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Bioprocessing of acorn by *Lactobacillus plantarum* and *Aspergillus oryzae* to reduce its tannin content

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ABSTRACT

Most area of forest in the Zagros Mountains has covered by oak trees that produced a nutritionally valuable fruit, commonly named acorns. Acorn contains high percentage of starch that can be used as a source of energy in animals and human's diets. Nevertheless, acorns contain a significant amount of tannin, thus restricting their use in animal diets. This experiment was done to investigate the bioprocessing efficacy of *Lactobacillus plantarum* and *Aspergillus oryzae* to reduce acorn tannin, in a completely randomized design with 9 treatments and 4 replicates. The treatments includes control (pre-bioprocessing at zero day, non-incubation), non-bioprocessing (without bacteria and fungi) but 5 or 10 days incubation, 5 or 10 days incubation with bacteria, 5 or 10 days incubation with fungi and 5 or 10 days mixed-incubation with bacteria and fungi. Phenolic and tannic compounds were analyzed by colorimetric assay. After undergoing bioprocessing with bacteria and/or fungal treatment, there was a significant decrease observed in total phenol, non-tannin phenol, condensed tannin, and hydrolysable tannin when compared to the control group (P<0.01). In Conclusion, the results of the current experiment showed that biodegradation of acorn tannins by *Lactobacillus plantarum* and *Aspergillus oryzae*is was an effective biosafety process. Furthermore, the degradation of phenols and tannins compounds was higher effective in the 10 days bioprocessing of fungal treatment.

Key words: *Aspergillus oryzae*, Bioprocessing, *Lactobacillus plantarum*, Oak, Tannin

INTRODUCTION

Oak trees of the genus *Quercus* ssp. cover a vast area of the Zagros Mountains, approximately 5 million hectares and comprises 40% of Iran's forests (Mehrnia *et al.*, 2013). The Zagros forest plays a crucial role on both a national and global scale by providing essential environmental services like carbon storage, air purification, and wildlife habitat. Additionally, oak trees within this forest produce acorns, a fruit with significant nutritional value and phytochemical content. Given their significant starch content, acorns can serve as a valuable energy source in both animal and human diets. (Rahi *et al.*, 2018; Morales, 2021; Szabłowska and Tańska, 2024).

Throughout centuries, indigenous populations of the Zagros region have utilized oak products for their nutritional, medicinal, and chemical properties, particularly in the process of converting skin into leather. Nevertheless, the high tannin content found in oak acorns has restricted their application in both animal and human diets. (Kirkpatrick and Pekins, 2002; Çalışlar, 2018; Besharati *et al.*, 2022).

The term tannin originates from the Celtic term for oak tree, subsequently evolving to denote the utilization of oak in the process of transforming animal hides into leather. Tannins are the second most abundant polyphenol after lignin, and mainly they function as defense compounds that protect plants against pests and other abiotic stresses, such as drought, heat, and high UV radiation (Mueller-Harvey and McAllan, 1992). Tannins are

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a type of bitter polyphenolic compounds characterized by diverse molecular weights and intricate structures. They can be categorized into two groups: condensed tannins and hydrolysable tannins. Condensed tannins consist of flavonoid polymers known as proanthocyanidins. On the other hand, hydrolysable tannins contain a polyhydric alcohol as their central component, with hydroxyl groups that are esterified with gallic acid. In some cases, these tannins may exhibit long chains of gallic acid originating from the central glucose core (Deshpande *et al*., 1986; Das *et al*., 2020).

The deleterious effects of tannins in the diet seem to be related to reduction in nutrient absorption due to the formation of complexes with proteins, and to a lesser degree with metal ions, amino acids, and polysaccharides (Majumdar and Moudgal, 1994; Sharma *et al*., 2021; Tong *et al*., 2022). Efficiency of microbial protein synthesis, diet palatability and feed intake, protein and starch digestibility as well as minerals and vitamins availability are negatively influenced by tannins. In addition, tannins adversely influence organs such as intestines, liver, kidney, spleen and pancreas in some animals such as poultry (Mangan, 1988; Makkar, 2003a; Waghorn, 2008; Naumann *et al*., 2017; Liu *et al*., 2019; Hassan *et al*., 2020).

Various methods such as sodium bicarbonate, polyethylene glycol, soaking in water, cooking or steaming have been reported to inactivation or removal of tannins or reduce their adverse effects, and therefore, to improve the nutritional value of tannin-rich feedstuffs (Makkar, 2003a; Frutos *et al*., 2004; Medugu *et al*., 2012; Houshmand *et al*., 2015). Now a days, bioprocessing has vast applies in livestock and poultry feed, using the enzymatic capabilities of microorganisms. Furthermore, *Lactobacillus plantarum* and *Aspergillus oryzae* are safe that have the potential for high enzymes to break down and eliminate tannin compounds (Barbesgaard et al., 1992; Paranthaman *et al*., 2008; Curiel *et al*., 2009, Abdel-Nabey *et al*., 2011). Such microorganisms have wide application in food industry (Khalil *et al*., 2021; Yilmaz *et al*., 2022; Sutaoney *et al*., 2023; Sun *et al*., 2024). Our prior research has indicated that bioprocessing of acorn by *Lactobacillus plantarum* results in a reduction of total phenol, non-tannin phenol, total tannin, condensed tannin and hydrolyzable tannin by 39%, 24%, 49%, 49.5%, and 59%, respectively (Bahaaldini *et al*., 2018). No report regarding the co-bioprocessing of acorn with *Aspergillus oryzae* and *Lactobacillus plantarum* was discovered by us. Consequently, we propose that the co-bioprocessing of acorn with *Lactobacillus plantarum* and *Aspergillus oryzae* may offer enhanced efficiency in reducing tannin compounds.

MATERIALS AND METHODS

The experiment was done in a completely randomized design with 9 treatments and 4 replicates. The treatments included non-incubation and pre-bioprocessing (CONTL-0DAY) or 5 and 10 days incubation of acorn flour without bacteria and fungi (non-bioprocessing, NOPR-05DAY, NOPR-10DAY), with L. plantarum (BAPR-05DAY, BAPR-10DAY), with A. oryzae (FUPR-05DAY, FUPR-10DAY) and with L. plantarum and A. oryzae (BFPR-05DAY, BFPR-10DAY). The moisture content of all samples was adjusted to 50% by sterile water. *Lactobacillus plantarum* and *Aspergillus oryzae* were obtained from the Persian Type Culture Collection (PTTC) of the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). For each gram of bioprocessing treatments, 10^7 CFU of *Lactobacillus plantarum* (PTCC 1058) and/or 10^6 spores of *Aspergillus oryzae* (PTCC 5164) were added and cultured in an incubator (Memert, Germany). Incubation was done in two thermal condition (27 °C and 37 °C).

Total phenol content was determined by Folin-Ciocalteu colorimetric methd. Samples (200 mg) were extracted with 70% aqueous solution of acetone in an ultrasonic bath for 20 min. Extracts were centrifuged (10 min, 3000×g at 4 °C) and supernatant collected. The absorbance was read at 725 nm wavelength (Makkar, 2003b).

Non-tannin phenols were determined following precipitation of tannin in extracts with polyvinylpyrrolidone (PVPP). Twenty mg PVPP in 200 µL water was mixed with 200 µL extracts. Solutions centrifuged at 3000×g for 10 min and absorbance of supernatant was read. Concentration of total tannins were calculated as the difference between total phenols and non-tannin phenols (Makkar, 2003b).

Condensed tannins concentrations were measured by HCl–butanol method. Extracts solution were diluted with 70% acetone in test tube and mixed with 3.0 ml butanol-HCl and 0.1 ml ferric reagent, vortex the solution, putted the samples in boiling water bath for 60 min and finally the optical absorbance read at 550 nm. Condensed tannins concentrations were calculated by formula: "absorbance×78.26×dilution factor". Hydrolysable tannin was estimated by subtracting condensed tannins from total tannin (Makkar, 2003b). The data underwent analysis through SAS software (2011). To compare the means, Duncan's multiple range test was employed with a significance level set at $P \leq 0.01$.).

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RESULTS

In relation to the entirety of outcomes, the presence of bacteria and/or fungi in the bioprocessing procedure exhibited a substantial reduction in the proportion of various components such as total phenol, total tannin, non-tannin phenol, condensed tannins, and hydrolysable tannins in acorn flour. This reduction was observed after 5 and 10 days of incubation at both 27 and 37 °C temperatures. The reduction was significant when compared to the pre-bioprocessing state and non-incubation conditions on day zero, as indicated in tables 1 and 2.

Table 1- Effects of experimental treatments on total phenol and non-tannin phenol (g/100 g Dry Matter) of acorn flour under two thermal incubation conditions

Treatments1	Total Phenol %		Non-Tannin Phenol %	
	27°C	37° C	27°C	37°C
CONTL-0DAY	7.90±0.01 ^{a}	7.90±0.01^{a}	1.69±0.01 ^{a}	1.69±0.01 ^{a}
NOPR-05DAY	7.88±0.00 ^{a}	7.88±0.00 ^{a}	1.69±0.01 ^{a}	1.68±0.00 ^{a}
NOPR-10DAY	7.83±0.00 ^{a}	7.85±0.00 ^{a}	1.69±0.00 ^{a}	1.69±0.00 ^{a}
BAPR-05DAY	4.66±0.03 ^{bc}	5.06±0.06 ^{b}	1.02±0.01 ^{b}	1.09±0.01 ^{b}
BAPR-10DAY	4.39±0.03 ^{bc}	4.81±0.04 ^{b}	1.01±0.02 ^{b}	1.07±0.01 ^{b}
FUPR-05DAY	4.75±0.06 ^{b}	4.78±0.03 ^{b}	1.03±0.01 ^{b}	1.06±0.01 ^{b}
FUPR-10DAY	3.72±0.03 ^{c}	3.88±0.03 ^{c}	0.89±0.01 ^{c}	0.94±0.01 ^{c}
BFPR-05DAY	4.79±0.04 ^{b}	4.54±0.04 ^{bc}	1.06±0.01 ^{b}	1.02±0.00 ^{b}
BFPR-10DAY	4.69±0.02 ^{b}	3.91±0.02 ^{c}	1.03±0.01 ^{b}	0.95±0.01 ^{c}
SEM	0.001	0.001	<0.001	<0.001

Means within the same column with different superscript letters differ significantly (P<0.01).
[1] CONTL-0DAY: Non-incubation and pre-bioprocessing in day 0, NOPR-05DAY: Non- bioprocessing and 5 days incubation, NOPR-10DAY: Non-bioprocessing and 10 days incubation, BAPR-05DAY: Bacterial bioprocessing under 5 days incubation, BAPR-10DAY: Bacterial bioprocessing under 10 days incubation, FUPR-05DAY: Fungal bioprocessing under 5 days incubation, FUPR-10DAY: Fungal bioprocessing under 10 days incubation, BFPR-05DAY: Bacterial and Fungal bioprocessing under 5 days incubation, BFPR-10DAY Bacterial and Fungal bioprocessing under 5 days incubation.

In compare with non-bioprocessing treatments, the highest decrease in total phenol, non-tannin phenol, total tannin, condensed tannins and hydrolysable tannins of acorn was observed by *Aspergillus oryzae* During a period of less than 10 days, the process of bioprocessing was carried out under two different temperature conditions.

Table 2- Effects of experimental treatments on total tannin, condensed tannin and hydrolysable tannin (g/100 g Dry Matter) of acorn flour under two thermal incubation conditions

Treatments1	Total Tannin %		Condensed Tannin %		Hydrolysable Tannin %	
	27° C	37° C	27° C	37° C	27° C	37° C
CONTL-0DAY	6.22±0.01^{a}	6.22±0.01^{a}	5.01±0.06^{a}	5.01±0.06 ^{a}	1.21±0.06^{a}	1.21±0.06 ^{a}
NOPR-05DAY	6.19±0.00 ^{a}	6.19±0.00 ^{a}	5.09±0.00 ^{a}	5.01±0.00 ^{a}	1.11±0.00 ^{a}	1.19±0.00 ^{a}
NOPR-10DAY	6.14±0.00 ^{a}	6.16±0.00 ^{a}	5.09±0.00 ^{a}	5.09±0.00 ^{a}	1.05±0.00 ^{a}	1.07±0.00 ^{a}
BAPR-05DAY	3.64±0.03 ^{b}	3.96±0.06 ^{b}	3.07±0.09 ^{b}	3.19±0.09 ^{b}	0.57±0.08 ^{b}	0.78±0.11^{b}
BAPR-10DAY	3.39±0.03 ^{bc}	3.75±0.04 ^{b}	2.84±0.09 ^{bc}	3.05±0.06 ^{b}	0.55±0.11 ^{b}	0.69±0.04 ^{b}
FUPR-05DAY	3.73±0.07 ^{b}	3.72±0.03 ^{b}	3.11±0.07 ^{b}	3.13±0.17 ^{b}	0.62±0.12 ^{b}	0.59±0.14 ^{b}
FUPR-10DAY	2.83±0.03 ^{c}	2.93±0.03 ^{c}	2.50±0.17 ^{c}	2.64±0.07 ^{c}	0.32±0.17 ^{c}	0.29±0.07 ^{d}
BFPR-05DAY	3.73±0.04 ^{b}	3.52±0.04 ^{b}	3.21±0.11 ^{b}	2.86±0.11 ^{bc}	0.52±0.13 ^{b}	0.67±0.13 ^{b}
BFPR-10DAY	3.66±0.02 ^{b}	2.97±0.02 ^{c}	3.03±0.04 ^{b}	2.58±0.17 ^{c}	0.63±0.05 ^{b}	0.38±0.17 ^{c}
SEM	0.001	0.001	0.009	0.009	0.010	0.010

Means within the same column with different superscript letters differ significantly (P<0.01).
[1] CONTL-0DAY: Non-incubation and pre-bioprocessing in day 0, NOPR-05DAY: Non- bioprocessing and 5 days incubation, NOPR-10DAY: Non-bioprocessing and 10 days incubation, BAPR-05DAY: Bacterial bioprocessing under 5 days incubation, BAPR-10DAY: Bacterial bioprocessing under 10 days incubation, FUPR-05DAY: Fungal bioprocessing under 5 days incubation, FUPR-10DAY: Fungal bioprocessing under 10 days incubation, BFPR-05DAY: Bacterial and Fungal bioprocessing under 5 days incubation, BFPR-10DAY Bacterial and Fungal bioprocessing under 5 days incubation.

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The statistical analysis revealed significant differences (P<0.01) in the results, as shown in tables 1 and 2. Specifically, when incubated at a temperature of 27 °C, the biodegradation percentages of various components by *Aspergillus oryzae* were as follows: 52.91% for total phenol, 47.34% for non-tannin phenol, 54.50% for total tannin, 50.10% for condensed tannins, and 73.55% for hydrolysable tannins (table 3). Similarly, under a temperature condition of 37 °C, the corresponding biodegradation percentages were 50.89% for total phenol, 44.38% for non-tannin phenol, 52.89% for total tannin, 47.31% for condensed tannins, and 76.03% for hydrolysable tannins (table 4).

Table 3- The percentage decreases of total phenol, non-tannin phenol, total tannin and condensed tannin in bacteria-fungal bioprocessing as compared with pre-bioprocessing/non-incubation of acorn under 27 °C temperature

Treatments[1]	Total Phenol	Non-Tannin Phenol	Total Tannin	Condensed Tannin	Hydrolysable Tannin
BAPR-05DAY	-41.01	-39.64	-41.48	-38.72	-52.89
BAPR-10DAY	-44.43	-40.24	-45.50	-43.31	-54.55
FUPR-05DAY	-39.87	-39.05	-40.03	-37.92	-48.76
FUPR-10DAY	-52.91	-47.34	-54.50	-50.10	-73.55
BFPR-05DAY	-39.37	-37.28	-40.03	-35.93	-57.02
BFPR-10DAY	-40.63	-39.05	-41.16	-39.52	-47.93

[1] BAPR-05DAY: Bacterial bioprocessing under 5 days incubation, BAPR-10DAY: Bacterial bioprocessing under 10 days incubation, FUPR-05DAY: Fungal bioprocessing under 5 days incubation, FUPR-10DAY: Fungal bioprocessing under 10 days incubation, BFPR-05DAY: Bacterial and Fungal bioprocessing under 5 days incubation, BFPR-10DAY Bacterial and Fungal bioprocessing under 10 days incubation.

When the incubation period is extended from 5 to 10 days at temperatures of 27 and 37 °C, the percentage of phenolic and tannin compounds in acorns remains unaffected by bacterial bioprocessing, as opposed to pre-processing and non-incubation methods (table 1 and 2). When the incubation period is extended from 5 to 10 days at temperatures of 27 and 37 °C, the percentage of phenolic and tannin compounds in acorns remains unaffected by bacterial bioprocessing, as opposed to pre-processing and non-incubation methods. However, as compared with 5 days incubation, the percentages of phenolic and tannin compounds of acorn after 10 days bioprocessed with *Aspergillus oryzae* under both temperature condition significantly (P<0.01) were reduced (table 1 and 2). Within 5 days treatments of bacterial and/or fungal bioprocessing, the percentages of phenolic and tannin compounds of acorn were not affected significantly (P<0.01) by co-bioprocessing, but 10 days co-bioprocessing was significantly (P<0.01) affected on the degradation of phenolic and tannin compounds (table 1 and 2).

Table 4- The percentage decreases of total phenol, non-tannin phenol, total tannin and condensed tannin in bacteria-fungal bioprocessing as compared with pre-bioprocessing/non-incubation of acorn under 37 °C temperature

Treatments[1]	Total Phenol	Non-Tannin Phenol	Total Tannin	Condensed Tannin	Hydrolysable Tannin
BAPR-05DAY	-35.95	-35.50	-36.33	-36.33	-35.54
BAPR-10DAY	-39.11	-36.69	-39.71	-39.12	-42.98
FUPR-05DAY	-39.49	-37.28	-40.19	-37.52	-51.24
FUPR-10DAY	-50.89	-44.38	-52.89	-47.31	-76.03
BFPR-05DAY	-42.53	-39.64	-43.41	-42.91	-44.63
BFPR-10DAY	-50.51	-43.79	-52.25	-48.50	-68.60

[1] BAPR-05DAY: Bacterial bioprocessing under 5 days incubation, BAPR-10DAY: Bacterial bioprocessing under 10 days incubation, FUPR-05DAY: Fungal bioprocessing under 5 days incubation, FUPR-10DAY: Fungal bioprocessing under 10 days incubation, BFPR-05DAY: Bacterial and Fungal bioprocessing under 5 days incubation, BFPR-10DAY Bacterial and Fungal bioprocessing under 10 days incubation.

DISCUSSION

Various chemical and physical methods have been reported to overcome the adverse effects of tannin in the animal feeds, however, in addition to economic and commercial benefits, biological methods considered as biosafety and eco-environmental friendly for detanninification and increasing nutritional value of tannin-rich feeds.

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The results of this experiment revealed the high efficiency of bioprocessing with *Lactobacillus plantarum* and/or *Aspergillus oryzae* for reducing acorn tannins and phenolic compounds. The ability of this food industrial-friendly microorganisms for tannin and phenolic degradation is duo to their tannase activity (Barbesgaard et al., 1992; Muñoz *et al*., 2024; Sun *et al*., 2024). The tannase is a valuable biotechnological enzyme which has been widely used to improve the safety, nutritional, and sensorial properties of foods (Aharwar and Parihar, 2018).

Tannin acyl hydrolase (E.C.3.1.1.20) commercially known as tannase is an intracellular or extracellular inducible enzyme that belongs the class hydrolase. It catalyzes the breakdown of ester and depside bonds of gallotannins, gallic acid esters, epigallocatechin-3-gallate, and produces glucose and phenolic acid like gallic acid (Biswas *et al*., 2022).The annase enzyme has wide application in the manufacture of gallic acid, pyrogallol, food and beverage processing, and also the management of tannery effluents and wastewater (Biswas *et al*., 2022).

Several species of fungi and bacteria are tannase producers, but unlike the *Lactobacillus plantarum* and *Aspergillus oryzae*, the most numerous of them are not bio-safe for food bioprocessing, because of their toxic potential for inducing adverse human health effects (Frisvad *et al*. 2018). In this regard, *Lactobacillus plantarum* and *Aspergillus oryzae* are bio-safe microorganism which are widely utilized in food industries (Barbesgaard *et al*., 1992; Frisvad *et al*., 2018; Sun *et al*., 2024).

Based on the present experimental result, the highest reduction of phenols and tannin compounds was observed in fungal bioprocessing under 10 days incubation, in both thermal conditions. Corresponding with our results, the investigation shown that fungi are more effective than bacteria in degrading phenolic compounds such as tannins (Ayed *et al*., 2017).

Tannins have deleterious or beneficial effects depending on their concentration, compounds, animal species and feed composition (Nawab et al., 2020). The deleterious effects of tannins in poultry were more obvious when tannins were supplemented more than one percent in their diets (Houshmand *et al*., 2015, Keshavarzi *et al*., 2017; Hidayat *et al*., 2021). However, the lower levels (0.05 to 0.5 %) of the tannin in poultry diets could improve grow rate and gut health due to their antimicrobial, antioxidants and anti-inflammatory potential (Choi and Kim, 2020). Obviously, ruminant animals have a higher tolerance to tannins than poultry due to evolutionary adaptability to tannin-rich feeds and rumen ecosystem. Low level of tannins in ruminants diets (up to 5 %) prevent bloating, improve the antioxidant status and reduce the rumen methane production, whereas higher concentrations reduced feed intake and nutrient digestibility (Makkar, 2003a; Smith *et al*., 2005; Addisu, 2016; Besharati *et al*., 2022).

Therefore, high-efficient reduction of acorn tannin content in this experiment not only provide the possibility of higher-level inclusion of this valuable native food source in the livestock diets, but also produce a functional food with beneficial health effects for utilization in human and animal nutrition.

CONCLUSION

The results of this experiment have been shown strongly that bioprocessing of acorn by *Lactobacillus plantarum* and *Aspergillus oryzae* are high efficacious for degradation of acorn phenolic and tannin compounds. Considering that the main factor known to limit the use of acorn in livestock diets is its tannin compounds, noteworthy reduction of the total tannin, concentrated tannin and hydrolyzable tannin of acorn by bioprocessing in this experiment is an opportunity to increase the level of supplementation of acorn in the diet of farmed animals.

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CONFLICT OF INTEREST

Authors certify that they have no entity with any financial interest in the subject matter or materials discussed in this manuscript.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript

DATA AVAILABILITY

The datasets produced and analyzed in the present study are not accessible to the public, however, they can be obtained from the corresponding author upon reasonable request.

ETHICAL CONSIDERATIONS

Along with the vital importance of providing safe food for humans, should always keep in mind the crucial of environmental protection and preservation, especially the extremely valuable the oak forest of Zagros region.

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