

The impact of polyphenols on *Bifidobacterium* growth

Daniela Gwiazdowska, Krzysztof Jus[✉], Joanna Jasnowska-Małecka and Katarzyna Kluczyńska

Department of Natural Science and Quality Assurance, Faculty of Commodity Science, Poznań University of Economics, Poznań, Poland

Polyphenols are a common group of plant based bioactive compounds, that can affect human health because of their antioxidant and antimicrobial properties as well as free-radical scavenging activity. An increasing interest is observed in the interaction between polyphenols and microbiota occurring in food and the human gut. The aim of the work presented here, was to evaluate the effect of some polyphenolic compounds on the growth of two strains of *Bifidobacterium*: *B. adolescentis* and *B. bifidum*. The influence of some flavonoids: naringinin, hesperidin, rutin, quercetin as well as phenolic acids: gallic, caffeic, *p*-coumaric, ferulic, chlorogenic, vanillic and sinapic was determined by a 96-well microtiter plate assay. In the experiments the effect of three different concentrations of polyphenols: 2, 20 and 100 µg/ml on the growth of *Bifidobacterium* strains was investigated. All tested compounds influenced the growth of the examined bacteria. Both stimulatory and inhibitory effects were observed in comparison to the positive control. The strongest impact on the growth of bifidobacteria was observed during the first hours of incubation. The constant inhibitory effect was observed for hesperidin and quercetin addition and was dose-dependent. *B. bifidum* showed a stronger dependence on phenolic acids content in the medium than *B. adolescentis* during the first hours of incubation.

Key words: dietary polyphenol, probiotic, inhibition, stimulation

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INTRODUCTION

Polyphenols comprise a heterogenous group of compounds characterized by hydroxylated phenyl moieties and generally classified into flavonoids and nonflavonoids. Flavonoids possess carbon skeleton of diphenylpropanes and two benzene rings (A and B) joined by the linear carbon chain, which forms a closed pyran ring (C) with benzene ring A. Depending on the oxidation state of the central pyran ring, flavonoids are divided into subclasses: flavonols, flavanols, flavones, flavanones, anthocyanidins and isoflavones and proanthocyanidins (Andrés-Lacueva *et al.*, 2009; Daglia, 2012). Nonflavonoids include phenolic acids, stilbenes and lignans. All of these groups comprise compounds differentiated in their structure. Phenolic acids are subdivided into derivatives of benzoic acid such as gallic acid and protocatechuic acid as well as derivatives of cinnamic acid such as ferulic and caffeic acid. In the group of stilbenes the most representative compound is resveratrol, occurring in *cis* and *trans* isomeric forms. These compounds are present in plants, where they play a role mainly as antifungal phytoalexins. Lignans are produced by oxidative

dimerization of two phenylpropane units. Their source in human diet comes mainly from linseeds, containing secoisolariciresinol and matairesinol (Adlercreutz & Mazur, 1997; D'Archivio *et al.*, 2007; Quideau *et al.*, 2011).

The main sources of polyphenols in human diet are fruits, vegetables and plant-derived beverages such as tea and red wine. Some of these compounds such as quercetin are found in all plant products, while others are specific to particular products for example phloridzin in apples or isoflavones in soya. Generally, different kinds of food contain complex mixtures of polyphenols (Sanoner *et al.*, 1999; Manach *et al.*, 2004).

In plant tissues, polyphenols are present mainly as glycosides, they also may be associated with different organic acids or may occur as complex polymerized molecules with high molecular weight such as tannins (D'Archivio *et al.*, 2007; Quideau *et al.*, 2011). These metabolites play an essential role in plant tissues especially as defense against plant pathogens and animal herbivore aggression. They are also produced as a response to different environmental stresses such as rainfall or ultraviolet radiation (Parr & Bolwell, 2000).

Polyphenols have been extensively studied for their potential role in the prevention of chronic diseases such as cardiovascular disease, cancer, osteoporosis, diabetes mellitus (Graf *et al.*, 2005; Arts & Hollman, 2005). Some data suggest that a diet rich in polyphenols has beneficial effect on human brain function and may provide protection in neurodegenerative diseases (Commenges *et al.*, 2000; Letenneur *et al.*, 2007). These compounds can affect human health mainly because of their antioxidant and antimicrobial properties as well as free-radical scavenging activity. Moreover consumption of food rich in polyphenols may support gut health. Therefore, an increasing interest is also observed in the interactions between polyphenols and microbiota occurring in food and human gut. Many phenolic compounds exert antimicrobial effect on various microorganisms including pathogenic bacteria and fungi. For example, catechins largely present in tea, inhibited the growth of *Vibrio cholerae*, *Campylobacter jejuni*, *Streptococcus mutans*, *Escherichia coli* and *Clostridium perfringens* (Ahn *et al.*, 1992; Diker *et al.*, 1991; Sakanaka *et al.*, 1992; Isogai *et al.*, 1998). Flavonols are active inhibitors against some Gram-positive bacteria, such as *Staphylococcus aureus*, *Lactobacillus acidophilus* and Gram-negative bacteria, such as *Prevotella* sp., *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Cushnie *et al.*, 2007). Also, phenolic acids displayed antibacterial

✉ e-mail: krzysztof.jus@ue.poznan.pl

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Abbreviations: LAB, lactic acid bacteria; PAs, phenolic acids; CFU, colony forming unit; MRS, de Man-Rogosa Sharpe; RSD, related standard deviation; NCFB, national collection of food bacteria

activity against Gram-positive (*S. aureus* and *L. monocytogenes*) and Gram-negative bacteria including *E. coli* and *Pseudomonas aeruginosa* (Saavedra *et al.*, 2010). However, some reports stated that phenolic compounds can also selectively stimulate the growth of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* and may therefore modulate gut microbiota (Lee *et al.*, 2006; Tzonis *et al.*, 2008). Bifidobacteria are members of the gut microbiota, known for their beneficial effect in human health. Their probiotic properties make them attractive as components of functional food and food supplements (Masco *et al.*, 2005). Taking into account that polyphenols and bifidobacteria are very important constituents of human diet, the relationship between them is particularly relevant. However, data concerning the effects of polyphenolic compounds on the bifidobacteria are insufficient. Therefore, the aim of the work presented here was the evaluation of the effect of some polyphenolic compounds on the growth of two strains of *Bifidobacterium*, i.e. *B. bifidum* and *B. adolescentis*.

MATERIALS AND METHODS

Microorganisms and culture conditions. Two strains of *Bifidobacterium*: *B. adolescentis* NCFB 2004 and *B. bifidum* NCFB 2235 were used in this study. Bacterial strains were routinely cultivated in de Man-Rogosa Sharpe broth (MRS) at 37°C for 24 h. Bifidobacteria suspensions for this study were prepared using log-phase cultures which were dissolved in MRS medium to obtain final concentration of bacterial suspension about 10⁵ CFU/ml.

Polyphenols. Flavonoids: naringin (naringenine-7-rhamnosidoglucoside), hesperidin (hesperetin-7-rutinoside), quercetin and its glycoside rutin (quercetin-3-O-rutinoside), as well as phenolic acids: gallic (3,4,5-trihydroxybenzoic acid), ferulic (trans-4-hydroxy-3-methoxycinnamic acid), chlorogenic (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)), sinapic (3,5-dimethoxy-4-hydroxycinnamic acid), *p*-coumaric (trans-4-hydroxycinnamic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid) and caffeic acid (3,4-dihydroxycinnamic acid) were purchased from Sigma-Aldrich (Germany). Pure polyphenols were dissolved in 80% ethanol to obtain a stock solution of 1 mg/ml. Stock solutions were kept at -20°C until analysis.

Assessment of polyphenols impact on *Bifidobacterium* growth. Experiments were performed using 96-well V-shaped microtiter plate assay. 135 µl of *Bifidobacterium* suspension in MRS broth was mixed with 15 µl of polyphenol solutions. The range of final concentration of polyphenol solutions were 2, 20 and 100 µg/ml. Microtiter plates were incubated at 37°C for 48 h. During the incubation, the absorbance was measured at 600 nm in a microplates reader after 0.5, 2, 14, 20, 24, 44 and 48 hours. The bacterial suspensions in MRS broth with addition of 8% ethanol were used as positive controls. Medium solutions with appropriate concentration of polyphenols were used as negative controls taking into account their influence on color and turbidity of blank samples. The value of the absorbance of blank samples (without bacteria) was subtracted from the value of sample absorbance (with bacteria). All experiments were performed in three parallel replications. Obtained results were expressed as a percentage of positive control in order to facilitate the comparison between bacteria species. The results were calculated with related standard deviation (±R.S.D.).

RESULTS

The impact of selected flavonoids e.g. naringinin, hesperidin, quercetin, rutin on the growth of *B. bifidum* and *B. adolescentis* is presented in Fig. 1. and the impact of phenolic acids: *p*-coumaric, ferulic, sinapic, caffeic, chlorogenic, gallic and vanillic is presented in Figs. 2 and 3. The examined polyphenols were used at the final concentration of 2, 20 and 100 µg/ml. The amount of ethanol (the polyphenols solvent) used at the final concentration of 8%, added to bacteria cultures, did not show any toxic effect. The turbidity of medium with polyphenols and without bacteria did not change significantly during 48-hour incubation at 37°C.

Comparing the results of the experiments, it can be clearly demonstrated that during 48 h incubation the tested polyphenols had the strongest impact on the growth of analyzed bifidobacteria during the first hours of incubation. Both, stimulatory and inhibitory effects were observed in comparison to the positive control (Figs. 1, 2, 3). The inhibitory effect during whole incubation time was observed only for hesperidin and quercetin addition. The effect was dose-dependent and the differences in concentration caused the biggest differences in growth rate of bacteria. The highest examined concentration of hesperidin and quercetin (100 µg/ml) reduced the growth of both *Bifidobacterium* species almost up to 10 and 20%, respectively (Fig. 1, B1, B2, C1, C2).

In the case of examined flavonoids, the strongest growth stimulation effect during the first hours of incubation was observed for *B. bifidum*. Rutin (100 µg/ml) increased growth up to 37%, and quercetin (by all examined concentrations), hesperidin (2 µg/ml) and naringin (20 and 100 µg/ml) up to about 20% in comparison to cultures without flavonoids. After 14 hours of incubation (after exponential phase), the growth rate of *B. bifidum* was at a similar level to the positive control, only the highest concentration of hesperidin and quercetin slowed down the growth of these bacteria, as it was already mentioned.

In the case of *B. adolescentis* no significant stimulation effect at the first two hours of incubation was observed, except for the quercetin at the concentration of 2 µg/ml. The impact of quercetin was dose-dependent. The highest quercetin concentration of 100 µg/ml showed inhibitory effect up to 35% (Fig. 1, C2). Naringin and hesperidin also slowed down the rate of growth depending on their concentration. Hesperidin at a higher concentration had the strongest inhibitory effect on *B. adolescentis* growth (up to 50%) at the first two hours. Rutin addition to the culture medium had no significant influence on the growth for all incubation times.

As polyphenolic acid are considered as antimicrobial compounds the effect of some derivatives of hydroxycinnamic and hydroxybenzoic phenolic acids (PAs) on *Bifidobacterium* growth was examined. In all cases of PAs, no significant inhibition effect on *Bifidobacterium* growth was observed. *B. bifidum* showed stronger dependence on PAs content in the medium than *B. adolescentis* during first two hours of incubation. The tests conducted, showed a significantly rapid, dose-dependent increase of cell numbers of *B. bifidum*, especially in the case of cumaric, caffeic and vanillic acid (Fig. 2, A1, C1, D1 and Fig. 3, A1). The strongest stimulatory effect was observed for the addition of cumaric acid (2, 20, 100 µg/ml) (Fig. 2, A1) which caused almost 20, 36 and 50% growth increase, respectively. Upon further incubation, the growth rate remained on a very similar level to the positive control. In the case of *B.*

adolescentis, during the first two hours of incubation no significant effect of polyphenolic acids on the increase of cell number was observed, except the stimulatory effect of vanillic (Fig. 3, A2) and caffeic (Fig. 2, D2) acid at the concentration of 20 µg/ml and the inhibitory effect of gallic acid (Fig. 3, B2). Probably, the phase of exponential growth of *B. adolescentis* started later than during the first two hours of incubation and any significant, neither stimulatory nor inhibitory effect was not registered. In general *B. adolescentis* was slightly inhibited by the presence of PAs in the incu-

bation medium in comparison to positive control and *B. bifidum* (Fig. 2 and Fig. 3).

DISCUSSION

The genus *Bifidobacterium* belonging to the *Actinobacteria* phylum comprises over 45 species/subspecies among which the best known are *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum*, *B. pseudocatenulatum*, *B. pseudolongum* and *B. animalis* subsp. *lactis* (Masco *et al.*, 2005; Turroni *et al.*, 2009). These bacteria are part of

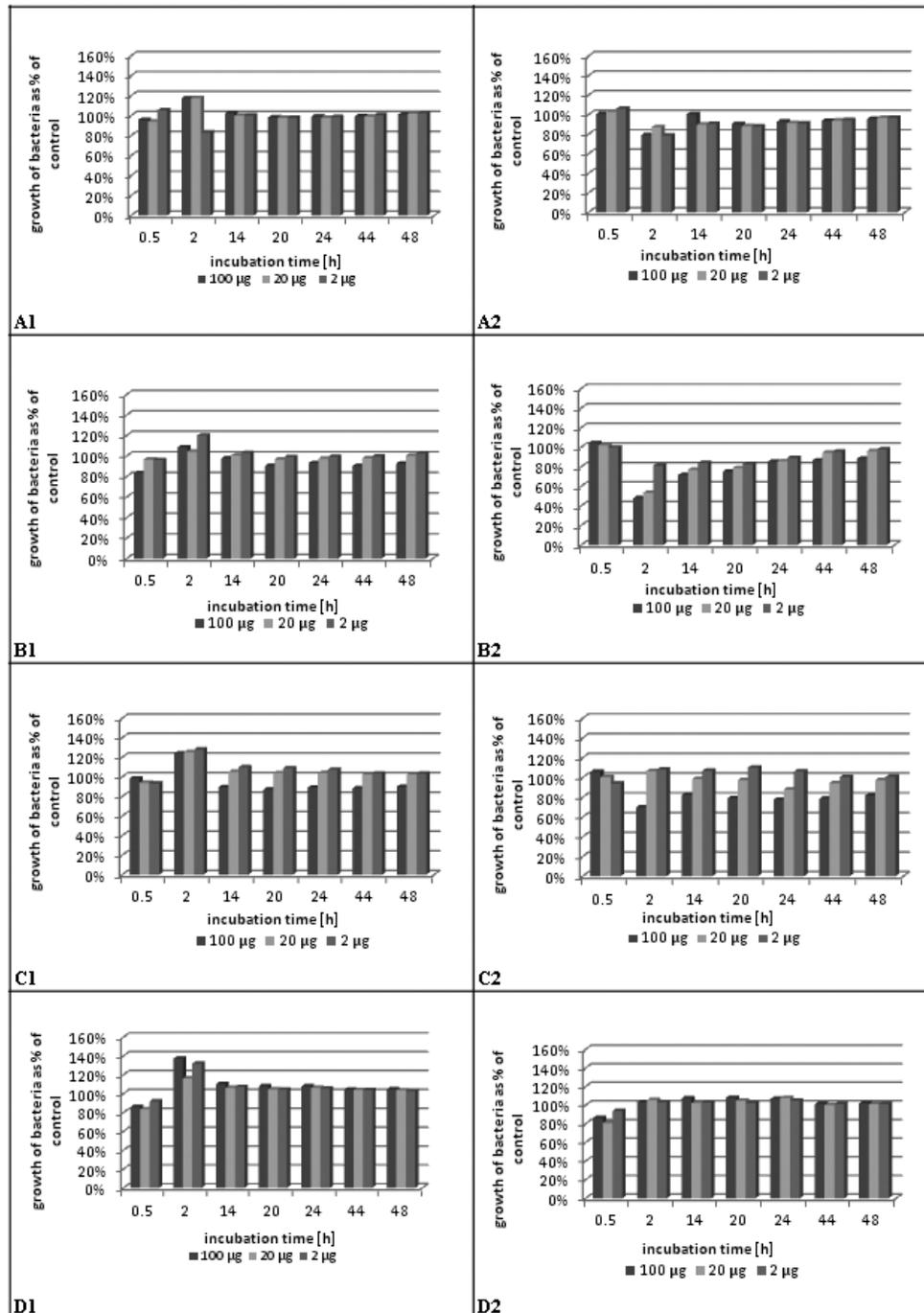


Figure 1. The effect of flavonoids on the growth of *Bifidobacterium* (A) naringinin, (B) hesperidin, (C) quercetin, (D) rutin, 1 — *B. bifidum*, 2 — *B. adolescentis*). The bars indicate the value of absorbance of samples containing bacterial cultures with polyphenols reduced by absorbance of the blank samples (MRS medium with polyphenol) and expressed as a percentage of positive control (absorbance of bacterial culture reduced by absorbance of the medium).

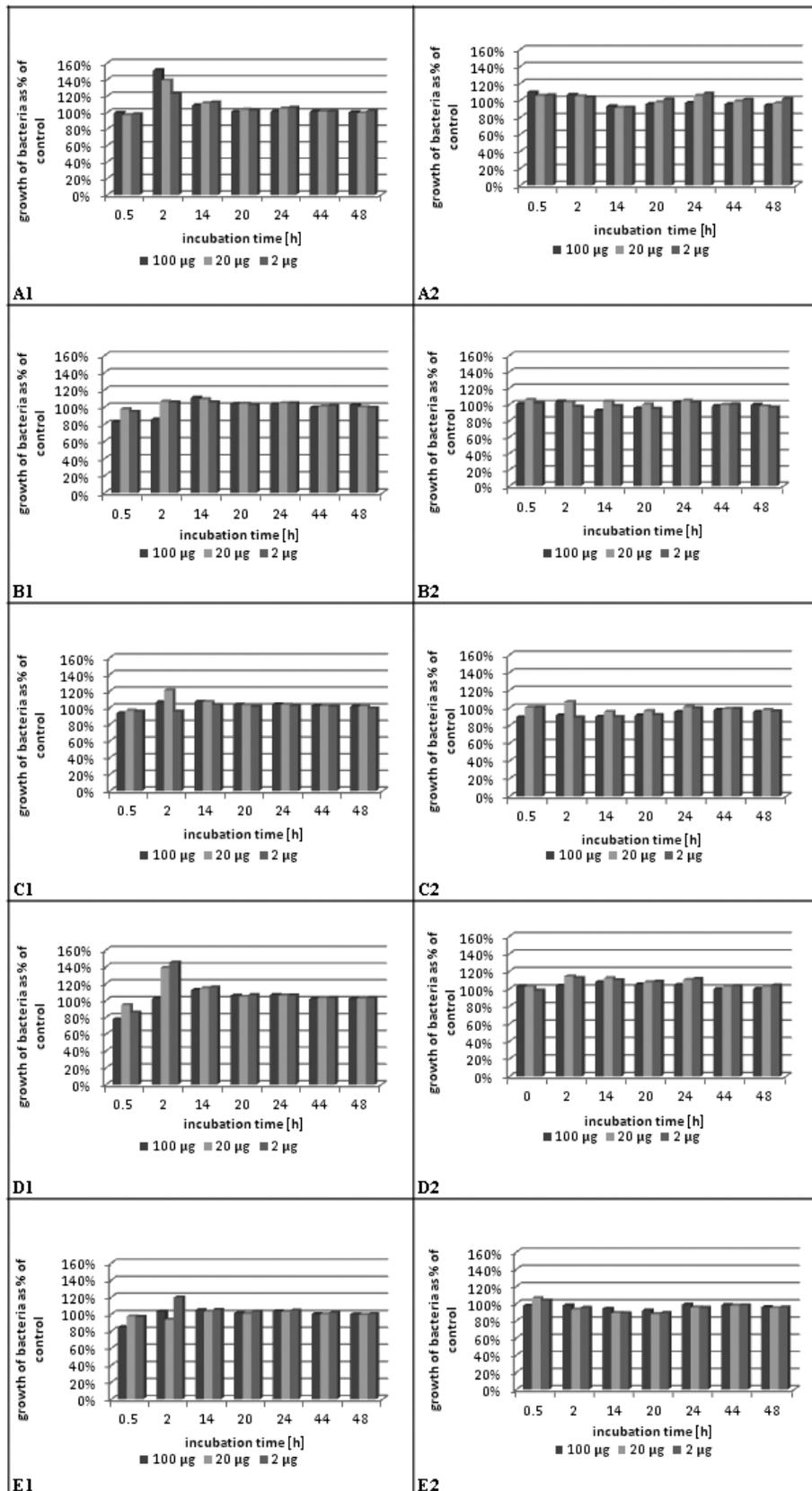


Figure 2. The effect of hydroxycinnamic phenolic acids on the growth of *Bifidobacterium*

(A) *p*-cumalic acid, (B) ferulic acid, (C) sinapic acid, (D) caffeic acid, (E) chlorogenic acid, 1 — *B. bifidum*, 2 — *B. adolescentis*. The bars indicate the value of absorbance of samples containing bacterial cultures with polyphenols reduced by absorbance of the blank samples (MRS medium with polyphenol) and expressed as a percentage of positive control (absorbance of bacterial culture reduced by absorbance of the medium).

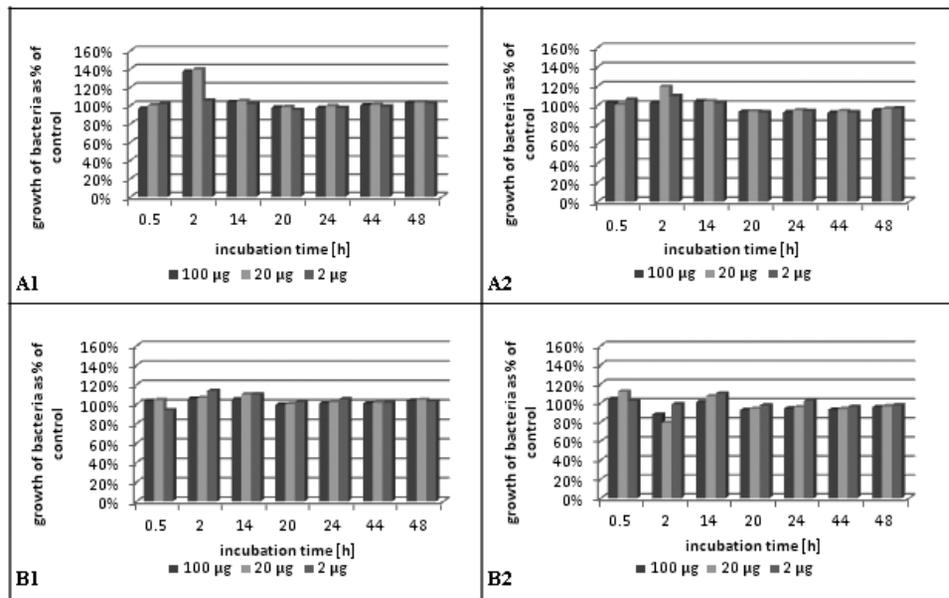


Figure 3. The effect of hydroxybenzoic phenolic acids on the growth of *Bifidobacterium*

(A) vanillic acid, (B) gallic acid, 1 — *B. bifidum*, 2 — *B. adolescentis*. The bars indicate the value of absorbance of samples containing bacterial cultures with polyphenols by absorbance of the blank samples (MRS medium with polyphenol) and expressed as a percentage of positive control (absorbance of bacterial culture reduced by absorbance of the medium).

normal gut microbiota of the gastrointestinal tract of humans and animals, often also found in functional foods and food supplements (Masco *et al.*, 2005; Margolles *et al.*, 2011). In recent years, *Bifidobacterium* genus has been a subject of many studies due to its important role within the human intestinal microbiota as well as the extensive use of certain strains in different probiotic products (Tojo *et al.*, 2014).

There are many studies concerning the influence of polyphenols on the growth and viability of lactic acid bacteria, however, mainly from *Lactobacillus* genera. The impact of these compounds on the gut microbiota is investigated since it is known that some polyphenols can inhibit growth of intestinal microorganisms. It is especially important taking into account possibility to inhibit pathogenic bacteria, while beneficial microorganisms are not inhibited or even stimulated. Most of the studies highlight the fact that the effect of polyphenolic compounds depends on the type of polyphenol, its form, concentration (Marsilio & Lanza, 1998; Rozès & Peres, 1998; Salih *et al.*, 2000), and also susceptibility of bacterial strain (Stead, 1993).

In the research conducted by Lee *et al.* (2006), tea polyphenolics including catechin, epicatechin, 3-O-methyl gallic acid, gallic and caffeic acid inhibited growth of pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides*, while the growth of *Bifidobacterium* as well as probiotic *Lactobacillus* sp. was less severely affected. Only caffeic acid inhibited growth of bifidobacteria, while in our research caffeic acid stimulated growth of bifidobacteria in the first hours of incubation. Tzounis *et al.* (2008) described stimulatory effect of catechin on the growth of *Bifidobacterium* sp. and less profound influence of epicatechin. Duda-Chodak (2012) investigated the influence of some flavonoids and phenolic acids on the growth of several chosen gut microorganisms including *Bacteroides galacturonicus*, *Lactobacillus* sp., *Enterococcus caecae*, *Bifidobacterium catenulatum*, *Ruminococcus gauvreauii*, *Escherichia coli*. The author had stated that naringenin and quercetin slowed down or even completely inhibited the

growth of all examined bacteria, while their glycosides (naringenin and rutin) did not exert inhibitory effect, but in some cases stimulated growth of microbiota. Similar observation was made in the case of hesperetin and hesperidin. Contrary to Tzounis *et al.* (2008), Duda-Chodak (2012) noticed that catechin did not influence the growth of most examined bacteria, however *Bifidobacterium catenulatum* was slightly inhibited by high concentration of catechin, while *E. caecae* was stimulated by higher doses of this compound.

Other authors also reported varying influence (dose and strain-dependent) of polyphenols on the growth of lactic acid bacteria including *Lactobacillus* and *Bifidobacterium*. Salih *et al.* (2000) reported the effect of hydroxycinnamic acids, their quinic esters and quinic acid on the growth of *L. plantarum* and stated that only hydroxycinnamic acids, among others including ferulic, *p*-coumaric and caffeic acid, influenced the bacterial growth. Differentiated effect of hydroxycinnamic acids on the growth of wine-spoilage LAB (*L. collinoides* and *L. brevis*) that related to concentration was noticed by Stead (1993). In his studies, caffeic, coumaric and ferulic acids markedly inhibited growth of LAB at concentration of 500 and 1000 mg/l, while at 100 mg/l, all compounds stimulated bacterial growth. It is worth noting that the strains of *L. collinoides* were more susceptible to polyphenol action than the *L. brevis* strain. Stead (1994) observed an effect of chlorogenic, gallic and quinic acids on the growth of *L. collinoides* and *L. brevis* dependent not only on concentration but also on growth phase of bacteria. Generally, the compounds tested at concentration of 100, 500 and 1000 mg/l stimulated growth of *L. collinoides* during early stages, however this effect was not observed for *L. brevis*. In the stationary phase, chlorogenic and gallic acids caused greater cell density of both strains, while quinic acid had a lesser influence. Dose-dependent effect of polyphenols on the growth of *Lactobacillus* and *Bifidobacterium* strains was reported by Duda-Chodak (2012). According to the author, *Bifidobacterium catenulatum* was fairly resistant to examined concentrations of polyphenols, while *Lactobacil-*

lus sp. was strongly inhibited by naringenin and quercetin at concentrations of 250 and 50 µg/ml, respectively.

In our research, all tested compounds influenced the growth of examined bacteria. Similar to other authors, the effect of phenolic compounds was differentiated and depended both on the concentration of polyphenol and the strain susceptibility. Both, stimulatory and inhibitory effects were observed in comparison to the positive control. It is worth noting that the effect of hesperidin and quercetin addition was the strongest dose-dependent and visible for all times of incubation. Generally, the influence of polyphenols on the growth of bifidobacteria was noticed during the first hours of incubation, which confirms the observations of other authors. Moreover, *B. bifidum* occurred to be more susceptible to phenolic compounds than *B. adolescentis*.

According to data based on *in vitro* and *in vivo* study, polyphenols may affect intestinal microbiota causing inhibition of some groups of bacteria and allowing others to thrive in the gut. That also indicates the influence of polyphenols on the bifidobacteria in the gut. Dolara *et al.* (2005) reported that in feces of rats whose diet for 16 weeks was supplemented with a dealcoholized, proanthocyanidin-rich red wine extract, *Bacteroides*, *Lactobacillus* and *Bifidobacterium* spp. were dominating, whereas *Bacteroides*, *Clostridium* and *Propionibacterium* spp. were predominant in control-fed rats. Sánchez-Patán *et al.* (2012) reported that wine extract rich in quercetin, flavan-3-ols and anthocyanins had no influence on the *Bifidobacterium*, *Lactobacillus/Enterococcus* spp., *Bacteroides* spp. and members of the domain *Bacteria*. Yamakoshi *et al.* (2001) evaluated the effect of proanthocyanidin-rich extract from grape seeds given to healthy adults for 2 weeks. This study stated that the number of bifidobacteria significantly increased, whereas the number of *Enterobacteriaceae* tended to decrease. Similarly, the research of a rat model indicated that the resveratrol commonly found in grapes increased the number of *Bifidobacterium* spp. and *Lactobacillus*, but diminished the number of enterobacteria (Larrosa *et al.*, 2009).

More detailed research is required in order to determine the differences in susceptibility to the polyphenols among strains of *Bifidobacterium*. The results obtained here indicate the differentiated activity of selected polyphenols towards tested bifidobacteria. The relationship between polyphenols and bifidobacteria is an essential one, taking into account that both of them are important bioactive compounds present in the human diet. Moreover, polyphenols may influence the viability of bifidobacteria in food products and diet supplements, as well as in human gastrointestinal tract.

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