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Dynamics of the yeast flora in artisanal country style and industrial dry cured sausage (yeast in fermented sausage)

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ABSTRACT

Yeast can act as an adjunct in the sausage-making process as a way to prevent or reduce excessive acidification during aging of products. Two kinds of process were studied: industrial and artisanal country style. Three hundred and fifty three yeast strains were isolated, characterized and identified by biochemical and molecular techniques. Evolution of pH, Aw, weight loss, bacterial growth and proteolytic and lipolytic activity was studied. Final pH in artisanal country style product was higher than in the industrial sausage. There was little difference noted between final weights of products but it was observed a lower yeast count in artisanal country style sausage. No relevant difference was observed in center or surface yeast count in both products. The biochemical assay identified six yeast genera and the molecular test confirmed four different genera, and further analysis showed predominance of the genera *Debaryomyces*. The relations between this four genera and isolation point (center or surface of sausage) were established. The presence of yeast in the center/surface of ART sausage was more prevalent than in the same places of industrial sausage.

1. Introduction

Dry-cured sausages are a popular meat product enjoyed by millions of consumers worldwide. Their acceptance by consumers relies mainly on sensory quality. Aroma is one of the most important parameters for product quality, and this is affected by raw materials, processing techniques and fermentation quality (Dirinck, Van Opstaele, & Vandendriessche, 1997; Sánchez-Peña, Luna, Gárcia-Gomez, & Aparicio, 2005).

The production of dry sausage was defined as an art based on ancient experience and the customs of each region. However, the most important aspect of modern fermented sausage manufacture, is to produce a product to the required standards (safety, consumer acceptability, shelf-life) in the minimum time so as to reduce holding periods which constitute a cost factor to production. Then for this purpose every day new technologies are development.

In the artisanal country style (ART) process, natural meat fermentation is allowed to occur over extended time periods, bringing about a gradual decrease in pH and drying-out of the product. This process has been progressively replaced by industrial rapid curing methods that use controlled drying chambers and starter cultures to guarantee the safety and quality of the final products (Coventry & Hickey, 1991).

There are reports in the literature which demonstrate that some species of yeasts contribute to flavor and texture development during the curing of various products (Arboles & Julia, 1999; Boissonnet et al., 1994; Deiana et al., 1990; Miteva, Kirova, Gadjeva, & Radeva, 1986; Viljoen & Greyling, 1995). Some studies have shown the influence of yeast strains on the development of the characteristic flavor of dry-cured meat products (Durá, Flores, & Toldrá, 2004; Flores, Durá, Marco, & Toldrá, 2004; Jessen, 1995; Martín, Córdoba, Aranda, Córdoba, & Asensio, 2006). Furthermore, differences in flavor development associated with particular yeast species and biotypes growing on ham have been recently reported (Andrade, Córdoba, Sánchez, Casado, & Rodríguez, 2009).

Yeasts are usually found in high numbers in dry-cured products, especially fermented sausages; even they are not added in methods of production. These high levels suggested that this micro-organism may play an important role in the maturation process (Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010). Despite the desirable effect that yeast may have on meat products, no special attention has been given to studying, identifying or quantifying the yeast species present in dry-cured fermented sausage.

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The contribution of yeast to flavor and aroma in a variety of products is directly attributed to the ability of some species to ferment different sugars and as a result produce ethanol, acetaldehyde, ethyl acetate and other compounds. Amino acid degradation leads to generation of volatile molecules during the processing, contributing to the typical flavor of dry-cured sausages (Durá et al., 2004). Yeast species also play a synergistic role by metabolizing the lactic acid present in fermented products, which causes a shift in pH toward neutrality and thus produces a sweeter end product.

The composition of yeast flora is an important determining factor for the quality and sensory characteristics of meat products. In this study, we endeavored to investigate yeast growth during the manufacturing process of ART and industrial style (IND) Spanish dry-cured sausage (Salchichón).

2. Materials and methods

2.1. Sausage manufacture and sampling

The formulation of the two batches of sausages included lean pork and pork fat (1:1), 2.8% (w/w) NaCl, 3.0% (w/w) lactose, 1.0% (w/w) sodium caseinate, 0.5% (w/w) sodium ascorbate, 0.02% (w/w) spices, 0.030% (w/w) KNO_3 (only for batch 1, ART) or 0.015% (w/w) $NaNO_2$ (only for batch 2, IND). Batch 2 also contained 0.01% of lactic acid starter MC-156 (*Lactobacillus sake*, *Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Staphylococcus xylosus* – from Textel, Europe Rhone Poulenc, Madrid, Spain).

The lean meat and fat were chilled at 4 °C for two days and kept at −5 °C overnight. They were then minced through a 6 mm mincer. The meat components and remaining ingredients were mixed under vacuum (Fatosa Mixer, Model AV80, 60 mm Hg) for two in both directions. Afterwardthe mixture was stuffed into 75/80 mm diameter collagen casing (Fibran). The final weight of each sausage was approximately 500 g.

The ART products were kept at 20–22 °C and 75% RH for 6 h and, then at 85–90 % RH and the same temperature for three days to promote product fermentation. The IND products were kept at 24–26 °C and 75% RH for 6 hand then at 85–90 % RH and the same temperature for three days for fermentation. The ART and IND sausages were dried at 12–15 °C and 70–75 % RH for 50 days.

Samples were taken for analysis at different stages during the processing: raw minced meat (0 day), after the initial pH drop/final fermentation (3rd day), at the beginning of curing (7th day), during curing (10/20/30 days) and at the end of curing (50th day). Four samples were taken each day for each treatment.

2.2. Microbiological analyses

For each sausage, samples of 25 g were taken aseptically from the inner part and 25 g were taken aseptically from the surface. The samples were homogenized with peptone water 0.1% (ADSA Micro, Barcelona, Spain) (w/v) in a blender (Stomacher Model 400) for 3 min. Serial decimal dilutions were made using the same medium, then plated in duplicate for yeast count in Rose Bengal chloramphenicol agar (ADSA Micro) and incubated at 27 °C for 48 h. Five representative yeast isolates were sub-cultured on yeast Malt Extract Agar (YM, ADSA Micro) and incubated for 48 h at 27 °C for control of purity by colony morphology and microscopy. The pure cultures were stored at 4 °C on Malt Extract Agar (MA, ADSA Micro) during the period of investigations.

Tributyrin Agar, Gelatin and Milk Agar were used as the medium to estimate lipolytic and proteolytic activity of yeast isolates (Besançon et al., 1992; Boissonnet et al., 1994).

2.3. Characterization and identification of isolates

2.3.1. Biochemical tests

The representative yeast isolates were classified according to the identification keys described by Van der Walt and Yarrow (1984) and by Barnett, Payne, and Yarrow (2000). The tests used for the genus and species determinations were: pseudomycelium or true mycelium formation on Corn Meal Agar (CMA, Difco), ascopore formation, carbohydrate fermentation and nitrate assimilation, formation of extracellular amyloidal compounds (starch test), production of ammonia from Urea (Urease test), assimilation of carbon compounds, resistance to 0.01 and 0.1% cycloheximide and growth on vitamin-free medium. Colony appearance, pigment and extracellular polysaccharide formations were noted by streaking onto MA.

2.3.2. Molecular characterization

2.3.2.1. PCR and analysis of rDNA. DNA was extracted and prepared according to the method used by Querol, Barrio, and Rámon (1992). The primers used to amplify the ITS region (ITS1-5′TCCGTAGGTGAACCTGCGG3′ and ITS4-5′TCCTCCGCTTATTGATATG C3′) were described by White, Bruns, Lee, and Taylor (1990). The separation of the fragments was performed by electrophoresis on 3% agarose gel in TAE buffer. The marker used was 100 bp DNA ladder (Biolabs, Inc), and bands were visualized in a Vilber Lourmat ultraviolet light transilluminator after gel had been stained with ethidium bromide.

2.3.2.2. Amplification and sequencing of mitochondrial DNA (mtDNA). The mitochondrial genes G_1C_1 A_1T_1 were amplified and sequenced according to Querol et al. (1992), with the following restriction enzymes: *Cfo*I, *Hae*III, *Hinf*I. Restriction enzymes were used according to the provider's recommendations (Boehringer Mannheim). The separation of the fragments was performed by electrophoresis on 1% agarose gel in TBE buffer. The marker used was DNAλ digested with PstI (Boehringer Mannheim). The bands were visualized in a Vilber Lourmat ultraviolet light transilluminator after gel had been stained with ethidium bromide.

2.3.3. Analytical methods

The pH of sausage was determined in each sample obtained by blending 10 g of sample with 30 mL of distilled water and measured in an Orion Research pH meter (Expandable Ion Analyzer EA920, Boston, USA).

Ten sausages were randomly picked from each batch, and the weight loss was measured by weighing the selected sausages each day throughout the processing period (day 0 to day 50). Water activity was measured with a Humtat-RC Novasina apparatus.

3. Results

3.1. Evolution of yeast flora and pH during sausage processing

The evolution of yeast flora during the processing of artisanal country style (ART) and industrial (IND) sausages is reflected in Fig. 1. The viable yeast count increased from an initial count of 3.43 log UFC/g to 5.03 on surface for ART samples and increased from 3.54 log UFC/g to 4.71 for IND samples, during the first seven days. A slight decrease in the viable count occurred between seven and 10 days, once again increasing during the next period. The viable yeast count between ART and IND sausages for surface count (continuous line) was very similar and corresponded to other reports in the literature (Banks & Board, 1987; Boissonnett et al., 1994; Fung & Liang, 1990; Molina, Silla, & Flores, 1990; Smith & Palumbo, 1973). The viable yeast count (point line), in the inner

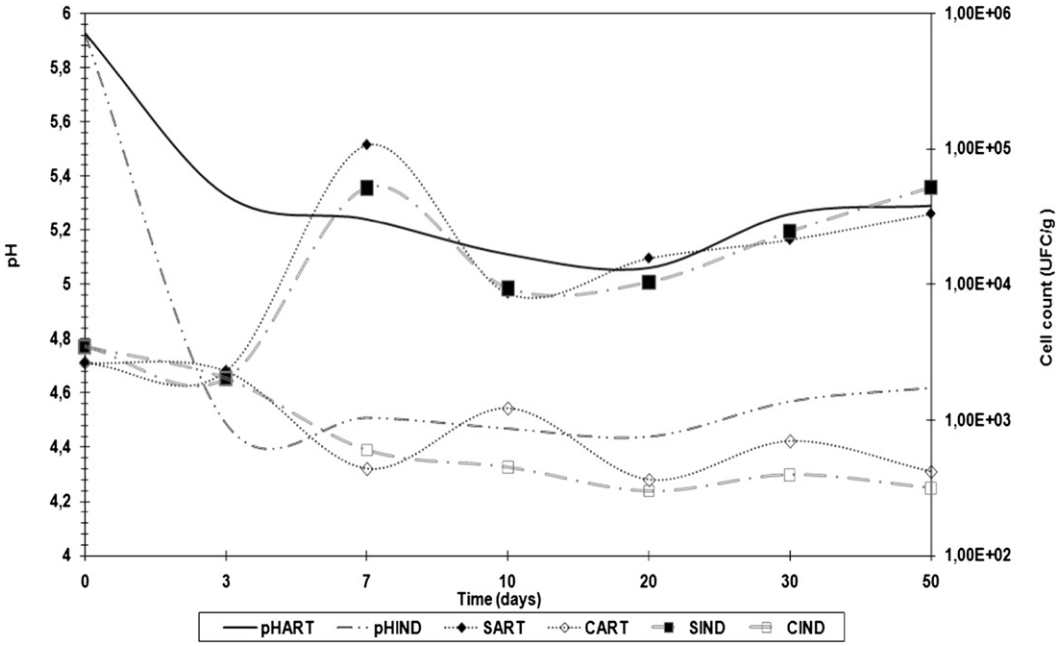

Fig. 1. Evolution of yeast flora and pH changes in the center (c) and surface (s) of sausages (ART and IND) during sausage processing time.

part of both batches of sausage decreased slightly but constantly (initially 3.5 log UFC/g) during the entire processing; it reached 2.5 log UFC/g viable cells in the final maturation period.

The fall in pH was significantly greater in IND sausages, decreasing from 5.9 to 4.4. For the ART sausages it fell from 5.8 to 5.3 during the first three days of processing. Subsequently, the pH increased, reaching 4.6 and 5.25 respectively at the end of the maturation period. There was a significant difference in the falls in pH between the two batches (Fig. 2). The final increase in the pH may be due to the formation of N non-protein compounds and basic ammonium ions coupled with buffering actions of protein (Astiasaran, Villanueva, & Bello, 1990; Demeyer, Verplaetse, & Gistelinck, 1987; Patrignani et al., 2007).

3.2. Evolution of weight loss and Aw

Total weight loss and Aw for the samples are shown in Fig. 2. Both types of sausage lost moisture regularly during processing and Aw changed little during the early part of the curing period, decreasing substantially only toward the end. The results obtained were very similar to those reported for analogous meat products (Andrade et al., 2010; Gökalp, 1986; Lücke, 1986), and these values are strongly dependent upon the characteristics of sausages and the curing period.

3.3. Biochemical characterization and identification of isolates

A total of 353 yeast strains were isolated from ART and IND sausages. The strains were identified by morphological and physiological characters, comparing the test results with tables from Van der Walt and Yarrow (1984) and Barnett et al. (2000). Morphological data were also established for all isolates, serving especially as identification criteria in cases where closely related species showed a high degree of similarity. Tables 1 and 2 show the distribution of yeast isolates by product processing style and location of isolating.

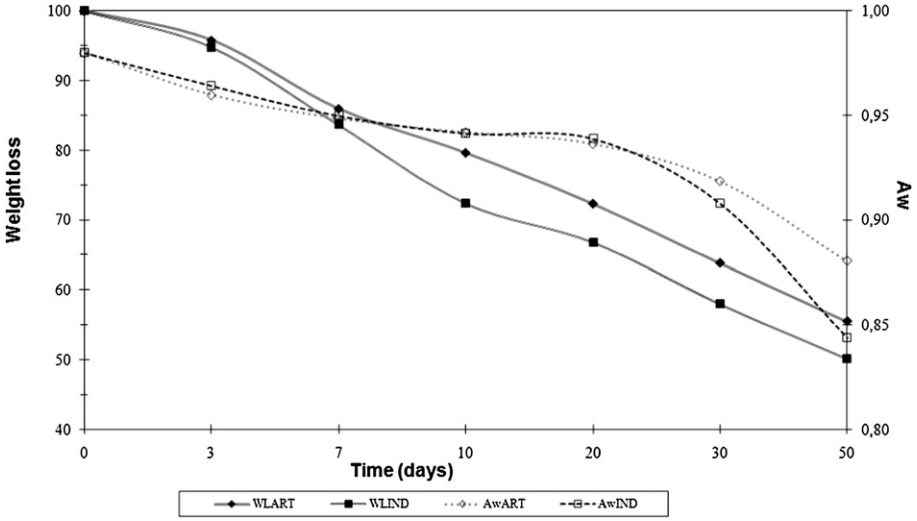

Fig. 2. Weight loss (Lw) and changes in Aw of sausages (ART and IND) during sausage processing.

Table 1
Frequency (%) of yeast strain distribution in ART sausages.

Species	Mixture	Artisanal country style											
		Center						Surface					
		3	7	10	20	30	50	3	7	10	20	30	50
Rhodotorula mucilaginosa	30.0	8.3	18.8	23.5	14.3	18.2	22.2	0.0	0.0	0.0	0.0	0.0	0.0
Yarrowia lipolitica	25.0	8.3	18.8	17.7	7.1	9.1	0.0	0.0	12.5	0.0	12.5	0.0	0.0
Candida parapsilosis	0.0	0.0	0.00	0.00	7.1	0.0	0.0	0.0	16.7	0.0	31.3	0.0	0.0
Debaryomyces spp	45.0	83.4	62.4	58.8	71.5	72.7	77.8	0.0	70.8	100	56.2	100	100

The yeast strains isolated represented both pigmented and non-pigmented yeasts. The majority of strains were identified as belonging to the genus *Debaryomyces*, followed by *Candida*, *Rhodotorula*, *Trichosporon*, *Yarrowia* and *Pichia* in ART and IND sausage. With the exception of *Rhodotorula* and *Trichosporon* the other species showed ascomycetous affinity. The occurrence of these strains, principally the genera, in association with meat products, corresponded with results in the literature, notably Banks and Board (1987), Molina et al. (1990), Dillon and Board (1991), Viljoen, Dykes, Callis, and Holy (1993), Boissonnet et al. (1994).

The evaluation of enzymatic characteristics of yeasts isolated in our study showed that all micro-organisms presented lipolytic activity. This may therefore play a significant role in the development of sensory characteristics, as has been reported in other studies (Andrade et al., 2010; Dillon & Board, 1991; Gökalp, 1986; Guerzoni, Lanciotti, & Marchetti, 1993; Lücke, 1986; Miteva et al., 1986). Additionally, their lipolytic activity may enable them to compete successfully with microflora (Aboukheir & Kilbertus, 1974; Dillon & Board, 1991; Guerzoni et al., 1993; Patrignani et al., 2007). The percentage and distribution of yeast strains showing proteolytic activity are reported in Table 3. These results showed that proteolytic yeasts were mostly isolated from ART sausage and from the center portion. This finding reflects the variety of strains isolated from this sausage and is in agreement with the results of Guerzoni et al. (1993) and Viljoen et al. (1993), which showed proteolytic activity principally associated with *Trichosporon* and *Yarrowia* strains.

3.4. Molecular identification of yeasts isolated

The ITS1 and ITS4 primers used to amplify a region of the rRNA gene repeat unit includes two noncoding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The PCR product of amplification of some isolates, taken as examples is shown in Fig. 3. As a result of this analysis, the isolates were grouped in three groups: P1, P2 and P3, respectively showing length variation in this region for the each group by 600–700 bp, 500–600 bp and 300–400 bp. These PCR products were digested with three restriction endonucleases: *Hin*fI, *Cfo*I, and *Hae*III. The approximate length of the amplified product and the restriction fragments observed after digestion with the three restriction endonucleases for all the strains studied are summarized in Fig. 4. After digestion it could be noted that there was a new pattern. The isolates of pattern P1 show a new behavior under restriction enzymes action, which were then placed in a new group, called P4 (Fig. 5). Table 4 shows the approximate molecular size of amplified products and their digestion with the three enzymes used. The restriction patterns obtained in this study are similar to those described by Guillamón, Sabaté, Barrio, Cano, and Querol (1998) and Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999) for the species *Rhodotorula mucilaginosa* (P1), *Candida parapsilosis* (P3) and *Yarrowia lipolytica* (P2). The isolates belonged to the group whose pattern, P4, is presented by Ramos, Valente, Hangler, and Leoncine (1998) and Esteve-Zarzoso et al. (1999) as containing isolates characterized as belonging to the genus *Debaryomyces*.

4. Discussion

Conditions adopted for fermentation, such as temperature and residence time in production chambers, are crucial for maturation of sausages. The pH changes occurring during the process are extremely important, since this parameter greatly affects drying, as well as the microflora responsible for the maturation of the meat mixture (Bacus, 1986; Moreno, 1979).

In our investigations the pH at the final stage of sausage maturation showed a slight increase, which may be due to the production of amines and ammonium at this stage of the process, in accordance with data obtained by Lücke (1986).

The results showed that despite a significant loss of water in the first days the Aw was little affected. Similar results have been found in other studies (Andrade et al., 2010; Palumbo, Zaika, Kissinger, & Smith, 1976; Patrignani et al., 2007). Yeasts are more tolerant to low Aw than bacteria and some extreme osmophilic yeasts that can grow in conditions as low as 0.62 Aw.

The composition and evolution of the micro-organisms in sausages depend on the initial microbial load of raw material and the adopted manufacturing technique, mainly temperature and period of maturation. The process is also determined by intrinsic factors such as low redox potential, degree of acidification and reduction of Aw in the product, which restricts the growth of some micro-organisms initially present, favoring the predominance of just a few genera. The levels found in our study coincide with those described in the literature (Molina et al., 1990; Saldanha da Gama,

Table 2
Frequency (%) of yeast strain distribution in IND sausages.

Species	Mixture	Industrial style											
		Center						Surface					
		3	7	10	20	30	50	3	7	10	20	30	50
Rhodotorula mucilaginosa	31.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Yarrowia lipolitica	15.8	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0
Candida parapsilosis	0.0	0.0	30.0	0.0	0.0	0.0	0.0	0.00	0.0	0.0	7.7	0.0	0.0
Debaryomyces spp	52.6	100	60.0	100	100	100	100	100	100	92.9	92.3	100	100

Table 3
Proteolytic yeast strains isolated according to sausage processing style (ART, IND) and sampling location (center (c), surface (s)).

Day	Total of proteolytic strains isolated (%)	Distribution according to					
		Sausage processing style		Location in sausage			
		ART	IND	ARTc	ARTs	INDc	INDs
Mixture	31.3	50	50	–	–	–	–
3	6.8	66.7	33.3	100	–	–	100
7	25.0	81.8	19.2	66.7	33.3	100	–
10	9.7	75	25.0	100	–	–	100
20	6.8	70	30.0	100	–	100	–
30	6.8	66.7	33.3	100	–	100	–
50	13.5	65.6	33.3	50	50	–	100

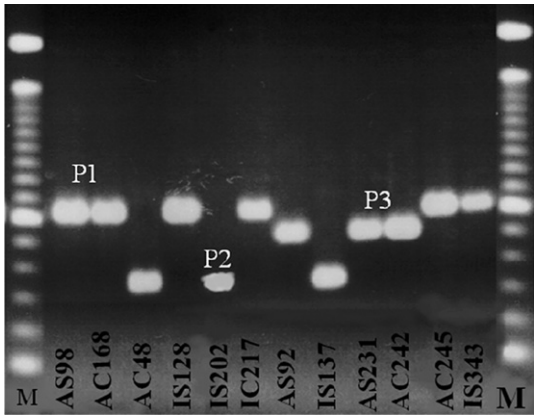

Fig. 3. Amplified region of rDNA 5.8S – ITS of some isolates (Codification of isolates: A – ART; I – IND; C – center; S – Surface; isolate number).

Malfeito-Ferreira, & Loureiro, 1997; Samelis, Stavropoulos, Kakouri, & Metaxopoulos, 1994; Viljoen et al., 1993).

The isolates were identified according to the traditional method for classifying yeast proposed by Barnett et al. (2000). The colony characteristics were classified into six genera, the majority belonging to the genus *Debaryomyces* followed by *Candida*, *Yarrowia*, *Rhodotorula*, *Pichia* and *Trichosporon*. Studies by Viljoen et al. (1993) and Saldanha da Gama et al. (1997) showed similar results in different meat products.

The aroma and flavor of sausages are the result of the interaction of numerous chemical compounds, added or not, mainly coming from chemical or enzymatic degradation of fats and proteins as reported by some authors (Durá et al., 2004; Flores et al., 2004; Flores, Marcus, Nieto, & Navarro, 1997; Toldrá, 2008). Preliminary assays for lipolytic and proteolytic activity of yeasts isolates from both the ART and the IND processes have shown a possible contribution of them for the flavor and aroma of the product during maturation. Proteolytic behavior observed was favored in absence of starter cultures. Similar results were previously reported by Saldanha da Gama et al. (1997), Patrignani et al. (2007) and Andrade et al. (2010). However, more tests are necessaries for understand their real contribution to sausage flavor.

After PCR and restriction studies, the yeasts were grouped into four genera and six previously unidentified isolates. Predominance of the genus *Debaryomyces* over other yeasts was confirmed by rDNA-RFLP, while showing yeast presence in both places sampled, the inner part and surface of the sausages. The results also confirmed a greater variety of yeast species in ART-type sausages than in IND sausages.

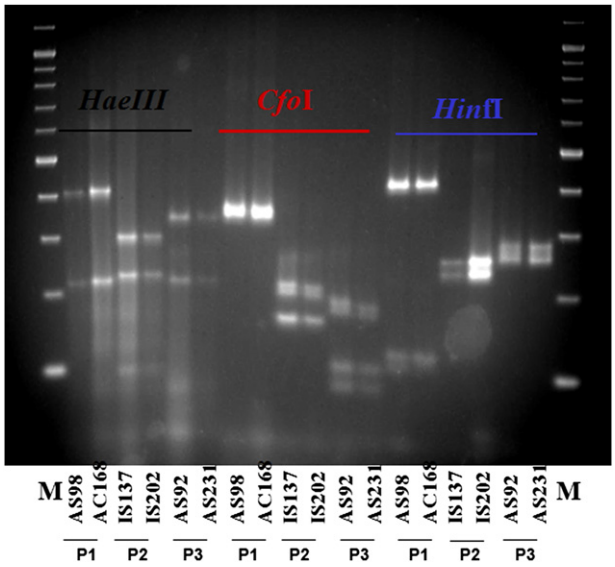

Fig. 4. Digestion of the amplified region of some isolates with restriction enzymes *Hae*III, *Cfo*I, and *Hinf*I, belonging to patterns – P1, P2, P3 (Codification of isolates: A – ART; I – IND; C – center; S – Surface; isolate number).

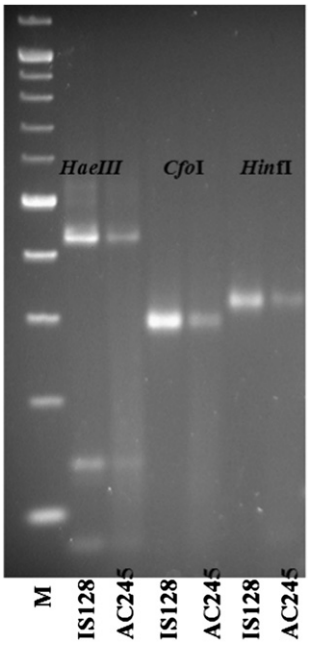

Fig. 5. Digestion of the amplified region with restriction enzymes *Hae*III, *Cfo*I, and *Hinf*I for isolates belonging to the new pattern P4 (Codification of isolates: A – ART; I – IND; C – center; S – Surface; isolate number).

Table 4
Approximate molecular size of amplified products digested.

Pattern	Amplified size	Restriction fragment (bp)			Species
		*Cfo*I	*Hae*III	*Hinf*I	
P1	690	300 + 250 + 120	400 + 220 + 50	350 + 240 + 50	*Rhodotorula mucilaginosa*
P2	370	210 + 160	370	180 + 100 + 90	*Yarrowia lipolitica*
P3	590	280 + 270 + 40	430 + 120 + 40	270 + 280	*Candida parapsilosis*
P4	650	300 + 300 + 50	420 + 140 + 90	320 + 320	*Debaryomyces* spp

5. Conclusions

The following conclusions were drawn from the results above: there are evident differences in yeast counts according to topographies from the same sample. The count is significantly lower in the inner part of the sausage, but this factor is not affected by the use of starter cultures. Significant differences can be observed in total yeast at the center and on the surface of the sausages in both the hand-made and industrial process. Restriction analysis showed that the technique is effective in checking the predominance of the *Debaryomyces* genus compared to other isolates.

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