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Morphological, Biochemical and Molecular Identification of the Yeast *Levica 25:* A Potential Ruminal Microbial Additive

[1]*Yoandra Marrero,* [2]*Yamicela Castillo,* [2]*M.Eduviges Burrola-Barraza,* [3]*Tamara Lobaina,*
[4]*Carlos A. Rosa,* [2]*Oscar Ruiz,* [2]*Everardo González-Rodríguez and* [5]*Luiz C. Basso*

[1]Departamento de Fisiología, Instituto de Ciencia Animal (ICA), Cuba
[2]Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua (UACH), México
[3]Departamento de investigaciones de Medios de Cultivo, Centro Nacional de Biopreparado (BIOCEN), Cuba
[4]Instituto de Ciências Biológicas (ICB), Universidade Federal de Minas Gerais (UFMG), Brasil
[5]Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de Sao Paolo (USP), Brasil

Abstract: *Levica 25* is a biological agent isolated from bovine rumen with potential use as an activator of ruminal fermentation because it stimulates total and cellulolytic populations and it reduces methanogenesis, which mitigates methane emissions to the environment. The aim of this study was to characterize the strain *Levica 25* using morphological, biochemical and molecular methods. Morphological and biochemical studies showed that this strain did not produce germ tubes, grew at a temperature of 45°C, absorbed cupric sulfate, reduced bismuth sulfite, was sensitive to actidione, formed blue colonies in the presence of chromogenic agents and presented profile number 2556175 in the API20C *AUX4* system. These data suggested that *Levica 25* may be a strain of *Candida tropicalis*. However, analysis of the D1/D2 region of rDNA26S demonstrated that *Levica 25* has only 96% homology with *C. tropicalis* and there are 11 substitutions involving 575 bases, which is equivalent to a 1.9% difference between the two sequences. Thus, *Levica 25* and *C. tropicalis* are different, albeit very close, species located in the same clade in the phylogenetic tree. The present results suggested that *Levica 25* is a new strain belonging to the genus *Candida*, which is very close to *C. tropicalis*.

Key words: Yeast · *C. tropicalis* · Ruminal fermentation · rDNA26S

INTRODUCTION

Biological products that are activators of ruminal fermentation [1] allow microbial ecosystems to be manipulated to improve nutrient efficiency in ruminants, especially in the consumption of fibrous diets. Such products are obtained from various microorganisms, particularly yeast [2]. In addition, the use of yeast not only has a positive impact on the microbial population and rumen fermentation indicators, but also improves the health and productivity of the animals [3, 4].

Saccharomyces cerevisiae [5] and *Issatchenkia orientalis* [6] are among the yeast most commonly used to trigger rumen fermentation. Apart from these, there are few genera of yeasts that are used for these purposes. In previous work, Marrero *et al.* [7,8] used biochemical and morphological assays to isolate and characterize a group of yeast strains from the rumen ecosystem that do not belong to the *Saccharomyces* genus. Among them, *Levica 25* was shown to produce greater stimulation of the total and cellulolytic populations of the rumen of cows with fibrous diets, than *S. cerevisiae* strains. In addition, the effect of the former was more long-lasting. In a separate set of studies, Galindo *et al*. [9] conducted *in vitro* studies that showed that *Levica 25* reduced the quantity of methanogen microorganisms and ruminal methanogenesis. These results suggested that it may be possible to use this yeast to improve the efficiency of energy utilization in ruminants, which could also help mitigate the impact of methane emissions on the environment.

All of the results hitherto presented point to the potential use of *Levica 25* in the development of a product that will work as an activator of ruminal

Corresponding author: Yoandra Marrero, Department de Fisiología, Instituto de Ciencia Animal (ICA), Cuba, carretera Central, Km 47 ½, Codigo Postal: 32700, San Jose de las Lajas, La Habana, Cuba. Tel: 53-47-994-33.

fermentation. Hence, the objective of the present study was to identify the strain *Levica 25* using morphological, biochemical and molecular assays.

MATERIALS AND METHODS

Morphological Assessment: Samples from a culture of the strain *Levica 25* were plated on Sabouraud dextrose agar plates. The plates were then incubated for 48 h at 30±2°C and the purity of the microbial strain was confirmed. The strain was macromorphologically characterized by observing colony characteristics such as color, texture and topography of the surface and edges, as previously described [10]. Micromorphological evaluation was performed via assessments of germ tube production [11] and formation of hyphae, blastoconidia and chlamydospores, while the strain was incubated in fetal calf serum (Invitrogen) at 37°C for 3 h.

Biochemical Evaluation: Detection of non *Saccharomyces* wild yeast was performed according to the procedure of the Bavaria brewery in Bogotá, Colombia, which evaluated the uptake of cupric sulfate [7]. In addition, the growth of the strain at 45°C was evaluated in Sabouraud dextrose agar for 48 h, in Nickerson media at 22°C for 48 h and in CromoCen CND-C and CromoCen CND-F media, for 48 h at 35°C.

Actidione or cycloheximide resistance was assessed in Sabouraud dextrose agar culture media supplemented with 0.5 g/L of cycloheximide (Sigma). The assimilation of carbon compounds was determined using API-20C AUX V4 strips (BioMerieux, France), according to the recommendations of the supplier. The results were interpreted according to the numerical profile generated from the reactions observed, using the Analytical Profile Index (API).

Molecular Characterization: Yeast DNA was obtained using PureLink™ Genomic DNA Mini Kits (Invitrogen), according to supplier's specifications. Molecular identification was based on amplification of the D1/D2 region of the rDNA26S gene using the nucleotides NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-'3) and NL-4 (5'-GGTCCGTGTTTCAAGACGG-'3), as described by Lachance *et al.* [12]. Polymerase chain reactions (PCRs) were performed in a final volume of 50 µL, containing 5 µL of 10× PCR buffer, 2 µL of 50 mM $MgSO_4$, 2 µL of 10 mM dNTP, 10 pmol of the primers NL1 and NL4, 200 ng of DNA and 1 U of High Fidelity Platinum® *Taq* DNA Polymerase (Invitrogen). Amplification was performed under the following program: initial denaturation at 95°C for 5 min; followed by 35 cycles of 15 s at 94°C, 25 s at 54°C, 20 s at 68°C; and finally 10 min at 68°C. The PCR products were purified and sequenced using DYEnamicTM (Amersham Biosciences, USA) in combination with the MegaBACE™ 1000 automated sequencing system. The obtained sequence was deposited in Genbank databases according to the program Bankit (NCBI) with access number JF894135. In addition, homology was searched for through the BLASTN program [13], with sequences of related species deposited in Genbank.

Phylogenetic analysis was performed with DAMBE software [14]. In addition, an alignment was conducted of the amplified D1/D2 region of *Levica 25* using the CLUSTALW program [15] against 23 close *Saccharomyces* and *Candida* yeast sequences taken from Genbank: *S. cerevisiae* (HM106430.1), *C. orthopsilosis* (FJ746061.1), *C. viswanathii* (EU589207.1), *C. tetrigidarum* (FJ614703.1), *C. frijolensensis* (EF120596.2), *C. tropicalis* (HM627137.1), *C. albicans* (GU319992.1), *C. castelli* (UG9876.1), *C. carpophila* (FM180531.1), *C. ernobii* (U70241.1), *C. ergastensis* (U45746.1), *C. fermentati* (DQ377634.1), *C. novergensis* (HM627085.1), *C. glabarata* (ABG18021.1), *C. lipolytica* (EU809454.1), *C. guillermondii* (GU373754.1), *C. dublinensis* (U57685.1), *C. parapsilosis* (EU326120.1), *C. kruisii* (DQ173679.1), *C. kefyr* (EU828791.1), *C. utilis* (DQ409141.1), *C. lisitaniae* (EU669469.1) and *C. famata* (DQ513292.1). The phylogenetic tree was constructed using Kimura's two parameter correction [16] genetic distance model with the neighbor-joining method [17].

RESULTS AND DISCUSSION

Phenotypic Characteristics: The primary identification of all yeast species begins with an evaluation of macroscopic criteria. The morphological characteristics of colonies isolated on Sabouraud dextrose agar allow one to conclude, presumptively, that a strain belongs to the genus *Candida* or *Saccharomyces*. In general, we observed complete colonies that were 2–3 mm in diameter, slightly convex, of a smooth, creamy consistency, white to cream in color and having a sweet smell that is typical of yeast (Fig. 1). It was found that the culture did not develop aerial mycelium, nor did it show extensions at the edges or the surface of the colonies; these features are characteristic of yeast of the genus *Geotrichum*, *Trichosporon,* or *Blastoschizomyces* [10].

Fig. 1: *Levica 25* colonies. The strain *Levica 25* was cultivated in Sabouraud dextrose agar for 48 h at 30°C and the colonies were observed under a Zeiss Stemi 2000-C stereoscope at 65× magnification.

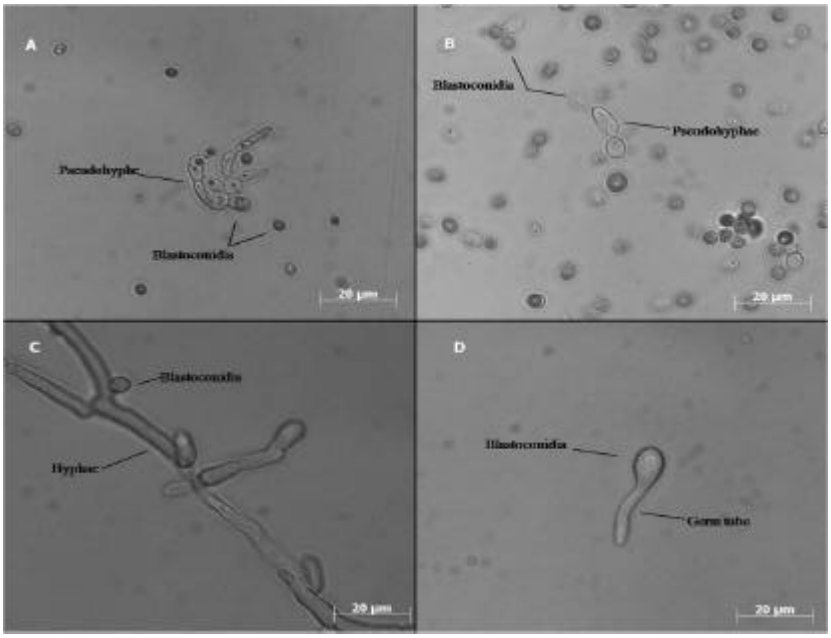

Fig. 2: Germ tube production. A and B. *Levica 25* (development of pseudohyphae). C and D. *C. albicans* (development of germ tube). The yeasts were incubated in the presence of fetal calf serum (Invitrogen) at 37°C for 3 h and were observed in a bright field under a Zeiss AxiosCop plus II microscope at 40× magnification.

Among other features observed in the colonies, we considered their appearance and color, which were essential to rule out their belonging to the genus *Cryptococcus*, which develops colonies of a mucoid consistency and the genus *Rhodotorula*, whose colonies are red-orange to orange due to the production of carotenoids [10].

Microscopic description of *Levica 25* in the presence of fetal calf serum showed the development of long pseudohyphae and the formation of few blastoconidia scattered at regular intervals or in small groups located alternately along the pseudohyphae. In addition, the presence of a germ tube was not found (Fig. 2, A and B), which distinguishes *Levica 25* from *C. albicans*, which if developed a germ tube (Fig. 2, C and D), but resembles the growth of *C. tropicalis* and *C. parapsilosis*, which can produce early pseudohyphae resembling germ tubes [18].

Fig. 3: Growth of *Levica 25* in chromogenic medium. The strain *Levica 25* was plated in CromoCen CND-F media and incubated at 35°C for 48 h.

Table 1: Biochemical characteristics of the strain *Levica 25*

Characteristic	Growth of *Levica 25*
Cupric sulfate assimilation	Positive
Bismuth sulfite reduction	Positive
Germ tube development	Negative
Growth at 45°C	Positive
Actidione resistance	Negative
Growth in chromogenic medium	Blue
API 20C AUX	Profile: 2556175

The growth of *Levica 25* in the presence of cupric sulfate was positive, indicating that the strain does not belong to the genus *Saccharomyces*. Also, when assessing the growth of *Levica 25* in Nickerson media, we observed colonies of a dark brown color, which were dense and smooth, showing that the strain was able to reduce bismuth sulfite [19]. In addition, growth at 45°C was positive, which is typical of the species *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. kefyr* [10]. On the other hand, *Levica 25* was unable to grow in the presence of actidione, a chemical that has strong inhibitory power for most species of *Candida*, with the exception of *C. albicans*, *C. dubliniensis* and *C. kefyr* [20]. In assessing the characteristics of the strain *Levica 25* in the chromogenic culture media CromoCen CND-C and CromoCen CND-F, we observed the growth of blue colonies (Fig. 3) and blue fluorescence under 366 nm ultraviolet light (data not shown), which is characteristic of *C. tropicalis*. Determination of the carbohydrates, which this organism is able to use as the sole source of nutrients, was performed using the API 20C AUX4 system. From the interpretation of the results, we obtained the profile 2556175, which corresponds to the species *C. tropicali*s with an identity of 95.7%, according to the API (Table 1).

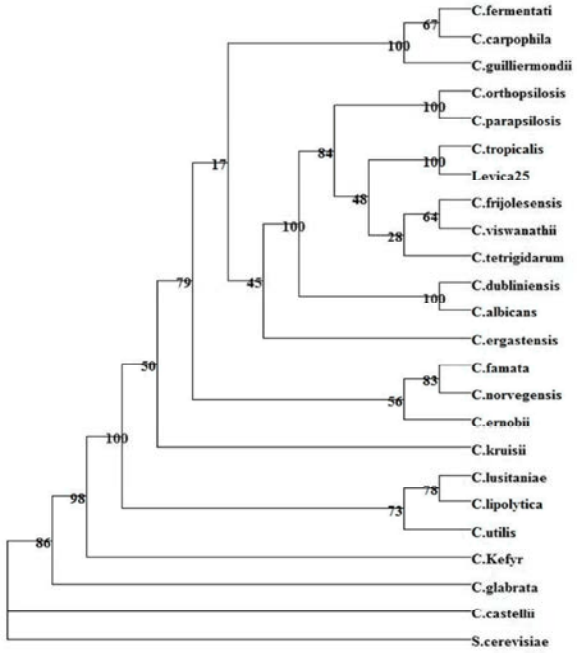

Fig. 4: Phylogenetic tree based on sequences of the D1/D2 region of the rDNA26S gene. The tree shows the position of *Levica 25* with respect to closely related *Candida* species. The tree was constructed based on the genetic distances obtained according to Kimura's two parameter model (1980) using the neighbor-joining method. The lengths of the branches are proportional to the number of substitutions per site. The numbers in the clades indicate the sampling percentage (bootstrap) derived from 1000 replicates.

Phylogenetic Analysis: To corroborate the data already obtained and to confirm whether *Levica 25* belongs to a strain of *C. tropicalis*, we amplified the D1/D2 region of *Levica 25*'s rDNA26S gene and compared it with the same sequence of *C. tropicalis* reported in GenBank (HM627137.1). The analysis showed that *Levica 25* has a 96% homology with *C. tropicalis*. The two sequences presented 11 substitutions in 575 bases, which is equivalent to a 1.9% difference. According to Kurtzman and Robnett [21], yeast strains that exhibit nucleotide substitution >1% in the D1/D2 region of the rDNA26S gene should be considered different species. A phylogenetic tree based on this D1/D2 region suggests that *Levica 25* is a sister species of *C. tropicalis*, since both are located in the same clade and both are, in turn, located in the same family together with *C. orthopsilosis*, *C. parapsilosis*, *C. frijolensis*, *C. viswanati* and *C. tetrigidarum* (Fig. 4).

In conclusion, the results reported herein strongly suggested that *Levica 25* is a new, as yet unreported, strain that is very close to *C. tropicalis*, sharing similarities in terms of their phenotypic characteristics, but differing with respect to the D1/D2 region of the rDNA26S gene.

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