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Synthesis of conjugated linoleic acid by the linoleate isomerase complex in food-derived lactobacilli

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Abstract

Aims—To assess strains of lactobacilli for their capacity to produce functional fatty acid-conjugated linoleic acid. To assess the linoleate isomerase for CLA production in the most efficient CLA producer.

Methods and Results—In this study, strains of food-derived lactobacilli were cultured in media with linoleic acid and CLA production was assessed. Most of the selected strains produced CLA at different levels, with *Lactobacillus plantarum* ZS2058 being the most efficient CLA producer converting over 50% of linoleic acid to c9, t11-CLA and t9, t11-CLA. Some intermediates 10-hydroxy-cis-12-octadecenoic acid, 10-oxo-cis-12-octadecenoic acid and 10-oxo-trans-11-octadecenoic acid were determined via GC-MS. The genes coding the multicomponent linoleate isomerase containing myosin-cross-reactive antigen, short-chain dehydrogenase/oxidoreductase and acetoacetate decarboxylase for CLA production in *Lact. plantarum* ZS2058 were cloned and expressed in *Escherichia coli*. With the mixture of recombinant *E. coli*, c9, t11-CLA and three kinds of intermediates were produced from linoleic acid, which were in line with those in the lactobacilli.

Conclusions—The ability for CLA production by lactobacilli exhibited variation. *Lactobacillus plantarum* and *Lact. bulgaricus* were the most efficient producers in the selected strains. *Lact. plantarum* ZS2058 converted linoleic acid to CLAs with 10-hydroxy-cis-12-octadecenoic acid, 10-oxo-cis-12-octadecenoic acid and 10-oxo-trans-11-octadecenoic acid as intermediates. The multiple-step reactions for CLA production catalysed by multicomponent linoleate isomerase in *Lact. plantarum* ZS2058 were confirmed successfully.

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Conflict of interest
We declare that we have no conflict of interest.

Significance and Impact of the study—Multicomponent linoleate isomerase provides important results for the illustration of the mechanism for CLA production in lactic acid bacteria. Food-derived lactobacilli with CLA production ability offers novel opportunities for functional foods development.

Keywords

conjugated linoleic acid; lactic acid bacteria; *Lactobacillus plantarum*; linoleate isomerase

Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA, 18:2 n-6) with conjugated double bonds. In the past several decades, CLA has attracted great interest because of its heath-associated activities including anticarcinogenic (Ip *et al.* 1991, 1994; Field and Schley 2004; Shen *et al.* 2013), anti-atherogenic (Lee *et al.* 1994; Valeille *et al.* 2004; McClelland *et al.* 2010), antidiabetic (Moloney *et al.* 2007; Castro-Webb *et al.* 2012; Rungapamestry *et al.* 2012), anti-inflammatory (Sugano *et al.* 1998; Coakley *et al.* 2006) and anti-obesity (Noone *et al.* 2002; Park *et al.* 2004; Sluijs *et al.* 2010).

CLA isomers occur naturally in ruminant meat and a variety of dairy food derived from ruminants as a minor component of the lipid fraction. CLAs are formed as intermediates during linoleic acid biohydrogenation to stearic acid by the anaerobic rumen bacteria. The complete biohydrogenation of linoleic acid by the anaerobic rumen bacterium (such as *Butyrivibrio fibrisolvens*) is a multi-step process (Kepler and Tove 1967). The first reaction occurs rapidly by linoleate isomerase, the conversion of linoleic acid to c9, t11-CLA, followed by the slower conversion to trans-11 vaccenic acid (Kepler *et al.* 1966). Vaccenic acid is known to be reduced to stearic acid by microbial activity other than that of *B. fibrisolvens* in the rumen. Vaccenic acid can also be converted to c9, t11-CLA by the delta-9 desaturase in the mammary tissue itself, providing another mechanism for its formation in milk (Griinari and Bauman 1999).

Additionally, it has also demonstrated that certain strains used in food fermentation possess the capacity to generate c9, t11-CLA. Two strains of *Propionibacterium freudenreichii* subsp. *freudenreichii* and one strain of *P. freudenreichii* subsp. *shermanii* can convert free LA to c9, t11-CLA (Jiang *et al.* 1998). Several bifidobacteria, isolated from the human gut and other sources, can produce c9, t11-CLA with LA in the medium (Coakley *et al.* 2003; Rosberg-Cody *et al.* 2004; Gorissen *et al.* 2010). Furthermore, several studies have reported the production of CLA isomers from LA by different lactic acid bacteria grown in MRS, skim milk and cheddar cheese (Lin *et al.* 1999; Alonso *et al.* 2003; Mohan *et al.* 2013; Ye *et al.* 2013).

To date, only three linoleate isomerases derived from *Lactobacillus reuteri* PYR8 (Rosson *et al.* 2004), *Clostridium sporogenes* (Peng *et al.* 2007) and *Propionibacterium acnes* (Liavonchanka *et al.* 2006) have been characterized. The linoleate isomerase from *Lact. reuteri* PYR8 was a myosin-cross-reactive antigen (MCRA), which was originally found in

Streptococcus pyogenes and predicted to have a polyunsaturated fatty acid isomerase function (Kil *et al.* 1994). Several putative linoleate isomerases, which were highly homologous to that from *Lact. reuteri* PYR8, were expressed in *E. coli*; unfortunately, none can produce CLA (Volkov *et al.* 2010; Rosberg-Cody *et al.* 2011), and instead of CLA, 10-hydroxy-cis-12-octadecenoic acid (10-HOE) was produced. A multiple-fraction linoleate isomerase was purified from *Lact. plantarum* AKU 1009a, which produced c9, t11-CLA, t10, c12-CLA and t9, t11-CLA, but no detailed results for the enzyme were reported (Kishino *et al.* 2011a). In a later study, the genes encoding the multicomponent enzyme machinery catalysing double bond migration in *Lact. plantrum* AKU 1009a were illustrated (Kishino *et al.* 2011b), with the transformed *E. coli* as the catalysts, t9, t11-CLA was produced at a significant level with c9, t11-CLA and 10-HOE. A multiple-step reaction for CLA production in *Lactobacillus* was hypothesized but without evidences for the putative intermediates. Recently, the mass spectra and NMR data for 10-hydroxyl-cis-12-octadecenoic acid, 10-oxo-cis-12-octadecenoic acid and 10-oxo-trans-11-octadecenoic acid, intermediates in CLA bioconversion catalysed by the multicomponent linoleate isomerase, were further demonstrated (Kishino *et al.* 2013).

In our previous study, MCRAs from several lactic acid bacteria were confirmed as fatty acid hydratase (Yang *et al.* 2013). In the present study, a selection of strains including different food-derived lactobacilli was assessed for CLA production from free linoleic acid. The genetic determinants for CLA production in the most efficient producer, *Lact. plantarum* ZS2058, were cloned in isolation in *E. coli*, and the ability of the resultant strains was then assessed for CLA production successfully.

Materials and methods

Strains, media and growth conditions

The lactic acid bacteria strains used in this study are detailed in Table 1. Strains of lactobacilli were cultured in de Man, Rogosa and Sharpe (MRS) medium consisting of 1·0% tryptone, 1·0% meat extract, 0·5% yeast extract, 2·0% glucose, 0·1% Tween 80, 0·2% K_2HPO_4, 0·5% sodium acetate, 0·2% diammonium citrate, 0·02% $MgSO_4{\cdot}7H_2O$ and 0·005% $MnSO_4{\cdot}H_2O$ (pH 6·5) under anaerobic conditions at 37°C for 48 h. When solid media were required, 2·0% agar was added to the MRS medium. For growth of bifidobacteria, 0·05% (w/v) L-cysteine hydrochloride was added to the MRS medium and cultured at 37°C for 48 h. *Escherichia coli* BL21 (DE3) carrying the plasmid pET28a was routinely cultured aerobically in Luria–Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) at 37°C in the presence of kanamycin (50 μg ml^{-1}) as a selective marker.

Fat extraction from bacterial supernatant fluids and pellets

Prior to examination of the strains for CLA production, each was subcultured twice in MRS broth. All strains were then cultured (1%) in broth spiked with 0·55 mg ml^{-1} free linoleic acid (99% purity; Sigma, St. Louis, MO). The linoleic acid was added as a 30 mg ml^{-1} stock solution containing 2% (v/v) Tween 80 and was previously filter sterilized through a 0·45-μm Minisart filter (Sigma) and stored in the dark at −20°C. The strains were incubated

anaerobically at 37°C in a modular atmosphere-controlled system (Whitley DG250 anaerobic workstation; Don Whitley Scientific, West Yorkshire, UK) that was continuously sparged with a mixture of 80% nitrogen, 10% carbon dioxide and 10% hydrogen.

Lipid extraction and methylation

After 48-h incubation, the cultures were centrifuged at 5000 ***g*** for 10 min at room temperature. The fat was extracted from the culture supernatant fluid as follows. The internal standard, C17:0 heptadecanoic acid (99% pure; Sigma), was added to 4 ml of the supernatant fluid to give a final concentration of 217 μg internal standard per sample. Four millilitre of isopropanol was added to the supernatant fluid, and the samples were vortexed for 30 s. Four millilitre of n-hexane was added to this mixture, vortexed and centrifuged at 5000 ***g*** for 5 min. The resultant hexane layer (containing lipids) was dried off under a stream of nitrogen.

The fat was extracted from the bacterial pellet as follows. The pellet from 10 ml of bacterial culture was washed in 2 ml saline solution (0·137 mol l^{-1} NaCl, 7·0 mmol l^{-1} K_2HPO_4 and 2·5 mmol l^{-1} KH_2PO_4). The cells were vortexed and centrifuged at 5000 ***g*** for 10 min and the washing step repeated twice. The cells were suspended in 2 ml saline solution, and then the samples were extracted completely as described above for the bacterial supernatant fluid.

Preparation of fatty acid methyl esters and gas chromatography analysis

Fatty acids were converted to corresponding methyl esters with (trimethylsilyl)-diazomethane (Sigma) as described previously (Yang *et al.* 2013). The FAMEs were extracted in n-hexane and separated on a Rtx-2560 column (100 m × 0·25 mm × 0·25 μm) using a gas chromatograph (GC2010 plus, Shimadzu, Kyoto, Japan) fitted with a QP2010 ultra mass spectrometer. Injections of 1 μl were administered automatically at a split ratio of 10 : 1. Helium was used as the carrier gas. The column temperature was set initially at 150°C, then increased to 200°C in increments of 5°C min^{-1} and maintained for 10 min, and finally increased to 240°C in a rate of 4°C min^{-1}. The 240°C was maintained for 10 min. The injector and detector were operated at 240°C. Electron energy of 70 eV and ion source temperature of 220°C were used. The CLA isomers were identified by retention time with reference to CLA standard mix (Sigma). The percentage conversion to CLA isomers was calculated by dividing the amount of CLA present in the broth after inoculation/ incubation with the amount of linoleic acid present in the spiked broth before incubation.

Cloning and expression of recombinant protein in *Escherichia coli*

Genomic DNAs were isolated rapidly from *Lact. plantarum* ZS2058 as described by Hoffman and Winston (1987). According to previous result (Kishino *et al.* 2011b), myosin-cross-reactive antigen (*mcra*, GenBank: JF747255.1), short-chain dehydrogenase/ oxidoreductase (*dh*, GenBank: KJ019513) and acetoacetate decarboxylase (*dc*, GenBank: KJ019514) were amplified from *Lact. plantarum* ZS2058 genomic DNA with specific primers according to their homologous gene in *Lact. plantarum* WCFS1 (GenBank: NC_004567). PCR was performed with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's protocol. PCR conditions were as follows: 30 cycles of 45 s of denaturation (94°C), 30 s of annealing (55°C) and 2 min of elongation (68°C). For

expression in *E. coli*, the three genes were amplified with the primers listed in Table 2 and cloned into the pET28a expression vector (Novagen, Darmstadt, Germany), yielding plasmids pET28a-*mcra*, pET28a-*dh* and pET28a-*dc* (*N*-His-tagged version).

For protein production, *E. coli* BL21 (DE3) Star strain (Invitrogen, Carlsbad, CA) harbouring pET28a-*mcra*, pET28a-*dh* and pET28a-*dc* plasmids, separately, was used. Bacteria were cultivated in LB medium supplied with kanamycin at 37°C until OD_{600} reached 0·6. At that point, IPTG was added to a final concentration of 0·05 mmol l^{-1}, and the culture was placed at 18°C for 10-h induction of protein expression. pET28a vector-inserted *E. coli* was used as negative control. Following induction, cells were harvested by centrifugation, washed with 20 mmol l^{-1} potassium phosphate buffer (KPB) (pH 6·5) and sonicated (Uilbra-Cell VCX500; Sonics & Materials Inc., Newtown, CT). The cell debris was removed by centrifugation, and the supernatant containing soluble proteins was collected. Ten micrograms of protein was subjected to SDS-PAGE (12% SDS-PAGE) followed by protein transfer to a PVDF membrane (Amersham Pharmacia Biotech, Amersham, UK); the immunoblots were developed with the use of anti-His antibody at a dilution of 1 : 2000 (Tiangen, Beijing, China). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Tiangen) secondary antibody diluted at 1 : 500 was used for the detection of specific antibody binding. The bands were visualized with enhanced chemiluminescence reagents (Kangwei, Beijing, China) according to the manufacturer instructions.

Activity assay and fatty acid analysis

For activity assay, the reaction was performed in a screw tube that contained 1 ml of reaction mixture (20 mmol l^{-1} KPB, pH 6·5) with 5 mmol l^{-1} NADH and 0·1 mmol l^{-1} FAD as cofactors, 0·5 mg ml^{-1} linoleic acid complexed with BSA as substrate and the recombinant *E. coli* cells under microaerobic conditions in a sealed chamber filled with N_2 and shaken (180 strokes min^{-1}) at 37°C for 6 h. All the transformed *E. coli* were suspended in KPB at a final concentration of 0·5 mg ml^{-1} wet cells. Fifty microlitre of each transformed *E. coli* suspension was used as catalyst in various combinations. The following reactions were carried out: (i) *E. coli* BL21(DE3)/pET28a-*mcra* recombinant suspension plus free linoleic acid, FAD and NADH; (ii) *E. coli* BL21(DE3)/pET28a-*mcra* recombinant suspension with FAD and NADH without linoleic acid; (iii) *E. coli* BL21(DE3)/pET28a-*mcra* recombinant suspension and *E. coli* BL21(DE3)/pET28a-*dh* recombinant suspension with substrate, FAD and NADH; (iv) *E. coli* BL21(DE3)/pET28a-*mcra* recombinant suspension and *E. coli* BL21(DE3)/pET28a-*dh* recombinant suspension without substrate but with cofactors; (v) mixture of *E. coli* BL21(DE3)/pET28a-*mcra* suspension, *E. coli* BL21(DE3)/pET28a-*dh* suspension and *E. coli* BL21(DE3)/pET28a-*dc* suspension plus linoleic acid, FAD and NADH; and (vi) all the three transformed *E. coli* suspension mixed with FAD and NADH without substrate. Following reaction, fatty acids were extracted, methylated and analysed as above.

Results

Screening of lactobacilli for conjugated linoleic acid production

In this study, a number of strains of lactobacilli (see Table 1) were assessed for the ability to generate CLA from free linoleic acid. The origin of these strains varied in vegetable fermentation and dairy fermentations to human intestinal isolates. Bifidobacteria previously reported to synthesize c9, t11-CLA from free linoleic acid, *Bifidobacterium animalis* subsp. *lactis* BB-12 (Coakley *et al*. 2003) and *Lact. reuteri* ATCC55739 (Rosson *et al.* 2004) were used as positive controls in this study.

Twenty-five selected strains were investigated for their ability to convert free linoleic acid (0·55 mg ml^{-1}) to CLA. The results demonstrated that c9, t11-CLA, t10, c12-CLA and t9, t11-CLA could be generated, but to varying levels ranging from 3 to 56% for CLA production. Most CLA isomers were found in the supernatant from the cultures rather than in the cell pellets. All nine strains of *Lact. plantarum* tested, except *Lact. plantarum* STIII, produced CLA. *Lact. plantarum* ZS2058 was the most efficient of the lactobacilli strains tested for the conversion of linoleic acid to CLA. There was a 37·78% conversion of linoleic acid into c9, t11-CLA and 16·57% conversion to t9, t11-CLA. *Lact. bulgaricus* CCFM3004, *Lact. bulgaricus* CCFM3029, *Lact. crispatus* CCFM5136, *Lact. gasseri* CCFM5115 and *Lact. helveticus* CCFM8310 converted 10–20% linoleic acid into three CLA isomers. Of the remaining strains in Table 1, some did not convert linoleic acid to CLA at any significant level, including *Lact. acidophilus*, *Lact. brevis*, *Lact. rhamnosus*, one strain of *Lact. casei* and one strain of *Lact. plantarum*. Although the concentration of 10-HOE differed (data not shown), it was produced in all the assessed strains.

Conjugated linoleic acid production by *Lactobacillus plantarum* ZS2058 in the presence of linoleic acid

As *Lact. plantarum* ZS2058 was the most efficient strain for linoleic acid conversion in this study, this strain was then studied in a lot more detail. Following culturing with linoleic acid, both c9, t11-CLA and t9, t11-CLA isomers were mainly found in cell supernatant, with very low concentration being recovered in the cell pellet (data not shown). Interestingly, t10, c12-CLA was not significantly produced in this strain. Indeed, c9, t11-CLA was the major isomer and represented over 65% in total CLAs. During CLA production, three kinds of intermediates were produced (Fig. 1), based on previous result (Yang *et al.* 2013), and the mass spectra in this study, the intermediate with retention time at 36·3 min, with significant mass fractions of 169 and 201 *m*/*z* were identified as 10-HOE (Figs 1 and 2a). As all the intermediates in the reaction were out of commercial standards, according to previous hypothesis (Kishino *et al.* 2011b), the fragmentation patterns of each intermediate were analysed. As structures of the two intermediates, 10-oxo-cis-12-octadecenoic acid and 10-oxo-trans-11-octadecenoic acid, were of high similarity, amount of mass fractions were identical, such as 171, 139 and 199 *m*/*z* (Fig. 2b, c). The significant difference between the two intermediates was the position of carbon double bond; therefore, 213 *m*/*z* was more notable in 10-oxo-cis-12-octadecenoic acid instead of 225 m/z in 10-oxo-trans-11-octadecenoic acid (Fig. 2b, c).

Expression and activity of the recombinant proteins

Based on the previous hypothesis (Kishino *et al.* 2011b), the three genes encoding myosin-cross-reactive antigen (*mcra*), short-chain dehydrogenase/oxidoreductase (*dh*) and acetoacetate decarboxylase (*dc*) were cloned from *Lact. plantarum* ZS2058. These three genes were overexpressed separately in *E. coli* BL21 (DE3) (Fig. 3a). Major bands with apparent molecular weights of 64·76 kDa (MCRA), 32·10 kDa (DH) and 30·71 kDa (DC) were visualized by SDS-PAGE and these corresponded to the expected molecular weights of the three proteins, which were absent in pET28a vector inserts. Immunoblotting with anti-His-tag antibodies confirmed that each single band contained a 6× His tag (Fig. 3b).

To assess the activity of the three recombinant proteins, different combinations of the resultant cells were tested for conversion of linoleic acid. In the case where the MCRA recombinant was used for reaction, linoleic acid was only converted to 10-HOE according to the retention time and mass fraction compared with that in *Lact. plantarum* ZS2058 (Fig. 4b). Addition of the *E. coli* cells containing DH protein resulted in the production of both 10-HOE and 10-oxo-cis-12-octadecenoic acid (Fig. 4c). While combination of all the three recombinant *E. coli* resulted in 10-HOE, 10-oxo-cis-octadecenoic acid, 10-oxo-trans-11-octadecenoic acid and c9, t11-CLA (Fig. 4d), about 13·66% of LA was converted to CLA by the recombinant *E. coli*.

Discussion

The reason for bacteria converting linoleic acid to CLA is unclear, and it remains uncertain as to why some strains of lactobacilli, in particular *Lact. plantarum*, exert superior CLA producers while some other do not produce CLA at a significant level. It has been reported that bioconversion of linoleic acid to CLA might be a key step for fatty acid detoxicification in bacteria (Jiang *et al.* 1998; Maia *et al.* 2007, 2010). In the present study, the ability of 25 strains of different food-derived lactobacilli to produce CLA from free linoleic acid was investigated. None of the *Lact. acidophilus*, *Lact. brevis* and *Lact. rhamnosus* tested converted linoleic acid to CLA at a significant level, while the range of *Lact. plantarum* exhibited considerable CLA production ability. From those strains, *Lact. plantarum* ZS2058, isolated from Chinese traditional fermented sauerkraut (Niu *et al.* 2007), was the most efficient producer of CLA, in which over 50% linoleic acid was converted to c9, t11-CLA and t9, t11-CLA as dominant isomers. The conversion percentages reported in the present study are in agreement with earlier studies (Alonso *et al.* 2003; Zeng *et al.* 2009; Li *et al.* 2012). Considering the potential health promotion of CLA, the discovery of food-grade lactobacilli with high ability to synthesize CLA, such as *Lact. plantarum* and *Lact. bulgaricus* strains in this study, may offer novel opportunities for developing health-promoting functional food safely with the multiple benefits of CLA and probiotics.

Following culturing with free linoleic acid in MRS medium, *Lact. plantarum* ZS2058 converted more than 50% linoleic acid to c9, t11-CLA and t9, t11-CLA. In addition, 10-HOE was produced during CLA production. Ogawa *et al.* (2001) firstly reported the 10-HOE accumulation in CLA production in *Lact. acidophilus*. With *Lact. acidophilus* AKU1137 grown in MRS medium containing free linoleic acid for four days, 10-HOE was significantly accumulated from linoleic acid in the first two days, while CLA was produced

slowly. Moreover, when the concentration of 10-HOE reached a high level, it was converted into CLA rapidly. Volkov *et al.* (2010) firstly reported the MCRA from *S. pypgenes* as a fatty acid double-bond hydratase, which converted linoleic acid into 10-HOE and 10, 13-dihydroxy-octadacenoic acid. MCRA from *Bifidobacterium breve* NCIMB 702258, a high CLA producer, was reported as a FAD-dependent fatty acid hydratase (Rosberg-Cody *et al.* 2011), which has a function in stress protection. Kishino *et al.* (2011a) reported the multiple-component enzymes for CLA production from *Lact. plantarum* AKU1009a. With different fractions from ultracentrifugation, 10-HOE was produced by a membrane-bound protein; further then c9, t11-CLA was produced while the membrane fraction was mixed with the other two unknown proteins from soluble fractions. Later, more MCRAs were reported as fatty acid hydratases from different bacteria (Joo *et al.* 2012; Kim *et al.* 2012; Yang *et al.* 2013).

Following culturing with free linoleic acid, 10-HOE, 10-oxo-cis-12-octadecenoic acid and 10-oxo-trans-11-octadecenoic acid combined with c9, t11-CLA and t9, t11-CLA were produced at different levels in *Lact. plantarum* ZS2058. The conversion of linoleic acid to 10-HOE and 10-oxo-octadecenoic acid derivatives was in agreement with previous hypothesis (Kishino *et al.* 2011b), in which CLA production was a multiple-step reaction: firstly, linoleic acid was converted to 10-HOE, then dehydrated and double-bond isomerized to 10-oxo-trans-11-octadecenoic acid, followed by rehydration and converted to CLA as the end product.

In the present study, MCRA was amplified and confirmed as fatty acid hydratase and played a role in linoleic acid hydration to 10-HOE. The latter two genes, *dh* and *dc*, were also expressed in *E. coli*. With the mixture of MCRA and DH recombinants, both 10-HOE and 10-oxo-cis-12-octadecenoic acid were converted, while the two recombinants were mixed with DC recombinant, all 10-HOE, 10-oxo-cis-12-octadecenoic acid, 10-oxo-trans-11-octadecenoic acid and c9, t11-CLA were produced. The present results were highly in agreement with a newest result (Kishino *et al.* 2013), in which *cla-hy*, *cla-dh* and *cla-dc* from *Lact. plantarum* AKU1009a were the genes in charge of linoleic acid bioconversion to CLA. Those results indicated that the mechanism for CLA production might be shared by different *Lactobacillus* species. The lactobacilli strain consisting the *mcra*, *dh* and *dc* genes for multiple-component linoleate isomerase genome might have the ability for CLA production, which needs further investigation.

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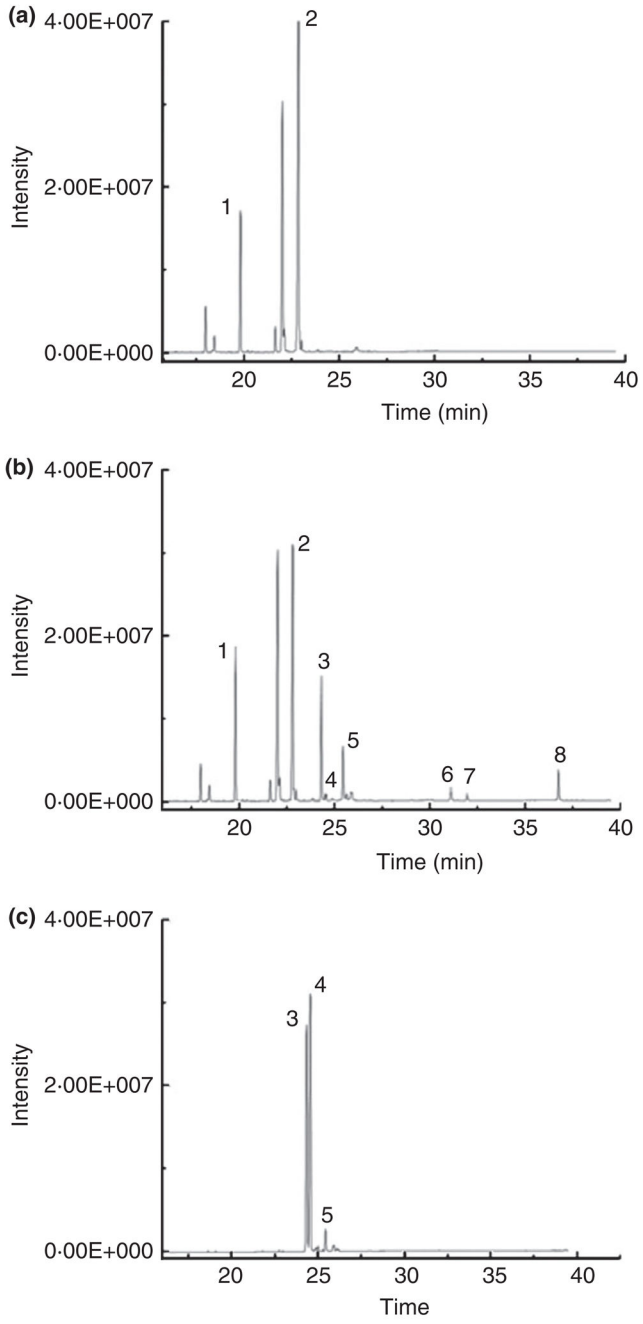

Figure 1.

GC-MS total ion chromatograms of the products from fatty acid profile of *Lactobacillus plantarum* ZS2058 grown in MRS plus 0·55 mg ml^{-1} linoleic acid. (a) *Lact. plantarum* ZS2058 at 0 h; (b) *Lact. plantarum* ZS2058 at 48 h; (c) CLA standard. (1) C17:0 (Internal standard); (2) linoleic acid; (3) c9, t11-CLA; (4) t10, c12-CLA; (5) t9, t11-CLA; (6) 10-oxo-cis-12-octadecenoic acid; (7)10-oxo-trans-11-octadecenoic acid; and (8) 10-hydroxyl-cis-12-octadecenoic acid.

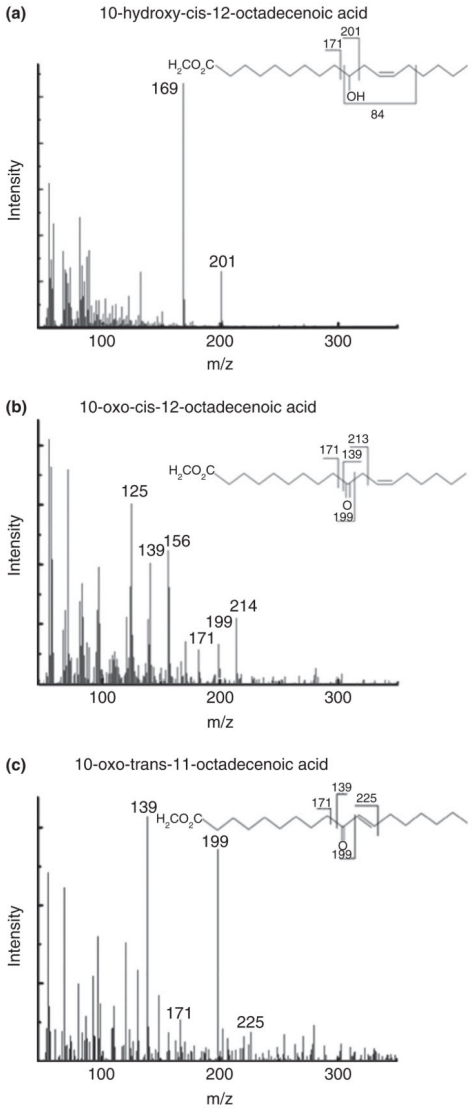

Figure 2.

Mass fractions of the intermediate products. (a) Mass spectra of 10-HOE and its fragmentation pattern. (b) Mass spectra of 10-oxo-cis-12-octadecenoic acid and its fragmentation pattern. (c) Mass spectra of 10-oxo-trans-11-octadecenoic acid and its fragmentation pattern.

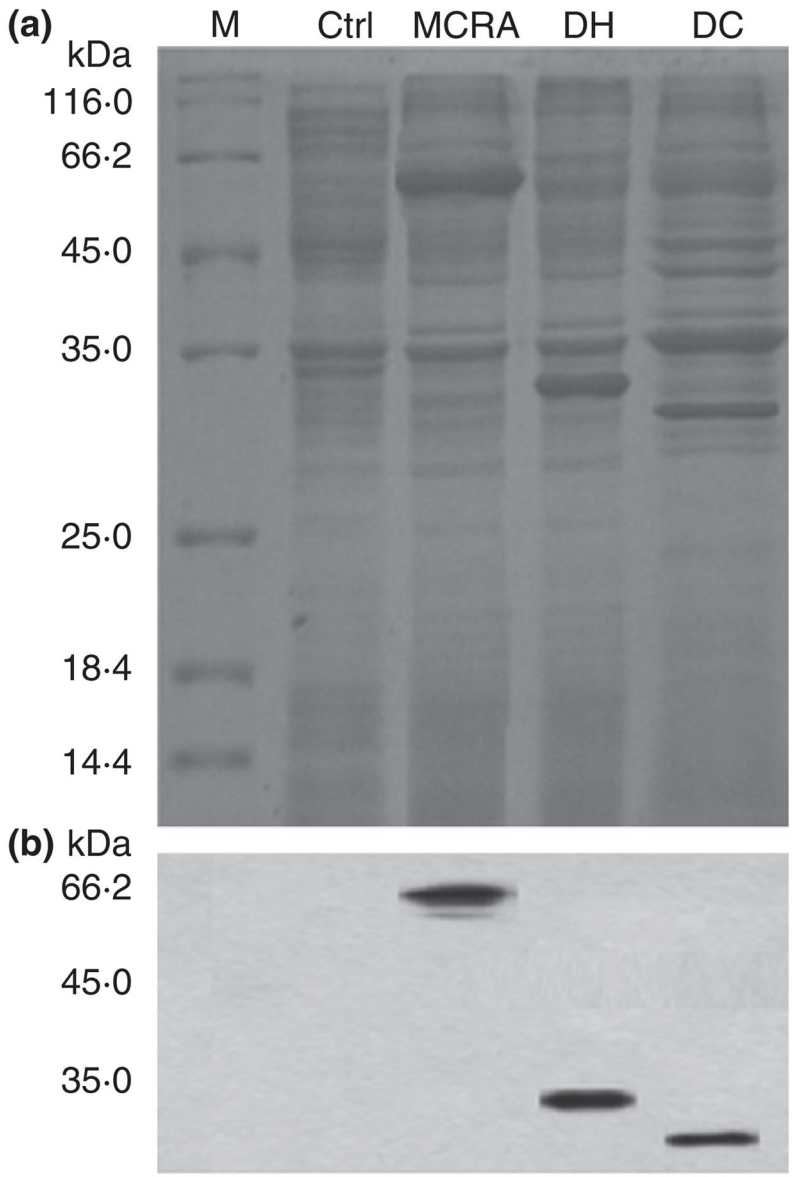

Figure 3.

SDS-PAGE and Western blot analyses of the recombinant proteins. (a) SDS-PAGE of control, MCRA, DH and DC; (b) Western blot of control, MCRA, DH and DC. Lane: M: Marker; Ctrl: *E. coli*/pET28a; MCRA; DH and DC.

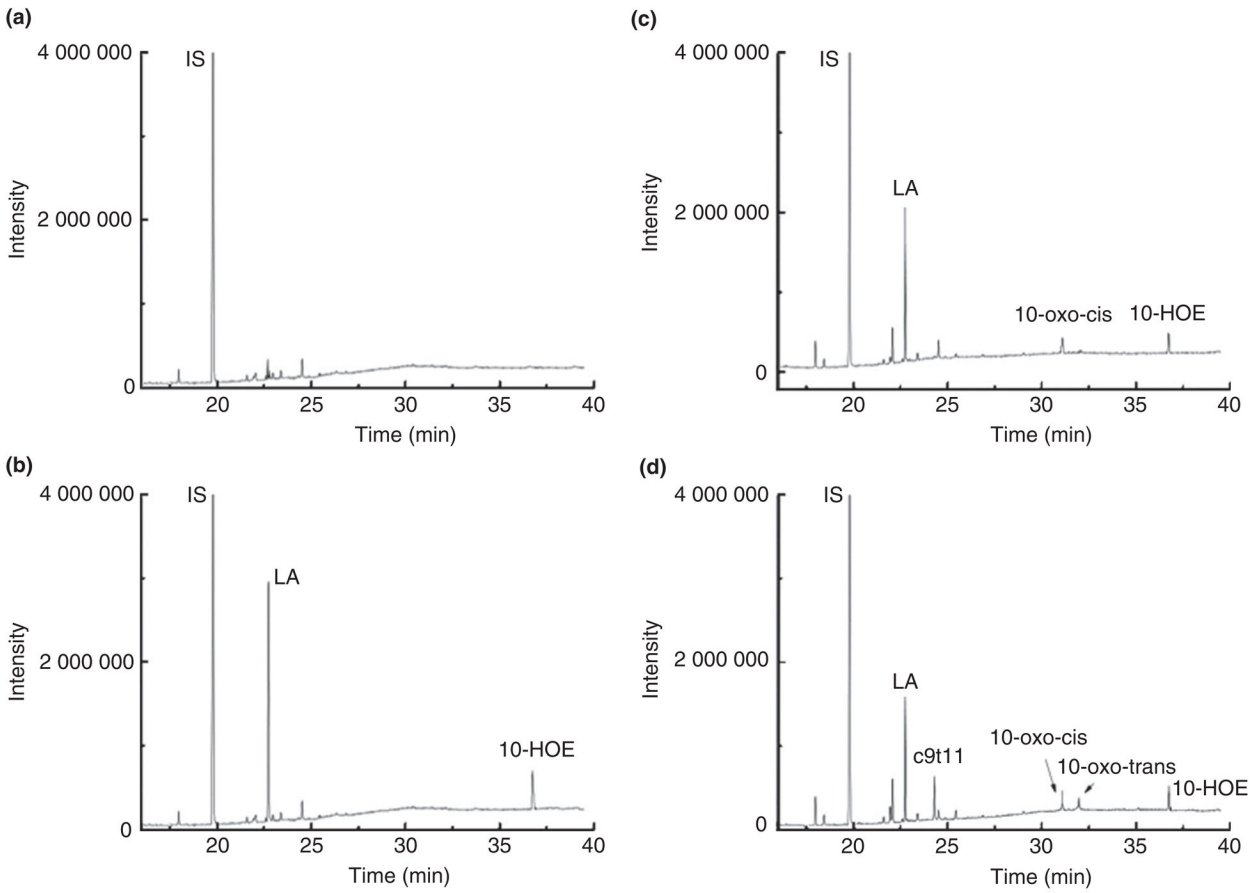

Figure 4.

Fatty acid profile after reaction of recombinant *E. coli* suspension in various combinations. (a) *E. coli*/pET28a-*mcra*, *E. coli*/pET28a-*dh* and *E. coli*/pET28a-*dc* plus FAD and NADH without linoleic acid. (b) *E. coli*/pET28a-*mcra* plus FAD and NADH with linoleic acid. (c) *E. coli*/pET28a-*mcra* and *E. coli*/pET28a-*dh* plus FAD and NADH with linoleic acid. (d) *E. coli*/pET28a-*mcra*, *E. coli*/pET28a-*dh* and *E. coli*/pET28a-*dc* plus FAD and NADH with linoleic acid. IS: internal standard (C17:0); LA: linoleic acid; 10-HOE: 10-hydroxy-cis-12-octadecenoic acid; 10-oxo-cis: 10-oxo-cis-12-octadecenoic acid; 10-oxo-trans: 10-oxo-trans-11-octadecenoic acid; c9, t11: c9, t11-CLA.

Table 1

List of selected strains in the present study

			CLA production from 0·55 mg ml^{-1} linoleic acid								
Species	**Strain**	**Biological Origin**	**c9,t11**	**S.D.**	**% converted**	**t10,c12**	**S.D.**	**% converted**	**t9,t11**	**S.D.**	**% converted**
Lactobacillus acidophilus	CCFM1006	Unknown	0·0039	0·0006	0·71	0·0035	0·0004	0·64	0·0082	0·0005	1·49
	CCFM1137	Unknown	0·0031	0·0001	0·56	0·0028	0·0003	0·51	0·0105	0·0008	1·91
	NCFM	Human GIT	0·0033	0·0003	0·60	0·0033	0·0002	0·60	0·0106	0·003	1·93
Lact. brevis	CCFM2135	Unknown	0·0035	0·0003	0·64	0·0026	0·0008	0·47	0·0127	0·001	2·31
	ATCC14869	Unknown	0·0046	0·0001	0·84	0·0038	0·0001	0·69	0·0081	0·0008	1·47
Lact. bulgaricus	CCFM3004	Yoghurt	0·0373	0·0009	6·78	0·0254	0·0001	4·62	0·0546	0·009	9·93
	CCFM3029	Unknown	0·0415	0·0001	7·55	0·0349	0·0004	6·35	0·0494	0·0007	8·98
Lact. casei	BD-II	Koumiss	0·0254	0·0009	4·62	0·0233	0·0008	4·24	0·0309	0·014	5·62
	CCFM7030	Raw milk	0·0229	0·0015	4·16	0·0211	0·0014	3·84	0·0336	0·003	6·11
	str. Zhang	Koumiss	0·0036	0·0005	0·65	0·0036	0·0001	0·65	0·0104	0·0003	1·89
	CCFM8236	Sauerkraut	0·0161	0·0002	2·93	0·016	0·0003	2·91	0·0355	0·007	6·45
Lact. crispatus	CCFM5136	Unknown	0·0165	0·0003	3·00	0·0151	0·0006	2·75	0·0307	0·0002	5·58
Lact. gasseri	CCFM5115	Yoghurt	0·015	0·0007	2·73	0·0135	0·0012	2·45	0·0331	0·0017	6·02
Lact. helveticus	CCFM8310	Yoghurt	0·0193	0·0017	3·51	0·0194	0·0003	3·53	0·0392	0·0015	7·13
Lact. plantarum	STIII	Sauerkraut	0·0023	0·0001	0·42	0·0026	0·0001	0·47	0·0069	0·0001	1·25
	ZS2058	Sauerkraut	0·2078	0·0033	37·78	0·0139	0·0001	2·54	0·0911	0·0026	16·57
	CCFM9047	Sauerkraut	0·0545	0·0006	9·91	0·0290	0·0002	5·27	0·0974	0·0005	17·71
	CCFM9187	Sauerkraut	0·0188	0·0004	3·42	0·0187	0·0005	3·40	0·0412	0·0005	7·49
	CCFM9225	Tibet Kefir	0·02	0·0011	3·64	0·02	0·0009	3·64	0·0383	0·0011	6·96
	CCFM9232	Sauerkraut	0·0326	0·0018	5·93	0·0239	0·0016	4·35	0·0672	0·0033	12·22
	CCFM9235	Sourdough	0·0264	0·0012	4·80	0·0241	0·0003	4·38	0·0464	0·0037	8·44
	CCFM9240	Unknown	0·0307	0·0014	5·58	0·0256	0·0015	4·65	0·049	0·0015	8·91
	CCFM9307	Milk Granule	0·0185	0·0009	3·36	0·0177	0·0004	3·22	0·0343	0·008	6·24
Lact. reuteri	CCFM4014	Tibet cheese	0·0317	0·0003	5·76	0·024	0·0009	4·36	0·0361	0·0004	6·56
	ATCC55739	Rat	0·3282	0·0048	59·68	0·0095	0·0028	1·74	0·0146	0·004	2·66
Lact. rhamnosus	GG	Human GIT	0·0033	0·0006	0·60	0·0032	0·0004	0·58	0·0072	0·0009	1·31
Bif. animalis	BB12	Unknown	0·1106	0·0005	20·10	0·0047	0·001	0·85	0·0587	0·0002	10·68

S.D., trial was carried out in triplicate; CCFM, Collection Centre of Food Microbiology, Jiangnan University, Wuxi, China; ATCC, American Type Culture Collection, Manassas, VA, USA.

Table 2

Primers used in this study

Name	Sequence (5′–3′)*	Restriction site
mcra-F	GTTCTCGAGAAAAGA-ATGGGGGCGTTATTTATG	*Xho I*
mcra-R	CGGCGGCCGCTTATCA-ATCAAACATCTTCTTAGTTGC	*Not I*
dh-F	CCGGAATTCATGAAAGATTTTAAAGATAAAGTTATGTTTATCACG	*EcoR I*
dh-R	CCCAAGCTTTTACATGATACCGTCCATGATGTGCA	*Hind III*
dc-F	CCGGAATTCATGGCAAGTTTTATTGCAAGTGATCA	*EcoR I*
dc-R	CCCAAGCTTCTAAATAATGTAAGTCGCTGCCTTGG	*Hind III*

*Restriction sites are underlined.