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*Full Length Research Paper*

# Evaluation of Two *Lactobacillus* Strains as Probiotics with Emphasis in Utilizing Prebiotic Inulin as Energy Source

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**Abstract**

**The aim of this experiment was to examine the capability of two *Lactobacillus* strains as probiotics with emphasis in utilizing prebiotic inulin as energy source. *Lactobacillus casei* strain AP and AG were isolated from fecal of Indonesian infant, and had been reported in previous experiments. Characteristics as probiotics was evaluated *in vitro* based on the ability to grow on media at low pH and the addition up to 1.5% bile salts, the ability to inhibit the growth of *Shigella flexnerii*, *Eschericia coli*, *Staphylococcus aureus* and *Enterococcus faecalis,* the ability to attach gastric mucin, and the ability to consume prebiotic inulin as the only carbon source. Results showed that 80% of cells survived to pH 2.0 for 90 minutes, while survival of these two strains after growing at media supplemented with 1.5% bile salts was more than 70%. These two strains showed the ability to inhibit the growth of *Shigella flexnerii, Eschericia coli*, *Staphylococcus aureus* and *Enterococcus faecalis.* Further study on the adherence ability showed high level (70%) attachment on gastric mucin. Both strains were also able to grow normally using prebiotic inulin as the only carbon source. It was concluded that *Lactobacillus casei* strains AP and AG are potential candidates as probiotics and subject to further *in vivo* assessment.**

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

## INTRODUCTION

Probiotics are defined as living microorganisms that contribute to beneficial effects on human health upon ingested in adequate doses (FAO/WHO, 2002). A number of beneficial effects in consuming probiotics have already been reported. Of these effects are alleviating lactose intolerance (Marteau *et al*., 2001; Heyman, 2000; Roberfroid, 2000), reducing concentration of serum cholesterol (Ooi and Liong, 2010; Anderson *et al*., 1999), decreasing the prevalence of allergy (Parvez *et al*., 2006), preventing and reducing risk of certain cancers (Xiao *et al*., 2006; Wollowski *et al*., 2001; Ohashi *et al.,* 2000), and stimulating the immune systems (Pareira *et al*., 2003; Nagao *et al*., 2000; Gill, 1998). To be functional as probiotics for human, bacterial strains must be of human origin, non pathogenic, survive to gastric acid and bile toxicity, able to attach to gut epithelial tissues, colonise gastrointestinal tract (GIT) and able to compete with pathogen, as well as having ability to modulate immune responses (Dunne *et al*, 2001; Dunne *et al*., 1999; Collins *et al*., 1998). Bacterial strains of genera lactobacilli and bifidobacteria have commonly been applied as probiotics for human consumption (Grajek *et*

*al*., 1995; Mercenier *et al*., 2003; Otieno, 2011; Roberfroid, 2000; Gomes and Malcata, 1999; Fuller, 1989). Lactobacilli are member of Lactic Acid Bacteria (LAB) with G + C content varied from 32 to 51%, while bifidobacteria is member of actinobacteria and phylogenetically separated from LAB with a G + C content ranging from 42% to 67% (Borriello *et al*., 2003; Biavati and Mattarelli, 2001). These two genera are typically chemoorganotrophic with bifidobacteria having metabolic similarity in producing lactate and other organic acids (Borriello *et al*., 2003; Biavati and Mattarelli, 2001).

The human gastrointestinal tract (GIT) is the best source of probiotics (Margolles *et al.,* 2009). GIT is the habitat of around 400 bacterial species of which 50 different genera co-exist (Simon and Gorbach, 2006). Favier *et al.* (2003) previously reported that the consumption of human milk oligossacharide (HMO) promotes the development of colonic microbiota in the newborn infants. The microbiota of breast-fed infants are dominated by bifidobacteria and lactobacilli as they growth were induced by HMO provided within breast milk (Boehm and Stahl, 2007; Favier *et al*., 2003). Human-origin probiotic strains isolated have been commercially presented. These include *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, and *Lactobacillus acidophilus* LA-1 (Dunne *et al.,* 2001).

Isolation and identification of human-origin *Lactobacillus sp.* isolated from fecal of Indonesian infants have previously been reported (Widodo *et al*., 2012a). Further experiments had successfully identified five isolates (Widodo *et al*., 2012b). Three of these isolates, namely AA, BE and BK, were identified as strains of *Pediococcus acidilactici* (Widodo *et al*., 2012b), while two of them, isolates AP and AG, were identified as strains of *Lactobacillus casei* (Widodo *et al*., 2012b). This study is a continuation from previous study with emphasis on the evaluation of the capability of two identified strains as probiotics *in vitro*, particularly their ability to utilize prebiotic inulin as carbon source.

## MATERIALS AND METHODS

### Bacterial strains

*Lactobacillus casei* strain AP and AG were obtained from previous experiments (Widodo *et al*., 2012b). Bacterial cells 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.

### Screening for probiotic capability *in vitro*

The capability of two strains as probiotic was examined on the basis of acid and bile tolerance, antimicrobial activity, adhesion assay, and the ability to grow on inulin-containing media. The survival of strain AP and AG in an extreme pH was examined by growing 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. Cell viability of bacterial culture was examined every 30 min by plating 100 µl on L-cysteine-supplemented MRS agar. The viable cells were counted and expressed as colony forming unit per mililiter (CFU/ml). The ability of strains to grown on bile-containing medium was performed according to Chou and Weimer (1999). One mililiter (1 ml) overnight healthy culture was innoculated into 9 ml MRS broth containing different concentration (0.3; 0.5; 1.0 and 1.5%) of bile salts (Sigma-Aldrich) and incubated at 37°C for 48 h. One hundreds microliter (100 µl) of the culture cells was platted into L-cysteine-supplemented MRS agar, incubated at 37°C for 48 h and viable cells were counted on MRS agar. Bile tolerance (%) was calculated based on the viable cells on MRS agar supplemented with bile salts divided with all viable cells on MRS agar. Antimicrobial activity was carried out acoording to Jacobsen *et al*., (1999) with modification. This was examined by means of well diffusion assay for bacteria to inhibit the growth of pathogenic *Shigella flexnerii*, *Eschericia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* (Jacobsen *et al*., 1999). *In vitro* adhesion assays were 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.

### The ability to utilize inulin as carbon source

The ability of strains to utilize inulin as carbon source was examined by growing on media with inulin as the only carbon cource, and accordingly as control the cells were also grown in MRS. The growth of bacterial cells at 37°C was monitored for 24 h and measured using spectrophotometry at Optical Density 620 nm ($OD_{620}$).

## RESULTS AND DISCUSSION

Isolation and identification *Lactobacillus casei* and *Pediococcus acidilactici* from fecal of Indonesian infants had previously been reported (Widodo *et al*., 2012a; Widodo *et al*., 2012b). In this study, further experiments were conducted to evaluate their ability to function as probiotics *in vitro*. While the competence of *Pediococcus* strains had previously been reported (Widodo *et al*., 2012b), this paper discuss more details regarding potential probiotics of *Lactobacillus casei* strain AP and

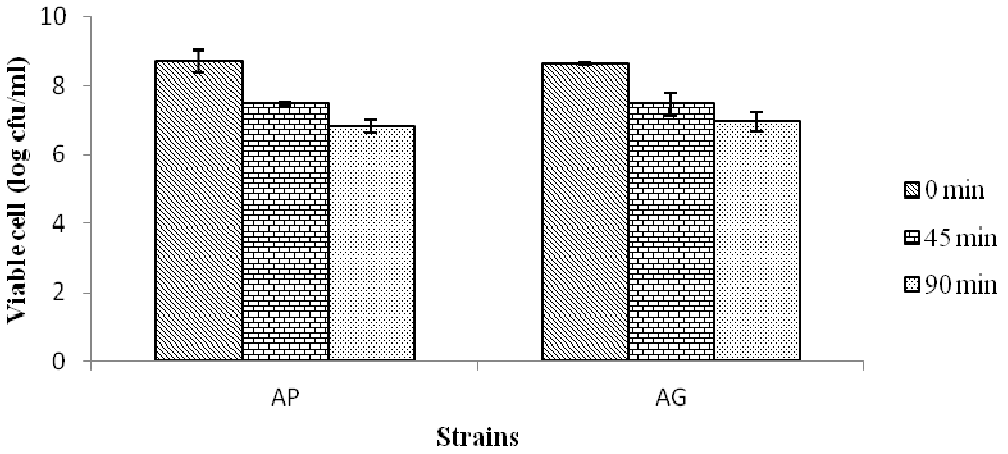


**Figure 1.** Cell viability of *Lactobacillus casei* strain AP and AG at pH 2.0 for 90 min

AG. This was carried out by examining cell survival on acid and bile salts, the ability to attach on gastric mucin and to inhibit the growth of pathogenic *Shigella flexnerii*, *Eschericia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*, as well as the ability to degrade prebiotic inulin *in vitro*.

Stomach is the main barrier where the secreted gastric acid plays a primary defence mechanism against the majority of ingested microorganisms (Chou and Weimer, 1999). To be function as probiotics, survival of *Lactobacillus* strains from bile and acid stress in stomach is vital. Examining the survival of *Lactobacillus* strain AP and AG in media having low pH and increased concentration of bile salts, as a representative condition of the stomach, is therefore of importance. This was performed by growing these two strains in MRS media at pH 2.0 for 90 min. The greater of cell survived from acid stress, the better in acid tolerance.

A decreased in cell viability of both *Lactobacillus* strain AP and AG was observed after growing for 90 min at pH 2.0 (Figure 1). Cell viability of *Lactobacillus casei* strain AP decreased from 8.72±0.33 to 6.82±0.18, while *Lactobacillus casei* strain AG decreased from 8.63±0.04 to 6.95±0.29 $\log_{10}$ CFU/ml (Figure 1). This data suggests that more than 50% of cells were survive after grown at pH 2.0 for 90 minutes. Acoording 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 minutes, suggesting that strain AP and AG are acid tolerance. To survive on acid condition, bacterial strains physiologically have to regulate their cytoplasmic or intracellular pH at a near neutral by using a number of transporters. One of the vital transporters in LAB is *Proton-translocating* ATP*ase* 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 at low extracellular pH may lose viability and cellular activity.

Another barrier for bacterial growth in the digestive tract is bile salts. As a surface active compound, bile penetrates and reacts with lipophilic side of bacterial cytoplasmic membrane causing a damage of membrane structure (Succi *et al.,* 2005). Bile also affects the structure and function of large macromolecules such as DNA and proteins leads to the damage of molecule.In this study, cell viability of *Lactobacillus casei* strain AP and AG after growing on media supplemented with up to 1.5% bile salt was also examined. Both strains experienced a growth decrease after growing for 48 hours in a media supplemented with 0.3; 0.5; 1.0; and 1.5 (%) bile salts (Figure 2). *Lactobacillus* strain AP experienced a decrease of cell viability from 9.60±0.01 to 7.38±0.03 $\log_{10}$ CFU/ml,while a decrease from 9.15±0.05 to 7.92±0.02 $\log_{10}$ CFU/ml was observed in strains AG suggesting cells survival of 70% after growing in 1.5% bile salt (Figure 2). A survival rate at 10.3% to 57.4% of human-origin *Lactobacillus acidophilus*, *Lactobacillus gasseri, Lactobacillus rhamnosus* and *Lactobacillus reuteri* after grown at 0.15% bile salts had been reported by Xanthopoulos *et al.* (2000). Meanwhile, study by Kim *et al.* (1999) showed a zero viability of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis*

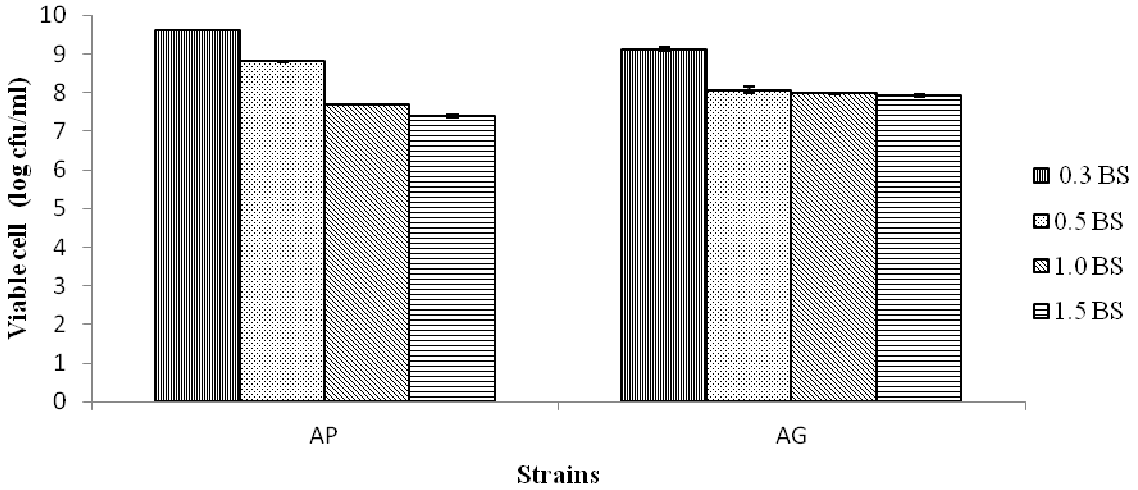


**Figure 2.** Cell viability of *Lactobacillus casei* strain AP and AG when grown under the addition of 1.5% bile for 48 hours

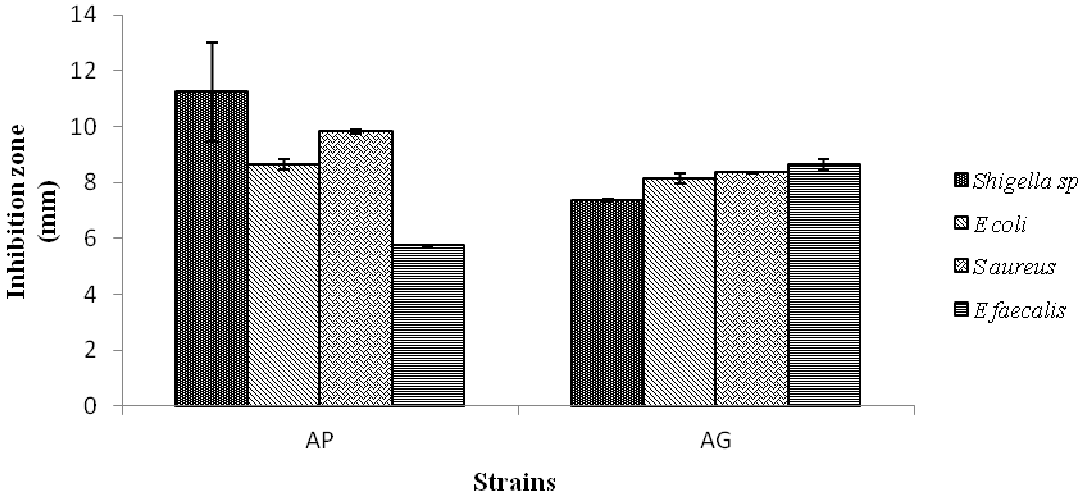


**Figure 3.** Inhibition zone of *Lactobacillus* strain AP and AG against *Shigella flexnerii, E. coli*, *Staphylococcus aureus* and *Enterococcus faecalis.*

when grown at 0.2% bile salt. Compared to these previous findings, *Lactobacillus casei*s train AP and AG are more tolerant to bile salts than *Lactobacillus* spp. and *Lactococcus*sp. According to Begley *et al*. (2004), bile tolerance was affected by the composition and structure of cell membrane. A number of mechanisms involved in bacterial resistance to bile salts had been reported, including the utilization of bile salt hydrolase (*bsh*) for bile exporter (Begley *et al*., 2006).

*Lactobacillus casei* strains AP and AG were also examined for antimicrobial activity against pathogenic bacteria *Shigella flexnerii, Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*. In this experiment, both strains were able to inhibit the growth of both Gram positive and Gram negative bacteria *in vitro* (Figure 3). The diameter of inhibition zone of *Lactobacillus* strain AP to *Shigella flexnerii* ATCC 12022, *Escherichia coli* FNCC 0091*, Staphylococcus aureus* FNCC 0047*,* and *Enterococcus faecalis* 99 EF were 11.25±1.77; 8.63±0.18; 9.83±0.07; and 5.74±0.01, respectively. Meanwhile, the diameter of inhibition zone of *Lactobacillus* strain AG to *Shigella flexnerii* ATCC

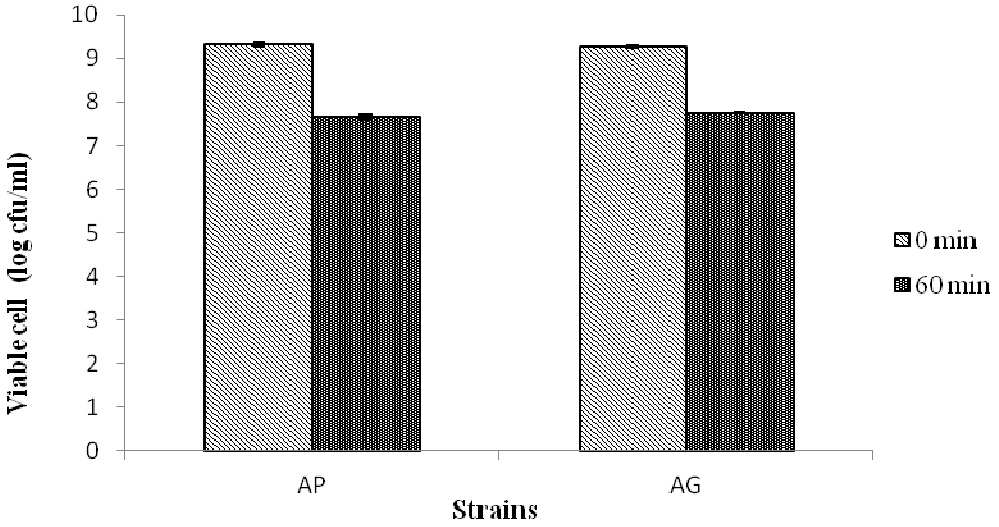


**Figure 4.** Adherence of *Lactobacillus casei* strain AP and AG in gastric mucin *in vitro*

12022, *Escherichia coli* FNCC 0091*, Staph-ylococcus aureus* FNCC 0047*,* and *Enterococcus faecalis* 99 EF were 7.37±0.03; 8.13±0.18; 8.37±0.02; and 8.63±0.18, respectively (Figure 3). Jacobsen *et al*. (1999) reported the ability of human-origin *Lactobacillus johnsoni* (*acidophilus*) LA1 and human clinical isolate *Lactobacillus plantarum* 22319 in inhibiting the growth of *E. coli*, *S. aureus* and *S. flexneri* with inhibition zone of between 2 to 5 mm. Other study on pathogenic growth inhibition of human-origin *Lactobacillus casei, Lactobacillus acidophilus* and *Lactobacillus reuteri* showed a wider inhibition zone (14-22 mm) compared to that of Jacobsen's findings (Awaisheh and Ibrahim, 2009). Previous study by Tharmaraj and Shah (2009) reported an average inhibition zone of *Lactobacillus casei* and *Lactobacillus paracasei* to *S. aureus* was 19 and 18 mm, respectively. The inhibition zone of two *Lactobacillus* strain AP and AG reported here is higher than *Lactobacillus* isolate reported by Jacobsen *et al.* (1999), within the range of human-origin *Lactobacillus sp.* reported by Awaisheh and Ibrahim (2009), but below the level of inhibition zone of *Lactobacillus casei* presented by Tharmaraj and Shah (2009).

Pathogenic inhibition by LAB has previously been reported due to the production of organic acids, $H_2O_2$, and bacteriocin (Silva *et al*., 1987). Bacteriocins produced by LAB have been reported to permeate the outer membrane of Gram-negative bacteria and to induce the inactivation of Gram-negative bacteria in conjunction with other growth-inhibiting factors, such as low temperature, organic acid and detergents (Alakomi *et al*., 2000). Some other LAB strains ribosomally synthesize antimicrobial peptides targeted to inhibit other Gram-positive bacteria (O'Sullivan *et al*., 2002), although such antimicrobial activity has a narrower inhibition spectrum than that of antibiotics (Morency *et al*., 2001).

Adherence on epithelial cells is one of the most important selection criteria for probiotics. Adherence to the mucosa of the epithelial cells allows probiotics colonization, immune stimulation, and competition with pathogens (Lahtinen and Ouwehand, 2009). Some of the probiotic effects exerted by probiotic lactobacilli are mediated by its interaction with the intestinal mucosa. The epithelial cells of the intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids with a large glycoprotein mucin being the main component. Bacteria colonizing the mucosa can be found both in the mucus layer and adhering to the epithelial cells (Brassart and Schiffrin, 2007). This layer is composed of a protein backbone of mucin, strongly O-glycosylated at the Ser and Thr positions (Roos and Johnsson, 2002).

In this study, adherence assays were carried out using gastric mucin from porcine stomach (Sigma) as the matrix. Figure 4 showed that 82.19±0.40% of cell *Lactobacillus casei* strain AP and 83.65±0.30% of strain AG was able to attach gastric mucin *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

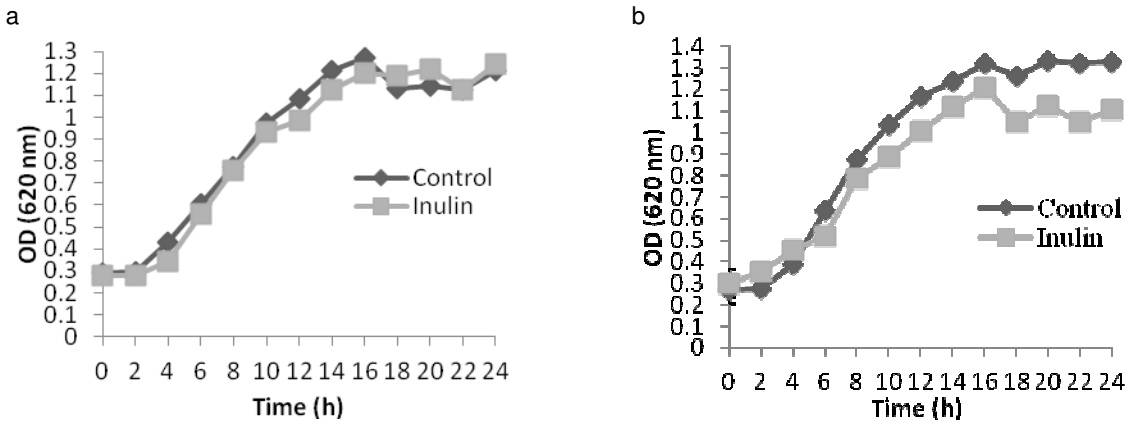


**Figure 5.** Growth curves of isolates: (a) AP and (b) AG, in MRS media (control) and media containing inulin 4% as carbon source (inulin)

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. Whereas Li *et al.* (2008) reported that *Lactobacillus fermentum* serotype I5007 had an adhesion index at 43.97% and showed the best competitive exclusion against *Salmonella typhimurium* IVDC C77-31and Eschericia soli serotype o8:K87.

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, two *Lactobacillus casei* strain AP and AG were grown on media containing prebiotics inulin as the only source of carbon. These two strains grew normally on inulin-containing media as the only carbon source, suggesting that they were able to utilize inulin as carbon source (Figure 5). Both *Lactobacillus* strain AP and AG had a normal growth curve in inulin-containing media comparable with that when grown on normal media (MRS).

Non-digestible oligosaccharides, such as fructo-oligosaccharides, galacto-oligosaccharides and inulin, are main utilized prebiotics for human consumption (Roberfroid, 2000). Fermentation of these carbohydrates in the GIT resulted in the generation of short-chain fatty acids, e.g. acetic acid, butyric acid and propionic acid, which function as energy sources (Grajek *et al*., 1995). Inulin is a polymer of fructose that consists glucosyl moiety and fructosyl moiety which are linked by β-(2,1)-D-fructosyl-fructose bonds, and terminated by a single glucose unit. Standard inulin has a degree of polymerization (DP) between 2 and 60. Polymers with DP below 10 were commonly considered as short-chained fructooligosaccharides, while longer-chained molecules were considered as inulin (Boeckner *et al*., 2001; Nines, 1999). Due to the presence of the β-(2,1) linkages, inulin is not digested by enzymes in the human GIT, contributing to its functional properties as prebiotic (Kalyani *et al*., 2010).

## CONCLUSION

In conclusion, two *Lactobacillus casei* strain AP and AG had a potential candidate as probiotic and subject to further investigation on its suitability in pro- and prebiotic application. Further study on health benefit both in animal models and human placebo controlled test was also recommended.

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