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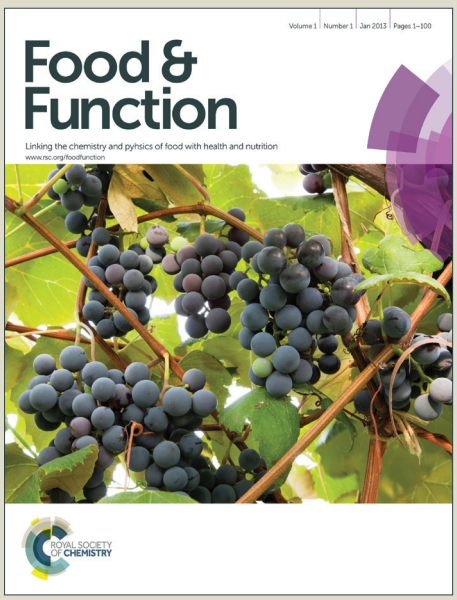

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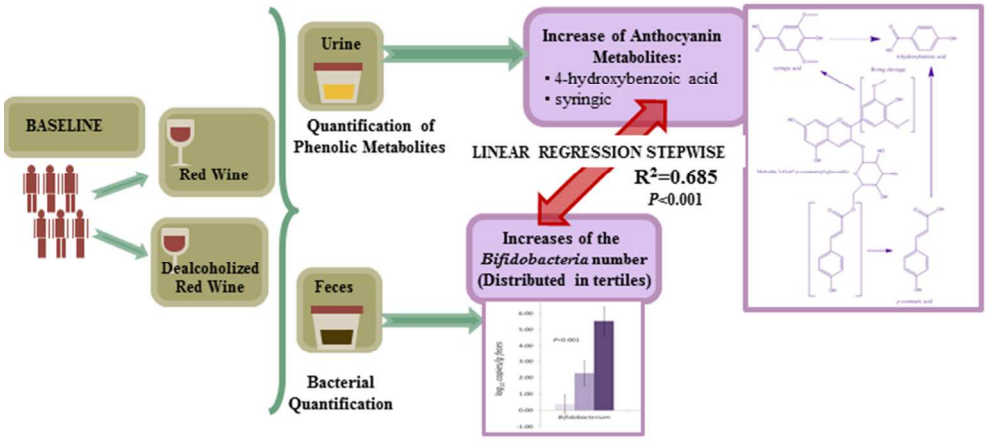

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High levels of *Bifidobacteria* are associated with increased levels of anthocyanin microbial metabolites: A randomized clinical trial

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ABSTRACT

The health benefits associated with the consumption of polyphenol-rich foods have been studied in depth, however, the full mechanism of action remains unknown. One of the proposed mechanisms is through microbiota interaction. In the present study, we aimed to explore the relationship between changes in fecal microbiota and changes in urinary phenolic metabolites after wine interventions. Nine participants followed a randomized, crossover, controlled interventional trial. After the washout period, they received red wine, dealcoholized red wine or gin for 20 days each. Polyphenol metabolites (n>60) in urine were identified and quantified by UPLC-MS/MS and the microbial content of fecal samples was quantified by from real-time quantitative PCR. Interventions with both red wine and dealcoholized red wine increased the fecal concentration of *Bifidobacterium*, *Enterococcus* and *Eggerthella lenta*, compared to gin intervention and baseline. When participants were categorized in tertiles of changes in fecal bacteria, those in the highest tertile of *Bifidobacteria* had higher urinary concentratio

65 microbiota incubated with polyphenols release phenolic metabolites whose presence
66 may modulate their growth.[16, 17]
67 For that reason, we embarked on a study to evaluate the associations between changes
68 in bacterial number produced at intestinal level and urinary changes in microbial
69 phenolic acids in a randomized, crossover, controlled intervention study divided in three
70 periods of 20 days each of RW, dealcoholized red wine (DRW) or gin consumption.
71

Materials and methods

Study subjects and design

The study was an open, randomized, crossover, controlled intervention trial[7] that involved 9 adult men aged between 45 and 50. The study design was divided into 3 consecutive periods of 20 days each with an initial washout period (baseline) during which the participants did not consume any alcohol or red wine. This period was followed in a random order by 3 consecutive periods during which the participants drank DRW (272 mL/d), or RW (272 mL/d, containing 30 g ethanol), or gin (100 mL/d, containing 30 g ethanol).

At baseline, and after each intervention period, participants provided fecal and 24 h urine samples, which were stored at -80 ºC until analysis. They were asked to maintain their dietary habits and pattern and lifestyle and to avoid alcoholic beverages during the whole study. No significant differences were observed in daily energy and dietary intake at the beginning of the study and after each intervention[7].

Participants had not received treatment for diabetes, hypertension, or dyslipidemia, any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 months before the start of the study or during the study (including the washout period). They did not have any acute or chronic inflammatory diseases, infectious diseases, viral infections, or cancer, and had not had a previous cardiovascular event at study entry. The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All the participants gave written informed consent. This trial was registered at controlled-trials.com as ISRCTN88720134.

Red wine, dealcoholized red wine and gin

The RW and DRW used in this study were elaborated with the Merlot grape variety, from the Penedès appellation (Catalonia). No differences in phenolic composition were found in the RW and DRW.[7]

Chemical and reagents

Available phenolic acids and flavanols and *β*-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO, USA), PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), and Extrasynthèse (Genay, France) as previously described.[14] HPLC grade solvents were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain) and Panreac Química, S.A.U. (Castellar del Vallès, Spain). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA).

Sample extraction

Microbial-derived and conjugated metabolites present in urine were analyzed using solid-phase extraction (SPE) with an Oasis® MCX and HLB 96-well plates (Waters, Milford, Massachusetts), respectively, as previously described.[14, 18, 19] Briefly, urine samples (1mL) were loaded onto the conditioned cartridge plate, washed and eluted with methanol or acidified methanol, respectively, and evaporated to dryness. Reconstitution of the residues was carried out with 100 µL of taxifolin in mobile phase.

UPLC-MS/MS Analysis

Metabolites in urine were analyzed by UPLC-MS/MS equipped with a binary solvent manager and a refrigerated autosampler plate (Waters Acquity UPLC system, Milford, MA, USA), coupled to an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, ionizing in negative mode (PE Sciex). An Acquity UPLC BEH C18 column (Milford, MA, USA) (1.7 µm, 2.1 mm × 5 mm), using a prefilter, working at 40 ◦C and with an injection volume of 5 µL, was used as described before.[14] Mobile phases used were: A (0.1% formic acid) and B (0.1% formic acid in acetonitrile) at a flow rate of 500 µL/min with the following proportions (v/v) of phase

A [t(min),%A]: (0,92); (2.5,50); (2.6,0); (3,0); (3.1,92); (3.5,92). The MS/MS parameters used were as previously described.[14] Phase II and microbial metabolites were quantified using the Multiple Reaction Monitoring (MRM) mode with a dwell time of 10 ms. Calibration curves were constructed with available standards in synthetic urine and subjected to the same procedure as the samples. If standard was not available, metabolites were quantified using the most similar compound standard curve and results were expressed as their equivalents.[14] The metabolites analyzed for this study are shown in the supplementary data. Quality parameters of the methodology accomplish with accuracy, precision and recovery <15%.[19]

DNA extraction from fecal samples and analysis of fecal microbiota by polymerase chain reaction (PCR)

Extraction of DNA was from 200 mg stools by using a QIAamp DNA Stool Mini Kit (Qiagen) and concentration and purity were estimated with a NanoDrop spectrophotometer (NanoDrop Technologies). For bacterial quantification to characterize the fecal microbiota, specific primers targeting different bacterial genera were used by PCR as previously described.[7] Briefly, the LightCycler 2.0 PCR sequence detection system, by using the FastStart DNA Master SYBR Green Kit (Roche Diagnostics), was used for quantitative PCR experiments. Comparison among Ct values obtained from the standard curves with the LightCycler 4.0 software was carried out to calculate bacterial concentration. Standard curves were created by using a serial 10-fold dilution of DNA from pure cultures, corresponding to 10^1–10^{10} copies/g feces. The data presented were the mean values of duplicate real-time qPCR analyses.

Statistical analysis

Before the statistical analysis, a cube root transformation and a range scaling of the data for phenolic data through the MetaboAnalyst Web-based platform was performed for normalization[14, 20] and the bacterial copy numbers were converted into logarithm

149 values.[7] We only considered bacteria with significant changes after both wines
150 compared to gin and baseline. These changes of bacteria were for two bacterial genera
151 (*Bifidobacterium*, *Enterococcus*) and one species *(Eggethella lenta*). Changes of
152 bacteria and phenolic acids after wine intervention were assessed checking the
153 difference compared to baseline. The procedure consisted in categorize the participants
154 based on tertiles of changes of bacterial genera or specie. To study the differences of
155 urinary metabolites through bacterial genera or specie tertiles, we used one-way analysis
156 of variance (ANOVA) (IBM SPSS Statistics software program for Windows version 20
157 (Chicago, IL)). If changes of metabolites and bacteria presented a significant Spearman
158 correlation, lineal regression stepwise analysis was performed in order to establish
159 which of these metabolites were predictors of bacterial changes. Statistical significance
160 was considered to be $P<0.05$.
161

Results

Tertiles of bacterial group changes after wine consumption

In this study, we considered the bacteria that showed significant modifications after both wine interventions and gin intervention compared to baseline.[7] No differences in number of bacteria (means±SD, $\log_{10}$ copies/g feces) were observed after DRW and RW: *Bifidobacterium* (9.93±1.85 and 9.88±1.78, respectively), *Eggerthella lenta* (9.84±1.65 and 9.97±1.77, respectively) and *Enterococcus* (6.94±1.5 and 7.10±1.1, respectively).[7] Tertiles of differences were calculated between bacterial number after wine interventions and baseline. The tertile distribution is presented in Fig. 1. The third tertile showed higher mean increases of *Bifidobacterium* (5.52±0.88 $\log_{10}$ copies/g feces), *Enterococcus* (2.83±0.51 $\log_{10}$ copies/g feces) and *Eggerthella lenta* (3.47±1.03 $\log_{10}$ copies/g feces) while the first tertile showed lower mean increases of *Bifidobacterium* (0.38±0.57 $\log_{10}$ copies/g feces), *Enterococcus* (0.50±0.81 $\log_{10}$ copies/g feces) and *Eggerthella lenta* (1.10±0.51 $\log_{10}$ copies/g feces). The increases through tertiles of bacteria were significant ($P<0.001$).

Relationship among changes in bacterial population and urinary phenolic metabolites

The tertiles of bacterial modifications were used to analyze phenolic metabolite changes after wine interventions (Table 1). From the lowest to the highest changes in *Bifidobacteria* tertiles, participants had a higher excretion of four phenolic metabolites related to anthocyanin metabolism (Figure 2): syringic, *p*-coumaric, 4-hydroxybenzoic and homovanillic acids. On the other hand, higher increases of *Eggerthella lenta* corresponded to lower excretion of hydroxycinnamates and syringic acid. In addition, higher increases of *Enterococcus* corresponded to lower excretion of 3,4-dihydroxyphenylacetic acid.

188 Additionally, correlation analysis indicated that only differences in *Bifidobacteria* were
189 significantly correlated with differences in syringic (r=0.537, P<0.022), *p*-coumaric
190 (r=0.621, P<0.006), 4-hydroxybenzoic (r=0.677, P<0.002) and homovanillic acids
191 (r=0.507, P<0.032). Linear regression stepwise analysis evaluating *Bifidobacteria*
192 changes included only two metabolites in the model (R^2=0.685; P<0.001), syringic acid
193 and 4-hydroxybenzoic acid. This model explained 68.5% of the *Bifidobacterium*
194 changes.
195

Discussion

The increased knowledge about the role of microbiota in human health and the possible modulation through food consumption is an interesting field for developing new products in the food industry such as probiotics and prebiotics.[21] Food has demonstrated the capacity to modulate the growth of intestinal bacteria in several clinical trials[8, 22] and produce bioactive metabolites.[23]

One of the main studied bacteria to be affected by food intake is *Bifidobacteria*. *Bifidobacterium* is one of the predominant genera in the human intestine, and it is considered health-promoting constituent of the microbiota.[24] In this study, the unique metabolites correlated to *Bifidobacterium* were those derived from anthocyanin degradation: 4-hydroxybenzoic, syringic, p-coumaric and homovanillic acid. The concentration of anthocyanins in wine is high but lower than flavanols, which are the main wine polyphenols.[15, 25] Nevertheless, their dietetic distribution in Mediterranean diets is more limited than flavan-3-ols and their metabolites have been proposed as excellent markers of wine consumption.[14] Anthocyanins were first supposed to have low bioavailability,[26] but in the last few years, studies with isotopically labeled anthocyanins have demonstrated that anthocyanins reach the colon where they are transformed, releasing new metabolites that differ from the original compound.[27]

Microbial metabolism of anthocyanins at colonic level involves reactions of breakage in the C-ring, resulting in hydroxylated aromatic compounds derived from the A-ring, and release of the B-ring in numerous phenolic acids, different depending on their hydroxylated pattern,[12] as well as deglycosylation.[28] Furthermore, *Bifidobacterium* enzymatic activity for polyphenols has not only been associated with ring fission,[28] but also hydrolysis[29] and glycosidase activity.[28] Figure 2 shows the principal origin of microbial metabolites derived from anthocyanin structure associated with *Bifidobacteria*

increase. One of these phenolic acids is 4-hydroxybenzoic acid, which has been proposed as a pelargonidin metabolite,[30] and comes from microbial degradation of *p*-coumaric[31] or could come from syringic acid demethylation, a reaction associated with certain intestinal bacteria.[11] Moreover, some studies have shown that the concentration of 4-hydroxybenzoic acid increased in plasma and urine after strawberry consumption by healthy volunteers[32] and in the urine of rats fed with wine powder.[33] Syringic acid may come from malvidin degradation described from *Lactobacillus* and *Bifidobacterium*.[28, 34] These two metabolites were the ones that entered the stepwise logistic regression, indicating that they were the strongest contributors to *Bifidobacteria* change after wine consumption. In the same study, *p*-coumaric was also formed when delphinidin and malvidin were incubated with these bacteria[28] via hydrolysis of *p*-coumaroyl-acylated anthocyanins, which are abundant in red wine (Fig. 2). Homovanillic acid has also been described as coming from malvidin glycoside degradation via demethoxylation and was one of the main urinary metabolites after berry purée consumption by humans.[35]

It is difficult to establish whether these compounds are primary anthocyanin metabolites or are derived from other sources. Homovanillic acid could also be formed from ferulic acid,[35] additionally *p*-coumaric acid could come from dehydroxylation of caffeic acid, and syringic from gallic acid.[36] Moreover, some of these metabolites, such as gallic acid, are also present in original wine composition.[15, 25]

Previous studies have already shown the role of anthocyanins in the bifidogenic effect as Guglielmetti *et al*. found after consumption of a wild blueberry drink by humans.[37] Biological effects associated with these changes have already been described. *Bifidobacterium* has been associated with antiobesity effects[38] and cholesterol regulation.[39] Metabolite 4-hydroxybenzoic could be responsible for the antioxidant

properties of polyphenol consumption, inhibiting tyrosine nitration through the formation of 4-hydroxy-3-nitrobenzoic acid, which is less reactive than nitrotyrosine.[40] Syringic acid has been proved to increase nitric oxide production[41] and *p*-coumaric acid has inhibitory activity over angiotensin-converting enzymes.[42] The biological activities attributed to the increase in metabolites could be responsible for benefits observed in blood pressure and improving plasma lipid profile or inflammation in this study.[22, 43]

The other bacteria species modified after red wine consumption was *Eggerthella lenta*, which is significantly abundant in intestinal microbiota.[24] Significant inverse associations were found between changes in *Eggerthella lenta* tertiles and changes in hydroxycinnamic acid concentrations and between changes in *Enterococcus* tertiles and changes in 3,4-dihydroxyphenylacetic acid concentration. This was probably due to the fact that both bacterial groups could be inhibited by phenolic compounds including gallic acid and resveratrol metabolites in *in vitro* studies.[44-46] In addition, *Enterococcus* genus bacteria have been inhibited by cloudberry intake.[47] Although we found significant inverse associations between *Eggerthella lenta* and *Enterococcus* tertiles and some phenolic acid concentrations, changes in *Eggerthella lenta* and *Enterococcus* cannot be predicted by phenolic acids changes.

Even one the main limitations of this study was the lack of washout periods between interventions, no carryover effect was observed, and the absence was therefore unlikely to affect the results obtained.[7] Moreover, the inclusion of washout periods between interventions would extend the study a further 6 weeks, making difficult to ensure compliance, so the subjects would be more inclined to withdraw from the study. [7] In addition, the limitations of this study suggest the need of future next steps that potentially will be to increase the number of subjects. And, future studies would be

designed to answer if changes in microbiota levels produced changes in phenolic acids concentration or inversely.

Conclusion

Bacteria changes after red wine consumption, with or without alcohol content have been associated with the excretion of phenolic metabolites. Specifically, *Bifidobacteria* increase correlates with increases in microbial metabolites derived from wine anthocyanins. Numerous *in vitro* studies have shown the ability of intestinal bacteria to metabolize polyphenols and release them to the medium. Those metabolites have been found in plasma, urine and tissues after food consumption. To our knowledge this is the first approach where colonic bacteria in feces and microbial metabolites present in biofluids are studied from the same volunteer in an *in vivo* study.

This study contributes with new data to understanding the role of phenolic compounds in the maintenance of intestinal health and opens the way to considering anthocyanins not only as new prebiotics, but also as being responsible for health benefits associated with the consumption of anthocyanin-rich food.

Acknowledgments

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Table 1 Changes in urinary phenolic metabolites after wine interventions (mean value ± standard deviation) according to changes in bacterial tertiles.

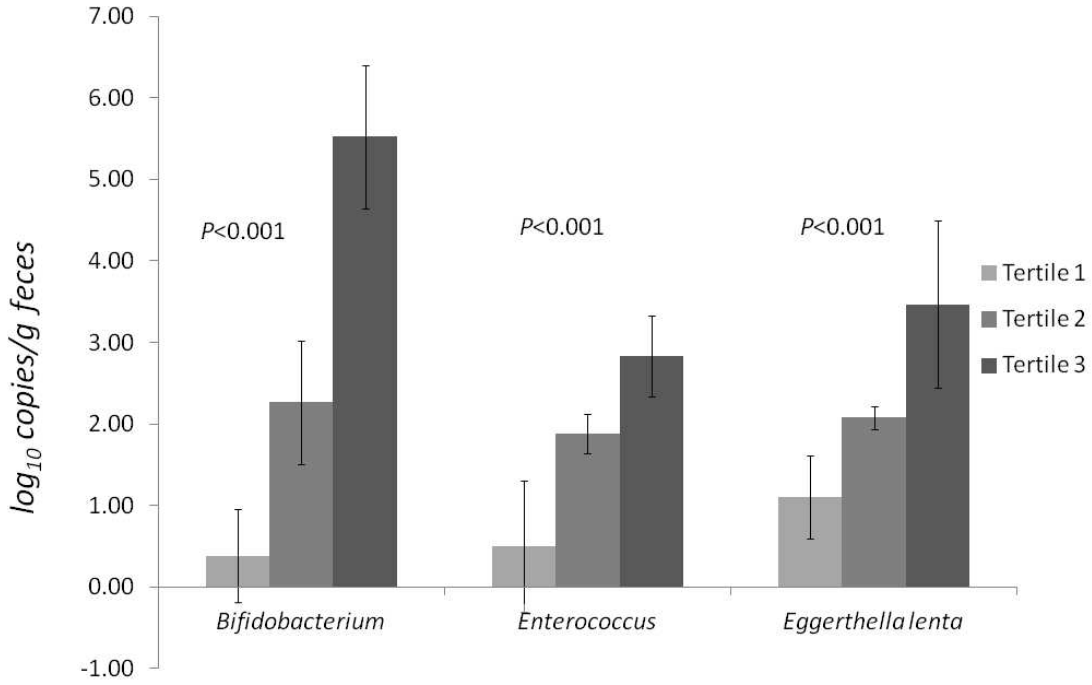
	Changes in bacterial population tertiles			*P*-trend
	Changes in Bifidobacterium tertiles [a]			
	1 (n=6) (<1.18)	2 (n=6) (1.18-4.47)	3 (n=6) (>4.47)	
4-Hydroxybenzoic acid[b]	-20.34±15.6	-2.87±22.07	18.04±34.2	0.013
Syringic acid	-0.91±1.75	-0.5±1.23	1.37±1.28	0.024
P-Coumaric acid	0.82±1.04	1.16±1.32	2.05±1.29	0.038
Homovanillic acid	-81.83±116.86	-29.65±89.66	20.28±93.29	0.043
	Changes in Enterococcus tertiles			
	1 (n=6) (<1.36)	2 (n=6) (1.36-2.07)	3 (n=6) (>2.07)	
3,4-(dihydroxyphenyl)acetic acid	-0.22±12.06	-1.88±6.94	-13.09±9.04	0.039
	Changes in Eggerthella lenta tertiles			
	1 (n=6) (<1.88)	2 (n=6) (1.88-2.29)	3 (n=6) (>2.29)	
Caffeic acid	3.36±2.69	0.69±2.12	-1.76±3.42	0.018
Ferulic acid	11.3±12.41	-3.58±3.12	-2.95±3.6	0.009
Syringic acid	1.16±1.21	-0.6±2.16	-0.6±1.07	0.037
Feruloylglycine	14.67±18.45	4.73±7.56	-5.46±7.41	0.018

[a] $\log_{10}$ copies/g feces
[b] µmol/24 h.

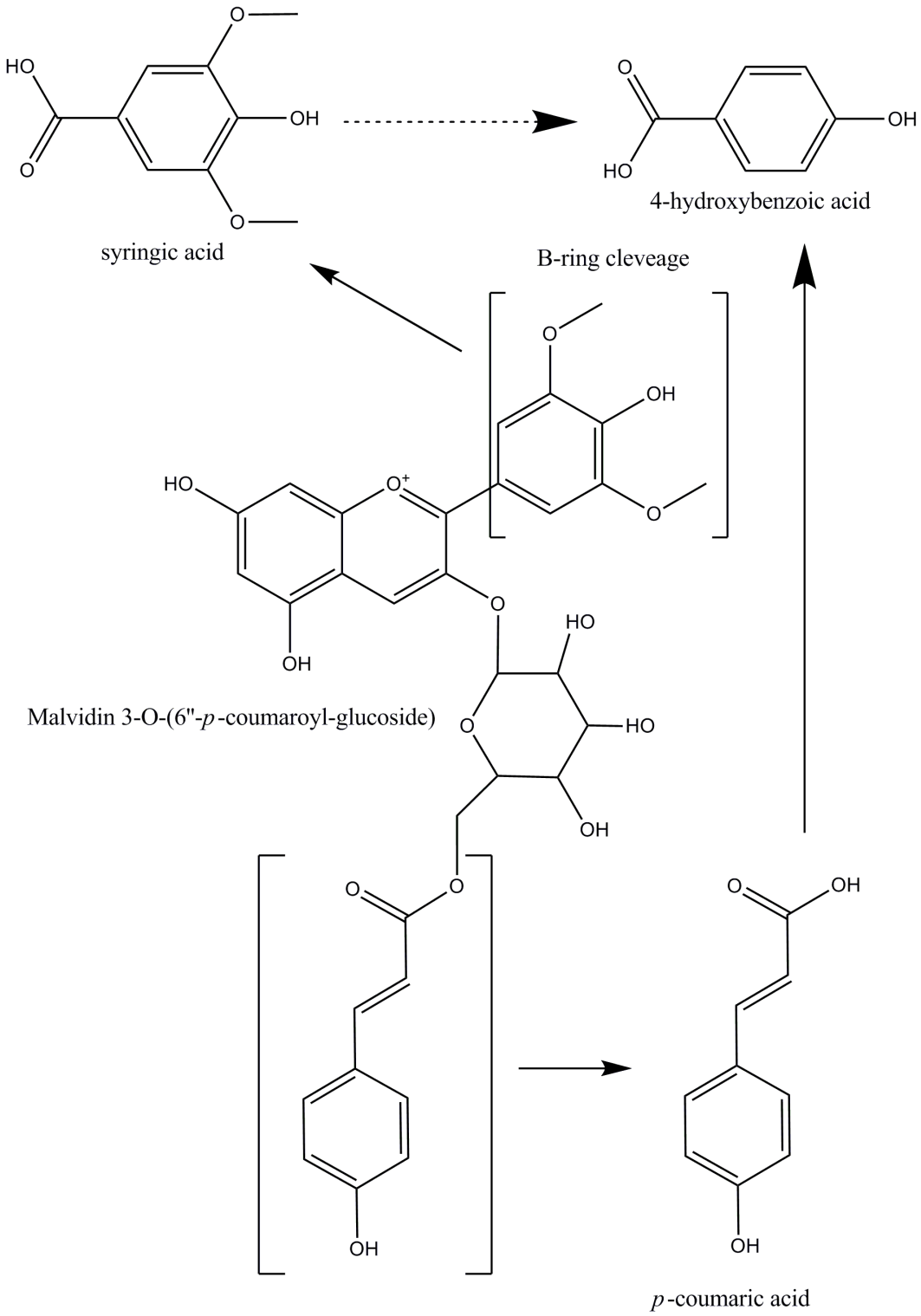
426 **Table 2** Stepwise linear regression model showing the best metabolite predictors of
427 *Bifidobacterium* change.
428

	B(SD)	β	*P*
Bifidobacterium			
Intercept	2.75 (0.37)		<0.001
4-Hydroxybenzoic acid	3.60 (1.27)	0.513	0.013
Syringic acid	3.63(1.51)	0.402	0.042

Fig. 1 Tertiles of bacterial differences of two genera (*Bifidobacterium*, *Enterococcus*) and one species (*Eggerthella lenta*) between wine interventions and baseline (mean±SD).



435
436 **Fig. 2** Proposed metabolic route of anthocyanin degradation by *Bifidobacteria*.



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