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Effects of dried chicory and Jerusalem artichoke on skatole-producing microbial populations of entire male pigs.

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Prevotellaceae and skatole production

Dried chicory as dietary supplement

Jerusalem artichokes as dietary supplement

Effects of inulin on diversity and composition of microbiomes

Novel skatole producers

[Type here]

Effects of dried chicory and Jerusalem artichoke on skatole-producing microbial populations of entire male pigs.

Abstract

In this study, we compared effects of inulin-rich plant based dietary supplements on the hindgut microbial communities of entire male pigs using 16S rRNA gene metabarcoding. The study was conducted using dried Jerusalem artichokes and dried chicory roots that were supplied to the basal diet one week before slaughter. Both of the experimental diets were formulated to contain the same amount of inulin and thus differed only in the composition and concentration of secondary metabolites within the plant material used in the study. Jerusalem artichokes was found to have no impact on microbial α- and β-diversity, while inclusion of dried chicory increased the abundance of microorganisms carrying predicted metabolic pathways that are capable of affecting host performance and gastrointestinal health. We have discovered a strong positive correlation between microorganisms in the Prevotellaceae family and skatole levels. We also identified several broadly distributed microorganisms from the Bacteroidetes phylum that share 16S rRNA similarity with the known skatole producers. Other verified skatole producers were not detected, were identified in single samples or showed no statistically significant correlations with skatole levels. Our findings suggests the the composition of plant secondary metabolites contained in the roots of chicory and Jerusalem artichokes is laying beneath the observed effects and require further detailed studies.

List of abbreviations

TRP: tryptophan.

IAA: indole acetic acid.

J. artichoke: Jerusalem artichoke.

QS: quorum sensing.

OUT: operational taxonomic unit.

AM: arithmetic mean.

Introduction

Successful commercial pig diets are formulated to have good pre-cecal protein digestibility as undigested protein that reaches the hindgut supports proliferation of harmful gut microflora. Protein fermentation in the hindgut is accompanied by release of toxic metabolites and is associated with increased rates of intestinal disorders in pigs [1, 2]. Skatole (CAS: 83-34-1) or 3-methylindole is an aromatic compound responsible for the distinctive odour of faeces and boar taint of the meat obtained from sexually mature male pigs. Indole and its derivatives serve as an intercellular communication signal that regulates microbial dormancy, biofilm formation, plasmid stability, drug resistance, and microbial virulence [3]. Skatole has been described as an oviposition cue for haematophagous insects and bees [4-7], but no physiological role has been described for skatole in vertebrates. Skatole that is produced in the lumen of the intestine is passively absorbed and then transported to the liver, where it is degraded via a two stage catabolic pathway [8]. Small quantities of skatole reach peripheral circulation and are oxidised into 3-methyleneindolenine. Adducts of 3-methyleneindolenine with DNA and proteins are responsible for the known toxic and carcinogenic properties of skatole [9-12]. The catabolism of skatole in the liver has been studied in great detail. In the liver, skatole first undergoes conversion by the CYP2E1 and CYP2A enzymes, followed by further modification with the ULT1A1 and UGT enzymes. The activities of CYP2E1 and CYP2A are inhibited by gonadal steroids, which explains the higher concentrations of skatole in the blood and tissue of male pigs [8]. Studies on *Clostridium drakei* and *Clostridium scatologenes* isolated from pig manure using enrichment culture techniques suggested that tryptophan (TRP) is converted to skatole through a two-step mechanism. During the first step, TRP is deaminated into indole acetic acid (IAA), and then, IAA is further decarboxylated into skatole [13]. Similar mechanisms for skatole production were suggested for *Lactobacillus* [14], *Pseudomonas* [15], *Prevotella*, *Actinomyces*, *Megasphaera* [16], *Bacteroides* [17] and *Olsenella* [18]. Recent metagenomics studies of microbial communities in pig manure have showed that slurry storage time have a strong impact on both the skatole concentrations and bacterial compositions. Longer storage is positively correlated with an increased level of skatole

and populations of *AC160630* (candidate taxon), *Acholeplasmatales*, *Mollicutes*, as well as various *Cloacamonas* species [19]. The last of which indicates that different bacterial groups are responsible for production of skatole in the pig hindguts and manure pits. A metagenomic study of the fresh pig manure showed a correlation between reduced crude protein content, reduced relative abundance of the *Bacteroides spp.* and decreased skatole levels suggesting that *Bacteroides spp.* are a main producer of skatole in pig intestines [20].

Inulin type fructans are fructose oligosaccharides containing terminal glucose units connected by β (2→1) glycosidic bonds. The inulins have a degree of polymerization that is ≤ 12, and they are not digested in the upper gastrointestinal tract [21]. Inulins have a sweet taste and attractive flavour [22] which in combination with high cecal digestibility makes these substances interesting prebiotic candidates for modulation of intestinal microbiota [23]. Inulin type fructans are found in more than 3500 plant species [24]. Industrial extraction is primarily done using the roots of chicory (*Cichorium intybus*), which contains approximately 20% inulin as a storage oligosaccharide[25]. An alternative source of inulin is Jerusalem artichoke (*Helianthus tuberosus*), which contains approximately 14% inulin [26].

In our previous studies, we evaluated the effects of chicory and J. artichoke as functional diet ingredients for modulation of skatole production in entire male pigs [23, 27]. Supplementation of both ingredients led to significant dose-dependent reduction of the skatole levels and led to a reduction in abundance of Clostridia and Enterobacteria accessed by cultivation. Addition of inulin increased the total amount of SCFA, acetic acid and valeric acid in faeces and was accompanied by pH reduction. In the current paper, we report a 16S metabarcoding study of the microbial populations in the hindgut of entire male pigs fed with diets containing dried chicory and J. artichoke and discuss our findings in the context of the prior data on experimentally verified skatole producers.

Materials and methods

Animal experiment and diets

A growth-performance experiment using inulin supplements was conducted at the Experimental Farm of the Norwegian University of Life Sciences, Aas, Norway. Detailed experimental design, diet formulations and digesta sampling are described in Vhile et al. [23]. Briefly, the diets were designed as follows: Diet I (negative control): low fibre, wheat and soybean meal based basal diet; diet II: basal diet with 9% chicory-inulin (6.3% pure inulin); diet III: and basal diet with 12.2% dried J. artichoke (6.3% pure inulin). The experiment was conducted using a total of 55 entire male pigs (Norwegian Landrace x Yorkshire) x (Norwegian Landrace x Duroc) from 11 litters. The average initial weight of the pigs was 25.3 kg, and their average final weight was 111.7 kg. A randomized block design was used in which the experimental animals were blocked by litter and by live weight. The experimental period lasted for an average of 91 days. The animals were fed a commercial diet (starting diet) for an average of 61 days, followed by the basal diet for 23 days, followed by a 7-day-period where the different dietary components were added in addition to the basal diet. The pigs were kept in temperature controlled rooms with pens designed for individual feeding and were fed twice daily, consistent with a restricted Norwegian feeding scale [28]. The pigs had free access to drinking water at all times.

Sample collection preparation and processing

Pigs were slaughtered at a commercial slaughterhouse within 2 h post-prandial. The entire digestive tract was taken out at the slaughter line, and samples from the *colon ascendens* were taken immediately. The samples were taken from 34 animals, kept on ice, mixed with RNAlater and frozen at -80°C until DNA extraction. Faecal samples from each animal were collected from the *rectum* one day before slaughter to determine the content of skatole, indole, and SCFA and their respective concentrations were previously reported by Vhile et al. [23].

The DNA extraction was performed using the QIAamp Fast DNA Stool kit (QIAGEN) according to the manufacturer's instructions. The excess RNAlater was removed by resuspending the faecal material in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by centrifugation for 5 min at 21,500 g in a fixed angle rotor. The extracted DNA was quantified, accessed for purity and used for 16S metabarcoding with primers specific to V1-V3 hypervariable region. The amplicons were generated using Illumina paired end 2x300 bp chemistry at GATC-Biotech, Germany. The raw sequencing data are available from NCBI under BioProject accession number: PRJNA474824 [29]. The metadata for the project is available in Additional file 4.

Analysis of bacterial communities

The analysis of microbial communities was performed using the Quantitative Insights into the Microbial Ecology, version 2 pipeline (QIIME2) [30]. The raw sequence reads were filtered by quality score to retain sequences with a median base quality score (base call) > Q20. The taxonomic analysis was performed using SILVA 128, 99%, bacterial 16S subset database [31]. The Naive Bayes classifier [32] was trained using the V1-V3 primer pair, 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGG), and the same primers as were used for generation of amplicons. For general differential abundance analysis, the operational taxonomic unit (OTU) table was created using the DADA2 pipeline [33], and the resulting table was filtered to retain features with frequencies above 5.6×10^1 per sample. Differential abundance analysis of the filtered table was done in QIIME2 using the Gneiss algorithm [34].

Functional predictions of microbial communities

Functional predictions of the present microbial communities were performed using the KEGG [35] database integrated into the PICRUSt 1.1.3 pipeline [36]. Predicted functions were collapsed into the KEGG level 3 pathways hierarchy using standard PICRUSt tools. The Shapiro–Wilk's test for

normality [37] and further analysis using two-tailed heteroscedastic t-test [38] were performed using the ggpubr 2.0 [39] package in R [40].

Detection of known and identification of new putative skatole producers

To identify the bacterial species contributing to the skatole production, the OTU table was treated without additional filtering. Differential abundance analysis was done in QIIME2 using the Gneiss algorithm [34]. The NCBI nucleic acid archive [41] was screened to find sequences associated with previously published, experimentally verified skatole producers. The 16S rRNA sequences were extracted and used for comparison with differentially abundant taxa from our study. Phylogenetic reconstruction was performed using the neighbour-joining algorithm with the bootstrap analysis options provided in the MEGA7.0 software package [42]. Bootstrap analysis [43] was performed using 10×10^3 replications. The relationship between relative abundance of differentially abundant taxa and measured skatole levels were studied in R [40] by calculation of the Pearson's correlation coefficient [44] and respective p-values [45] using the ggcorrplot 0.1.2 package [46]. The correlation coefficients with $p \leq 0.05$ were used to draw conclusions.

Results

Effects of inulin supplementation on diversity and composition of bacterial communities

Supplementation of inulin to the feed seven days before slaughter did not significantly affect the bacterial community evenness indexes (Pielou's Evenness) [47] or the community richness indexes (Faith's Phylogenetic Diversity) [48] at a level of confidence $P = 95\%$ (Figure 1).

Analysis of the weighted UniFrac distance metrics for the groups fed the basal diet and dried J. artichoke diet showed no significant differences in composition (PERMANOVA pseudo-F test: pseudo-F= 1.24, p= 0.27, q= 0.40). The differences in composition between the basal diet and the diet

supplied with dried chicory were statistically significant (Figure 2) indicating an effect of chicory on the microbial communities of the entire male pig hindgut (PERMANOVA pseudo-F test: pseudo-F= 2.53, p= 0.02, q= 0.05).

General taxonomic affiliations

Taxonomic analysis of the filtered samples (retaining features with frequencies above 5.6×10^1 per sample) have demonstrated that at the phylum level, pig hindgut microbial communities contained Bacteroidetes, Firmicutes, Cyanobacteria, Elusimicrobia and Spirochaetae. Bacteroidetes were the dominant phyla, representing 95.86% ± 2.88, 94.60% ± 3.39 and 96.77% ± 1.83 (median ± SD) of the bacterial populations in the hindgut of animals fed J. artichoke, chicory and basal diet, respectively. The second most dominant phyla, Firmicutes, represent 4.03% ± 2.57, 4.51% ± 3.30 and 3.23% ± 1.81 of the total biodiversity in the respective experimental groups. The three remaining phyla comprised less than 2% of the total biodiversity and were not present in all samples within feeding groups. Overall, we obtained 5 different phyla, 17 families, 40 genera and 53 species in all the filtered samples.

Functional Predictions of Microbial Communities

The functional predictions of microbial communities using the KEGG database integrated into the PICRUSt pipeline revealed an impact of the diets on 39 KEGG microbial metabolic pathways that potentially affected host performance during the feeding experiment. The respective OTUs were 1.4-2.1 times more abundant in the animals fed the diet containing dried chicory when compared to those that were fed the basal diet (Table 1). At the same time, we found no statistically significant differences (t-test: $p > 0.05$) in abundances of the same pathways in the group fed J. artichoke when compared with the basal diet. A complete list of the predicted functions can be found in the Additional file 1. In the current section, we report bacterial metabolic pathways that potentially affect animal growth performance. Within 39 outlined pathways, inclusion of dried chicory affected amino acid

metabolism, biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, glycan biosynthesis, lipid metabolism, metabolism of cofactors and vitamins, and metabolism of terpenoids and polyketides. It also affected bacterial membrane transport and production of signalling molecules. The most remarkable effect from inclusion of dried chicory was observed on microorganisms capable of producing natural tetracyclines. Abundance of those were, on average, 2.1 times higher (t-test: $p = 0.02$) in animals fed the diet with dried chicory compared to those fed the basal diet. The second and third highest fold changes in abundance were observed for microorganisms capable of steroid hormone biosynthesis (1.7-fold change; t-test $p=0.01$) and ansamycins biosynthesis (1.6-fold change; t-test $p= 0.03$). Inclusion of dried chicory into the diet also increased abundance of microorganisms capable of producing: vancomycin group antibiotics (1.4-fold change; t-test $p= 0.05$), butirosin and neomycin (1.4-fold change; t-test $p= 0.05$), novobiocin (1.4-fold change; t-test $p= 0.03$) and streptomycin type antibiotics (1.4-fold change; t-test $p= 0.04$). The remaining metabolic pathways were 1.5 (17 pathways) or 1.4 (15 pathways) times more abundant in the group fed the dried chicory (Table 1). Among those, butanoate biosynthesis pathways were 1.4 times more abundant (t-test $p= 0.01$) in the group fed chicory and biosynthesis pathways for other fatty acids were 1.4-1.5 times more abundant than in the group fed the basal diet.

Identification of experimentally verified skatole producers

The identification of experimentally verified skatole producers was performed using unfiltered datasets. The low abundance sequences with frequencies $< 5.6 \times 10^1$ per sample were found to belong to the Proteobacteria, Tenericutes, Fibrobacteres, Lentisphaerae, or Actinobacteria phyla, as well as several unknown phyla. Within all the unfiltered samples, we identified 11 different phyla, 31 families, 71 genera and 92 species. The Additional file 2 contains representative sequences from the current study. The Additional file 3 contains taxonomic descriptions for each representative sequence. The search of unfiltered samples for skatole producers belonging to *Olsenella* spp. demonstrated that members of the respective genus were present only in one sample. We identified 11 sequences in the

sample K37 taken from the hindgut of an animal fed a diet supplemented with dried chicory. In total, the sample contained 13363 unique sequences; thus, the *Olsenella* spp. represented only 0.08% of the microbial population in sample K37. *Lactobacillus* spp. were identified in 2 samples obtained from animals fed chicory, 2 samples from animals fed J. artichoke and 1 sample from an animal fed the basal diet. The sequences from the samples obtained from animals fed dried plant material belonged to uncultured species, while the sequences identified in the sample obtained from the animal fed the basal diet were identified as *Lactobacillus agilis*. In total, 35 out of 8911 unique sequences from sample K3 were identified as *L. agilis,* thus representing 0.39% of the sample biodiversity. The sequences identified as *Megasphaera* spp. were found in a single sample taken from the group fed the J. artichoke diet. Sample K50 contained 30 sequences identified as uncultured bacteria (0.47% of biodiversity in the sample, or 30 out of 6255 unique sequences). *C. drakei*, *C. scatologenes* and *Actinomyces* spp. were not detected in either of the samples.

Identification of new putative skatole producers

Identification of new putative skatole producers was performed using datasets obtained from the groups fed either the chicory or the basal diet. The dataset from the group fed J. artichoke was not used as we have observed no differences in α- and β-diversities between this group and the group that was fed the basal diet. Differential abundance analysis with Gneiss demonstrated that supplementation of dried plant material to the basal diet had very little effect on the bacterial species abundance in the hindgut of entire male pigs. Inclusion of inulin into the diet affected relative abundance of 2 groups of sequences belonging to Bacteroidetes and 1 group of sequences belonging to Firmicutes (α=95%). The sequences were found to be related to experimentally verified skatole producers described by Cook et al. (Figure 3). These sequences were less abundant in the samples obtained from pigs fed dried plant material compared to those obtained from animals fed the basal diet. Inclusion of dried plant material resulted in a 4.8-fold decrease in relative abundance of group 1

sequences, identified as members of Prevotellaceae UCG-003 (ID: 0f203eda5bd8c977b867ae477d39dcc6) for the samples taken from animals fed chicory (relative frequency = 1.1% ± 2.2; mean ± SD) when compared (p = 0.01) to the group fed the basal diet (relative frequency = 5.3% ± 4.5; mean ± SD). Another group of sequences belonging to Prevotellaceae UCG-003 was found only in the samples obtained from animals fed the basal diet (relative frequency = 0.7% ± 1.7; mean ± SD). The sequences identified as unknown members of the Lachnospiraceae family (ID: c43f93beed466360df24e7c46481e83a) were 8 times less abundant (p = 0.04) in samples obtained from animals fed chicory supplemented diets (relative frequency 0.1% ± 0.2; mean ± SD) compared to those fed the basal diet (0.8% ± 0.1; mean ± SD). The other differentially abundant sequences identified using Gneiss had no similarity with verified skatole producers, were present in single samples (ID: 4649b9e4407c1deb0d523f83299f38c2, 58d137fe6a2802d7765451ae147d6f27, 20be27460aada96ae334e8540e2827dc, 4649b9e4407c1deb0d523f83299f38c2) or had no statistically significant differences within the selected level of confidence, *P* = 95% (ID: ef5abcc30c758776c54d5d9b10999f1e and ID: 4e14d140c593833b74bccfb6ae720a1e).

A correlation analysis was performed between relative abundances of differentially abundant taxa and their measured skatole levels. We found five cases of positive correlation and one negative correlation within the group fed chicory and two cases of positive correlation within the group fed the basal diet (Figure 4). The Prevotellaceae NK3B31 group had nearly identical strong positive correlation coefficients with the skatole levels in both groups (chicory diet: $r = 0.74$, p = 0.01; basal diet: $r = 0.70$, p = 0.01). The relative abundance of the Prevotellaceae UCG-003 group was found to have a strong positive correlation ($r = 0.61$, p = 0.04) with skatole levels in the group fed the basal diet, while no association with skatole levels was found within the group fed chicory. The relative abundance of the Prevotellaceae UCG-003 group was 5.45 times higher in the group fed the basal diet compared to the group fed chicory.

Within the group fed chicory, Prevotellaceae UCG-003 showed a strong positive correlation with members of Lachnospiraceae (r = 0.61, p = 0.05) and Erysipelotrichaceae (r = 0.71, p = 0.02), as well as with unknown members of the order Bacteroidales (r = 0.77, p = 0.01). Within the group fed the basal diet, the Prevotellaceae UCG-003 had a strong negative correlation with the members of the Lachnospiraceae family (r = - 0.66, p = 0.02), and strong positive correlation with the Rikenellaceae RC9 group of gut microorganisms (r = 0.63, p = 0.03).

Discussion

A major objective of the current study was to evaluate the effect of fructan rich diets on the intestinal subpopulations of skatole producers in entire male pigs. Experimental animals were fed diets supplemented with dried J. artichoke or dried chicory one week before slaughter at a commercial facility. Both experimental diets were formulated to contain the same amount of inulin; thus, any observed differences were expected to be due to differences in the secondary metabolites contained within the dried plant material. We observed an effect of chicory on the intestinal microbial populations but did not observe any major impact on microbial α- or β-diversity for the diet supplemented with J. artichoke. Both experimental diets were normalised to contain 6.3% inulin, but differ in content of various plant secondary metabolites.

Chicory contains a number of bioactive compounds that reach their highest concentration in the roots of the plant. Studies of these bioactive compounds were mainly done using crude root extracts. Bioactive compounds extracted from chicory affected bacterial biofilm formation, adhesion of microbial cells, and inhibited growth of some microorganisms [49]. Chicory has a bitter taste that originates from sesquiterpene lactones (SLs), which play a key role in defending the plant against herbivore animals and serve as chemical signals to microorganisms [50].

J. artichoke has a sweet *flavour* and low soil quality requirements, and it has been successfully cultivated at an industrial scale in Nordic countries [51]. J. artichoke contains approximately 14%

inulin [26] and a number of bioactive compounds that can be categorised into four classes: terpenoids, flavonoids, coumarins and chromones [52]. Among those, at least 19 different compounds are defined as sesquiterpene lactones [53]. The biological activities of SLs are attributed to their a,b-unsaturated g-lactone moiety, while aldehyde, epoxy or peroxy groups modulate biological activity of the core [54].

Although detailed studies of the inhibitory activities of plant SLs on the gut microbiota are lacking, the studies of SLs on model microorganisms have shown various physiological effects. SLs from *Centratherum punctatum* have demonstrated growth inhibitory activities towards *Pseudomonas aeruginosa* and altered biofilm formation, elastase activity, and production of N-acyl-homoserine lactones (AHLs), which serve as bacterial intercellular communication signals. The inhibitory activities of the SLs from *C. punctatum* have a rather strong effect and reduced AHL production by 32-44% [55]. The SLs from *Vernonia blumeoides* were also capable of downregulating or even competitively inhibiting production of bacterial AHLs through the inhibition of the LuxS type of AHL synthases [56].

Several studies have reported the presence of the auto-induction systems (AI) AI-1 (LuxI type synthases) and AI-2 (LuxS type synthases) among the members of intestinal microbial communities, suggesting that these mechanisms are used for adaptation to changing dietary conditions [57, 58].

Taxonomic analyses performed in this study have demonstrated that at the phylum level, pig hindgut microbial communities were represented by Bacteroidetes and Firmicutes. These findings correlate well with data from other similar studies [59]. Both phyla have the highest number of experimentally verified skatole producers, belonging to the *Prevotella* and *Clostridium* species. Both use the AI-2 type quorum sensing (QS) system [57, 60, 61]. Changes in the activity of the AI-2 signalling system seem to affect production of short chain fatty acids (SCFAs) by Firmicutes and Bacteroidetes.

In this study, we observed that butanoate biosynthesis pathways were 1.4 times more abundant (t-test $p = 0.01$) and biosynthesis pathways for other fatty acids were 1.4-1.5 times more abundant in the

group fed chicory than in the group fed the basal diet. In total, the functional prediction revealed 39 metabolic pathways that potentially affected host performance during this feeding experiment. Among these, we identified pathways involved in amino acid metabolism, biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, glycan biosynthesis, lipid metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, bacterial membrane transport and production of signalling molecules.

The differential abundance analysis showed statistically significant differences in relative abundance of Prevotellaceae UCG-003 and Lachnospiraceae species. Pairwise comparison of partial 16S rRNA sequences of these taxa with 16S rRNA sequences of experimentally verified skatole producers showed similarity between the two. The relative abundance of Prevotellaceae UCG-003 was lowest in the group fed chicory, which correlated well with previously reported skatole levels and indicated that this group of microorganisms is responsible for variations in the skatole levels in the two experimental groups. The Prevotellaceae NK3B31 group demonstrated a positive correlation with the skatole levels in both compared groups. However, the relative abundance of the sequences identified as Prevotellaceae NK3B31 was nearly identical; thus, this group of microorganisms can be considered responsible for background skatole levels in our samples and seems to be not affected by bioactive compounds in the dried plant material.

Overall, skatole production has been described for *Clostridium* [13], *Lactobacillus* [14], *Pseudomonas* [15], *Prevotella, Actinomyces*, *Megasphaera* [16], *Bacteroides* [17] and *Olsenella* [18]. In our study, we found various species of Bacteroidetes and Clostridium, as well as *Megasphaera* and *Lactobacillus* spp., which were present in single samples. We did not, however, observe *C. drakei*, *C. scatologenes* and *Actinomyces* spp. in any of the samples; thus, we consider the members of Prevotellaceae UCG-003 and Prevotellaceae NK3B31 as the characteristic bacteria responsible for skatole production in our study.

Conclusions

In this study, we demonstrated that inclusion of dried Jerusalem artichoke had no significant effect on the microbial α- and β-diversity in the hindgut of entire male pigs compared to a basal diet. Inclusion of dried chicory significantly affected α- and β-diversity of the intestinal microorganisms. Supplementation of chicory increased the abundance of microorganism carrying predicted short chain fatty acid biosynthesis pathways and pathways for biosynthesis of cofactors, vitamins and other metabolites potentially influencing host performance and gastrointestinal health. Our analysis demonstrated that production of skatole in pig hindgut is associated with members of the Prevotellaceae family. Other described skatole producers were not detected or were only identified in single samples and thus were not able to contribute to production of skatole in the present study. Thus further efforts should be made to selectively control the Prevotellaceae spp. in the intestinal microbiomes of production animals.

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Figure captions

Figure 1. Alpha diversity of the entire male pig hindgut microbiota.

(A) Alpha-diversity is represented via Pielou's Evenness. (B) Faith's Phylogenetic Diversity plotted for experimental animals fed a J. artichoke supplemented diet, chicory supplemented diet, or commercial basal diet. The median values are represented by the lines inside the boxes; the whiskers represent the extrema, and outliers are represented as dots. Statistical testing showed no difference in the Pielou's Evenness (J. artichoke supplemented – Basal diet: H = 0, p = 1.0; Chicory – Basal diet: H= 1.09, p = 0.29). No differences were observed in the Faith's Phylogenetic Diversity indexes (J. artichoke supplemented – Basal diet: H =1.09, p = 0.3; Chicory – Basal diet: H= 1.09, p = 0.3) at a level of confidence *P* = 95%.

Figure 2. Beta diversity of the entire male pig hindgut microbiota.

Beta-diversity is represented via weighted UniFrac distances plotted for experimental animals fed a J. artichoke supplemented diet, a chicory supplemented diet or a commercial basal diet. The median values are represented by the lines inside the boxes, the whiskers represent the extrema and outliers are represented as dots. Statistical testing showed no difference in weighted UniFrac distances for the groups fed the J. artichoke supplemented diet and the basal diet (pseudo-F = 1.24, p = 0.26, q = 0.4, permutations = 999). The differences in β – diversity for groups fed the chicory supplemented diet and the basal diet were found to be statistically significant (pseudo-F = 2.53, p = 0.016, q = 0.048, permutations = 999).

Figure 3. **Evolutionary relationships of partial 16S rRNA sequences obtained in the current study with 16S rRNA sequences of experimentally verified skatole producers.** The red colour indicates sequences significantly reduced in the group fed the chicory supplemented diet. The partial 16S rRNA sequences of experimentally verified skatole produces were taken from the study by Cook et al. The respective NCBI accession numbers for the reference sequences are listed in parentheses. The evolutionary relationships were inferred using the Neighbour-Joining method. The numbers

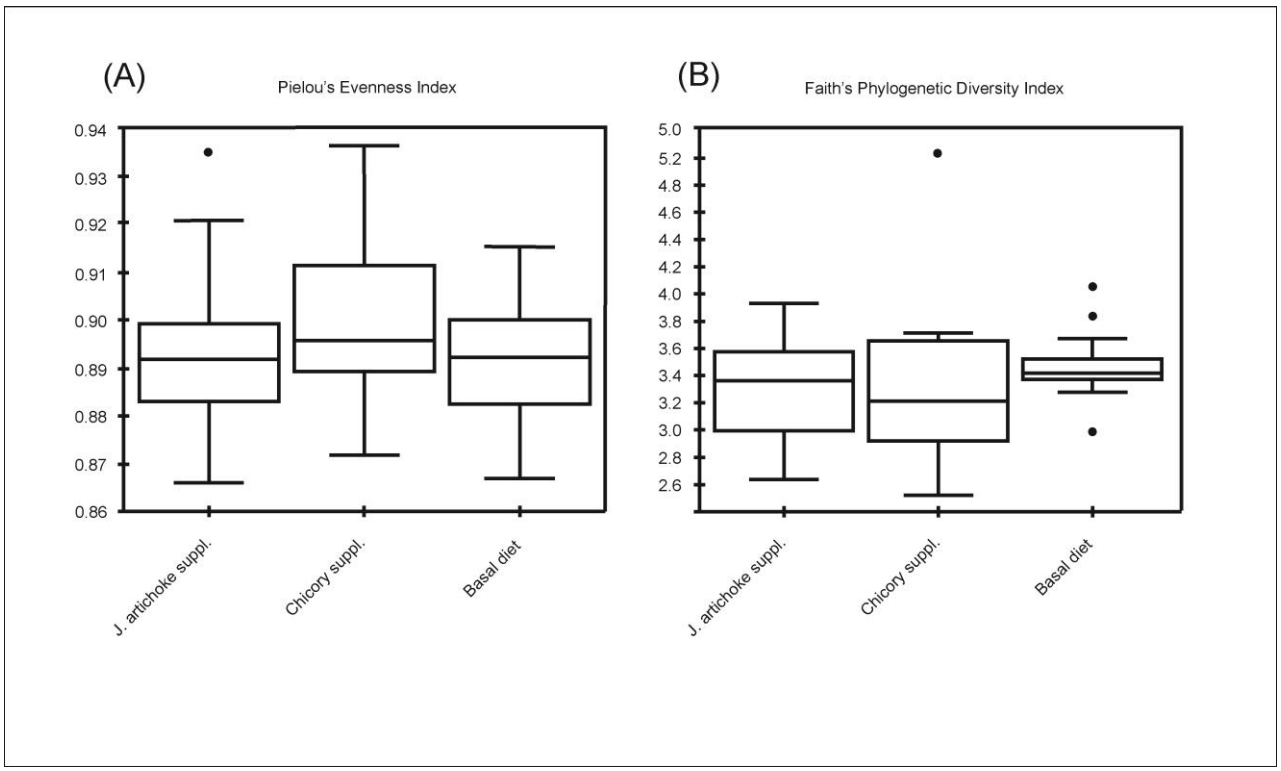
adjacent to the nodes of the tree represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10×10^3 replicates).

Figure 4.

Correlation heatmaps of bacterial genera and skatole levels in the colon. (A) Relative abundances of bacterial genera in the colon of the experimental animals fed a diet supplemented with chicory. (B) Relative abundances of bacterial genera in colon of the experimental animals fed the basal diet. The black-grey gradient represents the Pearson's correlation coefficients for significant correlations ($p \leq 0.05$).

Table 1. Functional Predictions of Microbial Communities. AM_{OTU} – represent arithmetic mean for OTU abundances for each predicted KEGG pathway; p-values are obtained from a two-tailed heteroscedastic t-test.

Figure_1 Click here to access/download;Figure;figure_1.docx



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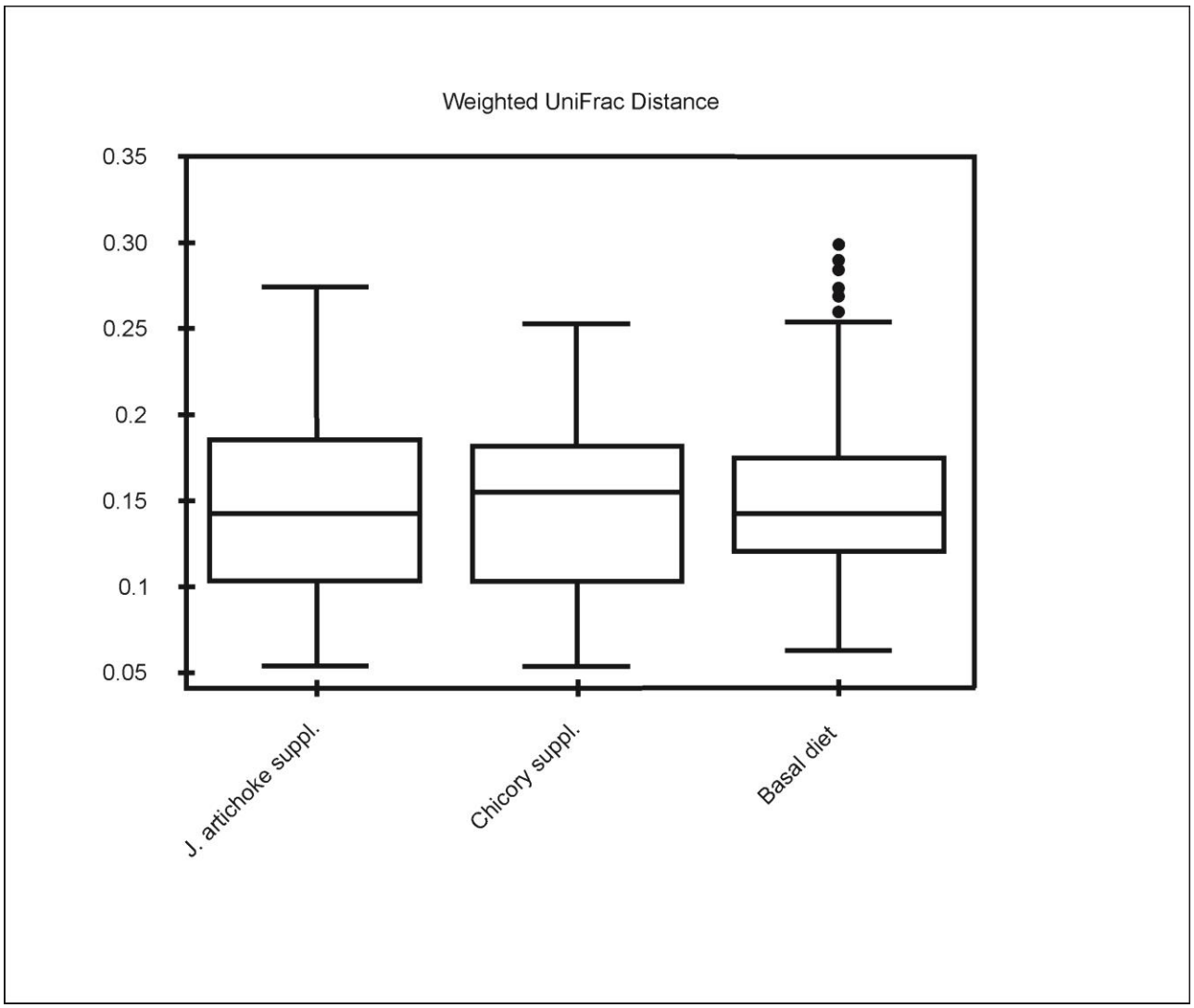

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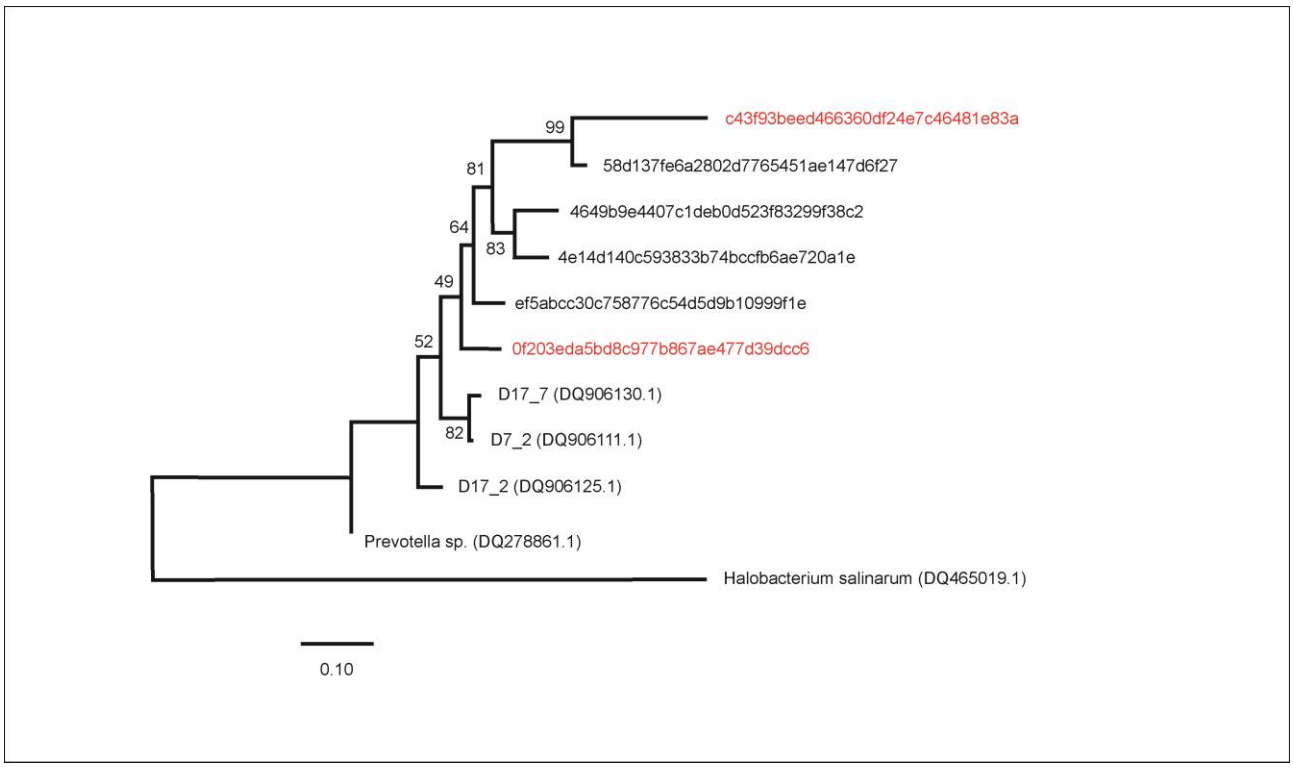

Figure_4

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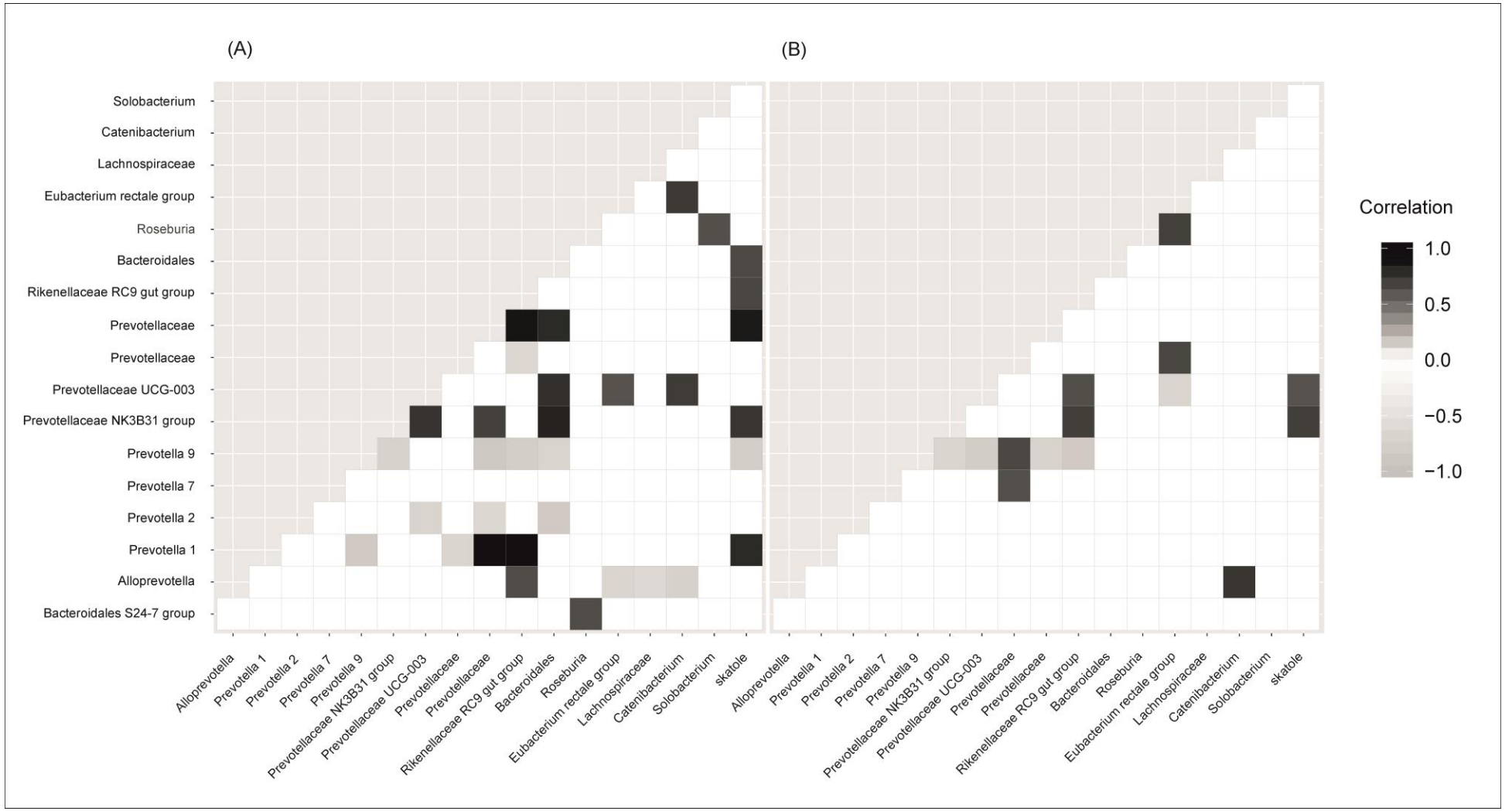

Biological Function	AM_{OTU}		Fold change	p-value	AM_{OTU}		Fold change	p-value	KEGG_Pathways
	J. art. suppl.	Basal diet			Chic. Suppl.	Basal diet			
Bacterial secretion system	17240	14569	1.2	0.34	20631	14569	1.4	0.03	Bacterial secretion system
Bacterial toxins	2194	1731	1.3	0.13	2615	1731	1.5	0.02	Bacterial toxins
Biosynthesis of ansamycins	2304	1782	1.3	0.16	2776	1782	1.6	0.03	Biosynthesis of ansamycins
Biosynthesis of unsaturated fatty acids	3529	2870	1.2	0.25	4281	2870	1.5	0.02	Biosynthesis of unsaturated fatty acids
Biosynthesis of vancomycin group antibiotics	2830	2348	1.2	0.31	3288	2348	1.4	0.05	Biosynthesis of vancomycin group antibiotics
Biotin metabolism	3949	3268	1.2	0.28	4685	3268	1.4	0.03	Biotin metabolism
Butanoate metabolism	10799	9283	1.2	0.35	12882	9283	1.4	0.04	Butanoate metabolism
Butirosin and neomycin biosynthesis	2210	1882	1.2	0.32	2639	1882	1.4	0.05	Butirosin and neomycin biosynthesis
Fatty acid biosynthesis	12512	10063	1.2	0.24	15362	10063	1.5	0.01	Fatty acid biosynthesis
Fatty acid metabolism	4328	3563	1.2	0.28	5268	3563	1.5	0.01	Fatty acid metabolism
Folate biosynthesis	17379	14581	1.2	0.34	20711	14581	1.4	0.03	Folate biosynthesis
Glycan biosynthesis	1636	1374	1.2	0.35	1969	1374	1.4	0.02	Glycan biosynthesis and metabolism
Glycerolipid metabolism	5776	4624	1.2	0.2	6867	4624	1.5	0.05	Glycerolipid metabolism
Glycerophospholipid metabolism	12899	10087	1.3	0.18	15454	10087	1.5	0.02	Glycerophospholipid metabolism
Isoquinoline alkaloid biosynthesis	3291	2755	1.2	0.33	3943	2755	1.4	0.03	Isoquinoline alkaloid biosynthesis
Lipid biosynthesis proteins	18748	15229	1.2	0.26	22692	15229	1.5	0.02	Lipid biosynthesis proteins
Lipid metabolism	4331	3431	1.3	0.22	5254	3431	1.5	0.02	Lipid metabolism
Lipopolysaccharide biosynthesis	19579	16375	1.2	0.34	23250	16375	1.4	0.03	Lipopolysaccharide biosynthesis
Lipopolysaccharide biosynthesis proteins	21823	18115	1.2	0.32	26008	18115	1.4	0.02	Lipopolysaccharide biosynthesis proteins
Lysine biosynthesis	21312	17663	1.2	0.27	25454	17663	1.4	0.03	Lysine biosynthesis
Metabolism of cofactors and vitamins	3386	2736	1.2	0.26	4101	2736	1.5	0.02	Metabolism of cofactors and vitamins
Methane metabolism	28865	24086	1.2	0.3	34530	24086	1.4	0.04	Methane metabolism
N-Glycan biosynthesis	1626	1370	1.2	0.36	1928	1370	1.4	0.03	N-Glycan biosynthesis

Nicotinate and nicotinamide metabolism	17977	14971	1.2	0.3	21681	14971	1.4	0.02	Nicotinate and nicotinamide metabolism
Nitrogen metabolism	22231	18265	1.2	0.27	26607	18265	1.5	0.03	Nitrogen metabolism
Novobiocin biosynthesis	4366	3682	1.2	0.33	5314	3682	1.4	0.03	Novobiocin biosynthesis
Pantothenate and CoA biosynthesis	18404	15151	1.2	0.28	22040	15151	1.5	0.03	Pantothenate and CoA biosynthesis
Peptidoglycan biosynthesis	33645	27720	1.2	0.28	40563	27720	1.5	0.02	Peptidoglycan biosynthesis
Polyketide sugar unit biosynthesis	8204	6808	1.2	0.3	9641	6808	1.4	0.04	Polyketide sugar unit biosynthesis
Starch and sucrose metabolism	29631	24071	1.2	0.24	35416	24071	1.5	0.04	Starch and sucrose metabolism
Steroid hormone biosynthesis	1111	787	1.4	0.13	1310	787	1.7	0.01	Steroid hormone biosynthesis
Streptomycin biosynthesis	12065	10083	1.2	0.31	14240	10083	1.4	0.04	Streptomycin biosynthesis
Terpenoid backbone biosynthesis	23230	19213	1.2	0.29	27901	19213	1.5	0.02	Terpenoid backbone biosynthesis
Tetracycline biosynthesis	528	371	1.4	0.22	795	371	2.1	0.03	Tetracycline biosynthesis
Zeatin biosynthesis	3347	2765	1.2	0.3	4028	2765	1.5	0.02	Zeatin biosynthesis

Supplementary_Material_3

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Supplementary Material

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