ELSEVIER

International Dairy Journal 12 (2002) 191–196

INTERNATIONAL DAIRY JOURNAL

www.elsevier.com/locate/idairyj

Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 for human intestinal applications

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Received 3 April 2001; accepted 27 August 2001

Abstract

Lactobacillus rhamnosus GR-1 and *L. fermentum* RC-14, previously characterized as urogenital probiotics were evaluated for human intestinal applications. RC-14 and GR-1 were tolerant to 0.3 and 0.5% (w/v) bile, respectively. Both strains were suspended in skim milk, stored as a frozen concentrate and administered in combination to five healthy women twice daily for 14 days. Faecal samples were analyzed and the *Lactobacillus* flora assessed by Randomly Amplified Polymorphic DNA (RAPD). Both strains were recovered from all subjects during the 14-day administration period and GR-1 was detected for at least 7 days post-administration in some individuals. No notable increases in serum IgG, IgA or IgM were observed and IL-2 and IL-4 were undetectable. Although IL-6 and IFN-γ levels increased slightly in some individuals, concentrations remained within normal ranges. Thus, *L. rhamnosus* GR-1 and *L. fermentum* RC-14 are bile tolerant and survive gastrointestinal transit without induction of systemic immune or inflammatory responses. These data together with the previously demonstrated probiotic properties of GR-1 and RC-14 make this strain combination potentially useful for human intestinal applications.

Keywords: Probiotic; Lactobacilli; Gastrointestinal tract

1. Introduction

There is considerable interest in the use of lactic acid bacteria as probiotic agents for the restoration and maintenance of a balanced urogenital and intestinal microflora. Defined as 'microbial cell preparations or components of microbial cells that have a beneficial effect on health and well being of the host' (Salminen, Ouwehand, Benno, & Lee, 1999), probiotics offer potential for immunomodulation and for the treatment and prevention of disorders and diseases, such as diarrhea, inflammatory bowel disease, urogenital infections, lactose maldigestion and cancer (Naidu, Bidlack, & Clemens, 1999). The use of probiotics in healthy populations has also been advocated to improve and maintain health and well-being. Oral administration is the most convenient route of delivery for probiotics and viable microorganisms are commonly added to foods, especially dairy products (Stanton et al., 1998). However, commercially available probiotic products are all too often unreliable (Hamilton-Miller, Shah, & Winkler, 1999) and there is a need for proper selection and characterization of strains intended for probiotic use.

For oral probiotics, survival during gastrointestinal transit (and therefore the ability to survive passage through the acidic stomach and bile) is important to convey probiotic benefits (Collins, Thornton, & O'Sullivan, 1998). Many studies have demonstrated intestinal transit of potentially probiotic microorganisms, with recovery from faecal samples usually used as an indication of survival (Ahrne, Johansson, & Molin, 1995; Fujiwara, Seto, Kimura, & Hashiba, 2001; Yuki et al., 1999; Goldin et al., 1992). However, bacterial

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0958-6946/02/$ - see front matter
PII: S0958-6946(01)00138-8

numbers can reach levels as high as 10^{10}–10^{12} CFU/g in the large intestine and specific diagnostic methodology must be used to differentiate exogenous probiotic strains from members of this indigenous microflora. Molecular techniques such as pulsed field gel electrophoresis (PFGE), ribotyping, denaturing gradient gel electrophoresis (DGGE), DNA probes and randomly amplified polymorphic DNA (RAPD) provide the means to do this (O'Sullivan, 1999, for review).

Lactobacillus rhamnosus GR-1 and *L. fermentum* RC-14 are probiotic agents with proven efficacy in the treatment and prevention of urogenital infections in women (Reid, 1999). When delivered to the urogenital tract by direct vaginal instillation these strains persist in the vagina for extended periods (Gardiner, Heinemann, Bruce, Beuerman, & Reid, 2002). Recent studies have also shown that RC-14 and GR-1 can be detected in the vaginal tract following oral ingestion, with entry to the vagina achieved through microbial colonization and ascension from the intestine (Reid et al., 2001). This provided preliminary evidence that GR-1 and RC-14 can survive passage through the human gastrointestinal tract (GIT), one of the criteria recommended for intestinal probiotics. However, this strain combination has not been investigated specifically for its potential as an intestinal probiotic preparation, neither has its effect on the immune system been evaluated.

L. fermentum RC-14 has previously been shown to bind to CaCo-2 cells (Reid, Servin, Bruce, & Busscher, 1993) and both RC-14 and *L. rhamnosus* GR-1 inhibit attachment and growth of various pathogens (Reid & Bruce, 2001). This, together with the preliminary evidence of intestinal transit survival, suggest that these combined strains may have a potentially probiotic effect in the GIT. Possible protective mechanisms could include inhibition of growth and adhesion of pathogens, as well as modulation of immunity, given the previous evidence of immunomodulation by probiotics (McCracken & Gaskins, 1999). The aim of the present study was therefore to evaluate a combination of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 as potential probiotics for human intestinal applications.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The probiotic strains used in this study, *L. fermentum* RC-14 and *L. rhamnosus* GR-1 have been well characterized and were selected as a result of extensive in vitro experimentation and human studies (Reid, 1999, for review). All lactobacilli were routinely cultured at 37°C in MRS broth (de Man, Rogosa, & Sharpe, 1960) (Merck, Darmstadt, Germany) under anaerobic conditions (anaerobic jars with BBL gas packs; Becton Dickinson & Co., Sparks, MD). To prepare probiotic frozen concentrates for use in human studies, cells of both *L. fermentum* RC-14 and *L. rhamnosus* GR-1 were harvested from overnight MRS broth cultures, washed in 0.9% (w/v) saline and resuspended in pasteurized skim milk in order to achieve a final cell concentration of $\geqslant 10^9$ viable bacteria per mL. This suspension was then aliquoted into 3 mL volumes and stored at −20°C until consumption.

2.2. Assessment of bile tolerance of lactobacilli

To investigate the tolerance of both *L. fermentum* RC-14 and *L. rhamnosus* GR-1 to bile, overnight MRS broth cultures of each of the *Lactobacillus* strains were serially diluted in 0.9% (w/v) saline and portions (100 μL) of appropriate dilutions were spread-plated onto MRS agar containing 0, 0.15, 0.3, 0.5, 0.8 or 1.0% (w/v) oxgall (Difco Laboratories, Detroit, MI). After 48 h of anaerobic incubation at 37°C the plates were examined and where colonies were present, their numbers and appearance were recorded.

2.3. Human study

Five healthy women aged between 47 and 54 (mean 50; SD 2.6) participated in the study which was approved by the Review Board for Health Sciences Research Involving Human Subjects at the University of Western Ontario, London, Ontario, Canada. Each subject signed an informed consent. Individuals were not included in the study if they were pregnant or breast-feeding, had a history of diabetes or intestinal disorders or were on immuno-suppressive or antibiotic therapy. Individuals consumed frozen concentrates containing *L. fermentum* RC-14 and *L. rhamnosus* GR-1 (prepared as described above) by defrosting the 3 mL probiotic concentrate immediately before use and mixing the contents with either milk or water or consuming the contents alone followed by either milk or water. Probiotic preparations were taken for 14 consecutive days twice daily (morning and evening) giving a total daily probiotic intake of $\geqslant 6 \times 10^9$ CFU. Faecal samples were obtained from each subject prior to (day 0), during (days 3, 7 and 14) and 7 days after (day 21) probiotic administration and blood samples were collected on day 0, 14 and 21. Each subject was asked to inform us of any adverse effects during the study.

2.4. Probiotic detection in faecal samples

Faecal samples were collected from subjects and held at 4°C until analysis (within 8 h). Weighed samples (approx. 0.3 g) were homogenized in 0.9% (w/v) saline solution as 10-fold dilutions and further diluted 10-fold in the same medium. Portions (100 μL) of appropriate

dilutions were spread-plated onto MRS agar and MRS agar containing 50 μg mL^{-1} tetracycline (Sigma Chemical Co., St. Louis, MO) or 15 μg mL^{-1} fusidic acid (Sigma) to aid in selection of RC-14 and GR-1, respectively. The plates were incubated anaerobically for 48 h at 37°C and colonies on the MRS plates were counted in order to enumerate total lactobacilli in the faecal samples. In addition, colonies on all agar plates were counted based on colony morphology and representative *Lactobacillus* colonies from each faecal sample (those suspected of being GR-1 and RC-14 as well as others) were selected and analyzed by RAPD PCR, as outlined below. RAPD fingerprints of these colonies were compared with those of the probiotic strains in order to determine whether they were GR-1, RC-14 or indigenous lactobacilli. Information obtained in this way from RAPD PCR analysis was correlated with plate count data, thereby allowing approximate quantification of the probiotic strains in the faecal samples.

2.5. Genetic fingerprinting of lactobacilli by RAPD PCR

RAPD PCR analysis was performed on each *Lactobacillus* strain and on intestinal isolates recovered from faecal samples of participating subjects. First, genomic DNA was isolated from 1.5 mL of overnight MRS broth cultures according to the method outlined by Coakley, Ross, and Donnelly (1996) which utilizes shearing with glass beads to lyse the bacterial cells. The extracted DNA was used as a template in subsequent PCR amplifications, which were performed in a total volume of 50 μL in a DNA thermal cycler (Eppendorf Scientific Inc., Westbury, NY). PCR reactions contained 1 μL of template DNA, 1 × *Taq* polymerase buffer (Gibco BRL Life Technologies, Burlington, Ontario, Canada), 4 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada), 2.5 units Platinum® *Taq* DNA polymerase (Gibco BRL) and 1 μM of each primer. Two primers of arbitrary nucleotide sequence (5′ACGAGGCAC3′ and 5′ACGCGCCCT3′) (Tilsala-Timisjarvi & Alatossava, 1998) were employed and these were synthesized by Gibco BRL. DNA was amplified for 40 cycles using the following temperature profile; denaturing at 94°C for 30 s, annealing at 36°C for 30 s and polymerization at 72°C for 2 min. The initial denaturation was performed at 94°C for 5 min and a final extension step of 72°C for 10 min was also employed. The PCR products (10 μL of each reaction) were analyzed on a 1.5% (w/v) agarose (Fisher Biotech, Fair Lawn, NJ) gel using 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) and ethidium bromide staining. A 100 bp ladder (Gibco BRL) was used as a molecular weight standard. Gels were run for approximately 2 h at 100 V and the DNA was visualized by UV transillumination.

2.6. Immunological analyses

Serum was prepared from blood samples, aliquoted and stored at −80°C until analysis. IgG, IgA and IgM concentrations were measured in the serum samples by particle-enhanced nephelometry with a Behring BNII analyser (Behring Diagnostics Ltd., Frankfurt, Germany) using a wavelength of 840 nm and a deflection angle of 90°. Rabbit antisera to human IgG, IgA and IgM (Dade Behring, Marburg, Germany) were used and relevant controls were included. Cytokines (IL-2, IL-4, IL-6 and IFN-γ) were quantified by ELISA using commercially available Quantikine® kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

3. Results and discussion

3.1. Bile tolerance of probiotic lactobacilli

In order to assess the suitability of *L. fermentum* RC-14 and *L. rhamnosus* GR-1 for intestinal use, we investigated their ability to tolerate bile in vitro. Bile tolerance is generally considered necessary for strains to survive passage through the small intestine and is usually tested in cultures intended for oral probiotic use (Collins et al., 1998). In the present study no reduction in viability was observed for GR-1 and RC-14 at oxgall concentrations of up to 0.5 and 0.3% (w/v), respectively. Both strains can therefore be considered bile tolerant given that a concentration of 0.3% is considered physiologically relevant (Dunne et al., 1999).

3.2. Probiotic survival in the human intestinal tract

L. rhamnosus GR-1 and *L. fermentum* RC-14 and B-54 (a strain almost identical to RC-14) have previously been shown to colonize the vaginal tract and prevent urinary tract infection in women, following oral and vaginal administration (Bruce & Reid, 1988; Reid, Bruce, & Taylor, 1992; Reid, 1999; Reid et al., 2001). To consider use of these probiotic strains for intestinal applications, they must be shown to survive passage through the GIT. In order to prove this, molecular tracking using RAPD was employed. RAPD is a PCR-based technique which generates reproducible DNA fingerprints for *L. rhamnosus* GR-1 and *L. fermentum* RC-14, enabling monitoring of these strains in the human vaginal tract (Gardiner et al., 2002). Other studies have used RAPD to determine probiotic survival in dairy products (Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998) and to track strains in the GIT following oral administration (Ahrne et al., 1995, Fujiwara et al., 2001). In the present study, this method successfully detected GR-1 and RC-14 in the human

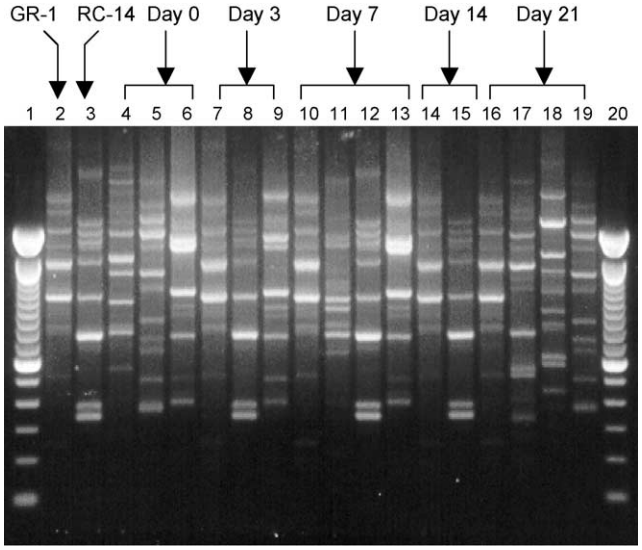

Fig. 1. RAPD fingerprints of *L. rhamnosus* GR-1, *L. fermentum* RC-14 and representative lactobacilli isolated from faecal samples of subject 228 before (day 0), during (day 3, 7 and 14) and 7 days after (day 21) daily oral administration of a *L. rhamnosus* GR-1/*L. fermentum* RC-14 combination in skim milk. Lanes 1 and 20 contain a 100 bp ladder.

intestinal tract following oral administration of this strain combination.

Faecal specimens collected at various time points before, during and after probiotic administration were analyzed and the *Lactobacillus* flora was assessed by RAPD. Fingerprints of the strains recovered from subject 228 are shown in Fig. 1 as an example. Prior to probiotic treatment (day 0), it was found that all subjects harbored lactobacilli at levels of $\sim 10^5$–10^7 CFU/g faeces but no strains were recovered which had RAPD fingerprints corresponding to GR-1 or RC-14 (Fig. 1, lanes 4–6; Table 1). Within 3 days GR-1 and RC-14 were detected in the faecal samples of all of the subjects, as evidenced by recovery of strains with RAPD fingerprints identical to those of the applied *Lactobacillus* strains (Fig. 1, lanes 7–8; Table 1). Thereafter, both strains were detectable as part of the dominant faecal *Lactobacillus* flora of all five individuals throughout the 14 day administration period (Fig. 1, lanes 10 and 12, 14 and 15). In fact, when RAPD PCR results were correlated with plate count data it was found that, depending on the particular individual RC-14 and GR-1 were present at $\sim 3 \times 10^3$–1.6×10^6 and $\sim 3 \times 10^3$–7.2×10^6 CFU/g, respectively during this period, thereby representing a substantial portion of the total faecal lactobacilli (which were present at between $\sim 5.5 \times 10^4$ and $\sim 1 \times 10^8$ CFU/g). In addition, GR-1 remained detectable at levels of $\sim 10^3$–10^4 CFU/g 7 days after probiotic administration had ceased in three subjects (Fig. 1, lane 16; Table 1), indicating that this strain is capable of persisting in the intestinal tract of some individuals for at least 1 week without further oral supplementation. Although the duration of colonization and persistence of probiotic strains may be limited, probiotic survival is generally considered important in order to confer associated health benefits. Given the differences in persistence observed between GR-1 and RC-14 in the present study and the fact that these strains express different anti-infective properties (Reid & Bruce, 2001), a good case can be made for use of multiple strains in intestinal probiotic preparations. However, studies concerning the effects of combined strains are limited, even though many probiotic products claim 'benefits' and report to contain more than one 'probiotic' strain.

Previous studies predicted that *L. rhamnosus* GR-1 and *L. fermentum* RC-14 may perform well as probiotics in the human GIT. Both strains are adherent to human epithelial cells (Reid, Cook, & Bruce, 1987; Reid et al., 1993) and are capable of surviving intestinal transit, as evidenced by vaginal colonization following oral ingestion (Reid et al., 2001). The results of the present study provide definitive molecular-based proof that GR-1 and RC-14 can survive in the human GIT following oral administration. Indeed, GR-1 compares well with other probiotic strains in terms of intestinal persistence (Dunne et al., 1999; Ahrne et al., 1995; Goldin et al., 1992). Further analyses are required to investigate the exact duration of its retention following cessation of ingestion. The finding that intestinal persistence of probiotic strains can be subject-dependent, indicates

Table 1
RAPD PCR detection of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 in faecal samples of subjects before (day 0), during (day 3, 7 and 14) and 7 days after cessation of (day 21) oral administration of a combination of these strains in skim milk for 14 days

Subject no.	Day 0	Day 3	Day 7	Day 14	Day 21
211	—[a]	RC-14 GR-1	RC-14 GR-1	RC-14 GR-1	—
215	—	RC-14 GR-1	RC-14 GR-1	RC-14 GR-1	—
227	—	RC-14 GR-1	RC-14 GR-1	RC-14 GR-1	GR-1[b]
228	—	RC-14 GR-1	RC-14 GR-1	RC-14 GR-1	GR-1
229	—	RC-14 GR-1	RC-14 GR-1	RC-14 GR-1	GR-1

[a] GR-1 or RC-14 not detected.
[b] Sample taken at day 19.

that this parameter may be influenced by host factors. Similar person-to-person variability has been reported for persistence of GR-1 and RC-14 in the vaginal tract (Gardiner et al., 2002) and for a *Bifidobacterium longum* strain in the GIT (Fujiwara et al., 2001).

While a complete analysis of the intestinal microflora was not the aim of this study, some interesting findings arose regarding indigenous intestinal lactobacilli. For example, a *Lactobacillus* strain present in the intestine prior to probiotic treatment in subject 228 (Fig. 1, lane 6), who subsequently became colonized by GR-1, was still detectable throughout probiotic treatment (Fig. 1, lanes 9 and 13). It was also possible to monitor other indigenous *Lactobacillus* strains; for example a strain detected on day 0 (Fig. 1, lane 5) was also recovered on day 21 (Fig. 1, lane 19). These results show that resident members of the *Lactobacillus* flora can also be monitored in the intestinal tract by RAPD.

3.3. Effect of probiotic administration on the immune system

Immunological parameters were assessed in order to investigate if these were influenced by oral administration of the *L. rhamnosus* GR-1/*L. fermentum* RC-14 combination. Analysis of blood samples taken from some subjects showed a slight increase in serum IgG following consumption of the probiotic strains for 14 days. However, levels remained below the upper limit of the normal range of values previously detected in healthy humans (Dati et al., 1996) (Fig. 2). Serum IgA and IgM levels remained unchanged throughout the study and were also within the range of normal values cited for healthy adults (Fig. 2) (Dati et al., 1996). In addition, all antibody titres (IgG, IgA and IgM) were comparable with those of control subjects who did not consume the probiotic product, demonstrating that probiotic therapy with GR-1 and RC-14 had no effect on systemic antibody levels in healthy individuals. Serum cytokines were also measured; IL-2, IL-6 and IFN-γ, because these are indicative of pro-inflammatory responses and IL-4, as this identifies a humoral response. Results showed that IL-2 and IL-4 were below detectable levels in all subjects at all time points and although IL-6 and IFN-γ levels did increase slightly in some individuals, levels remained within the normal range of values for healthy adults (data not shown). The fact that no substantial changes were observed for these cytokines indicated a consistent cytokine profile in the subjects ingesting the GR-1/RC-14 combination and suggests that probiotic ingestion did not modulate these immune parameters in healthy immunocompetent individuals.

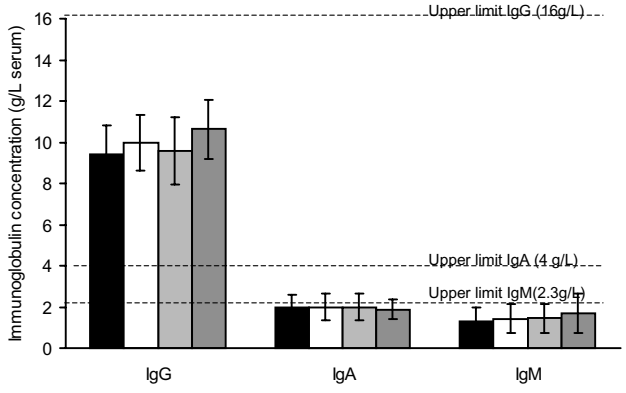

Fig. 2. IgG, IgA and IgM concentrations (g/L) in serum samples from individuals before (day 0; ■), during (day 14; □) and 7 days after (day 21; ▨) oral administration of the probiotic *L. rhamnosus* GR-1/*L. fermentum* RC-14 combination and in control subjects not consuming probiotic (▨). Results are mean (±SD) for 4 subjects. Upper limits of the normal ranges of IgG, IgA and IgM detected in healthy adults (Dati et al., 1996) are indicated by broken lines.

Probiotic bacteria have been shown to modulate host immunity with the main evidence for this emanating from studies showing an enhancement of non-specific immunity and increases in adjuvant-mediated responses to particular antigens or pathogens (McCracken & Gaskins, 1999). The latter is a different phenomenon because it refers to a situation where pathogens are present. The present study is of particular interest as it examined immune effects in healthy people. Given that probiotic products are ingested by many healthy people (e.g. 'Yakult' is reportedly consumed by over 24 million people each day), it is vital that there should be a better understanding of immune effects. The present finding that no adverse, potentially harmful systemic immunomodulatory or inflammatory effects are induced by GR-1 and RC-14 holds promise for their application as GIT therapeutics as well as dietary supplements for healthy people. This lack of inflammatory induction is in agreement with other studies (Dunne et al., 1999; Spanhaak, Havenaar, & Schaafsma, 1998). The study by Dunne et al. (1999) did, however report increased mucosal immunity as a result of ingestion of *L. salivarius* UCC 118.

In conclusion, we have demonstrated that *L. rhamnosus* GR-1 and *L. fermentum* RC-14 can be effectively delivered to the human GIT of healthy individuals by oral ingestion of a two-strain combination. Both strains survive gastrointestinal transit, as demonstrated by accurate molecular fingerprinting, and they do not induce systemic antibody or inflammatory responses. The persistence of GR-1 more so than RC-14 in the intestine following cessation of probiotic administration highlights the advantages of using combination probiotic therapy. Further characterization of the antagonistic effects of GR-1 and RC-14 against gastrointestinal pathogens, and of their in vivo effectiveness is necessary in order to confirm their clinical potential. However, the present findings provide a strong basis to explore their

therapeutic attributes and in time, these strains could potentially be a new orally administered probiotic product for human intestinal applications.

Acknowledgements

The technical assistance of Warren McDonald, Andrea Domonkos, Harry Georgeiou and Dominique Lam is greatly appreciated. We thank Joan Crosby for immunoglobulin measurements.

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