

Tormentic Acid Alters the Gut Microbiota of the Silkworm (*Bombyx mori*)

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Abstract

In recent years, phytochemicals have started to attract more attention due to their contribution to health and bioactivity. Microorganisms in the intestines of organisms contribute to the processing, function, and biotransformation of these substances. The silkworm (*Bombyx mori*) is one of the organisms used for the biotransformation of phytochemicals due to its controlled reproduction and liability to microbial manipulation. In this study, a bioactive compound, tormentic acid (TA), extracted from *Sarcopoterium spinosum* was used in the diet of silkworm and the alterations of intestinal microbiota of the silkworm were assessed. To do this, silkworms were fed on diet with various tormentic acid content, and 16S metagenomic analysis was performed to determine the alterations in the gut microbiota profile of these organisms. Diet with different TA content did not cause a change in bacterial diversity of the samples. Comprehensive comparisons between different feeding groups indicated increased abundance of bacteria associated with health, i.e., *Intestinibacter* spp., *Flavonifractor* spp., *Senegalimassilia* spp., through the utilization of bioactive substances such as flavonoids. In conclusion, it might be said that use of TA as a supplementary product might help ameliorating the infected gut and promoting the healthy gut and relieving the undesirable effects of medicines to gastrointestinal system.

Introduction

Herbal natural compounds include phenolic compounds and secondary plant metabolites, generally known as phytochemicals. These compounds have started to gain great importance with their positive effects against many diseases due to their bioactivity^{1,2}. Most of the natural compounds undergo microbial changes, namely biotransformation, in the small intestine before being transformed in the liver³. Biotransformation processes using mammalian, microorganism, plant cells and insect cell cultures or enzymes isolated from these play an important role in the discovery and development of new drug molecules⁴⁻⁶.

The silkworm (*Bombyx mori*), which feeds on mulberry (*Morus alba*) leaves, has been domesticated for thousands of years to produce economically important silk fibre⁷. Silkworm has provided a livelihood for humans for centuries and has been a highly productive source of biological and chemical substances^{8,9}.

In addition to the benefits of silkworm itself, many compounds are produced from its excrement, which are sources of treatment and food for other organisms (Liu J. et al. 2013, Liu X. et al. 2019; Li et al. 2019). Besides, it is possible to form bioactive compounds by feeding the silkworm with mulberry leaves enriched with different natural compounds by making use of the biotransformation ability of the silkworm^{13,14}.

Compounds that prevent liver damage, trigger tissue regeneration, or reduce the activity of toxic substances are called hepatoprotective agents. Some of the natural herbal compounds have antimicrobial, antidiabetic, antifungal and hepatoprotective effects^{15,16}. It is stated that tormentic acid

(TA), one of the terpenes found in the content of *Sarcopoterium spinosum*, has the potential to be a hepatoprotective agent, as well as its effectiveness in type 2 diabetes^{17,18}.

Many microorganisms live in the body of the silkworm. However, the microbiota of this insect model has not been well characterized to date⁷. With the advent of metagenomics technology, microbial communities of various species have been characterized and listed by genome studies on vertebrates and invertebrates¹⁹.

The silkworm gut has less microbial diversity compared to the other vertebrates due to controlled microenvironment for growth and development and shorter lifespan. In addition, the silkworm microbiota must be able to tolerate the alkalinity up to pH 11–12, which adversely affects the microbiota of most vertebrates, and the change of the peritrophic matrix during moulting also limits the microbial growth. In addition, the silkworm gut has low O₂ levels, thus it allows only the growth of facultative anaerobic microorganisms²⁰.

Diet has a great impact on gut microbiota profile of the organisms, and it is significantly associated with health and disease^{21,22}. It is also possible to manipulate gut microbiota with using different diet²³. Tormentic acid has been shown to take part in the treatment of bacterial disorders such as ulcerative colitis²⁴, and periodontal disease²⁵. Therefore, it could be a good candidate molecule for manipulation of the gut microbiota. In the present study, our aim was to investigate the effect of feeding silkworms with diet, consisting of different tormentic acid content, on the microbiota profile of silkworm gut.

Results

Intestine samples were collected from silkworms fed on diet with various tormentic acid content. The number of samples in each group is shown in Table-1.

Shannon and Chao1 alpha-diversity indexes of the samples are presented in Table 2. ClustVis webtool²⁶ was used to calculate principal components (PCs) and PC values were charted in 3D scatterplots in JMP Pro 13. Microbiota composition of the samples did not indicate any clear clustering (Fig. 1).

In total, 20 phyla were detected, and relative abundances of these phyla were charted in Fig. 2. Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria constitutes more than 90% of all phyla for all the samples.

Figure 3 shows the relative abundances of the 15 most prevalent genera in all groups. *Staphylococcus* spp. constituted approximately 30% of all genera in Control, RawExtract and TA_{rich} feeding groups. TA_{poor} and RawExtract groups had relatively more *Bacillus* spp. compared to TA_{rich} and Control groups. The TA_{poor} group also had relatively more *Enterococcus* spp. compared to the other groups.

The response screening analysis resulted in a more detailed analysis of the differences at genus level between the groups. Based on the response screening analysis, *Intestinibacter* spp. were significantly

more abundant in the gut microbiota of silkworm fed on TA_{rich} diet compared to the ones fed on raw extract (Fig. 4). TA_{rich} group samples also had significantly more prevalent *Intestinibacter* spp. when compared to the TA_{poor} group samples. Besides that, *Flavonifractor* spp. and some members of *Veillonella* spp. were significantly more prevalent in TA_{rich} group samples compared to the TA_{poor} group samples (Fig. 5).

TA_{rich} group samples had significantly more abundant *Senegalimassilia* spp. compared to the Control group samples (Fig. 6).

The silkworms fed on raw extract had significantly more abundant *Flavonifractor* spp. and *Leuconostoc* spp. in their gut compared to the ones fed on TA_{poor} diet. However, TA_{poor} group samples had significantly more prevalent *Coprobacter* spp. compared to the Raw Extract group samples (Fig. 7). There was no significant difference between TA_{poor} group and Control group samples (Supplementary Fig. 2).

Raw Extract group samples had significantly more prevalent *Hungatella* spp., *Senegalimassilia* spp., *Turicimonas* spp., *Catenibacterium* spp. compared to the Control group samples (Fig. 8).

Discussion

In the present study, we have investigated the alterations in the gut microbiota profile of silkworms fed on diet comprising different TA content using 16S rRNA gene sequencing. TA, a terpene, is a bioactive molecule with the potential to be a hepatoprotective agent, and it is also effective in type 2 diabetes. We have chosen the silkworm because it is an advantageous model organism due to its low gut microbial diversity compared to other organisms. Feeding silkworms with TA and evaluating changes in the gut microbiota profile of silkworms may provide insight into the biotransformation process of terpenes in the gut. Although there was a certain difference in the microbiota profiles of the feeding groups, there was no significant difference in bacterial diversity of these groups. This may be due to the controlled microenvironment in which silkworms are grown and the slight microbial changes that occur after feeding. In this study, fragments extracted from *Sarcopoterium spinosum* with different TA content were used in the diet of silkworms. The silkworm fed on TA_{rich} diet had significantly more *Intestinibacter* spp. compared to the ones fed on RawExtract and TA_{poor} diet. TA_{rich} group samples also had significantly more *Flavonifractor* spp. and some members of *Veillonella* spp. compared to TA_{poor} group samples. It was previously reported that the abundance of *Intestinibacter* spp. decreases and the abundance of *Escherichia* spp. increases in type 2 diabetes patients treated with metformin^{27–29}. Metformin is an effective medicine widely used for type 2 diabetes, and one of the adverse effects of metformin is gastrointestinal disorders^{30,31} which could be associated with increased abundance of *Escherichia* spp. Furthermore, current knowledge on the role of *Intestinibacter* spp. is scarce, but functional annotations suggest a role for these bacteria in mucus production through consumption of mucins^{28,32}, which are helpful for immune and metabolic responses. *Flavonifractor* spp. are known for their role in degradation of flavonoids³³ which are bioactive molecules like terpenes³⁴. *Veillonella* spp. are known as producers of propionate which supports health in the human gut³⁵. TA_{rich} group samples had significantly more

prevalent *Senegalimassilia* spp. compared to the control group. Like *Intestinibacter* spp., the abundance of *Senegalimassilia* spp. has been shown to decrease just after the metformin treatment. In conclusion, it can be said that TA, which is currently used in the treatment of type 2 diabetes, may have the potential to reverse the side effects of the widely used medication metformin. TA could also be given with metformin as a supplementary material to type 2 diabetes patients. To be able to assert this more precisely, further studies with larger sample size and appropriate experimental design are required.

Relative abundance of *Flavonifractor* spp. was significantly higher in RawExtract samples compared to TA_poor samples. On the other hand, TA_poor samples had higher abundance of *Coprobacter* spp., and *Leuconostoc* spp. than RawExtract samples. *Coprobacter* spp. were shown to be positively associated with dietary uptake of polyphenols³⁶, and *Leuconostoc* spp., members of lactic acid bacteria, have been shown to be associated with the production of extracellular polysaccharides³⁷.

RawExtract group samples had relatively higher abundance of *Hungatella* spp., *Senegalimassilia* spp., *Turicimonas* spp., *Catenibacterium* spp. compared to the Control group samples. *Hungatella* spp. play role in the conversion of carbohydrates into acetic acid which is involved in ATP synthesis pathways³⁸. *Catenibacterium* spp. were shown to be associated with the fermentation of fibre and production of short chain fatty acids which play promote the gut health³⁹. To date, there is no reported association between *Turicimonas* spp. and bioactive molecules.

In conclusion, microbiota profile of *Bombyx mori* could be manipulated with the diet containing the terpen, TA. Our results indicated that there was an increase in the relative abundance of bacteria associated with healthy gut in TA-rich diets. Hence, it might be said that TA could be used as a supplementary product to ameliorate and stabilize the healthy gut. It could also be used as a reversal agent to alleviate the adverse effects of medicines. On the other hand, the sample size is an important limitation for this study, but the results provide an insight into the microbiota changes in the case of TA supplementation to the silkworm diet. In brief, the results we report in this study, albeit on a small scale, may lead to potential future studies.

Methods

Sampling

Sarcopoterium spinosum plant was incubated at 60 °C for 6 hours in 70% ethanol at a solid-liquid ratio of 1:20. The solvent was removed in the evaporator and the extract was obtained in the water phase. A part of this extract was used in the diet of silkworm in RawExtract group. The remaining extract was taken into a separatory funnel and butanol was added. It was incubated until phase formation was observed and then the water phase and butanol phase were separated. The butanol phase was taken into a 50 ml tube as TA_poor fraction and the remaining extract was evaporated to remove the butanol. The remaining phase was subjected to liquid-liquid extraction using ethyl acetate. The ethyl acetate phase was used as TA_rich fraction.

Silkworm was reared at 26±2 °C with 75-85% humidity and normal daylight photoperiod. Silkworm in 5th instar were fed on mulberry leaves incubated with different fractions of *S. spinosum* extraction; RawExtract, TA_{rich}, TA_{poor}, and control. The silkworm larva was fixed with the help of a needle from the head and tail and the digestive system was opened by carefully cutting the shell. Silk glands were separated and placed in a sterile 15 ml tube for gastrointestinal microbiota analysis.

DNA Extraction

Microbial DNA was extracted using the EurXGeneMATRIX Tissue and Bacterial DNA purification kit (EurX Ltd., Poland) and following the manufacturer's instructions. The Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Fair Lawn, NJ, USA) was used to measure DNA concentrations before PCR.

16S rRNA gene amplification, and sequencing

The 341F (Illumina_16S_341F 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WG CAG), and 805R (Illumina_16S_805R 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) universal primers with adapter sequences were used ⁴⁰ for amplification of the V3-V4 hypervariable region of 16S rRNA gene.

For the first step PCR, 5 µl of amplicon PCR forward primer (1 µM), 5 µl of amplicon PCR reverse primer (1 µM), 2.5 µl of microbial DNA (5ng/µl) and 12.5 µl of 2X KAPA HotStart PCR Mix (Roche, Switzerland) were used at 95 °C initial denaturation for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were cleaned up with AgencourtAMPure XP beads (Beckman Coulter Genomics, Fullerton, CA, USA) following the manufacturer's protocol.

In a second PCR step, dual indices and Illumina sequencing adapters were attached using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2X KAPA HotStart PCR Mix, and 10 µl of nuclease free water with thermocycling at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The final PCR products were cleaned with AgencourtAMPure XP beads, and final concentrations of samples were measured with Qubit™ dsDNA HS Assay Kit. Amplicon libraries was sequenced on a lane of the Illumina® MiSeq platform.

Bioinformatics

Sequenced raw data was converted to FASTA format. Quality control of reads were performed using QIIME 2 software ⁴¹. Reads with Phred scores lower than 20, primer and barcode sequences, and chimeric sequences were filtered out using DADA2 software ⁴². Taxonomic assignment of each cluster was carried out using QIIME2 software to match a representative sequence from each OTU to a sequence from the GreenGenes database.

Statistical analyses.

Richness and evenness of the samples were analysed using Chao1 and Shannon diversity indexes. The indexes of groups were compared to each other using Student's t-test. Beta-diversity of the samples were analysed and compared using the first three principal components. Mean relative abundances of the twenty phyla and fifteen genera were charted compared for each pair of groups. Microbiota profiles of each group were compared to each other as previously described ⁴³. Briefly, Logfold changes (Log10) in relative abundance of the genera were calculated for each group, and pairwise comparisons between groups were performed. Robust response screening analysis was performed in JMP Pro 13 (SAS Institute Inc., Cary, NC) in order to evaluate the differences in genera relative abundance between pair of groups. A false discovery rate (FDR) correction was applied, and statistical significance was declared at FDR LogWorth of 1.3 (equivalent of a *p*-value of 0.05), and 2 (equivalent of a *p*-value of 0.01). Subsequently, the log fold change was plotted versus the Robust FDR LogWorth value using bubble plot graphs in JMPPro 13. Genera mean relative abundance defined the bubbles' size, and effect size was indicated by the bubbles' colouring.

Declarations

Author contributions

V.B. assisted supervision of the laboratory work, analysed the data, and wrote the manuscript. S.G. collected the samples, performed laboratory work, and wrote part of the manuscript. O. B. obtained the funding, designed, and supervised the study, and critically evaluated the manuscript.

Data availability

Sequences are available on the MG-RAST metagenomics analysis server at <https://www.mg-rast.org/mgmain.html?mgpage=project&project=mgp101832>.

Competing Interests

The authors declare no competing financial interests or other competing interests.

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Tables

Table 1. The number of samples in each group

Group	TA_rich	TA_poor	Control	RawExtract
Number	6	3	4	2

Table 2. Shannon and Chao1 alpha-diversity indexes of the samples (Mean ± s. d.).

Group	Shannon	Chao1
TA_rich	3.89±0.57	201.72±46.85
TA_poor	3.36±1.95	136.07±83.79
RawExtract	2.75±1.84	185.86±22.83
Control	4.36±2.28	157.67±25.13

Figures

Figure 1.

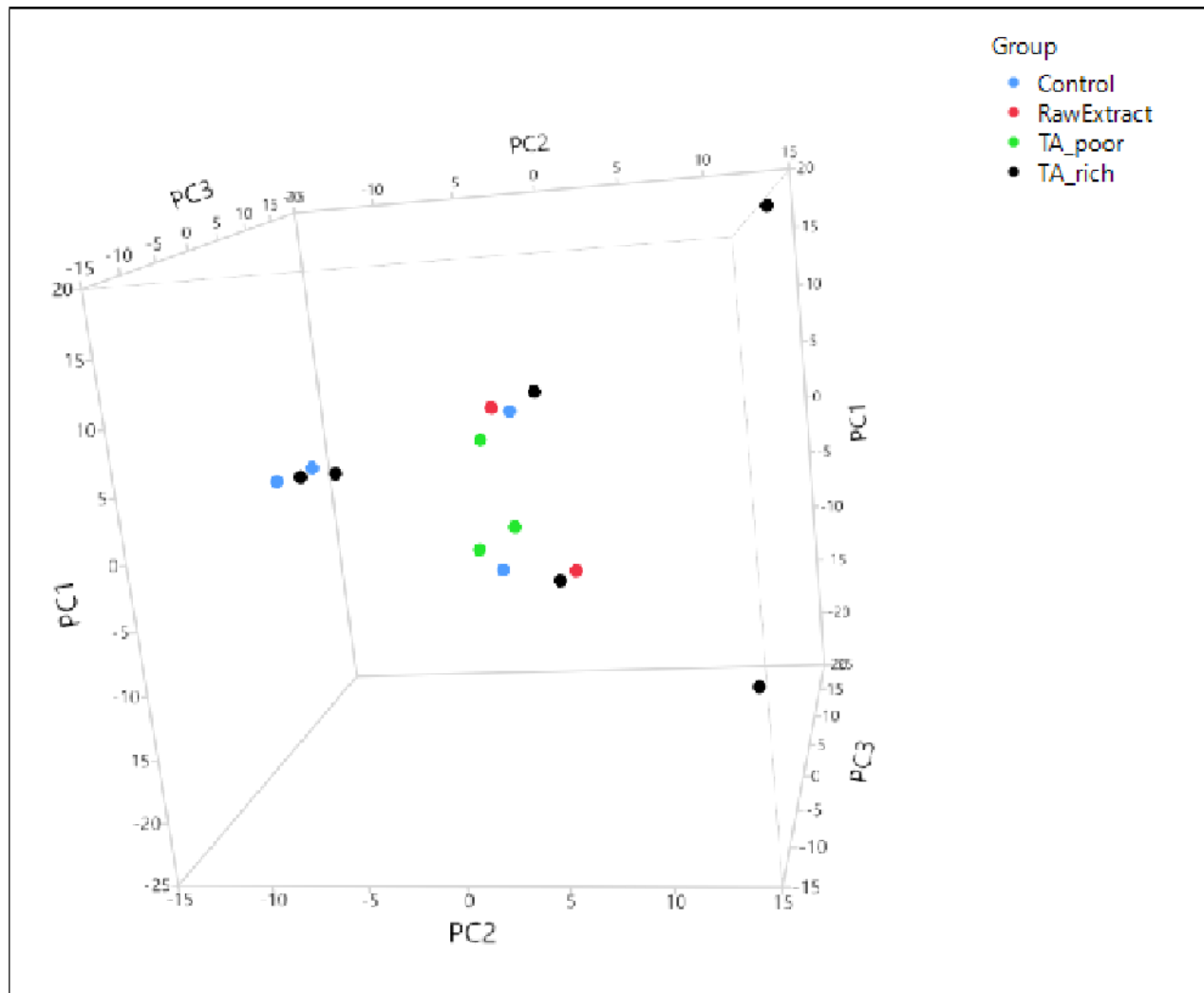


Figure 1

3D scatterplot of the first 3 PCs calculated for the microbiota composition of the samples.

Figure 2

Relative abundances of bacterial phyla in all feeding groups.

Figure 3

Figure 4

Comparison of the microbiota profiles of TA_rich vs RawExtract feeding groups (line at 1.3 (---) = p -value 0.05, line at 2 (—) = p -value 0.01 adjusted for FDR) The log fold change in genera relative abundances in samples from TA_rich group comparing to RawExtract group samples is plotted versus the corrected robust false discovery rate (FDR) LogWorth (i.e., $\log_{10}P$). Size of the circles represents the mean relative abundance of each genus, and colour represents the effect size.

Figure 5

Comparison of the microbiota profiles of TA_rich vs TA_poor feeding groups (line at 1.3 (---) = p -value 0.05, line at 2 (—) = p -value 0.01 adjusted for FDR) The log fold change in genera relative abundances in samples from TA_rich group comparing to TA_poor group samples is plotted versus the corrected robust false discovery rate (FDR) LogWorth (i.e., $\log_{10}P$). Size of the circles represents the mean relative abundance of each genus, and colour represents the effect size.

Figure 6

Comparison of the microbiota profiles of TA_rich vs Control feeding groups (line at 1.3 (---) = p -value 0.05, line at 2 (—) = p -value 0.01 adjusted for FDR) The log fold change in genera relative abundances in samples from TA_rich group comparing to Control group samples is plotted versus the corrected robust false discovery rate (FDR) LogWorth (i.e., $\log_{10}P$). Size of the circles represents the mean relative abundance of each genus, and colour represents the effect size.

Figure 7

Comparison of the microbiota profiles of TA_poor vs RawExtract feeding groups (line at 1.3 (---) = p-value 0.05, line at 2 (—) = p-value 0.01 adjusted for FDR) The log fold change in genera relative abundances in samples from TA_poor group comparing to RawExtract group samples is plotted versus the corrected robust false discovery rate (FDR) LogWorth (i.e., log₁₀P). Size of the circles represents the mean relative abundance of each genus, and colour represents the effect size.

Figure 8

Comparison of the microbiota profiles of RawExtract vs Control feeding groups (line at 2 (—) = p-value 0.01 adjusted for FDR) The log fold change in genera relative abundances in samples from RawExtract group comparing to Control group samples is plotted versus the corrected robust false discovery rate (FDR) LogWorth (i.e., log₁₀P). Size of the circles represents the mean relative abundance of each genus, and colour represents the effect size.

Supplementary Files

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