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16S ribosomal DNA analysis of the faecal lactobacilli composition of human subjects consuming a probiotic strain *Lactobacillus acidophilus* NCFM®

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Aims: The aims of this study were to evaluate the ability of exogenous *Lactobacillus acidophilus* strain NCFM® to survive through the human gastro-intestinal (GI) tract, and to evaluate the selectivity of Rogosa SL medium for faecal lactobacilli.

Methods and Results: The composition of the faecal lactobacilli of 10 healthy subjects was monitored for two weeks prior to, two weeks during and two weeks after the administration of the *Lact. acidophilus* strain NCFM® consumed with skim milk (daily dose 10^{10} viable cells). Fresh faecal samples were collected, processed and cultured on Rogosa SL selective medium for lactobacilli enumeration. Colonies demonstrating various morphologies were identified and purified for 16S ribosomal DNA sequence analysis for speciation of colonial genotype. The species composition of cultivable faecal lactobacilli changed considerably during consumption of the strain NCFM®.

Conclusions: The probiotic *Lact. acidophilus* strain NCFM® can survive through the human GI tract, but cannot colonize itself during the two-week consumption. Rogosa SL medium is selective for faecal lactobacilli. However, genetic analysis is required for colony speciation.

Significance and Impact of the Study: It is demonstrated that continuous consumption is necessary to maintain a high population of the probiotic strain, and that the Rogosa SL medium is reliable.

INTRODUCTION

The human colon harbours a large and complex microbial flora which is believed to include 10^{14} bacteria belonging to hundreds of bacterial species (Tannock 1999). The colonic microflora plays an important role in both health and disease of the human host. Probiotics, the live microbial food ingredients that benefit human health, are considered to promote beneficially the microbial balance of the host intestinal microflora (Klaenhammer 2000). The use of probiotics originated decades ago and has continued to increase, even though the scientific understanding of their function has not been fully elucidated (Holzapfel *et al.* 1998; Salminen *et al.* 1998). Recently, the health-promoting properties of *Lactobacillus rhamnosus* strain GG (Alander *et al.* 1999), strain DR20 (Tannock *et al.* 2000), *Lact. casei* strain Shirota (Spanhaak *et al.* 1998) and some other strains (Dunne *et al.* 1999) have been documented through well-designed clinical studies. However, few human studies are available for another well-characterized probiotic strain, *Lact. acidophilus* NCFM® (Klaenhammer and Kullen 1999; Reid 1999). Considering the wide applications of this strain in the USA (Klaenhammer 1998), study of the *in vivo* effects of this strain is important.

In order to study the effect of probiotics on the alteration of endogenous microflora, it is essential to monitor changes in the colonic microflora before, during and after the administration of the probiotic. Selective culturing methods are still widely used in these kinds of studies (McCartney *et al.* 1996; Spanhaak *et al.* 1998; Tannock *et al.* 2000). Reliance on selective media requires the specific media to have both superior sensitivity and superior selectivity if no further confirmation is conducted. Without the confirmation

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steps, the identity of colonies could be mistaken because of false faith in the selectivity of the media. In a recent study, the selectivity of Rogosa SL medium, a commonly-used lactobacilli selective medium, was found to be no better than MRS (de Mann, Rogosa and Sharpe) medium (Hartemink and Rombouts 1999). This conclusion raised questions regarding the reliability of Rogosa SL medium for the quantification of faecal lactobacilli.

In the present study, the hypotheses were that (i) it is necessary to confirm the identity of colonies that appear on Rogosa SL medium by determining the genus and species by molecular techniques, and (ii) consumption of *Lact. acidophilus* NCFM® will increase the faecal lactobacilli population and change the relative proportion of the various lactobacilli species. Therefore, the effect of lactobacilli consumption on the faecal lactobacilli of human subjects, and the identity of faecal cultivable lactobacilli, were examined.

MATERIALS AND METHODS

Experimental design

Ten human subjects participated in the study, which was approved by the University of Minnesota Institutional Review Board, Human Subjects Protection Committee. There were five males and five females, ages 20–47 years, including two vegetarians. The subjects maintained their lifestyle except that they excluded probiotics, prebiotics, alcohol or medications which could affect their intestinal microflora.

The study consisted of three periods: (1) an initial 2-week baseline period; (2) the 'premix' treatment period during which each subject consumes 10^{10} cells of *Lact. acidophilus* NCFM® (a generous gift from Rhodia Inc., Madison, WI, USA) premixed with 240 ml skim milk; and (3) a 2-week washout period immediately after the premix treatment. During each period, two to four faecal samples were collected and analysed.

Examination of faecal samples

Faecal samples were processed within 30 min after defecation. The entire sample was homogenized, serially diluted in PBS (phosphate-buffered saline), and appropriate dilutions spread plated in duplicate on Rogosa SL agar (Difco). The plates were incubated in an anaerobic chamber (Coy Laboratory, Grass Lakes, MI, USA) at 37 °C for 72 h and enumerated. Refer to Varcoe *et al.* (2002) for detailed experimental design and faecal analyses.

To document the various colony morphologies, digital images of the plates were taken by a Sony MVC FC-91 digital camera before the colonies were isolated. Colonies were numbered so that the identification obtained via 16S ribosomal DNA sequences would allow correspondence to the original colonies.

To analyse the dominant lactobacilli population of each subject, 10–20 colonies were picked at the ratio of various colony morphologies observed in the plates, i.e. if a particular colony morphology appeared in 10 out of 100 colonies present, 10% of the subsequent isolates would come from colonies of that specific morphology. Individual colonies were purified three times on MRS agar (Difco) and were stored at − 80 °C for further analysis.

Identification of *Lactobacillus* isolates

Bacterial DNA was extracted from overnight cultures by the method of Walter *et al.* (2000) with slight modification. Briefly, 100 mg sterile glass beads (Sigma, St Louis, MO, USA) were added to a sterile tube containing 400 μl Tris-EDTA (pH 8·0) buffered cell suspension. The tube was agitated for 2 min in a mini-bead beater (Biospec Products, Bartlesville, OK, USA). The crude DNA solution was extracted twice with 400 μl saturated phenol–chloroform–isoamyl alcohol 25:24:1 (pH 8·0) (Fisher). A 1·0 μl aliquot of the aqueous phase was used as PCR template in the following reactions.

Isolates from the treatment and washout periods were screened through *Lact. acidophilus* specific PCR using *Lact. acidophilus* specific primers. The *Lact. acidophilus* PCR negative isolates and baseline isolates (without *Lact. acidophilus* PCR screen) were analysed by partial sequencing of the 16S ribosomal DNA (position 55–536 in the *Escherichia coli* gene). All primers used in the study are listed in Table 1.

PCR was performed in 0·2 ml tubes in a PE2400 thermal cycler (PE, Foster City, CA, USA). The reaction mixture

Table 1 Primers used in the study

Primer pairs	Specificity	Sequence (5′-3′)	Expected product size (bp)
LACFOR	*Lactobacillus acidophilus*-specific	TCTTGACATCTAGRGCAATC	280
LACREV		GATTCGCTTGCCTTCGCAGG	
FOR*	Universal	GTTYGATYMTGGCTCAG	480
REV		GTATTACCGCGCCTGCTG	

*Used for 16 s ribosomal DNA sequencing.

(total volume 25 μl) consisted of reaction buffer (final concentrations, 10 mmol l^{-1} Tris-HCl (pH 9·0), 1·5 mmol l^{-1} $MgCl_2$ and 50 mmol l^{-1} KCl), 200 μmol l^{-1} concentration of each deoxynucleoside triphosphate, 1·0 μmol l^{-1} of each appropriate primer, 1·0 μl template and 1 U *Taq* DNA polymerase (Promega, Madison, WI, USA). For *Lact. acidophilus* species-specific PCR, the amplification conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 20 s, 72 °C for 20 s, and a final extension at 72 °C for 7 min. For PCR with universal primers, the annealing temperature was 55 °C. The specificity of *Lact. acidophilus* primers was confirmed experimentally (Sui, unpublished data) and by the RDP (Ribosome Database Project) probe match method (Maidak *et al.* 2000).

The PCR products were electrophoresed in 1% agarose gel and u.v. visualized after ethidium bromide staining. Before sequencing, the PCR products were excised from the agarose gel and purified using a gel extraction kit (Qiagen, Valencia, CA, USA). The sequencing process was accomplished by the University of Minnesota Advanced Genetic Analysis Center. The sequences obtained were searched against the GenBank DNA database using the BLAST function (Altschul *et al.* 1990), and against the RDP database by the sequence alignment method (Maidak *et al.* 2000) (alignment result as of April 2001).

The nucleic acid sequence accession numbers in GenBank are AF375886–AF375937.

RESULTS

Colonic lactobacilli profile change during the study

The mean baseline lactobacilli level of 10 subjects was 4·57 ± 0·87 cfu g^{-1} faeces, while the mean lactobacilli levels changed to 6·16 ± 0·72 and 5·08 ± 1·11 cfu g^{-1} faeces during the premix treatment period and the washout period, respectively.

Table 2 shows that the lactobacilli profiles changed considerably in almost all subjects. No strain of the species *Lact. acidophilus* was detected in any subject during the baseline period, while *Lact. acidophilus* isolates were frequently identified during the treatment and washout periods. During the premix treatment period, *Lact. acidophilus* became a dominant lactobacilli species present in the faecal samples (Table 2, subjects D, E, F, G and J), if not the only species isolated during this period (Table 2, subjects A and B). In the washout period, the numbers of *Lact. acidophilus* decreased and the lactobacilli population of most subjects returned to approximately that which was observed in their baseline periods.

Evaluation of selectivity of Rogosa SL medium

As shown in Table 2 and Fig. 1, some non-lactobacilli strains were isolated from the selective Rogosa SL plates. It was found that the same lactobacilli species could form various colonial morphologies (Fig. 1, *Lact. acidophilus* and *Lact. gasseri* colonies from different subjects, or from the same subject), and that different lactobacilli species could also form similar-appearing colonies (Fig. 1, *Lact. gasseri* from subject F, *Lact. oris* from J, *Lact. acidophilus* from E, *Lact. parabuchneri* from D).

DISCUSSION

Using molecular techniques, the lactobacilli composition changes for the subjects during the feeding study were observed. The data show that each individual had a unique composition of lactobacilli in the baseline period, similar to the findings of McCartney *et al.* (1996). Also, the subjects could be differentiated into groups depending on whether they harboured a relatively simple or a more complex baseline lactobacilli composition (such as subjects B and D *vs* F and J), similar to the findings of Tannock *et al.* (2000) and Kimura *et al.* (1997).

During the treatment period with a daily consumption of 10^{10} viable cells, the isolation frequency increased dramatically for *Lact. acidophilus*. It is assumed that the majority of the *Lact. acidophilus* isolates were derived from the feeding strain, since they were not present in any subjects during the baseline period. Even though the 16S ribosomal DNA sequence analysis technique used in this study provides a discrimination power to species level only, the log fold lactobacilli population changes strongly suggest that the exogenous feeding strain plays an important role in the population increase.

The isolation frequency decreased rapidly in the washout period, during which the probiotic consumption ceased. The *Lact. acidophilus* isolation frequency changing pattern indicates that the feeding strain can survive the gastrointestinal tract transit but probably did not colonize and grow during the two-week treatment period. Other researchers found similar temporary colonization of the same probiotic strain (Klaenhammer and Kullen 1999) or other probiotic strains (Fujiwara *et al.* 2001), even in prolonged (up to 6 months) feeding studies (Tannock *et al.* 2000). Thus, continuous consumption appears to be necessary to maintain a high population of the probiotic strain in the colonic microflora.

There were two vegetarian subjects participating in the study, subjects D and J. In contrast to non-vegetarian subjects, they did not have *Lact. acidophilus* as the predominant lactobacilli species during the premix treatment period. These vegetarian subjects tended to have a

Table 2 Isolates obtained during the study and their identity via 16S ribosomal DNA sequence analysis

	Isolates					
	Baseline period		Treatment period		Washout period	
Subject	Identity	Frequ.*	Identity	Frequ.*	Identity	Frequ.*
A	none†		***Lact. acidophilus*‡**	10/10	None†	
B	*Lact.* sp.	12/12	***Lact. acidophilus*‡**	8/8	*Lact. casei*	6/11
					Lact. rhamnosus	1/11
					Lact. jensenii	1/11
					Enterococcus avium	3/11
C	*Lact. gasseri*	12/26	*Lact. rhamnosus*	6/9	*Lact. rhamnosus*	4/5
	Lact. oris	10/26	***Lact. acidophilus*‡**	2/9	*Lact. vaginalis*	1/5
	Lact. rhamnosus	4/26	*Lact. vaginalis*	1/9	††	
			††			
D	*Lact. rhamnosus*	13/13	***Lact. acidophilus*‡**	3/14	*Lact. rhamnosus*	7/10
			Lact. rhamnosus	2/14	*Lact. fermentum*	1/10
			Lact. parabuchneri	2/14	*Lact. pentosus*	1/10
			Lact. vermiforme	1/14	*Staph. epidermidis*¶	1/10
			Lact. gasseri	1/14		
			Weissella cibaria	2/14		
			Staph. epidermidis¶	2/14		
			Leuc. mesenteroides	1/14		
E	*Lact. casei*	2/3	***Lact. acidophilus*‡**	15/18	***Lact. acidophilus*‡**	5/6
	Lact. gasseri	1/3	*Lact. parabuchneri*	2/18	*Kocuria kristinae*	1/6
			Lact. rhamnosus	1/18		
F	*Lact. vaginalis*	3/8	***Lact. acidophilus*‡**	11/17	***Lact. acidophilus*‡**	5/11
	Lact. gasseri	2/8	*Lact. vaginalis*	5/17	*Lact. vaginalis*	3/11
	Lact. rhamnosus	1/8	*W. confusa*§	1/17	*Lact. rhamnosus*	3/11
	Enteroccoccus avium	1/8				
	Staph. epidermidis¶	1/8				
G	*Lact. fermetum*	1/3	***Lact. acidophilus*‡**	15/19	*Lact. fermentum*	4/12
	Lact. rhamnosus	2/3	*Lact. rhamnosus*	4/19	*Lact. gasseri*	2/12
					Lact. bulgaricus	3/12
					Pediococcus acidilactici	3/12
H	*Lact. gasseri*	11/16	***Lact. acidophilus*‡**	1/1	††	
	Lact. rhamnosus	5/16	††			
I	††		***Lact. acidophilus*‡**	5/6	††	
			Lact. gasseri	1/6		
			††			
J	*Lact. gasseri*	4/17	***Lact. acidophilus*‡**	6/20	*Lact. fermentum*	7/14
	Lact. vaginalis	4/17	*Lact. vaginalis*	5/20	*Lact. rhamnosus*	3/14
	Lact. rhamnosus	2/17	*Lact. rhamnosus*	3/20	*Lact. gasseri*	2/14
	Lact. fermentum	2/17	*Lact. fermentum*	3/20	*Lact. casei*	1/14
	W. confusa§	4/17	*Lact. parabuchneri*	2/20	*Pediococcus acidilactici*	1/14
	Staph. epidermidis¶	1/17	*Lact. casei*	1/20		

*Number of specific isolates/number of total isolates.
†Lactobacilli population under detection limit.
‡Species of the feeding strain.
§Formerly *Lact. confusus*.
¶Not a normal intestinal bacteria species.
††Purification effort failed for majority of the isolates.

more complex faecal lactobacilli composition during the premix treatment period. Because of the small number of subjects, further investigation is needed to determine whether the diet played a role in the contrasting lactobacilli populations between vegetarian and non-vegetarian subjects.

Subject	Molecular Identification
A	*Lact. acidophilus* †
D	*Lact. acidophilus* †
E	*Lact. acidophilus* †
F	*Lact. acidophilus* †
G	*Lact. acidophilus* †
G	*Lact. acidophilus* †
I	*Lact. acidophilus* †
J	*Lact. acidophilus* †
J	*Lact. gasseri*
F	*Lact. gasseri*
I	*Lact. gasseri*
D	*Lact. gasseri*
D	*Lact. parabuchneri*
D	*Lact. rhamnosus*
J	*Lact. rhamnosus*
J	*Lact. oris*
J	*Lact. fermentum*
D	*Staphylococcus epidermidis*

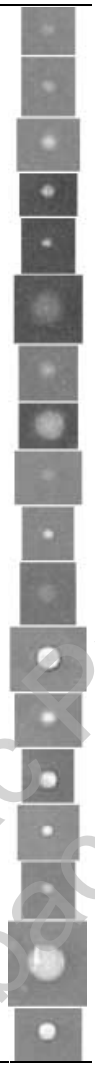

Fig. 1 Colony morphology and identification (all the images are in proportion). (†) Species of the feeding strain

A complete lactobacilli profile could not be determined for some subjects (Table 2). This occurred for subject I throughout the study, and for subjects C and H during the premix treatment and washout periods. In these periods, regular colonies appeared on faecal spread plates, giving faecal lactobacilli enumerations. However, extensive efforts to subculture the isolated colonies failed most of the time. It is worth noting that both C and H had noticeably high faecal lactobacilli populations during the baseline period (unpublished data) and a remarkably high isolation frequency of *Lact. gasseri*. *Lactobacillus gasseri* is closely related to the fed probiotic *Lact. acidophilus* and was included in the *Lact. acidophilus* complex (Kandler and Weiss 1984). The presence of a high *Lact. gasseri* population might or might not contribute to the difficulty encountered in obtaining isolates. Also, it is not known whether this occurred in subject I. Further study is needed to explain this phenomenon.

Since the human colon harbours a variety of lactobacilli and other acid-tolerant bacteria, it is not uncommon to observe colonies with different morphologies appearing on Rogosa SL plates. While selecting colonies to isolate and purify, an attempt was made to maintain the ratio of each colony morphology as they appeared on the plates, i.e. if a particular colony morphology appeared in 10 out of 100 colonies present, 10% of the subsequent isolates would come from colonies of that specific morphology. This ratio preference could introduce bias in species selection, but the isolates would still represent the faecal presumptive dominant lactobacilli species in proportion to their occurrence.

The isolation and purification procedures in this study did not exclude the possibility that other bacteria could grow on the selective Rogosa SL medium. As shown in Table 2 and Fig. 1, some non-lactobacilli strains were isolated from this medium, confirming that Rogosa SL medium is not selective enough to exclude all non-lactobacilli. As shown in Fig. 1, the uncertain relationship between colony morphology and colony identity made any attempt to differentiate colonies via colony morphology impossible on this medium. Further confirmation was obligatory when definitive colony identification was desired.

However, contrary to comments by Hartemink and Rombouts (1999), Rogosa SL medium still gave a reasonable level of selectivity and accuracy of lactobacilli counts overall. In this study, 269/284 isolates were confirmed as lactobacilli via 16S ribosomal DNA sequence analysis. Results from other researchers also confirm the selectivity of the Rogosa SL medium via molecular identification (Tannock *et al*. 2000; Walter *et al*. 2000). As one of the most commonly-chosen media for selective culturing of lactobacilli from faecal samples (Johansson *et al*. 1993; McCartney *et al*. 1996; Kimura *et al*. 1997; Ahrne *et al*. 1998; Spanhaak *et al*. 1998; Harmsen *et al*. 2000; Walter *et al*. 2001), Rogosa SL medium should be considered reliable, but its use requires caution and further confirmation.

In this study, colonic lactobacilli changes during the consumption of probiotic *Lact. acidophilus* NCFM® were determined using PCR and 16S ribosomal DNA identification, and Rogosa SL medium was evaluated for enumeration of lactobacilli from human faeces. The selectivity of Rogosa SL medium was reliable but required additional molecular confirmation of genus and species. The data showed changes in the colonic lactobacilli composition and population that were dramatic yet temporary.

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