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Ecological importance of cross-feeding of the intermediate metabolite 1,2-propanediol between bacterial gut symbionts

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Running Head: Cross-feeding of gut symbionts through 1,2-propanediol

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Abstract

Cross-feeding based on the metabolite 1,2-propanediol has been proposed to have an important role in the establishment of trophic interactions among gut symbionts, but its ecological importance has not been empirically established. Here, we show that *in vitro* growth of *Lactobacillus reuteri* ATCC PTA 6475 is enhanced through 1,2-propanediol produced by *Bifidobacterium breve* UCC2003 and *Escherichia coli* MG1655 from the metabolization of fucose and rhamnose, respectively. Work with isogenic mutants showed that the tropic interaction is dependent on the *pduCDE* operon in *L. reuteri*, which encodes for the ability to use 1,2-propanediol, and the L-fucose permease (*fucP*) gene in *B. breve*, which is required for 1,2-propanediol formation from fucose. Experiments in gnotobiotic mice revealed that, although the *pduCDE* operon bestows a fitness burden on *L. reuteri* ATCC PTA 6475 in the mouse digestive tract, the ecological performance of the strain was enhanced in the presence of *B. breve* UCC2003 and the mucus-degrading species *Bifidobacterium bifidum*. The use of the respective *pduCDE* and *fucP* mutants of *L. reuteri* and *B. breve* in the mouse experiments indicated that the trophic interaction was specifically based on 1,2-propanediol. Overall, our work established the ecological importance of cross-feeding relationships based on 1,2-propanediol for the fitness of a bacterial symbiont in the vertebrate gut.

Importance

Through experiments in gnotobiotic mice that employed isogenic mutants of bacterial strains that produce (*Bifidobacterium breve*) and utilize (*Lactobacillus reuteri*) 1,2-propanediol, this study provides mechanistic insight into the ecological ramifications of a trophic interaction between gut symbionts. The findings improve our understanding on how cross-feeding influences the competitive fitness of *L. reuteri* in the vertebrate gut and revealed a putative selective force that shaped the evolution of the species. The findings are relevant as they provide a basis to design rational microbial-based strategies to modulate gut ecosystems, which could employ mixtures of bacterial strains that establish trophic interactions or a personalized approach based on the ability of a resident microbiota to provide resources for the incoming microbe.

Introduction

The gut microbiota is a complex microbial community whose members form interdependent trophic relationships that determine the ecology of bacterial populations and their interplay with the host (1). One such interaction involves the exchange of products between microbes, otherwise known as cross-feeding (2). Cross-feeding of nutrients is central to the fermentative degradation of non-digestible dietary fibres and host-derived substrates, such as mucin or human milk oligosaccharides (HMO) (3-10). For example, glycans released from the partial degradation of HMO are utilized by other bifidobacterial species (11), and certain bifidobacteria and *Akkermansia muciniphila* liberate glycans from host mucins that are utilized by other inhabitants of the gut (6, 12). Similarly, *Ruminococcus bromii* releases carbohydrates from resistant starch fermentations into the gut environment (13). These interactions ultimately lead to the formation of dynamic metabolic networks essential in the ecology of the gut microbiome and the production of short chain fatty acids (SCFA), which are a key ecosystem service that benefits the host (14, 15).

Trophic interactions can also result from the exchange of metabolic end-products derived from fermentation processes. Hydrogenotrophic microbes utilize the molecular hydrogen produced by several fermentative organisms, a process important to maintain energy flux (16). In addition, cross-feeding based on intermediary metabolites such as lactate, acetate, succinate, and 1,2-propanediol is important for the production of SCFA (5). In the gut, 1,2-propanediol results from the microbial fermentation of deoxyhexose sugars that originate from the hydrolyzation of dietary fibre, fucosylated HMOs, and host mucins, such as rhamnose and fucose (17, 18). Several gut commensal bacteria (i.e.

75 *Bacteroides thetaiotaomicron, Roseburia inulinivorans, Escherichia coli,* and
76 *Bifidobacterium breve*) can produce 1,2-propanediol from the fermentation of either
77 rhamnose or fucose (3, 5, 17, 18). Other bacteria such as *Eubacterium hallii* and
78 *Lactobacillus reuteri* do not utilize fucose or rhamnose directly but metabolize 1,2-
79 propanediol (19-21) and convert it to propanol and propionate (21, 22). Cross-feeding of
80 1,2-propanediol between *Eu. hallii* and *Bifidobacterium* species based on metabolism of
81 mucin and HMO has been studied *in vitro* (3, 4, 6). Although cross-feeding based on
82 1,2-propanediol is considered important in gut ecosystems (5) and has been shown to
83 confer a fitness advantage *in vivo* for pathogens such as *Salmonella* spp. (23), its
84 ecological importance for bacterial gut symbionts, and the underlying mechanisms by
85 which it impacts bacterial performance in the gut, have not been empirically established
86 *in vivo* in a tractable experimental model.

87 In both *Eu. hallii* and *L. reuteri*, 1,2-propanediol is metabolized through the
88 cobalamin-dependent glycerol/diol dehydratase encoded in the *pduCDE* operon, found
89 in the *pdu-cbi-cob-hem* gene (*pdu*) cluster (19-21). The glycerol/dehydratase converts
90 1,2-propanediol to propionaldehyde and further to propanol and propionate and have
91 been shown to increase growth rates for *L. reuteri in vitro* (19, 24). In *L. reuteri*, the *pdu*
92 cluster is observed predominantly in two *L. reuteri* phylogenetic lineages that are
93 dominated by strains from human, herbivore, and chicken origin, but rarer in strains
94 from other hosts such as mice and rats (25). This distribution suggests that the cluster
95 constitutes an adaptation to the characteristics of the gastrointestinal tract of specific
96 hosts (26, 27). In the murine forestomach, which is densely colonized by *L. reuteri* (25),
97 fermentable mono- and disaccharides are in ample supply (28). In contrast, distal

98 portions of the human intestinal tract are characterized by low concentrations of mono-
99 and disaccharides. The *pdu* cluster may therefore constitute a colonization factor in the
100 distal regions of the gut, to take advantage of 1,2-propanediol produced by other
101 microbes (19, 25).

102 Most studies on diol-metabolism in *L. reuteri* have focused on the synthesis of the
103 antimicrobial intermediate β-hydroxypropionaldehyde (reuterin) from glycerol, which is
104 also mediated by the *pdu* operon (29). It remains unclear whether *L. reuteri* uses the
105 *pdu* operon to engage in cross-feeding interrelationships with other gut bacterial species
106 that produce 1,2-propanediol in its gastrointestinal habitat, and how this interaction
107 contributes to its ecological competitiveness. It was therefore the aim of this study to
108 characterize the ecological importance of 1,2-propanediol-cross-feeding between *L.*
109 *reuteri* and the 1,2-propanediol producers *Bifidobacterium breve* and *Escherichia coli,*
110 both *in vitro* and in the digestive tract of mice.

111

112 **Results**

113 **1,2-propanediol *enhances growth of L. reuteri in vitro***

114 We first sought to confirm the importance of 1,2-propanediol metabolism for *L.*
115 *reuteri L. reuteri* ATCC PTA 6475 (referred to as *L. reuteri* PTA 6475) growth *in vitro*.
116 The presence of 1,2-propanediol in the medium containing glucose improved growth
117 rates and increased the final cell density of the strain when compared to the growth
118 solely on glucose or to growth of a *pduCDE* delection mutant (referred to as *L. reuteri*
119 Δ*pduCDE*) (Fig. 1A). Neither *L. reuteri* PTA 6475 nor *L. reuteri* Δ*pduCDE* were able to

use 1,2-propanediol as the sole carbon source for growth (Fig. 1A). To confirm that the enhanced growth of *L. reuteri* PTA 6475 was due to 1,2-propanediol metabolism, the metabolic end-products in the supernatant were measured using HPLC. As shown in Figure 1B and C, *L. reuteri* PTA 6475 but not *L. reuteri* Δ*pduCDE* converted 1,2-propanediol to propanol and low concentrations of propionate. For *L. reuteri* PTA 6475 but not *L. reuteri* Δ*pduCDE*, utilization of 1,2-propanediol resulted in acetate production and decreased production of ethanol (Fig. 1D & E; Fig. S1A & B). These findings confirm that *L. reuteri* PTA 6475 is able to disproportionate 1,2-propanediol to propanol and propionate (22). However, 1,2-propanediol is not used as sole substrate but co-metabolized with glucose. Predominant production of propanol demonstrates that the reducing branch of the propanediol pathway is preferred over the oxidizing branch to regenerate electron acceptors, thus enhancing acetate formation and ATP production, and therefore growth (30).

Interspecies cross-feeding of 1-2-propanediol enhances growth of *L. reuteri in vitro*

We developed an experimental system to study cross-feeding between *L. reuteri* and gut bacteria that produce 1,2-propanediol. We chose *B. breve* and *E. coli* which are known to produce 1,2-propanediol from fucose and rhamnose, respectively, which are substrates not utilized by *L. reuteri*. Since growth rates and growth conditions differ among *L. reuteri*, *B. breve*, and *E. coli* are, cross-feeding was not studied in co-culture. Instead, *B. breve* and *E. coli* were first grown under their respective optimal conditions and on the specific substrates that result in the production of 1,2-propanediol. Spent supernatant obtained from these fermentations was then supplemented with glucose

and half-strength mMRS (conditioned media; see Materials and Methods) and used for analyzing growth kinetics and metabolite production of *L. reuteri* strains (Table 1). Conditioned media from an isogenic mutant of *B. breve* with an insertion mutation in *fucP* (encoding the L-fucose transporter) that is unable to metabolize fucose into 1,2-propanediol served as a control (Table 1).

B. breve UCC2003 and its *fucP* mutant *B. breve* UCC2003-fucP were grown in a medium containing cellobiose with and without fucose. Fucose does not support the growth of *B. breve* UCC2003 yet is co-utilized with cellobiose to produce 1,2-propanediol. Importantly, neither fucose or cellobiose are metabolized by *L. reuteri* (20). In the fucose/cellobiose-containing medium, *B. breve* UCC2003 and *B. breve* UCC2003-fucP reached similar cell density after 24 hours of growth (Fig. S2). *L. reuteri* PTA 6475 reached a significantly higher OD_{600} and showed elevated growth rate when grown in conditioned medium with supernatant of *B. breve* UCC2003 grown with fucose and cellobiose when compared to *L. reuteri* Δ*pduCDE* or *L. reuteri* PTA 6475 grown in conditioned medium of *B. breve* UCC2003 grown in the absence of fucose (Fig. 2A). The growth advantage of *L. reuteri* PTA 6475 was not observed in conditioned media from the *B. breve* UCC2003-fucP, even if grown in the presence of fucose (Fig. 2B). HPLC analysis confirmed the presence of 1,2-propanediol in the conditioned medium of *B. breve* UCC2003 but not *B. breve* UCC2003-fucP grown with fucose (Fig. S3A), and showed that enhanced growth of *L. reuteri* PTA 6475 was linked to the conversion of 1,2-propanediol to propanol, which was not detected in *L. reuteri* Δ*pduCDE* cultures (Fig. 2C & D). Propionate, acetate, and ethanol could not be quantified as unknown compounds in the conditioned media interfered with the metabolite analysis. Fucose

and cellobiose did not alter the growth kinetics of the *L. reuteri* strains when grown in glucose (Fig. S4A), and they did not serve as growth substrates on their own (Fig. S4B), confirming that the enhanced growth in *L. reuteri* PTA 6475 was not due to a direct effect of residual concentrations of these sugars in the spend supernatant.

1,2-propanediol results also from the fermentation of rhamnose by *E. coli* MG1655 (Fig S3B). Cross-feeding experiments revealed that *L. reuteri* PTA 6475 had a higher growth rate and reached a significantly higher cell density when grown in conditioned medium of *E. coli* grown on rhamnose when compared to *L. reuteri* Δ*pduCDE*, or *L. reuteri* PTA 6475 grown in conditioned medium from *E. coli* that did not contain rhamnose (Fig. 3A). Importantly, growth experiments of *L. reuteri* strains in media with rhamnose with or without glucose confirmed that rhamnose could neither be used as a carbon source nor alter growth (Fig. S4C & D). Metabolite analysis of fermentations conducted in the conditioned medium of *E. coli* grown on rhamnose revealed that *L. reuteri* PTA 6475, and not *L. reuteri* Δ*pduCDE,* could metabolize 1,2-propanediol produced by *E. coli* and form propanol, propionate, and acetate (Fig. 3B-D; Fig. S5). Metabolite interference in the conditioned media did not allow the quantification of ethanol by HPLC.

Together, these findings demonstrate that *L. reuteri* PTA 6475 utilizes 1,2-propanediol produced by *B. breve* and *E. coli* from the fermentation of deoxyhexose sugars as an electron acceptor, enhancing its growth capabilities.

Importance of 1,2-propanediol cross-feeding in the gastrointestinal tract

The ecological relevance of cross-feeding based on 1,2-propanediol in the gastrointestinal tract was investigated with a series of colonization experiments in gnotobiotic mice (Fig 4; Table S1 & S2). Germ-free Swiss-Webster mice (6-16 weeks old; male and female) were housed in individually ventilated cages (groups of 2-3). A fat-free diet was used in order to avoid possible confounding affects from the hydrolysis of dietary triglyceride fats, which is a source of glycerol (also utilized via the *pdu* cluster and enhances growth). Fecal cell numbers of colonizing strains were determined by selective plating (see Materials and Methods).

As described earlier, *B. breve* produces 1,2-propanediol from fucose. Host mucins are an intrinsic source of fucose in the gastrointestinal tract, but *B. breve* does not possess glycosidases required for mucin degradation (9). 1,2-Propanediol cross-feeding between *B. breve* and *L. reuteri* was therefore studied in triple-species associated mouse in the presence of the mucinolytic strain *B. bifidum* PRL2010, which is capable of degrading mucin and releasing fucose without producing 1,2-propanediol (Fig. 4A; Table S1) (9). We first tested wild-type and mutant *L. reuteri* strains separately. Mice were gavaged with an inoculum that contained *B. bifidum* PRL2010, *B. breve* UCC2003 or its *fucP* mutant, and either *L. reuteri* PTA 6475 or *L. reuteri* Δ*pduCDE* (Table S1, inocula A-D). In these experiments, the two *Bifidobacterium* strains formed stable populations of ~10^8-10^9 CFU/g (Fig. S6A-H), while the *L. reuteri* strains colonized at ~10^6-10^8 CFU/g (Fig. S7A). When colonizing with wild-type *B. breve* UCC2003, *L. reuteri* PTA 6475 formed higher populations than *L. reuteri* Δ*pduCDE* over the duration of the experiment. Contrary, fecal cell numbers of *L. reuteri* PTA6475 where consistently lower than *L. reuteri* Δ*pduCDE* when colonizing with *B. breve* UCC2003-

fucP (but differences did not reach statistical significance due to the high variation between mice) (Fig. S7A). However, normalized ratios (see Materials and Methods) between fecal cell numbers of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* were significantly higher in mice colonized with *B. breve* UCC2003 as compared to mice colonized with *B. breve* UCC2003-fucP (Fig. 5A). Interestingly, while the ratio between *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* was greater than 1 when the *L. reuteri* strains were co-colonized with *B. breve* UCC2003, it was substantially lower than 1 when co-colonized with *B. breve* UCC2003-fucP (Fig. 5A). These finding suggests that the *pdu* cluster confers a fitness burden on *L. reuteri* unless 1,2-propanediol is provided, under which the cluster becomes advantageous.

In a parallel set of experiments, we tested the importance of 1,2-propanediol cross-feeding in gnotobiotic mice that were gavaged with *B. bifidum* PRL2010, either *B. breve* UCC2003 or *B. breve* UCC2003-fucP, and both *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* (wild-type and mutant) in direct competition (Table S1, inocula E & F). In agreement with the experiments with only one *L. reuteri* strain, *Bifidobacterium* species formed stable populations that were comparable among groups (~10^8-10^9 CFU/g; Fig. S6I-L). *L. reuteri* formed stable populations (~10^6-10^8 CFU/g), and concordant with the experiments using single strains of *L. reuteri*, cell counts of *L. reuteri* PTA 6475 were higher than the mutant in mice colonized with *B. breve* UCC2003 as compared to the *B. breve* UCC2003-fucP (but differences were not statistically significant) (Fig. S7B). However, significant differences were observed between the relative proportions of the two *L. reuteri* strains. Specifically, *L. reuteri* PTA 6475 reached significantly higher proportions (>75 %) in mice colonized with *B. breve* UCC2003 as compared to *B. breve*

234 UCC2003-fucP (Fig. 5B). Interestingly, *L. reuteri* Δ*pduCDE* reached ~75 % of the total
235 *Lactobacillus* population in mice colonized with *B. breve* UCC2003-fucP (Fig. 5B),
236 supporting the notion that the *pdu* cluster is a fitness burden to *L. reuteri* unless 1,2-
237 propanediol is provided. Overall, these observations demonstrated that *B. breve*
238 UCC2003 can provide 1,2-propanediol as the result of a trophic chain from the
239 degradation of mucin by *B. bifidum* PRL2010 that facilitates colonization of *L. reuteri* in
240 the gastrointestinal tract (9).

241 A set of dual-associated gnotobiotic mouse experiments were also conducted to
242 test if the production of 1,2-propanediol, from the metabolism of rhamnose by *E. coli,*
243 influences the fitness of *L. reuteri* in the gastrointestinal tract. Mice were colonized
244 either with *E. coli* and *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* alone or in
245 competition. Rhamnose was provided to mice in the drinking water (Fig. 4B; Table S2).
246 Stable populations of *E. coli* were reached in all mice (~10^9 CFU/g), and the provision of
247 rhamnose led to an ~10-fold increase in fecal cell numbers of *E. coli* (Fig. S8). Contrary
248 to the findings with gnotobiotic mice colonized by bifidobacteria, *L. reuteri* Δ*pduCDE*
249 colonized with higher cell numbers and outcompeted *L. reuteri* PTA 6475 under all
250 conditions tested (Fig. 5C & D). This was indicated by ratios of less than 1 between *L.*
251 *reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in mice colonized with single *L. reuteri* strains
252 (Fig. 5C) and an enrichment of the mutant to ~60 % of the total *Lactobacillus* population
253 in competition experiments (Fig. 5D). The latter results resample those obtained with
254 the triple-species *Bifidobacterium* experiments in mice colonized with *B. breve*
255 UCC2003-fucP (Fig. 5), confirming the fitness burden of the *pdu* cluster.

256

Discussion

Cross-feeding between members of the gut microbiota based on 1,2-propanediol is considered an important ecological process that shapes the gut microbiome and its metabolism. However, conclusions have so far been derived from in vitro experiments and metagenomic predictions. In this study, we demonstrate that *L. reuteri* engages in trophic interactions with bacteria that are common in the human gut and provide 1,2-propanediol, which is used predominantly to regenerate reduced metabolic cofactors. Using isogenic mutants in both the bacterium that produces 1,2-propanediol and *L. reuteri*, we ensured that this cross-feeding interaction is in fact based on this metabolic intermediate. We further established that the *pduCDE* genes constitute a fitness burden for *L. reuteri* in the gut unless 1,2-propanediol is provided, which makes the cluster ecologically advantageous. Our findings therefore provide insight into both the ecological role of the *pdu* cluster in *L. reuteri* and how it evolved.

Our results demonstrate that *in vitro*, *L. reuteri* can obtain a growth advantage by cross-feeding from 1,2-propanediol derived from the fermentation of fucose and rhamnose by *B. breve* and *E. coli*, respectively. These findings extend previous work showing that *pdu* cluster-containing *L. reuteri* strains grow at a faster rate and to a higher cell yield in the presence of glycerol or 1,2-propanediol (19). Contrary to findings with *L. reuteri* DSM 20016, which produces propanol and propionate in equimolar concentrations through disproportionation when utilizing 1,2-propanendiol as a sole substrate (22), *L. reuteri* PTA 6475 produces propanol in excess over propionate (Figure 1B) and enhances acetate formation (Fig. 1D) when utilizing 1,2-propanendiol together with glucose. These metabolic patterns suggest that when *L. reuteri* PTA 6475

grows with glucose, 1,2-propanediol functions mainly as an electron acceptor, allowing the conversion of acetyl-phosphate to acetate and to generate an extra ATP (30). Apart from enhancing bacterial growth, 1,2-propanendiol metabolism by *L. reuteri* can therefore contribute to SCFA formation in the gut, which might have health implications as propionate and acetate impact host physiology, e.g. by contributing to gluconeogenesis in the liver, reducing cholesterol, and promoting satiety (5, 18).

Our experiments in gnotobiotic mice provide empirical evidence for the ecological relevance of 1,2-propanendiol cross-feeding for the ecological performance of a gut symbiont in the gastrointestinal tract. The use of the respective *pduCDE* and *fucP* mutants of *L. reuteri* and *B. breve* in the mouse experiments indicated that this trophic interaction was specifically based on 1,2-propanediol produced through the fermentation of fucose. Since *B. breve* cannot degrade mucins, and the mouse feed was devoid of fucose, the findings suggests that *L. reuteri* benefits from 1,2-propanediol resulting from interspecies trophic interactions between bifidobacteria, in which *B. bifidum* PRL2010 degrades host mucin and provides fucose for *B. breve* to produce 1,2-propanediol (9). The hydrolysis of mucin is suggested to play a key role in the facilitation of bacterial species in the gut microbiota and have been demonstrated *in vitro* (7, 31, 32) and with bacterial pathogens *in vivo* (e.g. *Salmonella* spp. and *Clostridium difficile*) (23, 33). Our data suggests that it also plays a role in cross-feeding interactions among symbionts or commensals, ultimately conferring a fitness advantage to *L. reuteri* in the murine gut through the provision of 1,2-propanendiol.

Although our experiments demonstrated 1,2-propanediol cross-feeding between bifidobacteria and *L. reuteri*, equivalent findings were not observed in mouse

303 experiments with *L. reuteri* and *E. coli.* This observation can potentially be attributed to
304 a phenomenon called carbon catabolite repression or the “all-or-none” effect in *E. coli*,
305 in which a hierarchy-based regulatory system controls the sequential uptake of carbon
306 sources (34). The mouse diet was highly saturated with glucose (34.4 % w/w) and, *in*
307 *vitro,* the presence of glucose suppresses the uptake of other carbohydrates in *E. coli*
308 (35), which is in agreement with our finding that *E. coli* MG1655 did not produce 1,2-
309 propanendiol from rhamnose while in the presence of glucose (Fig. S3B). Hence, it is
310 possible that the uptake and metabolism of rhamnose into 1,2-propanediol by *E. coli*
311 was suppressed in the murine gut.

312 Interestingly, the mouse experiments consistently revealed a fitness burden of
313 the *pduCDE* genes when 1,2-propendiol was absent. Fitness trade-offs are well
314 understood in the evolution of antibiotic resistance in bacteria, where resistance genes
315 are costly and lead to a reduction of growth (36), and are therefore often lost in the
316 absence of antibiotic pressure (37). Our findings indicate that genes that facilitate cross-
317 feeding interactions are also subjected to fitness trade-offs in that they are only
318 beneficial when the metabolite is provided. Such trade-offs have also been shown in
319 cross-feeding based on the exchange of carbohydrates. *Bacteroides ovatus* possesses
320 an enzyme system dedicated to the digestion of polysaccharides that does not directly
321 benefit itself, but rather cooperative members of the gut microbiota through reciprocal
322 cross-feeding. This enzyme system is energetically unfavorable and in the absence of a
323 reciprocating species, a knock-out mutant strain can outcompete the enzyme-encoding
324 wild-type (38). In *L. reuteri*, the fitness burden of the *pdu* genes provides a possible
325 explanation for the evolution of the *pdu* cluster (26). Although likely ancestral to

currently known lineages of *L. reuteri*, there is evidence of a deletion of the *pdu* cluster from most rodent strains (25, 26). Growth substrates are abundant in the forestomach and bacteria providing 1,2-propanediol are absent (25, 26), which might have led to loss of the *pdu* cluster as it was evolutionary advantageous, in agreement with the Black Queen Hypothesis (39). In the human distal gut, 1,2-propanediol is likely provided through other microbiota members, which may explain why the *pdu* cluster is conserved among these strains (26, 27).

Although cross-feeding based on 1,2-propandiol is only one of many trophic interactions that establish interactive networks within the gut microbiota, this study provides important information as it establishes the importance and consequences of cross-feeding for the ecological performance of the involved members. Such knowledge has repercussions for our understanding on the ecological and evolutionary forces that shape gut ecosystems and determine how they function (38, 40-42). In addition, an understanding of mutualistic interactions has important implications as it can be translated into improved microbial-based strategies to modulate gut microbiomes (i.e. probiotics). A challenge encountered in the field of probiotics is that gut ecosystems are homeostatic, resilient to change, and thus difficult to modulate, and most probiotics do not persist or change the resident community (43-46). One solution to this problem is the adoption of an ecological framework for probiotic applications (44). A consideration of the mutualistic and facilitative interactions between community members can be used for designing probiotic strain mixtures or personalized probiotic applications with the goal to achieve a more successful long-term persistence, which might be beneficial for certain applications. For example, 1,2-propanediol cross-feeding could be considered in

349 generating probiotic products by pairing *L. reuteri* with *Bifidobacterium* species that
350 release fucose from the degradation of host-derived substrates and convert it into 1,2-
351 propanediol (9, 12). Additionally, bifidobacteria are more prevalent in the infant gut (47,
352 48) and some strains only partly utilize HMOs, releasing fucose, possibly forming an
353 effective synergistic combination with *L. reuteri* (3, 32, 40). Furthermore, cross-feeding
354 of 1,2-propanediol derived from gut symbionts and *S. enterica* serovar Typhimurium has
355 been demonstrated to play a role in promoting pathogen expansion in the gut (23). *L.*
356 *reuteri* could play a therapeutic role in excluding pathogenic *Salmonella* during
357 gastroenteritis, by directly competing for the intermediary metabolite. Overall, this
358 information could not only be used to formulate probiotic mixtures and synbiotic
359 products, but potentially personalize probiotic applications based on the baseline
360 microbiome (43).

361

362 **Materials and Methods**

363 **Bacterial strains and culture conditions**

364 Bacterial strains used in this study are listed in Table 2. *L. reuteri* strains were grown
365 in de Man, Rogosa, and Sharpe (MRS) medium (Difco) in an anaerobic chamber (gas
366 mix of 5 % CO_2, 5 % H_2, and 90 % N_2). *B. bifidum* PRL2010, *B. breve* UCC2003, and *B.*
367 *breve* UCC2003-fucP were grown anaerobically in MRS medium supplemented with
368 0.05 % L-cysteine. *E. coli* were grown in Luria-Bertani (LB) broth with agitation. All
369 incubations were performed at 37 °C.

370 **Evaluation of the impact of 1,2-propanediol on *L. reuteri* ATCC PTA 6475 growth**

Overnight cultures of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* were inoculated at 1 % into 15 ml of half-strength mMRS (49) containing 25mM glucose alone, 50 mM of 1,2-propanediol (Sigma-Aldrich) alone, or 25mM glucose plus 50mM of 1,2-propanediol. Growth of cell cultures were monitored based on OD_{600} with a spectrophotometer every 3 h over the span of 12 h. One mL samples were collected for HPLC analysis every 3 h. All experiments were performed in triplicate at 37 °C under anaerobic conditions.

In vitro production of 1,2-propanediol and cross-feeding assay development

Pre-cultures of *B. breve* strains and *E. coli* were prepared as follows. Full-strength mMRS supplemented with 30 mM cellobiose ± 30 mM L-fucose were inoculated with 1 % of overnight cultures of *B. breve* UCC2003 or *B. breve* UCC2003-fucP. Full-strength mMRS containing 25 mM of glucose or 30 mM of L-rhamnose were inoculated with 1 % of overnight *E. coli* MG1655 cultures. These fermentations were conducted under anaerobic conditions for 24 h at 37 °C. Conditioned media were prepared from pre-cultures as follows. Cells were removed from pre-cultures by centrifugation (5000 x *g* for 10 minutes) after which the supernatant was collected. The supernatant was supplemented with half-strength mMRS from scratch (50 % w/v; mMRS dry reagents) and glucose (25 mM; dry reagent) to the supernatant. These were further adjusted to pH 6.6 and filter sterilized (0.22 µm), stored at 4 °C and used within 48 h. Conditioned media are described in Table 1. For growth experiments, conditioned media were inoculated with *L. reuteri* strains (1 % inoculation). Growth was monitored for 12 h by measuring OD_{600} with a spectrophotometer and 1 mL samples for HPLC analysis were collected every 3 h. All experiments were performed in triplicate under anaerobic conditions at 37 °C.

Experiments in gnotobiotic mice

All animal experiments were performed with the approval of the Animal Care and Use Committee (ACUC) of the University of Alberta (AUP 00002764). Germ-free Swiss-Webster mice (6-16 weeks of age, male and female) were bred and maintained in the Health Sciences Laboratory Animals Services (HSLAS) Facility at the University of Alberta. Mice were randomly selected and moved from a flexible-film isolator and housed in sterile, individually ventilated, positive-pressured biocontainment cages for the duration of the experiments (IsoCage P Biocontainment; Tecniplast). To avoid possible confounding effects of glycerol, which is also utilized by the *pdu* cluster-encoded diol/glycerol dehydratase (50) , an irradiated fat-free diet (34.4 % glucose and 34.4 % cornstarch; Teklad TD.180765) was used in order to minimize possible interference from the hydrolysis of triglyceride fats. After transfer to the biocontainment cages, mice were fed with the new diet for 3 d before colonization with the bacteria.

To study the cross-feeding of 1,2-propanediol in the gastrointestinal tract, groups of mice (*n=5*), 2 to 3 mice per cage, were assigned to receive either *Bifidobacterium-L. reuteri* triple-species mixtures (Table S1) or *E. coli-L. reuteri* double-species mixtures (Table S2). To test for cross-feeding of 1,2-propanediol produced from mucin derived fucose, mice were gavaged with *Bifidobacterium-L. reuteri* triple-species mixtures containing *B. bifidum* PRL2010, either *B. breve* UCC2003 or *B. breve* UCC2003-fucP, and either *L. reuteri* PTA 6475 or *L. reuteri* Δ*pduCDE* (single *L. reuteri* strains), or both strains in competition (Table S1). To test for cross-feeding of dietary rhamnose, we gavaged mice with *E. coli-L. reuteri* double-species mixtures containing *E. coli* and *L. reuteri* PTA 6475 or *L. reuteri* Δ*pduCDE* (single *L. reuteri* strains), or both strains in

competition (Table S2). Rhamnose was provided in the drinking water (2 % w/v). Each mouse was gavaged with 200 µL of the corresponding bacterial cell mixtures containing ~10^8 viable cells of each strain. Fecal pellets were collected from individual mice 1, 3, 5, and 7 days after inoculation and plated. Selective plating was used to enumerate bacterial cells in fecal samples as follows: Modified Rogosa agar plates were used to quantify *L. reuteri* strains (51). *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* were differentiated using a reuterin hydrazone detection assay (52). MacConkey agar was used for quantifying *E. coli*. *Bifidobacterium* were selected using *Bifidobacterium* selective iodoacetate mupirocin (BSIM) agar as previously described (48). *B. bifidum* PRL2010 and *B. breve* strains were differentiated based on colony morphology.

Metabolite analysis of post-fermentation

1,2-Propanediol, propanol, propionate, acetate, and ethanol were measured using HPLC. A BioRad Aminex HPX-87H column (300 mm x 7.8 mm) and a refractive index detector was used (HPLC-RI). Samples taken from fermentations were mixed with 70 % $HClO_4$ (0.005 % v/v), stored at 4 °C overnight to precipitate proteins, centrifuged (18,800 x g for 5 minutes), filtered (0.22 µm), and stored at -20 °C before injection into HPLC. Ten µL were injected and eluted with 5 mM H_2SO_4 at a flow rate of 0.4 ml/min at 70 °C. 1,2-Propanediol, propanol, propionate, acetate, and ethanol were quantified using external standards.

Statistical analysis

Statistical significance between *L. reuteri* growth curves were determined by two-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test ($\alpha = 0.05$).

439 An unpaired two-tailed Student's *t*-test was used to analyze significance between *B.*
440 *breve* growth in mMRS supplemented with cellobiose ± fucose.

441 Comparisons between *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* CFU recovered
442 from fecal samples over the duration of the gnotobiotic mice experiments were
443 performed by unpaired two-tailed Student's *t*-test. Tests were conducted between *L.*
444 *reuteri* strains that were associated with (i) *B. breve* UCC2003 or (ii) *B. breve* UCC2003-
445 fucPin the triple-species experiments and *E. coli-L. reuteri* double-species experiments
446 with rhamnose (iii) present or (iv) absent.

447 For the gnotobiotic mice experiments inoculated with the 'single *L. reuteri* strain'
448 mixtures (Table S1 & 2), CFU of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* recovered
449 from mouse feces were used to produce normalized ratios. Ratios were generated
450 using the formula (equation 1) below, where a_n is a CFU value for *L. reuteri* PTA 6475 -
451 from a single mouse - used in the comparison, b_n is the CFU value of *L. reuteri*
452 Δ*pduCDE* from each mouse in the group, and n_b is the total population of mice
453 inoculated with the mutant strain used in the experiment.

$$Normalized\ ratio = \frac{a_n}{\Sigma\left(\frac{(b_1 + b_2 + \cdots + b_n)}{n_b}\right)} \quad (1)$$

454

455 The formula was used to generate sets of ratios for the following comparisons from
456 the 'Single *L. reuteri* strain' mice experiments: (i) *B. breve* UCC2003 vs *B. breve*
457 UCC2003-fucP and (ii) *E. coli-L. reuteri* double-associated mice in the presence vs the

absence of rhamnose. Statistical significance between the sets of ratios were determined by Mann-Whitney U test (*P* value < *0.05*).

Fisher's exact test was used to determine statistical significance between *L. reuteri* population frequencies from murine groups inoculated with '*L. reuteri* strains in competition' mixtures (*P* value < *0.05*). This was performed between groups of mice from either: (i) *Bifidobacterium-L. reuteri* triple-species associations including *B. breve* UCC2003 vs *B. breve* UCC2003-fucP(ii) *E. coli-L. reuteri* double-species associations with murine diet supplemented with rhamnose vs without rhamnose. Statistical analyses were performed using GraphPad Prism 6.07.

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Author contributions

C.C.C. designed and conducted the experiments, collected and analyzed data, and wrote the manuscript. R.M.D. contributed to conceiving the project, the

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development of experimental approaches, supervision, intellectual leadership, and wrote the manuscript. X.B.L contributed to experimental designs and edited the manuscript. M.E.P.M contributed to the design of animal experiments, and contributed to the manuscript through editing and generation of figures. S.T. was responsible for animal husbandry and oversaw animal experiments. J.H.O. and J.P.V. generated the mutant *L. reuteri* ATCC PTA 6475 Δ*pduCDE* strains and contributed to manuscript editing. F.L. contributed to supervision and manuscript editing. D.V. provided the *Bifidobacterium bifidum* PRL2010, *Bifidobacterium breve* UCC2003, *Bifidobacterium breve* UCC2003-fucP strains, contributed to conceiving animal experiments, and gave technical and conceptual advice. M.G.G. oversaw analytical analysis and data interpretation, and contributed to supervision and manuscript editing. J.W. conceived project, contributed to the conceptualization of the experiments, supervised data analysis and interpretation, and wrote the manuscript.

494 Table 1. Media used for *in vitro* cross-feeding experiments

Fermenting Strain	Deoxyhexose Sugar	Other carbohydrates	Abbreviation	Purpose
B. breve UCC2003	N/a	30 mM Cellobiose	BM (C)	Control for *L. reuteri* growth absent of 1,2-propanediol production
	30 mM Fucose	30 mM Cellobiose	BM (CF)	For the study of effect of 1,2-propanediol produced from Fucose fermentation on *L. reuteri*
B. breve UCC2003-fucP	N/a	30 mM Cellobiose	B-fucP M (C)	Control for *L. reuteri* growth absent of 1,2-propanediol production
	30 mM Fucose	30 mM Cellobiose	B-fucP M (CF)	Control for *L. reuteri* growth absent of 1,2-propanediol production
E. coli MG1655	N/a	25 mM Glucose	EM (G)	Control for *L. reuteri* growth absent of 1,2-propanediol production
	30 mM Rhamnose	N/a	EM (R)	For the study of effect of 1,2-propanediol produced from Rhamnose fermentation on *L. reuteri*

495 N/a: not applicable

496

497

498

499 Table 2. Strains used in this study

Species (strain code)	Origin	Relevant features	Reference
Lactobacillus reuteri			
ATCC PTA 6475	Breast Milk	1,2-propanediol utilizer with complete *pdu* cluster	BioGaia; (53)
***L. reuteri* ΔpduCDE**	Isogenic mutant of PTA 6475	Deletion mutant of Glycerol/diol dehydratase genes (*pduCDE)*	(53)
Escherichia coli			
MG1655	Lab-derived strain	L-rhamnose utilizer, 1,2-propanediol producer	The Coli Genetic Stock Center (CGSC)
Bifidobacterium bifidum			
PRL2010	Infant stool	Mucin degrader, L-fucose producer	(32)
Bifidobacterium breve			
UCC2003	Infant stool	L-fucose utilizer, 1,2-propanediol producer	(54)
***Bifidobacterium breve* UCC2003-fucP**	Isogenic mutant of UCC2003	Insertion mutant of the L-fucose transporter gene (*fucP)*	(9)

500

501

Figures legends

Figure 1. Impact of 1,2-propanediol on growth and metabolism of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE.* (A) *L. reuteri* strains were grown in half-strength mMRS supplemented with either glucose (Glc; 25 mM), 1,2-propanediol (50 mM), or a mixture of the two. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.001$) in growth of *L. reuteri* PTA 6475 on glucose plus 1,2-propanediol compared to the other conditions. (B-C) Utilization of 1,2-propanediol and production of propanol and propionate by (B) *L. reuteri* PTA 6475 and (C) *L. reuteri* Δ*pduCDE* during growth on glucose in the presence of 1,2-propanediol. (D-E) Production of (D) acetate and (E) ethanol by the two strains during growth on glucose in the presence of 1,2-propanediol.

Figure 2. Growth and metabolites of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in conditioned media of *B. breve* UCC2003 and *B. breve* UCC2003-fucPgrown with cellobiose alone or with the addition of fucose. Growth curves of (A) *L. reuteri* strains in *B. breve* UCC2003 conditioned media and (B) *L. reuteri* in *B. breve* UCC2003-fucP conditioned media. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.001$) in growth of *L. reuteri* PTA 6475 grown in *B. breve* UCC2003 conditioned medium that had fermented cellobiose and fucose together compared to growth of *L. reuteri* PTA 6475 in *B. breve* UCC2003 conditioned medium without fucose or to *L. reuteri* Δ*pduCDE* grown in all conditioned media derived from *B. breve* UCC2003. (C-D) Utilization of *B. breve* derived 1,2-propanediol present in the conditioned media and production of propanol in cultures of (C) *L. reuteri* PTA 6475 and (D) *L. reuteri* Δ*pduCDE* grown in the conditioned medium of *B. breve* UCC2003 grown in the

presence of fucose. Propionate, acetate, and ethanol concentrations could not be determined due to interference of unknown compounds in the medium. Abbreviations: BM, *B. breve* UCC2003 conditioned media; B-fucP M, *B. breve* UCC2003-fucP conditioned media; (C), pre-culture fermentations of cellobiose only; (CF), pre-culture fermentations of cellobiose with added fucose (See Table 1 for more details about media used in the study).

Figure 3. Growth curves and metabolites of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in conditioned media of *E. coli* grown with glucose or rhamnose. (A) Growth of *L. reuteri* strains. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.01$) in growth of *L. reuteri* PTA 6475 grown in *E. coli* conditioned medium that had fermented rhamnose compared to *E. coli* conditioned medium that fermented glucose and to growth of *L. reuteri* Δ*pduCDE* in both *E. coli* conditioned media fermentations of either glucose or rhamnose. (B-C) Utilization of *E. coli*-derived 1,2-propanediol, and production of propanol and propionate by (B) *L. reuteri* PTA 6475 and (C) *L. reuteri* Δ*pduCDE* grown in conditioned medium from *E. coli* grown with rhamnose. (D) Comparison of acetate production by the two strains grown in conditioned medium from *E. coli* grown with rhamnose. Ethanol concentrations could not be determined due to interference of an unknown compound in the medium. Abbreviations: EM, *E. coli* conditioned media; (G), pre-culture fermentation of glucose only by *E. coli*; (R), pre-culture fermentation of rhamnose only by *E. coli*. (See Table 1 for more details about media used in the study).

548 **Figure 4.** Graphical illustration of hypothesized trophic interactions of 1,2-propanediol
549 in gnotobiotic mice. (A) In triple-species associated gnotobiotic mice (colonized by *B.*
550 *bifidum*, *B. breve*, and *L. reuteri*), *B. bifidum* liberates fucose from the degradation of
551 host mucin, which is metabolized by *B. breve* UCC2003 producing 1,2-propanediol, that
552 is subsequently utilized by *L. reuteri* PTA 6475. (B) In dual-species (*E. coli* and *L.*
553 *reuteri)* associated mice whose diet has been supplemented with rhamnose added
554 through the drinking water, *E. coli* metabolizes rhamnose producing 1,2-propanediol
555 that is subsequently utilized by *L. reuteri* PTA 6475.
556
557 **Figure 5.** Populations of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in the
558 gastrointestinal tract of triple-species and double-species associated gnotobiotic mice**.**
559 (A) Normalized ratios between *L. reuteri* PTA 6475 to *L. reuteri* Δ*pduCDE* obtained from
560 *Bifidobacterium-L. reuteri* triple-species associated gnotobiotic mice in which
561 colonization by *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* was tested separately. **"**+"
562 indicates mice colonized with *B. breve* UCC2003 and "-" indicates mice colonized with
563 *B. breve* UCC2003-fucP. (B) Percent CFU for *L. reuteri* PTA 6475 and *L. reuteri*
564 Δ*pduCDE* as measured in triple-species associated gnotobiotic mice in which the two *L.*
565 *reuteri* strains were tested in competition**.** (C) Normalized ratios between *L. reuteri* PTA
566 6475 and *L. reuteri* Δ*pduCDE* in *E. coli-L. reuteri* double-species associated gnotobiotic
567 mice in which colonization of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* was tested
568 separately. (D) Percent CFU for *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* mutant in
569 double-species associated gnotobiotic mice in which the two *L. reuteri* strains were
570 tested in competition**. "**+" indicates the presence of rhamnose (Rha) in the diet, while "-"

indicates absence of rhamnose in the diet. Statistical significance for ratios and percent abundance (% CFU) was determined using Mann-Whitney test and Fisher's exact test, respectively.

Table S1. Strains used in experiments with *Bifidobacterium-L. reuteri* triple-species associated gnotobiotic mice.

Table S2. Strains used in experiments with *E. coli-L. reuteri* double-species associated mice.

Figure S1. Concentrations of (A) acetate and (B) ethanol in media after growth of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* on glucose (Glc) in the presence or absence of 1,2-propanediol.

Figure S2. Growth of *B. breve* UCC2003 and *B. breve* UCC2003-fucP in mMRS supplemented with cellobiose (30 mM; Cell), fucose (30 mM; Fuc), cellobiose and fucose, or with no carbon source (No Carb) after 24 hours.

Figure S3. Total 1,2-propanediol concentrations in media after growth with (A) *B. breve* UCC2003 and *B. breve* UCC2003-fucP after fermentation of cellobiose (Cell) and cellobiose plus fucose (Cell + Fuc). (B) 1,2-propenediol concentrations in *E. coli* cultures after growth with glucose (Glc), rhamnose (Rha), or glucose and rhamnose (Glc + Rha).

Figure S4. Growth of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in the presence of deoxyhexose sugars. *L. reuteri* strains do not utilize (A & B) cellobiose (Cell), fucose (Fuc), or (C & D) rhamnose (Rha) as growth substrates or electron acceptors when cultured with glucose (Glc).

Figure S5. Acetate production of *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in the conditioned media of *E. coli* fermentation of glucose and rhamnose. In the symbol labels, *E. coli* conditioned media is abbreviated as EM, followed by fermentation of glucose by *E. coli* as indicated with (G), and rhamnose with (R) (See Table 1 for more details about media used in the study).

Figure S6. Quantification of *Bifidobacterium* strains in fecal samples from triple-species associated gnotobiotic mice. Fecal CFU of *B. bifidum* PRL2010 recovered from of gnotobiotic mice inoculated with (A) *B. breve* UCC2003 and *L. reuteri* PTA 6475 or (B) *B. breve* UCC2003 and *L. reuteri* Δ*pduCDE*. Fecal CFU of *B. bifidum* PRL2010 from gnotobiotic mice inoculated with (C) *B. breve* UCC2003-fucP and *L. reuteri* PTA 6475 or (D) *B. breve* UCC2003-fucP and *L. reuteri* Δ*pduCDE*. Fecal CFU of *B. breve* UCC2003 from gnotobiotic mice inoculated with (E) *B. bifidum* PRL2010 and *L. reuteri* PTA 6475 or (F) *B. bifidum* PRL2010 and *L. reuteri* Δ*pduCDE*. Fecal CFU of *B. breve* UCC2003-fucP from gnotobiotic mice inoculated with (G) *B. bifidum* PRL2010 and *L. reuteri* PTA 6475 or (H) *B. bifidum* PRL2010 and *L. reuteri* Δ*pduCDE*. Fecal CFU of *B. bifidum* PRL2010 from gnotobiotic mice inoculated with *L. reuteri* PTA 6475 and *L.*

reuteri Δ*pduCDE* in competition as well as with (I) *B. breve* UCC2003 or (J) *B. breve* UCC2003-fucP. (K) Fecal CFU of *B. breve* UCC2003 from gnotobiotic mice inoculated with *B. bifidum* PRL2010 and *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in competition. (L) Fecal CFU of *B. breve* UCC2003-fucP from gnotobiotic mice inoculated with *B. bifidum* PRL2010 and *L. reuteri* PTA 6475 and *L. reuteri* Δ*pduCDE* in competition.

Figure S7. Quantification of *L. reuteri* strains from triple-species and double-species associated gnotobiotic mice experiments. (A-B) CFU of *L. reuteri* recovered from feces of the *Bifidobacterium* triple-species gnotobiotic mice experiments containing either (A) single *L. reuteri* strains or (B) *L. reuteri* strains in competition. **"**+" indicates mice colonized with *B. breve* UCC2003 and "-" indicates mice colonized with the *fucP* mutant of *B. breve* UCC2003. (C-D) CFU of *L. reuteri* recovered from feces of *E. coli* double-species gnotobiotic mice experiments from either (C) single *L. reuteri* strains or (D) *L. reuteri* strains in competition. **"**+" indicates the presence of rhamnose (Rha) in the diet, while "-" indicates absence of rhamnose in the diet.

Figure S8. Quantification of *E. coli* from double-species associated gnotobiotic mice experiments. CFU of *E. coli* recovered from 'single *L. reuteri* strain' bacterial mixtures in the (A) presence of rhamnose in the diet, (B) absence of rhamnose in the diet, and from (C) *L. reuteri* in competition where rhamnose was either supplemented into the mouse diet or not.

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