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The Impact of Oil Type and Lactic Acid Bacteria on Conjugated Linoleic Acid Production

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ABSTRACT

This work was conducted to investigate the effect of oil type and lactic acid bacteria on the conjugated linoleic acid (CLA) production in MRS medium. The ability of eight strains of lactic acids bacteria; *Lactobacillus acidophilus* (P2, ATCC 20552), *Lactobacillus brevis* (P102), *Lactobacillus casei* (P9, DSMZ 20011), *Lactobacillus plantarum* (P1), *Lactobacillus pentosus* (P4), *Lactobacillus rhamnosus* (P5, TISTR 541), *Bifidobacterium longum* (BL) and *Bifidobacterium lactis* (P7, Bb-12) for the production of CLA in the MRS broth was investigated. Two vegetable oils (sun flower oil & linseed oil) and cod liver oil were used as substrates in MRS media. The oils were added to MRS in concentration of 10 mg/ml and incubated for three days at 37°C. The ability of lactic acid bacteria under the investigation as inhibitors were evaluated by the determination of the amount of conjugated linoleic acid at the end of the fermentation period. The results indicated that there were significant differences between the microorganisms in their ability to produce CLA. Furthermore, there were significant differences between oil types as substrate on the impact of CLA production. *Bifidobacterium lactis* showed the highest production of CLA (618.13 µg/ml) in MRS media fortified with cod liver oil. From the results, it can be concluded that there were positive impacts for both oils and lactic acid bacteria on the production of CLA. Therefore, the lactic acid bacteria grown in these oils can be utilized for probiotic production purposes and to produce other bioactive compounds.

INTRODUCTION

Essential fatty acids (EFAs) are fatty acids that cannot be synthesized in the human body and for that reason must be obtained from the diet or other external sources. The two essential fatty acids are α-linolenic acid (ALA) and linoleic acid (LA), which are also known as linseed oil acid [1]. α-linolenic acid is an omega-3 (*n*-3) fatty acid found in many common vegetable oils, and it has a lipid number of 18:3 with three *cis* double bonds 9,12,15. The first double bond is located at the third carbon from the methyl end of the fatty acid chain [2]. LA is a polyunsaturated omega-6 (*n*-6) fatty acid; it has a lipid number of 18:2 with two *cis* double bonds 9,12 and the first double bond is located at the sixth carbon from the methyl end [3,4].

Linoleic acid is transformed in the human body to the long-chain polyunsaturated fatty acids gamma-linolenic acid (GLA) and arachidonic acid (AA) [5]. Conjugated linoleic acid (CLA) is a term used to characterize positional and geometrical isomers of LA with conjugated double bonds [6,7] and are naturally occurring fatty acids that are produced by the ruminal bio-hydrogenation process in animal's tissues [8].

Although conjugated linoleic acid includes 28 isomers [9], the most commonly found are the *cis-9, trans-11 (c9t11)* and *trans-10, cis-12 (t10c12)* octadecadienoic acid (**Fig.** 1) [10]. Furthermore, the *c9t11* isomer has been shown to be the most active isomer because it is the only isomer incorporated into the phospholipid fraction of tissue of animals fed with a mixture of CLA isomers [11].

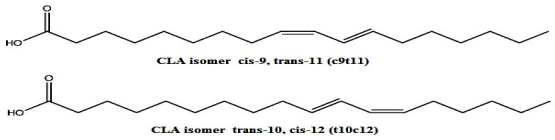

Fig. 1. Isomers of the conjugated linoleic acid (CLA) (photo from *examine.com*)

Conjugated linoleic acid can be synthesized chemically by alkaline isomerization of linoleic acid [12] and also via dehydration of vegetable oils as in castor oil [13] or through oxidation of LA free radicals in the presence of sulfur-rich proteins [14].

Conjugated linoleic acid is found naturally in the meat, milk of ruminants and some vegetables oils [8,15]. It can also be produced biologically by lactic acid bacteria (LAB). The strains which produce CLA are the most inhibited by LA while those that cannot produce CLA are the least affected [16,17]. The CLA produced by lactobacilli cultures is mainly present in the extracellular phase, and in some cases are present in the cellular membranes when cultures are grown in the presence of exogenous linoleic acid [18–21]. CLA shows protective properties against human malignant melanoma, colorectal and human breast cancer cells [22]. Both isomers also have controlling effects against the spread of colon cancer cells [23] and is also involved in body fat modulation [24].

Lactic acid bacteria refer to a large group of Gram-positive, acid-tolerant and bile resistant bacteria [25]. Interest in the possible health benefits associated with the genus lactobacillus has concentrated in controlling cancer [26] and lowering serum cholesterol level [27], although other diseases and conditions have been examined. *Lactobacillus reuteri*, a probiotic, has a high efficiency in the transformation of LA into CLA [28]. *Lactobacillus acidophilus* La-5 has been reported to be associated with the production of CLA during the fermentation of milk [29–31] and cream [32].

The low concentration of CLA found in foodstuffs does not seem to be sufficient for any therapeutic effect; for this reason, the purpose of this study was to screen the ability of some lactic acid stains for producing considerable amounts of CLA and to carry further investigation on the influence of types of oil on CLA production.

MATERIALS AND METHODS

Chemicals

Sunflower oil, flax oil (linseed oil) and cod liver oil were purchased from a customary local market in Cairo, Egypt. Total CLA (the isomer *cis-9,trans-11* and *cis10,trans12*) was purchased from Sigma Aldrich (*St. Louis, Missouri, USA*). All other chemicals and materials were of analytical reagent grade.

Microorganisms

Lactobacillus acidophilus (P2, ATCC 20552), *Lactobacillus brevis* (P102), *Lactobacillus casei* (P9, *DSMZ* 20011), *Lactobacillus plantarum* (P1), *Lactobacillus pentosus* (P4) and *Lactobacillus rhamnosus* (P5, TISTR 541), *Bifidobacterium longum* (BL) and *Bifidobacterium lactis* (P7, Bb-12) strains used in this study were obtained by personal communication with Dr. Hoda Marous, industrial biotechnology department, genetic engineering and biotechnology research institute, university of Sadat city, Egypt which were isolated from healthy, breast- feeding infants [33].

Growth and maintenance media

The standard lactobacillus strains were maintained on "de Man Rogosa and Sharpe" (MRS) medium, having the following composition (%, w/v): yeast extract 0.4; beef extract 0.8; glucose anhydrous 2.0; peptone 1.0; sodium acetate trihydrate 0.5; triammonium citrate 0.2; manganese sulfate tetrahydrate 0.005; magnesium sulfate heptahydrate 0.02, tween (80) 0.1; dipotassium hydrogen phosphate 0.2, agar 1.5; final pH (at 37˚C) was adjusted to 6.2±0.2.

Production media (Fermentation process)

The inocula were prepared by scratching the inoculated MRS slant (this step for each microbial strain separately) with a sterile needle and culturing in 10 ml of MRS broth for 24 h under Micro-aerobic conditions at 37˚C. These suspensions were used to inoculate sterile oily-fermentation medium which was composed of 10 mg/ml of each oil type (sunflower oil, flax oil and cod liver oil) with 1 % of Tween 80 (oil was mixed well with the Tween-80 before being added to the medium) in MRS broth medium until the total test volume reached 100 ml in a 250 ml Erlenmeyer flask. Flasks were then incubated on a rotary shaker (incubator shaker series Innova®43 New Brunswick scientific) at 120 rpm for 72 h at 37˚C. The control was done for each strain by the elimination of any kind of oil.

Sonication and preparation of cell free extracts

At the end of fermentation time, 0.5 ml trichloroacetic acid was added to the all fermentation cultures. The cultures were centrifuged at 6000 rpm for 20 min using (Centurion Scientific LTD Model 1020 series) to separate the cells from the culture filtrate.

The harvested cells were washed twice with physiological saline, then suspended in 50 ml of 5 mM-sodium phosphate buffer (SPB), pH 7.2, containing 0.2 M NaCl, and disrupted using sonicator (pulse 40/5 min) at 4˚C. The sonicated cells were centrifuged at 6000 rpm for 20 min and the precipitate was resuspended in the same buffer, sonicated and centrifuged again. The resulting precipitate was discarded and the clear supernatants were combined and used as the crude intracellular preparation "cell free extract" for next steps.

Total lipid extraction

The extraction of total lipid from cell free extract samples was performed according to the method described by Coakley *et al.* [34]. All treated samples were extracted with a 5-fold higher amount of n-hexane and mixed at 120 rpm for 6 h. Then the extraction mixtures were centrifuged at 6000 rpm for 10 min, the n-hexane phase was then collected and washed 3 times using the same volume of distilled water. Afterwards the n-hexane phase was recovered and the water content was removed finally with anhydrous-Na_2SO_4.

Determination of conjugated linoleic acid

The total amount of CLA was determined according to the Hong-wei Zhao *et al.,* [35] method. The samples were measured at 233 on a spectrophotometer (UV-200-RS LW Scientific) and compared to a CLA calibration curve. The CLA content was expressed as µg of CLA equivalent per 1 g of oil.

Statistical analysis

All the results were performed with at least four replicates. Differences between means were evaluated using one-way ANOVA and Duncan′s multiple range tests using the statistical

software Sigma Plot (version 13.0) *(Sigmaplot system requirements)*. Results are expressed as mean ± standard deviation (SD) and *P*-values less than 0.05 were considered statistically significant.

The effect of sunflower oil on conjugated linoleic acid production by lactic acid bacteria strains

The production of conjugated linoleic acid after three days of fermentation process using eight lactic acid bacteria strains (*Bifidobacterium longum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus casei*) grown on MRS medium containing 10 µg/ml of sunflower oil was studied and the obtained data were represented in **Fig. 2** and **Table 1**.

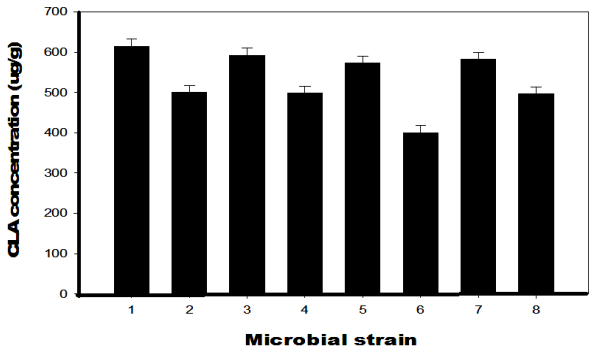

Fig. 2. The influence of sunflower oil on the production of total conjugated linoleic acid by different strains of lactic acid bacteria; values are mean of three replicates (p<0.05). **1**, *Bifidobacterium longum*; **2**, *Lactobacillus brevis*; **3**, *Lactobacillus rhamnosus*; **4**, *Bifidobacterium lactis*; **5**, *Lactobacillus acidophilus*; **6**, *Lactobacillus plantarum*; **7**, *Lactobacillus pentosus*; **8**, *Lactobacillus casei*.

Table 1. Shows the significant differences of each microbial strain in its ability to metabolize the oils and convert them to the conjugated linoleic acid.

Oil type	Conjugated linoleic acid (µg/ml)							
	Lactic acid strains							
	Bifido-bacterium longum	*Lacto-bacillus brevis*	*Lacto-bacillus rhamnosus*	*Bifido-bacterium lactis*	*Lacto-bacillus acidophilus*	*Lacto-bacillus plantarum*	*Lacto-bacillus pentosus*	*Lacto-bacillus casei*
Control treatment	359.57	334.29	365.29	385.34	348.91	328.15	332.79	335.66
Sunflower oil	*614.66	*500.23	**592.19	**498.22	*573.12	**400.32	**582.21	*496.33
Cod liver oil	*573.83	**442.19	**585.63	*618.13	*515.93	*432.55	*685.73	**484.26
Flax oil	**498.29	*592.29	*614.43	**492.22	**490.29	*488.12	*595.18	*600.2

Note: The values of the conjugated linoleic acid were the mean values of three replicates.
Multiple comparisons versus control group.
Means within the same column bearing different superscripts are significantly different (P< 0.05).
*significant correlation;
**non significant correlation

The results indicated that the *Bifidobacterium longum* strain has highest ability in the production of CLA (614.66 µg/g) then *Lactobacillus rhamnosus* strain (592.19 µg/g). Moreover, *Lactobacillus plantarum* strain has the lowest ability in the production of CLA (400.32 µg/g). There were significant differences (P<0.05) between the ability of microorganisms under investigation in the production of CLA. On the hand, there were significant differences between the microorganisms in their ability of production of CLA in the presence of sun flower oil and the control media (without addition of sunflower oil). From the obtained results, it could be concluded that there is a direct relation between the addition of sun flower oil and the production of CLA by the LAB under the investigation.

The results that were obtained are in agreement with previous results of Hosseini *et al.* [36] who found that the addition of sun flower oil has positive effects on increasing the capability of *L. plantarum* (AKU 1009a) in the production of CLA in the late log phase. Kishino *et al*. [37] also reported that sun flower oil contains large amount of LA of at least 69% and castor oil contains about 90% ricinoleic acid (hydroxy fatty acid). Ogawa *et al*. [38] also illustrated that probiotics have the ability to simultaneously produce the most bioactive fatty acid. Other results that were achieved by Akalin *et al*., [29] and Macouzet *et al*. [39] also indicated that *L. acidophilus* and *Bifidobacterium animalis* as probiotics of yoghurt and the probiotic *L. acidophilus (La-5)* produce CLA1 and CLA2.

The results here are also in agreement with the ability of six strains of *L. plantarum* isolated from local products reported by Li *et al.* [7]. For the meantime, 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. Because of the addition of external lipase in order to convert sunflower oil to LA is not profitable, the ability of strains to produce lipase in the presence of specified substrates was examined. In conclusion, according to the results of this study, biological CLA production from sunflower oil by different LAB stains can be much more effective and has economical value.

Effect of cod liver oil on conjugated linoleic acid production by lactic acid bacteria strains

The data presented in **Fig. 3** and **Table 1** indicated the effect of cod liver oil on conjugated linoleic acid production by lactic acid bacteria strains**.** It can be seen that the highest CLA production was obtained by *Lactobacillus pentosus* (685.73 µg/g) followed by *Bifidobacterium lactis* (618.13 µg/g) and the lowest CLA production (432.55 µg/g) was obtained by *Lactobacillus plantarum*. Also, the data in the same table (**Table 1**) indicated that there are significant differences (P<0.05) between the capabilities of the microorganisms in this study for the production of CLA.

In the same time, there are significant differences between the microorganisms in their ability of production of CLA in the presence of cod liver oil and control treatment without fortification of MRS media by cod liver oil at the end of incubation period. From the obtained results, it could be concluded that there are significant of the addition of cod liver oil on the production of CLA by the LAB under the investigation.

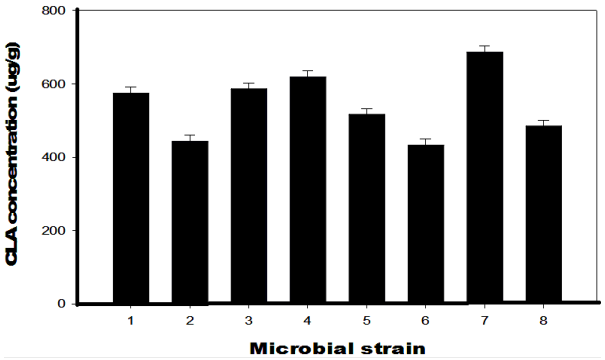

Fig. 3. The influence of cod liver oil on the production of total conjugated linoleic acid by different strains of lactic acid bacteria; values are mean of three replicates (p<0.05). **1**, *Bifidobacterium longum*; **2**, *Lactobacillus brevis*; **3**, *Lactobacillus rhamnosus*; **4**, *Bifidobacterium lactis*; **5**, *Lactobacillus acidophilus*; **6**, *Lactobacillus plantarum*; **7**, *Lactobacillus pentosus*; **8**, *Lactobacillus casei*.

The results in this study show that the higher CLA produced with cod liver oil might not be attributed to the fact that cod liver oil is a substrate containing linoleic acid (approximately 2.2%), but to the ability of LAB to produce LA when grown on MRS and enhance enzymes production needed for the CLA production. High CLA production could also be attributed to the role of cod liver oil as an additional nutrient source in the medium. The results obtained in this work are also in agreement with the results that is obtained by Vela Gurovic *et al*., [31] who found that LAB isolated from fish produce CLA without the addition of exogenous substrate and LA was detected in cultures grown on MRS at percentages of up to 18.3% of the total fatty acid. Also our results are in agreement with Kankaanpää *et al*. [40] that showed CLA production by lactobacilli at small percentages ranging from 2.7 to 3.4%. Other authors found up to 5% CLA in lyophilized LAB without the addition of exogenous substrate [41]. Kankaanpää *et al*. [40] also indicated that MRS broth contains small quantities of CLA in percentages of CLA ranging from 1.2 to 17 µg/ml using LAB strains which have the capability to produce linoleic acid and biotransform it into CLA could help to reduce the production costs of industrial processes via reduction of substrate amounts used.

Effect of flax oil on conjugated linoleic acid production by lactic acid bacteria strains

Fig. 4 and **Table 1** showed the influence of flax oil (linseed oil) on CLA production by different lactic acid strains. Data indicated that the *Lactobacillus rhamnosus* strain gave the highest ability for CLA production in MRS media (614.43 µg/g) followed by *Lactobacillus casei* strain (600.20 µg/g), *Lactobacillus pentosus* strain (595.18) and *Lactobacillus brevis* strain (592.29 µg/g); and the lowest CLA production (488.12 µg/g) was obtained by *Lactobacillus plantarum* strain. On comparing the results obtained with the negative control without the addition of flax oil as exogenous substrate to the media, it was found that *Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus pentosus, Lactobacillus brevis and Lactobacillus plantarum* strains all showed significant effects on increasing CLA production. On the other hand, the results indicated that there are significant differences (P<0.05) between the capabilities of the microorganisms in this study for the producing of CLA in the presence of flax oil. Similarly, there are significant differences between the microorganisms in their ability of production of CLA in the presence of flax oil and control treatment without fortification of MRS media by flax oil at the end of three days of the experimental period.

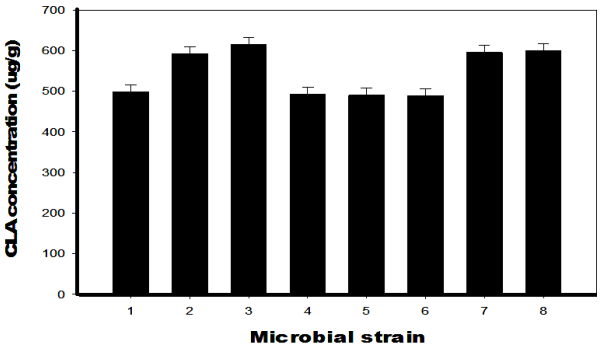

Fig. 4. The influence of flax oil on the production of total conjugated linoleic acid by different strains of lactic acid bacteria; values are mean of three replicates (p<0.05). **1**, *Bifidobacterium longum*; **2**, *Lactobacillus brevis*; **3**, *Lactobacillus rhamnosus*; **4**, *Bifidobacterium lactis*; **5**, *Lactobacillus acidophilus*; **6**, *Lactobacillus plantarum*; **7**, *Lactobacillus pentosus*; **8**, *Lactobacillus casei*.

From the results, it is concluded that there was a significant effect of the addition of flax oil on the production of CLA by the LAB under the investigation. The results that were obtained here are in agreement with the results that is obtained by Vela Gurovic *et al*. [31] who studied the effect of LAB isolated from fish gut on the production of CLA without the addition of exogenous substrate and CLA production by lactobacilli at small percentages ranging from 2.7 to 3.4%. Other studies achieved by authors Dionisi *et al*. [41] found that the CLA production up to 5% of CLA by LAB in the lyophilized state. However, reports from the literature also indicated that the MRS broth also contains small quantities of CLA.

CONCLUSION

From the obtained results, it is concluded that there are significant differences between the types of oils and microorganisms on the CLA production. The effect of oil may be due to the composition of its fatty acids. The cod liver oil was containing amount of linoleic fatty acid which was considered as efficient precursor in biosynthesis of certain enzymes which are responsible for production of CLA. The ability of lactic acid bacteria for producing of CLA refer to the ability of the strains to produce some specific enzymes that can formulate biotransformation of linoleic fatty acid into CLA. Depending on the obtained results we could conclude that there are positive effects for both oils and lactic acid bacteria on the production of CLA. Therefore, we should select the lactic bacteria and oils to perform probiotic purposes and to product of other benefit compounds.

CONFLICTS OF INTEREST

All authors declare that there are no financial/commercial conflicts of interest.

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