A THESIS FOR THE DEGREE

OF MASTER OF SCIENCE IN FOOD AND NUTRITION

호기-혐기 전환에 따른 acetic acid-ethanol 생산 변환을 나타내는

Lactobacillus fermentum G7 의 환경 조건에 따른 발효 특성

Effect of Environmental Conditions on Fermentation Pattern of *Lactobacillus*

fermentum G7 Showing Acetic acid-Ethanol Production Switch

by Aerobic-anaerobic Conversion

February 2017

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Abstract

Effect of Environmental Conditions on Fermentation Pattern of *Lactobacillus fermentum* G7 Showing Acetic acid-Ethanol Production Switch by Aerobic-anaerobic Conversion

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Lactobacillus fermentum G7 is a bacterium extracted from the feces of a healthy human that produces carbon dioxide. In this study, the physiological characteristics of this bacterium were examined when cultured under various temperature, sugar, and gas conditions through analysis of the concentrations of its typical fermentation products, including carbon dioxide, acetic acid, ethanol, and lactic acid. The strain showed the greatest growth when maltose was added to the culture medium, regardless of temperature and gas conditions. Neither aerobic nor anaerobic conditions greatly

influenced the amount of acetic acid and ethanol production when more than 4% glucose was added to the medium. The optimum growth temperature for both the growth and the fermentation product profiles of the strain was found to be 37 ℃.

L. fermentum G7 produced ethanol in anaerobic conditions but not in aerobic conditions. However, the bacterium produced a greater amount of acetic acid in aerobic conditions than in anaerobic conditions, instead of producing ethanol. Moreover, in anaerobic conditions, the highest concentration of ethanol was produced when lactose was added to the medium compared with other sugars. By contrast, no ethanol was produced when fructose was added to the culture medium, even in anaerobic conditions.

When the bacterium was cultured in a milk medium supplemented with the sugars glucose, fructose, maltose, and sucrose, significantly reduced amounts of fermentation products were produced in comparison to those detected in MRS broth culture. The production of lactic acid by *L. fermentum* G7 was lower than that of homofermentative bacteria, but high levels of acetic acid were produced, with an approximately 3-times higher level than that of other acetic acid-producing strains.

Moreover, *L. fermentum* G7 produced approximately 1,000 times the amount of carbon dioxide produced by the homofermentative bacteria, and also produced more carbon dioxide than another heterofermentative bacterium*, L. reuteri*.

Therefore, this bacterium shows great potential for applications as an acetic acid-producing strain in the production of dairy products, and is also expected to play a significant role as a carbon dioxide-producing strain in the production of soft drinks.

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MRS broth: de Man, Rogosa and Sharpe broth

PBS: Phosphate buffered saline

HPLC: High Performance Liquid Chromatography

DW: Distilled Water

1. Introduction

Lactobacillus fermentum is not only found in the intestines of humans and animals, but also in food such as sourdough (Vrancken, Rimaux, De Vuyst, & Leroy, 2008), kefir (Angulo, Lopez, & Lema, 1993), kumis (Wang, Chen, Liu, Yang, Airidengcaicike, & Heping, 2008), and yogurt (Jayashree, et al., 2013). *L. fermentum* has been used for the fermentation of various foods. Recently, several researchers reported that *L. fermentum* can be used as probiotics, due to its capacity for repressing harmful bacteria (Bao, et al., 2010) such as *Escherichia coli* O157, *Salmonella typhimurium*, *Shigella flexneri*, *Staphylococcus aureus*, and *Listeria monocytogenes*, which cause harmful effects in the human body. Moreover, *L. fermentum* alleviated colonic inflammation in rat model (Peran, et al., 2006). In addition, it was also reported that a strain of *L. fermentum* was able to lower concentration of serum lipids in subjects with elevated serum cholesterol (Simons, Amansec, & Conway, 2006).

Lactic acid bacteria(LAB) can use glucose in either the homofermentative or heterofermentative pathway. A molecule of glucose is degraded into two molecules of lactate by homofermentation, while a molecule of glucose is degraded into a molecule of lactate and a molecule of ethanol or acetic acid by heterofermentation depending in the presence of external electron acceptors (Zaunmüller, Eichert, Richter, & Unden, 2006). According to a report, maltose can be also used as an energy source by LAB (Egloff, Uppenberg, Haalck, & Herman, 2001). A molecule of maltose can be converted to two molecules of glucose by a maltose phosphorylase.

L. fermentum is known to belong to heterofermentation LAB. Heterofermentation is the process of fermentation by which bacteria produce more than one product, as the term 'hetero' means 'other' and/or 'different'. Heterofermentative LAB produce ethanol, acetic acid (Zaunmüller, Eichert, Richter, & Unden, 2006), and lactic acid. Some heterofermentative LAB can convert a molecule of fructose a molecule of mannitol (Pilone, Clayton, & Robert, 1991).

A strain of *L. fermentum* isolated from sourdough fermentation was able to utilize glucose, fructose, sucrose, and maltose respectively in the simulated sourdough broth at the pH of 5.5 (Vrancken, Rimaux, De Vuyst, & Leroy, 2008). It showed diverse fermentation profile depending on the sugar added in the broth. A strain of heterofermentative bacteria belonging to *L. plantarum* cultured in MRS broth at 30, 37, and 45℃ under both aerobic and anaerobic conditions showed difference in producing lactic acid, acetic acid and ethanol depending on the temperature and aeration (Smetankova, et al., 2012)

In the case of fermented food, fermentative bacteria play a critical role on affecting the taste of food, since the substances that they spend and produce are sugars, organic acids, sugar alcohols, and alcohols - all of which are important factors of the distinctive tastes of food (Thiele, Gänzle, & Vogel, 2002). Thus it is important to control the concentration of sugar, organic acid, and ethanol in accordance with the purpose of fermentation.

In this experiment, we cultured *L. fermentum* G7, which was isolated from fecal sample of healthy Chinese, in MRS broth with 2, 4, and 6 % glucose under aerobic and

anaerobic conditions to investigate the maximum amount of glucose which the bacteria are able to use, and how *L. fermentum* G7 metabolizes glucose differently depending on the presence of oxygen.

Additionally, different sugars were added to MRS broth without glucose in order to characterize the fermentation pattern of *L. fermentum* G7. The experimental groups were cultured in two different temperatures and two gas conditions to figure out how the temperature and aeration can affect growth and fermentation process of *L. fermentum* G7. Substrates and products of fermentation were measured by High performance liquid chromatography (HPLC).

Next, *L. fermentum* G7 was inoculated into milk which was supplemented with four different sugars independently – glucose, fructose, maltose, and sucrose. The inoculated milk was fermented at 37℃ under anaerobic conditions.

Lastly, the concentration of carbon oxide produced by *L. fermentum* G7 was measured to figure out which sugar the bacteria is able to use most effectively to produce carbon oxide.

2. Materials and Methods

2.1 Bacterial strains and cultures

   L. fermentum G7 was stored at -70℃ in MRS broth (BD, New Jersey, USA), supplemented with 50% of glycerol as cryoprotectant. It was inoculated into a 15 ml falcon tube (Corning, New York, USA) which contained MRS broth and cultured for 18 h under aerobic conditions. After being activated, it was centrifuged at the conditions of 8,000×g for 5 min. The supernatant was removed, and the cell pellet was re-suspended in 15 mL of PBS buffer. The composition of the PBS buffer is shown in Table 2.

Table 1. Composition of MRS broth medium

Component	Amount
Proteose Peptone No. 3	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g
D.W.	The final volume was adjusted to 1L

Table 2. Composition of Phsphate Buffered Saline

Ingredients	Contents
NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g
D.W.	The final volume was adjusted to 1L
	HCl was added to adjust pH to 7

2.2 Preparation of the media

The 2% (w/v) glucose, fructose, sucrose, maltose, and lactose were added respectively, to MRS broth without glucose (KisanBio, Seoul, South Korea). All sugars were obtained from Sigma-Aldrich (Missouri, USA). All broths were steam-sterilized under 121℃ for 15 min and cooled down to 4 ℃ in refrigerator afterwards.

The milk used in this experiment was Seoul milk (Seoul dairy cooperative, Seoul, South Korea), and 99% of the bacteria were already sterilized during the process in the factory. It was stored for 65 minutes for 30 minutes for further sterilization purposes.

Table 3. Composition of MRS broth supplemented with sugar

	MRS with glucose	MRS with fructose	MRS with sucrose	MRS with maltose	MRS with lactose
Sugar			20 g of sugar respectively		
Peptone (Peptospecial)			10.0 g		
Beef Extract			10.0 g		
Yeast Extract			5.0 g		
Triammonium citrate			2.0 g		
Sodium Acetate			5.0 g		
Magnesium Sulfate			0.2 g		
Manganese Sulfate			0.05 g		
Di-Potassium Phosphate			2.0 g		
D.W.			The final volume was adjusted to 1 L		

2.3 Characterization of cultivation of *L. fermentum* G7 in MRS broth.

2.3.1. Measurement of bacterial cell growth

The 200 μl of each broth, each with a different sugar added, were pipetted into three wells of 96 micro well plates (Corning, New York, USA) for triplication. The 2 μl of PBS solution containing activated *L. fermentum* G7 was inoculated into the wells. The 2 well plates were incubated in incubator at 37℃, and the others were in incubator at 30℃. One of two plates in each incubator was kept in anaerobic jar. OD600 was measured by ELISA reader (Bio-rad, California, USA) at 0, 3, 6, 9, 12, 18, 24, 30 h respectively

2.3.2. HPLC analysis of sugars and fermentation products

The 500μL of the cell suspension in PBS was inoculated into 50 ml of each broth added with different sugars. They were cultured with 4 replicates under 4 different conditions described in Table 4. The 100μl of each sample was regularly withdrawn from the broths and used for further analysis.

The concentration of sugars was determined by HPLC. YL9100 HPLC system (Younglin, Anyang, South Korea) was used, which was equipped with a YL9101 vacuum degasser, a YL9110 quaternary pump, a YL9131 column department, a 9170 RI detector, and a 9150 Autosampler.

An Aminex HPX-87H column, 300 x 7.8 mm column (Bio-rad, California, USA) was

used with 5 mM H2SO4 as mobile phase at a flow rate of 0.6ml/min. The column

temperature was constant at 26 ℃. All samples were filtered by a PVDF Acrodisc

syringe filter with 0.2 μm, 13 mm (Pall Corporation, Michigan, USA), and injected (5

μL). Calibration was carried out with external standards calculated by sugar solutions of

which the concentration is known. Samples were analyzed in a triplicate manner and the

results are described as the average of three independent measurements.

The concentration of organic acids and ethanol was also determined by HPLC. The

same HPLC machine as above was used, with a YMC-packTM Polyamine IITM 250 x

4.6 mm I.D. (Younglin, Anyang, South Korea). 75 % of acetonitrile solution(v/v) was

used as mobile phase at a flow rate of 1ml/min. The column temperature was kept at 26℃

constantly. The same samples were filtered the same way as above, and injected (5 μL).

Calibration was carried out with external standards of organic acids and ethanol

solution of which the concentration is known. Samples were analyzed in the same

manner as above.

Table 4. Culture conditions of *Lactobacillus fermentum* G7

Gas conditions	Temperature	Sugar
Aerobic conditions	37℃	Glucose
		Fructose
		Maltose
		Sucrose
		Lactose
	30℃	Glucose
		Fructose
		Maltose
		Sucrose
		Lactose
Anaerobic conditions	37℃	Glucose
		Fructose
		Maltose
		Sucrose
		Lactose
	30℃	Glucose
		Fructose
		Maltose
		Sucrose
		Lactose

Table 5. Conditions nof HPLC for analysis of organic acids and ethanol

Instrument	YL9100 HPLC system
Column	Aminex HPX-87H column 300 x 7.8 mm
Mobile phase	5 mM H2SO4
Flow rate	0.6 ml/min
Temperature	26 ℃
Detection	Refractive Index(RI)
Injection volume	5 μL

Table 6. Conditions of HPLC for analysis of sugars

Instrument	YL9100 HPLC system
Column	YMC-packTM Polyamine IITM 250 x 4.6 mm I.D.
Mobile phase	75 % of Acetonitrile solution(v/v)
Flow rate	1 ml/min
Temperature	26 ℃
Detection	Refractive Index(RI)
Injection volume	5 μL

2.4 Analysis of milk fermented by *L. fermentum* G7

The 500μL of the cell suspension in PBS was inoculated into 50 ml of each milk added with 2% glucose, fructose, maltose, and sucrose. The tubes which contained the milk were airtight. The tubes were in 37℃ for 60 h.

The concentration of lactic acid, ethanol and acetic acid was determined by HPLC the same way as 2.3.2.

2.5 Monitoring the concentration of carbon dioxide emitted by *L. fermentum* G7

The 500μL of the cell suspension was inoculated into 50ml of MRS broth. MRS broth with added glucose, fructose, and maltose respectively was contained in 50ml falcon tubes. The lid of the tube was loosely closed and the tube was put in an airtight box with portable digital carbon dioxide meter (Inparo, Gwang-Myeong, South Korea). Then, the airtight box was sealed completely and incubated at 37℃ for 24 h. The concentration of carbon dioxide was measured at every 20 minutes automatically.

3. Results and discussion

3.1. Analysis of the growth, glucose, and products of fermentation in MRS broth with additional 2, 4, 6% glucose

   3.1.1. Growth curve of *L. fermentum* G7 cultured in the MRS broth

   The growth curve of *L. fermentum* G7 in MRS broth is shown in Figure 1. *L. fermentum* G7 was cultivated in MRS broth with 2, 4, and 6% glucose concentration under aerobic and anaerobic conditions.

   The greatest growth was observed in the culture medium including 2% glucose under anaerobic conditions. Growth during the exponential phase was observed in the culture medium with 2% glucose regardless of the gas conditions. Of particular note, the growth rate decreased in the exponential phase as the concentration of glucose added increased. The growth rate in the exponential phase for the culture medium with 2%, 4%, and 6% glucose was 0.18, 0.16, and 0.15, indicating a decrease in growth at the exponential phase with an increasing sugar concentration.
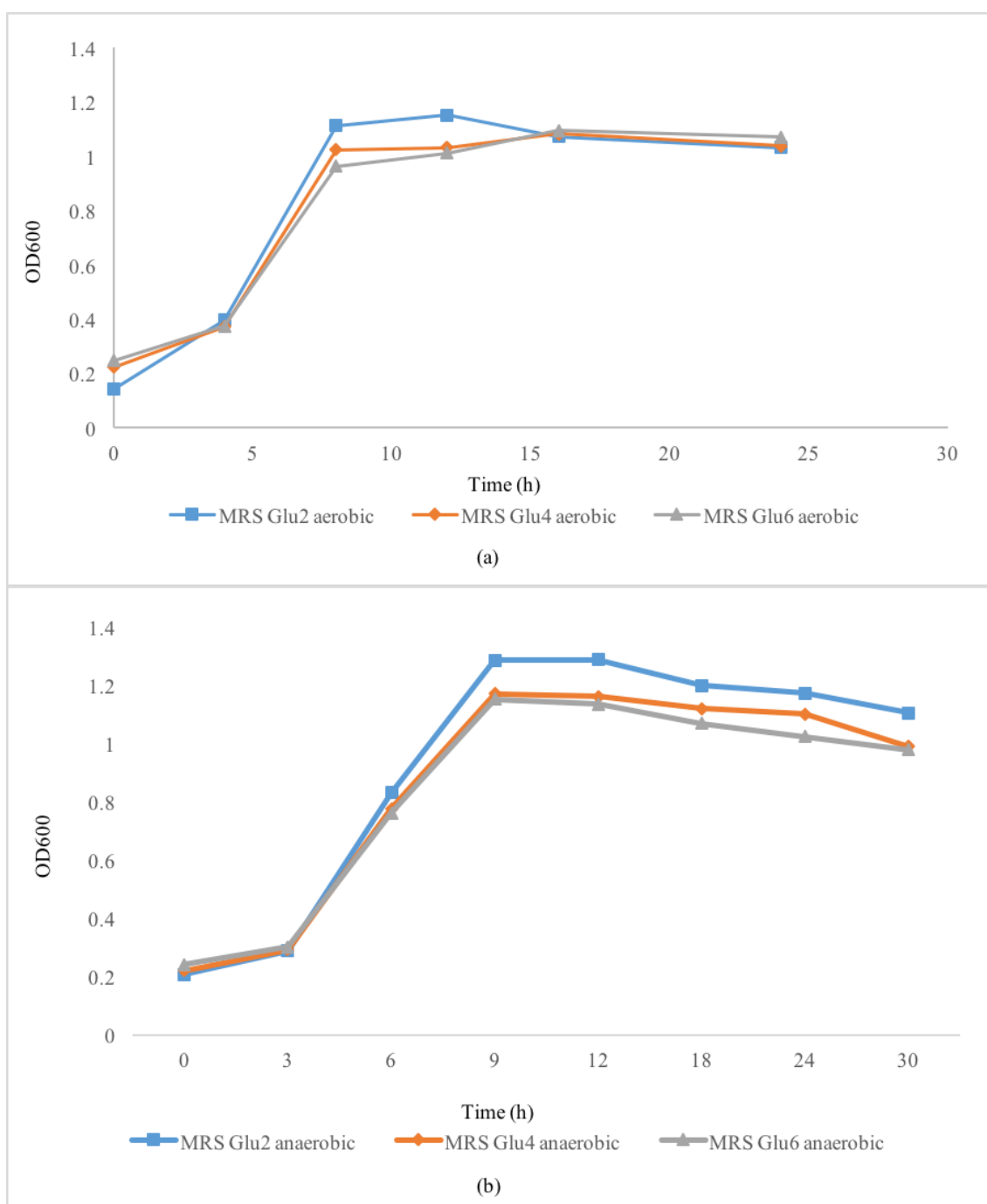
Figure 1. Growth curve of *L. fermentum* G7 in MRS broth with 2, 4, and 6% concentration of glucose at 37℃ under (a) aerobic and (b) anaerobic conditions

Table 7. Growth rate of *L. fermentum* G7 in MRS broth with added 2%, 4%, and 6% glucose

(unit : OD600/h)

Group	Growth rate in exponential phase(between 4 and 8 h)
MRS with 2% glucose, aerobic	0.18
MRS with 4% glucose, aerobic	0.16
MRS with 6% glucose, aerobic	0.15
MRS with 2% glucose, anaerobic	0.17
MRS with 4% glucose, anaerobic	0.15
MRS with 6% glucose, anaerobic	0.14

A previous study reported that the growth rates for certain yeasts decreased when the sucrose concentration of grape juice was increased from 2% to 3% (Charoenchai, Fleet, & Henschke, 1998). However, the growth rate of the bacterium *L. monocytogenes* was shown to increase when cultured in a soymilk medium with the addition of glucose and fructose at concentrations ranging from 0.5% to 10% each (Ariahu, Micheal, & Umeh, 2010). Therefore, conflicting results have been observed for different species of cultured bacteria. However, the growth rates of probiotics such as *Lactobacillus* and *Bifidobacterium* with respect to variations in different sugar concentrations have been scarcely reported. Thus, when considering developing products for which the bacterial number is significant, such as probiotics, further research is needed to clarify the relationship between the sugar concentration and growth rate of the bacterium.

3.1.2. HPLC analysis of glucose, and the products of fermentation

L. fermentum G7 was able to completely consume the existing sugar in a culture medium with the addition of 2% glucose. However, in the culture media with 4% and 6% glucose, only 3% of the glucose could be consumed.

Among the three culture media tested, *L. fermentum* G7 consumed the most lactic acid (1.63%) and ethanol (0.87%) in the medium with the addition of 4% glucose, representing a 0.27% and 0.38% increase compared to the lowest amount of lactic acid (1.25%) and ethanol (0.47%) produced from the 2% glucose culture medium. Importantly, although the sugar concentration doubled, the amount of fermentation products did not double in direct proportion. Moreover, in the culture medium with the addition of 6% glucose, lactic acid and ethanol were produced at 1.61% and 0.61%, respectively, which are even lower values than those produced from the culture medium with 4% glucose. In a previous experiment in which various concentrations of glucose were added to tea and tea fungus was utilized for fermentation, the levels of acid production tended to remain stable or even decreased with an increase in sugar concentration (Mi Ae, Jeong Ok, & Kyung Ho, 1996). However, this result is not transferable for bacteria that can be used as probiotics such as *Lactobacillus*. Therefore, further research is needed to examine the fermentation products from bacteria approved as probiotics processed with various sugars at different concentrations.
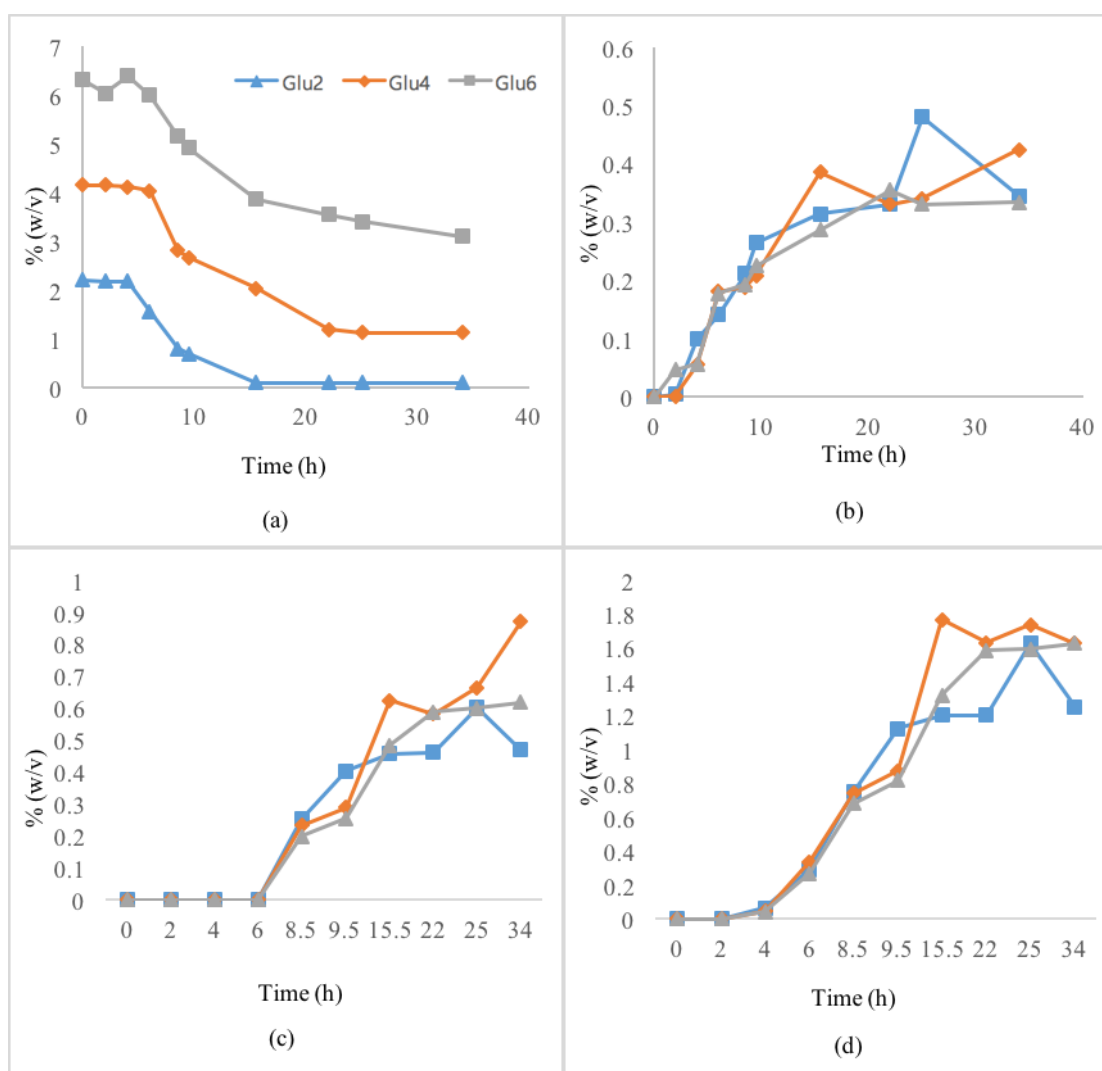
Figure 2. (a) Growth rate and production of (b) acetic acid (c) ethanol, and (d) lactic acid of *L. fermentum* G7 in MRS broth at 37℃ under anaerobic condition

3.2. Analysis of the growth, sugar and fermentation production under various sugar, temperature and gas conditions

3.2.1. Growth curve of *L. fermentum* G7 under the various conditions

L. fermentum G7 was capable of utilizing all of the sugar sources used in this experiment for its growth. It reached the peak point fastest at 37 ℃ under anaerobic conditions, around 9 h. However, the Cell density of *L. fermentum* G7 started to decrease right after it reached the peak under anaerobic conditions, whereas it retained the cell density of *L. fermentum* G7 after it reached the peak under aerobic conditions. Probably the bacteria which had been cultured under aerobic conditions was able to produce more energy via electron transport system.

Irrespective of the temperature and aerobic conditions, it was observed that the groups which had been cultured with added maltose and sucrose reached the highest cell density. Thus, *L. fermentum* G7 can utilize maltose and sucrose most efficiently for its rapid growth.

All of the groups which had lactose as their sole sugar source showed the lowest growth rate. This might be explained by the Leloir pathway, in which lactic acid bacteria spend 1 molecule of ATP to convert 1 molecule of α-D-galactose to 1 molecule of galactose-1-phosphate which turns into glucose. Since the bacteria spend extra time and energy converting galactose to glucose in order to create ATP, it may take more time to reproduce and reach the peak point.

Regardless of the temperature and aerobic conditions, the bacteria which used

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maltose and sucrose as their sugar source showed the highest growth rate in the

exponential phase. (Table 8) Those two groups showed very similar growth rate at both

temperatures under aerobic and anaerobic conditions. This implies that there is a

similarity between the pathway in which *L. fermentum* G7 utilizes maltose and the

pathway in which *L. fermentum* G7 utilizes sucrose.
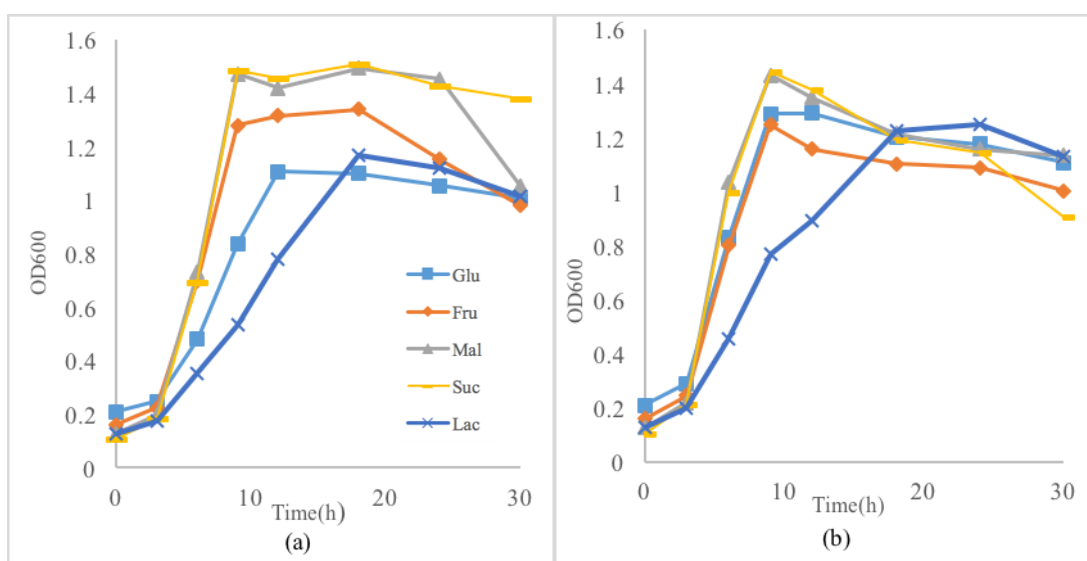
Figure 3. Cell density of *L. fermentum* G7 cells in MRS broth at 37℃ under (a) aerobic, and (b) anaerobic conditions
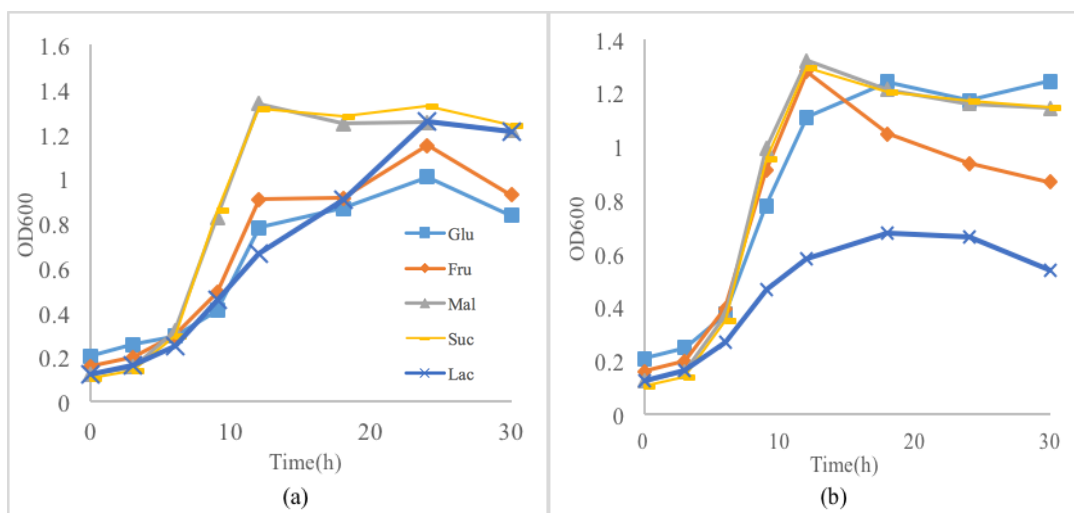
Figure 4. Cell density of *L. fermentum* G7 cells in MRS broth at 30℃ under (a) aerobic, and (b) anaerobic conditions

Table 8. Growth rate of *L. fermentum* G7 in MRS broth with different sugars in the exponential

phase at 37℃ and 30℃ under aerobic and anaerobic conditions (unit : OD600/h)

Group	37℃ aerobic	37℃ anaerobic	30℃ aerobic	30℃ anaerobic
MRS with 2% added glucose	0.098	0.167	0.124	0.122
MRS with 2% added fructose	0.176	0.167	0.139	0.147
MRS with 2% added maltose	0.213	0.201	0.169	0.158
MRS with 2% added sucrose	0.217	0.205	0.170	0.157
MRS with 2% added lactose	0.067	0.068	0.053	0.051

3.2.2. HPLC analysis of the sugars and products of fermentation

All of the fermented products identified in the bacteria culture in the two aerobic and anaerobic gas conditions with the temperature maintained at 37 ℃ or 30 ℃ with the addition of five different sugars to the MRS broth are shown in Figure 5. The bacterium produced more acetic acid, ethanol, and lactic acid when cultured at 37 ℃ than at 30 ℃ in the same sugar and gas conditions. One possible reason for the increased enzymatic activity of *L. fermentum* G7 at 37 ℃ than at 30 ℃ is that it was obtained from human feces. A strain of another LAB, *Lactococcus lactis*, which has been traditionally used in flour fermentation, was shown to more actively produce lactic acid at 37 ℃ than at 30 ℃ when other conditions were maintained (Åkerberg, Hofvendahl, Zacchi, & Hahn-Hagerdal, 1998). However, *L. fermentum* Ogi E1, obtained from maize sourdough (fermented dough of Nigerian bread), showed higher amylase activity at 40 ℃ than at 37 ℃ (Agati, Guyot, Morlon-Guyot, Talamond, & Hounhouigan, 1998)Experiments with *L. fermentum* 450 obtained from milk showed that the pH value, which is strongly correlated with acid production, decreased most rapidly at 40 ℃ (Han, Park, & Lim, 2015)
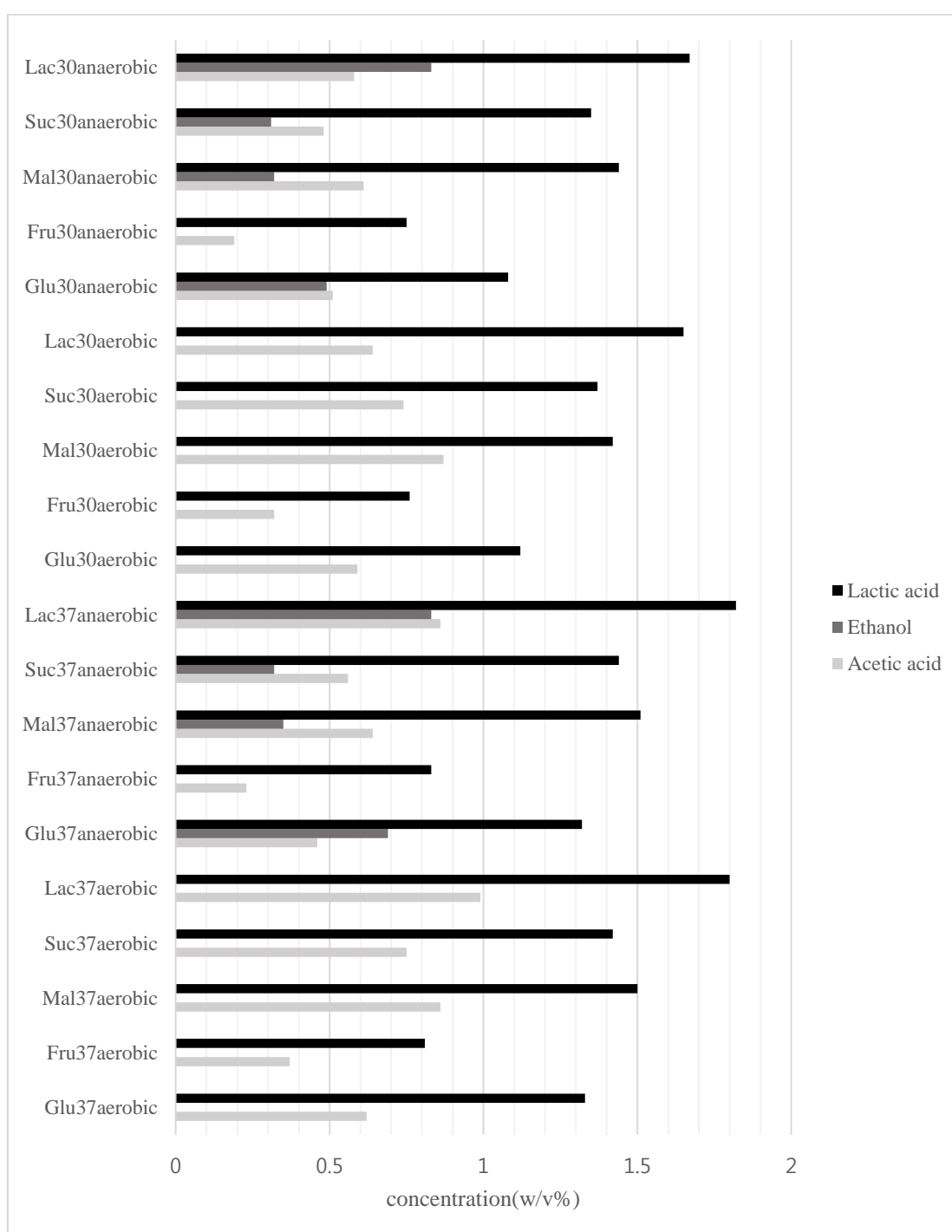
Figure 5. Concentrations of acetic acid, lactic acid, and ethanol produced by *L. fermentum* G7 during fermentation for 30 h of cultivation under various sugar, temperature, and gas conditions

Fermentation using *L. fermentum* in soymilk broth revealed the most rapid growth at 30 ℃, but the highest production levels of lactic acid and acetic acid at 37 ℃ (Garro, de Valdez, & de Giori, 2004). Thus, when the temperatures for optimum growth and maximum fermentation production differ, the temperature must be set according to the purpose of culturing the bacterium. However, in the present study, as described in section 3.2.1, the optimum growth and maximum fermentation products of the bacterium matched, indicating that 37 ℃ is the most suitable condition to culture the bacterium in regards to maximizing both factors.

Generally, LAB are categorized as facultative anaerobic bacteria, reflecting the fact that they can grow well in an environment without oxygen but can also grow in the presence of a minimal amount of oxygen. Recent research has revealed gene expression changes of LAB grown in the presence or absence of oxygen and depending on the oxygen concentration within the environment. For a strain of Lactococcus lactis subsp. lactis, expression of genes related to pyruvate metabolism was shown to change at the point in which all of the oxygen was consumed, resulting in a change in the type of acid produced (Larsen, et al., 2016). Moreover, a strain of *Lactobacillus plantarum* was shown to produce a greater amount of acetic acid in aerobic conditions than in anaerobic conditions (Zotta, et al., 2012). This effect was attributed to changes in the expression level of CcpA protein, which regulates the genes related to fermentation according to the presence of oxygen. This result corresponds to the determination of the fermentation products of *L. fermentum* G7 identified in this thesis. Similarly, a fermentation experiment with three strains of *L. plantarum* showed that the amount of fermentation products varied according to gas conditions; moreover, while less lactic

acid was produced in anaerobic conditions than in aerobic conditions, slightly more ethanol was produced in anaerobic conditions (Smetankova, et al., 2012). However, more detailed analyses at the gene and protein levels are necessary to elucidate the fundamental causes of these observed patterns. By contrast, although *L. plantarum* C17 produced more acetic acid in aerobic conditions than in anaerobic conditions, it produced less lactic acid (Zotta, Guidone, Ianniello, Parente, & Ricciardi, 2013). Unfortunately, direct comparison between prior studies and the present results is not possible because this is the first study to investigate changes in the fermentation products of *L. fermentum* according to gas conditions. However, previous research collectively suggests that *L. fermentum* G7 has a unique characteristic compared to other LAB by actively producing acetic acid in aerobic conditions and not ethanol, whereas in anaerobic conditions, it produces ethanol and less acetic acid.

L. fermentum G7 produced the highest concentration of ethanol when it was with added lactose under anaerobic conditions. In this case, the temperature did not affect production of ethanol at the point the samples were collected (30 hours).

Fructose was the only sugar source with which *L. fermentum* G7 was not able to produce ethanol. The group which used fructose also produced only small amounts of acetic acid and lactic acid when it was compared to the other sugar groups which used a different sugar. The fact that less total fermentative products were produced can be attributed to the conversion of fructose into a substance which was detected by HPLC, but not yet identified.

According to researchers (von Weymarn, Hujanen, & Leisola, 2002), certain strains

have been recognized as good producers of mannitol. Further research is required to identify the unknown substance.

Comparing the groups cultivated under anaerobic conditions with the groups cultivated under aerobic conditions, the former were able to produce ethanol. The latter showed no production of alcohol, but they were able to produce more acetic acid than those cultivated under anaerobic conditions. Both ethanol and acetic acid are made from acetyl phosphate. Ethanol is created by reducing acetyl CoA made from acetyl phosphate, and acetic acid is created by de-phosphorylate acetyl phosphate. Whereas the bacteria are able to utilize both pathways under anaerobic conditions (the product being acetic acid and ethanol), it appears that the bacteria shut down the ethanol-producing pathway under aerobic conditions, and thus acetic acid was the only product made from acetyl phosphate. However, since both acetic acid and ethanol are made from one substance, less acetic acid is made under anaerobic conditions than under aerobic conditions. Acetic acid production is promoted in the presence of oxygen according to researchers (Martínez-Anaya, Llin, Pilar Macías, & Collar, 1994). Our results are consistent with the aforementioned research, even though the bacteria used in the experiment are different. In earlier research (Kandler, 1983), it was found that the ratio acetate/ethanol depends on the oxidation-reduction potential of the system. If an additional hydrogen acceptor, e.g. O_2 is available, no ethanol is formed, but O_2 is reduced to H_2O_2 or H_2O.

Aerobic conditions did not significantly affect the production of lactic acid. The groups which were with the same added sugar produced very similar amounts of lactic

acid under both aerobic and anaerobic conditions. This suggests that the enzymes

involved in producing lactic acid are independent of oxygen, unlike the enzymes

involved in ethanol.

3.3. HPLC analysis of milk fermented by *L. fermentum* G7

The final concentrations of fermentative metabolites produced by *L. fermentum* G7 in milk at 37℃ under anaerobic conditions are shown in Figure 6.

Generally, there were fewer total fermentative metabolites produced in milk than those produced by the groups which were cultured in MRS broth at 37℃ under anaerobic conditions. In particular, there was a noticeable difference in the production of lactic acid. None of the groups cultured in milk produced more than 0.2% of lactic acid, whereas the lowest amount of lactic acid was 0.83%, which was produced by the group in MRS broth with fructose.

Ethanol production of *L. fermentum* G7 was less active in milk than in MRS broth. The bacteria grown in the MRS broth produced approximately two times more ethanol than the medium grown in milk media.

None of the groups showed aggregation of protein within 60 hours, meaning that the milk was not acidified enough to make proteins aggregate themselves. Rather, the fermented milk was liquid, and was slightly sticky.
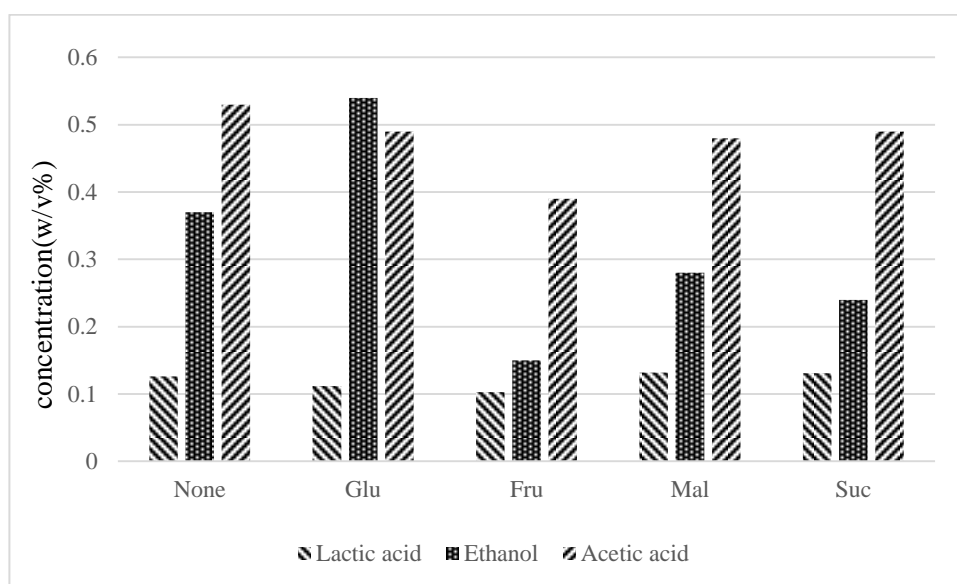
Figure 6. The final concentrations of fermentative metabolites produced by *L. fermentum* G7 in milk media at 37℃ under anaerobic conditions

Skim milk fermented with the homofermentative bacteria *Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, and *Lactobacillus acidophilus* produced a minimum of 0.5%(w/v) and maximum of 1.17%(w/v) lactic acid according to different types of added sugars, whereas *Bifidobacterium bifidum* produced a minimum of 0.05%(w/v) and maximum of 0.09%(w/v) of lactic acid, and also produced acetic acid at a minimum of 0.1%(w/v) and maximum of 0.18%(w/v) according to different types of added sugar (Chick, Shin, & Ustunol, 2001). Moreover, a probiotic strain of *Bifidobacterium longum* that can produce acetic acid was also shown to produce approximately 0.15%(w/v) acetic acid from fermented milk (Samona, Robinson, & Marakis, 1996).

The production of lactic acid by *L. fermentum* G7 was low compared to those of homofermentative bacteria mentioned above, but was high compared to that of *B. bifidum* (Samona, Robinson, & Marakis, 1996). Moreover, the production of acetic acid by *L. fermentum* G7 detected in the present study was approximately 3-times higher than that of *B. bifidum* and *B. longum*, at a minimum of 0.39%(w/v) and maximum of 0.52%(w/v). Therefore, this strain is expected to be more valuable as an acetic acid-producing bacterium than a lactic acid-producing bacterium for comestibles production using milk as the core ingredient.

3.4. Carbon dioxide production of *L. fermentum* G7 in MRS broth

L. fermentum G7 was cultured with different sugars in an airtight container for 24 h. The concentration curve of carbon dioxide produced during its growth and its differential curve is shown in Figures 7.

The highest concentration of carbon dioxide at the end point was shown when *L. fermentum* G7 was cultivated in MRS with glucose, followed by when it was cultivated in MRS with sucrose, and in MRS with fructose. We also confirmed that the highest concentration of ethanol was shown when *L. fermentum* G7 was cultivated in MRS with lactose, followed by in MRS with glucose, in MRS with sucrose, in MRS with maltose, and in MRS with lactose. Accordingly, it is suggested that the concentration of carbon dioxide which *L. fermentum* G7 produces is in proportion to the concentration of ethanol which *L. fermentum* G7 produces. This result corresponds to the findings about the fermentation pathway in which LAB utilize sugar. Carbon dioxide is produced when pyruvate is converted into acetyl CoA, which in turn is converted into ethanol or acetate. Thus there is a correlation between carbon dioxide and ethanol produced during fermentation.
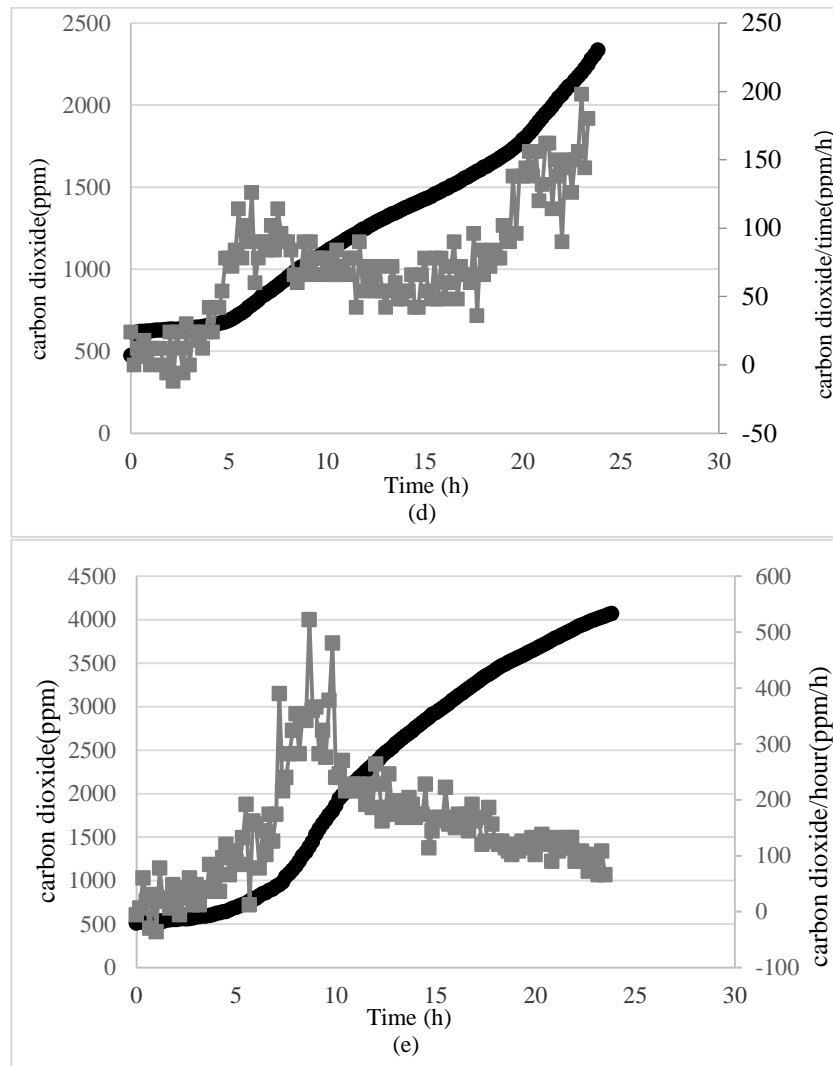
(a)

(b)

(c)
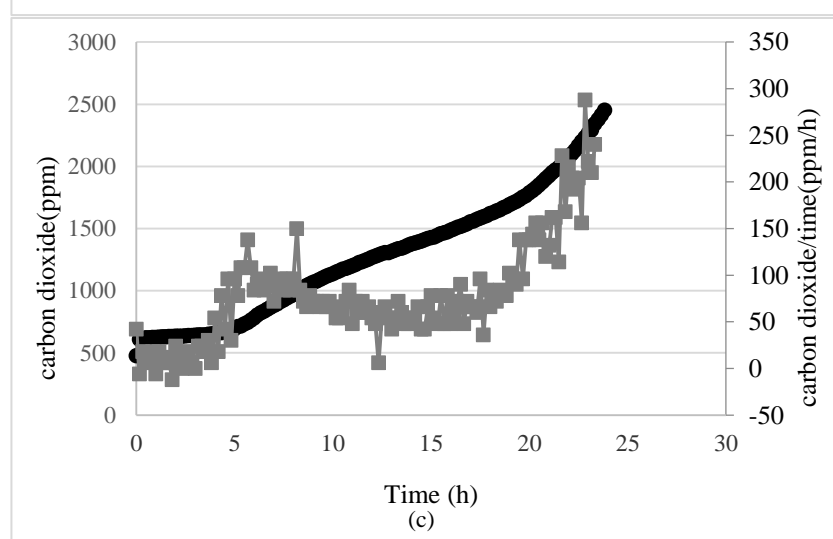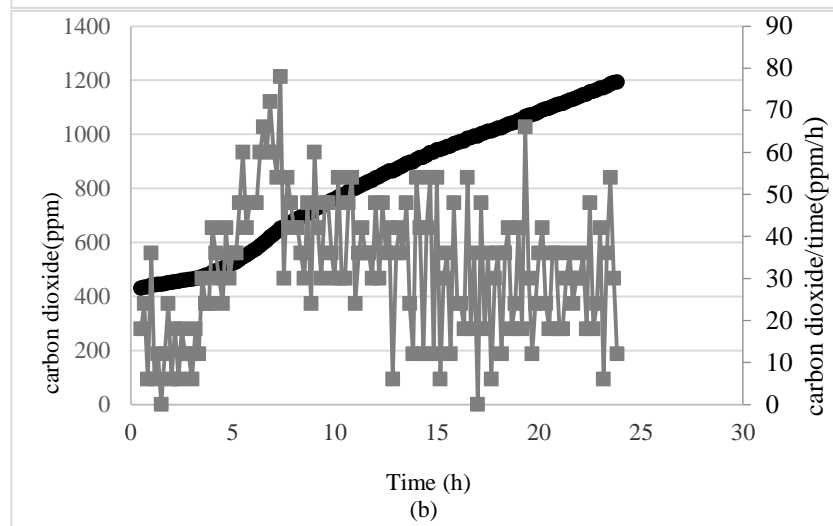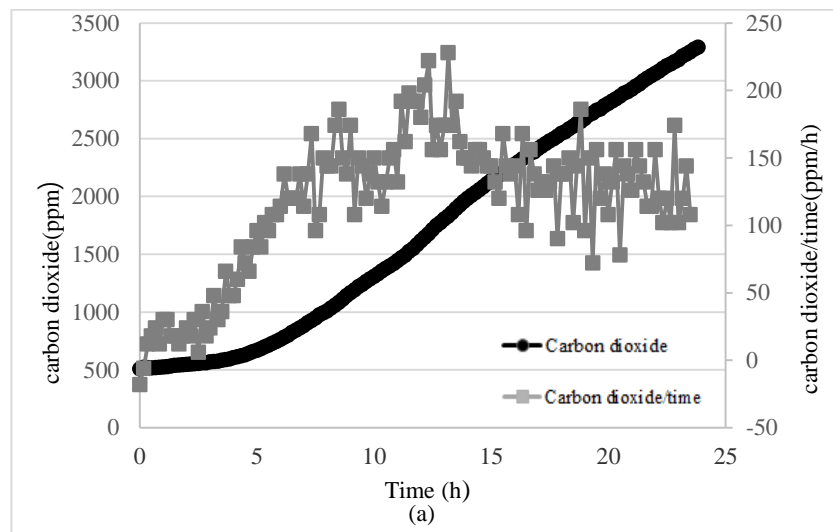
Figure 7. Concentration curves of carbon dioxide produced by *L. fermentum* G7 in MRS broth with (a) glucose, (b) fructose, (c) maltose, (d) sucrose, and (e) lactose in an airtight container and their differential curves

It was observed that the differential curve of carbon dioxide produced in MRS with monosaccharide had one peak, whereas the differential curve of carbon dioxide produced in MRS with disaccharide had two peaks. Disaccharide needed to be converted into monosaccharides in order to be utilized by bacteria. The first peak means that *L. fermentum* G7 degraded disaccharide into monosaccharides and used one that is preferable. The differential curve starts to go down as the preferable sugar gradually becomes depleted. Then the curve ascends again because *L. fermentum* G7 began to use the other sugar that is less preferred. This tendency was not found in the curves of monosaccharide, because *L. fermentum* G7 had only one kind of sugar it could utilize in MRS broth.

It was shown that that only 40 ppm of carbon dioxide was produced by the homofermentative bacterium Lactobacillus acidophilus within a 24-hour period, whereas 4000 ppm of carbon dioxide was produced by the heterofermentative bacterium *Lactobacillus reuteri* in 0.5% (w/v) tryptone milk within a 24-hour period. Similarly, when *L. fermentum* G7 was cultured in milk, approximately 4,100 ppm of carbon dioxide was produced (Østlie, Helland, & Narvhus, 2003). It Direct comparison to previous findings is difficult because the culturing conditions and environment are slightly different; however, considering that carbon dioxide production will be slightly lower without the addition of tryptone, it can be deduced that the amount of carbon dioxide produced by *L. fermentum* G7 is greater than that produced by *L. reuteri*.

4. Conclusion

In this study, *Lactobacillus* fermentum G7 isolated from Chinese human feces was cultivated under various conditions in order to investigate the fermentation profile of the strain. The maximum concentration of glucose that the bacteria were able to utilize is 3% in MRS broth. Adding glucose above 4% into MRS broth does not significantly affect the production of ethanol, lactic acid, and acetic acid. The bacteria were cultivated at 37℃ and 30℃, under aerobic and anaerobic conditions, in MRS broth with glucose, sucrose, maltose, sucrose, and lactose. It was found that the bacteria created more lactic acid, ethanol, and acetic acid in 37℃ than in 30℃. The bacteria produced ethanol under anaerobic conditions. The bacteria showed no production of alcohol under anaerobic conditions, but they were able to produce more acetic acid than those cultivated under anaerobic conditions. The bacteria produced more lactic acid, ethanol, and acetic acid in MRS broth with lactose, than in MRS broth with the other sugars. The bacteria cultivated in MRS broth with fructose did not produce ethanol under both aerobic and anaerobic conditions within 24 h. The bacteria cultivated in milk media produced less lactic acid, ethanol, and acetic acid than the bacteria cultivated in MRS broth. However, the fermentation products in milk were in an adequate range for fermented dairy products.

L. fermentum G7 has a distinctive fermentation feature compared to other LAB by actively producing acetic acid in aerobic conditions and not ethanol, whereas in anaerobic conditions, it produces ethanol and less acetic acid.

This bacterium shows great potential for applications as an acetic acid-producing strain in the production of dairy products, and is also expected to play a significant role as a carbon dioxide-producing strain in the production of soft drinks.

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국문초록

　　중국인의 변에서 분리된 균주가 활발한 기체를 생산 하는 것을 발견하였다. 이형발효 균으로 동정된 *Lactobacillus fermentum* G7 의 생장 및 발효대사 특성을 확인하기 위해 여러 가지 조건에서 배양을 시도 하였다.

　　2%, 4%, 그리고 6% 포도당 농도에서 균을 배양해 본 결과, 이 균의 최대 포도당 활용 농도가 3%이고, 4% 포도당 농도 이상의 배지에서는 당을 더 이상 사용하지 않는 것으로 나타났다.

　　두 가지 온도조건, 혐기와 호기, 그리고 5 개의 당 조건에서 균을 배양하였다. 혐기조건에서는 에탄올을 생산했지만, 호기조건에서는 에탄올을 생산하지 않고, 대신 혐기조건에서 보다 더 많은 아세트산을 생산하였다. 젖당을 첨가해준 배지에서 기른 균이 다른 배지에서 기른 균들보다 에탄올 및 다른 대사산물을 더 많이 생산하였다. 과당을 첨가해준 배지에서는 균이 에탄올을 생산하지 않았다.

　　우유배지에서 균을 배양한 결과, MRS 배지보다 대체적으로 발효산물의 생산량이 적었다. 하지만 여러 동형발효균들, 그리고 이형발효 균주인 *Bifidobacterium* 과는 구분되는 독특한 발효 산물 생산 비율을 보여주었다. .

　　이산화탄소 생산량 측정결과, 동형발효균과 비교하였을 때 1,000 배 정도 더 많은 이산화탄소를 생산했고, 이형발효균인 *L. reuteri* 와 비교하였을 때도 비슷하거나 더 많은 이산화탄소를 생산하였다.

　　이러한 발효 특징을 근거로 판단할 때, *Lactobacillus fermentum* G7 은 유제품 생산 시 acetic acid 생산균주로서의 사용이 기대되고, 또한 이산화탄소가 필요한 탄산 유제품 음료의 생산균주로서의 역할도 기대된다.

주요어: *Lactobacillus fermentum* G7, 이형발효, 우유 발효, 당, 가스 생산

학번: 2015-23322