Chapter 2
Lactic Acid Bacteria and Conjugated Fatty Acids

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2.1 Introduction

Conjugated fatty acid (CFA) refers to a group of positional and geometric isomers of polyunsaturated fatty acid possessing conjugated double bonds. Conjugated double bonds, conjugated triple bonds, and conjugated quadruple bonds are the typical conjugated fatty acid forms, in which conjugated octadecadienoic acid and conjugated octadecatrienoic acid are most common isomers, such as conjugated linoleic acid (CLA), conjugated linolenic acid (CLNA), and conjugated stearic acid CSA (Yang et al. 2015).

2.1.1 Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is a generic term of octadecadienoic acid with conjugated double bonds, referring to a group positional and geometric isomer of linoleic acid (LA), in which each conjugated double bond exists in two types, *cis* (*c*) and *trans* (*t*). In theory, according to the position of double bonds, 54 isomers of CLA could be synthesized; however, until now, only 28 isomers have been identified, including conjugated double bond on C_7,C_9, C_8,C_{10}, C_9, C_{11}, C_{10},C_{12}, C_{11}, and C_{13}. *c*9, *t*11-CLA (rumenic acid) was the most abundant CLA isomer, followed by *t*10,*c*12-CLA (Andrade et al. 2012).

CLA has attracted much attention due to its physiological effects, such as anti-inflammation, anticancer, reduction of atherosclerosis, anti-obesity, amelioration of

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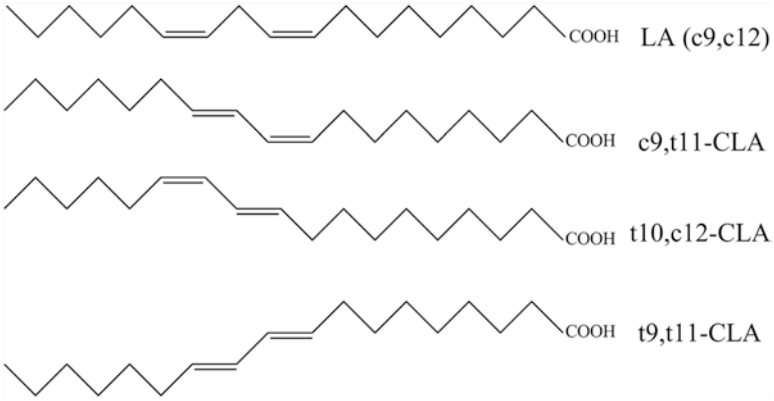

Fig. 2.1 Structure of linoleic acid and major CLA isomers

diabetes, promotion of bone growth, and immune regulation. As reported, the biological function was isomer-dependent, in which *c*9,*t*11-CLA and *t*10,*c*12-CLA were recognized as the CLA isomers with best physiological effects (Fig. 2.1). The major physiological functions of *c*9,*t*11-CLA were anti-cancer, anti-inflammation, and immune regulation, whereas *t*10,*c*12-CLA has significant benefits on anti-obesity and regulation of lipid metabolism. Additionally, *t*9,*t*11-CLA was reported with anti-inflammation function (Yang et al. 2015).

CLA naturally occurs in ruminant milk and issues; therefore, ruminant dairy and meat products are the main source of CLA in the daily diet, in which *c*9, *t*11-CLA comprised of 80–90% fatty acid of the total dairy lipids and *t*10,*c*12-CLA comprised of only 1% of total dairy lipids. Moreover, other CLA isomers, such as *t*7,*c*9-CLA, *c*8,*t*10-CLA, *t*10,*c*12-CLA, and *t*11,*c*13-CLA, could be detected in the milk (Jensen 2002). In ruminant animals, two major sources of CLA were reported: (1) CLA was mainly produced as one of the intermediates by some ruminant bacteria in the process of catalyzing LA into stearic acid (C18:0), and (2) numerous researches have reported that *c*9,*t*11-CLA could be generated by Δ9-dehydrogenase in the mammary gland with vaccenic acid (*t*11-C18:1) as substrate. Many studies on the CLA-producing mechanism in ruminant bacteria have been carried out (Kepler et al. 1966, 1971; Kepler and Tove 1967; Polan et al. 1964; Rosenfeld and Tove 1971) that LA could be quickly transformed into CLA by linoleic acid isomerase in ruminant bacteria and then transferred into vaccenic acid at a slower rate. After vaccenic acid was accumulated to a certain level, it would be further transformed to stearic acid. Other studies have demonstrated that some vaccenic acid in ruminant animals could be absorbed and then transported to other tissues. Vaccenic acid in the mammary bland could be further transferred into CLA through catalyzing by Δ9-dehydrogenase (Bauman et al. 2001). It has been identified that CLA generated from this process could comprise of 60–70% of total CLA in the milk (Corl et al. 2001).

2.1.2 Conjugated Linolenic Acid

Conjugated linolenic acid (CLNA) was one of the derivates from linolenic acid (LNA, C18:3) with conjugated double bonds, comprising of different isomers (Fig. 2.2). CLNA was firstly discovered in the nineteenth century; however, it was not attracted much attention due to rare awareness of its physiological effects. Till 1987, Nuteren and Christ-Hazelhof firstly identified the biological activity of CLNA when they studied the inhibitory effect of fatty acids derived from plant seeds on the synthesis of prostaglandin E2 (PGE2) (Nugteren and Christ 1987). Later, anti-cancer and anti-obesity activities of naturally CLNA from bifidobacteria were reported by other researchers (Coakley et al. 2009; Hennessy et al. 2012; Destaillats et al. 2005.) The bifidobacterial CLNA isomers analyzed included *c*9,*t*11,*c*15-CLNA, *t*9,*t*11,*c*15-CLNA, *c*9,*t*11,*c*13-CLNA, *c*9,*t*11,*t*13-CLNA, *c*6,*c*9,*t*11-CLNA, and *c*6,*t*9,*t*11-CLNA.

CLNA was widely distributed in nature, such as milk and ruminant meet. In addition, CLNA occurs in some plant seeds, for instance, pomegranate seeds, tung oil seeds, *momordica charantia* seeds, calendula seeds, etc. The CLNA isomers derived from plant seeds consist of many kinds of isomers, and thus proper

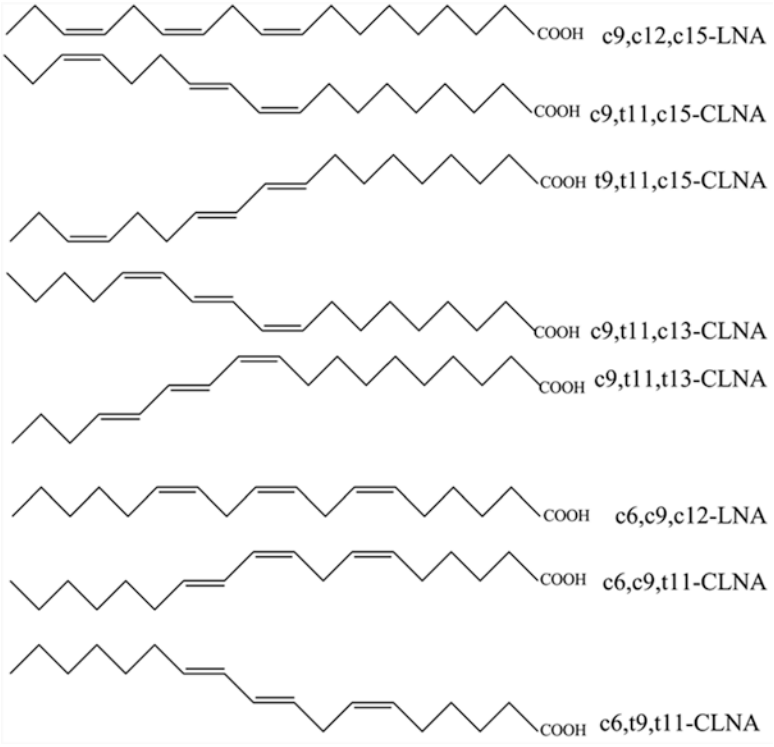

Fig. 2.2 Structure of α-linolenic acid, γ- linolenic acid, and conjugated linolenic acids

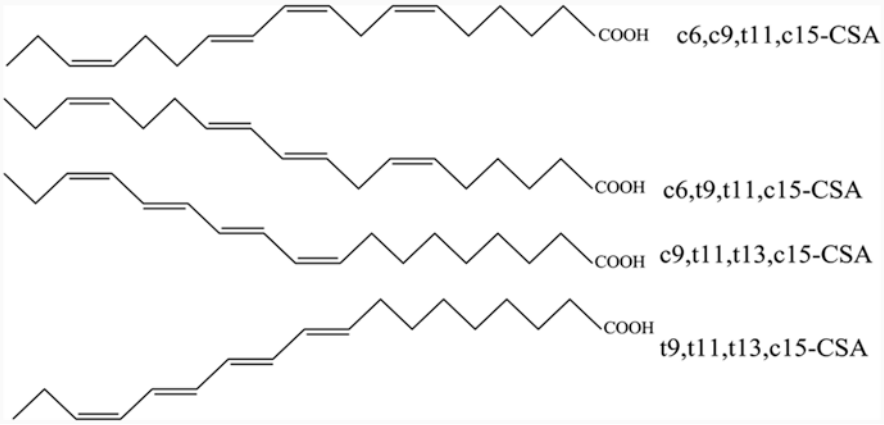

Fig. 2.3 Structure of conjugated stearidonic acids

separation methods would be key factors to obtain the pure isomers (Smith Jr. 1971). Recently, supercritical CO_2 fluid extraction and low-temperature-crystallization methods have been applied to separate CLNA. However, recent separation methods could not address the commercial requirements due to the limited amount of food grade plants.

2.1.3 Other Conjugated Fatty Acid

Despite CLA and CLNA, another conjugated fatty acid is conjugated stearidonic acid (CSA). CSA has been identified to possess many physiological activities, such as anti-tumor, antiatherosclerosis, and hypoglycemic activity. Besides, CSA could be applied in lipid peroxidation to evaluate the antioxidant agents. The identified isomers of CSA include *c*6,*c*9,*t*11,*c*15-CSA, *c*6,*t*9,*t*11,*c*15-CSA, *c*9,*t*11,*t*13,c15-CSA, and *t*9,*t*11,*t*13,*c*15-CSA (Fig. 2.3) (Hennessy et al. 2012).

2.2 Lactic Acid Bacteria with Conjugated Fatty Acid Production Ability

Microbial CFA producers have been studied for decades, which started in 1960s. Kepler et al. (1966, 1970, 1971), Kepler and Tove (1967) originally found that *Butyrivibrio fibrisolvens*, one of the ruminant bacteria, could convert LA to CLA. Then a variety of microbes showed the property of CLA production, especially that lactic acid bacteria could generate *c*9,*t*11-CLA and *t*9,*t*11-CLA. With the increase of research of CLA production in lactic acid bacteria, CLNA, CSA, and

other CFA were found in lactic acid bacteria metabolites. Lactic acid bacteria with CFA production ability include *Lactobacillus* (*L. plantarum*, *L. acidophilus*, *L. casei*, *L. reuteri*, *L. fermentum*, *L. bulgaricus*, *L. rhamnosus*), *Bifidobacterium* (*B. breve*, *B. longum*, *B. animalis* subsp. *lactis*), *Lactococcus lactis*, *Streptococcus thermophiles*, etc.

2.2.1 Conjugated Fatty Acid Production in Lactobacilli

Lactobacillus was widely reported with CFA production ability, especially CLA production, which consisted of almost each species of lactobacilli (Table 2.1).

2.2.1.1 *Lactobacillus plantarum*

L. plantarum was the most widely studied strain among lactobacilli with CLA-production ability. In 2002, Kishino et al. screened many lactic acid bacteria strains with CLA-production ability, including *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Propionibacterium* (Kishino et al. 2002), in which *L. plantarum* AKU1009a was the strain with the best CLA-generation ability. Further study demonstrated that *L. plantarum* AKU1009a could even transform ricinoleic acid into CLA directly. Interestingly, the washed cells of this strain could catalyze α-linolenic acid into *c*9,*t*11,*c*15-CLNA and *t*9,*t*11,*c*15-CLNA with a 40% of total conversion rate. Comparatively, it has a better ability of CLNA from γ-linolenic acid with a conversion rate up to 68%. CSA could be produced from stearidonic acid into *c*6,*c*9,*t*11,*c*15-CSA and *c*6,*t*9,*t*11,*c*15-CSA by the strain (Kishino et al. 2010). The concentration of ricinoleic acid utilized by the washed cells of *L. plantarum* JCM1551 was up to 2400 mg/L with *c*9,*t*11-CLA and *t*9,*t*11-CLA as the main isomers (Andrade et al. 2012). *L. plantarum* NCUL005 has also been reported to produce CLA with final concentration of 623 mg/L growing in MRS medium in the presence of free LA (Andrade et al. 2012). Furthermore, growing and washed cells of *L. plantarum* ZS2058 could both transform free LA into CLA with conversion rate as 54.3% and 46.75%, respectively (Yang et al. 2017).

Except growing cells in the MRS medium or washed cells in the proper reaction solution, the strains added into the fermentis medium including sunflower oils or soymilk could also be used as the CLA producers (Li et al. 2012). VSL3# was the most widely used probiotics including eight strains, and studies have reported that all the eight strains could produce CLA, in which the conversion rate of *L. plantarum* strain was about 60% with *c*9, *t*11-CLA and *t*9,*t*11-CLA as the main isomers (Ewaschuk et al. 2006).

Furthermore, the CLA production mechanism by lactobacilli was also identified. Shimizu et al. firstly found that LA was firstly transformed into 10-hydroxy-*cis*-12 octadecenic acid and 10-hydroxy-*trans*-12-octadecenic acid, and then these two intermediates were both transferred into CLA (Ogawa et al. 2001). Further analysis

Table 2.1 High conjugated fatty acid producers in lactobacilli

Strains	Substrate and conditions	Products	Isomers	Reference
L. acidophilus L1	LA; growth cells	CLA (131 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Alonso et al. (2003)
L. acidophilus La-5	LA; washed cells	CLA (388 mg/g)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Macouzet et al. (2009)
L. acidophilus F0221	LA; growth cells	CLA (161 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Li et al. (2011)
L. acidophilus ADH	LA; growth cells	CLA (630 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Xu et al. (2008)
L. acidophilus CCRC14079	LA; growth cells	CLA (105 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Lin et al. (1999)
L. acidophilus AKU1137	LA; washed cells	CLA (4900 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2001)
L. acidophilus IAM10074	LA; washed cells	CLA (600 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2005)
L. acidophilus AKU1122	LA; washed cells	CLA (120 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2005)
L. acidophilus CRL730	LA; growth cells	CLA (23.8%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	van Nieuwenhove et al. (2007)
L. acidophilus Q42	LA; growth cells	CLA (20%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
L. brevis IAM1082	LA; washed cells	CLA (550 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2005)
L. casei E5	LA; growth cells	CLA (111 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Alonso et al. (2003)
L. casei E10	LA; growth cells	CLA (80 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Alonso et al. (2003)
L. casei CRL431	LA; growth cells	CLA (175 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	van Nieuwenhove et al. (2007)
L. casei CRL87	LA; growth cells	CLA (17%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
L. curvatus LMG13553	LA; growth cells	CLA (1.6%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorissen et al. (2011)
	LNA; growth cells	CLNA (22.4%)	c9,t11,c15-CLNA; *t*9,*t*11,*c*15-CLNA	
L. paracasei IFO12004	LA; washed cells	CLA (200 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2005)
L. bulgaricus CCRC14009	LA; washed cells	CLA (16%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Lin et al. (2006)
L. pentosus IFO12011	LA; washed cells	CLA (30 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Ogawa et al. (2005)
L. plantarum AKU1009a	LA; washed cells	CLA (40,000 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Andrade et al. (2012)
	Ricinoleic acid; washed cells	CLA (1650 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	

(continued)

Table 2.1 (continued)

Strains	Substrate and conditions	Products	Isomers	Reference
L. plantarum JCM1551	Ricinoleic acid; washed cells	CLA (2400 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Andrade et al. (2012)
*L. plantarum*Ip5	LA; growth cells	CLA (142 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
L. plantarum NCUL005	LA; growth cells	CLA (623 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
L. plantarum ATCC8014	LA; growth cells	CLA (4.6%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorissen et al. (2011)
	LNA; growth cells	CLNA (26.8%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
*L. plantarum*ZS2058	LA; growth cells	CLA (54.3%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Yang et al. (20140
	LA; washed cells	CLA (46.75%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Xu et al. (2008)
L. plantarum DSM20179	LA; growth cells	CLA (240 mg/L)	*c*9,*t*11-CLA	Khosravi et al. (2015)
L. plantarum AKU1138	LA; washed cells	CLA (450 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Kishino et al. (2002)
L. plantarum LT2–6	LA; washed cells	CLA (52.4%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Zhang (2004)
L. plantarum A6-1F	LA; washed cells	CLA (276 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Zhao et al. (2011)
L. rhamnosus PL60	LA; washed cells	CLA (4438 mg/g)	*c*9,*t*11-CLA; *t*10,*t*12-CLA	Lee et al. (2006a)
L. rhamnosus C14	LA; growth cells	CLA (190 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	van Nieuwenhove et al. (2007)
L. reuteri ATCC55739	LA; growth cells	CLA (300 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Lee et al. (2003)
L. sakei LMG13558	LA; growth cells	CLA (4.2%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorissen et al. (2011)
	LNA; growth cells	CLA (60.1%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
L. sakei CG1	LNA; growth cells	CLNA (28.3%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	

has identified multiple enzymes that were involved in CLA production, including hydrogenase, oxidoreductase, and isomerase (Kishino et al. 2013).

In China, Zhou et al. firstly separated one strain, named *L. plantarum* ZS2058, from pickled vegetables in Sichuan province possessing high CLA-producing ability (Zhou et al. 2004). The optimal condition of CLA production by *L. plantarum* ZS2058 has also been reported by Xu et al. (2008), and furthermore, the separation

of linoleic acid isomerase from this strain has also been carried out (Gu et al. 2008). Yang et al. screened of the CLA generation by some lactobacilli strains, and results showed that *L. plantarum* ZS2058 possessed the highest conversion rate, and the enzymatic activity assay demonstrated that the process generating CLA by *L. plantarum* ZS2058 consisted of multiple reactions with 10-hydroxy-*cis*-12-octadecenic acid, 10-oxo-*cis*-12-octadecenic acid, 10-oxo-*trans*11-octadecenic acid, and 10-hydroxy-*trans*11-octadecenic acid as the substrates (Yang et al. 2014).

2.2.1.2 *Lactobacillus acidophilus*

The conversion of CLA by *L. acidophilus* CCRC14079 was firstly studied by Lin et al. (1999), in which results revealed that this strain could transform about 10% of free linoleic acid in the milk lipids into CLA. The highest conversion rate of CLA produced by this strain was obtained when the strain was cultivated in the medium in the presence of free LA for 24 h. *L. acidophilus* L1 and O16 were another two strains possessing CLA production ability with conversion rate more than 50% growing in the medium or dried skimmed milk system added with free linoleic acid. Washed cells of *L. acidophilus* AKU1137 could generate CLA with the final concentration of 4.9 g/L. In this study, large amount of hydroxyl fatty acids could also be detected, and both of the concentrations of CLA and hydroxyl fatty acids present positive linear relationship (Ogawa et al. 2001). Kim and Liu screened the CLA production ability by eight lactobacilli strains growing in the MRS medium and skimmed milk reaction system, and results demonstrated that *L. acidophilus* 96 could transform LA into CLA, while four strains could not generate CLA at this condition. Macouzet et al. reported that washed cells of *L. acidophilus* La-5 could accumulate CLA when the strain grew in the MRS medium added with free LA at the concentration of 0.4 g/L or 0.37% milk lipids. Other studies showed that limiting oxygen could reduce the ratio of the *c*9,*t*11-CLA and *t*9,*t*11-CLA without influencing the total amount of CLA (Kim and Liu 2010).

2.2.1.3 *Lactobacillus reuteri*

Rosson et al. (1999) reported that *L. reuteri* PYR8, separated from the rat intestine, could transform about 60% of free LA into *c*9,*t*11-CLA. Lee et al. optimized the reaction condition of producing CLA by *L. reuteri* ATCC55739 and found that the immobilized cells could produce 175 mg/L CLA with the concentration of free LA as 500 mg/L, possessing the production efficiency of 175 mg/(L.h), about 5.5 times than that produced by washed cells (Sun et al. 2003). Further study performed by Roman et al. showed that cholate could not influence the CLA production by *L. reuteri* ATCC55739 in vitro. Moreover, Hernandez et al. also investigated the effect of temperature, concentration of LA, oxygen, and pH on the CLA production by a *L. reuteri* strain (Hernandezmendoza et al. 2010). Results showed that the

concentration of CLA produced by this strain at different conditions showed significant difference, and the highest CLA conversion rate was obtained when the strain was cultivated at 10 °C for 20 h in a microanaerobic environment with free LA as the substrate at the concentration of 20 mg/mL. CLA production would decrease with pH decreasing from 6.5 to 5.5.

Rosson et al. (1999) first tried to separate the putative linoleic acid isomerase and also cloned these genes exogenously. However, the proteins cloned from *L. reuteri* were identified to produce trace of hydroxyl fatty acid, other than CLA, though Rosson modulated some other factors, which might influence the enzymatic activity, such as expression system.

2.2.1.4 *Lactobacillus casei*

In 2003, Alonso et al. discovered two *L. casei* strains which could produce CLA in the MRS medium or skimmed milk with the ratio of *c*9,*t*11-CLA exceeding 80% (Alonso et al. 2003). The study of van Nieuwenhove showed that *L. casei* CRL431 showed the highest CLA conversion rate (35.9%) among the eight studied strains (Nieuwenhove et al. 2010). Results also demonstrated that the concentration of CLA produced by these eight strains growing in the buffalo milk in the presence of free LA (200 mg/L) was two to three times than that in the MRS medium. Interestingly, all the tested eight strains could produce CLA with LA concentration up to 1000 mg/mL. The *L. casei* strain in the probiotics VSL#3 possessed the CLA conversion rate exceeding 60% with *c*9, *t*11-CLA and *t*10, *c*12-CLA as the main isomers.

2.2.1.5 Other Lactobacilli Strains

Recently, *L. rhamnosus* PL60 was the only strain in this species identified to produce CLA with t10,c12-CLA as the predominant isomer. In 2006, Lee investigated the physiological effect of *L. rhamnosus* PL60 in vivo. Results showed that comparative to the negative groups, the weight of the mice feeded with this strain decreased significantly, as well as the white adipose tissue. Further analysis identified that this strain possessed perfect anti-obesity effect due to its production of *t*10,*c*12-CLA. And this strain could also colonize in the gut of volunteers (Lee and Lee 2009).

Additionally, Romero-Pérez also reported a *L. paracasei* strain could also convert 85% of free LA into CLA (Romero-Pérez et al. 2013). Florence also revealed that the combination of *B. lactis*, *S. thermophilus*, and *L. bulgaricus* could increase the CLA in the dairy products (Florence et al. 2012). Other studies also reported that *L. sake* and *L. curvatus* could also transform ALA into CLNA with the conversion rate of 22.4% and 60.1%, respectively (Gorissen et al. 2011). Ewaschuk et al. also reported that the CLA conversion rate of *L. bulgaricus* and *S. thermophilus* tested in this study ranged 60–70% (Ewaschuk et al. 2006).

2.2.2 *Conjugated Fatty Acid Production in Bifidobacteria*

The first *Bifidobacterium* with CLA production was reported by Coakley (Coakley et al. 2003). In their study, 15 *Bifidobacterium* strains were screened for CLA generation in the medium in the presence of free LA, in which 9 strains showed perfect CLA-producing ability with *c*9, *t*11-CLA as the main isomer. Among all the tested strains, *B. breve* and *B. dentium* possessed higher CLA conversion rate, in which *B. breve* NCFB2258 could convert about 66% of free LA to *c*9,*t*11-CLA and 6.2% of LA to *t*9,*t*11-CLA. Moreover, nearly all the produced CLA existed in the supernatants of the medium. As bifidobacteria was one of the pioneer colonized species in neonates and infants fed with breast milk, a number of researchers isolated bifidobacteria from infants and analyzed their CLA production abilities. Chung (Chung et al. 2008) evaluated 150 bifidobacterial strains for their CLA-generation ability, and 4 strains among them could produce CLA with conversion rate exceeding 80%, especially the conversion rate of LA in one strain exceeded 90%. Additionally, 30 bifidobacteria were investigated for their CLA and CLNA production ability, and results demonstrated that the highest CLA conversion rate was 53%, which was 78% of CLNA conversion rate (Gorrisen et al. 2010). Major bifidobacterial CLA producers were listed in Table 2.2.

2.2.2.1 *Bifidobacterium breve*

Coakley and colleagues investigated many bifidobacteria strains for CLA production and found *B. breve* NCFB2257 and *B. breve* NCFB 2258 showed the highest CLA conversion rate of LA conversion up to 65% (Coakley et al. 2003). Rosberg-Cody and colleagues isolated and screened CLA producers from neonates' gut intestines and showed significant difference in CLA generation among different species, even different strains which belong to the same species, in which *B. breve* exhibited much higher conversion rate than all the other bifidobacterial species (Rosberg-Cody et al. 2004). Barrett et al. isolated from neonates healthy adults and elderly subjects suffered with *Clostridium difficile* infection and developed a rapid method for CLA producer screening. In their results, five strains could transfer free LA to CLA with conversion rate exceeding 20%, in which the highest conversion was 75% for a *B. breve* (Barrett et al. 2007). Chung et al. screened 100 and 50 bifidobacteria for CLA production, and only 4 strains could produce CLA with a conversion rate over 80%, in which *B. breve* LMC017 exhibited the highest conversion. This strain could convert 91.1% of free LA or 78.8% of LA monoglyceride into CLA. Another study revealed that the CLA conversion rate was substrate-dependent. For example, when different forms of LA (monolinolein, dilinolein, 50% safflower oil monolinolein, 90% safflower oil monolinolein) were added in the skim milk to serve as substrate for CLA production, the highest conversion rate of CLA by *B. breve* LMC520 was obtained when LA monolinolein or 90% safflower oil monolinolein as substrates (Choi et al. 2008)

Table 2.2 High conjugated fatty acids producers in bifidobacteria

Strains	Substrate and conditions	Products	Isomers	Reference
B. breve LMC017	LA; growth cells	CLA (474 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Chung et al. (2008)
B. breve LMG11040	LA; growth cells	CLA (44%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorrisen et al. (2010)
	LNA; growth cells	CLNA (65.5%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. breve LMG11084	LA; growth cells	CLA (53.5%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
	LNA; growth cells	CLNA (72%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. breve LMG11613	LA; growth cells	CLA (19.5%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
	LNA; growth cells	CLNA (55.6%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. breve LMG13194	LA; growth cells	CLA (24.1%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
	LNA; growth cells	CLNA (63.3%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. breve NCFB2257	LA; growth cells	CLA (231 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Coakley et al. (2003)
B. breve NCTC11815	LA; growth cells	CLA (215 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
B. breve NCIMB8815	LA; growth cells	CLA (242 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
B. breve NCIMB8807	LA; growth cells	CLA (128 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
B. breve pattern A	LA; growth cells	CLA (76.6%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Barrett et al. (2007)
B. breve NCFB2258	LA; growth cells	CLA (398 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Coakley et al. 2003
B. breve NCIMB702258	ALA; growth cells	CALA (199 mg/L)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	Hennessy et al. (2012)
	GLA; growth cells	CGLA (149 mg/L)	*c*6,*c*9,*t*11-CLNA; *c*6,*t*9,*t*11-CLNA	
	SA; growth cells	CSA (38 mg/L)	*c*6,*c*9,*t*11,*c*15-CSA; *c*6,*t*9,*t*11,*c*15-CSA	
B. breve NCIMB8807	LA; growth cells	CLA (277 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
	ALA; growth cells	CALA (272 mg/L)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. breve DPC6330	LA; growth cells	CLA (300 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
	ALA; growth cells	CALA (331 mg/L)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
	GLA; growth cells	CGLA (81 mg/L)	*c*6,*c*9,*t*11-CLNA; *c*6,*t*9,*t*11-CLNA	
	SA; growth cells	CSA (41 mg/L)	*c*6,*c*9,*t*11,*c*15-CSA; *c*6,*t*9,*t*11,*c*15-CSA	

(continued)

Table 2.2 (continued)

Strains	Substrate and conditions	Products	Isomers	Reference
B. breve KCTC10462	LA; growth cells	CLA (160 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Oh et al. (2003)
B. breve KCTC3461	LA; growth cells	CLA (350 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Song et al. (2005)
B. breve LMC520	LA; growth cells	CLA (280 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Choi et al. (2008)
	LNA; growth cells	CLNA (90%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	Park et al. (2011)
B. dentium	LA; growth cells	CLA (12.5%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Barrett et al. (2007)
B. bifidum LMG10645	LA; growth cells	CLA (40.7%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorrisen et al. (2010)
	LNA; growth cells	CLNA (78.4%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	
B. bifidum CRL1399	LA; growth cells	CLA (24.8%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	van Nieuwenhove et al. (2007)
B. animalis subsp. *lactis* Bb12	LA; growth cells	CLA (170 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Coakley et al. (2003)
B. longum DPC6320	LA; growth cells	CLA (205 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Hennessy et al. (2012)
B. longum	LA; growth cells	CLA (60.1%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Barrett et al. (2007)
B. infantis	LA; growth cells	CLA (18.1%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
B. pseudocatenulatum KCTC10208	LA; growth cells	CLA (135 mg/L)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Oh et al. (2003)
B. pseudolongum LMG11595	LA; growth cells	CLA (42.2%)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Gorrisen et al. (2010)
	LA; growth cells	CLNA (62.7%)	*c*9,*t*11,*c*15-CLNA; *t*9,*t*11,*c*15-CLNA	

Gorissen et al. assessed the ability of producing CLA and CLNA by 36 bifidobacteria with free LA and ALA as the substrate, respectively, and found that six strains could transfer LA into CLA, but the conversion ratio differed significantly, in which the CLA conversion rate by *B. breve* LMG 11613 was 19.5% while 53.5% by *B. breve* LMG11084 (Gorissen et al. 2011). Furthermore, *c*9,*t*11-CLA was the predominant isomer in those bifidobacteria strains comprising 51.3–82.2% of total CLA. Due to the high CLA production, bifidobacteria was chosen by some researches to be used as the starter cultures to increase the CLA content in dairy products. The study performed by Hennessy et al. showed that the conversion rate of *B. breve* NCIMB702258 in milk system consisting of different additives presented significant difference and finally the content of *c*9,*t*11-CLA produced by the strain with additives comprising of yeast extract, casein hydrolysate, peptone, acetate, butyrate, and propionate was comparable to that when the strain grew in expensive MRS

medium (Hennessy et al. 2010). Hennessy et al. also studied the ability of bifidobacteria to transfer different polyunsaturated fatty acids into their corresponding conjugated forms. Results clearly showed that the conversion rate was substrate-dependent, in which the conversion rate of CLA ranged from 12% to 97% with *c*9,*t*11-CLA and *t*9,*t*11-CLA as the major isomers, while for GLA and ALA, the conversion ratio ranged from 0–83% to 3.8–27%, respectively. In those strains they assessed, *B. breve* DPC 6330 showed the highest ability of producing conjugated fatty acids, which could convert 70% of LA into CLA, 90% of α-LNA into CLNA, 17% of γ-LNA into CLNA, and 28% of stearidonic acid into CSA, respectively. Ewaschuk et al. analyzed the commercial VSL#3 probiotics, which consisted of eight different strains, and found that both VSL#3 and each strain in it could generate CLA at a different level, in which *B. breve* showed the highest CLA-producing ability with 70% LA transferred to CLA (Ewaschuk et al. 2006).

2.2.2.2 *Bifidobacterium animalis*

Coakley and colleagues (2003) found that *B. animalis* Bb-12 could convert approximately 27% of LA into *c*9,*t*11-CLA when it grew in the MRS medium plus free LA. Rodriguez-Alcala et al. studied on the possible utilization of 22 probiotics including five bifidobacteria strains and selected two strains which could generate CLA in the skimmed milk with free linoleic acid or safflower oil. And the major isomers were c9,t11-CLA and t10,c12-CLA (Rodríguez-Alcalá et al. 2011). With the optimal condition, the conversion rate of CLA generated by *B. animalis* BLC was highest with free LA as substrate. *B. animalis* Bb12-1 could produce more CLA when ricinoleic acid served as substrate. These studies suggest that it's possible to utilize bifidobacteria strains as the starter cultures in the milk to increase the content of CLA with LA in different types as the substrate.

2.2.2.3 *Bifidobacterium longum*

With a rapid screening method for CLA production, Barrett et al. isolated a number of bifidobacteria strains from the feces of infant, health adults, and elder people infected with *C. difficile* which could generate CLA, and results revealed that four strains belonging to *B. longum* could produce CLA with a conversion ratio exceeding 20% (Barrett et al. 2007). Similar to *B. breve*, the main CLA isomers produced by *B. longum* was also *c*9,*t*11-CLA. Roberg-Cody and colleagues isolated a few strains belonging to *Bifidobacterium* genus with high CLA production ability and discovered that the CLA-producing ability among different bifidobacteria species present significant difference, in which *B. longum* strain could generate *c*9,*t*11-CLA and *t*9,*t*11-CLA in high conversion rate (Rosberg-Cody et al. 2011). Ewaschuk et al. studied the CLA production ability of each strain in VSL3# probiotics and found *B. longum* could transform ~70% of LA to CLA (Ewaschuk et al. 2006).

2.2.2.4 Other Bifidobacteria

B. breve, *B. longum*, and *B. animalis* were the widely studied *Bifidobacterium* species with CLA production. Other species have also been reported with CLA production ability. For example, Oh et al. isolated a number of *B. pseudocatenulatum* which could convert free LA to CLA with a high conversion rate (Oh et al. 2003). Rosberg-Cody et al. also reported that one *B. bifidum* strain possessed CLA-producing ability with conversion rate of 17.9% (Rosberg-Cody et al. 2011). Gorissen et al. found that *B. bifidum* LMG 10645 and *B. pseudocatenulatum* LMG11595 exhibited the capability of generating CLA and CLNA, and for *B. bifidum* LMG 10645, the *c*9,*t*11-CLA was up to 82% of total CLA it produced, while for *B. pseudocatenulatum* LMG11595, it was only 35.1% of total CLA. Furthermore, one *B. bifidum* strain could accumulate *c*9,*t*9-CLA in the skimmed milk in the presence of hydrolyzed soybean oil (Xu et al. 2004). Ewaschuk et al. reported that one *B. infantis* strain could produce *c*9,*t*11-CLA and *t*10,*c*12-CLA with a total conversion rate exceeding 70% (Ewaschuk et al. 2006).

2.2.3 Conjugated Fatty Acid Production by Other Lactic Acid Bacteria

Numerous of other lactic acid bacteria were reported to produce CLA, especially food fermentation involving lactococci and streptococci (Table 2.3).

Lc. lactis subsp. *cremoris* CCRC12586, *Lc. lactis* subsp. *lactis*, and *S. thermophilus* CCRC12257 could produce CLA in the skimmed milk in the presence of free LA (Lin et al. 1999). Kim and Liu found five *Lc. lactis* strains could transform LA into CLA in the skimmed milk, among which three strains could also generate CLA when they were cultivated in MRS medium (Kim and Liu 2010). Among all the strains analyzed, *Lc. lactis* I-01 presented the highest CLA-producing ratio when it grew in the MRS medium or skimmed milk at the concentration of 0.1 mg/mL (Kim and Liu 2010). Kishino et al. analyzed 250 lactic acid bacteria for CLA production, which were belonged to lactobacilli, streptococci, pediococci, leuconostoc, propionibacteria, bifidobacteria, and enterococci. They found that the strains tested of lactobacilli, propionibacteria, pediococci, and lactococci could produce a large amount of CLA in MRS medium and the predominant CLA was *c*9, *t*11-CLA isomer. Moreover, 10-hydroxy-*cis*-12-octadecenic acid (10-HOE) was detected during CLA production in the research, which was considered as an intermediate during LA conversion to CLA (Ando et al. 2003). Xu and colleagues (2004) reported that CLA production by *E. faecium* and *P. acidilactici* was substrate-dependent, in which some strains could produce CLA in the skimmed milk with hydrolyzed soybean oil as the substrate, rather than unhydrolyzed soybean oil. El-Salam and colleagues

Table 2.3 Other high CFA producers in lactic acid bacteria

Strains	Substrate and conditions	Products	Isomers	Reference
E. faecium M74	LA; washed cells	CLA (1 mg/g)		Xu et al. (2004)
E. faecium	Enzymatic sesame oil; washed cells	CLA (104 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	El-Salam et al. (2010)
E. faecium AKU1021	LA; washed cells	CLA (100 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	Kishino et al. (2002)
P. acidilactici AKU1059	LA; washed cells	CLA (1400 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
P. acidilactici	LA; washed cells	CLA (1 mg/g fat)		Xu et al. (2004)
Lc. lactis 210	LA; growth cells	CLA (2 mg/g fat)		Kim and Liu (2002)
Lc. lactis IO-1	LA; growth cells	CLA (4 mg/g fat)		
Lc. lactis LMG S19870	LA; growth cells	CLA (46 mg/L)		Rodríguez-Alcalá et al. (2011)
Lc. lactis subsp. *lactis* CCRC12586	LA; growth cells	CLA (63 mg/L)		Lin et al. (1999)
Lc. lactis subsp. *lactis* CCRC10791	LA; growth cells	CLA (78 mg/L)		
Lc. lactis subsp. *lactis*	Enzymatic sesame oil; washed cells	CLA (21.6 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	El-Salam et al. (2010)
Leu. mesenteroides subsp. *mesenteroides*	Enzymatic sesame oil; washed cells	CLA (198 mg/ml)	*c*9,*t*11-CLA; *t*9,*t*11-CLA	
S. thermophilus CRL728	LA; growth cells	CLA (33.9%)		van Nieuwenhove et al. (2007)
S. thermophilus CCRC12257	LA; growth cells	CLA (74 mg/L)		Lin et al. (1999)

demonstrated that some strains of lactobacilli, propionibacteria, lactococci, enterococci, and pediococci could grow well in the reconstituted milk plus 0.2% of enzymatic sesame oils and synthesize CLA with those hydrolyzed oil as substrate. Interestingly, they found the best CLA producers were *Lc. lactis* subsp. *lactis* strain and *Leu. mesenteroides* subsp. *mesenteroides*. Another study carried out by Rodriguez-Alcala showed that free LA and safflower oil could be utilized by *Lc. lactis* LMG 19870 as the substrate to generate CLA. CLA produced by the strain with free LA as substrate in the skimmed milk was up to 45.51 mg/L, whereas safflower oil served as substrate, and the concentration of CLA was 23.1 mg/L, nearly a half of that from free LA as substrate (Rodríguez-Alcalá et al. 2011).

2.3 The Mechanism for Conjugated Fatty Acid Production in Lactic Acid Bacteria

2.3.1 Conjugated Fatty Acid Production Mechanism in Lactobacilli

The reason why bacteria produced CLA was still unclear. The most accepted reason was biological detoxification, as those strains eliminate the toxic effect of free LA on the cells. However, this assumption was only identified by a few bacteria (Maia et al. 2007, 2010). In fact, LA was necessary for the growth of bacteria; however, the growth-promoting effect would be replaced by the stress effect when its concentration increased to some specific concentration. The relation between myosin-cross-reactive antigen (MCRA) and the anti-environmental stress has been already identified in bacteria. Recent studies have showed that most bacteria could produce 10-HOE from LA and then further conversion to CLA, suggesting that these transformations of LA into other substrates could decrease the toxic effect of free LA on the cells.

Compared with rumen bacteria, linoleic acid isomerase was believed as key factor in the mechanism of CLA production by lactic acid bacteria. Unfortunately, it remains unclear. Rosson et al. (1999) firstly tried to separate the putative linoleic acid isomerase and identified its function through overexpression and activity confirmation. Finally, a protein, with molecular weight as 67 kDa, was obtained and the optimal pH was 6.8~7.5. This protein was identified to be homology to myosin-cross-reactive antigen, widely present in the bacteria and predicted as fatty acid isomerase (Kil et al. 1994). However, when LA was catalyzed by the recombinant protein, only a trace of hydroxyl fatty acid was produced rather than CLA. Due to the instability structure of hydroxyl fatty acid, it would be degraded by heat, strong base (or acid), or even some methyl method, which would be the possible reasons why few studies reported the production of hydroxy fatty acids. Ogawa et al. found that LA would be firstly transformed into hydroxyl fatty acid by *L. acidophilus* AKU1137 and hydroxyl fatty acids was then quickly converted into CLA when its concentrated at a certain extent (Ogawa et al. 2001). With further GC-MS and NMR analysis, the hydroxyl fatty acids were confirmed as 10-hydroxy-*cis*-12-C18:1 (10-HOE) and 10-hydroxy-*trans*-11-C18:1. This was the first report to identify 10-HOE as the intermediate during CLA production in lactic acid bacteria. And they presumed that the pathway for CLA production involved hydration, dehydration, and isomerization.

Even though the 10-HOE was identified as the intermediate during CLA generation, the key enzymes involved in the following reactions were unclear. As high homologous to the putative linoleate isomerase purified by Rosson et al. (1999), myosin-cross-reactive antigen (MCRA) received more research of interests and firstly confirmed as fatty acid hydratase in *S. pyogenes*, where it was original found. Volkov and colleagues cloned the MCRA-encoding gene and expressed it in *E. coli*. With LA as substrate, it revealed that MCRA was fatty acid hydratase with FAD as

the cofactor. This enzyme could transform LA into 10-HOE and 10,13-dihydroxylstearic acid (10,13-diHOA), which was also identified to be related with the pathogenicity of this strain (Volkov et al. 2010).

Kishino et al. successfully separated a triple-component linoleic acid isomerase from *L. plantarum* AKU1009a through differential centrifugation. The first protein was identified to be membrane protein and involved in transforming LA into 10-HOE (Kishino et al. 2011). In combining the latter two separates, *t*9,*t*11-CLA could be produced, suggesting that the linoleic acid isomerase in lactobacilli was not a single enzyme; instead, three proteins were demanded for CLA production. Later on, the first protein was finally approved to be MCRA through the N-terminal sequence, recombinant technology, and enzymatic activity confirmation (Kishino et al. 2011). Though no other intermediates, except 10-HOE, could be detected in the enzymatic reaction, the author still presented the possible pathway for CLA production, including hydrogenation, dehydration, double bond migration, hydrogenation, and dehydration. These observations provided a novel direction in this field. In 2013, the detailed pathway of generating CLA by *L. plantarum* was elucidated (Kishino et al. 2013).

At the same time, Yang et al. (2013, 2014) showed that MCRA-encoding genes cloned from different lactic acid bacteria were approved to be fatty acid hydratase, neither linoleic acid isomerase. They found that *L. plantarum* ZS2058 could accumulate several intermediates during CLA production. Further analysis showed that those intermediates were 10-HOE, 10-oxo-*cis*-12-octadecenic acid, and 10-oxo-*trans*-11-ocradecenic acid. Through bioinformatics analysis and comparison with Kishino et al., the genetic determinates for CLA production in *L. plantarum* ZS2058 were fully confirmed. Their results showed that three proteins were involved, which were myosin-cross-reactive antigen, short chain dehydrogenase/oxidoreductase, and acetoacetate decarboxylase (Fig. 2.4) (Yang et al. 2014). Furthermore, the determinants for generating CLA in *L. plantarum* ZS2058 were knocked out based on the *cre-lox*-based system. Neither intermediate could be detected in the corresponding gene deletion mutant. Meanwhile all those mutants could recover the ability to convert LA into CLA when the corresponding gene was complemented, which indicated that the triple-component linoleic acid isomerase system was the unique pathway for CLA production in *L. plantarum* (Yang et al. 2017).

2.3.2 *Conjugated Fatty Acid Production Mechanism in Bifidobacteria*

To date, no detailed characterization of bifidobacterial production mechanism has been developed. Rosberg-Cody firstly cloned MCRA-encoding gene from *B. breve* NCFB2258, and then the gene was inserted into the vector (Rosberg-Cody et al. 2011). Recombinant *E. coli* strains with the vector inserted with *mcra* gene could only transform LA into 10-HOE, suggesting that *mcra* gene was linoleic acid

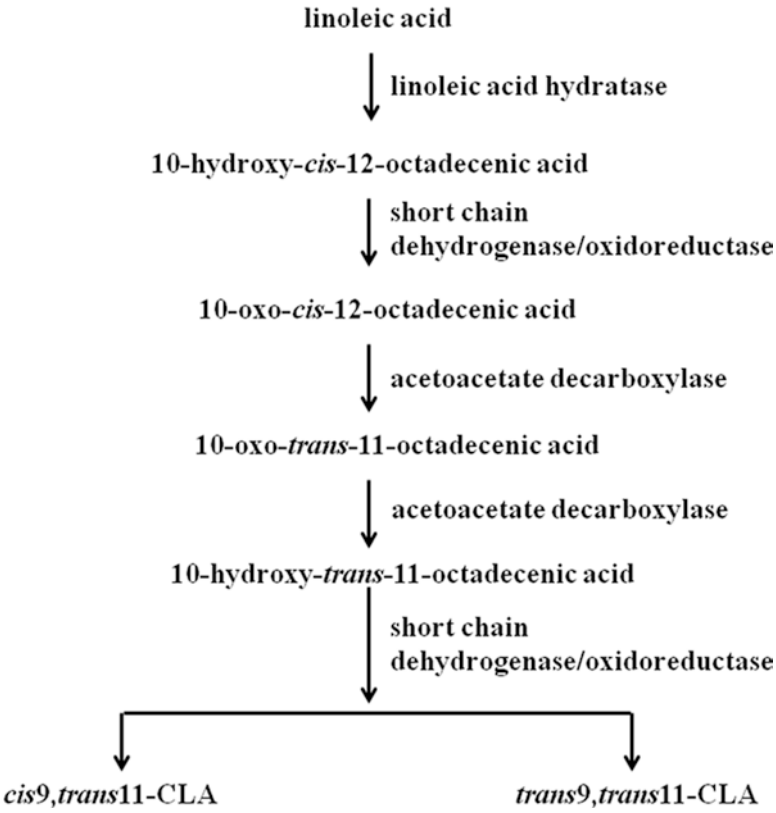

Fig. 2.4 CLA production pathway in *L. plantarum* ZS2058

hydratase, neither linoleic acid isomerase. The study demonstrated that MCRA from *B. breve* also utilized FAD as the cofactor, which was highly homologous to that from *L. reuteri* ATCC55739. Further study showed that deletion of *mcra* gene in *B. breve* NCFB2258 had no influence on CLA production (O'Connell et al. 2013) which indicated that MCRA was not involved in the converting LA in CLA by *Bifidobacterium*. Thus, identification of the bifidobacterial CLA production needs more investigation.

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