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Identification of *Pediococcus* Strains Isolated from Feces of Indonesian Infants With *in vitro* Capability to Consume Prebiotic Inulin and to Adhere on Mucus

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Abstract

The aim of this experiment was to identify isolates obtained from feces of Indonesian infants and to evaluate their capability as probiotics. Identification of isolates was carried out based on morphology, physiology and biochemical identifications, and molecular identification based on 16S rRNA sequence. Morphological and physiological identification was carried out based on Gram staining, shape, motility, spore formation and catalase production. Biochemical identifications based on production of CO_2 and NH_3 from glucose, the ability to grow on different temperature (10 and 45°C) and pH (4.4 and 9.6), and different salt concentration (6.5 and 18%). Probiotics capability of isolates was assayed on the ability to grow on low pH (pH 2.0), on different bile salts concentration (0.3; 0.5; 1.0 and 1.5%), the capacity to grow on media with inulin as the only carbon source, and *in vitro* adhesion ability on porcine mucin. Morphological, physiological and biochemical identification suggest that all of isolates belong to lactic acid bacteria. Further molecular identification of five isolates showed that isolates AA, BE and BK were strains of *Pediococcus acidilactici* (similarity 99%), while isolate AP and AG were strains of *Lactobacillus casei* (similarity 99-100%). Probiotic assays showed that more than 80% of cells of *Pediococcus acidilactici* isolates AA, BE and BK were viable after grown on pH 2.0 for 90 min, and around 80% of cells from the same isolates were survived on media supplemented with bile salt 1.5% for 2 h. All of isolates had high adhesion capacity as seen by more than 75% of cells attached on pig gastric mucin. Investigation of isolates to grow on inulin showed *Pediococcus acidilactici* isolate BE was able to consume inulin as the only carbon source. It is concluded that *Pediococcus acidilactici* isolate BE was a candidate probiotics and subject to further *in vivo* evaluation using animal models to examine their beneficial health effects.

Key word : *Pediococcus acidilactici, Lactobacillus casei,* human origin and probiotics.

Introduction

Lactic acid bacteria (LAB) is a group of bacteria with some species behaving as probiotics, especially from species *Lactobacillus* and *Bifidobacterium* (Margolles *et al.,* 2009). Probiotics are defined as microbial food supplements, which upon ingestion in certain numbers, exert beneficial health beyond regular basic nutrition (Parvez *et al*., 2006). Probiotics have been used for long times in food ingredients for human and animals without any side effects. Previous experiments have demonstrated the benefits of probiotics in maintaining the balance of bacteria in the digestive tract (Khan and Ansari, 2007), stimulating the immune system of the digestive tract (Pareira *et al*., 2003), and preventing cancers of the digestive tract (Wollowski *et al*, 2001; Xiao *et al*., 2006).

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The beneficial impacts of probiotics can be augmented with the addition of prebiotics, and this is defined as synbiotic. It is a synergetic application of probiotics and prebiotics, with probiotics improve physiological health effects, while prebiotics support the growth of probiotics in the digestive tract (Wells *et al.*, 2008). Prebiotic is non-digestible food components that provide specific substrates available for probiotics to increase their growth. Some fructose-containing prebiotics, such as inulin and oligofructose, have previously been reported to support the growth of probiotic *Bifidobacterium* and this was known as bifidogenic effects (Fooks *et al.*, 1999). Of the health-associated bifidogenic effects, included were growth inhibition of bacterial pathogen, absorbtion of toxic compounds, decrease on serum cholesterol, and the formation and release of feces (Niness, 1999). Several oligosaccharides have been demonstrated to function as prebiotics, including glucose-oligosaccharide (GOS), fructose-oligosaccharide (FOS), transgalacto-oligosaccharide (TOS), isomalto-oligosaccharide (IMO), and xylo-oligosaccharide (Gibson *et al.*, 1999 *cit* Fooks *et al.*, 1999). The addition of TOS, for instance, increased population of *Bifidobacterium* in mice (Djouzi dan Andreux, 1997). Meanwhile, growth inhibition of *Salmonella, E. coli, Shigella*, and *Camplyobacter* was reported as impacts of oligosaccharides supplementation on food (Gibson dan Wang, 1998).

The human gastrointestinal tract (GIT) is the best source for the isolation of probiotics (Margolles *et al.*, 2009). Several human-origin probiotic bacteria are now being exploited commercially, such as *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, and *Lactobacillus acidophilus* LA-1 (Dunne *et al.*, 2001). Being isolated and originally from intestinal tract of healthy human is of assurance that probiotics is ethically safe for human consumption (Angelis, 2006). Isolation and identification of human-origin *Lactobacillus sp.* isolated from feces of Indonesian infants have previously been reported (Widodo *et al.*, 2012). However, the identified and selected isolates showed lack of capability in degrading inulin as prebiotics, and thus incapable to be applied for synbiotic application. In this paper, we reported identification and selection of human-origin *Pediococcus acidilactici* from feces of Indonesian infants with potential capability in degrading inulin as prebiotics.

Materials and Methods

Identification of isolates

Six isolates originally obtained from feces of Indonesian infants were obtained from previous experiment (Widodo *et al.*, 2012). Isolates were purified by plating on De Man-Rogosa-Sharpe (MRS, Merck) agar suplemented with L-cysteine 0.5 g/L (Sigma) and incubated at 37°C for 48 h in aerobic condition. White colonies arisen on the media were subjected to morphological, physiological and biochemical identification (Holt *et al.*, 1994). Morphological and physiological identifications based on Gram staining, shape, motility, spore formation and catalase production. Biochemical identifications based on the production CO_2 and NH_3 from glucose, the ability to grow on different temperatures (10 and 45°C) and pH (4.4 and 9.6), and different salt concentrations (6.5 and 18%). Based on these preliminary screening, colonies with characteristics belong to LAB were selected and subject to molecular identification based on 16S rRNA gene.

Genomic DNA isolation

For genomic DNA isolation, an overnight 1.5 ml cell culture was harvested and suspended in 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5) containing lysozyme (30 mg/ml). The solution was mixed by inverting the tube followed by incubation at 37°C for 1 h and addition with 370 µl 1xTE buffer (pH 8) containing 1mg/ml Proteinase K. Fifty microliters (50 µl) of SDS solution (10%) were added and incubation was prolonged at 65°C for 1 h. After incubation, 167 µl of NaCl solution (5 M) were added and the solution

was incubated further for another 1 h. The solution was mixed with 400 µl chloroform and incubated at room temperature for 10 min followed by centrifugation at 13000 rpm for 10 min to separate DNA from other cellular components. The top aquaeous phase was transferred to new eppendorfs and the DNA was precipitated with isopropanol at -20°C for overnight. Presipitated DNA was harvested by centrifugation at 13000 rpm for 10 min, and washed with 500 µl ethanol (70%), and resuspended in Tris-EDTA (TE) buffer. The isolated DNA was resolved by electrophoresis in a 1.5% agarose gel in TBE buffer at 100 V for 30 min and visualized by ethidium bromide staining in UV-iluminator. Size was calculated by using 100 base pair (bp) DNA Ladder (Vivantis).

Amplification of 16S rRNA gene by polymerase chain reaction (PCR)

PCR was carried out to amplify ~518 bp fragment of the 16S rRNA gene using primers designed based on the conserved region of 16S rRNA gene. A combination of forward primers *plb16* (5-AGAGTTTGATCCTGGCTCAG-3) and reverse primers *mlb16* (5-GGCTGCT GGCACGTAGTTAG-3) were applied to amplify 16S rRNA sequence at positions 8 to 27 and 507 to 526, respectively (Martin *et al.*, 2009). PCR was performed using PCR thermal cycler (BOECO TC-SQ, Germany) with total volume of 25 µl consists of: 8 µl dH_2O (water free nuclease), 2.5 µl DNA template, 1 µl *plb16*, 1 µl *mlb16* and 12.5 µl KAPA Master Mix. The PCR condition was set as follows: pre denaturation at 96°C for 4 min, denaturation 96°C for 30 s, annealing 55°C for 30 s, elongation 72°C for 45 s (35 cycles) and final elongation 72°C for 10 min. Amplified bands were resolved by electrophoresis in a 1.5% (w/v) agarose gels in TBE buffer and visualized by ethidium bromide staining in UV-iluminator. Size was calculated by using 100 bp DNA Ladder (Vivantis).

DNA sequencing and phylogenetic analysis

The purified DNA of selected isolates were sequenced using 3730-XL Analyzer (Applied Biosystem) at 1st Base Sequencing, Malaysia. The obtained sequences were used to search high similarity sequences deposited in the NCBI database by using BLAST algorithm, and the identities of isolates were calculated on the basis of the highest score (>98%). Sequences were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) 5.05 to construct a phylogenetic tree by the neighbour-joining algorithm (Saitou and Nei, 1987).

Screening probiotics capability in vitro

Probiotic capability of selected isolates was examined on the basis of the ability to grow on low pH, the presence of bile salts, the ability to metabolize prebiotic (Inulin, Orafti), and adhesion assays. The survival of selected isolates in low pH was examined by growing isolates in MRS broth pH 2.0 according to Chou and Weimer (1999). One mililiter (1 ml) of overnight healthy culture was innoculated into 9 ml MRS broth (pH 2.0) and incubated at 37°C for 90 min. Viability of cells was examined every 45 min by plating on MRS agar. The ability of isolates to grow on bile-containing media was performed according to Chou and Weimer (1999). One mililiter (1 ml) of overnight healthy culture was innoculated into 9 ml MRS broth containing different concentration of bile salt (0.3; 0.5; 1.0 dan 1.5%) and incubated at 37°C for 2 h. One hundreds microliter (100 µl) of the isolates was platted into MRS agar, incubated at 37°C for 48 h and viable cells were counted. *In vitro* adherence ability was carried out according to Sanchez *et al*., (2010) and Roos and Jonsson (2002) with modification. Adhesion assays were performed in 96-well polystyrene microtiter plates (Corning) using gastric mucin from porcine stomach (Sigma) as the matrix.

Result and Discussion

Identification of isolates

Of six isolates, three were cocci in shape while three others were bacilli. Further physiological and biochemical identification

showed they were Gram positive, having no catalase production, non motil, non-spore formation, non-gas producer from glucose, growing well at 10 and 37°C, at pH 4.4 and 9.6, and growing at salt concentration 6.5% and 18% (Table 1). All of these characteristics preliminary suggest that six of isolates were belong to group of LAB. In general, LAB is characterized as Gram-positive, aerobic to facultatively anaerobic, asporogenous rods and cocci whitout oxidase enzyme, catalase, and benzidine negative, lack of cytochromes, do not reduce nitrates to nitrite, are gelatinase negative, and are unable to utilize lactate (Carr *et al.*, 2002).

The modern taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequence revealed that some taxa generated on the basis of phenotypic features do not correspond with the phylogenetic relations. Molecular approaches, especially polymerase chain reaction (PCR)-based methods, are regarded essential for specific characterization and detection of LAB strains (Gevers *et al.*, 2001). Therefore, genomic DNA of six isolates was isolated and subjected to PCR amplification using primers specifically recognized conserved region of 16S rRNA gene. The PCR products were visualized by agarose gel electrophoresis under UV light to check the presence of amplified bands of about 500 to 600 bp (Figure 1).

Figure 1 showed clearly amplified bands of ~518 bp that was generated from all isolates using PCR with specific primers. As the PCR amplification employed a conserved region within 16S rRNA gene of LAB, the presence of ~518 bp strongly

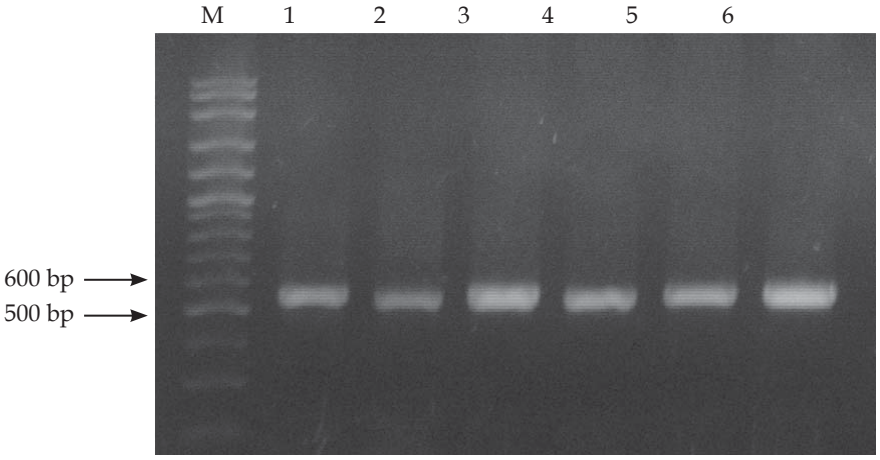

Figure 1. Amplified PCR products with 100 bp DNA ladder (M); 1. isolate AF, 2. isolate AP, 3. isolate AG, 4. isolate AA, 5. isolate BE and 6. isolate BK

Table 1. Morphological, physiological and biochemical identification of isolates

Isolates	Morphology and Physiology					Biochemical						
	Gram	Shape	Catalase	Motility	Spore formation	CO_2 production	Temperature (°C)		pH		Salt (%)	
							10	45	4.4	9.6	6.5	18
AF	+	Bacilli	-	-	-	-	-	+	+	+	+	+
AP	+	Bacilli	-	-	-	-	-	+	+	+	+	+
AA	+	Cocci	-	-	-	-	-	+	+	+	+	+
AG	+	Bacilli	-	-	-	-	-	+	+	+	+	+
BE	+	Cocci	-	-	-	-	-	+	+	+	+	+
BK	+	Cocci	-	-	-	-	-	+	+	+	+	+

suggests that all of isolates belong to LAB. This PCR-based molecular data supports previous morphological, physiological and biochemical data that all of isolates were member of LAB group. Apart from amplified band obtained from isolate 1AF that has been sequenced and presented previously (Widodo *et al*., 2012), the rest of amplified bands were than purified and subjected to DNA sequencing. The sequence data was then used to search homology with other sequence available in the database (www.ncbi.nlm.nih.gov).

Comparison of genetic relationship of isolates BK, BE, AA, AG and AP with partial sequences of 16S ribosomal RNA of some strains of *Lactobacillus sp*., *Pediococcus sp*. and *Bifidobacterium fermentum* as an out-group was illustrated using a dendogram and was shown in Figure 2. This figure 2 showed clearly that isolates BK, BE, and AA were genetically grouped within the cluster of *Pediococcus acidilactici*, and this strongly indicates these isolates were strains of *Pediococcus acidilactici*. Strains of *Pediococcus acidilactici* and *Pediococcus pentosaceus* isolated from human clinical sources have previously been reported (Barros *et al*., 2001), suggesting human GIT was abundant sources for *Pediococcus* sp. Apart from human GIT, *Pediococcus acidilactici* has previously been isolated from vacuum-packed fermented meat (Mandal *et al*., 2008), beef (Olaoye *et al*., 2008), and has been isolated from Indonesian traditional fish product (Lawalata *et al*., 2011).

Table 2 showed that Isolate AA, BE and BK had homology as seen with high similarity (99%) with sequence of *L. acidilactici* strain ZW001, *L. acidilactici* strain N8, and *L. acidilactici* strain YFPB7BMX. Supporting to figure 2, this data suggests that isolates AA, BE and BK are strains of *L. acidilactici*. According to Claverie and Notredame (2007), two or more sequences are categorized as homolog when more than 70% of nucleotide showing similarity.

Meanwhile isolates AG and AP were grouped within the cluster of strains of *Lactobacillus casei* and *Lactobacillus paracasei* (Figure 2). As isolate AG and AP were mixed within cluster *Lactobacillus casei* and *Lactobacillus paracasei* (Figure 2), further identification using species-specific primers is required to confirm of whether isolate AG was a strain of *L. casei* or *L. paracasei*. Chagnaud *et al*. (2001) have reported both conserved regions and variable zones of 16S

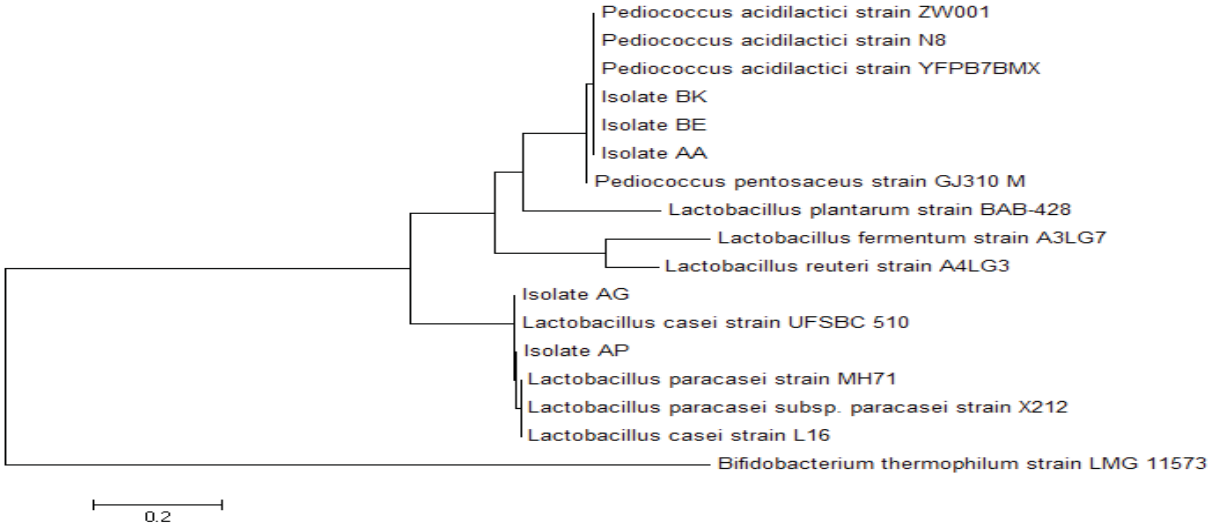

Figure 2. Phylogenetic tree showing genetic relationship of isolates BK, BE, AA, AG and AP with some species from genus *Pediococcus* and *Lactobacillus* based on 16S rRNA sequences.

Table 2. Comparison of 16S rRNA gene of isolates AA, BE and BK against Pediococcus database in the GenBank using BLAST program

Isolates	Similarity with sequence in the GenBank database (%)		
	P. *acidilactici* strain ZW001	P. *acidilactici* strain N8	P. *acidilactici* strain YFPB7BMX
AA	99	99	99
BE	99	99	99
BK	99	99	99

rRNA gene for the identification of LAB at species level, and this finding facilitated to the genetic differentiation between *L. casei* and *L. paracasei*. The finding of *Lactobacillus casei* in this study was supportive to previous experiment by Widodo *et al.* (2012) that identified *Lactobacillus casei* isolate 1AF from feces of Indonesian infants. The identification of *Lactobacillus casei* from feces indicates that human GIT is a favorable place for the growth of *Lactobacillus* sp. However, this paper does not discuss more details regarding *Lactobacillus casei* isolates due to fact that it has previously been presented (Widodo *et al.,* 2012). Rather, it emphasis on *Pediococcus acidilactici* isolates BK, BE and AA, and their probiotic capacity.

Screening for probiotic capacity in vitro

To be function as probiotics, bacterial strains must be non pathogenic, resistance to gastric acid and bile toxicity, adhere to gut epithelial tissues, be able to compete with pathogen and colonise GIT, and having ability to modulate immune responses (Collins *et al*., 1998; Dunne *et al*, 2001; Reid *et al.,* 2003). The ability of probiotics to exert beneficial effects in the lower small intestine and the colon is highly depend on the capability of strains to survive passage through the gastric acid as well as bile and pancreatic juice in the upper small intestine. The best effect is attained when probiotic strains capable to colonise the intestinal surface mucus layer since they can affect the intestinal immune system, displace enteric pathogens, provide antioxidants and antimutagens, and possibly other effects by cell signalling (Mottet and Michetti, 2005). It is therefore necessary to examine probiotic capacity of isolates by performing *in vitro* evaluation. The *in vitro* experiments was performed to monitor the ability of selected isolates BK, BE and AA to survive on acid, bile salts, to bind to the epithelial cells of GIT and the ability to utilize inulin as carbon source.

Being resistant to low pH is one of the major selection criteria for probiotic strains.

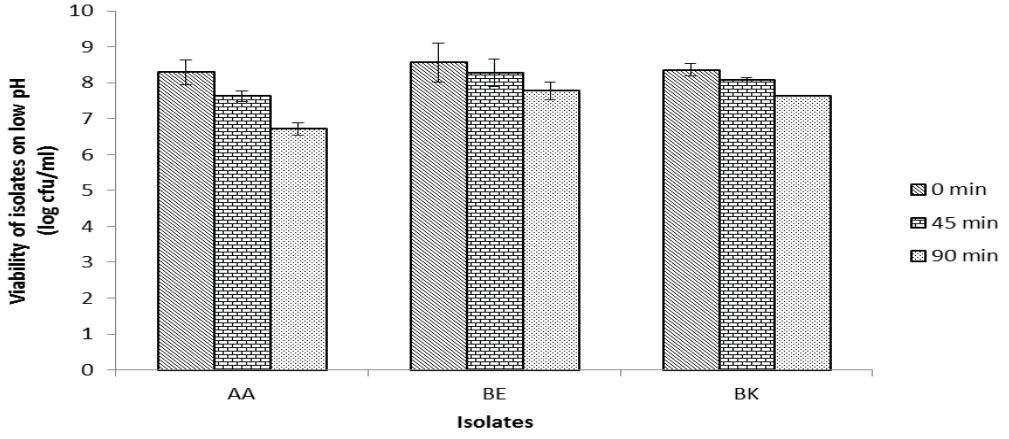

Figure 3. Viability of isolates AA, BE and BK on pH 2.0 for 90 min

In this study, selected isolates were grown at pH 2.0 for 90 min although in the real stomach pH can be as low as 1.0. Previous study revealed that a significant decrease in the viability of strains was frequently observed at pH 2.0 and below (Prasad *et al.*, 1998). Evaluation on the ability to grow on pH 2.0 showed that more than 80% of isolates AA, BE and BK were viable after grown for 90 min (Figure 3). Percentage of viability of isolates AA, BE dan BK on low pH for 90 min were 80.94±0.15; 90.67±0.38; and 91.27±0.06; respectively. According to Hutkins and Nannen (1993), bacterial strains were considered as acid resistant when more than 10% of cells survive under pH 2.0 for 90 min, suggesting that all of isolates tested here were acid tolerant. Total viable cells of isolates AA, BE and BK after growing at pH 2.0 for 90 min were 6.71±0.18; 7.77±0.25; and 7.63±0.21 log cfu/ml, respectively (Figure 3).

Apart from the ability to survive to the stressful conditions due to low pH in the stomach (pH 1.5 - 3.0), probiotics strains must also be able to resist bile salts secreted into the digestive tracts in the upper intestine (Chou and Weimer 1999). Bile is a surface active compound that penetrates and reacts with lipophilic side of cytoplasmic membrane of bacteria resulted in changes and damage of membrane structure. Hence, it is necessary

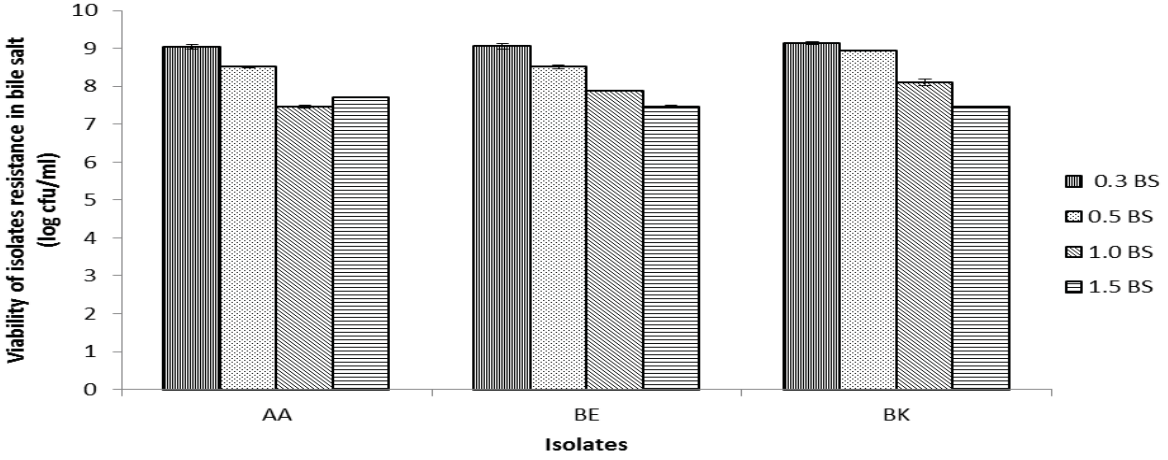

Figure 4. Viability of isolates AA, BE and BK on different bile concentration for 2 h

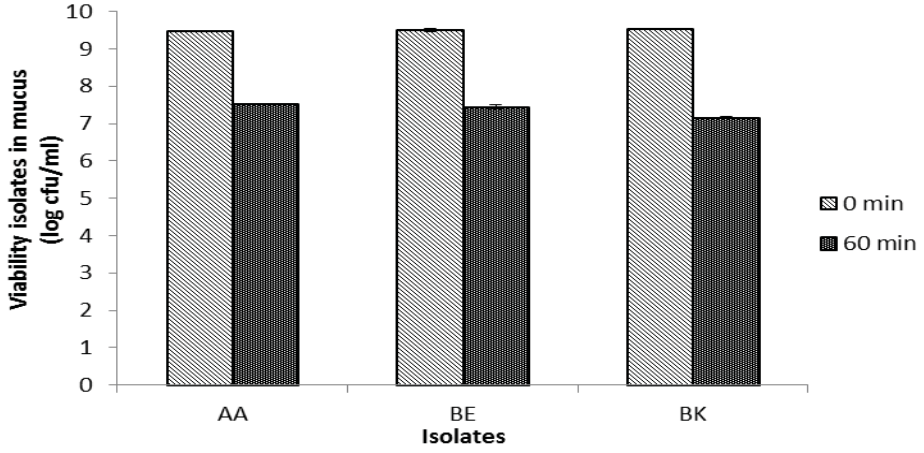

Figure 5. Adhesion ability of isolates AA, BE and BK in mucus for 60 min

to examine the capacity of selected isolates on different bile concentration. Although bile concentration in the human GIT varies, the mean intestinal bile concentration is believed to be 0.3 to 1.5% w/v and the residing time is suggested to be 2 h (Chou and Weimer , 1999). Viability of isolates AA, BE and BK on 0.3; 0.5; 1.0; and 1.5% for 2 h was presented in Figure 4. Figure 4 showed viability of around 80% of isolates AA, BE and BK after growing on bile-containing media at concentration up to 1.5%. Percentage of viable cells of isolates AA, BE dan BK on media supplemented with bile salt 1.5% for 2 h were 80.33±0.14% (from 9.42±0.05 to 7.71±0.01 log cfu/ml); 80.01±0.59% (from 9.33±0.04 to 7.47±0.02 log cfu/ml) and 79.81±0.33 (from 9.36±0.06 to 7.47±0.01 log cfu/ml), respectively. A survival of 10.3% to 57.4% of human-origin *Lactobacillus acidophilus*, *Lactobacillus gasseri, Lactobacillus rhamnosus* and *Lactobacillus reuteri* was demonstrated by Xanthopoulos *et al.* (2000) after growing at 0.15% bile salts. Compared to these previous findings, *Pediococcus acidilactici* isolates AA, BE and BK are more tolerant to bile salts than *Lactobacillus* spp. and *Lactococcus* sp. According to Begley *et al.* (2004), Composition and structure of the membrane play an important role in resistance to bile salts. There are several mechanisms involved in bacterial resistance to bile salts. One of them is the expression of bile salt hydrolase (*bsh*) genes for bile exporter (Begley *et al*., 2006).

Some of the beneficial effects exerted by probiotic are mediated by its interaction with the intestinal mucosa, and adhesion to the mucosa is considered to be a prerequisite. Bacteria colonizing the mucosa can be found both in the mucus layer and adhering to the epithelial cells (Brassart and Schiffrin, 2007). The epithelial cells of the intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids with large glycoprotein mucin being the main component. In this study, adhesion assays were performed using gastric mucin from porcine stomach (Sigma) as the matrix. Figure 5 showed that all of isolates had high adhesion capability as seen by more than 75% of cells were able to attach to the pig gastric mucin after 60 min. Percentage viability of isolates AA, BE and BK on mucus for 60 min were 79.56±0.03% (from 9.47±0.02 to 7.53±0.01 log cfu/ml); 78.29±0.18% (from 9.51±0.04 to 7.45±0.05 log cfu/ml), and 75.18±0.33% (from 9.53±0.01 to 7.17±0.02 log cfu/ml), respectively. Previous study by Xu *et al.* (2009) demonstrated adhesion yield less than 10% of *Pediococcus acidilactici* KACC 12307 to the Caco-2 cell. Adhesive value of *Pediococcus acidilactici* isolate AA (79.56%), BE (78.29%) and BK (75.18%) in this study was higher compared to other study.

The ability of probiotic LAB strains to survive in the GIT is promoted by oligosaccharides facilitating the metabolism and growth of LAB in the lumen. Dietary fibre, mainly oligosaccharides, fermented in the colon usually acts as prebiotics. The importance of prebiotics as growth enhancers of probiotic bacteria has been documented in humans (Crittenden *et al*., 2002). In this study, three selected *Pediococcus acidilactici* isolates AA, BE and BK were grown on media containing prebiotics inulin as the only source of carbon. Upon growing for 24 h, two isolates namely AA and BK were unable to grow normally as seen their growth declining after 6 h, suggesting the inability of isolates to consume inulin as the only carbon source (Figure 6). Meanwhile, isolates BE had a normal growth curve comparable when grown on normal media (MRS), suggesting that isolate BE were able to utilize inulin as carbon source. The most possible mechanism is that isolate BE consumed and fermented inulin using specific enzymes that were unknown.

Figure 6 also showed that isolate BE started their logarithmic phase of growth after 6 h of incubation and reaching the top of logarithmic after 16 h of incubation followed by a stationary phase, while isolates AA and BK were unable reach optimum logarithmic

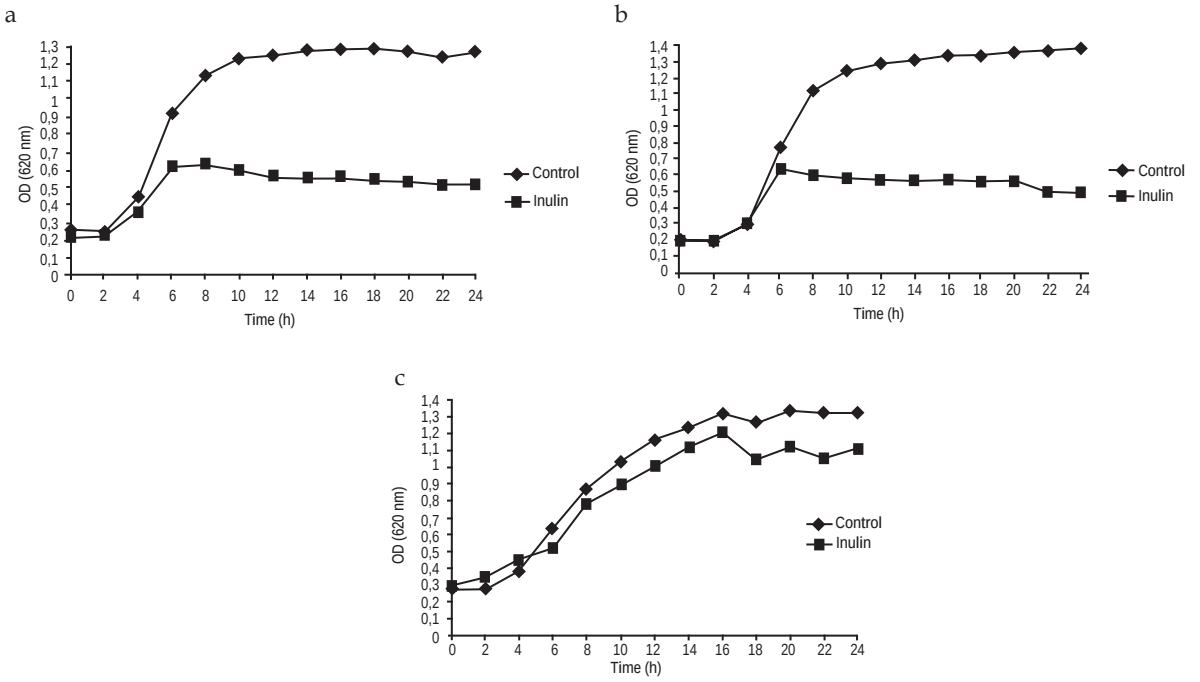

Figure 6. Growth curves of isolates: (a) AA, (b) BK, and (c) BE, in MRS media (♦) and media containing inulin 4% as carbon source (■)

phase and declining their growth after 6 h. Based on this capability in degrading inulin, the isolate BE was a potential candidate to be applied in a synbiotic consisting of probiotic and prebiotic application. The growth of isolate BE in this study on media containing prebiotic inulin was in agreement with the increase of the growth of bifidobacteria after the addition of inulin as previously reported by Muir (1999) and Ozer *et al.* (2005).

As the conclusion, identification of isolates showed that 3 isolates (AA, BE and BK) were strains of *Pediococcus acidilactici* and 2 isolates AP and AG were strains of *Lactobacillus casei*. Probiotic evaluation of strains of *Pediococcus acidiactici* demonstrated that more than 80% of cells of all of isolates were survived after growing on pH 2.0 for 90 min, around 80% cells were resistant to bile salt concentration up to 1.5% and having high ability (around 75%) to adhere on pig gastric mucin *in vitro*. Evaluation on their capability to grow on prebiotic inulin demonstrated that isolate BE was able to utilize inulin as the only carbon source and grew normally. This suggests that isolate BE is potential as probiotics as seen with the ability to resist low pH, bile salts, adhesion on mucus, and having capability to digest prebiotics.

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