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**Isolation of lactobacilli with probiotic properties**  
**from the human stomach**

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Running title: Gastric lactobacilli

**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.

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

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47     **INTRODUCTION**

48             Until the culture of *Helicobacter pylori* (Marshall and Warren, 1984), the  
49     human stomach was considered to be microbiologically sterile due to factors  
50     including low pH and digestive enzymes. The stomachs of mammals other than  
51     humans are frequently colonized by bacteria other than *Helicobacter*, and lactobacilli  
52     and streptococci appear to be especially prevalent (Roach *et al.*, 1977, Fuller *et al.*,  
53     1978, Yin and Zheng, 2005). However, there may be a greater microbial diversity in  
54     the human stomach than had previously been thought. Using 16s rRNA sequencing,  
55     Bik et al (Bik et al., 2006) detected 128 different bacterial phylotypes, including  
56     lactobacilli, in 23 human gastric biopsies. Roos et al (Roos *et al.*, 2005) successfully  
57     cultured lactobacilli from gastric biopsies from healthy humans, and identified and  
58     described four new *Lactobacillus* species, *L. gastricus*, *L. antri*, *L. kalixensis* and *L.*  
59     *ultunensis*. To date, these species have not been further described. We hypothesized  
60     that it might be possible to isolate other lactobacilli from the human gastric mucosa  
61     and that this niche might represent a reservoir for bacteria with beneficial traits.  
62     Lactobacilli that could survive the hostile gastric environment could have applications  
63     as probiotics, or in fermentations at particularly low pH to which formic acid is added  
64     such as silage (Nadeau *et al.*, 2000) or yoghurt (Cotter and Hill, 2003).

66     **MATERIALS AND METHODS**

67     **Culture of lactobacilli from gastric biopsies**

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

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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97 Oxoid) (MRS agar for *Lactobacillus sakei*, Brain Heart Infusion agar for *Listeria*  
98 *innocua*, Luria Bertani agar for *Salmonella enterica* and Colombia Base agar  
99 supplemented with 5% horse blood for *Helicobacter pylori*), then applying the  
100 *Lactobacillus* test strain as a standard inoculum of 5 µl of a 0.2 OD600 culture on top  
101 of a paper disk placed on the agar plate. Agar plates were incubated for 48-96 h after  
102 which time zones of clearance were measured. The ability of the strains to survive or  
103 grow at different pH's was determined by adjusting the OD600 of an overnight MRS  
104 culture to 0.2 in either MRS or MRS adjusted to pH 2 or pH 3 with hydrochloric acid  
105 (approx. 5 fold). Samples were removed from the culture after 4, 8 and 24 h and cell  
106 viability was determined using a spread plate method. *L. vaginalis* SR8 grew very  
107 poorly and did not survive sufficiently for this analysis to be carried out. The bile  
108 resistance levels of the strains were determined by inoculating 5 µl of an overnight  
109 culture of each strain (OD600 of approx. 1.0) onto MRS plates supplemented with  
110 either porcine or bovine bile (Sigma, St. Louis, MO) at concentrations varying from  
111 0-10% and observing the presence or absence of growth after 72 h.

112  
113 **RESULTS**

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

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

156  
157 **DISCUSSION**

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

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

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

191       It is noteworthy that lactobacilli have been demonstrated many times in the  
192 stomachs of other mammals including the pig, which is generally regarded as having  
193 the closest gastric physiology to that of humans. It is thought that lactobacilli survive  
194 in the pig stomach by adhering strongly to epithelial cells, to the extent that they can  
195 form highly-resistant biofilm-like structures (Tannock, 1992). If, as now seems  
196 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.

**ACKNOWLEDGEMENTS**

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210 Figure 1. 16S rRNA gene phylogeny of selected lactobacilli. The sequences from the  
211 five species described in the present work are arrowed. Novel lactobacilli previously  
212 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.

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

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
<i>L. acidophilus</i> NCTC4356	202	164	149	108	55	17
<i>L. fermentum</i> SR2	142	162	98	101	81	14
<i>L. fermentum</i> SR9	0	0	0	0	0	0
<i>L. fermentum</i> SR10	0	0	0	0	0	0
<i>L. gasseri</i> SR1	667	505	349	0.4	0.3	< 0.001
<i>L. gasseri</i> SR15	0	0	0	0	0	0
<i>L. gasseri</i> SR 17	0	0	0	0	0	0
<i>L. reuteri</i> SR11	< 1	0	0	0	0	0
<i>L. reuteri</i> SR14	< 1	0	0	0	0	0
<i>L. salivarius</i> SR16	< 0.1	0	0	0	0	0
<i>L. salivarius</i> UCC118	14	7	0	0	0	0
<i>L. vaginalis</i> SR8	ND	ND	ND	ND	ND	ND

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

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

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