Livestock Science 212 (2018) 99–110

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Identification, characterization and selection of autochthonous lactic acid bacteria as probiotic for feedlot cattle

Natalia C. Maldonado[1], Cecilia Aristimuño Ficoseco[1], Flavia I. Mansilla, Constanza Melián, Elvira Maria Hébert, Graciela M. Vignolo, Maria E. Fátima Nader-Macías*

Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán 4000, Argentina

ARTICLE INFO

Keywords:
Beneficial lactic acid bacteria
Feedlot steers
Molecular identification
Surface and functional properties

ABSTRACT

Livestock microbiota is becoming a focus of interest for veterinaries, animal nutritionists and microbiologists in view to select beneficial bacteria with impact in health and animal productivity. As resident adapted microorganisms, lactic acid bacteria (LAB) were isolated, identified and characterized from the homologous host to promote their permanence/efficiency acting as additives in feedlot cattle feeding. Cultivable LAB numbers from cattle feces (CF), pens soil (PS) and feed rations (FR) ranged from 5 to 6 log CFU/g during feedlot permanence. Isolates (500) were identified by $(GTG)_5$-PCR and sequence analysis of 16S rRNA, being represented by *Enterococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Weissella* genera and 20 different species. Genetic mapping showed that predominant LAB species in CF and PS samples were *Lactobacillus (Lb) mucosae* (34%), *Enterococcus (E) hirae* (26%) and *E. faecium-durans* (20%), while in FR *E. faecium-durans* (46%), *Pediococcus (P). pentosaceous, P. acidilactici* (17%) and *Lb. acidophilus* (11%) were mainly isolated. Surface characterization showed most of LAB as high hydrophilic, however several strains from CF and PS revealed strong hydrophobic and auto-aggregative character with a positive correlation between both superficial properties. Adhesion to polystyrene displayed variable biofilm formation patterns for *Enterococcus* and *Lactobacillus* strains depending on the presence of Tween in MRS medium. When antagonistic activity of isolated LAB against bovine relevant pathogens was evaluated, organic acids and hydrogen peroxide production were mostly responsible for inhibition; bacteriocin production was shown only by a *Lb. mucosae* strain. In addition, tolerance to acid and bile salts showed lactobacilli to withstand GIT conditions, while enterococci were more sensitive to low acid environment. On these bases, several *Lactobacillus* strains may be selected to explore their potential use as direct fed bacteria in feedlot cattle.

1. Introduction

Feedlots or landless systems for animal production are used throughout the world under temperate and tropical conditions as well as in developed and developing countries. Intensive production or feedlot systems use a high input system where intensive managements lead to very high growth rates or milk production (Sainz and Lanna, 2009). Livestock has been feed to make them “fatten” for hundreds of years, but the feedlot industry has increased a high development during the last century. Specifically in Argentina, cattle was displaced from traditional production areas in the plain pampa to other regions of the country because of the steady increase in soybean production (Guevara and Grünwaldt, 2012). Although the high costs associated with confinement feeding of cattle, when compared to grazing systems using renewable pasture resources feedlot systems result more sustainable (Galyean, 2010). The main objective for the feedlot cattle industry is to obtain a high meat production per animal, high meat quality and efficient feed conversion.

The main targets for intensive systems involve management of animal in artificial environments, prevention of disease spreading and rapid weight gain in a short time. The widespread use of antimicrobials in food animal production was linked to the development of antibiotics resistance in bacterial populations, which emerged as a global health crisis (WHO, 2012). The role that antibiotics use in livestock feeding plays in altering the prevalence of antibiotic-resistant organisms in humans triggered the ban since 2006 for antibiotics use as animal growth promoters in Europe. All around the world, multiple jurisdictions have responded by restricting antimicrobial use for these purposes, and by requiring a veterinary prescription to use them in food animals (Maron et al., 2013). This situation has prompted an interest in

* Corresponding author.
E-mail address: fnader@cerela.org.ar (M.E.F. Nader-Macías).
[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.livsci.2018.04.003
Received 29 September 2017; Received in revised form 5 April 2018; Accepted 6 April 2018

health and nutritional alternatives to avoid competitiveness losses (Seal et al., 2013). Among them, live direct-fed microbials also referred to as probiotics has gained considerable attention (Gaggía et al., 2010; Chaucheyras-Durand and Durand, 2010; Uyeno et al., 2015). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a physiological health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). The original concept of feeding bacterial probiotics to livestock was supported primarily on the beneficial post-ruminal effects, including improved establishment of beneficial gut microbiota (Fuller, 1989). In ruminants, the most significant effects of probiotics have been reported during specific animal stressful periods for the gut microbiota and the animal: at weaning, during lactation, and after the shift from high forage to high readily fermentable carbohydrates diets (Chaucheyras-Durand and Durand, 2010). In beef cattle, the main objective of probiotics is the promotion of health by avoiding and/or reducing ruminal acidosis, improving weight gain and feeding efficiency as well as reducing the elimination of human pathogens (Brashears et al., 2003; Chaucheyras-Durand and Durand, 2010; Uyeno et al., 2015). In addition, the supplementation of feedlot cattle diets with lactate-producing and/or lactate-utilizing bacteria have resulted in a reduction of acidosis risk and *Escherichia coli* O157:H7 fecal shedding (Galyean et al., 2000; Beauchemin et al., 2003; Callaway et al., 2009; Gressley et al., 2011). Probiotics are proposed to exert different mechanisms, including production of inhibitory compounds such as acids or bacteriocins, competitive exclusion, improvement of rumen fermentation parameters, blocking of quorum sensing, as immunomodulators or by other yet undefined mechanisms (Stanford et al., 2014; Nader-Macias et al., 2008). The most common marketed products for ruminants are live yeast (*Saccharomyces cerevisiae*) preparations and among bacterial probiotics, lactic acid bacteria (LAB), *Bifidobacterium, Propionibacterium* and *Bacillus* have been used in adult ruminants (Chaucheyras-Durand and Durand, 2012, Uyeno et al., 2015). Among LAB, *Lactobacillus (Lb.) acidophilus* (Peterson et al., 2007), *Lb. plantarum* (Qadis et al., 2014), *Lb. casei, Lb. lactis* (Stanford et al., 2014) and *Enterococcus faecium* (Emmanuel et al., 2007) were used as probiotics in feedlot cattle, although in some cases the host origin of these microorganisms was not specified. Probiotics effectiveness was reported to be host and strain dependent. While host specificity was regarded as a desirable property for probiotic bacteria and recommended as selection criteria, species specificity was considered important for temporary colonization needed for beneficial effects, such as immuno-stimulation (Dogi and Perdigón, 2006). Indeed, since inadequate and transient intestinal colonization of human LAB strains fed to cattle were reported (Ewaschuk et al., 2006), those LAB strains intended to be used as probiotics should be isolated from the same source or animal niche/environment where they are thought to exert their benefits. Therefore, the aim of this study was to analyze the predominant autochthonous LAB species present in rectal feces and feedlot environment (pens soil and feed rations), by means of molecular culture-dependent approaches, as a previous step to their characterization and selection to be further applied as probiotics.

2. Materials and methods

2.1. Animals and sample collection

Cattle used in this study were from Bradford and Brangus feedlot industry located in the Northern Province of Santiago del Estero (Argentina). Control of animal's health (vaccination against infectious organisms, respiratory diseases and parasites) was carried out according to the livestock preventive sanitary plan developed by the veterinary staff of the feedlot industry. Prior to sample collection, animals did not receive antibiotics treatment. Upon control, bovine cattle were stratified by weight and successively allocated in different feedlot pens, from an initial average body weight of 160–180 kg (6–7 months old) to approximately 350–360 kg (12–14 months old). During feedlot permanence (one cycle fattening), steers were feed usual diets consisting in three rations with different composition: initial/adaptation (one month), intermediate (2–5 months) and finishing (until slaughtered), composed by sorghum silage (63%, 57% and 17%, respectively), cracked corn grain (16.5%, 27.8% and 77.8%, respectively) and soy expeller (9.5% for adaption and intermediate and 3% for finishing ration). In addition, rations were supplemented with urea (0.5%), minerals/vitamins (1.7–2%) and occasionally wheat bran (8–10%). Three independent sampling (April, June and August 2014) were carried out for feedlot feces and environment analysis. Using a convenience-samplings scheme, rectal fecal samples (42) were collected from healthy animals having 0/6–7, 1/7–8, 2/8–9, 3/9–10, 4/10–11, 6/12 and 8/14 feedlot stay/age months. In addition, samples from pens soil (13) and feed rations (13) were also collected. Sampling was carried out by duplicate in sterile flasks (Deltalab, Spain) individually stored under refrigeration, transported to the laboratory and processed within 3 h of collection. The experiment was exempted from the institutional animal care and use committee because it did not involve direct experimentation on the animals.

2.2. Microbiological analysis and preliminary physiological characterization of isolates

Samples (5 g) were aseptically homogenized in 45 ml of saline-peptone water (8.5 g/l NaCl, 1 g/l bacteriological peptone) in a sterile plastic bag using Stomacher machine (Stomacher Lab-Blender 400, A.J. Seward Lab. London, UK) for 3 min, and decimal dilutions were then prepared in saline (NaCl 0.9 w/v). Microbial suspensions were plated in triplicate and incubated as follows: total bacteria (TB) on Plate Count Agar (PCA, Britania, Argentina) incubated aerobically (48 h at 30 °C and 37 °C); LAB on MRS (Biokar-France) medium (48 h at 30 °C) under restricted oxygen conditions by using Anaerocult® system (Merck, Germany). In addition, total coliforms (TC) were determined on McConkey agar (24 h at 37 °C) and molds and yeasts on H&L agar medium (Britania, Argentine) incubated in aerobiosis (3–5 days at 25 °C). Cycloheximide solution (0.1%) was added to agar media to prevent yeast development. Incubation temperature was 37 °C for feces samples while 30 °C was used for pens soil and food samples. For spore-forming bacteria (SFB) counts, the first dilution of each sample was heated for 15 min at 80 °C, cooled rapidly, spread onto PCA medium and incubated aerobically (24 h at 37 °C). For each sample and after counting, 10 to 15 well-isolated colonies were randomly picked from LAB medium plates and transferred to individual tubes containing 5 ml of the same broth media. The isolated cultures were re-streaked onto MRS agar plates and incubated at 37 °C for 48 h until isolated colonies of one morphology were obtained. Pure colonies were preliminary characterized as Gram positive and catalase negative and considered presumptively as LAB. The isolated cultures were maintained as frozen (−20 °C) stocks in a 10% (w/v) dilution of the corresponding broth medium supplemented with 20% (w/v) sterile glycerol. Isolates were subcultured in MRS broth at 37 °C for 24–48 h before used for further studies.

2.3. DNA extraction and PCR-based LAB identification

Genomic DNA was extracted according to Pospiech and Neumann (1995). Strain differentiation was performed by repetitive sequence-based (rep-PCR) fingerprinting by using $(GTG)_5$ primer (Gevers et al., 2001). The mastermix contained 4 µl of buffer 5X (Inbio Highway, Argentina), 2 µl of dNTPs 5 mM (Promega, Argentina), 2 U of Taq polymerase (Inbio Highway, Argentina), 1 µl of DNA template (50 ng), 2 µl of primer (GTG)5 10 µM (Sigma-Aldrich, Argentina) and 4 µl of $MgCl_2$ (25 mM). PCR reaction consisted of an initial denaturation at 94 °C 5 min; 30-cycle reaction of denaturation at 94 °C for 1 min, 1-min annealing at 40 °C, 8-min extension at 65 °C, and a final extension at 65 °C for 10 min. Amplification reactions have been carried out in a

thermocycler MyCyclerTM (Bio Rad). PCR-products were separated by electrophoresis on a 1.5% agarose gel. Genomic DNA of selected isolates in each cluster was used for amplification of the almost full-length 16S rRNA gene fragment using the primers MLB and PLB (Kullen et al., 2000) and sequenced at CERELA-CONICET through an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA). rRNA gene sequence alignments were performed using the multiple sequence alignment method and identification queries were fulfilled by a BLAST search (Altschul et al., 1990) in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). The identified strains were deposited at CERELA Culture Collection and a CRL number was assigned.

2.4. Lactic acid bacteria cell surface characterization

2.4.1. Hydrophobicity index

The hydrophobicity of the bacterial cell surface was evaluated by Microbial Adhesion To Hydrocarbons (MATH) according to Maldonado et al. (2012). Two different solvents were used in this study, xylene (nonpolar solvent) and toluene (acidic solvent). Briefly, LAB were grown (MRS) overnight, centrifuged (7000*g* for 10 min), washed (0.85% NaCl) and resuspended in the same solution (OD600:0.3–0.7; A0), 3 ml-suspension were mixed (60s) with 0.5 ml of each solvent, separately. After the two phase's separation, OD600 (A1) was determined again. The percentage of bacterial adhesion to solvents was calculated as (A0-A1/A0) × 100. Each measurement was performed in duplicate and experiments repeated twice with independent bacterial culture. The score of hydrophobicity applied was high (61–80%), medium (31–60%) and low (0–30%).

2.4.2. Auto-aggregation assay

Each LAB strain was grown for 16 h at 30 °C in 3 ml MRS and allowed to settle at room temperature for 2 h (Maldonado et al., 2012). The OD600 was determined at the initial time (ODinitial), and every hour up to 4 h. The data were obtained after 2 h sedimentation (OD2h). Autoaggregation percentage was calculated as [OD initial - OD2h/ODinitial)] × 100. The scores used were the same than for hydrophobicity.

2.4.3. Biofilm formation

Biofilm formation of isolated bacteria, previously selected by their surface properties, was evaluated as described by **Leccese Terraf et al. (2014)** in two different culture media: MRS and MRS-T (MRS without tween). Bacteria were subcultivated three times in both broth, and pellets were washed once with saline solution and then, suspensions of 1.5 DO560nm (2×10^8 CFU/ml) were prepared. Suspensions (200 µl) were inoculated into 5 ml of each broth media, and aliquots of 200 µl were placed in 96-well polystyrene microplates (ExtraGene, Taiwan). The microplates were then incubated for 72 h at 37 °C. To quantify biofilm formation, wells were washed with phosphate-buffered saline (PBS) and the remaining attached bacteria were stained for 30 min with 200 µl 0.1% (w/v) crystal violet in an isopropanol-methanol-PBS solution (1:1:18, v/v/v). Excess stain was rinsed twice with 200 µl distilled water per well. After the wells were air dried, the dye bound to the adherent cells was extracted with 200 µl 30% (v/v) glacial acetic acid and then OD570nm of each well was measured by using a microplate reader (VersaMax Molecular Devices, USA). Sterile medium was included as negative control and the biofilm forming strain *Lactobacillus reuteri* CRL1324 was used as positive control. All the experiments were performed by triplicate.

2.5. Inhibitory activity

The well diffusion assay was applied to evaluate the production of inhibitory substances in the supernatant fluid of LAB isolates. *Listeria monocytogenes* FBUNT and *Staphylococcus aureus* (clinical isolates from Facultad de Bioquímica Química y Farmacia, UNT, Argentina), *Escherichia coli* ATCC12900 and *Enterococcus faecalis* ATCC29212 were used as indicators strains. *E. faecalis* and *S. aureus* were grown in Brain Heart Infusion (BHI) (Britania, Argentina) for 24 h at 37 °C while *E. coli* and *L. monocytogenes* were cultured in Triptic Soy Broth (TSB) added with yeast extract (0.5%). Selected LAB were grown in MRS broth at 37 °C for 24 h and cell-free supernatants (CFS) were obtained by centrifugation (15.000*g*, 10 min); the CFS fluid was then adjusted to pH 7.0 with 1 N NaOH (Cicarrelli, Argentina). Neutralized CFS (5 ml) was spotted in plates containing 10 ml of BHI and TSB (1.5% agar) plus 10 ml of BHI soft agar (0.7%) inoculated with 107 CFU/ml of overnight culture of indicator strains. After 3 h at room temperature, the plates were incubated at 37 °C (30 °C for L. *monocytogenes*) for 24 h. Positive antimicrobial activity LAB supernatants were neutralized (NaOH 2 M) and later treated with catalase (1000 U/ml) (Sigma-Aldrich, St Louis, USA) to determine the chemical nature of the inhibitory substances (organic acids or hydrogen peroxide). Proteinase K (Sigma Chemical) was added to confirm bacteriocin production. Positive antagonistic activity was evidenced as an inhibition zone on the indicator organism lawn.

2.6. Tolerance to gastrointestinal conditions (pH and bile salts)

LAB tolerance to different pH was determined by inoculation in MRS broth previously adjusted to 3.0, 4.0 and 5.0 pH values with 0.1 N HCl (Cicarrelli, Argentina). For bile salts resistance, LAB strains were inoculated in MRS broth containing different bile salts (Oxgall, Fluka, Sigma-Aldrich, India) concentrations (0.1%, 0.25% and 0.5%). Bacteria were subcultivated three times, centrifuged and pelleted by centrifugation (5000 *g*, 10 min), washed three times with saline solution and then, suspensions of 0.9–1.0 DO560nm (2×10^8 CFU/ml) were prepared. Aliquots of 200 µl of MRS with different pH and bile concentrations were added to 96-well polystyrene microplates (ExtraGene, Taiwan) and 5 µl of each bacterial suspensions were inoculated. Growth was assessed by modifications in the DO_{560nm} at different time intervals (3, 6, 9, 24 and 48 h).

2.7. Statistical analyses

The results are expressed as the mean value (or log values) ± standard deviation of the data. The t-student test was applied to determine the differences ($P < 0.05$) of cultivable bacterial numbers during one fattening steers cycle. All *in vitro* assays were performed in duplicate or triplicate. Significant differences between means were determined by Tukey's test after analysis of variance (ANOVA) with Minitab Statistic Program, release 16.1.0 for Windows. *P* value of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Microbiological analyses

In view to select beneficial LAB to be used as probiotic, samples including rectal cattle feces (42), pens soil from different animal groups (11) and feed rations (9) along one cycle of feedlot steers fattening were analyzed (Table 1). Samples from cattle feces (CF) showed total bacteria ranging from 8.42 ± .0.72 upon arrival at the feedlot (0 month) to 7.13 ± 0.14 log CFU/g at 8 months. These values decreased during feedlot stage, showing differences ($p < 0.05$) from 4 months up to the end of the feedlot stage. LAB displayed values between 5.53 ± 0.88 at 0-months and 6.65 ± 0.29 log CFU/g in at 8-months feedlot stay respectively, with $p < 0.05$ at the end of the stage. In addition, total coliforms and spore-former bacteria numbers in fecal samples were counted at levels between 6.25 ± 0.24 to 6.97 ± 0.80 and 4.90 ± 0.31 to 5.32 ± 0.58 log CFU/g respectively, the higher numbers were detected initially and at 6-months feedlot permanence, respectively. TC population in feedlot CF showed higher numbers than

Table 1
Microbiological analyses (log CFU/g) from cattle feces (CF), pens soil (PS) and feed rations (FR) samples (S) during cattle permanence in the feedlot system.

Feedlot stay/animal age (months)	S	Total bacteria (TB)	*p*-value	Lactic acid bacteria (LAB)	*p*-value	Yeast/molds (Y/M)	*p*-value	Total coliforms (TC)	*p*-value	Spore-forming bacteria (SFB)	*p*-value
0/6-7	CF	8.42 ± 0.72	–	5.53 ± 0.88	–	5.35 ± 0.51	–	6.97 ± 1.08	–	4.90 ± 0.31	–
	PS	8.06 ± 0.58	–	4.58 ± 0.41	–	5.83 ± 0.16	–	4.86 ± 0.85	–	5.82 ± 0.61	–
	FR	9.29 ± 0.15	–	7.38 ± 0.46	–	6.00 ± 0.68	–	4.03 ± 0.10	–	5.68 ± 0.70	–
1/7-8	CF	8.20 ± 0.89	0,55	5.62 ± 0.74	0,468	5.29 ± 0.22	0,800	6.86 ± 0.10	0,756	4.95 ± 0.40	0,799
	PS	7.95 ± 0.72	0,850	6.00 ± 0.22	0,013	5.94 ± 0.01	0,342	5.04 ± 0.64	0,851	5.12 ± 0.04	0,352
	FR	7.08 ± 1.05	0,207	5.25 ± 0.58	0,138	6.20 ± 0.18	0,676	5.30 ± 0.40	0,142	5.49 ± 0.30	0,718
2/8-9	CF	8.05 ± 0.60	0,295	5.75 ± 0.65	0,527	5.25 ± 0.50	0,639	6.68 ± 0.93	0,420	5.99 ± 0.52	0,0
	PS	7.95 ± 0.48	0,807	6.16 ± 0.28	0,010	5.11 ± 0.27	0,026	5.00 ± 0.80	0,893	5.15 ± 0.65	0,481
	FR	7.08 ± 1.05	0,207	5.25 ± 0.58	0,138	6.20 ± 0.18	0,676	5.30 ± 0.40	0,142	5.49 ± 0.30	0,718
3/9-10	CF	7.87 ± 0.65	0,084	5.95 ± 0.71	0,124	5.17 ± 0.53	0,502	6.65 ± 0.51	0,451	5.00 ± 0.61	0,750
	PS	6.78 ± 0.66	0,086	6.13 ± 0.43	0,020	4.80 ± 0.68	0,285	4.54 ± 0.28	0,702	5.47 ± 0.30	0,589
	FR	7.08 ± 1.05	0,207	5.25 ± 0.58	0,138	6.20 ± 0.18	0,676	5.30 ± 0.40	0,142	5.49 ± 0.30	0,718
4/10-11	CF	7.75 ± 0.62	0,040	6.00 ± 0.91	0,176	5.12 ± 0.47	0,438	6.50 ± 1.30	0,512	5.10 ± 0.59	0,379
	PS	6.78 ± 0.57	0,072	6.04 ± 0.67	0,049	4.95 ± 0.23	0,141	4.92 ± 0.32	0,941	5.52 ± 1.00	0,718
	FR	7.08 ± 1.05	0,207	5.25 ± 0.58	0,138	6.20 ± 0.23	0,676	5.30 ± 0.40	0,142	5.49 ± 0.30	0,718
5/11-12	CF	7.70 ± 0.45	0,029	6.16 ± 0.48	0,115	5.14 ± 0.13	0,373	6.55 ± 0.95	0,676	5.15 ± 0.44	0,466
	PS	7.19 ± 0.15	0,132	5.94 ± 0.20	0,039	5.09 ± 0.20	0,153	4.53 ± 0.62	0,734	5.67 ± 0.70	0,823
	FR	7.38 ± 0.25	0,064	5.17 ± 0.80	0,172	5.30 ± 0.60	0,350	5.45 ± 0.15	0,054	5.17 ± 0.42	0,416
6/12	CF	7.50 ± 0.79	0,045	6.25 ± 0.82	0,143	5.08 ± 0.12	0,265	6.40 ± 0.60	0,198	5.18 ± 0.56	0,501
	PS	7.16 ± 0.30	0,151	6.14 ± 0.70	0,215	4.84 ± 0.65	0,284	4.04 ± 0.80	0,502	5.90 ± 0.32	0,892
	FR	7.38 ± 0.25	0,064	5.17 ± 0.80	0,172	5.30 ± 0.60	0,350	5.45 ± 0.15	0,054	5.17 ± 0.42	0,416
8/14	CF	7.13 ± 0.14	0	6.65 ± 0.29	0,009	4.90 ± 0.56	0,180	6.25 ± 0.24	0,079	5.32 ± 0.58	0,156
	PS	6.90 ± 0.68	0,298	6.30 ± 0.37	0,040	4.08 ± 0.34	0,096	4.28 ± 0.20	0,535	5.27 ± 0.97	0,518
	FR	7.38 ± 0.25	0,064	5.17 ± 0.80	0,172	5.30 ± 0.60	0,350	5.45 ± 0.15	0,054	5.17 ± 0.42	0,416

p-values were calculated by the t-Student test comparing different population in the samples from faeces, pens soil or feed rations during one steers- fattening cycle in the feedlot, referred to the viable bacterial numbers at the beginning of the study. In grey are indicated the results showing $p < 0.05$.

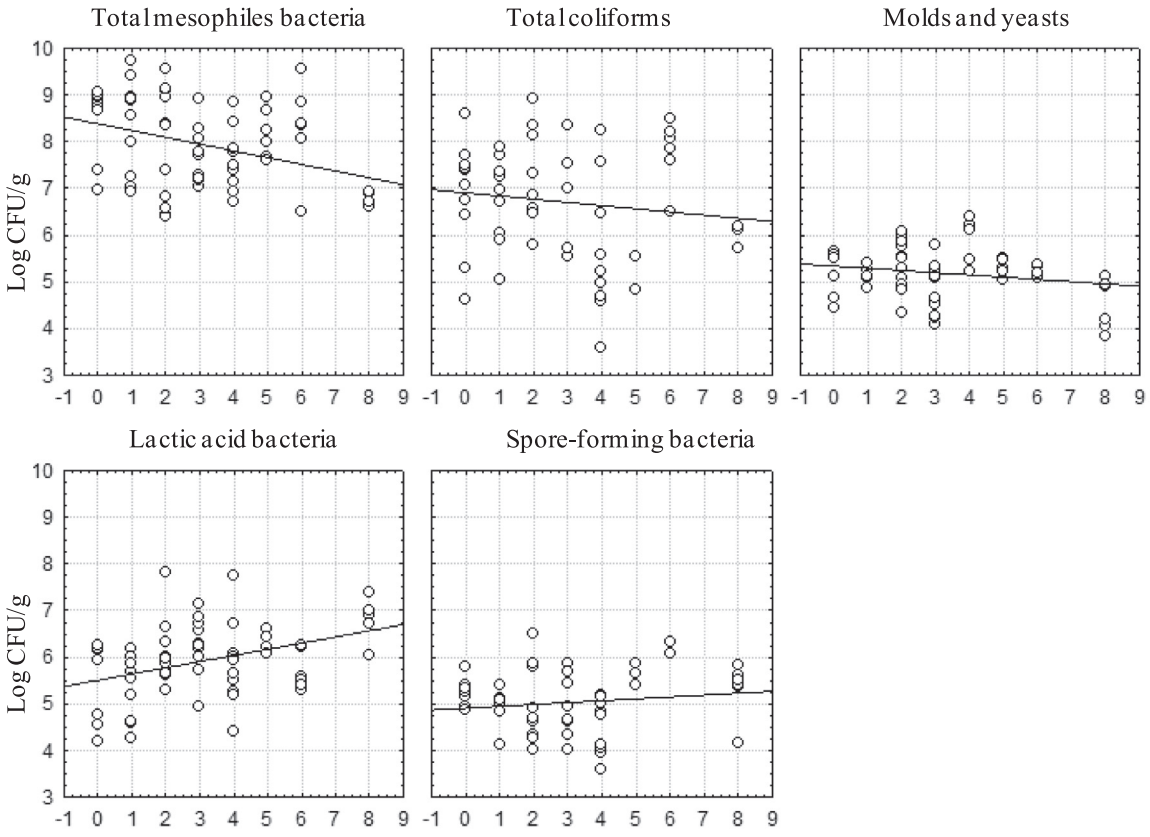

Fig. 1. Total mesophillic bacteria, total coliforms, molds and yeasts, lactic acid and spore-forming bacteria from cattle feces. Samples were collected from steers with different feedlot stay and age, along one fattening cycle.

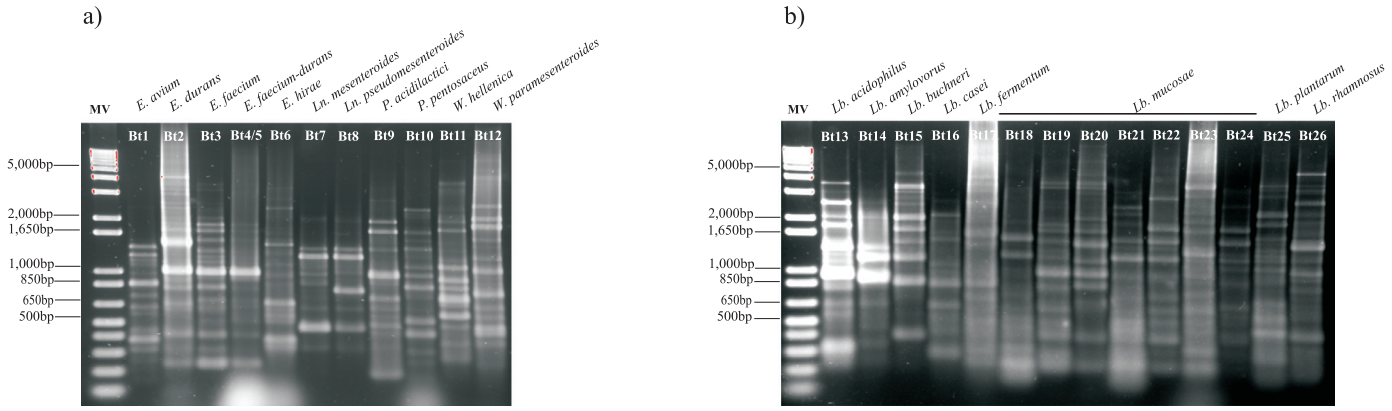

Fig. 2. PCR amplification of repetitive bacterial DNA elements fingerprinting using the (GTG)5 primer of lactic acid bacteria from steer's feedlot environment. M: Molecular weight marker (1 kb DNA ladder, Invitrogen). (a) $(GTG)_5$-RAPD profiles including the following biotypes: Bt1 (*E. avium*); Bt2 (*E. durans*); Bt3 (*E. faecium*); Bt4/5 (*E. faecium-durans*); Bt6 (*E. hirae*); Bt7 (*Ln. mesenteroides*); Bt8 (*Ln. pseudomesenteroides*); Bt9 (*P. acidilactici*); Bt10 (*P. pentosaceus*); Bt11 (*W. hellenica*) and Bt12 (*W. paramesenteroides*). (b) (GTG)5-RAPD profiles including Bt13 (*Lb. acidophilus*); Bt14 (*Lb. amylovorus*); Bt15 (*Lb. buchneri*); Bt16 (*Lb. casei*); Bt17 (*Lb. fermentum*); Bt18-Bt24 (*Lb. mucosae*); Bt25 (*Lb. plantarum*) and Bt26 (*Lb. rhamnosus*).

those of LAB, reaching similar values at the end of feedlot period (Table 1). Cultivable TB and LAB populations in feedlot steer's rectal feces were similar to that reported from 4-months old calves, cows and Native x Brahman crossbreeding (Maldonado et al., 2012; Adeniyi et al., 2015; Puphan et al., 2015). Nonetheless, Brashears et al. (2003) previously described for cattle manure 10^8–10^9 lactobacilli/g. In this study, a general tendency of microbial groups in CF samples during their permanence in feedlot is shown in Fig. 1. LAB and SFB populations exhibited an increasing mean trend, which was higher for LAB (0.156 *versus* 0.125 log CFU/g/month). With the exception of these two bacterial groups, a weak decrease in steer's feces bacterial numbers for TB, TC and molds and yeasts were evidenced throughout the feedlot process. On the contrary, a decrease in LAB, *Bifidobacterium* and *Enterobacteriaceae* populations was reported in Holstein calves as they aged (Uyeno et al., 2010; Maldonado et al., 2012). In addition, the somewhat high SFB counts found during the last feedlot stages is in agreement with that reported for dairy cows, 12-weeks Holstein calves and cattle at slaughterhouse (Bagge et al., 2010; Uyeno et al., 2010). Nevertheless, sequences related to the phyla *Firmicutes* were reported to predominate in Brazilian Nelore steer and dairy cow's feces as determined by metagenomic analysis (de Oliveira et al., 2013; Dill-McFarland et al., 2017). Changes in the intestinal bacterial communities of ruminants have been related to age, digestive tracts development and cattle management practices, diets transition from forage- to grain-based rations being the most important determinant of feedlot cattle microbiome (Uyeno et al., 2010).

Regarding feedlot pens soil (PS), a similar pattern than those from CF was found for TB and LAB populations, with maximal numbers of 8.06 ± 0.58 and 6.30 ± 0.37 log CFU/g (8 months), respectively (Table 1). In addition, TC and SFB were in the range of 4.04 ± 0.20 to 5.04 ± 0.64 log CFU/g and 4.72 ± 0.97 to 5.95 ± 0.70 log CFU/g, with maximal counts initially and during the last feedlot stages, respectively. LAB displayed a similar profile than that for CF, increasing its population whereas TC decreased during cattle permanence in feedlot. The LAB counts in pens soil were significant statistically different ($p < 0.05$) along the fattening cycle. In coincidence, higher numbers and diversity of LAB were also reported from henhouse and farms soil (Chen et al., 2005; Micallef et al., 2013). The similar distribution of the examined bacterial populations for PS and CF here described would suggest cattle manure as the main component. In addition, feed rations (FR) showed higher TB, LAB and SFB counts in the initial feedlot stage (9.29 ± 0.15 and 7.38 ± 0.46 log CFU/g, respectively) when adaptation/initial ration was delivered to cattle in coincidence with high silage percentage in initial and intermediate rations. Analysis of SFB population in fed diets showed values ranging between 5.17 ± 0.42 and 5.68 ± 0.70 log CFU/g, while TC exhibited an increase from 4.03 ± 0.10 to 5.45 ± 0.15 log CFU/g from adaptation to finishing rations. The rise of cracked corn grain and reduction in sorghum silage proportions in FR may account for the LAB numbers reduction in intermediate and finishing rations. The presence of LAB in vegetable matrices has been widely documented; silages were reported to contain LAB levels in the range of 10^8–10^9 CFU/g (Pang et al., 2011), while a wide LAB levels were reported among the epiphytic grains microbiota (De Vuyst and Neysens, 2005). In addition, a general decrease of molds and yeasts population was found in all three samples analyzed (Table 1). Higher levels of these populations were detected for FR when compared to CF and PS samples. In agreement, a significant mold diversity was identified in dairy cows feces (Dill-McFarland et al., 2017).

3.2. LAB identification and distribution among different samples from feedlot environment

Five hundred colonies from cattle feces, pens soil and feed rations recovered from MRS plates were considered as presumptive LAB, based on Gram staining and catalase test results. Analysis of LAB isolates were approached by repetitive sequence-based PCR (rep-PCR) fingerprinting analysis using $(GTG)_5$ primer coupled with partial 16S rRNA gene sequencing. First, rep-PCR analysis yielded 15 to 20 bands of molecular size ranging from 300 to 4000 bp corresponding to the genera *Enterococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Weissella*. Ascription of food isolates into species was based on the clusters derived from $(GTG)_5$-PCR analysis; strains showing identical rep-PCR band patterns were considered as one rep-PCR biotype. Isolates were grouped as belonging to 25 different $(GTG)_5$ biotypes (Fig. 2a, b). At least one representative from each biotype was identified by partial 16S rRNA gene sequencing. Biotype information for rep-PCR obtained with $(GTG)_5$ primer for LAB isolates is reported (Fig. 2a, b; Table 2). $(GTG)_5$ biotypes (Bt) were associated with *Enterococcus avium* (Bt1), *Enterococcus durans* (Bt2), *Enterococcus faecium* (Bt3), *Enterococcus faecium-durans* (Bt4/Bt5), *Enterococcus hirae* (Bt6), *Leuconostoc mesenteroides* (Bt7), *Leuconostoc pseudomesenteroides* (Bt8), *Pediococcus acidilactici* (Bt9), *Pediococcus pentosaceus* (Bt10), *Weissella hellenica* (Bt11) and *Weissella paramesenteroides* (Bt12), *Lactobacillus acidophilus* (Bt13), *Lactobacillus amylovorus* (Bt14), *Lactobacillus buchneri* (Bt15), *Lactobacillus casei* (Bt16), *Lactobacillus fermentum* (Bt17), *Lactobacillus mucosae* (Bt18 to Bt24), *Lactobacillus plantarum* (Bt25) and *Lactobacillus rhamnosus* (Bt26).

LAB species composition and their occurrence in CF (different animal ages/stay in the feedlot system), PS and FR, as determined by culture-dependent approaches, are summarized in Table 3. Results

Table 2
Feedlot LAB isolates biotypes and sequence information for rep-PCR obtained with $(GTG)_5$ primer.

CRL No.	Origin	Closest relative	Rep-PCR biotypes	Identity %	Accession No.
CRL2087	PS	*E. avium*	1	99	KX673997.1
CRL2047	CF/PS/FR	*E. durans*	2	98	KT205791.1
CRL2141	CF/PS/FR	*E. faecium*	3	99	KU9952991
CRL2153	CF/PS/FR	*E. faecium-durans*	4/5	99/99	KX609796.1/KU513402.1
CRL2068	CF/PS/FR	*E. hirae*	6	99	KU302755.1
StrainA5a/1	FR	*Ln. mesenteroides*	7	90	KT924430.1
Strain10/17	FR	*Ln. pseudomesenteroides*	8	98	LC119133.1
CRL2043	CF/PS/FR	*P. acidilactici*	9	97	KY883565.1
CRL2109	CF/PS/FR	*P. pentosus*	10	100	KR055464.1
ALIM1/2	FR	*W. hellenica*	11	94	KY883556.1
Strain6S4/2	CF/PS/FR	*W. paramesenteroides*	12	92	KX078328.1
CRL2074	CF/PS/FR	*Lb. acidophilus*	13	97	KX851523.1
CRL2044	CF/PS	*Lb. amylovorus*	14	96	KY810608.1
CRL2060	CF	*Lb. buchneri*	15	98	KR055508.1
CRL2088	PS	*Lb. casei*	16	96	KY786122.1
CRL2085	FR	*Lb. fermentum*	17	99	KY574532.1
CRL2069	CF/PS/FR	*Lb. mucosae*	18/24	99	MF425117.1
CRL2126	CF/FR	*Lb. plantarum*	25	95	CP020816.1
CRL2084	FR	*Lb. rhamnosus*	26	99	KY054577.1

Enterococcus (E); Leuconostoc (Ln); Weissella (W); Pediococcus (P); Lactobacillus (Lb); CF: cattle feces; PS: pens soil; FR: feed rations.

showed that LAB isolates were mostly recovered from fecal samples (256), the remaining being from feed rations (129) and pens soil (115) samples. When LAB composition was analyzed, enterococci (48.2%) and lactobacilli (38%) constituted the most representative genera, while a minor proportion of species belonged to *Pediococcus* (10.2%), *Weissella* (3%) and *Leuconostoc* (0.6%). *Lactobacillus* genus exhibited the highest diversity with eight species, followed by *Enterococcus* represented by four species and *Pediococcus, Weissella* and *Leuconostoc* with two different species each. LAB from CF mostly belonged to enterococci (*E. faeciun, E. durans, E. faecium-durans* and *E. hirae*) and lactobacilli were represented by *Lb. acidophilus, Lb. amylovorus, Lb. buchneri, Lb. mucosae, Lb. plantarum* and *Lb. rhamnosus* (Tables 2 and 3). The largest enterococci numbers were identified from 3-months samples, whereas lactobacilli population was maximal in feces from cattle between 2 and 4 months, with *Lb. mucosae* as the predominant species. LAB from CF samples varied depending on cattle permanence/age in the feedlot and the fed rations composition; *Lb. mucosae, E. hirae* and *E. faecium-durans* (105, 86 and 52 isolates, respectively) accounted for the main LAB populations. In CF samples, as the permanence in the feedlot progressed, some species disappeared while others were recovered, resulting in 11 LAB species identified throughout the 8-months feedlot period. An average of five LAB species were recovered from each residence time, this being maximal for 2-months CF samples (Table 3). The main presence of the phylum *Firmicutes* including *Enterococcaceae* and *Lactobacillaceae* families in beef and dairy feces was widely reported (de Oliveira et al., 2013; Klein-Jöbstl et al., 2014; Dill-McFarland et al., 2017).

On the other hand, LAB isolates from PS samples also exhibited higher enterococci population (60 isolates) than lactobacilli (38 isolates). Besides the minor pediococci and weissella numbers, the prevalent species were the same as those found from CF, as expected (Tables 2 and 3). The presence of *Lactobacillus, Enterococcus, Weissella* and *Pediococcus* is in agreement with that reported from farms floors, agricultural soils and plant rhizospheres (Chen et al., 2005). LAB

Table 3
Distribution of the different LAB species of among samples.

	Genera/species	Samples									Total isolates
		CF (feedlot permanence/animal age in months)							PS	FR	
		0/6–7	1/7–8	2/8–9	3/9–10	4/10–11	6/12	8/14			
Enterococcus	*avium*								1		1(1)
	faecium	1						3	4	4	12(3)
	durans	2			3		1			1	7(7)
	faecium-durans	7		5	11	1	16	6	25	43	114(0)
	hirae	2	11	15	14	11	1	22	30	1	107(10)
Lactobacillus	*acidipiscis*								2		2(2)
	acidophilus			1	1	1		3	1	11	18(3)
	amylovorus	1			2		2	1	2		8(7)
	buchneri					1					1(1)
	casei								1		1(1)
	fermentum									5	5(1)
	mucosae	2	5	43	16	28	10	1	31	5	141(25)
	plantarum								1	9	10(4)
	rhamnosus						1			3	4(2)
Leuconostoc	*mesenteroides*									2	2(0)
	pseudomesenteroides									1	1(0)
Pediococcus	*acidilactici*			1						18	19(4)
	pentosaceus		1	1					8	22	32(3)
Weisella	*hellenica*									1	1(0)
	paramesenteroides			1			1		9	3	14(1)
Total		15	17	67	47	42	32	36	115	129	500(75)

In brackets, the number of evaluated strains from each identified LAB species are indicated.

composition in FR showed a wide species diversity mainly coming from silage, maize grains and soy expeller. In addition to enterococci (49 isolates) and lactobacilli (33 isolates), *P. acidilactici* (18), *P. pentosaceus* (22), *W. paramesenteroides* (3), *W. hellenica* (1), *Ln. mesenteroides* (2) and *Ln. paramesenteroides* (1) were identified. *Lb. fermentum* and *W. hellenica* were only recovered from FR, while *E. avium* was only present in PS samples.

The dominance of species from *Enterococcus* genus in feedlot environment samples is closely related to their role as commensal inhabitants of the gastrointestinal tract (GIT) of warm-blooded animals, although the persistence of some species and strains in extra enteric habitats is expected (Byappanahalli et al., 2012). Of the enterococci recovered from CF samples (Tables 2 and 3), *E. hirae* predominated, its presence being consistent with previous reports from natural grazing animals, dairy/beef cattle, young calves and feedlot steers (Anderson et al., 2008; Soto et al., 2010; Jackson et al., 2011; Thamacharoensuk et al., 2013; Adeniyi et al., 2015). Similar to this study, *E. hirae*, was associated with different soils types (Chen et al., 2005; Abriouel et al., 2008; Micallef et al., 2013). The identification of *E. durans, E. faecium* and *E. faecium-durans* from CF as the second dominant population was in agreement to their wide presence in dairy/beef cattle and other warm-blooded animals fecal/manure samples (Anderson et al., 2008; Jackson et al., 2011; Byappanahalli et al., 2012; Adeniyi et al., 2015; Iseppi et al., 2015). However, as suggested by Beukers et al. (2015), they do not predominate since their prevalence declines after cattle enters the feedlot, diets and animal age may be contributing factors. Moreover, although in different proportions, the same *Enterococcus* species were present in PS and FR in coincidence with that previously reported (Chen et al., 2005; De Vuyst et al., 2014). Apart from those identified from CF, a strain of *E. avium* was retrieved from PS, being in accordance with its presence in animal feces, rhizosphere of fruit trees and irrigation ditch soils (Chen et al., 2005; Micallef et al., 2013; Thamacharoensuk et al., 2013). Enterococci from FR exhibited *E. faecium-durans* as the major population, in coincidence with that reported for raw and processed vegetable materials (Abriouel et al., 2008; Byappanahalli et al., 2012).

On the other hand, *Lactobacillus* that represented the second major population in feedlot environment, exhibited the widest diversity (Table 2). Of them, *Lb. mucosae* and *Lb. acidophilus* were recovered from the three evaluated feedlot samples. *Lb. mucosae* was by far the most frequently isolated specie and as described by Hammes and Hertel (2006) this obligate heterofermentative is an inhabitant of humans and animals intestines. Accordingly, it was reported from the gut of calves and swine/canine feces (De Angelis et al., 2006; Beasley et al., 2006; Soto et al., 2010; Maldonado et al., 2012; Mann et al., 2014). In addition, *Lb. acidophilus* mostly recovered from CF and FR has been previously reported for warm-blooded animals intestines and feces (Brashears et al., 2003; Silva et al., 2013; Mann et al., 2014), as well as during plant fermentation (Chang et al., 2010). The well-known acid tolerance, antimicrobial activity and host's immunoprotective role of *Lb. acidophilus*, may explain its probiotic use as direct-fed microbe (Hwang et al., 2015). In a lower proportion, *Lb. amylovorus* was identified from CF and FR samples; this specie was described as a major LAB in the GIT and feces of weaning pig (De Angelis et al., 2006; Mann et al., 2014). The presence of corn in feedlot diets likely influence the presence of *Lb. amylovorus* in CF and FR, which is involved in dietary starch degradation (Mann et al., 2014). In addition, the facultative heterofermenters *Lb. acidipiscis, Lb. casei* and *Lb. plantarum* were recovered from PS suggesting a cross-contamination, since these species were reported from silage, fermented soybean/wheat grains, and tropical grasses (Pang et al., 2011; De Vuyst et al., 2014; Khota et al., 2016). *Lb. rhamnosus* described as inhabitant of worm-blooded animals GIT (Brashears et al., 2003; Beasley et al., 2006) together with *Lb. fermentum* were isolated in FR samples, in correlation with their reported presence in cereal flours, fermented products and silage (Yousif et al., 2010; De Vuyst et al., 2014). The obligate heterofermenter *Lb. buchneri* (one strain), besides being spread in many different environments, was also reported from warm-blooded animals feces (Du Toit et al., 2003). Moreover, the presence of *P. acidilactici* and *P. pentosaceus* mostly recovered from FR samples agrees to that reported from cereals, fermented plant products and silages (Yousif et al., 2010; De Vuyst et al., 2014; Li et al., 2015). However, these LAB species were also recovered from calves gut and buffalo feces (Soto et al., 2010; Thamacharoensuk et al., 2013) and soil (Kaur and Tiwari, 2016), respectively. *Leuconostoc* and *Weissella* species that were also isolated from FR samples are in agreement to that reported in cereal grains/flours and silages (De Vuyst et al., 2014; Li et al., 2015). In particular, *W. paramesenteroides* recovered from CF and PS, agrees with that described for calves gut and buffalo feces (Soto et al., 2010; Thamacharoensuk et al., 2013) and soil samples (Chen et al., 2005).

3.3. Characterization and selection of LAB

Seventy five LAB strains from different feedlot environment sources representing 5 genera and 19 species (Table 2) were used for their surface and inhibitory characterization.

3.3.1. Surface characterization

MATHS partitioning method was applied for the evaluation of LAB cell surface properties by their affinity to toluene (apolar solvent) and xylene (polar solvent) with a polarity index of 2.3 and 2.4, respectively. Hydrophobicity and auto-aggregation, as surface properties, were assessed based on the principle that adhesion to the epithelial surface is the first step required for colonization of probiotic microorganisms (Ocaña and Nader-Macías, 2002). Affinity of isolated LAB for the two solvents is shown in Fig. 3. The high variation in the percentage of adhesion to toluene among strains reveals a great diversity in their hydrophobic character. However, most of LAB strains regardless their origin, presented surfaces with a clear hydrophilic character with affinity to apolar toluene below 40% (Fig. 3). However, *Lb. amylovorus* CRL2115, *E. hirae* CRL2089, *E. faecium* CRL2141, *Lb. mucosae* CRL2155 and *Lb. acidophilus* CRL2074 from CF and PS exhibited a toluene affinity >60% revealing higher hydrophobic character. In addition, *Lb. acidophilus* CRL2152, *Lb. amylovorus* CRL2116, *Lb. mucosae* CRL2070/2111 showed hydrophobicity level in the range of 50–60%. Based on their sedimentation characteristics, auto-aggregation at 2 h showed *Lb. amylovorus* CRL2116/2115 and *Lb. mucosae* CRL2069 with values >70% whereas percentages between 40% and 60% were found for *E. hirae* CRL2089/2071/2068, *Lb. amylovorus* CRL2065 and *Lb. mucosae* CRL2063/2070/2083/2111 (Fig. 3). In addition, positive Pearson correlations between hydrophobicity and auto-aggregation were higher (r: 0.96) for toluene than xylene (r: 0.74), *Lb. mucosae* CRL2069, *Lb. amylovorus* CRL2115/2116 and *E. hirae* CRL2089 exhibiting highest values. Results for surface characterization are in coincidence with that previously reported for lactobacilli from piglets and young calves feces (Iñiguez-Palomares et al., 2007; Maldonado et al., 2012). The low hydrophobic character found for LAB from feedlot environment agrees with that reported from fecal strains isolated from healthy dogs (Silva et al., 2013).

3.3.2. Production of antagonistic compounds

Several metabolic compounds produced by LAB, including organic acids, hydrogen peroxide and bacteriocins are able to exert antimicrobial effects against a range of pathogens. Inhibitory ability of LAB was evaluated using various Gram-positive (*L. monocytogenes, S. aureus* and *E. faecium*) and Gram-negative (*E. coli*) target bacteria. As shown in Fig. 4a, *E. hirae, Lb. acidophilus, Lb. amylovorus, Lb. mucosae,* and *Lb. plantarum* were among the most antagonistic against indicators used. Organic acid and H_2O_2 production was mostly responsible for inhibition, whereas bacteriocin/s were observed to be produced by *E. hirae* CRL2062/CRL2067/CRL2072/CRL2089, *E. durans* CRL2047 and *Lb. mucosae* CRL2112, these strains being inhibitory against *E. faecalis* (data

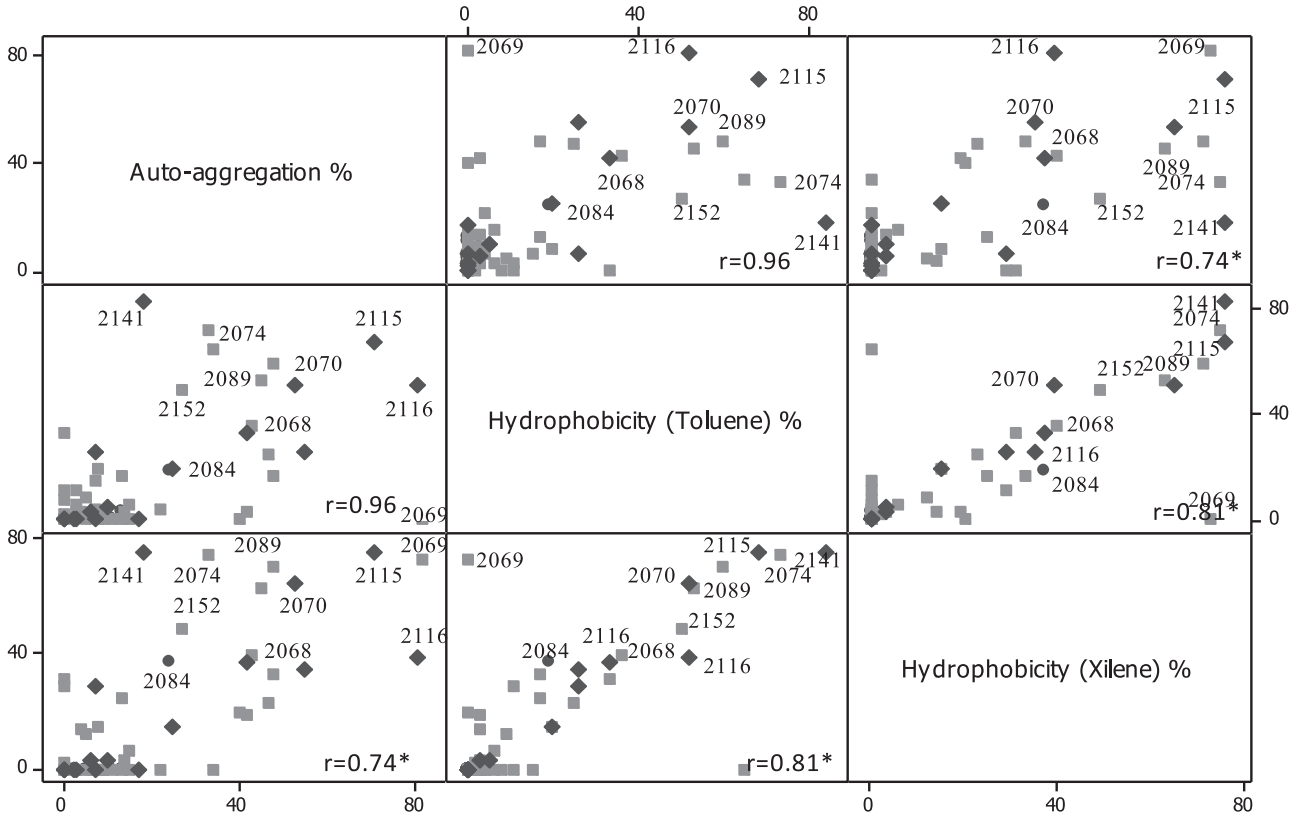

Fig. 3. Surface characterization of lactic acid bacteria isolated from steer's feedlot environment. Autoaggregation and hydrophobicity (toluene and xylene) indexes for cattle feces (■), pens soil (♦) and feed rations (●).

not shown). It is well known the ability of LAB to produce antimicrobial peptides (Nes et al., 2015). To our knowledge, this is the first report of bacteriocin production by a *Lb. mucosae* strain. Particularly, inhibition of *E. coli* was found when non-neutralized supernatants from *Lb. acidophilus* CRL2061/CRL2074/CRL2152, *Lb. amylovorus* CRL2044, *Lb. mucosae* CRL2155, *Lb. plantarum* CRL2103/2126/2142 and *P. acidilactici* CRL2046 were used (data not shown), while neutralized supernatants did not; this result is in correlation with the acidogenic capacity of these LAB. It is well known the inability of LAB bacteriocins to inhibit Gram negatives. On the other hand, hydrogen peroxide was produced by enterococci (*E. durans* CRL2047/2048; *E. faecium* CRL2102) and lactobacilli (*Lb. acidophilus* CRL2074/2152; *Lb. fermentum* CRL2085; *Lb. mucosae* CRL2112/2113/2114/2154/2070/2100/2101; *Lb. plantarum* CRL2126); H_2O_2 accumulated by LAB in cell suspensions is effective at reducing food-borne pathogens viable cells (Ito et al., 2003). Bacteriocinogenic LAB intended to be used as probiotics are of main interest since these antimicrobial compounds can be produced *in situ* in the gut by probiotic bacteria to combat pathogens (Cotter et al., 2013).

3.3.3. Tolerance to gastrointestinal conditions

Since probiotics must be able to survive the GIT environment, tolerance to acid and bile salts were investigated (Fig. 4b). Acidic conditions were differently tolerated by the assayed strains; *Lb. fermentum* was the only able to growth at pH 3.0 while with the exception of *E. avium* and *W. paramesenteroides* all other LAB tolerated pH > 4.0. Intolerance of enterococci and weissella to high acidic conditions agrees with their inability to adapt to acid stress as previously reported (De Vuyst et al., 2009). In coincidence to this study, *L. fermentum* strains from chicken and swine intestine were found as high acid tolerant being able to survive in gastric juice (Lin et al., 2007). Exposure to increasing concentration of bile salts (0.1 to 0.5%) showed the examined LAB were fully tolerant with the exception of *L. fermentum* that was intolerant to 0.5%. Although ruminal pH is often in the range of 5.8–6.2 in grain-adapted cattle, abomasum pH may be as low as 3.0 and probiotic strains may be able to survive this condition. In addition, bacteria from animal gut are constantly exposed to bile acids, thus high biotrasformation activity is required for effective gut colonization (Chae et al., 2012). As natural residents of GIT of ruminants, several *Lactobacillus* species were able to withstand the harsh conditions of the gut including bile and acid stresses, as previously reported from animal and human strains (Lin et al., 2007; Guo et al., 2010).

3.3.4. Biofilm formation

The ability of LAB (strains with high and low autoaggregative/hydrophobic profiles) for biofilm formation in MRS and MRS-T media was also studied in the pre-selected strains. Adhesion ability to polystyrene microtiter plates at 37 °C for enterococci and lactobacilli are shown in Fig. 5. Under the assayed conditions, variable biofilm formation patterns were obtained for *Enterococcus* strains; with the exception of *E. hirae* CRL2068/2072 and *E. durans* CRL2047 that failed to form biofilm in MRS and MRS-T, the remaining strains were able to adhere after 72 h to polystyrene growing in both media (Fig 5a). High level of biofilm formation ($OD_{570} \geq 1.0$) in MRS-T was obtained for *E. hirae* CRL2062/2089, *E. durans* CRL2048/2153 and *E. faecium* CRL2141, however maximal biofilmogenic ability was exhibited by *E. faecium* CRL2102 in MRS medium reaching OD_{570}: 2.5. *E. hirae* CRL 2089, *E. durans* CRL 2048 and E. *faecium* CRL 2141 showed higher biofilm formation ($p < 0.05$) in MRS-T than in MRS, while *E. hirae* CRL 2062 and *E. faecium* CRL 2102 did not show differences between MRS-T and MRS. On the other hand, a strong influence of culture media used to investigate *Lactobacillus* cell adhesion was found. Six out of 27 assayed lactobacilli strains showed polystyrene adhesion ($OD_{570} \geq 1.0$), among them, *Lb. mucosae* CRL2063/2083/2111/2154 in MRS medium, while *Lb. mucosae* CRL2112/2155 and *P. acidilactici* CRL2043 produced biofilm in MRS-T, maximal adhesion being detected for *Lb. mucosae* CRL2155 with $OD_{570} \geq 3$ (Fig. 5b). The biofilmogenic LAB strains were

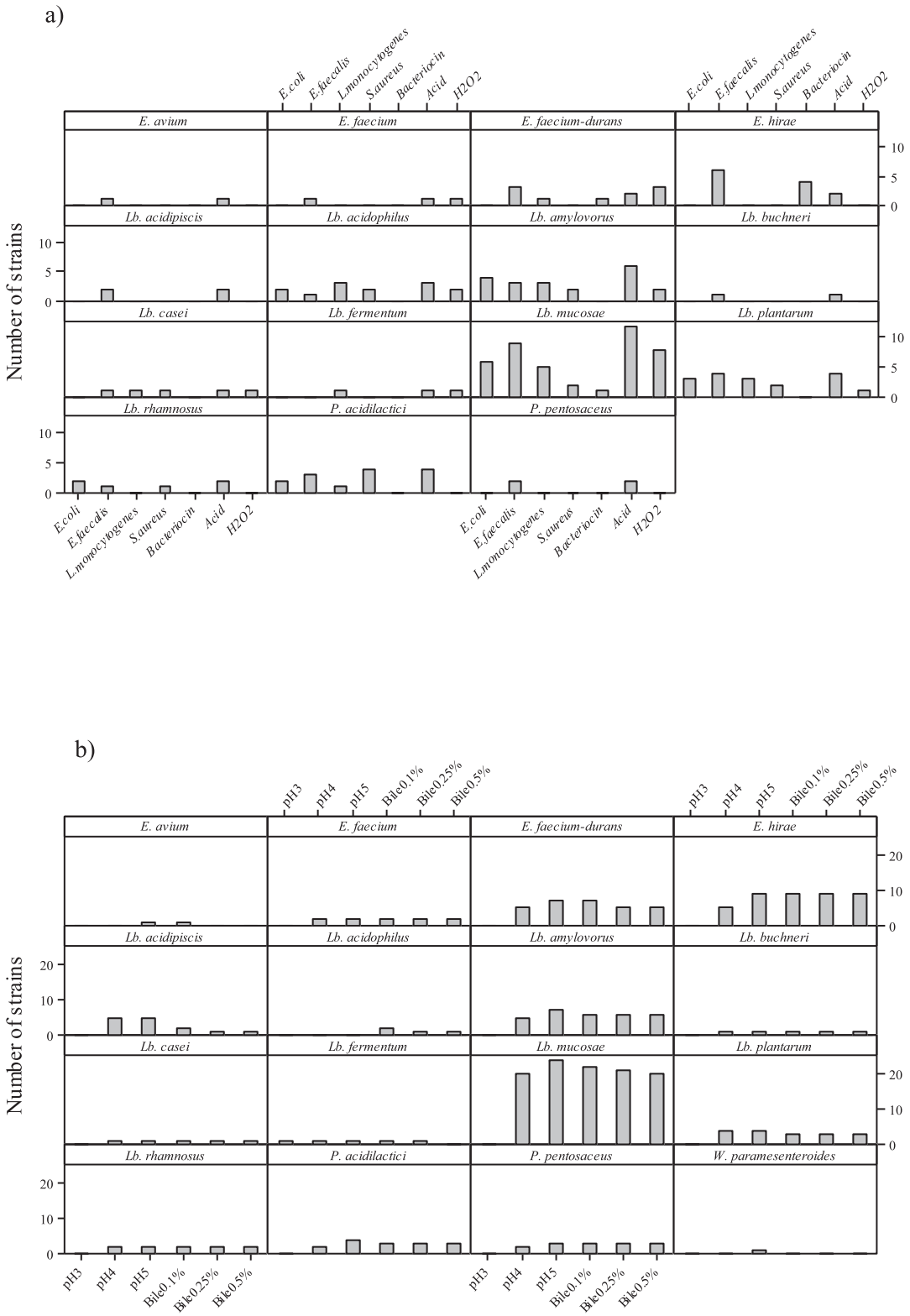

Fig. 4. Production of antagonistic compounds and tolerance to gastrointestinal (GIT) conditions of lactic acid bacteria isolated from feedlot steer's environment in number of strains of each specie. (a) Inhibitory properties (organic acids, hydrogen peroxide and bacteriocins) against Gram-positive (*L. monocytogenes, S. aureus, E. faecium*) and Gram-negative (*E. coli*) target bacteria. (b). Resistance to GIT conditions: pH (3.0–5.0) and bile salts concentration (0.1–0.5%).

mostly isolated from CF samples (7 out of 12). Culture media used to investigate biofilm formation led to different levels of adhesion by the assayed LAB, biofilm formation at 72 h being higher in MRS-T (Tween 80 omitted) than in MRS ($p < 0.05$). The presence of this emulsifier was reported to affect biofilm formation by LAB (Lebeer et al., 2007; Leccese Terraf et al., 2014).

4. Conclusions

This is the first report on the isolation, identification and diversity of

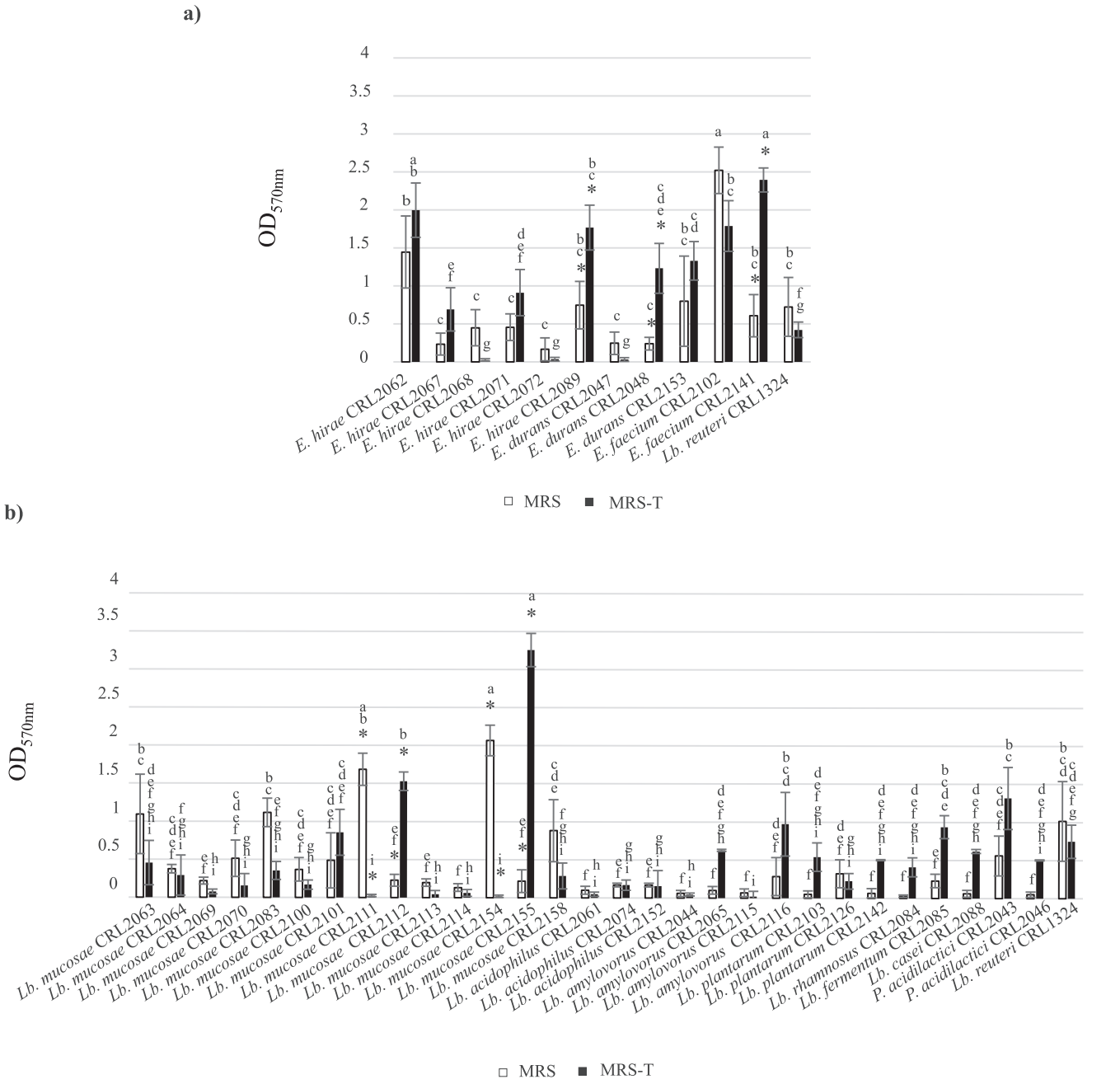

Fig. 5. Biofilm production by lactic acid bacteria from feedlot steer`s environment. (a) *Enterococcus* and (b) *Lactobacillus* and *Pediococcus* strains in MRS and MRS-T (MRS without Tween). The bars indicate the SD obtained from triplicate experiments. Different letters indicate significant differences ($p < 0.05$) between the strains at the same culture media condition assayed. Differences between the biofilm produced by each strain in the two culture media (with or without Tween) is indicated with *.

cultivable LAB population associated to feedlot cattle environment in Argentina. Molecular identification showed LAB representing five genera and twenty species, most of them recovered from cattle feces and in a minor extent from pens soil and feed rations. Based on LAB characteristics a significant correlation between hydrophobicity and autoaggregation as well as the ability to produce antimicrobial compounds was found for *Lb. mucosae, Lb. acidophilus, Lb. amylovorus, E. hirae* and *E. faecium* strains. *Lb. mucosae* CRL2069, *Lb. acidophilus* CRL2074, *Lb. fermentum* CRL2085 and *Lb. amylovorus* CRL2116 strains that also proved to resist GIT conditions were selected as potential probiotic candidates to be used as direct-fed bacteria in feedlot cattle industry. However, such probiotic activities and other properties related to safety and ruminal performance will be more deeply investigated.

Conflict of interest statement

The authors of the present study declare that they do not have any potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence the present work.

The authors of the manuscript declare that there is no conflict of interest. The research was supported by http://dx.doi.org/10.13039/501100002923 (PIP 545 and 744) and ANPCYT (PICT 1187) grants, and into an agreement CONICET-Molino TRIGOTUC S.A.

References

Abriouel, H., Ben Omar, N., Cobo Molinos, A., Lucas López, R., Grande, M.J., Martínez-Viedma, P., Ortega, E., Martínez Cañamero, M., Galvez, A., 2008. Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. Int. J. Food. Microbiol. 123, 38–49.

Adeniyi, B.A., Adetoye, A., Ayeni, F.A., 2015. Antibacterial activities of lactic acid bacteria isolated from cow faeces against potential enteric pathogens. African Health Sci. 5, 888–895.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anderson, J.F., Parrish, T.D., Akhtar, M., Zurek, L., Hirt, H., 2008. Antibiotic resistance of enterococci in American Bison (*Bison bison*) from a nature preserve compared to that of enterococci in pastured cattle. Appl. Environ. Microbiol. 74, 1726–1730.

Bagge, E., Persson, M., Johansson, K.-E., 2010. Diversity of spore-forming bacteria in cattle manure, slaughterhouse waste and samples from biogas plants. J. Appl. Microbiol. 109, 1549–1565.

Beasley, S.S., Manninen, T.J.K., Saris, P.E.J., 2006. Lactic acid bacteria isolated from canine faeces. J. Appl. Microbiol. 101, 131–138.

Beauchemin, K.A., Yang, W.Z., Morgavi, D.P., Ghorbani, G.R., Kautz, W., Leedle, J.A.Z., 2003. Effects of bacterial direct-fed microbials and yeast on site and extent of digestion, blood chemistry, and subclinical ruminal acidosis in feedlot cattle. J. Anim. Sci. 81, 1628–1640.

Beukers, A.G, Zaheer, R., Cook, S.R., Stanford, K., Chaves, A., Ward, M.P., McAllister, T.A., 2015. Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. Front. Microbiol. http://dx.doi.org/10.3389/fmicb.2015.00483.eCollection. 2015.

Brashears, M.M., Jaroni, D., Trimble, J., 2003. Isolation, selection, and characterization of Lactic Acid Bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7 in cattle. J. Food Prot. 66, 355–363.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. Enterococci in the environment. Microbiol. Mol. Biol. Rev. 76, 685–706.

Callaway, T.R., Carr, M.A., Edrington, T.S., Anderson, R.C., Nisbet, D.J., 2009. Diet, *Escherichia coli* O157:H7, and cattle: a review after 10 years. Curr. Issues Mol. Biol. 11, 67–80.

Chae, J.P., Valeriano, V.D., Kim, G.-B., Kang, D.-K., 2012. Molecular cloning, characterization and comparison of bile salt hydrolases from *Lactobacillus johnsonii* PF01. J. Appl. Microbiol. 114, 121–133.

Chang, J.-H., Shim, Y.Y., Cha, S.-K., Chee, K.M., 2010. Probiotic characteristics of lactic acid bacteria isolated from kimchi. J. Appl. Microbiol. 109, 220–230.

Chaucheyras-Durand, F., Durand, H., 2010. Probiotics in animal nutrition and health. Benef. Microbes 1, 3–9.

Chaucheyras-Durand, F., Chevaux, E., Martin, C., Forano, E., 2012. Use of yeast probiotics in ruminants: effects and mechanisms of action on rumen pH, fibre degradation, and microbiota according to the diet. In: Rigobello, E.C. (Ed.), Probiotics in Animals. INTECH, pp. 119–152.

Chen, Y.-S., Yanagida, F., Shinohara, T., 2005. Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. Lett. Appl. Microbiol. 40, 195–200.

Cotter, P.D., Ross, R.P., Hill, C., 2013. Bacteriocins a viable alternative to antibiotics? Nat. Rev. Microbiol. 11, 95–105.

De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A., Ragni, A., Gobbetti, M., 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Res. Microbiol. 157, 792–801.

de Oliveira, M.N.V., Jewell, K.A., Freitas, F.S., Benjamin, L.A., Tótola, M.R., Borges, A.C., Moraes, C.A., Suen, G., 2013. Characterizing the microbiota across the gastrointestinal tract of a Brazilian Nelore steer. Vet. Microbiol. 164, 307–314.

De Vuyst, L., Neysens, P., 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci. Technol. 16, 43–56.

De Vuyst, L., Vrancken, G., Ravyts, F., Rimaux, T., Weckx, S., 2009. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol. 26, 666–675.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H.-M., Weckx, S., 2014. Microbial ecology of sourdough fermentations: diverse or uniform? Food Microbiol. 37, 11–29.

Dill-McFarland, K.A., Breaker, J.D., Garret, S., 2017. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. Sci. Rep. 7. http://dx.doi.org/10.1038/srep40864.

Dogi, C.A., Perdigón, G., 2006. Importance of the host specificity in the selection of probiotic bacteria. J. Dairy Res. 73, 357–366.

Du Toit, M., Dicks, L.M.T., Holzapfel, W.H., 2003. Identification of heterofermentative lactobacilli isolated from pig faeces by numerical analysis of total soluble cell protein patterns and RAPD-PCR. Lett. Appl. Microbiol. 37, 12–16.

Emmanuel, D.G.V., Jafari, A., Beauchemin, K.A., Leedle, J.A.Z., Ametaj, B.N., 2007. Feeding live cultures of *Enterococcus faecium* and *Saccharomyces cerevisiae* induces an inflammatory response in feedlot steers. J. Anim. Sci. 85, 233–239.

Ewaschuk, J.B., Zello, G.A., Naylor, J.M., 2006. *Lactobacillus* GG does not affect D-lactic acidosis in diarrheic calves, in a clinical setting. J. Vet. Intern. Med. 20, 614–619.

FAO/WHO (Food and Agriculture Organization/World Health Organization), 2002. Guidelines for the Evaluation of Probiotics in Food. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Fuller, R., 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.

Gaggía, F., Mattarelli, P., Biavatiet, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol. 141, 15–28.

Galyean, M.L., 2010. Building sustainable beef production: addressing environmental and management challenges in intensive production systems. Rev. Argentina Prod. Anim. 30, 229–241.

Gevers, D., Huys, G., Swingset, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol. Lett. 205, 31–36.

Gressley, T.F., Hall, M.B., Armentano, L.E., 2011. Productivity, digestion, and health responses to hindgut acidosis in ruminants. J. Anim. Sci. 89, 1120–1130.

Guevara, J.C., Grünwaldt, E.G., 2012. Status of beef cattle production in Argentina over the last decade and its prospects. In: Javed, K. (Ed.), Livestock Production. INTECH, pp. 117–134.

Guo, X.H., Kim, J.M., Nam, H-M., Park, S-Y., Kim, J-M., 2010. Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. Anaerobe 16, 321–326.

Hammes, W.P., Hertel, C., 2006. The genera *Lactobacillus* and *Carnobacterium*. In: Dworkin, S., Falcow, E., Rosenberg, K-H., Schleifer, E., Stackebrandt, M. (Eds.), The Prokariotes, A Handbook on the Biology of Bacteria. Springer, pp. 320–403.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Berni Canani, R., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514.

Hwang, E-N., Kang, S-M., Kim, M-J, Lee, J-W., 2015. Screening of immune-active lactic acid bacteria. Korean J. Food Sci. Anim. 35, 541–550.

Iñiguez-Palomares, C., Pérez-Morales, R., Acedo-Félix, E., 2007. Evaluation of probiotic properties in *Lactobacillus* isolated from small intestine of piglets. Rev. Latinoam. Microbiol. 49, 46–54.

Iseppi, R., Messi, P., Anacarso, I., Bondi, M., Sabia, C., Condó, C., de Niederhausern, S., 2015. Antimicrobial resistance and virulence traits in *Enterococcus* strains isolated from dogs and cats. New Microbiol. 38, 369–378.

Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H., Toba, T., 2003. The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. Curr. Microbiol. 47, 231–236.

Jackson, C.R., Lombard, J.E., Dargatz, D.A., Fedorka-Cray, P.J., 2011. Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle. Lett. Appl. Microbiol. 52, 41–48.

Kaur, R., Tiwari, S.K., 2016. Isolation, identification and characterization of *Pediococcus pentosaceus* LB44 and *Weissella confusa* LM85 for the presence of Bacteriocin-Like Inhibitory Substances (BLIS). Microbiology 85, 540–547.

Khota, W., Pholsem, S., Higgs, D., Cai, Y., 2016. Natural lactic acid bacteria population of tropical grasses and their fermentation factor analysis of silage prepared with cellulase and inoculant. J. Dairy Sci. 99, 1–14.

Klein-Jöbstl, D., Schornsteiner, E., Mann, E., Wagner, M., Drillich, M., Schmitz-Esser, S., 2014. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front. Microbiol. 5, 1–7.

Kullen, M.J., Sanozky-Dawes, R.B., Crowell, D.C., Klaenhammer, T.R., 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J. Appl. Microbiol. 89, 511–516.

Lebeer, S., Verhoeven, T.L.A., Perea Vélez, M., Vanderleyden, J., De Keersmaecker, S.C.J., 2007. Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. Appl. Environ. Microbiol. 73, 6768–6775.

Leccese Terraf, M.C., Mendoza, L.M., Juárez Tomás, M.S., Silva, C., Nader-Macías, M.E., 2014. Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli. J. Appl. Microbiol. 117, 1761–1772.

Li, D., Nia, K., Pang, H., Wang, Y., Cai, Y., Jin, Q., 2015. Identification and antimicrobial activity detection of lactic acid bacteria isolated from corn stover silage. Asian Australas. J. Anim. Sci. 28, 620–631.

Lin, W.-H., Yu, B., Jang, S.-H., Tsen, H.-Y., 2007. Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. Anaerobe 13, 107–113.

Maldonado, N., Silva de Ruiz, C., Otero, M.C., Sesma, F., Nader-Macías, M.E., 2012. Lactic acid bacteria isolated from young calves – Characterization and potential as probiotics. Res. Vet. Sci. 92, 342–349.

Mann, E., Schmitz-Esser, S., Zebeli, Q, Wagner, M., Ritzmann, M., Metzler-Zebeli, B.U., 2014. Mucosa-associated bacterial microbiome of the Gastrointestinal Tract of weaned pigs and dynamics linked to dietary calcium-phosphorus. PLoS One 9. http://dx.doi.org/10.1371/journal.pone.0086950.

Maron, D.F., Smith, T.J.S., Nachman, K.E., 2013. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Global Health 9, 48–59.

Micallef, S.A., Rosenberg Goldstein, R.E., George, A., Ewing, L., Tall, B.D., Boyer, M.S., Joseph, S.W., Sapkota, A.R., 2013. Diversity, distribution and antibiotic resistance of *Enterococcus* spp. recovered from tomatoes, leaves, water and soil on U.S. Mid-Atlantic farms. Food Microbiol. 36, 465–474.

Nader-Macías, M.E.F., Otero, M.C., Espeche, M.C., Maldonado, N.C., 2008. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. J. Ind. Microbiol. Biotechnol. 35, 1387–1395.

Nes, I.F., Gabrielsen, C., Brede, D.A., Diep, D.B., 2015. Novel developments in bacteriocins from lactic acid bacteria. In: Mozzi, F., Raya, R., Vignolo, G. (Eds.), Biotechnology of Lactic Acid Bacteria. Novel Application. Willey Blackwell, UK, pp. 80–99.

Ocaña, V.S., Nader-Macías, M.E., 2002. Vaginal lactobacilli: self- and co-aggregating ability. Br. J. Biomed. Sci. 59, 183–190.

Pang, H., Qinb, G., Tan, Z., Li, Z., Wang, Y., Cai, Y., 2011. Natural populations of lactic

acid bacteria associated with silage fermentation as determined by phenotype, 16S ribosomal RNA and recA gene analysis. Syst. Appl. Microbiol. 34, 235–241.
Peterson, R., Klopfenstein, T.J., Folmer, J., Hinkley, S., 2007. Effect of *Lactobacillus acidophilus* Strain NP51 On *Escherichia Coli* 0157:H7 Fecal Shedding and Finishing Performance in Beef Feedlot Cattle. Faculty Papers and Publications in Animal Science Paper 540. DigitalCommons@University of Nebraska - Lincoln.
Pospiech, A., Neumann, B., 1995. A versatile quick-prep of genomic DNA from gram-positive bacteria. Trends Genet. 11, 217–218.
Puphan, K., Sornplang, P., Uriyapongson, S., Navanukraw, C., 2015. Screening of lactic acid bacteria as potential probiotics in beef cattle. Pakistan J. Nut. 14, 474–479.
Qadis, A.Q., Goya, S., Ikuta, K., Yatsu, M., Kmura, A., Nakanishi, S., Sato, S., 2014. Effects of a bacteria-based probiotic on ruminal pH, volatile fatty acids and bacterial flora of Holstein calves. J. Vet. Med. Sci. 76, 877–885.
Sainz, R.D., Lanna, D.P., 2009. Livestock production in feedlots/landless systems. In: UNESCO-EOLSS, Management of Agricultural, Forestry and Fisheries Enterprises, pp. 1–15.
Seal, B.S., Lillehoj, H.S., Donovan, D.M., Gay, C.G., 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim. Health Res. Rev. 14, 78–87.
Silva, B.C., Jung, L.R., Sandes, S.H., Alvim, L.B., Bomfim, M.R, Nicoli, J.R., Neumann, E., Nunes, A.C., 2013. In vitro assessment of functional properties of lactic acid bacteria isolated from faecal microbiota of healthy dogs for potential use as probiotics. Benef. Microbes 4, 267–275.
Soto, L.P., Frizzo, L.S., Bertozzi, E., Avataneo, E., Sequeira, G.J., Rosmini, M.R., 2010. Molecular microbial analysis of *Lactobacillus* strains isolated from the gut of calves for potential probiotic use. Vet. Med. Int. http://dx.doi.org/10.4061/2010/274987.
Stanford, K., Bach, S., Baah, J., McAllister, T., 2014. A mixture of *Lactobacillus casei, Lactobacillus lactis*, and *Paenibacillus polymyxa* reduces *Escherichia coli* 0157:H7 in finishing feedlot cattle. J. Food Prot. 77, 738–744.
Thamacharoensuk, T., Thongchul, N., Taweechotipatr, M., Tolieng, V., Kodama, K., Tanasupawat, S., 2013. Screening and characterization of lactic acid bacteria from animal faeces for probiotic properties. Thai. J. Vet. Med. 43, 541–551.
Uyeno, Y., Sekiguchi, Y., Kamagata, Y., 2010. rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. Lett. Appl. Microbiol. 51, 570–577.
Uyeno, Y., Shigemori, S., Shimosato, T., 2015. Effect of probiotics/prebiotics on cattle health and productivity. Microbes Environ. 30, 126–132.
WHO (World Health Organization), 2012. The evolving threat of antimicrobial resistance: options for Action. http://whqlibdoc.who.int/publications/2012/9789241503181_eng.pdf.
Yousif, N.M.K., Huch, M., Schuster, T., Cho, G-S., Dirar, H.A., Holzapfel, W.H., Franz, C.M.A.P., 2010. Diversity of lactic acid bacteria from Hussuwa, a traditional African fermented sorghum food. Food Microbiol. 27, 757–768.