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1 **Metabolic activity of probiotics – oxalate degradation**

2

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24 **Abstract**

25 Urinary tract stones are an important clinical problem in human and veterinary
26 medicine. Hyperoxaluria is the single strongest promoter of kidney stone formation. The
27 aims of the present study were to, (a) evaluate oxalate degradation by a range of
28 *Bifidobacteria* species and *Lactobacillus* species isolated from the canine and feline
29 gastrointestinal tract *in vitro* and, (b) to determine the impact of oxalate degradation by
30 selected strains *in vivo*. The bacteria were grown in oxalate-containing media and their
31 ability to degrade oxalate *in vitro* was determined using reverse-phased HPLC.
32 *Bifidobacteria* species and *Lactobacillus* species that degraded oxalate *in vitro* and
33 survived gastric transit were selected for further examination. The selected probiotics
34 were fed to rats for 4 weeks. Urine was collected at week's 0, 2 and 4 and oxalate levels
35 determined by HPLC. *In vitro* degradation was detected for 11/18 of the *Lactobacillus*
36 species. In contrast, the capacity to degrade oxalate was not detected for any of the 13
37 *Bifidobacterium* species tested. *Lactobacillus animalis* 223C, *Lactobacillus murinus*
38 1222, *Lactobacillus animalis* 5323 and *Lactobacillus murinus* 3133 were selected for
39 further investigation in a rat model. Urinary oxalate levels were significantly reduced
40 ($p < 0.05$) in animals fed *L. animalis* 5323 and *L. animalis* 223C but were unaltered when
41 fed *L. murinus* 1222, *L. murinus* 3133 or placebo. Probiotic organisms vary widely in
42 their capacity to degrade oxalate. *In vitro* degradation does not uniformly translate to an
43 impact *in vivo*. The results have therapeutic implications and may influence the choice of
44 probiotic, particularly in the setting of enteric hyperoxaluria.

45 1. Introduction

46 Hyperoxaluria complicated by renal tract stones is an important clinical problem
47 in humans, particularly those with enteric hyperoxaluria secondary to conditions such as
48 Crohn's disease (Kumar et al., 2004). In veterinary medicine, domestic animals, such as
49 cats and dogs, are particularly prone to oxalate stones. Currently, there is no successful
50 medical dissolution protocol, and renal stones must be removed or disrupted by physical
51 methods. Epidemiological studies over the last decade have associated a decrease in
52 struvite calculi with an increase in calcium oxalate renal stone formation (Hesse et al.,
53 1998; Lekcharoensuk et al., 2001). Acidification of commercial diets to maintain urine
54 pH between 6.0 and 6.4 reduces struvite crystal formation but increases the risk of
55 calcium oxalate formation in companion animals (Buffington and Chew, 1996). Oxalic
56 acid and its salts are widely distributed in dry commercially prepared dog food
57 (Hodgkinson, 1977; Stevenson et al., 2003). Increased dietary oxalate results in increased
58 urinary oxalate and calcium oxalate relative supersaturation in healthy adult dogs
59 (Stevenson et al., 2003).

60 While some components of the enteric bacterial flora, (such as *Oxalobacter*
61 *formigenes*) have oxalate degrading capacity, these organisms are not uniformly present
62 in all animals (Allison et al., 1986; Sidhu et al., 2001). However, dietary
63 supplementation with probiotics has emerged as a potential strategy for increasing the
64 degradation of dietary oxalate (Campieri et al., 2001; Weese et al., 2004). Therefore, the
65 purpose of our study was to screen a range of *Lactobacillus* species and *Bifidobacteria*
66 species derived from the feline and canine gastrointestinal tract for oxalate degradation
67 capacity *in vitro* and then to determine the impact of feeding such strains on urinary
68 oxalate excretion *in vivo*.

69

70 **2. Materials and methods**71 *2.1 Probiotic stain isolation*

72 The small intestine, caecum or colon of cats and dogs were dissected post mortem
73 and the removed tissue washed in Ringers solution (Oxoid, Basingstoke, Hampshire, UK)
74 to remove loosely adherent bacteria. The tissue was vortexed and homogenised in
75 Ringers solution to select adherent bacteria. The supernatants from the wash and vortex
76 steps were plated on de Man, Rogosa, Sharpe (MRS) agar (Oxoid, Basingstoke,
77 Hampshire, UK) supplemented with 20 µg/ml vancomycin (Sigma-Aldrich Chemie, St.
78 Louis, MO, USA) and Wilkins Chalgren Agar (Oxoid, Basingstoke, Hampshire, UK)
79 supplemented with 50 µg/ml mupirocin (Oxoid, Basingstoke, Hampshire, UK) for
80 *Lactobacillus* species and *Bifidobacteria* species, respectively. The plates were incubated
81 at 37°C in an anaerobic environment for 72 h. Isolated colonies were re-streaked to
82 ensure purity. Isolates from MRS agar + vancomycin plates were re-streaked on MRS
83 agar and isolates from Wilkins Chalgren Agar + mupirocin were re-streaked on
84 Reinforced Clostridia Agar (RCA: Oxoid, Basingstoke, Hampshire, UK) supplemented
85 with 0.05% (v/v) L-cysteine hydrochloride (Sigma-Aldrich Chemie, St. Louis, MO,
86 USA) for the purification of *Lactobacillus* species and *Bifidobacteria* species,
87 respectively. Following purification, single strain cultures were identified on the basis of
88 colony morphology, gram reaction, catalase activity and the Fructose-6-phosphate
89 phosphoketolase assay. Gram-positive, catalase negative rods were genetically
90 characterised using primers specific for the 16 S intergenic spacer region and
91 *Lactobacillus* species and *Bifidobacteria* species isolates were further examined.

92 *Lactobacillus* species strains were routinely cultured in MRS broth at 37°C in an
93 anaerobic environment for 24 h. *Bifidobacteria* species isolates were routinely cultured in
94 MRS broth supplemented with 0.05% (v/v) L-cysteine hydrochloride and incubated at
95 37°C in an anaerobic environment for 48 h. *Lactobacillus* species and *Bifidobacteria*
96 species stocks are maintained in 40% glycerol at -80° C (Alimentary Health Ltd.,
97 National University of Ireland, Cork, Ireland).

98

99 *2.2 Assaying Lactobacillus and Bifidobacteria isolates for growth in ammonium oxalate*
100 *media and determining oxalate-degrading capability*

101 The procedure for the determination of oxalate-degrading capacity of probiotic isolates
102 was based on the method previously described by Campieri et al. (2001). Briefly, 5 ml of
103 filtered sterilised ammonium oxalate solution [20 mM/l ammonium oxalate and 40 g/l
104 dextrose (Roquette, Lestrem, France)] was added to 5 ml of base media (Protease peptone
105 20 g/l, yeast extract 10 g/l, Tween 80 2 ml/l, KH₂PO₄ 4 g/l, NA acetate 10 g/l, di-
106 Ammonium-hydrogen-citrate 4 g/l, MgSO₄.7H₂O 0.1 g/l and MnSO₄ 0.1 g/l). All reagents
107 were supplied by either Sigma-Aldrich (St. Louis, MO, USA) or BDH Laboratory
108 supplies, Poole, UK; unless otherwise stated. Culture broths were inoculated at 2% into
109 base media and base media containing 20 mM ammonium oxalate. The base media was
110 supplemented with 0.05% (v/v) L-cysteine hydrochloride when inoculating
111 *Bifidobacteria* species and all cultures were incubated anaerobically at 37°C for 48 h. A
112 media control (ammonium oxalate base media) was prepared as above, but without the
113 inoculation of bacteria. Optical density (600 nm) and plate counts (colony forming
114 units/ml) were performed to determine growth of each strain in ammonium oxalate base

115 media, which was compared to growth in base media. Ammonium oxalate base media
116 cultures and the media control were centrifuged at 3000 rpm for 10 min and the
117 supernatants filter sterilised using 0.45 μ M filters (Sartorius AG, Goettingen, Germany).
118 The culture filtrates were stored at 4°C until plate counts were recorded and HPLC
119 analysis was performed on strains that grew in 20 mM ammonium oxalate base media.

120

121 *2.3 Chemicals and materials for HPLC*

122 All chemicals were of spectral or analytical grade. Unless otherwise stated, all
123 chemicals employed were obtained from Sigma-Aldrich (St. Louis, MO, USA) or BDH
124 Laboratory supplies, Poole, UK. HPLC grade water (Reagecon, Shannon, Ireland) was
125 utilised throughout the experiments. The procedure for the determination of oxalic acid in
126 samples by HPLC was based on the method previously described by Khaskhali et al.
127 (1996). The mobile phase was composed of 0.25% potassium dihydrogen phosphate and
128 0.0025 M tetrabutylammonium hydrogensulphate, buffered at pH 2.0 with
129 orthophosphoric acid. The mobile phase was filtered through a 0.2 μ m nylon membrane.
130 Aqueous oxalic acid standards were prepared in the range 0.02-20 mM. These solutions
131 were stable for 3 months at 4°C.

132

133 *2.4 Apparatus and chromatographic conditions*

134 Chromatographic analysis was performed using a Spectraseries 100
135 (Thermoseparation Products, Minnesota, USA) with a chromjet integrator, UV detector
136 and a Synergi Hydro-RP column, 4 μ m, 250 x 4.6 mm I.D. (Phenomenex, Cheshire, UK).
137 The analytic column was routinely cleaned by rinsing the column with: 94% water/5%

138 acetonitrile, tetrahydrofluran, 95% acetonitrile/5% water and mobile phase for 20 min
139 each. The column was purged by pumping the mobile phase at 4 ml/min for 3 min and
140 equilibrated by pumping the mobile phase to waste. The detector wavelength was fixed at
141 210 nm. The total cycle time was 35 min with 20 µl injections from each sample. At the
142 end of each run, acetonitrile: HPLC-grade water (65:35) was pumped through the column
143 for 15 min prior to storage.

144

145 *2.5 Preparation of filtrate samples*

146 20 mM, 15 mM, 10 mM, 5 mM and 2 mM ammonium oxalate standards were
147 prepared from 200 mM ammonium oxalate stock solution. All filtrates and standards
148 were diluted 1:50 in mobile phase and analysed using HPLC.

149

150 *2.6 Survival in a low pH environment.*

151 Probiotic strains must be capable of resisting the effects of a low pH environment.
152 Bacterial cells were harvested from overnight cultures, washed twice in phosphate buffer
153 (pH 6.5) and resuspended in the MRS broth adjusted with 1 N HCl to pH 2.5. The cells
154 were incubated anaerobically @ 37°C and their survival measured at intervals of 0, 30,
155 60, 120, 180, 240 and 360 min using the plate count method.

156

157 *2.7 Resistance to bile salts*

158 Resistance to bile was examined using MRS agar plates supplemented with 0.5,
159 1.0 and 5.0 % (w/v) porcine bile (Sigma-Aldrich Chemie, St. Louis, MO, USA).

160 *Lactobacillus* species probiotics were inoculated into MRS broth and incubated at 37°C

161 under anaerobic conditions for 24 h. Strains were spot inoculated (10 μ l) onto the various
162 concentrations of porcine bile plates and incubated at 37°C under anaerobic conditions
163 for 48 h. The growth rate on porcine bile plates were compared to the growth rate on
164 MRS agar plates and recorded.

165

166 *2.8 Tolerance to freeze drying process and stability*

167 The probiotic strains were grown overnight in MRS broth, centrifuged and
168 resuspended in cryoprotectant (18% reconstituted skim milk, 2 % sucrose). The mixtures
169 were then frozen at -20°C for 24 Hrs and then freeze dried for another 24 Hrs. The
170 mixtures were freeze-dried at a vacuum pressure of 133 x 10⁻³ mBar with a condenser
171 temperature of -53°C. All strains were examined for stability to freeze-drying and their
172 shelf life at room temperature was assessed for one month post-processing by MRS plate
173 counting techniques.

174

175 *2.9 Generation of spontaneous rifampicin-resistant variants of isolated probiotics*

176 Selected probiotics were streaked onto MRS agar for *Lactobacillus* species
177 isolates and RCA supplemented with 0.05% L-cysteine hydrochloride for *Bifidobacteria*
178 species isolates. All isolates were incubated at 37°C in an anaerobic environment for 48
179 h. Isolates were sub-cultured onto appropriate agar plates containing 100 μ g/ml
180 rifampicin and incubated at 37°C in an anaerobic environment for 72 h. Spontaneous
181 rifampicin resistant variants (Rif^R) were stocked in 40% glycerol (Sigma-Aldrich
182 Chemie, St. Louis, MO, USA), stored at -80 °C and checked for their continuous
183 resistance to 100 μ g/ml rifampicin by restreaking onto appropriate agar plates containing

184 100 µg/ml rifampicin and incubated at 37°C in an anaerobic environment for 48 h.
185 Growth curves of isolates and Rif^R isolates were performed to ensure the growth rate was
186 not altered.

187

188 *2.10 In vivo gastric transit of selected probiotic isolates*

189 15 female Spague-Dawley rats of similar age and weight were enrolled in the
190 study. Freeze dried Rif^R probiotic powders were resuspended in an appropriate volume of
191 water to ensure a does of ~ 9.8 x 10⁹ colony-forming units (cfu) for *L. animalis* 223C, *L.*
192 *murinus* 1222, *L. animalis* 5323 and *L. murinus* 3133 or 0 cfu control freeze dried product
193 for the placebo group. The resuspended powders were administered, ad libitum, for 6
194 days (n=3 animals per group). Rats were weighted daily and the volume of probiotic
195 consumed was calculated daily. Rat faecal pellets were collected prior to feeding (Day 0)
196 and on Days 1, 3 and 6 (post probiotic feeding). All faecal pellets were weighed and
197 resuspended in 1 ml Ringers (Oxoid, Basingstoke, Hampshire, UK). The colony forming
198 units/g was determined by plating onto MRS agar containing 100 µg/ml rifampicin, in
199 order to facilitate uncomplicated identification of the freeze dried Rif^R probiotics from all
200 other Lactobacilli.

201

202 *2.11 In vivo urinary oxalate levels using selected probiotics*

203 30 female Sprague-Dawley rats of similar age and weight were enrolled in the
204 study. Freeze dried probiotic powders were resuspended in an appropriate volume of
205 water to ensure a does of ~ 2 x 10⁹ cfu for *L. animalis* 223C, *L. murinus* 1222, *L. animalis*
206 5323 and *L. murinus* 3133 or 0 cfu control freeze dried product for the placebo group.

207 The resuspended powders were administered, ad libitum, for 4 weeks (n=6 animals per
208 group) Rats were weighed weekly and urine samples were obtained on Weeks 0, 2 and 4
209 by placing the animals in metabolic cages for a 24 h period.

210

211 *2.12 Preparation of urine samples*

212 10 ml of a 24 hour sample was obtained from the metabolic cage and placed in
213 polyethylene bottles to which 10 ml of 6 M hydrochloric acid was added as a
214 preservative. Deproteinisation of the samples was performed at ambient temperature by
215 mixing a homogeneous urine sample (10 ml) from each collection with 0.5 g crystalline
216 sulfosalicylic acid and after 10 min filtering the mixture through a 0.45 µm Minisart filter
217 (Khaskhali et al., 1996).

218

219 *2.13 Statistical analysis*

220 Statistical analysis of the in vitro results was performed using a paired student t-
221 tests. Changes in rat urinary oxalate excretion levels over time were assessed using a one-
222 way analysis of variance (ANOVA) with replicates.

223 **3. Results**224 **3.1 *In vitro* growth and oxalate degradation by probiotics of canine and feline origin.**

225 Thirteen *Bifidobacteria* species and 18 *Lactobacillus* species were included in the
226 *in vitro* assessment, which were identified using 16S intergenic spacer sequencing. **These**
227 **strains included 11 *B. longum* strains (feline-derived), 1 *B. globosum* strain (canine-**
228 **derived), 1 *B. animalis* strain (canine-derived), 1 *L. acidophilus* strain (feline-**
229 **derived), 5 *L. reuteri* strains (feline-derived), 8 *L. animalis* strains (7 canine-derived**
230 **& 1 feline-derived), 1 *L. salivarius* strain (canine-derived) and 3 *L. murinus* strains**
231 **(canine-derived).** All selected isolates grew in the presence of 20 mM ammonium
232 oxalate illustrating that oxalate at this concentration is not toxic to LAB. The average
233 cfu/ml of isolates, grown in the presence of 20 mM ammonium oxalate, was 2.3×10^8
234 cfu/ml. This was comparable to growth of isolates in base media. Supernatants from
235 isolates were subsequently analysed using HPLC. A media control (base media + 20 mM
236 ammonium oxalate) was included in order to provide a 20 mM ammonium oxalate
237 standard.

238 The ability of Lactic Acid Bacteria (LAB) to degrade oxalate was strain
239 dependant. No oxalate degradation was detected for any of the *Bifidobacterium* species
240 isolates when compared to the 20 mM ammonium oxalate media control (Fig. 1).
241 Oxalate degradation was detected for 11/18 (61%) of the *Lactobacillus* species when
242 compared to the ammonium oxalate media control (Fig. 2). *L. acidophilus*, *L. reuteri* and
243 *L. salivarius* isolates did not demonstrate oxalate degradation, but *L. animalis* and *L.*
244 *murinus* isolates demonstrated significant oxalate degradation. Two representative
245 isolates from *L. animalis* and two representative isolates from the *L. murinus* group were
246 selected for further examination in an *in vivo* rat model. Mean rate of *in vitro* oxalate

247 degradation for the selected strains was 0.15 mM/h (*L. animalis* 223C – **feline isolate**),
248 0.15 mM/h (*L. murinus* 1222 – **canine isolate**), 0.14 mM/h (*L. animalis* 5323 – **canine**
249 **isolate**) and 0.09 mM/h (*L. murinus* 3133 – **canine isolate**).

250

251 3.2 Assessment of gastric transit of probiotic bacteria in vitro

252 Prior to reaching the intestinal tract, probiotic bacteria must first survive transit
253 through the stomach, which involves survival to stomach and bile acids. The survival of
254 selected strains to a low pH environment was assessed by adding approximately 10^8
255 cfu/ml of *L. animalis* 223C, *L. murinus* 1222, *L. animalis* 5323 and *L. murinus* 3133 to
256 acidified MRS broth, pH 2.5. The results indicate that all selected probiotic strains have
257 the potential to successfully transit the human stomach, as strains were viable after 360
258 minutes in a low pH environment and the loss of viability was <1.5 logs (Fig 3).

259 The survival of probiotic strains upon exposure to deconjugated porcine bile was
260 examined using MRS agar plates supplemented with various concentrations of bile. All
261 selected strains survive up to 5.0 % bile acid (Table 1).

262

263 3.3 Stability of bacterial strains following the freeze-drying process

264 The putative probiotic strains were examined for their stability, following the
265 freeze-drying process, for 1 month at room temperature. *L. animalis* 223C, *L. murinus*
266 1222, *L. animalis* 5323 and *L. murinus* 3133 remained at high numbers post freeze-drying
267 and demonstrated no loss of activity during storage at room temperature (Fig 4).

268

269 3.4 In vivo gastric transit of selected probiotic isolates

270 Changes in rat weight were monitored daily during the gastric transit feeding trial.
271 No significant changes in body weight were detected for the duration of the trail. The
272 volume of Rif^R probiotic consumed ad libitum was recorded and the dose of Rif^R
273 probiotic consumed was calculated based on the dose of freeze-dried probiotic supplied
274 (Table 2). The average dose of probiotic consumed/day was 9.8×10^9 CFU. The
275 consumed probiotics survived gastric transit in this rat model (Fig 5). Prior to feeding
276 probiotics (Day 0), no Rif^R probiotics were detected on culture plates. This baseline
277 ensures the selectivity of the agar plates containing 100 µg/ml rifampicin. The Rif^R
278 probiotics were detected in faeces from all mice in the probiotic group within 1 day of
279 feeding. During the 6 day feeding study, the Rif^R probiotics were recovered at
280 approximately 4.6×10^9 bacteria per gram of faeces. Rif^R probiotics were not cultivated
281 from any of the rats in the placebo group. The amount of Rif^R probiotic consumed/day is
282 equivalent to the gastric transit of the probiotics/day. No significant difference was
283 observed between groups fed different probiotics or between transit levels on Day 1, 3 or
284 6.

285

286 3.5 *In vivo* oxalate degradation of selected probiotics in a rat model.

287 Sprague-Dawley rats (n=6/group) received 2×10^9 cfu/day of *L. animalis* 223C, *L.*
288 *murinus* 1222, *L. animalis* 5323 and *L. murinus* 3133 or placebo. During the study, 24 h
289 urine specimens were obtained on Week 0, Week 2 and Week 4 by placing the rats in
290 metabolic cages. **The mean urinary output per rat was 14.3mls over the 24 hours**
291 **(range 10.5 – 21.2mls)**. Rat weights were monitored for the duration of the study, and
292 demonstrated no significant difference when compared to the placebo control (Table 3).

293 Fig. 6 illustrates the trial results with urinary oxalate levels expressed as μM
294 oxalate **over a 24 hour period**. Urinary oxalate levels remained constant in the first
295 group of rats (not receiving a probiotic supplement). In contrast, rats consuming the
296 probiotic strains *L. animalis* 223C and *L. animalis* 5323 had decreased urinary oxalate
297 excretion. Rats consuming *L. murinus* 1222 and *L. murinus* 3133 did not have decreased
298 urinary oxalate excretion.

299

300 4. Discussion

301 The results of this study show that **some strains of *Lactobacillus*** but not
302 *Bifidobacteria* species degrade oxalate *in vitro* and reduce urinary oxalate excretion *in*
303 *vivo*. Several *L. animalis* and *L. murinus* isolates degrade ammonium oxalate *in vitro*
304 while four strains were selected for inclusion in the animal study, 2 representatives from
305 the *L. animalis* group (*L. animalis* 223C and *L. animalis* 5323) and 2 representatives from
306 the *L. murinus* group (*L. murinus* 1222 and *L. murinus* 3133). Both *L. animalis* strains (*L.*
307 *animalis* 223C and *L. animalis* 5323) reduced oxalate excretion in rats. All 4 selected
308 strains survived gastric transit.

309 Previous studies have demonstrated oxalate degradation by *O. formigenes*, a gram
310 negative, anaerobic bacterium that inhabits the gastrointestinal tracts of humans and
311 mammals (Allison et al., 1986; Dawson et al., 1980). The presence of *O. formigenes* has
312 been shown to reverse hyperoxaluria in a rat model and reduce urinary oxalate excretion
313 in humans (Duncan et al., 2002; Sidhu et al., 2001). It has been suggested that the
314 absence of *O. formigenes* in the gastrointestinal tract correlates with the number of
315 recurrences of oxalate stone disease (Sidhu et al., 1999). However, the establishment of

316 *O. formigenes* in a rat model was transient and the faecal population of *O. formigenes*
317 declined below the detectable limit once rats were placed on a normal diet (Sidhu et al.,
318 2001). Difficult isolation and transient colonisation of *O. formigenes* have resulted in
319 investigators screening for alternative oxalate-degrading bacteria in the intestine, such as
320 LAB (Campieri et al., 2001; Hokama et al., 2000; Hokama et al., 2005). *P. rettgeri* and
321 *E. faecalis* appear to have a mechanism of oxalate degradation similar to *O. formigenes*,
322 but they were unable to maintain their oxalate degrading ability when subcultured into
323 nutrient rich medium (Hokama et al., 2000; Hokama et al., 2005). We have shown, using
324 *in vitro* and *in vivo* models, that certain probiotics offer a therapeutic strategy to reducing
325 urinary oxalate excretion.

326 All four candidate strains tested degraded oxalate *in vitro*, but only two of these
327 strains degraded oxalate *in vivo*. It is unlikely that the inability of *L. murinus* 1222 and *L.*
328 *murinus* 3133 to degrade oxalate *in vivo* could be attributed to the physiological aspects
329 of the intestinal tract (gastric acidity, peristalsis, bile acids etc.) and the anti-microbial
330 defence mechanisms (adhesion, colonisation, nutrient competition etc.), as all four strains
331 transited the gut in equivalent amounts. Rather, the *L. animalis* and *L. murinus* strains
332 may interact with the host in a strain specific manner such as that demonstrated for
333 probiotic adherence to intestinal tissue and mucus (Ouweland et al., 1999). In addition,
334 the utilisation of oxalate as a substrate for *L. murinus in vivo* may not be allowable at a
335 genetic level due to phenomena such as quorum sensing. This highlights the importance of
336 carefully selecting strains using *in vitro* characteristics, in addition to using animal
337 models to observe the biological impact *in vivo*. **It is unlikely that the original source**
338 **of these strains has a significant impact on the excretion of oxalate in the rat studies**

339 as one of the successful strains was canine-derived (*L. animalis* 5323) while the other
340 was feline-derived (*L. animalis* 223C).

341 Our results suggest considerable variability in the ability of probiotics to degrade
342 oxalate, both *in vitro* and *in vivo*. We detected oxalate degradation for 61% of the
343 *Lactobacillus* species examined *in vitro*. In contrast, *Bifidobacterium* species appears not
344 to possess the mechanism of oxalate degradation demonstrated by *Lactobacillus* spp
345 when examined *in vitro*. Weese et al. (2004) also reported considerable variation in
346 oxalate degradation by different probiotics *in vitro*. They reported a mean oxalate
347 degradation of 17.7 % for 37 LAB, but they did not further identify the strains. Campieri
348 et al. (2001) previously reported variable *in vitro* oxalate degradation with *L. acidophilus*,
349 *L. plantarum*, *L. brevis*, *Streptococcus thermophilus* and *B. infantis*. They demonstrated
350 little or no oxalate degradation in *L. plantarum* and *L. brevis*, but *L. acidophilus*, *S.*
351 *thermophilus* and *B. infantis* degraded oxalate. However, the level of *in vitro* oxalate
352 degradation was low, with degradation of 5.26% of 10 mM/l ammonium oxalate and
353 2.18% of 20 mM/l ammonium oxalate and *in vivo* degradation was assessed in a mixture
354 of freeze-dried LAB (*L. acidophilus*, *L. plantarum*, *L. brevis*, *S. thermophilus*, *B.*
355 *infantis*). Why only some probiotics strains degrade oxalate remains unclear, fuelling a
356 desire to better understand the mechanism of oxalate degradation in probiotics. *O.*
357 *formigenes* has two oxalate degrading enzymes, oxalyl-coenzyme A decarboxylase (65
358 kDa) and formyl-coenzyme A transferase (48 kDa) (Kodoma et al., 2002). While these
359 oxalate degrading enzymes have been found in *Providencia rettgeri* and *Enterococcus*
360 *faecalis*, it is unknown if these enzymes have been found in LAB (Hokama et al. 2005;
361 Hokama et al. 2000).

362 The detected oxalate degradation in this study appears to be interspecies
363 dependent, with *L. animalis* and *L. murinus* degrading oxalate *in vitro* and *L. acidophilus*,
364 *L. reuteri* and *L. salivarius* demonstrating no oxalate degradation *in vitro*. Indeed, only *L.*
365 *animalis* strains and not *L. murinus* strains degraded oxalate *in vivo*. Other studies have
366 demonstrated considerable interspecies variation in metabolic activity; in particular the
367 ability to produce the health-promoting fatty acid conjugated linoleic acid (CLA) from
368 free linoleic acid (Coakley et al., 2003). They demonstrated considerable interspecies
369 variation, with *B. breve* and *B. dentium* being the most efficient CLA producers.

370

371 **5. Conclusion**

372 We have highlighted the metabolic potential of probiotics by examining one
373 specific metabolite, but mining the gut microbiota for further health promoting effects is a
374 viable option for future dietary management strategies of specific metabolic symptoms or
375 dysfunction. Future studies should also consider the development of an effective oxalate
376 degrading synbiotic (probiotic + prebiotic) by tailoring a prebiotic towards the specific
377 organism and investigating this combination using *in vitro* and *in vivo* studies (Weese et
378 al., 2004). Given that all rats tolerated the probiotic treatment well and strains *L. animalis*
379 223C and *L. animalis* 5323 in particular demonstrated superior oxalate degradative
380 capability, these strains are being further investigated as a probiotic food supplement for
381 the prevention and treatment of hyperoxaluria and renal stone formation.

382

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388

389 **Disclosures**

390 Alimentary Health is a multi-departmental university campus-based research
391 company, which investigates host-flora interactions. The content of this article was
392 neither influenced nor constrained by this fact.

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456 **Table 1.** Resistance of putative probiotic strains to porcine bile acids. Probiotic strains
457 were streaked onto MRS agar supplemented with porcine bile at 0.5, 1.0 and 5.0% (w/v).
458 Plates are incubated @ 37°C under anaerobic conditions and growth was recorded after
459 24-48 h. **Survival is illustrated as the mean percent of control (n=3; mean +/- SD).**

460

461 **Table 2.** Quantity of freeze-dried probiotic consumed ad libitum/day by each group
462 (n=3). The average dose of probiotics consumed /day was 9.8×10^9 CFU. **Doses are**
463 **illustrated as the mean dose/group +/- SD.**

464

465 **Table 3.** Animal weights for the placebo and test groups are illustrated over the 4
466 week feeding study. Body weight was not significantly influenced (compared to
467 placebo) by feeding probiotics to the animals. Results are illustrated as mean
468 (grams) per group (n=6) +/- SD.

469 **Fig. 1.** Lack of ammonium oxalate degradation by strains of *Bifidobacterium* species was
470 observed *in vitro*. No significant difference ($p>0.05$) was observed when compared to the
471 ammonium oxalate media control. The species examined were 11 *B. longum*, 1 *B.*
472 *globosum* and 1 *B. animalis*. **Results are expressed as mean +/- SD.**

473

474 **Fig. 2.** Degradation of ammonium oxalate by strains of *Lactobacillus* species *in vitro*. No
475 significant difference ($p>0.05$) was observed for 7 of the strains (*L. acidophilus*, *L.*
476 *reuteri*, *L. salivarius*). 11/18 strains (*L. animalis*, *L. murinus*) demonstrated significant
477 oxalate degradation ($p < 0.05$) when compared to the ammonium oxalate media control.
478 The detected oxalate degradation appears to be species dependent, with *L. animalis* and *L.*
479 *murinus* degrading oxalate and *L. acidophilus*, *L. reuteri* and *L. salivarius* demonstrating
480 no oxalate degradation *in vitro*. **Results are expressed as mean +/- SD.**

481 * $p<0.05$ versus control

482

483 **Fig. 3.** Survival of selected probiotics in a low pH environment. Bacterial cells
484 (approximately 10^8 cfu/ml) are resuspended into MRS broth adjusted with 1 N HCl to pH
485 2.5. Survival was measured at intervals of 0, 30, 60, 120, 180 and 360 min using the plate
486 count method. **Results are expressed as mean +/- SD.**

487

488 **Fig. 4.** Stability of putative probiotic strains during storage for 1 month at room
489 temperature. Selected probiotic strains were examined for their stability to freeze-drying
490 and their shelf life at room temperature for one month was assessed following the process

491 using the plate count method on MRS agar (n=2). **Results are expressed as mean +/-**
492 **SD.**

493

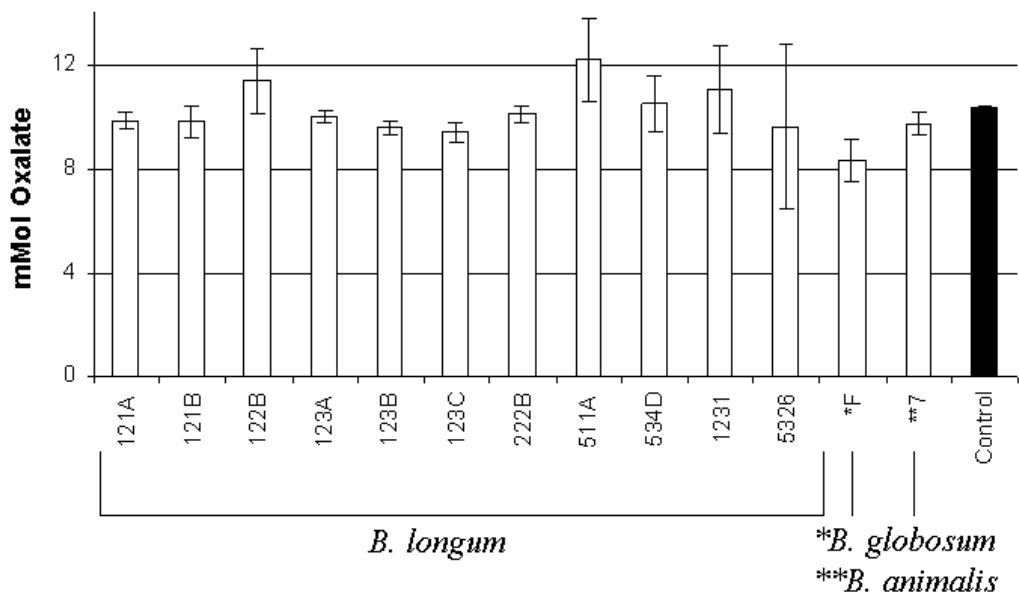
494 **Fig. 5.** Gastric transit of Rif^R freeze-dried probiotics. Freeze-dried Rif^R probiotics were
495 administered, ad libitum, at a dose of 9.8×10^9 CFU/dose to Sprague Dawley rats
496 (n=3/group). No Rif^R probiotics were detected on Day 0, which was prior to feeding and
497 confirms the selection of the Rif^R probiotics post feeding. Rif^R probiotics were detected
498 on Days, 1, 3 and 6 (post feeding) with no significant difference ($p>0.05$) observed
499 between groups fed probiotic or between the transit on Days 1, 3 and 6. **Results are**
500 **expressed as mean +/- SD.**

501

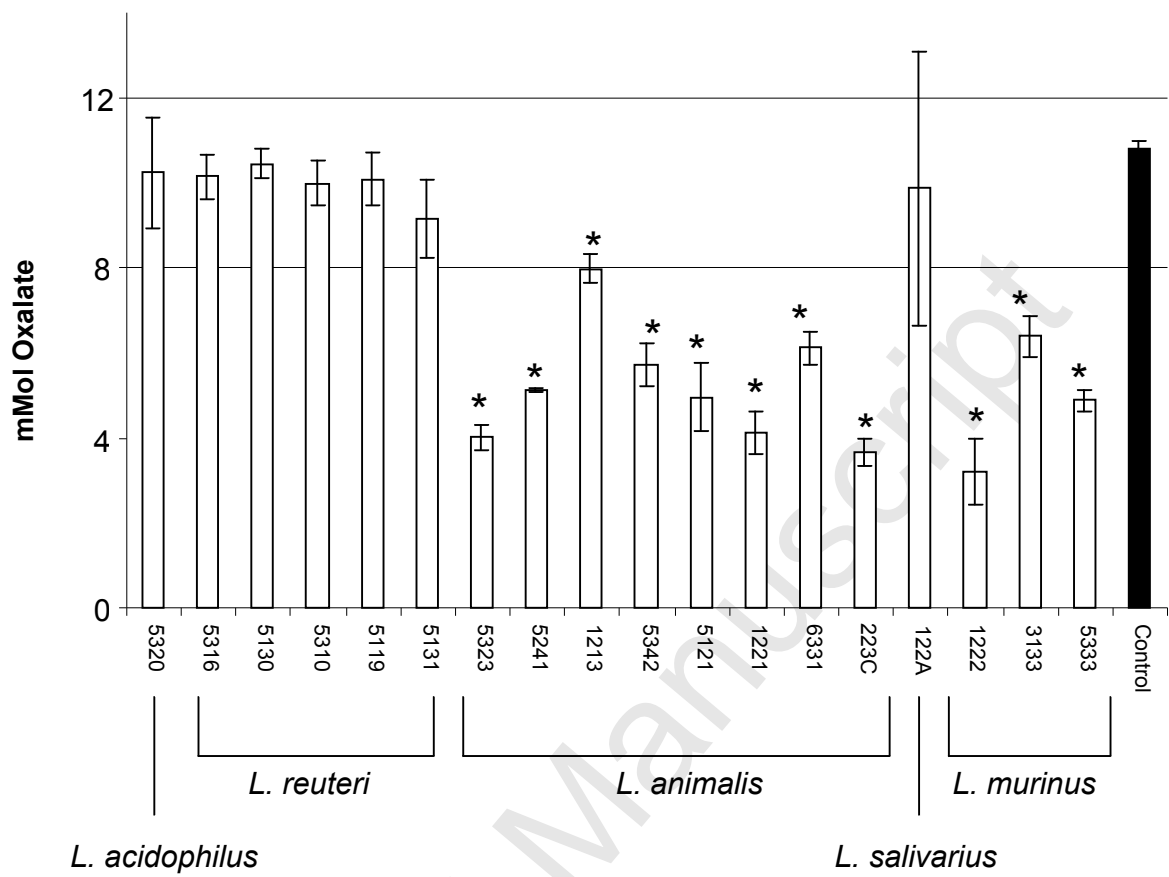
502 **Fig. 6.** Reduction of urine oxalate concentration by different strains of LAB *in vivo*.
503 Comparison of urine oxalate concentration ($\mu\text{M}/24$ hours) of rats before (Week 0) and
504 after probiotic or placebo treatment (n=6/group) revealed that *L. animalis* 223C and *L.*
505 *animalis* 5323 significantly reduced oxalate concentration when compared to placebo.
506 **Results are expressed as mean +/- SD.**

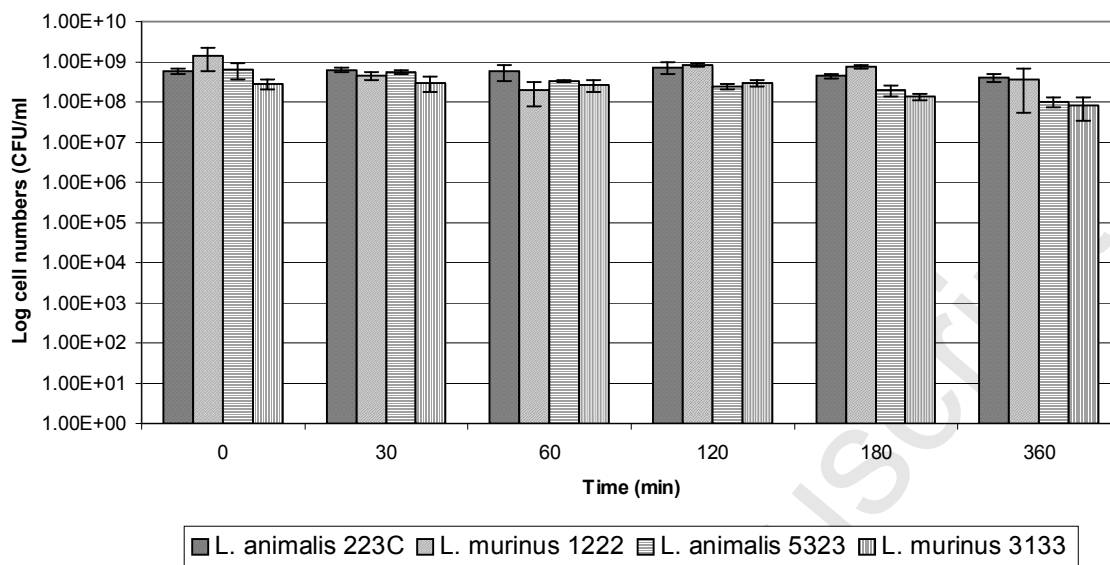
507 * $p<0.05$ versus placebo

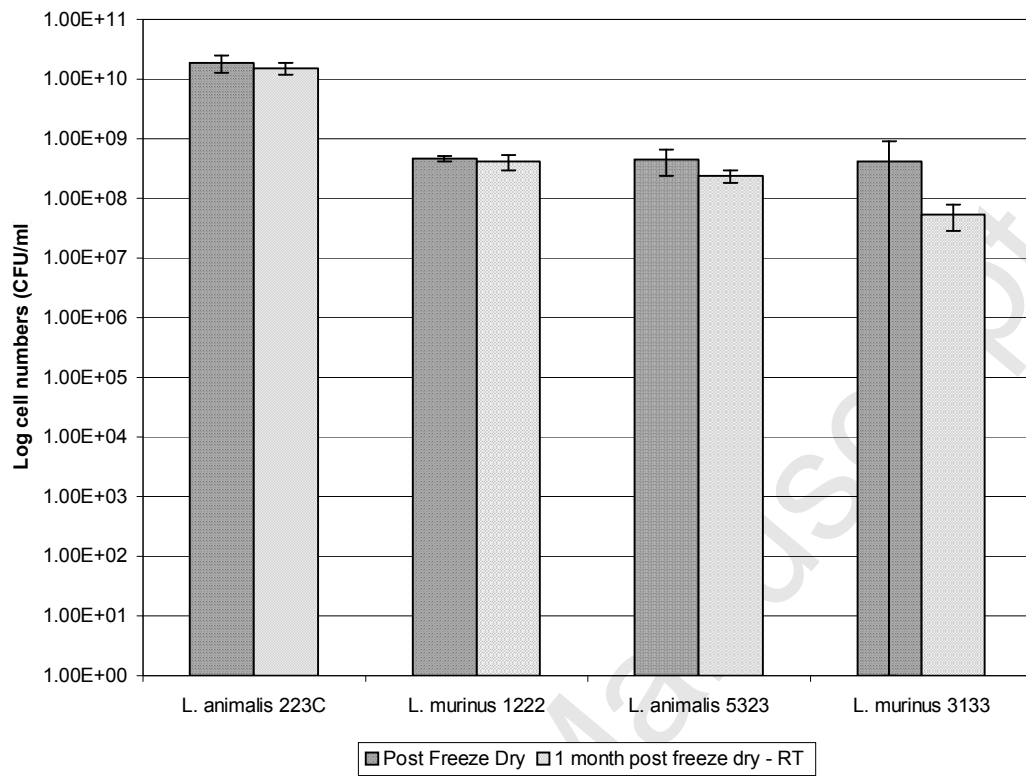
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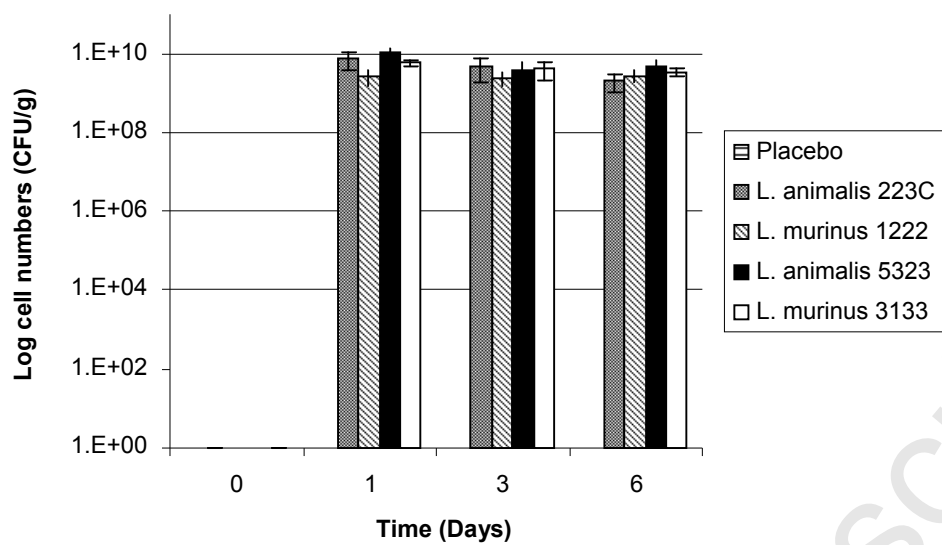


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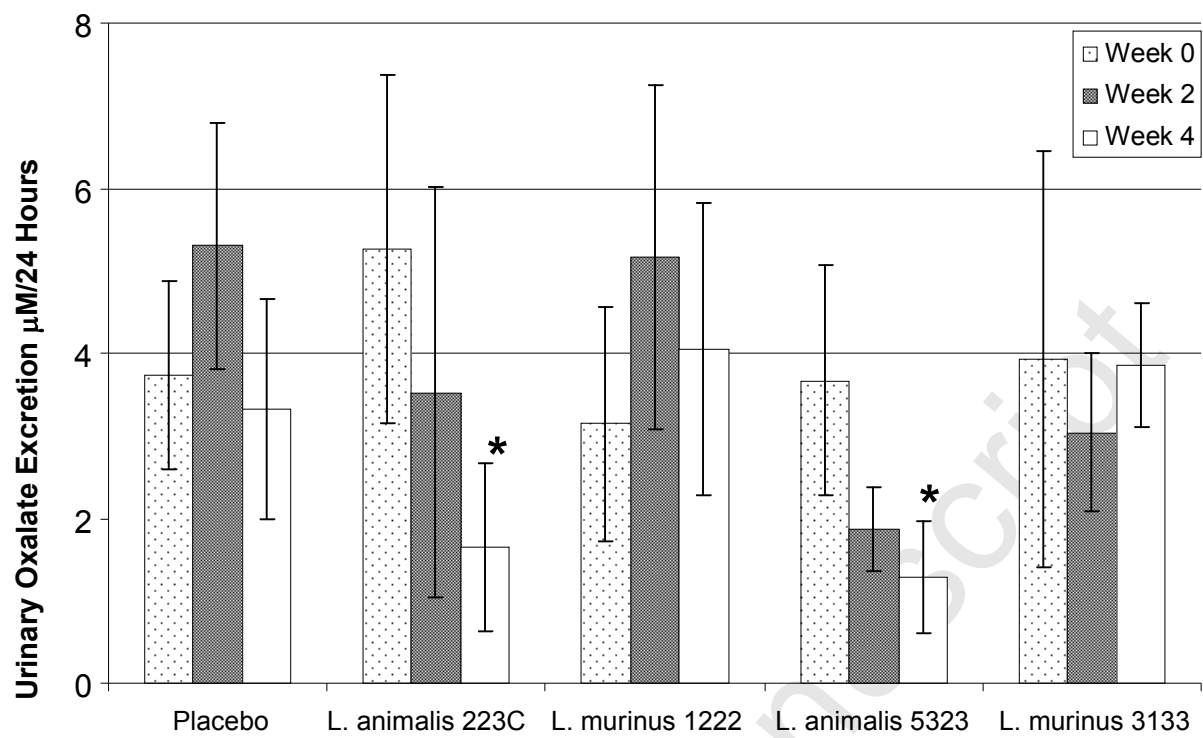








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| Strain ID | Percentage survival in porcine bile \pm standard deviation | | |
|-------------------------|--|-------------------|-------------------|
| | 0.5% Bile | 1.0% Bile | 5.0% Bile |
| <i>L. animalis</i> 223C | 66.6 \pm 0.00 | 66.6 \pm 0.00 | 49.95 \pm 23.55 |
| <i>L. murinus</i> 1222 | 100 \pm 0.00 | 83.3 \pm 23.55 | 66.6 \pm 0.00 |
| <i>L. animalis</i> 5323 | 100 \pm 0.00 | 66.6 \pm 0.00 | 49.95 \pm 23.55 |
| <i>L. murinus</i> 3133 | 66.6 \pm 0.00 | 49.95 \pm 23.55 | 49.95 \pm 23.55 |

| Group | Freeze dried probiotic (CFU/ml) | Volume consumed ad libitum/day (ml) | Probiotic dose/day (CFU, n=3) |
|----------------------------|--|--|--|
| Placebo | 0 | 19.00 ± 3.21 | 0 |
| <i>L. animalis</i> 223C | 6.8 x 10 ⁸ | 19.67 ± 1.67 | 1.29 x 10 ¹⁰ ± 1.43 x 10 ⁸ |
| <i>L. murinus</i> 1222 | 3.4 x 10 ⁸ | 20.67 ± 2.19 | 7.03 x 10 ⁹ ± 2.90 x 10 ⁹ |
| <i>L. animalis</i> 5323 | 7.8 x 10 ⁸ | 17.67 ± 3.71 | 1.38 x 10 ¹⁰ ± 1.61 x 10 ⁸ |
| <i>L. murinus</i> 3133 | 3.1 x 10 ⁸ | 17.67 ± 0.58 | 5.48 x 10 ⁹ ± 9.29 x 10 ⁷ |

| Group | Weight (grams) \pm SD | | |
|-------------------------|-------------------------|--------------|--------------|
| | Week 0 | Week 2 | Week 4 |
| Placebo | 129 \pm 5 | 140 \pm 4 | 156 \pm 4 |
| <i>L. animalis</i> 223C | 136 \pm 9 | 157 \pm 11 | 169 \pm 12 |
| <i>L. murinus</i> 1222 | 137 \pm 11 | 147 \pm 10 | 163 \pm 13 |
| <i>L. animalis</i> 5323 | 131 \pm 6 | 148 \pm 10 | 162 \pm 8 |
| <i>L. murinus</i> 3133 | 125 \pm 8 | 139 \pm 9 | 155 \pm 7 |