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Enhancing Acid Tolerance of the Probiotic Bacterium *Lactobacillus gasseeri* KCCC612 with Isomaltooligosaccharide

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ABSTRACT

Background: *Probiotic bacteria have successfully been used for the prevention and treatment of GI infe-ctions and diseases, such as enteropathogenic Escherichia coli infections and rotavirus inf-ections.* ***Objectives:*** *to access the acid tolerance of the probiotic bacterium Lactobacillus gasseri KCCC612 with isomaltooligosaccharide.* ***Materials and method:*** *within the artificial gastric juice environment we incubate the Lactobacillus gasseri KCCC612 with isomaltooligosaccharide (IMO) and assess their viability with change of time and PH.* ***Result:*** *viability of the Lactobacillus gasseri KCCC612 with isomaltooligosaccharide was increased in the artificial gastric juice with the proportion of the IMO concentration but decreased with incubation time. With the increase of the concentration of the addition of isomaltooligosaccharides in the artificial gastric juice at pH = 2, the survival rate of Lactobacillus gasseri increased, and with the increase of treatment time, the difference in survival rate decreased significantly, but showed a significant increase compared to the control. Viability of the Lactobacillus gasseri KCCC612 with isomaltooligosaccharide was decreased in low pH artificial gastric juice but significantly viable in acidic solution with pH of 2 and 3.* ***Conclusion:*** *The acid tolerance of Lactobacillus gasseri 612 in artificial gastric juice was experimentally tested by isomaltooligosaccharides and maybe used in the prevention and the treatment of the GI diseases for their inhibitive effect of H. pylori.*

Keywords: *Lactobacillus gasseri* KCCC612, Probiotic Bacteria, Isomaltooligosaccha-Ride, Stomach Acid Environment, Acid Stress.

Article Information

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INTRODUCTION

Probiotic bacteria have successfully been used for the prevention and treatment of GI infe-ctions and diseases, such as enteropathogenic Escherichia coli infections and rotavirus inf-ections[4]. Probiotics have also been shown to have beneficial effects against H. pylori infections. It is known that Lactobacillus gasseri, which is one of probiotics, has benefi-cial probiotic effects in the GI by suppressing H.pylori, which has been identified to be associated with the development of some diseases such as gastritis and peptic ulcers, chro-nic gastritis, and gastric cancer including difference digestive diseases.

Since Lactobacillus gasseri was found to have an inhibitory effect on Helicobacter pylori in the stomach in 1999, animal and clinical trials have been conducted to examine the anti-H. pylori effect of Lactobacillus gasseri,[3] with the result that oral administration of probiotic preparations with Lactobacillus gasseri as the major component has resulted to improve or to recovery of 80-90% of various digestive diseases, including gastric ulcers and duodenal ulcers. The gastric mucosa is not so acidic as in the gastric cavity, and it can colonize and gro-w by Lactobacillus gasseri[3]. In general, LAB with high acid production have involved several mechanisms for acid re-sistance, including metabolic changes that stabilize the intracellular pH in low pH enviro-nments, proton pumps, hydrophobic sensing of the surface of the biomass, and protecting and repairing the biomass components[2,5].

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However, these endogenous mechanisms may be ineffective when organisms are exposed to extremely low pH environments, such as in the human stomach. Therefore, the addition of external protective substances is essential for probiotics to over-come the extreme acidic environment before these microorganisms can pass through the stomach and settle safely in the target gastrointestinal tract.

The role of non-digestible oligosaccharides, including isomaltooligosaccharides, on the acid tolerance of Lactobacillus gasseri, has not been mechanistically investigated yet. Isomalto oligosaccharides (IMOs) are said to be the general name of branched oligosacch-arides with various combinations of glucose, such as a-1.6, a-1.1, a-1.2and a-1.3. IMO includes oligosaccharides such as isomaltose, trehalose, isomaltotriose, isomaltotetraose, and panose[10].

Because IMO is composed of a-1.6 bonds that are difficult to degrade by human digestive enzymes or acids, it can be difficult to digest and absorb in the stomach and small inte-stine after ingestion, as well as other functional oligosaccharides, reaching the lower dige-stive tract where bifidobacteria are present, and some enteric probiotics are available[10].

Lactobacilli can combat H. pylori through multiple strategies, including competitive adhesion,production of antimicrobial substances, coaggregation, immunomodulation, improvement of the epithelial barrier integrity, and inhibition of virulence gene expression (Qureshi et al., 2019). Lactobacilli are effective in eradicating the pathogen and are also responsible for reduced side effects due to antibiotic therapy (Zhang et al., 2015).However, the molecular mechanisms underlying the antagonistic effect of lactobacilli against H. pylori are still largely unknown, partly due to insufficient genetic tools, especially mutagenesis methods. In this study, we examined the protective effect of Lactobacillus gasseri on the addition of IMO against extremely low pH mimicking human stomach.

MATERIAL AND METHOD

Material:

- Lactobacillus gasseri KCCC612(National Institute for Conservation of Strains of the D.P.R.K).
- ***Isomaltooligosaccharide, saccharide mixture*** (containing 32% total IMO among dry matt-er) that was condensated high-concentration glucose by glucoamylase at high temper-ature, and was fermented with yeast Saccharomyces cerevisiae to remove glucose and then concentrated in vacuo[11].
- **The composition of the isomaltooligosaccharide (Total IMO in dry matter:** glucose 5.41%, disaccharides 55.35%, trisaccharides 24.55%, tetrasaccharides 14.69% of saccharides solution):
- ***Artificial gastric fluid:*** After 645 ml of 10% HCl, adjusted to pH around 1.2 with normal saline, pepsin-added to 2000 U/ml pepsin activity[1].
- ***MRS medium:*** glucose 2.0%, peptone 1.0%, yeast extract 0.5%, KH2PO4 0.2%, sodium acetate 0.5%, MgSO4 • 7H2O 0.02%, MnCl2 0.01%, pH 7.0.

Method:

Acid tolerance assay: Lactobacillus gasseri was incubated in MRS medium for 15 h until the optical density at 600nm(OD600) reached 2.0. Cells were spun down by centrifugation (6,000×g, 10 min, 4°C)and resuspended in IMO (pH 7.0) solution at indicated concentrations (w/v).

In order to mimic stomach acidic environment, hydrochloric acid was added as a proton donor to 0.9% (w/v) sodium chloride solution to achieve pH values of 1.0, 2.0, and 3.0, respectively. The cell suspension (0.5 ml) was added to acidic solutions (1.0 ml) at a final IMO concentration of 0.5%, 1.0%, 2.0%, 3.0%, and 5.0% (w/v), separately. Controls were cell suspension in 0.9% saline solution without IMO. The reaction systems were incubated for different time(30min, 60min) at 37°C, and viable cells were quantified by plating incubated cells on MRS agar and counting colony numbers. Three replications were performed for each condition.

RESULTS

1)IMO enhanced acid tolerance of L. gasseri at different concentration

Table 1. Protective effect of different concentration of IMO on L. gasseri at pH 2.0, (X ± SE)

IMO(%)	Biomass number(log10cfu/ml)			Survival rate(%)		
	0min	30min	60min	0min	30min	60min
0.5	8.04±0.11	7.73±0.08	7.53±0.08	100.00	49.19±3.99＊＊	30.99±2.02＊＊
1.0	8.19±0.06	7.92±0.08	7.69±0.14	100.00	54.22±2.89＊＊	32.74±5.44＊＊
2.0	8.24±0.06	8.02±0.05	7.82±0.08	100.00	60.76±2.04＊＊	38.11±2.27＊＊
3.0	8.31±0.04	8.16±0.05	7.92±0.09	100.00	70.83±2.02＊＊	41.84±5.36＊＊
5.0	8.64±0.04	8.52±0.06	8.28±0.05	100.00	75.41±3.87＊＊	43.66±0.71＊＊
Control	7.81±0.03	7.11±0.02	0	100.00	19.96±0.32＊＊	0

＊＊:p<0.01, n=3.

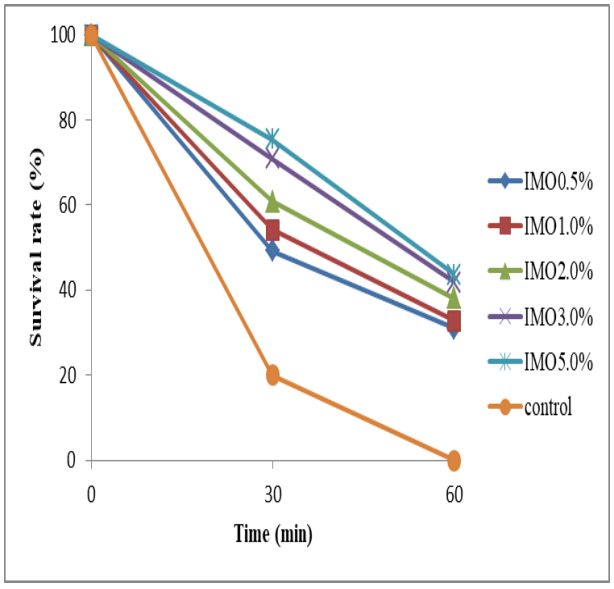

FIG.1: Protective effect of different concentration of IMO on L. gasseri at pH 2.0.

As shown in Table 1 and Fig. 1, with the increase of the concentration of the addition of isomaltooligosaccharides in the artificial gastric juice at pH = 2, the survival rate of Lactobacillus gasseri increased, and with the increase of treatment time, the difference in survival rate decreased significantly, but showed a significant increase compared to the control.

2)IMO enhance acid tolerance of L. gasseri at different pH

If the article presents direct quotations, excerpts from transcripts, or interview, use this format:**Table 2. The survival rate of *L. gasseri* under 3.0 % IMO protection at difference pH.**

pH		Biomass number (log_{10}cfu/ml)			Survival rate (%)		
		0 min	30 min	60 min	0 min	30 min	60 min
1.0	3% IMO	8.84±0.08	8.24±0.05	0	100.00	25.88±5.18＊＊	0
	Control	8.84±0.11	7.93±0.10	0		12.40±0.44	0
2.0	3% IMO	8.86±0.13	8.64±0.06	8.34±0.08		70.83±2.86＊＊	41.84±7.57＊＊
	Control	8.93±0.08	8.29±0.09	0		23.46±6.02	0
3.0	3% IMO	9.12±0.11	9.02±0.07	8.71±0.11		80.04±8.25＊＊	39.21±6.01＊＊
	Control	9.17±0.07	8.63±0.09	0		29.64±5.41	0

＊＊:P<0.01, n=3

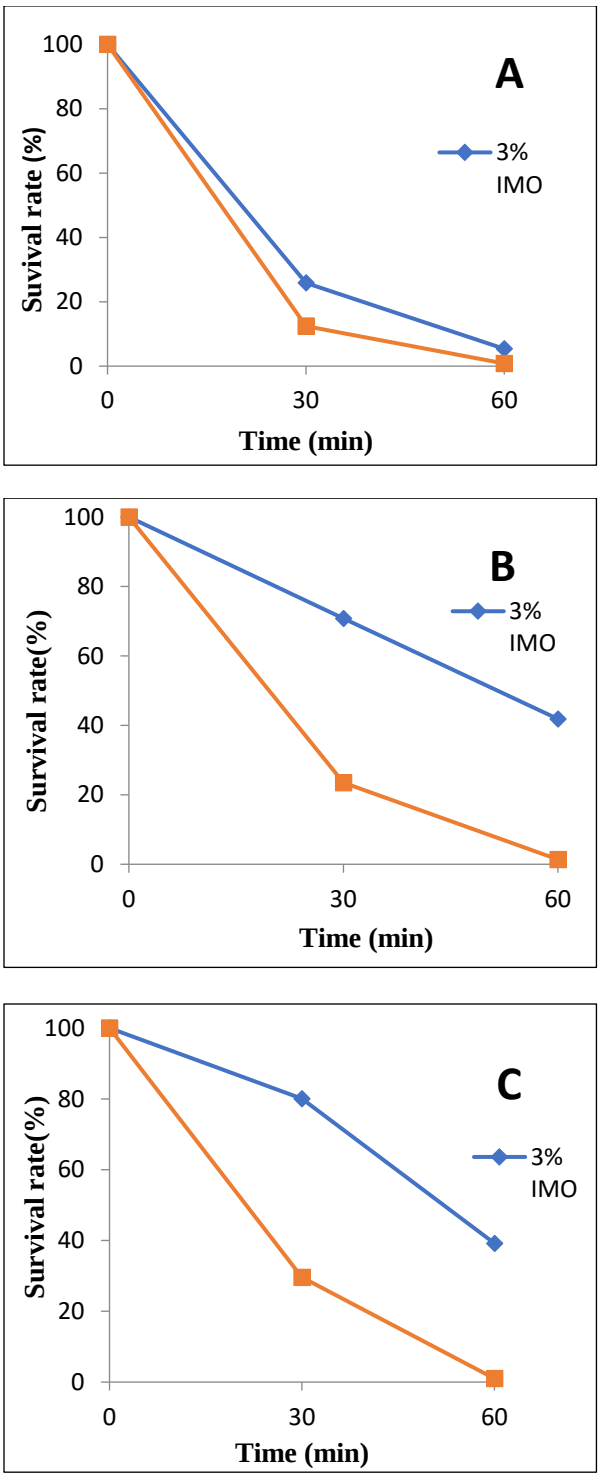

FIG.2: The survival rate of L. gasseri under 3.0 % IMO protection at (A) pH 1.0, (B) pH 2.0, and (C) pH 3.0

As shown in Table 2 and Fig. 2, survival of artificial gastric juice at 3% isomaltooligosaccharides was examined under pH conditions, and pH = 1.0 showed almost no viable counts at 60 min of treatment, and a significant increase at pH = 2 and 3.

DISCUSSION

Lactic acid bacteria have been used to produce fermented foods for the past several dec-ades and have been developed as probiotics and generally have been recognized as safe for health promoting functions in the human gastrointestinal tract. Probiotics must be viable in the gut at a high concentration of at least 106 cfu/ml to be beneficial to humans. Therefore, a more robust tolerance of lactic acid bacteria species against various extreme environments is essential for developing probiotic supplements. Lactobacilli are exposed to various stress conditions such as acid, temperature, osmotic pressure, and lyophilization in fermented food processing as well as in the gastrointestinal tract. Resistance of lactic acid bacteria to these stresses is crucial to identify potential candidates for application of lactic acid bacteria[7].

Bacteria, probiotic candidates, must survive in a variety of enzymatic or extremely acidic conditions in the entire gastrointestinal tract. Acid tolerance is generally considered as one of the criteria necessary for the selection of potential probiotics, as acid stress is given as a central issue for microbial survival. To date, the literature has not focused on the mechanisms of acid tolerance of lactic acid bacteria such as Lactococcus lactis, Lactobacillus bulgaricus, Lactobacillus casei, Lactob-acillus plantarum, and Lactobacillus reuteri[8]. Recently, acid stress due to the large amount of gastric acid in the gastrointestinal tract is a major survival challenge for probiotic species, and more sophisticated mechanisms and molecular levels from a physiological point of view have been developed by micro-organisms that have been applied and overcome acid stress, and a variety of approaches have been developed to elucidate the acid tolerance mechanisms in various microbial species at different levels. By comprehensively understanding

the mechanisms and mechanisms of microbial responses to acid stress, specific strategies can be devised for the improvement of microbial pro-duction factors and the biosynthesis of valuable chemicals. In the present study, we confirmed that the thermo-condensated isomaltooligosaccharides by glucoamylase enhanced the acid tolerance of

Lactobacillus gasseri 612, a member of the probiotic strain. We analyzed the survival of probiotic Lactobacillus gasseri 612 by bioassay with varying pH, concentration of isomaltooligosaccharides and acid treatment time to confirm the enhancement of probiotic acid tolerance of thermo-reverse synthesized isomaltooligosaccharides. We first tested the viability of L. gasseri exposed to pH 2.0 for 0, 30, and 60 minutes in the presence of 0.5-5.0% IMO. After 30-min incubation, the viability of cells was positively correlated with IMO concentration. At 3.0% and 5.0% IMO concentration, cell viability was 70.83±2.02% and 75.41±3.87%, respectively. After 60-min incubation, the survival rate under all IMO concentrations dropped to around 40%, with almost no viable cells in control group.

Statistical analysis of survival rate showed significantly difference in means bet-ween the groups with $P < 0.05$ both at 30-min and 60-min incubation, wherein the multiple comparison results stated that the groups of IMO concentration above 2% were significantly different from other test and control groups with P value < 0.05. Based on these results, IMO concentration of 3.0% was used for subsequ-ent experiments to further study its effect on acid tolerance. To test the effectiveness of 3.0% IMO protection against acid at different pH, we measured L. gasseri survival rate at pH 1.0, 2.0, and 3.0, representing the floating range of gastric juice. The cell survival rate at these tested pH values showed marked difference, with the statistical analysis showed there were significant variations in viable cell counts with $P < 0.05$ at both 30-min and 60-min test points in all groups (Fig. 2).

At pH 1.0, 30-minute incubation drastically reduced survival rate to approximately 25.88±5.18% and 12.40±0.44% in both 3.0% IMO and the control group, which further reduced to near zero after 60-minute incubation (Fig. 2), suggesting at pH 1.0, the high concentration of acid was lethal to L. gasseri and 3.0% IMO was ineffective in protecting L. gasseri against extremely low pH. At pH 2.0, 3.0% IMO significantly enhanced acid resistance of L. gasseri, resulting in more than 70.83±2.86% survival rate in 30 minutes and 41.84±7.57% in 60 minutes. At pH 3.0, 3.0% IMO led to the highest survival rate among all tested pH values that reached 80.04±8.25% after 30-minute incubation. The results indicated that 3.0% IMO could protect L.gasseri against varying concentration of acid, with the exception of minimal protection at extremely low pH.

The mechanisms of acid tolerance studied so far are : pH homeostasis, restriction of proton permeation, enhancement of proton pumps, consumption of protons within the cytoplasm, changes in cell membranes, regulation of metabolism, protection and repair of macromolecules. In the future, we have the task of mechanistically elucidating the principle by which the reverse-synthesized isomaltooligosaccharides enhance the acid tolerance of Lactobacillus gasseri 612. The mechanisms for enhancing acid tolerance of probiotics worldwide are still poorly understood and have been experimentally demonstrated to increase the acid tolerance of probiotics. The authors experimentally and genetically confirmed that IMO and glutamate enhanced the acid tolerance of probiotics, and the authors confirmed that some dairy products incl-uding whey proteins [6] and cereal materials including barley and wheat enhanced acid tolerance as carriers of probiotics[9].

Research on enhancing acid tolerance of probiotics is now in its infancy and will further study strategies to enhance acid tolerance of probiotics and support scientific elucidation of the mechanisms involved.This section is also a significant part of the research articles and is usually the longest part of an article. A discussion of the research presented in this section is the result—data analysis, such as statistical calculations or other methods for the achievement of its study. Please present the discussion narratively.

CONCLUSION

The acid tolerance of Lactobacillus gasseri 612 in artificial gastric juice was experimentally tested by isomaltooligosaccharides reverse synthesized by glucoamylase at high temperature, and the survival of probiotics increased significantly with increasing concentration of isomaltooligosaccharides, while the survival of probiotics decreased significantly with

increasing acid treatment time. In addition, the survival of probiotics decreased significantly with decreasing pH of artif-icial gastric juice, while the survival of probiotics decreased significantly with increasing acid treatment time.

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