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Survival of probiotic *Lactobacillus casei* and *Enterococcus fecium* in Domiati cheese of high conjugated linoleic acid content

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Abstract

Domiati cheese was made from reconstituted milk (20% total solids) containing normal (C) and high level (T) of conjugated linoleic acid (CLA) content respectively using mixed probiotic *Lactobacillus casei* and *Enterococcus fecium* (1:1) as starter. The cheeses were stored in brine at 25±5°C for 60 d storage. The added probiotics grow equally well in C and T cheeses. The total viable and *Lb casie* counts increased to a maximum after 15 d of storage and then decreased while the counts of *Enterococcus fecium* and total lactic acid continued to increase until the end of the storage period. The cheeses retained high counts of these probiotics (>10^7 cfu g^{-1}) throughout the storage period. The acidity, soluble nitrogen, free amino acid, and conjugated dienes increased significantly in Domiati cheese during storage with small differences between treatments except for the significantly higher conjugated dienes in T than C treatment.

Key words: Domiati cheese, Conjugated linoleic acid, Probiotics, Cheese microflora, *Lactobacillus casei*, *Enterococcus fecium*

Introduction

Functional foods are defined as foods that affect specific functions or systems in the human body, providing health benefits beyond energy and nutrients. The market of functional dairy products has developed markedly during the last decade (Granto et al., 2010). At the current time, the largest markets for functional foods and supplements are the United States, Europe, and Japan, accounting for 33.6%, 28.2% and 20.9% of sales in 2003, respectively with a yearly growth potential of 10% (Granto et al., 2010). This growth is mainly due to the segment of probiotic fermented milks. Analysis of the North American probiotics markets for human nutrition found that the probiotics sector earned revenues of US$ 698 million in 2006 and expected to reach US$ 1.70 billion in 2013, with an annual growth rate of 13.7%. Meanwhile, in Europe, consumption of probiotics is equally strong: between 2002 and 2007, consumption in Western Europe grew by an annual growth rate of 13% and by 18% in and consumption in Eastern Europe (Granto et al, 2010).

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However, the major challenge facing producers of probiotic fermented milk is to maintain the viability of probiotics from processing until it reaches the consumer. Probiotics have been reported to suffer from low viability in fermented dairy products due to several factors (Granto et al., 2010) such as inhibition by acid, peroxide and other metabolites.

Cheese has been used as an alternative form of dairy products for the delivery of probiotics. The high solids and fat content of cheese offer better protection for probiotics in simulated human gastrointestinal tract (Mäkeläinen et al., 2009). Also, the continuous proteolysis of cheese during ripening provides probiotics with needed nitrogen source for their growth and activity. Consumption of probiotic cheese creates a buffer against the high acidic environment in the gastrointestinal tract, and thus creates a more favourable environment for probiotic survival throughout the gastric transit, due to higher pH. Moreover, the dense matrix and relatively high fat content of cheese may offer additional protection to probiotic bacteria in the stomach (Gomes daCruz et al., 2009). Similar to other functional probiotic foods, maintaining the viability of probiotics during cheese processing and storage is a challenge.

Several technological hurdles have been described in the development of probiotic cheeses including the salting step and storage conditions (Gomes daCruz et al., 2009). Domiati cheese is

unique in that salt is added to milk before renneting, which would affect the survival of added probiotics. Domiati cheese is also stored in high salt brine (>8%) at ambient temperatures which would affect the viability of added probiotics during storage. Kasmo et al. (2004) found that *Lactobacillus acidophilus* survived to numbers > 10^7cfu g^{-1} in Turkish white cheese particularly when stored in vacuum without brine. Sarantinopoulos et al. (2002) found that the number of enterococci in Greek Feta cheese made with the use of *Enterococcus faecium* as an adjunct starter to increase until day 15 of ripening and then remained constant. El-Soda and Abd El-Salam (2002) reported that *Enterococcus faecium* and a combination of mesophilic and thermophilic lactobacilli developed the characteristic flavour of Domiati cheese made from pasteurized milk. Pirauzian et al. (2010) found that the inclusion of *Enterococcus faecium* improved the quality of ultrafiltered (UF) white cheese.

Conjugated linoleic acid (CLA) is a generic name that refers to a mixture of linoleic acid isomers in which the double bonds are conjugated. These isomers have received much attention recently for their beneficial health properties as anticarcinogens reducing potential cardiovascular diseases, immune modulation and body fat reduction (German abd Dillard, 2006). Research (Whitlock et al., 2006) has demonstrated that alteration of dairy cow diets by inclusion of sources of linoleic and linolenic acid (precursors for bacterial CLA) will increase yield of CLA and trans fatty acids in milk fat.

During the course of developing probiotic Domiati cheese with high level of conjugated linoleic acid (CLA) it was important to study the behaviour of the added probiotic under prevailing conditions in its manufacture and storage.

The aim of the present study was to investigate the survival of a combination *Lactobacillus casei* and *Enterococcus faecium* of potential probiotic characteristics in Domiati cheese made from milk of normal and high conjugated linoleic acid respectively. Additionally, changes in its composition during manufacture were followed.

Materials and Methods

Production of milk of high level in CLA content

The production of milk rich in CLA was carried out in the Experimental farm of the Dairy Science Department, South Dakota University, Brookings, USA as described by Whitlock et al. (2006). Two groups of multiparous Holstein cows were used. The first group received control diet (C) with 0% supplemental fat and second group received the C diet with 1% fish oil (FO) and 1% of extruded soy beans (ESB). In the fat supplemented diet FO replaced a portion of the cracked corn and the ESB replaced a portion of the cracked corn and a portion of the soybean in the control diet. Total mixed diets were formulated to be isonitrogenous at 18% crude protein and contain more than adequate amounts of major elements (NRC, 1989). Supplemental vitamin E was included in the diet at 53 IU/Kg of DM to help prevent oxidation of the milk because increased concentration of unsaturated fatty acids in milk fat may be more susceptible to oxidation. Cows were milked twice daily and the milk from each group was combined and spray dried using a pilot spray drier (Niro Atomizer, APV, Denmark). The milk powders had the following composition (Table 1).

Table 1. Chemical composition and properties of spray dried whole milk containing normal (Control) and high CLA contents.

Type of Milk	Moisture %	Fat %	Protein %	pH %	Acidity %	Solubility index %	Peroxide meq/kg Fat	Acid value ml 0.1 N KOH/100g fat
Control	3.37	28.64	24.21	6.62	0.25	2.25	4.3	1.90
High CLA	3.58	23.98	26.13	6.11	0.30	2.02	27.9	2.85

Milk fat was extracted from the powder and analyzed for fatty acids and results have been reported before (Abd El-Salam et al., 2011). The most obvious differences in the fatty acid composition of milk fat from animals receiving control and experimental diets were found in the CLA isomer *cis*9, *trans*11 of the two milks being 0.5 and 1.25 g 100 g^{-1} fat respectively. Also, the milk fat with high level of CLA contained slightly higher contents of the trans isomers of the fatty acids; $C_{15:1}$, $C_{16:1}$, $C_{17:1}$ and $C_{18:1}$ than the one with normal CLA content.

Source of cultures

Lctobacillus casei was obtained from Chr. Hansen's Lab., Denmark. Probiotic *Enterococcus faecium* isolated from traditional dairy products

was obtained from Dairy Microbiology Lab, National Research Center, Dokki, Cairo, Egypt (El-Shafei et al., 2002). The strains were maintained and transferred weekly in sterilized reconstituted skim milk and stored at 4°C. Cultures were propagated in sterilized reconstituted skim milk (10% TS) at 35°C for 12-16 hrs before its use in the manufacture of Domiati cheese.

Cheese manufacture

The manufacture and analysis of Domiati cheese was carried out in the Dairy Department, National Research Centre, Cairo, Egypt. Whole milk powders with normal and high levels of CLA content were reconstituted in pre-boiled water to give 20% total solids in the reconstituted milks. The milks were heated to 60°C for 30 min, homogenized using two stage laboratory homogenizer (Lab-20, Rannie, APV, Copenhagen) at 20 and 5 MPa for the 1st and 2nd stages respectively. The milk was cooled to 40°C, 8% NaCl were added and stirred until the salt was completely dissolved. Rennet (calf rennet powder, Ch. Hansen, Copenhagen, Denmark) was added at the ratio of 0.5 g kg^{-1} reconstituted milk and left to complete coagulation. The curd was ladled in rectangular frames (20 x 20 x 20 cm) lined with cheese cloth and the drained whey was collected to be used a brine for cheese storage and maturation. The fresh cheese was cut into cubes (~0.5 kg each), stored in salted whey in tightly closed containers at room temperature (20±5°C). Samples were taken from the fresh cheese and cheese stored for 15, 30 and 60d respectively for chemical and microbiological analysis. The whole experiment was repeated twice.

Methods of analysis

Sampling

Samples were taken aseptically by cutting a section from the cheese cubes with a sterilized sharp knife. About 1 cm of the surfaces of the obtained section, and the remaining part was divided into two portions. The first was kept for microbiological analysis and the second was kept frozen for chemical analysis.

Methods of chemical analysis and pH

The pH value of the cheese was determined at room temperature (20± 2°C) using digital pH meter (Hanna, Germany) equipped with a combined electrode. Cheeses were analyzed for total solids and fat contents and acidity according to A.O.A.C (1995) methods.

Determination of proteolysis

Cheeses were analyzed for soluble nitrogen (SN) content by Kjeldahl method (IDF, 1993) and total free amino acids (Folkertsma and Fox, 1992).

Determination of conjugated dienes

Analysis of conjugated dienes was carried as an index for changes in CLA content of cheese during storage. Five grams of the cheese sample were extracted with 6 ml chloroform:methanol (2:1). Two milliliters of chloroform were added to the cheese sample, mixed well, followed by 2 ml methanol and then 2 ml chloroform. The mixture was then centrifuged at 3000 x g for 15 min. The chloroform layer was withdrawn, dried with anhydrous sodium sulphate and then evaporated to dryness. The residue transferred to a capped tube, flushed with nitrogen and stored frozen until analyzed. The AOAC (1995) official method 957.13 was used for the determination of conjugated dienes in the extracted cheese lipids.

Microbiological analysis

The cheese samples were analyzed for total viable count (APHA, 1993), lactic acid bacteria (Elliker et al., 1956), *Lb. casei* on modified clbc medium (Nighswonger et al., 1996) and for *Enterococcus faecium* on kanamycin aesculin azide agar (Oxiod, 1998).

Statistical analysis

Two way independent factorial analysis of variance was carried out using Vassarstats computing website (Lowry (2009) (http://faculty.vassar.edu/lowry/anova2corr.html). Significance of differences between treatments (type of milk and starter), storage periods and their interactions were determined using Tukey test at significance level of 0.05.

Results and Discussion

Physicochemical characteristics

Domiati cheese from milk of high CLA content had lower total solids (TS) content than that from normal milk throughout the storage period. This can be attributed to the softer texture of cheese from milk containing high level of CLA content which results in retaining more moisture than cheese from normal milk. Jones et al. (2005) reported that cheeses of soft texture have been obtained from high CLA milk which agrees with the present finding.

Analysis of Domiati cheese (Table 2) revealed significantly lower fat content in cheese made from milk with high level of CLA content as compared to cheese from milk of normal CLA content. This

can be explained by the differences in the fat content of the two milks and increased losses of fat during the cheese manufacture from high CLA milk. Feeding animals rations supplemented with fish oil reduced the milk fat content (Whitlock et al., 2006) which would explain the lower fat in milk powder of high CLA level prepared in the present study. Also, milk of high CLA was reported to produce cheese of less firm texture than normal milk (Jones et al., 2005) which may explain the higher fat losses during the manufacture of Domiati cheese from milk of high CLA content.

The rate of acidity development in Domiati cheese from milk of high CLA content was slightly less than that from normal milk (Table 2) but the differences were not significant (Table 2). On the other hand, the pH of cheese from milk of high CLA was slightly higher, but not significant, than that from normal milk throughout the storage. This can be due to the differences in the developed acidity, and the higher buffer capacity of Domiati cheese from high CLA milk due to its high protein content.

The free amino acids (FAA) increased gradually in Domiati cheese from normal and high CLA milks during storage (Table 2). The development of FAA in the two cheeses was not significantly different (Table 3) indicating comparable proteolysis in the two cheeses.

The conjugated dienes content increased gradually in cheese from both treatments reaching 0.84 and 1.93 g 100 g^{-1} cheese fat after 60 d. This increase may be attributed to the growth and activity of the added probiotic starter which is capable of synthesizing CLA (Abd El-Salam et al., 2010). The differences in the conjugated dienes of Domiati cheese due to storage period and type of milk used were found significant (Table 2).

Table 2. Changes in the composition of Domiati cheese from milk with normal (C) and high (T) conjugated linoleic acid during storage (Average of two replicates).

Treatment	Storage Period			
	Fresh	15	30	60
		Total Solids (g $100g^{-1}$)		
C	35.75^{aA}	37.95^{bA}	39.07^{cA}	40.84^{dA}
T	35.37^{aA}	36.35^{bB}	38.11^{cB}	39.29^{dB}
		Fat (g $100g^{-1}$)		
C	17.50^{aA}	18.00^{aA}	19.00^{bA}	21.00^{cA}
T	13.00^{aB}	13.00^{aB}	14.00^{bB}	15.00^{cC}
		Acidity (%)		
C	0.28^{a}	0.74^{b}	0.79^{b}	1.21^{c}
T	0.26^{a}	0.70^{b}	0.85^{c}	1.20^{d}
		pH		
C	5.90^{a}	4.95^{b}	4.71^{bc}	4.04^{d}
T	5.97^{a}	5.17^{b}	4.74^{c}	4.25^{d}
		Soluble N (g $100g^{-1}$)		
C	0.34^{a}	0.34^{a}	0.36^{a}	0.36^{a}
T	0.36^{a}	0.36^{a}	0.39^{a}	0.39^{a}
		Free amino acids (mg $100g^{-1}$)		
C	24^{aA}	30^{bA}	54^{cA}	64^{dA}
T	30^{aB}	35^{bB}	63^{cB}	85^{dB}
		Conjugated dienes (g $100g^{-1}$ fat)		
C	0.39^{aA}	0.55^{bA}	0.69^{bcA}	0.84^{cdA}
T	0.84^{aB}	1.50^{bB}	1.64^{bcB}	1.93^{cdB}

* Small letters (a,b,c) different subscripts in the same row indicate significance

* Capital letters(A,B) different subscripts in the same column indicate significance

Effect of added probiotics on total viable and lactic acid bacteria counts

Figures 1 and 2 shows that the changes in the total viable count (TVC) and lactic acid bacteria (LAB) count from different treatments. The TVC increased to a maximum after 15 days of storage and then decreased slightly thereafter and statistical analysis showed that the effect of storage on TVC was highly significant as shown in Table 3. The changes in TVC during storage followed its normal pattern of changes during storage where TVC increased during the first two weeks of storage and then decreased after that (Abd El-Salam and Benkerroum, 2006). Slight but not significant differences were found in the TVC of Domiati cheese from milk of high and normal levels of CLA content (Figure 1 and Table 3). This indicates that the changes in CLA content of milk had no probable effect on the TVC of Domiati cheese. The count of LAB in Domiati cheese increased slightly throughout storage and these increases were highly significant (Table 3).

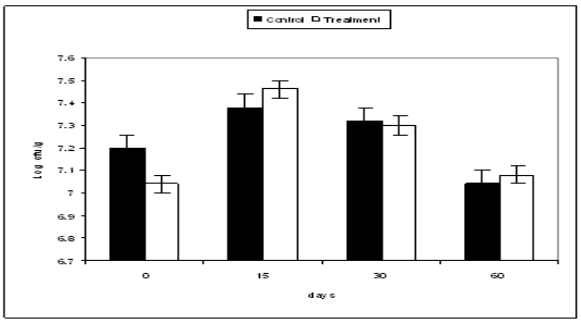

Figure 1. Changes in the total bacterial count (mean Log cfu g^{-1} ± Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

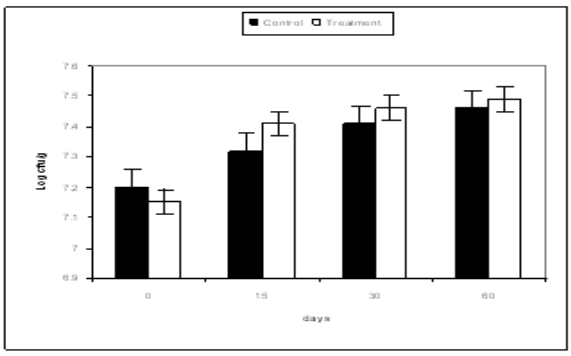

Figure 2. Changes in the total LBA count (mean Log cfu g^{-1}±Standard deviation) of Domiati cheese from milk containing normal and high CLA content

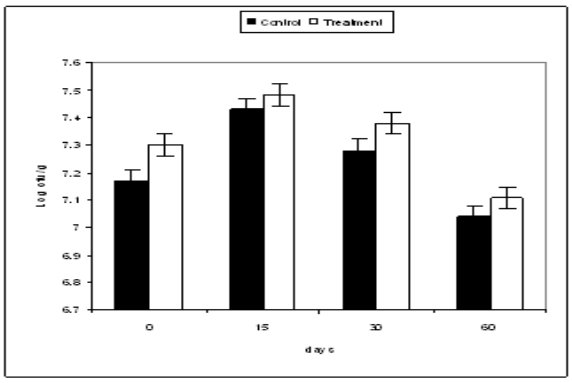

Figure 3. Changes in the total *Lb casei* count (mean Log cfu g^{-1}±Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

Previous studies showed that changes in LAB followed a similar pattern as TVC which was not found in the present study.

Table 3. Analysis of variance of chemical composition and microbiological quality of Domiati cheese as affected by storage and type of milk (treatment) used.

	Effect of Storage		Effect of treatment		Interaction (Storage x Treatment)	
	Probability	Tukey HSD test (0.05)	Probability	Tukey HSD test (0.05)	Probability	Tukey HSD test (0.05)
Total	<0.0001	0.84	0.0002	0.43	-*	-
Solids	<0.0001	0.57	<0.0001	0.29	0.016	0.99
Fat	<0.0001	0.11	-*	-	-*	-
Acidity	<0.001	0.31	-*	-	-*	-
pH	-*	-	-*	-	-*	-
Soluble N	<0.0001	1.6	<0.0001	0.82	<0001	2.8
Free AA**	<0.0001	0.16	<0.0001	0.08	0.008	0.28
Dienes	<0.0001	0.08	-*	-	0.008	0.14
TVC**	<0.0001	0.07	-*	-	-*	-
LAB**	<0.0001	0.08	0.006	0.04	0.02	0.14
Lb. casei	-*	-	-*	-	-*	-
E.faecium						

* not significant at P<0.05, Tukey HDS 0.05, absolute difference between two means required for significance at P 0.05.

** Free AA, free amino acids; TVC, total viable count LAB, lactic acid bacteria0

The changes in the LAB also followed a similar pattern to changes in *Enterococcus feacium* (Figure 4) which show that the count of LAB was affected by the added adjunct starter. On the other hand, the type of milk used had no significant effect on the count of LAB of Domiati cheese. Coakley et al. (2007) reported that elevating the CLA level in cheese did not affect the growth of starter bacteria or nonstarter lactic acid bacteria in cheese during ripening.

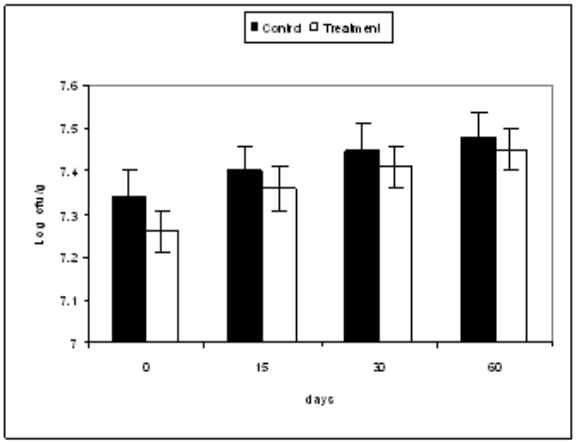

Figure 4. Changes in the total *Enterococcus feacium* count (mean Log cfu g^{-1}±Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

Survival of probiotics in Domiati cheese

After a storage period of 15 days, the viable count of *Lb casei* increased by 0.26 and 0.l8 log cycles cfu g^{-1} in Domiati cheese of normal and high level of CLA content respectively (Figure 2). However, the *Lb casei* count of normal and high level of CLA content decreased by 0.15 and 0.19 log cycles cfu g^{-1} respectively in comparison with the initial count after 2 mo of storage. The count of *Ent feacium* increased by 0.06 and 0.14 and 0.1 and 0.19 log cycles cfu g^{-1} after 15 d and 2 mo for Domiati cheese of normal and high levels of CLA respectively. The high viability of added probiotics during storage can be attributed to the high TS of Domiati cheese. High TS in milk was reported to offer protection for the growing microorganisms (Gün and Işţkli, 2007; Mahdian and Tahrani, 2007) which agree with the present finding. Also, *Ent. feacium* was reported as being able to tolerate the high salt in brined cheeses (Sarantinopoulos et al., 2002) which may explain the continuous increase of this probiotic in Domiati cheese during storage in brine. Donkor et al. (2007) reported that storage time play an important role in the extent of overall proteolytic activity, and consequent increase in the amount of liberated amino acids may cause a higher growth rate of probiotic bacteria even in an acidic environment.

Conclusions

Domiati cheese retained high counts of probiotics *Lb casei* and *Ent feactium* (> 10^7cfu g^{-1}) throughout the storage of 2 months indicating the high survival of the used probiotics under the storage conditions. Also, the level of CLA in Domiati cheese had no probable effect of the survival of the used probiotics. Therefore, the obtained Domiati cheese can be considered as a functional probiotic cheese with normal or high levels of CLA content.

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