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Influence of mastitis on D-amino acid content of milk

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Abstract. The California Mastitis Test was used as an indicator of mastitis. Five cows were chosen for each of the five scores from 0 to 4. Milk samples were analysed for free amino acids and free D-amino acids. The contents of free amino acids, free D-amino acids and the ratio of free D-amino acids to free amino acids increased significantly as score increased. The free D-amino acids content of foremilk (first milk jets) from nonmastitic cows (score = 0) was approximately five times that of samples drawn later from the same udders. Contents of free amino acids and free D-amino acids were highly associated with score and udder inflammation. Very low concentrations of free D-amino acids are normal for raw milk. Higher concentrations of free D-amino acids could be attributed to inclusion of foremilk and milk from cows having subclinical mastitis in the bulk tank milk.

Key words and phrases: free amino acids, D-amino acids, mastitis, foremilk, milk

1 Introduction

Foods have recently been shown to contain considerable quantities of D-AA [1, 4, 9, 10, 13, 15, 16, 18, 19] attributed to both processing technology and microbiological status of the foods. Milk and milk products have been reported [2, 3, 11, 20] to contain significant amounts of D-AA, which were generally attributed to biological status rather than to processing technology. The pertinent literature does not reveal an explanation for the presence of D-AA in raw milk from healthy cows.

The presence of D-AA in food products has been shown to cause a decrease in digestibility of protein [4, 9, 13, 18] and bioavailability of essential AA. It was suggested [9, 18] that some D-AA may provide the basis for formation of toxic products. Absorption rates of L-AA in the intestine were reported to be greater than those of the respective D-enantiomers [7]. Milk products produced by bacterial fermentation contained levels of D-AA [18], and the bacteria were implicated as a biological source of D-AA.

Because D-AA are often products of bacterial metabolism, and mastitis is an inflamation of the udder of bacterial origin, we investigated the influence of mastitis as a possible explanation of D-AA content of raw milk. The cow produces leukocytes to counteract the infection, and, in early studies, direct microscopic count of leukocytes was used to measure the severity of mastitis. Shalm and Noorlander [21] utilized a chemical reaction test that could be read in one of five classes (0 to 4) and was highly correlated with leukocyte count [17, 22]. The California Mastitis Test (CMT) has been used in many studies. Electronic procedures have been used to count cells, and the SCC has also served as the measure of mastitis.

Harmon [12] stated that the primary factor influencing SCC or CMT is intramammary bacterial infection. He also reported that cows with mastitis, as indicated by high SCC, produced milk that differed in composition from milk produced by cows with healthy udders. High SCC was associated with reduced casein and increased concentrations of whey protein, albumin, and immunoglobulin [12]. Another study [5] reported changes in mineral contents. Mastitic milk contained more sodium, chloride, calcium, iron, and manganese, and normal milk was higher in potassium, phosphorus, zinc, and copper. The protein and mineral contents of mastitic milk were similar to those of colostrum. In related studies [8, 17], the foremilk from nonmastitic cows was reported to have higher SCC or CMT than milk drawn later in the milking process.

The objectives of this research were 1) to determine the concentrations of

free AA and free D-AA in milk from cows having different CMT scores and 2) to compare the D-AA contents of foremilk and milk drawn later from cows having a negative CMT.

2 Material and methods

2.1 Assignment of cows and sampling milk

The CMT, as describes for use in Hungary [14] was used in a herd of 1020 Holstein-sired cows. For this experiments, 25 individual cows were identified with each having the same CMT score for all four quarters of the udder. There were five cows in each of the five groups based on CMT score: 0, 1, 2, 3, and 4. The CMT reaction [21] is disintegration of leukocytes when milk is mixed with the reagent (NaOH and an anionic surface active agent). In a negative sample (0 score), the mixture of milk and reagent remains liquid and produces no precipitate. As score increases, the degree of precipitation increases and, when score = 4, a distinct gel with central peak is formed. CMT score is based on number of leukocytes in milk, and mean counts for scores 0, 1, 2, 3, and 4, respectively, were 67, 118, 401, 1737, and 6964×10^3 per ml [17].

At the time of sampling, the amount of milk needed to conduct CMT on each quarter was drawn from each cow. For negative (0 score) cows, the sample was drawn from well-mixed complete yield of the udder with the remainder going to the bulk tank. For positive (CMT of 1, 2, 3, or 4) cows, a volume of 1 l was manually milked, mixed and sampled with the remainder being milked and discarded.

The comparison of D-AA contents of foremilk and later milk required the selection of 5 cows, each of which had negative CMT for all quarters. The foremilk sample consisted of two hand-milked jets from each of the four quarters of the udder. The udder was then milked out completely, and a sample was drawn from the well-mixed remainder.

All milk samples for both experiments were cooled immediately in ice-water and, within 2 h, were placed in a deep freeze at −25 °C. The samples were stored at −25 °C until preparations for AA analysis were initiated.

2.2 Preparation of milk samples for analysis

After defrosting and heating to 30 °C, the samples were centrifuged at 5000 g at room temperature for 20 min to skim the milk and deposit particulate matter at the bottom of the centrifuge tube. To 50 ml of sample, 50 ml of a 25%

trichloroacetic acid solution were added and the mixture was allowed to stand for 20 min. The resulting precipitate was centrifuged at room temperature for 30 min at 10 000 g. To determine total AA, pH of the supernatant was adjusted to pH 2.2 with 4 M sodium hydroxide. To conduct D-AA determinations, the pH was adjusted to pH 7. Using a 10 °C hot plate, the solutions were lyophilized. For total AA determinations, the resulting solid material was dissolved in 10 ml of citrate buffer at pH 2.2, and, for determinations of D-AA, dissolution was in 1 ml of twice-distilled water. Samples prepared were stored at −25 °C until analysis.

2.3 HPLC and ion-exchange column chromatography for the determination of D-AA and total free AA

Instruments. The chromatographic system was assembled from ISCO 100 DM syringe pumps (Isco Inc. Lincoln, Nebraska, USA) and a Rheodyne (Berkeley, California, USA) injector equipped with a 20 μl loop. The separation process was monitored and chromatograms stored on an ISCO Chem Research (Isco Inc. Lincoln, Nebraska, USA) system. The derivative formation and sample injection were performed manually. The excitation and observation wavelengths were 325 and 420 nm, respectively.

Reagents. Acetonitrile and methanol were purchased from Rathburn Ltd (Walkeburn, England). The AA standards, the *o*-phthalaldehyde and the TATG (2,3,4,6,-tetra-O-acetyl-1-thio-β-D-glucopyranozide) were obtained from Sigma Chemical Co., Inc. (St. Louis, MO). The buffers used for elution were prepared from mono- and disodium phosphate. The pH was adjusted with 4 M sodium hydroxide.

Synthesis of derivatives. The reaction was carried out in a 120 μl microvial which was placed in another vial (volume, 1.8 ml) that had Teflon^{R} coating, internal cover plate, and a screw cap. The sample (free AA or protein hydrolysate evaporated in a nitrogen atmosphere), dissolved in 90 μl borate buffer (0.4 M; pH 9.5), was mixed with 15 μl of reagent (8 mg of *o*-phthalaldehyde and 44 mg of TATG dissolved in 1 ml of methanol). The mixture was then homogenized by bubbling through approximately 100 μl of nitrogen and left standing for 6 min. Then, 25 μl of the reaction mixture were injected into the analytical column. After injection, the system was rinsed three times with approximately 100 μl of a 70:30 acetone-water (v/v) solution. Synthesis of derivatives was performed manually and mixing of reagent solution was made

with the aid of an IKA Vibro Fix instrument (Janke and Kunkel, IKA-WERK, Breisgau, Germany).

Separation and quantitation of the enantiomers. Separation of the enantiomers was made according to the method of Einarson et al. [6], using a reversed-phase analytical column packed with Kromasil octyl C-8 (250×5.6 mm internal diameter; 5 μm particle size, EKA Nobel AB, Bohus, Sweden). To increase the lifetime of the column, a safety column was fitted between the sample injector and the analytical column (RP-8, Newguard, 25×3.2 mm internal diameter, 7 μm particle size, EKA Nobel AB, Bohus, Sweden), and a cleaning column (C-18, 36×4.5 mm internal diameter, 20 μm particle size, Rsil, EKA Nobel AB, Bohus, Sweden) was installed between the pump and the sample injector. In order to separate the enantiomers, the two component gradient system had the following composition: A = 40% methanol in phosphate buffer (9.5 mM, pH = 7.05) and B=acetonitrile. The flow rate was 1 ml/min, and the elution of the gradient as a function of time is shown in *Table 1.*

Table 1: The elution gradient, as a function of time, for the two components used to separate and quantitate the enantiomers

Time	**A**[1]	**B**[2]
(min)	**%**	
0	95	5
10	95	5
35	83	17
55	72	28
56	67	33
74	67	33
75	62	38

[1]40% methanol in phosphate buffer (9.5 mM, pH = 7.05).
[2]Acetonitrile

Determination of total free AA was performed with an LKB Model 4101 automatic AA analyzer, following postcolumn derivative synthesis with ninhydrin.

3 Results

3.1 Free AA

Concentrations of total (D- and L-) free AA are shown in *Table 2* for the five CMT scores.

Table 2: Mean free AA (D- plus L-) contents of milk from cows with various California Mastitis Test scores

Amino acid (mg/100 ml)	CMT score[1]					SEM
	0	1	2	3	4	
Asp	0.12^{d}	0.89^{c}	1.13^{b}	1.38^{a}	1.45^{a}	0.021
Thr	0.12^{b}	0.16^{b}	0.18^{ab}	0.20^{ab}	0.24^{a}	0.010
Ser	0.23^{b}	0.27^{b}	0.31^{b}	0.49^{a}	0.52^{a}	0.014
Glu	0.96^{d}	2.53^{c}	3.19^{b}	4.63^{a}	4.71^{a}	0.043
Pro	0.05^{d}	0.09^{d}	0.42^{c}	0.78^{b}	0.89^{a}	0.016
Gly	0.04^{c}	0.06^{bc}	0.09^{b}	0.19^{a}	0.22^{a}	0.006
Ala	0.34^{d}	2.18^{c}	3.42^{b}	4.88^{a}	4.92^{a}	0.113
Cys	0.11^{c}	0.17^{b}	0.19^{b}	0.26^{a}	0.27^{a}	0.009
Val	0.67^{b}	0.17^{b}	0.74^{b}	0.81^{ab}	0.92^{a}	0.025
Met	0.01^{b}	0.01^{b}	0.02^{ab}	0.03^{a}	0.03^{a}	0.002
Ile	0.03^{d}	0.29^{c}	0.35^{b}	0.42^{a}	0.44^{a}	0.010
Leu	0.04^{d}	0.21^{c}	0.38^{b}	0.69^{a}	0.66^{a}	0.014
Tyr	0.09^{c}	0.11^{bc}	0.14^{ab}	0.17^{a}	0.18^{a}	0.007
Phe	0.10^{d}	0.13^{cd}	0.17^{bc}	0.21^{ab}	0.23^{a}	0.008
Lys	0.28^{d}	0.49^{c}	0.83^{b}	1.14^{a}	1.14^{a}	0.017
His	0.12^{b}	0.25^{b}	0.68^{a}	0.71^{a}	0.89^{a}	0.061
Arg	0.09^{d}	0.19^{c}	0.38^{b}	0.57^{a}	0.62^{a}	0.013
Total (sum)	3.40^{e}	8.73^{d}	12.62^{c}	17.56^{b}	18.32^{a}	0.097

[1]Five cows were in each group. 0 = no precipitate, 1 = slight precipitate, which disappears, 2 = distinct precipitate, but no gel formation, 3 = mixture thickens with some suggestion of gel formation and 4 = distinct gel with central peak.
[a,b,c,d,e]Means in the same row and having no superscripts in common differ ($P \leq 0.01$).

Based on analysis of variance and comparison of paired means, differences among the five CMT score groups were significant ($P \leq 0.05$) for each of the 17 AA. Variation among cows within CMT class was very small, as indicated by the small standard errors of means shown in *Table 2.* The milk samples with

CMT of 2, 3, or 4 had free AA composition similar to that of colostrum. This tendency has also been reported [5, 12] for protein components and minerals. Compared with concentrations of normal milk, the most spectacular increases were seen for Ile, Ala, Asp, Pro, and Leu which were more than 10 times the concentrations observed in milk samples scored 0 by the CMT.

The nature of differences among CMT classes was investigated by regression analysis. *Table 3* shows the significant ($P \leq 0.01$) linear contrast for each of the 17 free AA and for the sum. The concentration of free AA increased linearly for CMT scores 0 to 3. The differences between CMT scores of 3 and 4 were significant only for Pro and for the sum of all AA. This result tended to cause the quadratic contrast to account for more variation than the cubic contrast. Based on free AA contents, the CMT classifications could be reduced to four: 0 to 3.

Table 3: Regression of free amino acid content on CMT score: significance of linear (L), quadratic(Q), and cubic (C) orthogonal contrasts and proportion of variance (R^2) associated with regression.

Amino acid	F-Values[1]			R^2		
	L	Q	C	L	L, Q	L, Q, C
Asp	2149**	306**	27**	0.85	0.97	0.99
Thr	81**	0	1	0.80	0.80	0.81
Ser	318**	7*	14**	0.87	0.88	0.92
Glu	4995**	186**	11**	0.94	0.98	0.98
Pro	2171**	7*	113**	0.94	0.94	0.99
Gly	787**	18**	22**	0.90	0.92	0.96
Ala	1101**	63*	5*	0.92	0.97	0.98
Cys	221**	2	1	0.87	0.88	0.89
Val	55**	4	1	0.70	0.74	0.75
Met	80**	0	0	0.84	0.84	0.84
Ile	903**	171**	26**	0.80	0.95	0.98
Leu	1493**	23**	63**	0.92	0.93	0.97
Tyr	274**	1	2	0.84	0.85	0.86
Phe	171**	2	2	0.87	0.88	0.89
Lys	1972**	51**	66**	0.92	0.94	0.97
His	107**	4	1	0.91	0.94	0.95
Arg	1321**	5*	32**	0.96	0.96	0.99
Total (sum)	15921**	503**	76**	0.96	0.99	0.99

*$P \leq 0.05$, **$P \leq 0.01$
[1]F-Values (1, 20 d. f.)

A quadratic function of CMT score explained 99% of the variation in the sum of free AA and 97% of the variation in contents of Asp, Glu, and Ala. Conversely, the free AA content could be used as an indicator of severity of mastitis.

3.2 Free D-AA

Concentrations of free D-AA are shown in *Table 4.* Milk samples rated as negative score, CMT of 0, contained free D-Asp, D-Glu, and D-Ala. However, the quantities present (0.02, 0.05, and 0.04 mg/100 ml, respectively) were almost negligible compared with the quantities occurring in the milk samples that had CMT of 2, 3, or 4. For samples scored 1, D-Val, D-allo-Ile, D-Leu, and D-Lys were also present. D-Ser and D-Pro were identified in samples scored 2, 3 or 4. Not even traces could be identified of the D-enantiomers of other AA, which are the building blocks of proteins.

Table 4: Mean free D-AA contents of milk from cows with various California Mastitis Test scores.

D-AA	**CMT score**[1]					**SEM**
(mg/100 ml)	**0**	**1**	**2**	**3**	**4**	
D-Asp	0.02^{d}	0.17^{c}	0.23^{b}	0.32^{a}	0.32^{a}	0.008
D-Ser	0^{c}	0^{c}	0.02^{b}	0.04^{a}	0.04^{a}	0.003
D-Glu	0.05^{c}	0.08^{c}	0.99^{b}	1.48^{a}	1.53^{a}	0.027
D-Pro	0^{c}	0^{c}	0.04^{b}	0.09^{a}	0.10^{a}	0.005
D-Ala	0.04^{c}	0.05^{b}	1.13^{b}	2.32^{a}	2.41^{a}	0.020
D-Val	0^{b}	0.08^{ab}	0.09^{ab}	0.09^{ab}	0.12^{a}	0.017
D-allo-Ile	0^{d}	0.08^{c}	0.10^{bc}	0.12^{ab}	0.15^{a}	0.006
D-Leu	0^{d}	0.06	0.12^{b}	0.17^{a}	0.17^{a}	0.008
D-Lys	0^{b}	0.11^{c}	0.27^{b}	0.36^{a}	0.37^{a}	0.012
Total (sum)	0.11^{e}	0.63^{d}	2.99^{c}	4.99^{b}	5.21^{a}	0.018

Data expressed as in *Table 2.*
^{a,b,c,d,e}Means in the same row and having no superscripts in common differ ($P \leq 0.01$).

Free D-AA increased as CMT score increased for all nine free D-AA and for the sum of free D-AA. The nature of the regression pattern *(Table 5)* was slightly different; the cubic contrast was relatively more important than was the quadratic contrast. California Mastitis Test classes 3 and 4 had similar free D-AA concentrations, as was observed previously. However, differences in free D-AA contents of samples scored 0 and 1 also tended to be small. Therefore, the graphic expression of free D-AA content relative to CMT score tended to be sigmoid in shape. Thus CMT score can be used as an accurate predictor of concentration of free D-AA, but the prediction equation would be a cubic function: $y = a + bx + b_2x_2 + b_3x_3$.

Table 5: Regression of free D-amino acid content on CMT score: significance of linear (L), quadratic (Q), and cubic (C) orthogonal contrasts and proportion of variance (R^2) associated with regression.

D-AA	F-Values[1]			R^2		
	L	Q	C	L	L, Q	L, Q, C
D-Asp	692**	72**	0	0.87	0.96	0.96
D-Ser	148**	0	20**	0.80	0.80	0.99
D-Glu	5261**	13**	243**	0.89	0.90	0.98
D-Pro	638**	3	28**	0.86	0.87	0.94
D-Ala	25775**	14**	1246**	0.91	0.91	0.99
D-Val	216**	33**	34**	0.71	0.82	0.94
D-allo-Ile	333**	17**	13**	0.87	0.91	0.95
D-Leu	295**	17**	3	0.88	0.93	0.94
D-Lys	5195**	33**	12**	0.91	0.95	0.97
Total (sum)	9047**	27**	572**	0.94	0.94	0.99

Data expressed as in *Table 3.*

The increase in free D-AA could be easily attributed to the availability of larger amounts of free AA for conversion to D-AA. However, the concentration of free D-AA relative to free (D- and L-) AA increases as CMT increases *(Table 6).* Free D-Ala represented almost 50% of the total free Ala when CMT score was 3 or 4. Conversely, the increase in percentage D-Asp (17 to 23%) was small. For the sums of all AA, the percentage D-AA was only 3% for samples scored 0 compared with 28% for these scored 3 or 4 by CMT. Both absolute quantity and percentage (relative to total free) of D-AA increased as CMT increased.

Table 6: Free D-amino acid concentration as a percentage of free (D and L) amino acid content of milk from cows with various California Mastitis Test scores.

D-AA	CMT score[1]					SEM
(mg/100 ml)	0	1	2	3	4	
D-Asp	17.0^{c}	19.0^{bc}	20.7^{ab}	23.3^{a}	21.9^{ab}	0.92
D-Ser	0^{b}	0^{b}	6.6^{a}	8.6^{a}	7.7^{a}	0.74
D-Glu	5.6^{a}	3.0^{b}	31.1^{a}	31.9^{a}	32.5^{a}	0.79
D-Pro	0^{c}	0^{c}	8.4^{b}	11.8^{a}	11.1^{a}	0.89
D-Ala	12.9^{c}	2.2^{d}	33.1^{b}	47.6^{a}	49.1^{a}	1.03
D-Val	0^{b}	11.0^{a}	12.5^{a}	11.0^{a}	13.0^{a}	0.79
D-allo-Ile	0^{c}	27.6^{b}	27.6^{b}	29.3^{b}	34.7^{a}	1.61
D-Leu	0^{b}	29.1^{a}	31.7^{a}	24.4^{a}	25.8^{a}	1.91
D-Lys	0^{c}	22.0^{b}	32.6^{a}	32.0^{a}	32.8^{a}	1.81
Total (sum)	3.4^{d}	7.0^{c}	23.7^{b}	28.4^{a}	28.4^{a}	0.40

Data expressed as in *Table 2.*
^{a,b,c,d}Means in the same row and having no superscripts in common differ (P≤0.05).

3.3 Free D-AA content of foremilk

Foremilk has been shown to have higher CMT score and higher SCC [8, 17, 22] than that of milk drawn later in the milking process. The D-AA concentrations of foremilk and later milk from cows having CMT score of 0 are shown in *Table 7.*

Table 7: Free D-amino acid content of foremilk and mixed total milk from five cows having negative (zero) California Mastitis Test scores

D-AA	Milk sample			Conf. Limits[2]	
(mg/100 ml)	Foremilk	Milk	Difference[1]	LL	UL
D-Asp	0.132	0.021	0.111^{**}	0.086	0.136
D-Glu	0.214	0.053	0.161^{**}	0.124	0.198
D-Ala	0.203	0.043	0.160^{**}	0.111	0.209
D-allo-Ile	0.061	0	0.061^{**}	0.045	0.077
Total (Sum)	0.610	0.117	0.493^{**}	0.443	0.543

[1]Difference (foremilk-milk) calculated for each cow and averaged (D).
[2]Lower (LL) and upper (UL) 99% confidence limits for average difference (D) = D±t (S.E.D) with t5 d.f. and α= 0.01 and S.E.D = $\sqrt{s_{\mathrm{D}}^2/5}$
** P≤0.01

The foremilk was two jets of milk drawn from each quarter at the beginning of milking. Free D-AA contents of foremilk were approximately five times those of later drawn milk ($P \leq 0.01$). Negative (CMT score of 0) samples had extremely low concentrations of free D-AA in later milk. The free D-AA contents of foremilk from cows whose milk scored 0 was typical of the concentrations reported for milk from cows whose milk scored 1 *(Table 4).*

4 Implications

Based on the results, we concluded that very low concentrations of D-AA are normal for raw milk. Higher D-AA can be traced to inclusion of the bacteria-rich foremilk and milk from cows with subclinical mastitis in the bulk tank. Data presented here would support the hypothesis that D-AA content of raw milk is associated with mastitis and, consequently, with bacterial activity in the udder.

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