Different methods to identify yeast population associated with dry-cured lacón, a traditional meat product from North-West of Spain

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Abstract— **In the present study, three different methods were used to identify yeast isolated from dry-cured lacón, a traditional meat product from Galicia (North-West of Spain). A total of 145 strains were taken randomly from drying-ripening stage (from 7 to 84 days) and identified using the API 20C AUX kit (*BioMerieux-Vitek, Hazelwood, Mo*), restriction fragment length polymorphism (RFLP) analysis of 5.8S ribosomal RNA gene and two internal transcribed spacers (ITS1 and ITS2), and the last one, sequencing the same region restriction. Six different yeast species were identified representing five genera: *Debaryomyces, Candida, Cryptococcus, Rhodotorula* and *Rhodosporium*. Among the isolated lacón samples, *Debaryomyces hansenii* was the predominant specie (77,9%). Sequencing of the 5.8S-ITS1 region yielded the best results in terms of correct identification. The commercial identification kit API 20C AUX was able to correctly identify only 55,2% of the isolates.**

Keywords— **Yeast, ITS, 5,8S.**

I. INTRODUCTION

Lacón is a traditional raw-cured meat product made in Galicia (north-west of Spain), from the foreleg of the pig cut at the shoulder blade-humerus joint, following manufacturing processes similar to those used in the production of dry-cured ham.

The microbiological characterization, surface layers of lacón harbor a typical microbiota [1] which includes yeast like one of the most notable group of microorganisms. Yeasts are responsible for important biochemical changes during manufacture process that contribute to the aroma, taste and flavor to the final product. So far, typical yeast species found lacón belong genus *Candida*, being *Candida famata* (anamporh of *Debaryomyces hansenii*) the most prevalent species [2].

Classical identification of yeast species has been focused on morphological characteristics and physiological properties which is time consuming [3]. In recent years, rapid commercial systems for a rapid yeast identification have been developed as an alternative, being API 20C AUX system (bioMèrieux), one of the most used commercial kits [4].

Most recently, advances in genetic knowledge have been a revolution in the microbial identification. One of most successful PCR-based methods for yeast species identification is restriction fragment length polymorphism (RFLP) analysis of the 5.8S rRNA gene and the two flanking internal transcribed sequences (ITS1 and ITS2) which can reveal genetic variation between species [5]. This technique consists of direct PCR amplification using conserved oligonucleotide primers [6] followed by endonucleadse restriction analysis of the amplified product. In fact, Esteve-Zarzoso *et al*. [7] have established a database containing the 5.8S-ITS region endonuclease restriction patterns of 132 yeast species isolated from numerous sources.

The aim of this work was to compare three different methodologies (API 20C AUX, RFLP and sequencing of the 5.8S-ITS region) to identify yeast population present in lacón and establish which method could be more useful for routine analysis.

II. MATERIALS AND METHODS

1. Sampling

A total of 14 lacón pieces (4 kg each, in the fresh stage) were manufactured in our Centre. The process was as following: salting (3-5 days), post-salting (14 days), drying ripening (84 days). For microbial analysis, samples were taken from after 7, 14, 28, 56 and 84 days of drying ripening.

2. Yeast isolation and identification

Surface samples, approximately 2 mm thickness, were aseptically removed from different areas of the lean surface. Samples were weighed and diluted with peptone physiological solution (PPS) composed of 8,5 g NaCl, 1 g tryptone, 10 ml Tween 50 and 1000 ml distilled water and homogeneized in a masticator blender (IUL Instruments, Barcelona, Spain) for 2 min. For each sample, appropriate serial decimal dilutions were prepared in Peptone Water solution (0.1%) and 100 µl samples of appropriate dilutions were spread onto the surface of OGYE agar plates (Oxytetracycline Glucose Yeast Extract).

Morphologically different colonies were taken from OGYE agar plates in each sampling point. Isolates were purified by 4 alternate subcultures on MEB (Malt Extract Broth). The purified strains were, afterwards, maintained at -80ºC using a 20% of glycerol as cryoprotector agent.

3. API 20C AUX

The system consists of a disposable plastic strip with 20 cupules containing dehydrated reagents sufficient for 18 biochemical test, a negative control and a cupule containing glucose that serves as a positive control. Isolates were picked up with a sterile loop 5 days OGYE agar plates to be added to the medium provided to constitute a suspension. Suspensions were used to fill the cupules of the test strips following manufacturer´s instructions. A profile number based upon the reactions observed was generated at 30 ºC for 72 h of incubation for each strip. Identifications were made by reference to the API Analytical Profile Index (version 1.1) or by calling the computerized voice-activated system (version 1.1).

4. DNA extraction

Yeast cells from the pure cultures of the representative isolates were transferred to 1.5 ml tubes using a 4 mm inoculation rod.

DNA extraction was conducted using a EZNA™ Yeast DNA Kit (*OMEGA Bio-tek*) as directed by the manufacturer. Negative controls were processed in parallel to detect possible contaminations.

5. PCR amplification

The fungus-specific universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) were used to amplify the intervening 5.8S rRNA and the adjacent ITS1 and ITS2 regions [8]. All PCRs were carried out with a thermocycler (MyCycler, *BioRad*), and the PCR conditions were as follows: 50 µl reaction cocktail containing 25 µl of iQ™ Supermix (*BioRad*), 0,8 µM of each primer, with the remaining volume consisting o f distilled water and 5 µl of DNA template. The thermocycler program consisted of an initial denaturation at 94 ºC for 4 min followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 1 min; and a final extension step at 72 ºC for 4 min. Negative controls were included in all PCR experiments.

6. Restriction analysis

Aliquots (10 µl) of PCR products were digested with 1 U of restriction enzyme in 20 µl reaction volume in a Thermomixer Comfort basic (*Eppendorf*), using the manufacturer´s instructions and conditions. The restriction enzymes used were *Hha*I, *Hae*III and *Hinf*I (*Takara Bio Inc.*).

The amplified products and restriction fragments were analyzed by horizontal electrophoresis in 2% (w/v) agarosa gels stained with GelRed™ Nucleic Acid Gel Prestaining Kit (*Biotium*) and it was conducted with at 10 V/cm for 1 h. Fragment sizes were estimated by comparison against a DNA standard (DNA marker 100bp, Norgen). After electrophoresis, the gels were photographed under transilluminated UV light machine (GelDoc Imagen, *BioRad*)

7. DNA sequencing

Amplified products were purified using an Ultra Clean PCR Clean-Up Kit (*MOBIO latoratorie, Inc.*) before being sequenced. Sequencing was run on an ABI 3730xl capillary sequencer (*Applied Biosystems*).

Sequence comparisons were performed using the basic local alignment search tool (BLAST) in GenBank (www.ncbi.nlm.nih.gov/blast) after editing and trimming the sequences by BioEdit sequence alignment editor, version 7.0.0. The yeast isolates were identified based on a 99-100% similarity criterion.

III. RESULTS AND DISCUSSION

Yeast are an heterogeneous unicellular group of fungi which is part of the typical microbiota of the lacón [1,2].

These microorganisms contribute to the sensory characteristics of dry-cured lacón thanks to their proteolytic and lipolytic activity [8] and their role in volatile compounds generation [9]. Nevertheless, they can even cause economic losses since they grow like saprotrophs in food. Therefore, rapid and accurate identification methods are needed for wild environmental strains, to monitor biotechnological processes or to identify sources of food spoilage.

The aim of this study was to compare three methods (API 20C AUX, RFLP and sequencing of the 5.8S-ITS region) to identify a total of 145 yeast strain isolated from lacón. So far, yeast identification have been based on morphological and physiological approaches [3].

Table 1 Species identified by API 20C AUX and Sequencing 5.8S rRNA-ITS region

Species	***Identification method***			
	API 20C AUX		**Sequencing 5.8S rRNA-ITS region**	
	n	**%**	**n**	**%**
Debaryomyces hansenii	76	52,4	113	77,9
Candida guilliermondi	7	4,8	-	-
Candida lusitaniae	4	2,8	-	-
Candida parapsilopsis	3	2,1	-	-
Candida pelliculosa	3	2,1	-	-
Candida sphaerica	1	0,7	-	-
Candida tropicalis	2	1,4	-	-
Candida zeylanoides	24	16,6	12	8,3
Candida deformans	-	-	7	4,8
Candida spp.	-	-	4	2,8
Cryptococcus humicola	2	1,4	-	-
Cryptococcus laurentii	6	4,1	-	-
Cryptococcus curvatus	-	-	6	4,1
Kloeckera spp.	1	0,7	-	-
Kodamaea ohmeri	3	2,1	-	-
Rhodotorula mucilaginosa	1	0,7	2	1,4
Rhodotorula glutinis	2	1,4	-	-
Rhodosporidium babjevae	-	-	1	0,7
Stephanoascus ciferri	3	2,1	-	-
Tricosporum mucoides	7	4,8	-	-
TOTAL	**145**	**100**	**145**	**100**

Although many studies have demonstrated that API 20C AUX has 88-99% accuracy [3], our results, using this system, shown as only 55.2% of samples were correctly identified (Table 1). This commercial kit presents low identification rates due to any yeast isolates are not listed in its database since it was initially designed for clinical diagnosis [10] not for yeast species from other sources like food.

On the other hand, despite RFLP of the ITS-5.8S rDNA-ITS2 has been reported as a rapid and easy method for the differentiation of several yeast species [7], our results could not differentiate between phylogenetically related species such as *D. hansenii* and *C. zeylanoides*. The difference between the restriction patterns of this yeast pair is less than 15 bp what it could be seen in other accurate instrument as capillary electrophoresis but not by agarose gel electrophoresis as it is shown in the Fig. 1. Thus, this method has not enough detection sensitivity to be used as a routine analysis to discriminate yeasts species related to dry-cured lacón.

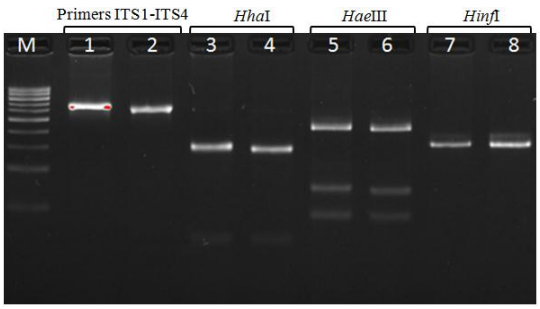

Fig. 1 Gel showing amplification by PCR-RFLP. Lane 1, 3, 5, 7: *D. hansenii*. Lane 2, 4, 6, 8: *C. zeylanoides*. Lane M: 100-bp DNA ladder.

The best results were obtained by sequencing of the 5.8S-ITS region with a 99-100% similarity. The yeast belonged to six species of the following five genera: *Debaryomyces*, *Candida*, *Cryptococcus*, *Rhodotorula* and *Rhodosporium* (Table 1). *D. hansenii* was the most frequently isolated species from the lacón with a contribution of 77.9%. It is previously reported that this species dominate the yeast composition of dry-cured meat products [11,12]. It is also remarkable, the presence of *Candida zeylanoides* (8.3%), a species associated to ham [11,12].

Surprisingly, 4,8% of samples were identified like *C. deformans*, a species previously considered synonymous of *Yarrowia lipolytica* [13]. *Y. lipolytica*

is a yeast of biotechnological importance found in meat fermented products [14] but not in raw-cured meat products. Nowadays, *C. deformans* is a taxon separate from *Y. lipolytica* although closely related [15]. Indeed, several authors considerer it like anamorphs stages of unnamed *Yarrowia* species [15].

Minority species were ocasioanlly found such as *Cryptococcus curvatus* (1.4%), *Rhodotorula mucilaginosa* (1.4%) and *Rhodosporidium babjevae* (0.7%). Although the first two have been previously reported in meat products [12,16], their role is still unknown. *R. babjevae* has been usually associated with marine environments [17] so it could be considered as pollution.

IV. CONCLUSIONS

In summary, the results demonstrate API 20C AUX and PCR-RFLP of the ITS-5.8S rDNA-ITS2 have not enough detection sensitivity to discriminate yeasts species from lacón. Probably it is necessary to combine these methods with sequence analysis of this region as the best means of reliable identification of yeast. It is important to note the high percentage of correct identification, resulting in 97% strains to species level since four isolates could only be identified to genera level (*Candida* spp.).

On the other hand, *D. hansenii* is the most prevalent yeast species present in lacón followed by other minority species.

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