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

--Manuscript Draft--

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Abstract:	Probiotics have gained significant attention as a potential strategy to improve health by modulating host-microbe interactions, particularly in situations where the normal microbiota has been disrupted. However, evidence regarding their efficacy has been inconsistent, with considerable inter-individual variability in response. We aimed to explore whether a common genetic variant that affects the production of mucosal α(1,2)-fucosylated glycans, present in around 20% of the population, could explain the observed interpersonal differences in the persistence of commonly used probiotics. Using a mouse model with varying α(1,2)-fucosylated glycans secretion (Fut2WTor Fut2KO), we examined the abundance and persistence of Bifidobacterium strains (infantis, breve and bifidum). We observed significant differences in baseline gut microbiota characteristics between Fut2WT and Fut2KO littermates, with Fut2WT mice exhibiting enrichment of species able to utilise α(1,2)-fucosylated glycans. Following antibiotic exposure, only Fut2WT animals showed persistent engraftment of Bifidobacterium infantis, a strain able to internalise α(1,2)-fucosylated glycans, while B. breve and B. bifidum, which cannot internalise α(1,2)-fucosylated glycans, did not exhibit this difference. In mice with an intact commensal microbiota, the relationship between secretor status and B. infantis persistence was reversed, with Fut2KO animals showing greater persistence compared to Fut2WT. Our findings suggest that the interplay between a common genetic variation and antibiotic exposure plays a crucial role in determining the dynamics of B. infantis in the recipient gut, which could potentially contribute to the observed variation in response to this commonly used probiotic species.
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Dear Dr. Neufeld,

We submit the manuscript titled "*Intestinal persistence of Bifidobacterium infantis is determined by interaction of host genetics and antibiotic exposure*" for consideration at The ISME Journal. While *Bifidobacterium* species have been found to play key roles in intestinal immunity (Microbiome. 2018;6(1):121; Nat Microbiol. 2021; 6(11):1367-82), their inconsistent efficacy in clinical trials (Lancet Gastroenterol Hepatol. 2019;4(2):81) and varied persistence within the gut microbiome (Cell Host Microbe. 2022; 30(5):712-25) highlights substantial inter-individual variation. Mechanistic understanding of this nexus is required to advance our understanding in delivering effective microbiome-based interventions.

We hypothesised that a key contributor to the inter-individual variability in bifidobacterial persistence was the effect of common genetic mutations that affect mucosal glycan composition. Specifically, mutations in the *FUT2* gene prevent the secretion of α(1,2)-fucosylated glycans into the mucosal lumen, limiting this key carbon source for the growth of many key taxa, including *Bifidobacterium* spp. (PNAS. 2008;105(48):18964-9).

We present the first study investigating whether the loss of *FUT2* functionality impacts persistence of three common *Bifidobacterium* species (*B. infantis*, *B. bifidum*, and *B. breve*). Utilising a mouse *Fut2*^{KO} model and considering cage effects, sex effects, and vertical transmission, we explore the intestinal temporal dynamics of colonisation of different *Bifidobacterium* spp., as well as the interaction between antibiotics and *Bifidobacterium* persistence.

We report intriguing findings that highlight the genetic and antibiotic specific considerations that impact bifidobacterial persistence. Specifically, as hypothesised, loss of *Fut2* impaired persistence of *B. infantis* in mice pre-exposed to antibiotics. However, in the absence of antibiotic pre-treatment, we found opposite phenomenon; *B. infantis* persisted longer in *Fut2*^{KO} mice. Delving into this further, we identified microbiome-specific differences between *Fut2*^{KO} and *Fut2*^{WT} mice that explained this effect.

This manuscript is not under consideration by any other journals. No authors have any conflicts of interest related to this work and all raw data and scripts have been made available.

Kind regards,

Professor Geraint Rogers
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Dear ISME J Editorial team,

On behalf of all authors, we kindly re-submit the revision of manuscript "*Intestinal persistence of Bifidobacterium infantis is determined by interaction of host genetics and antibiotic exposure*" for consideration to ISME J.

We would like to thank Senior Editor, Richard Lamont, the Editor-in-Chief, and all three Reviewers for their detailed assessment of our manuscript. Below we provide a point-by-point numbered list of each Reviewer comment in blue (e.g. **C1**) followed by our response in black (e.g. **R1**). Where these comments have resulted in revisions to the manuscript, we have copied this text into this response document, as appropriate. Our responses also include a line number to the revised section in the amended manuscript document.

All authors have contributed to the manuscript and this revision and have seen and approved the final version. We also submit a graphical abstract to accompany this manuscript.

Thank you for considering our manuscript for publication.

Sincerely,

Prof. Geraint Rogers

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Senior Editor (Richard Lamont)

C1: There is broad agreement among the reviewers that this is an interesting and well-performed study of genetic and antibiotic regulation of bifidobacterial persistence. The reviewers have, however, made a number of suggestions to improve the clarity and impact of the manuscript. Please pay particular attention to Reviewer 3's comments regarding the translational outlook related to personalized probiotic therapies in humans.

R1: We thank the editorial team for their consideration of this work. Below, we detail our responses to each of the reviewer comments.

Editor-in-Chief revision request

C2: Please use our ISME J Editor-in-Chief checklist while making revisions and indicate that this was done in the rebuttal letter uploaded during resubmission. Several of the recommended edits apply to this manuscript and, if missed, another round of revision will be required to address remaining issues. In addition, it is inappropriate to use Chao1 with DADA2/ASV data because singleton ASVs are deleted in this process and essential to the estimator. Replace with Observed ASVS or some other richness metric?

R2: All relevant Editor-in-Chief checklist revisions were adopted, including replacing Chao1 with Observed ASVs, as noted. We are big fans of this list and will use it for future publications.

REVIEWER #1

C3: In this manuscript, Wang et al. investigated the impact of secretor status on the efficacy of probiotic intervention using a murine model. Firstly, they generated Fut2-/- mice through heterozygous breeding and observed significant alterations in microbiota composition in the feces of male mice, particularly in the lower part of the small intestine. Subsequently, employing three *Bifidobacterium* species, they assessed how these species responded differently to the host's secretor status in terms of colonization efficacy. Notably, they found that *B. infantis*, which exhibited enhanced colonization in perturbed microbiota of WT mice, showed reduced colonization ability in mice with normal gut microbiota of WT mice. The findings underscore the significance of secretor status as one of the determining factors in response to probiotic administration. The experiments are carefully designed, notably avoiding cage effects caused by breeding, and the results are soundly presented. The referee has several comments, which the referee believes should be addressed before publication.

R3: We appreciate the time and care taken for this review.

C4: Fut2 introduces α-(1-2)-fucosyl linkage to non-reducing end Gal residues to form Fucα1-2Gal-O-R, the so-called H-antigens. It would be better to use the term 'H-antigens' instead of α-(1,2)-fucosylated glycans in the text.

R4: We acknowledge the reviewer's comment and agree. In the amended manuscript, we now describe the term H antigen at first use (lines 76-79) and use this term throughout.

C5: Introduction: It has been known that prevalence of prebiotic assimilation gene(s) in gut microbes is also an important factor determining the responder/nonresponder status (PMID: 34705611). The referee would suggest that the authors include these findings here from a broad perspective.

R5: We agree that this is important supporting literature for the rationale of this study. We have included a sentence to this effect (lines 100-104) and copied below.

> *the ability of a probiotic to colonise and persist in an individual may depend on the presence of secreted glycans and the ability of the introduced bacterial strain to utilize them. This is supported by studies identifying increased persistence of such glycan-utilising species when supplemented with exogenous oligosaccharides (20, 21).*

C6: Results: To the referee's knowledge, glycoside hydrolases that can hydrolyze H-antigens are classified into GH29, GH95, and GH151 in the CAZy database. The authors can extend their findings by examining the prevalence of these GHs in the genomes of each taxa whose abundance increased or decreased (whose persistence prolonged or not) by the secretor status, prebiotic supplementation (2'-FL) to the fecal cultures, or probiotic administration to mice. For example, *Candidatus Arthoromitus* possesses GH151.

R6: We appreciate the reviewer's insight and acknowledge that our expertise is not primarily in the field of glycoside hydrolases (GH). We are familiar with GH29 and GH95 families in H-antigen hydrolysis, but were unfamiliar of the more recent classification of GH151 as another H-antigen GH family. We agree that describing GH capabilities of taxa provides important context for this manuscript.

In response to the reviewer's comment, we have now included in the following sections:

- Introduction: a description of the relevant GHs (lines 98-100)
- Methods: an expanded description of why the three *Bifidobacterium* species were selected based on their GH capabilities (lines 163-168), as well as a description of our alignment of taxa names to H-antigen hydrolysis capacity (lines 223-225).
- Results: presentation of the baseline stool and tissue microbiota in the context of their GH function (lines 297-298)
- Discussion: a more comprehensive explanation of the particular α-1,2-L-fucosidases in each species and what this means for bifidobacterium persistence (lines 411-427).

In relation to the inclusion of α-1,2-L-fucosidase capacity in the results section, this was determined by comparing the genus-level taxonomic classification to genomes identified by CAZy as carrying either the GH29, GH95, or GH151 enzyme families. We performed this at the genus level due to the resolution constraints of 16S V4 amplicon sequencing, meaning that GH variation that exists between species within a single genus could not be captured. Similarly, some of the identified taxa were only assigned to the family level (e.g. *Lachnospiraceae* (UCG006) or *Oscillospiraceae* (uncultured)), further limiting the accurate description of H-antigen hydrolysis capability of the microbiome. Extensive culture-based analysis or metagenomic sequencing would be required for accurate GH characterisation.

In undertaking this analysis, we note multiple taxa with either GH29, GH95, or GH151, which we present in Supplementary Table S4. However, our analysis did not identify *Candidatus Arthoromitus* as possessing GH151, as the reviewer notes. As evidenced, the CAZy database does not show any of the three GHs as a component of the *Candidatus Arthoromitus* genome (e.g. www.cazy.org/b1794.html). A separate detailed search of the literature, including the KEGG pathway database, also did not yield any information relating to this.

C7: *B. bifidum* has a pronounced ability to degrade O-glycans *in vivo* by its extracellular GHs (PMID: 36864192). By contrast, it is believed that *B. infantis* does not degrade O-glycans, as it has no responsible extracellular GHs. Increase in the abundance of *B. infantis* may rely on cross-feeding by other gut microbes that can release mucin O-glycan oligosaccharides by the action of GH16 endo-O-glycanases. The authors can discuss the underlying mechanisms of the behavioral difference observed for these bacteria.

R7: The reviewer is correct in their description of intracellular versus extracellular localisation of GHs between species. While *B. infantis* also has transporters to enable internalisation of glycans (PMID: 31489370), this is still dependent on the release of oligosaccharides from the larger glycoproteins/glycolipids. It has been shown that, following antibiotics, there is an abundance of liberated sugars (PMID: 23995682), which may explain the results of enhanced *B. infantis* persistence following antibiotics, presented here. We have included a discussion of the various potential underlying mechanisms that explain persistence dynamics with and without antibiotics, and the role H antigens play in this process (lines 411-427 and copied below).

> *Bifidobacterial species that are commonly used as probiotics are relatively close phylogenetically but differ in their ability to use glycans, even at a strain level (44). We showed that neither B. breve (JCM 1192) nor B. bifidum (JCM 1255) differed in their abundance or persistence between secretor and non-secretor animals. In contrast, B. infantis (JCM 1222) persisted for significantly longer and showed a significantly higher abundance in secretor mice compared to non-secretor mice. This finding likely reflects differences in H antigen hydrolysis and catabolism capacities between species when administered as a probiotic. For example, B. infantis encodes GH29, GH95, and GH151 family intracellular α-1,2-L-fucosidases, along with fucose transporters to facilitate internalisation (46). While independent hydrolysis and catabolism of mucin-bound H antigens by B. infantis is not hypothesised (26), cross-feeding by organisms with extracellular α-1,2-L-fucosidases is likely, even following antibiotic supplementation (47). In contrast, B. bifidum, while expressing extracellular GH29 and GH95 α-1,2-L-fucosidases, does not consume fucose to facilitate growth (28). Finally, B. breve, encodes a separate GH95 intracellular α-1,2-L-fucosidase along with fucose transporters. While this species is capable of utilising the H antigen with support from cross-feeding (48), these findings suggest reduced persistence compared with B. infantis, when administered as a probiotic.*

Specific comments:

C8: Page 4, line 74: It would be better to use the term 'oligosaccharides', instead of polysaccharide, when referring to mucin O-glycans here.

R8: This has been amended.

C9: Page 4, line 87-90: Numerous papers describe differences in gut microbiota composition between secretors and non-secretors, some of which should be cited here.

R9: We acknowledge the large body of literature examining the role of secretor status on the composition of the gut microbiota. In response to this comment, we have included a recent, detailed examination of this across 7,738 participants (PMID: 35115690), along with a review that summarises the wider literature (PMID: 29079498).

C10: Page 9, line 189: It would be better to mention average read counts of NGS analysis.

R10: We have now included this information (line 206-207).

C11: Page 11, line 238: mBasal is not a minimal medium. Minimal media are synthetic media consisting of defined chemical composition.

R11: We have removed all references to mBasal as a minimal media.

REVIEWER #2

C12: This quite exhaustive and multi-faceted manuscript addresses whether genetic variation at the FUT2 allele can be linked to probiotic engraftment efficiency. It is a topical question, with a few large microbiome GWAS studies recently published, highlighting the role of secretor status and ABO in shaping microbiome composition and structure, in pigs and in humans. This manuscript specifically looks at bifidobacterial persistence after experimental colonization (by gavage) in KO FUT2 mutants in a mice line, which the authors create for this manuscript. A lot of valuable in vitro testing on glycan degradation vs the microbiota is shown. While it has been known for 10+ years that FUT2 polymorphism is associated with *Bifidobacterium* colonization in the human gut, a more precise foray into specific species and their metabolic abilities, or the role of antibiotics, is an interesting aspect of this present manuscript. I also appreciated the study of microbiota from faeces but also small and large intestines.

R12: We thank the reviewer for their comments and interest in this work.

General comment:

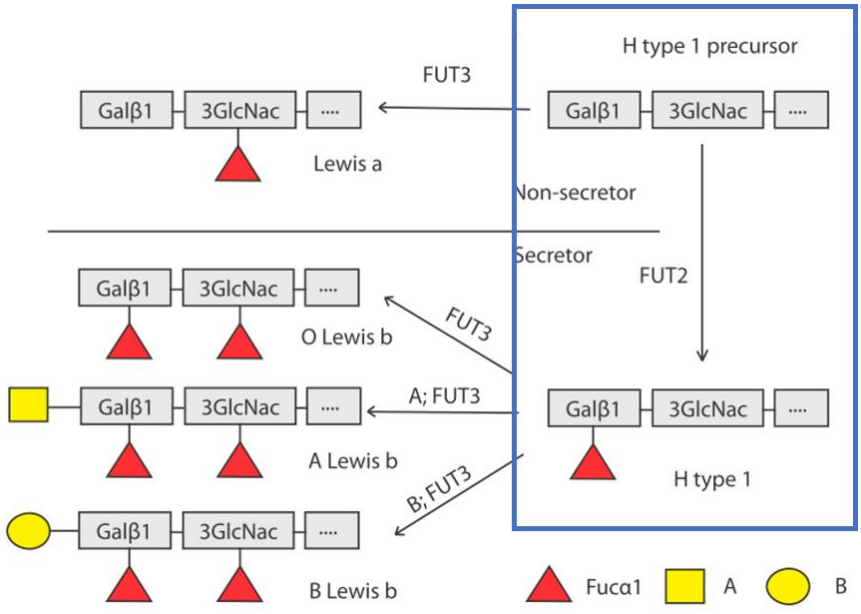
C13: I found the absence of consideration of blood antigens/ABO phenotypes in the interpretation a bit puzzling. (For instance, at lines 79-81 when authors say that "FUT2 controls the nature of the glycans secreted by mucosal surfaces". It does but it also doesn't, as it very much depends on what glycans there are to secrete). Indeed, a lot of the current

FUT2/microbiome hypothesis has to do with the fact that blood antigens (confusingly named as not just from blood cells) are secreted in the gut mucosa, and used by the microbiota there. It does mean that in blood type O individuals, the secretor status does not influence anything. This has been shown recently in a few publications (Qin et al Nat Gen 2022; Yang et al. Nature 2022; Zhernakova et al. Nature 2024). I am not familiar with blood types in mice, and authors did test for glycan utilisation in their model of study, but surely there are similarities and parallels to be drawn.

R13: We acknowledge the reviewer's query and would like to provide further clarification. Indeed, in humans, *FUT2* is responsible for the generation of the H antigen, which can be further modified to give the OLewis^b, ALewis^b, or BLewis^b antigens (provided in the figure below, PMID: 30845670). Each of these glycans can modulate the competitive advantage of particular microbes capable of cleaving the oligosaccharide constituents. In the absence of *FUT2*, these Lewis^b antigens are not displayed, leading to a Lewis^a antigen.

The reviewer correctly notes that the ability to secrete either A or B blood group glycans have been shown to influence key members of the gut microbiota in humans. In mice, however, there is no further modification of the H antigen by *Fut3*. Therefore, the experiments performed here represent the blue box of the below figure, with the terminal antigens of *Fut2*^{WT} being the H type 1 antigen, while those of *Fut2*^{KO} being the H type 1 precursor.

It should be noted that even in humans, a secretor O blood group and a non-secretor O blood group are not the same (as depicted below). The impact of this on the gut microbiome, in humans, is evidenced by studies reporting an association between H antigen concentrations and gut microbiome characteristics (e.g. PMID: 36678342).



Minor comments:

C14: Line 98-99: Authors did check later on the glycan-degrading abilities of the 3 strains they used *in vitro*, but could there be a reference to cite there to explain how it was established that some of the cited species degrade glycans or not there? Is there strain variation there? What bacterial genes are responsible? If not, is it something worth considering showing in this manuscript? (i.e. characterising a large collection of reference genomes for glycan-degrading pathways)? In general, I feel that for publication in a microbial ecology journal, there has to be some attempt to link the ecology of microbial glycan degradation to the presented results.

R14: We acknowledge that the submitted manuscript lacked detail around the genes responsible for glycan degradation. The need to discuss the role of glycoside hydrolases (GHs) was also noted by Reviewer #1 (C6). In response to both comments, we have included a description of GHs in the Introduction (lines 98-104), Methods (lines 163-168) as well as explanation of these in the context of the findings in the Discussion (lines 411-427). Both sections are copied below.

Introduction

> *Probiotic preparations typically contain Bifidobacteria (Bifidobacterium adolescentis, animalis, bifidum, breve and longum) and/or Lactobacilli (Lactobacillus acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus and salivarius). Both genera include species that encode the specific glycoside hydrolases (GHs), GH29, GH95, and GH151, which can utilise the H antigen. However, both genera also include species without this glycoside hydrolase capacity . Therefore, the ability of a probiotic to colonise and persist in an individual may depend on the presence of secreted glycans and the ability of the introduced bacterial strain to utilize them. This is supported by studies identifying increased persistence of such glycan-utilising species when supplemented with exogenous oligosaccharides (19, 20).*

Methods

> *This was confirmed by existing literature (24, 25) and the Carbohydrate-Active enZYmes (CAZy) database (26) showing that B. bifidum JCM 1255 encodes external GH29 and GH95 α-1,2-L-fucosidases, but does not consume fucose as a carbon source (27), B. infantis JCM 1222 encodes intracellular GH29, GH95 and GH151 α-1,2-L-fucosidases along with glycan transporters, and B. breve JCM 1192 encodes only the GH95 family.*

Discussion

> *Bifidobacterial species that are commonly used as probiotics are relatively close phylogenetically but differ in their ability to use glycans, even at a strain level (44). We showed that neither B. breve (JCM 1192) nor B. bifidum (JCM 1255) differed in their abundance or persistence between secretor and non-secretor animals. In contrast, B. infantis (JCM 1222) persisted for significantly longer and showed a significantly higher abundance in secretor mice compared to non-secretor mice. This finding likely reflects differences in H antigen hydrolysis and catabolism capacities between species when administered as a probiotic. For example, B. infantis encodes GH29, GH95, and GH151 family intracellular α-1,2-L-fucosidases, along with fucose transporters to facilitate internalisation (44). While independent hydrolysis and catabolism of mucin-bound H antigens by B. infantis is not hypothesised (25), cross-*

feeding by organisms with extracellular α-1,2-L-fucosidases is likely, even following antibiotic supplementation (45). In contrast, B. bifidum, while expressing extracellular GH29 and GH95 α-1,2-L-fucosidases, does not consume fucose to facilitate growth (27). Finally, B. breve, encodes a separate GH95 intracellular α-1,2-L-fucosidase along with fucose transporters. While this species is capable of utilising the H antigen with support from cross-feeding (46), these findings suggest reduced persistence compared with B. infantis, when administered as a probiotic.

C15: Line 147: for the sake of this manuscript (there seem to be prior references for this): how was the optimal gavage concentration determined?

R15: As the reviewer notes, we provide references to support the selection of gavage concentration. We did not, however, explain the reasoning for this dose, which we now include (lines 152-155 and copied below). To clarify, the referenced papers describe the safety and persistence of this dose, as well as immune modulation capability.

Mice received 5 × 10^7 colony forming units (CFU)/g of mouse of either B. infantis, B. bifidum, or B. breve daily for 5 days via oral gavage. A starting gavage concentration of 5 × 10^9 CFU/mL in PBS was prepared daily from fresh overnight cultures. The dose was selected based on previous reports of safety, persistence, and immune modulation capability (21, 22).

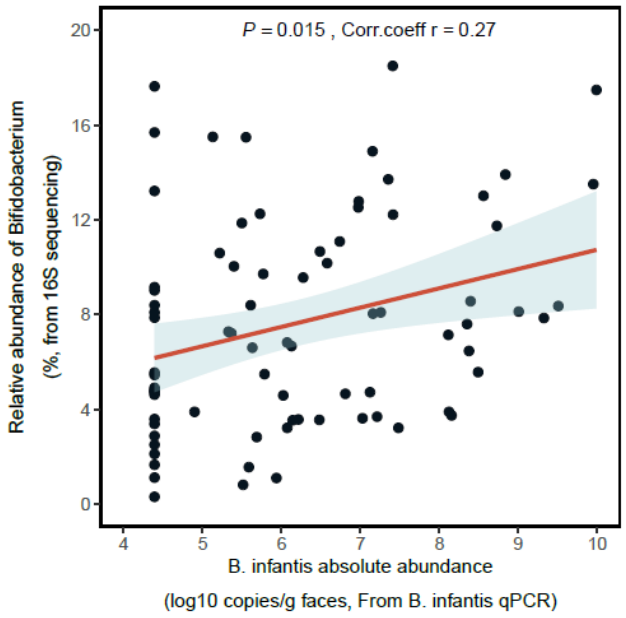
C16: Line 139: *Bifidobacterium* strains are generally considered to be highly resistant to some aminoglycosides (neomycin is one). Are the gavage strains resistant to ampicillin/neomycin or not? This could determine whether abundance patterns are independent or not from AB treatment.

R16: Our antibiotic mix contained a cocktail of ampicillin and neomycin, with this combination designed to deplete a wide range of bacteria. While the reviewer is correct that most *Bifidobacterium* strains are resistant to neomycin, the tested strains are sensitive to ampicillin (PMID: 17060530). We designed the experiment so that gavage with *Bifidobacterium* was immediately after ceasing antibiotic depletion to maximise opportunity for *Bifidobacterium* colonisation without competition from other bacteria. It is possible that this timing means that residual antibiotics in the intestine deplete *Bifidobacterium* over the first days of gavage. For this reason, we performed gavage for 5 days, a time period that extends beyond the activity spectrum of the administered antibiotics. Such an antibiotic combination is common for mouse models (e.g. PMID: 35284839, 29746836), as well as empiric for suspected sepsis in humans (PMID: 32232180).

C17: Line 189: it would be good to mention on how 16S is able to discriminate (or not) various strains of Bifidobacterium used (and if you tested it). You also did qPCR on the strains, so it's a good opportunity to comment on the correlation with 16S and perhaps even rRNA copy number in these species.

R17: We investigated the extent to which 16S amplicon sequencing could discriminate between different *Bifidobacterium* species. As was expected, level 7 resolution (species-level output) was unable to differentiate bifidobacterial, reflecting a well-recognised limitation of this approach.

In regard to the relationship between estimates of abundance derived by qPCR (absolute abundance) and 16S sequencing (relative abundance), we provide a correlation below. This analysis was limited to faecal samples where *Bifidobacterium* was gavaged and the mice had not received any antibiotics, as the relative abundance of *Bifidobacterium* neared 100% in all cases where mice that had undergone antibiotic exposure and no *Bifidobacterium* was detectable in naïve mouse stool without *Bifidobacterium* exposure. As shown below, we identified a significant correlation between the two methods. However, as evidenced by the Spearman's correlation co-efficient, while significant, the strength of the correlation was not high. This may be due to the difference in detection limit, with the qPCR showing reduced sensitivity. As the reviewer notes, amplicon sequencing uses the 16S gene, for which there are four copies in *Bifidobacterium* spp. (PMID: 22470420). The qPCR targeted the *groEL* gene (PMID:22307308), for which there is only a single copy (PMID: 12381787).



C18: Methods comment: Are results from littermates considered biological or technical replicates in these experiments?

R18: Give the heterogenous nature of the gut microbiome, even within littermates cohoused, we consider each mouse to be a biological replicate rather than a technical replicate. We did also conduct technical replicates for our *in vitro* experiments as well as assessment of *Bifidobacterium* spp. abundance, performed by qPCR.

C19: Line 275: Figure 1C does not seem to show any result in female mice

R19: We apologise for this error, where the text did not match the figure panels. We mistakenly referred to Figure 1C in text, instead of the correct panel (Figure 1B). This error occurred because we had previously had Figure 1A depicting the breeding pattern, which we removed from the figure prior to submission (but mistakenly did not correct the text).

In the amended manuscript, we have corrected this. This includes the re-addition of the Figure 1A depicting the breeding pattern (in response to Reviewer 3, C29).

REVIEWER #3

C20: The manuscript by Wang et al presents an interesting mechanistic study exploring how genotypic variation in the production of specific glycans can influence the microbiota colonization (specifically colonization by specific probiotic *Bifidobacteria*) and select for specific members with the given substrate utilization capabilities. The authors provide mechanistic evidence by providing evidence using pre-clinical models involving a range of mice experiments. However, I feel that the study falls short of providing a clear translational outlook of these findings on humans. And the authors either need to acknowledge this or additional evidence.

R20: We acknowledge the reviewer's concern regarding our description of the translational significance of our findings. We address these concerns in relation to their specific comments below.

C21: The is one primary reason which makes judging the translational applicability of these findings extremely confusing. The study is performed on mice. And, the authors see that when the probiotics are administered to individuals without prior antibiotic treatment, the strains that are able to utilize the specific glycan substrate a-1,2-fucosylated glycan show worse colonization in individuals who produce it. This means that in the absence of antibiotic exposure in mice, it is still the baseline microbiome that determines colonization (and not specific substrate utilization). Now, while mice is a simple system, in humans, even those with Ab treatment still contain a baseline microbiota (for some it can be reasonably high and recoverable) (https://pubmed.ncbi.nlm.nih.gov/32632261/) and besides probiotic, these Ab treated individuals are expected to have a range of other exposures including habitual diet. Thus, how would the probiotic perform in such competitive scenario?

R21: The reviewer highlights and important issue, and one the underpins the rationale for murine studies of the kind that we undertook. Mouse studies allow us to control virtually every exposure that might influence the outcome of the experiment, something that is clearly impossible in equivalent human investigations.

The reviewer cites a number of variables, including the extent and nature of antibiotic depletion of the gut microbiota and dietary intake, and there will be many others. In each case, the magnitude and directionality of the impact of this variance is likely to differ, resulting in turn in a great variability in probiotic colonisation. And indeed, similar murine studies have been used to explore the impact of other variables, including dietary intake, on probiotic dynamics.

The insight gained through these murine studies allows us both to identify effects that can be attributed to specific factors, when others are controlled, and to detect signal that might otherwise be difficult to distinguish from background. Importantly, this insight can be built on in the next logical step of such investigations – cohort studies in humans.

Within our revised manuscript, we now set out this translational pathway as a means to determine what the net impact of all exposures would be on probiotic performance (lines 440-453).

> *While this study was performed in mice, the effect of secretor status on bifidobacterium supplementation has important implications for probiotic strategies in humans. It is crucial to consider individual host traits and recent antibiotic exposure when designing a probiotic intervention (49). The findings here suggest that the 20% of the population who are non-secretors may have poorer persistence of H antigen utilising probiotics, such as B. infantis, compared to secretors following antibiotic exposure. Conversely, in the absence of recent antibiotic exposure, higher levels of microbial niche occupancy in secretors may hamper B. infantis persistence compared to non-secretors. An individualised supplementation with prebiotics may have potential as a means to optimise probiotic uptake in non-secretors. For example, previous studies have shown that supplementation with human milk oligosaccharides can enhance B. infantis engraftment (21), with successful supplementation shown to reduce intestinal inflammation in infants (50). Investigating additional α(1,2)-fucosylated glycans, given as prebiotics, may lead to improved outcomes of B. infantis supplementation in non-secretor individuals.*

C22: I also don't understand why did the authors not tested for other Bifidobacteria (like longum and adolescentis) that are more core to the gut microbiome than infantis?

R22: Clearly, there are a large number of bifidobacteria that we could have assessed but our goal was not to undertake a comprehensive characterisation of members of the genus. Rather, we selected three common 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 with a less defined α(1,2)-fucosylated glycans utilisation pathway. We felt that this approach provided fundamental insight and the ability to extrapolate the behaviour of other species.

C23: It is interesting to see that the microbiome stratification by genotype is observed only in males? Why? Is there a bias of the genotype towards males? If not, why? The authors don't probe the reasons for this observation. Does that mean the gut microbiome of females do not show any substrate-specific selection of microbiome members? If so, then again the previous observation comes forth. It is the other bacteria that drive colonization, the host genotype only contributes to a minor extent.

R23: This is indeed an interesting point. The reviewer raised the query of whether it is due to differential genotype expression between male and female mice. This is something we explored through the immunohistochemistry (Fig. S1), where both male and female mouse tissue was

stained for α(1,2)-fucosylated glycans. Here, the top and bottom panel of WT mouse tissue is female, while the middle is male. As can be seen, there is not any difference in stain intensity or coverage between the sexes. While we could not find any literature to directly support this, measures of mouse fertility and physiology have been previously shown to be unaltered by *Fut2* genotype in both male and female mice (PMID: 11713270).

While we remain uncertain regarding the precise mechanism, we hypothesise that this phenomenon may be due to the effect sex hormones play on the microbiome. Sex hormones are recognized as drivers of microbiome composition (PMID: 31636122). There is ongoing discussion regarding whether sex steroids regulate the composition or function of the gut microbiota, or conversely, if gut microbes influence sex steroid balance (PMID: 28778332). For example, Chen and Madak-Erdogan (2016) have proposed a potential interaction between the microbiome and estrogens, suggesting that gut microbes may metabolize endogenous or dietary estrogens, leading to the production of estrogenic metabolites that can impact host metabolism. This concept, referred to as the "estrobolome" (PMID: 22018233), encompasses enteric bacterial genes capable of metabolizing estrogens.

In addition to sex hormones, other factors, such as differences in the immune function, metabolism, as well as behavioural and dietary habits between male and female mice, might also impact the microbiome composition, effecting the strength of the signal of *Fut2* in female mice.

With respect to the implications of these findings, we understand the reviewer's query. The extent to which the sex x genotype interaction can be translated to humans is difficult to determine. Our PERMANOVA results showing that *Fut2* genotype has a stronger effect on the gut microbiome compared to sex (Table S3) suggests that genotype is an important variable. This, combined with the body of literature showing the *Fut2* effects baseline microbiota (e.g. PMID: 34419617, 33462482, 28642740, 25922665), forms a strong basis that secretor status impacts gut microbiology.

We include a paragraph in the Discussion, describing these potential explanations (lines 474-477).

> *The effect of sex on the relationship between secretor status and the gut microbiome is difficult to explain but may relate to variable intestinal expression of Fut2, which can be altered factors such as stress (57). In addition, independent interactions between sex hormones and the gut microbiome (58) may affect the relationship between Fut2 and the gut microbiome.*

C24: Figure 1: All comparisons should reported corrected p-values or FDR.

R24: We have taken care throughout the manuscript to adjust P values for multiple testing. Fig 1E reports the findings from a Linear discriminant analysis Effect Size (LEfSe) analysis. While the output from LEfSe analyses are often not presented with P values, we opted to report these for transparency. Several forums have discussed whether to adjust the LEfSe generated P values

for multiple testing (e.g. https://forum.biobakery.org/t/multiple-comparison-correcting/258/3). The suggestion from the BioBakery team is "*LEfSe still filters for features that a) pass the KW test and b) has strong LDA score support. The two together should provide enough evidence for biomarkers.*" In addition to a P value cut-off, we applied a LDA score cut-off of 2.

We do however appreciate the reviewer's concern that reporting unadjusted P values can misguide the readers. In light of this comment, we have removed the display of P values from LEfSe plots. This is in keeping with the conventional display of LEfSe outputs.

C25: Lines 288-291: References missing

R25: A reference has now been added.

C26: Commensal (lines 61-62): I would refrain from using the word commensal in this context.

R26: "Commensal" has now been removed.

C27: line 87: reflect a reduced in susceptibility.

R27: This mistake has been correct (amendment copied below).

> *The high carriage of these loss-of-function mutations is likely a result of positive selection from altered susceptibility to infections […]*

C28: I would encourage authors to highlight a bit more on the *Fut2* mutation and its prevalence across global population.

R28: We have expanded our description of the prevalence of Fut2 nonsense mutations in the population in the Introduction (lines 82-86), and the Discussion (lines 403-406), with both copied below.

Introduction

> *Across the human population, multiple nonsense single nucleotide polymorphisms (SNPs) are found within the FUT2 gene (15), leading to a "non-secretor" phenotype. The non-secretor phenotype, like the AB blood groups, is one of the more common functional mutations maintained in the population, with approximately one-fifth of people carrying homozygous loss-of-function FUT2 genes (15, 16).*

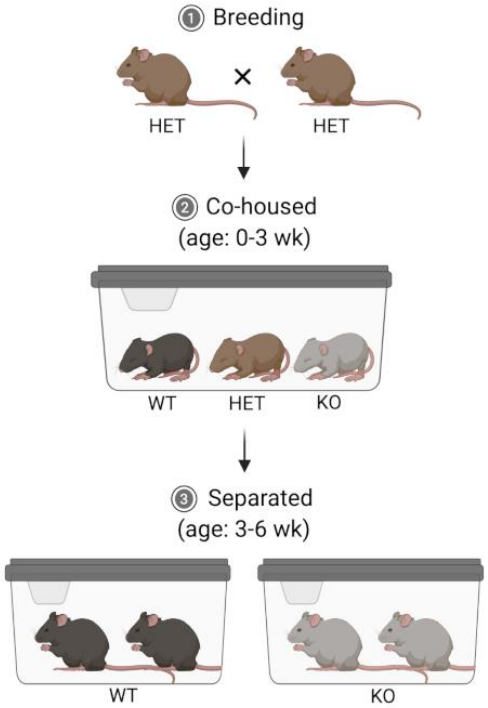
Discussion

> *Firstly, 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 α(1,2)-fucosylated glycans (H antigens).*

C29: The authors need to provide a detailed diagram of the experimental set-up. From the method description they have provided, it is hard to know the extent of co-housing of the different genotypes, etc.

R29: We have now included Figure 1A (below), which details the breeding and co-housing design. We have also expanded the corresponding Methods section to include a detailed description (lines 136-142).

> *Fut2 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. Fut2^{KO}, Fut2^{HET} and Fut2^{WT} littermates were co-housed from birth until weaning (~3 weeks), where they were genotyped by PCR amplicon melt curve using primers targeting the outer and inner regions of the Fut2 gene. Fut2^{KO} and Fut2^{WT} mice separated into cages after weaning based on sex and Fut2 genotype (Figure 1A). No experiments were performed on Fut2^{HET} mice. In all experiments, 6-week old, age- and sex-matched mice were used. Each experimental group consisted of at least 4 cages to control for cage effects.*



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

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ABSTRACT

Probiotics have gained significant attention as a potential strategy to improve health by modulating host-microbe interactions, particularly in situations where the normal microbiota has been disrupted. However, evidence regarding their efficacy has been inconsistent, with considerable inter-individual variability in response. We aimed to explore whether a common genetic variant that affects the production of mucosal α(1,2)-fucosylated glycans, present in around 20% of the population, could explain the observed interpersonal differences in the persistence of commonly used probiotics. Using a mouse model with varying α(1,2)-fucosylated glycans secretion (*Fut2*^{WT} or *Fut2*^{KO}), we examined the abundance and persistence of *Bifidobacterium* strains (*infantis, breve* and *bifidum*). We observed significant differences in baseline gut microbiota characteristics between *Fut2*^{WT} and *Fut2*^{KO} littermates, with *Fut2*^{WT} mice exhibiting enrichment of species able to utilise α(1,2)-fucosylated glycans. Following antibiotic exposure, only *Fut2*^{WT} animals showed persistent engraftment of *Bifidobacterium infantis*, a strain able to internalise α(1,2)-fucosylated glycans, whereas *B. breve* and *B. bifidum*, which cannot internalise α(1,2)-fucosylated glycans, did not exhibit this difference. In mice with an intact commensal microbiota, the relationship between secretor status and *B. infantis* persistence was reversed, with *Fut2*^{KO} animals showing greater persistence compared to *Fut2*^{WT}. Our findings suggest that the interplay between a common genetic variation and antibiotic exposure plays a crucial role in determining the dynamics of *B. infantis* in the recipient gut, which could potentially contribute to the observed variation in response to this commonly used probiotic species.

Keywords

Microbiome, secretor status, α(1,2)-fucosylated glycans, *Bifidobacterium*, *Bifidobacterium infantis*

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.

Various mechanisms enable the human gut to regulate commensal microbiota composition. One of the principal mechanisms involves the secretion of specific types of sugars that are utilised by beneficial microbial species. Many mucosal constituents and secreted factors are decorated with glycans (oligosaccharides), that are added by a diverse family of glycosyltransferase enzymes (12). Of these, the *FUT2* gene encodes a galactoside α(1,2)-fucosyltransferase, which adds a L-fucose monosaccharide to non-reducing end Gal residues to form Fucα1-2Gal-O-R glycans, termed the H antigens (13, 14). Expressed by multiple mucosal epithelial cell types, this H antigen, is a highly versatile structure that can be further modified to form many other important glycans, including the AB blood group glycans. Because *FUT2* controls the nature of the various α(1,2)-fucosylated glycans secreted by mucosal surfaces, it is commonly referred to as the "secretor" gene (13).

Across the human population, multiple nonsense single nucleotide polymorphisms (SNPs) are found within the *FUT2* gene (15), leading to a "non-secretor" phenotype. The non-secretor phenotype, like the AB blood groups, is one of the more common functional mutations maintained in the population, with approximately one-fifth of people carrying homozygous loss-of-function *FUT2* genes (15). This high carriage of loss-of-function mutations is likely a result of positive selection from altered susceptibility to infections by certain bacterial and viral pathogens (16). However, as fucosylated glycans are an important nutrient source for gut microbes, their absence in non-secretors has been shown to influence commensal microbiota composition (16, 17).

The intact commensal microbiota of secretor individuals is likely to be enriched for glycan utilising bacteria. In contrast, depletion of commensal taxa, for example, through antibiotic exposure, can provide a selective advantage to exogenous glycan utilisers that are absent in

non-secretors (9, 18). Probiotic preparations typically contain *Bifidobacteria* (*Bifidobacterium adolescentis*, *animalis*, *bifidum*, *breve* and *longum*) and/or Lactobacilli (*Lactobacillus acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *rhamnosus* and *salivarius*). Both genera include species that encode the specific glycoside hydrolases (GHs), GH29, GH95, and GH151, which can utilise the H antigen. However, both genera also include species without this glycoside hydrolase capacity. Therefore, the ability of a probiotic to colonise and persist in an individual may depend on the presence of secreted glycans and the ability of the introduced bacterial strain to utilize them. This is supported by studies identifying increased persistence of such glycan-utilising species when supplemented with exogenous oligosaccharides (19, 20).

We hypothesised that the interplay between secretor status, the glycan utilisation ability of the probiotic strain, and the presence of a disrupted commensal microbiota due to antibiotic exposure, would collectively influence the abundance and persistence of probiotic populations in the gut. To test this hypothesis, we introduced probiotic *Bifidobacterium* strains into a murine model of secretor/non-secretor status, with or without prior antibiotic depletion of commensal microbiota.

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.

Fut2^{KO}, *Fut2*^{HET} and *Fut2*^{WT} littermates were co-housed from birth until weaning (~3 weeks), where they were genotyped by PCR amplicon melt curve using primers targeting the outer and inner regions of the *Fut2* gene. *Fut2*^{KO} and *Fut2*^{WT} mice separated into cages after weaning based on sex and *Fut2* genotype (**Figure 1A**). No experiments were performed on *Fut2*^{HET} mice. In all experiments, 6-week old, age- and sex-matched mice were used. Each experimental group consisted of at least 4 cages to control for cage effects.

Antibiotic treatment

A cocktail of ampicillin (1 g/L, Sigma-Aldrich) and neomycin (0.5 g/L, Sigma-Aldrich) was provided to mice via drinking water for 7 days. Water bottle volume and mouse weight were monitored to assess water intake and antibiotic activity was confirmed by 16S qPCR (**Supplementary Table S2**) of faecal samples at day 7.

Probiotic supplementation

Mice received 5×10^7 colony forming units (CFU)/g of mouse of either *B. infantis*, *B. bifidum*, or *B. breve* daily for 5 days via oral gavage. A starting gavage concentration of 5×10^9 CFU/mL in PBS was prepared daily from fresh overnight cultures. The dose was selected based on previous reports of safety, persistence, and immune modulation capability (22, 23).

Bacterial strains

B. bifidum JCM 1255 (ATCC equivalent: 29521), *B. longum subspecies infantis* JCM 1222 (ATCC equivalent: 15697), *B. breve* JCM 1192 (ATCC equivalent: 15700) were obtained from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan). Utilisation of α(1,2)-fucosylated glycans was assessed by *in vitro* culture in mBasal media with/without supplementation of 5% w/v 2′-fucosyllactose (Layer Origin, Ithaca, New York, USA)

(**Supplementary Fig. S2**). This was confirmed by existing literature (24, 25) and the Carbohydrate-Active enZYmes (CAZy) database (26) showing that *B. bifidum* JCM 1255 encodes external GH29 and GH95 α-1,2-L-fucosidases, but does not consume fucose as a carbon source (27), *B. infantis* JCM 1222 encodes intracellular GH29, GH95 and GH151 α-1,2-L-fucosidases along with glycan transporters, and *B. breve* JCM 1192 encodes only the GH95 family.

Faecal and tissue collection

At least two faecal pellets were collected from separated mice at the beginning of the light phase, unless specified otherwise. For intestinal tissue collection, mice were sacrificed by CO_2 asphyxiation and laparotomy was immediately performed using a vertical midline incision. Once the digestive tract was exposed, separate dissection tools were used to dissect tissue into four parts: the proximal small intestine; distal small intestine; caecum and large intestine. For small and large intestine tissue segments, the luminal content was collected by instilling sterile PBS using a syringe barrel and the flushed mucosal tissue was collected into separate tubes. All collected faecal samples, organs, and luminal contents were immediately frozen on dry ice and stored at -80°C until further processing.

DNA extraction

DNA extraction of faecal samples

Faecal pellets were weighed, and 25 mg samples (±10 mg) were resuspended in 300 µL of cold phosphate buffered saline (PBS) (pH 7.2) by vortexing and pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. Microbial DNA was extracted from faecal samples using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously (28).

188

189 ***DNA extraction on mucosal tissue samples***

190 Mucosal tissue from the proximal small intestine, distal small intestine, and large intestine were
191 semi defrosted and 3 cm was removed from the tissue centre using sterile scalpel. The dissected
192 tissues were cut open longitudinally and mixed with 750 µL PowerSoil bead solution and 60
193 µL solution C1 in a PowerSoil bead tube. The bead tube was then incubated at 65°C for 10
194 mins prior to bead beating. The subsequent DNA isolation was performed using the
195 PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the
196 manufacturer's instructions as described previously (28).

197

198 **16S rRNA gene amplicon sequencing and bioinformatic processing**

199 Amplicon libraries of the V4 hypervariable region for 16S rRNA gene were prepared from
200 DNA extracts using modified universal bacterial primer pairs 515F and 806R (29). Amplicon
201 libraries were indexed, cleaned, and sequenced according to the Illumina MiSeq 16S
202 Metagenomic Sequencing Library Preparation protocol on a 2 × 300 bp Miseq reagent kit v3
203 at the South Australian Genomics Centre (SAGC), South Australian Health and Medical
204 Research Institute. Paired-end 16S rRNA gene sequence reads were analysed using QIIME2
205 version 2021.11.0 (30). Briefly, de-noising was performed on de-multiplexed sequences using
206 the DADA2 plugin (31), resulting in a mean read depth of 15,563 ± 2,719 for stool and 6,941
207 ± 4,503 for tissue. Taxonomic classification of amplicon sequence variants (ASVs) was
208 performed based on the V4 hypervariable region of the SILVA 16S rRNA gene reference
209 database (version 138) at 99% similarity (32). Sufficient coverage at this depth is confirmed
210 by the rarefaction curve, which reached an asymptote. Sequence data has been deposited in the
211 NCBI SRA under accession number PRJNA1011386.

212

213 **Microbiota characterisation**

214 The taxonomic relative abundance at the genus level was used to generate alpha diversity
215 (within-group) and beta diversity (between-group) measures. Alpha diversity metrics
216 (observed ASVs, Pielou's evenness, Shannon diversity, and Faith's phylogenetic diversity)
217 were obtained from QIIME2 at sampling depth of 9,883 reads (faecal samples) and 662 reads
218 (mucosal tissue samples). The Bray-Curtis dissimilarity index was calculated to compare
219 microbiome similarity between groups (beta diversity), using square-root transformed species
220 relative abundance data using the 'vegan' package in R. Non-metric multidimensional scaling
221 (nMDS) for all beta diversity measures were generated using the 'vegan' package in R. Core
222 taxa were defined as those present in more than 95% of samples, with a mean relative
223 abundance of >0.01%. Identification of taxa with α-1,2-L-fucosidases capability was
224 determined by comparing the genus-level taxonomic classification to genomes identified by
225 CAZy (26) as carrying either the GH29, GH95, or GH151 enzyme families.

226

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

228 Quantification of total bacterial load, *B. breve*, *B. infantis* and *B. bifidum* was performed by
229 SYBR Green-based qPCR assays (**Supplementary Table S2)**. For all qPCR assays, 1 µL of
230 DNA template was combined with 0.7 µL of 10 µM forward primer, 0.7 µL of 10 µM reverse
231 primer, 17.5 µL of 2×SYBR Green (Applied Biosystems, Waltham, Massachusetts, USA) and
232 15.1 µL nuclease-free water. All samples were run in triplicate (10 µL each replicate). Gene
233 copy quantification was performed using a standard curve generated from a known
234 concentration of a pure colony control. Any sample with a cycle threshold (CT) ≥40 cycles was
235 defined as 40 (limit of detection).

236

237 **Culture of *Bifidobacterium* strains**

All *Bifidobacterium* strains used in this study were cultured in De Man, Rogosa and Sharpe (Becton Dickinson, Franklin Lakes, NJ, USA) broth or agar supplemented with 0.34% (w/v) sodium ascorbate and 0.02% (w/v) cysteine-HCl (MRS-CS) and were grown under anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA) at 37 °C. The growth of *Bifidobacterium* was measured by optical density (600 nm) using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

In vitro glycan utilisation assay

Faecal pellets from untreated WT mice were incubated in mBasal media (mBasal; 10 g/L Trypton, 2 g/L yeast extract, 5 g/L NaCl, 0.2 g/L magnesium sulfate, 2 g/L dipotassium hydrogen phosphate, pH 6.4) with/without supplementation of 5% w/v of 2′-Fucosyllactose (Layer Origin, Ithaca, New York, USA) at 37°C under strict anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA). Bacterial biomass was measured by optical density (600 nm, OD_{600}) using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA). Bacterial colonies cultured from faecal samples were identified by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Billerica, Massachusetts, United States), as described previously (33).

Statistical analysis

Experimental mice were randomly assigned to different treatment groups. The investigators were not blinded to the experimental groups. No outliers have been removed from any of the data presented. All data analyses were performed using either R (R Foundation for Statistical Computing; version 4.1.0) or GraphPad Prism software (GraphPad Software, Inc.; version 9.00). For parametric data, unpaired Student's t test was used to compare data between two unpaired groups; One-way ANOVA was used to compare data among three or more unpaired

groups. For non-parametric data, Mann-Whitney *U* test was used to compare data between two unpaired groups; Kruskal-Wallis test was used to compared data among three or more unpaired groups. Differences in Bray-Curtis dissimilarity between groups was performed by permutational multivariate ANOVA (PERMANOVA) and pairwise PERMANOVA, using the "adonis" package in R, with 9,999. Linear discriminant analysis Effect Size (LEfSe) was applied to identify the abundant taxa in each site, using default parameters (34). Area under the curve (AUC) was calculated for *in vitro* growth experiments (using OD_{600} values) and bifidobacterial persistence in mice (using copies/ng faecal DNA). Log-rank test was employed to compare survival time differences based on bacterial qPCR detection. One-tailed tests were used where differences between groups were hypothesised to be in a single direction. Statistical outcomes with *P* value <0.05 were considered statistically significant. Core taxa plot was generated using GraphPad Prism, other data were visualised using R.

RESULTS

Fut2 shapes the faecal microbiota

Assessment of the faecal microbiota between SPF *Fut2*^{WT} and *Fut2*^{KO} littermates was performed at 6 weeks of age in both male and female mice (**Fig. 1A**). Faecal microbiota composition (**Fig. 1B**) differed significantly between *Fut2*^{WT} and *Fut2*^{KO} littermates (PERMANOVA: R^2=0.028; P=0.028, **Fig. 1C**) when male and female mice were assessed together. However, stratification according to sex identified a greater divergence according to genotype in male mice (PERMANOVA: R^2=0.12; P=0.021) compared to female mice (PERMANOVA: R^2=0.037; P=0.38, **Fig. 1C**). Exploration of this sex effect identified a significant interaction between sex and *Fut2* genotype (PERMANOVA: R^2=0.11; P=0.0068, **Supplementary Table S3**). These findings were unchanged after adjustment for cage effects (**Supplementary Table S3**). Given the interaction between sex and genotype, all subsequent experiments involved male mice only.

Potential relationships between secretor status and microbiota characteristics were then explored. While bacterial alpha-diversity measures did not differ substantially between differed between *Fut2*^{WT} and *Fut2*^{KO} groups (**Supplementary Fig. S3**), the membership of core microbiota (taxa present in ≥95% of samples at ≥0.01%) did (**Fig. 1D**). Specifically, *Oscillospiraceae* (unassigned) were exclusively core in *Fut2*^{WT}, whereas *Bifidobacterium*, Clostridia (UCG014), *Lachnoclostridium*, and *Parabacteroides* were exclusively core in *Fut2*^{KO} mice (**Fig. 1E**). Three bacterial genera had a significantly higher relative abundance in *Fut2*^{WT} mice, whereas 13 were more prevalent in *Fut2*^{KO} mice (**Fig. 1F**). Of these, *Lactobacillus*, a genus with the α-1,2-L-fucosidases GH29 and GH151 (**Supplementary Table S4**), was more abundant in *Fut2*^{WT} (LDA score [$\log_{10}$]=4.88). Together, these findings support previous reports of a relationship between *Fut2* and faecal microbiome characteristics.

The distal small intestine microbiota is influenced by *Fut2*

As *Fut2* is differentially expressed throughout the gastrointestinal tract (35), we then compared microbiota characteristics in tissue from the proximal small intestine, the distal small intestine, and the large intestine between *Fut2*^{WT} and *Fut2*^{KO} mice (**Fig. 2A**). Microbiota composition differed significantly between *Fut2*^{WT} and *Fut2*^{KO} groups in the distal small intestine (PERMANOVA: R^2=0.54; P=0.0071, **Fig. 2B**), coinciding with inducible *Fut2* expression, but not in the proximal small intestine (PERMANOVA: R^2=0.19; P=0.13, **Fig. 2B**) or the large intestine (PERMANOVA: R^2=0.16; P=0.18, **Fig. 2B**). Taxonomic assessment identified that *Candidatus* Arthromitus (LDA score [$\log_{10}$]=5.43) was more abundant in the distal small intestine in *Fut2*^{WT} compared to *Fut2*^{KO} mice, whereas *Lachnospiraceae* (unassigned) and *Acetatifactor* were more abundant in in the large intestine of *Fut2*^{WT} compared to *Fut2*^{KO} mice (LDA score [$\log_{10}$]=4.35, and LDA score [$\log_{10}$]=3.93, respectively) (**Fig. 2C**).

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

To further investigate the relationship between α(1,2)-fucosylated glycans and intestinal microbiology, faecal homogenate from *Fut2*^{WT} mice was grown in a mBasal media with or without the α(1,2)-fucosylated glycan, 2'-FL. Microbiota assessment following *in vitro* culture (**Fig. 3A**) confirmed the findings of the *in vivo* faecal microbiota analysis, with a significant difference in Bray-Curtis similarity between faecal cultures with and without 2'-FL (PERMANOVA: R^2=0.90; P<0.0001, **Fig. 3B**). This difference was marked by an enrichment of glycan-utilising genera (*Bacteroides*, *Enterococcus*, *Lactobacillus*), with compensatory decreases in the relative abundance of other taxa (**Fig. 3C**).

Analysis involving inoculation of solid basal media, either alone or supplemented with 2′-FL, with faecal homogenate from *Fut2*^{WT} mice further supported these findings. Specifically, proportional colony counts (**Supplementary Fig. S4, Supplementary Table S5**) showed 2′-FL led to enrichment of *Enterococcus faecalis* (66% with 2′-FL vs 4% without 2′-FL) and *Lactobacillus murinus/reuteri* (18% with 2′-FL vs 1% without 2′-FL), with a corresponding decrease in *E. coli* (16% with 2′-FL vs 95% without 2′-FL) (**Fig. 3D**). Enrichment of bacteria with 2′-FL supplementation was further confirmed by increased growth rate and bacterial density (median $AUC_{[mBasal]}$=15.1 [IQR=14.3, 16.5]; $AUC_{[mBasal+2'-FL]}$=35.6 [33.3, 38.7]; P<0.0001, **Fig. 3E**).

We next investigated whether differences in microbiota composition between *Fut2*^{WT} and *Fut2*^{KO} mice reflected selection for bacterial populations able to utilise α(1,2)-fucosylated glycans for growth in *Fut2*^{WT} animals. Faecal homogenates from *Fut2*^{WT} and *Fut2*^{KO} mice were used to inoculate mBasal media with or without 2′-FL supplementation. The increase in bacterial density between 2′-FL supplemented media and media alone was significantly greater when faecal homogenates were derived from *Fut2*^{WT} compared to *Fut2*^{KO} mice (median $AUC_{[WT]}$=20.5 [IQR=16.6, 23.5]; $AUC_{[KO]}$=16.7 [13.1, 19.7]; P=0.0051, **Fig. 3F**), consistent with a greater abundance of glycan-utilizing bacteria.

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

Based on the relationship between host glycan production and gut microbiota composition, we hypothesised that the colonisation dynamics (abundance and persistence) of 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 *B. infantis* (**Fig. 5A**). No significant difference in *B. infantis* persistence post-gavage was observed between $Fut2^{WT}$ and $Fut2^{KO}$ mice (**Fig. 5B**) and *B. infantis* was not detectable in intestinal tissue from either $Fut2^{WT}$ or $Fut2^{KO}$ mice at day 5 post-gavage (**Supplementary Fig.**

S5A). However, analysis of *B. infantis* abundance following gavage (based on AUC) revealed an effect that was opposite to that observed in antibiotic exposed mice, with *B. infantis* significantly higher in *Fut2*^{KO} mice compared to *Fut2*^{WT} (mean $AUC_{[WT]}$=3.8 [SD=0.6]; $AUC_{[KO]}$=5.6 [0.90]; *P*=0.00046; **Fig. 5C**).

Faecal levels of *Bifidobacterium* probiotics were assessed during the instillation period (samples collected two hours prior to gavage and six hours after gavage). Levels in post-gavage samples (6 hours) did not differ between *Fut2*^{WT} and *Fut2*^{KO} mice, consistent with the instillation of equal probiotic loads. However, at 22 hours post-gavage (2 hours prior to gavage), levels in *Fut2*^{KO} mice were significantly higher than in *Fut2*^{WT} mice (*P*<0.0001; **Supplementary Fig. S5B**). No cumulative effect was observed with repeat gavage and the decline in probiotic levels after 22 hours was comparable to that observed in the 24 hours 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. Firstly, 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 α(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.

411 Bifidobacterial species that are commonly used as probiotics are relatively close
412 phylogenetically but differ in their ability to use glycans, even at a strain level (44). We showed
413 that neither *B. breve* (JCM 1192) nor *B. bifidum* (JCM 1255) differed in their abundance or
414 persistence between secretor and non-secretor animals. In contrast, *B. infantis* (JCM 1222)
415 persisted for significantly longer and showed a significantly higher abundance in secretor mice
416 compared to non-secretor mice. This finding likely reflects differences in H antigen hydrolysis
417 and catabolism capacities between species when administered as a probiotic. For example, *B.*
418 *infantis* encodes GH29, GH95, and GH151 family intracellular α-1,2-L-fucosidases, along with
419 fucose transporters to facilitate internalisation (44). While independent hydrolysis and
420 catabolism of mucin-bound H antigens by *B. infantis* is not hypothesised (25), cross-feeding
421 by organisms with extracellular α-1,2-L-fucosidases is likely, even following antibiotic
422 supplementation (45). In contrast, *B. bifidum*, while expressing extracellular GH29 and GH95
423 α-1,2-L-fucosidases, does not consume fucose to facilitate growth (27). Finally, *B. breve*,
424 encodes a separate GH95 intracellular α-1,2-L-fucosidase along with fucose transporters.
425 While this species is capable of utilising the H antigen with support from cross-feeding (46),
426 these findings suggest reduced persistence compared with *B. infantis*, when administered as a
427 probiotic.
428
429 Secondly, we found that antibiotic exposure influenced the persistence of probiotics in a
430 secretor status-dependent manner. In the absence of microbiota depletion through antibiotic
431 exposure, it would be expected that other commensal bacteria would utilise available glycans
432 within the secretor gut. Moreover, such strains are highly adapted to an individual's gut
433 environment, making them likely to outcompete any exogenous glycan-utilisers that are
434 introduced. When we explored this directly, we found that in the absence of a prior period of
435 antibiotic exposure, the higher levels and greater persistence of *B. infantis* in secretors was

436 inverted, with these *B. infantis* being significantly higher in non-secretor mice. These findings
437 likely reflect the competitive exclusion of H antigen-utilising probiotics in the secretor gut and
438 highlight the importance of considering the ecological context in relation to probiotic impact.
439
440 While this study was performed in mice, the effect of secretor status on bifidobacterium
441 supplementation has important implications for probiotic strategies in humans. It is crucial to
442 consider individual host traits and recent antibiotic exposure when designing a probiotic
443 intervention (47). The findings here suggest that the 20% of the population who are non-
444 secretors may have poorer persistence of H antigen utilising probiotics, such as *B. infantis*,
445 compared to secretors following antibiotic exposure. Conversely, in the absence of recent
446 antibiotic exposure, higher levels of microbial niche occupancy in secretors may hamper *B.*
447 *infantis* persistence compared to non-secretors. An individualised supplementation with
448 prebiotics may have potential as a means to optimise probiotic uptake in non-secretors. For
449 example, previous studies have shown that supplementation with human milk oligosaccharides
450 can enhance *B. infantis* engraftment (20), with successful supplementation shown to reduce
451 intestinal inflammation in infants (48). Investigating additional α(1,2)-fucosylated glycans,
452 given as prebiotics, may lead to improved outcomes of *B. infantis* supplementation in non-
453 secretor individuals.
454
455 Determining the impact of secretor status on other species commonly considered beneficial
456 and marketed as probiotics is challenging due to their broad range of carbohydrate utilisation
457 capabilities (49). For instance, *Akkermansia muciniphila*, a mucin-degrading species, has been
458 associated with a reduced risk of chronic inflammatory diseases in humans and mice (50), and
459 is a potential target for probiotic development (47). However, its utilisation of mucin
460 glycoproteins, including the α(1,2)-fucosylated glycan, 2'-fucosyllactose (49), suggests that it

may also be affected by secretor status. While *Akkermansia* was not detected in the mice of this study, we found that *Candidatus* Arthromitus, another genus associated with immune modulation (51), was enriched in the distal small intestinal mucosal tissue of secretor mice. Genome annotation of *Candidatus* Arthromitus has indicated other fucose utilisation capabilities (52), indicating that the functional *FUT2* gene may promote colonisation by this species.

Our experiments involved SPF mice that were obtained through heterozygous mating. Such breeding was essential to allow comparison of $Fut2^{WT}$ and $Fut2^{KO}$ littermates from a maternal secretor lineage. The findings from this study are therefore independent of vertical transmission effects, which are known to influence the microbiome of the offspring (53, 54), and indicate that a change in gut microbiology occurred post-weaning. This difference in baseline gut microbiota composition between secretors and non-secretors was also only evident in male mice. The effect of sex on the relationship between secretor status and the gut microbiome is difficult to explain but may relate to variable intestinal expression of *Fut2*, which can be altered factors such as stress (55). In addition, independent interactions between sex hormones and the gut microbiome (56) may affect the relationship between *Fut2* and the gut microbiome.

Our study demonstrates a *Fut2*-dependent genetic determinant for inter-individual response to probiotic supplementation, which is affected by antibiotic exposure and glycan utilisation capabilities of the probiotic strain. With prior antibiotic exposure, *Fut2* functionality was associated with increased persistence of *B. infantis*, consistent with its ability to utilise the H antigen. However, without antibiotic exposure, *Fut2* functionality was associated with lower abundance of *B. infantis*, relating to difference in baseline microbiology and niche space occupation.

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AUTHOR CONTRIBUTIONS

The study was conceived by SW, SLT and GBR. SLT, GBR, JMC, and YW designed the experiments. YW and SLT performed the experiments; YW and SLT undertook the data generation and statistical analysis. GBR, JMC, AR, and LEP provided technical support and guidance. YW, JMC, SLT, and GBR drafted the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests, nor any financial or personal relationships with other people or organisations that could bias this study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All mice experiments were approved by the South Australia Health and Medical Research Institute Animal Ethics Committee (application numbers SAM-21-07 and SAM-21-036).

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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672 **FIGURES AND TABLES**

673

674 **Fig. 1: *Fut2* affects the faecal microbiota of male mice.** (**A**) Breeding and co-housing design.
675 *Fut2*^{HET} mice were bred to produce *Fut2*^{WT} and *Fut2*^{KO} littermates. Genotypes were co-housed
676 until weaning (~3 weeks), where mice were separated by sex and *Fut2* genotype until 6 weeks
677 of age. (**B**) Taxa bar plot of the faecal microbiota of *Fut2*^{WT} and *Fut2*^{KO} mice, also stratified by
678 sex. (**C**) Non-metric multidimensional scaling (NMDS) plot of faecal microbiota showing
679 significant separation between *Fut2*^{WT} and *Fut2*^{KO} male mice but not *Fut2*^{WT} and *Fut2*^{KO} female
680 mice. Significance: Permutational multivariate ANOVA. (**D**) The prevalence and mean relative
681 abundance of faecal taxa in *Fut2*^{WT} and *Fut2*^{KO} male mice, highlighting the "core" taxa present
682 in ≥95% of mice and at a mean relative abundance of ≥0.01%. (**E**) Venn diagram of core taxa
683 shared or different between *Fut2*^{WT} and *Fut2*^{KO} male mice. (**F**) Faecal taxa significantly
684 different between *Fut2*^{WT} and *Fut2*^{KO} male mice. Significance: Linear discriminant analysis
685 (LDA) Effect Size (LEfSe).

686

687 **Fig. 2: *Fut2* affects the intestinal microbiota.** (**A**) Taxa bar plot of the intestinal mucosal
688 microbiota of *Fut2*^{WT} and *Fut2*^{KO} mice. (**B**) Non-metric multidimensional scaling (NMDS)
689 plots of *Fut2*^{WT} and *Fut2*^{KO} intestinal mucosal microbiota. Significance: Permutational
690 multivariate ANOVA. (**C**) Taxa significantly different between *Fut2*^{WT} and *Fut2*^{KO} intestinal
691 mucosal tissue. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe). No taxa
692 differed between *Fut2*^{WT} and *Fut2*^{KO} proximal small intestine.

693

694 **Fig. 3: 2′-Fucosyllactose modifies the faecal microbiota *in vitro* and enhances growth of**
695 **α(1,2)-fucosylated glycan utilising bacteria.** (**A**) Taxa bar plot of the faecal microbiota of
696 *Fut2*^{WT} mice following anaerobic growth either with or without 2′-Fucosyllactose (2′-FL). (**B**)

Non-metric multidimensional scaling (NMDS) plot of faecal microbiota following anaerobic growth with or without 2′-FL. Significance: Permutational multivariate ANOVA. (**C**) Taxa significantly different between *Fut2*^{WT} faecal samples following anaerobic growth with or without 2′-FL. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe). (**D**) Comparison of colonies relative abundance of identified colonies following anaerobic growth with or without 2′-FL. **E. coli* or *Shigella*. (**E**) Optical density (OD) following *Fut2*^{WT} faecal bacteria cultured with or without 2′-FL. (**F**) OD of *Fut2*^{WT} or *Fut2*^{KO} faecal bacteria following growth with 2'-FL. OD normalised to growth in media without 2'-FL.

Fig. 4: *Bifidobacterium infantis*, but not *B. breve* or *B. bifidum*, persists longer in *Fut2*^{WT} mice following antibiotic pre-exposure. (**A**) Experimental design. (**B**) Persistence of detectable *Bifidobacterium* species in stool following antibiotic pre-exposure. Significance: log-rank test. (**C**) Bacterial copies of *Bifidobacterium* species in stool following antibiotic pre-exposure. Significance: T-test of area under the curve. (**D**) Bacterial copies of *B. infantis* in intestinal tissue mucosa following antibiotic pre-exposure. Significance: Mann-Whitney U test.

Fig. 5: *Bifidobacterium infantis* persists longer in *Fut2*^{KO} mice without antibiotic pre-treatment. (**A**) Experimental design. (**B**) Persistence of gavaged *Bifidobacterium infantis* in stool without antibiotic pre-treatment. Significance: log-rank test. (**C**) Bacterial copies of *B. infantis* in stool without antibiotic pre-treatment. Significance: T-test of area under the curve.

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

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ABSTRACT

Probiotics have gained significant attention as a potential strategy to improve health by modulating host-microbe interactions, particularly in situations where the normal microbiota has been disrupted. However, evidence regarding their efficacy has been inconsistent, with considerable inter-individual variability in response. We aimed to explore whether a common genetic variant that affects the production of mucosal α(1,2)-fucosylated glycans, present in around 20% of the population, could explain the observed interpersonal differences in the persistence of commonly used probiotics. Using a mouse model with varying α(1,2)-fucosylated glycans secretion (*Fut2*^{WT} or *Fut2*^{KO}), we examined the abundance and persistence of *Bifidobacterium* strains (*infantis, breve* and *bifidum*). We observed significant differences in baseline gut microbiota characteristics between *Fut2*^{WT} and *Fut2*^{KO} littermates, with *Fut2*^{WT} mice exhibiting enrichment of species able to utilise α(1,2)-fucosylated glycans. Following antibiotic exposure, only *Fut2*^{WT} animals showed persistent engraftment of *Bifidobacterium infantis*, a strain able to internalise α(1,2)-fucosylated glycans, whereas *B. breve* and *B. bifidum*, which cannot internalise α(1,2)-fucosylated glycans, did not exhibit this difference. In mice with an intact commensal microbiota, the relationship between secretor status and *B. infantis* persistence was reversed, with *Fut2*^{KO} animals showing greater persistence compared to *Fut2*^{WT}. Our findings suggest that the interplay between a common genetic variation and antibiotic exposure plays a crucial role in determining the dynamics of *B. infantis* in the recipient gut, which could potentially contribute to the observed variation in response to this commonly used probiotic species.

Keywords

Microbiome, secretor status, α(1,2)-fucosylated glycans, *Bifidobacterium*, *Bifidobacterium infantis*

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.

Various mechanisms enable the human gut to regulate commensal microbiota composition. One of the principal mechanisms involves the secretion of specific types of sugars that are utilised by beneficial microbial species. Many mucosal constituents and secreted factors are decorated with glycans (oligosaccharides), that are added by a diverse family of glycosyltransferase enzymes (12). Of these, the *FUT2* gene encodes a galactoside α(1,2)-fucosyltransferase, which adds a L-fucose monosaccharide to non-reducing end Gal residues to form Fucα1-2Gal-O-R glycans, termed the H antigens (13, 14). Expressed by multiple mucosal epithelial cell types, this H antigen, is a highly versatile structure that can be further modified to form many other important glycans, including the AB blood group glycans. Because *FUT2* controls the nature of the various α(1,2)-fucosylated glycans secreted by mucosal surfaces, it is commonly referred to as the "secretor" gene (13).

Across the human population, multiple nonsense single nucleotide polymorphisms (SNPs) are found within the *FUT2* gene (15), leading to a "non-secretor" phenotype. The non-secretor phenotype, like the AB blood groups, is one of the more common functional mutations maintained in the population, with approximately one-fifth of people carrying homozygous loss-of-function *FUT2* genes (15). This high carriage of loss-of-function mutations is likely a result of positive selection from altered susceptibility to infections by certain bacterial and viral pathogens (16). However, as fucosylated glycans are an important nutrient source for gut microbes, their absence in non-secretors has been shown to influence commensal microbiota composition (16, 17).

The intact commensal microbiota of secretor individuals is likely to be enriched for glycan utilising bacteria. In contrast, depletion of commensal taxa, for example, through antibiotic exposure, can provide a selective advantage to exogenous glycan utilisers that are absent in

non-secretors (9, 18). Probiotic preparations typically contain *Bifidobacteria* (*Bifidobacterium adolescentis*, *animalis*, *bifidum*, *breve* and *longum*) and/or Lactobacilli (*Lactobacillus acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *rhamnosus* and *salivarius*). Both genera include species that encode the specific glycoside hydrolases (GHs), GH29, GH95, and GH151, which can utilise the H antigen. However, both genera also include species without this glycoside hydrolase capacity. Therefore, the ability of a probiotic to colonise and persist in an individual may depend on the presence of secreted glycans and the ability of the introduced bacterial strain to utilize them. This is supported by studies identifying increased persistence of such glycan-utilising species when supplemented with exogenous oligosaccharides (19, 20).

We hypothesised that the interplay between secretor status, the glycan utilisation ability of the probiotic strain, and the presence of a disrupted commensal microbiota due to antibiotic exposure, would collectively influence the abundance and persistence of probiotic populations in the gut. To test this hypothesis, we introduced probiotic *Bifidobacterium* strains into a murine model of secretor/non-secretor status, with or without prior antibiotic depletion of commensal microbiota.

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.

Fut2^{KO}, *Fut2*^{HET} and *Fut2*^{WT} littermates were co-housed from birth until weaning (~3 weeks), where they were genotyped by PCR amplicon melt curve using primers targeting the outer and inner regions of the *Fut2* gene. *Fut2*^{KO} and *Fut2*^{WT} mice separated into cages after weaning based on sex and *Fut2* genotype (**Figure 1A**). No experiments were performed on *Fut2*^{HET} mice. In all experiments, 6-week old, age- and sex-matched mice were used. Each experimental group consisted of at least 4 cages to control for cage effects.

Antibiotic treatment

A cocktail of ampicillin (1 g/L, Sigma-Aldrich) and neomycin (0.5 g/L, Sigma-Aldrich) was provided to mice via drinking water for 7 days. Water bottle volume and mouse weight were monitored to assess water intake and antibiotic activity was confirmed by 16S qPCR (**Supplementary Table S2**) of faecal samples at day 7.

Probiotic supplementation

Mice received 5×10^7 colony forming units (CFU)/g of mouse of either *B. infantis, B. bifidum,* or *B. breve* daily for 5 days via oral gavage. A starting gavage concentration of 5×10^9 CFU/mL in PBS was prepared daily from fresh overnight cultures. The dose was selected based on previous reports of safety, persistence, and immune modulation capability (22, 23).

Bacterial strains

B. bifidum JCM 1255 (ATCC equivalent: 29521), *B. longum subspecies infantis* JCM 1222 (ATCC equivalent: 15697), *B. breve* JCM 1192 (ATCC equivalent: 15700) were obtained from the Japan Collection of Microorganisms (JCM; RIKEN, Saitama, Japan). Utilisation of α(1,2)-fucosylated glycans was assessed by *in vitro* culture in mBasal media with/without supplementation of 5% w/v 2′-fucosyllactose (Layer Origin, Ithaca, New York, USA)

(**Supplementary Fig. S2)**. This was confirmed by existing literature (24, 25) and the Carbohydrate-Active enZYmes (CAZy) database (26) showing that *B. bifidum* JCM 1255 encodes external GH29 and GH95 α-1,2-L-fucosidases, but does not consume fucose as a carbon source (27), *B. infantis* JCM 1222 encodes intracellular GH29, GH95 and GH151 α-1,2-L-fucosidases along with glycan transporters, and *B. breve* JCM 1192 encodes only the GH95 family.

Faecal and tissue collection

At least two faecal pellets were collected from separated mice at the beginning of the light phase, unless specified otherwise. For intestinal tissue collection, mice were sacrificed by CO_2 asphyxiation and laparotomy was immediately performed using a vertical midline incision. Once the digestive tract was exposed, separate dissection tools were used to dissect tissue into four parts: the proximal small intestine; distal small intestine; caecum and large intestine. For small and large intestine tissue segments, the luminal content was collected by instilling sterile PBS using a syringe barrel and the flushed mucosal tissue was collected into separate tubes. All collected faecal samples, organs, and luminal contents were immediately frozen on dry ice and stored at -80°C until further processing.

DNA extraction

DNA extraction of faecal samples

Faecal pellets were weighed, and 25 mg samples (±10 mg) were resuspended in 300 µL of cold phosphate buffered saline (PBS) (pH 7.2) by vortexing and pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. Microbial DNA was extracted from faecal samples using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously (28).

DNA extraction on mucosal tissue samples

Mucosal tissue from the proximal small intestine, distal small intestine, and large intestine were semi defrosted and 3 cm was removed from the tissue centre using sterile scalpel. The dissected tissues were cut open longitudinally and mixed with 750 µL PowerSoil bead solution and 60 µL solution C1 in a PowerSoil bead tube. The bead tube was then incubated at 65°C for 10 mins prior to bead beating. The subsequent DNA isolation was performed using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously (28).

16S rRNA gene amplicon sequencing and bioinformatic processing

Amplicon libraries of the V4 hypervariable region for 16S rRNA gene were prepared from DNA extracts using modified universal bacterial primer pairs 515F and 806R (29). Amplicon libraries were indexed, cleaned, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol on a 2 × 300 bp Miseq reagent kit v3 at the South Australian Genomics Centre (SAGC), South Australian Health and Medical Research Institute. Paired-end 16S rRNA gene sequence reads were analysed using QIIME2 version 2021.11.0 (30). Briefly, de-noising was performed on de-multiplexed sequences using the DADA2 plugin (31), resulting in a mean read depth of 15,563 ± 2,719 for stool and 6,941 ± 4,503 for tissue. Taxonomic classification of amplicon sequence variants (ASVs) was performed based on the V4 hypervariable region of the SILVA 16S rRNA gene reference database (version 138) at 99% similarity (32). Sufficient coverage at this depth is confirmed by the rarefaction curve, which reached an asymptote. Sequence data has been deposited in the NCBI SRA under accession number PRJNA1011386.

Microbiota characterisation

The taxonomic relative abundance at the genus level was used to generate alpha diversity (within-group) and beta diversity (between-group) measures. Alpha diversity metrics (observed ASVs, Pielou's evenness, Shannon diversity, and Faith's phylogenetic diversity) were obtained from QIIME2 at sampling depth of 9,883 reads (faecal samples) and 662 reads (mucosal tissue samples). The Bray-Curtis dissimilarity index was calculated to compare microbiome similarity between groups (beta diversity), using square-root transformed species relative abundance data using the 'vegan' package in R. Non-metric multidimensional scaling (nMDS) for all beta diversity measures were generated using the 'vegan' package in R. Core taxa were defined as those present in more than 95% of samples, with a mean relative abundance of >0.01%. Identification of taxa with α-1,2-L-fucosidases capability was determined by comparing the genus-level taxonomic classification to genomes identified by CAZy (26) as carrying either the GH29, GH95, or GH151 enzyme families.

Quantification of *Bifidobacterium* species and total bacterial load

Quantification of total bacterial load, *B. breve*, *B. infantis* and *B. bifidum* was performed by SYBR Green-based qPCR assays (**Supplementary Table S2)**. For all qPCR assays, 1 µL of DNA template was combined with 0.7 µL of 10 µM forward primer, 0.7 µL of 10 µM reverse primer, 17.5 µL of 2×SYBR Green (Applied Biosystems, Waltham, Massachusetts, USA) and 15.1 µL nuclease-free water. All samples were run in triplicate (10 µL each replicate). Gene copy quantification was performed using a standard curve generated from a known concentration of a pure colony control. Any sample with a cycle threshold (CT) ≥40 cycles was defined as 40 (limit of detection).

Culture of *Bifidobacterium* strains

All *Bifidobacterium* strains used in this study were cultured in De Man, Rogosa and Sharpe (Becton Dickinson, Franklin Lakes, NJ, USA) broth or agar supplemented with 0.34% (w/v) sodium ascorbate and 0.02% (w/v) cysteine-HCl (MRS-CS) and were grown under anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA) at 37 °C. The growth of *Bifidobacterium* was measured by optical density (600 nm) using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA).

In vitro glycan utilisation assay

Faecal pellets from untreated WT mice were incubated in mBasal media (mBasal; 10 g/L Trypton, 2 g/L yeast extract, 5 g/L NaCl, 0.2 g/L magnesium sulfate, 2 g/L dipotassium hydrogen phosphate, pH 6.4) with/without supplementation of 5% w/v of 2′-Fucosyllactose (Layer Origin, Ithaca, New York, USA) at 37°C under strict anaerobic conditions (75% N2, 20% CO2, 5% H2, Coy Laboratory Products, Grass Lake, Michigan, USA). Bacterial biomass was measured by optical density (600 nm, OD_{600}) using multimode plate reader (PerkinElmer, Waltham, Massachusetts, USA). Bacterial colonies cultured from faecal samples were identified by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Billerica, Massachusetts, United States), as described previously (33).

Statistical analysis

Experimental mice were randomly assigned to different treatment groups. The investigators were not blinded to the experimental groups. No outliers have been removed from any of the data presented. All data analyses were performed using either R (R Foundation for Statistical Computing; version 4.1.0) or GraphPad Prism software (GraphPad Software, Inc.; version 9.00). For parametric data, unpaired Student's t test was used to compare data between two unpaired groups; One-way ANOVA was used to compare data among three or more unpaired

groups. For non-parametric data, Mann-Whitney *U* test was used to compare data between two unpaired groups; Kruskal-Wallis test was used to compared data among three or more unpaired groups. Differences in Bray-Curtis dissimilarity between groups was performed by permutational multivariate ANOVA (PERMANOVA) and pairwise PERMANOVA, using the "adonis" package in R, with 9,999. Linear discriminant analysis Effect Size (LEfSe) was applied to identify the abundant taxa in each site, using default parameters (34). Area under the curve (AUC) was calculated for *in vitro* growth experiments (using OD_{600} values) and bifidobacterial persistence in mice (using copies/ng faecal DNA). Log-rank test was employed to compare survival time differences based on bacterial qPCR detection. One-tailed tests were used where differences between groups were hypothesised to be in a single direction. Statistical outcomes with P value <0.05 were considered statistically significant. Core taxa plot was generated using GraphPad Prism, other data were visualised using R.

RESULTS

Fut2 shapes the faecal microbiota

Assessment of the faecal microbiota between SPF *Fut2*^{WT} and *Fut2*^{KO} littermates was performed at 6 weeks of age in both male and female mice (**Fig. 1A**). Faecal microbiota composition (**Fig. 1B**) differed significantly between *Fut2*^{WT} and *Fut2*^{KO} littermates (PERMANOVA: R^2=0.028; P=0.028, **Fig. 1C**) when male and female mice were assessed together. However, stratification according to sex identified a greater divergence according to genotype in male mice (PERMANOVA: R^2=0.12; P=0.021) compared to female mice (PERMANOVA: R^2=0.037; P=0.38, **Fig. 1C**). Exploration of this sex effect identified a significant interaction between sex and *Fut2* genotype (PERMANOVA: R^2=0.11; P=0.0068, **Supplementary Table S3**). These findings were unchanged after adjustment for cage effects (**Supplementary Table S3**). Given the interaction between sex and genotype, all subsequent experiments involved male mice only.

Potential relationships between secretor status and microbiota characteristics were then explored. While bacterial alpha-diversity measures did not differ substantially between differed between *Fut2*^{WT} and *Fut2*^{KO} groups (**Supplementary Fig. S3**), the membership of core microbiota (taxa present in ≥95% of samples at ≥0.01%) did (**Fig. 1D**). Specifically, *Oscillospiraceae* (unassigned) were exclusively core in *Fut2*^{WT}, whereas *Bifidobacterium*, Clostridia (UCG014), *Lachnoclostridium*, and *Parabacteroides* were exclusively core in *Fut2*^{KO} mice (**Fig. 1E**). Three bacterial genera had a significantly higher relative abundance in *Fut2*^{WT} mice, whereas 13 were more prevalent in *Fut2*^{KO} mice (**Fig. 1F**). Of these, *Lactobacillus,* a genus with the α-1,2-L-fucosidases GH29 and GH151 (**Supplementary Table S4)**, was more abundant in *Fut2*^{WT} (LDA score [$\log_{10}$]=4.88). Together, these findings support previous reports of a relationship between *Fut2* and faecal microbiome characteristics.

300

301 **The distal small intestine microbiota is influenced by *Fut2***

302 As *Fut2* is differentially expressed throughout the gastrointestinal tract (35), we then compared
303 microbiota characteristics in tissue from the proximal small intestine, the distal small intestine,
304 and the large intestine between *Fut2*^{WT} and *Fut2*^{KO} mice (**Fig. 2A**). Microbiota composition
305 differed significantly between *Fut2*^{WT} and *Fut2*^{KO} groups in the distal small intestine
306 (PERMANOVA: R^2=0.54; P=0.0071, **Fig. 2B**), coinciding with inducible *Fut2* expression, but
307 not in the proximal small intestine (PERMANOVA: R^2=0.19; P=0.13, **Fig. 2B**) or the large
308 intestine (PERMANOVA: R^2=0.16; P=0.18, **Fig. 2B**). Taxonomic assessment identified that
309 *Candidatus* Arthromitus (LDA score [$\log_{10}$]=5.43) was more abundant in the distal small
310 intestine in *Fut2*^{WT} compared to *Fut2*^{KO} mice, whereas *Lachnospiraceae* (unassigned) and
311 *Acetatifactor* were more abundant in in the large intestine of *Fut2*^{WT} compared to *Fut2*^{KO} mice
312 (LDA score [$\log_{10}$]=4.35, and LDA score [$\log_{10}$]=3.93, respectively) (**Fig. 2C**).

313

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

315 To further investigate the relationship between α(1,2)-fucosylated glycans and intestinal
316 microbiology, faecal homogenate from *Fut2*^{WT} mice was grown in a mBasal media with or
317 without the α(1,2)-fucosylated glycan, 2'-FL. Microbiota assessment following *in vitro* culture
318 (**Fig. 3A**) confirmed the findings of the *in vivo* faecal microbiota analysis, with a significant
319 difference in Bray-Curtis similarity between faecal cultures with and without 2'-FL
320 (PERMANOVA: R^2=0.90; P<0.0001, **Fig. 3B**). This difference was marked by an enrichment
321 of glycan-utilising genera (*Bacteroides*, *Enterococcus*, *Lactobacillus*), with compensatory
322 decreases in the relative abundance of other taxa (**Fig. 3C**).

323

Analysis involving inoculation of solid basal media, either alone or supplemented with 2'-FL, with faecal homogenate from *Fut2*^{WT} mice further supported these findings. Specifically, proportional colony counts (**Supplementary Fig. S4, Supplementary Table S5**) showed 2'-FL led to enrichment of *Enterococcus faecalis* (66% with 2'-FL vs 4% without 2'-FL) and *Lactobacillus murinus/reuteri* (18% with 2'-FL vs 1% without 2'-FL), with a corresponding decrease in *E. coli* (16% with 2'-FL vs 95% without 2'-FL) (**Fig. 3D**). Enrichment of bacteria with 2'-FL supplementation was further confirmed by increased growth rate and bacterial density (median $AUC_{[mBasal]}$=15.1 [IQR=14.3, 16.5]; $AUC_{[mBasal+2'-FL]}$=35.6 [33.3, 38.7]; P<0.0001, **Fig. 3E**).

We next investigated whether differences in microbiota composition between *Fut2*^{WT} and *Fut2*^{KO} mice reflected selection for bacterial populations able to utilise α(1,2)-fucosylated glycans for growth in *Fut2*^{WT} animals. Faecal homogenates from *Fut2*^{WT} and *Fut2*^{KO} mice were used to inoculate mBasal media with or without 2'-FL supplementation. The increase in bacterial density between 2'-FL supplemented media and media alone was significantly greater when faecal homogenates were derived from *Fut2*^{WT} compared to *Fut2*^{KO} mice (median $AUC_{[WT]}$=20.5 [IQR=16.6, 23.5]; $AUC_{[KO]}$=16.7 [13.1, 19.7]; P=0.0051, **Fig. 3F**), consistent with a greater abundance of glycan-utilizing bacteria.

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

Based on the relationship between host glycan production and gut microbiota composition, we hypothesised that the colonisation dynamics (abundance and persistence) of 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 *B. infantis* (**Fig. 5A**). No significant difference in *B. infantis* persistence post-gavage was observed between *Fut2*^{WT} and *Fut2*^{KO} mice (**Fig. 5B**) and *B. infantis* was not detectable in intestinal tissue from either *Fut2*^{WT} or *Fut2*^{KO} mice at day 5 post-gavage (**Supplementary Fig.**

S5A). However, analysis of *B. infantis* abundance following gavage (based on AUC) revealed an effect that was opposite to that observed in antibiotic exposed mice, with *B. infantis* significantly higher in *Fut2*^{KO} mice compared to *Fut2*^{WT} (mean $AUC_{[WT]}$=3.8 [SD=0.6]; $AUC_{[KO]}$=5.6 [0.90]; *P*=0.00046; **Fig. 5C**).

Faecal levels of *Bifidobacterium* probiotics were assessed during the instillation period (samples collected two hours prior to gavage and six hours after gavage). Levels in post-gavage samples (6 hours) did not differ between *Fut2*^{WT} and *Fut2*^{KO} mice, consistent with the instillation of equal probiotic loads. However, at 22 hours post-gavage (2 hours prior to gavage), levels in *Fut2*^{KO} mice were significantly higher than in *Fut2*^{WT} mice (*P*<0.0001; **Supplementary Fig. S5B**). No cumulative effect was observed with repeat gavage and the decline in probiotic levels after 22 hours was comparable to that observed in the 24 hours 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. Firstly, 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 α(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.

Bifidobacterial species that are commonly used as probiotics are relatively close phylogenetically but differ in their ability to use glycans, even at a strain level (44). We showed that neither *B. breve* (JCM 1192) nor *B. bifidum* (JCM 1255) differed in their abundance or persistence between secretor and non-secretor animals. In contrast, *B. infantis* (JCM 1222) persisted for significantly longer and showed a significantly higher abundance in secretor mice compared to non-secretor mice. This finding likely reflects differences in H antigen hydrolysis and catabolism capacities between species when administered as a probiotic. For example, *B. infantis* encodes GH29, GH95, and GH151 family intracellular α-1,2-L-fucosidases, along with fucose transporters to facilitate internalisation (44). While independent hydrolysis and catabolism of mucin-bound H antigens by *B. infantis* is not hypothesised (25), cross-feeding by organisms with extracellular α-1,2-L-fucosidases is likely, even following antibiotic supplementation (45). In contrast, *B. bifidum*, while expressing extracellular GH29 and GH95 α-1,2-L-fucosidases, does not consume fucose to facilitate growth (27). Finally, *B. breve*, encodes a separate GH95 intracellular α-1,2-L-fucosidase along with fucose transporters. While this species is capable of utilising the H antigen with support from cross-feeding (46), these findings suggest reduced persistence compared with *B. infantis*, when administered as a probiotic.

Secondly, we found that antibiotic exposure influenced the persistence of probiotics in a secretor status-dependent manner. In the absence of microbiota depletion through antibiotic exposure, it would be expected that other commensal bacteria would utilise available glycans within the secretor gut. Moreover, such strains are highly adapted to an individual's gut environment, making them likely to outcompete any exogenous glycan-utilisers that are introduced. When we explored this directly, we found that in the absence of a prior period of antibiotic exposure, the higher levels and greater persistence of *B. infantis* in secretors was

436 inverted, with these *B. infantis* being significantly higher in non-secretor mice. These findings
437 likely reflect the competitive exclusion of H antigen-utilising probiotics in the secretor gut and
438 highlight the importance of considering the ecological context in relation to probiotic impact.
439
440 While this study was performed in mice, the effect of secretor status on bifidobacterium
441 supplementation has important implications for probiotic strategies in humans. It is crucial to
442 consider individual host traits and recent antibiotic exposure when designing a probiotic
443 intervention (47). The findings here suggest that the 20% of the population who are non-
444 secretors may have poorer persistence of H antigen utilising probiotics, such as *B. infantis*,
445 compared to secretors following antibiotic exposure. Conversely, in the absence of recent
446 antibiotic exposure, higher levels of microbial niche occupancy in secretors may hamper *B.*
447 *infantis* persistence compared to non-secretors. An individualised supplementation with
448 prebiotics may have potential as a means to optimise probiotic uptake in non-secretors. For
449 example, previous studies have shown that supplementation with human milk oligosaccharides
450 can enhance *B. infantis* engraftment (20), with successful supplementation shown to reduce
451 intestinal inflammation in infants (48). Investigating additional α(1,2)-fucosylated glycans,
452 given as prebiotics, may lead to improved outcomes of *B. infantis* supplementation in non-
453 secretor individuals.
454
455 Determining the impact of secretor status on other species commonly considered beneficial
456 and marketed as probiotics is challenging due to their broad range of carbohydrate utilisation
457 capabilities (49). For instance, *Akkermansia muciniphila*, a mucin-degrading species, has been
458 associated with a reduced risk of chronic inflammatory diseases in humans and mice (50), and
459 is a potential target for probiotic development (47). However, its utilisation of mucin
460 glycoproteins, including the α(1,2)-fucosylated glycan, 2'-fucosyllactose (49), suggests that it

may also be affected by secretor status. While *Akkermansia* was not detected in the mice of this study, we found that *Candidatus* Arthromitus, another genus associated with immune modulation (51), was enriched in the distal small intestinal mucosal tissue of secretor mice. Genome annotation of *Candidatus* Arthromitus has indicated other fucose utilisation capabilities (52), indicating that the functional *FUT2* gene may promote colonisation by this species.

Our experiments involved SPF mice that were obtained through heterozygous mating. Such breeding was essential to allow comparison of $Fut2^{WT}$ and $Fut2^{KO}$ littermates from a maternal secretor lineage. The findings from this study are therefore independent of vertical transmission effects, which are known to influence the microbiome of the offspring (53, 54), and indicate that a change in gut microbiology occurred post-weaning. This difference in baseline gut microbiota composition between secretors and non-secretors was also only evident in male mice. The effect of sex on the relationship between secretor status and the gut microbiome is difficult to explain but may relate to variable intestinal expression of *Fut2*, which can be altered factors such as stress (55). In addition, independent interactions between sex hormones and the gut microbiome (56) may affect the relationship between *Fut2* and the gut microbiome.

Our study demonstrates a *Fut2*-dependent genetic determinant for inter-individual response to probiotic supplementation, which is affected by antibiotic exposure and glycan utilisation capabilities of the probiotic strain. With prior antibiotic exposure, *Fut2* functionality was associated with increased persistence of *B. infantis*, consistent with its ability to utilise the H antigen. However, without antibiotic exposure, *Fut2* functionality was associated with lower abundance of *B. infantis*, relating to difference in baseline microbiology and niche space occupation.

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AUTHOR CONTRIBUTIONS

The study was conceived by SW, SLT and GBR. SLT, GBR, JMC, and YW designed the experiments. YW and SLT performed the experiments; YW and SLT undertook the data generation and statistical analysis. GBR, JMC, AR, and LEP provided technical support and guidance. YW, JMC, SLT, and GBR drafted the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests, nor any financial or personal relationships with other people or organisations that could bias this study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All mice experiments were approved by the South Australia Health and Medical Research Institute Animal Ethics Committee (application numbers SAM-21-07 and SAM-21-036).

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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672 **FIGURES AND TABLES**

673

674 **Fig. 1: *Fut2* affects the faecal microbiota of male mice.** (**A**) Breeding and co-housing design.
675 *Fut2*^{HET} mice were bred to produce *Fut2*^{WT} and *Fut2*^{KO} littermates. Genotypes were co-housed
676 until weaning (~3 weeks), where mice were separated by sex and *Fut2* genotype until 6 weeks
677 of age. (**B**) Taxa bar plot of the faecal microbiota of *Fut2*^{WT} and *Fut2*^{KO} mice, also stratified by
678 sex. (**C**) Non-metric multidimensional scaling (NMDS) plot of faecal microbiota showing
679 significant separation between *Fut2*^{WT} and *Fut2*^{KO} male mice but not *Fut2*^{WT} and *Fut2*^{KO} female
680 mice. Significance: Permutational multivariate ANOVA. (**D**) The prevalence and mean relative
681 abundance of faecal taxa in *Fut2*^{WT} and *Fut2*^{KO} male mice, highlighting the "core" taxa present
682 in ≥95% of mice and at a mean relative abundance of ≥0.01%. (**E**) Venn diagram of core taxa
683 shared or different between *Fut2*^{WT} and *Fut2*^{KO} male mice. (**F**) Faecal taxa significantly
684 different between *Fut2*^{WT} and *Fut2*^{KO} male mice. Significance: Linear discriminant analysis
685 (LDA) Effect Size (LEfSe).

686

687 **Fig. 2: *Fut2* affects the intestinal microbiota.** (**A**) Taxa bar plot of the intestinal mucosal
688 microbiota of *Fut2*^{WT} and *Fut2*^{KO} mice. (**B**) Non-metric multidimensional scaling (NMDS)
689 plots of *Fut2*^{WT} and *Fut2*^{KO} intestinal mucosal microbiota. Significance: Permutational
690 multivariate ANOVA. (**C**) Taxa significantly different between *Fut2*^{WT} and *Fut2*^{KO} intestinal
691 mucosal tissue. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe). No taxa
692 differed between *Fut2*^{WT} and *Fut2*^{KO} proximal small intestine.

693

694 **Fig. 3: 2′-Fucosyllactose modifies the faecal microbiota *in vitro* and enhances growth of**
695 **α(1,2)-fucosylated glycan utilising bacteria.** (**A**) Taxa bar plot of the faecal microbiota of
696 *Fut2*^{WT} mice following anaerobic growth either with or without 2′-Fucosyllactose (2′-FL). (**B**)

Non-metric multidimensional scaling (NMDS) plot of faecal microbiota following anaerobic growth with or without 2′-FL. Significance: Permutational multivariate ANOVA. (**C**) Taxa significantly different between *Fut2*^{WT} faecal samples following anaerobic growth with or without 2′-FL. Significance: Linear discriminant analysis (LDA) Effect Size (LEfSe). (**D**) Comparison of colonies relative abundance of identified colonies following anaerobic growth with or without 2′-FL. **E. coli* or *Shigella.* (**E**) Optical density (OD) following *Fut2*^{WT} faecal bacteria cultured with or without 2′-FL. (**F**) OD of *Fut2*^{WT} or *Fut2*^{KO} faecal bacteria following growth with 2'-FL. OD normalised to growth in media without 2'-FL.

Fig. 4: *Bifidobacterium infantis*, but not *B. breve* or *B. bifidum*, persists longer in *Fut2*^{WT} mice following antibiotic pre-exposure. (**A**) Experimental design. (**B**) Persistence of detectable *Bifidobacterium* species in stool following antibiotic pre-exposure. Significance: log-rank test. (**C**) Bacterial copies of *Bifidobacterium* species in stool following antibiotic pre-exposure. Significance: T-test of area under the curve. (**D**) Bacterial copies of *B. infantis* in intestinal tissue mucosa following antibiotic pre-exposure. Significance: Mann-Whitney U test.

Fig. 5: *Bifidobacterium infantis* persists longer in *Fut2*^{KO} mice without antibiotic pre-treatment. (**A**) Experimental design. (**B**) Persistence of gavaged *Bifidobacterium infantis* in stool without antibiotic pre-treatment. Significance: log-rank test. (**C**) Bacterial copies of *B. infantis* in stool without antibiotic pre-treatment. Significance: T-test of area under the curve.