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Cholesterol-lowering activity by lactic acid bacteria isolated from yogurt from Boyolali, Indonesia

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Abstract. *Nurcahyani I, Susilowati A, Pangastuti A. 2023. Cholesterol-lowering activity by lactic acid bacteria isolated from yogurt from Boyolali, Indonesia. Asian J Nat Prod Biochem 21: 34-45.* Hypercholesterolemia is considered a risk factor for cardiovascular disease, as it is characterized by increased cholesterol levels in the bloodstream that exceed the established normal range. At present, cardiovascular disease stands as the foremost cause of mortality worldwide, with a global death toll of 17.7 million. The employment of Lactic Acid Bacteria as a probiotic has the potential to reduce cholesterol levels via the enzymatic of Bile Salt Hydrolase (BSH), which facilitates the deconjugation of bile salts, as well as its capacity to assimilate cholesterol directly. The consumption of fermented dairy products that have undergone appropriate bacterial fermentation has been found to potentially contribute to a reduction in blood cholesterol. The region of Boyolali in Indonesia is known for its yogurt production; regularly consuming probiotic products has been suggested as a dietary approach to promoting long-term hypocholesterolemia effects. The objective of this research was to isolate and evaluate the efficacy of Lactic Acid Bacteria (LAB) in reducing cholesterol levels, and to identify the specific isolates derived from yogurt originating from Boyolali. The activity of the BSH enzyme was evaluated through qualitative testing by observing precipitation zones on the growth media. A quantitative assessment was also conducted using the UV-vis spectrophotometer method at λ570 nm. LAB isolates' activity in bile salt deconjugation and cholesterol assimilation was also measured using the UV-vis spectrophotometer method at λ660 nm and λ550 nm, respectively. The bacterial identification process was conducted through the examination of both macroscopic and microscopic morphology. The study employed a One-way Analysis of Variance (ANOVA) to compare the release of amino acids (specifically glycine and taurine), the release of free cholic acid, and the disparity in cholesterol levels between media that were inoculated with LAB and those that were not. The BSH enzyme activity was observed to be 1.11-3.82 U/mL for sodium glycocholate substrate and 0.85-3.13 U/mL for sodium taurocholic substrate. The recorded levels of bile salt activity were 0.52-1.26 µmol/mL for the sodium glycocholate substrate and 0.43-0.9 µmol/mL for the sodium taurocholic substrate. The percentage of assimilated cholesterol ranges from 38.69% to 71.98%.

Keywords: Bile salt hydrolase, lactic acid bacteria, lowering cholesterol

INTRODUCTION

According to the World Heart Federation (2003), Death from Cardiovascular Disease (CVD) jumped globallly from 12.1 million in 1990 to 20.5 million in 2021. Cardiovascular disease was the leading cause of death worldwidw in 2021. Hypercholesterolemia is considered a risk factor for cardiovascular disease. This condition is characterized by increased bloodstream cholesterol levels exceeding the normal range (Freed 1994). Individuals diagnosed with hypercholesterolemia are at a significantly elevated risk of experiencing a heart attack, with a threefold increase in risk compared to those with a typical blood lipid profile. Numerous methods have been employed to decrease cholesterol levels, including pharmacological agents to manage hypercholesterolemia. However, this intervention is associated with high costs and adverse effects, as Kumar et al. (2012) reported.

Recently, numerous investigations have been carried out on the functionality of Lactic Acid Bacteria (LAB), commonly known as probiotic bacteria, that can potentially lower cholesterol levels. Studies conducted by Zeng et al. (2010) and Ishimwe et al. (2015) have demonstrated the efficacy of diets incorporating LAB strains in reducing overall cholesterol levels and decreasing lipoprotein cholesterol concentration at lower densities. Prior studies have indicated that certain LAB strains can lower cholesterol levels. Specifically, *Lactobacillus casei* (Klaver and Van Der Meer 1993), *Lactobacillus plantarum, Lactobacillus paracasei* (Belviso et al. 2009), *Lactobacillus lactic* (Kimoto et al. 2002), and *Enterococcus faecium* (Hlivak et al. 2005; Ayyash et al. 2018) have been identified as such strains. The consumption of dairy products that have undergone fermentation by appropriate bacterial strains has been shown to reduce blood cholesterol. However, it is worth noting that the bacterial strains present in yogurt products are typically not indigenous to the human gastrointestinal tract. According to Lertcanawanichakul et al. (2015), regular intake of probiotic products may serve as a dietary intervention for inducing sustained hypercholesterolemic effects.

The process of cholesterol reduction by lactic acid bacteria is attributed to the enzymatic activity of Bile Salt Hydrolase (BSH), which deconjugates bile salts and facilitates cholesterol assimilation (Burhan et al. 2017).

The BSH enzyme is responsible for the hydrolysis of conjugated bile salts, which involves the amide bond cleavage in the conjugated bile salts. The liberation of free cholic acid occurs through the activity of the BSH enzyme, leading to the release of amino acid residues and deconjugated bile salts (Kumar et al. 2012). According to De Smet et al. (1994), unbound cholic acid and no cost attached to it exhibit lower solubility and reduced efficacy in lipid absorption within the intestinal tract. Consequently, it is eliminated from the body through fecal excretion. According to Kumar et al. (2012), cholesterol reduction can be achieved by deconjugating bile salts by LAB. According to Begley et al. (2006), the bile salts lost through fecal excretion increase the demand for cholesterol to facilitate the enteropathic synthesis of bile salts. Consequently, this process leads to a reduction in cholesterol within the body.

MATERIALS AND METHODS

Materials

Yogurt samples were obtained from Boyolali District, Central Java Province, Indonesia.

Procedure

Yogurt samples

The sample liquid was obtained from yogurt products from home production in Dusun 3, Kiringan, Boyolali District, Boyolali Regency, Central Java, with a YoGood trademark. In addition, for the yogurt milk starter, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were used.

Isolation of LAB from Boyolali Yogurt

Distilled water was used to dilute Boyolali yogurt at dilution levels ranging from 10^{-1} to 10^{-5}. In each dilution series, 100 µL of the sample liquid was applied onto the MRS (De Man, Rogosa, and Sharpe) agar surface, fortified with 0.5% calcium carbonate ($CaCO_3$). The agar plates were then incubated at 37°C for 1-2 x 24 hours. The colonies that exhibited distinct, clear zones on MRS agar were carefully selected and re-inoculated using the streak method to form quadrant streaks on MRS Agar. These cultures were then incubated under identical conditions. This process was repeated until uniform colonies were obtained. The resulting isolates were subjected to catalase testing, and those with negative catalase activity were identified as LAB isolates. The selected LAB isolates were subjected to inoculation on MRS Agar slant and subsequently preserved at 4°C as a stock culture. Furthermore, the culture was transferred to MRS Broth medium for subsequent examinations. The formation of a clear zone on MRS Agar media containing 0.5% CaCO3 is attributed to the interaction between bacterial acids and calcium in the media. Bacteria capable of producing clear zones are likely to be Lactic Acid Bacteria (LAB) due to their ability to produce lactic acid.

Qualitative measurement of BSH (Bile Salt Hydrolase) activity

Bile salt hydrolase activity was measured using the methodology described by Dashkevicz and Feighner (1989) and Ahn et al. (2003). MRS A medium was aseptically prepared using the following components: MRS B at a concentration of 525 g/L, agar at 7.5 g/L, and 0.3% bile salt and $CaCl_2$ at 0.375 g/L. The resulting medium was rendered sterile. Subsequently, the aseptic disc paper was immersed in LAB isolate that had undergone an 18-hour culture period, following which the disc paper was deposited onto MRS agar. The media containing LAB isolates underwent incubation for 72 hours at a temperature of 37°C. The BSH activity is distinguished by the emergence of a zone of bile salt precipitation encircling the colony on $CaCl_2$-containing agar media. This phenomenon arises from the interaction between cholic acid and $CaCl_2$, forming precipitated salt. The manifestation of a distinct area devoid of bacterial growth in the vicinity of the colony is indicative of the hydrolytic activity facilitated by the BSH enzyme.

Quantitative measurement of BSH (Bile Salt Hydrolase) activity

The BSH activity quantification was conducted through the assessment of free amino acids resulting from the conjugation of bile salts by LAB isolates, following the methodology described by Tanaka et al. (2000). After a 20-hour incubation period at 37°C, LAB cultures were subjected to cell harvesting via centrifugation at 9,700 xg for 15 minutes. The resulting cells were washed twice with sodium phosphate buffer (100 mM, pH 6.8), supplemented with dithiothreitol (10 mM), and resuspended in the same buffer. Subsequently, the cell's Optical Density (OD) was determined through 600 nm absorption.

The sonication process was employed to eliminate intracellular enzymes from the cell suspension. It was achieved by subjecting the suspension to ultrasonic waves for 60 seconds and cooling on ice for two cycles. The resulting mixture was centrifuged at a force of 9,700 x g for 15 minutes. A reaction mixture was assembled, comprising 1.8 mL of sodium phosphate buffer (100 mM, pH 6.0), 100 µL of conjugated bile salt (200 mM), 100 µL of dithiothreitol (10 mM), and 100 µL of cell-free extract. The mixture underwent incubation at a temperature of 37°C for 30 minutes. Subsequently, the control sample was subjected to a mixture of 200 µL and trichloroacetic acid (200 µL) to halt the reaction. Both sample types underwent centrifugation at a velocity of 9,700 x g for 15 minutes.

A 200 µL sample of supernatant was mixed with an equal volume of distilled water (200 µL) and subsequently combined with a ninhydrin reagent solution consisting of 1% ninhydrin solution (0.5 mL), 30% glycerol (1.2 mL) and 500 mM sodium citrate buffer (pH 5.5; 0.2 mL) to a total volume of 1.9 mL. Subsequently, the amalgam was subjected to vortexing and boiling for 14 minutes. Next, the solution was allowed to cool down, and its absorbance was gauged at a wavelength of 570 nm, with glycine and taurine employed as standards. A single unit of BSH activity (U/mL) is precisely characterized as the quantity of

enzyme that releases one micromole (1 µL) of amino acids from the substrate within a minute.

Measurement of deconjugated sodium glycocholate and sodium tauroccolate

A sterile MRS broth of 10 mL was enriched with sodium glycocholate (6 mM) and sodium taurocholate (6 mM). Subsequently, MRS B was inoculated with a 1% LAB isolate and incubated at 37°C for 20 hours. The liberation of unbound cholic acid is the foundation for the quantitative assessment of bile salt deconjugation. The culture that underwent incubation was subjected to pH adjustment by adding 1N NaOH, followed by centrifugation at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was collected and subjected to further pH adjustment by adding 10N HCL until a pH of 1.0 was achieved.

Subsequently, 1 milliliter (1 mL) of the supernatant was transferred and introduced into a solution containing 2 mL of ethyl acetate. Subsequently, the mixture was subjected to vortexing for 60 seconds and allowed to rest until the separation phase. Following this, a volume of 2 mL from the ethyl acetate layer was extracted and subsequently transferred to a glass tube, where it was subjected to evaporation at a temperature of 60ºC. The resulting residue was solubilized in a solution of 0.01N NaOH (1 mL) and subjected to vortex agitation. A solution containing 1% furfuraldehyde (1 mL) and H_2SO_4 (16N, 1 mL) was introduced into the mixture, which was subsequently subjected to vortexing for 1 minute. The mixture was then heated at 65°C in a water bath for 10 minutes. Following the cooling process, the mixture underwent the addition of glacial acetic acid and was subjected to vortexing for 1 minute. The solution's absorbance was assessed at a wavelength of 660 nm, and the quantity of cholic acid discharged was determined by employing a standard cholic acid gradient concentration curve, as per Shehata et al. (2019).

Cholesterol assimilation measurement

PEG 600 cholesterol was introduced into the MRS broth medium, resulting in a final concentration of 100 µg/mL. One percent LAB inoculum was introduced into MRS broth-Cholesterol-PEG 600 after incubating at 37°C for 24 hours. The mixture was then incubated for an additional 24 hours at 37°C. Next, to conduct cholesterol analysis, the LAB suspension underwent centrifugation at 4,000 rotations per minute for 10 minutes at 4°C. Then, 1 mL of the resulting supernatant was extracted for further examination.

Furthermore, 1 mL of the sample was treated with 33% potassium hydroxide (KOH) and 1 mL of absolute ethanol. Subsequently, the solution underwent vortexing for 1 minute. It was incubated at 37°C for 15 minutes, then left at ambient temperature. Subsequently, a mixture comprising 2 mL of deionized water and 2 mL of hexane was introduced into the solution, followed by vortexing for 1 minute while allowing for the separation phase at ambient temperature. Subsequently, 1 mL of the hexane layer was carefully dispensed into a glass tube and subjected to solvent evaporation using a water bath operating at 65°C. Following the formation of the residue, a volume of 2 mL containing 50 mg per deciliter of o-phthalaldehyde reagent prepared in acetic acid was introduced to the sample and subsequently homogenized. After mixing, 0.5 mL of sulfuric acid (H_2SO_4) was introduced to each tube, and the resulting solution was vortexed for 1 minute. Subsequently, the solution was incubated for 20 minutes at ambient temperature. The outcome entails the quantification of the solution's absorbance at a wavelength of 550 nm through the employment of a UV spectrophotometer. The cholesterol assimilation value was determined using the standard cholesterol curve and measuring the sample's absorbance value against varying cholesterol concentrations (ranging from 0 to 0.1 mg/mL) in MRS broth. The cholesterol assimilation value was calculated by subtracting the cholesterol value before incubation from the value of the LAB isolate sample after 24 hours of incubation, measured in units of µg/mL. In addition, the proportion of assimilated cholesterol calculations involved dividing the value of cholesterol assimilation by the value of cholesterol at 0 hours.

Cholesterol assimilation by LAB is determined using the following formula: (i) Cholesterol assimilation (µg/mL) = [cholesterol (µg/mL)]0h − [cholesterol (µg/mL)]24h. (ii) Cholesterol assimilation by each LAB isolate is also calculated in percentage: assimilated cholesterol (%) = [cholesterol assimilation (µg/mL)/ cholesterol (µg/mL)0h] × 100%.

Characterization of LAB isolates

The LAB isolates were characterized through morphological observations, which involved examining the growth of colonies on culture media. The characterization process included observations of the shape of the colonies when viewed from above, the surface of the colonies when viewed from above , the edges of the colonies when viewed from the side, and the color of the colonies (Dwidjoseputro 2005). The selected isolates underwent testing for catalase activity and gram staining on specific colonies. The catalase test involves the introduction of a 3% hydrogen peroxide solution to the bacterial inoculum on a glass surface. Bubbles indicate a positive catalase reaction, while the absence indicates a negative one. The bacterial isolates that were catalase negative were observed to lack the ability to produce the catalase enzyme, which is responsible for the decomposition of hydrogen peroxide into water and oxygen. In addition, using indicators such as the clear zone on $CaCO_3$-containing media and the catalase test facilitates the identification of LAB isolates.

Data analysis

The enzyme activity test results were analyzed using both quantitative and qualitative methods. The deconjugation and assimilation processes of cholesterol were quantitatively described during the study. The study conducted the quantitative analysis using one-way ANOVA to compare the release of amino acids, free cholic acid, and cholesterol in LAB-inoculated media.

RESULTS AND DISCUSSION

Characterization of lactic acid bacteria

This study collected LAB (Lactic Acid Bacteria) isolates from yogurt products produced in Dusun 3, Kiringan, Boyolali District, Central Java, Indonesia, under the brand name YoGood. The nomenclature of the isolate is derived from the commercial product appellation, succeeded by the isolate numeral corresponding to each bacterium that was effectively extracted. Thirteen isolates were obtained from yogurt isolation, which exhibited growth on MRS agar media supplemented with 0.5% $CaCO_3$. The formation of a clear zone surrounding the colony can be attributed to the alkaline properties of $CaCO_3$, which effectively counteracts the acid production by lactic acid bacteria. Clear zones in the isolate signify its classification as a lactic acid bacteria group member. It has been observed that lactic acid can hydrolyze CaCO3, forming a distinct zone on MRS agar media. This phenomenon has been documented in studies conducted by Sun et al. (2014) and Pisol et al. (2015).

Table 1 displays the outcomes of the morphological observations. The colonies generally exhibited a circular shape with flat edges, convex angles of elevation, smooth surfaces, and a milky white hue. The observations above align with the morphological attributes of the colonies that are presumed to be lactic acid bacteria. Specifically, the colonies exhibit a circular shape with flat edges and a white to yellowish-white hue (Kurnia et al. 2020). According to Candra et al. (2007), the isolates were identified as a single species (strain) based on their colony morphology as observed macroscopically.

Subsequently, the selected isolates underwent biochemical tests, specifically the catalase test. The catalase test is a diagnostic assay utilized to ascertain the presence or absence of catalase enzyme activity in a given bacterium. The test is conducted by adding 1-2 droplets of hydrogen peroxide to the bacterial colonies. The presence of bubbles characterizes Catalase positive bacteria upon observation, whereas the absence of bubbles identifies catalase-negative bacteria. According to Delvia et al. (2015), a positive catalase test signifies the ability of bacteria to break down harmful hydrogen peroxide molecules into water and oxygen. The findings of the catalase assay revealed that 13 isolates exhibited a negative catalase reaction, thereby suggesting their classification within the lactic acid bacteria cohort. The features above align with LAB's typical attributes: gram-positive, non-spore-forming, and lacking in catalase enzyme production (catalase negative). Most of the isolates exhibit facultative anaerobic characteristics and can generate lactic acid as the primary product of fermentation, as reported by Widodo (2003).

After going through the catalase test, 13 bacterial isolates were tested further in the form of a qualitative BSH (Bile Salt Hydrolase) enzyme test. By observing the formation of a precipitation zone in MRS A growth medium containing CaCl 0.37 g/L and 0.3% oxgall, 9 bacterial isolates were selected that could produce the BSH enzyme qualitatively. The nine isolates that passed the test were subjected to microscopic morphological observations. Gram staining was carried out as a colorant for the character of the isolates based on the structure of the bacterial cell wall. The observations of the ninth isolate had Gram-positive characters, which were observed under a microscope with 40-100x magnification, as shown in Table 2.

Gram staining determines whether bacteria are Gram-positive or gram-negative. Gram-positive bacteria have a purple tint on their cells because the bacterial cell wall has thicker peptidoglycan properties than gram-negative bacteria. The crystal violet color can be bound and maintained by thick peptidoglycan. Gram-negative bacteria, on the other hand, have a red color on their cells, indicating that they cannot bind crystal violet color and are solely stained by safranin (Yulvizar 2013). The findings revealed that nine bacterial isolates were Gram-positive bacteria with stem cell shape/bacillus. Lactic acid bacteria are known to b e Gram-positive bacteria. Bacteria isolated from Sumbawa mare milk are Gram-positive bacteria with variations in the shape of long and short bacilli (Sujaya et al. 2008). So, based on the data, it is clear that the nine bacterial isolates identified from yogurt from Boyolali are lactic acid bacteria.

Table 1. Morphological characteristics of bacterial colonies from Boyolali Yogurt, Boyolali, Indonesia

Isolate Name	Macroscopic Observations				Catalase Test
	Shape	Elevation	Border	Surface	
YG-1	Bulbous	Convex	Flat	Smooth	-
YG-2	Bulbous	Convex	Flat	Smooth	-
YG-3	Bulbous	Convex	Flat	Smooth	-
YG-4	Bulbous	Convex	Flat	Smooth	-
YG-5	Bulbous	Convex	Flat	Smooth	-
YG-6	Bulbous	Convex	Flat	Smooth	-
YG-7	Bulbous	Convex	Flat	Smooth	-
YG-8	Bulbous	Convex	Flat	Smooth	-
YG-9	Bulbous	Convex	Flat	Smooth	-
YG-10	Bulbous	Convex	Flat	Smooth	-
YG-11	Bulbous	Convex	Flat	Smooth	-
YG-12	Bulbous	Convex	Flat	Smooth	-
YG-13	Bulbous	Convex	Flat	Smooth	-

Table 2. Microscopic morphological characteristics of bacterial colonies from Boyolali Yogurt, Boyolali, Indonesia (via Gram staining)

Isolate name	Microscopic Observation	
	Shape	Gram
YG-2	Rod	+
YG-3	Rod	+
YG-4	Rod	+
YG-5	Rod	+
YG-8	Rod	+
YG-9	Rod	+
YG-10	Rod	+
YG-12	Rod	+
YG-13	Rod	+

Bile salt hydrolase enzyme activity

Nine isolates were successfully selected: YG-2, YG-3, YG-4, YG-5, YG-8, YG-9, YG-10, YG-12, and YG-13. The isolates selected had good growth, and a qualitative BSH (Bile Salt Hydrolase) enzyme assay was performed to assess the presence or absence of enzyme activity produced by the bacterial isolates. Nine LAB isolates on MRS agar media containing 0.3% oxgall demonstrated BSH enzyme activity. Bacterial isolates that manufacture BSH enzymes have a cloudy white precipitation zone and/or a clear zone around them (Dong et al. 2012) or cloudy white granular colony formation (Xiong et al. 2016). Figure 1 depicts the qualitative results of the BSH enzyme test.

Table 3 displays the obtained outcomes. All nine isolates can produce the BSH enzyme. The deposition of bile acids in the form of free bile acids/cholic acid observed in the precipitation zone surrounding the isolate on MRS agar growth media supplemented with oxgall is attributed to the deconjugation of bile acids by BSH enzymes, as reported by Sirilun et al. (2010). The study by Julendra et al. (2017) revealed that the MRS media with 0.3% oxgall content was utilized as a substrate for the BSH enzyme in the qualitative test. Additionally, the inclusion of CaCl2 in the media enhanced the activity of the BSH enzyme and facilitated the precipitation of the enzyme deconjugation outcomes. The manifestation of a transparent or haloed zone surrounding bacterial isolates indicates BSH enzyme activity resulting from the precipitation of unbound bile salts. The reason for this is that isolates exhibiting positive BSH possess the capacity to sustain their proficiency in the process of hydrolyzing conjugated bile salt compounds. According to Dong et al. (2012), positive strains for BSH exhibit greater efficacy in the hydrolysis of glycolic-conjugated bile salts than taurocholate-conjugated bile salts.

The nine isolates exhibiting qualitative enzyme activity will undergo quantitative testing to ascertain the enzyme activity levels in units per milliliter (U/mL). The findings indicated that the LAB isolates exhibited varying levels of BSH enzyme production ($P<0.05$). The results indicate that the enzyme can hydrolyze sodium glycocholate and sodium taurocholate substrates, as presented in Table 4. The activity of the BSH enzyme was observed on two different substrates, namely glycocholate and taurocholic. The resulting BSH activity on the glycocholate substrate ranged from 1.11 to 3.82 (U/mL), while on the taurocholic substrate, it ranged from 0.87 to 3.13 (U/mL). The YG-2 isolate exhibited the greatest enzyme activity, with a recorded value of 3.82 (U/mL) for the sodium glycocholate substrate and 3.13 (U/mL) for the taurocholic substrate. The YG-4 isolate exhibited the least BSH activity on the sodium glycocholate substrate, measuring at 1.11 (U/mL), while the YG-10 isolate exhibited the least BSH activity on the sodium taurocholic substrate, measuring 0.85 (U/mL).

Overall, the findings of this investigation suggest that the total activity of BSH enzymes on the glycocholate substrate was greater than that observed on the sodium taurocholate substrates (as presented in Table 4). This observation implies that the BSH enzymes exhibit more hydrolysis towards bile salts conjugated with glycine. Variations in enzyme activity can be attributed to the substrate specificity of the BSH enzyme, as noted by De Smet et al. (1994). The enzymatic substrate can be influenced by the existence of amino acid segments, such as glycine and taurine, as conjugates, or steroid side chains that serve as substrates for the BSH enzyme, as reported by Gilliland et al. (1985). According to Begley et al. (2005), bacterial strains that exhibit positivity for the BSH enzyme display distinct variations in their affinity and activity towards conjugated substrates. The differential affinity exhibited by certain bacterial strains towards specific substrates, particularly the hydrolysis of BSH, has been identified as a crucial factor for their survival in vivo (Prete et al. 2020). The study's findings suggest that the metabolism of glycine-conjugated bile acids is preferred by several bacterial strains over taurine.

The greater efficacy of BSH enzymes on glycocholate substrates in comparison to sodium taurocholate substrates can be attributed to the heightened toxicity of glycine-conjugated bile salts towards bacterial cells under low pH conditions in the intestinal tract, as opposed to taurine-conjugated bile salts, which exhibit lower toxicity. According to Miremadi et al. (2014) and Anwar and Sameer (2018), the ratio of taurine-conjugated bile salts to glycine-conjugated bile salts in the human digestive tract is smaller at 1:3. This results in higher efficiency of the BSH enzyme in the hydrolysis of glycolic bile acids during bile acid metabolism in vivo.

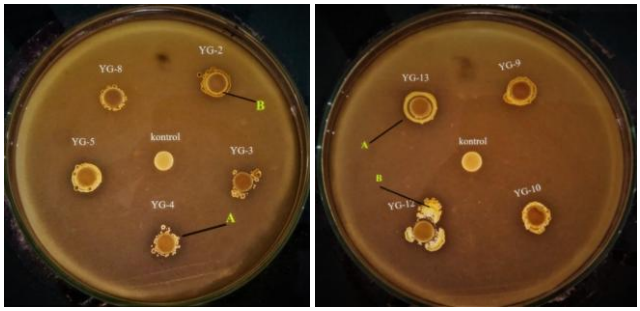

Figure 1. Qualitative BSH enzyme activity test results on MRS A media containing 0.3% oxgall. Note: A: Clear zone, B: Precipitation zone, Control: Without administration of LAB isolate

Table 3. Qualitative BSH enzyme activity by LAB isolates on MRS A media containing 0.3% Oxgall

Isolate name	BSH enzyme activity
YG-2	+
YG-3	+
YG-4	+
YG-5	+
YG-8	+
YG-9	+
YG-10	+
YG-12	+
YG-13	+

Note: Has BSH enzyme activity (+), does not have BSH enzyme activity (-)

Table 4. Quantitative BSH enzyme activity by LAB isolates on sodium glycocholate and sodium taurocholic substrates

Isolate name	BSH Enzyme Activity (U/mL) [1]			
	Sodium Glycocolate [2]		Sodium Taurocholic [2]	
	Sample	Control [3]	Sample	Control [3]
YG-2	3.82 ± 0.0478 [i]	0.32 ± 0.0095 [a]	3.13 ± 0.2003 [h]	0.16 ± 0.0000 [a]
YG-3	2.39 ± 0.2705 [g]	0.29 ± 0.0005 [a]	1.68 ± 0.1143 [e]	0.15 ± 0.0008 [a]
YG-4	1.11 ± 0.0269 [b, c]	0.37 ± 0.0005 [a]	1.55 ± 0.1362 [d, e]	0.16 ± 0.0064 [a]
YG-5	1.41 ± 0.1069 [c, d, e]	0.36 ± 0.0008 [a]	2.00 ± 0.2031 [f]	0.18 ± 0.0046 [a]
YG-8	1.51 ± 0.1518 [d, e]	0.33 ± 0.0009 [a]	2.47 ± 0.2206 [g]	0.18 ± 0.0002 [a]
YG-9	2.50 ± 0.2589 [g]	0.38 ± 0.0011 [a]	1.23 ± 0.0710 [c, d]	0.19 ± 0.0000 [a]
YG-10	2.56 ± 0.0499 [g]	0.36 ± 0.0086 [a]	0.85 ± 0.0007 [b]	0.19 ± 0.0000 [a]
YG-12	1.57 ± 0.1158 [d, e]	0.31 ± 0.0004 [a]	0.91 ± 0.0031 [b]	0.17 ± 0.0002 [a]
YG-13	3.03 ± 0.2234 [h]	0.28 ± 0.0002 [a]	0.87 ± 0.0594 [b]	0.16 ± 0.0004 [a]

Note: 1. One unit of BSH activity (U/mL) is measured based on the amount of enzyme that releases 1 µmol of amino acids from the substrate per minute. 2. Enzyme substrate used. 3. Control containing reaction mixture (cell-free extract, conjugated bile salt)+ Trichloroacetic acid to halt reaction. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a-i indicates the results in the column with different lowercase letters indicate a significant difference ($P \leq 0.05$)

The BSH enzyme activity generated by LAB obtained from yogurt originating from Boyolali was observed to be greater in amount compared to the BSH enzyme activity reported in prior investigations. Kumar et al. (2012) conducted a study wherein a Lactobacillus strain sourced from fecal matter was found to produce BSH enzyme activity on sodium glycocholate substrate. The enzyme activity was measured to be 0.517 U/mL by *L. plantarum* Lp21. On the other hand, *L. plantarum* Lp91 produced the highest BSH enzyme activity of 0.299 U/mL on sodium taurocholic substrate. Liong and Shah's (2005) investigation revealed that L. acidophilus exhibited a greater overall BSH enzyme activity than *L. casei* when tested on sodium glycocholate and taurocholic substrates. The enzymatic activity of BSH on substrates of sodium glycocholate was observed to range between 0.45-1.81 U/mL, while on substrates of sodium taurocholic, the activity ranged between 1.06-1.47 U/mL. In addition to the findings of Thakkar et al. (2016), it has been observed that LAB derived from multiple fermented food sources can generate BSH enzymes within the range of 0.048 to 0.097 µmol/mL per minute. The study by Bhat and Bajaj (2020) revealed that the LAB isolates M5 and M9, obtained from breast milk, exhibited enzyme activity capable of releasing 0.057 and 0.052 µmol/mL glycine per minute, respectively.

The activity variance of the resultant BSH enzymes is subject to the influence of the environmental conditions in which Lactic Acid Bacteria (LAB) thrive. Tanaka et al. (2000) and Begley et al. (2006) have reported that LAB isolates derived from the digestive tract and mammalian feces can produce BSH enzymes in an environment abundant in conjugated and deconjugated bile acids. Strains or species derived from environments lacking bile salts, such as dairy products and fermented vegetables, cannot synthesize BSH enzymes. As per prior literature, it was asserted that the function of BSH enzymes was exclusively associated with commensal bacteria present in the gastrointestinal tract. The study reported that all Lactobacillus strains obtained from the intestine could hydrolyze glycine and taurine-conjugated bile acids (Reyes et al. 2014).

Numerous studies have demonstrated that BSH enzyme activity can be produced by isolates derived from environments beyond the digestive tract. The study conducted by Prete et al. (2020) demonstrated through chromatographic analysis that *L. plantarum*, a food-borne strain, can modify the bile acid profile by producing unconjugated bile acids. The observed disparity in the reaction between the control and sample groups suggests that all strains have the potential to deconjugate bile acids, despite not being derived from the intestinal environment.

The capacity of *L. plantarum* to synthesize BSH enzymes and perform bile acid deconjugation can be regarded as an adaptive mechanism to its surroundings. The lactic acid bacterium, *L. plantarum*, can inhabit diverse ecological niches, such as the fermentation of milk, meat, and different kinds of vegetables, as well as the human gastrointestinal tract (De Vries et al. 2006). According to Klereebezem et al. (2003), *L. plantarum* has a genome size of 3.3Mb, one of the largest among lactobacilli. This characteristic allows the bacterium to adapt to diverse environmental niches. The genome of *L. plantarum* WCFSI, as reported by Molenaar et al. in 2005, is anticipated to contain four BSH-associated genes, denoted as bsh1 to bsh4, which are dispersed throughout the genome of the Lb strain. The study exclusively examined the bsh1 gene, which is accountable for the metabolism of bile acids in *L. plantarum*.

Out of the 13 isolates that were obtained from yogurt in the present investigation, it was observed that only 9 isolates exhibited the capacity to generate BSH enzyme activity on both sodium glycocholate and sodium taurocholate substrates. This finding demonstrates that the capacity to produce BSH enzymes was not universal among LAB isolates obtained from yogurt. The BSH enzyme activity level in YG-2 isolates was higher than that of several LAB strains previously studied in humans and the intestine. The production of enzyme activity by the nine isolates can be considered an adaptive mechanism employed by bacterial isolates to ensure their survival in the intestinal milieu. Prete et al. (2020) reported that BSH activity in *L. plantarum* isolated from food-borne sources was found to be equivalent to that of human isolates.

Bacterial isolates may possess one or multiple bsh-coding genes that exhibit activity within the intestinal milieu, despite the isolate's non-gastrointestinal origin. This phenomenon represents an adaptive mechanism of the isolate to the environment, facilitating the metabolism of bile acids. As per the findings of Reyes et al. (2014), it was observed that the bsh coding sequence was present across all significant phyla, and the enzymatic activity of the produced enzymes varied across different environments.

Christiaens et al. (1992) conducted a study to assess the BSH activity and growth of *L. plantarum* 80 obtained from forage grass, with regards to the existence of conjugated bile acids in vitro. The present investigation has demonstrated that the bsh LP80 gene exhibits homology with the DNA of intestinal lactobacilli, thereby leading to the expression of the BSH enzyme. The potential correlation between the silage ecosystem and the digestive ecosystem can be elucidated by the existence of the BSH enzyme in Lactobacillus strains that have been extracted from fodder grasses, as reported by Tannock (1990) and Van Renterghem et al. (1991).

The liquid containing bile salts is a surface-active compound that can permeate and interact with the lipophilic cytoplasmic membrane of bacteria, resulting in alterations and impairment of the bacterial membrane architecture. The surface active compounds can activate lipolytic enzymes that can interact with fatty acids present in the cell membrane, ultimately influencing the permeability of the cell and leading to cellular damage, as Astuti and Rahmawati (2010) stated. The crucial criterion to identify LAB isolates as potential probiotic candidates is their capacity to endure the gastrointestinal tract's harsh conditions. The resistance of LAB to oxgall, or bile salts, is closely linked to the activity of the BSH enzyme. This enzyme is responsible for the hydrolysis of conjugated bile salts into a deconjugated form, thereby mitigating the toxic effects of bile acids on bacterial cells (Zulfidin et al. 2018).

Lactic Acid Bacteria (LAB) possessing the capability to synthesize the BSH enzyme exhibit a favorable trait of thriving and establishing themselves in the distal portion of the small intestine, which is the site of enterohepatic circulation. De Smet et al. (1995) reported in an in vitro investigation that the activity of BSH serves as a resistance mechanism against intracellular acidification caused by conjugated bile salts. It is in line with the research by Bustos et al. (2012), who showed strains that were able to hydrolyze bile salts or had BSH enzymes (+) were more resistant to exposure to some conjugated bile salts and strains that did not have BSH enzymes (-) showed a significant percentage of cell death. From the process of hydrolysis of bile salts by BSH enzymes, conjugated bile salts will dissociate so that these substances can enter cells through active transport or passive diffusion, so that when inside the cells, conjugated bile salts will be converted into a weaker form for bacterial cells, namely deconjugated bile salts. The result of hydrolysis in the form of free amino acids released from the bile salt deconjugation process can be used as a carbon, nitrogen, or energy source by bacteria. According to Begley et al. (2006), the metabolic breakdown of glycine results in the production of ammonia and carbon dioxide, whereas the metabolic breakdown of taurine yields ammonia, carbon dioxide, and sulfate.

Apart from the function of BSH, whereby LAB can indirectly lower cholesterol levels, the enzymatic deconjugation of bile salts in the gastrointestinal tract can enhance the elimination of bile salts. According to Noh et al. (1997), the C-24 N-acyl amide bond connecting the bile acids and amino acids in conjugated bile salts can be cleaved by the BSH enzyme, leading to the formation of deconjugated bile salts. The deconjugation of bile salts reduces solubility and hinders absorption within the intestinal lumen, in contrast to their conjugated counterparts. Due to this enzymatic process, free bile acids are liberated and subsequently eliminated through fecal excretion (De Smet et al. 1994; De Rodas et al. 1996). Cholesterol, the precursor of bile salts, can substitute for the lost molecules of bile salts during the excretion process, thereby reducing the cholesterol level in the serum.

Several studies have demonstrated that LAB isolates possessing the capacity to synthesize BSH enzymes exhibit the potential to decrease Low-Density Lipoprotein (LDL) levels in the bloodstream. According to Ooi et al. (2010) and Malpeli et al. (2015), ingesting yogurt containing *L. reutei* CRL 1098 for four weeks has significantly reduced total cholesterol and LDL-c. Additionally, a six-week trial involving capsules containing BSH-active strains of *L. acidophilus* and inulin has significantly reduced LDL and total cholesterol, significantly. The BSH enzyme facilitates the formation of deconjugated bile salts, specifically free cholic acid, which are subsequently eliminated via the fecal route. This process reduces the amount of bile acid reabsorbed into the liver from the digestive tract, increasing the demand for cholesterol. Therefore, to maintain homeostasis of bile acids, the body will sequester surplus cholesterol within the system, thereby reducing cholesterol levels in the bloodstream. In the given circumstances, there will be a surge in the requirement for endogenous cholesterol synthesis, leading to the activation of LDL-α receptors in the liver. It, in turn, will result in an upsurge in the uptake of LDL-C by the liver, ultimately leading to a decrease in LDL-C concentrations (Nur et al. 2021; Bhat and Bajaj 2019).

Bile salt deconjugation activity

The findings indicated that the nine LAB isolates could deconjugate both types of bile salts utilized as substrates, as presented in Table 5. The LAB isolates exhibited deconjugation capabilities within the range of 0.52-1.26 (µmol/mL) on the sodium glycocholate substrate, while on the taurocholic substrate, their ability to deconjugate bile salts ranged from 0.43-0.9 (µmol/mL). The YG-2 isolate demonstrated the most significant deconjugation activity on the glycocholate substrate, yielding a value of 1.26 µmol/mL. Similarly, the YG-2 isolate exhibited the highest activity level on the taurocholic substrate, with a 0.9 µmol/mL value. The deconjugation potential of nine isolates was determined by measuring the liberation of free cholic acid. A positive correlation was observed between the amount of cholic acid released and the level of deconjugation activity.

Table 5. Deconjugation activity of sodium glycocolate substrate and sodium taurocholic substrate by LAB isolates

Isolate name	Deconjugation activity (µmol /mL) [1]	
	Sodium Glycocolate [2]	Sodium Taurocholic [2]
YG-2	1.26 ± 0.0026 [p]	0.90 ± 0.0015 [n]
YG-3	1.25 ± 0.0027 [p]	0.48 ± 0.0006 [d]
YG-4	0.93 ± 0.0040 [o]	0.80 ± 0.0010 [l]
YG-5	0.81 ± 0.0007 [l]	0.47 ± 0.0031 [d]
YG-8	0.65 ± 0.0006 [j]	0.67 ± 0.0018 [k]
YG-9	0.55 ± 0.0004 [g]	0.43 ± 0.0008 [c]
YG-10	0.52 ± 0.0013 [e]	0.48 ± 0.0012 [d]
YG-12	0.82 ± 0.0010 [m]	0.54 ± 0.0018 [f]
YG-13	0.61 ± 0.0014 [h]	0.64 ± 0.0007 [i]
Control [3]	0.20 ± 0.0031 [a]	0.12 ± 0.0014 [b]

Note: 1. Bile salt deconjugation activity is measured based on the amount of cholic acid released 1 µmol of amino acids from the substrate per milliliter (mL). 2. Substrate used. 3. Control, containing media + conjugated bile salt, without, LAB isolate. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a-p indicates the results in the column with different lowercase letters indicating a significant difference ($P \leq 0.05$)

Generally, the outcomes of the bile salt deconjugation examination revealed that the LAB isolates exhibited a greater capacity to liberate free cholic acid on glycocholate substrates than taurocholate substrates ($P \leq 0.05$). It suggests that the variance in substrates can influence the activity of bile salt deconjugation, as shown in Table 5. The results indicate a significant difference in the bile salt deconjugation activity between the two substrates, as evidenced by using distinct lowercase letters to denote each result in the column. The subsets in Duncan's table are represented by distinct alphabet letters for each column. The ANOVA test revealed a notable dissimilarity, indicating that sodium glycocholate substrates' deconjugation was more efficient than sodium taurocholic. A higher quantity of liberated cholic acid evidenced it.

The bile salt deconjugation test and the BSH enzyme activity test indicate greater activity on the glycocholate substrate than the sodium taurocholate substrate in this study. The observed variation in activity suggests that the BSH enzyme exhibits substrate recognition capabilities towards bile acids, specifically towards the cholicate steroid core and amino acid groups (glycine or taurine). The identification of substrates is primarily based on the amino acid groups they contain. According to Taranto and de Valdez (1999), most BSH enzymes exhibit greater efficacy in the hydrolysis of bile salts conjugated with glycine instead of taurine. Furthermore, examining the configuration of the BSH enzyme across diverse species has revealed the presence of specific residues within the enzyme's active site that serve as a crucial element in the binding of the substrate. The recognition of substrates by the active site and the provision of information regarding selective substrates in BSH enzymes have been reported in previous studies (Rossocha et al. 2005).

According to a study conducted by Liong and Shah (2005), it was found that most BSH enzymes exhibit higher efficacy in the hydrolysis of glycine-conjugated bile salts as compared to taurine-conjugated bile salts. The study observed that eleven strains of *L. casei* and *L. acidophilus* released a greater amount of cholic acid in deconjugating bile salts devoid of deconjugated sodium glycocholate, in contrast to sodium taurocholate. De Smet et al. (1995) observed that the BSH enzyme exhibits a higher specificity for the hydrolysis of Glycodeoxycholic Acid (GDCA) as compared to Taurodeoxycholic Acid (TDCA). The research conducted by Moser and Savage (2001) demonstrates that the steroid structure of bile salts plays a crucial role in determining the specific substrate specificity of BSH. Conversely, the analysis of conjugated bile acid hydrolases by *Clostridium perfringens* reveals a lack of specificity for particular substrates, which can be attributed to the absence of active site residues on the enzyme that is necessary for binding to the conjugated bile acid substrate (Rossocha et al. 2005). In essence, the BSH enzyme possesses specific active site residues that enable it to bind to the appropriate conjugated bile acid substrate.

It is a widely acknowledged fact that many probiotic strains possess multiple homologous BSH genes. Certain BSH genes found in bacteria can confer advantages, such as optimizing bacterial defense mechanisms in response to fluctuating environmental circumstances. The activity of enzymes towards substrates is subject to variation, further modulated by the BSH gene. The BSH gene exhibits specificity towards distinct bile salt compositions and protects varying durations of exposure to bile salts (Begley et al. 2006). The findings indicate that the isolates exhibited a greater capacity for deconjugating the two bile salts than in earlier investigations. Specifically, LAB isolates obtained from breast milk demonstrated the ability to deconjugate taurocholic bile salts within the range of 0.06-0.25 µmol cholic acid per mL, with the most notable performance being exhibited by *Pediococcus pentocaceus* 1-A38 (Nuraida et al. 2011). According to Pranata et al. (2016), *L. plantarum* 1.R.1.3.2 and *Lactobacillus acidophilus*, which were isolated from LAB, exhibited the ability to deconjugate taurocholic bile salts at a concentration of 0.58 µmol/mL. Similarly, Pato (2003) reported that six LAB isolates obtained from curd could release cholic acid at concentrations ranging from 0.21 µmol/mL to 0.45 µmol.

Probiotic bacteria that exhibit Bile Salt Hydrolase (BSH) activity can induce a hypocholesterolemic response by catalyzing the deconjugation of bile salts. The deconjugation of bile salts has been observed to decrease serum cholesterol levels by stimulating the production of bile acids to compensate for the loss of cholesterol in fecal matter. Additionally, this reaction has been found to diminish the solubility and absorption of cholesterol in the intestinal lumen, as reported by Lye et al. (2010). The solubility of cholesterol is greatly influenced by its interaction with bile salts, given its insolubility in aqueous solutions. According to Wang (2003), the efficacy of bile salts in emulsifying decreases when they are deconjugated. As a result, cholesterol becomes less soluble, which can hinder the formation of micelles in the intestinal tract.

During the deconjugation of bile salts, the resultant cholic acid reduces pH levels (<5.0) within the fermentation medium. This acidic bacterial environment can interfere with the formation of stable cholesterol micelles in the body. Consequently, cholesterol may

precipitate with free bile salts resulting from deconjugation (Klaver and Van der Meer 1993). The phenomenon of cholesterol deposition and subsequent cholic acid formation has been observed to impede cholesterol absorption within the gastrointestinal system potentially. The deposition of cholesterol alongside free bile acids represents a direct mechanism for reducing cholesterol levels, in addition to the assimilation of cholesterol by Lactic Acid Bacteria (LAB). The phenomenon of cholesterol deposition necessitates the presence of a BSH enzyme that exhibits optimal activity within a pH range of 5-6. Under pH less than 6 conditions, the deconjugated bile salts tend to coalesce with cholesterol, leading to precipitation. However, an elevation in the pH level of the medium to 7 can facilitate the dissolution of cholesterol back into the medium (Tahri et al. 1996).

Cholesterol assimilation activity

The selected lactic acid bacteria exhibit the trait of being capable of cholesterol assimilation. The direct assimilation of cholesterol within the intestine can diminish the absorption of cholesterol ingested from the digestive tract into the bloodstream. Therefore, using probiotics that possess hypocholesterolemic or cholesterol-lowering properties presents a viable option for averting the onset of cardiovascular disease.

The study revealed that nine distinct strains of Lactic Acid Bacteria (LAB) obtained from yogurt in Boyolali exhibited the capacity to lower cholesterol levels in the media. It was evidenced by the significant assimilation of cholesterol, with values ranging from 39.59 to 111.92 µg/mL, as presented in Table 6. The assimilated cholesterol value is a metric derived from the disparity between the quantity of cholesterol recorded during the initial incubation and the quantity recorded following a 24-hour incubation period. LAB YG-2 Isolate exhibited the greatest assimilated cholesterol value of 111.92 µg/mL, while YG-4 isolate demonstrated the lowest value of 39.59 µg/mL. A negative correlation exists between the disparity in cholesterol quantity in the media and the extent of cholesterol level reduction observed in the stated media.

Moreover, calculating the percentage of assimilated cholesterol involves dividing the difference into cholesterol levels in the media, previously determined, by the cholesterol level at the initial time. Table 6 and Figure 2 indicates that LAB isolates exhibit varying degrees of cholesterol assimilation, ranging from 38% to 71%. The YG-13 isolate yielded the highest percentage, measuring 71.98%, whereas the YG-4 isolate produced the lowest percentage, measuring 38.69%. The study's findings indicate that YG-13 exhibited the highest assimilation percentage value of 71.98% among the LAB isolates tested. However, it is noteworthy that the highest assimilated cholesterol value was observed in the YG-2 LAB isolate, which recorded a value of 111.92 µg/mL. It is important to note that the observed value difference could be attributed to variations in the total cholesterol level at 0 hours across the tested LAB isolates during measurement. Therefore, it is recommended to employ percentage calculations to compare the results accurately.

Table 6. Assimilated cholesterol value and percent assimilation of cholesterol by bacterial isolates

Isolate name	Assimilated cholesterol [1] (µg/mL)	% of Assimilated cholesterol [2]
YG-2	111.92 ± 0.3712 [f]	68.82 ± 0.3138 [e, f]
YG-3	60.93 ± 1.3867 [c]	54.59 ± 0.4368 [c]
YG-4	39.59 ± 0.0818 [b]	38.69 ± 0.0692 [b]
YG-5	103.24 ± 1.1330 [e,f]	62.59 ± 0.8818 [d, e]
YG-8	94.40 ± 0.0750 [e]	56.07 ± 0.0477 [c, d]
YG-9	77.28 ± 0.1531 [d]	48.83 ± 0.1108 [c]
YG-10	73.24 ± 10.373 [d]	53.88 ± 7.6343 [c]
YG-12	77.02 ± 0.2591 [d]	52.37 ± 0.1608 [c]
YG-13	72.20 ± 0.2095 [d]	71.98 ± 0.2091 [f]
Control [3]	4.84± 2.0721 [a]	3.98 ± 1.6124 [a]

Note: 1. Assimilated cholesterol is measured based on the difference in the amount of cholesterol released in µg per milliliter (mL) at 0-hour and 24 hours. 2. The percentage of cholesterol is measured by dividing the difference between the amount of assimilated cholesterol and the value of cholesterol at the 0-hour. 3. Control, containing media + cholesterol, without LAB isolate inoculation. Results are expressed as mean value of tripiclate (mean) ± SEM (standard error); n = 3. a- f indicates the results in the column with different lowercase letters indicating a significant difference ($P \leq 0.05$)

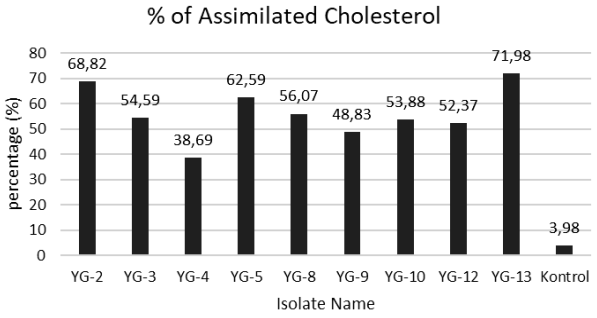

Figure 2. Assimilated cholesterol percentage

Statistically significant differences were observed between the control, cholesterol assimilation results, and cholesterol percentage ($P<0.05$). The graph illustrates that isolate YG-13 exhibited the highest percentage of assimilated cholesterol. The observed disparity in percentage between the isolate samples and the control can be attributed to the absence of LAB isolates in the control treatment, which was conducted using growth media that contained cholesterol. As a result, the control treatment did not exhibit any cholesterol assimilation activity. In the control group where LAB was not introduced, the total cholesterol levels remained elevated at the initial time point and after 24 hours of incubation. Consequently, the difference in total cholesterol levels was minimal when assimilated cholesterol was calculated, resulting in a low percentage. In the treatment group where LAB was administered, it was observed that the initial high levels of cholesterol decreased significantly after 24 hours. Furthermore, there was a substantial difference in the total cholesterol levels following the assimilation activity. This

finding demonstrates that incorporating LAB isolates can elicit a cholesterol-lowering effect in the medium.

The present investigation revealed a higher cholesterol level assimilation activity exhibited by the LAB isolates under examination than that demonstrated by LAB isolates obtained from prior research sources. According to a study by Nuraida et al. in 2011, LAB isolates obtained from breast milk exhibited cholesterol assimilation activity ranging from 0.86-14.97 µg/mL. The highest assimilation activity was observed in *Pediococcus pentosaceus* 1-A38. Burhan et al. (2017) conducted a study wherein they examined 8 LAB strains that were isolated from *dangke* (a traditional food from Enrekang, Sulawesi) and beef. The study yielded *L. fermentum* B111K and *L. plantarum* IIA-1A5 strains, which exhibited the potential to reduce cholesterol in vitro. The cholesterol assimilation rates of *L. fermentum* B111K and *L. plantarum* IIA-1A5 were 4.10% and 8.10%, respectively.

The variance in the cholesterol assimilation capacity of the bacteria examined in this investigation, as compared to prior studies, was impacted by the existence or nonexistence of bile salts in the testing milieu (Lye et al. 2010) and disparities in the cholesterol origins employed in the trial (Kusumawati 2002). Notably, cholesterol exhibits a considerably low solubility in water, thereby rendering it arduous to dissolve in water-based MRS B media. The limited solubility of cholesterol in aqueous solutions significantly impacts the bacterial assimilation of cholesterol. Lye et al. (2010) assert that the presence of bile salts impacts the capacity to eliminate cholesterol. Most tested strains exhibit the capacity to decrease or eradicate elevated cholesterol levels in test media containing bile salts, as opposed to control media lacking bile salts. Bile salts, classified as biological surfactants, can reduce surface tension, thereby enhancing the cellular attachment of cholesterol. These bile salts have a superior capacity to dissolve cholesterol than their solubility in aqueous solutions.

The present investigation yielded a comparatively elevated level of cholesterol assimilation activity in comparison to prior research endeavors. This outcome was attributed to the inclusion of 0.3% oxgall and pure cholesterol in the culture medium, whereas earlier studies solely employed pure cholesterol without supplementing bile salts. Consistent with the findings of Lye et al.'s (2010) investigation, it was observed that adding oxgall to the media resulted in a decrease in cholesterol concentrations. This effect was attributed to the activity of a *Lactobacilli* strain that was incubated at 37°C for 20 hours. Specifically, the strain *L. bulgaricus* FTDC 1311 exhibited the greatest cholesterol assimilation activity. The observed assimilated cholesterol activity in media containing cholesterol and the specified substance was 62.42 µg/mL. In contrast, media containing solely cholesterol exhibited an assimilated cholesterol activity of 27.89 µg/mL.

The variations observed in the cholesterol assimilation capacity among the bacterial strains examined were associated with the distinct characteristics of each strain, specifically the composition and physicochemical attributes of the peptidoglycan present in their respective cell walls. This finding was reported by Kimoto et al. (2002). Within the cholesterol assimilation mechanism, a portion of the cholesterol will undergo direct uptake or absorption, subsequently integrating with the bacterial cellular membrane. The study conducted by Lye et al. (2010) demonstrated the attachment of cholesterol by *L. bulgaricus* FTDC 1311, as evidenced by the use of Scanning Electron Microscopy (SEM). The direct binding of cholesterol to the surface of *lactobacilli* cells during fermentation was observed, resulting in a textured cell surface compared to the bacterial cell surface in the control group. These findings suggest probiotic conditions may induce *L. bulgaricus* FTDC 1311 cholesterol attachment. Kimoto et al. (2002) conducted research that revealed that the distribution of fatty acids in bacterial cells varied between media containing cholesterol and media lacking cholesterol. The interaction between cholesterol and the bacterial cell membrane can alter the fatty acid composition of the bacterial cell. This phenomenon has been widely discussed in various media outlets. According to Noh et al. (1997), Lactobacilli bacteria that can assimilate cholesterol exhibit greater resistance to interference from sonication. Therefore, it can be attributed to the fact that cholesterol absorption during the assimilation process can alter the composition of the bacteria's cell wall and cell membrane.

Lim et al. (2017) conducted a study wherein the qualitative analysis of pellet cells of LAB 4 and LAB 12 isolates was performed using fluorescence microscopy with the Neil Red staining method. The study results indicated that both LAB cells could assimilate cholesterol, as evidenced by the appearance of a higher intensity of red fluorescence on the surface of the bacterial cell from the media containing cholesterol compared to the control, wherein LAB isolates were incubated without cholesterol. The process of cholesterol assimilation by probiotic bacterial strains in the digestive tract has reduced cholesterol absorption by enterocytes. This reduction in cholesterol absorption has been linked to a decreased risk of developing coronary heart disease, as reported by Aquino et al. (2017). The inhibition of micelle formation in the intestine can occur due to cholesterol binding to the cell surface. The disruption of micelle formation results in reduced cholesterol entering the systemic circulation. Furthermore, decreasing cholesterol levels (Bhat and Bajaj. 2019).

The capacity to reduce cholesterol levels is a significant probiotic characteristic, as elevated serum cholesterol levels are recognized as a primary contributor to the heightened likelihood of developing metabolic disorders, such as cardiovascular disease, obesity, and coronary heart disease (Park et al. 2018). According to Wang et al. (2018), using probiotics is a non-pharmacological strategy that can mitigate the risk factors associated with cardiovascular disease by decreasing elevated levels of serum cholesterol within the human body. The consumption of probiotics derived from fermented products containing specific bacterial strains has been shown to elicit a reduction in levels of cholesterol concentration. According to Pan et al. (2011), it is necessary to regularly consume probiotic products to achieve long-term hypocholesterolemic effects,

as the strains responsible for these effects are not naturally present in the human digestive tract.

In conclusion, nine isolates obtained from yogurt originating from Boyolali exhibited in vitro cholesterol-lowering activity by producing BSH enzymes, deconjugating bile salts, and assimilating cholesterol. Therefore, of the nine LAB isolates, the YG-2 isolate exhibited the most significant BSH enzyme activity and bile salt deconjugation, measuring 3.82 U/mL and 1.26 (µmol/mL) for sodium glycocholate. The YG-13 isolates exhibited the highest cholesterol assimilation capacity, specifically 71.98%. The correlation between the activity of BSH enzymes and bile salt deconjugation was demonstrated by the higher activity observed against glycocholate substrates compared to sodium taurocholate.

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