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The Effect of GDL-induced pH Decrease on the Formation of Biogenic Amines in Meat

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ABSTRACT

The effect of pH on the formation of biogenic amines has mainly been studied in broths in which pH has been fixed before incubation. However, in the fermentation of dry sausage, pH quite rapidly decreases from the initial value to a certain level. In this study glucono-delta-lactone (GDL) was used to decrease pH in meat. Six minced meat samples were each divided into three portions (A-C): 0% (A), 0.5% (B), or 1.0% (C) of GDL was added and the samples were incubated at 20-22°C for 7 d. The amounts of biogenic amines (histamine, tyramine, putrescine, cadaverine, phenylethylamine, tryptamine, spermine, and spermidine) as well as pH, water activity, and the bacterial counts of lactic acid bacteria, fecal streptococci, coliforms, and total plate count were measured. Addition of GDL resulted in a significant decrease in pH and in the levels of histamine and putrescine as well as in the levels of fecal streptococci, coliforms, and total plate counts. Of 87 fecal streptococci, seven *Enterococcus faecalis* strains produced tyramine. All the coliforms and related strains isolated from violet red bile agar produced tyramine, putrescine, and cadaverine on agar plates. However, the proportion of histamine-positive strains of these strains, especially *Hafnia alvei*, increased from 0 to 57% during the incubation. The rate and level of pH decrease clearly affected amine formation in meat, indicating that the levels of, e.g., histamine produced could be decreased by optimizing the pH decrease during fermentation. Addition of GDL facilitates study of the effect of pH decrease without interactions between the starter culture and contaminant flora.

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (*1,19,23*). Amines such as putrescine, cadaverine, spermine, and spermidine are important in the regulation of nucleic acid function and protein synthesis and probably also in the stabilization of membranes (*4,27*). Some heterocyclic amines, such as tyramine and histamine, have vasoactive properties, and they can cause histamine poisoning or migraine (*1,9,18,20,31*).

Koessler et al. (*15*) proposed that biogenic amine formation is a protective mechanism for bacteria against acidic environments. The production of amines requires the availability of free amino acids and appropriate status of environmental factors such as pH and temperature. Many authors have reported the ability of different bacterial strains to produce biogenic amines or amine-decarboxylating enzymes. Amine-producing coliforms such as *Escherichia coli* and *Klebsiella oxytoca* and related bacteria like *Morganella morganii* and *Edwardsiella* spp. as well as lactic acid bacteria like *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus carnis, Lactobacillus curvatus, Lactobacillus divergens,* and *Lactobacillus hilgardii* have been isolated from meat and meat products (*8,32*).

There have been several reports of high levels of biogenic amines in fermented sausages obtained from retail markets (*3,22,23,25,26,32-34*). In fermented sausages the pH decreases during ripening. In vitro, the effect of pH on the formation of biogenic amines has mainly been studied by adjusting the initial pH value of the broth to different levels but not by decreasing the pH during the cultivation (e.g., *2,5,6,28,30*). These studies are important for basic knowledge of amine formation, but they may not reflect fermented products where pH changes as fermentation proceeds.

GDL (D-gluconic acid delta-lactone) is used in the meat industry for dry sausage manufacture, especially with starter cultures which do not contain lactic acid bacteria. It hydrolyzes spontaneously in water to gluconic acid and causes decrease in pH. GDL is, therefore, an interesting compound for studying the effects of pH decrease in meat without starter culture.

Karmas (*14*) proposed the Biogenic Amine Index (BAI) for measurement of the quality of raw and processed seafood: BAI = histamine + putrescine + cadaverine/1 + spermine + spermidine. A BAI value exceeding 10 is regarded as representing some kind of loss in quality. Because this index combines the results of five amines, it is a useful tool for the comparison of different meat samples.

The purpose of this study was to investigate the possible effect of pH decrease on amine formation in meat. GDL was used in order to avoid possible interactions between starter cultures and contaminant flora.

MATERIALS AND METHODS

Meat samples

Six minced meat samples were obtained from retail markets. Each sample was divided into three smaller samples (a' 300 g)

designated A, B, and C. No GDL was added to the samples A, and they served as negative controls for the GDL samples. To samples B 0.5% GDL and to samples C 1.0% GDL was added. The samples were then carefully mixed and transferred to plastic bags sealed by heat-jointing (room atmosphere). The bags were incubated at 20-22°C for 7 d.

Biogenic amines, bacteriological counts, and pH were determined on days 0, 1, 3, and 7 after addition of GDL. The plastic bags were resealed after each sampling.

Glucono-delta-lactone (GDL)

GDL (Gluconofin B) was obtained from Finnsugar Bioproducts, P.O. Box 105, SF-00241, Helsinki, Finland.

Biogenic amines

Biogenic amines were extracted from 2 g of sample with 20 ml of 0.4 M pechloric acid solution (Merck, 519). One milliliter of sample extract was derivatized with addition of 200 µl 2 N sodium hydroxide solution (EKA, 040980), 300 µl saturated sodium bicarbonate (Merck, 6346), and 2 ml of dansyl chloride solution [10 mg dansyl chloride (Serva) in 1 ml acetone]. The reaction mixture was incubated at 40°C for 45 min. After incubation the residual dansyl chloride was removed by addition of 100 µl ammonia (Merck, 5432). After 30 min, sample was adjusted to 5 ml with acetonitrile (Rathburn, HPLC-grade), centrifugated, and filtered with 0.45-µm filter (Millipore, SJHVL04NS).

Liquid chromatographic separations were performed with HP 1090 liquid chromatography using a Spherisorb ODS2 column (125 x 4 mm, 5 µm) and gradient elution program with a mixture of 0.1 M ammonium acetate (Merck, 1116) and acetonitrile. The gradient begun with 50% and ended with 90% acetonitrile in 19 min. The flow rate was 1.0 ml/min, and the column effluent was monitored at 254 nm.

The limits of determination for 2 g of sample were 1 mg/kg for tyramine, histamine, spermine, spermidine, and cadaverine and 2 mg/kg for phenylethylamine and putrescine.

For the production of histamine and tyramine, isolated lactic acid bacteria were adapted for 24 h at 30°C in MRS-broth (initial pH 6.3) containing 2.0 g/L L-tyrosine (Difco) or L-histidine-monohydrochloride (Merck). One hundred microliters of the broth was further transferred to another tube of the same medium and incubated at 30°C for 48 h. One milliliter of mixed broth culture was diluted to 10 ml with 0.4 M perchloric acid solution. Biogenic amines were determined from dilutions as their dansyl derivatives with the same high pressure liquid chromatography method as that used for the meat samples.

Bacterial counts

A 10-g sample of mixed minced meat was serially diluted with a diluent containing 0.1% peptone and 0.8% NaCl in sterile deionized water. Four different groups of bacteria were determined. Coliform bacteria were enumerated on Violet Red Bile Agar [VRB, Orion; ISO method No. 4832:1991 (E) (*12*)], fecal streptococci on Slanetz-Bartley agar [SB, Oxoid, NCFA method No. 68, 1978; incubated at 37°C for 48 h, (*21*)], aerobic plate count microorganisms on plate count agar [LAB M; International standard method No. 2293:1988 (*11*)], and lactic acid bacteria on de Man, Rogosa and Sharpe agar with sorbic acid (MRS-S, LAB M's MRS containing sorbic acid, Fluka (*24*); incubated at 20-22°C for 5 d anaerobically).

Amine agar

The strains isolated from VRB and SB plates were examined for decarboxylation of histidine, tyrosine, ornithine, and lysine on amine agar plates. The media containing 2% of tyrosine (T), histidine (H), ornithine (O), or lysine (L) were prepared according to the method of Joosten and Northolt (*13*) and autoclaved at 105°C for 5 min. Pure cultures of isolated bacterial strains were spread on agar plates which were incubated aerobically at 37°C for 48 h. Purple colonies were counted as positive for amine production. The reference strains were obtained from the National Veterinary Institute, Department of Food Hygiene, Finland: the results for *Escherichia coli* NVIF-085 were T+, H-, L+, and O+; *Enterococcus faecalis* NVIF-155 T+, H-, L-, and O-; *Enterococcus faecium* NVIF-070 T+, H-, L-, and O-; and *Klebsiella pneumoniae* NVIF-030 T+, H-, L+, and O+.

Strain identification

The tyramine-positive strains isolated from SB agar were identified by gram staining, catalase and oxidase test, growth in brain heart infusion broth (Difco) at 44.5°C for 48 h, growth in brain heart infusion containing 6.5% NaCl at 37°C for 72 h, and using the AP 120 Strep system. Some of the strains isolated from VRB agar were identified by the oxidase test and using lauryl tryptose mannitol broth with tryptophan (LTM, Oxoid), containing methylumbelliferyl-B-D-glucuronide (MUG, Oxoid BR71, one vial of 50 mg MUG per L LTM broth; modified SFS standard No. 4089, 1988) and incubated at 44.5°C for 24 h (*E. coli*). All non-*E. coli* strains, all the other strains isolated from VRB, and some *E. coli* strains according to results of LTM-MUG were identified by the oxidase test and the API 20E system.

pH and water activity (a_w)

The pH values were measured directly from the samples using an Orion Research Incorporated SA 520 pH/mV meter, with a Ross™ pH-electrode No. 8163 (Switzerland). After a 2-h adaptation period, a_w values were obtained at 25°C from a sample of 25-30 g using a Rotronic Hygroskop (Fattore Vitale & Co, Italy).

Statistics

The multiple analysis of variance, Mann-Whitney test (two-sided), and correlation were performed by Survo 84C (version 3.01, University of Helsinki, Department of Statistics) software on an IBM PC/70.

RESULTS

Parameters measured from initial samples

The initial parameter values of minced meat samples varied considerably, which was as intended in this study in order to obtain an estimate of the average quality of minced meat. The initial biogenic amine concentrations were: histamine 2-5 mg/kg, tyramine 1-46 mg/kg, putrescine 1-49 mg/kg, cadaverine 2-49 mg/kg, spermine 19-35 mg/kg, spermidine 2-4 mg/kg, tryptamine 1-17 mg/kg, and phenylethylamine 12-79 mg/kg. The pH values varied from 5.2 to 5.8, a_w values from 0.96 to 0.98 and the bacteriological counts of total aerobic plate count $\log_{10}$ 5.5-8.3 CFU/g, lactic acid bacteria $\log_{10}$ 4.6-8.2 CFU/g, fecal streptococci $\log_{10}$ 2.3-4.4 CFU/g, and coliforms $\log_{10}$ 2.5-7.8 CFU/g.

Biogenic amines, pH, and a_w during incubation

The amounts of biogenic amines except spermine and spermidine increased during incubation in all of the samples (Table 1). In particular, the increases of histamine, tyramine, putrescine, and cadaverine were considerable. The higher were the initial concentrations of amines in the sample, the higher were they also at the end of incubation (results not shown). Addition of GDL decreased significantly the levels of histamine and putrescine.

BAI was used to compare the results of different samples in this study. The means of BAI and pH during

TABLE 1. *The biogenic amines in fresh minced meat and in samples incubated for 1, 3, and 7 d.*

Day	GDL (%)	Histamine (mg/kg)		Putrescine (mg/kg)		Cadaverine (mg/kg)		Spermine (mg/kg)		Spermidine (mg/kg)		Tyramine (mg/kg)		Tryptamine (mg/kg)		Phenylethylamine (mg/kg)	
0	-	3		16		23		27		3		19		7		36	
1st	0.0	6		51		62		29		3		53		18		36	
	0.5	4	-	39	-	43	-	29	-	3	-	49	-	16	-	39	-
	1.0	4	-	37	-	39	-	28	-	4	-	39	-	16	-	37	-
3rd	0.0	22		95		113		28		3		88		43		46	
	0.5	6	-	77	-	85	-	26	-	4	-	83	-	32	-	47	-
	1.0	4	-	62	*	70	-	28	-	4	-	73	-	27	-	49	-
7th	0.0	126		236		386		26		3		131		73		69	
	0.5	19	*	214	-	318	-	27	-	5	-	219	-	77	-	64	-
	1.0	7	**	147	-	215	-	28	-	5	-	201	-	60	-	50	-

Means of six samples. The results of 0.5 and 1.0% GDL were compared to the negative control (0.0% GDL) each day by Mann-Whitney-test (two-sided). - Not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

incubation are presented in Fig. 1. Addition of GDL decreased the pH of the samples. The lower the pH after GDL addition on the first day, the lower was the BAI after 7 d of incubation. The highest BAI was seen in the samples without GDL already after the first day of incubation. In the analysis of the correlation between BAI and pH at the 0, 1st, 3rd, and 7th day of incubation, r was -0.84 ($p < 0.001$), 0.17 ($p > 0.05$), 0.43 ($p > 0.05$), and 0.74 ($p < 0.001$), respectively.

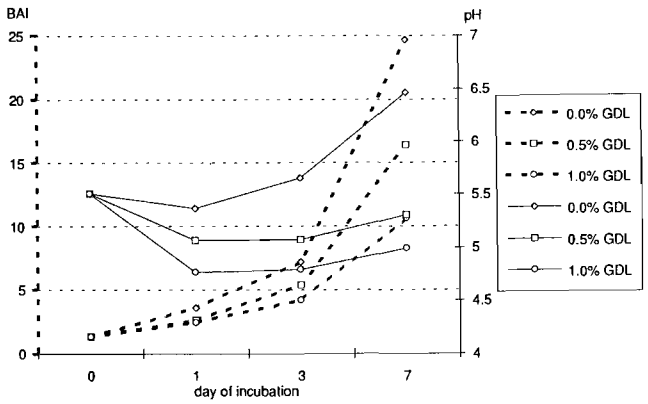

Figure 1. *The effect of GDL on the BAI (Biogenic Amine Index) and pH in minced meat samples during incubation at 20-22°C for 7 d. Each point represents the mean of six different samples (--- = BAI, _ = pH).*

The a_w values varied from 0.96 to 0.97 (1st day), from 0.96 to 0.98 (3rd day), and from 0.96 to 0.98 (7th day) during incubation. No differences between the samples A-C were observed.

Bacteriological results during incubation

Addition of GDL decreased significantly the levels of fecal streptococci, total aerobic bacteria, and coliforms (Table 2). However, it did not affect the levels of lactic acid bacteria, which is natural while these bacteria are aciduric.

Of the 87 strains isolated from SB agar, seven produced tyramine on the agar plates. They were all identified as *Enterococcus faecalis*; 97-100% of the 94 strains isolated from VRB produced tyramine, putrescine, and cadaverine. However, the strains isolated from the samples of different days differed from each other in the production of histamine from histidine. None of the strains isolated from the raw material produced histamine. During incubation of the minced meat samples, the proportion of histamine producing strains isolated increased: after the 1st day 11%, the 3rd day 17%, and the 7th day 57% of the isolates produced histamine on agar plates. GDL addition had no effect on this change in histidine decarboxylation.

Sixty of the isolates from VRB were identified as *Hafnia alvei*. The numbers of histamine-positive and histamine-negative *H. alvei* strains during incubation were: initial numbers 0 and 2 (29% of all the seven VRB strains isolated), after 1 d, 1 and 14 (54% of all the 28 VRB strains isolated); after 3 d, 3 and 18 (71% of all the 29 VRB strains isolated), and after 7 d, 12 and 10 (73% of all the 30 VRB strains isolated), respectively.

TABLE 2. *The bacteriological counts in fresh minced meat and in samples incubated for 1, 3, and 7 d.*

Day	GDL %	Fecal streptococci (CFU/g)		Lactic acid bacteria (CFU/g)		Total plate count (CFU/g)		Coliforms (CFU/g)	
0	-	3.6		6.6		6.8		4.9	
1st	0.0	4.5		8.2		7.9		6.6	
	0.5	3.9	-	8.0	-	7.7	-	6.0	-
	1.0	3.7	*	7.9	-	7.5	-	5.8	-
3rd	0.0	6.1		8.3		8.8		8.4	
	0.5	4.7	*	8.2	-	8.1	-	7.1	*
	1.0	4.0	**	8.1	-	8.0	*	6.7	**
7th	0.0	7.6		8.2		9.1		8.9	
	0.5	7.1	*	8.5	-	8.6	*	8.2	**
	1.0	6.3	**	8.1	-	8.1	**	7.4	**

Means of six samples. The results of 0.5 and 1.0% GDL were compared to the negative control (0.0% GDL) each day by Mann-Whitney-test (two-sided). - Not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Seventeen of the other isolates from VRB were identified as *E. coli* (none were histamine positive), 4 as *Enterobacter aerogenes* (none histamine positive), 3 as *Serratia liquefaciens* (all histamine positive), 3 as *Klebsiella oxytoca* (all histamine positive), 2 as *Enterobacter agglomerans* (one histamine positive and one negative), and 5 as *Enterobacter* sp. (two histamine positive and three negative). The GDL added did not affect the amounts of amine-positive strains detected.

Of the 24 isolated lactic acid bacterial strains, none produced histamine or tyramine in MRS-H or MRS-T broth.

Statistical results

The pH value correlated with all the amines except tyramine and phenylethylamine. According to multiple analysis of variance, the GDL level affected the pH, the histamine and putrescine values, and the numbers of coliforms. By contrast, the tyramine contents did not correlate with pH or with the GDL added.

DISCUSSION

Decrease in pH caused by added GDL clearly affected the levels of both amines and bacteria in minced meat samples. The greater was the pH decrease during the first day the less histamine and putrescine were formed, and the lower were the levels of fecal streptococci, total plate counts, and coliforms. According to these results, there seems to be a clear relationship between the levels of certain amines and bacterial counts. If a minced meat sample contains high levels of coliforms and fecal streptococci, high levels of histamine and putrescine also can be detected.

Great variations have been reported between the levels of amines detected in retail dry sausages (*23,29*). According to the results obtained in this study, one possible factor to explain this could be variation in the pH decrease between different types of sausages. Several factors influence the pH decrease of dry sausage, e.g., starter culture, contaminant flora, temperature/time combinations, and food additives used (*10,16,17*). The results of acidification with GDL cannot be directly applied to dry sausage fermentation because of the effect of the factors mentioned. However, the benefit of the use of GDL is that it permits study of the effect of pH decrease without interactions between starter culture and contaminant flora.

All the amine-positive bacterial strains isolated in this study were also known as traditional amine producers in fish or dairy products. However, the percentage increase of histamine-positive strains isolated from VRB, particularly of *H. alvei*, during incubation was exceptional. One reason for this phenomenon could be accelerated proteolysis increasing the amounts of histidine in meat samples during incubation. This would support the idea that histidine decarboxylase of these bacteria is an induced enzyme. The tyrosine aminotransferase (of *Trichoderma viride*), which is the initiator of the degradation of tyrosine, has already been found to be inducible, the best inducer being L-tyrosine (*7*).

It can be concluded that not only the initial pH value of the environment but also the decrease of pH during incubation affect the formation of biogenic amines in meat. It could be possible to optimize the process and starter cultures to decrease the formation of amines, especially histamine, during ripening of dry sausage.

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