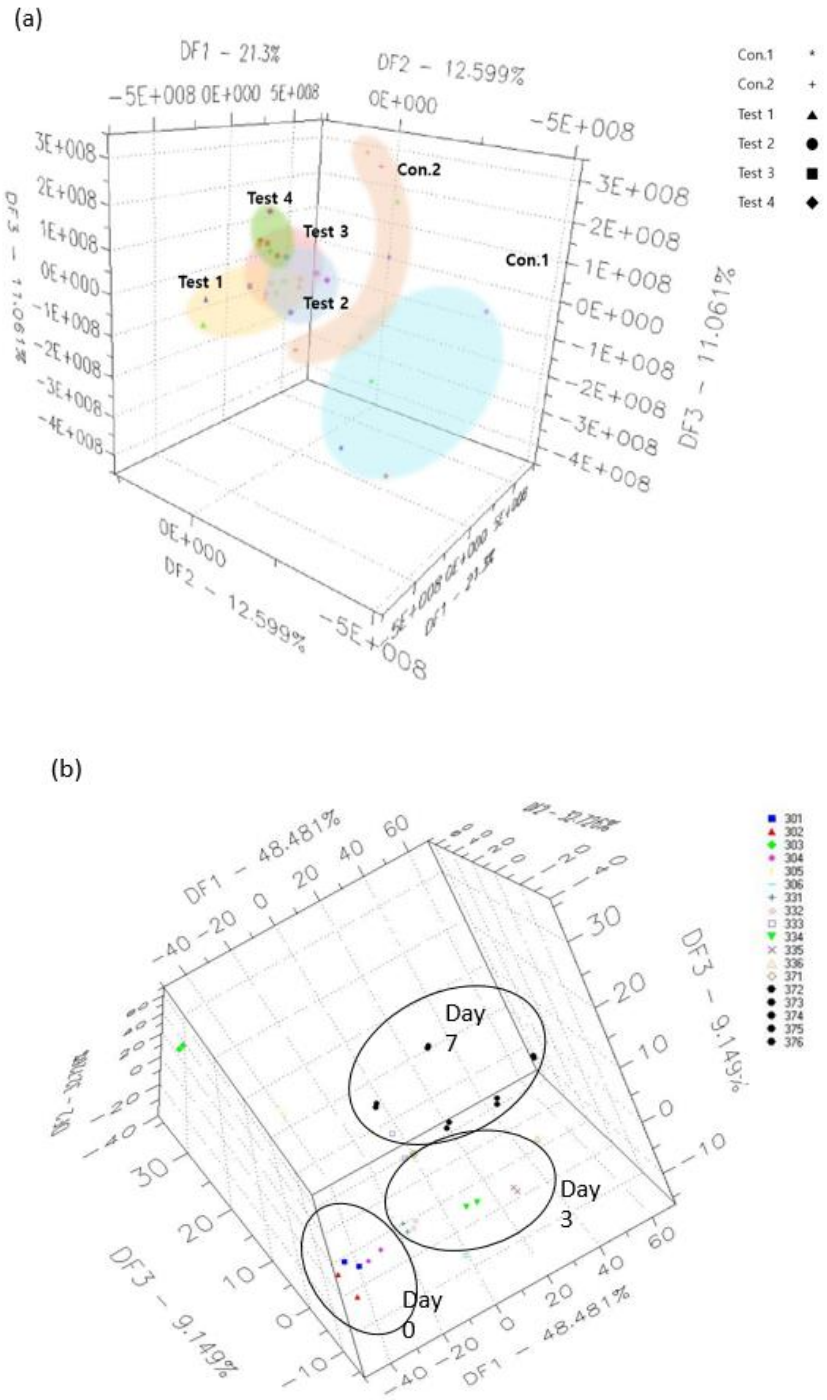

Figure 15. Discriminant Function Analysis (DFA) for the flavor pattern of the 10 ℃ ripened miniature cheeses (a) and 30 ℃ ripened miniature cheeses.

4.3.2.2. Flavor compounds analysis

All Miniature cheeses were analyzed by using electronic nose. There was clear difference in flavor profiles between control and test groups. Representative flavor compounds derived from investigated miniature cheeses were described with sensory descriptors in Table 13.

First, the all test groups of the 10 ℃ ripened miniature cheeses have more variety flavor profile than control groups in both DB-5 and DB-WAX column. In the DB-5 column, control groups showed ethanol, butane-2,3-dione, acetoin. Whereas, test groups showed 3-methyl-3-buten-2-one and methyl isothiocyanate including ethanol and butane-2,3-dione (Figure 16). In the DB-WAX column, 3-buten-2-one, acetoin, formic acid were formed in both of control and test groups (Figure 17). Flavor compounds are evolved during 28 days of ripening. Although the high level of methylethyl formate, 2-propenol, methylbutanone were detected at the 0 week, those three compounds almost disappeared at the last week. However, butane-2,3-dione, 3-methyl-3-buten-2-one, methyl isothiocyanate, formic acid increased during ripening period.

The flavor profile of 30 ℃ ripened miniature cheeses have more variety than 10 ℃ ripened miniature cheeses and also the test groups showed more flavor compounds than control groups in both DB-5 and DB-WAX column. In the DB-5 column, control miniature cheese

formed ethanol, butane-2,3-dione, acetoin, 1,3-dimethylcyclohexane, 3-methyl-3-butanol, heptafluorobutyrate. Test miniature cheeses also formed those 5 flavor compounds and additionally formed methylbutanone and methyl isothiocyanate (Figure 18). In the DB-WAX column, enflurane, 3-buten-2-one, butane-2,3-dione, methyl isothiocyanate, acetoin, formic acid, ethylene glycol were derived from 30 ℃ ripened control miniature cheeses, on the other hand, 1-propanol, 2-methyl-, isopropyl isothiocyanate, 3-formythiophene, methylbutanone were derived test miniature cheeses besides the flavor compounds of the control groups (Figure 19). Seven days of ripening at 30 ℃ were enough to evolved flavor compounds. High concentration of methylbutanone and enflurane was found at the initial ripening period of all test groups. Significant level of 3-buten-2-one was detected early period of ripening in the Test 1 and Test 2 groups but soon decreased. Some flavor compounds increased during the ripening period, for examples, butane-2,3-dione, acetoin, methyl isothiocyanate, 1,3-dimethylcyclohexane, 3-methyl-3-butanol, heptafluorobutyrate, formic acid, furfural, and ethylene glycol.

Among the all test groups, Test 1 which was composed of *L. lactic* subsp. *lactis* L5 and *L. lactis* subsp. *cremoris* C46 profiled more flavor compounds. Compared to other test groups at the point of last ripening day, Test 1 showed extra flavor compounds such as ethylbenxene-

d10, 2-methyl-2-butanol in 10 ℃ ripened cheeses and 2-nitropropane, nitromethane in 30 ℃ ripened cheeses. Other test groups formed similar composition of flavor compounds, however, their flavor intensity was different.

Table 13. Flavor profiling and expected sensory descriptors analyzed by electronic nose.

	Control group	Test group	Formula	Sensory descriptors	Reference
10℃	Acetoin	Acetoin	$C_4H_8O_2$	Butter, Coffee, Creamy	Arochembase[a]
	Butane-2,3-dione	Butane-2,3-dione	$C_4H_6O_2$	Butter, Caramelized, Creamy, Fruity, Pineapple	Arochembase
	Ethanol	Ethanol	C_2H_6O	Alcoholic, Ethanol, Pungent, Sweet	Arochembase
	Formic acid	Formic acid	CH_2O_2	Savory	FEMA[b]
	3-Buten-2-one	-	C_4H_6O	sweet	Arochembase
		1-Propanol, 2-methyl-	$C_4H_{10}O$	Alcoholic, Bitter, Chemical, Glue, Leek, Licorice, Solvent, Winey	Arochembase
		2,4-Dimethylfuran	C_6H_8O	Onion, Galic, Leek	Villière *et al.*, (2015)
		2-Propenol	C_3H_6O	Mustard	https://cameochemicals.noaa.gov/chemical/2357
		3-Methyl-3-buten-2-one	C_5H_8O	Alcoholic, Etheral	Arochembase
		Enflurane	$C_3H_2ClF_5O$	Etheral, Pungent	Arochembase
		Isopropyl isothiocyanate	C_4H_7NS	Savory	FEMA
		Methylbutanone	$C_5H_{10}O$	Camphor	Arochembase
		Methylethyl formate	$C_4H_8O_2$	Fruit	FEMA
		Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	Duque, Carmenza, *et al.*, (2001)
30℃	1,3-Dimethylcyclohexane	1,3-Dimethylcyclohexane	C_8H_{16}	Perfumery	Arochembase
	3-Buten-2-one	3-Buten-2-one	C_4H_6O	sweet	Arochembase
	3-Methyl-1-butanol, heptafluorobutyrate	3-Methyl-1-butanol, heptafluorobutyrate	$C_9H_{11}F_7O_2$	Fatty	Arochembase
	Acetoin	Acetoin	$C_4H_8O_2$	Butter, Coffee, Creamy	Arochembase
	Butane-2,3-dione	Butane-2,3-dione	$C_4H_6O_2$	Butter, Caramelized, Creamy, Fruity, Pineapple	Arochembase
	Enflurane	Enflurane	$C_3H_2ClF_5O$	Etheral, Pungent	Arochembase
	Ethylene glycol	Ethylene glycol	$C_2H_6O_2$	bittersweet	Berman *et al.*, (1957)
	Ethanol	Ethanol	C_2H_6O	Alcoholic, Ethanol, Pungent, Sweet	Arochembase
	Formic acid	Formic acid	CH_2O_2	Savory	FEMA
	Methyl isothiocyanate	Methyl isothiocyanate	C_2H_3NS	Nauseating, Toxic smell	Duque, Carmenza *et al.*, (2001)
		1-Propanol, 2-methyl-	$C_4H_{10}O$	Alcoholic, Bitter, Chemical, Glue, Leek, Licorice, Solvent, Winey	Arochembase
		2-Propenol	C_3H_6O	Mustard	https://cameochemicals.noaa.gov/chemical/2357
		3-Formylthiophene	C_5H_4OS	Floral, vegetable	Arochembase
		3-Methyl-3-buten-2-one	C_5H_8O	Alcoholic, Etheral	Arochembase
		Enflurane	$C_3H_2ClF_5O$	Etheral, Pungent	Arochembase
		Furfural	$C_5H_4O_2$	Almond, Bread, Sweet	Arochembase
		Isopropyl isothiocyanate	C_4H_7NS	Savory	FEMA
		Methylbutanone	$C_5H_{10}O$	Camphor	Arochembase

[a] Database provided by electronic nose manufacturer (Alpha M.O.S, France) which is linked with NIST database (http://webbook.nist.gov/chemistry).
[b] FEMA;Flavor & Extract Manufacturers Association

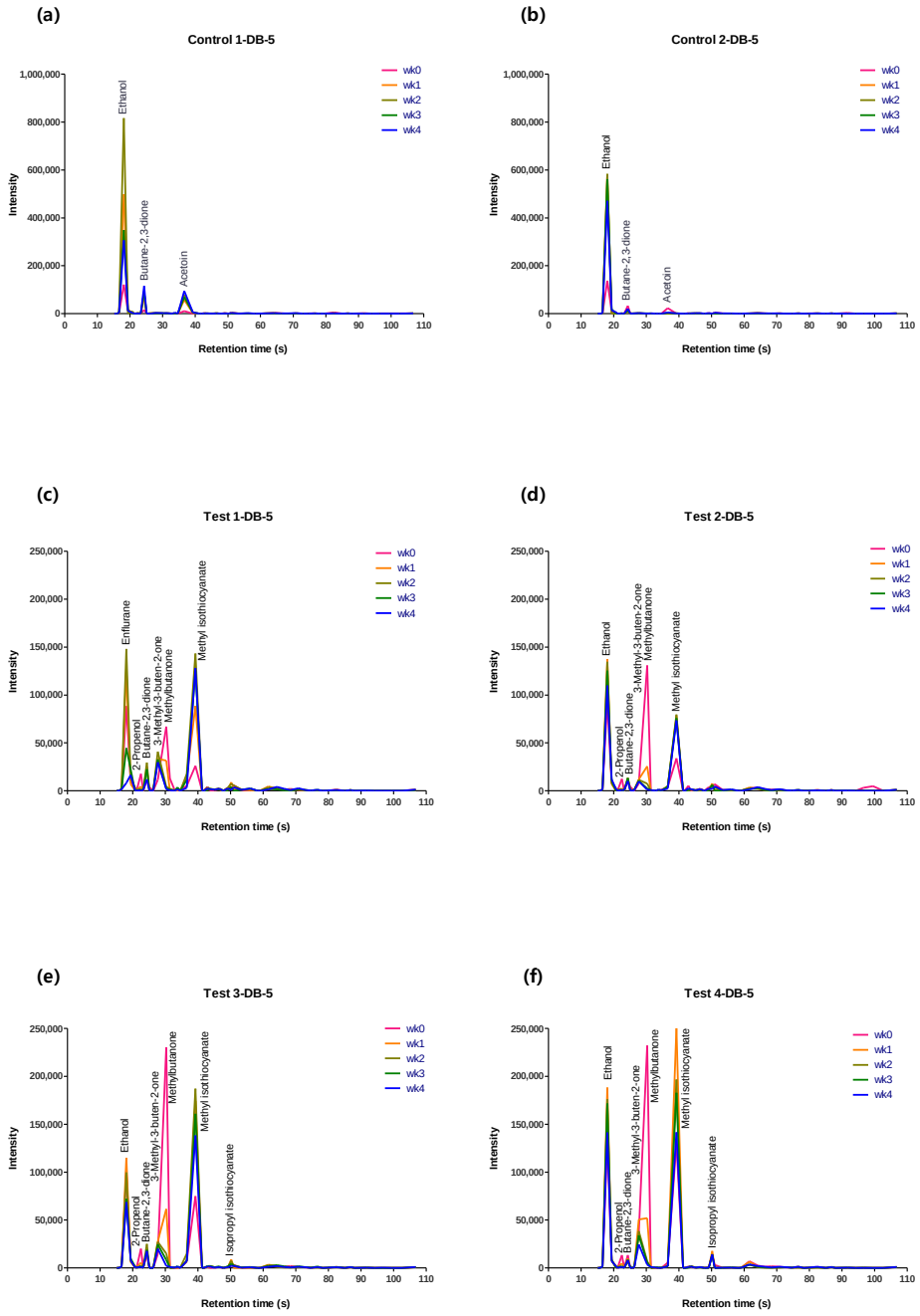

Figure 16. Chromatogram obtained from 10 ℃ ripened miniature cheese samples using DB-5 column. (a) Control 1; (b) Control 2; (c) Test 1; (d) Test 2; (e) Test 3; (f) Test 4.

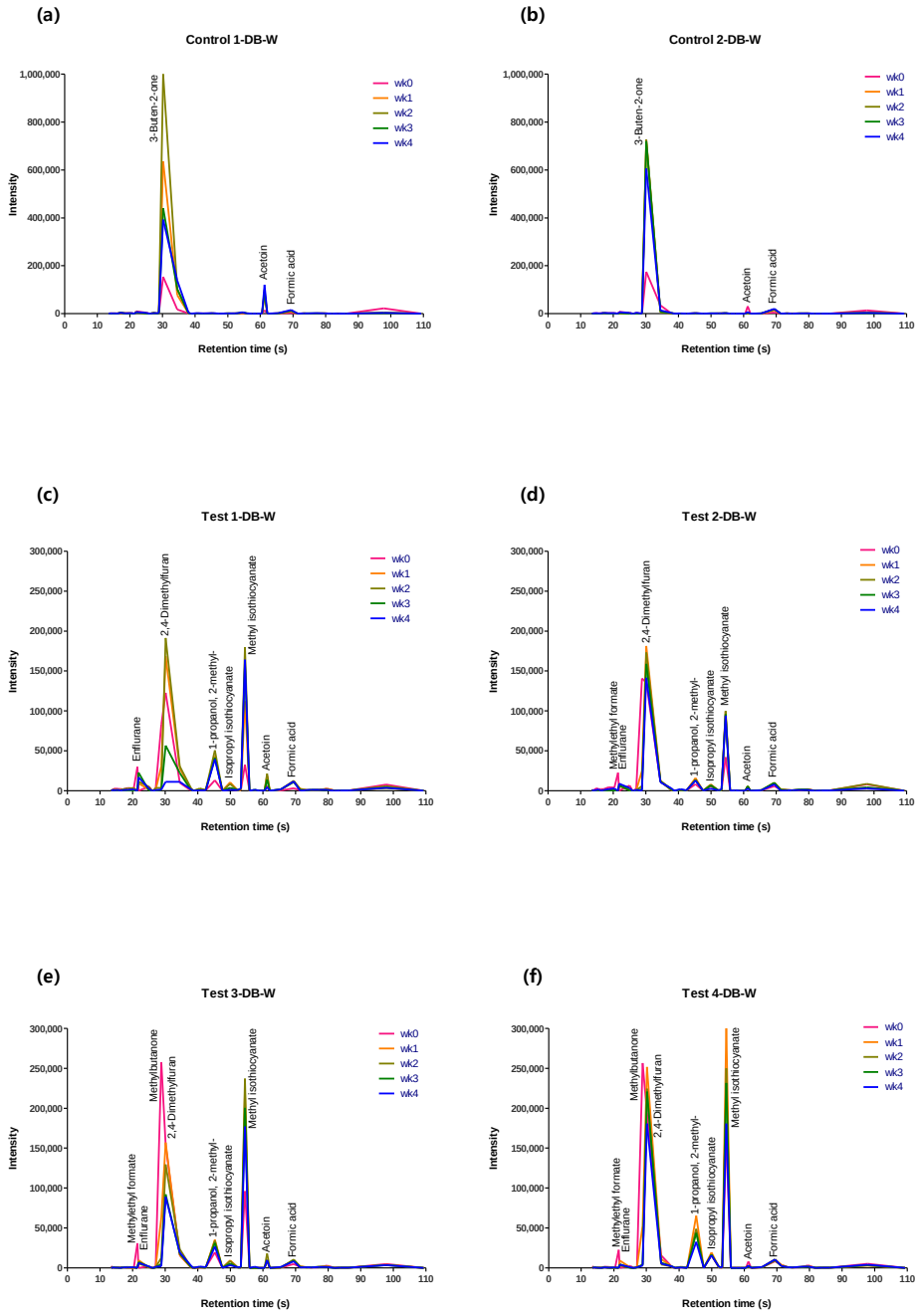

Figure 17. Chromatogram obtained from 10 ℃ ripened miniature cheese samples using DB-WAX column. (a) Control 1; (b) Control 2; (c) Test 1; (d) Test 2; (e) Test 3; (f) Test 4.

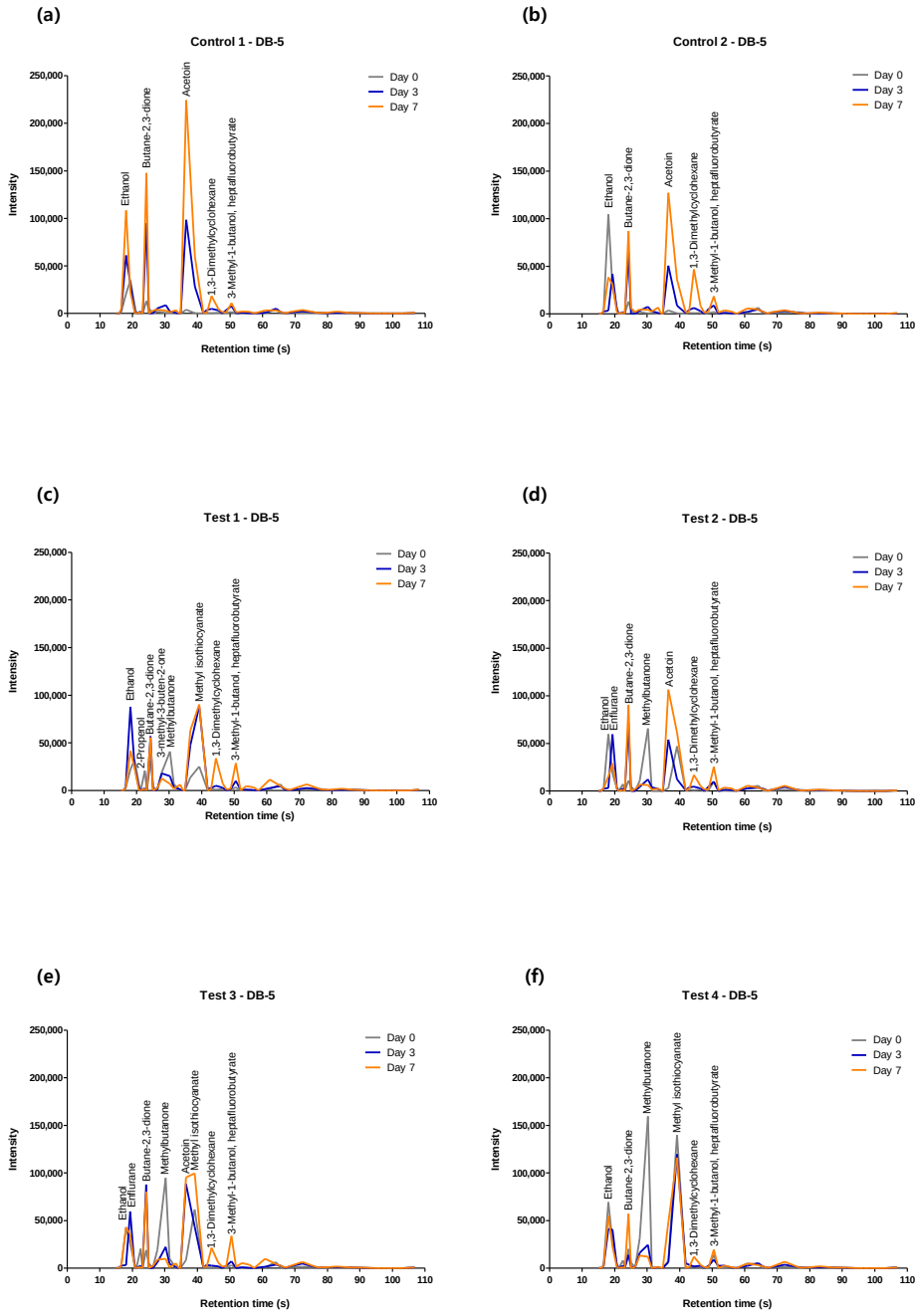

Figure 18. Chromatogram obtained from 30 ℃ ripened miniature cheese samples using DB-5 column. (a) Control 1; (b) Control 2; (c) Test 1; (d) Test 2; (e) Test 3; (f) Test 4.

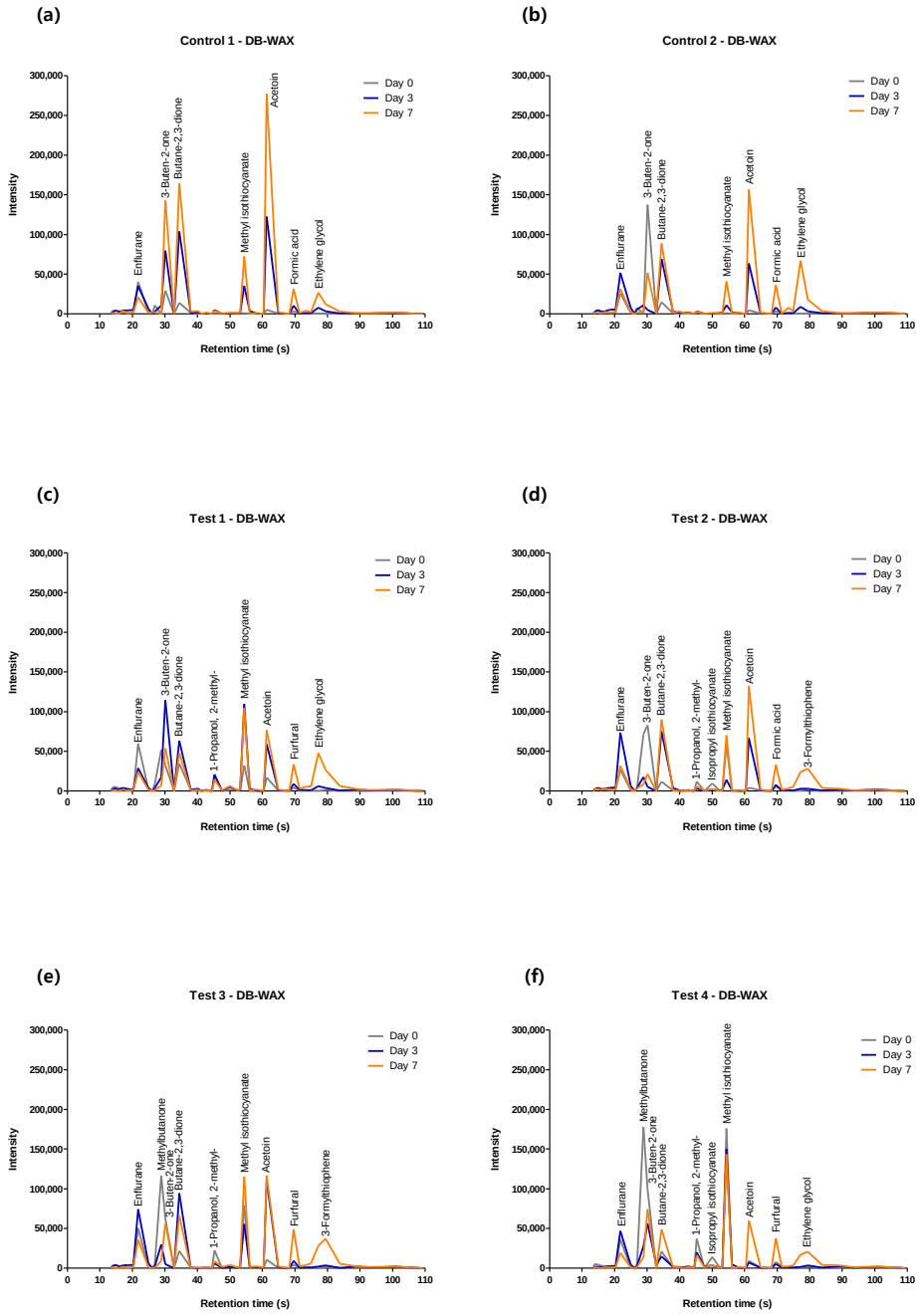

Figure 19. Chromatogram obtained from 30 ℃ ripened miniature cheese samples using DB-WAX column. (a) Control 1; (b) Control 2; (c) Test 1; (d) Test 2; (e) Test 3; (f) Test 4.

Chapter 5. Discussion

Cheese and cheese starter culture related studies were constantly researched in the world (Bosset and Gauch, 1993, Thage *et al.*, 2004, Di Cagno *et al.*, 2006, Lacroix *et al.*, 2010). However, it was rarely studied about development of new cheese starter culture, because of the complexity of their mechanisms in cheese production (H.E. Ayad *et al.*, 2000). Furthermore, the Korean cheese market has only just begun to grow. It is reason why the little research on cheese in South Korea. Therefore, it is significant that this is the first study on selection of cheese starter bacteria originated from Gangwon region and the first attempt to manufacture the Korean Gouda-type cheese.

Cheese starters differ in selection criteria from lactic acid bacteria for probiotics (Ong *et al.*, 2006). The basic selection criterion is the ability to form curd in milk through acid production (Cogan *et al.*, 1997, Curtin *et al.*, 2001, Kahala *et al.*, 2008). Rapid growth, autolysis ability, carbohydrate fermentation pattern and enzymatic profile are also the selection criteria (Hébert *et al.*, 2000, Bourdat-Deschamps *et al.*, 2004). In general, starter culture is used in Direct Vat Set (DVS) form when industrially used, so they should be stable in the freeze-drying process. And most of all, starter bacteria should contribute to the production of flavors in cheese (McSweeney and Sousa, 2000, McSweeney, 2004).

Cheese flavor is produced through complex process. According to

the Component Balance Theory of Cheese Flavor, it is the result of the correct balance and concentration of a wide variety of volatile flavor compounds (Mulder, 1952, Singh *et al.*, 2003). The flavor of cheese varies depending on the cheese types, and even the same type of cheese, their flavor varies depending on starter bacteria and the ripening condition (Sousa *et al.*, 2001, Van Leuven *et al.*, 2008). As the increased interest about Gouda cheese in Korea, the novel starter bacteria with flavor-forming ability were selected based on the traditional Gouda-type cheese flavor.

In the first step of the study, 164 isolates with acid-producing and coagulant ability were selected among 615 isolates originated from 80 samples. Starter bacteria with flavor-forming ability were selected by gene based strain selection. By using multiplex PCR assay, *adhE, bcaT, araT, estA, adh, citP*, and *kdcA* genes were detected in the selected 164 strains. In addition, GDH enzyme activity was assessed by enzymatic assay (Rijnen *et al.*, 2000). Those 8 of enzymes were related to flavor formation pathway (Liu *et al.*, 2008). Among the 164 strains, none was found to have all 8 enzymes. Only strains L56 expressed all genes except *citP* gene and 15 isolates expressed 5 genes except *citP* and *kdcA* genes. There was no isolate with *citP* gene, but *kdcA* gene was found in C46, L48, and L56. The fact that no *citP* gene was found in isolates was similar to previous studies (Exterkate *et al.*, 1997, Desmasures *et al.*, 1998, Drici *et al.*, 2010).

In bovine milk, *L. lactis* subsp. *lactis* is known to be a dominant LAB and has been extensively analyzed, but the *L. lactis* subsp. *lactis* biovar. *diacetylactis* which has *citP* gene has been more rarely detected. Whereas KdcA enzyme activity was only found in non-dairy lactococcal strains (Liu *et al.*, 2001). It is difficult to select strains with *kdcA* gene for starter culture because non-dairy origin strains often have no ability to form coagulant in milk (Ayad *et al.*, 1999).

Considering the overall results, the strains were selected and combined to complement each other (Ayad *et al.*, 2001). As a result, four combinations of *L. lactis* subsp. *lactis* L5 combined with *L. lactis* subsp. *cremoris* C46 (Test 1), *L. lactis* subsp. *lactis* L23 combined with *L. lactis* subsp. *cremoris* C46 (Test 2), *L. lactis* subsp. *lactis* L48 combined with *L. lactis* subsp. *cremoris* C46 (Test 3), and *L. lactis* subsp. *lactis* L56 combined with *L. lactis* subsp. *cremoris* C46 (Test 4) were prepared.

In general, Gouda-type cheese ripened from more than two months to several years (Van Leuven *et al.*, 2008). Long-term cheese ripening in the laboratory has a limit, so it is necessary to shorten the ripening period. Starter bacteria affect flavor formation at least 2 to 3 weeks after cheese production (Fox *et al.*, 2001). Thus, experiments were designed to ripening the miniature cheeses at 10 ℃ for 28 days, and ripening the miniature cheeses at 30 ℃ for 7 days in order to accelerate.

Flavor profile analyzed by using electronic nose was varied, as the starter bacteria, ripening temperature condition, and ripening period (Kalit *et al.*, 2014, Shiota *et al.*, 2015). Compared to control miniature cheese groups, test miniature cheese groups formed more flavor compounds such as 1-propanol, 2-methyl-, 2-propenol, 3-methyl-3-buten-2-one, enflurane, isopropyl isothiocyanate, methylbutanone, methyl isothiocyanate. Those flavor compounds were described as alcoholic, pungent, fruit, savory and so on.

Especially, methylbutanone and 1-propanol, 2-methyl- were not found in the control groups but found in the test groups. Those two flavor compounds are significant because they are seemed to be produced by target enzymes (Helinck *et al.*, 2004). First, methylbutanone was found only initial ripening period of test groups. This compound is usually derived from branched-chain amino acid, leucine. Aminotransferases such as BcaT and AraT transformed leucine into α-oxoisocaproate with the aid of GDH (Yvon *et al.*, 1997). Then KdcA convert α-oxoisocaproate into methylbutanone. It recognized as camphor flavor. Second, the flavor compound only found in the test groups was 1-propanol, 2-methyl-. It is derived from valine which is one of the branched-chain amino acid. Valine is transformed into α-oxoisovalerate by aminotransferases and then converted corresponding 2-methylpropanal by KdcA. Finally, 1-propanol, 2-methyl- is produced from 2-methylpropanal by Adh. It

was described as alcoholic, bitter, chemical, glue, leek, licorice, solvent, and winey flavor. The two flavor compounds demonstrated that miniature cheeses of test groups had a characteristic flavor profile. It means that the selected starter bacteria contributed to the cheese flavor formation by producing the target enzymes (Smit *et al.*, 2000, Fernández and Zúñiga, 2008).

Among the all test groups, flavor profile of Test 1 produced more flavor compounds compared to other test groups at the point of last ripening day. Other test groups formed similar composition of flavor compounds, however, their flavor intensity was different. Therefore, it was confirmed that starter bacteria have an important role for the characteristic flavor compounds and intensity in cheese (van Kranenburg *et al.*, 2002, Smit *et al.*, 2005b).

In this study, the Gangwon region originated starter bacteria with flavor-forming ability were selected and validated in Korean Gouda-type cheese model. In further studies, safety of the selected starter strains should be verified for industrial use. The long-term experiment of large-scale Gouda-type cheese also needed to evaluated actual flavor formation and its flavor formation mechanisms by starter bacteria should be more studied (Holland *et al.*, 2006, Taïbi *et al.*, 2011). The selection approach in this study can be used to screen novel starter bacteria and applied to develop new Korean semi-hard cheese such as Cheddar and Emmental.

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An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. Applied and environmental microbiology 63(2):414-419.

Abstract in Korean

치즈와 요구르트는 유산균 발효에 의해서 만들어지는 대표적인 유제품이다. 발효유제품을 만들기위해 사용하는 유산균을 스타터 컬처 (starter culture)라고 한다. 스타터 컬처는 주로 산 생성을 통해 우유의 응고를 촉진하며 풍미 성분 생산에도 기여한다. 치즈의 맛은 풍미 성분들의 복잡한 조화에 의해 결정된다. 풍미 성분들은 우유의 구성분에서 유래되는데 기본적으로 유당, 유단백질, 유지방이 있다. 이 유성분들이 당 분해 경로 (glycolysis), 단백질 분해 경로 (proteolysis), 지방 분해 경로 (lipolysis)를 거쳐서 풍미 성분으로 분해된다. 치즈 제조시에 유성분은 우유 자체에서 유래되는 효소와 렌넷 (rennet) 그리고 스타터 컬처 유래 효소에 의해서 1차 분해되고, 숙성되는 동안에 비(非) 스타터 유산균 (non-starter lactic acid bacteria), 곰팡이 등에 의해서 2차 분해된다. 스타터 유산균은 세 가지 경로 중 특히 아미노산 분해 과정과 연관성이 크다고 알려져 있다. 아미노산 분해 과정은 주요 풍미 생산 경로이다. 이 과정에 관여하는 특정 효소들 중 본 연구에서 주목한 효소들은 Bifunctional Alcohol/Aldehyde dehydrogenase (AdhE), Branched chain aminotransferase (BcaT), Aromatic aminotransferase (AraT), Esterase (EstA), Alcohol dehydrogenase (Adh), Citrate permease (CitP), Keto acid decarboxylase (KdcA) 이고, 이 효소들을 가진 균을 풍미 생산 능력이 있는 것으로 보았다. 산 생성을 통한 커드 형성능이 확인된 164개의 isolates 중에서 Multiplex PCR 기법을 이용해 풍미 생산 능력이 있는 균 44개를 선별했다. 결과들을 종합하여 최종적으로 가장 풍미 생산 능력이 뛰어날 것으로 여겨지는 네

개의 *Lactococcus lactis* subsp. *lactis* 균과 하나의 *Lactococcus lactis* subsp. *cremoris* 균을 선발했고, 이들의 안정적인 성장 및 autolysis 능력, 탄수화물 발효 패턴 및 효소 활성에 대한 특성을 확인했다. 일반적으로 스타터 박테리아가 산업적으로 이용될 때에는 Direct Vat Set (DVS) 형태로 이용되므로 Freeze-drying process에 안정성이 있는지도 평가했다. 각각의 선발균이 서로의 특성을 보완해 줄 수 있도록 스타터 컬처를 조합하고, 이를 이용해서 미니어처 고다 타입 치즈 모델을 만들었다. 미니어처 치즈 모델에서의 향미 성분을 Hercules Ⅱ electronic nose (Alpha M.O.S., France)로 분석해 선발균의 풍미 생산 능력을 검증했다. 산업적으로 주로 사용되고 있는 스타터 균주 (CHN-11, Chr.hansen, Denmark)와 비교하였을 때, 선발한 풍미 생산 균주가 보다 다양한 flavor profile을 가진 것으로 나타났다. 뿐만 아니라 Methylbutanone과 1-propanol, 2-methyl- 은 산업 균주에서는 나타나지 않는 독특한 향미 성분이었다. 테스트 그룹 간 비교에서는 *Lactococcus lactis* subsp. *lactis* L5와 *Lactococcus lactis* subsp. *cremoris* C46 조합의 풍미가 가장 다양했다.