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Original Article

Alteration of fatty acid profile of milk in Holstein cows fed *Bacillus coagulans* as probiotic: a field study

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Abstract

Background: Probiotics may improve milk quality and the general health status of animals. **Aims:** The effects of dietary *Bacillus coagulans* PRM101 on milk components, milk fatty acids (FA), and some health indicators of dairy cows were investigated. **Methods:** The probiotic was added to the feed of 12 Holstein cows (2 g/cow: 2×10^{11} CFU/cow) for 63 days compared to a control group fed on the basal ration (n=11). Milk and blood samples were taken on days 0, 21, 42, and 63. **Results:** The yields of milk and energy corrected milk (ECM; computed from milk weight and its fat and protein content) decreased linearly and similarly (P=0.60) in both groups. The treatment cows, however, showed quadratic increases in the weights of milk (P=0.03) and ECM (P=0.04) at d42 of the study. Energy corrected milk (d42, P<0.05) and crude protein content of milk (d42, P<0.05; d63, P<0.1) were higher in the cows receiving the probiotic. The proportions of heptadecanoic (C17:0; P=0.002) and linoleic (C18:2; P=0.077) acids in milk fat (g/100 g fat) were higher in the treatment cows on d63. Milk total antioxidant capacity (TAC), malondialdehyde (MDA), and similarly, amyloid A (AA) and haptoglobin (Hp) of milk and blood were not affected. Total antioxidant capacity and MDA were negatively correlated in the control group (r=-0.669, P=0.005). Heptadecanoic acid correlated negatively with milk MDA (r=-0.611, P=0.035) and positively (r=0.591, P=0.043) with serum Hp in the treatment cows. **Conclusion:** Dietary *B. coagulans* PRM101 may improve the proportions of C17:0 and C18:2 FA in milk. Some improvements in milk protein and the health status of the cows may also be anticipated.

Key words: Amyloid A, *Bacillus coagulans*, Haptoglobin, Heptadecanoic acid, Linoleic acid

Introduction

There are growing interests in nutrition and management of dairy cows for improving milk components and general health of the cows and responding to the interests of the processors and the consumers. Regards to human health, consumption of specific dietary fatty acids (FA) have been associated with lower risks of some diseases (Jenkins *et al*., 2015). For instance, conjugated linoleic acid (CLA) has potential health benefits such as decreasing the risks of diabetes (Castro-Webb *et al*., 2012), myocardial infarction (Smit *et al*., 2010), and breast and prostate cancers (Heinze and Actis, 2012). The odd-chain saturated fatty acids (OCS-FAs) have been suggested to reduce the risks of multiple sclerosis (Holman *et al*., 1995), Alzheimer's disease (Fonteh *et al*., 2014), type 2 diabetes (Santaren *et al*., 2014), and cancer (Vlaeminck *et al*., 2006). In human, OCS-FAs are thought to originate mainly from dairy fat (Brevik *et al*., 2005) or synthesized endogenously through special metabolic pathways (Jenkins *et al*., 2015), and their serum levels have been used as the markers of the intake of ruminant fat regards to the metabolic risk factors (Smedman *et al*., 1999; Warensjö *et al*., 2009).

Probiotics have been brought up as suitable agents for the improvement of ruminal and intestinal flora, milk yield and components (Vibhute *et al*., 2011; Retta, 2016), and immune status of the animals including cows (Xu *et al*., 2017). A number of studies have documented the effect of multi-strain bacterial preparations of probiotics on the milk FA. A mixture of *Lactobacillus reuteri*, *Lactobacillus alimentarius*, *Enterococcus faecium*, and *Bifidobacterium bifidum* increased the levels of oleic (18:1), linoleic (18:2), linolenic (18:3) acids, and cis-9, trans-11 CLA in goats' milk (Apas *et al*., 2015). A blend of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium thermophilum*, and *E. faecium* increased the contents of butyric and caproic acids in milk fat of ewes without affecting the levels of CLA, vaccenic acid (C18:1-trans 11), and omega-3 FA (Payandeh *et al*., 2017). Besides, some other beneficial

effects have been reported for probiotics used as feed additives. *Lactobacillus casei* and *Lactobacillus plantarum* have increased the milk yield of dairy cows without any substantial effects on milk fat, protein, and lactose (Xu *et al*., 2017). *Bacillus licheniformis* have also improved the milk yield and milk protein (Qiao *et al*., 2010), and *Bacillus subtilis* has increased milk yield in the study of Sun *et al*. (2013).

Bacillus coagulans has been known as a generally safe probiotic capable of enhancing the intestinal microbial flora both in animals and human (Cutting, 2011; Keller *et al*., 2011). The bacterium is a lactic acid spore forming Gram-positive, catalase positive, motile, and facultative anaerobe rod. The species grows well on the nutrient yeast extract salt medium (NYSM) and de Man Rogosa and Sharpe agar (MRS agar) following incubation for 18 h at 37°C. The spore resists the adverse environmental conditions, the low pH of the stomach, and also the bile acids (Hyronimus *et al*., 2000; Hong *et al*., 2005; Cutting, 2011). These characteristics make this probiotic attractive to apply in many areas of food technology (Keller *et al*., 2011).

Some *Bacillus* species probiotics have been reported with some positive responses in dairy cows. *Bacillus licheniformis*, but not *B. subtilis*, improved milk yield and milk protein in the study of Qiao *et al*. (2010). *Bacillus subtilis*, however, improved the milk yield in the study of Sun *et al*. (2013). *Bacillus coagulans* has been reported to increase milk crude protein in Holstein cows via an increase in the casein level without affecting whey proteins (Izadi *et al*., 2020). The present study was carried out to acquire some additional information on the effects of a pure culture of *B. coagulans* PRM101 on the yield of raw milk and energy corrected milk (ECM; computed from milk weight and its fat and protein content) as well as the FA profile of milk fat in Holstein cows under field condition. Antioxidant status of milk and acute phase proteins of blood and milk were also studied with respect to the general health of the animals.

Materials and Methods

Experimental design and samplings

In a commercial dairy farm, 23 lactating cows at various days in milk (DIM=31 to 267) were allocated in a treatment group (n=12; DIM=133 ± 59; parity = 1.8 ± 1.4) and a control (n=11; DIM=147 ± 76; parity = 2.0 ± 1.6) group. The average milk production of the treatment and the control cows at the beginning of the study were 34.42 ± 7.75 kg and 32.55 ± 6.03 kg, respectively (Table 2). Both groups were housed in open sheds, were fed a constant diet for about one month before and during the entire period of the study (63 days), and were milked three times a day. The basal diet (Table 1) was fed *ad libitum* as a total mixed ration (TMR) two times per day. The probiotic *B. coagulans* (PRM101; Parsilact®; Pardis Roshd Mehregan, Shiraz, Iran) was added daily to the TMR of the treatment group at a dose of 2 g per cow (2×10^{11} CFU/head) according to the manufacturer's advice. On days 0, 21, 42, and 63 of the study, composite milk samples (>200 ml) of individual cows were collected at milking times and then the blood samples of the cows were drained (3-4 h after the distribution of the morning feed) from coccygeal vessels in tubes without anticoagulant. The 24 h milk samples of each cow were refrigerated, mixed, and transferred cold to the laboratory and stored (without preservative) at -18°C for further analyses. The blood samples were also transferred to the laboratory in cold boxes. Blood sera were separated by centrifugation at 3000 × g and kept at -18°C for further analysis. The research was performed under the ethical procedure approved by Shiraz University, Shiraz, Iran (IACUC No.: 4687/63).

Milk fat and protein and ECM

Milk fat and protein were measured in fresh milk by Gerber (Kleyn *et al*., 2001) and Kjeldahl (AOAC, 2020) methods, respectively. The recorded milk for each cow

Table 1: The basal ration of the studied cows

Feed ingredients	% DM	Chemical composition	Concentration
Alfalfa hay	20.84	NEl (mcal/kg DM)	1.58
Corn silage	20.41	NDF (% DM)	31.8
Wheat straw	1.83	fNDF (% DM)	21.3
Beet pulp (dry, with molasses)	5.23	Crude protein (% DM)	17.71
Barley grain	13.54	Metabolizable protein (% DM)	10.79
Corn grain	13.11	Ether extract (% DM)	4.07
Wheat bran	2.04	Ca (% DM)	0.82
Vegetable oil	0.86	P (% DM)	0.43
Soybean meal	13.26		
Soybean seeds (roasted)	5.21		
Calcium carbonate	0.57		
Magnesium oxide	0.28		
Mineral mix	0.84		
Vitamin mix	0.28		
Sodium bicarbonate	0.85		
Salt	0.28		
Bentonite	0.56		

DM: Dry matter, NEl: Net energy for lactation, NDF: Neutral detergent fiber, and fNDF: Forage NDF

Table 2: Milk yield, protein, and fat in control and treatment cows

Economic factors	Groups of cows	Days of sampling				Average	P-value (trends of chances)	
		0	21	42	63	Days 21-63	L	Q
Milk (kg)	C	32.55±6.03[a]	30.41±5.02[a]	29.82±6.04[a]	29.01±5.73[a]	31.00±4.70	0.15	0.86
	T	34.42±7.75[a]	33.41±5.24[a]	33.96±6.45[a]	29.49±5.07[b]	32.39±4.58	0.03	0.03
ECM (kg)	C	31.59±7.21[a]	28.91±5.41[a]	27.54±5.31[a**]	26.42±4.69[a]	29.50±4.71	0.08	0.94
	T	33.81±8.39[a]	32.70±5.71[a]	33.60±6.83[a**]	29.27±5.36[b]	31.79±4.86	0.06	0.04
Fat (%)	C	3.44±0.48[a]	3.39±0.31[a]	3.40±0.30[a]	3.41±0.29[a]	3.38±0.29	0.78	0.49
	T	3.34±0.42[a]	3.33±0.37[a]	3.37±0.35[a]	3.38±0.34[a]	3.35±0.33	0.47	0.69
Crude protein (%)	C	2.98±0.13[a]	2.99±0.03[a]	3.01±0.08[a**]	2.99±0.20[a*]	3.00±0.12[*]	0.77	0.75
	T	3.03±0.17[a]	3.02±0.17[ab]	3.12±0.15[a**]	3.13±0.18[ac*]	3.11±0.16[*]	0.03	0.37

[a, b, c] Different letters within rows correspond to significant difference (P<0.05) between sampling days. ECM: Energy corrected milk, C: Control, T: Treatment, L: Linear, and Q: Quadratic. * (P<0.1) and ** (P<0.05) show differences between the control and the treatment groups

was then converted to ECM using the following equation (Tyrrell and Reid, 1965):

ECM = (0.323 × kg of milk) + (12.97 × kg of fat) + (7.2 × kg of protein)

ECM is an index for the amount of energy in the milk, based on the yield of milk and its fat and protein contents, adjusted to 3.5% fat and 3.2% protein.

Milk fatty acid profile

Milk fat was extracted and subjected to gas chromatography (GC) for investigating the probable changes in its FA profile. Briefly, twenty-five ml of the milk sample, corresponding to day 63 of sampling, was freeze-dried (Zirbus Technology, VaCo5, Germany) which yielded more than 2.5 g powder. The modified version of Folch's method was then used (Copley *et al*., 2005) in which 2.5 g of the freeze-dried preparation from each sample was added to a 25 ml chloroform-methanol mixture (2:1, v/v). The mixture was homogenized (2,500 rpm, 30 min) and sonicated for 20 min; then, 10 ml of saturated NaCl solution was added. The suspension was centrifuged at 4,000 rpm for 20 min at -4°C. The chloroform phase was recovered and transferred into a round 25 ml flask, and each fat extract was dried using a rotary evaporator at 45°C under vacuum (Kim *et al*., 2014). Afterwards, 0.5 g of the extracted fat from each sample was treated with 8 ml of methanolic solution of sodium hydroxide (NaOH, 0.5 N) and incubated at 85°C for 10 min. Nine ml of borontrifluoride (14%) was added over 2 min in a cooled flask. Hexane (4 ml) was added to the cooled flask and agitated. The mass up saponification flask was then added using a saturated solution of NaCl and left for 3 min. Fatty acids were extracted after adding 1 g of anhydrous sodium sulfate. The analyses of methyl esters of milk fat were performed by an Agilent Technologies 7890B GC with a 7955A Network mass-selective detector (Agilent Technologies, Waldbronn, Germany). Derivative fat extracts were separated by programmed temperature GC using a capillary column 60 m × 0.25 mm × 0.25 µm coated with DB-23 stationary phase (J&W Scientific, Agilent Technologies, GC conditions: injector temperature 300°C, injection volume 2 µL, split/splitless mode [split ratio 1:50]); the inlet column pressure was 210 kPa of He and the column temperature program was 70°C and it was maintained for 2 min, slope 25°C min-1 until 150°C, slope 5°C min-1 until 240°C and maintained there for 7 min. The separation time was 30 min. Standard mixtures of the identified FA were used for the determination of each peak. The retention time of the unknown sample was then compared to the standard values of each FA.

Milk total antioxidant capacity (TAC) and malondialdehyde (MDA)

Milk TAC and MDA were measured in the milk sera on days 0 and 63, separated after extraction of milk fat and precipitation of casein (HCl 0.1 N), centrifugation at 1000 × g for 3 min, and stored at -18°C. Milk TAC was determined using the commercial kit of ZellBio Company (Germany). The change in the color produced by the chromogenic substrate (tetramethylbenzidine) was measured colorimetrically (mmol/L). The total antioxidant capacity level was finally reported as the level of antioxidant in each sample compared to vitamin C action (as standard), at a wavelength of 490 nm (Hosseini-Zijoud *et al*., 2016). Milk MDA was measured after mixing 1 ml of the milk serum with 9 ml of 0.25 N HCl containing thiobarbituric acid (0.375%) and 15% trichloroacetic acid. Five µL of beta hydroxy-anisole (2% w/v, in absolute ethanol) was added as an antioxidant to the resulting mixture to prevent artificial lipid peroxidation during the assay. The mixture was placed in boiling water for 10 min. The samples were immediately cooled down and centrifuged at 4000 × g for 20 min. The supernatants were finally collected and their optical densities were recorded at 532 nm (Shimadzu UV-1601 spectrophotometer, Shimadzu Corp., Japan) (Basiri *et al*., 2015). Different concentrations of MDA were used to draw the standard curve.

Milk and blood amyloid A (AA) and haptoglobin (Hp)

Milk (days 0 and 63) and blood serum (day 63) levels of AA and Hp were measured by a quantitative sandwich enzyme immunoassay method using commercial cow-specific kits (Shanghai Crystal Day Biotech, Shanghai, China). The analytical sensitivity of this test was determined as 0.0156 mg/ml for Hp and 0.3 µg/ml for AA by the manufacturer.

Statistical analysis

Data (mean±SD) were assessed with the SPSS statistical software (version 16). The trends of changes in milk, ECM, fat, and crude protein were compared between groups using analysis of variance (ANOVA) for repeated measures. Within group changes were studied using the Bonferroni post-hoc test. The differences between groups at specific sampling days were analyzed for various measured parameters with independent sample t-test. The correlations of the measured indices were assessed by Pearson's correlation test. The p-values of <0.05 were considered statistically significant.

Results

The yields of milk and ECM showed decreasing patterns in both groups (Table 2) during the study (63 days) with no significant difference between groups. The treatment cows, however, showed quadratic increases in the levels of milk (P=0.03) and ECM (P=0.04) on day 42 of the study. On day 42, ECM was significantly higher (P<0.05) in the treatment group. The percentage of milk fat was almost similar and constant in both groups during the study (P>0.05). In the control group, crude protein level was rather constant, but in the treatment cows, it increased linearly during the study (P=0.03). On days 42 (P<0.05) and 63 (P<0.10), the milk protein content was higher in the treatment group compared with the control cows. The average milk protein for the whole period of the study tended (P=0.06) to be higher in the treatment cow.

The proportions of FA in milk fat (g/100 g fat) on day 63 of the study are shown in Table 3. The most prominent difference was detected in heptadecanoic acid (C17:0) that was significantly higher (P=0.002) in the treatment group. The other measured odd-chain FA (pentadecanoic acid, C15:0) was not different between groups. Lauric acid (C12:0) tended (P=0.097) to be lower and linoleic acid (C18:2) tended (P=0.077) to be higher in the treatment group compared to the control cows. The other measured FA were not statistically different between groups.

Milk TAC and MDA and the acute phase proteins (AA and Hb) of milk and blood serum were not different between groups (P>0.05; Table 4). There was a negative correlation between milk TAC and MDA in the control group (r=-0.669, P=0.005) but not in the treatment cows (r=-0.010, P=0.97). Heptadecanoic acid of milk showed a negative correlation with milk MDA (r=-0.611, P=0.035) and a positive relationship (r=0.591, P=0.043) with serum Hp in the treatment group.

Table 3: Milk fatty acid profile (g/100 g milk fat) of the control and the treatment cows at the end of the experiment (day 63)

Milk fatty acid	Groups		P-value
	Control	Treatment	
Butyric (C4:0)	2.29±0.08	2.31±0.11	0.648
Caproic (C6:0)	1.22±1.28	0.86±0.21	0.350
Caprylic (C8:0)	1.04±0.34	0.84±0.31	0.160
Capric (C10:0)	2.77±0.46	2.86±0.37	0.602
Lauric (C12:0)	4.75±1.73	3.85±0.43	0.097
Myristic (C14:0)	13.97±1.11	13.95±1.14	0.963
Pentadecanoic (C15:0)	2.13±0.31	2.20±0.29	0.582
Palmitic (C16:0)	32.60±3.98	33.66±2.45	0.445
Heptadecanoic (C17:0)	0.90±0.31	1.51±0.49	0.002
Stearic (C18:0)	7.00±2.39	8.12±1.49	0.184
Palmitoleic (C16:1)	2.77±1.08	2.48±0.59	0.449
Oleic (C18:1)	22.34±1.61	22.42±1.04	0.890
Linoleic (C18:2)	0.53±0.22	0.68±0.15	0.077
Arachidic (C20:0)	0.84±0.39	0.92±0.37	0.651
Other fatty acids	4.85±4.78	3.33±3.39	0.386

Discussion

The higher ECM in the treated cows on day 42 of the study and higher proportions of heptadecanoic acid (C17:0) and linoleic acid (C18:2) were the prominent findings of the present study. The higher ECM in the treatment cows would be partly the result of the positive affection of milk protein by the pure cultures of *B. coagulans* PRM101. In our previous report with a larger number of cows (33 heads), *B. coagulans* increased milk crude protein via an increase in casein without affecting milk yield and milk fat (Izadi *et al*., 2020).

The OCS-FA (C15:0 and C17:0) have been positively associated with human health (Khaw *et al*., 2012; Meikle *et al*., 2013; Jenkins *et al*., 2015). They have been suggested to have similar effects on that of polyunsaturated FA in increasing fluidity of membranes and meeting the homeostatic requirements of membrane functionality and reduce the risks of nervous system diseases (Holman *et al*., 1989; Holman *et al*., 1995).

The higher proportion of C17:0 in milk fat of the treatment cows would be the result of *De-novo* lipogenesis of *B. coagulans* PRM101 in the rumen as

Table 4: Indices of inflammatory and oxidant status in milk and blood of the control and the treatment cows on days 0 and 63 of study *

	Day 0		Day 63	
	C	T	C	T
Milk TAC (mmol/L)	19.69 ± 10.27	18.29 ± 7.33	28.63 ± 13.49	28.06 ± 14.32
Milk MDA (µmol/L)	14.55 ± 0.31	14.57 ± 0.26	14.17 ± 0.53	14.17 ± 0.56
Milk AA (µg/ml)	NM	NM	9.89 ± 0.49	9.80 ± 0.46
Milk Hb (ng/ml)	NM	NM	7.43 ± 0.26	7.60 ± 0.35
Serum AA (µg /ml)	NM	NM	5.15 ± 0.11	5.13 ± 0.13
Serum Hb (ng/ml)	NM	NM	89893 ± 221	89757 ± 824

* Differences are not significant (P>0.05). C: Control, T: Treatment, TAC: Total antioxidant capacity, MDA: Malondialdehyde, AA: Amyloid A, Hb: Haptoglobin, and NM: Not measured

well as the intestine, where it has been shown to be active (Cutting, 2011). The OCS-FA incorporated in milk fat synthesis are synthesized by rumen microbes (Vlaeminck *et al*., 2006), which produce C16:0 and C18:0 as the main FAs (Laliotis *et al*., 2010). These acids are then subjected to α-oxidation, losing their terminal carboxyl group and leaving C15:0 or C17:0, respectively (Jansen and Wanders, 2006). Certain bacteria use propionic acid as a starting compound to produce C17:0 (Dijkstra *et al*., 2011; French *et al*., 2012; Heck *et al*., 2012). Odd-chain saturated fatty acids may make up to 40% of the FA content of some bacterial species (Vlaeminck *et al*., 2006). Negligible amounts of OCS-FA (Croom Jr *et al*., 1981) can also be synthesized endogenously in adipose tissue and mammary gland of ruminants from propionate (Scaife *et al*., 1978; Pfeuffer and Jaudszus, 2016). The proportion of OCS-FA in milk fat has been reported to be about 1.5-2.5% of total FA (Dohme-Meier and Bee, 2012; Stefanov *et al*., 2013) with a ratio of C15:0 to C17:0 of almost 2:1 (Dewhurst *et al*., 2007; Fievez *et al*., 2012).

Regards to the higher proportion of linoleic acid (C18:2) in milk of the treated cows, *B. coagulans* PRM101 may affect the synthesis of this acid within the gastrointestinal tract. Milk linoleic acid (0.3 to 0.6% of total milk FA), occurs as a mixture of CLA isomers formed in the rumen from feed linoleic acid or during the bacterial biohydrogenation of unsaturated FA (Kelly *et al*., 1998; Chouinard *et al*., 1999). The higher linoleic acid could also be attributed to the action of the mammary gland for controlling the melting point of milk fat, physiologically performed through desaturation of FA, synthesis of short-chain FA, and positioning short-chain FA at the sn-3 position of glycerol (Dils, 1986; Jansen and Wanders, 2006). After the increase in C17:0 (melting point: 61.3°C), the formation of C18:2 (melting point: -5°C) may have been increased by desaturation of other C18 FA. The increased level of linoleic acid may also be effective in keeping the plasticity of the secretory cells to secrete liquid fat droplets at body temperature.

Regardless of un-affection of indices of oxidative status and acute phase proteins by the probiotic treatment in the present study (Table 4), the observed correlations (the results section; not shown in tables) could be interpreted as the possible effects of *B. coagulans* PRM101 on improving the health status of the cows through non-specific ways. The negative correlation between TAC and MDA in the milk of the treatment cows (r=-0.669, P=0.005) would be due to reduced conditions of oxidative stress. The negative correlation between C17:0 and MDA in milk (r=-0.611, P=0.035) and the positive relationship between milk C17:0 and serum Hp (r=0.591, P=0.043), both in the treatment group, could be pertained to the desirable effects of C17:0 or other metabolites produced by *B. coagulans* PRM101 on the oxidative conditions and inflammatory processes of the cows, respectively. However, such effects cannot be explained precisely by the results of this study and require further researches. In general, probiotics have been reported to be capable of conferring health benefits on the host animal (McFarland, 2015; Xu *et al*., 2017) through non-specific processes such as improving the resistance to diseases (Sarowska *et al*., 2013), helping eliminate the toxins produced in the metabolic processes (Jouany, 2006; Xu *et al*., 2017) or promoting nutrient absorption (Fecteau *et al*., 2016). Probiotics such as lactobacilli, insert their action possibly via the components of their cell wall (Xu *et al*., 2017) or produce substances such as organic acids and bacteriocins that are antagonistic to other organisms (Pinto *et al*., 2009).

Bacillus coagulans PRM101, when used as a feed probiotic in dairy cows, may improve the proportions of C17:0 and C18:2 FA in milk and the levels of milk protein and ECM. The overall health status of the cows may also be enhanced by reducing the conditions of oxidative stress and inflammatory processes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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