Dietary supplement based on stilbenes: A focus on gut microbial metabolism by the: In vitro simulator M-SHIME®

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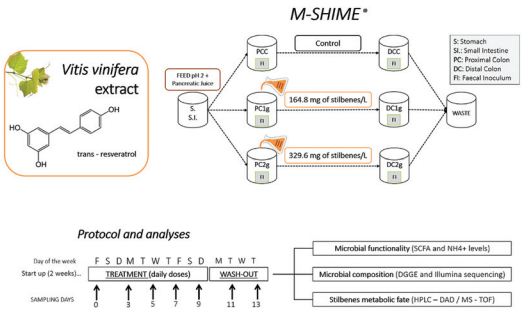
Camilla Giuliani, Massimo Marzorati, Marzia Innocenti, Ramiro Vilchez-Vargas, Marius Vital, Dietmar H. Pieper, Tom Van de Wiele and Nadia Mulinacci*

Effects of stilbenes on human microbiota were investigated in *in vitro* simulator technology M-SHIME® for the test of repeated daily intake.



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Food & Function

PAPER

Dietary supplement based on stilbenes: a focus on gut microbial metabolism by the *in vitro* simulator M-SHIME®

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Polyphenols and intestinal microbiota can influence each other, modifying metabolism and gut wellness. Data on this mutual effect need to be improved. Several studies on the biological activities of resveratrol and derivatives have been carried out, but the effects of a continuous administration of stilbenes on gut microbiota have not yet been investigated. This study evaluated the effects of an extract from *Vitis vinifera*, containing a combination of *t*-resveratrol and ε-viniferin, on intestinal microbiota, using the advanced gastrointestinal simulator M-SHIME®. A triple M-SHIME® experiment was performed using two concentrations of the extract (*i.e.* 1 and 2 g L^{-1}), simulating a continuous daily intake. The effects were evaluated in terms of microbial functionality (SCFA and NH_4^+) and composition (DGGE and Illumina sequencing), since the microbiological aspect has been less considered so far. The treatment induced changes in microbial functionality and composition. In fact, the levels of SCFA and NH_4^+ suffered a strong decrease (*i.e.* inhibition of the saccharolytic and proteolytic activity), while DGGE and Illumina showed important modifications of the microbiota composition, associated with an imbalance of the colonic microbiota (*i.e.* increase in the relative abundance of Enterobacteriaceae). HPLC-DAD-TOF-MS analyses demonstrated that the metabolism of *t*-resveratrol and other stilbenes was inhibited by continuous administration. Our results suggest M-SHIME® as an explorative tool to define the dosage of food supplements, in particular to simulate effective continuous administration in humans.

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1 Introduction

Resveratrol and its derivatives, polyphenols classified as stilbenes, are included in different foods and plants such as grapes, red wine, some kinds of tea, berries and peanuts.[1] They have many biological activities, such as antioxidant and anti-inflammatory effects, cardio protection and cancer prevention.[2–4] Although a large number of studies have been performed, results are contradictory.[5] Their metabolic fate after oral administration should be one explanation. Only 5–10% of dietary intake of polyphenols is absorbed in the small gut while the residual part reaches the large intestine.[6] Colon microbiota is able to metabolize stilbenes, until complete disappearance of the original molecules in short times.[4,7] Metabolites could maintain the same positive properties of the parent compound or be even toxic for both bacteria and humans.[8] Colonic absorption of stilbenes and their metabolites is estimated between 35% and 80% and the wide range is due to inter-individual microbial diversity and the consequent different metabolism.[9] At the same time, stilbenes' influence on gut microbiota is not much studied. From 2009 to 2014 at least 40 studies in humans were conducted on resveratrol,[10] but only one focused on the microbiota balance after a single administration.[9]

Polyphenols have a well-known *in vitro* antimicrobial activity and they are able to modify the composition and the activity of intestinal microbial communities. The main mechanisms involved are the creation of H-bonds and the ability to provide a huge stress to microbial cells.[6,8,11,12] Despite the antimicrobial activity of *t*-resveratrol studied *in vitro* on different bacterial species,[13] there is not much information about its effects on intestinal bacteria, composition and relative ratios between different microbial groups. So far, there have been few studies on stilbenes, even derived from different plants or synthesized, but still the data about their activity on

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intestinal microorganisms are incomplete.[14–16] Therefore, it is important to investigate more about the mutual effects of polyphenols and the microbial community in order to understand the mechanisms of interaction responsible for these effects.

According to different structures and related absorption, it is important to define the highest concentration that does not affect the microbial community, in order to enhance polyphenol intake.

Among the available simulators, the Mucus SHIME® (M-SHIME®) is a validated *in vitro* model useful to investigate changes occurring in the microbial community and adhesion of bacteria to the mucosal part in gastrointestinal tracts that are not easily accessible *in vivo*.

The aim was to investigate the effects of an extract from *Vitis vinifera*, containing *t*-resveratrol and ε-viniferin, on intestinal microbiota, using M-SHIME®. A triple M-SHIME® experiment was carried out using two different concentrations of the extract. The effects of the extract on the microbial communities were evaluated in terms of metabolism (SCFA and NH_4^+ concentrations) and taxonomic composition (DGGE and Illumina sequencing). HPLC-DAD and HLPC-DAD-TOF-MS analyses were performed to investigate the microbial metabolism of stilbenes.

2 Experimental

2.1 Abbreviations

All samples, in plots and throughout the text, were named according to abbreviations in Table 1.

2.2 *Vitis vinifera* extract

Vitis vinifera extract from grapevine shoots was purchased from Breko GmbH (Bremen, Germany). Stilbenes are declared in label to be around 30% of the total weight of the extract. The supplier also declared a total content of 2% of protein and 0.7% of fat, while the residual part is calculated as carbohydrates without any other addition of carriers to the extract. 40 g of powder was pre-treated with 100 mL of CH_2Cl_2 in order to eliminate lipophilic compounds, and the solvent was removed through vacuum filtration.

2.3 Batch experiments

Batch incubations of colonic microbial communities were performed to define experimental concentrations. Faecal slurries were incubated with the extract, as described previously.[17] Briefly, the faecal material obtained from 1 healthy volunteer (female, 30 years old) was suspended in phosphate buffer, and homogenized in a stomacher. The suspension was centrifuged and the supernatant was used as an inoculum. Different concentrations (0 for control, 1.5, 2.5, 3 and 4 g of extract per L) were selected for the experiments, and each dose was taken in duplicate using 1 mL of faecal slurry and 4 mL of feed added into Hungate tubes capped with butyl-rubber stoppers. Each tube contained a different concentration of the extract. The system was flushed with N_2 and incubated at 37 °C for 24 h. 5 mL of the sample was used for the extraction of SCFA and HPLC-DAD-TOF-MS analysis.

Table 1 Samples abbreviations

Abbreviation	Legend
PCC dX	Proximal colon control day X (0,3,5,7,9,11,13)
DCC dX	Distal colon control day X (0,3,5,7,9,11,13)
PC1g dX	Proximal colon 1 g L^{-1} day X (0,3,5,7,9,11,13)
DC1g dX	Distal colon 1 g L^{-1} day X (0,3,5,7,9,11,13)
PC2g dX	Proximal colon 2 g L^{-1} day X (0,3,5,7,9,11,13)
DC2g dX	Distal colon 2 g L^{-1} day X (0,3,5,7,9,11,13)

2.4 M-SHIME® experiments

The M-SHIME® contains, in addition to the traditional luminal microbial community, some mucin slots to host surface-attached microbes.[18,19] The slots (K1-carrier, AnoxKaldnes AB, Lund, Sweden) were submerged in mucin-agar, prepared by boiling mucin and agar in water until they formed a gel. The experiment was started by adding 500 mL of the selected feed to the proximal colon unit and 800 mL into the distal vessel. 80 mucin slots were included in both colon vessels. Inoculation of colon reactors was performed with 40 mL of a 1 : 5 dilution from fresh stools.[20] The vessels' pH was monitored through an electrode and maintained stable in narrow ranges during the experiment (5.6–5.9 in the PC and 6.5–6.9 in the DC). An initial incubation of 18 h was carried out for pH stabilization, then 140 mL nutritional medium and 60 mL pancreatic juice were supplied to all proximal colon compartments three times per day. The M-SHIME® was kept at 37 °C and under anaerobic conditions by flushing for 10 min with N_2. Three couples of PC and DC vessels ran simultaneously: one couple for control and the other for treatment with the extract [Fig. 1]. After a stabilization period of 2 weeks, daily doses of the extract were administrated into the PC vessel for 10 days [Fig. 1]. At the end of the treatment, a 4-days washout period was carried out. Two doses were used for the experiment, 1 and 2 g L^{-1}. Three times per week, 20 mL of liquid sample were collected from each colon vessel. 1 mL was centrifuged and the pellet was stored at −20 °C for DNA extraction. The residual liquid was stored at −20 °C for metabolic analyses.

There are pieces of evidence in the past that the SHIME®, as well as other chemostat models inoculated with a faecal microbial community, allows the creation of highly reproducible conditions when similar environmental factors are applied.[18,21] In this way, it is possible to increase the number of variables in the system (*i.e.* different diets or treatments; different colon region-specific conditions). The SHIME® has been designed to perform longitudinal studies to follow-up the adaptation of the microbial community composition and functionality. By collecting sequential samples, it is possible to have a higher number of observations of an experimental unit. For the purpose of this work, the experiment was so considered as if working with 1 individual.

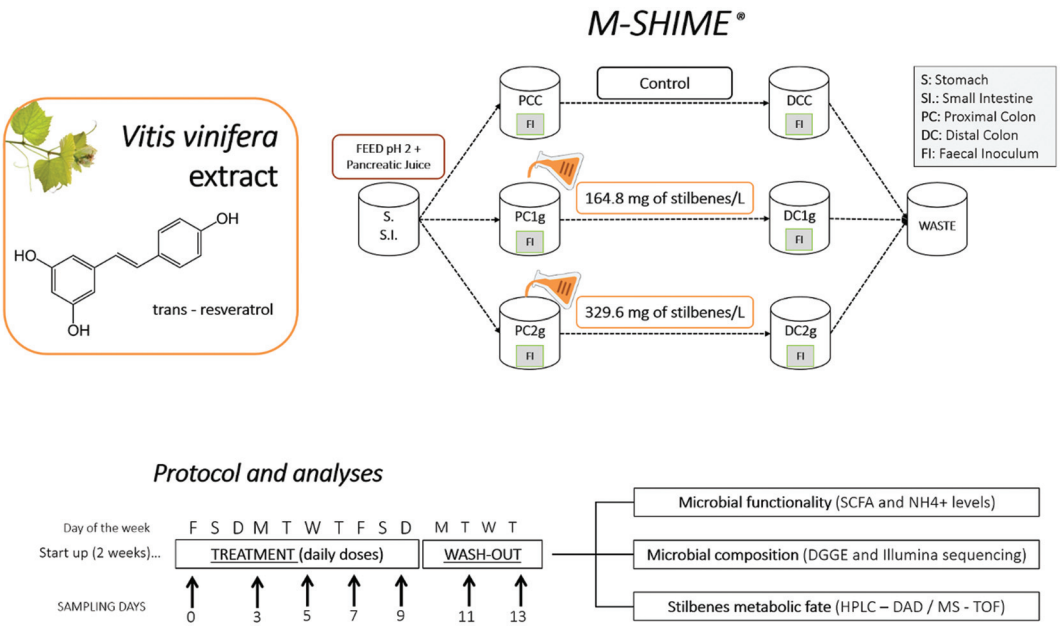

Fig. 1 M-SHIME® experiment design and protocol.

2.5 HPLC-DAD-TOF-MS analysis of colonic metabolites

For HPLC-DAD-TOF-MS analysis two samples from the PC control vessel were added with the extract at experimental concentrations, to evaluate time zero profiles (PCCVINEA1g and PCCVINEA2g). These samples were then compared with PC1g, DC1g, PC2g and DC2g collected during the experiment (d3, d5, d7, d9, and d13) and before the administration (PC1gd0, DC1gd0, PC2gd0 and DC2gd0) to identify any possible interference due to the feed. The samples for HPLC-DAD analysis were pre-treated in order to obtain a clean solution as described previously.[22] The supernatant was recovered and used for analysis. Qualitative analyses were performed using a HP 1100 liquid chromatograph coupled with a HP 6200 series MS/TOF (Agilent Technologies, USA). A 150 mm × 2 mm i.d., 4 μm, RP-18, Synergi Fusion column (Phenomenex, USA) was used. Eluents selected were (A) H_2O pH 3.2 with formic acid and (B) CH_3CN. The used multi-step linear solvent gradient was: 0–2 min, 20–20% B; 2–25 min, 20–50% B; 25–27 min, 50–100% B; 27–33 min, 100–100%; 33–35 min, 100–20%, equilibration time 10 min; flow rate 0.2 mL min^{-1}. The UV-Vis spectra were recorded in the range of 200–500 nm and the chromatograms were acquired at 240 nm, 254 nm, 280 nm, 307 nm, and 320 nm. MS spectra were acquired using a dual-ESI source in negative polarity with a 100 V fragmentor, 3800 V capillary voltage, and 350 °C gas temperature.

Quantitative analysis was carried out using a HP 1200 liquid chromatography equipped with a DAD detector (Agilent Technologies, USA), using the same column and method applied for qualitative analysis. The stilbenes content was determined by HPLC-DAD. A MilliQ water solution of *t*-resveratrol (Sigma Aldrich) 0.0214 mg mL^{-1} was used to create a calibration curve at 307 nm in the range of linearity 0.04–0.17 μg with R^2 = 0.999. The stilbenes content is expressed as *t*-resveratrol, as seen previously.[23]

2.6 Metabolite measurement

SCFA analysis was performed according to a standard method.[24] A liquid–liquid extraction with diethyl ether was applied on frozen samples, after the addition of H_2SO_4 and the internal standard. SCFA quantitative analysis was performed by capillary gas chromatography coupled with a flame ionization detector (GC-FID), to measure acetic, propionic, butyric, isobutyric, caproic, isocaproic, valeric and isovaleric acids.

NH_4^+ was analysed by steam distillation according to standard methods (4500-NH3 B; APHA, 1992). The determination of total ammoniacal nitrogen (TAN) in liquid luminal samples was performed through the quantification of NH_4^+ by the addition of MgO, distillation of NH_3 into boric acid solution and subsequent back-titration.

2.7 DNA extraction

Bacterial DNA from luminal samples was extracted as described earlier,[25] using a lysis buffer (TrisEDTA, NaCl, PVP40, SDS, water) and glass beads for FastPrep. DNA extract from mucin was obtained using bead beating with the same lysis buffer. Extraction was performed with phenol–chloroform and EtOH/NaOAc was used for precipitation.[26] The samples were dissolved in TrisEDTA 1× and stored at −20 °C. The concentration and quality were verified by using the Glomax Multi Detection system (Promega, USA) and 2% agarose gel electrophoresis.

2.8 PCR-denaturing gradient gel electrophoresis (DGGE)

In order to investigate composition changes in the microbial communities a PCR-DGGE was assessed as described previously.[20] All samples for PCR were prepared starting from 1 : 10 dilutions. A PCR-DGGE was carried out for total bacteria; two nested PCR protocols were performed for *Lactobacilli* and *Bifidobacteria*. Details are reported in Table 2. External PCR for *Lactobacilli* was performed using 159F/667R primers under the following conditions: initial denaturation 95 °C for 7 minutes; 35 cycles at 94 °C for 1 minute, 56 °C for 1 minute; 72 °C for 2 minutes and a final extension at 72 °C for 10 min.[27] External PCR for *Bifidobacteria* was performed using 164F/662R primers under the following conditions: initial denaturation 95 °C for 7 minutes; 35 cycles at 94 °C for 1 minute, 62 °C for 1 minute; 72 °C for 2 minutes and a final extension at 72 °C for 10 min.[28] Internal PCR (*Lactobacilli* and *Bifidobacteria*) and amplification for total bacteria of 16S rRNA gene were performed with primers 338F-GC and 518R. Cycling conditions were: initial denaturation 94 °C for 5 minutes; 30 cycles at 95 °C for 1 minute, 53 °C for 1 minute; 72 °C for 2 minutes and a final extension at 72 °C for 10 min.[29] PCR products were separated on denaturing gradient of polyacrylamide gel (DGGE). DGGE (Denaturing Gradient Gel Electrophoresis) was performed[30] using the INGENY system (Ingeny International BV, The Netherlands). PCR fragments were loaded on an 8% polyacrylamide gel in 1′TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). Polyacrylamide gels were prepared with 45–60% denaturing gradients for total bacteria and *Lactobacilli*, and 50–65% for *Bifidobacteria*. Electrophoresis was run for 16 hours at 60 °C and 120 V. Staining and analysis of the gels were performed as described previously.[31] The normalization and analysis of DGGE gel patterns were performed with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). During the processing, lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization and band classes were detected. A matrix of similarities for the densitometric curves of band patterns was calculated based on the Pearson product–moment correlation coefficient. A composite dataset was created by merging the information from all the band patterns in order to obtain a combined dendrogram – using the UPGMA linkage – containing the information from the gels on total bacteria, *Bifidobacteria* and *Lactobacilli*.

2.9 Illumina sequencing

Samples collected from all vessels (PCC, DCC, PC1g, DC1g, PC2g, DC2g) at different times (d0, start up; d9, end of the treatment; d13 after washout) were selected for Illumina sequencing. Genomic DNA was extracted as previously described[25] with the following modifications. The samples were suspended in 1 mL Tris/HCl (100 mM pH 8.0), supplemented with 100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) sodium dodecyl sulphate, transferred to a 2 ml Lysing Matrix E tube (Qbiogene, Alexis Biochemicals, Carlsbad, CA), subjected to mechanical lysis in a Fast Prep-24 instrument (40 s, 6.0 m s^{-1}) and purified as described. The V1–2 region of the 16S rRNA gene was amplified as previously described.[32] In the first 20 cycles of PCR reaction the 16S rRNA gene was enriched using the well-documented 27F and 338R primers[33,34] as previously specified.[35] Libraries were sequenced in a MiSeq platform (Illumina).

Paired-end raw sequences were assembled and aligned using mothur[36] and subsequently filtered as previously described.[32] Briefly, reads were clustered allowing for two mismatches using mothur. The dataset was then filtered to consider only those phylotypes that were present in at least one sample at a relative abundance >0.1% or were present in all the samples at a relative abundance >0.001%. A total of 1 591 358 reads were obtained, and grouped into 1747 phylotypes. Rarefaction curves and statistics were generated using the package vegan from the R program. All phylotypes were assigned a taxonomic affiliation based on the naïve Bayesian classification (RDP classifier)[37] with an 80% of confidence. The obtained tables, ranking the identified phylotypes and their abundance, have been used to describe their relative abundance – at order level – in each sample and to produce PCA (principal component analysis) graphs.

Table 2 Primer and cycling conditions used for PCR-DGGE

Primers	Sequences	Temperature-time program
Specific PCR for *Lactobacilli*[27]		
SGLAB0159f*	GGAAACAG(A/G)TGCTAATACCG	(a) 7′; 95 °C
SGLAB667r*	CACCGCTACACATGGAG	(b) 1′; 94 °C/1′; 56 °C/2′; 72 °C (35×)
		(c) 10′; 72 °C
Specific PCR for *Bifidobacteria*[28]		
BIF164f*	GGGTGGTAATGCCGGATG	(a) 7′; 95 °C
BIF662r*	CCACCGTTACACCGGGAA	(b) 1′; 94 °C/1′; 62 °C/2′; 72 °C (35×)
		(c) 10′; 72 °C
PCR for total bacteria[29]		
PRBA338F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGACTCCTACGGGAGGCAGCAG	(a) 94 °C, 5′
518R	ATTACCGCGGCTGCTGG	(b) 95 °C, 1′/53 °C, 1′/72 °C, 2′ (30×)
		(c) 72 °C, 10′

2.10 Live subject statement

All experiments were performed in compliance with the relevant laws and institutional guidelines. An informed consent was obtained from the healthy volunteer involved in the experiment.

3 Results

3.1 Stilbenes content

The total content of stilbenes was determined through HPLC-DAD-TOF-MS. A triplicate of an EtOH solution of the extract (1.03, 1.15 and 1.14 mg mL^{-1}) was prepared to quantify *t*-resveratrol, *trans*-ε viniferin and other stilbenes. Stilbenes were identified by comparing the UV and MS spectra and according to the supplier report.[38] *t*-Resveratrol and *trans*-ε viniferin resulted in more abundant compounds, with a concentration of 83.4 ± 1.63 and 61.6 ± 1.31 mg g^{-1} of extract respectively. The total content of stilbenes was assessed to be 164.8 mg g^{-1} of extract [Table 3]. Two main signals were recorded; they were identified as *t*-resveratrol (rt = 14.9 min; 227.068 *m*/*z*) and *trans*-ε viniferin (rt = 21.6 min; 453.13 *m*/*z*). Other minor compounds were also identified as the resveratrol dimer (rt = 13; 453 *m*/*z*), myabenol (rt = 23; 679.188 *m*/*z*) and the resveratrol tetramer (rt = 23.7 min; 905.244 *m*/*z*). The same results were obtained for both the tested concentrations.

Table 3 Total content of stilbenes determined in the extract and expressed as *t*-resveratrol. The data are the mean of triplicates

Compounds	Concentration (mg g^{-1} of extract)
Resveratrol dimer	3.40 ± 0.3
t-Resveratrol	83.43 ± 1.63
trans-ε viniferin	61.58 ± 1.31
Resveratrol tetramer	12.51 ± 0.22
Myabenol C	3.91 ± 0.02
Total	**164.82 ± 3.42**

3.2 Dosage selection

The doses were selected considering different criteria and taking into account previous human studies with stilbenes, including healthy volunteers and participants with medical conditions.[10] From the literature, there emerged a wide range of daily doses in terms of *t*-resveratrol (from 8 mg per day to 3000 mg per day) and periods of oral administration (from a single dose up to 1 year daily administration).[10] In light of these findings and excluding the lowest doses, a set of concentrations were selected for preliminary batch experiments. The following concentrations were used: 1.5, 2.5, 3 and 4 g of extract per L, all with the corresponding stilbenes content within the literature range.

After batch experiments, the higher doses (3 and 4 g L^{-1}) were excluded for the M-SHIME® experiment, considering the stilbenes' solubility in the media.

The lower concentrations (1.5 and 2 g L^{-1}) provided the *t*-resveratrol amount comparable to the mean values reported in other studies on oral administration. Finally, lower concentrations were not considered for the M-SHIME® test since the goal of this study was to enhance the administration of *t*-resveratrol and other stilbenes.

3.3 Batch experiments

SCFA levels, in controls and samples administered with the extract after 24 h, were investigated by GC-FID. Two replicates, A and B, were taken with four different concentrations: 1.5, 2.5, 3 and 4 g L^{-1} of extract.

The levels of the main SCFA in control samples are shown in Fig. 2. Despite the weak variations through different dosages, the SCFA levels of the treated batch were comparable

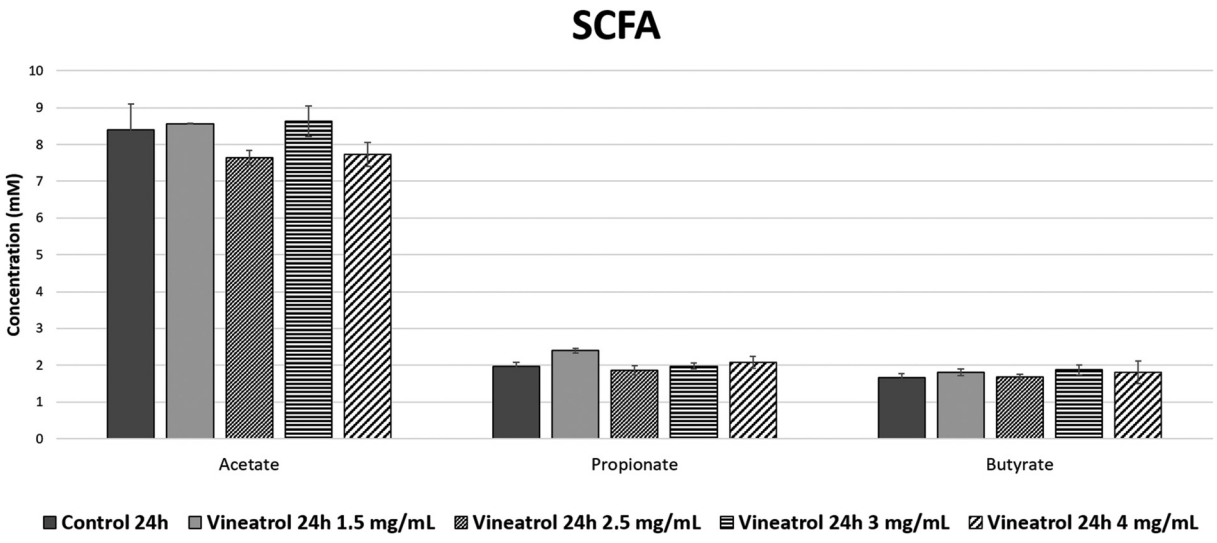

Fig. 2 Duplicates average of the main SCFA concentrations in samples from 24 h batch experiments.

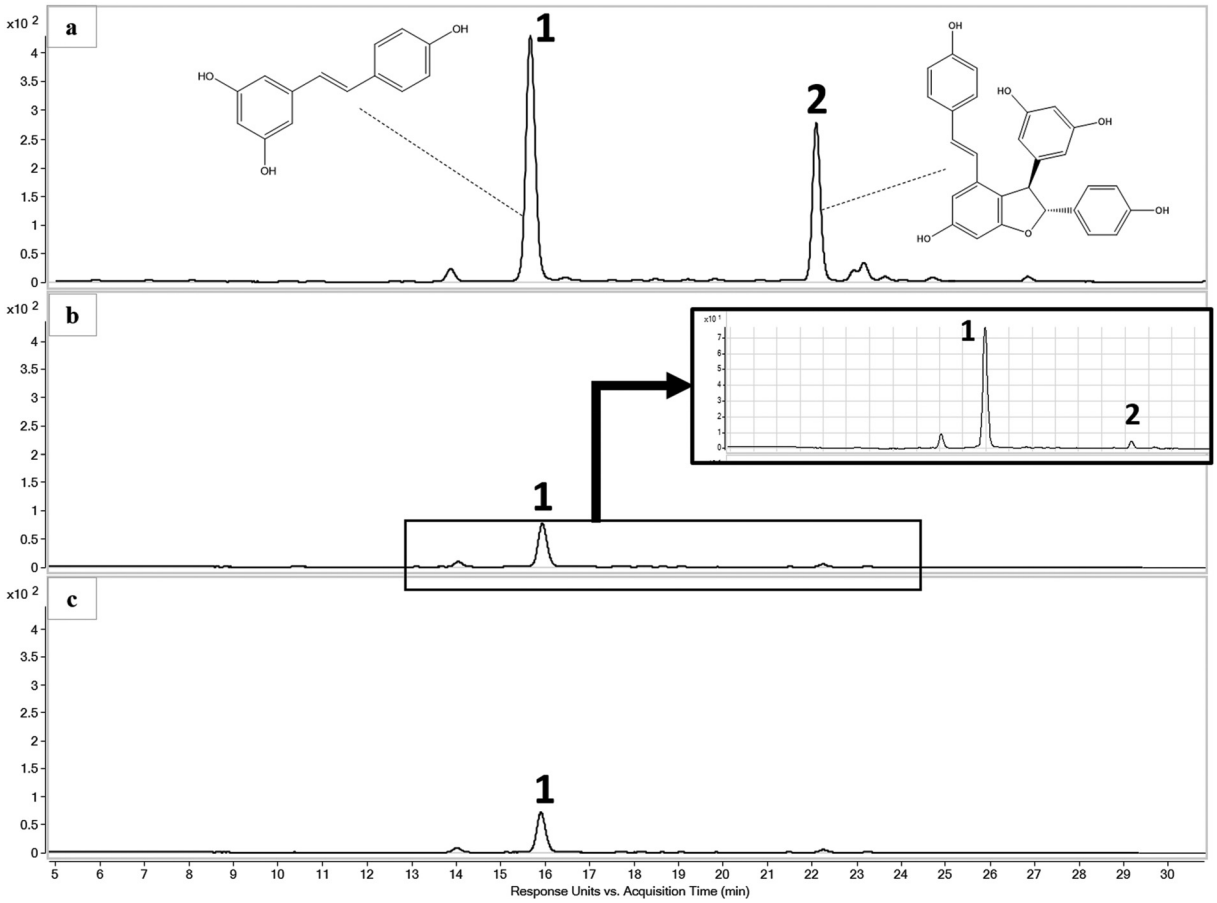

Fig. 3 HPLC-DAD profiles at 307 nm of samples from 24 h batch experiments. Control *t* 0 h (1), replicates A (2) and B (3) after 24 h at higher concentration (2.5 g L^{-1}) are reported at the same scale of Abs. The main compounds were identified as *t*-resveratrol (1) and *trans*-ε viniferin (2).

to the control samples and the microbial activity did not seem to be affected by the presence of the extract.

Stilbenes' metabolic fate was investigated comparing the control samples added with the extract and the samples treated by HPLC-DAD-TOF-MS analysis. Fig. 3 shows the profiles from the control and replicates A and B of the samples treated with 2.5 g L^{-1} of extract. The concentrations of the main components showed a strong decrease and the *trans*-ε viniferin's signal almost disappeared. The same trend was highlighted for the other concentrations indicating an interaction between stilbenes and the microbial community.

3.4 Effects on microbial metabolism in M-SHIME®: SCFA and NH_4^+

According to the batch findings and considering the stilbene solubility, the doses for the M-SHIME® experiments were assessed at 2 g L^{-1} and 1 g L^{-1}, as more suitable dosages, also for continuous administration for several days. The effects of the extract administration in the M-SHIME® were evaluated in terms of microbial functionality, in particular SCFA and NH_4^+ production.

SCFA levels significantly decreased after treatment with both selected doses [Fig. 4a]. PC and DC metabolism showed that similar trends and administration of the extracts provided the same effects, even with different doses. Subsequently, during the washout period, SCFA levels showed again an increasing trend. The same behaviour was observed for NH_4^+ concentration during and after treatment [Fig. 4b].

3.5 Effects on microbial composition in M-SHIME®

3.5.1 PCR-DGGE measurement. PCR-DGGE for total bacteria, *Bifidobacteria* and *Lactobacilli* was performed on samples collected immediately before starting the treatment, halfway and end of the treatment and after washout. DGGE images were processed through Bionumerics software to identify patterns and define the clusterization through a composite dataset.

Separation among the control and treated samples was defined [Fig. 5]. PC2g treated samples resulted to be different from the control and the starting point. In particular, correlation values of PC2g d5/d9/d13 compared with PC2g d0 and PCC were low, down to 40%, meaning that the microbial communities changed after administration of the extract. Patterns from DC2g vessels did not show the same evolution and showed 80% correlation, indicating smaller variations among d0 and samples collected during and after the treatment.

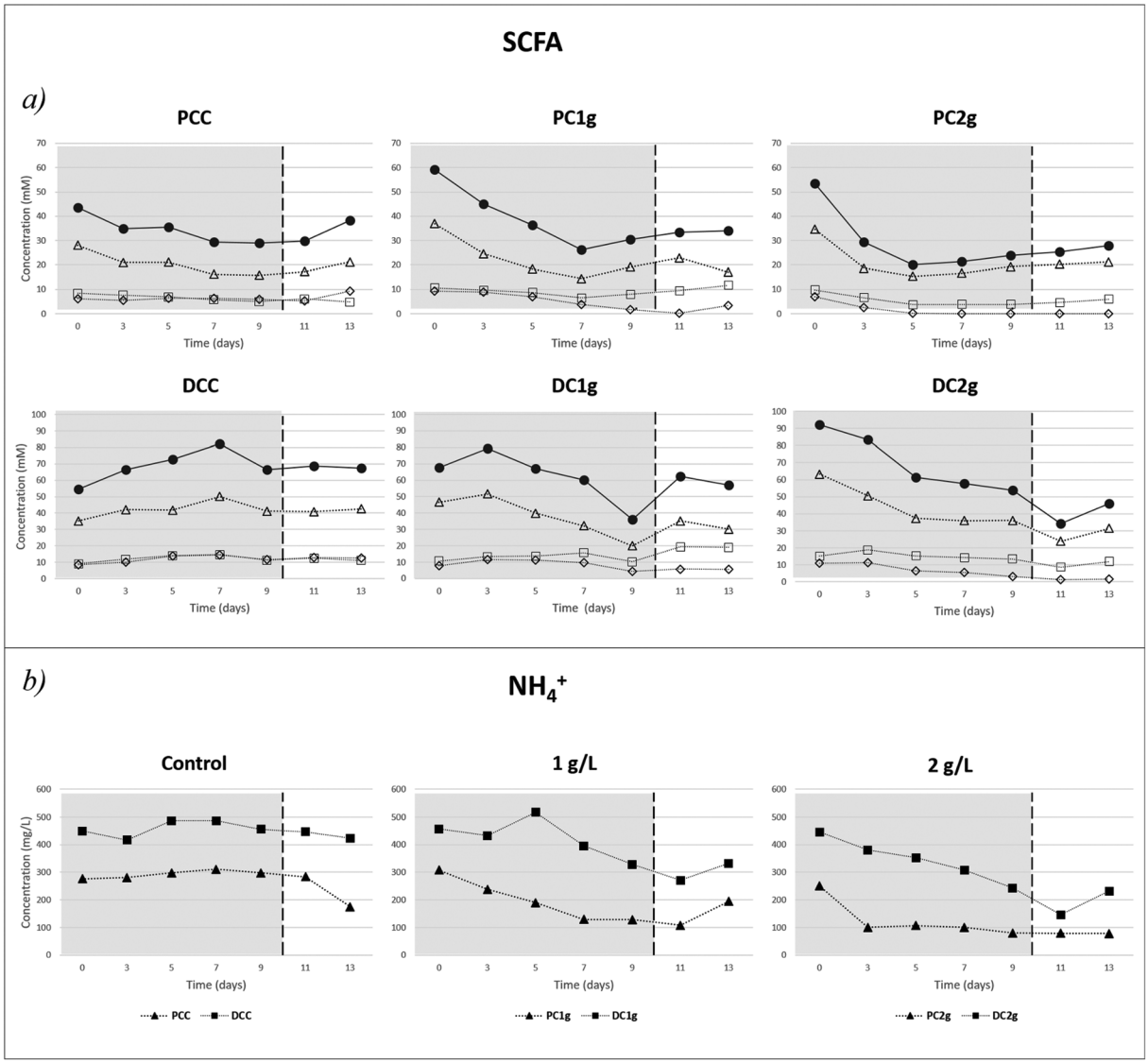

Fig. 4 (a) SCFA concentrations progression during the experiment in terms of acetate (△), propionate (□), butyrate (◊) and total SCFA (●). (b) NH_4^+ concentration during the treatment and washout in PC and DC. Grey areas of charts refer to the administration period.

PC samples treated with the lower concentration demonstrated a weak change compared to the starting point. However, correlation values are around 70% among d0 and following times, meaning that the administration of the extract poorly affected the composition of the microbial communities. The evolution of DC1g at time zero and during/after the treatment did not show significant variations, since the samples clustered together with high correlation (>80%).

In summary, the higher concentration of the extract had a stronger effect on the proximal vessel. For the lower dosage, the effect on PC was consistent with the higher one, but to a lower extent. Effects on DC2g and DC1g were not much evident during the treatment, probably due to lower concentrations reaching these colonic compartments. It is clear that the extract had an effect on the microbial composition at both the tested concentrations (2 and 1 g L^{-1}).

3.5.2 Illumina sequencing. As shown in Fig. 6 and 7, microbial communities from the control and treated vessels were compared at the order level. Despite the intrinsic variability, microbial communities treated with a higher dosage showed a strong change during the treatment. In PC2g d9 and PC2g d13 collected from the lumen, it is possible to observe that the order Enterobacteriales was enriched and the abundance of order Bacteroidales decreased during the treatment. This trend in the PC2g vessel was more intense in mucin than in lumen samples. The interruption of treatment did not provide a fast return to original conditions, since the microbial community from the lumen after the washout (PC2g d13) was similar to the microbial communities enriched during the treatment. In mucus samples, after the washout the composition returned close to starting point conditions. Comparable behaviour was observed also in samples DC2g d9 and DC2g

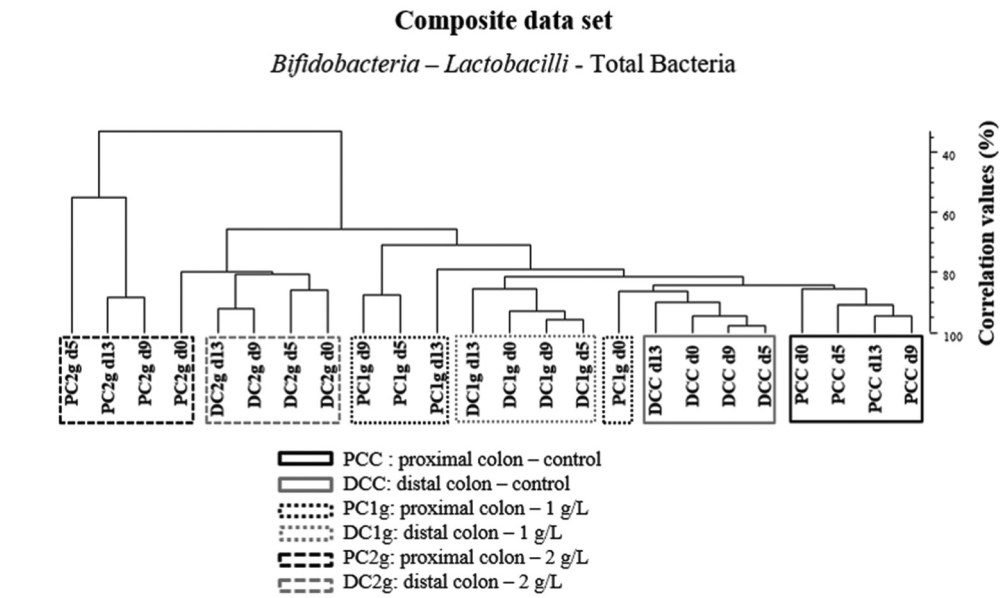

Fig. 5 Composite dataset of the DGGE patterns from PCR for total bacteria, *Bifidobacteria* and *Lactobacilli*.

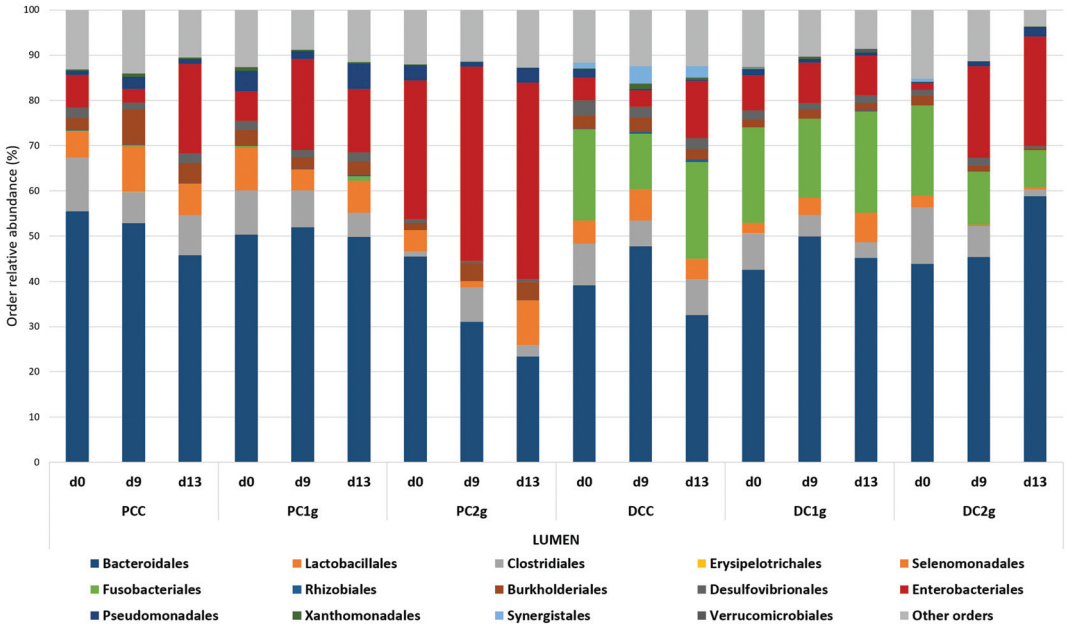

Fig. 6 Comparison of the microbial distribution data from Illumina sequencing of samples from the lumen, in terms of orders.

d13. The same taxonomic groups dominated the microbial communities after the treatment. The order Synergistales, which was abundant in the inoculum, was outcompeted during the treatment. Also the abundance of the order Erysipelotrichales decreased during the treatment.

In the samples treated with the lower concentration of the extract, a similar evolution was observed. PC1g d9 and PC1g d13, both lumen and mucus, presented the enrichment of the order Enterobacteriales and a corresponding decrease of the order Bacteroidales, but to a lesser extent than the PC2g samples. In this case, the 4 days washout seemed to be enough to revert the effects of the treatment. In DC1g samples, no significant changes were detected. As seen for DC2g, some less represented orders (*i.e.* Synergistales and Erysipelotrichales) were also outcompeted. Other Gram-positive commensal bacteria belonging to the order of Clostridiales, Bacillales and Lactobacillales were less affected by the treatment.

PCA for the lumen and mucus confirmed the previous results [Fig. 8]. In the lumen plot, PC and DC from the control

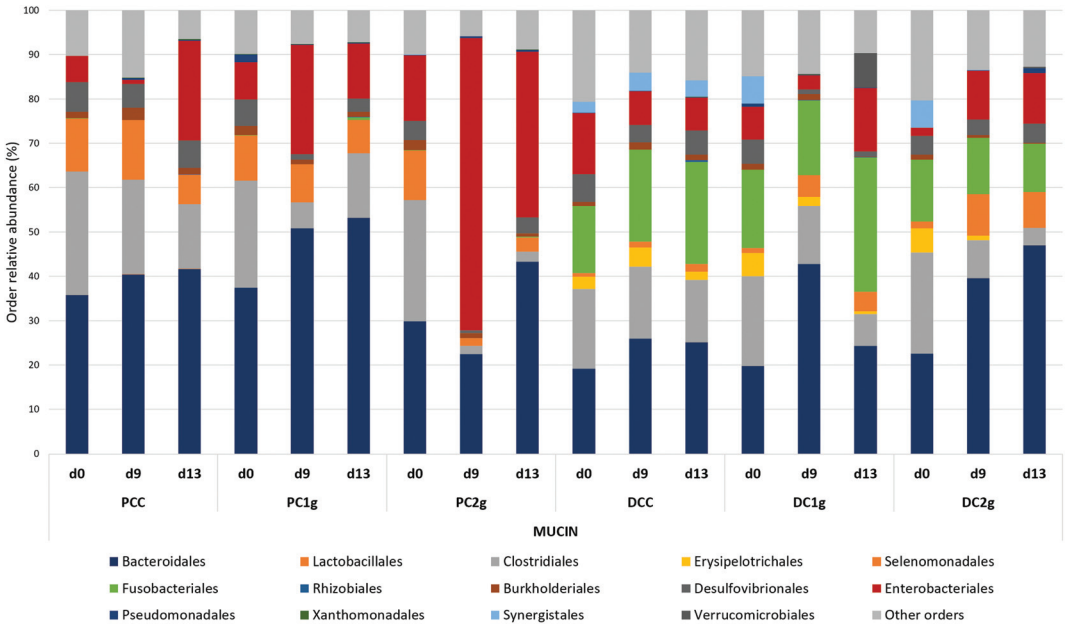

Fig. 7 Comparison of the microbial distribution data from Illumina sequencing of samples from mucin, in terms of orders.

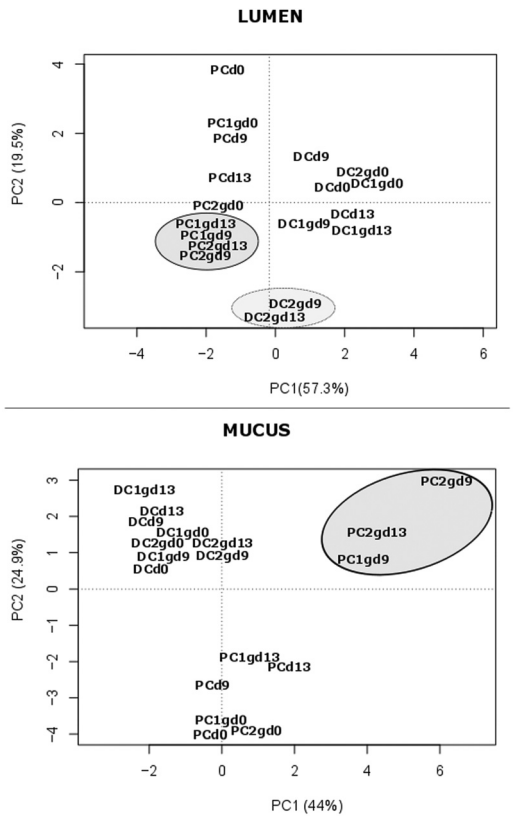

Fig. 8 PCA plots of microbial distribution for LUMEN and MUCUS samples based on Illumina sequencing.

and starting points were separated in two well-defined groups. PC-treated samples showed a significant clusterization. In the same way, DC2g d9/d13 formed a separate cluster from other DC samples. PCA for mucus samples was also consistent with other results. The group including PC2gd9/d13 and PC1g d9, formed a different cluster compared to the PC control and inoculum (d0) indicating that these communities are more affected by the treatment. The DC samples did not show specific patterns and were grouped together.

Calculation of the Shannon and richness indexes did not show significant differences among the samples (data not shown).

3.6 Stilbenes metabolic fate in M-SHIME®

The metabolic fate of *t*-resveratrol and other stilbenes was investigated by HPLC-DAD and HPLC-DAD-TOF-MS analysis. Samples were collected from PC and DC at different times, a blank and a control from PC were treated in order to purify the solution for the analysis.

The HPLC-DAD-TOF-MS analyses were performed to evaluate the differences in all the sample profiles and compounds' identity was confirmed by ESI-TOF analysis. No changes in chromatographic profiles at 307 nm and different sampling times were observed in terms of the relative concentration of the main stilbenes, PCCVINEA1, PCCVINEA2, and all collected samples from PC1g, DC1g, PC2g and DC2g vessels were then analysed to quantify the concentration of the main compounds, using the same elution method and the same column. The concentrations of samples collected at different times are reported in Fig. 9. The trends of all compounds were similar and consistent. In PC samples, concentrations increased quickly for both doses and reached a plateau, then decreased after 4 days washout. Samples from DC showed the same trend, with a slower increase, since the extract was added in the PC and just a part of this was transferred into the DC

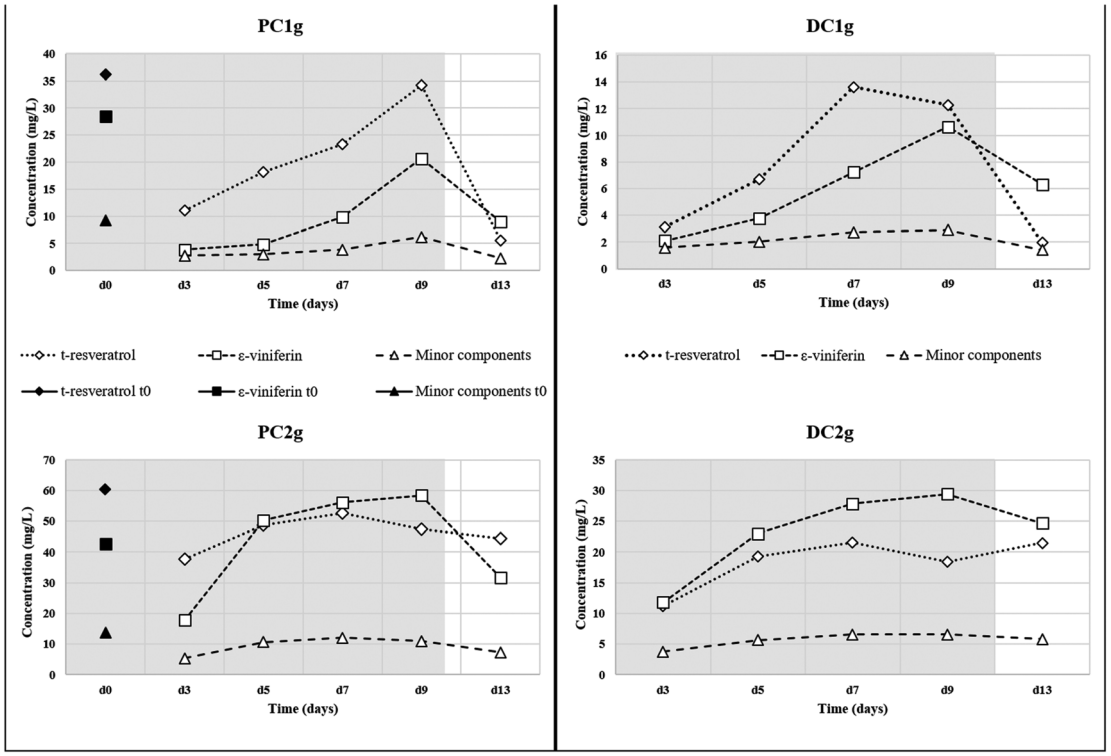

Fig. 9 Main stilbenes concentration during experiment, in PC and DC vessels administered with the extract.

vessel during each cycle. These consistent results confirmed that the extract composition did not change significantly and no relevant metabolism was detected.

4 Discussion

The aim of this work was to highlight the administration effects of a stilbene-enriched extract from *Vitis vinifera* on the intestinal microbial community using a gut simulator. Many aspects were evaluated to investigate the mutual effects of stilbenes in the dietary supplement and gut microbial community. It is reported that *t*-resveratrol and its derivatives suffer strong metabolism after a single oral administration, and their bioavailability is reduced, also due to the intestinal microbial functionality.[9,39,40] At the same time, *t*-resveratrol has a well-known antimicrobial activity,[13] while scant data are available on the *in vivo* metabolism of other stilbenes, such as viniferin. To the best of our knowledge, the continuous administration of *t*-resveratrol, or of a pool of stilbenes, was never investigated in terms of effects on the microbiome balance. Our aim was to conduct an ecological investigation on how the gut microbiota can react to the effect of 2 concentrations of the test product. The aspect taken into account was the biological variability and not the interindividual variability.

According to the common approach, doses of the extract for the treatment in *in vitro* simulator M-SHIME® were selected through preliminary batch experiments with single administration of different concentrations. The evaluation of the microbial functionality and stilbenes' metabolic fate were consistent with the literature, where the metabolization of *t*-resveratrol and other stilbenes after a single administration was clearly observed in humans.[9] SCFA levels were stable in the control and treated samples [Fig. 2], while the main stilbenes decreased, indicating that *t*-resveratrol and its derivatives suffered metabolization by microbiota [Fig. 3]. None of the tested concentrations inhibited the microbial functionality.

Two dosages were assessed for test with M-SHIME®, 1 g L^{-1} and 2 g L^{-1}, and microbial communities were studied. SCFA and NH_4^+ are the products from sugar fermentation and proteolysis and their levels are considered a clue of wellness of the microbial population [Fig. 4]. In PC and DC controls, their concentrations showed a regular trend all over the period of the experiment. On the other hand, SCFA and NH_4^+ levels were strongly reduced by the administration of the extract: PC1g, DC1g, PC2g and DC2g trends showed a decrease during the treatment, with a weak return to the starting value after the washout. In particular, the higher dose led to a more intense effect in both PC and DC samples. As expected, the effect of the administration was stronger in PC vessels, since the extract was added at this level. In DC samples, the decrease was weaker and after the washout the recovery of functionality was stronger. The result is most probably related to a broad antimicrobial activity of the tested product, which leads to a temporal inhibition of microbial species involved in both saccharolytic and proteolytic activity.

The second step of the study was to evaluate the changes in microbial composition. The preliminary study through DGGE on lumen samples highlighted significant differences among the control and treated communities [Fig. 5]. These modifications were then deeply investigated using Illumina sequencing on DNA extracted from lumen and mucus samples. The results obtained from sequencing were consistent with the alteration of the microbial functionality and pointed out that the communities treated with a higher dosage showed a strong change in the overall composition, in both the lumen and mucus materials [Fig. 6–8]. The variation after treatment with the extract was more evident for PC2g than for the DC2g community. In PC1g and DC1g, administered with the lower concentration, the evolution was similar, but to a lesser extent. Most important information is about the enrichment of Enterobacteriales and the decrease of the Bacteroidales order. In this aspect, it was observed that Gram-negative species – in line with their biological properties – were less sensitive to the potential anti-microbial activity of the extracts.

The results on microbial communities during the treatment were consistent with the information obtained from SCFA and NH_4^+ levels. In terms of communities, a 4-day washout was not sufficient for a return to the starting situation, in particular in PC2g (higher dosage) and SCFA and NH_4^+ levels showed only a weak increase after the treatment. Microbial functionality, in terms of SCFA and NH_4^+, seemed to recover quickly, while for the establishment of the communities' composition the resilience time was longer.

To the best of our knowledge, this is the first report on the repeated administration of stilbenes on human microbiota. To date, few works have been focused to evaluate a single dose effect,[9] where a more efficient resilience process of the microbial community was observed.

The evaluation of the stilbenes' metabolic fate was the last part of the work [Fig. 9]. Despite the positive results from preliminary batch experiments that confirmed the metabolization of *t*-resveratrol and derivatives after a single administration, a continuous treatment with repeated intake for several days led to inhibition of this metabolism. The main stilbenes were not transformed and new metabolites were not detected, as clearly shown by HPLC-DAD-MS-TOF analysis. At the same time, the stilbenes' concentration increased during the experiment. The toxicity should be ascribed to the complexity of molecules and their total amount and not only to the *t*-resveratrol content. Indeed, the extract contains also *trans*-ε viniferin, plus minor stilbenes. To date, this is the first report about the effects of these stilbenes on human microbiota after continuous administration.

5 Conclusions

The results obtained on the stilbenes' metabolic fate, together with the information on the microbial functionality and composition, pointed out a strong effect on microbial metabolism. Batch experiments, performed with a single dose, showed that the tested concentrations did not inhibit the microbial functionality. Despite the results obtained from batch experiments, a daily treatment with the extract for 10 days in an M-SHIME® led to an inhibition of metabolism and functionality, associated with an imbalance of microbial communities. The results obtained, in contrast to the single dose test, should be the consequence of the repeated administration of the extract.

To date, we cannot exclude an uptake and a consequent accumulation of stilbenes in some microorganisms. Therefore, the safer dosage of this extract would be lower than 1 g L^{-1}.

Our data highlighted the importance of simulating continuous administration in humans by means of dynamic *in vitro* systems such as the M-SHIME®, to better define the suitable dosage of a dietary supplement.

The high reproducibility of the SHIME® ensures the possibility to compare different test products *vs.* a blank in a highly reproducible way. At the same time, the SHIME® experiment consists of a long-term experiment that enables controlled *in vitro* studies to evaluate the modulatory effects of different dosages. For this type of study, the issue of the interindividual variability plays a more important role, and our work has to be considered as a case study and a starting point to plan a future clinical trial with several subjects.

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