Selection of Human-origin *Lactobacillus* strains as Probiotics with Capability in Synthesizing *Conjugated Linoleic Acid* and Alleviating Hyperglycemia in Rats (*Rattus norvegicus*) in vivo

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ABSTRACT : The objective of this study was to select *Lactobacillus* strains as potential probiotics with the capability to synthesize bioactive compounds *Conjugated Linoleic Acid* (CLA) and their ability to alleviate hyperglycemia in rats (*Rattus norvegicus*) in vivo. Five strains of Lactic Acid Bacteria consists of *Lactobacillus casei* strain AP and AG, and *Pediococcus acidilactici* strains AA, BE and BK were previously isolated and identified from faeces of infants who consumed breast milk as the only source of diet. In vitro evaluation those five strains showed their potential as probiotic based on their capability to grow on media with pH 2.0 and 1.5% concentration of bile salts, the ability to attach on gastric mucin in vitro, and their ability to inhibit the growth of pathogen. Evaluation on the ability to use prebiotic inulin as carbon source showed that *Lactobacillus casei* (strain AP and AG) and *Pediococcus acidilactici* strain BE had the ability to degrade inulin as a prebiotic. Evaluation of probiotic on their capability to synthesize *Conjugated Linoleic Acid* (CLA) from free linoleic acid showed that *Lactobacillus casei* strain AP was able to convert more than 60% of free linoleic acid to CLA in the media. Further in vivo studies using rats (*Rattus norvegicus*) showed that *Lactobacillus casei* strain AP had the ability to alleviate hyperglycemia. The ability of *Lactobacillus casei* strain AP in reducing hyperglycemia was comparable with that of metformin (anti-hyperglycemia drug) provided orally at level 45 mg/kg of body weight.

Keywords : Probiotics, *Lactobacillus*, Hyperglycemia, *Conjugated Linoleic Acid*

INTRODUCTION

Probiotics are defined as "living microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). The International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 recommended that probiotics definition was relevant and sufficiently accommodating for current and anticipated applications. Health-associated benefits to the hosts in consuming probiotics have already been reported, included here are the ability to reduce concentration of serum cholesterol (Ooi and Liong, 2010; Anderson *et al*., 1999), to prevent and reduce risk of certain cancers (Xiao *et al*., 2006; Wollowski *et al*., 2001; Ohashi *et al*., 2000), and to stimulate the immune systems (Pareira *et al*., 2003; Nagao *et al*., 2000; Gill, 1998). To be functional as probiotics and to guarantee as safe for human consumption, bacterial strains must be non pathogenic, survive to gastric acid and bile toxicity, able to attach and colonise gastrointestinal tract (GIT), and ideally must be originated from human (Dunne *et al*, 2001; Dunne *et al*., 1999). Bacterial member of genus *Lactobacilli* and *Bifidobacteria* have commonly been applied as probiotics for human consumption (Grajek *et al*., 2005; Mercenier *et al*., 2003; Otieno, 201; Roberfroid, 2000; Gomes and Malcata, 1999).

The human gastrointestinal tract (GIT) is the best source of probiotics (Margolles *et al*., 2009). Favier *et al*. (2003) previously reported that 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 their 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 *Lactobacillus casei* and *Pediococcus acidilactici* from fecal of Indonesian infants had previously been reported (Widodo *et al*., 2012a; Widodo *et al*., 2012b). Further experiments to evaluate their potential as probiotics had also been conducted (Widodo *et al*., 2012b; Widodo *et al*., 2014). Probiotic assays showed that more than 50% of cells were viable after grown on pH 2.0 for 90 min, and around 80% of cells from the same strains were survived on media supplemented with bile salt 1.5% for 2 h. All of strains had high adhesion capacity as seen by more than 75% of cells attached on pig gastric mucin in vitro. Investigation of selected strains to grow on inulin as the only carbon source showed *Lactobacillus casei* strain AP and AG, and *Pediococcus acidilactici* strain BE were able to consume inulin (Widodo *et al*., 2012b; Widodo *et al*., 2014). These three strains grew normally on inulin-containing media as the only carbon source, suggesting that they were able to utilize inulin as carbon source. This study is a continuation of previous studies with emphasize to select of *Lactobacillus* and *Pediococcus* strains with ability to synthesize *Conjugated Linoleic Acid* (CLA) and to evaluate their capability in alleviating hyperglycemia in rats (*Rattus norvegicus*) in vivo.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus casei strain AP and AG, and *Pediococcus acidilactici* BE were obtained from previous experiments (Widodo *et al*., 2012a; 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 microaerobic condition.

Growth media and CLA synthesis

Bacterial cells were grown on MRS broth and harvested at the logarithmic phase. The bacterial culture (2.5% v/v) were collected and inoculated on MRS broth supplemented with linoleic acid at 0.4 mg/ml (dissolved in 80% Tween solution), incubated at 37°C for 24 h. After 24 hours, samples (8 ml) were harvested by centrifugation at 5000 rpm for 30 minutes at 5°C. The supernatant were collected for CLA analysis using Gas Chromatography-Mass Spectrometry (GC-MS) according to Alonso *et al*., 2003).

Fat extraction

Fat extraction was carried out according to Alonso *et al*. (2003) with modification. Sample of supernatant (6 ml) was mixed with 12 ml isopropanol and homogenized. The solution was then mixed with 9 ml hexane and centrifuged at 23000 rpm for 5 minutes at 5°C. The upper layer solution was collected and filtered using natrium sulphate, and washed with 7 ml hexane. The hexane faction was then evaporated at room temperature (30 - 40°C) resulted in condensed fat. The esterification of fat residue was carried out with 300 µl 14% boron trifluoride (BF3) in methanol at 50-60°C for 2 h. After esterification, 800 µl hexane was added and sampled was analyzed using GC-MS.

GC-MS Analysis

Methil ester of CLA was analyzed using GC-MS according to Alonso *et al*, (2000) with modification. Sample analysis was carried out in a column AGILENTJ%W DB-1 (30 m x 0.25 mm i.d) with helium as the carier and ionizer El 70 Ev was applied. The coloumn temperature was set at 80°C, injection temperature at 310°C using split injection, pressure at 16.5 kPa, flow rate 40 mL/mins, flow rate in coloumn at 0.50 mL/mins with split rasio 73. Injection volume was 1 µl, and peaks of CLA were identified using retention time after spiking.

Study hyperglycemia in vitro in rats

Selected bacterial strains with capability in synthesizing CLA (*Lactobacillus casei* strain AP) and *Lactobacillus casei* strain AG (non synthesizing CLA) were applied as starters in dairy fermentation by inoculating 5% (v/v) of bacterial cultures into the pasteurised fresh milk and incubated at 60°C for 10 h. After fermentation, physic-chemical and microbiological qualities of the product was evaluated and 2 ml of the fermented products bearing total lactic acid bacteria 1x108 cfu/ml was applied for in vivo studies in rats (*Rattus norvegicus*). Rats were treated with 5 treatments as follows:

T1: rats were fed with standard feed and water provided at libitum.
T2: rats were fed with high content of fat and sucrose.
T3: rats were fed with high content of fat and sucrose, and after 129 days of treatments were fed with 2 ml milk fermented with L. *casei* strain AP.
T4: rats were fed with high content of fat and sucrose, and after 120 days were fed with 2 ml milk fermented with L. *casei* strain AG.
T5: rats were fed with high content of fat and sucrose, and after 120 days of treatments were fed with metformin at 45 mg/kg.

Variables measured were weight of rats and level of blood sugar at 0, 30, 60, 60, 90, 120 (before treatments), and 135 days (after treatments). Blood sugar was measured base on glucose oxidase (GOD-PAP) using DiaSys diagnostic systems Gmbh according to the manufacturer's instruction.

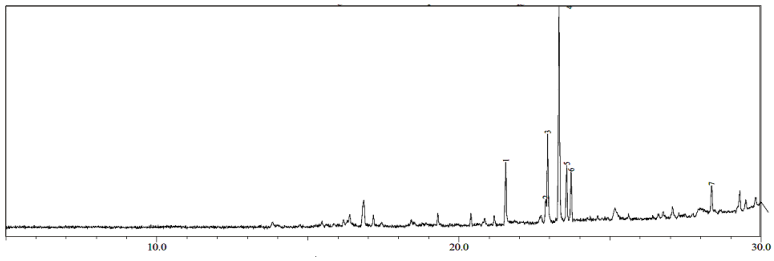
RESULT AND DISCUSSION

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; Widodo *et al*., 2014). In this study, further experiments were conducted to evaluate their ability to synthesize CLA in vitro and to function as anti-hyperglycemia in rats. Growth media used for CLA synthesis was MRS broth supplemented with 0.4 mg/ml linoleic acid as precursor for CLA synthesis. After fermentation, derivatization of products was carried out using boron trifluoride in methanol (BF3-metanol) followed by analyzed using GC-MS. Derivatisation converts linoleic acid to methylated linoleic that can be analyzed using GC-MS.

Figure 1[a] showed that samples prepared from products fermented with *Lactobacillus casei* strain AP had 4 peaks with similar molecular ions and mass-to-charge ratio (m/z) at 294. Mass spectra of peak number 3, 4, 5 and 6 were similar (data not shown), suggesting that those four peaks are isomers. Based on mass spectra, it was difficult to determine what compounds are peak number 3, 4, 5, and 6. To solve that problem, spiking or injecting compounds that has been known was carried out.

Spiking was carried out by injecting linoleic acid. The increasing peaks after spiking suggesting that those peaks were linoleic acids. Peak number 2 with retention time 22.912 was dramatically increased after spiking leading to the conclusion that this peak is linoleic acid (Fig 1[a] and 1b). On the other hand, peak number 3, 4, and 5 were proposed as isomers of linoleic acids or *Conjugated Linoleic Acid* (CLA) (Fig 1[a] and 1[b]).

a. Before spiking



b. After spiking with linoleic acid

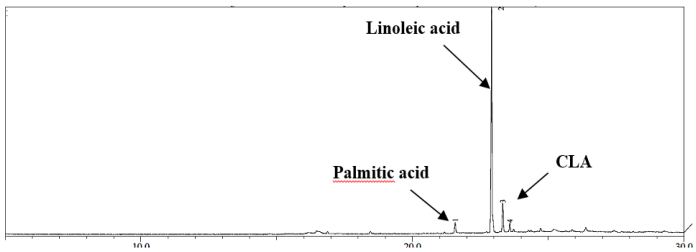


Figure 1. (a) GC-MS chromatogram of sample L. *casei* strain AP before spiking and (b) after spiking with linoleic acid.

GC-MS chromatogram showed that retention time of CLA isomers of peaks 4, 5 and 6 were detected at 23.310; 23.563, dan 23.710 minutes (data not shown) suggesting isomers separation. However, separated isomers could not be identified their geometric position and structure due to unavailability of comparable CLA standard.

Tabel 1. Percentage (%) of organic acids synthesized by L. *casei* strain AP

Peak number	Retention time	Proposed compounds	Percentage (%)
1	21.548	Palmitic acid	12.85
3	22.936	Palmitic acid	12.71
4	23.310	CLA	46.77
5	23.563	CLA	10.36
6	23.710	CLA	9.43

Tabel 1 showed that L. *casei* strain was able to do isomerization of linoleic acid resulting in production of CLA (66.56% of products). Meanwhile, hydrogenation of the rest linoleic acid was proposed argument why L. *casei* strain AP synthesize palmitic acids (25.56%) (Table 1).

According to Alonso *et al*. (2003), geometrical isomers of CLA that was converted from linoleic acids by human-isolated L. *casei* dan L. *acidophilus* were cis-9,trans-11; trans-10,cis-12 dan trans-9,trans-11. Alonso *et al*. (2003) also reported that L. *casei* E10 had the ability to manly synthesize CLA (80.14%) when grown on MRS broth supplemented with free linoleic acids at 0.2 mg/ml and incubated at 37°C for 24 hours. The level of CLA synthesized reported here is lower than that reported by Allonso *et al*. (2003), was likely due to differences on fermentation conditions.

CLA-synthesizing *Lactobacillus casei* strain AP was then selected as starters for milk fermentation, whilst non-synthesizing CLA *Lactobacillus casei* strain AG was also selected as negative control. The fermented product was fed to rats after being treated with high fat and sucrose for 120 days, and the treatment was prolonged for 15 days. Blood samples of rats before (120 days in high fat and sucrose) and after treated (15 days) was collected, and level of sugar was measured. The data of blood sugar before and after L. *casei* strain AP-containing fermented milk was presented at Figure 2.

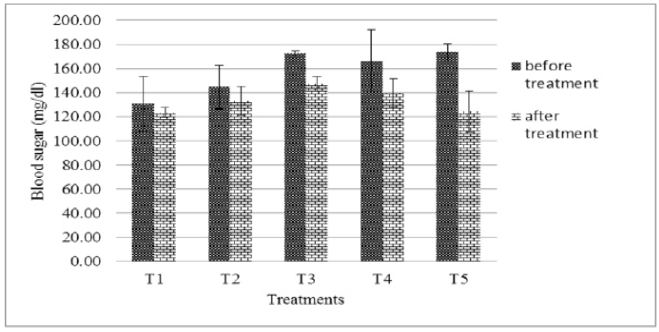


Figure 2. Level of blood sugar (mg/dl) before and after being fed with standard feed (T1), high fat and sucrose (T2), milk fermented with L. *casei* strain AP (T3), L. *casei* strain AG (T4) and metformin (T5).

Figure 2 showed that level of blood sugar in rats fed with milk fermented with *Lactobacillus casei* strain AP (T3) and metformin (T5) was reduced after 15 days of treatments, suggesting that *Lactobacillus casei* strain AP and metformin function as anti-hyperglycemia. Level of blood sugar in T3 decreased from 172.45±2.15 mg/dl to 147.20±6.01, while metformin (T5) reduced blood sugar from 173.53±6.55 to 124.18±16.90. Metformin is commercially known as antidiabetic medication to treat people with type 2 diabetes. On the other hand, untreated rats (T2) and rats fed with standard feed (T1) and fed with milk fermented with *Lactobacillus casei* strain AG had no effects on blood sugar reduction (Figure 3).

CONCLUSION

In conclusion, *Lactobacillus casei* strain AP had the ability to synthesize CLA on media supplemented with linoleic acid. Rats fed with milk fermented with *Lactobacillus casei* strain AP showed reduction on blood sugar after treatment for 15 days.

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