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Role of 10-hydroxy-*cis*-12-octadecenic acid in transforming linoleic acid into conjugated linoleic acid by bifidobacteria

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Abstract

10-hydroxy-*cis*-12 octadecenoic acid (10-HOE) is a type of octadecenoic acid with a hydroxyl on the C_{10} carbon. It is generated from linoleic acid (LA) catalyzed by linoleate hydratase in lactobacilli, which was initially named as myosin-cross-reactive antigen (MCRA). In lactobacilli, 10-HOE is the first intermediate in the production of conjugated LA (CLA). Although MCRA from bifidobacteria can generate 10-HOE, the precise role of 10-HOE in CLA production in bifidobacteria remains unknown. In the current work, 10-HOE and LA were added to the medium as the substrate both separately and synchronously to analyze their influence on CLA production. Using 10-HOE as the substrate, bifidobacteria were able to generate CLA by first converting it to LA, followed by CLA accumulation. Recombinant MCRA catalyzed the conversion of 10-HOE to LA, indicating that bifidobacterial MCRA can account for the reversible conversion between LA and 10-HOE. This is the first report to demonstrate the precise role of 10-HOE in the process of CLA production among bifidobacteria.

Keywords Linoleic acid · Conjugated linoleic acid · 10-Hydroxy-*cis*-12-octadecenoic acid · *Bifidobacterium*

Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid (LA) with conjugated double bonds (Yang et al. 2017a). Considerable research efforts have focused on CLA for its health-associated benefits, such as anti-carcinogenic, anti-obesity, and anti-inflammatory effects (Yang et al. 2015). *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA are the two major isomers reported to exhibit health-promoting activity.

A number of bacteria have been shown to possess CLA production ability, including *Butyrivibrio*, *Propionibacterium*, *Clostridium*, *Lactobacillus*, and *Bifidobacterium* (Yang et al. 2017a). For *Butyrivibrio*, the dominant CLA isomer is *cis*9, *trans*11-CLA; however, the detailed characterization of linoleate isomserase is still unclear. LA isomerase has been purified from *C. sporogenes* ATCC 25762, characterized as a membrane-bound enzyme with a molecular weight of 45 kDa (Peng et al. 2007). LA was transformed to *trans*10, *cis*12-CLA by *P. acnes* via a one-step reaction catalyzed by polyunsaturated fatty acid isomerase, which was the first LA isomerase with this structure to be elucidated (Liavonchanka et al. 2006). Among lactobacilli, *L. plantarum* is the most extensively reported species with a high capacity to produce CLA, and its precise CLA-biosynthesis pathway has been clarified (Kishino et al. 2013; Yang et al. 2017b). LA was first hydrated into 10-HOE at the Δ9Z double bond, catalyzed by myosin-cross-reactive antigen (MCRA), a 64-kDa membrane-associated protein. Then, 10-HOE was dehydrated to 10-oxo-*cis*-12-octadecenoic acid followed by isomerization, hy-

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drogenation, and dehydration, with CLA as the final product (Kishino et al. 2013; Yang et al., 2014). Furthermore, lactobacilli has been also reported to produce 10,13-hydroxystearic acid by a consecutive activity of two linoleate hydratases (Black et al. 2013).

MCRA is essentially a fatty acid hydratase first identified in *Streptococcus pyogenes* (Volkov et al. 2010). This protein is universal among bifidobacteria. The MCRA-coding gene from *B. breve* NCIMB 702258 was cloned and expressed in *Lactobacoccus* and *Corynebacterium*, and the recombinant protein was identified to be a FAD-dependent fatty acid hydratase (Rosberg-Cody et al. 2011). In addition, MCRA from *B. animalis* subsp. *lactis* BB-12 was reported to convert LA into 10-HOE (Yang et al. 2013). The MCRA from *B. breve* NCFB2258 was reported to be an oleate hydratase that played a role in stress tolerance, and knock-out of the *mcra* gene did not influence CLA generation (O'Connell et al. 2013). The precise role of 10-HOE in the CLA production process among bifidobacteria is still unknown.

In the current study, the relationships among LA, CLA, and 10-HOE were investigated to elucidate the role of 10-HOE in CLA production in bifidobacteria.

Materials and methods

Chemicals

LA and acetonitrile were purchased from Sigma-Aldrich (St. Louis, Marseilles) and J & K Scientific (Shanghai, China), respectively. All other chemicals used were of analytical grade and are commercially available.

Microorganism cultivation

Twenty-nine bifidobacterial strains were used in this study and deposited at the Culture Collection of Food Microorganisms (CCFM), Jiangnan University. All of the strains were first cultured in mMRS broth (pH 6.5) composed 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% $MgSO_4$, and 0.05% L-cysteine at 37 °C anaerobically (Electrotek 400TG, West Yorkshire, UK). *L. plantarum* ST-III, a non-CLA producer isolated from pickles (Yang et al. 2013), was used for 10-HOE purification.

E. coli JM109 (DE3) carrying the plasmid pOXO4 with *mcra* cloned from *B. breve* NCIMB 702258 (Rosberg-Cody

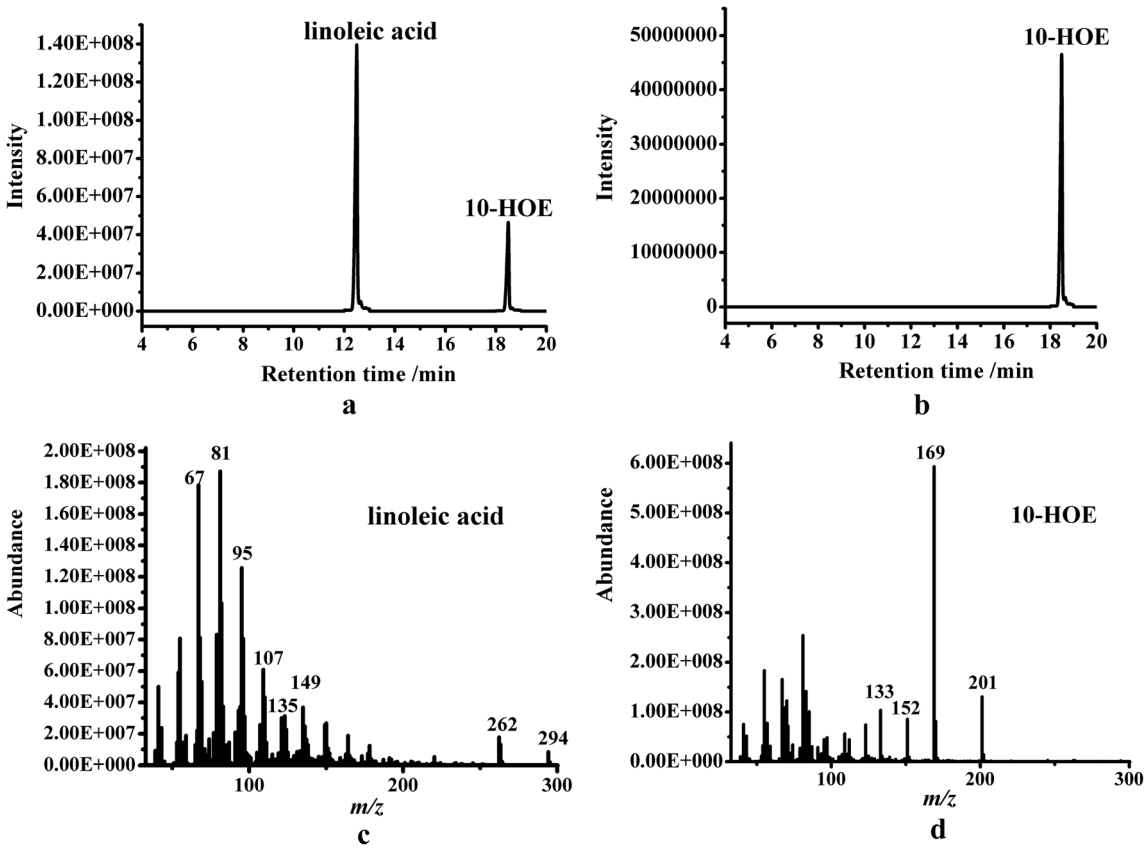

Fig. 1 GC-MS analysis of esterified fatty acid. **a** Fatty acid analysis extracted from medium-growing *L. plantarum* ST-III. **b** Identification of the purified 10-HOE. **c** Mass spectra of linoleic acid. **d** Mass spectra of 10-HOE

Table 1 CLA and 10-HOE generation by different bifidobacteria with LA as the substrate

Species	Strains	Source	CLA (mg/mL)	%	10-HOE (mg/mL)	%
B. adolescentis	CCFM743	Infant feces	ND	ND	0.125 ± 0.012	24.8
	CCFM744	Infant feces	ND	ND	0.156 ± 0.099	31.1
B. breve	CCFM622	Infant feces	0.408 ± 0.089	80.7	0.009 ± 0.001	1.8
	CCFM683	Infant feces	0.434 ± 0.098	85.9	0.003 ± 0.008	0.50
	CCFM684	Infant feces	0.421 ± 0.102	83.3	0.005 ± 0.002	0.91
B. longum subsp. *infantis*	CCFM644	Infant feces	0.171 ± 0.089	33.9	0.015 ± 0.003	2.99
	CCFM687	Infant feces	0.012 ± 0.008	2.4	0.079 ± 0.003	15.8
	CCFM689	Infant feces	ND	ND	0.043 ± 0.002	8.6
	CCFM751	Infant feces	0.032 ± 0.003	6.4	0.004 ± 0.001	0.85
	CCFM752	Infant feces	ND	ND	0.001 ± 0.010	0.26
	CCFM753	Infant feces	0.015 ± 0.002	2.9	0.004 ± 0.002	0.83
B. longum subsp. *longum*	CCFM642	Infant feces	0.116 ± 0.068	22.9	0.012 ± 0.002	2.2
	CCFM643	Infant feces	ND	ND	0.167 ± 0.097	33.1
	CCFM679	Infant feces	0.128 ± 0.019	25.4	ND	ND
	CCFM681	Infant feces	0.051 ± 0.007	10.1	0.009 ± 0.001	1.8
	CCFM686	Infant feces	0.039 ± 0.005	7.7	0.004 ± 0.001	0.72
	CCFM754	Infant feces	0.051 ± 0.002	9.9	0.017 ± 0.002	3.3
	CCFM755	Infant feces	0.034 ± 0.008	6.7	0.019 ± 0.003	3.8
	CCFM757	Infant feces	0.021 ± 0.009	4.2	0.006 ± 0.001	1.1
	CCFM758	Infant feces	0.041 ± 0.009	8.1	0.023 ± 0.002	4.5
	CCFM759	Infant feces	ND	ND	0.005 ± 0.001	0.93
	CCFM761	Infant feces	0.021 ± 0.009	4.3	0.007 ± 0.002	1.4
	CCFM762	Infant feces	0.175 ± 0.019	34.8	0.014 ± 0.003	2.8
B. pseudocatenulatum	CCFM680	Infant feces	0.186 ± 0.018	37.1	0.183 ± 0.019	36.3
	CCFM745	Infant feces	0.171 ± 0.089	33.9	0.156 ± 0.021	30.9
	MY45B	Infant feces	0.251 ± 0.098	49.9	0.164 ± 0.044	32.6
	CCFM747	Infant feces	0.097 ± 0.003	19.4	0.164 ± 0.018	32.5
	CCFM749	Infant feces	0.125 ± 0.029	24.9	0.197 ± 0.023	39.1
	CCFM750	Infant feces	0.072 ± 0.008	14.3	0.195 ± 0.045	38.7

ND not detected

et al. 2011) was routinely cultured aerobically in Luria-Bertani (LB) medium (10 g/mL tryptone, 5 g/L yeast extract, 10 g/mL NaCl) at 37 °C in the presence of chloramphenical (25 μg/mL) as a selective marker.

10-HOE preparation

L. plantarum ST-III was first cultured twice in MRS broth and then cultured (2%) with 0.50 g/L LA, which was then cultivated at 37 °C for 72 h. The LA stock solution was prepared as previously described (Yang et al. 2017b). Fermented cultures were centrifuged at 8000*g* for 5 min at room temperature. Fatty acids from the supernatant were extracted using isopropanol and n-hexane (Yang et al. 2017b). The upper hexane level was vacuum-concentrated. After the fatty acid mixture was re-dissolved with acetonitrile, 10-HOE separation was carried out using a liquid chromatogram (Waters 2695, Milford, MA) fitted with a Cosmosil column (Ultimate® XB-C30; 4.6 × 250 mm, 5 μm; Welch, Shanghai Co., Ltd) with pure acetonitrile as the mobile phase. Samples of 200 μL were injected into the column at a flow rate of 5 mL/min and column pressure of 1200 psi. A UV-detector was used to monitor the whole sample-collection process at 205 nm. 10-HOE fractions were analyzed using gas chromatography-mass spectrometry (GC-MS) to identify the purity. Purified 10-HOE fractions were then vacuum-concentrated and a 10-HOE stock solution was prepared by dissolving it in water with Tween-80 as the emulsifier.

Screening of bacterial CLA production by bifidobacteria

All of the tested strains were cultured (1%) in MRS broth including 0.05% (*w*/*v*) L-cysteine with LA (final concentration 0.5 mg/mL) or 10-HOE (final concentration 0.1 mg/mL) as the

Table 2 LA and CLA generation by different bifidobacteria with 10-HOE as the substrate

	Strains	Source	LA (mg/mL)	%	CLA (mg/mL)	%
B. adolescentis	CCFM743	Infant feces	0.016 ± 0.0002	15.5	ND	ND
	CCFM744	Infant feces	0.015 + 0.0006	14.6	ND	ND
B. breve	CCFM622	Infant feces	0.003 ± 0.0001	3.1	0.009 ± 0.002	8.3
	CCFM683	Infant feces	0.017 ± 0.0019	16.3	0.019 ± 0.014	18.3
	CCFM684	Infant feces	0.017 ± 0.0021	16.3	0.012 ± 0.016	11.5
B. longum subsp. *infantis*	CCFM644	Infant feces	0.002 ± 0.0001	2.1	ND	ND
	CCFM687	Infant feces	0.012 ± 0.003	11.5	0.0019 ± 0.0001	1.9
	CCFM689	Infant feces	0.006 ± 0.0001	5.9	ND	ND
	CCFM751	Infant feces	0.008 ± 0.0001	0.78	ND	ND
	CCFM752	Infant feces	0.016 ± 0.001	15.5	ND	ND
	CCFM753	Infant feces	0.006 ± 0.003	6.1	0.001 ± 0.0009	1.1
B. longum subsp. *longum*	CCFM642	Infant feces	0.019 ± 0.004	18.1	0.0002 ± 0.004	0.23
	CCFM643	Infant feces	0.011 ± 0.002	10.4	ND	ND
	CCFM679	Infant feces	0.005 ± 0.0001	4.7	ND	ND
	CCFM681	Infant feces	0.001 ± 0.001	1.3	0.007 ± 0.0005	6.3
	CCFM686	Infant feces	0.0006 ± 0.0009	0.53	0	ND
	CCFM754	Infant feces	0.001 ± 0.0006	1.3	ND	ND
	CCFM755	Infant feces	0.002 ± 0.0006	1.5	ND	ND
	CCFM757	Infant feces	0.016 ± 0.005	15.2	ND	ND
	CCFM758	Infant feces	0.004 ± 0.0017	3.8	ND	ND
	CCFM759	Infant feces	0.0009 ± 0.0003	0.87	ND	ND
	CCFM761	Infant feces	0.001 ± 0.0012	1.1	0.003 ± 0.006	3.3
	CCFM762	Infant feces	0.002 ± 0.0023	1.7	0.004 ± 0.006	3.6
B. pseudocatenulatum	CCFM680	Infant feces	0.009 ± 0.0004	8.4	ND	ND
	CCFM745	Infant feces	0.017 ± 0.0009	16.7	ND	ND
	MY45B	Infant feces	0.025 ± 0.0029	24.4	0.008 ± 0.001	8.2
	CCFM747	Infant feces	0.002 ± 0.0002	1.6	ND	ND
	CCFM749	Infant feces	0.004 ± 0.0002	3.6	0	ND
	CCFM750	Infant feces	0.007 ± 0.0003	6.4	0.0015 ± 0.002	1.4

ND not detected

substrate. Following incubation for 72 h, the fatty acid profiles of all of the tested cultures were detected as described above.

Variation investigation of LA, CLA, and 10-HOE during bifidobacterial CLA-producing process

B. breve CCFM683, which is also deposited at China General Microbiological Culture Collection Center and the corresponding number is CGMCC 11828, and *B. pseudocatenulatum* MY45B were selected to investigate the role of 10-HOE in CLA-producing process. LA or 10-HOE was added as the substrate at a concentration of 0.1 mg/mL. Cultures were sampled at 0 h, 5 h, 20 h, 48 h, and 72 h and the fatty acid of the supernatant was determined using GC-MS. Furthermore, LA and 10-HOE were synchronously used as the substrate, both at a concentration of about 0.05 mg/mL, and the fatty acid profile of the supernatant was evaluated.

LA production by *E. coli* JM109 (DE3) carrying plasmid pOXO4 with the *mcra* gene in the presence of 10-HOE

E. coli JM109 (DE3) harboring pOXO4-*mcra* was used to investigate the role of *mcra*-coding protein in transforming 10-HOE into LA. The strain was cultivated in LB medium with chloramphenicol added at 37 °C until OD_{600} reached 0.6. Isopropyl-beta-D-thiosulfan galactoside (IPTG) was then immediately added to a final concentration of 0.05 mmol/L and the culture was induced for protein expression at 18 °C for 10 h. pOXO4 vector-inserted *E. coli* JM109 (DE3) was used as a negative control. After induction, cells were harvested by centrifugation at 5000*g* for 5 min, washed twice with 20 mmol/L potassium phosphate buffer (KPB) (pH 6.5), and the cell pellets were stored at − 80 °C prior to use.

The reaction for activity assay was carried out in a screw tube containing 1 mL of the reaction mixture (20 mmol/L KPB, pH 6.5) with 5 mmol/L NADH and 0.1 mmol/L FAD as cofactors,

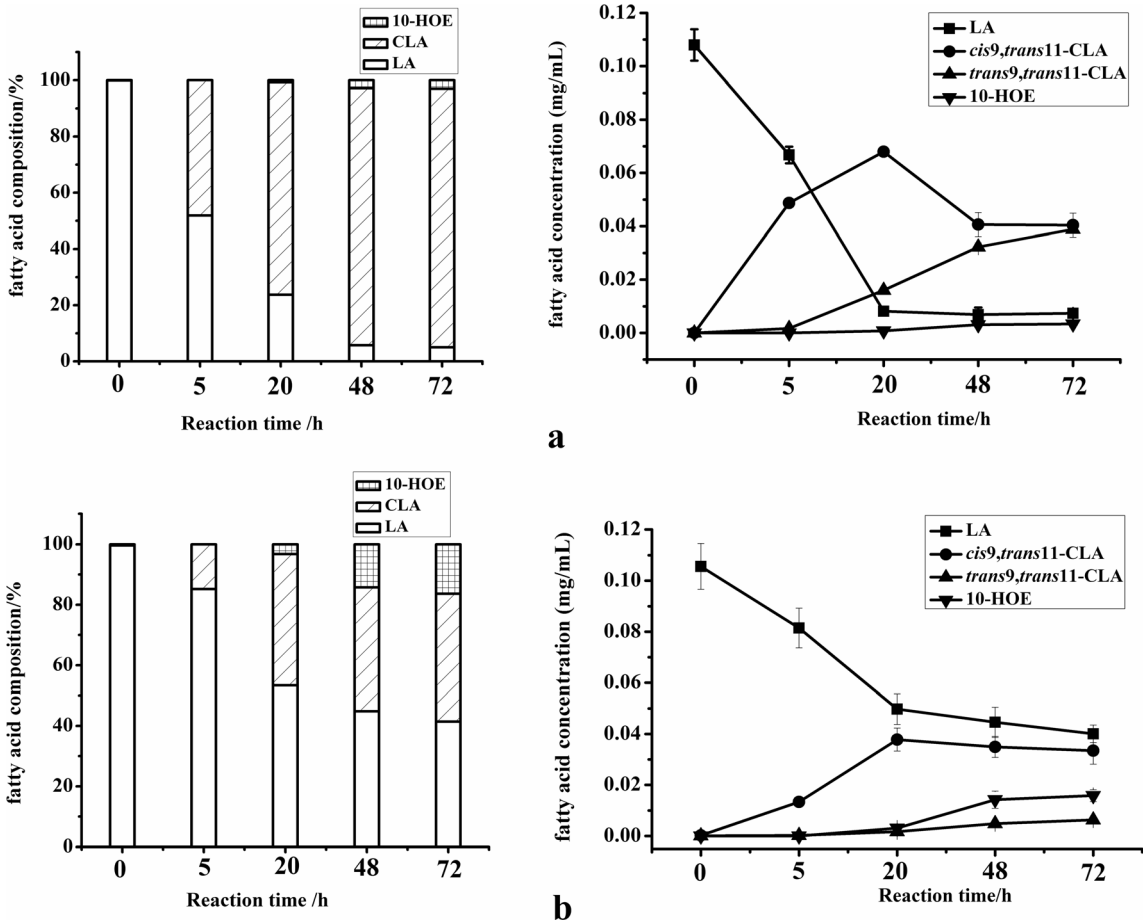

Fig. 2 Time courses of changes in LA, CLA, and 10-HOE during reaction with cells cultivated in mMRS medium with linoleic acid (0.1 mg/mL). **a** Time courses of changes in fatty acid composition during cultivation of *B. breve* CCFM683. **b** Changes in levels of LA, CLA, and 10-HOE for *B. breve* CCFM683. **c** Time courses of changes in fatty acid composition during cultivation of *B. pseudocatenulatum* My45B. **d** Changes in levels of LA, CLA, and 10-HOE for *B. pseudocatenulatum* My45B

0.1 mg/mL 10-HOE complexed with Tween-80 as the substrate, and 50 μL of transformed *E. coli* suspension (0.5 mg/mL wet cells) as the catalyst. The reaction mixture was kept under microaerobic conditions in a sealed chamber filled with N_2 and shaken (200 strokes/min) at 37 °C for 6 h.

Lipid analysis

Fatty acid extraction and methyl-esterification were carried out as previously described (Yang et al. 2013). Briefly, recovered fatty acid methyl esters with hexane were analyzed using GC (GC 2010 plus; Thermo Fisher, Waltham, Massachusetts) fitted with a QP2010 ultra mass spectrometer using a 5-MS column (30 m × 0.25 mm i.d. with 0.25 μm thickness) (Restek Corporation, Bellefonte, PA). The column temperature was set initially at 180 °C for 3 min, and then increased to 190 °C in increments of 10 °C/min, and maintained for 3 min. Then, the column temperature was increased to 220 °C in increments of 5 °C/min and maintained for 1 min. Finally, the column temperature was increased to 230 °C in increments of 2 °C/min and maintained for 18 min. The injector and detector were operated at 240 °C. Electron energy of 70 eV and ion source temperature of 230 °C were used. Heptadecanoic acid was used as the internal standard.

Statistical analysis

The experiments were carried out in triplicate and the results are presented as the mean ± standard deviation. Student's *t* test was used to determine the statistical differences. *p* values of < 0.05 were considered as statistically significant.

Results

10-HOE purification and identification

As *L. plantarum* ST-III can convert 10-HOE from LA without producing other derivates, it was chosen to purify 10-HOE. The conversion rate of 10-HOE from LA by this strain was 20.2% (Fig. 1a). The purity of the collected 10-HOE was

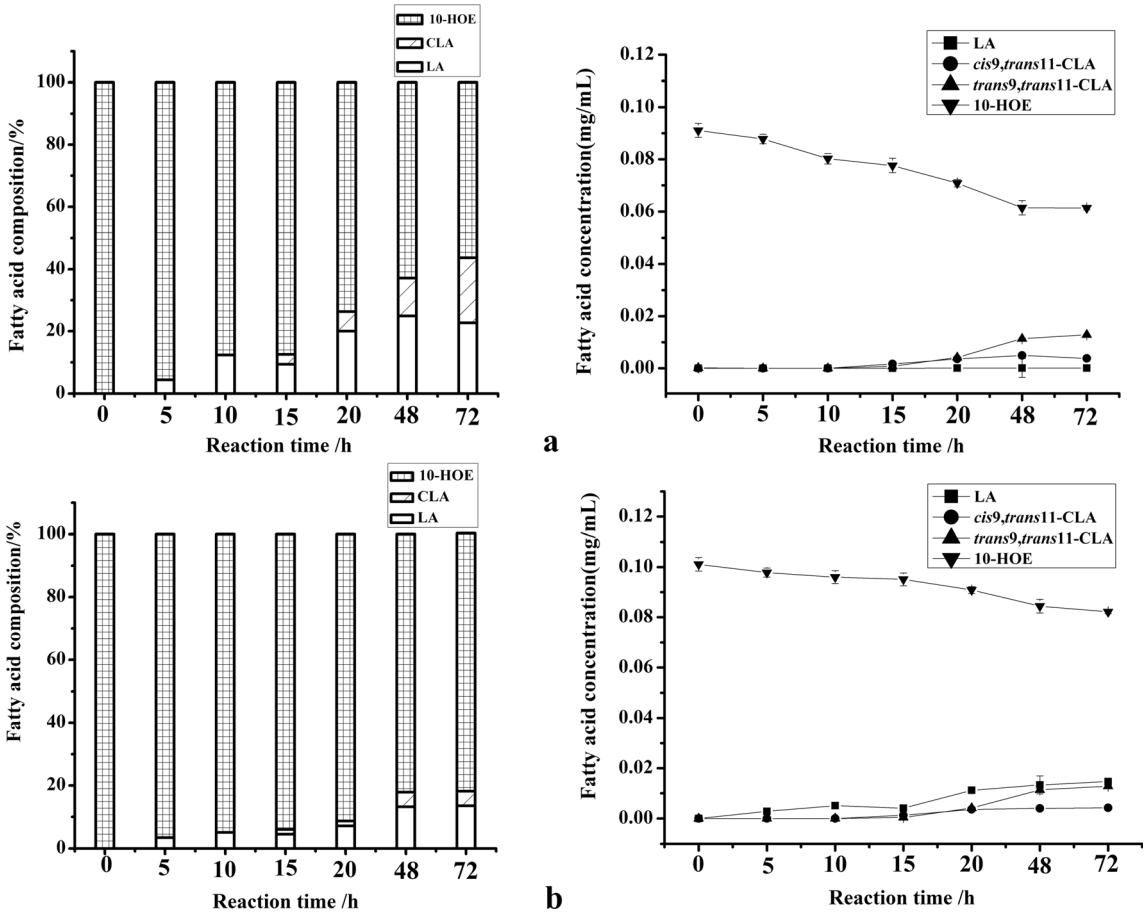

Fig. 3 Time courses of changes in LA, CLA, and 10-HOE during reaction with cells cultivated in mMRS medium with 10-hydroxy-*cis*-12-octadecenic acid (0.1 mg/mL). **a** Time courses of changes in fatty acid composition during cultivation of *B. breve* CCFM683. **b** Changes in levels of LA, CLA, and 10-HOE for *B. breve* CCFM683. **c** Time courses of changes in fatty acid composition during cultivation of *B. pseudocatenulatum* My45B. **d** Changes in levels of LA, CLA, and 10-HOE for *B. pseudocatenulatum* My45B

assessed as 100% by GC-MS (Fig. 1b). The qualitative measurement of LA and 10-HOE was based on its individual mass fragment. As reported in our previous studies, the typical mass fragment of linoleic acid was 81, 95, 107, 121, 135, 149, 262, and 294, while the mass fragment of 10-HOE was 133, 152, 169, and 201 (Fig. 1c and d). Ten liters of MRS medium containing LA-growing *L. plantarum* ST-III was cultivated for 72 h, and 0.81 g 10-HOE was obtained. The collected 10-HOE was used as the corresponding substrate in the following experiments.

Bifidobacterial CLA and 10-HOE production

To assess the CLA and 10-HOE production by bifidobacteria, 29 strains were examined. As shown in Table 1, only 23 strains converted LA into CLA to a certain extent, and the amount of CLA they produced varied widely, from 2.4% (for *B. logum* subsp. *infantis* CCFM687) to 85.9% (for *B. breve* CCFM683). Two *B. adolescentis* strains, CCFM743 and CCFM744, showed no CLA-producing ability. All of the tested strains also produced 10-HOE and the conversation rate ranged from 0.26 to 39.1%. *B. pseudocatenulatum* exhibited more efficient conversion of LA into 10-HOE (up to 39.1%) than other tested strains. Although *B. adolescentis* CCFM743 and CCFM742 could not convert LA into CLA, the 10-HOE conversion rate was 24.8% and 31.1%, respectively. In contrast, *B. breve* was identified to be the most efficient strain in CLA production, but the yield of 10-HOE was lower than 2%. These results suggest that CLA and 10-HOE could be converted from LA by bifidobacteria, but the conversion ability was strain-dependent.

10-HOE as the substrate to produce CLA

To analyze the role of 10-HOE in CLA production, all of the assessed strains were cultured in medium containing 10-HOE. The fatty acid analysis showed that CLA could be detected in 10 cultures. *B. breve* CCFM683 showed the highest conversion rate of up to 18.3% and another eight strains demonstrated conversion rates of less than 10%. Interestingly, LA could

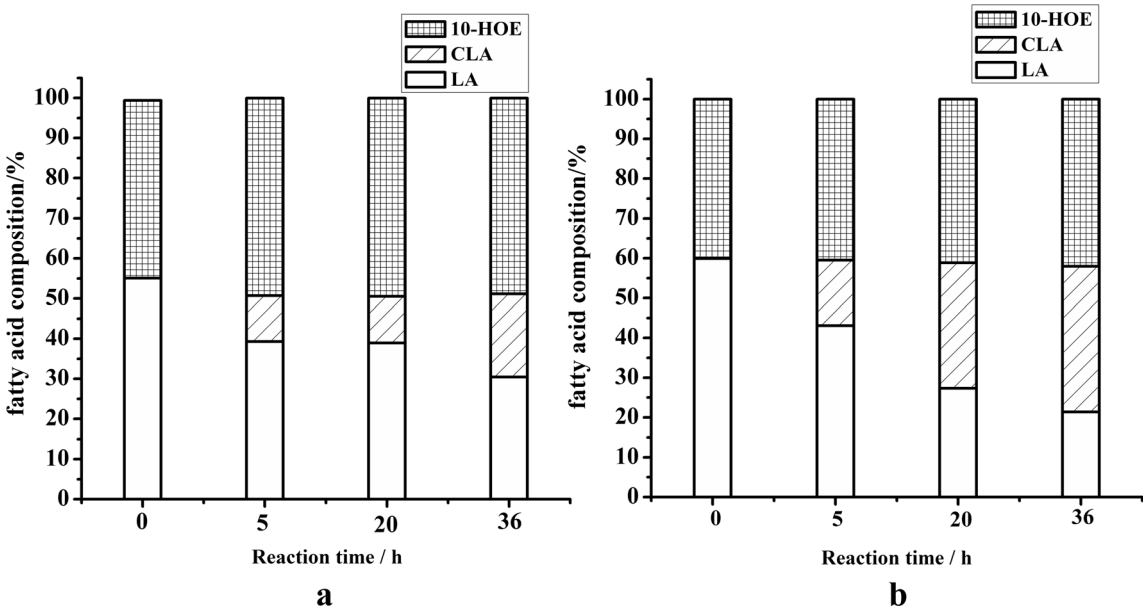

Fig. 4 Time courses of changes in LA, CLA, and 10-HOE during reaction with cells cultivated in mMRS medium with linoleic acid (0.05 mg/mL) and 10-hydroxy-*cis*-12-octadecenic acid (0.05 mg/mL). **a** Time courses of changes in fatty acid composition during cultivation of *B. breve* CCFM683. **b** Changes in levels of LA, CLA, and 10-HOE for *B. breve* CCFM683. **c** Time courses of changes in fatty acid composition during cultivation of *B. pseudocatenulatum* My45B. **d** Changes in levels of LA, CLA, and 10-HOE for *B. pseudocatenulatum* My45B

also be found in each culture (Table 2). *B. longum* subsp. *longum* CCFM642 produced the highest amount of LA with a conversion rate of up to 18.1%. These experimental results revealed that some bifidobacterial strains could also supply 10-HOE as the substrate to generate both CLA and LA.

LA acts as an intermediate during conversion of 10-HOE into CLA

To further examine the relationships among LA, CLA, and 10-HOE, *B. breve* CCFM683 and *B. pseudocatenulatum* MY45B were selected and LA, 10-HOE, or LA and 10-HOE were added as the substrate.

When LA was used as the substrate, CLA was first detected in cultures at 5 h. At this time, *B. breve* CCFM683 had converted nearly 50% of LA into CLA (Fig. 2a), whereas *B. pseudocatenulatum* MY45B had only transformed 12.5% (Fig. 2b). In contrast to LA, 10-HOE was markedly detected at 20 h rather than 5 h. At 20 h, the amount of 10-HOE had increased to 0.005 mg/mL for *B. breve* CCFM683 and to 0.003 mg/mL for *B. pseudocatenulatum* MY45B. Furthermore, *cis*9, *trans*11-CLA was the major isomer for both strains with a ratio of up to 52% for *B. breve* CCFM683 and 65.5% for *B. pseudocatenulatum* MY45B. Accordingly, the results clearly demonstrated that both strains could generate CLA and 10-HOE from LA, although they first accumulated CLA rather than 10-HOE.

Subsequently, 10-HOE was added to the medium as the substrate. Interestingly, LA could be observed earlier than CLA. At 5 h and 10 h, a significant amount of LA was found in the cultures growing both strains (Fig. 3), while no CLA could be detected. At this time interval, 10-HOE was only transformed into LA. At 10 h, the concentration of LA was 0.0113 mg/mL, but it decreased to 0.0085 mg/mL at 15 h. During this time, the concentration of CLA increased from 0 to 0.0025 mg/mL, while the amount of the 10-HOE remained unchanged. This result demonstrated that at this time, CLA was derived from the produced LA, not 10-HOE. In the following culturing time, we speculated that LA, CLA, and 10-HOE remained a dynamic balance. In contrast to the CLA isomer composition when LA was used as the substrate, *trans*9, *trans*11-CLA was the dominant isomer when 10-HOE was used as the substrate. At 72 h, the proportion of *trans*9, *trans*11-CLA in the total CLA was 77.2% for CCFM683 and 75.4% for MY45B. Based on these results and the findings using LA as the substrate, we hypothesized that LA probably acted as the intermediate when 10-HOE was transferred into CLA.

To further examine this hypothesis, we used a combination of LA and 10-HOE as the substrate (Fig. 4). For *B. breve* CCFM683, we found that LA decreased rapidly from 0.07 ± 0.0045 to 0.02 ± 0.0034 mg/mL while CLA increased to 0.04 ± 0.0049 mg/mL. However, the concentration of 10-HOE increased slightly from 0.0045 ± 0.0004 to 0.0047 ± 0.0001 mg/mL. Similarly, for *B. pseudocatenulatum* MY45B, the LA concentration decreased from 0.06 ± 0.0045 to 0.03 ± 0.0042 mg/mL, while CLA and 10-HOE increased to 0.02 ± 0.0041 mg/mL and 0.05 ± 0.0055 mg/mL, respectively.

Overall, our experimental evidence provides a solid basis for concluding that 10-HOE can be used as the substrate to generate CLA by bifidobacteria; however, LA acted as the intermediate in this case.

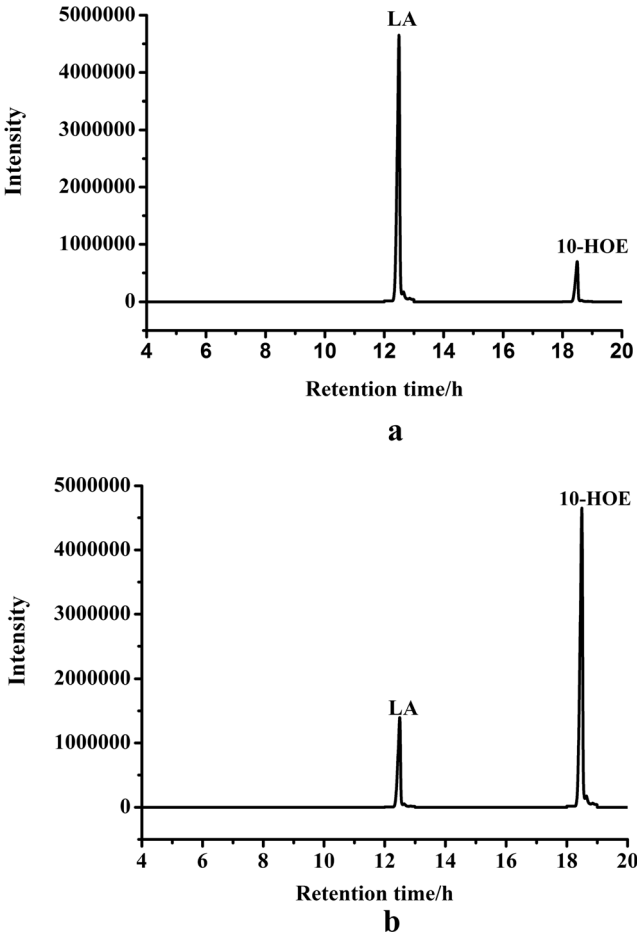

Fig. 5 Analysis of LA and 10-HOE during reaction with recombinant cells inserted with *mcra* gene with linoleic acid (0.1 mg/mL) and 10-hydroxy-*cis*-octadecenic acid (0.1 mg/mL) as substrate

MCRA catalyzes the reversible reaction between LA and 10-HOE

MCRA has been identified as an oleate hydratase among *B. breve*, which controls the transformation of LA into 10-HOE. We speculated that this protein may also be involved in the conversion of 10-HOE into LA. To clarify our speculation, we generated one *E. coli* variant including plasmid pOXO4 inserted with a *mcra* gene cloned from *B. breve* NCIMB 702258. *E. coli* variant including the empty plasmid pOXO4 was adopted as the control in this experiment. The *E. coli* pellets induced by IPTG were used as the catalyst suspended in KPB (pH 6.5), and a concentration of 0.1 mg/mL of LA or 10-HOE complexed with Tween-80 was added to the reaction mixture. When LA was used as the substrate, 0.03 mg/mL of 10-HOE was detected in the medium, as shown in Fig. 5a, while 0.02 mg/mL of LA was produced when 10-HOE was added to the reaction solution as the substrate. These results provide solid evidence that MCRA was responsible for the reversible reaction between LA and 10-HOE among *B. breve*.

Discussion

Bacterial bio-transformation of LA into CLA may be a key step in the detoxification of free fatty acids by bacteria (Jiang et al. 1998). In the current study, we assessed the ability of 29 bifidobacteria to convert free LA into CLA. None of the *B. adolescentis* strains investigated converted LA into CLA at a detectable level, whereas all *B. breve* strains showed considerable CLA production ability. *B. breve* CCFM683, isolated from neonate feces, was the most efficient CLA producer, converting over 85% of LA into *cis*9, *trans*11-CLA and *trans*9, *trans*11-CLA. It has previously been reported that *B. breve* might be one of the most efficient CLA producers among bifidobacteria (Coakley et al. 2003).

Accompanied by CLA, 10-HOE was also transformed from LA at different levels by these strains. *B. pseudocatenulatum* was the most efficient producer of 10-HOE with a conversion rate of up to 39.1%. 10-HOE has also been generated from LA by a number of other bacteria, such as *L. plantarum* (Yang et al. 2017b), *L. acidophilus* (Ogawa et al. 2001), *B. breve* (O'Connell et al. 2013), and *S. pyogenes* (Volkov et al. 2010). In addition to 10-HOE, some *L. plantarum* can also convert LA into 13,10-dihydroxy octadecanoic acid, a fatty acid containing 18 carbon atoms with 2 hydroxyls on the C_{10} and C_{13} carbon atoms, respectively (Black et al. 2013).

Previous studies have reported 10-HOE as the key intermediate during CLA production by *L. acidophilus* (Ogawa et al. 2001) and *L. plantarum* (Yang et al. 2017b). In contrast, the role of 10-HOE produced by *B. breve* in CLA generation might be different from that of other lactobacilli (O'Connell et al. 2013). It was reported that a mutant of the *mcra* gene did not influence the level of CLA produced by *B. breve* NCIMB702258, suggesting that 10-HOE is not the intermediate during conversion of LA into CLA by *B. breve*. For lactobacilli, 10-HOE first accumulated to some extent, and was then converted to 10-oxo-*cis*-12-octadecenoic acid, 10-oxo-*trans*-11-octadecenoic acid, 10-hydroxy-*trans*-11-octadecenoic acid, and finally CLA (Kishino et al. 2013). However, the results of our study deepen our understanding of the role of 10-HOE in CLA production by *B. breve* and *B. pseudocatenulatum*. In this case, 10-HOE can be used as the substrate to produce CLA, but first, it seemed to be transformed into LA, as shown in Fig. 6. Furthermore, we found that the major CLA isomer was *cis*9, *trans*9-CLA, when LA was the substrate, and was *trans*9, *trans*11-CLA, when 10-HOE was the substrate. More studies should be carried out to explain this difference. Moreover, we also observed a reversion reaction between LA and 10-HOE in *B. breve* and *B. pseudocatenulatum*.

Previous studies have demonstrated that MCRA was also responsible for LA generation from 10-HOE by *L.*

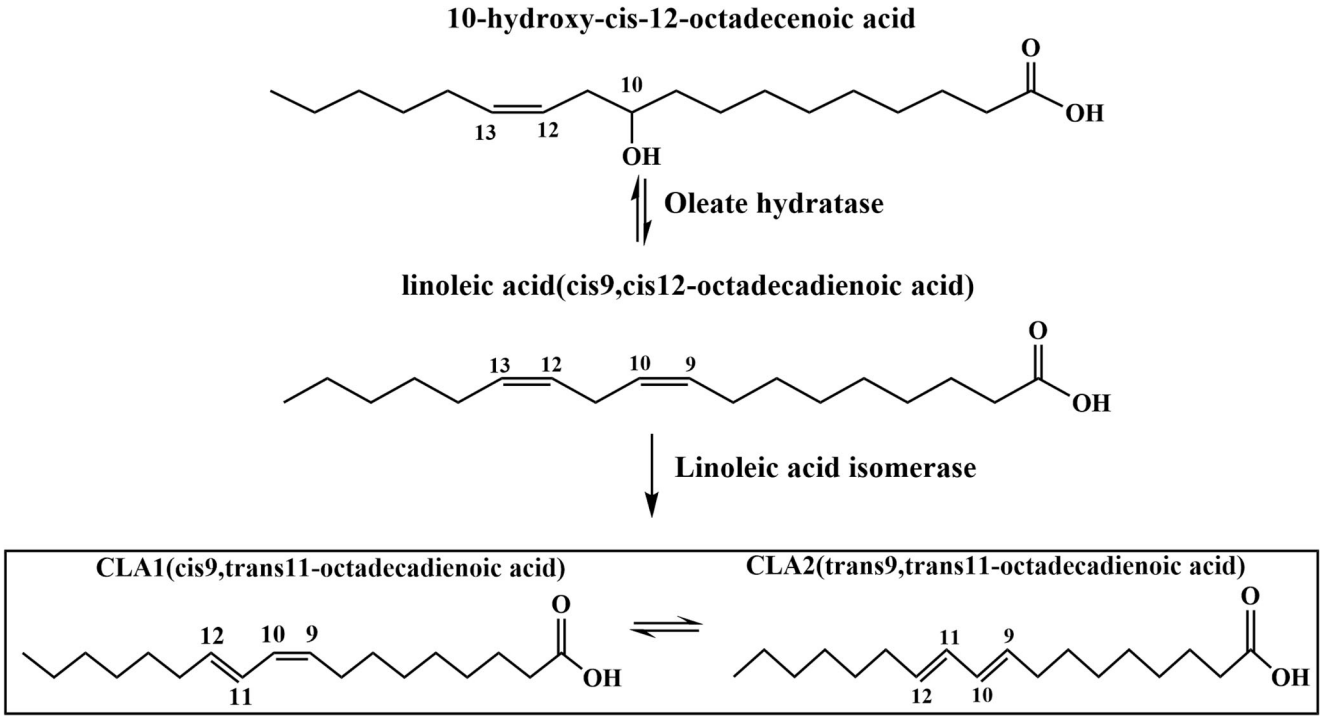

Fig. 6 Proposed pathway of CLA production by bifidobacteria

acidophilus (Kishino et al. 2013). Because the MCRA protein family has been identified to be highly conserved among different bacterial species (Volkov et al. 2010; Yang et al. 2013), we wondered whether MCRA from *B. breve* could also catalyze the reversible reaction between LA and 10-HOE. *E. coli* JM109 containing the *mcra* gene from *B. breve* NCIMB 702258 was used to clarify the role of MCRA in this reversible reaction. The results clearly revealed that the corresponding cell pellets could catalyze the reaction from 10-HOE to LA and from LA to 10-HOE, suggesting that MCRA accounted for the reversible transformation between LA and 10-HOE, which is clearly demonstrated in Fig. 6. Considering the highly conserved sequence of MCRA (Yang et al. 2013), we speculate that MCRA from other bifidobacterial species could also be responsible for the reversible reaction between LA and 10-HOE.

As far as we are aware, this is the first study to clarify the role of 10-HOE in CLA generation by bifidobacteria.

In conclusion, 10-HOE can be used as the substrate by bifidobacteria to produce CLA; however, in this case, LA acted as the intermediate during the transformation of 10-HOE into CLA, and MCRA was responsible for the reversible transformation between LA and 10-HOE.

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Conflict of interest The authors declare that they have no conflict of interest.

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