Master's Thesis of Science in Agriculture

Screening and Characterization of *β*-galactosidase producing *Bifidobacterium animalis* subsp. *lactis* HT 10-2 Isolated from Infant Feces

β－galactosidase를 생산하는 유아분변 유래 *Bifidobacterium animalis* subsp. *lactis* HT10－2의 선발과 특성 규명

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Abstract

In this study, 637 of *Bifidobacteria* and *Lactobacilli* colonies were obtained from Korean infants, and 10 isolates were selected by colorimetric assay for β-galactosidase activities. We investigated the probiotic potential of the isolates, and *Bifidobacterium animalis* subsp. *lactis* HT 10-2 was identified as a promising probiotic strain with high activity of β-galactosidase. The complete genome sequence of HT 10-2 reveals a single circular chromosome of 1,923,647 bp, with 1,613 predicted proteins-encoding 1,553 of coding sequence (CDS). The genes (*bgaA, ebgA, lacZ, and beta-galIII*) coding for β-galactosidase were possessed by both *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and HT 10-2. However, the enzyme activities of DSM 10140 and HT 10-2 have shown significant difference each other, HT 10-2 showed relatively higher β-galactosidase activity than DSM 10140 amounting to approximately 3 times. Forthermore, we investigated the relative mRNA expression of *bgaA, ebgA, lacZ,* and *beta-galIII* from HT 10-2 versus to thoses genes from DSM 10140 using the quantitative real time PCR (qRT-PCR). High transcriptional rate of the genes from HT 10-2 was observed compared to the genes from DSM 10140. ⊿Ct values indicated that mRNA expression from *lacZ of the* HT 10-2 were higher than that of *bgaA, ebgA, lacZ,* and *beta-galIII*. The β-galactosidase, expressed in *lacZ* gene, was targeted for isolation and

characterization. Finally, β-galactosidase with a molecular mass of 119 kDa was purified from crude cell extracts of the HT 10-2 using ammonium sulfate fractionation followed by ion exchange chromatography with 10 fold to a specific activity of 30,473.8 unit/ml. The temperature optimum of β-galactosidase activity was found to be 37 ℃ and the purified β-galactosidase was stable at 37 ℃ and the residual β-galactosidase activity still maintained 80.43 % and 78 % after treatment for 0.5 h and 1.0 h relatively. β-galactosidase activities were enhanced by by $CaCl_2$ (1.27 fold) and was remarkably enhanced by $MgSO_4$ (2.43 fold), $FeSO_4$ (3.00 fold), MnSO4 (2.75 fold) and $MgCl_2$ (2.17 fold), but some ions, such as KCl, NaCl, Na_2SO_4 and $CuSO_4$ inhibited the activities.

Keyword : Dairy product, β-Galactosidase, lactic acid bacteria, bifidobacteria, probiotics
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Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1. Study Background

β-galactosidases is one of the most important enzymes used in dairy industy for hydrolysis of lactose into glucose and galactose. Lactose is found in abundance in dairy products. Absorption of lactose requires β-galactosidases, termed lactase-phlorizin hydrolase (lactase), activity in the small intestinal brush border to split the bond linking the two of monosaccharides. However, 20 to 25% of the United States population maldigest milk because of its lactose content. (Johnson, Semenya *et al.* 1993). Lactose maldigestion is caused by a reduction in lactase (β-galactosidase) activity in the small intestine sometimes after weaning. In these "lactase nonpersistent" individuals, unhydrolyzed lactose passes into the large intestine, where it is fermented by the indigenous microflora into gases such as H_2, CH_4, and CO_2 and short-chain fatty acids. The excessive gas production and the osmotic effects of excessive undigested lactose can cause gastrointestinal symptoms, including flatulence, abdominal pain, and diarrhea (Heyman 2006). Lactose maldigesters digest and tolerate lactose in yoghurt better than an equivalent quantity of lactose in milk (Kolars, Levitt *et al.* 1984), but the importance of lactase activity present in yoghurt is not clear. Several authors emphasize the importance of the living bacteria of yoghurt or other fermented milks in connection with lactose digestion (Gilliland and Kim 1984).

However, in two of these studies, the tolerance of heat-treated yoghurt was not significantly inferior to that of fresh yoghurt with viable bacteria (Marteau, Flourie *et al.* 1990). The contribution of bifidobacteria to the improvement of lactose intolerance is currently speculative. One study has reported that ingestion of milk fermented with *Bifidobacterium bifidus* caused a moderate reduction in total excretion of breath hydrogen (Martini, Lerebours *et al.* 1991). No evidence is currently available on the improvement of lactose digestion by unfermented bifidus milk. We hypothesized that bifidobacteria could exert positive effects on lactose digestion because of their substantial β-galactosidases activity (Hughes and Hoover 1995).

In this paper, we screened the *Bifidobacterium animalis* subsp. *lactis* HT10-2 shown a high β-galactosidase activity and investigated its probiotic potiental. After identifing the genes coding β-galactosidase by the whole genenom sequence, we also purified and characterized the major active β-galactosidase from HT10-2.

Chapter 2. Review of Literature

2.1. Probiotics

Metchnikoff first introduced the concept of probiotics in 1908 that observed the long life of Bulgarian, who consumed fermented milk foods (Azizpour, Bahrambeygi *et al.* 2009). It was suggested that microorganisms in fermented milk provide the beneficial effects to the host. Probiotics, literally meaning 'for life', are micro-organisms proven to exert health-promoting influences in humans and animals. Several definition of probiotics can be found in the research papers (Azizpour, Bahrambeygi *et al.* 2009). According to the 2002 definition by the World Health Organization (WHO), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." However, there are several evidences indicating that non-viable microorganisms can also confer health benefits due to the fact that in many researches nowadays, the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. Two commonly used probiotic bacteria are *Bifidobacterium* spp. and *Lactobacillus* spp. (Nole, Yim *et al.* 2014). They are generally classified as lactic acid bacteria (LAB) and used for the food industry as well as a variety of health problems. LAB are the most important probiotics due to their long history of uses as starters for various fermented foods with few adverse effects on the health and generally recognized as safe (GRAS) (Adams 1999).

Importance of LAB has been also increased recently in livestock industry since antibiotics for farm animals have been banned in many countries. LAB are currently the main feed additive used in livestock (Solomons 1978).

Table 1. Definitions of probiotics.

Authors	Definition
Lilly and Stillwell (1965)	Growth promoting factors produced by microorganisms
Parker (1974)	Organisms and substances with beneficial effects for animals by influencing the intestinal mifcroflora
Fuller (1989)	A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance
Havenaar and Huis Int Veld (1992)	A mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora
IL SI Europe Working Group (1998)	A viable microbial food supplement which beneficially influences the health of the host
Naidu et al (1999)	A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract
Schrezenmeir and de Vrese (2001)	A preparation of a product containing viable, defined microorganism in sufficient numbers, which alter the microflora in a compartment of the host and by that exert beneficial health effects in this host
FAO-WHO (2002)	Live microorganisms which when administered in adequate amounts confer a health benefit on the host

2.1.1. The role of probiotics

The role of probiotic bacteria in dairy fermentations is to assist in the preservation of the milk by the generation of lactic acid and possibly antimicrobial compounds, the production of flavor compounds (e.g. acetaldehyde in yoghurt and cheese) and other metabolites that will improve the nutritional value of food (Parvez, Malik *et al.* 2006). Aside from these benefits improving food qualities, many researches have showed an important role of probiotics in host health because of competition in nutrition with pathogen and there are several possible functions of probiotics that include the production and secretion of antimicrobial substances (Gillor, Etzion *et al.* 2008), a stimulation of host immune responses (Isolauri, Sütas *et al.* 2001) and displacement of pathogen colonization (Stecher and Hardt 2011). Secretion of substances such as protein, short chain fatty acid (SCFA), organic acids, cell surface active components and DNA from these microbes exerts the same therapeutic effect in gastrointestinal disease. These therapeutic agents are known as pharmabiotics or probioactive (Soccol, Prado *et al.* 2015).

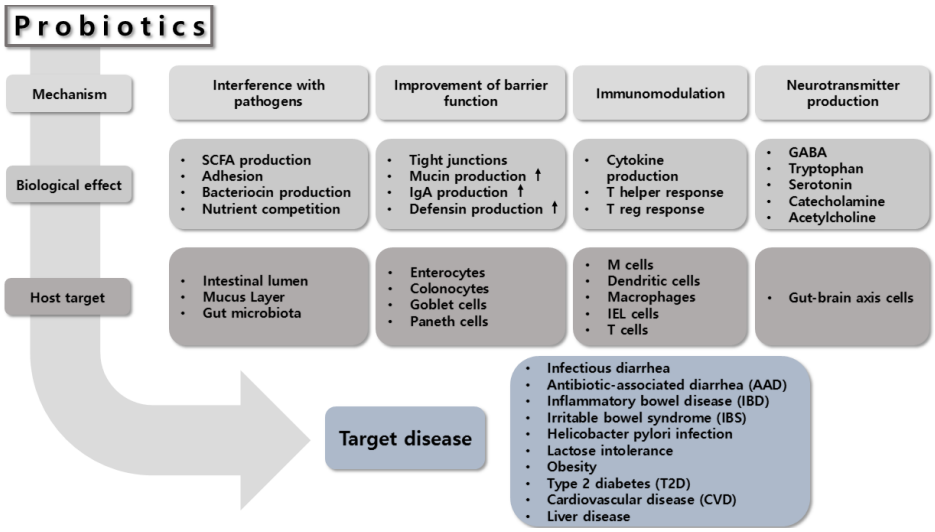

Figure 1. The main mechanisms of action of probiotics.

Known mechanisms of probiotic bacteria might impact on the intestinal microbiota (Sánchez, Delgado *et al.* 2016). First, these mechanisms include competition for dietary ingredients as growth substrates against harmful bacteria, bioconversion of, for example, sugars into fermentation products with inhibitory properties and production of growth substrates such as exopolysaccharide (EPS) (Nikolic, López *et al.* 2012) or vitamins (LeBlanc, Milani *et al.* 2013), for other bacteria. They can also inhibit pathogenic bacteria by antibacterial peptide (bacteriocin) and competitive exclusion for binding sites. Second, specific probiotics may enhance epithelial barrier function by modulating signaling pathways that lead to stimulated mucus or defensing production (Madsen, Cornish *et al.* 2001), or tight junction function (Ulluwishewa, Anderson *et al.* 2011) by preventing apoptosis (Yan and Polk 2002). Third, probiotics may modulate the

immunomodulatory capacity of the body, and the population size of the endogenous microbiota for this site is relatively small, allowing transient dominance of dietary microorganisms, including probiotics (Hill, Guarner *et al.* 2014). The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and a number of bile salt hydrolases (BSHs) have been identified and characterized (Begley, Hill *et al.* 2006).

2.1.2. Application of probiotics

Yogurt is one of the best-known of the foods that contain probiotics. Yogurt is defined by the Codex Alimentarius of 1992 as a coagulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophiles* (Bourlioux and Pochart 1988). Yogurt and fermented milks are considered to be the main carrier for the delivery of probiotics in the dairy industry. Even so, it is possible to obtain probiotic foods for several matrices, including both fermented and non-fermented products because dairy products may represent inconveniences due to their lactose and cholesterol content (Rivera-Espinoza and Gallardo-Navarro 2010). There is a genuine interest in the development of fruit juice-based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as health and refreshing food (Tuorila and Cardello 2002). Cereal gains are considered to be one of the most important sources of protein, carbohydrates, vitamins, minerals and fiber for people all over the world (Fenwick, Heaney *et*

al. 1983). Furthermore, they can be used as sources of non-digestible carbohydrates that besides promoting several beneficial physiological effects can also selectively stimulate the growth of Lactobacilli and Bifidobacteria present in the colon, thereby acting as prebiotics (Finegold, Sutter *et al.* 1983).

The criteria of probiotic include its total safety for the host; human origin; acid and bile resistance; survival in the gastrointestinal transit; production of antimicrobial substances; immune modulator activity; adhesion to epithelial cells; inhibition of pathogenic bacteria; resistance to antibiotics; tolerance to food additives and stability in the food matrix (Sanders 2008). Recently, the probiotics in use today have not been selected on the basis of all these criteria, but the most commonly used probiotics are strains of LAB such as *Lactobacillus*, *Bifidobacterium,* and *Streptococcus*, but new probiotic from other species and genera have recently been introduced (Parvez, Malik *et al.* 2006).

Table 2. The most commonly used species of lactic acid bacteria in probiotic.

Genus	Species
Lactobacillus	*L. acidophilus, L. casei, L. gasseri, L. delbrueckii sp bulgaricus, L. helveticus, L. fermentum, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L, salivarius*
Lactococcus	*Lc. Lactis*
Enterococcus	*E. faecium, E. faecalis*
Streptococcus	*S. thermophiles*
Bifidobacterium	*B. bifidum, B. breve, B. longum, B. animalis ssp. lactis*

2.1.3. *Bifidobacterium* spp.

Bifidobacteria were first isolated and described in 1899 – 1900 by Tissier, who described rod-shaped, nongas-producing, anaerobic microorganisms with bifid morphology, and present in the faeces of breast-fed infants. They are distributed in various ecological niches in the human gastrointestinal and genitourinary tracts, the exact ratio of which is determined mainly by age and diet (Mitsuoka 1990). The profile of constituent species changes; *B. infantis* and *B. breve*, typical of infants, are replaced by *B. adolescentis* in adults, whereas *B. longum* persists lifelong (Finegold, Sutter *et al.* 1983). The growth of bifidobacteria *in vitro* was stimulated by most of the oligosaccharides (Kohmoto, FUKUI *et al.* 1988), it may be concluded that oligosaccharides enhance the intestinal bifidobacteria to improve the intestinal flora, stool consistency, and lipid metabolism (Hughes and Hoover 1991). The studies on *Bifidobacterium* spp. revealed the beneficial effect to human body such as gastric protection and mucin production in an acute gastric injury rat model (Gomi, Harima-Mizusawa *et al.* 2013), allergen-induced lung inflammation in the mouse (MacSharry, O'Mahony *et al.* 2012), very-low-birthweight infants (Yamasaki, Totsu *et al.* 2012).

2.2. β-galactosidase

2.2.1. The function of β-galactosidase

The enzyme, β-galactosidase is a commercially important enzyme since it can hydrolysis of β-D-galactopyranosides such as

lactose which constitutes a substantial portion of daily carbohydrate intake (Hsu, Yu *et al.* 2005). There are a majority of substrates available of β-galactosidase which includes ganglioside Monosialotetrahexosylganglioside (GM1), lactosylceramides, lactose, and various glycoprotein (Kim and Rajagopal 2000). β-galactosidase enzyme hydrolyses lactose to galactose and glucose. As per the carbohydrate active enzymes database, this enzyme has been classified under the glycoside hydrolase 2 (GH 2) family of carbohydrate active enzymes. This enzyme has been widely used in the dairy industry due to the fact that lactose can cause undesirable 'sandiness' in frozen desserts and the comparatively low sweetness of lactose restricts its use (Kim and Rajagopal 2000). β-galactosidase is also associated with lactose intolerance which is widespread throughout the world. Absorption of lactose requires lactase activity in the small intestinal brush border to split the bond linking the two of monosaccharides. Lactase is found in the small intestine and localized to the tips of the villi, a factor of clinical importance (Heyman 2006). More than 70 % of the world's population suffer from the inability to utilize lactose because of the lack of lactase (β-galactosidase) (Lee and Krasinski 1998).

2.2.2. Enzyme sources

β-galactosidases are found in microorganisms, plants especially in almonds, peaches, apricots, apples and animal organs (Nagy, Kiss *et al.* 2001, Haider and Husain 2007). Microorganisms produce two

kinds of enzymes; extracellular which are released into the growth medium, and intracellular which are retained inside the cell wall. The major industrial enzymes are derived from *Aspergillus* spp. and *Kluyveromyces* spp. β-galactosidase from *Kluyveromyces lactis* is one of the most widely used enzymes (Zhou and Chen 2001, Jurado, Camacho *et al.* 2002, Lee, Kim *et al.* 2004, Klewicki 2007). *Bifidobacterium* are regarded as a probiotic organism and its β-galactosidases are used in food. *Bifidobacterium* together with *Lactobacillus* are the bacteria most applied as probiotics because of their potential health benefits. Bifidobacterium has been chosen as a model bacterium for studying fermentation of lactose by the colonic microbiotia (Arunachalam 1999)

Table 3. Microbial sources of β-galactosidas.

Source	Microorganism(s)
Fungi	*Alternaria alternata, A. palmi*
	Aspergillus foetidis, A. fonsecaeus, A. niger,
	A. oryzae, A. carbonarius
	Auerobasidium pullulans
	Beauveria bassiana
	Curvularia inaequalis
	Fusarium moniliforme, F. oxysporum
	Mucor meihei, M. pusillus
	Neurospora crassa
	Paecilomyces varioti
	Penicillium conescens, P. chrysogenum
	Rhizobium meliloti

	Saccharopolyspora rectivirgula
	Scopulariopsis sp.
	Streptomyces violaceus
	Trichoderma reesei
	Alternaria alternate, A. palmi
	Aspergillus foetidis, A. fonsecaeus, A. niger, A. oryzae, A. carbonarius
	Auerobasidium pulluans
	Beauveria bassiana
	Curvularia inaequalis
	Fusarium moniliforme, F. oxysporum
	Mucor meihei, M. pusillus
	Neurospora crassa
	Paecilomyces varioti
	Penicillium conescens, P. chrysogenum
	Rhizobium meliloti
	Saccharopolyspora rectivirgula
	Scopulariopsis sp.
	Streptomyces violaceus
	Trichoderma reesei
Yeasts	*Bullera singularis*
	Candida pseudotropicalis
	Saccharomyces anamensis, S. fragilis
	Kluyveromyces bulgaricus, K. fragilis, K. lactis, K. marxianus
Bacteria	*Arthrobacter sp.*
	Bacillus acidocaldarius, B. circulans, B. coagulans, B. subtilis, B. megaterium, B. stearothermophilus
	Bacteroides polypragmatus
	Bifidobacterium bifidum, B. infantis
	Clostridium acetobutylicum, C. thermosulfurogens

Corynebacteriu murisepticum

Enterobacter agglomerans, E. claceae

Escherichia coli

Klebsiella pneumoniae

Lactobacillus acidophilus, L. bulgaricus, L. kefiranofaciens, L. helviticus, L. lactis, L. sporogenes, L. thermophiles, L. delbrueckii

Leuconostoc citrovorum

Pediococcus acidilacti, P. pento

Propionibacterium shermanii

Pseudomonas fluorescens

Pseudoalteromonas haloplanktis

Streptococcus cremoris, S. lactis, S. thermophiles

Sulfolobus solfataricus

Thermoanaerobacter sp.

Thermus rubus, T. aquaticus, T. thermophiles

Vibrio cholera

Xanthomonas campestris

Source: references (Brandão, Nicoli *et al.* 1987, Adams, Yoast *et al.* 1994, Berger, Lee *et al.* 1995, Mahoney 1997, Hoyoux, Jennes *et al.* 2001, Nagy, Kiss *et al.* 2001, Cho, Shin *et al.* 2003, El-Gindy 2003)

Table 4. Some commercial sources of β-galactosidases.

Enzyme source	Trade name	Supplier
Bacteria		
Bacillus sp.	Novozym 231	Novozymes A/S, Bagsvaerd, Denmark
Escherichia coli	β-galactosidases	Sigma-Aldrich, UK
Yeasts		
Kluyveromyces lactis	Maxilact	DSM Food Specialties,

		Delft, The Netherlands
	Lactose	SNAM Progetti, Italy
	β−galactosidases	Sigma−Aldrich, UK
Saccharomyces fragilis	β−galactosidases	Sigma−Aldrich, UK
Kluyveromyces marxianus	Lactozyme	Novozymes A/S, Bagsvaerd, Denmark
Kluyveromyces sp.	Lactase NL	Enzyme Development Corporation, New York, USA
Candida pseudotropicalis	Neutral lactase	Pfizer, Milwaukee, USA
Fungi		
Aspergillus niger	Sumylact	Sumitomo Chemical, Japan
	Lactase	Valio Laboratory, Finlan
Aspergillus oryzae	Fungal lactase	Enzyme Development Corporation, New York, USA
	Biolactase	Biocon (US) Inc., Lexington, USA
	Lactase 2214C	Rohm, Darmstadt, Germany
	β−galactosidases	Sigma−Aldrich, UK

Source: references (Mahoney 1997, Roy and Gupta 2003, Jurado, Camacho *et al.* 2004)

2.2.3. Application of β−galactosidase in food

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry. (ROSENBERG 2006). Formation of galacto−oligosaccharides (GOS) during lactose

hydrolysis to favour the growth of intestinal bacterial microflora (Mahoney 1998). The β-galactosidases enzyme plays an important role not only in hydrolyzing simple disaccharides but also for transgalactosylation; synthesis of galactooligosaccharides (Hsu, Lee *et al.* 2007). The GOS belongs to prebiotics, non-digestible food ingredients that beneficially affect the host by stimulating the growth and activity of LAB in the intestine (Hill, Guarner *et al.* 2014). Improvement in the technological and sensorial characteristics of foods containing hydrolyzed lactose (Zadow 2012). High lactose concentration in nonfermented milk products such as ice cream and condensed milk can lead to excessive lactose crystallization resulting in products with sandy or gritty texture. The use of β-galactosidase enzyme prior to the condensing operation can reduce the lactose content that lactose is no longer a problem (Zadow 2012).

Table 5. Research reports on GOS production by β-galactosidases producing bacteria.

β-galactosidases	GOS and by-products	References
E. cloacae	GOS, glucose, galactose	(Lu, Xiao *et al.* 2009)
B. indica L3	Heteropolysaccharide-7	(Lu, Xiao *et al.* 2009)
P. expansum F3	GOS, glucose, galactose	(Li, Xiao *et al.* 2008, Li, Lu *et al.* 2009)
Lactobacillus ssp.	β-D-Gal-(1→6)-D-Glc, β-D-Gal-(1→6)-D-Lac β-D-Gal-(1→6)-D-Gal β-D-Gal-(1→3)-D-Lac β-D-Gal-(1→3)-D-Gal	(Splechtna, Nguyen *et al.* 2006)
B. longum BCRC 15708	Tri-, tetrasaccharides,	(Hsu, Lee *et al.*

	lactose, galactose, glucose	2007)
G. stearothermophilus KVE39	Lactosucrose, β−D galactopyranosyl−(1→3)− β−D galactopyranosyl−(1→4)−D−glucopyranoside(3'−galactosyl−lactose)	(Placier, Watzlawick *et al.* 2009)
L. reuteri	β−D−Gal−(1→6)−D−Glc, β−D−Gal−(1→6)−D−Gal, β−D−Gal−(1→3)−D−Gal, β−D−Gal−(1→6)−D−Lac, β−D−Gal−(1→3)−D−Lac,	(Splechtna, Nguyen *et al.* 2007)
L. bulgaricus	Sialyllactose, 14 other oligosaccharides	(Shene and Bravo 2007)
L. delbrueckii subsp. *bulgaricus*	Galactose, lactic acid, acetic acid, ethanol	(Shene and Bravo 2007)
B. infantis	GOS, lactose, monosaccharides	(Jung and Lee 2008)
Lactobacillus plantarum	β−D−Gal−(1→6)−D−Lac, β−D−Gal−(1→6)−D−Glc	(Iqbal, Nguyen et al. 2010)
Bacillus circulans	N−acetylactosamine, N−acetylglucosamine	(Kaftzik, Wasserscheid *et al.* 2002)

Source: references(Sako, Matsumoto *et al.* 1999)

2.3. Lactose intolerance

The interest of the dairy industry in lactose hydrolysis has been driven mainly by the fact that more than 70 % of the world's population suffer from the inability to digest lactose or lactose containing products due to the lactose intolerance symptom caused by the lack of β-galactosidases activity in the mucosa of the small intestine. Lactose is the dominant carbohydrate in milk, which is the significant natural source of lactose. Lactase located on the brush border of the small intestine can breakdown lactose into glucose and galactose and these monosaccharides are absorbed into the portal circulation. On the other hand, in people with lactose maldigestion, a portion of lactose is not digested in the small intestine; it passes into the large intestine where it is fermented by colonic microflora. The inability to completely digest lactose by the human population is termed as lactose intolerance. The symptoms of lactose intolerance are abdominal pain and distention, abdominal colic, diarrhea and nausea.

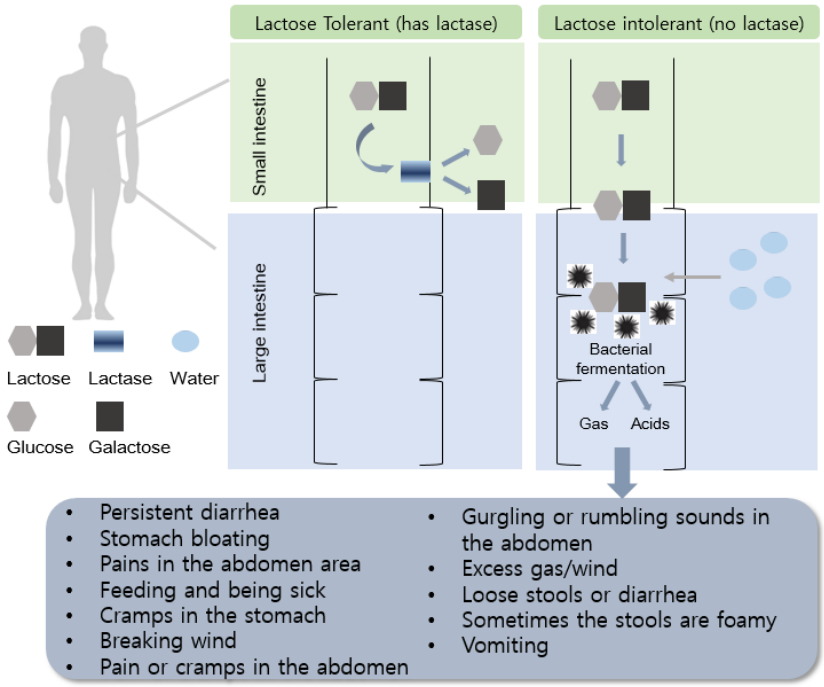

Figure 2. The role of lactase and lactose intolerance.

2.3.1. Absorption of lactose in small intestine

In normal physiological conditions, lactose is hydrolyzed by lactase also known as lactasephlorizin hydrolase and under its systemic name lactose – galactosehydrolase (EC 3.2.1.108), which is a brush-border membrane bound enzyme. Glucose and galactose are taken up by the intestinal cells and transported into the bloodstream (Fig. 2). A considerable part of glucose and most galactose is cleared by the liver after the first pass.

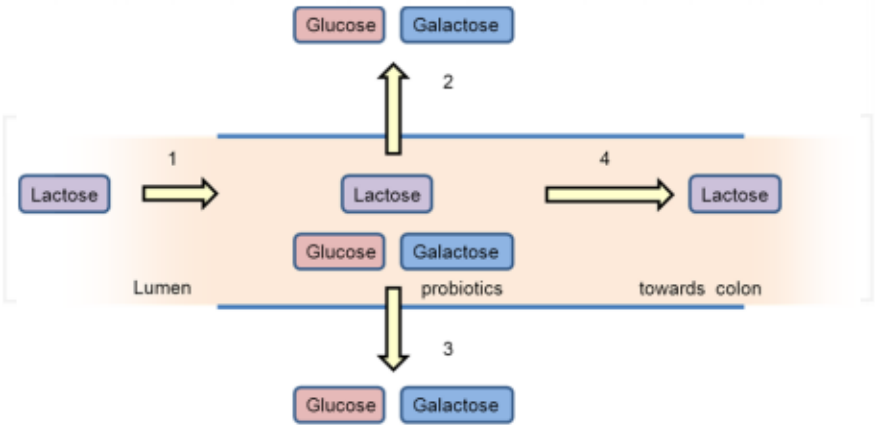

Figure 3. Small intestinal metabolism of lactose. Lactose enters the small intestine (1), lactose is then converted by lactase from the host (2) or by probiotics (3). Excess amounts of lactose spill over into the colon (4)

2.3.2. Colonic fermentation of lactose

Lactose which is spilled over into the colon can be hydrolyzed by the colonic bacterial enzyme β-galactosidase resulting in the formation of glucose and galactose. Glucose and galactose are subsequently converted into lactate as well as into the SCFA; acetate and propionate. Additionally, microbial biomass will be formed. The original substrate lactose, the intermediate products glucose and galactose, the final products can all contribute to the osmotic load in the intestine. This might lead to increased colonic transit time, altered fermentation profiles and ultimately to diarrhea.

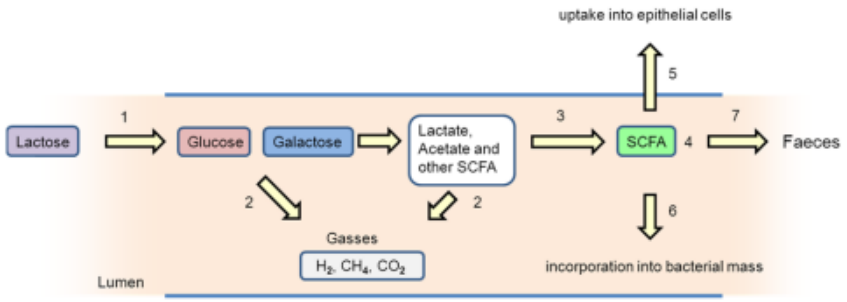

Figure 4. Colonic metabolism of lactose. Lactose enters the colon (1) and it fermented by the microbiota into glucose and galactose. Gasses such as hydrogen, methane and carbondioxide are formed (2). Lactate is also formed and converted in to short chain fatty acids (SCFA) (3,4), also in this stage gasses are formed (2). These SCFAs can be taken up by epithelial cells (5) or can be used by the microbiota (6) or excreted in the faeces (7)

2.3.3. Clinical symptoms of lactose intolerance

Symptoms of intestinal discomfort, abdominal pain and / or diarrhea can occur in case of lactose intolerance. These complaints are, however, not specific and can also be noticed in several other clinical conditions such as irritable bowel syndrome, coeliac disease, and crohn's disease. For proper treatment and correct interpretation of interventions accurate diagnosis of the underlying pathophysiology is therefore very important. The most direct diagnosis is the analysis of lactase activity. However, the enzyme activity derived from a small intestinal biopsy does not reflect the overall lactase activity in the small intestine because of the irregular character of the distribution of this enzyme. It can lead to false positive and negative estimation of the overall physiological capacity to hydrolyze lactose. Screening the

genotype of people with lactose intolerant-like symptoms can aid in the correct diagnosis of lactose intolerance. The lactase gene can contain single-nucleotide polymorphisms (SNP) in the promotor region which leads to a high capacity to digest lactose. The most common SNP C/T-13910 is found in many Northwest European people. Several methods have been developed to detect this most common SNP. For detection of all known SNPs, sequencing is the most reliable technique. Because it has a weak correlation between abdominal symptoms and lactase activity, genetics is not sufficient for a correct clinical diagnosis of adult lactose intolerance.

Chapter 3. Materials and methods

3.1. Isolation of *Bifidobacterium* spp. and *Lactobacillus* spp.

3.1.1. Bacteria isolation

Each fecal samples (1 g) was weighted to be inoculated into 9 ml of 0.85% NaCl solution added with 0.02 % L-cysteine and serially diluted in 10-fold increments. An amount of 0.1 ml of 0.85 % NaCl dilution was spread on TOS-Propionate agar with Lithium Mupriocin supplement (TOSm) plates and incubated at 37 ℃ for 48 h under anaerobic conditions used an anaerobic chamber (Coy Laboratory Products, AnnArbor, MI, USA). 0.1 ml of 0.85 % NaCl dilution was spread on MRS agar with vancomycin (20 mg/L) (MRSc) and incubated at 37 ℃ aerobically for 48 h. Single colonies with distinct morphologies from TOS-Propionate supplement agar was collected and transferred into BL broth tubes. Single colony with distinct morphologies from MRS agar with vancomycin collected and transferred into MRS broth tubes. Inoculated BL broth and MRS broth were incubated for 24 h at 37 ℃, at anaerobic and aerobic conditions, respectively.

3.1.2. Characterization of isolates

Overnight incubated MRS and BL broth of the isolates were streaked on BL and MRS agar plates, respectively. Streaked BL agar

plates were incubated at 37 ℃ anaerobically for 24 h, streaked MRS agar plates at 37 ℃ aerobically for 24 h for the catalase test. 3 % hydrogen peroxide (H_2O_2) were used for the catalase test.

Gram staining and observation of morphology All isolates were stained using gram stain method(Murray, Costilow *et al.* 1981) be observed with the microscope (Canon, USA) at a magnification of 1,000 ×.

Anaerobic As both *Bifidobacterium* and *Lactobacillus* are gram-positive, rod-shaped and catalase-negative bacteria, isolate strains selected from TOSm, were grown into MRS medium at aerobic and anaerobic condition for 48 h at 37 ℃. *Bifidobacerium* were discriminated from lactobacilli according to anaerobic requirement. All isolate strains with the physiological trait of *Bifidobacterium* and *Lactobacillus* were maintained at -80℃ in 20% glycerol for long-term storage.

3.2. Evaluation of β-galactosidase activity

3.2.1. Sample preparation

Frozen isolates were thawed for the enzyme activity assay. The culture activation and propagation were conducted by two successive transfers every 24 h into MRS broth for isolates from MRSc and BL broth for isolates from TOSm under aerobic and anaerobic conditions at 37 ℃, respectively. Each culture was centrifuged at 10,000 x g, 4 ℃ for 3 minutes and supernatant was obtained for extracellular β-galactosidase activity. For the preparation of intracellular cell-free

extracts, harvested pellets were washed twice with 1 ml of 1 x phosphate buffered saline and re-suspended. The screw-cap vials were filled with 2 g of 0.1 mm silica bead (Biospec, USA). Then 1 ml of cell suspension was added into the screw-cap vial and operated at 30 seconds with Mini-BeadBeater-16 (Biospec, USA).

3.2.2. β-galactosidase assay

Enzyme activity was determined using 0.4 ml of 5 mM p-Nitrophenyl β-D-galactopyranoside (Sigma, USA) with 0.1 ml of samples incubated at 37 ℃ for 20 minutes. The reaction was terminated by 0.5 ml of Na_2CO_3 (Sigma, USA) (Griffith and Wolf 2002). Enzyme activity was measured by spectrostar nano (bmg labtech, Germany) at 405 nm and compared with standard curve measured with 400, 200, 100, 50, 25 and 12.5 mM of 4-nitrophenolsolution (Sigma-Aldrich, USA). *Lactobacillus delbrueckii* subsp. *bulgaricus* KCCM 35463 was purchased from Korean culture center of microorganisms (KCCM) and used as control.

3.3. Identification

3.3.1. Detection of fructose-6-phosphate phosphoketolase gene (F6PPK)

Biochemical tests for the identification of members of the genus Bifidobacterium are now largely superseded by the use of the genus-specific PCR primers described by Kok and colleagues or Kaufman and colleagues (Kok, De Waal *et al.* 1996). Bifidobacteria were

selected by a PCR assay using the Bifidobacterium genus-specific primer pair (Forward-F6PPK: 5'- TGGCAGTCCAACAAGCTC -3'), Reverse-F6PPK: 5'- TAGGAGCTCCAGATGCCGTG -3') with AccuPowerPCR PreMix (Bioneer, Korea) using colony PCR method. The colony on each plate was picked up and suspended 0.04 ml of sterile distilled water and thoroughly suspended for using as Template DNA. Each μL of templates DNA and primers were added into AccPowerPCR PreMix tubes. 17 μL of distilled water were added into the tubes to a total volume of 0.02 μL. Amplification was carried out in a C1000 Touch thermal cycler (Bio-Rad, USA). The amplification consisted of one cycle at 95 ℃ for 3 min, 21 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 1 min, and a final cycle of 72 ℃ for 5 min. Amplified products were run on a 2.0 % agarose gel, and visualized under Universal Hood III (Bio-Rad) with Image Lab Software (Bio-Rad, USA).

Table 6. Oligonucleotide sequences.

Primer	Description	Sequence (5'→3')
GP-109	XFP-F1 :	TGGCAGTCCAACAAGCTC
GP-111	XFP-R1 :	TAGGAGCTCCAGATGCCGTG'

Table 7. Composition of AccPowerPCR PreMix.

Component	Reaction size (20 ml)
Top DNA polymerase	1 U
dNTP (dATP, dCTP, dGTP, dTTP,)	250 mM, respectively
Reaction Buffer with 1.5mM MgCl2	1X

Table 8. PCR condition for genus-specific PCR.

	Temperature(℃)	Time	
Initial denaturation	95	03:00	
Denaturation	95	00:30	
Annealing	58	0:30	20 cycle
Extension	72	1:00	
Final extension	72	05:00	

3.3.2. 16 S rRNA sequencing

The amplification of 16S rRNA gene of isolate strains was carried out using PCR. The PCR products were sequenced by Macrogen and Biofact Corporation (Korea). The DNA sequences were analyzed with the Internet BLAST Gene database (http://www.ncbi.nlm.nih.gov).

Table 9. Primers used for isolate strain identification by 16 S rRNA sequencing.

Primer name	Sequences	TM (℃)	GC (%)
27F-M	5'-AGA GTT TGA TCM TGG CTC AG-3'	55.3	47
1492R-M	5'-TAC GGY TAC CTT GTT ACG ACT T-3'	56.8	42
518F	5'-CCA GCA GCC GCG GTA ATA C-3'	63	63
805R	5'-GAC TAC CAG GGT ATC TAA TC-3'	53	45

3.4. Characterization of probiotic properties

3.4.1. Enzymatic profiles

Biochemical characteristics of isolates were determined using a commercial system, API ZYM (Bio-Merieux, France) according to manufacturer' s instructions with minor modification. Overnight cultured isolated were washed two times with 0.85 % NaCl. The initial suspension was adjusted at the OD_{600} of 1, and then inoculated into the test kits and incubated. The strips were incubated at 37 ℃ for 4 h and reagents added according to the manufacturer' s instructions. Enzymatic activity was recorded as positive if a score of 1 or greater was obtained after assessment of the color intensity using the

manufacturer’s color chart.

3.4.2. Safety assessment

Antibiotic susceptibility Antibiotic resistances of the isolates were tested with minimal inhibitory concentration (MIC) according to laboratory standards institute guidelines (Neville and O'Toole 2010). Ampicillin, kanamycin, streptomycin, erythromycin, tetracycline, and chloramphenicol were the antibiotics tested. This procedure involved preparing two-fold dilutions of antibiotics prepared in concentrations ranging between 2 and 512 μg /ml. Next, 100 ㎖ of each concentration of antibiotics were added in each well of 96-well microtiter plates, and 100 ㎖ of a double-enriched LSM media (90 % IsoSensitest and 10 % MRS) with 10^8 CFU / ml of isolates were inoculated in each well for final concentrations from 1 to 256 μg/ml. The microbiological cut-off values (μg/L) of antibiotics were derived from the European Food Safety Authority guidelines (EFSA, 2012).

Hemolytic activity The haemolytic activity of isolates was tested on brain heart infusion agar supplemented with 1 % (w/v) glucose, 0.03 % (w/v) L-arginine and 5 % (v/v) sheep blood (Birri, Brede *et al.* 2013). The isolates were streaked on the agar, followed by anaerobic incubation at 37 ℃ for 24 h. *Enterococcus faecalis* ATCC 29212 were used as positive control. Haemolytic activity was detected by the appearance of clear zone.

3.4.3. Functional assessment

Resistance to low pH Bacterial cells from overnight (18 h) cultures were harvested by centrifugation (10,000 × g for 3 min at 4 ℃). The cell pellet was washed and resuspended with 1 × PBS buffer solution. Inoculum was added in 12.25 ml of MRS broth containing 0.05 % L-cysteine, and measured optical density of 0.02 at 600 nm, adjusted pH at 2, 2.5, 3 and 3.5 with 0.1 N HCl. The viable CFU were counted in a colony counter. Samples were incubated at 37 ℃ for 2 h. After incubation, 1 ml of sample was withdrawn and serially diluted with 1 × PBS buffer solution, spread on MRS agar plates containing 0.05 % L-cysteine. The spread plates were incubated for 24 h at 37 ℃. The colonies grown on MRS agar was calculated to investigate survivability (Hyronimus, Le Marrec *et al.* 2000).

Bile resistance MRS broths containing 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 % (w/v) bile salt were prepared for bile tolerance. Next, 100 ㎖ of each concentration of bile salt was added in each well of 96-well microtiter plates, and 100 ㎖ of a MRS+L-cysteine (0.05 %) media with 10^8 CFU / ml of isolates were inoculated in each well for final concentrations from 0.1 to 0.8 mg/ml. The plates were incubated at 37 ℃ anaerobically for 48 h (Hyronimus, Le Marrec et al. 2000).

Bile salt hydrolase activity Bile salt hydrolase (BSH) activity of the cultures was detected using well diffusion method. 0.07 ml of overnight cultures were placed in 6-mm-diameter wells that had been cut in MRS agar plates containing 0.5% (w/v) sodium salt of taurodeoxycholic acid (Sigma, USA) and 0.37 g/L $CaCl_2$. Plates were

incubated at 37℃ for 3 days in an anaerobic chamber. Colonies with the diameter of the precipitation zones were measured (Du Toit, Franz *et al.* 1998).

3.5. Genomic comparison of *Bifidobacterium* spp.

3.5.1. Complete genome sequence

The genome of HT10-2 was sequenced by the Illumina Hiseq 4000 (Illumina, USA) from Macrogen (Korea). All the short reads were assembled using SOAPdenovo. The gaps of two contigs emerging during the scaffolding process by SOAPdenovo was closed, using the abundant pair relationships. Then a draft genome with one scaffolds was achieved. After whole genome is analyzed, the locations of protein coding sequences, tRNA genes, and rRNA genes were identified. Then their functions were annotated. Prokka is a pipeline that performs series of process automatically. At the end of the pipeline, Prokka gives gene bank of kenya (GBK) file as well as various types of files.

3.5.2. Quantitative real time PCR (qRT-PCR) analysis

We isolated total mRNA from 18 h cultured broths with HT 10-2 and DSM 10140, respectively, using TRIzol (TRI reagent). First-strand cDNA synthesis was carried out using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (TOYOBO, Japan) according to instructions of manufacturer. 0.5 ㎍ of total RNA and 2

μg of 4x DN Master Mix were put in 5.5 μg nuclease-free water. After Incubation at 37 ° C for 5 min., it was mixed with 2 μg 5x RT Master Mix II. Amplification was carried out in a C1000 Touch thermal cycler (Bio-Rad, USA). The reaction was incubated for 15 min at 37 ° C, and at 50 ° C for 5 min. Finally, for 5 min at 98 ° C. Synthesized cDNA was stored at -70 ° C for the experiment. Relative expression of *bgaA, ebgA, lacZ*, *bga III* and *tuf* mRNA was detected by real-time PCR with TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) kit (Ezynomics, Korea). Real-time PCRs were initiated by the denaturation step of 5 min at 95 ℃, followed by 40 cycles of amplification, which were performed according to the following thermal cycling protocol, denaturation for 5 s at 95 ℃, and annealing and extension for 1 min at 57 ℃. tuf gene was used as an endogenous control to normalize the expression of target transcripts. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Bustin and Mueller 2005).

Table 10. Sequences and information of primers used for Quantitative Real-Time Polymerase Chain reaction.

Genes	Primer	Sequences of primers	Product length	Tm (℃)	GC (%)	Reference
bgaA	bgA-F	CCTATGGGTTACAGCCTGCG	178	62.5	60	This study
	bgA-R	TGTGCCTGCGTTTCGAGC		62.5	61	
ebgA	ebgA-F	GGCCATTCTCGACCGCAT	180	62.1	61	This study
	ebgA-R	TCTTCGACATACCGCGCG		62.1	61	
lacZ	lacZ-F	CACAACATCAATGCCGTGCG	179	62.1	55	This study
	lacZ-R	TGCCAACGCACGTCGT'		62	62	
bga III	bga3-F	GTCGCAGAACACGCTCGA	177	62.3	61	This study
	bga3-R	ACTGCGGAGGTGGATTGCT		63.5	57	
tuf	tuf-R	CGGCAAGCTGCCGATCAAC	178	62.4	55	Sheu *et al., 2010*
	tuf-F	TCACGACAAGTGGGTTGCCA		63.5	63	

3.6. Characterization of β-galactosidase from HT 10-2

3.6.1. β-galactosidase isolation

Crude extract preparation *Bifidobacterium animalis* subsp. *lactis* HT10-2 was grown in MRS medium with 0.03 % (w / v) L-cystein-HCL (Sigma, USA) and grown under anaerobic conditions at 37℃ for 18 h and subcultured two times to be used as the inoculum. 1 % of an aliquot (20 ml) of the inoculum were transferred to Erlenmeyer flasks containing of 2 L of MRS medium with 0.05 % (w / v) L-cystein-HCL. The inoculated flask was cultured at 37℃. After 18 h, the cells were harvested by centrifugation at 4,000 × g for 10 min. The pellet was washed with 50mM sodium phosphate buffer (pH 7.0), centrifuged and the cells were used for isolation of β-galactosidase.

For the preparation of intracellular cell-free extracts, cells were broken with a bead beater as described above. Cellular debris was separated from the crude enzyme by centrifugation at 15,000 rpm for 15 minutes. Supernatant was collected and termed as cell free extract which was stored at 4℃ for further purification steps.

Ammonium sulfate precipitation The crude extract of *Bifidobacterium animalis* subsp. *lactis* HT10-2 was precipitated at 4℃ using ammonium sulfate saturation (30-90%). The resulting precipitates were suspended in 50 mM sodium phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer at 4 ℃. The ammonium sulfate fractionate (ASF) having the highest specific β-galactosidase activity was further used for purification (Nunoura, Ohdan *et al.* 1996).

DEAE-Sepharose Fast flow ion exchange chromatogram The dialyzed enzyme preparation was applied to DEAE-Sepharose (1.5 × 14 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was washed with the same buffer to remove unbound protein. A gradient of linearly increasing salt concentration was then applied to elute the protein from the column using 1.0M NaCl (Yoshimura, Matsushima *et al.* 1987).

Gel electrophoresis Purified enzyme was carried out on 10 × 8 cm, 1.5 mm thick, 10 well, 12% separating gel containing 30 % acrylamide. The electrode chamber buffer consisted of 0.025M Tris-base and 0.192M of glycine with pH 8.3. Loading sample preparation was prepared as below; 1 parte of 4× Laemmli sample buffer was diluted

with 3 parts sample and boiled. After polymerizing the stacking gel for 1 hour, 0.015 ml of loading sample was loaded into each well. The gels were run 80 V until each sample entered the running gel, after which the voltage was increased to 120 V. This voltage maintained for 80 min. The proteins were stained with Coomassie brilliant blue R-250 for 2 hours to detect protein bands and rinsed twice in distilled water. Destain solution was added to cover the gel (Groten 1997).

Table 11. Composition of running and stacking gel.

Running gel	Total 10ml	Stacking gel	Total 3ml
Solution components (10 %)		Solution components (5%)	
H_2O	4.0	H_2O	2.1
30 % Acrylamide mix	3.3	30 % Acrylamide mix	0.5
1.5 M This (pH 8.8)	2.5	0.5M This (pH 6.8)	0.38
10 % SDS	0.1	10% SDS	0.03
10 % Ammonium persulfate	0.1	10% Ammonium persulfate	0.03
TEMED	0.004	TEMED	0.003

3.6.2. Effects of pH and Temperature

The optimal pH was assessed in pH range of 5.0-7.5 with 50mM sodium phosphate buffer (pH 5.0-7.5) at 37 ℃. The effect of temperature was evaluated at 30 ℃ to 60 ℃ in 50 mM sodium phosphate buffer. The thermal stability of the purified enzyme

solution was evaluated by incubating at different temperature (37 ℃, 45 ℃, and 50 ℃) for 1 h and then enzyme activity assay at 37 ℃ (Han, Youn *et al.* 2014).

3.6.3. Effects of Metal ions

The effects of metal ions and on β-galactosidase activity were determined in the presence of various metal ion (1mM), including KCl, NaCl, Na_2SO_4, $MgSO_4$, $CuSO_4$, $FeSO_4$, $CaCl_2$, $MnSO_4$ and $MgCl_2$. The relative activity of the enzyme was compared with the activity measured in 50mM sodium phosphate buffer (pH 7.0) at 37 ℃ for 30 min (Han, Youn *et al.* 2014).

3.7. Statistical analysis

Data were analyzed using the one-way anova to evaluate differences in discrete variables between the samples.

Chapter 4. Results

4.1. Selection of β-galactosidase producing LAB

4.1.1. β-galactosidase assay

Fecal samples were collected from 81 Korean infants for potential *bifidobacterium* and *lactobacillus* probiotics isolation. 566 distinct isolates (195 bifidobacteria and 371 lactobacilli) were obtained by culture dependent methods using MRSc and TOSm. All isolates were also analyzed by the physiological traits of lactic acid bacteria; all isolates were found to be LAB. In this study, 563 isolates were tested for capability of β-galactosidase production. Fermentation was carried out in 5 ml of MRS broth containing 0.02 % L-cysteine for 24 h. Other fermentation parameters such as temperature (37 ℃), initial pH (6.5) and inoculum size (1 %) were fixed. 10 isolate strains were selected on the basis of high yield production of β-galactosidase by colorimetric assay using p-Nitrophenyl (PNP) β-D-galactopyranoside. The results of these experiments have shown that, 10 isolates have produced a significant amount of β-galactosidase (Fig 5). Among them, HT 10-2 (3,583.224 Unit/ml) is the most efficient β-galactosidase producer. HT 10-2 was significantly higher than the activities found in the other β-galactosidase-producing isolates.

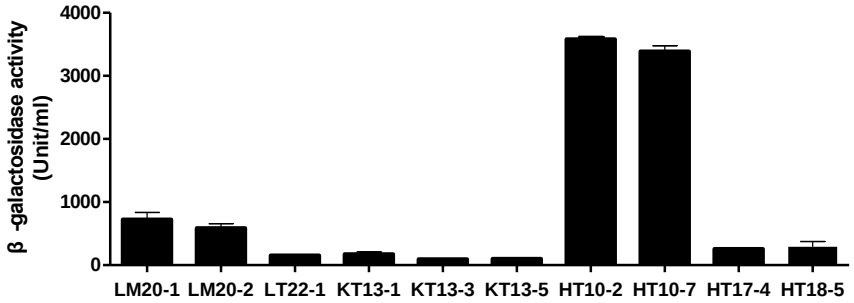

Figure 5. β−galactosidase activities from isolates. The data represent the means and standard deviation.

4.1.2. Identification

Bifidobacteria ferment hexoses by a fructose−6−phosphate phosphoketolase (F6PPK) shunt and are the only intestinal bacteria known to utilize this fermentation pathway. Demonstration of F6PPK activity in cellular extracts has been a useful method for differentiating *bifidobacteria* from morphologically similar bacteria. *Bifidobacteria* were selected by a PCR assay using genus−specific primer (GP 109, GP 111). A distinct product of 950 bp was observed on agarose gel electrophoresis for KT 13−1, KT 13−3, KT 13−5, HT 10−2, HT 10−7, HT 17−4. HT 18−5 and DSM 10140, while not detected for LM 20−1, LM 20−2, LT 22−1 and *Lactobacillus rhamnosus* ATCC 53103 (Fig 6).

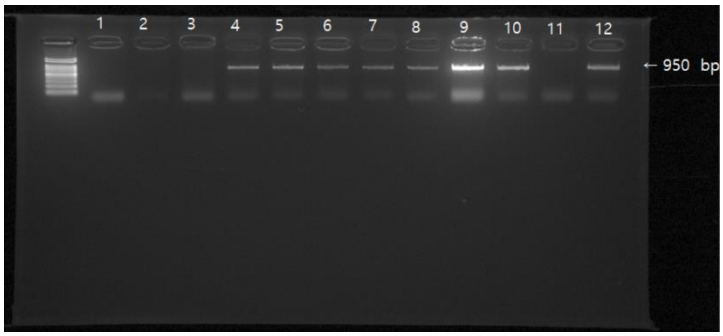

Figure 6. Agarose gel electrophoresis of PCR products obtained by PCR. Primer was designed to detect *f6ppk* Gene. From line 4 to 10 and 12, bands were detected. They were expected as *Bifidobacterium* spp. by 16 S rRNA sequencing.

1: LM 20-1, 2: LM 20-2, 3: LT 22-1, 4: KT 13-1, 5: KT 13-3, 6: KT 13-5, 7: HT 10-2, 8: HT 10-7, 9: HT 17-4, 10: HT 18-5, 11: *Lactobacillus rhamnosus* ATCC 53103, 12: *Bifidobacterium animalis* subsp. *lactis* HT 10-2

Three isolate strains, LM 20-1, LM 20-2 and LT 22-1, were identified as *Lactobacillus* spp. Also, the others, seven isolates, were identified as *Bifidobacterium* spp. by 16s rRNA sequence. It was consist with the results based on physiological traits and *xfp* gene PCR of isolate strains.

Table 12. Identification and characterization of isolates.

No	Strain	16s rRNA sequence analysis (Closest known species)	Catalase test	Gram staining	Morphology	Culture condition[a]		Curd formation[b]	XFP[c]
			(+/−)	(+/−)		Anaerobic	Aerobic	(+/−)	(+/−)
1	LM20−1	*L. reuteri*	−	+	Rod	+	+	+	−
2	LM20−2	*L. reuteri*	−	+	Rod	+	+	+	−
3	HT10−2	*B. animalis* subsp. *lactis*	−	+	Rod	+	−	+	+
4	HT10−7	*B. animalis* subsp. *lactis*	−	+	Rod	+	−	+	+
5	HT18−5	*B. bifidum*	−	+	Rod	+	−	+	+
6	HT17−4	*B. bifidum*	−	+	Rod	+	−	+	+
7	KT13−1	*B. longum*	−	+	Rod	+	−	+	+
8	LT22−1	*L. rhamnosus*	−	+	Rod	+	+	+	−
9	KT13−5	*B. animalis* subsp. *lactis*	−	+	Rod	+	−	+	+
10	KT13−3	*B. animalis* subsp. *lactis*	−	+	Rod	+	−	+	+

[a] Each isolate was incubated both aerobically and anaerobically. LM20−1, LM20−2 and LT22−1 were able to grow at both condition, other strains have oxygen sensitivity.
[b] Lactose in milk can be converted into glucose and galactose with formation of curd by LAB, which are potential to be yogurt starter
[C] XFP (Fructose 6−phosphate phosphoketolase) gene specific PCR

4.1.3. Enzymatic profiles

All strains showed strong β-galactosidase activity and KT 13-3, KT 13-5, HT 10-2 and HT 10-7, identified as *bifidobacteriaum amimalis* supsp. *lactis*, were also have β-glucosidase activity. Except for HT 18-5, α-galactosidase activity was observed with all isolates.

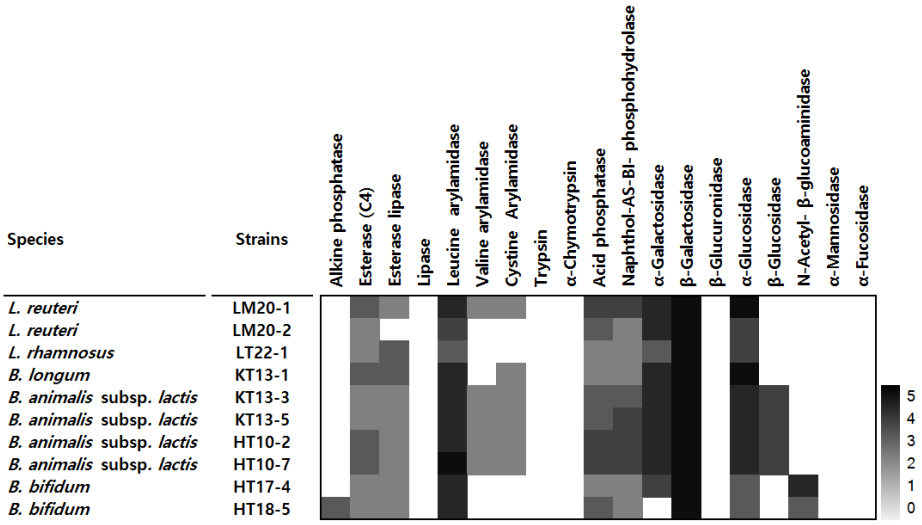

Figure 7. Enzymatic profiling of isolated strains. The colorimetric intensity is indicated by color gradients. White represents no reaction, darker color represent high activity (API ZYM kit, Korea)

4.1.4. Safety assessment

Antibiotic selection and determination of resistance or sensitivity of isolates against chosen antibiotics were performed according to the microbiololgical breakpoints. All bifidobateria strains except HT 18-5 were sensitive to all antibiotics, on the other hand, resistance to erythromycin was detected in strains HT18-5. Regarding

Lactobacillus strains, LM 20-1 and LM 20-2 were resistant to ampicillin, kanamycin and tetracycline and LT 22-1 showed resistance to kanamycin and streptomycin. None of the isolates exhibited hemolytic activity.

Table 13. Hemolytic activity of isolates.

Species	Strains	Hemolytic activity (+/-)
L. reuteri	LM20-1	-
L. reuteri	LM20-2	-
L. rhamnosus	LT22-1	-
B. longum	KT13-1	-
B. animalis subsp. *lactis*	KT13-3	-
B. animalis subsp. *lactis*	KT13-5	-
B. animalis subsp. *lactis*	HT10-2	-
B. animalis subsp. *lactis*	HT10-7	-
B. bifidum	HT17-4	-
B. bifidum	HT18-5	-
E. feacalis[a]	ATCC29212	+

[a] *Enterococcus faecalis* ATCC 29212 used as positive control

Table 14. Minimum inhibitory concentrations (μg/ml) of antibiotics to selected *Lactobacillus* spp. and *Bifidobacterium* spp..

Species	Strains	AMP	KAN	STR	ERY	TET	CHL
L. reuteri	LM20-1	>128	>128	128	≤1	>128	8
L. reuteri	LM20-2	>128	>128	128	≤1	>128	8
L. rhamnosus	LT22-1	≤1	>128	>128	≤1	64	16
B. longum	KT13-1	≤1	n.r.	16	≤1	2	8
B. animalis subsp. *lactis*	KT13-3	≤1	n.r.	128	≤1	16	8
B. animalis subsp. *lactis*	KT13-5	≤1	n.r.	128	≤1	32	4
B. animalis subsp. *lactis*	HT10-2	≤1	n.r.	64	≤1	16	2
B. animalis subsp. *lactis*	HT10-7	≤1	n.r.	64	≤1	16	2
B. bifidum	HT17-4	≤1	n.r.	64	≤1	32	4
B. bifidum	HT18-5	≤1	n.r.	32	>128	2	4
Suggested breakpoint in accordance to the European Food Safety Authority (EFSA)							
L. reuteri		2	64	64	1	16	4
L. rhamnosus		4	64	32	1	8	4
Bifidobacterium spp.		2	n.r.	128	1	8	4

AMP, KAN, STR, ERY, TET and *CHL* refer to ampicillin, kanamycin, streptomycin, erythromycin, tetracycline and chloramphenicol, respectively

n.r. = not required

4.1.5. Functional assessment

All 10 selected isolates were tested for survival in acid and bile salt environment. Survival following 2 h of incubation at pH values from 2.0 to 3.5 was observed for the strains, but KT 13-1, KT 13-3, KT 13-5, HT 17-4 and HT 18-5 showed lower survivability under pH 2 compared to other strains. HT 17-4 and HT 18-5 were even unable to grow under condition of pH 2.5, 3.0 and 3.5. *Lactobacilli*, LM 20-1, LM 20-2 and LT 22-1, were able to survive at pH 2.0, they showed strong survival rate comparing to the LGG positive control. The results said that HT 10-2 and HT 10-7 have strong resistance to acid stress. Growth of isolate strains was examined in MRS broth containing oxgall concentration from 0.1 to 0.8 %. Tolerance of bile salts seems to be an important character for the probiotic strain to grow and survive in the upper small intestine, where bile salt hydrolase activity of the probiotic strain may play a role in the enterohepatic cycle. All strains, except for KT 13-1, HT 17-4 and HT 18-5, were able to grow in MRS broth containing oxgall concentration from 0.1 to 0.8 %. BSH activity has been detected in *Lactobacillus* and *Bifidobacterium* spp. *Lactobacilli* and *bifidobacteria* are routinely used as probiotic strain. *Bifidobacterium animalis* subsp. *lactis* (KT 13-1, KT 13-3, KT 13-5, and positive control DSM 10140) exhibited high BSH ability, observed by precipitation zones in the BSH agar plate. However, other strains were unable to hydrolysis bile salt.

Table 15. Acid tolerance of selected *Lactobacillus* spp. and *Bifidobacterium* spp.

Species	Isolates	Acid tolerance[c] (%)							
		pH 2		pH2.5		pH3		pH3.5	
		0 h	2 h	0 h	2 h	0 h	2 h	0 h	2 h
Lactobacillus reuteri	LM20-1	100	92.21	100	100	100	100	100	100
Lactobacillus reuteri	LM20-2	100	94.67	100	98.67	100	100	100	100
Lactobacillus rhamnosus	LT22-1	100	88.61	100	100	100	100	100	98.75
B. longum	KT13-1	100	65.28	100	98.59	100	98.61	100	98.59
B. animalis subsp. *lactis*	KT13-3	100	63.89	100	100	100	100	100	100
B. animalis subsp. *lactis*	KT13-5	100	31.94	100	98.61	100	100	100	100
B. animalis subsp. *lactis*	HT10-2	100	92.75	100	97.10	100	97.10	100	98.55
B. animalis subsp. *lactis*	HT10-7	100	97.18	100	100	100	100	100	100
B. bifidum	HT17-4	100	52.11	100	53.52	100	53.52	100	53.52
B. bifidum	HT18-5	100	45.07	100	47.89	100	45.07	100	45.07
L. rhamnosus	ATCC 53103[a]	100	-	100	97.14	100	98.57	100	100
B. animalis subsp. *lactis*	DSM 10140[b]	100	92.42	100	98.51	100	100	100	97.01

[a]L. rhamnosus ATCC 53103

[b]*B. animalis* subsp. *lactis* DSM 10140 were used as reference cultures

[c]Cell survival at pH range from 2.0 to 3.5.

Table 16. Bile tolerance and BSH activity of selected *Lactobacillus* spp. and *Bifidobacterium* spp..

Species	Isolates	Bile tolerance (%)	BSH[d] Activity (+/−)
Lactobacillus reuteri	LM20−1	> 0.8	−
Lactobacillus reuteri	LM20−2	> 0.8	−
Lactobacillus rhamnosus	LT22−1	> 0.8	−
B. longum	KT13−1	< 0.1	−
B. animalis subsp. *lactis*	KT13−3	> 0.8	+
B. animalis subsp. *lactis*	KT13−5	> 0.8	+
B. animalis subsp. *lactis*	HT10−2	> 0.8	+
B. animalis subsp. *lactis*	HT10−7	> 0.8	+
B. bifidum	HT17−4	< 0.1	−
B. bifidum	HT18−5	< 0.1	−
L. rhamnosus	ATCC 53103[a]	> 0.8	−
B. animalis subsp. *lactis*	DSM 10140[b]	> 0.8	+

[a]L. rhamnosus ATCC 53103, [b]B. animalis subsp. lactis DSM10140 were used as reference cultureS

[c]Minimum inhibitory concentrations of oxgall (DifcoTM, USA)

[d]Bile salt hydrolase activity using (sodium salt of taurodeoxycholic acid, Sigma, USA)

4.2. Comparative analysis of HT 10-2 and DSM 10140

4.2.1. β-galactosidase activities

We compared β-galactosidase activity between HT 10-2 and DSM 10140 using the cololrimetric analysis. HT 10-2 showed relatively higher β-galactosidase activity than DSM 10140 amounting to approximately 3 times (Fig 8).

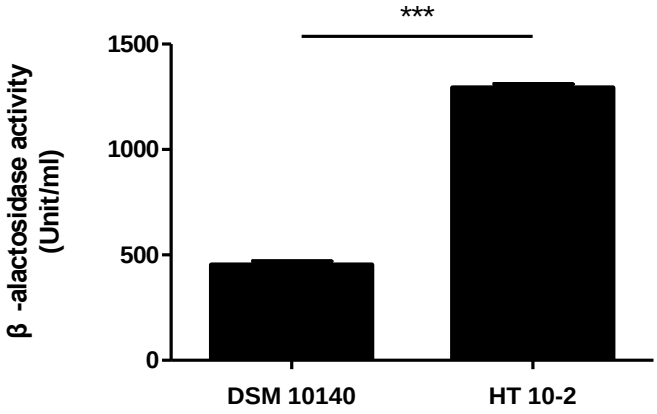

Figure 8. β-galactosidase activities of HT 10-2 and DSM 10140 in MRS media. Data were analyzed using the student's t test. Significance is indicated as follows: ***p ≤ 0.001.

4.2.2. Complete genome sequence

Quality control were confirmed by FastQC after raw sequence data were obtained from high throughput sequencing pipelines. They showed more than 90 % of bases with base quality. Two contigs were assembled, and then the gaps from each contig were closed again by SOAdenovo. Finally, we obtained one scaffold, whose size is

1,923,647 bp. Determination of the complete genome sequence of HT 10-2 reveals a single circular chromosome of 1,923,647 bp with 1,613 of predicted protein- encoding genes, 1,553 of coding sequence (CDS), 7 of rRNA operons, and 52 of tRNA genes.

Table 17. Summary of genome annotation.

Strain	Number of Contigs	Contigs sum	N50	Gene	CDS	GC contents (%)	ANIb (%)
DSM 10140	1	1.938.483	1.938.483	1,658	1,572	60.50	
HT 10-2	2	1,923,637	1,861,689	1,613	1,553	60.49	99.61

[a]Number of contigs: The number of contigs identified

[b]Contigs sume: The total number of bases in the contigs

[c]N50: Half of all bases reside in contigs of this size or longer

[d]CDS: Coding sequence

[e]GC contents: The percentage of guanine-cytosine base pairs

[f]ANIb: Average nucleotide identity (ANI) based on blast to reference strain, DSM 10140

HT 10-2 was identified as *Bifidobacterium animalis*. subsp. *lactis* since their sequence showed the highest pairwise similarity of 99.61 % for *Bifidobacterium animalis*. subsp. *lactis* DSM 10140. The phylogenetic tree of HT 10-2 and similar bacteria was constructed as shown in Figure 8. Both strains harbor four genes coding β-galactosidase known as *bgaA*, *ebgA*, *lacZ* and *beta-galIII*.

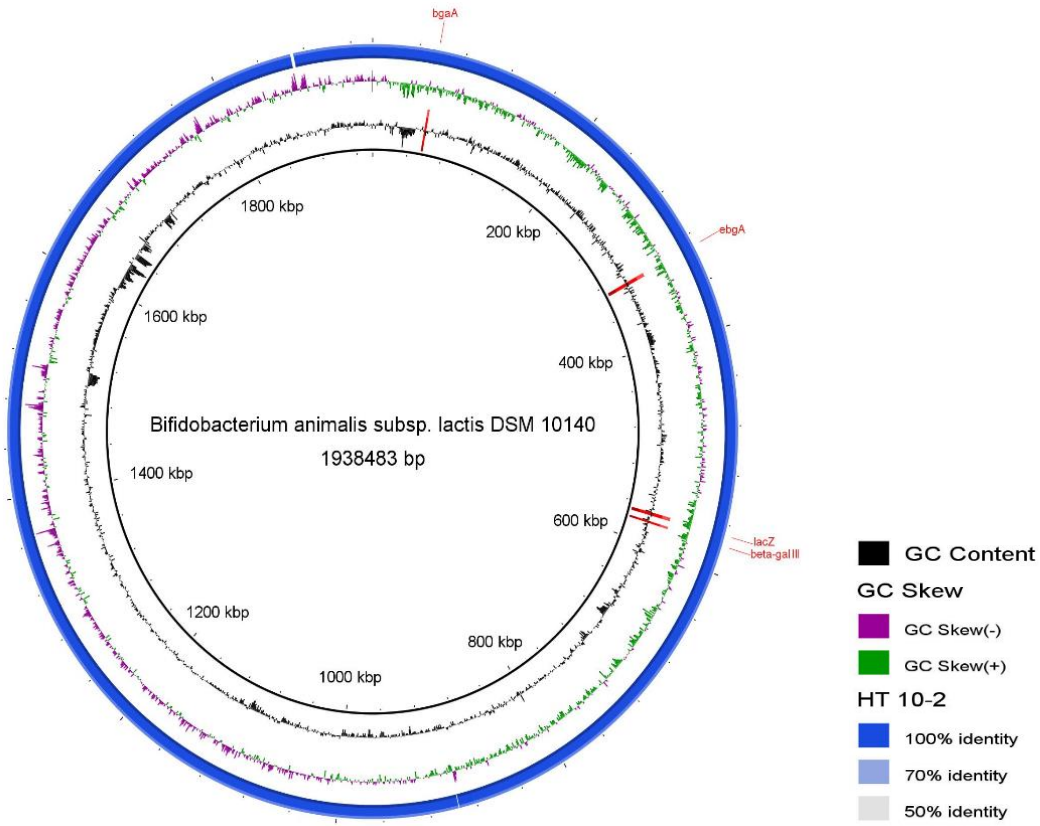

Figure 9. Complete genome sequence analysis and comparison with DSM 10140. BRIG images showing genomic regions shared by DSM 10140 and HT 10-2. The innermost rings show GC content (black) and GC skew (purple/green). The blue ring show BLAST comparison of HT 10-2 against DSM 10140.

4.2.3. Alignment of β-galactosidase coding genes

We aligned the sequences of genes coding β-galactosidase from HT 10-2 comparing to the genes from DSM 10140. There was no difference observed (Fig 10).

We investigated that promoter region of their *lacI* genes, regulatory genes of the *lac* operon, showed slightly differences (Fig 11). Reduction of two necleotide was observed in DSM 10140 compared to HT 10-2.

4.2.4. qRT-PCR analysis

We confirmed that *lacZ* is the major gene coding for β-galactosidase according to relatively high mRNA expression.

⊿Ct values of each genes from HT 10-2 indicated that *lacZ* codes the higher amount of β-galactosidase than others.

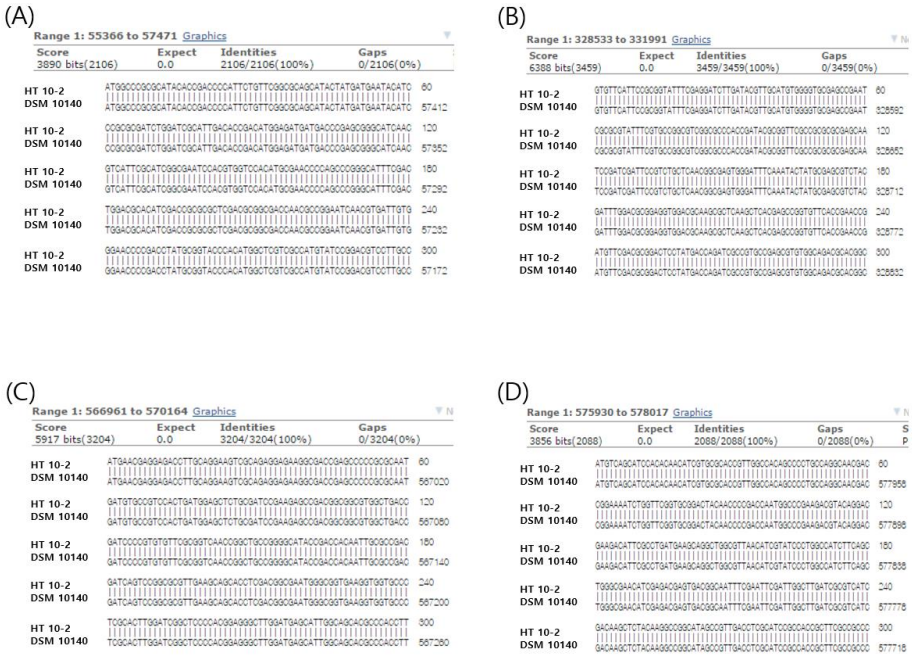

Figure 10. Comparison of nucleotide sequences for β-galactosidase coding genes. *bgaA* (A), *ebgA* (B), *lacZ* (C) and *beta-galIII* (D) of HT 10-2 and DSM 10140.

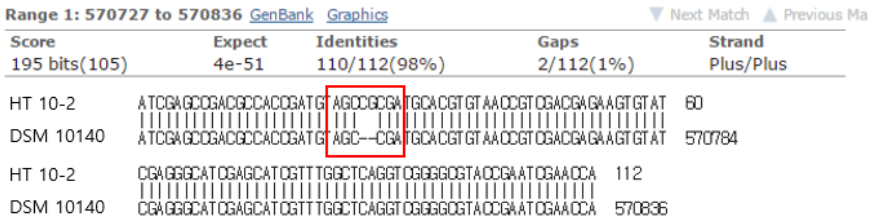

Figure 11. Comparison of nucleotide sequences for promoter region of *lacI* of HT 10-2 and DSM 10140.

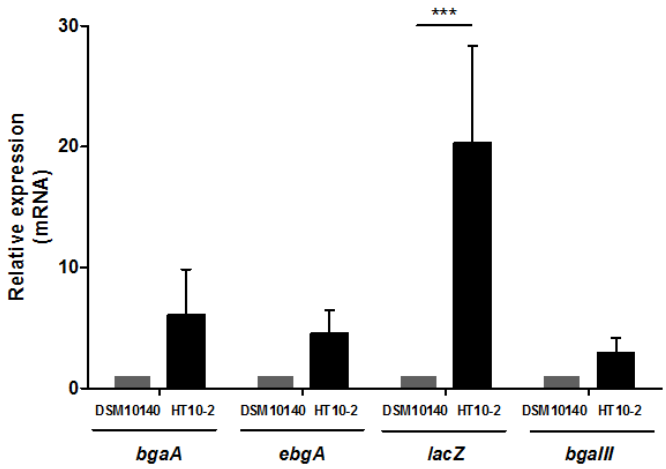

Figure 12. Relative expression of β-galactosidase with DSM 10140 and HT 10-2. The data represent the means and standard standard deviation of three replicates. Data were analyzed using the one-way anova. Significance is indicated as follows: *p < 0.05; **p < 0.01, and ***p < 0.001.

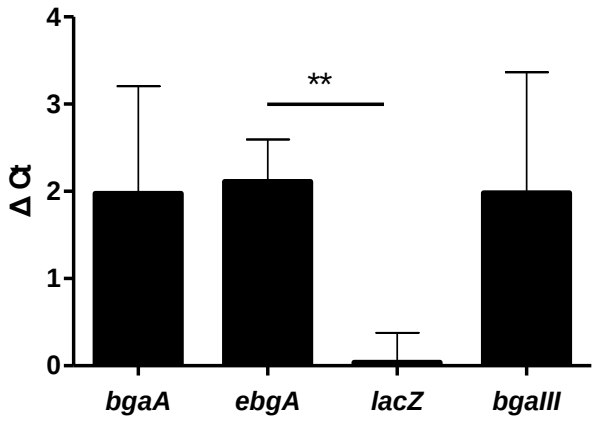

Figure 13. mRNA expression from the genes coding β-galactosidase. The data represent the means and standard standard deviation of three replicates. Data were analyzed using the one-way anova. Significance is indicated as follows: *p < 0.05; **p < 0.01, and ***p < 0.001.

4.3. Characterization of β-galactosidase from HT 10-2

The purification of β-galactosidase from *B. animalis* subsp. *lactis* HT10-2 achieved in one step summarized. This enzyme was purified by applying ammonium sulfate precipitation method and ion chromatography.

4.3.1. Ammonium sulfate precipitation

The crude extract with β-galactosidase activity was precipitated by ammonium sulfate added slowly for an hour on ice with a constant stirring using a magnetic stirrer. The highest enzyme activity was shown with 70 % ammonium sulfate saturation. However, it was purified to 11.4 fold with a yield 48.67 % using 40 % ammonium sulfate saturation due to the fact that the highest specific activity (34786.43 unit/mg) was observed from 40 % ammonium sulfate precipitation. It is well known that high molecule proteins have a tendency to be precipitated in low concentration of ammonium sulfate.

Table 18. Effect of varying saturations of ammonium sulfate on the degree of purification.

	Saturation % of ammonium sulfate							
	0	30	40	50	60	70	80	90
Enzyme activity (unit/ml)	8625.00	149.34	4198.03	4259.21	6219.74	8588.16	8159.21	6751.32
Protein content (mg/ml)	2.83	0.01	0.12	0.19	0.57	1.38	1.44	1.59
Specific activity (unit/mg)	3051.15	20346.34	34786.43	22022.81	10973.42	6228.72	5655.12	4254.67
Purification (fold)	1	6.66	11.40	7.22	3.60	2.04	1.85	1.39
Yield (%)	100.00	1.73	48.67	49.38	72.11	99.57	94.60	78.28

4.3.2. DEAE-Sepharose Fast flow ion exchange chromatogram

The dialyzed enzyme was applied to DEAE-Sepharose Fast flow ion exchange column. The bound proteins were then eluted with NaCl (0.1-1 M) solution. The elution profile indicated that fractions 10 to 20 showed β-galactosidase activity. The results showed that the enzyme was purified to homogeneity with a 68.51-fold increase in specific β-galactosidase activity with a yield of about 2.42 % from fraction 17 and 18.

Gel electrophoresis An extracellular β-galactosidase from *B. animalis* subsp. *lactis* HT10-2 has been purified to homogeneity by single chromatographic step, using ion-exchange chromatography. The molecular mass of the enzyme as determined by SDS-PAGE was 119 kDa.

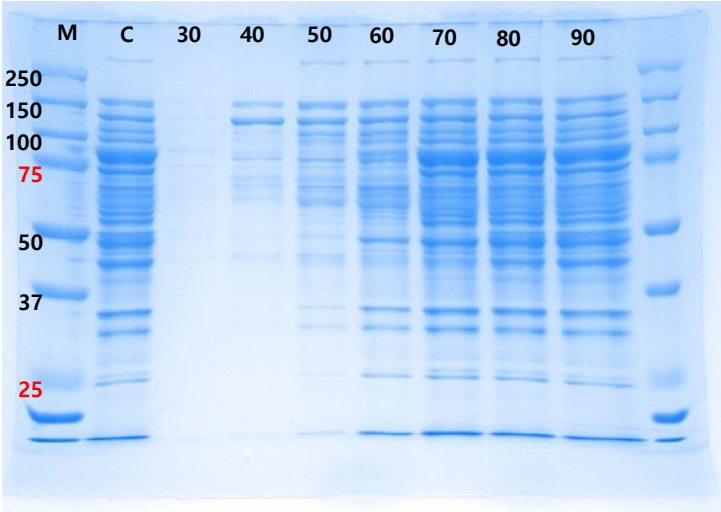

Figure 14. SDS electrophoresis in 10 % polyacrylamide gel of ammonium sulfate precipitation by 30−90 % of β−galactosidase from HT 10−2.

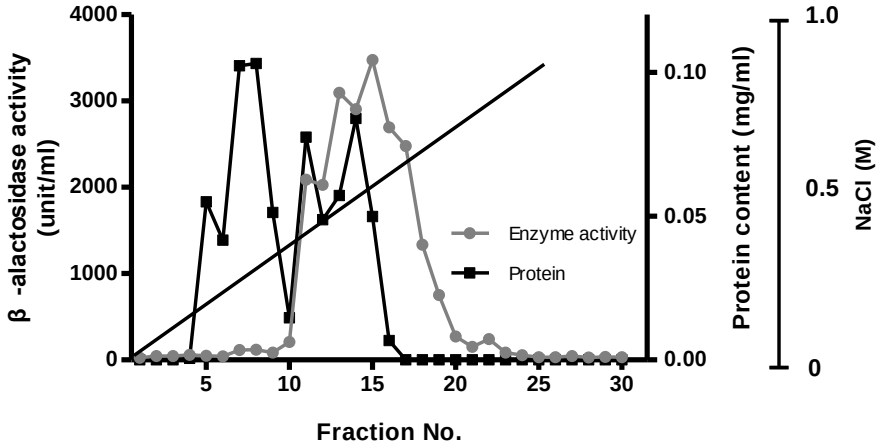

Figure 15. Elution profiles of ion exchange chromatography. Profile of β−galactosidase activity (405 nm) and protein content (595 nm) obtained after DEAE−Sepharose Fast flow chromatography (GE Healthcare Life Sciences^{TM}, Korea).

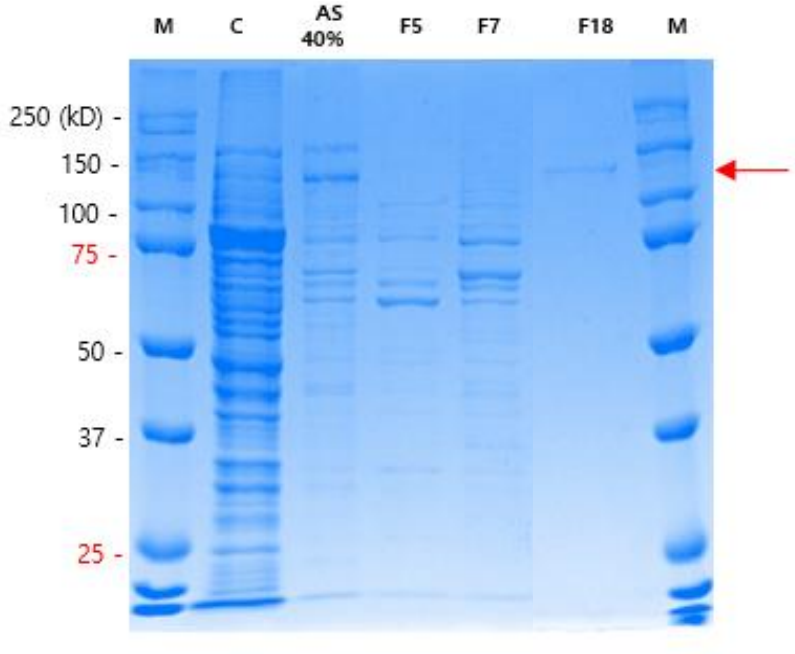

Figure 16. SDS electrophoresis in 10 % polyacrylamide gel of the each steps β-galactosidase purified from HT10-2.

M: molecular weight markers

C: crude enzyme

AS40%: 40% ammonium sulfate fractionation

F5: Fraction 5 (eluted with 0.2M NaCl)

F7: Fraction 7 (eluted with 0.3M NaCl)

F18: Fraction 18 (eluted with 0.6M NaCl)

The enzyme was purified 10-fold to a specific activity of 30,473.8 unit/ml, using standard assay conditions with PNPG as the substrate.

Table 19. Summary of purification of the β-galactosidase produced HT10-2.

Purification step	Total enzyme activity (unit)	Total protein content (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	86250	28.3	3047.7	1	100
Ammonium sulfate (40 % saturation)	41980.3	1.2	34983.6	11.48	48.7
DEAE-Sepharose Fast flow	1523.69	0.05	30473.8	10	1.8

4.3.3. Effects of pH and Temperature

The activities of β-galactosidase at different pH levels are shown. Our results showed that the optimal pH for hight activity was around 6 in 50 mM sodium phosphate buffer. However, β-galactosidase activities seemed to decrease except in pH 6.0. Optimum temperature for the β-galactosidase activities was found to be 37 ℃, and decreased over the optimal temperature. The activities of β-galactosidase sharply decreased at 45℃. The thermostability of the purified enzyme was investigated by pre-incubating the enzyme in 50 mM sodium phosphate buffer for 1 h and its remaining activity was determined every 30 min. The purified β-galactosidase was stable at 37 ℃ and the residual β-galactosidase activity still maintained 80.43 % and 78 % of the control after treatment at 37 ℃ for 0.5 and 1.0 h, respectively. On the other hand, purified β-galactosidase was unstable in the range of 45 ℃ - 50 ℃ with residual activities of 63.71-48.21 %, indicating that this enzyme was stable up to 37 ℃

4.3.4. Effects of Metal Ions

The results revealed that β-galactosidase activity was inhibited by metal ions, such as KCl, NaCl, Na_2SO_4 and $CuSO_4$ at the concentration of 1 Mm, but β-galactosidase activity slightly increased by $CaCl_2$ (1.27 fold) and was remarkably enhanced by $MgSO_4$ (2.43 fold), $FeSO_4$ (3.00 fold), $MnSO_4$ (2.75 fold) and $MgCl_2$ (2.17 fold).

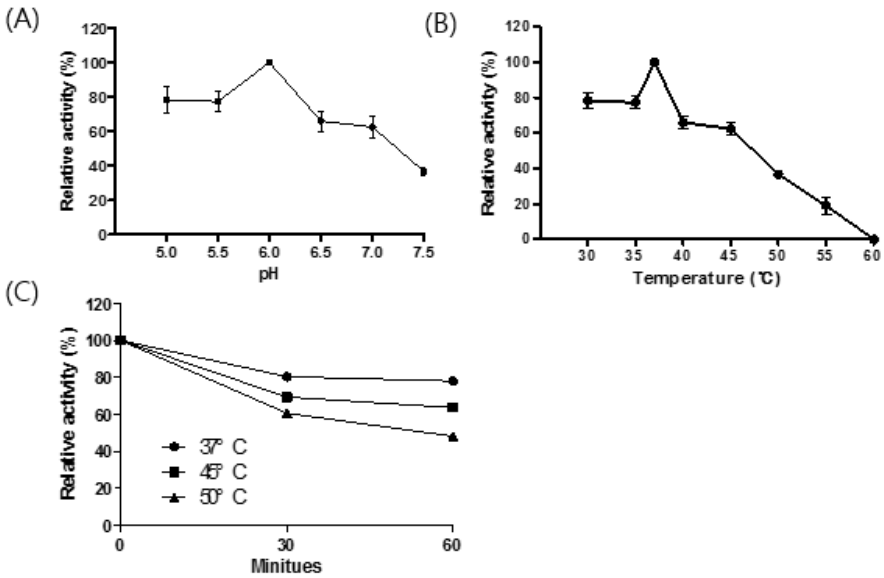

Figure 17. Effect of pH (A), temperature (B) and thermostability on the enzyme activities of β-galactosidase from HT 10-2. The data represent the means and standard deviation of three replicates. Data were analyzed using the one-way anova. Significance is indicated as follows: *p < 0.05; **p < 0.01, and ***p < 0.001.

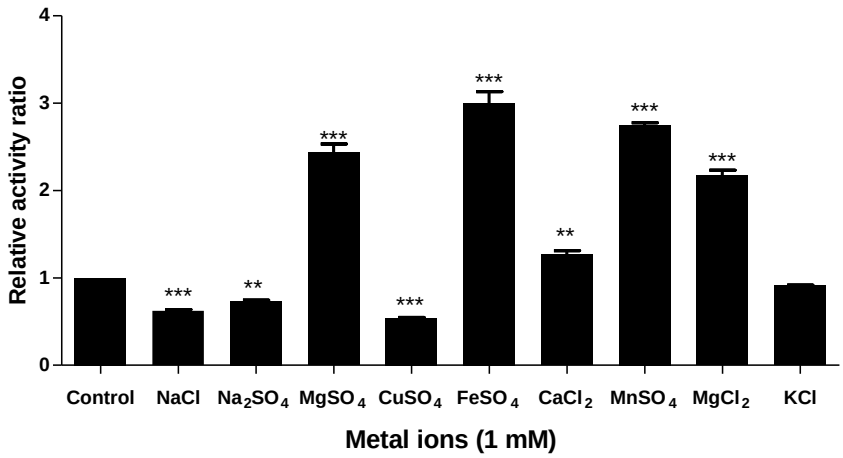

Figure 18. Effect of various metal ions on β-galactosidase activities. The data represent the means and standard deviation of three replicates. Data were analyzed using the one-way anova. Significance is indicated as follows: *p < 0.05; **p < 0.01, and ***p < 0.001.

Chapter 5. Discussion

In the present study, we screened the lactobacilli and bifidobacteria from Korean infant feces. Intake of breast milk for infants is associated with the increase of *bifidobacteria* and *lactobacilli* population, most common used probiotic bacteria (Tamburini, Shen *et al.* 2016). *Bifidobacteria* are regarded as high β-galactosidase producing bacteria in the other study (Ibrahim and O'Sullivan 2000). Among them, HT 10-2 was screened by a high β-galactosidase activity. Moreover, HT 10-2 could be potentially used as probiotics according to safty and functional assessment perfomed in this study. It was also revealed that other *B. animalis* subsp. *lactis* such as DN-173 has high survivality in digestive track and an effect on irritable bowel syndrome (IBS) (Bouvier, Meance *et al.* 2001). Therefore, *B. animalis* subsp. *lactis* is commonly used as starter for fermented milk.

We focused on high β-galactosidase activity of HT 10-2 which can hydrolyze the lactose in order to alleviate the symptoms of lactose intolerance. We assumed that there were be diffenceses of nucleotide sequences of β-galactosidase coding genes between HT 10-2 and DSM 10140 because HT 10-2 showed higher enzyme activity than DSM 10140 (Fig 8).

We aligned the sequences of genes coding β-galactosidase from HT 10-2 comparing to the genes from DSM 10140. There was no difference observed (Fig 10). To confirm the mRNA expression level of *lacZ* gene in HT 10-2 and DSM 10140, qRT PCR was performed.

qRT PCR analysis showed significantly higher mRNA expression level of *lacZ* in HT 10-2, which is a key reason of higher β-galactosidase activity of HT 10-2. We suspected that the factors which regulate transcription of *lacZ* gene might have subtle distinction between HT 10-2 and DSM 10140. We investigated that promoter region of their *lacI* genes, regulatory genes of the *lac* operon, showed slightly differences (Fig 11). We assumed that it might lead the β-galactosidase expression level to be differenct each other.

We purificated β-galactosidase for characterization. β-galactosidase from *B. bifidum* commonly had temperature and pH optima for substrate of 37 ℃ and pH 6.5, respectively (Dumortier, Brassart *et al.* 1994). It might be related to adaptation for human temperature, 37 ℃. However, β-galactosidase of *B. infantis* presents different characteristics (Hung and Lee 2002). Therefore, we assumed that optimal temperature and pH of β-galactosidase of *bifidobacteria* were associated with the environment where they were used to be, but it is also strain-specific. In this stduy β-galactosidase of HT 10-2 showed similar optimal temperature (37 ℃) and pH (6.5) on enzyme activities compared to *B. bifidum* (Dumortier, Brassart *et al.* 1994).

β-galactosidase was strongly stimulated and inhibited by various metal ions. Mg^{2+} and Mn^{2+} were found to enhance β-galactosidase activity in *E. coli* (Huber, Kurz *et al.* 1976), *Arthrobacter* B7 (Trimbur, Gutshall *et al.* 1994), *Bifidobacterium bifidum* 1901 (Garman, Coolbear *et al.* 1996), *Kluyveromyces lactis* (Cavaille and Combes 1995), and

Bacillus sp. MTCC 3088 (Chakraborti, Sani *et al.* 2000). These were simillar results from this study on β-galactosidase of HT 10-2 that $MgSO_4$ (2.43 fold), $FeSO_4$ (3.00 fold), MnSO4 (2.75 fold) and $MgCl_2$ (2.17 fold) inhanced β-galactosidase activity (Fig 18). In contrast, the studies on β-galactosidase of B. bifidum 1901 (Smart and Richardson 1987), and *Streptococcus thermophilus* (Garman, Coolbear *et al.* 1996) demonstrated that β-galactosidase were activated by Na^+ and K^+, which was opposed to the results of this study.

This study demonstrated that HT 10-2 has potential to be a probiotic strain with higher β-galactosidase activity. Since HT 10-2 was isolated from Korean infant feces and knowned as GRAS, this strain can be used in food. It was revealed that *lacZ* gene of HT 10-2 is a major gene coding β-galactosidase and this protein was purified and characterized. Although we screened the *bifidobacteria* with hight β-galactosidase activity to reduce the symptoms of lactose intolerance, *in vivo* or clinical demonstration should be performed to prove it. Therefore, further research on the effect of HT 10-2 on people with lactose maldigestion is needed in order to support the aim of this study.

References

Adams, M. (1999). "Safety of industrial lactic acid bacteria." Journal of Biotechnology **68**(2): 171-178.

Adams, R. M., S. Yoast, S. E. Mainzer, K. Moon, A. L. Palombella, D. A. Estell, S. D. Power and B. F. Schmidt (1994). "Characterization of two cold-sensitive mutants of the beta-galactosidase from Lactobacillus delbruckii subsp. bulgaricus." Journal of Biological Chemistry **269**(8): 5666-5672.

Arunachalam, K. D. (1999). "Role of bifidobacteria in nutrition, medicine and technology." Nutrition research **19**(10): 1559-1597.

Azizpour, K., S. Bahrambeygi, S. Mahmoodpour, A. Azizpour and S. Mahmoodpour (2009). "History and basic of probiotics." Research Journal of Biological Sciences **4**(4): 409-426.

Begley, M., C. Hill and C. G. Gahan (2006). "Bile salt hydrolase activity in probiotics." Applied and environmental microbiology **72**(3): 1729-1738.

Berger, J., B. Lee and C. Lacroix (1995). "Immobilization of β-galactosidases from Thermus aquaticus YT-1 for oligosaccharides synthesis." Biotechnology techniques **9**(8): 601-606.

Birri, D. J., D. A. Brede, G. T. Tessema and I. F. Nes (2013). "Bacteriocin production, antibiotic susceptibility and prevalence of haemolytic and gelatinase activity in faecal lactic acid bacteria isolated from healthy Ethiopian infants." Microbial ecology **65**(2): 504.

Bourlioux, P. and P. Pochart (1988). Nutritional and health properties of yogurt. Aspects of Nutritional Physiology, Karger Publishers**:** 217-258.

Bouvier, M., S. Meance, C. Bouley, J.-L. Berta and J.-C. Grimaud (2001). "Effects of consumptionof a milk fermented by the probiotic strain bifidobacterium animalis

DN-173 010 on colonic transit times in healthy humans." Bioscience and microflora **20**(2): 43-48.

Brandão, R. L., J. R. Nicoli and A. F. de Souza Figueiredo (1987). "Purification and characterization of a β-galactosidase from Fusarium oxysporum var. lini." Journal of dairy science **70**(7): 1331-1337.

Bustin, S. A. and R. Mueller (2005). "Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis." Clinical Science **109**(4): 365-379.

Cavaille, D. and D. Combes (1995). "Characterization of β-galactosidase from Kluyveromyces lactis." Biotechnology and Applied Biochemistry **22**(1): 55-64.
Chakraborti, S., R. Sani, U. Banerjee and R. Sobti (2000). "Purification and characterization of a novel β-galactosidase from Bacillus sp MTCC 3088." Journal of industrial microbiology & biotechnology **24**(1): 58-63.

Cho, Y.-J., H.-J. Shin and C. Bucke (2003). "Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from Bullera singularis." Biotechnology letters **25**(24): 2107-2111.

Du Toit, M., C. Franz, L. Dicks, U. Schillinger, P. Haberer, B. Warlies, F. Ahrens and W. Holzapfel (1998). "Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content." International journal of food microbiology **40**(1): 93-104.

Dumortier, V., C. Brassart and S. Bouquelet (1994). "Purification and properties of β-D-galactosidase from Bifidobacterium bifidum exhibiting a transgalactosylation reaction." Biotechnology and Applied Biochemistry **19**(3): 341-354.

El-Gindy, A. (2003). "Production, partial purification and some properties of β-galactosidase fromAspergillus carbonarius." Folia microbiologica **48**(5): 581-584.

Fenwick, G. R., R. K. Heaney, W. J. Mullin and C. H. VanEtten (1983). "Glucosinolates and their breakdown products in food and food plants." CRC Critical Reviews in

Food Science and Nutrition **18**(2): 123-201.

Finegold, S. M., V. L. Sutter and G. E. Mathisen (1983). "Normal indigenous intestinal flora." Human intestinal microflora in health and disease **1**: 3-31.
Garman, J., T. Coolbear and J. Smart (1996). "The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β-galactosidase from six strains of lactic acid bacteria." Applied microbiology and biotechnology **46**(1): 22-27.

Gilliland, S. E. and H. Kim (1984). "Effect of viable starter culture bacteria in yogurt on lactose utilization in humans." Journal of Dairy Science **67**(1): 1-6.
Gillor, O., A. Etzion and M. Riley (2008). "The dual role of bacteriocins as anti-and probiotics." Applied microbiology and biotechnology **81**(4): 591-606.

Gomi, A., N. Harima-Mizusawa, H. Shibahara-Sone, M. Kano, K. Miyazaki and F. Ishikawa (2013). "Effect of Bifidobacterium bifidum BF-1 on gastric protection and mucin production in an acute gastric injury rat model." Journal of dairy science **96**(2): 832-837.

Griffith, K. L. and R. E. Wolf (2002). "Measuring β-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays." Biochemical and biophysical research communications **290**(1): 397-402.

Groten, J. (1997). Reinigung, Charakterisierung und Immobilisierung der Levansucrase einer flockenden Mutante von Zymomonas mobilis ATCC 10988.
Haider, T. and Q. Husain (2007). "Preparation of lactose free milk by using ammonium sulphate fractionated proteins from almonds." J Sci Food Agric **87**: 1278-1283.

Han, Y. R., S. Y. Youn, G. E. Ji and M. S. Park (2014). "Production of α-and β-galactosidases from Bifidobacterium longum subsp. longum RD47." J Microbiol Biotechnol **24**: 675-682.

Heyman, M. B. (2006). "Lactose intolerance in infants, children, and adolescents." Pediatrics **118**(3): 1279-1286.

Hill, C., F. Guarner, G. Reid, G. R. Gibson, D. J. Merenstein, B. Pot, L. Morelli, R. B. Canani, H. J. Flint and S. Salminen (2014). "The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic." Nat Rev Gastroenterol Hepatol **11**: 506-514.

Hoyoux, A., I. Jennes, P. Dubois, S. Genicot, F. Dubail, J.-M. François, E. Baise, G. Feller and C. Gerday (2001). "Cold-adapted β-galactosidase from the Antarctic psychrophile Pseudoalteromonas haloplanktis." Applied and Environmental Microbiology **67**(4): 1529-1535.

Hsu, C.-A., S.-L. Lee and C.-C. Chou (2007). "Enzymatic production of galactooligosaccharides by β-galactosidase from Bifidobacterium longum BCRC 15708." Journal of Agricultural and Food Chemistry **55**(6): 2225-2230.

Hsu, C., R. Yu and C. Chou (2005). "Production of β-galactosidase by Bifidobacteria as influenced by various culture conditions." International Journal of Food Microbiology **104**(2): 197-206.

Huber, R., G. Kurz and K. Wallenfels (1976). "A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (E. coli) on lactose." Biochemistry **15**(9): 1994-2001.

Hughes, D. B. and D. G. Hoover (1991). "Bifidobacteria: their potential for use in American dairy products." Food technology (USA).

Hughes, D. B. and D. G. Hoover (1995). "Viability and enzymatic activity of bifidobacteria in milk." Journal of Dairy Science **78**(2): 268-276.

Hung, M.-N. and B. Lee (2002). "Purification and characterization of a recombinant β-galactosidase with transgalactosylation activity from Bifidobacterium infantis HL96." Applied microbiology and biotechnology **58**(4): 439-445.

Hyronimus, B., C. Le Marrec, A. H. Sassi and A. Deschamps (2000). "Acid and bile tolerance of spore-forming lactic acid bacteria." International journal of food microbiology **61**(2): 193-197.

Ibrahim, S. A. and D. O'Sullivan (2000). "Use of Chemical Mutagenesis for the Isolation of Food Grade β-Galactosidase Overproducing Mutants of Bifidobacteria, Lactobacilli and Streptococcus thermophilus1." Journal of dairy science **83**(5): 923-930.

Iqbal, S., T.-H. Nguyen, T. T. Nguyen, T. Maischberger and D. Haltrich (2010). "β-Galactosidase from Lactobacillus plantarum WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides." Carbohydrate research **345**(10): 1408-1416.

Isolauri, E., Y. Sütas, P. Kankaanpää, H. Arvilommi and S. Salminen (2001). "Probiotics: effects on immunity." The American journal of clinical nutrition **73**(2): 444s-450s.

Johnson, A. O., J. G. Semenya, M. S. Buchowski, C. O. Enwonwu and N. S. Scrimshaw (1993). "Correlation of lactose maldigestion, lactose intolerance, and milk intolerance." The American journal of clinical nutrition **57**(3): 399-401.

Jung, S. J. and B. H. Lee (2008). "Production and application of galacto-oligosaccharides from lactose by a recombinant β-galactosidase of Bifidobacterium infantis overproduced by Pichia pastoris." Food Science and Biotechnology **17**(3): 514-518.

Jurado, E., F. Camacho, G. Luzon and J. Vicaria (2002). "A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from Kluyveromyces fragilis." Enzyme and Microbial Technology **31**(3): 300-309.

Jurado, E., F. Camacho, G. Luzón and J. Vicaria (2004). "Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature." Enzyme and Microbial Technology **34**(1): 33-40.

Kaftzik, N., P. Wasserscheid and U. Kragl (2002). "Use of ionic liquids to increase the yield and enzyme stability in the β-galactosidase catalysed synthesis of N-acetyllactosamine." Organic process research & development **6**(4): 553-557.

Kim, J.-W. and S. Rajagopal (2000). "Isolation and characterization of β-

galactosidase fromLactobacillus crispatus." Folia microbiologica **45**(1): 29-34.
Klewicki, R. (2007). "Formation of gal-sorbitol during lactose hydrolysis with β-galactosidase." Food Chemistry **100**(3): 1196-1201.

Kohmoto, T., F. FUKUI, H. TAKAKU, Y. MACHIDA, A. Masaaki and T. MiTSUOKA (1988). "Effect of isomalto-oligosaccharides on human fecal flora." Bifidobacteria and Microflora **7**(2): 61-69.

Kok, R. G., A. De Waal, F. Schut, G. W. Welling, G. Weenk and K. J. Hellingwerf (1996). "Specific detection and analysis of a probiotic Bifidobacterium strain in infant feces." Applied and Environmental Microbiology **62**(10): 3668-3672.
Kolars, J. C., M. D. Levitt, M. Aouji and D. A. Savaiano (1984). "Yogurt—an autodigesting source of lactose." New England Journal of Medicine **310**(1): 1-3.

LeBlanc, J. G., C. Milani, G. S. de Giori, F. Sesma, D. Van Sinderen and M. Ventura (2013). "Bacteria as vitamin suppliers to their host: a gut microbiota perspective." Current opinion in biotechnology **24**(2): 160-168.

Lee, M.-F. and S. D. Krasinski (1998). "Human adult-onset lactase decline: an update." Nutrition reviews **56**(1): 1-8.

Lee, Y.-J., C. Kim and D.-K. Oh (2004). "Lactulose production by β-galactosidase in permeabilized cells of Kluyveromyces lactis." Applied Microbiology and Biotechnology **64**(6): 787-793.

Li, Y., L. Lu, H. Wang, X. Xu and M. Xiao (2009). "Cell surface engineering of a β-galactosidase for galactooligosaccharide synthesis." Applied and environmental microbiology **75**(18): 5938-5942.

Li, Z., M. Xiao, L. Lu and Y. Li (2008). "Production of non-monosaccharide and high-purity galactooligosaccharides by immobilized enzyme catalysis and fermentation with immobilized yeast cells." Process Biochemistry **43**(8): 896-899.

Lu, L.-l., M. Xiao, Z.-y. Li, Y.-m. Li and F.-s. Wang (2009). "A novel transglycosylating β-galactosidase from Enterobacter cloacae B5." Process Biochemistry **44**(2): 232-

236.

MacSharry, J., C. O'Mahony, K. H. Shalaby, B. Sheil, H. Karmouty-Quintana, F. Shanahan and J. G. Martin (2012). "Immunomodulatory effects of feeding with Bifidobacterium longum on allergen-induced lung inflammation in the mouse." Pulmonary pharmacology & therapeutics **25**(4): 325-334.

Madsen, K., A. Cornish, P. Soper, C. McKaigney, H. Jijon, C. Yachimec, J. Doyle, L. Jewell and C. De Simone (2001). "Probiotic bacteria enhance murine and human intestinal epithelial barrier function." Gastroenterology **121**(3): 580-591.

Mahoney, R. (1997). Lactose: enzymatic modification. Advanced Dairy Chemistry Volume 3, Springer**:** 77-125.

Mahoney, R. R. (1998). "Galactosyl-oligosaccharide formation during lactose hydrolysis: a review." Food chemistry **63**(2): 147-154.

Marteau, P., B. Flourie, P. Pochart, C. Chastang, J.-F. Desjeux and J.-C. Rambaud (1990). "Effect of the microbial lactase (EC 3.2. 1.23) activity in yoghurt on the intestinal absorption of lactose: an in vivo study in lactase-deficient humans." British Journal of Nutrition **64**(01): 71-79.

Martini, M. C., E. C. Lerebours, W.-J. Lin, S. K. Harlander, N. M. Berrada, J. M. Antoine and D. A. Savaiano (1991). "Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on in vivo lactose digestion." The American journal of clinical nutrition **54**(6): 1041-1046.

Mitsuoka, T. (1990). "Bifidobacteria and their role in human health." Journal of industrial microbiology **6**(4): 263-267.

Murray, R., R. N. Costilow, E. Nester, W. Wood, N. Krieg and G. Phillips (1981). "Manual of methods for general bacteriology." American society for microbiology, Washington, DC: 31.

Nagy, Z., T. Kiss, A. Szentirmai and S. Biró (2001). "β-Galactosidase of Penicillium

chrysogenum: Production, purification, and characterization of the enzyme." Protein expression and purification **21**(1): 24-29.

Neville, B. and P. O'Toole (2010). "Probiotic properties of Lactobacillus salivarius and closely related Lactobacillus species." Future Microbiology **5**(5): 759-774.

Nikolic, M., P. López, I. Strahinic, A. Suárez, M. Kojic, M. Fernández-García, L. Topisirovic, N. Golic and P. Ruas-Madiedo (2012). "Characterisation of the exopolysaccharide (EPS)-producing Lactobacillus paraplantarum BGCG11 and its non-EPS producing derivative strains as potential probiotics." International journal of food microbiology **158**(2): 155-162.

Nole, K. L. B., E. Yim and J. E. Keri (2014). "Probiotics and prebiotics in dermatology." Journal of the American Academy of Dermatology **71**(4): 814-821.

Nunoura, N., K. Ohdan, T. Yano, K. Yamamoto and H. Kumagai (1996). "Purification and characterization of β-D-glucosidase (β-D-fucosidase) from Bifidobacterium breve clb acclimated to cellobiose." Bioscience, biotechnology, and biochemistry **60**(2): 188-193.

Parvez, S., K. Malik, S. Ah Kang and H. Y. Kim (2006). "Probiotics and their fermented food products are beneficial for health." Journal of applied microbiology **100**(6): 1171-1185.

Placier, G., H. Watzlawick, C. Rabiller and R. Mattes (2009). "Evolved β-galactosidases from Geobacillus stearothermophilus with improved transgalactosylation yield for galacto-oligosaccharide production." Applied and environmental microbiology **75**(19): 6312-6321.

Rivera-Espinoza, Y. and Y. Gallardo-Navarro (2010). "Non-dairy probiotic products." Food microbiology **27**(1): 1-11.

ROSENBERG, Z. M.-M. (2006). "Current trends of β-galactosidase application in food technology." Journal of Food and Nutrition Research **45**(2): 47-54.

Roy, I. and M. N. Gupta (2003). "Lactose hydrolysis by Lactozym™ immobilized on cellulose beads in batch and fluidized bed modes." Process Biochemistry **39**(3): 325-332.

Sako, T., K. Matsumoto and R. Tanaka (1999). "Recent progress on research and applications of non-digestible galacto-oligosaccharides." International Dairy Journal **9**(1): 69-80.

Sánchez, B., S. Delgado, A. Blanco-Míguez, A. Lourenço, M. Gueimonde and A. Margolles (2016). "Probiotics, gut microbiota and their influence on host health and disease." Molecular Nutrition & Food Research.

Sanders, M. E. (2008). "Probiotics: definition, sources, selection, and uses." Clinical Infectious Diseases **46**(Supplement 2): S58-S61.

Shene, C. and S. Bravo (2007). "Whey fermentation by Lactobacillus delbrueckii subsp. bulgaricus for exopolysaccharide production in continuous culture." Enzyme and Microbial Technology **40**(6): 1578-1584.

Smart, J. and B. Richardson (1987). "Molecular properties and sensitivity to cations of β-galactosidase from Streptococcus thermophilus with four enzyme substrates." Applied microbiology and biotechnology **26**(2): 177-185.

Soccol, C. R., M. R. M. Prado, L. M. B. Garcia, C. Rodrigues, A. B. P. Medeiros and V. T. Soccol (2015). "Current developments in probiotics." Journal of Microbial & Biochemical Technology **2015**.

Solomons, I. (1978). "Antibiotics in animal feeds—human and animal safety issues." Journal of animal science **46**(5): 1360-1368.

Splechtna, B., T.-H. Nguyen and D. Haltrich (2007). "Comparison between discontinuous and continuous lactose conversion processes for the production of prebiotic galacto-oligosaccharides using β-galactosidase from Lactobacillus reuteri." Journal of agricultural and food chemistry **55**(16): 6772-6777.

Splechtna, B., T.-h. Nguyen, M. Steinböck, K. D. Kulbe, W. Lorenz and D. Haltrich

(2006). "Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from Lactobacillus reuteri." Journal of Agricultural and Food Chemistry **54**(14): 4999-5006.

Stecher, B. and W.-D. Hardt (2011). "Mechanisms controlling pathogen colonization of the gut." Current opinion in microbiology **14**(1): 82-91.

Tamburini, S., N. Shen, H. C. Wu and J. C. Clemente (2016). "The microbiome in early life: implications for health outcomes." Nature Medicine **22**(7): 713-722.

Trimbur, D. E., K. R. Gutshall, P. Prema and J. E. Brenchley (1994). "Characterization of a psychrotrophic Arthrobacter gene and its cold-active beta-galactosidase." Applied and environmental microbiology **60**(12): 4544-4552.

Tuorila, H. and A. V. Cardello (2002). "Consumer responses to an off-flavor in juice in the presence of specific health claims." Food Quality and Preference **13**(7): 561-569.

Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells and N. C. Roy (2011). "Regulation of tight junction permeability by intestinal bacteria and dietary components." The Journal of nutrition **141**(5): 769-776.

Yamasaki, C., S. Totsu, A. Uchiyama, H. Nakanishi, K. Masumoto, Y. Washio, K. Shuri, S. Ishida, K. Imai and S. Kusuda (2012). "Effect of Bifidobacterium administration on very-low-birthweight infants." Pediatrics International **54**(5): 651-656.

Yan, F. and D. B. Polk (2002). "Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells." Journal of Biological Chemistry **277**(52): 50959-50965.

Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim and E. J. Leonard (1987). "Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines." Proceedings of the National Academy of Sciences **84**(24): 9233-9237.

Zadow, J. (2012). Whey and lactose processing, Springer Science & Business Media.

Zhou, Q. Z. and X. D. Chen (2001). "Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from Kluyveromyces lactis." Biochemical Engineering Journal **9**(1): 33-40.

Abstract in Korean

이 실험에서는 한국인 유아분변에서 총 637개의 bifidobacteria 와 lactobacilli가 선발하였다. 선발된 bifidobacteria와 lactobacilli 에 관하여 비색분석법을 통해 베타갈라토시데이스의 활성을 측정하였다. 3개의 락토바실리와 7개의 비피도박테리아가 높은 베타갈락토시데이스의 활성을 보였다.

선발된 10개의 균을 가지고 프로바이오틱스 가능성을 실험하였다. 항생제 내성, 용혈성, 내산 및 내담즙성 실험을 통해 *Bifidobacterium animalis* subsp. *lactis* HT 10-2균주가 효소 활성도 높고 프로바이오틱스로서의 안전성 기능성도 높은 균주로 선발되었다. 선발된 균주는 유전체 분석을 통해서 1,923,647 bp, 1,613, predicted protein-coding 유전자, 1,553 coding sequence를 보유하는 것으로 확인되었다. 그리고 *Bifidobacterium animalis* subsp. *lactis* DSM 10140 균주의 뉴클레오타이드를 기준으로 하여 상동성을 비교하였을때 91.66%의 높은 상동성을 보이는 것으로 밝혀 졌다. 두 균주는 베타갈락토시데이스를 발현하는 *bgaA, ebgA, lacZ* 그리고 *beta-galII* 총 4개의 coding 유전자를 보유하고 있었으며, 실질적으로 비색분석법으로 확인한 결과 HT 10-2균주의 효소활성이 약 3배정도 높은 것으로 밝혀졌다.

두 유전자의 mRNA 상에서의 유전자 발현량을 확인하기 위해 quantitative real time PCR (qRT-PCR)을 이용하여 두 유전자의 mRNA 발현량을 비교하였다. HT 10-2 내에서의 베타갈락토시데이스 코딩진들의 발현량이 DSM 10140에서 보다 더욱 높은 것으로 나타났고, 특히 *lacZ* 유전자에서의 mRNA의 량이 높은 것으로 나타났다. HT 10-2 내에서의 서로 다른 4가지 유전자들의 발현량을 비교해본 결과 lacZ의 발현량이 다른 3가지 유전자 보다 높았다. 위와 같은 결과로 HT 10-2의 베타갈락토시데이스 활성은 *lacZ*유전자에서 코딩되는 단백질에 의한 것으로 생각 되었고, 그 단백질을 타켓으로 단백질 정제를 하였다. 40% 황산

암모늄 침전과 이온교환 크로마토그래피를 통해서 119kDa의 정제된 효소를 얻을 수 있었고 약 10-fold와 30473.8 unit/ml의 비활성을 보였다. 이 효소활성의 최적온도는 37℃, pH 6.0 이였으며 37 ℃ 1시간 동안 처리하였을 때 80.43% 유지 하는 것으로 나타났다. 또한 베타갈락토시데이스의 활성을 도와주는 보조인자로는 농도 0.1 M의 $FeSO_4$ 가 약 3배가량 효소활성을 높였고, $MgSO_4$ (2.17 fold), $MnSO_4$ (2.75 fold), $MgCl_2$ (2.17 fold)등이 효소활성을 증가 시켰다.

결과적으로, 이 연구를 통하여 베타갈락토시데이스의 활성이 높은 HT 10-2를 선발하였고 그 균의 프로바이오틱스 가능성을 밝혔다. 또한 HT 10-2의 유전자 분석을 통하여 *lacZ*가 이 단백질을 발현하는 중요 유전자라는 것을 찾았고 단백질 정제를 통하여 그 효소를 정제하였다. HT 10-2는 높은 베타갈락토시스의 활성으로 보아 유당불내증 개선효과를 예상 할 수 있다.

주요어: 유제품, 베타갈락토시데이스, 유산균, 비피도박테리아, 프로바이오틱스