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Formation of amino acid (L-leucine, L-phenylalanine) derived volatile flavour compounds by *Moraxella phenylpyruvica* and *Staphylococcus xylosus* in cured meat model systems

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Abstract

A bacterial strain isolated from Danish immersion curing brine, *Moraxella phenylpyruvica* 0100, and a commercial meat starter culture, *Staphylococcus xylosus* DD34, were tested for their ability to form characteristic volatile compounds in minimal medium with the added amino acid L-leucine or L-phenylalanine under different environmental conditions (pH 5.5 and 6.0; 0 and 210 ppm nitrate; pre-incubation with and without agitation) and compared with respect to their ability to form volatile compounds in cured meat extracts and vacuum-packed cured meat cuts. The characteristic cured meat aroma precursors/compounds 3-methylbutanal and 3-methylbutanol were found to be formed in cured meat extracts and vacuum-packed cured meat cuts inoculated with *M. phenylpyruvica*. These volatiles are most probably formed by metabolic conversion of the amino acid L-leucine by *M. phenylpyruvica*, as they were also produced in minimal media with added L-leucine inoculated with this organism. The characteristic L-phenylalanine derived compound, benzaldehyde, formed by *M. phenylpyruvica* in minimal medium in the presence of nitrate (210 ppm), was not produced in any noticeable amount in cured meat extracts or vacuum-packed cured meat inoculated with *M. phenylpyruvica*. In contrast, benzacetaldehyde, which has been described as a possible metabolic product of the microbial conversion of L-phenylalanine, was found to be a characteristic volatile compound formed in cured meat extracts and vacuum-packed cured meat inoculated with *M. phenylpyruvica*, indicating an alternative metabolic pathway for L-phenylalanine by this organism in a cured meat environment. Even though *S. xylosus* was able to form volatile compounds characteristic of cured meats (3-methylbutanal, 3-methylbutanol) in minimal media with added L-leucine, this bacterial strain seemed not to be able to produce these characteristic volatiles in the studied cured meat systems. The present data imply that *M. phenylpyruvica*, in particular, is a potential meat starter for ensuring superior flavour development in cured meat.

Keywords: Aroma; Cured meat; L-Leucine; L-Phenylalanine; 2-Methylbutanal; 3-Methylbutanal; *Moraxella phenylpyruvica*; *Staphylococcus xylosus*; Volatile compounds

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1. Introduction

Consumer perception of meat products is closely related to sensory properties, taste being the criterion with highest priority, closely followed by aroma and texture (Touraille, 1992). These three sensory attributes all contribute to the flavour of a meat product. In a study of the relationship between sensory data and acceptability, it was found that the more pronounced meat flavour a meat product possessed, the more it was considered acceptable by the consumer (Horsfield and Taylor, 1976). This is further supported by recent consumer surveys, which showed that superior flavour makes the consumer rebuy meat products (Verplaetse, 1994). These findings clearly show the importance of sensory attributes, and in particular flavour, in relation to meat products.

Inferior flavour development in bacon products seems to be an increasing problem (Anonymous, 1992). This might be due to the implementation of new, rapid and thereby economically advantageous processing technologies, e.g. the cure-in-bag (CIB) process, at the expense of traditional manufacturing methods, e.g. the Wiltshire curing process, as indicated by the findings of Andersen and Hinrichsen (1995).

Studies of the micro-organisms isolated from old back slopping curing brines suggest that these have been essential factors in the superior flavour development in traditionally produced bacon products (Hinrichsen and Andersen, 1994). Subsequently, the meat industry has shown interest in finding suitable micro-organisms which in combination with modern processing technologies can be used as starter cultures with a view to produce superior flavour in cured meat products based on whole muscles, e.g. bacon and ham products.

Microbial formation of essential flavour compounds/precursors in meats is supposed to be associated with the metabolism of amino acids, peptides and proteins (Verplaetse, 1994). This is based on the fact that hitherto most of the aroma compounds known to give rise to the characteristic aroma of fermented meat products are derived from the protein fraction of the meat (Lücke, 1994). This is further supported by investigations showing that proteolytic activity is essential to flavour development in meat products (Hammes and Knauf, 1994).

Amino acids are released from the protein matrix of meat and milk products through the action of proteolytic enzymes. They might thereafter be involved in the basic taste together with small peptides, but amino acids do not contribute directly to the typical flavours associated with the volatile fraction of these products. However, they can contribute indirectly to these typical flavours since they are precursors of volatile compounds such as aldehydes, acids, alcohols, esters and thiols. Aromatic, branched-chain and sulfurous amino acids, in particular, are known to be precursors of potential aroma compounds (Yvon et al., 1997).

Fermented foods are, in general, known for their characteristic flavour, and enzymatic degradation of amino acids in these food products is believed to generate aroma compounds and thereby to be involved in the complex process of flavour development (Dunn and Lindsay, 1985; Hammes and Knauf, 1994). Amino acid degradation in fermented products is due mainly to microbial action, although non-catalyzed chemical degradation may occur during ripening. To improve and control the action of microbial enzymes during ripening of fermented products, it is essential to study the microbial transformation of amino acids into aroma compounds. Intensive studies have been performed within this area in connection with dairy starters (Visser, 1977; Law and Mulholland, 1995; Engels and Visser, 1996), whereas hardly any studies have been performed within the area of meat starters, until now.

Recently, the amino acid L-leucine was proposed to be the possible precursor in the microbial formation of 3-methylbutanal in cured meats (Hinrichsen and Andersen, 1994). 3-Methylbutanal is known to be able to give rise to bacon-like flavour via reactions with sulphur-containing compounds (Wiener, 1972; Shu et al., 1985). L-Phenylalanine is another amino acid which likewise has been proposed as a potential precursor in the microbial formation of aroma compounds, especially in cheese (Lee and Richard, 1984; Lee et al., 1985). *Streptococcus lactis* var. *maltigenes* has been shown to metabolize L-phenylalanine to phenylacetaldehyde (Sheldon et al., 1971), which in diluted samples has the characteristic aroma of hawthorn, and due to its chemical nature it is expected to contribute further to flavour formation after reaction with other food constituents.

In the present study, the abilities of the potential meat starter *Moraxella phenylpyruvica* and the com-

mercial meat starter *Staphylococcus xylosus* to produce volatile compounds through metabolic conversion of L-leucine and L-phenylalanine in minimal media were investigated in order to elucidate the possible role of these bacteria in amino acid derived flavour formation. Additionally, the effects of previous sub-culturing with and without agitation in enriched media, pH (5.5, 6.0) and the presence of nitrate (210 ppm) on the formation of volatile compounds were analysed. Moreover, the formation of volatile compounds by *M. phenylpyruvica* and *S. xylosus* in cured meat extracts was investigated including the same factorial design as in the minimal media to determine whether this medium allows the formation of bacterial-formed volatiles derived from the amino acids L-leucine and L-phenylalanine. Finally, the formation of volatile compounds in cured meat cuts inoculated with *M. phenylpyruvica* and *S. xylosus* was investigated, and the results are discussed in relation to the findings used in the model systems.

2. Materials and methods

2.1. Growth media

Minimal media. Minimal media were composed of 0.5 g l^{-1} yeast extract (Oxoid, Hampshire, UK), 40.0 g l^{-1} NaCl (Riedel-de Haën AG, Seelze, Germany) and 14.2 g l^{-1} Na_2HPO_4 (Merck, Darmstadt, Germany) either with or without 0.34 g l^{-1} KNO_3 (Merck, Darmstadt, Germany). The pH of the media was adjusted to 5.5 or 6.0 using 4 N HCl. Subsequently, these minimal media plus the same media with added 0.5% L-leucine or L-phenylalanine (Sigma, St. Louis, MO, USA) were sterile filtered (0.25 μm) in 5 ml fractions into an appropriate number of 10 ml odour-free, sterilised plastic tubes.

Cured meat extracts. Pieces of deboned and derinded *M. longissimus dorsi* from pigs (Danish Grade A) were, 48 h post-mortem, mixed in a ratio of 1:2 with buffer solutions (4.08 g l^{-1} KH_2PO_4 (Riedel-de Haën AG, Seelze, Germany) and 40.0 g l^{-1} NaCl (Riedel-de Haën AG, Seelze, Germany) with and without 0.34 g l^{-1} KNO_3 (Merck, Darmstadt, Germany)) and the pH was adjusted to 5.5 or 6.0 using 4 N HCl. Subsequently, the meat was homogenised using a Nelco Mixer resulting in meat slurries. The four different meat slurries were subsequently centrifuged for 20 min (2250*g* at 5°C, Cryofuge 20-3, Heraeus Ceraeus, Osterode, Germany) and the supernatants were filtered through filter paper (Whatman No. 1) before further 1:1 dilution with the respective buffer solutions with and without KNO_3. Finally, these solutions were centrifuged for 1 h (50 000*g*, 5°C) and the supernatants were sterile filtered (0.25 μm) in 5 ml fractions into an appropriate number of 10 ml odour-free, sterilised plastic tubes.

Meat cuts. Forty-eight hours post-mortem, deboned and derinded *M. longissimus dorsi* from pigs (Danish Grade A) were trimmed to 15 mm of back fat. Meat cuts were halved yielding samples of approximately 2 kg. All operations were performed at a temperature of 4°C.

2.2. Micro-organisms

For experiments in minimal media and cured meat extracts, two bacterial strains, one isolated from Danish immersion curing brines, *Moraxella phenylpyruvica* 0100, and one commercial starter culture, *Staphylococcus xylosus* DD34 (Chr. Hansen A/S, Hørsholm, Denmark), were grown in brain heart infusion (BHI) medium (Difco, Detroit, MI, USA) including 4% (w/v) NaCl. They were incubated at 20°C for 2 days with or without agitation (200 rpm). Cells were harvested by centrifugation (12 000*g*, 15 min, 5°C) and re-suspended in 4% (w/v) sterile saline solution to an optical density at 620 nm (OD_{620}) of 0.7 [approximately 10^8 colony-forming units (CFU) g^{-1}, determined as described later].

In experiments with whole meat cuts, *M. phenylpyruvica* was grown as mentioned above and subsequently re-suspended in curing brine (18% w/v NaCl, 0.10% w/v $NaNO_2$) to $OD_{620} = 0.7$, while a freeze-dried culture of *S. xylosus* DD34 was re-suspended in curing brine (18% w/v NaCl, 0.10% w/v $NaNO_2$) to a concentration of $> 10^{10}$ CFU g^{-1}.

2.3. Inoculation and incubation

Minimal media. Minimal media [5 ml, combinations of pH (5.5, 6.0), with/without nitrate (210 ppm) and amino acid (0.5%)] were inoculated

(7 days at 20°C) with 0.5 ml cell suspension sub-cultured in BHI medium either with or without agitation in a $2 \times 2 \times 2 \times 2$ factorial design for each of the tested strains with a final cell concentration of 10^7 CFU ml^{-1}. This was performed with both L-leucine and L-phenylalanine added to the minimal media.

Cured meat extracts. Meat extracts [5 ml, combinations of pH (5.5, 6.0) and with/without nitrate (210 ppm)] were inoculated (7 days at 20°C) with 0.5 ml cell suspension sub-cultured in BHI medium either with or without agitation in a $2 \times 2 \times 2$ factorial design for each of the tested strains with a final cell concentration of 10^7 CFU ml^{-1}.

Cured meat cuts. Meat cuts were injected with curing brine, curing brine containing *M. phenylpyruvica* 0100 or curing brine containing *S. xylosus* DD34 to a gain of 10–15% (w/w) using a hand-injector (Boss Garant, Essen, Germany). Immediately after injection and inoculation the pork loins were vacuum-packed one by one in vacuum bags (Cryovac BC2, 200×500 mm, W.R. Grace A/S, Herlev, Denmark) and stored at 20°C for 3 or 7 days before analysis.

2.4. Chemical analysis

Cured meat cuts were analysed for sodium chloride, nitrate and nitrite after 3 and 7 days of storage at 20°C. Sodium chloride was determined by potentiometric titration with $AgNO_3$ in an auto-titrator (Radiometer, Denmark) according to NMKL (1986), and results are given as %(w/w). Nitrate and nitrite were measured spectrophotometrically at 546 nm using the diazotization reaction of sulphanilamide and subsequent coupling with *N*-(1-naphthyl)ethylenediamine (NMKL, 1982). Results are given as ppm KNO_3 and $NaNO_2$.

2.5. Extraction of volatile compounds

Minimal media (5 ml), cured meat extract or 30 g of cured pork loin pre-minced using a household blender were transferred to a 500 ml conical flask with a Dreschel head joined to a trap packed with 250 mg of Tenax (Tenax TA mesh 60–80, Chrompack Inc., Raritan, NJ, USA). As internal standard an aqueous solution of 1-chloroheptane (1 ml, 16.7 mM) in a small cup was placed in the flask. The flask was sealed with parafilm and equilibrated for 30 min at 50°C. Subsequently, volatile compounds were purged onto the Tenax trap with ultra-pure nitrogen for 10 min at a flow rate of 60 ml min^{-1}. Volatile compounds were thermally desorbed from the Tenax trap (250°C, 30 min) in a ATD400 automatic thermal desorption system (Perkin-Elmer Corp., Norwalk, CA, USA) and re-trapped on a Tenax-packed cold trap maintained at -30°C.

2.6. Head space gas chromatographic analysis of volatile compounds

Injection into the GC column was performed by thermal desorption of the trap at 300°C for 2 min with a split of 1:24.

A Perkin-Elmer capillary gas chromatograph 8120 connected to a Varian Star chromatography work station (Varian Version 4.4, Walnut Creek, CA, USA) was used in the GC analyses. A flame ionisation detector (FID) held at 250°C was used.

Chromatographic separations were performed with a DB-1701 capillary column (J&W Scientific, Folsom, CA, USA, 30 m × 0.25 mm i.d., film thickness 1 μm) using He as carrier gas (linear flow 29.17 cm s^{-1}). The oven temperature was held at 35°C for 10 min, raised from 35 to 150°C at 3°C min^{-1}, held at 150°C for 5 min and subsequently raised from 150 to 250°C at 30°C min^{-1} with a final holding time of 5 min. Volatile compounds were identified using Kovats' indices (Kovats, 1965) and reconfirmed by comparison with the GC-MS data of Hinrichsen and Pedersen (1995).

All samples from minimal media and cured meat extracts were analysed in duplicate after 7 days incubation at 20°C, while each cured meat cut was analysed in triplicate (one slice from each end and one from the centre) after 3 and 7 days storage at 20°C.

2.7. Bacterial sampling

Cured meat extracts were examined after 7 days incubation at 20°C and cured meat cuts after 3 and 7 days incubation at 20°C for aerobic viable cell counts. After appropriate dilutions in 0.1% Peptone (Difco, Detroit, MI, USA) including 4% NaCl (w/v), plate count agar (Difco) with added 4% (w/v) NaCl

was used as plating medium. Incubation was carried out for 5 days at 20°C.

2.8. Statistical analysis

Volatile compounds (peak areas) found by GC analysis were analysed using multivariate statistical methods. Chromatograms of volatile compounds from minimal media, cured meat extracts and cured meat cuts were all examined using principal component analysis (PCA) in Unscrambler (CAMO A/S, 1994). Moreover, selected volatile compounds found in minimal media and cured meat extracts were examined using partial least squares (PLS) regression. This was done to elucidate which factors affected the formation of these compounds. All chromatographic data were transformed (square root) prior to statistical analysis.

Chemical data were tested for linearity and normal distribution and subsequently analysed by analysis of variance using SAS statistical software (SAS, 1988). When significant F-values were obtained, the method of least square means was used to determine significant differences ($P < 0.05$) between means.

3. Results

3.1. Minimal media

Samples with added L-leucine and incubated 7 days at 20°C with either *Moraxella phenylpyruvica* or *Staphylococcus xylosus* all had a characteristic cheesy aroma varying in intensity depending on the preceding sub-culturing with and without agitation, pH and presence or absence of nitrate (results not shown). *M. phenylpyruvica* produced the most intense cheesy aroma independently of the presence or absence of nitrate and preceding sub-culturing conditions, but with higher intensity at low pH. In contrast, nitrate seemed to stimulate the formation of a cheesy aroma by *S. xylosus* independently of the other environmental factors. Samples with added L-phenylalanine and incubated 7 days at 20°C with *M. phenylpyruvica* had a characteristic scented/almond-like aroma in the presence of nitrate, but this became insignificant in the absence of nitrate. *S. xylosus* did not form any characteristic aroma under these conditions (results not shown).

Representative gas chromatograms of volatile compounds in the head space of minimal media with and without incubation of bacteria and the presence of L-leucine and L-phenylalanine after 7 days incubation are shown in Figs. 1 and 2 and the corresponding volatile compounds are indicated in Table 1. Approximately 40 different volatile compounds were isolated from the different minimal media with and without the presence of bacteria sub-cultured either with and without agitation, amino acids, and nitrate at pH 5.5 and 6.0. Principal component analysis of all volatile compounds isolated from the different minimal media showed that 79% of the total variance in the data could be described by the two first principal components (Fig. 3A). The scores plot shows that the chromatograms obtained can be

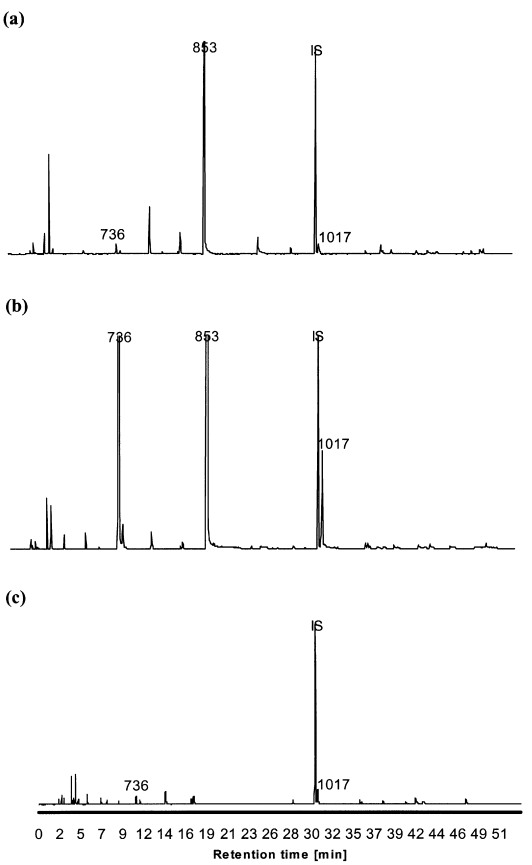

Fig. 1. Typical gas chromatograms obtained from minimal media with added L-leucine inoculated with *S. xylosus* (a), *M. phenylpyruvica* (b) or without inoculation of bacteria (control) (c). Samples analysed after 7 days incubation at 20°C (numbers assign Kovats indices; IS, internal standard).

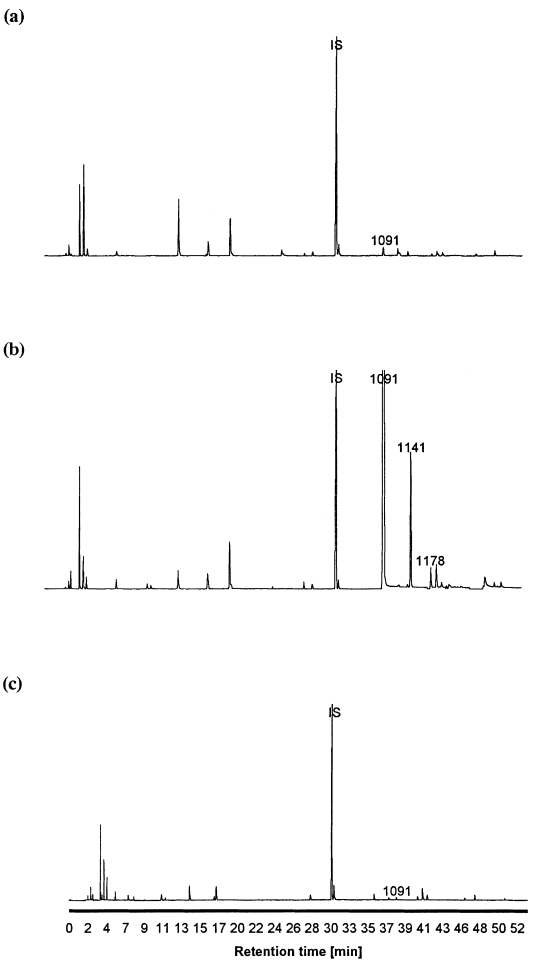

Fig. 2. Typical gas chromatograms obtained from minimal media with added L-phenylalanine inoculated with *S. xylosus* (a), *M. phenylpyruvica* (b) or without inoculation of bacteria (control) (c). Samples analysed after 7 days incubation at 20°C (numbers assign Kovats indices; IS, internal standard).

Table 1
Characteristic volatile compounds formed from L-leucine or L-phenylalanine in minimal media

Amino acid	Volatile compound	Kovats' index
L-Leucine	3-Methylbutanal	736
	3-Methylbutanol	853
	3-Methylbutanoic acid	1017
L-Phenylalanine	Benzaldehyde	1091
	Benzonitrile	1141
	2-Hydroxybenzaldehyde	1178

divided into three separate groups (I, II and III), as indicated in Fig. 3A. Group I exclusively contains chromatograms obtained from the head space of minimal media with added L-phenylalanine and nitrate and inoculated with *M. phenylpyruvica*. Group II includes all samples with added L-leucine, while Group III includes chromatograms from all control samples plus samples with added L-phenylalanine incubated with *S. xylosus*. The loadings plot (Fig. 3B) illustrates the volatile compounds responsible for the grouping observed in the scores plot. The loadings plot clearly shows that the volatile compounds with Kovats' indices (KI) 736, 853 and 1091 differ from origo. Closer analyses showed a correlation between KI 736 and KI 853 and, moreover, a slight correlation between these and a volatile compound near origo with a KI of 1017. In contrast, none of these correlated with the volatile compound with a KI of 1091. However, this compound correlated slightly with two other volatile compounds with KI 1141 and 1178. Comparison between the scores and loadings plot revealed that the volatile compound with KI 1091 and thus also the compounds with KI 1141 and 1178 are characteristic for gas chromatograms obtained from samples of Group I in the scores plot. Moreover, gas chromatograms obtained on the basis of the head space analyses of minimal media containing L-leucine and incubated either with *M. phenylpyruvica* or *S. xylosus* (Group II) are characterised by the presence of volatile compounds with KI 736 and 853, together with the correlated compound with a KI of 1017.

Table 1 presents an outline of the volatile compounds found to be characteristic of the head space of minimal media including L-leucine (Group II) or L-phenylalanine (Group I) in the presence of either *M. phenylpyruvica* or *S. xylosus* as based on the obtained Kovats' indices and comparison with GC-MS data.

For the volatile compounds found to originate either from L-leucine or L-phenylalanine in the presence of *M. phenylpyruvica* or *S. xylosus*, PLS regression analysis made it possible to estimate the effects of preceding sub-culturing with and without agitation in enriched media, pH and nitrate on the formation of volatile compounds. These results are shown in Table 2 and Table 3 together with the respective concentrations of the characteristic vola-

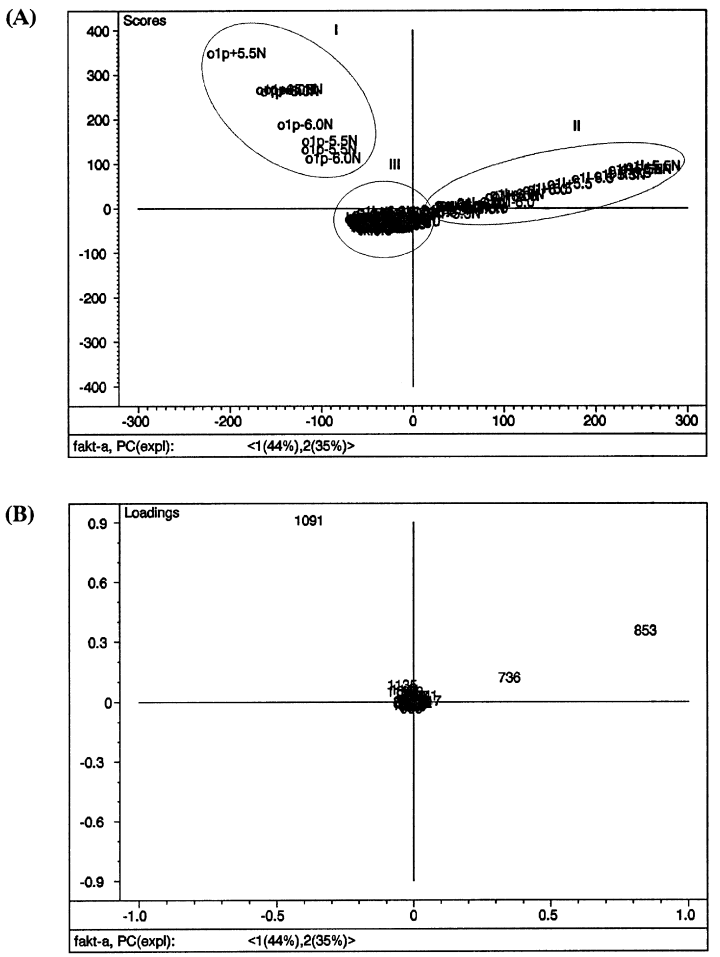

Fig. 3. (A) Scores plot after principal component analysis of gas chromatograms obtained from minimal media. 01, 34 and K assign *M. phenylpyruvica, S. xylosus* and control, respectively, + /- designate with or without agitation during preincubation, 5.5 or 6.0 designate pH in medium and N the presence of nitrite. (B) Loadings plot. The numbers assign Kovats indices of isolated volatiles.

tile compounds. In general, the concentrations of the specific volatile compounds formed by *M. phenylpyruvica* were at least an order of magnitude higher than the concentrations of those formed by *S. xylosus*. High pH (6.0) had a significant negative effect on the production of 3-methylbutanol and 3-methylbutanoic acid, while nitrate had a positive influence on the production of 3-methylbutanoic acid by *M. phenylpyruvica* inoculated in minimal media with added L-leucine. Preceding sub-culturing (with or without agitation) did not influence the production of any of the three characteristic volatile compounds produced by *M. phenylpyruvica* in the minimal media containing L-leucine. In contrast, agitation during sub-culturing stimulated the production of 3-methylbutanol and suppressed the formation of 3-methylbutanal by *S. xylosus* during incubation in minimal media with added L-leucine. *M. phenylpyruvica* was also able to convert L-phenylalanine into characteristic volatile compounds (benzaldehyde, benzonitrile and 2-hydroxybenzaldehyde), whereas *S. xylosus* did not produce a noticeable amount of characteristic volatiles under the same conditions. The presence of nitrate (210 ppm) was

Table 2
The effect of pH, nitrate concentration and preincubation of bacteria culture on quantities of selected volatile compounds formed by *M. phenylpyruvica* and *S. xylosus* in minimal media with added leucine (leu) and how these factors influence the quantity of volatile compounds, as determined by PLS regression

Volatile compound		Factor combinations	*Moraxella phenylpyruvica*		*Staphylococcus xylosus*	
			Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P[a]	Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P
3-Methylbutanal	Preincubation	pH 5.5 + nitrate[b]	184±11	Leu (43.4)	22±0	Leu (7.6)
	without agitation	pH 5.5	340±30	Leu *pH*nitrate (−9.4)	4±4	Agitation (−1.6)
		pH 6.0 + nitrate	403±67		46±2	Leu*agitation (−1.6)
		pH 6.0	929±36		17±0.5	
	Preincubation	pH 5.5 + nitrate	1245±853		97±0.5	
	with agitation	pH 5.5	577±94		5±0	
		pH 6.0 + nitrate	380±15		15±2	
		pH 6.0	1260±46		5±0.5	
3-Methylbutanol	Preincubation	pH 5.5 + nitrate	5066±542	Leu (86.6)	62±4	Leu (22.9)
	without agitation	pH 5.5	4137±695	pH (−38.1)	67±12	Agitation (21.8)
		pH 6.0 + nitrate	600±14	Leu*pH (−33.7)	117±14	Leu*agitation (13.0)
		pH 6.0	676±186		100±1	
	Preincubation	pH 5.5 + nitrate	4499±1048		501±15	
	with agitation	pH 5.5	2138±208		527±184	
		pH 6.0 + nitrate	1218±41		670±13	
		pH 6.0	1095±266		854±158	
3-Methylbutanoic acid	Preincubation	pH 5.5 + nitrate	27±13	L-leu (5.5)	10±10	Leu (4.5)
	without agitation	pH 5.5	10±0.5	Nitrate (4.4)	ND[c]	
		pH 6.0 + nitrate	ND	pH (−5.1)	63±63	
		pH 6.0	ND		35±35	
	Preincubation	pH 5.5 + nitrate	126±40		15±6	
	with agitation	pH 5.5	ND		5±5	
		pH 6.0 + nitrate	3±0.5		4±0.5	
		pH 6.0	ND		3±3	

[a]Significant effect of factors determined from normal probability plot of β-coefficients. A positive value indicates a positive effect on the formation of the volatile compound and a negative value indicates a negative effect on the formation of the volatile compound.
[b]210 ppm.
[c]Not detectable.

required for the metabolic conversion of L-phenylalanine into volatiles by *M. phenylpyruvica*. Moreover, this metabolic pathway was stimulated by agitation of *M. phenylpyruvica* during sub-culturing.

3.2. Cured meat extract

To further investigate the ability of the two bacteria to form characteristic volatile compounds in quantities that may affect the aroma in meat products in an environment more similar to meats, both strains were inoculated into sterile cured meat extracts in which the same environmental factors were varied as in the minimal media. As also observed in minimal media with added L-leucine, a characteristic odour, typical cheesy/malty, was registered in cured meat extracts inoculated with either of the two bacteria (results not shown). Cured meat extracts inoculated with *M. phenylpyruvica* had a more pronounced odour compared with samples inoculated with *S. xylosus*, while control samples without bacteria did not evolve any noticeable odour. Bacterial counts in samples inoculated with *M. phenylpyru vica* or *S. xylosus* were in the region 7.5–8.3 log CFU ml^{-1} after 7 days incubation at 20°C, independent of pre-incubation conditions. This indicates significant, but moderate, growth during the incubation period.

Fig. 4 shows representative gas chromatograms

Table 3
The effect of pH, nitrate concentration and preincubation of bacteria culture on quantities of selected volatile compounds formed by *M. phenylpyruvica* and *S. xylosus* in minimal media with added L-phenylalanine (phe) and how these factors influence the quantity of volatile compounds, as determined by PLS regression

Volatile compound		Factor combinations	*Moraxella phenylpyruvica*		*Staphylococcus xylosus*	
			Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P[a]	Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P
Benzaldehyde	Preincubated	pH 5.5 + nitrate	1874±222	Phe (67.0)	12±0	Nitrate (3.3)
	without agitation	pH 5.5	10±4	Nitrate (65.3)	ND[b]	Phe*nitrate (2.0)
		pH 6.0 + nitrate	2229±1201	Phe*nitrate (57.6)	16±13	Phe*pH (2.0)
		pH 6.0	7±7	Nitrate*agitation (20.3)	3±3	Phe (1.0)
	Preincubated	pH 5.5 + nitrate	144±2630	Phe*nitrate*agitation (19.6)	5±2	
	with agitation	pH 5.5	23±6	Agitation (18.7)	7±2	
		pH 6.0 + nitrate	535±141		3±1	
		pH 6.0	21±3		5±0.5	
Benzonitrile	Preincubated	pH 5.5 + nitrate	116±3	Nitrate (13.4)	ND	
	without agitation	pH 5.5	ND	Phe (11.1)	ND	
		pH 6.0 + nitrate	63±33	Phe*nitrate (9.4)	ND	
		pH 6.0	ND		ND	
	Preincubated	pH 5.5 + nitrate	278±106		ND	
	with agitation	pH 5.5	4±4		ND	
		pH 6.0 + nitrate	81±20		ND	
		pH 6.0	ND		ND	
2-Hydroxybenzaldehyde	Preincubated	pH 5.5 + nitrate	7±0	Agitation (4.6)	1±1	Agitation (2.9)
	without agitation	pH 5.5	ND	Phe (3.4)	ND	Phe*agitation*pH (1.5)
		pH 6.0 + nitrate	1±1	Nitrate (2.8)	ND	
		pH 6.0	ND		ND	pH (−1.1)
	Preincubated	pH 5.5 + nitrate	29±5		4±4	
	with agitation	pH 5.5	6±1		4±1	
		pH 6.0 + nitrate	16±1		5±1	
		pH 6.0	7±2		3±1	

[a] Significant effect of factors determined from normal probability plot of β-coefficients. A positive value indicates a positive effect on the formation of the volatile compound and a negative value indicates a negative effect on the formation of the volatile compound.
[b] Not detectable.

from cured meat extracts inoculated with the two bacteria. PCA analysis of gas chromatograms obtained from cured meat extracts inoculated with either *M. phenylpyruvica* or *S. xylosus* showed that the first two principal components could describe 56% of the total variance between the 60 volatile compounds detected in cured meat extracts. The scores plot separates the chromatograms into five distinctive groups (Fig. 5A). Group I contains cured meat extracts inoculated with *S. xylosus* and with added nitrate. All samples without nitrate inoculated with *S. xylosus* are found in group II. Groups III and IV contain samples inoculated with *M. phenylpyruvica* pre-incubated with and without agitation, respectively. Finally, group V contains all control samples. The loadings plot (Fig. 5B) shows the distribution of the volatile compounds responsible for the grouping observed in the scores plot. Two volatile compounds differ considerably from origo, ethanol (KI 589) and 3-methylbutanal (KI 736). The latter, 3-methylbutanal (KI 736), correlates with 2-methylbutanal (KI 741), 2-methylpropanal (KI 630) and butane-2-one (KI 687). Moreover, the volatile compounds 3-methylbutanol (KI 853) and benzacetaldehyde (KI 1185) correlate with 3-methylbutanal (KI 736), although to a lesser extent. Ethanol (KI 589) and 3-methylbutanal (KI 736) are situated perpendicularly with respect to each other, indicating that these compounds are not correlated. However, ethanol (KI 589) correlates with pentanal (KI 780), 2-octenal (KI 1176) and n-pentanol (KI 882), and to some extent also with 3-methylbutanol (KI 853), pentanal (KI 602) and an unidentified compound (KI 561).

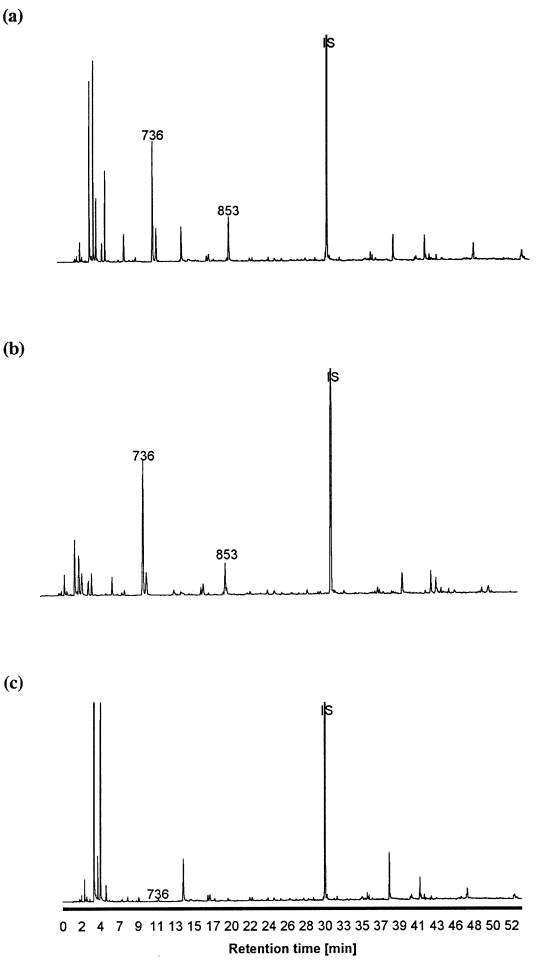

Fig. 4. Typical gas chromatograms obtained from cured meat extracts inoculated with *S. xylosus* (a), *M. phenylpyruvica* (b) or without inoculation of bacteria (control) (c). Samples analysed after 7 days incubation at 20°C (numbers assign Kovats indices; IS, internal standard).

A comparison of the scores and loadings plots reveals that cured meat extracts inoculated with *M. phenylpyruvica* pre-incubated without agitation (group III) are correlated with the presence of 3-methylbutanal (KI 736) and associated volatiles (2-methylbutanal, 2-methylpropanal, butane-2-one, 3-methylbutanol and benzacetaldehyde). Cured meat extracts inoculated with *M. phenylpyruvica* pre-incubated with agitation (group IV) also correlate with the presence of these volatile compounds, however to a much smaller extent. Group II, consisting of samples inoculated with *S. xylosus* and without nitrate, correlates with the presence of both ethanol (KI 589) and 3-methylbutanol (KI 853), whereas group I, consisting of cured meat extracts inoculated with *S. xylosus* and added nitrate, correlates mostly with the presence of ethanol (KI 589), pentanal (KI 780) and 2-octenal (KI 1176). All control samples are situated in the opposite direction to 3-methylbutanal (KI 736), suggesting a negative correlation between these samples and the presence of 3-methylbutanal (KI 736).

Characteristic volatile compounds from minimal media recognised in cured meat extracts were subsequently submitted to PLS regression. Table 4 shows the analysed concentrations of the volatiles and the effect of different factors on the formation of the respective volatile compounds by the two bacteria in cured meat extract.

3.3. Cured meat cuts

Finally, the ability of the bacterial strains to form characteristic aroma precursors/compounds in a meat product was tested. The bacterial strains were added to meat cuts, which were subsequently processed similar to cure-in-bag (CIB) bacon in a pilot plant trial. The samples were stored at 20°C to ensure optimal conditions for the added bacterial strains. The aerobic mesophilic bacterial counts and chemical data during storage are shown in Table 5. These data show that the high storage temperature caused substantial bacterial growth and consequently spoilage making the samples unacceptable for consumption. However, head space analysis for volatile compounds from the products revealed the presence of characteristic volatiles, as illustrated by the gas chromatograms in Fig. 6.

Principal component analysis showed that 76% of the total variance in the data could be described by the two first principal components. The analysis was performed using the average of three gas chromatographic analyses of volatile compounds from each cured meat cut. Samples in the scores plot could be divided into four groups as illustrated in Fig. 7A. Groups I and II consist of samples inoculated with *S. xylosus* and control samples stored for 3 and 7 days, respectively. Groups III and IV include samples inoculated with *M. phenylpyruvica* stored for 7 and 3 days, respectively. The loadings plot showed a

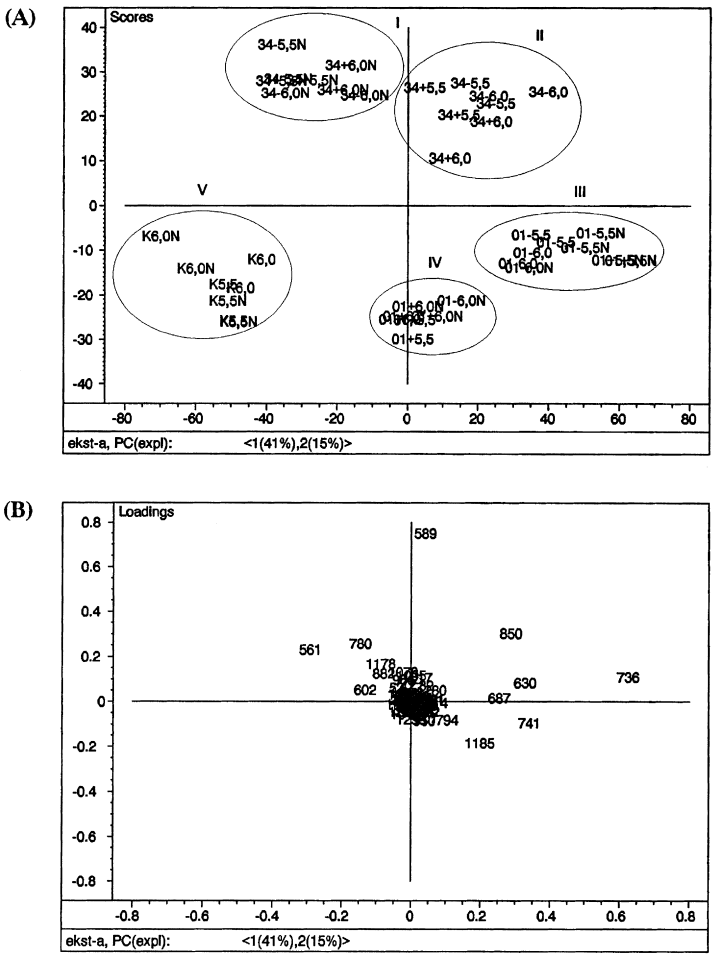

Fig. 5. (A) Scores plot after principal component analysis of gas chromatograms obtained from cured meat extracts. 01, 34 and K assign *M. phenylpyruvica, S. xylosus* and control, respectively, + /- design with or without agitation during preincubation, 5.5 or 6.0 the pH of the extract and N the presence of nitrite. (B) Loadings plot. The numbers assign Kovats indices of isolated volatiles.

number of volatile compounds situated away from origo (Fig. 7B). Of these, the most important compounds were 3-methylbutanal (KI 736), 2-methylbutanal (KI 741), dimethyl disulphide (KI 809), n-propanol (KI 672), butane-2-one (KI 685), butane-2-ol (KI 706), 3-methylbutanol (KI 853) and an unidentified compound (KI 666). Volatiles were all tentatively identified according to their Kovats' indices.

A comparison of the two plots showed a correlation between cured meat cuts inoculated with *M. phenylpyruvica* and the presence of the volatile compounds 3-methylbutanal (KI 736) and 2-methylbutanal (KI 741) after 3 days of storage. This correlation became even more pronounced after 7 days of storage. Moreover, meat cuts inoculated with *M. phenylpyruvica* or *S. xylosus* showed a weak correlation with the formation of 3-methylbutanol (KI 853) after 7 days of storage. In contrast, cured meat cuts inoculated with *S. xylosus* or control samples showed a strong correlation with the typical spoilage compound dimethyl disulphide (KI 809) and the unknown compound (KI 666), together with a weaker correlation with n-propanol (KI 672), butane-2-one (KI 685) and butane-2-ol (KI 706) after 7 days of storage.

Table 4
The effect of pH, nitrate concentration and preincubation of bacteria culture on quantities of selected volatile compounds formed by *M. phenylpyruvica* and *S. xylosus* in cured meat extract and how these factors influence the quantity of volatile compounds, as determined by PLS regression

Volatile compound		Factor combinations	*Moraxella phenylpyruvica*		*Staphylococcus xylosus*	
			Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P[a]	Quantity of volatile compound ($n = 2$) (ng dodecane equivalents)	P
3-Methylbutanal	Preincubation	pH 5.5 + nitrate[b]	227±14	Agitation (−13.9)	ND[c]	Nitrate (−23.3)
	without agitation	pH 5.5	213±5	pH (−12.5)	147±4	
		pH 6.0 + nitrate	68±44		12±18	
		pH 6.0	125±43		221±43	
	Preincubation	pH 5.5 + nitrate	136±4		ND	
	with agitation	pH 5.5	40±4		72±3	
		pH 6.0 + nitrate	24±27		4±0	
		pH 6.0	12±0		126±50	
3-Methylbutanol	Preincubation	pH 5.5 + nitrate	46±0	Agitation (−5.7)	78±6	Agitation (2.9)
	without agitation	pH 5.5	72±3	Nitrate (4.8)	49±5	Nitrate (−2.2)
		pH 6.0 + nitrate	33±11	Nitrate*agitation (1.3)	71±16	pH (−2.1)
		pH 6.0	56±0		48±2	
	Preincubation	pH 5.5 + nitrate	54±2		76±8	
	with agitation	pH 5.5	17±0		93±3	
		pH 6.0 + nitrate	22±18		67±0	
		pH 6.0	12±0		61±8	
3-Methylbutanoic acid	Preincubation	pH 5.5 + nitrate	12±18	No factor with	11±3	Nitrate*pH (−3.5)
	without agitation	pH 5.5	3±4	significant effect	5±1	Nitrate (1.9)
		pH 6.0 + nitrate	ND		4±6	
		pH 6.0	2±3		4±1	
	Preincubation	pH 5.5 + nitrate	6±1		7±6	
	with agitation	pH 5.5	ND		4±1	
		pH 6.0 + nitrate	ND		4±5	
		pH 6.0	ND		2±2	

[a]Significant effect of factors determined from normal probability plot of β-coefficients. A positive value indicates a positive effect on the formation of the volatile compound and a negative value indicates a negative effect on the formation of the volatile compound.
[b]210 ppm.
[c]Not detectable.

Table 5
Bacterial counts and chemical data for curing brines and vacuumed-packed cured meat cuts stored at 20°C

Bacon sample	Brine	Cured meat cuts ($n = 2$)					
		Day 3			Day 7		
	Bacterial counts (log CFU ml^{-1})	Bacterial counts (log CFU g^{-1})	Nitrite (ppm)	NaCl % (w/w)	Bacterial counts (log CFU g^{-1})	Nitrite (ppm)	NaCl % (w/w)
Inoculated with *M. phenylpyruvica*	7.8	7.2	23±5	2.4±0.3	8.1	18±3	2.6±0.3
Inoculated with *Staph. xylosus*	8.7	7.4	25±5	2.5±0.3	8.3	< 10	2.5±0.3
Control	2.8	7.3	20±2	2.3±0.3	8.2	< 10/14[a]	2.3±0.1

[a]One bacon sample contained < 10 ppm, whereas the other control bacon sample contained 14 ppm.

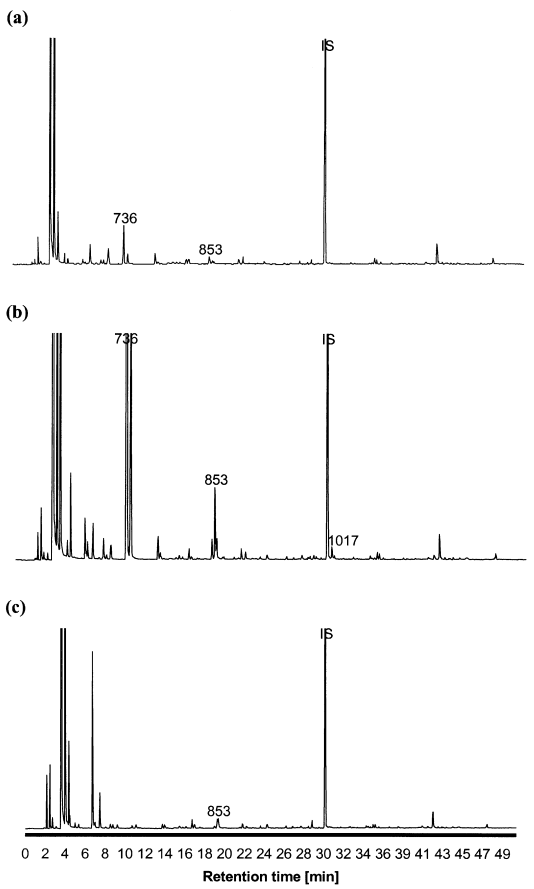

Fig. 6. Typical gas chromatograms obtained from cured meat cuts inoculated with *S. xylosus* (a), *M. phenylpyruvica* (b) or without inoculation of bacteria (control) (c). Samples analysed after 7 days incubation at 20°C (numbers assign Kovats indices; IS, internal standard).

4. Discussion

A basic understanding of the conversion of amino acids and other flavour precursors into flavour compounds via metabolic processes in starter cultures will facilitate the choice of specific starters in the production of foods where the presence of specific flavour compounds are desirable.

Amino acids are reported to be essential substrates for the microbial formation of characteristic flavour precursors/compounds in meats (Hammes and Knauf, 1994; Dainty and Blom, 1995). However, little data is available regarding which amino acids are involved and how they enter the metabolism of starters in fermented foods and contribute to overall flavour development. The present study has attempted to elucidate whether *Moraxella phenylpyruvica* and *Staphylococcus xylosus* can metabolize L-leucine and L-phenylalanine into potential flavour compounds in cured meats.

The three characteristic volatile compounds formed from L-leucine in minimal media are known to posses pungent and unpleasant odours in high concentrations, whereas in low concentrations they are described as apple-like and fruity (Burdock, 1994). The registered odours of the minimal media with added L-leucine and inoculated with either *M. phenylpyruvica* or *S. xylosus* fall within these aroma descriptions, as they varied from pronouncedly cheesy, nearly unpleasant, when high concentrations of 3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid were present, to more desirable lightly cheesy/malty odours in samples where only low concentrations of 3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid were present. Both 3-methylbutanal and 3-methylbutanol have been isolated from cured meat products, e.g. Wiltshire bacon (Andersen and Hinrichsen, 1995), dried sausages (Stahnke, 1994), Parma ham (Hinrichsen and Pedersen, 1995) and Spanish Serrano ham (Flores et al., 1997). It is therefore likely that these volatile compounds contribute to the desirable flavour in meat products. Microbial deamination and decarboxylation of amino acids may result in methyl branched aldehydes (Hemme et al., 1982), which can subsequently be reduced to the corresponding alcohols by microbial alcohol dehydrogenases resulting in a mixture of aldehydes and alcohols (Berlitz and Grosch, 1987). The present data show that *M. phenylpyruvica* produced higher amounts of these characteristic volatile compounds than *S. xylosus* in minimal media with added L-leucine. Furthermore, the difference in the alcohol/aldehyde ratio between the two bacterial cultures indicates considerable differences in alcohol dehydrogenase activity with *S. xylosus* giving rise to an approximately 10-fold higher alcohol/aldehyde ratio than *M. phenylpyruvica*. The alcohol/aldehyde ratio of methyl-branched volatile compounds has been found to be significant for aroma development in fermented milk products, where a high alcohol/aldehyde ratio results in an inferior aroma (Sheldon et al., 1971). It may be speculated that the same phenomenon is valid for aroma formation in fermented meat prod-

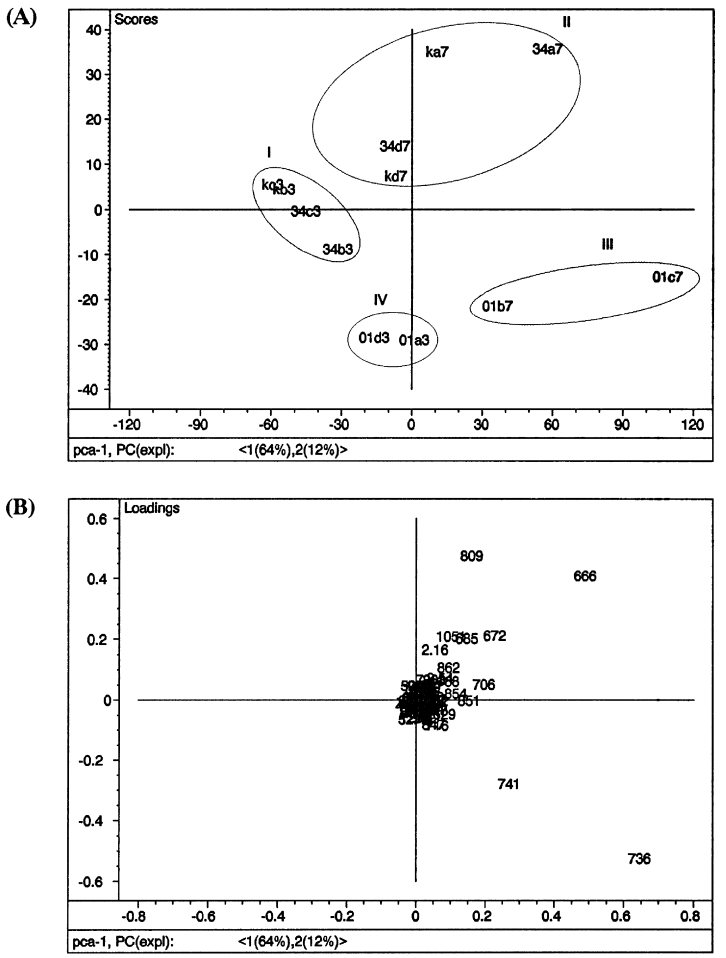

Fig. 7. (A) Scores plot after principal component analysis of gas chromatograms obtained from cured meat cuts. 01, 34 and k assign *M. phenylpyruvica, S. xylosus* and control, respectively, a, b, c and d the sample and 3 and 7 assign days of storage before analysis was performed. (B) Loadings plot. The numbers assign Kovats indices of isolated volatiles.

ucts. The much lower average aroma threshold of aldehydes (3-methylbutanal: 0.06 $\mu g\ g^{-1}$ in water) than the corresponding alcohols (3-methylbutanol: 3.20 $\mu g\ g^{-1}$ in water) (Margalith, 1981) should be mentioned.

Benzaldehyde and benzonitrile both possess the characteristic odour of almond, while 4-hydroxybenzaldehyde possesses an 'agreeable' odour and 2-hydroxybenzaldehyde possess an almond-like odour (Burdock, 1994; Merck Index, 1989). This corresponds to the characteristic pleasant-sweet almond odour of minimal media with added L-phenylalanine and nitrate and inoculated with *M. phenylpyruvica* which was registered. Benzaldehyde has been identified in Wiltshire bacon (Andersen and Hinrichsen, 1995), and benzonitrile has been found in cured pork (Mottram, 1984). Mottram (1984) isolated a number of organic nitrile compounds in bacon including benzonitrile and phenylacetonitrile, which were both found to increase in concentration with increasing addition of nitrite to the product. Similar reactions may account for the formation of benzonitrile in minimal media with added L-phenylalanine and nitrate in the present study, indicating

that the presence of benzonitrile is a combination of metabolic transformation of L-phenylalanine and the subsequent chemical reaction with nitrate/nitrite rather than a direct metabolic product of L-phenylalanine.

Only *M. phenylpyruvica* was able to convert the amino acid L-phenylalanine into these characteristic volatile compounds [benzaldehyde (KI 1091), benzonitrile (KI 1141) and 2-hydroxybenzaldehyde (KI 1178)] in minimal media, whereas *S. xylosus* did not use this metabolic pathway. Studies of the microbial enzymes involved in the metabolism of aromatic amino acids demonstrated variable activity of aminotransferases with an affinity for aromatic amino acids in strains of coryneforme bacteria (Lee et al., 1985; Fazel and Jensen, 1979). The present results show that *S. xylosus* did not possess noticeable enzymatic activity involved in the metabolism of aromatic amino acids under the given conditions, while *M. phenylpyruvica* exhibited high enzymatic activity regarding conversion of L-phenylalanine. This is in agreement with the data of Bøvre (1985), who states that this organism is L-phenylalanine deaminase positive. Both principal component analysis and PLS regression implied that nitrate played a vital role in the degradation of L-phenylalanine by *M. phenylpyruvica*. The large quantities of benzaldehyde in samples inoculated with *M. phenylpyruvica* indicate that this bacteria does not carry the enzymes involved in the further degradation of the aromatic ring structure. The formation of 2-hydroxybenzaldehyde may be part of a detoxification process by the bacteria, where benzaldehyde is hydroxylated in order to increase the solubility, and thereby facilitate excretion from the cell (Stryer, 1988).

From Table 2 and Table 3 it is evident that various factors have different effects for each bacteria on the amount of characteristic volatile compounds produced and only *M. phenylpyruvica* was able to metabolize L-phenylalanine. It is therefore obvious that the two bacteria used different pathways in the metabolic conversion of these amino acids under the given conditions.

The fact that inoculation of cured meat extracts with *M. phenylpyruvica* and *S. xylosus* gave rise to cheesy and malty odours and the formation of characteristic volatiles found to be formed from L-leucine in minimal media inoculated with these bacterial strains, strongly suggests that these organisms are able to form potential aroma precursors and compounds from L-leucine in a cured meat environment.

In contrast to inoculation of minimal media, inoculation of cured meat extracts with *M. phenylpyruvica* was influenced by the pre-incubation conditions, with agitation during pre-incubation resulting in lower 3-methylbutanal concentrations. A possible explanation might be a change in cell morphology of the culture during pre-incubation with agitation which was observed in this part of the study. Cells changed from small coccoid rods (normal morphology) to elongated filament shapes. Similar observations have been made for various other bacteria genera, e.g. *Vibrio* sp. (Östling et al., 1993) and *Halomonas* sp. (Vreeland, 1980). Morphological changes could be caused by limitation of the supply of nutrients and subsequent starvation, thus triggering alternative metabolic pathways leading to changes in cell structure. If normal cells and cells with changed morphology differ in metabolic pathways, it would explain the observed difference in the amount of 3-methylbutanal from cells of *M. phenylpyruvica* pre-incubated with and without agitation.

In accordance with observations made in minimal media containing L-phenylalanine, nitrate also had a positive effect on benzaldehyde formation in cured meat extract inoculated with *M. phenylpyruvica*. However, this compound was only formed in very low concentrations (data not shown). Instead, benzacetaldehyde was found to be a characteristic compound present in noticeable concentrations when cured meat extracts were inoculated with *M. phenylpyruvica*. This compound may originate from metabolic conversion of L-phenylalanine as described for *Lactococcus lactis* (Yvon et al., 1997) with subsequent reduction of the formed phenylacetate to benzacetaldehyde, and indicates an alternative catabolic pathway of L-phenylalanine by *M. phenylpyruvica* than that observed in the minimal media.

The present data show that *M. phenylpyruvica* most probably deaminates and decarboxylates the methyl-branched amino acids, L-leucine, iso-L-leucine and valine, into the methyl-branched aldehydes, 3-methylbutanal, 2-methylbutanal and 2-

methylpropanal, in cured meat extract. In contrast, *S. xylosus* forms more common fermentation products, e.g. ethanol, under similar conditions. The ability of *M. phenylpyruvica* to form methyl-branched aldehydes may prove beneficial in relation to aroma development in meat products, as several studies have shown that these volatile compounds can participate in the formation of a desirable aroma in high quality meat products (Barberi et al., 1992; Careri et al., 1993; Hinrichsen and Pedersen, 1995).

Despite the undefined microbial growth and unacceptable sensory quality of the cured meat cuts, the analysis of volatile compounds indicated that *M. phenylpyruvica* influenced the amount of characteristic volatile compounds 3-methylbutanal and 2-methylbutanal relevant to aroma development in meat products. Cured meat cuts injected with *S. xylosus* did not differ from control samples, indicating that *S. xylosus* cannot be expected to contribute to aroma formation in vacuum-packed bacon. The ability of *M. phenylpyruvica* to affect the aroma in a product similar to CIB bacon is exceptional, as only few starter cultures are capable of sustaining normal metabolism when injected into whole muscles with simultaneous limitation of oxygen availability as a result of the packaging procedure (vacuum). In fact, this is, to our knowledge, the only organism which has been reported to have a substantial beneficial impact on aroma formation in vacuum-packed whole muscle meat products.

A process for the production of whole muscle meat products, which incorporates spraying and injecting a brine containing starter culture, has been described (Karmas, 1976). In the early stages of this process, different temperature/time combinations are included, e.g. short storage at elevated temperature followed by storage at normal chilling temperatures for a longer period of time. It is likely that a similar procedure applied to CIB bacon could prevent the natural flora from proliferating, and simultaneously allowing *M. phenylpyruvica* to improve the aroma of the final product.

In conclusion, the present study has shown that *M. phenylpyruvica* is able to metabolize the amino acids L-leucine and L-phenylalanine into characteristic aroma precursors and compounds both in minimal media and in more complex cured meat systems. In comparison, the capacity of *S. xylosus* is significantly less.

Acknowledgements

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