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ORIGINAL ARTICLE

Conjugated linoleic acid production from various substrates by probiotic *Lactobacillus plantarum*

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Abstract Conjugated linoleic acid (CLA, a colloquial term referring to a collection of geometrical and positional isomers of linoleic acid) has attracted the attention of scientists due to its special properties. In this study, we used sunflower oil and castor oil as cost-effective substrates (in comparison with linoleic acid) for CLA production. A novel method using bacterial lipase in order to convert sunflower oil and castor oil to free fatty acids was investigated. In addition, the effect of some significant parameters on CLA production, such as substrate type and concentration, incubation time, and probiotic lipase on sunflower oil and castor oil were evaluated. Among the five studied probiotic strains, washed cells of *Lactobacillus plantarum* (ATCC 8014) produced the highest concentration of CLA isomers. Analysis of results revealed that produced CLA was a mixture of two bioactive isomers including *cis*-9, *trans*-11- CLA (0.38 $mg \cdot ml^{-1}$) and *trans*-10, *cis*-12-CLA (0.42 $mg \cdot ml^{-1}$) from 8 $mg \cdot ml^{-1}$ sunflower oil by bacterial lipase conversion to linoleic acid. Hence, the capacity of *L. plantarum* (ATCC 8014) to produce bioactive isomers of CLA from cost-effective substrates is of interest for development of probiotic supplements such as CLA and other health-food products.

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Introduction

Linoleic acid (C18:2) is an essential fatty acid that is only available through consumption in the diet (Das 2006). Conjugated linoleic acid (CLA) is a term used to describe positional and geometrical isomers of linoleic acid with conjugated double bonds (Moon et al. 2008). Among them, *cis*-9, *trans*-11-CLA (CLA1) and *trans*-10, *cis*-12-CLA (CLA2) are the most active isomers (Kepler et al. 1966; Adamczak et al. 2008). They exhibit several important physiological effects, including anticancer (Kelley et al. 2007), antioxidant, anti-atherosclerosis (McLeod et al. 2004), and anti-obesity (Whigham et al. 2007) activities, as well as normalization of impaired glucose tolerance in animals and humans (Iannone et al. 2009) and improved immune system function (Bhattacharya et al. 2006). Natural sources of CLA isomers are milk and meat of ruminants, which produce CLA by the activity of their ruminal bacteria (Macouzet et al. 2009). Chemical production of CLA results in accumulation of other components and a wide range of unknown CLA isomers in the human body. In contrast, microbial methods, which produce active isomers, have the potential to increase the health of the host and even reduce disease (Gorissen et al. 2011). Today biotechnological methods enable us to increase the CLA concentration in dietary supplements, too (Adamczak et al. 2008). Many strains from various genera of *Lactobacillus, Lactococcus, Streptococcus, Propionibacterium, Bifidobacterium,* and *Butyrivibrio* are able to produce CLA from linoleic acid under specific growth conditions (Sieber et al. 2003). These bacteria have a special enzyme, linoleic acid isomerase, with the capacity to convert linoleic acid or ricinoleic acid to CLA isomers (Liavonchanka and Feussner

2008). In addition, many of these genera are considered to have probiotic effects. Probiotics play an essential role in human health. Hence, presence of probiotics which produce CLA in the gastrointestinal tract can play an important role in maintenance of human health (Ewaschuk et al. 2006). In comparison with purified linoleic acid, castor oil and sunflower oil are economical sources for CLA production. Sunflower oil typically has at least 69 % linoleic acid and castor oil contains about 90 % ricinoleic acid (hydroxy fatty acid) (Ogawa et al. 2005). In this study, sunflower oil and castor oil are used as cost-effective substrates for CLA production. Also, a novel method of using the bacterial lipase in order to convert sunflower oil and castor oil to free fatty acids is investigated. In addition, the effects of several parameters on CLA production such as substrate forms and concentration and incubation time are evaluated.

Material and methods

Chemicals and solutions

Standard samples of CLA1 and CLA2 methyl esters, linoleic acid, and lipase NO. L-1754 (E.C. 3.1.1.3) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Sunflower oil, castor oil, and all other chemicals and solutions used were purchased from a local store in Tehran, Iran.

Microorganisms and growth conditions

Lactobacillus acidophilus (ATCC 4356), *L. reuteri* (ATCC 23272), and *L. plantarum* (ATCC 8014) were obtained from the Microbial Collection of Iran, Tehran. *Lactobacillus brevis* and *L. buchneri* were isolated from cheese and yoghurt, respectively (Kermanshahi and Peymanfar 2012). The standard strains were subcultured twice under microaerobic conditions at 37 °C for 24 h in Man Rogosa Sharpe (MRS) broth (Merck, Frankfurt, Germany). They were stored at −80 °C in MRS medium supplemented with 25 % (v/v) glycerol as a cryoprotectant.

Fermentation

Each strain was cultured in 10 ml of MRS broth for 24 h under microaerobic conditions at 37 °C. 1 ml of this culture was inoculated to 5 ml of MRS broth (pH 6.3) mixed with 750 $\mu g \cdot ml^{-1}$ of linoleic acid and Tween 80 (1 %, Merck, Frankfurt, Germany) and incubated under microaerobic conditions at 37 °C for 24 h.

Preparation of washed cells

A pure active culture was obtained by picking a single colony from the MRS agar plate and growing in 10 ml of MRS broth for 24 h, under microaerobic conditions at 37 °C. Then, 1 % of each strain subculture was inoculated into 100 ml of MRS broth containing 0.6 % of linoleic acid (linoleic acid was mixed with 0. 8 ml Tween 80 before being added to the medium). The prepared media were incubated under microaerobic conditions at 37 °C. After 24 h, cells were harvested by centrifugation (8,000×*g*, 15 min), washed twice with 8.5 $mg \cdot ml^{-1}$NaCl and then used as washed cells.

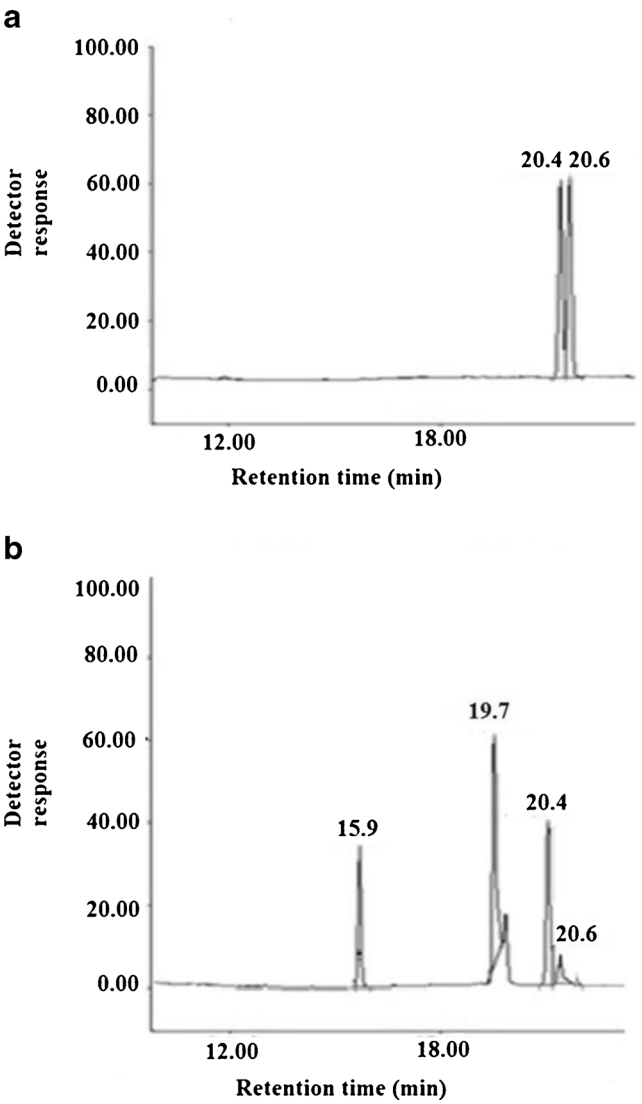

Fig. 1 GC chromatograms of methyl ester standards of CLA1 at 20.4 min and CLA2 at 20.6 min; b methyl ester of CLA1 and CLA2 produced by *L. plantarum* resting cells in the presence of 4 $mg \cdot ml^{-1}$ linoleic acid; linoleic acid at 19.7 min and internal standard at 15.9 min. Values are Mean ± SE (n=3), P<0.05

Reaction conditions

The reaction mixture contained 1 ml of 100 mmol·l^{-1} potassium phosphate buffer (pH 6.5), 4 mg of linoleic acid, and 22 % washed cells. Sunflower oil and linoleic acid as substrates were mixed with Tween 80 (1 % w/v) before adding them to the reaction mixture, because Tween 80 disperses lipids in the reaction mixture completely. Reactions were carried out microaerobically under an O2–adsorbed atmosphere in a sealed chamber and gently shaken at 37 °C for 72 h.

Lipid extraction and methylation

Following incubation, the reaction mixture was mixed with 3.75 ml of chloroform: methanol (1:2, v/v) and vortexed well according to the Bligh Dyer method. Next, 1.25 ml of chloroform was added, and the mixture was vortexed again. The mixture was centrifuged (1,900×*g*, 5 min) to give a two-phase system. The bottom phase was dried with anhydrous sodium sulfate and evaporated in a rotary evaporator at 30 °C. To produce fatty acid methyl esters, the residues were saponified with 2 ml of 1 mol·l^{-1} sodium hydroxide in methanol in a screw- capped tube at 100 °C for 15 min, and then cooled at room temperature. The free fatty acids were methylated with 6 ml of 4 % hydrochloric acid in methanol at 60 °C for 20 min. Then, the mixture was mixed with 2 ml of hexane: water (1:1, v/v) and centrifuged (4,000×*g*, 20 min, 4 °C). The organic layer was dried under a stream of nitrogen at room temperature. The residue was dissolved in 1 ml of n-hexane for quantification of CLA1 and CLA2 by capillary gas chromatography.

Gas chromatography analysis

Fatty acid methyl esters (FAME) were analyzed by a PU-4410 system (Philips, London, England) fitted with a capillary column (Bp 10.25 m by 0.22 mm inside diameter, Philips, London, England). Initially the column temperature was 150 °C at 1 min and raised to 250 °C at a rate of 5 °C min$^{-1,}$ and the final temperature was 230 °C.

Statistical analysis

All values are expressed as Mean ± standard error of mean. Analyses were performed using the statistical software SPSS (Version 13.0 SPSS). Differences between means were evaluated using 1-way ANOVA. Differences were considered significant at $P<0.05$. Each parameter was performed in three replications.

Results

Screening of probiotics producing CLA isomers from linoleic acid

Probiotic growing cells and resting cells were examined to produce CLA from linoleic acid. As Fig. 1 shows, fatty acid classes were analyzed by gas chromatography. Table 1 depicts no significant CLA isomers (CLA1+ CLA2) (<0.15±0.07 mg·ml^{-1}) ($P<0.05$) in the fermentation mixture of all growing cells. Meanwhile, resting cells produced significant concentratison of CLA isomers (CLA1+ CLA2) (> 0.2±0.04 mg·ml^{-1}) ($P<0.05$) in the reaction mixture. Among these probiotics, resting cells of *L. plantarum* produced the highest amounts of two CLA isomers, *cis*-9, *trans*-11- CLA (0.41±0.05 mg·ml^{-1}) ($P<0.05$) and *trans*-10, *cis*-12-CLA (0.12±0.01 mg·ml^{-1}) ($P<0.05$). Hence, optimization experiments were followed by *L. plantarum* ATCC 8014.

Table 1 Comparison of probiotic growing cells and resting cells in the production of CLA isomers

Strain	Origin	Fatty acids [mg·ml^{-1}]					
		Growing cells			Resting cells		
		LA	CLA1	CLA2	LA	CLA1	CLA2
L. reuteri	ATCC 23272	3.89±0.03	nd	nd	3.38±0.02	0.003±0.03	0.002±0.01
L. acidophilus	ATCC 4356	3.02±0.02	0.15±0.02	nd	2.56±0.04	0.25±0.06	0.08±0.04
L. plantarum	ATCC 8014	2.37±0.02	0.1±0.04	0.02±0.01	1.87±0.05	0.41±0.05	0.12±0.01
L. brevis	Isolated from cheese	2.83±0.03	0.098±0.04	nd	2.55±0.03	0.034±0.01	0.026±0.01
L. buchneri	Isolated from yoghurt	2.53±0.05	0.15±0.03	nd	2.69±0.07	0.008±0.01	0.033±0.02

Concentration of fatty acids is expressed per millilitre of reaction mixture

Values are Mean ± SE ($n=3$), $P<0.05$

LA linoleic acid, *CLA1 cis*-9, *trans*-11, *CLA2 trans*-10, *cis*-12, *nd* not detected

Lipase effect

Meanwhile, fatty acids are the only substrates of linoleate isomerase; triacylglycerols should convert to fatty acids by lipase at first. By addition of lipase NO. L 1754 (100 $U \cdot ml^{-1}$) ($P<0.05$) to the reaction mixture, castor oil as substrate is converted to ricinoleic acid and CLA isomers (0.24± 0.05 $mg \cdot ml^{-1}$ CLA1 and 0.39±0.08 $mg \cdot ml^{-1}$ CLA2) ($P<0.05$) were produced. In the same conditions, CLA isomers (0.29±0.12 $mg \cdot ml^{-1}$ CLA1 and 0.30±0.09 $mg \cdot ml^{-1}$ CLA2) ($P<0.05$) were produced from sunflower oil as a substrate. Since lipase activity has been characterized in *L. plantarum* ATCC 8014 in the previous studies, CLA production is evaluated in the absence of lipase NO. L 1754. CLA isomers were not detected in the reaction mixture of castor oil, but bacterial lipase converted sunflower oil to linoleic acid and CLA isomers (0.31±0.05 $mg \cdot ml^{-1}$ CLA1 and 0.35±0.04 $mg \cdot ml^{-1}$ CLA2) ($P<0.05$) were produced (Fig. 2). There are no significant differences between produced CLA isomers under Lipase NO. L 1754 and bacterial lipase activity in the reaction mixture.

Effect of substrate concentration

Numerous concentrations (1, 4, 8, 12 $mg \cdot ml^{-1}$) of linoleic acid, sunflower oil, and castor oil in the reaction mixture for producing CLA were examined. As Fig. 3 shows, in the linoleic acid mixture (12 $mg \cdot ml^{-1}$), the highest CLA isomer concentrations were 0.16±0.04 $mg \cdot ml^{-1}$ ($P<0.05$) CLA1 and 0.45±0.03 $mg \cdot ml^{-1}$ ($P<0.05$) CLA2. Even so, in the sunflower oil reaction mixture (8 $mg \cdot ml^{-1}$ and 12 $mg \cdot ml^{-1}$), the most top produced CLA isomers were 0.38±0.05 $mg \cdot ml^{-1}$ ($P<0.05$) CLA1, 0.42±0.03 $mg \cdot ml^{-1}$ ($P<0.05$) CL;A2 and 0.29±0.01 $mg \cdot ml^{-1}$ ($P<0.05$) CL,A1 and 0.53±0.06 $mg \cdot ml^{-1}$ ($P<0.05$) CLA2, respectively. However, the ratio between CLA1 and CLA2 was not balanced in the 12 $mg \cdot ml^{-1}$ sunflower oil reaction mixture. The highest CLA isomers in the castor oil reaction mixture (8 $mg \cdot ml^{-1}$ and 12 $mg \cdot ml^{-1}$) were 0.42±0.07 $mg \cdot ml^{-1}$ ($P<0.05$) CLA1, 0.37±0.03 $mg \cdot ml^{-1}$ ($P<0.05$) CL;A2 and 0.73±0.09 $mg \cdot ml^{-1}$ ($P<0.05$) CLA1, 0.10±0.01 $mg \cdot ml^{-1}$ ($P<0.05$) CLA2, respectively. However, there is no balance between CLA1 and CLA2 concentrations in the 12 $mg \cdot ml^{-1}$ sunflower oil reaction mixture.

Effect of incubation time

The time course of CLA production from linoleic acid, sunflower oil, and castor oil was monitored at 24, 48, 72, and 96 h. CLA production trends were similar in sunflower oil, castor oil, and linoleic acid mixtures (Fig. 4). There were no significant differences by ANOVA between total produced CLA (CLA1+CLA2) 0.66±0.05 $mg \cdot ml^{-1}$ ($P<0.05$) after 72 h in the castor oil and sunflower oil (4 $mg \cdot ml^{-1}$) reaction

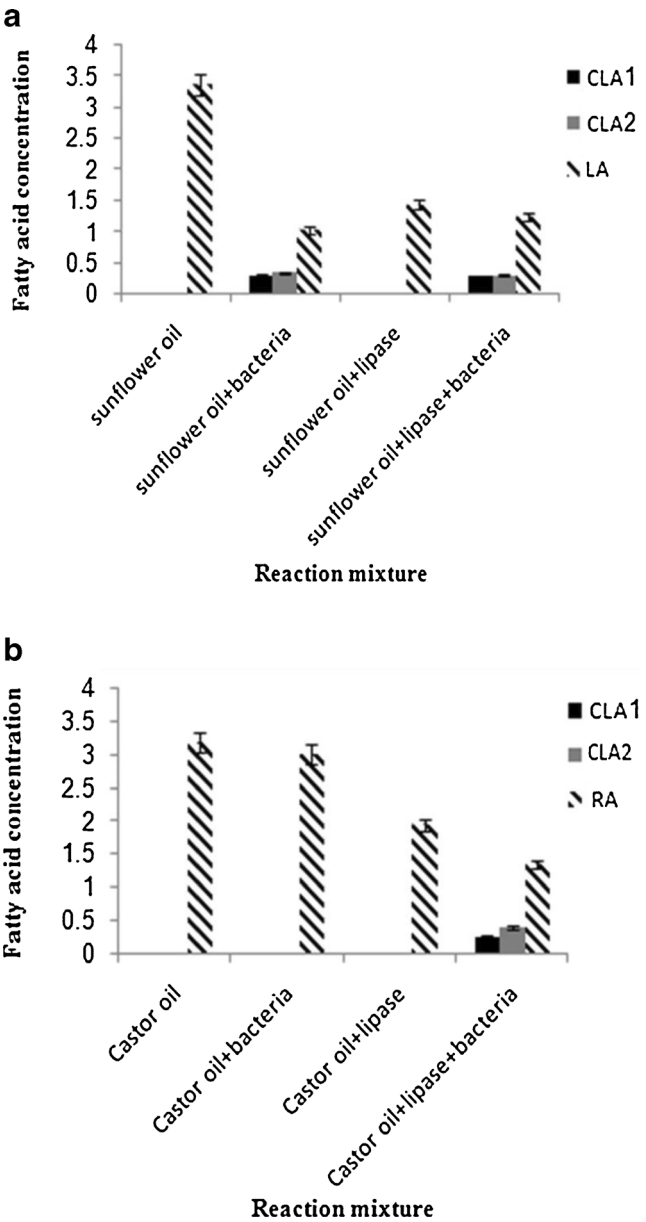

Fig. 2 Effect of lipase NO. L-1754 (100 $U \cdot ml^{-1}$) on the CLA1 and CLA2 production in the presence of 4 $mg \cdot ml^{-1}$ sunflower oil and b castor oil. Values are Mean ± SE ($n=3$), $P<0.05$

mixtures. Separately, longer incubation time tended to increase CLA1 up to 72 h, and dramatically decreased at 96 h. However, the CLA2 ratio in the total CLA increased with prolonged incubation time. Therefore, the proportions of CLA isomers can be varied depending on the incubation time.

Discussion

In this study, we investigated the capacity for CLA production in five probiotic strains. The resting cells of the late log phase

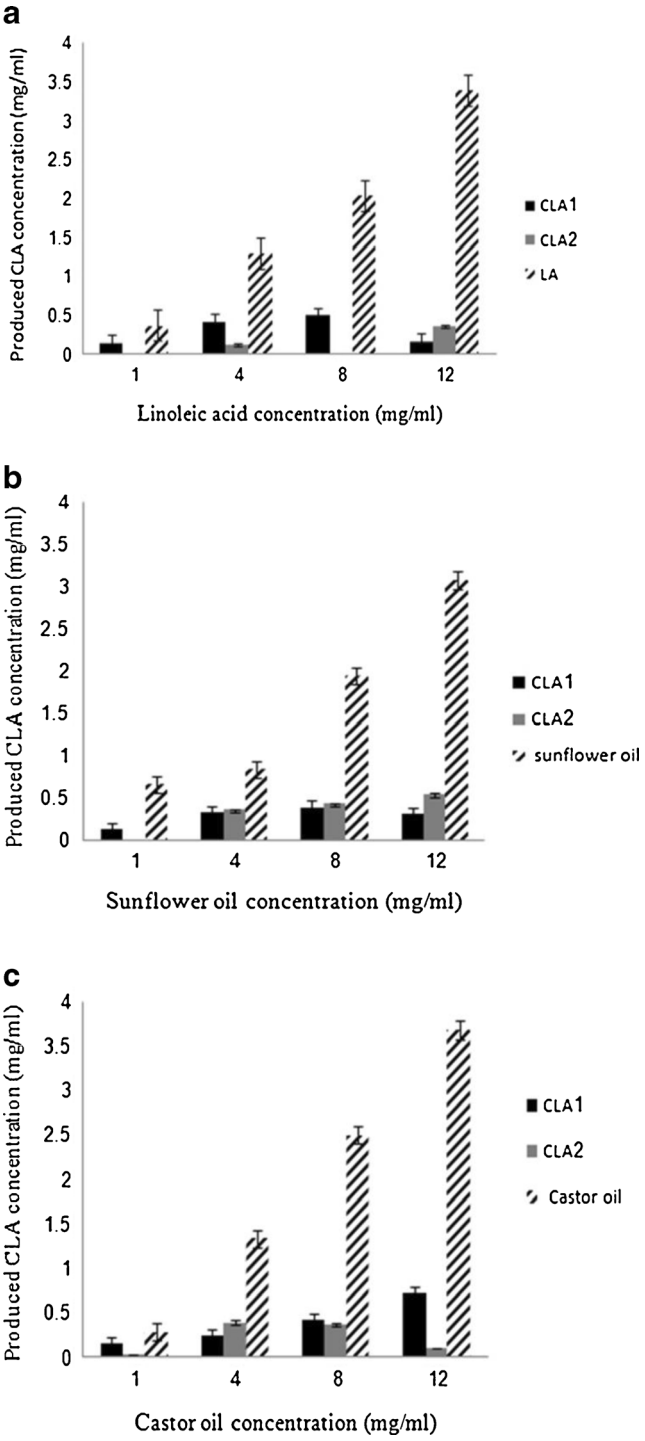

Fig. 3 Effect of substrate concentration on the production of CLA isomers. The reaction mixture contained (1, 4, 8, 12 mg·ml^{-1}) of a linoleic acid and b sunflower oil and c castor oil. Values are Mean ± SE (n=3), P<0.05

depicted considerable CLA productivity, but further cultivation resulted in a decrease in CLA production. Conversely, when we incubated probiotics as growing cells in MRS broth medium containing linoleic acid, no significant amount of CLA was detected. These results are in agreement with those

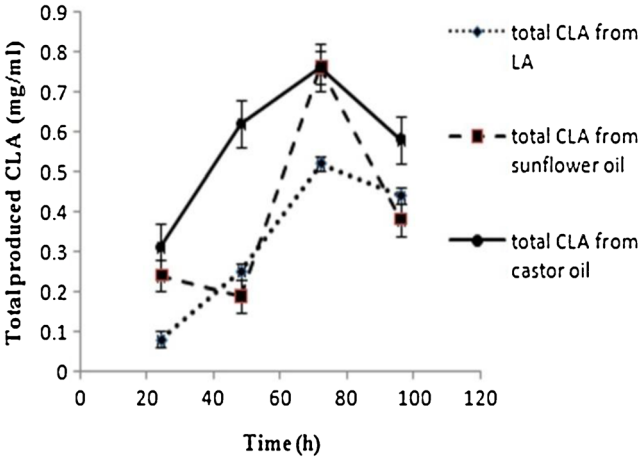

Fig. 4 Effect of incubation time on the production of CLA isomers. The reaction mixtures contained 4 mg·ml^{-1} of linoleic acid, sunflower oil, and castor oil, separately. Values are mean ± SE (n=3), P<0.05

reported by Kishino et al. (2002) that *L. plantarum* (AKU 1009a) produced a little CLA in MRS broth, but produced higher amounts in resting cells. This is probably due to the toxicity of unsaturated fatty acids to living cells, especially Gram-positive bacteria and probiotics. The results of this study show that probiotics have the ability to simultaneously produce the most bioactive isomers such as *cis*-9, *trans*-11-CLA (CLA1) and *trans*-10, *cis*-12-CLA (CLA2). Akalın et al. (2007) and Macouzet et al. (2009) also reported that *L. acidophilus*, *Bifidobacterium animalis,* as probiotics of yoghurt, and the probiotic *L. acidophilus* (La-5) produced CLA1 and CLA2. This result is in agreement with the ability of six strains of *L. plantarum* isolated from local products reported by Li et al. (2012). Meanwhile, strains which are not probiotic usually produce CLA1 and *trans*-9 and *trans*-11-CLA, or can produce only one of these biologically active isomers.

Since the addition of external lipase in order to convert castor oil and sunflower oil to ricinoleic acid and linoleic acid, respectively, is not profitable, the ability of strains to produce lipase in the presence of specified substrates was examined. Lipase activity is specifically substrate-dependent. As results depict, lipase activity of *L. plantarum* resulted in the conversion of sunflower oil to free linoleic acid, but not with castor oil as the substrate.

Another important factor in CLA production is substrate concentration. According to Ando et al. (2004) CLA production from castor oil by *L. plantarum* (JCM 1551) increased by raising the concentration of castor oil up to 20 mg·ml^{-1} and reached a plateau at the increase to 30 mg·ml^{-1}. Therefore, the type of substrate and its concentration have a deep effect on the ratio between produced CLA isomers.

In conclusion, according to the results of this study, biological CLA production from linoleic acid, sunflower oil, or castor oil by lactic acid bacteria can be much more isomer-

selective than chemical methods. Also, by changing the reaction conditions of CLA production, the isomer ratio could be controlled. We presented here the comparison of different types of substrates for CLA production and isomer proportion for the first time. The ability of *L. plantarum* to produce CLA isomers is of interest for the development of probiotic supplements or health-food products, but further studies should be performed to maximize the enrichment in CLA and enhance the health functionality of probiotic products.

Conflict of interest The authors declare that they have no conflict of interest.

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