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Addition of α-ketoglutarate enhances formation of volatiles by *Staphylococcus carnosus* during sausage fermentation

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Abstract

The effect of leucine and α-ketoglutarate addition on transamination of branched-chain amino acids was studied in model minces inoculated with *Pediococcus pentosaceus* and *Staphylococcus carnosus*. Leucine addition changed the ratio of volatile breakdown products of leucine, isoleucine and valine but not the total amount of the volatiles and it was concluded that the amount of free amino acids does not limit transamination of amino acids. The addition of α-ketoglutarate resulted in increased levels of methyl-branched aldehydes and insignificant positive changes in methyl-branched acid production. The results were verified in real fermented sausages with no, low (0.09% w/w) and high (0.36% w/w) addition of added α-ketoglutarate since the levels of the flavour intensive methyl-branched aldehydes and acids were drastically increased in sausages added α-ketoglutarate. The catabolism of phenylalanine was also induced by α-ketoglutarate and there were further indications of increased transamination of aspartate. A triangular test showed that the flavour of sausages with no and low addition of α-ketoglutarate could be clearly distinguished from one another. Altogether the results presented in this paper point to glutamate dehydrogenase, the enzyme catalyzing regeneration of α-ketoglutarate, as a key enzyme in catabolism of amino acids and thereby also in aroma formation during sausage processing.
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Keywords: Fermented sausage; Volatiles; α-Ketoglutarate; Leucine; Glutamate dehydrogenase; *Staphylococcus carnosus*

1. Introduction

There is a general trend in modern sausage manufacturing towards faster acidification and shorter processing time. This has the obvious advantages of increased processing capacity and reduced costs, but it also has some drawbacks with respect to aroma formation. The importance of microorganisms and in particular staphylococci in aroma formation during sausage ripening has been recognised (Berdagué, Monteil, Montel, & Talon, 1993; Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996; Stahnke, 1995; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2002) and it has been shown that fast acidification limits growth and activity of staphylococci (Tjener, Stahnke, Andersen, & Martinussen, 2003). Often the flavour of traditional, slowly fermented sausages is preferred over the typical flavour of fast fermented sausages, and therefore acceleration of flavour forming reactions in staphylococci used as starters in fast fermentations is of great interest.

Aroma compounds originating from microbial breakdown of amino acids are known to be of major importance for the overall flavour of fermented sausages. Especially the breakdown of leucine, isoleucine and valine into methyl-branched aldehydes and acids has been linked to dry sausage odour (Montel et al., 1996; Stahnke, 1995; Stahnke et al., 2002) and recently the pathway leading to the formation of those compounds has been studied in *Staphylococcus* (Beck, Hansen, & Lauritsen, 2002; Larrouture, Ardaillon, Pépin, & Montel, 2000; Madsen, Beck, Ravn, Vrang, Hansen, & Israelsen, 2002). The branched-chain amino acid aminotransferase encoded by *ilvE* catalyzes the conversion of three methyl-branched amino acids into methyl-branched keto acids (exemplified in Fig. 1(a) by

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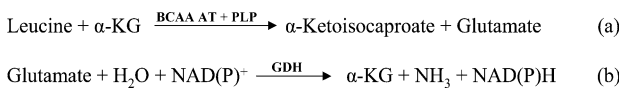

Fig. 1. Reaction scheme for transamination of leucine (a) and regeneration of α-ketoglutarate (b). Transamination of isoleucine and valine is parallel to the transamination of leucine. α-KG, α-ketoglutarate; BCAA AT, Branched-chain amino acid aminotransferase; PLP, Pyridoxal-5′-phosphate; GDH, glutamate dehydrogenase.

the conversion of leucine). The keto acids are subsequently converted into the corresponding aldehydes, alcohols, or acids. The transamination step requires pyridoxal 5′-phosphate as co-factor and a keto acid, for instance α-ketoglutarate, as amino group acceptor. The α-ketoglutarate is regenerated by glutamate dehydrogenase (GDH) in NAD^+ or $NADP^+$ dependent reaction (Fig. 1(b)) (Rhodes, 2003). An *ilvE* deletion mutant of *Staphylococcus carnosus* produced very low amounts of the branched-chain amino acid breakdown products thus demonstrating the importance of the *ilvE* encoded transaminase in branched-chain amino acid catabolism (Madsen et al., 2002).

Theoretically the transamination step can be accelerated by: (i) increasing the amount of transaminases, (ii) increasing the amount of precursors (amino acids and α-ketoglutarate) or (iii) increased levels of co-factors (pyridoxal-5′-phosphate) and redox equivalents (NAD^+, $NADP^+$).

The objective of the present study was to investigate ways of accelerating the transamination of branched-chain amino acids for the evolution of methyl-branched aroma compounds during fermentation of minces in a model system and in fermented sausages. Two approaches were tested: (1) addition of leucine and (2) addition of α-ketoglutarate. Addition of leucine and α-ketoglutarate would reveal whether the amounts of free branched-chain amino acids (leucine) or amino group acceptors (α-ketoglutarate) are rate-limiting factors in transamination of branched-chain amino acids (Fig. 1).

2. Materials and methods

Model minces and sausages were all produced with the following composition: pork shoulder (32% w/w), beef back rib (32% w/w), pork back fat (31.3% w/w), NaCl with 0.6% w/w $NaNO_2$ (1.7% w/w), NaCl (1.0% w/w), potato starch (1.25% w/w), glucose (0.4% w/w), sodium ascorbate (0.05% w/w) and $MnSO_4 \cdot H_20$ (0.1 μg/g Mn). [1] Freeze-dried starter cultures of *Pediococcus pentosaceus* PC-1 and *S. carnosus* MIII (Chr. Hansen A/S, Denmark) were added to levels of 5×10^6 CFU/g.

[1] Manganese was added as substitute for the amount typically supplied by spices.

2.1. Model mince trials

In trial 1, minces were produced with 0% or 0.02% w/w L-leucine (Merck, Germany) and in trial 2 minces were produced with 0%, 0.09% or 0.36% w/w di-sodium-α-ketoglutarate (Sigma Chemical Co., USA). Portions of 250 g of mince were stuffed into plastic beakers (polyethylene, 450 ml, $d = 70$ mm, Berry Plastics, USA), vacuum stopped, sealed with lids and fermented in a water bath at 24 °C for 7 days. Minces added leucine were produced in duplicate and minces with added di-sodium-α-ketoglutarate in triplicate for aroma analysis. Furthermore one mince of each type was used for continuous pH-registration.

2.2. Sausage trial

Three sausage batches were produced with addition of no (0% w/w), low (0.09% w/w) or high (0.36% w/w) amounts of di-sodium-α-ketoglutarate (Sigma Chemical Co., USA). The sausages were stuffed into 48 mm cellulose casings (SFK amba, Denmark), fermented and ripened in a climate chamber (Multimat MC1000, Deutsch, Germany) with initial temperature and humidity of 24 °C and 95% RH. Temperature and humidity were gradually reduced to 17 °C and 75%RH during the first 11 days of production and then kept constant for the following 10 days. Fifteen minutes of smoke from beech chips was applied at day 2, 3 and 6. After fermentation sausages were vacuum packed and stored at −50 °C until volatile or sensory analysis.

2.3. pH

Sausage pH was measured at day 0, 1, 2, 3, 5, 7, 9, 14, 17, and 21 (Knick Portamess®, Knick Elektronische Meßgeräte GmbH, Germany, with Metrohm 6.0226.100 electrode, Metrohm Ltd., Switzerland). pH of model minces was followed continuously with 3 HA405-DXX-S8/120 electrodes per beaker (Mettler Toledo GmbH, Switzerland) connected to an AAC-2 PC-logger (INTAB Interface Teknik AB, Sweden).

2.4. Volatile analysis

To 100 g of model mince 20 g of NaCl was added, mixed with a hand held mixer and 3×30 g were transferred to 150 ml cylindrical glass bottles. 100 g of sausage was cut into cubes (of approx. 1 cm^3), 20 g of NaCl added, frozen with liquid nitrogen, homogenized with a domestic chopper (Braun Multiquick professional MR500, Braun 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, the 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: 250 °C for 3 min onto Tenax TA® cold trap (20 mg, −30 °C), second desorption: 250 °C for 60 seconds, line temp: 225 °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 i.d. DB 1701 (1 μm film) fused silica capillary column (J&W Scientific, 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 to the NBS75k-database.

Standard addition curves were made for quantification of 19 components in sausages. A stock-solution was prepared in methanol (10 g/l) and diluted with methanol to concentrations of 0, 0.5, 1.0 and 1.5 g/l. 10 microliter of dilution was added to 100 g of sausage prior to the addition of NaCl. Curves were based on two triplicate analyses per level of standard addition.

2.5. Sensory analysis

A forced choice triangular test of the sausages was executed according to ISO (1983). Sausages were homogenised with a domestic chopper and served at room temperature in 100 ml plastic vials. Eleven trained panellists evaluated the flavour of sausages with no and low addition of α-ketoglutarate twice.

3. Results and discussion

Two approaches to accelerate transamination of branched-chain amino acids were tested in model minces and the most promising approach was thereafter tested in dry sausage fermentation.

Important characteristics of the three meat fermentations are shown in Table 1. The model minces were relatively similar, but they differed from the sausages in terms of fermentation time, weight loss and acidification profile. The slower acidification in sausages was due to a lower fermentation temperature (Sections 2.1 and 2.2) and probably also the lower water activity caused by drying. For a more thorough discussion of the model system compared to sausages see Tjener et al. (2003).

Sausages as well as model minces were analysed for 32 volatile compounds related to sausage flavour, and of those compounds 19 were quantified by standard addition in the sausage experiment. The unquantified volatile responses presented below are all Log_{10} values of single ion counts. Only levels of methyl-branched aldehydes and acids are presented for the model mince experiments, whereas data for all 32 compounds are given in the sausage experiment in order to examine the effect of α-ketoglutarate on other pathways as well. Quantified volatile responses are specified in the text when relevant.

3.1. Effect of leucine addition

By free amino acid analysis it was found that the addition of 0.02% w/w leucine corresponded to a five-fold increase of the initial level (data not shown). The effect of leucine addition on the amount of methyl-branched volatiles is seen in Fig. 2. Although not all changes were significant ($\alpha = 0.05$), the levels of 3-methyl butanal and 3-methyl butanoic acid increased marginally when leucine was added whereas the levels of 2-methyl butanal, 2-methyl propanal, 2-methyl butanoic acid and 2-methyl propanoic acid (arising from isoleucine and valine) were slightly decreased. This indicates that transamination of the three branched-chain amino acids is not limited by lack of free branched-chain amino acids during fermentation. Thus, the addition of free leucine only changed the relative amounts of leucine, isoleucine and valine to be transaminated but not the total transaminase activity.

However, it should be noted that since the odour notes and sensory threshold values are different for the catabolites of the three branched-chain amino acids, the addition of one of those amino acids may lead to changes in aroma profile although the total transaminase activity is unaltered.

A number of sausage fermentations with added proteases have been conducted over the years in order to accelerate flavour formation (Ansorena, Astiasarán,

Table 1
Characteristics of model and sausage fermentations

System additions	Mince model leucine	Mince model α-ketoglutarate	Sausages α-ketoglutarate
Fermentation time (week)	1	1	3
Initial pH	5.79	5.77	5. 78
Time to pH 5.30	52 h	40 h	~4 days
Final pH	4.97	4.89	5.30
Weight loss	–	–	30.0%

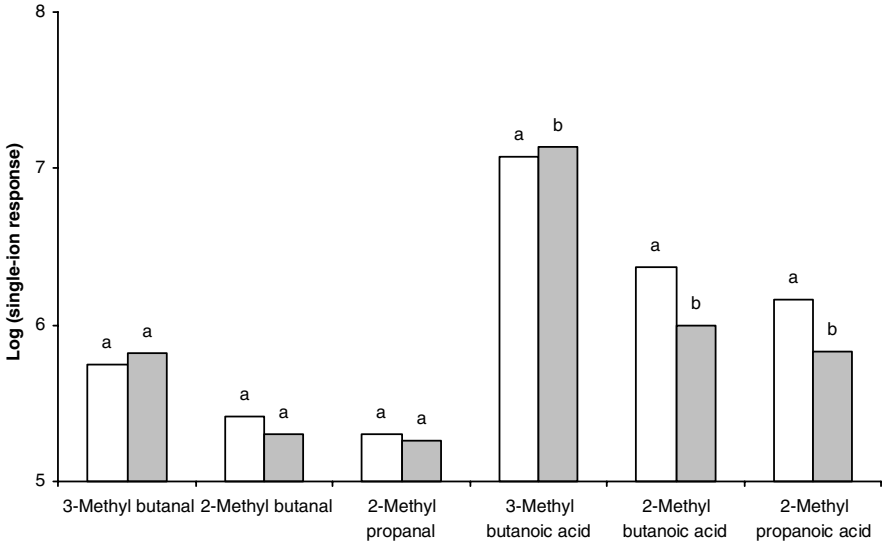

Fig. 2. Levels of methyl-branched aldehydes and acids in minces without (□) or with (■) leucine (0.02% w/w) added. Letters a and b indicate whether volatile levels are significantly different according to Duncan's multiple range test ($\alpha = 0.05$) (Montgomery, 1997).

& Bello, 2000; Ansorena, Zapelena, Astiasarán, & Bello, 1998; Díaz, Fernández, García de Fernando, Hoz, & Ordoñez, 1993; Díaz, Fernández, García de Fernando, Hoz, & Ordoñez, 1996; Díaz, Fernández, García de Fernando, Hoz, & Ordoñez, 1997; Zapelena, Ansorena, Zalacain, Astiasarán, & Bello, 1998; Zapelena, Astiasarán, & Bello, 1999; Zapelena, Zalacain, Peña, Astiasarán, & Bello, 1997). In two of those studies a weak positive influence on the sensory profile of sausages was detected (Zapelena et al., 1999; Zapelena et al., 1997), but in all other studies the protease activity increased the levels of free amino acids without altering any of the sensory properties, except texture. The current leucine addition experiment and the proteinase addition experiments thus lead to the same conclusion: volatile production and flavour formation from amino acid catabolism are not limited by the content of specific free amino acids.

3.2. Effect of α-ketoglutarate addition to model minces

The effect of adding α-ketoglutarate to model mince fermentations is seen in Fig. 3. The addition clearly increased the level of 2- and 3-methyl butanal whereas the influence on 2-methyl propanal was insignificant. Due to very high analytical uncertainties the effect of α-ketoglutarate on the levels of methyl-branched acids was not significant, but the average values clearly indicated a positive correlation to α-ketoglutarate. Analysis of volatile acids is unfortunately often associated with high uncertainties (Mateo & Zumalacárregui, 1996; Tjener et al., 2003). Nevertheless, the results suggest that addition of α-ketoglutarate is one way of increasing the levels of the flavour intensive methyl-branched aldehydes and perhaps also the acids, and it was decided to test α-ketoglutarate addition in fermented sausage production.

3.3. Effect of α-ketoglutarate addition to sausages

Results from volatile analysis of sausages with no, low (0.09% w/w) or high (0.36% w/w) addition of α-ketoglutarate are presented in Fig. 4. The addition of α-ketoglutarate significantly increased the levels of methyl-branched alcohols, aldehydes and acids, but also the levels of ethanol, 2-phenyl ethanol, phenylacetaldehyde, benzaldehyde, diacetyl, 4-methyl-2-pentanone and the four ethyl esters.

The increased levels of methyl-branched alcohols, aldehydes and acids illustrate the importance of the α-keto acid in degradation of methyl-branched amino acids. For the aldehydes the increases were approximately one order of magnitude when increasing the added α-ketoglutarate level from no to low and from low to high.

In addition, the level of 4-methyl-2-pentanone was increased by the α-ketoglutarate addition. This compound has been suggested to arise from leucine degradation (Stahnke, 1999), and the observed increase is thus in accordance with that hypothesis.

The levels of phenylacetaldehyde, benzaldehyde and 2-phenylethanol were also positively correlated to the amount of α-ketoglutarate and these compounds are known metabolites of phenylalanine catabolism (Yvon & Rijnen, 2001). The increased levels of those compounds indicate the presence of aromatic amino acid aminotransferase activity in *S. carnosus*. This enzyme is found in, e.g., *Lactococcus* and also here the transami-

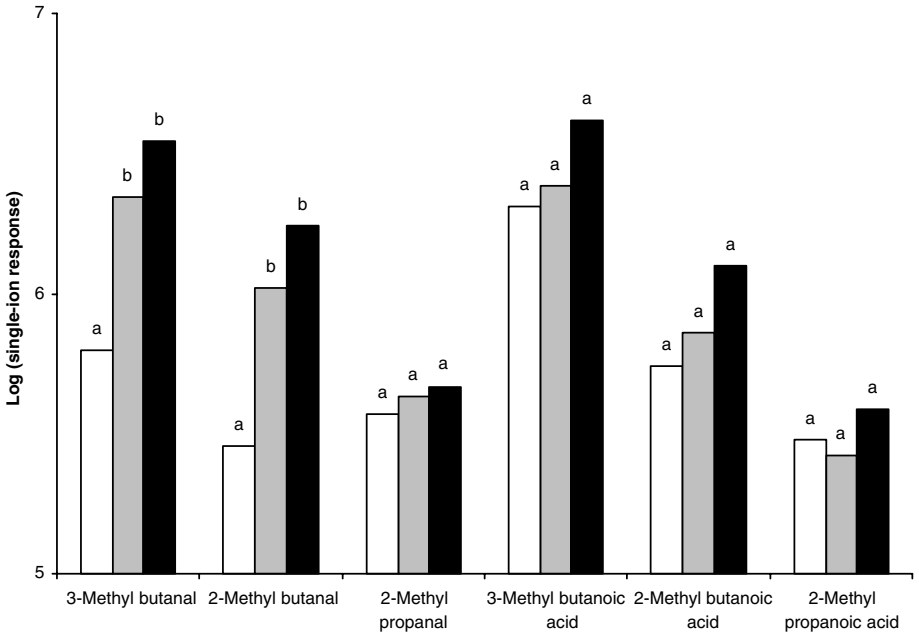

Fig. 3. Levels of methyl-branched aldehydes and acids in minces with or without α-ketoglutarate added. (□) No addition; (▒) 0.09% w/w; (■) 0.36% w/w. Letters a, b and c indicate whether volatile levels are significantly different according to Duncan's multiple range test ($\alpha = 0.05$) (Montgomery, 1997).

nase activity is increased by α-ketoglutarate (Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997).

Ethanol is formed from pyruvate and the positive correlation between α-ketoglutarate and ethanol levels indicates that more pyruvate is formed because of α-ketoglutarate addition. Two possible causes of increased pyruvate formation are increased transamination of alanine and conversion of α-ketoglutarate to pyruvate.

The positive impact of α-ketoglutarate on the levels of ethyl esters is most likely due to the higher ethanol levels. A strong correlation between ethanol and ethyl ester levels was earlier reported for a number of very different fermentations in this model system (Tjener, Stahnke, Andersen, & Martinussen, 2004).

Diacetyl is formed from acetolactate, a compound synthesised from pyruvate (Kandler, 1983) or from catabolism of leucine and valine (Banks et al., 2001). Yet another possibility is the formation of diacetyl from aspartate degradation previously discovered in *Lactobacillus* (Kieronczyk, 2002).

As opposed to diacetyl the level of 2-butanone was unaffected by α-ketoglutarate addition. This is in contrast to the suggested formation pathway of this compound by reduction of diacetyl via acetoin (Margalith, 1981).

Dimethyldisulphide and dimethyltrisulphide levels were increased by α-ketoglutarate addition though the increases were insignificant from low to high level addition. The sulphides are most likely formed from methanethiol, a known elimination product of methionine (Yvon & Rijnen, 2001) and the observed increase caused by α-ketoglutarate thus not readily explained. Methional is also a product of methionine degradation (McSweeney & Sousa, 2000) but it was not significantly affected by α-ketoglutarate addition.

The aliphatic alcohol, aldehydes and acids showed no positive correlation to α-ketoglutarate addition. On the contrary, 1-hexanol, nonanal and decanal levels were slightly lower in sausages with a high level of α-ketoglutarate. The methylketones 2-pentanone and 2-heptanone, possibly arising from decarboxylation of β-keto- or hydroxy acids, showed no clear correlation to the α-ketoglutarate level, and in summary the fatty acid metabolism seemed to be very little affected by the α-ketoglutarate addition.

3.4. Sensory analysis

In a triangular test of sausages with no and low (0.09% w/w) addition of α-ketoglutarate 17 of 22 evaluations were correct and sausages thus significantly different ($p < 0.001$) with respect to flavour. Sausages added the highest level (0.36% w/w) of α-ketoglutarate had such a markedly different odour that it was unnecessary to test them in a triangular test.

Quantification by standard addition showed that the absolute amounts of the branched-chained aldehydes for the three levels of α-ketoglutarate were 151, 1857 and 9753 μg/kg of 3-methyl butanal, 28, 516 and 5140 μg/kg of 2-methyl butanal and 17, 110 and 1626 μg/kg of 2-methyl propanal. Retronasal sensory threshold values

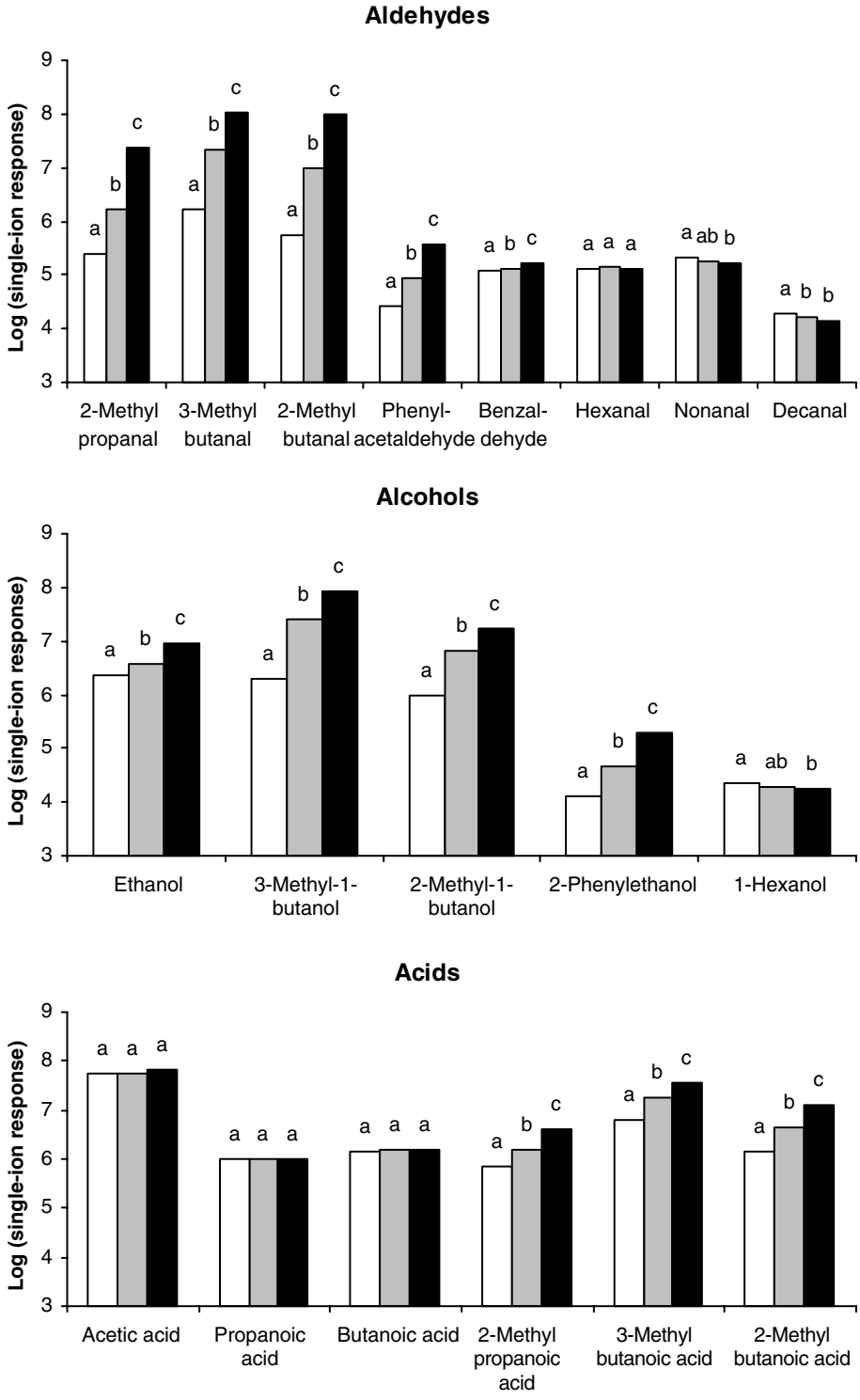

Fig. 4. Levels of volatiles in sausages with or without supplementary α-ketoglutarate added. (□) No addition; (▒) 0.09% w/w; (■) 0.36% w/w. Letters a, b and c indicate whether volatile levels are significantly different according to Duncan's multiple range test ($\alpha = 0.05$) (Montgomery, 1997).

for these compounds are below 1 μg/L in water (Rychlik, Schieberle, & Grosch, 1998), and it is therefore not surprising that the sausages had a very different flavour when α-ketoglutarate was added.

Quantification of phenylacetaldehyde showed levels of 39, 141 and 535 μg/kg. When compared to a stated orthonasal sensory threshold in water of 4 g/kg (Rychlik et al., 1998), it seems likely also that phenylacetaldehyde

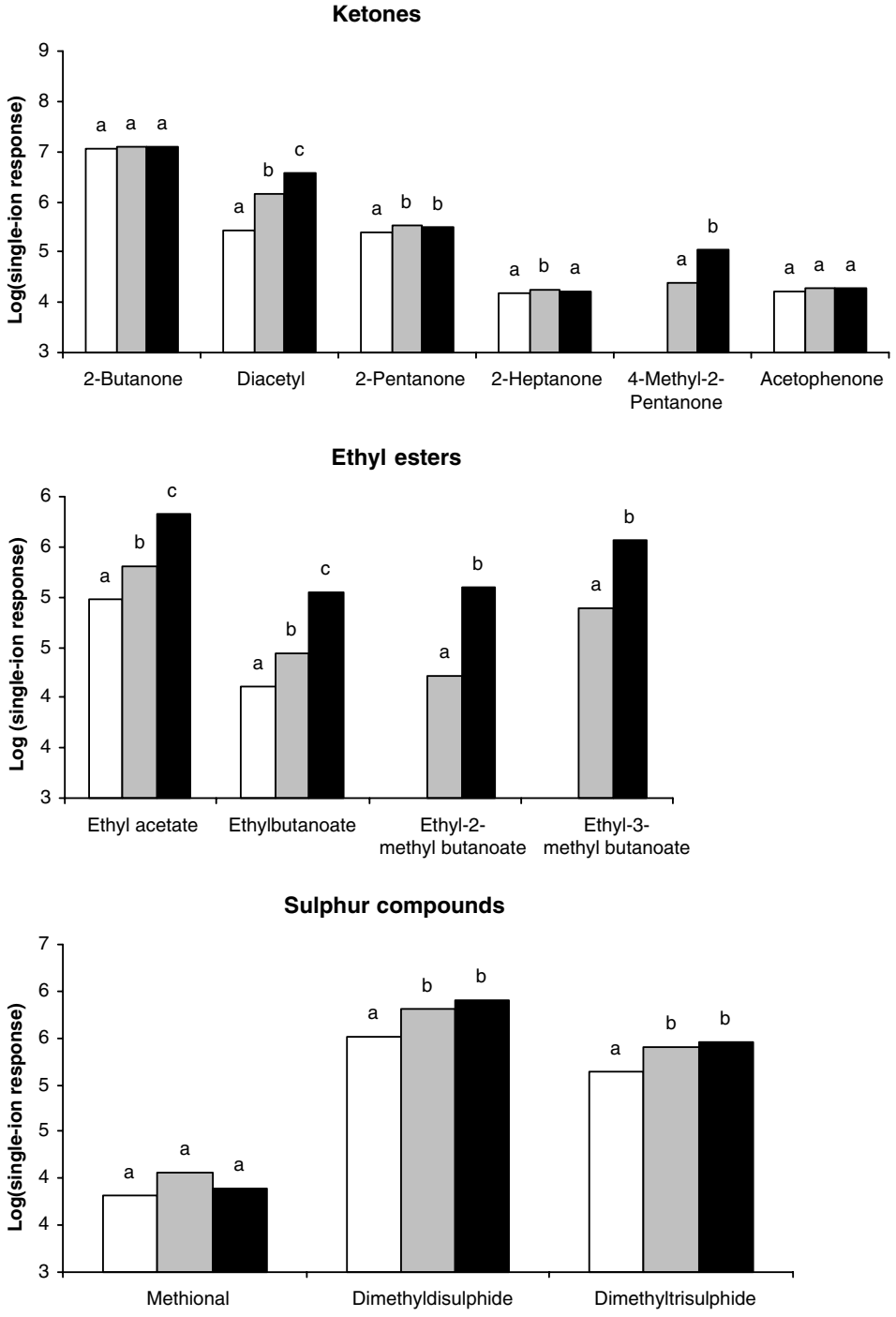

Fig. 4. *(continued)*

contributed to the sensory differences detected in the triangle test mentioned above.

3.5. General discussion

The addition of α-ketoglutarate clearly increased degradation of the amino acids leucine, isoleucine, valine and phenylalanine whereas the addition of leucine did not have an overall effect on the transamination. This showed that the limiting factor in transamination of branched-chain amino acids by staphylococci during meat fermentation is the amount of the amino group acceptor and not the amount of the amino acids. These results are in accordance with studies of α-ketoglutarate addition in relation to cheese ripening where the addition resulted in an increased degradation of amino acids and intensified cheese aroma (Banks et al., 2001; Yvon, Berthelot, & Gripon, 1998).

The results suggest that the activity of GDH (Fig. 1) should be subject to further attention. Rijnen, Courtin, Gripon, and Yvon (2000) constructed a *gdh*$^+$ *Lactococcus lactis* able to convert the same amount of amino acids in a cheese model system as the wild-type with added α-ketoglutarate. From the published sequences of staphylococci (*Staphylococcus aureus* Mu50 and *Staphylococcus epidermidis* ATCC12228) it appears that staphylococci do have GDH enzymes (Kuroda et al., 2001; Zhang et al., 2002), but as seen in Fig. 1 the activity of GDH is dependent on NAD(P)$^+$. The organism used in the study of Rijnen et al. (2000) was apparently able to supply enough NAD(P)$^+$ to facilitate the regeneration of α-ketoglutarate but whether this is true for staphylococci during sausage ripening is unknown.

4. Conclusion

Addition of leucine to model minces prior to fermentation results in slightly increased levels of 3-methyl butanal and 3-methyl butanoic acid, but it does not increase the total transamination of branched-chain amino acids. Addition of α-ketoglutarate highly enhances conversion of amino acids into flavour compounds by *S. carnosus* during sausage fermentation. Since GDH is responsible for the regeneration of α-ketoglutarate from glutamate it is suggested that GDH is the rate-limiting enzyme in transamination of amino acids under the conditions typical for dry sausage processing.

Acknowledgements

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