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Lactobacillus equigenerosi Strain Le1 Invades Equine Epithelial Cells

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Lactobacillus equigenerosi strain Le1, a natural inhabitant of the equine gastrointestinal tract, survived pH 3.0 and incubation in the presence of 1.5% (wt/vol) bile salts for at least 2 h. Strain Le1 showed 8% cell surface hydrophobicity, 60% auto-aggregation, and 47% coaggregation with *Clostridium difficile* C6. Only 1% of the cells adhered to viable buccal epithelial cells and invaded the cells within 20 min after contact. Preincubation of strain Le1 in a buffer containing pronase prevented adhesion to viable epithelial cells. Preincubation in a pepsin buffer delayed invasion from 20 min to 1 h. Strain Le1 did not adhere to nonviable epithelial cells. Administration of *L. equigenerosi* Le1 (1×10^9 CFU per 50 kg body weight) to healthy horses did not increase white blood cell numbers. Differential white blood cell counts and aspartate aminotransferase levels remained constant. Glucose, lactate, cholesterol, and urea levels remained constant during administration with *L. equigenerosi* Le1 but decreased during the week after administration.

Horses are hindgut fermenters; i.e., most of the microbial activity takes place in the cecum and colon (13). Production of large quantities of saliva (10 to 12 liters per day) helps to transport the feed through a 1.2- to 1.5-m-long esophagus and buffers the digesta (13). Transition of digesta through the stomach is relatively rapid, although a large portion remains for 2 to 6 h in the anaerobic fundic (lower) section of the stomach. Digesta reaches the cecum and colon approximately 3 h after feeding (13). The pH of the cecum and colon is approximately 6.0 and forms the ideal condition for lactic acid bacteria, anaerobic bacteria, fungi, and protozoa to degrade residual starch, hemicelluloses, and pectins (4, 17). Survival at low pH, tolerance to bile salts, and adherence to epithelial cells and mucus are considered important characteristics for any strain to survive the gastrointestinal tract (GIT) (8, 14).

The equine gut is host to only a selected few species of lactic acid bacteria, e.g., *Lactobacillus equigenerosi*, *Lactobacillus buchneri*, *Lactobacillus hayakitensis*, *Lactobacillus kitasatonis*, *Lactobacillus delbrueckii*, *Lactobacillus equi*, *Lactobacillus johnsonii*, *Lactobacillus mucosae*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Enterococcus faecalis*, and *Enterococcus faecium*. Of these species, *L. equigenerosi*, *L. equi*, and *L. hayakitensis* have not been isolated from other niches and are considered host specific (11, 23). Several *Clostridium* spp. have been isolated from horses. Although naturally present, toxins produced by clostridia, especially *Clostridium difficile*, may lead to death (2, 3, 16, 20, 21).

Little is known about the survival of lactic acid bacteria in the equine gastrointestinal tract (GIT) and its effect on the host. This study sheds some light on the survival of *L. equigenerosi* at different pHs and in the presence of bile salts and on the ability of the cells to aggregate, auto-aggregate, and coaggregate with *C. difficile*. The adhesion of *L. equigenerosi* Le1 to equine epithelial cells was studied to determine if the two cell types have a unique association. We also report on changes in blood cell numbers, aspartate aminotransferase, glucose, lactate, cholesterol, and urea of horses that have been administered *L. equigenerosi* Le1.

MATERIALS AND METHODS

Isolation and identification of bacteria. Feces collected from healthy horses, receiving no probiotic treatment, were inoculated (10%, wt/vol) into De Man Rogosa (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) and incubated at 37°C for 24 h. From this, plates with MRS

agar (Biolab) were streaked and incubated at 37°C for 48 to 72 h in anaerobic flasks with gas-generating envelopes (Anaerobic system BR0038B; Oxoid Ltd., Basingstoke, Hants, United Kingdom). Smooth cream-colored colonies of approximately 1 to 2 mm in diameter were selected. Colonies with oval-shaped morphology, typical of that described for *L. equigenerosi* (11), were restreaked to obtain pure cultures. The selected isolates were tested for growth in MRS broth adjusted to pH 4.0 and pH 3.5 and in MRS broth supplemented with 2.5% and 5.0% (wt/vol) NaCl. Species identity was confirmed by 16S rRNA gene sequencing, using primers 8F and 1512R (Table 1). DNA was isolated according to the method of Endo and Okada (10). This isolate that corresponds to the description of *L. equigenerosi* was selected for further studies.

Samples of gut contents collected from a diseased horse, diagnosed with clinical signs of colic, were streaked onto *Clostridium difficile* agar base CM0601, supplemented with the selective reagent SR0096 (Oxoid Ltd., Cambridge, England). The plates were incubated in anaerobic flasks at 37°C for 6 days. Gray-white colonies, resembling the morphology of *Clostridium difficile*, were selected, Gram stained, and tested for catalase activity by dropping 5% (vol/vol) H_2O_2 onto the colonies.

Tolerance to pH and bile salts. Strain Le1, confirmed as being *L. equigenerosi*, was cultured in MRS broth for 18 h at 37°C and then inoculated (100 µl) into 10 ml MRS broth preadjusted with DL-lactic acid to pH 2.0, 2.6, 3.0, 4.0, 5.0, and 5.4. The pH of the medium was determined after autoclaving and readjusted if necessary. In another experiment, the strain was inoculated (100 µl) into 10 ml MRS broth, supplemented with 0.5%, 1.0%, and 1.5% (wt/vol) Oxbile (Oxoid). Incubation was for 2 h at 37°C, followed by serial dilution in sterile saline and plating onto MRS agar. Bile salt hydrolase (BSH) activity was determined by streaking strain Le1 onto MRS agar supplemented with 0.37 g $CaCl_2$ and 0.5% (wt/vol) taurodeoxycholic acid (Sigma, St. Louis, MO). Plates were incubated at 37°C for 24 h, and BSH activity was recorded as positive if a precipitate formed around the colonies. Incubation was always anaerobic in anaerobic flasks with gas-generating envelopes (Oxoid).

Cell surface hydrophobicity, auto-aggregation, and coaggregation. Hydrophobicity, auto-aggregation, and coaggregation were determined according to the methods described by Botes et al. (5). *L. equigenerosi* Le1

TABLE 1 DNA primers used in this study

Primer	Sequence (5′ to 3′)	Annealing temp (°C)	Reference
16S rDNA			
8F	CACGGATCCAGACTTTGATYMTGGCTCAG	54	12
1512R	GTGAAGCTTACGGYTAGCTTGTTACGACTT		
DGGE			
Lac1	AGCAGTAGGGAATCTTCCA	61	36
Lac2GC	CGCCCGGGGCGCGCCCCGGGCGGCCCGGG GGCACCGGGGGGATTYCACCGCTACACATG		36
Lac3	AGCAGTAGGGAATCTTCGG	61	10

was grown anaerobically in MRS broth for 18 h at 37°C, and the cells were harvested (10,000 × g, 10 min, 4°C) and washed twice with quarter-strength Ringer's solution (1.5 g NaCl, 0.02 g KCl, 0.03 g CaCl$_2$, and 0.03 g NaHCO$_3$). The optical density (OD) of the cell suspension was recorded at 580 nm (reading 1). To the cell suspension an equal volume of *n*-hexadecane was added and mixed for 2 min, and the mixture was left at 26°C for 30 min to separate into two phases. One milliliter of the top phase was carefully extracted, and the OD was recorded at 580 nm (reading 2). The level of hydrophobicity was expressed as a percentage value, calculated by using the equation of Doyle and Rosenberg (9):

$$\% \text{ hydrophobicity} = \left[(\text{OD}_{580} \text{ reading } 1 - \text{OD}_{580} \text{ reading } 2)/\text{OD}_{580} \text{ reading } 1\right] \times 100$$

Auto-aggregation of *L. equigenerosi* Le1 was determined by washing the cells, as described before, and resuspending them in sterile saline (0.8%, wt/vol, NaCl). The cell density was adjusted to 0.3 (measured at 660 nm). One milliliter of the cell suspension was transferred to a sterile 2-ml plastic cuvette and centrifuged for 2 min at 2,000 × g. The cell density in the supernatant was recorded immediately after centrifugation (OD$_{660}$0) and 60 min later (OD$_{660}$60). The percentage of cells that aggregated was calculated using the equation of Malik et al. (22):

$$\% \text{ auto-aggregation} = \left[(\text{OD}_{660}0 - \text{OD}_{660}60)/\text{OD}_{660}0\right] \times 100$$

Coaggregation of *L. equigenerosi* Le1 and *C. difficile* C6 was determined as follows. Strain Le1 was cultured anaerobically in MRS broth for 18 h at 37°C and strain C6 anaerobically in DRCM broth (Biolab) for 24 to 48 h at 37°C. Cells were harvested (10,000 × g, 10 min, 18°C) and washed with sterile saline (0.8%, wt/vol, NaCl). The cells were resuspended in sterile saline, and the cell density (OD$_{660}$) of each was adjusted to 0.3. Equal volumes of the two cell suspensions were mixed and allowed to stand for 20 min at 25°C. The cell density of the combined cell suspension was recorded (OD$_{660}$Tot) to represent the initial OD. Cells were then harvested (2,000 × g, 2 min, 18°C) to determine the OD of the nonaggregated cells in the supernatant (OD$_{660}$S). The percentage coaggregation was calculated using the equation of Malik et al. (22):

$$\% \text{ coaggregation} = \left[(\text{OD}_{660}\text{Tot} - \text{OD}_{660}\text{S})/\text{OD}_{660}\text{Tot}\right] \times 100$$

To determine how many cells of *L. equigenerosi* Le1 coaggregated with *C. difficile* C6, the number of viable cells in the pellet was determined. The pellet was resuspended in sterile saline, serially diluted, and then plated onto MRS agar and DRCM (Biolab) agar, respectively. Incubation was at 37°C (24 h for MRS plates and 48 to 72 h for DRCM plates).

Adhesion to epithelial cells. Fresh buccal (cheek) epithelial cells were collected from healthy horses. The swabs were immediately suspended in minimal essential medium (MEM) Earle's base (Highveld Biological Pty. Ltd., Kelvin, Johannesburg, South Africa). A sterile eight-chamber slide (Nalge Nunc International, Rochester, NY) was seeded with 200 µl epithelium cells per well. *L. equigenerosi* Le1 was cultured in MRS broth for 18 h at 37°C. The cells were harvested (10,000 × g, 5 min, 18°C), washed in sterile distilled water, and resuspended in 1 ml sterile phosphate-buffered saline (PBS) to 1 × 10^4 CFU ml^{-1} (OD$_{600}$ = 1.5). From this suspension 150 µl (1.5 × 10^3 CFU) was added to each of the wells with epithelial cells

and the plates were incubated for 2 h at 37°C. Bacterial cells that did not adhere to the epithelial cells were withdrawn, serially diluted in sterile saline, plated onto MRS agar, and incubated anaerobically at 37°C for 24 h. Adherence of the bacteria to epithelial cells was expressed as a percentage value, calculated by using the equation

$$\% \text{ adhesion} = \left[(\text{CFU ml}^{-1}_{120} - \text{CFU ml}^{-1}_{0})/\text{CFU ml}^{-1}_{120}\right] \times 100$$

CFU ml$^{-1}_0$ refers to the initial number of bacteria and CFU ml$^{-1}_{120}$ to the number of bacteria that adhered to the epithelial cells after 2 h.

In another experiment, the bacterial cells were stained to differentiate viable from dead cells. To 1 ml washed cell suspension (1 × 10^4 CFU), 1.5 µl SYTO 9 (3.34 mM stock solution), and 1.5 µl propidium iodide (20 mM stock solution) from the LIVE/DEAD BacLight bacterial viability kit, L34856 (Leiden, The Netherlands), were added, and the suspensions were incubated for 15 min at 25°C. The epithelium-seeded wells, containing either viable or dead buccal epithelial cells, were each inoculated with 150 µl (1.5 × 10^3 CFU) of the bacterial suspension. Images of the bacteria and epithelial cells were recorded using an Olympus cell^R system, attached to an IX-81 inverted fluorescence microscope equipped with an F-view-II cooled charge-coupled-device (CCD) camera (Soft Imaging Systems). Cells were excited by exposure to 472 nm and 572 nm using a Xenon-Arc burner (Olympus Biosystems GMBH). Emission was collected using a UBG triple-bandpass emission filter cube. For the z-stack image frame acquisition, an Olympus UPlan Apo N 100× oil objective and the cell^R imaging software were used. Images were processed and background subtracted using the cell^R software. Three-dimensional images were obtained using the cell^R software with a step width of 0.26 µm represented in fence view and either a 60× (Olympus PlanApo) or a 100× (UPlanApo N) oil immersion objective. Specific conditions were maintained for z-stack analysis. Parameters for image acquisition were defined and maintained for all experiments. Z-stacks were processed and displayed in the slice view mode, using the cell^R software. For the time lapse series, images were acquired over 20 min with a cycle time of 5 s.

The adhesion experiment was repeated but with cells of *L. equigenerosi* Le1 that had been pretreated with pronase and pepsin, respectively. Cells were grown in MRS broth to 1 × 10^4 CFU ml^{-1}, harvested, washed, and resuspended in PBS as described before. One milliliter of the cell suspension was resuspended in either 500 µl pronase or 500 µl pepsin (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 2 h at 37°C. The pronase and pepsin solutions were prepared by suspending 5.0 mg of each in 1 ml sterile PBS. The cells were then harvested (14,000 × g, 10 min, 4°C), washed using 2 volumes of 0.8 ml sterile PBS to remove residual enzymes, and resuspended in 1 ml sterile PBS. The cells were stained with BacLight as described before, and adhesion to epithelial cells was studied under the fluorescence microscope. Images were taken immediately after adding strain Le1 to the epithelial cells and 20 min, 1 h, and 2 h later.

Competition with *Clostridium difficile* for adherence to viable epithelial cells. To determine if *L. equigenerosi* Le1 has the ability to compete with *C. difficile* C6 for adherence to viable epithelial cells, the adhesion experiment was repeated but with a mixed bacterial cell suspension. Epithelium-seeded wells were inoculated with a combination of 100 µl (approximately 1 × 10^3) of strains Le1 and C6, respectively. After 2 h of incubation, bacterial cells that did not adhere to the epithelial cells were withdrawn and plated onto MRS and DRCM agar. The percentage of adhesion to the epithelial cells was determined as described before. The experiment was repeated with cells that had been stained with BacLight, and adhesion to epithelial cells was studied as described before. Images were taken after 20 min, 1 h, and 2 h of contact between the bacteria and the epithelial cells.

Administration of *Lactobacillus equigenerosi* Le1 to horses. Four healthy crossbreed horses, stabled at the Welgevallen experimental farm of the University of Stellenbosch, were included in this study. Permission to perform the research was granted by the Ethical Committee (ethical clearance number 2009B03002). The horses were stabled separately in 9-m^2 quarters and were fed high-quality alfalfa, hay, and a commercial

energy-rich feed supplement twice a day. The horses were dewormed 4 weeks before the onset of the experiment.

L. equigenerosi Le1 was cultured in 10% (wt/vol) sterile molasses at 37°C for 48 h. Viable cell numbers were determined by plating onto MRS agar, and the dosage was calculated based on 1×10^9 CFU per 50 kg body weight. Each horse received 20 ml of a cell suspension early morning and another 20 ml 10 h later. The experiment was conducted over 24 days, consisting of a first 7-day period before dosing the horses with strain Le1, a second 10-day period during dosage, and a third 7-day postadministration period.

Screening of feces for the presence of *Lactobacillus equigenerosi* Le1. Fecal samples were collected from the horses on each of the 24 days of the trial. Serial dilutions of the samples were prepared in sterile saline and plated onto MRS agar and modified MRS agar, i.e., MRS agar adjusted to pH 4.0 and supplemented with 2.5% (wt/vol) NaCl. Microbial DNA was isolated from fecal samples collected on days 6, 4, and 1 before administering strain Le1 to the horses, on days 2, 4, and 7 during the dosage period, and every second day for 1 week following the last dosage. The ZR fungal/bacterial DNA kit (Zymo Research, CA) was used. DNA was amplified using primers Lac1, Lac2GC, and Lac3 (Table 1). PCR conditions were as described by Walter et al. (36). PCR products were analyzed with denaturing gradient gel electrophoresis (DGGE), using a DCode system (Bio-Rad Laboratories, Hercules, CA). The denaturing gradient of the 8% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio, 37.5:1) ranged from 35% to 50%. In this case a 100% denaturant represents 40% formamide and 7 M urea. Electrophoresis was performed at 70 V for 16 h in a Tris-acetate-EDTA buffer. Immediately after electrophoresis, the gels were stained with SYBR green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME) for approximately 30 min. Clearly visible bands on the DGGE gels were excised with sterile toothpicks, and the DNA fractions were amplified by using the same primer sets and conditions used to amplify DNA for DGGE.

Blood analyses. Three Vacutainers of whole blood were drawn from the jugular vein of each of the horses every third day of the trial, starting 1 week before dosage with *L. equigenerosi* Le1. The first was anti-coagulated with EDTA and kept at room temperature until analysis (within 4 h) for full and differential white blood cell (WBC) counts, using the Celldyne 3700CS hematology analyzer with the veterinary software package. The second sample was collected in serum separating tubes (SST) and kept on ice until centrifugation, after which serum was separated and stored at −80°C. Stored serum was used to determine aspartate aminotransferase (AST), glucose, cholesterol, lactate, and urea levels. The third sample, collected in sodium fluoride, was kept on ice until analysis for glucose and lactate levels using standard automated pathology laboratory procedures. Blood chemistry tests were conducted by Pathcare (Pty) Ltd. (Stellenbosch, Western Cape, South Africa). Statistical analysis was performed on the blood data by using two-way analysis of variance (ANOVA). Variability between groups was noted when P was ≤0.05 and the F-value was more than the F-crit value.

RESULTS

Identification of bacteria. A total of 120 colonies were selected from MRS agar plates that were incubated anaerobically at 37°C. From these colonies, 10 were smooth and cream-colored and had an oval-shaped morphology. An isolate selected from one of these colonies grew in MRS broth adjusted to pH 4.0, but not in broth at pH 3.5, and in MRS broth supplemented with 2.5% (wt/vol) NaCl, but not in the same medium supplemented with 5.0% (wt/vol) NaCl. This isolate, designated Le1, corresponded to the description of *L. equigenerosi* and was selected for further studies. The 16S rRNA gene sequence shared 100% homology with its closest relative (GenBank accession no. AB425935, deposited as *L. equigenerosi*). Gray-white colonies isolated from Clostridium dif-

ficile agar base supplemented with the selective reagent SR0096 were Gram positive and catalase negative.

Resistance to pH and bile salts. *L. equigenerosi* Le1 cell numbers of 1×10^8 CFU ml^{-1} were recorded in MRS broth at pH 6.4. No significant changes in cell numbers were recorded after 2 h of incubation in MRS broth adjusted to pH 5.4 or pH 5.0. However, after 2 h at pH 4.0 the cell numbers decreased to 7×10^6 CFU ml^{-1}. At pH 3.0 numbers decreased to 5×10^6 CFU ml^{-1}. No viable cells were recorded after 2 h of incubation in medium adjusted to pH 2.0 or pH 2.6. Cell numbers decreased from 1×10^8 CFU ml^{-1} in MRS broth without bile salts to 5×10^6 CFU ml^{-1} after 2 h in the same medium supplemented with either 0.5%, 1.0%, or 1.5% (wt/vol) bile salts. No BSH activity was recorded.

Cell surface hydrophobicity, auto-aggregation, and coaggregation. *L. equigenerosi* Le1 displayed 8% hydrophobicity and an auto-aggregation level of 60%. Forty-seven percent of the Le1 cells coaggregated with *C. difficile* C6.

Adhesion to epithelial cells. Only 1% of *L. equigenerosi* Le1 cells adhered to viable epithelial cells, as determined from growth on MRS agar plates. Visual images of adhesion of *L. equigenerosi* Le1 to viable and nonviable buccal epithelial cells, as distinguished by propidium iodide-positive nuclei, are shown in Fig. 1. Cells of *L. equigenerosi* Le1 clearly adhered to viable epithelial cells (Fig. 1A) and invaded the cells within 20 min (Fig. 1B and E). No bacterial cells adhered to (Fig. 1C) or entered (Fig. 1D and F) nonviable epithelial cells. Bacterial cells pretreated with pronase did not adhere to or invade epithelial cells (Fig. 2), as indicated by the lack of bacterial aggregates at the cell membrane or within its cytoplasm. However, bacteria pretreated with pepsin adhered to and invaded viable epithelial cells after 1 h of incubation and remained in the cells for at least another hour (Fig. 3). In the presence of *Clostridium difficile* C6, 3% of *L. equigenerosi* Le1 cells adhered to viable epithelial cells, as determined from growth on MRS agar plates. Competition between the cells for adherence to the epithelial cells was clearly visible in the fluorescent images (Fig. 4).

Presence of *L. equigenerosi* Le1 in the intestine. The number of total lactic acid bacteria, recorded from growth on MRS agar, remained between 10^7 and 10^8 CFU g^{-1} feces, irrespective of the sampling point during the trial. However, only 20% of the colonies grew on modified MRS agar. Results obtained with DGGE confirmed the presence of *L. equigenerosi* in all four horses before they were administered strain Le1 and during the 10-day dosage period but not in the week after dosage (Fig. 5).

Blood analyses. Results of blood analyses are shown in Table 2. The white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts remained more or less constant ($P > 0.05$) throughout the trial. A statistically significant ($P \leq 0.05$) decrease in levels of red blood cell (RBC) counts, hemoglobin, and hematocrit was recorded for 7 days immediately after treatment compared to average values prior and during treatment. The average platelet count decreased nonsignificantly from 2.0×10^{11} liter^{-1} before and during administration of strain Le1 to 1.8×10^{11} liter^{-1} after administration, i.e., during the last 7 days of the trial. The aspartate aminotransferase levels remained between 291 and 283 U liter^{-1}, thus without significant variation. Cholesterol levels remained constant before and during administration with *L. equigenerosi* Le1 but decreased significantly ($P \leq 0.05$) from 2.3 mmol liter^{-1} to 2.2 mmol liter^{-1} during the 7-day postadministration period. Glucose levels decreased significantly ($P \leq 0.05$)
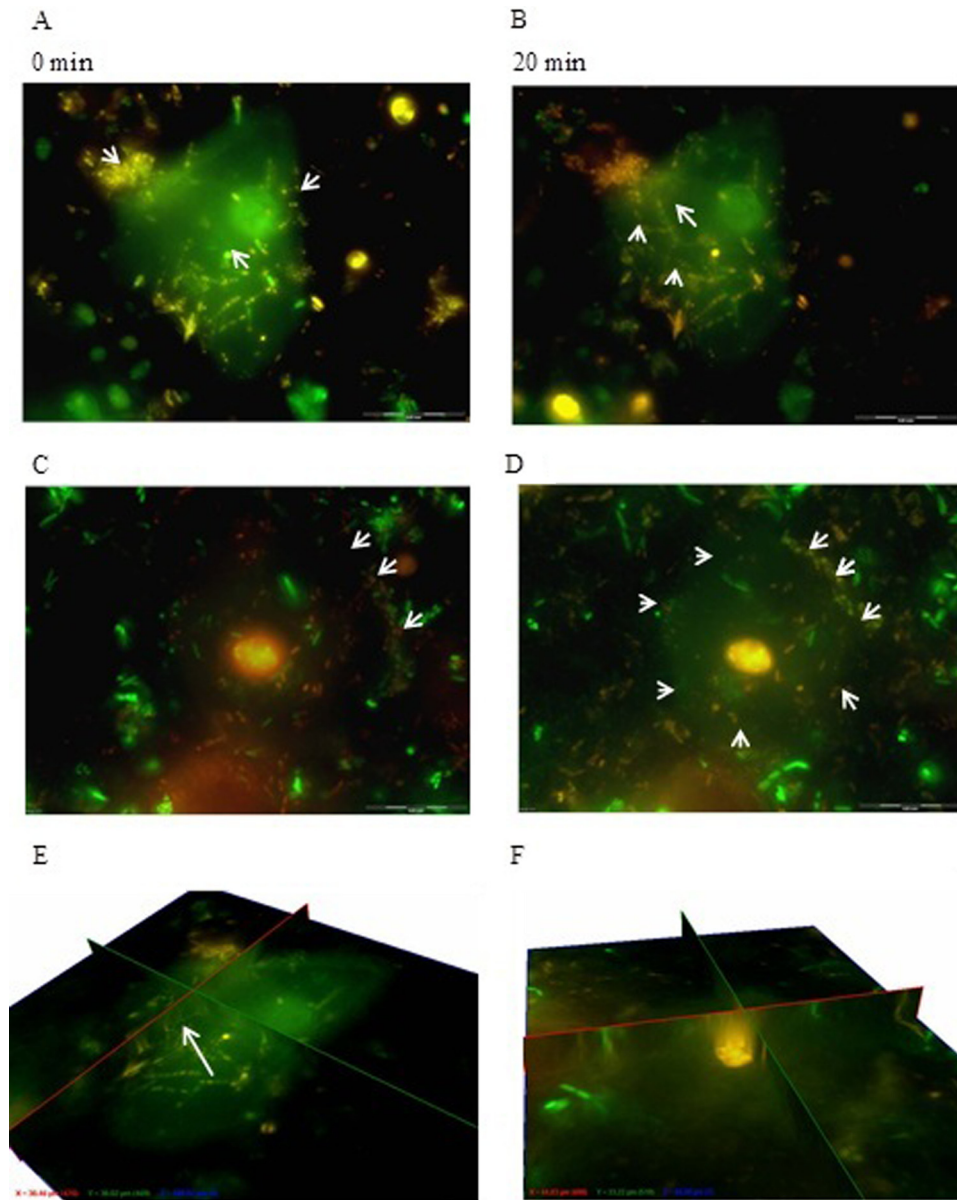
FIG 1 Adhesion of *L. equigenerosi* Le1 to viable buccal epithelial cells (indicated by arrows), as observed with z-stack immediately after addition of the bacteria (A) and 20 min later (B). Adhesion to nonviable buccal epithelial cells is shown immediately after addition of the bacteria (C) and 20 min later (D). (E and F) Fence view images taken 20 min after strain Le1 had been in contact with viable and nonviable buccal epithelial cells, respectively. Nuclei of viable cells stained green with BacLight, and nuclei of nonviable cells stained orange or red.

from 5.5 mmol liter^{-1} before and during administration to 4.6 mmol liter^{-1} in the week postadministration. Lactate levels remained more or less constant before and during administration (between 0.8 and 0.9 mmol liter^{-1}) but decreased ($P \leq 0.05$) to 0.7 mmol liter^{-1} during the week postadministration. Urea levels decreased from 5.3 mmol liter^{-1} before administration to 4.6 mmol liter^{-1} during administration and 4.3 mmol liter^{-1} in the week postadministration.

DISCUSSION

The pH within the equine stomach varies between 2.6 and 5.4 (25). Digesta rapidly passes onto the rest of the GIT, but a portion remains in the stomach for 2 to 6 h (13). During fermentation of

carbohydrates, lactic acid is produced, which lowers the intestinal pH to 2.6 (13). Only 5% of *L. equigenerosi* Le1 survived pH 3.0 after 2 h, as shown by a decrease in cell numbers from 1×10^8 CFU ml^{-1} to 5×10^6 CFU ml^{-1}. Survival in MRS broth at pH 4.0 was slightly better (7%), as shown by a decrease from 1×10^8 CFU ml^{-1} to 7×10^6 CFU ml^{-1}. These results conform to the description of the species, i.e., growth at pH 4.0 but no growth at pH 3.5 (11). *L. equigenerosi* Le1 did not survive 2 h in MRS broth adjusted to pH 2.0 and 2.6, suggesting that the cells would be killed in the stomach. However, strain Le1 was originally isolated from feces, suggesting that it did survive conditions in the GIT. A complex environment such as digesta may provide some protection to the cells. The duodenum is less acidic and approximately pH 3.7, de-
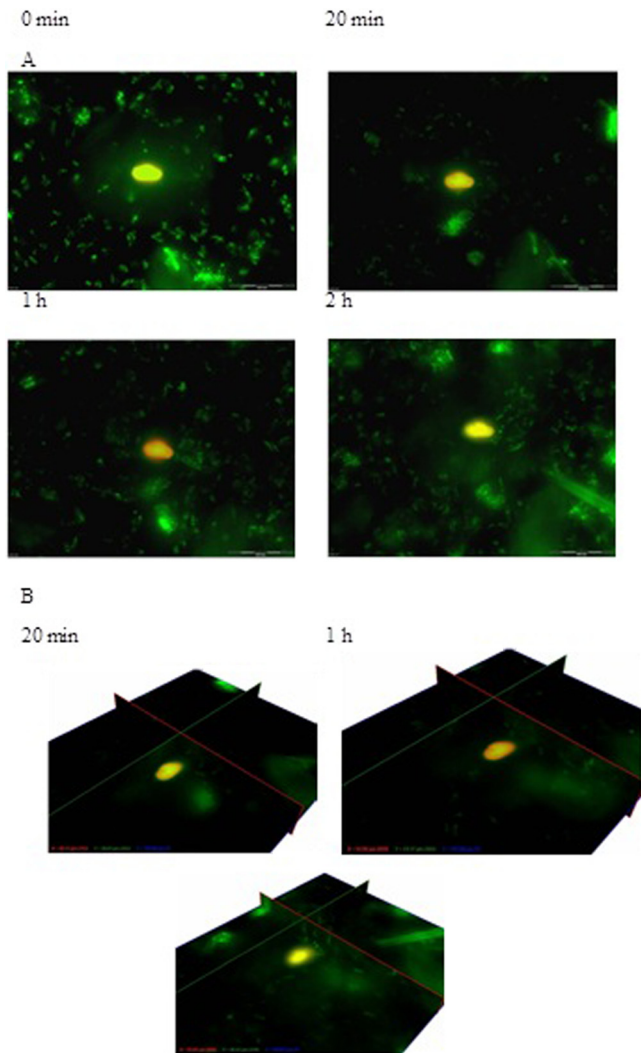
FIG 2 Adhesion of *L. equigenerosi* Le1, pretreated with pronase, to buccal epithelium cells. (A) Z-stack images were taken immediately after addition of the bacteria and 20 min, 1 h, and 2 h later. (B) Fence view images were taken after 20 min, 1 h, and 2 h. Nuclei of viable cells stained green with BacLight, and nuclei of nonviable cells stained orange or red.

FIG 3 Adhesion of *L. equigenerosi* Le1, pretreated with pepsin, to buccal epithelium cells immediately after addition of the bacteria and 20 min, 1 h, and 2 h later. Z-stack (A) and fence view (B) images taken after 20 min, 1 h, and 2 h. Nuclei of viable cells stained green with BacLight, and nuclei of nonviable cells stained orange or red.

pending on the diet (25). However, secretion of bile in the duodenum may repress bacterial growth. Strain Le1 survived bile salt concentrations of 1.5% (wt/vol) for 2 h *in vitro* and may survive passage through the small intestine. No BSH activity was recorded for strain Le1, suggesting that tolerance to bile salts has to be attributed to other factors.

The 8% hydrophobicity recorded for strain Le1 compares with the 13% published for *L. casei* Shirota, but it is far below the 54% recorded for *L. rhamnosus* GG, 50% for *L. plantarum* 423, 45% for *L. casei* MB1, and 28% for *L. johnsonii* La1 (5). Cell surface hydrophobicity is thought to play an important role in adherence between lactic acid bacteria and to epithelial cells or mucus (32). *Lactobacillus* strains with high cell surface hydrophobicity adhered in high numbers to porcine enterocytes (32). However, in other studies (5, 24, 32) no correlation was found between cell surface hydrophobicity and adhesion to mucus or epithelial cells. These discrepancies may be ascribed to differences in methods used or to the influence of unknown physiological factors.
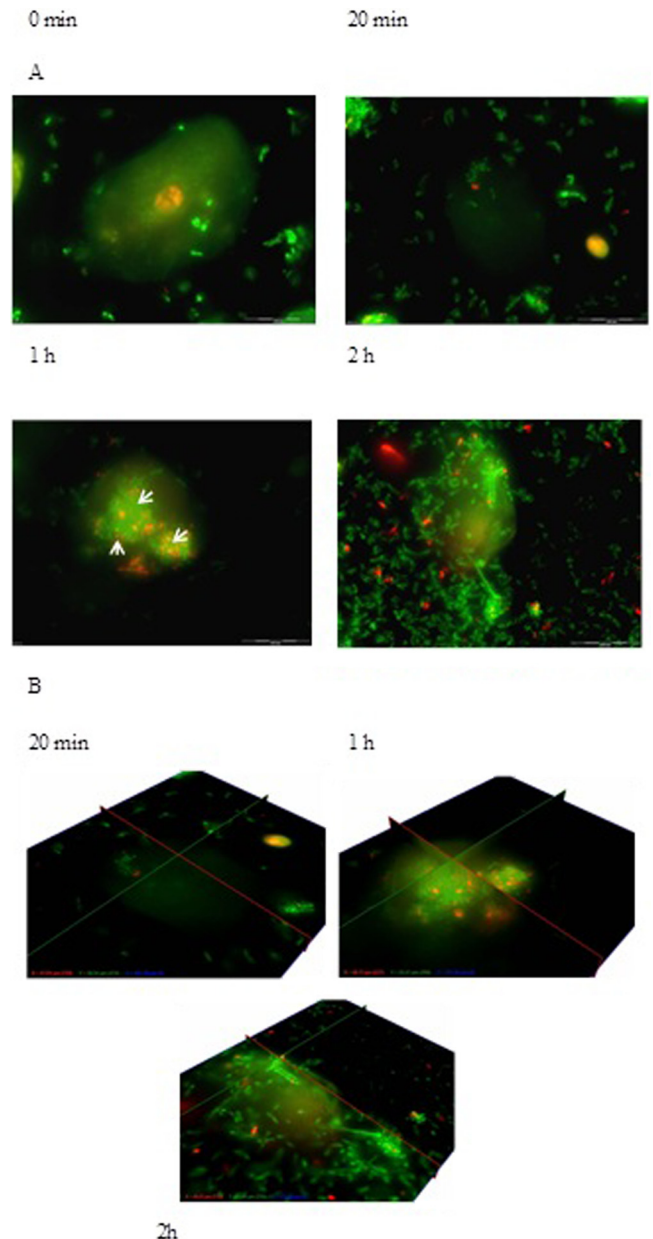
Auto-aggregation plays an important role in biofilm formation (6). The auto-aggregation value of 60% suggests that *L. equigenerosi* Le1 forms strong aggregates, which may increase adhesion to epithelial cells. The level of auto-aggregation compares well with the 48% to 64% values recorded for the probiotic strains *Enterococcus mundtii* ST4SA, *L. plantarum* 423, *L. casei* Shirota, *L. johnsonii* La1, *L. casei* MB1, and *L. rhamnosus* GG (5).

Coaggregation with a pathogen is important in that it may prevent the pathogen from binding to mucus or epithelial cells. In the case of bacteriocin-producing strains, coaggregation forces the cell closer to the target (28). Approximately half (47%) of the *L.*
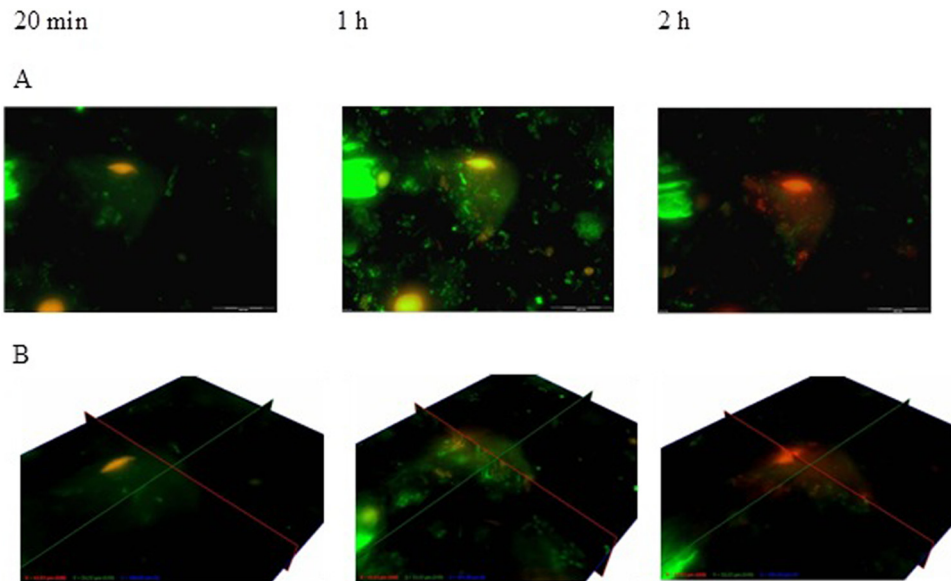
FIG 4 Adhesion of *L. equigenerosi* Le1 to viable buccal epithelial cells in the presence of *Clostridium* sp. C6 after 20 min, 1 h, and 2 h. (A) Z-view. (B) Fence view. Nuclei of viable cells stained green with BacLight, and nuclei of nonviable cells stained orange or red.

equigenerosi Le1 cells coaggregated with *C. difficile* C6. Botes and coworkers (5) reported 48% coaggregation of *L. rhamnosus* GG with *Listeria monocytogenes* ScottA. *L. johnsonii* La1, on the other hand, did not coaggregate with *L. monocytogenes* ScottA (5). Co-aggregation seems to be species and, most probably, strain specific. *L. casei* Shirota did not coaggregate with *L. monocytogenes* ScottA, whereas *L. casei* MB1 coaggregated with *L. monocytogenes* ScottA (5). Strain Le1 does not produce bacteriocins. It would

thus be interesting to determine how many of the coaggregated *C. difficile* cells remained viable and if they could recolonize the GIT. These questions need to be addressed with *in vivo* studies on healthy horses.

Adhesion of lactic acid bacteria to epithelial cells has been reported in many studies. Most of the *in vitro* studies have been done with colon adenocarcinoma cell lines Caco-2 and HT-29 (5, 15, 18, 24, 31, 35). Adhesion of *L. equigenerosi* Le1 to viable buccal epithelial cells is not surprising, although interaction between the cells, based on the low percentage of adhesion (1%), is probably very weak. As far as we could determine, this is the first report on
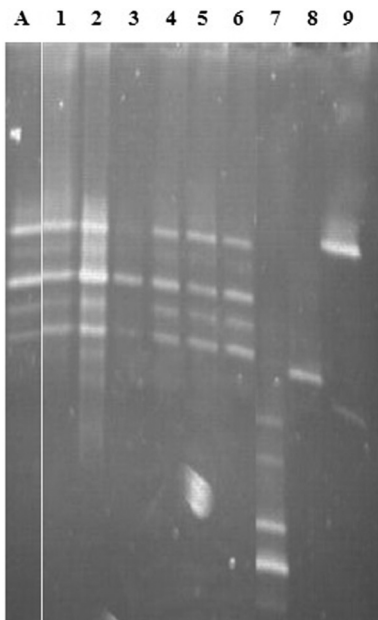

FIG 5 Composite gel, showing the DNA banding patterns of *L. equigenerosi* Le1 in feces, as recorded with DGGE. Lane A, *L. equigenerosi* Le1; lanes 1 to 3, 6 days, 4 days, and 1 day, respectively, before administration of strain Le1; lanes 4 to 7, days 2, 4, and 7 during administration of strain Le1; lanes 7 to 9, days 12, 14, and 16 after administration of strain Le1.

TABLE 2 Blood analysis profile of horses before, during, and after administration with *L. equigenerosi* Le1

Cell type[a]	Number or percentage of cells[b]		
	B	D	A
WBC	7.6×10^9 liter^{-1}	7.5×10^9 liter^{-1}	7.3×10^9 liter^{-1}
NEU	3.3×10^9 liter^{-1}	3.6×10^9 liter^{-1}	3.4×10^9 liter^{-1}
LYM	3.3×10^9 liter^{-1}	3.1×10^9 liter^{-1}	2.8×10^9 liter^{-1}
MONO	3.7×10^8 liter^{-1}	3.6×10^8 liter^{-1}	3.4×10^8 liter^{-1}
EOS	1.3×10^8 liter^{-1}	1.1×10^8 liter^{-1}	1.2×10^8 liter^{-1}
BASO	1.5×10^8 liter^{-1}	1.4×10^8 liter^{-1}	1.6×10^8 liter^{-1}
RBC	7.7×10^{12} liter^{-1}	7.7×10^{12} liter^{-1}	7.1×10^{12} liter^{-1}
HGB	13.5 g dl^{-1}	13.5 g dl^{-1}	12.4 g dl^{-1}
HCT	64.9%	64.9%	60.5%
PLT	2.0×10^{11} liter^{-1}	2.0×10^{11} liter^{-1}	1.8×10^{11} liter^{-1}
AST	291 U liter^{-1}	291 U liter^{-1}	283 U liter^{-1}
Cholesterol	2.3 mmol liter^{-1}	2.3 mmol liter^{-1}	2.2 mmol liter^{-1}
Glucose	5.5 mmol liter^{-1}	5.5 mmol liter^{-1}	4.6 mmol liter^{-1}
Lactate	0.8 mmol liter^{-1}	0.9 mmol liter^{-1}	0.7 mmol liter^{-1}
Urea	5.3 mmol liter^{-1}	4.6 mmol liter^{-1}	4.3 mmol liter^{-1}

[a] WBC, white blood cells; NEU, neutrophils; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; AST, aspartate aminotransferase.
[b] B, D, and A refer to before, during, and after administration of *L. equigenerosi* Le1. Values are averaged from three repeats.

the invasion of lactic acid bacteria into epithelial cells. The fact that *L. equigenerosi* Le1 did not adhere to or invade dead epithelial cells suggests that the physiological condition of the cells plays an important role in cellular interaction. The inability of pronase-treated cells to adhere to, or invade, viable epithelial cells suggests that a specific protein on the surface of strain Le1 plays a key role. Invasion of viable epithelial cells after treatment of the bacteria with pepsin suggests that the protein on the surface of *L. equigenerosi* Le1 that may be involved in adhesion to epithelial cells is less sensitive to pepsin. In another study (5), treatment of *L. plantarum* 423 and *E. mundtii* ST4SA with pepsin, pronase, and trypsin led to a decrease in adhesion to Caco-2 cells.

Only a few cells (1%) of *L. equigenerosi* Le1 and *C. difficile* C6 adhered to viable epithelial cells. The increase in adherence of *L. equigenerosi* Le1 to epithelial cells (from 1 to 3%) in the presence of *C. difficile* C6 may be ascribed to the high level of aggregation (47%) recorded between the two strains. Coaggregation with *C. difficile* C6 could have enforced physical contact between *L. equigenerosi* Le1 and the epithelial cells. No visual signs of the opposite, i.e., an increase in adherence of *C. difficile* C6 to epithelial cells in the presence of *L. equigenerosi* Le1, were observed; this rules out the possibility of *L. equigenerosi* Le1 stimulating the adhesion of *C. difficile* C6 to epithelial cells. Ramiah et al. (27) have reported the opposite. In their study, *L. plantarum* 423 prevented the adherence of *Clostridium sporogenes* LMG 13570 to Caco-2 cells.

L. equigenerosi Le1 is present in feces but at low numbers, as recorded from growth on modified MRS agar plates. Furthermore, *L. equigenerosi* cell numbers in the feces remained more or less constant during the trial. This suggests that many of the *L. equigenerosi* cells administered either have not survived transit or have colonized the GIT. The absence of DNA bands specific for *L. equigenerosi* (Fig. 5) in feces sampled after the 10-day administration period does not mean that no cells were present. According to Endo et al. (11), cell numbers below 1×10^3 CFU g^{-1} feces cannot be detected with DGGE.

Pathogenicity is usually recorded as an increase in WBC counts (7, 29). Constant levels recorded for WBC and neutrophil counts suggest that the equine immune system did not recognize *L. equigenerosi* Le1 as a pathogen. Furthermore, the stability in lymphocyte, monocyte, eosinophil, and basophil numbers recorded throughout the trial suggests that *L. equigenerosi* Le1 did not elicit an allergic response. The decreases in RBC count, hemoglobin, and hematocrit levels observed during the 7 days after administration of *L. equigenerosi* Le1 have also been observed in horses that were not administered *L. equigenerosi* Le1 (controls). The reason for this phenomenon is not clear. It might well be that the RBC, hemoglobin, and hematocrit levels were abnormally high at the beginning of the trial due to stress, accompanied by an elevated heart rate. The lower RBC, hemoglobin, and hematocrit levels recorded at the end of the trial (last 7 days) may thus present normal levels. The opposite findings in hemoglobin and hematocrit levels, from low to high, have been reported when piglets were administered enterocin-A, a bacteriocin produced by *Enterococcus faecium* EK13 (33). *L. equigenerosi* Le1 does not produce a bacteriocin.

High levels of aspartate aminotransferase are usually observed in horses with clinical signs of acute colic (26, 34). The constant levels recorded in our study correlate with the observation that all horses were healthy throughout the trial. *L. equigenerosi* Le1 did not initiate colic.

The reason why cholesterol levels decreased during the week after administration with *L. equigenerosi* Le1 is not known. Similar results were reported when *Enterococcus faecium* EK13 was administered to piglets (33). The results suggest that strain Le1 has anti-cholesterol properties. Although the decrease in cholesterol levels was significant ($P \leq 0.05$), the values (2.3 mmol liter^{-1} to 2.2 mmol liter^{-1}) fall within the range that has been recorded for healthy horses (25) and are not considered to be biologically significant. Cholesterol and bile salt metabolism are closely linked. Bile salts are deconjugated during enterohepatic circulation by bile salt hydrolase (BSH) (EC 3.5.1.24), and the free bile acids, including glycine and taurine, are excreted in the feces. *Lactobacillus equigenerosi* Le1 did not display extracellular BSH activity, which correlates with the finding that cholesterol levels remained constant during administration. This may also explain why no drastic decrease in cholesterol levels has been recorded after 1 week of administration with strain Le1.

Blood glucose and lactate levels vary according to the physiological state of the animal; e.g., higher glucose levels have been reported in horses at the start of training (34). Elevated glucose levels may cause colic (13). Lactate levels usually increase with exercise but are also related to diet (37). If not controlled, abnormally high levels of lactate in the blood may lead to lactic acidosis (1). The gradual decrease in blood glucose and lactate levels in animals that have been administered *L. equigenerosi* Le1 is viewed as a positive change.

High levels of urea in blood may lead to encephalopathy. Salminen and coworkers (30) have shown that *L. acidophilus* may lower blood ammonia levels. Similar findings have been reported for *Enterococcus faecium* SF68 (19). Our results have shown that *L. equigenerosi* Le1 has similar properties.

Conclusions. *Lactobacillus equigenerosi* Le1 survived low pH and bile salts *in vitro*, suggesting that the species may survive similar conditions in the GIT. However, the steep decline (an estimated 80%) in viable cell numbers during the administration period and the further decline in cell numbers to an estimated level of below 1×10^3 CFU g^{-1} feces suggest that many of the cells did not survive conditions in the lumen. The findings that *L. equigenerosi* Le1 has high auto-aggregation properties and that the cells invade epithelial cells suggest that the species is well adapted to colonize the equine GIT. Strain Le1 is not pathogenic and did not elicit an allergy response. The decreases in glucose, lactate, cholesterol, and urea levels are considered positive attributes. *Lactobacillus equigenerosi* Le1 may be used as an equine probiotic.

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