Kafkas Univ Vet Fak Derg
25 (2): 137-146, 2019
DOI: 10.9775/kvfd.2018.20290

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

In Vitro Antimicrobial Effect of Phenolic Extracts and Resistant Starch on *Escherichia coli, Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp.

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Article ID: KVFD-2018-20290 Received: 02.06.2018 Accepted: 26.12.2018 Published Online: 30.12.2018

How to Cite This Article

Karamati Jabehdar S, Mirzaei Aghjehgheshlagh F, Navidshad B, Mahdavi A, Staji H: *In vitro* antimicrobial affect of phenolic extracts and resistant starch on *Escherichia coli, Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp.. *Kafkas Univ Vet Fak Derg,* 25 (2): 137-146- 2019. DOI: 10.9775/kvfd.2018.20290

Abstract

The present study aimed to evaluate the antimicrobial activity of Grape Pomace Extract (GPE), Pistachio Peel Extract (PsPE), and Pomegranate Pomace Extract (PPE) with or without Resistant Starch (RS) as a prebiotic on gut microflora representative's *in vitro* conditions. For this purpose, the Resistant Starch (Fibersol2), grape pomace, pistachio peel, and pomegranate pomace were provided and the extracts of by-products were prepared. Folin-Ciocalteu method was used to determine the total phenolic content of extracts. The antimicrobial activity of extracts ± Resistant Starch against *Escherichia coli, Streptococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. were evaluated using Minimum Inhibitory Concentration (MIC) method. The total tannin and phenolic compounds of pomegranate pomace were more than the others. The results of MIC showed that 1600 and 3200 ppm of pistachio peel extract inhibited the *E. coli* growth. The growth inhibition of *Streptococcus* spp. by Resistant Starch was equal to 400 ppm dilution. *Streptococcus* did not grow in 50, 100, and 200 ppm of pistachio peel extract + Resistant Starch treatment. The dilution of 800, 1600, and 3200 ppm of grape pomace extract could prevent the growth of *Lactobacillus* spp., while *Bifidobacterium* increased in all treatments dilution except in 3200 ppm pistachio peel extract and 50 and 100 ppm pistachio peel extract + resistant starch.

Keywords: *Phenol, Resistant starch, Antimicrobial activity, Bacteria, MIC*

Fenolik Ekstrakt ve Dirençli Nişastanın *Escherichia coli, Streptococcus* spp., *Bifidobacterium* ve *Lactobacillus* spp. Üzerine İn-vitro Antimikrobiyal Etkisi

Öz

Bu çalışma Üzüm Posası Ekstraktının, Fıstık Kabuğu Ekstraktının ve Nar Posası Ekstraktının Dirençli Nişasta ile birlikte veya ayrı olarak *in vitro* şartlarda mide mikroflorası bileşenlerine bir prebiyotik olarak antimikrobiyal etkilerini araştırmak amacıyla yapılmıştır. Bu amaçla, dirençli nişasta (Fibersol 2), üzüm posası, fıstık kabuğu ve nar posası elde edilerek ekstraktları hazırlandı. Ekstraktlardaki fenolik miktarını belirlemek amacıyla Folin-Ciocalteu metodu kullanıldı. Minimum inhibe edici konsantrasyon metodu kullanılarak *Escherichia coli, Streptococcus* spp., *Lactobacillus* spp. ve *Bifidobacterium* spp. etkenlerine karşı ekstraktlar ± dirençli nişastanın antimikrobiyal etkisi araştırıldı. Nar posasının toplam tanin ve fenolik bileşikleri diğerlerinden daha fazlaydı. Minimum inhibe edici konsantrasyon sonuçları, 1600 ve 3200 ppm düzeyindeki fıstık kabuğu ekstraktının *E. coli* üremesini inhibe ettiğini gösterdi. Dirençli nişastayla birlikte Streptococcus'u büyüme inhibisyonu 400 ppm dilusyonda gerçekleşti. *Streptococcus* dirençli nişastayla birlikte 50, 100 ve 200 ppm fıstık kabuğu ekstraktı uygulamasında üremedi. Üzüm posası ekstraktının 800, 1600 ve 3200 ppm dozları *Lactobacillus* spp. üremesini önlerken *Bifidobacterium* spp. üremesi 3200 ppm fıstık kabuğu ekstraktı ile 50 ve 100 ppm fıstık kabuğu ekstraktı ile birlikte dirençli nişasta uygulamaları haricinde tüm uygulamalarda arttı.

Anahtar sözcükler: *Fenol, Dirençli nişasta, Antimikrobiyal aktivite, Bakteri, Minimum İnhibe edici konsantrasyon*

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INTRODUCTION

Polyphenols are known as natural compounds that could be found in foods like fruits, vegetables, cereals, etc.[1]. Phenolic compounds are not involved in metabolic pathways of plants, and they are a kind of secondary plant substances [2]. These compounds can act as anti-inflammatory, antimicrobial, and antioxidant factors [3]. In this regard, using natural antibacterial compounds such as residual plant rich extracts in phenolic compounds, as food preservatives, has increased due to concerns about food safety [4]. The industrial by-products were used in livestock feed too. Since animal's intestinal microorganisms can have an effect on the energy harvested of diet, adjustment of gut microbiota opens up an opportunity for promoting digestive health [5]. Recently, researchers have paid a lot of attention to the industrial pomaces especially those containing phenolic compounds [6]. Colonic microbiota has an effect on the absorption of dietary polyphenols in small intestine [7]. Some bacterial species (e.g. *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp. etc.) are catalyzing the metabolism of phenolic [8] and some of phenolic extracts like Grape Pomace Extract (GPE) [9], Pistachio Peel Extract (PsPE) [10] and Pomegranate Pomace Extract (PPE) [11] have bioactive properties including antimicrobial activity. Not only can the colonic microbiota have an effect on using phenolic compounds, but also it can change by prebiotic substrates. One of the reasons for the effectiveness of prebiotic is that prebiotics are fermented using the intestinal flora, and the commensal microorganisms increase. Therefore, diseases decrease by moderating the intestinal microflora and controlling the pathogenic microorganisms [12].

The term of Resistant Starch (RS) was defined by Asp [13] as "the starch or starch degradation products that escapes digestion in the small intestine and may be completely or partially fermented in the large intestine". Hence, RS is one of the substrates that increases the concentrations of beneficial bacteria through diet. The prebiotic properties of RS can be due to its non-digestibility of carbohydrate fractions for cecal and colonic microbiota that influence the host gut health in animal studies [14]. RS is used by *Lactobacilli Bifidobacteria* and promotes the *Lactobacilli Bifidobacteria* colonization, and it can also reduce the intestinal pathogen levels [15].

However, there are several reports about antimicrobial activity of phenolic extracts of different food sources against common animal colonic bacteria. But there are no available reports about synchronic effect of phenolic compounds and prebiotics on these bacteria. Therefore, this investigation was carried out to evaluate the antibacterial effect of GPE, PsPE and PPE phenolic compound extracts on *E. coli*, *Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp. as common animal's gut microflora with or without RS.

MATERIAL and METHODS

Raw Material

Pomegranate pomace and grape pomace were purchased from Nariran Co., Saveh and SunSunShahd Co., Urmia, Iran, respectively. Pistachio peel was purchased from Nut and Pistachio Peel Commerce Co., Mashhad, Iran. The peels and pomaces were air-dried under ambient conditions. Then, they were milled (0.5 mm) and stored in 4°C for the following tests and extractions. The RS (Fibersol2) was purchased from Karen Nutrilife Co., Yazd, Iran.

Preparing Extracts and Determining Total Phenolic Content

To prepare the extracts, 50 g of air-dried and powdered pomegranate pomace, grape pomace and pistachio peel were extracted separately with 300 mL of methanol (99.5%), and kept 30-32 h at room temperature by shaking every 30 min. Then, the extracts were filtered through Whatman 42 mm and kept at water bath under sterile air condition. Afterwards, the extracts were collected and weighed after combination and evaporation of all methanolic fractions. Finally, the extracts were kept at -20°C for the next experiments. It should be noted that Folin-Ciocalteu method was used to determine the total phenolic content [16].

Determining Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the extracts in combination or without RS was determined through National Committee for Clinical Laboratory standards suggestion (NCCLS, 2000) by using Micro Broth Dilution method (96-well plates) in duplicates. Briefly, in order to prepare the stock solution, 0.02 g of each extract and 0.02 g of RS was added separately to 2 mL sterile Brain Heart Infusion (BHI) Broth medium, and it was vortexed well to reach a final concentration of 10^4 ppm. Two-fold dilutions were prepared to obtain concentrations of 50, 100, 200, 400, 800, 1600, and 3200 ppm to each extract in 2 mL BHI broth + DMSO (dimethyl sulfoxide). The standard bacterial strains including *E. coli* ATCC 35218, *Streptococcus* spp. (*S. sobrinus* ATCC 33478), *Bifidobacterium* spp. ATCC 29521, and *Lactobacillus* spp. *(L. acidophilus* ATCC 43121) were cultivated on LB broth for the activation of bacteria (Luria Bertani Broth, Sigma-Aldrich). Then, the bacterial suspensions were prepared in turbidity equal to 0.5 McFarland standard tubes (5×10^5 cfu/mL). Then, 200 µl of each dilution (GPE, PsPE, PPE, RS, GPE + RS, PsPE + RS, PPE + RS) with 6 µL of bacterial suspensions of each bacterium was added to each well. Finally, the plates were incubated at 37°C for 24 h in aerobic atmosphere (except for *Bifidobacterium* strain that was incubated in anaerobic condition). After incubation period, ELISA Microplate Reader was used to measure the absorbance of each well at 630 nm (BIOTEK ELX 800). MIC came to be the lowest concentration of extracts (with or without RS) which prevented visible growth of bacteria [17].

Statistical Analysis

The data was recorded at 0 h (at the time of innoculation) and 24 h (after incubation), and analyzed through t-tests (P≤.05) using SAS (9.1) to determine the difference between the growth of bacteria in two-hour intervals.

RESULTS

Since phenolic compounds of grape pomace, pomegranate pomace, and pistachio peel play an important role in their antibacterial activity, the tannin and total phenolic compounds of their extracts were measured. As shown in *Table 1*, total tannin and phenolic compounds of pomegranate pomace were more than the others, while phenolic compounds of grape pomace and tannin content of pistachio peel were the lowest.

The results MIC on GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for *E. coli* are shown in *Table 2*. According to this table, *E.coli* could grow in culture media containing all dilution of GPE, PPE, RS, GPE + RS, PPE + RS, PsPE + RS. While the dilution of 1600 and 3200 ppm of PsPE could restrain its growth. In *Table 3*, the MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS are shown for *Streptococcus* spp., according to which, 200, 1600 and 3200 ppm dilution of GPE could act as an inhibiting factor for growing *Streptococcus* spp., while *Streptococcus* spp. could not grow in 50 and 800 ppm dilution of PsPE; RS prevented the *Streptococcus* spp. growth in 400 ppm dilution. Therefore, the MIC of RS for *Streptococcus* spp. was 400 ppm dilution (80 µL of medium + extract or RS in 2 mL BHI Broth + DMSO). 1600 ppm of GPE + RS also could prevent *Streptococcus* spp. growth. On the other hand, *Streptococcus* spp. bacteria did not grow in 50,100 and 200 ppm of PsPE + RS, while it could grow in 400, 800, 1600, and 3200 ppm.

The results of MIC treatments on *Lactobacillus* spp. are shown in *Table 4*, according to which, 200, 800, 1600 and 3200 ppm of GPE could prevent *Lactobacillus* spp. growth. This bacterium did not grow in 800 and 600 ppm of PsPE treatment. On the other hand, 100, 200, 400, and 3200 ppm of RS could prevent *Lactobacillus* growth. 1600 ppm and 3200 ppm of GPE + RS and PsPE + RS treatment also prevented the growth of *Lactobacillus*, respectively. In *Table 5*, the MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for *Bifidobacterium* spp. are shown. Accordingly, *Bifidobacterium* increased in all treatment dilutions except 3200 ppm PsPE and 50 and 100 ppm PsPE + RS *(Table 6)*.

Table 1. *The phenolic compounds of grape pomace, pomegranate pomace, and pistachio peel (% of DM)*

Extracts	Tannin	Total Phenol
Grape pomace	2.167	2.700
Pomegranate pomace	3.643	14.939
Pistachio peel	1.906	11.739

DISCUSSION

The by-products of food industry and some peels are tannin-rich sources which are used in animal feed in many developed countries. The antimicrobial activity of peels has been confirmed against pathogenic bacteria [18]. It appears that polyphenols show prebiotic like effects on modulation of the gut microbiota [19]. Several *in vitro* studies have shown that some polyphenols can change the composition of gut microbiota, while some bacteria may be inhibited; others can be developed [20]. Shoko et al.[21] noticed the antimicrobial activity of methanol extract from grape seeds. Similarly, Tzounis et al.[22] reported that phenolic compounds significantly increased the growth of *E. coli*, while the growth of *Bifidobacterium* and *Lactobacillus* were unaffected. Yamakoshi et al.[23] also discussed that a proanthocyanidin-rich (a type of phenolic compound) extract from grape seeds significantly increased the number of *Bifidobacteria* of gastrointestinal tract. *Bifidobacteria* is one of the potentially beneficial bacteria due to its beneficial effects on the immune system and metabolism [24] which is a non-pathogenic bacterium [25]. Secondary metabolites of plants and by-products such as phenolic compound, carotenoids, flavonoids etc. with biological activity may have some resistance mechanisms including enzymatic inactivation, target site modifications, and decrease in intracellular drug accumulation [26]. In this regard, Tabasco et al.[27] argued that using different phenolic extracts and sensitivity of *Bifidobacteria* is different, and *B. lactis* showed the highest sensitivity towards the phenolic extract. Mir Ahmadi and Davari [28] reported that the antimicrobial effects of tea leaf extract against *E.coli* were 750 ppm. In their study about the effects of phenolic compounds on probiotic and pathogenic bacteria, Pacheco-Ordaz et al.[29] concluded that phenolic compounds, without affecting the viability of probiotics *(L. rhamnosus, L. acidophilus)*, can selectively restrain the growth of pathogenic bacteria *(E. coli, S. typhimurium)*. Similarly, Vega-Vega et al.[30] evaluated the effects of rich extracts of the phenolic compounds on the growth of some pathogenic bacteria and reported the antimicrobial benefits of the mixture of phenolic extracts.

The factors which may have a crucial impact on the bacterial growth include the structure of polyphenols, the microorganism strain, and the estimated dosage [31]. The differences of bacterial resistant to polyphenolic compounds are possibly due to bacteria wall composition differences. For example, Puupponen-Pimia et al.[32] reported that the Gram-positive bacteria are more sensitive to polyphenols than Gram-negative bacteria. Hence, in the present study, *E. coli* as Gram-negative bacteria can easily grow in the culture medium containing the phenolic extract. Kemperman et al.[33] stated that the mode of action of polyphenols on bacteria may be due to binding

Table 2. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for E. coli*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.076	0.643	0.1745	Equal	0.0007	*
GPE 100	0.059	0.572	0.2103	Equal	<.0001	*
GPE 200	0.097	0.593	0.0935	Equal	0.0047	*
GPE 400	0.152	0.599	<.0001	Unequal	0.0263	*
GPE 800	0.320	0.659	0.3642	Equal	0.0007	*
GPE 1600	0.374	0.784	0.4027	Equal	0.0050	*
GPE 3200	0.698	0.940	0.2717	Equal	0.0156	*
PPE 50	0.101	0.669	0.3119	Equal	<.0001	*
PPE 100	0.120	0.712	0.2615	Equal	0.0004	*
PPE 200	0.139	0.776	<.0001	Unequal	0.0010	*
PPE 400	0.134	0.811	0.3119	Equal	0.0005	*
PPE 800	0.315	0.924	0.3390	Equal	0.0004	*
PPE 1600	0.361	1.066	0.3140	Equal	0.0108	*
PPE 3200	0.877	1.516	0.8731	Equal	0.0013	*
PsPE 50	0.108	0.649	0.1491	Equal	0.0002	*
PsPE 100	0.152	0.585	0.1463	Equal	0.0009	*
PsPE 200	0.229	0.592	0.6500	Equal	0.0015	*
PsPE 400	0.377	0.626	0.9234	Equal	0.0137	*
PsPE 800	0.659	0.776	0.8997	Equal	0.0492	*
PsPE 1600	1.115	1.028	0.3469	Equal	0.1314	NS
PsPE 3200	1.327	1.226	0.8810	Equal	0.1564	NS
RS 50	0.070	0.923	0.0953	Equal	0.0006	*
RS 100	0.071	0.988	<.0001	Unequal	0.0578	*
RS 200	0.066	0.862	0.0493	Equal	0.0093	*
RS 400	0.071	0.876	0.0352	Equal	0.0124	*
RS 800	0.073	0.857	0.0369	Equal	0.0077	*
RS 1600	0.068	0.821	0.0235	Equal	0.0201	*
RS 3200	0.071	0.812	0.0368	Equal	0.0134	*
GPE+RS 50	0.073	0.5345	0.2290	Equal	0.0001	*
GPE+RS 100	0.0745	0.6115	0.0977	Equal	0.0001	*
GPE+RS 200	0.0795	0.5005	0.1474	Equal	0.0026	*
GPE+RS 400	0.0985	0.4705	0.1807	Equal	0.0023	*
GPE+RS 800	0.124	0.476	0.2784	Equal	0.0027	*
GPE+RS 1600	0.1724	0.6	0.681	Equal	0.0043	*
GPE+RS 3200	0.2755	0.7355	1.000	Equal	<.0001	*
PPE+RS 50	0.073	0.786	0.0707	Equal	0.0006	*
PPE+RS 100	0.0855	0.615	0.2103	Equal	<.0001	*
PPE+RS 200	0.1045	0.6505	0.3276	Equal	0.0003	*
PPE+RS 400	0.1235	0.745	0.0163	Equal	0.0039	*
PPE+RS 800	0.157	0.852	0.8193	Equal	<.0001	*
PPE+RS 1600	0.2315	0.9315	0.7312	Equal	0.0002	*
PPE+RS 3200	0.3375	1.0557	0.4778	Equal	0.0006	*
PsPE+RS 50	0.093	0.494	0.3119	Equal	<.0001	*
PsPE+RS 100	0.1125	0.8325	0.0137	Equal	0.0041	*
PsPE+RS 200	0.1615	0.7215	0.0606	Equal	0.0004	*
PsPE+RS 400	0.245	0.924	<.0001	Unequal	0.0009	*
PsPE+RS 800	0.4255	1.3055	0.2627	Equal	0.0006	*
PsPE+RS 1600	0.6505	1.632	0.3417	Equal	0.0004	*
PsPE+RS 3200	1.2535	2.007	0.3060	Equal	0.0105	*

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 3. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for Streptococcus spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.0845	0.3105	0.1409	Equal	0.0004	*
GPE 100	0.0965	0.3425	0.2331	Equal	0.0031	*
GPE 200	0.1335	0.137	0.3119	Equal	0.6285	NS
GPE 400	0.1555	0.2615	0.4973	Equal	0.0074	*
GPE 800	0.2725	0.6405	0.9556	Equal	0.0263	*
GPE 1600	0.49	0.512	0.8959	Equal	0.6617	NS
GPE 3200	0.7485	0.7575	0.2313	Equal	0.7525	NS
PPE 50	0.1065	0.2685	0.6289	Equal	0.0021	*
PPE 100	0.1195	0.2765	0.6881	Equal	0.0003	*
PPE 200	0.1585	0.3925	0.0386	Unequal	0.0446	*
PPE 400	0.2275	0.454	0.2845	Equal	0.0025	*
PPE 800	0.304	0.619	0.7487	Equal	0.0012	*
PPE 1600	0.557	0.8675	0.1371	Equal	0.0036	*
PPE 3200	0.796	1.161	1.000	Equal	0.0005	*
PsPE 50	0.146	0.144	1.000	Equal	0.2929	NS
PsPE 100	0.237	0.251	<.0001	Unequal	0.0454	*
PsPE 200	0.331	0.3725	0.9152	Equal	0.0160	*
PsPE 400	0.627	0.7855	0.7487	Equal	0.0032	*
PsPE 800	1.005	1.128	0.7897	Equal	0.0612	NS
PsPE 1600	1.526	1.29	0.1521	Equal	0.0112	*
PsPE 3200	1.91	1.67	0.9810	Equal	0.0090	*
RS 50	0.08	0.3585	<.0001	Unequal	0.0034	*
RS 100	0.082	0.371	<.0001	Unequal	0.0044	*
RS 200	0.0785	0.2955	0.0948	Equal	0.0231	*
RS 400	0.0825	0.0905	0.1409	Equal	0.2193	NS
RS 800	0.0815	0.0835	1.0000	Equal	0.1056	NS
RS 1600	0.0865	0.09	0.7112	Equal	0.5354	NS
RS 3200	0.0865	0.0895	1.000	Equal	0.0513	NS
GPE+RS 50	0.705	0.295	0.1896	Equal	0.0020	*
GPE+RS 100	0.291	0.3035	0.0509	Equal	0.0030	*
GPE+RS 200	0.0855	0.3135	0.0411	Unequal	0.0430	*
GPE+RS 400	0.1095	0.3165	0.1889	Equal	0.0423	*
GPE+RS 800	0.162	0.32	0.6358	Equal	0.0062	*
GPE+RS 1600	0.263	0.288	0.9470	Equal	0.5384	NS
GPE+RS 3200	0.364	0.407	0.5903	Equal	0.0106	*
PPE+RS 50	0.089	0.276	<.0001	Unequal	0.0086	*
PPE+RS 100	0.102	0.2135	0.1016	Equal	0.0124	*
PPE+RS 200	0.1135	0.28	0.1583	Equal	0.0143	*
PPE+RS 400	0.1335	0.3035	1.000	Equal	<.0001	*
PPE+RS 800	0.1755	0.3515	0.3476	Equal	0.0054	*
PPE+RS 1600	0.3565	0.623	0.7776	Equal	0.0005	*
PPE+RS 3200	0.4715	0.8195	0.6573	Equal	0.0037	*
PsPE+RS 50	0.098	0.101	0.8193	Equal	0.6094	NS
PsPE+RS 100	0.112	0.1135	0.5903	Equal	0.3118	NS
PsPE+RS 200	0.195	0.21	0.9152	Equal	0.2937	NS
PsPE+RS 400	0.316	0.37	1.000	Equal	0.0061	*
PsPE+RS 800	0.486	0.561	1.000	Equal	0.0404	*
PsPE+RS 1600	0.966	1.0955	0.4243	Equal	0.0111	*
PsPE+RS 3200	1.450	1.287	0.2268	Equal	0.1651	*

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 4. *The MIC results of GPE, PPE, PsPE, RS, GPE+RS, PPE+RS, PsPE+RS for Lactobacillus spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.0795	0.181	0.1059	Equal	0.0035	*
GPE 100	0.1005	0.1855	0.0977	Equal	0.0491	*
GPE 200	0.118	0.3165	0.0777	Equal	0.2257	NS
GPE 400	0.171	0.249	0.5325	Equal	0.0156	*
GPE 800	0.248	0.3135	0.3261	Equal	0.0945	NS
GPE 1600	0.4615	0.6765	0.0700	Equal	0.2992	NS
GPE 3200	0.839	0.9455	0.6711	Equal	0.2704	NS
PPE 50	0.104	0.2125	0.0727	Equal	0.0251	*
PPE 100	0.13	0.183	<.0001	Unequal	0.1644	NS
PPE 200	0.1535	0.252	0.6402	Equal	0.0132	*
PPE 400	0.217	0.331	0.2397	Equal	0.0334	*
PPE 800	0.322	0.466	0.4475	Equal	0.0120	*
PPE 1600	0.497	0.746	0.2290	Equal	0.0020	*
PPE 3200	0.789	1.166	0.9636	Equal	0.0095	*
PsPE 50	0.112	0.114	0.9674	Equal	0.8729	NS
PsPE 100	0.157	0.163	0.8193	Equal	0.3530	NS
PsPE 200	0.2715	0.3155	0.1154	Equal	0.0154	*
PsPE 400	0.4715	0.5575	0.9092	Equal	0.0131	*
PsPE 800	0.7485	0.8735	0.4097	Equal	0.1123	NS
PsPE 1600	1.284	1.252	0.5074	Equal	0.0876	NS
PsPE 3200	1.795	1.636	0.3119	Equal	0.0007	*
RS 50	0.081	0.307	0.5903	Equal	<.0001	*
RS 100	0.08	0.288	<.0001	Unequal	0.0972	NS
RS 200	0.077	0.2735	0.0359	Unequal	0.1136	NS
RS 400	0.08	0.319	<.0001	Unequal	0.0690	NS
RS 800	0.081	0.301	0.1104	Equal	0.0108	*
RS 1600	0.0815	0.2815	1.000	Equal	<.0001	*
RS 3200	0.083	0.3	<.0001	Unequal	0.0701	NS
GPE+RS 50	0.079	0.152	1.000	Equal	0.0015	*
GPE+RS 100	0.0845	0.144	0.8591	Equal	0.0029	*
GPE+RS 200	0.0865	0.16	0.8846	Equal	0.0028	*
GPE+RS 400	0.1085	0.1415	0.6457	Equal	0.0235	*
GPE+RS 800	0.1975	0.146	<.0001	Unequal	0.0309	*
GPE+RS 1600	0.2765	0.315	0.8877	Equal	0.5374	NS
GPE+RS 3200	0.396	0.4665	0.6123	Equal	0.0322	*
PPE+RS 50	0.0875	0.183	0.5903	Equal	0.0001	*
PPE+RS 100	0.1045	0.1455	0.3390	Equal	0.0188	*
PPE+RS 200	0.12	0.1725	0.5325	Equal	0.0087	*
PPE+RS 400	0.1535	0.219	0.2615	Equal	0.0333	*
PPE+RS 800	0.2025	0.2955	0.0848	Equal	0.0065	*
PPE+RS 1600	0.381	0.49	1.000	Equal	0.0027	*
PPE+RS 3200	0.4975	0.693	0.7776	Equal	0.0087	*
PsPE+RS 50	0.0895	0.0905	1.000	Equal	0.8586	NS
PsPE+RS 100	0.1375	0.1425	1.000	Equal	0.0194	*
PsPE+RS 200	0.2275	0.233	0.4845	Equal	0.1778	NS
PsPE+RS 400	0.2845	0.3355	0.2726	Equal	0.0493	*
PsPE+RS 800	0.487	0.608	<.0001	Unequal	0.0368	*
PsPE+RS 1600	0.8705	0.977	0.4441	Equal	0.0030	*
PsPE+RS 3200	1.385	1.195	0.0238	Unequal	0.1742	NS

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0h and 24 h (P>0.05)*

Table 5. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for Bifidobacterium spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.085	0.5585	<.0001	Unequal	0.0181	*
GPE 100	0.097	0.527	0.0993	Equal	0.0055	*
GPE 200	0.151	0.552	0.3895	Equal	0.0097	*
GPE 400	0.194	0.591	0.6881	Equal	0.0002	*
GPE 800	0.323	0.752	0.0771	Equal	0.0059	*
GPE 1600	0.551	0.938	0.7172	Equal	0.0290	*
GPE 3200	0.857	1.217	0.2513	Equal	0.0005	*
PPE 50	0.1135	0.567	0.0848	Equal	0.0003	*
PPE 100	0.146	0.6645	0.0652	Equal	0.0014	*
PPE 200	0.179	0.7725	0.1037	Equal	0.0017	*
PPE 400	0.2365	0.8895	0.2513	Equal	0.0001	*
PPE 800	0.3715	1.0685	0.2661	Equal	0.0006	*
PPE 1600	0.6425	1.267	0.7014	Equal	0.0040	*
PPE 3200	0.955	1.483	0.5432	Equal	0.0001	*
PsPE 50	0.1375	0.1535	1.000	Equal	0.0171	*
PsPE 100	0.2065	0.2525	1.000	Equal	0.0002	*
PsPE 200	0.3475	0.5465	0.8417	Equal	0.0008	*
PsPE 400	0.600	0.888	0.3922	Equal	0.0016	*
PsPE 800	0.8485	0.515	0.1583	Equal	0.0013	*
PsPE 1600	1.429	1.671	0.4568	Equal	0.0003	*
PsPE 3200	1.983	2.047	0.1556	Equal	0.3811	NS
RS 50	0.0725	0.6275	0.1807	Equal	<.0001	*
RS 100	0.0775	0.6305	0.0143	Equal	0.0064	*
RS 200	0.0795	0.6115	0.0153	Equal	0.0060	*
RS 400	0.0795	0.6095	0.0161	Equal	0.0055	*
RS 800	0.079	0.6225	0.0877	Equal	0.0064	*
RS 1600	0.079	0.6305	0.0331	Equal	0.0048	*
RS 3200	0.0805	0.633	0.0277	Equal	0.0017	*
GPE+RS 50	0.074	0.537	0.2513	Equal	0.0001	*
GPE+RS 100	0.079	0.5175	0.1104	Equal	0.0062	*
GPE+RS 200	0.0865	0.506	0.1095	Equal	0.0048	*
GPE+RS 400	0.11	0.499	0.1409	Equal	0.0022	*
GPE+RS 800	0.1395	0.528	0.1059	Equal	0.0022	*
GPE+RS 1600	0.22	0.7265	0.2374	Equal	0.0028	*
GPE+RS 3200	0.336	0.8185	0.3625	Equal	0.0020	*
PPE+RS 50	0.0875	0.558	0.2103	Equal	0.0004	*
PPE+RS 100	0.11	0.525	<.0001	Unequal	0.0383	*
PPE+RS 200	0.128	0.6335	0.0499	Unequal	0.0318	*
PPE+RS 400	0.164	0.7355	<.0001	Unequal	0.0228	*
PPE+RS 800	0.2365	0.9295	0.2673	Equal	0.0020	*
PPE+RS 1600	0.3585	0.4855	0.5115	Equal	0.0009	*
PPE+RS 3200	0.5	1.174	0.3613	Equal	0.0014	*
PsPE+RS 50	0.088	0.085	0.8193	Equal	0.6094	NS
PsPE+RS 100	0.1205	0.123	0.8193	Equal	0.4226	NS
PsPE+RS 200	0.166	0.21	0.2513	Equal	0.0132	*
PsPE+RS 400	0.2705	0.396	<.0001	Unequal	0.0004	*
PsPE+RS 800	0.4915	0.8705	0.3390	Equal	0.0056	*
PsPE+RS 1600	0.7935	1.28	0.7487	Equal	0.0007	*
PsPE+RS 3200	1.345	1.752	0.4501	Equal	0.0119	*

**:Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 6. *Observed bacterial growth in different dilutions of extracts±RS (brief)*

Bacterial Strains		Dilutions						
		50	100	200	400	800	1600	3200
E. coli	GPE	+	+	+	+	+	+	+
	PPE	+	+	+	+	+	+	+
	PsPE	+	+	+	+	+	-	-
	RS	+	+	+	+	+	+	+
	GPE+RS	+	+	+	+	+	+	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	+	+	+	+	+	+	+
Streptococcus spp.	GPE	+	+	-	+	+	-	-
	PPE	+	+	+	+	+	+	+
	PsPE	-	+	+	+	-	+	+
	RS	+	+	+	-	-	-	-
	GPE+RS	+	+	+	+	+	-	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	-	-	+	+	+	-
Lactobacillus spp.	GPE	+	+	-	+	-	-	-
	PPE	+	-	+	+	+	+	+
	PsPE	-	-	+	+	-	-	+
	RS	+	-	-	-	+	+	-
	GPE+RS	+	+	+	+	+	-	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	+	-	+	+	+	-
Bifidobacterium spp.	GPE	+	+	+	+	+	+	+
	PPE	+	+	+	+	+	+	+
	PsPE	+	+	+	+	+	+	-
	RS	+	+	+	+	+	+	+
	GPE+RS	+	+	+	+	+	+	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	-	+	+	+	+	+

+ The bacteria grew; - The bacteria did not grow

polyphenols to cell membranes of bacteria. Therefore, it can disturb the function of membrane, and prevent cell growth. Hattori et al.[34] also reported that polyphenols can produce hydrogen peroxide and change the permeability of microbial membrane. On the other hand, some of phenolic compounds can interact with lipids and proteins and change the permeability of the membrane [33].

A study done by Roozegar et al.[35] showed that the leaf extract of *P. atlantica* was phenol compound-rich which was implied to associate with antibacterial properties. This extract had an antimicrobial effect on *Streptococcus* spp. In his evaluation of the antimicrobial activity of pomegranate pomaces, Al-Zoreky [36] found that 80% of methanolic extract of peels were a strong inhibitor for *E. coli*. In another study, Hosseini et al.[37] stated that *P. atlantica* extracts has an antibacterial activity against *S. mutans*. The pomegranate fruit skin extracts were shown by Sadeghian et al.[38] as a strong antimicrobial activity against the microorganisms (e.g. *S. aureus*; *P. aeruginosa*; *C. albicans)*. Rodriguez et al.[39] noticed that *L. plantarum* has several enzymatic activities such as tannase, phenolic acid decarboxylase, and benzyl alcohol dehydrogenase that make it able to have an effect on degradation of some phenolic compounds. Importantly, RS is a type of prebiotic that functions by binding the bacteria to the granule surface [40]. This could improve the viability of beneficial bacteria such as *Bifidobacteria* at the end of the digestive area [41]. Wronkowska et al.[42] stated that RS has beneficial effects on the growth of *Bifidobacteria* in the intestine. Our findings are similar to the findings of Li [43] who mentioned RS as a prebiotic based on its ability to enrich *Bifidobacterium* and *Lactobacillus* spp. Therefore, RS is completely fermented by gut microflora and selectively

used by *Lactobacilli Bifidobacteria* followed by decrease in intestinal pathogen levels [15]. Roberfroid et al.[44] showed that RS can stimulate the growth of *Bifidobacterium* and *Lactobacillus* ssp. as beneficial bacteria. However, our study indicates that RS had an effect on *Bifidobacterium,* but it could not stimulate the growth of *Lactobacillus* ssp.

We concluded that, on the one hand, PsPE could inhibit *E. coli* growth, while GPE + RS, PPE + RS and PsPE + RS were inactive against *E.coli*. On the other hand, GPE and RS were active against *Streptococcus* spp. growth. The MIC of GPE and RS was 1600 and 400 ppm, respectively. GPE inhibited *Lactobacillus* spp. growth. *Bifidobacterium* (as beneficial bacteria) could increase in all mixtures of extracts and RS. Since the industrial by-products of this study are used in animal feed, their individual phenolic compounds can be identified and quantified. It will be beneficial for optimizing extraction to be used with RS as a prebiotic in livestock industry.

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