

## RESEARCH PAPER

# ***In-vitro* Screening and Characterisation of Conjugated Linoleic Acid Producing Lactic Acid Bacteria**

B.H. Joshi<sup>1\*</sup>, R.M. Dhingani<sup>1</sup> and S.M. Patel<sup>2</sup>

<sup>1</sup>Department of Food Quality Assurance, College of Food Processing Technology and Bioenergy, Anand Agricultural University, Anand, Gujarat, India

<sup>2</sup>Shree P.M. Patel Institute of Integrated M. Sc. in Biotechnology, Anand, Gujarat, India

\*Corresponding author: bhavesh@aau.in

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### Abstract

Conjugated Linoleic Acid (CLA) isomers have attracted significant attention due to their important functional traits with plenty of health prospects to humans. Many Lactic Acid Bacteria (LAB) demonstrate the ability to produce CLA isomers C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 from the Linoleic Acid (LA) *in-vitro*. In the present study, 16 LAB with ability to produce CLA were isolated from diverse ecosystem. Three LAB isolates, CMLAB2, BMLAB1 and PMLAB1 had shown their potential to convert *in-vitro* free LA to CLA. The FAME extracts of CLA produced in fermentation experiments by these isolates were studied. The isolates CMLAB2, BMLAB1 and P2MLAB1 were found to produce 21.21; 14.68; 6.35 µg/ml of CLA respectively. Among these isolates, CMLAB2 isolate was found to produce physiologically important and most studied c9, t11CLA and t10, c12 CLA isomers in significant amount *in-vitro*. Upon characterization of this isolate, it was identified as a species of Lactobacilli.

**Keywords:** Lactic acid bacteria, Conjugated linoleic acid, FAME, C18:2 *cis*9, *trans*-11 isomer

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Food is fundamental to our health, and the health of the nation as a whole. This necessitates the development of foods and food ingredients with enhanced nutritional and functional properties. There is an increasing demand for food products with higher levels of polyunsaturated fatty acids (PUFA), as well as enriched in fatty acids with potentially positive health effects such as Omega-3 Fatty Acids or Conjugated Linoleic Acid (CLA).

CLA is one of the most often investigated functional components in particularly meat and dairy products according to research published during the last decades (Nieuwenhove *et al.* 2012). It is a mixture of geometric and positional isomers of linoleic acid (LA) with a conjugated double bond system (Pariza *et al.* 2001). It recently has gained attention

because of its possible human health benefits, such as potential anti-carcinogen, antioxidants, reduced risk of atherogenesis, adipogenesis, diabetogenesis, inflammation, bone density loss and immune dysfunction (Pariza *et al.* 1979; Miller *et al.* 2001; Roche *et al.* 2001; Pariza, 2004; Wahle *et al.* 2004; Bhattacharya *et al.* 2006). In view of this, an increasing interest in CLA has been aroused in scientific research field.

CLA is found in many foods, such as milk, natural and processed cheeses, meat products and plant oil; however food stuff originating from ruminants, especially beef and dairy products are the major dietary sources of CLA. The most desirable source of CLA, however, would be through the natural enrichment of fermented food products by the action of food grade micro-organisms. Although low levels

of CLA have been observed in the fermented dairy products, the origin of the LA transformation is generally uncertain and conflicting data have been reported on the capacity of some strains to produce CLA (Sieber *et al.* 2004). CLA could also be produced by microorganisms *in vitro*. It has been reported that CLA could be bio-synthesized by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens* from free linoleic acid (Kepler *et al.*, 1966; Sanchez *et al.*, 2004). In recent years, many papers reported that many other probiotics such as *Propionibacterium freudenreichii*, *Lactococcus Lactis*, *Lactobacillus salivarius*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum* etc. commonly used as a dairy starter culture, could also be able to convert free linoleic acid to its conjugated form.

Even though animal sources of foods such as beef and dairy products naturally have CLA, the concentration of CLA is low (Rule *et al.* 2002). Therefore, studies on enhancing CLA level in meat and dairy products by dietary manipulation and direct addition have increased in recent years (Marco *et al.* 2009; Martin *et al.* 2008).

Nowadays, CLA production by probiotic bacteria has received special interest in the research field (Nieuwenhove *et al.* 2012). However, the most common approach of previous studies for evaluating the potential of CLA production by food grade bacteria is *in vitro* setup. There is few previous study related to enhancing the amounts of CLA isomers in a meat system by the use of starter culture (*L. sakei* LMG 13558) which showed the ability to produce CLA *in vitro* (Gorissen *et al.* 2012). Present research was attempted to isolate and select, most efficient CLA producing bacteria followed by characterisation selected isolates and the CLA and the results are reported here.

## MATERIALS AND METHODS

### Samples collection

Seven commercial preparations of mango pickle procured from the local market having different vegetable oil in its composition were used for

the isolation. Besides the mango pickles several fermented dairy products such as curd, *lassi*, Swiss cheese (Emmental Cheese), yoghurt etc. as well as woman milk were also attempted to isolate LAB. All samples were procured and processed under aseptic condition. In all, 12 samples were collected.

### Enrichment for LAB

The samples (1 g) were weighed and enriched in 10 ml of MRS broth. The composition of MRS borth (g/l) was; proteose peptone (10 g), beef extract (10 g), yeast extract (5 g), dextrose (20 g), polysorbate 80 (1 g), ammonium citrate (2 g), sodium acetate (5 g), magnesium sulphate (0.10 g), manganese sulphate (0.05 g), dipotassium phosphate (2 g). The final pH was 6.5 ± 0.2. These were incubated at 37°C for 48 h. These enriched samples were further used for the isolation of LAB.

### Isolation of LAB

Enriched broth from each tube was used for isolation of LAB on MRS agar plates using pour plate technique. Then plates were incubated at temperature of 37°C for 24 h. Individual colonies were picked up and purified on MRS agar plates. Single purified distinct colony of the each LAB isolates were preserved at 5°C for further studies. Sub-culture of the preserved slants and master plates were carried out at regular interval.

### Screening of CLA producing LAB isolates

**Fermentation:** LAB strains were activated by repeatedly growing it on MRS medium. Then, 0.5 ml of the active culture (about 10<sup>7</sup>cfu/ml) was inoculated again in MRS medium containing 0.5 mg/ml LA. It was incubated at 37°C for 72 h. All the experiments were performed in triplicate.

**Lipid Extraction from culture supernatant:** Cultures were centrifuged at 5000 rpm for 10 min at 4°C after 72 h of incubation in MRS broth. Then, 3 ml of the supernatant was added to 6 ml isopropanol and vortexed the mixture for 1 min; 5 ml hexane was added to this mixture and again vortexed the mixture

for 1 min and finally centrifuged it at 2000 rpm for 5 min at 4°C. The hexane selectively extract the lipid (Alonso *et al.*, 2003; Bligh and Dyer, 1959).

**Estimation of CLA by UV Spectrophotometer:** CLA in the hexane layer was quantified using a UV/VIS spectrophotometer (Shimadzu, Japan) at 233 nm. Measurements were obtained in triplicate from 2 ml of the lipid extract in hexane placed into quartz cuvettes. In order to verify the suitability of this method, a standard curve was constructed for the absorbance at 233 nm versus CLA (C18:2 c9, t11) concentration.

#### Identification of CLA by GC analysis

CLA isomers produced by selected LAB were identified. 3 ml hexane extract from each culture was evaporated and dried on sodium sulphate; resultant Fatty Acid Methyl Esters (FAMES) were directly trans methylated with sulphuric acid in methanol (Christie, 2003) and analyzed using a 6890N GC system equipped with an FID, HP5% phenyl methyl silixane capillary column (30m× 0.32 mm i.d. and 0.25 µm film thickness) and HP5973 mass selective detector (Agilent Technologies, USA). Helium was used as a carrier gas at a flow rate of 1.5 ml/min. The oven temperature was held at 70°C for 2 min, then increased to 230°C at 8°C/min and maintained at this temperature for 20 min. Injector and detector temperatures were 250°C and 280°C, respectively. FAMES were identified by comparing the retention times with those of a standard FAME mixture (C18:2 c9, t11 & t10, c12) Sigma-Aldrich, purity > 99.0% by GC) using probability merge search software and the National Institute of Standards and Technology MS spectra search program.

#### Characterization of selected LAB Strain isolate

Morphological characterization of efficient CLA producing isolate CMLAB2 was carried out by Gram staining, spore staining etc. Cultural characteristics such as size, shape, texture, margin, elevation, opacity and pigmentation of the colony on MRS agar plate were studied. Selected biochemical characterizations of CMLAB2 isolate was carried out following the

Bergey's Manual of Systemic Bacteriology for its identification.

## RESULTS AND DISCUSSION

### Isolation of CLA producing LAB

Isolation of bacteria was carried out from the samples. This was followed by their screening for ability to produce CLA. The results are presented in Table 1.

**Table 1:** Potential CLA producing LAB isolates

Source	CLA producing LAB Isolates
Curd	CMLAB1 and CMLAB2
Yoghurt	YHMLAB1
Female Breast Milk	BMLAB1
Emmental cheese (Swiss cheese)	ECMLAB1
Lassi	LMLAB2 and LMLAB2
Pickle 1	P1MLAB1, P1MLAB2 and P1MLAB3
Pickle 2	P2MLAB1
Pickle 3	P3MLAB1
Pickle 4	P4MLAB1
Pickle 5	P5MLAB1
Pickle 6	P6MLAB1
Pickle 7	P7MLAB1

### Screening for CLA Producing LAB

All the isolates were assayed for the CLA production from linoleic acid. They were incubated in MRS broth containing free LA (0.5 mg/ml) at 37 °C for 72 h. Subsequently, CLA production was assessed by using UV-spectrophotometric analysis at 233 nm. A standard curve was prepared by plotting CLA concentration versus absorbance at 233 nm. An increase in CLA concentration from 0 to 0.08 mg/ml coincided with a linear increase in absorbance from 0 to 2 with 0.99 R<sup>2</sup> demonstrated the suitability of graph.

After extensive screening from different samples, 16 CLA producing LAB isolates were obtained (Table 1). These isolates were further screened out based

on their ability to convert free linoleic acid into CLA in fermentation assay. The result of % conversion efficiency of potential 16 LAB isolates is shown in Figure 1. Out of the 16 CLA producers CMLAB2 was found to convert maximum of 48.36±1.2% LA into CLA isomers. While, isolates BMLAB1 and P2MLAB1 were also found to convert free LA into CLA in significant amount (Fig. 1). Rest of the isolates had shown less than 25 % conversion rate.

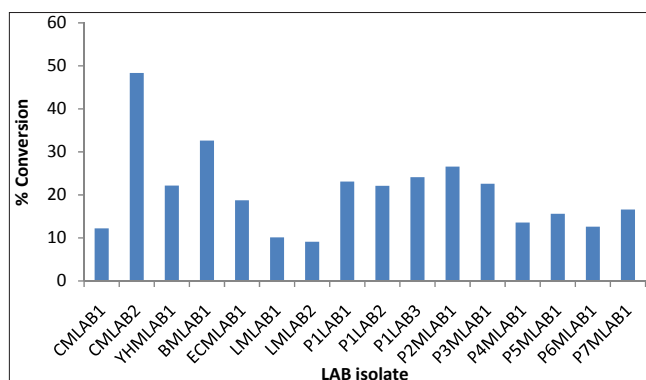


Fig. 1: Screening of LAB isolates based on % conversion of free LA to CLA

Looking to overall results, the isolates CMLAB2, BMLAB1 and P2MLAB1 were found superior to others and thus were further investigated.

**Identification CLA isomers using GC analysis**

The secondary screening was carried out based on the GC analysis. The CLA production by three selected isolates i.e. CMLAB2, BMLAB1 and P2MLAB1 were studied. The isolates were grown in the MRS broth containing LA (0.5 mg/ml) and after incubation the lipid extraction was carried out from the fermented broth. This FAME extracts were investigated for the presence of CLA qualitatively as well as quantitatively. The results showed that the isolate CMLAB2, BMLAB1 and P2MLAB1 were able to produce 21.21; 14.68; 6.35 µg/ml of CLA in reaction mixture respectively. The c9, t11CLA isomer and t10, c12 CLA isomers were confirmed in the CMLAB2 and BMLAB1 FAME analysis. The retention times of c9, t11CLA isomer and t10, c12 CLA isomer were 20.58 and 20.37 min. respectively. While, significant production of these

isomers were not found in the FAME of P2MLAB1 isolate. CMLAB2 isolate was found to produce c9, t11 isomer most efficiently than the BMLAB1. Looking to this prospect, the isolate CMLAB2 was further investigated for its identification by morphological, cultural and biochemical characterization.

**Characterization of selected LAB isolate CMLAB2**

Selected most efficient CLA producing LAB isolates CMLAB2 was identified based on Bergey’s manual of determinative bacteriology. The results are summarized in Table 2.

**Morphological characterization:** CMLAB2 isolate was Gram positive, non-spore forming, rod-shaped, about 0.1-1.1 µm × 2.5 µm in dimension which is shown by arrows in Fig. 2. Cells occur in one plane with tendency to form chain (Table 2).

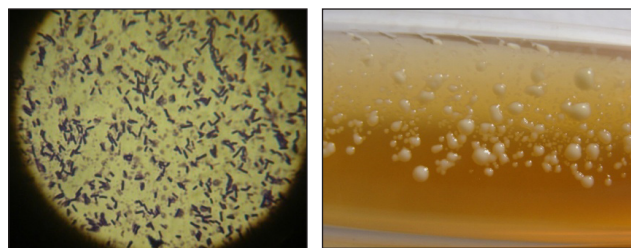


Fig. 2: Morphological characterization of CMLAB2 isolate

**Cultural and Biochemical characterization:** The result of cultural and biochemical characterization are summarized in Table 2. The CMLAB2 isolate is found to be mesophilic, catalase negative, facultative hetero-fermentative *Lactobacilli* (Table 2). Based on the results of morphological, cultural and biochemical characteristics, the CMLAB2 was tentatively identified on as species of *Lactobacilli*.

Table 2: Characterization of selected CMLAB2 isolate

Morphological Characteristics	
Size	Small
Shape	Short rod
Arrangement	Single and few in chain
Gram reaction	Gram positive
Endospore	Absent

Cultural characteristics	
Size	Small
Shape	Round
Consistency	Moist
Texture	Smooth
Margin	Entire
Elevation	Convex, Slightly raised
Opacity	Opaque
Odour	Nil
Motility	Not found
Biochemical characteristics	
Salt tolerance:	
7% NaCl	+
10% NaCl	+
Temperature of growth:	
4 °C	-
40 °C	+
45 °C	+
50 °C	-
Acid Production from:	
L - arabinose	+
Galactose	+
Gluconate	+
Inulin	-
Lactose	+
Maltose	+
Melezitose	+
Melibiose	+
Raffinose	+
Sucrose	+
Salicin	+
Sorbitol	+
Catalase	-
Voges - Proskauer test	-
Arginine hydrolysis	-
Growth in acetate agar	+

+ Positive ; - Negative

## CONCLUSION

LAB were isolated from the twelve diverse ecosystems. Total 16 LAB isolates were found to produce CLA in fermentation experiment followed by its spectroscopic analysis. Isolate CMLAB2 was found to most efficient in terms of its ability to

convert free LA into CLA. The CLA extract produced by this potential isolate was analysed for FAME by using GC and potential of the isolate for production of physiologically important c9, t11CLA and t10, c12 CLA isomers in significant amount was confirmed. Looking to the better prospects of this isolate, it was characterized by morphological, cultural and biochemical potentials and was tentatively identified as species of *Lactobacilli*.

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