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Production of conjugated linoleic acid by lactic acid bacteria; important factors and optimum conditions

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ABSTRACT

Conjugated linoleic acid (CLA) has recently attracted significant attention as a health-promoting compound. CLA is a group of positional isomers of linoleic acid (LA) with a conjugated double bond naturally occurring in dairy and ruminant meat products. Microbial biosynthesis of CLA is a practical approach for commercial production due to its high safety and purity. There are some factors for the microbial CLA production such as strain type, microbial growth phase, pH, temperature and incubation time, based on which the amount and type of CLA can be controlled. Understanding the interplay of these factors is essential in optimizing the quantity and composition of microbial CLA, as discussed in the current study. Further exploration of CLA and its influences on human health remains a dynamic and evolving area of study.

1. Introduction

Conjugated linoleic acid (CLA) is a general term for a mixture of positional and geometric isomers of linoleic acid (LA) (18: 2), in which two double bonds are conjugated (Wang, Li, Meng, Tong, & Liu, 2022). *Cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA are the most abundant CLA isomers, accounting for approximately 95 % of all isomers (den Hartigh, 2019; Suksatan et al., 2022). Double-conjugated fatty acids (FAs) have long been identified in different amounts in dairy products and other foods derived from ruminants. Conjugated FAs are common in nature, and are usually part of the products of fat metabolism by bacteria that form in the rumen of cattle, sheep, and other ruminants (Zongo et al., 2021). In recent years, consumers have shifted to healthier foods, and it is expected that the trend will increase in the future (Arjeh, Akhavan, Barzegar, & Carbonell-Barrachina, 2020). Vegetable fats and oils are a source of energy in the diet of many people around the world. A lack of fats in the diet causes a feeling of fat hunger. Fats prevent premature hungry after a meal by delaying the digestion of food. Some unsaturated FAs are essential components of the diet, and their complete elimination causes fat-related diseases that appear as flaking of the skin, weight loss, kidney ulcers and eventually death. In humans, infants need essential fatty acids (EFAs), and a diet without fats causes itchy skin and eczema that goes away with the consumption of fats (Olejnik et al., 2023). EFAs are also involved in normal pregnancy and lactation. These compounds act as radiation resistance agents and prevent capillary rupture.

Today, researchers have found that foods, in addition to providing essential nutrients, contain components that may be helpful in maintaining health and preventing chronic diseases (Caballero et al., 2021 (Arjeh, et al., 2022). The components in animal products are each associated with a specific effect on human health, which is why the interest in conjugated FAs has increased significantly. Studies showed that CLA has anti-cancer properties (Dachev et al., 2021). Further research has shown that anti-cancer isolates are composed of conjugated octadecadienoic acid isomers in which double bonds are separated by a methylene (–CH_2–) group, instead of a single carbon-to-carbon bond. These isomers were generally referred to as CLA (Yurawecz, 2003). It has also been shown that CLA can have a variety of health effects such as inhibiting atherosclerosis, increasing immune function, liver metabolism and blood sugar (Chen & Park, 2019). But the most credible findings about the health effects of CLA include its anti-cancer and

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reduction in body fat effects. Some reported data suggest that these effects may be isomer-dependent; the *c*9, *t*11, and *t*10, *c*12 isomers are more important in terms of health (Amiri, Mokarram, Khiabani, Bari, & Khaledabad, 2020; Koba & Yanagita, 2014). The immune system is the central defense against many diseases, especially cancer. The anticancer activity of CLA could be the result of enhanced immune function, which has been associated with promoting *T*-cell (Lymphocyte) proliferation and increased IgA secretion and macrophage function against certain cancers (Kumar, Bhatia, & Arora, 2009; Marín, Meléndez, Aranda, & Ríos, 2018). In this regard, *Trans*-10, *cis*-12-CLA (t10c12-CLA) has been shown to alter immune function through modulating the activity of monocytes and macrophages (Kang, Lee, Jeung, & Yang, 2007).

Dairy products and meat are among the foods that naturally have the highest levels of CLA. The content of CLA in milk and meat is influenced by various factors including animal breed, age, diet and dietary supplements affecting the diet (Dhiman, Nam, & Ure, 2005). The amount of CLA in food can be increased by using various enzymatic, chemical and microbial methods. Microbial CLA production is recognized as an environmentally friendly method (Salamon et al., 2015). The naturally synthesized form of CLA has better properties than its chemically synthesized form, because most of the natural isomers are of *c*9, *t*11 type, which has a high biological activity; while in the chemical synthesis of CLA, four types of isomers are produced (Das, Holland, Crow, Bennett, & Manderson, 2005; Wang, et al., 2022). The recommended dose of CLA is approximately 3 g/day, and through its consumption, many health effects are manifested in humans (Gangidi & Proctor, 2004). Therefore, considering the importance of CLA in the diet and its effects on human health, it is necessary to study the safe production of CLA and the factors affecting the process.

2. Conjugated linoleic acid; an overview

2.1. Chemical structure

CLA is an 18-carbon fatty acid with two double bonds. Fatty acids with two double bonds have four fewer hydrogen atoms than similar saturated fatty acids and their general formula is $C_nH_{2n-4}O_2$, of which LA (*c*9, *c*12 octadecadienoic acid) with the chemical formula ($C_{18}H_{32}O_2$) is the most important fatty acid. As shown in Fig. 1A, CLA is the geometric (*cis* and *trans*) and positional (8 to 13) isomer of LA, in which the double bonds are conjugated (Khanal et al., 2005). More than 12 CLA isomers are found naturally in dairy and ruminant meat products, but *c*9, *t*11 and *t*10, *c*12 are two isomers (Fig. 1B) of high physiological importance (Banni, et al., 2001; Kadamne, Castrodale, & Proctor, 2011; Lee, Paek, Lee, Park, & Lee, 2007).

2.2. CLA production precursors

2.2.1. Vaccenic acid 18: trans-11

Vaccenic acid is a FA with the chemical formula $C_{18}H_{34}O_2$ and the IUPAC name "Octadec-11-enoic acid- (E)" which is one of the precursors of CLA production. Vaccenic acid is found in milk fat and is considered a functional ingredient because it is a precursor to the endogenous synthesis (Fig. 1C) of rumenic acid (c9, t11 CLA) in cattle and humans (Caballero et al., 2021).

2.2.2. Ricinoleic acid 18: 1n 9

Ricinoleic acid is another FA with the chemical formula $C_{19}H_{25}NO_4$ and the systematic name "12-hydroxy *c*9-18: 1", which is found in abundance in castor oil. According to Fig. 1D, there are two possible pathways for the production of CLA from ricinoleic acid by lactic acid bacteria (LAB): (i) direct conversion of ricinoleic acid to CLA by dehydration at position 11; (ii) three-step conversion through LA; through dehydration at position 12 and sequential isomerization of LA.

2.3. Health benefits of CLA

CLA was first discovered as an anti-cancer compound (Yurawecz, 2003), but subsequent studies have shown several health effects for it, including reducing body fat, inhibiting atherosclerosis, increasing the immune system function, improving liver metabolism, hypoglycemia and bone health (den Hartigh, 2019; Koba & Yanagita, 2014; Watkins, Li et al., 2004). Isomers of CLA have several properties, and the isomers of rumenic acid (*c*9, *t*11 CLA) and *t*10, *c*12 CLA are the most biologically active isomers which can lower blood cholesterol, prevent atherosclerosis and carcinogenesis (den Hartigh, 2019). A summary of the health benefits of CLA are listed in Table 1.

As explained, there is a growing interest in incorporating CLA into food products. Despite its increasing demand in the food industry, CLA is very sensitive to light, heat, and oxygen, which can produce unpleasant odors and flavors during processing and storage, limiting its use in the food industry. Therefore, CLA should be provided in protected forms such as oil-in-water emulsions (Li, et al., 2022). In terms of industrial application, the utilization of CLA-producing LAB improves the functional potential of dairy products, e.g., fermented milks. Such innovative dairy products are considered as newer business growth drivers for the food industry (Dahiya & Puniya, 2018). Besides, the structure of CLA creates special conditions for its wider applications in various industries. For example, CLA is highly reactive towards polymerization, which makes it suitable for various industrial uses such as conjugated drying oils that have faster drying rates, better resistance to water, and improved toughness (Chen, Zhang, Zheng, & Zheng, 2017).

Although CLA consumption has health benefits, there are also potential risks associated with CLA consumption. The effects of CLA on weight loss and other health parameters have shown variability across studies, and not all individuals may respond in the same way. For example, high doses of CLA supplements may lead to side effects such as digestive issues, hepatic steatosis and induction of colon carcinogenesis in humans, and potential liver problems (Asbaghi, Shimi, Shiraseb, Karbasi, Nadery, Ashtary-Larky, et al., 2022; Jaudszus, Moeckel, Hamelmann, & Jahreis, 2010; Putera et al., 2023). Therefore, long-term safety of CLA has not been extensively studied. Before using CLA supplements, it is important to consulted with a healthcare professional or registered dietitian to determine the appropriate dosage and assess potential risks and benefits tailored to specific health goals and needs.

2.4. CLA natural resources

CLA isomers are found naturally in a variety of foods. Meat products, tissue fat, and dairy products have higher levels of CLA due to the production of this FA during bio-hydrogenation in ruminants (Jaglan, Kumar, Choudhury, Tyagi, & Tyagi, 2019; Paszczyk & Czarnowska-Kujawska, 2022). The CLA content in beef and dairy products varies from 3 to 10 mg/g fat (Dhiman, Nam, & Ure, 2005). The extent of CLA in human milk (1.7–36.4 mg/g fat), sheep (10.8–29.7 mg/g fat), goat (6.1–10.35 mg/g fat), buffalo (4.4–7.0 mg/g fat) and cattle (0.7–10.1 mg/g fat) are different from each other (Uniacke-Lowe & Fox, 2012). The content of CLA in different foods is presented in Fig. 2.

The optimal dosage of CLA for health benefits is not definitively established, and it can vary depending on individual factors. In general, CLA is considered to be a safe and effective supplement for most people when used at the directed dose. Studies have used doses ranging from 1 to 10 g/day (Asbaghi et al., 2022; Putera et al., 2023). Dietary sources of CLA, such as meat and dairy products, provide only low amounts of CLA, making it challenging to obtain therapeutic doses from food. Hence, supplementation is often considered to achieve higher CLA consumption.

3. CLA production methods

CLA production methods can be divided into three groups: chemical,

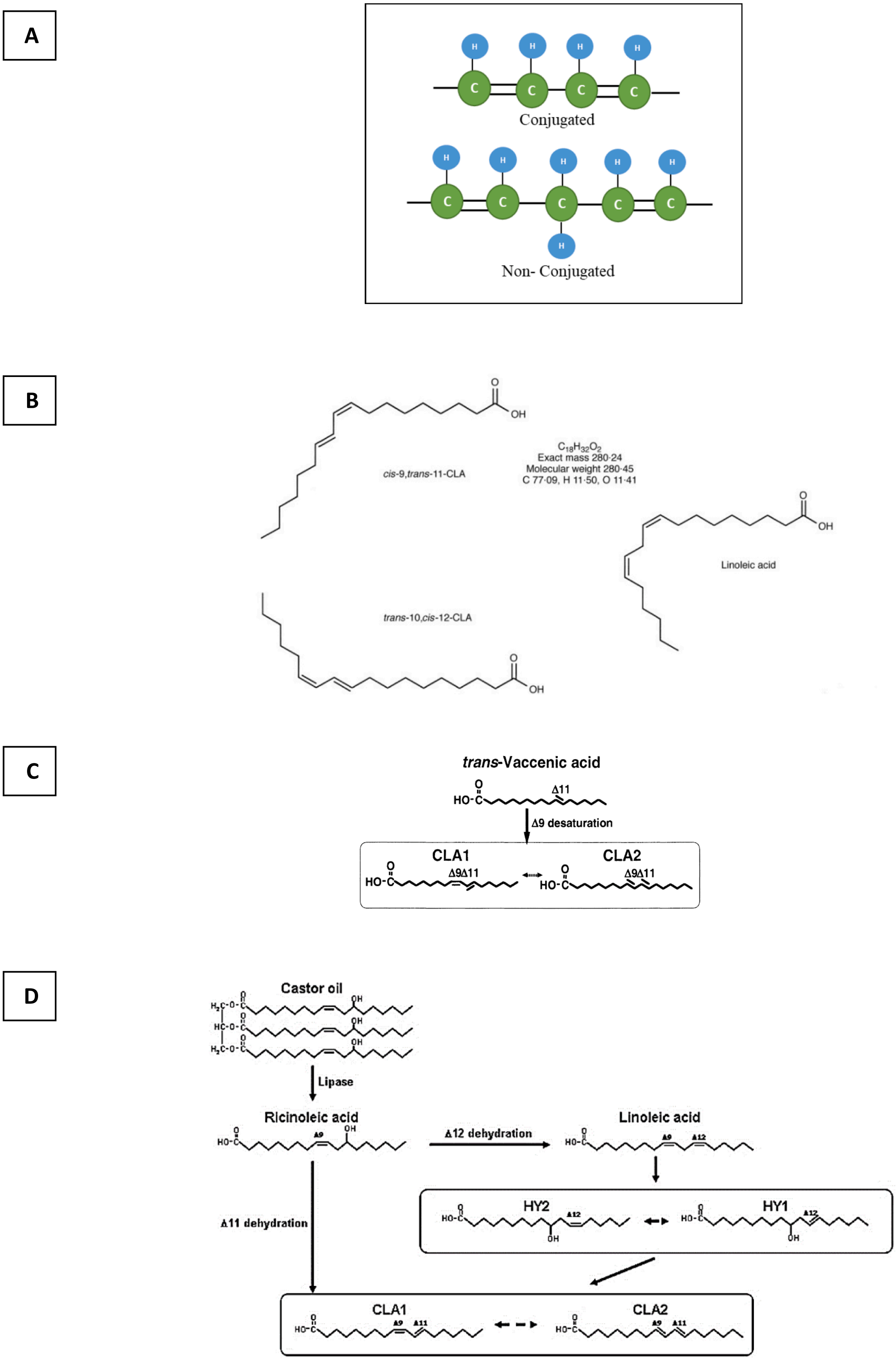

Fig 1. (A) General structure of conjugated and unconjugated bonds; (B) Linoleic acid and its important conjugated isomers; reproduced with permission from (Roche, Noone, & Gibney, 2001); (C) Isomerization of vaccenic acid to CLA isomers (Ogawa, Kishino, Ando, Sugimoto, Mihara, & Shimizu, 2005); (D) Conversion of ricinoleic acid in castor oil triglycerides to CLA from two different pathways (Kishino, Ogawa, Yokozeki, & Shimizu, 2009).

Table 1
Some health effects reported for CLA.

Health effect	Description	Reference
Anti-cancer properties	*c*9, *t*11 isomer is an effective isomer in creating this effect. A diet containing 0.1, 1 and 5.5 % of the mixture of CLA isomers can reduce the number of tumors by 32, 56 and 60 %, respectively.	(Caballero, Ríos-Reina, & Amigo, 2021; Gnadig, Xue, Berdeaux, Chardigny, & Sebedio, 2003; Huang, Luedecke, & Shultz, 1994; Knekt, Järvinen, Seppänen, Pukkala, & Aromaa, 1996; Zeng, et al., 2020)
Reducing body fat	CLA, while increasing body mass (protein), reduces fat. The action mechanism is probably to increase fat lipolysis, and to reduce the accumulation of FAs in adipose tissue.	(DeLany, Blohm, Truett, Scimeca, & West, 1999; Ibrahim & El-Sayed, 2021; Koba & Yanagita, 2014; Lehnen, da Silva, Camacho, Marcadenti, & Lehnen, 2015; Park & Pariza, 2007)
Improving immune system function	Consumption of diets containing CLA can increase the body's immune factors (antibodies, white blood cells and the enzyme lysozyme). The *c*9, *t*11 and *t*10, *c*12 CLA isomers have different effects on immune function and the population of immunoglobulin subtypes.	(Aydin, 2005; O'Shea, Bassaganya-Riera, & Mohede, 2004; Viladomiu, Hontecillas, & Bassaganya-Riera, 2016; Zhang, Guo, & Yuan, 2005)
Inhibition of atherosclerosis	Increasing CLA in the diet could inhibit atherosclerosis and, in addition, severely regenerate clogged arteries.	(Kritchevsky, et al., 2004; S Toomey, Roche, Fitzgerald, & Belton, 2003; Toomey, Harhen, Roche, Fitzgerald, & Belton, 2006)
Bone health	The effective isomers are *c*9, *t*11 and *t*10, *c*12. CLA, along with dietary calcium, can prevent osteoporosis by affecting the hormonal and enzymatic systems associated with bone.	(Dilzer & Park, 2012; Park, et al., 2013; Watkins, Li, Lippman, Reinwald, & Seifert, 2004)
Reduction of inflammation	CLA can reduce inflammation by affecting cells involved in the inflammatory process (such as T cells, macrophages, and monocytes).	(Ávila, et al., 2020; Bassaganya-Riera & Hontecillas, 2010; Butz, Li, Huebner, & Cook, 2007; Gebauer, Destaillats, Dionisi, Krauss, & Baer, 2015)
Decrease of LDL cholesterol and increase of blood HDL	Many studies have confirmed the effect of CLA on lowering LDL and increasing HDL, but some studies have reported the opposite, so the effect of CLA on blood cholesterol still needs to be studied.	(Brouwer, Wanders, & Katan, 2010; Derakhshande-Rishehri, Mansourian, Kelishadi, & Heidari-Beni, 2015; Gebauer, et al., 2015; LeDoux, et al., 2007)
Increasing energy	CLA probably increases the energy available to the body by increasing oxidation in fats.	(Choi, Jung, Park, & Song, 2004)
Reducing blood platelet clotting	Although lab studies confirm the effect of CLA on reducing platelet clotting, human studies suggest that short-term use of CLA in humans is unlikely to produce this effect.	(Benito, et al., 2001; Kung & Yang, 2006; Uniacke-Lowe & Fox, 2012)

microbial and enzymatic methods, as described in the following sections.

3.1. Chemical production of CLA

In general, chemical methods of CLA production can be divided into three classes, including ricinoleic acid dehydration (Berdeaux et al., 1997), alkaline isomerization (Reaney et al., 1999) and process catalysis with heterogeneous metals (Gnadig et al., 2003). Chemical methods require temperatures > 100 °C, use more chemicals, and have the potential to form by-products, such as cyclic, oxidized, and polymerized compounds. In addition, due to the lower selectivity of chemical catalysts, the product obtained is usually a mixture of CLA isomers with a purity < 40 % for each of the isomers (Saebo, 2003).

3.2. Enzymatic production of CLA

Linoleate isomerase is an enzyme that converts LA into CLA. By extracting and purifying the enzyme and using it in the production of CLA, more selectivity in the reaction and production of desirable isomers can be achieved. However, since some types of these enzymes are stabilized on the membrane and resistant to solubilization, they are very difficult to separate and purify. For this reason, many attempts in this field have not been very successful (Irmak et al., 2006).

3.3. Microbial production of CLA

Since the discovery of *B. fibrisolvens* in the rumen of ruminants as a CLA-producing bacterium, there have been many reports of CLA production by various bacteria. In addition to rumen bacteria, some other bacteria, such as *Propionibacterium*, *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Enterococcus*, *Closteridium*, *Pediococcus* and *Megasphaera* are also capable of producing CLA (Adamczak et al., 2008). In this method, bacteria are used as biocatalysts to convert LA (or ricinoleic acid) into CLA. After production, pure CLA or a mixture of FAs containing CLA is obtained. CLA can be used as a capsule or dietary supplement or in the production of activator feed (Ogawa et al., 2005). Fig. 3 summarizes the production of CLA in cow rumen and cow's milk fermented products. It should be noted that lipolytic and proteolytic reactions are favorable for the formation of CLA. Lipolysis produces free fatty acids, as the precursors of CLA, which are formed via the microbiological hydrogenation pathway. Proteolysis breaks down proteins into low molecular weight compounds that act as hydrogen donors (Lin, Boylston, Luedecke, & Shultz, 1999). In addition, proteolysis is a pre-requisite for the growth of LAB and the subsequent degradation of proteins, leading to the release of peptides and free amino acids (Olivo, et al., 2021). In this regard, Moslemi, Moayedi, Khomeiri, and Maghsoudlou (2023) observed a positive correlation between the amount of proteolysis and CLA synthesis.

The CLA content in milk and meat is affected by several factors, such as animal's breed, age, diet, and animal keeping conditions (Dhiman, Nam, & Ure, 2005). Ruminant diet supplementation with sunflower oil, fish oil and soybean oil (with a high percentage of C18:2, C18:3) has been reported as a good strategy for enhancing CLA in dairy cows (Dohme-Meier & Bee, 2012; Lounglawan & Suksombat, 2011). In the animal rumen, ester linkages are first catalyzed by microbial lipase and then bio-hydrogenation of unsaturated FAs by linoleate isomerase occurs. Linoleate isomerase is an enzyme involved in the formation of conjugated double bonds of LA as well as α and γ-linolenic acids (Weiss, Martz, & Lorenzen, 2004). During fermentation in dairy products, CLA precursor FAs (such as vaccenic, LA and ricinoleic acid) or oils containing a high level of these FAs turn into CLA. The microbial mechanism of CLA production is unclear, but a number of theories have been proposed. It was illustrated that the presence of high levels of free LA inhibits bacterial growth because the production of CLA is considered as a detoxification mechanism for the bacterial cell (Yang et al., 2017).

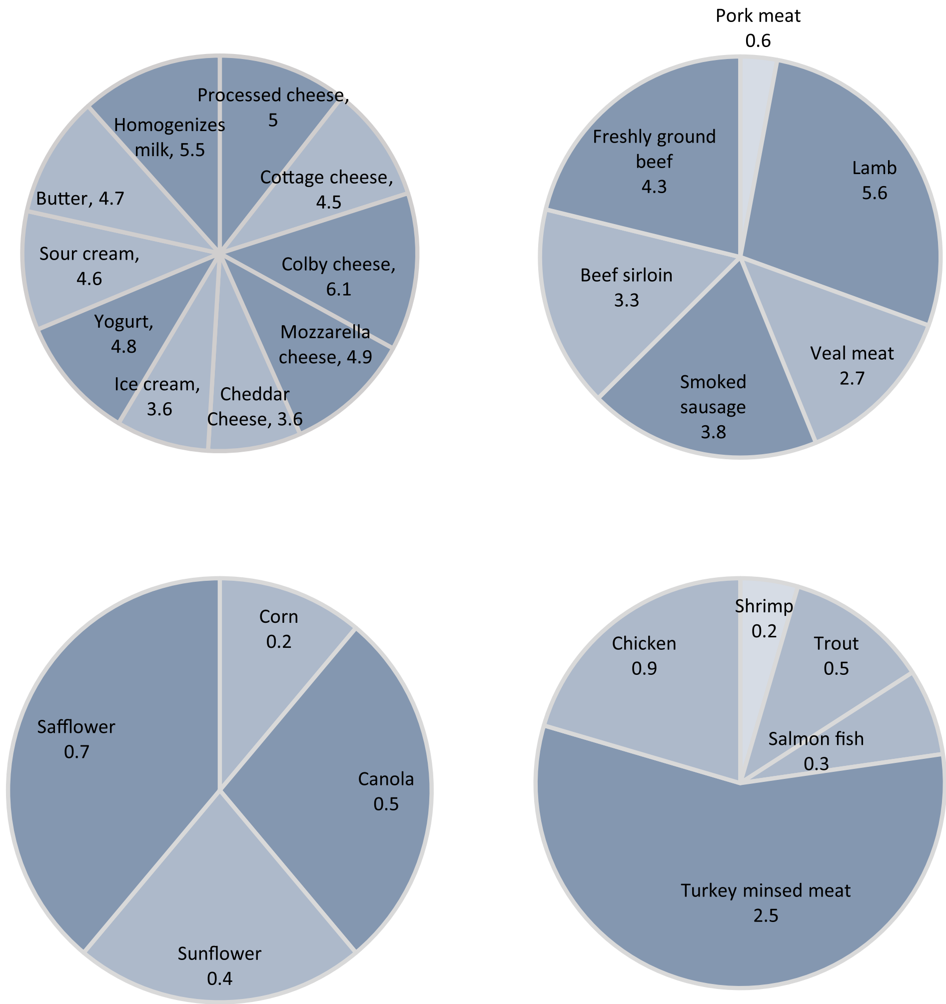

Fig 2. The extent of CLA reported in different foods; adapted from (Chinnadurai & Tyagi, 2011; Guo, 2013; Kumar, Sharma, Bhadwal, Sharma, & Agnihotri, 2018; Shaikh, et al., 2018).

In general, bio-isomerization of LA into CLA is carried out in three ways: (i) using CLA-producing bacteria with the aim of producing functional fermented foods; (ii) production of CLA by microbes using pure substrate such as LA or ricinoleic acid; (iii) extraction of enzymes for the conversion of LA to CLA from bacteria with potential CLA production, such as *Lactobacillus, Lactococcus, Propionibacterium, Bifidobacterium, Megasphaera elsdenii* and *Butyrivibrio fibrisolvence*.

In terms of efficiency, different CLA production methods are comparable as follows (Boyaval, Corre, Dupuis, & Roussel, 1995; Farmani, et al., 2010; Kabara, Swieczkowski, Conley, & Truant, 1972):

- Cost-effectiveness: chemical > microbial > enzymatic method
- Product purity: chemical < microbial < enzymatic method

Table 2 presents some advantages and disadvantages of CLA production methods.

As it turns out, each of the chemical, microbial, and enzymatic methods has several advantages and disadvantages. However, the use of food as a functional product to deliver CLA into the body is still preferred because food is one of the most essential human needs. On the other hand, the consumption of micronutrients in the form of drugs is less popular. Therefore, the best way to deliver micronutrients is to enrich/fortify food products. In this regard, various formulated products such as iron-enriched flour or vitamin D-containing oils have been successfully produced in the past. Therefore, the use of microbial and fermentation methods to produce functional food products can be a good way to deliver CLA to individuals in the form of a daily diet (Blasi & Cossignani, 2019).

Today, various strategies have been proposed to increase the efficiency of microbial production of CLA and eliminate its disadvantages. LA, as one of the main precursors of CLA production, causes toxicity in bacteria (Kim, 2003). This compound stimulates the production of CLA in bacteria. The conversion of LA into a less toxic compound is the most important strategy of bacteria to reduce its toxicity; hence, by converting it to CLA, bacteria reduce its toxic effects. One way to reduce the toxicity of LA is to use washed or resting (immobile) cells; bacterial-washed cells are alive but do not grow. Bacterial cells at the end of the logarithmic phase or at the beginning of the stationary phase are considered washed cells; therefore, in this phase, they are less sensitive to the toxicity of LA (Ogawaet al., 2005). The precursors of CLA production, such as oils and FAs (LA and ricinoleic acid), must be dissolved in the medium to be consumed by bacteria. The use of supplementary compounds, such as bovine serum albumin (BSA), Tween 80, starch and lipid compounds including cholesterol, lecithin and surfactants of FAs, can act as dispersing agents, which concurrently reduce the antimicrobial effects of FAs (Kabara et al., 1972).

3.3.1. *CLA-producing bacteria*

CLA production by bacterial strains is influenced by various factors. Studies have shown that most strains with the potential to produce CLA are from LAB (lactic acid bacteria) (Wu & Li, 2018). The term of LAB is used for bacteria that cause fermentation and coagulation of milk, and

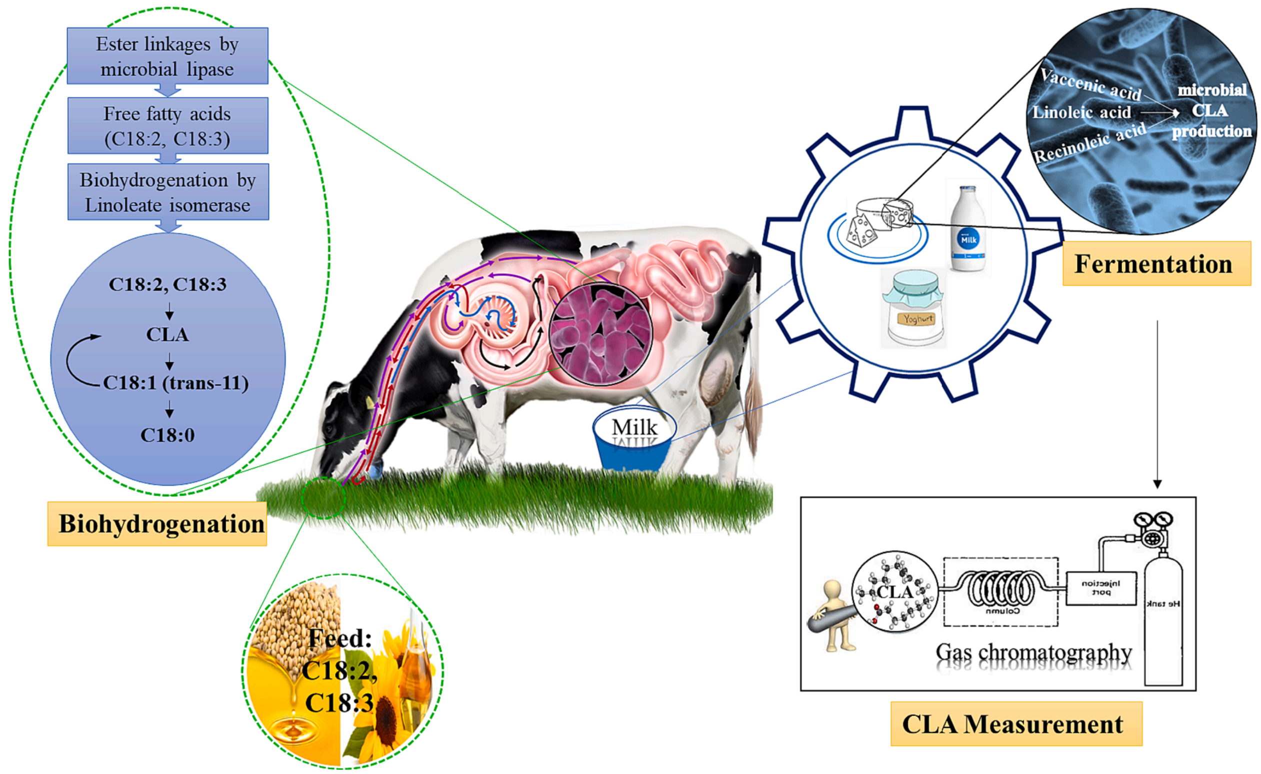

Fig 3. Production of CLA in cow rumen and cow's milk fermented products.

Table 2
Advantages and disadvantages of CLA production methods.

Methods	Advantages	Disadvantages
Chemical	Better control Larger scale More profit margins	Low selectivity Less isomeric purity producing more by-products Consuming more chemic
Microbial	Production of biologically active isomers No need for a cofactor Production of functional foods using microbial fermentation	Bacterial sensitivity to CLA precursors Low solubility of CLA precursors Lower efficiency Production of by-products Cost of production
Enzymatic	More purity Less by-product	More cost Low enzyme stability Requires cofactor

produce lactic acid from lactose. LAB are one of the most important microbial groups used in food fermentation; they help to create the texture of fermented products (Blasi & Cossignani, 2019). Interestingly, probiotic bacteria have been used for centuries in the form of dairy foods containing LAB (Song et al., 2021). Among LAB, *Lactobacilli* are more commonly known CLA-producing strains (Song, Kim, Kim, & Baik, 2021; Tapia, et al., 2019; Tyagi, Kumar, Choudhury, Tyagi, & Tyagi, 2020). In the meantime, many studies have been performed on *L. plantarum* strains; it was revealed that using *L. plantarum* cells as a catalyst, the production of CLA from LA in optimal conditions reached to 40 mg/L. This was the highest amount of CLA produced among *Lactobacillus* bacteria (Ogawa, et al., 2005; Tapia, et al., 2019).

Table 3 reports the amount of CLA production in LAB, ranging from 1 up to 40,000 mg/L. In addition to *lactobacilli*, other LABs can also produce CLA. Coakley, Ross, Nordgren, Fitzgerald, Devery, and Stanton (2003) examined the different strains of *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Bifidobacterium* in CLA-free LA medium and found that *B. breve* and *B. dentium* were the most efficient CLA producers among the strains used, and 65 % of LA was converted to *c*9, *t*11 CLA (Coakleyet al.,

Table 3
The extent of CLA production in lactic acid bacteria.

Lactic acid bacteria	CLA (mg/L)	Reference
Bifidobacterium		
B. breve	99–600	(Chung, et al., 2008; Coakley, et al., 2003; Lee, Hong, & Oh, 2003; Ogawa, et al., 2005; Song, et al., 2005; Van Nieuwenhove, Oliszewski, González, & Chaia, 2007)
B. adolescentis	3–4	
B. bifidum	1–207	
B. angulatum	1	(Coakley, et al., 2003)
B. infantis	4–25	
B. lactis	170	
B. pseudolongum	214	(Gorissen, et al., 2010)
B. animalis	7–48	(Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011)
B. pseudocatenulatum	23–135	(Coakley, et al., 2003)
Lactobacillus		
L. plantarum	7–40000	(Ando, Ogawa, Kishino, & Shimizu, 2004; Hou, et al., 2011; Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002; Lee, et al., 2007)
L. casei	100–175	(Van Nieuwenhove, et al., 2007)
L. acidophilus	4–4900	(Alonso, Cuesta, & Gilliland, 2003; Li, et al., 2011; Ogawa, Matsumura, Kishino, Omura, & Shimizu, 2001; Rodríguez-Alcalá, et al., 2011)
L. delbrueckii	78	(Lin, 2000)
L. rhamnosus	1–190	(Van Nieuwenhove, et al., 2007; Xu, Boylston, & Glatz, 2004)
L. reuteri	119–300	(Lee, et al., 2003; Roman-Nunez, Cuesta-Alonso, & Gilliland, 2007)
L. casei	11–80	(Alonso, et al., 2003)
L. curvatus	8	(Gorissen, et al., 2011)

2003). Alonso et al. (2003) studied two bacterial strains of *Lactobacillus acidophilus* and two bacterial strains of *Lactobacillus casei* for CLA production in culture medium containing free LA. The results showed that all strains were able to produce CLA, and the highest amount of CLA was between 130 and 180 μg/mL after 24 h in medium containing 0.02 % LA. In addition, an increase in CLA in skim milk was also observed. Therefore, they found that the use of CLA-producing LAB in fermented dairy products may have potential health benefits. Therefore, if CLA-producing bacteria are included in starter cultures, the produced CLA could be readily available for absorption through the gastrointestinal tract (Alonso et al., 2003).

3.3.2. Other CLA-producing bacteria

In addition to LAB, some butyric acid and propionic acid bacteria are also known as CLA-producing strains. *Propionibacterium freundenreichii*, *P. shermanii*, *P. thoenii* and *Butyrivibrio fibrisolvens* A38 are bacteria whose ability to produce CLA has been proven. (Jiang, Björck, & Fonden, 1998; Kim, 2003; Rainio, Vahvaselkä, Suomalainen, & Laakso, 2002). As mentioned earlier, CLA production varies from species to species and is not limited to a specific class of microbes; some strains of *Lactobacillus*, *Propionibacterium*, *Bifidobacterium* and *Enterococcus* were studied for CLA production by Deng et al. (2007); they found that *propionibacteria* had the highest CLA production among these species (Deng et al., 2007). *Butyrivibrio fibrisolvens* A38 is one of the most active rumen bacteria in the production of CLA. It has been shown that LA has an inhibitory effect not only on bacterial growth, but also on hydrogenation of LA, and this effect is more pronounced at higher concentrations of LA. CLA levels increase significantly when LA is added to the medium with a inhibitory agent such as glycolytic and iodoacetate (Kim, 2003; Mei, et al., 2021).

4. Factors affecting CLA production by bacteria

There are numerus factors that can affect CLA production. The pH of the culture medium, the type of bacterial strain, the type and concentration of the substrate, the temperature and time of fermentation, and the physical conditions of the culture medium all can affect the extent and type of isomers produced (Dahiya & Puniya, 2018; Özer & Kılıç, 2021). In the following, we will discuss some of these important factors.

4.1. Microbial strains

Microbial strains do not have the same potential for substrate conversion and CLA production (Gao, et al., 2021; Palachum, Choorit, & Chisti, 2018; Srivastava, et al., 2021; Zahed, Khosravi-Darani, Mortazavian, & Mohammadi, 2021). Kishinoet al., (2009) compared over 250 strains of LAB from 14 genera in terms of CLA production. They found that strains of the *Enterococcus*, *Pediococcus*, *Propionibacterium* and *Lactobacillus* produce significant amounts of CLA from LA. All strains produced two specific isomers called *c*9, *t*11-18: 2 (CLA1) and *t*9, *t*11-18: 2 (CLA2). Among the evaluated bacterial strains, *L. plantarum* strain AKU 1009a displayed the highest potential for CLA production (Kishinoet al., 2009). Ando et al., (2003) also reviewed 250 strains of LAB, belonging to the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Propionibacterium*, *Bifidobacterium*, *Aquaspirillum*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Butyrivibrio*, *Lactococcus* and *weissella*, for CLA production. They found that CLA producing strains belonged to *Lactobacillus*, *Propionibacterium*, *Streptococcus*, *Leuconostoc* and *Pediococcus*, and *L. plantarum* had the highest CLA production among all strains. Overall CLA production, 21 % was CLA1 and 79 % was CLA2 (Ando et al., 2003).

4.2. Bacterial growth phase

It has been discovered that CLA production depends on the bacterial growth phases; in some of them, CLA is produced in the logarithmic phase and in others in the stationary phase. The production potential of CLA by *L. lactis* in culture medium and milk containing sunflower oil was studied. The results showed that at all concentrations of sunflower oil, when the oil was added at the beginning of incubation (the cells are in the growth stage), bacteria produced more CLA than when the cells were in a stationary phase (Kim & Liu, 2002). In another study, production of CLA in probiotic yogurt and the optimization of the production process by mixing the starter and sunflower oil as a source of LA have been investigated. Temperature of 37 °C, sunflower oil at 0.5 mL/L milk and pH = 5 were estimated as the optimal conditions for CLA production in yogurt. Increasing the temperature and oil content had a decreasing effect on the production of CLA, while adding oil at the end of the growth phase increased the production rate. Under these conditions, 10.07 mg CLA/g milk fat was produced, which indicates a 161 % increase compared to regular yogurt (Khorasani, 2005).

4.3. Substrate

Studies have shown that the type of carbon source used for bacterial growth affects the extent and type of CLA produced. For example, when different sources of carbon (e.g., glucose, lactose or a combination of glucose-lactose) were used for CLA production by *L. plantarum* in biofilm reactors, the extent of biomass and CLA was changed. The combination of lactose-glucose in the ratio of 1:1 resulted in the highest amount of biomass (3.66 g/L) and the highest concentration of CLA (37.08 μg/mL) (Razmjooei, et al., 2020).

4.4. Triglyceride esters

LA is one of the most important substrates for the production of CLA in bacterial cultures. The form of LA in the substrate can be free or esterified in the triglyceride structure. According to studies, LAB use only the free form of LA to produce CLA and cannot use LA in the ester structure or triacylglycerol form (Kishino et al., 2009). In addition, ricinoleic acid is another substrate used by LABs to produce CLA. It has been revealed that bacteria use its free form to produce CLA, and they cannot use the esterified form and triacylglycerol of ricinoleic acid (Kishino et al., 2009). But, some studies have shown that the free or esterification forms of LA did not influence the CLA production. In a study, three strains of *Propionibacterium freundenreichii* ssp. *shermanii*, which converts free LA to CLA *in vitro*, were used as auxiliary strains along with *Geotrichum candidum* and *Yarrowia lipolytica* to make cheese. It was observed that total CLA levels (esterified and free) were similar in control and experimental cheeses and remained unchanged during the four months of ripening. The addition of LA-rich safflower oil increased the concentration of free LA produced in the cheese, but the CLA content did not change (Das et al., 2005).

4.5. Type and quantity of oil

Vegetable oils could be a more suitable substrate for CLA production depending on the content of CLA precursor FAs (Turek & Wszołek, 2021). Accordingly, castor, sunflower, safflower, and sesame oils are the most common vegetable oils used as a microbial substrate for CLA production. Ogawa et al., (2005) used castor oil as a substrate to produce CLA by LAB. Castor oil is rich in triacylglycerol ricinoleic acid, which was used as a substrate for CLA production using lipase enzyme and oil hydrolysis (to release ricinoleic acid as a substrate for CLA production). This study showed that *L. plantarum* AKU 1009a had the highest CLA production among the studied strains. *L. plantarum* AKU 1009a firstly produced α- and γ-linolenic acids from conjugated trienoic FAs. Trienoic FAs produced from α-linolenic acid include *c*9, *t*11, *c*15-octadecatrienoic acid (18:3) and *t*9, *t*11, *c*15-18: 3 and those from γ-linolenic include *c*6, *c*9, *t*11-18:3 and *c*6, *t*9, *t*11-18:3. Conjugated trienoic FAs produced from α- and γ-linolenic acids were converted to *t*10, *c*15-18:2 and *c*6, *t*10-18:2 by *L. plantarum* AKU 1009a, respectively (Ogawa et al., 2005).

Van Nieuwenhove et al., (2007) investigated the effect of adding sunflower oil on CLA production in buffalo cheese. The results showed that the level of CLA in cheese was higher than raw milk, especially after ripening period. In addition, they found that sunflower oil increased the CLA content of fresh cheese. CLA can also be formed under optimal conditions in milk containing sunflower oil during fermentation by LAB. In another study, Kim and Liu (2002) evaluated the factors and methods that increase the amount of CLA in fermented milk. They studied 14 LAB strains to produce CLA using sunflower oil (containing 70 % LA) as a substrate. Based on the obtained data, it was found that among the screened strains, *Lactococcus lactis* I-01 had the highest ability to produce CLA. The optimum concentration of sunflower oil for CLA production was estimated as 0.1 g/L milk, which was 0.25 % of total milk fat. The results showed that the formation of CLA in fermented milk can be affected by many factors, such as bacterial strain, cell number, optimal substrate concentration and incubation time at neutral pH (Kim & Liu, 2002).

4.6. Lipase enzyme

Researchers have found that oil, either intact or lipolyzed, could have a significant effect on CLA content. Oil lipolysis could be due to endogenous oil lipase or external lipase added to the product. Studies performed by Vahvaselkä and Laakso (2010) showed that by introducing oat meal (water activity = 0.7), lipolytic activity started after three weeks and LA was released; then, in the lipolyzed form of oats, *Propionibacterium freundenreichii* was cultured and the CLA content was studied. It was revealed that the content of CLA produced by bacteria in the fermented substrate was significantly higher than the non-fermented substrate. Therefore, fermentation is mediated by the activity of endogenous enzymes and the production of LA, which is a precursor for CLA production; it can be another effective factor in CLA production.

Hosseini et al., (2015) used sunflower and castor oils as cost-effective substrates (compared to LA) for CLA production. In this study, bacterial lipase was used to produce free FAs from sunflower and castor oils. In addition, they examined the effect of some significant parameters on CLA production, such as substrate type and concentration, incubation time, and the effect of probiotic lipase on sunflower and castor oils. Among the 5 probiotic strains studied, *L. plantarum* (ATCC 8014) cells produced the highest concentration of CLA isomers. Their results also showed that the produced CLA was a mixture of two bioactive isomers, including *c*9, *t*11-CLA (0.38 mg/mL) and *t*10, *c*12-CLA (0.42 mg/mL), which were obtained from 8 mg/mL of sunflower oil in the presence of bacterial lipase. Therefore, they discovered the important capability of *L. plantarum* (ATCC 8014) to produce active CLA isomers from cost-effective substrates for the production of probiotic supplements such as CLA and other bioactive compounds (Hosseini et al., 2015).

4.7. pH

The pH of medium plays an important role in the fermentation process, as it can alter the surface charge of microorganisms, thereby affecting their growth and the internal metabolic pathways responsible for nutrient uptake. It is now well understood that *lactobacilli* convert LA to CLA through the activity of LA isomerase, and this enzyme is sensitive to pH. Studies have shown that at pH = 5.0, CLA isomers gradually increased for 48 h after incubation and then decreased. The highest quantity of CLA isomers produced at this pH included the *c*9, *t*11: 16.0; *t*10, *c*12: 4.36 g/mL and *t*9, *t*11: 5.51 g/mL. Similarly, at pH = 6 and 7, an increasing trend in CLA biosynthesis was observed for the first 48 h; the isomers produced at pH = 6 and 7 were included, (*c*9, *t*11: 31.97 and *t*10, *c*12: 5.94 and t9, *t*11, 9.99 g/mL) and (*c*9, *t*11: 24.21 and *t*10, *c*12: 4.04 and *t*9, *t*11: 6.31 g/mL), respectively (Dahiya & Puniya, 2018). Dahiya and Puniya (2018) showed that the highest quantity of CLA was produced at pH = 6 (compared to pH = 5 and 7). This may be due to the slower growth of strains at this pH, which in turn affects linoleate isomerase.

Researchers have shown that the maximum CLA biosynthesis from LAB will be attained in the optimal pH range, which is mainly related to better enzyme performance. For instance, Ando et al., (2003) found that the maximum CLA synthesis from *L. plantarum* JCM 1551 cells was at pH = 6.5. In another study, Li et al. (2013) demonstrated that efficient production of CLA with *L. acidophilus* F0221 from LA substrate was possible when the initial pH of the medium was in the range of 6.0–7.0. However, data showed that *lactobacilli* have the ability to produce CLA at a wide pH range; lower (pH = 5.0) and higher (pH = 7.0). These results indicate that *lactobacilli* have the potential to produce CLA in different conditions and this varies from one strain to another (Dahiya & Puniya, 2018).

4.8. Incubation time

Some works have revealed that CLA production could increase over time, but gradually decreases over long periods of time. As an example, Dahiya and Puniya (2018) observed that CLA levels increased up to 48 h after incubation, but after this time its production decreased. Incubation for > 48 h reduces the density of living cells, which could be due to excessive accumulation of *c*9, *t*11-CLA, since it inhibits bacterial activity. In another study, at higher incubation time from 24 to 72 h, a gradual increase in *c*9, *t*11-CLA biosynthesis was observed, but in any case, long-term fermentation seems to reduce the production of *c*9, *t*11-CLA (Wang et al., 2022). The ability of *L. plantarum* Ip15 to synthesize CLA in MRS liquid culture medium was evaluated by adding 0.2 mg/mL LA; the results showed that CLA increased to 48.7 μg/mL within 48 h and then, it dropped quickly. In addition, data showed that with increasing incubation time up to 48 h, CLA production increased and then, a decreasing trend. They found that in the medium inoculated with *L. acidophilus* and *Streptococcus thermophilus*, the maximum synthesis of CLA was 120.37 μg/mL. Therefore, according to the different stages of bacterial growth, LA concentration and isomeric activity of linoleate, the highest amount of CLA can be obtained at a specific time (Wang et al., 2022).

5. Conclusions and perspectives

In recent years, CLA has attracted a lot of attention due to its health-promoting properties, such as anti-cancer, anti-inflammatory, anti-obesity, immune-boosting function, and anti-diabetic activity. The present study was a critical review of different CLA production strategies in which microorganisms with high potential in CLA production, factors affecting microbial production and CLA-rich sources were compared and studied. Due to many health-promoting effects of CLA for humans, it is recommended to use it in the diet. However, the testing of CLA in clinical trials is still limited, and further research is needed in the future. It is also necessary to provide reliable information considering the functional activities of CLA in dairy products in future research so that the consumer can choose the product with more knowledge and confidence. In terms of production costs, chemical methods are likely to be economical than fermentation methods. However, the quality and purity of microbial CLA are far higher than the product of chemical processes. Therefore, future studies should focus more on optimizing the microbial production process and screening species with a high production capacity and selectivity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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