IN VITRO pH TOLERANCE, BILE SALT RESISTANCE AND ANTIMICROBIAL ACTIVITY OF Lactobacillus plantarum ISOLATED FROM CROSSBRED CATTLE

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RESEARCH NOTE

IN VITRO pH TOLERANCE, BILE SALT RESISTANCE AND ANTIMICROBIAL ACTIVITY OF *Lactobacillus plantarum* ISOLATED FROM CROSSBRED CATTLE

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ABSTRACT

This research was done to evaluate the characteristics and probiotic potential of lactic acid bacteria (LAB) isolated from the small intestine of ten three year-old male Ongole crossbred cattle. Ten-centimeter samples were obtained from each small intestine, wastes were removed then samples were placed in sterile sample bottles, and immediately taken to the laboratory for bacterial isolation. The LAB isolates were subjected to low pH tolerance (pH 2 and 4), bile salt resistance, and antimicrobial activity against enteric pathogens *Staphylococcus aureus* and *Eschericia coli*. Biochemical assay indicated that isolate was gram positive, rod-shaped, catalase negative, and capable of fermenting glucose, mannitol, xylose, rhamnose, sucrose, lactose, arabinose, raffinose and sorbitol. Biochemical and morphological identification suggests that the isolate was *Lactobacillus plantarum* WPL 117 (strain number of control indicator organisms was *Lactobacillus plantarum* ATCC 14917). This isolate was able to survive at low pH (2 and 4), tolerated 0.3% bile salts, and capable of inhibiting *S. aureus* and *E. coli*. Thus, this isolate can be considered a probiotic candidate for further study.

Key words: antimicrobial activity, bile salt, lactic acid bacteria, pH tolerance

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INTRODUCTION

Lactic acid bacteria (LAB) have been widely used as a preservative supplement in food and feed industry, and have been known to reduce the use of antibiotics in food products for humans and feed products for livestock. This is due to their ability to produce potent bacteriocins, which, are antimicrobial peptide substances (Woraprayote *et al*., 2016; Seddik *et al*., 2017). LAB can be found in different environments: in animal gut, human gut, food and water (Ahmed, 2003). *Lactobacillus plantarum,* a widely used probiotic, is among the LAB that can ferment a variety of carbohydrates. It is also used as a starter culture for food and feed fermentation (Siezen and van Hylckama Vlieg, 2011; da Silva Sabo *et al*. 2014).

A probiotic is a non-pathogenic living microorganism, which, when consumed in adequate amounts, can provide health benefits to its host (FAO/WHO, 2006). There are a number of benefits to using probiotics: increased utilization of nutrients, decreased use of antibiotics, reduction in serum cholesterol level (Guo *et al*., 2010), and

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promotion of balance in gut microbiota (Saez-Lara *et al.*, 2015). Addition of *L. casei* probiotic in chicken feeds was found to improve feed consumption (g/hen) and increase egg mass (g/hen/day) and egg weight (g) (Griggs and Jacob, 2005). Benefits seen in the study include maintenance of normal intestinal microbiota and improved nutrition by detoxifying hazardous compounds in feeds and denaturing potentially indigestible components in the diet with hydrolytic enzymes amylases and proteases (Fuller, 1989; Balcazar *et al.*, 2006; Suzer *et al.*, 2008).

Lactic acid bacteria are the most common microorganisms used as probiotics in livestock production, including species from the genera *Lactobacillus, Bifidobacterium, Pediococcus*, and *Leuconostoc* (Garcia *et al.*, 2016; Lee *et al.*, 2016). *Lactobacillus* consists of 135 species, 27 subspecies and a heterogeneous group (Bernardeu *et al.*, 2008). The small intestines of healthy Ongole crossbreed beef cattle may contain lactic acid bacteria which can be used as probiotics. For this reason, this study sought to find and characterize a new strain of lactic acid bacteria isolated from Ongole crossbreed beef cattle, capable of surviving in low pH, bile salts, and possess antimicrobial activity – conditions that define a probiotic. Identification of isolates for probiotic use can contribute in increasing livestock productivity.

MATERIALS AND METHODS

Animals

Ten healthy 3-year old, 300-400 kg, male Ongole crossbreed beef cattle from a slaughterhouse in Surabaya, Indonesia were used in the study. Cattle were considered apparently healthy based on nutrition and overall health management and deworming frequency of every three months.

Sample collection and cultivation

Slaughtering of cattle was carried out in accordance to Halal regulations. After slaughtering, all internal organs were removed, and 10 cm samples of small intestines were collected. Wastes were removed and samples were placed into sterile sample bottles, and immediately taken to the laboratory for isolation process.

Collection and cultivation of samples were adopted from Rajoka *et al.* (2018), with some modifications. Samples were diluted in PBS solution (0.1 M, pH 7.4) (Merck, Germany). One hundred µl diluted samples were spread onto sterilized de Man Rogosa Sharpe (MRS) agar media (Merck, Germany), incubated at 37°C for 3 days to obtain single colonies and select for further characterization.

Screening and identification of LAB isolates

Selected LAB isolates were subjected to biochemical assay, morphological examination, catalase test and gram staining. Isolates that were observed as rod-shaped, catalase negative, and gram positive were suspended on MRS broth (Merck, Germany) and supplemented with 20% glycerol at -80°C. Prior to assay, LAB isolates were grown in MRS broth medium for further experiments (Leite *et al.*, 2015).

In vitro pH tolerance, bile salt resistance and antimicrobial activity

Bile salt and acid tolerance were determined, with some modifications according to the methods described by Rajoka *et al.* (2018). The isolates were grown in MRS broth at 37°C for 24 h and subcultured (1%, v/v) in sterilized MRS medium. For *in vitro* pH tolerance, overnight cultures of isolates were spotted on MRS agar plates adjusted to pH 2.0 and pH 4.0 with 3 M HCl solution (Merck, Germany). Colonies that survived were counted after incubation at 37°C for 24 h.

Bile tolerance assay was conducted using modified methods of Lee *et al.* (2016). Overnight cultures of LAB isolated were inoculated (1% v/v) in MRS medium 1% (w/v) Oxgall. Overnight cultures of isolates were spotted on MRS agar plates supplemented with 0.3% bile salts, specifically 50% cholic acid sodium salt and 50% deoxycholic acid sodium salt (Sigma-Aldrich, 48305). Plates were incubated under microaerophilic conditions at 37°C for 24 h. Precipitated bile salts around the colonies denote positive result. This procedure was performed twice.

Antimicrobial assay

Antimicrobial assay was carried out based on the methods of Adeniyi *et al*. (2015), with some modifications. Isolated bacterial culture (200 µl) was inoculated in MRS broth at 37°C and incubated for 24 h under microaerophilic conditions. After incubation, a loopful of isolate was inoculated on MRS agar plate and incubated at 37°C for 24 h in facultative aerobic conditions. MRS agar plates were then overlaid with approximately 0.2 ml x 10^7 CFU/ml of overnight broth culture of *E. coli* (APEC/ Avian pathogenic *Eschericia coli*) and *S. aureus* (Avian pathogenic *Staphylococcus aureus)* assays, inoculated in 10 ml of MRS agar, and incubated at 37°C under facultative aerobic conditions. A clear zone in the agar plate indicates bacteriocin inhibition (Ravi *et al*., 2015).

RESULTS AND DISCUSSION

Lactic acid bacteria were successfully isolated from the samples using a selective medium of MRS agar. Identification classified the lactic acid bacteria *Lactobacillus plantarum* WPL 117 as gram positive, catalase-negative and rod-shaped. These results show similarities with the studies done by Ahmed (2003) and Leite *et al*. (2015), wherein isolates had the same biochemical characteristics, and lactic acid was the metabolic end product from carbohydrate fermentation. Based on this study, five similar LAB strains were isolated from the intestine wastes, and all isolated strains underwent gram staining, catalase test and morphological examination, until one isolate that matched the desired characteristics was selected for optimization. Table 1 shows the biochemical characteristics of the isolate *L. plantarum* WPL 117.

The *L. plantarum* WPL 117 isolate was able to ferment glucose, mannitol, xylose, rhamnose, sucrose, lactose, arabinose, raffinose and sorbitol. Positive reaction signifies the presence of enzymatic activity. Some lactic acid bacteria have the enzymes β-glucosidase (β-Glu), β-galactosidase (β-Gal) (de Vrese *et al*., 2001) and enzymes that can hydrolyze lactose (Roy and Ward, 1990). *Lactobacillus plantarum* C182 have enzymes, including α-galactosidase (α-Gal), β-Gal, α-glucosidase (α-Glu), and β-Glu 6.14, 118.45, 52.38, 168.25 (U/mg of protein). Characteristics that define lactic acid bacteria are tolerance to acidic conditions and bile salt. Therefore, the ability of the isolates to survive in acidic conditions and bile salt were tested *in vitro*.

Table 2 shows the survival rate of *L. plantarum* WPL 117 to acid and bile salt tolerance after 24 h of incubation at pH 2 and pH 4. *In vitro* low pH tolerance study revealed that isolates at pH 2 and 4 showed equal viability compared to pH 7 (control), suggesting that *L. plantarum* WPL117 strain can survive in simulated gastrointestinal tract conditions. This is in agreement with the study done by Argyri *et al.* (2013) where they reported that four *L. plantarum* strains demonstrated survival at low pH after 3 h of exposure (highest final population >8 log cfu/ml). Bactericidal effect in the GIT occurs at pH under 2.5 (Surono, 2003). Corcoran *et al.* (2005) reported that *Lactobacillus* resistance to low pH can be attributed to its F0F1-ATPase activity. *Lactobacillus* can produce lactic acid and inhibit pathogenic bacterial growth by creating acidic conditions.

Meanwhile, bile salt is toxic to cells, and it tends to damage the structure of cell membrane. This is why tolerance to bile salt is considered one of the essential properties, which enable lactic acid bacteria strains to survive in the gastrointestinal tract (Rajoka *et al*., 2018). Their resistance to bile salt and acidic condition contributes to their overall ability to withstand harsh conditions in the GIT(de Vrese *et al*., 2001).

This study showed that *L. plantarum* WPL117strain was resistant to bile salts. Biomass (cell dry matter) of the isolate was 22.6 mg/100 ml. This value indicates that the isolate can hydrolyze the bile salt and thus, tolerates it to a certain level. Presence of the biomass after growth in MRS agar plate supplemented with 0.3% bile salt supports this claim.

One of the conditions that qualifies a lactic acid bacteria as a probiotic is resistance to 0.3% bile salts, since this concentration is relatively the same as that found in the

Table 1. Biochemical characteristics of *L. plantarum* WPL 117 isolated from crossbred cattle.

Substrate	Reaction	Substrate	Reaction	Substrate	Reaction
Lysine	–	Urease	–	Rhamnose	+
Ornithine	+	VP	–	Sucrose	+
H_2S	–	Citrate	+	Lactose	+
Glucose	+	TDA	–	Arabinose	+
Mannitol	+	Gelatine	–	Adonitol	–
Xylose	+	Malonate	+	Raffinose	+
ONPG	+	Inositol	–	Salisin	–
Indole	–	Sorbitol	+	Arginine	–

Table 2. Survival rate of *L. plantarum* WPL 117 isolated from crossbred cattle to low pH and bile salt.

Survival of *L. plantarum* WPL 117	Biomass (cell dry weight) (mg/100 ml)			
	MRS broth control (pH 7)	MRS broth (pH 2)	MRS broth (pH 4)	MRS broth (*ox bile salt)*
	50.2	50.1	49.9	22.6

intestine (Leite *et al.,* 2015). In this study, isolate WPL 117 was found resistant to 0.3% bile salts. This result is similar with other studies, which showed that five *L. plantarum* strains were resistant to bile salts after having exhibited partial bile salt hydrolase activity. *L. plantarum* was found similar with probiotic *L. casei* Shirota strains and *L. rhamnosus* GG (Argyri *et al.*, 2013). The study of Rajoka *et al*. (2018) showed that 13 isolates of *Lactobacillus* sp. in MRSc medium supplemented with 0.5 and 1% bile salt after 12 h incubation showed resistance to various concentrations of bile salt. This suggests that increasing bile salt concentration translates to a corresponding decrease in growth rate of lactic acid bacteria.

The ability of crude bacteriosin produced by the isolated strain *L. plantarum* WPL 117 was evaluated *in vitro*. Table 3 shows the diameter of inhibition zone of the isolate. Result demonstrates that crude bacteriosin from *L. plantarum* WPL 117 was able to inhibit *E. coli* and *S. aureus*. Bacteriocin-producing strains may be used as protective cultures to improve food safety. Likewise, the purified or crude form of these antimicrobial agents may also be applied directly as food preservatives. Different bacteriocins produced by *L. plantarum* are isolated from fermented food products, with particular emphasis on their genetic and biochemical properties. A number of bacteriocins including plantaricin A, plantaricin B, plantaricin C, plantaricin F, plantaricin BN, plantaricin S and T, plantaricin SA6, and C19 are produced by *L. plantarum* (Olasupo, 1996). *Lactobacillus* has been considered safe for human and livestock use, particularly in dairy cow farming (Tagg and Dierksen, 2003; Maragkoudakis *et al.*, 2006).

This study found that the isolated *Lactobacillus plantarum* WPL 117 survived at low pH (pH 2 and pH 4), was resistant to 0.3% bile salts, and exhibited antimicrobial activity against *E. coli* and *S. aureus*, qualifying it as a potential probiotic. It is recommended to conduct molecular and *in vivo* test on animals to verify its potential as a probiotic.

Table 3. Inhibition zone of crude bacteriosin from *L. plantarum* WPL 117 isolated from crossbred cattle.

Diameter of inhibition zone (mm)		
Crude bacteriosin (mm)	*Escherichia coli*	20
	Staphylococcus aureus	9

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