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Effect of tannin-degrading bacteria isolated from the rumen of some ruminants on the *in vitro* digestibility and gas production of fruits residues silage

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Abstract

While severe shortage of feed has presented livestock industry with a major challenge, millions of tons of fruit by-products (FBPs) are discarded annually, as valuable sources of energy and numerous nutrients. However, some of FBPs contain high tannins that must be treated for use in animal feeding. This study was investigated the effect of tannin-degrading bacteria isolated from the rumen of some ruminants on the *in vitro* digestibility and gas production of FBPs silage. The FBPs, including pomegranate peel (PP), and the pulps of lemon (LP), grape (GP), and orange (OP) were ensilaged with tannin-degrading bacteria (enzyme activity: 10.46 - 8.60 U mL^{-1}) isolated from the rumen of male goat (*Escherichia coli* GHMGHE41), deer (*Escherichia fergusonii* GHMGHE44), ram (*E. fergusonii* GHMGHE30), and camel (*Klebsiella aerogenes* GHMGHE38). After anaerobic incubation (30 days; 39.00 ˚C), PP + camel strain silage showed the highest dry matter, and the lowest natural acid detergent fiber and pH. The camel strain increased crude protein content of LP silage to the highest level, and decreased acid detergent fiber of GP silage to the lowest level. The highest digestibility was observed for LP + goat strain silage (50.37%) compared to the uninoculated OP silage (42.73%). The maximum ammonia ($N\text{-}NH_3$) and minimum level of pH were recorded for the silages of LP + goat strain and PP + CR strain, respectively. Overall, the current results showed that tannin-degrading *E. coli* GHMGHE41 and *K. aerogenes* GHMGHE38 were able to improve the digestibility of LP and PP silages as ingredients in ruminants' diets.

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Introduction

Currently, livestock section is one of the fastest growing agricultural subsectors in worldwide and demand for its products is rapidly increasing in all countries. Since many of them are facing a shortage of animal feed, unconventional alternate resources could play an important role in combating this challenge.[1,2] Fruit and vegetable processing, packing, distribution and, consumption generate a huge quantity of fruits wastes which can act as valuable resources of energy and nutrients, such as soluble sugars, fiber, amino acids, proteins, organic acids, lipids, oils, and minerals, and help to bridge the gap between demand and supply of feed for livestock. In addition, their use in animal feeding can also reduce the cost of ration, giving higher profits to farmers.[1,2] Agricultural and food-industry residues constitute a major proportion (almost 30.00%) of worldwide agricultural production.[3] These wastes mainly comprise lignocellulosic materials, fruit and vegetable wastes, and sugar-industry herbs and leaves, as well as animal and fish byproducts.[3] According to the reports of United Nations Food and Agriculture Organization, about 1.30 billion tons of food products are wasted annually in world, which is one third of the food produced in the world.[4] The fruit and vegetable wastes are estimated up to 60.00% of the food waste, posing both environmental and economic challenges, affecting the human health due to the penetrance in the water and soil resources.[5,6] In Iran, internal statistics and estimates also showed that the amount of fruit waste is estimated from 40.00 to 50.00% from the stage of cultivation in the field to household consumption.[5] Also,

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Iran's share in agricultural waste production is about 2.70 or 35.00 million tons of the total 1.30 billion tons of global production, mainly including rice, bread, vegetables, and fruits.[5] Thus, an appropriate approach for managing fruit and vegetable wastes is a necessary need in Iran, because sustainable earth without pollution has become a part of technical-economic, strategic, and management decisions of countries.[5] In recent decades, interesting strategies have emerged that can significantly manage environmental challenges result from agriculture waste and lack of animal feed.[6] If appropriate technologies can be used for their valorization by enriching nutrients, it will be easier to provide energy and protein in animal diets.[3] Technologies available for protein enrichment of these wastes include solid substrate fermentation, ensiling, and high solid or slurry processes. Technologies to be developed for the reprocessing of these wastes need to take account of the peculiarities of individual wastes and the environment in which they are generated, reprocessed, and used. In particular, such technologies need to deliver products that are safe, not just for animal feed use, but also from the perspective of human feeding.[3,6] However, the presence of anti-nutritional content may adversely affect the use of these peels as animal feeds, such as the presence of limonene, tannins, and essential oil; therefore, they must be treated before using as an animal feed.[6,7] Nowadays, ensiling is a promising technology for converting fruit waste to animal feed using anaerobic microbial fermentation and acidification condition. It is an important pre-treatment method that preserves more than 90.00% of feed energy due to the severe shortage of animal feedstock.[8] In ruminants' diets, the main citrus wastes are supplemented as fresh pulp and peel, dried, meal, molasses, silage, citrus peel liquor, and cull or excess fruit. These wastes can be used as a high energy feed for ruminants to enhance growth and lactation, with fewer negative effects than high starch feeds.[9] Fruit wastes, like apple pomace, mango fruit waste, banana peels, pineapple waste, and citrus by-products have been recommended to be used in ruminants and non-ruminants feeding.[1] As previously indicated, substitution of raw lucerne with raw citrus lemon by-product in silage improved *in vitro* apparent digestibility and gas production.[2] The fruit by-products (FBPs) contain useful components for animal digestion processes, while they are an attractive source of energy, like readily fermentable carbohydrates and fats.[10] However, some secondary metabolites, such as tannins, saponins, oxalates, cyanogens, and phytic acid may suppress ruminal enzymes, digestive processes, and protozoa population, and also can form complexes with microbial proteins and structural sugars.[11] As frequently reported, these dietary supplementations might reduce animal growth efficiency due to the decline in the availability of nutrients.[11] To decrease or remove tannin, microbial silage additives have been suggested as a cost-effective technique, and enzyme addition to silages has received considerable attention over the past decade. Their primary function is to break down forage fiber during fermentation, rendering the silage more digestible during feed out. The breakdown of fiber into soluble sugars also helps bacteria produce lactic acid, leading to the lower silage pH.[10] Common enzyme-based silage additives include cellulases, hemicellulases, xylanases, amylases, pectinases, and tannases. Reportedly, they are typically utilized to enhance the quality of silage feeds, especially since they are environmentally safe and widely accepted.[10-15] In a study, inoculation of *Lactobacillus Buchneri* and fresh whey on alfalfa silage was led to reduce pH and improve silage quality.[15]

To this aim, the current study was evaluated the effect of tannin-degrading bacteria isolated from the rumen of some ruminants on the *in vitro* digestibility and gas production of fruits residues silage. For this purpose, the FBPs, such as pomegranate peel (PP), and the pulps of lemon (LP), grape (GP), and orange (OP), were combined with tannin-degrading strains isolated from the rumen of wild and domestic ruminants and incubated anaerobically at 39.00 ˚C for 30 days. At the end of ensilaging, the gas production and nutritional value of FBPs were evaluated.

Materials and Methods

Study location. This study was carried out at the Microbiology and Nutrition Laboratories of the Department of Animal Science, Faculty of Agriculture, University of Birjand, Birjand, Iran, in August 2022.

Fruits pulps. The FBPs including LP, OP, PP, and GP were collected from local fruit-juice shops in Birjand, Iran. They were checked to be free of all internal organs, such as seeds and dried in shade at room temperature (~ 25.00 ˚C; 10 day). Then, they were crushed into smaller pieces of 3.00 - 4.00 cm using a gardening shear and their moisture content was adjusted to 70.00% of fresh weight.[14,15]

Tannase inoculant. Tannase-producer strains (TPSs) were previously isolated from the rumen of male animals, including urial rams (R: *Ovis vignei*), fallow deer (D: *Dama dama*), Balochi camels (C: *Camelus dromedarius*), and Cashmere goats (G: *Capra hircus*). Four superior isolates with probiotic potential were used as silage inoculum (SI).[16-18] By modified method,[19] TPSs were cultured in nutrient broth (Merck, Darmstadt, Germany) tubes at 39.00 ˚C for 24 hr and then, 1.00 mL of overnight cultures were serially diluted 10-fold in sterile phosphate buffer solution. The tubes containing nutrient broth without bacteria were considered as controls and the numbers of viable bacteria were determined on nutrient agar plate. The survival rate was calculated as log values of colony forming units *per* mL.

Preparation of silage. The chopped FBPs were completely mixed with SI (1/00 v/w) and vacuum-sealed in sterile plastic bottles. For control group, distilled water was added as silage additive. Three replicates were considered for each treatment and the weight of each bottle was recorded before storage. The samples were fermented under anaerobic conditions at 39.00 ˚C for 30 days. Experimental treatments consisted of LP, OP, PP, and GP without additive, LP, OP, PP, and GP plus *Klebsiella aerogenes* GHMGHE38 (CR), LP, OP, PP, and GP plus *Escherichia fergusonii* GHMGHE44 (DR), LP, OP, PP, and GP plus *Escherichia coli* GHMGHE41 (GR), and LP, OP, PP, and GP plus *E. fergusonii* GHMGHE30 (RR).

Chemical composition analysis. At the end of 30 days, the bottles were opened and sampled for measuring the pH, dry matter (DM), ash, crude protein (CP), ethereal extract (EE), neutral detergent fiber (NDF), and acid detergent fiber (ADF) of silage FBPs. The chemical composition of the dried-shade FBPs and ensilaged FBPs was characterized using Association of Official Analytical Collaboration methods.[20] The filtrated fluid of each bottle was collected to measure pH using a mobile pH meter (GM761 Benetech; Shenzhen Jumaoyuan Science and Technology Co., Shenzhen, China); 200 g of fresh and silage FBPs were mixed separately with 800 mL of distilled water. The mixture was homogenized for 3 min and filtered by four layers cheesecloth. For DM, the fresh and silage FBPs samples were dried in a forced draft oven (65.00 ˚C for 3 days) and ground to pass a 1.00 mm screen. The CP content of the samples was determined by the Kjeldahl method.[20] The NDF and ADF of the silage FBPs were measured using an ANKOM A200 Fiber Analyzer (ASCOFOOD; Lotus Tajhiz Caspian Co., Mashhad, Iran).[21]

***In vitro* digestibility.** The *in vitro* digestibility of silage FBPs was estimated by gas production technique.[22] Before the morning feeding, rumen fluids were collected from Holstein dairy cattle *via* a rumen fistula. The cows were fed with wheat straw and commercial concentrate at a 50 / 50 ratio. Thermos flasks containing rumen fluid were continuously purged with a CO_2 gas to stabilize anaerobic conditions and taken within 20 min to the laboratory. After filtering by four-layers cheesecloth, the rumen fluid was diluted with pre-warmed artificial saliva (McDougall buffer; 9.80 g NaHCO3, 4.63 g Na2HPO4, 0.57 g KCl, 0.47 g NaCl, 0.118 g MgSO4·7H2O, 0.053 g CaCl2), and flushed with CO_2 (with a ratio of 2 / 1 buffer: rumen fluid). Then, 0.30 g of each ground FBP (1.00 mm mesh) was placed into a 100 mL glass vial and 60.00 mL of rumen fluid was dispensed. The vials were flushed with CO_2 gas and sealed with a rubber plug and an aluminum cap. They were placed in a water bath with temperature of 39.00 ˚C for 48 hr and shaken frequently every 45 min. Eventually, a glass electrode pH meter was used to determine the level of pH, and proximate composition of the FBPs, including DM, CP, NDF, ADF, EE, and ash was determined.[20,21]

Statistical analysis. A completely randomized experimental design [5.00 (SI) × 5.00 (FBPs) factorial treatments] was used. Analysis of variance (ANOVA) was applied for pH, DM, CP, EE, ash, NDF, and ADF in the FBPs silages. The ANOVA was performed using the general linear model (GLM) by SAS Software (version 9.2; SAS Institute, Cary, USA), according to the following model:

$$Yij = \mu + \alpha i + \beta j + (\alpha\beta)ij + eij$$

where, *Yij* is the observations for dependent variables, μ is overall mean, αi is the fixed effect of treatment, βj is the fixed effect of time, $\alpha\beta ij$ is the interaction between treatment and time, and *eij* is the residual error. Numbers of *j* were different among parameters (pH, DM, CP, EE, NDF, and ADF). The *p*-value of less than 0.05 on least-squares means was considered statistically significant.

Results

Tannase-producing strains and their viability. In the current study, four TPSs were used in FBPs ensilaging, that were Gram-negative bacteria belonging to the *Klebsiella* and *Escherichia* genera, and their phylogenetic tree is shown in Figure 1.

As shown in Table 1, the highest tannase activity was recorded for *E. coli* GHMGHE41 (10.46 U mL^{-1}) isolated from the goat rumen, and the lowest activity was obtained by *K. aerogenes* GHMGHE30 (8.59 U mL^{-1}) isolated from the ram rumen (RR; $p < 0.05$). On the other hand, TPSs used as silage additives must have acceptable viability, as *K. aerogenes* GHMGHE38 (CR) and *E. fergusonii* GHMGHE44 (DR) showed the strongest and weakest survival rate, respectively ($p < 0.05$).

Chemical characterization of the FBPs. There were significant differences between the chemical composition of PP, GP, LP, and OP. The OP showed the highest values in DM and ADF, whereas the lower amounts were observed for GP and PP, respectively ($p < 0.05$). The highest values of the ash, EE, NDF, and pH were seen for GP, but the lowest numbers were obtained for OP, OP, OP, and LP, respectively ($p < 0.05$). Compared to the LP, the minimum content of CP was recorded for PP ($p < 0.05$). In general, the DM content of fresh FBPs ranged from 301.01 to 224.75, ash from 204.85 to 103.25, CP from 153.44 to 82.49, EE from 51.677 to 15.981, NDF from 726.66 to 410.22, and ADF from 325.83 to 246.71 (Table 2).

Nutrient composition of FBPs after ensilaging. Ensilaging process affected the chemical components of the FBPs, and there was a significant interaction between the bacterial strains and FBPs for all components, except ash and EE ($p < 0.05$; Table 3). The PP treated with *K. aerogenes* GHMGHE38 isolated from the camel rumen strain (PPCR) had the highest DM, while GP treated with RR strain showed the lowest amount of DM ($p < 0.05$). For CP

content, the highest amount was observed for LP treated with CR strain, but the lowest amount was recorded for PPCR ($p < 0.05$). Also, no bacteria-LP (LPNB) silage had the maximum levels of NDF, ADF, and pH, while the minimum amounts were seen for PPCR, GP treated with CR strain (GPCR), and PPCR silages, respectively ($p < 0.05$).

Fermentation characteristics of the FBPs silage. According to the Table 4, the no bacteria-grape pulp (GPNB) silage had the higher pH during 12 and 24 hr of incubation ($p < 0.05$) than PPCR (during 12 hr) and PP treated with RR strain (PPRR; during 24 hr) silages. Furthermore, the LPNB (after 12 hr) and LP treated with GR strain (LPGR; after 24 hr) silages had the highest N-NH_3, while GPNB and PPCR silages showed the lowest amounts of N-NH_3 during 12 and 24 hr, respectively ($p < 0.05$).

***In vitro* digestibility of the FBPs silage.** Based on the Figure 2, the FBPs type and bacterial strain interactions affected the *in vitro* DM digestibility (IVDMD) in all the treatments ($p < 0.05$).

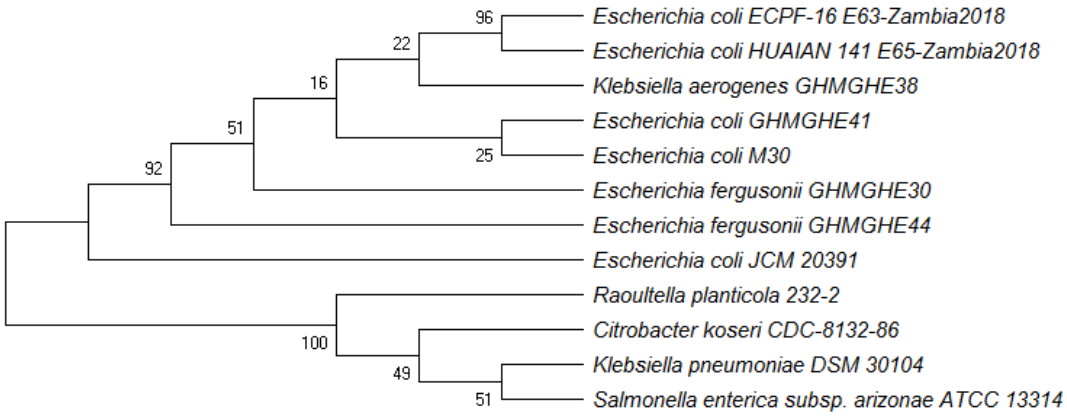

Fig. 1. Phylogenetic reconstruction of the tannase-producing strains isolated from the rumen of ram, deer, goat, and camel.[16-18] The symbols showed at nodes are fraction of the bootstrap values from 500 replications with values only above 50.00%. The isolates with GHMGHE suffix are the tannae-producing strains. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length of 0.17425494 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions *per* site. This analysis involved 12 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + non-coding. All positions containing gaps and missing data were eliminated (complete deletion option). There were 162 positions in the final dataset. Evolutionary analyses were conducted in MEGA Software (version X; Biodesign Institute, Tempe, USA).[23]

Table 1. Tannase assay and viability rate of tannase-producing strains (n = 3).

Animals	Isolates	Tannase activity (U mL^{-1})	Colony forming unit *per* mL	Accession No.
Goat	*Escherichia coli* GHMGHE41	10.46^{a}	8.97^{b}	891627.1
Deer	*Escherichia fergusonii* GHMGHE44	9.39^{ab}	8.91^{b}	891630.1
Ram	*Escherichia fergusonii* GHMGHE30	8.60^{b}	8.92^{b}	891616.1
Camel	*Klebsiella aerogenes* GHMGHE38	9.17^{b}	9.53^{a}	891624.1
Standard error of means		0.2534	0.1202	-
***p*-value**		0.0053*	0.0175*	-

* indicates significant different at the level of $p < 0.05$.

Table 2. The chemical composition (g kg^{-1}) of the fruit by-products before ensilage.

By-products	DM	Ash	CP	EE	NDF	ADF	pH
Pomegranate peel	230.65^{b}	156.56^{b}	82.49^{d}	38.33^{b}	441.49^{b}	246.71^{b}	4.00^{b}
Grape pulp	224.75^{b}	204.85^{a}	145.70^{b}	51.68^{a}	726.66^{a}	249.16^{b}	4.54^{a}
Lemon pulp	245.33^{b}	218.11^{a}	153.44^{a}	31.56^{c}	440.28^{b}	269.65^{b}	3.94^{b}
Orange pulp	301.01^{a}	103.25^{c}	108.91^{c}	15.98^{d}	410.22^{b}	325.83^{a}	4.24^{ab}
Standard error of means	7.7774	6.2592	0.6752	0.4471	10.8570	7.6505	0.0751
***p*-value**	0.0004	0.0001	0.0001	0.0001	0.0001	0.0003	0.0019

DM: Dry matter; CP: Crude protein; EE: ethereal extract; NDF: Neutral detergent fiber; and ADF: Acid detergent fiber.
^{abc} Different letters in the same line indicate statistical differences at $p < 0.05$.

Table 3. Nutrient composition (g kg^{-1}) of fruit by-products treated with tannase-producing bacteria after 30 days of ensiling (n = 4). Values reported are means of three ensilaged fruit by-products FBPs samples.

Effects			DM	Ash	CP	EE	NDF	ADF	pH
Main effects	Waste	PP	249.75^{a}	162.28^{b}	77.13^{d}	36.51^{b}	329.54^{c}	164.88^{c}	3.44^{c}
		GP	219.39^{b}	215.04^{a}	116.00^{b}	36.47^{b}	383.88^{b}	134.05^{d}	3.56^{b}
		LP	225.01^{b}	209.98^{a}	128.30^{a}	56.40^{a}	627.87^{a}	396.62^{a}	3.76^{a}
		OP	228.08^{b}	101.57^{c}	96.73^{c}	14.25^{c}	324.76^{c}	255.51^{b}	3.80^{a}
	Standard error of means		2.3798	2.3337	1.9578	36.5126	1.7295	2.9979	0.0120
	Additive	GR	228.13^{bc}	169.29	107.87	36.75	414.24^{b}	235.59^{b}	3.55^{b}
		DR	232.26^{b}	172.30	101.47	34.27	414.09^{b}	238.22^{b}	3.56^{b}
		RR	219.01^{cd}	173.28	106.45	36.63	406.52^{b}	232.75^{b}	3.54^{b}
		CR	256.61^{a}	172.81	105.76	36.32	407.77^{b}	229.07^{b}	3.52^{b}
		NB	216.77^{d}	173.41	101.16	35.57	439.93^{a}	253.19^{a}	4.03^{a}
	Standard error of means		2.6607	2.6092	2.1888	1.0479	1.9336	3.3518	0.0135
Interaction effects	**PP**	GR	235.05^{bcde}	160.51^{b}	91.00^{ef}	37.25^{b}	322.75^{g}	166.72^{de}	3.44^{fg}
	PP	DR	252.05^{b}	162.16^{b}	75.51^{fg}	34.79^{b}	322.05^{g}	172.08^{de}	3.39^{fg}
	PP	RR	238.63^{bcd}	165.98^{b}	77.92^{fg}	36.10^{b}	323.79^{g}	159.11^{def}	3.50^{ef}
	PP	CR	306.68^{a}	159.58^{b}	65.80^{g}	37.08^{b}	316.41^{g}	149.11^{def}	3.31^{g}
	PP	NB	216.35^{cde}	163.16^{b}	75.43^{fg}	37.34^{b}	362.68^{b}	177.36^{d}	3.56^{def}
	GP	GR	220.19^{cde}	216.05^{a}	113.71^{bcde}	36.75^{b}	394.23^{cd}	134.55^{ef}	3.48^{efg}
	GP	DR	207.56^{e}	223.32^{a}	108.72^{cde}	35.60^{b}	378.13^{def}	141.34^{def}	3.45^{fg}
	GP	RR	205.73^{e}	214.68^{a}	121.10^{abcd}	37.03^{b}	357.74^{f}	127.09^{f}	3.50^{ef}
	GP	CR	253.26^{b}	214.73^{a}	124.73^{abcd}	37.64^{b}	384.30^{cde}	122.81^{f}	3.53^{ef}
	GP	NB	210.19^{de}	206.43^{a}	111.74^{bcde}	35.33^{b}	404.99^{c}	144.46^{def}	3.83^{bc}
	LP	GR	241.95^{bc}	202.08^{a}	134.40^{ab}	58.62^{a}	619.87^{b}	390.35^{b}	3.49^{ef}
	LP	DR	228.26^{bcde}	209.50^{a}	121.45^{abcd}	51.91^{a}	627.17^{b}	398.24^{ab}	3.65^{cde}
	LP	RR	216.40^{cde}	214.18^{a}	118.30^{abcd}	59.89^{a}	618.59^{b}	383.58^{b}	3.41^{fg}
	LP	CR	221.48^{cde}	208.15^{a}	140.74^{a}	57.24^{a}	616.08^{b}	376.51^{b}	3.47^{efg}
	LP	NB	216.95^{cde}	216.00^{a}	126.61^{abc}	54.34^{a}	657.64^{a}	434.42^{a}	4.80^{a}
	OP	GR	215.32^{cde}	98.52^{c}	92.36^{ef}	14.37^{c}	320.13^{g}	250.77^{c}	3.87^{b}
	OP	DR	241.15^{bc}	94.22^{c}	100.19^{def}	14.77^{c}	329.01^{g}	241.23^{c}	3.79^{bc}
	OP	RR	215.29^{cde}	98.26^{c}	108.49^{cde}	13.50^{c}	325.97^{g}	261.21^{c}	3.73^{bcd}
	OP	CR	245.01^{bc}	108.79^{c}	91.74^{ef}	13.34^{c}	314.29^{g}	267.82^{c}	3.75^{bc}
	OP	NB	223.60^{bcde}	108.06^{c}	90.85^{ef}	15.28^{c}	334.40^{g}	256.53^{c}	3.90^{b}
	Standard error of means		5.3214	5.2184	4.3778	2.0958	3.8672	6.7036	0.0319
***p*-value**		Waste	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
		Bacteria	0.0001	0.7913	0.1439	0.4533	0.0001	0.0007	0.0001
		Interaction	0.0001	0.3136	0.0030	0.7376	0.0001	0.0061	0.0001

DM: Dry matter; CP: Crude protein; EE: ethereal extract; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; GR: *Escherichia coli* GHMGHE41 isolated from the goat rumen; DR: *Escherichia fergusonii* GHMGHE44 isolated from the deer rumen; RR: *E. fergusonii* GHMGHE30 isolated from the ram rumen; CR: *Klebsiella aerogenes* GHMGHE38 isolated from the camel rumen; NB: No bacteria; PP: Pomegranate peel; GP: Grape pulp; LP: Lemon pulp; OP: Orange pulp.

[a-g] Means within columns with different superscript letters have a significant difference at $p < 0.05$.

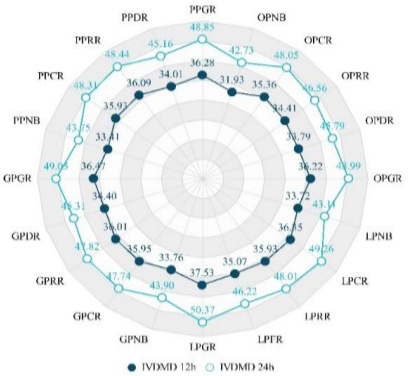

Fig. 2. *In vitro* dry matter digestibility (IVDMD) of the fruit by-products ensilaged with tannase additive after 30 days. GR: *Escherichia coli* GHMGHE41 isolated from the goat rumen; DR: *Escherichia fergusonii* GHMGHE44 isolated from the deer rumen; RR: *E. fergusonii* GHMGHE30 isolated from the ram rumen; CR: *Klebsiella aerogenes* GHMGHE38 isolated from the camel rumen; NB: No bacteria; PP: Pomegranate peel; GP: Grape pulp; LP: Lemon pulp; OP: Orange pulp.

Table 4. *In vitro* fermentation characteristics of fruits by-products treated with tannase-producing bacteria after 30 days of ensilaging. Values reported are means of three ensilaged fruit by-products samples.

Effects			pH		$N\text{-}NH_3$	
			12 hr	24 hr	12 hr	24 hr
Main effects	Waste	PP	7.46^{c}	7.38^{b}	6.43	7.10^{d}
		GP	7.57^{a}	7.48^{a}	6.70	8.22^{b}
		LP	7.47^{bc}	7.40^{b}	6.87	14.31^{a}
		OP	7.51^{b}	7.40^{b}	6.80	7.80^{c}
	Standard error of means		0.0117	0.0102	0.1606	0.0281
	Additive	GR	7.51^{ab}	7.43^{b}	6.90	9.51^{a}
		DR	7.48^{b}	7.36^{c}	6.42	9.48^{a}
		RR	7.50^{ab}	7.39^{bc}	6.66	9.56^{a}
		CR	7.47^{b}	7.41^{b}	6.93	9.17^{b}
		NB	7.54^{a}	7.48^{a}	6.59	9.07^{b}
	Standard error of means		0.0131	0.0114	0.1795	0.0314
Interaction effects	PP	GR	7.52^{abcd}	7.48^{abc}	6.05^{b}	7.16^{k}
	PP	DR	7.42^{def}	7.31^{ab}	6.49^{b}	7.47^{jk}
	PP	RR	7.45^{cdef}	7.29^{e}	6.70^{ab}	7.65^{hij}
	PP	CR	7.36^{f}	7.32^{de}	6.77^{ab}	6.82^{l}
	PP	NB	7.55^{abcd}	7.49^{ab}	6.15^{b}	6.40^{m}
	GP	GR	7.52^{abcd}	7.43^{bcd}	7.32^{ab}	8.28^{d}
	GP	DR	7.57^{abc}	7.49^{ab}	6.49^{b}	8.33^{d}
	GP	RR	7.57^{abc}	7.44^{bc}	6.73^{ab}	8.19^{de}
	GP	CR	7.55^{abcd}	7.48^{abc}	7.09^{b}	8.18^{de}
	GP	NB	7.64^{a}	7.57^{a}	5.87^{b}	8.11^{def}
	LP	GR	7.55^{abcd}	7.45^{bc}	6.78^{ab}	14.58^{a}
	LP	DR	7.45^{cdef}	7.32^{de}	6.07^{ab}	14.31^{abc}
	LP	RR	7.50^{bcde}	7.44^{bc}	6.42^{b}	14.46^{ab}
	LP	CR	7.37^{ef}	7.39^{bcde}	6.67^{ab}	14.18^{bc}
	LP	NB	7.48^{bcdef}	7.41^{bcde}	8.41^{a}	14.04^{c}
	OP	GR	7.47^{bcdef}	7.36^{cde}	7.45^{ab}	8.00^{defg}
	OP	DR	7.49^{bcdef}	7.32^{de}	6.62^{ab}	7.82^{fghi}
	OP	RR	7.50^{abcde}	7.37^{bcde}	6.81^{ab}	7.93^{efgh}
	OP	CR	7.59^{ab}	7.47^{abc}	7.20^{ab}	7.50^{ij}
	OP	NB	7.51^{abcd}	7.47^{abc}	5.94^{b}	7.74^{ghij}
	Standard error of means		0.0262	0.0228	0.3591	0.0628
p-value		Waste	0.0001	0.0001	0.2395	0.0001
		Bacteria	0.0016	0.0001	0.2318	0.0001
		Interaction	0.0001	0.0001	0.0005	0.0001

GR: *Escherichia coli* GHMGHE41 isolated from the goat rumen; DR: *Escherichia fergusonii* GHMGHE44 isolated from the deer rumen; RR: *E. fergusonii* GHMGHE30 isolated from the ram rumen; CR: *Klebsiella aerogenes* GHMGHE38 isolated from the camel rumen; NB: No bacteria; PP: Pomegranate peel; GP: Grape pulp; LP: Lemon pulp; OP: Orange pulp.

[a-m] Means within columns with different superscript letters have a significant difference at $p < 0.05$.

Discussion

The use of biocatalysts, enzymes, as a silage additive for animal feed has been considered in many tropical and subtropical countries. Due to the lack of feed, agricultural residues have been used in livestock feed, which contain some anti-nutritional compounds, such as tannins. Thus, the current study used the TPSs isolated from the rumen of deer, goat, camel, and ram to prepare tannase additive for ensilaging high-tannin FBPs. Herbivores, such as goats, deer, and antelopes, are among several browsing animal species that may degrade tannin by their digestive tract microbes.[16-18] Among them, goats due to the high walking power can travel to vast areas in search of forages (rich in tannin), while other animals are not capable for that.[24,25] Compared to goats, urial rams mainly graze on grass and feed small number of tannin-rich twigs and leaves. Hence, the higher tannase activity observed by *E. coli* can be due to the diet of its host, goat. Since microbial tannase potential can be changed based on source of isolation, diet, host animal, enzyme assay methods, and culture media composition, it needs to be validated.[16] In this study, the identified strains and genera were in consistent with previous studies that explored the TPSs, like *Streptococcus bovis* in the rumen of sika deer,[26] *Klebsiella pneumonia* and *Acinetobacter baumannii* in the rumen of fallow deer,[27] and *Streptococcus pneumonia*, *S. bovis*,[28] *Klebsiella, Pseudomonas, Bacillus, Escherichia*, *Enterobacter*,[29] and *Streptococcus gallolyticus* in the gout rumen.[30] Also, a bacterial additive must be able to survive in silage

conditions and maintain its activity. In the current study, *K. aerogenes* GHMGHE38 had the best survival rate that may result from its peritrichous flagella. Also, *K. aerogenes* produces diverse metabolites that can increase its survival rate and growth;[31] however, non-motile genera have relatively higher survival rates.[32] Meanwhile, the lower survival of *Escherichia* strains may be due to the structural differences in their cell wall.[33]

After determining the chemical properties, the lowest content of CP was measured for PP, which was probably due to its storage in a high temperature environment. In a study, GP contained 42.60% DM, 9.80% CP, 5.10% EE, 6.90% ash, 67.40% NDF, and 35.10% ADF.[34] Also, analysis of proximate composition of red grape pomace revealed the values of 33.50%, 9.50%, and 25.00% for DM, CP, and NDF, respectively.[35] It has been reported that persimmon skin has 25.10% DM, 4.50% CP, and 22.50% NDF.[36] The DM, CP, NDF, and ADF of sweet potato vines were also found to be 25.60, 15.20%, 57.20, and 34.60%, respectively.[37] National Research Council has recorded 9.00 - 12.00% CP and 5.00 - 7.00% EE for grape pomace.[38] The variation in the FBPs chemical composition depends on the different factors, such as climatic conditions, region, culture, species, harvesting methods and period, plant maturity stage, storage environment, production methods, and components proportions (seeds and pulp) of FBPs.[10] For this reason, it is very difficult to compare the results of different studies. Some authors have stated that the DM of FBPs should be between 250.00 and 400.00 g kg^{-1} to avoid high nutrient losses.[34] Thus, the FBPs used in present experiment had a suitable DM content for ensiling. In this study, the pH of FBPs was varied between 3.90 to 4.50, which can be from the volume of organic acids in each FBP. By others the pH of grape pomace, wild grape pomace, and persimmon skin was reported to be 3.86, 3.55, and 6.04, respectively.[34,36,37] In a study, the pH measured for sweet potato vines was also 5.63.[39] Another study has reported that the pH of GP is equal to 4.82.[10] In the present study, nutrient composition of FBPs was improved after ensilaging. The highest DM was recorded for PPCR, which is may be resulted from the type of substrate. Since CR strain was isolated on tannic acid plate agar, a hydrolysable tannin, it showed better tannase activity in PP silage with high level of hydrolysable tannin. While, the lowest DM was observed in GP treated with RR strain, which is probably due to the high levels of dense lignin and tannin in GP and the inability of RR isolate to degrade them.[40] In this study, the highest and lowest CPs were measured respectively for LP treated with CR strain and PPCR silages, which can be caused by the amount of CP in fresh LP and PP wastes. Also, the maximum values of NDF, ADF, and pH were observed in LPNB silage, and the minimum amounts for PPCR, GPCR, and PPCR silages, respectively. Here, the lack of inoculation of TPSs in LPNB silage can be the cause of high levels of pH, NDF, and ADF. However, CR strain was able to reduce all three factors in the PPCR and GPCR silages due to the successful cellulase secretion. Thus, its cellulase activity was previously confirmed.[17] The decreases in NDF and ADF content could have occurred under the influence of bacterial enzyme saccharification and release of fermentable sugars for lactic acid fermenters. Anaerobic silage of organic materials, such as lignocellulose, facilitates their fermentation and increases fermentation energy.[40] Acceptable silage quality is usually confirmed by a low pH value of 3.70 - 4.30,[41] as previously a pH value of 4.20 has been defined as an upper threshold for a positive evaluation of a silage.[10] In the current study, the decrease in pH in PPCR silage may have associated with the favorable fermentation of tannins and increase of organic acids, such as gallic acid. Also, low pH may be linked with a synergistic effect between rumen fluid microbes and TPSs. It has been demonstrated that acids produced during the ensiling process can quickly reduce the pH, inhibit harmful microbial growth, and preserve the forage nutrients. To optimize fermentation, it is essential to use additives in the ensiling process. As previously reported, tannin-degrading microorganisms are important contributors to the fermentation process, because they use the tannin content to produce energy, releasing tannin from binding with protein and increasing the protein availability in the silage.[22] Also, the most common parameters to evaluate the quality of silage fermentation are *in vitro* digestibility, pH, and N-NH_3 contents. The current study reported that the GPNB silage had higher pH than PPCR (12 hr) and PPRR (24 hr) silages. It is likely due to the high ash and NDF contents of the GP waste that could not be easily degraded by rumen fluid microbes, leading to higher pH. On the other hand, this finding may be due to the buffering effect of GP phenolic compounds and absence of TPSs. Moreover, the low pH of PPCR and PPRR silages may be resulted from the effective tannase activity of CR and RR strains. Correspondingly, these isolates have been screened using tannic acid substrate, a hydrolysable tannin that PP is rich for that.[42] Compared to the LPNB (12 hr) and LPGR (24 hr), GPNB and PPCR silages showed the lowest amounts of N-NH_3 during 12 and 24 hr. This observation probably resulted from the high amounts of acids in the LP waste, that rapidly declined pH, suppressed undesirable fermentation, and conserved more nutrient substrate.[43] Reportedly, the chemical composition and physiological properties of epiphytic bacteria of fruit waste are the most effective factors affecting silage quality.[44] Others have suggested that citrus flavonoids possess potential synergistic effects on mitigating ruminal CH_4 emissions by cows and improving nitrogen utilization.[45] After reviewing, it was found that fruits peel could improve nutrient digestibility, rumen pH, and N-NH_3 concentrations. In a study, the white dragon fruit peel, a good source of tannins and

saponins, improved nutrient digestibility, pH, and N-NH_3 by decreasing protozoa and methane production both *in vitro* and *in vivo*.[1] Also, the utilization of dragon fruit peel powder at the level of 10.00% improved pH and N-NH_3 levels in goats.[46] It has been indicated that *in situ* microbial inoculation of avocado peel silage increases fermentation substrate, but pH of the silage is not affected by inoculation.[47] It was observed that inoculated avocado peel silage had low fiber compared to the control and its degradation was enhanced.[47] It has also been clarified that the tannase additive has the potential to rapidly fall the pH of silage, stop the harmful microbial activity, reduce the CP degradability, and ultimately decline the nutrient loss.[44] The IVDMD is an important factor to estimate energy of ruminant feed, as those physical properties of ensiling substrates have a significant role in digestibility efficiency of silages. This study indicated that the FBPs type and bacterial strain interactions affected the IVDMD in all the treatments, as the best and least digestibilities were observed in LPGR and no bacteria-OP silages, respectively. These findings may be attributable to the high ADF content of the OP waste, in other words, its high lignin, and lack of tannase additive. The high lignin feed had the low digestibility coefficient.[47] Based on the finding, the FBPs, such as PP, OP, LP, and GP can be effectively used as silage for supplying animal feed shortage. The use of different tannase silage additives, including *E. coli* GHMGHE41, *E. fergusonii* GHMGHE44, *E. fergusonii* GHMGHE30, and *K. aerogenes* GHMGHE38 isolated from the rumen liquor could improve the chemical composition of tropical FPBs silages. Among additives, CR strain especially had enhanced CP amount, and reduced NDF and ADF contents. Also, the *in vitro* rumen digestibility of FBPs silages was greater by 50.37% *via* LP silage inoculated by GR strain compared to the uninoculated OP silage (42.73%) by GR strain addition, *E. coli* GHMGHE41.

In conclusion, administration of bacterial tannase enzyme during FBPs ensiling allows to effectively feed fermentation, and these silages can represent an important alternative feed containing digestible nutrient for ruminants. However, further advanced *in vitro* techniques and research demonstrating the *in vivo* utilization are needed to gather more data.

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Conflict of interest

The authors declare no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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