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Research Article

The Tolerance of *Lactobacillus paracasei* and *Lactobacillus curvatus* Originated from Bovine Colostrum towards Acidity and Bile Salts as a Probiotics Candidate

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Abstract: The study conducted to obtain *Lactobacillus* species from bovine colostrum as probiotic candidate that have resistant character towards acidity and bile salt condition. Research done experimentally with Factorial Completely Randomized Design Pattern. The results were analyzed using Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Results showed that *L. paracasei* and *L. curvatus* have tolerance of pH 2 with colony population by 0.07×10^2 CFU/mL. *L. paracasei* and *L. curvatus* also have tolerance as well towards 0.3% of bile salt concentration with colony population by 4.05×10^{10} CFU/mL and 0.5% of bile salt concentration with colony population by 4.02×10^{10} CFU/ml. *L. paracasei* and *L. curvatus* have character as probiotic because grew well at high acidity and bile salts condition.

Keywords: Acidity, bile Salt, *Lactobacillus paracasei*, *Lactobacillus curvatus*, probiotic

INTRODUCTION

Probiotic bacteria are non-pathogenic microorganisms that can control microbial populations in the human digestive system and has a benefit to its host. The competition mechanisms that can control population of pathogenic bacteria in the digestive system provided by probiotic bacteria (De Smet *et al*., 1995; Bezkorovainy, 2006). Main source of probiotics can be obtained from wide variety of food products made from milk, such as colostrum, cottage cheese, yogurt, kefir, or milk powder enriched with probiotics (Gómez Zavaglia *et al*., 1998).

Colostrum is a yellow-colored solution produced by the mammary glands of cattle between 24-96 h after birth. Colostrum will not produce after 4-5 days of birth because it will turn into milk (Kusumawati, 2002). Colostrum contains bifigenic factor proved to be N-acetylglucosamine that can support the growth of lactic acid bacteria (*Lactobacillus* sp., *Lactococcus* sp., *Bifidobacteria*) (Kashket, 1987; Salminem *et al*., 2004).

Lactobacillus sp. is one of the excellent bacteria that often used as a probiotic culture. These bacteria have the criteria for probiotic bacteria such as viability at high acidity and bile salt tolerance (Kusumawati, 2002). The study aimed to isolated *Lactobacillus* sp. originated from bovine colostrum that have character of acidity and bile salts tolerance so it can be used as probiotic bacteria.

MATERIALS AND METHODS

Material: Acetic acid, bile salts (Oxoid), *L. paracasei* and *L. curvatus* strain originated from bovine colostrum, NaCl 0.9%, NaOH 1N, Nutrient Agar/NA (Oxoid) and Nutrient Broth/ NB (Oxoid).

Method:

Tolerance of acidity test method: *L. paracasei* and *L. curvatus* incubated at NB at 30°C for 24 h. Set the acidity level pH 2, 4, 6 by 20 mL of NB using 1% acetic acid and 1% NaOH as a buffer. Each treatment inoculated with 10% starter during the logarithmic phase (log) into acidified NB. Control (blank) was prepared using NB with pH that set without addition of starter. Treatment incubated for 6 hours. At pH 6 condition, we do eight dilution series (10^{-8}). Total Plate Count (TPC) method used to count the population of bacteria with NA after incubation for 24 hour (Hartati, 2007).

Tolerance of Bile Salts Test Method: *L. paracasei* and *L. curvatus* incubated at NB at 30°C for 24 h. Set the bile salt level of 0.3 and 0.5% by NA using (Oxoid) bile salt. One mL of 10 serial dilutions (10^{-10}) liquid

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culture grown on plates with pour plate method. Total Plate Count (TPC) method used to count the population of the bacteria with NA after incubation for 24 h (Buntin *et al*., 2008).

RESULTS AND DISCUSSION

Tolerance of acidity: Probiotic Bacteria generally have high tolerance to acidic pH in the range of 2-4. This is because the stomach has very low pH conditions ranging from 2-4 (Gómez Zavaglia *et al*., 1998). Results of Analysis of Variance (ANOVA) showed that the pH gave significant effect on colony growth resistance of each test bacteria (*L. paracasei* and *L. curvatus*), but no interaction between pH and the type of bacteria. The lower levels of pH, the number of bacterial population growth will decrease more. Results of the Analysis of Variance (ANOVA) was performed Duncan's Multiple Range Test which can be shown in Table 1.

At pH two, the number of bacteria population was 0.07×10^2 CFU/mL, which indicates that the grown bacteria have tolerance towards low pH. At pH four, the average population of bacteria was 0.498×10^2 CFU/mL. Both bacteria still grown at pH 2 and pH 4, which indicates that the bacteria can be candidate as probiotic bacteria (Hardianingsih *et al*., 2006).

Condition of digestive tract especially the human being was in the low pH range. Stress happens to the bacterial cell when it enters the digestive tract and exposed to acid. Potential probiotic bacteria must be resistant to the low pH conditions of the stomach in the range of 2-4 (Hardianingsih *et al*., 2006). Kashket (1987) and Kimoto *et al*. (2004) states that *Lactobacillus* is bacteria that resistant towards acidic conditions.

Acidic conditions on the media obtained by adding acetic acid in liquid medium (Nutrient Broth). Exposure to high acidic conditions may result damage to the cell membrane and the release of intracellular components that are capable of causing bacterial cell death. Bacteria that are tolerance towards low pH has a resistance to membrane damage due to decrease of extracellular pH. *Lactobacillus* is lactic acid bacteria and probiotic bacteria that can live in wide pH range. The main defense of the bacteria towards the environment is cell membrane structure consisting of two layers of fat (lipid bilayer). Hartati (2007) states that *L. casei* and *L. paracasei* can grow at extreme low pH conditions that making it suitable to be candidate as probiotic bacteria.

The difference in susceptibility to the cytoplasmic membrane of bacteria will determine how big the bacterial tolerance to low pH. To survive in an acidic environment, *Lactobacillus* must maintain intracellular pH higher than the extracellular pH. Intracellular pH of Lactic acid bacteria such as *Lactobacillus* could dynamically changes along with decrease in extracellular pH (Drouault *et al*., 1999; Hill, 1995). Drouault *et al*., (1999) suggested that the enzyme might also affect the growth of *Lactobacillus* as lactic acid bacteria in a state of low pH. The enzymes that affect the resistance of lactic acid bacteria at low pH are protease. Protease enzyme needed by *Lactobacillus* to grow and producing acid in the manufacture of fermented products such yogurt (De Angelis *et al*., 2001). According Bezkorovainy (2006) in acid condition, *Lactobacillus* is able to maintain the acidity of the cytoplasm so that proteins and enzymes inside the cell can still work optimally. Based on the results, *L. paracasei* and *L. curvatus* potential as probiotic candidates because the ability to grow at acidic pH range.

Table 1: Duncan's multiple range test (level of 5%) acidity into bacteria population

Treatment	Median of Population Colony (CFU/mL)	Result test
pH 2	0.07×10^2	C
pH 4	0.498×10^2	B
pH 6	5.28×10^9	A

Description: different letters in different columns indicate significant differences ($p<0.05$) among treatment

Table 2: Median of bacteria population into bile salts 0.3% and 0.5% condition (10^8 CFU/mL)

Treatment	*L. paracasei*	*L. curvatus*
Bile salts 0.3%	3.87	4.24
Bile salts 0.5%	4.76	3.29

In accordance with Bezkorovainy (2006), the effect of probiotic bacteria in the intestinal environment can be occur when the population of the bacteria reach at least 10^9-10^{11} CFU/mL at low pH. *L. paracasei* and *L. curvatus* obtained from bovine colostrum has the population below the standard for probiotic acitivities. Acclimatization or optimization needed if *L. paracasei* and *L. curvatus* originated from bovine colostrum will used as probiotic, so that the population of bacteria can meet the standards.

Tolerance of bile salts: The test performed by growing *L. paracasei* and *L. curvatus* isolates in NA that added by bile salts with concentration of 0.3% and 0.5%. Concentration of 0.3 and 0.5% selected as the concentration equivalent to the concentration of bile salts in duodenum physiologically (Gómez Zavaglia *et al*., 1998). According to analysis of variance (ANOVA) at concentration of 0.3% bile salts and 0.5% there is no significance difference between the growth of *L. paracasei* and *L. curvatus* at the bile salt concentration of 0.3% and 0.5% so it does not proceed with the test Duncan's Multiple Range. At bile salt concentration of 0.3% and 0.5%, *L. paracasei* and *L. curvatus* grew optimally with not significantly different amount. The results of the average population of *L. paracasei* and *L. curvatus* in the bile salt concentration of 0.3 and 0.5% can be seen in Table 2.

The average population of *L. paracasei* at concentrations of 0.3% bile salts was 3.87×10^{10} CFU/mL and at concentrations of 0.5% bile salts were 4.76×10^{10} CFU/mL. Meanwhile the growth of the bacterial population of *L. curvatus* at concentration of 0.3% bile salts was 4.24×10^{10} CFU/mL and at

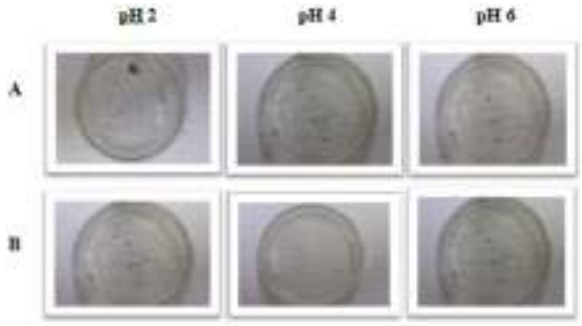

Fig. 1: A: *L. paracasei* population growth on pH 2, 4 and 6 agar plates medium; B: *L. curvatus* population growth on pH 2, 4 and 6 agar plates medium

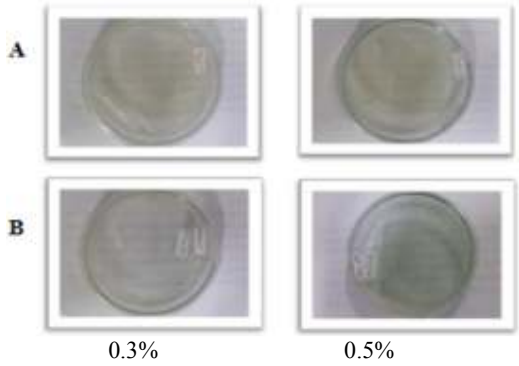

Fig. 2: A: *L. paracasei* population growth on bile salts 0.3% and 0.5% agar plates medium; B: *L. curvatus* population growth on bile salts 0.3% and 0.5% agar plates medium

concentration of 0.5% bile salts were 3.29×10^{10} CFU/mL.

Results showed that both bacteria grew well with the same relative population in the media with addition of bile salts concentrations of 0.3% and 0.5%. This shows that both bacteria have a strong character as a probiotic candidate because it can grow optimally at each concentration of bile salts tested. In order to survive and grow in the gastrointestinal tract, *Lactobacillus* as a probiotic culture must be able to pass various environmental conditions such as high concentration of bile salts in the upper intestinal tract where bile secreted into the intestine. Bile is a mixture of bile acids, cholesterol, fatty acids, phospholipids, bile pigments and lipolytic enzymes that activated by surface-active characteristics of bile (Xiao *et al*., 2003). Bile combination is bactericidal for commensal microorganisms in the human body except for the few inhabitants of the gut that are resistant to bile salts (Kimoto *et al*., 2004). Both bacteria tested at bile salts concentrations of 0.3 and 0.5% that is the critical concentration and high enough bile salts amount to test the isolates that were resistant to bile salts (Xiao *et al*., 2003).

Good tolerance to bile salts is presumably due to the role of polysaccharides as one a constituent of the cell wall of gram-positive bacteria. *Lactobacillus* known as the group of lactic acid bacteria has a resistance to mixed bile salts (Saarela *et al*., 2000; Salminen *et al*., 1999). According Bezkorovainy (2006) liquid bile salts are surface-active compounds that can penetrate and react with the cytoplasmic side of the membrane which are lipophilic which can cause changes in bacterial membrane structural damage. *Lactobacilli* have bile salt hydrolase enzyme activity to hydrolyze bile salts. This enzyme is able to alter the physical and chemical capabilities possessed by bile salts, so it is not toxic to the *Lactobacillus*. This is what allows some *Lactobacillus* isolates resistant to bile salts circumstances (Fig. 1 and 2).

CONCLUSION

Based on the results it can be concluded that *L. paracasei* and *L. curvatus* have ability to tolerate high aciditiy environment and high concentration of bile salts, so that can be candidate as probiotic.

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