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ORIGINAL PAPER

Conjugated linoleic acid producing potential of lactobacilli isolated from the rumen of cattle

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Abstract Lactobacilli isolated from the rumen of cattle were subjected to morphological and biochemical characterizations followed by PCR-based identification. Among isolates, *Lactobacillus brevis* was found to be the most prevalent species in the rumen. For in vitro conjugated linoleic acid (CLA) production, the two isolates of *L. brevis* and one each of *Lactobacillus viridescens* and *Lactobacillus lactis* were selected. The sunflower oil (i.e., 0.25, 0.5 and 1.0%; a rich source of linoleic acid) was added to skim milk as a substrate for CLA production by isolates at 37 °C/12 h. *L. brevis* 02 was found to be the most potential CLA producer (10.53 mg CLA/g fat) at 0.25% concentration of sunflower oil followed by *L. brevis* 01 (8.27 mg CLA/g fat). However, at higher level of sunflower oil (i.e., 1.0%), *L. lactis* was the highest CLA producer (9.22 mg/g fat) when compared to *L. brevis* and *L. viridescens*. The results indicated that *L. brevis* and/or CLA production was inhibited with increasing concentration of sunflower oil in skim milk. In contrast, *L. lactis* and *L. viridescens* could tolerate the increasing concentrations of sunflower oil and produced higher CLA. Overall, *L. brevis* extends a possibility to be used as a direct-fed microbial for ruminants to increase the CLA content in milk, however, in vivo trials are needed for validation of results obtained.

Keywords Conjugated linoleic acid · *Lactobacillus brevis* · *Lactobacillus viridescens* · *Lactobacillus lactis* · Sunflower oil · Rumen

Introduction

Milk and meat from ruminants represent an important source of nutrients providing high energy, proteins, essential fatty acids, minerals, vitamins, etc., in human diets. The functional foods exert beneficial effects in addition to routine nutrients, for example, the presence of conjugated linoleic acid (CLA) in various food products of ruminant origin. CLA, a fatty acid with two conjugated unsaturated double bonds at different positions, is a mixture of positional and geometric isomers of linoleic acid. It is formed mainly as an intermediate during the biohydrogenation of linoleic acid in the rumen [15] or from endogenous conversion of transvaccenic acid in mammary gland [6, 10]. An ever increasing research and industrial interest in CLA is associated with its various health benefits (i.e., anticarcinogenic, antiatherogenic, antidiabetic, antiadipogenic, etc.), to the consumers [22]. The major areas of biohydrogenation of CLA within the rumen microbial ecosystem (i.e., microorganisms involved, identification of intermediates, biochemistry of key enzymes and development of mathematical models to predict outcomes) are summarized in a recent review [12]. Out of more than a dozen of the isomers, *cis*-9, *trans*-11 isomer of CLA has been found to exhibit potential health benefits to the host because of its

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predominant incorporation into the phospholipids of cell-membrane. A number of CLA producing bacteria [12] have been isolated from the rumen including novel strains of *Butyrivibrio fibrisolvens* [8, 9] and *Megasphaera elsdenii* [18], human intestine [1] and dairy products [5]. Nam and Garnsworthy [23] confirmed that the major product of linoleic acid metabolism by rumen fungi was *cis*-9, *trans*-11-CLA, suggesting that these fungi could influence CLA content of ruminant products. Most of the lactic acid bacteria, namely, *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, L. delbrueckii* ssp. *lactis, Lactococcus lactis* ssp. *cremoris, L. lactis* ssp. *lactis* and *Streptococcus thermophilus,* and rumen bacteria *B, fibrisolvens* [17] have the ability to convert linoleic acid into CLA [20, 21, 24]. *M. elsdenii* strains differ greatly in their ability to produce *trans*-10, *cis*-12 CLA and hence, CLA production is not a phylogenetically conserved trait [18]. The different aspects of CLA formation and impact of microbial cultures for increasing CLA in dairy products were reviewed by Sieber et al. [29].

Recently, a rapid method based on spectrophotometer for screening of bacteria that are able to convert free linoleic acid to CLA was developed by Barrett et al. [2]. Animals are the richer sources of CLA than plants and generally, foods from ruminants contain higher CLA than foods of non-ruminants origin. Microbial fermentation in the rumen has also been found to increase the CLA production [10]. Hence, there is a possibility to further increase the CLA content in milk and milk products by manipulating the rumen microbial fermentation. This can be achieved either by supplementing the ruminants diets with plant oils that are rich in linoleic acid or by administering the microbes into the rumen having the ability to convert linoleic acid into CLA or both, ultimately leading to the production of foods of animal origin with enhanced CLA. Keeping all these points in view, an effort was made in the present study to isolate and characterize CLA producing lactobacilli of rumen origin, and to determine their in vitro CLA producing potential in presence of sunflower oil, a linoleic acid rich substrate of CLA.

Materials and methods

Lactobacilli were isolated, using MRS agar with incubation at 37 °C for 24 h, from the rumen liquor of fistulated cross-bred male calves (Sahiwal × Holstein-Friesian; age ≈ 3 years; weight ≈ 250 kg; fed on standard diet of wheat straw, green fodder maize and concentrate mixture) maintained at cattle yard, NDRI, Karnal. The rumen liquor collected in a thermos flask was strained through four layers of muslin cloth and centrifuged at 2,000*g* for 10 min to remove the feed particles, protozoa and fungi. The identification of the isolates was ascertained by Gram's staining, cell shape, catalase test, growth at 15 and 45 °C, gas from glucose, arginine hydrolysis, aesculin hydrolysis, and nitrate reduction test. The species of lactobacilli isolates were identified by sugar utilization patterns using CHL media [11]; and the final identity was assigned by PCR-based methods using specific primers. The genomic DNA, from three isolates showing maximum CLA producing ability, was extracted by the method of Pospiech and Neikmann [26]. The PCR reaction conditions were: reaction buffer, 10× with $Mgcl_2$, 2.5 µl (1×); dNTPs mix, 2 µl (0.2 mm); primer (forward), 2 µl (0.1–1 mm); primer (reverse), 2 µl (0.1–1 mm); Taq DNA polymerase, 1 µl (0.5–3 U); template DNA, 1–10 µl (100 ng–1 µg) and sterile milli Q water, variable. The PCR cycling steps for LbLMA-1 and R16-1 were: initial denaturation, 95 °C/5 min; denaturation, 95 °C/30 s; annealing, 55 °C/30 s; extension, 72 °C/30 s; final extension 72 °C/7 min and number of cycles, 30. The genus-specific primers used along with their sequences were: primer region, 16s rRNA; primer sequence, (F)5′CTCAAAACTAAACAAAGTTTC3′, (R)5′CTTGTACACACCGCCCGTCA3′; annealing temperature, 55 °C and product size, 250 bp. The species-specific primers used were: for *L. brevis*, Bre1/Bre2; (F) 5′CTTGCACTGATTTTAACA3′, (R)5′GGGCGGTGTGTACAAGGC3′; for *L. lactis* Lac1/Lac2, (F)5′CCTCTTCGCTCGCCGCTACT3′, (R)5′A CAGATGGATGGAGAGCAGA3′; annealing temperature, 59.0 and 60.0 °C and product size (bp), 350 and 500, respectively. PCR amplified products obtained with different templates were electrophoresed on the agarose gel following the standard procedures [28].

For CLA production, sunflower oil was chosen as a substrate because it contains a high concentration (67%) of linoleic acid. The experiment was set up as per the following treatments: Control 1, skim milk; Control 2, skim milk + hemin (0.01%) + glucose (0.3%) + sunflower oil (0.25–1.0%); and treatments, skim milk + hemin (0.01%) + glucose (0.3%) + sunflower oil (0.25–1.0%) + *Lactobacillus* isolates (overnight grown culture at 1%) [16]. The incubations were carried out in culture tubes containing 10 ml of media at 37 °C for 12 h and samples were processed for further analysis of CLA.

Fat from milk/curd was extracted by the method of Bligh and Dyer [3]. For this, fresh sample (3 ml) was first vortexed with 3 ml of methanol and 1.5 ml of chloroform. This mixture was then vortexed with 1.5 ml chloroform for an additional 2 min and the homogenate was centrifuged at 5,000*g* for 10 min. The upper (methanol–water) layer was removed through aspiration and the bottom layer (chloroform layer) was passed through anhydrous sodium sulphate on Whatman filter paper No.1. The filter paper was rinsed with 3 ml of chloroform and the extract (≈4.5 ml) was

Table 1 The morphological, physiological and biochemical characterization of *Lactobacillus* isolates of rumen origin

Isolate (s)	Gram's staining, maltose	Cell shape (rods)	Catalase test, growth at 45 ºC, nitrate reduction, sorbitol, mannose	Growth at 15 ºC	Gas from glucose, arginine hydrolysis, arabinose	Aesculin hydrolysis	Raffinose, lactose, trehalose, salicin, galactose	Melibiose	Species
1	+ve	Very short	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
2	+ve	Thin	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
3	+ve	Long thin	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
4	+ve	Thick	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
5	+ve	Small chains	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
6	+ve	Long thin	−ve	+ve	−ve	+ve	−ve	−ve	*L. viridescens*
7	+ve	Long pair	−ve	−ve	−ve	+ve	+ve	+ve	*L. lactis*
8	+ve	Thick	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
9	+ve	Thick	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
10	+ve	Short	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
11	+ve	Very short	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
12	+ve	Thin	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
13	+ve	Thin	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
14	+ve	Small chains	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*
15	+ve	Thick	−ve	+ve	+ve	−ve	−ve	+ve	*L. brevis*

+ve = positive; −ve = negative

evaporated to dryness under vacuum and then under the stream of nitrogen. The extracted fat was hydrolyzed [31] with 1 ml of 1 N methanolic sodium hydroxide in a boiling water bath for 15 min and then cooled to room temperature for 5 min. Hydrochloric acid (1 ml, 2 N) and 2 ml chloroform were added to the tube containing methanolic sodium hydroxide and vortexed for 4 min, followed by centrifugation at 5,000*g* for 10 min. The organic layer (lower layer) was collected and evaporated to dryness under vacuum and then under the stream of nitrogen.

A working stock solution of CLA in acetonitrile that gave a concentration of 4 µg of CLA in 20 µl of standard to be injected was prepared. The hydrolyzed fat was dissolved in acetonitrile containing 0.14% acetic acid and separation of CLA was carried out in a HPLC system (Waters Chromatography Division, Milford, USA) using C_{18} μ Bondapak where mobile phase was acetonitrile/water/acetic acid (70:30:0.12 v/v/v) at a flow rate of 1.5 ml/min. The peak of CLA was eluted at 12–16 min with the injection volume of 20 µl. The retention time was determined at 234 nm, and compared with a standard CLA (Sigma Chemical Co., St. Louis, MO, USA). After calculating the amount of standard CLA using peak area, the area of sample peak was calculated as CLA content using heptadecanoic acid as the internal standard. The results were expressed as mean ± SE ($n = 3$) and the test of significance ($P < 0.05$) was employed using analysis of variance [30].

Results and discussion

Fifteen isolates based on colonial morphology (cream color, oval shape, ≈0.5-mm diameter) were randomly picked up from a number of colonies after plating rumen liquor on MRS agar. All the isolates were found to be Gram-positive rods of varying shape, catalase negative, no growth at 45 °C, nitrate reduction negative and maltose positive while sorbitol and mannose negative. Thirteen isolates except number 6 and 7 produced gas from glucose, arginine hydrolysis positive, arabinose positive and aesculin hydrolysis negative. Similarly, except isolate 7 all the isolates could grow at 15 °C and were negative for raffinose, lactose, trehalose, salicin and galactose. On the other hand, all the isolates were melibiose positive except isolate 6 (Table 1). Based on these biochemical assays, all the isolates were identified as *Lactobacillus brevis* except isolate 6 and 7 that were identified as *Lactobacillus viridescens* and *Lactobacillus lactis*, respectively. The data obtained from morphological, physiological, biochemical and sugar utilization tests for genus and species identification were confirmed with 'Bergey's Manual of Determinative Bacteriology' [11].

After biochemical identification, two isolates of *L. brevis* were randomly selected along with *L. lactis* and *L. viridescens* for their CLA production potential in vitro using skim milk as media containing varying concentrations of sunflower oil, a rich source of linoleic acid (Table 2). Control 1

Table 2 The production of CLA by *Lactobacillus* isolates of rumen origin at different levels of sunflower oil supplementation in skim milk at 37 °C/12 h

Isolate(s)	CLA (mg/g fat)			
	Sunflower oil (%) in skim milk			
	Control 1	0.25	0.5	1.0
Control 2	Nil	Nil	Nil	Nil
L. viridescens	Nil	1.83 ± 0.08^{ap}	5.23 ± 0.02^{bp}	5.67 ± 1.04^{bq}
L. lactis	Nil	1.89 ± 0.05^{ap}	8.27 ± 0.02^{bq}	9.22 ± 0.04^{cr}
L. brevis 01	Nil	8.27 ± 0.02^{cq}	5.73 ± 0.02^{bp}	1.66 ± 0.02^{ap}
L. brevis 02	Nil	10.53 ± 0.02^{cr}	8.41 ± 0.02^{bq}	1.96 ± 0.04^{ap}

Control 1: skim milk without sunflower oil

Control 2: skim milk + glucose (0.3%) + hemin (0.01%) + sunflower oil (0.0–1.0%) without inoculums

a–c: means (±SE, $n = 3$) within same row with different superscripts differ significantly ($P < 0.05$)

p–r: means (±SE, $n = 3$) within same column with different superscripts differ significantly ($P < 0.05$)

with no sunflower oil and with and without inoculums in the skim milk showed no CLA content after analysis. Similarly, Control 2 with varying levels of sunflower oil but without inoculums also showed the similar results of absence of CLA in the medium, supporting the fact that CLA content in media is neither due to sunflower oil nor due to the inoculums alone in the media.

Ogawa et al. [25] reported that *L. acidophilus* producing high levels of CLA were obtained by cultivation in a medium containing linoleic acid, indicating that the enzyme system for CLA production is induced by linoleic acid. Hence, the CLA productivity of washed cells obtained by cultivation with linoleic acid was much higher than that of the cells obtained by cultivation without linoleic acid. This might be due to the induction of enzymes catalyzing the CLA formation by linoleic acid during cultivation of cultures. *L. viridescens* was shown to convert sunflower oil (i.e., 0.25, 0.5 and 1.0% in media) into CLA at the rate of 1.83, 5.23 and 5.67 mg/g fat, respectively. In case of *L. lactis*, the CLA content was 1.89, 8.27 and 9.22 mg/g fat with respective sunflower oil levels. However, it appears to be in contrast of Jiang et al. [13], who reported that the free linoleic acid in media inhibits the growth of propionibacteria, and hence, CLA production; and suggested that the conversion of free linoleic acid to CLA might be a detoxification mechanism by bacteria. Kim and Liu [16] also reported an optimal concentration of sunflower oil in whole milk at the rate 0.1 g/L and beyond 0.2 g/L has no effect on CLA production, which was in agreements with the antimicrobial effect of free linoleic acid on propionic acid bacteria by Boyaval et al. [4] and Jiang et al. [13]. In present study, when *L. viridescens* and *L. lactis* that have the tendency of producing higher CLA with increased sunflower oil, were compared, the latter was found to be better. This was in close agreement with Qiu et al. [27], who observed that more linoleic acid in a fermentor produced more CLA. Kelly et al. [14] also studied that sunflower oil and linseed oil in ruminant diet enhances CLA production in milk. Linoleic acid addition, to either the washed cells of *L. delbrueckii* ssp. *bulgaricus* or the crude enzyme extract, improved CLA production, indicating the presence of linoleic acid isomerase activity in the culture [20]. Yadav et al. [32] reported that CLA content increased in probiotic dahi by lipolysis of natural milk fat during fermentation even without addition of external linoleic acid and remained stable during storage. This indicated that strains *L. acidophilus* and *L. casei* utilized linoleic acid produced by the lipolysis of milk fat acted as a substrate for CLA synthesis in probiotic dahi. Kishino et al. [19] reported that in presence of lipase, castor oil in which the main fatty acid component is ricinoleic acid was also a substrate for CLA.

In contrast to the above results of enhanced level of CLA with enhanced sunflower oil, it was noticed that *L. brevis* 01 and 02 produced a higher amount of CLA at lower level of sunflower oil, and decreased with increasing amount of linoleic acid in media. The *L. brevis* 01 produced CLA at a concentration of 8.27, 5.73 and 1.66 mg/g fat in the decreasing order with increasing sunflower oil concentration, i.e., 0.25, 0.5 and 1.0%, respectively. A similar trend of CLA production, i.e., 10.53, 8.41 and 1.96 mg/g fat was noticed with the overall highest CLA producer, i.e., *L. brevis* 02. These results indicate that linoleic acid inhibits the bacterial growth leading to lower CLA produced and supported well with the work of Jiang et al. [13] and Kim and Liu [16].

The two potential CLA producing isolates identified biochemically as *L. brevis and L. lactis* were followed for genus-specific PCR identification that showed positive 250 bp bands on agarose gel (1%) thereby, establishing their identity as lactobacilli. Our results are in complete agreement with those of Dubernet et al. [7]. After ascertaining the identity of the two isolates as *Lactobacillus* on the

basis of genus-specific PCR assays, the strains were further identified at species level by subjecting to species-specific primers. The primer Bre1/Bre2 could amplify 350-bp products, indicating that this isolate belongs to *L. brevis.* While primer Lac 1/Lac 2, specific for *L. lactis* amplified a positive 500-bp product. Hence, after consolidation of the species-specific PCR results, the ruminal isolates with maximum CLA producing ability were identified as *Lactobacillus brevis* and *Lactobacillus lactis.*

From the present work, it could be concluded that the rumen constitute a rich source of CLA producing potential lactobacilli. *L. brevis* was found to be the most prevalent species, and a potential CLA producer with an ability to produce significant level of CLA at lower concentration of sunflower oil. As there is a high demand and scope for CLA enriched health-promoting foods because of various functional properties associated with CLA, the isolate *L. brevis* 02 extends a possibility to be used as direct-fed microbial for ruminants in order to increase the CLA content in milk, and hence, in vivo feeding trials are needed to validate the results obtained under laboratory conditions.

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