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Human Origin *Lactobacillus casei* Isolated from Indonesian Infants Demonstrating Potential Characteristics as Probiotics *in vitro*

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Abstract

The aim of this experiment was to isolate and identify Lactic Acid Bacteria (LAB) from infant faeces and subsequent evaluation of its potential probiotics. LAB was isolated from faeces of infants who consumed breast milk as the only source of diet on *L-cysteine*-supplemented MRS Agar, and incubated on 37°C for 48 hours. Colonies grew on this media were then identified based on morphological, physiological and molecular approaches. Morphological and physiological identifications based on Gram staining, shape, motility, spore formation, catalase, CO_2 and NH_3 production, and the ability to grow on temperature at 10°C and 45°C. Molecular identification based on the amplification of 16S rRNA gene. The potential application of selected isolates for probiotics was evaluated based on the ability to grow on media with low pH and the addition of 0.5% bile salts, the ability to inhibit the growth of pathogenic *Bacillus cereus* and *Eschericia coli*, and *in vitro* adherence ability. On the basis of morphological, physiological and molecular analysis of 16S rRNA gene, it was concluded that the selected isolate 1AF was a strain of *Lactobacillus casei*. Evaluation of probiotic *in vitro* showed that 60.4% of cells were resistant to pH 2.0 for 90 minutes. Survival of isolate 1AF after growing at 0.5% bile salts was 70.8%. The selected isolate 1AF showed the ability to inhibit the growth of *Eschericia coli* and *Bacillus cereus* with inhibitory zone of 12.00±1,00 and 15.33±1.53 mm, respectively. *In vitro* study on the adherence value of isolate to solid plate was found at 46.5%. It is concluded that *Lactobacillus casei* isolate 1AF is a potential candidate as probiotics and subject to further *in vivo* evaluation.

Keywords : *Lactobacillus casei*, human-origin, probiotics

Introduction

According to FAO/WHO (2002), Probiotics are defined as live microorganisms that provide beneficial effects on human health. Bacterial strains commonly used as probiotics are member of genera *Lactobacillus* and *Bifidobacterium* (Fuller, 1989; Gomes and Malcata, 1999; Roberfroid, 2000; Mercenier *et al*., 2003; Grajek *et al*., 1995). These two genera are typically chemoorganotrophic and ferment carbohydrate with lactic acid as a major end product (Fuller, 1989). *Lactobacillus* belongs to the group of Lactic Acid Bacteria (LAB) with G + C content between 32 and 51%, while *Bifidobacterium* is part of *Actinobacteria* phylum and phylogenetically distinct from LAB with a G + C content ranging from 42% to 67% (Gomes and Malcata, 1999; Biavati and Mattarelli, 2001; Borriello *et al*., 2003). Bifidobacteria are often included as LAB due to metabolic similarity, such as fermentative and producing lactate among other acids (Biavati and Mattarelli, 2001; Borriello *et al*., 2003). Of the 106 species in *Lactobacillus*, 56 are potential for probiotic, while of 30 species

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in *Bifidobacterium*, 8 are potential for probiotic (Otieno, 2011).

Health-associated benefits of consuming probiotics have previously been reported. These include the capability to alleviate lactose intolerance (Heyman, 2000; Roberfroid, 2000; Marteau *et al*., 2001), to reduce serum cholesterol concentration (Anderson *et al*., 1999), to decrease the prevalence of allergy (Parvez *et al*., 2006), to prevent and reduce risk of certain cancers (Ohashi *et al.,* 2000; Wollowski *et al*., 2001; Xiao *et al*., 2006), and to stimulate the immune systems (Gill, 1998; Nagao *et al*., 2000; Pareira *et al*., 2003). To perform as probiotics, bacterial strains must have specific criteria. Of these criteria including human origin, non pathogenic, resistance to gastric acid and bile toxicity, adherence to gut epithelial tissues, ability to compete with pathogen and colonise gastrointestinal tract (GIT), and the ability to modulate immune responses (Collins *et al*., 1998; Dunne *et al*., 1999; Dunne *et al*, 2001).

A number of bacteria strains for probiotics have been isolated from different sources, and one of the best source is from GIT (Margolles *et al*., 2009). GIT has been reported to host around 400 species of bacteria of 50 different genera co-exist (Lisal, 2006). It has previously been reported that the development of colonic microbiota in the newborn infants is strongly associated with the consumption of human milk oligossacharide (HMO) (Favier *et al*., 2003). The intestinal tract of breast-fed infants is rich sources for *Bifidobacterium* and *Lactobacillus* as induced by HMO provided within breast milk (Boehm and Stahl, 2007; Favier *et al*., 2003). LAB have previously been isolated from human intestine (Heilig *et al*., 2001), however human origin isolates obtained from Indonesian infants consuming breast milk has never been reported. As such, isolation, identification and selection of LAB for probiotics from this source is of rationale. In this paper, we report the isolation and identification of human origin *Lactobacillus casei* isolated from faeces of Indonesian infants with potential capability as probiotics.

Materials and Methods

Sample collection

Samples used in this experiments were faeces of breast-fed infants aged less than 1-month old and born normally from healthy mother who did not consume antibiotics. Fecal samples were collected aspetically in sterilized tubes and kept in ice (±4°C) during transportation to laboratory and until ready for further analysis.

Bacterial isolation and cellular identification

Bacterial isolation was carried out in an anaerobic condition (Beerens *et al*., 1980) with modification. Tenfold serial dilution of the fecal samples were prepared with buffer NaCl 0.85%, and 0.1 ml of each dilution was plated onto De Man-Rogosa-Sharpe (MRS; Oxoid) agar supplemented with L-cysteine 0.5 g/l (MRS-Cys). The plates were incubated at 37°C for 48 h in an anaerobic condition by injecting nitrogen. Between 10 and 15 isolates from each sample were randomly selected, grown in MRS-Cys broth, harvested and stored at -80°C in the presence of glycerol (30%, v/v). White colonies grown on the plate after incubation were subjected to morphological and physiological identification (Holt *et al*., 1994), including Gram staining, shape, spore formation, motility, catalase test, the growth at 10° and 45°C, and production of CO_2 and NH_3. Based on these preliminary screening, colonies with characteristics belong to LAB were selected and subject to molecular identification using 16S rRNA gene amplification.

Genomic DNA extraction

Genomic DNA of the selected isolates was extracted from 1.5 ml of overnight cell culture, suspended in 400 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH7.5) containing lysozyme (30 mg/ml). The solution was mixed by inverting the tube, and

incubated at 37°C for 1 h. Fifty microliters of SDS solution (10%) were added and followed by incubation at 65°C for 1 h. After incubation for 1 h, 167 µl of NaCl solution (5M) were added and incubation was prolonged for another 1 h. DNA was separated from cellular debris with 400 µl chloroform and incubated at room temperature for 10 min followed by centrifugation at 13000 rpm for 10 min. Top aquaeous phase was transfered to new eppendorf 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. Agarose gel electrophoresis was carried out on 0.8% agarose gel in TAE buffer at 80 V for 1 h. Size was calculated by using 1 Kb DNA Ladder (Promega, USA).

PCR amplification of 16S rRNA genes

Amplification of a 518-bp fragment of the 16S rRNA gene was carried out using PCR with primers designed based on the conserved region of 16S rRNA gene. Forward primers *plb16* (5-AGAGTTTGATCCTGGCTCAG-3) and reverse primers *mlb16* (5-GGCTGCTGGCACGTAGTTAG-3) are located at positions 8 to 27 and 507 to 526 in the 16S rRNA gene sequence of *Escerichia coli*, respectively (Martin *et al*., 2009). Amplification of DNA was performed using PCR thermal cycler (BOECO TC-SQ, Germany). The PCR condition was set as follows: denaturation at 96°C for 30 s, annealing 55°C for 30 s, elongation at 72°C for 45 s (30 cycles) and a final extension at 72°C for 4 min. Amplified bands were resolved by electrophoresis in a 1.6% (w/v) agarose gels and visualized by ethidium bromide staining.

DNA sequencing and phylogenetic analysis

The purified DNA was sequenced using Applied Biosystem 3730-XL Analyzer at 1st Base Sequencing, Singapore. The resulting was used to search sequence deposited in the NCBI database by using the 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. Phylogenetic tree of the sequence was constructed by the neighbor-joining algorithm (Saitou and Nei, 1987). The root position on the unrooted tree was estimated by using *Pediococcus acidilactici* strain DSMZ 20238 as the outgroup strain.

Screening for probiotic capacity in vitro

Probiotic potential of the selected isolates was examined on the basis of pH and bile tolerance, antimicrobial activity, and adhesion assay. The survival of selected isolates in extreme pH was examined by growing in MRS broth pH 2.0 according to Chou and Weimer (1999). One microliter (1 µl) of overnight healthy culture was innoculated into 9 µl MRS broth (pH 2.0) and incubated at 37°C for 90 min. Viability of cells was examined every 30 min by plating on MRS agar. The ability of isolates to grown on bile-containing medium was performed according to Chou and Weimer (1999). Selected isolates were grown at 37°C for 24 h and 100 µl of the culture was platted into MRS agar and MRS agar supplemented with 0.5% bile salt. Plates were incubated at 37°C for 48 h and viable cells were counted. Bile tolerance (%) was calculated based on the viable cells on MRS agar supplemented with 0.5% bile salt divided with all viable cells on MRS agar. Antimicrobial activity of the selected isolates was carried out acoording to Jacobsen *et al*., (1999) with modification. The potential application of selected isolates for probiotics was also evaluated based on the ability to inhibit the growth of pathogenic *Bacillus cereus* and *E. coli* (Jacobsen *et al*., 1999), and in vitro adherence ability (Dewanti and Wang, 1995).

Results and Discussion

Faeces of breast-fed infants were reportedly recognized with high

population of Bifidobacteria and Lactobacilli (Bezkorovainy, 2001). This condition was due to the presence of HMO that creates supportive environment for the growth of those bacteria (Stark and Lee, 1982). Previous experiments had reported that effective human probiotic should be of human origin, and this is sufficiently different from animals and environments.

In this experiment, we isolated and identified 17 isolates with morphological and physiological characteristics belongs to LAB (Table 1). According to Orla-Jensen (1942) as cited by Franz *et al.* (2010), LAB is characterized as Gram-positive, aerobic to facultatively anaerobic, non motile, asporogenous rods and cocci, and fermentate carbohydrate to produce lactic acid as the main product. Of the 17 isolates, only 11 isolates showed differences on both morphological and physiological (Table 1) suggesting they were from different groups. *Lactobacillus* is the main genus with rod in shape, while *Lactococcus, Streptococcus, Enterococcus* and *Leuconostoc* are cocci (Axelsson, 2002). The CO_2 production from glucose is an indication of heterofermentative bacteria,

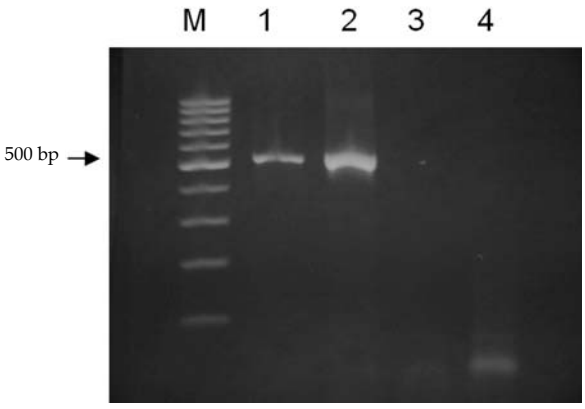

Figure 1. Amplified bands of 518 bp of 16S rRNA of isolate (1) 1A7, (2) 1AF, (3) 1AL, and (4) 1AA.

whilst homofermentative bacteria were unable to produce CO2 (Margolles *et al.*, 2009; Axelsson, 2002). Table 1 showed that isolates 1AB, 1AH and 1AK were heterofermentative as shown with CO_2 production, while the rest were homofermentative as shown without CO_2 production. The selected isolates showed growth differences when grown at 10°C and 45°C. According to Axelsson (2002), genus *Enterococcus* was able to grow at 10°C and 45°C, while genus *Lactococcus*

Table 1. Morphological and physiological identification of isolates

Isolates	Gram	Shape	CO_2 production	Growth		Catalase	Motility	Spore formation
				10°C	45°C			
1AA[a]	+	Bacilli	-	+	-	-	-	-
1AB[b]	+	Bacilli	+	+	+	-	-	-
1AC[c]	+	Bacilli	-	+	+	-	-	-
1AD[d]	+	Cocci	-	+	-	-	-	-
1AE[a]	+	Bacilli	-	+	-	-	-	-
1AF[a]	+	Bacilli	-	+	-	-	-	-
1AG[e]	+	Bacilli	-	-	+	-	-	-
1AH[f]	+	Bacilli	+	+	+	-	-	-
1AI[g]	+	Bacilli	-	-	-	-	-	-
1AK[h]	+	Bacilli	+	+	-	-	-	-
1AL[i]	+	Bacilli	-	-	-	-	-	-
1AN[c]	+	Bacilli	-	+	+	-	-	-
1BE[d]	+	Cocci	-	+	-	-	-	-
1BH[d]	+	Cocci	-	+	-	-	-	-
1BK[d]	+	Cocci	-	+	-	-	-	-
1BL[j]	+	Cocci	-	-	+	-	-	-
1A7[k]	+	Cocci	-	-	-	-	-	-

Note : [a,b,c,d,e,f,g,h,i,j,k)] Isolates with similar superscript show similarity on both morphology and physiology.

and *Vagococcus* was able to grow at 10°C but unable to grow at 45°C. Genus *Streptococcus* was recognized with the unability to grow at 10°C, and the growth at 45°C was variable among species.

Of the 11 different isolates, 4 isolates namely 1AA, 1AF, 1AL and 1B7 were chosen as representative and then subjected to molecular identification based on the amplification of 16S rRNA gene using universal primer of plb16 and mlb16. These pair of primers was designed to amplify the variable region V1 and V2 of the 16S rRNA gene of a number LAB strains (Martin *et al.*, 2009). Approximately 518-bp fragment of 16S rRNA gene was amplified and this was presented in Figure 1.

Figure 1 showed that out of 4 isolates tested only two isolates produced the amplified bands of 518 bp, that were isolates 1A7 and 1AF. The amplified band was then subjected for sequencing. Of two amplified bands, only amplified band of isolate 1AF generated a high quality sequence that was applied for sequence comparison. The comparison was carried out using BLAST program for homology searching of multiple sequences in the GenBank, and the result was presented in Table 2.

Table 2. Comparison of 16S rRNA gene of 1AF isolates against Lactobacillicaeae (txid3395) database in the GenBank using BLAST program.

Isolates	Similarity (%)
L. casei strain 43-197	99%
L. casei strain Lc18	99%
L. casei ATCC 334	99%
L. casei strain Shirota	99%
L. casei strain Zhang	99%
L. casei ATCC 4646	99%

Isolate 1AF showed high homology as seen with high similarity (99%) with sequence of *L. casei* strain 43-197, *L. casei* strain Lc18, *L. casei* ATCC 334, *L. casei* strain Shirota, *L. casei* strain Zhang, and *L. casei* strain 4646 (Table 2). Two or more sequences are categorized as homolog when more than 70% of nucleotide showing similarity (Claverie and Notredame, 2007). Comparison of genetic relationship of isolate 1AF with several partial sequences and complete genome of some strains of *Lactobacillus sp.*, *Lactococcus*

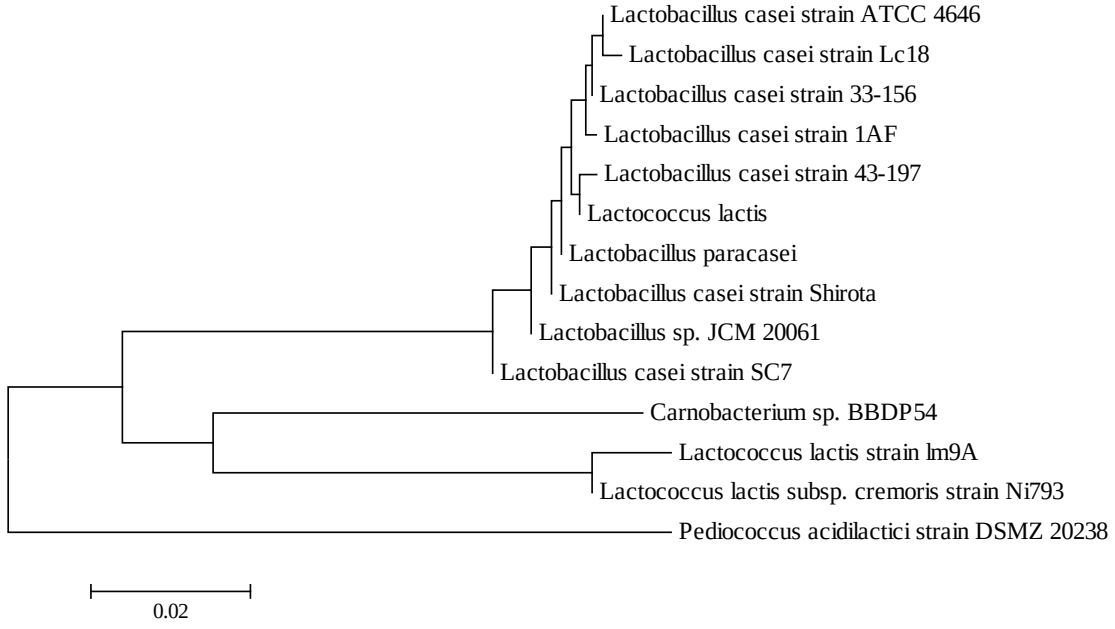

Figure 2. Phylogenetic tree showing genetic relationship of *Lactobacillus casei* isolate 1AF and other species from genus *Lactobacillus* and other bacteria based on 16S rRNA sequences.

sp., and *Pediococcus sp.* was illustrated using a dendogram and was shown in Figure 2. Figure 2 showed *Lactobacillus casei* isolate 1AF was grouped within the cluster of *Lactobacillus casei*, and this strongly suggests that isolate 1AF was a strain of *Lactobacillus casei*. In contrast, isolate 1AF out of cluster with *Pediococcus acidilactici* strain DSMZ 20238, *Lactococcus lactis subsp. cremoris* strain Ni793 and *Lactococcus lactis* strain lm9A (Figure 2), suggesting that isolate 1AF was not part of genus *Pediococcus* and *Lactococcus*. The isolation of *Lactibacillus casei* in this study was in line with previous reports on the isolation of *Lactobacillus* sp. from faeces of infants (Heilig *et al*., 2001). Some of these strains have been applied commercially, included *Lactobacillus rhamnosus* GG, *Lactobacillus casei* strain Shirota, and *Lactobacillus acidophilus* LA-1 (Dunne *et al.,* 2001).

To asses probiotic capacity of *Lactobacillus casei* isolate 1AF, *in vitro* evaluation was carried out. The *in vitro* experiments was performed to monitor the ability of selected isolates to survive on acid, bile salts, the ability to bind to the epithelial cells of GIT, and the ability to inhibit the growth of pathogenic *Eschericia coli* and *Bacillus cereus* (Greene and Klaenhammer, 1994). Prior to arrive at GIT, probiotic bacteria have to be survive through the stomach where the secretion of gastric acid constitutes a primary defence mechanism against the majority of ingested microorganisms (Chou and Weimer, 1999). It is therefore important to examine survival of *Lactobacillus casei* isolate 1AF in human gastric juice as a representative condition of the stomach. The results showed that the growth of *Lactobacillus casei* 1AF decreased from 8.67±0.10 to 5.24±0.15 log CFU/ml after growing at pH 2,0 for 90 minutes (Figure 3). The data suggest that more than 50% of cells were survive after grown at pH 2.0 for 90 minutes. Kim *et al.* (1999) reported the lost of viability of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis* when grown at pH 2.0 suggesting that *Lactobacillus casei* isolate 1AF is more acid

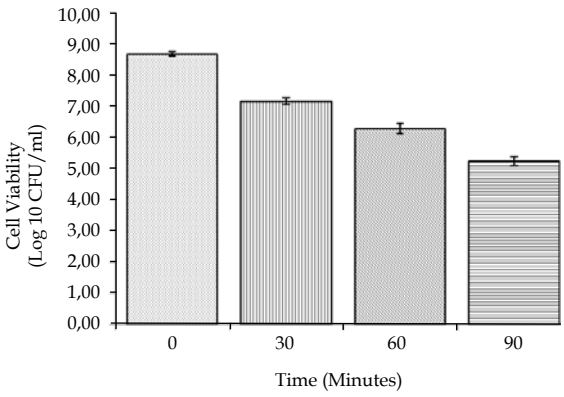

Figure 3. Cell viability of *Lactobacillus casei* 1AF when grown at pH 2.0 for 90 min

tolerant compared to *Lactococcus lactis* subsp. cremoris. Bacterial strains were considered as acid resistant when more than 10% of cells survive under pH 2.0 for 90 min (Hutkins and Nannen, 1993). The ability of bacteria to regulate their cytoplasmic or intracellular pH is the most important physiological requirements of the cells for growing under low pH. *Proton-translocating ATPase* is protein transporter in LAB that maintains pH homeostatis by means of pumping H^+ out of cells (Hutkins and Nannen, 1993). Using this transporter, *Lactobacillus casei* was reportedly able to maintain internal pH at 5.1 to 6.4 when grown at external pH 3.8 (Nannen and Hutkins, 1991). Bacterial cells unable to maintain a near neutral intracellular pH during growth or storage at low extracellular pH may lose viability and cellular activity. Moreover, acid resistant bacteria showed a greater resistance to the membrane's damage due to the decline in extracellular pH compared to non acid-resistant bacteria (Hutkins and Nannen, 1993).

Bile salts are secreted into the digestive tract and one of barrier for bacterial growth. Bile has properties as a surface active compound that penetrates and reacts with lipophilic side of cytoplasmic membrane of bacteria resulted in changes and damage of membrane structure (Succi *et al*., 2005). Stress on bile salts affect the structure and function of large macromolecules such as

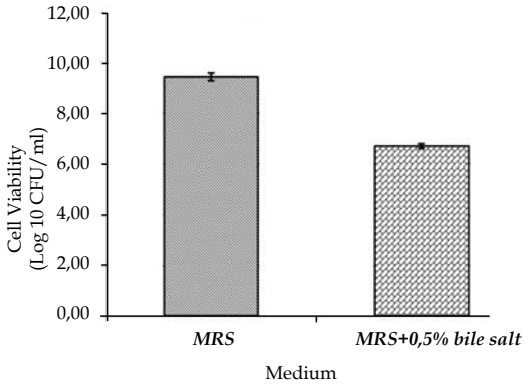

Figure 4. Cell viability of *Lactobacillus casei* 1AF when grown at normal condition on MRS broth and under the addition of 0.5% Oxbile for 48 h

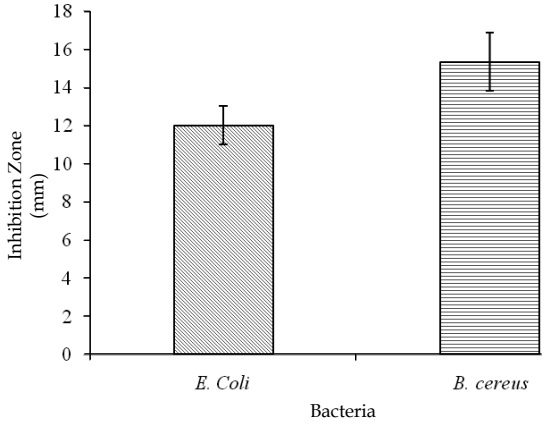

Figure 5. Inhibition zone of *Lactobacillus casei* 1AF against *E. coli* and *B. cereus*

DNA and proteins as well as stress to the acid and bile salts damage the molecule. In this study, the ability of *Lactobacillus casei* isolate 1AF to grow on media supplemented with 0.5% bile salt was also assessed. The result showed a decrese of cell viability from 9.46±0.16 to 6.70 ±0.04 $\log_{10}$ CFU/ml after growing with the addition of 0.5% bile salt for 48 h (Figure 4). This data suggest survival of 70% of cells following treatment with 0.5% bile salt. Previous studies by Xanthopoulos *et al.*(2000) reported survival of 10.3% to 57.4% of human-origin *Lactobacillus acidophilus*, *L. gasseri, Lactobacillus rhamnosus* and *Lactobacillus reuteri* after grown at 0.15% bile salts. Another study by Kim *et al.* (1999) reported the lost of viability of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis* when grown at 0.2% bile salt. Compared to these previous findings, *Lactobacillus casei* isolate 1AF is more tolerant to bile salts than *Lactobacillus* spp. and *Lactococcus* sp. Composition and structure of the membrane play an important role in resistance to bile salts (Begley *et al.,* 2004). 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).

In this study, *L. casei* 1AF was further assayed for antimicrobial activity against pathogenic bacteria *Bacillus cereus* and *Escherichia coli*. *L. casei* 1AF was found capable for significantly inhibiting the growth of both Gram positive and Gram negative bacterial cultures *in vitro*. The average diameter of the inhibition zone of *Escherichia coli* and *Bacillus cereus* were 12.00 ± 1.00 mm and 15.33 ± 1.53 mm, respectively (Figure 5). Savadogo *et al.* (2004) reported a 10-mm growth inhibition of *Bacillus cereus* 13569 LMG and a 9-mm growth inhibition of *Eschericia coli* 105182 CIP as a result of bacteriocin produced from LAB isolated from Burkina Faso's fermented milk. Another study by Awaisheh and Ibrahim (2009) found that human origin LAB, namely *Lactobacillus casei, Lactobacillus acidophilus* and *Lactobacillus reuteri*, had the highest inhibition zone (14-22 mm) against pathogenic bacteria compared to vegetable origin LAB (*Lactobacillus plantarum*). The inhibition zone of *Lactobacillus casei* isolate 1AF is higher than that of LAB isolated from fermented milk in Burkina Faso Savadogo *et al.* (2004) and within the range of human origin LAB reported by Awaisheh and Ibrahim (2009). In this study we did not elaborate the mechanisms of inhibition. Previous study has revealed that pathogenic inhibition by LAB was due to the production of organic acids, H_2O_2, and bacteriocin (Silva *et al*., 1987).

Adherence of probiotic on GIT and epithelial cells is one of the most important selection criteria for probiotics. Adherence to the mucosa of the digestive tract allows

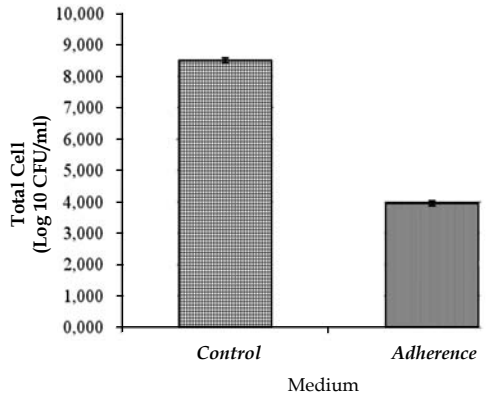

Figure 6. The ability of *Lactobacillus casei* isolate 1AF to adhere steel plate *in vitro*

probiotics colonization, immune stimulation, and competition with pathogens (Lahtinen and Ouwehand, 2009). The difficulty to evaluate the ability on attachment of probiotics to the digestive tract and epithelial cells in vivo was overcome with the use of *in vitro* methods. Stainless steel plate is one *in vitro* method to measure the attachment ability. Using this method, *Lactobacillus casei* isolate 1AF demonstrated the adherence ability at 3.95±0.08 $\log_{10}$ CFU/mm^2 out of total cells 8.50±0.07 $\log_{10}$ CFU/ml (Figure 6). This data suggest 46.5% of cell *Lactobacillus casei* isolate 1AF was able to attach stainless steel plate *in vitro*. Previous study by Duary *et al*. (2011) demonstrated the ability of indigenous probiotic *Lactobacillus* strain Lp9 and Lp91 to adhere to Caco2 and HT-29 colonic adenocarcinomal human intestinal epithelial cell lines *in vitro*. They reported that *Lactobacillus* Lp91 was the most adhesive strain to HT-29 and Caco2 cell lines with adhesion values of 12.8% and 10.2%, respectively. *Lactobacillus delbrukeii* CH4 was the least adhesive with value of 2.5% and 2.6% on HT-29 and Caco2 cell lines. Another study by Lewandowska *et al*. (2005) demonstrated adhesion yield 2.02% of *Lactobacillus casei* strain Shirota, 4.91% of *Lactobacillus acidophilus* LC1, 33.81% of *Lactobacilus rhamnosus* GG, and 3.89% of *Lactobacillus helveticus* to the Caco-2 Cells. Adhesive value of *Lactobacillus casei* isolate 1AF in this study was higher (46.5%) compared to other study, and this is likely due to the use of stainless steel plate instead of using human intestinal epithelial cell lines.

In conclusion, on the basis of morphological, physiological and molecular analysis of 16S rRNA gene, the selected isolate 1AF was identified as a strain of *Lactobacillus casei*. *In vitro* data indicate that the selected isolate of *Lactobacillus casei* 1AF is a potential candidate as probiotic and subject to further investigation on its health benefit both in animal models and human placebo controlled test.

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