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Characterization and effect of year of harvest on the nutritional properties of three varieties of white lupine (*Lupinus albus* L.)

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Abstract

BACKGROUND: Three cultivars of *Lupinus albus* L. (Lutteur, Lublanca and Multitalia) were assessed for proximate composition, fatty acids, alkaloids and *in vitro* fermentation characteristics over three harvest years.

RESULTS: The chemical composition varied greatly during the three harvest years. Crude protein content ranged from 353 to 456 g kg^{-1} dry matter (DM), neutral detergent fiber content from 209 to 321 g kg^{-1} DM and lignin content from 3.0 to 63.9 g kg^{-1} DM. Lublanc showed the highest crude protein (417 g kg^{-1} DM) and lignin (35 g kg^{-1} DM) contents. High levels of lipids (89.9 g kg^{-1} DM) and starch (93.3 g kg^{-1} DM) were found in all samples. Alkaloid content ranged from 3.63 to 165 mg per 100 g. Lutteur and Lublanc showed more favorable *n*-3/*n*-6 polyunsaturated fatty acid ratios (from 0.44 to 0.73) and lower values of the anti-quality factor 'erucic acid' (from 5.8 to 20.9 g kg^{-1}) than Multitalia. Lutteur showed higher degradability (897 g kg^{-1}), gas production (330 mL g^{-1} organic matter (OM)) and volatile fatty acid production (117 mmol g^{-1} OM) than the other varieties.

CONCLUSION: The present data suggest *L. albus* L. cv. Lutteur to be a promising crop as food thanks to its high nutritive traits and most constant yield over time.

Keywords: alkaloid; chemical composition; digestibility; fatty acids; *in vitro* gas production; nutritional index

INTRODUCTION

World agriculture is facing two challenges: ensuring adequate food production for an ever-increasing human population and protecting natural resources from pollution.[1] Seed legumes are strategically important not only because they decrease the marked deficit of high-protein feedstuff[2] but also because they increase the sustainability of crop–livestock systems through the safeguarding of soil fertility, the reduction of greenhouse gas emission and the reduction of nitrogen fertilizer use.[3] Recently, Leguminosae seeds have been considered as an alternative protein source to soybean meal in animal feeding[4] owing to the controversy related to the use of genetically modified organisms (GMOs).[5,6] Among legumes, lupine appears an interesting and promising crop since it represents a resource for agriculture in human and animal nutrition as well as a solution for both challenges. In fact, this plant has some traits that make it a valuable alternative crop: it has a winter cycle, a high grain productivity for food and feed destination, a limited phosphorus requirement compared with other crops[7] and a high content of protein deriving from nitrogen fixed from the atmosphere[8] compared with other winter legumes[8,9] and is also an excellent rotation crop able to enrich soil with nitrogen.[10]

Lupine seeds are a valuable nitrogen and energy source owing to their high content of crude protein (300–500 g kg^{-1}) and oil (50–100 g kg^{-1}), which vary as a function of species and variety. In humans, oil quality is interesting from a nutritional point of view,[11] so that, for many years, lupine has been studied as an ingredient in functional and healthy food products owing to its hypocholesterolemic potential[12,13] in relation to the fatty acid profile.[1] On the other hand, in ruminant nutrition, some authors[14] reported that diets for buffalo containing lupine seeds have lower protein content (358 g kg^{-1} dry matter (DM)) and *in situ* degradability (818 g kg^{-1}) than soya solvent extract (526 g kg^{-1} DM and 897 g kg^{-1} respectively). This can partly be explained by the presence of antinutritional factors (ANFs) including protease inhibitors, alkaloids, oligosaccharides and/or fibrous material. All these factors limit the accessibility of legume seed protein to digestive enzymes.[15] Among ANFs, lupines not genetically selected contain more than 150 alkaloids of the quinolizidine, piperidine and indole groups which are known to appear at concentrations up

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to 60 g kg^{-1}, conferring resistance to pathogens and grazing.[16] Nowadays, the economic sustainability of this legume depends on its ability to maintain or reduce its alkaloid content and to increase its seed yield.[17] In fact, the increased selection of sweet varieties that have very low levels of quinolizidine alkaloids and favorable protein and fiber contents[18] as well as a high level of α-linolenic acid and a favorable *n*-3/*n*-6 polyunsaturated fatty acid ratio has amplified the possibility of using white lupine seed in human or livestock nutrition.

Lupine is widely cultivated in Central and Eastern Europe and in Australia, but it is well adapted to the Mediterranean climate[2] and is cultivated in countries with very different latitudes and climate characteristics (from Lithuania to Morocco),[19] which can have a great influence on both quantitative and qualitative traits of agricultural products. Nevertheless, there is a lack of information on the influence of environmental conditions on the protein content and fatty acid profile of lupine.[20] Final users need to know whether the quality of different grain lots of the some variety can be considered homogeneous or, on the contrary, may vary depending on the growing environment. If the environment exerts a marked influence, preference could be given to seed lots that are produced under environmental conditions capable of maximizing the requested quality trait.[19]

This study was therefore designed to increase the knowledge regarding the nutritional properties and *in vitro* digestibility of three varieties of economic importance: two selected varieties of white lupine (*Lupinus albus* L.) currently cultivated in the Mediterranean area, and the Multitalia variety, an Italian old sweet variety, less genetically selected, used as control. The study also examined the impact of three harvest years on the characteristics of these cultivars in order to promote the use of this crop within the Mediterranean food chain. The main hypothesis was that nutritional properties can vary among lupine varieties and harvest years.

MATERIALS AND METHODS

Experimental design

Three cultivars of *L. albus* L. (Lublanc, Lutteur (selected for their low alkaloid content) and Multitalia (the historical cultivar used as control)) harvested in three successive years were used in this study. The cultivars for each harvest year were named LB1, LB2, LB3 (cv. Lublanc, years 1, 2, 3 respectively), LT1, LT2, LT3 (cv. Lutteur, years 1, 2, 3 respectively) and MU1, MU2, MU3 (cv. Multitalia, years 1, 2, 3 respectively).

All samples were analyzed for proximate composition (including starch), fatty acid profile, alkaloid content and *in vitro* fermentation kinetics and characteristics.

Plant material and environmental conditions

The lupine samples were from an agronomic trial[21] carried out over three years (2006–2009) in a flat area of southern Italy (Piana del Sele, Pontecagnano, SA, Italy; longitude 14° 52′ E, latitude 40° 64′ N, altitude 28 m a.s.l., annual mean temperature ranging from 15 to 23 °C, annual average rainfall 770 mm) on a silt–clay soil with sub-alkaline pH, normal salinity, average critical salt concentrations, traces of limestone, low organic matter and nitrogen contents and high phosphorus and potassium contents. Each year, lupine seeds were sown in the last days of November at 60 plants m^{-2} with a distance of 30 cm between rows and 5.5 cm between plants within each row, following a randomized block experimental design with four parcel repetitions. Seeds from 30 plants for each parcel were randomly collected at the end of April the following year. After collection, all samples were stored under the same conditions at 4 °C until the analyses were carried out.

Overall, the total number of samples was 3 (varieties) × 3 (years) × 4 (parcel repetitions) × 3 (analytical replicates) = 108. Only field replicates were used for the statistical comparison.

Chemical composition

All samples were ground to pass a 1 mm screen (Brabender Wiley mill, Brabender OHG, Duisburg, Germany) and analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to AOAC methods 2001.12, 978.04, 920.39, 978.10 and 930.05 respectively.[22] Neutral detergent fiber (NDF) was determined as described by Van Soest *et al.*[23] and corrected for ash. Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined as described by Goering and Van Soest.[24] Starch content was measured after acid hydrolysis by polarimetric detection.[25]

Extraction and identification of alkaloids

Alkaloids were extracted as described by Muzquiz *et al.*[26] and Oboh *et al.*[27] The alkaloids in the sample extracts were extracted with dichloromethane and analyzed by high-resolution gas chromatography/mass spectrometry (HRGC/MS)[28] using an Agilent 6890 N gas chromatograph coupled to an Agilent 5973 inert mass-selective detector (Agilent Technologies, Palo Alto, CA, USA). An HP-5 capillary column (30 m length × 0.25 mm i.d., 0.25 µm film thickness; Agilent J&W GC Column) was used. Samples (1 µL) were injected in split mode (1:10), the injector temperature was 240 °C and the HRGC/MS interface temperature was 250 °C. Spectral acquisition was from *m/z* 50 to 300 and the source operated in electron impact mode at 70 eV. The column temperature was programmed to rise at 5 °C · min^{-1} from 150 to 235 °C (held for 15 min). Helium was used as carrier gas at an average linear velocity of 35 cm · s^{-1} and a flow rate of 1 mL · min^{-1}. Alkaloid quantification was performed in full-scan mode by the internal standard method using caffeine as analytical standard.[28] In the standard solutions the limit of quantification (signal/noise ratio >7) was 0.2 mg kg^{-1} for sparteine and 0.4 mg kg^{-1} for all other alkaloids.[18]

Extraction and identification of fatty acid methyl esters

In order to analyze the fatty acid profile, the crude oil from lupine seeds was extracted by the method of Boschin *et al.*[20] The fatty acid methyl esters (FAMEs) of lupine seeds were prepared by direct transesterification.[29] The FAMEs were analyzed by gas chromatography with flame ionization detection (GC-FID) using an Agilent 6890 N gas chromatograph with a split/splitless injector, a flame ionization detector and an Omegawax 250 fused silica capillary column (30 m length × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA), as described by Chiofalo *et al.*[1] Identification of fatty acids was made by comparing the relative retention times of FAME peaks of samples with those of standards from Supelco. Chromatogram peak areas were acquired and calculated using Chemstation software (Agilent Technologies). Concentrations of individual fatty acids were expressed as g kg^{-1} total FAMEs identified. Fatty acids were grouped into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The chromatographic analysis was replicated three times for each sample.

Owing to the relevance for human health, on the basis of the identified fatty acids, the atherogenic index (AI) and thrombogenic index (TI) were calculated using the equations proposed by Ulbricht and Southgate.[30]

In vitro gas production

The fermentation characteristics and kinetics were studied using the *in vitro* gas production technique by incubating the three varieties of lupine at 39 °C under anaerobic conditions with buffered rumen fluid.[31] Each test sample was ground (1 mm screen) and weighed (1.0043 ± 0.025 g) in triplicate into 120 mL serum flasks, then 74 mL of anaerobic medium was added.[32] Rumen fluid was collected in a pre-warmed thermos at a slaughterhouse authorized according to EU legislation[33] from six mature cows fed a total mixed ration containing corn silage, oat hay and concentrate (NDF 435 g kg^{-1} DM and crude protein 120 g kg^{-1} DM). The collected material was rapidly transported to the laboratory, where it was pooled, flushed with CO_2, filtered through cheesecloth and added to each flask (5 mL) within 1 h of collection. Three flasks containing no substrate were incubated as blanks to correct for organic matter (OM) disappearance and gas and end-product production.

Gas production of fermenting cultures was recorded 22 times (at 2–24 h intervals) during the period of incubation (96 h) using a manual pressure transducer (Cole-Palmer Instrument Co., Vernon Hills, IL, USA). For each flask, the gas production profiles were fitted to the sigmoid model described by Groot *et al.*:[34]

$$G\left(\text{mL g}^{-1}\right) = A/\left(1 + B/t\right)^{C}$$

where G is the total gas produced (mL g^{-1} OM) at time t (h), A is the asymptotic gas production (mL g^{-1} OM), B is the time at which one-half of the asymptote is reached (h) and C is the switching characteristic of the curve. The maximum fermentation rate (R_{max}) and the time at which it occurred (T_{max}) were calculated according to the following formulae:[35]

$$R_{max}\left(\text{mL h}^{-1}\right) = \left(AC^{B}\right) B \left(T_{max}^{-B-1}\right) / \left[\left(1 + C^{B}\right)\left(T_{max}^{-B}\right)^{2}\right]$$

$$T_{max}\left(\text{h}\right) = C\left[\left(B - 1\right)/\left(B + 1\right)\right]^{1/B}$$

After 96 h of incubation, the fermentation liquor was analyzed for pH and sampled for end-product analysis. At the end of fermentation, the extent of sample disappearance, expressed as organic matter digestibility (dOM, g kg^{-1}), was determined by the weight difference of the incubated OM and the undegraded filtered (sintered glass crucibles, porosity #2; Schott Duran, Mainz, Germany) residue burned at 550 °C for 5 h. The cumulative volume of gas produced after 96 h of incubation was related to the incubated OM (OMCV, mL g^{-1}).

For volatile fatty acid (VFA) determination, fermenting liquors were centrifuged at 12 000 × *g* for 10 min at 4 °C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Vlotho, Germany). A 1 mL aliquot of supernatant was then mixed with 1 mL of oxalic acid (0.06 mol L^{-1}). VFA were measured by gas chromatography (ThermoQuest 8000 Top, Rodano Italia SpA, Milan, Italy; fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness), using an external standard solution composed of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids, as described by Calabrò *et al.*[36] Using VFA data, the following indices were estimated: total VFA (tVFA) = acetic + propionic + butyric + isobutyric + valeric + isovaleric, A/P = acetic/propionic ratio and BCP (branched-chain fatty acid proportion) = (isobutyric + isovaleric)/tVFA.

Data processing

The nutritive value of the studied lupine cultivars was estimated as metabolizable energy (ME, MJ kg^{-1} DM) using the equation proposed by Menke and Steingass[37] for this kind of feed:

$$\text{ME} = -2.3 + 0.1335 \times \text{CP} + 0.0121 \times \text{EE} + 0.0281 \times \text{NDF} + 0.0055 \times \text{GP24}$$

where CP, EE and NDF are the respective contents (g kg^{-1} DM) of crude protein, ether extract and structural carbohydrates in the samples and GP24 is the gas obtained *in vitro* (mL per 200 mg incubated DM) after 24 h of incubation.

Chemical composition data, fatty acid contents, alkaloid contents, fermentation characteristics (gas and VFA production, OM degradability and pH) and model parameters (A, B, t_{max} and R_{max}) were subjected to analysis of variance (PROC GLM)[38] to detect the effects of lupine cultivar and harvest year according to the following model:

$$y_{ijk} = \mu + \text{CV}_i + \text{HY}_j + (\text{CV} \times \text{HY})_{ij} + \varepsilon_{ijk}$$

where y is the single datum, μ is the mean, CV is the cultivar effect (i = 1, 2, 3), HY is the harvest year effect (j = 1, 2, 3), CV × HY is the first-order interaction and ε is the error term.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the three varieties of lupine is shown in Table 1. As expected, on average, the CP content was high (396 g kg^{-1} DM), the level of lipids was high (89.9 g kg^{-1} DM) and the starch content was low (93.3 g kg^{-1} DM) in all samples. As reported by Abreu and Bruno-Soares,[39] the high protein content in *Lupinus* spp. is partly due to the low level of starch, which is replaced by fat as the main energy source. As expected, lupine seeds showed more dietetically beneficial structural carbohydrates (NDF 242 g kg^{-1} DM) compared with other leguminous seeds (peas, faba beans, soy beans); this fraction is mainly represented by pectin-like material.[40] The ME value, estimated from chemical and gas production data, differed slightly between seeds (mean 14.5 ± 0.60 MJ kg^{-1} DM). It is necessary to underline that, in ME calculation, fat and fiber levels as well as other nutrient availability have a negative influence on microorganism activity in the rumen and consequently on *in vitro* fermentation.[41] Overall, the slight difference observed between the results of this experiment and the values reported in the literature[42] can be attributed to the cultivars used and the sites and climatic conditions of their growth.

In general, small differences emerged between samples as a result of harvest year or variety effect. In particular, comparing the lupine seeds as a function of harvest year, it is possible to note that, in year 2, better seeds in terms of highest CP (419 g kg^{-1} DM), EE (98.2 g kg^{-1} DM) and starch (96.9 g kg^{-1} DM) contents as well as lower contents of structural carbohydrate fractions (NDF 206 g kg^{-1} DM, ADL 5.1 g kg^{-1} DM) were obtained; this trend was clearer for LB and MU. These results are partially due to the lower seed yields in year 2 (38, 37 and 38 q ha^{-1}) compared with year 1 (60, 59 and 38 q ha^{-1}) and year 3 (52, 51 and 38 q ha^{-1}) for MU, LB and LT respectively, as reported in the agronomic trial,[21] which probably led to a higher concentration of nutrients. It is likely that the variation in rainfall (84.7, 66.5 and 82.2 mm) and temperature (12.2, 11.0 and 11.9 °C) recorded during the growing season by the Italian Department of Meteorology in the three harvest years

Table 1. Chemical composition (g kg^{-1} DM) and nutritive value (MJ kg^{-1} DM) of three varieties of lupine

Sample	DM	CP	CF	EE	NDF	ADF	ADL	Hemicellulose	Starch	Ash	ME
LB1	933A	369C	143A	67.8C	321A	257A	36.4B	63.9B	81.1B	43.3AB	14.0
LB2	913B	456A	100C	99.5A	213B	99.1C	4.9C	113A	125A	46.0A	14.2
LB3	902C	423B	130B	87.2B	212B	155B	63.9A	56.5B	87.2B	41.1B	15.4
LT1	943A	353B	129B	57.3B	299A	235A	28.4A	63.7B	92.2A	41.9A	13.9
LT2	922B	408A	114C	106A	216B	95.4C	7.50B	120A	115A	40.1ABa	14.8
LT3	908C	346B	140A	102A	209B	173B	3.20B	36.2C	66.9B	36.7Bb	14.6
MU1	940A	367C	110B	95.4Ba	278A	193A	3.10B	85.0A	111A	39.2Ba	14.5
MU2	903B	389B	129A	89.2Bb	210C	159B	3.00B	50.6B	50.5B	34.9Bb	13.6
MU3	905B	447A	129A	105A	244B	159B	28.1A	85.4A	111A	42.3A	15.2
SEM	1.05	4.60	2.04	1.69	5.78	6.48	4.13	5.98	4.19	0.87	1.13
Prob. *t*											
CV	***	***	***	***	NS	NS	***	NS	NS	***	***
HY	***	***	NS	***	***	***	***	***	NS	NS	***
CV × HY	***	***	***	***	***	***	***	***	***	***	***

DM, dry matter; CP, crude protein; CF, crude fiber; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; hemicellulose = NDF − ADF; ME (MJ kg^{-1} DM), metabolizable energy estimated according to Menke and Steingass.[37] LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B, C) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; NS, not significant.

(2007, 2008 and 2009 respectively) partly influenced these results. Moreover, the lowest nutritive value (lower CP and high fiber content) in year 1 is probably due to damage from a spring hailstorm, mainly for LB and MU. As reported by Sij *et al.*[43] in a field study with guar simulating hail damage, plant defoliation due to the wet weather resulted in rapid deterioration of bean quality. Comparing the three varieties during the three harvest years, the highest CP (456 g kg^{-1} DM) and starch (125 g kg^{-1} DM) contents were found in LB2, which in the agronomic trial had the lowest production (37 q ha^{-1}) and was ready for harvest latest in the season.[21] However, LB also showed a very high lignin value (mean 35.1 g kg^{-1} DM), which is difficult to explain. The highest value for EE was found in MU (mean 96.5 g kg^{-1} DM), and interesting differences among the three varieties were also observed for the lipid content of the seeds. These results are in accordance with those reported by Boschin *et al.*[20] for white lupine seed cv. Luxe grown in 13 Italian environments and by Uzun *et al.*[44] for white lupine seed.

Alkaloid content

As reported in Table 2, six alkaloids were detected and quantified: sparteine, lupanine, 13α-hydroxylupanine, α-isolupanine, angustifoline and multiflorine. The chromatographic analysis allowed the quantification of eight alkaloids, but only six of these were identified. All identified alkaloids levels were significantly influenced by harvest year, cultivar and their interaction. The analysis of the results showed that LT3 had a much lower alkaloid content than the safe limit of toxicity (20 mg per 100 g) indicated for human and animal consumption by the health authorities of the UK, France and Australia.[45] As expected, very low concentrations of quinolizidine alkaloids were also observed in LB and LT, except for LB2, where the alkaloid level was higher than the limit of toxicity (67.9 mg per 100 g). MU showed higher levels of alkaloids (136–165 mg per 100 g) than LT (9.15–14.5 mg per 100 g) and LB (3.63–67.9 mg per 100 g); the data are in accordance with those reported by Gresta *et al.*[18] for *L. albus* cv. Multitalia (166 mg per 100 g). Considering that plant breeders have developed so-called 'sweet lupines' with low alkaloid content (<50 mg per 100 g) for human and animal diets,[46] LT and LB can be considered 'sweet lupines' in comparison with the bitter MU, the historical variety less selected by geneticists. Lupanine was the most represented and sparteine the least represented alkaloid; these data are slightly higher than those observed by Reinhard *et al.*[46] in the alkaloid profile of *L. albus*, by Boschin *et al.*[45] in nine alkaloid-poor varieties of *L. albus* and *Lupinus angustifolius* grown in two climatically contrasting Italian sites and by Gresta *et al.*[18] in nine sweet varieties of *L. albus*, *Lupinus luteus* and *L. angustifolius* cultivated in the Mediterranean region. Angustifoline has been observed at low levels in *L. albus* as reported by Wink *et al.*;[47] nevertheless, a high quantity was found in MU. On the whole, the comparison among the varieties of *L. albus* cultivated in the same environment showed that the alkaloid levels of the six LB and LT samples were considerably lower those of the three MU samples; a large part of this difference may be ascribed to the marked breeding improvement of the new varieties.

Fatty acid profile

The content of fatty acids (SFA, MUFA and PUFA) is reported in Tables 3 and 4. In the three varieties of lupine, eight SFA were identified (Table 3); among these, the most represented was palmitic acid, found at significantly higher ($P < 0.001$) levels in seeds of LT (mean 83.2 g kg^{-1}), which also showed significant differences ($P < 0.001$) in relation to harvest year and the interaction of cultivar and harvest year (CV × HY), followed by stearic acid, which showed no significant differences in relation to all variables. Palmitic acid content was similar to that observed by Uzun *et al.*[44] in *L. albus* seeds (76 g kg^{-1}), while stearic acid content was similar to that reported in seeds of other leguminous genera which can be used in animal and human nutrition, such as *Hedysarum*, *Lathyrus*, *Gonocytisus*, *Trigonella*, *Onobrychis*, *Lens*, *Pisum*, *Astragalus* and *Vicia*.[48–51] Concerning long-chain SFA (arachidic, behenic and lignoceric acids), significant differences were observed in relation

Table 2. Composition of quinolizidine alkaloids (mg per 100 g) of three varieties of lupine

Sample	Sparteine	Lupanine	13α-Hydroxylupanine	NI 1	α-Isolupanine	Angustifoline	NI 2	Multiflorine	Total
LB1	0.036AB	2.68Ba	0.381B	0.110Bb	0.105B	0.067Bb	0.226B	0.021B	3.63C
LB2	0.026A	56.1A	2.58A	2.230A	0.467A	0.754A	1.060A	4.630A	67.9A
LB3	0.017B	10.6Bb	0.684B	0.437Ba	0.124B	0.336Ba	0.323B	0.333B	12.9B
LT1	0.042B	5.13	1.13B	0.689	0.187	0.572	0.624b	0.785	9.15
LT2	0.096A	8.92	1.90A	0.662	0.228	0.623	0.771ab	1.280	14.5
LT3	0.064AB	8.18	1.44AB	0.601	0.177	0.764	0.692a	1.320	13.2
MU1	0.174A	111B	6.18Ba	6.55A	1.110A	4.30A	2.09A	14.40A	146B
MU2	0.200A	134A	6.50A	5.44B	1.200A	3.93B	2.06A	10.90B	165A
MU3	0.074B	109B	5.49Bb	5.12C	0.802B	3.25C	1.71B	10.40B	136C
SEM	0.013	1.840	0.157	0.076	0.057	0.068	0.035	0.019	1.99
Prob. *t*									
CV	***	***	***	***	***	***	***	***	***
HY	**	***	***	***	***	***	***	***	***
CV × HY	**	***	***	***	***	***	**	***	***

NI 1, not identified 1; NI 2, not identified 2. LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B, C) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{**}P < 0.01$.

to cultivar, harvest year and their interaction. Moreover, the sum of long-chain SFA (arachidic, behenic and lignoceric acids) in harvest year 2 showed significantly higher values in LB and MU (66.8 and 66.6 g kg^{-1} respectively) than in LT (55.9 g kg^{-1}). Such findings are interesting from a nutritional point of view, since oils with high levels of long-chain SFA were reported to be difficult to digest in both humans and animals.[52]

Seven MUFA were identified and quantified (Table 4); among these, the most represented were oleic acid, which showed significantly higher ($P < 0.001$) values in LT, particularly in LT1 and LT3, and gadoleic acid, showing significantly higher ($P < 0.001$) levels in LB and MU, particularly in MU1. The statistical analysis showed a significant effect of cultivar ($P < 0.001$) on oleic acid content and a significant effect of harvest year and cultivar ($P < 0.001$) and their interaction ($P < 0.01$) on gadoleic acid content.

Concerning erucic acid, which is considered an anti-quality factor for animal and human metabolism, the average relative content was significantly higher ($P < 0.001$) in MU and LB than in LT; in particular, among the years of harvest, the highest level was observed in LB2 and MU (1, 2 and 3), while the lowest level was found in LT1 and LT3. As regards oleic acid, our results are similar to those reported by Uzun *et al.*[44] in *L. albus* seeds (476 g kg^{-1} on average). The content of erucic acid in our samples was lower than that observed by Bhardwai *et al.*[53] in white lupine seed cv. Lunoble (23.6 g kg^{-1}) and cv. Lucyanne (27.3 g kg^{-1}), by Boschin *et al.*[20] in white lupine seed cv. Luxe grown in 13 Italian environments (39–53 g kg^{-1}) and by Volek and Marounek[54] in white lupine seed cv. Amiga (36.9 g kg^{-1}). Although the effects of erucic acid on human health are controversial,[55] the government regulation of the European Union limits the level of erucic acid for human consumption to a maximum of 50 g kg^{-1} of the total level of fatty acids in the fat component.[56] Moreover, a content of erucic acid up to 30 g kg^{-1} is not considered detrimental to human health.[57] In this regard, the values obtained in this study are below the maximum level fixed for this acid. The FAO/WHO has developed for rapeseed oil the definition of 'erucic acid-free oil' when the content is lower than 10 g kg^{-1} and of 'oil with a low erucic acid content' when the content is lower than 20 g kg^{-1}.[57] In this respect, all LT samples harvested in the three years of our study may be defined as 'erucic acid-free'.

Among PUFA (Table 4), linoleic acid was the most represented in all species, with a major content, on average, in LT and MU and significant differences ($P < 0.001$) in relation to cultivar and HY × CV, followed by α-linolenic acid, which showed a significantly higher ($P < 0.001$) level in LB and MU and a significant effect in relation to harvest year and HY × CV ($P < 0.05$). These data are in accordance with those reported by Uzun *et al.*[44] in *L. albus* seeds for linoleic acid (203 g kg^{-1}) and linolenic acid (92 g kg^{-1}). The high content of essential fatty acids (C18:2n6 and C18:3n3) found in lupine oil is typical of many leguminous genera[48] and suggests that this legume seed could be used in feed mixtures for farm animals to improve the nutritional quality of their products.[11]

Table 5 presents the fatty acid classes, the fatty acid ratios and the quality indices AI and TI. From a nutritional point of view, LB and LT showed higher values of the *n*-3/*n*-6 PUFA ratio, with a significant effect of harvest year, cultivar and their interaction ($P < 0.001$), whereas MU showed higher values of the ratio UFA/SFA, with a significant effect of harvest year ($P < 0.05$), cultivar ($P < 0.01$) and their interaction ($P < 0.05$), and of the ratio PUFA/SFA, with a significant effect of cultivar and HY × CV ($P < 0.01$). Finally, AI and TI, strictly relating to the fatty acid profile, showed significantly lower values in MU; AI was significantly influenced by harvest year, cultivar and their interaction ($P < 0.001$), whereas TI was significantly influenced only by cultivar ($P < 0.001$). The *n*-3/*n*-6 PUFA ratio, quite different among lupine cultivars, was in line with the observations of Boschin *et al.*[20] in white lupine seed cv. Luxe grown in 13 Italian environments (0.45–0.63).

Studies on the relationship between the *n*-3/*n*-6 PUFA ratio and the pathogenesis of many diseases indicate that the optimal ratio may vary with the disease or condition under consideration; this is consistent with the fact that many diseases are multigenic and multifactorial. The *n*-3/*n*-6 profiles of lupine seeds are in the range from 1:1 to 1:4, which is considered optimal for human and animal nutrition.[58] On average, the high content of essential fatty acids, α-linolenic acid and linoleic acid and the low value of the quality

Table 3. Mean values of saturated fatty acid content (g kg^{-1} total fatty acid methyl esters identified) in three varieties of lupine ($n = 3$)

Sample	14:0	15:0	16:0	17:0	18:0	20:0	22:0	24:0
LB1	1.27B	0.55B	73.9B	0.53	24.6	10.1B	38.2B	9.40B
LB2	1.36A	0.61A	78.3A	0.55	21.5	12.3A	43.9A	10.6A
LB3	1.30B	0.64A	79.6A	0.51	21.3	12.4A	43.4A	10.9A
LT1	1.00A	0.72Aa	84.5A	0.62	23.8	12.5A	36.6Aa	11.3A
LT2	0.79Bb	0.63Bc	81.4B	0.57	23.8	11.5B	34.2b	10.2B
LT3	0.85Ba	0.68Ab	83.7A	0.58	29.4	11.8B	33.0B	10.1B
MU1	0.94b	0.55b	67.9B	0.51	22.4	9.60A	36.5B	9.20B
MU2	0.98a	0.60a	72.7A	0.51	23.3	11.7B	44.1A	10.8A
MU3	0.7014	0.58	71.8A	0.52	22.0	11.7B	42.1A	9.80AB
SEM	0.01	0.01	0.45	0.02	3.66	0.14	0.67	0.21
Prob. *t*								
HY	NS	*	***	NS	NS	***	***	*
CV	***	***	***	***	NS	***	***	*
CV × HY	***	***	***	NS	NS	***	***	***

LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B) $P < 0.01$ or (a, b, c) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{*}P < 0.05$; NS, not significant.

Table 4. Mean values of unsaturated fatty acid content (g kg^{-1} total fatty acid methyl esters identified) in three varieties of lupine ($n = 3$)

Sample	16:1	17:1	18:1n9	18:1n7	20:1n9	20:1n7	22:1n9	18:2n6	18:3n3	20:2n6
LB1	5.27A	0.68A	475	23.7	42.7a	1.14	19.6ab	17.0	97.1ab	3.19
LB2	5.56A	0.62B	465	20.6	41.4Ab	1.20	20.9a	170	100a	3.34
LB3	4.40B	0.62B	467	24.7	40.2Bb	1.05	18.7b	170	94.6b	3.27
LT1	5.94A	0.68	502a	27.4	25.9	0.86	5.8B	175B	83.7	2.03
LT2	4.33B	0.68	494b	26.4	25.8	0.83	9.4A	189A	83.9	2.04
LT3	4.72B	0.71	491ab	26.7	24.9	0.85	6.1B	188A	85.8	1.90
MU1	4.05b	0.67	469	21.7	44.5A	01.08	21.5	195A	91.2Bb	4.06A
MU2	4.76a	0.66	469	22.3	41.5Bb	01.15	21.5	178B	96.1A	3.58B
MU3	4.73a	0.66	466	2.3.9	42.5Ba	01.20	21.0	179B	94.9a	3.65B
SEM	0.17	0.02	3.44	1.59	0.32	0.03	0.53	1.26	0.95	0.06
Prob. *t*										
HY	*	NS	NS	NS	***	NS	**	NS	*	*
CV	**	*	***	*	***	***	***	***	***	***
CV × HY	***	NS	NS	NS	**	*	NS	***	*	**

LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

indices of the three lupine varieties make the lipid fraction of this crop suitable for animal and human nutrition. On the whole, variations in the environmental conditions when the seeds were collected may also have exerted a key role, since it is known that the environment can significantly affect the synthesis of fatty acids in plants.[52,59]

In vitro fermentation characteristics

The values shown in Tables 6 and 7 indicate that fermentation was substantial in terms of OM digestibility (dOM 892 g kg^{-1}), gas production (OMCV 317 mL g^{-1} OM) and total VFA production (115 mmol g^{-1} OM). However, according to Calabrò *et al.*,[41] the *in vitro* characteristics of lupine are lower than those of other Leguminosae seeds (peas, faba beans, soy beans) because of the chemical composition (high content of protein, fiber and lipids and low starch level). For all samples, the final pH after 96 h of incubation indicated that the buffering capacity of the medium was sufficient to maintain the pH above 6.4, the necessary value to ensure optimal activity of the cellulolytic bacteria.[37] The high BCP ((isobutyric + valeric)/tVFA) values (mean 0.072) are due to the intense degradability of amino acids (valine and leucine) of this Leguminosae seed,[60] whose fermentation leads to the production of these volatile fatty acids.

As a whole, all fermentation parameters were only slightly affected by harvest year, cultivar and their interaction. Regarding the influence of harvest year on the *in vitro* data, year 2 showed a significantly lower ($P < 0.001$) real gas production value (OMCV 294 mL g^{-1} OM) compared with years 1 and 3 (326 and 331 mL g^{-1} OM respectively) but a significantly higher ($P < 0.001$) total VFA

Table 5. Mean values of fatty acid class content (g kg^{-1} total fatty acid methyl esters identified), ratios and quality indices in three varieties of lupine ($n = 3$)

Sample	SFA	MUFA	PUFA	*n*-3 PUFA	*n*-6 PUFA	UFA/SFA	PUFA/SFA	*n*-3/*n*-6	AI	TI
LB1	159	568a	273	97.1AB	171	5.31a	1.73	0.552Ab	0.094B	0.150
LB2	168	555b	276	100A	1716	4.94ab	1.65	0.567Aa	0.101A	0.151
LB3	170	556b	261	94.6B	180	4.88b	1.61	0.530B	0.103A	0.156
LT1	171	568ab	261B	83.7	177B	4.86	1.53b	0.730A	0.107Aa	0.170
LT2	163	562a	275A	83.9	192A	5.10	1.69a	0.438Ba	0.101B	0.168
LT3	170	555b	275A	85.8	190A	4.90	1.63ab	0.453Bb	0.105Ab	0.180
MU1	148Bb	562	290A	91.2Bb	199A	5.78Aa	1.97A	0.458B	0.084B	0.139
MU2	165A	558	278B	96.1A	191B	5.08B	1.69B	0.528A	0.092A	0.147
MU3	160a	563	277B	94.9a	182B	5.27b	1.74B	0.521A	0.090A	0.143
SEM	3.62	3.11	1.98	0.95	0.128	0.130	0.043	0.0042	0.00065	0.0064
Prob. *t*										
HY	NS	*	NS	*	NS	*	NS	***	***	NS
CV	*	NS	***	***	***	**	**	***	***	***
CV × HY	NS	NS	***	*	***	*	**	***	***	NS

SFA (saturated fatty acids) = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0; MUFA (monounsaturated fatty acids) = C16:1 + C17:1 + C18:1n9 + C18:1n7 + C20:1n9 + C20:1n7 + C22:1n9; PUFA (polyunsaturated fatty acids) = C18:2n6 + C18:3n3 + C20:2n6; *n*-3 PUFA, *n*-3 polyunsaturated fatty acids; *n*-6 PUFA, *n*-6 polyunsaturated fatty acids; UFA/SFA, unsaturated/saturated fatty acid ratio; PUFA/SFA, polyunsaturated/saturated fatty acid ratio; *n*-3/*n*-6, *n*-3/*n*-6 polyunsaturated fatty acid ratio; AI, atherogenic index; TI, thrombogenic index. LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

Table 6. Mean values of *in vitro* gas production characteristics in three varieties of lupine ($n = 3$)

Sample	dOM (g kg^{-1})	OMCV (mL g^{-1})	*A* (mL g^{-1})	*B* (h)	T_{max} (h)	R_{max} (mL h^{-1})
LB1	872	319Ab	370AB	9.48A	0.423	31.6
LB2	899	276B	336B	7.85B	0.423	33.3
LB3	882	352Aa	396A	9.32A	0.850	31.0
LT1	923A	341a	387	9.61B	0.633	31.9a
LT2	924A	312b	375	12.5A	0.617	23.7Bb
LT3	843B	338	374	8.45B	0.660	34.7A
MU1	905	318	366A	9.23A	0.423	32.3
MU2	883	293	318B	7.25B	0.843	32.6
MU3	898	304	347AB	9.61A	1.14	27.3
SEM	16.9	9.56	12.1	0.40	0.375	2.59
Prob. *t*						
CV	NS	*	**	***	NS	NS
HY	NS	***	**	NS	NS	NS
CV × HY	*	*	NS	***	NS	*

dOM, organic matter digestibility; OMCV, cumulative volume of gas related to incubated OM; *A*, potential gas production; *B*, time at which *A*/2 was formed; T_{max}, time at which maximum rate was reached; R_{max}, maximum fermentation rate. LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

production (127 mmol g^{-1} OM) compared with years 1 and 3 (111 and 107 mmol g^{-1} OM respectively). On the other hand, the OM degradability was not statistically different between the three harvest years. Not easy to explain is the very high value of acetate (50.9% tVFA, $P < 0.01$) in year 2 compared with years 1 and 3 (38.2 and 36.4% tVFA respectively).

Comparing the *in vitro* fermentation characteristics of the three varieties, very few differences appeared. In particular, LT showed the significantly highest ($P < 0.01$) real gas production (OMCV 330 mL g^{-1} OM) and MU the significantly highest ($P < 0.01$) time to reach the maximum fermentation rate (T_{max} 0.80 h). No significant difference was detected for VFA between lupine varieties.

Fermentation kinetics

As shown in Fig. 1, gas production in the first 12 h of incubation did not allow discrimination of the studied legume seeds. Consequently, in the first 12 h of fermentation, the effect of harvest year was not clear and no consistent difference was seen between

Table 7. Mean values of *in vitro* end-products (mmol g^{-1}) in three varieties of lupine ($n = 3$)

Sample	pH	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	tVFA	A/P	BCP
LB1	6.66B	35.3B	25.2	3.69a	24.9	9.09	5.06	103B	1.40B	0.085A
LB2	6.72A	67.6A	24.8	2.39b	19.9	7.03	3.82	126Aa	2.73A	0.050B
LB3	6.68	39.8B	24.5	3.67a	23.6	9.40	5.40	106b	1.63B	0.086A
LT1	6.66	53.5A	27.2	3.11	21.1	7.37	4.19	116a	1.99ab	0.062b
LT2	6.63	58.9A	27.5	3.43	27.8	9.07	5.02	132Ab	2.27a	0.063b
LT3	6.62	33.9B	26.0	3.96	26.5	9.66	5.40	105B	1.31b	0.088a
MU1	6.63	36.5Bb	29.9	3.85a	27.1a	9.88a	5.58	113	1.23B	0.084Aa
MU2	6.70a	66.6A	26.3	2.62b	18.5b	6.29b	3.83	124	2.54Aa	0.052B
MU3	6.66b	43.8a	25.4	3.52ab	24.3ab	8.81ab	4.60	110	1.72b	0.074b
SEM	0.0162	4.63	1.81	0.319	2.51	1.05	0.62	4.76	0.239	0.0068
Prob. *t*										
CV	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
HY	*	***	NS	**	NS	NS	NS	NS	***	***
CV × HY	*	*	NS	NS	NS	NS	NS	NS	NS	NS

tVFA, total volatile fatty acids; A/P, acetic/propionic ratio; BCP, branched-chain fatty acid proportion. LB1, LB2 and LB3, Lublanc variety in harvest years 1, 2 and 3; LT1, LT2 and LT3, Lutteur variety in harvest years 1, 2 and 3; MU1, MU2 and MU3, Multitalia variety in harvest years 1, 2 and 3. For each variety, means within a column with different letters are significantly different at (A, B) $P < 0.01$ or (a, b) $P < 0.05$. SEM, standard error of mean. CV, cultivar; HY, harvest year.
Significance: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

varieties. This overlapping of profiles could be a consequence of the high levels of hemicelluloses and pectic substances in lupine seeds.[61] In fact, hemicelluloses and pectins are generally the more rapidly fermentable energy sources for gut bacteria, though it also depends on where they are located within the plant cell wall (readily accessible or not). As expected, for all substrates, the fermentation process started immediately and finished rapidly within 36 h of incubation, showing on average a high value of fermentation rate (R_{max} 30.9 mL h^{-1}) reached in a short time (T_{max} 0.67 h). The highest gas production curve was always detected for LB2; in the first 24 h, LT2 showed the slowest process, which subsequently accelerated; conversely, MU2 and LB2 started soon but slowed from 36 h until the end of incubation. The fermentation rate profiles were similar among all lupine seeds; the highest fermentation rate was reached by LT3 (R_{max} 34.7 mL h^{-1}) at 0.660 h (T_{max}) and the lowest by LT2 (R_{max} 23.7 mL h^{-1}) at 0.617 h (T_{max}).

In vitro fermentation

It is known that the chemical composition and the presence of other nutrients in feed influence the *in vitro* fermentation of incubated substrates. In our study, on the one hand, the relatively high content of protein, fat and fiber (in some cases highly lignified) and the low level of starch and, on the other hand, the good level of hemicelluloses were responsible for the fermentation process trend (high fermentation rate reached in a short time) and its extent (high amount of gas and VFA produced and OM degradability).

Moreover, the lowest gas production in LB2 ($P < 0.01$) and MU2 ($P < 0.001$) should be due to the significantly higher values of long-chain SFA (sum of arachidic, behenic and lignoceric acids) in LB and MU (66.8 and 66.6 g kg^{-1} respectively) compared with that in LT (55.9 g kg^{-1}) in harvest year 2. As reported by many authors, the presence of antinutrient substances (quinolizidine alkaloids) might contribute to reducing microbial activity and thus gas production, OM degradability and VFA production. In particular, as reported by Rubio *et al.*,[62] gastrointestinal lactobacilli (*Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus salivarius* and *Lactobacillus brevis*) numbers were consistently increased in all intestinal sections of chickens fed dehulled lupine-based diets compared with whole lupine-based diets. Concerning lupanine and sparteine, Aguiar *et al.*[63] reported that these quinolizidine alkaloids decreased gas production and apparent digestibility, with the effect of lupanine being greater than that of sparteine. Probably also in our experiment, the levels of lupanine (Table 2) in LB2 and MU2, which were significantly higher than those in LB1 and LB3 ($P < 0.01$) as well as those in MU1 and MU3 ($P < 0.01$), influenced potential gas production (Table 6). However, except for the bitter MU, the variety less selected by geneticists, the lupanine content was too low to play any important biological activity in either human or animal metabolism.[64]

In our trial, the presence of quinolizidine alkaloids seemed to have no effect on OM degradability; this result could be due to the level or type of these substances in the lupine cultivars.[63] On the other hand, erucic acid, considered an ANF, as well as the quinolizidine alkaloids,[63] could have negatively influenced gas production and also apparent digestibility, which, in fact, appeared lower in LB2 and MU2 than in LT2. The absence of a correlation between OM degradability, gas production and VFA production should be due to the variation in OM composition of these feeds;[61] in particular, the high content of nitrogen compounds in the tested substrate can improve OM disappearance and microorganism activity but not gas production. According to other authors,[61–63] the presence of ANF substances (lupanine in particular) also had a negative effect on the fermentation kinetics, particularly at the beginning of incubation. In our case, LB2, characterized by a high lupanine content (56.1 mg per 100 g, $P < 0.01$), showed the slowest fermentation kinetics (*B* 7.85 h).

CONCLUSION

Chemical characteristics among lupine cultivars Lublanc, Lutteur and Multitalia varied slightly as function of harvest year, whereas alkaloid and fatty acid contents were more strongly influenced by cultivar than by harvest year. *Lupinus albus* cultivated in a Mediterranean environment showed a high-quality fatty acid

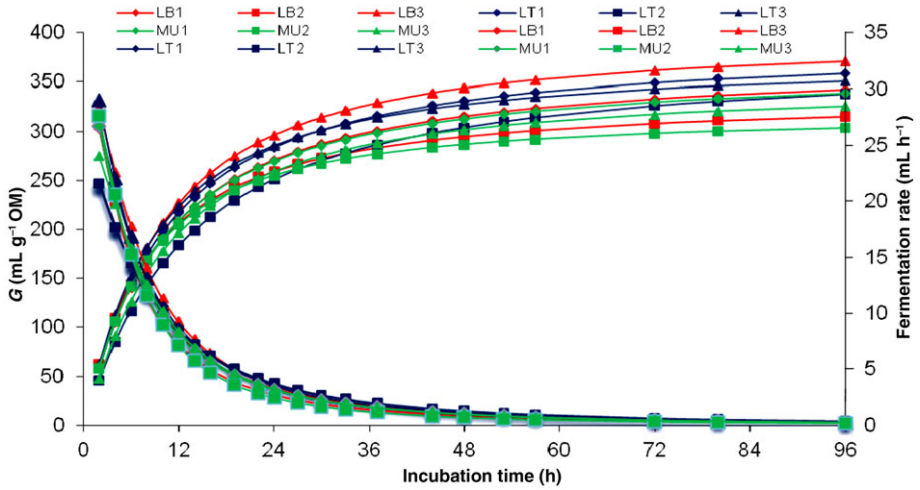

Figure 1. *In vitro* gas production (*G*) and fermentation rate over time in three varieties of lupine. The figure shows the potential gas related to incubated organic matter (OM) produced during 96 h of *in vitro* incubation and the fermentation rate calculated according to the Groot *et al.*[34] model for white lupine varieties Lublanc (LB), Lutteur (LT) and Multitalia (MU) harvested in consecutive years 1, 2 and 3.

profile in terms of linoleic and α-linolenic acids. This underlines the importance of choosing the best cultivar to ensure a favorable nutritional profile in order to improve the quality of animal products as well as human health. *In vitro* fermentation characteristics were little influenced by alkaloid content, thus indicating that the negative influence on microbe activity depends on the quality and quantity of ANFs.

The present data suggest *L. albus* cv. Lutteur to be a promising crop as food thanks to its high nutritive traits. Comparing the three varieties, Lutteur showed higher degradability, gas production and VFA production and lower alkaloid, long-chain SFA and erucic acid levels. Furthermore, despite the low crop yield, it was the most constant over time.

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