Kafkas Univ Vet Fak Derg
22 (3): 451-455, 2016
DOI: 10.9775/kvfd.2015.14445

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Short Communication

Effect of Tween 80 on Conjugated Linoleic Acid Production by *Lactobacillus* Strains in Reconstituted Skim Milk Powder [1]

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[1] *This study was presented as a poster presentation in I. International Bioengineering Conference BIOENG '14, November 27-29, 2014, İstanbul, Turkey*

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Article Code: KVFD-2015-14445 Received: 04.10.2015 Accepted: 16.02.2016 Published Online: 17.02.2016

Abstract

In this study, three conjugated linoleic acid (CLA)-producing strains of *Lactobacillus* were cultured up to 36 in reconstituted skim milk powder (10%) supplemented with 2000 µg/ml of free linoleic acid (LA) and various concentrations of Tween 80. During the incubation, total CLA levels in the culture supernatants were determined by UV-spectrophotometry. The CLA levels significantly ($P<0.05$) increased with the addition of 5 and 20 mg/ml Tween 80. However, increasing Tween 80 concentration from 20 to 40 mg/ml did not appear to enhance CLA levels. Similar increase patterns were observed in the growth rate and CLA production of *Lactobacillus* strains during the incubation.

Keywords: *Conjugated linoleic acid, Lactobacillus, Skim milk, Tween 80*

Lactobacillus Suşlarının Rekonstitüe Yağsız Süt Tozunda Konjuge Linoleik Asit Üretimine Tween 80'in Etkisi

Özet

Bu çalışmada üç adet konjuge linoleik asit (KLA) üreten *Lactobacillus* suşu 2000 µg/ml linoleik asit (LA) ve çeşitli konsantrasyonlarda Tween 80 ilave edilmiş rekonstitüe yağsız süt tozu (%10) içinde 36 saate kadar kültüre edilmiştir. İnkübasyon boyunca kültür süpernatantlarında toplam KLA düzeyleri UV-spektrofotometre ile tespit edilmiştir. KLA düzeyleri 5 ve 20 mg/ml Tween 80 ilavesi ile önemli ($P<0.05$) seviyede artmıştır. Bununla birlikte, Tween 80 konsantrasyonunun 20 mg/ml'den 40 mg/ml'ye arttırılması KLA düzeyinde bir artışa neden olmamıştır. İnkübasyon esnasında *Lactobacillus* suşlarının gelişme hızı ve KLA üretimlerinde benzer artış oranları gözlenmiştir.

Anahtar sözcükler: *Konjuge linoleik asit, Lactobacillus, Yağsız süt, Tween 80*

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective name that refers to a wide variety of positional and geometric isomers of linoleic acid (LA) with conjugated double bonds at several positions from C7 to C14. In recent years, CLA, especially its isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12, has attracted attention among food and medical scientists due to its various beneficial biological effects. It has been reported in animal models to have anticarcinogenic, anti-atherogenic, anti-inflammatory and anti-diabetic activity and the ability to reduce the catabolic effects of immune stimulation [1].

Dairy products derived from ruminant animals are the major source of CLA in human diet. The major isomer of CLA in milk fat is *cis*-9, *trans*-11, and it represents 80% of the total CLA [2]. The CLA levels of various dairy products were reported in the range of 0.55-9.12 mg/g of fat [3]. These values are clearly lower compared to the recommended daily intake of CLA for appreciation of health benefits. Recommended therapeutic daily intake of CLA for an anticarcinogenic response in humans were reported in the range of 3.0-3.5 g/day based on diet and cancer risk studies and on the amount of CLA required for an anticarcinogenic response extrapolated from rats to humans [4,5]. Therefore, the development of strategies to increase CLA levels in dairy products is an ongoing subject of research.

Results of previous studies showed that many strains of lactic acid bacteria, especially from the genera *Lactobacillus*, *Bifidobacterium* and *Lactococcus* are able to efficiently

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convert LA to CLA in either synthetic medium or milk [6]. Therefore, the incorporation of suitable strains of lactic acid bacteria and free LA into processed foods is a reasonable option to increase CLA concentration in the human diet [7]. Antibacterial effect of free LA is the main challenge in the use of lactic acid bacteria for the purpose of increasing the CLA levels in foods. The growth rate and CLA production of lactic acid bacteria may be negatively affected by the presence of higher concentrations of free LA [8].

It is well documented that Tween 80 (polyoxyethylene sorbitan monooleate), a nonionic surfactant is used widely as an additive in foods, pharmaceutical preparations, and as an emulsifier, dispersant or stabilizer [9]. Also, Tween 80 has ability to neutralize the antimicrobial effects of several substances including free fatty acids and positive effect on CLA production by lactic acid bacteria [8,10]. According to our knowledge, the effect of Tween 80 concentration on CLA-producing bacteria has not previously been reported. Therefore, in this study, the effect of various concentration of Tween 80 on growth rate and CLA production of selected *Lactobacillus* strains were evaluated in reconstituted skim milk powder supplemented with free LA.

MATERIAL and METHODS

Microorganisms and Growth Conditions

The *Lactobacillus* strains used were previously isolated from Tulum cheese, which is a traditional Turkish goat's milk cheese. The isolates were primarily evaluated based on colony and cell morphology, Gram reaction and catalase test. Presumptive *Lactobacillus* strains were identified by API 50 CHL biochemically-based identification system (bioMérieux, Marcyll'Etoile, France). After initial screening for CLA conversation in MRS broth (Lab M, Bury, UK), three strains from different *Lactobacillus* species (*L. rhamnosus*, *L. plantarum* and *L. brevis*) were assayed in this study.

Spray dried skim milk powder (1.1% fat) was kindly provided by Enka Milk Company (Konya, Turkey). Autoclaved reconstituted skim milk powder (10%) supplemented with 2000 µg/ml of LA (Sigma-Aldrich, Milwaukee, WI, USA) and 0, 5, 20 or 40 mg/ml of Tween 80 (Merck, Darmstadt, Germany) was designated as test medium. Stock cultures of microorganisms maintained in brain heart infusion broth (Liofilchem, Roseto Degli Abruzzi, Italy) supplemented with 20% glycerol at -18°C were activated in MRS broth. The activated cultures were inoculated into skim milk (10%) at a ratio of 1% (v/v) and incubated at 37°C. Thereafter, 0.1 ml of overnight cultures were transferred to 10 ml test media and incubated at 37°C up to 36 h. All incubations were done under anaerobic conditions. At the end of incubation times of 12, 24 and 36 h, total CLA levels and *Lactobacillus* counts in cultures were determined.

Enumeration of Lactobacillus

Lactobacillus counts were performed by pour plate technique using MRS agar (Lab M). Dilutions of culture were made by peptone salt diluent (Lab M). One milliliter of the dilutions was transferred in two sterile plates per dilution and 15 ml MRS agar was then added per plate. After incubation at 30°C for 72 h, colonies on plates were counted and *Lactobacillus* counts in cultures were calculated as log CFU/ml.

Total CLA Assay

Lipid extraction from cultures was carried out by a procedure described by Barrett et al.[6] with some minor modifications. After specified incubation period, 1 ml of cultures was centrifuged at 18600 *g* for 2 min (Hettich, Tuttlingen, Germany). Then, the supernatants were mixed with 2 ml of isopropanol by vortexing and allowed to stand for 3 min. The lipids were extracted by addition 3 ml of hexane and vortexing. Final solutions were centrifuged at 6000 *g* for 3 min and the resulting supernatants were used in CLA quantitation assay.

The UV-spectrophotometric method was used for quantification of total CLA in the supernatants of Lactobacillus cultures. According to the literature [10,11] the most widely used methods for analysis of CLA in milk and microbiological media are gas chromatography (GC) and silver-ion high-performance liquid chromatography (Ag+-HPLC). However, the chromatographic methods can be considered as laborious and time-consuming for total CLA quantification. Alternatively, conjugated double bonds of CLA isomers can be detected using a UV-spectrophotometer at a wavelength of 233 nm [11]. It was previously reported that UV-spectrophotometry is a simple, rapid, cheap and accurate measurement method for CLA analysis. However, it should be noted that UV-spectrophotometry is not able to distinguish between CLA isomers [10].

Spectrophotometric assay based on the procedure of Rodríguez-Alcalá et al.[11] was used to quantitate the total CLA concentration in the culture supernatants. Absorbances were obtained using a UV-spectrophotometer at a wavelength of 233 nm (Thermo Scientific, Waltham, MA, USA) from 2 ml of the lipid extracts in hexane placed into quartz cuvettes. The measurements were compared to a calibration curve constructed with solutions of *cis*-9, *trans*-11 CLA isomer (Sigma-Aldrich) in hexane. Culture supernatants containing CLA at a concentration exceeding linear concentration range were diluted with appropriate volumes of hexane.

The calibration curve constructed using the *cis*-9, *trans*-11 CLA isomer, demonstrated that an increase in the CLA concentration up to 50 µg/ml coincided with a linear increase (R^2>0.99) in the absorbance up to 2.2. The UV-spectrophotometric method was extremely sensitive

and could detect and quantitate CLA concentration in the supernatant (2.3 µg/ml) obtained from sterile skim milk medium. The precision of method was evaluated by the multiple analyses of supernatants with different CLA contents. The relative standard deviations of repeatability were in range of 6.2-11.1%, respectively.

Statistical Analysis

Results of three independent trials were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS Inc., Chicago, USA). Mean values were compared using the Duncan grouping test at $P<0.05$.

RESULTS

The changes in total CLA level in the supernatants of *Lactobacillus* cultures grown in test medium supplemented with free LA (2000 µg/ml) and Tween 80 (0, 5, 20 or 40 mg/ml) during 36 h incubation are shown in *Fig. 1*. The level of CLA in supernatant of sterile skim milk was determined as 2.3 µg/ml. CLA concentrations in supernatants obtained from test medium without addition of Tween 80 significantly ($P<0.05$) increased from 2.3 to 37-64 µg/ml after 24 h of incubation. However, CLA levels in Tween 80-free test medium remained relatively constant during 36 h of incubation. CLA concentrations of the culture supernatants obtained after 36 h incubation of *Lactobacillus* strains in test medium with 5 mg/ml of Tween 80 ranged from 97 to 189 µg/ml. The levels of CLA in culture supernatants of test medium supplemented with 20 mg/ml of Tween 80 reached up to 198-327 and 233-384 µg/ml at the end of 24 and 36 h incubations, respectively. The CLA levels observed in culture supernatants obtained from test medium with 40 mg/ml of Tween 80 after 36 h of incubation were in the range of 244 to 357 µg/ml. At the end of 36 h incubation, the highest CLA levels were observed in test media inoculated with *L. brevis*. Our results showed that CLA conversion by selected Lactobacillus strains in skim milk with free LA was significantly ($P<0.05$) enhanced with the addition of 5 µg/ml of Tween 80, and increasing concentration of Tween 80 to 20 µg/ml resulted in a significant ($P<0.05$) increase in CLA conversions. However, CLA levels did not change significantly ($P>0.05$) at the two higher Tween 80 concentrations (20 and 40 mg/ml).

The spectrophotometric results obtained revealed that CLA production by *Lactobacillus* strains in Tween 80-added test media were biphasic, indicating an initial rapid increase in the CLA levels during the 12 h of incubation, followed by gradually slower production rates. CLA is an intermediate in the bio-hydrogenation of polyunsaturated fatty acids [12]. Therefore, in addition to changes in the available amount of free LA, decrease in the CLA production rates during the incubation period can be attributed to the further transformation of CLA to saturated fatty acids.

Microbial growth curves of *Lactobacillus* strains in test media for up to 36 h of incubation are given in *Fig. 2*. The growth patterns of selected strains observed in test media were similar to those of CLA conversions. The numbers of *Lactobacillus* strains in test medium without the addition of Tween 80 increased from 4.1-4.7 to 5.3-6.2 log CFU/ml during incubation. *Lactobacillus* counts in test medium with 5 mg/ml of Tween 80 after 36 h of incubation were in the range of 7.6 to 8.1 log CFU/ml. A final *Lactobacillus* counts in 20 and 40 mg/ml Tween 80-added test medium were determined as 8.7-8.8 and 8.5-8.8 log CFU/ml, respectively. Plate counts showed that growth of *Lactobacillus* strains in the presence of 2000 µg/ml of free LA was increased significantly ($P<0.05$) through the addition of Tween 80. However, counts of *Lactobacillus*

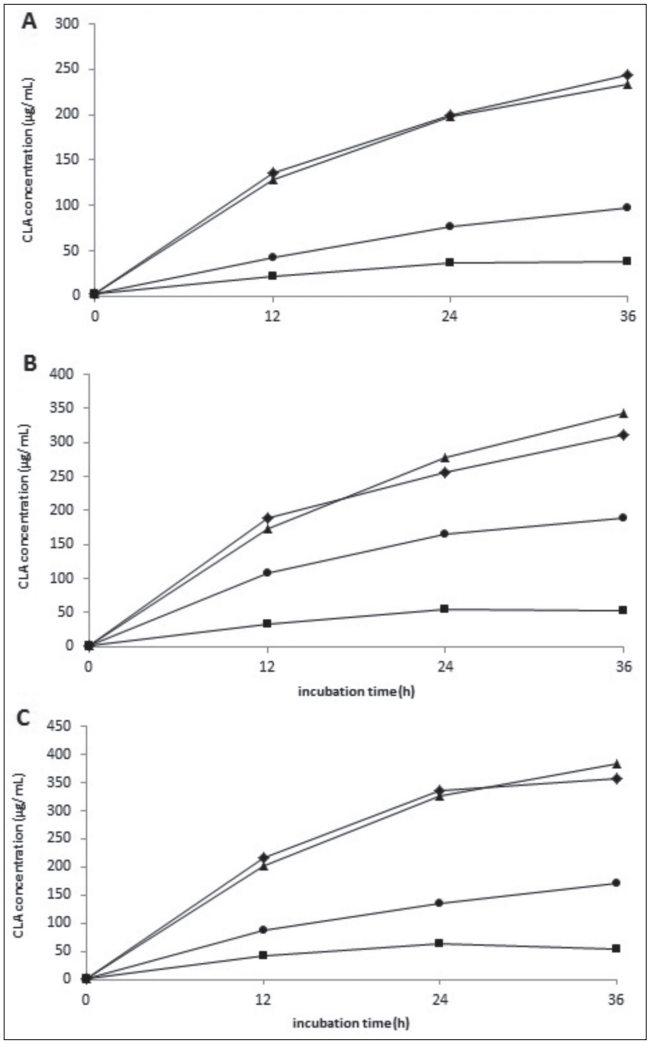

Fig 1. Total CLA concentration (µg/ml) of the culture supernatants obtained after incubation of the selected *Lactobacillus* strains ((A) *L. rhamnosus*; (B) *L. plantarum*; (C) *L. brevis*) in skim milk with free LA (2000 µg/ml) and Tween 80 ((▲) 0 mg/ml; (●) 5 mg/ml; (♦) 20 mg/ml; (■) 40 mg/ml) for 12, 24 and 36 h

Şekil 1. Serbest LA (2000 µg/ml) ve Tween 80 ((▲) 0 mg/ml; (●) 5 mg/ml; (♦) 20 mg/ml; (■) 40 mg/ml) içeren yağsız sütten *Lactobacillus* suşlarının ((A) *L. rhamnosus*; (B) *L. plantarum*; (C) *L. brevis*) 12, 24 ve 36 saat inkübasyonu sonunda elde edilen kültür süpernatantlarındaki toplam KLA konsantrasyonu (µg/ml)

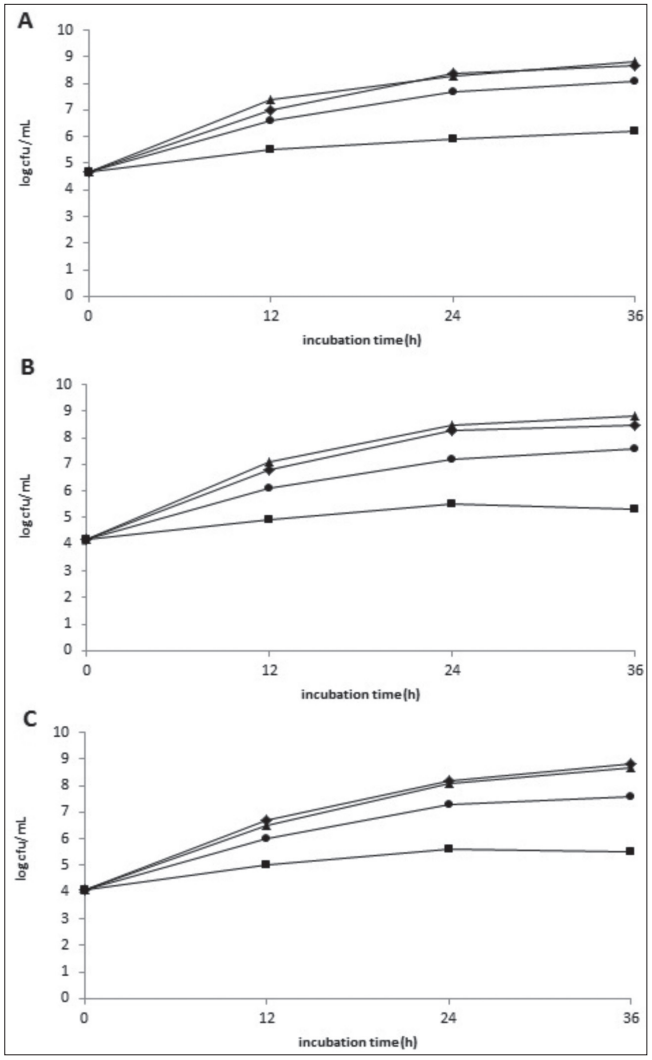

Fig 2. Plate counts of the selected *Lactobacillus* strains ((A) *L. rhamnosus*; (B) *L. plantarum*; (C) *L. brevis*) after 12, 24 and 36 h incubation in skim milk with free LA (2000 µg/ml) and Tween 80 ((▲) 0 mg/ml; (●) 5 mg/ml; (♦) 20 mg/ml; (■) 40 mg/ml)

Şekil 2. Serbest LA (2000 µg/ml) ve Tween 80 ((▲) 0 mg/ml; (●) 5 mg/ml; (♦) 20 mg/ml; (■) 40 mg/ml) içeren yağsız sütte *Lactobacillus* suşlarının ((A) *L. rhamnosus*; (B) *L. plantarum*; (C) *L. brevis*) 12, 24 ve 36 saat inkübasyonu sonunda sayısı

strains did not change significantly (P>0.05) when Tween 80 concentration increased from 20 to 40 mg/ml.

DISCUSSION

The antimicrobial effect of free LA against Gram positive bacteria, including Lactobacillus strains, has been previously documented in several studies [13]. Lin et al.[14] reported that growth of six lactic acid bacteria in skim milk was negatively affected by addition of free LA (1000 and 5000 µg/ml). In study of Jiang et al.[15], a negative correlation was found between the growth of CLA-producing strains of Propionibacterium and the LA concentration in MRS broth. Similarly, Boyaval et al.[16] reported that 100 µg/ml of LA had a strong bacteriostatic effect on the growth and metabolism of *Propionibacterium freudenreichii* ssp. shermanii in a lactate-yeast extract based medium. In study of Talon et al.[17], plate counts of the six lactic acid bacteria in the culture media with 500 µg/ml of LA remained at their initial levels of inoculation or decreased during the incubation.

The possible antibacterial mechanism for free fatty acids was postulated to proceed via disruption of the bacterial cell membrane resulting in a change in membrane permeability. Cell membranes have long been regarded as a primary target for the antimicrobial effect of free fatty acids. Exposure to free fatty acids causes the detrimental effects on bacterial cells such as formation transient or permanent pores, leakage of intracellular materials, inhibition of enzyme activity, impairment of nutrient uptake and the generation of toxic per-oxidation [13]. Generally, Gram-positive bacteria are more sensitive to long chain un-saturated free fatty acids than Gram-negatives [18].

In accordance with results of present study, neutralization of the antimicrobial properties of free fatty acids by Tween 80 was previously evidenced by several authors [16]. Li et al.[19] reported that Tween 80 can act as protective agent against leakage of intracellular materials. However, the precise mechanism of the Tween 80 in neutralizing the antibacterial activity of free fatty acids is still unclear. In study of Rainio et al.[8], negative effects of free LA (1000 µg/ml) on growth rate and CLA production of *P. freudenreichii* ssp. shermanii could be tolerated by the addition of 15 mg/ml Tween 80. Van Nieuwenhove et al.[7] reported that CLA production by lactic acid bacteria was positively affected by neutralization of negative effects of LA on bacterial metabolism. Wang et al.[10] suggested that Tween 80 in the formulation of MRS broth (1 mg/ml) reduces the antimicrobial effect of fatty acids against CLA-producing bacteria and thus stimulates their growth and CLA conversation.

In conclusion, the results obtained from this study suggest that addition of Tween 80 to milk together with free LA is a very feasible strategy to increase CLA conversion by lactic acid bacteria. Thus, the CLA content of fermented dairy products can be enhanced considerable by the use of CLA-producing lactic acid bacteria. Alternatively, a concentrated form of CLA obtained from synthetic media or milk can be used as a food additive. Further studies are still required to determine the commercialization potential of this strategy in terms of applicability and acceptable daily intake of Tween 80.

Acknowledgement

The authors would like to thank Mr. Mehmet Zeki Öçlü, Director of Kemiteks Chemical Company, for supply of chemicals used.

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