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Method for Screening of Bacterial Strains Biosynthesizing Specific Conjugated Linoleic Acid Isomers

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ABSTRACT: A simple and accurate method for screening of bacterial strains with the ability to convert free linoleic acid into specific conjugated linoleic acid (CLA) isomers has been developed by combining the ultraviolet spectral scan and capillary electrophoresis analysis. The ultraviolet spectral scan was carried out for preliminary screening of bacterial strains with the capacity to biosynthesize CLA, and the absorption peak at 228−235 nm was used for assessing the possible production of CLA by bacteria. The capillary electrophoresis analysis was used as the follow-up confirmation to definitively conclude CLA production and the composition of CLA isomers. Linoleic acid at the concentration of 25 μg/mL, which showed little inhibitory effect on the growth of bacteria, was used for initial screening of CLA-producing strains. The strains with the ability to produce specific CLA isomers can be selected quickly from a large number of bacteria by this high-throughput method.

KEYWORDS: *Conjugated linoleic acid, biosynthesis, bacteria, ultraviolet spectral scan, capillary electrophoresis*

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of octadecadienoic acid with conjugated double bonds. There are about 20 types of CLA isomers with different positional (7,9; 8,10; 9,11; 10,12; and 11,13) and geometric (*cis*,*cis*; *cis*,*trans*; *trans*,*cis*; and *trans*,*trans*) combinations. The mixture of CLA isomers has demonstrated various potential health benefits, including anticarcinogenic, antiatherogenic, antidiabetic, and immunity-enhancing activities.[1−3] Recently, more and more studies showed that each CLA isomer has unique bioactive properties. *cis*9,*trans*11-CLA has the anticarcinogenic biological activity in Caco-2 and MCF-10A cells,[4,5] whereas *trans*10,*cis*12-CLA appears to be specifically responsible for enhancement of energy metabolism in Chang liver cells and reducing liver lipid content in Zucker rats.[6,7]

CLA occurs naturally in fat of dairy products and meat from ruminant animals at a level lower than 10 mg/g of fat usually.[8] The CLA originating from the ruminant products predominantly consists of *cis*9,*trans*11-CLA (over 80% of total CLA), with a small amounts of *trans*10,*cis*12-CLA and other isomers. For the production of CLA on a large scale, chemical isomerization has been developed. However, the product consists of nearly 20 types of CLA isomers.[9] Recent studies have focused on enzymatic isomerization for the purpose of producing high-purity *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA. Some bacterial strains, such as *Lactobacillus plantarum* AKU 1009a and *Bifidobacterium breve* LMC 520, have been reported to biosynthesize *cis*9,*trans*11-CLA.[10,11] At the same time, *Propionibacterium acnes* G449 and *Megasphaera elsdenii* YJ-4 have been found to produce *trans*10,*cis*12-CLA.[12,13] However, all of these strains were screened by gas chromatography (GC) and/or high-performance liquid chromatography (HPLC). The two methods are time-consuming, labor-intensive, and usually take about 1 h for analyzing one sample. Therefore, these reported methods cannot meet the requirements for rapid screening of CLA-producing bacteria from large numbers of bacterial isolates.

In the present paper, we described a method to screen bacterial strains with the ability to produce specific CLA isomers. A ultraviolet (UV) spectral scan method was used for preliminary assessment of the possible production of CLA, and the follow-up capillary electrophoresis (CE) analysis was implemented to detect the composition of CLA isomers.

MATERIALS AND METHODS

Reagents. Linoleic acid and CLA were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Standard CLA isomers (*cis*9,*trans*11-CLA, *trans*9,*trans*11-CLA, and *trans*10,*cis*12-CLA) were purchased from Nanchang Huaxing Bioscience Co. (Nanchang, Jiangxi, China). MRS medium was obtained from Beijing Land Bridge Technology Co. (Beijing, China). All other reagents were of analytical grade.

Strains and Cultivation. The bacterial strains used in this study are detailed in Table 1. All of the strains were obtained from the Chinese General Microorganism Culture Collection (CGMCC). Strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus salivarius* ssp. *thermophilus*, and *Lactobacillus acidophilus* were cultured in MRS broth and incubated at 37 °C for 24 h. *Lactobacillus casei* ssp. *casei* was cultivated in MRS broth at 30 °C for 24 h. For growth of *M. elsdenii*, medium was prepared as previously described by Kim et al.,[13] and the strain grew under O_2-free CO_2 at 37 °C for 24 h in Hungate-type tubes. The remaining propionibacteria were cultured in sodium lactate medium under O_2-free CO_2 at 37 °C for 24 h in Hungate-type tubes.[14] Cell growth was measured at 600 nm and incubated for 24 h. Linoleic acid was prepared as a 5 mg/mL aqueous solution in 1% (v/v) Tween 80. For screening of CLA-producing strains, linoleic acid was added to the medium to give a final concentration of 25 μg/mL.

Fatty Acid Extraction. After incubation for 24 h, the fatty acids were extracted from the fermentation broth as follows. A total of 1 mL of sample and 2 mL of isopropanol were mixed vigorously; 1.5 mL of hexane were then added; and the mixture was vortexed for 2 min

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Table 1. Screening of Strains for Their Capacity To Produce CLA

strains	UV absorption peak at 228−235 nm[a]	CLA in the fermentation broth assayed by CE (μg/mL)		
		*cis*9,*trans*11-CLA	*trans*9,*trans*11-CLA	*trans*10,*cis*12-CLA
Lactobacillus delbrueckii ssp. *bulgaricus* CGMCC 1.1480	−	−	−	−
Streptococcus salivarius ssp. *thermophilus* CGMCC 1.1855	−	−	−	−
Lactobacillus acidophilus CGMCC 1.1854	+	13.01 ± 0.61	5.74 ± 0.30	−
Lactobacillus casei ssp. *casei* CGMCC 1.574	+	9.35 ± 0.55	−	−
Propionibacterium thoenii CGMCC 1.2228	−	−	−	−
Propionibacterium acidipropionici CGMCC 1.2232	−	−	−	−
Propionibacterium freudenreichii ssp. *shermanii* CGMCC 1.2227	+	13.25 ± 0.57	4.17 ± 0.27	−
Megasphaera elsdenii CGMCC 1.2720	−	−	−	−

[a]−, absorption peak/CLA isomer not detected; +, absorption peak detected.

before being centrifuged at 4000*g* for 5 min. The upper layer was then collected for CLA analysis.

UV Spectral Scan. The fatty acid extraction samples were scanned from 200 to 300 nm with a UV/vis spectrophotometer (Ultrospec 4300 Pro, Amersham Pharmacia Biotech, Sweden). A characteristic absorption peak of conjugated double bonds at 228−235 nm indicated the possible presence of CLA in the extraction sample. The CLA-containing samples were then dried with nitrogen at room temperature for about 2 min for further detection of the composition of CLA isomers.

CE Analysis. The fatty acids were dissolved in 200 μL of borate solution (80 mM, pH 9.0) prepared as previously described.[15] The dissolved samples were degassed for 5 min by sonication before CE analysis. CLA isomers were identified by a Beckman P/ACE MDQ capillary electrophoresis system (Beckman, Fullerton, CA) equipped with a photodiode array detection system and a coolant-cooled capillary cartridge. Analyses were carried out on untreated fused-silica capillaries of 61 cm (50 cm effective length) × 100 μm inner diameter (Yongnian Optical Fiber Factory, Hebei, China). Pressure injection was performed using 3447.38 Pa for 5 s. The CLA isomers were analyzed at the capillary temperature at 15 °C and the separation voltage at 20 kV. The electropherograms were recorded and integrated by a personal computer with 32 Karat software, version 5.0 (Beckman).

Statistical Analysis. All data were in triplicate and expressed as the mean ± standard deviation. Analysis of variance was performed by ANOVA procedures using the SPSS Statistical Package (SPSS 13, SPSS Inc., Chicago, IL). Post-hoc analyses were carried out using Duncan's test for multiple comparisons at the significance level of 0.05.

RESULTS AND DISCUSSION

UV Spectral Scan. In this study, eight bacterial strains were assessed for their capacity to produce CLA from free linoleic acid. According to the characteristic absorption peak of conjugated double bonds at 228−235 nm, a UV spectral scan method was developed to detect CLA in fatty acids extracted from fermentation broth. CLA (Sigma-Aldrich) has a maximum UV absorbance at 233 nm (Figure 1A), while linoleic acid only has an absorption peak at 213 nm (Figure 1B). The fatty acids extracted from fermentation broth of *L. acidophilus* CGMCC 1.1854 have two absorption peaks at 212 and 233 nm (Figure 1C), which means that *L. acidophilus* CGMCC 1.1854 may have the ability to produce CLA. The two absorption peaks at 212 and 233 nm were also observed in the samples of *L. casei* ssp. *casei* CGMCC 1.574 and *P. freudenreichii* ssp. *shermanii* CGMCC 1.2227, respectively. However, for *S. salivarius* ssp. *thermophilus* CGMCC 1.1855, there is a shoulder at 233 nm (Figure 1D), the possibility of CLA production needs to be confirmed by the followed CE analysis. With this approach, a total of three of the eight bacterial strains assayed in this study were found to have a UV absorption peak at 228−235 nm (Table 1).

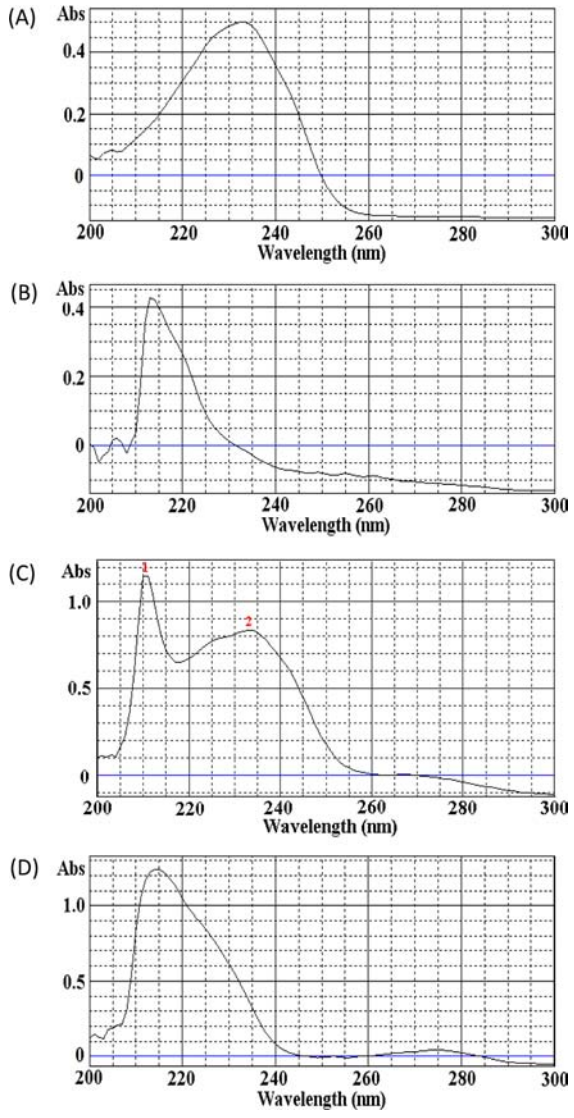

Figure 1. UV spectral scan data of different samples: (A) CLA (a mixture of *cis*- and *trans*-9,11- and -10,12-CLA, Sigma-Aldrich), (B) linoleic acid (Sigma-Aldrich), (C) fatty acids extracted from the fermentation broth of *L. acidophilus* CGMCC 1.1854, and (D) fatty acids extracted from the fermentation broth of *S. salivarius* ssp. *thermophilus* CGMCC 1.1855.

Linoleic acid serves as the substrate in the biosynthesis of CLA by bacteria. It was suggested that the conversion of linoleic acid to CLA might be a detoxification mechanism in bacteria and a stronger linoleic acid tolerance implies a higher

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Table 2. Inhibitory Effect of Free Linoleic Acid on the Growth of Bacteria

strains	cell density (OD_{600}) at different concentrations of linoleic acid (μg/mL)			
	0	25	50	100
Lactobacillus delbrueckii ssp. *bulgaricus* CGMCC 1.1480	0.306 ± 0.021 a[a]	0.294 ± 0.024 a	0.276 ± 0.020 a	0.197 ± 0.023 b
Streptococcus salivarius ssp. *thermophilus* CGMCC 1.1855	0.313 ± 0.029 a	0.299 ± 0.032 a	0.208 ± 0.028 b	0.121 ± 0.020 c
Lactobacillus acidophilus CGMCC 1.1854	0.333 ± 0.028 a	0.326 ± 0.025 a	0.282 ± 0.017 a	0.157 ± 0.034 b
Lactobacillus casei ssp. *casei* CGMCC 1.574	0.325 ± 0.033 a	0.314 ± 0.028 a	0.237 ± 0.031 b	0.147 ± 0.029 c
Propionibacterium thoenii CGMCC 1.2228	0.287 ± 0.025 a	0.275 ± 0.016 a	0.248 ± 0.023 a	0.177 ± 0.019 b
Propionibacterium acidipropionici CGMCC 1.2232	0.305 ± 0.024 a	0.293 ± 0.030 a	0.265 ± 0.026 a	0.185 ± 0.026 b
Propionibacterium freudenreichii ssp. *shermanii* CGMCC 1.2227	0.323 ± 0.024 a	0.312 ± 0.026 a	0.293 ± 0.024 ab	0.248 ± 0.035 b
Megasphaera elsdenii CGMCC 1.2720	0.316 ± 0.022 a	0.294 ± 0.027 a	0.205 ± 0.024 b	0.108 ± 0.018 c

[a]Values within rows followed by different letters are significantly different ($p < 0.05$).

Table 3. Screening of CLA-Producing Lactic Acid Bacteria Isolated from Sourdough and Fermented Vegetable

strains	UV absorption peak at 228−235 nm[a]	CLA in the fermentation broth assayed by CE (μg/mL)		
		*cis*9,*trans*11-CLA	*trans*9,*trans*11-CLA	*trans*10,*cis*12-CLA
L. helveticus NC2	−	−	−	−
L. helveticus NC14	−	−	−	−
L. maltaromicus NC1	−	−	−	−
L. maltaromicus NC19	−	−	−	−
L. delbrueckii ssp. *bulgaricus* NC4	−	−	−	−
L. delbrueckii ssp. *delbrueckii* NC17	+	11.24 ± 0.54	4.36 ± 0.28	−
L. vaccinostercus NC6	−	−	−	−
L. vaccinostercus NC10	−	−	−	−
L. amylophilus NC12	−	−	−	−
L. amylophilus NC20	−	−	−	−
L. fructosus NC18	−	−	−	−
L. brevis NCL912	+	8.47 ± 0.48	6.12 ± 0.35	−
L. brevis ZDY26b	−	−	−	−

[a]−, absorption peak/CLA isomer not detected; +, absorption peak detected.

CLA productivity.[16] The concentration of linoleic acid higher than 600 μg/mL was once used in screening of CLA-producing bacterial strains from bifidobacteria and lactic acid bacteria.[17,18] However, the performance of the strains varied when grown in different concentrations of linoleic acid, with some being inhibited at concentrations of linoleic acid above 100 μg/mL.[19,20] In the present study, the effects of the different concentrations of free linoleic acid on the growth of the screened bacteria were studied by measuring cell density. All of the bacterial strains grew well in the medium supplemented with 25 μg/mL linoleic acid (Table 2). The growth of *S. salivarius* ssp. *thermophilus* CGMCC 1.1855, *L. casei* ssp. *casei* CGMCC 1.574, and *M. elsdenii* CGMCC 1.2720 were significantly inhibited as the concentration of linoleic acid increased to 50 μg/mL. However, all of the strains were significantly inhibited with the addition of 100 μg/mL linoleic acid. Therefore, lower linoleic acid levels are beneficial for initial screening of CLA-producing strains from a large quantity of bacteria.

A spectrophotometric method has been reported by Rodríguez-Alcalá et al. and Barrett et al. for rapid screening of CLA-producing strains from lactic acid bacteria[18] and bifidobacteria,[21] respectively. They only detected the absorbance at 233 nm for qualitative and quantitative analysis of CLA produced by bacteria. CLA has four types of geometric isomers, including *trans*,*trans*; *cis*,*trans*/*trans*,*cis*; and *cis*,*cis*, with the characteristic absorption peak at 228.5, 231.5, and 234.5 nm, respectively.[15] Therefore, the UV spectral scan method was carried out for rapid screening of bacterial strains with the capacity to produce CLA, and the absorption peak at 228−235 nm was used for assessing the possible presence of CLA.

The fact that some fermented dairy products contain higher levels of CLA than non-fermented counterparts creates the possibility of producing fermented dairy products with high levels of CLA. Some CLA-producing strains of lactic acid bacteria, bifidobacteria, and propionibacteria have been isolated from fermented milk and cheese.[18,22] The development of enzyme-catalyzed reactions makes it possible to produce specific CLA isomers, such as *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA.

To select bifidobacteria that can be used as a CLA-producing starter strain for milk fermentation, the GC analysis method was used for screening and identification.[23] Among the 150 screened bifidobacteria from human intestines, only 9 strains showed the capacity to produce CLA. The GC method was also used by Kishino et al. in screening of CLA-producing strains.[24] A total of 18 strains of lactic acid bacteria were selected from more than 250 samples. These reports indicate that only a very low percentage of the strains have the capacity to produce CLA in nature. Thus, the GC-based screening process is time-consuming and laborious for large quantities of bacterial strains. The UV spectral scan method can detect 96 samples in several minutes using a 96-well plate spectrophotometer. Potential strains for producing CLA can be selected rapidly from a large number of bacteria by this method. Hence, the UV spectral scan method can meet the requirements for high-throughput screening of CLA-producing bacteria.

CE Analysis. A rapid and accurate method for analysis of CLA isomers was developed in the previous work. All seven CLA isomers were successfully separated in 15 min at the separation voltage of 30 kV.[15] Because a spectrophotometric method cannot distinguish between isomers of CLA because

the determination is based on the measurement of the conjugated double bonds in fatty acids, the CE analysis was used for further confirmation and detection of the composition of CLA isomers (Table 1). A lower separation voltage at 20 kV was used in this study because of the limitation of the high voltage power of the instrument. Three standard CLA isomers were completely separated in 30 min (Figure 2A). All of the samples of fatty acid extraction were assayed without methylation by CE. Two CLA isomers, *cis*9,*trans*11-CLA and *trans*9,*trans*11-CLA, were generated by *P. freudenreichii* ssp. *shermanii* CGMCC 1.2227 (Figure 2B). The same result was observed in the fatty acids extracted from *L. acidophilus* CGMCC 1.1854. However, it is interesting that *L. casei* ssp. *casei* CGMCC 1.574 synthesized only *cis*9,*trans*11-CLA, as shown in Figure 2C. *S. salivarius* ssp. *thermophilus* CGMCC 1.1855 produced an unknown compound with the retention time at 34.5 min (Figure 2D). The other four samples, which did not have an absorption peak at 228−235 nm, were also analyzed by CE. It was found that the four strains did not produce any CLA isomers, as shown in Figure 2E.

As we can see, there is an absorbance of 0.425 at 233 nm in Figure 1D. However, subsequent CE analysis only found an unknown compound having an absorbance at 233 nm with the retention time at 34.5 min, as shown in Figure 2D. Hence, it is not sufficient to screen CLA-producing strains based on UV analysis alone. It indicated that the UV spectral scan method can be used as a quick, preliminary assessment of the possible CLA-producing strains from a large number of bacteria. A follow-up confirmation, such as the use of CE analysis, must be implemented to definitively conclude CLA production and the composition of CLA isomers.

In the present study, lactobacilli and propionibacteria showed the capacity to biosynthesize *cis*9,*trans*11-CLA and *trans*9,*trans*11-CLA. This is in agreement with some of the previous reports.[24−26] The ratio of *cis*9,*trans*11-CLA/*trans*9,*trans*11-CLA is variable between species and may be affected by fermentation conditions, such as the concentration of linoleic acid and oxygen.[16,27] A strain of *M. elsdenii* YJ-4 isolated from ruminal bacteria has been reported to produce *trans*10,*cis*12-CLA mainly.[13] However, the strain of *M. elsdenii* CGMCC 1.2720 tested in this study cannot generate *trans*10,*cis*12-CLA or any other CLA isomer.

The GC and HPLC methods have been used popularly in analysis of CLA isomers, but the CLA must be methylated before identification and quantification of individual CLA isomers. Methylation of CLA is time-consuming, and *cis*,*trans*/*trans*,*cis* isomers of CLA can be converted to *trans*,*trans* isomers.[28,29] For the purpose of obtaining a full resolution of all of the CLA isomers in the sample, three columns in series were used in the HPLC method with the analysis time up to 60 min.[18,30] Recently, a nuclear magnetic resonance spectroscopy method was developed for measuring the total CLA content.[31] It is fast and simple, but it cannot analyze the composition of CLA isomers. Therefore, the CE method is simple, rapid, and accurate in the analysis of CLA isomers and has advantages over the existing conventional GC and HPLC methods.

Ruminant-derived foods are the major source of CLA in the diet. A wide range of CLA isomers are found in the fat of dairy products and meat of ruminant, with *cis*9,*trans*11-CLA as the major isomer.[32] It is considered that these CLA isomers are mainly biosynthesized by ruminal bacteria during metabolism of dietary linoleic acid.[33,34] In comparison to the large number of species of ruminal bacteria, only very few CLA-producing

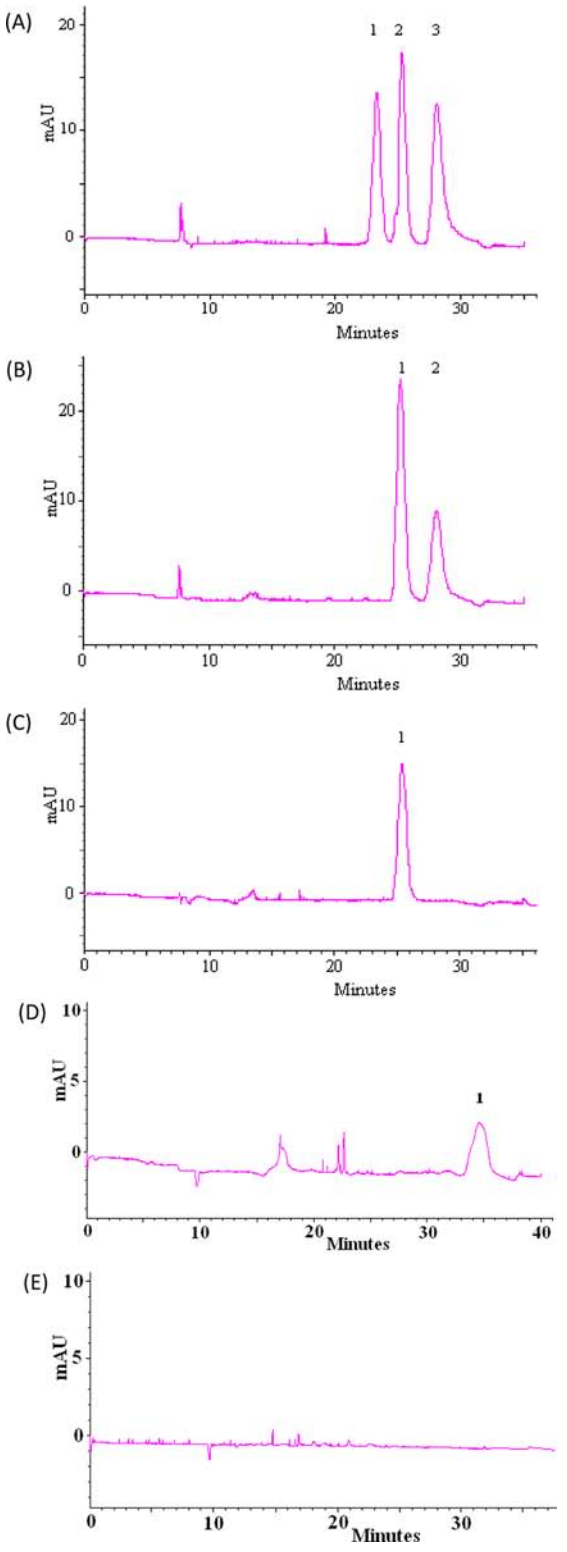

Figure 2. Chromatograms of the CLA isomers assayed by CE: (A) standard CLA isomers (peaks 1, 2, and 3 are *trans*10,*cis*12-CLA, *cis*9,*trans*11-CLA, and *trans*9,*trans*11-CLA, respectively), (B) fatty acids extracted from the fermentation broth of *P. freudenreichii* ssp. *shermanii* CGMCC 1.2227 (peaks 1 and 2 are *cis*9,*trans*11-CLA and *trans*9,*trans*11-CLA, respectively), (C) fatty acids extracted from the fermentation broth of *L. casei* ssp. *casei* CGMCC 1.574 (peak 1 is *cis*9,*trans*11-CLA), (D) fatty acids extracted from the fermentation broth of *S. salivarius* ssp. *thermophilus* CGMCC 1.1855 (peak 1 is an unknown compound), and (E) fatty acids extracted from the fermentation broth of *P. thoenii* CGMCC 1.2228.

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strains, such as *Butyrivibrio fibrisolvens* MDT-5,[35] *M. elsdenii* YJ-4,[13] and *P. acnes* G449,[12] have been isolated from rumen because of the lack of a high-throughput screening method. The present study will provide a new powerful approach for us to disclose the biosynthesis of specific CLA isomers in rumen.

Practical Application. The new method was used to screen CLA-producing lactic acid bacteria isolated from sourdough and fermented vegetable previously.[36,37] The UV spectral scan was used first as the preliminary assessment of the possible production of CLA by lactic acid bacteria. Among the 13 tested bacterial strains, only *L. delbrueckii* ssp. *delbrueckii* NC17 and *L. brevis* NCL912 had an absorption peak at 233 nm. The CLA isomers were identified as *cis*9,*trans*11-CLA and *trans*9,*trans*11-CLA by the following CE analysis (Table 3). It showed that this method is simple and reliable. Next, we will use this method to screen bacterial strains producing *trans*10,*cis*12-CLA from ruminal bacteria.

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Notes

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