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Production of Organic Acid and Short-Chain Fatty Acids (SCFA) from Lactic Acid Bacteria Isolate on Oligosaccharide Media

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Abstract

The growth of microorganisms in food, one of which is lactic acid bacteria (LAB), can produce metabolites beneficial to health. It is essential to study the results of LAB metabolism to improve the quality of a functional food product. This study aimed to evaluate the isolates *Lactobacillus acidophilus* FNCC 0051 and *Lactobacillus rhamnosus* R23 to metabolize oligosaccharides as a carbon source so that the final fermentation product can benefit health especially in lowering cholesterol. In vitro testing was carried out on MRS media with or without oligosaccharides, either singly or in a combination consisting of galactooligosaccharides (GOS), fructooligosaccharides (FOS), inulin (IN), inulin hydrolyzate (HI), or their combination as prebiotics by adding 0.3 % oxbile (bile salt) and inoculated with 1% v/v LAB isolate culture and incubated at 37°C for 24 hours. The results showed that the main product of oligosaccharide metabolism by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 produced several organic acids (lactic acid), including short-chain fatty acids (SCFA) (acetic acid, propionic acid, and butyric acid). The single and combined carbon sources affected the proportion of lactic acid and acetic acid produced by *L. acidophilus* FNCC0051 ($p<0.05$). However, they did not affect the proportions of propionic acid and butyric acid. While in *L. rhamnosus* R23 ($p<0.05$), the presence of a single carbon source significantly affected the proportions of lactic acid, acetic acid, propionic acid, and butyric acid, while the combination of oligosaccharides affected the proportions of lactic acid and butyric acid produced. SCFA is the main product of prebiotic metabolism, but the characteristics of the acid produced have not been identified. The fermentation pattern is thought to be related to molecular weight, chain length, and oligosaccharide structure. Short-chain molecules, such as FOS generally ferment more rapidly than long-chain molecules such as inulin. The results of this study indicate that both isolates can be used as probiotics in the development of symbiotic products with the addition of oligosaccharides, which have a physiological effect in lowering cholesterol levels.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide [1]. According to the World Health Organization (WHO) data, cardiovascular disease causes 17.6 million deaths every year [2]. The pattern and lifestyle of modern society without regulating daily food consumption patterns and controlling the intake of balanced nutrition cause cardiovascular disease. Maintaining blood cholesterol levels at normal levels is very necessary to reduce the risk of coronary heart disease. One of the efforts to maintain blood cholesterol levels is to consume functional foods naturally and more economically. The development of functional foods based on probiotics and prebiotics with hypocholesterolemic functions continues to be developed.

Lactic acid bacteria (LAB) are a group of probiotic bacteria that produce lactic acid as the main metabolic product during carbohydrate fermentation. Lactic acid bacteria are included in the group of bacteria that are beneficial to humans because they are generally recognized as safe (GRAS). Probiotics are live and beneficial microorganisms because they provide health benefits to the host after being consumed in sufficient quantities, primarily by increasing the proliferation of native digestive microflora [3]. Probiotic bacteria are non-pathogenic bacteria generally found in the human gastrointestinal tract and protect the intestines from pathogenic bacteria. Probiotics can be used as food adjuvants and provide several health benefits, including preventing diarrhea [4], preventing constipation [5], stimulating the immune system [6], overcoming lactose intolerance [7], preventing the risk of colon cancer [8], reduces allergy symptoms [9], facilitates mineral absorption [10], and lowers cholesterol levels [1, 11]. An increase in the number of beneficial bacteria in the colon requires an indigestible carbohydrate source (prebiotic) to be used as a substrate for the growth of probiotic bacteria in the colon.

Prebiotics are fermentable selective ingredients that produce specific changes in the gastrointestinal microbiota's composition and/or activity, thereby benefiting the host's health [12]. Prebiotic effects refer to pathological and physiological effects in both experimental and interventional studies in humans, specifically related and correlated with selective changes in gut microbiota composition [13]. Dietary fiber, especially soluble fiber, is an undigested component, showing some prebiotic activity. These compounds include types of prebiotics such as inulin, fructooligosaccharides, and galactooligosaccharides (GOS) [14], lactulose, lactose, soybean oligosaccharides, isomaltooligosaccharides, palatinose, xylooligosaccharides, and glucooligosaccharides [15]. Fructooligosaccharides, inulin, oligofructose, lactulose, and galactooligosaccharides have been identified as prebiotics due to resistance to gastric acidity, hydrolyzed by mammalian enzymes, and fermented by the gastrointestinal microflora to more selectively stimulating the growth and/or activity of beneficial gut bacteria [16]. The presence of dietary fiber (prebiotics) is required in the intestine. If it is not available, then anaerobic bacteria will draw energy from protein fermentation [17]. Protein metabolism leads to the production of toxic and potentially carcinogenic compounds (such as ammonia or phenolic compounds) [18].

Prebiotic carbohydrates (oligosaccharides) are fermented in the large intestine by colon bacteria, producing short-chain fatty acids such as butyric, acetic, and propionic acids. Prebiotic fermentation involves various metabolic processes by anaerobic microbes into organic compounds and the production of short-chain fatty acids (SCFA). It also produces metabolites for microbial growth, with the by-products of bacterial fermentation including methane (CH_4), hydrogen (H_2), and carbon dioxide (CO_2) [19]. Moens and De Vuyst [20] found that butyrate is the primary fermentation product of inulin, whereas acetate is produced from fructooligosaccharides [21].

The combination of prebiotics and probiotics has a synergistic effect because, in addition to increasing the growth of beneficial bacterial strains in the large intestine, it also has a physiological effect on the health of the digestive tract. This study aims to investigate the production of organic compounds and SCFA produced by *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* isolates in their ability to grow on oligosaccharide compounds, in providing physiological effects on health, one of which is in their ability to lower cholesterol.

2. Methodology

2.1. Materials and media preparation

LAB isolates used in this study were local isolates isolated from breast milk *L. rhamnosus* R23 from the SEAFAST Center Laboratory, IPB University, Indonesia, and commercial isolate *L. acidophilus* FNCC 0051, which is an isolate from human digestion, obtained from Gadjah Mada University, Indonesia. The oligosaccharides used in this study were commercial inulin ORAFTI®, FOS from ORAFTI®, commercial GOS from JINAO® (Anhui China), and inulin hydrolyzate (Chemical Research Center Laboratory-LIPI). Chemicals used for the preparation of LAB isolate culture media and growth fermentability testing of LAB isolates on prebiotics were physiological NaCl, MRS Agar (Merck), MRS Broth (Merck), MRSC broth without glucose (MRS-C): peptone (Difco), yeast extract, Tween 80, K_2HPO_4, Na-acetate, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, cholesterol PEG-600 (polyoxyethanyl-cholesteryl sebacate, Sigma), ox bile, o-phtaldealdehyde, KOH (33% w/v), hexane, concentrated acid sulfate, 96% ethanol and for the determination of organic compounds and SCFA in the form of standard lactic acid, acetic acid, propionic acid, and butyric acid.

2.2. In vitro fermentation stages

LAB isolates were inoculated in MRS broth-based media, which contained either four oligosaccharides single oligosaccharides each: 5% GOS, 5% FOS, 5% inulin, and 5% inulin hydrolyzate, as well as a combination of two oligosaccharides as a carbon source consisting of 5 % FOS + 5% GOS, 5% FOS + 5% inulin, 5% FOS + 5% inulin hydrolyzate, 5% GOS + 5% inulin, 5% GOS + 5% inulin hydrolyzate, 5% inulin + 5% inulin hydrolyzate. As a control, media based on MRS broth without sugar and glucose was used. MRS broth medium contains 0.30% ox bile and 80µg/mL water-soluble cholesterol solution. Previously, a stock solution of water-soluble cholesterol (10 mg/mL) was prepared, which had been sterilized with a 0.22 m cellulose acetate filter membrane. As much as 1% (v/v) of LAB culture was inoculated into an MRS broth medium containing 0.3% ox bile and incubated at 37°C for 24 hours [22]. Furthermore, centrifugation was carried out at a speed of 1000 gauge, at a temperature of 4°C, and for 10 minutes, until a pellet (sediment) and supernatant were obtained. The organic acid and SCFA concentrations were measured in the media.

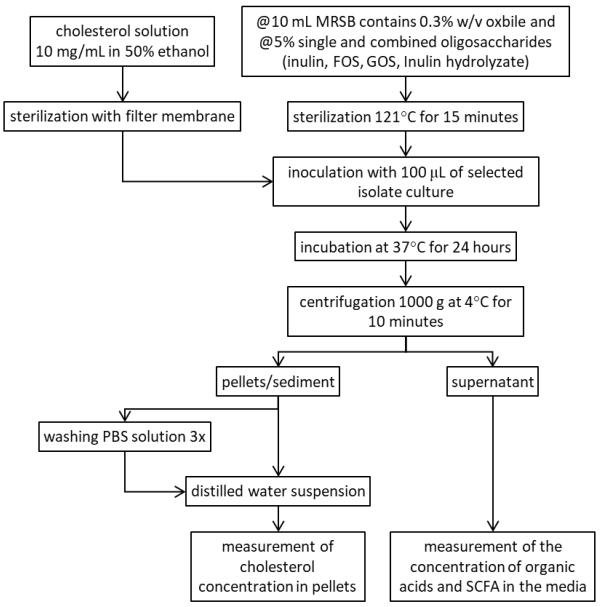


Figure 1. Diagram of LAB fermentation process the ability of selected local LAB isolates to metabolize oligosaccharides

2.3. Sample analysis

LAB (Viable microorganism) population was calculated by Standard Plate Count (SPC) [23]. The result of prebiotic fermentation with probiotic LAB in the form of metabolites then measured the profile of organic acids (lactic acid), including SCFA consisting of acetic, butyric, and propionic acids using high-performance liquid chromatography (HPLC) [24]. The pH was determined at the beginning of incubation for 0 hours and at the end of incubation for 24 hours using a digital pH meter with a combination of standard glass electrodes with pH 4 and 7 standard buffer solutions (Gmbh, pH 330; Weilheim, Germany). The total sugar consumed [25], and the total acid yield was calculated based on the amount of organic acid produced against the total sugar used by bacteria. Determination of cholesterol-binding ability refers to the literature [26, 27, 28].

2.4. Determination of organic acids and SCFA profile

The 24-hour fermentation containing LAB isolates and prebiotics was centrifuged at 2714 gauge at 4°C for 15 minutes. The supernatant was filtered using a syringe filter sterile cellulose acetate 25 mm/0.45 m and put into an autosampler vial. Samples were prepared in duplicate and ready for analysis with HPLC brand Young Lin YL 9170 with refractive index detector, Aminex HPX-87H column (7.8 mm × 300 mm) (Bio-Rad), and the analysis temperature was maintained at 35°C, mobile phase 5 mM H_2SO_4 with a flow rate of 0.5 mL/min and the volume injected for each analyzer was 10 L. Each standard of lactic acid, acetic acid, propionic acid, and butyric acid was also prepared. The preparation of standard solutions was carried out by taking pure standard solutions with different volumes and dissolved in distilled water up to 10 mL so that several different concentrations of solutions were obtained with a concentration range of 100-1500 mM by diluting each standard solution until 7 (seven) concentrations were available (mM). The seven concentration solutions of each standard were then injected into the HPLC column with a volume of at least two times the volume of the loop sample, which was 10 µL. The injection results were in the form of a chromatogram in the form of peaks indicating the area and concentration of the injected solution. Calculation of the final concentration (mM) followed the equation:

$$\text{Final concentration}\left(\text{mM}=\frac{\mu\text{mol}}{\text{mL}}\right) = \frac{\text{standard solution volume}(\mu\text{L})\cdot\frac{1\,\text{mL}}{1000\,\mu\text{L}}\cdot\text{density}\left(\frac{\text{g}}{\text{mL}}\right)\cdot\frac{\text{purity}}{\text{molecular weight}\left(\frac{\text{g}}{\text{mol}}\right)}\cdot\frac{1000\,\text{mmol}}{1\,\text{mol}}\cdot\frac{1000\,\mu\text{mol}}{1\,\text{mmol}}}{\text{final volume of solution (mL)}}$$

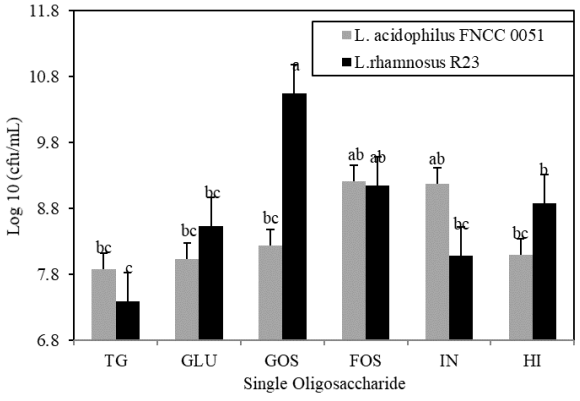
2.5. Statistical analysis

Statistical analysis using ANOVA General Linear Model with Minitab Statistical Software, Release 16 for Windows was used to determine the significance of the variation ($P < 0.05$) between the mean in each trial. The Honestly Significant Difference value in the Tukey test is used to determine a significant difference.

3. Results and Discussion

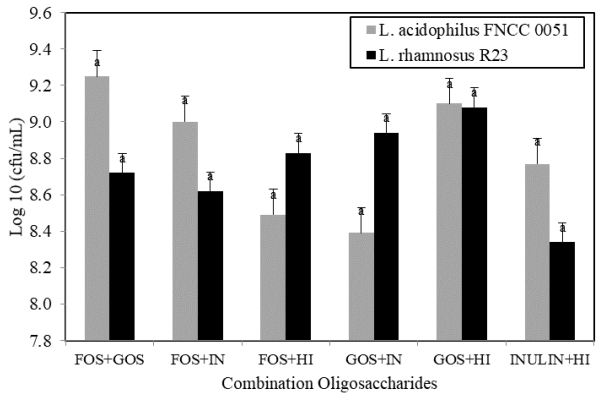
3.1. Effect of single and combined oligosaccharides on LAB growth and pH value

L. acidophilus FNCC 0051 and *L. rhamnosus* R23 can grow in an oligosaccharide medium as a single and combined carbon source containing cholesterol and oxbile (bile salts) indicated by the growth of total LAB (log10 CFU/mL), which is presented in Figure 2 and Figure 3. The growth of *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 was influenced ($p<0.05$) by the type of single carbon source, but the different combinations of oligosaccharides did not affect the growth of LAB isolates ($p>0.05$).



Note: *) TG = No Glucose, GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences ($P<0.05$)

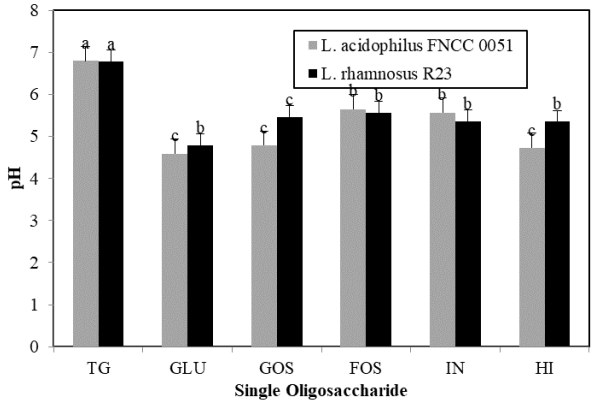
Figure 2. LAB population (log10 CFU.mL^{-1}) on media with a single oligosaccharide containing cholesterol for 24 hours of incubation



Note: *) FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences (P<0.05)

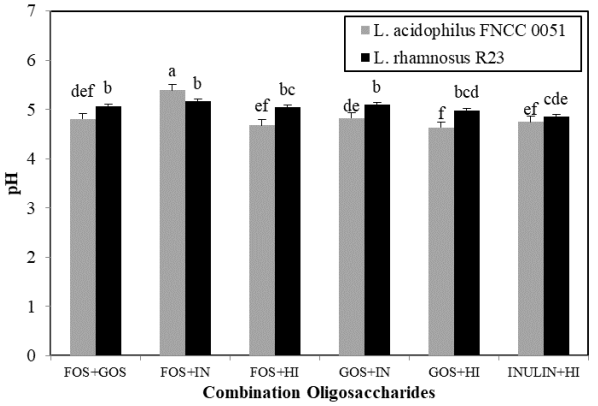
Figure 3. LAB population (log10 CFU.mL^{-1}) with a combination of oligosaccharides and medium containing cholesterol for 24 hours of incubation

The availability of adequate nutrients in the substrate is utilized by LAB to grow and develop. Molecules of complex oligosaccharide compounds are broken down into simpler units before these compounds enter the cell to be used as metabolic substrates in the synthesis of cell components. The highest population of *L. rhamnosus* R23 was obtained from single media GOS, which was log 10.54±1.10 CFU/mL, while the highest population of *L. acidophilus* FNCC 0051 was obtained from media containing a combination of FOS and GOS, which was 9.25 CFU/mL. This can be influenced by the effectiveness of prebiotics, namely the ability of each isolate to produce enzymes to hydrolyze oligosaccharides. FOS is hydrolyzed by the enzyme -fructokinase [29] and the enzyme -galactosidase, which plays a role in the breakdown of GOS [30]. The presence of growth indicates the possibility that the tested isolates can produce this enzyme. The decrease in pH value by the activity of *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 showed a significant difference (p>0.05) or affected oligosaccharides and glucose compounds (non-prebiotic).



Note: *) TG = No Glucose, GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences (P<0.05)

Figure 4. The pH value of media with a single oligosaccharide containing cholesterol for 24 hours of incubation



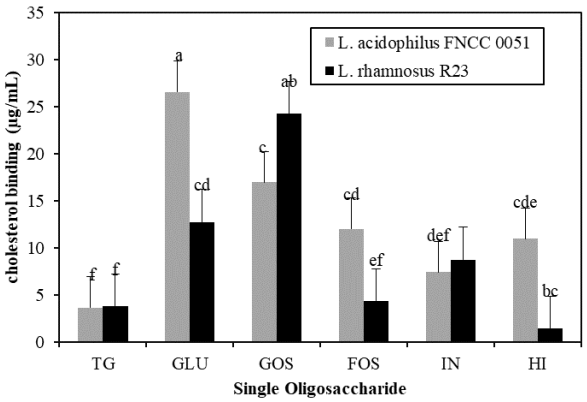
Note: *) FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences (P<0.05)

Figure 5. pH value in media with a combination of oligosaccharides containing cholesterol for 24 hours of incubation

The decreasing pH value indicated the ability of LAB isolates to grow on oligosaccharides from pH 6.8 to the range of pH 5.65-4.59 on single media (Figure 4). In the combination medium, it reached a pH range of 5.39-4.63 (Figure 5). [31] reported that in inulin fermentation by *L. acidophilus* for 24 hours, there was a decrease in the pH value and an increase in lactic acid levels, both in the treatment without inulin and inulin administration. The formation of lactic acid lowers the pH value. Changes in pH occurred during the fermentation process due to the metabolism of LAB in the medium [32]. The final pH value of the media was significantly affected (p<0.05) by the type of single carbon source and the combination of oligosaccharides. This happens because enzymes influence LAB isolates, which metabolize available nutritional sources to produce lactic acid as the end product of LAB metabolism. The pH value also influences LAB population growth because pH affects the function of membranes, enzymes, and other cell components, especially those containing protein elements. Too extreme changes in pH values affect the stability of the protein, which the protein undergoes a coagulation process at its isoelectric point. Changes in pH also affect cell permeability and enzyme synthesis [33].

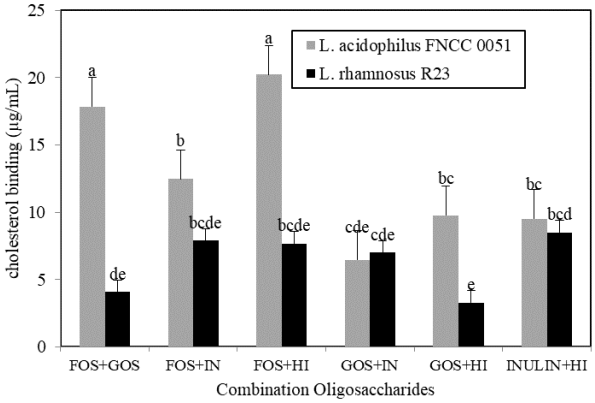
3.2. Effect of single and combined oligosaccharides on cholesterol binding to cells

The ability of LAB isolates to bind cholesterol in a medium with a single carbon source and a combination of oligosaccharides containing cholesterol and oxbile (bile salts) is shown in Figure 6 and Figure 7.



Note: *) TG = No Glucose, GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences (P<0.05)

Figure 6. Binding of cholesterol in cell pellets with medium containing single oligosaccharides for 24 hours of incubation



Note: *) FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Letter differences indicate significant differences (P<0.05)

Figure 7. Binding of cholesterol in cells with a medium containing a combination of oligosaccharides for 24 hours

The concentration of cholesterol absorbed by cells or binding of cholesterol to the cell surface of *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 was influenced by the type of carbon source, either single or mixed ($p<0.05$). The highest cholesterol-binding ability was found in *L. rhamnosus* R23 isolate, which occurred in a single oligosaccharide medium with a GOS medium. The results were not significantly different from glucose medium (non-prebiotic). Meanwhile, the binding of cholesterol to cells was significantly affected by the combination of carbon sources ($p<0.05$). In combination with oligosaccharides, the highest cholesterol-binding occurred in *L. acidophilus* FNCC 0051 and the combination of FOS medium with inulin hydrolyzate. The results were not significantly different from the combination of FOS and GOS. Oligosaccharide media can affect the growth of bacterial cells. This suggests that the possible mechanism of action on the LAB isolates is (1) the binding of cholesterol through the transfer of cholesterol from the media into the cellular membrane during LAB cell growth [34]. Since the lipids of Gram-positive bacteria were found mainly in the cell membrane, the decrease in cholesterol from the media by the isolates was due to the incorporation of cholesterol into the cell membrane. It may have changed the fatty acid composition of the bacterial cells [35].

The ability of cholesterol metabolism for cell growth related to cholesterol assimilation can be influenced by factors such as type of media, presence of bile salts, bacterial growth phase, viability, and a number of bacterial cells [34]

3.3. Effect of LAB fermentation on oligosaccharide media on organic acids and production of SCFA

The composition of organic acids produced by the fermentation process of *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 isolates on media containing oligosaccharides and cholesterol are presented in Table 1 and Table 2. The type of carbon source or oligosaccharide used as the fermentation medium affects the composition of organic acids, including SCFA (acetic acid, propionic acid, and butyric acid). The type of single carbon source significantly affected the proportion of lactic acid and acetic acid produced by *L. acidophilus* isolate FNCC0051 ($p<0.05$). However, it did not affect the proportion of propionic acid and butyric acid. In contrast, in *L. rhamnosus* R23, a single carbon source affected ($p<0.05$) the proportion of organic acids, including SCFA. The combination of carbon sources produced by *L. acidophilus* FNCC0051 affected the proportion of lactic acid ($p<0.05$), but not the composition of SCFA (acetic, propionic, and butyric acids). The type of combination of carbon sources affects the proportion of lactic and butyric acid produced by *L. rhamnosus* R23.

Table 1. Production of SCFA from fermented LAB isolates on single oligosaccharide media containing cholesterol and bile salts

Carbon source	*L. acidophilus* FNCC 0051				*L. rhamnosus* R23			
	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric Acid (mM)	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric Acid (mM)
Single								
GLU	142.95±3.74^{a}	68.15±8.81^{a}	3.83±1.26^{a}	4.66±0.74^{a}	108.62±0.08^{a}	39.98±0.25^{ab}	4.19±0.03^{c}	14.52±0.04^{c}
GOS	0.61±0.07^{d}	0.99±0.34^{b}	0.44±0.13^{a}	5.89±2.46^{a}	72.89±0.51^{b}	38.56±0.18^{b}	19.60±0.14^{b}	12.43±0.17^{d}
FOS	44.89±2.10^{c}	64.58±0.26^{a}	4.70±1.51^{a}	5.55±0.97^{a}	16.56±0.09^{e}	34.48±0.07^{c}	20.48±0.15^{a}	10.80±0.26^{e}
IN	50.83±6.11^{c}	60.83±2.45^{a}	4.12±1.24^{a}	6.11±0.84^{a}	50.10±0.00^{c}	41.42±1.03^{a}	3.08±0.02^{d}	22.05±0.06^{a}
HI	107.90±0.68^{b}	61.17±5.45^{a}	5.29±1.48^{a}	5.71±0.89^{a}	45.45±0.00^{d}	38.22±0.00^{b}	2.67±0.00^{e}	21.34±0.00^{b}

Note: *) GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. The average value (n = 2) the difference in letters in the same column shows a significant difference (P<0.05)

Table 2. Production of SCFA from LAB isolate fermentation on combined oligosaccharide combination media containing cholesterol and bile salts

Carbon source	*L. acidophilus* FNCC 0051				*L. rhamnosus* R23			
	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric Acid (mM)	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric Acid (mM)
Combination								
FOS+GOS	114.25±3.74^{a}	59.72±5.20^{a}	6.60±3.76^{ab}	4.12±0.54^{b}	96.20±0.00^{a}	41.43±0.00^{a}	3.42±0.00^{a}	16.56±0.00^{a}
FOS+IN	61.58±0.06^{b}	60.56±1.82^{a}	6.21±0.07^{ab}	4.87±0.12^{ab}	59.13±0.59^{b}	42.01±0.13^{a}	4.24±0.26^{a}	6.56±0.28^{d}
FOS+HI	107.75±2.28^{a}	54.91±1.70^{a}	6.72±2.14^{ab}	5.73±0.35^{ab}	53.51±0.04^{b}	41.39±0.54^{a}	4.32±0.26^{a}	5.86±0.33^{d}
GOS+IN	114.32±0.84^{a}	55.55±2.09^{a}	5.52±1.58^{ab}	5.26±0.47^{ab}	90.09±0.26^{a}	41.22±1.33^{a}	4.52±0.11^{a}	9.97±0.21^{b}
GOS+HI	0.50±5.06^{c}	1.15±0.03^{b}	0.00±0.00^{b}	6.96±0.91^{a}	95.55±13.2^{a}	43.83±6.63^{a}	4.65±0.76^{a}	9.04±1.21^{bc}
IN+HI	112.83±0.36^{a}	64.53±2.64^{a}	10.84±2.71^{a}	6.89±0.61^{a}	80.64±0.02^{a}	44.01±1.27^{a}	3.68±0.01^{a}	7.67±0.09^{cd}

Note: *) GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. The average value (n = 2) the difference in letters in the same column shows a significant difference (P<0.05)

The highest proportion of lactic acid produced by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 was produced from media with a glucose carbon source. In *L. acidophilus* FNCC 0051, the highest proportion of lactic acid was produced in the single oligosaccharide inulin hydrolyzate and the combination of FOS medium with GOS, FOS with inulin hydrolyzate, GOS with inulin, and inulin with inulin hydrolyzate. Meanwhile, for *L. rhamnosus* R23, in the combination media of FOS with GOS, GOS with inulin, and GOS with inulin hydrolyzate. The high proportion of lactic acid indicates that the two bacteria belong to the group of homofermentative bacteria. Where the factors that affect the utilization of prebiotic oligosaccharides by probiotic bacteria depend on the species. The ability of each isolate to produce enzymes that can hydrolyze oligosaccharides is a factor that affects the effectiveness of prebiotics. β-fructosidase enzymes in hydrolyzing FOS [29] and β-galactosidase enzymes play a role in the breakdown of GOS [30]. The lowest proportion of lactic acid produced by *L. acidophilus* on GOS media is not in line with the LAB growth population on GOS media compared to other media. This is likely to occur because the metabolites from the activity of *L. acidophilus* isolates containing cholesterol undergo incorporation of cholesterol into cell membranes and change the composition of fatty acids into bacterial cells. This is in accordance with the study of Ooi and Liong [28], which showed that the ability of cholesterol-binding/cholesterol transfer from the medium to the cellular membrane varied significantly among different bacterial strains.

A higher proportion of acetic acid from lactate was produced from FOS media and inulin by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 activity from FOS media. Isolate of *L. acidophilus* FNCC 0051 on single carbon source medium containing GOS (Table 1). In the combination of carbon source media of GOS with inulin hydrolyzate, *L. acidophilus* isolate was more dominated by butyric acid than the total concentration of organic acids produced. In the media containing the combination of FOS with inulin by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23, the proportions of lactic acid and acetic acid were comparable, as well as in the medium with the combination of FOS with inulin hydrolyzate (Table 2).

Several studies have reported that differences in the final product produced depend on the type of strain, carbon source, culture medium, and growth medium [36]. Prebiotics is a source of SCFA both in vitro and in vivo, although the relative yield per gram of fermented substrate has not been studied. However, there is no specific characteristic that distinguishes the SCFA production pattern. Another study [37] reported that starch consistently produced relatively more butyrate while oligofructose and inulin were the lowest productions. Arabinogalactan and polydextrose produce relatively more propionate, whereas oligofructose produces acetate predominantly. Another study also mentioned that in an in vitro study consuming 20 g of oligofructose daily for four weeks, the molar ratio of acetate: propionate: butyrate at 12 hours was 63:12:25 [38].

This study showed that the high proportion of lactic acid in glucose produced by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 indicated that the two bacteria belonged to the group homofermentative bacteria in fermenting glucose. Homofermentative bacteria use the process of glycolysis through the Embden Meyerhof Pathway (EMP), which oxidizes glucose to 2 pyruvic acids, producing 2 ATP. The NADH produced in this pathway is used to reduce pyruvate to lactic acid. Nuraida *et al.* [39] stated that the results of identifying several LAB isolated from breast milk, including *L. rhamnosus* R23, based on the physiological and biochemical characteristics of the LAB isolates were classified as homofermentative bacteria. However, according to Oliveira *et al.* [36], Lactobacillus rhamnosus is a heterofermentative facultative bacteria that ferment hexoses such as lactose and fructose to lactic acid, while pentoses produce a mixture of lactate and acetic acid.

Table 3 and Table 4 present the total acid and total sugar consumed, and yield of organic acids produced by *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 from single and combined carbon sources in a medium containing cholesterol. The decrease in total sugar indicated that bacteria could utilize prebiotic oligosaccharides as the single sugar contained in the media for their growth. The growth of LAB in the media converts sugar into lactic acid and other metabolites [15]. The total sugar consumed by LAB isolates during incubation showed a significant difference ($p<0.05$) in all media containing oligosaccharides. This indicates that prebiotic oligosaccharides as a single carbon source or combination of oligosaccharides can be utilized by LAB for their growth.

Table 3. Total acid, total sugar consumed, and yield of organic acids fermented by LAB isolates on single oligosaccharide media containing cholesterol

Carbon source	*L. acidophilus* FNCC 0051			*L. rhamnosus* R23		
	Total acid (mM)	Total sugar consumed (g/L)	The yield of Organic acids (mM/g)	Total acid (mM)	Total sugar consumed (g/L)	The yield of Organic acids (mM/g)
Single						
GLU	219.59±9.81^{a}	13.94±1.02^{ab}	15.81±1.18^{a}	167.32±0.23^{a}	35.89±1.95^{a}	4.66±0.01^{b}
GOS	7.93±2.06^{d}	8.06±3.29^{b}	1.08±0.36^{c}	143.49±0.65^{b}	19.06±3.18^{b}	7.53±0.03^{a}
FOS	119.66±0.64^{c}	13.88±5.06^{ab}	9.37±3.13^{b}	82.32±0.42^{e}	19.86±6.39^{b}	4.15±0.02^{c}
IN	121.88±5.73^{c}	19.61±2.01^{a}	6.26±0.65^{b}	116.65±0.95^{c}	30.17±8.75^{ab}	3.87±0.03^{d}
HI	180.06±6.72^{b}	13.22±0.63^{ab}	13.64±0.67^{a}	107.68±0.00^{d}	30.56±4.73^{ab}	3.52±0.00^{e}

*) GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Differences in letters in the same column indicate significant differences (P<0.05)

Table 4. Total acid, total sugar consumed, and yield of organic acids fermented by LAB isolates on combination media of oligosaccharides containing cholesterol

Carbon source	*L. acidophilus* FNCC 0051			*L. rhamnosus* R23		
	Total acid (mM)	Total sugar consumed (g/L)	The yield of Organic acids (mM/g)	Total acid (mM)	Total sugar consumed (g/L)	The yield of Organic acids (mM/g)
Combination						
FOS+GOS	184.68±12.16^{a}	13.17±2.58^{c}	14.40±2.83^{a}	157.61±0.00^{a}	102.80±1.77^{a}	1.53±0.00^{cd}
FOS+IN	133.22±1.70^{b}	28.22±7.42^{ab}	4.99±1.55^{c}	111.94±1.27^{bc}	61.00±8.54^{b}	1.84±0.02^{c}
FOS+HI	175.10±3.05^{a}	14.67±3.71^{c}	12.52±3.48^{ab}	105.08±0.66^{c}	103.39±1.35^{a}	1.02±0.01^{d}
GOS+IN	180.65±4.98^{a}	11.83±2.92^{c}	15.94±4.14^{a}	145.8±0.97^{ab}	109.33±9.72^{a}	1.30±0.01^{cd}
GOS+HI	8.60±1.30^{c}	19.33±4.10^{bc}	0.46±0.11^{c}	153.07±1.76^{a}	47.50±3.75^{b}	3.22±0.46^{b}
IN+HI	195.09±9.80^{a}	32.22±4.26^{a}	6.13±0.87^{bc}	136.00±1.19^{abc}	20.89±5.11^{c}	6.69±0.06^{a}

*) GLU = Glucose (Non prebiotic), FOS = fructooligosaccharides, GOS = Galactooligosaccharides, IN = Inulin, HI = Inulin Hydrolyzate. Differences in letters in the same column indicate significant differences (P<0.05).

The yield of organic acids in a single or combined medium fermented by *L. acidophilus* FNCC 0051 was higher than that of *L. rhamnosus* R23. The largest organic acid yield was obtained by the activity of *L. acidophilus* on the combination of GOS and inulin media, which was 15.94±4.14 mM/g. Meanwhile, by the activity of *L. rhamnosus* R23 on GOS media, the highest organic acid yield was 7.53±0.03 mM/g. The type of LAB influences the yield of organic acids produced. This is related to the ability of *L. acidophilus* FNCC 0051 isolate to metabolize oligosaccharide compounds influenced by the structure of the DP substrate (degree of polymerization) and the extracellular enzymes produced by the isolate in breaking down oligosaccharides. Lactic acid bacteria isolates more easily metabolize the low degree of polymerization. FOS and GOS have a low degree of polymerization (DP), i.e., DP 2-7 and DP 2-8, respectively, inulin with a high DP (DP 2-60) and inulin hydrolyzate have a low DP range of 2-7 [40]. The resulting enzyme is inductive, produced when the appropriate substrate is in the LAB growth environment [30]. Several studies have shown that the SCFA profile depends on the physicochemistry of the substrate or the chemical structure of carbohydrates [41]. This is in accordance with Wei *et al.* [42], where the fermentability and prebiotic effects were reported to be related to their physicochemical characteristics, including solubility, monosaccharide composition, molecular weight (Mw), and the content and position of substitute groups.

4. Conclusion

L. acidophilus FNCC 0051 and *L. rhamnosus* R23 can use oligosaccharides as a carbon source for their growth and produce several organic compounds, including SCFA (acetate, propionate, and butyrate). The production of organic acids produced in a medium containing cholesterol in the presence of single or combined oligosaccharides generally results in a high proportion of lactic acid and acetic acid compared to other organic acids. *L. acidophilus* FNCC 0051 in metabolizing oligosaccharide compounds produced a higher concentration of acetic acid than *L. rhamnosus* R23. Cholesterol binding to cells was influenced by the type of combination of carbon sources, with the best results in medium with single oligosaccharide GOS in *L. rhamnosus* isolate and medium with combined oligosaccharide FOS with GOS, and FOS with Inulin on *L. acidophilus* isolates. For this reason, *L. acidophilus* FNCC 0051 and *L. rhamnosus* R23 isolates with probiotic properties were able to provide physiological effects in lowering cholesterol levels.

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