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Effect of growth phase and dry-cured sausage processing conditions on *Debaryomyces* spp. generation of volatile compounds from branched-chain amino acids

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Abstract

The generation of volatile aroma compounds by *Debaryomyces* spp. from branched-chain amino acids (BCAAs) was investigated. The yeast, that was a natural isolate from sausages, was grown in a meat model system and incubated with leucine, isoleucine and valine under various conditions of interest in dry-cured sausages processing. In the meat model system, *Debaryomyces* spp. showed ability for growing and, simultaneously, raising the pH, using lactate, generating ammonia and several volatile compounds, and altering the free amino acids content. The production of volatile compounds from BCAAs by *Debaryomyces* spp. was negatively affected by the presence of salt, although pH reduction to 4.5 increased the yield of alcohols and aldehydes. Cells transition from exponential to stationary growth phase diminished alcohol and aldehyde production, but increased acid generation. Thus, it can be expected that the addition of *Debaryomyces* spp. as a starter culture can modify the flavour pattern of dry-cured sausages.

Keywords: Volatile compounds; Debaryomyces; Yeast; Amino acids; Dry-cured sausage

1. Introduction

Amino acid degradation, during the processing of dry-cured sausages, leads to the generation of volatile molecules that make an important contribution to the typical flavour of these products. Some volatile compounds resulting from branched-chain amino acids (BCAAs, i.e., Leu, Ile and Val) metabolism have very low threshold values (Berdagué, Monteil, Montel, & Talon, 1993; Montel, Masson, & Talon, 1998). 2- and 3-methyl-1-butanol and, especially, 2- and 3-methyl-butanal and 2-methyl-propanal are significant components of dry-cured sausage aroma. Other compounds, such as 2-methyl-propanoic and 2- and 3-methyl-butanoic acids and their derived esters also play a significant role in the sausage flavour (Berdagué et al., 1993; Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996; Søndergaard & Stahnke, 2002). In fact, several esters are essential for the appropriate aroma since they add a fruity note and/or mask rancid odours (Stahnke, 1994).

In recent years, the metabolism of BCAAs has prompted a great interest, particularly in yeasts such as *Saccharomyces cerevisiae*, due to the outstanding contribution of branched-chain alcohols generated through yeast fermentation to the flavour of alcoholic beverages (Eden, van Nedervelde, Drukker, Benvenisty, & Debourg, 2001; Nykänen, 1986; ter Schure, Flikweert, van Dijken, Pronk, & Verrips, 1998; van Iersel, Eppink, van Berkel, Rombouts, & Abee, 1997; Yoshimoto, Fukushige, Yonezawa, & Sone, 2002). Moreover, recent studies on *Debaryomyces hansenii* and *Kluyveromyces lactis* have shown their influence on the development of cheese aroma (Arfi, Spinnler, Tache, & Bonnarme, 2002).

However, in spite of the apparent potential of *D. hansenii* in flavour generation from amino acid metabolism, Olesen and Stahnke (2000) observed little effect of this yeast in the production of volatile compounds in

Abbreviations: BCAA, branched-chain amino acid; PDMS, polydimethylsiloxane; SPME, solid phase microextraction.

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model systems and in fermented sausages. According to these authors, the presence of *D. hansenii* caused a reduction in the concentration of some flavour components (i.e., ethyl 3-methyl-butanoate), although the results of this study were not conclusive, since the garlic powder present in both the model systems and sausages inhibited *D. hansenii* growth.

The improvement of dry-cured sausage aroma is of major economical interest since the final flavour of the product has a considerable influence on consumer choice. Yeasts can participate in enhancing flavour through their amino acid converting capabilities. *Debaryomyces* spp. has been found in fermented sausages with no evidence of toxic effects. Therefore, the aim of this work was to investigate the generation of volatile aroma compounds by *Debaryomyces* spp. grown in a meat model system and incubated with Val, Leu and Ile under representative conditions of dry-cured sausage processing. Moreover, the yeast effectiveness in modifying ammonia, lactic acid and amino acid concentrations in the meat model system was also studied. The results of these investigations will be very helpful in establishing the suitability of the *Debaryomyces* spp. strain as starter culture for dry-cured sausage production.

2. Material and methods

2.1. Meat model system

The meat model system consisted of a medium containing (in g/l): meat extract (Scharlau Chemie S.A., Barcelona, Spain), 10; glucose, 10; DL-lactic acid, 10, NaCl, 30. The medium pH was adjusted to pH 4.5 with NaOH before autoclaving.

2.2. Yeast strain, growth conditions and sampling

Debaryomyces spp. CECT 11815 was originally isolated from the indigenous flora of dry-cured sausages (Santos Mendoça, 2000). The microorganism was cultured at 27 °C, without shaking, in 250 ml Erlenmeyer flasks containing 100 ml of the meat model system medium. Following one subculture in the same medium, but without NaCl and adjusted to pH 6.7, the yeast was inoculated at 10^6 cfu/ml.

At different culture times (0, 3, 5, 7, 10, 12 and 14 days), three flasks were taken and cells were removed by centrifugation (8000*g*, 10 min, 4 °C). The supernatant was used for the analysis of ammonia, D- and L-lactic acids, and free amino acids. The mean ± standard deviation of the concentration was calculated using three replicates for each culture time. For volatile compound determination, aliquots of 7.5 ml of the supernatant were placed in a 15 ml vial for headspace analysis by SPME and sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA). All samples were maintained at −20 °C until further analysis.

2.3. Determination of ammonia

Two millilitres of 1 M perchloric acid were added to 0.5 ml of culture medium supernatant in order to precipitate proteins and other medium components. Then, the mixtures were neutralised with KOH and water was added up to 10 ml. The samples were kept at 4 °C for 20 min, and, after that, they were filtered through glass wool. The ammonia present in the filtrates was analysed by the method of Bergmeyer and Beutler (1985), based on the NADH oxidation via glutamate dehydrogenase. The change in absorbance at 340 nm was followed in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The results were expressed as mg of ammonia per L of initial medium.

2.4. Determination of D- and L-lactic acids

For measuring D- and L-lactic acid concentrations, interfering proteins present in the medium supernatant were precipitated at 80 °C for 15 min and removed by centrifugation (10,000*g*, 10 min, 4 °C). D- and L-lactic acids contents of the supernatants were determined using the methods of Gawehn (1984) for D-lactate and Noll (1984) for L-lactate, modified and combined as follows: a reaction mixture containing 250 mM glycylglycine/40 mM glutamate buffer, pH 10.0, 4 mM NAD^+, 14 U of glutamate–pyruvate transaminase/ml, and sample solution, was treated with 50 U of D-lactate dehydrogenase/ml. The molar amount of NADH formed, which is directly proportional to the D-lactate molar concentration, was followed by means of the increase in absorbance at 340 nm in an Ultrospec 3000 spectrophotometer. When the conversion of D-lactate into pyruvate was finished (unchanging absorbance at 340 nm), 50 U of L-lactate dehydrogenase/ml were added to the reaction mixture. In this way, concentration of L-lactate was determined by pyruvate oxidation coupled to NAD^+ reduction, which caused an increase in absorbance at 340 nm. The acid concentration was expressed as g/l of initial medium.

2.5. Determination of free amino acids content

The free amino acid concentration present in the supernatant of the culture medium was analysed as phenylthiocarbamyl derivatives by reverse-phase HPLC with a previous deproteinisation of the samples with acetonitrile (Aristoy & Toldrá, 1991). The amino acid content was expressed as mg/l of initial medium.

2.6. Analysis of volatile compounds

The samples, placed in 15 ml sealed vials, were thawed at room temperature and left at 35 °C for 30 min with stirring (1100 rpm) to equilibrate their headspace. Subsequently, the extraction of volatile compounds was performed by SPME, using a 75 μm Carboxen-PDMS fibre (Supelco), while maintaining the sample at 35 °C stirred (1100 rpm) for 3 h.

The volatile compounds adsorbed by the fibre were desorbed in the injection port of a 5890 Series II Plus gas chromatograph (Hewlett Packard, Palo Alto, CA) for 6 min at 220 °C with the purge valve off (splitless mode). The compounds were separated in a DB-624 capillary column (J & W Scientific, Folsom, CA; 30 m, 0.25 mm i.d., film thickness 1.4 μm) and identified in a 5972 Series Mass-Selective detector (Hewlett Packard). Helium was used as carrier gas at a linear velocity of 27.3 cm/s. The GC oven temperature programme began when the fibre was inserted and was as follows: the temperature was held at 38 °C for 13 min, ramped to 110 °C at 3 °C/min, then to 210 °C at 20 °C/min, and finally held for 10 min at 210 °C. The GC/MS interface was maintained at 240 °C. Mass spectra were obtained by electron impact at 70 eV. Mass spectral data were acquired in the range 25–400 amu.

The individual volatile compounds were identified through their mass spectra by comparison with Nist'98 library, Kovats indices of authentic standards, and previously published values (Flores, Grimm, Toldrá, & Spanier, 1997; Gianelli, Flores, & Toldrá, 2002).

The quantification was based on total ion current (TIC) and the results expressed as mean ± standard deviation, calculated from three replicates analysed at each culture time and at each incubation condition.

2.7. Preparation of cell suspensions

Cells in exponential or stationary growth phase (cultured for 3 or 10 days, respectively) were harvested by centrifugation (10 min at 8000*g*, 4 °C), washed once with cold water, and suspended in water at a ratio of 1 ml of water per gram of the cells (wet weight). The suspensions were stored at −80 °C until further use.

The dry weight of the cell suspensions was determined as the difference between the suspension weight before and after its drying at 105 °C, and was expressed as a percentage of the initial suspension weight. The final result was the mean of two replicates.

2.8. Incubations

The cell suspensions were thawed at 25 °C and diluted in water up to a proportion of 0.082 g of cells (dry weight)/ml. Then, aliquots of 1 ml of the resulting suspensions were incubated with L-Leu, L-Ile and L-Val under different conditions of pH (4.5 or 5.5) and presence/absence of 3% (w/v) of salt and 1% (w/v) of lactate (Table 1). Incubations were performed in 30 ml (2 cm diameter) capped tubes, with 100 U/min oscillatory shaking in an Unitronic 320 OR incubator (J.P. Selecta, Barcelona, Spain), for 6 h at 27 °C. The incubations were terminated by placing the tubes in ice and adding 1 M citric acid up to a final pH of 3. All incubations were performed in triplicate. Blanks having the same composition but without being incubated (addition of 1 M citric acid at zero time), were included.

The incubated samples and blanks were employed for the study of the influence of growth phase, pH, salt and lactate on the generation of volatile compounds from BCAAs by *Debaryomyces* spp. For this purpose, blanks and incubated samples were centrifuged (2000*g*, 15 min, 4 °C) after volume equilibration with cold water. Aliquots were taken from the supernatants and the pH adjusted to 5.5 with 5 M NaOH to avoid potential chemical conversion of reaction products under too acid conditions. After that, cold water and solutions of 21% NaCl, 14% DL-lactic acid/NaOH, pH 5.5, and 1 M citric acid/NaOH, pH 5.5, were added as needed to make equal volumes, and salt, lactate and citrate concentrations

Table 1
Incubation conditions of *Debaryomyces* spp. cell suspensions

Sample	TA[a] (ml)	TB[b] (ml)	LA[c] (ml)	LB[d](ml)	Salt[e] (ml)	Water (ml)
A1	4.5	–	–	–	–	1.5
A2	4.5	–	0.5	–	–	1
A3	4.5	–	–	–	1	0.5
A4	4.5	–	0.5	–	1	–
B1	–	4.5	–	–	–	1.5
B2	–	4.5	–	0.5	–	1
B3	–	4.5	–	–	1	0.5
B4	–	4.5	–	0.5	1	–

[a] TA, 77.8 mM potassium hydrogen phthalate/NaOH buffer, pH 4.5, containing 15.6 mM L-Leu, 15.6 mM L-Ile and 15.6 mM L-Val.
[b] TB, 77.8 mM potassium hydrogen phthalate/NaOH buffer, pH 5.5, containing 15.6 mM L-Leu, 15.6 mM L-Ile and 15.6 mM L-Val.
[c] LA, 14% DL-lactic acid solution/NaOH, pH 4.5.
[d] LB, 14% DL-lactic acid solution/NaOH, pH 5.5.
[e] Salt, 21% NaCl solution.

in all samples. Aliquots of 7.5 ml of each mixture were stored at −20 °C in 15 ml headspace vials sealed with PTFE-faced silicone septum (Supelco) until headspace analysis of the volatile compound, using the SPME technique described above.

3. Results and discussion

3.1. Behaviour of *Debaryomyces* spp. in a meat model system

Debaryomyces spp. was cultured in a meat model system containing meat extract, glucose, DL-lactic acid and NaCl, with an initial pH lower than 4.5, and with limited aeration (static culture). The yeast showed a significant growth, together with an increase in the medium pH of almost 4 units (see Fig. 1(A)), assimilation of lactic acid, especially the D-isomer (Fig. 1(B)), and ammonia generation at the last culture stages (Fig. 1(C)). Also, a consumption of free amino acid, as well as an important production of these compound, depending on the yeast growth phase and the medium

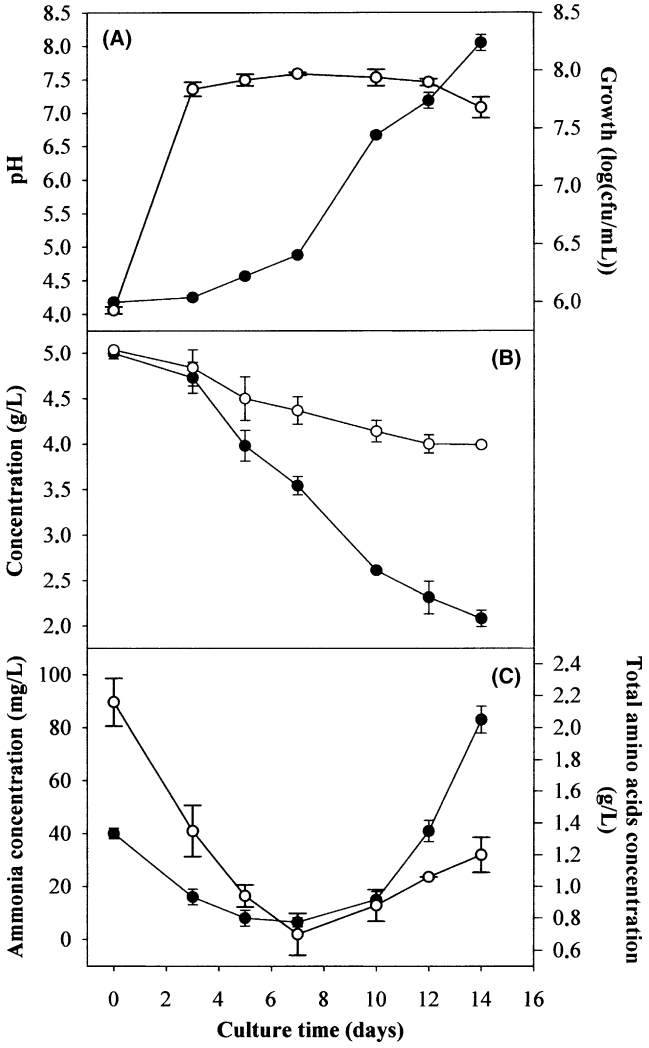

Fig. 1. *Debaryomyces* spp. static culture in the meat model system at 27 °C. Evolution of: (A) growth (○) and medium pH (●); (B) D-lactate (●) and L-lactate (○) concentrations; (C) ammonia (●) and total free amino acid (○) concentrations.

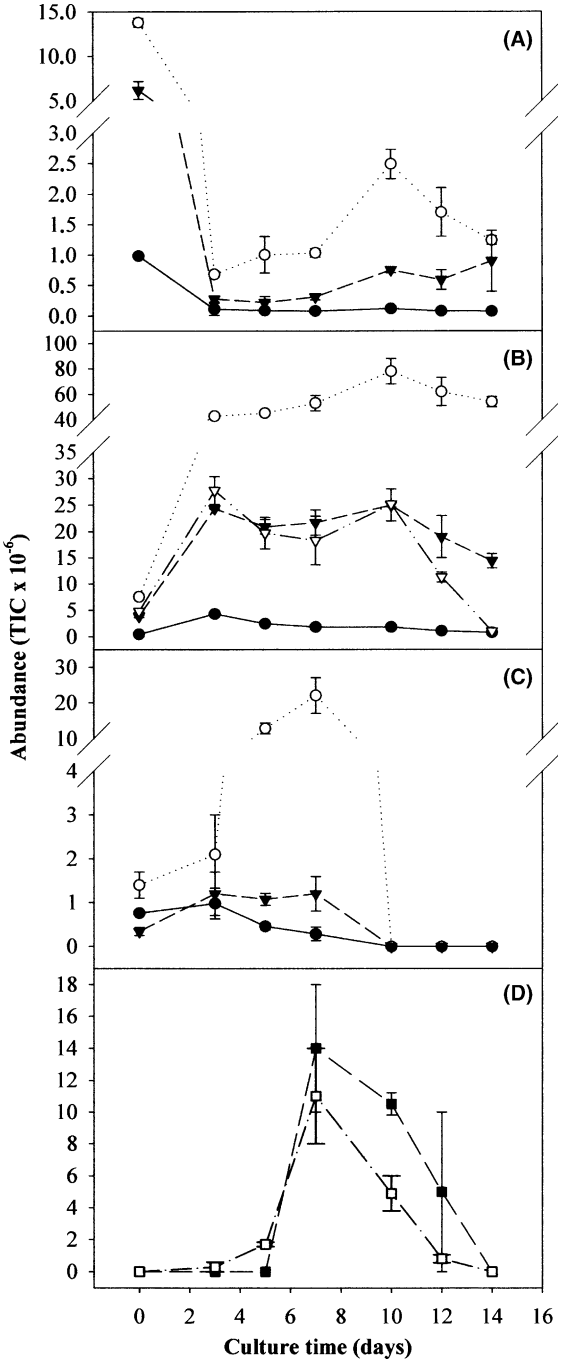

Fig. 2. Generation of volatile aldehydes (A), alcohols (B), acids (C), and esters (D) during the growth of *Debaryomyces* spp. in the meat model system at 27 °C. Branched-chain volatile compounds derived from L-valine (●, – –), L-leucine (○, · · · · · · ·), and L-isoleucine (▲, – – –); ethanol (▽, – – · · – –); ethyl 3-methyl-butanoate (■, – – – –); methyl 3-methyl-butanoate (□, – – · – –).

conditions (Fig. 1(C)), was detected. Moreover, *Debaryomyces* spp. was able to generate several volatile aldehydes, alcohols, acids and esters derived from BCAAs (Val, Leu and Ile). The production patterns of these volatile compounds varied through the culture time (Fig. 2).

Regarding the growth of *Debaryomyces* spp. in the adverse conditions of the meat model system (very acid initial pH, high NaCl concentration, oxygen deficiency), a clear exponential growth phase was observed in coincidence with the 3 first days of culture, in which the cfu number increased by nearly 2 logarithmic units (Fig. 1(A)). In this period, lactate consumption was negligible, but ethanol production was observed (Fig. 2(B)), so it can be assumed that the yeast fermented the glucose of the medium. However, since *Debaryomyces* species are respiratory yeasts (Gancedo & Serrano, 1989), it seems probable that most of the glucose was assimilated (i.e., metabolised through the tricarboxylic acid cycle) requiring consumption of oxygen. *Debaryomyces* spp. could also utilize, in this way, other carbon sources present in the medium, such as free amino acids. Additionally, during the exponential growth phase, the amino acids were used as nitrogen sources, together with ammonia, since a reduction in their concentrations was observed (Fig. 1(C)). Moreover, yeast could obtain free amino acids from peptides and proteins present in the meat extract, due to the possible presence of proteases or proteolytic activity in *Debaryomyces* spp., as described for *D. hansenii* (Santos et al., 2001; Bolumar, Sanz, Aristoy, & Toldrá, 2003a, 2003b).

In yeasts, the end-products of Val, Leu and Ile degradation are the branched-chain alcohols 2-methyl-1-propanol (isobutyl alcohol), 3-methyl-1-butanol (isoamyl alcohol) and 2-methyl-1-butanol (active amyl alcohol), respectively. These compounds are known as "fusel" or higher alcohols (Dickinson, Harrison, Dickinson, & Hewlins, 2000). Recent studies on the catabolism of the BCAAs in *S. cerevisiae* have shown that the conversion of their derived 2-oxoacids to their corresponding alcohols seems to take place through the so-called Ehrlich pathway. This is a catabolic route in

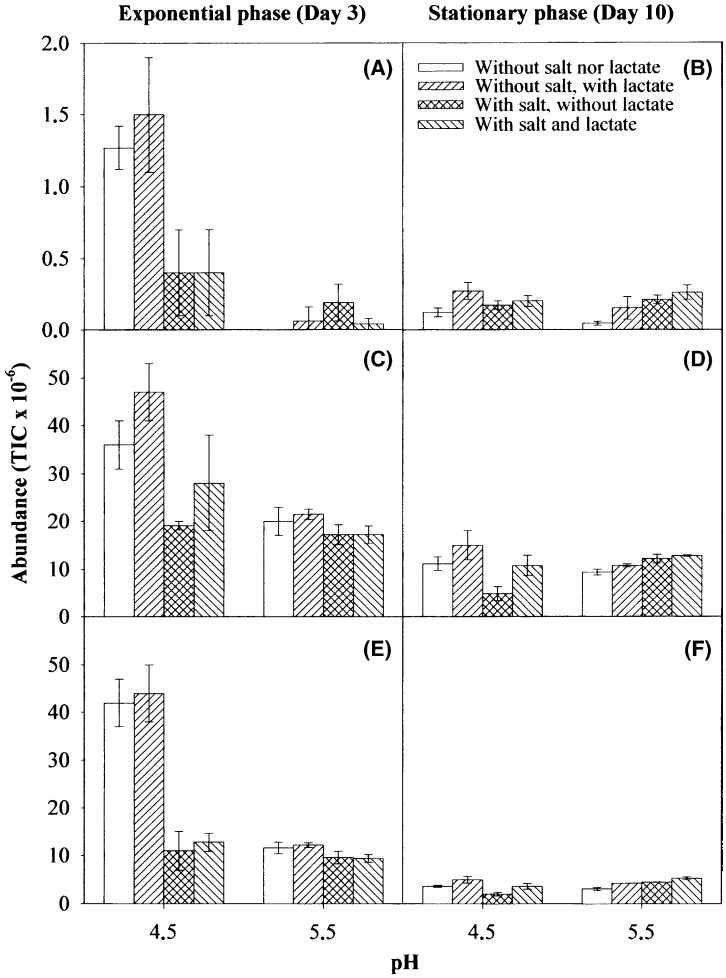

Fig. 3. Generation of 2-methyl-1-propanol (A,B), 3-methyl-1-butanol (C,D), and 2-methyl-1-butanol (E,F) by exponential and stationary growth phase cells of *Debaryomyces* spp. incubated in the presence of Val, Leu and Ile under different conditions of pH, salt and lactate.

which the branched-chain 2-oxoacids are decarboxylated and then, the resulting aldehydes are reduced to give branched-chain alcohols (ter Schure et al., 1998). The enzyme(s) involved in the metabolism of the branched-chain aldehydes to the fusel alcohol end-products are unknown, although they are presumed to be alcohol dehydrogenases (Dickinson et al., 2000).

In our case, the exponentially growing cells yielded a high production of fusel alcohols and decreased amounts of their precursor aldehydes (Figs. 2(A) and (B)). Moreover, generation of branched-chain volatile acids and methyl 3-methyl-butanoate was observed (Figs. 2(C) and (D)). A general increase in branched-chain volatile aldehyde production was detected between days 7 and 10 (Fig. 2(A)). These compounds could originate from 2-oxoacid decarboxylation, acid reduction and/or alcohol oxidation (Larrouture, Masson, Talon, & Montel, 1998). Moreover, in this culture period (days 7 to 10), a general decrease in the amounts of volatile acids and esters was observed in the meat model system (Figs. 2(C) and (D)). It seems probable that yeast was using these compounds as carbon sources.

In consequence, the evolution of the volatile compounds derived from the BCAAs, which have a great impact on dry-cured sausage aroma (Stahnke, 1994, 1995), displayed important variations throughout the culture time, and this circumstance could be related to several factors, such as the cell growth phase, the medium pH, and the presence of substances, such as salt and lactate, in the medium. Therefore, an investigation of the effects caused by these factors on the generation of the volatile alcohols, aldehydes, and acids derived from Val, Leu and Ile was conducted.

3.2. Generation of volatile compounds from branched-chain amino acids by Debaryomyces spp.

In order to establish the influence of dry-cured sausage conditions on volatile compound production from BCAAs by *Debaryomyces* spp., yeast cells grown in the meat model system and harvested on day 3 (exponential growth phase) and day 10 (stationary growth phase) were incubated with Val, Leu and Ile at pH 4.5 or 5.5 and in the presence/absence of salt and lactate. Harvest days were selected, taking into account that, despite the different yeast behaviour observed when the microorganism was either in the exponential growth phase or in the stationary phase on both culture days 3 and 10, maximum volatile compound generation occurred; particularly, alcohols and aldehydes were detected (Fig. 2).

Results (Figs. 3 and 4) show that the production of alcohols and aldehydes in the incubations with BCAAs was higher when day 3-harvested cells were employed than when stationary phase cells were used, which can be easily explained, since cells growing exponentially had a more active Ehrlich pathway than stationary phase cells. The presence in the yeast of some pyruvate decarboxylases and alcohol dehydrogenases intended

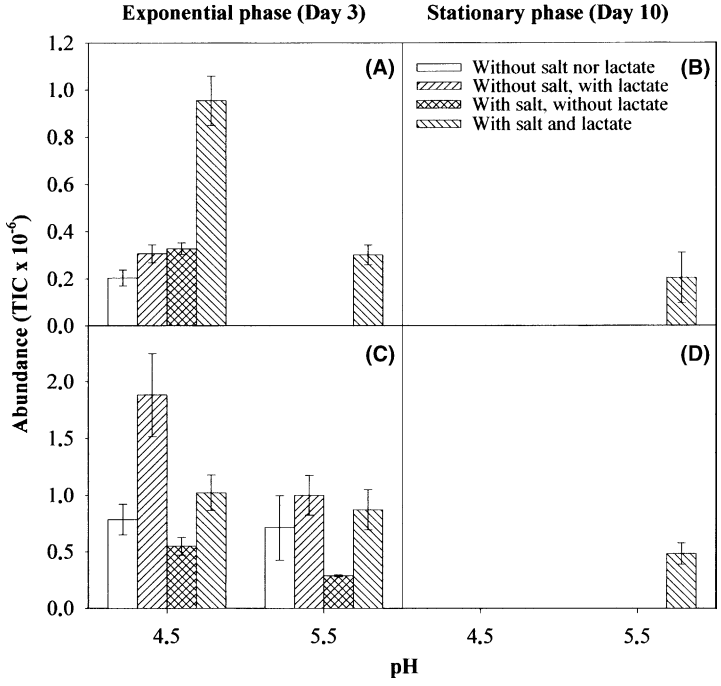

Fig. 4. Generation of 3-methyl-butanal (A,B) and 2-methyl-butanal (C,D) by exponential and stationary growth phase cells of *Debaryomyces* spp. incubated in the presence of Val, Leu and Ile under different conditions of pH, salt and lactate.

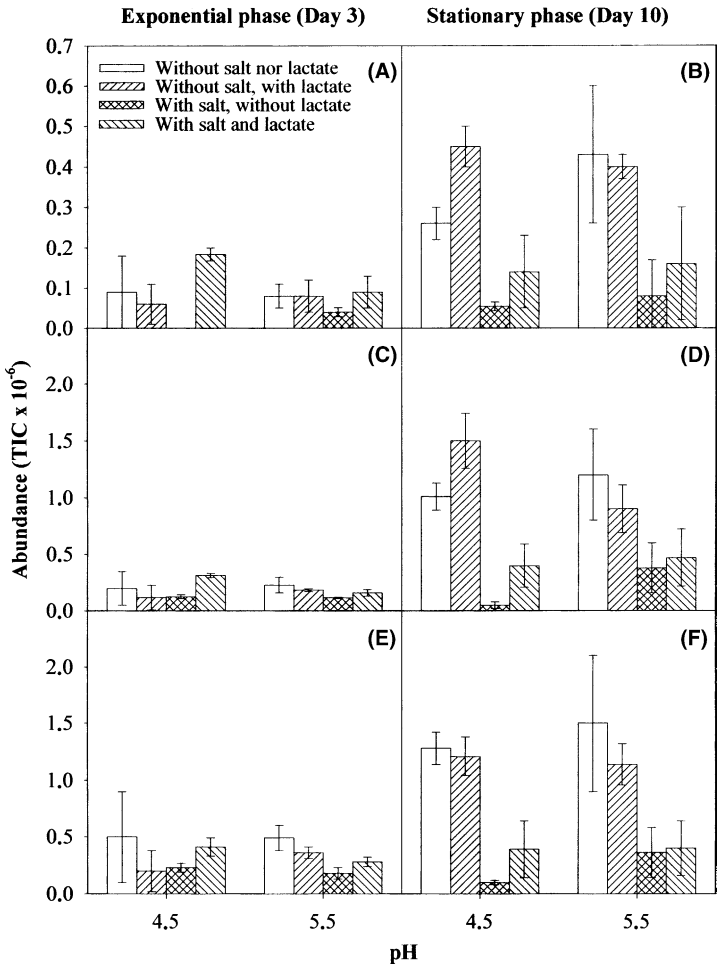

Fig. 5. Generation of 2-methyl-propanoic acid (A,B), 3-methyl-butanoic acid (C,D), and 2-methyl-butanoic acid (E,F) by exponential and stationary growth phase cells of *Debaryomyces* spp. incubated in the presence of Val, Leu and Ile under different conditions of pH, salt and lactate.

for ethanol production (Fig. 2(B)), together with the remaining decarboxylase activity on Leu, could account for the alcohol generation by cells harvested on day 10. Moreover, these cells yielded higher proportions of volatile acids than cells growing exponentially (Fig. 5), suggesting that stationary phase cells did not have suitable alcohol dehydrogenases for converting all the generated aldehydes into alcohols effectively, thus deviating a great part of the aldehydes toward acid production.

The most evident effect of pH was registered on *Debaryomyces* spp. cells growing exponentially since, as a general rule, when pH was increased from 4.5 to 5.5 a negative influence was observed on the alcohol and aldehyde production, especially in the case of 3-methylbutanal (Figs. 3 and 4). This negative effect of the lower acid pH could be related to the fact that cells harvested on day 3 came from a medium with a pH of 4.2 (Fig. 1(A)) and, consequently, they were better adapted to an acid environment.

The presence/absence of salt was very influential in several of the studied situations. Generally, the addition of salt to the incubation media was detrimental for the generation of volatile compounds from BCAAs by *Debaryomyces* spp. This effect was very significant on the alcohol production at pH 4.5 when exponentially growing cells were employed (Fig. 3), and it could be related to the energy requirements when yeast is growing in the presence of salt (Lucas, da Costa, & van Uden, 1990; Lages, Silva-Graça, & Lucas, 1999), since those requirements reduce the amount of energy available for protein synthesis. The high production of branched-chain volatile acids by stationary *Debaryomyces* spp. cells when salt was absent, was also highly significant (Fig. 5). These cells were still able to consume BCAAs as nitrogen source and this fact, combined with the lower osmotic stress (i.e., a more relaxed energetic situation), could lead the yeast to invest more energy in protein synthesis, and thus, its nitrogen requirements increased.

On the other hand, lactate had interesting effects on 3-methyl-1-butanol and aldehyde productions. In the case of the alcohol, higher generation was detected in the presence than in the absence of lactate, especially at pH 4.5 (Figs. 3(C) and (D)), and this fact highlights the

Debaryomyces spp. preference for using Leu as nitrogen source when the medium contains other assimilable carbon sources. The increase in 2-methyl-butanal production by yeast cells harvested on day 3 when lactate was added to the incubation media (Fig. 4(C)) could have a similar explanation; more intense use of Ile as nitrogen source due to a higher concentration of assimilating carbon in the medium. Finally, it has to be pointed out that production of the aldehyde derived from Val could not be detected in any of the assayed conditions.

In conclusion, the behaviour of *Debaryomyces* spp. in the meat model system indicated that the yeast strain had real potential to influence the composition of meat products such as dry-cured sausages, but its action was highly dependent on the changing medium conditions, as well as the cell state. Moreover, the study of the generation capabilities of volatile compounds from BCAAs demonstrated that *Debaryomyces* spp. can modify the aroma pattern of dry-cured sausages since, although the generation of these compounds was, as a general rule, negatively affected by the presence of salt, the pH reduction to 4.5 increased the alcohol and aldehyde yields and did not influence acid generation. On the other hand, despite that the cell transition from exponential to stationary growth phase decreased alcohol and aldehyde production, it increased acid generation at the same time. So, it can be expected that the addition of *Debaryomyces* spp. as a starter culture in dry-cured sausage processing contributes to a gradual release of volatile compound, starting with the generation of alcohols and aldehydes and finishing with acid production.

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