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Production of Conjugated Linoleic Acid from Safflower Oil as Precursor by Probiotic Cultures

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Abstract: Four potentially probiotic strains of *lactobacillus* and *Bifidobacterium* were evaluated for their ability to produce conjugated linoleic acid (CLA) from safflower oil in vitro. All the four strains were found to be capable of converting linoleic acid (LA) to CLA when using lipolysed safflower oil as a precursor for free linoleic acid. Production of CLA by four probiotics bacterial strains increases in presence 0.6% lipolysed safflower oil as maximum level for 48 h at 37°C and *Lb. plantartum* has higher CLA content in MRS broth media than *Lb. acidophilus*, *Lb. casei* and *B. lactis.* Also, Supplementation with amounts higher than 0.6% lipolysed safflower oil reduced the CLA content. The research on the ability of converting CLA of probiotics cultures could be basis for the future research and development of fermented dairy products.

Keywords: Conjugated linoleic acid, Safflower oil, Probiotic, *lactobacillus*, *Bifidobacterium.*

1. Introduction

CLA is an isomers group of more than 28 isomers of LA wherein the isomers *cis*-9, *trans*-11 (rumenic acid) and *trans*-10, *cis*-12 are the most abundant [1]. In recent years, the CLA has attracted more attention in food science and health due to the impact of the CLA-isomers was believed to have several biological activities [2]. Consumption of CLA by human can cause several health benefits such as antihypertensive effect, atherosclerosis, antioxidant activity, cancer preventing, immune response, lipid metabolism and promotes the body weight loss [3, 4 and 5]. Furthermore, consumption of a diet with dose CLA 3.2 g/day may enhance a decrease in body weight and fat [6]. *De Almeida et al.* [7] found that CLA enriched butter with *cis*-9, *trans*-11 isomer inhibited hyperinsulinemia and enhanced serum HDL cholesterol levels in rats. Furthermore *Davoodi et al.* [8] reviewed many researches through years ago which focused on the CLA, as a component of milk fat,

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showed some anti-carcinogenic effects against colorectal cancer [9], breast cancer [10 and 11], prostate cancer [12]. Recently, *Virsangbhai et al.* [13] reported that CLA is a potent fat-soluble antioxidant.

CLA formation intensity of strains, as in the case of the other metabolites, is influenced by external conditions as well as by genetic traits. Optimization of fermentation to achieve the highest yield and to maintain this level, as far as possible, until the product is consumed is an indispensable task when CLA-enriched fermented functional foods are to be produced. Several trials were done to increase the CLA concentration based on the ability of some probiotic strains such as Lactobacilli and Bifidobacteria to use free LA [14] with using 0.2% lipolyzed sesame oil as precursor and 0.4% lipolysed milk sunflower oil [15].

One of strategies to induce CLA concentration is using vegetable oils rich in monounsaturated fatty acids, especially LA (i.e., high oleic sunflower) in different dairy foods [16]. However, the adequate ratio for each used oils to stimulate CLA synthesis by various strains differed [17].

Safflower (*Carthamus tinctorius L.*) oil is colorless, flavorless, and rich in the essential *n*–6 (omega-6) fatty acid; approximately 78% LA in form c9c12-linoleate [18]. Safflower oil has shown many beneficial health effects decreased fat accumulation in rats when compared to beef tallow diet [19]. The presence of CLA in safflower oil has effectively shown to decrease body mass and adipose tissues as demonstrated in clinical trials [18]. Further safflower oil has been found effective in fat-induced insulin resistance [20]. Currently several applications of safflower oil in the food industry are presented owing to higher mono and polyunsaturated fatty acids. So, we can use safflower oil as a new precursor to production of CLA by probiotic cultures.

This study investigated the potential factors affecting CLA production by four candidate probiotic *Lactobacillus* and *Bifidobacterium* strains with lipolysed safflower oil as a precursor for CLA and their ability to convert lipolysed safflower oil to CLA and also, their tolerance to lipolysed safflower oil was evaluated by addition of different concentrations of lipolysed safflower oil in MRS broth.

2. Materials & Methods

2.1. Materials:

Probiotic strains (*Lb. plantarum* LpU4, *Lb. acidophilus* 200711A1/ CCFM6, *Lb. casei* CCFM137 and *B. lactis* NFM7) were supplemented by China Industrial Microbiology Culture Collection Center (CICC) and Culture Collection of the Laboratory of lipids Biotechnology, Wuxi, China. Safflower oil was obtained from Agricultural Science and Technology Co., China. Palatase®20000 L is a microbial lipase enzyme (food grade) derived from *Rhizomucor miehei* was obtained from Novozymes Co., China. All other chemicals used were of analytical grade.

2.2. Methods:

2.2.1. Preparation of lipolysed milk with safflower oil:

Lipolysed safflower oil was prepared according to *Abd El-Salam, et al.* [14]. Lipase powder (Palatase®20000 L from Novozymes Co., China.) was dissolved in a minimum quantity of distilled water, added to the safflower oil to give a lipase/oil ratio of 1:100 and stirred for 1 min at speed No. 6 by a stirrer (JANKE & KUNKEL IkA® - Labortechnik, ultra-turrax T50, Germany). The treated safflower oil was incubated at 40°C. The pH of lipolysed safflower oil was adjusted to 7.0 and sterilized before use.

2.2.2. Preparation of culture medium to screening cultures for the production of CLA:

Aliquot 0.02 ml of the lipolysed safflower oil was aseptically transferred to 9.98 ml of sterilized MRS broth to give 0.2% lipolysed oil in the medium. To study the effect of added lipolysis oil on the growth and activity of selected micro-organisms 0, 0.02, 0.04, and 0.06 and 0.08 ml of lipolysed safflower oil were added to sterilized MRS broth to give 0, 0.2, 0.4, 0.6 and 0.8 % lipolysed oil in the medium respectively and mixed well by vortex. The starter cultures were prepared twice by subculturing in MRS broth. After the second subculture, the medium was inoculated with 1 % (v/v) culture followed by incubation at 37°C in still condition. Culture without lipolysed safflower oil as a control. Growth was monitored by samples withdrawn at different time intervals for the analysis of CLA production. During this experiment, different treatments were analysed for the effect of lipolysed safflower oil on the bacterial growth was monitored by the viable cells plating count ($CFU.ml^{-1}$), O.D 600, the change in pH value of the medium and CLA concentration when 0, 4, 8, 12, 24, 48 and 72 h. of incubation at 37°C.

2.2.3. Preparation of probiotic strains:

Prior to the experiment, the cultures were sub-cultured at least three times in MRS broth inoculated with 2% and incubated for 18 h. at 37°C near the end of the logarithmic phase. Between subcultures, the cultures were maintained at 2°C. Each tested culture was incubated until stationary phase at the corresponding temperatures employed in the experiment after being inoculated at a density of 10^6 $CFU.ml^{-1}$.

2.2.4. Microbiological Analysis:

2.2.4.1. Enumeration of lactic acid bacteria and *Bifidobacterium*:

MRS agar media [21] was used for the enumeration of *lactobacillus* lactic acid bacteria and *Bifidobacterium*, after incubation at 37°C for 3 days under aerobic conditions.

2.2.5. Chemical analysis:

2.2.5.1. Determination of conjugated dienes:

To determine the conjugated dienes in the culture broth media (10 ml) were vortexed for 30 second with 20 ml of chloroform: methanol (2:1, v/v) and the homogenate was centrifuged at 4500

rpm for 5 min at 4°C. The separated organic phase (chloroform layer) was withdrawn and transferred to a tube by micropipettor, and then the chloroform layer passed through anhydrous sodium sulphate (Na_2SO_4) on Whatman filter paper Grade No. 1. The Whatman filter paper was rinsed with 3 ml of chloroform, and the extract (4.5 ml) was evaporated to dryness. The sample was mixed with 10 ml of hexane for further quantification. The determination of CLA was carried out by a UV spectrum analysis method described by *Rosson and Grund* [22]. The absorbance of the prepared extract was measured at 233 nm using a UV/VIS spectrophotometer (T80 UV/VIS spectrophotometer PG Instruments LTD., Felsted, Dunmow, UK) and 1 cm quartz cuvettes at room temperature. The standard CLA concentrations in hexane used for calibration and the absorbance was measured at 233 nm. The concentration of CLA in each sample was calculated, based on the standard curve.

2.2.6. pH value:

The pH values of the culture broth media were measured using a pH meter (Jenway, 3505, Jenway Ltd., Felsted, Dunmow, Essex, UK).

3. Results and Discussion:

3.1. Screening of tolerant probiotic's bacteria to lipolysed safflower oil and CLA-producing in MRS broth:

3.1.1. Bacterial growth in MRS broth:

Table (1) and Figure (1) show the inhibitory effect of lipolysed safflower oil on bacterial growth in MRS broth supplemented with increasing levels of lipolysed safflower oil for 72 h. at 37°C by determined the change of O.D 600 values of probiotic's cultures.

As the incubation time increased, a large increase in rate of O.D 600 values were observed for all the probiotic's bacteria in MRS broth, which gives an indication of the increasing of the bacterial count and their vitality. It is observed that a large increase in O.D 600 values of *Lb. acidophilus* and then *Lb. plantarum* and *B. lactis* next and the least *Lb. casei.*

Generally, bacterial growth and metabolism is not affected by low level of lipolysed safflower oil in the MRS broth media. But the bacterial growth inhibition at high lipolysed safflower oil doses, all probiotic's cultures growth observed decreased at 0.8% lipolysed safflower oil dose and it is noticed that the optimal level of lipolysed safflower oil for all probiotic's bacteria growth is 0.6%.

Salamon, et al. [15] concluded that, there can be an optimal LA intake for each bacterium (in our case in 100µl sunflower oil /100 ml milk), above which LA can act as growth inhibitor, reducing the amount of CLA, and there are bacterial strains that react with maximal production of CLA upon addition of optimal amount of LA, in fact, the CLA content can decrease even below the value of the starting milk.

Kim and Liu [23] reported that the CLA production of *Lactococcus lactis* I-01in milk depends on various factors, i.e. substrate (sunflower oil) concentricity, pH, incubation time and

culture conditions. When sunflower oil was added initially, growing cells produced more CLA than cells in the stationary phase; however, stationary cells were capable of producing more CLA when sunflower oil was added shortly before the end of the incubation period.

Table (1): Change of O.D 600 values of probiotic's cultures in MRS broth at increasing levels of lipolysed safflower oil for 72 h. at 37°C.

Strains		lipolysed safflower concentration%				
	Time (h)	Control	0.2%	0.4%	0.6%	0.8%
Lb. plantarum	**0**	0.124	0.128	0.133	0.134	0.14
	4	0.185	0.207	0.242	0.289	0.42
	8	0.521	0.577	0.711	0.868	0.811
	12	0.86	0.880	0.995	1.220	0.840
	24	0.955	0.911	1.022	1.115	0.613
	48	0.618	0.588	0.588	0.718	0.412
	72	0.388	0.401	0.415	0.521	0.333
Lb. acidophilus	**0**	0.225	0.277	0.289	0.296	0.315
	4	0.722	0.758	0.761	0.791	0.744
	8	0.932	0.941	0.945	0.977	0.811
	12	1.132	1.254	1.246	1.488	0.983
	24	1.233	1.377	1.345	1.340	1.085
	48	1.012	1.206	1.555	1.611	0.98
	72	0.833	0.870	0.966	1.781	0.810
Lb. casei	**0**	0.04	0.450	0.053	0.067	0.071
	4	0.062	0.720	0.061	0.119	0.099
	8	0.301	0.408	0.433	0.430	0.381
	12	0.512	0.644	0.802	0.911	0.714
	24	0.433	0.516	0.633	0.618	0.558
	48	0.318	0.418	0.459	0.406	0.278
	72	0.299	0.256	0.221	0.195	0.173
B. lactis	**0**	0.082	0.109	0.115	0.116	0.121
	4	0.108	0.147	0.277	0.188	0.163
	8	0.416	0.477	0.713	0.780	0.621
	12	0.866	0.911	0.898	1.011	0.588
	24	0.699	0.704	0.888	0.931	0.591
	48	0.633	0.698	0.736	0.761	0.256
	72	0.522	0.660	0.702	0.611	0.203

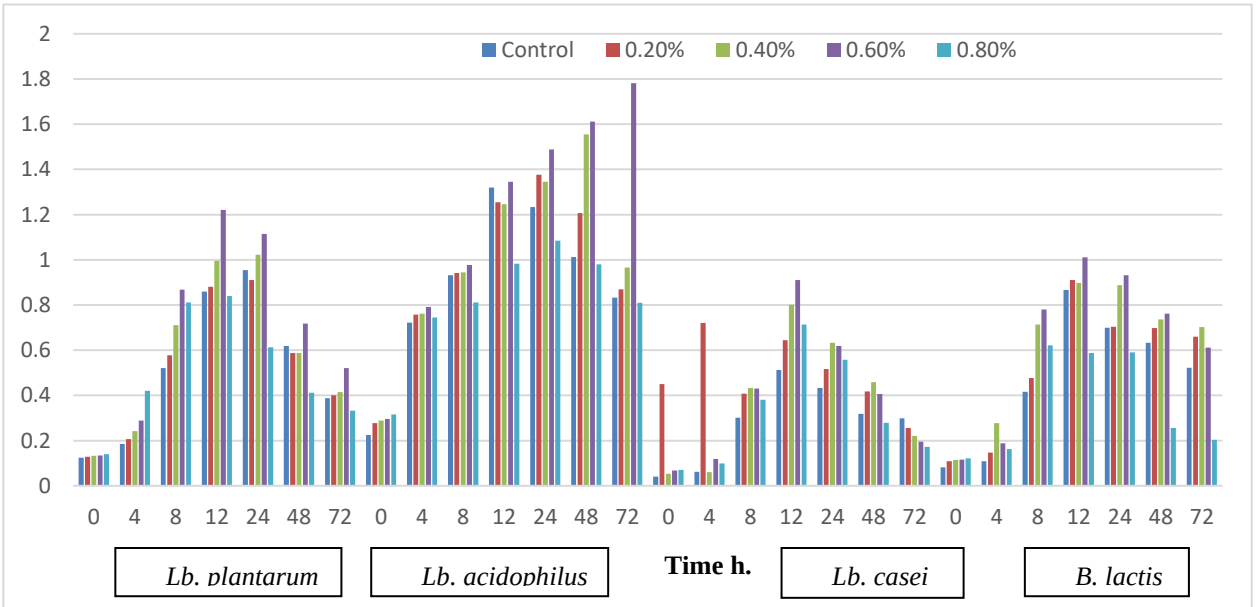


Fig (1): Change of O.D 600 values of probiotic's cultures in MRS broth at increasing levels of lipolysed safflower oil for 72 h. at 37°C.

3.1.2. Change of pH value of probiotic's cultures:

Table (2) shows the changes of pH values of probiotic's cultures grown in MRS broth at for 48 h. at 37°C. The changes of pH values affected by the growth of probiotic's activity in MRS broth during incubation. The attained data revealed that all pH values of different strains observed decreased with different rates as a result of further lactic acid formed through microbial fermentation process. Generally, all probiotic cultures (*Lb. plantarum, Lb. acidophilus, Lb. casei* and *B. lactis*) had observed lower pH values. This may be due to the post acidification by the growth of microorganism's activity. Similar findings were reported by [24 and 25].

Table (2): Change of pH value of probiotic's cultures grown in MRS broth (Control treatment) for 48 h. at 37°C.

Strains	Time (h)	Control
Lb. plantarum	**0**	6.57
	4	6.36
	8	5.78
	12	5.35
	24	5.19
	48	5.16
Lb. acidophilus	**0**	5.89
	4	5.33
	8	4.67
	12	4.17
	24	3.93
	48	3.77
Lb. casei	**0**	6.64
	4	6.62
	8	6.22
	12	5.47
	24	5.25
	48	5.18
B. lactis	**0**	6.35
	4	6.22
	8	5.57
	12	4.93
	24	4.74
	48	4.57

3.1.3. Bacterial growth and CLA production in MRS broth:

Table (3 & 4) and Figure (2) shows the Change of CLA content (mg/ml media) and TVBC (log CFU.ml^{-1}) of probiotic's cultures in MRS broth at increasing levels of lipolysed safflower oil for 48 h at 37°C.

It is observed that in all selected strains the higher percentage of CLA was determined at level 0.6% of lipolysed safflower oil and all strains showed the highest CLA production near stationary phase. Similar finding was reported by *Van Nieuwenhove et al.* [26], who studied the ability to produce CLA from free LA by strains of Lactobacilli, Bifidobacteria and Streptococci and found the most tolerant strain to LA was *Lb. casei*, and *Lb. rhamnosus* produced the maximum level of CLA at high LA concentrations (800 µg ml^{-1}).

Table (3): Change of probiotic's cultures counts (log CFU.ml^{-1}) in MRS broth (Control treatment) for 48 h. at 37°C.

Strains	Time (h)	Control
Lb. plantarum	**0**	4.4
	4	7.0
	8	7.9
	12	8.0
	24	7.6
	48	6.5
Lb. acidophilus	**0**	7.5
	4	8.5
	8	9.0
	12	9.3
	24	9.2
	48	9.2
Lb. casei	**0**	4.7
	4	6.1
	8	7.5
	12	7.6
	24	7.7
	48	9.2
B. lactis	**0**	6.6
	4	7.8
	8	8.3
	12	8.5
	24	8.5
	48	7.9

It is noted that, table (4 & 5) and Figure (3) shows *Lb. plantarum* had the higher CLA production at level 0.6% of lipolysed safflower oil followed by *Lb. acidophilus* while *Lb. casei* and *B. lactis* was the lowest CLA producer, varying CLA production level from 99.73 to 77.03 mg/ml media after incubation for 24 h. at 37°C, respectively. Similar findings were reported by *Song et al.* [27] who reported that *Lb. plantarum* increased CLA concentration than *Lb. acidophilus*. Also, *Kim and Liu* [23] reported that the *Lb. plantarum* strains had the strongest CLA conversion capability during logarithmic phase.

Also, the amount of CLA produced varied inversely with lipolysed safflower oil concentration. all probiotic's cultures observed decreased in CLA production at level 0.8% lipolysed safflower oil in MRS broth. It is known that LA has antibacterial effects at high concentrations [28 and 29].

Studies by *Jiang et al.* [30] found that the formation of CLA during growth by 45 Propionibacteria was related to the amount of LA in the medium up to 0.20%, between 0.20 and 0.60% the production remained almost constant. In another research 0.20% LA added to MRS broth resulted in production of more CLA after 24 h incubation time than in broth containing 0.4% by active strains of lactobacilli [31]. Thus, based on the observations found in this experiment and the literature review, it was suggested to add 0.20% LA to medium in future experiments with washed cells.

Table (4): Change of CLA content (mg/ml media) of probiotic's cultures in MRS broth at increasing levels of lipolysed safflower oil for 48 h at 37°C.

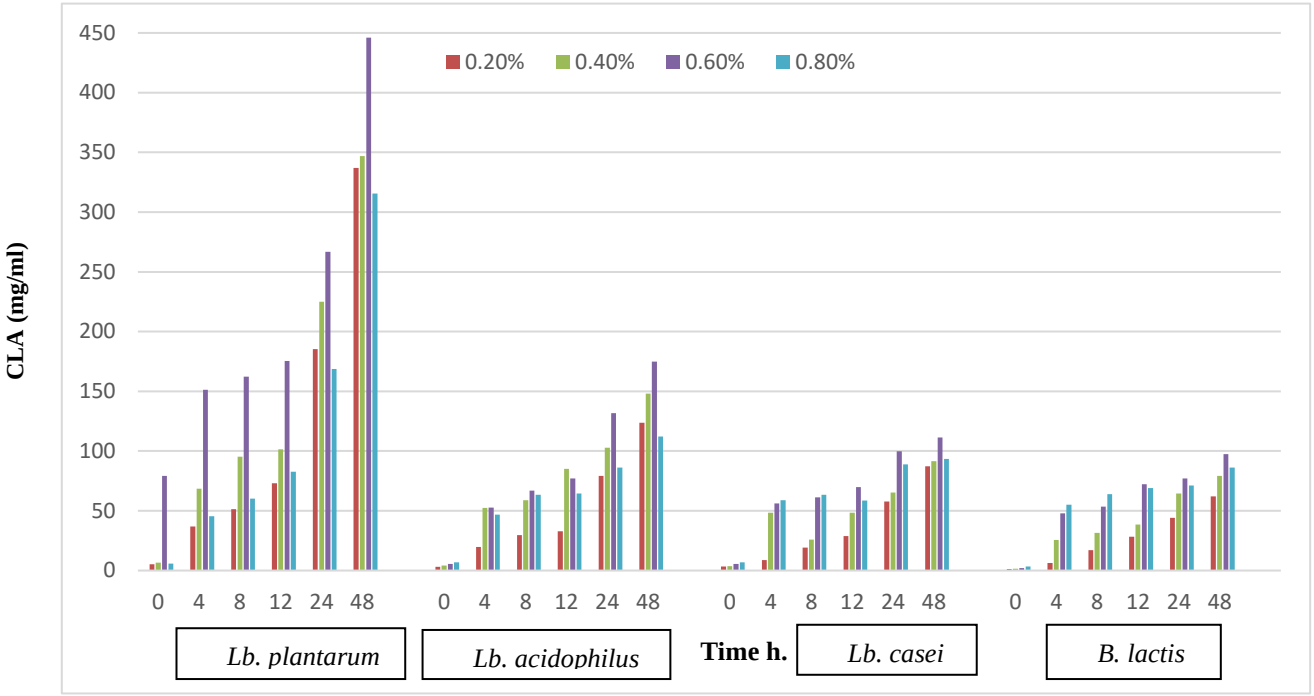
Strains	lipolysed safflower concentration%					
	Time (h)	**Control**	**0.2%**	**0.4%**	**0.6%**	**0.8%**
Lb. plantarum	**0**	0	5.23	6.73	79.2	5.8
	4	0	37.00	68.44	151.2	45.5
	8	0	51.22	95.34	162.4	60.2
	12	0	73.12	101.56	175.4	82.6
	24	0	185.33	224.86	266.7	168.7
	48	0	336.87	346.87	446.1	315.5
Lb. acidophilus	**0**	0	3.18	4.17	5.40	6.9
	4	0	19.7	52.34	52.60	46.7
	8	0	29.58	58.77	67.00	63.3
	12	0	32.76	85.11	77.00	64.5
	24	0	79.19	102.7	131.86	86.3
	48	0	123.58	148.2	174.86	112.2
Lb. casei	**0**	0	3.46	3.67	5.4	7.0
	4	0	8.66	48.33	56.3	58.8
	8	0	19.15	25.8	61.2	63.3
	12	0	28.77	48.3	69.8	58.6
	24	0	57.65	65.3	99.73	88.9
	48	0	87.34	91.6	111.32	93.4
B. lactis	**0**	0	1.23	1.41	2.1	3.4
	4	0	6.34	25.5	47.77	55.0
	8	0	17.00	31.5	53.56	64.0
	12	0	28.32	38.5	72.23	69.0
	24	0	44.12	64.4	77.03	71.3
	48	0	62.12	79.2	97.54	86.3

Several research groups have explored the capability of *Lactobacillus* strains to convert LA to CLA [31, 32 and 33]. *Alonso et al.* [31] tested two strains of *Lb. acidophilus* and two strains of *Lb. casei* for their ability to produce CLA. Each of these *Lactobacillus* strains converted 40 to 66 percent of LA present to CLA in the spent reaction medium.

Also, several research found similar trend for the obtained results by *Abd El-Salam et al.* [14] used different probiotics in reconstituted skim milk containing 0.2% lipolysed sesame oil on CLA production and found that *Lb. plantarum* had higher CLA value than that of *Lb. acidophilus* and *Lb. casei*.

Also, *Al-Hindi and Abd El Ghani* [34] evaluated the availability of several *Lb. casei* and *B. bifidium* in MRS media, MRS with skim milk with 1% hydrolysed soybean oil on the CLA concentration. It was found that the highest CLA was correlated by using skim milk, lipolysed oil and using *Lb. casei*. *Van Nieuwenhove, et al.* [26] reported that the CLA production at different LA levels was strain dependent on the power of biotransformation for these probiotics.

Fig (2): Change of CLA content (mg/ml media) of probiotic's cultures in MRS broth at increasing levels of lipolysed safflower oil for 48 h at 37°C



Throughout the course of these bioconversion reactions, the highest concentrations of CLA are obtained for the highest concentration of *Lb. plantarum* cells. The concentration of CLA produced appears to be linear in the concentration of *Lb. plantarum* cells followed by *Lb. acidophilus* and then *Lb. casei* and *B. lactis*. The same trend was noticed by *Crowley* [35] with *Lb. reuteri* strain. This variance and inconsistency are possibly because CLA production is strain-specific within a given genus and species. The reason why bacteria would convert LA to CLA is unclear. *Jiang et al.* [30] proposed that conversion of LA into CLA may be a detoxification mechanism to avoid growth inhibitory effect of fatty acid.

4. Conclusion

Based on these results, concluded that the environmental conditions that observed affect the maximal production of CLA are the presence of growth nutrients, the atmospheric conditions and addition of optimal amount of LA precursor. Production of CLA by four probiotics strains increases in presence 0.6% lipolysed safflower oil as maximum level for 48 h at 37°C. It was found that *Lb. plantarum* has higher CLA content than *Lb. acidophilus*, *Lb. casei* and *B. lactis*. Also, Supplementation with amounts higher than 0.6% lipolysed safflower oil reduced the CLA content for all four probiotics bacterial strains.

5. Competing Interests

The authors declare that they have no competing of interests in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this paper.

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