

PRELIMINARY EVALUATION OF FENUGREEK (*TRIGONELLA FOENUM-GRAECUM*) SEED GUM AS A POTENTIAL PREBIOTIC FOR GROWING RABBITS IN TUNISIA: EFFECTS ON *IN VIVO* FAECAL DIGESTIBILITY AND *IN VITRO* FERMENTATION

ZEMZMI J. ^{*,†}, RÓDENAS L. [†], BLAS E. [†], ABDOULI H. ^{*}, NAJAR T. [‡], PASCUAL J.J. [†]

^{*}Laboratory of Improvement & Integrated Development of Animal Productivity & Food Resources, Higher School of Agriculture of Mateur, University of Carthage, Route de Tabarka, 7030, MATEUR, Tunisia Republic.

[†]Institute for Animal Science and Technology, Universitat Politècnica de València, Camino de Vera, s/n, 46022, VALENCIA, Spain.

[‡]National Agronomic Institute of Tunisia, University of Carthage, Avenue de la République BP 77-1054, AMILCAR, Tunisia Republic.

Abstract: This study aims to determine the effect of dietary inclusion of fenugreek seed gum (FSG), rich in galactomannans, on nutrient apparent digestibility and caecal environment, as well as on *in vitro* caecal fermentation of Tunisian growing rabbits. Three experimental diets were formulated, including 0, 0.25 and 0.5% of FSG (FSG0, FSG0.25 and FSG0.5, respectively) for the *in vivo* trial and 0, 0.125, 0.25, 0.5 and 100% of FSG (FSG0, FSG0.125, FSG0.25, FSG0.5 and FSG100, respectively) for the *in vitro* trial. In the *in vivo* trial, 45 weaned rabbits 31 d old (15 per treatment) were housed in individual cages until 94 d of age. Apparent digestibility coefficients were determined at two ages, from 38 to 41 and from 56 to 59 d old, and caecal traits were recorded after slaughtering. In the *in vitro* trial, the five experimental diets were incubated with a rabbit caecal inoculum. Gas production was measured and modelled until 72 h and the fermentation traits were measured. Apparent faecal digestibility coefficients of main nutrients and main caecal environment traits were not significantly affected by the dietary inclusion of FSG ($P > 0.05$). However, animals fed with FSG showed lower caecal pH (-0.15 ; $P < 0.05$) values. Regarding the *in vitro* fermentation, FSG100 increased asymptotic gas production ($+11.25$, $P < 0.001$), sharpness of the switching characteristic of the profile ($+1.98$, $P < 0.001$) and the maximum substrate degradation rate (RM) ($+0.188$, $P < 0.001$), but decreasing the time after incubation at which half of the asymptotic amount of gas has been formed (-5.86 , $P < 0.001$) and at which RM occurs (-4.53 , $P < 0.01$). Likewise, FSG100 significantly decreased caecal pH (-1.035 , $P < 0.001$), lactic acid (-9.51 , $P < 0.069$) and $N-NH_3$ concentrations (-12.81 , $P < 0.001$). Meanwhile, it increased the total volatile fatty acids (VFA) production ($+43.15$, $P < 0.001$). Gradual dietary inclusion of FSG from 0 to 0.5% only significantly increased total VFA production in the caecum ($+100$ mmol/L per percentage point of FSG inclusion; $P < 0.05$). In conclusion, FSG is highly and rapidly *in vitro* fermented by rabbit caecal bacteria. However, dietary inclusion of FSG up to 0.5%, might be insufficient to affect the apparent digestibility and fermentation profile of growing rabbits to a great extent.

Key Words: rabbit, *in vivo*, *in vitro*, fenugreek seed gum, faecal digestibility, caecal fermentation.

INTRODUCTION

One of the main limitations and concerns in rabbit farming is the frequency of digestive disorders, especially after weaning. Rosell *et al.* (2009) reported that 54.1% of the urgent visits to commercial rabbit farms in Spain and Portugal during 1997 to 2007 were due to digestive tract diseases. Under these circumstances, increasing the dietary soluble fibre content has shown to be an effective strategy to improve the integrity of the intestinal mucosa and modulate the intestinal microbiota of rabbits (Gómez-Conde *et al.*, 2007, 2009; Trocino *et al.*, 2013a). Thus, the inclusion of 20 to

Correspondence: J. Zemzmi, zemji@doctor.upv.es. Received January 2020 - Accepted June 2020.
<https://doi.org/10.4995/wrs.2020.12994>

30% of beet pulp (rich in soluble fibre) promotes caecal fermentation, promoting butyrate proportion, which is usually related to a reduced mortality associated with digestive disorders (Martínez-Vallespín *et al.*, 2011, 2013).

An alternative to a high inclusion of soluble fibre-rich raw materials, which also increases other fibrous fractions or could increase the amount of protein linked to fibre, is the direct inclusion of a small amount of an isolated soluble fibre so that, acting as a prebiotic, it could allow us to obtain these same effects (Falcão-e-Cunha *et al.*, 2007). To this end, some works (Bónai *et al.*, 2010; Volek and Marounek, 2011) have evaluated the dietary inclusion of inulin-type fructans from chicory extracts, which seems to affect caecal fermentative activity in growing rabbits, but without relevant positive effects on the growing performance and digestive health of the animals. In other works, the inclusion of mannan-oligosaccharides (Bovera *et al.*, 2010) afforded a reduction in rabbit mortality and an improvement in growth performance, whereas supplementation with gluco-oligosaccharides (Gidenne, 1995) had no effect on the caecal fermentation pattern and even a negative effect on morbidity and mortality. Lack of consistency in the results obtained with prebiotics could be explained by differences in the trial conditions, the nature of the prebiotic used or the amount of prebiotic added to feed (Falcão-e-Cunha *et al.*, 2007).

Trigonella foenum-graecum, known as fenugreek, is an annual herb of the Leguminosae family, originating in the Near East and widely produced in India and Northern Africa as a spice and forage crop. In Tunisia, fenugreek is widely cultivated in the North-western, Northern and North-eastern regions. However, despite its potential interest as forage, fenugreek use remains limited, perhaps because some works (Bartley *et al.*, 1981; Sewell *et al.*, 1999; Korman *et al.*, 2001; Mazza *et al.*, 2002) have associated its intake with an unpleasant taste in cattle's meat and milk, as well as a strong odour in human sweat and urine. Fenugreek seeds contain 30.6 and 20.6% of soluble and insoluble dietary fibre, respectively (Naidu *et al.*, 2011). The main component of its soluble fibre is a galactomannan, which is a polysaccharide structurally composed of a main chain of β - (1,4)-linked d-mannopyranose, where 83.3% of the main chain is substituted at C-6 with a single residue of α - (1,6)-D-galactopyranose (Jiang *et al.*, 2007). Fenugreek seed gum (FSG), which corresponds to the soluble fibre fraction, is obtained by aqueous extraction followed by ethanol precipitation (Zemzmi *et al.*, 2017), which eliminates the odour problems of the product. This FSG contains 63.5% of this galactomannan, which can be a potential prebiotic due to its resistance to acid and pancreatic digestion and its high fermentability (Majeed *et al.*, 2018).

As an initial approach, the aim of this study was to determine the effect of dietary inclusion of FSG on the nutrient apparent digestibility and caecal environment of growing rabbits, as well as on *in vitro* caecal fermentation, to evaluate the potential of this product as a prebiotic for Tunisian growing rabbits.

MATERIALS AND METHODS

Galactomannan extraction

Following the recommendations of Zemzmi *et al.* (2017), fenugreek seeds were ground to pass a 2 mm diameter mesh, defatted in hexane-isopropanol (3/2, v/v), extracted in distilled water for 24 h and precipitated with ethanol 95°. Then, the FSG extracted was washed with acetone and finally freeze-dried and ground into a white powder.

Diets

Experimental diets were obtained using as basis a commercial feed, including as main ingredients alfalfa hay, wheat bran, soybean meal and barley grain. Using this basal diet, three experimental diets were formulated and pelleted including 0, 0.25 and 0.5% of FSG (FSG₀, FSG_{0.25} and FSG_{0.5}, respectively) for the *in vivo* trial and 0, 0.125, 0.25, 0.5 and 100% of FSG (FSG₀, FSG_{0.125}, FSG_{0.25}, FSG_{0.5} and FSG₁₀₀, respectively) for the *in vitro* trial. Chemical composition of the experimental diets is presented in Table 1.

Animals and housing

The trial was carried out at the experimental farm of the Higher School of Agriculture of Mateur (Carthage University, Tunisia Republic). The experimental procedure was carried out following the recommendations of the European Group

Table 1: Chemical composition of the experimental diets (% dry matter), including different levels of fenugreek seed gum (FSG), used both in the *in vivo* and *in vitro* fermentation trials.

	<i>In vivo</i> trial diets			<i>In vitro</i> fermentation trial diets				
	FSG ₀	FSG _{0.25}	FSG _{0.5}	FSG ₀	FSG _{0.125}	FSG _{0.25}	FSG _{0.5}	FSG ₁₀₀
FSG (g/kg)	0.00	2.50	5.00	0.00	1.25	2.50	5.00	1000
Dry matter (DM, %)	90.2	89.5	88.7	93.7	92.4	92.9	93.02	91.3
Ash	10.1	9.82	10.1	9.48	9.36	9.47	9.55	1.27
Crude protein	17.1	16.9	17.2	17.0	17.1	16.8	16.9	22.2
Neutral detergent fibre	42.0	42.4	43.4	41.7	43.0	43.1	41.6	73.1
Acid detergent fibre	24.6	24.5	25.3	24.7	25.9	26.0	25.1	13.3
Lignin detergent fibre	3.30	3.15	3.50	4.92	4.06	3.81	3.30	0.00
Starch	12.4	11.4	12.0	12.3	14.3	14.3	13.6	0.00
Total dietary fibre	46.0	46.2	48.8	–	–	–	–	–
Soluble fibre	6.90	6.56	6.89	–	–	–	–	–
Ether extract	2.65	2.63	2.73	–	–	–	–	–

on Rabbit Nutrition (Fernández-Carmona *et al.*, 2005) and the experimental protocols followed the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes.

Forty-five weaned crossbreed rabbits (New Zealand White×Californian), at 31 d of age, were randomly assigned to one of the three treatments (15 rabbits/group). Animals were housed in individual cages (78×46×23 cm) from 31 to 94 d of age, equipped with a feeder and a drinker to provide free access to feed and water. The lighting schedule was 16 h light/8 h dark throughout the experimental trial. Weaned rabbits had a similar live weight at the beginning of the trial (552.12±12.72, 545±13.17 and 550.13±12.73 for FSG₀, FSG_{0.25} and FSG_{0.5}, respectively). Mortality and morbidity were controlled daily. Ten non-morbid rabbits per treatment with a similar live weight were used to determine the individual apparent faecal digestibility coefficients for dry matter (DM), organic matter, crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF). Apparent digestibility coefficients for ether extract (EE), total dietary fibre (TDF) and soluble fibre (SF) were determined from the pool of faeces from each treatment. The digestibility trial was performed at two ages using the same group of animals, from 38 to 41 and from 56 to 59 d of age. Following the recommendations of Perez *et al.* (1995), faeces and feed intake were controlled for 4 d, by covering the bottom of the cage with perforated plastic bags to eliminate urine. Faeces were stored in identified sealed plastic bags and frozen at –20°C until analysis. At 94 d of age, all animals were slaughtered between 8:00 and 10:00 am. After skin removal, pH value of caecal content was measured and an aliquot of caecal content was placed in a 45 mL plastic tube with 20 µL of 10% HgCl₂ to stop caecal fermentation and stored at –20°C for subsequent determination of VFA profile, N-NH₃ and lactic acid content.

In vitro fermentation

The gas production kinetics of FSG diets were determined in an *in vitro* fermentation trial. Two New Zealand 70 d old rabbits were fasted overnight, with free access to water, before their slaughter between 8:00 and 10:00 am. Caecal contents from both animals were collected, mixed and stirred for few minutes. Finally, 100 mL of the mixed caecal content were homogenised under CO₂ bubbling in 400 mL of solution A (including 10 g/L KH₂PO₄, 0.5 g/L Mg₂SO₄·7H₂O, 0.5 g/L NaCl and 0.1 g/L CaCl₂·2H₂O) and 8 mL of solution B (including 15 g/L Na₂CO₃ and 0.25 g Cysteine-HCl) and the inoculum solution obtained was filtered through six layers of cheesecloth (Marten and Barnes, 1980). Propylene sterile syringes of 60 mL were used for the incubation. Twenty mL of inoculum solution was included in each syringe with 200 mg of treatment (FSG₀, FSG_{0.125}, FSG_{0.25}, FSG_{0.5} and FSG₁₀₀). A total of 24 syringes were used in this trial, four syringes for each of the five treatments, as well as another four for the blanks (inoculum solution without substrate). Syringes were quickly placed in an oven and incubated at 39°C for 72 h. Gas production

was recorded at 1, 3, 6, 8, 12, 18, 22, 30, 36, 42, 48, 54, 60, 66, and 72 h post inoculation. At the end of incubation (72 h), 0.1 mL of HgCl₂ 10% was added to each syringe to stop fermentation. After direct measurement of pH, syringe content was transferred to 45 mL plastic tubes and centrifuged at 3000 rpm for 10 min. Supernatant was transferred in another tube and stored at 4°C to determine total VFA, N-NH₃ and lactic acid concentration, as well as the VFA profile. Gas production values at each measurement time were corrected for gas produced at this time by the corresponding blanks. Gas production data were fitted to the monophasic model described by Groot *et al.* (1996):

$$G = \frac{A}{1 + \left(\frac{B}{t}\right)^C} \quad (1)$$

In this equation, G (mL g⁻¹ DM) is the amount of gas produced per gram of DM incubated, at time t after incubation; A (mL g⁻¹ DM) is the asymptotic gas production; B (h) is the time after incubation at which half of the asymptotic amount of gas has been formed; and C is the constant determining the sharpness of the switching characteristic of the profile. Maximum substrate degradation rate (R_M) and the time after the start of the incubation at which R_M occurs (t_{R_M}) were also calculated as:

$$t_{R_M} = B(C-1)^{1/C} \quad \rightarrow \quad R_M = \frac{Ct_{R_M}^{(C-1)}}{B^C + t_{R_M}^C} \quad (2)$$

Chemical analysis

The AOAC (2002) methods were used for DM (934.01), ash (942.05), CP (990.03, Dumas method, CN628 Elemental Analyzer, LECO, St. Joseph, MI, USA) and EE (920.39, with acid-hydrolysis of samples prior to the extraction). Starch content was determined according to Batey (1982) in a two-step enzymatic procedure with solubilisation and hydrolysis of maltodextrins with thermo-stable α-amylase followed by complete hydrolysis with amyloglucosidase (both enzymes from Sigma-Aldrich, Steinheim, Germany), and the resulting D-glucose was measured by the hexokinase/glucose-6 phosphate dehydrogenase/NADP system (kit D-glucose-HK Megazyme Int. Ireland Ltd., Wicklow, Ireland). The TDF content was determined by a gravimetric-enzymatic method, procedure 991.43 of Van Soest *et al.* (1991), with α-amylase, protease and amyloglucosidase treatments (Megazyme TDF R.30.K-TDFR-100A/200A), correcting for ash and CP. The NDF, ADF and lignin detergent fibre (ADL) fractions were analysed sequentially according to Mertens (2002), AOAC procedure 973.18 (2002) and Robertson and Van Soest (1981), respectively, with a thermo-stable α-amylase pre-treatment and expressed exclusive of residual ash, using a nylon filter bag system (Ankom, Macedon, NY, USA). The SF content was determined as proposed by Van Soest *et al.* (1991), by subtracting the NDF corrected for CP from the TDF content. Determination of VFA was based on the method described by Jouany (1982). Samples were filtered through 0.45 μm cellulose syringe filters. Next, 100 μL of an internal standard solution (0.4 g of 4-methylvaleric acid diluted in 100 mL of deionised water) and 0.1 mL of a preservative (5% H₃PO₄ and 1% HgCl₂ in deionised water) were added to 0.9 mL of filtrate. One μL from each sample was injected into a gas chromatograph (Fisons 8000 series, Milan, Italy) equipped with a split/split less injector and FID detector. The separation of VFA was done in a DB-FFAP capillary column (30 m×0.25 mm×0.25 μm of film thickness, J&W Scientific, Folson, CA, USA). The carrier gas was N₂ at a constant pressure of 120 kPa. Injector and detector temperatures were set at 200°C and 245°C respectively. The initial oven temperature was set at 110°C held for 5 min and increased to 230°C at 8.5°C/min and finally maintained at that temperature for 10 min. Finally, VFA were identified by comparing their retention times with a standard (46975-U from Supelco®, Bellefonte, PA, USA). N-NH₃ concentration was determined using the nesslerisation spectrophotometric method adapted by Koroleff (1966). The VFA and N-NH₃ concentrations were expressed as mmol/L of the liquid phase of caecal digesta. Lactic acid was determined according to the method of Borshchevskaya *et al.* (2016), where an aliquot of the samples was diluted 20 times with deionised water, 50 μL from the dilution was added to 2 mL of a 0.2% solution of FeCl₃, the mixture was stirred and absorbance was measured at 390 nm against a reference solution (2 mL of a 0.2% FeCl₃). Colour solution was stable for 15 min. All *in vitro* traits values were corrected by their corresponding blanks.

Statistical analysis

All the traits in this work were statistically analysed with a General Linear Model (GLM) procedure from SAS (2009). For the digestibility traits, the model included the effect of FSG inclusion (0, 0.25 and 0.5%), the age (38 and 56 d) and their interaction as main effects. However, for the caecal environment and *in vitro* traits, the model only included the effect of FSG inclusion (0, 0.25 and 0.5%, or 0, 0.125, 0.25 and 0.5%, respectively) as main effect. An orthogonal contrast to evaluate the effect of FSG inclusion $[FSG_0 - (FSG_{0.25} + FSG_{0.5})/2]$ was performed for digestibility and caecal traits, while possible linear effect of FSG inclusion (from 0 to 0.5%) was evaluated for the *in vitro* traits.

RESULTS

All animals were perfectly adapted to the experimental diets and no morbidity or mortality problems were recorded throughout the growing period. No significant differences were observed for the final body weights at 94 d of age, which were 2360 ± 32 , 2428 ± 32 and 2376 ± 32 for FSG_0 , $FSG_{0.25}$ and $FSG_{0.5}$, respectively. During the digestibility trial, daily feed intake obviously increased with the age of the growing rabbits (+28% from 38 to 56 d; $P < 0.001$), but was not affected by the inclusion level of FSG in the diet (Table 2). Apparent faecal digestibility coefficients (d) of main nutrients were not significantly affected by the dietary inclusion of FSG. However, dNDF and dADF values at 56 d were significantly higher than those observed at 38 d of age (+0.049 and +0.048 percentage points, respectively; $P < 0.05$), which could explain the higher dDM with the age observed (+0.021; $P = 0.051$). No significant interaction was observed between the inclusion of FSG and age during the digestibility trial. The effect of dietary inclusion of FSG on the caecal traits is presented in Table 3. No significant effect of the dietary inclusion of FSG on any of the traits related to the caecal environment was observed. However, animals fed with FSG showed lower caecal pH (-0.15 ; $P = 0.037$) and slightly higher caproic acid concentration in the caecum ($+0.48\%$; $P = 0.093$) than those fed with FSG_0 . Table 4 shows the effect of dietary inclusion of FSG on the gas production parameters estimated throughout incubation and the *in vitro* traits controlled at the end of this incubation. In comparison to the experimental diets, pure FSG (FSG_{100}) significantly affected the main gas kinetic parameters (Figure 1a), increasing A, C and R_M traits, but decreasing B and $t_{B,M}$ traits ($P < 0.001$). However, gradual dietary inclusion of FSG (from 0 to 0.5%) had no effect on any of these kinetic parameters (Figure 1b). Similarly, FSG_{100} significantly decreased caecal pH and total VFA production, as well as lactic acid and N-NH₃ concentrations compared to the values observed with the experimental diets ($P < 0.001$). Finally, gradual dietary inclusion of FSG

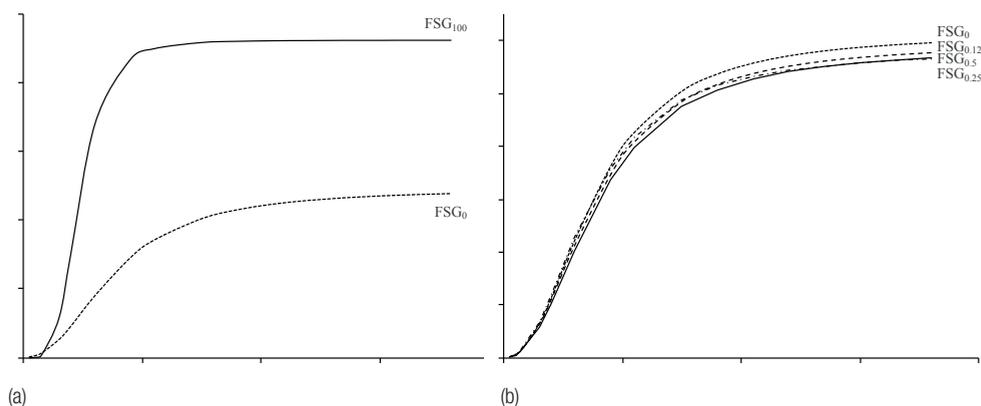


Figure 1: Evolution of gas production during the *in vitro* incubation of fenugreek seed gum (FSG) and the experimental diets with caecal inocula: (a) Comparison of pure FSG (FSG_{100}) with the basal diet (FSG_0); (b) Comparison of the four experimental diets including 0, 0.125, 0.25 and 0.5% of FSG (FSG_0 , $FSG_{0.125}$, $FSG_{0.25}$ and $FSG_{0.5}$, respectively). Curves fitted to the monophasic model of Groot et al., 1996 $[Gt = A/(1+(B/t)^C)]$. For coefficients description see Table 4.

Table 2: Effect of the dietary inclusion of fenugreek seed gum (FSG) and the age of growing rabbits on feed intake (g dry matter/d) and *in vivo* apparent digestibility coefficients of nutrients.

	FSG level (%)				Age (d)			P-value		
	FSG ₀	FSG _{0.25}	FSG _{0.5}	SEM	38	56	SEM	FSG	Age	FSG×Age
Feed intake	105	102	103	1.50	91.0	116.	1.20	0.349	0.001	0.279
Digestibility coefficients										
Dry matter	0.527	0.538	0.526	0.007	0.520	0.541	0.007	0.430	0.051	0.517
Organic matter	0.528	0.542	0.526	0.007	0.524	0.539	0.006	0.269	0.129	0.512
Crude protein	0.634	0.658	0.647	0.010	0.649	0.644	0.009	0.262	0.655	0.262
Neutral detergent fibre	0.209	0.224	0.234	0.010	0.198	0.247	0.010	0.239	0.006	0.751
Acid detergent fibre	0.057	0.062	0.083	0.012	0.043	0.091	0.011	0.273	0.017	0.827
Ether extract	0.859	0.859	0.859	–	0.865	0.853	–	–	–	–
Total dietary fibre	0.252	0.270	0.267	–	0.250	0.276	–	–	–	–
Soluble fibre	0.534	0.574	0.559	–	0.568	0.543	–	–	–	–

SEM: standard error of the means.

from 0 to 0.5% significantly increased total VFA production (+100 mmol/L per percentage point of FSG inclusion; $P=0.038$) and slightly decreased lactic acid concentration in the caecum (–14 mmol/L per percentage point of FSG inclusion; $P=0.091$).

DISCUSSION

The results of this work show that FSG inclusion in diet up to 0.5% does not produce any significant effect on the apparent faecal digestibility of DM or its main components. However, we did observe that the values obtained for the digestibility of all fibrous fractions (TDF, SF, NDF and ADF) were always between 2 and 3% higher when the diets included FSG. Although galactomannan is not expected to affect the digestibility of the dietary components that are mainly digested in the small intestine, it could be expected that high fermentability of galactomannans in the caecum

Table 3: Effect of fenugreek seed gum (FSG) dietary inclusion on caecal pH, total volatile fatty acids production (VFA), VFA profile, lactic acid and the ammonia N in growing rabbits.

	FSG level (%)			SEM	P-value
	FSG ₀	FSG _{0.25}	FSG _{0.5}		
Caecal pH*	5.82	5.69	5.65	0.0581	0.096
Caecal VFA (mmol/L)	50.8	46.6	50.01	2.77	0.534
VFA profile (% of total VFA):					
Acetic acid	75.9	76.2	76.3	0.944	0.915
Butyric acid	12.5	12.7	13.0	0.639	0.868
Propionic acid	7.96	7.01	6.79	0.538	0.264
Isobutyric acid	0.350	0.423	0.339	0.0544	0.519
Valeric acid	1.54	1.40	1.34	0.109	0.428
Isovaleric acid	0.853	0.957	0.823	0.104	0.649
Caproic acid*	0.790	1.25	1.29	0.231	0.234
Heptanoic acid	0.0436	0.0637	0.0743	0.0143	0.304
Lactic acid (mmol/L)	0.0589	0.0636	0.0578	0.00503	0.690
Propionic/butyric ratio	0.637	0.529	0.591	0.0495	0.375
Caecal N-NH ₃ (mmol/L)	5.70	5.43	5.71	0.192	0.528

SEM: Standard error of the means; * Contrast FSG₀ – [FSG_{0.25}+FSG_{0.5}]/2: Caecal pH ($P=0.037$) and caproic acid ($P=0.093$).

Table 4: Effect of fenugreek seed gum (FSG) dietary inclusion in rabbit diets and pure FSG on the gas production parameters of monophasic model [Groot *et al.*, 1996; $G_t = A/(1+(B/t)-C)$]1 and some *in vitro* traits after incubation.

	FSG level (%)					SEM	P-value
	FSG ₀	FSG _{0.125}	FSG _{0.25}	FSG _{0.5}	FSG _{1.00}		
Gas kinetic parameters:							
A (mL/g DM)	12.3 ^a	11.9 ^a	11.6 ^a	11.7 ^a	23.1 ^b	0.72	<0.001
B (h)	15.3 ^b	15.5 ^b	14.6 ^b	15.8 ^b	9.44 ^a	0.72	<0.001
C	2.28 ^a	2.24 ^a	2.28 ^a	2.29 ^a	4.25 ^b	0.07	<0.001
t _{R_M} (h) ³	17.0 ^b	17.1 ^b	16.2 ^b	17.6 ^b	12.5 ^a	0.94	0.010
R _M (h ⁻¹) ²	0.07 ^a	0.07 ^a	0.08 ^a	0.07 ^a	0.26 ^b	0.01	<0.001
<i>In vitro</i> traits ⁴ :							
ΔpH	-0.52 ^b	-0.49 ^b	-0.49 ^b	-0.48 ^b	-1.53 ^a	0.026	<0.001
ΔVFA (mmol/L) ⁵	56.7 ^a	46.7 ^a	75.9 ^{ab}	106.9 ^b	114.7 ^b	17.7	<0.001
ΔLactic acid (mmol/L) ⁶	14.0 ^b	9.73 ^b	5.89 ^{ab}	6.64 ^{ab}	-0.43 ^a	3.23	0.069
ΔN-NH ₃ (mmol/L)	2.91 ^b	2.96 ^b	2.76 ^b	2.76 ^b	-9.96 ^a	0.362	<0.001

¹G_t (mL/g DM), amount of gas produced per gram of DM at t time of incubation; A (mL/g DM), asymptotic gas production; B (h), time to achieve half of the asymptotic amount of gas; C, constant determining the sharpness of the switching characteristic of the profile.

²R_M: maximum substrate degradation at time.

³Time after the start of the incubation at which R_M occurs.

⁴Change with respect to the blank (caecal inoculum without treatment).

⁵Total volatile fatty acids.

⁶Lineal effect of FSG dietary inclusion: VFA (P=0.038) and lactic acid (P=0.091).

^{a,b} Means in a row not sharing the same letter are significantly different at P<0.05.

SEM: standard error of the means.

could promote the fibrolytic microbiota of the caecum, having an effect on the use of the rest of the fibrous fractions. In fact, Trocino *et al.* (2013b), in a meta-study on soluble fibre in rabbits, showed that an increase in dietary SF is positively correlated with an improvement in the digestibility of the remaining fibrous fractions. However, contrary to expectations, other studies have observed a lower cellulose digestibility in rabbits (Volek *et al.*, 2007) and pigs (Lipinski *et al.*, 2005) when mannan-oligosaccharides were included in the diet. On the other hand, although the faecal digestibility of NDF and ADF increased with the age of growing rabbits, no interaction with FSG inclusion was observed. Increasing digestive efficacy with age is a well-known effect. Evans and Jebelian (1982) observed that the digestibility of ADF increased by 0.95 percentage points per week between 5 and 12 wk of age. Moreover, comparing nutrient digestibility of growing rabbits and rabbit females (Read *et al.*, 2017), digestibility of NDF and ADF was higher in rabbit females due to the higher digestive capacity of adult animals.

Most of the water-soluble polysaccharides and oligosaccharides are not digested in the small intestine but are rapidly fermented in the hindgut (Volek and Marounek., 2011). In our work, we observed that the dietary inclusion of FSG up to 0.5% did not lead to major significant changes in the caecal environment of growing rabbits at 94 d of age. The only changes observed were a slight reduction in the caecal pH and a non-significant increase in some VFA, especially caproic acid. Some previous studies have observed that a reduction in caecal pH could inhibit the growth of some pathogenic bacteria, such as *E. coli* in rabbits (Gidenne and Licois, 2005), and an increase in the concentration of caproic acid in the caecum could also help to reduce the count of *E. coli* and *C. perfringens* (Skriverova *et al.*, 2006; 2008). For that reason, it would be interesting to continue exploring the possible effect of FSG on the caecal environment with a greater inclusion in the diet, as inclusion up to 0.5% has not been able to produce relevant changes. *In vitro* gas production can reflect the extent to which substrates are fermented by gut microorganisms (Schofield, 2000). Water-soluble fibres, like the galactomannan of FSG, seem to be highly fermentable in the rabbit caecum, leading to high gas production, the production of a greater amount of VFA and reduction of the N-NH₃ free at the final inoculum. Compared to the rabbit diets used in this work (Figure 1a), kinetic traits obtained for the pure FSG were characterised by higher substrate degradation rate, lower time to achieve the maximum rate of degradation and

clearly higher asymptotic gas production, which confirms the potential of FSG to be used by the fibrolytic microbiota of the rabbit's caecum. Recently, Ocasio-Vega *et al.* (2018) compared gas production kinetics and *in vitro* traits of different fibrous substrates (cellobiose, pectin, sugar beet pulp and wheat bran). When compared to a highly insoluble fibre (wheat straw), the other fibrous substrates lead to a higher gas production after *in vitro* incubation with a rabbit caecal inoculum, and the higher the SF content, the higher the gas production. Similar results were observed by Abad-Guamán *et al.* (2018) when comparing gas production of different substrates (pectin, sugar beet pulp and wheat straw) in a caecal inoculum for 28 h, where the volume of gas was proportional to the amount of substrate fermented. The higher microbial activity associated with the increased availability of a fermentable substrate is also usually associated with promoted microbial protein synthesis and reduced N-NH₃ level in the caecum (Carabaño *et al.*, 2009; Trocino *et al.*, 2013a). However, dietary inclusion of FSG up to 0.5% did not affect any of the kinetic parameters of the gas production model (Figure 1b). Bovera *et al.* (2010), comparing *in vitro* gas production of rabbit diets including mannan-oligosaccharides (MOS) up to 0.15% or supplemented with antibiotics, observed higher gas production and fermentation rate of MOS diets compared to the diet with antibiotics when gas production was fitted to the same model used in the present work (Groot *et al.*, 1996). However, a linear increase in MOS inclusion from 0.05 to 0.15% did not affect gas production, although it seems to increase R_m and reduce. These results seem to indicate that the inclusion of low levels of fermentable substrates does not excessively affect *in vitro* gas production. The lack of differences in the kinetics traits observed with the inclusion of FSG may be due to a higher dilution of the inoculum in this work (2:1 and 4:1 in Bovera *et al.* (2010) and this work, respectively). In any case, dietary inclusion of FSG up to 0.5% caused a linear increase in the observed increase in the VFA content of the caecum during *in vitro* fermentation, due to its high fermentability, but without any relevant effect on the VFA profile. It can be concluded that pure FSG is highly and rapidly fermented in the rabbit caecum, decreasing the pH and N-NH₃ concentration and increasing total VFA level when it was *in vitro* incubated in a caecal inoculum. However, dietary inclusion of FSG up to 0.5%, although it linearly increased the total VFA level after *in vitro* incubation and decreased the caecal pH of young rabbits, did not affect the apparent digestibility and fermentation profile of the animals to any great extent. Based on these results, it would be convenient to carry out more studies on the fermentation pattern of this galactomannan, and on how its greater inclusion can affect growth performance, digestive health and microbiota in growing rabbits.

Acknowledgements: This study is supported by the Interministerial Commission for Science and Technology (CICYT) of the Spanish Government (AGL2017-85162-C2-1R), the Universitat Politècnica de València (Project 20180290; Spain), and the Higher School of Agriculture of Mateur of the Carthage University (Tunisia Republic). Grant assistance for Jihed Zemzmi from the Carthage University is also gratefully acknowledged.

REFERENCES

- Abad-Guamán R., Larrea-Dávalos J.A., Carabaño R., García J., Carro M.D. 2018. Influence of inoculum type (ileal, caecal and faecal) on the *in vitro* fermentation of different sources of carbohydrates in rabbits. *World Rabbit Sci.*, 26: 227-240. <https://doi.org/10.4995/wrs.2018.9726>
- AOAC. 2002. Official methods of analysis of the AOAC International. 17th ed. AOAC International, Gaithersburg, MD USA.
- Bartley G.B., Hiltz M.D., Anderson B.D., Clairemont A.C., Maschke S.P. 1981. "Maple-syrup" urine odor due to fenugreek ingestion. *New Engl. J. Med.*, 305: 467. <https://doi.org/10.1056/NEJM198108203050823>
- Batey I.L. 1982. Starch Analysis Using Thermostable Alpha-Amylases. *Starch/Stärke*, 34: 125-128. <https://doi.org/10.1002/star.19820340407>
- Boletín Oficial del Estado. 2013. Real Decreto 53/2013, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. *BOE*, 34: 11370-11421.
- Bónai A., Szendrő Zs., Matics Zs., Fébel H., Kametler L., Tornóyos G., Horn P., Kovács F., Kovács M. 2010. Effect of inulin supplementation and age on growth performance and digestive physiological parameters in weaned rabbits. *World Rabbit Sci.*, 18: 121-129. <https://doi.org/10.4995/WRS.2010.5883>
- Borshchevskaya L.N., Gordeeva T.L., Kalinina A.N., Sineokii S.P. 2016. Spectrophotometric determination of lactic acid. *J. Anal. Chem.*, 71: 755-758. <https://doi.org/10.1134/S1061934816080037>
- Bovera F., Marono S., Di Meo C., Piccolo G., Iannaccone F., Nizza A. 2010. Effect of mannanoligosaccharides supplementation on caecal microbial activity of rabbits. *Animal*, 4: 1522-1527. <https://doi.org/10.1017/S1751731110000558>
- Carabaño R., Villamide M.J., García J., Nicodemus N., Llorente A., Chamorro S., Menoyo D., García-Rebollar P., García-Ruiz A.I., De Blas J.C. 2009. New concepts and objectives for protein-amino acid nutrition in rabbits: a review. *World Rabbit Sci.*, 17: 1-14. <https://doi.org/10.4995/wrs.2009.664>
- Evans E., Jebelian V. 1982. Effects of age upon nutrient digestibility by fryer rabbits. *J. Appl. Rabbit Res.*, 5: 8-9.

- Falcão-E-Cunha L., Castro-Solla L., Maertens L., Marounek M., Pinheiro V., Freire J., Mourão J.L. 2007. Alternatives to antibiotic growth promoters in rabbit feeding: A review. *World Rabbit Sci.*, 15: 127-140. <https://doi.org/10.4995/wrs.2007.597>
- Fernández-Carmona J., Blas E., Pascual J.J., Maertens L., Gidenne T., Xicatto G., García, J. 2005. Recommendations and guidelines for applied nutrition experiments in rabbits. *World Rabbit Sci.*, 13: 209-228. <https://doi.org/10.4995/wrs.2005.516>
- Gidenne T. 1995. Effect of fibre level reduction and gluco-oligosaccharide addition on the growth performance and caecal fermentation in the growing rabbit. *Anim. Feed Sci. Technol.*, 56: 253-263. [https://doi.org/10.1016/0377-8401\(95\)00834-9](https://doi.org/10.1016/0377-8401(95)00834-9)
- Gidenne T., Licois D. 2005. Effect of a high fibre intake on the resistance of the growing rabbit to an experimental inoculation with an enteropathogenic strain of *Escherichia coli*. *Animal Sci.*, 80: 281-288. <https://doi.org/10.1079/ASC41570281>
- Gómez-Conde M.S., García J., Chamorro S., Eirás P., Rebollar P.G., De Rozas A.P., Badiola I., De Blas C., Carabaño R. 2007. Neutral detergent-soluble fiber improves gut barrier function in twenty-five-day-old weaned rabbits. *J. Ani. Sci.*, 85: 3313-3321. <https://doi.org/10.2527/jas.2006-777>
- Gómez-Conde M.S., Pérez de Rozas A., Badiola I., Pérez-Alba L., de Blas C., Carabaño R., García J. 2009. Effect of neutral detergent soluble fibre on digestion, intestinal microbiota and performance in twenty five day old weaned rabbits. *Livest. Sci.*, 125: 192-198. <https://doi.org/10.1016/j.livsci.2009.04.010>
- Groot J.C.J., Cone J.W., Williams B.A., Debersaques F.M.A., Lantinga E.A. 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. *Anim. Feed Sci. Technol.*, 64: 77-89. [https://doi.org/10.1016/S0377-8401\(96\)01012-7](https://doi.org/10.1016/S0377-8401(96)01012-7)
- Jiang J.X., Zhu L.W., Zhang W.M., Sun R.C. 2007. Characterization of Galactomannan Gum from Fenugreek (*Trigonella foenum-graecum*) Seeds and its rheological properties. *Int. J. Polym. Mater. Po.*, 56: 1145-1154. <https://doi.org/10.1080/00914030701323745>
- Jouany J.P. 1982. Volatile Fatty Acid and Alcohol Determination in Digestive Contents, Silage Juices, Bacterial Cultures and Anaerobic Fermentor Contents. *Sciences des Aliments*. 2: 131-144.
- Korman S.H., Cohen E., Preminger A. 2001. Pseudo-maple syrup urine disease due to maternal prenatal ingestion of fenugreek. *J. Pediatr. Child Health*, 37: 403-404. <https://doi.org/10.1046/j.1440-1754.2001.00617.x>
- Koroleff F. 1966. Direct spectrophotometric determination of ammonia in precipitation. *Tellus XVIII.*, 2. <https://doi.org/10.3402/tellusa.v18i2-3.9343>
- Lipinski K., Purwin C., Tywoczuk J., Zduńczyk Z., Wroblewska M., Laniewska-Trokenheim L., Warminska-Radyko L. 2005. Effects of feed additives on nutrient digestibility and the bacterial status of faeces in pigs. *J. Anim. Feed Sci.*, 14: 369-372. <https://doi.org/10.22358/jafs/70582/2005>
- Majeed M., Majeed S., Nagabhushanam K., Arumugam S., Natarajan S., Beede K., Furqan A. 2018. Galactomannan from *Trigonella foenum-graecum* L. Seed: Prebiotic application and its fermentation by prebiotic *Bacillus coagulans* strain MTCC 5856. *Food Sci. Nutr.*, 6: 666-673. <https://doi.org/10.1002/fsn3.606>
- Marten G.C., Barnes R.F. 1980. Prediction of energy digestibility of forages with *in vitro* rumen fermentation and fungal enzyme systems. In: W.J. Pigden, C.C. Balch, M. Graham (Eds.), *In Proc.: The International Workshop on Standardization of Analytical Methodology for Feeds*, Int. Dev. Res. Center, Ottawa, Ont., Canada. 61-71.
- Martínez-Vallespín B., Martínez-Paredes E., Ródenas L., Cervera C., Pascual J.J., Blas E. 2011. Combined feeding of rabbit female and young: Partial replacement of starch with acid detergent fibre or/and neutral detergent soluble fibre at two protein levels. *Livest. Sci.*, 141: 155-165. <https://doi.org/10.1016/j.livsci.2011.05.014>
- Martínez-Vallespín B., Martínez-Paredes B., Ródenas L., Moya J.M., Cervera C., Pascual J.J., Blas E. 2013. Partial replacement of starch with acid detergent fibre and/or neutral detergent soluble fibre at two protein levels: Effects on ileal apparent digestibility and caecal environment of growing rabbits. *Livest. Sci.*, 154: 123-130. <https://doi.org/10.1016/j.livsci.2013.02.012>
- Mazza G., Di Tommaso D., Foti S. 2002. Volatile constituents of Sicilian fenugreek (*Trigonella foenum-graecum* L.) seeds. *Sciences des Aliments*, 22: 249-264. <https://doi.org/10.3166/sda.22.249-264>
- Mertens D.R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. *J. AOAC Int.*, 85: 1217-1240.
- Naidu M.M., Shyamala B.N., Naik J.P., Sulochanamma G., Srinivas P. 2011. Chemical composition and antioxidant activity of the husk and endosperm of fenugreek seeds. *LWT-Food Sci. Technol.*, 44: 451-456. <https://doi.org/10.1016/j.lwt.2010.08.013>
- Ocasio-Vega C., Abad-Guamán R., Delgado R., Carabaño R., Dolores Carro M., Javier García. 2018. Effect of cellobiose supplementation and dietary soluble fibre content on *in vitro* caecal fermentation of carbohydrate-rich substrates in rabbits. *Arch. Anim. Nutr.*, 72: 221-238. <https://doi.org/10.1080/1745039X.2018.1458459>
- Perez J.M., Lebas F., Gidenne T., Maertens L., Xicatto G., Parigi-Bini R., Dalle Zotte A., Cossu M.E., Carozzolo A., Villamide M.J., Carabaño R., Fraga M.J., Ramos M.A., Cervera C., Blas E., Fernández J., Falcão-e-Cunha L., Freire J. 1995. European reference method for *in vivo* determination of diet digestibility in rabbits. *World Rabbit Sci.*, 3: 41-43. <https://doi.org/10.4995/wrs.1995.239>
- Read T., Gidenne T., Combes S., Labatut D., Bricard D., Bébin K., Fortun-Lamothe L. 2017. Digestibilité comparée chez le lapin: effets de l'âge, de l'état et du stade physiologiques. In *Proc.: 17èmes Journées de la Recherche Cunicole*, 21-22 Novembre, 2017. Le Mans, France. 117-180.
- Robertson J. B., Van Soest P. J., James W. P. T., & Theander O. 1981. The analysis of dietary fiber in food. The detergent system of analysis and its application to human foods. *James, WPT and Theander, O., eds. Marcel Dekker, New York, NY*, 123-158.
- Rosell J.M., de la Fuente L.F., Badiola J.I., Fernández de Luco D., Casal J., Saco M. 2009. Study of urgent visits to commercial rabbit farms in Spain and Portugal during 1997-2007. *World Rabbit Sci.*, 17: 127 -136. <https://doi.org/10.4995/wrs.2009.652>
- SAS. 2009. SAS/STAT User's Guide (Release 9.2). *SAS Institute. Inc., Cary NC, USA*.
- Schofield P. 2000. Gas production methods. *Farm Animal Metabolism and Nutrition. J.P.F. D'Mello, ed. CAB International, Wallingford, Oxon, U.K.* 209-232. <https://doi.org/10.1079/9780851993782.0209>

- Sewell A. C., Mosandl A., Bohles H. 1999. False diagnosis of maple syrup urine disease owing to ingestion of herbal tea. *Massachusetts Medical Society*, 341: 769. <https://doi.org/10.1056/NEJM199909023411020>
- Skrivanova E., Marounek M., Benda V., Brezina P. 2006. Susceptibility of *Escherichia coli*, *Salmonella* sp. and *Clostridium perfringens* to organic acids and monolaurin. *Veterinární Medicina*, 51: 81-88. <https://doi.org/10.17221/5524-VETMED>
- Skrivanova E., Molatova Z., Marounek M. 2008. Effects of caprylic acid and triacylglycerols of both caprylic and capric acid in rabbits experimentally infected with enteropathogenic *Escherichia coli* O. *Vet. Microbiol.*, 126: 372-376. <https://doi.org/10.1016/j.vetmic.2007.07.010>
- Trocino A., Fragkiadakis M., Majolini D., Tazzoli M., Radaelli G., Xiccato G. 2013a. Soluble fibre, starch and protein level in diets for growing rabbits: Effects on digestive efficiency and productive traits. *Anim. Feed Sci. Technol.*, 180: 73-82. <https://doi.org/10.1016/j.anifeedsci.2013.01.007>
- Trocino A., García J., Carabaño R., Xiccato G. 2013b. A meta-analysis of the role of soluble fiber in diets for growing rabbits. *World Rabbit Sci.*, 21: 1-15. <https://doi.org/10.4995/wrs.2013.1285>
- Van Soest P.J., Robertson J.B., Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.*, 74: 3583-3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Volek Z., Marounek M., Skrivanova V. 2007. Effect of a starter diet supplementation with mannan-oligosaccharide or inulin on health status, caecal metabolism, digestibility of nutrients and growth of early weaned rabbits. *Animal*, 1: 523-530. <https://doi.org/10.1017/S1751731107685012>
- Volek Z., Marounek M. 2011. Dried chicory root (*Cichorium intybus* L.) as a natural fructan source in rabbit diet: effects on growth performance, digestion and caecal and carcass traits. *World Rabbit Sci.*, 19: 143-150. <https://doi.org/10.4995/wrs.2011.850>
- Zemzmi J., Mabrouki S., Abdouli H., Najjar T. 2017. Preliminary characterization of fenugreek seed gum for use as prebiotic in rabbits' nutrition. In *Proc.: the 20th International Symposium on housing and diseases of rabbits, fur providing animals and pet animals, 17-18 May, 2017, Celle, Germany.* 179-186.
-