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The Ability of Some Starter Cultures to Increase the Conjugated Linoleic Acid Level in Cow and Camel Milks

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The ability of 12 bacterial strains to increase the level of conjugated linoleic acid (CLA) in cow and camel milk was evaluated. Sunflower oil was added to both milk types at different concentrations to obtain a final concentration of 300, 600, 900, 1200 and 1500 μg linoleic acid (LA) /ml. *Bifidobacterium angulatum* DSM 20098 formed the highest level of CLA in both milk types followed by *Bifidobacterium longum* subsp. *infantis* DSM 20088, and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20080, respectively. Moreover, *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 and *Propionibacterium jensenii* DSM 20278 produced the highest levels of CLA in cow milk. The CLA levels produced by these strains in cow milk were 13.06 and 10.14 mg /g fat when LA was 300 μg /ml, while it was 11.03 and 5.75 mg /g fat in camel milk when LA was 1200 μg /ml, respectively.

Key words: Starter cultures; Conjugated linoleic acid; Camel milk.

Conjugated linoleic acid (CLA) is receiving attention because of its potential and beneficial biological effects. It has been shown to enhance the immune system[33, 8], reduce body fat[18, 10], promote growth[6], create anticarcinogenic effect[4], stimulate bone formation and bone density[27,38], reduce body fat while enhancing lean body mass[25], inhibit platelet aggregation[3,17], possess anti-proliferative effect[15] and decrease insulin resistance[34]. The CLA isomers are formed during biohydrogenation of linoleic acid in the rumen, and through conversion of vaccenic acid in the mammary gland[32]. The human body cannot produce CLA. However, human blood and tissue contain low concentration of CLA, which may be driven directly from dietary sources such as whole milk, fermented dairy products and meat of ruminants[35]. CLA occurs naturally in a variety of foods including meat, poultry, sea food, cheese, butter, milk and vegetable oils[12]. [26]reported the levels of CLA in different dairy products. Fontina Valdostana had the highest amount of CLA (8.11 mg /g fat), followed by Pecorino cheese (7.77 mg / g fat), Swiss Emmental (7.66 mg /g fat) and sheep yoghurt (6.92 mg /g fat). High levels of CLA were also found in fermented milk and yoghurt of mountain pasture and organic yoghurt (6.15, 6.06 and 6.05 mg /g fat, respectively). Yoghurts made from mountain area showed high average of c9, t11 CLA than those from prairie district[31]. Considerable research had been focused on the formation of CLA in MRS medium inoculated with linoleic acid (LA)[37, 19, 21, 35, 16, 24], or in fermented cow milk[26,7,30]. Few researches were performed in buffalo milk[36, 40]. According to our knowledge, no studies were performed on the formation of CLA in camel milk. The purpose of this study was to compare the ability of some starter cultures in increasing

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the level of conjugated linoleic acid in camel and cow milk.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Twelve bacterial cultures (*Lactobacillus acidophilus* DSM 9126, *L. acidophilus* DSM 20079, *L. acidophilus* DSM 20242, *L. delbrueckii* sub sp. *bulgaricus* DSM 20081, *L. delbrueckii* subsp. *bulgaricus* DSM 20080, *Bifidobacterium infantis* DSM 20088, *B. angulatum* DSM 20098, *S. thermophilus* DSM 20617, *Propionibacterium freudenreichii* sub sp. *freudenreichii* DSM 20271, *P. freudenreichii* sub sp. *shermanii* DSM 20270, *P. freudenreichii* subsp. *shermanii* DSM 4902 and *Propionibacterium jensenii* DSM 20278) were obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ), GmbH, Germany. All strains of *Lactobacillus* and Bifidobacteria were grown anaerobically at 37°C for 24h in deMan–Rogosa–Sharpe (MRS) medium (Difco Laboratories, Detroit, Mich., USA), and 0.05% L-cysteine was added to MRS media when Bifidobacteria were grown. *S. thermophilus* strain was aerobically grown at 40°C, while Propionibacteria were grown at 25°C for 72 h.

Bacterial growth was monitored in MRS medium under suitable condition for strains as mentioned above by measuring the increase in optical density at 620 nm after dilution with 1% peptone solution, using a Beckman DU 640 spectrophotometer (Beckman Instrument Inc., Fullerton, Calif. USA).

Milk fermentation

Full cow and camel milk (3% fat) were used as media for growth of the above mentioned bacterial cultures. Sunflower oil (66% linoleic acid)[36] was added to obtain a final concentration of 300, 600, 900, 1200 and 1500 µg linoleic acid/ml milk. Milk samples were heated at 65°C in water bath, and then homogenized by homogenizer (Danish Turnkey Dairies LTD. Rannie, Denmark) at ~14 MPa and autoclaved at 121°C for 15 min. The sterilized milk samples were inoculated with 1% active culture which, counted about 10^7 - 10^8 cfu ml^{-1}, for all strains, then incubated for 24h except propionibacteria which incubated for 72h. under desire conditions which, mentioned above.

Determination of conjugated linoleic acid (CLA)

Fat contents of fermented milk samples were extracted according to[13]. The extracted lipids were hydrolyzed to free fatty acids by adding 2ml 0.5 N NaOH in methanol heated at 50°C for 30 min[22]. Methyl esters were prepared in the dark with 14% boron trifluoride methanol solution (Sigma Aldrich Chemie GmbH, Steinheim, Germany). Conjugated linoleic acid methyl ester was monitored with Gas Chromatography Mass spectrometer 6890 (GC-Mass 6890, Agilent Technologies, Wilmington, USA). The instrumentation used for the analyses is as follows: a Hewlett-Packard GC-5MS fused silica capillary column (30m, 0.25mm i.d., 0.25 mm film thickness and mass selective detector (MSD) - agilent technologies 5975). The injection volume was 1µL. The temperature of GC oven was programmed from 175 to 220°C at the rate of 5°C/min. The injector and detector temperatures were 300°C. Helium was used as the carrier gas and the flow rate was 0.9ml/min. The mode of Pulsed Splitless was used. A standard CLA was obtained from Sigma, (St. Louis, MO, USA). The standard contains about 42% of a mixture of cis-9; trans-11 and trans-9; cis-11 which occurs as one peak; trans-10; cis-12 which is about 44%; cis-10; cis-12 which is about 10%; and the balance which is about 5% of a mixture of other isomers including trans-9, trans-11, *etc*. Fig. 1 shows the elution times of CLA isomers.

Statistical analysis and design

All experiments were performed in triplicate. Mean values and standard deviations of the mean are shown in the figures. All data were subjected to analysis of variance using Duncan's multiple range test and SAS program[29]. Overall differences among experiment means were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Results in Fig. 2 show the concentrations of CLA in cow and camel milk. It can be seen that the addition of sunflower oil had no significant effect *($P < 0.05$)* on CLA level in both milks at all LA added levels (300-1500 µg/ml milk) when compared with control samples (zero linoleic acid)[30], observed that there was no correlation between CLA content of milk products and the linoleic acid

content, or any other unsaturated fatty acid. The concentration of CLA was 4.56 and 4.17 mg/g fat in camel and cow milk, respectively. These results are in good agreement with those obtained by[20], who showed that the concentration of CLA in full cream cow milk was 4.5 mg /g fat.[30], reported that the CLA concentration in Turkish dairy products ranged from 1.50–5.60 mg /g fat.

Effect of Bifidobacteria addition on CLA formation

Bifidobacteria are normal inhabitants of the human digestion tract, and are now being used as probiotics in the production of fermented dairy products. In this study, the possible role of two strains of Bifidobacteria (*Bifidobacterium longum* subsp. *infantis* DSM 20088 and *Bifidobacterium angulatum* DSM 20098) was assessed for CLA formation in cow and camel milk. Results in Fig. 3 show that the addition of *B.longum* subsp. *infantis* DSM 20088 to cow milk had no significant effect on formation of CLA at zero concentration LA. However, the levels of CLA significantly increased with increasing LA from 300-1500 µg /ml, and the highest increase values were obtained at 900-1500 µg LA /ml (3.37-3.76 mg /g fat) (Fig. 3). These results are not consistent with those reported by[6], who found that *Bifidobacterium infantis* NCFB 2205 did not convert linoleic acid to CLA at any significant level in MRS medium inoculated with LA. This discrepancy may be attributed to the

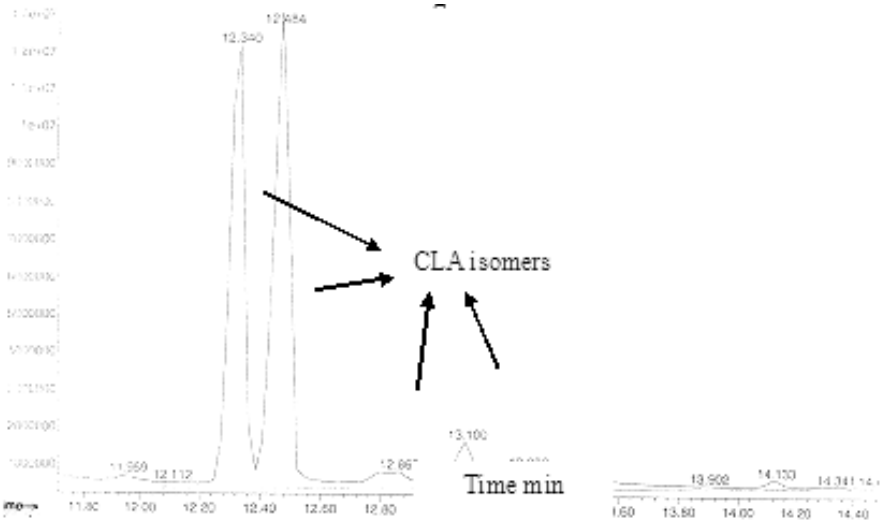

Fig. 1. CLA isomers measured in standard solution by GC-MS.

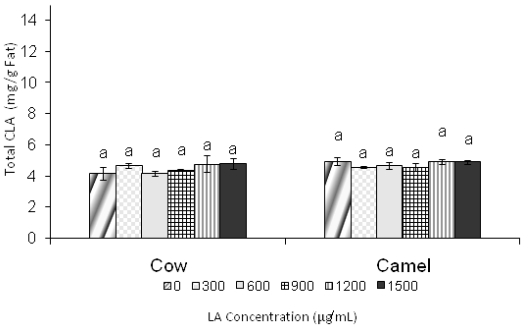

Fig. 2. CLA concentration in cow and camel milk supplemented with different concentrations of lenoleic acid, means with same letters show no significant differences.

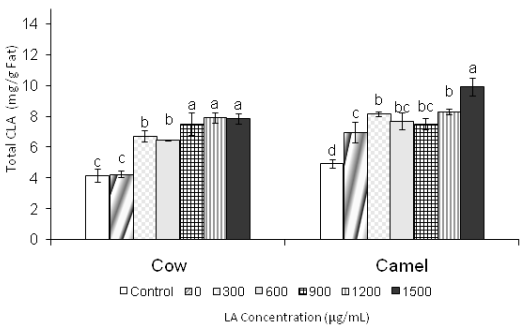

Fig. 3. Effect of *Bifidobacterium longum* subsp. *infantis* DSM 20088 addition on CLA formation in milk. a, b, c, d means with different letters show significant differences.

different strain or/and the different medium (in this study, cow and camel milk was used). However[11], found that the conjugated linoleic level increased 1.4 times when milk yoghurt was inoculated with *Bifidobacterium animalis* subsp. *lactis* HN019. Moreover[28], reported that the highest levels of CLA production by *Bifidobacterium animalis* was 36.3 µg /ml at 24 h of incubation when sunlower oil was added as a substrate to skim milk. Nevertheless, when free LA was used as substrate, the highest rate of bioconversion of CLA was obtained at 21.6 µg /ml at 24 h of incubation time. Moreover, addition of *B. longum* subsp. *infantis* DSM 20088 to camel milk increased CLA content in all samples under studies (0-1500 µg LA /ml). The highest value was obtained at 1500 µg LA /ml (4.97 mg /g fat) (Fig. 3). The higher CLA levels that occurred in camel milk than those in cow milk may be attributed to the higher level of free linoleic acid in camel milk[5], or to the higher viability of Bifidobacteria in camel milk than in cow milk[1]. Inoculation of cow and camel milk at zero concentration of LA by *B.angulatum* DSM 20098 did not increase CLA. However, addition of *B. angulatum* DSM 20098 strain formed the most CLA at a linoleic acid concentration of 900 µg /ml in cow milk (4.39 mg /g fat) (Fig. 4). At the same linoleic acid concentration (900 µg /ml) in camel milk, *Bifidobacterium angulatum* DSM 20098 strain produced more CLA (4.51 mg /g fat) (Fig. 4).

CLA formation by propionibacteria

Four Propionibacteria strains were tested for CLA formation in cow and camel milk. Two strains of propionibacteria (*Propionibacterium*

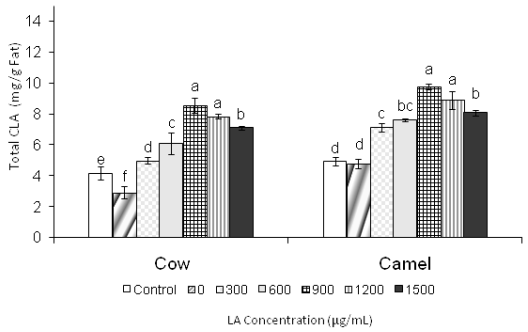

Fig. 4. Effect of *Bifidobacterium angulatum* DSM 20098 addition on CLA formation in milk. A: cow milk, B: camel. [a, b, c, d, e, f] means with different letters show a significant differences

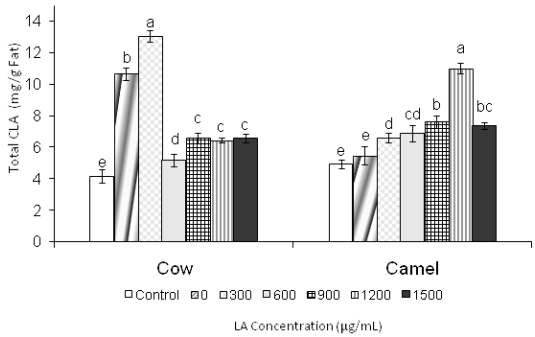

Fig. 5. Effect of *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 addition on CLA formation in milk. a, b, c, d, e means with different letters show significant differences.

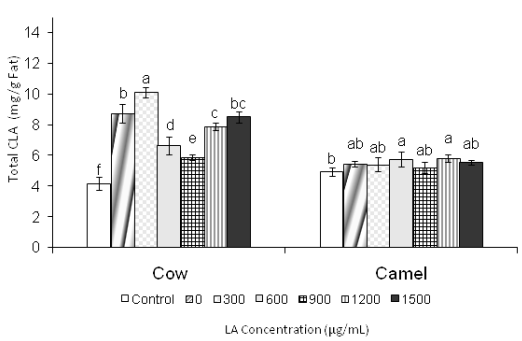

Fig. 6. Effect of *Propionibacterium jensenii* DSM 20278 addition on CLA formation in milk. a, b, c, d, e, f means with different letters show significant differences.

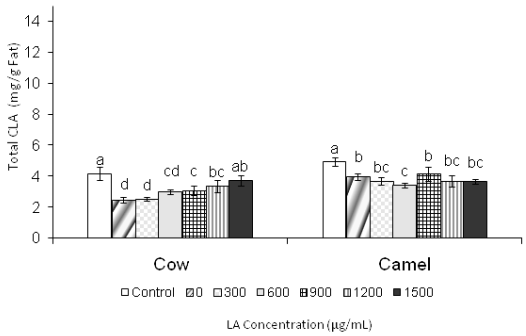

Fig. 7. Effect of *Lactobacillus acidophilus* DSM 20079 on CLA formation in milk. a, b, c, d means with different letters show significant differences.

freudenreichii sub sp. *freudenreichii* DSM 20271 and *P. jensenii* DSM 20278) were able to form CLA in both milks (Figs. 5 and 6). In contrast, *P. freudenreichii* sub sp. *shermanii* DSM 20270 and *P. freudenreichii* sub sp. *shermanii* DSM 4902 showed no such activity[37], assessed two strains of *Propionibacterium freudenreichii* sub sp. *shermanii* and *P. freudenreichii* sub sp. *freudenreichii* for their ability to produce CLA in sodium lactate medium (SLM), MRS medium and skim milk. Also, they found that both strains were able to produce CLA in the three media supplemented with different concentrations of sunflower oil. The maximum production of CLA (78.8 µg /ml) was observed after 36 h of incubation in MRS containing 12 mg/ml of sunflower oil by *P. freudenreichii* subsp. *shermanii* (the higher concentration of used LA may be the reason for the discrepancies at 12000 µg /ml). With the two former strains, the CLA formation was studied at different concentrations of free linoleic acid (0 - 1500 µg /ml). Inoculation of cow milk by *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 significantly increased formation of CLA at all LA concentrations. This strain reached the highest CLA formation at zero and 300 µg LA /ml milk (6.51 and 8.89 mg /g fat, respectively) (Fig. 5) As the linoleic acid concentration increased from 600 to 1500 µg /ml, CLA formation decreased in comparison with zero and 300 µg LA /ml. The addition of the same strain to camel milk also, significantly increased CLA formation at LA concentration of 300 – 1500 µg / ml. The highest value was obtained at 1200 µg /ml (6.08 mg /g fat) However, the results seem to be in contrast with the findings of[13] as they reported that the free linoleic acid in media inhibits the growth of propionibacteria, and hence, CLA formation. In addition[9], reported that yeast lipase and propionibacteria, together in wash curd and dry-salted cheese, were not able to increase the CLA content in the presence of free linoleic acid[14], reported that an optimal concentration of sunflower oil in whole milk at the rate of 100 µg /ml and beyond 200 µg /ml had no effect on CLA production. It has been suggested that the conversion might have resulted from the action of the LA isomerase enzyme[19],[39], found that higher CLA production was achieved by Propionibacteria strains on hydrolyzed soy oil in fermented milks after 14 days. Inoculation of *P. jensenii* DSM 20278 into cow milk significantly ($P<0.05$) increased formation of CLA at all LA concentrations under studies (0-1500 µg / ml). The maximum increase was at 300 µg /ml (5.97 mg /g fat). However, CLA slightly increased (0.82-0.87 mg /g fat) with addition of *P. jensenii* DSM 20278 to camel milk at LA concentrations of 600 and 1200 µg /ml, respectively (Fig. 6).

Effect of *Lactobacillus acidophilus* on CLA formation

Three strains of *Lactobacillus acidophilus* (*Lactobacillus acidophilus* DSM 9126, *L. acidophilus* DSM 20079 and *L. acidophilus* DSM 20242) were used. Results showed that none of them was able to increase CLA levels in control (zero linoleic acid) and treated (equivalent to 300-1500 µg LA/ml milk) cow or camel milk samples. Results in Fig. 7 showed the effects

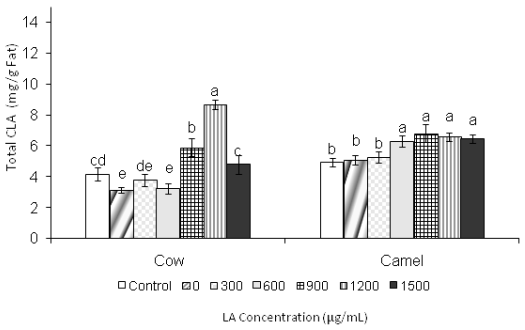

Fig. 8. Effect of *Lactobacillus delbreuckii* subsp. *bulgaricus* DSM 20080 on CLA formation in milk. a, b, c, d, e means with different letters show significant differences.

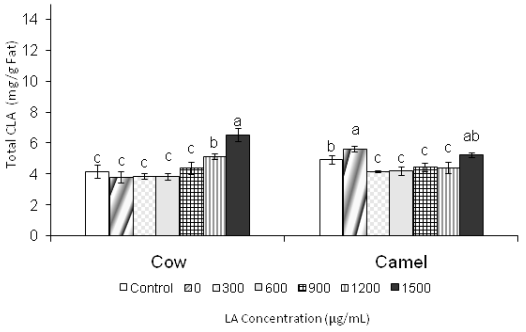

Fig. 9. Effect of *Streptococcus thermophilus* DSM 20617 addition on CLA formation in milk. a, b, c, means with different letters show significant differences.

of *Lactobacillus acidophilus* DSM 20079 as an example for cow and camel milk, respectively. These results are in good agreement with those obtained by[13], who evaluated 19 strains of *Lactococcus, Lactobacillus, streptococcus* and propionibacteria in vitro system with free LA. Only propionibacteria demonstrated the ability to form CLA. However, *Lactobacillus acidophilus* was capable of producing CLA in wide range when LA was added at a concentration of 1000 µg /ml to sterilized skim milk and 24h incubation as reported by[20] (105.5 µg/ ml milk) and[28], (from 2.31- 15.29 µg /ml milk depending on the *lactobacillus* strain used). Moreover, *Lactobacillus acidophilus* AKU 1137 was able to produce CLA from LA[23]. The discrepancies between these results and those obtained by the above authors are that in these studies, sunflower oil was added as a source of LA (in esterified form) while it was added as free linoleic acid in the others. Free LA may be more accessible for biohydrogenation than esterified one[14] claimed that LA isomerase was inactivated by lower pH. It is noteworthy to mention that *Lactobacillus acidophilus* strains are highly lactic acid producer.

Effect of *Lactobacillus delbreuckii* subsp. *bulgaricus*

Two strains of *Lactobacillus delbreuckii* subsp. *bulgaricus* DSM 20080 and DSM20081 were examined for CLA production in cow and camel milk. Results in Fig. 8 show that *Lactobacillus delbreuckii* subsp. *bulgaricus* DSM 20080 was not able to increase CLA level in cow milk with the addition of LA at concentration of 0, 300 and 600 µg /ml, however, production was occurred when LA was increased to above 600 µg/ ml. The higher increase in CLA was obtained with 1200 µg /ml LA (4.53 mg/g fat) while it was 1.75mg/ g fat at 900 µg /ml LA. Increasing linoleic acid addition from 1200 to 1500 µg /ml showed little enhancement on CLA production in comparison with control sample (Fig. 8)[20], found that CLA increased from 21.5 to 86.5 µg /ml skim milk when LA addition increased from 0 to 1000 µg /ml inoculated milk with *L. delbrueckii* subsp *bulgaricus* for 24h. The addition of *Lactobacillus delbreuckii* subsp. *bulgaricus* DSM 20080 to camel milk had no significant effect ($P < 0.05$) on the formation of CLA with the addition of LA at zero and 300 µg /ml, and it significantly increased with an increased LA to 600 µg /ml. The increase of linoleic acid addition from 600 to 1500 µg /ml did not show any significant CLA increase in milk samples ($P < 0.05$). Inoculation of cow and camel milk with *Lactobacillus delbreuckii subsp. bulgaricus* DSM 20081 did not show any significant increase ($P < 0.05$) in CLA concentration at all LA concentrations (0-1500 µg /ml) except that at 1200 µg /ml in camel milk, there was an increased 1 mg/g fat (data not shown)[23], postulated that first, 10-hydroxy-cis-12- and 10-hydroxytrans-12-octadecenoic acids would be formed from linoleic acid. These hydroxy fatty acids are intermediate compounds for CLA production from linoleic acid. It was noted that not all lactic acid cultures are able to produce these intermediate compounds.

Effect of *Streptococcus thermophilus* DSM 20617 addition on CLA formation

Addition of *S. thermophilus* DSM 20617 to cow milk increased CLA formation only at 1200 and 1500 µg LA/ml but not at lower concentrations (Fig. 9). This strain had the highest CLA-producing activity at 1500 µg LA /ml (2.38 mg/g fat equivalent to 71.4 µg CLA /ml milk). These results are comparable with those obtained by[20], who found that CLA increased to 73.5 µg /ml when *S. thermophilus* was added to skim milk containing LA at concentration of 1000 µg/ml. However, *S. thermophilus* was not able to increase CLA in camel-treated samples but at zero concentration of LA, CLA increased about 0.7 mg/g fat.

CONCLUSION

The production of functional foods containing CLA has been reported as important food with biological properties. Therefore, the ability of starter cultures (lactobacilli, streptococci, bifidobacteria and propionibacteria) which are used normally in fermented dairy production was assessed. It can be concluded that *P. freudenreichii* sub sp. *freudenreichii* DSM 20271 is a promised strain for the production of CLA in fermented cow and camel milk, where the highest level of CLA in both milks was obtained compared to the other strains. In addition, *Bifidobacterium longum* subsp. *infantis* DSM 20088 and *B. angulatum* DSM 20098 showed high potential as CLA producers. Since the formation

of CLA is greatly affected by the condition of assessment, *i.e*. type and concentration of substrate, fermentation time and bacterial strains. Future works are needed to improve and achieve the optimal production conditions of CLA.

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