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EFFECT OF MICROORGANISMS ON THE D-AMINO ACID CONTENT OF MILK

DJELOVANJE MIKROORGANIZAMA NA SADRŽAJ D-AMINOKISELINA U MLIJEKU

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ABSTRACT

In the course of our experiments it was established that certain microbe species causing mastitis (inflammation of the udder) (Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Staphylococcus aureus, Pasteurella multocida, Corynebacterium bovis, Arcanobacter pyogenes and Pseudomonas aeruginosa) contributed to the D-aspartic acid, D-glutamic acid and D-alanine contents of milk to a different extent. However the examination of amino acids was only partially suitable for identification of pathogen microbe species causing mastitis. Out of D-amino acids of peptidoglycann the D-glutamic acid content provides the possibility for identifying the microbes. Based on the D-aspartic acid content only Mastitest-negative milk sample and the species Staphylococcus aureus can be identified. On the basis of the D-alanine content microbes examined by us, with the exception of the species Escherichia coli, Streptococcus aureus and Pseudomonas aeruginosa could be identified. The free amino acid contents of milk derived from mastitic udder with mastitis caused by the individual bacterial species did not differ significantly. There are, however, such free amino acids whose proportion is characteristic of the given microbe species. The species Streptococcus uberis produces the least glycine, for the Escherichia coli is typical the very high phenylalanine content. Milk derived from mastitic udder with mastitis caused by Pseudomonas aeruginosa contained most of the free lysine.

Keywords: D-amino acids, free amino acids, mastitis, microorganisms, bacterial species, milk

INTRODUCTION

Our foodstuffs can contain, due to either technological interventions or to changes in the microbiological conditions of the foodstuff a substantial quantity of D-amino acids (Gandolfi et al., 1992; Brückner and Hausch, 1990; Fuse et al., 1984). Several publications have reported on D-amino acids in milk and dairy products making it clear that they are mainly result of microbia

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activity and that the technological intervention plays here only a minor role.

It appears proven that D-amino acids present in traces in a mixed milk drawn from healthy cows are the result of a bacterial infection evolved during subclinical mastitis and they get into the milk as metabolism products of bacteria.

Earlier we already examined the changes occuring in milk composition due to mastitis and worked out a method for determination of the proportion of milk of abnormal composition drawn to the healthy milk (Csapó et al., 1986). We decided to examine the D-amino acid contents of milk derived from the udder infected according to different degrees of Mastitest. In this series of examinations it was established that the D-amino acid content of commercially obtainable milk could be caused by the first milk flows rich in bacteria to the mixed milk on the one hand, and the presence of bacteria causing inflammation of the udder on the other hand, their metabolism products, and after death of the bacterium the D-amino acid contents of peptidoglycans being in the cell wall. It was also established that according to the degrees of the Mastitest the quantity of total free and free D-amino acids increased in the milk (Pohn and Csapó, 2002).

Based on our earlier investigations in this experiment we aimed at establishing whether microbe species causing mastitis could be identified on the basis of the free amino acid and free D-amino acid content of milk as this would provide the possibility for introducing a new diagnostic method for identification of microorganisms causing mastitis.

Conditions of the sampling

Milk samples derived from the mastitic udder were obtained from Holstein-Friesian cows of three cow farms (Lajoskomárom, Mosdós, Kaposvár). Samples are taken into sterilized 2·10 cm^3 pots. For Mastitest-negative cows a sample was taken from the mixed milk of the completely milked out udder, while in case of Mastitest-positive cows first two milk flows (around 10–12 cm^3 each) were separately drawn and the Mastitest was carried out. For amino acid analysis only samples with +++ and ++++ were used. Half of the samples was cooled immediately after sampling in iced water, placed into a deep freeze within 2 hours and stored at –25 °C until preparation for the analysis. The other half of the samples was stored in a refrigerator at +4°C, and was forwarded to the National Veterinary Hygienic Station for bacteriological examination within 12 hours.

Preparations of milk samples for amino acid analysis

Sample preparation as well as analysis are carried out at the University of Kaposvár, Faculty of Animal Science, Department of Chemistry and Biochemistry. After defrosting and warming to 30 °C milk samples were centrifuged at 8.000 g for 10 min in order to remove the figural elements and milk fat. Subsequently, to 50 cm^3 of sample 50 cm^3 of 25% trichloroacetic acid were added, left standing for 20 min, and centrifuged at 10.000 g for 10 min. The pH of the supernatant was adjusted to be 7 with 4.0 M NaOH for the determination of both the free amino acid and free D-amino acid contents. The obtained solutions were lyophilized at 10 °C, and the dried remainder was solved for the determination of free amino acid content in 10 cm^3 sodium acetate buffer (pH=7), and for the determination of the free D-amino acid content in 1 ml bidistilled water, respectively. Prepared samples were stored at –25 °C until being analyzed.

Instrument, chemicals

For determination of the free amino acid and free D-amino acid content derivatization and analysis were carried out using a Merck-Hitachi HPLC apparatus, for collecting and evaluating the measured data D-7000 HPLC System Manager software was used. Chemicals used in sample preparation, derivatization and analysis were of analytical grade (p.a.). O-phthaldialdehyde (OPA) and 1-thio-β-D-glucose tetra-acetate (TATG) were obtained from Sigma (St. Louis, USA), and 2-mercapto-ethanol from Merck (Darmstadt, Germany). Solvents (acetonitrile, methanol) used for the analysis were purchased from Merck and were of HPLC gradient grade quality. Eluent buffers

were prepared from sodium dihydrogen phosphate, di-sodium hydrogen phosphate and sodium acetate. The pH was adjusted with 4.0 M NaOH.

Determination of free D-amino acids

During derivatization from amino acids enantiomers diastereomer pairs were formed using o-phthaldialdehyde (OPA) and 1-thio-β-D-glucose tetra-acetate (TATG) as per the method of Einarsson et al. (1987). The reaction was carried out in a 1.5 cm^3 vial. 465 µl of sample were dissolved in 205 µl of borate buffer (0.4 M; pH=9.5) and mixed with 25 µl of the reagent (8 mg OPA and 44 mg TATG solved in 1 cm^3 of methanol) and left standing for 6 min. 20 µl of this mixture were injected onto the analytical column. The derivatives were detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm). For the separation of the enantiomers Superspher 60 RP-8e analytical column (C8, 125x4 mm, 4 µm) and a three-component gradient system (consisting of methanol, acetonitrile and phosphate buffer) were used. Flow rate was 1 cm^3/min.

Determination of free amino acids

During derivatization from amino acids cyclic derivatives were formed with o-phthaldialdehyde (OPA) and 2-mercapto-ethanol as per the following: 465 µl of sample were dissolved in 205 µl of borate buffer (0.4 M; pH=9.5) and was mixed with 105 µl of the reagent (100 mg OPA were dissolved in 9 cm^3 methanol and 1 cm^3 of borate buffer, and to this solution 100 µl 3.0 M 2-mercapto-ethanol was added) and left standing for 3 min. 20 µl of this mixture were injected onto the analytical column. The derivatives were detected with a fluorescence detector (excitation wavelength: 325 nm, emission wavelength: 420 nm). Separation of the free amino acids was performed with a LiCrospher RP analytical column (C18, 125 x 4 mm, 4 µm) and a two-component gradient system (consisting of methanol and sodium acetate buffer). Flow rate was 1 ml/min.

Statistical evaluation of the results

Statistical evaluation of the results was carried out using SPSS 10.0 statistical software. Difference in free the D-amino acid contents of the bacterial groups was examined one-way analysis of variance. Precondition of variance analysis was met as no significant difference was found in variance of data of species examined ($p>0.01$). For comparison of mean values of bacterial species a Student-Newman-Keuls test was used.

RESULTS

Bacteriological examination of milk samples

In the milk samples originating from mastitic udder eight bacterial species were identified in the bacteriological examination. The identified, in our country typical mastitis pathogen microbes are as follows: *Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Staphylococcus aureus, Pasteurella multocida, Corynebacterium bovis, Arcanobacter pyogenes and Pseudomonas aeruginosa.*

Examination of amino acid enantiomers

Enantiomer pairs of aspartic acid, glutamic acid and alanine were measured in milk samples derived from mastitic udders with Mastitest degree of +++ and ++++ where mastitis was caused by the above eight bacterial species (Table 1). Since in peptidoglycans of cell walls of bacteria and in metabolism products of bacteria these three amino acids are present in the highest concentration their quantities can be evidenced without any doubt. Free D-amino acid and free amino acid content of samples with +++ and ++++ do not differ significantly, therefore they are suitable for examination of amino acid content jointly.

Table 1. The D-Asp, D-Glu and D-Ala content of milk samples with mastitis of various bacterial origin in percentage of total free amino acids

Tablica 1. Sadržaj D-Asp, D-Glu i D-Ala u uzorcima mlijeka s mastitisom različitog bakterijskog podrijetla u postotku ukupnih slobodnih masnih kiselina

Milk samples / Uzorci mlijeka	D-amino acid* / D-aminokiselina*		
	D-Asp (n=3)	D-Glu (n=3)	D-Ala (n=3)
Negative, non mastitic sample / Negativan, nemastitičan uzorak	13.53	6.13	10.75
Identified bacterial species from mastitic samples / Identificirane vrste bakterija iz mastitičnih uzoraka			
Streptococcus dysgalactiae	22.46	21.83	49.37
Escherichia coli	31.96	41.40	34.47
Staphylococcus aureus	40.94	28.82	38.39
Pasteurella multocida	26.29	47.88	43.49
Streptococcus uberis	22.99	34.84	26.26
Corynebacterium bovis	23.50	38.43	40.90
Arcanobacter pyogenes	25.77	32.36	46.48
Pseudomonas aeruginosa	25.48	44.88	36.76

*D-aminosav% = $\frac{\text{D-As} \cdot 100}{\text{D-As + L-As}}$

Based on data shown in Table 1 it can be established that the D-aspartic acid content of bacteria-free, Mastitest-negative milk sample significantly differ from that of milk samples containing bacterial species. Out of the microbe species the D-aspartic acid content of the species *S. aureus* was significantly higher than that of other species, the species *Str. dysgal., Str. uberis, Corynebact. bovis, Pseud. aeruginosa, Arc. pyogenes* and *Past. multocida* do not differ from each other based in the D-aspartic acid content, however. Based on the percentage of D-aspartic acid therefore only the Mastitest-negative, bacteria-free milk sample and the species *S. aureus*, could be identified. Thus, it can be concluded that D-aspartic acid is not suitable for the identification of the pathogen microbes.

Examining average values of the D-glutamic acid contents of the species groups it can be established that this amino acid is suitable for the identification of the microbe species because between the groups the difference in quantity of the amino acid in question is significant. This means that on the basis of examination of glutamic acid enantiomers identification of the pathogen microbe species would be possible. Based on examination of the average values of D-alanine it can be established that the species *E. coli, S. aureus* and *Pseud. aeruginosa* cannot be identified on the basis of this amino acid, the D-alanine content of the other species show significant differences, however.

Examination of the free amino acid contents

Examination of free amino acids was carried out from the same samples from which the enantiomers were determined. Results of the analysis of free amino acids are summarized in Table 2 where the free amino acid content is given in percentage of the total free amino acid content.

Having examined the quantity of Asp, Glu and Ala it was established that the quantity of Glu varied between 14–25%, the proportion of Asp and that of Ala was less than 10%. The species *Str. dysgalactiae* contained significantly more Asp than the bacterium-free, Mastitest-negative milk sample. As an effect of the species *Corynebact. Bovis* and *E. Coli the* Asp content was significantly lower compared to Mastitest-negative milk sample.

Table 2. The free amino acid content of the individual microbe species in the percentage of the total free amino acid content

Tablica 2: Sadržaj slobodnih masnih kiselina pojedinih vrsta mikroorganizama u postotku ukupnog sadržaja slobodnih masnih kiselina

AMINO ACIDS / AMINO-KISELINE %	NEGATIVE/ NEGATIVNE (N=3)	BACTERIAL SPECIES / BAKTERIJSKE VRSTE (N=3)							
		E. coli	Staph. aureus	Past. multocida	Str. dysgal.	Str. uberis	Corynebact. bovis	Arcanobact. pyogenes	Pseud. aeruginosa
Asp	5.30	2.57	4.25	4.10	9.26	6.83	3.60	4.83	5.96
Ser	8.80	6.42	4.86	5.51	7.27	7.62	6.09	5.48	8.65
Glu	25.00	14.04	15.32	18.88	15.39	21.50	24.87	25.66	15.96
His	2.00	4.36	1.02	2.15	2.13	2.02	2.61	2.36	2.33
Gly	13.90	10.98	12.80	13.61	10.47	8.47	13.40	14.23	10.45
Arg	5.80	4.35	8.64	6.91	4.17	5.22	4.20	4.87	3.56
Thr	2.00	1.17	5.45	3.47	2.38	3.37	2.98	0.92	2.26
Ala	6.80	10.13	4.84	7.36	6.73	7.27	7.12	7.87	5.87
Tyr	3.50	4.01	8.61	4.62	5.1	4.18	4.01	3.97	4.84
Met	1.30	1.35	1.22	2.36	1.12	1.78	1.56	1.08	1.58
Val	8.80	11.26	8.00	9.41	12.57	7.36	10.90	7.68	10.78
Phe	2.70	5.33	3.53	3.37	3.24	2.32	1.62	2.42	3.75
Ile	3.00	5.80	3.26	3.56	2.61	5.36	2.69	3.01	1.12
Leu	4.00	9.61	7.36	6.68	6.72	6.91	4.39	6.22	7.39
Lys	7.10	8.64	10.85	8.02	10.84	9.80	10.42	9.41	15.55

There was no significant difference between samples containing other bacterium species and the Mastitest-negative milk sample. Based on the Glu content it could be established that within the individual bacterium groups (Group 1: *Str. dysgalactiae, S. aureus; Group 2: Arc. pyogenes, Str. uberis, Corynebact. bovis; Group 3: E. coli, Pseud. aeruginosa, P. multocida)* there was no significant difference in the amino acid contents. However, Group 2 contains significantly more Glu than the bacterium species in Group 1 and Group 3. There was no substantial difference in the Glu content between Group 1 and Group 3. The Mastitest-negative milk sample contained significantly more Glu than mastitic milk samples where mastitis was triggered out by bacterium species of Group 1 and Group 3. The Glu contents in Group 2 and Mastitest-negative milk sample did not differ from each other significantly.

On the basis of the Ala content it can be established that mastitic milk with mastitis caused by the species *E. coli* contains significantly more amino acids than the Mastitest-negative milk sample. The other species do not differ significantly from the Mastitest-negative milk sample. Out of the bacteria species Ala contents of *E. coli* and *S. aureus* differ considerably. Having examined average percentage value of Ser, His and Gly it was established that out of the three amino acids the percentage of Gly ranged between 8–14%, that

of Ser between 5–9%, and thequantity of His was 2% on the average in examined milk samples. There was no significant difference in Ser content between the milk samples. In the mastitic milk sample with mastitis caused by *E. coli* significantly more His was found while in that with mastitis caused by *S. aureus* significantly less His than the Mastitest-negative milk sample. On the ground of the Gly content the milk derived from a mastitic udder with mastitis triggered out by *Str. uberis* differs significantly from the rest of the samples.

The Tyr and Thr content of milk infected by *S. aureus* significantly differs from all of the other samples. Mastitic milk samples with mastitis caused by *Arc. pyogenes* and *E. coli* do not differ from each other in the Thr content but they significantly do from the other samples. On the basis of the Arg content it can be established that there is no substantial difference between mastitic milk samples with mastitis caused by *S. aureus* and *P. multocida*, these samples differ, however, significantly from all of the other milk samples. In the Met content no significant difference could be found between the milk samples. In the case of the Phe content it could be established that mastitic milk with mastitis due to the species *E. coli* significantly differed from the other milk samples. Val contents of milk from udder inflamed by *Str. dysgalactiae* significantly differ from those of both mastitic milk sample with mastitis caused by *S. aureus, Arc. pyogenes* and *Str. uberis* and Mastitest-negative milk sample. Milk from mastitic udder with mastitis caused by *Pseud. aeruginosa* contains significantly more Lys than all of the other milk samples. Lys contents of milk derived from mastitic udder with mastitis caused by *S. aureus, Arc. pyogenes, Str. uberis* and *Corynebact. bovis* significantly increased compared to Mastitest-negative milk sample, there was no significant difference between the species. Except *Corynebact. bovis* each species contains significantly more Leu than Mastitest-negative milk sample. In case of inflammation caused by *Str. uberis* and *E. coli* Ile contents of milk significantly increased in comparison with the other species, no significant deviation could be observed, however, in Ile contents of these two milks.

CONCLUSIONS

As a result of our experiments we established that the D-aspartic acid content of bacteria-free, Mastitest-negative milk sample significantly differed from that of bacterial species examined; among the individual microbe species only *S. aureus* differed significantly from the others, however. D-glutamic acid can be suitable for the identification of the microbes as the quantity of this amino acid differs to a significant extent in mastitic milk samples with mastitis caused by the individual microbes. Based on the D-alanine content significant differences could also be established with the exception of *E. coli, S. aureus,* and *Pseud. aeruginosa*.

The free amino acid content of milk derived from mastitic udder with mastitis due to the individual bacterial species does not differ significantly. There are, however, such free amino acids whose proportion is characteristic of the given microbe species. Based on the glycine content it can be said that mastitic milk sample with mastitis caused by *Str. uberis* significantly differs from the other samples. The Phe content of milk infected by the *E. coli* significantly differ from the other milk samples. Milk derived from mastitic udder inflamed by *Pseud. aeruginosa* contains significantly more lysine than all of the other milk samples.

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SAŽETAK

U našim je pokusima utvrđeno da su neke vrste mikroorganizama što uzrokuju mastitis *(Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Staphylococcus aureus, Pasteurella multocida, Corynebacterium bovis, Arcanobacter pyogenes* i *Pseudomonas aeruginosa)* djelovale u različitoj mjeri na sadržaj D-alaninske kiseline, D-asparitske kiseline i D-glutaminske kiseline u mlijeku. Međutim, djelovanje aminokiselina samo je djelimično prikladno za identificiranje patogenih vrsta mikroorganizama, uzročnika mastitisa. Od D-aminokiselina peptidoglikana sadržaj D-glutaminske kiseline omogućuje identificiranje mikroorganizama. Na temelju sadržaja D-asparitske kiseline mogu se identificirati samo Mastitis-negativni uzorci mlijeka i vrsta *Staphylococcus aureus*. Na temelju sadržaja D-alanina u mikroorganizmima koje smo ispitali, s iznimkom vrste Escherichia coli, moguće je utvrditi Staphylococcus aureus i Pseudomonas aeruginosa. Sadržaj slobodnih kiselina u mlijeku iz vimena s mastitisom uzrokovan pojedinačnim vrstama bakterija značajno se ne razlikuje, međutim postoje takve slobodne aminokiseline čiji omjer je karakterističan za te vrste mikroorganizama. Vrsta Streptococcus uberis proizvodi najmanje glicerina, a za Escherichia coli tipičan je vrlo visok sadržaj fenilanina. Mlijeko dobiveno iz mastitičnog vimena čiji je uzročnik Pseudomonas aeruginosa sadrži najviše slobodnog lizina.

Ključne riječi: D-aminokiseline, slobodne aminokiseline, mastitis, mikroorganizmi, vrste bakterija, mlijeko