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Production of Free Conjugated Linoleic Acid by Fermentation Performed Using *Lactobacillus casei* and *Bifidobacterium bifidum*

[1]*Rashad R. Al-Hindi and* [2]*Salem Abd El Ghani*

[1]Biological Sciences Department, Faculty of Science,
King Abdulaziz University, Jeddah, Saudi Arabia
[2]Dairy Science Department, National Research Centre, Dokki, Giza, Egypt

Abstract: This study investigated the potential factors affecting conjugated linoleic acid (CLA) production by 24 candidate probiotic Bifidobacterium and Lactobacillus strains. Strains were cultured in de Man, Rogosa and Sharpe (MRS) medium, MRS with cysteine hydrochloride, or skim milk with 1% hydrolyzed soybean oil (SO) at 37°C for 24h. Quantitative and qualitative analyses of CLA were performed by UV spectrometry and gas chromatography/mass spectrometry, respectively. Of the strains examined, 10 were CLA producers, with two strains each of *B. bifidum* and *L. casei* producing the highest amounts of CLA in skim milk, ranging from 80-90 and 125-153 µg CLA/g lipid, respectively. CLA isomers produced by the two species were qualified as C18:2 cis-9,trans-11 and C18:2 trans-10, cis-12, respectively. *B.bifidum* was characterized as a C18:3 conjugated linolenic acid producer. CLA production was significantly affected by 1.5%SO, while extending the fermentation time was detrimental. Characterization of the CLA-producing potential of these strains can be applied by the dairy industry to the production of new types of probiotic-containing foods for human consumption.

Key words: CLA Production · Probiotic Bacteria · UV Spectrophotometer · GC/MS

INTRODUCTION

Conjugated linoleic acids (CLAs) are a family of at least 28 isomers of linoleic acid (LA;octadecadieonic acid) mainly found in meat and dairy products derived from ruminant materials [1]. Chemically, CLA contains two conjugated double bonds with only one single bond between them [2, 3] and originates as a bio hydrogenation intermediate produced by rumen bacteria from linoleic acid in the forage to stearic acid and by the action of Δ desaturase enzyme on trans-11 vaccenic acid [4, 5]. Thus, the meat and milk of ruminants naturally contain CLA, especially in the lipid fraction [6]. CLA isomers have been identified as cis-cis, trans-trans, cis-trans and trans-cis isomers of 7,9; 8,10;9,11;10,12 and 11,13C18 diene acids. However, only five of these (9c, 11t-18:2; 10t, 12c-18:2; 9c, 11c-18:2; 9t, 11t-18:2; and 11c, 13t-18:2) are commercially available [7]. Notably, the principal isomer is cis-9, trans-11 (c9, t11 CLA; rumenic acid, RA), which constitutes about 80% of total CLA in food [8]. Other isomers such as trans-10, cis-12 (t10, c12 CLA) are also present, but in lesser amounts. RA and t10, c12 CLAs are reported as the most bioactive isomers with many health benefits for humans [9-11].

CLA isomers have anti carcinogenic, anti atherogenic, anti diabetic, anti-obesity and antioxidant activities and modulate bone formation, immune response and gene expression [12]. Most of these experiments have been performed in mice; however, studies have also shown these effects in humans, including a recent report on the protective effects of CLA against inflammatory bowel disease, also known as Crohn's disease [13].

A3.0 g/day intake of c9, t-11 CLA isomer has been recommended for a protective effect against cancer [14]. Approaches for increasing dietary intake of CLA isomers have been proposed. For example, cheese and yogurt are effective vehicles for CLA [15, 16]. Probiotic bacteria, including species of the genera Lactobacillus and Bifidobacterium were used in this regard [17, 18]. Other species of probiotic bacteria, such as *Lactococcus lactis*1-01,*Propionibacterium freudenreichii* subsp. *shermanii* and *P.freudenreichii* subsp.*freudenreichii*

Corresponding Author: Salem Abd El Ghani,Department of Dairy Science, National Research Centre, Elbohoth Str., Dokki, Giza, Egypt. Tel: +202 3337 1499, Fax +202 3337 1615, E-mail: ghani43@hotmail.com.

have been screened for their ability to produce high quantities of CLA [19, 20]. Interest in bifidobacteria and lactobacilli as CLA producers has burgeoned in the last two decades and its beneficial probiotic effects for humans have been highlighted [21, 22]. Unique strains of these probiotic bacteria that have a high capacity for synthesizing CLA and conjugated linolenic acid (CLnA) from LA and linolenic (LnA) fatty acids (respectively, have been characterized [23, 24].

In this study, 24 candidate probiotic bacterial strains of the Bifidobacterium and Lactobacillus isolated from traditionally fermented milk products in Saudi Arabia and Egypt were tested for their ability to synthesize CLA. Hydrolyzed soybean oil (SO; 1.0%) was a source of LA for bacteria cultured in de Man, Rogosa and Sharpe (MRS) medium, with or without cysteine hydrochloride or skim milk for fermentation at 37°C for 24 h. Owing to its simplicity and rapidity, a UV spectrophotometric method was used to screen for CLA-producing bacteria [24-26]. The optimum substrate concentration and incubation time were determined based on the quantity of CLA produced, using skim milk supplemented with 0.5%,1.5% and 2.0% SO as a source of LA and fermentation at 37°C for 24 and 48h. The molecular profiles of CLA isomers produced by each strain were obtained by gas chromatography-mass spectrometry (GC-MS). Bacterial growth was monitored as colony forming units (cfu) per ml and changes in pH at 0, 24 and 48 h fermentation at 37°C were evaluated. The aim of the study was to screen unique strains of lactic acid bacteria capable of production of bioactive compounds in fermented milk namely conjugated linoleic acid.

MATERIALS AND METHODS

Reagents, Media and Growth Conditions: SO (National Co. for Soy Products, Alexandria- Egypt),used as a source of LA and skim milk powder (Arla Foods, Visby J, Denmark) were purchased from a supermarket in Giza City, Egypt. MRS (Himedia, Mumbai, India) supplemented with 0.5 g/l cysteine hydrochloride(MRS-C medium) was used to propagate bifidus strains at 37°C in an anaerobic jar using the AnaeroGen AN0035 kits (Oxoid, Hampshire, UK), while lactobacilli were propagated in MRS medium at 37°C under microaerophilic conditions generated using the Campy Gen CN0035 kit (Oxoid). The strains were stored in MRS-C or MRS medium, respectively, containing 25% v/v glycerol as a cryo-protectant at-80°C. Prior to use, the strains were activated by transferring to the appropriate medium and incubating at 37°C for two 18-h periods. Standard CLA methyl ester (Sigma-Aldrich, St. Louis, MO, USA), hexane and all other solvents were of high performance liquid chromatography grade and potassium hydroxide (KOH) and hydrochloric acid were of analytical grade. Sodium sulfate (Sisco Research Lab., Mumbai, India was used to prepare hydrolyzed SO. Gum Arabic (Sigma-Aldrich) was used as an emulsifying agent to disperse SO in the media.

Bacterial Strains: This study evaluated 24 candidate probiotic strains, including eight and 16 strains, respectively, of Bifidobacterium and Lactobacillus. Of these, 18 strains were provided by the Biological Sciences Department, Faculty of Science at King Abdulaziz University (Jeddah, Saudi Arabia) where they were isolated and identified (data not shown); four strains were obtained from the culture collection of the Dairy Science Department of the National Research Centre (Giza, Egypt); and two strains were donated by the Dairy Science Department, Faculty of Agriculture at Cairo University (Giza, Egypt). Detailed descriptions of these strains are shown in (Table1).

Preparation of SO: Hydrolyzed SO used as an LA substrate in fermentation studies was prepared according to a published method [24] with minor modifications. Briefly, 40g SO were hydrolyzed with 6.6% ethanol in 120 ml of 3 N KOH at 70°C, stirred and maintained in a shaking incubator for 24 h. The mixture was then neutralized by adding 32 ml of 12 N hydrochloric acid and refluxing at 80°C for 3h in a temperature-controlled water bath. The mixture was cooled to 50°C and the upper layer was passed through sodium sulfate to recover the hydrolyzed SO. A gum Arabic aqueous solution (15%v/v) was prepared and added at 1:1 (v/v) to the substrate to facilitate its dispersion in MRS, MRC-C and skim milk. The mixture was autoclaved at 121°C for 15 min, cooled to room temperature (30°C) and stored until use in the fermentation experiments.

Determination of free FA Methyl Ester (FAME) of SO: Free FAs of SO were converted into FAMEs using a GC Auto System XL (Perkin Elmer, Waltham, MA, USA) equipped with a flame ionization detector (FID) according to a previously described method [27]. The conditions for the GC analysis were as follows: fused silica capillary column DB-6 (60m× 0.32 mm inner diameter, i.d.);initial oven temperature of 150°Cwas increased to 240°C at 3°C/min; and injector and detector temperatures at 230°C and 250°C, respectively. Helium was used as a carrier gas at a flow rate of 1 ml/min.

Table 1: Candidate CLA-producing probiotic bacterial strains cultured in MRS, MRS- C media or skim milk with hydrolyzed SO[a]

Strain	CLA production (mg/g lipid) MRS/MRS-C	Skim milk	Source of strains[c]
Bifidobacteriumanimalis1KAU	-[b]	-	Commercial set and stirred fermented milk (KAU)
B.bifidum 2KAU	++	+++	
B. breve 3KAU	++	++	
B. breve 4 KAU	-	-	
B. longum 5 KAU	-	-	
B. bifidum 1NRC (Christian Hansen, Denmark)	++	++	Goat milk (NRC)
B. longum 2NRC	-	-	
B. animalis Bb-12Cu	-	-	
Lactobacillusacidophilus 6KAU	++	++	Traditional soft cheese and raw camel milk (KAU)
L. acidophilus 7KAU	-	-	
L. brevis 8KAU	++	++	
L. casei 9KAU	+++	+++	
L. casei 10KAU	++	++	
L. casei 11KAU	-	-	
L. fermentum 12KAU	-	-	
L. helveticus 13KAU	-	-	
L. plantarum 14KAU	--	--	
L. plantarum 15KAU	+	+	
L. rhamnosus 16KAU	-	-	
L. reuteri 17KAU	-	-	
L. reuteri 18KAU	-	-	
L. acidophilus 3NRC	+	++	Traditional yogurt (NRC)
L. casei 4NRC	+++	+++	
L. casei 2CU	-	-	CU

[a]Cells were grown anaerobically in MRS, MRS-C,or skim milk with 1% hydrolyzed SO for 24h at 37°C.

[b]-, no CLA; +,= 2 mg CLA mg/g fat; ++, 2-5 mg CLA/g fat; +++,= 5 mg CLA/g fat.

[c]Sources:CU, Dept. of Dairy Science, Cairo University, Egypt; KAU, Dept. ofBiological Sciences, King Abdulaziz Univ., Saudi Arabia; NRC, Dept. of Dairy Science, National Research Center, Egypt.

CLA, conjugated linoleic acid; MRS, de Man, Rogosa and Sharpe medium; MRS-C, medium supplemented with 0.5 g/l cysteine hydrochloride; SO, soybean oil.

Screen for CLA-Producing Strains

Fermentation: Lactobacilli and bifidus strains were activated twice for 18h at 37°C in MRS or MRS-C medium, respectively, by inoculating 0.5ml of the active culture (about 10^7 cfu/ml)in 10 ml of medium containing 1% emulsified SO and incubating at 37°C for 24h. Similarly, tubes containing 10 ml homogenized skim milk (12% w/v) supplemented with 1%SO were inoculated with 0.5 ml of the active culture. All experiments were repeated thrice.

Lipid Extraction from Culture Supernatant: Lipid was extracted according to previously described methods for cultures grown in MRS [28, 29]. Cultures were centrifuged at 5000 rpmfor10 min at 4°C;3 ml of the supernatant was added to 6 ml isopropanol and vortexes for 1 min; 5ml hexane was added to the mixture and vortexes for 1 min and centrifuged at 2000 rpm for 5min at 4°C.

Measurement of CLA by UV Spectrophotometer: CLA in the hexane layer was quantified using a UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan) at 233 nm. Readings were obtained in triplicate and a standard curve was generated for the absorbance at 233 nm versus the CLA (C18.2 c9,t11) concentration (0-30 mg/ml) up to an absorbance of 230 nm [25].

Identification of CLA Isomers by GC/MS: CLA isomers produced by two strains each of *B. bifidum*(2KAU and1NRC) and *Lactobacillus casei* (9KAUand 4NRC) were identified. Briefly, 3 ml hexane extract from each culture was evaporated and dried on sodium sulfate; resultant FAMEs were directly trans methylated with sulphuric acid in methanol [30] and analyzed using a 6890N GC system equipped with an FID, HP5% phenyl methyl silixane capillary column (30m× 0.32 mm i.d. and 0.25 µm film thickness) and HP5973 mass selective

detector (Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas at a flow rate of 1.5 ml/min. The oven temperature was held at 70°C for 2 min, then increased to 230°C at 8°C/min and maintained at this temperature for 20 min. Injector and detector temperatures were 250°C and 280°C, respectively. FAMEs were identified by comparing the retention times with those of a standard FAME mixture (Sigma-Aldrich, purity > 99.0% by GC) using probability merge search software and the National Institute of Standards and Technology MS spectra search program.

Effect of Substrate Concentration and Incubation Time on CLA Production: CLA production by bacterial strains was measured by adding 0.5 ml of active cultures to tubes containing 10 ml skim milk supplemented with SO concentrations ranging from 0.5%-2.0% and incubation at 37°C for 24or 48 h. At the end of each incubation period,5 ml of the mixture was removed for lipid extraction and CLA production was determined using a spectrophotometer at 233nm. Skim milk tubes containing the bacterial culture without SO were the controls.

Bacterial Growth: The viable count (cfu/ml) was determined for incubation times of 0, 24 and 48 h at 37°C. Serial dilutions of fermented bifidus and *Lactobacillus* suspensions with 1.5%SOwere plated on MRS or MRS-C agar, respectively; plates were incubated aerobically at 37°C for 48h. Counts were reported as $\log_{10}$cfu/ml.

Measurement of pH: The pH of soy milk cultures was monitored using a digital pH meter (Hanna Instruments, Portugal).Readings were recorded for incubation times of 0, 24 and 48hat 37°C.

Statistical Analysis: The study designed as a three-way factorial experiment with screened bacterial isolates, lipid concentration and incubation time as the main factors was performed thrice. Analysis of variance was performed with Duncan's multiple range test using SAS version 8.2 (SAS Institute Inc., Cary, NC, USA).P> 0.05 was statistically significant.

RESULTS AND DISCUSSION

CLA Production by Candidate Probiotic Bacterial Strains: Totally, 24 bacterial isolates, widely used in the dairy industry, were examined for CLA production. Bifidobacterium and Lactobacillus spp. are recognized as safe and hence used in the processing of cheese, fermented milk, butter and sour cream. Moreover, certain bifidus and lactobacilli strains are known for their probiotic characteristics. Recent studies have focused on obtaining CLA-producing bacteria from different genera and sources. Bifidobacteria are known to generate CLA and CLnA from LA and LnA, respectively [17, 18, 23, 24, 31, 32]. Here, Lactobacillus produced CLA, is consistent with the findings of previous reports [19, 33]. However, bifidobacteria were shown to be CLA-producer a finding contrary studies reported by Salamon *et al*.[34]. This inconsistency is possibly because CLA production is strain-specific within a given genus and species.

Conjugated double bonds have an absorbance peak at 232-234 nm [26]. A standard curve of CLA UV absorption was generated and CLA production by candidate strains was calculated by the equation $R^2 = 0.9993$; $y = 0.0994x - 0.23$.

Of the 24 strains examined, only 10 (41.67%) were CLA producers. However, strains differed in their capacity to produce CLA in synthetic media and skim milk (Table 1). Notably, culturing in skim milk enhanced CLA production from SO in the various strains as compared to synthetic media with the same substrate concentration. Kim and Liu [19] and Xu *et al*. [20] reported a similar observation. Therefore, the 10 producer strains could be distributed into low (+), moderate (++) and high producer (+++) groups according to the quantity of CLA produced by the different strains. We selected four isolates for further studies based on their high productivity of CLA in skim milk: *B.bifidum* 2KAU, *B.bifidum* 1NRC, *Lactobacillus casei* 9KAU and *Lactobacilluscasei* 4NRC (Table 1). We quantified CLA produced by the isolates using UV spectrophotometer because the method has been reported to be inexpensive, accurate and quick for CLA analysis [24, 25].

Identification of FAMEs of SO: Using SO as a source of free LA in skim milk medium inoculated with probiotic bacteria is a new trend for developing fermented milk with a non- conventional food source that is more palatable, nutritive and less costly [35]. LA isomerase isolated from *B. fibrisolvens* isomerizes LA of hydrolyzed safflower oil into CLA [8]. Studies evaluating CLA synthesis by bacteria have used free LA [36]) or that derived from lipolyzed sesame oil [37]. Sunflower oil with 70% LA in esterifies form was nearly effective as free LA for CLA synthesis by *Lactococcus lactis* 1-01 [19].

Here, SO was used as a source of LA and LnA, both are CLA precursors and was hydrolyzed to liberate the FAs before being added to the media. The FAME profiles of the resultant SO (Table 2) contained 50.11% LA, 7.52% LNA and 3.76% stearic acids, which play a principal role

Table 2: FAME profiles of hydrolyzed SO used as a source of LA for fermentation

Peak	FA*	Area (%)**
1	Palmitoleic acid (C16.1)	2.12
2	Palmitic acid(C16.0)	17.92
3	Margaric acid(C17.0)	1.82
4	LA (C18.2)	50.11
5	Oleic acid(C18.1)	16.75
6	LnA(C18.3)	7.52
7	Stearic acid (C18.0)	3.76

*FAMEs were identified by gas chromatography-mass spectroscopy based on the total known FAs (retention time in min).

**The amount of the FA was evaluated by the peak area in the chromatograph.

FA, fatty acid; FAME, FA methyl ester; LA, linoleic acid; LnA, linolenic acid; SO, soybean oil.

Table 3: Total CLA content (µg/g lipid) of cultures grown in skim milk at different SO concentrations for 24 or 48 h at 37°C

Strains	Time (h)	Control	0.5%	1.0%	1.5%	2.0%
*B. bifidum**2KAU	24	ND	15.80^{bC}±0.30	80.25^{bD}±0.37	95.02^{A}±0.98	36.44^{aD}±0.38
	48	ND	27.24^{aC}±0.39	86.42^{aD}±0.40	92.63±0.82	28.41^{bD}±0.41
*B. bifidum*1NRC	24	ND	32.21^{aB}±1.13	92.30^{C}±1.02	102.32^{C}±1.66	65.88^{aC}±0.70
	48	ND	24.43^{bD}±0.40	94.62^{C}±1.10	101.42±0.40	59.32^{bC}±0.59
*L. casei***9KAU	24	ND	106.01^{bA}±0.61	153.75^{bA}±0.34	141.22^{A}±0.91	138.02^{A}±0.79
	48	ND	142.06^{aA}±1.15	184.24^{aA}±0.73	139.30±0.78	136.02^{A}±0.05
*L. casei*4NRC	24	ND	104.2^{bA}±1.55	125.31^{bB}±0.62	132.05^{bB}±0.61	124.33^{B}±2.23
	48	ND	135.82^{aB}±0.28	165.21^{aB}±0.57	136.61^{a}±0.28	122.54^{B}±0.38

Values are expressed as mean±SE (n = 3). Lower case and uppercase superscript letters denote non-significant values (P>0.05) within columns and rows, respectively. ND, no CLA was detected for any strain at any SO concentration.

**Bifidobacterium bifidum*; ***Lactobacillus casei*;CLA, conjugated linoleic acid; KAU = King Abdulaziz University, Saudi Arabia; NRC = National Research Center, Egypt; SO, soybean oil.

in microbial production of CLA. Other free FAs such as palmitic, oleic, palmitolic and margaric acids were also present at 17.92%, 16.75%, 2.12% and 1.82%.LA and LnA fatty acid content in SO were 50-11% and 7.52% of the total fatty acids, respectively, in agreement with other reports [38].

Effect of Concentration and Incubation Time on CLA Production: The four selected isolates (*B. bifidum* 2KAU and 1NRC and *Lactobacillus casei* 9KAU and 4NRC) were inoculated in skim milkcontaining 0%, 0.5%, 1.0%, 1.5% and 2.0% SO, incubated at 24 and 48h at 37°C. The controls showed that LA is required for CLA production by the tested *B. bifidum* and *Lactobacillus casei* strains. Neither LA nor LnA is sufficiently found in skim milk to initiate CLA synthesis activity, which was not observed even when these FAs were added exogenously to the medium. However, when 0.5% SO was added to the skim milk, *B.bifidum* 2KAU produced 15.80±0.30 and 27.24±0.39µg/ml CLA at 24 and 48h, respectively, representing an increase of 72.41% between the two incubation temperatures (Table 3). The same trend was observed for *B.bifidum* 1NRC. The amount of CLA produced by *Lactobacillus casei* 9KAU and4NRCwas markedly higher than that by the bifidus strains at 24 and 48h of incubation, consistent with other investigators such as Knapp & Melly [39] and Xu *et al*. [20] and with 1.0% SO,the same percentage increase was observed between the two time points for both bifidus and lactobacilli. At 1.5%SO, CLA production was higher than at 1.0% for both cultures of *B.bifidum*; this was also true for *Lactobacillus casei* 4NRC; but for *Lactobacillus casei* 9KAU, the amount of CLA produced varied inversely with SO concentration. The CLA yield was lowest at 2.0% SO for both strains of *B.bifidum*, while the decrease was less apparent for *Lactobacillus casei*. It is known that LnA has antibacterial effects at high concentrations [40, 41] and therefore the present findings may be explained by a similar bactericidal effect by LA on these strains, especially against *B. bifidum*, possibly due to variations in metabolic activities between strains.

CLA Isomers: *B.bifidum* 2KAU produced *c*-9, *t*-11 and *t*-10, *c*-12 CLA and *c*-9, *t*-11, *t*13 CLnA in quantities of 74.12, 15.20 and 4.75 µg/g lipid, respectively (Table 4). *B.bifidum* 1NRC yielded 82.88 and 23.77 µg/g lipid, respectively, of the two CLA isomers and 7.92 µg/g fat of the CLnA isomer. *Lactobacillus casei* 9KAU synthesized 115.80 and 21.18 µg/g lipid and *Lactobacillus casei* 4NRC produced 105.64 and 26.41 µg/g lipid of *c*9, *t*11 and *t*10, *c*12, respectively.

Table 4: Concentration of CLA isomers (μg/g lipid) produced in skim milk by four strains at 37°C for 24 h

Strains	cis-9, trans-11 CLA	trans-10,cis-12 CLA	cis-9,trans-11,trans-13 CLnA
*B.bifidum**2KAU	74.12†	15.20	4.75
B.bifidum 1NRC	82.88	23.77	7.92
*L. casei*** 9KAU	115.80	21.18	ND
L. casei 4NRC	105.64	26.41	ND

†Results represent the mean of three readings.

**Bifidobacterium bifidum*; ***Lactobacillus casei*; CLA, conjugated linoleic acid; CLnA, conjugated linolenic acid; KAU = King Abdulaziz University, Saudi Arabia; NRC = National Research Center, Egypt.

Table 5: Viable cell counts of selected strains in sterilized skim milk with 1.5% SO after fermentation or 24 and 48 h at 37°C

	Log_{10} (cfu/ml)†		
Strains	0 h	24 h	48 h
*B.bifidum** 2KAU	7.04[h]±0.03	7.52±0.47	7.43±1.20
B.bifidum 1NRC	7.10±05	7.37±0.20	7.25±0.32
*L. casei*** 9KAU	7.28±0.42	9.30±0.02	9.65±0.04
L. casei 4NRC	7.45±0.06	9.21±0.12	9.58±0.36

Results represent the mean±SE of three readings.

†Log_{10} colony-forming unit per ml culture.

**Bifidobacterium bifidum*; ***Lactobacillus casei*. KAU = King Abdulaziz University, Saudi Arabia; NRC = National Research Center, Egypt.

Table 6: Changes in culture pH of selected strains in sterilized skim milk with 1.5% SO after fermentation for 24 and 48 h at 37°C

	pH value		
Strains	0 h	24 h	48 h
*B.bifidum** 2KAU	5.88	5.87	5.80
*B.bifidum*1NRC	5.89	5.88	5.79
*L. casei*** 9KAU	5.77	5.25	4.25
*L. casei*4NRC	5.76	5.36	4.40

Results represent the mean of three readings.

Bifidobacterium bifidum*;*Lactobacillus casei*. KAU = King Abdulaziz University, Saudi Arabia; NRC = National Research Center, Egypt. SO, soybean oil.

Growth and Acid Production in Skim Milk: Changes in growth at 0, 24 and 48 h at 37°C were measured for the four selected isolates with the highest CLA production at 0.15% SO (Table 5). *B.bifidum* 2KAU and 1 NRC showed only slight increases in cell count at 24and 48 h over the initial value at 0h, consistent with previous reports [20].. Bifidobacteria, strict anaerobes, require bifidogenic growth factors, which explains their slow development in skim milk. However, total polyunsaturated FAs of hydrolyzed SO, which accounted for 76.32% of the CLA produced (Table 2), may have had a role owing to their bactericidal effects [40, 42]. A sharp increase in the *Lactobacillus casei* 9KAU counts and 4NRC cultures was observed after 24h, but the rate of increase slowed between 24 and 48h. Thus, *Lactobacillus casei* apparently tolerated environmental conditions in skim milk better than *B.bifidum* strains.

The pH of skim milk at 0 h was slightly less than that of fresh milk (6.4-6.8) and decreased slightly after 24 and 48h incubation at 37°Cfor *B.bifidum* 2KAU and1NRC (Table 6). This was expected due to the slow growth of bifidobacteria, as discussed above. However, similar to previous findings reported by [Xu *et al*., 20], the counts for *Lactobacillus casei* 4KAU and 4NRC increased sharply and continuously from 0 to 24 and up to 48h, evident by the decrease in pH due to the increased metabolite accumulation, mainly lactic acid.

CLAs have many health benefits as demonstrated by animal model studies, owing to anticarcinogenic, antiatherogenic, antidiabetic and antioxidant activities, as well as roles in promoting bone formation and modulating immune properties and gene expression (reviewed by Kumar *et al*.,12).

The CLA requirement for cancer prevention in humans, estimated from animal studies, is 3g/day [14]. This is considerably higher than the estimated average daily intake of only 0.1-1.5 g as reported by Mushtaq, Mangiapane & Hunter [3]. To address this, several strategies for increasing levels of dietary CLA have been investigated. To this end, dairy starter and adjunct bacteria used to produce fermented milk and cheese are promising; thus, altering the properties of dairy starter cultures or screening for probiotic bacteria of different genera for high CLA-producing strains has been attempted [17, 31].

Various species of lactic acid bacteria can synthesize CLA from LA in milk with or without adding substrates during processing [5]. However, it is highly recommended that a source of LA and LnA is included along with adjunct bacteria capable of converting these essential FAs to conjugated isomers during industrial processing of fermented milk. Different species of lactobacilli, such as *Lactobacillus acidophilus, Lactobacillu casei* and *Lactobacillus plantarum* have been documented as unique CLA producers [28, 39]. However, further research is needed to identify and exploit the properties of other probiotic bacteria capable of producing large amounts of CLA and CLnA isomers. Additionally, it is proposed to modify the definition of probiotic bacteria to recognize the ability of a given strain to produce bioactive compounds such as different antimicrobials, as well as PUSFA and di- and tripeptides, as a unique feature.

In this study, Bifidobacterium and Lactobacillus spp. were screened to identify those with the ability to synthesize CLA—an essential polyunsaturated FA—from hydrolyzed SO in skim milk and the effect of hydrolyzed SO concentration and incubation time on CLA production was evaluated. A unique strain of *B. bifidum* was selected that produced CLA and CLnA from LA and LnA, respectively, during fermentation of hydrolyzed SO. The isolation and identification of these and similar strains are important for the dairy industry to satisfy recent consumer interest in different probiotic-containing foods and ongoing studies are applying molecular methods to further characterize the biochemical properties of these isolates.

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