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Yeast Species Associated with Orange Juice: Evaluation of Different Identification Methods†

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Five different methods were used to identify yeast isolates from a variety of citrus juice sources. A total of 99 strains, including reference strains, were identified using a partial sequence of the 26S rRNA gene, restriction pattern analysis of the internal transcribed spacer region (5.8S-ITS), classical methodology, the RapID Yeast Plus system, and API 20C AUX. Twenty-three different species were identified representing 11 different genera. Distribution of the species was considerably different depending on the type of sample. Fourteen different species were identified from pasteurized single-strength orange juice that had been contaminated after pasteurization (PSOJ), while only six species were isolated from fresh-squeezed, unpasteurized orange juice (FSOJ). Among PSOJ isolates, *Candida intermedia* and *Candida parapsilosis* were the predominant species. *Hanseniaspora occidentalis* and *Hanseniaspora uvarum* represented up to 73% of total FSOJ isolates. Partial sequence of the 26S rRNA gene yielded the best results in terms of correct identification, followed by classical techniques and 5.8S-ITS analysis. The commercial identification kits RapID Yeast Plus system and API 20C AUX were able to correctly identify only 35 and 13% of the isolates, respectively. Six new 5.8S-ITS profiles were described, corresponding to *Clavispora lusitaniae*, *Geotrichum citri-aurantii*, *H. occidentalis*, *H. vineae*, *Pichia fermentans*, and *Saccharomycopsis crataegensis*. With the addition of these new profiles to the existing database, the use of 5.8S-ITS sequence became the best tool for rapid and accurate identification of yeast isolates from orange juice.

Citrus is the most economically important tree fruit crop in the world (26). The majority of citrus arrives to market in the form of processed products, such as single-strength orange juice and frozen juice concentrates. In Florida, one of the world's top producing regions, more than 96% of all oranges are processed into orange juice. In the 1999–2000 season, Florida produced more than four billion liters of single-strength orange juice (2), and as such, the Florida citrus juice industry is considered an important food processing industry in the United States. Economic losses due to juice spoilage are minimized by good sanitation procedures before and during citrus processing. Pasteurization, concentration, or low-temperature storage protocols help to reduce the number of microorganisms in the final product. However, these products are not free of microbiological spoilage problems, especially nonpasteurized single-strength juices.

Citrus juices are acidic beverages (ca. pH 3 to 4) with high sugar content (~15° Brix). Under these conditions, acidolactic bacteria, molds, and yeasts comprise the typical microbiota present in citrus juices. Lactic acid bacteria are the primary spoilage bacteria in fruit beverages; however, their numbers are greatly reduced after pasteurization, concentration, and refrigeration. Molds and yeasts tolerate high-osmotic and low-pH conditions and grow at refrigeration temperatures and can therefore cause spoilage in the processed product. Typical yeast species found in citrus juices are *Candida parapsilosis*, *Candida stellata*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, and *Zygosaccharomyces rouxii*, although species from the genus *Rhodotorula*, *Pichia*, *Hanseniaspora*, and *Metschnikowia* are also common (14). Despite the economic importance of citrus juices, there are few reports investigating the yeast species associated with them (7, 23, 24). A detailed study of citrus juice microbiota is needed so that factors involved in spoilage can be assessed and methods can be developed to aid in rapid identification of spoilage microorganisms.

Traditionally, identification and characterization of yeast species has been based on morphological traits and their physiological capabilities (3, 16). This conventional methodology requires the evaluation of some 60 to 90 tests, resulting in a complex, laborious, and time-consuming process. In recent years, rapid kit identification methods have been developed to overcome the complexity of traditional methods (8, 20, 28). One of these methods, the API 20C AUX system (bioMèrieux, Lyon, France), has been widely used and consists of 19 assimilation tests. A recently developed kit, the RapID Yeast Plus system (Remel, Lenexa, Kans.), enables identification in only 4 h. This method, although based on physiological properties, does not require yeast growth for biochemical test evaluation and dramatically reduces identification time. Unfortunately, all yeast identification kits were originally designed for clinical diagnosis and their application is generally restricted to few yeast species.

In the last decade, microbial identification has undergone a revolutionary change by the introduction of PCR-based methodologies. These techniques were first used for bacterial identification but have since been adapted for yeasts. One of the

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most successful 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 (ITS) (29). This technique consists of direct PCR amplification using conserved oligonucleotide primers against the 26S and 18S rRNA genes, followed by endonuclease restriction analysis of the amplified product. Because ribosomal regions evolve in a concerted fashion they have low intraspecific polymorphism and high interspecific variability (15). Consequently, RFLP analysis of the 5.8S-ITS region is an excellent tool for yeast identification (5, 13). Recently, an extensive work by Esteve-Zarzoso et al. (9) established a database containing the 5.8S-ITS region endonuclease restriction patterns of 132 yeast species isolated from numerous sources. This 5.8S-ITS database combines reference yeast strains from different origins and can be more useful for environmental or wild yeast strain identification than the clinically oriented commercial databases.

The first objective of this work was to investigate the yeast species present in pasteurized single-strength orange juice (PSOJ) and fresh-squeezed orange juice (FSOJ). A second objective was to compare different methodologies for yeast identification and establish which method could be more useful for routine analysis. In this sense, we used two commercial identification methods based on phenotypic traits (API 20C and RapID Yeast Plus systems) and two DNA sequence-based protocols (5.8S-ITS profiles and partial sequence of the 26S rRNA gene). We decided to utilize the partial sequence of the 26S rRNA gene since it has a universally accepted role in yeast taxonomy and the available database includes all yeast species described to date.

MATERIALS AND METHODS

Yeast strains and growth conditions. A total of 92 wild yeast strains were used in the study. Fifty-two strains were isolated from fifteen different samples of experimental FSOJ extracted from sound and defective fruit. Oranges were surface decontaminated by placing the whole fruit in an 80°C water bath for 2 min. The oranges were then sliced and juiced by hand into sterile bottles under aseptic conditions. The juice was plated onto orange serum agar (Difco Laboratories, Detroit, Mich.) or acidified potato dextrose agar (Difco) and incubated for 48 h at 30°C. Three to five colonies were randomly selected from each batch. Thirty-two strains came from problematic commercial PSOJ that had been contaminated after pasteurization and were provided by different citrus juice processors. The following seven reference strains were also included: *Candida intermedia* (CECT 11054), *Kluyveromyces thermotolerans* (CECT 1962), *Pichia fermentans* (CECT 1455), *Pichia pastoris* (ATCC 28485), *Rhodotorula mucilaginosa* (CECT 10033), *S. cerevisiae* (ATCC 4132), and *T. delbrueckii* (CECT 11146). Four strains of yeasts from pasteurized single-strength apple juice (SSAJ), two from single-strength grapefruit juice (SSGJ), and two from grape must (GM) were also included to increase the diversity of strains studied. Cells were routinely cultivated on acidified potato dextrose agar for 48 h at 30°C. All strains were grown on yeast-peptone-dextrose agar (Difco) medium for direct PCR amplification. Sabouraud-dextrose agar (Emmons) was employed for the RapID Yeast Plus system and API 20C AUX.

API 20C AUX. Identification was accomplished as directed by the manufacturer (bioMèrieux). Molten (50°C) API basal medium ampoules were inoculated with yeast cells picked from individual colonies and the resulting suspension was standardized to turbidity equal to a no. 2 McFarland standard. Each cupule was inoculated and trays were incubated for 72 h at 30°C. Cupules showing turbidity significantly greater than that of the negative control were considered positive. Identification was made by generating a microcode and using the API 20C Analytical Profile index. Morphology on cornmeal agar (Difco) was also evaluated as suggested by the manufacturer.

RapID Yeast Plus system. Strains were plated onto Sabouraud-dextrose agar (Emmons) and incubated at 30°C for 48 h. Yeast cells were resuspended in RapID Yeast Plus (Remel) inoculation fluid to achieve a visual turbidity that met the manufacturer's recommendation (no. 3 McFarland turbidity standard). The entire contents of the inoculation fluid were transferred into the reaction panel and incubated at 30°C for 4 h. After reading the strips, six-digit microcodes were constructed and used for species identification according to instructions provided within the RapID Yeast Plus Code book.

Classical identification. Initial carbohydrate assimilation was assayed with API 20C AUX strips (bioMèrieux). Nitrogen utilization, fermentation patterns, growth at 37°C, growth in 50 or 60% glucose, and growth with 0.1% cycloheximide were assessed as necessary. Keys and descriptions by Barnett et al. (3) were used to identify yeast isolates.

5.8S-ITS analysis and sequencing. Oligonucleotide primers used for PCR amplification were synthesized according to the method of White et al. (29). PCR conditions for ITS amplification were described previously by Esteve-Zarzoso et al. (9). Cells were collected from a single colony with a sterile toothpick and resuspended in 100 μl of PCR mixture containing 0.5 μM primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), 0.5 μM primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), 10 μM deoxynucleotides, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and 1× buffer (Eppendorf, Hamburg, Germany). PCR conditions were as follows: initial denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension step of 10 min at 72°C. PCR products (approximately 0.5 to 1.0 μg) were digested without further purification with *Cfo*I, *Hae*III, and *Hin*fI restriction endonucleases (Promega, Madison, Wis.). Amplified products and their restriction fragments were electrophoresed on 1.5 and 3% agarose gels, respectively, in 1× TAE (Tris-acetic acid-EDTA) buffer. Gels were stained with ethidium bromide, visualized, and photographed under UV light. Fragment sizes were estimated by comparison against a DNA standard (100-bp ladder; Promega). When the profile obtained did not match any established restriction patterns published in the existing database (9), 5.8S-ITS fragments were sequenced using ITS1 and ITS4 primers (Sequencing Core, University of Florida). Amplified products were purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad, Hercules, Calif.) before being sequenced. The presence or absence of restriction digestion sites within DNA sequences was analyzed using the Omiga 2.0 software package (Oxford Molecular Ltd., Oxford, United Kingdom).

26S rRNA gene sequencing. Amplification of partial 26S rRNA gene sequences was carried out using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (19). PCR amplification, product purification, and sequencing were carried out as described above. Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database (1). An isolate was ascribed to the species showing the highest matched sequence identity. DNA sequences were analyzed using the Omiga 2.0 software package (Oxford Molecular Ltd.).

Analysis of the data. An isolate was considered correctly identified when at least two methods ascribed it to the same species. When this situation did not occur, putative identification was based on a partial sequence of the 26S rRNA gene. Each identification method was evaluated (i) for its ability to identify the isolates to species level, (ii) for its ability to identify the isolates to genus level, (iii) for its discrepant identification, and (iv) for its failure to provide identification.

Nucleotide sequence accession numbers. 5.8S-ITS sequences of *G. citri-aurantii*, *Saccharomycopsis crataegensis*, and *P. fermentans* were submitted to GenBank under accession no. AF411060, AF411061, and AF411062.

RESULTS

Yeast species present in orange juice. A total of 92 yeast strains isolated from PSOJ, FSOJ, SSGJ, SSAJ, and GM were identified in this study (Table 1). Twenty-three species were identified, representing 11 genera. *Hanseniaspora uvarum* (27%), *Hanseniaspora occidentalis* (15%), *Pichia kluyveri* (9%), *C. intermedia* (7%), and *C. parapsilosis* (6%) were more common among isolates. *S. cerevisiae* represented only 3% of the total isolates. *H. uvarum* was the only species isolated from both pasteurized and nonpasteurized orange juice samples. The number of samples from SSAJ, SSGF, and GM was too low to be representative; however, except for one SSGJ strain identified as *R. mucilaginosa*, isolates belonged to different species than those from orange juice. Strains isolated from FSOJ and contaminated PSOJ dif-

TABLE 1. Source and incidence of yeast species isolated in this study

Species	No. of isolates from:					% of total
	PSOJ[a]	FSOJ[b]	SSGJ[c]	SSAP[d]	GM[e]	
Candida intermedia	7					7.6
Candida parapsilosis	6					6.5
Candida tropicalis	2					2.2
Candida stellata		1				1.1
Candida zeylanoides	2					2.2
Clavispora lusitaniae	3					3.3
Geotrichum citri-aurantii	1					1.1
Issatchenkia orientalis	1					1.1
Hanseniaspora occidentalis		14				15.2
Hanseniaspora uvarum	1	24				27.2
Hanseniaspora vineae					1	1.1
Metschnikowia pulcherrima				2		2.2
Pichia anomala	1					1.1
Pichia fermentans		2				2.2
Pichia jadinii	1					1.1
Pichia kluyveri		9				9.8
Pichia stipitis	1					1.1
Rhodotorula minuta					1	1.1
Rhodotorula mucilaginosa	1		1			1.1
Saccharomyces cerevisiae	3					3.3
Saccharomyces uvarum			1	2		2.2
Saccharomycopsis crataegensis		2				2.2
Torulaspora delbrueckii	2					2.2
Total (of 92)	32	52	2	4	2	

[a] PSOJ, pasteurized and subsequently recontaminated single-strength orange juice.
[b] FSOJ, fresh-squeezed single-strength orange juice.
[c] SSGJ, pasteurized and subsequently recontaminated single-strength grapefruit juice.
[d] SSAJ, pasteurized and subsequently recontaminated single-strength apple juice.
[e] GM, grape must.

TABLE 2. Performance of five different identification methods used with 99 strains, including reference strains

Identification method	No. of correctly identified isolates[a] at:		No. of misidentified isolates[b]	No. of unidentified isolates[c]
	Species level	Genus level		
26S rRNA sequence	94	98		1
Classical identification	74	89	9	1
ITS profiles	69	74	2	23
RapID Yeast Plus	33	42	21	36
API 20C	12	13	4	82

[a] Isolate was ascribed to the same species by at least two methods.
[b] Identification was in disagreement between two or more methods.
[c] Identification was not possible or was only supported by partial sequence of the 26S rRNA gene.

fered in species composition. Fourteen different species were identified in PSOJ whereas only six species were found in FSOJ. Predominant species of PSOJ isolates were *C. intermedia* (22%) and *C. parapsilosis* (19%). The main species isolated from FSOJ was *H. uvarum*, representing more than 46% of the total FSOJ isolates, followed by *H. occidentalis* (27%) and *P. kluyveri* (17%). The remaining isolates were ascribed to *C. stellata*, *P. fermentans*, and *Saccharomycopsis crataegensis* and totaled less than 10% of the FSOJ strains. At the genus level, *Hanseniaspora* spp. constituted more than 73% of the FSOJ isolates, whereas *Candida* spp. represented more than 53% of the PSOJ isolates.

Comparison of the identification methods studied. The reliability of each identification method is shown in Table 2. The proportion of correctly identified yeast isolates among the identification test methods varied between 12 and 94% at the species level. Nucleotide sequence of the 26S rRNA gene provided the highest correct identification percentage. With this method, only one isolate, identified as *Issatchenkia orientalis* by partial sequence of the 26S rRNA gene, was not corroborated by any other method and was considered unidentified. Three strains were ascribed to the species *H. uvarum* but they were identified only to genus level by the remaining techniques. Finally, a PSOJ isolate was identified as *Geotrichum citri-aurantii* and was only identified to the genus level by classical techniques. Identifications of remaining isolates were confirmed by any one of the other methods and thus considered correct. Sequence differences were slight or nonexistent and further consideration as separate species was not warranted. The majority of partial 26S rRNA gene sequences showed identities of 98% or higher to sequences in the GenBank database. Most sequences (70%) perfectly matched sequences present in the database; some (24%) had 99% identity and even fewer had 98% identity (4%). Only the two strains identified as *P. fermentans* showed 95% identity with sequences in the databases. However, since both strains shared exactly the same sequence and classical methods identified both as *P. fermentans*, we considered this identification to be correct.

The method resulting in the second highest correct identification percentage was classical methodology. Seventy-four percent of the isolates were correctly identified at the species level and up to 89% were correctly identified at the genus level. Nine strains were misidentified (9%) and only one strain (1%) could not be identified. The unidentified strain was ascribed to the species *C. parapsilosis* by 26S rRNA gene sequence and 5.8S-ITS analysis. Misidentified isolates are shown in Table 3.

Analysis of the 5.8S-ITS endonuclease restriction pattern enabled us to correctly identify 69% of the strains at the species level and 74% at the genus level (Table 2). Only two species (2%) were misidentified (Table 3) and 23 isolates (23%) could not be identified. These 23 isolates represented new 5.8S-ITS patterns that have not been previously described. Using the restriction endonucleases *Cfo*I, *Hae*III, and *Hin*fI, we found six different profiles corresponding to *Clavispora lusitaniae*, *G. citri-auriantii*, *H. occidentalis*, *Hanseniaspora vineae*, *P. fermentans*, and *Saccharomycopsis crataegensis*. The 5.8S-ITS amplified products of these isolates were further sequenced, confirming the presence and position of experimental restriction sites (Table 4). 5.8S-ITS sequences of *G. citri-auriantii* and *Saccharomycopsis crataegensis* were not found in GenBank and therefore were submitted as new sequences. Alignments of published *Clavispora lusitaniae*, *H. occidentalis*, and *H. vineae* 5.8S-ITS regions with our amplified ITS regions resulted in identities of higher than 95% and were not considered significantly different. However, alignment of the three existing *P. fermentans* 5.8S-ITS sequences with those from FSOJ isolates resulted in a lower degree of identity, ranging from 70 to 88%. Although the *P. fermentans* 5.8S-ITS pattern was already described by Esteve-Zarzoso et al. (9), the pattern

TABLE 3. Misidentification results obtained with the different methods assayed

Method	Incorrect identification	Correct identification[a]
Classical identification	*Candida diversa*	*Pichia kluyveri* (3/9)
	Pichia membranifaciens	*Issatchenkia orientalis* (1/1)
	Saccharomyces cerevisiae	*Saccharomycopsis crataegensis* (2/2)
	Zygosaccharomyces rouxii	*Hanseniaspora occidentalis* (3/14)
5.8S-ITS pattern	*Candida skinneri*	*Issatchenkia orientalis* (1/1)
	Candida tropicales	*Pichia stipitis* (1/1)
RapID Yeast Plus	*Blastoschizomyces capitatus*	*Pichia fermentans* (1/2)
	Candida apicola	*Issatchenkia orientalis* (1/1)
	Candida guilliermondii	*Kluyveromyces thermotolerans* (CECT 1962)
	Candida krusei	*Pichia kluyveri* (4/9)
	Candida lambica	*Pichia fermentans* (1/2)
		Pichia fermentans (CECT 1455)
	Candida utilis	*Torulaspora delbrueckii* (CECT 11146)
	Clavispora lusitaniae	*Candida parapsilosis* (1/6)
		Metschnikowia pulcherrima (1/2)
	Hansenula anomala	*Candida parapsilosis* (1/6)
	Hanseniaspora uvarum	*Pichia kluyveri* (1/9)
	Saccharomyces cerevisiae	*Torulasporula delbrueckii* (2/2)
		Pichia pastoris (ATCC 28485)
	Trichosporon beigelii	*Cryptococcus laurentii* (1/1)
API 20C AUX	*Candida krusei*	*Pichia pastoris* (ATCC 28485)
	Candida magnoliae	*Torulaspora delbrueckii* (CECT 11146)
	Candida sphaerica	*Kluyveromyces thermotolerans* (CECT 1962)
	Trichosporon mucoides	*Candida intermedia* (CECT 11054)

[a] Numbers in parentheses are the number of times the correct organism was incorrectly identified/total number of isolates in the species.

we obtained was very different. Consequently, we described a new 5.8S-ITS pattern for the species *P. fermentans*.

The RapID Yeast Plus system was able to accurately identify only 33 and 42% of the isolates at the species and genus level, respectively. Up to 36% of the strains could not be identified and 21% were misidentified (Tables 3 and 4). Twenty new RapID Yeast Plus profiles were found in this study and are listed in Table 5. Some species were not included in the RapID Yeast Plus system database, such as *C. stellata*, *Metchnikovia pulcherrima*, *Pichia* spp., and *Saccharomycopsis crataegensis*. We also found new RapID Yeast Plus profiles of isolates belonging to yeast species such as *C. intermedia* or *S. cerevisiae* already present in the database.

Only 12% of isolates were correctly identified to the species level by API 20C AUX, and the rate of unidentified strains reached 82%. Four reference strains, *P. pastoris* (ATCC 28485), *T. delbrueckii* (CECT 11146), *K. thermotolerans* (CECT 1962), and *C. intermedia* (CECT 11054), were misidentified. The isolates correctly identified belonged to the genera *Candida*, *Rhodotorula*, and *Saccharomyces*.

DISCUSSION

In recent years, several identification methods have been proposed as alternatives to cumbersome classical yeast identification techniques. Among these methods, commercial miniaturized systems such as Vitek, API 32C, API 20C AUX (bioMèrieux), Yeast Star (Clarc Laboratories, Heerlen, The Netherlands), Auxacolor (Sanofi, Paris, France), and RapID Yeast Plus system (Remel) were designed to shorten the identification time of clinical yeast isolates and are extensively used in clinical diagnosis (8, 12, 25, 28). However, yeast importance is not confined to human pathogenesis. This large and divergent group of microorganisms has an important role in food science for its beneficial activities (i.e., wine and bread making) but also for the economic losses yeasts can cause when growing

TABLE 4. Nucleotide fragment length of new 5.8S-ITS profiles described in the study

Species	AP[a] (bp)	Fragment length(s) (bp)[b] after restriction endonuclease analysis with:		
		*Cfo*I	*Hae*III	*Hin*fI
Clavispora lusitaniae	360	210, 90, 60	360	180, 180
Geotrichum citri-aurantii	380	380	380	190, 190
Hanseniaspora occidentalis	750	330, 320, 100	640, 110	250, 170, 110, 100
Hanseniaspora vineae	700	250, 150, 110	700	370, 350
Pichia fermentans	450	120, 84, 70	260, 80	260, 120
Saccharomycopsis crataegensis	650	580, 70	650	320, 320

[a] AP, 5.8S-ITS amplified product size.
[b] Profiles did not consider restriction fragments under 60 bp.

TABLE 5. New RapID Yeast Plus profiles described in the study

Species	RapID Yeast Plus profile	No. of isolated strains with new profile/ total
Candida intermedia	506033	CECT 11054[a]
	746017	3/7
	746417	2/7
	766007	1/7
	776017	1/7
Candida stellata[b]	771106	1/1
Geotrichum citri-aurantii	46406	1/1
Hanseniaspora occidentalis[b]	504406	1/14
	544406	12/14
	704406	1/14
Hanseniaspora uvarum	774402	2/24
Metschnikowia pulcherrima[b]	146017	1/2
Pichia anomala[b]	566406	1/1
Pichia jadinii[b]	776436	1/1
Pichia kluyveri[b]	104406	2/9
Pichia stipitis[b]	307037	1/1
Rhodotorula mucilaginosa	742077	2/2
Saccharomyces cerevisiae	736206	1/3
	776002	1/3
Saccharomycopsis crataegensis[b]	106407	2/2

[a] This reference strain gave a new profile but was not one of the study isolates.
[b] Species is not present in the RapID Yeast Plus database.

as saprotrophs in food and manufactured goods. For this reason, rapid and accurate identification methods are needed for environmental strains to monitor biotechnological processes or to identify sources of food spoilage. Unfortunately, commercial kits for yeast identification were designed based on clinical strains and their use for identification of wild yeasts is often inconclusive.

Recently, new identification methods, mainly based on nucleic acid sequences, have been adjusted to include environmental yeast isolates. Some of these are highly discriminative, such as those using mitochondrial DNA restriction analysis (10, 21), randomly amplified polymorphic DNAs (22), karyotyping (10), and intron splice site-specific PCR amplification (4), and have been used mainly for intraspecific characterization and strain identification. Other powerful identification methods with a wider scope are Fourier-transform infrared spectroscopy (17), 5.8S-ITS restriction pattern analysis (9), and nonradioactive dot blot DNA reassociation (6). Although each method offers different advantages, the analysis of the 5.8S-ITS pattern allows the shortest identification time and relies on a published database that includes most common food-borne yeast species.

The first objective of this study was to investigate the yeast species composition in two types of orange juice, PSOJ (problematic commercial juice contaminated after pasteurization) and FSOJ. Although all yeast species isolated from juice can be considered typical inhabitants of this particular medium or its surroundings (3), striking differences in species composition were found between PSOJ and FSOJ. The PSOJ yeast population was more diverse and included some typical fermentative yeast species, such as *S. cerevisiae*, *C. intermedia*, or *T. delbrueckii*. In contrast, the FSOJ yeast population consisted mostly of *H. uvarum* and *H. occidentalis*. Apparently, these mild fermentative species do not tolerate pasteurization well or physicochemical conditions are not appropriate for their recovery in the processed product. In addition, their numbers could be overcome by better-adapted species. Another possibility that could explain the differences in composition is that the method we used in this study for fruit surface sterilization eliminates more contaminants than the typical washing and sanitizing method used in the industry. This is likely, since most commercial FSOJ facilities use chemical sprays, rather than hot water dips, for fruit surface sanitation prior to juice extraction. None of the species isolated from PSOJ were isolated from FSOJ, with the exception of one strain of *H. uvarum* isolated from PSOJ, suggesting that their number was either too low to be detected in FSOJ or PSOJ was subjected to contamination during processing. It should be noted that commercial pasteurized orange juice does not typically contain viable yeast cells, and PSOJ strains evaluated in this study are from problematic commercial juices that had been contaminated after the pasteurization step.

The citrus juice industry lacks rapid and accurate tools to identify spoilage yeasts from both processed and unprocessed products. The availability of these tools for processors is critical since the identity of microorganisms present in juice will determine appropriate measures to avoid or minimize economic losses. Furthermore, there are few studies about the composition and incidence of yeast species associated with citrus products. In this study, we compared the performance of narrower identification methods, typified by two commercial methods (API 20C and RapID Yeast Plus), with analysis of the 5.8S-ITS region and contrasted them with broader identification tools, typified by a classical identification methodology as well as a partial sequence of the 26S rRNA gene. Overall, the results demonstrate good reliability of the 5.8S-ITS analysis as a routine technique for identification of orange juice yeast isolates. This method allows identification in less than 8 h from colony isolation since no specific medium for cell growth or DNA extraction is required. Another notable characteristic of the 5.8S-ITS analysis is the low percentage of misidentifications, resulting in only two strains being misidentified in our case. In contrast, there were 23 strains that had restriction digest profiles that were not present in the published database and could not be identified using this technique. In these cases, species assignment was based on partial sequence of the 26S rRNA gene and/or classical methods. These 23 isolates displayed six different patterns that were sequenced to confirm empirical restriction sites. Sequencing of new 5.8S-ITS profiles revealed the existence of three unique sequences not present in GenBank. Based on identification using other methods, the three new 5.8S-ITS profiles corresponded to the species *G. citri-auriantii*, *P. fermentans*, and *Saccharomycopsis crataegensis*. Although *P. fermentans* was already present in the 5.8S-ITS database, our two isolates showed a very different pattern. In fact, alignment of all available 5.8S-ITS *P. fermentans* patterns gave a range of sequence identities from 70 to 88%. This type of discrepancy has been reported for other yeasts for which several 5.8S-ITS profiles were assigned to the same species (9). Interestingly, our *P. fermentans* strains showed the lowest identity (95%) when comparing their 26S rRNA partial gene sequences with the GenBank sequences, although they were accurately identified by classical techniques. Further investiga-

tion will be needed to decide if *P. fermentans* presents unusual intraspecific variability or if our orange juice isolates may belong to a new species.

As expected, commercial methods yielded the lowest number of correct results, since the major part of the species found in juices are not present in their databases. Nevertheless, new profiles can always be added to an existing database, as has been shown in the case of the 5.8S-ITS (9–11). The simplicity and rapidity of these commercial methods may be attractive enough to use in the food industry if the developed databases were robust. In the case of the RapID Yeast Plus system, up to 20 new microcodes could be added to the existing database. Based on our results, 10 new microcodes were assigned to species already present in the database. In fact, all PSOJ *C. intermedia* isolates displayed different RapID Yeast Plus results from those already ascribed to this species, underscoring the biochemical differences that may exist between clinical and environmental isolates. However, even if the RapID Yeast Plus profile database is extended with the addition of new environmental isolates, the misidentification percentage is too high to be recommended for citrus industry quality control laboratories.

As has been shown by several authors, a polyphasic approach may be the best way to achieve proper microbial identification (22, 27). Integration of different classes of data and information leads to a consensus type of taxonomy and overcomes the limitations of each single identification method, thereby improving the reliability of the whole determination. This appears to be especially true for yeast identification since yeast taxonomy is incomplete and present-day classification is based on strains (3). Although taxonomic descriptions should be as complete as possible, clinical diagnosis and industrial quality control laboratories demand rapid yeast identification methods. Classical identification relies on numerous sets of data and is still considered the standard method for yeast identification despite requiring an extended period of time and qualified personnel to achieve a proper identification. Commercial identification kits are faster, simpler to perform, and do not require special equipment. On the other hand, they rely on only a few tests, limiting their application in identifying environmental strains, although their usefulness for clinical isolates has been reported (8, 12, 20).

Yeast identification based on 5.8S-ITS restriction analysis has proven to be a rapid, reliable, and accurate tool for environmental yeast identification (9–11, 13). In our study, this technique provided good results in terms of time and accuracy, but the existent database should be updated with the typical microbiota found in citrus juices. After we updated the previous database with the six new 5.8S-ITS profiles described in this study, up to 98% of isolates would be correctly identified. However, as more profiles are added to the database identification will become increasingly difficult due to no or slight differences between the 5.8S-ITS profiles. Unfortunately, similar or identical 5.8S-ITS patterns do not necessarily belong to related species (9). Furthermore, it has to be considered that one single mutation in the 5.8S-ITS region could lead to the loss or gain of a restriction site, resulting in a completely different pattern. One promising alternative to overcome such an occurrence would be to sequence either the 26S rRNA gene or the 5.8S-ITS region and contrast them with the presently available databases. Both regions, but especially the 26S rRNA gene (18), have been shown to provide enough variability to distinguish between most yeast species due to their high taxonomic value. The sequencing time requirement and cost are still too high to facilitate use in common quality control labs but may be affordable in the future. Until that time, we propose the use of 5.8S-ITS analysis as the best method for rapid and accurate identification of yeasts isolated from citrus juices, although we certainly recommend utilizing classical methodologies or 26S rRNA gene sequencing for further corroboration.

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