Microbial Production of Conjugated Linoleic Acid and Conjugated Linolenic Acid Relies on a Multienzymatic System

Ana S. Salsinha,^a Lúcia L. Pimentel,^{a,b,c} Ana L. Fontes,^{a,c} Ana M. Gomes,^a Luis M. Rodríguez-Alcalá^{a,d}

^aUniversidade Católica Portuguesa, Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Porto, Portugal

^bCentro de Investigação em Tecnologias e Sistemas de Informação em Saúde, Faculdade de Medicina da Universidade do Porto, Porto, Portugal

^cUnidade de Investigação de Química Orgânica, Produtos Naturais e Agroalimentares, Universidade de Aveiro, Aveiro, Portugal

^dCentro de Investigación en Recursos Naturales y Sustentabilidad, Universidad Bernardo O'Higgins, Santiago de Chile, Chile

SUMMARY	1
INTRODUCTION	2
CLA and CLNA Bioactivity	2
CFA and Rumen Biohydrogenation	2
Other CFA-Producing Bacteria	4
Screening Methods for Possible Candidates	4
LINOLEATE ISOMERASE	5
Using LAI as a Screening Tool	6
Homology and Phylogenetic Analyses	6
MYOSIN-CROSS-REACTIVE ANTIGEN PROTEIN	8
Homology and Phylogenetic Analysis	10
FATTY ACID HYDRATASE ACTIVITY	10
THE REQUIREMENT OF COFACTORS	11
CLA PRODUCTION: THE ACTION OF A MULTICOMPONENT SYSTEM?	12
METABOLITES FROM CLA/CLNA PRODUCTION	13
Hydroxy FAs as Intermediate Metabolites	13
Multienzymatic Complexes and Possible Synthesis Pathways	16
Hydratases Are Not Substrate Specific	16
CHALLENGES AND FUTURE PERSPECTIVES	17
ACKNOWLEDGMENTS	18
REFERENCES	18

SUMMARY Conjugated linoleic acids (CLAs) and conjugated linolenic acids (CLNAs) have gained significant attention due to their anticarcinogenic and lipid/energy metabolism-modulatory effects. However, their concentration in foodstuffs is insufficient for any therapeutic application to be implemented. From a biotechnological standpoint, microbial production of these conjugated fatty acids (CFAs) has been explored as an alternative, and strains of the genera *Propionibacterium*, *Lactobacillus*, and *Bifidobacterium* have shown promising producing capacities. Current screening research works are generally based on direct analytical determination of production capacity (e.g., trial and error), representing an important bottleneck in these studies. This review aims to summarize the available information regarding identified genes and proteins involved in CLA/CLNA production by these groups of bacteria and, consequently, the possible enzymatic reactions behind such metabolic processes. Linoleate isomerase (LAI) was the first enzyme to be described to be involved in the microbiological transformation of linoleic acids (LAs) and linolenic acids (LNAs) into CFA isomers. Thus, the availability of *lai* gene sequences has allowed the development of genetic screening tools. Nevertheless, several studies have reported that LAIs have significant homology with myosin-cross-reactive antigen (MCRA) proteins,

Published 29 August 2018

Citation Salsinha AS, Pimentel LL, Fontes AL, Gomes AM, Rodríguez-Alcalá LM. 2018. Microbial production of conjugated linoleic acid and conjugated linolenic acid relies on a multienzymatic system. *Microbiol Mol Biol Rev* 82:e00019-18. <https://doi.org/10.1128/MMBR.00019-18>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Luis M. Rodríguez-Alcalá, lalcala@porto.ucp.pt.

A.S.S. and L.L.P. contributed equally.

which are involved in the synthesis of hydroxy fatty acids, as shown by hydratase activity. Furthermore, it has been suggested that CLA and/or CLNA production results from a stress response performed by the activation of more than one gene in a multiple-step reaction. Studies on CFA biochemical pathways are essential to understand and characterize the metabolic mechanism behind this process, unraveling all the gene products that may be involved. As some of these bacteria have shown modulation of lipid metabolism *in vivo*, further research to be focused on this topic may help us to understand the role of the gut microbiota in human health.

KEYWORDS linoleate isomerases, myosin-cross-reactive antigen protein, hydratases, genetic screening, lactic acid bacteria, bifidobacteria, rumen biohydrogenation, conjugated linoleic acid, conjugated linolenic acid, bifidobacterium

INTRODUCTION

Currently, conjugated fatty acids (CFAs), namely, conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA), represent promising bioactive compounds that may be used for the promotion of human health and well-being. The fact that they can be obtained through microbial synthesis opens interesting possibilities for the elaboration of functional food products.

However, the molecular basis of their production is currently uncertain, and conflicting data have been reported about the possible enzymes involved (1) as well as their regulatory mechanisms (2).

This review aims to collect and summarize the available information regarding the identified genes and/or proteins involved in CLA and CLNA production by bifidobacteria and lactic acid bacteria (LAB) and, consequently, the possible enzymatic reactions behind such processes. This will certainly help in the development of more-efficient screening strategies and the optimization of such production.

CLA and CLNA Bioactivity

CLA (particularly rumenic acid [RA]) and CLNA have been gaining significant attention due to their potential health benefits (3). Specifically, CLA isomers, namely, RA and C_{18:2} *t*10, *c*12, have been associated with protection against carcinogenesis (e.g., colon cancer metastasis) (4, 5), reduction of atherosclerosis lesions (6, 7), body fat modulation (8), antihypercholesterolemic effects (9), and immunity enhancement while also reducing inflammation (10, 11).

On the other hand, commercial mixtures of RA and C_{18:2} *t*10, *c*12 (i.e., CLA-containing oils) were found to exert negative effects, such as impairment of both medium- and long-term spatial learning and memory in rats with brain lesions (12) or increased insulin resistance in male Wistar rats fed either high- or low-fat diets (13). The latter effect has also been observed in humans but, in general, seems to be associated with C_{18:2} *t*10, *c*12 (14, 15).

Regarding CLNA isomers, their effects have been reviewed as being associated with anticancer, anti-inflammatory, antioxidant, and antiobesity activities (16, 17).

CFA and Rumen Biohydrogenation

CLA and CLNA are positional and geometric isomers of linoleic acid (LA) (C_{18:2} *c*9, *c*12) and α -linolenic acid (LNA) (C_{18:3} *c*9, *c*12, *c*15), respectively, presenting conjugated double bonds in their chemical structures. Both isomers can be produced by microorganisms. CLA has been vastly studied, and two pathways have been described. The first pathway is biohydrogenation of LA in the rumen (18), while the second is Δ^9 -desaturation of *trans*-vaccenic acid (TVA) (C_{18:1} *t*11) in adipose, but mainly in mammary gland, tissues (19–21).

Regarding the mechanism occurring in the rumen (Fig. 1), dietary fat first undergoes lipolysis to release free fatty acids (FAs) (22, 23). Polyunsaturated FAs (PUFAs) are then isomerized and hydrogenated into saturated fatty acids (SFAs) as end products, namely, stearic acid (C_{18:0}). During this process, LA is mainly isomerized to C_{18:2} *c*9, *t*11 CLA (RA). Other isomers are also formed and further hydrogenated to several *trans* C_{18:1} fatty

FIG 1 Scheme of LA and LNA biohydrogenation. Bold arrows indicate the principal pathway, and broken lines indicate alternative pathways. SA, stearic acid.

acids, mainly TVA. Finally, through a biohydrogenation mechanism, the $C_{18:1}$ isomers are converted to $C_{18:0}$ in the rumen.

Concerning CLNA, LNA biohydrogenation involves reactions similar to those described above for LA, producing both CLA and CLNA in different proportions. After lipolysis, released LNA is isomerized at the *cis*-12 position, producing $C_{18:3}$ c9, t11, c15 (rumelenic acid [RLA]), which was identified for the first time in milk fat (24). RLA is consequently reduced to $C_{18:2}$ t11, c15 and further converted to TVA, $C_{18:1}$ c15, and $C_{18:1}$ t15. Other pathways have been proposed, namely, the transformation of isorumenic acid (iRLA) ($C_{18:3}$ c9, t13, c15) to $C_{18:2}$ c9, t13 and $C_{18:2}$ t13, c15 and, finally, to $C_{18:1}$ t13 (24).

On the other hand, other research works have pointed out that some resulting compounds have not yet been identified. When assaying the quantitative and qualitative transfer of ^{13}C to CFA, a mixed microbial population from the rumen of a dairy cow was tested in batch cultures in the presence of $[^{13}\text{C}]\text{LNA}$ (25). During the incubation time (48 h), both nonconjugated and conjugated 18:2 and 18:3 isomers were enriched with ^{13}C . Interestingly, for $C_{18:2}$, six unknown isomers were reported, while for $C_{18:3}$, there were 10 plus 2 conjugated forms listed. Formation of RA and isomers $C_{18:2}$

t10, c12 plus t9, t11; t9, c11; c9, c11; t11, t13; t8, t10; and c10, c12 was also observed. Accordingly, it was concluded that ruminal microbes are capable of transforming LNA into both CLA and 18:3 compounds. Finally, these intermediates are then hydrogenated into stearic acid (C_{18:0}) (17, 26), and fatty acids are absorbed in the gut and transported via the bloodstream to different body tissues (27, 28).

The above-mentioned PUFA biohydrogenation pathways have been proposed to be a detoxifying mechanism for bacteria. By this process, growth-inhibiting free PUFAs are transformed into less-toxic SFAs (29, 30). Several studies have been performed in order to deepen the understanding of the effects of these compounds on the bacterial cell and tentatively unravel mechanisms associated therewith. In this respect, it was observed that Fab I, an enoyl-acyl carrier protein (ACP) reductase that catalyzes the final and rate-limiting step of chain elongation in bacterial fatty acid synthesis, was inhibited by LNA (31).

This hypothesis seems to be supported by investigations performed with other organisms: for example, the *cis*-12 linoleate isomerase (LAI) gene of *Fusarium graminearum* (FgLAI12), responsible for the conversion of LA into RA, was characterized (32). Assays of gene deletion mutants showed, among other effects, a reduction of mycelial growth. Thus, wheat plant produces LA as a response to *Fusarium graminearum* infection.

It has also been demonstrated that γ -linolenic, arachidonic, and eicosapentaenoic acids modified membrane-bound proteins, ATPase, and the histocompatibility complex as well as brought about the control of FA binding proteins (33, 34). Based on those observations, it was hypothesized that the bacterial-growth-inhibitory effect of FA, namely, LA, is related to high bacterial membrane permeability due to its surfactant action (35). Interestingly, the growth of *Butyrivibrio fibrisolvens* in the presence of LA and LNA was initiated only when these PUFAs were converted to TVA (36). Reported data showed that toxicity was mediated via an energy metabolism impairment, as a significant decrease in the acyl-CoA pools in LA-containing cultures was reported.

Other CFA-Producing Bacteria

As mentioned above, CLA concentrations in their natural sources are relatively low: 0.55 to 9.2 mg CLA/g fat in dairy products and 1.2 to 17 mg CLA/g fat in ruminant meat, with RA being the main isomer (37). Regarding CLNA, reported concentrations have been 1 to 3 mg CLNA/g in milk fat (38, 39) and 9 to 27 mg CLNA/g in beef fat (40) or 4 to 48 mg/g in goat meat (41). The most common isomers within this group of compounds, found in those products, were RLA, iRLA, and C_{18:3} c9, t11, t15 (17, 42).

However, a problem arises if we consider that concentrations in these above-mentioned foodstuffs are not high enough for any significant therapeutic effect, as described in the literature: the effective dose of CLA in humans has been indicated to be 3 to 6 g/day (43), while that of CLNA has been reported to be 2 to 3 g/day (42, 44).

Thus, while exploring new ways to increase the levels of these bioactive lipids, other CFA-producing bacteria were found. Strains of the genera *Propionibacterium*, *Clostridium*, and *Lactobacillus* synthesized these compounds in promising quantities, and their use for commercial biosynthesis was further evaluated (27). Indeed, throughout the years, several species of CLA-producing bacteria have been isolated from rumen, intestine, and fermented foods and used as starter cultures as a means to elaborate functional dairy products (46). In addition, *Bifidobacterium* has been described as being the most interesting genus for the *in situ* production of CFA, and therefore, bifidobacterium strains have attracted significant attention (47, 48).

Screening Methods for Possible Candidates

Previous research works aiming to find possible producing strains were generally based on measurements of CFA substrate transformation capability. Thus, different quantification methods have been assayed, such as those using Ag⁺ high-performance liquid chromatography (Ag⁺-HPLC) coupled with diode array detectors (DADs) (49, 50). Others have proposed spectrophotometric determinations combined with Ag⁺-HPLC

for the qualitative determination of isomers (51, 52). However, gas chromatography (GC) methods are the most commonly assayed, as reported by Varga-Visi et al. (53), Nzali et al. (54), Alonso et al. (55), and Villar-Tajadura et al. (47).

Nevertheless, independently of the quantification method, the growth conditions (e.g., culture medium or inoculum level), and the initial precursor substrate concentration tested, it was observed that conversion rates are variable, and consequently, the results obtained are often inconsistent. Alonso et al. (55) tested 4 *Lactobacillus* strains for their ability to produce CLA in MRS broth and in skim milk supplemented with linoleic acid. The best results for CLA production ranged between 80.14 and 131.63 $\mu\text{g/ml}$ and were obtained with MRS broth supplemented with 0.02% substrate, after 24 h of incubation at 37°C. In skim milk, the level of CLA obtained, under the same incubation conditions and with the same substrate concentration, was lower (54.31 to 116.53 $\mu\text{g/ml}$). Different results were obtained by Kim and Liu (56), who screened lactic acid bacteria for their capacity to produce CLA from LA in MRS medium and whole milk. In that study, cells produced more CLA in whole milk than in MRS medium. Similarly, a higher rate of LA conversion in buffalo milk than in MRS medium was determined by Van Nieuwenhove et al. (57) when assessing lactic acid bacteria. The rate of conversion of LA to CLA ranged from 17.0% to 35.9% in MRS broth containing 200 $\mu\text{g/ml}$ LA. In buffalo milk, the percentage of conversion varied from 39.0% to 95.1%.

Moreover, analysis methods for CFA are usually expensive and time-consuming: besides the cumbersome sample preparation, involving lipid isolation from bacterial cells or extracellular contents and further derivatization into fatty acid methyl ester (FAME), subsequent data treatment steps are time-demanding. This is without considering that some derivatization procedures can alter the CLA/CLNA profile (58).

In addition, quantification-based screenings to find producing strains are trial-and-error studies, since all available strains have to be tested for production. Therefore, when considering a selection strategy, a faster, standardized, and cost-effective approach is required. Accordingly, a genetic approach searching for the presence of involved enzymes could be a useful strategy to improve the selection process. Through this approach, only potentially producing strains, containing the involved enzymes, would be selected for the following production test. This step could minimize time as well as resources used for the assay.

Given this approach, the following section is dedicated to the described and proposed enzymes involved in LA and LNA metabolism to produce CLA and CLNA.

LINOLEATE ISOMERASE

In 1967, an enzyme catalyzing the isomerization of LA and LNA in *B. fibrisolvans*, for the production of RA, was described (59). Further investigations demonstrated that the transformation of these FAs into the corresponding conjugated forms was performed by a membrane-bound linoleate isomerase (LAI) enzyme (26, 60). In this case, the hydrogenation of LA produced an RA isomer as the first intermediate, where the double bond at the carbon 12 position was transferred to carbon 11.

Once a new enzyme is discovered, protein characterization is an important step, especially when the mechanisms behind an enzymatic process are not fully understood. Thereafter, several LAI proteins were characterized, namely, that from *Clostridium sporogenes* ATCC 25762 (61). This LAI was found to be very unstable, especially after being solubilized by detergents. Due to the observed instability mentioned above, the enzyme was suggested to be an integral membrane protein. Consequently, a major drawback in the characterization of this LAI relies on its protein membrane association features, since its hydrophobic structure hinders its solubilization (59).

In contrast, *Propionibacterium acnes* LAI (PAI) presents a substrate specificity similar to those of the *B. fibrisolvans* and *Clostridium sporogenes* proteins, but it is an intracellular soluble cytoplasmic protein capable of converting LA to C_{18:2} Δ^{10} , Δ^{12} (62). Therefore, PAI solubility would allow the easy isolation and purification of the protein. For instance, Liavonchanka et al. (63) described up to six crystal structures where PAI-CLA and PAI-CLNA complexes were characterized. Three determinants for the

regiospecificity and stereospecificity of PUFA isomerases were also reported, which can serve as a guide for the study of PUFA substrate specificity. Additionally, those authors suggested that this information can serve as a framework for future investigations where plant oils are directly converted to CFA.

However, there is a lack of information regarding the LAI molecular mechanism behind the ability of lactic acid bacteria (LAB) to convert LA into CLA. Potential LAI enzymes from selected *Lactobacillus*, *Bifidobacterium*, and *Leuconostoc* strains have been compared at the genetic, amino acid sequence, and functional levels. These comparative studies have confirmed membrane-bound features of the LAI protein and the presence of some conserved motifs, namely, a putative flavin adenine dinucleotide (FAD) binding domain (64). As a result of all of these studies, to date, different LAI protein sequences have been annotated in GenBank (Table 1), including sequences of *P. acnes*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactococcus lactis* subsp. *lactis*, *Bifidobacterium dentium*, *Bifidobacterium breve*, *Rhodococcus erythropolis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Propionibacterium freudenreichii* subsp. *shermanii*, *B. fibrisolvens*, and *C. sporogenes* strains (17).

Finally, one should take into consideration that LAI activity is affected by several factors, such as pH and temperature. For example, Gorissen et al. (65) reported that for *Lactobacillus sakei* LMG 13558, optimal CFA production conditions were pH 6.6 at 30°C, while for assays at pH 5.5, no conversion was obtained. On the other hand, Hennessy et al. (66) reported that for *Bifidobacterium breve* NCIMB 702258, the concentrations of yeast extract, sodium acetate, and L-Cys-HCl were positively correlated with CFA synthesis.

Using LAI as a Screening Tool

Both the availability of *lai* sequences and the identification of conserved domains among different strains open new ways for the development of genetic screening tools to identify possible CLNA and CLA producers. Macouzet et al. (64) studied, for the first time, genomic DNA from industrial and other common *Lactobacillus* species (*L. acidophilus* ATCC 832, *L. reuteri* ATCC 55739, and *L. gasseri* ATCC 33323) by PCR and immunoblot analysis. Positive hybridization of strains with a probe based on a sequence from *L. reuteri lai* demonstrated that this gene is indeed present in different *Lactobacillus* species. Furthermore, all these *lai*⁺ bacteria were able to synthesize detectable levels of CLA.

Other authors, such as Gorissen et al. (65), also applied genetic screening by designing primers based on the *L. sakei* 23k and *L. plantarum* AS1.555 *lai* sequences. In agreement with what was reported by Macouzet et al. (64), a putative LAI gene was found only in the genomes of strains showing CLA- and CLNA-producing capacities, confirming the potential for using *lai*-based screening as a tool to identify possible producers.

Nevertheless, it is important to consider that the design of primers based solely on the genome of a specific strain may bias this type of genotypic screening. In fact, as reported, primers designed based only on LAI gene sequences from *L. sakei* and *L. plantarum* identified bacteria belonging mainly to these two species (65).

Homology and Phylogenetic Analyses

Homology and phylogenetic analyses are techniques that have been extensively used in order to understand the potential of other species as CFA producers as well as the possible involvement of homologous proteins.

Considering this, Farmani et al. (67) performed an *in silico* analysis where the primary structure and physicochemical properties of different LAIs were analyzed. Based on these results, LAIs were proposed to be classified into four groups. The first group (group I [GI]) comprised the enzyme from *Propionibacterium acnes*, PAI, since it did not show any similarity to other LAIs. The remaining proposed groups were composed of *L. reuteri*, *L. acidophilus*, and *L. lactis* subsp. *lactis* LAIs (GII); *L. plantarum* and *R. erythropolis* LAIs (GIII); and *B. dentium* and *B. breve* LAIs (GIV). The division into these

TABLE 1 Isomerase enzymes identified to be involved in the linoleic and/or linolenic acid conjugation mechanism

Organism tested	Protein annotation	Substrate	Isomer(s) produced	Nucleotide sequence accession no. (GenBank)	Protein accession no.	Reference(s)
<i>Propionibacterium acnes</i>	PAI	LA	r10, c12 CLA		PDB accession no. 2BAB (PAI + LA); 2BAC (PAI + LNA); and 2B9W , 2B9X , 2B9Y , and 2BA9 (PAI)	63
<i>Clostridium sporogenes</i> ATCC 25762	LAI	LA	c9, r11 CLA			61
<i>Propionibacterium acnes</i> ATCC 6919	<i>P. acnes</i> linoleic acid isomerase	LA	r10, c12 CLA			86, 112
<i>Lactobacillus acidophilus</i> ATCC 832	LAI (reannotated as MCRA)	LA	r10, c12 CLA; c9, r11 CLA; <i>trans</i> , <i>trans</i> CLA	GU474813	GenBank accession no. ADD22720.1	64
<i>B. breve</i> LMG 13208	LAI (reannotated as MCRA)	LA	r10, c12 CLA and <i>trans</i> , <i>trans</i> CLA	GU474814	GenBank accession no. ADD22721.1	64
<i>Lactobacillus curvatus</i> LMG 13553	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR773883	GenBank accession no. CBY89653.1	65
<i>Lactobacillus plantarum</i> ATCC 8014	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR732045	GenBank accession no. CBY45494.1	65
<i>Lactobacillus plantarum</i> IMDO 130201	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR732046	GenBank accession no. CBY45495.1	65
<i>Lactobacillus plantarum</i> LMG 6907	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR732048	GenBank accession no. CBY45497.1	65
<i>Lactobacillus plantarum</i> LMG 13556	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR732047	GenBank accession no. CBY45496.1	65
<i>Lactobacillus plantarum</i> LMG 17682	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR73884 (partial sequence) FR732051	GenBank accession no. CBY89654.1	65
<i>Lactobacillus sakei</i> LMG 13558	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA		GenBank accession no. CBY45500.1	65
<i>Lactobacillus sakei</i> CG1	LAI	α -LNA	c9, r11, c15; t9, r11, c15 CLNA	FR732050	GenBank accession no. CBY45499.1	65
<i>Fusarium graminearum</i>	<i>cis</i> -12 LAI gene (<i>FgLA12</i>)	LA	c9, r11 CLA C ₁₈₂		GenBank accession no. CBY45499.1	32

groups was made considering that each group shared similar percent identities with other LAIs. In addition, classification into two different families can also be performed, GI LAIs and myosin-cross-reactive antigen (MCRA)-like LAIs, the latter of which comprises GII, GIII, and GIV. It was observed that members of MCRA-like LAIs have significant homology with MCRA proteins, contrarily to *P. acnes*.

All these LAI proteins are also different from each other in terms of product specificity. PAI is a C_{18:2} Δ^6 10, Δ^{12} isomerase, while MCRA-like LAIs are mainly RA isomerases (67). Additional studies conducted by homology searches and phylogenetic analyses have reported that the MCRA protein of *Staphylococcus aureus* shows over 50% homology with LAIs from *L. acidophilus* and *L. reuteri* PYR8 (68). The high LAI sequence identity with MCRA proteins justifies the growing interest in the study of the latter as a possible element involved in the FA conjugation mechanism.

MYOSIN-CROSS-REACTIVE ANTIGEN PROTEIN

MCRA from *Streptococcus pyogenes* M49, with PUFA isomerase activity, was the first MCRA to be identified (69). With the exception of PAI, LAIs from the above-mentioned species show significant homology with MCRA proteins.

These proteins were found to play a role in bacterial stress tolerance. Different experiments testing tolerance to butanol (70), ethanol (71), lactate, acetate, and salt (72) showed that the presence of a MCRA protein in the tested bacteria resulted in increased tolerance to these factors. In fact, in a recent *in silico* analysis, the *LBA649* gene in the *L. acidophilus* NCFM genome was predicted to encode a putative MCRA cell surface protein (72). The data obtained indicated that *LBA649* allows *L. acidophilus* NCFM to grow in stressful environments, such as in the presence of porcine bile, sodium lactate, and sodium chloride.

Nevertheless, MCRA comprise a family of enzymes found in a wide range of bacteria, especially LAB. Currently, there is increasing interest in MCRA, due to their high homology with LAIs, namely, that from *L. reuteri* PYR8 (90).

However, and despite several studies (Table 2), their role in the production of CFA is not completely clear. Ogawa et al. observed that when assaying CLA production from washed cells, hydroxy fatty acids also appeared (73, 74). Those authors proposed a pathway involving hydration/dehydration steps. In further experiments, a MCRA from *L. plantarum* AKU 1009a was found to be associated with the cell membrane fraction, hydrating LA into 10-hydroxy-*cis*-12-octadecenoic acid (10-HOE) (75). Later, it was confirmed elsewhere that MCRA plays a role in the conversion of LA and oleic acid (OA) (C_{18:1} Δ^9) to their respective 10-hydroxy derivatives (76). Furthermore, MCRA from *S. pyogenes* (77) and CFA-producing *B. breve* (68) were described as FAD-containing hydratases using C₉ and C₁₈ free fatty acids as the substrates.

This brings up the question, What is, in fact, the association between CLA and hydroxy acids? The above-mentioned work of Yang et al. (76) focused on studying MCRA proteins from *Lactobacillus rhamnosus*, *L. plantarum*, *L. acidophilus*, and *Bifidobacterium animalis* subsp. *lactis* and confirmed their CLA production capacities through 10-HOE accumulation. All the enzymes were located in the cytoplasm, showing homologies of >30% with MCRA from *S. pyogenes* M49 and at least 29% with MCRA from *B. breve* NCIMB 702258. Based on these results, those authors predicted the presence of a FAD binding motif in all the MCRA proteins assayed. This would be in agreement with the fact that MCRA from *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* NCFM presented an extra FAD-dependent oxidoreductase domain, which is also present in PAI (63).

The heterologous expression of MCRA proteins in *Escherichia coli*, for example, may contribute to unraveling their role in CFA metabolism. Volkov et al. (77) observed that when the MCRA protein from *S. pyogenes* was heterologously expressed in *E. coli*, it acted as a hydratase, but no CLA-forming isomerase activity was detected. Elsewhere, the *B. breve* NCIMB 702258 gene encoding the MCRA protein was cloned and transformed into *Corynebacterium glutamicum* and *Lactococcus lactis* (68): the cultures expressing this gene were found to accumulate increasing amounts of 10-HOE and

TABLE 2 Myosin-cross-reactive antigen enzymes identified to be involved in the linoleic and/or linolenic acid conjugation mechanism^a

Strain studied	Protein annotation	Substrate(s)	Product(s)	GenBank nucleotide sequence accession no.	GenBank protein accession no.	Reference
<i>Bifidobacterium breve</i> NCIMB 702258	MCRA	LA	10-HOE	HQ593838	ADY18551.1	68
<i>Streptococcus pyogenes</i> M49	MCRA	LA	10-Hydroxy and 10,13-dihydroxy derivatives	CP000829.1	ACI60731.1	77
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> Bb-12	MCRA	LA and OA	10-HOE and 10-HOA	CP001853.1	ADC85468	76
<i>Lactobacillus rhamnosus</i> LGG	MCRA (reannotated as oleate hydratase)	LA and OA	10-HOE and 10-HOA	NC_013198.1	YP_003170249.1 (original removed version), WP_005714981.1 (100% homology to an oleate hydratase)	76
<i>Lactobacillus plantarum</i> ST-III	MCRA	LA and OA	10-HOE and 10-HOA	NC_014554.1	YP_003923433 (original removed version)	76
<i>Lactobacillus acidophilus</i> NCFM	MCRA	LA and OA	10-HOE and 10-HOA	CP000033.3	AAV42528.1	76

^a10-HOE, 10-hydroxy-*cis*-12-octadecenoic acid; OA, oleic acid; 10-HOA, 10-hydroxy-octadecanoic acid.

10-hydroxy-octadecanoic acid (10-HOA) in the culture medium. The protein was shown to be a FAD hydratase enzyme. Accordingly, it was proposed that a first step includes the hydration of LA to 10-hydroxy $C_{18:1}$ and the subsequent dehydration and isomerization of these hydroxy fatty acids to the RA and $C_{18:2}$ Δ^9 , Δ^{11} isomers. Thus, the absence of CLA formation in heterologous strains may be explained by the fact that the MCRA gene would be involved only in the formation of CLA precursors.

On the other hand, O'Connell et al. (71) stated that there is no involvement of MCRA proteins in the LA isomerization process. Those authors created and analyzed an insertion mutant of the *B. breve* NCFB 2258 MCRA-encoding gene. No difference was observed in the amounts of CLA produced by the mutant and the wild-type strains. Those authors thus proposed that this protein is an oleate hydratase that is involved in solvent stress protection and responsible for the breakdown of oleic acid to 10-hydroxystearic acid (71). Furthermore, 14 bifidobacterial strains were identified as having a homolog of the *B. breve* NCIMB 702258 MCRA gene (78). In that study, the best CLA producers were positive for the MCRA gene, but no hydroxylated forms of LA were detected. Moreover, this gene was identified in both producing and nonproducing species. Accordingly, no correlation between the MCRA gene and CLA metabolism can be assumed, at least for bifidobacterial strains. Consequently, the role of MCRA proteins in the LA conjugation process remains unsolved, identifying the need for future studies.

Homology and Phylogenetic Analysis

Most of these proteins were annotated as cross-myosin-reactive antigens considering their homology to the *S. pyogenes* 67-kDa protein that was isolated and sequenced because of its ability to bind to myosin antigens (69).

A phylogenetic analysis of MCRA from *B. breve* NCIMB 702258 along with both LAI and oleate hydratase homologs was performed (68). *B. breve* NCIMB 702258 MCRA was placed between both groups, appearing, however, to be evolutionarily closer to the hydratase group. This relationship supports the presence of hydratase activity in some MCRA proteins, as was found in many of the above-mentioned studies. In fact, alignment analysis of putative LAI genes showed that there were some cases where the deduced translation products, generally classified as members of the MCRA superfamily, were associated not with LAI activity but with a presumed oxidoreductase (64). In fact, there are studies where a close evolutionary relationship between tested MCRA proteins and known oleate hydratase homologs was observed (76). Nowadays, the molecular functions of proteins continue to be predicted by using homology-based computational methods. These methods rely on the principle that homologous proteins may share similar functions. However, most protein families have sets of proteins with different functions (79). This suggests that predicting a protein function based on homology is not completely reliable.

FATTY ACID HYDRATASE ACTIVITY

Hydratases are enzymes catalyzing (de)hydration reactions of double bonds (80). As discussed above, some MCRA proteins have exerted FA hydratase activity, reported mainly for *Lactobacillus* bacteria. Recently, *L. acidophilus* NTV001, a gut bacterium, was shown to have a high ability to produce 13-hydroxy-*cis*-9-octadecenoic acid (13-HOE) from LA (81). During those studies, two hydratases were identified: fatty acid hydratase 1 (FA-HY1) and FA-HY2 (Table 3). The first one was responsible for the production of 13-HOE from LA, while the other, FA-HY2, catalyzed the production of 10-HOE. They were classified as belonging to the MCRA family of proteins and presenting FAD binding motifs at their N-terminal regions. These results support the possible hydratase role of some MCRA proteins, considering the close phylogenetic relationship between some identified MCRA proteins and hydratase proteins; as discussed above, other studies also corroborate this relationship.

On the other hand, oleate hydratases and LAI act on related substrates and share substantial sequence similarity but catalyze different reactions: a putative isomerase (LAI) shared 99.5% identity with a *L. acidophilus* hydratase (LAH) (82). These two

TABLE 3 Hydratase enzymes identified to be involved in the linoleic and/or linolenic acid conjugation mechanism

Strain studied	Protein annotation	Substrate	Product	GenBank nucleotide sequence accession no.	GenBank protein accession no.	Reference
<i>Lactobacillus acidophilus</i> NTV001	FA-HY1 FA-HY2	LA	13-HOE 10-HOE	LC030242 LC030243	BAX29644.1 BAX29645.1	81
<i>Lactobacillus reuteri</i> LTH2584	Linoleate 10-hydratase	LA	10-HOE	KX827285	AOZ57082.1	83
<i>Lactobacillus plantarum</i> TMW1.460	Linoleate 10-hydratase	LA	10-HOE	KX827286	AOZ57083.1	83
<i>Lactobacillus hammesii</i> DSM 16381	Linoleate 10-hydratase	LA	10-HOE	KX827287	AOZ57084.1	83
<i>Lactobacillus spicheri</i> LP38	Linoleate 10-hydratase	LA	10-HOE	KX827288	AOZ57085.1	83

enzymes differed by only three residues, but LAH catalyzes water addition, while LAI isomerizes double bonds.

Lactobacillus plantarum can express a linoleate 10-hydratase to produce antifungal 10-HOE with 13-HOE activity also. When this gene was compared with four others encoding putative LAHs from lactobacilli (*L. reuteri* LTH2584, *L. plantarum* TMW1.460, *L. hammesii* DSM16381, and *L. spicheri* Lp38), the results highlighted that there were FAD-dependent 10-LAHs (Table 3) belonging to the MCRA family (83). Interestingly, there is no evidence that 10-LAH and 13-LAH, or enzymes able to produce both 10-HOE and 13-HOE, were distinguished by phylogenetic and sequence analyses. These results suggest that significant genetic homology or a close phylogenetic relationship is not sufficient for an enzymatic function to be accurately predicted. Therefore, studies focused on protein recombinant expression can be very helpful.

Furthermore, knowledge of the structural and mechanistic relationships between CLA and hydroxy fatty acid formation is still scarce (84). Nevertheless, the crystal structure of a LAH was characterized, revealing the protein structure as a homodimer with each protomer comprised of four domains: three of them form the FAD binding and substrate binding sites, and the fourth domain is located at the C terminus and covers the entrance to the hydrophobic substrate channel. It is this domain that, in the presence of LA, undergoes conformational changes, allowing the entrance of the substrate to the hydrophobic substrate binding channel. That study confirmed that LAH is active only toward free fatty acids and requires a 9Z double bond. Among the identified structures of homologs, PAI was found to share a similar biological function with LAH (85).

THE REQUIREMENT OF COFACTORS

As mentioned above, several conserved motifs have been identified by MCRA protein sequence-structure analysis. Macouzet et al. (64) reported a putative FAD binding domain in some *Bifidobacterium* strain sequences, and Rosberg-Cody et al. (68) described a noncanonical flavin binding motif at the N terminus of the *B. breve* MCRA protein. Such findings are in agreement with previous studies regarding the same enzyme in *P. acnes*, where a FAD binding domain was detected in the N-terminal region (86). Therefore, it has been proposed that substrate activation and hydrogen transfer are assisted by FAD. In fact, it was observed that *S. pyogenes* MCRA is a FAD-containing hydratase that adds water to 9Z and 12Z double bonds of C₁₆ and C₁₈ fatty acids (77).

Furthermore, redox cofactors, such as NADH together with FAD or NADPH, seem to be required to obtain full functionality of these proteins (75). According to this, it has been hypothesized that direct oxidation and/or the reduction of the 10-HOE hydroxyl group requires NAD(P)H or that NAD(P)H is necessary for FAD reduction (75). In turn, this FAD domain was proposed to transfer electrons from the PUFA substrate to O₂ or other redox cofactors (76).

Indeed, this FAD cofactor has been found in both oleate hydratases and linoleate isomerases but performs different functions: in the case of isomerases, it is involved in

TABLE 4 Multicomponent enzymatic systems identified to be involved in the linoleic and/or linolenic acid conjugation mechanism

Strain studied	Protein annotation	Substrate	Product(s)	GenBank nucleotide sequence accession no.	GenBank protein accession no.	Reference(s)
<i>Lactobacillus plantarum</i> AKU 1009a	CLA-HY	LA	CLA intermediate 10-HOE	AB671229	BAL42246.1	84
	CLA-DH			AB671230	BAL42247.1	84, 87
	CLA-DC			AB671231	BAL42248.1	84
	CLA-ER			AB812091	BAO04454.1	30
<i>Lactobacillus plantarum</i> ZS2058	MCRA	LA	CLA intermediates 10-HOE, 10-oxo- <i>cis</i> -12-octadecenoic acid, and 10-oxo- <i>trans</i> -11-octadecenoic acid	JF747255.1	AEF13381.1	90, 91
	Short-chain dehydrogenase/oxidoreductase			KJ019513	AI119315.1	
	Acetoacetate decarboxylase			KJ019514	AI119316.1	

a redox-based mechanism, while for hydratases, it has been proposed to have a simple structural or charge-stabilizing function (82).
However, to the best of our knowledge, the role of the FAD binding residues in the above-mentioned proteins is still not fully understood.

CLA PRODUCTION: THE ACTION OF A MULTICOMPONENT SYSTEM?

In terms of CLA production mechanisms, most of the reviewed studies focused on analysis of a single gene product. However, the LA conjugation process as a detoxifying mechanism in bacterial cells seems to be an organized response performed by more than one gene. Supporting such a hypothesis, LAI from *L. plantarum* was found to be a novel multicomponent enzyme system (Table 4) localized in both the cell’s membrane and soluble fractions (75). Cell extracts were fractionated into membrane (UP) and soluble (US) fractions. The UP fraction exerted hydrating and dehydrating activities, but CLA was produced from LA and 10-HOE only when both UP and US fractions were present. Considering these results, 10-HOE would effectively be a reaction intermediate from LA during the hydration step by the membrane fraction. Subsequently, CLA would be further produced from 10-HOE and would involve the whole enzymatic system (Fig. 2). The membrane fraction is comprised of the hydratase/dehydratase (CLA-HY) enzyme, while the soluble fraction contains the dehydrogenase (CLA-DH) and isomerase (CLA-DC) enzymes. Furthermore, the *cla-dh* and *cla-dc* genes were found to be located

FIG 2 Schematic representation of the proteins (families) possibly involved in the fatty acid conjugation process. The multienzymatic system described is based on that proposed by Kishino et al. (75).

in a cluster with another gene, *cla-er* (75). By *cla-er* cloning in *E. coli* and using mass spectrometry (MS) and nuclear magnetic resonance (NMR), it was observed that by using the four identified enzymes, CLA-HY, CLA-DH, CLA-DC, and CLA-ER (enone reductase), as catalysts, RA and C_{18:2} Δ^9 , Δ^{11} ; 10-HOE; C_{18:2} Δ^{10} ; and oleic acid (OA) were obtained from LA (30). This multicomponent enzymatic system consists of multiple reactions where the four enzymes perform different functions. CLA-HY works as a catalyst for the hydration of LA and dehydration of 10-HOE (87), CLA-DH plays a role in the oxidation of hydroxyl groups and the reduction of oxo groups (88), CLA-DC is involved in the migration of double bonds, and the final saturation step is carried out by CLA-ER (30).

More recently, other genes have been suggested as alternatives for CFA metabolism. *Lactobacillus plantarum* α -enolase (Table 4), a protein belonging to the conserved family of enolases, was characterized and predicted to have a side role in LA biohydrogenation, catalyzing the formation of bioactive RA through the dehydration and isomerization of 10-HOE (89). This enzyme did not transform LA directly but instead targeted 10-HOE. This therefore implies the existence of a more complex metabolic process, involving not only LAI or MCRA proteins but a set of proteins working in a coordinated manner. In fact, those authors proposed that this enolase protein works along with the above-mentioned linoleate isomerase multiple complex.

Following these results and rationales, Yang et al. (90) performed a study where strains of food-derived lactobacilli were assessed for CLA production. *Lactobacillus plantarum* ZS2058 was identified as the most efficient producer, showing 37.8% conversion of LA into RA and 16.6% conversion into C_{18:2} Δ^9 , Δ^{11} . Genes encoding the multicomponent linoleate isomerase were cloned and expressed in *E. coli* (Table 4): myosin-cross-reactive antigen (*mcra*), short-chain dehydrogenase/oxidoreductase (*dh*), and acetoacetate decarboxylase (*dc*). By overexpressing these three genes separately, the activity of the corresponding recombinant proteins was assessed by using different combinations of the transformant cells: when the MCRA recombinant protein was studied, LA was able to be converted only to 10-HOE, and in *E. coli* cells producing the DH protein, both 10-HOE and 10-oxo-*cis*-12-octadecenoic acid were produced. When all three different transformed *E. coli* cell types were combined, 10-HOE, 10-oxo-*cis*-12-octadecenoic acid, 10-oxo-*trans*-11-octadecenoic acid, and RA were produced (90).

Moreover, in a subsequent study, the same group confirmed, through a gene deletion mutant, that the multiple-component LAI (MCRA, DH, and DC) was responsible for the *L. plantarum* ZS2058 CLA-producing capacity (91). The *mcra* gene deletion mutant failed to produce CLA or any intermediate. Regarding the *dh* deletion mutant, 10-HOE was detected, while both 10-HOE and 10-oxo-*cis*-12-octadecenoic acid were converted in the *dc* deletion mutant. The three genes were then overexpressed in the corresponding deletion mutants, which resulted in each strain recovering the ability to convert LA to CLA. In fact, other species, including *Bifidobacterium bifidum*, *B. breve*, *Bifidobacterium longum*, *Lactobacillus casei*, *L. plantarum*, *Lactobacillus brevis*, *Lactobacillus johnsonii*, *L. reuteri*, *L. rhamnosus*, *L. lactis*, and *Lactococcus cremoris*, contained these three genes (91).

METABOLITES FROM CLA/CLNA PRODUCTION

In the sections above, evidence pointing out enzymes involved in the microbiological production of CLA and CLNA is discussed. These above-described research works suggest that strains of lactic acid bacteria and bifidobacteria do not follow the same isomerization/biohydrogenation pathway of rumen bacteria but follow one where hydroxy FAs are intermediate metabolites (Fig. 3 and 4).

Hydroxy FAs as Intermediate Metabolites

The first studies on this topic were conducted under microaerobic conditions with washed cells of *Lactobacillus acidophilus* AKU 1137. In a first study, the authors reported that this strain was able to transform LA into CLA and that 10-HOE and 10-hydroxy-*trans*-12-octadecenoic acids were produced as intermediate metabolites (74). In those

FIG 3 Pathways of CLA production involving hydroxy fatty acids. Compounds labeled in green are initial substrates for CLA synthesis, compounds labeled in blue are intermediate metabolites, and compounds labeled in yellow are CLA isomers. The following enzyme names appear as in the original papers: hydratase and oxidase (94), 10-LAH/13-LAH (100), 12-LAH (80), lipoxigenase (LOX) (101), DH/DC (91), enolase (89), and CLA-ER/CLA-DH/CLA-ER (30).

studies, this *Lactobacillus* strain was grown in the presence of 10-hydroxy-*trans*-12-octadecenoic, producing C_{18:2} t9, t11. This behavior was further confirmed in a second study, where, using *Lactobacillus plantarum* AKU 1009a, the authors pinpointed a similar pathway during the transformation of LA into CLA (92). Accordingly, it was proposed that other compounds from this family of FAs, such as ricinoleic acid (ROL) (12-hydroxy-*cis*-9-octadecenoic acid), would be produced by these bacteria. Data obtained after assessing castor oil (which has a high concentration of ROL) agreed with this hypothesis (93). Thus, ROL can be transformed into LA by Δ^{12} dehydration (via 10-HOE and 10-hydroxy-*trans*-12-octadecenoic acids) or by Δ^{11} dehydration to directly produce RA or C_{18:2} t9, t11 (73).

However, rumen bacteria can also perform hydrogenation of ROL to 12-OH-octadecenoic acid and afterwards oxidation to 12-oxo-octadecanoic acid (94). Moreover, although the concentration of LA decreased during *in vitro* batch incubations of

FIG 4 Microbial production of CLNA and hydroxy fatty acids from LNA. Compounds labeled in green are initial substrates for CLNA synthesis, compounds labeled in blue are putative intermediate metabolites, and compounds labeled in yellow are CLNA isomers. Dotted arrows indicate putative pathways. FA-HY1 from *Lactobacillus acidophilus* appears as in reference 81.

castor oil with rumen fluid during 72 h, concentrations of RA and TVA did not show significant variations. This can be explained from the perspective that CFA synthesis in the rumen arises from the isomerization of the respective substrates (95, 96).

Previous investigations testing sheep rumen fluid incubated with ROL also reported no CLA production but the synthesis of 10-HOE by bacteria other than *B. fibrisolvens*

(97). On the other hand, results from the latter work showed that the use of LA as the substrate and deuterated water led to the protonation of carbon 13 of 9,11 isomers. In similar experiments with gut microbiota, it was proposed that the formation of C-13-labeled CLA was consistent with a hydration/dehydration/rearrangement mechanism involving 10-OH octadecenoic acids (98).

Multienzymatic Complexes and Possible Synthesis Pathways

As discussed above, evidence shows the existence of a multienzymatic complex, and studies have confirmed that at least lactobacilli synthesize CFA through a mechanism that may not involve only LAI (84, 90, 91): the initial step is the hydration of LA by 10-LAH, resulting in 10-HOE acid that is oxidized by a short-chain hydrogenase/oxidoreductase (DH), which is afterwards transformed into 10-oxo-*trans*-11-octadecenoic acid by the action of an acetoacetate decarboxylase (DC) and reduced by DH (10-hydroxy-*trans*-11-octadecenoic), while finally, LAH leads to the production of RA and C_{18:2} *t*9, *t*11 (Fig. 3). On the other hand, Ortega-Anaya and Hernández-Santoyo (89) recently reported that in *Lactobacillus plantarum* ATCC 8014, CLA synthesis is accomplished by a multifunctional enolase through dehydration/isomerization of 10-hydroxy-*cis*-12-octadecenoic acid.

Until now, studies of the microbial production of hydroxy fatty acids have focused on those related to the synthesis of CFA. However, some authors have also found that *L. plantarum* and *L. acidophilus* can convert LA into 13-hydroxy-*cis*-9-octadecenoic acid or 10,13-dihydroxy-octadecanoic acid by action of both 10-LAH and 13-LAH (99, 100). However, the biological significance of such specific compounds for these microorganisms is not completely understood. Nevertheless, it has been found that *Lactobacillus hammesii* (101) and *Lactobacillus plantarum* TMW1.460Δlah (102) have an antifungal defense mechanism using coriolic acid (13-hydroxy-9,11-octadecadienoic acid).

Hydratases Are Not Substrate Specific

The specificity of such hydratase enzymes seems not to be solely limited to LA, as hydratases from *Lactobacillus acidophilus* NTV001 (81) and *Lactobacillus plantarum* AKU 1009a (103) can perform hydration using several PUFAs from palmitoleic acid (C_{16:1} *c*9) to DHA (C_{22:6} *c*4, *c*7, *c*10, *c*13, *c*16, *c*19), yielding different hydroxy FAs (Table 5).

While the role of these compounds in CLA production is clear for lactobacilli, research works involving bifidobacterial strains have confirmed their LA/LNA-transforming capacity (47, 104, 105), but the currently available data suggest that no other metabolites are involved (Fig. 4).

Thus, *Bifidobacterium breve* 2258 was previously identified as an RA and C_{18:2} *t*9, *t*11 producer (106). However, while MCRA converts oleic acid into 10-hydroxystearic acid, the enzyme is not involved in CFA synthesis (71). Furthermore, an interesting strain is *Bifidobacterium breve* NCIMB 702258, which, besides CLA, can also transform LNA into C_{18:3} *c*9, *t*11, *c*15 and C_{18:3} *t*9, *t*11, *c*15; γ LNA (i.e., C_{18:3} *c*6, *c*9, *c*12) into C_{18:3} *c*6, *c*9, *t*11; and stearidonic acid (C_{18:4} *c*6, *c*9, *c*12, *c*15) into C_{18:4} *c*6, *c*9, *t*11, *c*15 (107). In the specific situation of CLNA compounds, a putative pathway with 10-hydroxy-*cis*-12-*cis*-15-octadecadienoic or 10-hydroxy-*cis*-6-*cis*-12-octadecadienoic acids as intermediates can be suggested (Fig. 4). Indeed, the *Bifidobacterium breve* NCIMB 702258 genome also encodes a MCRA with hydratase activity, thus producing 10-OH-plamitic acid using palmitoleic acid (C_{16:1} *c*9) as the substrate, 10-hydroxystearic acid from oleic acid, and 10-hydroxy-*cis*-12-octadecenoic acid from LA (68). However, LNA did not lead to the formation of any hydroxy FA, and this proposed mechanism is most likely to be found in lactobacillus strains.

Nevertheless, while *Lactobacillus plantarum* AKU 1009a produced CLNA from both LNA and γ LNA, the presence of hydroxy FA was not detected (108). Accordingly, it may be hypothesized that CLNA results from direct isomerization, as in the conjugating mechanism of *Butyrivibrio fibrisolvens*, but action through a multienzymatic system cannot be discarded.

TABLE 5 Microbial production of hydroxy fatty acids from PUFA

Fatty acid	Hydroxy FA ^a		
	<i>Lactobacillus acidophilus</i> NTV001 ^b	<i>Lactobacillus plantarum</i> AKU 1009a ^c	<i>Bifidobacterium breve</i> 702258 ^d
C _{14:1} c9			—
C _{16:1} c9	10-Hydroxy-16:0	10-Hydroxy-16:0	10-Hydroxy-16:0
C _{18:1} c9	10-HOA	10-HOA	10-HOA
C _{18:1} t9			—
12-Hydroxy- <i>cis</i> -9-18:1		10,12-Hydroxy-18:0	
C _{18:1} c11	12-Hydroxy-18:0		—
C _{18:1} t11	No products detected		
C _{18:2} c9, c12	13-Hydroxy- <i>cis</i> -9-18:1	10-Hydroxy- <i>cis</i> -9-18:1	10-Hydroxy- <i>cis</i> -9-18:1
C _{18:2} t9, t12	No products detected		
C _{18:3} c5, c9, c12	13-Hydroxy- <i>cis</i> -5, <i>cis</i> -9-18:2		
C _{18:3} t5, c9, c12	13-Hydroxy- <i>trans</i> -5, <i>cis</i> -9-18:2		
C _{18:3} c6, c9, c12	13-Hydroxy- <i>cis</i> -6, <i>cis</i> -9-18:2 10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-18:2		
C _{18:3} c9, c12, c15	13-Hydroxy- <i>cis</i> -9, <i>cis</i> -15-18:2	10-Hydroxy- <i>cis</i> -9, <i>cis</i> -15-18:2	—
C _{18:4} c6, c9, c12, c15	13-Hydroxy- <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -15-18:3	10-Hydroxy- <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -15-18:3	
C _{20:2} c11, c14	15-Hydroxy- <i>cis</i> -11-20:1		
C _{20:3} c5, c8, c11	12-Hydroxy- <i>cis</i> -5, <i>cis</i> -8-20:2		
C _{20:3} c5, c11, c14	15-Hydroxy- <i>cis</i> -5, <i>cis</i> -11-20:2		
C _{20:3} c8, c11, c14	15-Hydroxy- <i>cis</i> -8, <i>cis</i> -11-20:2 12-Hydroxy- <i>cis</i> -8, <i>cis</i> -14-20:2		
C _{20:3} c11, c14, c17	12-Hydroxy- <i>cis</i> -14, <i>cis</i> -17-20:2 15-Hydroxy- <i>cis</i> -11, <i>cis</i> -17-20:2		
C _{20:4} c5, c8, c11, c14	15-Hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11-20:3		
C _{20:4} c8, c11, c14, c17	12-Hydroxy- <i>cis</i> -8, <i>cis</i> -14, <i>cis</i> -17-20:3		
C _{20:5} c5, c8, c11, c14, c17	No products detected		
C _{22:6} c4, c7, c10, c13, c16, c19	14-Hydroxy- <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -16, <i>cis</i> -19-22:5		

^a— indicates that fatty acids were assayed, but no product was detected. Blank cells indicate fatty acids that were not assayed in the different bacteria.

^bSee reference 81.

^cSee reference 87.

^dSee reference 68.

CHALLENGES AND FUTURE PERSPECTIVES

The current strategy to identify CLA/CLNA-producing bacteria is the assessment of production under generalized growth and substrate concentration conditions by chromatographic/spectrophotometric techniques. However, these methods of analysis require laborious and time-consuming sample preparation, thus being unsuitable for routine screening. On the other hand, genetic approaches appear to be a more efficient alternative, although these methods require an understanding of enzymatic mechanisms upholding CFA synthesis.

As illustrated in Fig. 4, to date, there are several proteins possibly involved in this

process. Nevertheless, these processes are still not fully described, and some results seem to be contradictory. For example, some recent reports found an absence of production in strains positive for the LAI gene (109). This may be related to results reported elsewhere showing that CFA synthesis is regulated by various factors, such as pH or temperature (56, 65). Besides, through a microarray analysis, *mcra* expression was found to be upregulated by transcription factors induced by stress (110). Consequently, the lack of CFA production for a certain strain might result not from production incapacity but instead from inappropriate growth conditions leading to a stress response, negatively affecting this synthesis on a molecular basis.

Furthermore, one of the major challenges, when considering a genomic approach, is the fact that predicted enzymatic function follows the principle of genetic homology with other annotated proteins without considering that members of the same protein family can exert different functions (79). Thus, the putative LAI from *Lactobacillus acidophilus* did not isomerize LA, and it was concluded that the protein was misannotated in the UniProt database (82).

With this in mind, after determining that a gene is possibly involved in CLA/CLNA production, subsequent steps should be directed to confirm such a role. These types of studies have shown interesting results, since after insertional mutagenesis, none of the integration mutants were able to grow (64). This may be due to the fact that translation products of the studied genes are essential for the bacteria: if this conjugation process works as a detoxifying mechanism, these genes may not be involved in just LA/LNA isomerization but may be integrated in a more complex stress response. Further investigations aiming to confirm this hypothesis could test antisense RNA to reduce both transcript and, consequently, protein levels, without totally inactivating the gene; all this needs to be combined with transcriptomic/proteomic/lipidomic analysis.

Finally, results suggesting a multicomponent enzymatic system point to different challenges that need to be addressed, namely, (i) unveiling strong evidence that may uphold whether proteins or a multienzymatic system is responsible for both LA and LNA transformation and (ii) characterization of their kinetic parameters. This will allow us to understand, for instance, why some strains have a greater ability to isomerize LA to CLA and LNA to CLNA than others. LAI of *B. fibrisolvens* MDT-10 catalyzes the isomerization of LA to RA, and CLA reductase (CLA-R) is responsible for subsequent hydrogenation to TVA (111). On the other hand, the MDT-5 strain presents a lack of CLA-R activity (19). Instead, MDT-5 rapidly isomerizes LA and LNA to CLA and CLNA (RA and RLA). Sequence analysis of the *cla-r* gene product in MDT-5 compared with that of MDT-10 revealed a difference in four consecutive amino acids, and this mutated part was found to be closely associated with the active center of the enzyme (3).

Moreover, several studies regarding *Lactobacillus* and *Bifidobacterium* have shown that inhibition of growth by LA and LNA is strain and not species dependent (104, 109). This shows that characterization of these enzymes cannot be carried out according to homology but should consider strain specificity.

ACKNOWLEDGMENTS

This work was financed by national funds via the Fundação para a Ciência e a Tecnologia (FCT), under the project Pro-TECh-CLnA—Microbial Production of Bioactive Conjugated Linolenic Acid Isomers To Obtain Functional Ingredients and Foods (reference PTDC/AGR-TEC/2125/2014). Financial support for L.L.P. and A.L.F. was provided by fellowships SFRH/BPD/119785/2016 and SFRH/BD/117721/2016, respectively, granted by the Portuguese government through the FCT. We also acknowledge the scientific collaboration under FCT project UID/Multi/50016/2013.

REFERENCES

1. Sieber R, Collomb M, Aeschlimann A, Jelen P, Eyer H. 2004. Impact of microbial cultures on conjugated linoleic acid in dairy products—a review. *Int Dairy J* 14:1–15. [https://doi.org/10.1016/S0958-6946\(03\)00151-1](https://doi.org/10.1016/S0958-6946(03)00151-1).
2. Liavonchanka A, Feussner I. 2008. Biochemistry of PUFA double bond isomerases producing conjugated linoleic acid. *Chembiochem* 9:1867–1872. <https://doi.org/10.1002/cbic.200800141>.
3. Fukuda S, Suzuki Y, Komori T, Kawamura K, Asanuma N, Hino T. 2007.

- Purification and gene sequencing of conjugated linoleic acid reductase from a gastrointestinal bacterium, *Butyrivibrio fibrisolvens*. *J Appl Microbiol* 103:365–371. <https://doi.org/10.1111/j.1365-2672.2006.03247.x>.
4. Soel SM, Choi OS, Bang MH, Yoon Park JH, Kim WK. 2007. Influence of conjugated linoleic acid isomers on the metastasis of colon cancer cells in vitro and in vivo. *J Nutr Biochem* 18:650–657. <https://doi.org/10.1016/j.jnutbio.2006.10.011>.
 5. Rodríguez-Alcalá LM, Castro-Gómez MP, Pimentel LL, Fontecha J. 2017. Milk fat components with potential anticancer activity—a review. *Biosci Rep* 37:BSR20170705. <https://doi.org/10.1042/BSR20170705>.
 6. Kritchevsky D, Tepper SA, Wright S, Czarnecki SK. 2002. Influence of graded levels of conjugated linoleic acid (CLA) on experimental atherosclerosis in rabbits. *Nutr Res* 22:1275–1279. [https://doi.org/10.1016/S0271-5317\(02\)00432-3](https://doi.org/10.1016/S0271-5317(02)00432-3).
 7. Lee KN, Kritchevsky D, Pariza MW. 1994. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis* 108:19–25. [https://doi.org/10.1016/0021-9150\(94\)90034-5](https://doi.org/10.1016/0021-9150(94)90034-5).
 8. Park Y, Pariza MW. 2007. Mechanisms of body fat modulation by conjugated linoleic acid (CLA). *Food Res Int* 40:311–323. <https://doi.org/10.1016/j.foodres.2006.11.002>.
 9. Rodríguez-Alcalá LM, Fontecha J, de la Hoz L, da Silva VSN, Carvalho JE, Pacheco MTB. 2013. CLA-enriched milk powder reverses hypercholesterolemic risk factors in hamsters. *Food Res Int* 51:244–249. <https://doi.org/10.1016/j.foodres.2012.12.013>.
 10. Pariza MW. 2004. Perspective on the safety and effectiveness of conjugated linoleic acid. *Am J Clin Nutr* 79:1132S–1136S. <https://doi.org/10.1093/ajcn/79.6.1132S>.
 11. Wahle KWJ, Heys SD, Rotondo D. 2004. Conjugated linoleic acids: are they beneficial or detrimental to health? *Prog Lipid Res* 43:553–587. <https://doi.org/10.1016/j.plipres.2004.08.002>.
 12. Geddes RJ, Hayashi K, Bongers Q, Wehber M, Anderson IM, Jansen AD, Nier C, Fares E, Farquhar G, Kapoor A, Ziegler TE, VadakkadathMeethal S, Bird IM, Atwood CS. 2017. Conjugated linoleic acid administration induces amnesia in male Sprague Dawley rats and exacerbates recovery from functional deficits induced by a controlled cortical impact injury. *PLoS One* 12:e0188611. <https://doi.org/10.1371/journal.pone.0188611>.
 13. Bezan P, Holland H, de Castro G, Cardoso J, Ovidio P, Calder P, Jordao A. 2018. High dose of a conjugated linoleic acid mixture increases insulin resistance in rats fed either a low fat or a high fat diet. *Exp Clin Endocrinol Diabetes* 126:379–386. <https://doi.org/10.1055/s-0043-118348>.
 14. Riserus U, Smedman A, Basu S, Vessby B. 2004. Metabolic effects of conjugated linoleic acid in humans: the Swedish experience. *Am J Clin Nutr* 79:1146S–1148S. <https://doi.org/10.1093/ajcn/79.6.1146S>.
 15. Riserus U, Vessby B, Arner P, Zethelius B. 2004. Supplementation with trans10cis12-conjugated linoleic acid induces hyperproinsulinaemia in obese men: close association with impaired insulin sensitivity. *Diabetologia* 47:1016–1019. <https://doi.org/10.1007/s00125-004-1421-8>.
 16. Yuan G-F, Chen X-E, Li D. 2014. Conjugated linolenic acids and their bioactivities: a review. *Food Funct* 5:1360–1368. <https://doi.org/10.1039/c4fo00037d>.
 17. Fontes AL, Pimentel LL, Simoes CD, Gomes AMP, Rodriguez-Alcala LM. 2017. Evidence and perspectives in the utilization of CLNA isomers as bioactive compound in foods. *Crit Rev Food Sci Nutr* 57:2611–2622. <https://doi.org/10.1080/10408398.2015.1063478>.
 18. Jenkins TC. 1993. Lipid metabolism in the rumen. *J Dairy Sci* 76:382–426. [https://doi.org/10.3168/jds.S0022-0302\(93\)77727-9](https://doi.org/10.3168/jds.S0022-0302(93)77727-9).
 19. Fukuda S, Suzuki Y, Murai M, Asanuma N, Hino T. 2006. Augmentation of vaccenate production and suppression of vaccenate biohydrogenation in cultures of mixed ruminal microbes. *J Dairy Sci* 89:1043–1051. [https://doi.org/10.3168/jds.S0022-0302\(06\)72171-3](https://doi.org/10.3168/jds.S0022-0302(06)72171-3).
 20. Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela K, Bauman DE. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ^9 -desaturase. *J Nutr* 130:2285–2291. <https://doi.org/10.1093/jn/130.9.2285>.
 21. Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, Griinari JM. 2002. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. *Am J Clin Nutr* 76:504–510. <https://doi.org/10.1093/ajcn/76.3.504>.
 22. Jenkins TC, Wallace RJ, Moate PJ, Mosley EE. 2008. Board-invited review: recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J Anim Sci* 86:397–412. <https://doi.org/10.2527/jas.2007-0588>.
 23. Lourenço M, Ramos-Morales E, Wallace RJ. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal* 4:1008–1023. <https://doi.org/10.1017/S175173111000042X>.
 24. Destailhats F, Trotter JP, Galvez JM, Angers P. 2005. Analysis of alpha-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. *J Dairy Sci* 88:3231–3239. [https://doi.org/10.3168/jds.S0022-0302\(05\)73006-X](https://doi.org/10.3168/jds.S0022-0302(05)73006-X).
 25. Lee Y-J, Jenkins TC. 2011. Biohydrogenation of linolenic acid to stearic acid by the rumen microbial population yields multiple intermediate conjugated diene isomers. *J Nutr* 141:1445–1450. <https://doi.org/10.3945/jn.111.138396>.
 26. Van Nieuwenhove C, Terán V, González S. 2012. Conjugated linoleic and linolenic acid production by bacteria: development of functional foods, p 55–80. In Rigobelo EC (ed), *Probiotics*. InTech, London, United Kingdom.
 27. Gorissen L, Leroy F, De Vuyst L, De Smet S, Raes K. 2015. Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. *Crit Rev Food Sci Nutr* 55:1561–1574. <https://doi.org/10.1080/10408398.2012.706243>.
 28. Ferlay A, Bernard L, Meynadier A, Malpuech-Brugere C. 2017. Production of trans and conjugated fatty acids in dairy ruminants and their putative effects on human health: a review. *Biochimie* 141:107–120. <https://doi.org/10.1016/j.biochi.2017.08.006>.
 29. Polan CE, McNeill JJ, Tove SB. 1964. Biohydrogenation of unsaturated fatty acids by rumen bacteria. *J Bacteriol* 88:1056–1064.
 30. Kishino S, Takeuchi M, Park S-B, Hirata A, Kitamura N, Kunisawa J, Kiyono H, Iwamoto R, Isobe Y, Arita M, Arai H, Ueda K, Shima J, Takahashi S, Yokozeki K, Shimizu S, Ogawa J. 2013. Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. *Proc Natl Acad Sci U S A* 110:17808–17813. <https://doi.org/10.1073/pnas.1312937110>.
 31. Zheng CJ, Yoo JS, Lee TG, Cho HY, Kim YH, Kim WG. 2005. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Lett* 579:5157–5162. <https://doi.org/10.1016/j.febslet.2005.08.028>.
 32. Zhang YZ, Wei ZZ, Liu CH, Chen Q, Xu BJ, Guo ZR, Cao YL, Wang Y, Han YN, Chen C, Feng X, Qiao YY, Zong LJ, Zheng T, Deng M, Jiang QT, Li W, Zheng YL, Wei YM, Qi PF. 2017. Linoleic acid isomerase gene FgLA12 affects sensitivity to salicylic acid, mycelial growth and virulence of *Fusarium graminearum*. *Sci Rep* 7:46192. <https://doi.org/10.1038/srep46192>.
 33. Zhang X, Li M, Wei D, Wang X, Chen X, Xing L. 2007. Disruption of the fatty acid Δ^6 -desaturase gene in the oil-producing fungus *Mortierella isabellina* by homologous recombination. *Curr Microbiol* 55:128–134. <https://doi.org/10.1007/s00284-006-0641-1>.
 34. Needleman P, Turk J, Jakschik B, Morrison A, Lefkowitz J. 1986. Arachidonic acid metabolism. *Annu Rev Biochem* 55:69–102. <https://doi.org/10.1146/annurev.bi.55.070186.000441>.
 35. Greenway D, Dyke K. 1979. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *J Gen Microbiol* 115:233–245. <https://doi.org/10.1099/00221287-115-1-233>.
 36. Maia MR, Chaudhary LC, Bestwick CS, Richardson AJ, McKain N, Larson TR, Graham IA, Wallace RJ. 2010. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. *BMC Microbiol* 10:52. <https://doi.org/10.1186/1471-2180-10-52>.
 37. Shokryzadan P, Rajion MA, Meng GY, Boo LJ, Ebrahimi M, Royan M, Sahebi M, Azizi P, Abiri R, Jahromi MF. 2017. Conjugated linoleic acid: a potent fatty acid linked to animal and human health. *Crit Rev Food Sci Nutr* 57:2737–2748. <https://doi.org/10.1080/10408398.2015.1060190>.
 38. Plourde M, Destailhats F, Chouinard PY, Angers P. 2007. Conjugated alpha-linolenic acid isomers in bovine milk and muscle. *J Dairy Sci* 90:5269–5275. <https://doi.org/10.3168/jds.2007-0157>.
 39. Lerch S, Shingfield KJ, Ferlay A, Vanhatalo A, Chilliard Y. 2012. Rapeseed or linseed in grass-based diets: effects on conjugated linoleic and conjugated linolenic acid isomers in milk fat from Holstein cows over 2 consecutive lactations. *J Dairy Sci* 95:7269–7287. <https://doi.org/10.3168/jds.2012-5654>.
 40. Mapiye C, Aalhus JL, Turner TD, Rolland DC, Basarab JA, Baron VS, McAllister TA, Block HC, Uttaro B, Lopez-Campos O, Proctor SD, Dugan MER. 2013. Effects of feeding flaxseed or sunflower-seed in high-forage diets on beef production, quality and fatty acid composition. *Meat Sci* 95:98–109. <https://doi.org/10.1016/j.meatsci.2013.03.033>.
 41. Ebrahimi M, Rajion MA, Goh YM. 2014. Effects of oils rich in linoleic and

- alpha-linolenic acids on fatty acid profile and gene expression in goat meat. *Nutrients* 6:3913–3928. <https://doi.org/10.3390/nu6093913>.
42. Bialek M, Czauderna M, Bialek A. 2017. Conjugated linolenic acid (CLnA) isomers as new bioactive lipid compounds in ruminant-derived food products. A review. *J Anim Feed Sci* 26:3–17.
 43. Fuke G, Nornberg JL. 2017. Systematic evaluation on the effectiveness of conjugated linoleic acid in human health. *Crit Rev Food Sci Nutr* 57:1–7. <https://doi.org/10.1080/10408398.2012.716800>.
 44. Shinohara N, Tsuduki T, Ito J, Honma T, Kijima R, Sugawara S, Arai T, Yamasaki M, Ikezaki A, Yokoyama M, Nishiyama K, Nakagawa K, Miyazawa T, Ikeda I. 2012. Jacaric acid, a linolenic acid isomer with a conjugated triene system, has a strong antitumor effect in vitro and in vivo. *Biochim Biophys Acta* 1821:980–988. <https://doi.org/10.1016/j.bbalip.2012.04.001>.
 45. Reference deleted.
 46. Fukuda S, Suzuki Y, Murai M, Asanuma N, Hino T. 2006. Isolation of a novel strain of *Butyrivibrio fibrisolvens* that isomerizes linoleic acid to conjugated linoleic acid without hydrogenation, and its utilization as a probiotic for animals. *J Appl Microbiol* 100:787–794. <https://doi.org/10.1111/j.1365-2672.2006.02864.x>.
 47. Villar-Tajadura MA, Rodríguez-Alcalá LM, Martín V, Gómez De Segura A, Rodríguez JM, Requena T, Fontecha J. 2014. Production of conjugated linoleic and conjugated α -linolenic acid in a reconstituted skim milk-based medium by bifidobacterial strains isolated from human breast milk. *Biomed Res Int* 2014:725406. <https://doi.org/10.1155/2014/725406>.
 48. Patterson E, Wall R, Lisai S, Ross RP, Dinan TG, Cryan JF, Fitzgerald GF, Banni S, Quigley EM, Shanahan F, Stanton C. 2017. Bifidobacterium breve with alpha-linolenic acid alters the composition, distribution and transcription factor activity associated with metabolism and absorption of fat. *Sci Rep* 7:43300. <https://doi.org/10.1038/srep43300>.
 49. Robinson NP, MacGibbon AKH. 2000. Determination of the conjugated linoleic acid-containing triacylglycerols in New Zealand bovine milk fat. *Lipids* 35:789–796. <https://doi.org/10.1007/s11745-000-0587-x>.
 50. Czauderna M, Kowalczyk J, Wąsowska I, Niedźwiedzka KM. 2003. Determination of conjugated linoleic acid isomers by liquid chromatography and photodiode array detection. *J Anim Feed Sci* 12:369–382. <https://doi.org/10.22358/jafs/67717/2003>.
 51. Barrett E, Ross RP, Fitzgerald GF, Stanton C. 2007. Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. *Appl Environ Microbiol* 73:2333–2337. <https://doi.org/10.1128/AEM.01855-06>.
 52. Rodríguez-Alcalá LM, Braga T, Malcata FX, Gomes A, Fontecha J. 2011. Quantitative and qualitative determination of CLA produced by Bifidobacterium and lactic acid bacteria by combining spectrophotometric and Ag⁺-HPLC techniques. *Food Chem* 125:1373–1378. <https://doi.org/10.1016/j.foodchem.2010.10.008>.
 53. Varga-Visi É, Salamon R, Lóki K, Csapó J. 2012. Gas chromatographic analysis of conjugated linoleic acids. *Alimentaria* 5:52–62.
 54. Nzali H, Tchiegang C, Mignolet E, Turu C, Larondelle Y, Meurens M. 2012. Study of bioconversion of conjugated linolenic acid (CLNA) of Ricinodendron heudelotii (Bail.) seed in male rats into conjugated linoleic acid (CLA) using UV-Vis spectrometry and gas chromatography. *Asian J Biochem* 7:194–205. <https://doi.org/10.3923/ajb.2012.194.205>.
 55. Alonso L, Cuesta EP, Gilliland SE. 2003. Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. *J Dairy Sci* 86:1941–1946. [https://doi.org/10.3168/jds.S0022-0302\(03\)73781-3](https://doi.org/10.3168/jds.S0022-0302(03)73781-3).
 56. Kim YJ, Liu RH. 2002. Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. *J Food Sci* 67:1731–1737. <https://doi.org/10.1111/j.1365-2621.2002.tb08714.x>.
 57. Van Nieuwenhove CP, Oliszewski R, González SN, Pérez Chaia AB. 2007. Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk. *Lett Appl Microbiol* 44:467–474. <https://doi.org/10.1111/j.1472-765X.2007.02135.x>.
 58. Castro-Gómez P, Fontecha J, Rodríguez-Alcalá LM. 2014. A high-performance direct transmethylation method for total fatty acids assessment in biological and foodstuff samples. *Talanta* 128:518–523. <https://doi.org/10.1016/j.talanta.2014.05.051>.
 59. Kepler C, Tove S. 1967. Biohydrogenation of unsaturated fatty acids. *J Biol Chem* 242:5686–5693.
 60. Palmquist DL, Lock AL, Shingfield KJ, Bauman DE. 2005. Biosynthesis of conjugated linoleic acid in ruminants and humans, p 179–217. In Taylor SL (ed), *Advances in food and nutrition research*. Academic Press, Cambridge, MA. [https://doi.org/10.1016/S1043-4526\(05\)50006-8](https://doi.org/10.1016/S1043-4526(05)50006-8).
 61. Peng SS, Deng M-D, Grund AD, Rosson RA. 2007. Purification and characterization of a membrane-bound linoleic acid isomerase from *Clostridium sporogenes*. *Enzyme Microb Technol* 40:831–839. <https://doi.org/10.1016/j.enzmictec.2006.06.020>.
 62. Rosberg-Cody E, Johnson MC, Fitzgerald GF, Ross PR, Stanton C. 2007. Heterologous expression of linoleic acid isomerase from *Propionibacterium acnes* and anti-proliferative activity of recombinant trans-10, cis-12 conjugated linoleic acid. *Microbiology* 153:2483–2490. <https://doi.org/10.1099/mic.0.2006/001966-0>.
 63. Liavonchanka A, Hornung E, Feussner I, Rudolph MG. 2006. Structure and mechanism of the *Propionibacterium acnes* polyunsaturated fatty acid isomerase. *Proc Natl Acad Sci U S A* 103:2576–2581. <https://doi.org/10.1073/pnas.0510144103>.
 64. Macouzet M, Lee BH, Robert N. 2010. Genetic and structural comparison of linoleate isomerases from selected food-grade bacteria. *J Appl Microbiol* 109:2128–2134. <https://doi.org/10.1111/j.1365-2672.2010.04844.x>.
 65. Gorissen L, Weckx S, Vlaeminck B, Raes K, de Vuyst L, de Smet S, Leroy F. 2011. Linoleate isomerase activity occurs in lactic acid bacteria strains and is affected by pH and temperature. *J Appl Microbiol* 111:593–606. <https://doi.org/10.1111/j.1365-2672.2011.05087.x>.
 66. Hennessy AA, Ross RP, Devery R, Stanton C. 2009. Optimization of a reconstituted skim milk based medium for enhanced CLA production by bifidobacteria. *J Appl Microbiol* 106:1315–1327. <https://doi.org/10.1111/j.1365-2672.2008.04098.x>.
 67. Farmani J, Safari M, Roohvand F, Razavi SH, Aghasadeghi MR, Noorbazargan H. 2010. Conjugated linoleic acid-producing enzymes: a bioinformatics study. *Eur J Lipid Sci Technol* 112:1088–1100. <https://doi.org/10.1002/ejlt.201000360>.
 68. Rosberg-Cody E, Liavonchanka A, Göbel C, Ross RP, O'Sullivan O, Fitzgerald GF, Feussner I, Stanton C. 2011. Myosin-cross-reactive antigen (MCRA) protein from *Bifidobacterium breve* is a FAD-dependent fatty acid hydratase which has a function in stress protection. *BMC Biochem* 12:9. <https://doi.org/10.1186/1471-2091-12-9>.
 69. Kil KS, Cunningham MW, Barnett LA. 1994. Cloning and sequence analysis of a gene encoding a 67-kilodalton myosin-cross-reactive antigen of *Streptococcus pyogenes* reveals its similarity with class II major histocompatibility antigens. *Infect Immun* 62:2440–2449.
 70. Liu S, Qureshi N. 2009. How microbes tolerate ethanol and butanol. *N Biotechnol* 26:117–121. <https://doi.org/10.1016/j.nbt.2009.06.984>.
 71. O'Connell KJ, Motherway MOC, Hennessy AA, Brodhun F, Ross RP, Feussner I, Stanton C, Fitzgerald GF, Van Sinderen D. 2013. Identification and characterization of an oleate hydratase-encoding gene from *Bifidobacterium breve*. *Bioengineered* 4:313–321. <https://doi.org/10.4161/bioe.24159>.
 72. O'Flaherty SJ, Klaenhammer TR. 2010. Functional and phenotypic characterization of a protein from *Lactobacillus acidophilus* involved in cell morphology, stress tolerance and adherence to intestinal cells. *Microbiology* 156:3360–3367. <https://doi.org/10.1099/mic.0.043158-0>.
 73. Ogawa J, Kishino S, Ando A, Sugimoto S, Mihara K, Shimizu S. 2005. Production of conjugated fatty acids by lactic acid bacteria. *J Biosci Bioeng* 100:355–364. <https://doi.org/10.1263/jbb.100.355>.
 74. Ogawa J, Matsumura K, Kishino S, Omura Y, Shimizu S. 2001. Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecanoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl Environ Microbiol* 67:1246–1252. <https://doi.org/10.1128/AEM.67.3.1246-1252.2001>.
 75. Kishino S, Ogawa J, Yokozeki K, Shimizu S. 2011. Linoleic acid isomerase in *Lactobacillus plantarum* AKU1009a proved to be a multi-component enzyme system requiring oxidoreduction cofactors. *Biosci Biotechnol Biochem* 75:318–322. <https://doi.org/10.1271/bbb.100699>.
 76. Yang B, Chen H, Song Y, Chen YQ, Zhang H, Chen W. 2013. Myosin-cross-reactive antigens from four different lactic acid bacteria are fatty acid hydratases. *Biotechnol Lett* 35:75–81. <https://doi.org/10.1007/s10529-012-1044-y>.
 77. Volkov A, Liavonchanka A, Kamneva O, Fiedler T, Goebel C, Kreikemeyer B, Feussner I. 2010. Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial virulence. *J Biol Chem* 285:10353–10361. <https://doi.org/10.1074/jbc.M109.081851>.
 78. Raimondi S, Amaretti A, Leonardi A, Quartieri A, Gozzoli C, Rossi M. 2016. Conjugated linoleic acid production by bifidobacteria: screening,

- kinetic, and composition. *Biomed Res Int* 2016:8654317. <https://doi.org/10.1155/2016/8654317>.
79. Engelhardt BE, Jordan MI, Repo ST, Brenner SE. 2009. Phylogenetic molecular function annotation. *J Phys Conf Ser* 180:12024. <https://doi.org/10.1088/1742-6596/180/1/012024>.
 80. Demming RM, Fischer MP, Schmid J, Hauer B. 2018. (De)hydratases—recent developments and future perspectives. *Curr Opin Chem Biol* 43:43–50. <https://doi.org/10.1016/j.cbpa.2017.10.030>.
 81. Hirata A, Kishino S, Park S-B, Takeuchi M, Kitamura N, Ogawa J. 2015. A novel unsaturated fatty acid hydratase toward C16 to C22 fatty acids from *Lactobacillus acidophilus*. *J Lipid Res* 56:1340–1350. <https://doi.org/10.1194/jlr.M059444>.
 82. Fibinger MPC, Freiherr von Saß GJ, Herrfurth C, Feussner I, Bornscheuer UT. 2016. A directed mutational approach demonstrates that a putative linoleate isomerase from *Lactobacillus acidophilus* does not hydrate or isomerize linoleic acid. *Eur J Lipid Sci Technol* 118:841–848. <https://doi.org/10.1002/ejlt.201500444>.
 83. Chen YY, Liang NY, Curtis JM, Gänzle MG. 2016. Characterization of linoleate 10-hydratase of *Lactobacillus plantarum* and novel antifungal metabolites. *Front Microbiol* 7:1561. <https://doi.org/10.3389/fmicb.2016.01561>.
 84. Kishino S, Park SB, Takeuchi M, Yokozeki K, Shimizu S, Ogawa J. 2011. Novel multi-component enzyme machinery in lactic acid bacteria catalyzing CC double bond migration useful for conjugated fatty acid synthesis. *Biochem Biophys Res Commun* 416:188–193. <https://doi.org/10.1016/j.bbrc.2011.11.022>.
 85. Volkov A, Khoshnevis S, Neumann P, Herrfurth C, Wohlwend D, Ficner R, Feussner I. 2013. Crystal structure analysis of a fatty acid double-bond hydratase from *Lactobacillus acidophilus*. *Acta Crystallogr D Biol Crystallogr* 69:648–657. <https://doi.org/10.1107/S0907444913000991>.
 86. Deng M-D, Grund AD, Schneider KJ, Langley KM, Wassink SL, Peng SS, Rosson RA. 2007. Linoleic acid isomerase from *Propionibacterium acnes*: purification, characterization, molecular cloning, and heterologous expression. *Appl Biochem Biotechnol* 143:199–211. <https://doi.org/10.1007/s12010-007-8075-1>.
 87. Takeuchi M, Kishino S, Hirata A, Park SB, Kitamura N, Ogawa J. 2015. Characterization of the linoleic acid $\delta 9$ hydratase catalyzing the first step of polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a. *J Biosci Bioeng* 119:636–641. <https://doi.org/10.1016/j.jbiosc.2014.10.022>.
 88. Takeuchi M, Kishino S, Park SB, Kitamura N, Ogawa J. 2015. Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a. *J Mol Catal B Enzym* 117:7–12. <https://doi.org/10.1016/j.molcatb.2015.03.020>.
 89. Ortega-Anaya J, Hernández-Santoyo A. 2016. Production of bioactive conjugated linoleic acid by the multifunctional enolase from *Lactobacillus plantarum*. *Int J Biol Macromol* 91:524–535. <https://doi.org/10.1016/j.jbiomac.2016.05.105>.
 90. Yang B, Chen H, Gu Z, Tian F, Ross R, Stanton C, Chen Y, Chen W, Zhang H. 2014. Synthesis of conjugated linoleic acid by the linoleate isomerase complex in food-derived lactobacilli. *J Appl Microbiol* 117:430–439. <https://doi.org/10.1111/jam.12524>.
 91. Yang B, Qi H, Gu Z, Zhang H, Chen W, Chen H, Chen YQ. 2017. Characterization of the triple-component linoleic acid isomerase in *Lactobacillus plantarum* ZS2058 by genetic manipulation. *J Appl Microbiol* 123:1263–1273. <https://doi.org/10.1111/jam.13570>.
 92. Kishino S, Ogawa J, Omura Y, Matsumura K, Shimizu S. 2002. Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. *J Am Oil Chem Soc* 79:159–163. <https://doi.org/10.1007/s11746-002-0451-4>.
 93. Kishino S, Ogawa J, Ando A, Omura Y, Shimizu S. 2002. Ricinoleic acid and castor oil as substrates for conjugated linoleic acid production by washed cells of *Lactobacillus plantarum*. *Biosci Biotechnol Biochem* 66:2283–2286. <https://doi.org/10.1271/bbb.66.2283>.
 94. Alves SP, Araujo CM, Queiroga RC, Madruga MS, Parente MOM, Medeiros AN, Bessa RJB. 2017. New insights on the metabolism of ricinoleic acid in ruminants. *J Dairy Sci* 100:8018–8032. <https://doi.org/10.3168/jds.2017-13117>.
 95. Baldin M, Rico DE, Green MH, Harvatine KJ. 2018. Technical note: an in vivo method to determine kinetics of unsaturated fatty acid biohydrogenation in the rumen. *J Dairy Sci* 101:4259–4267. <https://doi.org/10.3168/jds.2017-13452>.
 96. Meynadier A, Zened A, Farizon Y, Chemit M-L, Enjalbert F. 2018. Enzymatic study of linoleic and alpha-linolenic acids biohydrogenation by chloramphenicol-treated mixed rumen bacterial species. *Front Microbiol* 9:1452. <https://doi.org/10.3389/fmicb.2018.01452>.
 97. Wallace RJ, McKain N, Shingfield KJ, Devillard E. 2007. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J Lipid Res* 48:2247–2254. <https://doi.org/10.1194/jlr.M700271-JLR200>.
 98. McIntosh FM, Shingfield KJ, Devillard E, Russell WR, Wallace RJ. 2009. Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria. *Microbiology* 155:285–294. <https://doi.org/10.1099/mic.0.022921-0>.
 99. Yang B, Gao H, Stanton C, Ross RP, Zhang H, Chen YQ, Chen H, Chen W. 2017. Bacterial conjugated linoleic acid production and their applications. *Prog Lipid Res* 68:26–36. <https://doi.org/10.1016/j.plipres.2017.09.002>.
 100. Kim KR, Oh HJ, Park CS, Hong SH, Park JY, Oh DK. 2015. Unveiling of novel regio-selective fatty acid double bond hydratases from *Lactobacillus acidophilus* involved in the selective oxyfunctionalization of mono- and di-hydroxy fatty acids. *Biotechnol Bioeng* 112:2206–2213. <https://doi.org/10.1002/bit.25643>.
 101. Black BA, Zannini E, Curtis JM, Gänzle MG. 2013. Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. *Appl Environ Microbiol* 79:1866–1873. <https://doi.org/10.1128/AEM.03784-12>.
 102. Liang N, Cai P, Wu D, Pan Y, Curtis JM, Gänzle MG. 2017. High-speed counter-current chromatography (HSCCC) purification of antifungal hydroxy unsaturated fatty acids from plant-seed oil and *Lactobacillus* cultures. *J Agric Food Chem* 65:11229–11236. <https://doi.org/10.1021/acs.jafc.7b05658>.
 103. Takeuchi M, Kishino S, Park SB, Hirata A, Kitamura N, Saika A, Ogawa J. 2016. Efficient enzymatic production of hydroxy fatty acids by linoleic acid $\Delta 9$ hydratase from *Lactobacillus plantarum* AKU 1009a. *J Appl Microbiol* 120:1282–1288. <https://doi.org/10.1111/jam.13088>.
 104. Gorissen L, Raes K, Weckx S, Dannenberger D, Leroy F, De Vuyst L, De Smet S. 2010. Production of conjugated linoleic acid and conjugated linolenic acid isomers by *Bifidobacterium* species. *Appl Microbiol Biotechnol* 87:2257–2266. <https://doi.org/10.1007/s00253-010-2713-1>.
 105. Yang B, Chen H, Stanton C, Chen YQ, Zhang H, Chen W. 2017. Mining bifidobacteria from the neonatal gastrointestinal tract for conjugated linolenic acid production. *Bioengineered* 8:232–238. <https://doi.org/10.1080/21655979.2016.1222996>.
 106. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. 2003. Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *J Appl Microbiol* 94:138–145. <https://doi.org/10.1046/j.1365-2672.2003.01814.x>.
 107. Hennessy AA, Barrett E, Ross RP, Fitzgerald GF, Devery R, Stanton C. 2012. The production of conjugated α -linolenic, γ -linolenic and stearidonic acids by strains of bifidobacteria and propionibacteria. *Lipids* 47:313–327. <https://doi.org/10.1007/s11745-011-3636-z>.
 108. Kishino S, Ogawa J, Ando A, Yokozeki K, Shimizu S. 2010. Microbial production of conjugated γ -linolenic acid from γ -linolenic acid by *Lactobacillus plantarum* AKU 1009a. *J Appl Microbiol* 108:2012–2018. <https://doi.org/10.1111/j.1365-2672.2009.04609.x>.
 109. Renes E, Linares DM, González L, Fresno JM, Tornadijo ME, Stanton C. 2017. Production of conjugated linoleic acid and gamma-aminobutyric acid by autochthonous lactic acid bacteria and detection of the genes involved. *J Funct Foods* 34:340–346. <https://doi.org/10.1016/j.jff.2017.05.014>.
 110. Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, Berger-Bachi B, Projan S. 2004. Microarray-based analysis of the *Staphylococcus aureus* SigB regulon. *J Bacteriol* 186:4085–4099. <https://doi.org/10.1128/JB.186.13.4085-4099.2004>.
 111. Kepler CR, Hirons KP, McNeill JJ, Tove SB. 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *J Biol Chem* 241:1350–1354.
 112. Rosson RA, Grund AD, Deng MD, Sanchez-Riera F. December 2004. Linoleate isomerase. US patent 6,743,609.