



Research Article

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Comparison of conjugated linoleic acid production in *Bifidobacterium* sp. and *Lactobacillus acidophilus* probiotic yoghurt

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ABSTRACT

The aim of this study was to assess the strains of probiotic bacteria in yoghurt for their ability to produce CLA from free linoleic acid. The effect of process variables was investigated on increasing of CLA in probiotic yoghurt. Two different set of microorganism consists of *L. acidophilus*, *B. lactis* and traditional yoghurt starters and in second group *L. acidophilus*, *B. breve* was studied as probiotics besides starter culture. Results showed that in both sets, the highest amount of CLA was obtained by addition of 2% non-fat dry milk, the addition of safflower oil (1.4 ml/l milk) in pH=6.0, incubation temperature of 37°C, and termination of incubation at pH=4.5. In the most suitable condition, the amount of CLA in probiotic yoghurt containing *B. lactis* increased by 450% from an average of 0.04 mg/ml in non-treated yoghurt to 0.22 mg/ml fat in the probiotic yoghurt containing safflower oil. But in the case of *B. breve*, the content of produced CLA was increased 10 fold from 0.04 to 0.4 mg/ml. Anyway, the viability of *L. breve* was less than *L. acidophilus* by 2 log CFU/ml. So for further investigation encapsulation of *B. breve* is suggested to reach to a product with the high amount of CLA and enough viable cells. No significant difference between product and control was observed in the ranking sensory test.

Keywords: Probiotic yoghurt, Conjugated linoleic acid, *L. acidophilus*, *B. lactis*, *B. breve*, Taguchi method

INTRODUCTION

Production of conjugated linoleic acid (CLA), as a unique fatty acid, in probiotic yoghurt has many health-promoting beneficial properties. CLA as a stereoisomer of linoleic acid (LA) is an omega-6 fatty acid naturally present in meat from ruminants, milk fat and dairy products such as yoghurt, butter, and cheese [18]. Cis9/trans11 isomer of CLA is an active and dominant isomer in milk (75- to 90%), and its importance was approved after the discovery of its anti-carcinogenic properties in laboratory animals [11, 24]. CLA possesses several health beneficial properties including antioxidant, anticancer, anti-hypertensive, anti-cholesterol and anti-diabetic activities, as well as adjusting the immune system.

Formation of CLA takes place in the livestock rumen during biohydrogenation by the intestinal bacteria. The content of CLA in dairy products can be also improved through biosynthesis by some bacteria including lactic acid bacteria (LAB) and bifidobacteria via converting LA. Microbial production of this fatty acid can be achieved also by applying LA isomerase in the presence of a source of LA. Different methods based on gas chromatography and spectrophotometric methods are reported for CLA detection [4,16].

Production of CLA has been formerly reported in different products e.g. Dahi [3], cheddar cheese [21], MRS media [12, 15], milk [27], non-fat probiotic yoghurt [22], and different culture conditions and species [1, 17, 19, 21, 25, 29].

Coakley et al. [2009] reported the inhibitory effect of conjugated α -LA produced by *Bifidobacterium (B.) breve* NCIMB 702258 on SW480 colon cancer cells [7].

Lee et al. [2007] investigated on the anti-obesity activity of trans-10, cis-12 CLA-producing *Lactobacillus* in mice [20]. Xu et al. [2008] estimated the biohydrogenation kinetics of *Lactobacillus (L.) acidophilus* on the production of CLA from LA [28]. They elucidated the pathway from LA to stearic acid, known as biohydrogenation. Although there are plenty of research about probiotic health beneficial properties e.g. toxin and heavy metal removal [29] and application in food enrichment [6], anyway, till now, there is no report about the evaluation of independent variables on CLA production in probiotic yoghurt.

In the present study, the effect of supplementation of safflower oil (as a rich source of LA) on CLA yield using a mixed culture of *Streptococcus (S.) thermophilus* and *Lactobacillus (L.) delbrueckii (d.) bulgaricus* as well as commercial ABY starter and probiotic of *L. acidophilus* and *B. lactis* was investigated. The impact of independent variables was determined separately by Taguchi design. Amount and time of incorporation of safflower oil, time, and temperature of incubation, and also the amount of added non-fat dry milk on the yield of CLA in probiotic yoghurt were studied, and the most suitable condition for CLA production was obtained. The next purpose of this study was to compare different sets of co-culture to determine their synergistic impact on CLA production in probiotic yoghurt.

EXPERIMENTAL SECTION

Sample Preparation

One litre of non-fat dry milk (10% dry matter) was prepared and pasteurised at 90°C for 30 minutes. After cooling a magnetic stirrer for 15 minutes to 35°C, 50 g of ABY- 1 starter was added to the milk and mixed by The starter culture was inoculated at 0.4% v/v to the milk, aseptically (recommended by Danish Christian Hansen Co). Profile of pH changes was obtained for all trials by sampling in 30 minutes interval. At this stage, 0.1% safflower oil was added to the inoculated milk. Following the incubation, for 1 and/or 2 h and achieving the right pH, the samples were cooled down in ice water and transferred to the refrigerator adjusted at 0°C to stop the bacterial activities and maintaining the pH at a constant value. Finally, the yield of CLA in the produced probiotic yoghurt was measured [21, 23].

Considering the experimental design, eight trials with three replicates were performed to determine the yield of CLA in each probiotic sample just after production and during one-week storage. The amounts of yoghurt fat, non-fat dry matter, pH, acidity (as lactic acid), probiotic count and sensory evaluation of the final product were determined in both probiotic sets. Extraction and purification of CLA were performed according to the method reported by Lin [21-23].

Microbial analysis

Two sets of inoculation were prepared

S. thermophilus, *L. d. bulgaricus*, *L. acidophilus* and *B. lactis*;

S. thermophilus, *L. d. bulgaricus*, *L. acidophilus* and *B. breve*.

MRS- bile agar medium was used for selective cell count of each of the two probiotic species. Incubation at 37°C for 72 h in aerobic condition resulted in the growth of *L. acidophilus*, while *B. lactis* grew in anaerobic condition [5,10].

Statistical methods and experimental design

Experimental design was done by applying Taguchi method as shown in Table 1. For eight variables in two levels, the design of experiments with eight trials and two replicates was performed. The data analysis was done using the Qualitek-4 software according to Taguchi method. Therefore, the effect of each variable and the best condition to get the maximum yield was achieved.

Data are expressed as means \pm SEM. Analyses were performed using the statistical software SPSS (version 13.0, SPSS). Differences between the means were evaluated using 1-way ANOVA, and differences among the treatment means were assessed using Tukey's test when variances were unequal. The results of sensorial evaluation were

analysed by a non-parametric Kolmogorov-Smirnov test using the SPSS software (ver. 19). Differences were considered significant at $P < 0.05$.

Table 1. An L8 Taguchi array for evaluation of effect of 5 process variables (in two levels) on CLA production in probiotic yoghurt

Trial Number	Skim milk (%w/w)	Time of oil addition	Temperature (°C)	Added oil (%v/v)	End of incubation at pH
1	2	At first	37	0	4.5
2	2	At first	40	1.4	5
3	2	At pH 6	37	1.4	5
4	2	At pH 6	40	0	4.5
5	4	At first	37	0	5
6	4	At first	40	1.4	4.5
7	4	At pH 6	37	1.4	4.5
8	4	At pH 6	40	0	5

Sensory evaluation

Sensory evaluation was conducted by 9 trained panelists to compare probiotic yoghurt with and without CLA by ranking test. The samples and control were presented separately to panelists, then they were asked to rank quality parameters e.g. off-flavor, saltiness, sourness, oral viscosity, mouth feel and texture smoothness, as well as opacity, aroma intensity stringiness, and overall acceptance [14, 16].

RESULTS AND DISCUSSION

The results of this study showed that by incorporation of non-fat dry milk to the raw milk, CLA yield may be increased. The addition of 2% v/v non-fat dry milk results in a significant increase of CLA (Figure 1a) due to the role of proton donation of proteins in non-fat dry milk during the oxidation mechanism and formation of free LA [22, 23]. However, the addition of non-fat dry milk up to 4% v/v did not cause any more significant increase in the CLA yield. Figure 1b shows that incubation temperature of 37°C results in more increase in CLA yield in comparison to 40°C. During the production process of probiotic yoghurt, increasing the incubation temperature is more favorable for the growth of *L. d. bulgaricus* as a strong acid producer. Thus, accumulation of lactic acid has adverse effects on CLA yield.

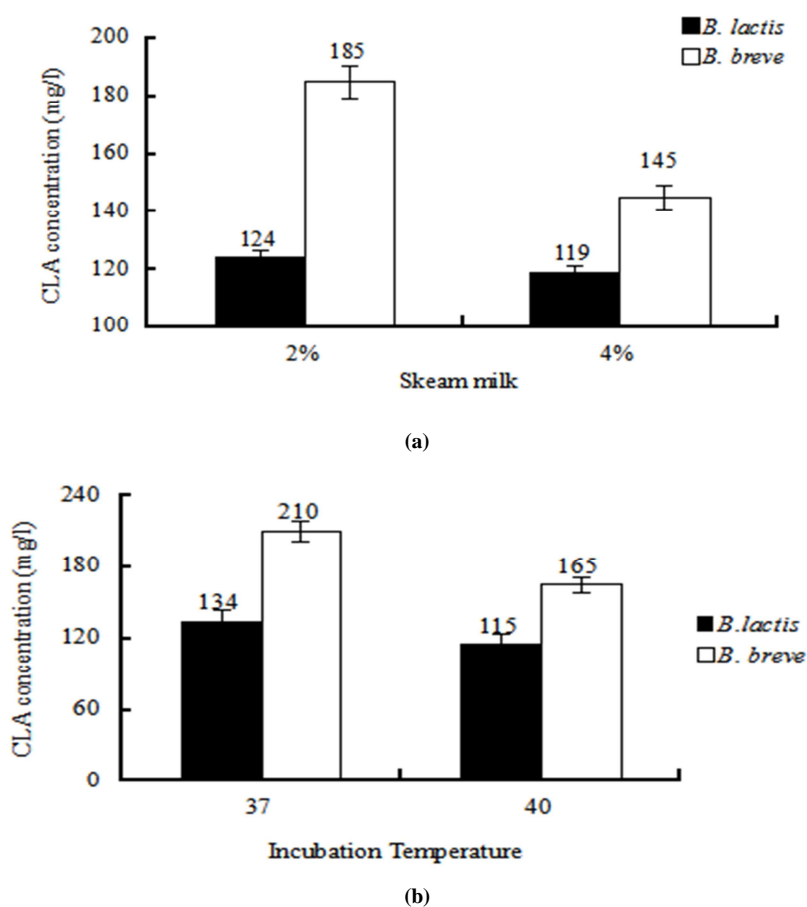


Figure 1. The effect of (a) skim milk incorporation, (b) time of Safflower oil addition, on CLA production in probiotic yoghurt

The addition of 0.14% safflower oil as a rich source of LA leads to more production of CLA. Suitable time for oil addition was obtained when pH reached to 6 and resulted in a relatively higher yield of CLA (Figures 2a and 2b). Other investigators have also reported a correlation between the amount of available LA and CLA yield [12].

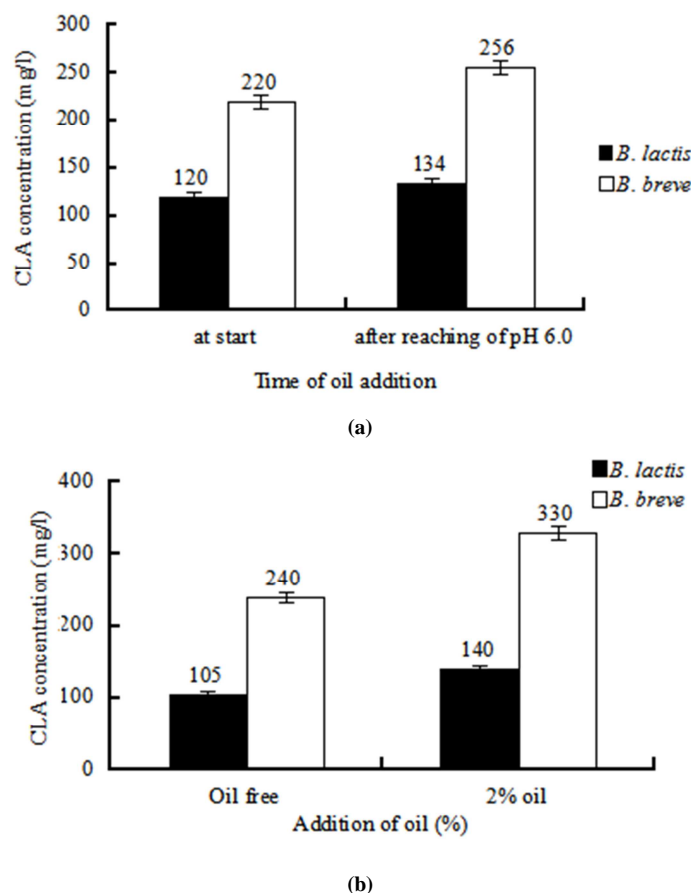


Figure 2. The effect of (a) incubation temperature, (b) amount of safflower oil addition on CLA production in probiotic yoghurt

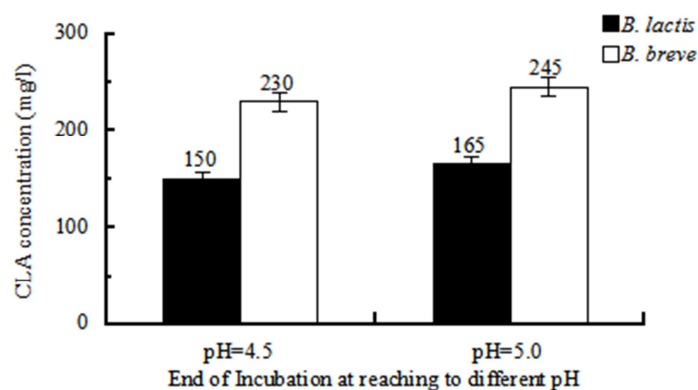


FIGURE 3. The effect of incubation time on CLA production in probiotic yoghurt

It was found that the best time for termination of incubation is at pH 4.5 in which probiotic bacteria are survived, suitable flavour and aroma are maintained in yoghurt and undesirable effects of lactic acid produced by *L. d. bulgaricus* are inhibited (Figure 3a). These results are in agreement with those reported by Kim and Liu [2002]. Moreover, application of safflower oil and mixed cultures of probiotic bacteria *L. acidophilus* and *B. lactis* along with traditional yoghurt starters *S. thermophilus* and *L. d. bulgaricus* caused an increase in CLA yield. Organoleptic evaluation of probiotic yoghurt containing safflower oil versus blank probiotic yoghurt showed no significant difference in total acceptance. Therefore, the addition of 0.14% safflower oil had no undesirable effect on the organoleptic properties of yoghurt (Table 2).

TABLE 2. Ranking test among control and probiotic yoghurt[†] containing CLA [‡]

Sample	Sensory parameters							Total acceptance
	Smoothness	Viscosity	Aroma	Flavor& off-flavor	Sourness	Saltiness	Mouthfeel	
CLA, control	NS [†]	NS	Control> CLA	CLA> control	Control> CLA	NS [†]	CLA> control	NS [†]

[†] Condition of production: 0.2% v/v non-fat dry milk, incubation at 37°C, the addition of 1.4 ml/l safflower oil at pH 6, and termination of incubation at pH 4.5. The amount of CLA in probiotic yoghurt containing *B. breve* and control was 11.4×10^{-3} and 7.1×10^{-3} % of total fat, respectively

[‡] The sensory evaluation was conducted after 1 h cold storage

* Not significant ($p < 0.05$)

According to the research results, the best conditions to get maximum yield of CLA in yoghurt production are the addition of 0.2% v/v non-fat dry milk, and incubation at 37°C, the addition of 1.4 ml/l safflower oil at pH 6, and termination of incubation at pH 4.5. The CLA content in the extracted fat from 2.5 ml of yoghurt (often dissolving in 5 ml of solvent) was increased. In fact, the maximum amount of CLA in produced probiotic yoghurt containing *B. lactis*, *B. breve*, and blank yoghurt was 9.23×10^{-3} , 11.44×10^{-3} and 7.07×10^{-3} % of total fat, respectively.

The growth curve (Figure 4) of two set of probiotics and their ability to produce CLA was obtained over time (Figure 5). The maximum cell count was achieved at 28 h for both inocula, and maximum conversion of sunflower oil to CLA was at 24 and 28 h, for *B. lactis* and *B. breve*, respectively (Figures 4 and 5). Production of CLA was growth-associated and at the late stationary phase of bacterial growth.

The concentration of CLA increased following about 24 h incubation with a corresponding decrease in cell viability (Figure 1). It seems that, in the late stationary phase, oil was further converted to CLA. The reason of LA conversion to CLA is unclear, but it has been suggested that such isomerization reaction may function as a detoxification mechanism in bacteria [15].

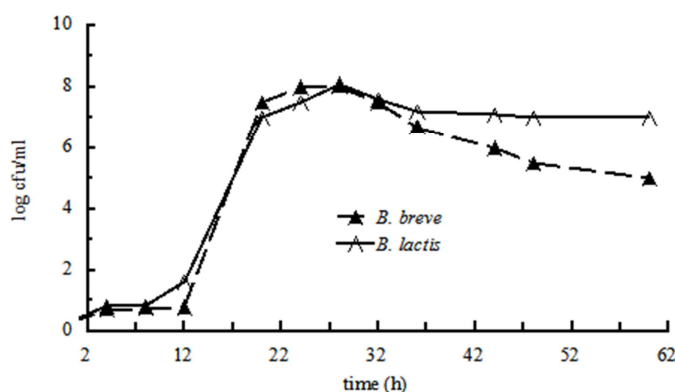


Figure 4. The growth of two sets of probiotic in yoghurt over time in the presence of 4% skim milk 1.4% v/v sunflower oil added at pH=6 and 37°C

Effect of non-fat dry milk

Addition of 2% non-fat dry milk caused an increase in CLA yield; however, by adding 4% non-fat dry milk, no significant change in CLA yield was observed. Non-fat dry milk acts as a source of hydrogen donor and isomerization will increase at the first stage of bio-hydrogenation. So LA will be converted to CLA [27].

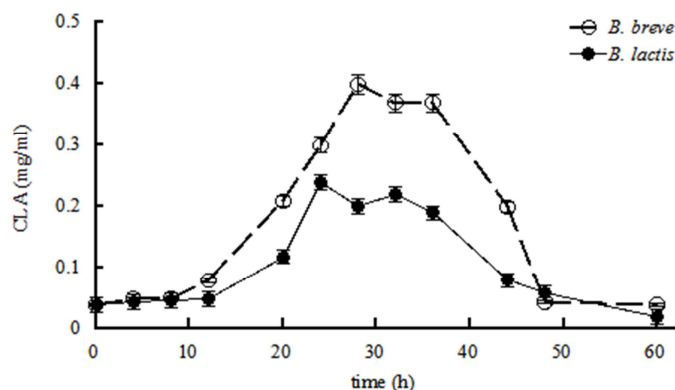


Figure 5. Conjugated linoleic acid (CLA) production of probiotic in yoghurt over time in the presence of 4% skim milk 1.4% v/v sunflower oil added at pH=6 and 37°C

Effect of time of safflower oil addition

The results of this study revealed that addition of safflower oil was suitable for CLA production after a pH of 6 was reached (Figure 2a). It is to be noted that growth of bacteria in yoghurt container is a kind of batch culture, and production organic acid is related to the growth of bacterial cells. Yoghurt acidity has a reverse linear correlation in the normal range of milk pH down to 4.5. Tables 3 demonstrate the pH change in the yoghurt samples containing 2 and 4% (w/w) of non-fat dry milk during two different incubation temperatures. This result indicates no significant difference between yoghurt containing different amount of non-fat dry milk in both temperatures ($P>0.05$). It seems that kinetics of lowering pH is proportional to kinetic of bacterial growth [5]. On the other hand, bio-hydrogenation process needs energy, which is less available in old cells [17]. According to the results of this study and the pH variations in probiotic yoghurt, the amount of produced CLA was higher at the final stage of the logarithmic growth phase. Kim and Liu [2002] reported that addition of sunflower oil to 2.5% fat milk, which was fermented by lactic starters such as traditional yoghurt starter 10-60 minutes prior to termination of incubation, resulted in an increase of CLA yield in these products. They also showed that the highest CLA was produced during the bacterial logarithmic growth phase.

Effect of incubation temperature

Formation of CLA in the produced probiotic yoghurt was higher when the incubation temperature was 37°C as compared to 40°C. By increasing the incubation temperature, *L. d. bulgaricus* had a better condition for growth, so its higher activity and acid production led to the suppression of probiotic bacteria in the mixed yoghurt starter. Kim and Liu [2002] also reported a decreased CLA yield due to acid formation and lowering of pH to 4.6.

TABLE 3. pH changes in the yoghurt samples containing 2 and 4% (w/w) of non-fat dry milk during two different incubation temperatures

Incubation Time (h)	pH in 37°C	pH in 40°C	Incubation Time (h)	pH in 37°C	pH in 40°C
2% (w/w) of non-fat dry milk		4% (w/w) of non-fat dry milk			
0	6.60	6.66	0	6.65	6.58
30	6.49	6.44	30	6.47	6.42
60	6.37	6.28	60	6.34	6.20
90	6.19	6.14	90	6.20	6.14
120	5.88	5.76	120	6.02	6.00
150	5.70	5.66	150	5.74	5.63
180	5.33	5.20	180	5.36	5.30
210	5.06	5.00	210	5.06	5.03
240	4.83	4.78	240	4.87	4.80
270	4.61	4.58	270	4.62	4.52

Effect of safflower oil addition

The addition of 0.14% v/v safflower oil increased the CLA yield (Figure 2b). Lin et al. also reported that by the addition of 0.1% v/v of LA to the milk used for yoghurt production, the CLA yield increased from 0.71 to 2095 mg/g of non-fat yoghurt [22], [23]. Aghajani [2012] also showed that addition of 0.1% v/v of sunflower oil to the milk used for the production of plain yoghurt by common yoghurt starter resulted in an increase in CLA yield from 4.5 to 7.0 mg/g [2].

Effect of termination time of incubation

As mentioned before, Figure 3a shows that termination of incubation at pH 4.5 resulted in more accumulation of CLA in probiotic yoghurt. At this stage, probiotic bacteria are survived and favourable taste and aroma are formed in the yoghurt while the distractive effect of high lactic acid produced by *L. d. bulgaricus* is prevented. Kim and Liu [2002] also reported that increasing the amount of lactic acid in the fermented 2.5% milk by lactic starters caused reduction of pH and CLA yield [17].

Aneja & Murthi [1990] studied the production of CLA in a fermented product called Dahi and found a significant increase in CLA content from 5.5 to 26.5 mg/g in the fat [3]. Lin et al [1995] showed that incorporation of non-fat dry milk accelerates the isomerization reaction in the first step of bio-hydrogenation and results in the conversion of LA to CLA [21]. Also increasing the temperature from 70°C to 85°C caused higher yield of CLA production in cheddar cheese. Jiang & Fonden [1998] studied the production of CLA by various lactobacillus bacteria in MRS media in which 25 µg/mL of CLA was added [15]. Kim and Liu [2002] studied the production of CLA in milk fat by 14 species of LAB [17]. They reported that CLA formation was related to the enzymatic conversion of LA; however, it was interrupted after bacterial growth in milk and reduction of pH due to inactivation of *isomerase*. Shantha, et al. [1992] investigated the parameters effective on the formation of CLA in milk products [27]. They reported that incorporation of whey proteins and non-fat dry milk increases the content of this fatty acid in cheese and non-fat yoghurt. Lin et al. [2003] also investigated the influence of LA and oligosaccharides addition on the CLA yield in non-fat probiotic yoghurt containing *L. acidophilus*. The results showed that CLA yield increased from 0.71 to 2.95 µg/g of non-fat yoghurt containing 0.1% of LA. Gorissen, et al. [2010] conducted screening of 36 different *Bifidobacterium* strains for the ability of biotransformation of free LA (0.5 mg ml⁻¹) in MRS broth [12]. Strains belonging to *Bifidobacterium* Sp. showed different yields of CLA production varying from 19.5 to 53.5%. The CLA isomers produced were further identified with Ag⁺-HPLC. LA was mainly converted into t9t11-CLA and c9t11-CLA. A rapid method for identifying bacteria, which convert free LA to CLA based on spectrophotometric detection of CLA, has been reported [4], which can facilitate high-throughput screening of bacterial isolates. Kishino [2002] selected *L. plantarum* AKU 1009a as a potential strain for CLA production from LA [19]. Under the best conditions of fermentation, 33% molar yield (12% w/v) was achieved in 108 h. The produced CLA was about half of the total FA, and contains a mixture of two CLA isomers, cis-9, trans-11 (or trans-9, cis-11)-octadecadienoic acid and trans-9, trans-11-octadecadienoic acid. Coakley et al. [2009] reported the inhibitory effect of conjugated α-LA produced by *B. breve* NCIMB 702258 on SW480 colon cancer cells. The cells were cultured in the presence of the extracted fermented oil (10–50 µg/ml) for 5 days. The results showed a four fifth reduction of cells at a concentration of 180 µM, (P≤0.001), compared with a reduction of the only half with α-LA (P≤0.01). Abd El-Salam et al. [2010] conducted a screening experiment for strains of potentially probiotic *Lactobacillus*, *Propionibacteria*, *Leuconostoc*, *Lactococcus*, *Enterococcus*, and *Pediococcus* to examine their ability to convert LA to CLA in medium containing 0.0–1% lipolysis oil [1]. *Leuconostoc mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis biovar deacetylates* gave maximum dienes in a medium containing 0.6 and 0.8% lipolysis oil, respectively. Lee et al. [2007] showed that trans-10, cis-12-CLA-producing *Lactobacillus* can be applied for obesity treatment in mice [20]. Park et al. [2009] optimised culture conditions to improve CLA production by *B. breve* LMC 520 [26]. A maximal yield of CLA (up to 90% conversion) was obtained after 24 h incubation in culture medium containing 1 mM LA at pH 5.5 under anaerobic condition. Ogawa et al. [2005] showed that castor oil, as a rich source of ricinoleic acid acts as a substrate for CLA production by LAB [19]. Xu et al. [2008] estimated the bio-hydrogenation kinetics of *L. acidophilus* on the production of CLA from LA and described pathway of bio-hydrogenation [28]. Till now, there is no report about the evaluation of independent variables on CLA production in probiotic yoghurt.

The amount of LA available for CLA production in the colon depends on ingested amount and efficacy of absorption in the small intestine, but usually reports indicated that an amount of ~20 mg LA per day may be excreted in human gastrointestinal tract [9]. It suggests that following by probiotics colonization in human colon, the substrate is available for microbial production of CLA.

CONCLUSION

The results of the present study demonstrated that use of a rich source of LA such as safflower oil, which contains more than 77% LA, in the presence of yoghurt LAB starters along with two probiotic bacteria (e.g. *L. acidophilus* and *B. lactis* or *B. breve*) increased CLA yield in the produced probiotic yoghurt. Between two sets of microorganism, inoculation containing *B. breve*, causes increased production yield in compare to *B. lactis*, while the survival of probiotics reduces 2 log phases.

It was also concluded that the best conditions for promoting the amount of CLA in probiotic yoghurt are the addition of 2% v/v non-fat dry milk, incubation at 37°C, the addition of 1.4×10⁻³% v/v of safflower oil at pH 6, and termination of incubation when pH 4.5 is obtained.

The amount of CLA in probiotic yoghurt containing *B. lactis* increased by 450% (from 0.04 to 0.22 mg/ml), but in the case of *B. breve*, the content of produced CLA was increased 10 fold from 0.04 to 0.4 mg/ml.

Sensory evaluation of probiotic yoghurt containing safflower oil showed no significant difference as compared to the blank probiotic yoghurt. These results provide a novel opportunity to develop foods with anti-obesity activity.

By consideration of the numerous health benefits of CLA, it can be investigated whether the anti-carcinogenic properties of some probiotics are linked to their ability of CLA production. Also, because of the many beneficial health effects of CLA, the ability of new mutant of *Lactobacillus* and *Bifidobacterium*. To synthesise CLA can be considered as a novel probiotic trait. Furthermore, the encapsulation of bifidobacteria in any edible film or capsules is recommended to increase cell viability. The ability to synthesise CLA may offer novel opportunities for the development of health-promoting functional foods, with the benefits of enriched CLA and probiotic bacteria. Also, use of different extracts as an antioxidant in dairy beverages is recommended to enrich with conjugated linoleic acid as well as masking any undesirable taste of some probiotic dairy products.

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