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Minimum Inhibitory Concentrations of Phenolic Extracts and Resistant Starch for *Clostridium perfringens*: *In vitro* Study

Samira Karamati Jabehdar[1], Farzad Mirzaei Aghjehgheshlagh[1*], Bahman Navidshad[1], Ali Mahdavi [2], Hamid Staji[3], Nemat Hedayat Evrigh[1]

[1] *Department of Animal Science, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, IRAN*
[2] *Department of Animal Science, Faculty of Veterinary Medicine, Semnan University, Semnan, IRAN*
[3] *Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, IRAN*

Abstract

BACKGROUND: *Clostridium perfringens*, as a bacterial agent causing foodborne illnesses, is of great importance in the food industry. On the other hand, the increasing concern of antibiotic resistance is forcing humans to find an alternative to antibiotics.

OBJECTIVES: This study aimed to evaluate the antimicrobial activity of the extracts of grape pomace, pistachio peel, and pomegranate pomace against *Clostridium perfringens* (*C. perfringens*) in the presence or absence of resistant starch (RS) as a prebiotic.

METHODS: The RS (Fibersol-2) was purchased, and the extracts of grape pomace, pistachio peel, and pomegranate pomace were prepared. The total phenolic content and tannin of extracts were determined by Folin-Ciocalteu and standard tannic acid method, respectively. The antimicrobial activity of the extract with or without RS was evaluated using the minimum inhibitory concentration (MIC) against *C. perfringens*.

RESULTS: Our findings showed that 100 ppm of pistachio peel extract could act as an inhibition factor against the growth of *C. perfringens*. The RS alone was not able to prevent *C. perfringens* growth. In contrast, 400 ppm dilution of RS+grape pomace extract could restrain *C. perfringens* growth. In contrast, the pomegranate pomace extract with and without RS could not inhibit its growth. On the other hand, the RS±pistachio peel extract could not prevent *C. perfringens* growth, in comparison with other treatments.

CONCLUSIONS: We concluded that grape pomace extract, both with and without RS, effectively prevented *C. perfringens* growth.

KEYWORDS: Antimicrobial activity, *Clostridium Perfringens*, Phenolic compounds, Prebiotic, Resistant starch

Correspondence
Farzad Mirzaei Aghjehgheshlagh, Department of Animal Science, Faculty of Agriculture and Natural Resources, Mohaghegh Ardabili University, Ardabil, IRAN
Tel: +98 (453) 1505039, Fax: +98 (453) 1505035, Email: f_mirzaei@uma.ac.ir
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Introduction

Clostridium perfringens (*C. perfringens*) is a gram-positive, anaerobic, and rod-shaped bacterium that survives longer than vegetative cells, such as coliforms (e.g., *Escherichia coli* and *Enterococci*) (Gerba, 2015). This bacterium is distributed in nature and could be found in the intestine of animals and humans (Taghi Akhi *et al*., 2015). The ingested *C. perfringens* can produce enterotoxin in the intestine, which is capable of binding to the epithelial cells of the intestine leading to damaged cell membranes of the host. As a result, glucose absorption is prevented, while secretion of sodium and chloride increases due to altered permeability. In animal nutrition, reduced nutrient uptake causes a decline in feed efficiency, and removing *C. perfringens* from the intestines could improve growth performance (Zaffarano, 2003).

Feeding antibiotics for improving livestock performance has been associated with antibiotic resistance concerns. Resistant bacteria will be transported from animals to humans through food consumption (Zaffarano, 2003). Foodborne illness is the central problem of pathogenic resistant bacteria (Chan *et al*., 2018). Antibiotic-resistant *C. perfringens* strains are becoming a significant health concern due to their role in bacterial foodborne illnesses. However, increasing concern about antibiotic resistance is forcing farmers to find alternatives (Modi *et al*., 2014). These alternatives include probiotics and prebiotics, which can prevent the disease and improve growth characteristics. To alter the intestinal microbiota, the consumption of prebiotic carbohydrates like resistant starch (RS) is recommended (Herrmann *et al*., 2017). This type of carbohydrate is not digested in the upper gastrointestinal tract. According to Liu *et al*. (2020), Dietary fiber isolated from sweet potato residues, as a type of RS, significantly decreases the concentrations of *C. perfringens*.

On the other hand, the food industry has an ever-growing interest in using natural antimicrobials due to the health risk of chemical additives, which can improve food stability and safety against pathogens (Santas *et al*., 2010). Some chemicals, including phenolic compounds, which are a significant group of biologically active chemicals found in some foods, plants, and residual plants are generally recognized as safe (GRAS) (Lambert *et al*., 2001) and are often used as natural preservatives in food. These compounds are utilized as natural antimicrobials and have a great potential for controlling the growth of pathogens (Cetin-Karaca and Newman, 2015). In addition to their antimicrobial activity, they are of particular interest as natural alternatives to synthetic preservatives in food (Bouarab-Chibane *et al*., 2019).

Moreover, phenolic compounds and flavonoids are synthesized by many plants and fruit species that are utilized in traditional medicine or diets (Tungmunnithum *et al*., 2018). Kim *et al*. (2011) reported the antimicrobial activity of some plant-derived phenolic compounds. In a study by Jianu *et al*. (2012), the thymol derived from dill seeds had a strong antimicrobial impact on *C. perfringens*. However, there are no available reports about the synchronic effect of phenolic compounds and prebiotics on *C. perfringens* as a pathogenic bacterium. Therefore, this investigation was carried out to evaluate the antibacterial effect of the phenolic compound of the extracts of grape pomace, pistachio peel, and pomegranate pomace on *C. perfringens* in the presence or absence of RS.

Materials and Methods

Providing Material and Preparing Extracts

Pistachio peel (from Nut and Pistachio Peel Commerce Co., Mashhad), Pomegranate pomace (from Naariran Co., Saveh), and grape pomace (from SunSunShahd Co., Urmia) were purchased. The RS (Fibersol2) was purchased

from Karen Nutrilife Co., Yazd, Iran. For preparing the extracts, 50 g of air-dried and powdered (0.5 mm) pomegranate pomace, grape pomace, and pistachio peel were extracted separately with 300 mL methanol 99.5% and were kept and shaken every 30 min at room temperature for 30-32 h. Afterwards, the extracts were filtered through Whatman 42 mm and located in a water bath under sterile air condition. Finally, the extracts were collected and weighted after combining and evaporating all methanolic fractions.

Determining the Total Phenolic Compounds and Total Tannin Content

The Folin-Ciocalteu and standard tannic acid method were used to determine the total phenol and tannin content (Makkar, 2000). Briefly, tannins containing extracts were transferred into the test tube at three different quantities of 0.02, 0.05, and 0.1 ml. Next, 1.25 ml sodium carbonate solution and 0.25 ml Folin-Ciocalteu reagent were added and vortexed well. Absorbance was recorded at 725 nm after keeping at room temperature for 40 min. The total phenols were measured based on a standard calibration curve and expressed on a dry matter basis. Afterwards, the tannins were removed from the extract. For this aim, 100 mg Polyvinylpyrrolidone (PVPP) was poured into the test tube, 1 ml distilled water was added, vortexed well, and kept at 4ºC for 15 min. The test tube was centrifuged at 3000 rpm for 10 min and the supernatant was collected. The phenolic content of this supernatant was calculated according to the Folin-Ciocalteu method. This non-tannin compound was expressed based on dry matter. After calculating total phenolic and non-tannin compounds, the result of subtracting the non-tannin from the total phenolic was considered as total tannin.

Determining Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the extracts with or without RS was determined according to the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 2000) using the broth microdilution method (96-well plate) in duplicates. Briefly, 0.02 g of each extract and 0.02 g of RS were added separately to a 2 mL sterile brain heart infusion (BHI) broth medium and were vortexed well to reach a final concentration of 10^4 ppm as the stock solution. Two-fold dilutions were prepared to obtain the concentrations of 50, 100, 200, 400, 800, 1600, and 3200 ppm of each extract in 2 mL BHI broth + dimethyl sulfoxide. The standard strain of *C. perfringens* ATTC 13124 was cultivated on Luria-Bertani (LB) broth to activate the bacteria (Sigma-Aldrich, Germany). Afterwards, the bacterial suspension was prepared in turbidity equal to 0.5 McFarland standard tubes (5×10^5 CFU/mL). Then, 200 µl of each dilution of grape pomace extract, pistachio peel extract, pomegranate pomace extract, grape pomace extract+RS, pistachio peel extract+RS, pomegranate pomace extract+RS with 6 µL of bacterial suspension of *C. perfringens* was added to each well. Finally, the plate was incubated at 37ºC for 24 h in anaerobic conditions. After the incubation period, an enzyme-linked immunosorbent assay microplate reader was applied to measure the absorbance of each well at 630 nm (BIOTEK ELX 800, USA). The MIC was the lowest concentration of extracts with or without RS that prevented the visible growth of bacteria (Andrews, 2001).

Statistical Analysis

The data were recorded at 0 and 24 h (i.e., the times of inoculation and after incubation) and analyzed by the t-test ($P\leq0.05$) using the SAS software version 9.1 4 (Statistical Analysis Systems, Cary, NC, USA) for determining the difference between the growth rates of bacteria at two-hour intervals.

Results

The total phenolic compounds of grape pomace, pomegranate pomace, and pistachio peel extracts were 2.7%, 14.94%, and 11.74% of dry

matter, respectively. The total phenolic compounds of pomegranate pomace and grape pomace were the highest and lowest, respectively. The tannin contents of grape pomace, pomegranate pomace, and pistachio peel extracts were 2.167%, 3.634%, and 1.906% of dry matter, respectively. Therefore, the highest tannin content was observed in pomegranate pomace.

The MICs of the extracts of grape pomace, pomegranate pomace, and pistachio peel for *C. perfringens* are shown in Table 1. According to this table, *C. perfringens* could grow in a culture media containing diverse dilutions of grape pomace extract, except 800 ppm. As shown in Table 1, *C. perfringens* did not grow in 100 and 200 ppm of pomegranate pomace extract. In contrast, the MIC of pistachio peel extract showed that 100 ppm of pistachio peel extract could inhibit the growth of *C. perfringens*.

Table 1. The Minimum Inhibitory Concentration results of grape pomace extract, pomegranate pomace extract, and pistachio peel extract for *Clostridium perfringens*

	Dilution	Maen-0h	Mean-24h	f-value	v. equal test	T-value	significant
Grape Pomace Extract	50	0.087	0.087	<.0001	Unequal	1.0000	NS
	100	0.1215	0.159	0.1003	Equal	0.1880	NS
	200	0.122	0.156	0.1409	Equal	0.0642	NS
	400	0.174	0.2515	0.2966	Equal	0.1250	NS
	800	0.2785	0.338	0.7487	Equal	0.0222	*
	1600	0.443	0.526	0.8705	Equal	0.1400	NS
	3200	0.8	0.9185	0.8562	Equal	0.0754	NS
Pomegranate Pomace Extract	50	0.102	0.223	0.1209	Equal	0.0291	*
	100	0.1075	0.228	0.0883	Equal	0.0792	NS
	200	0.1295	0.311	0.3349	Equal	0.0792	NS
	400	0.1755	0.3705	0.6289	Equal	0.0014	*
	800	0.4165	0.6015	0.4097	Equal	<.0001	*
	1600	0.3725	0.569	0.3711	Equal	0.0007	*
	3200	1.0035	1.167	0.6962	Equal	0.0067	*
Pistachio Peel Extract	50	0.112	0.117	0.7487	Equal	0.2999	NS
	100	0.128	0.1435	0.5903	Equal	0.0052	*
	200	0.2055	0.2975	0.3390	Equal	0.0038	*
	400	0.2975	0.357	0.9252	Equal	0.0101	*
	800	0.503	0.8455	0.6500	Equal	0.0017	*
	1600	0.903	1.4465	0.5096	Equal	0.0007	*
	3200	1.384	1.927	0.4320	Equal	0.0098	*

*: Significant difference in bacterial growth between 0h and 24h ($P \leq 0.05$)

NS: Not Significant difference in bacterial growth between 0h and 24h ($P > 0.05$)

The MICs of RS are presented in Table 2 which indicates that RS could not inhibit the growth of *C. perfringens* in all dilutions. As shown in Table 3, RS + grape pomace extract prevented *C. perfringens* growth in 400, 800, 1600, and 3200 ppm dilutions. Therefore, the MIC of RS + grape pomace extract for *C. perfringens* was 400 ppm dilution. The MICs of RS + pomegranate pomace extract for *C. perfringens* revealed that the combination of RS and pomegranate pomace extract could not inhibit *C. perfringens* growth. However, the dilutions of 50 and 100 ppm of RS + pistachio peel extract could restrain its growth (Table 4).

Table 2. The Minimum Inhibitory Concentration results of Resistant Starch for *Clostridium perfringens*

Dilution	Maen-0h	Mean-24h	f-value	v. equal test	T-value	Significant
50	0.088	0.3675	0.9023	Equal	0.0003	*
100	0.083	0.388	0.1688	Equal	0.0025	*
200	0.087	0.369	<.0001	Unequal	0.0045	*
400	0.0865	0.3685	1.0000	Equal	<.0001	*
800	0.08	0.362	0.4097	Equal	0.0005	*
1600	0.0775	0.3795	0.2513	Equal	<.0001	*
3200	0.0775	0.3675	0.1228	Equal	0.0029	*

*: Significant difference in bacterial growth between 0h and 24h ($P \leq 0.05$)

NS: Not Significant difference in bacterial growth between 0h and 24h ($P > 0.05$)

Table 3. The Minimum Inhibitory Concentration results of grape pomace extract, pomegranate pomace extract, and pistachio peel extract + Resistant Starch for *Clostridium perfringens*

	Dilution	Maen-0h	Mean-24h	f-value	v. equal test	T-value	Significant
grape pomace extract + Resistant Starch	50	0.08	0.4165	0.1335	Equal	0.0072	*
	100	0.086	0.336	0.0606	Equal	0.0070	*
	200	0.0995	0.1795	0.1651	Equal	0.0204	*
	400	0.1275	0.173	0.1666	Equal	0.1409	NS
	800	0.17	0.2135	<.0001	Unequal	0.0656	NS
	1600	0.291	0.3355	0.1491	Equal	0.0351	NS
	3200	0.415	0.4605	0.4568	Equal	0.0087	NS
pomegranate pomace extract + Resistant Starch	50	0.0915	0.2495	0.1295	Equal	0.0234	*
	100	0.104	0.2445	0.0688	Equal	0.0169	*
	200	0.118	0.25	0.1521	Equal	0.0345	*
	400	0.151	0.329	0.5325	Equal	0.0030	*
	800	0.221	0.3885	0.6067	Equal	0.0107	*
	1600	0.3335	0.499	0.9252	Equal	0.0117	*
	3200	0.5295	0.735	0.3753	Equal	0.0197	*
pistachio peel extract	50	0.0965	0.095	0.8591	Equal	0.6855	NS
	100	0.1145	0.12	0.8591	Equal	0.2280	NS

Dilution	Maen-0h	Mean-24h	f-value	v. equal test	T-value	Significant
200	0.151	0.1745	0.5903	Equal	0.0023	*
400	0.2135	0.2655	0.6199	Equal	0.0325	*
800	0.3435	0.5445	0.4097	Equal	0.0030	*
1600	0.5895	0.9885	1.0000	Equal	0.0003	*
3200	0.942	1.456	0.3119	Equal	<.0001	*

*: Significant difference in bacterial growth between 0h and 24h ($P \leq 0.05$)

NS: Not Significant difference in bacterial growth between 0h and 24h ($P > 0.05$)

Table 4. Growth of *Clostridium perfringens* in different dilutions of phenolic extract ±Resistant Starch (brief)

	Dilution						
	50	100	200	400	800	1600	3200
grape pomace extract	-	-	-	-	+	-	-
pomegranate pomace extract	+	-	-	+	+	+	+
pistachio peel extract	+	+	+	+	+	+	+
RS	+	+	+	+	+	+	+
grape pomace extract + RS	+	+	+	-	-	-	-
pomegranate pomace extract + RS	+	+	+	+	+	+	+
pistachio peel extract + RS	-	-	+	+	+	+	+

+: bacterial growth

- : Lack of bacterial growth

Discussion

The structure of polyphenol, the microorganism strain, and the evaluated dosage are some factors that affect bacterial metabolism and growth (Hervert-Hernandez and Goni, 2011). The outer membrane of gram-negative bacteria is a lipopolysaccharide membrane (Kalambhe *et al*., 2017). Consequently, gram-positive bacteria are more sensitive to polyphenols due to their wall composition (Ghimire *et al*., 2017).

Recent findings demonstrated that phenolic compounds may bind to bacterial cell membranes and disturb their function leading to the inhibition of cell growth (Kemperman *et al*., 2010). Singh *et al*. (2019) argued that polyphenols generate hydrogen peroxide and can alter the microbial membrane permeability. In addition, polyphenols can bind bacterial cell membranes and alter membrane function resulting in prevention from their growth (Singh *et al*., 2019).

Bouarab-Chibane *et al*. (2019) noticed that hydrogen bonding of hydroxyl groups of polyphenols (e.g., catechins and theaflavins) to lipid bilayers of cell membrane controls the antimicrobial mechanism of polyphenols. The configuration of these polyphenols is influenced by molecular structure at the time of binding to the bilayer surface, and they form hydrogen bonds with the lipid head groups. Selma *et al*. (2009) reported that the main genera involved in the metabolism of many phenolics (e.g., isoflavones, flavonols, flavones, and flavan-3-*ols*) are *C.* and *Eubacterium*.

Dolara *et al*. (2005) found a shift in fecal bacterial composition from *Bacteroides*, *Clostridium*, and *Propionibacterium* spp. to *Bacteroides*, *Lactobacillus*, and *Bifidobacterium* spp. in rats that consumed proanthocyanidin-rich grape extract. Larrosa *et al*. (2009) and Tzounis *et al*. (2008) stated that the growth of some *Bifidobacteria* and *Lactobacilli* were stimulated or remained comparatively unaltered by phenolic compounds, such as resveratrol. However, the growth of *C. perfringens* was inhibited by catechin and epicatechin, as the types of polyphenols. Yamakoshi *et al*. (2001) evaluated the growth inhibitory activity of grape seed extract against *C. perfringens*. They stated that the growth of *C. perfringens* was not prevented by the phenolic extract.

Bouarab-Chibane *et al*. (2019) stated that the phenolic compounds of plant extracts are natural alternatives to synthetic preservatives in food. Li *et al*. (2015) reported that pomegranate extract increased the growth of Lactobacilli and *bifidobacteria*. On the other hand, it inhibited the growth of the *Bacteroides fragilis* group, *Clostridia*, and *Enterobacteriaceae* in stool cultures. In another study carried out by Rosas-Burgos *et al*. (2016), the most sensitive strains to the constituents of pomegranate by-products were gram-positive intestinal pathogenic species, such as *C. perfringens*. Naziri *et al*. (2012) demonstrated that the different antibacterial activities of the methanolic extract of pomegranate peel may be due to the variations in the antibacterial substances, namely tannins and phenolic substances. Kavak *et al*. (2010) investigated *Pistacia terebinthus* extract, as a potential antioxidant, antimicrobial, and possible β-glucuronidase inhibitor. These authors concluded that *Pistacia terebinthus* leaf extract had antimicrobial activity against *Staphylococcus aureus* as a gram-positive bacteria, while it did not have sufficient antimicrobial activity against *E. coli*.

Tzounis *et al*. (2011) suggested that phenolic compounds (flavan-3-*ol* monomers) may influence the bacterial population of the large intestine even in the presence of carbohydrates and proteins. It appears that polyphenols have a prebiotic effect on the modulation of gut microbiota and exert antimicrobial activities against pathogenic gastrointestinal bacteria (Kawabata *et al*., 2019). As mentioned before, the host physiology is dependent on gut microbiota (Umu *et al*., 2013), and the distinct physicochemical and metabolic properties of fibers result in a different impact on community composition from the ingestion of dietary (Umu *et al*., 2015). Therefore, the prebiotic characteristics of RS may be due to the non-digestibility of carbohydrate fractions for colonic bacteria that influence the host gut health (Spencer, 2011). The weight of the total gastrointestinal tract increased by RS consumption in the animals. Elevated bacterial mass, fermentation end-products (Slavin, 2013), and augmented metabolically active tissue in the colon (Souza da Silva *et al*., 2014) result from the mentioned effect of RS. The prebiotics selectively stimulate the growth of beneficial bacteria, such as *Lactobacilli* and *bifidobacteria* (Samal *et al*., 2015), while suppressing the growth of toxogenic and proteolytic bacteria, including *C. perfringens*, *Streptococcus* spp., and *Staphylococcus* spp. (Samarasinghe *et al*., 2003; Rohin *et al*., 2014). We found that although RS did not affect growth prevention of *C. perfringens*, grape pomace extract and RS had a synchronic inhibitory effect on this strain.

Conclusion

We concluded that the grape pomace extract prevented *C. perfringens* growth. The RS had no inhibitory effect on the growth of this bacterium. However, the treatments of RS + pomegranate pomace extract and RS pistachio peel extract could not inhibit the ,growth of *C. perfringens.* On the other hand the RS + grape pomace extract could well

suppress the growth of *C. perfringens* by a synchronic inhibitory effect.

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Conflict of Interest

The authors declared no conflict of interest.

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مجله طب دامی ایران، ۱۳۹۹، دوره ۱۵، شماره ۱، ۹۳-۱۰۳

حداقل غلظت بازدارندگی عصاره فنولی و نشاسته مقاوم بر *کلستریدیوم پرفرینجنس*: مطالعه آزمایشگاهی

سمیرا کرامتی جبه‌دار[۱]، فرزاد میرزائی آقجه قشلاق[۱*]، بهمن نویدشاد[۱]، علی مهدوی[۲] ، حمید استاجی[۳] و نعمت هدایت ایوریق[۱]

[۱]گروه علوم دامی، دانشکده کشاورزی و منابع طبیعی، دانشگاه محقق اردبیلی، اردبیل، ایران

[۲]گروه علوم دامی و صنایع غذایی، دانشکده دامپزشکی، دانشگاه سمنان، سمنان، ایران

[۳]گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه سمنان، سمنان، ایران

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زمینه مطالعه: نقش *کلستریدیم پرفرینجنس* در ایجاد بیماری‌هایی که از طریق خوراک منتقل می‌شوند، مسئله مهمی است که در صنعت خوراک انسان و دام وجود دارد. افزایش نگرانی‌ها دربارهٔ مقاومت آنتی بیوتیکی انسان را وادار به یافتن جایگزین‌هایی برای آنتی‌بیوتیک‌ها کرده است.

هدف: هدف از تحقیق حاضر بررسی فعالیت ضد میکروبی عصارهٔ تفاله انگور، عصارهٔ پوستهٔ پسته و عصارهٔ تفالهٔ انار در حضور یا عدم حضور نشاسته مقاوم به‌عنوان پری بیوتیک بر *کلستریدیوم پرفرینجنس* بود.

روش کار: برای این هدف، نشاسته مقاوم (فایبرسول ۲) فراهم شده و عصارهٔ تفالهٔ انار، پوستهٔ پسته و تفالهٔ انار آماده شد. میزان فنول کل و تانن عصاره‌ها به ترتیب به‌وسیله روش فولین سیوکالتو و استاندارد اسید تانیک تعیین شد. فعالیت ضد میکروبی عصاره‌ها در ترکیب یا بدون نشاسته مقاوم با استفاده از روش حداقل غلظت بازدارندگی علیه باکتری *کلستریدیوم پرفرینجنس* ارزیابی شد.

نتایج: نتایج نشان داد که ۱۰۰ پی‌پی‌ام از عصارهٔ پوستهٔ پسته توانست به‌عنوان یک بازدارندهٔ رشد برای *کلستریدیوم پرفرینجنس* عمل کند. نشاسته مقاوم به تنهایی قادر به ممانعت از رشد *کلستریدیوم پرفرینجنس* نبود. درحالی‌که ۴۰۰ پی‌پی‌ام از مخلوط عصارهٔ تفالهٔ انگور و نشاستهٔ مقاوم از رشد *کلستریدیوم پرفرینجنس* ممانعت کرد؛ در مقابل، عصارهٔ پوستهٔ انار در هر دو حالت بدون نشاسته مقاوم و در ترکیب با نشاسته مقاوم مانع رشد این باکتری نشد. از سویی دیگر، عصارهٔ پوستهٔ پسته در مخلوط با نشاسته مقاوم و بدون نشاسته مقاوم در مقایسه با سایر تیمارها نتوانست از رشد باکتری *کلستریدیوم پرفرینجنس* جلوگیری کند.

نتیجه‌گیری نهایی: عصارهٔ تفالهٔ انگور در هر دو حالت، همراه با نشاسته مقاوم و بدون نشاسته مقاوم، توانست در ممانعت از رشد *کلستریدیوم پرفرینجنس* موثر باشد.

واژه‌های کلیدی: پری بیوتیک، ترکیبات فنولی، فعالیت ضد میکروبی، *کلستریدیوم پرفرینجنس*، نشاسته مقاوم

نویسندهٔ مسئول: فرزاد میرزائی آقجه قشلاق ، گروه علوم دامی، دانشکده کشاورزی و منابع طبیعی، دانشگاه محقق اردبیلی، اردبیل، ایران. ایمیل: f_mirzaei@uma.ac.ir