In vitro Rumen Fermentation and Gas Production: Influence of Different by-product Feedstuffs

M. H. Delavar¹*, A. M. Tahmasbi¹, M. Danesh-Mesgaran¹ and R. Valizadeh¹

¹Department of Animal Science, Ferdowsi University of Mashhad, P O Box 91775-1163, Mashhad, Iran.

ABSTRACT

Aims: To determine the chemical composition and estimation of nutritive value of different by-product feedstuffs (BPF) using in vitro gas production technique.

Place and Duration of Study: Department of Animal Science, between February 2013 and June 2013.

Methodology: In an anaerobic batch culture system, 50 ml of buffered rumen fluid was dispensed into a 125-ml serum bottle containing 0.2 g dry matter (DM) of the experimental treatments. Experimental treatments included five by-products (pomegranate peel and seed, apple pomace, walnut hull, almond hull). All bottles were purged with anaerobic CO₂, sealed and placed in a shaking water bath for 96 h at 38.6°C. Gas production of each bottle was recorded at 3, 6, 9, 12, 24, 36, 48, 72 and 96h of the incubation and then gas released. The batch cultures were repeated in three incubation runs. The biomass residues were centrifuged and the pellet was dried at 65°C for the determination of the residual DM and in vitro DM disappearance (IVDMD).

Results: The total tannins and phenol content were higher (P<0.01) in almond hull and pomegranate peel than in the other BPF. The total tannin content ranged from 0.34% in apple pomace to 9.78% in almond hull. The total phenol of pomegranate peel, pomegranate seed, apple pomace, walnut hull and almond hull were 10.9, 1.20, 0.76, 3.80, 3.71 and 3.68 mg g⁻¹ DM, respectively.

*Corresponding author: Email: mh_delavar@yahoo.com;
and 10.6%, respectively. The rate (c) and cumulative gas volume (b) was significantly higher (P<0.01) for apple pomace than the other feedstuffs. There were significant differences (P<0.01) among feedstuffs about lag time. Apple pomace showed higher (P<0.01) organic matter digestibility (OMD), in vitro dry matter digestibility (IVDMD), metabolizable energy (ME), short chain fatty acid (SCFA) and lower (P<0.01) pH than the other feedstuffs.

**Conclusion:** The higher values obtained for the potential gas production in apple pomace will indicate a better nutrient availability for rumen microorganisms (P<0.01).

**Keywords:** Almond hull; apple pomace; pomegranate peel; pomegranate seed; walnut hull.

### 1. INTRODUCTION

Most by-product feedstuffs (BPF) result from the processing of commercial crops, the food processing industry, and the fiber industry [1]. Increased disposal costs in many parts of the world lead to increase interest in BPF as alternative feeds for ruminants [2]. By-products feedstuffs utilization as animal feed not only lowers feed shortage in the area but also reduce the risk of environmental pollution [2,3]. While some by-products have been successfully fed for decades (e.g., corn distiller's grains or sugar beet pulp), other by-products (e.g., apple pomace, pomegranate hull, almond hull and walnut hull) are not consistently used because of the uncertainty regarding availability, poor handling characteristics, storage properties, palatability or intake [1]. Increasing agricultural industrial units for producing pomegranate juice leads to the accumulation of pomegranate peel and the annual production of this by-product approximately 120,000 metric tons in Iran [3]. Pomegranate fruit is consists of three parts: the seeds, the juice and the peels which include the husk and interior network membranes [4]. Apple pomace is the main by-product resulting from apple juice processing and containing peel, seeds and solid parts [5]. Apple pomace is a rich source of pectin besides other nutrients like carbohydrates, dietary fibres, minerals and vitamin C [5]. Almond hull is the by-product obtained by drying that portion of the almond fruit that surrounds the hard shell [6]. Low moisture content makes almond hull attractive for livestock feed by reducing transportation costs and allowing for long-term storage [6]. Walnut processing yields several by-products (walnut meal and hull). A fibrous product called walnut hull possibly a mixture of kernel particles and ground shells [7]. There is a very little information available regarding the nutritive value of pomegranate peel, pomegranate seed, apple pomace, almond hull and walnut hull produced in Iran. The aim of this study was to determine the chemical composition including tannin content of BPF and gas production characteristics using in vitro gas production technique.

### 2. MATERIALS AND METHODS

#### 2.1. Samples Collection and Treatments

Fresh BPF (pomegranate peel, pomegranate seed, apple pomace, walnut hull, almond hull) samples were collected from the agro-industrial unites in Khorasan-Razavi province, Iran. Air-dry samples were ground in a Wiley mill to pass a 1-mm screen. Chemical analysis and in vitro gas production (GP) evaluated at the laboratories of Department of Animal Science, Ferdowsi University of Mashhad, Iran. The by-product samples were analyzed for dry matter (DM), organic matter (OM), crude protein (CP), ether extracts (EE) and ash by standard procedures [8]. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were
determined according to Van Soest et al. [9]. The total phenolics (TP) content was determined according to the Folin-Ciocalteu assay and total tannins were determined by methods previously described [10]. Tannic acid (Merck GmbH, Darmstadt, Germany) was used as the standard to express the amount of total phenols and total tannins.

Rumen fluid was collected manually before the morning feeding from three adult ruminally-fistulated sheep (49.5 ± 2.5 kg, body weight) and was maintained at 39°C until strained through 3 layers of cheesecloth. Animals were fed 0.6 kg of alfalfa hay and 0.4 kg of concentrate (24% corn grain, 20.4 barley grains, 27% soybean meal, 13.8% canola meal, 13.8% wheat bran, 0.3% calcium carbonate, 0.5% mineral and vitamin premix, and 0.2% salt) mixture. The filtrated rumen fluids from the three animals were mixed in equal proportion and held under CO₂ in a water-bath at 39°C prior to in vitro inoculation. Procedure of in vitro batch culture was performed according to the Menke and Steingass [11]. In an anaerobic condition, 50 ml of buffered rumen fluid was dispensed with pipetor pump into a 125-ml serum bottle containing 0.2 g DM of the experimental treatments. Treatments were: 1) pomegranate peel, 2) pomegranate seed, 3) apple pomace, 4) walnut hull, and 5) almond hull. For each treatment, 200 mg of the sample was placed in a bottle (5 bottles per treatment) each filled with 50 ml of buffered rumen fluid [ratio of buffer to rumen fluid was 2:1]. Bottles were gently shaken after each recording.

All bottles were purged with anaerobic CO₂ for 5 second, sealed with rubber stoppers and aluminum caps and placed in a shaking water bath for 96 h at 39°C. To prevent accumulation of gas produced, head space gas pressure of each bottle was recorded using a pressure transducer [12] at 3, 6, 9, 12, 24, 36, 48, 72 and 96 h of the incubation and then gas released. The batch cultures were repeated in three incubation runs. Gas pressure was measured manually by using a digital pressure gauge (model SEDPGB-0015-PG5 sensor unit, SenSym, Milpitas, Calif). After 96 h incubation, the bottles were respectively transferred to refrigerator to stop fermentation, and then opened. Total in vitro gas produced was corrected to blank incubations which contained only rumen fluid. In addition to in vitro GP, the bottles were transferred to an ice bath to stop fermentation after 24 h of the incubation, and then opened to measure medium pH using a pH meter (Metrhom pH meter, Model 691). The filtrated (42 µm pore size) residual was oven dried (60°C for 48 h) and used to calculate in vitro dry matter disappearances (IVDMD).

### 2.2. Calculations and Statistical Procedure

Gas pressure was converted into volume using an experimentally calibrated curve. Data of cumulative GP data were fitted to the exponential equation $Y= B (1- e^{- Ct})$, where B is the GP from the fermentable fraction (mL), the GP rate constant C (mL/h), t the incubation time (h) and Y is the gas produced at time t. In vitro DM disappearance (IVDMD) was calculated as the difference between initially incubated DM and residual DM, corrected by blanks. Metabolizable energy (MJ/kg) content and feed OM digestibility (g/kg⁻¹ OM) were estimated using equations [11], given below:

$$\text{Metabolizable energy (MJ/kg⁻¹ DM)} = 2.20 + 0.136\text{GP} + 0.057\text{CP} + 0.0029\text{CP}^2$$

$$\text{Organic matter digestibility (OMD) (\%)} = 14.88 + 0.889\text{GP} + 0.45\text{CP} + 0.065 \text{ ash}$$

Where, GP is 96 h net gas production (mL/ 200 mg of incubated DM), CP is crude protein (%). Short chain fatty acids (SCFA) (mmol/ 200 mg of incubated DM) were calculated according to the equation from Getachew et al. [13].
SCFA (mmol/200 mg of incubated DM) = 0.0222 GP - 0.00425

Where, GP - 96 h net gas production (ml/200 mg of incubated DM). Data were statistically analyzed using GLM procedure of SAS [14] with flowing statistically model; 
\[ y = \mu + T_i + e_{ij} \]
where \( y \) = depended variable, \( \mu \) = overall mean, \( T_i \) = effect of BPF and \( e_{ij} \) = residual error. Significant means were compared using the Duncan’s multiple range tests. Mean differences were considered significant at \( P<0.05 \).

3. RESULTS AND DISCUSSIONS

The chemical composition of the BPF is presented in Table 1. The chemical composition of a given BPF varied with source of the feedstuffs in the current study (Table 1). The CP content ranged from 4.80 % in pomegranate peel to 11.2% in pomegranate seed. The CP content was higher (\( P<0.01 \)) in pomegranate seed than in the other BPF. Chemical compositions of pomegranate seeds in the current study were inconsistent with findings of Taher-Maddah et al., [15] and Mirzaei-Aghsaghali et al., [3]. Feizi et al., [16] reported that DM, OM, CP, crude fiber, and EE values of pomegranate seeds were 94.8, 96.8, 11.4, 38.9, and 1.0%, respectively. These differences in chemical composition of by-products may be due to a difference in cultivar, growing conditions, varieties, and different de-hulling process methods [15]. The CP concentration of the pomegranate peel, pomegranate seed, walnut and almond hulls, used in this experiment would be inadequate for the requirements of maintenance and production of ruminants. Thus, it is recommended that treating these BPF with urea as a cheap source of nitrogen would reduce the need to provide supplementary ruminal CP for these classes of livestock. The NDF and ADF contents were higher (\( P<0.01 \)) in pomegranate seed than in the other BPF. The TT of pomegranate peel, pomegranate seed, apple pomace, walnut hull and almond hull were 9.73, 0.66, 0.34, 2.34, and 9.78%, respectively. The total phenol content ranged from 0.76% in apple pomace to 10.9% in pomegranate peel. The total tannins and phenol content were higher (\( P<0.01 \)) in almond hull and pomegranate peel than in the other BPF. This result is in agreement with findings of Taher-Maddah et al [15]. Kamalak et al. [17] reported that total and soluble condensed tannins, NDF and ADF were negatively correlated with estimated parameters of gas production. The results in our study are consistent with those of Feizi et al. [16] who obtained that pomegranate peel tannins have negative effect on in vitro rumen fermentation.

Table 1. Chemical composition of by-product feedstuffs

| Item      | Treatment          | S.E.M | P-value |
|-----------|--------------------|-------|---------|
|           | Pomegranate peel   |       |         |
| DM        | 93.4\(^c\)         |       | 0.13    |
| CP        | 4.80\(^d\)         |       | 0.01    |
| Ash       | 6.40\(^b\)         |       | 0.01    |
| ADF       | 21.2\(^a\)         |       | 0.01    |
| NDF       | 31.6\(^d\)         |       | 0.01    |
| TP        | 10.9\(^a\)         |       | 0.01    |
| SP        | 1.17\(^d\)         |       | 0.01    |
| TT        | 9.73\(^b\)         |       | 0.01    |
|           | Pomegranate seed   |       |         |
| DM        | 94.6\(^c\)         |       |         |
| CP        | 11.2\(^a\)         |       |         |
| Ash       | 11.2\(^a\)         |       |         |
| ADF       | 46.0\(^a\)         |       |         |
| NDF       | 64.0\(^a\)         |       |         |
| TP        | 1.20\(^a\)         |       |         |
| SP        | 0.56\(^d\)         |       |         |
| TT        | 0.66\(^d\)         |       |         |
|           | Apple pomace       |       |         |
| DM        | 95.2\(^a\)         |       |         |
| CP        | 6.80\(^b\)         |       |         |
| Ash       | 6.80\(^b\)         |       |         |
| ADF       | 36.4\(^a\)         |       |         |
| NDF       | 48.0\(^a\)         |       |         |
| TP        | 0.76\(^d\)         |       |         |
| SP        | 0.42\(^d\)         |       |         |
| TT        | 0.34\(^d\)         |       |         |
|           | Walnut hull        |       |         |
| DM        | 95.1\(^a\)         |       |         |
| CP        | 5.40\(^c\)         |       |         |
| Ash       | 5.40\(^c\)         |       |         |
| ADF       | 24.4\(^a\)         |       |         |
| NDF       | 48.5\(^c\)         |       |         |
| TP        | 3.80\(^b\)         |       |         |
| SP        | 1.50\(^a\)         |       |         |
| TT        | 2.30\(^b\)         |       |         |
|           | Almond hull        |       |         |
| DM        | 94.5\(^a\)         |       |         |
| CP        | 6.40\(^b\)         |       |         |
| Ash       | 6.40\(^b\)         |       |         |
| ADF       | 27.6\(^c\)         |       |         |
| NDF       | 58.6\(^b\)         |       |         |
| TP        | 10.6\(^a\)         |       |         |
| SP        | 0.82\(^c\)         |       |         |
| TT        | 9.78\(^a\)         |       |         |

DM= Dry matter; CP= Crude protein; EE= Ether extract; ADF= Acid detergent fiber; NDF= Neutral detergent fiber; TP= Total phenol; SP= Simple phenol; TT= Total tannins.
Gas production volumes (ml/200mg DM) from in vitro incubation of different BPF samples at different incubation times are shown in Table 2 and Fig. 1. The cumulative volume of gas production increased with increasing time of incubation. Ranges of GP characteristics reported in this study may partly due to difference in CP, NDF and ADF contents. The rate and cumulative gas volume at each sampling time was affected by different BPF (Table 2). These finding indicate that fraction of substrate and degradability of BPF are different. The gas produced is directly proportional to the rate at which substrate degraded [18]. The maximum gas volume (b) was highest for apple pomace (P<0.01), greater for pomegranate seed (P<0.01) than for almond hull or walnut hull and lowest for pomegranate peel. The reason of more GP volume (b) in apple pomace may be caused by present of high level of pectin and non-structural carbohydrates [1]. Maheri-Sis et al. [19] reported that variation in chemical components (i.e., starch, soluble sugars, non-fiber carbohydrate, OM, CP, and NDF) of same feeds in different studies can be result in variation of in vitro gas production volume.

Table 2. Gas production characteristics and estimated parameters of different by-product feedstuffs

| Item               | Treatments       | S.E.M | P-value |
|--------------------|------------------|-------|---------|
|                    | Pomegranate seed |       |         |
| B                  | 64.37±           |       |         |
| C                  | 0.034±           |       |         |
| Lag time, h        | 0.76±            |       |         |
| ME                 | 8.27±            |       |         |
| OMD                | 43.24±           |       |         |
| SCFA               | 0.88±            |       |         |
|                    | Pomegranate hull |       |         |
| B                  | 48.59±           |       |         |
| C                  | 0.071±           |       |         |
| Lag time, h        | 0.47±            |       |         |
| ME                 | 8.93±            |       |         |
| OMD                | 59.74±           |       |         |
| SCFA               | 1.05±            |       |         |
|                    | Apple pomace     |       |         |
| B                  | 83.17±           |       |         |
| C                  | 0.073±           |       |         |
| Lag time, h        | 0.38±            |       |         |
| ME                 | 11.39±           |       |         |
| OMD                | 70.40±           |       |         |
| SCFA               | 2.25±            |       |         |
|                    | Walnut hull      |       |         |
| B                  | 58.66±           |       |         |
| C                  | 0.046±           |       |         |
| Lag time, h        | 0.62±            |       |         |
| ME                 | 7.81±            |       |         |
| OMD                | 51.63±           |       |         |
| SCFA               | 0.86±            |       |         |
|                    | Almond hull      |       |         |
| B                  | 61.86±           |       |         |
| C                  | 0.062±           |       |         |
| Lag time, h        | 0.56±            |       |         |
| ME                 | 9.53±            |       |         |
| OMD                | 64.78±           |       |         |
| SCFA               | 1.82±            |       |         |

B= the gas production from the fermentable fraction (mL g⁻¹ DM), C= Rate constant of gas production during incubation (ml/h⁻¹); ME= Metabolizable energy (MJ/kg⁻¹ DM); OMD= Organic matter digestibility (%DM); SCFA= Short chain fatty acid (mmol/L). Within rows, means with different letters are significantly different (P < 0.05). S.E.M= standard error of the mean.

Fig. 1. Pattern of in vitro gas production (fitted with exponential model) affected by different by-product feedstuffs at different incubation times.
The rate of GP (c) was highest for apple pomace (P<0.01), followed by pomegranate peel, almond hull or walnut hull (P<0.01), and lowest for pomegranate seed (P<0.01). High rate of GP possibly affected by carbohydrate fractions which readily available to the microbial population. There were significant differences among the experimental feedstuff about the lag time. Lag time ranged from 0.76 in pomegranate seed to 0.38 in Apple pomace. Lag time probably is not affected by the time required for bacterial attachment to new substrate per se, which occurs very rapidly; lag time probably is related more to proper digestion of feed and subsequent, long-term colonization by microbes. Ude’n reported that in vitro lag time generally decreased as particle size of forages decreased. The extent of digestion in vitro may be decreased by addition of concentrate or by low pH. However, increasing buffer strength to buffer VFA production can increase lag time, probably because of high osmolality (20). So, Ranges of lag time reported in this study may partly due to difference in chemical composition of feedstuff.

The values for the ME and OMD ranged from 7.81 in walnut hull to 11.39 (MJ/kg-1 DM) in apple pomace and from 43.24 in pomegranate peel to 70.40 (%DM) in apple pomace, respectively. Menke and Steingass [11] suggested that gas volume at 24 h after incubation has been relationship with ME in feedstuffs. In the current study, low ME content of pomegranate peel and walnut hull can be resulted from its low rate of gas production and extent of gas production. The SCFA was highest for apple pomace (2.25 mmol/L) and lowest for walnut hull (0.86 mmol/L) in this study. Short chain fatty acids (i.e., acetic, propionic, butyric, and valeric) are produced during rumen fermentation and supply up to 80% of their maintenance energy requirements [3]. There is a highly significant correlation between SCFA and gas production [13,21] and this correlation use to estimate the SCFA from gas values, which is an indicator of energy availability to the animal. Thus, it can be concluded that, higher SCFA production in gas production technique is the reliable index of gas production and energy content of tested materials [15].

The values of IVDMD and pH of different BPF are presented in Table 3. The IVDMD was varied in five BPF particularly high in apple pomace and pomegranate seed had significantly lower (P<0.01) values of IVDMD (Table 3). The higher (P<0.01) IVDMD observed in this study may be due to the low level of tannin in apple pomace which suggest that it could be a valuable protein supplement in ruminant diets [22].

**Table 3. In vitro dry matter disappearance (IVDMD) and pH of different by-product feedstuffs using batch culture**

| Item     | Treatments          | S.E.M | P-value |
|----------|---------------------|-------|---------|
|          | Pomegranate seed    |       |         |
| IVDMD    | 39.92^a             |       | 0.62    | 0.01    |
| pH       | 6.69^a              |       |         |
|          | Pomegranate peel    |       |         |
| IVDMD    | 59.24^c             |       |         |
| pH       | 6.48^c              |       |         |
|          | Apple pomace        |       |         |
| IVDMD    | 74.88^a             |       |         |
| pH       | 6.35^a              |       |         |
|          | Walnut hull         |       |         |
| IVDMD    | 52.84^d             |       |         |
| pH       | 6.58^d              |       |         |
|          | Almond hull         |       |         |
| IVDMD    | 70.28^e             |       |         |
| pH       | 6.44^e              |       |         |

Within rows, means with different letters are significantly different (P < 0.05). S.E.M= standard error of the mean.

The presence of tannins and phenolic compounds affected the nutritive value of these BPF to a different degree. Pomegranate seed and apple pomace contained traces of tannins (<1%), almond hull contained medium levels of tannins (>1%) and pomegranate peel and walnut hull contained high levels of tannins (>2%). This result is consistent with the findings of Seresinhe and Iben [23] and Ammar et al. [24], indicate that NDF and ADF were significant and negatively correlated with in vitro digestibility. In the current study, pH was highest for pomegranate seed (6.69) and lowest for apple pomace (6.35) and the lower
(P<0.01) pH observed in apple pomace may be due to its high NSC content. Bae et al., [25] reported that apple pomace contains abundant NSC and energy.

4. CONCLUSION

Chemical composition and in vitro digestibility can be considered useful indicators for the preliminary evaluation of the likely nutritive value of previously uninvestigated BPF. The results of current study based on chemical composition, IVDMD, ME, OMD and SCFA indicated that by-products can be used as replacement feedstuffs in diet for ruminants. It can be said that apple pomace has a potentially relative nutritive value in ruminants under in vitro conditions. However, there is a need for in vivo studies to support the in vitro findings.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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