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Research Article

Inhibitory Activity of Fermented Milk Multi Strain Lactic Acid Bacteria Against *Staphylococcus Aureus* ATCC 25923

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ABSTRACT

The aim of the study was to investigate inhibitory activity of fermented milk multi strain lactic acid bacteria (LAB): *Lactobacillus casei* Shirota, *Lactobacillus acidophilus*, and *Lactobacillus bulgaricus* against *Staphylococcus aureus* ATCC 29523 at variable concentration milk using diffusion method. The multi strain culture of LAB were inoculated on skimmed and diary cow milk (DCM) at concentration of 50% and 100%; in which carbon, amino acid, fat and water concentration were different. Effect of the milk and its concentration on the inhibitory activity against the test bacteria was observed as well as decreasing milk acidity and increasing of milk viscosity after 24 hours incubation. Results showed that the performance of LAB in two kinds of milks at 50% and 100% concentrations were different. On the lower milk concentration, the acidity was lower than on the 100% milk, while on the higher milk concentration, the viscosity was bigger than on the 50% concentration. The fermented milk of *L. cassei* Sirota did not show inhibitory activity. The multi strain of LAB on the DCM at concentration of 50% and 100% showed antibacterial activity againts *Staphylococcus aureus* ATCC 25923, while the skimmedmed milk fermentation showed lower inhibitory activity on both concentrations.

Keywords: *Lactobacillus acidophilus, L. bulgaricus, L. casei* Shirota, skimmed milk, diary cow milk.

Introduction

Probiotic is known as live microbial feed or food supplements; by which beneficially affect the host obtained by improving intestinal microbial balance. FAO-WHO has adopted the definition of the probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Anonymous, 2002). These foods are well suited to promote the health image of probiotics since fermented food and dairy products in particular already have a positive health effect (Helland et al., 2004). Most commonly they have been lactobacilli such as *Lactobacillus acidophilus, Lactobacillus bulgaricus and Lactobacillus casei*. The use of probiotic bacterial cultures stimulates the growth of preferred micro-organisms, crowds out potentially harmful bacteria, and reinforces the body's natural defence mechanisms (Saarela et al., 2000).

Most of probiotic products are daily consumed; the most popular is yogourt that made from milk as a fermented substrate and prebiotic. Prebiotic is an alternative for probiotics cofactors (Grajek et al., 2005). They are defined as non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of probiotic bacteria in the colon. This role is played by fermentable carbohydrates that stimulate the growth of some Gram-positive bacteria (Grajek et al., 2005). The growth of probiotic bacteria in DCM and skimmed milk has been reported. The DCM consists of water 87%, milk fat 3.45%, amino acid 26.5%, lactose 4.6%, mineral, acids (citrate, formate, acetate, lactate, oxalate), enzymmes (peroxidase, catalase, phospatase, lipase), gas (O_2, N_2), and vitamins (vit. A, vit. C, vit. D, thiamin, riboflavin) while the skimmed milk has the same compositions unless the milk fat was under 1% (Anonymous, 2008). The main end products of carbohydrate metabolism by the probiotics are short-chain fatty acids, namely acetic, butyric, and propionic acids, by which the host of organism used as an energy source.

The physiological effects related to probiotic bacteria include the reduction of gut pH, production of some digestive enzymes and vitamins, production of antibacterial substances, e.g., organic acids, bacteriocins, hydrogen peroxide, diacetyl, acetaldehyde, lactoperoxidase system, lactones and other unidentified substances, reconstruction of normal intestinal microflora disorders caused by diarrhoeas, antibiotic therapy and radiotherapy, reduction of cholesterol level in the blood, stimulation of immune functions, suppression of bacterial infections, removal of carcinogens, improvement of calcium absorption as well as the reduction of faecal enzyme activity (Grajek et al., 2005). Immunomodulator effect of LAB combined with guava extract has been proven in vitro (Isnaeni, 2016).

In this work, two types of LAB fermented milk were produced in order to evaluate inhibitory activity of *L. casei* Shirota in single and mixed culture. Two types of fermented milk were prepared; one based on DCM milk and the other prepared in skimmed milk. Both of milk was made in 50% and 100% concentrations. Furthermore, the effect of adding lactobacilli was studied in relation to pH and viscosity produced during fermentation. The LAB fermented milks were analysed for pH, viscosity, and

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inhibitory activity against *Staphylococcus aureus* ATCC 25923 after 24 hours fermentation.

Materials and Methods

Lactobacillus casei Shirota, Lactobacillus acidophilus, Lactobacillus bulgaricus and *Staphylococcus aureus* ATCC 25923 are obtaines from Institute of Tropical Disease, Airlangga University. MRS (Difco), Nutrient Agar (Difco), and skimmed milk were obtained from market, while the DCM was obtained from Jemursari animal husbandry.

Preparation of LAB starter

Fresh SCM and skimmed milk were pasteurated at 90 °C for 10 minutes, then allowed to cool to 45 °C. Starter of LAB was made by inoculating two ml of *L. casei* Shirota and mixed culture of LAB inoculum consisted of *L. casei* Shirota, *L. acidophilus*, and *L. bulgaricus* to 10 mL of milk and then incubating for 24 hours. Each stater of LAB was added to 100 mL pasteurized milk at 45 °C, and incubated for 24 hours at 37 °C.

Preparation of LAB fermented milk

LAB stater (50 mL) of was trasfered into 1000 mL of homogenized pasteurized DCM and skimmed milk, then divided into 10 by pouring 100 mL into sterile Erlenmeyer flask and incubated for 24 hours at 37 °C. Preparation of LAB fermented milk using 50% DCM and skimmed milk was carried out in the same manner. Dilution of the milks was done by sterile water at the same temperature.

Analysis of pH and viscosity of LAB fermented milk

Samples were taken every 3 hours for observation of acidity, viscosity, and inhibitory activity test. The pH levels were measured during fermentation using pHmeter Schott and the viscosity were measured by viscometer cup and bob.

Preparation of test media

Inhibitory activity was observed according to Kirby Bauer difussion method using well/hole as a reservoir. Suspension of the test bacteria was prepared by inoculated Staphylococcus aureus ATCC 29523 in fresh slant NA medium, incubated at 37 °C for 24 hours. Ten mL of saline was added to the culture, vortexed and the Optical Density (OD) of the bacteria suspension was measured by spectrophotometer at 580 nm, adjusted the suspension to obtain 25% transmitant. Test media Nutrient Agar was prepared in two layers, 10 mL for base layer without test bacteria addition and 8 mL seed layer inoculated by 5 µL of suspension or inoculum of the test bacteria. The holes on reservoir were made by perforator in 7 mm diameter.

Inhibitory activity test

Each reservoir well was filled by 50 µL of sample, incubated at 37 °C for 24 hours. The inhibitory activity was observed as a clear zone around the reservoir. Diameter of the growth inhibitory zone (mm) was measured by digital caliper.

Results and discussions

The presented results showed changes in pH and fermented milk viscosity. The DCM fermentation produced lower pH values significantly than skimmed milk, depend on concentrations of LAB in milk. One of the fermented milk products using LAB is lactic acid in addition to other organic acids. This is what causes a decrease in pH. Laurens et al (2001) reported that lactic acid present in yogurt is then produced from the glucose moiety of lactose rather than the galactose moiety. Thus, galactose accumulates in fermented milk products. Free galactose can later be utilized by *L. bulgaricus* (Laurens et al., 2001). The pH level obtained in 50% DCM fermented with *L. casei* Shirota alone was significantly lower than all other pH values measured (Table I and Table II).

Table I. Characteristic of *Lactobacillus casei* Shirota fermented milk

hours	*Characteristic of Lactobacillus casei Shirota fermented by*							
	DCM 50%		DCM 100%		Skimmed milk 50%		Skimmed milk 100%	
	pH ± SD	Viscosity ± SD (dPas)	pH ± SD	Viscosity ± SD (dPas)	pH ± SD	Viscosity ± SD (dPas)	pH ± SD	Viscosity ± SD (dPas)
0	6.80±0.00	0.30±0.00	6.80±0.00	0.30±0.00	6.80±0.00	0.30±0.00	6.80±0.00	0.30±0.00
3	4.89±0.08	0.30±0.00	4.81±0.06	0.37±0.06	4.99±0.40	0.30±0.00	5.25±0.31	0.30±0.00
6	4.45±0.18	0.47±0.06	4.57±0.13	0.67±0.06	4.61±0.04	0.40±0.06	5.06±0.34	0.40±0.00
9	4.32±0.06	0.57±0.06	4.34±0.07	0.80±0.00	4.46±0.09	0.47±0.06	4.85±0.18	0.46± 0.06
12	4.14±0.05	0.70±0.1	4.31±0.04	0.90±0.00	4.26±0.08	0.50±0.10	4.57±0.12	0.56± 0.06
18	3.94±0.11	0.86±0.06	4.05±0.03	1.00±0.00	4.16±0.06	0.66±0.06	4.38±0.06	0.70±0.10
24	3.82±0.17	0,97 ±0,06	4.03±0.01	1.50±0.00	4.08±0.09	0.70±0.00	4.19±0.08	0.85± 0.06

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Table II. Characteriztic of *L. casei* Shirota, *L. acidophilus*, *L. bulgaricus* mixed culture fermented milk

Chracteristic of L. casei Shirota, L.acidophilus and L.bulgaricus mixed culture fermented by

Hours	DCM 50%			DCM 100%		
	pH ± SD	Viscosity ± SD (dPas)	Activity ± SD	pH ± SD	Viscosity ± SD (dPas)	Activity ± SD
0	6.80±0.00	0.30±0.00	-	6.80±0.00	0.30±0.00	-
3	4.30±0.09	0.50±0.00	14.27 ± 1.40	4.72±0.33	0.33± 0.04	12.93 ± 0,61
6	4.15±0.04	0.63 ±0.04	14.03 ± 1.60	4.60±0.07	1.33 ±0.24	14.67 ± 0,66
9	4.04±0.07	0.86 ±0.07	16.23 ± 0.78	4.52±0.13	1.50 ±0.00	14.14 ± 1,96
12	4.07 ±0.07	1.33 ±0.04	16.96 ± 0.47	4.42±0.06	2.00 ±0.00	14.97 ± 1,64
18	3.97 ±0.07	1.33 ±0.24	17.20 ± 0.68	4.34±0.04	2.00 ±0.00	15.40 ± 1,53
24	3.96 ±0.07	1.73 ±0.04	17.23 ± 0.89	4.27±0.03	2.33 ±0.24	16.07 ± 1,40

Hours	Skimmed milk 50%			Skimmed milk 100%		
	pH ± SD	Viscosity ± SD (dPas)	Activity ± SD	pH ± SD	Viscosity ± SD (dPas)	Activity ± SD
0	6.80±0.00	0.30±0.00	10.53 ± 0.00	6.80±0.00	9.07± 6.47	-
3	4.95 ±0.03	0.30±0.00	10.13 ± 7.19	4.91±0.06	4.03 ± 5.71	9.07± 6.47
6	4.62 ±0.04	0.43 ±0.04	14.17 ± 2.95	4.63±0.09	4.03 ± 5.71	4.03 ± 5.71
9	4.47 ±0.09	0.53 ±0.04	14.58± 3.39	4.49±0.12	13.37± 1.36	4.03 ± 5.71
12	4.28 ±0.07	0.63 ±0.04	14.36 ± 2.93	4.44±0.15	10.06 ±7.21	13.37±1.36
18	4.21±0.01	0.83 ±0.04	13.40 ± 4.81	4.29±0.02	10.73 ± 7.68	10.06 ±7.21
24	4.18 ±0.01	0.93 ±0.24	10.53 ± 0.00	4.30±0.01	9.07± 6.47	10.73 ± 7.68

Decrease in pH value was faster in milk with 50% concentration, as compared to milk 100% concentration even though 100% milk contained twice the amount of lactose that converted into lactic acid. These low pH values could be attributed to the milk concentration that added into the formula obtained a low pH value.

Increasing viscosity was followed by the addition of substrate concentration. The concentration of milk affected the viscosity through total solidity in the substrat. Increasing concentration mainly increases the lactose, protein, and fat content. These components would increase the substrate viscosity. High total solidity would inhibit the growth of probiotic bacteria despite serving more energy sources to convert.

The antimicrobial effect exerted by LAB is the production of lactic acid that led to reduced pH value, chemical substance (bacteriocins, acetic acid, diacetyl, hydrogen peroxide, fatty acids, aldehydes, and other compounds). The results of the direct inhibition test indicated that *L. casei* Shirota alone did not give antibacterial effects. The LAB that involved in the fermentation of traditional beverages had an antimicrobial property against various food-pathogens and the inhibitory products were extracellular and diffusible. The observed inhibitory property of LAB was influenced by the medium they grew in (Erdogrul et al., 2006).

The single culture did not show any antibacterial activity against *Staphylococcus aureus* ATCC 25923 whereas the average pH values with the mix culture were insignificantly different (Figure 1 and Figure 2). The single probiotic produced a weak chemical substance that limited to inhibit pathogenic bacteria while the multiculture *Lactobacilli* on milk with their different metabolic product (organic acid, bacteriocins, other antimicrobial substance) were resulted in antibacterial activity profiles. Isnaeni et al (2014) reported the inhibitory activity of LAB mixed cultures against pathogenic microorganisms. Growth and metabolism products of the probiotic bacteria could be affected by the nutrition on DCM that more substances content than skimmed milk. The presence of natural prebiotics such as fruit juice can help inhibit LAB activity against pathogenic bacteria (Sholeha, 2014).

The inhibition zone in DCM was larger than skimmed milk. Milk fat that presents on DCM can be assumed as a prebiotic. The milk fat increased the viability of *Lactobacilli* showed that based on DCM the inhibition zone much larger significantly than based on skimmed milk. These lead to assume that substantial amount of enzyme that present on DCM were much more than skimmed milk. The rate of the catalyzed reaction is directly proportional to the substance concentration. As the substrate concentration is increased, a point of an enzyme substrat complex and the velocity of a reaction will be maximum, thus can help energy metabolism and produce of chemical substance that profitable to antibacterial activity (Boyd, 1994).

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The milk concentration did not give a significant inhibition zone against *Staphylococcus aureus* ATCC 25923. The diameter of inhibition zones of 50% and 100% DCMs were insignificantly different. They give the same average of diameter while skimmed milk gave narrower diameter.

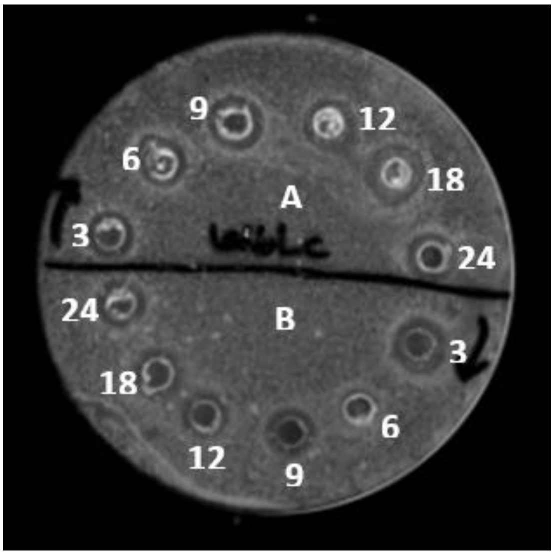

Figure 1. Inhibitory activity of *L. casei* Shirota, *L. acidophilus* and *L. bulgaricus* fermented milk by DCM: A= 100%, B= 50% after 3 hours, 6 hours, 9 hours, 12 hours, 18 hours, 24 hours.

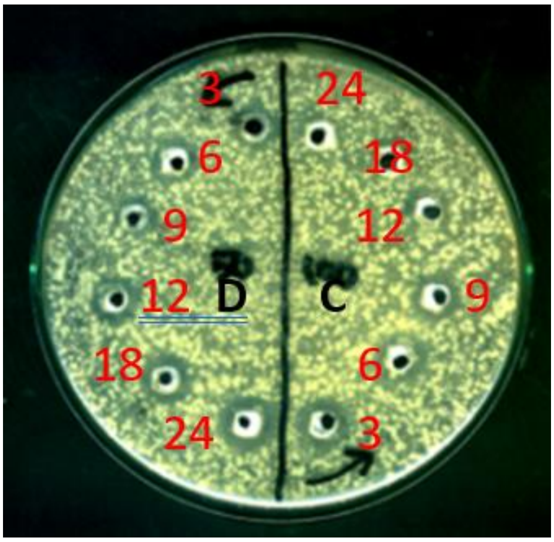

Figure 2. Inhibitory activity of *L. casei* Shirota, *L. acidophilus* and *L. bulgaricus* fermented by skimmed milk: C= 100%, D= 50% after incubation 3 hours, 6 hours, 9 hours, 12 hours, 18 hours, and 24 hours

The milk concentration did not give a significant inhibition zone against *Staphylococcus aureus* ATCC 25923. The diameter of inhibition zones of 50% and 100% DCMs were insignificantly different. They give the same average of diameter while skimmed milk gave narrower diameter.

The beneficial effects of living microbes (probiotics) containing food on human health, and in particular of milk products like fermented milk on children and other high-risk populations, are being increasingly promoted by health professionals. It has been reported that these probiotics can play an important role in immunological, digestive, and respiratory functions and could have a significant effect in alleviating infectious disease in children (WHO-FAO, 2006). Various dosage forms of LAB microparticles have been proven to be active invitro inhibiting the growth of pathogenic microbes (Sugiyartono, 2014). Future research related to the use of fermented milk products that rely on the role of lactic acid bacteria or probiotics is very important, especially for human health.

4. Conclusion

The dairy cow milk and skimmed milk showed good media for fermentation of the *Lactobacilli. Lactobacillus casei* Shirota as a single culture did not show inhibitory activity against the test bacteria. The mixture of *Lactobacillus casei* Shirota, *Lactobacillus acidophilus, Lactobacillus bulgaricus* antibacterial exhibited inhibitory activity against *Staphylococcus aureus* ATCC 25923. The maximum inhibitory activity was reached after 24 hours fermentation. The pH values decrased after 3-24 hours fermentation. Viscosity affected the inhibitory activity and achieved maximum after 24 hours fermentation.

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