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Improving the production of conjugated linoleic acid from sunflower oil by lactic acid bacteria spp: Effect of calcium carbonate supplementation in fermentation medium

Koka Zongo*, Fehmi Yazici & Ahmet Hilmi Çon

Food Engineering Department, Graduate School of Sciences, Ondokuz Mayis University, Samsun-55270, Turkey

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Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid with various positional and geometric isomers of linoleic acid (LA) present mainly in food items and produced endogenously in non-ruminants and humans or through fermentation process. It is associated with health-beneficial effects and subject to more research on its natural sources (ruminant-derived foods) and strategies to increase the content in various foodstuffs. Although several studies have reported the most common intake value of 0.8 g/day (0.6 to 3.0 g/day), research for raising in situ concentration should focus on strategies such as *in vitro* bioconversion of its precursors by bacteria and supplementation of LA rich-oils in foods fermentation process. In this study, the ability of some lactic acid bacteria (LAB) from diverse samples to produce CLA from sunflower oil and the effect on production yield of fermentation medium supplementation with carbonate calcium were investigated. Results showed that ten *Limosilactobacillus fermentum* and *Enterococcus faecium* produced *trans*-10, *cis*-12-CLA isomer accounted for at least 85% of total CLA ranging from 4.64 to 5.22 µg/mL. Despite the fermentation medium supplementation with $CaCO_3$ enhanced the production yield, the residual LA inhibitory effect on bacteria growth governing CLA biosynthesis process was not mitigated. So, although our LAB strains can produce CLA, the more the LA concentration goes up, the more the conversion rate downgrades. Further studies on strains behavior in a wide range of LA concentrations will help establish a stable relationship between bacteria and LA in the presence of $CaCO_3$.

Keywords: Biosynthesis, Conjugated linoleic acid, Inhibition, Lactic acid bacteria, Linoleic acid, Mitigation

The recent emergence of conjugated linoleic acid market and the overwhelming consumer interest in its beneficial effects have sped up research on its natural sources[1–4]. CLA is a polyunsaturated fatty acid mainly observed in ruminant food items such as milk and meat that the consumption is a suitable way to increase its bioavailability[5]. It refers to a mixture of LA with several minor isomers (*cis*-8, *cis*-10 CLA; *cis*-9, *cis*-11-CLA; *cis*-10, *cis*-12-CLA; and *cis*-11, *cis*-13-CLA) and some significant isomers containing unique conjugated double bonds. Perhaps two major CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) are the most abundant naturally occurring biologically active compounds that have been shown to reduce body fat accretion, inhibit carcinogenesis and enhance the immune system in animal models experimnts[6].

Approved to be generally recognized as safe (GRAS) for foods, these two isomers have been the object of several studies. They were reported to improve high-density lipoprotein (HDL) or good cholesterol composition and function and be involved in cardiovascular diseases and cancers[6,7]. More CLA benefits and evaluations showing LAB ability to convert free linoleic acid (LA) or some vegetable oils LA into CLA have been reviewed in literature[8,9]. As a naturally occurring molecule, CLA is majorly present in ruminant-derived foods (6.6 mg/g in meat to 29.6 mg/g FAME in bovine milk)[1]. However, to benefit to human health, the recommended daily intake of *cis*-9, *trans*-11 CLA (80% of total dietary CLA) should be around 3g for a 70 kg person and both isomers mixture administration to healthy overweight persons is required to be more bioactive against obesity[10,11]. Because adults consume only half of the recommended daily intake, extensive research on improving CLA availability in foodstuffs has been undertaken[1,6,12].

To this purpose, using *Lactobacillus* spp and sunflower oil (69% linoleic acid) or castor oil (90% ricinoleic acid) for CLA production has been found both oils as cost-effective alternatives substrates

*Correspondence:
E-mail: kokazongo2@gmail.com

compared to free LA to produce competitive CLA[4]. Trials about enrichment of foodstuffs using various strategies, including bacterial strains, animal feeding, or fortification, are well reported[9,12,13]. However, although various attempts were undertaken, results are far below expectations since some technological barriers hinder the biosynthesis process[14,15]. As mentioned by previous research, a pool of some circumstances (low concentrations of free LA in dairy products, lack of the lipolytic activity of targeted bacteria) and environmental conditions (temperature and pH) could seriously affect CLA production[16]. For example, the free linoleic acid at 2 mg/mL concentration inhibits bacterial growth restraining bacteria from starting CLA production[16,17]. But interestingly, using the polyoxymethylene sorbitan monooleate (Tween 80) detergent in the fermentation medium and suitable free LA concentrations (optimized and low quantity) successfully promoted CLA production yield. Otherwise, utilizing higher free LA concentration as substrate results in decreasing yield that affects the end product cost remaining excessive and not accessible to consumers[18]. Then, with projections to formulate CLA enriched staple food, the present study aimed to investigate the ability of lactic acid bacteria growing in the presence of LA to produce CLA (mainly two major and bioactive isomers *cis*-9, *trans*-11-CLA, and *trans*-10, *cis*-12-CLA) from sunflower oil and the effect of carbonate calcium (an antacid) on the production yield.

Materials and Methods

Isolation and maintenance of potential CLA-producing LAB

Twenty-one biological matrices (Fig. 1) including kaşar cheese (n = 7) and sucuk (n = 3) from Turkey, adjuevan (fermented *Chloroscombrus chrysurus*, n=6) from the Ivory Coast, and goat (*Capra aegagrus hircus*, n = 5) rumen from India were collected between October 2017 and December 2018. The experiments were carried out at Ondokuz Mays University (Turkey) and National Institute of Food Technology, Entrepreneurship and Management, Thanjavur, formerly Indian Institute of Food Processing Technology (India). Bacterial isolation was carried out according to previously published method[19]. Samples (25 g) were homogenized in a stomacher (Smasher, AES/BioMérieux, France) after being diluted with 225 mL buffer peptone water. To select presumptive lactobacilli, ten-fold dilutions (10^{-3} to 10^{-8}) of each sample were plated in duplicate on MRS agar, sealed in anaerobic jars with 5% carbon dioxide (CO_2) or Anaérocult A (Merck), and incubated at 30°C for 96 h. For presumptive lactococci isolation, the same dilutions were parallelly plated on M17 agar under aerobic conditions at 25°C for 72 h. Ten colonies from each countable plate were sub-cultured twice in the same medium to get pure culture. Subsequent research focused on catalase-negative, Gram-positive, and nitrate-negative pure cultures. Isolates were regularly grown in MRS broth under anaerobic or aerobic conditions whether they were isolated from MRS or M17 agar. Isolates were stored at −80°C in 30% sterile glycerol. The isolates sensitivity to linoleic acid was assessed on MRS agar supplemented with LA. The aptitude of isolates to develop in the presence of LA was evaluated using 0.2 mL (1.2×10^9 CFU/mL) of an overnight grown culture disseminated on MRS agar supplemented with 100 g/mL of LA[20]. The plates were then incubated for 48 h under aerobic conditions at 25°C or anaerobic conditions at 30°C. For maintenance, isolates were sub-cultured in modified MRS at 25 or 30°C for 24 h. All candidates growing in the presence of LA were considered for subsequent investigations

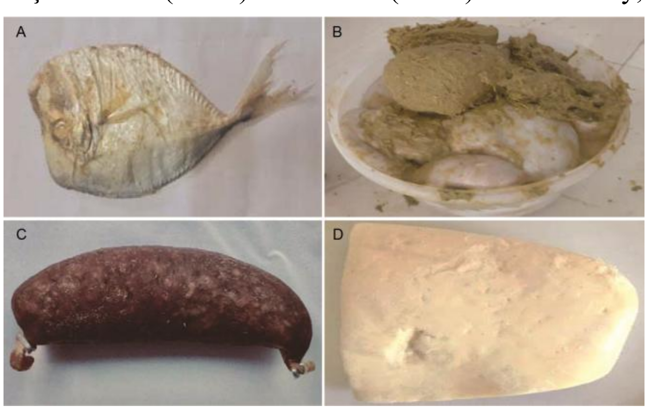

Fig. 1 — Biological matrices include (A) adjuevan (fermented *Chloroscombrus chrysurus*) from the Ivory Coast; (B) goat (*Capra aegagrus hircus*) rumen from India (C) sucuk; and (D) kaşar cheese from Turkey.

Genotyping and Identities confirmation

Genomic DNA extraction

Isolates (n = 30) growing on MRS in the presence of LA were sub-cultured for 20 h in modified MRS (mMRS) broth under aerobic or anaerobic conditions at 25 or 30°C. Two milliliters of each sub-cultured isolate (1.2×10^9 CFU/mL) were transferred to a microtube and centrifuged for 10 min at 10,000 × g. According to the manufacturer procedure, total genomic DNA was extracted and purified DNA was kept at −20°C until used (Invitrogen, USA).

PCR amplification and Phylogenetic analysis

16S rRNA gene was amplified in 0.2 mL PCR tubes using the universal primers including 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3')[21]. The PCR reaction consisted of 35 cycles of denaturation at 95°C for 3 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min was performed in a final 50 µL reaction volume using a BioRad T100 thermal cycler. *Fructilactobacillus sanfranciscensis* ATCC 27651T was used as a positive control. A tube containing chemicals without a DNA sample served as the negative control. 16S rRNA amplicons were separated at 100 V on 1% agarose (Sigma) gel containing 2 µL ethidium bromide (0.5 µg/mL, Sigma) and photographed with UV light in GelDoc System (VilberLourmat, Quantum ST5). A gene ruler DNA ladder was used to determine the size of DNA pieces (Thermo Fisher Scientific, 50 bp). PCR products were purified with the PureLink PCR purification Kit (Thermo Fisher Scientific) and single-sequenced using three universal primers (518F; Mg5F; 800R) (Macrogen Europe BV, the Netherlands). ChromasPro Version 2.1.8 (Technelysium Pty Ltd, Australia) was used to edit and compile ABI format chromatogram files of 16S rRNA sequences. The EZbiocloud server (URL-13) algorithm was used to identify isolates. The MEGA X program performed an alignment and phylogenetic analysis using the Muscle and Maximum Parsimony methods in a *p*-distance model (bootstrap 1000) to validate identities and displayed evolutionary connection between isolates[22].

Carbohydrates fermentation

Straight strains with the highest resemblance (≥99%) were submitted to carbohydrate fermentation tests for proper identification**.** Peptone (10 g), NaCl (5 g), yeast extract (3 g), carbohydrates (10 g), and 0.2% bromothymol blue solution (10 mL) were added to the fermentation broth per liter to create a fermentation medium. A 0.2 mL overnight grown culture (1.2×10^9 CFU/mL) was used to inoculate 5 mL of fermentation medium incubated for 48 h at 25 or 30°C.

Production and determination of CLA

An emulsion stock of 1% of sunflower oil was prepared by stirring the sunflower oil in a sterile Tween 80 solution (2%). The mMRS broth (0.1% sunflower oil), which the pH adjusted to 6 was inoculated with 1mL (1.2×10^9 CFU/mL) of an 18 h old culture. The mixture was incubated at 25 or 30°C for 48 h. Suitable controls were only cells or fatty acids; LA was quantified using spectrophotometric and chromatographic assays.

Quantification of CLA through Spectrophotometric assay

CLA was quantified using a rapid screening method through the ultraviolet (UV) spectrophotometer at 233 nm as previously described[23]. Briefly, 2 mL culture was centrifuged at 10000 × g at 4°C for 15 min. Four mL isopropanol was vortexed with the supernatant for 30 s, and the mixture was left to stand for 10 min. The sample was thoroughly vortexed with 3 mL hexane for 30 s, then allowed to stand for 10 min . An aliquot of 2 mL of the upper layer containing fatty acids was taken to estimate the CLA content at 233 nm using the Cary 60 UV-Vis spectrophotometer (Agilent, Palo Alto, CA) against hexane layer containing LA served as a blank. The samples total CLA was determined using an external standard curve drawn from CLA serial concentration up to 125 µg/mL

Isomerization and Identification of CLA bioactive isomers through RP-HPLC

Reverse-phase High-Pressure Liquid Chromatography (RP-HPLC) was used to assess the type of CLA isomers. Before RP-HPLC analysis, both reference standards (methyl (9,11) and (10,11) conjugated) obtained from Nu-Chek-Prep and Sigma were isomerized with iodine (I_2) using 5 and 10 mg of each CLA reference picked up and dissolved in 2 mL petroleum ether in a screw-capped glass test tube[24,25]. A few drops of I_2 solution (6 mg I_2/100 mL petroleum ether) were added until the light pink color appeared. Test tubes were exposed to sunlight for 30 min and shaken for 10 s with 5 mL aqueous (0.1 N) sodium thiosulfate ($Na_2S_2O_3$) to remove the I_2. This step was repeated until a transparent solution was achieved. The organic phase was dried over anhydrous sodium sulfate (Na_2SO_4), evaporated under nitrogen, and resuspended in 2 mL acetonitrile (CH_3CN) containing 0.14% of acetic acid (CH_3COOH). Parallelly, CLA produced by isolates was extracted through 5 mL postassium hydroxide (KOH) in methanol (2N) with 3 mL cultured broth and incubated in a water bath for 30 min at 60°C, left to stand at room temperature (25°C) for 10 min. Excess KOH was then neutralized adding 5 ml HCL (2N). Five mL hexane was added to samples and centrifuged at 4,100 x g for 15 min. The upper layer was collected and evaporated under the vacuum, and the residue was resuspended in 0.5 mL

CH_3CN containing 0.14% CH_3COOH. CLA isomers and metabolites were separated using an Agilent 1200 Infinity Series High-Pressure Liquid Chromatograph (Agilent, Palo Alto CA) equipped with a variable wavelength UV detector. C-18 Inertsil ODS-3 column (5µm particle size, 250 × 4.6 mm, GL Science Inc. USA) with CH_3CN/H_2O (80/20, *v/v*) mobile phase flowing at 1.5 mL/min was used[26]. Identification of CLA isomers was performed by comparing the retention times of bacteria-produced CLA compound peaks with those obtained from reference standards. UV spectrum of all compounds was drawn from 200 to 260 nm.

Carbonate Calcium ($CaCO_3$) effect on CLA production

As some barriers affect CLA production, we hypothesized that calcium salt could remove the residual LA inhibition and enhance LA production. So, mMRS broth was inoculated using 1 mL ($1.2x10^9$ CFU/mL) of a 20 h old culture of *Lactobacillus fermentum* AFKB7N, *Lactobacillus fermentum* AFKM26, *Lactobacillus* AFKS3, and *Lactobacillus fermentum* AFKSC1). After 24 h incubation period, the broth pH was adjusted to pH 6 with $CaCO_3$ (10%). Sunflower oil (0.1 – 0.4%) dispersed in 2.5% tween 80 was added to reaction mixtures, vortexed once within 24 h, and incubated at 30°C for 48 h. The $CaCO_3$-free medium was inoculated in parallel with the relevant strains.

Statistical analysis

Analyses were performed using statistical software SPSS (Version 24.0 SPSS). Subgroup differences were tested with a statistical significance at *P*-value < 0.05 using a 1-way ANOVA test. Values are expressed as mean ± standard error of the mean. Each parameter was performed in three replications.

Results and Discussion

Isolation and maintenance of potential CLA-producing LAB

Based on the colonial morphology, total pure isolates (n = 538) growing on MRS agar (n = 424) and M17 agar (n = 114) were found to be Gram-positive and catalase-negative including presumptive bacilli (79%) and cocci (21%). Thirty, classified as nitrate-negatives and growing on MRS agar supplemented with 100 µg/mL LA, were potential presumptive LAB**.** Bacterial ability to resist LA and growth in LA supplemented medium was assessed according to previously described methodology and ascribed to strains types, media, and fermentation conditions[20,27]. For example, fortifying milk products with fish oil depleted the total LAB population within 16 days while *Lactobacillus acidophilus* ADH, *Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* growth has not been influenced, constrary to the growth of *Bifidobacterium longum* B6 held back at 0.5 and 1 mg/mL[28]. Similarly, *Lactobacillus viridescens* and *Levilactobacillus brevis* 01 isolated from cattle rumen were reported to tolerate LA differently in skim-milk[29]. Using sunflower oil (0.25, 0.5, and 1.0%) as substrate in media, *Levilactobacillus brevis* 01 produced (8.27, 5.73, and 1.66 mg) while *Lactobacillus viridescens* generated (1.83, 5.23, 5.67 mg) CLA per gram oil. Isolates ability to continuously growth on media supplemented with LA and use or tolerate this free fatty acid varies according to the type of bacteria, culture conditions and related inhibition threshold. Additional investigations using a wide range of LA concentrations could then help in better understanding sensitivity discrepancies between our bacterial strains.

Genotyping and Identities confirmation of thirty elected isolates

All presumptive LAB were submitted to 16S rRNA gene sequencing. Out of 21 identified, 12 strains were redundant, and 10 considered for further analysis. Amplification of 16S rRNA sequences using 27F and 1541R universal primers generated PCR products length higher than 1.2 kB. Ten strains depicting phenotypic and genotypic diversities were classified into three species (Fig. 2). 16S rRNA gene sequences from all isolates were at least 1.5 kB, except AFK_2-01 and AFK_2-04 strains corresponding to 1.2 and 1.3 kB, respectively. Isolates were found belonging to two genera including *Lactobacillus* and *Enterococcus* sp with over 99% similarity. Over the ten strains, the genus *Limosilactobacillu*s spp accounting for 7 strains was dominant (Table 1) with two sub-groups: group I (*Lm*. AFK-1-7, L7, S3, SC1) and group II (M26, M10, B7N) (Fig. 2). Blast results of 16S rRNA genes against deposited genes in the EZbiocloud database suggested that these two sub-groups are closest to *Limosilactobacillus fermentum* CECT562 than *Limosilactobacillus gorillae* KZ01 with a variation ratio of 3/1497 vs. 25/1495 in nucleotides. Remaining three strains were classified as *Enterococcus faecium* (AFK-2-04 and 2-09) and AFK-201 was not identified completely but likely to be *Lactobacillus* spp. Strains AFK_2-04 and AFK_2-09 16S rRNA genes were more connected with *Enterococcus faecium* LMG11423^T (>99.63%) than *Enterococcus lactis*

BT159^T (>99.23% similarity). Since subgroups are very closer, the carbohydrate fermentation test was performed to make proper differentiation[30]. Results in Table 2 showed a clear distinction of phenotypic characteristics confirming a diversity of genotypes (species) similar to 16S rRNA sequencing analysis results. Results are not surprising since that fermentation profiles are identical to those described in Bergey's Manual on Determinative Bacteriology[31]. Moreover, in agreement with previous descriptions, the present study reported that *Limosilactobacillus fermentum* and *Enterococcus faecium* strains did not ferment inulin, sorbitol, L-rhamnose, and glycerol. *Limosilactobacillus fermentum* strains were found in all matrices while *Enterococcus* spp were only found in cheeses. The predominance of *Enterococcus* spp in cheese has been ascribed to milk processing in which some farm-specific characteristics and the direct contact between the milking parlor and the hay in or the bedding area appear to promote milk contamination with *Enterococcus faecalis,* or *Enterococcus faecium* strains[32]. *Limosilactobacillus fermentum* are naturally occurring microflora for fermented milk products. For example, *Limosilactobacillus fermentum* strains have been reported belonging to sausage and adjuevan (fermented *Chloroscombrus chrysurus*) flora[33,34] and widely present in spontaneously fermented cereals and other plant materials or dairy products, manure and sewage feces, and the human vagina.

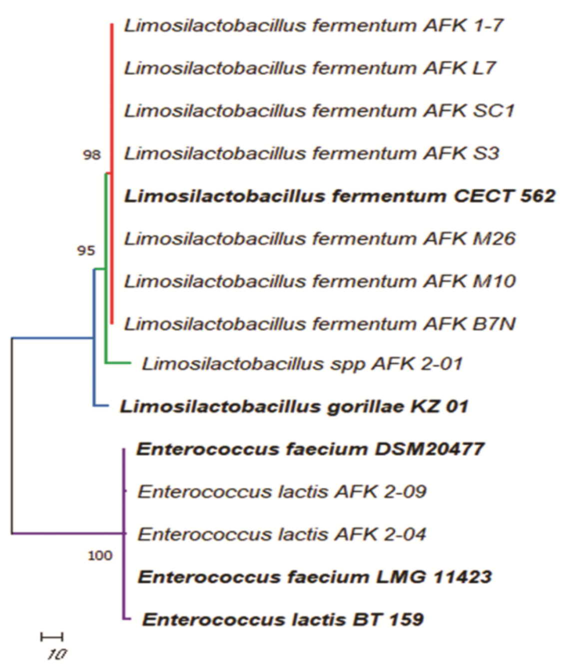

Fig. 2 — Dendrogram of LAB based on 16S rDNA gene sequences

CLA producing bacteria and Quantification of CLA

A standard curve was drawn using quadruple reading of CLA serial concentration (0 -125 µg/mL) absorbances in a Cary 60 UV-vis spectrophotometer (Agilent, Palo Alto, CA) at 233 nm. Total CLA amount was determined according to an established relationship formula between absorbances and CLA concentrations (R^2 = 0.9998; y = 0.1566 × + 0.017). As shown in (Table 3), the amounts of CLA were ranged from 4.64 to 5.22 µg/mL and were found to be quite similar with no statistical difference from the overall group was observed (P >0.05). However, *Limosilactobacillus fermentum* AFK_2-01, AFK_B7N, and AFK_S3 strains are likely to produce higher CLA than the remaining strains. Since the difference between CLA produced by *Enterococcus faecium* and *Limosilactobacillus* spp may be due to the total count of *Enterococcus faecium* that was generally lower. It is reported that *Enterococcus* spp produce minor CLA compared to other LAB. The results obtained in the present study are in agreement with some previous studies which reported the abilities of *Limosilactobacillus fermentum* and *Enterococcus*

Table 1 — LAB isolates related to matrices of isolation

Isolates	Matrices			
	Kasar Cheese[a]	Sucuk[b]	Adjuevan[b]	Goat Rumen[c]
Limosilactobacillus fermentum AFK L7				x
Limosilactobacillus fermentum AFK B7				x
Limosilactobacillus fermentum AFK SC1			x	
Limosilactobacillus fermentum AFK S3		x		
Limosilactobacillus fermentum AFK M26	x			
Limosilactobacillus fermentum AFK M10	x			
Limosilactobacillus fermentum AFK 1-7	x			
Lactobacillus spp AFK 2-01				x
Enterococcus faecium AFK 2-04	x			
Enterococcus faecium AFK 2-09	x			

faecium strains to convert LA into CLA[35,36]. Ham *et al*., 2002[36] screened 34 LAB isolated from 19 feces samples of healthy babies and first reported the ability of *Lactobacillus fermentum* to convert LA into CLA. Herzallah, 2013[35] fed chickens with two commercially balanced rations rich in fat (starter diet for four weeks and finisher diet at the 5th week) and 1 mL LAB bacterial culture, suggested supplementation of diet with LAB enhanced poultry meat and hen eggs enrichment in CLA (0.53 to 0.85 mg per gram of fat). Another study evaluating the potential of 283 lactobacilli isolated from various sources (dairy products, human breast milk, healthier persons fecal samples, and collection cultures) to convert LA into CLA found that 57 isolates including

Limosilactobacillus fermentum could produce CLA (19.5-79 µg/mL) in pure and skimmed milk MRS media supplemented with 0.5 mg LA per liter[37]. *Enterococcus faecium* also has been reported to convert LA into CLA[38]. However, although it is commonly reported that LAB possess the ability to convert vegetable oils LA into CLA[16], the conversion of LA into CLA is not systematically tributary to a given bacterial genus or species but should be dependent on strain capacity. Herein, the potential ability of lactic acid bacteria grown on LA to produce was hypothesized and the results showed that *Limosilactobacillus fermentum* and *Enterococcus faecium* produce CLA effectively.

Characterization of bacterial CLA isomers through RP-HPLC

Bioactive CLA isomers were identified by comparing chromatograms and spectra from isomerized standards with those produced by bacteria and validated in agreement with previous methods[24,25]. Results were presented in (Figs 3 and 4). More isolates produced more *trans*-10, *cis*-12-CLA than the *cis*-9, *trans*-11-CLA isomer at a variable rate (data not shown) differing from that reported in the literature. Figure 4 showed the clear separation of spectra corresponding to both isomers from CLA standards and those generated through bacterial LA biohydrogenation reaction. Figure 4A & B revealed

Table-2 — Carbohydrate fermentation profile of our CLA-producing lactobacilli

Species	Carbohydrates									
	Glucose	*Galactose*	*Sorbitol*	*Xylose*	*Inulin*	*Sucrose*	*Lactose*	*Trehalose*	*L-Rhamnose*	*Glycerol*
Limosilactobacillus fermentum AFK B7N	+	+	-	+	-	*w*	+	+	-	-
Limosilactobacillus fermentum AFK 1-7	+	+	-	-	-	*w*	+	*v*	-	-
*Lactobacillus spp*AFK 2-01	+	+	-	+	-	+	+	-	*w*	-
Enterococcus faecium AFK 2-04	+	+	-	*v*	-	*w*	+	+	-	-
Enterococcus faecium AFK 2-09	+	+	-	*v*	*v*	+	-	+	-	*w*
Limosilactobacillus fermentum AFK L7	nd	+	-	+	-	+	+	+	-	-
Limosilactobacillus fermentum AFK M10	+	+	-	+	-	-	+	+	-	-
Limosilactobacillus fermentum AFK M26	+	+	-	+	-	*v*	+	-	-	-
Limosilactobacillus fermentum AFK S3	nd	+	-	+	-	+	+	+	*w*	*w*
Limosilactobacillus fermentum AFK SC1	+	+	-	+	-	+	+	+	-	-

nd: not determined; w: weak; v: variable

Table 3 — Different CLA (µg. mL^{-1}) produced by isolates using $CaCO_3$ supplemented sunflower (0.1%) medium after 48 h. Values of CLA concentration are means (n=3)

Isolates	N	CLA production (µg/mL)	
		With$CaCO_3$^{a}	Without$CaCO_3$^{b}
Limosilactobacillus fermentum AFK M10	5	5,85^c±0,56	4,90^d±0,32
Limosilactobacillus fermentum AFK M26	3	6,15^c±0,72	4,64^d±0,32
Limosilactobacillus fermentum AFK S3	4	6,23^c±1,15	5,01^d±0,07
Limosilactobacillus fermentum AFK SC1	3	5,46^c±0,44	5,07^d±0,28
Limosilactobacillus fermentum AFK B7N	3	5,64^c±0,15	4,85^d±0,23
Limosilactobacillus fermentum AFK 1-7	3	5,52^c±0,21	4,77^d±0,28
Limosilactobacillus fermentum AFK L7	4	5,91^c±0,54	5,22^d±0,47
Lactobacillus spp_2-01	4	5,29^c±0,22	5,03^d±0,12
Enterococcus faecium AFK 2-04	5	5,93^c±0,53	5,06^d±0,29
Enterococcus faecium AFK 2-09	3	6,21^c±0,39	5,01^d±0,32

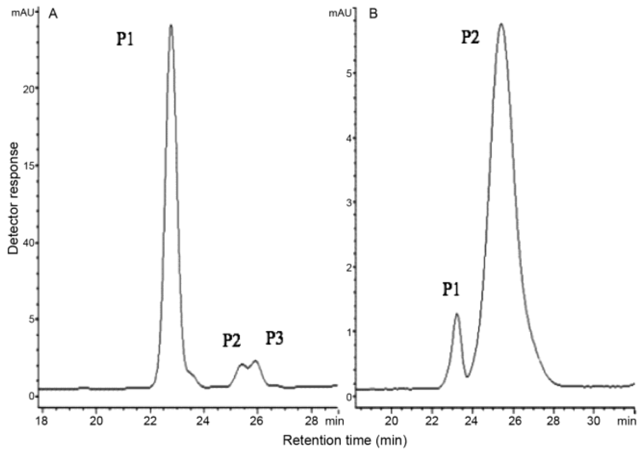

Fig. 3 — Partial chromatograms of CLA produced by *Limosilactobacillus fermentum* AFK M26 using the (A) sunflower oil ; and (B) Sigma CLA reference standard. Peaks (P) appear at 22,7-23,5 and 24-28 min

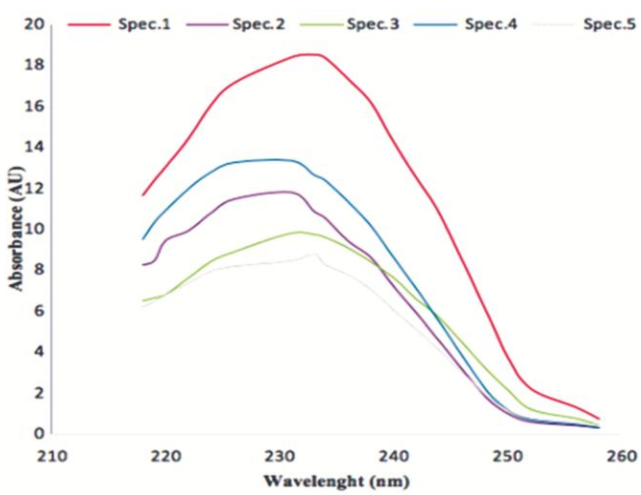

Fig. 4 — The spectra of CLA produced by *Limosilactobacillus fermentum* AFK M26 using the sunflower oil and Sigma CLA reference standard. The spectra of reference standards peak 1&2 (Figure 3B) correspond to Spec. 1&2 in Figure 4 and the peaks 1, 2&3 (Figure 3A) to Spec.3, 4 & 5 (Figure 4) obtained from the CLA produced by isolates

that absorbance increases from spectrum 4, 2, 3, 5, and 1 while peaks (*P*) eluted between 22.7 and 28 min. Since C18:2 molecules with two conjugated double bond systems have a maximum UV absorption at 233 nm (*t*, *t*-, *c*, *t*- or *t*, *c*-, and *c*, *c*- isomers absorb at 230, 232, and 234 nm, respectively)[24,26], each peak from produced CLA isomers was effectively associated with the corresponding comparing retention times, elution orders, maximum absorbance value, and wavelength. The peaks 1 & 2 (Fig. 4.) from CLA standards HPLC analysis have maximum absorption around 232 and 233 nm, respectively. Similarly, peak 1 having equal absorbance with CLA standard peak 1 showed both peaks corresponded to the same compound (*trans*-10 *cis*-12-CLA isomer). Sample peaks *viz*. 3 & 4 with maximum and same absorbance corresponded to standard peak 2, equivalent to *cis*-9, *trans*-11-CLA isomer which absorbed maximally at 233 nm (Fig. 4). Standard peak 2 equaled to *cis*-9, *trans*-11-CLA isomer and peaks 2 & 3 from samples CLA isomers might be a mixture of *cis*-9, *trans*-11 and *trans*-9, *cis*-11-CLA isomers. In brief, all targeted CLA isomers were identified and characterized contrary to authors who failed to separate *cis*-9, *trans*-11 and *trans*-10, *cis*-12-*CLA* isomers on an Inertsil ODS-2 column. Success in the present study in resolving both isomers using an Inertsil ODS-3 column may be associated with the column design[26]. Generally, an Intersil OSD-2 column is used instead of a C-18 Inertsil ODS-3 (5µm particle size, 250 × 4.6 mm) one. The OSD-3 column contrary to OSD-2 provides high retentivity useful in separating structurally similar analytes since the lengthier the column, the longer the retention time will be, permitting better separation of those indistinguishable isomers. *Cis*-9, *trans*-11 and *trans*-10, *cis*-12-*CLA* isomers can then be better separated using an OSD-3 column.

Compared to previous works using vegetable oils as substrate, our strains (*Limosilactobacillus fermentum* and *Enterococcus faecium*) species produce less CLA (4.64-5.22 µg/mL concentrated in *trans*-10,*cis*-12-CLA isomer) than past work (13.44-84.07 µg/mL)[39]. Thus, using vegetable oils castor oil (≈ 90% ricinoleic acid) or sunflower oil (≈ 55-70% linoleic acid) as alternative substrates with our strains to produce specific CLA isomer constituted a new approach[4,16]. The formation of a given CLA isomer could be elucidated as an integrated reaction ascribed to various factors including incubation time that controlled and optimized favor to better production[16,40]. For example, a suitable incubation period (up to 3h) of *Lactobacillus reuteri* PYR8 allowed significant *cis*-9, *trans*-11-CLA formation. Otherwise, this isomer will decrease gradually while the concentration of *trans*-9 and *trans*-11 CLA increases when reaching an incubation period of 4 to 24 h[41]**.** For better knowledge, further research is needed to extend our knowledge of CLA isomer formation conditions and the glucose influence on isomers ratio.

Effect of carbonate calcium ($CaCO_3$) on CLA production

Results showing the effect of carbonate calcium were presented in (Table 3 & Fig. 5). Twenty-four hours incubation period of culture supplemented with

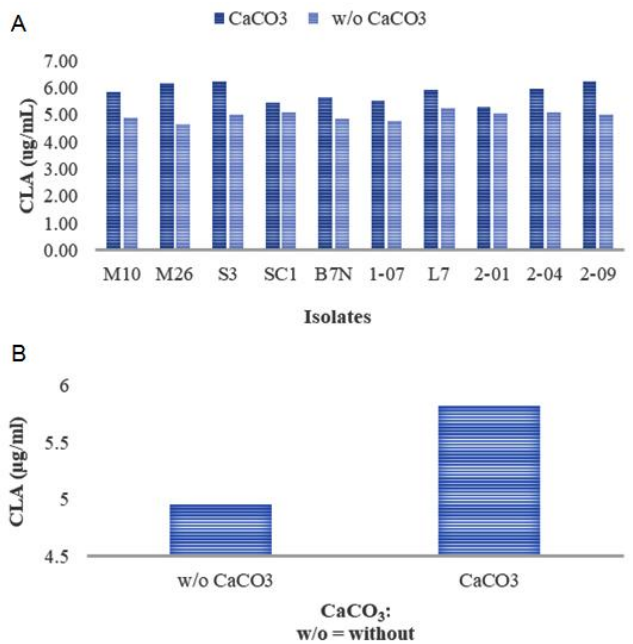

Fig. 5 — The effect of carbonate calcium ($CaCO_3$) on CLA production by isolates: Isolates individual performance (A) all bacteria global performance; and (B) in CLA production with or without $CaCO_3$

$CaCO_3$ raised the pH values which favors biohydrogenation pathway to form more CLA because when the medium pH is at pH 6, isolates have multiplied enough to support inhibition effect upon addition of LA[40,42]. For example, *Lactobacillus plantarum* GSI 303 produced more CLA (6.36 mg/g oil) at pH 6.5, 37 °C than at lower pH[17]. It was highligthed that activity of *Lactobacillus plantarum* GSI 303 could be more active at neutral pH than lower pH. So in medium optimized conditions, namely in pH above 5, LAB could be more active in producing higher CLA quantity[16].

Nevertheless, statistical analysis using SPSS *vs* 24 ANOVA's Tukey test did not show a significant difference between isolates individual performance to convert LA (0.1%) into CLA (P >0.05, Table 3.). However, when summing CLA quantity produced by all isolates upon $CaCO_3$ addition, there is a statistical difference (P <0.05, Fig. 5) allowing to state addition of $CaCO_3$ to the fermentation medium can improve CLA production. Figure 5A showed each isolate performance in CLA production with $CaCO_3$ supplemented and isolates global performance (Fig. 5B). The $CaCO_3$ supplementation to 24 h old inoculated culture was investigated in order to show whether it can mitigate LA inhibitory effect on bacteria growth. The results pointed out the more the

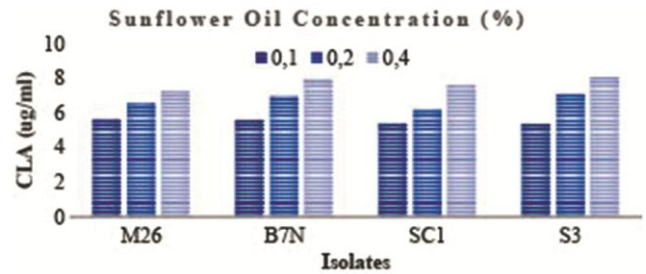

Fig. 6 — CLA production at sunflower oil different concentrations

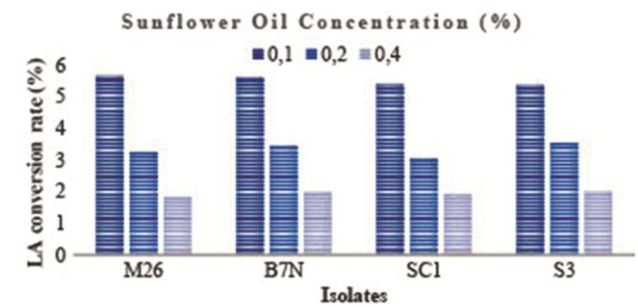

Fig. 7 — LA conversion rate at sunflower oil concentrations (0.1>0.2 >0.4%)

sunflower oil concentration increased, the better the CLA production was (Fig. 6). However, looking at the conversion rate in (Fig. 7), more the LA concentration went up, the more the rate downgraded, assuming some residual LA inhibition effect on the bacterial growth rate[43]. Therefore, $CaCO_3$ supplementation in a fermentation medium although improving the CLA production does not mitigate LA inhibitory effect on CLA biosynthesis and all bacteria do not respond equally to this supplementation.

Conclusion

Ability of lactic acid bacteria isolated from various matrices for CLA production using sunflower oil and effects of $CaCO_3$ on the production yield were examined. Ten *Limosilactobacillus fermentum* and *Enterococcus faecium* were found to produce more CLA, particularly *trans*-10, *cis*-12-*CLA* isomer accounted for at least 85% of total CLA ranging from 4.64 to 5.22 µg/mL. Fermentation medium supplementation with $CaCO_3$ improved CLA production but failed to mitigate the residual LA inhibitory effect on the biosynthesis process. This study, to our knowledge, is the first reporting *Limosilactobacillus fermentum* ability to predominantly convert the sunflower oil LA into *trans*-10, *cis*-12-CLA isomer. The findings provide a substructure for further investigations on the relevance of using these strains as probiotics and subsequently enriching, other than milk products, a staple food with *trans*-10, *cis*-12-CLA from a cost-effective substrate. Addition of $CaCO_3$ into fermentation to produce more CLA for human consumption could be an alternative to formulate a cost-effective functional food product. Further studies on given bacterial strain behavior in a wide range of LA concentrations are needed to establish a stable relationship between bacteria and LA inhibition on CLA following $CaCO_3$ addition.

Conflict of interest

All authors declare no conflict of interest.

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