Ann Microbiol (2015) 65:1087–1096
DOI 10.1007/s13213-014-0954-4

ORIGINAL ARTICLE

Adhesion of *Lactobacillus reuteri* strain Lr1 to equine epithelial cells and competitive exclusion of *Clostridium difficile* from the gastro-intestinal tract of horses

Leon M. T. Dicks · Marlie Botha · Ben Loos · Carine Smith

Received: 3 June 2014 / Accepted: 29 July 2014 / Published online: 22 August 2014

Abstract *Lactobacillus reuteri* Lr1, isolated from healthy horses, remained viable after 2 h at pH 2.0 and in the presence of 1.5 % (w/v) bile. Strain Lr1 survived passage through the equine gastro-intestinal tract (GIT). However, no viable cells of *L. reuteri* Lr1 were detected on the third day after administration, suggesting that the strain did not colonise the GIT for longer than two days. Strain Lr1 adhered to non-viable, but not to viable, buccal epithelial cells in vitro. Adherence of strain Lr1 to buccal epithelial cells increased 25 % after treatment of the bacterial cells with pepsin. Treatment with pronase prevented the adhesion to epithelial cells. This suggested that specific proteins on the cell surface of *L. reuteri* Lr1 are involved in adhesion to epithelial cells. Strain Lr1 aggregated with *Clostridium difficile* C6, isolated from the GIT of a horse that died from severe colic. Adherence of *C. difficile* C6 to epithelial cells declined from 60 % to 3 % when challenged with *L. reuteri* Lr1 and the number of viable clostridia decreased tenfold during dosage. Red blood cell, haemoglobin and haemocrit levels were significantly ($P \leq 0.05$) lower after dosage with *L. reuteri* Lr1. Cholesterol and glucose levels were mildly elevated for one day during dosage, but decreased significantly thereafter to levels similar than before dosage. Genes encoding adhesion to collagen, production of aggregation substances, cytolysin and β hemolysin III, resistance to vancomycin A, B and C, and gelatinase activity were not detected, suggesting that *L. reuteri* Lr1 is a potential probiotic that may be used to control *C. difficile* cell numbers in the GIT.

Keywords Horses · Epithelial cells · *Lactobacillus reuteri* · *Clostridium difficile*

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Introduction

Many strains of lactic acid bacteria (LAB) are well known for their probiotic properties and are commercially available (Dicks and Botes 2010). Survival at pH below 3.0, tolerance to bile salts of 0.3 to 1.5 % (w/v), and adherence to epithelial cells and mucus are considered important probiotic properties (Dicks and Botes 2010; Dicks et al. 2014). Probiotic strains have been known to stimulate the functioning of epithelial cells, increase the production of immunoglobulins, and decrease the production of inflammatory cytokines (Kopp-Hoolihan 2001; Parvez et al. 2006). Little is known about the immunological effect LAB have on horses. Botha (2011) showed that direct contact of probiotic cells with equine epithelial cells may stimulate the immune response in horses. A few studies showed that bacteriocins (ribosomally translated antimicrobial peptides) of LAB may elicit an immune response (reviewed by Dicks and Botes 2010).

Yuyama and co-workers (2004) reported alleviation of diarrhoea when horses were fed probiotic strains belonging to the species *Lactobacillus equi*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, and *Lactobacillus salivarius*. Bernard and co-workers (2011) claimed that an equine strain of *L. reuteri* isolated from the stratified squamous epithelium of the stomach and mucosa of the colon inhibited the growth of *Salmonella* spp. Similar findings were

reported by Ward et al. (2004), but with a combination of strains ascribable to *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Enterococcus faecium*. Botha and co-workers (2012) reported the invasion of *Lactobacillus equigenerosi* Le1 into buccal epithelial cells. When the authors administered *L. equigenerosi* Le1 to horses at 1×10^9 CFU/50 kg body weight per day, white blood cell numbers and aspartate aminotransferase levels remained unchanged. However, a slight decrease in glucose, lactate, cholesterol, and urea levels was reported during the week following administration with *L. equigenerosi* Le1 (Botha et al. 2012).

Clostridium difficile survives in faeces for up to four years from the first infection, is almost always associated with equine colic, and was isolated from foals with acute diarrhoea (Båverud et al. 2003). Preliminary studies (Botha 2011) have shown that *L. reuteri* Lr1, isolated from a healthy horse, inhibited the growth of *C. difficile* C6, isolated from a horse that died from acute colic.

In this study we report on the ability of *L. reuteri* Lr1 to compete with *C. difficile* for adhesion to epithelial cells, to survive in the equine gastro-intestinal tract (GIT), and to compete efficiently with *Clostridium* spp. in the GIT. The safety of *L. reuteri* Lr1 was evaluated by screening for the presence of genes encoding virulence factors such as adhesion to collagen, production of aggregation substances, cytolysin and β hemolysin III, resistance to vancomycin, and gelatinase activity. Changes in blood cell numbers, cholesterol, glucose, lactate, urea, and aspartase aminotransferase were monitored during and after dosage with *L. reuteri* Lr1.

Materials and methods

Horses

Four healthy crossbreed horses, between three and 16-years-old, each weighing between 450 kg and 500 kg and 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 in separate quarters and were fed high quality lucern, hay, and a commercial energy-rich feed supplement twice a day. The horses were dewormed four weeks before the onset of the experiment.

Bacterial strains and growth conditions

Lactobacillus reuteri Lr1 was isolated from horses that received a balanced diet (Botha 2011). The strain was maintained by culturing in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C. *Clostridium difficile* C6 was isolated from the GIT of a horse that died from severe colic (Botha et al. 2012) and was maintained by culturing in Differential Reinforced Clostridial Medium (DRCM; Merck, Darmstadt, Germany). Incubation was at 37 °C in anaerobic flasks with gas generating envelopes (Anaerobic system BR0038B, Oxoid Ltd., Basingstoke, Hants, UK).

pH and bile salt tolerance of *L. reuteri* Lr1

Three sets of test tubes, each containing 10 ml MRS broth (Biolab), were prepared. The first set of medium was adjusted to pH 2.0, 2.5, 3.0, 4.0, and 5.0, using 1 M HCl. The second set was adjusted to the same pH values, but with concentrated DL-lactic acid. The pH of the media was determined after autoclaving and readjusted by adding either HCl or DL-lactic acid. The third set of the medium was supplemented with 0.5 %, 1.0 % and 1.5 % (w/v) Oxbile (Oxoid). Each of the test tubes was inoculated with 100 μl of an 18 h-old culture of *L. reuteri* Lr1 in MRS broth. After 2 h of incubation at 37 °C, the cultures were serially diluted in sterile saline (0.8 %, w/v, NaCl), plated onto MRS agar and incubated at 37 °C. Colonies were counted after 24 h of incubation.

Cell surface hydrophobicity of *L. reuteri* Lr1, auto-aggregation and co-aggregation with *C. difficile* C6

Hydrophobicity, auto-aggregation and co-aggregation were determined according to the methods described by Botes et al. (2008a). *L. reuteri* Lr1 was grown in MRS broth for 18 h at 37 °C, the cells 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). An equal volume of *n*-hexadecane was added to the cell suspension, mixed for 2 min and left at 26 °C for 30 min to separate into two phases. One ml of the top phase was carefully extracted and the OD reading recorded at 580 nm (reading 2). Hydrophobicity was expressed as a percentage value, calculated by using the equation of Doyle and Rosenberg (1995)

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

Auto-aggregation of *L. reuteri* Lr1 cells was determined by washing the cells in quarter-strength Ringer's solution, as described before. Cells were resuspended in sterile saline and the cell density adjusted to 0.3 (at 660 nm). One ml of

the cell suspension was transferred to a sterile 2 ml plastic cuvette and centrifuged for 2 min at 2,000*g*, 18 °C. 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. (2003):

$$\%\,\text{Auto}-\text{aggregation} = [(\text{OD}_{660}\,0 - \text{OD}_{660}60)/\text{OD}_{660}0] \times 100.$$

Co-aggregation of *L. reuteri* Lr 1 and *C. difficile* C6 was determined as follows. Strain Lr1 was cultured in MRS broth for 18 h at 37 °C and strain C6 in DRCM broth for 24 to 48 h at 37 °C under anaerobic conditions as described before. Cells of both strains were harvested (10,000*g*, 10 min, 18 °C), washed with sterile saline and equal volumes of the two cell suspensions mixed. The cell density of the supernatant was recorded (OD_{660}Tot) to represent the initial OD. Cells were then harvested (2,000*g*, 2 min, 18 °C) and the OD of the cell-free supernatant determined (OD_{660}S). The percentage co-aggregation was calculated using the equation of Malik et al. (2003):

$$\%\,\text{Co}-\text{aggregation} = [(\text{OD}_{660}\text{Tot} - \text{OD}_{660}\text{S})/\text{OD}_{660}\text{Tot}] \times 100.$$

Adhesion of *L. reuteri* Lr1 to buccal epithelial cells

Epithelial cells collected by swabs from the inside of the cheeks of healthy horses were suspended in Minimal Essential Medium (MEM) Earle's Base (Highveld Biological PTY LTD, Kelvin, Johannesburg, SA) and were used to seed the wells of an eight-chamber slide (Nalge Nunc International, Rochester, NY, USA). Each well received 200 μl epithelial cells (approximately 1×10^3 epithelial cells ml^{-1}). *Lactobacillus reuteri* Lr1 cells were prepared by suspending 200 μl of an 18 h-old culture (OD_{600nm}=1.5) into 1 ml sterile distilled water. The cells were harvested (10,000*g*, 5 min, 18 °C) and resuspended in 1 ml sterile PBS. To this suspension, 1.5 ml SYTO 9 (3.34 mM stock solution) and 1.5 ml propidium iodide (20 mM stock solution; LIVE/DEAD BacLight bacterial viability kit, L34856) were added and the cells incubated for 15 min at 25 °C. The epithelium-seeded wells were each inoculated with 150 μl (1×10^4 CFU ml^{-1}) of the labelled bacterial suspension and incubated for 2 h at 37 °C. At specific time points, bacterial cells that did not adhere to the epithelial cells were carefully withdrawn with a micropipette, serially diluted and plated onto MRS agar (Biolab). Colonies were counted after 24 h of incubation at 37 °C. The percentage cells that adhered to the epithelial cells were calculated as follows: % Adhesion = [(CFU $ml^{-1}{}_{120}$ − CFU $ml^{-1}{}_0$) / CFU $ml^{-1}{}_{120}$]×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. The experiment was repeated with non-viable buccal epithelial cells, i.e., cells collected from swabs that were suspended in MEM Earle's Base and left at 25 °C for three days.

In a separate experiment, *L. reuteri* Lr1 was grown in 10 ml MRS broth (Biolab) for 18 h, harvested (10,000*g*, 5 min, 18 °C) and washed with 0.8 ml sterile PBS. The cells were suspended in 500 μl pepsin (5.0 mg ml^{-1}, Roche Diagnostics GmbH, Mannheim, Germany), incubated at 37 °C for 1 h, harvested (14,000*g*, 10 min, 4 °C), washed in two volumes 0.8 ml sterile PBS, and resuspended in 1 ml. The experiment was repeated with pronase (5.0 mg ml^{-1}, Roche). Adhesion experiments on the pepsin and pronase treated cells were performed as described before.

Images of the bacteria were recorded using an Olympus Cell^R system, attached to an IX-81 inverted fluorescence microscope equipped with a F-view-II cooled CCD camera (Soft Imaging Systems), as described by Botha et al. (2012). Adherence of the bacteria to epithelial cells was expressed as a percentage value, calculated by using the equation % Adhesion = [(CFU ml_T^{-1}) / (CFU ml_0^{-1})] x 100 (Botha et al. 2012). CFU/ml_0 refers to the initial number of bacteria and CFU/ml_T to the number of bacteria that adhered to the epithelial cells after exposure for a specific time.

Competition between *L. reuteri* Lr1 and *C. difficile* C6 for adhesion to epithelial cells

To determine if *L. reuteri* Lr1 has the ability to compete with *C. difficile* C6 for adherence to epithelial cells, the adhesion experiment was repeated. In this case, wells seeded with buccal epithelial cells were inoculated with 100 μl (1×10^6 CFU ml^{-1}) viable cells of *L. reuteri* Lr1 plus 100 μl (1×10^6 CFU ml^{-1}) viable cells of *C. difficile* C6. After 2 h of incubation at 37 °C, bacteria that did not adhere to epithelial cells were carefully withdrawn, plated onto MRS agar and DRCM agar and incubated at 37 °C for 24 h. Adhesion to epithelial cells was calculated from the difference in the cell numbers recorded before and after exposure to epithelial cells, and it was expressed as a percentage.

Screening for virulence genes in *L. reuteri* Lr1

Strain Lr1 was cultured in MRS broth for 18 h. Genomic DNA was isolated using the ZR Fungal/Bacterial DNA kit (Zymo Research, CA, USA). Plasmid DNA was isolated using the Qiagen plasmid midi kit (Qiagen, Inc., Valencia, USA). Genomic and plasmid DNA were amplified with primers detecting genes encoding adhesion to collagen, production of aggregation substances, cytolysin and β hemolysin III, and resistance to vancomycin A, B, and C. The primers used and PCR conditions were as described by Botes et al. (2008b).

Gelatinase activity was tested by streaking active growing cells onto MRS agar, supplemented with 3 % (w/v) gelatin (BDH Laboratory Supplies, Poole, England). After 24 h of incubation at 37 °C, the plates were stored at 4 °C for 5 h and examined for opaque zones surrounding the colonies.

Administration of *L. reuteri* Lr1

Lactobacillus reuteri Lr1 was grown in MRS broth, as described before, the cells were harvested (10,000*g*, 5 min, 18 °C) and then resuspended in sterile 10 % (w/v) molasses to obtain a final cell concentration of 1×10^9 CFU per 20 ml. Each horse received 20 ml of the cell suspension, daily for seven days. The dosage of approximately 1×10^8 CFU per 50 kg body weight represents the original cell numbers of *L. reuteri* Lr1 that were isolated from a healthy 450 kg horse (Botha 2011).

Isolation of *L. reuteri* Lr1 and *Clostridium* spp from faeces

Faecal samples were collected from each horse seven days before administration with *L. reuteri* Lr1, and on each successive day for the following 17 days of the experiment. Serial dilutions of the samples were prepared in sterile saline and plated onto MRS agar, DRCM agar, and *Clostridium difficile* agar base CM0601 supplemented with the selective reagent SR0096 (Oxoid). MRS agar plates were incubated as described before. DRCM and CM0601 plates were incubated at 37 °C for six days in anaerobic flasks with gas-generating envelopes (Oxoid). All colonies that developed on DRCM plates were counted. On CM0601 plates, grey-white colonies, resembling the morphology of *C. difficile*, were counted. Colonies randomly selected from DRCM and CM0601 plates were Gram stained and tested for catalase activity by dropping 5 % (vol/vol) H_2O_2 onto the colonies. Colonies on MRS plates were overlaid with 1×10^6 CFU of 24 h-old cells of *C. difficile* C6, suspended in soft (0.8 %, w/v, agar) DRCM medium, and incubated for a further 24 h in anaerobic flasks with gas-generating envelopes. Colonies surrounded by a halo, indicating that growth of *C. difficile* C6 was repressed (Botha 2011), were regarded as *L. reuteri* Lr1 and counted. The identity of the organism was confirmed by isolating the DNA from a few randomly selected colonies and performing 16S rRNA sequencing. DNA was isolated using the ZR Fungal/Bacterial DNA kit (Zymo Research, CA, USA) and amplified with primers 8 F (CACGGATCCAGACTTTGATYMTGGCTC AG) and 1512R (GTGAAGCTTACGGYTAGCTTGTTA CGACTT), according to Felske et al. (1997). Reaction mixtures were as described by Endo and Okada (2005). PCR amplification was as described by Walter et al. (2001). Sequencing of the 16S rRNA gene was done using an automatic sequencer (ABI Genetic Analyzer 3130X1, Applied Biosystems, SA) and BigDye Terminator chemistry (Biosystems, Warrington, England). Sequences were subjected to Blast analysis on the GenBank.

Blood analysis

Blood was drawn from the jugular vein of each of the horses before dosage with *L. reuteri* Lr1, daily during the seven days of administration and thereafter for ten consecutive days. One blood sample was kept in EDTA, one sample was kept without an anticoagulant (SST tubes), and another sample stored in the presence of sodium fluoride. The SST tubes were centrifuged and the serum stored at −80 °C. Samples with EDTA were used to determine full blood counts and white blood cell (WBC) counts, using the Celldyne 3700CS Haematology Analyser with Veterinary Software Package. Sodium fluoride tubes were used for glucose and lactate analysis. Stored serum was used to determine aspartate aminotransferase (AST), glucose, cholesterol, lactate, and urea levels. The tests were conducted by Pathcare Pathologists, Stellenbosch, South Africa.

Statistical analysis

Statistical analyses of the results obtained from the blood samples were done using two-way analysis of variance (ANOVA). Variability between groups was noted when $P\leq0.05$ and the F-value was more than the F-crit value.

Results

Resistance of *L. reuteri* Lr1 to pH and bile

Cell numbers of *L. reuteri* Lr1 declined from 2×10^8 CFU ml^{-1} in MRS broth (control tube) to 8×10^6 CFU ml^{-1}, after 2 h of exposure to pH 2.0 with HCL. No viable cells were recorded after 2 h in MRS broth adjusted to pH 2.0 with DL-lactic acid. Cell numbers recorded after 2 h of exposure in MRS broth, adjusted to pH 2.5, 3.0, 4.0, and 5.0 with HCl, ranged between 1×10^6 and 1×10^7 CFU ml^{-1}. Lower cell numbers (1×10^3 to 1×10^7 CFU ml^{-1}) were recorded after 2 h of exposure in MRS broth that was adjusted with DL-lactic acid ranging from pH 2.5 to 5.0.

Cell numbers of *L. reuteri* Lr1 decreased from 2×10^8 CFU ml^{-1} in MRS broth pH6.4 (control tube) to 1×10^6 CFU ml^{-1} after 2 h in the same medium with added 0.5 %, 1.0 %, or 1.5 % (w/v) bile. The same decline in cell numbers was recorded after 2 h of exposure to 1.0 % and 1.5 % (w/v) bile.

Hydrophobicity, auto-aggregation and co-aggregation

Lactobacillus reuteri Lr1 revealed no hydrophobic properties (no hydrophobicity), but the cells did aggregate with each other (22 % auto-aggregation). A higher percentage of the cells (47 %) aggregated with *C. difficile* C6.

Adhesion to buccal epithelial cells

Lactobacillus reuteri Lr1, harvested from MRS broth (Biolab) and suspended in PBS, did not adhere to viable buccal epithelial cells (Fig. 1). However, approximately 10 % of the cells adhered to non-viable buccal epithelial cells after 20 min (image B, Fig. 2). No further increase in adherence was recorded during the following 100 min. Treatment of *L. reuteri* Lr1 with pepsin did not increase adherence to non-viable epithelial cells (not shown). However, 25 % bacteria adhered to viable cells pretreated with pepsin after 20 min of exposure (image B and E, Fig. 3). Treatment of *L. reuteri* Lr1 with pronase did not facilitate adhesion to non-viable (not shown) or viable (Fig. 4) epithelial cells.

Competition between *L. reuteri* Lr1 and *C. difficile* C6 for adherence to buccal epithelial cells

Clostridium difficile C6 adhered strongly (60 %) to buccal epithelial cells (Fig. 5). However, when combined with *L. reuteri* Lr1 in the same cell suspension, strain Lr1 competed with *C. difficile* C6 for adhesion to epithelial cells. After 20 min of exposure, only 3 % of *C. difficile* C6 cells adhered to the epithelial cells (images B and E, Fig. 5). The increase in adherence of *L. reuteri* Lr1 to the epithelial cells was difficult to determine as some bacterial cells stained less than others, but was estimated to be between 30 % and 40 % within the first 20 min (Fig. 5). Based on cell counts, the number of *L. reuteri* Lr1 and *C. difficile* C6 cells that adhered to epithelial cells did not change with further incubation.

Virulence of *L. reuteri* Lr1

Genes encoding adhesion to collagen, aggregation substance (AS), cytolysin, non-cytolysin (β hemolysin III), and vancomycin A, B, C1, C2, and C3 were not amplified. No gelatinase activity was recorded.

Isolation of *L. reuteri* Lr1 and *Clostridium* spp. from faeces

No viable cells of *L. reuteri* Lr1 were detected in faeces during the seven days before dosage started. Cell numbers of *L. reuteri* Lr1 in faeces increased from 1×10^4 CFU g^{-1} faeces after the first day of dosage to 1×10^6 CFU g^{-1} faeces after seven days of administration, but declined to 1×10^2 CFU g^{-1} at day 9 (two days after the last dosage). No viable cells of *L. reuteri* Lr1 were detected thereafter. Colonies surrounded with growth inhibition zones were identified as *L. reuteri* by matching sequences in GenBank (97 % sequence homology). Cell numbers of clostridia declined from 1×10^2 CFU to 10 CFU g^{-1} faeces during dosage with *L. reuteri* Lr1. Two days after the last dosage, clostridia cell numbers increased to 1×10^2 CFU g^{-1} faeces.

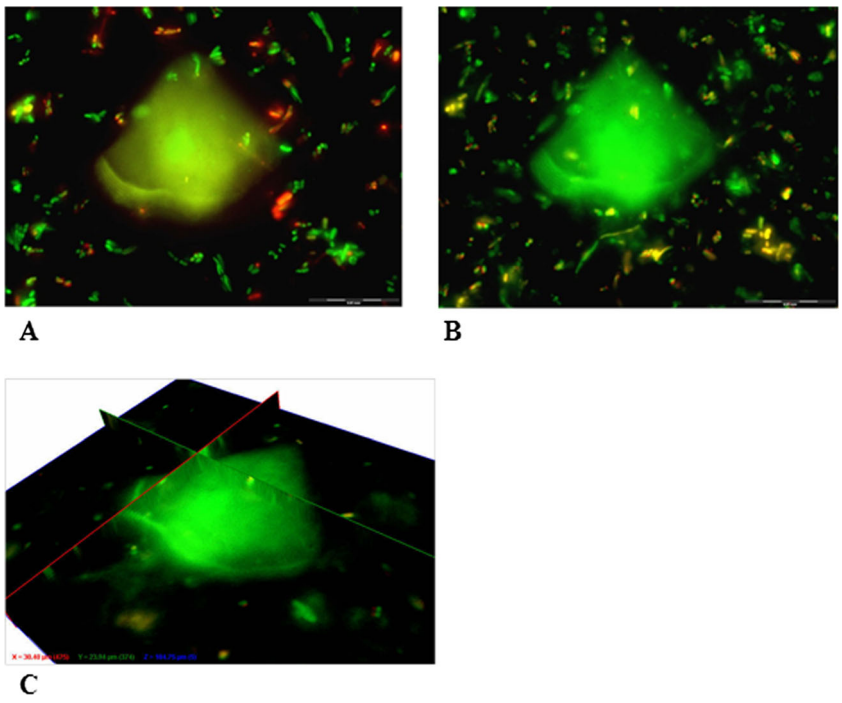

Fig. 1 Fluorescent microscopy images showing the interaction between *L. reuteri* Lr1 and viable buccal epithelial cells. **a**: Z-stack view at time zero, immediately after contact. **b**: Z-stack view recorded 20 min later. **c**: Fence view recorded after 20 min of contact

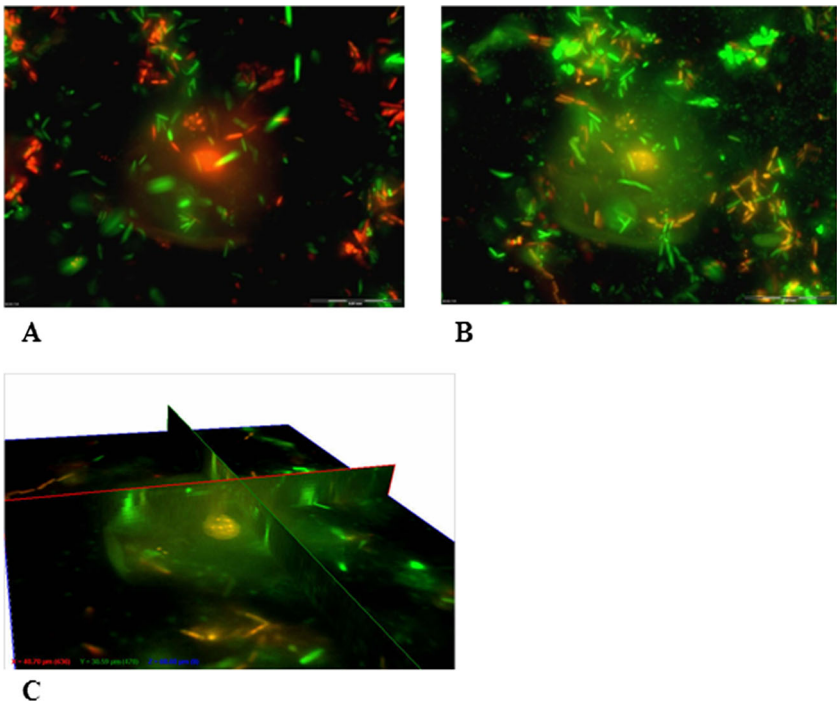

Fig. 2 Fluorescent microscopy images showing the interaction between *L. reuteri* Lr1 and non-viable buccal epithelial cells. **a**: Z-stack view at time zero, immediately after contact. **b**: Z-stack view recorded 20 min later. **c**: Fence view recorded after 20 min of contact

Blood analyses

The blood biochemical profiles (average of four horses) are listed in Table 1. No statistically significant variations ($P>0.05$) in red and white blood cell numbers, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelet counts, and aspartate aminotransferase levels were recorded before, during, and after administration with *L. reuteri* Lr1. No statistically significant changes in red blood, haemoglobin, and haemocrit numbers were recorded before and during dosage. However, the red blood cell, haemoglobin, and haemocrit levels were significantly ($P\leq0.05$) lower after dosage with *L. reuteri* Lr1 compared to before dosage. Hemoglobin levels remained constant during dosage with

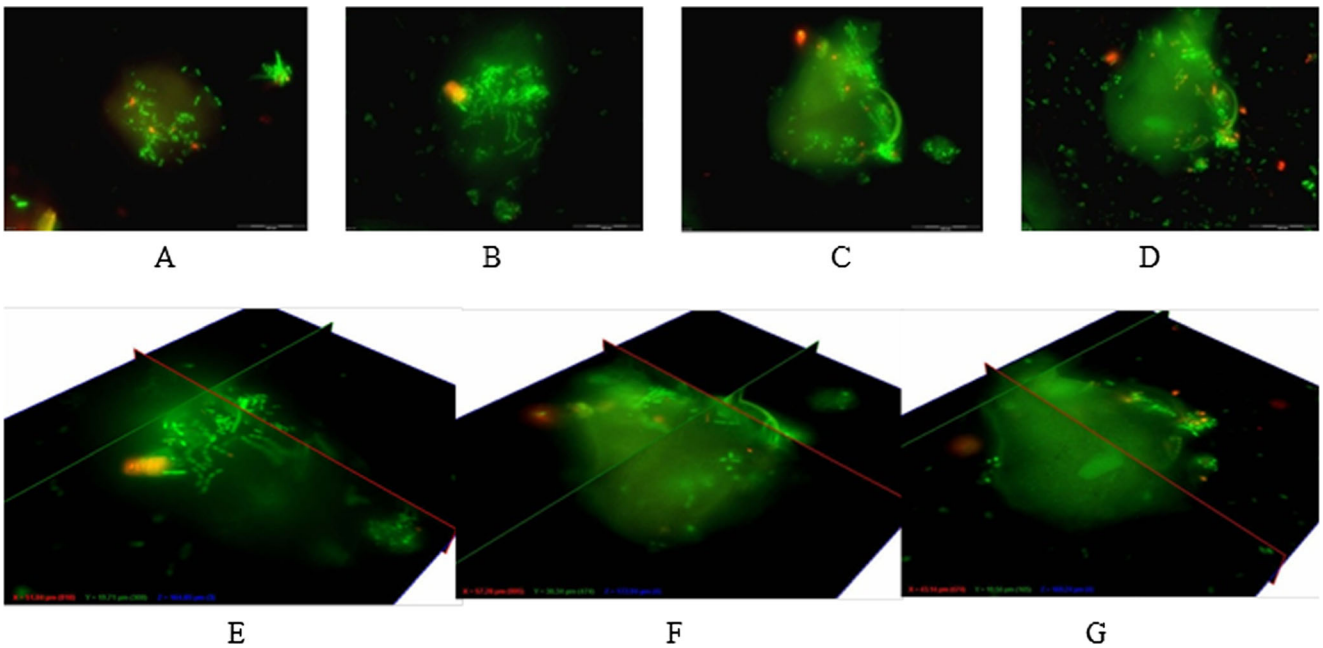

Fig. 3 Fluorescent microscopy images showing the interaction between *L. reuteri* Lr1 and viable buccal epithelial cells after strain Lr1 was treated with pepsin. **a**–**c**: Z-stack view at time zero and after 20 min, 60 min and 120 min, respectively. **e**–**g**: Fence view recorded after 20 min, 60 min and 120 min, respectively

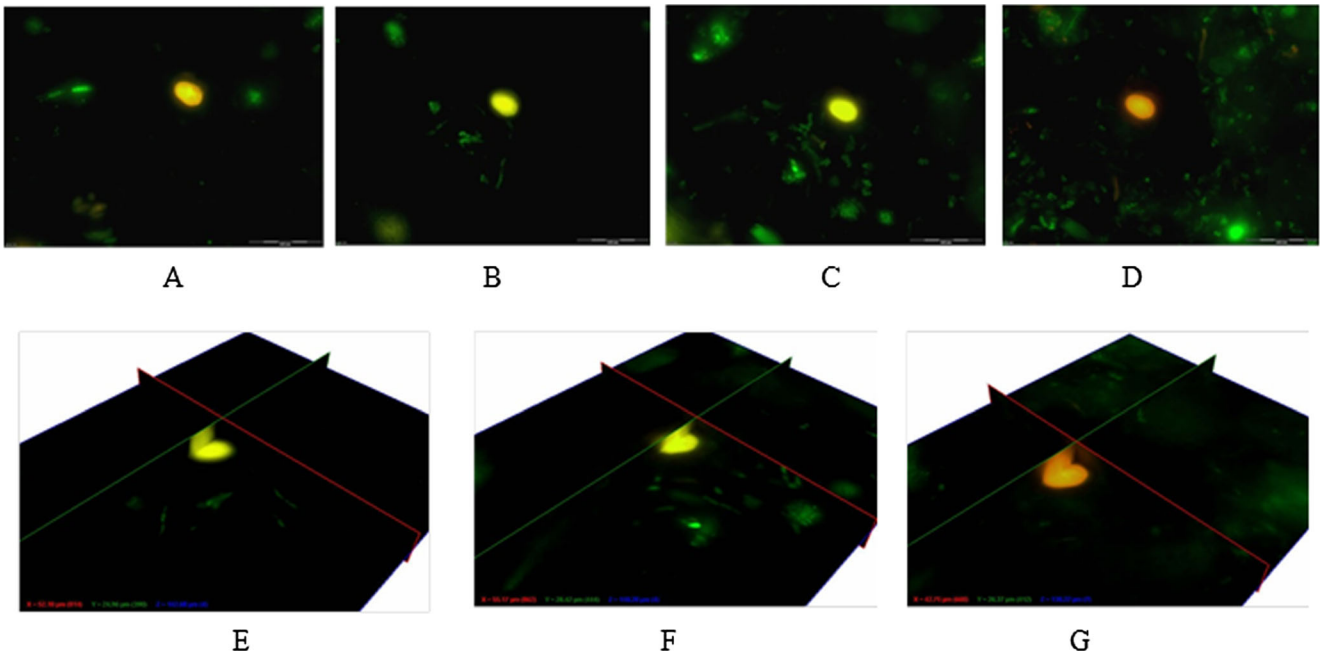

Fig. 4 Fluorescent microscopy images showing the interaction between *L. reuteri* Lr1 and viable buccal epithelial cells after strain Lr1 was treated with pronase. **a**–**d**: Z-stack view at time zero and after 20 min, 60 min and 120 min, respectively. **e**–**g**: Fence view recorded after 20 min, 60 min and 120 min, respectively

L. reuteri Lr1 but decreased significantly after dosage. Cholesterol and glucose levels were mildly elevated for one day during dosage (not shown), but decreased significantly thereafter to levels similar to that recorded before dosage. Lactate levels were slightly higher for one day during dosage (not shown), but decreased thereafter to levels similar to that recorded before dosage, or slightly lower. Urea levels decreased significantly during dosage with *L. reuteri* Lr1.

Discussion

In the anaerobic fundic section of the equine stomach, carbohydrates are fermented to lactic acid, mostly by *Lactobacillus* and *Streptococcus* spp. (de Fombelle et al. 2003), and the pH decreases to approximately 2.6 (Frape 2010). *L. reuteri* has been isolated from epithelial cells of the oesophageal section of the stomach (Yuki et al. 2000). *Lactobacillus reuteri* Lr1

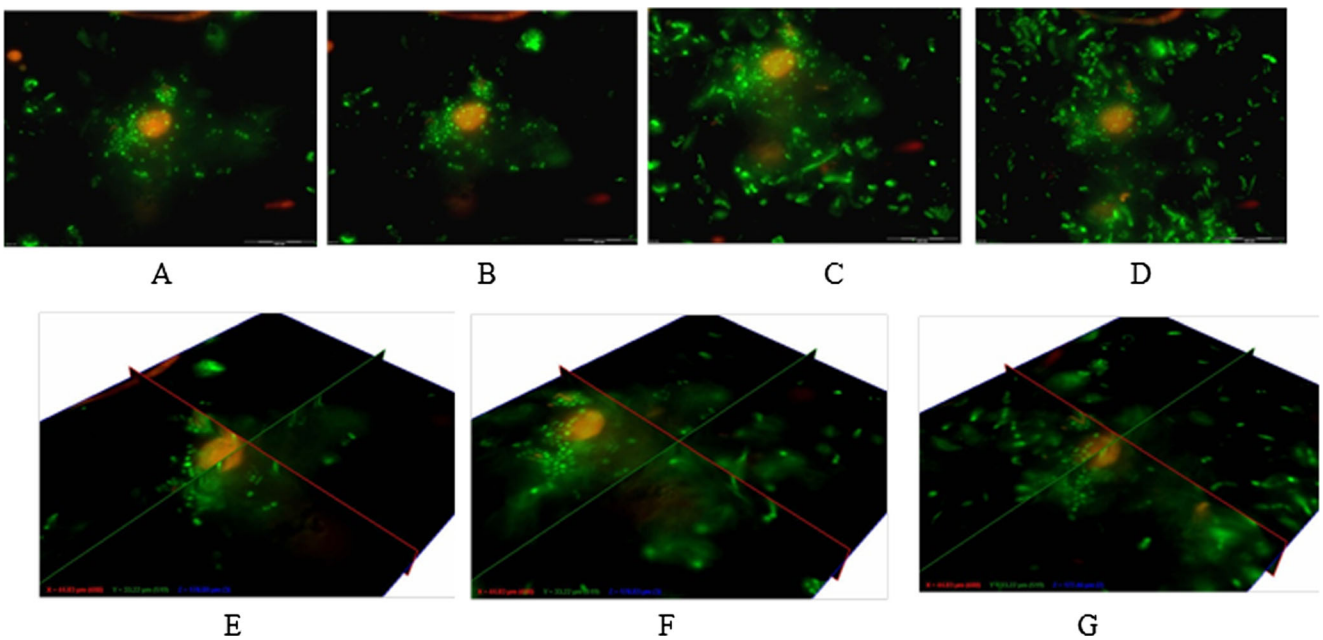

Fig. 5 Fluorescent microscopy images showing the competition between *L. reuteri* Lr1 and *C. difficile* C6 for adhesion to viable buccal epithelial cells. **a**–**d**: Z-stack view at time zero and after 20 min, 60 min and 120 min, respectively. **e**–**g**: Fence view recorded after 20 min, 60 min and 120 min, respectively

Table 1 Statistical analysis of blood biochemical profiles before, during, and after *L. reuteri* Lr1 administration

Variables with units	Mean and SD			Variance (*P* values)		
	$X_B \pm SD_B$	$X_D \pm SD_D$	$X_A \pm SD_A$	B vs. D	B vs. A	D vs. A
WBC (10^9/l)	7.65±1.80	7.50±0.89	7.26±0.90	*	*	*
NEU (10^9/l)	3.29±0.77	3.56±0.18	3.42±0.01	*	*	*
LYM (10^9/l)	3.34±0.87	3.13±0.10	2.76±0.37	*	*	*
MONO (10^9/l)	0.37±0.09	0.36±0.00	0.34±0.00	*	*	*
EOS (10^9/l)	0.13±0.07	0.11±0.04	0.11±0.07	*	*	*
BASO (10^9/l)	0.12±0.11	0.12±0.01	0.22±0.09	*	*	*
RBC (10^{12}/l)	7.71±0.65	7.74±0.39	7.15±0.33	*	**	*
HGB (g/dl)	13.5±0.6	13.5±0.71	12.4±0.74	*	**	**
HCT (%)	64.9±2.9	64.9±2.53	60.5±2.47	*	**	*
PLT (10^9/l)	204±56	200±12.90	183±35.53	*	*	*
AST (UI/l)	291±31	291±1.06	283±10.43	*	*	*
Cholesterol (mmol/l)	2.33±0.15	2.34±0.05	2.24±0.16	*	**	**
Glucose (mmol/l)	5.40±0.79	5.50±0.39	4.65±0	*	**	**
Lactate (mmol/l)	0.83±0.33	0.94±0.16	0.72±0.15	*	*	**
Urea (mmol/l)	5.25±3.28	4.65±0.02	4.28±0.32	**	*	*

SD: Standard deviation

B: before administration, D: during administration, A: after administration

WBC: White blood cells, NEU: neutrophils, LYM: lymphocytes, MONO:mMonocytes, EOS: eosinophils, BASO: basophils, RBC: red blood cells, HGB: haemoglobin, HCT: haematocrit, PLT: platelets, AST: aspartate aminotransferase

* $P>0.05$

** $P\leq0.05$

survived 2 h in the presence of HCl, suggesting that cells may enter the duodenum in a viable state.

Bile secreted in the first section of the duodenum suppresses the growth of many microorganisms (Colville and Bassert 2008). However, de Fombelle and co-workers (2003) reported high bacterial cell numbers (10^9 CFU ml^{-1} digesta) in the duodenum of horses. *Lactobacillus reuteri* Lr1 survived bile concentrations as high as 1.5 % (w/v, in vitro). It is, thus, safe to assume that a large percentage of cells in the duodenum would remain viable and enter the lower GIT. The increase in cell numbers of *L. reuteri* Lr1, from 1×10^4 CFU g^{-1} faeces on day 1 to 1×10^6 CFU g^{-1} faeces on day 7, indicates that the strain survived passing through the GIT.

One of the key properties of a probiotic is the ability to compete with pathogens for binding to epithelial cells or mucus (Schillinger et al. 2005). Adherence is facilitated by the presence of surface located aggregation substances, such as carbohydrates, cell-surface proteins, haemagglutins, lipoteichoic acids, and S-layer proteins (reviewed by Dicks and Botes 2010). The level at which cells aggregate (co-aggregation) is usually an indication of adhesion properties. Almost half of the cells (47 %) of *Lactobacillus reuteri* Lr1 aggregated with *C. difficile* C6 and competed for binding to buccal epithelial cells, but only to non-viable cells (Fig. 2). Adherence to viable epithelial cells was only recorded after the bacterial cells were treated with pepsin (Fig. 3). Pepsin cleaves peptide bonds between hydrophobic and aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Dunn 2001) and would, thus, denature specific proteins on the surface of *L. reuteri* Lr1. By doing this, other receptors on the bacterial cell surface, including proteins not denatured by pepsin, may be exposed. Adhesion to non-viable epithelial cells may be facilitated by structures different from that required for adhesion to viable epithelial cells.

Treatment of *L. reuteri* Lr1 with pronase did not facilitate adhesion to dead or viable epithelial cells (Fig. 4). Pronase is a combination of serine-type proteases, Zn^{2+} endopeptidases, Zn^{2+}-leucine aminopeptidases, and Zn^{2+} carboxypeptidase and denatures many different proteins (Sweeney and Walker 1993). This supports our hypothesis that cell surface proteins are involved in the adhesion of *L. reuteri* Lr1 to epithelial cells. The mucus binding protein (Mub) and mucus adhesion promoting protein (MapA) of *Lactobacillus reuteri* have been described in detail (Satoh et al. 2000; Roos and Jonsson 2002). Wang and coworkers (2008) identified a protein on the surface of a strain of *L. reuteri* that facilitated adhesion to porcine mucin and human enterocyte-like HT-29 cells.

The aggregation of *L. reuteri* Lr1 to *C. difficile* C6 suggested that the bacteria may compete for binding to epithelial

cells. Sixty percent of *C. difficile* C6 cells adhered to epithelial cells in vitro (Fig. 5). In the presence of *L. reuteri* Lr1, only 3 % of *C. difficile* C6 cells adhered to the epithelial cells, suggesting that *C. difficile* C6 was outcompeted by *L. reuteri* Lr1. This may be the case in vivo, but will have to be confirmed with further experiments.

The decline in cell numbers of clostridia in the faeces (from 1×10^2 CFU g^{-1} to 10 CFU g^{-1}) during administration with *L. reuteri* Lr1 indicates that the strain may be used to control *Clostridium* infections in the GIT. The decline in *Clostridium* cell numbers was, however, only observed during administration with *L. reuteri* Lr1, suggesting that the probiotic will have to be administered on a daily basis.

In humans and animals probiotics play a role in the stimulation of specific-immune and non-specific immune responses (Parvez et al. 2006). Probiotics may increase cytokine levels and natural killer cell activity, activate macrophages, change systemic T cell balances, and increase immunoglobulin levels (Kopp-Hoolihan 2001; Parvez et al. 2006). In addition, probiotic bacteria promote the proper functioning of the epithelium barrier (Kopp-Hoolihan 2001). Neutrophil and white blood cell numbers remained stable during and after dosage with *L. reuteri* Lr1, which suggests that the innate immunity of the horses were not altered and that *L. reuteri* Lr1 did not cause inflammation. Eosinophil levels were also consistent, suggesting that strain Lr1 did not elicit an allergy response. The lower red blood cell numbers, and lower hemoglobin and hemocrit levels recorded during and after dosage with *L. reuteri* Lr1 is most probably due to longer stabling hours and less exercise. Horses that are routinely exercised have higher red blood cell numbers (Kopp-Hoolihan 2001).

The general decrease recorded in blood glucose levels was expected. Blood glucose levels in exercised horses are normally higher than in resting horses (Tateo et al. 2008). However, blood glucose levels have to be carefully monitored. High glucose levels may cause colic (Frape 2010). It would be interesting to monitor changes in the glucose levels of racing horses that receive *L. reuteri* Lr1.

The decrease in cholesterol in horses that received *L. reuteri* Lr1 correlated with results obtained by Strompová et al. (2006). However, unlike our findings, the authors reported an increase in cholesterol levels for the first six days during treatment. Probiotics administered to calves decreased cholesterol levels for as long as three weeks during administration (Frizzo et al. 2010).

The decrease in lactate levels is characteristic of resting horses (Zobba et al. 2011). High blood lactate levels may lead to acidosis, a phenomenon associated with irritated or damaged mucosa (Al Jassim et al. 2005). Acidosis often leads to laminitis, which is symptomatic of toxins secreted from the GIT into the blood stream (Biddle et al. 2013) or colic (Pagan 1998), but this is usually associated with blood glucose concentrations in excess of 200 mg dl^{-1} (Thrall et al. 2004).

Aspartate aminotransferase levels remained stable and corresponded with results reported by Strompová et al. (2006). Aspartate aminotransferase levels are usually high in horses with acute colic (Patton et al. 2009) or when stabled for too long (Tateo et al. 2008).

Urea levels decreased gradually during and after dosage with *L. reuteri* Lr1. Urea is secreted into the ileum and transported to the caecum where it is hydrolysed by bacteria to ammonia (Dicks et al. 2014). Excessive levels of ammonia can lead to ammonia toxicity (Frape 2010).

None of the virulence genes screened for were detected in *L. reuteri* Lr1, suggesting the strain is a safe probiotic. Yuyama and co-workers (2004) administered *L. equi*, *L. crispatus*, *L. johnsonii*, *L. reuteri* and *L. salivarius* to horses. The authors reported better growth and increased resistance against diarrhoea.

The inability of *L. reuteri* Lr1 to adhere to viable epithelial cells and variation in adherence recorded after treatment with proteolytic enzymes suggest that the strain does not form a strong association with the GIT. This hypothesis is supported by an increase in viable cell numbers of *L. reuteri* Lr1 in faeces during administration and an absence of viable cells three days after the last dosage. Strain Lr1 did, however, prevent the adherence of *C. difficile* to epithelial cells (in vitro) and decreased the number of viable clostridia isolated from faeces tenfold when administered to horses. *L. reuteri* Lr1 may, thus, be used to control *C. difficile* cell numbers in the GIT. The demand for equine probiotics is on the increase, especially with the white paper published by the Equine Research Coordination Group in May 2013 (http://www.aaep.org/images/files/AntibioticResistancePaper(Final).doc) that warns against the abuse of antibiotics and the developing of antibiotic-resistant bacteria.

Acknowledgements The assistance of Vino Pretorius, part time technical assistant, is much appreciated.

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