Intestinal persistence of *Bifidobacterium infantis* is determined by interaction of host genetics and antibiotic exposure

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21 **ABSTRACT**

22 Probiotics have gained significant attention as a potential strategy to improve health by

23 modulating host-microbe interactions, particularly in situations where the normal microbiota

24 has been disrupted. However, evidence regarding their efficacy has been inconsistent, with

25 considerable inter-individual variability in response. We aimed to explore whether a common

26 genetic variant that affects the production of mucosal $\alpha(1,2)$-fucosylated glycans, present in

27 around 20% of the population, could explain the observed interpersonal differences in the

28 persistence of commonly used probiotics. Using a mouse model with varying $\alpha(1,2)$-

29 fucosylated glycans secretion ($Fut2^{WT}$ or $Fut2^{KO}$), we examined the abundance and

30 persistence of *Bifidobacterium* strains (*infantis, breve,* and *bifidum*). We observed significant

31 differences in baseline gut microbiota characteristics between $Fut2^{WT}$ and $Fut2^{KO}$ littermates,

32 with $Fut2^{WT}$ mice exhibiting enrichment of species able to utilise $\alpha(1,2)$-fucosylated glycans.

33 Following antibiotic exposure, only $Fut2^{WT}$ animals showed persistent engraftment of

34 *Bifidobacterium infantis*, a strain able to internalise $\alpha(1,2)$-fucosylated glycans, whereas *B.*

35 *breve* and *B. bifidum*, which cannot internalise $\alpha(1,2)$-fucosylated glycans, did not exhibit this

36 difference. In mice with an intact commensal microbiota, the relationship between secretor

37 status and *B. infantis* persistence was reversed, with $Fut2^{KO}$ animals showing greater

38 persistence compared to $Fut2^{WT}$. Our findings suggest that the interplay between a common

39 genetic variation and antibiotic exposure plays a crucial role in determining the dynamics of

40 *B. infantis* in the recipient gut, which could potentially contribute to the observed variation in

41 response to this commonly used probiotic species.

42

43 **Keywords**

44 Microbiome, secretor status, $\alpha(1,2)$-fucosylated glycans, *Bifidobacterium*, *Bifidobacterium*

45 *infantis*

2

INTRODUCTION

Host-microbiome interactions play a pivotal role in shaping human physiology. The intestinal microbiome in particular is an important regulator of innate and adaptive immunity [1], metabolic control [2], the central nervous system [3], as well as contributing to energy and nutrient harvest [4], and suppressing pathogen proliferation [5]. Given the association between disruption of the commensal gut microbiota and adverse outcomes, there is significant interest in approaches that facilitate its restoration following perturbation. Among the most well-established of these approaches is the ingestion of viable commensal bacteria in the form of probiotics.

Probiotics can be defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" [6]. Most commonly, these take the form of individual strains or multi-strain consortia, of well-characterised commensal bacteria, prepared either as liquid suspensions or in freeze-dried capsules. The principal concept underlying the use of probiotics is that the introduction of live bacteria can re-establish physiological homeostasis by modifying the composition or behaviour of the gut microbiota, or by directly providing regulatory cues to the host. Despite substantial evidence supporting the efficacy of probiotics in principle, their use remains poorly supported by empirical data in many physiological or health contexts [7, 8]. Further, substantial inter-individual variance in probiotic persistence has been noted, in part explained by variation in colonisation resistance by the microbiome [9, 10]. Consequently, the global probiotics market, which is projected to reach USD 73.9 billion by 2030 [11], is dominated by direct-to-consumer sales, with little or no consideration is given to recipient traits that might substantially influence probiotic efficacy.

71 Various mechanisms enable the human gut to regulate commensal microbiota composition.

72 One of the principal mechanisms involves the secretion of specific types of sugars that are

73 utilised by beneficial microbial species. Many mucosal constituents and secreted factors are

74 decorated with glycans (oligosaccharides), that are added by a diverse family of

75 glycosyltransferase enzymes [12]. Of these, the *FUT2* gene encodes a galactoside α(1,2)-

76 fucosyltransferase, which adds a L-fucose monosaccharide to non-reducing end Gal residues

77 to form Fucα1-2Gal-O-R glycans, termed the H antigens [13, 14]. Expressed by multiple

78 mucosal epithelial cell types, this H antigen, is a highly versatile structure that can be further

79 modified to form many other important glycans, including the AB blood group glycans.

80 Because *FUT2* controls the nature of the various α(1,2)-fucosylated glycans secreted by

81 mucosal surfaces, it is commonly referred to as the "secretor" gene [13].

82

83 Across the human population, multiple nonsense single nucleotide polymorphisms (SNPs)

84 are found within the *FUT2* gene [15], leading to a "non-secretor" phenotype. The non-

85 secretor phenotype, like the AB blood groups, is one of the more common functional

86 mutations maintained in the population, with approximately one-fifth of people carrying

87 homozygous loss-of-function *FUT2* genes [15]. This high carriage of loss-of-function

88 mutations is likely a result of positive selection from altered susceptibility to infections by

89 certain bacterial and viral pathogens [16]. However, as fucosylated glycans are an important

90 nutrient source for gut microbes, their absence in non-secretors has been shown to influence

91 the composition of commensal microorganisms [16, 17].

92

93 The intact commensal microbiota of secretor individuals is likely to be enriched for glycan

94 utilising bacteria. In contrast, depletion of commensal taxa, for example, through antibiotic

95 exposure, can provide a selective advantage to exogenous glycan utilisers that are absent in

96 non-secretors [9, 18]. Probiotic preparations typically contain *Bifidobacteria*

97 (*Bifidobacterium adolescentis*, *animalis*, *bifidum*, *breve,* and *longum*) and/or Lactobacilli

98 (*Lactobacillus acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*,

99 *rhamnosus,* and *salivarius*). Both genera include species that encode the specific glycoside

100 hydrolases (GHs), GH29, GH95, and GH151, which can utilise the H antigen. However, both

101 genera also include species without this glycoside hydrolase capacity. Therefore, the ability

102 of a probiotic to colonise and persist in an individual may depend on the presence of secreted

103 glycans and the ability of the introduced bacterial strain to utilize them. This is supported by

104 studies identifying increased persistence of such glycan-utilising species when supplemented

105 with exogenous oligosaccharides [19, 20].

106

107 We hypothesised that the interplay between secretor status, the glycan utilisation ability of

108 the probiotic strain, and the presence of a disrupted commensal microbiota due to antibiotic

109 exposure, would collectively influence the abundance and persistence of probiotic

110 populations in the gut. To test this hypothesis, we introduced probiotic *Bifidobacterium*

111 strains into a murine model of secretor/non-secretor status, with or without prior antibiotic

112 depletion of commensal microbiota.

113

MATERIALS AND METHODS

Details of reagent catalogue numbers and resource links are provided in **Supplementary Table S1**.

Mouse model

Establishment of a Fut2 knockout mice

A *Fut2*^{KO} mouse line was developed using CRISPR/Cas9 technology in C57BL/6 mice (IMSR_JAX:000664) by South Australian Genome Editing (SAGE). Briefly, a 1230 bp region of the *Fut2* exon region was excised using targeted CRISPR guide sequences. Gene knock out was confirmed by Sanger sequencing and phenotype confirmed by α(1,2)-fucosylated glycan staining of intestinal biopsies using Ulex Europaeus lectin 1 (**Supplementary Fig. S1**), as described previously [21]. Littermate *Fut2*^{WT} and *Fut2*^{KO} mice (six weeks of age, gender-matched) were obtained by mating heterozygous male and female mice originating from F1 heterozygotes.

Breeding and housing

All mice were bred and maintained under specific and opportunistic pathogen free (SPF) conditions at 22°C ± 2°C, under a 12 h light-dark cycle, at the South Australian Health and Medical Research Institute (SAHMRI). All mice were housed in individually ventilated cages, fed an identical diet (Teklad Global 18% Rodent Protein Diet, Envigo, Huntingdon, UK), maintained under the Federation of European Laboratory Animal Science Associations (FELASA) standards and routinely screened using a SNP genotyping panel.

Heterozygous x heterozygous breeding was performed to allow for *Fut2*^{KO} and *Fut2*^{WT} littermates, while also standardising effects of *Fut2* that occur through vertical transmission.

139   *Fut2*[KO], *Fut2*[HET], and *Fut2*[WT] littermates were co-housed from birth until weaning (~3

140   weeks), where they were genotyped by PCR amplicon melt curve using primers targeting the

141   outer and inner regions of the *Fut2* gene. *Fut2*[KO] and *Fut2*[WT] mice separated into cages after

142   weaning based on sex and *Fut2* genotype (**Figure 1A**). No experiments were performed on

143   *Fut2*[HET] mice. In all experiments, 6-week old, age- and sex-matched mice were used. Each

144   experimental group consisted of at least 4 cages to control for cage effects. Given the

145   heterogeneous nature of the gut microbiome, each mouse was considered as a biological

146   replicate rather than a technical replicate, even within cohoused littermates.

147

148   *Antibiotic treatment*

149   A cocktail of ampicillin (1 g/L, Sigma-Aldrich) and neomycin (0.5 g/L, Sigma-Aldrich) was

150   provided to mice via drinking water for 7 days. Water bottle volume and mouse weight were

151   monitored to assess water intake. Antibiotic activity was confirmed by qPCR targeting 16S

152   rRNA gene (**Supplementary Table S2**) of faecal samples at day 7.

153

154   *Probiotic supplementation*

155   Mice received 5×10^7 colony forming units (CFU)/g of mouse of either *B. infantis, B.*

156   *bifidum*, or *B. breve* daily for 5 days via oral gavage. A starting gavage concentration of $5 \times$

157   10^9 CFU/mL in PBS was prepared daily from fresh overnight cultures. The dose was selected

158   based on previous reports of safety, persistence, and immune modulation capability [22, 23].

159

160   **Bacterial strains**

161   *B. bifidum* JCM 1255 (ATCC equivalent: 29521), *B. longum subspecies infantis* JCM 1222

162   (ATCC equivalent: 15697), *B. breve* JCM 1192 (ATCC equivalent: 15700) were obtained

163   from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan). Utilisation of

164 α(1,2)-fucosylated glycans was assessed by *in vitro* culture in mBasal media with/without

165 supplementation of 5% w/v 2′-fucosyllactose (Layer Origin, Ithaca, New York, USA)

166 (**Supplementary Fig. S2**). This was confirmed by existing literature [24, 25] and the

167 Carbohydrate-Active enZYmes (CAZy) database [26] showing that *B. bifidum* JCM 1255

168 encodes external GH29 and GH95 α-1,2-L-fucosidases, but does not consume fucose as a

169 carbon source [27], *B. infantis* JCM 1222 encodes intracellular GH29, GH95, and GH151 α-

170 1,2-L-fucosidases along with glycan transporters, and *B. breve* JCM 1192 encodes only the

171 GH95 family.

172

173 **Faecal and tissue collection**

174 At least two faecal pellets were collected from separated mice at the beginning of the light

175 phase, unless specified otherwise. For intestinal tissue collection, mice were sacrificed by

176 CO_2 asphyxiation and laparotomy was immediately performed using a vertical midline

177 incision. Once the digestive tract was exposed, separate dissection tools were used to dissect

178 tissue into four parts: the proximal small intestine; distal small intestine; caecum; and large

179 intestine. For small and large intestine tissue segments, the luminal content was collected by

180 instilling sterile PBS using a syringe barrel and the flushed mucosal tissue was collected into

181 separate tubes. All collected faecal samples, organs, and luminal contents were immediately

182 frozen on dry ice and stored at -80°C until further processing.

183

184 **DNA extraction**

185 *DNA extraction of faecal samples*

186 Faecal pellets were weighed, and 25 mg samples (±10 mg) were resuspended in 300 µL of

187 cold phosphate buffered saline (PBS) (pH 7.2) by vortexing and pelleted by centrifugation at

188 $10,000 \times g$ for 10 min at 4°C. Microbial DNA was extracted from faecal samples using the

189 PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the

190 manufacturer's instructions as described previously [28].

191

DNA extraction on mucosal tissue samples

193 Mucosal tissue from the proximal small intestine, distal small intestine, and large intestine

194 were semi defrosted, and 3 cm was removed from the tissue centre using sterile scalpel. The

195 dissected tissues were cut open longitudinally and mixed with 750 µL PowerSoil bead

196 solution, and 60 µL solution C1 in a PowerSoil bead tube. The bead tube was then incubated

197 at 65°C for 10 mins prior to bead beating. The subsequent DNA isolation was performed

198 using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to

199 the manufacturer's instructions as described previously [28].

200

16S rRNA gene amplicon sequencing and bioinformatic processing

202 Amplicon libraries of the V4 hypervariable region for 16S rRNA gene were prepared from

203 DNA extracts using modified universal bacterial primer pairs 515F and 806R [29]. Amplicon

204 libraries were indexed, cleaned, and sequenced according to the 16S Metagenomic

205 Sequencing Library Preparation protocol. Paired-end sequencing was performed using MiSeq

206 Reagent Kit v3 (600-cycle kit) (Illumina) on a MiSeq Sequencing System (Illumina), at the

207 South Australian Genomics Centre (SAGC). Paired-end 16S rRNA gene sequence reads were

208 analysed using QIIME2 version 2021.11.0 [30]. Briefly, de-noising was performed on de-

209 multiplexed sequences using the DADA2 plugin [31], resulting in a mean read depth of

210 $15,563 \pm 2,719$ for stool and $6,941 \pm 4,503$ for tissue. Taxonomic classification of amplicon

211 sequence variants (ASVs) was performed based on the V4 hypervariable region of the

212 SILVA 16S rRNA gene reference database (version 138) at 99% similarity [32]. Sufficient

213 coverage at this depth is confirmed by the rarefaction curve, which reached an asymptote.

9

214 Sequence data has been deposited in the NCBI SRA under accession number

215 PRJNA1011386.

216

217 **Microbiota characterisation**

218 The taxonomic relative abundance at the genus level was used to generate alpha diversity

219 (within-group) and beta diversity (between-group) measures. Alpha diversity metrics

220 (observed ASVs, Pielou's evenness, Shannon diversity, and Faith's phylogenetic diversity)

221 were obtained from QIIME2 at sampling depth of 9,883 reads (faecal samples) and 662 reads

222 (mucosal tissue samples). The Bray-Curtis dissimilarity index was calculated to compare

223 microbiome similarity between groups (beta diversity), using square-root transformed species

224 relative abundance data using the 'vegan' package in R. Non-metric multidimensional scaling

225 (nMDS) for all beta diversity measures were generated using the 'vegan' package in R. Core

226 taxa were defined as those present in more than 95% of samples, with a mean relative

227 abundance of >0.01%. Identification of taxa with α-1,2-L-fucosidases capability was

228 determined by comparing the genus-level taxonomic classification to genomes identified by

229 CAZy [26] as carrying either the GH29, GH95, or GH151 enzyme families.

230

231 **Quantification of *Bifidobacterium* species and total bacterial load**

232 We investigated the extent to which 16S amplicon sequencing could discriminate between

233 different *Bifidobacterium* species. As expected, level 7 resolution (species-level output) was

234 unable to differentiate bifidobacterial strains, reflecting a well-recognised limitation of this

235 approach. Given that, quantification of total bacterial load, *B. breve*, *B. infantis*, and *B.*

236 *bifidum* was performed by SYBR Green-based qPCR assays (**Supplementary Table S2**). For

237 all qPCR assays, 1 µL of DNA template was combined with 0.7 µL of 10 µM forward

238 primer, 0.7 µL of 10 µM reverse primer, 17.5 µL of 2×SYBR Green (Applied Biosystems,

239 Waltham, Massachusetts, USA), and 15.1 µL nuclease-free water. All samples were run in

240 triplicate (10 µL each replicate). Gene copy quantification was performed using a standard

241 curve generated from a known concentration of a pure colony control. Any sample with a

242 cycle threshold (CT) ≥40 cycles was defined as 40 (limit of detection).

243

244 **Culture of *Bifidobacterium* strains**

245 All *Bifidobacterium* strains used in this study were cultured in De Man, Rogosa and Sharpe

246 (Becton Dickinson, Franklin Lakes, NJ, USA) broth or agar supplemented with 0.34% (w/v)

247 sodium ascorbate and 0.02% (w/v) cysteine-HCl (MRS-CS) and were grown under anaerobic

248 conditions (75% N_2, 20% CO_2, 5% H_2, Coy Laboratory Products, Grass Lake, Michigan,

249 USA) at 37 °C. The growth of *Bifidobacterium* was measured by optical density (OD_{600})

250 using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

251

252 ***In vitro* glycan utilisation assay**

253 Faecal pellets from untreated WT mice were incubated in mBasal media (mBasal; 10 g/L

254 Trypton, 2 g/L yeast extract, 5 g/L NaCl, 0.2 g/L magnesium sulfate, 2 g/L dipotassium

255 hydrogen phosphate, pH 6.4) with/without supplementation of 5% w/v of 2′-Fucosyllactose

256 (Layer Origin, Ithaca, New York, USA) at 37°C under strict anaerobic conditions (75% N_2,

257 20% CO_2, 5% H_2, Coy Laboratory Products, Grass Lake, Michigan, USA). Bacterial

258 biomass was measured by optical density (OD_{600}) using multimode plate reader

259 (PerkinElmer, Waltham, Massachusetts, USA). Bacterial colonies cultured from faecal

260 samples were identified by matrix assisted laser desorption/ionization time-of-flight

261 (MALDI-TOF) (Billerica, Massachusetts, United States), as described previously [33].

262

263 **Statistical analysis**

264 Experimental mice were randomly assigned to different treatment groups. The investigators

265 were not blinded to the experimental groups. No outliers have been removed from any of the

266 data presented. All data analyses were performed using either R (R Foundation for Statistical

267 Computing; version 4.1.0) or GraphPad Prism software (GraphPad Software, Inc.; version

268 9.00). For parametric data, unpaired Student's t test was used to compare data between two

269 unpaired groups; One-way ANOVA was used to compare data among three or more unpaired

270 groups. For non-parametric data, Mann-Whitney U test was used to compare data between

271 two unpaired groups; Kruskal-Wallis test was used to compared data among three or more

272 unpaired groups. Differences in Bray-Curtis dissimilarity between groups was performed by

273 permutational multivariate ANOVA (PERMANOVA) and pairwise PERMANOVA, using

274 the "adonis" package in R, with 9,999. Linear discriminant analysis Effect Size (LEfSe) was

275 applied to identify the abundant taxa in each site, using default parameters [34]. Area under

276 the curve (AUC) was calculated for *in vitro* growth experiments (using OD_{600} values) and

277 bifidobacterial persistence in mice (using copies/ng faecal DNA). Log-rank test was

278 employed to compare survival time differences based on bacterial qPCR detection. One-

279 tailed tests were used where differences between groups were hypothesised to be in a single

280 direction. Statistical outcomes with P value <0.05 were considered statistically significant.

281 Core taxa plot was generated using GraphPad Prism, other data were visualised using R.

282   **RESULTS**

283   *Fut2* **shapes the faecal microbiota**

284   Assessment of the faecal microbiota between SPF $Fut2^{WT}$ and $Fut2^{KO}$ littermates was

285   performed at 6 weeks of age in both male and female mice (**Fig. 1A**). Faecal microbiota

286   composition (**Fig. 1B**) differed significantly between $Fut2^{WT}$ and $Fut2^{KO}$ littermates

287   (PERMANOVA: R^2=0.028; P=0.028, **Fig. 1C**) when male and female mice were assessed

288   together. However, stratification according to sex identified a greater divergence according to

289   genotype in male mice (PERMANOVA: R^2=0.12; P=0.021) compared to female mice

290   (PERMANOVA: R^2=0.037; P=0.38, **Fig. 1C**). Exploration of this sex effect identified a

291   significant interaction between sex and *Fut2* genotype (PERMANOVA: R^2=0.11; P=0.0068,

292   **Supplementary Table S3**). These findings were unchanged after adjustment for cage effects

293   (**Supplementary Table S3**). Given the interaction between sex and genotype, all subsequent

294   experiments involved male mice only.

295

296   Potential relationships between secretor status and microbiota characteristics were then

297   explored. While bacterial alpha-diversity measures did not differ substantially between

298   differed between $Fut2^{WT}$ and $Fut2^{KO}$ groups (**Supplementary Fig. S3**), the membership of

299   core microbiota (taxa present in ≥95% of samples at ≥0.01%) did (**Fig. 1D**). Specifically,

300   *Oscillospiraceae* (unassigned) were exclusively core in $Fut2^{WT}$, whereas *Bifidobacterium*,

301   Clostridia (UCG014), *Lachnoclostridium*, and *Parabacteroides* were exclusively core in

302    $Fut2^{KO}$ mice (**Fig. 1E**). Three bacterial genera had a significantly higher relative abundance

303   in $Fut2^{WT}$ mice, whereas 13 were more prevalent in $Fut2^{KO}$ mice (**Fig. 1F**). Of these,

304   *Lactobacillus,* a genus with GH29 and GH151 that comprise α-1,2-L-fucosidases

305   (**Supplementary Table S4**), was more abundant in $Fut2^{WT}$ (LDA score [$\log_{10}$]=4.88).

13

306   Together, these findings support previous reports of a relationship between *Fut2* and faecal

307   microbiome characteristics.

308

309 Distal small intestine microbiota influenced by *Fut2*

310   As *Fut2* is differentially expressed throughout the gastrointestinal tract [35], we then

311   compared microbiota characteristics in tissue from the proximal small intestine, the distal

312   small intestine, and the large intestine between $Fut2^{WT}$ and $Fut2^{KO}$ mice (**Fig. 2A**).

313   Microbiota composition differed significantly between $Fut2^{WT}$ and $Fut2^{KO}$ groups in the

314   distal small intestine (PERMANOVA: R^2=0.54; P=0.0071, **Fig. 2B**), coinciding with

315   inducible *Fut2* expression, but not in the proximal small intestine (PERMANOVA: R^2=0.19;

316   P=0.13, **Fig. 2B**) or the large intestine (PERMANOVA: R^2=0.16; P=0.18, **Fig. 2B**).

317   Taxonomic assessment identified that *Candidatus* Arthromitus (LDA score [$\log_{10}$]=5.43) was

318   more abundant in the distal small intestine in $Fut2^{WT}$ compared to $Fut2^{KO}$ mice, whereas

319   *Lachnospiraceae* (unassigned) and *Acetatifactor* were more abundant in in the large intestine

320   of $Fut2^{WT}$ compared to $Fut2^{KO}$ mice (LDA score [$\log_{10}$]=4.35, and LDA score [$\log_{10}$]=3.93,

321   respectively) (**Fig. 2C**).

322

323 *Fut2*-microbiota relationships can be recapitulated *in vitro* through glycan exposure

324   To further investigate the relationship between α(1,2)-fucosylated glycans and intestinal

325   microbiology, faecal homogenate from $Fut2^{WT}$ mice was grown in a mBasal media with or

326   without the α(1,2)-fucosylated glycan, 2'-FL. Microbiota assessment following *in vitro*

327   culture (**Fig. 3A**) confirmed the findings of the *in vivo* faecal microbiota analysis, with a

328   significant difference in Bray-Curtis similarity between faecal cultures with and without 2'-

329   FL (PERMANOVA: R^2=0.90; P<0.0001, **Fig. 3B**). This difference was marked by an

14

330 enrichment of glycan-utilising genera (*Bacteroides*, *Enterococcus*, *Lactobacillus*), with

331 compensatory decreases in the relative abundance of other taxa (**Fig. 3C**).

332

333 Analysis involving inoculation of solid basal media, either alone or supplemented with 2'-FL,

334 with faecal homogenate from $Fut2^{WT}$ mice further supported these findings. Specifically,

335 proportional colony counts (**Supplementary Fig. S4, Supplementary Table S5**) showed 2'-

336 FL led to enrichment of *Enterococcus faecalis* (66% with 2'-FL vs 4% without 2'-FL) and

337 *Lactobacillus murinus/reuteri* (18% with 2'-FL vs 1% without 2'-FL), with a corresponding

338 decrease in *E. coli* (16% with 2'-FL vs 95% without 2'-FL) (**Fig. 3D**). Enrichment of bacteria

339 with 2'-FL supplementation was further confirmed by increased growth rate and bacterial

340 density (median $AUC_{[mBasal]}$=15.1 [IQR=14.3, 16.5]; $AUC_{[mBasal+2'-FL]}$=35.6 [33.3, 38.7];

341 *P*<0.0001, **Fig. 3E**).

342

343 We investigated whether differences in microbiota composition between $Fut2^{WT}$ and $Fut2^{KO}$

344 mice reflected selection for bacterial populations able to utilise α(1,2)-fucosylated glycans for

345 growth in $Fut2^{WT}$ animals. Faecal homogenates from $Fut2^{WT}$ and $Fut2^{KO}$ mice were used to

346 inoculate mBasal media with or without 2'-FL supplementation. The increase in bacterial

347 density between 2'-FL supplemented media and media alone was significantly greater when

348 faecal homogenates were derived from $Fut2^{WT}$ compared to $Fut2^{KO}$ mice (median

349 $AUC_{[WT]}$=20.5 [IQR=16.6, 23.5]; $AUC_{[KO]}$=16.7 [13.1, 19.7]; *P*=0.0051, **Fig. 3F**), consistent

350 with a greater abundance of glycan-utilizing bacteria.

351

352 **Probiotic bifidobacterial species differentially colonise $Fut2^{WT}$ and $Fut2^{KO}$ mice**

353 Based on the relationship between host glycan production and gut microbiota composition,

354 we hypothesised that the colonisation dynamics (abundance and persistence) of

15

bifidobacterial probiotic species, introduced following antibiotic depletion, would differ

between $Fut2^{WT}$ and $Fut2^{KO}$ recipients (**Fig. 4A**). We utilised strains of three *Bifidobacterium*

species that interact with glycan in different ways; *B. infantis*, an intracellular α(1,2)-

fucosylated glycan-utiliser, *B. bifidum*, an extra-cellular α(1,2)-fucosylated glycan utiliser,

and *B. breve*, a species that does not utilise α(1,2)-fucosylated glycans (characteristics that

were confirmed by *in vitro* growth assays, **Supplementary Fig. S2**).

The persistence of species that do not internalise and degrade α(1,2)-fucosylated glycans (*B.

bifidum* and *B. breve*) did not differ substantially when introduced to $Fut2^{WT}$ or $Fut2^{KO}$ mice

(**Fig. 4B-4C**). In contrast, the abundance and persistence of *B. infantis*, an intracellular glycan

utiliser, differed significantly according to the genotype of the recipient (**Fig. 4B-4C**). Ten

days post-gavage, *B. infantis* was detected in 7 out of 8 $Fut2^{WT}$ mice but 0 out of 8 $Fut2^{KO}$

mice (Log-rank test: chi-q=11.4; P=0.00074, **Fig. 4B**). The overall abundance of *B. infantis*

was also significantly higher in $Fut2^{WT}$ compared to $Fut2^{KO}$ mice in the following 14 days

post-gavage (mean $AUC_{[WT]}$=31.1 [SD=4.1]; $AUC_{[KO]}$=26.2 [5.2]; P=0.028, **Fig. 4C**).

A difference in *B. infantis* persistence was also evident in intestinal tissue assessed five days

post-gavage. *B. infantis* was significantly more abundant in $Fut2^{WT}$ large intestine (median

$Fut2^{WT}$ =$\log_{10}$ 6.7 copies/g tissue [IQR=$\log_{10}$ 6.0, $\log_{10}$ 6.8]; $Fut2^{KO}$ =$\log_{10}$ 2.6 [IQR=$\log_{10}$

2.6, $\log_{10}$ 3.9]; P=0.011), and numerically, though not significantly, more abundant in $Fut2^{WT}$

proximal (P=0.085) and distal small intestine (P=0.094) (**Fig. 4D**).

Prior antibiotic exposure profoundly affects the *Fut2*-probiotic relationship

To test whether the relationship between *Fut2* genotype and probiotic strain characteristics

were independent of antibiotic exposure, we supplemented non-antibiotic exposed mice with

380   *B. infantis* (**Fig. 5A**). No significant difference in *B. infantis* persistence post-gavage was

381   observed between *Fut2*^{WT} and *Fut2*^{KO} mice (**Fig. 5B**), and *B. infantis* was not detectable in

382   intestinal tissue from either *Fut2*^{WT} or *Fut2*^{KO} mice at day 5 post-gavage (**Supplementary**

383   **Fig. S5A**). However, analysis of *B. infantis* abundance following gavage (based on AUC)

384   revealed an effect that was opposite to that observed in antibiotic exposed mice, with *B.*

385   *infantis* significantly higher in *Fut2*^{KO} mice compared to *Fut2*^{WT} (mean AUC$_{[WT]}$=3.8

386   [SD=0.6]; AUC$_{[KO]}$=5.6 [0.90]; *P*=0.00046; **Fig. 5C**).

387

388   Faecal levels of *Bifidobacterium* probiotics were assessed during the instillation period

389   (samples collected two hours prior to gavage and six hours after gavage). Levels in post-

390   gavage samples (6 hours) did not differ between *Fut2*^{WT} and *Fut2*^{KO} mice, consistent with the

391   instillation of equal probiotic loads. However, at 22 hours post-gavage (2 hours prior to

392   gavage), levels in *Fut2*^{KO} mice were significantly higher than in *Fut2*^{WT} mice (*P*<0.0001;

393   **Supplementary Fig. S5B**). No cumulative effect was observed with repeat gavage and the

394   decline in probiotic levels after 22 hours was comparable to that observed in the 24 hours

395   post cessation of installation.

DISCUSSION

Although probiotics have shown great potential in modifying host-microbiome interactions [36], their actual performance has been disappointing in many clinical contexts [37]. Previous studies have investigated the reasons for this underperformance, relating to cohort-level effects and variation in response between individuals. Mode of delivery and dose have both been shown to contribute to overall efficacy [38, 39], whereas the habitual diet of the recipient, particularly fibre intake, is an important determinant of probiotic response [40]. The influence of factors that shape the gut microbiome on the abundance and persistence of probiotics is unsurprising [41, 42], given the ability of resident gut microbiota to competitively exclude introduced populations [10]. Indeed, exposure to antibiotics, a factor that greatly impacts the gut microbiome, has been shown to considerably influence probiotic effects at a microbiological level [9, 43]. However, while common *FUT2* genetic variants are known to help shape intestinal microbiology [16, 17], the effect of secretor status on probiotics had not been described.

Our study highlights several important points in relation to inter-individual variance in intestinal microbiology and probiotic efficacy. With 20% of the global population also homozygous for a non-functional *FUT2* gene [15], our findings suggest that these "non-secretor" individuals will also experience different probiotic population dynamics compared to "secretor" individuals, if the probiotic taken contains one of the many bacterial species able to utilise $\alpha(1,2)$-fucosylated glycans (H antigens). In our study, this was reflected in the significantly greater transience of *B. infantis* in the faecal and intestinal microbiome of non-secretor (*Fut2*[KO]) mice compared with secretor mice following antibiotic exposure.

18

420   Bifidobacterial species that are commonly used as probiotics are relatively close

421   phylogenetically but differ in their ability to use glycans, even at a strain level [44]. We

422   showed that neither *B. breve* (JCM 1192) nor *B. bifidum* (JCM 1255) differed in their

423   abundance or persistence between secretor and non-secretor animals. In contrast, *B. infantis*

424   (JCM 1222) persisted for significantly longer and showed a significantly higher abundance in

425   secretor mice compared to non-secretor mice. This finding likely reflects differences in H

426   antigen hydrolysis and catabolism capacities between species when administered as a

427   probiotic. For example, *B. infantis* encodes GH29, GH95, and GH151 family intracellular α-

428   1,2-L-fucosidases, along with fucose transporters to facilitate internalisation [44]. While

429   independent hydrolysis and catabolism of mucin-bound H antigens by *B. infantis* is not

430   hypothesised [25], cross-feeding by organisms with extracellular α-1,2-L-fucosidases is

431   likely, even following antibiotic supplementation [45]. In contrast, *B. bifidum*, while

432   expressing extracellular GH29 and GH95 α-1,2-L-fucosidases, does not consume fucose to

433   facilitate growth [27]. Finally, *B. breve*, encodes a separate GH95 intracellular α-1,2-L-

434   fucosidase along with fucose transporters. While this species is capable of utilising the H

435   antigen with support from cross-feeding [46], these findings suggest reduced persistence

436   compared with *B. infantis*, when administered as a probiotic.

437

438   We found that antibiotic exposure influenced the persistence of probiotics in a secretor status-

439   dependent manner. In the absence of microbiota depletion through antibiotic exposure, it

440   would be expected that other commensal bacteria would utilise available glycans within the

441   secretor gut. Moreover, such strains are highly adapted to an individual's gut environment,

442   making them likely to outcompete any exogenous glycan-utilisers that are introduced. When

443   we explored this directly, we found that in the absence of a prior period of antibiotic

444   exposure, the higher levels and greater persistence of *B. infantis* in secretors was inverted,

19

445   with these *B. infantis* being significantly higher in non-secretor mice. These findings likely

446   reflect the competitive exclusion of H antigen-utilising probiotics in the secretor gut and

447   highlight the importance of considering the ecological context in relation to probiotic impact.

448

449   It should be noted that our antibiotic mix contained a cocktail of ampicillin and neomycin,

450   designed to deplete a wide range of bacteria. While most *Bifidobacterium* strains are resistant

451   to neomycin, the tested strains are sensitive to ampicillin [47]. We designed the experiment

452   so that gavage with *Bifidobacterium* was immediately after ceasing antibiotic depletion to

453   maximize colonisation without competition from other bacteria. It is possible that residual

454   antibiotics in the intestine deplete *Bifidobacterium* over the first days of gavage. For this

455   reason, we performed gavage for 5 days, a time period that extends beyond the activity

456   spectrum of the administered antibiotics. Such an antibiotic combination is common for

457   mouse models [48, 49], as well as empiric for suspected sepsis in humans [50].

458

459   While this study was performed in mice, the effect of secretor status on bifidobacterium

460   supplementation has important implications for probiotic strategies in humans. It is crucial to

461   consider individual host traits and recent antibiotic exposure when designing a probiotic

462   intervention [51]. The findings here suggest that the 20% of the population who are non-

463   secretors may have poorer persistence of H antigen utilising probiotics, such as *B. infantis,*

464   compared to secretors following antibiotic exposure. Conversely, in the absence of recent

465   antibiotic exposure, higher levels of microbial niche occupancy in secretors may hamper *B.*

466   *infantis* persistence compared to non-secretors. An individualised supplementation with

467   prebiotics may have potential as a means to optimise probiotic uptake in non-secretors. For

468   example, previous studies have shown that supplementation with human milk

469   oligosaccharides can enhance *B. infantis* engraftment [20], with successful supplementation

470 shown to reduce intestinal inflammation in infants [52]. Investigating additional α(1,2)-

471 fucosylated glycans, given as prebiotics, may lead to improved outcomes of *B. infantis*

472 supplementation in non-secretor individuals.

473

474 Determining the impact of secretor status on other species commonly considered beneficial

475 and marketed as probiotics is challenging due to their broad range of carbohydrate utilisation

476 capabilities [53]. For instance, *Akkermansia muciniphila*, a mucin-degrading species, has

477 been associated with a reduced risk of chronic inflammatory diseases in humans and mice

478 [54], and is a potential target for probiotic development [51]. However, its utilisation of

479 mucin glycoproteins, including the α(1,2)-fucosylated glycan, 2'-fucosyllactose [53],

480 suggests that it may also be affected by secretor status. Although *Akkermansia* was not

481 detected in the mice of this study, we found that *Candidatus* Arthromitus, another genus

482 associated with immune modulation [55], was enriched in the distal small intestinal mucosal

483 tissue of secretor mice. Genome annotation of *Candidatus* Arthromitus has indicated other

484 fucose utilisation capabilities [56], indicating that the functional *FUT2* gene may promote

485 colonisation by this species.

486

487 Our experiments involved SPF mice that were obtained through heterozygous mating. Such

488 breeding was essential to allow comparison of $Fut2^{WT}$ and $Fut2^{KO}$ littermates from a maternal

489 secretor lineage. The findings from this study are therefore independent of vertical

490 transmission effects, which are known to influence the microbiome of the offspring [57, 58],

491 and indicate that a change in gut microbiology occurred post-weaning. This difference in

492 baseline gut microbiota composition between secretors and non-secretors was also only

493 evident in male mice. The effect of sex on the relationship between secretor status and the gut

494 microbiome is difficult to explain but may relate to variable intestinal expression of *Fut2*,

495   which can be altered factors such as stress [59]. In addition, independent interactions between

496   sex hormones and the gut microbiome [60] may affect the relationship between *Fut2* and the

497   gut microbiome.

498

499   We acknowledge the importance of considering blood antigens/ABO phenotypes in

500   interpreting the influence of *FUT2* gene on the gut microbiome, as indicated by recent studies

501   [61, 62]. Indeed, in humans, *FUT2* is responsible for the generation of the H antigen, which

502   can be further modified to give the OLewis[b], ALewis[b], or BLewis[b] antigens [63]. Each of

503   these glycans can modulate the competitive advantage of particular microbes capable of

504   cleaving the oligosaccharide constituents. In the absence of *FUT2*, these Lewis[b] antigens are

505   not displayed, leading to a Lewis[a] antigen. While our study did not address these blood type

506   variations, it should be noted that even in humans, a secretor O blood group and a non-

507   secretor O blood group are not the same. The impact of this on the gut microbiome is

508   evidenced by studies reporting an association between H antigen concentrations and gut

509   microbiome characteristics [58].

510

511   Our study demonstrates a *Fut2*-dependent genetic determinant for inter-individual response

512   to probiotic supplementation, which is affected by antibiotic exposure and glycan utilisation

513   capabilities of the probiotic strain. With prior antibiotic exposure, *Fut2* functionality was

514   associated with increased persistence of *B. infantis*, consistent with its ability to utilise the H

515   antigen. However, without antibiotic exposure, *Fut2* functionality was associated with lower

516   abundance of *B. infantis,* relating to difference in baseline microbiology and niche space

517   occupation.

529 **AUTHOR CONTRIBUTIONS**

530 The study was conceived by SW, SLT and GBR. SLT, GBR, JMC, and YW designed the

531 experiments. YW and SLT performed the experiments; YW and SLT undertook the data

532 generation and statistical analysis. GBR, JMC, AR, and LEP provided technical support and

533 guidance. YW, JMC, SLT, and GBR drafted the manuscript. All authors approved the final

534 version of the manuscript.

535

536 **DECLARATION OF INTERESTS**

537 The authors declare that they have no competing interests, nor any financial or personal

538 relationships with other people or organisations that could bias this study.

539

540 **ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

541 All mice experiments were approved by the South Australia Health and Medical Research

542 Institute Animal Ethics Committee (application numbers SAM-21-07 and SAM-21-036).

543

DATA AVAILABILITY STATEMENT

All 16S rRNA gene sequencing data have been deposited to the NCBI SRA and are available

under accession number PRJNA1011386. All qPCR data is available through FigShare

(https://figshare.com/s/9fa55ea8b65304d9f722). Data processing, statistical analysis and

visualisation is available through GitHub (https://github.com/Yiming-Wang-1992).

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709

FIGURES AND TABLES

Fig. 1: *Fut2* affects the faecal microbiota of male mice. (**A**) Breeding and co-housing

design. $Fut2^{HET}$ mice were bred to produce $Fut2^{WT}$ and $Fut2^{KO}$ littermates. Genotypes were

co-housed until weaning (~3 weeks), where mice were separated by sex and *Fut2* genotype

until 6 weeks of age. (**B**) Taxa bar plot of the faecal microbiota of $Fut2^{WT}$ and $Fut2^{KO}$ mice,

also stratified by sex. (**C**) Non-metric multidimensional scaling (NMDS) plot of faecal

microbiota showing significant separation between $Fut2^{WT}$ and $Fut2^{KO}$ male mice but not

$Fut2^{WT}$ and $Fut2^{KO}$ female mice. Significance: Permutational multivariate ANOVA. (**D**) The

prevalence and mean relative abundance of faecal taxa in $Fut2^{WT}$ and $Fut2^{KO}$ male mice,

highlighting the "core" taxa present in ≥95% of mice and at a mean relative abundance of

≥0.01%. (**E**) Venn diagram of core taxa shared or different between $Fut2^{WT}$ and $Fut2^{KO}$ male

mice. (**F**) Faecal taxa significantly different between $Fut2^{WT}$ and $Fut2^{KO}$ male mice.

Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe).

Fig. 2: *Fut2* affects the intestinal microbiota. (**A**) Taxa bar plot of the intestinal mucosal

microbiota of $Fut2^{WT}$ and $Fut2^{KO}$ mice. (**B**) Non-metric multidimensional scaling (NMDS)

plots of $Fut2^{WT}$ and $Fut2^{KO}$ intestinal mucosal microbiota. Significance: Permutational

multivariate ANOVA. (**C**) Taxa significantly different between $Fut2^{WT}$ and $Fut2^{KO}$ intestinal

mucosal tissue. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe). No

taxa differed between $Fut2^{WT}$ and $Fut2^{KO}$ proximal small intestine.

Fig. 3: 2′-Fucosyllactose modifies the faecal microbiota *in vitro* and enhances growth of

α(1,2)-fucosylated glycan utilising bacteria. (**A**) Taxa bar plot of the faecal microbiota of

$Fut2^{WT}$ mice following anaerobic growth either with or without 2′-Fucosyllactose (2′-FL).

735 (**B**) Non-metric multidimensional scaling (NMDS) plot of faecal microbiota following

736 anaerobic growth with or without 2′-FL. Significance: Permutational multivariate ANOVA.

737 (**C**) Taxa significantly different between *Fut2*^{WT} faecal samples following anaerobic growth

738 with or without 2′-FL. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe).

739 (**D**) Comparison of colonies relative abundance of identified colonies following anaerobic

740 growth with or without 2′-FL. *E. coli* or *Shigella.* (**E**) Optical density (OD) following

741 *Fut2*^{WT} faecal bacteria cultured with or without 2′-FL. (**F**) OD of *Fut2*^{WT} or *Fut2*^{KO} faecal

742 bacteria following growth with 2˙-FL. OD normalised to growth in media without 2˙-FL.

743

744 **Fig. 4: *Bifidobacterium infantis*, but not *B. breve* or *B. bifidum,* persists longer in *Fut2*^{WT}**

745 **mice following antibiotic pre-exposure.** (**A**) Experimental design. (**B**) Persistence of

746 detectable *Bifidobacterium* species in stool following antibiotic pre-exposure. Significance:

747 log-rank test. (**C**) Bacterial copies of *Bifidobacterium* species in stool following antibiotic

748 pre-exposure. Significance: T-test of area under the curve. (**D**) Bacterial copies of *B. infantis*

749 in intestinal tissue mucosa following antibiotic pre-exposure. Significance: Mann-Whitney U

750 test.

751

752 **Fig. 5: *Bifidobacterium infantis* persists longer in *Fut2*^{KO} mice without antibiotic pre-**

753 **treatment.** (**A**) Experimental design. (**B**) Persistence of gavaged *Bifidobacterium infantis* in

754 stool without antibiotic pre-treatment. Significance: log-rank test. (**C**) Bacterial copies of *B.*

755 *infantis* in stool without antibiotic pre-treatment. Significance: T-test of area under the curve.

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