ELSEVIER

Meat Science 66 (2003) 211–218

MEAT SCIENCE

www.elsevier.com/locate/meatsci

A fermented meat model system for studies of microbial aroma formation

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Received 23 October 2002; received in revised form 8 April 2003; accepted 15 April 2003

Abstract

A fermented meat model system was developed, by which microbial formation of volatiles could be examined. The model was evaluated against dry, fermented sausages with respect to microbial growth, pH and volatile profiles. Fast and slowly acidified sausages and models were produced using the starter cultures *Pediococcus pentosaceus* and *Staphylococcus xylosus*. Volatiles were collected and analysed by dynamic headspace sampling and GC–MS. The analysis was primarily focused on volatiles arising from amino acid degradation and a total of 24 compounds, of which 19 were quantified, were used for multivariate data analysis. Growth of lactic acid bacteria was comparable for model and sausages, whereas survival of *S. xylosus* was better in the model. Multivariate analysis of volatiles showed that differences between fast and slowly acidified samples were identical for model and sausage. For both sausage and model, fast-acidified samples had a high content of ketones, sulphides and methyl-branched acids, whereas slowly acidified samples had the highest content of methyl-branched alcohols, aldehydes, their ethyl esters, phenylacetaldehyde and methional. Furthermore, model repeatability with respect to pH, microbial growth and volatile profiles was similar to sausage production. Based on these findings, the model system was considered valid for studies of aroma formation of meat cultures for fermented sausage.

Keywords: Fermented sausage; Model; Volatiles; *Staphylococcus xylosus*; *Pediococcus pentosaceus*

1. Introduction

Various *Staphylococcus* spp. are often used as starter cultures in production of dry, fermented sausages due to their nitrate reductase activity and flavour enhancing capacity. Their main contribution to flavour formation is believed to be related to carbohydrate degradation, amino acid catabolism, and fatty acid β-oxidation (Berdagué, Monteil, Montel, & Talon, 1993; Montel, Reitz, Talon, Berdagué, & Rousset, 1996). Additionally, *Staphylococcus* often possesses catalase activity, which indirectly could alter flavour formation by preventing chemical oxidation of fatty acids (Talon, Walter, Chartier, Barrière, & Montel, 1999). The levels of volatiles produced by *Staphylococcus* are highly strain dependent (Søndergaard & Stahnke 2002; Larrouture, Ardaillon, Pépin, & Montel, 2000; Stahnke 1999a; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2002) and furthermore, ingredient levels and processing conditions have a great impact on their growth and aroma formation (Stahnke, 1995a, 1995b). Though volatile production by staphylococci has been studied for several years, the relation between specific *Staphylococcus* strains, processing conditions and volatile production is far from fully understood. Since sausage production is a time and labour intensive process requiring advanced smoke and climate chambers, an extensive investigation of volatile production of various *Staphylococcus* spp. under different growth conditions would be almost impossible without the introduction of a model system.

Several workers have studied the volatiles from amino acid degradation by staphylococci in a liquid medium model (Beck, Hansen, & Lauritsen, 2002; Larrouture et al., 2000; Masson, Hinrichsen, Talon, & Montel, 1999; Olesen & Stahnke, 2002). However, in all these studies the formation of methyl-branched acids was favoured compared with the flavour important methyl-branched aldehydes, which is in contrast to the levels detected in several

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0309-1740/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0309-1740(03)00093-7

sausage studies (Berdagué et al., 1993; Johansson, Berdagué, Larsson, Tran, & Borch, 1994; Stahnke, 1995b). Olesen and Stahnke (2002) suggest disparity in redox balance and diffusion of aldehydes into the lipid phase of sausages as possible causes for this elevated acid/aldehyde ratio.

In a study by Molly, Demeyer, Johansson, Raemaekers, Ghistelinck, and Geenen (1997) it was shown that endogenous meat enzymes are involved in protein breakdown and thereby supply the small peptides that are precursors for microbial production of some important aroma compounds. The study also showed that the activity of the important cathepsin D like enzymes was strongly pH dependent. Thus, a meat-based model with a realistic pH profile would provide a nutritional situation close to sausage conditions and, compared with a liquid model system, probably also lead to volatile profiles closer to sausage volatile profiles.

The objective of this study was to develop and evaluate a model system based on minced meat fermentation as a substitute for sausage processing with respect to investigations of microbial growth and aroma formation. The model was based on several considerations: (1) using the model has to be considerably simpler and faster than sausage production. (2) Changes in processing conditions must be reflected in volatile profiles from model minces in the same way as in sausages, but profiles do not have to be identical. (3) Model repeatability must be at least as good as the repeatability within sausages.

2. Materials and methods

2.1. Sausage and model mince production

Sausage and model minces were produced according to the recipe in Table 1 starter cultures (freeze-dried *Pediococcus pentosaceus* PC-1 and *Staphylococcus xylosus* DD-34, Chr. Hansen A/S, Denmark). $MnSO_4H_2O$ were added to levels of 0.31×10^{-3} and 3.39×10^{-3} g/kg for slow and fast acidification, respectively[1] (in this case fast and slow acidification is defined as pH decreasing to below 5.3 within 30 h and no earlier than 40 h, respectively). The mince was stuffed into 48-mm casings (cellulose, SFK amba, Denmark) or plastic beakers (Polyethylene, 450 ml, $d=70$ mm, Berry Plastics, USA) giving sausages and model minces an initial weight of approximately 700 and 300 g, respectively. The sausages were fermented in a climate chamber (Multimat MC1000, Deutsch, Germany) at the settings given in Table 2. Model minces were vacuum stopped, sealed with a lid and incubated in a water bath at 24 °C for 7 days.

Table 1
Recipe for model minces and sausages

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)	316.0
NaCl with 0.6% w/w $NaNO_2$	17.0
Potato starch	12.5
NaCl (vacuum salt)	10.0
Glucose	4.0
Sodium ascorbate	0.5
$MnSO_4H_2O$[a]	0.31 or 3.39×10^{-3}
Total	1000.0

[a] Low and high level for slow and fast acidification, respectively.

Duplicate samples of sausage and model mince were taken at day 0, 1, 3, 7, 14 and 21 (day 14 and 21 sausage only), vacuum packed and stored at −50 °C for a maximum of 8 weeks until aroma analysis. Parts of the samples were used for bacteriological studies immediately after sampling (see later). After 3 weeks of fermentation, the remaining sausages were vacuum packed and stored at 5 °C until sensory analysis.

2.2. Determination of cell counts

Cell counts of LAB and Micrococcaceae were determined by 10-fold dilution of 35 g of sample with subsequent pour plating in MRS (de Man, Rogosa, Sharpe, Oxoid, UK) and S-110 (Difco, Germany) agar media, respectively. Plates were incubated for 3 days anaerobically (MRS) and for 2 days aerobically (S-110) at 30 °C.

2.3. pH-measurements

Sausage pH was measured at day 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17 and 21 (Knick Portamess®, Knick Elektronische Meßgeräte GmbH, Germany, with Metrohm

Table 2
Fermentation and drying conditions in the climate chamber

Time (days)	Temperature (°C)	Humidity (% RH)	Smoke[a] (min)
1	24	95	
2	22	95	15
3	20	92	15
4	20	90	
5	18	85	
6	18	83	15
7	18	80	
8	17	80	
9	17	78	
10	17	78	
11	17	75	
.	.	.	
.	.	.	
21	17	75	

[a] Smoke was generated with beech chips.

[1] The addition of manganese (0–3.39×10^{-3} g/kg) was tested in a preliminary experiment, described in Section 3.

6.0226.100 electrode, Metrohm Ltd., Switzerland) whereas pH of model minces was measured continuously until day 7 with HA405-DXX-S8/120 electrodes (Mettler Toledo GmbH, Switzerland) connected to an AAC-2 PC-logger (INTAB Interface Teknik AB, Sweden). pH was followed in two fast and two slowly acidified sausages and model minces. pH of model minces was measured with three electrodes per beaker, and sausage pH was measured three times per sausage on each day of analysis.

2.4. Volatile analysis

One hundred grams of model meat (5 °C) was mixed with 20 g of NaCl and 30 g was transferred into three cylindrical glass bottles (150 ml). One hundred grams of sausage was cut into cubes (approx. 1 cm^3), 20 g of NaCl added, treated with liquid nitrogen, homogenized with a domestic chopper (Krups Speedy Pro, Krups GmbH, Germany), and transferred to glass bottles (3×30 g). 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 to Tenax TA® tubes (200 mg, 60/80 mesh, Chrompack, Holland) and purged with nitrogen (grade $N_2 > 99.999\%$, flow rate 50 ml/min) for 30 min at 42 °C. Prior to sampling, Tenax TA® tubes were conditioned by purging with helium (99.9995%, flow rate 75 ml/min) for 20 min at 340 °C.

Tenax TA® tubes were desorbed by thermal desorption (ATD50, Perkin-Elmer Ltd, UK) in a two-step manner (first desorption: 200 °C for 3 min onto Tenax TA® cold trap (20 mg, −30 °C), second desorption: 200 °C for 60 s, line temp: 200 °C) and automatically injected into a GC (Hewlett-Packard 5890 series II, Agilent Technologies, USA). Separation was performed on a 30 m×0.25 mm id. DB 1701 (1 μm film) fused silica capillary column (J & W Sci., USA), detection by an MS detector (Hewlett-Packard 5972, ionisation energy 70 eV, 3.4 scans/s, source 160 °C, scan range 33–250 AMU). GC oven program 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 with the NBS75k-database.

Standard addition curves were made for quantification of 19 components. A stock-solution was prepared in methanol (10 g/l). This was diluted with methanol to concentrations of 0, 0.5, 1.0 and 1.5 g/l. A 10 μl of dilution was added to 100 g of sample of sausage or model mince (fast fermented, day 3) prior to the addition of NaCl. Curves were based on double triplicates for sausages as well as models.

2.5. Sensory analysis

A triangular test of the sausages based on odour (no tasting) was executed according to ISO (1983) at day 14 and 35. Each time, 10 trained panellists evaluated 2 sets of test samples. Fast and slowly acidified sausages were homogenized with a domestic chopper and served at 13 °C in 100-ml plastic vials.

2.6. Multivariate data analysis

Data were analysed by principal component regression (PCR) with The Unscrambler® software (version 7.6, CAMO A/S, Norway). The model data matrix consisted of 36 samples vs. concentrations or peak areas in Log_{10} scale of the 24 volatiles listed in Table 3 (*X*-variables) and a set of binary variables (*Y*-variables) describing the experimental design. The sausage data matrix consisted of 60 samples and the same set of variables as the model data matrix.

3. Results and discussion

A fermented meat model system has been developed. Though relatively closely related to fermented sausage, the model is considerably easier to handle. Fermentation time is reduced to only 1 week and because no climate chamber is needed, fermentations at several temperatures can run simultaneously. Furthermore, uninoculated model mince can be stored frozen and later thawed in appropriate portions, supplementary ingredients and starter culture added before being finally placed in a water bath. Thus, the model gives a lot of flexibility compared with sausage production.

However, the growth conditions in sausage and model differ with respect to several important growth parameters. The model mince is placed in a sealed container and therefore has a constant water content. The temperature in the model, in contrast to sausages, is kept at 24 °C throughout fermentation. These differences in environmental conditions will be reflected in growth and volatile production of the starter cultures.

3.1. Addition of manganese

Many lactic acid bacteria have a high requirement for manganese (Archibald, 1986), which, in sausage production, is often covered by the levels present in the added spices. The model mince does not contain spices and addition of manganese was therefore necessary. In a preliminary experiment various levels of $MnSO_4$ were added to model minces and pH was monitored continuously for 7 days (data not shown). Based on the pH profiles from this experiment, it was decided to add 0.31 and 3.39 mg of $MnSO_4H_2O$/kg of mince (0.1 and 1.1 ppm Mn^{2+}) to obtain slow and fast fermentation, respectively. The correlation between acidification profile and amount of added Mn^{2+} was in good accordance with the findings of Hagen, Næs, and Holck (2000).

Table 3
Concentration ranges and estimated sensory importance of quantified volatiles in model minces (day 1–7) and sausages (day 1–21)

Compound	Sausage	Model	Sensory importance in sausages[a]	Model and sausage	
	Concentration μg/kg	Concentration μg/kg		CV triplicates	CV duplicate samples
2-Methyl-1-butanol	1–20	3–15	–	12	15
3-Methyl-1-butanol	6–66	18–261	–	12	17
2-Phenylethanol	0–129	0–1523	–	24	19
1-Hexanol	1–22	3–96	–	17	24
2-Methyl propanal	0–7	2–17	+	17	17
2-Methyl butanal	1–17	1–23	+	14	18
3-Methyl butanal	3–42	4–73	+	13	18
Benzaldehyde	4–16	12–94	?	12	15
Phenylacetaldehyde	0–132	0–376	+	20	26
Hexanal	10–201	18–1260	+	18	37
2-Methyl propanoic acid	nq[b]	nq	?	12	15
2-Methyl butanoic acid	nq	nq	?	50	28
3-Methyl butanoic acid	nq	nq	?	40	34
Ethyl-2-methyl butanoate	0–6	0–6	?	19	38
Ethyl-3-methyl butanoate	0–1	0–1	?	13	0
Ethyl butanoate	0–12	0–32	+	18	25
Dimethyltrisulphide	nq	nq	?	37	33
Dimethyldisulphide	0–17	2–44	+	17	8
Methional	nq	nq	?	32	32
Acetophenone	0–6	0–28	–	23	42
Diacetyl	5–63	193–781	+	16	15
2-Butanone	2–39	31–3632	–	16	18
2-Pentanone	0–5	4–24	?	18	25
4-Methyl-2-pentanone	0	0–9	?	18	24

[a] (–) Concentrations below sensory threshold, (+) concentrations above sensory threshold at the end of ripening, (?) not quantified or threshold not found (Rychlik, Schieberle, & Grosch, 1998).
[b] nq = Not quantified. For these volatiles, the integrated single ion responses are used for multivariate data treatment.

3.2. Repeatability of pH and cell counts

In order to determine model repeatability the following experiment was conducted: Six minces were inoculated and pH was followed (three electrodes per mince) for 7 days. In this period standard deviation of pH measurements increased from 0.04 to 0.06 pH units. However, the final variation was comparable with the batch variations found by Demeyer et al. (2000) on four types of industrial dry, fermented sausages. Gimeno, Astiasarán, and Bello (2001) found variations of 0.2–0.5 within five batches.

In the same experiment LAB and staphylococci cell numbers were determined in six minces fermented for 7 days. A relative standard deviation of 10 and 35% was found for LAB and staphylococci, respectively. This variability is within the variation found in fermented sausages by others (Montel, 1999; Sanz, Flores, Toldra, & Feria, 1997).

3.3. Cell counts and pH

Fig. 1 shows that the pH profiles in sausages and models were comparable, especially for the fast-acidified samples. After day 3 the difference between pH in slowly acidified sausages and model minces increased. This could be explained by the lowering of temperature and/or water activity in sausages that would delay lactic acid production by *P. pentosaceus* in the sausage. At day 21, pH of slowly acidified sausages had reached the same level as the fast-acidified sausages did at day 2, which indicates that all available sugar had been metabolized.

Fig. 2 shows that growth of LAB in models and sausages was quite similar, and the correlation between acidification rate and LAB growth rate is clear. However, for slow acidification a fast growth during the first days of fermentation was seen in the model. This observation could be explained by the high temperature and/or water activity in the model minces during the first days of fermentation.

While LAB numbers were the same in sausages and model minces, the survival of *S. xylosus* was generally better in model minces (Fig. 3). The decrease in viable cell counts of *S. xylosus* during sausage fermentation was in accordance with the findings of Johansson et al.,

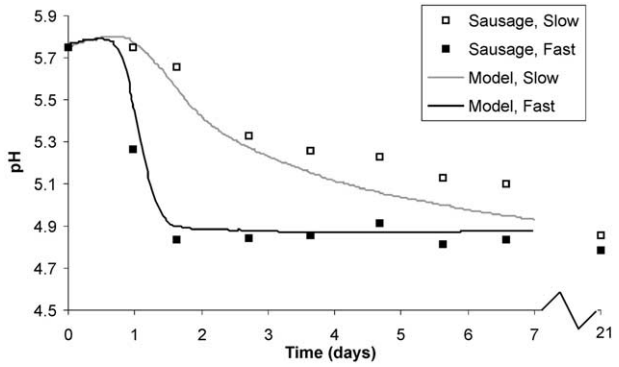

Fig. 1. Comparison of pH in fast and slowly acidified models and sausages. Points and curves are averages of three measurements in each of two models/sausages.

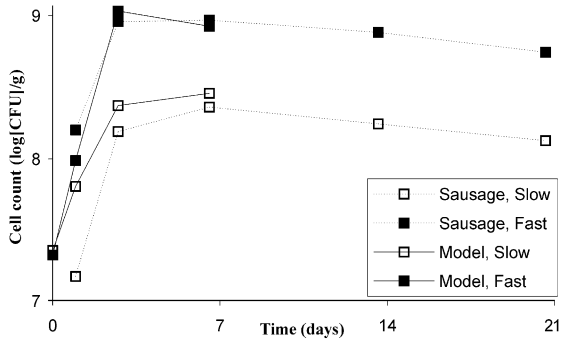

Fig. 2. Lactic acid bacteria cell counts in fast and slowly acidified models and sausages. Each point is the average count of two samples.

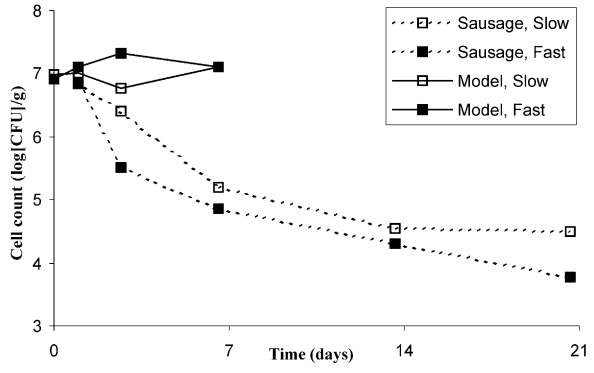

Fig. 3. Staphylococci cell counts in fast and slowly acidified models and sausages. Each point is the average count of two samples.

(1994), Gardini, Martuscelli, Crudele, Paparella, and Suzzi (2002) and others. The decrease is often ascribed to decreasing pH and oxygen depletion, but these factors alone cannot explain the difference in staphylococci cell numbers between model and sausage, since they were no worse in sausage compared to the model. Sørensen and Jakobsen (1996) showed that growth of *S. xylosus* was positively correlated to temperature (10–30 °C) and negatively correlated to water activity (1–9% w/v NaCl), and differences in these factors probably explain the different survival rates of *S. xylosus* in the model and sausage.

3.4. Volatiles

More than 350 different volatiles have been identified from various dry, fermented sausages (Stahnke, 2002). Many of these compounds originate from spices and only serve to complicate analysis of volatiles formed by bacteria. Therefore, the model mince was produced without spices. A further simplification was the focus on volatiles related to amino acid degradation, though representative volatiles of other origins were included, for example, hexanal (auto-oxidation of lipids) and 2-pentanone (incomplete β-oxidation of fatty acids).

Results from volatile analyses of sausages and model minces are listed in Table 3. The coefficient of variation was 22% on average for triplicates and 24% between replicate samples. The coefficients of variation are based on both sausage and model data, since their separate average values were tested not to be significantly different (paired *t*-test, $P > 0.01$). The variation for triplicates is a measure of the combined uncertainty associated with sample preparation, aroma collection, GC–MS analysis and peak integration. The large variations for acids and sulphur compounds are in agreement with the results of Mateo and Zumalacárregui (1996) and in general, variations are comparable with those found by Stahnke et al. (2002). Due to the large variations for acids and sulphur compounds, standard addition curves were not made for these compounds, except for dimethyldisulphide.

To our knowledge, the present study is the first to quantify volatiles in fermented sausages by standard addition. Though other workers have quantified or semi-quantified volatiles in fermented sausages, the reported amounts from different studies are generally difficult to compare, since the various studies present data in different arbitrary units. Thus, workers have reported detector responses (FID, single-ion MS or total-ion MS) in units of response of a given compound divided by response of a known amount of an internal or external standard compound (heptane, octane, dodecane, 2-methyl-1-pentanol, fenchone, methyl undecanoate) (Berdagué et al., 1993; Edwards, Ordóñez, Dainty, Hierro, & de la Hoz, 1999; Mateo & Zumalacárregui, 1996; Misharina, Andreenkov, & Vashchuk, 2001; Schmidt & Berger, 1998; Sunesen, Dorigoni, Zanardi, & Stahnke, 2001).

The 19 quantified compounds were generally present in higher amounts in the model minces compared with sausages. This was probably caused by (1) the higher number of *Staphylococcus* (Fig. 3), (2) increased activity of staphylococci in the model due to the high temperature and water activity, (3) model minces are fermented in closed containers, i.e. no evaporation. The high concentration of volatiles in the model minces is generally

advantageous, since many components are present in amounts close to the detection limit. On the other hand, 2-butanone and diacetyl were present in amounts exceeding the linear interval of the MS-detector, and care should be taken when interpreting the amount of these components in the model.

The concentrations of aldehydes (except benzaldehyde), diacetyl and dimethyldisulphide in the sausages were all above sensory threshold values in oil or water matrices (Rychlik, Schieberle, & Grosch, 1998) and are likely to have an impact on the overall aroma of the analysed products. The not quantified acids and sulphur compounds shown in Table 3 all have relatively low threshold values (0.2–50 μg/l) and may also influence the overall aroma (Rychlik et al., 1998).

Fig. 4 shows a PCR correlation-loading plot of sausage volatile data from day 1 to 21. Most of the analysed components are positively correlated with the age of the sausages, i.e. those components are formed during sausage fermentation and/or maturation. Principal component 1 (PC1) represents this variation. PC2 is associated with differences in aroma profiles between fast and slowly acidified sausages. The compounds related to fast acidification are ketones, sulphides and methyl-branched acids, whereas slow acidification is related to methyl-branched alcohols and aldehydes, their ethyl esters, phenylacetaldehyde and methional. The inverse correlation between methyl-branched aldehydes and acids in relation to acidification was also shown by Stahnke (1995b).

The difference in volatile profiles seen in Fig. 4—especially regarding compounds of sensory importance (Table 3)—between fast and slowly acidified sausages was expected to yield sausages with different aroma. Slow and fast fermented sausages were indeed perceived as being different according to triangular tests (odour) performed by a sensory panel after 2 weeks ($0.01 < P < 0.05$) and again after 5 weeks ($P < 0.001$).

Fig. 5 is a PCR correlation-loading plot of model volatiles from day 1 to 7. In opposition to analysis of sausage data, 2 principal components, PC1 and PC2, explain the variation related to sample time (PC2 not shown in Fig. 5). PC3 represents variation associated with differences between fast and slowly acidified model samples. As in Fig. 4, fast acidification is correlated with ketones, sulphides and methyl-branched acids and slow acidification with methyl-branched alcohols, aldehydes, their ethyl esters, phenylacetaldehyde and methional. Figs. 4 and 5, however, are not identical. For instance 2-butanone and diacetyl are strongly correlated with fast acidification in sausages, but almost uncorrelated with acidification rate in the model. As mentioned earlier, the high levels of these components cause MS-detector overload and care should be taken when interpreting their position in Fig. 5. Despite the differences between Figs. 4 and 5, the information to be extracted from multivariate data analysis regarding microbial activity under different growth conditions—here fast and slow acidification—is very similar for sausage and model data. Hence, the model is a well-suited system for investigation of microbial activity under various growth conditions. The model has several advantages over sausages: First of all, it is easier to handle and requires no climate

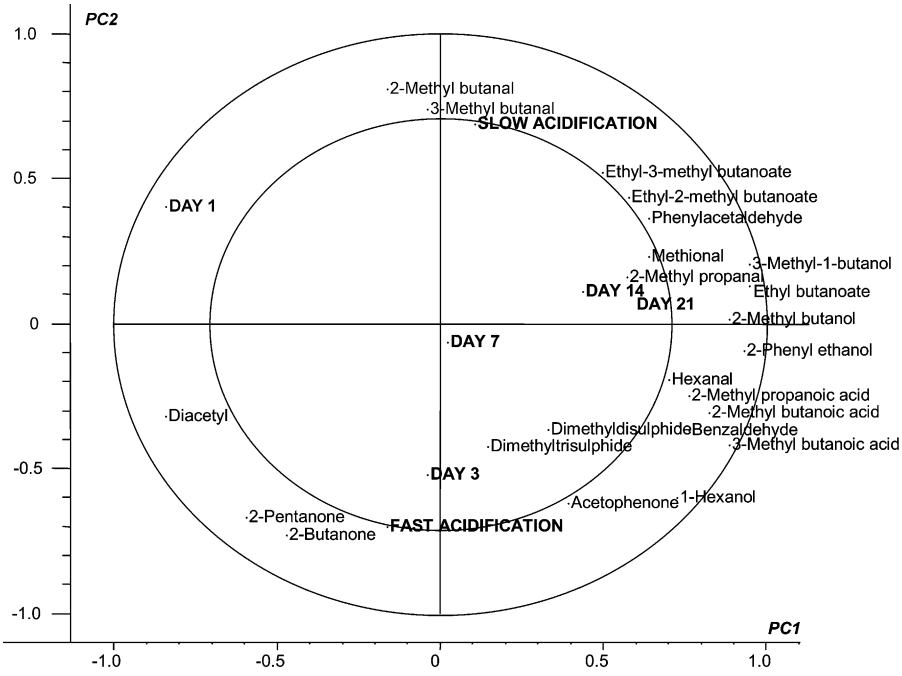

Fig. 4. Principal component regression (PCR). Correlation loadings for volatiles in sausages, day 1–21. Inner and outer circle represents 50 and 100% explained variance for a given variable. Principal component 1 (PC1) explains 44 and 17% of variability in *X*- and *Y*-matrices, respectively. PC2 explains 19% of the variability in both *X*- and *Y*-matrices.

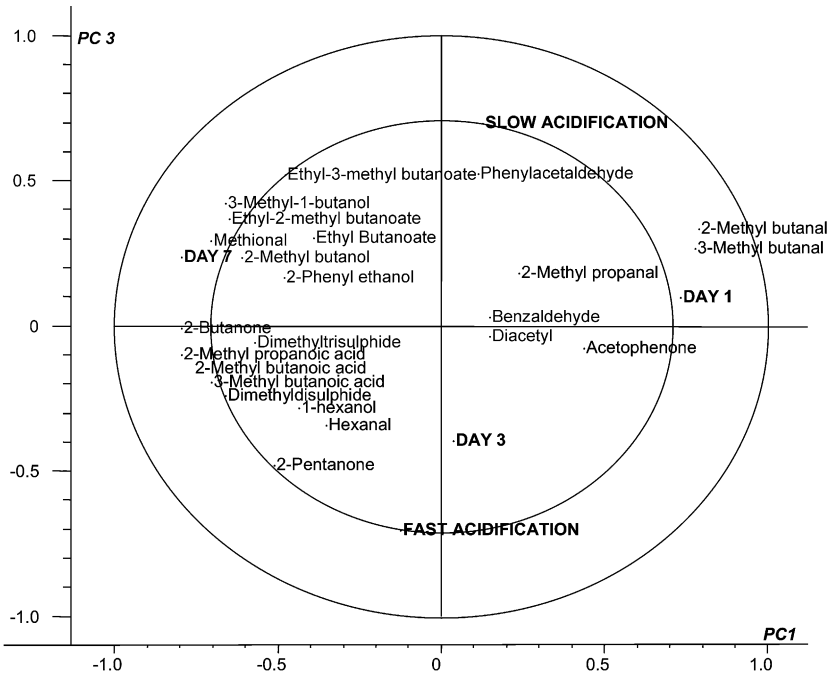

Fig. 5. PCR correlation loadings for volatiles in model minces, day 1–7. Inner and outer circle represents 50 and 100% explained variance for a given variable. PC1 explains 42 and 29% and PC3 10 and 31% of the variability in *X*- and *Y*-matrices.

chamber, secondly it reduces processing time to 1 week. The model is, however, less applicable to sensory analyses, which is the only significant drawback of the system.

Methyl-branched aldehydes were among the few components not positively correlated with age of sausages and model minces. An increase in the level of methyl-branched aldehydes in the early stages of ripening followed by a decrease towards the end of ripening was also seen by others (Mateo & Zumalacárregui, 1996; Sunesen et al., 2001). The aldehydes are probably oxidized to the corresponding acids over time (Beck et al., 2002).

All the compounds correlating with slow acidification are degradation products (or derivatives thereof) of the amino acids valine, isoleucine, leucine, methionine, and phenylalanine. The degradation of these amino acids during sausage fermentation has been ascribed to the activity of *Staphylococcus* (Masson et al., 1999; Stahnke, 1999a; Vergnais, Masson, Montel, Berdagué, & Talon, 1998). Factorial experiments have shown a significant effect of pH on growth and volatile production of *Staphylococcus* (Søndergaard & Stahnke; 2002; Stahnke 1999b) but to our knowledge it has never been shown that two different pH profiles of industrial relevance influence the production of volatiles of sensory importance.

4. Conclusion

This study shows that (1) it is possible to generate aroma profiles in model minces that reflect changes in microbial growth conditions in the same way as in sausages and (2) slow acidification is correlated with formation of methyl-branched aldehydes, methional and phenylacetaldehyde which are all positive contributors to dry sausage flavour.

Acknowledgements

The work was supported by the Danish Academy of Technical Sciences grant EF865. Professor Harald Martens is acknowledged for supervision of multivariate data analysis.

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