Lactobacillus rhamnosus strain GG is a potential probiotic for calves

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Abstract

Diarrhea is a common occurrence in neonatal calves. Several veterinary probiotics claiming to prevent or treat calf diarrhea are available, but have not been well studied. This study assessed the capability of *Lactobacillus rhamnosus* strain GG (LGG) to maintain viability in the gastrointestinal tract of calves. We also determined whether LGG can be administered in an oral rehydration solution (ORS) without compromising the efficacy of the ORS or the viability of LGG, and whether LGG produces D-lactate or not. To investigate the intestinal survival of LGG, 15 calves were randomized into 3 groups and LGG was administered orally with their morning milk feeding on 3 consecutive days at a low (LD), medium (MD), or high (HD) dosage. Fecal samples were collected on days 0 (control), 1, 2, 3, 5, and 7 and incubated for 72 h on deMan, Rogosa, Sharpe agar. Twenty-four hours after the 1st feeding, LGG was recovered from 1 out of 5 calves in the LD group, 4 out of 5 calves in the MD group, and 5 out of 5 calves in the HD group. To determine if LGG caused the glucose levels in the ORS to drop below effective levels, 1.5 L of the ORS was incubated with LGG for 2 h at 37°C and the glucose concentration was measured every 20 min using a glucose meter. This ORS was then further incubated for 10 h and aliquots analyzed by high performance liquid chromatography to determine if D-lactate was produced by LGG. Glucose concentrations did not change over the 2 h of incubation, and no D-lactate was produced after 48 h. The LGG maintained viability in ORS. Therefore, this study demonstrated that LGG survives intestinal transit in the young calf, produces no D-lactate, and can be administered in an ORS.

Résumé

Les veaux nouveau-né présentent fréquemment de la diarrhée. Plusieurs probiotiques vétérinaires s'affichant capable de prévenir ou traiter la diarrhée chez les veaux sont disponibles mais n'ont pas été bien étudiés. La présente étude a pour but d'évaluer la capacité de la souche GG de Lactobacillus rhamnosus *(LGG) à maintenir la viabilité du tractus gastro-intesinal des veaux. Nous avons également déterminé si LGG peut être administré dans une solution orale de réhydratation (ORS) sans compromettre l'efficacité de l'ORS ou la viabilité de LGG, et si LGG produit ou non du D-lactate. Pour étudier la survie intestinale de LGG, 15 veaux ont été répartis au hasard en 3 groupes et LGG administré par voie orale trois jours consécutifs avec le repas de lait du matin à une dose faible (LD), moyenne (MD) ou élevée (HD). Des échantillons de matière fécale ont été prélevés aux jours 0 (témoin), 1, 2, 3, 5 et 7, et incubés pour 72 h sur agar deMan, Rogosa, Sharpe. Vingt-quatre heures après le premier repas, LGG a été retrouvé chez 1 des 5 veaux du groupe LD, 4 des 5 veaux du groupe MD, et chez les 5 veaux du groupe HD. Afin de déterminer si LGG causait une diminution du taux de glucose dans l'ORS sous le niveau efficace, 1,5 L d'ORS a été incubé avec LGG pendant 2 h à 37 °C et la concentration de glucose mesurée à toutes les 20 min à l'aide d'un glucomètre. Cette préparation d'ORS était par la suite incubée pendant une période de 10 h supplémentaires et des aliquots analysés par chromatographie (HPLC) afin de déterminer si du D-lactate était produit par LGG. Aucun changement dans les concentrations de glucose ne fut détecté pendant l'incubation de 2 h, et aucune production de D-lactate ne fut détectée après 48 h d'incubation. Le LGG demeura viable dans l'ORS. Ainsi, cette étude démontre que LGG peut survivre le transit intestinal chez les jeunes veaux, ne produit pas de D-lactate et peut être administré avec une ORS.*

(Traduit par Docteur Serge Messier)

Introduction

At birth, the gastrointestinal tract is sterile. Microbes introduced from the environment and from fecal and vaginal flora during parturition colonize the gastrointestinal tract and remain relatively stable throughout life, although changes presumably occur periodically, most notably at weaning (1). It is difficult to modify enteric bacterial flora permanently once they are established; however, temporary modifications can be made using antibiotics or by the introduction of probiotics (2,3). Probiotics are defined as living microorganisms, which, upon ingestion in adequate numbers, exert health benefits beyond inherent general nutrition (4). The knowledge that the normal intestinal flora has a protective function against infection provides the basis for their use (5,6). The use of probiotics in both human and animal medicine has increased as the demand for alternative, non-traditional treatments increases. Furthermore, the widespread use of antibiotics since the 1950s has raised concerns about the development of antibiotic-resistant populations, and prompted investigation into alternative therapies.

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Lactobacillus rhamnosus strain GG (LGG) (ATC 53103) is a probiotic that has been isolated from the human gastrointestinal tract, and has been extensively studied (2,7–11). This probiotic has been shown to be resistant to acid and bile, have strong adhesive properties to human and rabbit intestinal mucosal cells, suppress bacterial enzyme activity, and produce antimicrobial substances (12,13). Randomized, placebo-controlled studies have confirmed that LGG is successful in humans for the treatment of infectious diarrhea (14), antibiotic-associated diarrhea (7,8) traveller's diarrhea (15), and *Clostridium difficile* diarrhea (16). A criterion initially regarded as essential in defining a probiotic was that it be isolated from the target species. This has recently been challenged, as cross-species colonization has been demonstrated for LGG, in rabbits (13), rats (17), and dogs (18).

Acute enteric infections are the single most important cause of morbidity and mortality in neonatal calves (19). Although progress has been made in developing vaccines, improving herd management practices and treatment protocols; calf diarrhea continues to cause considerable economic loss to the livestock enterprises of many beef and dairy producers (20). Antibiotics are a common treatment for gastroenteritis in calves, but growing concern due to antibiotic residues in food products of animal origin and the emergence of antibiotic-resistant pathogens has prompted interest in veterinary probiotics (21). Veterinary probiotics do exist, but few have been adequately studied (22). To investigate the potential effectiveness of LGG in treating neonatal calf diarrhea, it must first be determined if this microbe is capable of surviving transit through the gastrointestinal tract of the calf, which differs from dogs and humans by the presence of the ruminoreticulum.

The decision to feed LGG in an oral rehydration solution (ORS), which contains 110 mmol/L of glucose, may have an effect on the efficacy of the ORS treatment since LGG ferments glucose, but not lactose. Glucose present in ORS capitalizes on the sodium-glucose transporter system on the apical membrane of the enterocyte to increase water absorption. If LGG reduces glucose concentration significantly, the ORS may be less effective. The viability of LGG in ORS was also assessed.

Lactobacillus rhamnosus has been shown to only produce L-lactate (23), but lactate isomer production in subspecies LGG has not been assessed. Formation of D-lactate may have implications in calf diarrhea, since D-lactic acidosis is often present (24) and D-lactate is poorly metabolized by mammals (25).

The primary objective of this study was to determine if LGG is capable of surviving transit through the gastrointestinal tract of the calf, and would thus be a candidate for probiotic therapy in calf diarrhea. Additional objectives were to determine if LGG can be administered in an ORS and to ensure D-lactate is not a product of LGG fermentation.

Materials and methods

Lactobacillus rhamnosus strain GG recovery in feces

To determine whether or not LGG is capable of surviving transit through the calf gastrointestinal tract, a prospective, randomized study was carried out, using 15 healthy Holstein calves (1 to 42 d old) from the Dairy Barn, University of Saskatchewan. Prior to the study, calves had received colostrum from their dams, were reared on pooled milk, had free access to calf starter and hay, but not water. None had previously suffered from enteric disease. The calves were randomized into 3 groups and orally administered LGG capsules (CAG Functional Foods, Omaha, Nebraska, USA) mixed into their normal 08:00 milk feeding, on 3 consecutive days. The LGG was mixed into the milk with a metal whisk. The 3 groups were fed dosages of either 2×10^{10} colony forming units (CFU) (low dose [LD]; 1 capsule), 1×10^{11} CFU (medium dose [MD]; 5 capsules), or 2×10^{11} CFU (high dose [HD]; 10 capsules). Calves were housed in separate stalls, thereby preventing comingling. Fecal samples were collected following perineal massage directly into 100 mL tubs at 08:30 on days 0 (control), 1, 2, 3, 5, and 7 and stored at −70°C until analysis. The researcher performing LGG colony identification was blind to treatment and the day of sample collection at the time of analysis. One gram of each fecal sample was serially diluted to in phosphate buffered saline solution and 100 μL aliquots of dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were inoculated onto deMan, Rogosa, Sharpe (MRS) agar and incubated microaerophilically for 72 h at 37°C. Colonies were identified as LGG based on colonial morphology (large, white, creamy, opaque) and Gram stain appearance (small uniform rods, in chains) (9). Twenty colonies were randomly selected from those showing the characteristic colony morphology and Gram stain and identified using a biochemical identification assay (API 50 CHL; BioMerieux, St. Laurent, Quebec). Mean growth of LGG on MRS was determined and groups compared using analysis of variance (ANOVA) of $\log_{10}$ residuals of LGG level ($P < 0.05$; Analysis ToolPak, Microsoft Excel, Mississauga, Ontario).

This study was approved by the Animal Care Committee of the University of Saskatchewan, and was carried out in accordance with the guidelines specified by the Canadian Council on Animal Care.

Administration medium

To determine if LGG decreases the glucose levels in ORS to below effective levels (a decrease in concentration of 10 mmol/L or more), 1.5 L of ORS (Revibe; Wyeth-Ayerst Canada, Guelph, Ontario) was incubated with LGG (1×10^{11} CFU) at 37°C for 2 h, representing approximate transit time to the small intestine. One millilitre aliquots were taken in duplicate every 20 min over the 2 h period, and glucose concentration was measured using a glucose meter (Accusoft Advantage; Roche, Laval, Quebec). To assess the effect of ORS on LGG, 1 capsule of LGG (2×10^{10} CFU) was dissolved in 50 mL normal saline. Two millilitre of the solution were mixed into 2 L of ORS. Ten millilitre aliquots of the solution were incubated at room temperature and at 37°C for 12 h. One hundred microliter aliquots of the solution were taken at time 0, 1 h, 2 h, and 12 h. Aliquots were serially diluted, plated on MRS agar plates, and incubated at 37°C for 72 h. Colonies were counted.

Lactate enantiomer production

The LGG (2×10^7) was incubated at 37°C for 48 h in 4 tubes each containing 10 mL of MRS broth. Aliquots of 100 μL were taken in duplicate at 0, 1, 4, 12, 24, and 48 h. High performance liquid chromatography (HPLC) was used for the stereospecific analysis of

Table I. Amount and frequency of *Lactobacillus rhamnosus* strain GG (LGG) recovered from the feces of healthy calves administered different dosages of the probiotic

	LD (n = 5) 2×10^{10}		MD (n = 5) 1×10^{11}		HD (n = 5) 2×10^{11}	
Group dose	mean CFU range	F (out of 5)	mean CFU range	F (out of 5)	mean CFU range	F (out of 5)
Day 0	0	0	0	0	0	0
Day 1	2.4×10^4	2	3.2×10^5	4	1.1×10^6	5
	0 to 1.2×10^5		0 to 9.0×10^5		3.0×10^4 to 2.0×10^6	
Day 2	3.0×10^4	2	5.8×10^5	5	2.3×10^7	4
	0 to 1.1×10^5		1.4×10^4 to 1.9×10^6		0 to 1.1×10^8	
Day 3	1.9×10^4	3	1.2×10^5	3	1.4×10^6	3
	0 to 6.0×10^4		0 to 5.3×10^5		0 to 6.0×10^6	
Day 5	1.7×10^4	3	1.0×10^4	1	2.0×10^6	3
	0 to 8.0×10^4		0 to 5.2×10^4		0 to 1.7×10^6	
Day 7	3.5×10^4	2	0	0	2.0×10^6	1
	0 to 1.7×10^4				0 to 1.0×10^5	

CFU/g — mean colony forming units of LGG per gram of feces; F — frequency of colonization (number of colonized calves/total calves); LD — low dose group (49, 14, 14, 4, and 5 days old); MD — medium dose group (48, 22, 15, 17, and 10 days old); HD — high dose group (32, 13, 9, 6, and 1 days old)

lactate enantiomers using a CHIRALPAK MA(+) column (Chiral Technologies, Exton, Pennsylvania, USA) and 2 mM copper sulphate in 1% acetonitrile as the mobile phase (26). Prior to analysis, samples were ultrafiltered through a centrifugal filter (Ultrafree-MC; Millipore, Milford, Massachusetts, USA) with a 10 000 dalton cutoff. A calibration curve was generated using D- and L-lactate concentrations ranging from 0.5 mmol/L to 10 mmol/L. Malonic acid (7 mM) was used as the internal standard. The HPLC system employed a Waters 715 Ultra WISP autosampler, a Waters 600 controller, and a Waters 486 Tunable Absorbance UV detector (Waters, Mississauga, Ontario).

Results

Lactobacillus rhamnosus strain GG recovery in feces

All calves rapidly consumed the entire dose of LGG and no adverse or unusual effects were noted by barn staff or researchers. The MRS is not selective for LGG, and numerous other gram positive rods and cocci were recovered on MRS; however, LGG was easily identified by the unique large, white, creamy, opaque morphology of its colonies combined with its gram positive bacillary staining pattern. The results of biochemical testing confirmed the identity of the species selected, as randomly selected samples of presumed LGG colonies had the carbohydrate fermentation pattern of LGG including absence of the ability to ferment lactose. No LGG was recovered from the control (day 0) samples. Twenty-four hours after the 1st feeding, LGG was recovered in 1 out of 5 calves in LD, 4 out of 5 calves in MD, and 5 out of 5 calves in HD. Three days after cessation of feeding, LGG was recovered from 3 out of 5 calves in LD, 1 out of 5 calves in MD, and 3 out of 5 calves in HD (Table I). Of those calves with LGG present in the feces, levels recovered ranged from 10^4 to 10^7 CFU/g (Figure 1). By day 7, LGG was present in the feces of only 3 of the total 15 calves. Overall, no statistically significant difference was observed in mean CFU/g recovered between the LD group and MD group or the MD group and HD group; the HD group was significantly higher ($P < 0.05$) than the LD group. No relationship existed between the age or sex of the calf and the presence of or extent of colonization.

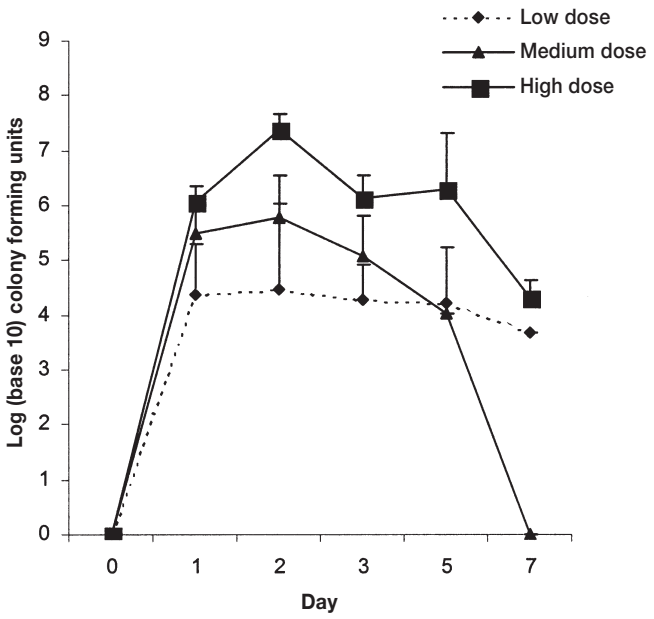

Figure 1. *Lactobacillus rhamnosus* strain GG recovery per gram of feces in low (2×10^{10} colony forming units [CFU]), medium (1×10^{11} CFU), and high (2×10^{11} CFU) dose groups. (n = 4 at each time point)

Choice of administration medium

Glucose concentration in ORS did not change over the 2 h incubation with LGG. The ORS had an initial glucose concentration of

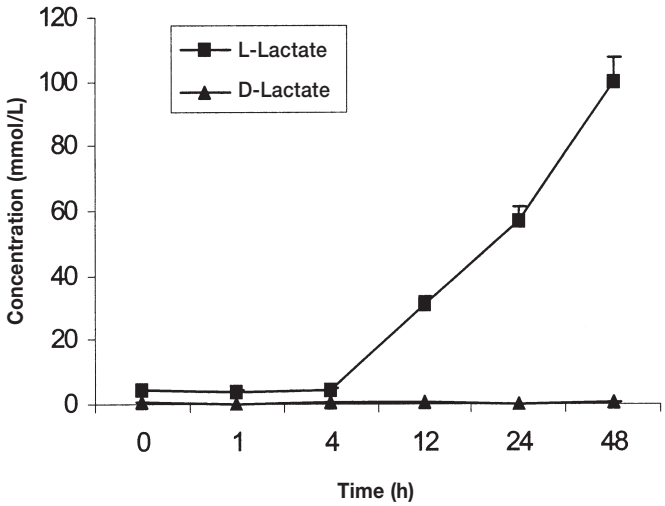

Figure 2. Lactate enantiomer production by *Lactobacillus rhamnosus* strain GG over 48 h. (n = 4 at each time point)

135 mmol/L. The mean glucose concentration over the 7 time points was 128 ± 5 mmol/L, ranging from 122 mmol/L to 135 mmol/L during LGG incubation. After 2 h of incubation with LGG, concentration decreased, very slightly, to 127 mmol/L. The number of LGG colonies did not decrease after 2 h of incubation in ORS; a slight reduction (~15%) in viability was noted after 12 h incubation. No difference was noted in viability between incubation at 37°C and room temperature.

Lactate enantiomer production

No D-lactate was present in the LGG fermented ORS at any time point. L-lactate was present at 0 h, at levels below the limit of quantification of the assay (< 0.5 mmol/L). An increase in L-lactate was observed after 12 h and continued to increase to > 100 mmol/L after 48 h (Figure 2).

Discussion

This study demonstrates that LGG survives gastrointestinal transit in calves and, therefore, may be a suitable probiotic for the treatment of gastroenteritis. *Lactobacillus rhamnosus* strain GG was present in the feces of some calves at each dosage, however, at the highest dose (2×10^{11} CFU) LGG was present in the feces of all calves in that group. For clinical purposes, this higher dose would be the propitious choice.

Colonization implies adherence and multiplication on mucosal epithelium. Fecal levels are frequently used to assess probiotic colonization, but may underestimate colonization relative to mucosal biopsy (27). Since the intention of this study was to measure colonization over time, and biopsies would likely impact colonization as a result of anesthetic-induced reductions in motility, fecal LGG output was the measurement of choice. It is also possible that the probiotic may have been growing in intestinal contents, but not adhering to mucosal cells. However, both in vitro (28) and in vivo (27) studies indicate that LGG adheres very well to human mucosal cells. Further investigation of mucosal adherence in calves is required to definitively determine if mucosal colonization occurs.

Fecal counts were higher in calves (group mean, 10^4 CFU/g) after administration of 2×10^{10} CFU than in dogs (10^2 CFU/g) administered 1×10^{10} CFU (18), but lower than humans (10^6 CFU/g) administered 1×10^{10} CFU (5). Although fecal LGG counts did not remain high several days after administration, this may not affect the therapeutic potential of LGG for calf diarrhea, since the most common pathogens affect calves in the first 5 d of life. Interestingly, a similar study in equines noted more prolonged colonization in foals (up to 11 d after cessation of administration) than in adult horses (up to 3 d after cessation of administration) (29). Further investigation is required to determine if this phenomenon occurs in bovines as well.

Some variation was likely present in the dosages administered, due to manufacturing processes. The manufacturer claims each capsule contains at least 2×10^{10} CFU, and independent analysis has revealed slightly higher amounts (22). Our analysis indicated slightly lower amounts (1.6×10^{10} CFU).

D-lactic acidosis is commonly observed in neonatal calf diarrhea (up to 20 mmol/L in serum) (24), and gastrointestinal floral imbalance with D-lactate production and absorption is the likely source. Some lactobacilli ferment substrate to D-lactate, which likely contributes to D-lactic acidosis. The lack of D-lactate production by LGG indicates further that LGG is not contraindicated for the treatment of gastroenteritis in calves. Since glucose concentrations were not reduced in ORS after 2 h of fermentation and LGG viability was not decreased by 2 h of incubation in ORS, this is a suitable medium for administering LGG. However, LGG dissolved in ORS is best administered immediately and not left to sit overnight, as a slight decrease in viability occurs.

Diarrhea is the most common ailment afflicting neonatal calves, causing a great deal of economic loss to livestock producers. Mortality rates have decreased over the past several decades, however, as antibiotic resistant pathogens become more prevalent, clinicians require alternative therapies. This study demonstrates that future in vivo studies are warranted to investigate the efficacy of LGG as a therapy for diarrhea in calves.

Acknowledgments

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