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1 ORIGIN OF METABOLITES IN MASTITIS MILK

2

3 **The origin of the volatile metabolites found in mastitis milk**

4

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13

14 **Abstract**

15 The possibility to detect mastitis pathogens based on their volatile metabolites was  
16 previously studied. Because the origin of the metabolites is unknown, the formation of  
17 volatile metabolites by 5 mastitis pathogens inoculated in milk of healthy cows was studied.  
18 The volatile metabolites from inoculated samples were compared to those of mastitis milk  
19 samples from which the inoculated pathogens were isolated. Most metabolites formed in the  
20 inoculated samples were similar to the metabolites formed in mastitis samples, both in  
21 presence and in amount. Prediction by a neural network showed that the similarity between  
22 the inoculated samples and mastitis samples was sufficient for correct prediction of the  
23 pathogen in the inoculated sample. The main difference between the inoculated samples and  
24 the mastitis samples was the absence of ethyl esters of free fatty acids in inoculated samples.

25 This could be explained by disturbance of the milk-blood barrier, allowing the transfer of  
26 esterase from the cows' blood to the milk in cows with mastitis.

27

28 *Keywords:* mastitis, pathogens, metabolites, inoculation, artificial neural networks

29

## 30 **1. Introduction**

31 Determination of mastitis causing pathogens is of great interest, both for choice of  
32 treatment as well as for herd level management measures to prevent the spread of mastitis.  
33 Currently, determination of mastitis pathogens is generally done by bacteriological culturing  
34 (NMC, 2004). Previous research (Hettinga et al., 2008b) showed that results of a method for  
35 mastitis pathogen identification based on the analysis of volatile metabolites in milk samples  
36 were comparable with bacteriological culturing. The two main advantages of this method are  
37 the total analysis time and the cost of analysis.

38 Specific volatile metabolites detected in mastitis milk samples are formed by the pathogen  
39 itself. For example, the formation of 2- and 3-methylbutanal, as well as branched free fatty  
40 acids by *S. aureus* have been described by Ritter and Hanni (1960). In mastitis samples with  
41 *S. aureus*, other, not previously described metabolites, like ethyl esters of free fatty acids,  
42 were also detected. Whether these components are formed by *S. aureus* or are the result of  
43 other processes occurring during mastitis is not known.

44 Detected metabolites may, for example, also be formed by enzymes which travel from the  
45 blood through the blood-milk barrier to the milk, or by enzymes associated with endothelial  
46 cells of the udder tissue. To determine whether the volatile metabolites originate from the  
47 pathogens or from other sources, inoculation of milk samples from healthy cows can be used.

48 The goal of this study was to compare the volatile metabolites from mastitis samples with  
49 those from inoculated samples containing the same pathogen, to get insight in the mechanism

50 of volatile metabolite formation, and to evaluate whether inoculated samples can be used as a  
51 model for mastitis samples in studying volatile metabolites.

52

## 53 **2. Materials and methods**

54

### 55 *2.1 Milk samples*

#### 56 *2.1.1 Mastitis samples*

57 The clinical mastitis samples in this study were the same as described in our previous  
58 publication (Hettinga et al., 2008b). If one of the 5 pathogens of interest (*S. aureus*, CNS, *S.*  
59 *uberis*, *S. dysgalactiae*, or *E. coli*) was cultured, the remainder of the milk sample (approx. 5  
60 mL) was frozen at -20°C for later analysis of the volatile metabolites. Also, the pathogen  
61 cultured from the mastitis samples was frozen at -80°C for the inoculation procedure.  
62 Bacteriological culturing was carried out according to NMC protocols (NMC, 2004). Because  
63 these were milk samples from routine mastitis diagnosis, the milk samples have been  
64 incubated overnight at 37°C.

65

#### 66 *2.1.2 Inoculated samples*

67 Fifteen milk samples from 3 cows (5 samples/cow), without clinical signs of mastitis and  
68 with low SCC (<75,000/ml cow SCC), were supplied by the “De Ossekampen”, the university  
69 farm of Wageningen University and Research Centre (Wageningen, The Netherlands). After  
70 disinfection of the teat and forestripping, 10 mL foremilk samples were taken into sterile 12  
71 mL tubes. Directly after taking these samples, they were transported to the laboratory and  
72 inoculated, in the same tube, with one of the five pathogens cultured from the mastitis  
73 samples (3 samples per pathogen). Inoculation was performed using BHI broth (CM0225,

74 Oxoid, Cambridge, UK) containing  $10^9$  CFU/mL, diluted to a final inoculation level of  $10^5$   
75 CFU/mL in the milk sample.

76 The inoculated samples were incubated at  $37^\circ\text{C}$  for 8 h. After incubation, the samples were  
77 cooled on ice to approximately  $0^\circ\text{C}$ . Five mL of the sample was subsequently transferred to a  
78 headspace vial. The headspace vials were frozen at  $-20^\circ\text{C}$  for later analysis of the volatile  
79 metabolites.

80

### 81 *2.2 Analysis of volatile metabolites*

82 The volatiles were analyzed using solid-phase microextraction (SPME) combined with gas  
83 chromatography/mass spectrometry (GC/MS) according to Hettinga et al. (2008b).

84

### 85 *2.3 Statistical analysis*

86 SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical  
87 analysis. An ANOVA was performed for comparison of inoculated and mastitis samples, with  
88 a Welch test in the case of unequal variances.  $P < 0.05$  was considered statistically significant.

89 The obtained data were used in the prediction module of NeuralTools (Palisade, Ithaca,  
90 NY, USA) using the previously developed probabilistic neural network (PNN) to identify the  
91 mastitis causing pathogen (Hettinga et al., 2008b).

92

## 93 **3. Results**

94

### 95 *3.1 Analysis of volatile metabolites*

96 The results of the analysis of the mastitis samples have been described elsewhere (Hettinga  
97 et al., 2008b). The results of the inoculated samples were compared with the mastitis samples  
98 per pathogen.

99 Table 5.1 contains an overview of the results of the comparison between the mastitis  
100 samples and the inoculated samples.

101

### 102 3.2 Comparison of volatile metabolites per pathogen

#### 103 3.2.1 *Staphylococcus aureus*

104 When comparing the data of the inoculated samples with the mastitis samples, especially the  
105 group of ethyl esters of the free fatty acids stands out. Four of the ethyl esters were not found  
106 at all in the inoculated samples, only ethyl acetate was detected in one of the samples.  
107 Another difference between the inoculated and mastitis samples is that the samples inoculated  
108 with *S. aureus* contained a higher amount of dimethyl sulfide. Finally, hexanoic acid was  
109 found in the inoculated samples but not in the mastitis samples.

110

#### 111 3.2.2 CNS

112 Just like *S. aureus*, samples inoculated with CNS contained hexanoic acid, whereas the  
113 mastitis samples did not. Additionally, the concentration of dimethyl sulfide and 2-butanone  
114 were significantly higher for the inoculated samples compared to the mastitis samples. The  
115 inoculated samples did not contain isopentanol, acetoin, and ethyl esters of branched fatty  
116 acids whereas some of the mastitis samples did contain these components. These differences  
117 were, however, not significant.

118

#### 119 3.2.3 *Streptococcus uberis*

120 The absence of the ethyl esters and the presence of hexanoic acid in the inoculated samples  
121 compared to the mastitis samples was similar to the other pathogens. Additionally, 2-  
122 methylbutanal and acetoin were absent in the inoculated samples. Furthermore, the inoculated

123 samples contained a higher concentration of dimethyl sulfide compared to the mastitis  
124 samples.

125

### 126 3.2.4 *Streptococcus dysgalactiae*

127 In the inoculated samples, the ethyl esters, 2-methylbutanal, isopentanol, and 3-  
128 methylbutanoic acid were all absent. Hexanoic acid was present in all inoculated samples, but  
129 not in the mastitis samples. Like the other inoculated samples, samples inoculated with *S.*  
130 *dysgalactiae* contained a higher concentration of dimethyl sulfide compared to mastitis  
131 samples.

132

### 133 3.2.5 *E. coli*

134 The samples inoculated with *E. coli*, like the inoculated *S. aureus* samples, had very limited  
135 formation of ethyl esters of free fatty acids. As for other pathogens, the presence of hexanoic  
136 acid in inoculated samples was also seen in the samples inoculated with *E. coli*. In addition to  
137 these two differences, a 100-fold increase in dimethyl sulfide was seen for the inoculated  
138 samples. This increase was much higher than for the other pathogens.

139

### 140 3.3 Prediction by PNN

141 The results of the inoculated samples were used in the prediction module of the previously  
142 developed PNN. Of the 15 inoculated samples, 14 were correctly classified (93%). Only 1 of  
143 the *E. coli* samples was incorrectly classified.

144

## 145 4. Discussion

146 Comparing the mastitis samples to the inoculated samples, the majority of the volatile  
147 metabolites were present in both types of samples in similar amounts. The prediction of the



148 PNN showed that the similarity between the inoculated samples and mastitis samples was  
149 sufficient for correct prediction of the pathogen in the inoculated sample.

150 Looking at the 20% of metabolites which were different between the inoculated samples  
151 and mastitis samples, some differences were seen for all 5 pathogens. The main difference  
152 was that in inoculated samples, ethyl esters of free fatty acids were absent, or significantly  
153 lower. This may be explained by the activity of the esterase enzyme. Esterases are enzymes  
154 which are produced by the cow and occur in the blood (McSweeney and Sousa, 2000). In case  
155 of mastitis, this enzyme can probably transfer from the blood to the milk due to disturbance of  
156 the blood-milk barrier (Raulo et al., 2002). These enzymes would then not be present in the  
157 milk of healthy cows, thus explaining the absence of the ethyl esters in inoculated samples.

158 Hexanoic acid was detected in all inoculated mastitis samples, but not in any of the 50  
159 mastitis samples. However, in most of the mastitis samples, ethyl hexanoate was detected.  
160 Hexanoic acid is a precursor for the formation of ethyl hexanoate. Possibly, the hexanoic acid  
161 in the mastitis samples was converted by esterase to ethyl hexanoate.

162 In the inoculated samples, a higher dimethyl sulfide concentration was found compared to  
163 the mastitis samples. The relative amount of dimethyl sulfide found in all inoculated samples  
164 ( $6 \times 10^5$  to  $1 \times 10^6$  arbitrary units of area), except for the sample inoculated with *E. coli*, was  
165 similar to what was found in the milk of healthy cows (Hettinga et al., 2008a). The observed  
166 difference between inoculated samples and mastitis samples may be due to different sample  
167 handling, because dimethyl sulfide is easily lost due to its high volatility (Patton, 1956). This  
168 does, however, not explain the high concentration of dimethyl sulfide in the samples  
169 inoculated with *E. coli*. Dimethyl sulfide most likely is a metabolite formed by *E. coli* and  
170 was also probably lost in the mastitis samples due to handling. The formation of dimethyl  
171 sulfide by *E. coli* may be useful for the identification of *E. coli* in mastitis samples.

172 In some of the samples, especially those inoculated with CNS and *S. dysgalactiae*, an  
173 increased concentration of 2-butanone was detected. However, the concentration of 2-  
174 butanone still falls within the normal range of this component for fresh raw milk (Hettinga et  
175 al., 2008a).

176

## 177 **5. Conclusions**

178 When comparing the whole pattern of volatile metabolites, it can be concluded that most  
179 metabolites were present in both inoculated as well as mastitis samples, in similar  
180 concentrations. The prediction of the PNN showed that the similarity between the inoculated  
181 samples and mastitis samples was sufficient for correct prediction of the pathogen in  
182 inoculated samples (93% correct).

183 The absence of ethyl esters was the most striking difference between the two types of  
184 samples, and this could be explained by the absence of transfer of the esterase enzyme from  
185 the cow's blood to the milk of a healthy cow.

186 Therefore, it can be concluded that the metabolites found in mastitis samples are mainly  
187 formed by the pathogens and that the ethyl esters of free fatty acids are, probably, formed by  
188 the esterase enzyme from cow's blood.

189 Finally, because of the similarities between inoculated and mastitis samples, inoculation  
190 can be used to obtain samples for further research on the use of volatile metabolites in mastitis  
191 diagnosis.

192

## 193 **6. References**

194 Acree, T. E. and H. Arn. Website: <http://www.flavornet.org/>. Accessed: 20 Oct., 2008.

195

196 Barkema, H. W., Schukken, Y. H. Lam, T. J. G. M. Beiboer, M. L. Wilmink, H. Benedictus,  
197 G., Brand. A., 1998. Incidence of clinical mastitis in dairy herds grouped in three categories  
198 by bulk milk somatic cell counts. *J. Dairy Sci.* 81, 411-419.  
199

200 Hettinga, K.A., H.J.F van Valenberg and A.C.M. van Hooijdonk. 2008a. Quality control of  
201 raw cows' milk by headspace analysis. *International Dairy Journal* 18:506-513.  
202

203 Hettinga, K. A., H. J. F. van Valenberg, T. J. G. M. Lam, and A. C. M. van Hooijdonk.  
204 2008b. Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *Journal of*  
205 *Dairy Science* 91:3834-3839.  
206

207 Le Rouw, Y., J. M. Girardet, G. Humber, F. Laurent, and G. Linden. 1995. Proteolysis in  
208 samples of quarter milk with varying somatic cell counts. 2. Component PP3 and b-Casein-IP  
209 (as105 and f29-107) of the proteose-peptone fraction. *Journal of Dairy Science* 78:1298-1305.  
210

211 Marquardt, R. R., T. L. Forster, G. R. Spencer and G. H. Stabenfeldt. 1965. A-Esterase  
212 activities of milk from cows with experimentally induced mastitis. *Journal of Dairy Science*  
213 49:631-635.  
214

215 McSweeney, P. L. H. and M. J. Sousa. 2000. Biochemical pathways for the production of  
216 flavour compounds in cheeses during ripening: a review. *Lait* 80:293-324.  
217

218 NMC. 2004. Microbiological procedures for use in the diagnostics of bovine udder infection  
219 and determination of milk quality. National Mastitis Council, Inc., Madison, WI.  
220

- 221 Patton, S., D. A. Forss and E. A. Day. 1956. Methyl sulfide and the flavor of milk. Journal of  
222 Dairy Science 39:1469-1470.  
223
- 224 Raulo, S. M., T. Sorsa, T. Tervahartiala, T. Latvanen, E. Pirilä, J. Hirvonen and P. Maisi.  
225 2002. Increase in milk metalloproteinase activity and vascular permeability in bovine  
226 endotoxin-induced and naturally occurring *Escherichia coli* mastitis. Veterinary Immunology  
227 and Immunopathology 85:137-145.  
228
- 229 Ritter, W. and H. Hanni. 1960. The application of gas chromatography in dairying. II.  
230 Detection and determination of volatile fatty acids in dairy products and cultures.  
231 Milchwissenschaft 15:296-302.  
232
- 233 Sampimon, O. C., R. G. M. Olde Riekerink, and T. J. G. M. Lam. 2008. Prevalence of  
234 subclinical mastitis pathogens and adoption of udder health management practice on Dutch  
235 dairy farms: preliminary results. Pages 39-46 in Proceedings of Mastitis Control 2008 'From  
236 Science to Practice', The Hague, The Netherlands.  
237
- 238 Zechman, J. M., S. Aldinger, and J. N. Labows jr. 1986. Characterization of pathogenic  
239 bacteria by automated headspace concentration-gas chromatography. Journal of  
240 Chromatography 377:49-57.  
241  
242

243 **Table 1:** Amount (arbitrary units of area) of volatile metabolites detected in mastitis samples and inoculated samples containing *S. aureus*,  
 244 coagulase-negative staphylococci (CNS), *S. uberis*, *S. dysgalactiae*, and *E. coli* (n=10 for mastitis samples, n=3 for inoculated samples).

Metabolite	<i>S. aureus</i>		CNS		<i>S. uberis</i>		<i>S. dysgalactiae</i>		<i>E. coli</i>	
	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated
Acetaldehyde	$7 \times 10^6$	$2 \times 10^7$	$6 \times 10^4$	$1 \times 10^5$	$3 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$1 \times 10^5$	$3 \times 10^5$	$2 \times 10^5$
Ethanol	$1 \times 10^8$	$3 \times 10^7$	$1 \times 10^5$	$4 \times 10^4$	$1 \times 10^6$	0	$6 \times 10^6$	0	$1 \times 10^8$	$4 \times 10^7$
Dimethyl sulfide	$9 \times 10^4$	$1 \times 10^{6a}$	$3 \times 10^4$	$6 \times 10^{5a}$	$2 \times 10^5$	$9 \times 10^{5a}$	$2 \times 10^5$	$8 \times 10^{5a}$	$1 \times 10^5$	$2 \times 10^{7a}$
2,3-Butadione	$1 \times 10^6$	$3 \times 10^6$	$5 \times 10^4$	$3 \times 10^3$	$6 \times 10^5$	$1 \times 10^4$	$1 \times 10^5$	$4 \times 10^3$	$2 \times 10^4$	$4 \times 10^{5a}$
2-Butanone	$2 \times 10^6$	$3 \times 10^6$	$3 \times 10^5$	$3 \times 10^{6a}$	$1 \times 10^6$	$3 \times 10^6$	$7 \times 10^5$	$5 \times 10^{6a}$	$5 \times 10^5$	$4 \times 10^6$
Eth. acetate	$1 \times 10^6$	$4 \times 10^{4a}$	0	0	$5 \times 10^4$	0 <sup>a</sup>	$2 \times 10^4$	0 <sup>a</sup>	$7 \times 10^5$	$4 \times 10^5$
3-Meth.butanal	$3 \times 10^7$	$8 \times 10^7$	$3 \times 10^6$	$2 \times 10^6$	$9 \times 10^4$	$8 \times 10^5$	$5 \times 10^5$	$5 \times 10^5$	$5 \times 10^4$	$3 \times 10^5$
2-Meth.butanal	$4 \times 10^5$	$2 \times 10^6$	$6 \times 10^4$	$6 \times 10^4$	$3 \times 10^3$	0	$3 \times 10^3$	0	0	0
2-Pentanone	$8 \times 10^4$	$1 \times 10^5$	$8 \times 10^3$	$4 \times 10^4$	$5 \times 10^4$	$1 \times 10^5$	$7 \times 10^4$	$7 \times 10^4$	$1 \times 10^6$	$6 \times 10^4$
Acetic acid	$6 \times 10^6$	$4 \times 10^7$	$5 \times 10^5$	$2 \times 10^6$	$3 \times 10^6$	$2 \times 10^6$	$3 \times 10^6$	$3 \times 10^6$	$1 \times 10^7$	$9 \times 10^6$
Isopentanol	$7 \times 10^6$	$2 \times 10^6$	$1 \times 10^6$	0	0	0	$1 \times 10^5$	0	$4 \times 10^6$	0
Acetoin	$8 \times 10^5$	$5 \times 10^5$	$3 \times 10^3$	0	$4 \times 10^3$	0	0	0	0	0
Eth. butyrate	$7 \times 10^5$	0 <sup>a</sup>	0	0	$7 \times 10^4$	0 <sup>a</sup>	$1 \times 10^5$	0 <sup>a</sup>	$3 \times 10^5$	$2 \times 10^4$
Butyric acid	$3 \times 10^6$	$4 \times 10^5$	$2 \times 10^5$	$4 \times 10^5$	$8 \times 10^5$	$3 \times 10^5$	$1 \times 10^6$	$4 \times 10^5$	$1 \times 10^6$	$2 \times 10^6$
Eth. 2-meth.but.	$2 \times 10^4$	0 <sup>a</sup>	$4 \times 10^2$	0	0	0	0	0	0	0
Eth. 3-meth.but.	$1 \times 10^5$	0 <sup>a</sup>	$5 \times 10^2$	0	$3 \times 10^3$	0	$3 \times 10^3$	0	0	0
3-Meth.but.	$1 \times 10^6$	$4 \times 10^4$	0	0	0	0	$7 \times 10^2$	0	0	0
2-Meth.but.	$6 \times 10^6$	$1 \times 10^5$	0	0	0	0	0	0	0	0
2-Heptanone	$2 \times 10^5$	$4 \times 10^4$	$2 \times 10^4$	$4 \times 10^4$	$1 \times 10^5$	$3 \times 10^4$	$1 \times 10^5$	$4 \times 10^4$	$3 \times 10^5$	$5 \times 10^4$
Hexanoic acid	0	$3 \times 10^{5a}$	0	$2 \times 10^{5a}$	0	$1 \times 10^{5a}$	0	$2 \times 10^{5a}$	0	$3 \times 10^{6a}$
Eth. hexanoate	$4 \times 10^5$	0 <sup>a</sup>	$8 \times 10^2$	0	$6 \times 10^4$	0	$9 \times 10^4$	0	$2 \times 10^5$	0 <sup>a</sup>

245 <sup>a</sup> significant difference between mastitis samples and inoculated sample ( $p < 0.05$ )

246