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The pH-unrelated influence of salt, temperature and manganese on aroma formation by *Staphylococcus xylosus* and *Staphylococcus carnosus* in a fermented meat model system

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Abstract

The influence of manganese (0.01–0.1–1.0 μg/g), temperature (15–24 °C) and salt (3–4% w/w) on volatile formation in model minces inoculated with *Pediococcus pentosaceus* and either *Staphylococcus xylosus* or *Staphylococcus carnosus* was studied in a full factorial experiment. In order to study the direct, pH-unrelated effect of the parameters, data were analysed by use of multiple linear regression and partial least-squares regression both before and after transformation of the volatile responses into pH-orthogonal (pH-unrelated) responses. By using the pH-orthogonalised data, the overall interpretability of the experiment was increased, and new cause-and-effect relations were suggested.

Approximately 50% of the total variance in volatile levels was due to differences caused by *S. xylosus* and *S. carnosus*, and another 30% was related to differences in pH development. The remaining 20% covered pH-orthogonal effects of manganese, temperature and salt plus the experimental noise. From this, it was concluded that most of the variation in volatile profiles caused by manganese, temperature and salt was in fact directly or indirectly caused by changes in lactic acid bacterial activity and pH.

Keywords: Fermented sausage; Orthogonalisation; Volatiles; pH; Salt; Temperature; Manganese; *Staphylococcus xylosus*; *Staphylococcus carnosus*; *Pediococcus pentosaceus*

1. Introduction

Microbial aroma formation plays a central role in the overall flavour of fermented sausages. The influence of different *Staphylococcus* starter cultures on the level of volatiles was first shown by Berdagué et al. (1993), and later, Montel et al. (1996) established the first correlation between the use of *Staphylococcus* starter cultures, volatile production and sensory characteristics. The authors showed that model minces inoculated with *S. xylosus* or *S. carnosus* developed the highest dry-cured odour and the highest level of 3-methyl butanal, methylketones and ethyl esters compared to minces inoculated with other Micrococcaceae.

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Stahnke (1995b) combined volatile analyses and sensory evaluations of sausages inoculated with *S. xylosus* and found salami odour to correlate with methylketones, ethyl esters, 2- and 3-methyl butanal and high numbers of *S. xylosus*. Recently, the pathways and several key enzymes involved in production of these compounds have been identified (Engelvin et al., 2000; Larrouture et al., 2000; Beck et al., 2002; Madsen et al., 2002) and the impact of various sausage relevant growth parameters as temperature, pH, salt, nitrate, nitrite and ascorbate on staphylococcal aroma formation in liquid media has been investigated (Talon et al., 1998; Masson et al., 1999; Fadda et al., 2002; Olesen et al., 2004; Olesen and Stahnke, 2004).

Parallel to this research, the volatile production of staphylococci under various processing conditions has been studied during real meat fermentations. Aroma formation by staphylococci during sausage fermentation is, however, a complex process strongly influenced by process parameters as ingredient levels, temperature, pH, drying conditions and fermentation time (Stahnke, 1995a, 1999; Mateo and Zumalacárregui, 1996; Misharina et al., 2001; Olesen et al., 2004). Most of these factors do also affect growth and activity of lactic acid bacteria (LAB) and in turn the pH profile that again influences volatile formation by the staphylococci. Biochemical processes as proteolysis and lipolysis supplying precursors for microbial aroma formation are also influenced by processing parameters (Toldrá et al., 1997). Thus, the change of a given growth parameter will affect several mechanisms, and the resulting effect on volatile formation is a sum of all those reactions. For instance, a recent study concludes that salt has a positive effect on the formation of methyl-branched aldehydes at the initial phase of fermentation, not because salt or decreased water activity as such improves branched chain amino acid breakdown, but because it has a greater negative effect on LAB and their acidification than on the staphylococci (Olesen et al., 2004). The conclusion may very well be right, but strictly speaking, it is only hypothetical, and other causes could possibly contribute to this effect as well. This example is by no means exceptional, and numerous studies have resulted in this type of hypothetical cause-and-effect relations based on multiple linear regression or partial least-squares regression of volatile responses (Stahnke, 1995a, 1999; Demeyer et al., 2000; Søndergaard and Stahnke, 2002).

The scope of this article is to demonstrate a practical way of reducing complexity in cause and effect patterns by multivariate data analysis, thereby increasing the interpretability of data, in this case, by subtracting variations due to pH, revealing the direct effect of the investigated parameters (salt, temperature, manganese, *Staphylococcus* strain) on volatile responses in a fermented meat model system. Three parameters with major influence on pH development were chosen. Temperature and salt due to their importance in sausage fermentations and manganese as an ingredient with the ability to create great variety in pH (Archibald, 1986; Hagen et al., 2000).[1]

The data analysis presented in this paper builds on the assumption of 100% correlation between LAB activity and pH, whereby continuous pH registrations become a simple measure of LAB activity during meat fermentations. Finding and extracting the correlation between this measure and volatile profiles of minces fermented under various processing conditions leaves a matrix of design parameters vs. volatile responses orthogonal to any effects of pH. Apart from LAB activity, this operation also removes other variations due to pH, i.e., the effect of pH on meat biochemistry and volatile production by staphylococci.

2. Materials and methods

2.1. Experimental design

The four variables, temperature (15 or 24 °C), salt (3% or 4% w/w), manganese (0.01, 0.1 or 1.0 μg/g Mn) and *Staphylococcus* strain (*S. xylosus* or *S. carnosus*), were varied according to a full factorial design. For each combination of growth parameters and *Staphylococcus* strain, three model minces were produced, two for analysis of volatiles and one for continuous pH registration.

2.2. Meat fermentations

Model minces were produced according to the recipe in Table 1 added extra salt (0 or 10 g/kg) and

[1] Addition of manganese to sausage as an ingredient is not legal.

Table 1
Recipe for model minces

Ingredient	g/kg
Pork shoulder (15–20% w/w fat)	320.0
Beef back rib (15–20% w/w fat)	320.0
Pork back fat (80–90% w/w fat)	313.0
NaCl with 0.6% w/w $NaNO_2$ (570016)[a]	17.0
NaCl (570005)[a]	13.0
Potato starch (540001)[a]	12.5
Glucose (530064)[a]	4.0
Sodium ascorbate (550580)[a]	0.5
Manganese[b]	10^{-5}, 10^{-4} or 10^{-3}
Total	1000.0

[a] Numbers refer to supplier order numbers (SFK Food A/S, Viborg, Denmark).

[b] Concentrations of pure manganese. The compound added was $MnSO_4 \cdot H_2O$ (Merck no. 1.05941.0250, Merck, Darmstadt, Germany).

starter cultures (freeze-dried *Pediococcus pentosaceus* PC-1, 5×10^6 CFU/g, and either *Staphylococcus xylosus* DD-34, 10^7 CFU/g, or *Staphylococcus carnosus* MIII, 10^7 CFU/g, Chr. Hansen, Hørsholm, Denmark). Model minces were stuffed into plastic beakers (polyethylene, 450 ml, $d = 70$ mm, Berry Plastics, Evansville, USA), vacuum stopped[2], sealed with a lid and incubated in water baths at 15 or 24 °C for 7 days. After incubation, minces were stored at −50 °C until analysis of volatiles.

2.3. Continuous pH registration

pH of the model minces was measured continuously until day 7 with three electrodes per beaker (Mettler Toledo, HA405-DXX-S8/120, Mettler Toledo, Greifensee, Switzerland) connected to a PC-logger system (INTAB AAC-2, INTAB Interface Teknik, Stenkullen, Sweden).

2.4. Analysis of volatiles

Before analysis, the model minces were thawed overnight at 5 °C. From each model mince, a sample of 100 g was mixed with 20 g of NaCl (NaCl 0277, J.T. Baker, Phillipsburgh, NJ, USA), and 30 g was transferred into three cylindrical glass bottles (150 ml). Bottles were sealed with a glass stopper and placed in a 42 °C water bath for equilibration. After 30 min, glass stoppers were replaced by glass purge heads connected with Swagelok® unions/Teflon ferrules (Swagelok, Solon, OH, USA) to Tenax TA® tubes (200 mg, 60/80 mesh, Chrompack/Varian, Palo Alto, CA, USA) and purged with a flow rate of 50 ml/min (N_2 5.0, AGA, Ballerup, Denmark) for 30 min at 42 °C. Prior to sampling, Tenax TA® tubes were conditioned by purging with a flow rate of 75 ml/min (He 5.0, AGA, Ballerup) for 30 min at 340 °C. Tenax TA® tubes were desorbed by thermal desorption (ATD50, Perkin-Elmer, Beaconsfield, UK) in a two-step manner (first desorption: 250 °C for 3 min onto Tenax TA® cold trap (20 mg, −30 °C), second desorption: 250 °C for 60 seconds, line temperature: 225 °C) and automatically injected into a GC (Hewlett-Packard 5890 series II, Agilent, Palo Alto, CA, USA). Separation was performed on a 30-m × 0.25-mm i.d. DB 1701 (1-μm film) fused silica capillary column (J&W Sci., Köln, Germany), detection by an MS detector (ionisation energy 70 eV, 3.4 scans/s, source 160 °C, scan range 33–250 AMU, Hewlett-Packard 5972, Agilent). GC oven programme was 35 °C, 1 min, 4 °C/min until 175 °C, 10 °C/min from 175 to 260 °C, 260 °C for 5 min. Identification was based on MS spectra compared to the NBS75k-database (National Bureau of Standards database in Hewlett-Packard Chemstation software, Agilent) and integration based on single ion responses.

2.5. Data analysis

For the purpose of linearisation, the design variable manganese and the integrated GC-MS peak areas were $\log_{10}$ transformed before regression analyses. For each set of design parameters, the responses were based on triplicate aroma analyses of duplicate minces. A mean value of those six data points were used in all further data treatment.

pH orthogonalisation of volatile responses was performed by principal component regression (PCR) where the *X*-matrix consisted of samples vs. pH readings from 0 to 7 days (average readings of 5 h), and *Y*-matrix consisted of samples vs. volatile responses. The impact of pH on volatile responses was explained by three principal components. pH-

[2] Vacuum stopping: beaker with mince put into a vacuum bag, vacuum applied and bag removed.

orthogonalised responses were the remaining variance in the *Y*-matrix after extraction of the first three principal components. The pH orthogonalisation was performed with The Unscrambler® software (The Unscrambler®, version 7.6, CAMO, Oslo, Norway).

Discriminant partial least-squares regression was performed twice with an *X*-matrix of samples vs. unweighted volatile responses and samples vs. unweighted pH-orthogonal volatile responses, respectively. The *Y*-matrix consisted in both cases of samples vs. design parameters and pH readings from 0 to 7 days (average readings of 5 h). Design variables were weighted with 1/(standard deviation) and pH readings with 1/(1000 standard deviations) in order to make the pH readings "passive" variables, i.e., without influence on the regression between volatile levels and design parameters, just making them visible in the plots. Discriminant partial least-squares regression was performed by use of The Unscrambler® software (The Unscrambler®, version 7.6, CAMO).

Multiple linear regression and significance testing of the correlation between design factors and volatile responses before and after pH orthogonalisation were carried out by stepwise exclusion of insignificant (p>0.05) factors (SAS JMP 5.0, SAS Institute, Cary, NC, USA).

3. Results and discussion

The model system used in this study was introduced and thoroughly discussed by Tjener et al. (2003). In that article, it has been demonstrated that even if volatile profiles of model minces and sausages are different, their variations due to changes in a growth parameter are comparable and the model system thus suited for this type of studies. The primary reasons for using this model system instead of sausage fermentations are shorter ripening time and increased flexibility with respect to changes in processing conditions (Tjener et al., 2003).

In the present study, the model system is applied to determine the pH-related and the pH-unrelated influence of salt, temperature and manganese on volatile formation by *S. carnosus* and *S. xylosus* during meat fermentation.

Fig. 1 shows acidification profiles as a function of temperature, manganese and salt. Acidification profiles were similar for *S. xylosus* and *S. carnosus* when growth parameters were identical, and therefore, average values are presented in Fig. 1. The design parameters were deliberately chosen so that large variations in pH profiles were to be expected. This was done in order to ensure a strong correlation between design and pH, which is a prerequisite for successful pH orthogonalisation.

3.1. Volatile analysis

The volatiles studied were only a fraction of the volatiles typically identified in fermented meat (Mateo and Zumalacárregui, 1996; Ansorena et al., 2001; Sunesen et al., 2001). In the present study, more than 80 volatile compounds could be identified in the model minces, but most of them were omitted from data analysis because of their non-microbial origin or low sensory impact. The 26 volatiles chosen for this study are presented in Table 2. Most of the volatiles were selected because of their proven relation to sausage aroma and metabolism of staphylococci, but a few were chosen as indicators of carbohydrate metabolism and lipid oxidation as well. The uncertainty of the volatile responses used for multiple linear regression (MLR) and discriminant partial least-squares regression (D-PLSR) was 2.8%. This uncertainty was calculated as an average of relative standard deviations of duplicate responses (each sample based on triplicate analysis) for all experimental conditions and all volatile compounds investigated. An average uncertainty for each of the volatiles was also calculated. For all compounds, the uncertainty was between 1% and 6%.

3.2. pH orthogonalisation

Fig. 2 illustrates the relation between design variables and the volatile responses through D-PLSR. Principal component 1 (PC1) describes 52% of the variance in volatile levels. The variance in PC1 is mainly related to the design variable strain, i.e., to differences in volatile profiles of *S. xylosus* and *S. carnosus*. PC2 is related to the design variables manganese and temperature and explains 31% of the variability in volatile responses. The pH readings are well explained by PC2, and from this, it may be concluded that approximately 31% of the variability

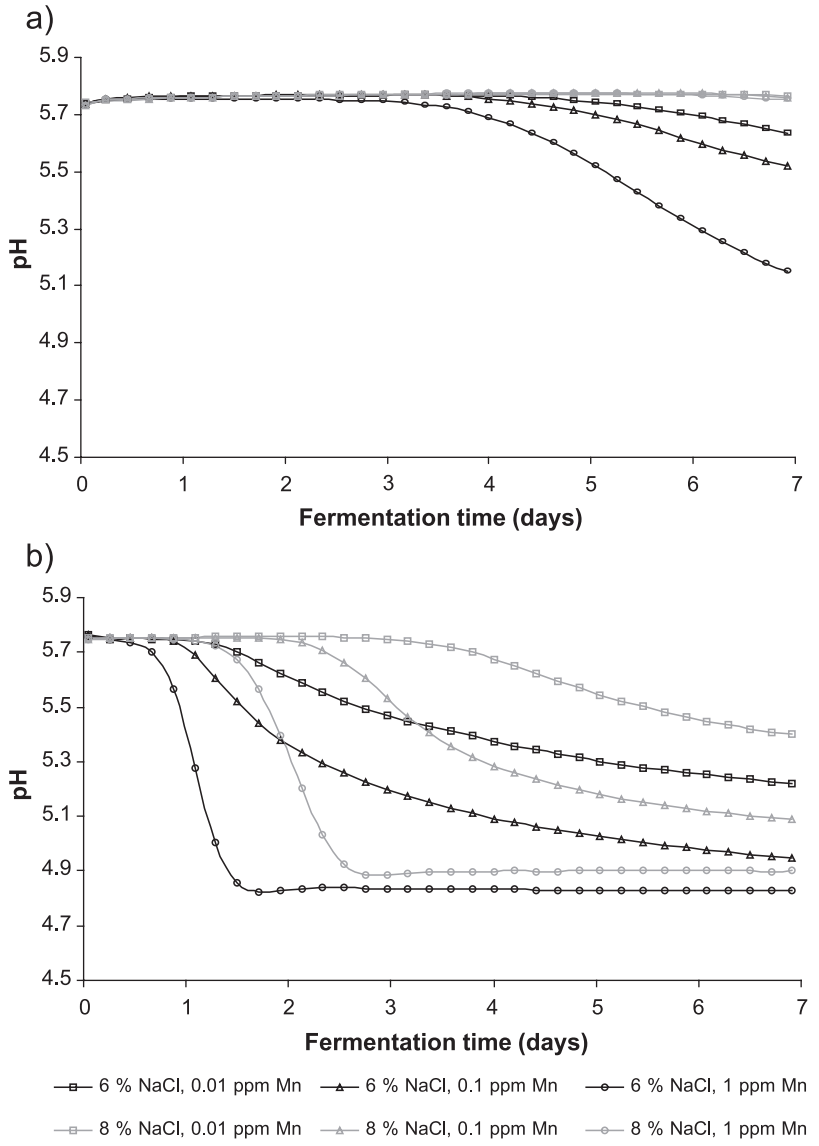

Fig. 1. pH curves based on average recordings of minces inoculated with *S. xylosus* and *S. carnosus*. Fermentation temperatures (a) 15 and (b) 24 °C. Each curve is based on two model mince fermentations with three pH electrodes per mince.

is related to pH. The variability of pH_7 (pH after 7 h of fermentation) to around pH_{42} is less than 50% explained, and the reason for this is the small and random variance that characterises the pH curves before a real pH decline is registered (Fig. 1). As acidification takes on, the variability in pH readings increases in a structured way correlated to the experimental design factors.

The design variable salt is not well explained by PC1 and PC2, but it is correlated to PC3 that explains only 4% of the total variation in volatile responses (data not shown).

In Fig. 3, D-PLSR of pH-orthogonalised volatile responses vs. design variables is shown. The pH-orthogonalisation step is fully described in Section 2.5 and serves the purpose of reducing data complex-

Table 2
Summary of multiple linear regression of raw and pH-orthogonalised volatile responses[a,b]

	Strain[c]		Manganese		Temperature		Salt		Interactions[d]	
	Raw	Orth.[e]	Raw	Orth.	Raw	Orth.	Raw	Orth.	Raw	Orth.
2-Methyl propanal			− − −	− −	−	++	+		− M × T	
2-Methyl butanal			− − −	− −	−	+	++	+		
3-Methyl butanal	+++	+++	− − −	−	− −	+	++	+		
Phenylacetaldehyde	+++	+++	− − −	− −			−	− −	++S × St	++S × St
Hexanal							−			
Decanal										
2-Methyl propanoic acid	++	+++			++		−	−		
2-Methyl butanoic acid	+++	+++			+		−	−		++S × St
3-Methyl butanoic acid	+++	+++			++		−	−		++S × St
Acetic acid	+	++	++		+++		−			
Ethanol			− − −	− − −	−		−	−		
2-Methyl-1-butanol	+++	+++	− −	−					+M × St	+M × St
3-Methyl-1-butanol	++	++	−							
2-Phenyl ethanol	++	+++					−	−		
1-Hexanol				−	+					
Ethyl acetate	++	++	− − −	− −			− − −	− −		
Ethyl butanoate			− − −	− − −			− −	− −		
Ethyl-3-methyl butanoate	+++	+++	− − −	−			− −		− M × T	
Ethyl-2-methyl butanoate	++	+++	− − −	− −	−			− − −	++S × St	++S × St
2-Butanone			++		++					
Diacetyl	− − −	− − −		+	−	− −		+	− T × St	− − T × St
2-Pentanone	+++	+++	+++	++	+		++	+++	++S × St	+S × St
4-Methyl-2-pentanone								+		
Methional					++					
Dimethyldisulphide					+++	+	−	−		− S × T
Dimethyltrisulphid			++		+++					

[a] Significance levels: +++ , ++ and + represent *p*-values below 0.001, 0.01 and 0.05, respectively.
[b] The symbols + or − indicate positive or negative impact on volatile level, respectively.
[c] Positive effect of the factor strain indicates a positive influence of *S. carnosus* compared to *S. xylosus*.
[d] M: manganese, T: temperature, S: salt, St: strain.
[e] Orth.: pH-orthogonalised data.

ity and enhancing interpretability regarding the effect of design variables on volatile formation. The effect of pH orthogonalisation is visualised by comparison of Figs. 2 and 3. In Fig. 2, pH values are lying in the fourth quadrant strongly influenced by the model, whereas in Fig. 3, pH readings are placed in the centre of the correlation loading plot indicating very weak correlation to volatile responses and design parameters. Thus, the pH orthogonalisation has removed all the variance in volatile responses explained by pH (the variance in *Y*-matrix explained by PC1, PC2 and PC3 in the PCR described in Section 2.5, corresponding to 30% of total variance), and the remaining structure is caused by direct variations due to the investigated parameters; strain, salt, temperature and manganese (Fig. 3).

The purpose of showing Figs. 2 and 3 was merely to illustrate the effect of pH orthogonalisation. Much information about design parameters' effects on volatile formation is indeed found in the figures, but a full discussion of those effects are presented in relation to the MLR of the same data in Section 3.3.

3.3. Design parameters' direct and indirect effect on volatile levels

Table 2 displays the results of MLR of raw and pH-orthogonalised data. Note that when structure in variance is removed from a data matrix, in this case, 30% of the total variance, the noise-to-structure ratio is increased in the resulting matrix, and therefore, the

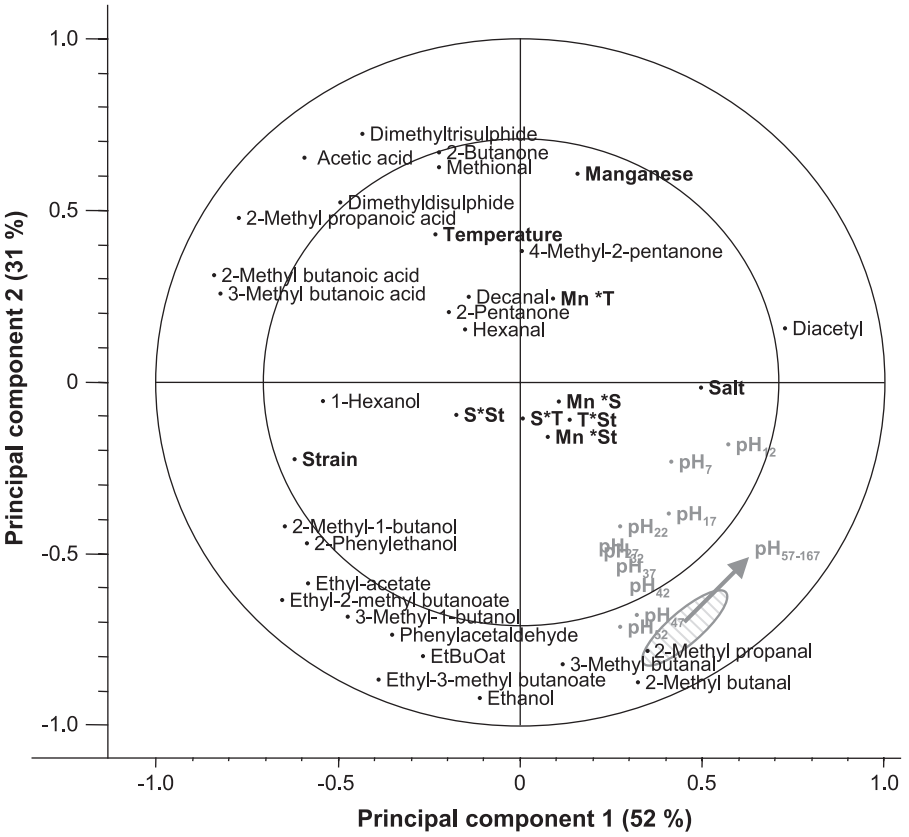

Fig. 2. Correlation loading plot of discriminant partial least-squares regression of volatile responses vs. design. pH measurements are included as passive variables (pH_x: average pH reading to fermentation time, × hours). Inner and outer circle represents 50% and 100% explained variance for a given variable. High level of the variable strain corresponds to *S. carnosus*. M: manganese, T: temperature, S: salt, St: strain.

magnitude of the significance levels from MLR on raw and pH-orthogonalised data cannot be directly compared.

3.3.1. Effect of S. xylosus and S. carnosus

The significant effects of strain were exactly the same in raw and pH-orthogonalised data. This was also to be expected, as strain is orthogonal to pH readings in Fig. 2. *S. carnosus* was positively correlated to the level (and thus producing more than *S. xylosus*) of methyl-branched alcohols, acids, their ethyl esters, 3-methyl butanal, 2-phenylethanol and phenylacetaldehyde which are all compounds related to amino acid catabolism (McSweeney and Sousa, 2000; Madsen et al., 2002; Stahnke, 2002). 2-Methyl butanal had an insignificant positive correlation ($p = 0.12$ and 0.07 in raw and pH-orthogonal data, respectively) to *S. carnosus*, whereas 2-methyl propanal had an insignificant negative correlation ($p = 0.09$ and 0.07 in raw and pH-orthogonal data, respectively). *S. carnosus* also produced higher levels of 2-pentanone, whereas minces inoculated with *S. xylosus* had the highest content of diacetyl. It has also been shown in numerous other studies that *S. carnosus* generally produces more volatiles than *S. xylosus* in liquid media (Larrouture et al., 2000; Olesen et al., 2004) as well as in model minces (Montel et al., 1996; Søndergaard and Stahnke, 2002) and dry sausages (Olesen et al., 2004).

With only one exception, the interaction effects involving strain were positive, indicating greater robustness of *S. carnosus* than *S. xylosus* towards high levels of salt and manganese. The exception was diacetyl, which, in opposition to most other com-

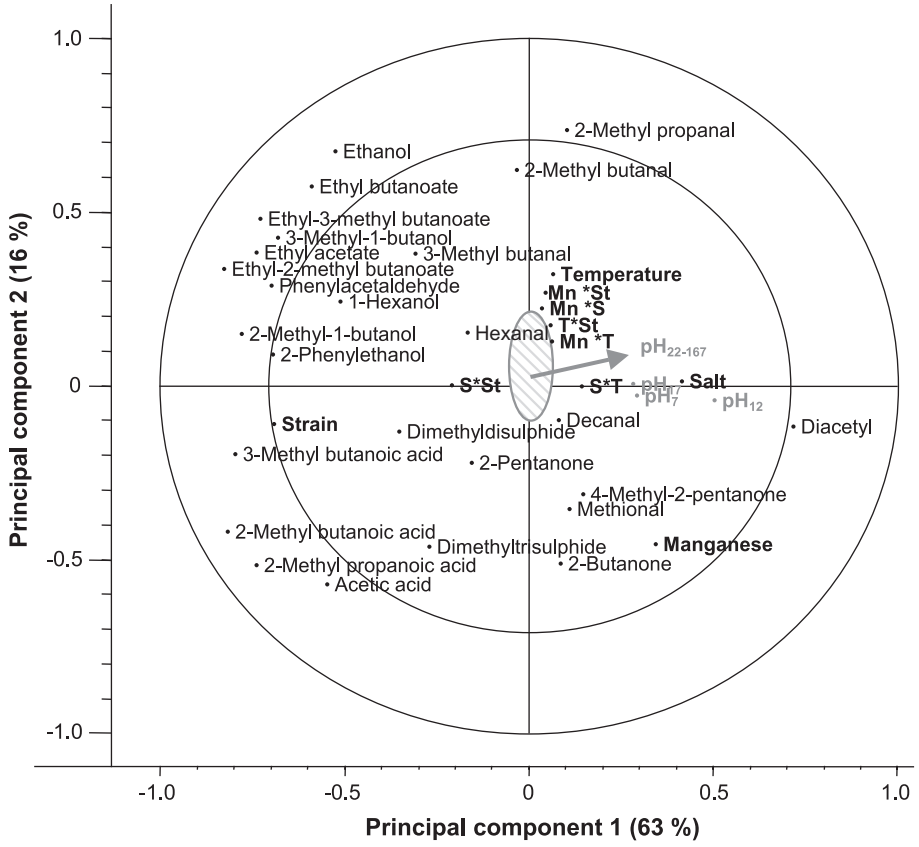

Fig. 3. Correlation loading plot of discriminant partial least-squares regression of pH-orthogonalised volatile responses vs. design. pH measurements are included as passive variables (pH_x: average pH reading to fermentation time, × hours). Inner and outer circle represents 50% and 100% explained variance for a given variable. High level of the variable strain corresponds to *S. carnosus*. M: manganese, T: temperature, S: salt, St: strain.

pounds, disappeared during fermentation (Olesen et al., 2004; Tjener et al., 2003). The generally higher activity of *S. carnosus* compared to *S. xylosus* mentioned above may have led to increased need for NAD^+ regeneration, for instance, through reduction of diacetyl to acetoin and 2,3-butanediol. The explanation of the negative interaction ($-T \times St$) may therefore be a higher increase in NAD^+-dependent activity of *S. carnosus* compared to *S. xylosus* when temperature is increased from low to high level.

3.3.2. Effect of manganese

Manganese plays an important role in LAB's defence mechanism against oxygen, and the activity of LAB is therefore dependent on the presence of manganese (Archibald, 1986). As previously described (Hagen et al., 2000; Tjener et al., 2003), manganese showed a strong effect on pH in this study (Fig. 1), and it was therefore expected that much of the variation caused by this parameter would disappear after pH orthogonalisation. The effects of manganese found by MLR on raw data were all in accordance with an earlier study carried out in this model system as well as sausages, and in that study, the effects were all ascribed to differences in pH (Tjener et al., 2003).

In the present study, manganese had a positive effect on acetic acid and 2-butanone levels before pH orthogonalisation, and those effects were not present in the pH-orthogonal data. This indicates that LAB are the main contributors to the production of these compounds.

However, the level of many compounds related to staphylococcal metabolism, i.e., methyl-branched alcohols and aldehydes, phenylacetaldehyde, ethanol and ethyl esters, remained negatively influenced by manganese after the pH orthogonalisation. This indicates that manganese not only influenced the activity of LAB but also the activity of the staphylococci. A ^{31}P and ^{13}C nuclear magnetic resonance study of glucose metabolism in *Staphylococcus aureus* revealed that intracellular orthophosphate and lactate were complexed with manganese (Ezra et al., 1983), which may have had a negative effect on energy generation. Though very hypothetical, this effect might contribute to the explanation of the general negative influence of manganese on volatile production by staphylococci. In contrast to the general negative effect observed here, Olesen and Stahnke (2004) reported a small but positive effect of manganese on the formation of methyl-branched alcohols and acids by *S. xylosus* in liquid medium. For *S. carnosus*, the same study revealed no effect of manganese on the formation of methyl-branched alcohols and acids (Olesen and Stahnke, 2004).

A positive effect of manganese on the level of 2-pentanone was seen before and after pH orthogonalisation and may be ascribed to increased lipid oxidation caused by high levels of this transition metal (Aust et al., 1985; Schaich, 1992).

3.3.3. Effect of temperature

In the present study, temperature was highly correlated to acidification (Figs. 1 and 2), and it is therefore not surprising that many effects related to temperature are absent in the MLR of pH-orthogonalised data (Table 2).

In the raw data, methyl-branched acids were positively correlated to temperature, which is in accordance with studies in both liquid media (Olesen and Stahnke, 2004), model minces (Stahnke, 1999) and dry sausages (Stahnke, 1995a). This could be a consequence of (1) increased amino acid degradation activity by staphylococci (Olesen and Stahnke, 2004), (2) lower end-pH, leading to enhanced extraction during analysis,[3] (3) higher level of precursors (Waade and Stahnke, 1997) or more likely (4) a combination of the previously mentioned effects. The fact that the positive effect of temperature on the methyl-branched acids was absent in orthogonalised data indicates that differences in end-pH contribute significantly to the variation in the level of these compounds.

Temperature had a negative effect on the level of methyl-branched aldehydes, which could be explained by inhibition of staphylococcal activity because of accelerated acidification. After pH orthogonalisation, temperature has a positive effect on the level of methyl-branched aldehydes. Thus, when the negative, pH-related effects of temperature are removed, the expected positive effect of this factor on activity of staphylococci appears.

The levels of acetic acid and 2-butanone were positively affected by temperature, but those effects disappeared by the pH orthogonalisation. This indicates a strong correlation between the formation of acetic acid and 2-butanone and LAB activity, as previously mentioned in Section 3.3.2.

The sulphur compounds methional, dimethyldisulphide and dimethyltrisulphide probably originating from breakdown of methionine and cysteine by staphylococci (Beck et al., 2002) were positively affected by temperature before pH orthogonalisation. After orthogonalisation, the effects were almost removed, indicating that formation of those volatiles was strongly affected by pH, though it is not readily explainable how decreased pH will increase degradation of sulphurous amino acids. Another interpretation of these data is that the degradation of sulphurous amino acids by staphylococci is related to temperature in the same way as acid production by LAB and the positive effect by temperature therefore removed together with pH effects by pH orthogonalisation.

3.3.4. Effect of salt

As seen in Fig. 1, salt had a negative effect on acidification, probably caused by inhibition of LAB activity. However, according to Fig. 2, there was no strong correlation between salt and pH readings, and based on this, it is reasonable that most significant effects related to salt remained after pH orthogonalisation as shown in Table 2.

Salt had a negative effect on the level of most volatiles indicating a general inhibition of volatile

[3] With pK_a of 4.8, approximately 10% of acids are protonated (volatile) at pH 5.75 and 50% at pH 4.8, which means that higher amounts of acids will be extracted during analysis at low pH.

formation by this ingredient. The level of methyl-branched aldehydes was, however, positively correlated to salt. A similar effect was seen in a recent sausage experiment by Olesen et al. (2004), and it was here hypothesised that because salt inhibits LAB activity and acidification, the more salt tolerant staphylococci would take advantage of the less acidic environment and increase amino acid degradation. This hypothesis may very well be right, but it does not account for the positive effect on the level of 2- and 3-methyl butanal seen after pH orthogonalisation. However, staphylococci incorporate relatively large proportions of iso- and ante-iso-fatty acids into the cell membrane, and degradation products of methyl-branched amino acids are precursors for such branched-chain fatty acids (Kaneda, 1991; Beck et al., 2004). The proportion of straight- to branched-chain fatty acids changes upon variations in growth conditions such as water activity and thus renders a possibility for salt to influence amino acid breakdown in a pH-orthogonal manner. This explanation is indeed hypothetical and should be verified or rejected by further studies.

Phenylacetaldehyde that is not related to cell membrane synthesis varied in the opposite way of the methyl-branched aldehydes, although it is formed by the same sort of mechanisms (Yvon and Rijnen, 2001). This supports the idea of a specific, pH-orthogonal cause-and-effect relation between methyl-branched aldehydes and salt, which counteracts the general negative impact of salt on activity of staphylococci.

The level of 2-pentanone was strongly positively correlated to the level of salt before and after pH orthogonalisation. A plausible explanation is that salt may increase lipid oxidation and/or reduce activity of antioxidative enzymes (Ladikos and Lougovois, 1990; Aguirrezábal et al., 2000; Hernandez et al., 2002) and thereby provide more β-keto- or hydroxyacids for decarboxylation. However, in a resting cell study, the decarboxylation reaction was shown to be slightly inhibited by salt (Fadda et al., 2002).

Except for the positive effect on 2-pentanone, Stahnke (1995a) found the same effects of salt as mentioned above in dried sausages. The 2-pentanone effect was found by Stahnke (1999) for *S. carnosus* but not for *S. xylosus* in a model system similar to the present. This corresponds to the positive Strain × Salt interaction also seen in Table 2.

3.4. General discussion of pH orthogonalisation

The pH orthogonalisation removes 30% of the variation in volatile responses, and further, approximately 50% was explained by differences between *S. carnosus* and *S. xylosus* (Fig. 2). The effects of salt, manganese and temperature found by MLR of pH-orthogonal data were thus only responsible for the remaining 20% (inclusive noise). In other words, 50% of the variation was explained by salt, manganese and temperature, and of those 60% (30% of total variation) was related to pH. The pH-orthogonal effects may therefore be of relatively little importance in the overall picture of bacterial aroma formation. Nevertheless, the pH orthogonalisation may contribute to a deeper understanding of the impact of growth parameters on volatile formation, e.g., effects of manganese and salt earlier ascribed to pH appears to be incompletely explained by these factors and thus promoting the search for additional explanations. Overall, the interpretability of design parameters' effect on volatile responses was significantly increased by use of pH orthogonalisation.

4. Conclusion

This study introduced pH orthogonalisation as a tool for reduction of complexity and thereby enhancement of interpretability of volatile responses of a designed experiment. The pH orthogonalisation was used to demonstrate that most of the variation in volatile profiles caused by the design variables manganese, temperature and salt was in fact directly or indirectly caused by changes in LAB activity and pH. Furthermore, the pH orthogonalisation served to confirm or refute earlier proposed cause-and-effect relations, and in some cases, new relations were proposed.

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