A Thesis for the Degree of Master of Science

Investigation of microbial diversity in Korean sourdough and its monitoring by real-time quantitative PCR

실시간 정량적 중합효소연쇄반응법을 이용한
한국형 사워도우 내의 미생물 모니터링

By

Hyun-Wook Baek

Department of Agricultural Biotechnology

Seoul National University

August 2014

A Thesis for the Degree of Master of Science

Investigation of microbial diversity in Korean sourdough and its monitoring by real-time quantitative PCR

Advisor: Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science

By

Hyun-Wook Baek

Department of Agricultural Biotechnology

Seoul National University

August 2014

農學碩士學位論文

실시간 정량적 중합효소연쇄반응법을 이용한 한국형 사워도우 내의 미생물 모니터링

指導敎授 徐 鎭 浩

이 論文을農學碩士學位論文으로 提出함

2014年 8月

서울大學校大學院
農生命工學部食品生命工學專攻
白賢煜

白賢煜의農學碩士學位論文을認准함

2014年 8月

委員長문태화 (인)

副委員長서진호 (인)

委員유상렬 (인)

ABSTRACT

Sourdough is a bread product made by a long fermentation of dough using naturally occurring *lactobacilli* and yeasts. The yeasts generate carbon dioxide which leavens the dough, and the lactic acid bacteria (LAB) produce lactic acid which contributes to flavor of sourdough.In comparison with breads made with cultivated yeasts, it usually has a mildly sour taste because of the lactic acid produced by*lactobacilli*.Although sourdough has a good preservability and texture, it is not appealing to many Koreans because of a sour taste.

Recently, a modified form of sourdough has been developed by inoculating Nuruk as a starter, hereafter called the Korean sourdough. In order to further improve the Korean sourdough, it is necessary to understand the dynamics of microbial populations during the sourdough fermentation. Among many microorganisms present in the Korean sourdough, LAB are of special interest because various properties of sourdough are related to the metabolites of LAB such as acetic acid and lactic acid. In order to obtain useful information to modulate the microbiota in the sourdough for desirable properties, it is important to develop a rapid and reliable quantification method to detect LAB in the sourdough.

In this study, a quantitative assay using a real-timepolymerase chain reaction (qRT-PCR) method was developed. First, the identification of the LAB in the Korean sourdough was performed by a randomly amplifiedpolymorphic DNA

polymerase chain reaction (RAPD-PCR) along with partial 16S rRNA sequencing. *Lactobacillus sanfrnaciscensis* was the most dominant species, representing 56% of the isolates followed by *Lb. curvatus* (27%) and *Lb. brevis* (9%). The LAB such as *Lb. plantarum*.*Lb. sakei* and *Pediococcus pentosaceaus* were also detected.

Next, several primers were designed to detect individual species, based on the sequence information obtained from National Center for Biotechnology Information (NCBI). It was demonstrated that each primer set bound to its target microorganisms without cross reactivity. The newly developed method showed a strong correlation with the result obtained by a colony-forming counting method, which has beenrecognized as a reliable method but requires tedious procedures and a long time. In addition, the spike recovery values werehigher than 95 percent, even in the presence of interfering substances such as high concentrations of sugars and proteins in the sourdough. Above all, the total time required for the assay was shorter than four hours, which allows arapid and real time monitoring of the microbial populations.

Finally, the qRT-PCR method was applied to examining the changes in microbial populations during the sourdough fermentation. It was found that *Lb. curvatus* decreased throughout the fermentation. It seemed that a poor tolerance against acids as well as the inefficient maltose utilization ability of *Lb. curvatus* might be attributed to the reduced population.

To sum up, the dynamics of microbial populations during the Korean

sourdough fermentation could be rapidly assayed by the newly developed qRT-PCR method. It would provide useful information to improve the properties of sourdough by monitoring and controlling the microbiota.

Keywords: sourdough, lactic acid bacteria(LAB), mixed culture, identification, microbial diversity, randomly amplified polymorphic DNA (RAPD), culture-independent microbial analysis, quantitative real-time PCR.

Student Number: 2012-23380

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

1.1. Sourdough

Sourdough has been used in bread production for more than 500 years. It is a mixture of ground cereals, such as wheat or rye, and water that is spontaneously fermented(Prajapati JB *et al.*2003). Three types of sourdough are used in traditional and industrial processes. Type I sourdoughs are manufactured by traditional technique and characterized by continuous daily propagation to keep the microorganisms in an active metabolic state (Gaggiano M *et al.* 2007). A second type of sourdoughs based on their inocula is the result of the addition of a starter culture to the flour-water mixture (de Vuyst L *et al.* 2014). Lastly, type III sourdoughs are dried doughs in powder form,initiated by defined starter cultures**.**

Main function of sourdough was to leaven the dough to produce a more gaseous dough piece and as such a more aerated bread(Decock P *et al*. 2005). This is because that different microorganisms, including bacteria and yeasts, are simultaneously present in sourdough (Corsetti A *et al*. 2007). Indeed, sourdough fermentations are known to improve rheological, organoleptic and nutritional properties of the final product (Hansen A *et al*. 2005) to prevent their microbial spoilage and to retard the staling process (Corsetti A *et al.* 1998). Most of these effects are due to the growth of LAB, which, during dough leavening, carries out different metabolic activities, such as lactic acid fermentation, proteolysis and synthesis of volatile as well as anti-mold compounds.

Until now, more than 60 different LAB species have been isolated and identified from sourdough fermentations (Wouters D *et al.* 2013), however, these LAB were isolated with geographic variations depending mainly upon raw materials. Despite this, ina recent review, only a research onKorean sourdoughartificially inoculated with *Leuconostoc citreum*has been reported (de Vuyst L *et al.* 2014).Following this, Korean sourdough has not drawn much attention yet and thus scarce literature is currently available regarding this product. For this reason,Korean sourdough should be studied not only as a microbial ecosystem but also towards industrial standardization.

1.2. Korean sourdough fermentation with mixed culture system

Industrial food productions were achieved by pure starter culture isolation techniques(Lee CH*et al*., 2004). It also includes a sourdough fermentation with the predominant key bacterium, *Lb. sanfranciscensis*. However, many of the traditional fermentation starters, especially in the Orient, are a naturally fermented mixed culture system(Postollec F *et al.* 2011, Sieuwerts S *et al.* 2008). Furthermore, consumer perception of food has changed to prefer the traditional fermented products made by a naturally fermented starter culture (Rhee SJ *et al.* 2011).In accordance with these customer preferences, several attempts have been made to produce sourdoughsstarted with a naturally fermented starter culture in Korea. One of the most significant attempts is that nuruk is used as the main microbial sources of starter culture in sourdough. To produce Korean sourdough, nuruk extract was added to a mixture of wheat and rye at the first stage of the fermentation (**Figure1**). Consequently, sensory evaluation of sourdoughs made this way proved that it is more preferred by Korean people than other sourdoughs because of a mild sour taste. However, this has met a new challenge after 10^{th} propagation of the sourdough fermentationbecause of the sudden changes in sourdough characteristics from that point on (**Figure 2**).

Originally, nurukhas been widely used in Korea asa traditional fermentation starter for rice wineand made with wheat flour and grits fermented spontaneously

by various microorganisms(Kim SY *et al.* 2010).Therefore,different microorganisms originated from raw materials as well as nurukare simultaneously present in Koreansourdough, and this microbiota greatly contributes to the broad diversity of the characteristics of products through variable dynamics during processing (Beresford TP *et al.* 2001, Wouters J *et al.* 2002). It is surmised thatthese dynamics are largely influenced by interactions between microorganismsand have a marked impact on the survival, growth and activity of the different microbial populations during processing (Charlet M *et al.* 2009, Irlinger F *et al*. 2009). However, the physiological characteristics of microbial strains present in the Korean sourdough fermentation and their interactions for growth and biosynthesis of functional compounds are now under investigateion.It is necessary to study Korean sourdough as a microbial ecosystem in order to not only characterize the sudden changes of sourdough but also apply towards commercial standardization. The present study is the first step towards this direction.

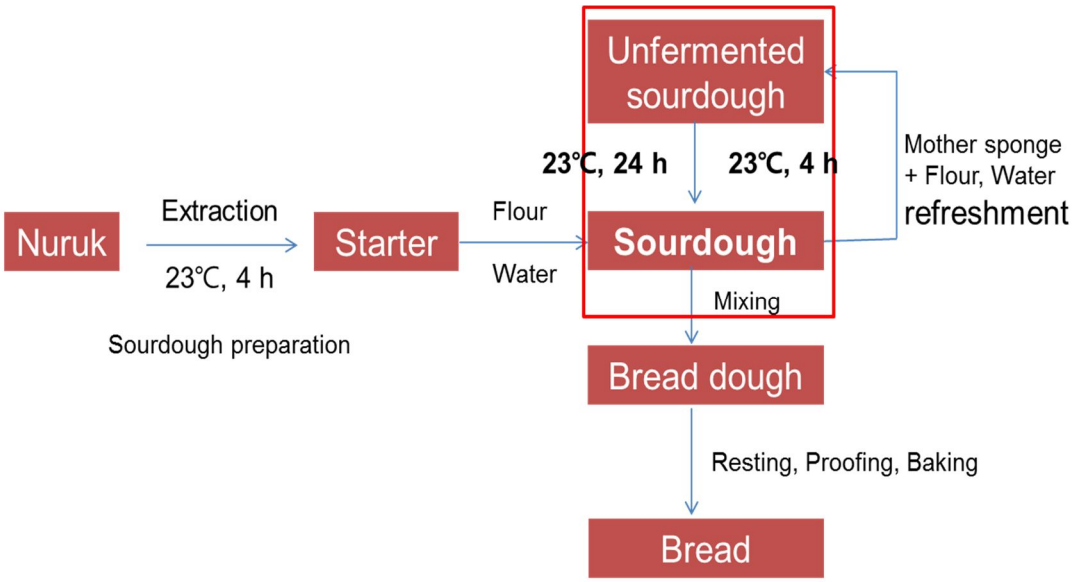


Figure 1. Flow sheet of sourdough propagation in this study

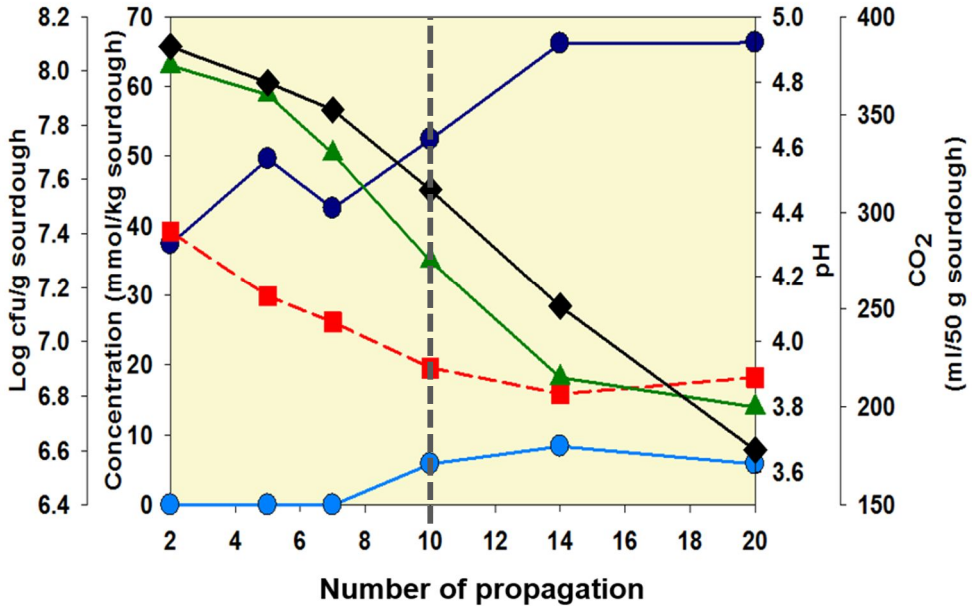


Figure 2 Profile of metabolitesof Korean sourdough inoculated with nuruk a function of number of propagation. Symbols: pH (–■–), Lactic acid (–●–), Acetic acid (–●–), CO_2production (–▲–), total yeast count(–◆–).The vertical dotted line indicates the 10^{th} of propagation with the sudden changes of sourdough characteristics.

1.3. Culture-independent method

In many studies on other fermented foods, the population dynamics of microbial food ecosystems have been studied principally through microbiological analysis(Giraffa G 2004). On the other hand, little has been known about population dynamics during sourdough fermentation, becausewell-defined starter cultures,such as *Lb. sanfrancsicensis*, were used that dominated the entire fermentation process (van der Meulen R *et al.* 2007). In this study, however, nuruk consisting of several undefined microorganisms wasadded tosourdough at the initial stage of fermenatation. Given this peculiarities of the Korean sourdough ecosystem, it is important to investigate the changes in microbial diversity during sourdough fermentation.However, the physiological characteristics of microbial strains isolated from traditional fermentation starters and their interactions for growth and biosynthesis of functional compounds are now under investigation (Leroy F and De Vuyst L 2004).It is therefore of primary importance to reliably quantify populations, in terms of dynamic changes, in order to understand the role of each microbial population.Such questions have mainly been addressed using culture-dependent methods based on the growth of microorganisms on culture media. This approach is generally labor intensive, time-consuming and more or less specific of a microbial group, depending on the microbial groups targeted and the complexity of the microbiota studied. Only strains that can grow under the

specificconditions will be monitored. Moreover, these methods fail to characterize minorpopulations when a dominant population grows on the same culture medium (Nocker A and Camper AK 2009).

For these reasons, culture-independent methodshave rapidly been recognized as a valuable alternative in many food matrics(Ercolini D 2004). These methods are based on the direct analysisof DNA or RNA extracted from the foods matrix withoutculturing step. Several reviews (Ndoye B *et al.* 2011) have recently been publishedfocusing on the culture-independent methods available to investigatemicrobial communities in terms of diversity, dynamicsand activity, providing a good overview of the benefits andlimitations of the methods. Among culture-independent methods,real-time quantitative PCR (qPCR) represents a powerful tool forthe quantification of microbial populations through the measurementof targeted gene numbers (Postollec F *et al.* 2011). According to Ndoye, DNA extraction efficiency, selection of the target region and distinction between live and dead microorganisms are key factorsto take into consideration when using a culture-independent method(Ndoye B *et al.* 2011). However, qPCR detects both viable and dead cells. A method that distinguish physiologically active population is not yet available.

1.4. Research objectives

For monitoring population dynamics during the Korean sourdough fermentation, a quantification method should be available to specifically determine each bacterial population in a mixed culture.

This study was focused on investigation of microbial changes in Korean sourdough by applying a novel quantitative method.

The specific objectives of this study are as follows.

(1) To investigate microbial diversity, especially focused on Lactic acid bacteria, of Korean sourdough inoculated with nuruk.

(2) To develop quantitative PCR assays for the quantification of lactic acid bacteria originated from Korean sorudough.

(3) To assess the applicability of qPCR for direct quantification in Korean sourdough.

2. Materials and Methods

2.1. Microorganisms, growth and storage conditions

Table 1 lists microorganisms used in this study. *L. sanfranciscensis* strains were grown at 30℃ in SDB broth (2% maltose, 0.3% yeast extract, 1.5% fresh yeast extractives, 0.03% Tween 80, 0.6% trypticase, final pH 5.6) as described by Kline *et al*. (Kline L *et al*.1971). Other bacterial strains were grown at 37℃ in MRS (de Man Rogosa and Sharpe, Difco). For isolation of bacterial strains from sourdough, the SDB agar (1.5~2% Bacto^{TM} Agar, BD) plate was used. All microorganisms were stored at -80℃ in the same medium supplemented with glycerol (15% v/v) until further use.

2.2. Genomic DNA preparation from bacteria

Microorganisms for genomic DNA extraction were grown on the appropriate medium and temperature in an anaerobic condition until the cell number reached 10^8-10^9. Cells were centrifuged at 13000rpm for 2 min. and the supernatants were discarded. The chromosomal DNA was extracted by using the genomic DNA prep kit (SolGent, Korea) and the DNA purity was measured by Nanovue plus (GE Healthcare Life Science, USA).

Table 1. List of microorganisms used in this study.

Species	Strain designation	Source
Lactobacillus sanfranciscensis	ATCC 27651	Sanfrancisco sourdough
Lactobacillus sanfranciscensis	JHS 55	This study
Lactobacillus sanfranciscensis	JHS 101	This study
Lactobacillus plantarum	ATCC 8014	N.I.
Lactobacillus brevis	DSM 6235	Spoiled beer
Lactobacillus paracasei	ATCC 25302	Milk products
Lactobacillus sakei subsp. sakei	ATCC 31063	Pickled cabbage
Lactobacillus casei	ATCC 393	Cheese
Lactobacillus lactis subsp. lactis	ATCC 19435	N.I.
Pediococcus pentosaceus	ATCC 33314	Sake mash
Leuconostoc mesenteroides subsp. mesenteroides	ATCC 9135	N.I.
Lactobacillus crustorum	KACC 16344	Artisan wheat sourdough
Leuconostoc citreum	ATCC 49370	Honeydew of rye ear
Lactobacillus curvatus	ATCC 25601	Milk
Lactobacillus paralimentarius	JCM 10415	Japanese sourdough
Lactobacillus buchneri	ATCC 4005	Tomato pulp

ATCC - American Type Culture Collection, Rockville, Maryland, USA; **DSM** –Deutsche Sammlung für Mikroorganismen und Zelkulturen, Braunschweig,Germany; **KACC** – Korean Agricultural Culture Collection, Suwon, Korea;**JCM** – Japan Collection of Microorganisms, Tsukuba, Japan;**N.I.** – Not Informed.

2.3. Multiplex-Randomly Amplified polymorphic DNA

2.3.1. PCR conditions

A polymerase chain reaction (PCR) was performed with the Accupower™ PCR PreMix (Bioneer Co., Daejon, Korea) in a GeneAmp PCR System 2400 (Applied Biosystems, CA, USA. Each PCR solution was composed of 10 pmol of combined primers, and 70~100ng of extracted genomic DNA as template in a final volume of 20**μ**l. The primers used in this study are described in **Table 2**. PCR amplification was carried out as following; 1 cycle of 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 40°C for 1 min, 72 °C for 2 min, 1 cycle of 72 °C for 10 min(Venturi M *et al.* 2012).

2.3.2. Electrophoresis

10 **μ**l of the amplified gene was separated on a 1.5% (w/v) agarose gel by electrophoresis in TAE buffer for 1.5h at 90V. After complete separation of the desired DNA bands from the gel, distinct DNA fragments were stained with ethidium bromide and visualized by ultraviolet trans-illumination. Gel images were captured as TIFF format files with an i-MAX^{TM} Gel Image Analysis System (CoreBio, Korea)

2.3.3. Amplicon analysis

The patterns were converted, normalized and further analyzed byusing the BioNumerics 7.1 version software (Applied Math, Belgium). As described by Zapparoli (Zapparoli G *et al.* 1998), from the two RAPD–PCR profiles, aunique dendrogram was obtained by combining patterns from thedifferent PCR amplifications. The comparison of combined RAPD patternswas done with the Unweighted Pair Group Method using ArithmeticAverages (UPGMA) clustering method and the band-based Dicesimilarity coefficient.

Table 2. List of oligonucleotide primers used for RAPD-PCR reactions

Primer name	Sequence (5` 3`)	References
RD1	GCTTAAGGAGGTGATCCAGCC	Weisburg et al. (1991)
OPL-05	ACGCAGGCA	Sesena et al. (2005)
P1	ACGCGCCCT	De Angelis et al. (2001)

2.4. Real-time quantitative PCR

2.4.1. PCR conditions

DNA used in this study and the corresponding strains are listed in **Table 1.** DNA was extracted according to the method 2.2.

2.4.2. Primer design

The primers specific to*Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis* were designed based on sequence information obtained from National Center for Biotechnology Information (NCBI). The BLAST search tool (Basic Loval Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check the in silico specificity of each primer set. The primer sequences and amplicon size are shown in **Table 3**. All primers were synthesized by Bioneer (Daejoen).

Table 3. List of oligonucleotide primers used in this study for quantification of *Lactobacillus brevis, Lb. curvatus*and *Lb. sanfranciscensis* by quantitative PCR.

Target species	Primer	Sequence (5`- 3`)	Target sequences[a]	Amplicon size (bp) Amplicon *T*m (℃)
Lb. brevis	q_BreF	ACGGTTTCCGTACTAGCGTTCTTTC	Hypothetical (LVISKB_0189)	162
	q_BreR	ACCCATCATCGCAAGAACTGAC		86.5~87
Lb. curvatus	q_CurF	GACCCATGCCTTTAATACGCATAG	CRL705 contig00107	129
	q_CurR	CTGAAATAACCACTATAGCCACCCC		84~84.5
Lb. sanfranciscensis	q_SanfF	CAGTTAACTTTTGCGAGTCAGCAG	Hypothetical (LSA 03860)	120
	q_SanfR	CGTCAGGTTCCCCACATAACTC		78~78.5

2.4.3. Real-time PCR conditions

For each primer set, different annealing temperatures ranging from 58 to 63˚C were tested to determine the most effective PCR amplification conditions. The ideal primer set should yield the lowest Ct value, a high final fluorescence value and a melting curve showing a single peak, thus ensuring a single amplicon is being generated. For all primers, the best results were obtained at a temperature of 61.5˚ C. qPCR reactions were performed in a CFX Manager™ real-time thermal cycler (Bio-Rad Laboratories, Inc., USA). All PCR amplifications were carried out in a final volume of 25 µl containing 12.5 µl of SYBR® Premix Ex Taq (Takara Bio., Japan), 8.5 µl of sterile distilled water, 2 µl of template DNA (<500 ng), 0.4 µM of each primer. Thermal cycling conditions consisted of 1 cycle at 95˚C for 30 s and 40 cycles at 95˚C for 5 s followed by 61.5˚C for 30 s. A melting-curve analysis was performed at the end of each PCR assay by gradually heating from 65 to 95˚C and continuously measuring the fluorescence to confirm a single PCR-product. All samples were analyzed in duplicate. Thermal cycling, fluorescence data collection and data analysis were carried out with CFX Manager™ Software v 3.0 (Bio-Rad Laboratories, Inc., USA). The cycle threshold value (Ct) was defined as the PCR cycle at which the fluorescence signal exceeded the background level. Absolute quantification of *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis* in sourdough samples was performed by interpolation on a standard regression curve of Ct values

generated from genomic DNA extracted from reference strain cultures.

2.4.4. PCR specificity check

Firstly, the specificity of each primer set was tested againsttarget and non-target bacteria DNA samples commonly found insourdough and Nuruk and/or phylogenetically related to thetarget species (**Table 1**). This specificity was further evaluated usingthe melting peak analysis of the qPCR product. The melting analysisof the PCR product obtained should produce a single peak with aunique Tm value, while no specific peak was detectable in thenegative controls. With the exception of *Lb. sanfrancisensis* (907ng), positive and negative control DNA samples wereset at 60~200ng in the reaction mixture.Secondly, the specificity was checked in the presence of highamounts of non-target bacterial DNA. Equal quantities of non-targetDNA or 10, 100, 10^3,10^4,10^5 times more than the target DNAwere added to the reaction mixture.

2.4.5. Standard curve

Ten-fold serial dilutions, ranging from 10^3 to 10^9 copies/**μ**l, ofthe DNA extracted

from *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis*were performed and the DNAamplified. Standard curves were obtained by plotting the Ct valuesagainst the target gene copy number/**µ**l. Each standard curve wascarried out in triplicate. The reliability criteria were the correlationcoefficient and the amplification efficiency. The correlation coefficientR^2 is a measure of how well the data fit to a straight line,indicating both the agreement between replicates and the linearrange of the assay. The qPCR efficiency was calculated according tothe equation $E = 10^{(-1/b)} - 1$, where b is the slope of the linear fit.The quantification limit was defined as the lowest concentration atwhich linearity was maintained. Intra-assay repeatability wasevaluated using a coefficient of variation (CV%) based on Ct valuesand calculated at different concentrations from standard curves forat least three duplicate samples in the same PCR run. The CV% wasalso used to estimate inter-assay reproducibility when calculatedfor at least three independent PCR runs.

2.4.6. Calculation of the gene copy number

All extracted DNA were quantified using a spectrophotometer and copy numbers were calculated for all standards by the following formula (Dhanasekaran S *et al.* 2010).

$$\text{Number of copies/}\mu\text{l} = \frac{6.022 \times 10^{23}\ \text{(molecules/mole)} \times \text{DNA concentrations (g/}\mu\text{l)}}{\text{Number of bases pairs} \times 660\ \text{daltons}}$$

6.022×10^{23} (molecules/mole): Avogadro's number

660 Da: Average weight of a single base pair.

2.5. Analysis of sourdough fermentation

2.5.1. Laboratory sourdough production and propagation

Wheat and rye were provided by SPC Corporation. Characteristics of these materials used for sourdough production are simply depicted in table 1. Sourdough production and propagation were established based on the protocol used for bread making in SPC. The production of sourdoughs was carried out according to thefollowing protocol. Wheat flour MGPB-01 (225g), rye R1800 (25g), tap water (250ml), and cellsuspension (ca.5ml), containing about 9 log CFU/ml of the above individual strains (final cell number in the dough of ca. 6~7log CFU/g ofdough), were used to prepare 500 g of dough [dough yield (dough weight X100)/(flour weight) = 200] with a continuous mixer for 5 min (Chopin & Co., Boulogne, Seine,

France). A control sourdough without starter was also produced under the sameconditions. The sourdoughs wereincubated at 23°C for 5 h. After fermentation, sourdoughswere stored at 4°C for about 19 h and further used for propagation. Sourdough propagation was carried out according to the back-slopping protocol.Each of the above sourdoughs was individually used as the starter (inoculumrate, 25% [wt/wt]) to ferment a mixture of flour MGPB-01 (225 g), rye R1800 (25g), and tapwater (250ml) having a dough yield of 200. Sourdoughs were fermented at23°C for 5 h and stored at 4°C for about 19 h between back-slopping. Sourdoughswere sampled and propagated daily for 7 to 14 days.

Table 4. Characteristics of flour and rye used for the production of laboratory sourdoughs

Trade name	Supplier and country of manufacturer	Moisture(%)	Ash (% of dry matter)	Protein (% of dry matter)
MBPG-01	Korea	11.9	0.46	11.2
R1800	Finland	11.3	1.29	8.3

2.5.2. Determination of pH, total titratable acidity (TTA) and viable cell counts

The pH was measured from an aliquot of 10 g of sourdough blended with 90 ml of distilled water. To determine the total titratable acidity, the suspension was titrated against 0.1 N NaOH, using phenolphthalein as an indicator. TTA was expressed as percent lactic acid. Cell counts for LAB were determined by plating appropriate dilutions of the dough onto mMRS and SDB agar plates containing cycloheximide (0.2 g/ml).

2.5.3. Preparation of cell pellet from sourdough

For extraction of total DNA from sourdough samples, 10 g of sourdough was mixed with 90 mL of sterile saline (8.5 g NaCl/L). An aliquot of 50 mL was centrifuged at 4 °C for 5 min at 200×g to remove solids. Cells were harvested by centrifugation for 15 min at 5000×g and cell pellets were stored at −20 °C until use. Frozen cell pellets were thawed, washed three times with 1 mL of sterile phosphate- buffered saline (8.0 g NaCl, 0.2 g KCl,1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per L, pH 7.4), and DNA was extracted by using the genomic DNA prep kit (SolGent, Korea) and the DNA purity was measured by Nanovue plus (GE Healthcare Life Science, USA).

2.5.4. Metabolite detection in sourdough

For analysis of metabolites in sourdough, 10g of sourdough sample was diluted withsterile distilled water and homogenized by a stomacher (AES, France) for 10 min. Mixed samples were centrifuged at 13,000 rpm for 2 min and the supernatants were used.Concentrations of maltose, glucose, fructose, acetate, lactate and ethanolwere measured by for a high performance liquid chromatography (Agilent 1100LC, U.S.A) with the Carbohydrate Analysis column (Phenomenex, USA). The carbohydrate analysis ion exclusion column heated at 60°C was applied to analyze the 20 **μ**l of diluted sample. HPLC operation conditions were set according to the instruction manual of the column supplier.H_2SO_4 (5mM) solution was used as mobile phase at a flow rate of 0.6 mL/min. Detection was made with a reflective index detector.

3. Results and Discussions

3.1. Identification of lactic acid bacteria from Korean sourdough

3.1.1. Isolation of LAB from Korean sourdough and raw materials (nuruk, wheat and rye)

Sourdoughs are produced in many countries with local variations depending primarily on raw materials. In accordance with a recent report(De Vuyst L *et al.* 2014), Up to now, more than 60 species of *lactobacilli* have been isolated from sourdoughs. However, only one experimental data of Korean sourdough started with *Leuconostoc citreum*has been reported in 2012(Hansen A *et al.*2005). In this study, several colonies grown on SDB and MRS agar plates were isolated from Korean sourdough inoculated with undefined nuruk. As a result, *Lb. curvatus, P. pentosaceus, Lb. sakei, Lb. plantarum,* and *Pediococcus acidilactici* were isolated from nuruk. Only *P. pentosaceus* was found in wheat, the main material of sourdough. *P. pentosaceous* and *Lb. curvatus* were isolated from rye. Total 6 species of *P. pentosaceus,Lb. cuvatus, Lb. brevis, Lb. plantarum, Lb. sakei* were isolated from fermented sourdough (**Table 6**). According to Huys, obligately heterofermentative *lactobacilli* are characteristic for sourdough fermentation processes, because of highly adapted carbohydrate metabolism. In the present study, facultatively heterofermentative or obligately homofermentative LAB were usually found in nuruk, wheat and rye. Conversely, obligately heterofermentative LAB

such as *Lb. sanfranciscensis* and *Lb. brevis* are almost solely associated with sourdoughs.

3.1.2. Identification of isolates by RAPD and 16S rRNA sequencing

In order to overview of fermentation flora, a total of 120 LAB isolates collected from 11th propagation of Korean sourdough were analyzed for RAPD fingerprinting together with reference strains commonly found in sourdough fermentations. M. Venturi (Venturi M *et al.* 2012) evaluated the suitability of literature primers used individually or in dual combination and four RAPD reactions were selected on the basis of their differentiating capability, type ability, reproducibility, and ease of interpretation. Two multiplex reactions with the combined primers OPL-05+RD1 and P1+RD1 were selected in this study for differentiating the isolated strains from Korean sourdoughs in reference to the result that the use of multiplex RAPD-PCR reactions increased the degree of reproducibility according to other authors. **Figure 3** depicts a digitized picture of the generated banding patterns after cluster analysis. The patterns consisted of four to ten main fragments sized between 3.0Kb to approximately 0.4 Kb. To obtain the optimal molecular grouping, applying the UPGMA algorithm with the Pearson correlation coefficient, 10 main clusters with the 60% similarity value were

obtained. Some representative strains were selected and confirmed for identification of species by 16S rRNA sequencing. Clusters A and B included the most dominant strains of *Lb. sanfrnaciscensis*. Clusters G and H belonged to *Lb. curvatus*, and I and J included the strain of *Lb. brevis*. Because these strains were from sourdough samples, *Lb. sanfrnaciscensis Lb. curvatus* and *Lb. brevis* were considered as dominant strains from Korean sourdough, representing 56%, 27% and 9% respectively of the total isolates.

Table. 5. Lactic acid bacteria isolated from raw materials (wheat, rye and nuruk) and sourdough.

Origin	Isolated lactic acid bacteria	Metabolism gruop
Nuruk	*Lb. curvatus*	Facultatively heterofermentative
	P. pentosaceus	Obligately homofermentative
	Lb. sakei,	Facultatively heterofermentative
	Lb. plantarum,	Facultatively heterofermentative
	Pediococcus acidilactici	Obligately homofermentative
Wheat	*P. pentosaceous*	Obligately homofermentative
Rye	*P. pentosaceous,*	Obligately homofermentative
	Lb. curvatus	Facultatively heterofermentative
Sorudough	*P.pentosaceous*	Obligately homofermentative
	Lb. brevis	Obligately heterofermentative
	Lb. sanfranciscensis	Obligately heterofermentative
	Lb. curvatus	Facultatively heterofermentative
	Lb. plantarum	Facultatively heterofermentative
	Lb. sakei	Facultatively heterofermentative

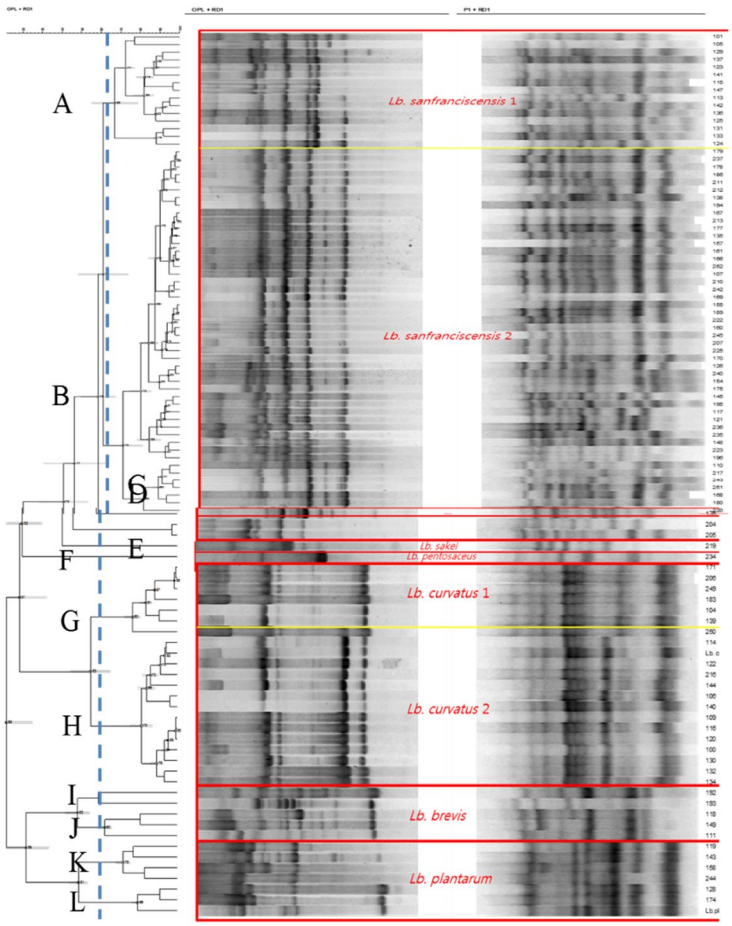


Figure 3. UPGMA dendogram derived from integration and comparisons of the two combined RAPD-PCR patterns obtained with primer OPL-05 + RD1 and P1+RD1 for isolates from sourdough. The vertical dotted line indicates the 65% similarity level. A to L are clusters based on cutoff value of 65%.

Table 6. Species distribution of the 120 total isolates from sourdough analyzed by RAPD-PCR and 16S rRNA sequencing. Underlined number means the identificated by 16S rRNA sequencing

Species	Isolates
Lb. brevis	102,111,118,149,192,240,154,243,175,193,
Lb. curvatus	100,104,106,109,112,114,116,120,122,127,130,132,134,138,139,140,144,146,156,159,171,172, 183,206,216,238,241,249,250,253
Lb. sanfranciscensis	101,105,107,108,110,113,115,117,121,123,124, 125,126,129,131,133,135,136,137,141,142,145, 147,148,160,161,194,195,196,197,204,205,207, 220,222,223,225
Lb. plantarum	119,128,143,158,174,244
Pediococcus pentosaceus	176, 224
Lactobacillus sakei	219

3.2. Development of quantitative method to monitor lactic acid bacteria

3.2.1. Primer design

In the previous chapter, *Lb. sanfrnaciscensis Lb. curvatus* and *Lb. brevis* were considered as prevalent strains from Korean sourdough, representing 56%, 27% and 9% respectively of the total isolates. Therefore, these hetero-fermentative LAB are regarded as crucial species with regard to monitoring unexplored Korean sourdough fermentation. However, methods forspecifically and simultaneously quantifying these species existing in sourdough are lacking.

In this study, three specific primer sets for quantifying those species were designed, amplifying under 162 base pairs (bp) fragments, according to S. Dhanasekaran who proposed that primers giving 60 to 150 bp amplicon sizes are considered ideal for real-time quantitative PCR(Dhanasekaran S *et al.,* 2010).

Quantitative PCR assays with primers targeting 16S *r*RNA, the*rec*A or *tuf* gene have been reported for the quantification of some *lactobacilli* (Grattepanche *et al.* 2005, Friedrich*et al*. 2006 and Achilleos *et al.* 2013). 16S ribosomal RNA represents the most important target for studying bacterial ecology. However, the fact that 16S rRNA genes are limited by the presence of variable copy numbers in bacterial genomes and sequence variations within closely related taxa (Větrovský T *et al.* 2013). Besides, two *tuf* genes or up to three *tuf*-like genes have

recentlybeenidentified in some Gram positive bacteria. In addition, the choice of targeting genes for *Lb. curvatus*quantification was restricted due to the lack of reliably annotated sequences. Therefore, all designed primers in this study targeted for unannotated genes in this study.

3.2.2. Primers specificity

Primer specificity is especially important when real-time PCR is associated with SYBR Green. This is because intercalating dye is not specific and detects all double stranded DNA fragments.The specificity of each primer set was assessed by qPCR amplification of DNA from a total of 12 different bacterial species, mainly covering bacteria that are commonly found in Nuruk and sourdough. **Figure 4** and **Table 7**show that the Ct values ranged from 10.62 to 13.53 under the optimal condition with the amount of DNA present. Non-target DNA showed Ct values over 27 because the amplification plot did not cross the threshold fluorescence level. Therefore, a single peak was also observed on the amplicon melting curve for each primer set, showing the specificity of the amplification. Therefore, each primer set amplified a unique locus of the genome. *Lb. brevis, Lb. curvatus and Lb. sanfranciscensis* primers amplified a 162 bp, 129 bp and 120 bp fragment from DNA with a melting temperature (Tm) 87℃, 78℃ and 84.5℃ respectively. In

many case qPCR is applied to DNA extracted from a complex microbiota in which the target species are not necessarily the dominant ones. The specificity of each primer set was checked when the target DNA was mixed with non-target DNA. In the present study, the amplification of *Lb. curvatus, Lb. brevis* and *Lb. sanfrnaciscensis* DNA was tested for a mixture of all three strains. The amplification was not affected by high amount of non-target DNA (**Table 8**). No significant difference in Ct values was observed in the presence of non-target DNA up to 10^4 times more added. The differences of Ct value ranged from 0 to 0.2 for *Lb. brevis*, from 0.02 to 0.21 for *Lb. curvatus* and from 0.01 to 0.2 for *Lb. sanfranciscensis*, showing that the real-time qPCR assay developed was specific for these strains.Specificproducts were detected as separate distinct meltingpeaks in a meltingcurveanalysis.

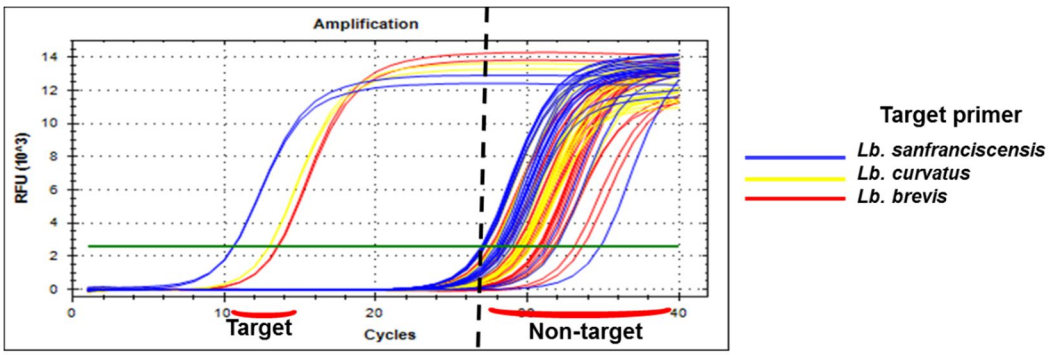


Figure 4. Phase of PCR amplification curve for comparison of Ct values between target species and non-target species.

Table 7. Bacterial DNA used in this study and primer specificity expressed as cycle threshold (Ct values) obtained from DNA in each PCR well. Ct values in bold indicate values for target DNA.

Bacterial DNAa	**DNA concentration**	***Lb. brevis***	***Lb. curvatus***	***Lb. sanfranciscensis***
Lb. sakei subsp. sakei	54	29.57	30.36	29.31
Lb. paraplantarum	41.5	27.60	30.28	28.30
Lb. casei	35	30.15	31.90	31.61
Lb. lactis subsp. lactis	35	28.52	29.75	31.91
Lb. curvatus	70	31.29	**13.00**	27.06
Lb. brevis	25.5	**13.53**	27.76	27.07
Pediococcus Pentosaceus	42	29.70	29.02	28.00
Lb. plantarum	37	30.83	29.64	28.41
Lb sanfranciscensis	907	33.39	29.80	**10.62**
Lb. buchneri	22.5	30.09	27.19	28.17
Lb. paralimentarius	111	31.79	28.56	28.66
Leuconostoc mesenteroides	32	29.24	29.60	27.20
Negative control : DDW	0	34.00	37.41	35.94

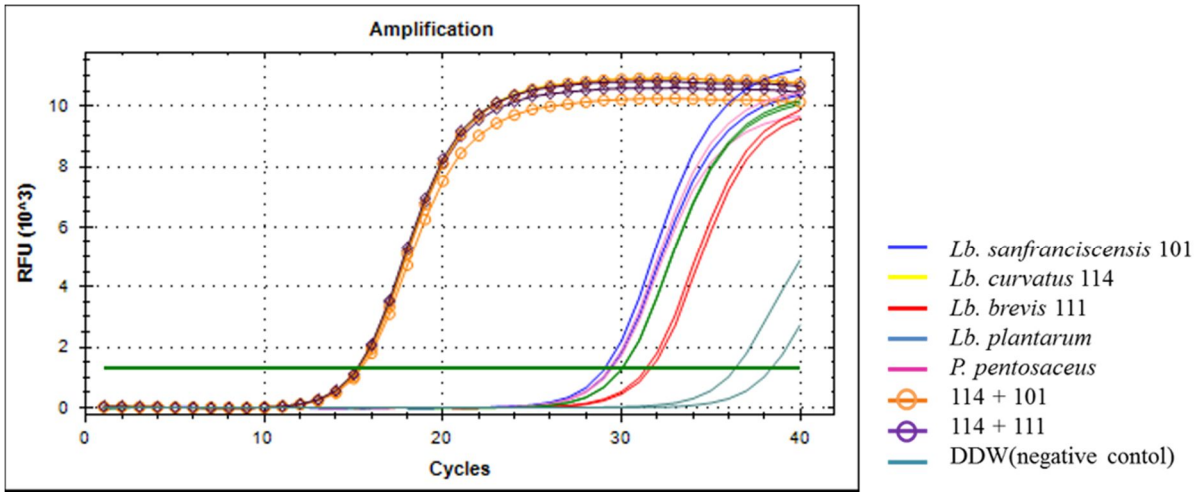


Figure 5. Cycle threshold (Ct) values obtained from target DNA only and mixed with non-target DNA

Table 8. Cycle threshold (Ct) values obtained from target DNA only and mixture of target DNA and non-target DNA in the ratio of 1to 10,000. Ct values in bold indicate values for target DNA only.

Target DNA		Non-target DNA			
Log copies /μl	Ct value	Target DNA : non-target DNA	Ct value	Target DNA : non-target DNA	Ct value
Lb. brevis		*Lb. curvatus*		*Lb.sanfranciscensis*	
7.47	**12.24**	1:1	12.25	1:1	12.24
6.47	**16.06**	1:10	16.07	1:10	16.14
5.47	**19.58**	$1:10^2$	19.62	$1:10^2$	19.78
4.47	**23.32**	$1:10^3$	23.4	$1:10^3$	23.48
3.47	**27.03**	$1:10^4$	27.04	$1:10^4$	27.05
Lb.curvatus		*Lb. sanfrnaciscensis*		*Lb. brevis*	
7.92	**10.51**	1:1	10.48	1:1	10.53
6.92	**13.25**	1:10	13.36	1:10	13.43
5.92	**16.45**	$1:10^2$	16.58	$1:10^2$	16.66
4.92	**19.93**	$1:10^3$	20.05	$1:10^3$	20.11
3.92	**23.31**	$1:10^4$	23.48	$1:10^4$	23.51
Lb. sanfranciscensis		*Lb brevis*		*Lb.curvatus*	
7.62	**12.96**	1:1	13.01	1:1	12.87
6.62	**16.49**	1:10	16.55	1:10	16.48
5.62	**20.00**	$1:10^2$	20.14	$1:10^2$	20.03
4.62	**23.54**	$1:10^3$	23.67	$1:10^3$	23.59
3.62	**27.31**	$1:10^4$	27.1	$1:10^4$	27.16

3.2.3. Validation of qPCR assays

Standard curves for the quantification of *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis* were obtained from 10-fold serial dilutions of the genomic DNA of *Lb. brevis* 111*, Lb. curvatus* 114 and *Lb. sanfranciscensis* 101 (Figure 3). A good linear correlation between the Ct values and the number of the genome copies was obtained for all standard curves with R^2 values ranging from 0.995 to 0.999. These correlation coefficients demonstrated that the qPCR assays were reliable over a range of 5 log units. According to the run, the qPCR efficiency ranged over 85% for all target species. The intra-assay repeatability and inter-assay reproducibility for each primer set are also checked. The intra- and inter-assay CV% values (mean over standard deviation×100) were low for each primer set. The intra-assayrepeatability varied from 0.06% to 1.76% for the *Lb. brevis* primer set, from 0.00% to 1.45% for the *Lb. curvatus* primer set and from 0.00% to 1.95% for the *Lb. sanfranciscensis* primer set according to the concentration of gene copies/**µ**l. Meanwhile, inter-assay CV% values were silightly higher than intra-assay CV% values.

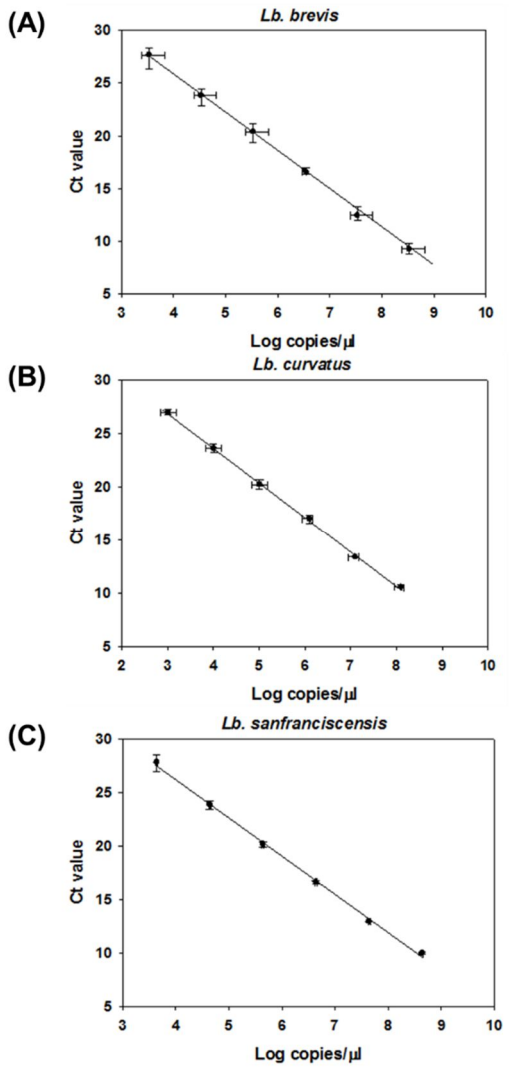


Figure 6. Standard curves generated from the threshold cycle (Ct) values plotted against the estimated logarithm each target gene concentration (copies/**μ**l) for *Lb. brevis* (a), *Lb curvatus* (b) and *Lb. sanfranciscensis* (c). Ten-fold serial dilutions of DNA extracted from a *Lb. brevis* 111, *Lb. curvatus* 114 and *Lb. sanfranciscensis* 101 culture were performed.

3.2.4. Quantification in sourdough samples

Sourdough samples were analyzed to assess the applicability of qPCR for direct quantification of *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis* in sourdough samples. First of all, the cell numbers of these strains from pure culture obtained by qPCR were then compared with the number of cells obtained by a traditional culture method, mMRS plates counting. The **figure 7** shows that the *Lb. brevis* cells numbered from 7.2 to 7.3 log cells/ml by plate count method and from 7.9 to 8.0 log copies/ml by qPCR. For *Lb. curvatus*, the number of cells ranged from 6.8 to 6.9 log cells/ml by plate counting and from 6.7 to 6.9 log cells/ml by qPCR. Also, *Lb. sanfranciscensis* counts obtained by plate count method ranged from 6.7 to 6.9 log cells/ml and from 7.3 to 7.4 log cells/ml by qPCR.Recovery was defined as the closeness of relationship between the number of cells determined by qPCR and the number of cells determined by plate counting, calculated as a percentage of the qPCR count (log10) versus the plate count (log10). According to recovery, qPCR counts were higher than plate counts for *Lb. brevis* and *Lb. sanfranciscensis*. Conversely, only for *Lb. curvatus*, the amount of cells from plate countswasalmost equal amount of qPCR counts. This means that DNA could be easily extracted from *Lb. brevis and Lb. sanfranciscensis*. Furthermore, qPCR methodcould detectboth viable but non-culturable (VBNC) cells and dead cells that do not grow in culture media.

Food samples, such as sourdough, are highly complex matrices, with high numbers of PCR inhibitors and background microflora that could interfere with the ability of real-time PCR to quantify *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis*. Therefore, it is important to study the accuracy of the real-time PCR method developed and its suitability for further applicationsto sourdough products. With that aim, real-time qPCR data were analyzed both strains harvested from pure culture and from artificially inoculated sourdough containing an equal amount of cells. As a result, the quantification of strains from broth numbered 8.0 log and 7.9 log by sourdough sample, 6.8 log from broth and 6.7 log from sourdough, and 7.9 log from broth and 7.5 log from sourdough inoculated by *Lb. brevis*.*Lb. curvatus* and *Lb*. *sanfranciscensis*, respectively(**Figure 8**). However, the statistical analysis performed showed no significant differences between pure culture and sourdough samples. This indicates the reliability of the designed real-time PCR technique to count target strains in a sourdough sample, and also the reliability of the pre-PCR treatment to minimize the interference of the inhibitors present in food samples.

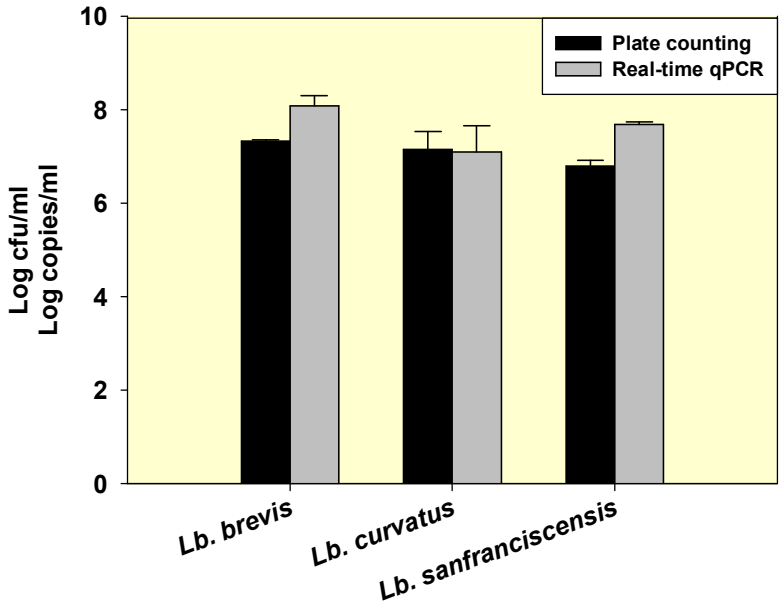


Figure 7. Comparison of quantification methods between plate counts and qPCR

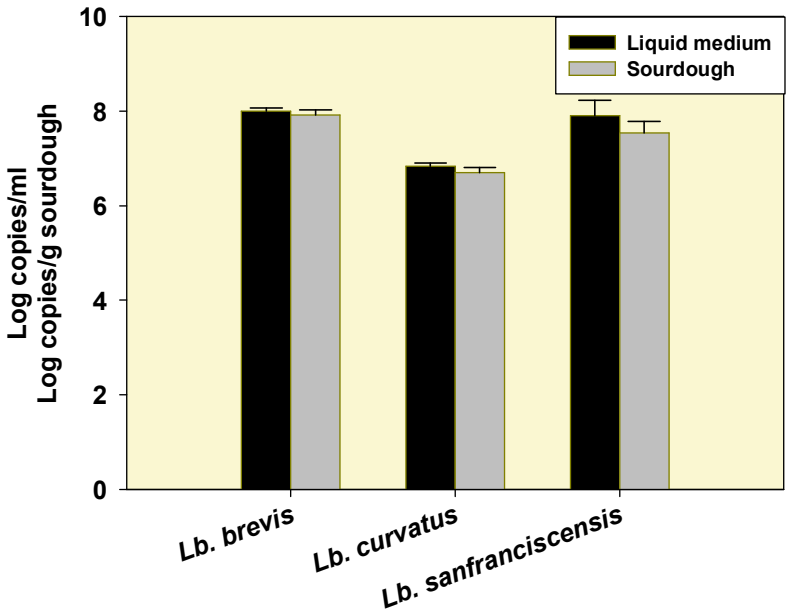


Figure 8. DNA extraction yield from liquid cultures and sourdough samples.

3.3. Application of qPCR method to sourdough samples

In the previous Chapter, it is proved that the qPCR method for quantifying *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis*is valid. In this study, fermented Korean sourdough samples were analyzed to monitor the population dynamics of *Lb. brevis, Lb. curvatus* and *Lb. sanfranciscensis* by the newly developed qPCR method. As indicated in Introduction (**Figure 1**), Korean sourdoughs were inoculated with nuruk, and were fermented at23°C for 5 h and stored at 4°C for about 19 h between back-slopping. Sourdoughswere sampled right before new back slopping. As shown in **Figure 9**, it was observed that *Lb. curvatus* was the most dominant strain, existing about 9.5 log copies/g sourdough at the first propagation of sourdough. It has a possibility that *Lb. curvatus* was originated from nuruk, according to the results of isolation (**Table 5**). However, the number of *Lb. curvatus* continued to decline with increasing the number of propagation. Conversely, the number of *Lb. sanfranciscensis*dramatically increased with the number of propagation. For *Lb. brevis*, log 6 to 7 (copies/g sourdough) were quantified at the first propagation. These results are correspond to PCR-DGGE during this fermentation, conducted at Chung-Buk National University (**Figure 10**). Therefore, it is verified again that the qPCR method developed for three strains is reliable and also by the comparison of the results between PCR-DGGE and qPCR method.

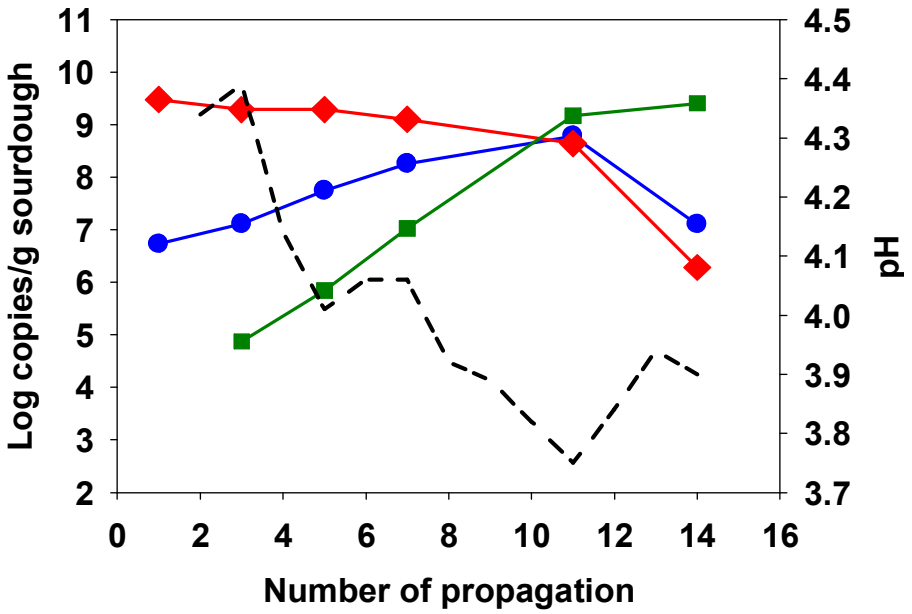


Figure 9. Quantification of *Lb. brevis*(—●—), *Lb. curvatus*(—◆—) and *Lb. sanfranciscensis*(—■—) from sourdough started with nuruk by qPCR method. Dotted plot means pH value.

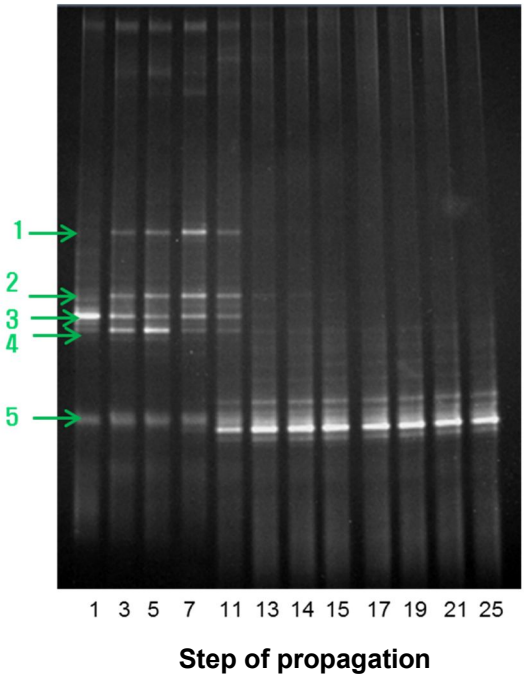


Figure 10. PCR-DGGE profiles of bacterial communities associated to sourdough fermentation inoculated with nuruk. 1: *Leuconostoc mesenteroides*, 2: *Lactobacillus brevis*, 3: *Lb. curvatus*, 4: *Pediococcus pentosaceus*, 5: *Lb. sanfranciscensis*

3.4. Monitoring dominant populations during Korean sourdough fermentations

At the start of sourdough fermentation, total LAB counts of the samples were 9.3 log cfu/g sourdough on MRS medium and 8.9 log cfu/g sourdough on SDB medium. However, LAB numbers in MRS gradually decreased during sourdough fermentation for 20 days. Conversely, LAB numbers in SDB gradually increased after the first day of fermentation. Also, the pH and gas production decreased (based on data from SPC).However, it is difficult to explain what happened in sourdough based on these data only. To identify dominant populations of*Lb. curvatus, Lb. sanfranciscensis* and *Lb. brevis,* qPCR method was conducted to Korean sourdough fermenation.A growth of *Lb. sanfranciscensis* consistently increased during 11 days, becoming the most dominant strain in the sourdough.For *Lb. curvatus* and *Lb. brevis*, a sudden decrease was noticed after the 11^{th} propatation of sourdough fermentation.

Three important LAB of Korean sourdough were simultaneously inoculated to monitor their population changes during sourdough fermentation and propagation of doughswas carried out for 7 days. The results are shown in **Figure 11**. In common with the results of sourdough started with nuruk (Korean sourdough),*Lb. curvatus*showed a significant decrease after the first day of fermentation.

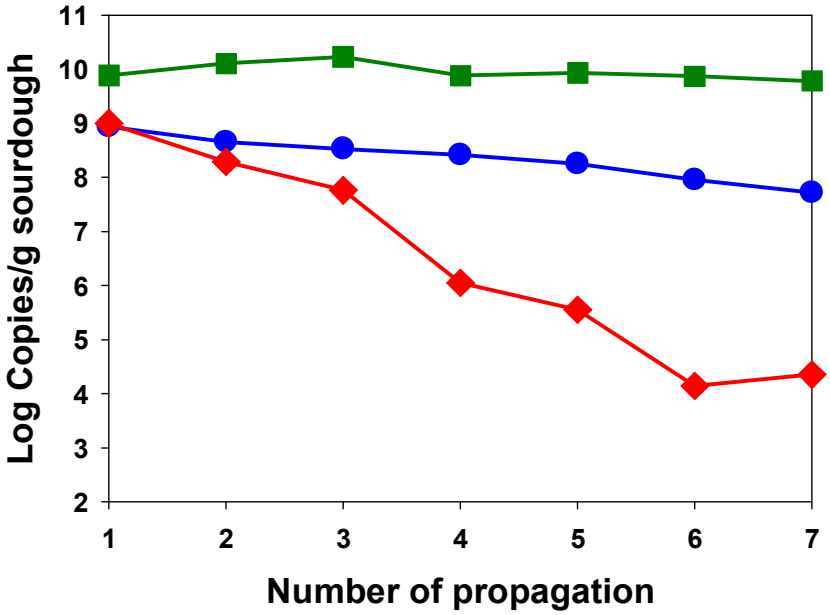


Figure 11. Quantification of *Lb. brevis*, *Lb. curvatus* and *Lb. sanfranciscensis* from artificially inoculated sourdough by qPCR method. (●: *Lb. brevis*, ◆: *Lb. cuvratus*, ■: *Lb. sanfranciscensis*) Results refer to cell concentration at the end of each propagation step.

3.5. Investigation on the causes of the decline growth of *Lb.curvatus*

The qRT-PCR method was used to examine thechangesin microbial populationsduring the sourdough fermentation. It was found that *Lb. curvatus* decreased ththroughout the fermentation (**Figure 11**).To monitor the interactions among the sourdough LAB, the effects of the combination of LAB on sourdough characteristics were monitored over a 24 h sourdough fermentation.The results of sourdough fermentation obtained by mixed starters with dual combinations are presented in **Figure 12**. Initial sourdough pH (6) and TTA (3) values were quite similar for each combination of LAB starters and lactic and acetic acids and ethanol were absent at the beginning of the process. At the end of the sourdough fermentation, the pH value of the sourdoughs inoculated with each combined LAB startersdecreased over the incubation period from 6.0 to about under 4.0. A stable final pH was reached after 12–16 h. In particular, the sourdoughs containing *Lb. sanfranciscensis* were more acidic in comparison to sourdough made with *Lb. curavatus* and *Lb. brevis*. Utilization of starters containing *Lb. sanfranciscensis* strains has resulted in a more rapid decrease of the pH value than when the inoculation was performed with *Lb. curvatus* strains. These observations could be assigned to a difference in LAB population since initial LAB cell numbers varied from log 5.5 copies/g sourdough to log 6.5 copies/g sourdough and reached about log 8.5 copies/g sourdough for *Lb. sanfranciscensis* but log 6.5 copies/g sourdough for *Lb. curvatus*after 16h.The number of *Lb. curvatus*cells decreased slightlyduring

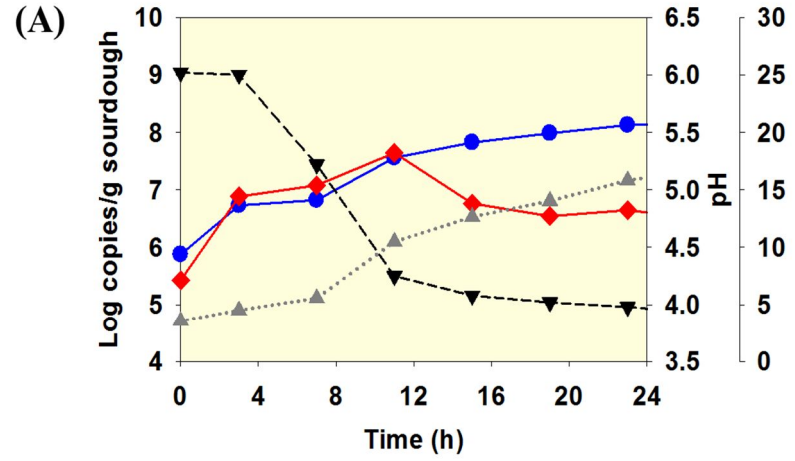
the acidification period.

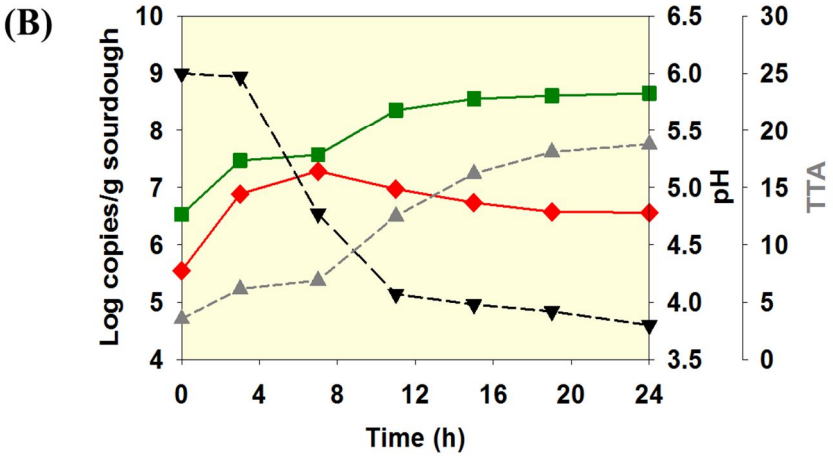
To investigate the effects of pH on growth of *Lb. curvatus*, three strains were cultured in mMRS at pH 4.0. The pH used in this study was 4.0, since the final aciditiy of sourdough fermentation was 4.0 on average. The resultsareshown in **Figure 13A**. *Lb. sanfranciscensis* and *Lb. brevis* could grow at lowpH (4.0), whereas *Lb. curvatus*could not grow in the condition of low pH.Although the three strains were able to grow in mMRS at pH 5.4, the specific growth rate of *Lb. curvatus* was the lowest in comparison with the other strains (**Figure 13B**)

Sourdough is characterized by various complex interactions among the microorganisms present, as well as interactions between these organisms and their environment (Leroy F *et al.* 2006). The predominance of specific microorganisms is related to their metabolic features, including the ability to ferment the available sugars(Gobbetti M 1998). According to Oura, wheat starch is enzymatically degraded into maltose and glucose, mainly because of amylases on damaged starch granules (Oura E *et al.* 1982).Therefore, the effects of maltose and glucose on the growth of three strains were assessed, in order to determine the role of maltose and glucose in the process of sourdough fermentation.

It has beenknown that *Lb. sanfranciscensis*is physiologically well adapted to the sourdough environmentdue toutilization of specific amino acids and peptides and presence of maltose phosphorylase (Gobbetti M and Corsetti A 1997). Likewise, in

this study,*Lb. sanfranciscensis*was able to utilize both glucose and maltose. Almost all of the maltose or glucose were consumed completely during the early stages of fermentation (by 12 hours), eliciting a rapid increase in the total cell numbers(**Figure 14C, Figure 15C**).Besides, *Lb. sanfranciscensis* produced 25 g/L of lactic acid, 1 g/L of acetic acid and 5 g/L of ethanol in mMRS broth containing glucose or maltose. On the other hand,*Lb. brevis* was incapable of fermenting glucose, although it did utilize a total amount of maltose (20g/L). Meanwhile,a slow growth rate of *Lb. curvatus*was observed at sugar (maltose, glucose) concentration of 20 g/L,suggetsting inefficient maltose and glucose utilization ability. Sugar consumption rate for *Lb. curvatus* was lower than that of other strains. As a result, a poor tolerance against acid as well as the inefficient maltose utilization ability of *Lb. curvatus* might be attributed to the reduced population.


(A)
Log copies/g sourdough
pH
Time (h)
10
9
8
7
6
5
4
6.5
6.0
5.5
5.0
4.5
4.0
3.5
30
25
20
15
10
5
0
0
4
8
12
16
20
24


(B)
Log copies/g sourdough
pH
TTA
Time (h)
10
9
8
7
6
5
4
6.5
6.0
5.5
5.0
4.5
4.0
3.5
30
25
20
15
10
5
0
0
4
8
12
16
20
24

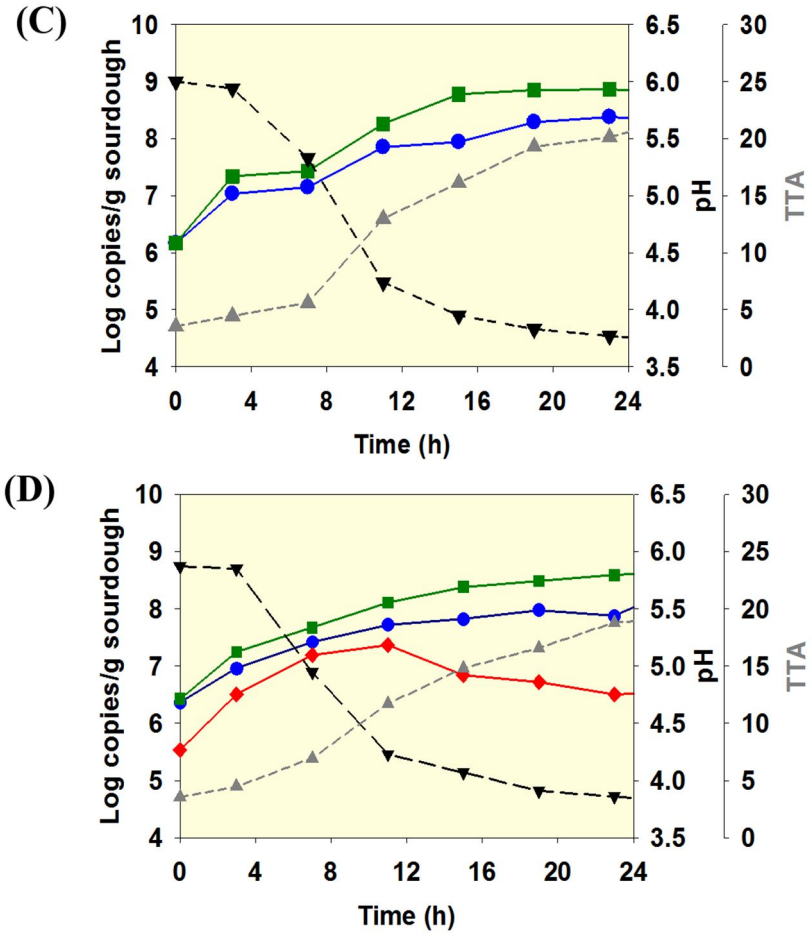


Figure12. Result of sourdough fermentation inoculated with thevarious combinations of(A); *Lb. brevis* and *Lb. curvatus*, (B); *Lb. curvatus* and *Lb. sanfranciscensis*, (C) ; *Lb. brevis* and *Lb. sanfranciscensis*, (D); *Lb. brevis*, Lb. curvatus and *Lb.sanfranciscensis*. (: *Lb. brevis*, : *Lb. cuvratus*, : *Lb. sanfranciscensis*, : pH, : Total titrable acidity)

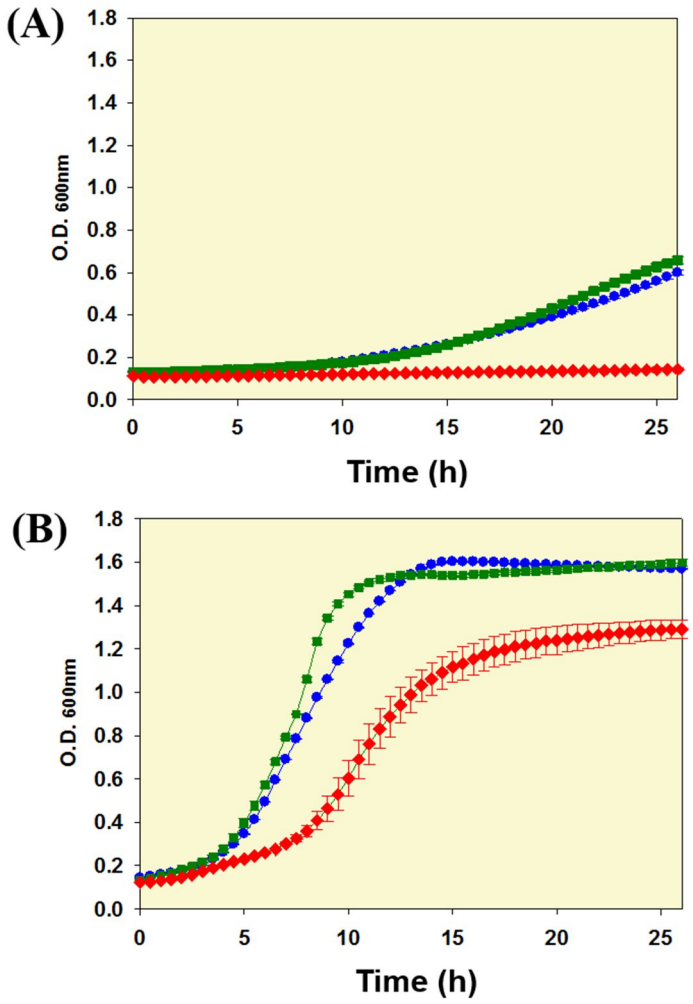
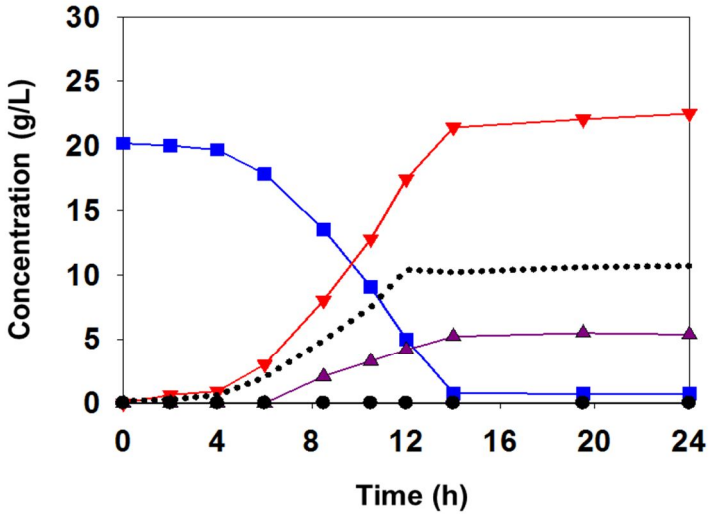
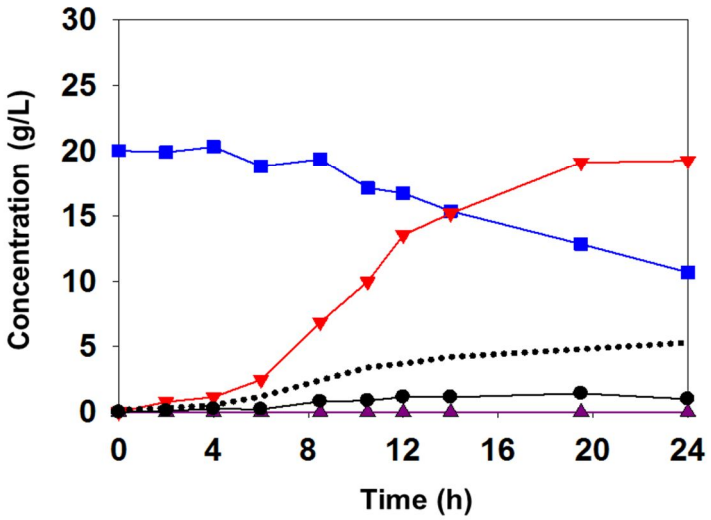


Figure 13. Growth curves for each strain at initial pH 4.0 (A) and pH 5.4 (B) in mMRS mediumat 23℃. (●: *Lb. brevis*, ◆: *Lb. cuvratus*, ■: *Lb. sanfranciscensis*)

(A)


30
25
20
15
10
5
0
Concentration (g/L)
0
4
8
12
16
20
24
Time (h)

(B)


30
25
20
15
10
5
0
Concentration (g/L)
0
4
8
12
16
20
24
Time (h)

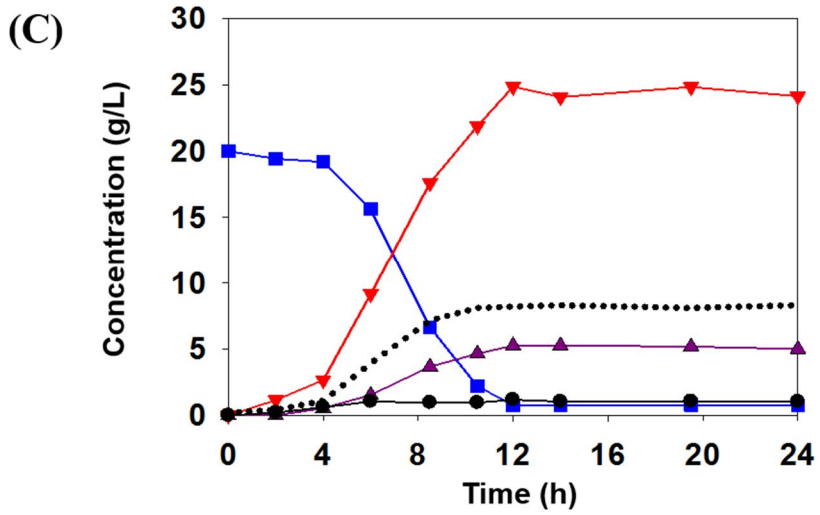
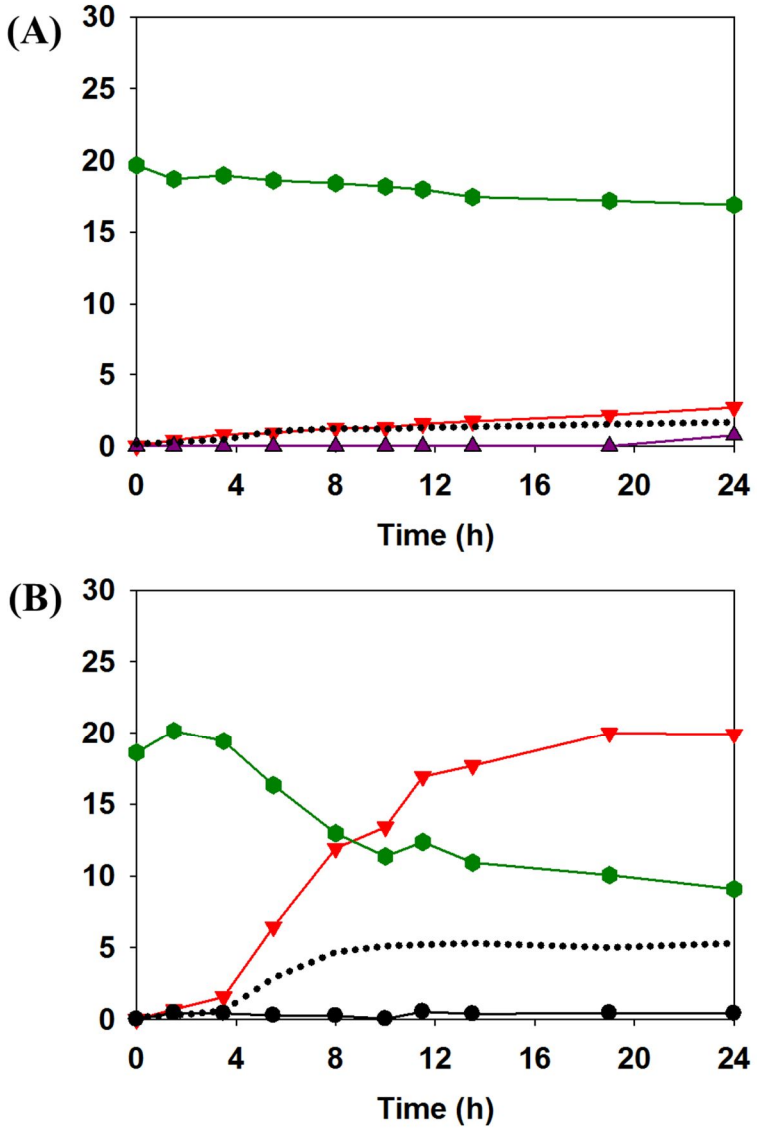


Figure 14. Comparison of maltose consumption and growth by the strains.. (A); *Lb. brevis*, (B); *Lb. curvatus*, (C); *Lb. sanfranciscensis*in mMRS with 20g/L maltose.(: Maltose, : Lactic acid, : Ethanol, : Acetic acid, : O.D at 600 nm)


(A)
30
25
20
15
10
5
0
0
4
8
12
16
20
24
Time (h)
(B)
30
25
20
15
10
5
0
0
4
8
12
16
20
24
Time (h)

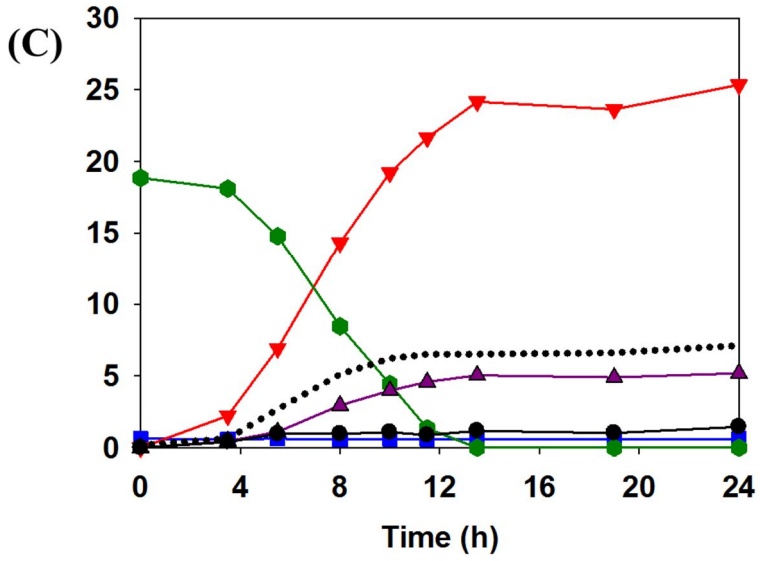


Figure 15. Comparison of glucose consumption and growth by the strains. (A); *Lb. brevis*, (B); *Lb. curvatus*, (C); *Lb. sanfranciscensis*in mMRS with 20g/L glucose.(: glucsoe, : Lactic acid, : Ethanol, : Acetic acid, ····· : O.D at 600 nm)

Table 9. Summary of flask-batch fermentations by sourdough LAB in mMRS medium with 20g/L maltose or glucose.

Carbon source	Strains	Specific growth rate(h^{-1})	Carbon source consumption rate (g/L· h)	Lactic acid concentration (g/L)	Acetic acid concentration (g/L)	Ethanol concentration (g/L)
Maltose	*Lb.brevis*111	0.41	1.39	22.5	0.1	5.36
	*Lb. curvatus*114	0.25	0.39	19.24	0.94	0
	*Lb. sanfranciscensis*101	0.51	1.65	24.86	1.04	5.27
Glucose	*Lb.brevis*111	0.1	0.12	2.7	0.1	0.76
	*Lb. curvatus*114	0.44	0.48	19.98	0.1	0
	*Lb. sanfranciscensis*101	0.50	1.52	25.34	1.45	5.18

4. Conclusions

The following conclusions can be drawn in this thesis.

(1) Investigation of microbial populations by RAPD-PCR unveiled that *Lb. sanfranciscensis, Lb. curvatus* and *Lb. brevis*were dominant in Korean sourdough inoculated with nuruk.

(2) The newly real-time PCR method developed provides a highly sensitive and specific tool to monitor*Lb. brevis, Lb. curvatus,* and *Lb. sanfranciscensis* during sourdough fermentation.

(3) Utilization of maltose and weak tolerance against acids might contribute to the reduction of population size of during the fermentation of Korean sourdough.

REFERENCES

Beresford TP, Fitzsimons NA, Brennan NL and Cogan TM (2001). Recent advances in cheese microbiology. International Dairy Journal **11**(4): 259-274.

Charlet M, Duboz G, Faurie F, Le Quéré JL and Berthier F (2009). Multiple interactions between *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* strongly affect their growth kinetics during the making of hard cooked cheeses. International Journal of Food Microbiology **131**(1): 10-19.

Corsetti A, Gobbetti M, Balestrieri F, Paoletti F, Russi L and Rossi J (1998). Sourdough lactic acid bacteria effects on bread firmness and stalin. Journal of Food Science **63**(2): 347-351.

Corsetti A and Settanni L (2007). *Lactobacilli* in sourdough fermentation. Food Research International **40**(5): 539-558.

De Vuyst L, Van Kerrebroeck S, Harth H, Huys G, Daniel H-M and Weckx S (2014). Microbial ecology of sourdough fermentations: Diverse or uniform? Food Microbiology **37**: 11-29.

Decock P and Cappelle S (2005). Bread technology and sourdough technology. Trends in Food Science and Technology **16**(1): 113-120.

Dhanasekaran S, Doherty T and Kenneth J (2010). Comparison of different standards for real-time PCR-based absolute quantification. Journal of Immunological Methods **354**(1): 34-39.

Ercolini D (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods **56**(3): 297-314.

Gaggiano M, Di Cagno R, De Angelis M, Arnault P, Tossut P, Fox PF and Gobbetti M (2007). Defined multi-species semi-liquid ready-to-use sourdough starter. Food Microbiology **24**(1): 15-24.

Giraffa G (2004). Studying the dynamics of microbial populations during food fermentation. FEMS Microbiology Reviews **28**(2): 251-260.

Gobbetti M (1998). The sourdough microflora: interactions of lactic acid bacteria and yeasts. Trends in Food Science and Technology **9**(7): 267-274.

Hansen A and Schieberle P (2005). Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. Trends in Food Science and Technology **16**(1-3): 85-94.

Irlinger F and Mounier J (2009). Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology **20**(2): 142-148.

Kim SY, Yoo KS, Kim JE, Kim JS, Jung JY, Jin Q, Eom HJ and Han NS (2010). Diversity analysis of lactic acid bacteria in Korean rice wines by culture-independent method using PCR-denaturing gradient gel electrophoresis. Food Science and Biotechnology **19**(3): 749-755.

Kline L and Sugihara TF (1971). Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species

responsible for the souring activity. Applied Microbiology**21**(3): 459-465.

Lee CH (2004). Creative fermentation technology for the future. Journal of Food Science-Chicago- **69**(2): CRH31-CRH32.

Leroy F and de Vuyst L (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science and Technology **15**(2): 67-78.

Leroy F, de Winter T, Adriany T, Neysens P and de Vuyst L (2006). Sugars relevant for sourdough fermentation stimulate growth of and bacteriocin production by *Lactobacillus amylovorus* DCE 471. International Journal of Food Microbiology **112**(2): 102-111.

Ndoye B, Rasolofo EA, LaPointe G and Roy D (2011). A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. Dairy Science and Technology **91**(5): 495-524.

Nocker A and Camper AK (2009). Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiology letters **291**(2): 137-142.

Oura E, Suomalainen H and Viskari R (1982). 4. Breadmaking. Economic Microbiology.

Postollec F, Falentin H, Pavan S, Combrisson J and Sohier D (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiology **28**(5): 848-861.

Prajapati J B and Nair BM (2003). The history of fermented foods. Fermented Functional Foods. CRC Press, Boca Raton, New York, London, Washington DC: 1-25.

Rhee SJ, Lee JE and Lee CH (2011). Importance of lactic acid bacteria in Asian fermented foods. Microbial Cell Factories **10**(1): S5.

Sieuwerts S, de Bok FAM, Hugenholtz J and van Hylckama Vlieg JET (2008). Unraveling microbial interactions in food fermentations: from classical to genomics approaches. Applied and Environmental Microbiology **74**(16): 4997-5007.

Van der Meulen R, Scheirlinck I, Van Schoor A, Huys G, Vancanneyt M, Vandamme P and De Vuyst L (2007). Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Applied and Environmental Microbiology **73**(15): 4741-4750.

Venturi M, Guerrini S, Granchi L and Vincenzini M (2012). Typing of *Lactobacillus sanfranciscensis* isolates from traditional sourdoughs by combining conventional and multiplex RAPD-PCR profiles. International Journal of Food Microbiology **156**(2): 122-126.

Wouters D, Bernaert N, Conjaerts W, Van Droogenbroeck B, De Loose M and De Vuyst L (2013). Species diversity, community dynamics, and metabolite kinetics of spontaneous leek fermentations. Food Microbiology **33**(2): 185-196.

Wouters J, Ayad EHE, Hugenholtz J and Smit G (2002). Microbes from raw milk

for fermented dairy products. International Dairy Journal **12**(2): 91-109.

Zapparoli G, Torriani S, Pesente P and Dellaglio F (1998). Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. Letters in Applied Microbiology **27**(5): 243-246.

국문 초록

사워도우(sourdough)는 밀, 호밀 등의 곡물의 가루와 물을 혼합하여 만든 반죽으로, 유산균과 효모에 의한 발효와 숙성을 거쳐 제조된다.사워도우는 유기산에 의한 신 맛을 내기 때문에 산성 반죽이라고도 불린다.사워도우 내의 유산균은 젖산발효를하며 유기산을 발생시키는 주요 미생물로서 사워도우의식품공학적 특성과 밀접한 관련이 있다. 발효 생산물인 젖산과 초산은 사워도우의 저장성을 높이며,전통적인 방법으로 제조한 사워도우는 유럽인들에게는 그 풍미가 익숙하지만,한국인에게 강한 신 맛으로 인식되고 있다.이에 한국의 전통 술 발효에 쓰이는 누룩을 스타터로 밀가루에 접종하여 새로운 한국형 사워도우를 제조하는 연구가 진행되었다.

본 연구에서는 누룩을 접종한 새로운 사워도우(이하 한국형 사워도우로 지칭)에 존재하는 다양한 미생물을 분리하였다.그 중 우점종으로 존재하는 세 종의 유산균들을 정량하는 분석 방법을 구축한 후, 이 방법을 이용하여 사워도우 내유산균들을 모니터링하였다.

먼저 한국형 사워도우 내 유산균의 다양성을 파악하고자 사워도우로부터 다양한 미생물을 분리하였다.분리한 미생물들을 대상으로 RAPD-PCR을

이용하여 어떤 미생물들이 사워도우에 있는지를 파악하였다. 연쇄중합효소반응 밴드의 유연관계 분석을 통해 총 여섯 그룹으로 나눌 수 있었다.추가적으로 16S rRNA 시퀀싱을 실시하여, 분리 미생물들 중 *Lb. sanfranciscensis*가 사워도우에 가장 많이 존재함을 확인하였다(58%). 이외, *Lb. curvatus* (27%), *Lb. brevis* (9%), *Lb. plantarum*, *Lb. sakei*, *Pediococcus pentasaceus*도 분리· 동정되었다.

사워도우 발효와 숙성 기간 동안 유산균들의 개체수 변화는 사워도우의 특성과 밀접한 연관 관계가 있다.본 연구에서는 실시간 정량적중합효소연쇄반응법을 이용하여 *Lb. sanfranciscensis, Lb. curvatus, Lb. brevis*을특이적이며 정량적으로 측정할 수 있는 방법을 개발하였다. 이를 위해 National Center for Biotechnology Information(NCBI) 에서 제공되는 유전자 서열 정보에 기반하여 교차 반응성 없이 목적 미생물의 특정 유전자를 증폭하는 프라이머를 설계하였다. 이러한 중합효소연쇄반응법을 통해 얻은 결과는 기존의 생균수 측정 결과와 높은 상관성이 있음을 확인하였다.또한 단백질과 당과 같은 저해물질이 존재하는 사워도우에서 DNA를 추출하여 측정하였을 때도 그 결과가 신빙성이 있음을 확인하였다.

마지막으로 본 연구를 통해 개발된 방법을 한국형 사워도우 모니터링에

적용하였고,이 때 *Lb. curvatus*의 개체수는 발효 계대차수가 증가함에 따라 감소함을 관찰하였다.이 현상의 원인을 조사하고자, 각 미생물들의 내산성 및 사워도우에 주로 존재하는 탄소원인 말토스배지에서말토스소비속도와비성장속도를비교하였다. *Lb. brevis*의경우 1.39g/L· h의 말토스 소비속도,0.36/h의 비성장속도를 나타내었다.그리고*Lb. sanfranciscensis*의경우 1.65g/L· h, 0.51/h의말토스소비속도와비성장속도를나타낸반면, *Lb. curvatus*는 0.39 g/L· h의말토스소비속도와 0.26/h의비성장속도를나타내었다. 특히*Lb. curvatus*는말토스배지에서말토스소비속도와비성장속도가다른두균주에 비해 현저히 낮았으며, 또한 낮은 내산성을 보였다. 이는 *Lb. curvatus*의 개체수 감소의 한 원인으로 추정된다.

요약하면,본 연구에서 개발한 실시간 정량적 중합효소반응법을 통해 한국형 사워도우에 존재하는 대표적인 유산균의 개체수 변화를 신속하게 파악할 수 있었다.이런 방법은 사워도우 내의 미생물 다양성을 조절하거나 사워도우의 특성을 개선시키는데에 이용 될 수 있는 유용한 정보를 제공 할 것이다.

주요어: 사워도우, 유산균, 혼합배양, 균의 분리 동정, 미생물 다양성,

유전적 다양성 유연관계, 배양독립적 미생물 분석 방법, 실시간정량적 중합효소연쇄반응.

학 번: 2012-23380