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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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26 **ABSTRACT**

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

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

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

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

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

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

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3 72 informed consent was obtained from all subjects. Biopsies were homogenized and
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5 73 spread on Rogosa agar (Oxoid, UK) for selective culture of lactobacilli. Agar plates
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8 74 were incubated anaerobically at 37°C for at least three days, after which visible
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10 75 colonies, if present, were selected and cultured anaerobically in de Man, Rogosa,
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12 76 Sharpe (MRS) (Oxoid) broth at 37°C. Carbohydrate fermentation profiles were
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15 77 assessed by API 50 CH kit (bioMerieux, Marcy l'Etoile, France).
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79 **16s rRNA sequencing and phylogenetic analyses**

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

94 **Antimicrobial activity, acid and bile tolerance**

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

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3 97 Oxoid) (MRS agar for *Lactobacillus sakei*, Brain Heart Infusion agar for *Listeria*
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5 98 *innocua*, Luria Bertani agar for *Salmonella enterica* and Colombia Base agar
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8 99 supplemented with 5% horse blood for *Helicobacter pylori*), then applying the
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10 100 *Lactobacillus* test strain as a standard inoculum of 5 µl of a 0.2 OD600 culture on top
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12 101 of a paper disk placed on the agar plate. Agar plates were incubated for 48-96 h after
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14 102 which time zones of clearance were measured. The ability of the strains to survive or
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16 103 grow at different pH's was determined by adjusting the OD600 of an overnight MRS
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18 104 culture to 0.2 in either MRS or MRS adjusted to pH 2 or pH 3 with hydrochloric acid
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20 105 (approx. 5 fold). Samples were removed from the culture after 4, 8 and 24 h and cell
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22 106 viability was determined using a spread plate method. *L. vaginalis* SR8 grew very
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24 107 poorly and did not survive sufficiently for this analysis to be carried out. The bile
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26 108 resistance levels of the strains were determined by inoculating 5 µl of an overnight
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28 109 culture of each strain (OD600 of approx. 1.0) onto MRS plates supplemented with
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30 110 either porcine or bovine bile (Sigma, St. Louis, MO) at concentrations varying from
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32 111 0-10% and observing the presence or absence of growth after 72 h.
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41 **RESULTS**

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43 114 Lactobacilli were successfully cultured from 4/12 gastric biopsies. There was
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45 115 no correlation between biopsies positive for lactobacilli and i) the sex of the patient;
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47 116 ii) the age of the patient; iii) the *H. pylori* status of the patient; iv) the disease status of
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49 117 the patient. Three different species (*L. fermentum*, *L. gasseri* and *L. vaginalis*) were
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51 118 isolated from one biopsy; two species (*L. fermentum* and *L. reuteri*) and (*L. salivarius*
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53 119 and *L. gasseri*) were isolated from two other biopsies and only one species (*L.*
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55 120 *gasseri*) was isolated from the remaining positive biopsy. Sequence identity to
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57 121 published sequences was at least 99% in all cases, and sequences from all strains of
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4 122 the same species were identical. We identified that two different strains of *L.*
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6 123 *fermentum* and two different strains of *L. reuteri* were present in one biopsy by
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8 124 comparing the carbohydrate fermenting capability of the four isolates using API
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10 125 analysis. A phylogenetic analysis of the 16S sequences from the ten species showed
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12 126 that, with the exception of *L. salivarius*, all isolates are members of group A or B of
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14 127 the *Lactobacillus* 16S phylogeny (Canchaya et al., 2006) (Fig. 1). Interestingly, the
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16 128 recently described gastric lactobacilli *L. antri*, *L. gastricus*, *L. ultunensis* and *L.*
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18 129 *intestinalis* (Roos et al., 2005) are all contained within these same two groups. This
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20 130 may indicate a phylogenetic relationship between lactobacilli capable of persisting in
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22 131 the human gastric environment.

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27 132 The ten isolated lactobacilli were screened for their ability to inhibit growth of
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29 133 two Gram-positive (*L. sakei* and *Listeria innocua*) and two Gram-negative (*S. enterica*
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31 134 and *H. pylori*) bacteria (Table 1). The three *L. fermentum* strains inhibited growth of
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33 135 both Gram-positive indicator organisms, and strain SR2 also inhibited growth of *H.*
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35 136 *pylori*. *L. salivarius* SR16 was the only other strain capable of inhibiting growth of *H.*
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37 137 *pylori*. *L. fermentum* and *L. salivarius* both produce bacteriocins (Yan and Lee, 1997,
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39 138 Claesson *et al.*, 2006), and this is one potential source of the inhibitory effect. Both
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41 139 strains of *L. reuteri* inhibited growth of only *L. sakei*, possibly due to the production
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43 140 of reuterin (Talarico and Dobrogosz, 1989).

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48 141 Lactobacilli cultured from the stomach might arguably be transient
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50 142 (allochthonous) rather than long-term colonizers (autochthonous). To investigate this
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52 143 we examined the acid resistance of the ten lactobacilli from the human stomach
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54 144 (Table 2). *L. fermentum* SR2 exhibited 100% survival, and *L. gasseri* SR1 cell
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56 145 numbers increased three-fold in MRS pH 3 after 24 h. This compared favourably
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58 146 with the acid-tolerant control species *L. acidophilus* ATCC4356 (Lorca et al., 1998).

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3 147 In contrast, no cells of *L. salivarius* UCC118 were viable after this time, even though
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5 148 *L. salivarius* UCC118 has been previously shown to have probiotic qualities in a
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8 149 number of *in vivo* studies (McCarthy *et al.*, 2003, Sheil *et al.*, 2004). *L. fermentum*
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10 150 SR2 and *L. acidophilus* ATCC4356 showed < one log decrease in viability after 24 h
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12 151 in MRS pH 2. None of the other strains exhibited significant acid tolerance. The ten
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14 152 strains also varied greatly in their ability to grow on porcine and bovine bile (Table 3).
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16 153 The type strain *L. acidophilus* NCTC4356 was the most tolerant to both bile types. Of
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18 154 the gastric strains, *L. reuteri* SR11 was the most resistant; it grew on MRS plates
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20 155 containing 10% bovine and 0.5% porcine bile.
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27 **DISCUSSION**

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29 158 We have shown that lactobacilli can be cultured from human gastric tissue.
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31 159 Although these organisms are abundant in the upper and lower gastrointestinal tract, it
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33 160 is generally thought that they do not persist for any significant length of time in the
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35 161 stomach (O'Hara and Shanahan, 2006). The main source of lactobacilli is food, and
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37 162 since a patient must fast for at least 12 h before a gastric endoscopy is performed, the
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39 163 bacteria we isolated may have survived in the stomach for at least this length of time.
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41 164 During fasting, the gastric pH can drop as low as 1.5 (Drasar *et al.*, 1969) indicating
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43 165 that these strains may have an intrinsic *in vivo* resistance to low pH. Although we
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45 166 cannot refute the possibility that the strains may have been introduced into the
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47 167 stomach at a later time-point via saliva, our *in vitro* experiments show that two of the
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49 168 strains are capable of surviving at least 24 h at low pH. Resistance to bile is
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51 169 considered a valuable probiotic trait and although the gastric lactobacilli were not
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53 170 particularly bile-tolerant, this is not perhaps surprising in this case because bile first
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55 171 enters the gastrointestinal tract in the duodenum, and is only present in the stomach if
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3 172 duodeno-gastric reflux occurs. Porcine bile contains a higher level of glycine
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5 173 conjugated bile salts than bovine bile (Coleman *et al.*, 1979). These salts are more
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8 174 toxic to lactobacilli (De Smet *et al.*, 1995). It is not surprising therefore that all strains
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10 175 survived less well in the presence of porcine bile.
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13 176 The relative abundance of lactobacilli in the human stomach has several
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15 177 implications. Lactobacilli have been shown to have beneficial effects in the
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17 178 alleviation of many human conditions (O'Mahony *et al.*, 2005, Zocco *et al.*, 2006). If
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19 179 these organisms are capable of surviving for a significant length of time in the
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21 180 stomach, probiotic treatments might also be useful in the treatment of gastric
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23 181 disorders. Indeed a number of trials have already shown that this might be the case
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25 182 (Johnson-Henry *et al.*, 2004, Sykora *et al.*, 2005). Furthermore, in a conventional
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27 183 probiotic setting, lactobacilli capable of surviving in the stomach for extended periods
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29 184 of time will be more aciduric, ensuring not only that more cells survive gastric transit
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31 185 to reach the intestine, but also allowing for greater survival and shelf-life in fermented
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33 186 dairy products. The presence of significant numbers of bacteria other than *H. pylori*
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35 187 in the human stomach may well have implications for the human health, and the
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37 188 culture-independent analysis of the gastric metagenome of 23 subjects supports this
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39 189 notion (Bik *et al.*, 2006). Metagenomic analysis of a much larger cohort of subjects
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41 190 with a range of disease pathologies is required to address this question.
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48 191 It is noteworthy that lactobacilli have been demonstrated many times in the
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50 192 stomachs of other mammals including the pig, which is generally regarded as having
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52 193 the closest gastric physiology to that of humans. It is thought that lactobacilli survive
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54 194 in the pig stomach by adhering strongly to epithelial cells, to the extent that they can
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56 195 form highly-resistant biofilm-like structures (Tannock, 1992). If, as now seems
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58 196 likely, lactobacilli are more prevalent in the human stomach, it is also possible that
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3 197 this site may be home to other novel, previously unidentified lactobacilli (Roos *et al.*,
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5 198 2005) which may also form biofilms.
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8 199 In conclusion, this work describes what is, to our knowledge, the first isolation
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10 200 of *L. fermentum*, *L. gasseri*, *L. vaginalis*, *L. reuteri* and *L. salivarius* from the human
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12 201 stomach, and suggests this site may be a novel source for new organisms with
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14 202 probiotic and other beneficial properties.
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5 210 Figure 1. 16S rRNA gene phylogeny of selected lactobacilli. The sequences from the
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8 211 five species described in the present work are arrowed. Novel lactobacilli previously
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10 212 isolated from the human gastric mucosa (Roos *et al.*, 2005) are boxed.
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For Peer Review

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

	<i>L. sakei</i>	<i>Listeria innocua</i>	<i>S. enterica</i>	<i>H. pylori</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	-	+	-	-

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

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	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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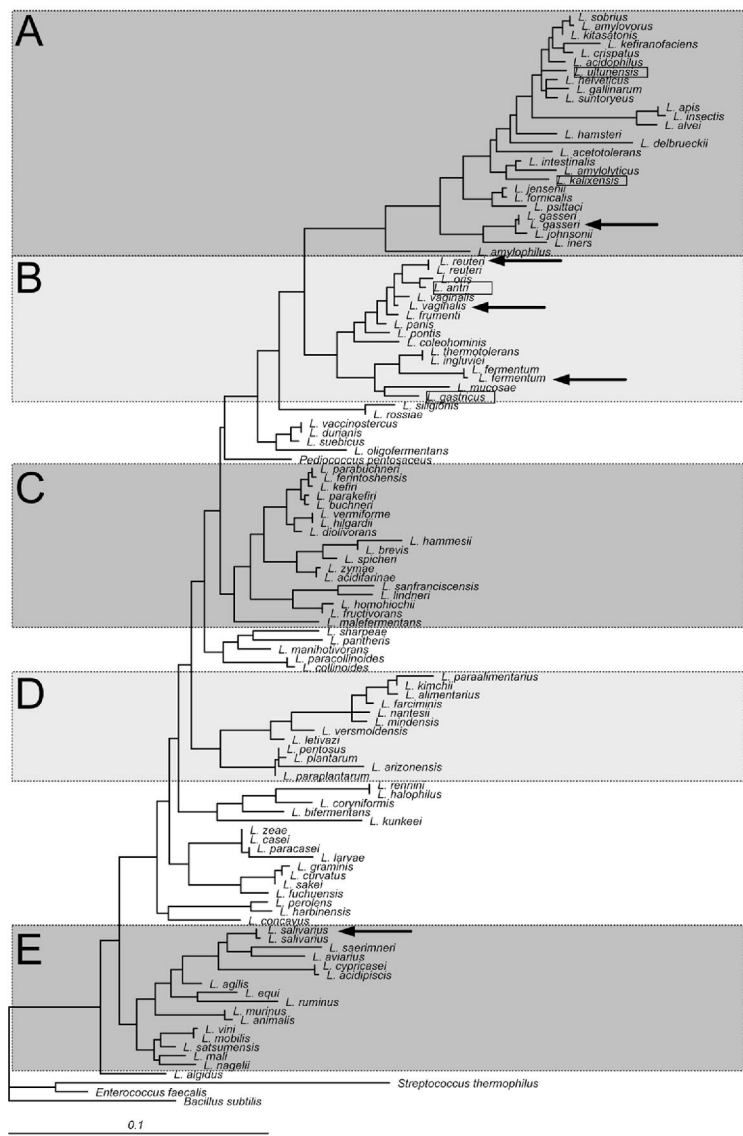
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For Peer Review

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