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# INFLUENCE OF TWO PROBIOTIC *LACTOBACILLUS* STRAINS ON CLA CONTENT IN MODEL RIPENING CHEESES

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Key words: *Lactobacillus,* model ripening cheeses, CLA

The aim of the study was to determine the influence of probiotic strains *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* DN-114001 on the content of CLA in model ripening cheeses. Lactococci and lactobacilli count was determined. Model cheeses with probiotic bacteria were subjected to ripening during 8 weeks at a temperature of 6°C. Fatty acids methyl esters including CLA (18:2 *cis*-9, *trans*-11) were analysed using gas chromatography coupled with mass spectrometry. The content of CLA in fresh cheeses was between 680 and 750 mg/100 g of fat. In the investigated model cheeses there were no essential changes in the CLA content during the eight weeks of ripening.

## INTRODUCTION

The occurrence of linoleic acid isomers with conjugated double bonds (CLA) in milk fat of the ruminant animals results from the activity of rumen microflora. Strains of *Butyrivibrio* and *Megasphaera genera* carry out the enzymatic biohydrogenation of unsaturated fatty acids from the group of C18 (linoleic acid, linolenic acid), where 18:2 *cis*-9, *trans*-11 isomer is an indirect product. This acid can also be generated by desaturation of vaccenic acid (*trans*-11 18:1) in mammary glands of ruminants [Bauman *et al*., 1999; Sieber *et al.,* 2004]. In dairy products, 18:2 *cis*-9, *trans*-11 CLA isomer is a predominant CLA isomer in milk fat. Research works from the last decade indicate that some strains of lactic acid bacteria can also possess the ability to convert the linoleic acid to CLA. Those bacteria are usually used as starter cultures of the fermentation processes or as microorganisms with probiotic proprties, *e.g*. *Lactobacillus* and *Bifidobacterium* [Lin, 2000; Alonso *et al*., 2003; Coakley *et al.,* 2003; Sieber *et al*., 2004; Yadav *et al*., 2007]. Most of the authors point that only free form of linoleic acid (LA) can be utilised by bacteria as a substrate in the mentioned biotransformation, however other authors observed that the CLA content increased when the source of LA contained the esterified form of this fatty acid, mostly in acylglycerides, *e.g.* in milk fat or sunflower oil [Alonso *et al*., 2003; Xu *et al*., 2004; Van Nieuwenhove *et al*., 2007]. Thanks to the usage of microorganisms with the capacity of CLA production, functional products with enlarged CLA content could be elaborated.

The aim of this research was to determine the content of CLA in model cheeses after different time of ripening and subsequently to verify whether the addition of probiotic cultures of *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* DN-114001 can influence CLA concentration in the fatty acids constitution in a model of ripening cheeses.

## MATERIALS AND METHODS

**Strains.** Probiotic strains included in this study were: *Lactobacillus acidophilus* La-5 (lyophilized monoculture, Chr. Hansen), *Lactobacillus casei* DN-14001 (isolated from Actimel, Danone). Before inoculation, the strains were cultured in the MRS broth medium using 1.0% inocula and 24 h incubation at 37°C in order to activate the strains. *Lactococcus lactis* subsp. *lactis* R-603 (DVS, Chr. Hansen) was utilised as a starter culture for cheese manufacture. Before inoculation the strains were cultured in the M17 broth medium using 1.0% inocula and 24 h incubation at 30°C.

**Material.** Four model cheese products were made. The control cheese was prepared only with starter cultures of the fermentation process – *Lactococcus lactis* subsp. *lactis (*R-603*).* Other chesses were made as a combination of R-603 with the probiotic bacteria *Lactibacillus acidophilus* La-5 and/or *Lb. casei* DN-114001. Combinations of microorganisms in model cheeses are shown in Table 1. Model cheeses were produced in aseptic conditions. Ingredients for model cheese production – cream UHT (30%), skim milk powder, NaCl (2% of model cheese mass) and sodium citrate (0.3% of model cheese mass) were weighed to Schott's glass bottles (1 L capacity). Then, 250 mL of sterile water was added. The content of protein to fat was standardized (1:1). After warming to 31°C temperature in a water bath, the cheese mass was inoculated with bacterial cultures. For cheeses, inoculum of *Lactococcus lactis subsp. lactis* was between 7.77 log and 8.11 log. In the case of *Lactobacillus* strains the inoculum was

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TABLE 1. Combination of microorganisms in a model of ripening cheeses.

| Letter symbols of model cheeses | Combination of microorganisms in model cheeses |
|---|---|
| A (control) | *Lactococcus lactis* subsp. *lactis* R603 |
| B | *Lactococcus lactis* subsp. *lactis* R603 + *Lactobacillus casei* DN-114001 |
| C | *Lactococcus lactis* subsp. *lactis* R603 + Lactobacillis acidophilus La-5 |
| D | *Lactococcus lactis* subsp. *lactis* R603 + *Lactobacillus casei* DN-114001 + Lactobacillis acidophilus La-5 |

about $10^7$ cfu/g of the model cheese mass. The model cheese masses were thermostated at 31°C for additional 40 min. Then, rennet was added (1:13000, Marzyme, Chr. Hansen). After curd formation (about 50 min) the curd was cut and scalded (41°C/ 45 min). So prepared model cheeses were subjected to ripening for 8 weeks at a temperature of 6°C. The final mass of the model cheese was approx. 600 g. Samples for microbiological and physicochemical analyses were taken every second week of ripening (in 2, 4, 6, 8 week) beginning with the time 0, which means after cheese production. Samples intended for fatty acid methyl ester (FAME) analyses were kept at a temperature of – 21°C until analysed.

**Survival assay.** *Lactococcus* count was determined using 10-fold serial dilutions and M17-agar of pH 7.2 (Merck). Aerobic incubation was carried out at 30°C±1°C for 72 h. *Lactobacillus* count was determined using 10-fold serial dilutions and MRS-agar, pH 5.4. The gas packages for anaerobic cultivation were used (Anaerocult, Merck). Anaerobic incubation was carried out at 42°C for 72 h. Microbiological analyses were performed in duplicate for each cheese. Results were given as log cfu/g of the model cheese.

**Chemical analysis.** Physicochemical analyses of fresh model cheeses (at time 0) were performed according to Polish Standard [PN-73/A-86232]. The nitrogen analysis was performed with the Kjeldahl method and the protein content was calculated using 6.38 factor. Fat content was estimated with Gerber method and the calculation of the fat content in the dry mass was made. Content of the water and dry mass was analysed by drying at 102°C. Measurement of pH was carried using a pH- meter LPH33OT, TOCUSSEL.

Fatty acid methyl ester analysis was performed according to Christie [1993] in triplicate for model cheeses from each time of ripening. Before the fat extraction, 500 $\mu$g of internal standard (triglyceride of heneicosanoic acid, Nu-Chek Prep.) was added to test samples. Fat was extracted with chloroform:methanol (2:1 v/v) and the saturated KCl solution was added. After vortexing, the chloroform layer was collected and the solvent was evaporated under nitrogen. Next, fat was dissolved in hexane. Transesterification of fatty acids was performed with 0.5 mol/L KOH in methanol (37°C /30 min). FAMEs, including CLA (18:2 *cis*-9, *trans*-11), were analysed using gas chromatography coupled with mass spectrometry (GC/MS-QP2010, Shimadzu) using a polar column SP-2560 (100 m x 0.2 $\mu$m x 0.25 mm), Supelco. Samples were injected in splitless mode in following conditions of GC: injector temperature: 240°C, column programme temperature: start temperature: 40°C, the isotherm of minutes, then temperature rise about 10°C/min to 160°C, isotherm of 20 min, following the rise of the temperature about 4°C/min to 180°C – isotherm 20 min and temperature rising 6°C/min to 230°C – isotherm 15 min. Helium was used as a carrier gas with the flow rate of 1.10 mL/min. Conditions of the mass spectrometer: ion source temperature 200°C; interface 200°C; energy of ionization 70eV, detector voltage 1.13 kV. The range of scanning of the quadrupole mass filter 50- 500 m/z. The identification of CLA (18:2 *cis*-9, *trans*-11) was done comparing the retention time (RT) and mass spectrum with standard of this isomer (Nu-Chek Prep.) Quantitative calculations were performed on the basis of internal standard. Results were given as mg/100 g of fat of model cheese.

**Statistical analysis.** Analyses of significant statistical differences for CLA content in samples of cheese from different times of ripening were estimated with analysis of variance ($\alpha$= 0.05) using Statgraphics 4.1. Each analysis was performed in triplicate. The presented results were obtained from one series of cheeses made.

## RESULTS AND DISCUSSION

Produced model cheeses contained about 23% of fat in dry matter. The water constituted approx. 58% of the cheese mass. That characteristics made those models similar to semi-skimmed soft cheeses. The content of protein was about 11% of cheese mass. After producing the cheeses (time 0), pH was at a level of 6.5-6.4 units for the control cheese and cheeses with probiotic bacteria. During the ripening, pH decreased as a result of the fermentation and the production of organic acids. In the eighth week, pH of cheese with *Lactococcus lactis* (model A) was about 5.7 while that of cheeses with *Lactobacillus* strains pH attained the value of approx. 5.3 (models B, C) and over 5.5 for model D.

The number of viable cells of lactococci after the production of cheeses was at a similar level in all models, approx. $1x10^8$ cfu/g of the cheese (Figure 1). After 2 weeks of ripening, the number increased by about 1 log cycle in all investigated cheeses, attaining the level of 8.5 log cycles. After the mentioned time of ripening the number of viable cells of lactococci decreased in control cheese by 0.2 log cycle after eight weeks. Another tendency was observed in models with probiotic bacteria. In models where *Lb. acidophilus* and *Lb. casei* where added (separately or in combination), after 4 weeks of ripening, the number of lactococci increased again. It was by 0.7 log cycle till 8 week of ripening, but in model D, where all microorganisms were added, the rate of viable cells number increase was slower as compared with models B and C. According to literature, those relations could depend on the metabolic activity of nonstarter *Lactobacilli* that could contribute to liberation of components necessary for *Lactococcus* growth, *e.g.*, amino acids [Muehlenkamp-Ulate & Warthesen, 1999]. At this stage of investigation we did not determine the proteolytic activity of the used strain but it will be done in the future. Ong *et al.* [2006] pointed out that addition of probiotic adjunct to Cheddar cheese did not affect primary proteolysis,

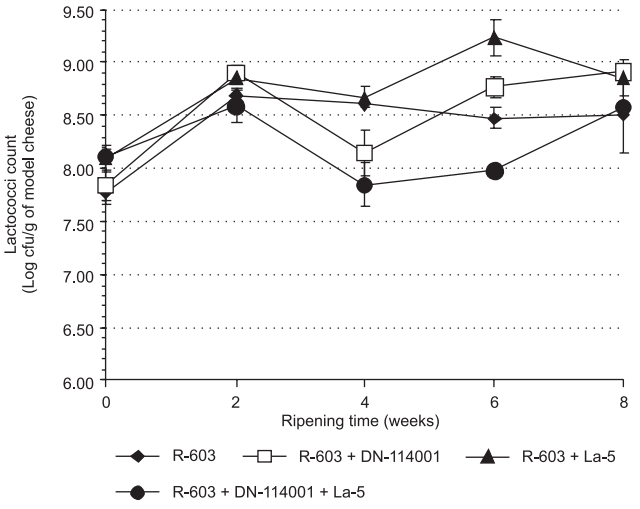


FIGURE 1. Changes of lactococci count during ripening of model cheeses at 6°C.

but the level of secondary proteolysis, which was related to significantly higher amino acid concentration. Vinderola *et al.* [2002] noticed possible stimulation between probiotic bacteria and starter cultures. In the applied conditions of ripening of model cheeses we did not observe any inhibitory influence of *Lactobacillus* strains on *Lactococcus* starter bacteria.

Probiotic bacteria remained viable during the whole period of ripening at a temp. of 6°C. Figure 2 shows changes in viable counts of lactobacilli during cheese ripening. After cheeses manufacture, the number of lactobacilli was about 7.30 log cycle. In model B, where *Lb. casei* were incorporated and in model D with R603, *Lb. casei* and *Lb. acidophilus,* until 2 weeks of ripening a reduction was observed in the number of lactobacilli. Decrease in the number of these bacteria was from approx. $3x10^7$ cfu/g of model cheese (time 0) to about 6.4 of log cycles. After this process we noticed a slow increase in the count of viable cells of *Lactobacillus* to approx. 7.2 log cycles in the discussed chesses. In model C (*Lb. acidophilus*), during the period of ripening, the number of viable cells remained at a rather stable level (approx. $10^7$ cfu/g). The greatest increase of the number of lactobacilli from about 7.3 log to above 8.3 log appeared in model D, where *Lactobacillus casei* and *Lb. acidophilus* were present. Results of *Phillips et al.* [2006] showed good survival of probiotic bacteria, *i.e.* $10^6$-$10^7$ cfu/g of the cheese after 32 weeks of ripening. All probiotic-containing chesses developed by Ong *et al.* [2006] showed a similar pattern, which means that ripening cheeses could be a good vehicle for health-promoting bacteria.

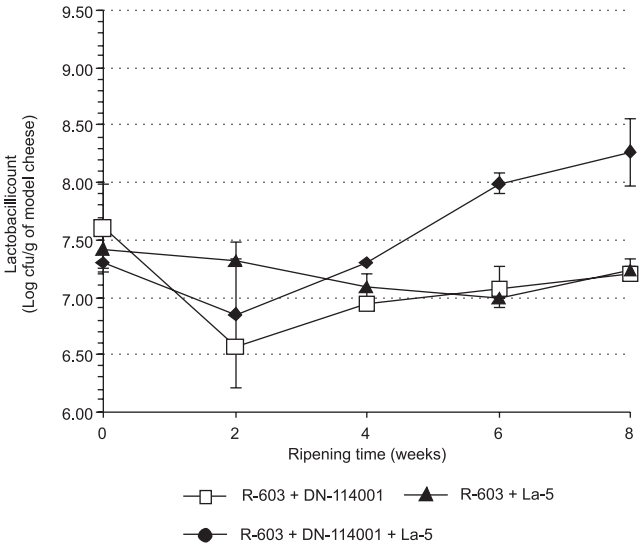


FIGURE 2. Changes of lactobacilli count during ripening of model cheeses at 6°C.

The CLA (18:2 *cis*-9, *trans*-11) content after model cheeses preparation was between 680 mg/100 g of the fat, in model C and D, and 750 mg/100 g of the fat of rest cheeses. Figure 3 presents results for CLA analysis in model cheeses during ripening. The conjugated linoleic acid content in fat of cheeses with *Lb acidophilus* was lower comparing with CLA content in fat of the other cheeses. There is no unequivocal answer which indicates the reason of that change. The content of CLA has not been analysed in the cheeses matrix before the fermentation process, which means before curd formation. These results would be helpful in interpretation of received findings now. It seems that possible alteration of the CLA content could follow during the fermentation, because during cheeses ripening there were no statistically significant changes. In cheese with *Lb. acidophilus* the pH after the fermentation process (time 0) was 6.37, when in the cheese with *Lb. acidophilus* and *Lb. casei* it reached 6.32. Higher pH was noticed in control cheese (6.5) and in cheese with *Lb. casei (*6.40). These findings could point to higher metabolic activity of microorganisms in cheeses with *Lb. acidophilus.* There is possibility that the activity could influence lipolytic changes, and, as a consequence, the hydrolysis of ester bonds of CLA in acylglycerides. In this case, CLA isomers could be liberated as free fatty acids. The methylation procedure used in the research did not methylate the free fatty acids to methyl esters, which were subjected to chromatographic analysis. Also some chemical or oxidative factors could affect the CLA concentration. Further investigation will be carried to verify the discussed changes and to determine their character.

Zlatanos *et al.* [2002] made a comparison of CLA content in different types of cheeses. According to their results,

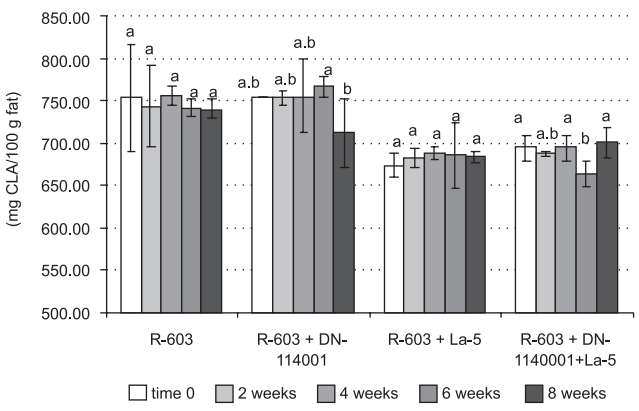


FIGURE 3. Changes of CLA (18:2 *cis*-9, *trans*-11) content in fat of model cheeses ripening at 6°C. Explanatory notes: a, b– the same letters at average values for model cheese indicate lack of the statistical significant differences ($p>0.05$). For each cheese analyses were made in triplicate.

usually cheeses contain from 450 to 950 mg of CLA per 100 g of fat. Jiang *et al.* [1997] noticed the CLA concentration in cheddar cheeses produced with the addition of different strains of *Lactococcus lactis* was between 500 and 700 mg/100 g of fat. Differences could be due to different CLA content in milk used during cheese production, different production conditions, starter cultures used or conditions and lengths of the time of cheeses ripening. However the mentioned authors did not notice any essential influence of production conditions of cheeses and the kind of microorganisms used on the content of CLA in Swedish ripening cheeses. Das *et al*. [2005] suggested that in complex cheese matrix, fatty acids may not have a good contact with bacteria cells which influence biochemical changes. Also low water content could affect bacterial isomerase activity. Shantha *et al*. [1992] observed increasing concentration of C18:2 *cis*-9, *trans*-11 isomer in ripening cheeses. They postulated that the content of CLA isomers was probably affected by different factors of the processing. The concentration of acids with conjugated bound (approx. 8.8 mg CLA/g of fat) in ripening cheeses was higher as compared with not processed milk – below 1 mg CLA/ 1 g of fat [Lin, 2000]. Fritshe *et al.* [1999] observed that in Emmenthal cheeses produced by them with probiotic bacteria addition the quantity of CLA slightly increased during ripening. In investigation of Yadav *et al.* [2007] the CLA content was significantly increased in probiotic dahi samples during fermentation. The authors produced their yogurt without addition of external linoleic acids, which indicated that *Lactobacillus* strains could utilize milk fat as a substrate for CLA bioproduction, being higher lipolytic comparing with traditional dahi cultures. CLA amount depended on the adjunct strain used by Van Nieuwenhove *et al.* [2007]. For buffalo cheeses with *Lactobacillus* and *Bifidobacterium,* CLA content was similar to that of raw milk, but increased during ripening. In the present work, in the investigated cheeses there were no significant changes in the CLA content during the eight weeks of ripening at the storage temperature of 6°C. These research take into account only the analysis of fatty acids occurring in the cheese fat in esterified form, mainly as triglycerides. Some authors noticed that lactic acid bacteria can produce CLA from fatty acids bounded in triglycerides, that is why the first step of our work was to investigate potential changes only in the content of esterified fatty acids. Results of this work can show that the applied *Lactobacillus* strains do not utilize unsaturated fatty acids in the form of esters for CLA biosynthesis or conditions of cheese ripening were not favourable for the strains applied. The isomerase of the linoleic acid is situated in cytoplasm and the bacterium cell membranes and shows substrate specificity to the system of double bonds in the configuration of *cis*-9, *cis*-12. Probably isomerase demands the presence of free carboxyl groups of fatty acids [Bauman *et al*., 1999]. The death or lysis of bacterial cells facilitate release of intracellular enzymes and could facilitate the course of reactions catalyzed with them.

At this stage of research and under ripening conditions applied we did not notice any significant influence of the applied *Lactobacillus* strains on the CLA content in the investigated model cheeses. Further studies should be carried to find lipolysis level and possible subsequent conversion of milk fat to CLA by the microorganisms used, also under ripening conditions more convenient for *Lactobacillus* strains.

## CONCLUSIONS

1. There was no inhibitory influence of *Lactobacillus* strains on *Lactococcus* starter bacteria in the applied conditions of cheese ripening.

2. Probiotic bacteria remained viable during the whole period of ripening of produced model cheeses and their count was between $10^7$-$10^8$ cfu/g of cheese after 8 weeks.

3. The lower CLA content was noticed in cheeses with *Lb. acidophilus* compared with cheeses without addition of La-5, which needs verification.

4. In the investigated model cheeses we did not ascertain any significant changes in CLA content during the eight weeks of ripening at the storage temperature of 6°C.

5. To verify the received results, additional analyses should be done during the cheese ripening, especially analysis of free fatty acids composition.

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## WPŁYW DWÓCH PROBIOTYCZNYCH SZCZEPÓW Z RODZAJU *LACTOBACILLUS* NA ZAWARTOŚĆ CLA W MODELACH SERÓW DOJRZEWAJĄCYCH

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W niniejszej pracy podjęto próbę określenia wpływu wybranych kultur probiotycznych *Lactobacillus acidophilus* La-5 i *Lactobacillus casei* DN-114001 na zawartość CLA w puli kwasów tłuszczowych modelowych serów dojrzewających. Analizowano również zmiany liczby żywych komórek mikroorganizmów zastosowanych do produkcji serów modelowych. Sery modelowe dojrzewały przez 8 tygodni w temperaturze 6°C. Estry metylowe kwasów tłuszczowych, w tym CLA (18:2 *cis*-9, *trans*-11) oznaczano metodą chromatografii gazowej sprzężonej ze spektrometrem masowym. Po wytworzeniu serów zawartość CLA wynosiła w granicach 680–750 mg/ 100 g tłuszczu. W czasie ośmiotygodniowego dojrzewania w temperaturze 6°C w badanych serach modelowych nie stwierdzono istotnych zmian w zawartości CLA.