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Complete genome-based approach and substrate change analysis on *Lactobacillus fermentum* L1 producing high-yield conjugated linoleic acid

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Abstract

A total of 300 strains of lactic acid bacteria (LAB) were screened for conjugated linoleic acid (CLA) production. The major isomers of CLA in the fermentation broth of the strain with the highest CLA production were analysed by gas chromatography; and whole-genome sequencing analysis was performed to explore the reasons for CLA production. A total of 65 strains of CLA-producing bacteria belonging to four species of LAB were found. The *Lactobacillus fermentum* L1 with the highest CLA production (1002.486 × 10^{-3} mg/mL) was isolated from the fermented dough. The gene number of *L. fermentum* L1 was 2034, and the GC content in the gene region was 53.18%. Moreover, there were high numbers of genes involved in the function of metabolism. In addition, there were two major isomers of CLA (C18:2 10t,12c and C18:2 9c,11t) in the fermentation broth of *L. fermentum* L1. The change in C:18 fatty acids and the linoleic acid metabolism pathway of *L. fermentum* L1 revealed that C18:2n6c was the substrate of CLA. The present work provides a scientific basis for the application of CLA-producing with LAB.

Introduction

The demand for high-fat food with potentially positive health effects, in the form of ω-3 fatty acids or conjugated linoleic acid (Choi and Song, 2005), has increased with the improvement in the living standards of people. Therefore, CLA has gradually become an emerging research recently (Kim *et al.,* 2016). CLA collectively refers to a group of positional isomers of octadecadienoic-conjugated dienoic acid (Diez *et al.,* 2007). CLA has multiple physiological functions, such as anti-carcinogenesis (Arab *et al.,* 2016), anti-obesity (Oh *et al.,* 2017), anti-inflammatory (Olson *et al.,* 2017), anti-diabetic (Song *et al.,* 2016), and enhancing human immunity (Nugent *et al.,* 2005).

CLA is commercially produced by chemical isomerisation of linoleic acid (LA), but the chemical process resulted in an unexpected by-products (Silva-Ramírez *et al.,* 2016). Technically, using LAB to produce CLA is a perfect approach. Several microbial CLA producers have been reported, including lactic acid bacteria (LAB), such as *Lactobacillus plantarum* (Yang *et al.,* 2017b), *Bifidobacterium breve* (Park *et al.,* 2009), and *B. longum* (Barrett *et al.,* 2007). Many strains can produce CLA, and among them, the one belonging to the genus *Lactobacillus* have the highest potential (Özer *et al.,* 2016). The precise CLA-producing mechanisms in *Propionibacterium acnes* and *L. plantarum* have been completely illustrated (Yang *et al.,* 2017a), but the precise CLA-producing mechanisms in other species of LAB needs further investigation.

Yunnan Province of China is known as the 'kingdom of the plant' and the 'kingdom of the animal'. There are 25 kinds of ethnic minorities live in this place, and 534 kinds of ethnic foods have been produced under the influence of ethnic food culture (Pan *et al.,* 2004). These ethnic foods contain a large amount of LAB. In recent years, some researchers have isolated and identified LAB from Yunnan ethnic foods, such as sour whey (Wang *et al.,* 2018), and douchi (Liu *et al.,* 2014). Subsequently, we have successfully isolated and identified 10,400 strains of LAB from ethnic foods, milk, faeces, and breast milk samples in Yunnan Province. These bacteria should not only be used to ferment products, but other capabilities need to be excavated. Studies on screening of CLA-producing LAB that were isolated from Yunnan ethnic foods have not been reported yet. Therefore, we randomly selected 300 strains of bacteria from 10,400 strains of LAB to explore and study whether the LAB isolated from Yunnan ethnic foods can produce CLA.

Genomic analysis is a way to understand the

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genetic characteristics of an organism (Kostic *et al.,* 2012). By analysing and comparing the genetic sequences of the organism, we can understand the metabolic pathway, protease sequence, drug resistance gene, anti-cancer gene, and many more (Meereis and Kaufmann, 2004). Gene Ontology (GO) provides ontology of defined terms representing gene product properties. The ontology covers three domains namely, cellular component, molecular function, and biological process (Gene Ontology Consortium, 2006). Kyoto Encyclopedia of Genes and Genomes (KEGG) connects known information on molecular interaction networks, information about genes and proteins generated by genome projects (including the gene database), and information about biochemical compounds and reactions (including compound and reaction databases). These databases are different networks, known as the protein network (Wixon and Kell, 2000). In the present work, based on the analytical methods aforementioned, we analysed genome distribution of GO annotations and enzymes involved in KEGG linoleic acid metabolism pathway analysis of the strains with the highest CLA production, and to study the reasons for the CLA production of this strain.

Materials and methods

Microorganisms

A total of 300 strains of LAB were screened for CLA production. These bacteria were isolated from different kinds of ethnic foods, milk, faeces, and breast milk in Yunnan Province, China. They were preserved at the Key Laboratory of Food Processing and Safety Control in Yunnan Agricultural University.

Chemicals

Isomers of CLA (C18:2 10t,12c and C18:2 9c,11t) were obtained from Nu-check prep (USA). Linoleic acid (95%) and all other chemicals were purchased from Sigma-Aldrich (3050 Spruce Street, St Louise, USA).

Screening of LAB for CLA production

Inoculation of 10% of the bacteria in the De Man Rogosa and Sharpe (MRS) broth were carried out, and incubated for 24 h at 37°C in a 50 mL centrifuge tube. The cells were collected by centrifugation (Lee *et al.,* 2013) at 4,000 rpm for 30 min, and then washed for three times with 5 mL of NaCl (0.85%). Subsequently, washed cells cultured in the MRS broth were supplemented with 0.04% LA in 15 mL of the medium in screw-capped tubes, and incubated under O_2-limited conditions in sealed tubes for 48 h at 37°C with shaking. Then, they were centrifuged at 4,000 rpm for 30 min, followed by aspiration of 10 mL of the supernatant to a 250 mL separating funnel. Hexane (20 mL) was added to the separating funnel containing the aspirate, and the extraction was oscillated for 5 min. The extract was allowed to stand for 20 min, and the process was repeated for five times. Later, the pellet was discarded, and the extract was collected in a clean beaker. Anhydrous sodium sulphate was added to absorb excess water after the extraction, and a 0.22 μm-membrane filter was used to retain impurity and bacteria. The CLA production was detected using an ultraviolet (UV) spectrophotometer at a wavelength of 232 nm (Rodríguez-Alcalá *et al.,* 2011). The content of CLA was calculated based on the standard curve:

$$y = 0.0781x - 0.0508 \quad (0.02\mu g/mL25\mu g/mL, R^2 = 0.9993) \qquad \text{(Eq. 1)}$$

Whole-genome sequencing analysis

Bacteria were cultivated in the MRS broth. The cells were collected by centrifugation (Eppendorf, NY, USA) after 48 h of incubation at 37°C. DNA was extracted, manipulated, and sequenced. Genomic library screening and sequence analysis were performed by the Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The Glimmer 3.02 (http://www.cbcb.umd.edu/software/glimmer/) software was used to predict the gene of *L. fermentum* L1. The basic local alignment search tool (BLAST) algorithm (blastx/blastp 2.2.28+) was used to compare the predicted genes obtained with the KEGG gene data. The specific biological pathways involved in the genes were obtained based on the KEGG Orthology (KO) number.

Lipid analyses

The control group was not inoculated with LAB. Meanwhile the experimental group was inoculated with 10% LAB, and after 24 h of fermentation, the fatty acids were extracted as follows. The fatty acid extract was added to 5 mL of 5% hydrochloric acid-methanol solution (v/v) and reacted at 100°C for 1 h (Uribe *et al.,* 2014). The fatty acid methyl ester was extracted with 5 mL of *n*-hexane for five times. The extract was dried over anhydrous sodium sulphate, and filtered with a 0.22 μm-membrane filter. The same conditions were used for the control group without LAB. The samples were analysed using the gas chromatography method, which was improved based on the study by Pandit *et al.* (2012). The sample was dissolved in 100 mL of *n*-hexane. An SP-2560 fused silica capillary column (SUPELCO) with a dimension of 110 × 0.25 mm^2 and 0.2 μm film thickness was

Table 1. CLA produced by different sources of lactic acid bacteria.

Number	Total CLA production (10^{-3} mg/mL)	Species	Source
L1	1002.486 ± 1.186^{A}	LF	Fermented dough
L2	557.150 ± 2.117^{B}	LF	Thick broad-bean sauce
L3	198.228 ± 1.263^{C}	LF	Milk
L4	69.442 ± 0.884^{D}	LF	Breast milk
L5	65.073 ± 1.636^{E}	LF	*Schisandra chinensis* soaked wine
L6	53.928 ± 1.820^{E}	LP	Pickled garlic
L7	53.868 ± 2.253^{E}	LP	Cow dung
L8	52.443 ± 0.829^{EF}	LP	Dry-cured yak meat
L9	52.251 ± 0.968^{EF}	LF	Goat milk cake
L10	51.807 ± 1.004^{EF}	LF	Milk residue
L11	51.073 ± 0.702^{F}	LD	Milk cake
L12	50.947 ± 1.146^{F}	LD	Milk residue
L13	49.722 ± 0.823^{FG}	LP	Sweet rice wine
L14	47.856 ± 1.655^{GH}	LF	Cow dung
L15	46.326 ± 1.001^{HI}	LF	Breast milk
L16	44.604 ± 0.960^{I}	LF	Xuan Wei ham
L17	40.261 ± 1.140^{J}	LP	Milk
L18	40.051 ± 1.065^{J}	LP	Pickled radish
L19	38.559 ± 2.071^{JK}	LF	Cattle feed
L20	37.789 ± 0.777^{JKL}	LF	Pickled vegetable paste
L21	37.036 ± 1.062^{KLM}	LR	Milk residue
L22	35.407 ± 3.038^{LMN}	LF	Bacon
L23	34.994 ± 0.938^{MN}	LF	Xuan Wei ham
L24	34.810 ± 1.037^{MNO}	LF	Sheep manure
L25	34.667 ± 0.740^{MNO}	LP	Milk
L26	34.195 ± 2.404^{NOP}	LR	Milk cake
L27	32.158 ± 1.182^{OPQ}	LF	Goats' milk
L28	31.924 ± 1.082^{PQ}	LF	Thick broad-bean sauce
L29	31.419 ± 1.314^{QR}	LP	Cow dung
L30	31.011 ± 1.101^{QRS}	LF	Sheep manure
L31	28.904 ± 1.611^{RST}	LF	Breast milk

L32	28.680 ± 2.307^{ST}	LP	Pickled radish
L33	28.237 ± 1.186^{T}	LP	Pickled radish
L34	27.919 ± 1.231^{T}	LF	Bovine whey
L35	27.130 ± 1.057^{TU}	LF	Pickles
L36	26.978 ± 1.015^{TU}	LF	Pig manure
L37	25.101 ± 0.870^{UV}	LF	Milk
L38	24.751 ± 2.249^{UV}	LF	Horse manure
L39	24.083 ± 0.939^{VW}	LP	Milk
L40	23.765 ± 1.025^{VWX}	LF	Breast milk
L41	23.640 ± 0.805^{VWX}	LF	Yogurt
L42	23.403 ± 1.980^{VWX}	LF	Pickled ginger
L43	22.979 ± 0.674^{VWX}	LF	Cow dung
L44	22.543 ± 0.873^{VWXY}	LF	Yak dung
L45	22.271 ± 1.174^{VWXY}	LF	Sheep manure
L46	21.794 ± 0.752^{WXYZ}	LP	Pickled cole flowers
L47	21.768 ± 2.536^{WXYZ}	LP	Yogurt
L48	21.355 ± 1.721^{WXYZa}	LP	Pickled capsicum
L49	$21.128 \pm 2.501^{WXYZab}$	LF	Pipa meat
L50	20.880 ± 1.541^{XYZab}	LF	Milk
L51	20.022 ± 1.206^{YZabc}	LF	Milk
L52	19.958 ± 0.915^{YZabc}	LF	Bacon
L53	19.734 ± 1.606^{YZabc}	LF	Sheep manure
L54	18.886 ± 1.181^{Zabcd}	LF	Dry-cured beef
L55	18.644 ± 1.423^{abcd}	LF	Pickles
L56	18.422 ± 2.465^{bcd}	LF	Pig manure
L57	17.975 ± 2.160^{cde}	LP	Pickled radish
L58	17.798 ± 1.247^{cde}	LP	Cow dung
L59	17.561 ± 2.577^{cde}	LP	Bacon
L60	17.529 ± 2.584^{cde}	LP	Breast milk
L61	16.408 ± 1.143^{def}	LF	*Dregea sinensis* Hemsl
L62	15.527 ± 1.935^{efg}	LP	Milk
L63	13.941 ± 2.111^{fgh}	LF	Milk
L64	12.949 ± 1.041^{gh}	LF	Xuan Wei ham
L65	11.997 ± 2.097^{h}	LF	Dry-cured beef

LD = *L. delbrueckii*; LF = *L. fermentum*; LP = *L. plantarum*; and LR = *L. reuteri*. ^{Aa} indicates significant difference; mean values with different superscripts are significantly different.

used for the analysis. The injection volume was 1 µL, and helium was the carrier gas used at a flow rate of 2.0 mL/min. The initial temperature was adjusted to 120°C and held for 8 min, then raised to 200°C at the rate of 6°C/min and held for 15 min, later increased at the rate of 4°C/min and held for 10 min until it rises to 220°C, and finally increased at the rate of 4°C/min and held for 5 min until it rises to 240°C. The split ratio was 50:1. The area normalisation method was used for the calculation.

Statistical analysis

All data were analysed by SPSS 21.0 statistical analysis software. An analysis of variance (ANOVA) procedure and Duncan's multiple range tests were used to analyse the difference in CLA productions of different strains. The difference in the content of the same fatty acids was analysed by Independent Samples *t*-Test. Differences were considered significant at $p < 0.05$.

Results and discussion

CLA-producing LAB

In the present work, we screened 65 strains of CLA-producing LAB (Table 1). Some of the CLA-producing LAB were isolated from ethnic foods in Yunnan Province of China, such as thick broad-bean sauce, *Schisandra chinensis* soaked wine, pickled garlic, dry-cured yak meat, goat milk cake, milk residue, milk cake, sweet rice wine, Xuan Wei ham, pickled radish, pickled vegetable paste, bovine whey, pickles, pickled ginger, pickled cole flowers, pickled capsicum, Pipa meat, dry-cured beef, and *Dregea sinensis* Hemsl. In addition, we found that from the entire CLA-producing LAB (Table 1), 42 strains were *L. fermentum*, 19 strains were *L. plantarum*, two strains were *L. delbrueckii*, and two strains were *L. reuteri*. In previous research, many CLA-producing LAB have been screened from milk, dairy products, and faeces (Vela Gurovic *et al.,* 2014; Dahiya and Puniya, 2018). *L. reuteri* was the first LAB species reported with CLA production capability, which was isolated from milk (Yang *et al.,* 2017a). Ham *et al.* (2002) isolated 34 strains of LAB from 19 faecal samples of healthy infants. Pandit *et al.* (2012) isolated a *Lactobacillus* strain from cheese. From Table 1, it is apparent that the total CLA produced by *L. fermentum* L1 reached 1002.486×10^{-3} mg/mL, while *L. fermentum* L65 yielded the lowest CLA production (11.997×10^{-3} mg/mL). *L. fermentum* L1 was isolated from the fermented dough (Table 1) in Dali City, Yunnan Province. This fermented dough was used to make the special foods of Bai ethnic known as Xizhou-Pizza. Meanwhile *L. fermentum* L65 was isolated from the dry-cured beef, which was the main meat product of Muslims in Yunnan Province of China. Therefore, it can be found that the ability of same species of LAB, which were isolated from two kinds of samples to produce CLA was different. Another important findings was that as compared to strains with high CLA-producing under the same fermentation conditions, the total CLA produced by *L. fermentum* L1 (Table 1) was higher than the *L. fermentum* DDHI27 (500×10^{-3} mg/mL) that was isolated from the faecal sample of a healthy infant (Dahiya and Puniya, 2018). In addition, the transformation ratio of LA into CLA by *L. fermentum* L1 was similar to those of some bacteria, such as *L. acidophilus* Q42 (20.0%) and *L. casei* CRL87 (17.0%) (Van Nieuwenhove *et al.,* 2010). It means that under the same fermentation conditions, the CLA production of *L. fermentum* L1 could reach the level of other strains with high CLA production.

Genome properties of L. fermentum L1

The gene number of *L. fermentum* L1 was 2034 (Table 2), and the total gene length was 1723611 bp with a GC content of 53.18% in the gene region, and the average gene length was 847.4 bp. Moreover, the genes of *L. fermentum* L1 statistical distribution of GO annotations (Figure 1) showed that the biological process enriched most genes, especially in the metabolic process. This means that there is strong ability of metabolism in *L. fermentum* L1. The stronger the metabolic capacity of organisms, the more favourable they are to resist the harm of toxic substances (Juárez-Jiménez *et al.,* 2010). Because of LA damage to the cell wall, LAB converts LA to CLA through some metabolic process (Glaesser *et al.,* 1996). The reason of *L. fermentum* L1 with high CLA production may be because there is a strong ability of metabolism.

The substrate changes

There were two major isomers of CLA in the fermented broth of *L. fermentum* L1 (Table 3) namely C18:2 10t,12c (9.877%) and C18:2 9c,11t (5.742%). Another important observation was the change in concentration of C18:1n9c and C18:2n6c. The proportion of C18:1n9c and C18:2n6c in control was 6.159 and 93.841%, respectively. In the fermented broth of *L. fermentum* L1, the proportion of C18:1n9c and C18:2n6c was 6.832 and 77.594%, respectively. As compared to the control, the content of C18:1n9c, C18:2 10t,12c, and C18: 2 9c,11t in the experimental group increased by 0.673, 9.877, and 5.742%, respectively, while the content of C18:2n6c decreased by 16.247%. Kishino *et al.* (2002) reported that the production of CLA was positively correlated with the

Table 2. Genome features of *L. fermentum* L1.

Sample	*L. fermentum* L1
Gene number	2034
Gene total length (bp)	1723611
Gene average length (bp)	847.4
Gene density (kb)	1.02
GC content in gene region (%)	53.18
Gene / Genome (%)	86.14
Intergenetic region length (bp)	277216
GC content in intergenetic region (%)	44.14
Intergenetic length / Genome (%)	13.86
G + C content (%)	51.93

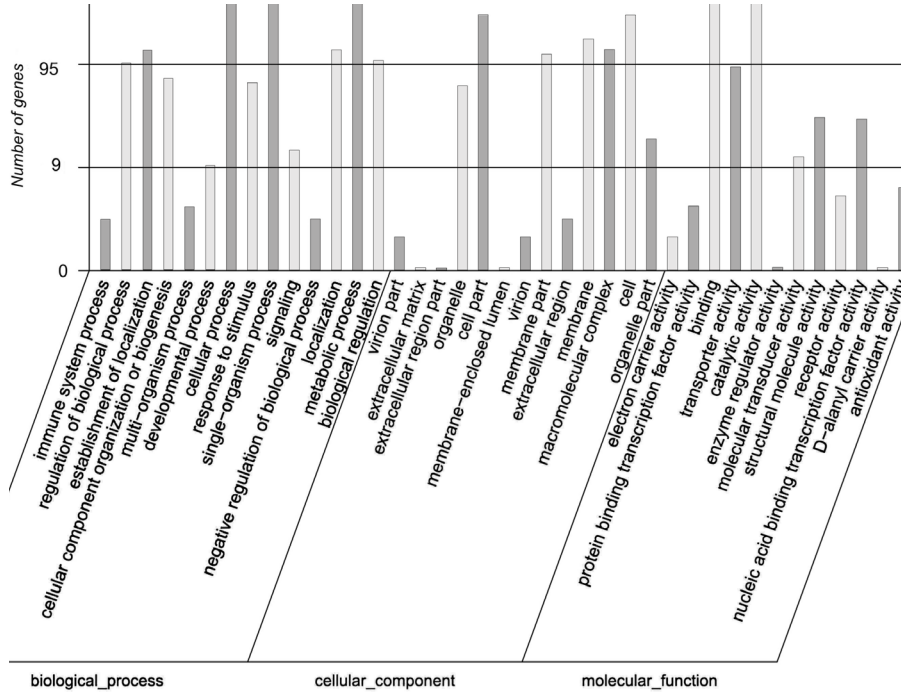

Figure 1. The genes of *L. fermentum* L1 statistical distribution of GO annotations.

Table 3. C:18 fatty acid proportion (%) in the fermented broth of *L. fermentum* L1.

Fatty acid	Control	*L. fermentum* L1
C18:1n9c	6.159 ± 0.024^{b}	6.832 ± 0.062^{a}
C18:2n6c	93.841 ± 0.024^{a}	77.594 ± 0.039^{b}
C18:2 10t,12c	–	9.877 ± 0.037
C18: 2 9c,11t	–	5.742 ± 0.087

Mean values with different superscripts are significantly different.

increase. In other words, the C18:2n6c is the substrate of CLA production. From the LA metabolism pathway map of *L. fermentum* L1 (Figure 2), it showed that 10 types of enzymes were related to LA metabolism based on the LA metabolism reference pathway (www.-kegg.jp/kegg-bin/show_pathway?map00591); and we found that these enzymes were linoleoyl-CoA desaturase (1.14.19.3), acyl-lipid delta12-acetylenase (1.149933), secretory phospholipase A2 (3.1.1.4), linoleate 8R-lipoxygenase / 9,12-octadecadienoate 8-hydroperoxide 8R-isomerase (5.4.4.5 and 1.13.1160), linoleate 8R-lipoxygenase / 9,12-octadecadienoate 8-hydroperoxide 8S-isomerase (5.4.4.6), linoleate 10R-lipoxygenase (1.13.1162), linoleate 9S-lipoxygenase (1.13.1158), lipoxygenase (1.13.1112), arachidonate 15-lipoxygenase (1.13.1133), and 13-HODE : NAD+ oxidoreductase (1.1.1-). With the participation of these enzymes, LA

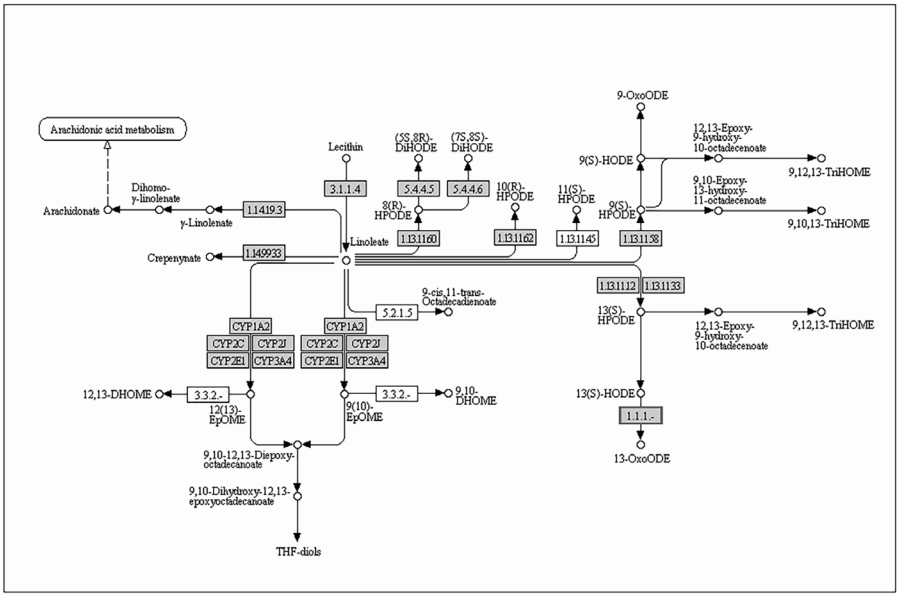

Figure 2. Linoleic acid metabolism map of *L. fermentum* L1. Rectangular nodes represent gene products (such as enzymes or RNA regulators), and all grey background gene products belong to the KO classification system (proteins that are highly similar in sequence and have similar functions in the same pathway). The white background gene products are not included in the KO classification system. Circular nodes represent compounds (i.e., substrates or products); white background rounded rectangles represent other pathways associated with this pathway. Arrows indicate the direction of enzyme reaction or direction of information transfer, the solid line indicates direct action, and the dotted line indicates indirect action.

can be converted into other fatty acids, such as CLA, γ- linolenate, and crapenynate. This findings validates our results that LA is a substrate for CLA production. It is known that C18:2 10t,12c and C18:2 9c,11t are the most widely studied (Dipasquale *et al.,* 2018), and provide significant contribution to health benefits associated with CLA (Banni, 2002). In the present work, C18:2 10t,12c and C18:2 9c,11t can be produced by *L. fermentum* L1, hence, *L. fermentum* L1 is worthy of further research.

It has been reported that one of the reasons of CLA-producing is biohydrogenation of LA to CLA by the catalytic action of linoleate isomerase (Hao *et al.,* 2015). In addition, Yang *et al.* (2013) found that the catalysing reaction from the LA to 10-hydroxy-cis-12-octadecacenic resulted from linoleate isomerase. From the linoleate isomerase database (www.genome.jp/dbget-bin/get_linkdb?-t+uniprot+ec:5.2.1.5), 11 kinds of linoleate isomerase have been found. The original strains of these 11 kinds of linoleate isomerase are: *L. lactis subsp*. (D2BQ64), *Aspergillus niger* (E2PSY), *Streptococcus salivarius* (F8LL17), *L. plantarum* (F9USW0), *L. acidophilus* (H2E787), *L. pentosus* (I8RCV7), *Carnobacterium maltaromaticum* (K8EWC0), *L. acidophilus* (Q307I4), *L. plantarum* (Q38IA5), *L. plantarum* (Q88ZZ7), and *Bifidobacterium longum* (T2I262). These results imply that the linoleate isomerase in *L. fermentum* has not been reported. So far, the mechanism for CLA synthesis in *L. fermentum* is unknown. In future research, we will try to study the enzymes involved in CLA production and the mechanism of CLA production in *L. fermentum* L1.

In addition to the studies aforementioned, we found that there were some genes related to folate metabolism and seleno compound metabolism in *L. fermentum* L1. It means that under suitable conditions, *L. fermentum* L1 can produce folate or seleno compound. More interestingly, because of *L. fermentum* L1 with high CLA production, we can use this strain to ferment some functional products with high CLA, folic, and seleno compound. If these functional products can be successfully produced, many consumers will be attracted. Now, we are using *L. fermentum* L1 to ferment yogurt and cheese with enhanced levels of CLA. In addition, we are going to develop yogurt rich in folate and seleno compound by the fermentation of *L. fermentum* L1.

Conclusion

Through screening of CLA-producing strains from a large number of LAB, the present work found that CLA-producing LAB can also be obtained from other samples except for milk, dairy products, and faeces. C18:2n6c is a substrate for LAB fermentation to produce CLA. In addition, gene function and enzymes affect CLA production of LAB. With the increase in the numbers of people pursuing CLA-rich foods, we do believe that *L. fermentum* L1 can be

used as an adjunct culture in developing fermented CLA-rich products. The present work complements the scientific basis for the application of functional foods by LAB, and provides the theoretical for the formation of CLA in *L. fermentum*.

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