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EFFECT OF TOTAL GERM NUMBER IN RAW MILK ON FREE AMINO ACID AND FREE D-AMINO ACID CONTENT OF VARIOUS DAIRY PRODUCTS

DJELOVANJE BROJA UKUPNIH MIKROORGANIZAMA SIROVOG MLIJEKA NA SADRŽAJ SLOBODNIH AMINOKISELINA I SLOBODNIH D-AMINOKISELINA RAZLIČITIH MLIJEČNIH PROIZVODA

Csilla Albert[1], Katalin Lóki[2], Szidónia Salamon[1], Beáta Albert[1], Péter Sára[2], Jánosné Csapó[2], János Csapó [1,2]

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ABSTRACT

In the course of our researches we examined the free amino acid and free D-amino acid content of milk samples with different germ numbers and composition of dairy products made from them. Total germ number of milk samples examined varied from $1.25 \cdot 10^6$ to $2.95 \cdot 10^6$. It was established that with an increase in germ number the concentration of both free D-amino acids and free L-amino acids increased, however, increase in the D-amino acid content was bigger considering its proportion. There was a particularly significant growth in the germ number range of $1.5 \cdot 10^6$ to $2.9 \cdot 10^6$. In the course of analysis of curds and cheese samples produced using different technologies we have came the conclusion that in fresh dairy products and in those matured over a short time there was a close relation between total germ number and the free D-amino acid and free L-amino acid contents, ratio of the enantiomers was not affected by the total germ number, however. In dairy products, however, where amino acid production capability of the microbial cultures considerably exceeded production of microorganisms originally present in the milk raw material, the free amino acid content of the milk product (both D- and L-enantiomers) seemed to be independent of the composition of milk raw material.

Keywords: D-amino acids, free amino acids, dairy products, germ number, milk, cheese

INTRODUCTION

From our earlier examinations (Csapó et al., 1986; 1995; Pohn and Csapó, 2002) it is obvious that the free amino acid and free D-amino acid content of milk is significantly influenced by the technology, in the first and 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 have been reported in several publications (Gandolfi et al., 1992; Brückner and Hausch, 1990 a, b; Fuse et al., 1984). 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 (Man and Bada, 1987). 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 (Boehm and Bada, 1984).

[1] MSc Csilla Albert, MSc Szidónia Salamon and MSc Beáta Albert - Sapientia Hungarian University of Transylvania, Csíkszereda Campus, Csíkszereda, RO-4100 Szabadság tér 1
[2] MSc Katalin Lóki, MSc Péter Sára[2], Dr. Jánosné Csapó[2] and Prof.Dr.János Csapó - University of Kaposvár, Faculty of Animal Science, Kaposvár, H-7400 Guba S. u. 40

In the countries recently joining the European Union milk producers are reduced to occasionally produce various dairy products complying with the standards of milk with germ number of several million considered to be unsuitable for human consumption in EU countries. Because of the above we aimed at examining the total free and free D-amino acid content of milk with various total germ numbers on the one hand in order to establish a relationship between germ number and the total free and free D-amino acid content 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 made from it.

MATERIAL AND METHODS

Milk samples examined

Milk with different total germ 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 germ number of obtained milk samples varied from $1.23 \cdot 10^6$ to $2.95 \cdot 10^6$. As a control sample milk with total germ 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 germ number milk samples were cooled down to –25°C and were kept at this temperature until the preparation for chemical analysis.

Determination of total germ 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 by rapid rotation. A 10^{-1} dilution was prepared (for the dilution 0.85% sodium chloride solution was used sterilized in autoclave beforehand). 1 cm^3 of the pasteurized milk sample was added to 9 cm^3 of sterile dilutant water, and then 1 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 °C for 24 h, and the developed cultures were directly counted with the use of a culture counter.

Dairy products examined

From the dairy company yoghurt, Sana, curds, and cheeses of types Telemea, Dalia and Rucăr were obtained for analysis. The company documentation showed which dairy product milk of what average total germ number was made from, so the examined products could be sorted as per germ number. Out of dairy products examined curds, yoghurt, Sana and Telemea were considered for products matured over a short time while cheeses Dalia and Rucăr as products matured over a longer time. The examined milk products were made by keeping the Rumanian standards and specifications as well as hygienic regulations.

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 cheese sample analysis, so much cheese was homogenized with distilled water that the dry matter content of the mixture obtained was similar to milk between 12–15%. Subsequently, milk-like homogenized samples were treated like milk samples. The milk samples stored deep freeze were after defrosting and warming up to 30 °C centrifuged at 8.000 g for 10 min in order to remove the figural elements and milk fat. Subsequently, to 25 cm^3 of sample 25 cm^3 of 25% trichloro acetic acid was 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 the total free amino acid content. Prepared samples were stored at –25 °C until being analyzed.

Determination of total free amino acids and free D-amino acids

Determination of the free amino acid and free D-amino acid content was 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-mercapto-ethanol, the formed derivatives were separated on a Licrospher (C18) analytical column (dimensions: 125·4 mm; particle size: 4μm) 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 in a Superspher (C8) 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.

RESULTS

The total free and free D-amino acid content of milk with various total germ number by 50.000 total germ number units is shown in Table 1.

Table 1. Total free amino acid and free D-amino acid content of milks with different total germ numbers (mg/100 g sample) and proportion of D-amino acids/(D/D+L)·100/

Tablica 1. Ukupan sadržaj slobodnih aminokiselina i slobodnih D-aminokiselina u mlijeku s različitim ukupnim brojem mikroorganizama (uzorak mg/100 g) i omjer D-aminokiselina /(D/D+L)·100/

Germ numbers / Broj mikro-organizama 10^6	Amino acid / Aminokiselina								
	Aspartic acid / Asparti nska kiselina			Glutamic acid / Glutaminska kiselina			Alanine / Alanin		
	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

It was established that the L-aspartic acid content of the control milk sample was 0.12 mg/100 g, the D-aspartic acid content as 0.015 mg/100 g and the proportion of D-aspartic acid to total free amino acids was 11.11%. The L-glutamic acid content of the same sample was 0.96 mg/100 g, the D-glutamic acid content 0.053 mg/100 g, the proportion of D-glutamic acid was 5.23%. The L-alanine content of the control sample was 0.32 mg/100 g, the D-alanine content was 0.043 mg/100 g, and proportion of D-alanine was 11.85%. In case of samples received from the dairy company between total germ number of $1.25 \cdot 10^6$ and $1.53 \cdot 10^6$ 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 germ number.

This minimal change continued up to total germ number of $2.20 \cdot 10^6$ where there was an explosion in both total free amino acid quantity and free D-amino acid quantity, and this sudden increase also referred to the proportion of D-amino acids to the total free amino acids. It appeared that up to the germ number of 1.5–1.6 million there were no significant changes in the free amino acid and free D-amino acid contents of milk. Afterwards, subsequent to a short period there was an explosion. In summary, in case of each examined free amino acids concentration of both free D-amino acids and free L-amino acids increased, however, increase of D-amino acids was higher in its proportion considered since for aspartic acid compared to the control milk up to the germ number of $2.95 \cdot 10^6$, 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 germ number, in the next phase of our research we examined what effect increased quantity of free D- and L-amino acids had on the composition of dairy products from this raw milk. As in examination of he free amino acid content of milk, in milk products the focus was on aspartic acid, glutamic acid and alanine since these three amino acids are in peptidoglycan which makes the cell walls of bacteria, and when released they make a major part of the D-amino acid contents of milk products. After bacteria die, subsequent to the lysine these amino acids contribute to the formation of taste, aroma and nutritional value of dairy products. Knowing the relationship between total germ number of milk raw material and D-amino acid concentration it can be assumed that the milk raw material can affect composition of dairy products made from it. In order to prove this hypothesis composition of 4 Sana, 4 Dalia, 3 Telemea, 2 curds, 1 Rucăr and 1 yoghurt made from 4 milks of different total germ number was examined. We do not want to draw any definitive conclusions from our examinations because of the low sample number in the case of curds, Rucăr and yoghurt, so the results are published here only for orientation. Results are shown in Table 2.

Table 2. The total free and free D-amino acid content (mg/100 g sample) of dairy products made from milk with various total germ numbers and proportion of D-amino acids/(D/D+L)·100/

Tablica 2. Ukupan sadržaj slobodnih kiselina i slobodnih D-aminokiselina (uzorak mg/100) mliječnih proizvoda od mlijeka s različitim ukupnim brojem mikroorganizama i omjerom D-aminokiselina (D/D+L) · 100

Germ numbers/ Broj mikro-organizama 10^6	Dairy products/ Mliječni proizvod	Amino acid / Aminokiselina								
		Aspartic acid / Aspartinska kiselina			Glutamic acid / Glitaminska kiselina			Alanine / Alanin		
		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	3.324	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

The four Sana cheeses were made from milk with the total germ number of 1.23, 1.35, 1.53 and 2.95 million. For the three cheeses produced from milk of lower total germ numbers the free L-aspartic acid content ranged between 0.55-0.73 mg/100 g, while in the raw milk with total germ number of 2.95 million it increased to 1.13 mg/100 g. In the same samples the D-aspartic acid contents varied between 0.25-0.32 mg/100 g, its highest value was 0.54 mg/100 g for the product made of raw milk with the highest germ number. The proportion of the D-aspartic acid in the total aspartic acid content varied from 31.3 to 32.4%. The quantity of L-glutei acid was 1.62 mg/100 g in Sane produced from raw milk with the lowest total germ number, then up to a germ number of $1.5 \cdot 10^6$ it increased to 2.55 mg/100 g, and in the raw material with the highest total germ number to 4.56 mg/100 g. In the same samples quantity of D-glutei acid increased from 0.58 mg/100 g to 0.83 mg/100 g, and for the milk raw material with the highest total germ number to 1.54 mg/100 g. The proportion of D-glutei acid varied between 22.4 and 26.4%. The quantity of L-almandine increased in samples produced from raw materials with total germ numbers listed before from 0.70 mg/100 g to 1.27 mg/100 g, and in the producer made of milk with the highest total germ number to 1.74 mg/100 g. The quantity of D-almandine was in these samples 0.46; 0.79 and 1.25 mg/100g, the proportion of D-almandine in the total quantity of almandine varied between 37.6 and 41.9%.

Thus, for Sane the conclusion can be drawn that with increasing the total germ number of milk raw material the quantity of both D- and L-enantiomers increases in all the three amino acids, and this increase becomes substantial after the germ number of $1.5 \cdot 10^6$ as Sana produced from milk with total germ number of nearly 3 million contains the most of both L- and D-amino acids. No significant changes could be experienced regarding D- and L-ratios within the individual amino acids. The proportion of D-glutamic acid is the least within total free amino acids with 24-25%, followed by that of D-aspartic acid with 30-32%, and finally by that of D-alanin which is almost 40%.

In the cheese Dalia, the free amino acid content of cheeses produced from milk with total germ number of 1.25; 2.00; 2.80 and $2.91 \cdot 10^6$ was analyzed. the free L-aspartic acid content in the cheese made from milk with the lowest total germ number were 13.42; in the cheese produced from milk with the highest total germ number it was 15.17 mg/100 g, whereas the concentration of D-aspartic acid was 5.59 and 6.23 mg/100 g, respectively.The percentage of D-aspartic acid varied from 27.11 to 29.42%. Practically there was only a little difference in the L-glutamic acid content of Dalia cheeses produced from milk with different total germ number, it varied from 41.38 to 48.25 mg/100 g, the same applies to the quantity of D-glutamic acid which ranged from 12.79 to 13.52 mg/100 g. The proportion of D-glutamic acid varied from 21.85 and 24.62%, and appeared to be similar to aspartic acid independent of germ number of milk raw material. In the cheese Dalia the quantity of L-alanine varied from 21.71 to 27.35 mg/100 g; that of D-alanine from 15.62 to 17.80 mg/100 g. The percentage of D-alanine exceeded with the exception of one sample of 40%, having ranged between 39.43 and 41.85%. Having examined the three D-amino acid proportions we have come to the conclusion similar to that of Sana since the proportion of free D-glutamic acid to total free glutamic acid varied from 21.85 to 24.62%, that of D-aspartic acid varied from 27.11 to 29.42%, and for D-alanine from 39.43 to 41.85%.

In Telemea products made of milk with total germ numbers of 1.32; 1.66 and 2.20 millions were analyzed. In this total germ number range, with the exception of L-glutamic acid there was an increase in all amino acids and enantiomers, but since the total germ number range was not wide enough, definitive conclusions similar to those in the case of the two previous dairy products could not be drawn from our investigations. In the total germ number examined the quantity of L-aspartic acid was 0.86–1.50 mg/100 g; that of D-aspartic acid 0.39–0.61 mg/100 g, the quantity of L-glutamic acid was 3.06–3.49 mg/100 g; that of D-glutamic acid 0.75–0.94 mg/100g, the quantity of L-alanine was 1.69–1.97 mg/100 g; that of D-alanine 1.07–1.35 mg/100g. Similarly to the previous two cheeses percentage of D-glutamic acid was found to be the lowest with 19.73–22.54%, whereas the quantity of D-aspartic acid ranged between 28.99–31.14%, and the proportion of D-alanine between

38.81–40.6%. It appears that in Telemea there is no relation between total germ number of milk raw material and the examined products made from milk raw material.

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

CONCLUSIONS

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

Having examined the relationship between the qualities of dairy products made from milk raw material of different germ number and total germ number it was established that the percentage of D-amino acids in the total free amino acid content was not affected by either the total germ number of milk raw material. or the fact what kind of dairy it is about. The proportion of D-aspartic acid was found to be around 30% for most of the examined dairy products, although in the case of Sana and the curds this was a little higher, while for Dalia somewhat lower. Percentage of D-glutamic acid varied between 18–27%, this ratio was higher in Sana than Dalia and the lowest in Telemea. The proportion of D-alanine was around 40% for each dairy product independently of total germ number of the milk. Out of the examined three amino acids the proportion of D-glutamic acid was the smallest, that of D-alanine is the biggest, while D-aspartic acid had a value between these two, nearer to that of D-glutamic acid.

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

REFERENCES

1. Boehm, M.F., Bada, J.L. (1984): Racemization of aspartic acid and phenylalanine in the sweetener aspartame at 100 °C. Proc. Natl. Acad. Sci., 81. 5263-5266.
2. Brückner, H., Hausch, M. (1990a): D-amino acids in dairy products: Detection, origin and nutritional aspects. I. Milk, fermented milk, fresh cheese and acid curde cheese. Milchwissenschaft, 45. 357.
3. Brückner, H., Hausch, M. (1990b): D-amino acids in dairy products: Detection, origin and nutritional aspects. II. Ripened Cheeses. Milchwissenschaft, 45. 421.
4. Csapó, J., Martin, T.G., Csapó-Kiss, Zs., Stefler, J., Némethy, S. (1995): Influence of udder inflammation on the D-amino acid content of milk. Journal of Dairy Science, 78. 2375-2381.
5. Csapó, J., Csapó-Kiss, Zs., Máté, J., Juricskay, I. (1986): Kísérletek a masztitiszes tej részarányának meghatározására elegytejekből. Állattenyésztés és Takarmányozás, 35. 337.
6. Fuse, M., Hayase, F., Kato, H. (1984): Digestibility of proteins and racemization of amino acid residues in roasted foods. J. Jpn. Soc. Nutr. Food Sci., 37. 348.
7. Gandolfi, I., Palla, G., Delprato, L., DeNisco, F., Marchelli, R., Salvadori, C. (1992): D-amino acids in

milk as related to heat treatments and bacterial activity. J. Food Sci., 57. 377-379.

8. Man, H., Bada, J.L.(1987): Dietary D-amino acids. Ann. Rev. Nutr., 7. 209-225.

9. Pohn, G., Csapó, J. (2002): Free D-amino acid content of milk from mastitic udder. Acta Agraria Kaposváriensis, 6. 149-157.

SAŽETAK

U tijeku naših istraživanja ispitali smo sadržaj slobodnih kiselina i slobodnih D-aminokiselina u uzorcima mlijeka s različitim brojem mikroorganizama i sastav njihovih mliječnih proizvoda. Ukupan broj mikroorganizama ispitanih uzoraka mlijeka varirao je od $1,25 \cdot 10^6$ do $2,95 \cdot 10^6$. Ustanovljeno je da je s porastom broja mikroorganizama porasla i koncentracija slobodnih D-aminokiselina i slobodnih L-aminokiselina, međutim, porast sadržaja D-aminokiselina bio je viši s obzirom na njegov omjer. Osobito značajan rast broja mikroorganizama bio je u rasponu od $1,5 \cdot 10^6$ do $2,9 \cdot 10^6$. Tijekom analize uzoraka usirenog mlijeka i proizvedenog sira primjenom različitih tehnologija došli smo do zaključka da je za svježe mliječne proizvode i one koji su dozrijevali kratko vrijeme postojala uska veza između ukupnog broja mikroorganizama i sadržaja slobodnih D-aminokiselina i slobodnih L-aminokiselina, no međutim na omjer enanciomera nije djelovao ukupan broj mikroorganizama. Međutim u mliječnim proizvodima gdje sposobnost proizvodnje aminokiselina mikroskopskih kultura znatno prelazi proizvodnju mikroorganizama koji se prvotno nalaze u sirovom mlijeku, sadržaj slobodnih aminokiselina mliječnog proizvoda (l D- i L-eman-ciomeri) čini se da ovisi o sastavu sirovog mlijeka.

Ključne riječi: D-aminokiseline, alobodne aminokiseline, mliječni proizvodi, broj mikroorganizama, mlijeko, sir