The effect of pyridine-alkaloid secondary metabolites on sunbirds gut microbiome

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Research

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Abstract

Background: Sunbirds (*Cinnyris osea*) feed on nectar of the tobacco tree (*Nicotiana glauca*), which contains the secondary metabolites nicotine and anabasine. These toxic pyridine-alkaloids presumably serve to deter nectar thieves and allow only specific pollinators to feed on nectar. The floral nectar contains low level of proteins and hence nectarivores may have problem balancing their nitrogen requirements. Our aim was to understand whether the gut microbiome of nectarivores contains bacteria that are able to degrade plant secondary metabolites and how the presence of secondary metabolites in nectar shapes the gut microbiome of nectarivores.

Results: Sunbirds (n=16) that were held in captivity were randomly divided into control and treatment groups. The control group was fed with artificial nectar while the treatment group was fed with artificial nectar with the addition of nicotine and anabasine. Excreta were collected from each of the 16 birds on day 0 and at the end of weeks 2, 4 and 7. Nonmetric multidimensional scaling analysis (nMDS) demonstrated that the secondary metabolites significantly changed the gut microbiota composition and diversity of those sunbirds that were fed nectar with added pyridine alkaloids. *Aeromonas, Enterobacteriaceae*, *Exiguobacterium, Klebsiella*, *Lactococcus*, *Leuconostoc*, *Rothia* and *Salmonella* were found to contribute to the differences in gut microbiota between the control and treated birds (birds feed with Nicotine and Anabasine). Four weeks after starting to feed the birds with added nicotine and anabasine, we found that genera with the ability to degrade these pyridine-type metabolites proliferated from a total relative abundance of only 0.3% to 52.7% of the gut bacterial community.

Conclusion: Sunbirds feed on *Nicotiana* nectar that contains toxic pyridine-alkaloids. Nonetheless, the symbiotic gut microbiota which is changing dynamically towards enabling the degradation of these secondary metabolites, supply the birds with nitrogen that enables the synthesis of essential amino acids. The current study contributes to our understanding of the complex relationships between plants, birds and their microbiomes.

Background

Microorganisms are omnipresent in the universe in different ecological niches and perform various functions. One important environment is the gut of animals, which harbours a complex microbial community that is important for physiology, immune system development, digestion and detoxification reactions in their hosts [1–3]. The majority of the studies on microbiomes have focused on mammalian hosts — largely ruminants and humans [4]. Studies on gut-microbiome diversity and functions in avian hosts have been largely overlooked [3]. Of those limited number of studies on avian gut microbiomes, most were carried out on domestic birds like chickens and turkeys [3], rather than on wild birds. Furthermore, only a few studies have examined the gut bacteria of passerine birds [5–8]. As far as we know, only a single study has been carried out on the gut bacteria of nectarivorous birds (hummingbird, Calypte anna), using culture-dependent techniques to investigate nitrogen-recycling bacteria [9].

The importance of specific bacteria for digestive recycling in avian species with large ceca and well-developed gastrointestinal microbiotas has been well documented [10]. However, most nectarivorous and frugivorous birds have only vestigial ceca. For example, hummingbirds, arguably the most specialized avian nectarivores, have no ceca and perform extremely fast digestion throughout the entire digestive tract, which may make the colonization by bacteria challenging [3,11,12]. Therefore, it has been assumed that the gastrointestinal tracts of birds that feed on nectar do not have the structures needed to house intensive microbiota, as is presumably required for effective digestive recycling [3]. However, our previous study [13]suggested that, despite lacking large ceca, the frugivorous yellow-vented bulbul (Pycnonotus xanthopygos) possesses a rich gut microflora, including bacterial species that are capable of degrading uric acid.

The widespread existence of 'toxic nectar,' or nectar with secondary metabolites, is puzzling given that the function of nectar is to attract mutualists, such as legitimate pollinators [14]. Nevertheless, plant secondary metabolites may play an adaptive role as mediators of mutual plant-animal interactions, such as pollination and seed dispersal, and thus may increase plant fitness [15–17]. The 'direct toxicity hypothesis', suggests that the plant secondary metabolites control or filter out nectar robbers and allow only the appropriate pollinators to feed on nectar [18,19].

Nectarivores may have a problem balancing their nitrogen. Nitrogen can be a limiting resource for nectarivores, which require it for protein, nucleotides and the synthesis of other nitrogenous compounds [20]. Floral nectar, particularly of hummingbird-pollinated plants, contains low levels of proteins [21]. Tsahar et al. [13] showed that nectar-feeding birds have low nitrogen requirements, but the mechanisms that these animals use to conserve nitrogen remain unclear. Studies on the gut bacteria of insects demonstrated that these bacteria compensate for dietary deficiencies in essential amino acids by using various mechanisms, such as the recycling of nitrogenous waste, to nutritionally upgrade the diet of their host [22]. A similar mechanism was reported in hummingbirds by Preest et al. [9], who found the decomposition of nitrogenous compounds by intestinal bacteria. A metagenome analysis of cecal samples from sage-grouse (Centrocercus urophasianus) by Khol et al. [23], discovered rich genes associated with the metabolism of plant secondary metabolites. They suggested that the gut microbiota of specialist avian herbivores play a similar role to that of the gut microbiota of mammals in degrading plant secondary metabolites.

We hypothesized that plant secondary metabolites are mediators of the interactions between plants and nectar consumers through the gut microbiome of the latter. Thus, our aim was to understand (i) whether the gut microbiome of nectarivores contains bacteria that are able to degrade plant secondary metabolites; (ii) how the presence of secondary metabolites in nectar shapes the gut microbiome of nectarivores; and (iii) how the time elapsed from initial exposure to secondary metabolites in the birds' diet affects the dynamics of the composition of the gut microbiome. To accomplish these goals, we based our study on the natural relationships occurring between the tobacco tree (Nicotiana glauca), whose nectar contains pyridine alkaloids (mainly anabasine and nicotine), and its avian nectar consumers in the Old World (orange-tufted sunbirds, Cinnyris osea). In an experimental setup with captive

birds fed with artificial nectar and with or without the addition pyridine alkaloids, we were able to demonstrate that sunbirds' gut microbiota include bacterial species that are able to degrade these secondary metabolites. The results showed that the composition of the bacterial microbiome changes significantly when the birds were fed nectar with added nicotine and anabasine compared to the control birds; moreover, significant changes were observed over time.

Results

Sunbirds feeding experiment

Sixteen birds were adapted to the laboratory conditions were randomly divided into two groups. The control group was fed the artificial nectar without any additional nutrients while the treatment group was fed the same artificial nectar with the addition of nicotine and anabasine (0.5 ppm and 5 ppm, respectively) (Fig. 1). Excreta were collected from each of the 16 birds on day 0 and at the end of weeks 2, 4 and 7 (Fig. 1). The excreta samples were used for culture-dependent and culture-independent bacterial analyses (more details can be found in the Methods).

Culture-independent approach

A total of 3 638 528 quality reads were generated from 66 cloacal samples from sunbirds, with an average count of 55 129 reads per sample (after size filtering, quality control, and chimera removal). Overall, 812 operational taxonomic units (OTUs) were identified based on 97% nucleotide sequence identity between reads. After removing rare OTUs, 331 were left. The rarefaction curves of each sample reached an asymptotic level (Fig. S1), suggesting that our sampling efforts were sufficient to obtain a full estimate of OTU richness.

The effect of treatment and time on gut-microbiome composition and diversity

nMDS analysis demonstrated dynamic changes between the gut microbiota composition of sunbirds from each feeding group and over time (Fig. 2). Significant differences (ANOSIM) were found between the composition of the sunbirds' gut bacterial communities in the control and treatment groups. The maximal difference was observed after four weeks (R = 0.353, P = 0.013), with the average of 49.9% Bray-Curtis dissimilarity between the control and treatment groups. Fewer differences were observed after two weeks (ANOSIM R = 0.29, P = 0.006) and after seven weeks (R = 0.329, P = 0.002), with the averages of 32.9% and 31.8% Bray-Curtis dissimilarities, respectively (Fig. 2; Tables 1, S1).

Significant differences in the composition of the sunbirds' gut microbiota were also observed over time, both in the control and treatment groups (Table 1). Moreover, the changes over time were more pronounced in the control group than in the treatment group. The differences in the bacterial community composition groups between the samples at day 0 and after two weeks in the control group were larger than in the treatment group (ANOSIM control groups - R = 0.517, P = 0.001; treatment groups - R = 0.488, P = 0.001). The same pattern was observed between the samples after two and four weeks (control, R =

0.755, P = 0.001, and R = 0.393, P = 0.001, respectively) and between four and seven weeks (R = 0.691, P = 0.001 and R = 0.202, P = 0.009, respectively) (Table 1).

The Shannon H diversity index of the control group decreased over time, whereas in the treatment group, it increased until the fourth week and then decreased drastically (Fig. 3). After the beginning of the feeding experiment, in week 2, the Shannon H index was higher in the treatment group than in the control group for each sampling time (Fig. 3). The highest difference between the two groups in the Shannon index was observed after four weeks and the lowest difference occurred after seven weeks (Fig. 3). A mixed model ANOVA showed that time had a significant effect on Shannon diversity (all birds pooled, $F_{3,42}$ = 3.88, P = 0.016). The interaction of time X treatment was also significant ($F_{3,42}$ = 5.66, P = 0.002), indicating that the two groups (control and treatment) demonstrated different diversity patterns over time (Fig. 3).

Bacterial composition in the sunbirds' gut

Proteobacteria, *Firmicutes* and *Actinobacteria* were the three dominant phyla detected in the experiments conducted at different time intervals (Fig. 4a). In the naïve birds (at day 0), the *Proteobacteria* were the relatively dominant phyla (49% in 0WC and 56% in 0WT), but they decreased thereafter with time. After two weeks *Firmicutes* became dominant and were more abundant in the control (63% in 2WC) than in the treatment group (53% in 2WT). At the end of the fourth week, *Proteobacteria* (28%) and *Firmicutes* (48%) abundances were greater in the treatment group (4WT) compared to the control group (4WC) 14% and 33%, respectively. By contrast, *Actinobacteria,* which were less abundant in the previous time intervals, showed the highest prevalence at week four and were more abundant in the control than in the treatment group (52% in 4WC and 21% in 4WT). After seven weeks, *Firmicutes* was the most dominant phylum in both the control (61% in 7WC) and the treatment groups (65% in 7WT), followed by *Proteobacteria*, with a relative abundance of 32% in 7WC and 24% in 7WT (Fig. 4a).

A total of eight genera displayed mean relative abundances of over 1% across all samples, and the genera with the highest relative abundances are represented in Fig. 4b. *Acinetobacter* was one of the dominant genera in the initial naïve birds, with a relative abundance of 25% in 0WC and 18% in 0WT; however, its abundance decreased over time, until it reached less than 1% in both groups. After two weeks, the dominant genera in both the treatment and control groups were *Lactococcus* (36% in 2WC and 35% in 2WT), unclassified *Enterobacteriaceae* (28% in 2WC and 32% in 2WT), and *Leuconostoc* (18% in 2WC and 12% in 2WT). *Rothia* became the most abundant genus in the fourth week and was more abundant in the control (51% in 4WC) than in the treatment group (21% in 4WT). After seven weeks, *Lactococcus* (35% in 7WC and 49% in 7WT) and unclassified *Enterobacteriaceae* (29% in 7WC and 19% in 7WT) became the most abundant genera (Fig. 4b).

Ten OTUs were found to contribute to most of the significant differences between the bacterial community compositions in the control and treatment groups at various time intervals (Fig. 5). During the three different time intervals of sampling, the main (common) discriminating bacterial genera between the control and treatment groups were *Lactococcus* (OTU 1), unclassified *Enterobacteriaceae* (OTU 2),

Rothia (OTU 3), *Leuconostoc* (OTU 4), *Klebsiella* (OTU 7) and *Salmonella* (OTU 9) (Fig. 5). Differences in *Lactococcus* relative abundances contributed most to the dissimilarities between the microbial community structures of the control and treatment groups (8.46% of the total dissimilarity, on average), followed by *Rothia* (7.81% of the dissimilarity, on average) (Table S1). *Leuconostoc* had an average contribution of 6.69% of the dissimilarities and unclassified *Enterobacteriaceae* contributed another 5.96%. Other contributing genera were *Klebsiella* (1.10%) and *Salmonella* (1.75%) (Fig. 5).

Culture-dependent approach

A total of 146 nicotine/anabasinedegrading strains were isolated from the sunbird excreta. At day 0, 28 were isolated from all 16 birds. After four weeks, 118 isolates were isolated (Table 2), 51 from the control birds and 67 from the treated birds. The isolates belonged to 6 bacterial classes and 14 genera. The majority of the isolates at day 0 belonged to *Gammaproteobacteria* class and to four species in the genus *Enterobacter*. After four weeks, five bacterial classes were identified from the control group and six classes from the treatment group. The genera *Methylorubrum* and *Roseomonas* were isolated only from the nicotine/anabasine–treated birds. Interestingly, all isolates were able to degrade both nicotine and anabasine, although they were enriched and isolated on only one kind of medium (M9 with nicotine or M9 with anabasine) (Table 2).

The proliferation of specific genera during treatment

All statistical parameters indicated that the most significant differences were observed in the bacterial communities in the excreta of the treatment group compared to those of the control group. (Figs. 2, 3, 5; Tables 1, 2, S1). Thus, we followed the changes in the Illumina results for the OTUs of the gut bacterial community between day 0 (0WT) and the fourth week (4WT). All the OTUs with increased relative abundances between 0WT and 4WT were recorded. We found that the abundances of 45 genera increased from time 0 to the fourth week (Illumina results at the OTU level, data not shown). Of these 45 genera, 19 were either reported as nicotine-degrading bacteria in the literature or were isolated by us as nicotine- and anabasine-degrading species in the culturable part of the current study (Tables 2, 3). Interestingly, these 19 genera comprised only a very minor part of the bacterial communities in the excreta at the beginning of the experiment (0WT, 0.30% abundance out of the OTUs) and proliferated up to 52.72% after four weeks of treatment (4WT) with the addition of the pyridine-alkaloid food additives (Table 3). The two most dominant genera at week four, whose representative species were also isolated in the current study, were *Lactococcus* and *Enterobacter*, which were below detection in the Illumina sequencing results at day 0 and reached the high abundances of 29.54% and 18.38%, respectively, in the fourth sampling week.

Discussion

The effect of diet on gut microbiome

The study of birds' gut microbiomes is increasingly important for ecological and evolutionary perspectives because of its relevance to host fitness, longevity, disease resistance and adaptation [36]. Here we explored the effect of secondary metabolites on the gut microbiota of sunbirds, whereupon we fed the control birds with artificial nectar and added nicotine and anabasine to the nectar of the treated birds. nMDS analyses of the Illumina 16S rRNA gene sequencing demonstrated significant differences between the bacterial communities of control and treated sunbirds' guts (Fig. 2; Table 1). These differences reveal that diet influenced the composition of the gut microbiome.

A similar observation has been reported by Baurhoo et al. [37] regarding the impact of different types of diet on the composition of the gut microbial community of broiler chickens. They fed the chickens with two types of mannan-oligosaccharides and two types of antibiotics. The mannan-oligosaccharides diet significantly affected the composition of the gut microbiota of broilers compared to the control and antibiotic diets [37]. Borda Molina et al. [38] studied the effects of diets supplemented with the minerals phosphorous, calcium and phytase on the gut microbiome of broiler chickens and found that the phosphorous-supplemented diet significantly influenced the microbial community compared to the calcium-supplemented diet.

Diet affects not only the microbiome composition but also its diversity. Lower Shannon diversity was observed in chickens that were fed the calcium diet, compared to chickens that were fed the control diet [38]. Our results showed that the gut-microbiome diversity of sunbirds that were fed with added nicotine and anabasine was higher than the control group (Fig. 3). Pyridine alkaloids may stimulate both positive and negative responses on various bacteria. Hence, the higher diversity of the gut microbiome of the treatment group may reflect a transitional state of the gut microbiome, in which the suppressed bacteria are still present alongside the appearance of new bacterial species.

The effect of time on the gut microbiome

Our study demonstrated that not only diet but also time significantly affected the gut-microbiome composition and diversity (Figs. 2, 3; Table 1). A large-scale study based on 16S rRNA gene-sequencing, which was conducted on faecal samples of newborn piglets at various time intervals (days 1, 7, 14, 21, 28, 35, 120 and 180), showed relatively apparent temporal and spatial changes in the gut microbiome [39]. In another study, caecal and faecal microbiomes of broiler chickens, which were sampled at various time points between one and six weeks, showed significant changes over time. Significant differences were observed both in richness and diversity in the six-week-old birds compared to three- and one-week-old birds [40].

A similar observation was reported by Ji et al. [41]. Four healthy human individuals and six individual housed mice were fed two different diets (low-fat, high-plant polysaccharide, LFPP; and high-fat, high-sugar, HFHS). The diets, especially the LFPP diet, significantly affected the short-term dynamics of the gut microbiota, whereas in the long term, the dynamics of the microbiota decreased and was stabilized [41]. We observed similar dynamics when the diversity of the gut microbiota of birds that were fed with nicotine and anabasine increased between the second and the fourth week and then decreased after

seven weeks (Fig. 3). Furthermore, the pyridine-alkaloid diet showed a marked effect on the composition of the gut microbiome after four weeks, but after seven weeks, the composition of the gut microbiome was more closely resembled to that at the second week (Fig. 2).

Proteobacteria, *Firmicutes* and *Actinobacteria* were relatively abundant phyla in the sunbirds' gut microbiota in both the control and the treatment groups at different time intervals (Fig. 5a). This pattern bears high resemblance to the microbiota composition of other neotropical birds [42]and also to that of Darwin's finch [43]. *Proteobacteria* and *Firmicutes* were the dominant phyla in the sunbirds treated with nicotine and anabasine after four weeks. Similar results were observed in gull species that were treated with glucocorticoids [44].

The effect of secondary metabolites on the gut microbiome

In a recent study, Aizenberg et al. [45] studied the influence of nicotine on the bacterial community composition of nectar of the wild-type *N. glauca* and the wild-type and transgenic *N. attenuata*. They [45] found that the elimination of nicotine from the transgenic *N. attenuata* (in which the nicotine biosynthesis was silenced), significantly affected the bacterial community of the nectar compared to the wild-type plants. Although the bacterial community composition in *N. glauca* nectar was different from that of *N. attenuata*, both in its wild-type and transgenic forms, the two wild-type plants that contained nicotine in their nectar were more similar compared to the transgenic *N. attenuata* plant that was manipulated to not produce nicotine. Thus, nicotine manipulation in *Nicotiana* plants affects the microbiome of the floral nectar [45].

Nicotine can be toxic even in a relatively low concentration and, at the same time, this metabolite can be the source of nutrients if enzymes are present that can degrade this molecule. Here we demonstrated that pyridine alkaloids that are added to artificial nectar in concentrations that naturally occur in the floral nectar from which sunbirds feed affect the sunbirds' microbiome composition and diversity and shape the gut microbiome of these nectarivorous birds.

In nature, the birds consume plant products and invertebrates that contain secondary metabolites that are toxic when absorbed [46]. According to Dearing et al. [47] birds may harbour bacteria that can detoxify these toxic metabolites. The plant saponins was found to be degraded by bacteria in the crop of *Opisthocomus hoazin* [48], and several mycotoxins were found to be metabolized by microbial communities from the intestines of chickens [49]. A caecal metagenome analysis of the *Centrocercus urophasianus* revealed that its microbiota was enriched with genes related to the metabolic pathways that can degrade phenols to pyruvates as well as genes responsible for xenobiotic degradation and the metabolism of terpenoids [23]. Kohl et al. [23]also found genes that were responsible for the biosynthesis of essential amino acids. They suggested that these bacterial genes can assist the host in maintaining nitrogen balance. Similarly, we suggest that the gut microbiome of sunbirds degrades pyridine alkaloids and thus promotes nitrogen balance in sunbirds.

There are various reports of nicotine degradation by different bacterial genera; for example; *Delftia, Klebsiella, Stenotrophomonas Pseudomonas* and *Lactobacillus* [28–30,32,35]. Here we have also isolated various bacterial genera (Table 2) that showed the ability to grow on nicotine and anabasine as sole carbon and nitrogen sources and, thus, to degrade these compounds. The following bacterial genera that were isolated and identified by us in the current study have, as far as we know, never been reported as nicotine and or anabasine-degrading bacteria: *Chryseobacterium*, *Enterobacter*, *Exiguobacterium, Lactococcus, Methylobacterium* and *Kocuria* (Table 2).

Similarly using caffeine as a sole source of carbon and nitrogen, Ceja-Navarro et al. [50] isolated bacteria from the gut of the insect *Hypothenemus hampei* that had the ability to degrade the toxic alkaloid caffeine. The majority of the caffeine-degrading isolates belong to the genus *Pseudomonas*. In our study, *Pseudomonas* isolates were also the prominent genus among nicotine-and anabasine degrading isolates (Table 2). Ceja-Navarro et al [50] showed that the gut bacterial community of *H. hampei* metabolized and detoxified caffeine and thus promoted the reproduction and fitness of the host. In our study, we found a significant change in the bacterial community composition, in which a number of genera with the ability to degrade the toxic metabolites nicotine and anabasine (Table 3) proliferated from 0.3% of the total bacterial community to 52.7% after four weeks of feeding sunbirds with these secondary metabolites, in the same concentrations as they occur in their natural food, *N. glauca* nectar. These results clearly indicate that these bacteria play an important role in the detoxification and degradation of nicotine and anabasine.

Conclusion

The current study contributes to our understanding of the complex relationships between plants, birds and their microbiomes. Sunbirds feed on *Nicotiana* nectar that contains toxic pyridine-alkaloid secondary metabolites, which presumably serve to deter nectar thieves. Nonetheless, when sunbirds feed on this nectar, their symbiotic gut microbiota detoxify these toxic secondary metabolites and provide sunbirds with valuable nutrients like nitrogen. The nectar is very poor in protein content and the bacterial community, which is changing dynamically towards enabling the degradation of the secondary metabolites supplies the birds with nitrogen and enables the synthesis of amino acids, which are essential for protein synthesis. For further verification of this theory, a metagenomic study should be performed on a similar set of experiments like the current one.

Materials And Methods

Studied Organisms

The *orange-tufted* sunbird (*Cinnyris osea*) is a small passerine bird weighing 6–7 g. It has a long, slender, decurved bill (1.4–2.0 cm in length) with a long tongue, which allows it to feed mainly on floral nectar and arthropods [51]. Like other nectarivorous birds, the sunbirds feed on high carbohydrate foods with low-

protein content and have high sugar-absorption efficiencies despite the rapid speed that food moves through their gut [52].

Nicotiana glauca is native to Argentina and Bolivia and is also found in other parts of South America, Hawaii, Australia and in the east Mediterranean region, including Israel. *N. glauca* is a small tree with long, tubular yellow flowers that bloom almost throughout the year in warm climates. The nectar of *N. glauca* is rich in sugar with a mean sugar equivalent concentration of 20% ± 0.3%. The nectar contains the toxic pyridine alkaloids nicotine and anabasine in the concentrations of 0.50 ± 0.12 ppm and 5.0 ± 0.8 ppm, respectively [53]. The pollination of *N. glauca* depends on pollinating vectors because its stamens are shorter than the stigma [54]. *N. glauca* has a relatively long corolla and, hence, mainly depends on birds with long bills, such as sunbirds and hummingbirds, for pollination. The sunbirds in Israel are the main pollinators of *N. glauca* (60% of them are legitimate visitors, where they feed on nectar from the front of the flower) [55].

Ethical statement

All methods were performed in accordance with the relevant guidelines and regulations. Sunbirds were captured in Israel according to the regulations and with the permission of Israel Nature and Parks Authority (permission #2016/41432). All experimental procedures and animal care were approved by the Committee of Animal Experimentation of the University of Haifa (permit #477/16, expiration date September/2020). In total, 16 adult sunbirds were captured between December 2017 and January 2018 with mist nets and held in captivity for about 12 weeks. Each bird was held in a separate cage in a room with a controlled temperature (25 °C) and 12h:12h light:dark conditions. After the experiment ended, the birds were set free.

Sunbirds feeding experiment

After capture, the 16 birds were adapted to the laboratory conditions that included a room temperature of 25 °C, 12h:12h light:dark and fed with artificial food (Sunbird nectar special formula for Nectariniidae; Aves & Avian, Lot nr IS240718; Reg.nr. NL113333, Raalte, Netherlands) and water, for a period of four weeks.

After four weeks of captivity, the birds were randomly divided into two groups by assigning eight birds to each group (that is, each bird was assigned randomly, but the cages stayed in the same spot and were not moved). The control group (eight birds) was fed the artificial nectar mentioned above, without any additional nutrients. The treatment group (eight birds) was fed the same artificial nectar with the addition of nicotine and anabasine (Sigma Aldrich, Rehovot, Israel), in concentrations that naturally occur in *N. glauca* (0.5 ppm and 5 ppm, respectively) (Fig. 1). The concentrations of the pyridine alkaloids were based on Tadmor-Melamed et al. [53]. Fresh artificial nectar and water were supplied every day to both groups.

Excreta (cloacal fluid and faeces) were collected from each of the 16 birds on day 0 (before adding nicotine and anabasine to their feed) and at the end of weeks 2, 4 and 7 (Fig. 1). The excreta collection procedure was as follows: a new, clean piece of baking paper was spread on the bottom of each cage so that the cloacal fluids/faeces would not get contaminated with the cage surface; once the bird left its excreta it was immediately collected using a sterile tip into a sterile Eppendorf tube.

The excreta samples were used for DNA extraction for microbiome analyses (culture-independent) and for isolating nicotine/anabasine–degrading bacteria, as described below.

Culture-independent approach

Cloacal fluids/faeces (excreta) samples for culture-independent analyses were kept at –20 ºC until DNA was extracted using DNeasy Blood and Tissue isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

16S rRNA gene library The Genomic DNA was PCR-amplified using primers targeting the V4 region of the 16S rRNA gene. The primers were: CS1_515F (ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA) and CS2_806R (TACGGTAGCAGAGACTTGGTCTGGACTACHVGG-GTWTCTAAT). Primers were synthesized by Sigma Aldrich (Rehovot, Israel) and contained 5' common sequence tags [56]. The amplification was performed in 25 µl reaction volume using the Emerald Amp MAX HS PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan). Primer concentrations were 0.5 ng/µl. The PCR experimental conditions were as follows: 95 °C for 5 min, followed by 28 cycles of 30 s at 95 °C, 45 s at 55 °C, and 30 s at 68 °C. A final elongation step of 7 min at 68 °C was included. The amplification products were verified by running in agarose gel electrophoresis. The PCR products were stored at –20 °C.

Illumina sequencing. Illumina MiniSeq sequencing was performed at the DNA Services Facility, University of Illinois, Chicago. The sequencing protocol was followed exactly as described by Aizenberg *et al* [45]. Before sequencing the samples, a second PCR amplification was performed in a 10 µl reaction in 96 wells plate. The master mix used for the reaction was made using the 2X AccuPrime SuperMix II (Thermo Fisher Scientific, Massachusetts, United States). A final concentration of 400 nM of each primer was used, and each respective well in the 96 wells plate received a separate primer set with a unique 10-base barcode (Fluidigm, South San Francisco, CA, USA; item #100-4876). The unique barcodes in separate reactions were used for the positive control and a second no-template control reaction with only Access Array Barcode library primers were used. The amplification conditions were 95 °C for 5 min, followed by 8 cycles at 95 °C for 30s, 60 °C for 30s and 68 °C for 30s. A final, 7 min elongation step was performed at 68 °C. The amplified products of positive and negative controls and selected samples were validated using Qubit fluorometric quantitation with the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, United States. After finding the quality of amplification, the samples were collected in equal volume and purified in solid phase reversible immobilization (SPRI). The final quality control was performed using Agilent 2200 TapeStation and Qubit analysis, prior to dilution to 6 pM for emulsion PCR. Pooled, diluted libraries were sequenced on an Illumina MiSeq instrument, using the MiSeq 600-cycle

sequencing kit version 3, and analyzed with Casava 1.8 (pipeline 1.8). The reads were 150 nucleotides in length and PhiX DNA serves as a spike-in control. Barcode sequences from Fluidigm were provided to the MiSeq server, and sequences were automatically binned according to 10-base multiplex identifier sequences. Raw reads were recovered as FASTQ files.

Sequence analysis. Bioinformatics was performed using the DADA2 pipeline. Data analysis was performed on unrarefied data. Amplicon sequence variants (ASV) were selected using DADA2 [57]. ASVs were filtered when they were not assigned to the Domain Bacteria or were assigned to the family Mitochondria, the class of Chloroplast or the phylum of Cyanobacteria/Chloroplast. Taxonomy was assigned using the Ribosomal Database Project 16S rRNA gene sequence database [58]. All subsequent ecological analyses were completed using PhyloSeq and other R packages [59].

Sequences were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/) under the BioProject accession number PRJNA548382.

Statistical analysis. The sample rarefaction analysis was performed using the iNEXT R package [60]. The non-metric multidimensional scaling analysis (nMDS), based on the Bray-Curtis dissimilarity matrix, was analysed in PRIMER7 in order to test whether the bacterial community differs between the treatment and control groups. An analysis of similarity (ANOSIM) was performed between the treatment and control groups at various time intervals. The Shannon H' diversity index was analysed in PAST. Mixed model ANOVA was carried out to determine the effects of time and treatment on the Shannon H diversity index of the microbiome using SPSS23. No violations of data sphericity were detected (Mauchly's test of sphericity: $p > 0.05$). Similarity percentages routine (SIMPER) was applied using the PRIMER7 software. The average Bray-Curtis dissimilarity in the structure of the microbial community between the control and treatment groups was calculated for each time point separately. The contributions from each bacterial genus to the overall dissimilarity between the groups (with a 90% cut-off) are presented in the results.

Culture-dependent approach

Isolation of nicotine/anabasine–degrading bacteria. The nicotine/anabasine–degrading bacteria were isolated at two different sampling time points: (i) cloacal excreta were collected from the naïve birds, which were fed with artificial nectar at day 0 (0 weeks control group, 0WC; 0 weeks treatment group, 0WT; n = 8 at each group; hereafter, weeks control and weeks treatment will be mentioned as WC and WT, respectively) (Fig. 1); (ii) four weeks after starting to add nicotine and anabasine to the special food in the treatment group (4WC and 4WT) (Fig. 1). The excreta were collected as described above. Collected samples were immediately cultured. For enriching nicotine- or anabasine-degrading bacteria, we used the M9 minimal medium ($Na_2HPO_4.7H_2O$ – 64g, KH_2PO_4 – 15g, NaCl – 2.5g, H_2O – 1L), sterilized by autoclaving. To 200 ml of this mixture, we added 700 ml water, 2 ml sterile 1M $MgSO_4$ and 100 µl 1M $CaCl_2$; and the whole solution was adjusted to 1 l with H_2O, with the addition of either 0.1% nicotine or 0.1% anabasine as the only carbon and nitrogen sources. Samples of 100 µl of the collected faeces were

incubated in 500 µl M9 minimal medium at 37 ºC for 3 to 6 hours. After this enrichment incubation, samples were inoculated on agar plates with the same medium but with the addition of 2% agar. Plates were incubated at 37 ºC for 5 to 7 days.

Nicotine- and anabasine-degrading bacterial colonies were picked and streaked five times on *Luria-Bertani* (LB, HiMedia Laboratories, Mumbai, India) agar plates. Their ability to grow on nicotine or anabasine as the only carbon and nitrogen sources was verified again by growing them on M9 agar plates with nicotine or anabasine as carbon or nitrogen sources. Pure bacterial isolates were kept in LB broth with 30% glycerol at –80 ºC.

The bacterial isolates were identified by amplifying and sequencing a 1501-bp internal fragment of 16S rRNA gene, in accordance with Senderovich et al. [61]. Purified PCR products were sequenced at MCLAB (South San Francisco, CA, USA) and analyses of all sequences was carried out using the EzTaxon website (http://eztaxon-e.ezbiocloud.net/) [62]. The sequences were deposited in the GenBank database under the accession numbers: MK348690–MK348835.

Declarations

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Author contributions

M.G., I.I. and M.H. conceived of and designed the experiments. M.G. performed the experiments. M.G., M.L., Y.S. and M.H. analysed the data. M.H. and I.I. contributed reagents/materials and analysis tools. M.G. wrote the article. M.H. and I.I. reviewed and commented on the article.

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Availability of data and materials

The Sequence data of this study are available under the BioProject accession number PRJNA548382 in National Center for Biotechnology Information (NCBI) Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/). The 16s rRNA sequences are available in the GenBank database under the accession numbers: MK348690–MK348835.

Competing interests

The authors declare that they have no conflicts of interest.

References

1. Smith P, Willemsen D, Popkes M, Metge F, Gandiwa E, Reichard M, et al. Regulation of life span by the gut microbiota in the short-lived african turquoise killifish. Elife. 2017;6:1–26. https://doi.org/10.7554/eLife.27014.002.
2. Brucker RM, Bordenstein SR. Response to Comment on "The hologenomic basis of speciation: Gut bacteria cause hybrid lethality in the genus *Nasonia*." Science. 2014;345:1011. https://doi.org/10.1126/science.1256708.
3. Kohl KD. Diversity and function of the avian gut microbiota. J Comp Physiol B Biochem Syst Environ Physiol. 2012;182:591–602. https://doi.org/10.1007/s00360-012-0645-z.
4. Mizrahi I. The Role of the Rumen Microbiota in Determining the Feed Efficiency of Dairy Cows. In: Beneficial Microorganisms in Multicellular Life Forms**,** Rosenberg E, and Gophna U, editors. Berlin, Heidelberg: Springer Berlin Heidelberg 2011. p. 203–210.
5. Klomp JE, Murphy MT, Smith SB, Mckay JE, Ferrera I, Reysenbach A. Cloacal Microbial Communities of Female Spotted Towhees Pipilo maculatus : Microgeographic Variation and Individual Sources of Variability. J. Avian Biol. 2008;39:530–8. https://doi.org/10.1111/j.0908-8857.2008.04333.x.
6. Benskin CM, Rhodes G, Pickup RW, Wilson K, Hartley IR. Diversity and temporal stability of bacterial communities in a model passerine bird, the zebra finch. Mol Ecol. 2010;19:5531–44. https://doi.org/10.1111/j.1365-294X.2010.04892.x.
7. Lewis WB. Gut Microbiota of Migratory Passerines at Stopover. The University of Southern Mississippi, Master thesis. 2015. https://aquila.usm.edu/masters_theseshttps://aquila.usm.edu/masters_theses/116
8. Hird SM, Carstens BC, Cardiff SW, Dittmann DL, Brumfield RT. Sampling locality is more detectable than taxonomy or ecology in the gut microbiota of the brood-parasitic Brown-headed Cowbird (*Molothrus ater*). PeerJ. 2014;2:e321. https://doi.org/10.7717/peerj.321.
9. Preest MR, Folk DG, Beuchat CA. Decomposition of Nitrogenous compounds by intestinal bacteria in hummingbirds. The Auk. 2003:120:1091-101. https://www.jstor.org/stable/4090280.
10. Son JH, Karasawa Y. Effect of removal of caecal contents on nitrogen utilisation and nitrogen excretion in caecally ligated chickens fed on a low protein diet supplemented with urea. Br Poult Sci. 2000;41:69–71. https://doi.org/10.1080/00071660086420.
11. Clench MH. The avian cecum: Update and motility review. J Exp Zool. 1999;283:441–7. https://doi.org/10.1002/(SICI)1097-010X(19990301/01)283:4/5%3C441::AID-JEZ13%3E3.0.CO;2-8.
12. Nicolson SW, Fleming PA. Nectar as food for birds: The physiological consequences of drinking dilute sugar solutions. Plant Syst Evol. 2003;238:139–53. https://doi.org/10.1007/s00606-003-0276-7.

13. Tsahar E, Arad Z, Izhaki I, Rio CM del. Do Nectar- and Fruit-Eating Birds Have Lower Nitrogen Requirements than Omnivores? An Allometric Test. The Auk. 2006;123:1004–12. https://doi.org/10.1093/auk/123.4.1004.
14. Faegri K, Pijl LV. The Principles of Pollinaton Ecology. *Pergamon press*. 1979. https://trove.nla.gov.au/version/44491847.
15. Adler LS, Irwin RE. Ecological costs and benefits of defenses in nectar. Ecology. 2005;86:2968–78. https://doi.org/10.1890/05-0118.
16. Izhaki I. Emodin - A secondary metabolite with multiple ecological functions in higher plants. New Phytol. 2002;155:205–17. https://doi.org/10.1046/j.1469-8137.2002.00459.x.
17. Tsahar E, Friedman J, Izhaki I. Impact on fruit removal and seed predation of a secondary metabolite, emodin, in *Rhamnus alaternus* fruit pulp. Oikos. 2002;99:290–9. https://www.jstor.org/stable/3547910.
18. Adler LS. The Ecological significance of toxic nectar. Oikos. 2000;91:409–20. https://doi.org/10.1034/j.1600-0706.2000.910301.x.
19. Detzel A, Wink M. Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochmicals. Chemoecology. 1993;4:8-18. https://doi.org/10.1007/BF01245891.
20. Klasing KC. Comparative avian nutrition. Wallingford 1998. 1:350pp. https://www.cabdirect.org/cabdirect/abstract/19501100562.
21. Michel P, Pérez-Emán J, Mata A. The Bananaquit, a Neotropical passerine nectar feeding bird, has a high protein requirement relative to other nectarivorous birds. J Ornithol. 2013;154:1039–47. https://doi.org/10.1007/s10336-013-0972-1.
22. Ben-Yosef M, Aharon Y, Jurkevitch E, Yuval B. Give us the tools and we will do the job: Symbiotic bacteria affect olive fly fitness in a diet-dependent fashion. Proc Biol Sci. 2010;277:1545–52. https://doi.org/10.1098/rspb.2009.2102.
23. Kohl KD, Connelly JW, Dearing MD, Forbey JS. Microbial detoxification in the gut of a specialist avian herbivore, the Greater Sage-Grouse. FEMS Microbiol Lett. 2016;363:1–6. https://doi.org/10.1093/femsle/fnw144.
24. Hylin JW. Microbial degradation of nicotine. I. Morphology and Physiology of *Achromobacter nicotinophagum* N. sp. J Bacteriol. 1958;76:36–40. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC290150/.
25. Sabourmoghaddam N, Zakaria MP, Omar D. Evidence for the microbial degradation of imidacloprid in soils of Cameron Highlands. J Saudi Soc Agric Sci. 2015;14:182–8. http://dx.doi.org/10.1016/j.jssas.2014.03.002.
26. Lei L, Xia Z, Liu X, Wei HL. Occurrence and variability of tobacco rhizosphere and phyllosphere bacterial communities associated with nicotine biodegradation. Ann Microbiol. 2015;65:163–73. http://dx.doi.org/10.1007/s13213-014-0847-6.
27. Yang Y, Chen T, Ma P, Shang G, Dai Y, Yuan S. Cloning, expression and functional analysis of nicotinate dehydrogenase gene cluster from *Comamonas testosteroni* JA1 that can hydroxylate 3-

cyanopyridine. Biodegradation. 2010;21:593–602. https://doi.org/10.1007/s10532-010-9327-2.

28. Ma G. Diversity and phylogenetic analyses of nicotine-degrading bacteria isolated from tobacco plantation soils. African J Microbiol Res. 2012;6:6392–8. https://doi.org/10.5897/AJMR12.994.
29. Ruan A, Min H. Studies on microbiological degradation of tobacco tar. J Environ Sci Heal. 2005;40:2073–83. https://doi.org/10.1080/10934520500232704.
30. Chaudhary N, Qazi JI, Gill A. Isolation and Optimization of Tobacco Decomposing *Bacillus* and *Lactobacillus* Sp. Caspian J. Env. Sci. 2007;5:45–9. https://cjes.guilan.ac.ir/article_979_0.html.
31. Qiao L, Wang JL. Microbial degradation of pyridine by *Paracoccus* sp. isolated from contaminated soil. J Hazard Mater. 2010;176:220–5. https://doi.org/10.1016/j.jhazmat.2009.11.016.
32. Gaekwad I, Vinuchurkar AS. Isolation and Identification of Nicotine Utilizing Bacterial Species From Tobacco Leaves. Int Res J Pharm. 2018;9:103–6. https://doi.org/10.7897/2230-8407.09115.
33. Xia Z, Lei L, Zhang HY, Wei HL. Characterization of the ModABC molybdate transport system of *Pseudomonas putida* in nicotine degradation. Front Microbiol. 2018;9:1–9. https://doi.org/10.3389/fmicb.2018.03030.
34. Wang H, Zhi XY, Qiu J, Shi L, Lu Z. Characterization of a novel nicotine degradation gene cluster ndp in *Sphingomonas melonis* TY and its evolutionary analysis. Front Microbiol. 2017;8:1–16. https://doi.org/10.3389/fmicb.2017.00337.
35. Hu H, Wang L, Wang W, Wu G, Tao F, Xu P, et al. Regulatory Mechanism of Nicotine Degradation in *Pseudomonas putida*. mBio. 2019;10:1–13. https://doi.org/10.1128/mBio.00602-19.
36. Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK. Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. Mol Ecol Resour. 2018;18:424–34. https://doi.org/10.1111/1755-0998.12744.
37. Baurhoo B, Ferket PR, Zhao X. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult Sci. 2009;88:2262–72. https://doi.org/10.3382/ps.2008-00562.
38. Borda-Molina D, Vital M, Sommerfeld V, Rodehutscord M, Camarinha-Silva A. Insights into broilers' gut microbiota fed with phosphorus, calcium, and phytase supplemented diets. Front Microbiol. 2016;7:1–13. https://doi.org/10.3389/fmicb.2016.02033.
39. Liu Y, Zheng Z, Yu L, Wu S, Sun L, Wu S, et al. Examination of the temporal and spatial dynamics of the gut microbiome in newborn piglets reveals distinct microbial communities in six intestinal segments. Sci Rep. 2019;9:1-8. http://dx.doi.org/10.1038/s41598-019-40235-z.
40. Oakley BB, Kogut MH. Spatial and temporal changes in the broiler chicken cecal and fecal microbiomes and correlations of bacterial taxa with cytokine gene expression. Front Vet Sci. 2016;3:1–12. https://doi.org/10.3389/fvets.2016.00011.
41. Ji BW, Sheth RU, Dixit PD, Vitkup D. Macroecological relationships in the dynamics of gut microbiota. bioRxiv. 2018. https://doi.org/10.1101/370676.

42. Hird SM, Sánchez C, Carstens BC, Brumfield RT. Comparative gut microbiota of 59 neotropical bird species. Front Microbiol. 2015;6:1-16. https://doi.org/10.3389/fmicb.2015.01403.
43. Michel AJ, Ward LM, Goffredi SK, Dawson KS, Baldassarre DT, Brenner A, et al. The gut of the finch: uniqueness of the gut microbiome of the Galápagos vampire finch. Microbiome. 2018;6:167. https://doi.org/10.1186/s40168-018-0555-8.
44. Noguera JC, Aira M, Pérez-Losada M, Domínguez J, Velando A. Glucocorticoids modulate gastrointestinal microbiome in a wild bird. R Soc Open Sci. 2018;5:171743. http://dx.doi.org/10.1098/rsos.171743.
45. Aizenberg-Gershtein Y, Izhaki I, Santhanam R, Kumar P, Baldwin IT, Halpern M. Pyridine-type alkaloid composition affects bacterial community composition of floral nectar. Sci Rep. 2015;5:1–11. http://dx.doi.org/10.1038/srep11536.
46. Karasov WH, and Rio MD. *Physiological Ecology : How Animals Process Energy, Nutrients, and Toxins*. Princeton: Princeton UP, 2007. Print. https://press.princeton.edu/books/hardcover/9780691074535/physiological-ecology.
47. Dearing MD, Foley WJ, McLean S. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annu Rev Ecol Evol Syst. 2005;36:169–89. https://doi.org/10.1146/annurev.ecolsys.36.102003.152617.
48. García-Amado M, Michelangeli F, Gueneau P, Perez M, Domínguez-Bello M. Bacterial detoxification of saponins in the crop of the avian foregut fermenter *Opisthocomus hoazin*. J Anim Feed Sci. 2007;16:82–5. https://doi.org/10.22358/jafs/74460/2007.
49. Young JC, Zhou T, Yu H, Zhu H, Gong J. Degradation of trichothecene mycotoxins by chicken intestinal microbes. Food Chem Toxicol. 2007;45:136–43. https://doi.org/10.1016/j.fct.2006.07.028.
50. Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, et al. Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. Nat Commun. 2015;6:1–9. http://dx.doi.org/10.1038/ncomms8618.
51. Markman S, Pinshow B, Wright J, Kotler BP. Food Patch Use by Parent Birds : To Gather Food for Themselves or for Their Chicks ? Journal of Animal Ecology. 2004;73:747–55. https://www.jstor.org/stable/3505283.
52. Roxburgh L, Pinshow B. Nitrogen requirements of an Old World nectarivore, the orange-tufted sunbird *Nectarinia osea*. Physiol Biochem Zool. 2000;73:638–45. https://www.jstor.org/stable/10.1086/317747.
53. Tadmor-Melamed H, Markman S, Arieli A, Distl M, Wink M, Izhaki I. Limited ability of Palestine Sunbirds *Nectarinia osea* to cope with pyridine alkaloids in nectar of Tree Tobacco *Nicotiana glauca*. Funct Ecol. 2004;18:844–50. https://www.jstor.org/stable/3599112.
54. Galetto L, Bernardello L. Nectar secretion pattern and removal effects in three species of *Solanaceae*. Can J Bot. 1993;71:1394–8. https://doi.org/10.1139/b93-167.
55. Ollerton J, Watts S, Connerty S, Lock J, Parker L, Wilson I, et al. Pollination ecology of the invasive tree tobacco *Nicotiana* glauca: comparisons across native and non-native ranges. Journal of

Pollination Ecology. 2012;9:85–95. http://dx.doi.org/10.26786/1920-7603%282012%2912.

56. Moonsamy P V., Williams T, Bonella P, Holcomb CL, Höglund BN, Hillman G, et al. High throughput HLA genotyping using 454 sequencing and the Fluidigm Access Array™ system for simplified amplicon library preparation. Tissue Antigens. 2013;81:141–9. https://doi.org/10.1111/tan.12071.
57. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–583. https://dx.doi.org/10.1038%2Fnmeth.3869.
58. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42:D633–42. https://dx.doi.org/10.1093%2Fnar%2Fgkt1244.
59. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:1-11. https://doi.org/10.1371/journal.pone.0061217.
60. Hsieh TC, Ma KH, Chao A. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods Ecol Evol. 2016;7:1451–6. https://doi.org/10.1111/2041-210X.12613.
61. Senderovich Y, Izhaki I, Halpern M. Fish as reservoirs and vectors of *Vibrio cholerae*. PLoS One. 2010;5:e8607. https://doi.org/10.1371/journal.pone.0008607.
62. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: A prokaryotic 16s rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 2012;62:716–21. https://doi.org/10.1099/ijs.0.038075-0.

Tables

Table 1. Analysis of similarities (ANOSIM) for the pairwise test between all the treatments (See also Fig. 2). Samples were taken at 0, 2, 4 and 7 weeks; W, Week of sampling; C, control; T, treatment. The number before the letter W - indicates the week of sampling.

Sampling time (numbers) Sampling groups (WC or WT)	R Statistic	*p* value
0WC vs 0WT	0.012	0.366
0WC vs 2WC	0.517	0.001
0WC vs 2WT	0.616	0.002
0WC vs 4WC	0.835	0.002
0WC vs 4WT	0.801	0.001
0WC vs 7WC	0.759	0.001
0WC vs 7WT	0.86	0.001
0WT vs 2WC	0.392	0.001
0WT vs 2WT	0.488	0.001
0WT vs 4WC	0.668	0.001
0WT vs 4WT	0.629	0.002
0WT vs 7WC	0.562	0.001
0WT vs 7WT	0.711	0.001
2WC vs 2WT	0.291	0.006
2WC vs 4WC	0.755	0.001
2WC vs 4WT	0.443	0.001
2WC vs 7WC	0.222	0.005
2WC vs 7WT	0.579	0.001
2WT vs 4WC	0.758	0.001
2WT vs 4WT	0.393	0.001
2WT vs 7WC	0.433	0.001
2WT vs 7WT	0.662	0.001
4WC vs 4WT	0.353	0.013
4WC vs 7WC	0.691	0.001
4WC vs 7WT	0.743	0.002

4WT vs 7WC	0.321	0.004
4WT vs 7WT	0.202	0.009
7WC vs 7WT	0.329	0.002

Table 2. Nicotine and anabasine degrading bacterial isolates. Isolates were cultured at time 0 before nicotine and **anabasine** were added to the birds' food (0WC, 0WT) and 4 weeks after starting the addition of nicotine and anabasine to the food (4WC and 4WT). Isolates were identified by sequencing the 16S rRNA gene. All sequences of the identified isolates were deposited in the GenBank with the accession numbers MK348690 - MK348835.

Class	The Closest species identity in the GenBank database	0 Weeks (0WC, 0WT) n=16	Nicotine anabasine treatment (4^{th} week)	
			Control group 4WC n=8	Treatment group 4WT n=8
Gammaproteobacteria	*Pseudomonas geniculata*	1	-	-
	Pseudomonas aeruginosa	3	11	17
	Pseudomonas hibiscicola	-	-	1
	Delftia lacustris	-	1	-
	Acinetobacter pittii	8	1	3
	Acinetobacter junii	1	-	2
	Acinetobacter bereziniae	-	3	-
	Enterobacter hormaechei subsp. oharae	2	-	-
	Enterobacter tabaci	7	-	-
	Enterobacter hormaechei subsp. xiangfangensis	2	7	10
	Enterobacter ludwigii	1	-	-
	Enterobacter hormaechei subsp. hormaechei	-	2	1
	Stenotrophomonas pavanii	-	2	2
	Stenotrophomonas rhizophila	-	3	3
	Stenotrophomonas maltophilia	-	2	-
	Klebsiella michiganensis	-	1	1
	Klebsiella quasipneumoniae subsp. similipneumoniae	-	1	-
	Klebsiella grimontii	-	3	4
Flavobacteria	*Chryseobacterium gleum*	2	5	2

Bacilli	*Exiguobacterium indicum*	1	2	2
	Lactococcus lactis subsp. hordniae	-	-	1
Actinobacteria	*Brevibacterium sanguinis*	-	4	1
	Kocuria palustris	-	2	
Alphaproteobacteria	*Methylorubrum thiocyanatum*	-	-	1
	Methylorubrum populi	-	-	1
	Roseomonas mucosa	-	-	1
Sphingobacteria	*Sphingobacterium spiritivorum*	-	-	13
	Sphingobacterium multivorum	-	1	1

Table 3. A list of the genera from the sunbird's excreta Illumina sequencing results that their abundances increased from time zero (0WT) to the 4th week sampling time (4WT) and that have the ability to degrade nicotine or anabasine (evidence for the degradation abilities are either from the current study or from the literature). The table shows the relative genera abundances in the birds' excreta at 0WT and 4WT sampling times. In parallel to the Illumina analyses, we have isolated at the same dates, bacterial strains that were able to use nicotine and or anabasine as a sole carbon and nitrogen sources (these genera are marked in bold; see also Table 2). After 4 weeks of nicotine and anabasine addition to the birds' food, these genera proliferated from a relative total abundance of only 0.3% to 52.7% out of the bacterial excreta community, demonstrating the effect of the edition of nicotine and anabasine to the birds' food.

Genus	0WT (%)	4WT (%)	Reference
Achromobacter	0.000	0.003	[24]
Brevibacterium	**0.001**	**0.036**	[25]
Brevundimonas	0.000	0.031	[26]
Chryseobacterium	**0.000**	**0.078**	Current study
Comamonas	0.000	0.009	[27]
Delftia	**0.000**	**0.005**	[28]
Enterobacter	**0.000**	**18.382**	Current study
Exiguobacterium	**0.040**	**0.159**	Current study
Klebsiella	**0.241**	**2.639**	[29]
Kocuria	**0.000**	**0.003**	Current study
Lactobacillus	0.000	0.007	[30]
Lactococcus	**0.000**	**29.544**	Current study
Methylobacterium	**0.007**	**0.016**	Current study
Paracoccus	0.000	0.005	[31]
Pseudomonas	**0.013**	**0.927**	[32]
Rhodobacter	0.000	0.002	[33]
Sphingobacterium	**0.001**	**0.160**	[28]
Sphingomonas	0.000	0.046	[34]
Stenotrophomonas	**0.002**	**0.672**	[35]
Total percentage	0.305	52.724	

Figures

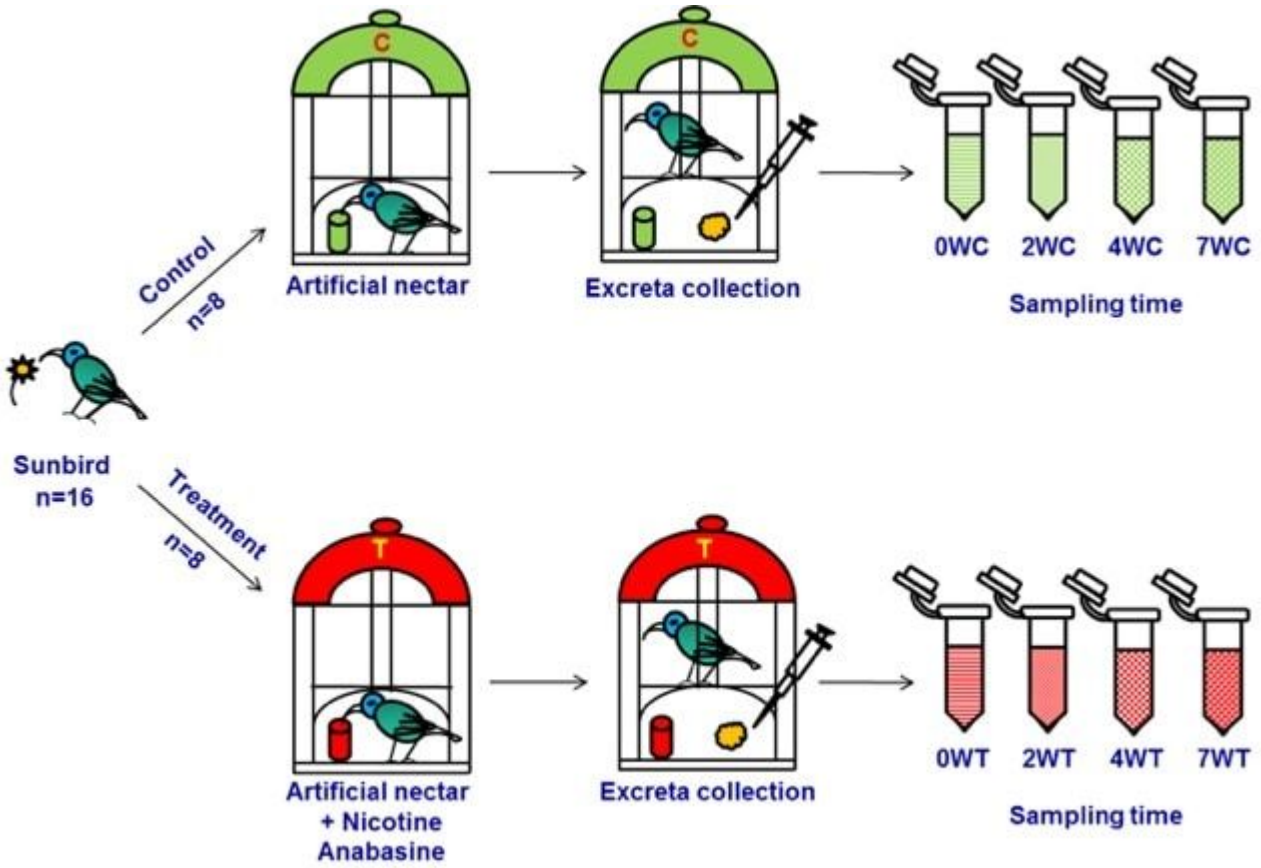

Figure 1

The setup of the feeding experiment. 16 naive sunbirds were captured and divided into two groups (i) Control group that were fed with sunbird artificial nectar free of alkaloids (ii) Treatment group that were fed with artificial nectar with the addition of nicotine (0.5 ppm) and anabasin (5 ppm). Excreta samples were collected from both groups at 0, 2, 4 and 7 weeks. All samples were used for generating a 16S rRNA libraries for bacterial community analyses using Illumina sequencing. Excreta that were collected at day 0 and at 4 weeks from the treatment group were used for culturing bacterial isolates that can degrade nicotine or anabasine. W, sampling week; C, control; T, treatment

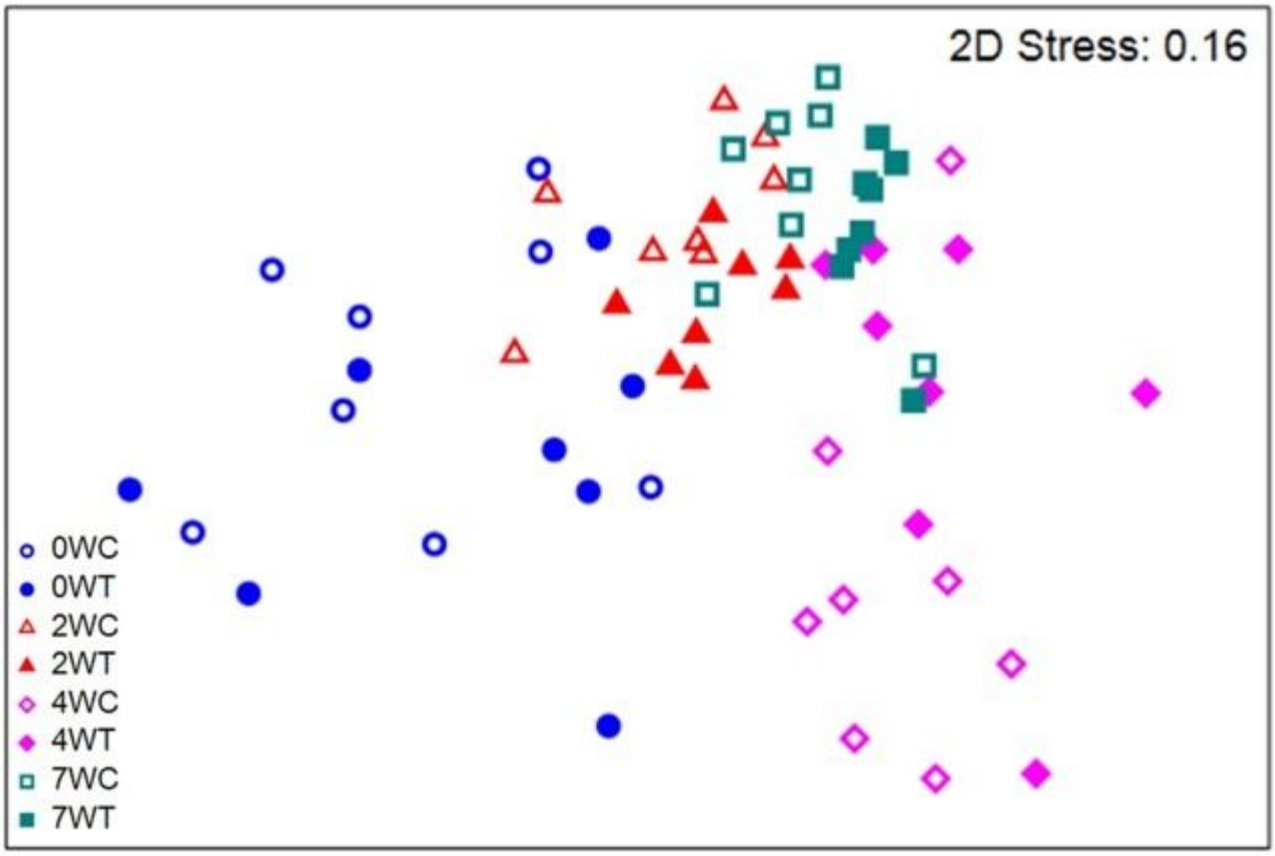

Figure 2

Non-metric Multidimensional scaling analysis (nMDS) with Bray-Curtis dissimilarity matrix. The figure demonstrates the dynamic changes in the gut microbiota composition of sunbirds from the different feeding groups. Control group (C) with no addition of secondary metabolites and treatment group with the addition of nicotine and anabasine (T). Each treated group contained 8 birds that were held in separate cages. Excreta samples were collected at day 0 and after 2, 4 and 7 weeks. Each sampling time is colored differently; day 0 – blue; week 2 - red; week 4 - pink; week 7 - green. All controls symbols are empty shapes and all treatment symbols are full shapes. Significant differences were found in the microbiota composition between the control and the treatments at each sampling time and also between the same treatment in the different time intervals (See Table 1 for the statistical analyses). W, sampling week; C, control; T, treatment

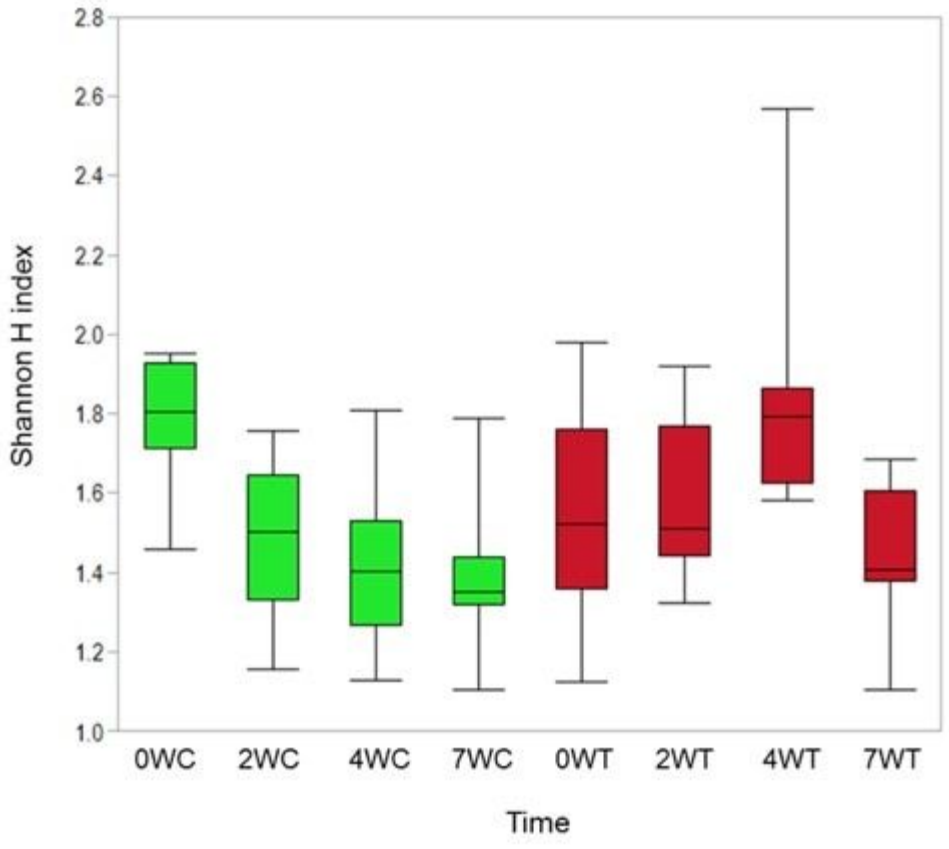

Figure 3

Comparison of alpha diversity between control at different time intervals and between treatment at different time intervals. Shannon H diversity indices shows a significance with a p value of 0.016 and $F_{3,42} = 3.877$. W, sampling week; C, control; T, treatment

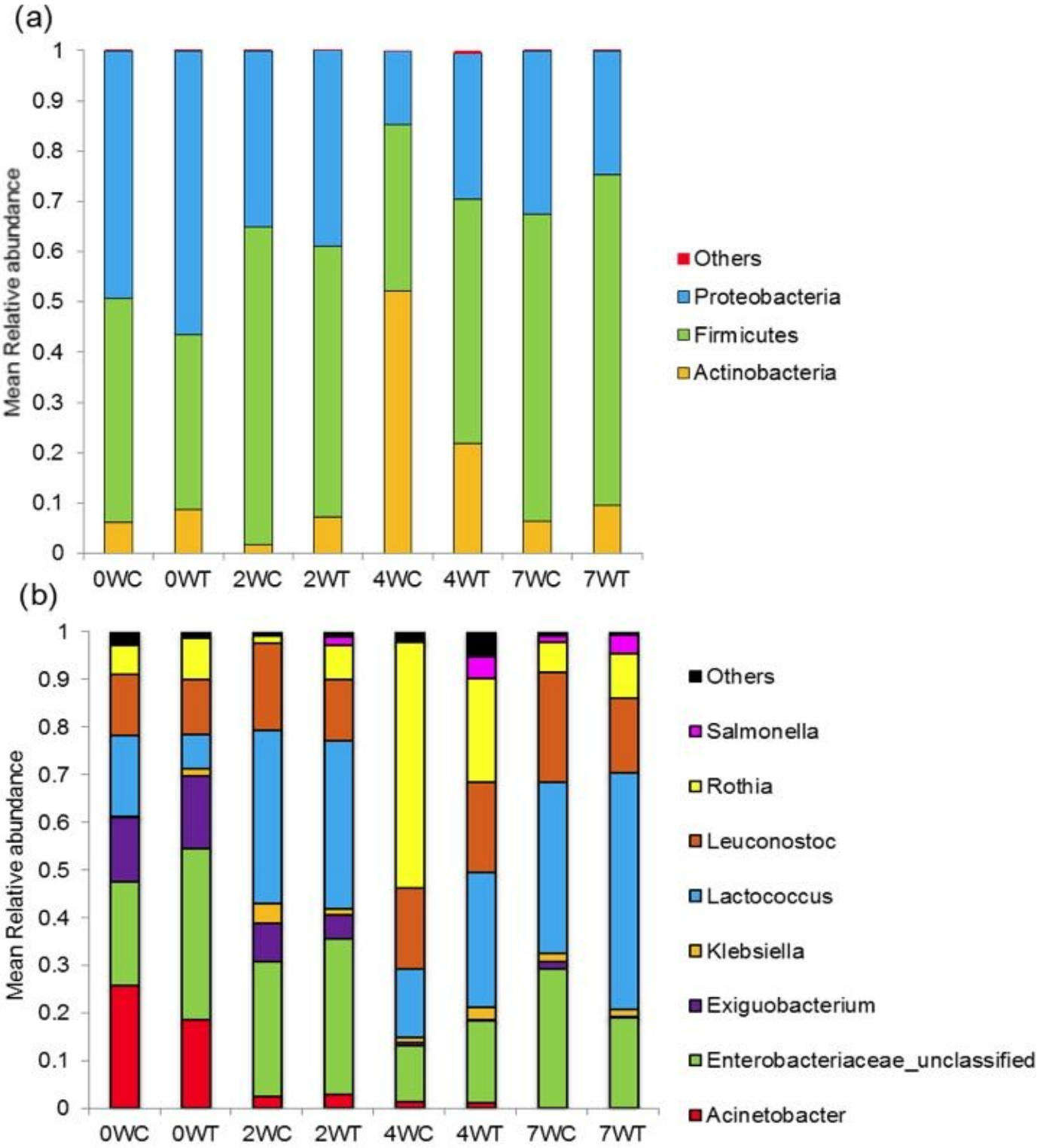

Figure 4

Mean relative abundance of different bacterial phyla (a) and genera (b) in the control and treatment groups. W, sampling week; C, control; T, treatment

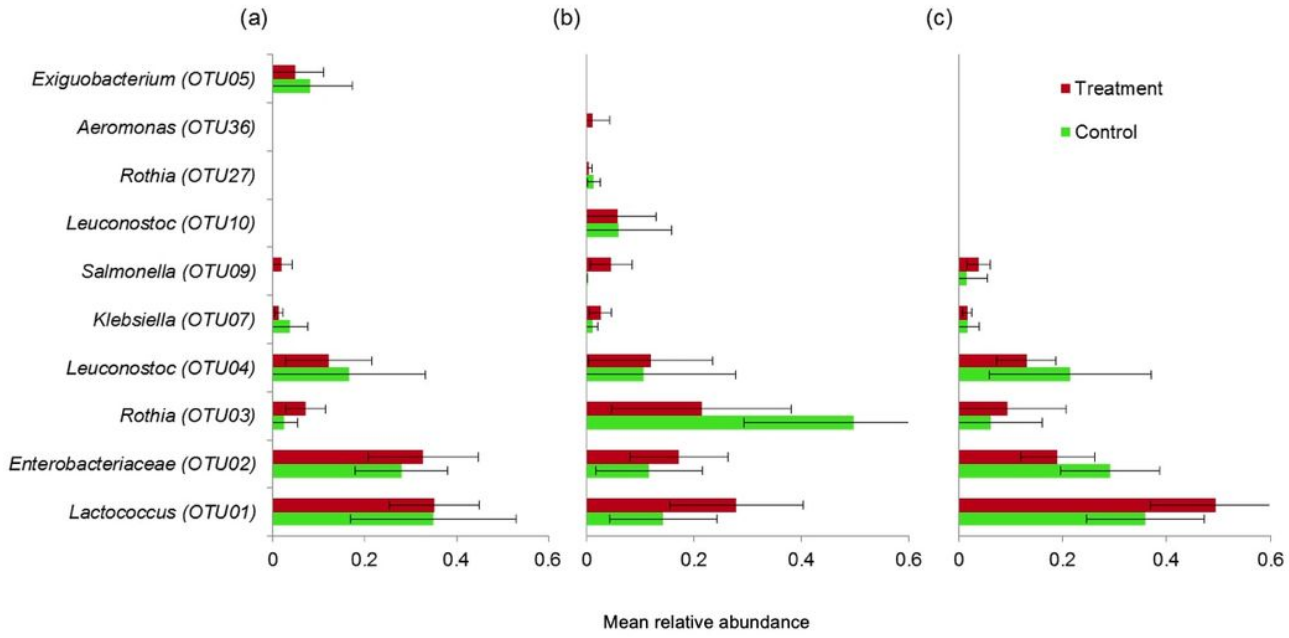

Figure 5

The relative abundances of 10 genera that contributed most saliently to differences among the control and the treatment groups at various time intervals – (a) 2, (b) 4 and (c) 7 weeks. Bars indicate means ± STD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiles.zip