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Identification and selection of non-*Saccharomyces* strains isolate from brazilian apple must

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ABSTRACT: *This study aimed to know the biodiversity of non-Saccharomyces yeasts in Brazilian apples must with potential to improve of the aromatic quality of ciders. The strains were isolated from thirty-five (35) Gala and Fuji apple musts from different locations from south region of Brazil. Forty-five (45) strains were isolate and identified by PCR analysis. Results indicated ten (10) species: Candida oleophila, Candida parapsilosis, Candida tropicalis, Clavispora lusitaniae, Hanseniaspora guilliermondii, Hanseniaspora uvarum, Lodderomyces elongisporus, Pichia anomala, Pichia fermentans and Rhodotorula mucilaginosa. The genus Rhodotorula sp., Lodderomyces sp. and Clavispora sp. constituted 71.2% of the strains identified. The following strains, C. oleophila, R. mucilaginosa, P. fermentans, H. uvarum and H. guilliermondii were selected in qualitative tests due the fruity aroma production by trained team in the aromatic assessment of cider.*
Key words: *apiculate yeast, biodiversity, fruity aroma, PCR analysis.*

Identificação e seleção de leveduras não-*Saccharomyces* isoladas de mostos de maçãs brasileiras

RESUMO: *Este estudo teve como objetivo conhecer a biodiversidade de leveduras não-Saccharomyces em maçãs com potencial para a melhoria da qualidade aromática da sidra brasileira. As cepas foram isoladas de trinta e cinco (35) mostos de maçã Gala e Fuji de diferentes locais da região Sul do Brasil. Quarenta e cinco (45) cepas foram isoladas e identificadas por análise de PCR. Os resultados indicaram dez (10) espécies: Candida oleophila, Candida parapsilosis, Candida tropicalis, Clavispora lusitaniae, Hanseniaspora guilliermondii, Hanseniaspora uvarum, Lodderomyces elongisporus, Pichia anomala, Pichia fermentans e Rhodotorula mucilaginosa. Três desses gêneros (Rhodotorula sp., Lodderomyces sp. e Clavispora sp.) juntos constituíram 71,2% das cepas identificadas. Entre estas cepas, C. oleophila, R. mucilaginosa, P. fermentans, H. uvarum e H. guilliermondii foram selecionadas em teste qualitativo devido a produção de aroma frutado, indicando potencial para a produção de compostos aromáticos na sidra.*
Palavras-chave: *levedura apiculada, biodiversidade, aroma frutado, análise por PCR.*

Cider is a beverage obtained by the total or partial alcoholic fermentation (1.5 to 8.0°GL) of varietal apple musts or blends of table and/or industrial apples. Fermentation can be natural or with inoculum of *S. cerevisiae* in the apple must (SANTOS et al., 2015). However, in both cases cider-making is not a sterile process. Many other yeast species belonging to several non-*Saccharomyces* genera occur in apple must and can contribute, at the beginning of fermentation, to the sensory characteristics of cider, mainly with fruity aroma, one of the most important indicators of its quality (VALLES et al., 2007; PIETROWSKI et al., 2012; COUSIN et al., 2017).

However, in sulphited crushed apple or/ and apple must (>50mg $SO_2.L^{-1}$) or apple must inoculated with a high population of *Saccharomyces cerevisiae* (>10^7 cfu.mL^{-1}) and high temperatures (>20°C, processing in the southern hemisphere) cider has neutral sensory notes or a slightly yeast aroma (SANTOS et al., 2015). Conversely, apple musts without the addition of sulphite, with a low initial population of *Saccharomyces* sp. and control of temperature, "fruity" or "floral" notes appear due to the formation of esters and fusel alcohols by non-*Saccharomyces* strains (NOGUEIRA et al., 2008; PIETROWSKI et al., 2012).

Studies have shown a growing interest in the industrial application of non-conventional yeasts, due to their ability to positively contribute to the flavour and aroma of wines, ciders, fermented

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fruit beverages and distillates (PADILLA et al., 2016, HU et al., 2018). Thus, the objective of this study was to know the biodiversity of non-*Saccharomyces* yeasts in Brazilian apple must and to verify the presence of strains with technological potential for cidermaking.

Isolation of non-*Saccharomyces* yeasts was performed on freshly extracted apple musts. For the experiments, thirty-five (35) samples of Gala and Fuji apples (5.0kg) were obtained from several producers from southern states of Brazil (Paraná, Santa Catarina and Rio Grande do Sul).

Fruits were selected but they were not washed and sanitized. The apple musts were obtained by milling, which was followed by centrifugation in a juicer (Philips Walita, 700W, Rio de Janeiro, BR). From each apple must, serial dilutions up to 10^{-4} were prepared in 0.1% peptone water that had been previously sterilized in autoclave (Phoenix, model AV75, 4726 Series, São Paulo) at 121°C for 15min. Dilutions were plated on the surface of YA-LYS (11.75g/L of yeast extract and 20.0g/L of agar-agar from HiMedia Laboratories Pvt. Ltd, India and 2.3g/L of lysine, Biotec, Brazil) and incubated at 25°C for 48h (Quimis oven, model 316B24, Series 6520, Ohio, US) (KURTZMAN et al., 2003). In order to obtain pure cultures, the colonies were visually differentiated and then inoculated in the same culture medium for purification and isolation. The colonies isolated were grown in rosa bengala chloranphenicol (Merck) medium, which inhibits the growth of bacteria by the presence of 0.1% of the antibiotic chloramphenicol (ZOTT et al., 2008). Conservation of the isolated yeasts occurred in inclined tubes with YMA medium (Yeast Malt Agar, HiMedia Laboratories Pvt. Ltd, India) at a temperature of 7-10°C; recovery for selection of the strains occurred in GPYB medium (Merck, Germany) that consists of 40g/L D-glucose, 5g/L yeast extract, 5g/L peptone and 15g/L agar (KURTZMAN et al., 2003).

Total DNA was extracted using the technique described by BRANDÃO et al. (2011). To obtain PCR fingerprinting, was used the Micro/mini satellite-primed PCR (MSP-PCR) fingerprinting method. The PCR reactions were performed according to LIBKIND et al. (2003). Isolated yeast with identical patterns of DNA bands were grouped and considered as the same species. Isolates of each molecular group formed by PCR-fingerprinting were identified by sequencing the D1/D2 domains of the largest sub-unit of the rRNA gene. The D1/D2 domains of the largest sub-unit of the rRNA gene were amplified according to the protocol previously described by LACHANCE et al. (1999) using the primers NL-1 (5´-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5´-GGTCCGTGTTTCAAGACGG-3'). Products obtained after the PCR reactions were purified and sequenced using a MegaBace^{tm} automated sequencer (Amersham Biosciences, USA). Sequences obtained were compared with those deposited in the GenBank database using the Basic Local Alignment Search Tool (ALTSCHUL et al., 1997).

For the selection of isolated yeast a pasteurized apple must (80-85°C/20min) was processed according to ALBERTI et al. (2016). A colony of the pure cultures was inoculated in 250mL of apple must and incubated for 72h at 20°C. Turbidity and production of carbon dioxide were evaluated as indicators of fermentative activity. Selection of strains that produced fruity/floral aromas was performed by simple olfactory analysis by trained team (ten cider experts) using the free profile descriptive sensory technique (STONE et al., 2012). The evaluated attributes of aroma, after smelling the sample twice, were fruity, floral, yeast, pasteurized apple must (control) and atypical cider aroma. The yeast strains that show fruity and floral aroma were selected.

Identification of isolated yeasts (non-*Saccharomyces* strains) obtained from thirty-five (35) samples of apple must, can be observed in table 1. 84.9% of the strains were identified by molecular taxonomy, corresponding to forty-five (45) selected strains. Some strains were not identified, due to problems with amplification of the extracted DNA. Identified yeast strains were: thirteen (13) belonged to the genus *Rhodotorula* sp., 9 to *Lodderomyces* sp., 10 to *Clavispora* sp., 5 to *Candida* sp., 4 to *Hanseniaspora* sp. and 4 to *Pichia* sp. (Table 1). Three of these genus (*Rhodotorula* sp., *Lodderomyces* sp. and *Clavispora* sp.) together they constituted 71.2% of the strains identified.

Three species of *Candida* sp. were reported in the present study. *Candida tropicalis* and *Candida oleophila* had already been detected in apple juice and cider (COTON et al., 2006) and the *Candida parapsilosis* in cider (BEDRIÑANA et al., 2010). *Clavispora lusitanea* and *Candida parapsilosis* are human pathogens. The *Lodderomyces elongisporus* strain is associated with bloodstream infections (DÖĞEN et al., 2017). Thus, the presence of these species may pose a risk to people who consume unpasteurized apple juice.

Hanseniaspora uvarum has been isolated from apple must, cider and wine (COTON et al., 2006; VALLES et al., 2007; BEDRIÑANA et al., 2010;

Table 1 - Identification and diversity of non-*Saccharomyces* yeasts isolated and selected of fresh apple must.

Code of isolated yeasts	Size of fragment [(a)]	Name of species	GenBank Number	Similarity [(b)] (%)
2/5/37	613	*Candida oleophila*	EU326130	98
45	570	*Candida parapsilosis*	AB617999	95
46	570	*Candida tropicalis*	HM589856	95
3/4/6/9/41	517	*Clavispora lusitaniae*	AB617983	100
10/12	517	*Clavispora lusitaniae*	AB617983	98
7/11/15	541	*Clavispora lusitaniae*	GU460176	99
42/43	578	*Hanseniaspora guilliermondii*	AB618029	99
53	564	*Hanseniaspora guilliermondii*	EU386743	93
52	590	*Hanseniaspora uvarum*	GU080043	99
20/38/40/44/4748/49/50/51	588	*Lodderomyces elongisporus*	HM357469	99
1/8	561	*Pichia anomala (Wickerhanomyces anomalus)*	HQ199214	100
22/39	581	*Pichia fermentans*	GQ121617	99
24	571	*Rhodotorula mucilaginosa*	EF174506	99
28/29/30/31/33/34	564	*Rhodotorula mucilaginosa*	HQ116535	99
23/36	564	*Rhodotorula mucilaginosa*	HQ116535	98
25/26/32	614	*Rhodotorula mucilaginosa*	EU563932	97
35	561	*Rhodotorula mucilaginosa*	HQ199212	99

Note: [(a)]Value for the number of base pairs per fragment; [(b)]Percentage of identical nucleotides in the sequence obtained from the D1/D2 region of the 26S rRNA gene and sequence found in Genbank.

VARELA, 2016). In this study, the *Hanseniaspora guilliermondii* (*Kloeckera apiculata* or *Kloeckera apis*) species was isolated from apple must, but it had been previously isolated from grapes, grape must and wine (VARELA, 2016; ÇELIK et al., 2017). The genus *Hanseniaspora* sp. has been successfully used to improve the aromatic quality of wine (PADILLA et al., 2016) and cider (PIETROWSKI et al., 2012; COUSIN et al., 2017), indicating the importance of this discovery in Brazilian apple must. In addition, *H. uvarum* can be preserved lyophilized for up to 12 months without losing viability (percentage of living cells) and vitality (fermentative capacity) (PIETROWSKI et al., 2015).

The yeast strains that not modify the primary aroma of the pasteurized apple must or produced an unpleasant aroma (atypical cider aroma), were discarded. The five (5) non-*Saccharomyces* and non-pathogenic yeasts (*Candida oleophila* [Nº5]; *Rhodotorula mucilaginosa* [Nº32]; *Pichia fermentans* [Nº39]; *Hanseniaspora uvarum* [Nº52] and *Hanseniaspora guilliermondii* [Nº 53]) were selected in qualitative tests due the fruity or floral aroma production by trained team in the aromatic assessment of cider. Therefore, this study indicated new microorganisms that can be used to improve the aroma quality of cider.

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DECLARATION OF CONFLICTS OF INTEREST

All the co-authors reported that they have no conflict of interest with the publication of this manuscript.

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