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The influence of *Debaryomyces hansenii* and *Candida utilis* on the aroma formation in garlic spiced fermented sausages and model minces

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Abstract

The influence of the yeast starter cultures *Debaryomyces hansenii* and *Candida utilis* on fermented meat aroma was studied in model minces and in commercial-type fermented sausages. Volatile compounds from model minces and sausages were collected using diffusive and dynamic headspace sampling respectively and were identified by gas chromatography/mass spectrometry (GC/MS). A triangle test was carried out on the sausages to detect whether the yeast influenced the sausage odour. *C. utilis* demonstrated high metabolic activity in the model minces, producing several volatile compounds, in particularly esters. *C. utilis* also seemed to ferment the amino acids valine, isoleucine and leucine into compounds important for the aroma of sausages. *D. hansenii* on the contrary, had very little effect on the production of volatile compounds in the model minces. In the sausage experiment both yeast cultures died out before the ripening process ended and the sensory analysis showed only a slight difference between the sausages. A fungistatic test of the garlic powder added to the sausages indicated that garlic inhibits the growth of the yeast starter cultures.

Keywords: Fermented sausages; Starter cultures; Yeast; *Candida utilis*; *Debaryomyces hansenii*; Aroma; Garlic

1. Introduction

Yeast's role in fermented sausages is not a well-researched area. Nevertheless *Debaryomyces hansenii* is presently sold as a commercial starter culture for sausage manufacture. Yeast is naturally found on the hides of animals, from where it is easily spread to the fresh meat during slaughtering (Dillon & Board, 1991). Meat is a good nutritional growth media. The high salt concentration (low water activity) combined with the acidic environment in a typical fermented sausage favours the growth of certain yeast species since competing bacteria are repressed (Beuchat, 1987). Yeast has, therefore, often been detected in these products. According to Leistner and Bem (1970), Metaxapoulus, Stavropoulus, Kakouri and Samelis (1996) and Encinas, López-Diaz, García-López, Otero and Moreno (2000) the halotolerant *D. hansenii* is the dominant yeast species, but a considerable number of other species can also be found. Yeast has been detected in concentrations up to 10^5 cfu/g in the finished products (Encinas et al., 2000; Samelis, Aggelis & Metaxapoulus, 1993). In this context it should be remembered that the biomass of a yeast cell is approximately 100 times greater than the biomass of a bacterial cell (Dillon & Board, 1991).

According to Geisen, Lücke and Kröckel (1992) yeasts' requirement for oxygen restricts them to mainly growing near the surface of fermented sausages. Fermentative yeast species however, can thrive under low oxygen tensions since they only require oxygen for production of cell wall constituents such as sterols and fatty acids (Deak & Beuchat, 1996).

It is claimed that yeast stabilises the red colour of fermented sausages and improves the aroma, adding a yeast like aroma to the sausage. This is for example the case for certain Italian sausages (Coretti, 1973, 1977; Leistner & Bem, 1970). It is believed that yeast delays rancidity and protects the red nitroso-myoglobin from breakdown by degrading peroxides and consuming oxygen thus stabilising the appealing red colour of fermented sausages (Lücke & Hechelmann, 1987).

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However, unlike staphylococci and *Candida utilis*, *D. hansenii* does not reduce nitrate to the colour generating nitrite (Barnett, Payne & Yarrow, 1990).

Both *D. hansenii* and *C. utilis* have been shown to initially proliferate in sausages and then slowly decline. However, after 30 days of ripening viable yeasts can still be found (Gadjeva, Miteva, Nestorou & Radeva, 1983; Gehlen, Meisel, Fischer & Hammes, 1991).

All previously examined yeast species are capable of forming alcohols such as 2-methylpropanol, 2- and 3-methylbutanol and 2-phenylethanol (Stam, Hoogland & Laane, 1998). The reduction of carbonyls into alcohols is favoured by some yeasts (Peppard & Halsey, 1981) and many yeast species can produce volatile esters (Stam et al., 1998; Westall, 1997). However, it has never been directly examined how yeast influences the volatile profile of fermented sausages, though the sensory properties of sausages with added yeast starter cultures have been evaluated by Gadjeva et al. (1983) and Miteva, Kirova, Gadjeva and Radeva (1986). They found that addition of *C. utilis* starter culture improved the aroma and taste of the final product, smoked as well as unsmoked. In addition it was shown by Miteva et al., 1986 that *C. utilis* hydrolysed lipids in fermented sausages. According to Gehlen et al. (1991) the addition of *D. hansenii* to fermented sausages results in increased ammonia concentration and a lower content of lactate and acetate, thus raising pH in the sausage. But *D. hansenii* might inhibit the growth of staphylococci, which is not desirable.

Westall (1997) examined the aroma production of *D. hansenii* on yeast malt agar. The total number and concentration of volatile compounds were somewhat low compared to many other yeast species. However, compounds such as 2-methylpropanol, 3-methylbutanol and acetic acid were produced. *D. hansenii* is lipolytic, but the lipolytic activity is inhibited by low pH and low temperatures (Sørensen, 1997).

The aim of this study was to investigate the aroma formation of *D. hansenii* and *C. utilis* when grown in fermented sausage model minces and real fermented sausages.

2. Materials and methods

2.1. Experimental design

For both sausages and model minces three different batches were produced. A reference batch and two batches of similar composition to the reference, but with addition of either *D. hansenii* or *C. utilis* starter cultures. Both minces and sausages were modelled to simulate an average Danish fermented sausage. However, it should be stressed that the model minces were not real sausages, mainly due to differences in fermentation/ripening conditions. Therefore, no direct comparison can be made between the two. The purpose of the model minces was to examine the aroma generating potential of the yeast starter cultures in a controlled fermented meat media.

2.2. Preparation of model minces

The preparation of the meat used for the model minces was carried out as described by Stahnke (1999) with the only exception that the pork back was frozen at −40°C. After mincing of the meat and fat all other ingredients were added except for the yeast starter culture. The mixture was minced again to ensure a thorough mixing, split into three batches and to two of them yeast was added. All batches were divided into three 200 g portions and put into Erlenmeyer flasks mounted with two horizontal glass tubes closed with caps (Swagelok®, USA). All flasks were closed with Teflon capped glass stoppers and incubated for 7 days at 25°C (see Section 2.4).

The final composition of the model minces was as follows: lean pork 62% ($^{w}/_{w}$), pork back fat 31% ($^{w}/_{w}$), added water 3% ($^{w}/_{w}$), NaCl 3% ($^{w}/_{w}$), glucose 0.75% ($^{w}/_{w}$), Californian garlic powder (SFK, Denmark) 0.18% ($^{w}/_{w}$), sodium ascorbate 500 ppm, $NaNO_2$ 100 ppm, *Pediococcus pentosaceus* (floracarn SPX, Chr. Hansen, Denmark) $\approx 2\times10^7$ cfu/*g*, *Staphylococcus xylosus* (floracarn SPX, Chr. Hansen, Denmark) $\approx 2\times10^7$ cfu/g. For the batches added yeast starter culture, *D. hansenii* (LAF 3, Chr. Hansen, Denmark) $\approx 1\times10^6$ cfu/g or *C. utilis* (CUM-10D, Texel, France) $\approx 1\times10^6$ cfu/g were added.

2.3. Sausage manufacturing

All ingredients were added to a rotating meat chopper, starting with the partly frozen meat and lard. The homogenised chopped meat was stuffed into 60 mm fibre casings (Fuji fibre casings, SFK, Denmark).

The composition of the fresh sausages was as follows: pork meat (15–20% fat) 65% ($^{w}/_{w}$), pork back fat (80–90% fat) 31.5% ($^{w}/_{w}$). NaCl 2.7% ($^{w}/_{w}$), dextrose monohydrate 0.5% ($^{w}/_{w}$), white pepper 0.20% ($^{w}/_{w}$), Californian garlic powder (SFK, Denmark) 0.18% ($^{w}/_{w}$), sodium ascorbate 500 ppm, $NaNO_2$ 100 ppm, *Pediococcus pentosaceus* (floracarn SPX, Chr. Hansen, Denmark) $\approx 5\times10^6$ cfu/*g*, *Staphylococcus xylosus* (floracarn SPX, Chr. Hansen, Denmark) $\approx 5\times10^6$ cfu/g. For the batches with yeast starter culture *D. hansenii* (LAF 3, Chr. Hansen, Denmark) $\approx 1\times10^5$ cfu/g or *C. utilis* (CUM-10D, Texel, France) $\approx 1\times10^5$ cfu/g were added. The sausages were fermented and dried for a total of 21 days in a humidity regulated chamber (Multimat® MC1000, Deutsch, Germany). The drying and temperature programmes were: 0–11 days: 95–75% R.H., 12–21 days: 75% R.H., 0–8 days: 24–17°C, 9–21 days:

17°C. The sausages were smoked (beech wood) for 1/2 an hour on the second day and for 1 h on the sixth day. After 21 days the sausages were wrapped in alu-foil, vacuum packed and stored at 5°C.

2.4. Collection of volatile compounds

Model minces: after the first 7 days of incubation, volatile compounds were collected by passive diffusion over a period of an additional 7 days. During this period the flasks were stored at 17°C. Collection of the volatile compounds were done by fixing two Tenax TA® tubes (200 mg, 60/80 mesh, Chrompack, Holland) onto each Erlenmeyer flasks with Swagelok® unions/Teflon ferrules.

Sausages: volatile compounds from the finished sausages were collected using dynamic headspace sampling. A hundred and ten grams of sausage were cut into smaller pieces, cooled with liquid nitrogen and pulverised in a domestic food processor. Thirty grams of sausage powder was weighed into a 125 ml wash bottle (in triplicate) and the samples equilibrated for 1 h in a water-bath at 25°C. The samples were purged with N_2 (99.999%, 90 ml/min) for 30 min in a heating cabinet at 30°C and the extracted volatiles trapped onto Tenax TA®.

Tenax TA® tubes were cleaned prior to sampling by purging with helium (99.9995%, flow rate 75 ml/min) for 15 min at 340°C. All glassware used in the aroma collection were thoroughly cleaned with water and 96% ($^{v}/_{v}$) ethanol and conditioned at 140°C for at least 24 h. Blanks were made by fixing tubes to empty flasks.

2.5. Analysis of volatile compounds

The Tenax TA® tubes were thermally desorbed at 200°C (75 ml/min, 3 min) and cryofocused onto 20 mg of Tenax TA® at −30°C (ATD 400, Perkin-Elmer Ltd, UK). The cold trap was desorbed at 250°C, transferring (line temperature 200°C) the volatile compounds into a GC-column (DB-1701, 1 μm, 30 m, 0.25 mm, J&W Sci., USA) installed in a GC–MS (HP 5890 Series II — 5972, USA). The GC-programme was: 35°C for 1 min, 4°C/min to 175°C, 10°C/min to 260°C, 5 min. MS-parameters were: Ionisation energy 70 eV, scanning frequency 2.80 scans/s, scan range 33–300 AMU. Identification was based on mass spectrum compared to the NBS75k-database (HP) and retention indices of authentic compounds. Quantification was based on either the total or single ion chromatogram on an arbitrary scale (eV).

2.6. Data treatment

Three Tenax tubes with 5.0 μl 0.01% *n*-octane standard were included in each GC–MS run. All peak areas were divided by the averaged area of the octane standards and multiplied by 1×10^3. Analysis of variance was used to verify any effects of yeast addition and Duncan's multiple range test was used to detect differences between means (Montgomery, 1997). The relationship between the volatiles and yeast addition was investigated by discriminating partial least squares regression (D-PLSR), using Unscrambler (Version 6.11, CAMO A/S, Trondheim, Norway). The Y-matrix consisted of the three batches, and the X-matrix of the logarithmic_{10} peak areas.

2.7. Sensory evaluation

Odour differences between the finished sausages were evaluated by triangle tests, split in two sessions. The tests were carried out in a sensorial test room, under red illumination so visual differences could not be spotted. For each sample two sausages slices were put in a closed plastic beaker and equilibrated 1 h at 25°C before serving. An untrained panel of 12 judges was used. The first six judges tested the three sausage combinations against each other, but in a different serving and sniffing order. All combinations were used. The design was duplicated in reverse order for the last six judges. In addition to the triangle test the judges were asked to describe the aroma of an unknown sample of each sausage.

2.8. Water activity

Water activity (Novasina aw-CENTER, Switzerland) of the model minces was measured on the fermented minces (14 days) and on day 0, 2, 6, 13 and 20 for the sausages (Novasina TH1 TH2, Switzerland).

2.9. Microbiological analysis

For the model minces the microbial count was determined at day 0 and 14 (the start/end of the incubation period), for the sausages at day 0, 1, 2, 3, 6, 13 and 20 days in the production period. The yeast concentration was determined by plate counting onto YM (yeast-malt) agar (added 5 ppm chloramphenicol and chlortetracycline) for model minces and YGC (yeast-glucose-chloramphenicol) agar for sausages. The amount of staphylococci and lactic acid bacteria was determined on MSA (manitol salt agar, Difco, Germany), and MRS-agar (de Man, Rogosa, Sharpe, Oxoid, UK) respectively. Incubation conditions were: YM: 3 days at 25°C, MSA: 3 days at 30°C and MRS: 5 days anaerobic incubation at 25°C.

2.10. Fungistatic test of garlic powder

This test was constructed specifically to test the garlic powder's ability to inhibit yeast growth. The growth of

Table 1
Concentration of yeast, staphylococci and lactic acid bacteria in model minces

	Yeast (cfu/g)		Staphylococci (cfu/g)		L.A. bacteria (cfu/g)	
Batch	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
Reference	<50	<50	2.2×10^7	6.1×10^5	2.7×10^7	1.9×10^8
+ *D. hansenii*	4.7×10^5	6.8×10^3	2.2×10^7	$<5\times10^3$	2.7×10^7	9.9×10^7
+ *C. utilis*	1.2×10^6	6.6×10^6	2.2×10^7	1.2×10^5	2.7×10^7	1.4×10^8

the two yeast cultures was examined at concentrations of 0.00, 0.20 and 0.40% ($^{w}/_{w}$) of Californian garlic powder (SFK, Denmark) at three concentrations of NaCl (1, 5 and 9%). pH in all media was adjusted to 4.9 with 0.1 M HCl. Each combination was repeated six times so $3\times3\times6=54$ measurements were made for each yeast species; 0.2% garlic powder corresponds approx. to the concentration used in the sausages and minces, while the 5% salt conc. approx. corresponds to the amount of salt in the water phase of the fresh sausages. Garlic powder was poured into sterile test tubes, 100 μl 70% ($^{v}/_{v}$) ethanol was added to extract potentially hydrophobic fungistatic compounds. Tubes were equilibrated for $1\frac{1}{2}$ h. Then 9.9 ml YM-media (added 5 ppm chloramphenicol and chlortetracycline) with the desired salt concentration was added.

Each tube (except blank controls) was inoculated with 10 μl of a 10^{-1} dilution of yeast in YM-media (yeast propagated in YM-media at 25°C for 2 days), giving an initial concentration of yeast in the media of 6×10^2 cfu/g for *D. hansenii*, and 8×10^3 cfu/g for *C. utilis*. After mixing, the tubes were left to stand for 1 h so the insoluble garlic powder could sediment. Hereafter, 250 μl of the supernatant was pipetted into each well in sterile microtitter plates (NUNCLEON™ Δ Surface, NUNC™, Denmark). The controls were inoculated on a separate plate. The finished plate was covered with sterile foil, and placed in a box filled with wet paper to prevent the wells from drying out.

The plates were incubated at 25°C. The optical density (620 nm) in the wells was measured at day 1, 2, 3, 4, 8, 16, 25 and 35 in a microtitter reader (Anthos reader 2001, Anthos Labtec Instruments, Austria). Growth in a well was defined by the optical density (OD_{well}−average OD_{blind}) exceeding 0.10 (visual growth).

3. Results and discussion

3.1. Microbiological analysis

During the incubation period two model mince flasks had to be discarded (one reference due to growth of fungi, and one with added *C. utilis* due to lack of yeast growth), reducing the total number of model minces to seven. The pH in the media was unaffected by the yeast since the minces soured evenly in all three batches. Acidity increased from pH 5.5 in the fresh minces to pH 5.0–4.7 at the end of the experiment, depending on whether the pH was measured in the centre or top of the minces.

The concentration of *C. utilis* in the model minces increased slightly during the incubation, while the concentration of *D. hansenii* dropped by more than a factor of 50 (Table 1). This drop was somewhat surprising since *D. hansenii* in a previous experiment had shown excellent growth ($>10^7$ cfu/g) in model minces (data not shown). Those minces, however, had a considerably higher water content (70% as compared to 50%), half the addition of bacterial starter culture, and no addition of garlic powder. In the sausages this drop was even more pronounced. The concentration of yeast declined within 3 days (Fig. 1). Both *D. hansenii* and *C. utilis* disappeared within 6 and 20 days respectively. As previously described both *D. hansenii* and *C. utilis* have been shown to initially proliferate and survive 30 days in sausages (Gadjeva et al., 1983; Gehlen et al., 1991). The disappearance of both yeast species from the sausages and the poor survival of *D. hansenii* in the model minces clearly pointed out, that at least one factor in the minces and sausages was detrimental to the yeasts' survival.

C. utilis is not very tolerant to low water activities, with an a_w minima for growth around 0.94 (Beuchat, 1987). Since a_w of the fermented minces was 0.94 and a_w of the sausages (Fig. 2) dropped below 0.90 during the ripening of the sausages, this could explain the difficulties for *C. utilis* to proliferate. It is, however, noteworthy (Figs. 1 and 2) that the concentration of *C. utilis* clearly had declined after 6 days, even though the a_w was still above 0.95.

On the other hand, *D. hansenii* is very tolerant to low water activities with an a_w minima for growth of 0.84 in saline solutions (Deak & Beuchat, 1996). According to Conner and Beuchat (1984) essential oils of garlic are a potent inhibitor of yeast growth, especially for *D. hansenii*. In addition, the dried garlic powder used in this study is a rather concentrated product compared to fresh garlic. In other studies where *D. hansenii* and *C. utilis* starter cultures have been reported to proliferate there was no report of garlic addition to the sausages (Gadjeva et al., 1983; Gehlen et al., 1991; Miteva et al., 1986). However, Encinas et al. (2000) report that Spanish garlic spiced "Chorizo" sausages, contain high

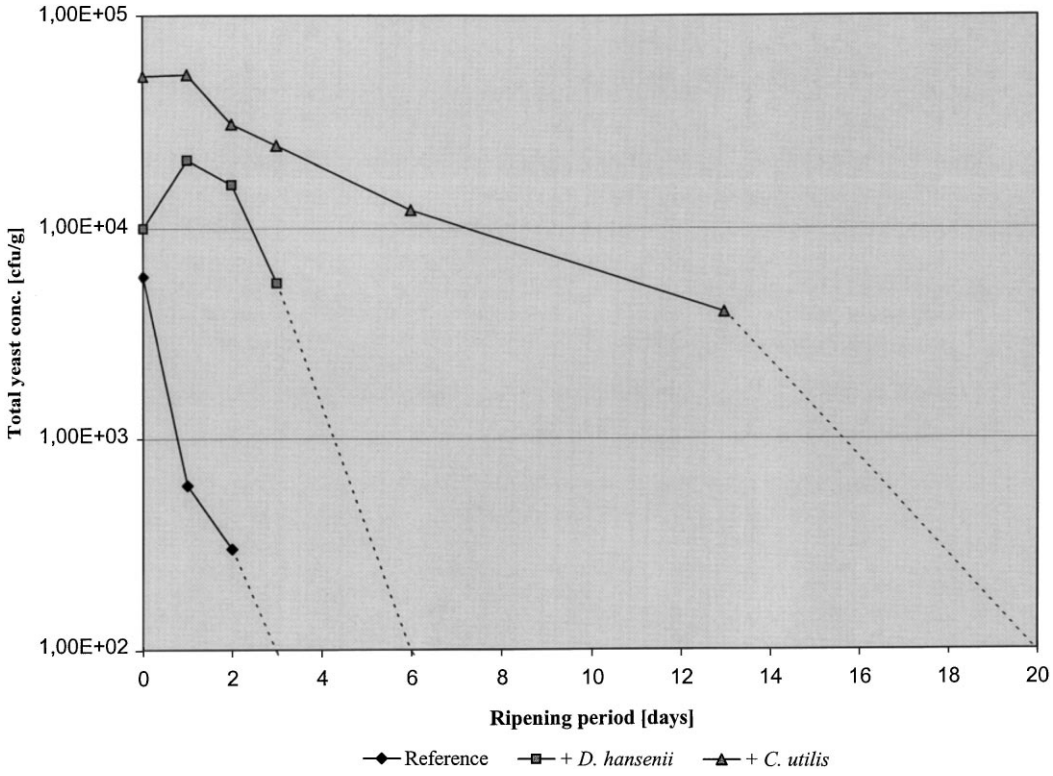

Fig. 1. Changes in yeast concentration during the sausages ripening. Dotted line indicates final measurement below detection limit (100 cfu/g).

concentrations of yeast (up to 10^5 cfu/g) in the finished products.

The number of lactic acid bacteria and staphylococci in the minces (Table 1) were within the range of what could be expected in fermented sausages depending on the growth conditions (Lücke, 1998), but the staphylococci seemed to be inhibited in the minces with added *D. hansenii*. This strengthens the results of Gehlen et al. (1991), that *D. hansenii* may inhibit staphylococci. One should notice that the inhibition of staphylococci might affect the aroma profile of the minces with added *D. hansenii*. In the sausage experiment the concentration of staphylococci was completely unaffected by the yeast addition (data not shown).

3.2. Analysis of volatile compounds from model minces

A total of 44 compounds were identified in the model minces (Table 2). Various esters (15) were the most numerous, but also sulphur compounds (11), alcohols (6), ketones (4), carboxylic acids (4), aldehydes (3) and a single nitrile were found in the sausages. Most of these compounds have previously been identified in sausages of various kinds (Berdagué, Monteil, Montel & Talon, 1993; Mateo & Zumalacárregui, 1996; Schmidt & Berger 1998; Stahnke, 1995a).

Among the non-sulphur volatiles there were only few major differences between the reference minces and the minces with added *D. hansenii*. However, the concentration of ethyl-3-methyl butanoate (29) was significantly higher in the reference minces than in the minces with *D. hansenii*. The same is perhaps true for 2- and 3-methylbutanoic acid (37 and 36) and ethyl-2-methyl butanoate (28), but the differences were not significant (Table 2 and Fig. 3). These differences might be caused by the low concentration of staphylococci in the minces with *D. hansenii*, since *S. xylosus* has been reported to be capable of producing those two esters and 3-methylbutanoic acid (Vergnais, Masson, Montel, Berdagué & Talon, 1998).

The difference in concentration between the primarily garlic-derived sulphur compounds (compound nos. 1, 8, 16, 20, 30, 35, 38, 40, 41, 42 and 44) is noteworthy when all the compounds are compared as a whole (Fig. 3). There is a tendency for the high molecular weight sulphur compounds to be associated with the minces with *D. hansenii*, while the low molecular weight compounds seem associated with the reference minces. However, the differences are not significant. All taken together, *D. hansenii's* production of volatile compounds in the minces seems thus highly limited. Nevertheless, in minces were *D. hansenii* proliferates it could perhaps make a different impact on the aroma.

The concentration of ethyl acetate (9) was almost 50 times higher than in the reference minces. In addition *C. utilis* produced seven esters, which were not even produced in the other minces (Table 2). One of these esters was 2-phenylethyl acetate (43) that is most likely derived

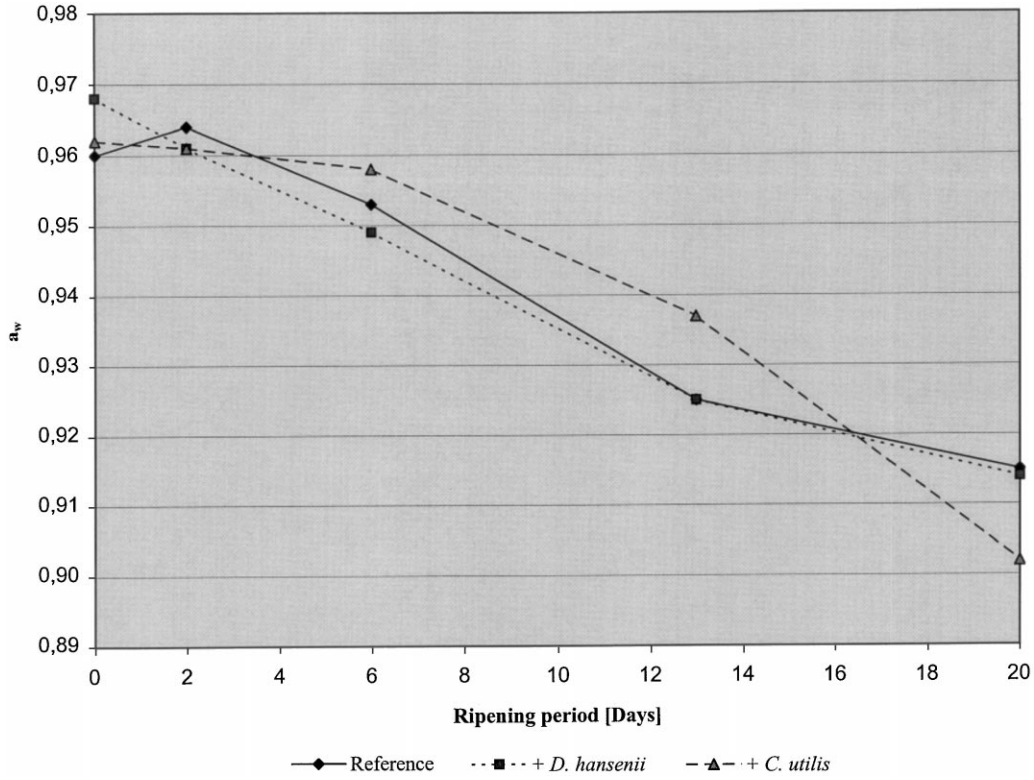

Fig. 2. Changes in water activity during the sausages ripening.

from 2-phenylethanol. This would indicate breakdown of phenylalanine, since yeast is known to convert this amino acid into 2-phenylethanol (Stam et al., 1998). Ethyl esters have strong fruity odours (Fenaroli, 1995; Stahnke, 1994) so a high concentration of fruity smelling esters is not desirable in sausages. However, neither Gadjeva et al. (1983) or Miteva et al. (1986) reported fruity odour notes in sausages with added *C. utilis* starter culture. Nevertheless, in lower concentrations esters may have a positive influence on sausage aroma (Stahnke, 1994, 1995b).

Significant higher concentrations of 2-methyl-1-propanol (14), 2-methylbutanal (15), 2-methyl-1-butanol (25) were found in minces with added *C. utilis* (Table 2). The concentration of 2- and 3-methylbutanoic acid (37 and 36), 2-methylpropanoic acid (32), 2-methylpropanal (7), 3-methyl-1-butanol (24) appeared to be higher (Table 2 and Fig. 3), but the differences were not significant. In addition, higher concentrations of ethyl-2-methyl butanoate (28), ethyl-3-methyl butanoate (29), ethyl-2-methyl propanoate (21), 2-methylbutyl acetate (34), 3-methylbutyl acetate (33) (difference not significant), 2-methylpropyl acetate (23), could be found in the minces with added *C. utilis* (Table 2 and Fig. 3).

The appearance of those specific volatile compounds indicates an increased breakdown of the amino acids valine, isoleucine and leucine. Valine and leucine can be degraded directly into methyl branched acids (Dickinson, Harrison & Hewlins, 1998; Dickinson et al., 1997). It has generally been believed that methyl branched alcohols are generated through the Ehrlich pathway. According to this pathway valine, isoleucine and leucine are transaminated into their respective alfa-keto acids and then decarboxylatated into the corresponding branched-chain aldehyde, and reduced into the alcohol. However, recent studies of the degradation of valine and leucine by *Saccharomyces cerevisisae* questions the exact pathway for the decarboxylation of the alfa-keto acid and the generation of the hypothesised branched chain aldehyde (Dickinson et al., 1998, 1997).

Valine cannot be used to increase the biomass of *C. utilis*, and only some of the valine which the yeast metabolises, is converted into 2-methylpropanol and (Derrick & Large, 1993), so additional metabolites must be generated. In the sausage minces *C. utilis* apparently degraded valine into 2-methylpropanoic acid and the respective acetate and ethyl esters, in addition to 2-methylpropanol. This metabolic fate also seems to be shared by the two other methyl branched amino acids; leucine and isoleucine. It should, however, be noted that these changes could be a more complicated result of microbial interactions between *C. utilis* and various bacteria, most likely staphylococci.

The minces with *C. utilis* also contained significantly less acetone (3), more 2-propanol (5), acetoin (26) and no acetonitrile (6) (Table 2 and Fig. 3). It is unknown whether that would affect the aroma in any way. Taken together, all these results indicated that *C. utilis*

Table 2
Volatile compounds identified in model minces and the mean peak area of each batch

Identified compounds	No.	I.D.[a]	Reference[b]	+ *D. hansenii*	+ *C. utilis*	ANOVA[c]
Carbondisulfide	1	MS	8354a	12 049b	9277a	*
Ethanol (45)[d]	2	MS/KI	6422	6865	7655	N.S
Propanone	3	MS	2598a	2694a	1707b	*
Methyl acetate (74)	4	MS	6	6	276	N.S
2-Propanol (45)	5	MS/KI	18a	19a	65b	**
Acetonitrile	6	MS	23	44	0	N.S
2-Methylpropanal	7	MS/KI	58	39	132	N.S
Allylmercaptan	8	MS	1171	516	1005	N.S
Ethyl acetate	9	MS/KI	2103a	2395a	96 759b	**
2,3-Butandione (86)	10	MS/KI	2	0	4	–
2-Butanone (72)	11	MS/KI	18	17	37	N.S
2-Butanol	12	MS/KI	0	0	0	–
iso-Propyl acetate	13	MS/KI	0	0	410	–
2-Methyl-1-propanol	14	MS/KI	70a	104a	511b	*
2-Methyl butanal	15	MS/KI	15a	20a	91b	**
Allylmethylsulfide[e]	16	MS	787	695	683	N.S
Ethyl propanoate	17	MS/KI	0	0	136	–
n-Propyl acetate	18	MS/KI	0	0	1149	–
Acetic acid	19	MS	795	788	1062	N.S
Dimethyldisulfide	20	MS/KI	3016	1813	1483	N.S
Ethyl-2-methyl propanoate	21	MS/KI	0	0	158	–
sec-Butyl acetate	22	MS	0	0	27	–
2-Methylpropyl acetate	23	MS/KI	0	0	490	–
3-Methyl-1-butanol (70)	24	MS/KI	42	64	81	N.S
2-Methyl-1-butanol (70)	25	MS/KI	0	15	37	*
3-Hydroxy-2-butanone (45)	26	MS	67a	75a	268b	**
Ethyl butanoate	27	MS/KI	49	58	99	N.S
Ethyl-2-methyl butanoate	28	MS/KI	22a	14a	62b	**
Ethyl-3-methyl butanoate	29	MS/KI	58b	37a	146c	***
3,3′-Thiobis-1-propen[e]	30	MS	348	220	266	N.S
Ethyl 2-hydroxypropanoate	31	MS	27	32	39	N.S
2-Methylpropanoic acid	32	MS	16	11	40	N.S
3-Methylbuthyl acetate	33	MS/KI	11	18	3798	N.S
2-Methylbuthyl acetate	34	MS	0	0	473	–
Methyl-2-propenyldisulfide[e]	35	MS	2492	1568	1366	N.S
3-Methylbutanoic acid	36	MS	41	20	62	N.S
2-Methylbutanoic acid	37	MS	18	8	32	N.S
Dimethyltrisulfide	38	MS	3045	4132	2848	N.S
Benzaldehyde	39	MS/KI	34	74	14	N.S
Diallyldisulfide[e]	40	MS	2042	1417	1503	N.S
Methyl-2-propenyltrisulfide[e]	41	MS	2590	3210	2050	N.S
Dimethyltetrasulfide	42	MS	678	957	540	N.S
2-Phenylethyl acetate	43	MS	0	0	37	–
2-Dipropenyltrisulfide	44	MS	829	958	704	N.S

[a] MS: identification based on mass spectra. KI: identification based on Kovats retention indices on authentic compounds (retention value within ±5).
[b] Letters show results of Duncan's multiple range test ($P < 0.05$).
[c] Analysis of variance, N.S = no significant difference, $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).
[d] Number in parentheses corresponds to the ion used for quantification. Compounds without parentheses have been quantified by TIC (total ion current).
[e] Uncertain identification.

displayed a considerable alcohol dehydrogense and esterase activity in the model minces.

When comparing these data to what would happen in real sausages, one should keep in mind that there is no drying of the minces, and, therefore, the water content is stabile, compared to real sausages. Also, the oxygen tension in the minces is different from real sausage.

2- and 3-Methylbutanal have been described as important for "correct" salami aroma. Also the cheesy smelling 2- and 3-methylbutanoic acid and 2-methylpropanoic acid might be of importance for the sausage

Table 3
Volatile compounds identified in sausages and the mean peak area of each batch

Identified compounds	No.	I.D.[a]	Reference[b]	+ *D. hansenii*	+ *C. utilis*	ANOVA[c]
Ethanol	1	MS	1032	1119	1294	N.S
Propanone	2	MS/KI	470	457	558	N.S
Methyl acetate	3	MS	414	450	373	N.S
2-Propanol (45)[d]	4	MS/KI	96	107	125	N.S
2-Methylpropanal	5	MS/KI	106	100	155	N.S
Allylmercaptane	6	MS	5208	5430	5571	N.S
Ethyl acetate	7	MS/KI	1488	1692	1994	N.S
2,3-Butandione (86)	8	MS/KI	8	12	14	N.S
2-Butanone (72)	9	MS/KI	55	57	82	N.S
Methylthiiran[e]	10	MS	133a	142b	158c	***
2-Butanol	11	MS	148	155	162	N.S
Benzene	12	MS	145	156	155	N.S
3-Methyl butanal	13	MS/KI	515	638	819	N.S
2-Methyl butanal	14	MS/KI	107	116	150	N.S
Allylmethylsulfide[e]	15	MS	3988	3932	4339	N.S
Ethyl propanoate	16	MS/KI	20	21	27	N.S
2-Pentanone (86)	17	MS/KI	8	7	7	N.S
Acetic acid (60)	18	MS	942	1309	1393	N.S
Pentanal (58)	19	MS	9	14	12	N.S
Methyl butanoate (74)	20	MS/KI	9	10	11	N.S
1-Methylthio-1-propene[e]	21	MS	320	329	360	N.S
Octane	22	MS/KI	238	254	288	N.S
Dimethyldisulfide (94)	23	MS/KI	44	51	48	N.S
1-Hydroxy-2-propanone (43)	24	MS	698	795	1127	N.S
Toluene	25	MS	166	174	184	N.S
3-Methyl-1-butanol (70)	26	MS/KI	27	29	31	N.S
3-Hydroxy-2-butanone (45)	27	MS	232	624	582	N.S
Ethyl butanoate	28	MS/KI	109	118	144	N.S
Propanoic acid	29	MS/KI	121	184	237	N.S
Hexanal	30	MS/KI	164	159	300	N.S
Cyclopentanone	31	MS/KI	169	182	186	N.S
1-Hydroxy-2-butanone	32	MS	202	238	305	N.S
3,3′-Thiobis-1-propene[e]	33	MS	1182	1566	1476	N.S
2-Methylpropanoic acid	34	MS	62a	75a	91b	*
Alfa-pinene	35	MS	436	422	575	N.S
Butanoic acid	36	MS/KI	191	280	362	N.S
2,3-Butandiol	37	MS/KI	359	821	819	N.S
2,3-Butandiol	38	MS/KI	271	362	402	N.S
2,2,4,6,6-Pentamethylheptane	39	MS	200	200	279	N.S
Methyl-2-propenyldisulfide[e]	40	MS	484	564	587	N.S
Beta-pinene	41	MS	470a	463a	602b	*
2-Furanmetanol	42	MS	979	1113	1240	N.S
Beta-Myrcene	43	MS	139	151	174	N.S
2-Methyl-2-cyclopenten-1-one	44	MS	257	275	284	N.S
3-Carene	45	MS	799a	789a	1010b	*
Alfa-Pellandrene	46	MS	149	150	191	N.S
1-(-2furanyl)-etanone[e]	47	MS	211	230	240	N.S
Limonene	48	MS	506a	511a	637b	*
Benzaldehyde	49	MS/KI	68a	72a	90b	*
3-Methyl-2-cyclopenten-1-one	50	MS	164a	187b	203b	*
Butyrolactone	51	MS	155	186	198	N.S
Diallyldisulfide[e]	52	MS	2561a	2896b	3440c	**
Phenol	53	MS	89	101	110	N.S
2-Methoxyphenol	54	MS	470	527	572	N.S
2-Methoxy-4-methylphenol	55	MS	92	112	116	N.S
2-Dipropenyltrisulfide	56	MS	87	79	111	N.S
Caryophyllene	57	MS	80	88	98	N.S

[a] MS: identification based on mass spectra. KI: identification based on Kovats retention indices on authentic compounds (retention value within ±5).
[b] Letters show results of Duncan's multiple range test ($P < 0.05$).
[c] Analysis of variance, N.S = no significant difference, $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).
[d] Number in parentheses corresponds to the ion used for quantification. Compounds without parentheses have been quantified by TIC (total ion current).
[e] Uncertain identification.

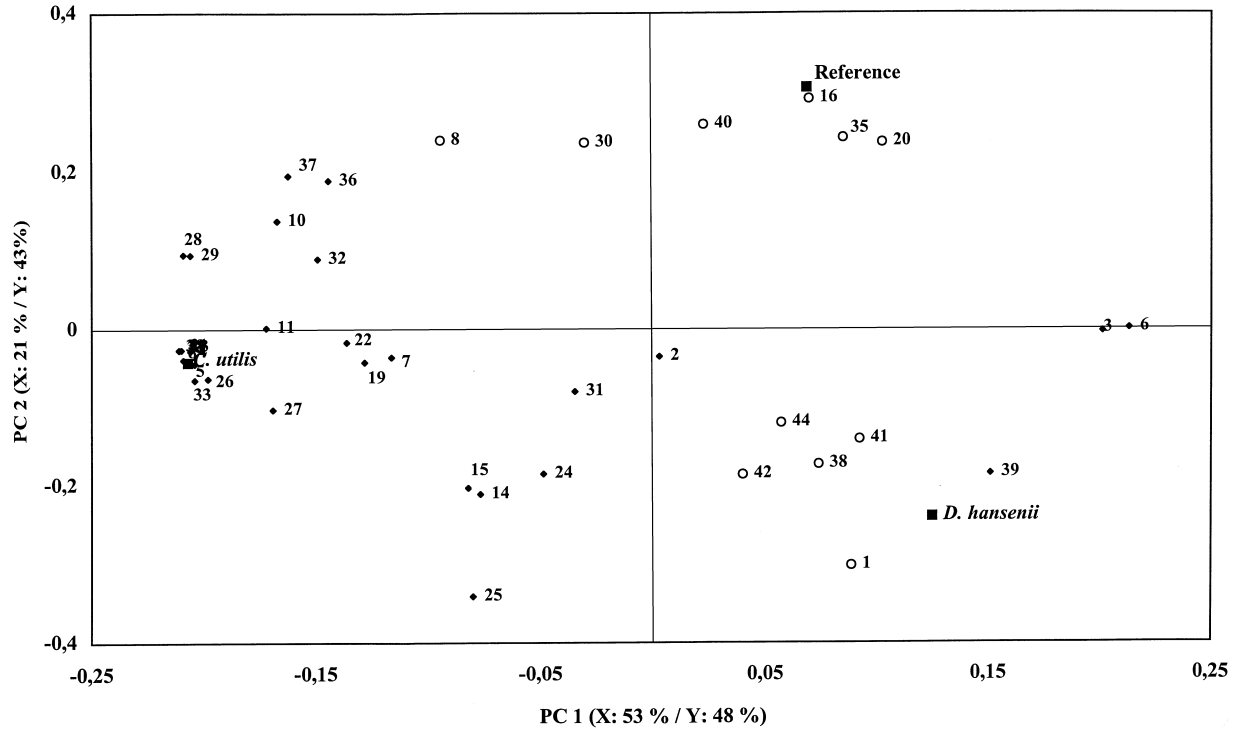

Fig. 3. Loading weights plot from the D-PLSR on volatile profile of model minces. X-loadings relate to the volatile compound matrix and Y-loadings to the design matrix. Sulphur compounds marked by open data point.

flavour (Stahnke, 1995b). If the ester synthesis of *C. utilis* is reduced, the concentration of these odorous acids and aldehydes might be increased. The degradation of valine, leucine and isoleucine could perhaps help to explain the improved aroma reported by Gadjeva et al. (1983) and Miteva et al. (1986) in sausages added *C. utilis* culture.

3.3. Analysis of volatile compounds from sausages

The aroma analysis resulted in the identification of 57 volatile compounds in all sausages (Table 3). Many of these volatile compounds were originating from pepper and smoke (terpenes and aromatic compounds).

Except for the esters, almost all compounds found in the minces could be found in the sausages. However, four of the esters found in the reference minces and eleven of the esters found in the *C. utilis* added minces, was not detected in any of the sausages. This could indicate that the model mince media favoured ester production, perhaps because the higher water content favoured microbial activity. The sausages contained both 2- and 3-methylbutanal contrary to the minces where 3-methylbutanal was not detected.

Among the sausages, the only noteworthy difference was a slightly but significant higher concentration of certain terpenes, 2-methylpropanoic acid and benzaldehyde in the sausages with added *C. utilis* compared to the reference sausage (Table 3). The concentration of the methyl-branched aldehydes and butanoic acid also appears to be somewhat higher in these sausages. All in all, this could weakly indicate that *C. utilis* metabolises valine, leucine and isoleucine as it did in the model minces, but to a much lesser extent.

The D-PLSR plot (Fig. 4) indicates a higher concentration of most volatiles in the sausages with added *C. utilis* culture. Especially terpenes seem to be correlated to the adition of *C. utilis* culture. It also indicates a difference between the reference and the

Table 4
Result of triangle test of sausages

Tested batches	Answers correct/total	Level of significance[a]
Reference vs. + *D. hansenii*	3/12	N.S
Reference vs. + *C. utilis*	3/12	N.S
+ *D. hansenii* vs. + *C. utilis*	8/12	*

[a] N.S = no significant difference, $P < 0.05$ (*).

Table 5
Fungistatic test of garlic powders inhibition of *D. hansenii* and *C. utilis*

Concentration		Number of wells (out of six) with yeast growth															
Garlic	NaCl	*D. hansenii* Day no.								*C. utilis* Day no.							
$^{w}/_{w}$	$^{w}/_{w}$	1	2	3	4	8	16	25	35	1	2	3	4	8	16	25	35
0.00%	1%	0	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	5%	0	3	6	6	6	6	6	6	0	6	6	6	6	6	6	6
	9%	0	0	4	6	6	6	6	6	0	0	0	0	0	1	4	4
0.20%	1%	0	0	0	0	0	0	0	0	0	0	0	0	0	2	5	5
	5%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.40%	1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

sausages with added *D. hansenii* culture. Despite these results, there are no big differences in the concentration of any volatile compound among the sausages (Table 3).

3.4. Sensorial analysis of sausages

No obvious differences could be found in the odour between the sausage batches. The test panel described all sausages as having a typical smoked sausage odour with a hint of garlic/onion. The triangle test (Table 4) clearly showed that the reference sausages were not different from the sausage with added yeast starter cultures. Nevertheless, there seemed to be a slight difference between the sausages with different yeast starter culture, indicating that the combined differences of these two sausages from the reference sausage were barely detectable. The members of the test panel all agreed that the odours of the sausages were very similar.

3.5. Fungistatic effect of garlic

The microbiological results of the minces and sausages combined with present knowledge suggested that the dried garlic powder used in this study inhibited the

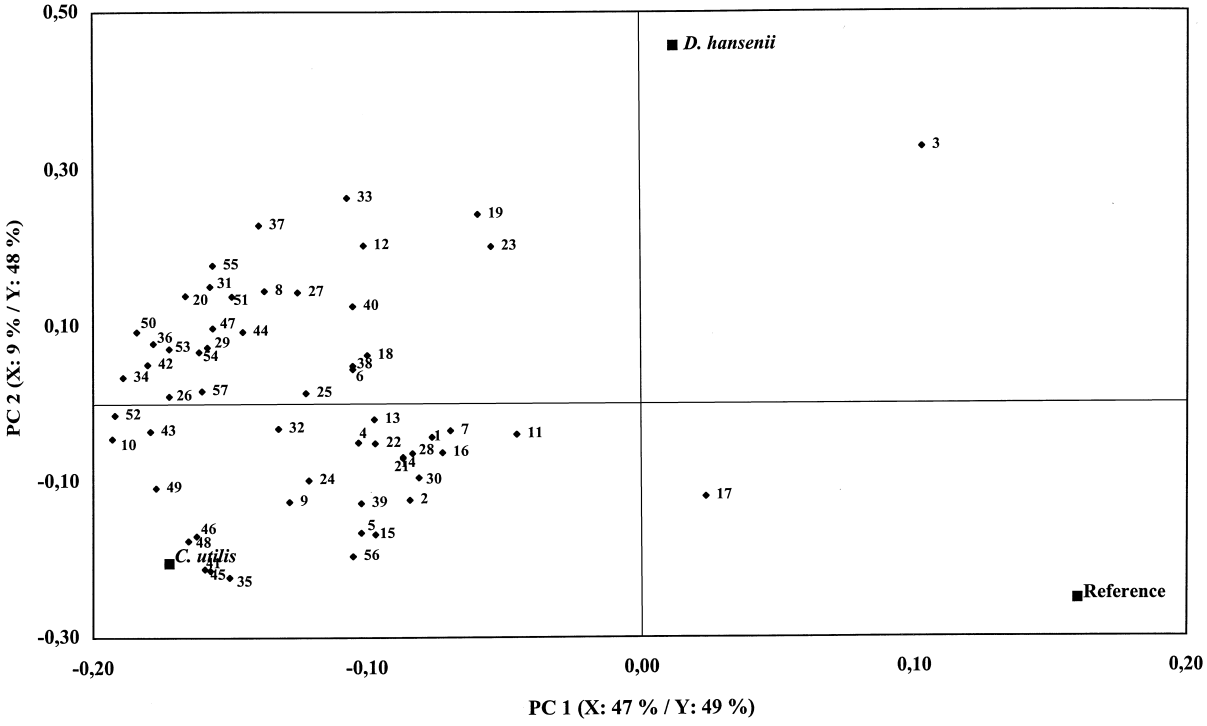

Fig. 4. Loading weights plot from the D-PLSR on volatile profile of sausages. X-loadings relate to the volatile compound matrix and Y-loadings to the design matrix.

growth of *D. hansenii* and *C. utilis*. To determine this a fungistatic test was set up. To better simulate the saline environment in a sausage, increasing concentrations of NaCl were used in the growth media.

Despite the low pH both yeast thrived in the garlic free media as long as the saline concentration did not exceed 5% (Table 5). Even though the salt addition lowered the growth rate of both yeast, only *C. utilis* had major problems growing in 9% NaCl, which were in accordance with *C. utilis*'s much lower tolerance of low water activities compared to *D. hansenii*. The addition of garlic powder, however, strongly affected both *D. hansenii* and *C. utilis*. Whereas *D. hansenii* was completely inhibited, *C. utilis* seemed to be somewhat more resistant. Garlic's inhibitory effect appeared to be cumulative with the increased concentration of NaCl for *C. utilis* (Table 5). The reason why the yeasts die off in the sausages, but survive in the sausage minces could be due to the drying of the sausages (water content is constant in the minces, since water cannot escape from the flasks), which increases the concentration of garlic and NaCl. In addition, the inoculation level was lower in the sausages compared to the minces and the sausages were smoked, which is known to suppress fungi (Geisen et al., 1992). However, the results clearly show that dried garlic powder has at least a fungistatic potential, and perhaps also a fungicidal potential.

4. Conclusion

The research conducted on sausage minces indicated that *C. utilis* has a considerable potential for producing several odorous compounds, especially alcohols and esters, of which many probably we derived from the amino acids isoleucine, leucine, valine and phenyl alanine. *D. hansenii* on the contrary, showed very limited production of volatile compounds. However, the growth environments in this study showed to be very unfavourable for *D. hansenii*, and so *D. hansenii* might prove far more interesting under more favourable growth conditions.

Both yeast species failed to survive in the sausage experiment and they also failed to markedly affect the sensory properties. The fungistatic test showed that addition of garlic powder was the most likely reason for the poor survival of the yeast in the minces and especially in the sausages.

Based on these results, the beneficial value of adding *D. hansenii* and *C. utilis* starter cultures to garlic spiced sausages for the improvement of the sausage aroma, must be considered somewhat dubious. The growth results of Encinas et al. (2000) seem to contradict these results. But the garlic used in their study might have been fresh or used in lower concentrations compared to the present experiment. Neither should it be forgotten that yeast strains found in Spanish sausages might be better adapted to a garlic spiced environment than the strains used in this study.

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