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CHARACTERIZATION OF *Enterococcus* BACTERIA ISOLATED FROM BOVINE COLOSTRUM AS PROBIOTICS

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Abstract

Aims of the research was to characterize probiotic properties of Enterococcus bacteria isolated from bovine colostrum. The research done experimentally with factorial pattern of Completely Randomized Design including two factors such type of bacteria and the characters of probiotics and replicated three times. Parameters of the research was pH tolerance, bile salts, antimicrobial activity, the ability of auto-aggregation. Data analyzed by ANOVA and Duncan's Multiple Range Test (DMRT). Two Enterococcus bacteria isolated from the colostrum which identified with the API 20 Strep were known as E. faecalis and E. faecium. The result showed that E. faecalis and E. faecium were pH 4 tolerant with the population of 1.623 x 10^2 and 1.316 x 10^2 CFU/ml. Availability to survive at high bile salt concentrations (0.3% and 0.5%) which shown by 15.683 x 10^{10} and 23.667 x 10^{10} CFU/ml population. Enterococcus faecalis and E. faecium also had antimicrobial properties which indicated by clear zone diameter towards E. coli (9.7 and 10.3 mm), S. thypimurium (9.7 and 10 mm), and L. monocytogenes (9 and 12 mm). The inherent ability of the isolated Enterococcus was better than the test bacteria (E. coli, S. thypimurium, Listeria) with the auto-aggregation values were 61.783% and 60.425%.

***Key words** : Characterization, Probiotic, Enterococcus, Colostrum.*

INTRODUCTION

Bovine colostrum has known contain nutrients, antimicrobial agents, and antibodies (Kelly, 2003). The components can be used to treat health problems and infections caused by bacteria, viruses, parasites, and fungi. Beside bovine colostrum could be given as a nutritional supplement for diarrhea and sinusitis, it also improve patient immunological factors (Rawal, et al., 2008). The ability was not only caused by immune factors, but also indigenous microorganisms that live inside the bovine colostrum.

Indigenous microorganisms contained in colostrum were diverse and several of them are round-shaped bacteria (cocci). Cocci bacteria in colostrum can act as beneficial bacteria. Catalase-negative cocci bacterial was lactic acid bacteria (LAB) that already known has a role as probiotic.

Probiotics are living microorganisms that gave health benefit for the host when given in adequate amounts (Shinde, 2012). There are not all microorganisms could be called as probiotics, the characterization is needed to determine selected microorganism as a probiotic.

Probiotics has ability to survive in digestive tract conditions such as acidic conditions and can grow in the presence of bile salt exposure. In a normal condition filled stomach has a pH of 4.0-5.0 while the pH of empty stomach was 1.5-2.0 (Shinde, 2012). At pH 1.5, some of probiotics *Lactobacillus* strain showed 55% loss of viability, while at pH of > 2 the strain retained relative constant viability (Vamanu, 2014). In the human intestine, probiotic should survive 0.3% bile salt concentration (Jacobsen, et al., 1999). Meanwhile, bile salt concentration of 0.5% with 4h of exposure gave maximum decrease in probiotics viability (Vamanu, 2014). Both are

main character for the selection of probiotics microorganisms.

Preliminary studies has been done to isolate two cocci bacterial, gram-positive, catalase negative and have been identified by API-50 CHL kit (bioMerieux) as *E. faecalis* and *E. faecium*. *E. faecalis* and *E. faecium* were *Enterococcus* bacteria that have been widely used as probiotic (Fuller, 1989). *Enterococcus* has resistant to gastric acid and bile salts (Moreno, et al., 2006). The study aims to characterized isolated *Enterococcus* ability in surviving low pH and bile salts so that could recomended as probiotic.

MATERIALS AND METHODS

Research design and statistical analysis

Low pH resistance test done experimentally using completely randomized design (CRD) with 2 x 3 factorial pattern and each treatment was performed 3 replications. The first factor is type of probiotic bacteria candidate (*E. faecalis, E. faecium*) and second factor was pH levels (2,4,6). While, bile salts resistance test done with CRD experimental method with 2 x 2 factorial pattern with 3 replications. Probiotic bacteria candidate (*E. faecalis, E. faecium*) as first factor and bile salts (Oxoid) level (0.3%, 0.5%) as second factor. Data analyzed using Analysis of Variance (ANOVA) with level of 95% and treatment effect analyzed by Duncan's Multiple Range Test (DMRT) (Gomez, 1995).

Probiotic Characterization

Parameter measured by Total Plate Count method of grown bacteria on MRS-Agar (Oxoid) with acetic acid addition for low pH resistance test and MRS-Agar (Oxoid) with various bile salts addition for bile salts (Oxoid) resistance test (Hardianingsih, et al., 2006; Gomez, 1995).

RESULTS AND DISCUSSIONS

Probiotic resistance towards low pH

Resistance to acid used as indicators of probiotic bacteria abilitiy to survive in the human stomach (pH of 2-4). Results showed that pH and *Enterococcus* type significantly affected the *Enterococcus* total colonies, so that it proceeds with the DMRT (Table 1). The decreased of pH also decreased the number of bacterial colonies. Highest number of colonies has shown by *E. faecalis* at pH 6 with a number of 40.633 x 10^8 CFU / ml. Both types of bacteria could not grew at pH 2, but still grew at pH 4. The number of colonies of *E. faecalis* at pH 4 was 1.32 x 10^2 CFU / ml and the number of colonies of *E. faecium* was 1.62 x 10^2 CFU / ml. Gastric acidity vary from pH 2-5, filled stomach has pH range of 4-5 and if empty can reach pH of 2, so that both strains could still be categorized as a probiotic (Jacobsen, et al., 1999). Generally, microorganisms grew in pH range of 6.5-7.5 (Pelczar, 2006). *Enterococcus faecalis* and *E. faecium* could grew in a wide range of pH 4.6 to 9.9 and optimally grew at the pH of 7.5 (Fisher and Philips, 2009).

Table 1. Duncan Multiple Range Test (DMRT) Probiotics Tolerance towards Low pH

pH	Bakteri (CFU/ml)	
	E. *faecalis*	E.*faecium*
2	nd C a	nd C a
4	1,32 x 10^2 B B	**1,62 x 10^2 B** **a**
6	**40,633 x 10^8 A** **A**	27,433 x 10^8 A b

Description:

The same uppercase letter read vertically showed no significant difference

The same lowercase letters read vertically to the side showed no significant difference

nd = not determined (number of colonies = 0 CFU / ml)

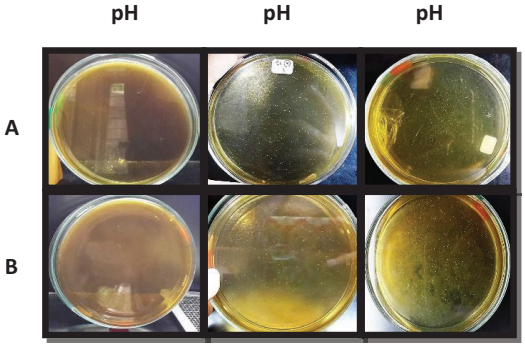

Figure 1. (A) *Enterococcus faecalis* colonies at the pH of 2, 4, 6. (B) *Enterococcus faecium* colonies at the pH of 2, 4, 6.

Enterococcus are less resistant to acid, when external pH <3.0, magnesium ions out of the cell and lead to death less than 4 hours (Bender, et al., 1986). The number of cocci-shaped Lactic Acid (LAB) including *Enterococcus* would declined when the pH of the medium decreased into 5 (Hutkins and Nannen, 1993). Acidity level below 5 will disrupt the structure of the cell membrane, the cell membrane becomes saturated by hydrogen ions thus limiting membrane transport and intracellular component out. Poisoning that occurs at low pH cause by acid substances did not decompose into the cell that resulting in ionization and pH of cells changed then inhibit growth and may even kill microorganisms (Hutkins and Nannen, 1993). Only small number of *E. faecium* could grew at pH of 0.5, 1, 2, and 3 then dead after 4 hours incubation (Chavarin, et al., 2003). None of the LAB could grew at pH of 2.5 more than 4 hours incubation while the incubation of the research was done 6 hours so that the result showed that no colonies found at the media with the pH of 2 (Jacobsen, 1999, Chavarin, et al., 2013).

Tested probiotic candidates showed that have ability to survive in low pH conditions and has tolerance to acid (Jacobsen, 1999). If the bacteria survived and active in gastric acid condition can be regarded as probiotic bacteria (Salminen and von Wright, 2004). Based on the results, *E.faecalis* and *E. faecium* can be said probiotics because survived at the pH of 4. *Enterococus faecalis* and *E. faecium* isolated from bovine colostrum did not resistant to gastric acid if consumed when the stomach empty, but if consumed when the stomach filled they have opportunity to survive.

Probiotic resistance towards bile salts

The results on Table 2 showed that there was no significant effect between the interactions of bile salt concentrations (0.3% and 0.5%) and *Enterococcus* type, while significant effect found at type of probiotic bacteria (*E. faecalis* and *E. faecium*) treatments. *Enterococcus faecalis* and *E. faecium* has ability to grow in different bile salt concentration of 0.3% and 0.5%. *Enterococcus faecalis* has greater ability to grow on both concentration of bile salts than *E. faecium* with total colonies of 23.667 x 10^{10} CFU / ml.

Bile salt tolerance was an indicator of probiotic bacteria's ability to survive in the upper gastrointestinal tract. *Enterococcus faecalis* and *E. faecium* were able to grow on the medium with the addition of 0.3% and 0.5% bile salts that indicated selected bacteria were able to survive and grow in the upper intestinal tract where bile salts secreted. Both strains were able to grew more in the bile salt concentration of 0.3% compared to 0.5%, but did not show significant difference. Higher concentration of bile salts resulting in higher amount of bacterial cell death. Bacteria resistant ability to bile salts caused by peptidoglycan layer and thicker wall owned by gram-positive bacteria so that protected from lysis when exposed to bile salts. In addition, lipid components owned by Gram-positive bacteria kept the membrane structure and decreased cell leakage caused by bile salts (Kimoto, et al., 2002).

Table 2 Average probiotics colonies with different bile salts treatments

Probiotics	Average colonies (CFU/ml)	$p < 0.05$
E. faecium	15,683 x 10^{10}	b
E. faecalis	23,667 x 10^{10}	a

Description: Different letter showed significant difference

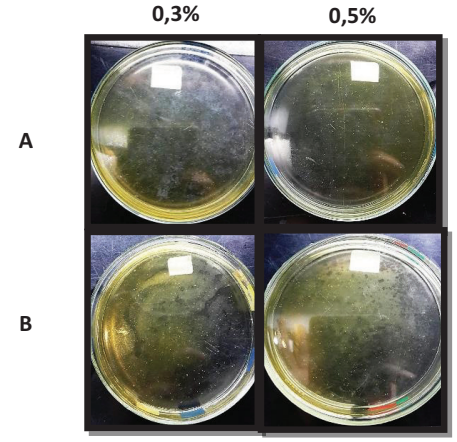

Figure 2. (A) *Enterococcus faecalis* colonies on 0.3% dan 0.5% bile salts added media. (B) *Enterococcus faecium* colonies on 0.3% dan 0.5% bile salts added media.

The concentration of bile salts in the small intestine ranged from 0.2 to 2% (w/v) depend on the organims, type and amount of food ingested, while the equivalent concentration of bile salts in the intestine was 0.5% (Kristoffersen, 2007; Puspawati, 2010). Bile salt concentration of 0.3% was high enough for probotic selection that were resistant to bile salts, so that the bile salt concentration of 0.3% also called as the critical concentration (Jacobsen, 1999).

Bacteria ability to grow in unfavorable environmental conditions, such as the presence of bile salts is one of probiotics bacteria characteristics. *Enterococcus* have been targeted as probiotic because the bacteria have characteristics such as resistance to bile salts (Araujo and Ferreira, et al., 2013). According to the results, concentrations of bile salts until 0.5% did not inhibit bacteria growth. *Enterococcus faecalis* and *E. faecium* can be categorized as probiotics because they could grew on critical concentration of 0.3% or higher of 0.5%.

CONCLUSIONS

Enterococcus faecalis and *E. faecium* isolated from bovine colostrum had resistant to low pH until 4 and bile salt concentration until 0.5%. Both character was main character that must be owned by the probiotics bacteria. Therefore isolates of *E. faecalis* and *E. faecium* have probiotic characteristics. However both bacteria should be tested further to determine other properties of probiotics, such as inhibition ability towards pathogenic bacteria, adhesion to the intestinal epithelial cells, and pathogenicity test.

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