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,

A drawing of a plane

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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.

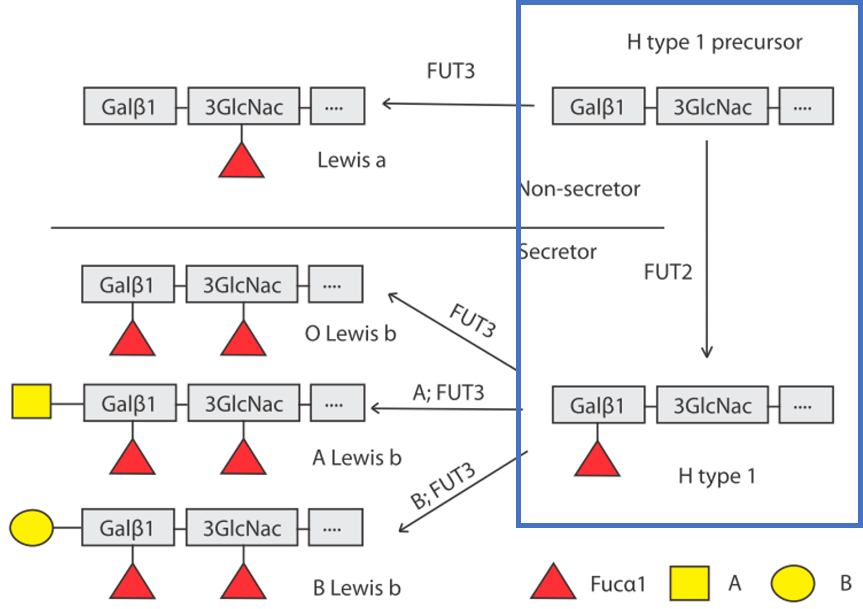
General comment:

**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:** Weacknowledge 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 OLewisb, ALewisb, or BLewisb 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 Lewisb antigens are not displayed, leading to a Lewisa 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 × 107 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 × 109 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:** Weinvestigated 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).

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**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 Fut2KO and Fut2WT littermates, while also standardising effects of Fut2 that occur through vertical transmission. Fut2KO, Fut2HET and Fut2WT 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. Fut2KO and Fut2WT mice separated into cages after weaning based on sex and Fut2 genotype (Figure 1A). No experiments were performed on Fut2HET 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.*

A diagram of mice in a container

Description automatically generated