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Identification of Conjugated Linoleic Acid in Milk Fermented by Probiotics Originating in the Gastrointestinal Tract

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ABSTRACT

One of the benefits of probiotics is their ability to synthesize conjugated linoleic acid (CLA) as a functional bioactive compound. The objective of this study is to identify CLA synthesized in milk fermented with the probiotic *Lactobacillus casei* strain AP and *Lactobacillus casei* strain AG as starter cultures. Fermented milk products were analyzed using gas chromatography-mass spectrometry (GC-MS) to characterize the CLA formed. The result of GC-MS in milk fermented using *Lactobacillus casei* strain AG was detection of a CLA compound with a retention time of 41.467, whereas in milk fermented using *Lactobacillus casei* strain AP, linoleic acid, but not CLA, was detected.

Keywords: CLA, Fermented milk, *Lactobacillus casei* strain AG, *Lactobacillus casei* strain AP, Probiotic

Introduction

Milk is a food composed of various nutrients in balanced proportions. Milk contains 87.3% water, 3.9% fat, 3.25% protein, 4.6% lactose, 0.65% minerals, and 0.18% acids, as well as enzymes and vitamins (Soeparno, 2015). Fresh milk in general is easily spoiled, especially by spoilage microorganisms that are widespread in the environment. Fresh milk must be preserved to avoid spoilage and contamination with pathogenic bacteria (Tamime, 2006).

Fresh milk can be processed into various products, including milk powder, yogurt, cheese, kefir, sour milk, sweetened condensed milk, pasteurized milk, and sterilized milk (Widodo, 2003). One food processing technique than can extend the shelf life of milk is acidification by means of fermentation. Fresh milk is usually fermented by specific starter cultures until the milk is acidified and the proteins coagulated. Usually, milk fermentation is carried out by a member of the lactic acid bacteria (LAB) as a starter culture that will degrade lactose into lactic acid, thus lowering the pH value of the milk (Tamime, 2006).

BAL cultures that are often used as starter cultures for milk fermentation are those of the genera *Lactobacillus, Streptococcus*, and *Pediococcus*. Some species of these bacteria can function as probiotics. *Lactobacillus casei* is one species of LAB that has been used commercially to produce fermented milk (Makinen and Bigret. 2004). A culture of the *Lactobacillus casei* strain Shirota has been developed for the fermentation of milk produced by the Yakult Honsha company in Japan. *Lactobacillus casei* strain Shirota has been tested in experimental animals and showed a positive stimulatory effect on the immune system by reducing digestive system disorders (Heimbach, 2012).

The use of probiotics in fermentation processes can increase the functionality of food for human health. The fermentation process carried out by probiotics produces a variety of bioactive metabolites, including short-chain fatty acids and conjugated linoleic acid (CLA). In addition, fermentation generates organic acids and hydrogen peroxide as antimicrobial components (Ross *et al*., 2010).

Goat milk fermentation using *Lactobacillus bulgaricus* has been shown to increase the CLA content of milk (Kishino *et al*., 2013). CLA is an unsaturated fatty acid with a long chain of conjugated double bonds. CLA has positive effects on human health. The positive effects of CLA include slowing of the degenerative processes in humans, inhibition of atherosclerosis, inhibition of carcinogenesis, and modulation of the immune system (Parodi, 1999).

Widodo *et al.* (2012a; 2012b) previously isolated LAB strains from the human gastrointestinal tract. The bacteria, identified as *Lactobacillus casei* strain AP and *Lactobacillus casei* strain AG, demonstrated probiotic activity *in vitro* (Widodo *et al.*, 2012a; Widodo *et al.,* 2012b). These bacteria had also been used as starter cultures for acidifying milk during fermentation (Widodo *et al*., 2017). In this study we investigate

the capability of *L. casei* AP and *L. casei* AG to synthesize CLA from linoleic acids during milk fermentation.

Materials and Methods

Bacterial strains and culture preparation

The bacteria strains used in this study were *Lactobacillus casei* strain AP and *Lactobacillus casei* strain AG obtained from previous studies (Widodo *et al.*, 2012a; Widodo *et al.,* 2012b). Both strains were grown in de Man Ragosa Sharpe (MRS; Oxoid) with or without linoleic acid supplementation at 0.4 mg/ml (Sigma).

For culture preparation, bacterial cells were grown in sterile MRS media supplemented with L-cysteine HCl 0.05% (w/v) and incubated overnight at 37ºC under microaerophilic conditions. Bacterial cells were harvested, and 5% (v/v) of the culture was inoculated into pasteurized milk supplemented with skim milk powder (4%, w/v) and incubated at 37ºC until curd formation. Five percent (5%) of this culture was transferred to pasteurized milk supplemented with skim milk powder (4%, w/v) and incubated at 37ºC until curd formation. This starter culture was used for further milk fermentation.

CLA synthesis *in vitro*

Milk fermentation and fat extraction. Fresh cow's milk was pasteurized at 85ºC for 30 minutes. After cooling at 40 °C, 5% (v/v) bulk starter was inoculated into the pasteurized milk and then incubated at 37ºC for 6 hours. A 6-ml sample was then centrifuged at 5000 rpm for 30 minutes at 5 °C. The supernatant was removed and then transferred to conical tubes for CLA analysis (Alonso *et al*., 2003).

Fat extraction was performed according to Alonso *et al.* (2003). Briefly, fat was extracted using isopropanol and hexane, followed by esterification using 14% boron trifluoride (BF3) in methanol at 50–60ºC for 2 h. After esterification, hexane was added to the samples, which were then analyzed by gas chromatography-mass spectrometry (GC-MS).

CLA analysis

CLA methyl esters were analyzed by GC-MS according to Alonso *et al*. (2003), with modifications. GC-MS analyses were performed using AGILENTJ% W DB-1 (30 m x 0.25 mm i.d.) columns with helium as the carrier gas and ionizing EI 70 Ev under the following conditions: heater temperature 80ºC, injection temperature 310ºC, injection model split, pressure 16.5 kPa, total flow rate 40 mL/min, and a column flow rate 0.50 mL/min, with a split ratio of 73.2 mL injection volume. The CLA peak was identified by retention time comparison with CLA standard (Sigma).

Result and Discussion

Linoleic acid detection in milk fermented by *Lactobacillus casei* AP

The chromatogram of milk fermented by *Lactobacillus casei* strain AP using GC is shown in Figure 1. Figure 1a shows chromatograms of

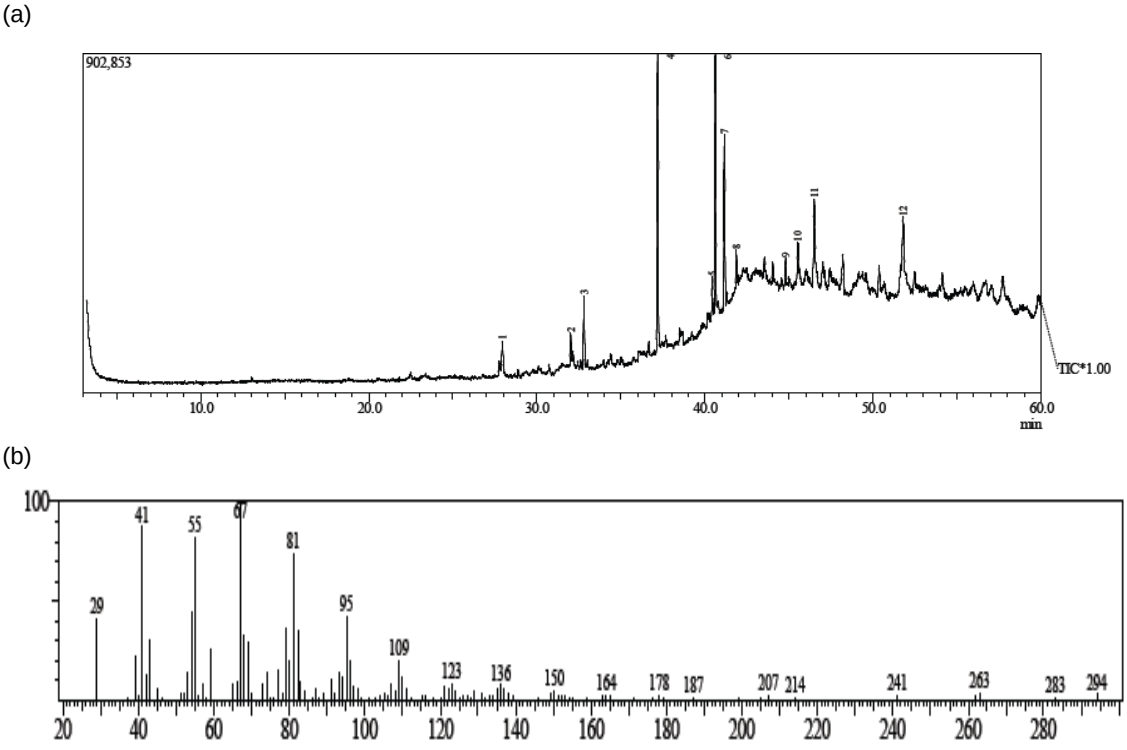


Figure 1. (a) Chromatograms and (b) mass spectra of milk fermented by *Lactobacillus casei* AP.

the 12 peaks in milk fermented with *Lactobacillus casei* strain AP that could be analyzed based on their mass spectra (MS). Of the 12 peaks obtained, the fifth peak had a retention time of 40.424, which is close to the standard retention time of linoleic acid, which is 41.872. MS analysis of the fifth peak also showed spectra with abundances (m/z) of 150, 164, 178, and 220, in accordance with the standards of linoleic acid (Figure 1b). Therefore, milk fermented using *Lactobacillus casei* strain AP had detectable linoleic acid, but no CLA was detected in the product.

CLA detection in milk fermented by *Lactobacillus casei* AG

The chromatogram of milk fermented by *Lactobacillus casei* AG is presented in Figure 2. The area of the 19th peak, which had a retention time of 40.467, was 1.23%, and the area of the 23rd peak, which had a retention time of 41.467, was 0.45%, as shown in Figure 2.

Figure 2 shows that there were 30 peaks in the chromatogram of milk fermented by *Lactobacillus casei* AG. The 19th peak is a linoleic acid compound with a retention time of 40.467, and the 23rd peak is a CLA compound with a retention time of 41.467. Analysis of the 30 peaks of the MS is presented in Figure 3. The analysis of MS of milk fermented by *Lactobacillus casei* AG in Figure 3 shows that there are two compounds with a molecular weight (m/z) of 294, but they have different retention times. The compound represented by the 19th peak is linoleic acid, in agreement with the linoleic acid standard, with a retention time of 40.467, and the compound represented by the 23rd peak is CLA, with a retention time of 41.467.

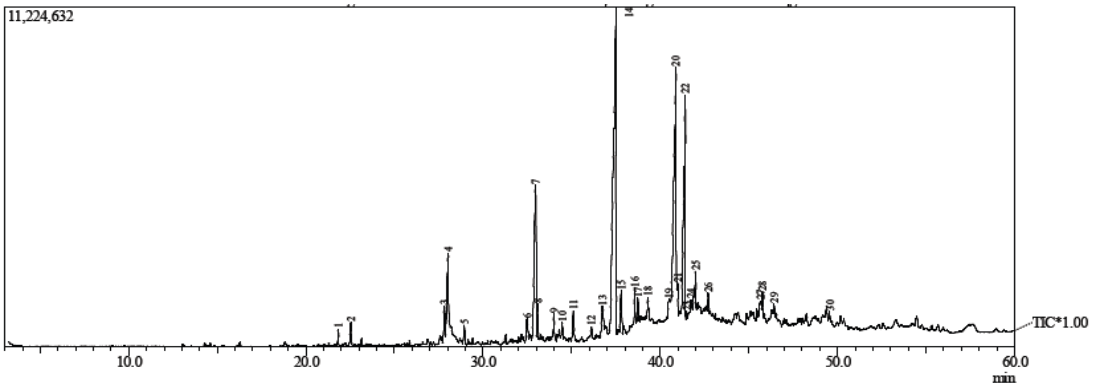
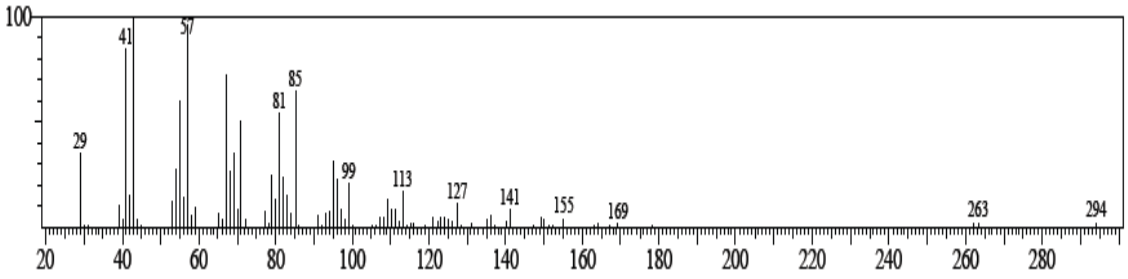


Figure 2. Chromatogram of milk fermented by *Lactobacillus casei* AG.

(a) Nineteenth peak



(b) Twenty-third peak

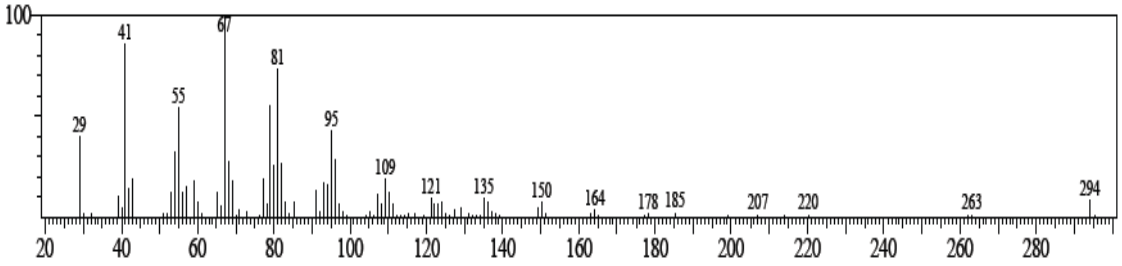


Figure 3. The 19th and 20th peaks of mass spectra of milk fermented by *Lactobacillus casei* AG.

Conclusions

Using GC-MS technology, linoleic acid and CLA were detected in milk fermented using *Lactobacillus casei* AG, while in milk fermented using *Lactobacillus casei* AP, only linoleic acid was detected. The content of CLA in milk fermented using *Lactobacillus casei* AG was 0.45%.

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