Bioproduction of Conjugated Linoleic Acid in Yogurt by Probiotic Bacteria

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Abstract: Conjugated linoleic acid as unique fatty acid of milk fat has beneficial properties including antioxidant, anticarcinogen, antidiabetic, antiblood pressure, stimulating the body immune system and reducing cholesterol. The aim of this study was assessing ability of some probiotics for biotransformation of linoleic acid to conjugated linoleic acid. Effect of process variables were investigated on production of conjugated linoleic acid in probiotic yogurt. An 8 run Plackett-Burman design was used to study the effect of 7 variables included: addition of supplements (whey powder, the amount of added grape seed oil), fermentation (temperature, pH, incubation time) and inoculum condition (age and size) on biomass and conjugated linoleic acid production in probiotic yogurt containing strains of *Lactobacillus acidophilus* La5, *Bifidobacterium bifidum* and *Propionibacterium freudenerchii* grown in medium containing free linoleic acid. The highest amount of conjugated linoleic acid was obtained by addition of 8% w/v whey powder, addition of 4%v/v grape seed oil in pH=6.0, inoculation of 0.7%v/v Inoculum of 36 h age and fermentation at temperature of 35°C for 27 h. This research showed that at the optimized conditions, the amount of conjugated linoleic acid in probiotic yogurt was increased by 40% from an average of 8.01 mg/g fat in non-treated yogurt to 11.03 mg/g fat of probiotic yogurt containing grape seed oil.

Keywords: Probiotic yogurt, Conjugated linoleic acid, *Lactobacillus acidophilus* La5, *Bifidobacterium bifidum*, *Propionibacterium freudenerchii*, Plackett-Burman Design.

1. INTRODUCTION

Conjugated linoleic acid (CLA) is a geometrical isomer of linoleic acid which can be found naturally in meat and milk fat [1]. Cis-9 transe-11 isomer of CLA is the most biologically active and dominant isomer in milk fat which include 75-90% of the total CLA isomers in milk fat [2]. Studies on the effect of omega-6 fatty acids on human health started in 1998. Numerous health-promoting properties associated with particular isomers of CLA are reported [3, 4]. This unique fatty acid shows numerous health benefits, including antiatherogenic [5, 6], antidiabetic [3], anti-inflammatory, anticarcinogenic and antiobesity [7, 8] properties antioxidant, adjusting the body immune system [9], anti-hyper blood pressure, cholesterol reducing, and reduction of body fat [10, 11]. These anticarcinogenic effects of CLA e.g. mammary [12] and prostate tumors [13], as well as inhibitor of skin papillomas [4], colon aberrant crypt foci [14], and metastasis of human breast cancer cells in animal models [15] are attributed either to mixtures of CLA isomers or to the pure c9, t11 isomer [16]. Kinetics of biohydrogenation in *Lactobacillus acidophilus* for production of CLA has been also studied [17].

Microbial production of CLA can be achieved by intestinal bacteria. Fermentative production of CLA in milk was firstly reported in 1989 and 1990 [18, 19]. Then several research was conducted on the impact of some process variables on CLA production including incorporation of non- fat dry milk, temperature [20], presence of LA in different media [21], reaching to acidic pH [22] whey incorporation, LA and oligosaccharides addition [23], time and microorganism e.g. probiotic strain of Lactobacilli, Bifidobacteria, Propionibacteria, Leuconostoc, Lactococcus, Enterococcus, and Pediococcus [24-28]. Also effect of process variables e.g. air, additives, and pH, addition of prebiotics [29] and castor oil, (as a rich source of in the triacylglycerol form of ricinoleic acid) [30]. Rapid detection of CLA producing bacteria was reported by spectrophotometric [31].

Our previous study showed that the highest amount of CLA was obtained by addition of 4% whey powder, addition of grape seed oil in pH=6.0, amount of added oil was 4%v/v milk, incubation temperature of 35°C and termination of incubation at pH=4.8 [30, 31]. The amount of CLA in probiotic yogurt increased from 7.07 mg/g in non-treated yogurt to 9.23 mg/g fat.

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In the present study, three probiotic strains *Lactobacillus acidophilus* La5, *Bifidobacterium bifidum* and *Propionibacterium freudenerchii* were compared from the view of CLA production yield. Then the effect of process variables on production yield was investigated separately by Plackett-Burman design (PBD) using a mixed culture of selected probiotic.

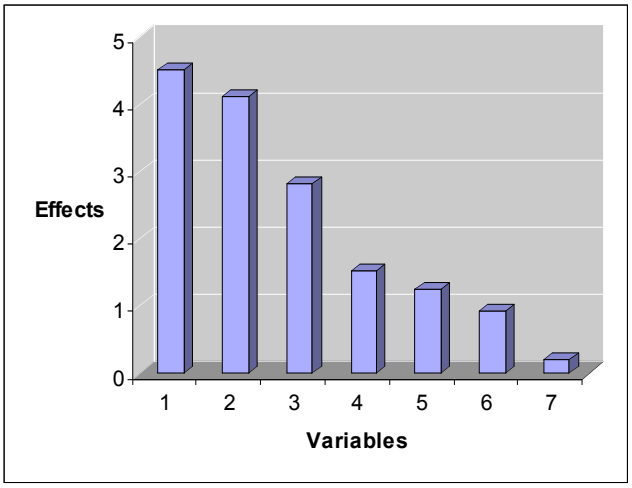

Figure 1: Impact of different variables on CLA production in Plackett-Burman design, (1: Inoculum age, 2:whey, 3: time, 4:oil, 5: Inoculum size, 6: Temperature, 7:pH).

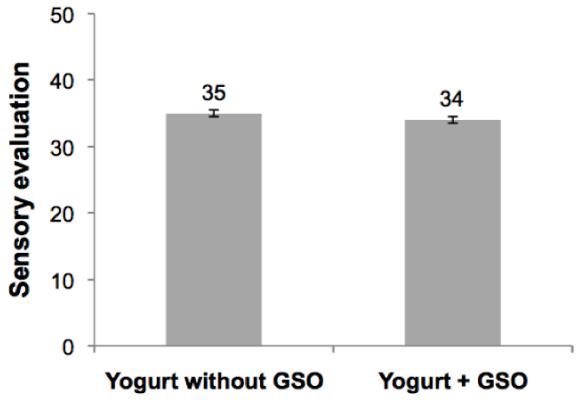

Figure 2: Sensory evaluation of yogurt with and without grape seed oil (GSO) in terms of flavor, taste and texture.

2. MATERIALS AND METHODS

2.1. Sample and Microorganism Preparation

Reconstituted milk made of 10% milk solid non fat was pasteurized for 30 min at 90°C, and cooled to 35° C to prevent possible heat shock to probiotic bacteria. Inoculum size of 10^6 probiotic was added into the reconstituted milk and was slowly stirred by magnetic stirrer for 15 min till it dissolved completely. Packs of starter cultures which were supplied by Chr-Hansen (Horsholm, Denmark) were kept in a -18 °C freezer. This process was performed alongside the flame and under sterile conditions. An amount of 0.4% or 0.8 % of inoculums was added to the milk to produce probiotic yogurt. The dilution degree of powder dissolved in milk was as the recommended amount stated on the package of Christian Hansen (equivalent to 0.000048). Samples were prepared by addition of starter culture including the probiotic bacteria into milk with 3% fat [20, 21]. Whey powder and grape seed oil was purchased from Gela dairy product Industry (Amol, Iran) and Azran-sanat (Esfahan, Iran)

Samples were checked every half an hour and their pH reduction was monitored according to preliminary experimental design. At this stage, grape seed oil was added to the samples. The amount of addition was 0.1 % of the volume of the inoculated milk. After the incubation according to experimental design, produced probiotic yogurt samples placed inside a container full of ice. Thereafter, they were transferred to the freezer and subjected to approximately 0 °C. Consequently after about 30 min incubation temperature decreased to 20°C to prevent bacterial activity and further decrease in pH. Samples were allowed to remain at this condition for about two hours to ensure the temperature is fixed around 4 °C. It should be noted that probiotic bacterial inoculums were not used for subsequent experiments in order to prevent and reduce the loss of viability of them. After measuring the CLA content of probiotic yogurt production, sample with the highest amount of CLA content was separated for further 8 final main tests including evaluation of the CLA content immediately after production and also after one week storage of probiotic yogurt, measuring yogurt fat, non-fat dry matter, pH, acidity (in terms of lactic acid bacteria), probiotic bacteria counts, and sensory evaluation were performed on the final product (each test was performed in triplicates) [20, 21, 29, 32, 33].

2.2. Chemical Analysis

Extraction and separation of CLA were conducted according to the method used by Lin *et al.* [34, 35]. A 2.5 gram of yogurt sample was mixed with 10 mL of chloroform-methanol in a 2:1 volume ratio (V/V) and was centrifuged for 3 min at 2800 rpm and the resulting mixture was centrifuged again for 20 min at -8 C° in 4000 rpm. Thereafter, three phases composed of three layers was specified including the surface (blue), middle (yogurt dry matter) and the lower organic layer containing the fatty acids associated with chloroform

and methanol. The bottom layer was removed by Pasteur pipette and after addition of 0.3g of sodium sulfate was kept inside the refrigerator overnight. This phase consisted of three new phases; top layer which was remained from the previous aqueous phase; the middle layer which was a mixture of chloroform-methanol with a mixture of fatty acids, and last layer which consisted of sodium sulfate. At this stage, the supernatant was removed by using Pastor Pipette. The middle phase was separated from sodium sulfate by performing decantation and then used in our experiment [20, 21, 29].

To remove the mixture of organic solvents (chloroform-methanol) rotary evaporator was used and all the solvents of fatty acids were completely dried off. At the end, what remained was only a mixture of fatty acids. Thereafter, in order to saponify fatty acids 1 mL solution of 1N sodium hydroxide in methanol was added into the solution and then it was left in the COD reactor for 15 min at 100 °C. After cooling it back to the room temperature 6 mL of 4% hydrochloric acid solution in methanol was added to the solution in order to methylate the fatty acids and then incubated for 20 min in water bath at 60 °C.

2.3. Methyl Ester Forming for Gas Chromatography Analysis

At this stage, 2 mL deionized water was added to the sample and homogenized for 15 min by using a vortex mixer. As a result, polar bonds between methanol and water caused release of methyl ester. Then 5mL N-hexane was added and the sample was homogenized for 15 min by using a vortex mixer. This caused transfer of methyl ester from aqueous to organic phase. The organic phase was washed out twice and aqueous phase was completely removed. Finally, one gram of anhydrous sodium sulfate was added to the organic phase and 1 µl of this sample was injected in gas chromatographic columns (Capillary BP10) in a philips scientific model 4410 gas chromatography (UK). Column used was 25 m in length and 0.22 mL in diameter; and with a thickness of 0.25 micro liters. The initial temperature of column was 150°C with 1 min holding time, injection temperature at 250°C, the final temperature 230°C and 10 min duration, with a temperature ramp of 5°C in 1 min.

2.4. Microbiological Methods

Probiotic strain including *L. acidophilus* La5, *Propionibacterum freudenreichii*, *Bifidobacterium bifidum* were tested for their ability to convert linoleic acid to conjugated linoleic acid. Growth and CLA production were followed during incubation for 37 h in reconstituted skim milk containing 0.2% addition of safflower oil in pH=6.0, and amount of added oil was 1.4 ml/l milk. MRS-bile agar medium was used for selective count for probiotic. 0.15% bile integral part was added to the basal medium which is MRS-agar medium. Therefore, growth of traditional bacterial culture was prevented in yogurt. Incubation in aerobic conditions at 35 ° C for 72 hours led to absolute growth of *L. acidophilus*.

2.5. Statistical Methods

Plackett-Burman experimental design was used to study the effect of 7 variables in 8 treatment combinations as Table **1** shows. PBD is one of the highly fractional designs which allows for the study of $k = (N-1)/(L-1)$ factors, each with L levels with N experimental trials. The usefulness of the design lies in the fact that in determining the effects of one variable, the net effects of changing other variables cancel out so that the effect of each variable on the system can be independently determined. As it is known, in the PBD only two levels can be considered for each factor. Table **1** shows the factors that include some medium components, environmental factors, and inoculum condition. The corresponding levels in this table were chosen on the basis of the preliminary tests. Table **2** shows selected experimental factors and a PBD for conducting eight experimental trials. All the trials were done in triplicate. The elements, + (high level) and - (low level) represent the two different levels of the independent factors examined. The most important result obtained from this design is screening of 7 variables and identification of the most important ones CLA production in probiotic yogurt.

Table 1: Seven Variables and Their Levels selected for Evaluation of Their Impact by Plackett-Burman Design on CLA Production

	Variables	Lower level(-)	Higher level (+)
A:	Whey powder (% w/v)	2	4
B:	oil % (v/v)	2	4
C:	pH	4.5	6
D:	Time (h)	18	27
E:	Seed size (% v/v)	0.4	0.8
F:	Temperature (˚C)	35	39
G:	Seed age (h)	20	35

Table 2: Experimental Plackett-Burman Matrix and Yield of CLA Production[a]

Run	A	B	C	D	E	F	G	CLA yield (g/l)
1	1	-1	-1	1	-1	1	1	7.34 ± 0.5
2	1	1	-1	-1	1	-1	1	6.76 ± 0.6
3	1	1	1	-1	-1	1	-1	0.30 ± 2.2
4	-1	1	1	1	-1	-1	1	3.98 ± 0.5
5	1	-1	1	1	1	-1	-1	2.26 ± 5.5
6	-1	1	-1	1	1	1	-1	0.45 ± 6.7
7	-1	-1	1	-1	1	1	1	9.34 ± 1.9
8	-1	-1	-1	-1	-1	-1	-1	6.39 ± 2.7

[a]All data are the average of three replications with standard deviations of the means.

3. RESULTS AND DISCUSSION

The results of 8 trials conducted by PBD are summarized in Table **2**. The coefficients for the seven variables were determined and were used for calculation of predicted yield which are shown in Table **3**. Table **3** also gives a comparison of the experimentally determined citric acid production yield and productivity to those predicted. Then the student's *t*-test was performed to determine the significance of each variable employed (*t*-value =coefficient/S_b). A significant level of 0.05 was acceptable. Statistical calculations for PBD of CLA production from date sugar has summarized in Table **3**.

Based on the results of this study, addition of 4% whey powder to whole milk increased CLA content. This increase could be due to proteins role in mechanism of oxidation of linoleic acid and formation of linoleic acid radical. It can be interesting to search whether further increase in the amount of whey powder may cause more increase in CLA content. This result is supported by previous report [32, 33] who shows that addition of skim milk into dairy products led to higher production of CLA. It may be due to high protein content in whey powder. Proteins can act as hydrogen donors and increase isomerisation of linoleic acids in first step of bio-hydrogenation. As a result, linoleic acid radicals converted to CLA.

Results showed that in probiotic yogurt containing *L. acidophilus* and *B. lactis* along with traditional yogurt bacterial culture higher amount of CLA was produced at 35°C compared to 40 °C. Increase in temperature may lead suitable condition for growth of *L. bulgaricus* as a strong acid producer in process of production of

Table 3: Statistical Results of Impact of Seven Variables on CLA Production Using Plackett-Burman Design[a]

Factors	Effect	Coefficient	*t*-value	P-value
A (whey)	4.1233	2.0617	6.24	0.000
B (oil)	1.5385	0.7692	2.33	0.033
C (pH)	-1.2650	-0.6325	-1.92	0.074
D (time)	2.8117	1.4058	4.26	0.001
E (Inoculum size)	0.2000	0.1000	0.30	0.766
F (temperature)	-0.9433	-0.2467	-0.75	0.466
G (Inoculum age)	4.5083	2.2542	6.83	0.000

[a]Tabulated *t*-value for degree of freedom 6 and α = 0.05 is 2.447.

Table 4: Chemical Characteristics of Yogurt Containing the Highest Amount of CLA

Character	CLA 0.1% w/w		Acidity 0.01 % w/w	pH	Solid lipid	
	at first	after 1 week			%	(w/w)
Yogurt containing CLA	9.23	9.18	0.76	4.8	11.24	0.03

probiotic yogurt. However accumulation of produced lactic acid had a detrimental impact on CLA. By increase in temperature form 35°C to 39°C, *L. delbrueckii* subspecies *bulgaricus* in mixed culture had a more suitable condition as an active acid producer inhibited the probiotic bacterial growth. Kim *et al.,* [2002] also reported by decrease in pH of culture to 4.6 the amount of produced CLA in final product decreased.

Therefore, addition of 0.4% of inoculated milk grape seed oil as a rich source of linoleic acid caused higher CLA amount. There is a direct relationship between the degree of conjugated linoleic and linoleic acid in fermented dairy products. A report by Kim *et al.* [2002], supported this hypothesis. This recent study showed that use of grape seed oil besides using mixed cultures of probiotic bacteria *L. acidophilus* and *B. lactis* along with traditional bacteria to produce yogurt (*S. salivarius* subspecies *thermophilus* and *L. delbrueckii* subspecies *bulgaricus*) increased the CLA. Kim *et al.,* [22] reported that the addition of sunflower oil to the valve 2.5% fat fermented with lactic acid starters including regular starters of yogurt, 10-6 min right before incubation increased CLA content in the final product. CLA produced by cells in logarithmic growth phase were reported as highest value. Addition of 4% of inoculated milk Grape seed oil in probiotic yogurt was effective in CLA production. Lim *et al*. also showed that by addition of 0.1% linoleic acid into milk, the amount of CLA production was increased from 0.71 to 2.95 micro grams in each gram of non fat yogurt [34]. Addition of sunflower oil to the milk for production of yogurt containing *S. salivarius* and *L. delbrueckii* subspecies *bulgaricus* the amount of CLA increased from 4.8 mg to 7 mg fat in final product. Using a rich resource of linoleic acid such as grape seed oil which contains more than 77% linoleic acid in presence of lactic acid bacteria that are commonly associated with production of probiotic yogurt along with two probiotic bacteria i.e. *L acidophilus* and B. *lactis*, caused increase in the amount of produced CLA [32, 33].

The better condition to terminate the incubation is when the pH of the dairy product reached to 6. In acidic condition CLA production may be inhibited [35-37]. At incubation time of 37 h, probiotic bacteria were survived and their growth related metabolite production could be developed. Moreover, this can inhibit the detrimental impact of lactic acids produced by *L. delbrueckii* subspecies *bulgaricus*. It should be noted production of organic acids in yogurt is directly related to bacterial growth.

Inoculum age and size of 36 h and 0.8% lead to more production of CLA. Bio-hydrogenation is a process which needs energy to take place, thus, it seldom occurs in old cells. According to curve obtained from pH changes in probiotic yogurt, the produced CLA was higher in logarithmic phase and addition of substrate rich in fat at pH 6 was the most efficient factor.

Sensory Evaluation

Sensory evaluation was conducted by 30 untrained panelists on probiotic yogurt with and without grape seed oil in order to compare the taste, texture and flavor between them. Samples and control were presented to panelists, separately. For this test, a non parametric Kolmogorov-Smirnov method was used and data were analysed by SPSS software 12.

According to the results taken from this section, there was no difference between the two samples with or without grape seed oil in terms of flavor, taste and texture. Therefore, addition of grape seed oil limited to the amount used in this study had no any adverse and unpleasant effect on the flavor, taste and texture of yogurt samples.

4. CONCLUSION

The best conditions to increase CLA in probiotic yogurt in present study were as follows: the amount of added whey powder 4%of the volume of milk inoculated, incubation temperature 35 °C, the amount of grape seed oil added 4% of inoculated milk, oil addition at pH 6, and time of incubation termination 27, Inoculum age 36 h and size 0.8% respectively.

Concentration of CLA was 188 ppm as measured in the fat extracted from 2.5mL yogurt dissolved in 5 mL solvent. Considering the percentage of milk fat used in this study (3%) produced yogurt contained 0.075 g fat. Thus in optimum condition, amount of CLA was 9.23×10^3 (%w/w) and in control sample it was 7.07×10^3 (%w/w) fat. No much research conducted on this issue. Many researchers had also considered the positive role of probiotic bacteria along with traditional bacteria of yogurt to increase the CLA in yogurt without fat. During fermentation, the majority of CLA isomers were in the culture supernatant, but with washed cells obtained at the early stationary phase, 30 h, about 40% was detected in the cellular lipid.

Experimental design such as Taguchi [38] and PBD [39] can be used successfully in biotechnological research to screen the process variables influencing on biomass and metabolite production. The second step will be optimization and response surface methodology.

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