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ScienceDirect
Food Chemistry 103 (2007) 313–318
Food Chemistry
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# Production of conjugated linoleic acid by *Propionibacterium freudenreichii*

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Received 5 April 2006; received in revised form 14 June 2006; accepted 25 July 2006

**Abstract**

Two strains each of *Propionibacterium freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* were tested for their ability to produce conjugated linoleic acid (CLA) in sodium lactate medium (SLM), De Man-Rogosa-Sharpe (MRS) medium and skim-milk. Data showed that both strains were able to produce CLA in three media supplemented with different concentrations of sunflower oil. Maximum production of CLA (78.8 μg/ml) was observed after 36 h of incubation in MRS containing 12 mg/ml of sunflower oil by *P. freudenreichii* ssp. *shermanii*. Moreover, the growths of both strains were inhibited by sunflower oil and a positive relationship between CLA production and ability to tolerate sunflower oil was observed. At the same time, it was also observed that the inhibitory effects on *P. freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* in three media follow the order SLM > skim-milk > MRS and SLM > MRS > skim-milk, respectively. Micro aerobic conditions were in favour of increasing the amounts of CLA. The amounts of CLA increased from 0 to 36 h under micro aerobic conditions and no significant ($p > 0.05$) increases in total CLA levels were observed after 80 h of incubation. Results showed that *P. freudenreichii* may have potential for producing CLA.


*Keywords:* Conjugated linoleic acid; *P. freudenreichii*; SLM; MRS; Skim-milk

## 1. Introduction

Conjugated linoleic acid (CLA) is a collective term used to describe the mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The double bonds can be in several positions from C7 to C13, as either *cis* or *trans* isomers, and there is a wide variety of isomers. Among the isomers, the c9 t11-CLA is the predominant one in natural lipids and it constitutes 90% of the total isomers. Food products originating from ruminants, especially beef and dairy products are the major dietary sources of CLA. Studies show that it is formed as an intermediate during the bio-hydrogenation of linoleic acid by ruminant bacteria and c9 t11-CLA is also called rumenic acid (Gangidi & Proctor, 2004). CLA may also be formed from the endogenous conversion of tranvaccenic acid (t-11 $C_{18:1}$) by $\Delta^9$-desaturase in mammary gland (Kim & Liu, 2000). In addition, many animal studies have proved that CLA has extensive health benefits in anticarcinogenic activity, diabetes, obesity and immune stimulation (Alonso, Cuesta, & Gilliland, 2003; Donna, 2000; Lin, Boylston, & Luedecke, 1999; Lin, Lin, & Lee, 1999). c9 t11-CLA and t10 c12-CLA are the most important bioactive isomers because they can be incorporated into the phospholipid fraction of tissues (Michihiro, Akira, & Masao, 1997).

It is estimated, from animal studies, that a daily intake of 3 g/d of CLA may be effective for cancer prevention. Dairy products (0.55–9.12 mg/g fat) are the principal dietary source of CLA, so the current human intake is less than the daily intake recommended for optimal beneficial effects (Lin, Hung, & Cheng, 2005). The level of CLA in dairy products may vary due to different processing

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doi:10.1016/j.foodchem.2006.07.065

parameters, such as the heat treatment during pasteurization and starter cultures used (Lin, Boylston et al., 1999; Lin, Lin et al., 1999). It is reported that the starter cultures may increase the CLA content in cheese and yogurt. Studies show that *Propionibacterium freudenreichii* (CCRC 11076), *Lactobacillus acidophilus* (CCRC 14079, 1.1854), *L. casei* (Bs5, Bs7), *L. lactis* (CCRC 12586), and *L. lactis lactis* (CCRC 10791) can produce CLA from free linoleic acid (Hu and Zhang, 2004; Jiang et al., 1998; Kim and Liu, 2002; Lin, Boylston et al., 1999; Lin, Lin et al., 1999; Lin & Lin, 2000; Shao, Bian, & Ma, 2001).

Propionibacteria are gram-positive, shot rods that grow under anaerobic conditions. They are essential in the development of the characteristic flavour and eye formation in Swiss-type cheeses (Jiang et al., 1998). The aim of this study was to evaluate the ability of *P. freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* to produce CLA in SLM, MRS and skim-milk, and to study the effects of sunflower oil, $O_2$in the medium and fermentation time on the production of CLA by the cultures.

## 2. Materials and methods

### 2.1. Materials

Sunflower oil (Standard Foods Co., Shanghai, China) was purchased from a supermarket. Skim-milk was provided by Beijing Sanjuan Foods Co. Ltd. Conjugated linoleic acid (CLA) was obtained from Sigma Chemical Co.. Hexane was GC pure and all other solvents or chemicals used were of analytical grade.

### 2.2. Culture of cells

*Propionibacterium freudenreichii* ssp. *shermanii* (CGMCC 1.2227) and*Propionibacterium freudenreichii* ssp. *freudenreichii* (CGMCC 1.2236) were from the Chinese General Microorganism Culture Collection (CGMCC). The strains were sub cultured twice under micro aerobic conditions at 30 °C for 48 h in sodium lactate medium (SLM) containing 1.5% peptone, 0.5% yeast extract and 1% DL-sodium lactate. Two percent (v/v) concentrations of the subcultures were inoculated into SLM, De Man-Rogosa-Sharpe (MRS, Difco Lab, Detroit, MI, USA) and 12% (W/V) skim-milk before the experiment and incubated towards the end of the logarithmic phase.

### 2.3. Fermentations

Two percent (v/v) concentrations of *P. freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* were inoculated into SLM, MRS and skim-milk supplemented with different levels of sunflower oil. The sunflower oil was added as a micellar solution into the media. The micellar solution was prepared as follows: 300 mg of sunflower oil and 0.36 ml of polyoxyethylene sorbitan monooleate were dissolved in 5 ml of deoxygenated deionised water while stirring (Rainio, Vahvaselkä, & Suomalainen, 2002). The micellar solution was filterated through a 0.22 μm filter and stored at −20 °C before use. Biomass was measured with a U-3010 spectrophotometer (Hitachi, Japan). The samples were centrifuged at 4500 rpm for 10 min and the pellet was washed twice in saline solution (0.9% NaCl) before measuring the optical density at 600 nm in 1 cm i.d. quartz cuvettes at room temperature.

### 2.4. Lipid extraction and analysis

Samples of the bacterial suspensions (10 ml) were mixed with 20 ml of chloroform: methanol (2:1, v/v) and centrifuged at 4500 rpm for 5 min at 4 °C. The organic phase was separated, and dehydrated with anhydrous $Na_2SO_4$, then concentrated under vacuum at 30 °C. The sample was mixed with 10 ml of hexane for further quantification.

The determination of CLA was carried out by a UV spectrum analysis method described by Rosson and Grund (2001). The absorbance was measured in 1 cm quartz cuvettes at room temperature. The sample was scanned from 200 nm to 350 nm and the CLA peak was identified at 233 nm. The standard CLA concentrations in hexane used for calibration were 0–13.8 μg/ml and the absorbance was measured at 233 nm. The concentration of CLA (μg/ml) in each sample was calculated, based on the standard curve.

### 2.5. Statistical analysis

Each treatment was performed in three replications. The data from those replications were subjected to one-way ANOVA and Duncan's multiple range tests (SAS 6.0) and a significance level of 0.05 was used.

## 3. Results and discussion

### 3.1. Analysis of CLA

The absorbance peak of conjugated double bonds is at 232–234 nm. The standard curve of CLA UV absorption is was shown in Fig. 1. In the concentration range 0–13.8 μg/ml a linear relationship of absorbance at 233 nm with standard CLA concentration was observed.

CLA can be analyzed by many methods, such as gas–liquid chromatography (GC), silver–ion high-performance liquid chromatography ($Ag^+$-HPLC), and nuclear magnetic resonance (NMR). The GC and $Ag^+$-HPLC method can separate *cis* /*trans* and *trans*/*trans* isomers of CLA with a good resolution, but the CLA must be methylated in order to achieve GC and $Ag^+$-HPLC identification and quantification of individual CLA isomers. The disadvantage of methylation is that it may isomerize conjugated double bonds. At the same time the spectrophotometry method has advantages over other methods by avoiding methylation and its low analytical cost. Zhang, Fan, and Ma (2002) assayed the concentrations of CLA, methyl ester

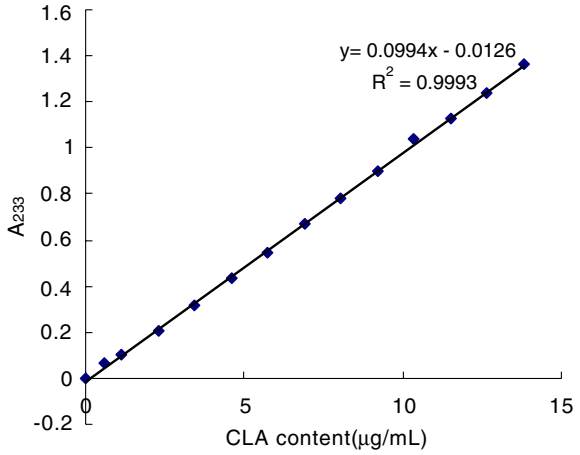


Fig. 1. Standard curve of CLA UV absorbance at 233 nm at room temperature.

of CLA and conjugated linoleic acid triglycride by combining the spectrophotometry and GC method. Results showed that UV spectrophotometry was a cheap, accurate and quick method for CLA analysis (Zhang et al. 2002).

### 3.2. Effects of substrate concentration on CLA production in different media

Sunflower oil was used as the fat substrate. It is tolerant of both low and high temperature (Niu, Huang, & Guo, 2004). Sunflower oil has a high concentration of linoleic acid (60–70%) and it is an economical source of linoleic acid. Fig. 2 shows the effects of sunflower oil on the CLA contents in different media. For *P. freudenreichii* ssp. *shermanii*, the concentration of CLA increased from 3.41 μg/ml in the control to 73.9 μg/ml at the sunflower oil level of 9.6 mg/ml in SLM and decreased significantly when the concentration of sunflower oil further increased. Regarding the strain *P. freudenreichii* ssp. *freudenreichii*, the formation of CLA was nearly constant when the concentration of sunflower oil was between 0 and 6 mg/ml and decreased when more sunflower oil was added. Moreover, significantly higher CLA levels were obtained in SLM and MRS than in skim milk for both strains. In MRS, the highest level of CLA was reached for *P. freudenreichii* ssp. *shermanii* (78.8 μg/ml) and*P. freudenreichii* ssp. *freudenreichii* (24.8 μg/ml). The reason is not very clear but perhaps the Tween-80 in MRS stimulates the growth of bacteria.

Meanwhile, two strains showed substantial differences regarding the formation of CLA in the same media. *P. freudenreichii* ssp. *shermanii* produced more CLA than *P. freudenreichii* ssp. *freudenreichii* in SLM and MRS, and *P. freudenreichii* ssp. *freudenreichii* produced more CLA in skim-milk. It was reported that microorganisms were able to form CLA by the action of linoleic acid (LA) isomerase. LA isomerase activity was observed in many CLA-forming bacteria, such as *Lactobacillus reuteri*, *Propionibacterium acnes* and *Clostridium sporogenes*. Studies

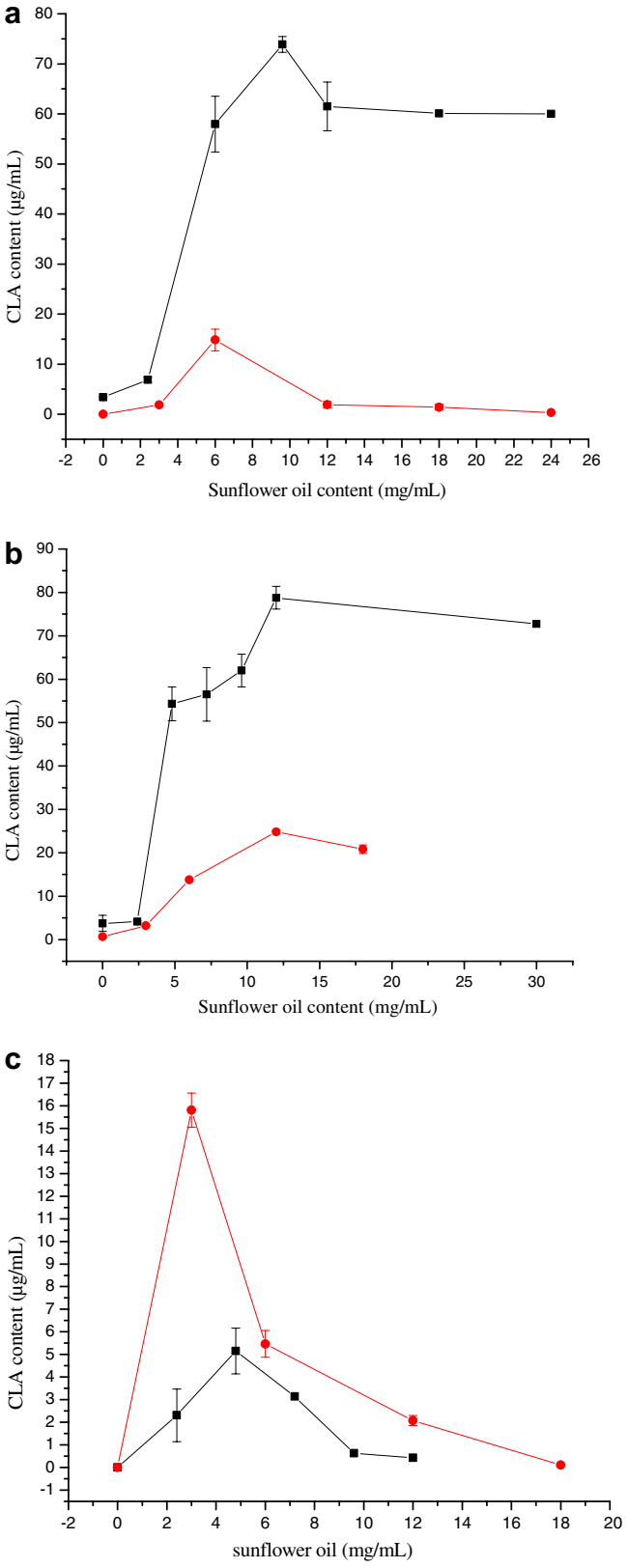


Fig. 2. Effect of different contents of sunflower oil on the formation of CLA in different media. Values are means ± SD of three independent experiments. *P. freudenreichii* ssp. *shermanii* (■); *P. freudenreichii* ssp. *freudenreichii* (●); SLM (a); MRS (b); skim-milk (c). Conditions: 2% (v/v) inoculation at 30 °C for 24 h under micro aerobic conditions.

showed the properties of LA isomerase extracted from different bacteria made a lot of difference. The molecular weights of LA isomerase isolated from *L. reuteri*, *P. acnes* and *C. sporogenes* were 68 kDa, 45 kDa and 55 kDa, respectively (Rosson & Grund, 2001). In our study, *P. freudenreichii* ssp. *shermanii* and*P. freudenreichii* ssp. *freudenreichii* showed different abilities to form CLA which indicated that the isomerase activities of both strains might be different. Further study on the mechanism of CLA production by propionibacterial strains are in progress.

### 3.3. CLA production in different media during incubation

The pattern of CLA production during incubation by *P. freudenreichii* ssp. *shermanii* in three media was similar to that of *P. freudenreichii ssp. freudenreichii* (Fig. 3). There is no significant increase ($p > 0.05$) in total amounts of CLA formed by strains *P. freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* from 0 h to 9 h in SLM and MRS containing 12 mg/ml of sunflower oil. The amounts of CLA were significantly higher ($p < 0.05$) in *P. freudenreichii* ssp. *shermanii* culture than in *P. freudenreichii* ssp. *freudenreichii* after 10 h of incubation (Fig. 3a and b). For *P. freudenreichii* ssp. *shermanii,* the amounts of CLA reached a maximum at 48 h, 36 h and 48 h, respectively, in SLM, MRS and skim-milk and then gradually decreased as the incubation time extended. The highest amount of CLA (78.8 μg/ml) was obtained in MRS supplemented with 12 mg/ml of sunflower oil at 36 h. Regarding the strain *P. freudenreichii* ssp. *freudenreichii*, the highest level of CLA production was obtained at 24 h in the three media.

CLA is an intermediate in the bio-hydrogenation of polyunsaturated fatty acid and it is generally accepted that CLA in lactic acid and rumen bacteria comes from the incomplete bio-hydrogenation of LA (Alonso et al., 2003). When reaction time was extended, CLA was further transformed to saturated fatty acids or some of the CLA were oxidized, and thus induced the loss of CLA (Lin, Lin, & Wang, 2002; Cao, Wei, & Zeng, 2004).

### 3.4. Effects of substrate on the growth of Propionibacterium freudenreichii

The effects of sunflower oil on the growth of *P. freudenreichii* ssp. *shermanii* and *P. freudenreichii* ssp. *freudenreichii* were studied in SLM, MRS and skim-milk by measuring the changes in absorbance at 600 nm ($A_{600}$). Fig. 4 shows that the inhibitory effects on the growth of *P. freudenreichii* were significantly different in the three media. *P. freudenreichii* ssp. *shermanii* grew better in MRS and skim-milk than in SLM and, regarding *P. freudenreichii* ssp. *freudenreichii,* the inhibitory effects were stronger in SLM than in MRS and skim-milk.

Additionally, it is interesting to note that a positive relationship between CLA production and ability to tolerate sunflower oil was observed. *P. freudenreichii* ssp. *freud-*

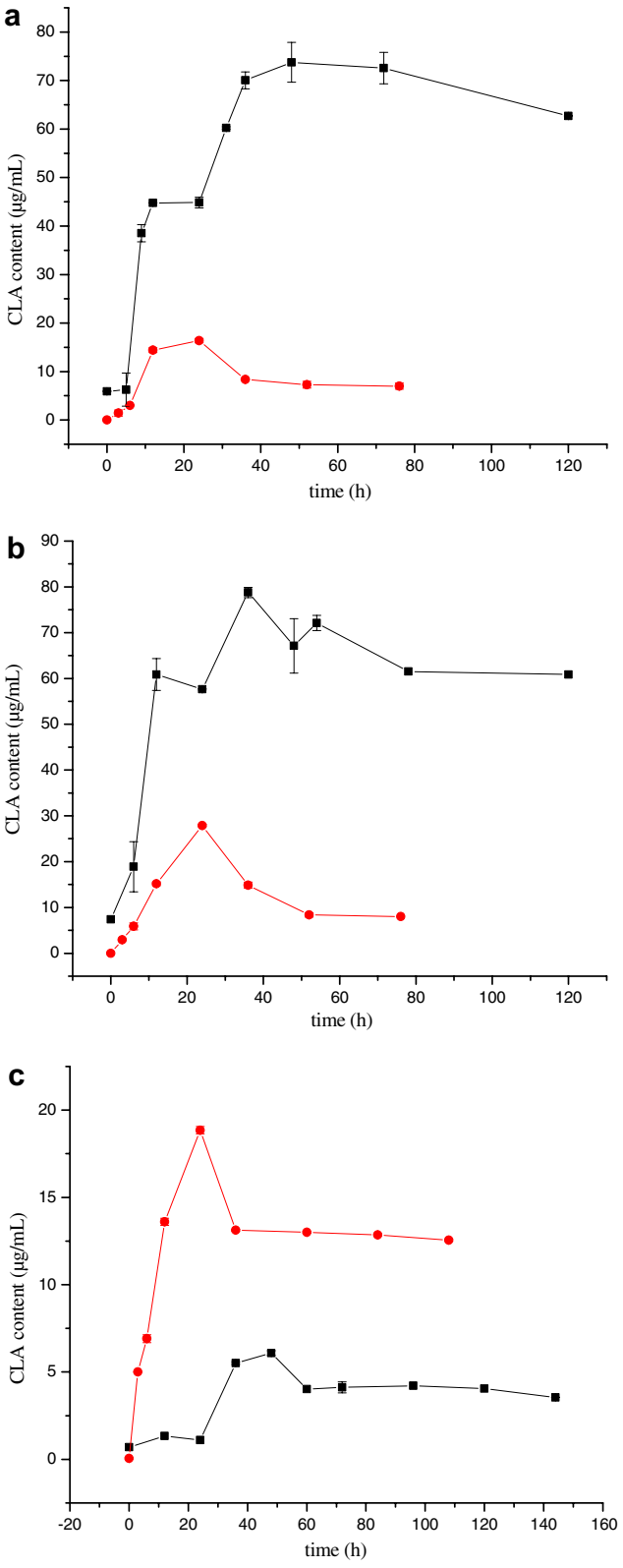


Fig. 3. CLA formation in different media during incubation. Values are means ±SD of three independent experiments. *P. freudenreichii* ssp. *shermanii* (■); *P. freudenreichii* ssp. *freudenreichii* (●); SLM (a); MRS (b); skim-milk(c). Conditions: 2% (v/v) inoculation, 30 °C for both strains under micro aerobic conditionS. For *P. freudenreichii* ssp. *shermanii*, sunflower oil content is 9.6 mg/ml, 12 mg/ml, 4.8 mg/ml in SLM, MRS and skim-milk, respectively. For *P. freudenreichii* ssp. *freudenreichii*, sunflower oil content is 6 mg/ml, 12 mg/ml, 3 mg/ml in SLM, MRS and skim-milk, respectively.

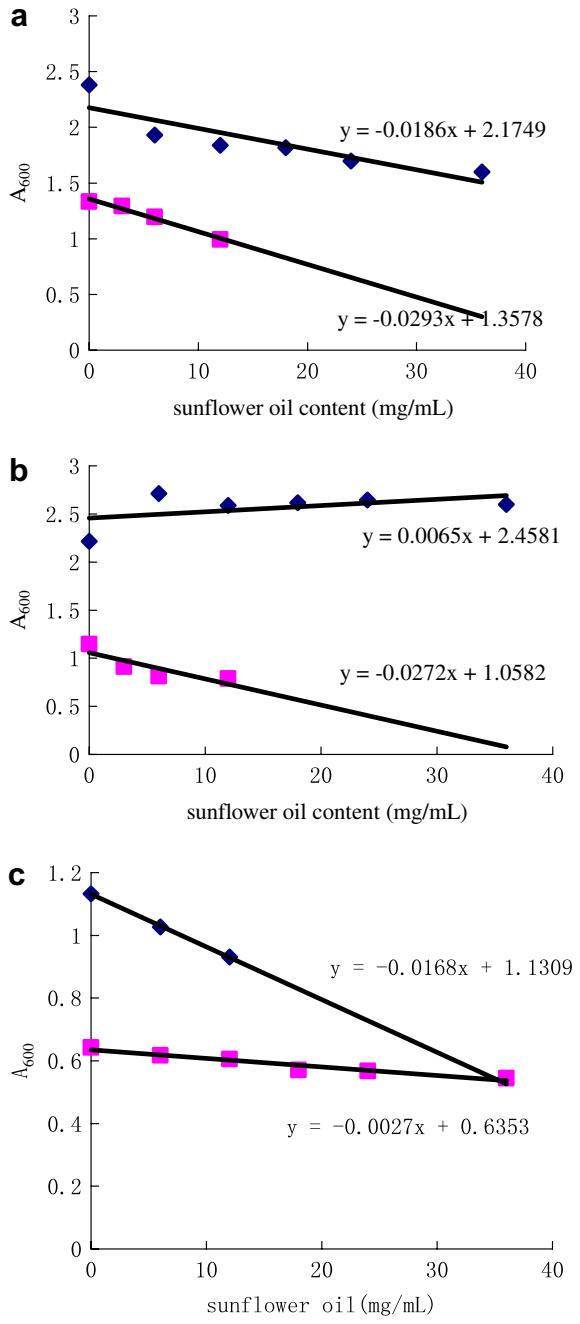


Fig. 4. Effects of different concentrations of sunflower oil on the growth of*P. freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *shermanii* in different media. *P. freudenreichii* ssp. *shermanii* (◆); *P. freudenreichii* ssp. *freudenreichii* (■); SLM (a); MRS (b); skim-milk (c). Conditions: 2% (v/v) inoculation, 30 °C, 24 h for both strains. Samples were measured in 1 cm i.d quartz cuvettes at 600 nm at room temperature. Control was the culture without bacteria.

*enreichii* produced less CLA in SLM and MRS than did *P. freudenreichii* ssp. *shermanii*, but the inhibitory effects of sunflower oil on *P. freudenreichii* ssp. *freudenreichii* were stronger. In skim-milk, *P. freudenreichii* ssp. *freudenreichii* produced more CLA and it was less susceptible to sunflower oil.

The antibacterial activity of LA has been recognized for a long time. Nieman (1954) reported that free fatty acids generally disturbed the permeability of cytoplasmic membranes of gram-positive bacteria and the conversation of free fatty acids to CLA might function as a detoxification mechanism (Nieman, 1954). At the same time, many studies have shown that free fatty acids had inhibitory effects on the growth of CLA-forming bacteria, such as *Lactobacillus acidophilus*, *P. freudenreichii*,*L. plantarum* (Alonso et al., 2003; Jiang et al., 1998; Lin, Boylston et al., 1999; Lin, Lin et al., 1999; Lin, 2000). In our study, *P. freudenreichii* converted LA, existing in sunflower oil to CLA by the action of linoleic acid isomerase. The growths of *P. freudenreichii* ssp. *shermanii* and*P. freudenreichii* ssp. *freudenreichii* were significantly inhibited by sunflower oil in the three media used. Similar observations were made in other studies with *L. lactis* I-01 in MRS (Kim & Liu, 2002). Furthermore, our data have shown that MRS might be a better medium for CLA production. The reason for this perhaps is that Tween-80, not only stimulates the bacteria growth, but also neutralizes the antimicrobial effect of fatty acids.

### 3.5. Effects of $O_2$ on the CLA production

Fig. 5 showed CLA production by *P. freudenreichii* ssp. *shermanii* under aerobic and micro aerobic conditions. Reactions were carried out in $N_2$-filled test tubes and in test tubes without $N_2$. Micro aerobic conditions benefited CLA production. The amounts of CLA increased from 0 to 36 h and reached a maximum at 36 h of incubation, then decreased. No significant ($p > 0.05$) increases in CLA contents were observed after 80 h of incubation under micro aerobic conditions. Total amounts of CLA were highest at 12 h under the conditions without $N_2$, but decreased significantly ($p < 0.05$) after 48 h of incubation. The results observed in this study are consistent with the opinion that

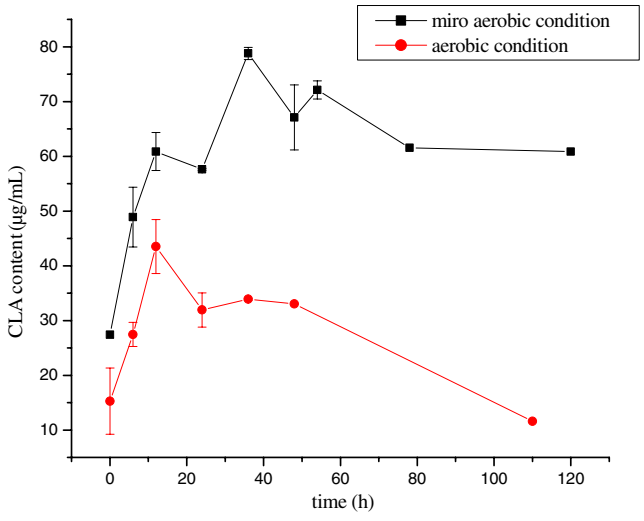


Fig. 5. Effects of $O_2$ on the production of CLA. Values are means ±SD of three independent experiments Conditions: 2% (v/v) *P. freudenreichii* ssp. *shermanii* was inoculated into MRS containing 12 mg/ml of sunflower oil.

oxygen promotes oxidative metabolism (Zhang, Hu, & Liu, 2004).

It has been proposed that LA is first converted to CLA by isomerase in the process of biohydrogenation, and further transformed to saturated fatty acid. Micro aerobic conditions might inhibit β-oxidation and protect the double bonds of CLA, which results in higher CLA production.

## 4. Conclusions

In this study, it was demonstrated that two strains of *P. freudenreichii* used as starter cultures in dairy industry were capable of producing CLA from sunflower oil in SLM, MRS and skim-milk. *P. freudenreichii* ssp. *shermanii* showed a higher ability to produce CLA in SLM and MRS than did *P. freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *freudenreichii* produced more CLA in skim-milk than did *P. freudenreichii* ssp. *shermanii*. The highest amounts of CLA (78.8 μg/ml) were obtained by *P. freudenreichii* ssp. *shermanii* in MRS. At the same time, the inhibitory effects on the bacterial growth were shown in three media and a positive correlation between CLA production and the ability to tolerate sunflower oil was observed. Micro aerobic conditions were effective in CLA formation and accumulation. The results show a potential for producing dairy products enriched with CLA. However, the pathway of CLA production needs to be studied further and CLA production by other starter cultures, such as lactic acid bacteria, is also a future interest.

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