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A survey of yeasts in traditional sausages of southern Italy

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Abstract

The evolution of the yeast population during manufacturing and ripening of 'salsiccia sotto sugna', a typical salami of the Lucania region (southern Italy), was investigated. Four different batches, produced in four farms in Lucania, were studied. Each batch showed a specific yeast population, and the most frequently isolated yeasts belonged to *Debaryomyces hansenii* and its anamorph *Candida famata*, and *Rhodotorula mucilaginosa*. *Yarrowia lipolytica* was isolated from three sausage batches. The *Y. lipolytica* isolates were further characterised, in particular for their lipolytic activity on pork fat. Lipolytic activity was maximal at pH 5.5, with oleic and palmitic acids as major free fatty acids produced. The use of randomly amplified polymorphic DNA-polymerase chain reaction allowed the detection of a high genetic heterogeneity among the isolates phenotypically assigned to the species *Y. lipolytica*.

Keywords: Yeast; Sausage; Ripening; Lipolytic activity

1. Introduction

In Europe, dry fermented sausages have a long tradition originating from Mediterranean countries during Roman times [1]. Many types of fermented sausages have been developed and processing conditions, as well as ingredients and additives, vary among the different types. Industrial production of fermented sausages is increasing and, as a consequence, the introduction of starter cultures has become essential in order to shorten the ripening period, ensure colour development, enhance the flavour and improve product safety [2].

However, the use of commercially available starters, mainly constituted of lactic acid bacteria and micrococci, may also produce an impoverishment of flavour and aroma and a loss of peculiar organoleptic characteristics found in naturally fermented sausages. For this reason, in several European countries, artisanal sausages are still preferred by the consumer and are manufactured by relying on an unknown 'factory flora' [3]. Lactic acid bacteria, micrococci and coagulase-negative staphylococci have the most relevant role in the fermentative process and ripening, but also yeasts and moulds can be involved. Though there are several reports on the yeast populations in various meat products [4], studies on the yeast biodiversity in sausages are limited. The earliest studies on salami [5] reported that *Debaryomyces hansenii* was the most commonly isolated yeast. Most recently, several researches confirmed these results, but other yeast genera were found, such as *Candida*, *Pichia*, *Rhodotorula*, *Hansenula* (synonym of *Pichia*) and *Torulopsis* (synonym of *Candida*) [6–8]. On the basis of this, *D. hansenii* was used as a starter with positive effects on the development of a characteristic yeast flavour and stabilisation of the reddening reaction. *D. hansenii* and its imperfect form *Candida famata* are now used in starter preparations and should be added to the sausage mixture at a concentration of 10^6 cfu g^{-1} [9]. The yeast *Yarrowia lipolytica*, the perfect form of *Candida lipolytica*, has also frequently been isolated from fresh beef

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[4,7] and sausages [10]. Due to its lipolytic and proteolytic activity, this species can have a high technological potential [11].

The present study investigated the growth patterns and biodiversity of yeasts isolated from 'salsiccia sotto sugna' (SSS), an artisanal salami produced in the Lucania region (southern Italy) [12], and involved in its ripening processes. Moreover, the yeast isolates belonging to the species *Y. lipolytica* were studied, and characterised for their genetic polymorphism and lipolytic activity, in order to evaluate their suitability to be used as starter cultures in a productive process.

2. Materials and methods

2.1. Manufacturing of sausages

Dry sausages were produced, according to the traditional protocol for SSS, at farm level in four different areas of the Lucania region in southern Italy: Cancellara, Genzano, Viggianello and Rotonda. The composition of the sausage mixture was always strictly related to the traditional farmer recipes as reported by Amato et al. [12]. In the traditional production of SSS, pork meat and fat are chopped and thoroughly mixed with salt, pepper and other seasonings. The sausage mixture is stuffed in natural casings from the cleaned small intestine of pigs and then spiked to allow entrapped air to escape. The sausages are stored for some days in a warm room to dry (about 24°C at 80% relative humidity (RH) for 24 h and then 18–22°C at 75–80% RH for 5 days) and then stored for 2 months in low-temperature rooms (15–20°C at 80–85% RH). At the end of ripening, the sausages are put in pots, covered with melted pork fat and stored in a cool room (about 10°C). For microbiological and physico-chemical analyses, samples were taken at the stuffing and after different times of ripening (1, 3, 7, 14, 28 and 60 days) and microbiologically analysed according to the procedures described by Samelis et al. [3]. For each sample, three replicates were analysed.

2.2. Isolation and identification of yeasts

Yeasts isolated during production and ripening of sausages were randomly selected from high-dilution Sabouraud agar plates (Oxoid, UK), supplemented with 200 ppm of chloramphenicol (Sigma, USA) to inhibit bacterial growth. Purity was checked by streaking yeasts on Sabouraud agar and pure cultures were kept on agar slants on the same medium at 4°C. Isolates were identified at species level according to Kurtzman and Fell [13] and API ID 32C (Biomerieux, France). Growth in media with high NaCl concentration, at different temperatures, hydrolysis of urea, lipolytic and proteolytic activity were tested according to Yarrow [14].

2.3. Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis

Yeast cells were grown overnight in 5 ml of YEPD medium (1% yeast extract, 2% peptone, 2% glucose, w/v). The DNA was isolated by the method of Querol et al. [15], except that the lytic enzyme from *Rhizoctonia solani* (Sigma, Italy) was used to digest the cell wall.

Amplification reactions were performed with primers M13 and RF2 as described by Andrighetto et al. [16]. RAPD-PCR profiles were analysed with the pattern analysis software package Gel Compar Version 4.0 (Applied Maths, Belgium). The final dendrogram was obtained by means of the unweighted pair group method using arithmetic average clustering algorithm.

2.4. Determination of lipolytic activity of Y. lipolytica

Qualitative tests on pork fat agar were carried out according to Kouker and Jaeger [17].

Quantitative assays for lipolytic activity were performed through the following procedure: *Y. lipolytica* isolates were grown on pork fat agar medium, containing (w/v) 1% yeast extract, 1% peptone, 0.5% NaCl, 2% agar and 5% pork fat. The pH was adjusted at the desired value by adding HCl or NaOH. Lipolytic activity was determined as free fatty acids (FFA) produced after 3 and 6 days of incubation at 28°C. Lipid extraction and FFA determination were carried out on 1 g of homogenate culture medium following the procedure proposed by Lencioni et al. [18]. FFA analysis was performed using a Carlo Erba Mega 5160 gas chromatograph (Carlo Erba, Italy), equipped with a flame ionisation detector and a cold-on-column injector. The sample (1 μl) was injected into a Nukol wide-bore column (15 m × 0.53 mm ID) having a film thickness of 0.5 μm (Supelco, USA). The carrier gas (helium) flow rate was set at 20 ml min^{-1}, the detector temperature at 220°C. The oven temperature was programmed from 90°C to 195°C at 8°C min^{-1}.

The data reported for FFA in each condition are the means of three repetitions. The variability coefficient, expressed as the percentage ratio between the standard deviation and the mean, was always lower than 5%.

3. Results and discussion

3.1. Yeasts isolated during the ripening processes

Fig. 1 shows the changes in the numbers of yeasts during fermentation and ripening of sausages. A great variability was observed in the different samples. The yeast counts before casing ranged from not detectable to about 10^4 cfu g^{-1}, and increased during the first days of fermentation up to 10^5–10^6 cfu g^{-1} in the four farms. Ninety-four yeasts were isolated during the production and ripening

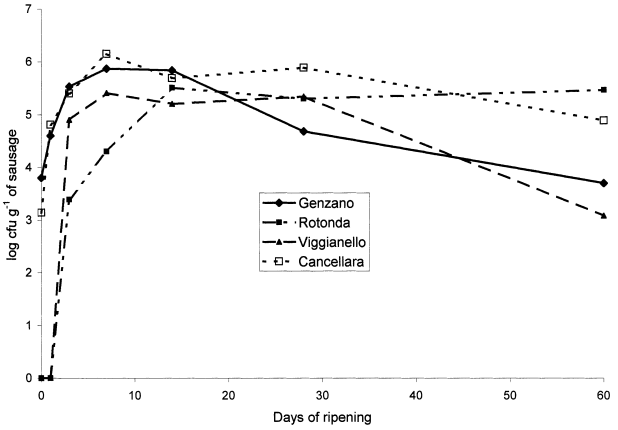

Fig. 1. Evolution of the number of yeasts in four different batches of SSS.

steps and identified. Table 1 reports the percentages of isolates attributed to the different yeast species in the sausages produced in the four different farms. The species *D. hansenii* and its anamorph *C. famata* were isolated from all the sausages and were the dominant species in three batches out of four, representing 52% of the total isolates. The anamorph form was prevalent and represented by the two varieties *C. famata* var. *famata* (37 strains) and *C. famata* var. *flareri* (six strains). Only one of these strains was able to assimilate xylose in spite of the fact that *D. hansenii* is known to assimilate xylose [13]. However, similar discrepancies have been previously reported in *D. hansenii* strains isolated from Greek dry sausages [19]. The attribution of these xylose-negative isolates to the species *D. hansenii* was confirmed by RAPD-PCR analysis (data not shown). Isolates of *Rhodotorula mucilaginosa* were the most frequent yeasts found in sausages produced in Genzano, whereas *Y. lipolytica* was isolated from sausages produced in three of the farms, with the exception of Rotonda, and represented 8.5% of the total isolates.

It is interesting to note that samples with a low initial number of yeasts at the beginning of fermentation were characterised by higher biodiversity than those in which the yeasts were initially present at significant levels. In fact, in Rotonda and Viggianello samples, the yeasts were found to belonged to 10 and 12 different species, respectively.

Table 2 reports some important physico-chemical and taxonomic characteristics of the yeasts isolated from SSS. Most of the isolates could grow in the presence of a high NaCl concentration and at a low temperature; this ability could offer a fundamental ecological advantage for the growth of *D. hansenii*, *R. mucilaginosa*, and *Y. lipolytica* in sausages. Most of the isolates belonging to these species were also positive in urease activity. Hydrolysis of urea is a characteristic of a few ascogenous yeasts, such as *Schizosaccharomyces pombe*, *Y. lipolytica* and *Lipomyces* spp. [20]. Only the *R. mucilaginosa* and *Y. lipolytica* isolates possessed a significant ability to hydrolyse pork fat in vitro. In addition, *Y. lipolytica* isolates showed an enhanced proteolytic activity in skim milk and were further studied, in order to better understand their characteristics and their impact on the final quality of sausages.

Table 1
Yeast species isolated from four different batches of sausages during ripening

Species	Production site			
	Genzano	Rotonda	Cancellara	Viggianello
C. boidinii	–[a]	5.9	–	3.7
C. cariosilignicola	–	–	–	3.7
C. citrea	–	5.9	–	3.7
C. diversa	–	5.9	–	3.7
C. domercqiae	–	5.9	–	–
C. edax	–	5.9	–	–
C. famata	26.3	29.4	83.9	25.9
C. hellenica	–	11.8	–	–
C. maltosa	–	–	–	3.7
C. pintolopesii	–	5.9	–	3.7
C. silvanorum	–	–	3.2	–
C. sphaerica	–	–	3.2	–
C. valvidiana	–	5.9		–
D. hansenii	–	–	–	22.2
R. mucilaginosa	58.7	17.6	–	3.7
R. glutinis	5.3	–	–	3.7
S. cerevisiae	–	–	–	11.1
Y. lipolytica	10.6	–	9.7	11.1
Number of isolates	19	17	31	27

The frequency is expressed as percentage of isolates for each species.
[a]No isolate.

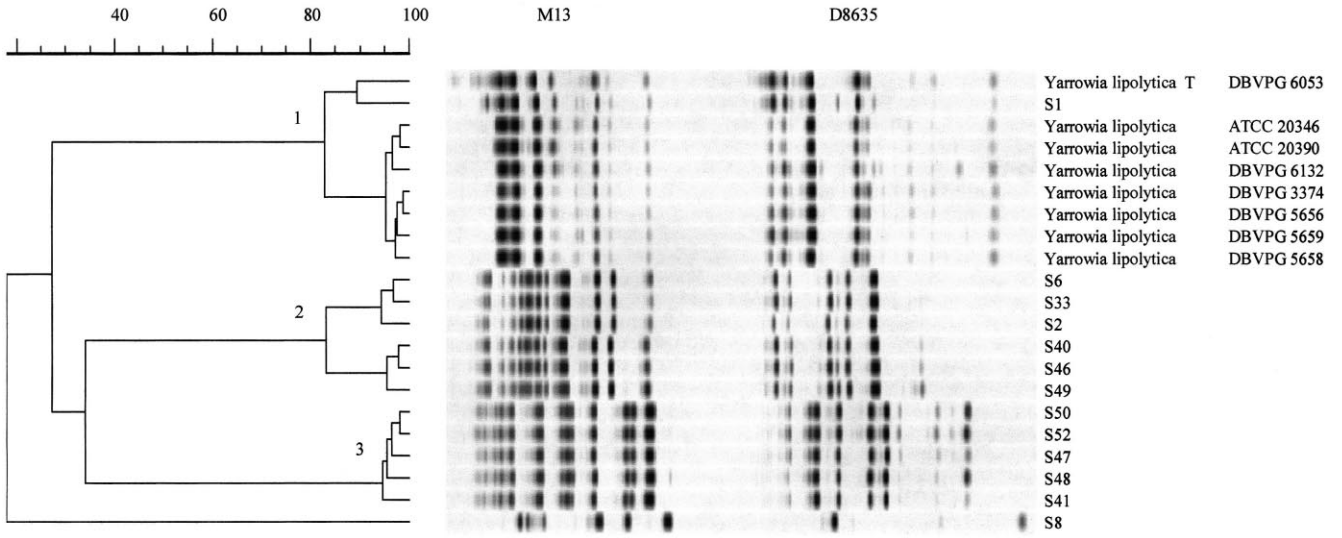

Fig. 2. Cluster analysis of RAPD-PCR patterns generated with M13 and RF2 primers. *Y. lipolytica* type and reference strains were obtained from the Dipartimento di Biologia Vegetale of Perugia University (DBVPG) and American Type Culture Collection (ATCC).

3.2. Phenotypic and genetic characteristics of *Y. lipolytica* from dry sausages

Eight yeasts isolated from the sausages were phenotypically identified as *Y. lipolytica*. All the strains assimilated erythritol, lactic and citric acids, possessed enhanced lipolytic and proteolytic activity, had resistance up to 10% NaCl and to 100 ppm actidione, and were able to grow at 10°C. Some phenotypic differences were observed in mannitol (37.5% of positive isolates) and succinic acid assimilation (25%). Three strains were able to grow at 37°C.

Y. lipolytica strains were studied at the genetic level by means of RAPD-PCR using the primers M13 and RF2. In order to have a larger pool of strains, besides the strains studied in detail at the biochemical level and five other strains (S40, S41, S46, S47, S52) previously isolated from SSS [12], seven reference strains from different origins and the type strain of the species were included in the RAPD-PCR analysis. The dendrogram derived from the combined analysis of the M13 and RF2 RAPD-PCR profiles showed the presence of three distinctive clusters and one independent strain (Fig. 2). Cluster 1 grouped one strain isolated from sausages (strain S1) as well as all the reference strains and the type strain of the species, while the remaining sausage strains, except strain S8, were all grouped in clusters 2 and 3. These two groups of strains isolated from the sausages showed very different RAPD-PCR profiles (34% similarity) and were also quite far from the type and the reference strains of the species (27% of similarity), even if some common bands in their M13 profile were detected. However, on the basis of their phenotypic characteristics and API ID 32 C analysis, all the strains were clearly assigned to the species *Y. lipolytica*. The low similarity of the studied strains with the type and the reference strains of the species could be related to the particularly selective habitat present in this kind of sausage. In order to better understand this high intraspecific

Table 2
Evaluation of some characteristics of the yeasts isolated from sausages

Characteristic	*D. hanseniil/C. famata*	*R. mucilaginosa*	*Y. lipolytica*	*Candida* spp.
Number of strains tested	49	15	8	28
Resistance to NaCl:				
5%	49[a]	15	8	16
10%	45	14	8	7
15%	43	0	0	4
Growth at:				
10°C	45	15	8	17
37°C	8	12	3	11
40°C	0	0	0	0
Proteolysis on skim milk	0	0	8	0
Pork fat hydrolysis	0	15	8	0
Urease	30	15	8	9

All the tests were carried out according to Yarrow [14].
[a]Number of strains positive for the characteristic tested.

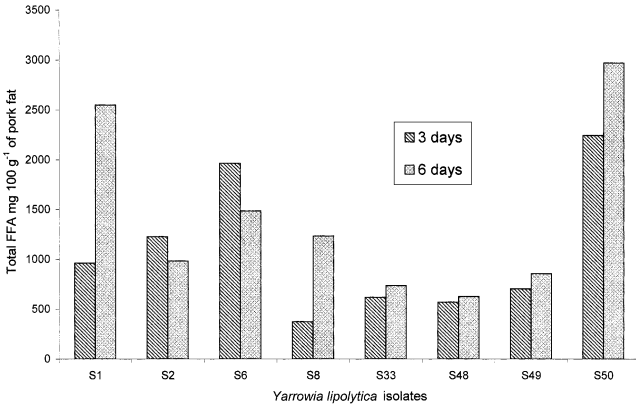

Fig. 3. Amount of FFA detected in a medium at pH 7.0, inoculated with eight *Y. lipolytica* strains and incubated for 3 and 6 days at 28°C.

genetic heterogeneity, a larger number of strains need to be investigated.

3.3. Lipolytic activity of Y. lipolytica on pork fat

The typical flavour of dry sausages is due to acids produced during fermentation, salt and different compounds derived from the catabolism of sugar, lipids and proteins. Among the flavour products identified in dry sausages, the oxidation products of lipids account for about 60% of the total compounds which influence the flavour [21]. The distinctive flavours of these products were found to be related, at least in part, to hydrolytic and oxidative changes occurring in the lipid fraction during ripening [21]. On the basis of these considerations, the isolates of *Y. lipolytica*, which were characterised by the higher lipolytic potential, were further investigated by analysing the FFA hydrolysed from the pork fat by this yeast at pH 7.0, according to the method of Muderhawa et al. [22]. These authors found that this pH value was optimum for the lipolytic activity of *Y. lipolytica* growing on pork fat. In a medium containing pork fat (5%), and adjusted at pH 7.0, the cell numbers of the eight *Y. lipolytica* isolates increased from 10^5 cfu g^{-1} up to 10^7 and 10^8 after 3 and 6 days of incubation, respectively.

All the strains were able to hydrolyse pork fat, even if at different levels (Fig. 3). The isolates S1, S8 and S50 produced higher amounts of FFA in the medium after 6 days. The isolates S33, S48 and S49 produced relatively low amounts of FFA, without significant differences in relation to the incubation time. The isolates S2 and S6 were responsible for a slight decrease of total FFA in the medium after 6 days of incubation. The decrease of total FFA content after 6 days of growth could be due to the fact that the unsaturated FFAs can be further metabolised by *Y. lipolytica* or oxidised to flavour compounds [23].

However, the relatively low total FFA amounts detected in these conditions induced to verify the effect of pH values on the lipolytic activity of *Y. lipolytica*. For these further analyses, the strain S2 was chosen because it is characterised by a mean production of total FFA at pH 7.0 among the strains tested.

The isolate was grown at different pH, ranging from 5.0 to 7.0, and the amount of total FFA, as well as the amounts of the principal fatty acids, were determined (Table 3). The highest lipolytic activity was observed at pH 5.5, while it was strongly reduced at other pH values. The activity of many microbial lipases appears to be very sensitive to pH even though Linfield et al. [24] found that between pH 4.8 and 7.2 no appreciable change occurred in the extent of lipolysis due to a *Candida rugosa* lipase. On the other hand, an optimum pH of 6.5 for lipases of *D. hansenii* has been determined by Sørensen and Samuelsen [25]. All the FFA detected under the analytical conditions adopted in this work (C16:0, C18:0, C18:1, C18:2) were found at their maximum concentration in the medium at pH 5.5. Only the fatty acid C16:1 was produced in higher proportion at pH 6.5, but its presence was rather limited with respect to the total amount. The major products of lipolytic activity of *Y. lipolytica* S2 were, in decreasing order, oleic, palmitic, stearic, linoleic and myristic acids. These results clearly indicate a specificity of the lipolytic enzymes for the positions *sn*1 and *sn*3 of the triglycerides. In fact, these are the positions of the triglycerides most frequently occupied by unsaturated fatty acids in pork fat. Nearly 30% of oleic and linoleic acids are esterified in the position *sn*1 of the triglycerides and about 50–60% of the same fatty acids appear in position *sn*3 [26]. The differ-

Table 3
FFAs expressed as mg per 100 g (and relative percentages) produced by *Y. lipolytica* S2 grown on pork fat at different pH levels at 28°C

FFA	pH				
	5	5.5	6	6.5	7
Total FFA	6333	29 777	11 388	1 733	294
C14:0	0.0 (0.0%)	104 (0.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
C16:0	1 592 (25.1%)	8 986 (30.2%)	2 142 (18.8)	0 (0.0%)	61 (21.0%)
C16:1	0 (0.0%)	96 (0.3%)	236 (2.1%)	243 (14.1%)	0 (0.0%)
C18:0	1 318 (20.8%)	5 575 (18.7%)	1 868 (16.4)	444 (25.6%)	98 (33.4%)
C18:1	3 168 (50.0%)	13 717 (46.0%)	6 817 (59.8%)	1 044 (60.3%)	134 (45.6%)
C18:2	255 (4.1%)	1 298 (4.4%)	324 (2.9%)	0 (0.0%)	0 (0.0%)
Saturated FFA	2 910 (45.9%)	14 665 (49.3%)	4 010 (35.2%)	444 (25.6)	160 (54.4)
Unsaturated FFA	3 423 (54.1%)	15 112 (50.7%)	7 377 (64.8%)	1 288 (74.4%)	134 (45.6%)

ences observed for oleic and linoleic acids may be related to a specificity for fatty acid structure, in particular for oleic acid, because both positional and structural specificity are known to occur in microbial lipases [27]. However, when the mean percentages of major fatty acids produced by *Y. lipolytica* S2 were compared with the mean relative percentages of fatty acids present in pig fat triglycerides, it appeared that this lipase was not 1–3-specific. In fact, no significant difference was evidenced between the percentage of the fatty acid produced by *Y. lipolytica* S2 and the typical fatty acid composition of pork fat, with the exception of the fatty acid C18:2, which was present in the pH 5.5 medium, to a lesser extent (about 4.2%) with respect to the pork fat mean composition (11.0%). On the other hand, a slight increase was observed for C18:0 (17.9% against 14.0% of pork fat) and C16:0 (28.9% against 25.0% of pork fat).

Under the same analytical conditions, acetic acid was also monitored. This organic acid, being a metabolic product of *Y. lipolytica*, can be related with the growth extent of the yeast in the medium via β-oxidation. Its concentration was relatively constant, independent of pH, with a mean value of about 1600 mg per 100 g. Only in the medium at pH 7.0, its amount fell under the value of 1000 mg per 100 g.

Table 3 also shows the relative percentage of saturated and unsaturated FFAs produced by *Y. lipolytica* S2 from pork fat. The saturated and unsaturated fatty acids were present at similar concentrations at pH 5.5, while at the other pH values the saturated fatty acids were at lower concentrations. This indicates a possible tendency of these lipases to favour, at pH 5.5, the liberation of saturated FFA rather than unsaturated. This tendency could have a positive effect by reducing the possibility of rancidity, because this mainly involves polyunsaturated FFAs. Similar results were obtained by Zalacain et al. [28], who added lipase from *Candida cylindracea* to a traditional formulation of a dry fermented sausage. Microbial and pancreatic lipases have been used to accelerate flavour development of fermented sausages [25,29,30]. Therefore, it appears possible to use *Y. lipolytica* as a starter to the traditional formulation of dry fermented sausages. It can give rise to a shortening of the ripening time in relation to the evolution of the lipid fraction and also in relation to protein breakdown, taking into account its proteolytic activity.

In conclusion, dry fermented sausages produced by natural fermentation were generally characterised by the presence of a relevant number of yeasts. Among the dominant species isolated from SSS, common phenotypic characteristics can be identified, such as tolerance to NaCl, growth at a low temperature and urease activity. These characteristics, together with lipolytic activity of several isolates, may contribute to the prevalence and colonisation of the habitat of salami by particular specialised yeasts. The presence of these yeasts can cause an increased ammonia production, a higher FFA, an increase in pH and a decrease in lactic acid content in the sausages, contributing to the characteristics of the final product. In addition, lipolytic activity may be modulated by the presence of a specific yeast species, such as *Y. lipolytica*.

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