Dietary supplementation with strawberry induces marked changes in the composition and functional potential of the gut microbiome in diabetic mice

By: Chrissa Petersen, Umesh D. Wakhande, Divya Bharat, Kiana Wong, Jennifer Ellen Mueller, Sree V. Chintapalli, Brian D. Piccolo, Thunder Jalili, Zhenquan Jia, J. David Symons, Kartik Shankar, and Pon Velayutham Anandh Babu

Petersen, C., Wankhade, U. D., Bharat, D., Wong, K., Mueller, J. E., Chintapalli, S. V., Piccolo, B. D., Jalili, T., Jia, Z., Symons, J. D., Shankar, K., Anandh Babu, P. V. (2019). Dietary supplementation with strawberry induces marked changes in the composition and functional potential of the gut microbiome in diabetic mice. *Journal of Nutritional Biochemistry 66*, 63-69. https://doi.org/10.1016/j.jnutbio.2019.01.004

Abstract:

Gut microbiota contributes to the biological activities of berry anthocyanins by transforming them into bioactive metabolites, and anthocyanins support the growth of specific bacteria, indicating a two-way relationship between anthocyanins and microbiota. In the present study, we tested the hypothesis that strawberry supplementation alters gut microbial ecology in diabetic *db/db* mice. Control (*db/+*) and diabetic (*db/db*) mice (7 weeks old) consumed standard diet or diet supplemented with 2.35% freeze-dried strawberry (*db/db* + SB) for 10 weeks. Colon contents were used to isolate bacterial DNA. V4 variable region of 16S rRNA gene was amplified. Data analyses were performed using standardized pipelines (QIIME 1.9 and R packages). Differences in predictive metagenomics function were identified by PICRUSt. Principal coordinate analyses confirmed that the microbial composition was significantly influenced by both host genotype and strawberry consumption. Further, α-diversity indices and β-diversity were different at the phylum and genus levels, and genus and operational taxonomical units levels, respectively ($P<.05$). At the phylum level, strawberry supplementation decreased the abundance of *Verrucomicrobia* in *db/db* + SB *vs. db/db* mice ($P<.05$). At the genus level, *db/db* mice exhibited a decrease in the abundance of *Bifidobacterium*, and strawberry supplementation increased *Bifidobacterium* in *db/db* + SB *vs. db/db* mice ($P<.05$). PICRUSt revealed significant differences in 45 predicted metabolic functions among the 3 groups. Our study provides evidence for marked changes in the composition and functional potential of the gut microbiome with strawberry supplementation in diabetic mice. Importantly, strawberry supplementation increased the abundance of beneficial bacteria *Bifidobacterium* which play a pivotal role in the metabolism of anthocyanins.

Keywords: Strawberry | Diabetic mice | Gut microbiome | *Bifidobacterium* | *Berry fruits* | *Anthocyanins*

Article:

Funding: This work was supported by the National Institute of Food and Agriculture (grant 2018-67018-27510), University of Utah research start-up fund, University of Utah Seed Grant and College of Health Pilot Grant (to P.V.A.B.); the University of Utah Undergraduate Research Opportunities Program award (to K.W., J.E.M.); American Heart Association (AHA: 16GRNT31050004) and the National Institutes of Health (NIH: R03AGO52848) (to J.D.S.); and USDA CRIS 6206-51000-010-05S (to K.S.).

1. Introduction

Anthocyanins are one of the major flavonoid compounds present in many fruits and vegetables including blueberries and strawberries [1]. Anthocyanins are glycosides comprised of an aglycone component (anthocyanidins such as cyanidin, delphinidin, malvidin, peonidin, pelargonidin and petunidin) and a sugar moiety (glucose, xylose, galactose and arabinose) [1]. Epidemiological and clinical studies support the beneficial health effects of berry anthocyanins such as reducing the risk of cardiovascular disease [2], [3], [4], [5]. The health benefits of berry anthocyanins are ascribed to their antihypertensive, antioxidant and antiatherosclerotic effects [3], [4]. Anthocyanins are extensively metabolized by digestive enzymes and intestinal microbiota in humans, suggesting that a significant portion of the biological activity of anthocyanins may be linked to their metabolites [6], [7]. Data from our laboratory and others suggest that the circulating metabolites mediate the beneficial vascular and endothelial effects of anthocyanins [8], [9], [10].

Strawberry is an excellent source of dietary anthocyanins, and the most commonly found anthocyanins in strawberries are the glycosidic derivatives of pelargonidin and cyanidin [5]. Epidemiological study demonstrated that habitual consumption of 2–3 servings (~160–240 g) of strawberries per week reduces the risk of myocardial infarction in humans [2]. Further, strawberry intake attenuated postprandial inflammation in overweight adults and reduced inflammatory molecules in humans with cardiovascular risk factors [11], [12], [13]. Recently, we showed that dietary supplementation of strawberry at a nutritional dosage (equivalent to two human servings) reduced vascular inflammation and ameliorated vascular dysfunction in diabetic mice [10]. Further, our study suggested that circulating metabolites mediate the vascular effects of strawberry anthocyanins [10]. Gut microbiota contributes significantly to the biological activities of berry anthocyanins by transforming them into more readily absorbable bioactive metabolites [6], [7]. Interestingly, anthocyanins promote intestinal colonization and support the growth of specific groups of bacteria, indicating a two-way relationship between anthocyanins and microbiota [7], [14]. Indeed, anthocyanins may act as prebiotics and play a role in reshaping the gut microbiome, which enhances the host microbial interaction to provide beneficial health effects in humans [7].

Healthy gut microbiota plays a major role in converting anthocyanins into bioactive metabolites. Therefore, it is one of the important factors required for the potential biological activities of anthocyanins. Anthocyanins in strawberry extracts were shown to enhance the beneficial effects of diets with fructooligosaccharides (a constituent of dietary fiber which has prebiotic properties) in the rat cecal environment [15]. However, to our knowledge, the effect of strawberry on gut

microbiota in general and diabetes in particular is unknown. In the present study, we tested the hypothesis that dietary supplementation of strawberry induces changes in the composition and functional potential of the gut microbiome in diabetic *db/db* mice.

2. Materials and methods

2.1. Experimental animals

db/db mice homozygous for the diabetes spontaneous mutation (*Lepr*^{db}) with C57BL/6J background manifest morbid obesity, pancreatic cell atrophy and chronic hyperglycemia. These leptin-receptor-deficient *db/db* mice are a widely used type 2 diabetic animal model which exhibits dysbiosis of gut microbiota [16], [17], [18]. Six-week-old male diabetic *db/db* mice and control *db/+* mice (stock no. 000642) were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The mice were held under humane conditions in the animal facility at the University of Utah and acclimated for a week before experiments were performed. Mice were housed 4 per cage and maintained under artificial light in a 12-h light/dark cycle, 23°C±1°C and 45%±5% humidity. The Institutional Animal Care and Use Committee at the University of Utah approved the animal experiment protocols which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

2.2. Experimental groups

The freeze-dried strawberry powder was provided by FutureCeuticals (Momence, IL, USA). The customized pelleted diets (control diet and strawberry supplemented diet) were prepared as reported in our recent study and supplied by Dyets Inc. (Bethlehem, PA, USA) [10]. The strawberry-supplemented diet was adjusted to compensate for the fiber and additional sugars provided by the freeze-dried strawberry powder. The composition of the control diet and strawberry supplemented diet was reported in our recent study [10]. The amount of freeze-dried strawberry powder used in this study was calculated based on the Food and Drug Administration recommendation for the extrapolation of doses from humans to animals by normalization to body surface area [19]. The nutritional dose of freeze-dried strawberry powder was based on average human consumption. The dose used in these studies [2.35% freeze-dried strawberry powder in the diet pellets (w/w)] is equivalent to two human servings of fresh strawberries (~160 g strawberries) [10]. After 1 week of acclimation, diabetic mice (7 weeks old) were divided into 2 groups and received control diet (n=8) or 2.35% freeze-dried strawberry-supplemented diet for 10 weeks (n=8). *db/+* mice fed control diet for 10 weeks served as controls (n=8). At the end of the 10-week treatment, mice were euthanized, and colon contents were collected, snap-frozen in liquid nitrogen and stored at −80°C.

2.3. Microbial community profiling using 16S rRNA amplicon sequencing

Genomic DNA was extracted from colon contents using the DNeasy PowerSoil Kit (Qiagen, MD, USA). Fifty nanograms of genomic DNA was utilized for amplification of the V4 variable region of the 16S rRNA gene using 515F/806R primers. Forward and reverse primers were dual-indexed as described by Kozich *et al.* [20] to accommodate multiplexing of up to 384 samples

per run. Paired-end sequencing (2× 250 bp) of pooled amplicons was carried out on an Illumina MiSeq [21] with ~30% PhiX DNA.

2.4. Bioinformatics analysis

Processing and quality filtering of reads were performed using scripts in QIIME (v1.9.1) [22] and other in-house scripts. Paired reads were stitched with PEAR, an overlapping paired-end reads merging algorithm which evaluates all possible paired-end read overlaps, minimizing false-positive hits [23]. Reads were further filtered based on Phred quality scores and for chimeric reads using USEARCH61 [24]. Filtered reads were demultiplexed within QIIME, and samples with less than 5000 reads were excluded from further analysis. UCLUST was used to cluster sequences into operational taxonomical units (OTUs based on>97% identity) [24]. OTU picking was performed using open-reference method which encompasses clustering of reads against a reference sequence collection and also performs *de novo* OTU picking on the reads which fail to align to any known reference sequence in the database [25]. To eliminate erroneous mislabeling, the resulting OTU tables were checked for mislabeling sequences [26]. Representative sequences were further aligned using PyNAST with the Greengenes core-set alignment template [27]. Construction of the phylogenetic tree was performed using the default (FASTTREE) method in QIIME [28].

All samples were clustered based on their between-sample distances using UPGMA, and subsequent jack-knifing was performed by resampling methods. Comparisons of intergroup and intragroup diversity were performed using analysis of variance (ANOVA) including correction for multiple comparisons. OTU reads were summed at genus levels and then assessed for group differences with negative binomial regression using the DESeq2 package. PICRUSt was used to identify differences in predictive metagenome function [29]. OTUs were normalized by the predicted 16S copy number, and functions were predicted with the use of GreenGenes 13_5 databases for KEGG Orthologs.

2.5. Statistical analysis

Microbiota OTU reads were imported into R version 3.2.1, and all statistical analyses were performed using the vegan and phyloseq packages unless specifically noted. OTU richness was measured by Chao1, and evenness was measured by several diversity indices (Shannon, Simpson, Inverse Simpson and Fisher). Group differences in α-diversity (richness and diversity) were assessed by ANOVA. Between-specimen diversity (β-diversity) was assessed by calculating a matrix of dissimilarities using the Bray–Curtis method and then visualized using nonmetric multidimensional scaling. Group differences in β-diversity were assessed using permutational multivariate analysis of variance with 500 permutations. Group differences among genus-level OTUs were assessed by pairwise comparisons on read counts using negative binomial Wald tests from the DESeq2 package. OTU relative abundance is given as median % relative abundance when described in text. All statistical tests used on 16S-rRNA gene sequencing data were considered significant at $P \leq .05$. All tests were corrected for multiple comparisons using the false discovery rate correction by Benjamini and Hochberg. Associations among selected variables were assessed with Spearman's correlations. An in-house-developed R-based shiny app (DAME) was developed to facilitate procedures and statistical analysis

described above [30]. All statistical analyses were performed and figures were made using R. Correlations between bacterial abundance with predicted metagenomic function were performed using the corrplot package in R and utilized bacteria at family or genus levels as described in the specific comparison. Statistical significance was determined at $P \leq .05$.

3. Results

3.1. α-Diversity and β-diversity

In the present study, a total of 480,321 reads were assessed. A total of 320 of OTUs were assigned to taxonomic classifications of 6 phyla, 17 families and 30 genera. Principal coordinate analysis of unweighted UniFrac distances performed on the OTU abundance matrix showed that the *β*-diversity of gut microbial communities was significantly different between the groups and microbial composition was significantly influenced by both genotype (*db/db*) and strawberry consumption (Fig. 1A).

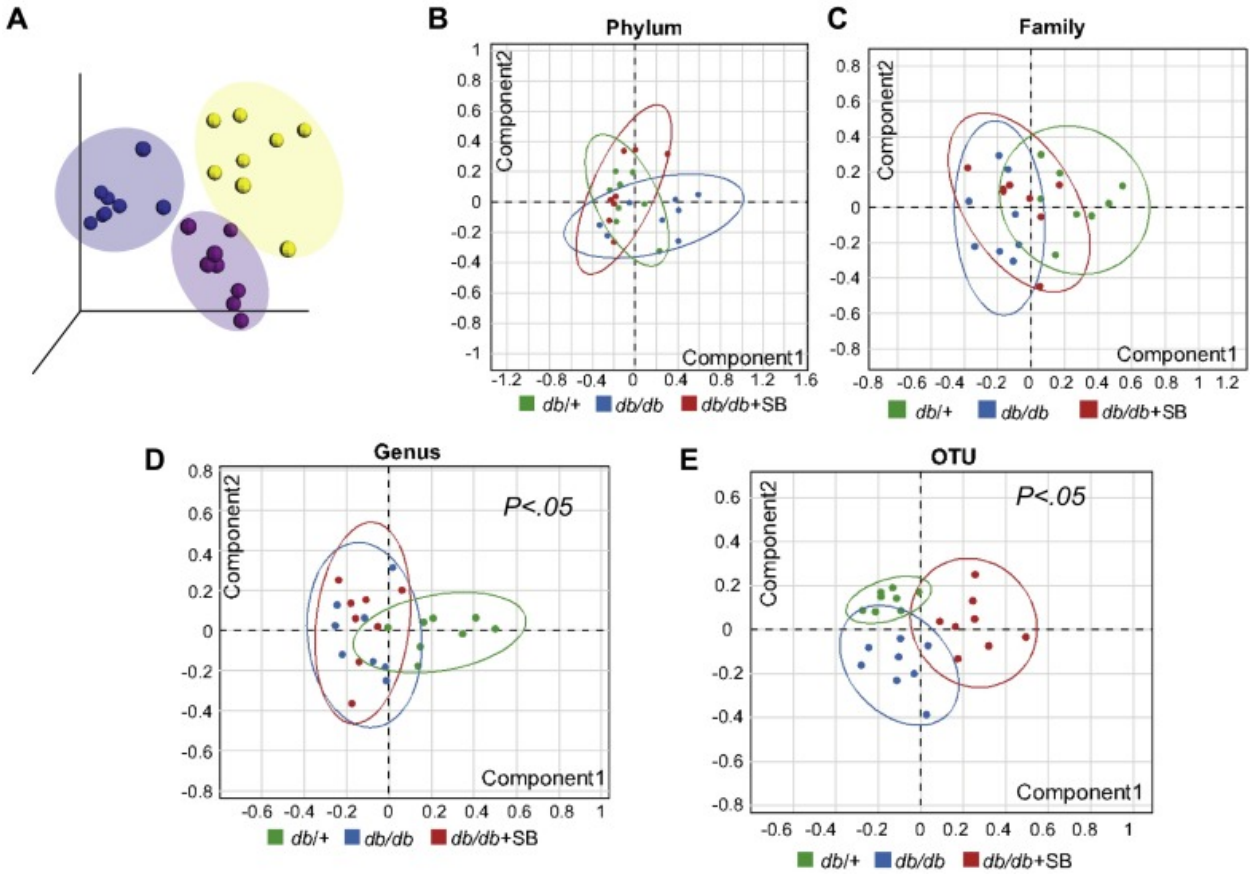

Fig. 1. (A) Principal component analysis plot. Nonmetric multidimensional scaling analysis of the OTU abundance matrix of β-diversity of gut microbial communities at phylum (B), family (C), genus (D) and OTU (E) levels. Values are mean±S.E.M.; *n=8*. *db/+*, standard-diet-fed control mice; *db/db*, standard-diet-fed diabetic mice; *db/db* + SB, strawberry-fed diabetic mice.

Measurements of α-diversity are indicative of phylogenetic species richness and evenness within a sample. Indices of α-diversity such as ACE, Chao1, Fisher, InvSimpson, Observed, Shannon

and Simpson were measured. At the phylum level, α-diversity indices ACE, Chao1, Fisher and Observed were significantly different at phylum level among groups (Table 1). At the genus level, α-diversity indices such as Fisher and Observed were significantly different among groups (Table 2). β-Diversity represents compositional differences between samples (Fig. 1B–E). β-Diversity, a measure of global microbial composition, was significantly different at the genus and OTU levels (Fig. 1D and E). β-Diversity was influenced by genotype and diet at OTU levels (Fig. 1E).

Table 1. α-Diversity indices at phylum level

Index	***db/+***	***db/db***	***db/db* + SB**	***P* value**
ACE	5.167±0.144	6.017±0.217	6.187±0.162	.006
Chao1	5.125±0.125	5.75±0.164	5.875±0.125	.002
Fisher	0.486±0.016	0.542±0.018	0.565±0.013	.006
InvSimpson	2.129±0.103	2.364±0.187	2.025±0.041	.173
Observed	5.125±0.125	5.75±0.164	5.875±0.125	.002
Shannon	0.807±0.044	0.943±0.084	0.778±0.043	.142
Simpson	0.524±0.019	0.558±0.036	0.505±0.01	.305

Data are expressed as means±S.E. (*n*=8). *db/+*, standard-diet-fed control mice; *db/db*, standard-diet-fed diabetic mice; *db/db* + SB, strawberry-fed diabetic mice.

Table 2. α-Diversity indices at genus level

Index	***db/+***	***db/db***	***db/db* + SB**	***P* value**
ACE	23.63±0.501	22.069±0.858	25.4±3.57	.554
Chao1	23.062±0.538	21.562±0.741	22.562±1.551	.590
Fisher	2.539±0.069	2.244±0.062	2.247±0.066	.006
InvSimpson	3.108±0.322	3.421±0.449	2.491±0.062	.139
Observed	22.625±0.596	20.625±0.532	20.25±0.491	.012
Shannon	1.463±0.071	1.48±0.109	1.257±0.025	.095
Simpson	0.657±0.03	0.668±0.046	0.597±0.01	.266

Data are expressed as means±S.E. (*n*=8).

3.2. Relative abundance of microbiota at the phyla level

The distribution of bacterial taxa and the relative abundance of bacteria at the phyla level are shown in Fig. 2A–G. Bacterial sequences were distributed among six bacterial phyla including *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia*. The taxonomic abundance indicated a significant ($P<.05$) decrease in *Actinobacteria* in *db/db* mice compared with *db/+* mice (Fig. 2B). Strawberry supplementation did not increase the abundance of *Actinobacteria* in *db/db* + SB mice (Fig. 2B). The most abundant phyla were *Bacteroidetes* and *Firmicutes* (>90%). In the present study, the abundance of *Bacteroidetes* and *Firmicutes* was similar among three groups (Fig. 2C and D). The taxonomic abundance indicated a significant increase ($P<.05$) in *Proteobacteria* in *db/db vs. db/+* mice, but strawberry supplementation did not alter the abundance of *Proteobacteria* in *db/db* + SB mice *vs. db/+* mice (Fig. 2E). The abundance of *Tenericutes* was similar among the groups (Fig. 2F). There was a nonsignificant increase in *Verrucomicrobia* in *db/db* compared with *db/+* mice, but this was significantly reduced ($P<.05$) in *db/db* + SB mice compared with *db/db* mice (Fig. 2G).

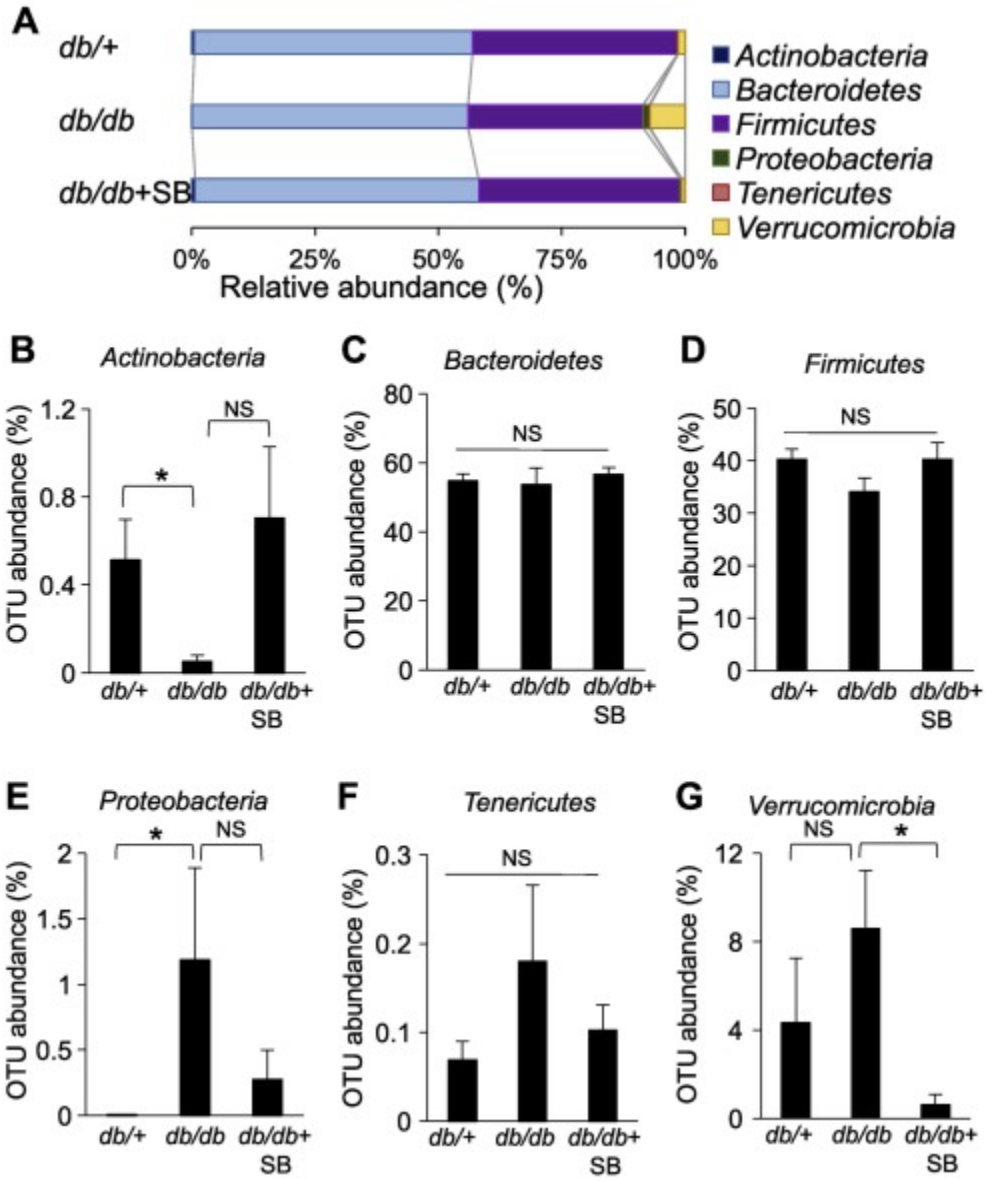

Fig. 2. (A) The relative abundance of bacterial population at phylum level. The abundance of *Actinobacteria* (B), *Bacteroidetes* (C), *Firmicutes* (D), *Proteobacteria* (E), *Tenericutes* (F) and *Verrucomicrobia* (G) in *db/+*, *db/db* and *db/db* + SB mice treated for 10 weeks. **P*<.05; values are mean±S.E.M.; *n=8*. NS, nonsignificant.

3.3. Relative abundance of microbiota at the genus level

The abundance of *Bifidobacterium*, which belong to the phylum *Actinobacteria*, is significantly decreased in *db/db* mice as compared to *db/+* mice (*P*<.05) (Fig. 3A). However, strawberry supplementation significantly decreased the abundance of *Bifidobacterium* in *db/db* + SB mice (*P*<.05) (Fig. 3A). The abundance of *Bacteroides* was unchanged between *db/+* and *db/db* mice (Fig. 3B). Strawberry supplementation significantly increased *Bacteroides* in *db/db* + SB mice (*P*<.05) (Fig. 3B). *Dehalobacterium* and *Dorea,* which belong to *Firmicutes* phyla, were significantly increased in *db/db* mice compared with *db/+* mice (*P*<.05) (Fig. 3C and D).

Lactobacillus, *SMB53* and *Turicibacter*, which belong to the phylum *Firmicutes*, were significantly decreased in *db/db* mice compared with *db/+* mice ($P<.05$) (Fig. 3E–G). Strawberry supplementation did not change the abundance of *Dehalobacterium*, *Dorea*, *Lactobacillus*, *SMB53* and *Turicibacter* in *db/db* + SB mice compared with *db/db* mice. *Akkermansia* is significantly reduced in *db/db* + SB mice compared with *db/db* mice ($P<.05$) (Fig. 3H). In addition, the unassigned genera belong to the families *Enterobacteriaceae* and *Ruminococcaceae* were increased whereas *Peptostreptococcaceae* was decreased in *db/db* mice compared with *db/+* mice ($P<.05$) (data not shown). Strawberry supplementation did not alter these genera in *db/db* + SB mice compared with *db/db* mice.

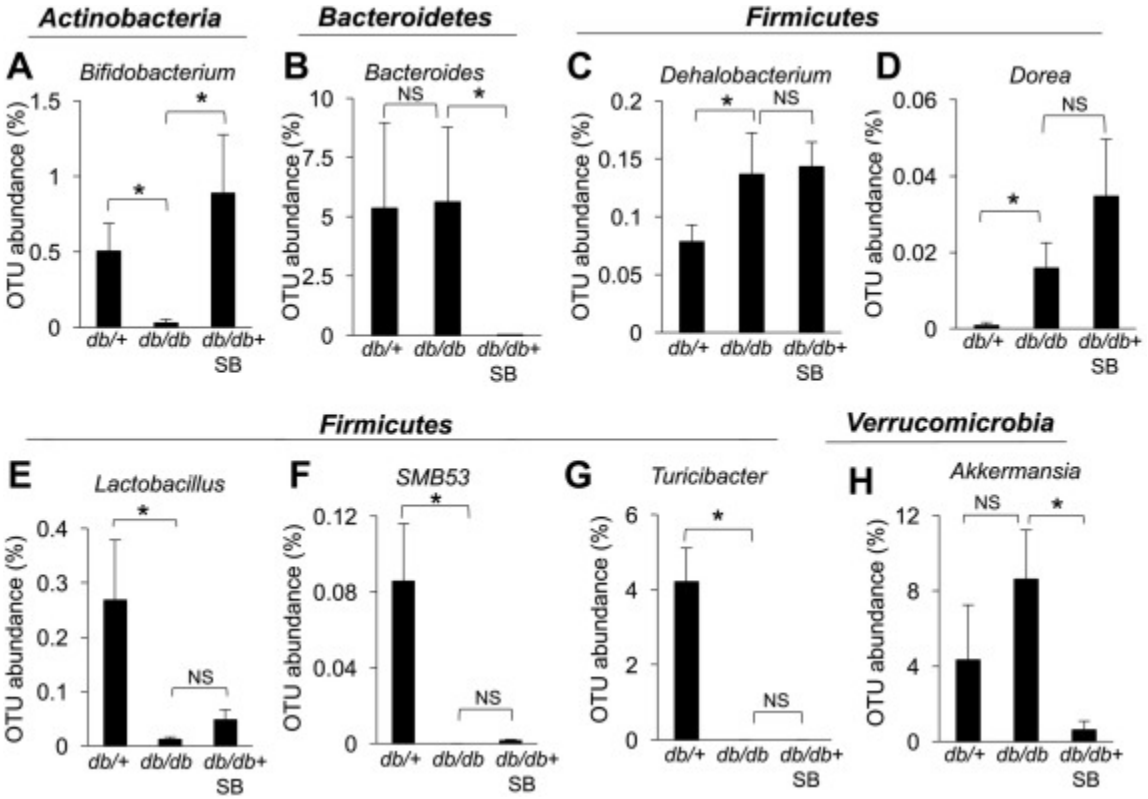

Fig. 3. The abundance of *Bifidobacterium* (A), *Bacteroides* (B), *Dehalobacterium* (C), *Dorea* (D), *Lactobacillus* (E), *SMB53* (F), *Turicibacter* (G) and *Akkermansia* (H) in *db/+*, *db/db* and *db/db* + SB mice treated for 10 weeks. **P*<.05; values are mean±S.E.M.; *n=8*.

3.4. Predicted metabolic pathways

Predicted functional metagenomic profiles based on KEGG pathways were generated using PICRUSt. Comparison between groups revealed significant differences in 45 predicted metabolic functions ($P<.05$) (Fig. 4). Most of these features revealed similar abundances between control *db/+* compared to *db/db* + SB. The correlations between bacterial abundance with predicted metagenomic function indicate that G-protein-coupled receptors, insulin signaling pathway, fatty acid elongation in mitochondria, glycerophospholipid metabolism, lipid biosynthesis protein, fatty acid biosynthesis and steroid hormone biosynthesis were significantly different among the three groups. Specifically, lipid biosynthesis proteins, insulin signaling pathway and the

phosphatidylinositol signaling pathway were modified in *db/db* + SB compared with *db/db* mice (*P*<.05).

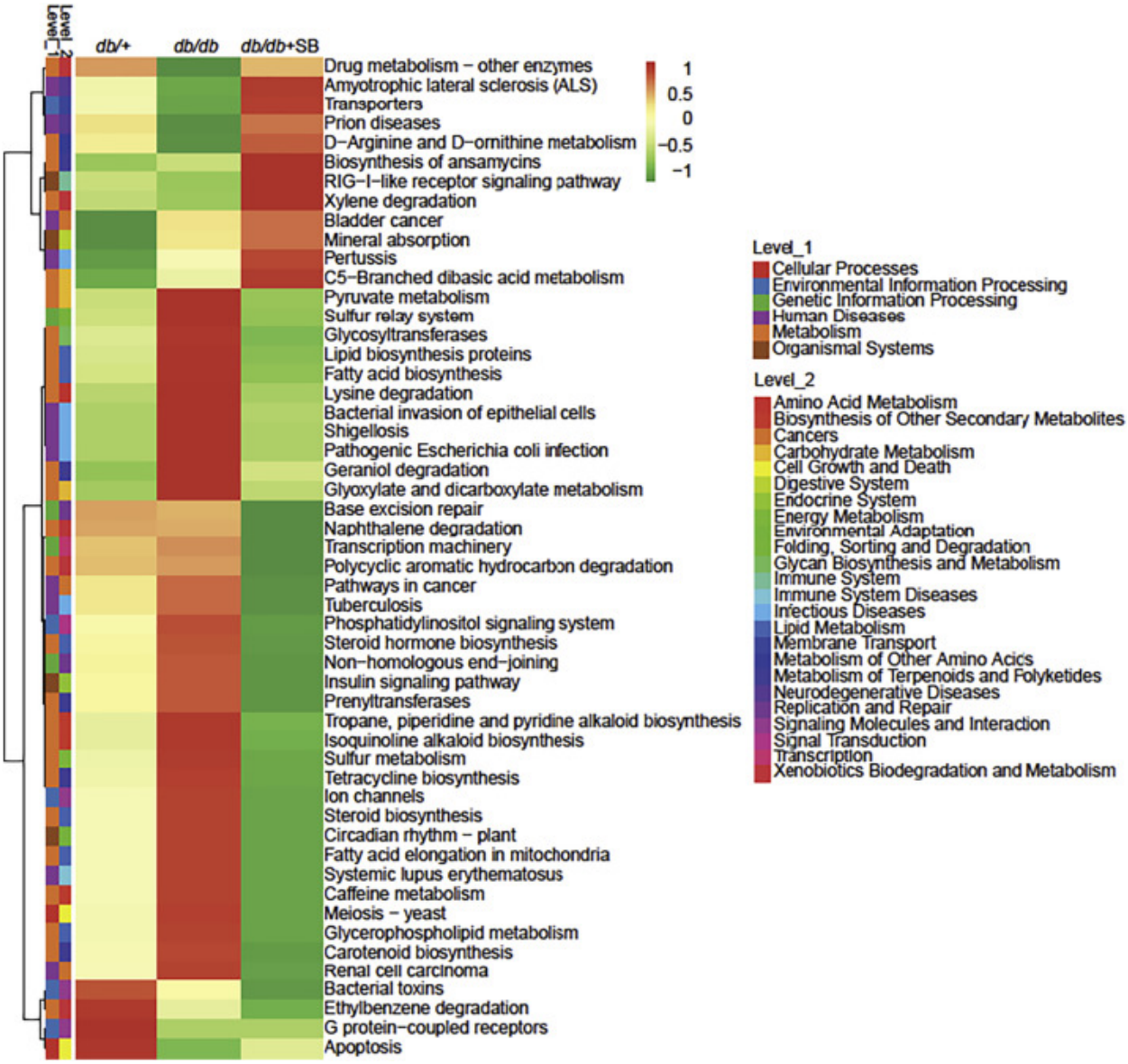

Fig. 4. Heat map for the effect of strawberry supplementation on functional potential of gut microbiome in diabetic mice. Metabolic pathways from KEGG module predictions using 16S rRNA data with PICRUSt and sequenced shotgun metagenome (*n=8)*.

4. Discussion

The gut microbiome plays a pivotal role in the metabolism of anthocyanins and is one of the important mechanisms of the health-promoting properties of anthocyanins. We investigated the effect of dietary supplementation of strawberry on gut microbiota in diabetic mice. In our study, strawberry supplementation at a nutritional dosage induced marked changes in the composition and functional potential of the gut microbiome in diabetic *db/db* mice.

Evidence from epidemiological and clinical studies indicates beneficial cardiovascular effects following the consumption of strawberries [2], [5], [12], [13]. Our group has also recently reported that dietary supplementation of strawberry attenuates vascular inflammation and improves vascular dysfunction in diabetic mice [10]. It has been speculated that such cardiovascular benefits may be mediated by circulating metabolites of the bioactive compounds of strawberries. These are produced by human digestive enzymes and intestinal microbiota, and highlight the potential importance of microbiota [31], [32], [33]. A human study indicated that 21 polyphenolic metabolites appear in the plasma following the consumption of strawberries[31]. We recently assessed the role of circulating metabolites of strawberry in mediating the vascular effects of strawberry. We used serum that was obtained from strawberry-fed mice (serum containing circulating metabolites of strawberry) and control-diet-fed mice (control serum) for this study. In our study, serum from strawberry-fed mice reduced high-glucose- and palmitate-induced endothelial inflammation in mouse aortic endothelial cells, indicating the possible role of circulating metabolites in mediating the vascular effects of strawberry [10].

Intestinal microbiotas play a major role in the conversion of anthocyanins to metabolites. Therefore, they modulate the biological activities of dietary anthocyanins. The commensal bacteria such as *Bifidobacterium* and *Lactobacillus* possess β-glucosidase activity and have the ability to metabolize anthocyanins into phenolic metabolites [34], [35]. Indeed, a recent randomized clinical study showed that high levels of *Bifidobacterium* are associated with increased urinary concentrations of anthocyanin metabolites [35]. On other hand, anthocyanins act as prebiotics and support the growth of *Bifidobacterium* and *Lactobacillus,* indicating a two-way relationship between anthocyanins and gut microbiota [36]. Hence, a healthy microbiome is essential to benefit from the effects of anthocyanins as intestinal microbiota plays a key role in the conversion of parent anthocyanins into metabolites.

In our study, *db/db* mice exhibited marked changes in the microbial abundance at phylum and genus levels. This is consistent with previous studies that showed the compositional changes in gut microbiota at phylum and genus levels in both type 1 and type 2 diabetes [37], [38], [39]. *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* were significantly altered among the three groups at the phylum level. There was a significant decrease in the abundance of *Actinobacteria* in *db/db* mice compared with *db/+* mice. *Actinobacteria* represent only a small percentage but still are pivotal in the maintenance of gut homeostasis [40]. The classes of *Actinobacteria*, importantly *Bifidobacterium*, are widely used as probiotic, indicating their beneficial effects in many pathological conditions [40]. *Actinobacteria* were shown to decrease in type 1 diabetic children compared with healthy children [37]. Though strawberry supplementation increased the *Actinobacteria* in *db/db* + SB mice in our study, the difference did not reach significance. Consistent with a previous study, *db/db* mice exhibited a significant increase in *Proteobacteria* compared with control mice [38]. A bloom of *Proteobacteria* in the gut is an indication of an unstable gut microbial community and/or gut dysbiosis [41]. The levels of *Proteobacteria* were shown to increase in type 2 diabetic mice and diabetic patients [38], [39]. There was a nonsignificant increase in *Verrucomicrobia* in *db/db* compared with *db/+* mice, but it was significantly reduced in *db/db* + SB mice compared with *db/db* mice.

At the genus level, many of the bacterial genera were altered in *db/db vs. db/+* and *db/db vs. db/db* + SB mice. The abundance of *Bifidobacterium* and *Lactobacillus* was significantly

decreased in *db/db* mice compared to *db/+* mice. *Bifidobacterium* and *Lactobacillus* are considered beneficial bacteria and are associated with positive effects for the host in the large intestine. Evidence shows that the etiology and development of type 2 diabetes are closely associated with changes in the gut microbiota including a decrease in the abundance of *Bifidobacterium* and *Lactobacillus* [42], [43]. These microbes modulate lipid and glucose metabolism, improve insulin resistance, reduce low-grade inflammation, improve the gut barrier function and stimulate the host immune system [7], [44], [45]. Indeed, the *Bifidobacterium* abundance was shown to be lower in overweight, obese or type 2 diabetic patients than in lean subjects [46], [47]. Further, a significant decrease in the number of *Lactobacillus* and *Bifidobacterium* was reported in children with diabetes [37]. In the present study, strawberry supplementation significantly increased the abundance of *Bifidobacterium* in *db/db* + SB mice compared to *db/db* mice but did not change the abundance of *Lactobacillus.* A recent study showed that incubating malvidin-3-glucoside, one of the major berry anthocyanins, with fecal slurry enhanced the growth of *Bifidobacterium* and *Lactobacillus* and exhibited a synergic effect to support the growth of beneficial bacteria when mixed with other anthocyanins [36]. Further, blueberry consumption for 6 weeks was shown to increase *Bifidobacterium* in human volunteers [48]. These studies indicate that the anthocyanins can act as a prebiotic to support the growth of beneficial gut bacteria such as *Bifidobacterium*. An increase in the *Bifidobacterium* can enhance the bioactivity of strawberry as it increases bioavailability of metabolites of anthocyanins [35].

Akkermansia was nonsignificantly increased in *db/db* mice compared to *db/+* mice but was significantly reduced in *db/db* + SB mice compared with *db/db* mice. Studies suggest that *Akkermansia muciniphila* possesses anti-inflammatory properties, although the underlying mechanisms are unknown [49]. *Akkermansia muciniphila* was shown to protect against atherosclerosis and prevent the development of high-fat-diet-induced obesity by improving the gut barrier and metabolic inflammation in animal models [50]. Interestingly, in our study, strawberry supplementation reduced *Akkermansia* in *db/db* + SB mice. Given that we have previously shown that strawberries significantly improve vascular function and indices of vascular inflammation, the biological relevance of *Akkermansia* in the *db/db* model remains unclear.

PICRUSt revealed significant differences in 45 predicted metabolic functions among the 3 groups. Specifically, lipid biosynthesis proteins, the insulin signaling pathway and the phosphatidylinositol signaling pathway were modified in *db/db* + SB *vs. db/db*. Evidence suggests that oral supplementation of prebiotics (fermented oligosaccharides) and/or probiotics may improve metabolic disorders such as obesity and type 2 diabetes [51], [52]. A recent study showed that strawberry extracts modulate the effects of fructooligosaccharides on microbiota in the gastrointestinal tract [53]. To our knowledge, the present study is the first to show the complex interaction between strawberry and intestinal microbiota in diabetes.

Our findings indicate that dietary supplementation of strawberry at a nutritional dosage induced marked changes in the composition and functional potential of gut microbiota in *db/db* mice. Importantly, strawberry supplementation increased the abundance of *Bifidobacterium,* which plays a pivotal role in the metabolism of anthocyanins and the formation of metabolites. Hence, the reported beneficial health effects of strawberry could be due to an increased abundance

of *Bifidobacterium,* which may enhance the metabolism of strawberry and the bioactive metabolites formed by *Bifidobacterium* metabolism. However, mechanistic studies are still needed to provide evidence for the prebiotic effects of strawberry anthocyanins and to understand how this could modulate the biological activity of metabolites. Our results promote further exploration into the analysis of strawberry metabolites and correlating the functional aspects of strawberries with metabolites and microbiome. In conclusion, our study provides a strong proof of concept for further considering strawberry as an adjunct therapy to improve intestinal microbiota and thereby to prevent or reverse the complications associated with diabetes.

References

[1] He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. Ann Rev Food Sci Technol 2010;1:163–87.

[2] Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. Circulation 2013;127:188–96.

[3] Afrin S, Gasparrini M, Forbes-Hernandez TY, Reboredo-Rodriguez P, Mezzetti B, Varela-Lopez A, et al. Promising health benefits of the strawberry: a focus on clinical studies. J Agric Food Chem 2016;64:4435–49.

[4] Li D, Wang P, Luo Y, Zhao M, Chen F. Health benefits of anthocyanins and molecular mechanisms: update from recent decade. Crit Rev Food Sci Nutr 2017;57:1729–41.

[5] Giampieri F, Forbes-Hernandez TY, Gasparrini M, Alvarez-Suarez JM, Afrin S, Bompadre S, et al. Strawberry as a health promoter: an evidence based review. Food Funct 2015;6:1386–98.

[6] Morais CA, de Rosso VV, Estadella D, Pisani LP. Anthocyanins as inflammatory modulators and the role of the gut microbiota. J Nutr Biochem 2016;33:1–7.

[7] Faria A, Fernandes I, Norberto S, Mateus N, Calhau C. Interplay between anthocyanins and gut microbiota. J Agric Food Chem 2014;62:6898–902.

[8] Bharat D, Cavalcanti RRM, Petersen C, Begaye N, Cutler BR, Costa MMA, et al. Blueberry metabolites attenuate lipotoxicity-induced endothelial dysfunction. Mol Nutr Food Res 2018;62:201700601.

[9] Rodriguez-Mateos A, Rendeiro C, Bergillos-Meca T, Tabatabaee S, George TW, Heiss C, et al. Intake and time dependence of blueberry flavonoid-induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic insights into biological activity. Am J Clin Nutr 2013;98:1179–91.

[10] Petersen C, Bharat D, Cutler BR, Gholami S, Denetso C, Mueller JE, et al. Circulating metabolites of strawberry mediate reductions in vascular inflammation and endothelial dysfunction in db/db mice. Int J Cardiol 2018;263:111–7.

[11] Edirisinghe I, Banaszewski K, Cappozzo J, Sandhya K, Ellis CL, Tadapaneni R, et al. Strawberry anthocyanin and its association with postprandial inflammation and insulin. Br J Nutr 2011;106:913–22.

[12] Djurica D, Holt RR, Ren J, Shindel AW, Hackman RM, Keen CL. Effects of a dietary strawberry powder on parameters of vascular health in adolescent males. Br J Nutr 2016;116:639–47.

[13] Basu A, Fu DX, Wilkinson M, Simmons B, Wu M, Betts NM, et al. Strawberries decrease atherosclerotic markers in subjects with metabolic syndrome. Nutr Res 2010;30:462–9.

[14] Jamar G, Estadella D, Pisani LP. Contribution of anthocyanin-rich foods in obesity control through gut microbiota interactions. Biofactors 2017;43:507–16.

[15] Fotschki B, Juskiewicz J, Jurgonski A, Kolodziejczyk K, Milala J, Kosmala M, et al. Anthocyanins in strawberry polyphenolic extract enhance the beneficial effects of diets with fructooligosaccharides in the rat cecal environment. PLoS One 2016;11:e0149081.

[16] Zheng J, Yuan X, Cheng G, Jiao S, Feng C, Zhao X, et al. Chitosan oligosaccharides improve the disturbance in glucose metabolism and reverse the dysbiosis of gut microbiota in diabetic mice. Carbohydr Polym 2018;190:77–86.

[17] Garcia-Mazcorro JF, Lage NN, Mertens-Talcott S, Talcott S, Chew B, Dowd SE, et al. Effect of dark sweet cherry powder consumption on the gutmicrobiota, short-chain fatty acids, and biomarkers of gut health in obese db/db mice. PeerJ 2018;6:e4195.

[18] Mei X, Zhang X, Wang Z, Gao Z, Liu G, Hu H, et al. Insulin sensitivity-enhancing activity of Phlorizin is associated with lipopolysaccharide decrease and gut microbiota changes in obese and type 2 diabetes (db/db) mice. J Agric Food Chem 2016;64:7502–11.

[19] Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7:27–31.

[20] Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 2013;79:5112–20.

[21] Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 2013;10:57–9.

[22] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.

[23] Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 2014;30:614–20.

[24] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1.

[25] Rideout JR, He Y, Navas-Molina JA, Walters WA, Ursell LK, Gibbons SM, et al. Subsampled open-reference clustering creates consistent, comprehensive OUT definitions and scales to billions of sequences. PeerJ 2014;2:e545.

[26] Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian community-wide culture-independent microbial source tracking. Nat Methods 2011;8:761–3.

[27] McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 2012;6:610–8.

[28] Price MN, Dehal PS, Arkin AP. FastTree 2 — approximately maximum-likelihood trees for large alignments. PLoS One 2010;5:e9490.

[29] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 2013;31:814–21.

[30] Piccolo BD,Wankhade UD, Chintapalli SV, Bhattacharyya S, Luo C, Shankar K. Dynamic Assessment of Microbial Ecology (DAME): a web app for interactive analysis and visualization of microbial sequencing data. Bioinformatics 2018;34:1050–2.

[31] Sandhu AK, Miller MG, Thangthaeng N, Scott TM, Shukitt-Hale B, Edirisinghe I, et al. Metabolic fate of strawberry polyphenols after chronic intake in healthy older adults. Food Funct 2018;9:96–106.

[32] Mullen W, Edwards CA, Serafini M, Crozier A. Bioavailability of pelargonidin-3-Oglucoside and its metabolites in humans following the ingestion of strawberries with and without cream. J Agric Food Chem 2008;56:713–9.

[33] Azzini E, Vitaglione P, Intorre F,Napolitano A, Durazzo A, Foddai MS, et al. Bioavailability of strawberry antioxidants in human subjects. Br J Nutr 2010;104:1165–73.

[34] Avila M, Hidalgo M, Sanchez-Moreno C, Pelaez C, Requena T, Pascual-Teresa S. Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. Food Res Int 2009;42:1453–61.

[35] Boto-Ordonez M, Urpi-Sarda M, Queipo-Ortuno MI, Tulipani S, Tinahones FJ, Andres-Lacueva C. High levels of Bifidobacteria are associated with increased levels of anthocyanin microbial metabolites: a randomized clinical trial. Food Funct 2014;5:1932–8.

[36] Hidalgo M, Oruna-Concha MJ, Kolida S, Walton GE, Kallithraka S, Spencer JP, et al. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. J Agric Food Chem 2012;60:3882–90.

[37] Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Med 2013;11:46.

[38] Geurts L, Lazarevic V, Derrien M, Everard A, Van Roye M, Knauf C, et al. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. Front Microbiol 2011;2:149.

[39] Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, et al. Gut microbiota in human adults with type 2 diabetes differs from nondiabetic adults. PLoS One 2010;5:e9085.

[40] Binda C, Lopetuso LR, Rizzatti G, Gibiino G, Cennamo V, Gasbarrini A. Actinobacteria: a relevant minority for the maintenance of gut homeostasis. Dig Liver Dis 2018;50:421–8.

[41] Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 2015;33:496–503.

[42] Upadhyaya S, Banerjee G. Type 2 diabetes and gut microbiome: at the intersection of known and unknown. Gut Microbes 2015;6:85–92.

[43] Moreno-Indias I, Cardona F, Tinahones FJ, Queipo-Ortuno MI. Impact of the gut microbiota on the development of obesity and type 2 diabetes mellitus. Front Microbiol 2014;5:190.

[44] Philippe D, Favre L, Foata F, Adolfsson O, Perruisseau-Carrier G, Vidal K, et al. Bifidobacterium lactis attenuates onset of inflammation in a murine model of colitis. World J Gastroenterol 2011;17:459–69.

[45] Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091–103.

[46] Wu X, Ma C, Han L, NawazM, Gao F, Zhang X, et al. Molecular characterisation of the faecal microbiota in patients with type II diabetes. Curr Microbiol 2010;61:69–78.

[47] Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[48] Vendrame S, Guglielmetti S, Riso P, Arioli S, Klimis-Zacas D, Porrini M. Six-week consumption of a wild blueberry powder drink increases Bifidobacteria in the human gut. J Agric Food Chem 2011;59:12815–20.

[49] Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J 2017;474: 1823–36.

[50] Zhao S, Liu W, Wang J, Shi J, Sun Y, Wang W, et al. Akkermansia muciniphila improves metabolic profiles by reducing inflammation in chow diet-fed mice. J Mol Endocrinol 2017;58:1–14.

[51] Delzenne NM, Cani PD. Gut microbiota and the pathogenesis of insulin resistance. Curr Diab Rep 2011;11:154–9.

[52] Everard A, Matamoros S, Geurts L, Delzenne NM, Cani PD. Saccharomyces boulardii administration changes gut microbiota and reduces hepatic steatosis, low-grade inflammation, and fat mass in obese and type 2 diabetic db/db mice. MBio 2014;5:e01011–4.

[53] Jurgonski A, Juskiewicz J, Fotschki B, Kolodziejczyk K, Milala J, Kosmala M, et al. Metabolism of strawberry mono- and dimeric ellagitannins in rats fed a diet containing fructo-oligosaccharides. Eur J Nutr 2017;56:853–64.