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Improvement in the nutritional composition and nutrient digestibility of pomegranate (*Punica granatum* L.) seed by *Bacillus subtilis* and *Aspergillus niger* solid-state fermentation

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ABSTRACT

This study was conducted to investigate the effect of *Aspergillus niger* and *Bacillus subtilis* on nutritional composition and *in vitro* digestibility of pomegranate (*Punica granatum* L.) seed (PGS). The PGS was fermented by *A. niger* ATCC 20345, *A. niger* ATCC 9142 and *B. subtilis* ATCC 21556 (10^5, 10^5 spores and 10^{10} cfu per kg PGS, respectively). Both *A. niger* and *B. subtilis* increased (P<0.001) crude protein (CP), ether extract (EE) and ash content but decreased (P<0.001) crude fiber (CF), hemicellulose (HC), nitrogen-free extract (NFE), neutral detergent fiber (NDF) and acid detergent fiber (ADF) in PGS. Besides, *in vitro* nutrient digestibility of PGS was increased (P<0.001) by *A. niger* and *B. subtilis*. The results of the study showed that *A. niger* and *B. subtilis* pose a serious opportunity to improve the nutritional quality and nutrient digestibility of PGS. *Aspergillus niger* ATCC 200345 gives better nutritional improvement in PGS with highest CP, EE, lowest CF, ADF and average NDF and HC among the treatment groups.

Keywords:
Agricultural residue
Solid-state fermentation
In vitro digestibility
Nutritional improvement
Aspergillus niger
Bacillus subtilis

Bacillus subtilis ve *Aspergillus niger*'in nar (*Punica granatum* L.) çekirdeğinin besin madde kompozisyonu ve sindirilebilirliği üzerine etkisi

ÖZET

Bu çalışma *Aspergillus niger* ve *Bacillus subtilis*'in nar (*Punica granatum* L.) çekirdeğinin besin madde kompozisyonu ve *in vitro* sindirilebilirliği üzerine etkilerini araştırmak üzere yapılmıştır. Nar çekirdeği, *A. niger* ATCC 20345, *A. niger* ATCC 9142 ve *B. subtilis* ATCC 21556 ile fermente edilmiştir (sırasıyla 10^5, 10^5 spor/kg and 10^{10} cfu/kg nar çekirdeği). *A. niger* ve *B. subtilis* nar çekirdeğinin ham protein (HP), ham yağ (HY), ve ham kül (HK) içeriğini artırırken (P<0.001) ham selüloz (HS), hemiselüloz, nitrojensiz öz madde (NÖM), nötral deterjan fiber (NDF), asit deterjan fiber (ADF) içeriğini azaltmıştır (P<0.001). Ayrıca nar çekirdeğinin *in vitro* besin madde sindirilebilirliği *A. niger* ve *B. subtilis* fermantasyonu ile artmıştır (P<0.001). Çalışmanın bulguları *A. niger* ve *B. subtilis*'in nar çekirdeğinin besin madde kompozisyonu ve sindirilebilirliğini iyileştirmede önemli bir potansiyele sahip olduğunu göstermiştir. *Aspergillus niger* ATCC 200345, diğer mikroorganizmalarla karşılaştırıldığında en yüksek HP ve HY, en düşük HS, ADF ve ortalama NDF ve hemiselüloz değerleriyle en iyi besinsel kompozisyon değişikliğine sebep olmuştur.

Anahtar Sözcükler:
Tarımsal atıklar
Katı kültür fermantasyonu
In vitro sindirilebilirlik,
Besinsel zenginleştirme
Aspergillus niger
Bacillus subtilis

1. Introduction

Utilization of agricultural residues has become having particular attention in recent years in order to reduce the dependence of poultry nutrition on the cereals that are used in human nutrition (Saki et al., 2014). Agricultural residues, which can be supplied at cheap prices, can reduce feed costs by using in poultry

diets. In addition, environmental pollution caused by agricultural residues can also be lowered.

Pomegranate (*Punica granatum* L.) is one of the most ancient edible fruits (Manterys et al., 2016). Annual pomegranate production of Turkey reached 656,200 tons in 2016 (TUIK, 2016). Pomegranate fruit consists of 48% shell and %52 edible portion (Sarica, 2011). Edible part consists of %78 juice and %22 seed (Zarei et al., 2011).

Pomegranate seed (PGS) is rich in unsaturated and conjugated fatty acids (Wang et al., 2010). Therefore, PGS improved the fatty acid composition of liver (Białek et al., 2017) and adipose tissue (Manterys et al., 2016) in broiler chicks. The PGS has also antioxidant effect on broiler chicks and laying hens (Saki et al., 2014; Ahmed et al., 2015). It can increase egg production (Saki et al., 2014) and improve egg yolk color (Kostogrys et al., 2017) Moreover, harmful effects of mycotoxins on broiler chicks can be eliminated with pomegranate feeding (Hussein, 2015). It also improved the immune functions of mice (Yamasaki et al., 2006).

The PGS contains 13.7% crude protein (CP), 39.4% crude fiber (CF) and 29.6% ether extract (EE) on dry matter basis (Rowayshed et al., 2013). High CF content of PGS is thought to limit its use in animal nutrition. Solid-state fermentation is a unique biotechnological process having great potential for recycling agro-industrial residues into useful animal feeds. It can enhance the nutrient composition (Altop et al., 2018a) decrease the antinutritional components (Sun et al., 2012) and also improve the nutrient digestibility (Shi et al., 2017) of agricultural wastes. *Aspergillus niger* and *Bacillus subtilis* are used as probiotics in animal nutrition and highly preferred for solid state fermentation (Raimbault, 1998; Teng et al., 2012). This study aimed to determine the possibilities of improving the nutritional quality of PGS by using *A. niger* (ATCC 200345 or ATCC 9142) or *B. subtilis* (ATCC 21556).

2. Materials and Methods

2.1 Microorganisms and substrate

The PGS was obtained from a juice factory in Turkey. *A. niger* (ATCC 200345 [A1] and ATCC 9142 [A2]) and *B. subtilis* (ATCC 21556 [B]) strains were supplied from the American Type Culture Collection (ATCC).

2.2 Preparation of fermented pomegranate seed (FPGS)

The PGS was milled to pass through a 2 mm sieve and sterilized by autoclaving at 121°C for 15 min. The nutritional salt (glucose: urea:$(NH4)_2SO_4$:peptone: KH_2PO_4:MgSO4.7H_2O=4:2:6:1:4:1) was added to support microbial development. *A. niger* and *B. subtilis* were cultured in Potato Dextrose Agar and Tryptic Soy Broth and inoculated at 10^5 spores and 10^{10} cfu per kg PGS, respectively. Afterwards, samples were incubated at 60 °C for 48 hours and dried at room temperature for 6 days till reaching %90 dry matter.

2.3 Determination of main nutritional components

CP (method, 976.06), EE (method, 920.29), ash (method, 942.05), and CF (method, 973.18) were determined according to AOAC (2000) before and after fermentation. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses were conducted as reported by Van Soest et al. (1991). Hemicellulose (HC) was calculated as NDF minus ADF.

2.4 Determination of in vitro digestibility

In vitro digestibility of samples were determined according to Yegani et al. (2013). Seeds were milled to 11 mm and weighed to 500 mg into *in vitro* flasks. Subsequently, 25 ml of 0.1 M phosphate buffer and 10 ml of 0.2 M HCl were added to each flask. Flasks were shaken to make sure the samples were mixed with the solution. Then, 1 ml porcine pepsin (25 mg/ml, P-7000, Sigma-Aldrich) was added into the flasks. To minimize the effects of the possible bacterial fermentation during the incubation phase, chloramphenicol (0.5 ml) was added into each flask. Flasks were again shaken and then placed into a shaking incubator (100 rpm, 41 °C) for 2 h.

After the incubation, 5 ml of 0.6 M NaOH and 10 ml 0.2 M phosphate buffer were added into each flask. Flasks were shaken and 1 ml of porcine pancreatin containing amylase, lipase, and protease (100 mg/ml, P-1750; Sigma-Aldrich) was added. Flasks were shaken and incubated for 4 h (100 rpm, 41 °C). Flasks were removed from the incubator and 5 ml of 20% sulfosalicylic acid was added into each flask. Flasks were left for 30 min at room temperature. Then, flask contents were filtered using Whatman no. 54 filter papers (Whatman Inc., Florham Park, NJ). Filter papers were dried overnight at 80 °C before being used for the filtration. The residues collected in filter papers were also dried overnight at 80 °C. *In vitro* dry matter digestibility was calculated by the following formula:

$$\textit{In vitro}\ \text{dry matter digestibility}\ (\%) = 100 - \left(\frac{\textit{in vitro}\ \text{residue weight}\ (g)}{\text{sample weight}\ (g)}\right) \times 100$$

2.5 Statistical analysis

All the experiments were carried out in a completely randomized design with three replicates and the results were expressed as means with pooled standard error of means. Differences between treatments were tested using ANOVA and Duncan's multiple range test (SPSS 21.0 Statistics). The level of statistical significance was declared at $P \leq 0.05$. Orthogonal contrast tests were

applied to test whether means of A. niger groups and B. subtilis group differed significantly for each parameters.

3. Results and Discussion

3.1 Nutritional composition

Nutrient composition of PGS and FPGS is presented in Table 1. The CP content of PGS was increased (P<0.001) by solid-state fermentation. *A. niger* had higher (P<0.001) increase in CP than *B. subtilis*. Similar to the results of the present study, *A. niger* increased CP contents of pomegranate peel (Aguilar et al., 2008), shea nut (Dei et al., 2008), mango kernel (Kayode and Sani, 2008), palm kernel cake (Iluyemi et al., 2006; Lawal et al., 2010), sour cherry kernel (Güngör et al., 2017) and grape seed (Altop et al., 2018b). *B. subtilis* also increased CP content of rapeseed meal (Fazhi et al., 2011), cottonseed meal (Sun et al., 2012), soybean meal (Teng et al., 2012), mixed feed (Shi et al., 2017), napiergrass and pangolagrass (Hsu et al., 2013) and *Leucaena leucocephala* leaf (Bairagi et al., 2004). Increase in CP content may be due to the microbial protein produced by *A. niger* and *B. subtilis* (Raimbault, 1998; Shi et al., 2017).

Microorganisms have the capability to produce microbial lipid and can increase the EE content of the substrates (Hui et al., 2010). The EE content of PGS was increased (P<0.001) by *A. niger* and *B. subtilis*. FPGS by *A. niger* had higher (P<0.001) EE content compared with the FPGS by *B. subtilis* in this study. Similar findings have been reported from the *A. niger* fermentation study on shea nut (Dei et al., 2008). However, no change was reported in EE content of pomegranate peel (Aguilar et al., 2008), palm kernel (Iluyemi et al., 2006; Lawal et al., 2010), sour cherry kernel (Güngör et al., 2017) and grape seed (Altop et al., 2018b). Moreover, fermentation increased EE content of mango kernel (Kayode and Sani, 2008), sour cherry kernel (Güngör et al., 2017) and grape seed (Altop et al., 2018b). Similarly, *B. subtilis* was reported to increase EE content in rapeseed meal (Fazhi et al., 2011) and soybean meal (Teng et al., 2012) although EE was changed in mixed feed (Shi et al., 2017) and also decreased in cottonseed meal (Sun et al., 2012) by *B. subtilis*.

Ash content of PGS was increased (P<0.001) by solid-state fermentation of *A. niger* or *B. subtilis* but reached the highest values at *A. niger* groups. This result is consistent with the studies on *A. niger* fermentation in pomegranate peel (Aguilar et al., 2008), shea nut (Dei et al., 2008), mango kernel (Kayode and Sani, 2008), sour cherry kernel (Güngör et al., 2017) and grape seed (Altop et al., 2018b). *B. subtilis* also increased ash content of cottonseed meal (Sun et al., 2012), mixed feed (Shi et al., 2017), napiergrass and pangolagrass (Hsu et al., 2013). These results may be due to the relative increase of ash content because of the decrease in NFE, CF, NDF and ADF content of pomegranate seed by fermentation.

Microorganisms prefer soluble carbohydrates to other nutrients for using as a carbon source (Papagianni, 2007). The NFE content of pomegranate seed was decreased (P<0.001) by fermentation in this study. The lowest NFE was obtained (P<0.001) from FPGS-A2 group. This result is in line with the studies on pomegranate peel (Aguilar et al., 2008), shea nut (Dei et al., 2008), mango kernel (Kayode and Sani, 2008), sour cherry kernel (Güngör et al., 2017) and grape seed (Altop et al., 2018b).

Table 1. Chemical composition of unfermented and fermented pomegranate seeds

Nutrients	PGS	FPGS-B	FPGS-A1	FPGS-A2	SEM	P	Contrast[1]
Crude Protein	16.12^{c}	29.79^{b}	31.82^{a}	32.63^{a}	2.023	***	***
Ether Extract	1.55^{c}	3.62^{b}	5.72^{a}	6.17^{a}	0.561	***	***
Ash	3.05^{c}	7.42^{b}	8.19^{a}	8.33^{a}	0.653	***	***
NFE	41.12^{a}	30.88^{b}	28.65^{c}	26.49^{d}	1.704	***	***
Crude Fiber	38.16^{a}	28.29^{b}	25.62^{d}	26.39^{c}	1.518	***	***
Hemicellulose	13.72^{a}	10.75^{c}	12.44^{b}	10.73^{c}	0.406	***	NS
NDF	51.38^{a}	44.08^{b}	41.32^{b}	37.77^{c}	1.559	***	**
ADF	37.66^{a}	33.32^{b}	28.87^{c}	27.04^{c}	1.284	***	***

***: <0.001, **: 0.01, NS: not significant

[1]Planned orthogonal contrast FPGS-B vs. FPGS-A1 and FPGS-A2

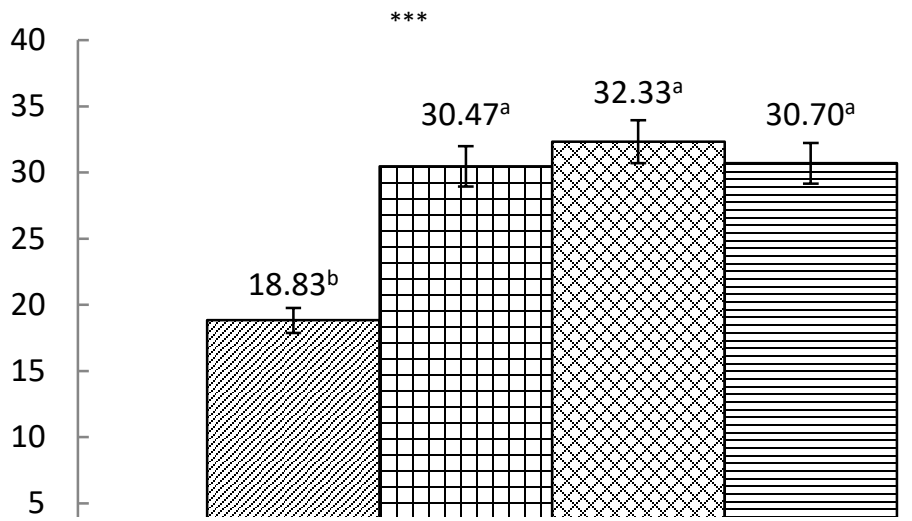
PS: unfermented pomegranate seed, FPGS-B: *Bacillus subtilis* (ATCC 21556), FPGS-A1: *Aspergillus niger* (ATCC 200345), FPGS-A2: *Aspergillus niger* (ATCC 9142), SEM: standard error of means

The CF, HC, NDF, and ADF were decreased (P<0.001) by *A. niger* or *B. subtilis* solid-state fermentation in this study. *A. niger* provided lower CF (P<0.001), NDF (P<0.01) and ADF (P<0.001) content compared with *B. subtilis*. However there was no significant difference (P>0.05) between FPGS-B and FPGS-A groups with regard to HC content. The FPGS-A1 had the lowest (P<0.001) CF content in the treatment groups. Cellulase enzyme that breaks down the structural carbohydrates can be produced by *A. niger* (Xie et al., 2016) and *B. subtilis* (Ritter et al., 2018) in solid state fermentation. The decrease in structural carbohydrates in PGS can be attributed to the production of cellulase enzyme during fermentation. Similarly, *A. niger* decreased structural carbohydrate content in CF, NDF and ADF in palm kernel cake (Iluyemi et al., 2006; Lawal et al., 2010), shea nut (Dei et al., 2008) and grape seed (Altop et al., 2018b). Similarly, *B. subtilis* diminished CF in cottonseed meal (Sun et al., 2012) and *L. leucocephala* leaf (Bairagi et al., 2004), decreased NDF in mixed feed (Shi et al., 2017)

3.2. In vitro digestibility

In vitro dry matter digestibility of unfermented and fermented pomegranate seed is given in Figure 1. The PGS had a low *in vitro* digestibility (18.83%) in this study. Taher-Maddah et al. (2012) also reported a lower *in vitro* digestibility (34.62%) for PGS in ruminants. Cellulose is one of the major factors affecting the nutrient digestibility of feedstuffs (Graminha et al., 2008). Lower digestibility may be due to high CF content of PGS. *In vitro* dry matter digestibility of FPGS groups was increased compared with PGS in this study. Teng et al. (2012) reported that *in vitro* CP digestibility of soybean meal increased by *B. subtilis* fermentation. Similarly, Shi et al. (2017) reported increase *in vitro* CP and amino acid digestibility in mixed feed by *B. subtilis*.

Dry matter digestibility was increased (P<0.001) by solid-state fermentation in the present study. Various enzymes such as protease, cellulose and lipase were synthesized by *A. niger* and *B. subtilis* during fermentation (Wu et al., 2015; Ritter et al., 2018). Improvement in the nutrient digestibility may be due to the reduction of structural carbohydrates as well as the production of enzymes that help the nutrient digestion. Teng et al. (2012) reported that *B. subtilis* increased *in vitro* CP digestibility of soybean meal more than *Aspergillus oryzae*. However, *in vitro* digestibility of FPGS was similar in *A. niger* and *B. subtilis* groups in the present study. This may be due to differences in substrates, microorganisms and culture conditions between two studies.



***: P<0.001; PGS: unfermented pomegranate seed; FPGS-B: *Bacillus subtilis* (ATCC 21556); FPGS-A1: *Aspergillus niger* (ATCC 200345); FPGS-A2: *Aspergillus niger* (ATCC 9142).

Figure 1. *In vitro* dry matter digestibility of fermented and unfermented pomegranate seeds

4. Conclusions

The present study showed that *B. subtilis* and *A. niger* can be used to improve the nutritional composition and to increase the digestibility of PGS by increasing CP, EE, ash and decreasing CF, NFE, HC, NDF and ADF content. Best results were obtained from the *A. niger* ATCC 200345 by causing highest CP, EE and lowest CF, ADF content. Animal experiments should be conducted to determine the effects of PGS and FPGS on the growth performance, digestibility and etc. in the future.

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