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Grapevine extracts and their effect on selected gut-associated microbiota: *In vitro* study

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Abstract: The biological activity of polyphenol substances contained in food supplements prepared from *Vitis vinifera* can affect the microorganisms present in the digestive tract in terms of their representation and activity of the individual species. This study deals with resveratrol and two polyphenol-rich extracts (extract from *V. vinifera* canes and the commercial product Regrapex-R-forte) and their effect on selected gut microbiota (*Bifidobacterium animalis* subsp. *lactis* Bb-12, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei Lafti* L-26, *Citrobacter freundii* DBM 3127, *Escherichia coli* DBM 3125). The effect of the studied agents on planktonic and biofilm growth of the microorganisms was determined as minimum inhibitory concentration (MIC_{80}) and minimum biofilm inhibitory concentration ($MBIC_{80}$), respectively. The extracts induced metabolic activity as well as total biofilm biomass production in probiotic strain *L. acidophilus* LA-5 while successfully inhibiting the growth of opportunistic pathogenic microorganisms *C. freundii* DBM 3127 and *E. coli* DBM 3125.

Keywords: anti-biofilm activity; gut microbiota; polyphenols; resveratrol; *Vitis vinifera*

Polyphenols are an important group of antioxidants, which are significant for the human health and are therefore sought out by consumers. Since there is a great demand for natural antioxidants, many food supplements are prepared as extracts of higher plants (Kolouchová et al. 2005). Studies showed that plant-derived wastes (by-products of agriculture or horticulture) could also be considered as renewable sources of polyphenols that can be valorized (Ravindran & Jaiswal 2016; Kolouchová et al. 2018). Grapevine (*Vitis vinifera*) by-products represent a rich source of polyphenols. In the following years, these *V. vinifera* extracts could be used in pharmaceutical and food industries as additives or food supplements (Lavelli et al. 2004). However, little is known about the effect of grapevine extracts on gut microbiota even though the importance of gut microbiota for human health has been proved (Sankar et al. 2015).

An *in vitro* study by Lee et al. (2006) has shown that the intake of food rich in different polyphenols can modulate the composition of the microbial population in the human body. Furthermore, Li et al. (2015) exam-

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ined the effect of pomegranate juice rich in polyphenols on multiple intestinal bacterial species and observed an increase in the mean count of *Bifidobacterium* spp. and *Lactobacillus* spp. and a decrease of *Bacteroides fragilis* and *Clostridium* spp. and *Enterobacter* spp.

This study deals with the *in vitro* effect of three biologically active agents (*trans*-resveratrol, extract from *V. vinifera* canes and the commercial extract Regrapex-R-forte) on probiotic bacteria *Bifidobacterium animalis* subsp. *lactis* Bb-12, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* Lafti L-26 and opportunistic pathogens *Citrobacter freundii* DBM 3127 and *Escherichia coli* DBM 3125 closely related to the human digestive tract.

MATERIAL AND METHODS

Bacteria and culture conditions. Strains of probiotic bacteria *Bifidobacterium animalis* subsp. *lactis* Bb-12, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* Lafti L-26 (DSM Food Specialties, Netherland), strains *Citrobacter freundii* DBM 3127 and *Escherichia coli* DBM 3125 [Collection of Microorganisms of the Department of Biochemistry and Microbiology, UCT Prague (DBM)] were used in an experiment. The microorganisms were stored cryopreserved at –70 °C in sterile 50% glycerol.

The probiotic strains were cultivated aerobically in MRS broth (20 g L^{-1} glucose, 10 g L^{-1} peptone, 10 g L^{-1} meat extract, 5 g L^{-1} yeast extract, 1 mL Tween 80, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} sodium acetate, 2 g L^{-1} ammonium citrate, 0.2 g L^{-1} $MgSO_4 \cdot 7\ H_2O$ and 0.2 g L^{-1} $MnSO_4 \cdot H_2O$) at 37 °C, 150 rpm. *Citrobacter freundii* DBM 3127 and *Escherichia coli* DBM 3125 were cultivated in LB broth (tryptone 10 g L^{-1}, NaCl 10 g L^{-1}, yeast extract 5 g L^{-1}) at 37 °C, 150 rpm. The LB medium was adjusted to pH 7 and MRS to pH 6.5. The pH was adjusted with 20% NaOH or 25% H_2SO_4 solutions. For the inoculum, 250 mL Erlenmeyer flasks containing 100 mL of the medium were inoculated with a cryopreserved stock culture and the bacteria were cultivated (150 rpm, 37 °C) for 24 h until the exponential phase.

Biologically active agents and preparation of *Vitis vinifera* cane extract. Regrapex-R-forte was kindly provided by Interpharma Praha, Czech Republic. *Trans*-resveratrol (cat. No. R5010). was purchased from Sigma-Aldrich (USA). Stock solutions were prepared by dissolving the powdered form of Regrapex-R-forte/resveratrol in 40% (v/v) ethanol.

The *V. vinifera* cane extract was prepared from pruned *V. vinifera* canes, from both white and red varieties (Rheinriesling, Pinot gris and Pinot noir), which were obtained during the dormancy period in January 2017 from vineyards in the Czech Republic. The dry canes were stored for 6 months in dry and dark environment at room temperature (Houille et al. 2015) and afterward cut into 2 cm sections and ground to particles of 2–3 mm in size (Zelmer 32Z012; Zelmer, Poland). A static solid-liquid extraction [24 h in dark, laboratory temperature, ratio 1 : 4 (w/w)] with 40% (v/v) ethanol p.a. – water solution was applied. The extract was separated from the solids and the solvent was removed on a rotary evaporator at 40 °C. After rotary evaporation, the residue was dissolved in 40% (v/v) ethanol to obtain a 10-fold concentrated extract. The concentrated extract was stored in the dark at 4 °C.

Determination of total polyphenolic content. The amount of total soluble polyphenols in the *V. vinifera* cane extracts was determined by Folin-Ciocalteu method in a microtiter plate. In each well, 15 µL of the extract was mixed with 165 µL of a 10-fold diluted Folin-Ciocalteu reagent. The mixture was homogenized and after 3 min, 60 µL of 2M Na_2CO_3 solution and 80 µL of distilled water were added. The mixture was incubated 60 min in darkness at laboratory temperature and the absorbance was measured at 700 nm. Gallic acid (Sigma-Aldrich, Germany) was used as a standard.

HPLC analysis. The concentrations of *trans*-resveratrol and *trans*-polydatin (Sigma-Aldrich, Germany) in extracts were determined by RP-HPLC using a gradient of acetonitrile and demineralized water according to a previously described methodology (Gharwalova et al. 2018). The separation and quantification were carried out with an 1 100 series HPLC system equipped with a DAD detector (Agilent, USA) and a reversed-phase 125 × 4 mm Watrex, Nucleosil 120-C18 column at 25 °C. The standards were dissolved in 40% (v/v) ethanol and stored in the dark at 4 °C.

UHPLC-HRMS/MS analysis. The *V. vinifera* cane extract composition was studied by ultrahigh-performance liquid chromatography coupled to high-resolution tandem mass spectrometry (UHPLC-HRMS/MS) consisting of an Acquity UPLC® (Waters, USA) liquid chromatograph coupled to a Synapt G2 mass spectrometer (Waters, USA) as described previously by Paldrychová et al. (2019). The HRMS/MS detection took place using electrospray ionization (ESI) operated in positive and negative ionization modes (scan range from 100 to 1 200 m/z).

Determination of planktonic minimum inhibitory concentration. Determination of minimum concentration inhibiting 80% of the visible planktonic cell growth

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(MIC_{80}) was performed according to the definition by Andrews (2001) as the lowest concentration that causes at least an 80% decrease in growth after 24 h of cultivation. The growth in microtiter plates was investigated by monitoring the medium optical density in a Bioscreen C microcultivation device (Oy Growth Curves Ab Ltd., Finland). The results were expressed as the concentration of total polyphenols present in the extract (determined by Folin-Ciocalteu method).

The inoculum was prepared as described above. Then it was centrifuged (10 min, 10 °C, 9 000 g) and the optical density at 600 nm (OD_{600}) was adjusted to 0.100 ± 0.015 using a complex medium. Afterwards, 30 µL volume of the cell suspension was pipetted into the microtiter plate. The pipetting of the stock solutions of biologically active agents (Regrapex-R-forte, *trans*-resveratrol, *V. vinifera* cane extract) was done so as to obtain the desired concentrations (1–150 mg L^{-1} for *trans*-resveratrol and Regrapex-R-forte; 1–30 mg L^{-1} for *V. vinifera* cane extract) in the individual wells. The concentration of the solvent (40% ethanol) in the well did not exceed 3% to avoid cell growth inhibition by the solvent (verified by control experiments). Sterile medium was added into each well to obtain the total volume of 280 µL (in each well).

Controls without agent (with and without the presence of the solvent) were also included. Each experiment was performed in five replicates.

Determination of biofilm inhibitory concentration. The minimum biofilm inhibitory concentration ($MBIC_{80}$) was determined as the lowest concentration that causes at least an 80% decrease in metabolic activity of the examined bacterial strain {determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay} after 24 h of cultivation in the presence of biologically active agents (Sabaeifard et al. 2014). The results were expressed as the total concentration of polyphenols present in the extract (determined by Folin-Ciocalteu method).

The inoculum was prepared as described above and after 24 h centrifuged (10 min, 10 °C, 9 000 g). The biofilm cultivation was performed in 96-well microtiter plates. Into each well, aliquots of 200 µL of standard cell suspensions (OD_{600} = 0.800 ± 0.015) in growth media and the appropriate biologically active agent were transferred. The microtiter plate was covered with a lid fastened with semipermeable parafilm and it was placed into an incubator with temperature control (37 °C). After 24 h, each well was washed three times with saline (0.9% NaCl) and biofilm formation on the bottom of wells was evaluated by MTT assay and crystal violet staining method.

Controls without agent (with and without the presence of the solvent) were also included. Each experiment was performed in eight replicates.

MTT assay. The method was adopted and modified according to Riss et al. (2013). After biofilm cultivation and washing, 60 µL of glucose solution (57.4 mg mL^{-1}) and 50 µL of MTT solution (1 mg mL^{-1}) were added into each well. The plate was incubated in the dark for 1–4 h (according to microbial strain requirements) in an incubator with temperature control (37 °C). After incubation, 100 µL of sodium dodecyl sulphate solution (160 g L^{-1}) was added into the wells and the plates were incubated once more in the dark for another 60 min (150 rpm, 37 °C). From each well, 100 µL of the homogenized solution was transferred into a 96-well microtiter plate and absorbance in each well at 570 nm was measured spectrophotometrically using an automated microtiter plate reader (Tecan, Switzerland).

Crystal violet staining. The total biofilm biomass was quantified using crystal violet staining. This dye binds to negatively charged molecules and polysaccharides which are contained in the extracellular matrix (Peeters et al. 2008). In the quantification process, each well was filled with 200 µL of filtered 0.1% crystal violet solution. After 20 min of incubation at room temperature, the wells were washed three times with saline (0.9% NaCl). Crystal violet bound to the biofilm biomass was released by adding 200 µL of 96% ethanol. After 10 min, a 100 µL volume of the coloured solution was transferred from each well into a new 96-well microtiter plate to measure absorbance (580 nm) using an automated microtiter plate reader (Tecan, Switzerland).

Statistical analysis. Dixon's Q test was performed to detect and omit outliers in datasets obtained by the crystal violet staining and the MTT assay.

RESULTS AND DISCUSSION

Extract characterization. Resveratrol had either no effect on *E. coli* strains (O'Connor et al. 2011) or was effective only at higher concentrations, i.e. 400 mg L^{-1} (Bostanghadiri et al. 2017). Resveratrol was acknowledged to have health beneficial properties in the past; however, in most studies these effects were found only after application of high doses (1–10 mg kg^{-1} per day) (Larrosa et al. 2009). Taguri et al. (2006) in their study found the MIC_{100} for pure resveratrol against *C. freundii* ATCC 8090 to be 2 133 mg L^{-1}.

Regrapex-R-forte is standardized to contain a certified amount (at least 35%) of the resveratrol complex

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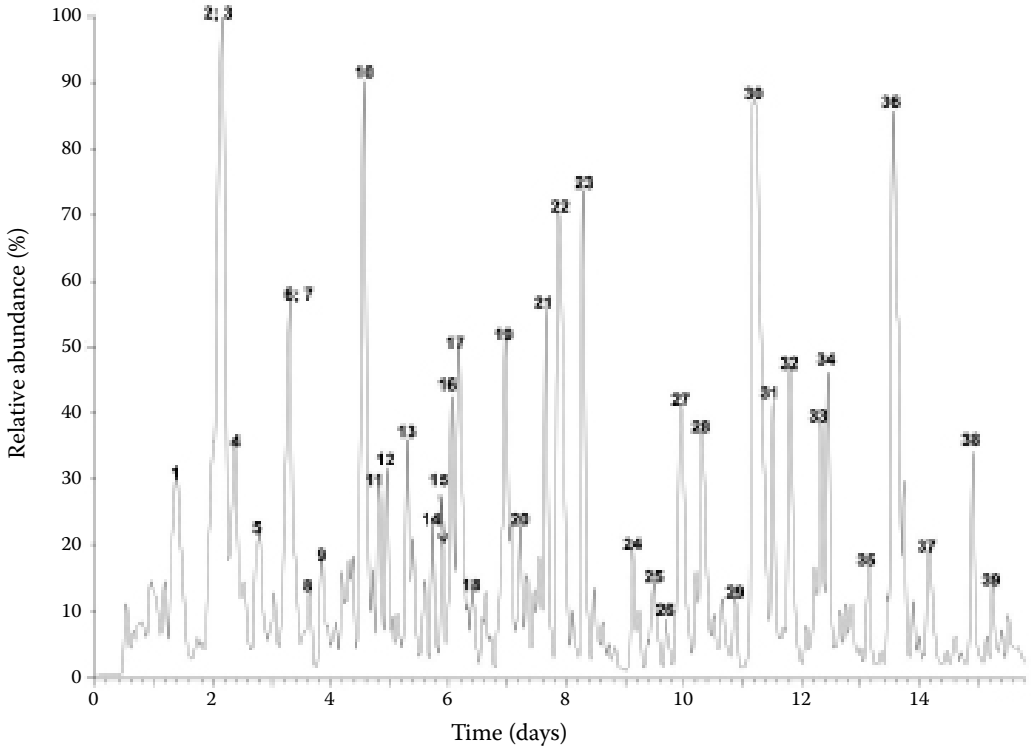

Figure 1. UHPLC-HRMS/MS analysis of *Vitis vinifera* cane extract in ESI^+
Peaks are identified in Table S1 – see Eletronic Supplemenary Material (ESM)

(i.e. the combined content of resveratrol and its glycoside polydatin), where resveratrol should account for 9% at least. The *trans*-resveratrol content in Regrapex-R-forte determined in this study by HPLC was 10.3%. However, Regrapex-R-forte contains many biologically active substances besides resveratrol (Paldrychova et al. 2019), such as gallic acid, procyanidin and quercetin.

The *V. vinifera* cane extract used in the experiment was found to contain 730 mg L^{-1} of *trans*-resveratrol. The *V. vinifera* cane extract, analysed by UHPLC-HRMS/MS [Figure 1, identification in Table 1S – see Eletronic Suplementary Material (ESM)], was found to contain several biologically active substances including procyanidins, viniferins, catechins and quercetin.

Determination of planktonic minimum inhibitory concentration. MIC_{80} of *trans*-resveratrol was not determined within the observed concentration range (0–150 mg L^{-1}) for any of the tested microorganisms (Table 1). Both extracts decreased the planktonic growth of the tested gut bacteria. The most sensitive bacteria proved to be *C. freundii* DBM 3127: 0.50 g L^{-1} and 0.18 g L^{-1} (concentration expressed as the concentration of total polyphenols) for Regrapex-R-forte and cane extract, respectively.

Table 1. Determination of MIC_{80} concentrations of the commercial extract Regrapex-R-forte, *Vitis vinifera* cane extract and *trans*-resveratrol for some gut bacteria

MIC_{80} (g L^{-1})	Microorganisms				
	E. coli DBM 3125	*C. freundii* DBM 3127	*L. acidophilus* LA-5	*L. casei* Lafti *L-26*	*B. animalis* subsp. *lactis Bb-12*
Regrapex-R-forte	0.67	0.50	> 0.80	0.67	0.67
V. vinifera cane extract	0.35	0.18	0.35	0.35	0.53
Trans-resveratrol	> 0.15	> 0.15	> 0.15	> 0.15	> 0.15

The concentrations for Regrapex-R-forte and *V. vinifera* cane extract are expressed as the concentration of total polyphenols in the extracts

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Table 2. Determination of $MBIC_{80}$ concentrations of the commercial extract Regrapex-R-forte and *Vitis vinifera* cane extract for some gut bacteria

$MBIC_{80}$ (g L^{-1})	Microorganisms		
	E. coli DBM 3125	*C. freundii* DBM 3127	*L. acidophilus* LA-5
Regrapex-R-forte	> 1.12	1.12	> 1.12
V. vinifera cane extract	0.53	0.53	> 0.53

The concentrations are expressed as the concentration of total polyphenols in extracts

Determination of biofilm inhibitory concentration. The effect of *V. vinifera* cane extract and Regrapex-R-forte on the biofilm formation of three selected bacterial strains (*E. coli* DBM 3125, *C. freundii* DBM 3127, *L. acidophilus* LA-5) was tested. The nhibition of the metabolic activity of biofilm cells was determined as the minimum biofilm inhibitory concentration ($MBIC_{80}$; see Table 2). The cane extract effectively inhibited the metabolic activity of the biofilm cells of both pathogenic bacterial strains (*E. coli* DBM 3125, *C. freundii* DBM 3127) at a concentration of 0.53 g L^{-1} (concentration expressed as the concentration of total polyphenols). For both opportunistic pathogenic strains, the increasing Regrapex-R-forte concentration resulted in a decreased metabolic activity. For *C. freundii* DBM 3127, the $MBIC_{80}$ value was 1.12 g L^{-1}. For *E. coli* DBM 3125, the $MBIC_{80}$ value could not be determined in the given concentration range, however, the concentration of 1.12 g L^{-1} (concentration expressed as the concentration of total polyphenols) decreased the metabolic activity of this strain by 50%. When applying the *V. vinifera* cane extract to *L. acidophilus* LA-5, an increase in the total biofilm biomass and the metabolic activity of the biofilm cells could be observed (Figure 2B), especially at lower concentrations of total polyphenols (0.09–0.18 g L^{-1}).

The same effect on *L. acidophilus* LA-5 was witnessed when applying the commercial extract Regrapex-R-forte (Figure 2A). This increase in both biofilm biomass and metabolic activity of the probiotic strain could have also other beneficial effects with regard to the finding that *L. acidophilus* also inhibits the growth of pathogenic *Helicobacter pylori* (Chatterjee et al. 2003). These results are also supported by a study of Chatterjee et al. (2003), in which resveratrol inhibited the growth of *H. pylori* and at the same time promoted the growth of *L. acidophilus*. In addition, two studies on human subjects demonstrated that the intake of tea extract rich in catechins (Goto et al. 1999) or intake of proanthocyanidin-rich grape seed extract (Yamakoshi et al. 2001) led to an increase in *Bifidobacterium* spp. and a decrease in the number of undesirable bacteria, representatives of *Enterobacteriaceae* and *Clostridiaceae*, in the faeces of the tested subjects. In general, polyphenols are prone to being metabolised by some gut microbiota (especially *Bifidobacterium* spp. and *Lactobacillus* spp.), which leads to the production of smaller compounds that can be more easily absorbed across the intestinal

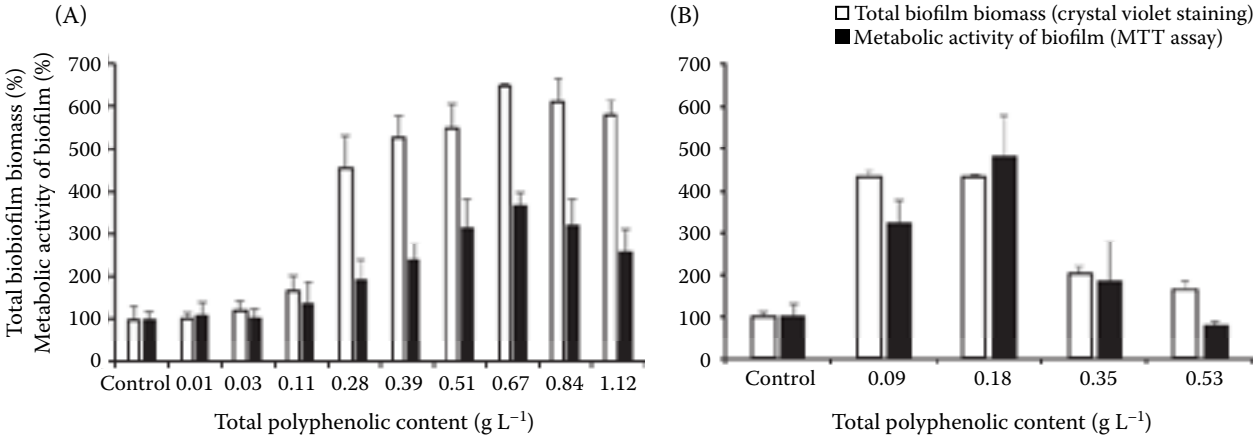

Figure 2. The influence of Regrapex-R-forte (A) and *Vitis vinifera* cane extract (B) on *Lactobacillus acidophilus* LA-5 biofilm formation

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wall (Zhou et al. 2016; Ma & Chen 2020). Furthermore, resveratrol was found to protect *Lactobacillus reuteri* against protein carbonylation (promoted by reactive oxygen species) through various mechanisms including direct scavenging of reactive oxygen species, upregulation of the dhaT gene and promoting the synthesis of sulphur containing compounds (Arcanjo et al. 2019).

CONCLUSION

The *V. vinifera* cane extract prepared from plant waste obtainable from viticulture was proved to have similar antimicrobial properties against opportunistic bacterial pathogens *E. coli* DBM 3125, C. freundii DBM 3127 as the commercial extract Regrapex-R-forte, which was fortified to contain a certified amount of the resveratrol complex. Both extracts were able to positively influence the growth of probiotic strain *L. acidophilus* LA-5. Further in vivo studies would be needed to confirm the prebiotic benefits of these nutraceuticals.

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