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Enhanced meat chicken productivity in response to the probiotic *Bacillus amyloliquefaciens* H57 is associated with the enrichment of microbial amino acid and vitamin biosynthesis pathways

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Abstract

Aims: Sub-therapeutic use of antibiotics as a growth promoter in animal diets has either been banned or voluntarily withdrawn from use in many countries to help curb the emergence of antibiotic-resistant pathogens. Probiotics may be an alternative to antibiotics as a growth promoter. We investigated the effects of a novel probiotic strain, *Bacillus amyloliquefaciens* H57 (H57) on the performance and microbiome-associated metabolic potential.

Methods and Results: Broiler chickens were fed either sorghum- or wheat-based diets supplemented with the probiotic H57. The growth rate, feed intake, and feed conversion in supplemented birds were compared with those in non-supplemented control. Caecal microbial metabolic functions were studied with shotgun metagenomic sequencing. H57 supplementation significantly increased the growth rate and daily feed intake of meat chickens relative to the non-supplemented controls without any effect on feed conversion ratio. In addition, relative to the non-supplemented controls, gene-centric metagenomics revealed that H57 significantly altered the functional capacity of the caecal microbiome, with amino acid and vitamin synthesis pathways being positively associated with H57 supplementation.

Conclusions: *Bacillus amyloliquefaciens* H57 improves the performance of meat chickens or broilers and significantly modifies the functional potential of their caecal microbiomes, with enhanced potential capacity for amino acid and vitamin biosynthesis.

Significance and impact of the study:

This study explored the impact of *Bacillus amyloliquefaciens* H57 on poultry productivity and microbiome functions.
A feed supplement like H57, with the ability to enhance weight gain through modulation of intestinal microbial functions, has the potential to be an alternative to antibiotic growth promoters and provide substantial benefits to the poultry industry.

Keywords: *bacillus amyloliquefaciens h57*, probiotic, alternative to antibiotic growth promoters, metagenomic shotgun sequencing, microbiome, broilers, chickens

Introduction

The avian gastrointestinal tract (GIT) harbours complex microbial communities that influence host health and productivity. Chicken diets have been supplemented with sub-therapeutic doses of antibiotics for >50 years (Jones and Ricke 2003, Dibner and Richards 2005) to promote growth and control enteric pathogens. However, with the emergence of antibiotic resistant pathogens in humans and animals, this practice has been banned in many countries or is being voluntarily phased out in others (Casewell et al. 2003, Dibner and Richards 2005, Castanon 2007). Probiotics are emerging alternatives to antibiotic growth promoters (AGPs), as several products have been shown to improve meat production, and control or prevent enteric pathogens (El Jeni et al. 2021, Krysiak et al. 2021, Shini and Bryden 2022).

Probiotics exert their effect through several proposed mechanisms, including modifications to the host microbiome (Bajagai et al. 2016, Ma and Suzuki 2018). Advances in DNA sequencing technology have revealed the chicken intestinal microbiota in unprecedented resolution. Chicken gut microbiota consists of hundreds of microbial species (Oakley et al. 2014, Shang et al. 2018). However, there are limited studies on their functional capability, as the majority have focussed on describing microbial community composition using 16S rRNA gene (16S) sequencing (Oakley et al. 2014). In addition, 16S rRNA based profiling methods have revealed that the

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composition of the resident chicken intestinal microbiota, as well as the effect of probiotics on community structure, are highly variable (Stanley et al. 2013, Oakley et al. 2014, Pan and Yu 2014, Waite and Taylor 2014, Mancabelli et al. 2016). Furthermore, studying microbiota with 16S rRNA gene sequencing has some limitations due to multiple copies of markers in many species, choice of several variable regions and distinct primers making studies difficult to compare, different analysis pipelines and taxonomic databases (Bajagai et al. 2022). Instead, a shotgun metagenomic sequencing approach can give more reliable information about the microbiota profile and its function (Durazzi et al. 2021).

Bacillus amyloliquefaciens strain H57 (H57) is a spore-forming member of the *Firmicutes* phylum isolated from lucerne leaf (*Medicago sativa*) and initially selected for its ability to prevent mould development in hay (Brown and Dart 2003). This strain increased nitrogen retention in ewes fed H57-treated hay (Brown and Dart 2003), improved the performance of pregnant ewes fed H57 inoculated pellets, (Le et al. 2017) and reduced the incidence of diarrhoea in dairy calves (Le et al. 2017). This probiotic improved intestinal mucosa integrity and alleviated subclinical necrotic enteritis in chickens (Shini et al. 2020).

Here, we compared the effects of wheat and sorghum-based diets with or without H57 on the performance and intestinal microbiome of broiler chickens. These cereals are the primary dietary energy sources in the Australian poultry industry. We studied the impacts of diet and H57 supplementation on the composition and functional capacity of microbial communities associated with the caecum using shotgun metagenomic sequencing. The Caecum is the most populous compartment of the GIT (Apajalahti et al. 2004, Bjerrum et al. 2006, Sergeant et al. 2014) and the main site of microbial fermentation of undigested carbohydrates into short-chain fatty acids (Annison et al. 1968, Józefiak et al. 2004). We tested the hypothesis that H57 supplementation would increase chicken productivity and alter the functional capacity of the caecal microbiome.

Materials and methods

Experimental design and dietary treatments

A total of 288, 1-day-old, male Ross 308 broiler chicks were randomly allocated to 24 pens with 12 chicks per pen in an environment-controlled shed in the Poultry Research Unit on the Gatton Campus of the University of Queensland. All pens were randomly assigned to one of four treatment diets: sorghum-control, sorghum-H57, wheat-control, and wheat-H57, resulting in six replicate pens for each treatment (72 chicks total). The sorghum-control was a diet formulated with sorghum as the primary source of energy, and wheat-control was a diet with wheat as the main source of energy (Supplementary Table S1). All diets were formulated as starter (0–14 days) and grower (15–21 days) diets to meet or exceed recommended dietary requirements of Ross 308 Broilers (Aviagen 2014b). The treatment diets contained 8×10^7 colony forming units (cfu) of H57 per gram of feed in starter diets and 5×10^7 cfu per gram in grower diets designed to provide at least 10^9 cfu H57 per bird per day. The H57 was mixed in sodium bentonite as a carrier and an equivalent quantity of sodium bentonite was also mixed in the control diet. The birds were raised with free access to feed and water following recommended husbandry practices for Ross broilers (Aviagen 2014a). The experiment was approved by the Animal Ethics Committee of the University of Queensland (approval No. SAFS/159/16/ARC).

Sample and data collection

The experiment was run for 21 days with weekly measurement of body weight (BW) and feed intake. One randomly selected chicken from five randomly selected pens in each treatment were euthanised by intracardial injection of sodium pentabarbitone on day 13 and caecal contents were aseptically collected by squeezing the digesta into sterile 1.5 ml Eppendorf tubes. The caecal samples were immediately frozen in liquid nitrogen, transported with dry ice, and stored at −80°C.

DNA extraction, library preparation, and sequencing

The microbial genomic DNA from the caecal contents was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN), and concentration was measured using a Qubit 2.0 fluorometer with a dsDNA Broad Range Assay kit (Thermo Fisher Scientific Inc, Victoria, Australia).

A paired-end indexed library for shotgun metagenomic sequencing was prepared using the Illumina Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) as per the 'manufacturer's instructions. The DNA library was cleaned with Agencourt AMPure XP beads (Beckman Coulter Australia Pty Ltd, Lane Cove, NSW, Australia), and the quality of the library was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The DNA libraries were normalized, pooled, and sequenced at the Australian Centre for Ecogenomics using Illumina NextSeq 550 sequencing system (Illumina, San Diego, CA, USA) with 2×150 bp configuration.

Data analyses

The performance data [body weight, average daily weight gain, average daily feed intake (ADFI), and feed conversion ratio (FCR)] were analysed by one-way ANOVA using GraphPad Prism (v9.2.0) considering individual birds as observation units and individual pens as experimental units. The normality of distribution and homogeneity of variances were confirmed before applying the statistical tests. 'Tukey's multiple comparison test was used for *post hoc* pairwise comparison of means.

Bioinformatics

Quality of the shotgun sequence reads was tested with fastQC, and quality trimming was done with trimmomatic. Sequence reads were then aligned to the UniREF100 reference protein database using DIAMOND v0.8.30 (19). Protein sequences in the reference database were then grouped according to their assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) Orthology (KO), and the number of reads aligned to each protein sequence was collated accordingly by KO functions. The KOs with zero mapped reads across all samples were removed. The collated KO counts table was annotated using the KEGGREST (Tenenbaum and Maintainer 2022) package in R v3.3.1 (R Core Team 2016).

The resulting KO count matrix (each row representing a KO identifier and each column representing a sample) was then analyzed with the DESeq2 package (Love et al. 2014) in

Table 1. Effects of *B. amyloliquefaciens* strain H57 when added to sorghum and wheat-based diets to feed meat chickens from 1 to 21 days of age.

Comparisons[1]	Variables	Control (Mean)	H57 (Mean)	Mean Diff.	95.00% CI of diff.	Adjusted *P* Value
S-CT vs. S-H57	BW (g/bird)	922	1027	−105.1	−162.0 to −48.18	<0.001
	Average daily gain (ADG) (g/bird/day)	39.6	46.6	−7.03	−10.13 to −3.932	<0.001
	FCR (g feed/g bird)	1.26	1.26	−0.004	−0.075 to 0.066	0.998
	ADFI (g/bird/day)	48.9	56.4	−7.473	−10.16 to −4.785	<0.001
W-CT vs. W-H57	BW (g/bird)	930	994	−63.96	−120.9 to −7.052	0.025
	ADG (g/bird/day)	40.5	43.9	−3.458	−6.558 to −0.3585	0.027
	FCR (g feed/g bird)	1.29	1.27	0.015	−0.055 to 0.086	0.923
	ADFI (g/bird/day)	50.9	54.3	−3.4	−6.088 to −0.712	0.011

[1]S-CT = Sorghum control, S-H57 = Sorghum H57, W-CT = Wheat control, W-H57 = Wheat H57

R to identify differentially abundant KOs between treatments (Control and H57). Variance-stabilizing transformation (Lin et al. 2008) was applied to the DESeqDataSet of KO counts to reduce the heteroskedasticity and its subsequent effect in downstream data analysis. Association of the KO counts with treatment diets (Control and H57) was tested by permutational multivariate analysis of variance (PERMANOVA) of variance-stabilized data using adonis() function in the vegan package (Oksanen et al. 2016) in R. Principal Component Analysis (PCA) ordination of the KO counts was performed using vegan (Oksanen et al. 2016) to visualize compositional similarities and differences in KO counts between samples.

Analysis of higher level metabolic pathway was done by using HUMAaN2 v2.8.1 (Franzosa et al. 2018), by aligning the reads with UniRef90 database. The HUMAaN2 annotated pathways were grouped according to metacyc database and analyzed with Linear discriminant analysis Effect Size (LEfSe) to identify differentially enriched pathways between control and H57 treatment (Segata et al. 2011).

Taxonomic analyses

The taxonomic profile of the microbiota was analyzed with MetaPhlAn2 v2.7.7, which is an efficient tool for mining metagenome sequences for characterizing the taxonomic profile (Truong et al. 2015). The resultant taxonomy matrix with relative abundances of taxa obtained from the MetaPhlAn2 was analyzed with the vegan (Oksanen et al. 2013) package in R to find the overall significance of the difference. The association of individual taxa with the experimental conditions (control and H57) both in the sorghum and wheat group was analysed with Microbiome Multivariable Association with Linear Models (MaAsLin2) (Mallick et al. 2021).

Results

Influence on bird performance

Birds appeared clinically normal throughout the experiment and the probiotic H57 significantly enhanced the growth rate of chickens irrespective of diet (Table 1 and Fig. 1). For the sorghum-based diet, H57 improved the average daily BW gain from day 0 to 21 by 18% (39.6 vs. 46.6 g/bird/day, $P < 0.001$) compared with the controls, resulting in a bodyweight at day 21 of 922 vs. 1027 g ($P < 0.001$). Similarly, the growth rate of birds fed the wheat-based diet supplemented with H57 also improved, with an overall difference of 8.4% (40.5 g/bird/day vs. 43.9 g/bird/day) between the control and H57 fed birds from days 0 to 21; the final bodyweight was 930 vs. 994 g ($P = 0.025$).

Feed intake also significantly increased in response to H57 treatment, irrespective of diet (Table 1 and Fig. 2). For the sorghum-based diet, the ADFI per bird throughout the 21 days of the trial was 15.3% greater for H57 than controls (48.9 vs. 56.4 g, $P < 0.001$). The feed intake for wheat fed birds for the same period was 6.1% greater for the H57 group (50.9 vs. 54 g, $P = 0.011$). Feed use efficiency measured as FCR showed no difference between treatments for both the sorghum and wheat-based diets over the 21 days of the experiment.

Effects of the H57 on BW (BW in g) and ADG (ADG in g) of chickens. S-CT, S-H57 = Sorghum H57, W-CT, W-H57. A = BW of chicks at day 0, B = BW at day 7, C = BW at day 14, D = BW at day 21, E = ADG at day 7, F = ADG at day 14, G = ADG at day 21. $P < 0.0001$****, $P < 0.001$***,and $P < 0.05$*, ns. Bars are standard errors of the means (SEM) and each dot represents the mean value for a replicate.

Influence of H57 on metagenome-inferred microbial functions

A molecular function count matrix, with rows representing KO (Kanehisa and Goto 2000) identifiers and columns representing samples, was created by aligning the sequences to the UniREF100 database (Suzek et al. 2007) and collating counts by KO. The differentially abundant KO groups were identified using DESeq2 (Love et al. 2014). The KO table was transformed by applying the variance stabilizing transformation function of Deseq2. The overall differences in functional capacity between groups were analyzed with PERMANOVA of the DESeq2 normalized count table using the vegan (Oksanen et al. 2016) package in R. There were significant differences in overall KO composition between the control and H57 treatments for both sorghum ($P < 0.01$) and wheat ($P < 0.05$) based diets, indicating that H57 supplementation altered the functional capacity of the caecal microbiome irrespective of diet (Fig. 3, Fig. S1).

Heatmap showing the top 50 DESeq2 differentially abundant KO in sorghum group. The heatmap cell color is based on row z-scores (number of SDs a cell value lies above or below the mean). Rows represent KO numbers and columns represent samples. Both rows (KOs) and columns (samples) are clustered with hierarchical clustering with clustering trees on left (KOs) and at top (samples).

The DESeq2 analyses revealed that for sorghum-diet, 376 molecular functions (KOs) were differentially abundant ($P_{adj} < 0.05$) between H57-fed and control birds (Fig. 4). Among the differentially abundant functions, 369 functions were over-represented in the control and seven were

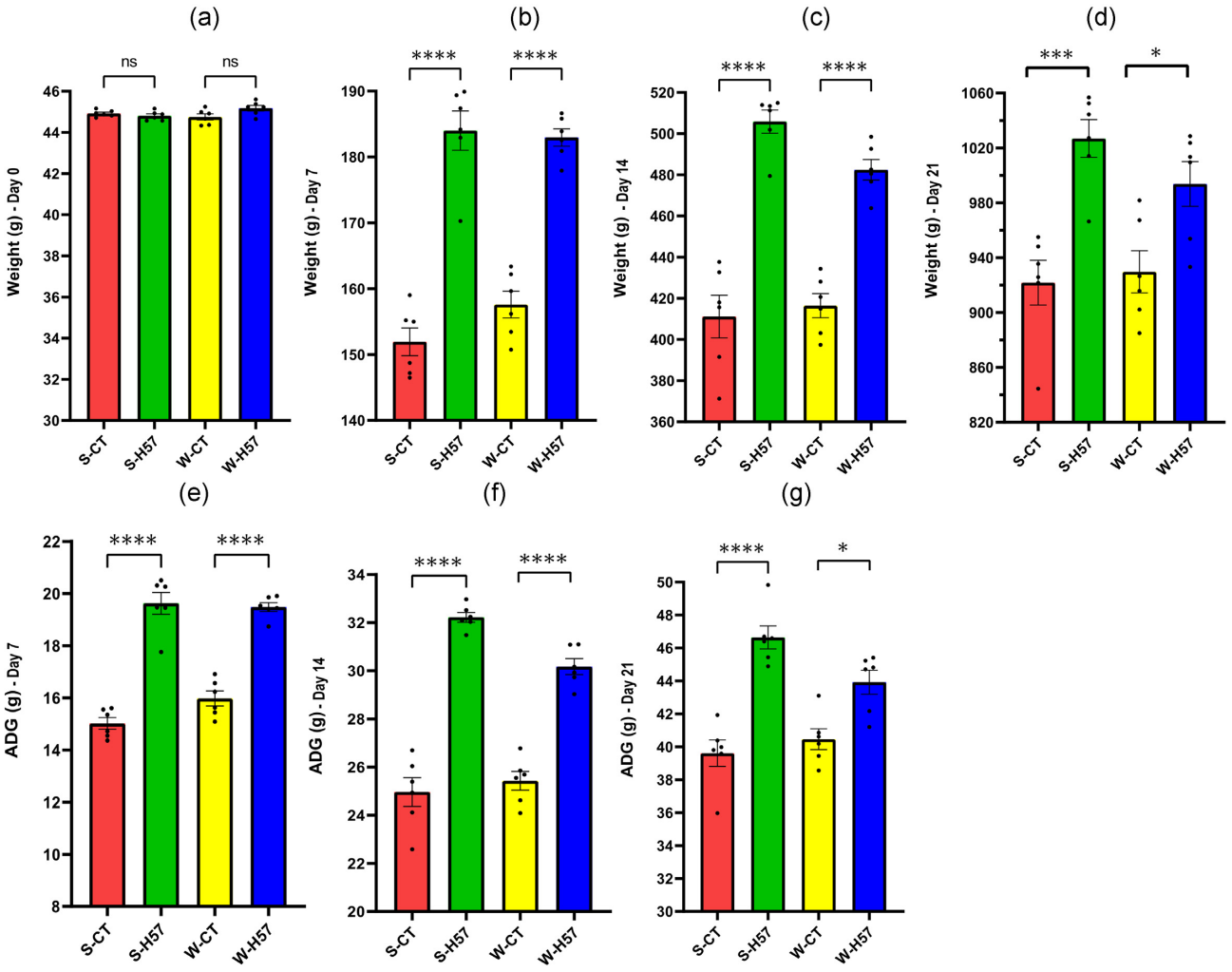

Figure 1. Effects of the H57 on BW (BW in g) and ADG (ADG in g) of chickens. S-CT, S-H57 = Sorghum H57, W-CT, W-H57. A = BW of chicks at day 0, B = BW at day 7, C = BW at day 14, D = BW at day 21, E = ADG at day 7, F = ADG at day 14, G = ADG at day 21. $P < 0.0001$****, $P < 0.001$***, and $P < 0.05$*, ns = not significant. Bars are SEM and each dot represents the mean value for a replicate.

over-represented in H57-fed birds (complete list in Supplementary Table S3). Similarly, 126 molecular functions (KO) were differentially abundant (*Padj* < 0.05) between control and H57 within the wheat-diet group (Fig. 5). Among these differentially abundant functions, 36 were over-represented in control and 90 were over-represented in the H57-fed birds (complete list in Supplementary Table S4).

Higher-level metabolic pathways clustered according to the MetaCyc database (Caspi et al. 2020) obtained from HUMAnN2 (Franzosa et al. 2018) was analyzed with Linear Discriminant Analysis Effect Size (LEfSe) (Segata et al. 2011) to identify the differentially abundant pathways. There were 34 metabolic pathways differentially abundant between control and H57-fed birds fed sorghum, and all but two of these pathways had an LDA score higher than three (Fig. 6). Among these pathways, 15 were overabundant in the H57 group and 19 were underabundant in the H57 group.

Intriguingly, among 15 differentially abundant pathways enriched in the caecal microbiota of birds fed the H57 sorghum diet, seven pathways were related to the biosynthesis of essential amino acids. More importantly, the top five enriched pathways with the largest effect sizes are part of the essential amino acid synthesis pathways. The amino acid pathways enriched in the H57 group are L-isoleucine biosynthesis pathway III, L-isoleucine biosynthesis I from threonine, L-valine biosynthesis, L-arginine biosynthesis IV, L-arginine biosynthesis I via L-ornithine, superpathway of arginine and polyamine biosynthesis, and L-lysine biosynthesis I.

The LEfSe analyses identified a total of 52 metabolic pathways enriched in either control or H57 group in birds fed wheat (Fig. 7). Among these pathways, 36 were overabundant in the H57 group and 16 were underabundant in the H57 group. A total of 22 pathways had an LDA score higher than three.

Five pathways related to amino acid biosynthesis are enriched in the H57 group for the wheat-based diet. Notably, the top two enriched pathways with the largest LEfSe effect size were amino acid synthesis pathways for L-asparate and L-proline. Metabolic pathways for amino acids L-tryptophan and L-arginine were also enriched in H57 fed chickens. Contrary to the sorghum group, biosynthesis pathways for valine and isoleucine were less abundant in the H57 group than for the control wheat-based diet.

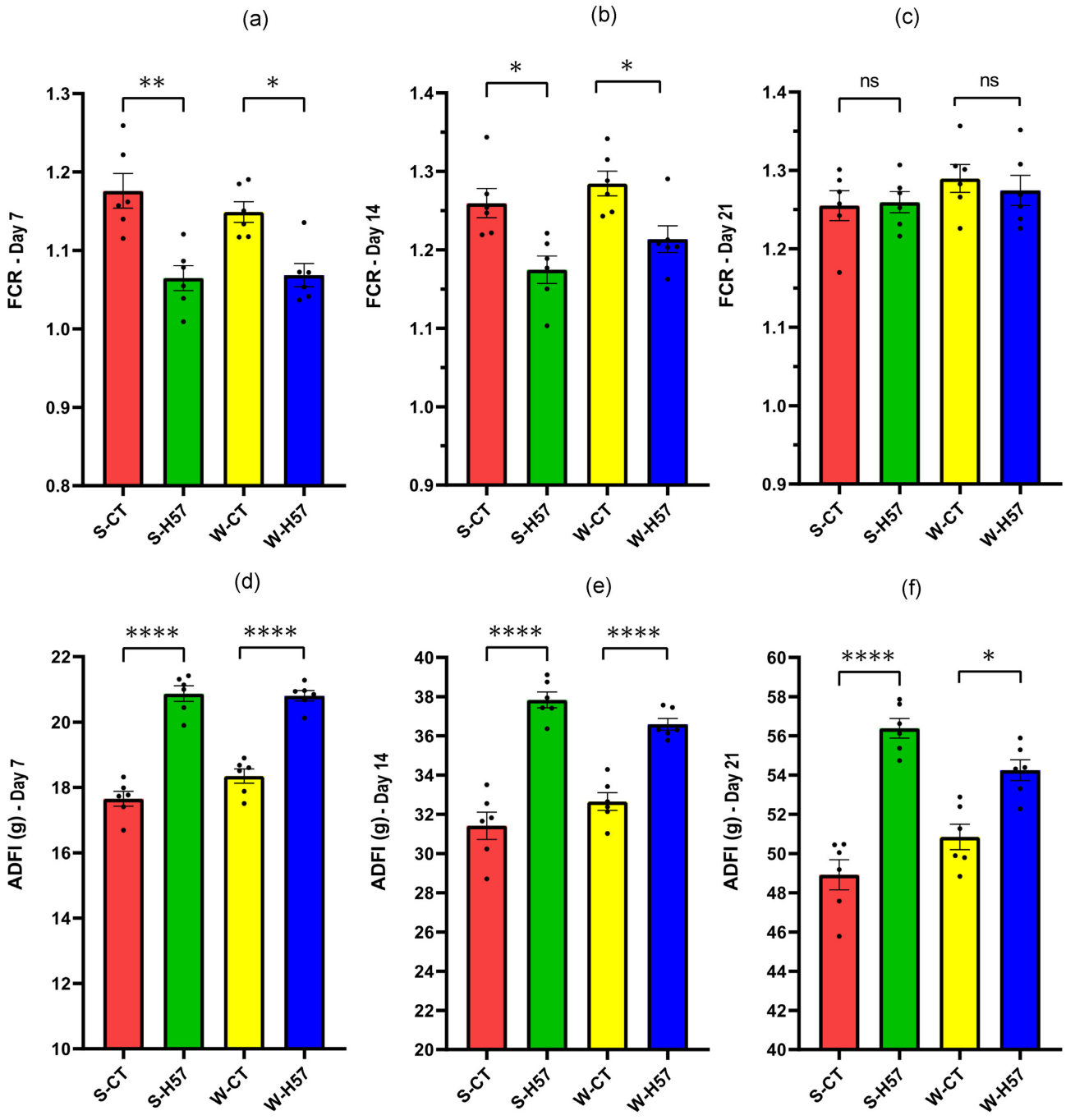

Figure 2. Effects of H57 on FCR and ADFI (ADFI in g) of chickens. S-CT, S-H57 = Sorghum H57, W-CT, W-H57. A = FCR day 7, B = FCR at day 14, C = FCR at day 21, D = ADFI at day 7, E = ADFI at day 14, F = ADFI at day 21. $P < 0.0001$****, $P < 0.01$**, and $P < 0.05$*, ns. Error bars are SEM; each dot represents a replicate's mean value.

Influence of H57 on the chicken gut microbiota profile

The relative abundances of microbial taxa recovered from the metagenome using MetaPhlAn2 differed significantly between diets ($P = 0.001$, PERMANOVA) (Fig. 8). Between the control and H57 treatments, however, there were no significant differences in community composition, irrespective of diet and statistical method [$P > 0.05$, PEMANOVA, LEfSe, and Microbiome Multivariable Associations with Linear Models (MaAsLin2)] (Supplementary Figure S2).

Discussion

This study has demonstrated that probiotic *B. amyloliquefaciens* strain H57 improves the performance of broiler chickens and changes the functionality of the caecal microbiome, especially in relation to amino acid and vitamin synthesis, supporting the hypothesis of this study. The significant increase in growth rate and feed intake indicates the possibility of using this probiotic as an alternative to AGPs in chickens. Our results are in general agreement with Ahmed et al. (2014) and Lei et al. (2015), who reported positive effects of *B. amyloliq-*

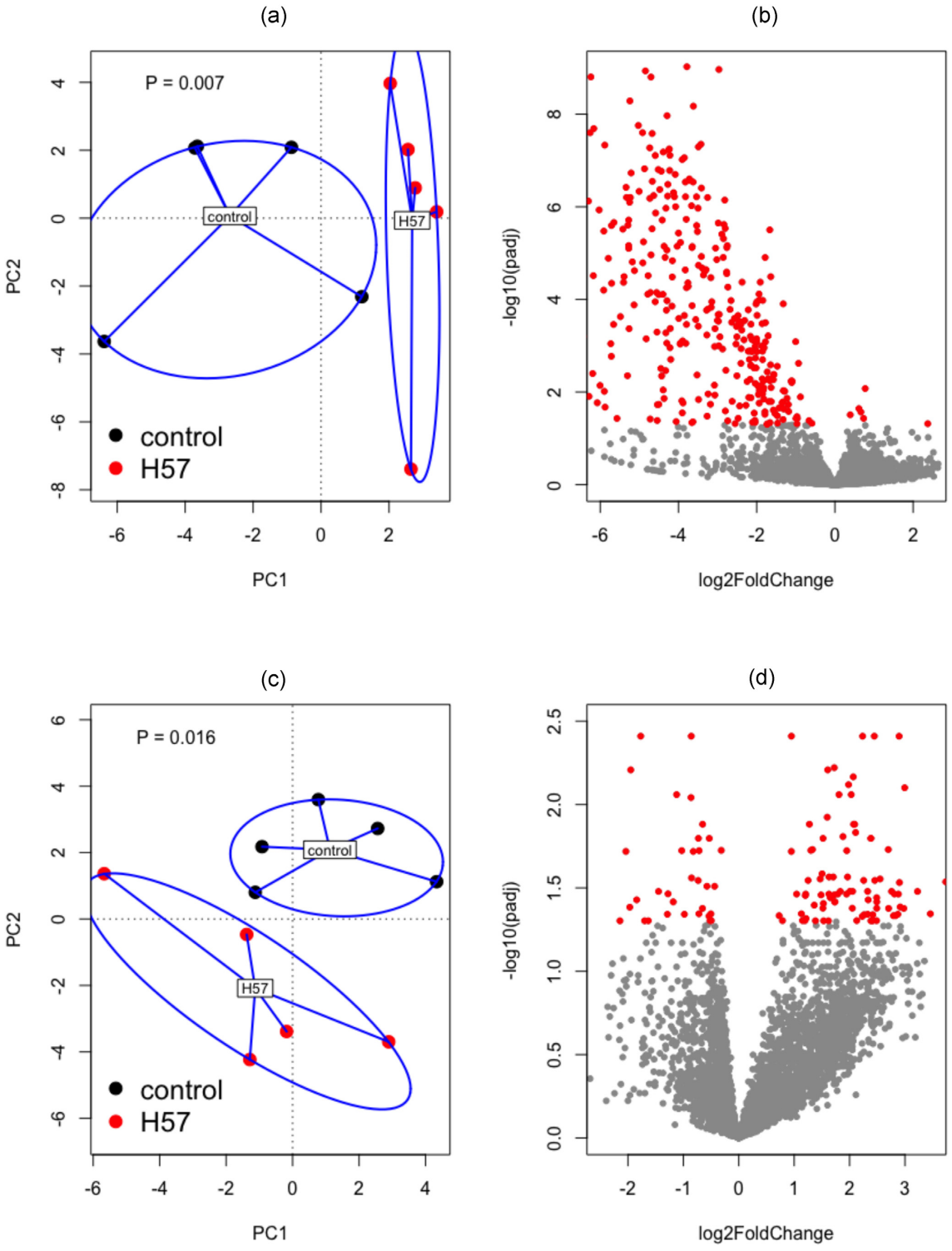

Figure 3. Effects of H57 on KO profiles associated with the caecal microbiome at day 13. (a) PCA ordination of variance-stabilizing transformed KO function matrix in the birds fed a sorghum-based diet. The points represent the individual sample (bird), blue lines connect each sample to the group centroid with an ellipsoid enclosing all samples. *P* value is calculated with PERMANOVA. (b) Volcano plot of KO functions with the sorghum diet showing DESeq2 differentially abundant functions (red). (c) PCA ordination of variance-stabilizing transformed KO function matrix in birds fed a wheat-based diet. The points represent the individual samples (birds), blue lines connect each sample to the group centroid with an ellipsoid enclosing all samples. *P* value is calculated with PERMANOVA. (d) Volcano plot of KO functions within the wheat diet showing DESeq2 differentially abundant functions (red).

uefaciens supplementation on BW gain, feed intake, and FCR of broiler chickens fed a corn and soybean-based diet. In a recent study, supplementation of broiler feed with *B. amyloliquefaciens* LFB112 improved the growth rate of broilers (Ahmat et al. 2021). In earlier studies, broilers fed with a commercial probiotic product containing *B. amyloliquefaciens* had increased growth rates (Ortiz et al. 2013) and improved feed efficiency over the 42-day growth period (Diaz

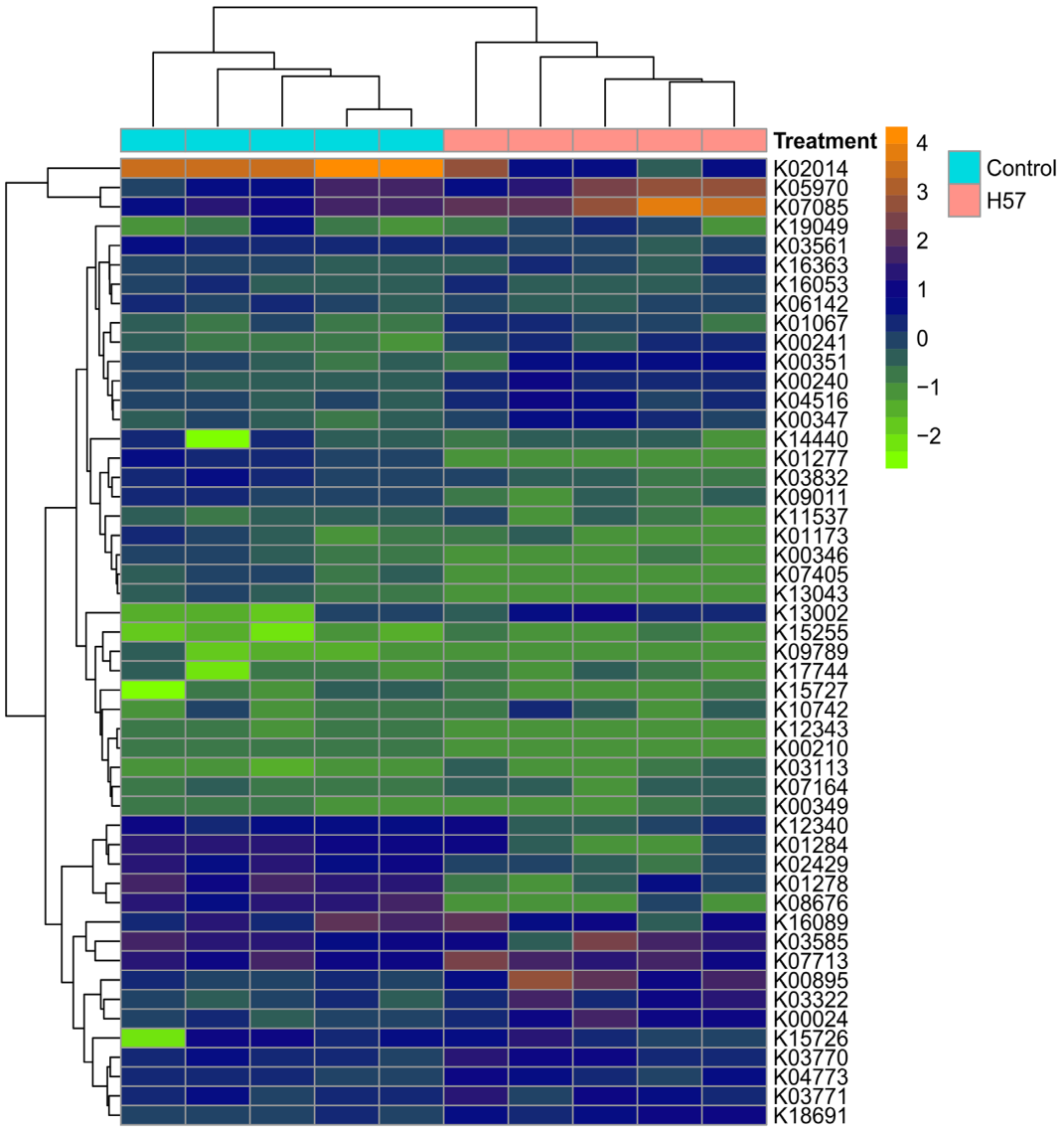

Figure 4. Heatmap showing top 50 DESeq2 differentially abundant KO in sorghum group. The heatmap cell color is based on row z-scores (number of SDs a cell value lies above or below the mean). Rows represent KO numbers and columns represent samples. Both rows (KOs) and columns (samples) are clustered with hierarchical clustering with clustering trees on left (KOs) and at top (samples).

2008, Ortiz et al. 2013). Likewise, birds fed H57 and challenged with subclinical necrotic enteritis had improved feed efficiency compared to unsupplemented birds (Shini et al. 2020).

Several studies describe the effects of probiotics on the intestinal microbiota of chickens that concentrate on modulating microbial profiles (Yadav and Jha 2019). Balancing the intestinal microbiota has been proposed as a possible mode of action for potential benefits from probiotics (Ma and Suzuki 2018). However, this study shows that probiotics can modulate microbial functions without significantly affecting the microbiota profile. Therefore, studies of the effects on microbial function are as critical, if not more important than studies on microbial community composition, when seeking to elucidate the mode of action of probiotics.

Amino acids are important nutrients with versatile physiological functions in addition to their role in protein synthesis and play crucial roles on growth, productivity and health of chickens (He et al. 2021). Importantly, isoleucine, valine, arginine, and lysine are essential amino acids, which cannot be synthesised endogenously by the bird and must be supplied in the feed. The pathways for the synthesis of these amino acids were enriched in H57 supplemented birds in the sorghum group. However, pathways for valine and isoluceine synthesis were not enriched by H57 in the wheat group. This indicates that the effect of H57 varies with the diet or the resident intestinal microbial profile as the caecal microbial profiles of sorghum and wheat fed birds were significantly different (Fig 8). It is a commercially important characteristic of H7 that it has been able to improve the growth rate across two diets with different ingredient composition.

There is growing interest in low protein diet formulation in the poultry industry based on environmental sustainability and cost reduction concerns. The approach is to formulate low crude protein diets with specific synthetic amino acid supplements to balance the amounts of specific essential amino acids in the diet (Pack et al. 2003, Van Harn et al. 2019, Liu et al. 2021). Our study opens up the possibility of supplementing

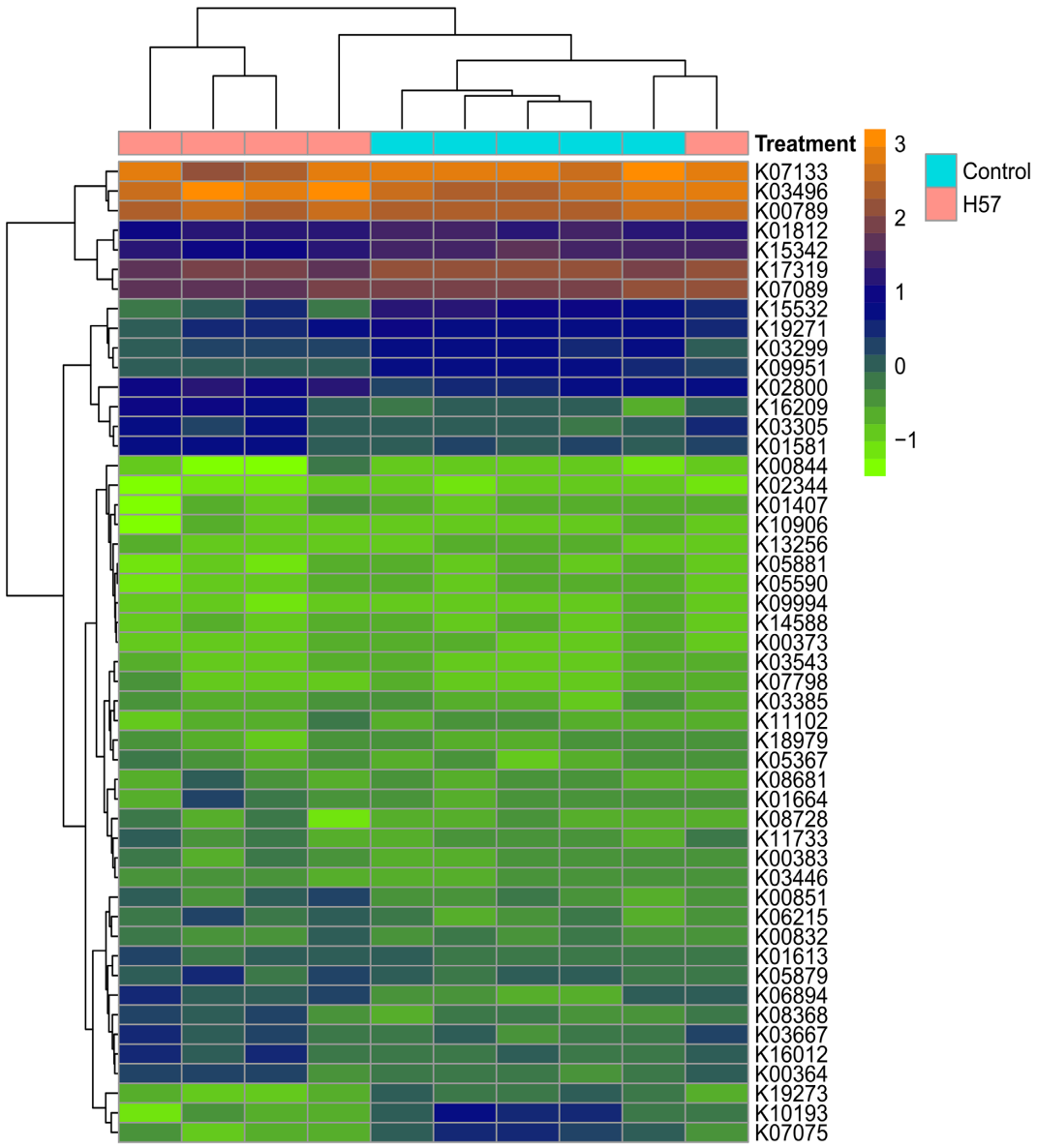

Figure 5. Heatmap showing top 50 DESeq2 differentially abundant KO functions in the wheat group. The heatmap cell colour is based on row z-scores (number of SD a cell value lies above or below the mean). Rows represent KO numbers. Both rows (KOs) and columns (samples) are clustered with hierarchical clustering with clustering trees on left (KOs) and at top (samples).

low-protein chicken diets with selected probiotics to enhance microbial amino acid synthesis.

Another important group of metabolic pathways enriched in the H57 group in the wheat-based diet is vitamin synthesis, particularly vitamins from the B-complex group, which are essential co-factors for enzymes in intermediatory metabolism. A total of seven metabolic pathways responsible for the synthesis of thiamine (vitamin B1), riboflavin (vitamin B2), biotin (vitamin B7 or vitamin H), and folate (vitamin B9) were significantly enriched in the H57 group. A deficiency of thiamine in chickens can cause loss of appetite, reduced body weight, and clinical signs of cardiac and neurological dysfunction (Burgos et al. 2006). A deficiency of riboflavin may lead to curled-toe paralysis in chickens due to myelin degeneration of the sciatic nerve (Burgos et al. 2006). A diet deficient in biotin can result in impaired growth, reduced feed use efficiency and development of the fatty liver and kidney syndrome (FLKS) on wheat based diets (Bryden 1991), while folate deficiency can also reduce appetite and growth rate and result in poor feathering (Burgos et al. 2006). Thus, H57 can indirectly improve the production of these vitamins in the caeca by enriching the intestinal environment with the required metabolic pathways to produce vitamins. Interestingly, Sabo et al. (2020) isolated probiotic species from intestinal samples of poultry that produced B-vitamins and used these isolates to inoculate poultry (Sabo et al. 2020).

For both amino acids and vitamins there is need to quantify the amount of these nutrients produced as a result of supplementing diets with specific probiotics and locating where they are produced in the GIT and if available to the bird. Many studies have shown that the uptake of amino acids (Denbow 2015) and B vitamins (Heard and Annison 1986, Bryden 1989) from the caeca in birds is limited. However, retrograde movement of digesta from the caeca into the small

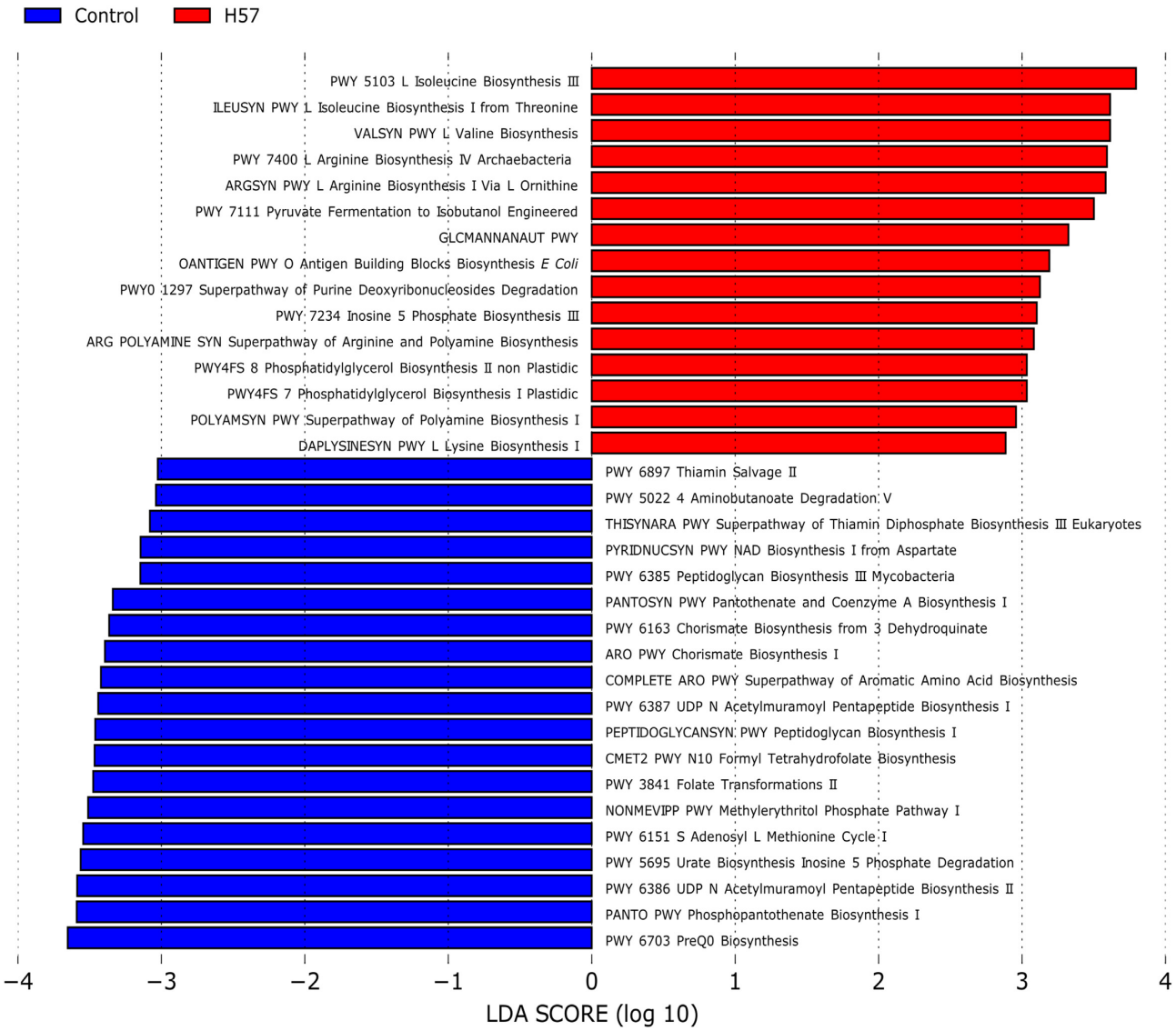

Figure 6. Comparison of functional pathways in control and H57 in the sorghum diet group with LEfSe analysis. The histograms show the LDA scores of differentially abundant pathways, which explain the differences between two communities (control and H57). The higher the LDA score, the higher the contribution of the specific pathway to explain the difference between the two communities compared. Red = metabolic pathways over-represented in the H57 group. Blue = metabolic pathways over-represented in the control group.

intestine (Denbow 2015) would subject bacterial cells to the normal processes of digestion and absorption. Moreover, birds practising coprophagy may benefit from nutrients produced in the caeca, as Whitehead and Bannister (1980) have estimated that broilers can obtain ~10% of their B-vitamin requirements from this practice.

In addition to inducing the caecal microbiota to produce amino acids and B-vitamins, *B. amyloliquefaciens* produces a large range of extracellular metabolites eg. enzymes such as α-amylase, proteases, cellulase, and xylanase (Gould et al. 1975, Breccia et al. 1998, Gracia et al. 2003, Lee et al. 2008), antimicrobial and antifungal lipopeptides eg. surfactin, fengicin, bacillumycin D, iturin A (Koumoutsi et al. 2004, Ongena and Jacques 2008, Chen et al. 2009, Arrebola et al. 2010), polyketides eg. macrolactin, difficidin, bacillaene, chlorotetain (Rapp et al. 1988, Chen et al. 2006, Schneider et al. 2007) and bacteriocins (Ulyanova et al. 2011). Strain H57 has genes to encode for many of these exogenous metabolites ,including several carbohydrate active enzymes such as glycoside hydrolases, lipopeptides (surfactin, iturin, bacillomycin D and fengycin) and antibiotic polyketides (macrolactin, difficidin and bacillaene) (Schofield et al. 2016). It is not known if any of these compounds benefit poultry nutrition and how they might influence poultry GIT microbiome composition and function, especially related to the changes described in this study.

Most studies on the effects of diet supplements (probiotics, phytogens, prebiotics etc.) focus on changes in the microbial profile by sequencing 16S rRNA genes (Wang et al. 2017, Baldwin et al. 2018, Yadav and Jha 2019, Bajagai et al. 2020, Jha et al. 2020). However, this study has demonstrated that probiotics can affect metabolic functional pathways of the intestinal microbes without significantly altering the microbiota population. Although studying microbiota by sequencing and analysis of the 16S rRNA gene has been the method of choice for the last decade, this method is not without limitations and challenges (Bajagai et al. 2022). Sequencing of total DNA, as

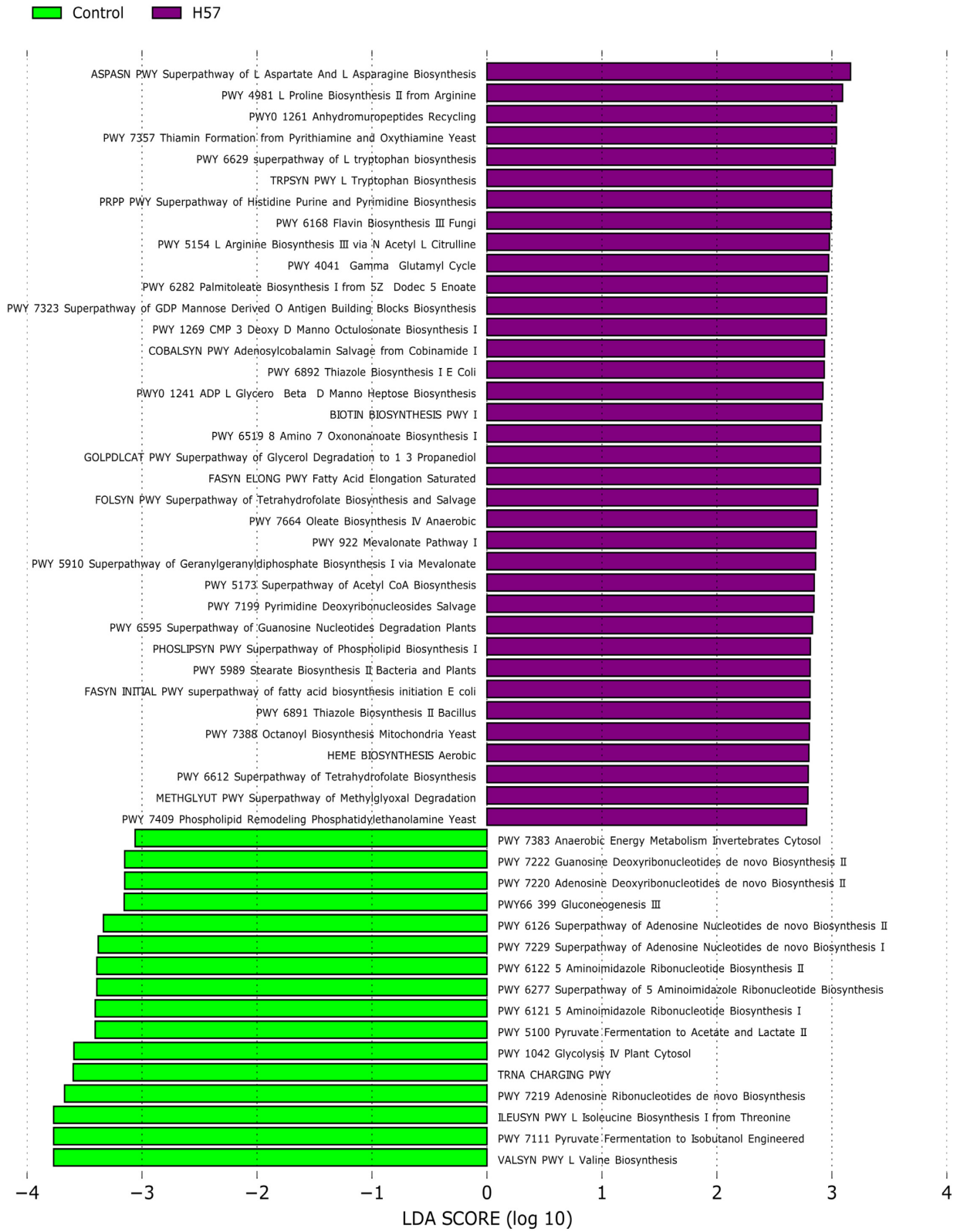

Figure 7. Comparison of functional pathways in control and H57 in the wheat diet group with LEfSe analysis. The histograms show the LDA scores of differentially abundant pathways which explain the differences between two communities (control and H57). The higher the LDA score, the higher the contribution of the specific pathway to explain the difference between the two communities compared. Purple = metabolic pathways over-represented in the H57 group. Green = metabolic pathways over-represented in the control group.

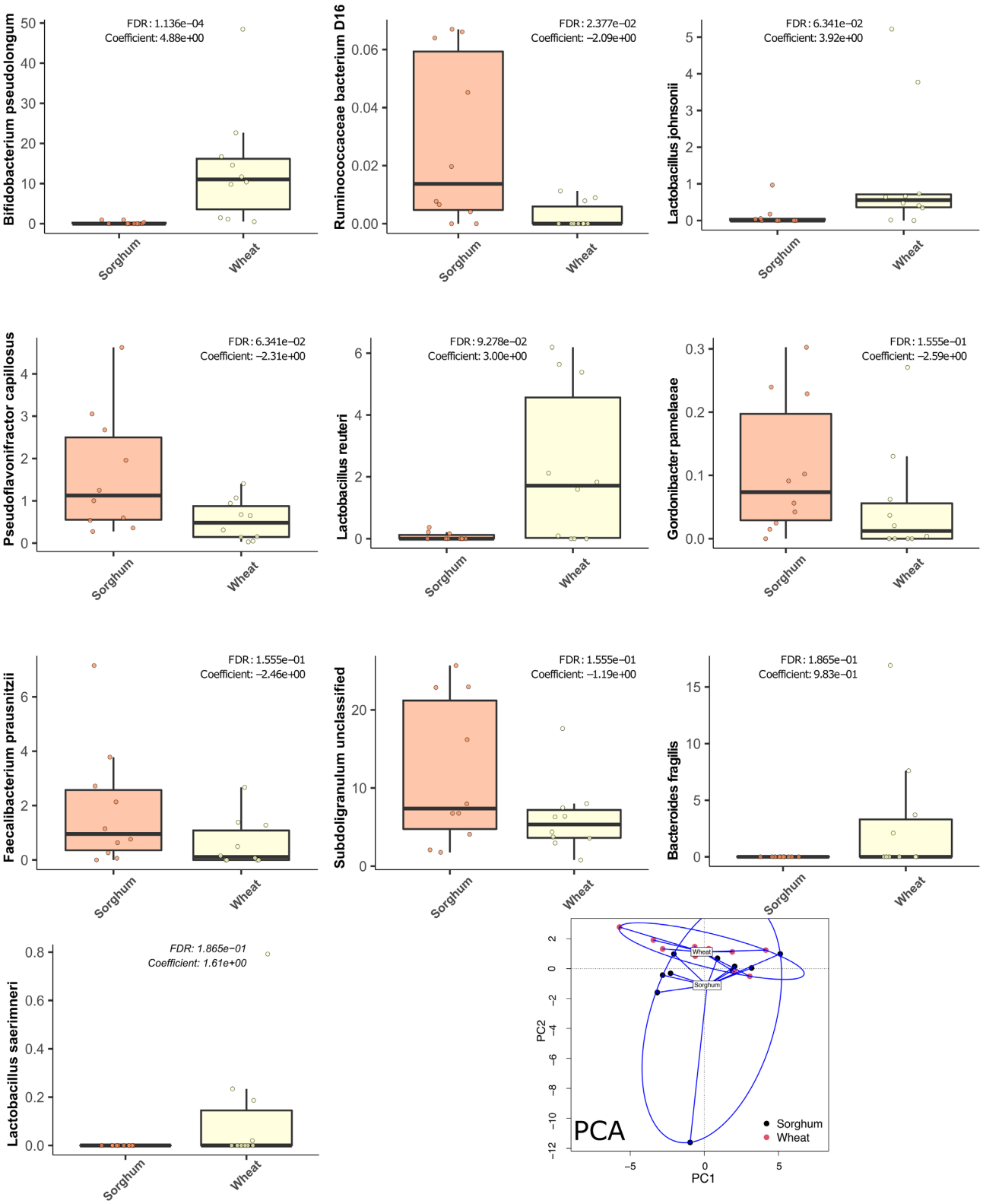

Figure 8. Differential abundant taxa between sorghum and wheat group. Microbial species association with feed type was analysed with Microbiome Multivariable Associations with Linear Models (MaAsLin2). The Y-axis gives species of bacteria and their relative abundance, which are associated with feed type (sorghum or wheat). The false discovery for multiple testing controlled *P* value (FDR) and corresponding coefficient are also presented for each taxon. PCA ordination of relative abundance of microbial species in sorghum and wheat group. The points represent the individual sample (bird), blue lines connect each sample to the group centroid with an ellipsoid enclosing all samples.

we did in this study, can be an effective tool to unravel the complexity of the intestinal ecosystem and study the mode of action of probiotics and other dietary supplements. It is important to find out if intestinal microbial metabolites such as the amino acids and vitamins, shown potentially enhanced by H57 in this study are taken up by the host and used to improve bird performance. Moreover, it is clearly demonstrated that these effects were different in sorghum and wheat-based diets.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

The authors declare no conflicts of interest in relation to this research work. The funding agencies played no role in the study design, data collection, analysis, interpretation, or writing of the manuscript.

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Yadav Sharma Bajagai (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft), Yun Kit Yeoh (Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing), Xiuhua Li (Investigation, Methodology, Resources, Supervision, Writing – review & editing), Dagong Zhang (Data curation, Investigation, Methodology), Paul G. Dennis (Conceptualization, Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing), Diane Ouwerkerk (Data curation, Methodology, Project administration, Writing – review & editing), Peter J. Dart (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing), Athol V. Klieve (Conceptualization, Data curation, Methodology, Resources, Writing – review & editing) and Wayne L. Bryden (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing).

Data availability

The dataset supporting the conclusions of this article is available in the NCBI SRA repository, PRJNA835154, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA835154.

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