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Isolation of lactobacilli with probiotic properties from the human stomach

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Running title: Gastric lactobacilli

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ABSTRACT

Aims: Recent evidence suggests that the human gastric microbiota is much more diverse than previously thought. The aim of the present study was to assess the potential for isolating lactobacilli from the human stomach.

Methods and results: Lactobacilli were selectively cultured from gastric biopsies from 12 patients undergoing routine endoscopy. Lactobacilli were present in 4/12 biopsies. We isolated, in total ten different strains representing five species (*Lactobacillus gasseri, L. fermentum, L. vaginalis, L. reuteri* and *L. salivarius*). The ten isolates varied greatly in their ability to inhibit the growth of two Gram-positive bacteria and two Gram-negative bacteria. Furthermore the acid and bile resistance profiles of the ten isolates spanned a wide range.

Conclusions: Five different *Lactobacillus* species were cultured from human gastric biopsies for the first time.

Significance and impact: Diverse *Lactobacillus* species are more prevalent in the human stomach than previously recognized, representing an untapped source of bacteria with beneficial probiotic and/or biotechnological properties.

Key words: Lactobacilli, stomach, probiotics, *Helicobacter pylori*, bile, acid

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INTRODUCTION

Until the culture of *Helicobacter pylori* (Marshall and Warren, 1984), the human stomach was considered to be microbiologically sterile due to factors including low pH and digestive enzymes. The stomachs of mammals other than humans are frequently colonized by bacteria other than *Helicobacter*, and lactobacilli and streptococci appear to be especially prevalent (Roach *et al.*, 1977, Fuller *et al.*, 1978, Yin and Zheng, 2005). However, there may be a greater microbial diversity in the human stomach than had previously been thought. Using 16s rRNA sequencing, Bik et al (Bik et al., 2006) detected 128 different bacterial phylotypes, including lactobacilli, in 23 human gastric biopsies. Roos et al (Roos *et al*., 2005) successfully cultured lactobacilli from gastric biopsies from healthy humans, and identified and described four new *Lactobacillus* species, *L. gastricus, L. antri, L. kalixensis* and *L. ultunensis.* To date, these species have not been further described. We hypothesized that it might be possible to isolate other lactobacilli from the human gastric mucosa and that this niche might represent a reservoir for bacteria with beneficial traits. Lactobacilli that could survive the hostile gastric environment could have applications as probiotics, or in fermentations at particularly low pH to which formic acid is added such as silage (Nadeau *et al.*, 2000) or yoghurt (Cotter and Hill, 2003).

MATERIALS AND METHODS

Culture of lactobacilli from gastric biopsies

Gastric biopsies were collected from twelve patients (seven female and five male, aged 29 to 67 with an average age of 50.5 and a median age of 54) undergoing routine upper gastrointestinal endoscopy at Cork University Hospital, Ireland. This study was approved by the Ethics Committee of Cork University Hospital and

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informed consent was obtained from all subjects. Biopsies were homogenized and spread on Rogosa agar (Oxoid, UK) for selective culture of lactobacilli. Agar plates were incubated anaerobically at 37°C for at least three days, after which visible colonies, if present, were selected and cultured anaerobically in de Man, Rogosa, Sharpe (MRS) (Oxoid) broth at 37°C. Carbohydrate fermentation profiles were assessed by API 50 CH kit (bioMerieux, Marcy l'Etoile, France).

16s rRNA sequencing and phylogenetic analyses

DNA was extracted from lactobacillus isolates using a phenol chloroform method (Flynn et al., 2002) and near-complete 16s rRNA gene fragments were PCR amplified with primers 27F and 1492R (Gurtler and Stanisich, 1996). PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Crawley, UK) and sequenced using the same forward and reverse primers as above (MWG Biotech, Ebersberg, Germany). The 16S sequences were aligned and approximately 1400 bp of each sequence was subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences of one strain from each species were deposited in Genbank (accession numbers EF460495, EF460496, EF460497, EU099039 and EU099040). Phylogenetic analysis of various *Lactobacillus* 16S sequences was performed using PhyML (Guindon and Gascuel, 2003) with the general time-reversible (GTR) model. Sequences were aligned with CLUSTALW (Thompson et al., 1994) using default parameters and gaps were removed manually.

Antimicrobial activity, acid and bile tolerance

Growth inhibition experiments were performed in a standardized protocol by spreading a lawn of the indicator bacterial culture onto an appropriate agar plate (all

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Oxoid) (MRS agar for *Lactobacillus sakei*, Brain Heart Infusion agar for *Listeria innocua*, Luria Bertani agar for *Salmonella enterica* and Colombia Base agar supplemented with 5% horse blood for *Helicobacter pylori*), then applying the *Lactobacillus* test strain as a standard inoculum of 5 µl of a 0.2 OD600 culture on top of a paper disk placed on the agar plate. Agar plates were incubated for 48-96 h after which time zones of clearance were measured. The ability of the strains to survive or grow at different pH's was determined by adjusting the OD600 of an overnight MRS culture to 0.2 in either MRS or MRS adjusted to pH 2 or pH 3 with hydrochloric acid (approx. 5 fold). Samples were removed from the culture after 4, 8 and 24 h and cell viability was determined using a spread plate method. *L. vaginalis* SR8 grew very poorly and did not survive sufficiently for this analysis to be carried out. The bile resistance levels of the strains were determined by inoculating 5 µl of an overnight culture of each strain (OD600 of approx. 1.0) onto MRS plates supplemented with either porcine or bovine bile (Sigma, St. Louis, MO) at concentrations varying from 0-10% and observing the presence or absence of growth after 72 h.

RESULTS

Lactobacilli were successfully cultured from 4/12 gastric biopsies. There was no correlation between biopsies positive for lactobacilli and i) the sex of the patient; ii) the age of the patient; iii) the *H. pylori* status of the patient; iv) the disease status of the patient. Three different species (*L. fermentum, L. gasseri* and *L. vaginalis*) were isolated from one biopsy; two species (*L. fermentum* and *L. reuteri*) and (*L. salivarius* and *L. gasseri*) were isolated from two other biopsies and only one species (*L. gasseri*) was isolated from the remaining positive biopsy. Sequence identity to published sequences was at least 99% in all cases, and sequences from all strains of

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the same species were identical. We identified that two different strains of *L. fermentum* and two different strains of *L. reuteri* were present in one biopsy by comparing the carbohydrate fermenting capability of the four isolates using API analysis. A phylogenetic analysis of the 16S sequences from the ten species showed that, with the exception of *L. salivarius,* all isolates are members of group A or B of the *Lactobacillus* 16S phylogeny (Canchaya et al., 2006) (Fig. 1). Interestingly, the recently described gastric lactobacilli *L. antri, L. gastricus, L. ultunensis* and *L. intestinalis* (Roos et al., 2005) are all contained within these same two groups. This may indicate a phylogenetic relationship between lactobacilli capable of persisting in the human gastric environment.

The ten isolated lactobacilli were screened for their ability to inhibit growth of two Gram-positive (*L. sakei* and *Listeria innocua*) and two Gram-negative (*S. enterica* and *H. pylori*) bacteria (Table 1). The three *L. fermentum* strains inhibited growth of both Gram-positive indicator organisms, and strain SR2 also inhibited growth of *H. pylori*. *L. salivarius* SR16 was the only other strain capable of inhibiting growth of *H. pylori*. *L. fermentum* and *L. salivarius* both produce bacteriocins (Yan and Lee, 1997, Claesson *et al.*, 2006), and this is one potential source of the inhibitory effect. Both strains of *L. reuteri* inhibited growth of only *L. sakei*, possibly due to the production of reuterin (Talarico and Dobrogosz, 1989).

Lactobacilli cultured from the stomach might arguably be transient (allochthonous) rather than long-term colonizers (autochthonous). To investigate this we examined the acid resistance of the ten lactobacilli from the human stomach (Table 2). *L. fermentum* SR2 exhibited 100% survival, and *L. gasseri* SR1 cell numbers increased three-fold in MRS pH 3 after 24 h. This compared favourably with the acid-tolerant control species *L. acidophilus* ATCC4356 (Lorca et al., 1998).

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In contrast, no cells of *L. salivarius* UCC118 were viable after this time, even though *L. salivarius* UCC118 has been previously shown to have probiotic qualities in a number of *in vivo* studies (McCarthy *et al.*, 2003, Sheil *et al.*, 2004). *L. fermentum* SR2 and *L. acidophilus* ATCC4356 showed < one log decrease in viability after 24 h in MRS pH 2. None of the other strains exhibited significant acid tolerance. The ten strains also varied greatly in their ability to grow on porcine and bovine bile (Table 3). The type strain *L. acidophilus* NCTC4356 was the most tolerant to both bile types. Of the gastric strains, *L. reuteri* SR11 was the most resistant; it grew on MRS plates containing 10% bovine and 0.5% porcine bile.

DISCUSSION

We have shown that lactobacilli can be cultured from human gastric tissue. Although these organisms are abundant in the upper and lower gastrointestinal tract, it is generally thought that they do not persist for any significant length of time in the stomach (O'Hara and Shanahan, 2006). The main source of lactobacilli is food, and since a patient must fast for at least 12 h before a gastric endoscopy is performed, the bacteria we isolated may have survived in the stomach for at least this length of time. During fasting, the gastric pH can drop as low as 1.5 (Drasar et al., 1969) indicating that these strains may have an intrinsic *in vivo* resistance to low pH. Although we cannot refute the possibility that the strains may have been introduced into the stomach at a later time-point via saliva, our *in vitro* experiments show that two of the strains are capable of surviving at least 24 h at low pH. Resistance to bile is considered a valuable probiotic trait and although the gastric lactobacilli were not particularly bile-tolerant, this is not perhaps surprising in this case because bile first enters the gastrointestinal tract in the duodenum, and is only present in the stomach if

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duodeno-gastric reflux occurs. Porcine bile contains a higher level of glycine conjugated bile salts than bovine bile (Coleman *et al.*, 1979). These salts are more toxic to lactobacilli (De Smet *et al.*, 1995). It is not surprising therefore that all strains survived less well in the presence of porcine bile.

The relative abundance of lactobacilli in the human stomach has several implications. Lactobacilli have been shown to have beneficial effects in the alleviation of many human conditions (O'Mahony *et al.*, 2005, Zocco *et al.*, 2006). If these organisms are capable of surviving for a significant length of time in the stomach, probiotic treatments might also be useful in the treatment of gastric disorders. Indeed a number of trials have already shown that this might be the case (Johnson-Henry *et al.*, 2004, Sykora *et al.*, 2005). Furthermore, in a conventional probiotic setting, lactobacilli capable of surviving in the stomach for extended periods of time will be more aciduric, ensuring not only that more cells survive gastric transit to reach the intestine, but also allowing for greater survival and shelf-life in fermented dairy products. The presence of significant numbers of bacteria other than *H. pylori* in the human stomach may well have implications for the human health, and the culture-independent analysis of the gastric metagenome of 23 subjects supports this notion (Bik *et al.*, 2006). Metagenomic analysis of a much larger cohort of subjects with a range of disease pathologies is required to address this question.

It is noteworthy that lactobacilli have been demonstrated many times in the stomachs of other mammals including the pig, which is generally regarded as having the closest gastric physiology to that of humans. It is thought that lactobacilli survive in the pig stomach by adhering strongly to epithelial cells, to the extent that they can form highly-resistant biofilm-like structures (Tannock, 1992). If, as now seems likely, lactobacilli are more prevalent in the human stomach, it is also possible that

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this site may be home to other novel, previously unidentified lactobacilli (Roos *et al*., 2005) which may also form biofilms.

In conclusion, this work describes what is, to our knowledge, the first isolation of *L. fermentum*, *L. gasseri*, *L. vaginalis, L. reuteri* and *L. salivarius* from the human stomach, and suggests this site may be a novel source for new organisms with probiotic and other beneficial properties.

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Figure 1. 16S rRNA gene phylogeny of selected lactobacilli. The sequences from the five species described in the present work are arrowed. Novel lactobacilli previously isolated from the human gastric mucosa (Roos *et al*., 2005) are boxed.

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Table 1. Antimicrobial properties of ten lactobacilli from the human gastric mucosa. The ability of the strains to inhibit growth of two Gram-positive and two Gram-negative bacteria was tested three times in duplicate using different cultures in a standard plate overlay inhibition assay. + indicates degree of inhibition of the indicator strain by the lactobacilli, - indicates no inhibition.

	L. sakei	*Listeria innocua*	*S. enterica*	*H. pylori*
L. fermentum SR2	+++	+	-	++
L. fermentum SR9	++	+	-	-
L. fermentum SR10	++	+	-	-
L. gasseri SR1	-	-	-	-
L. gasseri SR15	-	-	-	-
L. gasseri SR 17	-	-	-	-
L. reuteri SR11	++	-	-	-
L. reuteri SR14	++	-	-	-
L. salivarius SR16	+++	-	-	+
L. vaginalis SR8	-	+	-	-

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Table 2. Acid resistance of two control strains and ten gastric lactobacilli isolated from the human gastric mucosa. Values tabulated are the cell numbers at indicated time points expressed as a percentage of the cell numbers at time zero. Experiments were repeated twice in duplicate and values averaged. ND = not determined.

	Growth at pH 3 (%)			Growth at pH 2 (%)		
	4 h	**8 h**	**24 h**	**4 h**	**8 h**	**24 h**
L. acidophilus NCTC4356	202	164	149	108	55	17
L. fermentum SR2	142	162	98	101	81	14
L. fermentum SR9	0	0	0	0	0	0
L .fermentum SR10	0	0	0	0	0	0
L. gasseri SR1	667	505	349	0.4	0.3	< 0.001
L. gasseri SR15	0	0	0	0	0	0
L. gasseri SR 17	0	0	0	0	0	0
L. reuteri SR11	< 1	0	0	0	0	0
L. reuteri SR14	< 1	0	0	0	0	0
L. salivarius SR16	< 0.1	0	0	0	0	0
L. salivarius UCC118	14	7	0	0	0	0
L. vaginalis SR8	ND	ND	ND	ND	ND	ND

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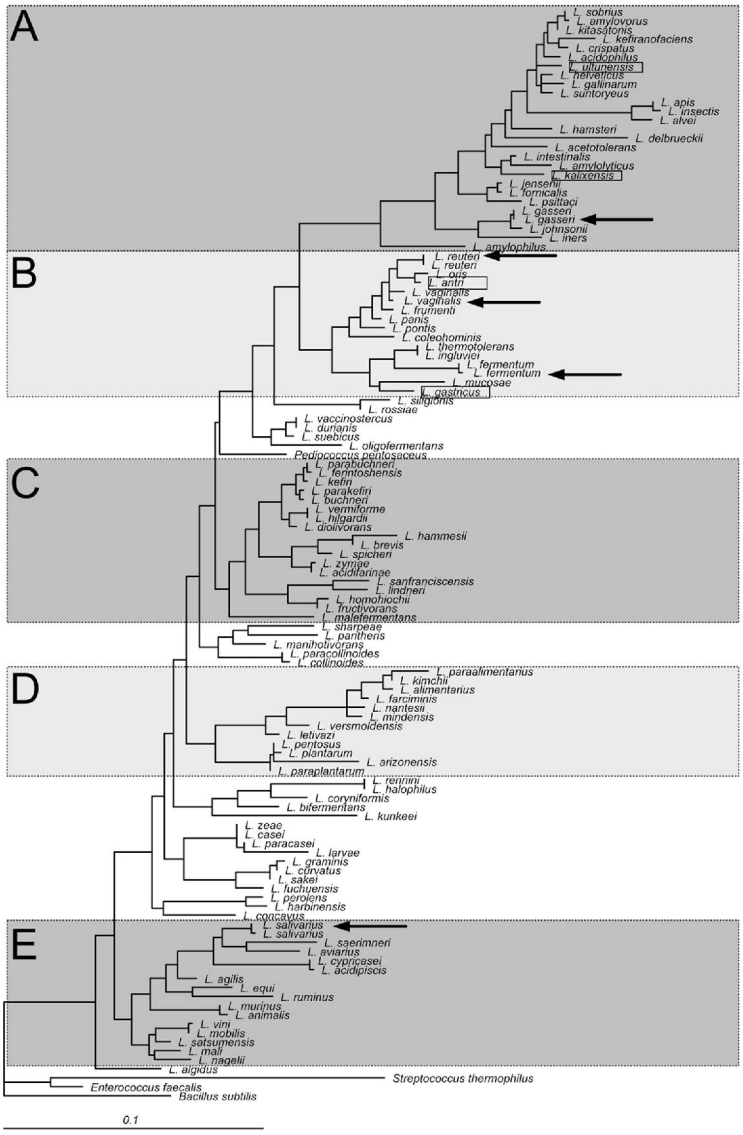
Table 3. Bile tolerance of two control strains and ten lactobacilli isolated from the human gastric mucosa. Three independent cultures of each strain were grown anaerobically for 72 h on MRS plates supplemented with either bovine or porcine bile. Values tabulated are the highest bile concentration at which growth was observed.

	Bovine bile	**Porcine bile**
L. acidophilus NCTC4356	10 %	7.5 %
L. fermentum SR2	0.5 %	0.25 %
L. fermentum SR9	1 %	0.1 %
L .fermentum SR10	7.5 %	0.25 %
L. gasseri SR1	0.5 %	0.25 %
L. gasseri SR15	5 %	0.25 %
L. gasseri SR 17	1 %	0.1 %
L. reuteri SR11	10 %	0.5 %
L. reuteri SR14	10 %	0.3 %
L. salivarius SR16	10 %	0.25 %
L. salivarius UCC118	1 %	0.25 %
L. vaginalis SR8	0 %	0 %

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