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# Precision glycan supplementation: A strategy to improve performance and intestinal health of laying hens in high-stress commercial environments

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## Abstract

In the dynamic world of animal production, many challenges arise in disease control, animal welfare and the need to meet antibiotic-free demands. Emerging diseases have a significant impact on the poultry industry. Managing gut microbiota is an important determinant of poultry health and performance. Introducing precision glycans as feed additives adds another dimension to this complex environment. The glycans play pivotal roles in supporting gut health and immunological processes and are likely to limit antibiotic usage while enhancing intestinal well-being and overall poultry performance. This study explores precision glycan product as a feed additive supplemented at a continuous dose of 900 g per tonne of feed, in a free-range production system on a large commercial farm. Forty thousand 17-week-old pullets were randomly allocated to one of two separated sections of the production shed, with individual silos and egg-collecting belts. The flock performance, gut microbiota and its functionality were analysed throughout the laying cycle until 72 weeks of age. The results demonstrated that introducing precision glycans improved a range of performance indicators, including reduced cumulative mortality, especially during a major smothering event, where the birds pile up until they suffocate. There was also significantly increased hen-housed egg production, reduced gut dysbiosis score and undigested feed, increased number of goblet cells and improved feed conversion ratio. Additionally, microbiota analysis revealed significant changes in the composition of the gizzard, ileum content, ileum mucosa, and caecal and cloacal regions. Overall, the findings suggest that precision glycans have the potential to enhance poultry egg production in challenging farming environments.

KEYWORDS

glycans, microbiota, poultry, precision biotics

## 1 | INTRODUCTION

Animal production systems face numerous challenges concerning disease control and animal welfare. Common diseases, such as colibacillosis and salmonellosis, traditionally managed through antibiotics, are re-emerging in antibiotic-free environments (Van et al., 2017). The demand for antibiotic-free animal products, with the increasing popularity of free-range farming alternatives, is causing novel challenges in animal health management (Falker-Gieske et al., 2020).

Animal production strives to ensure a sustainable and affordable food supply that meets high feed safety standards and the preferences of environmentally conscious consumers. This response to consumer demand reshaped poultry production toward the open and free-range systems, which in turn introduced novel and re-emerging diseases. Gut microbiota, the master regulator of organ and immune system health, plays a pivotal role in both controlling and developing devastating diseases in pigs and poultry (Pourabedin & Zhao, 2015; Szafraniec et al., 2020). The interaction between raw materials and additives in poultry feed and the gut microbiota can significantly impact animal health and welfare. There is a clear need for antibiotic alternatives that do not cause significant disturbance to the microbiome but can still provide a high level of pathogen control.

Prebiotics, complex carbohydrates that the host's enzymes cannot digest, serve as a food source for probiotics and beneficial intestinal bacteria. Polysaccharides, including glycans, are complex polymers abundant in plants, animals or microbes. In addition to the ingested or supplemented complex carbohydrates, the host produces its own glycans, with mucin-type O-glycans particularly relevant for poultry gut health. These mucin-type O-glycans are the main components of mucins, forming a protective slimy layer over epithelial cells that can trap and prevent bacterial invasion. Secreted by goblet cells, the mucus layer acts as a barrier between the resident microbiota, including pathogens, and the immune cells patrolling the intestinal structure (Bergstrom & Xia, 2013).

Glycans are not limited to the gut; they are present on the cell surfaces of all living organisms and play a crucial role in immunological processes. Glycans are abundant in both the microbiome and the host, where they contribute to immunological pathogen recognition, immune system activation and self/nonself differentiation (Zhou & Cobb, 2021). Secretory IgA (SIgA), rich in glycosylation, forms a protective coating around bacteria. This coating supports the growth of beneficial bacteria while inhibiting the growth of pathogens (Raskova Kafkova et al., 2021). SIgA has been proposed to control the intestinal microbiota through glycan-mediated innate immune interactions (Corthésy, 2013). The growing body of evidence highlighting the beneficial roles of glycans in gut health and the increasing interest in intestinal health has driven research on the role of glycans in intestinal homoeostasis.

Synthetic glycans have been tested for various purposes, including as vaccine candidates against major gut pathogens like *Clostridium difficile* (Broecker et al., 2016). Controlled synthesis of homogeneous precision glycans is crucial for developing glycan-based drugs, and advancements in analytical chemistry and technology have made precision glycans a reality (Wang et al., 2021).

The precision glycans, also referred to as precision biotics (PBs), utilised in this study were selected since they can increase the metabolic synthesis of short-chain fatty acids (SCFAs) and reduce the amine synthesis related to amino acid degradation with beneficial performance outcomes. Furthermore, and as shown by Bortoluzzi et al. (2023), a microbiome protein metabolism index (MPMI) was elaborated to measure the shift in the microbiome pathways toward a beneficial increase in protein utilisation, which was ultimately positively correlated with improvements in the feed conversion ratio (FCR) and final body weight. Similar FCR and body weight improvements in broilers were observed by Yan et al. (2023) from incrementing the MPMI by downregulating pathways related to protein fermentation and putrefaction when using a precision biotic.

This was confirmed by a meta-analysis published on the effects on broiler performance, where the activation of these selected metabolic pathways positively impacted the productivity of chickens, reducing FCR and improving final body weight. Furthermore, it was supported by in vivo metagenomic changes in SCFA and amino acid metabolism pathways, corresponding to the in vitro selection criteria already mentioned (Walsh et al., 2021) and also ex vivo SCFA increase in broilers (Bortoluzzi et al., 2023).

Improvements in zootechnical performance extend beyond the avian gastrointestinal tract and can have an influence on the surrounding environment. Impairment on gut health can manifest through the amelioration of litter quality, characterised by altering pH and ammonia. Consequently, improvement in intestinal health contributes to a decline in foot pad lesions, ultimately enhancing both animal welfare and overall performance (Jacquier et al., 2022).

The health impact of using PB can also improve broiler resilience, reflected under typical commercial environments with enteric challenges. As presented by Blokker et al. (2022), the modulation of microbiome pathways can improve broiler resistance to coccidiosis by maintaining gut epithelium morpho functionality, downregulating the expression related to gut inflammation and promoting the cycle or re-generation of new gut cells and enterocyte growth.

This study aims to investigate the effects of novel precision-made glycans on intestinal health in layer chickens, focusing on their impact on disease resistance, animal welfare, management strategies and environmental sustainability.

The PB addition to the birds utilised in this study improved zootechnical key performance indicators. The cumulative mortality was significantly lower ($p < 0.0001$) at the end of the trial; the PB group delivered 3.55 more eggs per bird initially placed, and there was a consistent FCR improvement in both kg feed/dozen and kg feed/kg eggs ($p < 0.0001$). No significant differences were observed in the egg quality parameters.

The findings from this study will provide valuable insights for developing novel strategies to enhance animal welfare, optimise management practices and ensure the production of safe and environmentally conscious poultry products.

## 2 | MATERIALS AND METHODS

### 2.1 | Animal trial

The research presented in the document was approved by the CQ University's Animal Ethics Committee under approval number 0000022879. All animal manipulations were performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes and reported according to guidelines and regulations of *Animal Research: Reporting of In Vivo Experiments* (ARRIVE). Additionally, the commercial farm where the experimental procedures were performed complies with the 4th edition '*Code of Practice for the Welfare of Domestic Poultry*' standards under the laws operating in Australia's States and Territories.

### 2.2 | Experimental design

The project was conducted at a layer hens aviary free-range farm in Queensland, Australia, focusing on studying the effects of a glycan intervention in HyLine Brown hens, a commonly used breed in the Australian egg industry.

The study used a single flock of pullets from the same rearing farm and shed. This ensured that the birds were of the same age and had been subjected to consistent management practices and feed diets. The layout of the two sheds allowed for the division of the flock into two sides. Each side had independent silos and feed lines, enabling one side to receive the glycan treatment while the other served as the control (CT) group. The precision glycan used in this study was Symphiome™ (DSM-Firmenich) incorporated at a rate of 900 g per tonne of finished feed.

At each designated time point, a total of 40 birds, 20 from each group, were euthanised using cervical dislocation. Gut scoring (according to De Gussem, 2010) was performed by a certified poultry veterinarian, and intestinal and liver samples were collected for 16S amplicon sequencing and histology. Sampling was performed at 28, 50 and 72 weeks of flock age timepoints, and for each sampling point, we analysed ileum content and ileum mucosa-associated microbiota, caecal and gizzard content microbiota, as well as microbiota from 100 cloacal swabs collected from each treatment at each timepoint. Samples were also collected for histopathology of the ileum and liver.

### 2.3 | DNA extraction

The DNA extraction process used a cell lysis protocol adapted for 16S microbiota analysis described by Yu and Morrison (2004). The lysate was then purified using a DNA spin purification kit (Enzymax LLC, Cat# EZC101). A NanoDrop spectrophotometer was used to assess the DNA quality and quantity. A custom barcoding system was employed for polymerase chain reaction (PCR) amplification along with NEBNext® High-Fidelity 2X PCR Master Mix, which contains the original formulation of Q5® High-Fidelity DNA Polymerase.

Once the DNA extraction and PCR amplification steps were completed, the amplicon library was prepared and purified following the protocol provided by the manufacturer (Illumina). The sequencing process was performed using the Illumina MiSeq platform and 2 × 300 bp paired-end sequencing.

### 2.4 | Amplicon sequencing data analysis

Data analysis was carried out using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software (Bolyen et al., 2019). The sequencing errors were corrected, and the data were cleaned of chimeric sequences using the Dada2 algorithm and recommended settings (Callahan et al., 2016). Provisional taxonomy assignments were made using the SILVA database (Quast et al., 2012). The Qiime analysis outputs were analysed using a range of R software packages, including Phyloseq, Microecco, Vegan and Microbiome.

### 2.5 | Histological analysis

The tissue samples of ileum and liver were collected and preserved by fixing them in 10% buffered formalin solution. Paraffin embedding, deparaffinisation and rehydration processes were outsourced to a commercial medical pathology company, PathCare (Rockhampton).

The further processing was outsourced to the Veterinary Laboratory Services at The University of Queensland, Gatton, Australia. The ileum slides were stained using the Periodic Acid-Schiff-Alcian Blue staining method; the liver slides were stained with haematoxylin and eosin stain, and all slides were scanned using Panoptiq™ software (ViewsIQ) and Nikon Eclipse Ci-L Plus biological microscope.

Key morphometric parameters for the ileum included villus height (the distance from the tip to the bottom of the villi), villus width (mean value between the basal and apical villi width) and crypt depth (the distance between the crypt neck and its base). Also, the goblet cells were counted in the villi and crypts. These parameters were measured using ten slides per group and ten villi per slide and analysed using GraphPad Prizm 9 software and a nonparametric Mann–Whitney *t*-test.

To calculate the villus surface area, a widely accepted equation proposed by Rubio et al. (2010) was used: Villus surface area [$\mu m^2$] = $\pi$ × Villus height [μm] × Villus width [μm]. This equation allows for the estimation of the surface area based on the dimensions of the villus.

### 2.6 | Gut scoring analysis

All the birds randomly selected for necropsy and gut sampling at every age point were also used to perform a gut scoring analysis. The analysis was carried out using a 'Macroscopic Scoring System For Bacterial Enteritis In Broiler Chickens And Turkeys' (De Gussem, 2010). The system evaluates gut ballooning, dilated

blood vessels, wall thickness, inversion reflex, abnormal content and undigested feed.

### 2.7 | Zootechnic analysis

Performance measures routinely collected by the farm staff on a daily basis included mortality (both daily and cumulative count of dead birds), rate of lay (ROL, calculated as the percentage of eggs produced daily relative to the number of birds in the shed), cumulative hen-housed eggs (HHEs) (total number of eggs laid compared to the initial number of birds placed at the point of lay), grams of feed consumed per bird per day (measured using weight cells under the shed's silos to obtain the total feed used per day and per shed), feed conversion (kg of feed required to produce one dozen eggs and 1 kg of eggs), bird body weight (weekly measurements taken at various locations in the shed to obtain a representative sample, randomly weighing 100 birds per shed), cumulative dirty eggs (eggs excluded from the first graded eggs due to their unsuitability for consumption), egg weight (weekly samples collected and measured), eggshell thickness (weekly samples collected and thickness measured in millimetres using an ultrasound metre), eggshell Haugh units (weekly samples collected to assess egg protein quality by correlating the height of the egg white with the total egg weight), and average yolk colour (evaluated on a scale of 1–16 according to the DSM yolk colour fan, version 2020).

These performance measures were collected routinely by the farm staff for all operating poultry sheds to assess various aspects of poultry production and egg quality.

## 3 | RESULTS

### 3.1 | Animal health and performance

After the end of the trial at late lay, the PB group outperformed the CT group in several key performance indicators. PB had significantly lower cumulated mortality, as shown in Figure 1, 0.36% percentual points lower versus control. Overall cumulated mortality was significantly lower in the PB group (Paired Wilcoxon test $p < 0.0125$). In both groups, there was an exacerbated increase in mortality from 36 weeks until 50 weeks, during the smothering events. Looking into the cumulative morality only during the smothering event (Figure 1) we observed significantly ($p < 0.0112$) higher mortality in CT. Further dissection of the mortality data by week confirms an overall significant reduction in mortality in PB group originated from these events. Furthermore, the mortality data with smothering event removed does not show a significant difference ($p = 0.2424$).

HHEs were significantly higher in the PB group at the end of the trial at late lay, producing 3.55 more HHE (Paired Wilcoxon test $p < 0.0001$). There were significant differences in the FCR. At the end of the experiment at 72 weeks (late lay), there was a reduction of nine FCR points feed kg/dozen eggs and 15 FCR points feed kg/kg egg produced in the PB (Figure 1). There were no significant differences between groups on the average ROL or egg quality indicators at the end of the trial or at flock depletion.

### 3.2 | Histomorphology and gut scoring

At the 28 and 50 weeks timepoints, the number of goblet cells was significantly higher in the treated group ($p = 0.005$ and $p < 0.0001$ respectively); this increase is more pronounced in the region of villi than in the region of the crypt. At the 72-week timepoint, there was no significant difference in the number of goblet cells between the treated and the CT group. Goblet cells mucin granules show mostly blue coloration in both groups at all timepoints, indicating the high content of acidic mucins (Figure 2).

The analysis of liver samples timepoint showed no pathological changes in tissue structure in both groups.

The gut scoring showed significant differences with higher dysbiosis scores in CT during early and mid lay ($p = 0.001$ and 0.0001 respectively; Figure 2); CT birds had higher presence of undigested feed and loss of gut integrity. No significant differences were found during late lay.

### 3.3 | Alterations in the gizzard microbiota

The relative microbiota alpha diversity detected in the gizzard had significant differences in some sampling points (Supporting Information S1: Figure S1A,B). The observed richness decreased in both CT and PB groups from early lay to mid lay (significantly in CT, $p = 0.0001$). A similar trend was identified in Shannon Entropy diversity: a significant decrease in CT from early lay to mid lay ($p = 0.001$), and an increase from mid lay to late lay in both CT and PB ($p = 0.0001$ and 0.00001 respectively).

The most abundant known genera (Figure 3) commercially relevant identified throughout the trial were *Staphylococcus*, *Lactobacillus*, *Gallibacterium*, *Corynebacterium*, *Curtobacterium*, *Ruminococcus*, *Enterococcus* and *Avibacterium*.

During early and late lay, the predominant relevant genus was *Lactobacillus*. During mid lay, there was a temporal shift in the microbiota in both PB and CT, with a substantial increase in the abundance of the *Staphylococcus*. These differences are evident in the barchart shown in Figure 3. This overgrowth of *Staphylococcus* was significantly higher in CT than in PB, temporary, and cleared by late-lay.

There was a significant dissimilarity between groups during late lay (Figure 4 and Table 1) and the mid lay, in line with the observed *Staphylococcus* overgrowth (unweighted UniFrac [UWUF], $p = 0.013$, Table 1).

We used LEfSe analysis to present differentially abundant taxa in all sampled gut origins and all three production stages. Due to the size and complexity of the figures, all LEfSe data are provided in Supporting Information S2: File S2. Figures show only significantly

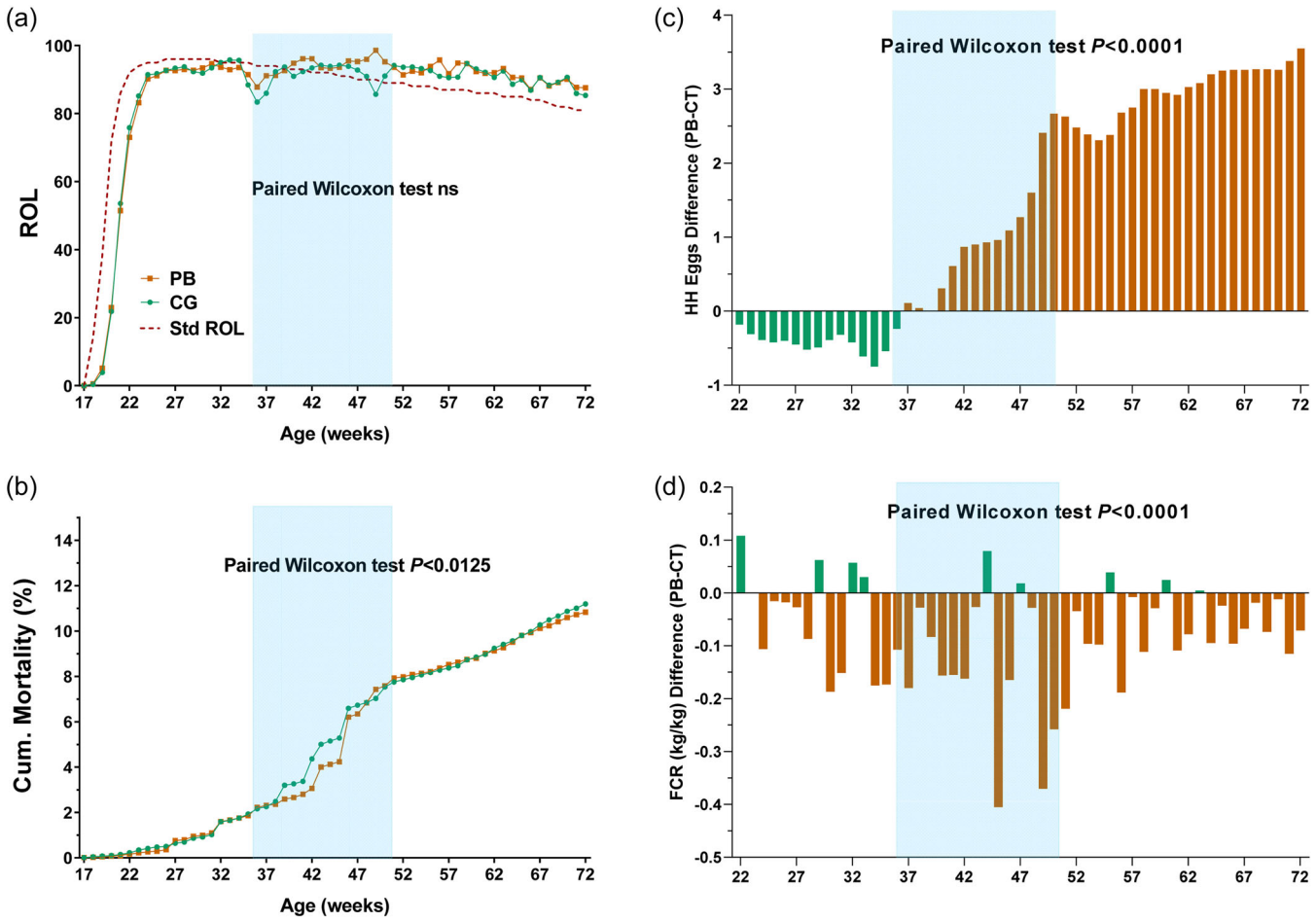


**FIGURE 1** Layer health and performance parameters summarising data collected from early lay at 17–72 weeks (late lay). Performance measures are shown as the temporal rate of lay (ROL) (a), cumulative mortality (b) and hen-housed eggs and feed conversion differences (c) and (d), respectively, shown as precision biotic (PB)-control (CT). The light blue area represents the period of smothering events. [Color figure can be viewed at wileyonlinelibrary.com]

affected ($p < 0.01$ and linear discriminant analysis (LDA) score > 3.5) taxa. Significant CT and PB differences in the relative abundance were noted at the early lay in several production-relevant genera. *Lactobacillus* was enriched in the gizzard of PB birds during early lay and was significantly higher in control birds during mid and late lay. *Staphylococcus* was associated with CT birds during an early and mid lay, and with PB in late lay. Thus, CT birds were significantly more affected by an unexplained overgrowth of *Staphylococcus* during mid lay (Figure 3). *Gallibacterium* was another genus affected by PB differently across the birds' age, increased in CT at early lay, then higher in PB in mid lay and significantly higher in CT in late stage.

## 3.4 | Alterations of microbiota in the ileum content

The most production-relevant abundant genera identified in ileum throughout the trial were *Lactobacillus*, *Staphylococcus*, *Escherichia-Shigella*, *Enterococcus*, *Gallibacterium* and *Clostridium senso stricto 1* (Supporting Information S1: Figure S2).

The relative abundance and the Beta diversity were less affected by *Staphylococcus* overgrowth compared to gizzard during mid lay (Supporting Information S1: Figure S3). There was a significant dissimilarity between groups during mid and late lay (UWUF, $p = 0.008$ and 0.032 respectively—Supporting Information S1: Table S1).

Although not statistically significant, ileum observed richness increased in both groups from early lay to mid lay and from mid lay to late lay. Similar results were found with the Shannon Entropy diversity index (Supporting Information S1: Figure S1C,D).

## 3.5 | Alterations in ileum mucosa

The number of ileum mucosa samples was adequate during early and mid lay, but it was reduced in both groups in late lay because of low DNA yield on some of the swabs. The most abundant genera observed in ileum mucosa was *Lactobacillus*, *Enterococcus*, *Clostridium senso stricto 1 and Staphylococcus* (Supporting Information S1: Figure S4). *Lactobacillus* was the predominant genus identified in both groups during early, mid, and late lay. Beta diversity separates the late lay ileum mucosa microbial community from the rest of the samples (Supporting Information S1: Figure S5). There was a

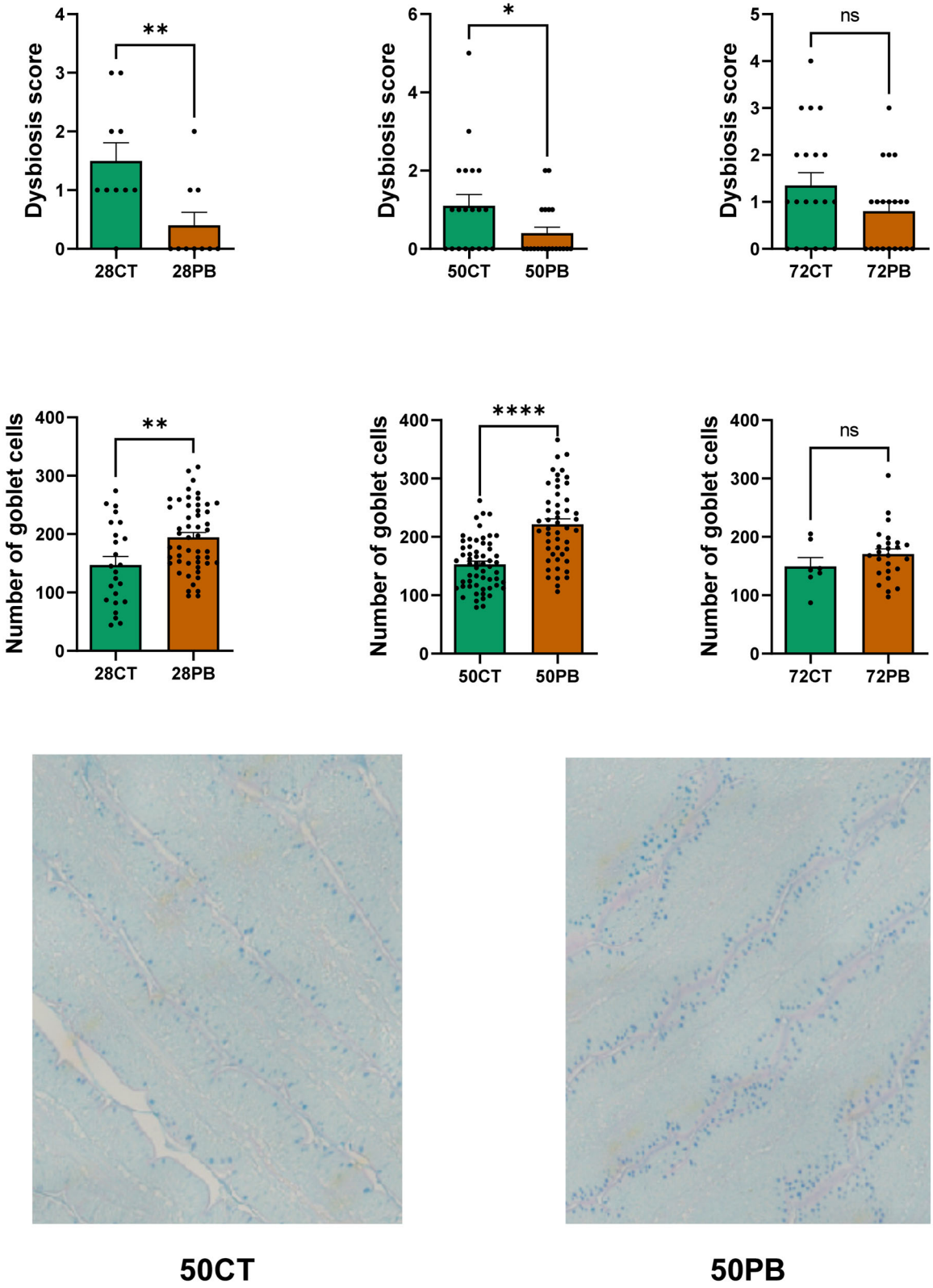


**FIGURE 2** Ileum gut scoring (dysbiosis) barchart, goblet cell numbers and histology. 28, 50 and 72 represent sampling points in weeks. CT, control; PB, precision biotic. [Color figure can be viewed at wileyonlinelibrary.com]

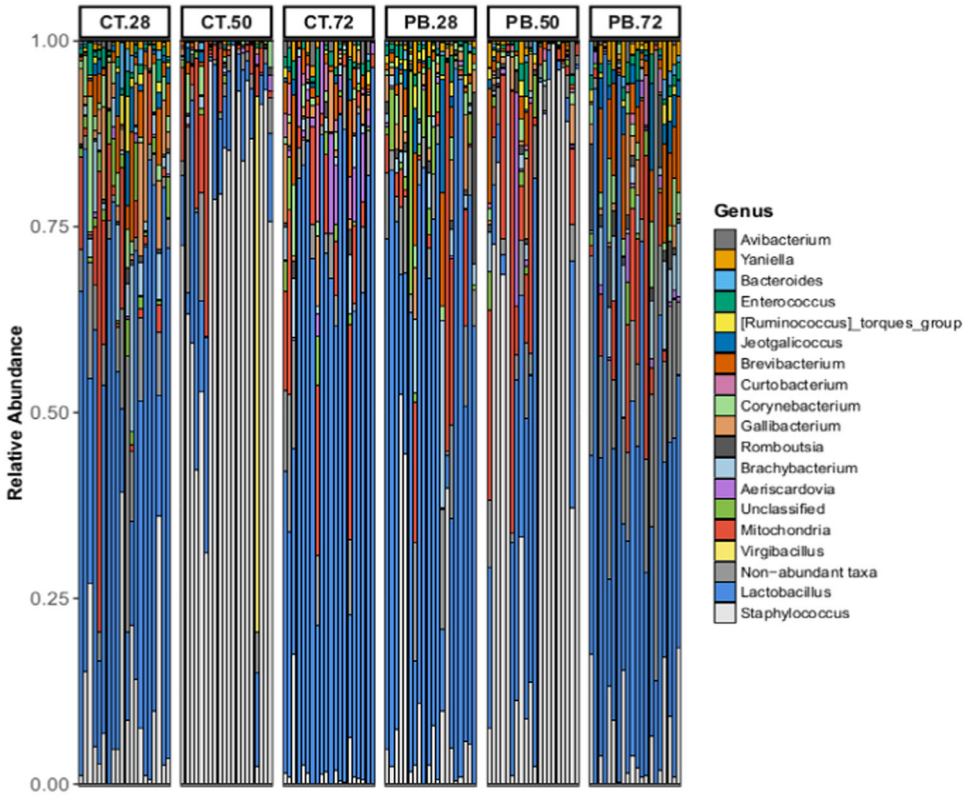


**FIGURE 3** Barchart showing major gizzard genus level microbiota over the main production stages. CT, control; PB, precision biotic. [Color figure can be viewed at wileyonlinelibrary.com]

significant group-to-group dissimilarity at early lay (UWUF and weighted UniFrac [WUF], $p = 0.01$ and 0.02 respectively) (Supporting Information S1: Table S2).

The Observed Richness increased in both groups from early lay to mid lay and to late lay (Supporting Information S1: Figure S1E). No significant differences were observed in Shannon Entropy diversity between groups throughout the trial (Supporting Information S1: Figure S1F). Precision glycan introduced significant (LEfSe $p < 0.05$; LDA > 3.5) taxa abundance differences in ileum mucosa, with *Lactobacillus* increased in CT in early and mid lay and then in PB in the late lay (Supporting Information S2: File S2).

## 3.6 | Alterations in the caecal microbiota

No significant differences were observed between groups throughout the trial in caecal Alpha diversity (Supporting Information S1: Figure S6).

Beta diversity was affected by the production stage in both groups (Figure 5). Significant changes were identified during early lay (UWUF, $p = 0.012$), mid lay (Bray Curtis and UWUF, $p = 0.049$ and 0.012 respectively), and late lay (Bray Curtis, UWUF and WUF, $p = 0.001$, 0.003 and 0.003 respectively; Supporting Information S1: Table S3.

The observed richness had a significant increase in PB compared to CT during late lay ($p = 0.00001$) (Supporting Information S1: Figure S1G). No significant differences were identified between groups throughout lay in the Shannon Diversity Index (Supporting Information S1: Figure S1H).

In the caecal intestinal section, there were significant differences in the relative abundance at early and mid lay of typical SCFA producers (Supporting Information S2: File S2).

## 3.7 | Swab microbiota

Beta diversity in caecal swabs was significantly affected in both groups (Supporting Information S1: Figure S7) throughout the trial in Bray Curtis, weighted and UWUF parameters (Supporting Information S1: Table S4).

The caecal observed richness and diversity were significantly affected in both groups throughout the duration of the study (Supporting Information S1: Figure S1I,J). CT had a significant richness increase from mid lay to late lay ($p = 0.0001$) and PB from early lay to late lay ($p = 0.001$). There was a lower richness during early lay in PB compared to the CT ($p = 0.001$). The Shannon Entropy index showed a significant increase in diversity in CT from early lay ($p = 0.001$) and mid lay ($p = 0.001$) towards late lay. A significant diversity drop in PB versus CT during late lay ($p = 0.0001$) Significant differences in the relative abundance at the early lay were observed in LEfSe analysis with the increase of *Lactobacillus* in PB during the early and mid lay and a contrary significant (LEfSe $p < 0.05$;

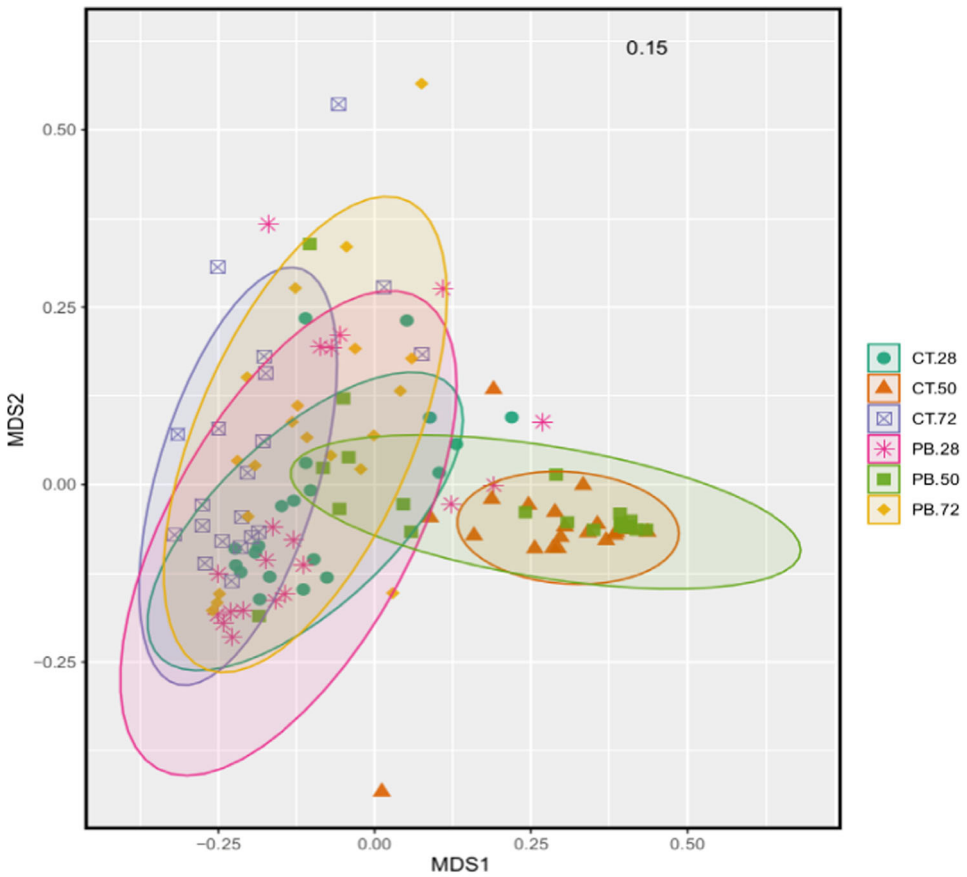


**FIGURE 4** Gizzard microbial community MDS plot. CT, control; MDS, multidimensional scaling; PB, precision biotic. [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 1** Permutational multivariate ANOVA (PERMANOVA) group differences in gizzard measured by Bray Curtis, unweighted UniFrac (UWUF) and weighted UniFrac (WUF).

| Groups | Measure | *R2* | *p*-Value | Significance |
|---|---|---|---|---|
| CT.28 versus PB.28 | Bray | 0.015352 | 0.721 | |
| CT.50 versus PB.50 | Bray | 0.048466 | 0.107 | |
| CT.72 versus PB.72 | Bray | 0.066672 | 0.011 | * |
| CT.28 versus PB.28 | UWUF | 0.040389 | 0.062 | |
| CT.50 versus PB.50 | UWUF | 0.054539 | 0.013 | * |
| CT.72 versus PB.72 | UWUF | 0.039287 | 0.086 | |
| CT.28 versus PB.28 | WUF | 0.013838 | 0.733 | |
| CT.50 versus PB.50 | WUF | 0.042766 | 0.152 | |
| CT.72 versus PB.72 | WUF | 0.079707 | 0.01 | ** |

Abbreviations: CT, control; PB, precision biotic.

LDA > 3.5) reduction in the late lay (Supporting Information S2: File S2). *Escherichia* was significantly higher in CT than in PB during an early and mid lay period and in PB in late lay. During mid lay *Clostridium senso stricto 1* and *Gallibacterium* were higher in PB than in CT (Supporting Information S2: File S2).

## 3.8 | Specific taxonomical changes

Although many changes in different gastrointestinal sections have already been described, *Lactobacillus*, *Escherichia-Shigella* and *Campylobacter* showed notable changes in both CT and PB groups. *Lactobacillus* showed a consistently higher presence in PB during early lay across all gut sections. Similar results were observed during mid lay, except in ileum mucosa and cloacal swabs where CT presented a higher presence of *Lactobacillus*, and mixed results were present during late-lay.

*Campylobacter* showed variable abundance during early lay, but it was considerably higher in ileum content in CT. During mid lay there was a low presence of *Campylobacter* except in ileum content and mucosa, where CT had a considerably higher presence. Mixed results were shown during late lay but considerably higher in ileum content and mucosa in CT, and in caeca was higher in PB.

*Escherichia-Shigella* had a low presence during early lay, except in ileum content and cloacal swabs, where it was higher in CT. Mid lay showed similar results with low presence except in ileum content and cloacal swabs, also higher in CT. Mixed results were present during late lay but, there was a considerably higher presence in cloacal swabs in PB.

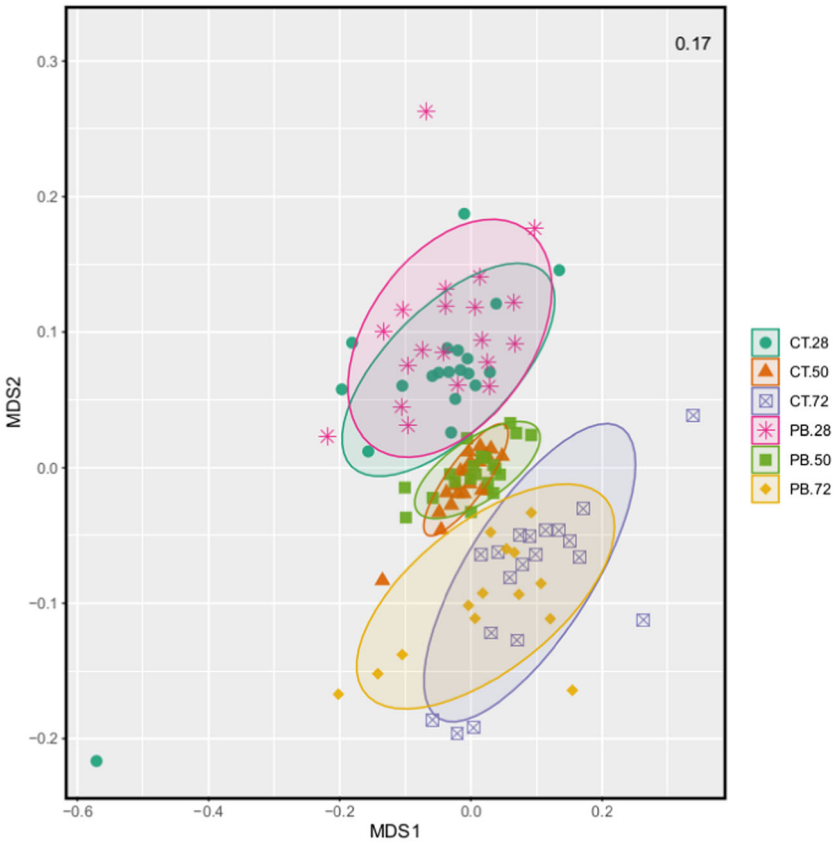


**FIGURE 5** Caecal microbial community MDS plot. CT, control; MDS, multidimensional scaling; PB, precision biotic. [Color figure can be viewed at wileyonlinelibrary.com]

## 4 | DISCUSSION

The present study illustrates how precision glycans can potentially modulate the microbiota composition in commercial layer hens, enhancing their performance on the ROL, cumulative mortality, feed conversion and overall bird resilience during the peak of the production phase.

At the beginning of the trial, both groups exhibited adequate pullet conditions. Upon reaching the lay phase, although slightly delayed, both groups demonstrated expected performance compared to the genetic standard. This trend continued through peak production, with expected performance indicators for a challenging free-range environment.

However, differences emerged after the initial lay phase. The group receiving precision glycans (PB) exhibited an improved feed conversion, that could be attributed to enhanced gastrointestinal tract function. This is supported by the macroscopic clear improvement in the gut functionality with a conserved intestinal structure, lower undigested feed, lower abnormal content detected and histological findings that showed improved gut health in PB, with significantly increased goblet cell count in the treated groups at time points of 28 and 50 weeks. Goblet cells produce mucin and can make a favourable environment for commensal microbiota, which can utilise glycoproteins as a major source for their nutrition (Marcobal et al., 2013), which is a source of mucin that promotes favourable conditions for beneficial commensal microbiota.

PB outperformed CT in egg production, with PB producing more eggs despite also encountering mortality due to random smothering events. Although both groups were affected, PB experienced significantly lower mortality (Figure 1). Smothering has not been comprehensively studied, and no clear causal factor has been identified, but some triggers can be age, time of day, temperature fluctuations and litter condition (Bright & Johnson, 2011). It is a significant cause of increased mortality and an overall flock stress factor, with close to 60% of free-range main producers affected at different levels in the UK (Barrett et al., 2014). Nevertheless, none of these factors were reported, and it can be assumed that both groups shared similar conditions for potential smothering occurrence. Furthermore, the performance differences could not be related to these events, as it was also pointed out by Herbert et al. (2021).

Interestingly, during mid lay, right after the ease of these smothering events, there was a temporal change in alpha diversity at the gizzard level with a marked overgrowth of *Staphylococcus* genus in both groups during mid lay. *Staphylococcus* can colonise poultry, where it can contribute to more than 10% of the total bacterial strains present in chickens (Marek et al., 2016). It can also be part of the chicken litter as a resident microbiota, given its robust growth in similar phosphate levels (Vadari et al., 2006). An overgrowth of this genus can lead to intestinal infections in layer hens, which could cause inflammation and intestinal damage, leading to diarrhoea, reduced nutrient absorption and weight loss. *Staphylococcus* can also lead to lameness (Szafraniec et al., 2022) and skin infections known as 'focal ulcerative dermatitis' in free-range

layers (Ayala et al., 2023; Gornatti-Churria et al., 2018). Furthermore, *Staphylococcus agnetis*, has been linked to lameness, mortality and endocarditis in broiler breeders (Szafraniec et al., 2020). However, it should be noted that *Staphylococcus* was never identified or suspected as an issue in this production system history. It is quite possible that we saw an overgrowth of commensal *Staphylococcus*. This overgrowth could act as an indicator of major alterations of the microbiota during a stressful period such as smothering events. Furthermore, the *Staphylococcus* overgrowth was not observed at ileum mucosa at any stage, which could be explained by the high abundance of *Lactobacillus* detected, which normally has enhanced colonisation properties (Kobierecka et al., 2017).

Focusing on the early lay, there were clear differences between the groups across all sampled gut sections. PB showed a prevalence of LEfSe biomarkers related to lactic acid bacteria commonly associated with probiotics. *Lactobacillus* was consistently higher in PB in all gut sections during early lay. In contrast, CT showed the prevalence of potential pathogenic groups such as *Escherichia-Shigella* and *Campylobacter* with a considerably higher presence in ileum content and cloacal swabs during early and mid lay.

While the effects of PB supplementation on microbiota were notable during early and mid lay, there was no clear distinction between the groups in late lay. This could be further supported by the resilience of the microbiota in older layer hens, potentially posing challenges for modulation once the microbiota becomes established (Ricke et al., 2022). Furthermore, Videnska et al. (2014) delineated a late-lay stage of caecal microbiota development characterised by an unaltered ratio at a phylum level, unlike in earlier stages.

## 5 | CONCLUSIONS

Overall, it can be concluded that precision glycans serve as a suitable and commercially viable tool to improve poultry egg production, even in challenging environments such as free-range egg farming, with the ability to assist antibiotic-free poultry production goals. The use of precision glycans helps restore the potential of breed genetics for optimum performance and offers economic benefits to the industry. Although the impact of PB on microbiota exhibited its highest benefits during early and mid lay, its effects were minimal in late lay. Despite this variation, the enhancements in bird health and performance indicators resulting from supplementation were enduring and consistent across all stages of the trial. This affirms that relying solely on assumptions about microbiota community and gut health from 16S amplicon data or even more accurate taxonomy identification methods is suboptimal. Therefore, traditional indicators such as performance, egg quality, gut scoring and histology continue to be essential and indispensable in gut health studies. Our data confirms enormous differences between gut sections previously reported by a large-scale study in layers. Further research is necessary to understand the underlying mechanisms better and optimise the application of precision glycans in poultry production.

## AUTHOR CONTRIBUTION

**Friedrich Petranyi**: Conceptualisation; methodology; data curation; investigation; writing—original draft preparation. **Maria M. Whitton**: Methodology; data curation, investigation; writing—reviewing and editing. **Edina Lobo**: Methodology; data curation; investigation; writing—reviewing and editing. **Santiago Ramirez**: Conceptualisation; methodology; data curation; investigation; writing—reviewing and editing. **Anita Radovanović**: Methodology; data curation; investigation; writing—reviewing and editing. **Yadav S. Bajagai**: Methodology; formal analysis; supervision; project administration; investigation, writing—reviewing and editing. **Dragana Stanley**: Conceptualisation; methodology; data curation; supervision; investigation, writing— reviewing and editing.

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## CONFLICTS OF INTEREST STATEMENT

Friedrich Petranyi and Edina Lobo are on PhD scholarship projects funded by DSM-Firmenich. Friedrich Petranyi and Santiago Ramirez are DSM-Firmenich employees, and Yadav S. Bajagai and Dragana Stanley supervise DSM-Firmenich funded projects. The remaining authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Genebank at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1034760. Data associated with this study has been deposited at the GenBank: www.ncbi.nlm.nih.gov/genbanksequencing database under the accession number PRJNA1034760.

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