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Effect of microorganisms on free amino acid and free D-amino acid contents of various dairy products

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Abstract. Free amino acid and free D-amino acid contents of milk samples with different microorganism numbers and composition of dairy products produced from them were examined. Total microorganism number of milk samples examined varied from 1.25×10^6 to 2.95×10^6. It was established that with an increase in microorganism number concentration of both free D-amino acids and free L-amino acids increased, however, increase in D-amino acid contents was bigger considering its proportion. There was a particularly significant growth in the microorganism number range of 1.5×10^6 to 2.9×10^6. Based on analysis of curds and cheese samples produced using different technologies we have come to the conclusion that for fresh dairy products and for those matured over a short

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time there was a close relation between total microorganism number and free D-amino acid and free L-amino acid contents. At the same time it was found that the ratio of the enantiomers was not affected by the total microorganism number. For dairy products, however, where amino acid production capability of the microbial cultures considerably exceeds production of microorganisms originally present in the milk raw material, free amino acid contents of the milk product (both D- and L-enantiomers) seem to be independent of the composition of milk raw material.

1 Introduction

From our earlier examinations [4, 5, 9] it is obvious that free amino acid and free D-amino acid contents of milk are significantly influenced by the technology, in the first place, however, by the microbiological condition of milk raw material. It is known that D-stereoisomer amino acids are not or not easily utilized by the human organism, their harmful effects were reported in several publications [2, 3, 6, 7]. It is also known that presence of D-amino acids in the proteins reduces digestibility and in bigger volumes they can act as growth inhibitors [8]. In nutritional scientific respect an important fact is that D-amino acids and peptides containing D-amino acids have a different taste than the corresponding L-stereoisomers [1].

In case of countries recently joined the European Union milk manufacturers are reduced to occassionally produce various dairy products complying with the standards out of milk with microorganism number of several millions considered to be unsuitable for human consumption in EU countries. Because of the above we aimed at examinating of total free and free D-amino acid contents of milk with various total microorganism numbers in order to establish a relationship between microorganism number and total free and free D-amino acid contents of milk. Subsequently we were trying to answer the question how free amino acid contents of milk raw material influenced free amino acid composition of dairy products manufactured from it.

2 Material and methods

2.1 Milk samples examined

Milks with different total microorganism numbers and dairy products were obtained from a dairy company in Székelyland out of those mixed milk samples from which the company produced consumption milk and various dairy products. Total microorganism number of obtained milk samples varied from 1.23×10^6 to 2.95×10^6. As a control sample milk with total microorganism number less than 100.000 was used, obtained from the cattle farm of the University of Kaposvár, Faculty of Animal Science, and which was taken from a mixed milk of around 100 Holstein-Friesian cows having lactation milk production of around 10.000 liters. Subsequent to the sampling and determination of the total microorganism number milk samples were cooled down to $-25\,^\circ$C and kept at this temperature until the preparation for chemical analysis.

2.2 Determination of total microorganism number

For determination of the microbe number direct counting of the bacteria was applied. The milk sample taken into a sterile test tube was thoroughly mixed through a rapid rotation movement. A 1/10 dilution was prepared (for the dilution 0.85% sodium chloride solution was used that was sterilized in autoclave beforehand). One cm^3 of the pasteurized milk sample was added to $9\,\text{cm}^3$ of sterile dilutant water, then $1\,\text{cm}^3$ of the thoroughly mixed diluted sample was pipetted onto a sterile Petrifilm plate with a culture medium. The Petrifilm plate was incubed at $37\,^\circ$C for 24 h, and the developed colonies were directly counted with the use of a culture counter.

2.3 Dairy products examined

The examined dairy products included yoghurt, Sana, curds and some types of cheeses (Telemea, Dalia and Rucăr), all obtained from a Transylvanian dairy company for analysis. The company documentation showed which dairy product from milk of what average total microorganism number was produced, so the examined products could be sorted one by one as per microorganism number.

Sana is a soured dairy product, manufactured by lactic acidic coagulation of milk using a lyophilized culture mixture (consisting of *Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus lactis diacetilactis).* Telemea is a feta-type cheese, produced by mixed coagulation i.e. using both a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactobacillus bulgaricus),* and rennet (chymosin). During its production Telemea is matured over 2 days in a brine of 20–21%, at 12–14 °C. Dalia is a semi-hard cheese, produced by mixed coagulation, using a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus acidophilus)* and rennet (chymosin). Dalia is matured over 2 weeks at 14 °C at a relative humidity of 75–80%. Cow's curds were produced by mixed coagulation using a lyophilized culture mixture (consisting of *Streptococcus thermophilus, Lactococcus lactis lactis, Lactococcus lactis cremoris, Lactococcus diacetilactis)* and chymosin.

Out of dairy products examined, curds, yoghurt, Sana and Telemea are considered as products matured over a short time, while cheeses Dalia and Rucăr as products matured over a longer time. The examined milk products were manufactured by keeping the Romanian standards and specifications as well as hygienic regulations.

2.4 Sample preparation

Preparation of milk and dairy products for analysis. Preparation of the samples was carried out at the University of Kaposvár, Faculty of Animal Science, Department of Chemistry and Biochemistry. In case of cheese sample analysis, as much cheese was homogenized with distilled water so that the dry matter contents of the mixture obtained similarly to milk be between 12–15%. Subsequently, the completely milk-like homogenized samples were treated as they had been milk samples. The milk samples stored deep-frozen were after defrosting and warming up to 30 °C centrifuged at 8.000 g for 10 min in order to remove the cellular elements and milk fat. Subsequently, to 25 cm^3 of sample 25 cm^3 of 25% trichloroacetic acid were added, left standing for 20 min, and centrifuged at 10.000 g for 10 min. The supernatant was poured down and its pH was adjusted to be 7 with 4.0 M NaOH. The obtained solution was lyophilized at −10 °C, and the residue (pH = 7) was solved in sodium acetate buffer for determination of total free amino acid contents. Prepared samples were stored at −25 °C until analysis.

Determination of total free amino acids and free D-amino acids. Determination of free amino acid and free D-amino acid contents were carried out using a Merck-Hitachi HPLC instrument, for collecting and evaluating the measured data D-7000 HPLC System Manager software was used.

For determination of total free amino acids cyclic derivatives were formed from the amino acids with *o*-phthaldialdehyde and 2-mercaptoethanol, the formed derivatives were separated on a Licrospher (C-18) analytical column (dimensions: 125×4 mm; particle size: 4m μm) using a gradient system consisting of methanol and sodium acetate buffer. Derivatives were detected at an excitation wavelength of 325 nm and emission wavelength of 420 nm. For determination of free D-amino acids diastereomer derivatives were formed from the amino acid enantiomers with *o*-phthaldialdehyde and 1-thio-β-D-glucose tetraacetate, the enantiomers were separated in the above described system on a Superspher (C-8) analytical column using a gradient system consisting of methanol, acetonitrile and phosphate buffer, the derivatives were detected at an excitation wavelength of 325 nm and at emission wavelength of 420 nm.

3 Results

Free L-amino acid and free D-amino acid contents of milks with various total microorganism numbers by 50.000 CFU, are presented in *Table 1.*

Table 1: Free L-amino acid and free D-amino acid contents of milks with different total microorganism numbers (mg/100 g sample) and proportion of D-amino acids ((D/D+L)×100)

Total CFU $\times 10^6$	Amino acid								
	Aspartic acid			Glutamic acid			Alanine		
	L	D	ratio	L	D	ratio	L	D	ratio
0.1	0.12	0.015	11.11	0.96	0.053	5.23	0.32	0.043	11.85
1.23	0.34	0.042	10.99	1.22	0.084	6.44	0.67	0.102	13.21
1.53	0.54	0.087	13.88	1.47	0.124	7.78	0.91	0.235	20.52
2.00	0.84	0.145	14.72	2.79	0.455	14.02	1.69	0.454	21.17
2.20	0.88	0.257	22.60	2.80	0.715	20.32	1.85	0.942	33.73
2.95	1.48	0.321	21.97	4.53	1.534	25.30	4.83	2.419	33.37

CFU: Colony Forming Unit

It was established that in the control milk sample proportion of D-aspartic acid to total free aspartic acid was 11.11%, proportion of D-glutamic acid was 5.23%, and that of D-alanine was 11.85%. In case of samples with total microorganism numbers between 1.25×10^6 and 1.53×10^6 there was no substantial change in the quantity of either free L-amino acids or free D-amino acids, although both concentration of free L-amino acids and proportion of D-amino acids grew continuously with increasing total microorganism number. This minimal change continued up to total microorganism number of 2.20×10^6 where there was an explosion in both total free amino acid quantity and free D-amino acid quantity, and this sudden increase also applied to the proportion of D-amino acids to the total free amino acids. It appears that up to a microorganism number of 1.5×10^6–1.6×10^6 there are no significant changes in free amino acid and free D-amino acid contents of milk. Afterwards, subsequent to a short period there is an explosion. In summary, in case of each examined free amino acids concentration of both free D-amino acids and free L-amino acids increases, however, increase of D-amino acids is bigger in its proportion considered since for aspartic acid compared to the control milk up to the microorganism number of 2.95×10^6 this proportion increased from 11.11% to 21.97%, for glutamic acid from 5.23% to 25.30% and for alanine from 11.85% to 33.37%.

After having determined the development of milk raw material composition as a function of microorganism number, we examined what effect the increased quantity of free D- and L-amino acids had on the composition of dairy products produced from this raw milk. Again, it was focused on aspartic acid, glutamic acid and alanine since these three amino acids are contained in peptidoglycan that compose cell wall of bacteria, and when released they give a major part of D-amino acid contents of milk products. After bacteria die, subsequent to the lysis these amino acids contribute to the formation of taste, aroma and nutritional value of dairy products. Knowing the relationship between total microorganism number of milk raw material and D-amino acid concentration it can be assumed that the milk raw material can affect composition of dairy product manufactured from it. In order to prove this hypothesis composition of 4 Sana, 4 Dalia, 3 Telemea, 2 curds, 1 Rucăr and 1 yoghurt, manufactured from 4 milks with different total microorganism numbers was examined. We do not want to draw any definitive conclusions from our examinations because of the low sample number in case of curds, Rucăr and yoghurt, results are published here only for orientation. Results are presented in *Table 2.*

The four Sana were manufactured from milks with total microorganism numbers of 1.23, 1.35, 1.53 and 2.95×10^6. Based on the obtained results the conclusion can be drawn that with increasing total microorganism number of milk raw material the quantity of both D- and L- enantiomers increases for all the three amino acids. This increase becomes substantial after a microorganism number of 1.5×10^6 as Sana produced from milk with total microorganism number of nearly 3×10^6 contains the most of both L- and D-amino acids. No significant changes could be experienced regarding D- and L-ratios of the individual amino acids. Proportion of D-glutamic acid is the least with 22.4–26.4%, followed by that of D-aspartic acid with 31.3–32.4%, and by that of D-alanine with 37.6–41.9%.

For the cheese Dalia, free amino acid contents of cheeses produced from

Table 2: Free L-amino acid and free D-amino acid contents (mg/100 g sample) of dairy products manufactured from milk with various total microorganism numbers and proportion of D-amino acids ((D/D+L)×100)

Total CFU $\times 10^6$	Dairy products	Amino acid								
		Aspartic acid			Glutamic acid			Alanine		
		L	D	ratio	L	D	ratio	L	D	ratio
1.228	Sana	0.552	0.251	31.34	1.624	0.583	26.41	0.698	0.462	39.81
1.351	"	0.567	0.259	31.42	2.144	0.619	22.39	0.861	0.519	37.63
1.530	"	0.725	0.320	30.64	2.548	0.834	24.65	1.265	0.790	38.42
2.945	"	1.132	0.543	32.43	4.556	1.542	25.09	1.735	1.251	41.90
1.250	Dalia	13.419	5.593	29.42	42.535	12.791	23.12	21.706	15.621	41.85
2.000	"	15.309	6.142	28.63	43.049	12.852	22.99	26.379	17.601	40.02
2.800	"	16.754	6.231	27.11	48.247	13.439	21.85	27.347	17.803	39.43
2.912	"	15.170	6.324	29.42	41.381	13.516	24.62	24.816	17.004	40.66
1.320	Telemea	0.861	0.389	31.14	3.057	0.752	19.73	1.688	1.071	38.81
1.664	"	1.027	0.428	29.42	3.493	0.841	19.41	1.904	1.223	39.12
2.200	"	1.504	0.610	28.99	3.212	0.935	22.54	1.973	1.349	40.60
1.560	Curds	0.081	0.038	32.14	0.458	0.109	19.23	0.187	0.124	41.62
1.684	"	0.101	0.051	33.51	0.492	0.112	18.54	0.213	0.133	38.43

CFU: Colony Forming Unit

milk with total microorganism number of 1.25; 2.00; 2.80 and 2.91×10^6 were analyzed. Proportion of D-aspartic acid varied from 27.11 to 29.42%, that of D-glutamic acid from 21.85 and 24.62%, and appeared to be, similarly to aspartic acid, independent of microorganism number of milk raw material. Percentage of D-alanine exceeded with the exception of one sample 40%, ranging between 39.43 and 41.85%.

In case of Telemea, products manufactured from milks with total microorganism numbers of 1.32; 1.66 and 2.20×10^6 were analyzed. In this total microorganism number range with the exception of L-glutamic acid there was an increase for all amino acids and enantiomers, but since the total microorganism number range was not wide enough in this case, definitive conclusions similar to those in case of the two previous dairy products could not be drawn from our investigations. Similarly to the previous two cheeses, percentage of D-glutamic acid was found to be the lowest with 19.73–22.54%, whereas quantity of D-aspartic acid ranged between 28.99–31.14%, and proportion of D-alanine between 38.81–40.60%. It appears that in case of Telemea there is no relation between total microorganism number of milk raw material and the examined products manufactured from the milk raw material.

In case of the two curds, one Rucăr, and one yoghurt of course no conclusions can be drawn on the effect of microorganism number. Compared the amino acid composition of the curds to that of all of the other dairy products it can be established that the quantity of both D- and L-amino acids is less by almost one order of magnitude than that of the other products examined, while proportion of the D-amino acids shows only a slight difference compared to the others.

Summarized the results of our investigations, we can say that in case of the milk raw material with increasing total microorganism number concentration of both free D-amino acids and L-amino acids increases, however, the increase for the D-amino acids is bigger its proportion regarded since compared to the control sample the ratio of D-amino acids increases to a multiplied value.

Examined the relationship between the quality of dairy products manufactured from milk raw material of different total microorganism numbers it was established that the percentage of D-amino acids to the total free amino acid contents was not affected by either the total microorganism number of the milk raw material or the fact what kind of dairy it was about. Proportion of D-aspartic acid was found to be around 30% for most of the examined dairy products, although in case of Sana and the curds this was a little more, while for Dalia somewhat less. Percentage of D-glutamic acid varies between 18–27%, this ratio is higher for Sana than for Dalia and the lowest for Telemea. Proportion of D-alanine is around 40% for each dairy products independently of total microorganism number of the milk. Out of the examined three amino acids proportion of D-glutamic acid is the smallest, that of D-alanine is the biggest, while D-aspartic acid has a value between these two, nearer to that of D-glutamic acid.

For fresh dairy products and for those are matured for a short time (Sana, yoghurt, curds, Telemea) a relationship can be established between total microorganism number and D-amino acid contents and this relation applies in most cases also to the L-enantiomers. Despite the fact that total microorganism number has a substantial effect on concentration of both enantiomers, ratio of the enantiomers is not affected by the total microorganism number. For those dairy products, however, which are matured over a longer time and for those where amino acid production capability of microbial cultures significantly exceeds production of microorganisms originally present in the milk raw material no effect of the milk raw material can be expected, thus, free amino acid contents of the milk products seem to be independent of the composition of milk raw material.

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