

DOCTORAL (PhD) DISSERTATION THESES

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**THE EFFECT OF PRODUCTION PARAMETERS ON
FATTY ACID COMPOSITION, MAINLY ON THE
CONJUGATED LINOLEIC ACID CONTENT OF MILK
AND DAIRY PRODUCTS, MANUFACTURED IN
SEKLER'S COUNTY**

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1. BACKGROUND OF THE RESEARCH, OBJECTIVES

In the last decade an intense research activity was effectuated in the area of prebiotics and probiotics possessing highly significant benefic effects on human health. These researches aim the attention on conjugated linoleic acid (CLA) mainly found in the milk and muscle of the ruminant animals, and implicit, in the dairy products obtaining by milk processing. These compounds had been proved to have benefic healthy effects, since by their regular consumption may be prevented and in some case, even repressed.

From our food palette, mainly the milk and the dairy products have possessed such special fatty acid composition, out of which many fatty acids are health protective. Among these, high importance may be attributed to short chain fatty acids and especially CLA, with many demonstrated pharmacologic effects. Our research was focused on studies of milk, made by two major considerations. One hand, the concentration of the CLA is the highest in milk, caused by microbiological processes from rumen, and other hand, this is one of the consumed foods in high volumes by the population. The high consumed quantities are results of tradition and the relatively low price, which confer accessibility to a large circle of consumers.

A number of researchers has studied the possibilities of increase the CLA contents of dairy products. Among the proposed methods can be mentioned the utilization of selected microbial strains in fermentation (pure or mixed cultures), addition of high linoleic acid content substrate, and the optimal selection of technology involving thermal processes. Related to mentioned researches, we attended to analyze the CLA content of milk as well as the fatty acid composition of dairy products (fermented milk varieties, cheese and butter). In parallel, we analyzed the fatty acid composition of butter and margarine consumed in Szekler region and we followed the variation of fatty acid in cheeses during the maturation and storage process.

The main research objectives of the present dissertation can be grouped and defined as follows:

1. The fatty acid composition and the CLA content of the milk fats obtained from cows with different genotype, as well as the variation of milk fat composition during the lactation period.

2. The effect of different bacterial cultures mixture and substrates on fatty acid composition and CLA content of dairy products.

2.1. The effect of selected pure bacterial cultures on fatty acid composition and CLA content.

2.2. The effect of sunflower oil – as a high linoleic acid containing substrate – on CLA content.

2.3. The effect of linoleic acid addition of the substrate on CLA content.

3. The effect of production technology on fatty acid composition and CLA content of the dairy products.

3.1. Comparison of the fatty acid composition of the different brands of butters and margarines.

3.2. The effect of conventional and microwave thermal treatment on fatty acid composition of dairy products.

4. The variation of fatty acid composition of cheeses during the storage.

2. MATERIALS AND METHODS

2.1. The examined cow races, collection of milk probes and the probe storages

The milk samples were collected from selected 9 cows throughout a year from two cow genotype: Black Holstein Friesian, Hungarian Simmental and throughout nine months for Red Holstein Friesian (from three animals from each race). During the summer period (from 10th May until 15th October) the animals were kept on the pasture and the grass was supplemented by necessity with fodder that contained dairy concentrate, corn and offal wheat. During winter alfalfa and grass hay were supplied ad libitum and the diet consisted of 3.5 kg of dairy forage, sliced sugar-beet, corn silage and mineral supplementation. The bulk milk of the individual cows was obtained by manual milking sampled and in the case of each breed three milk samples were drawn that means 100 cm³ of milk per probe. The samples were immediately cooled with water and stored frozen at -85 ± 1 °C until the chemical analysis.

2.2. The obtaining of the fermented dairy products

For obtaining of the fermented dairy product, as raw material a milk mixture aquired by Lactis Company. The pasteurization was realised at the temperature of 78 °C in 50 second. The pasteurized milk was inoculated with pure or mixed culture of *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus lactis* subsp. *cremoris*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Lactobacillus acidophilus* and *Bifidobacterium lactis*. The mixed culture was obtained in conformity of comertialized and applied variations in dairy product

industry. In the fermentation process the optimal temperature and fermentation time combination was applied, to maintain the optimal multiplying and growth condition for the bacterial consortia.

In the forthcoming stage the CLA producing capacity of pure strains of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum* was investigated, using additivated substrate with sunflower oil (linoleic acid content: 62.7 rel. %). In 100 cm³ of pasteurized milk a variable amount (50, 100, 150, 200, 300, 400, 600, 1000 and 1500 µl) of sunflower oil and 1 cm³ mother souring mixture was added. The probe was incubated throughout 24 hour in isothermic condition (38 °C), and the pH, cell number and fatty acid composition were measured.

2.3. The determination of fatty acid composition and CLA content of butter and margarine probes

The fatty acid composition and CLA content of four types of butter and twenty type of margarine was measured. The products were procured from local commerce in Harghita County. The fat content of the butter varied between 65–80% (w/w), and of margarines between 25–67% (w/w).

2.4. The microwave-processed milk and dairy products

The examined samples was the following: a) milk sample with fat contents of 3.6%(w/w); b) commercially obtainable cheeses: 1) Dalia (semi-hard, obtained by mixed coagulation with rennet addition and acidulation); 2) Telemea (a type of feta cheese, fermented with *Lactobacillus acidophilus*, coagulated with enzymatic rennet); c) a commercially obtainable butter of trade name Alpenbutter with fat content of 82% (w/w). The combination between of heating power and treatment duration (for microwave heat treatment) and the heating time

(for conventional heat plate) was chosen as usual in daily kitchen technologies. Conventional heat treatment was carried out on a laboratory hot plate, for 2 and 8 min, respectively, after the boiling point was reached. For microwave treatment we applied a treatment over 1, 2, 4, and 8 min using a microwave oven (electrical power of 450 W). Subsequent to the heat treatment the samples were cooled down immediately and stored at -85 ± 1 °C until prepared for analysis.

2.5. The variation of fatty acid composition of cheeses during the storage

In order to establish the variation of fatty acid composition of the cheeses during the storage, four types of cheese (Dalia, Rucăr, Penteleu and Telemea) were procured from the Primulact Factory, where the processing technology is the standard industrial method usually applied in Szekler region. Probes were collected throughout a period of five months in intervals of three weeks. The cheeses were stored at the temperature of $+4\pm 1$ °C, since the prevailing probes at the temperature of -85 ± 1 °C until prepared for analysis. All the probes were analysed together at the finish of the storage experiment.

2.6. The determination of fatty acid composition and the CLA content of milk and dairy products

The homogenized sample (containing about 0.5–1 g fat) was weighed into a flask, concentrated hydrochloric acid was added and it was extracted with petrolether, followed by evaporation to dryness of the organic phase. Then sodium hydroxide in methanol was added, and the mixture was boiled and trans-esterified with methanolic boron trifluoride. From dehydrated probe an amount of 0.5–1 µl was injected into the gas chromatograph. The standard for fatty acid methyl esters „, Supelco 37

component FAME Mix” was purchased from Sigma-Aldrich. The gas chromatographic condition: Instrument: Varian 3380 CP gas chromatograph. Column: 100 x 0.25 mm, CP-Sil 88 (FAME) phase. Detector: FID 270 °C. Injector: splitter, 270 °C. Carrier gas: H₂, 235 kPa. Temperature program: 140 °C for 10 min; at 10 °C/min up to 235 °C; isotherm for 26 minutes.

For determination of the CLA content in sample preparation two methods were used:

◆ *Methylation with sodium methylate*

The fats was extracted with a mixture of hexane:*i*-propanol 3:2 (vol), the extract was filtered on membrane filter, and the anhydrided filtrate evaporate to dryness on rotavapor apparatus. One aliquot from the hexane base solution was treated with methanolic sodium methylate. The reaction mixture was extracted with water, and from dried hexane phase a probe was taken for chromatographic determination of the CLA content.

◆ *Methylation with sodium methylate (NaOCH₃) and boron trifluoride catalysator mixture (BF₃)*

The probes were homogenized and 100 µl methanolic sodium methylate solution was added, stirred and heated on water bath and was treated with methanolic boron trifluoride solution. After finishing the transesterification 500 µl hexane and NaCl was added. and well stired. The phase separation was enhanced with centrifugation. Finally, for chromatographic analysis a probe from hexanic phase was preveled.

The standard of CLA methyl ester used for preparation of the original solution and for calibration series was purchased from Sigma-Aldrich. The gas chromatographic apparatus and the column were presented earlier. The applied temperature program: maintain at 140 °C for 10 min; gradient at 5 °C/min up to 235 °C; and finally isothermic for 30 minutes.

2.7. Microbial analysis

During the study of CLA production capacity of pure strains *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum* using sunflower oil additivated milk as substrate, we effectuate bacterial counting also. For microbial counting the Breed method was applied: A known volume of microbial cell suspension is spread uniformly with the inoculating needle over a glass slide covering a specific area. The smear is then dried in air and fixed by heating with flame. An alcoholic defatting and a methylene blue staining was applied. The stained preparate was examined microscopically under oil immersion lens, and the cells are counted.

2.8. Statistical analysis

The evaluation of the results was carried out by SPSS for Windows 16.0 (SPSS Inc., 2006) statistical program package. For demonstration that the fatty acid composition of the milk fat was influenced by whether the seasons or the cow species a multifactor variance analysis was applied.

The difference between the fatty acid composition of milk products, fermented by starter culture mixtures, and that of the raw milk was analysed by one factor variance analysis. For comparison of the mean of the variables the Dunett test was applied at 5% confidence level.

The heat induced change in the fatty acid composition of fermented milk products, produced with sunflower oil and with different starter cultures, and different milk and milk products and margarine was evaluated by one factor variance analysis. The Student-Newmann-Keuls-test was applied for comparison of the means of the investigated variables at 5% significance level. The statistically significant change in the fatty acid

composition during the storage was investigated by one factor variance analysis. The Student-Newmann-Keuls-test was applied for comparison of the means of the investigated variables at 5%-os significance level.

3. RESULT AND DISCUSSION

3.1. The variation of fatty acid composition and the CLA content of the milk fats obtained from cows with different genotype during the lactation period.

Examined the milk fatty acid composition of cow's milk from the genotypes Black Holstein Friesian, Red Holstein Friesian and Hungarian Simmental, no significant difference was observed. By study of the seasonal effects on milk composition, we obtain that the seasonal variation of fatty acid composition is significant ($P < 0.05$), except some components, namely lauric, miristoleic, pentadecanoic and palmitoleic acids. Higher fluctuation was observed for CLA composition, this phenomenon may relate to seasonal variation of forage composition and with the difficulties of the analytical determination. Briefly, we found, that the majority of saturated fatty acids present minimal values in summer period, and maximal values in winter and early spring period. The concentration of unsaturated fatty acids (including CLAs) is higher in summer period and in all case their minimal values are reached in winter and early spring. Except the aforementioned compounds, the seasonal difference between the summer and winter period is significant ($P < 0.05$). The maximum of the CLA content was reached in August, a mean value for the cow races was 1.35%. In the period of June-September the CLA content of the all probes exceeded 1.2%, and decreased monotonic in autumn, to the final value 0.75–0.80% in winter. As results from above, the milk collected in summer – indifferent from genotype – contains substantial higher amount of linoleic, linolenic, oleic acids and CLA, in comparison with the winter and early spring collected milk. For this reason the milk collected in the summer possesses higher benefic effects on human health, and appropriate for human consumption. In conclusion, the higher CLA content of milk collected in the summer may be explained by the higher unsaturated fatty acid

content of the fresh forage and the effect of higher level of UV irradiation given by outdoor pasture.

3.2. The fatty acid composition and the CLA content of fermented dairy products

3.2.1. Fermented dairy products obtained with selected pure bacterial strains

Comparing the fatty acid composition of the raw milk, pasteurized milk, and fermented milk obtained by seven different pure and mixed (in different variation) bacterial strains probes, can conclude that the composition of the raw and pasteurized milk is identical within the margin of error of the analytical method. The processing of the experimental data (the fatty acid composition of the fermented products) by one-way analysis of variance and comparing with composition of raw milk, result that the microorganism does not have any significant impact on the fatty acid composition of the products for the majority of fatty acids.

Except a few minor fatty acids, for all examined probes the results were practically overlapped and the measured significant differences are so low, that these do not present any influence on nutritional value of the fermented dairy products. A slightly more deviation from the control was obtained using mixed culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* with *Lactobacillus acidophilus*. In this case, the fermented product contains significantly higher quantity of short-chain and middle-chain fatty acids and linoleic acid respectively, comparative with the raw milk. For each mixed culture occur few (one or two) minor fatty acid, for which quantity differ significant from their content in the raw milk, but for us interested CLA no significant difference from control was detected.

From above presented results we conclude, that the CLA content of the raw milk decreases neither during the pasteurization, nor after the inoculation process, since the CLA content of the fermented products was quite the same like the initial CLA content of the raw material.

3.2.2. The effect of sunflower oil addition of the substrate on CLA content of fermented dairy products obtained with selected bacterial strains

By examination of CLA production of three *Lactobacillus* strains (*L. acidophilus*, *L. actobacillus casei* and *L. plantarum*) from substrate with added sunflower oil in different concentration, we demonstrate that there is an optimal value for additive quantity for all of the three selected pure strains. At added linoleic acid in optimal quantities the CLA production of the strains was maximal. The initial concentration in CLA of the raw milk substrate was 118 mg/100 g fat, and during pasteurization, this value remained quite unchanged. The different strains had different response to substrate-added sunflower oil, as follows: for *Lactobacillus acidophilus* the maximum concentration of CLA (178–180 mg/100 g fat) was obtained at additive concentration of 100–150 μ l oil/100 ml substrate, for *Lactobacillus plantarum* the correspondent values was 189 mg CLA/100 g fat at 100 μ l oil/100 ml substrate. The further increase of oil quantity for both strains a continuous decrease in CLA production was observed: for *Lactobacillus acidophilus* to minimal level of 84–90 and for *Lactobacillus plantarum* to 115–117 mg CLA/100 g fat. In the case of *Lactobacillus casei* already at 50 μ l/100 ml oil additive result the final CLA concentration (139–140 mg/100 g fat) and this value level remained almost constant, independent of further linoleic acid addition.

Based on experimental results, we conclude that there are some lactobacterial strains which produce a weak response to substrate addition with sunflower oil (*Lactobacillus casei*), and other strains which increase their CLA production (*Lactobacillus acidophilus* and

Lactobacillus plantarum) and may not to be excluded the existence of strains for that the CLA production are inhibited by addition of higher quantity of linoleic acid, decreasing the final CLA content of the final product. Based on our experiments, the addition of sunflower oil before fermentation in 100 µl oil/100 ml substrate, in case of industrially utilized strains, the optimal CLA production may be obtained.

3.3. The fatty acid composition and the CLA content of butter and margarine brands

The fatty acid composition and CLA content of four types of butter and twenty type of margarine were measured. The mean contents in saturated fatty acid of margarine was 33.2%, and of butters was 64.6%, expressed as relative weight (or chromatographic) percent of total fatty acids. The butters may contain high amount of short-chain fatty acids, in contrast to margarines, where they occur only in traces. The margarines contain in average 66.5% of unsaturated fatty acid and the butters only 35.0%. The main fatty acid in both products was oleic acid, in quantities of 26.95% (butter), and 36.19% (margarines). The margarines contain higher amount of linoleic acid in comparison with butters, although no important difference was observed for compositions in other unsaturated fatty acids. The elaidic acid content of butters was 1.81% and that of margarines 0.99%. The margarines, practically do not contain CLA, butters contain in average 1% CLA.

Accordingly, comparing the composition of the two nutritional fats result, that the butter contain substantially more saturated fatty acid and oleic acid than margarine, since in margarines dominates the linoleic acid. The elaidic trans fatty acid occurs both in butter and margarines only in low concentration. The margarines do not contain CLA at all, since the CLA content in butter is practically equal with the CLA content of the milk fat.

3.4. The variation of fatty acid composition of milk and dairy products produced by conventional and microwave thermal treatment

The fatty acid composition of the examined heat treated milk and dairy products (cheeses Dalia and Telemea, butter with 80% fat content) was measured. Neither at conventional (electric heating plate), nor at microwave (microwave oven) heating no substantial changes (except the oleic and elaidic acid) were detected, this does not imply any risk for human health. The combination between of heating power and treatment duration (for microwave heat treatment) and the heating time (for conventional heat plate) was chosen as usual in daily kitchen technologies. Heat treatment was carried out for 2 and 8 min, respectively, the applied energy level does not lower substantially the amount of the essential fatty acids, and there should not be any concern about the formation of any harmful artifact during the heat treatment processes. In case of oleic-elaidic acid isomerisation, the increase of elaidic (trans configuration) and decrease of oleic (cis configuration) content were observed in both heat processing cases. Prolonged heat processing treatment may cause important increase of elaidic acid quantity.

3.5. The variation of fatty acid composition of cheeses during the storage

By determination of CLA content variation during the storage of three semihard (Dalia, Rucăr, Penteleu) and one feta type (Telemea) cheese, we found the following: for Dalia the initial CLA content (0.047 g CLA/100 g fat) rise to 0.115 g CLA/100 g fat at the final of the week 21 of storage. For Penteleu these values were 0.034 and 0.089 and for Rucăr

0.042 and 0.128, respectively. In the case of Telemea we obtained values between 0.038 to 0.199 g CLA/100 g fat.

For Rucăr sortiment, the concentration of conjugated linoleic acid changed significantly ($P < 0,05$) during storage periode, in contrary with the other investigated cheese sortiments, but after 18th week storage the similar decreasing tendency was observed like for the other sortiments.

By statistical analysis of the mean value of the CLA content of the studied cheese sortiments may be affirmed, that between the Dalia and Penteleu sortiments do not exist significant differences ($P < 0,05$). In contrary the sortiments Rucăr and Telemea differs significantly from the rest of the investigated cheese sortiments.

For every cheese sortiment, the maximal value of CLA content was obtained in the 15th-18th week of storage, and after this period the CLA content remained constant or decreased.

4. CONCLUSION

4.1. The variation of fatty acid composition and the CLA content of the milk fats obtained from cows with different genotype during the lactation period

Following the seasonal variation of the milk fat composition of three cow races (Simmental, Red Holstein Friesian and Black Holstein Friesian), we were unable to establish any significant difference between the fatty acid content of milk obtained from these three different cow genotypes kept in same housing and feeding conditions. In contrast, for all studied races, the seasonal variation in milk composition is important. The amount of saturated fatty acids, in general, shows a minimum in summer and has maximal value in winter and early spring. For unsaturated fatty acids, including CLA, the opposite tendency was shown: maximum values in summer (august) are reached. The value in summer was significantly higher than in winter and early spring (an increase of 60–70% compared with the winter minimal value). The consumption of the summer milk, in conclusion, is healthier, given the higher CLA and unsaturated fatty acid content.

4.2. The variation of fatty acid composition of fermented dairy products during the steps of processing technology

For the compositional change of fermented dairy products obtained with examined lactobacterial cultures (pure and mixed) we do not find any important change compared to the original composition of the raw material, despite the fact, that for some minor components, the variation was significant. The CLA content also remained unchanged during the

fermentation process. The CLA content of milk remain unchanged both during thermal pasteurization, and the fermentation following inoculation, this processes don't affect the CLA content.

By addition of sunflower oil some of the used lactobacterial strains CLA contents increased by 35–40%, but other part only by 20%. For two strains the addition 50–150 $\mu\text{l}/100\text{ cm}^3$ oil was the optimal value, and for further addition the CLA production decreased considerably. In the case of one strain, the supplementation below the optimal value doesn't change the CLA content.

4.3. The fatty acid composition and the variation of composition during the stages of obtaining process of milk, butter and margarine

We established that the butter contains significantly higher quantity of saturated fatty acids, oleic acid and CLA, like the margarines (with main components linoleic and oleic acid). During the thermal processing the fatty acid content of the examined products (milk, butter, chees), except the oleic and elaidic acid content, did not change substantially. Although, after a prolonged thermal processing the quantity of oleic acid decreased, parallely with increase of elaidic acid content, but the changes are not so pronounced, to present any risk for human health.

4.4. The variation of fatty acid composition of cheeses during the storage

During the storage period (between the semihard cheese sortiments) the highest CLA value was obtained for the Rucăr sortiment, and the lowest for the Penteleu sortiment. By statistical analysis of the mean value of the CLA content of the studied cheese sortiments may be affirmed, that between the Dalia and Penteleu sortiments do not exist significant differences ($P < 0,05$). In contrary the sortiments Rucăr and Telemea differs significantly from the rest of the investigated cheese sortiments.

The variation tendency of the CLA contents during storage periode concentration of conjugated linoleic acid was the same for all sortiments: until the 18th week the CLA content increase, and after that decrease, but significant changes was evidentiated only for Rucăr sortiment.

5. NEW SCIENTIFIC RESULTS

We established that the seasonal variation of cow's milk composition in fatty acids of the cow's race breeds in Hungary (Hungarian Spotted, Black Holstein-Friesian and Red Holstein-Friesian) is important. In the summer, the cow's milk contains significantly higher quantity of unsaturated fatty acids, including CLA, in comparison with winter period. We did not find any significant difference between the fatty acid content of milk obtained from the different cow races maintained in same housing and feeding conditions.

The examined industrially applied bacterial cultures for milk fermentation in Harghita County do not change the original fatty acid composition of the milk fat, and the CLA content also remains unchanged during the fermentation process.

By addition of substrate with sunflower oil rises the CLA production of the *Lactobacillus* strains. In the cases of some bacterial cultures the optimal amount of the supplemented linoleic acid was established. The overdosing of oil may cause significant decrease in CLA contents.

During the conventional and microwave thermal processing with variable duration of milk, dairy products and the margarines, a decrease of oleic acid and a slightly increase of elaidic acid contents were observed, but the changes are so small, that they do not present any risk health.

The CLA content of the examined cheeses (except Rucăr brand) was not significantly affected by the storage time. During the storage of cheese the CLA contents initially increase, and after attaining a maximum, an abrupt decrease follows.

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