Identification of *Levica* yeasts as a potential ruminal microbial additive

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ABSTRACT: The objective of this study was to identify and characterize yeast strains isolated from the ruminal ecosystem that are capable of enhancing fermentation in bovines that consume high-fibre diets recommended by livestock feed guidelines in Cuba. The yeasts were isolated from the rumen of Holstein cows that had been fed a biofermented product. Isolated colonies were purified, identified, and characterized using biochemical and molecular methods, and their effects on ruminal fermentation were compared by measuring *in vitro* gas production. Thirteen new strains enhancing gas production with potential use as additives in ruminal fermentation were identified and named *Levica*. These strains grew successfully in detection medium for non-*Saccharomyces* wild yeasts and had long survival periods in the rumen. PFGE analysis found four karyotypes and homology of D1/D2 domain of gene 26S rDNA sequence was similar to that of *I. orientalis*, *R. mucilaginosa*, *P. guilliermondii*, and *C. tropicalis*. Phylogenetic analysis classified the strains into clades A and B. Clade A was further divided into groups AI, AII, BI, and BII. The AI cluster contained *Levica* (*L*)*23, L24, L29*, *L33*, and formed a monophyletic group with *I. orientalis*, while group AII contained *L18* and formed a monophyletic group with *R. muciloginosa*. The BI cluster contained *L13*, *L15, L17, L27, L28,* and *L32*, all derived from *P. guilliermondii*. Cluster BII was composed only of *L25* located in a separate subclade, forming a monophyletic group with *C. tropicalis*. The most useful strain for preparing microbial feed products to improve ruminal fermentation was *L25* because it showed an increase in gas production.

Keywords: fermentation; bovine; rumen ecosystem

In recent decades interest has grown in the use of yeasts as livestock feed additives. In bovines with high-fibre diets the importance of altering the microbial ecosystem to improve the efficiency of nutrient use has been demonstrated. Around the world one of the most frequent methods of improving ruminal fermentation is the use of biological products called direct-fed microbial feed additives (DFM) obtained from various microorganisms, especially yeasts (Lila et al., 2004; Chaucheyras-Durand et al., 2008).

The addition of these microorganisms to a diet can have a favourable effect on the microbial population and on fermentation indicators in the rumen, thus improving animal health and productivity (Jouany, 2001; El-Ghani, 2004; Stella et al., 2007). The yeast *S. cerevisiae* has been used in some Latin American countries as a supplement in high-fibre diets. Introduction of this yeast has altered fermentation patterns resulting in more efficient utilization and availability of nutrients (Angeles et al., 1995; Mendoza et al., 1995; Doležal et al., 2005), higher cellulolytic bacterial counts (Newbold et al., 1998), higher ruminal and lower lactic acid concentration (Williams et al., 1991), greater digestion of ruminal dry matter (DM),

organic matter (OM), and neutral detergent fibre (NDF) both *in vivo* and *in vitro* (Williams et al., 1991; Wohlt et al., 1998; Biricik and Turkman, 2001), and higher degradation of acid detergent fibre (ADF) and nitrogen (Doreau and Jouany, 1998). It is important to note that most yeast additive products used in these countries must be imported from developed countries that do not face the same livestock management and feed challenges. The need exists to obtain additives within each country that respond to local conditions related to dairy production, based on medium- and low-quality fibre diets. Thus the objective of this study was to identify and characterize yeast strains extracted from the ruminal ecosystem that can enhance ruminal fermentation in bovines with high-fibre diets as recommended by livestock feed guidelines in Cuba.

MATERIAL AND METHODS

Animal management

Three ruminally cannulated 5-year old Holstein cows with an average weight of 458 kg were stabled in individual cubicles. These animals were used only for experimental purposes and not for milk production. Over 12 days, the animals were fed a diet based on concentrate (6 g DM/kg true protein (TP)), fresh king grass fodder (10 kg/100 kg TP), 42% biofermented product (Vitafer, Institute of Animal Science (ICA), San José de las Lajas, Cuba) enriched with yeast (10^8 CFU/ml), and water as required. Rumen fluid was sampled at 4, 8, 12, and 24 h after food consumption and transferred to the laboratory in hermetically sealed sterile bottles.

Isolation of ruminal yeasts

Yeasts were isolated according to the methodology of Marrero et al. (2005). Isolation media included malt agar, Sabouraud broth, and Sabouraud agar, all supplemented with streptomycin and chloramphenicol (0.1% w/v), unless otherwise stated. Briefly, rumen fluid dilutions (10^{-3}–10^{-5}) were filtered under CO_2, seeded in malt agar roll tubes under strict anaerobic conditions, and incubated at 39°C for 24 h. Cultures exhibiting different macroscopic characteristics were inoculated into Sabouraud broth, incubated at 30°C for 2–7 days, then streaked onto Sabouraud agar and incubated at 30°C for 48–72 h. Isolated colonies were inoculated into both Sabouraud media and malt agar roll tubes, incubated at 30°C for 24–72 h, and stored at 4°C for further use.

Biochemical characterization

To determine whether the isolated yeasts belong to the genus *Saccharomyces*, yeast isolates were grown on malt agar extract (3.2 g/l) with bacteriological grade agar (19 g/l), peptone (1.8 g/l), dextrose (10 g/l), K_2HPO_4 (1 g/l), NH_4Cl (0.5 g/l), $CuSO_4$ (5% w/v) at 30°C for 72 h. *Saccharomyces cerevisiae* L/25-7-13 from the Cuban Institute for Research on Sugarcane Derivatives (ICIDCA) was used as the control. To determine their oxygen requirements, strains were grown in malt extract broth, malt extract agar, and thioglycollate media at 30°C for 48 h. Urea hydrolysis, starch fermentation, and high osmotic pressure tests were performed according to methods described in Marrero et al. (2005). Carbohydrate fermentation tests were performed in triplicate according to Wickerham (1952). Growth in glucose, galactose, sucrose, lactose, and inositol media was evaluated by measuring absorbance at 460 nm after 7, 14, and 21 days of incubation.

Molecular characterization

Genomic DNA was isolated with PureLink™ Genomic DNA Mini Kits (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Region D1/D2 of gene 26S rDNA was amplified using oligonucleotides NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') according to Lachance et al. (1999). PCR reactions contained 5 µl 10X High Fidelity PCR Buffer, 2 µl 50mM $MgSO_4$, 2 µl 10mM dNTPs, 10 pmol of NL1 and NL4 primers, 200 ng DNA, 1U Platinum® Taq DNA Polymerase High Fidelity in a final volume of 25 µl (Invitrogen). Amplification was carried out as follows: 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 a final extension at 68°C for 10 min. PCR products were purified and sequenced using DYEnamic™ in combination with the MegaBACE™ 1000 automated sequencing system (GE Healthcare, Wakeusha,

USA). Sequences were submitted to the GenBank database with the BankIT program (NCBI) and analyzed using BLASTn (Altschul et al., 1997). Phylogenetic analyses were performed using PHYLIP (Felsenstein, 1993) and ClustalW (Thompson et al., 1994) programs. In addition to the yeasts isolated in this study, the D1/D2 region of 26S rDNA of 26 closely related yeasts was included in the analyses. The yeasts are *S. cerevisiae* (FJ665617.1), *I. orientalis* (JX537791.1), *I. occidentalis* (EF643590.1), *I. scutulata* (U76349.1), *I. terricola* (U76345.1), *P. membranifaciens* (AB045135.1), *C. rugopelliculosa* (U71069.1), *P. nakasei* (U75728.1), *C. pseudolambica* (U71063.1), *I. hanoiensis* (AY163900.1), *C. ethanolica* (U71073.1), *P. deserticola* (U75734.1), *P. galeiformis* (U75738.1), *P. guilliermondii* (FJ468466.1), *C. carpophila* (FM180531.1), *C. fermentati* (DQ377634.1), *C. tropicalis (*JX532155.1), *C. albicans* (KC113635.1), *C. dubliniensis* (U57685.1), *C. maltosa* (AB557868.1), *C. parapsilosis* (EU326120.1), *C. viswanathii* (JN031570.1), *R. mucilaginosa* (KC160628.1), *R. glutinis* (HM190197.1), *R. slooffiae* (KC006818.1), *R. fujisanensis* (KC006647.1), and *R. pallida (*AF189962.1). The phylogenetic tree was generated using Kimura's two-parameter correction model for genetic distance (Kimura, 1980) and the neighbour-joining method (Saitou and Nei, 1987).

Karyotyping of yeast isolates

A portion (1–4 mg of wet weight) of each yeast isolate was treated according to the protocol of Blondin and Vézinhet (1988). Transverse alternating-field electrophoresis (TAFE), a method similar to pulsed-field gel electrophoresis (PFGE), was performed using a GeneLine instrument (Beckman Coulter Inc., Fullerton, USA) set at 170 mA for 4 h with 4 s pulses, followed by 150 mA for 18 h with 60 s pulses according to Basso et al. (2008).

Gas production

The fermentative capacity of the isolated yeasts was assessed using the *in vitro* gas production method of Theodorou et al. (1994). Isolates were grown in 10 ml of malt extract broth at 30°C for 24 h, then subcultured (1 : 40 dilution) in malt extract broth, and incubated at 30°C for 24 h. 4 ml of the culture (equivalent to 20 mg DM/ml) were transferred to 50 ml glass bottles containing 0.2 g *Cynodon nlemfuensis* (stargrass) with a chemical composition of 7.26% crude protein (CP), 74.57% NDF, 10.11% ash, 0.42% calcium, and 0.18% phosphate. The glass bottles were shaken at 39°C and 120 rpm. Gas production was measured at 2, 4, 6, 8, 10, 12, and 24 h by bubble displacement in a 10 ml syringe. Data was analyzed by multivariate analysis and fully randomized Analysis of Variance (5 groups with 4 repetitions).

RESULTS AND DISCUSSION

Isolation and characterization of *Levica* strains

In this study, by the addition of biofermented product to cows' feed, 13 yeasts named *Levica* were isolated from the ruminal ecosystem and given corresponding isolate numbers. A key requirement for a strain to be used as DFM is that it remains viable in the rumen for sufficient time to stimulate ruminal metabolism (Lee et al., 2002). In addition to a proven effect upon ruminal fermentation, stability of the newly selected yeast strains within the rumen is important, too. Although it is known that many yeast strains cannot effectively colonize the rumen, certain strains can remain metabolically active in rumen fluid for up to 48 h (Kung et al., 1997; Chaucheyras-Durand et al., 2005) and live cells may be recovered from the faeces of animals up to several days after their initial incorporation in the diet (Chaucheyras-Durand et al., 2008). The *Levica* yeast strains proved in this study were retained for up to 12 h after ingestion (data not shown). These results imply that *Levica* yeast strains have a longer survival period in the rumen, making them potentially useful as biological products that could be activators of ruminal microbial fermentation.

While the morphological characteristics of the yeast colonies may be highly complex and difficult to describe, it was observed that *Levica* (*L*)*18* was the only strain to present a pink coloration, unlike the other strains which were white. In addition, *Levica* strains *L13, L15, L17, L23, L25, L27, L28,* and *L32* presented a creamy appearance and regular borders, while *L24, L29, L33,* and *L37* had irregular borders (data not shown). During growth in liquid media most of the strains showed sediment, with the exception of *L24* and *L29* which formed

a complete film. Strains *L18*, *L23, L32,* and *L37* presented both types of growth in liquid media (data not shown). All the strains showed growth across their entire medium, both in malt extract and in thioglycollate. Also, cultures kept under strict anaerobic conditions exhibited diffuse growth. Strain *L18,* exhibiting pink pigmentation in aerobic conditions, was white under anaerobic conditions.

The tests for urea hydrolysis, starch fermentation, and high osmotic pressure revealed that all isolated strains were ureolytic, and showed growth under high osmotic pressure conditions (Table 1). Metabolism of carbohydrates was determined by evaluating growth of the isolates in media containing carbohydrate. Fermentation was analyzed by evaluating gas production by isolates in media containing carbohydrate. All *Levica* strains grew in glucose media but only *L13*, *L15*, *L17,* and *L27* were able to ferment glucose. All strains except *L37* grew in saccharose media, while only *L17* and *L18* were able to ferment saccharose. Strains *L13*, *L15*, *L17*, *L18*, *L27*, *L28*, *L29*, *L32*, *L33,* and *L37* grew in galactose, but only *L13*, *L15*, *L17,* and *L18* fermented galactose. For galactose, strains *L23*, *L29,* and *L25* could not assimilate it, while *L18*, *L13*, *L15,* and *L17* were able to ferment it. On the other hand, none of the strains was able to assimilate or ferment lactose. Considering that carbohydrate fermentation and assimilation are key differentiating characteristics for yeast species, it can be stated that there is a high species variability in the set of yeast strains (Table 1).

Molecular characterization

Pulsed-field gel electrophoresis (PFGE) has proven to be a powerful method of differentiating the yeast strains species by generating a band pattern specific to the karyotype of each strain (Basso et al., 2008). PFGE analysis of the *Levica* strains identified four different band profiles (Figure 1). Karyotype I is composed of strains *L23*, *L24*, *L29*, *L33,* and *L37. L18* (karyotype II) has a unique band profile. Karyotype III contains *L13*, *L15, L17, L27, L28,* and *L32*. The karyotype of *L25* (karyotype IV) showed no similarity to any other *Levica* strain. To know the similitude with other yeasts reported, from each isolate the D1/D2 domain of gene 26S rDNA was amplified, sequenced, and compared to sequences in GenBank (Table 2). Karyotype II strains *L24* and *L23* had 99% homology with *I. orientalis*, while *L29* and *L33* exhibited 98 and 96% homology, respectively. *L37* had 86% homology with *I. orientalis* and 83% similarity to *I. occidentalis*, while *L18* had 98% identity with *R. mucilaginosa*. Karyotype II *Levica* strains showed above 90% homology with the yeasts *P. guilliermondii*, *C. carpophila,* and *C. fermentati* (Table 2). Phylogenetic analysis classified the

Table 1. Biochemical characterization of *Levica* strains from bovine ruminal ecosystem

Levica strain	Fermentation							Assimilation					Urea hydrolysis	High osmotic pressure
	glucose	saccharose	maltose	galactose	raffnose	lactose	starch	glucose	saccharose	galactose	lactose	inositol		
13	+	-	-	+	-	-	+	+	+	-	-	+	+	+
15	+	-	+	+	+	-	+	+	+	+	-	-	+	+
17	+	+	+	+	+	-	+	+	+	+	-	-	+	+
18	-	+	-	+	+	-	+	+	+	-	-	+	+	+
23	-	-	-	-	-	-	+	+	-	+	-	-	+	+
24	-	-	-	-	-	-	+	+	+	+	-	+	+	+
25	-	-	-	-	-	-	+	+	+	+	-	+	+	+
27	+	-	-	-	-	-	+	+	+	+	-	-	+	+
28	-	-	-	-	-	-	+	+	+	+	-	-	+	+
29	-	-	-	-	-	-	+	+	+	+	-	+	+	+
32	-	-	-	-	±	-	+	+	+	+	-	+	+	+
33	-	-	-	-	-	-	+	+	+	+	-	-	+	+
37	-	-	-	-	-	-	+	+	+	-	-	+	+	+

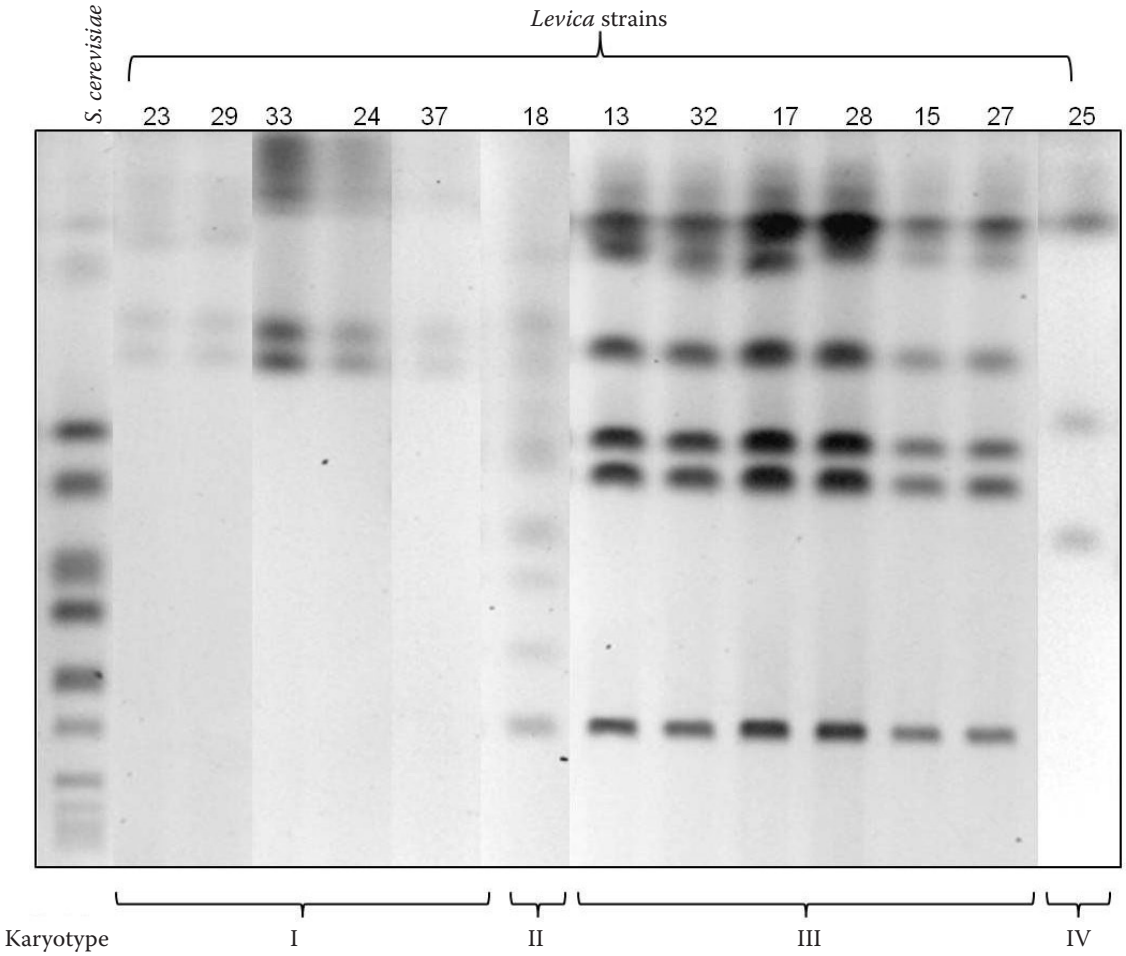

Figure 1. Karyotypes of *Levica* strains obtained by PFGE/TAFE

strains into clades A and B (Figure 2). Clade A had a bootstrap value of 70.7%, while that of clade B was 83.5%. Clade A was divided into groups AI and AII. The AI cluster contained all the strains with karyotype I (72.9% bootstrap) and was divided into subgroups AI.1 and AI.2. *L23, L24*, *L29,* and *L33* clustered in AI.1 (95.5% bootstrap) and were organized monophyletically with *I. orientalis*. In this group, *L23* and *L29* were in the same subclade with a bootstrap of 86.0%. In concordance with these, Lee et al. (2002) isolated the *I. orientalis* DY252 strain with the potential to be used as a microbial feed additive in ruminants. Group AII contained *L18* and formed a monophyletic group with *Rhodotorula muciloginosa* (Figure 2), an yeast that is found in air, soil, water, and dairy products (Guamán-Burneo and Carvajal-Barriga, 2009). They form pink or orange colonies on Sabouraud dextrose agar at 35°C (García-Suárez et al., 2011) similar to *L18* that forms pink colonies on Sabouraud agar (data not shown). According to Jimoh et al. (2011), *R. muciloginosa* is able to assimilate glucose, saccharose, and galactose, as does *L18* (Table 1). However *L18* is unable to assimilate inositol (Table 1), while *R. muciloginosa* has this capacity (Jimoh et al., 2011). Analyzed transitions and transversions in the D1/D2 domain of 26S rDNA of *L18* and *R. muciloginosa* show 1.9% nucleotide substitutions. Kurtzman and Robnett (1998) stated that yeast strains showing nucleotide substitutions >1% in the region D1/D2 of 26S rDNA are different, thus *L18* and *R. muciloginosa* are not the same strain. Clade B was divided into subgroups BI and BII. Cluster BI contained karyotype III strains *L13*, *L15, L17*, *L27*, *L28,* and *L32*, all derived from *P. guillermondii* with bootstrap value of 99.7% (Figure 2). *P. guillermondii* is the yeast widely distributed in nature, present in the soil, plants, and water which can behave as a human opportunistic pathogen (Vaughan-Martini et

Table 2. Percent identity of 26S rRNA gene: *Levicas* strains vs. yeasts

Yeast (GenBank Accession No.)	Strain (GenBank Accession No.)					
	Karyotype I					
	Levica23 (JF894138.1)	*Levica29* (JF894140.1)	*Levica33* (JF894146.1)	*Levica24* (JF894147.1)	*Levica37* (JF894144.1)	
I. orientalis (JX537791.1)	99	98	96	99	86	
I. occidentalis (EF643590.1)	92	91	89	92	83	
I. scutulata (U76349.1)	91	90	88	91	ns	
I. terricola (U76345.1)	86	85	85	87	ns	
P. membranifaciens (AB045135.1)	91	90	88	91	82	
C. rugopelliculosa (U71069.1)	91	91	89	92	ns	
P. nakasei (U75728.1)	86	86	85	87		
C. pseudolambica (U71063.1)	92	92	89	92	86	
I. hanoiensis (AY163900.1)	89	89	88	90	82	
C. ethanolica (U71073.1)	89	88	88	90	81	
P. deserticola (U75734.1)	90	89	87	90	ns	
P. galeiformis (U75738.1)	90	90	88	90	ns	
	Karyotype II					
	Levica18 (JF894148.1)					
R. mucilaginosa (KC160628.1)	98					
R. glutinis (HM190197.1)	93					
R. slooffiae (KC006818.1)	87					
R. fujisanensis (KC006647.1)	85					
R. pallida (AF189962.1)	87					
	Karyotype III					
	Levica13 (JF894136.1)	*Levica32* (JF894142.1)	*Levica17* (JF894137.1)	*Levica27* (JF894143.1)	*Levica15* (JF894141.1)	*Levica28* (JF894145.1)
P. guilliermondii (FJ468466.1)	96	97	90	98	99	95
C. carpophila (FM180531.1)	96	97	90	98	99	95
C. fermentati (DQ377634.1)	95	97	90	98	98	95
	Karyotype IV					
	Levica25 (JF780515.1)					
*C. tropicalis (*JX532155.1)	97					
C. albicans (KC113635.1)	91					
C. dubliniensis (U57685.1)	92					
C. maltosa (AB557868.1)	95					
C. parapsilosis (EU326120.1)	95					
C. viswanathii (JN031570.1)	93					

ns = nonsignificant similarity

al., 2005). Cluster BII was composed only of *L25* located in a separate group forming a monophyletic subclade with *C. tropicalis* bootstrap at 99.8% (Figure 2). *C. tropicalis* has been used in biotechnology, due to its ability to degrade hydrocarbons, ferment cocoa beans (Ardhana and Fleet, 2003), degrade polyphenols in wast water (Ettayebi et al., 2003), and produce polyhydroxybutyrate (Priji et

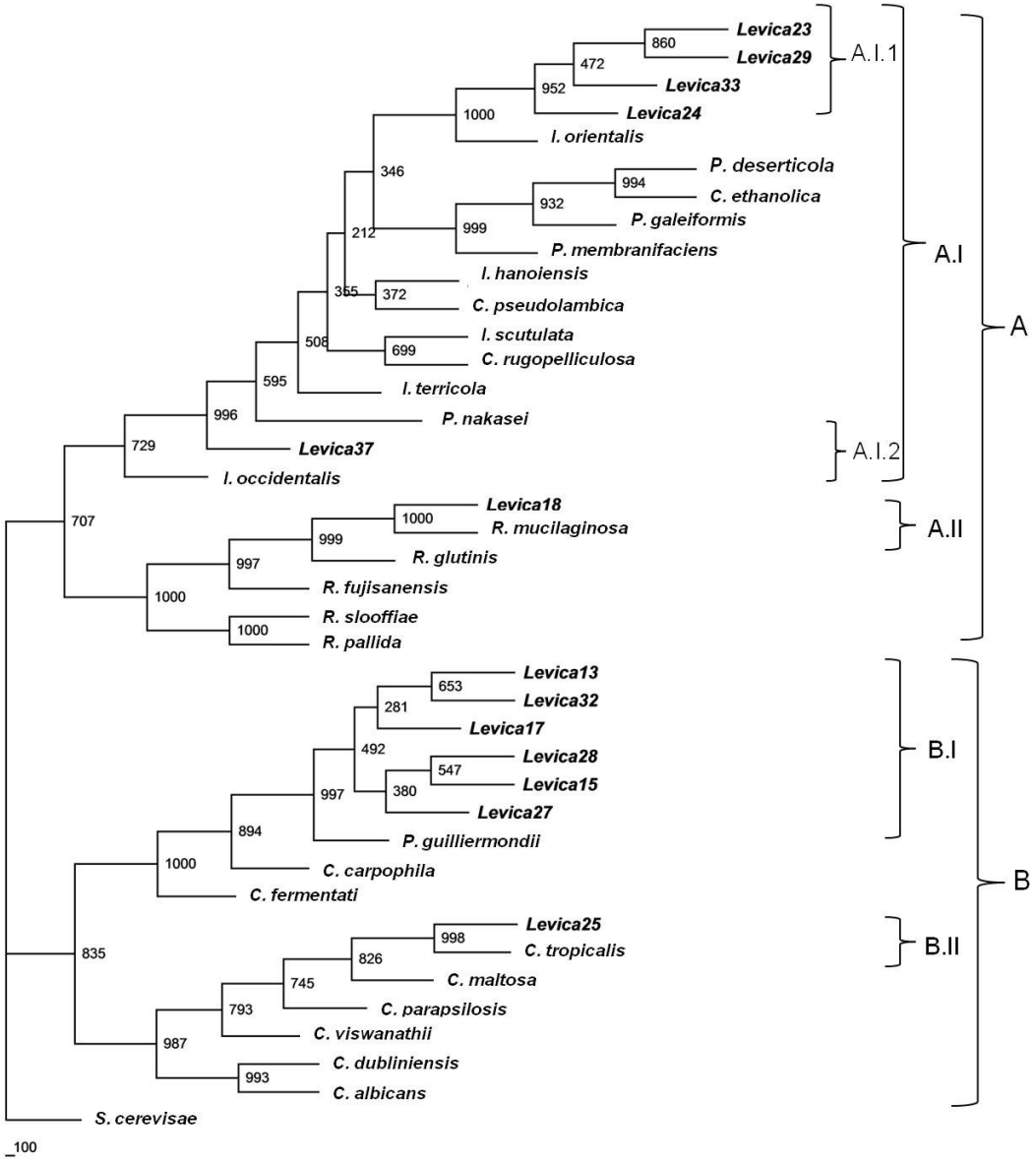

Figure 2. Phylogenetics of *Levica* strains isolated from bovine ruminal ecosystem

The majority consensus tree was built according to genetic distances generated by Kimura's two-parameter method with neighbour-joining. Branch length is proportional to number of substitutions per site. Numbers in clades indicate resampling (bootstrap) derived from 1000 replicates

al., 2013). Further, *C. tropicalis* has been isolated from the rumens of goats (Priji et al., 2013). Due to their genetic similarity and because both karyotypes were identical, it was assumed that *L25* and *C. tropicalis* were the same yeast. However our biochemical tests indicated the opposite, showing marked differences in metabolism of the strains; *C. tropicalis* is able to ferment glucose and maltose, and can assimilate galactose (Hirimuthugoda et al., 2006), while *L25* cannot do that (Table 1). Also *L25* has 1.9% nucleotide substitutions in the region D1/D2 of gene 26S rDNA and when compared to *C. tropicalis* it is obvious that these strains are different from each other.

Gas production

The *in vitro* gas production technique is a useful alternative for studying degradation kinetics of various feeds (Theodorou et al., 1994). To detemine which *Levica* strain most effectively ferments cellulose, *Levica* isolates were individually inoculated with the substrate *C. nlemfuensi*

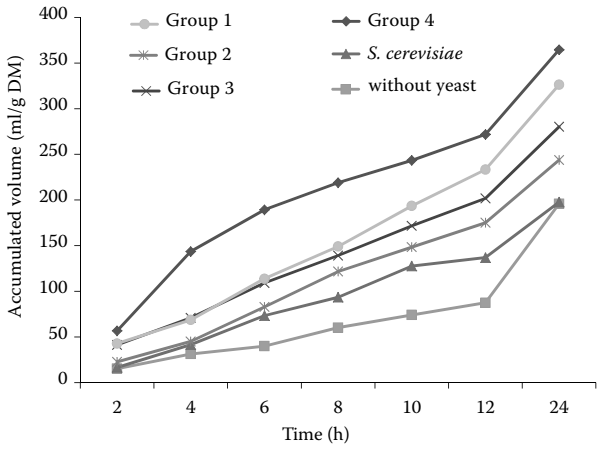

Figure 3. Gas production of yeast strain groups in DM degradation of *C. nlemfuensis*

(stargrass) and gas levels were measured after 24 h of fermentation (Figure 3). Using multivariate analysis on the gas production data, the *Levica* strains formed four groups. Group I contained strains *L17*, *L18*, *L23, L24*, *L27*, *L28*, *L33,* and *L37*. Group II contained strains *L29* and *L32*. Group 3 contained *L13* and *L15*. Group 4 had only *L25*. Results indicated that *L25* had higher gas production at all measurement times than the other strains (Figure 3). Statistical analysis verified that the yeast strain with the highest gas production ($P < 0.05$) was *L25* at 70.83 ml. In addition, all *Levica* strains produced more gas than *S. cerevisiae* (Table 3).

CONCLUSION

Thirteen new *Levica* yeast strains potentially useful as additives in ruminal fermentation were isolated and identified. Of these, the strain *Levica25* showed the greatest increase in gas production. These results are encouraging and the fact that none of the strains belongs to the genus *Saccharomyces* increases their usefulness in the development of products enhancing ruminal fermentation in bovines on high-fibre diets.

Table 3. Accumulated gas production after 24 h of fermentation

Group	Strain	Gas production accumulated volume (ml)	SE
1	*Levica17* *Levica18* *Levica23* *Levica24* *Levica27* *Levica33* *Levica37*	53.27[d]	0.85
2	*Levica22* *Levica32* *Levica29*	49.07[b]	
3	*Levica13* *Levica15*	61.53[c]	
4	*Levica25*	70.83[e]	
Controls	without yeast *S.cerevisiae*	28.90[a]	

[a–e]different letters denote differences with $P < 0.05$ (Duncan, 1955)

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