Eur Food Res Technol (2018) 244:735–745
https://doi.org/10.1007/s00217-017-2997-9

ORIGINAL PAPER

Polyphenol content and in vitro evaluation of antioxidant, antimicrobial and prebiotic properties of red fruit extracts

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Received: 1 August 2017 / Revised: 8 October 2017 / Accepted: 14 October 2017 / Published online: 28 October 2017

Abstract A diet rich in fruit provides nutrients that are vital for human health. In particular, the anthocyanins contained in fruit have received attention due to their health-promoting properties and antioxidant, anti-inflammatory and antimicrobial activities. In this study, total phenolic, anthocyanin, flavonoid content and antioxidant activity were determined in extracts from plum skins, Italian red grape skins, and different parts of elderberry. Furthermore, it was analysed the activity of the fruit extracts in inhibiting several pathogens and in stimulating the growth of three probiotic strains and one blend SYNBIO®. All extracts show a good content of anthocyanins, exhibited high antioxidant activity and significantly inhibited the pathogens tested. The extracts had no inhibitory activity on the probiotic strains, but rather, they stimulated the growth of all the probiotics. In order to test the potential prebiotic properties of these anthocyanin-rich red fruit extracts, the kinetic growth of all probiotics was monitored, and it was found that probiotics in presence of elderberry and plum extracts had a significant increase in mean doubling time. The combined formulation of elderberry extracts and each of the four probiotics showed higher antioxidant activity compared to that of the extract alone, indicating the ability of probiotics to increase the antioxidant activity of the elderberry extracts. The fruit extracts used in this study can be considered beneficial for human health, due to their high content of polyphenols, particularly anthocyanins. Based on their antioxidant and antimicrobial activities, these fruit extracts can be considered good candidates for designing new functional foods and beverages, as well as nutraceuticals. Moreover, the ability of the fruit extracts to stimulate the growth of the probiotics merits further study to ascertain whether they can be defined as prebiotics.

Keywords Plant extracts · Polyphenols · Anthocyanins · Antioxidant/antimicrobial activity · Probiotics · Prebiotics

Introduction

Various traditional and folk medicines have used many antimicrobial substances from such sources as microorganisms, animals and plants [1].

More recent interest in natural food ingredients has been an important factor in expanding studies on less known horticultural plants. Various berries containing high amounts of bioactive phytochemicals, which have noticeable long-term physiological effects, have been the focus of a number of recent studies [2, 3].

Generally, fruit extracts have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory conditions [4, 5]. Moreover, fruits and berries are important dietary sources of such phenolic compounds as phenolic acids, flavonoids, stilbenes and lignans [6]. While little is known about the quantity of polyphenols consumed daily throughout the world, some information is available about the most studied group, the flavonols, the consumption of which has been estimated at 20–25 mg/day in the United States, Denmark and Holland [7]. In Italy, consumption ranges from 5 to 125 mg/day, with a mean value of 35 mg/

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day [8]. People who eat several servings of fruits and vegetables a day likely have a total polyphenol intake of about 1 g/day. Polyphenols have received much attention since being reported to have a positive influence on human health [9]. Epidemiological studies have repeatedly shown an inverse association between the risk of chronic human diseases and the consumption of a polyphenol-rich diet [10]. Polyphenol consumption limits the incidence of coronary heart diseases, as these substances are potent inhibitors of LDL oxidation, which is considered to be a key mechanism in the development of atherosclerosis [7].

Polyphenols may be protective against cardiovascular diseases because of their antioxidant, anti-platelet, and anti-inflammatory effects, and also because they increase HDL and improve endothelial function [11]. Moreover, other epidemiological studies and associated meta-analyses strongly suggested that long-term consumption of diets rich in plant polyphenols offered some protection against development of cancers, diabetes, osteoporosis and neurodegenerative diseases [12, 13].

Such fruits as grapes, apples, pears, cherries and berries contains up to 200–300 mg polyphenols per 100 g of fresh weight. In food, polyphenols may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability. The distribution of phenolics in plants at the tissue, cellular and sub-cellular levels is not uniform [14].

Among the polyphenols, the flavonoids are the most studied, mainly because a sub-group, the anthocyanins, have shown great bioactivity [14]. The anthocyanins exhibit a wide range of antioxidant protection and therapeutic benefits, including improved visual acuity and cognitive behaviour, as well as anti-inflammatory, antimicrobial and anti-age properties [15, 16]. Berry anthocyanins offer therapeutic and pharmacologic benefits, in particular, significant chemoprotective, anti-platelet aggregation and anti-angiogenic properties. Berry anthocyanins have been identified as novel cardioprotectants, beneficial in reducing age-associated oxidative stress, improving neuronal and cognitive brain function, as well as ocular health, and protecting genomic integrity [15, 17]. The purple-black fruits of elderberries (*Sambucus* spp. L.) are one of the richest sources of anthocyanins and phenolic compounds among small fruits [16]. Recently, extracts of both European, black or common elderberry (*Sambucus nigra* L.) and American elderberry (*Sambucus canadensis* L.) demonstrated significant chemopreventive properties, showing the potential to control enzymes commonly associated with various forms of cancer [18]. Because red grape (*Vitis vinifera* L.) contains a high amount of anthocyanins, which provide interesting colour, it is used in the food industry as an ingredient in healthy juices that are visually appealing to consumers [19]. The concentration and profile of anthocyanins in red grapes varies with species, variety, maturity, seasonal conditions, production area, viticultural practice and yield. Plums are the most numerous and diverse groups of fruit tree species [20], but the amount of research data does not reflect this abundance and there is only limited knowledge about anthocyanin composition in European plums (*Prunus domestica* L.).

In the production of probiotic functional foods, polyphenols do not interfere with the fermentation processes that take place in the food matrix, but they can play a key role in stimulating the metabolic activity of microorganisms, as well as improving viability in vitro and in vivo of probiotic starter cultures [21].

The majority of dietary anthocyanins are not absorbed at the upper GI tract, and hence reach the intestinal microbiota where they are biotransformed into their metabolites. This conversion is often essential for absorption and modulates the biological activity of these dietary compounds, not only due to the direct bioactivity of the products of microbiota metabolism, which is different from that of the parent compounds, but also because of their prebiotic activity in modulating the microbiota composition [22]. The phenolic influence on growth and viability of lactic acid bacteria was studied by Rodriguez and co-workers [23]. Only a few studies are available regarding the effect of polyphenols on starter and probiotic bacteria growth and viability. *Lactobacillus casei*, *L. plantarum*, *L. fermentum*, *L. rhamnosus*, *L. acidophilus*, *L. bulgaricus*, *Bifidobacterium lactis*, *B. breve*, *B. infantis* and *B. bifidum* [24] are the most studied probiotic species. In a study of the effect of phenolic compounds with antioxidant properties on *L. casei*, as a representative of probiotic microorganisms, it was shown that pure antioxidant compounds and fruit extracts had a stimulatory effect on its growth [25].

The first of three objectives of this study was to evaluate the total content of phenolics, anthocyanins, and flavonoids in extracts from Italian red grape skins, plum skins and elderberry parts, specifically the skin alone, the fruit (pulp, skin and seeds), and the skin and seeds together. Second, the antioxidant activity and inhibitory capacity of the five fruit extracts toward some pathogenic bacteria were evaluated. Third, the ability of the five fruit extracts to stimulate growth of some probiotic bacteria was tested in order to determine their prebiotic potential.

Materials and methods

Fruit extract preparation and characterization

The plums (*Prunus domestica* L.) and Italian red grapes (*Vitis vinifera* L.) were purchased from a local market, while the elderberry fruits (*Sambucus nigra* L.) were collected from the local botanical garden. Each fruit type was transported to the lab and manually peeled. Each type of

fruit underwent washing, drying, lyophilizing and grinding, that is grape peel, plum peel, and three categories of elderberry parts. Specifically, they were washed three times with distilled water, dried at room temperature and then lyophilized using an ALPHA 1–4 LD plus Freeze Dryer (Martin Christ, Osterode, Germany). The freeze-dried products were ground using a mini-blender (Philips, Suffolk—Glemsford, England) and stored at − 20 °C.

The smell, colour and granule dimensions of the lyophilized fruit powders were noted, the latter two visually, and the water activity and moisture content were determined, the former with the use of an AQUA LAB 4TE Decagon Device (Hopkins Ct. Pullman, USA) and the latter with the use of an Ohaus Thermo-balance moisture analyser (Nanikon, Switzerland).

Ethanolic fruit extract

All fruit extracts were prepared using 70% ethanol to reach a final concentration of 250 mg/ml, then filtered and stored at − 20 °C.

Determination of total phenolic content

Total phenolic content was determined in aliquots of each fruit extract by a modified Folin-Ciocalteu assay [26], using gallic acid as reference standard. All spectrophotometric data were acquired using a Shimadzu UV–visible spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany). Absorbance was read at 765 nm and results were expressed as gallic acid µg/ml equivalents using a gallic acid standard curve.

Determination of monomeric anthocyanin content

Total monomeric anthocyanin content was determined spectrophotometrically using the pH-differential method [27]. Two salt solutions were employed, namely potassium chloride, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M), and the absorbance was read at 510 and 700 nm. Total anthocyanins were calculated using the following equation:

$$\text{Total anthocyanins (mg/l)} = \frac{A \times M_W \times \text{DF} \times 10^3}{\varepsilon \times 1},$$

where $A = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$, M_W (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu), DF = dilution factor, l = path length in cm, ε = 26,900 molar extinction coefficient, in $l \times mol^{-1} \times cm^{-1}$ for cyd-3-glu and 10^3 = factor for conversion from g to mg. Results of monomeric anthocyanins were expressed as cyanidin-3-glucoside equivalents in mg/l.

Determination of total flavonoid content

The total flavonoid content of the fruit extracts was determined using a modified colorimetric method [28]. Briefly, 0.25 ml of diluted fruit extracts were mixed with 1.25 ml of distilled water and, subsequently, with 0.075 ml of 5% sodium nitrite solution and allowed to react for 5 min. Then 0.15 ml of 10% aluminium chloride was added and allowed to further react for 6 min before 0.5 ml of 1 M sodium hydroxide was added. Distilled water was added to bring the final volume of the mixture to 3 ml. The absorbance of the mixture was immediately measured at 510 nm against a prepared blank using a Double Beam UV/visible UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The flavonoid content was determined by a catechin standard curve and expressed as µg/ml catechin equivalents.

Antioxidant activity assay

Antioxidant activity was determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl) method [29] in which samples were allowed to react with 3.9 ml of daily prepared DPPH solution for 90 min at room temperature, in a dark place. The absorbance (A) of the resulting solution was measured at 517 nm. Blank values were subtracted from samples and standard values and a linear regression for the Trolox standards were constructed. For the antioxidant activity, the inhibition (%) was calculated with the following formula: $I(\%) = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$. The higher the inhibition, the higher the antioxidant activity.

Antimicrobial activity against pathogens and probiotic bacteria

Pathogens/probiotic strains and growth conditions

Standard pathogenic bacteria were purchased from ATCC and DSM, while *Listeria* strain was isolated from foods by IZS (Istituto Zooprofilattico Sperimentale, Umbria-Marche, Italy). The probiotic strains *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502® [30], their combination, named SYNBIO®, as well as *Lactobacillus plantarum* IMC 509 [31, 32], were provided by Synbiotec S.r.l. (Camerino, Italy). All the strains, their origins and cultural conditions are described in Table 1.

Agar well diffusion method

Potential mechanisms involved in the inhibition of pathogenic bacterial growth were investigated by a well diffusion assay [32]. A concentration of 0.5 McFarland

Table 1 Target strains used in this study, their origin, cultural and growth conditions

	Strain	Strain code	Origin	Cultural and growth conditions
Gram positives	*Bacillus cereus*	DSM[a] 345	Culture collection	TSB[b], 24 h at 37 °C, aerobiosis
	Lactobacillus paracasei	IMC 502®	Human intestinal tract[c]	MRS[d], 48 h at 37 °C, aerobiosis
	Lactobacillus plantarum	IMC 509	Human intestinal tract	MRS, 48 h at 37 °C, aerobiosis
	Lactobacillus rhamnosus	IMC 501®	Human intestinal tract	MRS, 48 h at 37 °C, aerobiosis
	Listeria monocytogenes	306	Isolated from food	TSB, 24 h at 37 °C, aerobiosis
	Staphylococcus aureus	ATCC[e] 25923	Culture collection	TSB, 24 h at 37 °C, aerobiosis
	SYNBIO®		Probiotic strain combination[c,f]	MRS, 48 h at 37 °C, aerobiosis
Gram negative	*Escherichia coli*	ATCC 13706	Culture collection	TSB, 24 h at 37 °C, aerobiosis
Yeast	*Candida albicans*	ATCC 10261	Culture collection	SAB[g], 24 h at 37 °C, aerobiosis

[a] DSM-German collection of microorganisms and cell cultures

[b] TSB-Tryptone soy broth (OXOID, Basingstoke, UK)

[c] SYNBIOTEC Srl Camerino (MC), Italy

[d] MRS-de Man, Rogosa, Sharpe broth (OXOID)

[e] ATCC-American type culture collection

[f] The 1:1 combination of *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®

[g] SAB-Sabouraud broth (OXOID)

(1.5×10^8 CFU/ml) of each target strain was prepared, then inoculated on the surface of the specific media (Table 1), after which 10 µl of each fruit extract was added. The 70% ethanol was used as control sample. After incubation the inhibition zone was measured. The results are expressed as difference between the inhibition zone given by the fruit extracts (IZ_{FE}) and the inhibition zone given by the ethanol (IZ_{Et}), in order to have the exclusive effect of inhibition of just the active compounds extracted from the lyophilized fruit powder.

Minimum inhibitory concentration (MIC)

The initial concentration of the fruit extracts was of 250 mg/ml and a minimum concentration of 0.24 mg/ml was reached. MICs were determined by a modified version of the broth micro-dilution method [33].

Growth kinetics of probiotic strains

Experimental design

Investigation of the growth kinetics of each culture was conducted using a modified de Man, Rogosa, Sharpe broth (mMRS) fermentation by *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, SYNBIO® and *L. plantarum* IMC 509, in the presence of the fruit extracts (10 g/l). MRS broth medium (Liofilchem, Roseto degli Abruzzi, Italy) containing glucose at a concentration of 10 g/l was used as positive control (CP). In particular, the maximum specific growth rate (µ) for each culture was calculated using the following equation:

$\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_2 and X_1 are the cell concentrations at times t_2 and t_1. Mean doubling time (Td) was calculated as $Td = \ln 2/\mu$ [34].

Optical density and pH determination

The optical density (OD) of each culture was monitored each hour using a Double beam UV/visible spectrophotometer at $\lambda = 560$ nm, while the pH was measured with a JENWAY 3510 pH meter (Stone Staffordshire, UK) every hour, in triplicate.

Determination of probiotic bacteria viability

The viability of probiotic bacteria was determined by the standard plate method and expressed as CFU/ml. For each sample, 500 µl were taken and serially diluted in saline solution. Ten-fold serial dilutions were used in counting bacteria by the pour plate technique in MRS agar, after aerobic incubation at 37 °C for 72 h.

Antioxidant and antimicrobial activities of new functional combinations

Preparation of fruit extract

To determine antioxidant and antimicrobial activity, the extracts of plum skin, Italian red grape skin and different parts of elderberries previously prepared were used in combination with several probiotic strains: *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, their combination 1:1 (SYNBIO®) and *L. plantarum* IMC 509.

Preparation of cell-free supernatants of probiotic strains

Cell free supernatant (CFS) of the probiotic strains was prepared to be tested as follows. Lactobacilli were grown in MRS broth for 24–48 h at 37 °C. Each culture was centrifuged at 10,000 rpm for 30 min and filtered using a 0.20 µm porous membrane (Sigma, St. Louis, MO). In order to determine the antioxidant and antimicrobial activity of the *Lactobacillus* strains in combination with the fruit extracts, each CFS probiotic strain was mixed 1:1 with each fruit extract.

Antioxidant and antimicrobial activity

The antioxidant activity of each fruit extract, CFS of probiotic lactobacilli, and their combination 1:1 (fruit extract: probiotic CFS) was investigated by the DPPH method, described previously, while their bacterial-inhibitory activity was investigated by agar well diffusion method against all pathogen strains described in Table 1.

Statistical analysis

All experiments were carried out in duplicate and each sample was analysed in duplicate. The results were expressed as mean ± standard deviation (SD). For each experiment and where applicable, a negative and a positive control were prepared. Significant differences between mean values were determined by Tukey's test after one-way analysis of variance (1-way ANOVA) using the GraphPad PRISM® 5.1 program. A *P* value less than 0.05 was considered statistically significant.

Results and discussions

Lyophilized fruit powder characterization

The visual description (colour and aspect), the water activity [35] and moisture content determination are shown in Table 2. Regarding the water activity, elderberry skin and seeds extract showed the lowest value (0.195), while the highest Aw was registered for the plum skin extract (0.412).

Determination of total phenolic content

The total phenolic content, expressed as µg of gallic acid per g of extract powder, was determined using a gallic acid standard curve and is presented in Table 3. Regarding the concentration of gallic acid in the extracts, the highest amount was shown in the elderberry skin extract (1005.02 µg/g), followed by the elderberry fruits and Italian red grape skin extracts, with a concentration in gallic acid of 474.95 and 275.39 µg/g, respectively. As for the plum skin and the elderberry skin and seeds extract, the concentration of the total phenolics did not exceed 122 µg/g.

Table 2 Physical and chemical properties of the lyophilized fruit powders

Extracts	Colour	Aspect	Water activity	Moisture content (%)
Elderberry fruits	Dark violet	Small particles	0.269 ± 0.003	4.62
Elderberry skin	Dark violet	Small particles	0.396 ± 0.001	6.84
Elderberry skin and seeds	Dark violet	Small particles	0.195 ± 0.010	4.45
Italian red grape skin	Blue–violet	Fine and smooth particles	0.375 ± 0.006	6.92
Plum skin	Bright burgundy	Fine and smooth particles	0.412 ± 0.001	9.37

Table 3 Concentration of total polyphenols (TP), total anthocyanins (TA), total flavonoids, antioxidant activity and TA/TP ratio of the fruit extracts

Fruit extracts	Total phenolics (TP) content (µg gallic acid/g of powder)	Total anthocyanins (TA) content (µg cya-3-glu/g of powder)	Total flavonoid content catechin equivalents (µg/g of powder)	Antioxidant activity (%) inhibition of DPPH	TA/TP ratio
Elderberry fruits	474.95 ± 8.67	381.14 ± 0.38	163.66 ± 2.84	90.19 ± 1.56	0.75
Elderberry skin	1005.02 ± 54.09	284.28 ± 0.57	534.51 ± 14.50	83.53 ± 1.30	0.28
Elderberry skin and seeds	122.39 ± 4.95	102.99 ± 1.23	47.28 ± 1.42	62.60 ± 1.38	0.84
Italian red grape skin	275.39 ± 1.65	236.95 ± 13.41	140.74 ± 9.66	89.86 ± 1.49	0.86
Plum skin	82.69 ± 0.83	42.15 ± 3.12	29.39 ± 2.84	35.01 ± 4.09	0.51

Mean of two determinations ± standard deviation

Determination of monomeric anthocyanin content

The total monomeric anthocyanin of all the tested samples ranged between 42.15 and 381.14 µg/g of extract powder. The elderberry fruit extract had the highest concentration in cyanidin-3-glucoside (381.14 µg/g), followed by elderberry skin and Italian red grape skin extracts with a value of 284.28 mg/ml of cyanidin-3-glucoside and 236.95 µg/g, respectively. The plum skin extract showed the smallest content of monomeric anthocyanins (42.15 µg/g), as reported in Table 3.

Determination of total flavonoid content

The flavonoid content was correlated with the total phenolic content and ranged from 29.39 µg of catechin/g in the plum skin extract to 534.51 µg of catechin/g in the elderberry skin extract (Table 3).

Determination of antioxidant activity

The antioxidant activity of all fruit extracts was tested determining the anti-radical activity against free-radical DPPH. All extracts displayed antioxidant activity and the highest values were demonstrated by the elderberry fruit and Italian red grape extracts, with 90.19 and 89.86%, respectively (Table 3). The elderberry skin extract also provided very good antiradical activity against DPPH, with a percentage of 83.53%, while the plum skin extract, with a value of 35.01%, showed the lowest antiradical activity.

Antimicrobial activity against pathogens and probiotic bacteria

Agar well diffusion method

Figure 1 shows the antimicrobial activity of fruit extracts against several potential pathogen strains (Gram-positives, Gram-negatives and yeast). The antimicrobial activity of an active compound has been classified on the basis of the pathogen's resistance or sensitivity, according to measurement of inhibition zone [36]: when IZ < 5 mm it is considered resistant (*R*), when IZ = 5–20 mm it is considered sensitive (*S*) and when IZ > 20 mm it is considered super-sensitive (SS).

As shown in Fig. 1, all extracts showed inhibitory activity against the potential pathogen strains tested. The extracts had very good growth inhibitory activity against *B. cereus* DSM 345, especially the elderberry skin and seeds extract, which had an inhibition zone higher than 18 mm. Regarding *S. aureus* ATCC 25923 and *E. coli* ATCC 13706, the extracts displayed almost the same inhibitory activity, the inhibition zone ranging between 10 and 15 mm. Except for *C. albicans* ATCC 10261, which was resistant to all fruit extracts, the other pathogens exhibited sensitivity when in contact with the fruit extracts.

Figure 1 also indicates that the fruit extracts seem to stimulate growth of probiotic strains. All the probiotics were stimulated in the presence of fruit extracts, except *L. paracasei* IMC 502®, which was slightly stimulated by the presence of extracts or, in some cases, such as elderberry skin and elderberry skin and seeds extracts, was slightly inhibited. The elderberry skin extract expressed the highest stimulatory effect on the growth of *L. rhamnosus* IMC 501®. The other fruit extracts also stimulated the growth of *L. rhamnosus* IMC 501®, except for elderberry skin and seeds extract, which instead expressed slight inhibition. In the case of *L. plantarum* IMC 509 and SYNBIO®, which is the 1:1 combination of *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®, there was a tendency to growth stimulation in the presence of fruit extracts.

Determination of minimum inhibitory concentration (MIC)

Table 4 shows the MIC of all 5 fruit extracts and ethanol tested to evaluate the inhibitory effect level on several microorganisms. Among the Gram-positive bacterial strains, *B. cereus* DSM 345 and *S. aureus* ATCC 25923 were highly sensitive to all three elderberry extracts and the Italian red grape extract, with values ≤ 15.63 mg/ml. Regarding Gram-negative bacteria, *E. coli* ATCC 13706 was sensitive to elderberry skin and Italian red grape skin extracts, with a MIC value of 7.81 mg/ml, while *C. albicans* ATCC 10261

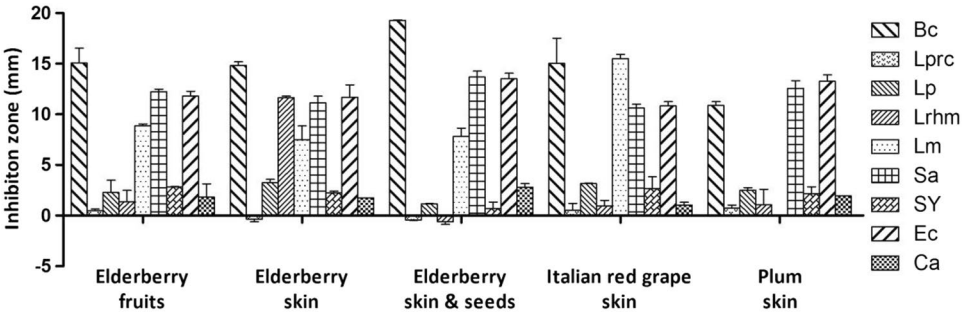

Fig. 1 Effect on growth of pathogenic and probiotic bacteria (*Bc B. cereus* DSM 345, *Lprc L. paracasei* IMC 502®, *Lp L. plantarum* IMC 509, *Lrhm L. rhamnosus* IMC 502®, *Lm L. monocytogenes* 306, *Sa S. aureus* ATCC 25923, SY-SYNBIO®, *Ec E. coli* ATCC 13706, *Ca C. albicans* ATCC 10261)

Table 4 Antimicrobial activity of fruit extracts on several bacterial strains expressed as minimal inhibitory concentration (MIC)

	Strains	Fruit extracts' MIC (mg/ml[a])					
		Elderberry skin	Plum skin	Elderberry skin and seeds	Elderberry fruits	Italian red grape skin	Ethanol 70%[b]
Gram positives	*B. cereus* DSM 345	7.81	15.63	7.81	7.81	7.81	15.63
	L. paracasei IMC 502®	15.63	15.63	15.63	15.63	31.25	15.63
	L. plantarum IMC 509	15.63	15.63	15.63	15.63	62.5	15.63
	L. rhamnosus IMC 501®	15.63	15.63	15.63	15.63	31.25	15.63
	List. monocytogenes 306	7.81	7.81	7.81	7.81	7.81	7.81
	S. aureus ATCC 25923	15.63	15.63	7.81	7.81	7.81	15.63
	SYNBIO®	15.63	15.63	31.25	15.63	31.25	15.63
Gram negative	*E. coli* ATCC 13706	7.81	15.63	15.63	15.63	7.81	15.63
Yeast	*C. albicans* ATCC 10261	3.91	7.81	7.81	7.81	7.81	7.81

[a] Mean of two determinations

[b] Negative control for the samples

was inhibited only by elderberry skin extract at a concentration of 3.91 mg/ml.

Growth curve of several probiotic strains

Growth kinetics

Table 5 shows fermentation starting time, according to the fermentation process of probiotics, and all the growth curves followed the tendency of the positive control (glucose), in which the pH decreased as the lactobacilli grew and multiplied. Table 5 shows that all five fruit extracts can be used by the probiotic strains tested as prebiotic substrate, with behaviour similar to that of the positive control. Table 6 reports the mean doubling time (hours) of each probiotic strain and of the SYNBIO® combination when grown in mMRS enriched with one of the fruit extracts, that is, mMRS with elderberry skin, mMRS with plum skin, mMRS with elderberry skin and seeds, mMRS with elderberry fruits and mMRS with Italian red grape skin. Mean doubling time was used as a measure of the effectiveness of the polyphenol/anthocyanin-rich fruit extracts in modulating the growth rate. In general, doubling times of the tested probiotics in the presence of the fruit extracts were similar to those of the positive control.

Determination of probiotic concentration

Probiotic bacterial concentration, expressed as CFU/ml, was monitored during the process of fermentation of all probiotics tested, using different modified media enriched with fruit extracts, as described above. Overall, the presence of fruit extracts improved the probiotic counts. In all cases, the bacterial concentration followed the same trend as the positive control (glucose) (Table 7).

Table 5 Fermentation starting time (h) of *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, SYNBIO® and *L. plantarum* 509 in mMRS enriched with several fruit extracts

Fruit extracts	Fermentation starting time (hours)			
	L. rhamnosus IMC 501®	*L. paracasei* IMC 502®	SYNBIO®	*L. plantarum* IMC 509
Elderberry fruits	10 ± 0.16*	8 ± 0.05	7 ± 0.09	3 ± 0.08*
Elderberry skin	12 ± 0.06	8 ± 0.10	9 ± 0.05	5 ± 0.03
Elderberry skin and seeds	14 ± 0.03	8 ± 0.07	8 ± 0.01	5 ± 0.14
Italian red grape skin	4 ± 0.08*	4 ± 0.12*	5 ± 0.11*	3 ± 0.10*
Plum skin	15 ± 0.11	8 ± 0.06	8 ± 0.12	5 ± 0.06
Positive control[a]	11 ± 0.05	7 ± 0.01	7 ± 0.10	4 ± 0.05

* Significantly lower difference ($P < 0.05$) from the control by 1-way ANOVA test

[a] mMRS containing glucose 10 g/l

Antioxidant and antimicrobial activities of new functional combinations

Antioxidant activity

As illustrated in Fig. 2, the combined formulation with plum skin extract and all four probiotics showed higher antioxidant activity than did the extract alone. The same situation was also observed for the elderberry skin and seeds combined with the probiotic strains. Even if each individual probiotic strain demonstrated low antioxidant activity, the ability to increase the antioxidant activity of the plum skin extract and the elderberry skin and seeds extract was significantly higher than the extract activity alone.

Antimicrobial activity

Applying the previous agar well diffusion method, the antimicrobial activity of the combinations of the five fruit extracts with each probiotic strains was analysed. The combination between probiotics and fruit extracts did not demonstrate potentiated/increased antimicrobial activity against any of the pathogen strains tested (data not shown).

Conclusions

Consumers are increasingly interested in foods that contain high levels of bioactive compounds that help prevent various diseases. Flavonoid compounds of plant origin have gained considerable attention due to their functional and nutritional benefits, including anti-inflammatory, antimicrobial activity and other salutary effects [37].

The results of this study provide enough evidence that fruit extracts that are rich in polyphenols, especially anthocyanins, have beneficial effects on human health. The fruit extracts were tested for their composition in bioactive compounds, such as polyphenols, flavonoids and anthocyanins.

Table 6 Doubling time of *Lactobacillus* strains in mMRS enriched with fruit extracts

Probiotic strains	Mean doubling time (h)[a]					
	Elderberry fruits	Elderberry skin	Elderberry skin and seeds	Italian red grape skin	Plum skin	Control
L. rhamnosus IMC501®	1.07 ± 0.02	1.21 ± 0.06	1.40 ± 0.21*	1.14 ± 0.10	1.20 ± 0.02	0.99 ± 0.05
L. paracasei IMC502®	1.33 ± 0.07	1.36 ± 0.05	1.74 ± 0.25	1.27 ± 0.05	1.53 ± 0.12	1.46 ± 0.02
SYNBIO®	1.53 ± 0.26	1.25 ± 0.05	1.37 ± 0.11	1.07 ± 0.06	1.37 ± 0.09	1.05 ± 0.04
L. plantarum IMC 509	1.36 ± 0.02*	1.52 ± 0.08*	1.72 ± 0.04*	1.18 ± 0.06	1.67 ± 0.14*	0.99 ± 0.02

Control: mMRS with glucose

* Significantly different ($P < 0.05$) from the control by 1-way ANOVA test

[a] Measurement are mean ± SD of 3 replicates

Table 7 Viable counts (expressed as Log CFU/ml) of *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, SYNBIO® and *L. plantarum* IMC 509 in mMRS containing different fruit extracts

Probiotic strains	Time (h)	Probiotic concentration (Log CFU/ml ± standard deviation)					
		Elderberry fruits	Elderberry skin	Elderberry skin and seeds	Italian red grape skin	Plum skin	Positive control[a]
L. rhamnosus IMC 501®	Start[b]	6.00 ± 0.03	5.97 ± 0.05	6.05 ± 0.16	6.10 ± 0.04	6.13 ± 0.04	6.03 ± 0.03
	End[c]	8.81 ± 0.03	8.46 ± 0.09	8.23 ± 0.17	8.75 ± 0.18	8.64 ± 0.01	9.08 ± 0.13
L. paracasei IMC 502®	Start	6.16 ± 0.02	6.05 ± 0.06	6.21 ± 0.04	6.18 ± 0.05	6.14 ± 0.08	6.13 ± 0.02
	End	8.43 ± 0.14	8.28 ± 0.01	7.96 ± 0.30	8.56 ± 0.04	8.12 ± 0.07	8.20 ± 0.02
SYNBIO®	Start	6.06 ± 0.02	6.04 ± 0.00	6.10 ± 0.01	6.01 ± 0.11	6.11 ± 0.11	6.09 ± 0.06
	End	8.06 ± 0.36	8.45 ± 0.09	8.30 ± 0.20	8.82 ± 0.04	8.31 ± 0.03	8.96 ± 0.17
L. plantarum IMC 509	Start	6.50 ± 0.00	6.47 ± 0.09	6.41 ± 0.09	6.43 ± 0.05	6.43 ± 0.06	6.44 ± 0.02
	End	8.72 ± 0.03	8.44 ± 0.02	8.17 ± 0.05	8.97 ± 0.08	8.25 ± 0.10	9.49 ± 0.04

[a] Positive control: MRS

[b] Concentration values (± SD) of the inoculum

[c] Concentration values (± SD) at fermentation end

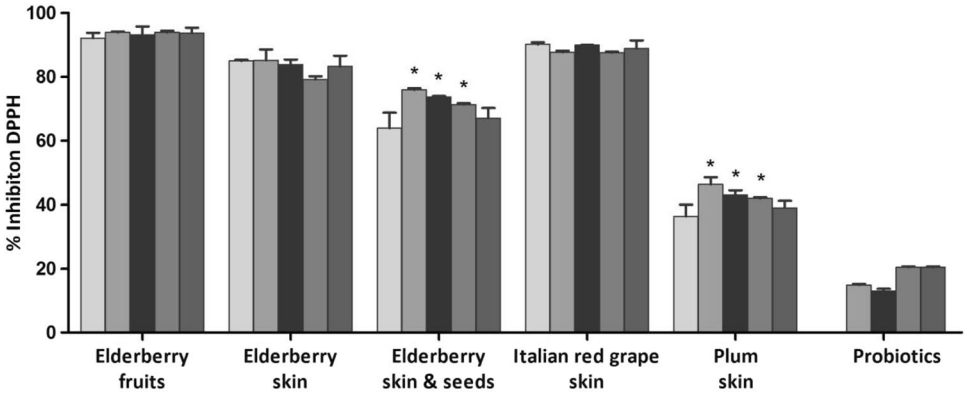

Fig. 2 Antioxidant activity of fruit extracts in combination with probiotics (filled square-Fruit extract alone, filled square-Fruit extract with *L. rhamnosus* IMC 502®, filled square-Fruit extract with *L. paracasei* IMC 502®, filled square-Fruit extract with SYNBIO®, filled square-Fruit extract with *L. plantarum* IMC 509)

Among all the fruit extracts, the highest concentration in polyphenols and flavonoid compounds was found in elderberry skin extract, followed by the elderberry fruits and Italian red grape skin extracts. High concentrations of anthocyanins, expressed as cyanidin-3-glucosides, were found in elderberry skin and elderberry fruits extracts, followed by Italian red grape skin extract. Regarding the anthocyanin content, a lower concentration was found in plum skin extract and elderberry skin and seeds extract. It is known that the higher concentration in phytochemicals, especially anthocyanins, the higher the antioxidant activity. All the fruit extracts expressed strong antioxidant capacity, being able to combat the free radicals that are the major contributors to aging and diseases like cancer, heart disease, decline of the brain function and decline of the immune system. As expected, the highest antioxidant activity was expressed by the elderberry fruits, elderberry skin and Italian red grape skin extracts, followed by the extracts of elderberry skin and seeds and of plum skin.

All fruit extracts also revealed antimicrobial properties with an inhibitory effect against all the pathogenic strains tested, except for *C. albicans* ATCC 10261, which was resistant, given an inhibition zone less than 5 mm. The highest inhibition was observed in the cases of *B. cereus* DSM 345, *S. aureus* ATCC 25923 and *E. coli* ATCC 13706. The elderberry skin and seeds extract had a very good inhibitory effect against the growth of *B. cereus*, with an inhibition zone of almost 20 mm, thus making *B. cereus* almost super-sensitive in the presence of this extract. The fruit extracts had a moderate inhibitory effect against *L. monocytogenes* 306.

While all 5 fruit extracts had an inhibitory effect against pathogens, it was interesting and surprising to note that they had a stimulatory effect on the growth of the probiotic strains tested (*L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, their 1:1 combination, named SYNBIO® and *L. plantarum* 509). The highest stimulatory effect on growth was observed in the case of *L. rhamnosus* IMC 501® in the presence of elderberry skin extract. The fruit extracts did not have a good stimulatory effect on *L. paracasei* IMC 502®, but there was a satisfactory stimulatory effect on the SYNBIO® blend of *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®, specifically in the presence of fruit extracts rich in anthocyanins, the highest stimulatory effect being observed in the presence of elderberry fruit extract, followed by the Italian red grape skin extract.

To verify the real stimulatory effect of fruit extracts on the growth of probiotics, the growth curves of *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, the SYNBIO® combination and *L. plantarum* 509 were determined in order to evaluate the prebiotic potential of the extracts. All fruit extracts were used as carbon source by the probiotics during the in vitro fermentation, thus their growth were stimulated in the presence of these anthocyanin-rich fruit extracts. Growth curves similar to those of the positive control were observed in the media enriched with Italian red grape skin extract for all probiotics tested and a similar trend was also observed in media enhanced with elderberry fruit extract. The pH followed the normal trend-line of fermenting lactobacilli, the values decreasing with the growth of probiotic cells.

When considering antimicrobial treatments for diseases, it is important to reflect on the possible impact upon beneficial microorganisms, because many antibiotic treatments cause a detrimental reduction in the natural microflora population, and thus the overpopulation of non-beneficial bacteria. This research demonstrates that the probiotic species have the resistance and, surprisingly, were stimulated by the fruit extract treatments, compared to pathogenic species. The results suggest that anthocyanins and their metabolites may exert a positive modulation on the intestinal bacterial population.

Therefore, the present findings indicate that anthocyanins appear to be stable in food matrices. With the goal of creating a functional food product or a synbiotic food formulation, the antioxidant capacity and antimicrobial activity of several probiotic strains in combination with several anthocyanin-rich fruit extracts were tested. The results

demonstrate improved antioxidant potential of the probiotic-fruit extract combinations.

When it comes to pro-health food, maximal retention of bioactive compounds during processing is strongly desired, since the beneficial biological properties of food are to some extent due to their active constituents. Quantification of the anthocyanin profile provides useful information in the effort to optimize the fruit extracts, as well in the choice of processing techniques, storage methods and raw materials that best limit losses and changes, in order to improve the pro-health potential of these red fruit extracts.

The results of this study indicate that all five fruit extracts tested, in particular the elderberry extracts and the Italian red grape extract, have high phenolic (anthocyanin) content and elevated antioxidant activity. Further studies are needed to determine how anthocyanins from different fruit sources behave after consumption and to explore the relationship between a polyphenol/anthocyanin-rich diet and gut microbiota composition.

In conclusion, elderberry, like all red foods, can be considered quite beneficial for human health because of the antioxidant and antimicrobial compounds it contains.

In fact, the elderberry investigated here showed appreciable antioxidant activities and supported the growth of *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®, SYNBIO® combination and *L. plantarum* 509. Therefore, this variety of plant can be very useful in reducing free radical induced damage, enhancing immune-competence and reducing inflammation mediated pathologies.

While interesting and in some cases surprising, the results of this study have limitations, dictated by the in vitro testing, single culture microbial growth and inhibition experiments, and focus on chemical antioxidant capacity. They do not mimic the complex, competitive gut environment or take fully into account biological antioxidant activity.

An in vivo study on human subjects will follow to confirm these interesting results and to provide more information on the significant bioactive qualities of elderberry.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements All authors declare that this article does not contain any studies with human or animal subjects.

References

1. Hleba L, Vuković N, Horská E, Petrová J, Sukdolak S, Kačániová M (2014) Phenolic profile and antimicrobial activities to selected microorganisms of some wild medical plant from Slovakia. Asian Pac J Trop Dis 4:269–274
2. Diaconeasa Z, Leopold L, Rugina D, Ayvaz H, Socaciu C (2015) Antiproliferative and antioxidant properties of anthocyanin rich extracts from blueberry and blackcurrant juice. Int J Mol Sci 16:2352–2365
3. Kraujalyte V, Venskutonis PR, Pukalskas A, Cesoniene L, Daubaras R (2013) Antioxidant properties and polyphenolic compositions of fruits from different European cranberrybush (*Viburnum opulus* L.) genotypes. Food Chem 141:3695–3702
4. Okigbo RN, Ogbonnaya UO (2006) Antifungal effects of two tropical plant leaf extracts (*Ocimum gratissimum* and *Aframomum melegueta*) on postharvest yam (*Dioscorea* spp.) rot. Afr J Biotechnol 5:727–731
5. Shariff N, Sudarshana MS, Umesha S, Hariprasad P (2006) Antimicrobial activity of *Rauvolfia tetraphylla* and *Physalis minima* leaf and callus extracts. Afr J Biotechnol 5:946–950
6. Spencer JP, El Mohsen MMA, Minihane AM, Mathers JC (2008) Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. Br J Nutr 99:12–22
7. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747
8. Pietta P, Simonetti P, Roggi C (1996) Dietary flavonoids and oxidative stress. In: Kumpulainen JT, Salonen JT (eds) Natural antioxidants and food quality in atherosclerosis and cancer prevention. Royal Society of Chemistry, London, pp 249–255
9. Jakobek L, Seruga M, Medvidovic-Kosanovic M, Novak I (2007) Anthocyanin content and antioxidant activity of various red fruit juice. Deutsche Lebensmitlel-Rund 2:58–64
10. Scalbert A, Manach C, Morand C, Remesy C, Jimenez L (2005) Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr 45:287–306
11. García-Lafuente A, Guillamón E, Villares A, Rostagno MA, Martínez JA (2009) Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflamm Res Off J Eur Histamine Res Soc 58:537–552
12. Arts IC, Hollman PC (2005) Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 81:317S–325S
13. Graf BA, Milbury PE, Blumberg JB (2005) Flavonols, flavones, flavanones, and human health: epidemiological evidence. J Med Food 8:281–290
14. Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270–278
15. Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C, Scalbert A (2003) Metabolism of dietary procyanidins in rats. Free Radic Biol Med 35:837–844
16. Ozgen M, Scheerens JC, Reese RN, Miller RA (2010) Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (*Sambucus canadensis* L.) accessions. Pharmacogn Mag 6:198–203
17. Zafra-Stone S, Bagchi M, Bagchi D (2007) Health benefits of edible berry anthocyanins: novel antioxidant and anti-angiogenic properties. In: Shahidi F, Ho CT (eds) Antioxidant measurement and applications. ACS Symposium Series 956, American Chemical Society, Washington, D.C, pp 337–351
18. Thole JM, Kraft TF, Sueiro LA, Kang YH, Gills JJ, Cuendet M, Pezzuto JM, Seigler DS, Lila MA (2006) A comparative evaluation of the anticancer properties of European and American elderberry fruits. J Med Food 9:498–504
19. Siro I, Kapolna E, Kapolna B, Lugasi A (2008) Functional food. Product development, marketing and consumer acceptance-a review. Appetite 51:456–467
20. Blazek J (2004) A survey of the genetic resources used in plum breeding. In: VIII international symposium on plum and prune genetics, breeding and pomology 734:31–45

21. Servili M, Rizzello CG, Taticchi A, Esposto S, Urbani S, Mazzacane F, Di Maio I, Selvaggini R, Gobbetti M, Di Cagno R (2011) Functional milk beverage fortified with phenolic compounds extracted from olive vegetation water, and fermented with functional lactic acid bacteria. Int J Food Microbiol 147:45–52
22. Faria A, Fernandes I, Norberto S, Mateus N, Calhau C (2014) Interplay between anthocyanins and gut microbiota. J Agric Food Chem 62:6898–6902
23. Rodriguez H, Curiel JA, Landete JM, de la Rivas B, de Felipe FL, Gomez-Cordoves C (2009) Food phenolics and lactic acid bacteria. Int J Food Microbiol 132:79–90
24. Rapeanu G, Bahrim G, Stanciuc N (2014) Microorganism metabolic activity stimulation by polyphenols. In: Watson RR, Preedy VR, Zibadi S (eds) Polyphenols in human health and disease. Academic Press, Elsevier, pp 513–521
25. Duda-Chodak A, Tarko T, Statek M (2008) The effect of antioxidants on *Lactobacillus casei* cultures. Acta Scientiarum Polonorum Technol Aliment 7:39–51
26. Arnous A, Makris DP, Kefalas P (2002) Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. J Food Compos Anal 15:655–665
27. Lee J, Durst RW, Wrolstad RE (2005) Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J AOAC Int 88:1269–1278
28. Dewanto V, Wu X, Adom KK, Liu RH (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 50:3010–3014
29. Kedare SB, Singh RP (2011) Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol 48:412–422
30. Verdenelli MC, Ghelfi F, Silvi S, Orpianesi C, Cecchini C, Cresci A (2009) Probiotic properties of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces. Eur J Nutr 48:355–363
31. Coman MM, Cresci A (2014) Probiotics and prebiotics for human health: innovations and new trends. Edizioni Accademiche Italiane trademark of OmniScriptum GmbH & Co., KG
32. Coman MM, Verdenelli MC, Cecchini C, Silvi S, Orpianesi C, Boyko N, Cresci A (2014) In vitro evaluation of antimicrobial activity of *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and SYNBIO® against pathogens. J Appl Microbiol 117:518–527
33. Nasuti C, Coman MM, Olek RA, Fiorini D, Verdenelli MC, Cecchini C, Silvi S, Fedeli D, Gabbianelli R (2016) Changes on fecal microbiota in rats exposed to permethrin during postnatal development. Environ Sci Pollut Res Int 23(11):10930–10937
34. Coman MM, Verdenelli MC, Cecchini C, Silvi S, Vasile A, Bahrim GE, Orpianesi C, Cresci A (2013) Effect of buckwheat flour and oat bran on growth and cell viability of the probiotic strains *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and their combination SYNBIO®, in synbiotic fermented milk. Int J Food Microbiol 167:261–268
35. Aoi W, Naito Y, Yoshikawa T (2006) Exercise and functional foods. Nutr J 5:15
36. Homayouni A, Alizadeh M, Alikhah H, Zijah V (2012) Functional dairy probiotic food development: trends, concepts, and products. In: Probiotics. InTech
37. Peng M, Aryal U, Cooper B, Biswas D (2015) Metabolites produced during the growth of probiotics in cocoa supplementation and the limited role of cocoa in host-enteric bacterial pathogen interactions. Food Control 53:124–133