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Dried Sausages Fermented with *Staphylococcus xylosus* at Different Temperatures and with Different Ingredient Levels. Part IV. Amino Acid Profile

C. Waade & L. H. Stahnke

Department of Biotechnology, Technical University of Denmark, build. 221, Lyngby 2800, Denmark

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ABSTRACT

Sausages with added Staphylococcus xylosus *were fermented at different temperatures and with different added levels of salt, glucose, nitrite, nitrate and* Pediococcus pentosaceus *in accordance with a six-factor fractional design. The amounts of individual amino acids were measured and the effects of temperature and different ingredients on the amino acid profile were tested using multiple linear regression and analysis of variance. Also, the amino acid profile was correlated to the level of volatile compounds by partial least squares analysis. The study showed that the level of free amino acids was significantly affected by the different factors. High fermentation temperature and nitrite content increased the amount, while high contents of salt, nitrate, glucose and* P. pentosaceus *lowered the amount of free amino acids. In general, temperature and nitrate had the greatest influence. It was shown that the amounts of the volatile compounds, 2-methyl propanal, 2- and 3-methyl butanal, were inversely correlated with the amounts of valine, isoleucine and leucine, respectively, indicating that those volatiles were degradation products of the latter.* © *1997 Elsevier Science Ltd*

INTRODUCTION

During fermentation and ripening of fermented sausage, the concentration of water soluble nitrogenous compounds like small proteins, peptides and free amino acids steadily increases due to proteolytic activity of microbial and endogenic enzymes (Dierick *et al.*, 1974; Lois *et al.*, 1987; DeMasi *et al.*, 1990; Naes *et al.*, 1991; Johansson *et al.*, 1994*a*).

The amino acids liberated have been investigated by several workers (Dierick *et al.*, 1974; Dineva *et al.*, 1984; DeMasi *et al.*, 1990; and many others). Depending on sausage type and ripening period, the amounts of free amino acids in fermented sausages are in the range of 300–1400 mg per 100 g of dry weight (Langner, 1969; Wardlaw *et al.*, 1973; Dierick *et al.*, 1974; DeMasi *et al.*, 1990), though levels up to 2200 mg have been measured in Spanish sausages (Astiasaran *et al.*, 1990).

Some workers have studied the effect of microbial growth on the content of free amino acids. Verplaetse *et al.* (1992, 1995) showed that the addition of antibiotics to sausage mince reduced the level of free amino acids by up to 60–70%, indicating that more than half of

the breakdown of peptides into amino acids is caused by microorganisms. Similarly, Sajber *et al.* (1971) detected 50% more free amino acids in sausages fermented with the starter culture 'Bactoferment' than in sausages with added antibiotics. DeMasi *et al.* (1990) showed that *Pediococcus pentosaceus*, *P. acidilactici* and *M. varians* significantly affected the level of free amino acids in fermented sausages, creating three different amino acid profiles. Dierick *et al.* (1974) did not see a striking effect on the amino acid pattern but found that the addition of starter culture slightly increased the content of free amino acids.

The effect of different processing parameters on the proteolytic activity has been studied by several workers. For example: Klement & Cassens (1974) and Lee & Song (1987) showed that proteolytic activity increased with increasing temperature. Verplaetse *et al.* (1992) found that low pH increased the breakdown of myosin and actin. Toldrá *et al.* (1993) showed that salt concentrations higher than 2·5% (w/v) inhibited all investigated peptidases to some degree. At levels of 200 mg/kg, nitrite had a slight inhibiting effect (72–95% of the activity remained). Klement & Cassens (1974) found that sausages with added 0·3% (w/w) glucose had higher levels of non-protein nitrogen (NPN) than sausages without glucose, perhaps due to a final lower pH.

The purpose of the present study was to investigate the influence of temperature and basic sausage ingredients on the content of free amino acids in sausages fermented with *Staphylococcus xylosus*. a starter culture widely used in fermented sausages. The experimental design was set up as a fractional factorial design examining fermentation temperature, concentration of salt, nitrite, nitrate and glucose, and addition of another starter culture, the acidifying *P. pentosaceus*.

MATERIALS AND METHODS

Detailed information on experimental design, sausage production, chemical, bacteriological and sensory analyses and on the analysis of volatiles are given in the three proceeding papers (Stahnke, 1995*a*,*b*,*c*). Table 1 sums up the experimental design and the factorial levels.

Amino acid analyses

Analyses of free amino acids were performed on sausage samples after 2, 4 and 8 weeks of fermentation/drying.

Sample preparation

The free amino acids were extracted by a modified technique from Aristoy & Toldrá (1991). Frozen sausage was blended for 1 min and 5-g samples diluted 1:30 (W/V) with 0.1M cold HCl. The suspension was homogenized at 0–5°C for 6 min (Stomacher, Seward Laboratory, U.K.) and centrifuged at 13 000 *g* for 25 min at 4°C. Solidified fat was removed and the supernatant filtrated (Whatmans no. 4), diluted 1:10 (V/V) and ultra filtrated (10 kDa cutoff, 10 ml stirred cell, Filtron Technology, U.S.A). The resulting extract was analysed by HPLC. Three replicate extracts with duplicate HPLC analysis were made.

HPLC analysis

Free L-amino acids were separated by reverse-phase HPLC and detected as ortho-phtaldialdehyde-mercaptoethanol (OPA-ME) derivatives. HPLC system: Waters WISP M712 autosampler, M510 pumps, SIM A/D interface module, TCM column heater (45°C), fluorescence detector M420 (flow cell 8 μl, lamp 4 W, excitation 334 nm, emission 425 nm) and Maxima 810 and 820 software (Waters, U.S.A.). Derivatization was done automatically in the autosampler in a coiled capillary tube (i.d. 1·5 mm × 150 mm). Sample

TABLE 1
Experimental Design[a]

Batch no	*Factor*					
	Fermentation temperature	Pediococcus pentosaceus	*$NaNO_2$*	*KNO_3*	*Glucose*	*Salt*
A	0	0	1	0	1	1
B	0	1	1	0	0	0
C	0	0	0	1	0	1
D	0	1	0	0	1	1
E	0	0	0	0	0	0
F	0	1	1	1	0	1
G	0	1	0	1	1	0
H	0	1/2	1/2	1/2	1/2	1/2
I	0	0	1	1	1	0
J	1	1	0	0	0	1
K	1	0	1	0	0	1
L	1	1	1	1	1	1
M	1	0	0	1	1	1
N	1	0	1	1	0	0
O	1	0	0	0	1	0
P	1	1	0	1	0	0
Q	1	1	1	0	1	0
R	1	1/2	1/2	1/2	1/2	1/2
Factor levels						
Low	15°C	0[b]	50 ppm	0	0	1.5%
High	25°C	4.2×10^7 cfu/g	300 ppm	0.20%	1.0%	3.5%

[a]0, low level; 1, high level, 1/2, centre level.
[b]Minces did not have added *Pediococcus pentosaceus*, but a natural flora of $2{\cdot}5\times10^3$ cfu/g lactic acid bacteria was present from the very beginning.

volume was 10-30 μl, OPA-ME 90 μl (100:1, PO532, M6250, Sigma), reaction time 1 min. Column: Nova-Pak C18 column, 3·9 mm i.d. × 150 mm, pore size 60 Å, particle size 4 μm (no. 36975, Waters, USA). Eluents: A: 88% v/v 0·1M NaAc (p.a., pH = 7·8, Merck no. 6267), 9·5% v/v methanol (gradient grade, Lichrosolv, Merck 6007), 2·5% v/v tetrahydrofuran (Lichrosolv, Merck 8181); B, methanol (gradient grade, Lichrosolv, Merck 6007). The eluents were filtered (0·2 μm Minisart®, Sartorius, Göttingen, Germany) and degassed with helium (grade N46, Hede Nielsen, Denmark) before use. Gradient: 0–10 min, 100% A; 10–16 min, 80% A, 20% B; 16–21 min, 75% A, 25% B; 21–31·5 min, 60% A, 40% B; 31·5–34·5 min, 100% B; 34.5–48 min, 100% A.

Statistical analyses

The influence of the different factors on the amounts of the individual free amino acids were tested using multiple linear regression and analysis of variance (MODDE, version 2·0, UMETRI AB, Umeå, Sweden). The relationship between volatiles (GC peak areas) and amino acid profile was evaluated by 'partial least squares analysis' (PLS) using a multivariate analysis program (UNSCRAMBLER, version 5·52, CAMO A/S, Trondheim, Norway). The dependent variables (Y-matrix) were the relative GC peak areas in the logarithmic$_{10}$ scale. The independent variables (X-matrix) were the amino acid data (mg/100g protein).

RESULTS AND DISCUSSION

Tables 2–4 list the results from the amino acid analyses on sausage samples from weeks 2, 4 and 8. The concentration levels are similar to the levels found by other workers (Dierick *et al.*, 1974; DeMasi *et al.*, 1990) and are clearly increasing with time: 20–40% from week 2 to 4 and from week 4 to 8. Otherwise it is difficult to see any trends from the tables due to the experimental design.

Tables 5–7 show the results from the regression analyses of the amino acid data. The tables display the factors that significantly affect the concentration of the individual free amino acids. All amino acids were affected in the same way at all sampling times, except in four cases. Those cases were not possible to explain.

The values of the coefficients are proportional to the importance of the individual factors; however, the magnitude of the coefficients rather than their exact numerical values should be considered when evaluating the results.

Interaction effects were only significant after 2 and 4 weeks of ripening. All interaction effects at 4 weeks and most at 2 weeks were between temperature and salt, nitrate and glucose (TEM*SAL, TEM*NAT, TEM*GLU). At 2 weeks, interaction effects between *P. pentosaceus*, glucose and salt (PED*GLU, PED*SAL) and between nitrite, salt, temperature and glucose (NIT*SAL, NIT*TEM, NIT*GLU) seemed likely, though one has to keep in mind that interaction effects are confounded and be careful when interpretating the results (cf. Table 2 in Stahnke, 1995*a*). It is also important to realize that the factor effects found in this experiment are only valid in the inspected ingredient (factor) span shown in Table 1, even though some factors may have the same effect outside the tested range.

Temperature

Tables 5–7 show that temperature is the factor that affects the level of free amino acids to the greatest extent and that increasing temperature raises their amounts. Several workers have already shown that proteolytic activity increases at raised temperature. Johansson *et al.* (1994*b*) found a higher proteolytic activity at higher temperature in meat/fat mixtures. Spicher & Nierle (1984) showed that a rise in temperature from 25 to 35°C doubled the content of some amino acids in sourdough while the content of others was little or not affected.

The increased amino acid content may be caused by an increase in the activity of microbial and endogenic proteolytic enzymes and from increased production of the microbial enzymes. The interaction effects between temperature, glucose and nitrate (−TEM*GLU, +TEM*NAT) show that the increasing effect of temperature may not only be a temperature effect but also an effect caused by reduced pH. High temperature, high amounts of glucose and low amounts of nitrate reduced the pH according to a proceeding paper (cf. Table 5 in Stahnke, 1995*a*). pH-lowering has been shown to increase the activity of endogenic proteinases (Verplaetse, 1994).

It seems unlikely that the added *S. xylosus* is responsible for increased production of microbial proteases, as the growth of this bacteria in the mince was inhibited by raised temperature, though to a lesser extent if salt concentration was high [high salt concentration probably favoured the very salt-tolerant *S. xylosus* at the expense of other bacteria (Stahnke, 1995*a*)]. However, the interaction effect, +TEM*SAL, shows that high salt content increases the positive effect of temperature, indicating that salt has an effect on proteolysis. This is discussed further below.

TABLE 2
Free Amino Acids (mg/100g protein) after 2 Weeks of Ripening[a]

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*
Aspartic acid	17·1	22·2	8·4	15·6	21·1	7·8	27·2	12·5	13·5	32·5	11·9	7·2	12·1	19·7	27·9	16·1	32·2	15·3
Glutamic acid	54·0	82·7	56·4	75·9	84·2	62·1	93·3	58·5	42·0	169·6	92·9	86·9	81·2	87·7	136·6	88·7	140·3	79·6
Serine	39·6	47·4	39·9	45·5	50·4	49·5	56·1	44·3	32·9	50·0	42·1	52·3	50·6	32·6	40·5	39·1	57·6	51·7
Histidine	123·5	114·0	94·4	81·5	93·0	122·7	109·3	94·8	70·8	86·1	81·2	72·2	79·3	82·2	68·9	90·3	84·8	63·2
Glycine	38·9	58·0	34·9	42·8	58·1	50·3	57·2	36·6	28·0	73·3	50·9	54·1	58·8	57·9	73·8	51·1	80·0	56·8
Arginine	21·9	34·3	24·3	20·4	35·8	26·7	23·8	23·9	20·7	39·3	28·8	26·6	22·7	21·8	28·6	33·4	42·4	32·2
Threonine	38·9	23·0	33·7	8·7	28·7	24·4	10·1	4·2	20·9	Tr	62·6	17·8	50·7	20·9	Tr	16·0	26·5	10·8
Alanine	67·2	76·8	69·8	80·1	92·7	99·7	99·0	92·9	83·1	119·0	124·8	126·3	136·9	145·3	164·4	167·0	93·4	175·4
Tyrosine	22·7	24·6	23·5	28·5	30·8	24·9	32·3	22·6	17·7	52·7	41·4	35·6	36·9	36·8	29·0	38·1	53·5	38·1
Methionine	12·7	16·8	13·1	17·4	20·6	14·0	15·6	9·1	10·5	29·0	25·6	21·3	23·1	20·1	32·0	30·8	34·6	24·0
Valine	25·7	39·7	26·3	28·2	44·6	30·6	37·0	28·4	24·3	58·3	44·4	40·5	41·7	40·7	40·0	45·4	61·6	43·8
Tryptophan	5·0	7·2	3·6	5·3	9·7	5·3	6·0	3·5	4·5	12·7	9·0	7·4	8·4	3·0	15·7	6·4	14·0	6·9
Phenylalanine	22·6	32·6	24·1	31·7	33·2	26·7	25·1	23·6	17·9	65·6	56·0	50·1	46·7	32·2	29·0	23·0	68·9	46·9
Isoleucine	22·9	32·1	22·3	29·5	34·9	26·5	24·0	24·4	18·6	49·7	35·9	37·8	39·0	31·8	49·0	34·8	55·7	37·6
Leucine	12·7	34·0	12·0	8·3	37·2	19·7	22·3	18·5	11·1	34·5	15·6	14·0	12·6	9·0	30·2	12·4	9·3	21·1
Lysine	41·8	58·2	46·6	48·5	50·3	46·0	55·8	41·3	35·1	120·0	86·5	76·2	81·2	81·1	106·6	92·6	113·2	75·7
Total	567·0	703·6	533·3	567·9	725·3	636·9	694·1	539·1	451·6	992·3	809·6	726·3	781·8	722·8	872·2	785·2	968·0	779·1

[a]L-amino acids. Duplicate analysis of three replicates. Tr, trace.
[b]Multiply with the factor 0·42g dry matter/g protein to get the unit mg/100g dry matter.

TABLE 3
Free Amino Acids (mg/100g protein) after 4 Weeks of Ripening[a]

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*
Aspartic acid	Tr	45·3	Tr	11·3	52·4	Tr	39·2	m	25·0	71·6	34·1	20·0	25·2	38·0	64·9	31·9	65·9	45·2
Glutamic acid	85·9	186·0	98·0	142·0	184·6	35·1	154·4	m	93·9	323·0	203·7	212·9	203·0	153·2	285·5	197·2	284·4	178·9
Serine	61·3	107·6	53·9	77·3	107·1	63·9	83·9	m	71·1	103·6	103·6	118·3	120·5	112·3	182·9	92·4	128·7	110·5
Histidine	74·3	119·1	71·0	80·0	106·4	64·4	77·6	m	53·4	226·9	161·2	158·0	170·8	158·7	212·1	158·1	154·1	170·6
Glycine	48·5	133·5	45·9	77·1	107·0	57·9	92·0	m	60·1	148·1	32·7	105·6	106·7	110·2	189·0	110·6	154·8	125·5
Arginine	51·9	Tr	52·6	43·3	57·0	44·4	41·6	m	53·7	32·7	136·6	72·2	122·1	73·2	66·2	33·0	64·7	90·0
Threonine	38·9	83·8	37·8	54·0	77·2	40·4	60·5	m	47·2	100·9	73·3	76·1	73·0	72·5	100·5	79·4	103·0	88·4
Alanine	201·7	221·7	171·4	203·5	221·4	197·3	216·2	m	188·6	323·5	345·0	347·6	363·3	375·4	357·5	375·8	365·8	360·2
Tyrosine	33·7	73·0	44·9	59·9	62·8	33·5	60·5	m	44·8	103·5	75·3	73·6	72·9	67·6	100·5	87·1	108·1	85·3
Methionine	21·0	59·8	19·9	28·0	39·8	20·9	31·9	m	22·1	68·2	45·2	45·2	44·2	38·9	67·1	45·3	73·7	55·1
Valine	48·3	108·5	46·2	62·9	101·2	48·7	75·7	m	58·6	145·6	103·9	94·4	89·9	93·0	154·1	99·1	127·4	124·9
Tryptophan	6·7	18·8	4·7	9·0	12·2	4·8	9·5	m	7·7	21·8	72·8	36·4	Tr	9·4	26·1	12·1	15·8	26·4
Phenylalanine	41·5	90·5	38·8	50·9	65·9	38·8	58·4	m	39·5	146·0	103·1	96·5	92·7	80·5	122·4	83·2	131·1	107·5
Isoleucine	50·1	86·6	36·5	53·4	75·5	39·9	61·0	m	43·6	124·4	78·3	82·0	81·5	119·5	123·7	79·0	110·3	100·3
Leucine	77·0	171·7	67·1	96·5	128·6	75·2	114·6	m	76·8	246·6	174·5	162·5	151·9	136·3	233·5	149·6	235·0	187·8
Lysine	53·1	121·5	54·8	94·5	88·2	69·3	96·6	m	74·0	238·9	163·4	146·9	144·7	152·5	224·2	162·5	193·2	171·8
Total	893·9	1627·4	843·5	1143·6	1487·3	834·5	1273·6	m	960·1	2425·3	1906·7	1848·2	1862·4	1791·2	2510·2	1796·3	2316·0	2028·4

[a]L-amino acids. Duplicate analysis of three replicates. Tr, trace; m, missing.
Multiply with the factor 0middot;42g dry matter/g protein to get the unit mg/100g dry matter.

TABLE 4
Free Amino Acids (mg/100g protein) after 8 Weeks of Ripening[a]

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*
Aspartic acid	32·8	121·1	30·6	36·0	92·2	19·8	21·8	29·3	143·9	161·1	58·1	63·0	74·8	125·2	162·6	90·9	93·0	277·3
Glutamic acid	212·5	174·8	122·9	175·4	175·5	138·2	183·5	153·7	121·8	426·9	141·4	260·4	240·4	194·2	446·3	283·2	267·1	555·5
Serine	127·1	106·1	74·6	91·4	96·6	70·4	79·8	75·6	70·4	155·4	133·4	135·5	143·9	121·8	217·4	113·4	143·9	213·2
Histidine	237·3	169·1	159·6	129·2	114·5	81·9	88·2	81·9	70·4	326·6	296·1	258·3	101·9	220·5	356·0	251·0	214·2	343·4
Glycine	147·6	129·2	70·4	99·8	116·6	56·7	93·5	75·6	62·0	199·5	119·7	134·4	133·4	156·5	275·1	151·2	152·3	293·0
Arginine	88·2	78·8	70·4	60·9	64·1	37·8	60·9	50·4	43·1	130·2	97·7	96·6	101·9	96·6	168·0	109·2	111·3	194·3
Threonine	128·1	10·5	81·9	30·5	18·9	35·7	5·3	50·4	34·7	7·4	133·4	47·3	69·3	25·2	12·6	5·3	17·9	4·2
Alanine	344·7	265·8	242·3	245·7	257·6	264·8	257·2	271·7	250·3	416·7	398·2	359·7	366·1	393·0	498·5	432·6	362·5	540·8
Tyrosine	64·1	47·3	42·0	49·4	45·2	30·5	48·3	34·7	35·7	144·9	119·7	109·2	106·1	77·7	123·9	108·2	108·2	189·0
Methionine	34·7	42·0	26·3	32·6	37·8	23·1	33·6	26·3	15·8	91·4	80·9	66·2	65·1	57·8	111·3	69·3	75·6	146·0
Valine	135·9	165·6	94·5	108·9	154·2	77·7	122·1	91·2	99·0	292·2	224·1	191·7	184·5	226·2	406·2	255·0	239·4	383·1
Tryptophan	9·9	15·4	8·1	9·7	9·6	5·9	10·3	8·2	7·1	32·3	25·7	17·5	19·4	19·6	48·4	26·1	22·7	43·8
Phenylalanine	75·6	77·7	47·3	57·8	66·2	42·0	62·0	47·3	39·9	167·0	140·7	125·0	112·9	99·8	207·9	131·3	141·8	262·5
Isoleucine	77·7	76·7	48·3	59·9	67·2	43·1	59·9	48·3	43·1	142·8	120·8	109·2	106·1	98·7	189·0	113·4	121·8	223·7
Leucine	176·7	519·3	180·5	112·2	570·3	269·7	297·9	274·9	191·0	519·8	222·6	195·8	179·6	144·4	421·6	186·4	754·4	294·5
Lysine	181·7	168·0	119·7	140·7	123·9	110·3	123·9	113·4	102·9	319·2	253·1	199·5	190·1	196·4	246·8	231·0	207·9	387·5
Total	2074·6	2167·4	1419·4	1440·1	2010·4	1307·6	1548·2	1432·9	1331·1	3533·4	2565·6	2369·3	2195·5	2253·6	3891·6	2557·5	3034·0	4351·8

[a]L-amino acids. Duplicate analysis of three replicates.
Multiply with the factor 0·42 g dry matter/g protein to get the unit mg/100g dry matter.

TABLE 5
Main Effects and Two-Factor Interactions after 2 Weeks of Ripening

Amino acid	*Significant effects*[a]							
	Regression coefficients of main effects						*Two-factor*[b] *interactions*	R^{2c}
	TEM	*PED*	*NIT*	*NAT*	*GLU*	*SAL*		
Aspartic acid	+2***	−2**	+2**	−2**		−6***		0·93
Glutamic acid	+20***	−10◆	+15**	−15**			−10TEM·NAT*	0·83
Serine	+2**		+6***	−1⊥			−4NIT·SAL**	0·90
Histidine	+9***		+7*					0·69
Glycine	+10***			−6*	−2*		+5TEM·GLU	0·88
Arginine	+2*		+3***	−4***		−2***	+3TEM·NIT*, −2TEM·NAT*, +2TEM·GLU*	0·98
Threonine			+8*		−3*			0·77
Alanine	+25***	−7*		−7*	−6*	−6*	−7PED·GLU*, +9PED·SAL**	0·97
Tyrosine	+7***		+4**	−3**			+3NIT·GLU**	0·83
Methionine	+6***	−2**		−3***		−2**		0·93
Valine	+8***	−2*	+3*	−5***	−1*	−3*	−2TEM·NAT*, +2TEM·GLU*	0·94
Tryptophan	+2***	−1◆		−2***			−2TEM·NAT*, +1TEM·GLU*	0·91
Phenylalanine	+8***			−4**		+6***	+7TEM·SAL***	0·95
Isoleucine	+8***		+2*	−5**	−1*		+1TEM·GLU*	0·96
Leucine			+4*	−7***	−1*	−5**	−4TEM·NAT*	0·85
Lysine	+23***		−6*				−4TEM·NAT*	0·80

[a]***, $P \leq 0{\cdot}001$; **, $P \leq 0{\cdot}01$; *, $P \leq 0{\cdot}05$; ◆, $P \leq 0{\cdot}10$. TEM, fermentation temperature; PED, *Pediococcus pentosaceus*, NAT, KNO_3; NIT, $NaNO_2$; GLU, glucose; SAL, salt. + or − in front of a factor indicates whether the factor increases or decreases the amount of the amino acid.
The unit of the coefficients is mg/100g protein; cf. Table 2–4.
[b]Two-factor interactions are confounded; cf. Table 2 in Stahnke (1995*a*).
[c]R^2, the percentage of the variation explained by the model.

Pediococcus pentosaceus

Tables 5–7 show that *P. pentosaceus* had a significant effect on the level of, respectively, 7, 5 and 4 amino acids after 2, 4 and 8 weeks of fermentation/drying. The levels of methionine and tryptophane were both affected at week 2 and 8 and glutamic acid at all times. The levels of the other amino acids were not consistently influenced by the starter culture. However, the bacteriological analyses showed that naturally occuring lactic acid bacteria were growing in the mince, reaching a level similar to the added amounts in most of the sausage batches without added *P. pentosaceus* (Stahnke, 1995*a*). This means that the effect of added *P. pentosaceus* may be obscured. Also, the inherent lactic acid bacteria growing together with *P. pentosaceus* may influence the level of free amino acids in a different way than the added *P. pentosaceus*, blurring the picture even more.

Addition of *P. pentosaceus* decreased the level of free amino acids. There may be several reasons for that.

The proteolytic enzymes were perhaps inhibited. But this does not seem likely, since the chemical analyses showed that addition of *P. pentosaceus* decreased the pH of the mince

TABLE 6
Main Effects and Two-Factor Interactions after 4 Weeks of Ripening

Amino acid	*Significant effects*[a]							
	Regression coefficients of main effects						*Two-factor interactions*[b]	R^{2c}
	TEM	*PED*	*NIT*	*NAT*	*GLU*	*SAL*		
Aspartic acid	+8*			+35***		−28*		0·63
Glutamic acid	+45***	−23**		−24**			+19TEM·SAL 0·94	
Serine	+17**	−18**	+14*					0·78
Histidine	+37***	−20*						0·73
Glycine	+25***			−17**	−2*	−17**	+10TEM·GLU*	0·81
Arginine	+13*		+14*					0·79
Threonine	+15***	−3*	+5**	−9***	−8*	−8***	+4TEM·GLU*, +4TEM·SAL**	0·99
Alanine	−77***				−8*	−11***		0·99
Tyrosine	+17***	−5**		−8***	−0·5*	−7***	+2TEM·GLU*, −2TEM·NAT*	0·99
Methionine	+13***		+4*	−7**	−1*	−4*	+3TEM·GLU	0·98
Valine	+22***			−15***		−11**		0·92
Tryptophan	+13***			−4*		+8**	+11TEM −SAL***	0·99
Phenylalanine	+28***		+6*	−15***	−2*		+5TEM·GLU*, +6TEM·SAL***	0·98
Isoleucine	+20***			−12**		−7*		0·94
Leucine	+43***		+13*	−27***		−12*		0·96
Lysine	+49***		+11*	−17**		−9*	−9TEM·NAT*	0·90

[a] ***, $P \leq 0{\cdot}001$, **$P \leq 0{\cdot}01$, *$P \leq 0{\cdot}05$. TEM, fermentation temperature; PED, *Pediococcus pentosaceus*; NAT, KNO_3; NIT, $NaNO_2$; GLU, glucose; SAL, salt. + or − in front of a factor indicates whether the factor increases or decreases the amount of the amino acid. The unit of the coefficients is mg/100g protein, cf Tables 2–4.

[b] Two–factor interactions are confounded; cf. Table 2 in Stahnke (1995*a*).

[c] R^2, the percentage of the variation explained by the model.

(Stahnke, 1995*a*). As mentioned above, pH-lowering has been shown to increase the activity of endogenic proteinases in general (Verplaetse, 1994).

P. pentosaceus may have catabolized the free amino acids. This does not seem probable though, since a proceeding paper (Stahnke, 1995*b*) showed that the levels of volatile compounds like 2-methyl propanal, 3- and 2-methyl butanal (possible breakdown products from the amino acids valine, leucine and isoleucine, respectively) were reduced and not increased by the addition of *P. pentosaceus*.

P. pentosaceus inhibited the growth of proteolytic microorganisms. This seems to be the most likely reason. The bacteriological analyses showed that *P. pentosaceus* inhibited the growth of *S. xylosus*, which is proteolytic according to the manufacturer (Anon., 1990). This is also indicated by the interaction effects, PED*SAL and −PED*GLU, at week 2, since high salt level inhibits *P. pentosaceus* [no growth above 7% 'salt in water', Anon. (1990)] and the addition of glucose increases the growth of *P. pentosaceus*. Inherent lactic acid bacteria possessing proteolytic activity would be inhibited as well. *Lactobacilli* have been shown to be proteolytic (Law & Kolstad, 1983).

TABLE 7
Main Effects and Two-Factor Interactions after 8 Weeks of Ripening

Amino acid	*Significant effects*[a]							
	Regression coefficients of main effects						*Two-factor interactions*[b]	R^{2c}
	TEM	*PED*	*NIT*	*NAT*	*GLU*	*SAL*		
Aspartic acid	+20**			−8*		−27**		0·87
Glutamic acid	+71***	−23◆		−41**				0·80
Serine	+25***			−13**				0·83
Histidine	+69***			−29*				0·78
Glycine	+33***			−21**				0·74
Arginine	+24***	−6◆		−6*				0·90
Threonine	+13***			−22***		+25***		0·96
Alanine	+20***			−4*				0·96
Tyrosine	+34***			−9**				0·94
Methionine	+22***	−3◆		−7◆				0·96
Valine	+59***			−77**		−51***		0·92
Tryptophan	+7***	−1◆		−3*				0·94
Phenylalanine	+38***			−13***				0·93
Isoleucine	+35***			−14**				0·92
Leucine				−102****		−77**		0·88
Lysine	+49***			−23***				0·92

[a] ***, $P \leq 0{\cdot}001$, **, $P \leq 0{\cdot}01$, *, $P \leq 0{\cdot}05$, ◆, $P \leq 0{\cdot}10$. TEM, fermentation temperature; PED, *Pediococcus pentosaceus*, NAT, KNO3; NIT, $NaNO_2$; GLU, glucose; SAL, salt. + or − in front of a factor indicates whether the factor increases or decreases the amount of the amino acid.
The unit of the coefficients is mg/100g protein; cf. Table 2–4.
[b] Two-factor interactions are confounded; cf. Table 2 in Stahnke (1995*a*).
[c] R^2, the percentage of the variation explained by the model.

Nitrite

Nitrite increased the level of free amino acids after 2 and 4 weeks of ripening, but had no effect after 8 weeks. At 2 and 4 weeks the levels of 11 and 7 amino acids, respectively, were significantly affected. This means that the amino-acid-producing mechanisms were accelerated by nitrite and/or the degradation of amino acids into other compounds reduced.

Other workers did not see an accelerating effect of nitrite, but rather the opposite. However, their results came from the study of meat systems other than fermented sausages. Toldrá *et al.* (1993) found a slight inhibiting effect of 200 ppm nitrite towards aminopeptidase activities in meat extracts — 72 to 95% of the enzyme activity remained. Lee & Song (1987) did not see an effect of nitrite on the content of free amino acids in pork salted with 0–300 ppm nitrite at 4 or 15°C. Tsumakasa *et al.* (1989) found a reduced level of free amino acids when nitrite was added to minced pork, brine-salted for 10 days at 5, 10 and 15°C.

The volatile analysis in this study showed that the content of 3-methyl butanal and 2-methyl propionic acid (possible breakdown products of leucine and valine, respectively) was reduced by nitrite (Stahnke, 1995*b*). This shows that the positive influence of nitrite on the amounts of free amino acids could be caused by nitrite inhibiting the amino acid degradation. It seems possible that *S. xylosus* is important to this degradation since nitrite exerted an inhibitory effect on that starter culture (Stahnke, 1995*a*; see also below under

Nitrate). In addition, the interaction effects between nitrite and salt, temperature and glucose at week 2 (−NIT*SAL, NIT*TEM, NIT*GLU) show that low salt content, high temperature and addition of glucose increased the positive effect of nitrite even more (i.e. decreased the amino acid degradation to a higher degree). Those conditions would favour lactic acid bacteria at the expense of *S. xylosus* (Stahnke, 1995*a*), indicating that inhibition of *S. xylosus* was indeed responsible for the reduced breakdown of amino acids when nitrite is present.

Nitrate

Addition of nitrate decreased the level of free amino acids at all times, either by inhibiting the proteolytic activity in the mince or by increasing the breakdown of amino acids.

Since nitrite and nitrate levels are very much interrelated it may be difficult to differentiate between a nitrite and a nitrate effect. Minces with added nitrate contain a higher level of nitrite over a longer period of time due to conversion of the nitrate reservoir into nitrite (Terrell, 1977; Roca & Incze, 1990). Nitrite is able to inhibit, for example, *Micrococci* spp. when present in amounts of only 200 ppm at pH = 6·0, while *Lactobacilli* are more resistant. The inhibition effect is even higher at lower pH due to conversion of nitrite into nitrous acid (Ingram, 1973). This means that the decreased level of free amino acids perhaps was due to an inhibition of proteolytic microorganisms. The effect of high nitrate/nitrite level may not have been seen as a nitrite factor effect because of the much lower nitrite levels compared to nitrate levels — 300 ppm versus 2000 ppm [converted into 550–1970 ppm nitrite depending on sausage batch (cf. Table 3 in Stahnke, 1995*a*)]. The chemical analyses showed that the addition of nitrate increased the pH of the mince, indicating that lactic acid bacteria were indeed inhibited. *Lactobacilli* have been shown to posses proteolytic activity (Law & Kolstad, 1983).

As mentioned above, several workers found that free amino acid levels fell when nitrate was added to meat systems. Piotrowski *et al.* (1970) showed that addition of nitrate to raw, salted ham reduced the level of free amino acids by 26%. They explained the decrease as caused by the Van-Slyke reaction converting free amino acids into a-hydroxy acids by a reaction between free amino acids and nitrous acid. Also, nitrous acid can react with nitrogen in the side chains of tryptophan and histidine and the F-amino group in lysine (Cassens *et al.*, 1979). In fact, Sebranek *et al.* (1973) traced 20–30% of radiolabeled nitrite added to model mince to small water soluble compounds after 40 days of ripening.

Glucose

The effect of glucose was not great and only significant for the levels of six amino acids at 2 and 4 weeks, but not at 8 weeks. In general, glucose addition decreased their amounts. However, the interaction effect (+TEM*GLU) shows that, while **low** temperature together with glucose addition decreased the level of free amino acids more than glucose alone, **high** temperature in combination with glucose slightly increased their amounts (the coefficients for TEM*GLU are slightly higher than for GLU). This indicates that the level of free amino acids is controlled by many mechanisms, differently affected by glucose.

The chemical analyses showed that the addition of glucose slightly increased the pH at **low** temperature (15°C) (cf. Table 5 in Stahnke, 1995*a*). Since the amino-peptidases of the meat are less active at high pH (Verplaetse, 1994), their activity would be reduced at those conditions, lowering the level of free amino acids as seen in the experiment. Oppositely, Toldrá *et al.* (1993) showed that glucose increased the activity of amino-peptidases with up to 80%. However, in his study the temperature was higher than in our study, 37°C, and the interaction effect of temperature not studied. As mentioned above, glucose addition

increased the level of amino acids when the temperature was high (25°C). This effect might be greater at higher temperatures. In our study the effect of high temperature together with glucose signifies that proteolytic activity originating from microbial activity may also be of importance. High temperature and glucose addition would favour lactic acid bacteria that may possess proteolytic activity (Law & Kolstad, 1983).

Salt

Salt reduced the level of, respectively, 7, 11 and 4 amino acids after 2, 4 and 8 weeks of ripening. In a few cases the interaction effect, +TEM*SAL, was significant, indicating that salt had a greater negative effect at low temperature.

In general, salt inhibits proteolysis. This has been shown for meat enzymes (Kudryashov *et al.*, 1987; Sarraga *et al.*, 1989; Rico *et al.*, 1991). It is likely that inhibition is less pronounced at high temperature due to the positive influence of temperature on proteolysis (+TEM*SAL). On the other hand, high concentrations of salt degrade the muscle structure (Katsaras *et al.*, 1990), making it more susceptible to attack by proteases and peptidases.

The analysis of volatile content showed that amounts of 2- and 3-methyl butanal (breakdown products of isoleucine and leucine) were increased by high salt level. This would also decrease the amounts of the respective amino acids.

The effect of salt was only significant for four amino acids after 8 weeks. This may be caused by the fact that after 8 weeks of ripening the salt levels in both 'high' and 'low' salt batches are so high that the enzymatic activity in any case is inhibited.

Relationship between volatile compounds and free amino acids

Figure 1 shows a loading plot from the partial least squares analysis (PLS) of free amino acids and volatile compounds after 4 weeks of ripening. A table of the volatiles was shown

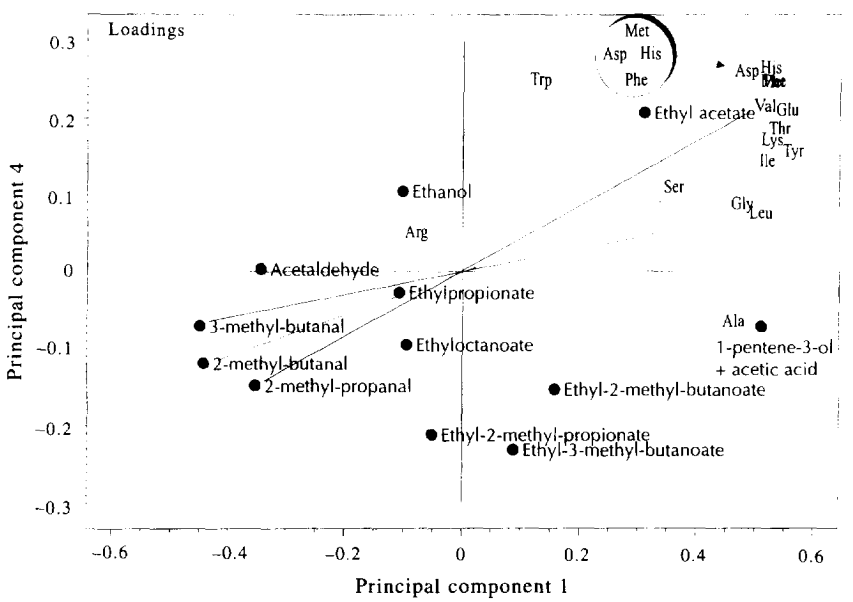

Fig. 1. Loading plot from PLS analysis on amino acid and volatiles content. The variation of both data sets explained by the principal components are given in the text.

in a preceding paper (cf. Table 3 in Stahnke, 1995*b*). The plot displays the relationship between the level of volatile compounds and the amounts of individual amino acids. Volatiles placed close to the centre of the plot are less affected by the amino acid profile than volatiles far from the centre. Volatiles placed in the origo are not influenced by the amino acid levels and have been removed in order to create a better model. In total, the model explains 62 and 78%, respectively, of the variation in the data sets of the amino acids and the volatiles. The first principal component (PC) in the PLC plot explains 61% of the variation in the volatile profile and 17% of the variation in the amino acid profile. The fourth PC explains 12 and 15%, respectively.

This means that 61% of the volatile profile in the direction of PC 1 can be explained by the level of certain amino acids.

Figure 1 shows that all of the amino acids except of arginine and alanine are placed in the first quadrant, while most of the volatile compounds are placed in the third and fourth. This indicates that the concentration of the shown volatiles are inversely correlated to the level of amino acids. As mentioned in preceding paragraphs, the volatiles 2-methyl propanal, 2- and 3-methyl butanal most likely arise from the amino acids valine, isoleucine and leucine. This is supported by the plot. The correlation coefficients between valine and 2-methyl propanal, isoleucine and 2-methyl butanal, leucine and 3-methyl buatanal are 0.80, 0.92 and 0.85, respectively. In addition, the plot indicates a relationship between the mentioned amino acids and the corresponding ethyl esters.

CONCLUSIONS

The present work investigated the effect of different processing parameters on the level of free amino acids in dried sausages fermented with the starter culture *S. xylosus*.

It was shown that increasing fermentation temperature and concentration of nitrite raised the content of free amino acids, while the addition of nitrate, glucose, the *P. pentosaceus* and high levels of salt lowered their amount. Temperature had the greatest effect, followed by nitrate and nitrite. The effects of the other parameters were lower and less consistent.

The level of free amino acids depends on several mechanisms taking place in the mince. When combining all of the factor effects, it appeared that sausages produced in a modern way (added salt, glucose, nitrite, *P. pentosaceus* and fermented at high temperature) are likely to contain much higher levels of free amino acids than sausages produced in an old-fashioned way (added salt and nitrate and fermented at low temperature). The way the different processing parameters affected their content gave hints as to which mechanisms were dominating in certain types of sausages.

In addition, it was shown that the content of valine, leucine and isoleucine were inversely correlated to the level of the volatile compounds 2-methyl propanal, 3-methyl butanal and 2-methyl butanal, respectively. This strongly indicates that the volatiles are degradation products of those amino acids. A previous paper showed that 2-methyl propanal, 2- and 3-methyl butanal were of importance to sausage flavour (Stahnke, 1995*c*); therefore, it seems possible that controlling the level of free amino acids is a prospect of controlling the level of some aroma compounds important to dried sausage flavour. This is a subject that needs to be further studied in detail.

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