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DEPARTMENT OF AGRICULTURAL, ENVIRONMENTAL AND FOOD SCIENCES

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Studies on sustainable feedstuffs in livestock productions of inner areas in Centre-South Italy

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“The choice of a student depends on their own aptitude,
but also on the luck of meeting a great teacher.”

- Rita Levi Montalcini -

And I, I had this privilege..

“La scelta di uno studente dipende dalla sua inclinazione,
ma anche dalla fortuna di incontrare un grande docente.”

- Rita Levi Montalcini -

Ed io ho avuto questo privilegio..

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Preface

The research work carried out during the Doctoral Course in Agricultural Technology and Biotechnology, and discussed in the present thesis dissertation, is focused on the sustainable animal productions in Centre-South Italy inner areas, emphasizing the role that nutrition and animal management can have in districts particularly suited to agricultural activities, as Molise region and neighbouring territories. Starting from the need to protect the intrinsic natural characteristics of these marginal areas, with traditional vocation to livestock farming and seriously endangered by abandonment and depopulation phenomena as well as decreased productivity, in this study it was proposed to address novel issues to investigate the sustainable contribute of animal nutrition and the production activities related to it.

The research activities carried out during the presented doctoral course, are reported in five main chapters. In Chapter 1, the evolutive dynamics of two relevant pasture areas of Molise region were investigated thanks to several literature data which, since 1990, have reported all the variations occurred on plant and animal diversity, as well as on soil and climate. Because pasture is one of the main sources of feed supply for herbivorous, as well as a scenario for the economic, social, and cultural development, it could be a tool of biodiversity and environmental safeguard, which should not be underestimated. Moreover, it must be noted that the use of mountain territories for grazing is the most important and common land use, crucial for preservation from degradation phenomena, such as erosion and hydrogeological instability, and for the reinforcement of human-animal-environment system.

The following Chapters 2, 3 and 4 are dedicated to an innovative non-smoking tobacco cultivar that, for the first time, has been introduced in the diets of livestock animals. After a detailed description of the multipotentialities application and use of *Nicotiana tabacum* L., cv. *Solaris* as biofuel and biomethane producers (Chapter 2), the introduction of its co-products as feedstuffs have been described and subsequently tested in animal nutrition. Particularly, in Chapter 3, the storage attitude of cv. *Solaris* whole plant was tested through ensiling practice. The ensiled biomass was then studied as ingredient of growing heifers' diet, and the effect of the dietary treatment has been investigated on productive and reproductive performances, as well as on animal's welfare indices. Furthermore, another co-product of *Solaris* tobacco, *i.e.*, seed cake obtained from the cold oil extraction, was included in diet of growing lambs as partial replacement of soybean meal, as reported in Chapter 4. Besides assessing the palatability of this alternative protein source, lamb's growth performances were evaluated. Both on-field trials were carried out in two farms of Molise region, confirming the important link of the present Ph. D. thesis with the territory.

Finally, in Chapter 5, the growth performances of broiler chickens reared under semi-intensive conditions were evaluated by comparing two different dietary treatments and two different genotypes. Starting from the dietary ingredients traditionally used, the total flacked soybean percentage was replaced by pea bean. Besides the effects of dietary treatments and genotypes on growth performances, the two diets were also compared from economic and environmental point of view. On this regard, by the means of an open-source FAO software, the annual global warming potential of the studied diets has been estimated.

All the presented studies are connected by the same common thread to support the needed changes in the Italian and worldwide agricultural sector. In recent years, there has been a convergence towards agricultural practices defined as conservative and green, with the aim to make more sustainable and eco-compatible the entire agri-food chain, from the field to the table.

Another common feature of this thesis work is certainly the multidisciplinary approach. So that, synergic work has been carried out with soil scientists in relation to the study of Molise's pasture, and with agronomy and plant pathology researchers for the aspects related to tobacco cv. *Solaris* cultivation and ensiling, besides the cooperation with the animal scientists in the small ruminants and poultry trials.

In addition to the collaboration within the Dipartimento Agricoltura, Ambiente, Alimenti, the external cooperation with the Scuola di Bioscienze e Medicina Veterinaria of University of Camerino deserves mention also for the studies on mineral components of milk from different species (cow, buffalo, and donkey), although not directly linked to the research activity here presented.

Last but not the least, the collaboration with the private company Sunchem BV, patent holder of tobacco cv. *Solaris*, allowed the realization of several trials involving this innovative and multifunctional plant studied for the environmentally friendly recovery of tobacco cultivation knowledge in inner areas of Centre-South Italy.

Some sections of this thesis dissertation have been published as articles on indexed journals, book chapter, and conference proceedings. Particularly, some data reported and discussed in Chapter 1 and Chapter 5 have been disseminated as conference proceeding, data presented in Chapter 2 and Chapter 3 have been partially published as journal articles and as conference proceedings.

The complete list of scientific contributions is given at the end of the present thesis.

Chapter 1

Evolution of biodiversity and ecosystem conservation of two Molise region pasture areas

Introduction

Total world lands used for agricultural practices and productions is less than 40 % of which, about 26 % is classified as pasture generally located in marginal areas, not suitable for intensive crops farming (Godfray *et al.*, 2010; Pornaro *et al.*, 2019). Modern society benefits from the ecosystem functions and services that mountain pastures provide, *i.e.*, food and feed production, conservation of biodiversity and landscape, carbon storage, preservation of water quality and environment, regulation of climate changes, cultural and hedonistic perception (Silva *et al.*, 2008; FAO, 2016; Pittarello *et al.*, 2019). Pastures are included in a vulnerable ecosystem affected by several kind of degradation, caused by natural and anthropic activities (Pornaro *et al.*, 2019). For instance, an intensive land use changes occurred in last century on pasture areas because of the several socio-economic changes in the Mediterranean regions (Pittarello *et al.*, 2019). On this regard, especially during last decades, Centre-South Italy inner areas have been characterized by a gradual land abandonment in particular of hilly and mountainous districts in favor of flat areas where the major production activities and services are gathered. This has contributed to a progressive decline of the extensive and small-scale farming systems with low-income levels, determining the abandonment of lands, marginalization, and depopulation phenomenon resulting in several negative consequences as increased of soil erosion, and therefore loss of plant and animal diversity (Renwick *et al.*, 2013; Ievoli *et al.*, 2017; Quaranta *et al.*, 2020).

Mediterranean basin could be considered as a large hotspot of biodiversity thanks to the high number of plant species and grazing animals. Mountain pasture represents an important feed resources for grazing animals that strongly affect the vegetation dynamics and landscape diversity (Cosentino *et al.*, 2014). For these reasons, to preserve and regulate the grassland ecosystem integrity, grazing management must be considered as necessary tool for a sustainable pasture utilization, *i.e.*, respectful of plant diversity, nutrient flows, animal wastes control, and physical and biological degradation phenomena occurred on soil (Salimei *et al.*, 2005; Sollenberger *et al.*, 2020). Among the main factors influencing the complex pasture ecosystems, special attention should be devoted to herbivorous species, animal size, seasonal or continuous grazing regime, topography, and climate changes responsible, for instance, to the population dynamics and forage nutritive values (Gao and Camel, 2020; Miraglia *et al.*, 2020). According to Launchbaugh (2020), herbivores may have different feeding behaviors to fulfil their nutrient requirements for growth and reproductive performances. Grasslands are an important source of energy in terms of cellulose, hemicellulose, lignin, and other polymers and grazing species evolved to achieve energy from plant cell walls thanks to the symbiotic relationship with the gastroenteric microbiota. According to Pulina *et al.* (1999), practical and flexible models for the assessment of environmentally friendly grazing pressure, *i.e.*, compatible with both the environment preservation and animal's behavior and requirements, are needed in pasture management. Uncontrolled grazed areas, frequently over-used or under-used, may have a negative role towards plant productivity and quality, that affect animals' forage intake and behavior. The average number of grazing animals should be therefore managed aiming to improve the preservation of pasture landscape and vegetal and animal diversity, while exploiting the role of animal productions as main co-factor of agriculture ecosystem services, and the traditional practices related to the food chains.

Aim

Based on the need to protect the natural traits of a territory characterized by generalized abandonment, depopulation and decrease of productive activities, and considering the natural agricultural-zootechnical vocation of many areas of the Molise region, the study of two interesting pasture areas and their evolution in last decades was performed through a multidisciplinary approach aiming to investigate the botanical composition of pastures and the relative productivity.

Materials and Methods

Description of the studied areas

The present study was carried out on pasture areas of Frosolone (41°36′ N 14°27′ E) and Montenero Val Cocchiara (41°43′ N 14°04′ E) municipalities, located up to 850 m of altitude, in Isernia province. In both cases pasture areas are owned by the respective municipality and their use is regulated by the agrarian law (*fida pascolo*).

Since 1990, several studies were carried out on soil, plant, and animal characteristics of selected grazing areas, traditionally used in summer season by cattle, sheep, goat, horse, and donkey, but also populated by wildlife, such us wild boar and deer. The first studies related to the Apennines pasture areas of Molise region were carried out between 1990 - 1992 by the local Provincial Breeders Association (APA) in collaboration with Agriculture and Forestry Department of Molise region with the main goal to describe the most important regional pasture areas and to create an inventory of grazing resources. Several parameters, *i.e.*, climate, soil texture and fertility, soil productivity and water availability, plants and grazing animal characteristics, pasture yield, and eventual degradation phenomena occurred on pasture, were studied and recorded through datasheets (De Renzis *et al.*, 1992; Di Rocco *et al.*, 1992).

On the same areas, in 2000 - 2005 period, the potential stocking rate of homogeneous grazing unit areas (GUAs) was assessed through the application of a simulation model studied by Pulina *et al.* (1999) for the rangeland ecosystem of Southern Africa and later adapted to the Mediterranean areas (Salimei *et al.*, 2005). The model, based on the forage availability, expressed as dry matter (DM) per ha, of herbaceous, shrubs, and wood species, simulates with nutritional approach a sustainable grazing pressure of multispecies herding, as a tool supporting decision-making in pasture management (Salimei *et al.*, 2005).

More recently, the Regional Agency for Agricultural, Rural and Fishing Development (ARSARP) repeated the APA study in the same pasture sites of Molise region aiming to monitor eventual land use changes (Colonna and Rosati, 2015).

Among the pasture investigated areas, Frosolone site, included in the ancient transhumance road acknowledged by UNESCO as Intangible Cultural Heritage (UNESCO, 2019), is located between 1 200 m and 1 400 m a.s.l., and is characterized by sloping surfaces (10 - 20 % of slope) and flat areas completely covered by natural pasture where animals commonly graze from May to November. Pasture surface, about 1 000 ha of utilized agricultural area (UAA), is divided into four mains areas, *i.e.*, Terzo Scampisco, Terzo Cervaro and Terzo di Mezzo, where only cattle, horse and donkey can graze, and Pesco la Messa, where only sheep and goat have access. According to the *fida pascolo* regulation, the utilization of these pasture areas is reserved to farmers residing in Frosolone municipality upon declaration of grazing animal species and numbers (maximum 50 bovine heads, 500 sheep/goat and 15 horse/donkey, on a year basis), whose good health status must be ensured. Annual payment of a fee is required for pasture utilization (1 euro/sheep and goat head, 10 euro/cattle head, and 35 euro/horse and donkey head) and for water utilization (0.65 euro, 8.05 euro and 13.5 euro, respectively for small ruminants, cattle and equines).

From a climatic point of view, the average monthly temperature registered in Frosolone area over a 30-years period from 1987 to 2017, ranged (minimum - maximum values) from 8 °C in January to 20 °C in July/August, while the average monthly precipitations ranged from 40 mm of July to around 130 mm in November (Fatica *et al.*, 2019). According to USDA Soil Taxonomy (Soil Survey Staff, 2010), Frosolone soils classified as Hamplumbrets and Hampludalfs, are characterized by low calcium and carbonate content with acid/sub-acid reaction and have moderate slow permeability and high water holding capacity (Fatica *et al.*, 2019). The grassland composition is typical of southern Apennine geographical region with a greater presence of grass plants than legume plants. Nevertheless, especially alongside the animal roads, weed species as *Cardus* spp. and *Cirsium* spp., and *Pteridium aquilinum* L. are present.

Montenero Val Cocchiara area is an intermontane basin, originally located at the bottom of ancient lake, at about 850 m a.s.l., crossed by Zittola river and surrounded by wooden hills. Montenero valley, approximately 3 km of length and 1 km of width for a total of 900 ha of UAA, is partially covered by peat layers with local outcropping and is characterized by cold-humid winters and hot-dry summer, typical of Mediterranean regions. During the 30-years period (1987 - 2017), the average temperature ranged from a minimum of 4 °C recorded in January, to a maximum of 21 °C registered in July, while the average rainfalls ranged from 40 mm during the summer season to 180 mm during the winter season (Fatica *et al.*, 2019). Soils of Montenero peatland, usually flooded by the Zittola river from October to April, are classified as Eutrochrept due to the presence of gley features in B and C horizons (Fatica *et al.*, 2019). They have clay-silty texture, weakly to moderate alkaline reaction, slow permeability, high organic matter content and high water holding capacity, so that the growth of grazed plant species, mainly Gramineae and Legume families, is allowed together with weed species as *Juncus articulatus* L. and *Ranunculus acris* L.

Moreover, this area is under the Sites of Community Importance (SIC) protection programme aiming to preserve habitats and local breeds, *i.e.*, autochthonous Pentro horse, and plant communities, *i.e.*, *Salix pentandra* L. and *Dactylorhiza incarnata* L. (Miraglia *et al.*, 2008; Fatica *et al.*, 2019). Considering the protection program, the utilization of pasture areas is strongly related to the regulations defined by the Authority (*fida pascolo)*. Montenero Val Cocchiara pasture areas may be utilized throughout the year (from 15 May to 14 May next year), only by residing farmers upon the payment of an annual fee based on the number of grazing cattle and horses (around 1 300 heads in 2020). Moreover, based on climatic conditions, at the beginning of grazing season (May/June) municipality authorized each farmer to harvest the turf through a public allocation of the plots. Only after the biomass collection, animals could graze on the peatland.

Sampling design

Three sampling sites were identified respectively on Frosolone within Terzo Scampisco and Terzo di Mezzo areas, *i.e.*, Acqua Spruzza (AQ, 41°36’10’’ N 14°24’3’’ E), Colle Campo di Fave (CF, 41°35’55’’ N 14°23’12’’ E), and Piana di Santa Maria (SM, 41°36’18’’ N 14°23’17’’ E) (Figure 1.1), and on Montenero Val Cocchiara, *i.e.*, Bocca del Pantano (BP, 41°42’7’’ N 14°04’54’’ E), Ponte di Pietra (PP, 41°42’37’’ N 14°05’36’’ E), and Zittola (ZT, 41°43’18’’ N 14°05’32’’ E) (Figure 1.2).

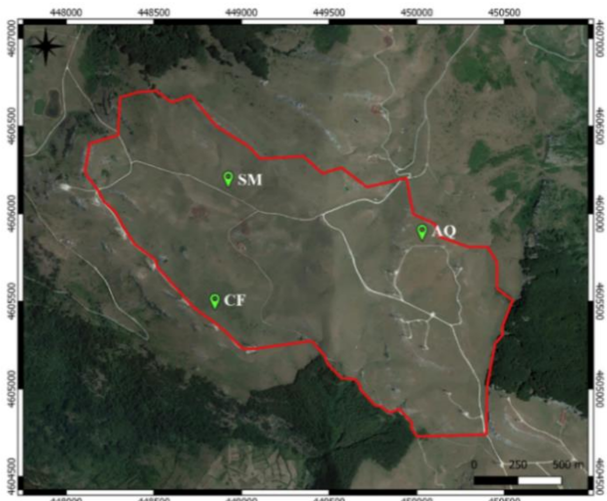

Figure 1.1 - Spatial representation and geographical localization of AS, CF, and SM sampled sites on Frosolone area (Source: Google Earth, 2021).

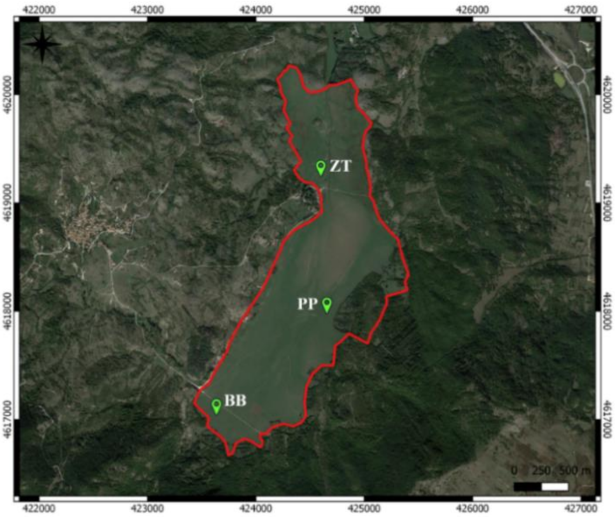

Figure 1.2 - Spatial representation and geographical localization of BP, PP and ZT sampled sites on Montenero Val Cocchiara area (Source: Google Earth, 2021).

During the 2019 - 2020 grazing season, several transepts (0.5 m^2, n = 36) representative of areas of Frosolone and Montenero Val Cocchiara underwent, from May to July, vegetation samplings to perform biomass production evaluation and phytosociological investigation (Figure 1.3). More in details, for each sampled site within the same studied area (about 200 m^2 of pasture surface), three representative transepts were made twice during the same grazing period, after 20 days.

As a part of a larger interdisciplinary study, soil investigations were also carried out in collaboration with the soil scientists of the Department of Agricultural, Environmental and Food Sciences.

Figure 1.3 - Example of transept (0.5 m^2) before (left) and after (right) manual plant collection. Site data were reported on blackboard. Courtesy Antonella Fatica.

Vegetation analysis and animal data collection

Average monthly temperatures (°C) and average monthly rainfalls (mm) for the period 2019 - 2020 have been recovered from the historical archives of Dipartimento IV Servizio di Protezione Civile (Regione Molise, 2021), and taking the climatological data online (3BMeteo, 2020).

Each sampled site was georeferenced, and the topographic data were processed to digitize the characteristics as altitude, slope, and general aspect of area. Geographic coordinates of central point were taken by GPS technology.

Vegetation was sampled by manual cutting around 3 cm up to the soil level to simulate the grazing activities. Phytosociological evaluations were performed according to Zurigo-Montpellier Sigmatist method (Braun-Blanquet, 1964), and plants coverage was estimated using the conventional abundance/dominance scale, *i.e.*, r = rare species, + = coverage < 1 %, 1 = coverage from 1 to 5 %, 2 = coverage > 5 - 25 %, 3 = coverage > 25-50 %, 4 = coverage > 50 - 75 %, 5 = coverage > 75 - 100 %, according to Cislaghi *et al.* (2019). Successively, plant species were identified according to the Pignatti (1982) dichotomous key.

Plant samples (n = 36), stored in a plastic bag, were analysed for DM and ash contents according to the official methods (AOAO, 2000).

Grazing data recordings for period 2012 - 2020 were recovered only for Frosolone area from the official archive. Adult bovine units (ABU) were then calculated, and for 2019 and 2020 also the grazing index (GI) was estimated as ratio between ABU and pasture productivity, according to Quaranta *et al.* (2020). Briefly, each ABU is equivalent to one cattle, one horse/donkey, or 6 sheep/goat (Quaranta *et al.*, 2020).

In year 2020, during the first sampling occurred on the first week of June, no animals were present on pasture due to the Brucellosis outbreak, which caused the loss of about 600 heads including cattle, sheep, and goat from October 2019 to April 2020 (Mazzeo *et al.*, 2020). Animals came back to the pasture only at the end of June 2020.

To describe the pasture evolution both on plants and animal point of view, data about pasture DM and ash contents, and number of grazing animals were compared to the literature references.

Statistical analyses of the results

All data were subjected to descriptive statistic (Microsoft© Excel© for Office 365 ProPlus Version 1903 -11425.202429) and results are reported as mean ± standard deviation (SD).

Results and Discussion

Climatic characterization of investigated areas

In Figures 1.4 and 1.5 are reported the thermo-pluviometric characterization respectively for Frosolone and Montenero Val Cocchiara areas. To better describe the climatic characterization of studied areas, data about the monthly temperature (°C) and rainfalls (mm) are presented as average values 2019 - 2020.

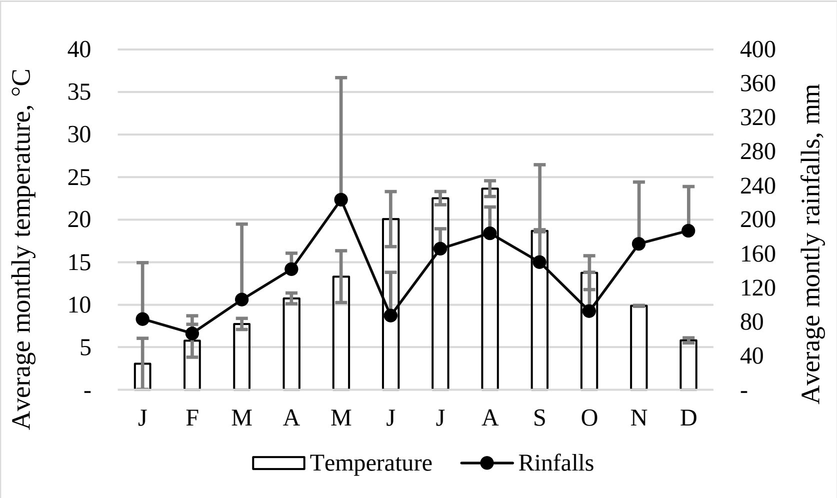

Figure 1.4 - Climatic characterization of Frosolone sampling area for 2019 - 2020 period.

As already pointed out, the studied areas are generally characterized by cold-humid weather condition, typical of Apennine zone: in more details, Frosolone average monthly temperature ranged from minimum value registered in January (3.0 °C) to maximum value of August (23.6 °C), while the average monthly rainfalls ranged (minimum - maximum value) from 66.3 mm in February to 223.5 mm in May, for a total average annual precipitation accounting for 1 658 mm.

Focusing on the investigated grazing period (May - July), respectively for the average monthly temperature and the average monthly rainfalls, were recorded 13.3 °C in May, 20.1 °C in June and 22.5 °C in July, and 223 mm in May, 87 mm in June and 166 mm in

July (Figure 1.4). Moreover, May 2020 was characterized by higher temperature and rainfall compared to May 2019, while an inverse situation was observed in June and July. Montenero Val Cocchiara average monthly temperature ranging from the minimum value of 2.6 °C recorded in January, to the maximum value of 23.1 °C recorded in August (Figure 1.5). The rainiest month was November (266 mm), while the driest was February (74 mm), for a total average annual precipitation accounting for 1 858 mm.

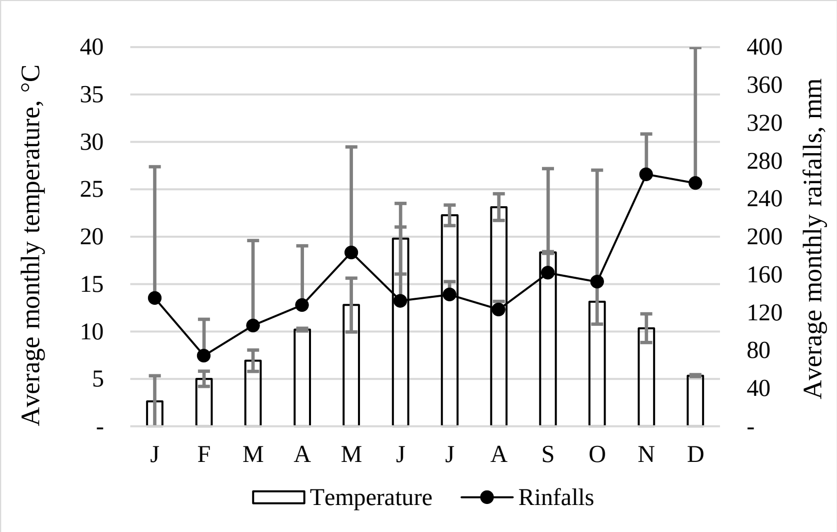

Figure 1.5 - Climatic characterization of Montenero Val Cocchiara sampling area for 2019 - 2020 period.

Giving details about the investigated period, the average monthly temperature ranged from 12.8 °C of May and 23.1 °C of August, nevertheless May 2019 was colder than the same month of 2020 (- 4.0 °C), but June and July were hotter than the same period of 2020, respectively + 5.3 °C and + 1.5 °C. Regarding the average monthly precipitation levels, during 2019 both June and July has been characterized by more rainfalls than 2020, while May 2020 was more rainy than the same 2019 period (+ 15.4 mm).

Topographical and botanical description, and forage chemical composition

Topographical description and plant community analyses were reported respectively in Table 1.1 for Frosolone and in Table 1.3 for Montenero Val Cocchiara areas.

Table 1.1 - Topographical description and plant community analyses of Frosolone sampling sites (n = 18). Data from literature [α] are reported for comparisons.

	Sampled sites						1992 [α]
	2019			2020			
	AQ	CF	SM	AQ	CF	SM	Frosolone area
Altitude, m a.s.l.	1300	1300	1290	1300	1300	1290	1200 - 1400
Slope, %	10	10	-	10	10	-	
Exposition	S	NE	-	S	NE	-	
Vegetal analyses							
Turf height, cm	< 5	> 20	< 2	< 10	25	< 15	> 40
Turf land cover, %	90	95	85	90	100	100	> 90
Number of species	15	15	11	20	15	18	42
Total average production, t DM/ha		**3**			**4.3**		**1.2 - 2.2**
Average turf composition, %							
Gramineae	70	55	65	60	65	45	40
Leguminosae	10	5	10	20	5	30	25
Composite	5	15	5	10	15	10	20
Labiatae	< 5	15	5	< 5	10	5	-
Other	10	10	1	5	5	10	15
Braun-Blanquet Scale *							
Achillea millefolium L.	1	2	+	1	2	3	2
Agrostis spp.	+	+	r	+	2	2	1
Bromus erectus Hds.	1	2	2	+	2	3	2
Cardus spp.	1	3	+	1	1	3	1
Crateagus monogyna Jacq.	-	r	1	1	2	2	-
Cynara cardunculus L.	1	+	r	1	r	r	-
Cynodon dactylon L.	3	3	2	3	1	1	-
Cynosurus cristatus L.	2	r	-	1	3	2	2
Festuca spp.	1	1	r	2	2	2	2
Galium spp.	2	1	-	1	3	4	1
Lolium perenne L.	2	-	+	1	+	+	1
Phleum spp.	2	1	-	2	3	+	1
Plantago spp.	+	+	-	-	-	-	+
Pteridium spp.	-	-	-	-	-	-	4
Rosa canina L.	1	1	2	1	1	2	1
Scrophularia spp.	r	1	-	-	2	1	-
Trifolium spp.	1	2	1	2	2	1	2

[α] Di Rocco *et al.*, 1992.
AQ = Acqua Spruzza; CF = Colle Campo di Fave; SM = Piana di Santa Maria; Braun-Blanquet scale: r = rare species, + = coverage < 1 %, 1 = coverage from 1 to 5 %, 2 = coverage > 5 - 25 %, 3 = coverage > 25-50 %, 4 = coverage > 50 - 75 %, 5 = coverage > 75 - 100 %.

In Frosolone area, probably due to the absence of the animals at first sampling occurred in 2020, higher average turf height and land cover (%) than 2019 were observed, and more plant species were identified compared to 2019 (Table 1.1). Gramineae was the plant family more represented (about 60 %), while the same percentage (around 15 %) were found for Leguminosae, Composite and Labiatae families. The most abundant herbaceous species were *Achillea millefolium* L., *Cardus* spp., *Festuca* spp., *Galium* spp., *Trifolium* spp. (Table 1.1).

When considering the references data showed in Table 1.1, the decrease occurred in the last 30-years for both turf height and total plant species appears evident, while the turf cover did not vary. Furthermore, in 2019 - 2020 period some herbaceous and shrub weed species as *Cardus* spp., *Crataegus monogyna* Jacq., *Cynodon dactylon* L., *Rosa canina* L. and *Rumex crispus* L. were found especially along the animal roads, near the drinkers, and in the rest areas, according to previous findings (Salimei *et al.*, 2005; Colonna and Rosati, 2015). Total average pasture production ranged from 3 t DM/ha in 2019, which is consistent with values (2.9 t DM/ha) reported by Salimei *et al.* (2005), and 4.3 t DM/ha in 2020, which accounts for almost twice the maximum productivity (2.2 t DM/ha) reported by Di Rocco *et al.* (1992).

Pasture samples collected during 2019 showed a DM content higher than values observed in 2020 in the same areas (Table 1.2). Particularly, during 2019, AQ site showed the highest DM and ash contents (43.9 g/100 g and 7.15 g/100 g DM respectively), while SM showed the lowest value of both DM (37.0 g/100 g) and ash contents (5.34 g/100 g DM) (Table 1.2). In 2020, AQ and CF sites showed similar value of pasture DM content, while higher DM content was observed for SM samples (31.6 g/100 g DM), which were characterized also by the lowest ash content, consistently with AQ samples (7.2 g/100 g DM) (Table 1.2), possibly related to the rainfall levels recorded during the investigated period. The average DM values observed in 2019 - 2020 period was in the line with those reported for the same areas in 2002 - 2003 period, *i.e.*, 30.2 (± 1.02 SEM) g/100 g and 34.1 (± 1.10 SEM) g/100 g (Salimei *et al.*, 2005).

Table 1.2 - Dry matter and ash contents (± SD) of Frosolone pasture samples for 2019 and 2020 (n = 18).

	2019			**2020**		
	AQ	**CF**	**SM**	**AQ**	**CF**	**SM**
DM, g/100 g	43.9 ±0.96	40.0 ±4.13	37.0 ±1.95	25.4 ±2.05	26.1 ±1.16	31.6 ±7.98
Ash, g/100 DM	7.15 ±1.20	7.70 ±0.55	5.34 ±0.30	7.24 ±0.09	7.95 ±1.33	7.24 ±0.57

AQ = Acqua Spruzza; CF = Colle Campo di Fave; SM = Piana di Santa Maria; DM = dry mater.

Considerable turf differences were reported also in Montenero Val Cocchiara case study between 2019 and 2020 (Table 1.3).

Table 1.3 - Topographical description and plant community analyses of Montenero Val Cocchiara sampling sites (n = 18). Data from literature [α] are reported for comparisons.

	Sampled sites						1992 [α]
	2019			2020			
	BP	**PP**	**ZT**	**BP**	**PP**	**ZT**	**Montenero VC area**
Altitude, m a.s.l.	850	820	820	850	820	820	830 - 850
Slope, %	-	-	-	-	-	-	
Exposition	-	-	-	-	-	-	
Vegetal analyses							
Turf height, cm	< 5	< 5	< 2	20	55	< 5	5
Turf land cover, %	95	95	85	90	100	100	100
Number of species	10	10	8	20	15	18	20
Total average production, t DM/ha		**0.6**			**5**		**1.5 - 2**
Average turf composition, %							
Graminacea	45	60	15	30	25	20	40
Leguminosae	35	15	20	50	60	40	25
Compositae	10	10	30	10	5	15	20
Labiatae	< 5	10	5	< 5	5	5	-
Other	5	5	30	<5	< 5	20	15
Braun-Blanquet Scale *							
Achillea millefolium L.	r	+	r	1	2	r	1
Anthoxanthum odoratum L.	+	1	r	1	1	r	2
Cardus spp.	1	+	2	1	r	2	+
Convolvulus arvensis L.	1	1	r	2	2	r	+
Cynara cardunculus L.	r	r	1	r	r	2	-
Cynosurus cristatus L.	1	1	+	1	2	+	1
Festuca spp.	1	1	+	1	2	+	2
Lolium perenne L.	+	2	r	2	3	r	1
Lotus corniculatus L.	r	r	4	r	r	4	2
Mentha arvensis L.	+	+	r	1	2	r	+
Phleum pratense L.	1	1	r	2	2	r	+
Picris spp.	r	r	+	r	1	r	+
Ranunculus spp.	+	1	2	1	3	2	1
Trifolium spp.	+	+	r	2	3	1	1

[α] Di Rocco *et al.*, 1992.
BP = Bocca del Pantano; PP = Ponte di Pietra; ZT = Zittola; Braun-Blanquet scale: r = rare species, + = coverage < 1 %, 1 = coverage from 1 to 5 %, 2 = coverage > 5 - 25 %, 3 = coverage > 25-50 %, 4 = coverage > 50 - 75 %, 5 = coverage > 75 - 100 %.

In 2020 the first sampling was made before the mechanical hay harvest, when animals were not allowed to graze, while in 2019 both samplings were carried out when the animals already had access to the pasture area. The different values reported for ZT site, where turf height was less than 2 cm, can be explained by the fact that animals can graze all over the years in this area. During samplings was indeed observed that the only plants not grazed by the animals were *Cardus* spp., and *Ranunculus* spp. Compared to 1990 data, the presence of *Lolium perenne* L., *Phleum pratense* L. and *Trifolium* spp. has increased (Table 1.3). It is worth noting that pasture productivity in 2020 has reached very high yield level (5 t DM/ha) compared to 0.6 t DM/ha reported for 2019, and to the maximum value of 2 t DM/ha reported by Di Rocco *et al.* (1992). However, the 2020 average pasture production were found very close to the average values reported by Salimei *et al.* (2005).

As showed in Table 1.4, the DM content of Montenero Val Cocchiara pasture samples were found higher in 2019 than in 2020, although falling within the range of 23.2 (± 1.8 SEM) g/100 g and 32.7 (± 2.58 SEM) g/100 g reported by Salimei *et al.* (2005), probably due to the climatic condition observed. For ZT site only one sampling was possible in 2020 because of a severe overgrazing phenomenon. Furthermore, always referring to this site, the highest DM and ash contents were observed in 2019, while the lowest DM content in 2020 (Table 1.4).

Table 1.4 - Dry matter and ash contents (± SD) of Montenero Val Cocchiara pasture samples for 2019 and 2020 (n = 18).

	2019			2020		
	BP	**PP**	**ZT**	**BP**	**PP**	**ZT**
DM, g/100 g	41.7 ±5.36	24.8 ±3.51	49.8 ±6.85	27.5 ±3.84	25.4 ±1.36	16.7 ±0.00*
Ash, g/100 DM	11.1 ±4.02	9.12 ±1.04	14.4 ±2.88	9.04 ±1.94	9.58 ±3.51	11.3 ±0.00*

* Only one sampling was possible in 2020 due to overgrazing phenomenon.
BP = Bocca del Pantano; PP = Ponte di Pietra; ZT = Zittola; DM = dry matter.

Forage intake is affected by plant resources availability, rate of intake, time of grazing, stocking rate, and grazing species, since equines are able to exploit large amount of fibrous forage, often with a low nutritive value (Miraglia *et al.*, 2020).

For both studied areas, values of pasture yield and DM content of grazed species were consistent with values reported in literature for Apennine area (Cavallero *et al.*, 2002; Cislaghi *et al.*, 2019). As possible consequence of the abandonment of mountainous areas related to a progressive decrease of pastoral activities, a general decline of turf quality was observed especially in terms of plant biodiversity along with a progressive recolonization by forest, as also observed during last decades in Molise region (Colonna and Rosati, 2015), and in inner Mediterranean areas (Pornaro *et al.*, 2013).

Frosolone and Montenero Val Cocchiara sites showed areas overgrazed and areas underutilized, both responsible of a general soil degradation and morphological changes on vegetal species, which assumed a creeping and prostate habit with the roots arranged superficially (Gusmeroli, 2004). During the growth phase, some sugars synthetized by the plant through photosynthetic activity are used as energy reserves that will be used in flowering and in the eventual re-growth phase. When plants are cut too early, or if subsequent cuts are too close, plants lose the ability to vegetate again because not able to accumulate the necessary energy in its reserve organs (Pinheiro Machado, 2004; Mancini, 2019); accordingly, overgrazing has negative effects on both plant cover and soil structure (Fatica *et al.*, 2019; Miraglia *et al.*, 2020). In the investigated areas, the uncontrolled grazing pressure seems to be the major cause of progressive changes occurred in the floristic composition of pasture, with a consequent rapid decrease of palatable species, and average turf cover, leading to the increase of uncovered soil surfaces likely subjected to water and wind erosion phenomena, as reported for several Mediterranean pasture areas (Lu *et al.*, 2015; Quaranta *et al.*, 2020). On the other hand, as a direct consequence of pasture underutilization, shrub and wood biomass increases, resulting in reduction in the diversity of herbaceous layers and increased fire risk (Arnaez *et al.*, 2011; Pornaro *et al.*, 2013), besides a not homogeneous manure distribution. Changes occurring in Central Apennine areas are also related to modify of land use, responsible of soil loss due to water erosion, sensible reduction of top-soil and worsening soil physical properties, *i.e.*, increase the compaction and reduction of porosity, that were negatively affected by repeated and uncontrolled passage of machinery and animal (ISPRA, 2015; Frate *et al.*, 2018; Rosskopf *et al.*, 2020).

Animal consistency data and grazing index

During the 2012 - 2020 investigated period, grazing animal population of Frosolone area has varied between a minimum value of 2 267 heads in 2014 to a maximum value of 2 470 heads in 2019 (Table 1.5). More in details, cattle and sheep heads remained almost constant, equines decreased their numbers, while goats increased (Table 1.5). Furthermore, between 2019 and 2020 an overall decrease of grazing animals has been registered due to the above-mentioned brucellosis outbreak, that negatively affected the local farm activities, already heavily damaged by the effects of COVID-19 lockdown

(Mazzeo *et al.*, 2020). Finally, as Table 1.5 shows, grazing animal number has more than halved in 30 years. This trend confirms once again how the progressive land abandonment of the mountains is affecting less favoured areas of Molise region, characterized by depopulation and progressive population ageing (Ievoli *et al.*, 2017).

Table 1.5 - Frosolone grazing animal consistency (heads of animals) between 2012 to 2020. Data from literature [α] were reported for comparisons.

	Time period									
	2012	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**1992 [α]**
Cattle	1 127	1 118	1 106	1 116	1 065	1 119	1 107	1 178	1 182	2 126
Sheep	1 070	1 088	963	963	1 115	1 012	1 048	1 060	1 038	2 327
Goat	35	49	61	61	75	80	83	142	65	354
Horse	152	157	136	136	104	89	93	88	77	288
Donkey	4	3	1	1	2	2	2	2	-	-
Total heads	2 388	2 415	2 267	2 277	2 361	2 302	2 333	2 470	2 362	5 095
Total ABU	1 449	1 449	1 397	1 407	1 350	1 374	1 372	1 448	1 424	2 816
GI, head/ha	1.4	1.4	1.4	1.4	1.3	1.4	1.4	1.4	1.4	2.8

[α] Di Rocco *et al.*, 1992.
ABU = adult bovine unit, GI = grazing index, calculated considering 1 000 ha of utilized agricultural area.

As expected, also the total ABU and relatives GI have undergone a general halving, when compared to 1992 literature data (Table 1.5). The observed ratio "head per hectare" ranges between 1 - 1.5 UBA/ha, which is a recommended value for Molise pasture areas (Miraglia *et al.*, 2000) also considering the limit of nitrogen soil concentration of 180 kg N/ha, and 2 UBA/ha (Piccirilli *et al.*, 2001). In recent years, livestock productions have recorded a steep decline in terms of grazing index and pasture density (Quaranta *et al.*, 2020). Notwithstanding, as result of inappropriate grazing practice, overgrazing is the main responsible for soil and vegetation damage (Imbrenda *et al.*, 2013), consequently the adoption of appropriate strategies as rotational grazing, weed control, and fertilization are becoming crucial also in natural pasture (Fatica *et al.*, 2019; Miraglia *et al.*, 2020).

Conclusions

Pasture should be considered as a heterogeneous biological entity that can change easily with pedo-climatic conditions and animal and human uses. Molise region is characterized by pasture areas strategic from an economic, social, environmental, and cultural point of view, but incredibly vulnerable and threatened of land abandonment and soil degradation. The presented study is focused on two areas of Molise region of relevant interest, as UNESCO Intangible Cultural Heritage (Frosolone) and NATURA 2000 Site of Community Importance (Montenero Val Cocchiara), where the agricultural and zootechnical vocations are firmly rooted.

The extensive literature review concentrated mainly in three different time periods (1900 - 1992, 2000 - 2005 and 2014), has allowed the collection of several data used as basis for the presented study. The multidisciplinary investigation including climatic changes, botanical and productive characteristics of the turf, and grazing livestock population has allowed to monitor the evolution of the two areas over a 30-years period.

As a major concern, the number of plant species has decreased over the last decades, while a progressive expansion of herbaceous and shrubby weed species was observed as possible consequence of non-homogeneous pasture utilization. Moreover, animal numbers in Frosolone area have recorded a sensible decrease. These negative trends reflect the abandonment of less productive and marginal lands in favour of a more productive intensification in flat areas, which characterizes Italian and Mediterranean inner districts.

Considering the multiple benefits that grazing brings to the local community, in terms of landscape and traditional culture, relationship with territories, and added nutritional value of traditional foods, it seems essential to re-evaluate the role of pasture and its ecosystem services in order to address the good practices towards more sustainable management. As a contribute to this pressing aspect of rural development, a new grazing management approach that allows to limit the negative effects induced by overgrazing and/or pasture underutilization, should evaluate the qualitative and quantitative availability of forages, also including shrub and wood species, so that the nutrient requirements of grazing animals can be fulfilled in the respect of both animal welfare and production, and environment. Finally, the introduction of near-infrared spectroscopy (NIRs) as innovative, rapid, and economical method for evaluating the pasture value, could represent a promising tool for the development of pasture areas.

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Chapter 2

Study on *Nicotiana tabacum* L., cv. *Solaris* biomass

Introduction

Nicotiana tabacum L., herbaceous plant belonging to *Solanaceae* family with a height ranging between of 0.9 m to 1.8 m, is characterized by large oval leaves, white and pink flowers, green capsules that contain spherical brown seeds with a diameter of 0.5 mm (Usta *et al.*, 2011). Traditionally, tobacco leaves are the main commercial product largely used by the tobacco processing industry for cigarettes production all around the word. Several factors can affect tobacco cultivation, *i.e.*, soil moisture, available water capacity, air moisture and temperature, and intensity of lighting. Tobacco plant is suitable for cultivation in areas with warm-humid climate conditions, where soils are deep, basically sandy and/or with a medium texture, and with an average pH from 6 to 8 (MIPAAF, 2005). According to FAOSTAT data (2021), during the 2019 the word arable lands cultivated with tobacco plant were more than 3,6 million of ha, which allowed a tobacco unmanufactured world yield around 6,7 Mt/year. The largest global tobacco producers in 2019 were China (2.7 Mt), Brazil (0.7 Mt), India (0.6 Mt), USA (0.4 Mt) and Indonesia (0.2 Mt), while Italy is the largest tobacco producer in the European Union, with an amount of 21 % of total European tobacco production that, for 2019, accounted for about 0.18 Mt (FAOSTAT, 2021). Tobacco cultivation in Italy is mainly concentrated in four regions: Campania, Umbria, Veneto, and Toscana where the cultivation know-how is highly qualified, from both agricultural machinery and cultivation techniques point of view (Fatica *et al.*, 2019; Sifola *et al.*, 2021). However, especially due to the generalized crisis affecting the global smoking tobacco sector during last decades, together with worldwide interest shifting to radically new non-carbon and renewable energy sources (Amicarelli *et al*., 2020; Moriarty and Honnery, 2021), a new cultivar of *Nicotiana tabacum* named *Solaris*, was developed in 15 years of research for maximizing the production of oil-containing seeds used as biofuel or biomass sources (Grisan *et al.*, 2016). This no-food and GMO-free cultivar developed as energy tobacco has been patented (PCT/IB/2007/053412) in 119 countries and granted in over 75 countries, including USA, Italy, Russia, Australia, and some Africa and Euro-Asia states (Fogher 2008, Fogher *et al.*, 2011; Fatica *et al.*, 2019). Particularly, in 1997 began a breeding programme with the aim of increasing tobacco seeds production through the selection of a variety characterised by seed and oil yields per hectare significantly higher than the traditional tobacco smoking plants and the common oilseed crops (Fogher *et al.*, 2011). The industrial use of *Solaris* as sustainable bioenergy product, tested for the first time in North and Central Italy, showed strong vegetative capacity and short cultivation cycle so that, about 90 days from the transplanting, seeds can be harvested by apexes flower collection (Grisan *et al.*, 2016). Cold-press extraction of cv. *Solaris* seeds supplies oil suitable as biofuel with an average yield of 38 % - 40 % (Fogher *et al.*, 2011). As co-product of oil extraction, the seed cake, given the chemical and nutritional characteristics, may be useful as a high protein and energy ingredient in piglets' and in poultry diets, as reported in literature (Rossi *et al.*, 2013; Sipokazi, 2018). According to Grisan *et al.* (2016), tobacco *Solaris* could regrow after the first seeds collection so that, during the same vegetative season (about 150 days after transplanting), a second harvest of *Solaris* seeds and/or green biomass can be possible if pedo-climatic conditions allow it. In this way, both seed cake and biomass could enter in a circular supply chain including other alternative sectors (Andrianov *et al.*, 2010), such as biomethane production and animal feeding.

Nicotiana tabacum cv. *Solaris* compared with smoking tobacco varieties (Figure 2.1) maximizes the production of flowers/seeds reducing leaf growth, minimising the nicotine content (Grisan *et al.*, 2016; Fatica *et al.*, 2019). Due to the peculiar characteristics here summarised, the cultivation of cv. *Solaris*, as source of biofuel and co-products, *i.e.*, protein seed cake and biomass, fits perfectly with the goals of promoting development of alternative renewable energy sources, also providing several agricultural residuals or “wastes” as sources of additional income. For these reasons, the cultivation of cv. *Solaris* must be considered from a circular bioeconomy point of view, especially within the inner area of Centre-South Italy traditionally suited both to tobacco cultivation and animal breeding.

Figure 2.1 - Traditional cultivar of smoking tobacco (left) compared to *Nicotiana tabacum* L., cv. *Solaris* (right). Courtesy Antonella Fatica.

Aim

Aiming to contribute to new advances of knowledge about *Nicotiana tabacum* L., cv. *Solaris* and its potential secondary products, the chemical characteristics of this innovative culture were investigated for the first time as potential source of biomass for animal feeding, energy production or food supplement, based on data recorded in different areas of Italy during three-year cultivation period.

Materials and Methods

General design of Nicotiana tabacum cv. Solaris cultivation protocol

According to a randomized blocks design, the *Solaris* cultivation was performed from 2016 to 2018 in experimental fields covering an area of 600 m^2 located in Vicenza (45°17′25.4″ N 11°30′09″ E), Chieti (42°10’0” N 14°20’0” E), and Perugia (42°59’0” N 12°25’0” E) provinces, adopting the conventional tobacco farming technique of the selected area.

Tobacco plants were transplanted after tillage carried out by plowing (maximum depth 50 cm). Insecticide products (z-cypermethrin) were buried on the ground to counter attacks of terrestrial nocturnal insects in the transplantation stage. According to the selected densities, 3.6 plants/m^2 and 4.8 plants/m^2, plants were mechanically transplanted in May, at a distance between rows of 90 cm and of 31 cm or 23 cm along the row, respectively. After transplant, weeding was performed using clomazone based product, and fungicide and insecticide treatments were carried out during the cultivation season to protect the maturing capsules. A total amount of 120 kg of nitrogen, 60 kg of phosphorus, and 70 kg of potassium were distributed by fertigation system, based on soil analysis. Drip irrigation was carried out according to the weather conditions of each cultivation area, and a total amount of water ranging from 1 000 m^3 ha^{-1} to 1 450 m^3 ha^{-1} was distributed. Data about monthly temperatures (°C) and rainfalls (mm) for the period 2016 - 2018 were provided by ARPA Veneto (2019), HORT@ Agrometeorological Network (2019), and Regional Hydrographic Service (2019), for Vicenza, Chieti, and Perugia provinces, respectively.

Nicotiana tabacum cv. Solaris yield and analytical investigation

In each cultivation area, seed harvest was carried out from the end of July to the first decade of August (80 - 90 day after transplanting), while biomass was harvested depending on altitude, temperatures, and rainfalls. Seed was harvested manually by cutting floral apexes, while green biomass was harvested through a common shredding machine. At the end of each harvest, seeds and biomass were weighted and yields per hectare were recorded.

During three-year period, *Nicotiana tabacum* cv. *Solaris* biomass samples (n = 15) were collected by differentiating respectively whole plant, floral apex, and stem-leaf samples. After plant manual cutting, all samples underwent natural drying and subsequently chopping at a maximum length of 4 cm. For all samples (n = 15) collected during the three-year period in the studied areas, official methods have been applied for the analyses (in three replicates) of moisture, pH, crude protein (CP), ether extract (EE), and ash contents (Reg. EC 152/2009), NDF, ADF, ADL, starch, and sugars (glucose, fructose, sucrose) contents (AOAC, 1995, 2000). Total alkaloids content, expressed as nicotine (UNI EN ISO 2881/1992), was investigated on whole plant samples. Data on fiber fractions were subjected to further evaluations, *i.e.*, hemicellulose = NDF - ADF, cellulose = ADF - ADL, available fiber = NDF - ADL.

On samples collected in 2016 (n = 5) further analyses in three replicates were also carried out including KOH soluble protein content, amino acid concentration after hydrolysis (lysine, threonine, leucine, isoleucine, valine, histidine, proline, arginine, phenylalanine, tyrosine, glycine, serine, alanine, aspartic acid, glutamic acid) (Reg. EC 152/2009), and macro-minerals (Ca, P, Mg, K, Na and chlorides) (AOAC, 1995, 2000; Reg. EC 152/2009) contents. All analyses listed were carried out by an accredited laboratory

(Mérieux NutriSciences Corporation, Resana, Italy). Finally, the biochemical methane potential (BMP) has been tested by a certified laboratory (CRPA LAB, Reggio Emilia, Italy), according to anaerobic digestion batch procedure (UNI EN ISO 11734/2004) on whole plant samples collected in 2017 and 2018.

Statistical analyses of the results

All data were subjected to descriptive statistic (Microsoft© Excel© for Office 365 ProPlus Version 1903 -11425.202429).

The effects of density on cv. *Solaris* yield and analytical components underwent analysis of variance (IBM SPSS, data editor ver. 25). Since non-significant effects were detected on the investigated parameters, the two densities were not further considered.

All results are therefore reported as mean ± standard deviation (SD), on dry matter (DM) basis except for BMP results reported on organic matter (OM) basis.

Results and Discussion

Climatic characterization of cultivation areas

Figures 2.2, 2.3, 2.4 depict the environmental conditions recorded in the investigated areas during the study. As Figure 2.2 shows, Vicenza province was characterized by an average minimum temperature (2.5 °C) and an average maximum temperature (24.8 °C) respectively registered in December and July, while the average monthly rainfalls ranged from 24 mm of December to 95 mm of February (Figure 2.2), for a total annual average rainfall of 770 mm. In 2016 the hottest month was July, while both for 2017 and 2018 the highest temperatures were registered in August, despite the average annual temperature was just over 14 °C. The rainiest months were February, November, and March respectively in 2016, 2017 and 2018, while August 2017 was the lowest rainy month, with only 2.5 mm of rain.

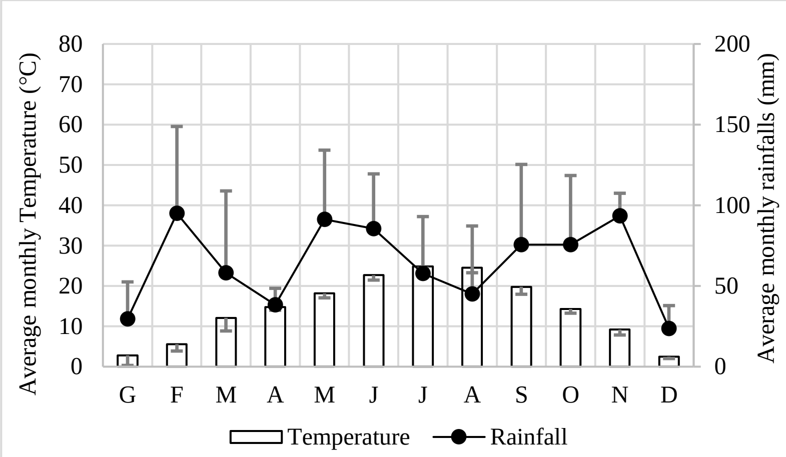

Figure 2.2 - Monthly temperature (°C) and rainfalls (mm) for Vicenza province in years 2016, 2017, and 2018 (mean values ± SD).

As far as the Chieti province is concerned (Figure 2.3), monthly temperature and rainfall ranged from a minimum of 8.1 °C in January to a maximum of 25.7 °C in July, and from 41.5 mm of August to 116 mm of November, respectively. The average annual precipitation was equal to 893 mm with the rainiest months observed in July (111 mm), January (262 mm), and February (161 mm) respectively for 2016, 2017, 2018. The highest temperatures were recorded in July 2016 and 2018, and in August 2017.

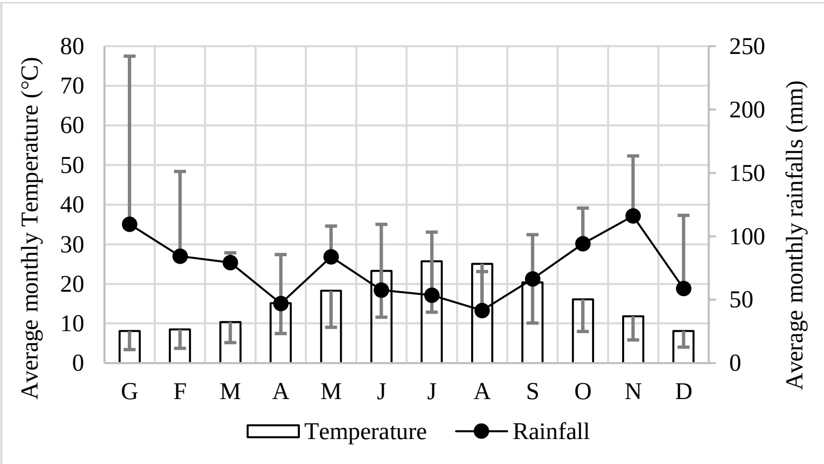

Figure 2.3 - Monthly temperature (°C) and rainfalls for Chieti province in years 2016, 2017, and 2018 (mean values ± SD).

Finally, Figure 2.4 reports the thermo-pluviometric characterization of Perugia province. The average monthly temperature and rainfalls ranged from a minimum of 5.1 °C in December to a maximum of 25.3 °C in July, and from 24 mm in July to 98 mm in March, respectively. Total average annual rainfall was equal to 731 mm. February, December, and March were the rainiest months in 2016, 2017 and 2018, respectively. Consistently with data from Chieti province, July was the hottest month in 2016 and 2018, while August was the hottest one in 2017.

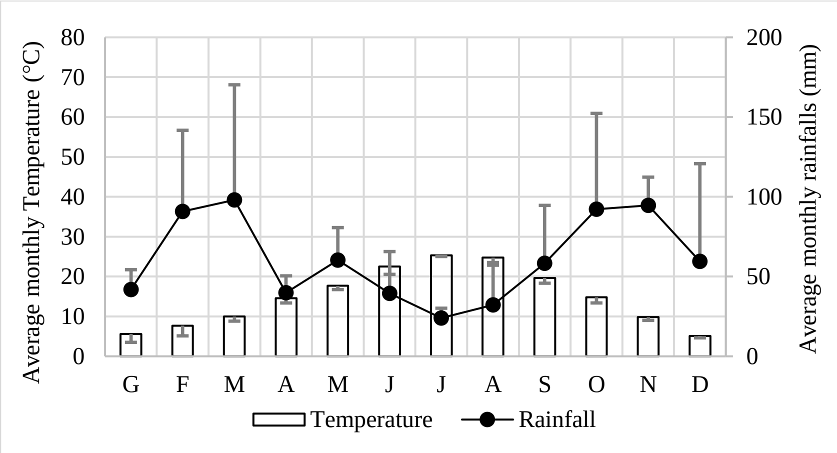

Figure 2.4 - Monthly temperature (°C) and rainfalls (mm) for Perugia province in years 2016, 2017, and 2018 (mean values ± SD).

Yields and chemical composition of Nicotiana tabacum cv. Solaris

Nicotiana tabacum cv. *Solaris* seed production averaged per year 2.1 (± 0.16) t DM/ha, consistently with values previously reported for North and Centre Italy (Grisan *et al.*, 2016). The cv. *Solaris* green biomass production averaged 70.8 t/ha per year, ranging (minimum - maximum value) from 65 t/ha to 76.6 t/ha.

The chemical composition of cv. *Solaris* whole plant, floral apex, and stem-leaf is reported in Table 2.1. Biomass samples (n = 15) were characterized by a low DM content, *i.e.*, 19.32 (± 3.78) g/100 g, while the OM content was 80.1 g/100 g DM, 82.8 g/100g DM, and 85.7 g/100g DM respectively for stem-leaf, whole plant, and floral apex (Table 2.1). The inflorescence CP content, in average 24.8 g/100g DM, was close to the values reported in literature for the seed cake (Rossi *et al.*, 2013), while CP values detected for whole plant (19.0 g/100 g DM) and for stem-leaf (16.0 g/100g DM) were intermediate between the protein content of alfalfa (*Medicago sativa* L.) dehydrated pellets (17.6 g/100 g DM), and sugar beet (*Beta vulgaris* L.) dried pulp (9.2 g/100 g DM) (Kirkpatrick *et al.*, 1983; Afshar and Naser, 2008). It is worth noting that the protein levels of whole plant and stem-leaf were close to the values reported for the common sainfoin (*Onobrychis sativa* Lam.) and Italian sainfoin (*Hedysarum coronarium* L.) among the legume plants, and for Italian ryegrass (*Lolium italicum/multiflorum* Lam.), cocksfoot (*Dactylis glomerata* L.) and sorghum (*Sorghum vulgare* L.) among grass plants, but also for rapeseed (*Brassica napus* L.) (Van Soest 1994; Dell'Orto and Savoini 2005; Cevolani 2005; Crovetto 2006). Lower crude protein contents are reported for grass plants, such as triticale (*x Triticosecale*), barley (*Hordeum vulgare* L.) and green maize (*Zea mays* L.), while higher values are found in legumes such as alfalfa, vetch (*Vicia sativa* L.), white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.) (Dell'Orto and Savoini 2005; Cevolani 2005; Crovetto 2006; INRA, 2018). As expected for this energy tobacco variety, ether extract content (Table 2.1) was higher in floral apex (14.2 g/100g DM) than in both steam-leaf (3.06 g/100g DM) and whole plant (7.92 g/100 g DM). Despite this, the ether extract content of *Solaris* steam-leaf was very close to the values reported by Crovetto (2006) for legume and grass plants (in average 2.64 g/100 g DM and 2.88 g/100 g DM, respectively). The high dietary lipids content, especially rich in unsaturated long fatty acids, could negatively affect the anaerobic digestion of biomass as a result of the microbial activities inhibition (Hagos *et al.*, 2017). For the same reason it is well known that the lipid content of ruminant diets should not exceed 5 - 7 % of DM to prevent DM intake and fiber fermentation depression phenomena (Lucas and Loosli, 1944; Beauchemin *et al.*, 2008). Values of ash content varied among the investigated plant fraction and were found higher compared to the values reported for grass plant (9.25 g/100 g DM) and legume plant (10.4 g/100 g DM) (Crovetto, 2006). However, higher ash content (16 - 20 g/100 g DM) was reported for cabbage (*Brassica oleracea* L.) both forage and biogas biomass, sugar beet leaves and necks, and rapeseed (Van Soest, 1994; Cevolani, 2005; Crovetto, 2006). Considering the cell wall composition, NDF, ADF, and ADL values were similar in the studied plant parts (Table 2.1). Moreover, NFD contents were close to the value reported for legume plant (49.3 g/100 g DM), and ADF levels were higher when compared to 33.5 g/100 g DM and 34.3 g/100 g DM, reported for grass and legume plants, respectively (Cevolani, 2005; Crovetto, 2006). The ADL content of cv. *Solaris* whole plant (12.9 g/100 g DM) was found higher than average values reported for grass and legume plants (Cevolani, 2005; Crovetto, 2006), likely due to the late plant harvest. The cell wall components reported in Table 2.1, hemicellulose, cellulose and available fiber besides lignin provide essential information as they affect differently the anaerobic ecosystems and influence the gastrointestinal fill (NDF) of a forage, main responsible for the voluntary intake in ruminants, especially in some physiologically phases, such as the lactation onset (Van Soest, 1994). Hemicellulose content ranged among 8.48 g/100 g DM, 9.14 g/100 g DM, and 11.4 g/100 g DM respectively for inflorescence, whole plant, and stem-leaf, while cellulose content was higher in steam-leaf (28.1 g/100 g DM) than in whole plant (24.6 g/100 g DM) (Table 2.1). It is interesting noting that these values were very close to the values reported for legume plant forages (Van Soest, 1994; Cevolani, 2005; Crovetto, 2006). As showed in Table 2.1, floral apex samples were characterized by higher starch (2.32 g/100 g DM) and total sugars (0.87 g/100 g DM) contents, which could reduce methanogenesis during the anaerobic fermentation according to Hagos *et al.* (2017). The content of non-structural carbohydrates (sugars and starch) suggests that cv. *Solaris* could be suitable to the conservation by ensiling practice (Kung *et al.*, 2018).

Finally, the investigated total alkaloids content, expressed as nicotine, was 0.30 (± 0.20) g/100 g DM of whole plant, markedly lower that values reported for the traditional smocking cultivar (Tassew and Chandravanshi, 2015), which is a fundamental prerequisite for a potential use of this cultivar as an ingredient of herbivore diets.

Table 2.1 - Chemical components (g/100 g DM) of cv. *Solaris* whole plant, floral apex, and stem-leaf (mean values ± SD) [a].

	Whole plant				**Floral apex**				**Stem-leaf**			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
OM	82.8	2.36	78.1	85.7	85.7	2.37	82.7	89.9	80.1	3.83	74.6	83.8
CP	19.0	2.36	14.8	23.2	24.8	1.96	22.4	27.8	16.0	2.64	12.6	20.1
EE	7.92	4.06	1.93	15.5	14.2	6.99	3.19	20.3	3.06	1.53	1.67	4.76
Ash	17.2	2.36	14.3	21.2	14.3	2.37	10.1	17.3	19.9	3.83	16.2	25.4
NDF	46.6	4.07	39.4	52.3	46.0	10.8	31.3	65.7	47.6	6.96	38.1	58.7
ADF	37.5	4.99	28.6	45.7	37.5	8.67	22.9	50.5	36.3	7.49	25.7	46.4
ADL	12.9	3.87	5.69	20.7	17.7	7.11	7.10	30.0	8.14	2.57	5.34	11.3
Available fibre	33.8	4.14	26.0	40.0	28.2	5.85	19.9	35.7	39.5	4.80	32.7	47.3
Hemi-cellulose	9.14	2.07	6.44	12.9	8.48	4.28	1.89	15.1	11.4	2.73	6.87	14.7
Cellulose	24.6	4.31	19.0	33.2	19.8	2.88	15.7	24.1	28.1	5.02	20.4	35.0
Starch	1.75	0.73	0.95	3.61	2.32	1.77	1.26	5.45	1.67	0.75	0.89	2.56
∑Sugars	0.56	0.88	0.03	3.05	0.87	1.41	0.22	3.75	0.26	0.42	0.01	1.18

[a] Values are means of 15 samples (in three replicates).
SD = standard deviation; Min = minimum; Max = maximum; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin.

Taken together, the results suggest that cv. *Solaris* can be considered an innovative biomass suitable as forage whose estimated net energy content for lactation in dairy cattle (Sauvant *et al.*, 2004) account for approximately 5.33 MJ NEl/kg DM and 5.83 MJ NEl/kg DM for whole plant and steam-leaf, respectively, and energy content for growth ranges 5.48 - 5.94 MJ NEg/kg DM.

Nitrogenous and mineral components

Nicotiana tabacum cv. *Solaris* KOH protein soluble content varied among the different plant samples, *i.e.*, was equal to 4.93 (± 0.91) g/100 g DM in stem-leaf, 6.83 (± 1.04) g/100 g DM in whole plant, up to 11.0 (± 1.08) g/100 g DM in floral apex. KOH protein soluble content is considered an index of protein quality for monogastric, and it is known that about 50 % of soluble proteins in plant leaves is mainly represented by the enzyme ribulose-1,5-bisphosphate carboxylase (RuBisCo) which is of interest in the food industry (Andersson and Backlund, 2008; Kobbi *et al.*, 2017).

Moreover, Table 2.2 shows for the first time the amino acid content of cv. *Solaris* whole plant, confirming the interest toward this plant as an interesting source of alternative proteins suitable for food and feed preparation. The cv. *Solaris* biomass lysine content (0.63 g/100 g DM) was close to values reported for grass plant (0.79 g/100 g DM) (Cevolani, 2005). Moreover, cv. *Solaris* levels of leucine, isoleucine, valine, and histidine were very close to values reported in literature both for legume and grass plants (Antongiovanni and Gualtieri, 2002; Cevolani, 2005). Arginine content (3.74 g/100 g DM) was found in cv. *Solaris* whole plant higher than values reported for both grass (0.85 g/100 g DM) and legume (1.20 g/100 g DM) plants, while the phenylalanine + tyrosine content (1.12 g/100 g DM in cv. *Solaris* whole plant) was close to the values reported in average for grass (1.33 g/100 g DM) plants, but lower than those reported for legume (2.06 g/100 g DM) plants (Antongiovanni and Gualtieri, 2002; Cevolani, 2005).

Table 2.2 - Amino acid profile content (g/100 g DM) of cv. *Solaris* fractions (mean values ±SD)[a].

	Whole plant		**Floral apex**		**Steam-leaf**	
	Mean	SD	Mean	SD	Mean	SD
Lysine	0.63	0.29	0.83	0.53	0.51	0.05
Threonine	0.69	0.21	0.91	0.29	0.57	0.08
Leucine	1.19	0.35	1.53	0.42	0.99	0.15
Isoleucine	0.65	0.14	0.85	0.21	0.52	0.03
Valine	0.84	0.21	1.11	0.27	0.68	0.04
Histidine	0.29	0.08	0.44	0.14	0.23	0.02
Proline	0.94	0.98	1.37	1.60	0.42	0.08
Arginine	3.74	0.31	1.58	0.14	0.65	0.08
Phenylalanine	0.72	0.19	0.98	0.28	0.58	0.03
Tyrosine	0.4	0.11	0.56	0.18	0.34	0.12
Glycine	0.92	0.30	1.16	0.40	0.73	0.06
Serine	0.69	0.21	0.96	0.33	0.58	0.06
Alanine	0.91	0.23	1.13	0.34	0.78	0.07
Aspartic acid	1.89	0.95	2.95	2.50	1.18	0.10
Glutamic acid	2.06	0.70	3.08	0.78	1.39	0.16

[a] Values are means of 5 samples (in three replicates).
SD = standard deviation.

Forage macro minerals concentration could be variable as influenced by plant species, soil fertility and climatic conditions (Given *et al.*, 2000). Many types of forage show deficiency in macro minerals such as sodium, phosphorus, and magnesium. However, with an adequate levels of soil organic matter, the forage phosphorus content can be enough to meet the animals' requirement, while the forage magnesium content is generally characterized by a low availability for new-growth plants (Lardy and Waterman, 2020). Legume forages are typically rich in minerals and are able to provide greater mineral intake levels compared than grass forages (Lardy and Waterman, 2020), so that estimating the correct ration between anions and cations in forage is essential to limit the occurrence of diseases especially in the cow's *peripartum* phase (Wildman *et al.*, 2007). As far as the mineral content of cv. *Solaris* whole plant is concerned (Table 2.3), attention should be paid to the calcium content which is almost double compared to legume hays (1.30 ± 0.35 g/100 g DM), while the phosphorus content is consistent with values observed in sainfoin, alfalfa, maize, barley, fescue (*Festuca* spp.), permanent meadows, as well as in sugar beet leaves and necks (Van Soest 1994; Cevolani 2005; Dell'Orto and Savoini 2005; Crovetto 2006). Because of the high Ca/P ratio, the eventual use of cv. *Solaris* as forage in ruminant diets would require attention in balancing the dietary mineral components, while its use in late pregnancy diet would be avoided for the above-mentioned effects on the *peripartum* phase.

Magnesium and potassium cv. *Solaris* contents (Table 2.3) were slightly higher than what detected in legume forages, *i.e.*, 0.24 (± 0.10) g/100 g DM and 2.12 (± 0.42) g/100 g DM, respectively for Mg and K (Van Soest 1994; Cevolani 2005; Dell'Orto and Savoini 2005; Crovetto 2006). Magnesium uptake by plants is affected by soil magnesium content. Wet weather and low air temperature could influence the plant Mg content, increasing the susceptibility of grazing animals to the development of hypomagnesemia tetany (NRC, 2005). On the other hand, manure or fertilizers rich in potassium can results in an excess of K content in forages, causing problems with calcium and magnesium metabolisms especially during *peripartum* period, *i.e.*, udder edema (NRC, 2005). Finally, the sodium value reported in Table 2.3 ranges between values reported for legume plant (0.02 ± 0.01 g/100 g DM) and grass plant (0.05 ± 0.04 g/100 g DM) (Cevolani, 2005; Crovetto, 2006), while chloride content is much more variable and generally reflects soil chloride content and fertilization practices, ranged for common crop plants and alfalfa, corn, wheat from 0.2 to 1.2 g/100 g DM (NRC, 2005).

Table 2.3 - Major minerals content (g/100 g DM) of cv. *Solaris* fractions (mean values ± SD) [a].

	Whole plant		Flowering apex		Steam-leaf	
	Mean	SD	Mean	SD	Mean	SD
Ca	2.48	0.57	1.35	0.31	3.35	0.94
P	0.27	0.04	0.39	0.03	0.21	0.04
Mg	0.62	0.16	0.51	0.11	0.73	0.13
K	4.33	1.28	3.96	0.66	4.13	0.94
Na	0.044	0.028	0.037	0.027	0.052	0.041
Cl	1.52	0.28	1.09	0.14	1.69	0.35

[a] Values are means of 5 samples (in three replicates).
SD = standard deviation; Ca = calcium; P = phosphorus; Mg = magnesium; K = potassium; Na = sodium; Cl = chloride.

Biochemical methane production

Consistently with the described compositional data, the biochemical methane potential (BMP) tested for the first time on whole plant samples by anaerobic digestion showed quite encouraging results, summarised by an average biogas production of 290 (± 75) Nm^3/t OM (or volatile solids) whose 58 % as biomethane. Among lignocellulosic crops evaluated for biogas/methane production under BMP operating conditions, for switchgrass (*Panicum virgatum* L.) is reported a production of 257 - 297 Nm^3 biogas/t

OM (Bhange *et al.*, 2015) and 125 Nm^3 CH_4/t OM (Frigon and Guiot, 2010), consistently with values observed for grass silage (128 - 140 Nm^3 CH_4/t OM), and gliricidia (*Gliricidia sepium* Jacq.) leaves (165 - 180 Nm^3 CH_4/t OM), while higher values are reviewed for alfalfa and rapeseed crops (240 Nm^3 CH_4/t OM) (Frigon and Guiot, 2010). Much lower values than cv. *Solaris* are reported for rice (*Oryza sativa* L.) straw (74 Nm^3 biogas/t OM), cotton (*Gossypium* spp.) stalks and mixed green leaves biomass (180 Nm^3 biogas/t OM) (Bhange *et al.*, 2015).

Conclusions

Preliminary investigation about yield and analytical components of *Nicotiana tabacum* L., cv. *Solaris* biomass as forage confirms the multiple and sustainable potentialities of its plant, adding value to its attitude already tested as source of renewable energy (biofuel from oilseed) and animal feed (alternative protein from seed cake), according to the circular bioeconomy view. The chemical components investigated showed a good quality of cv. *Solaris* fractions in terms of crude protein and fibrous carbohydrates contents, although presenting higher levels of lignin, close to what is observed in legume plants. The content of non-structural carbohydrates also suggests that it is more feasible to preserve cv. *Solaris* by ensiling the biomass. Furthermore, as key factors of anaerobic digestion, analytical components in whole plant and stem-leaf biomass portions were more balanced respect to what has emerged for the floral apex. From a nutritional standpoint the observed high Ca to P ratio needs to be balanced in the diet of lactating ruminants, and when its use is intended in ruminant nutrition. The very low level of alkaloids represents a fundamental prerequisite for the use of this cultivar as an ingredient of the diet for herbivores. Besides its possible use as forage, cv. *Solaris* biomass from whole plant showed interesting performances also as both biogas/biomethane source, according to the biochemical methane potential test, and alternative source of protein for food and feed industry.

Taken together these first results, although need to be confirmed by further studies, allow to propose *Nicotiana tabacum* cv. *Solaris* as a valid candidate for a competitive repositioning of innovated tobacco cultivation, especially in the marginal areas traditionally suited to tobacco and devoted to animal breeding. In the meantime, the suggested multiple potentialities of cv. *Solaris* raise questions about management of the crop, with reference to timing and harvesting mode, storage techniques and the development of various strategies depending on the final destination of products and derivatives.

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Chapter 3

Nicotiana tabacum L., cv. *Solaris* ensiled biomass used as forage: analytical characteristics and effects on growth, welfare, and follow-up of Holstein heifers

Introduction

Several food chains cannot be considered sustainable due to their environmental impacts, mainly related to the primary production, and to the negative externalities arising from the human demand for food and non-food supplies (Notarnicola *et al*., 2015). Most of the agriculture research are therefore focused on multipurpose crops, according to the concept of a circular economy (FAO, 2019). Within this context, new cultivar of *Nicotiana tabacum* L., named *Solaris*, has been developed as a nongenetically-modified energy plant (Fogher, 2008). The innovative cv. *Solaris* maximizes the production of inflorescences and seeds with reduced leaf production and nicotine content (Fatica *et al.*, 2019). Due to its short vegetative cycle, strong vegetative capacity, and great pedo-climatic adaptations, *Nicotiana tabacum* cv. *Solaris* has been suggested as a multifunctional plant for biomethane production, animal nutrition, and for the food industry with a circular economy approach of particular interest in marginal areas traditionally suited to tobacco cultivation (Grisan *et al.*, 2016; Poltronieri and D'Urso, 2016; Fatica *et al.*, 2019).

The global dairy sector is currently under high pressure due to the variable feed costs and incomes from farmgate milk prices, which implies a minor consideration of heifer management compared to that of milking herds (Boulton *et al.*, 2017). Heinrichs (1993) reported that the costs for raising heifers were approximately twenty percent of total dairy farm costs. Therefore, a strategy leading to reduced costs may be represented by raising dairy heifers that calve at a younger age without negative effect on the subsequent milk production (Van Amburgh *et al.*, 1998; Brown *et al*., 2005).

The isoenergetic and isonitrogenous partial replacement of dietary grains and oilseed ingredients with by-products, *i.e.*, secondary products obtained after harvesting or processing principal commodities (Grasser *et al.*, 1995), could also be considered a sustainable strategy leading to the decrease of bovine feeding costs (Bocquier and González-García, 2010). Since nutrition could have a direct effect on general animal health and welfare status, it is essential to select diet ingredients according to the animal nutritive needs and physiological status, also considering the economic balance of farming activities. For this reason, study about metabolic mechanisms plays a fundamental role in animal nutrition allowing to understand how the chemical components and energy contained in feedstuffs could be available to the animals, ensuring the fulfilment of the animal needs (Kaneko *et al.*, 2008). Metabolisms, which includes all biochemical events that occur from feed intake to excretion, involve the changes of major constituents of the diet, *i.e.*, carbohydrates, proteins, and lipids. Consequently, both chemical analysis of dietary ingredients and nutritional and hematologic profile of animals are needed to monitor animal health and welfare as related to productive and reproductive capacity (Stefani *et al.*, 2011). Several sources of variability, other than feedstuffs, *i.e.*, age, sex, gestation and lactation stage, milk yield, and season period time, could affect the concentration of plasma constituents, including nutrients, metabolites, and hormones (Herdt, 2000; Wood and Quiroz-Rocha, 2010; Yehia *et al.*, 2020; Kovačević *et al.*, 2021). More in detail, aging may affect several blood parameters so that, for instance, plasma levels of total protein, albumin, haematocrit, and magnesium are reported to increase from weaning to puberty, while glucose, calcium, phosphorus, and potassium contents decrease during the same period (Bertoni, 1985). During the first week of age neutrophils are predominant, but lymphocytes become predominant at about the second week, while in adults the concentration of both neutrophils and lymphocytes decreases, and lymphocytes are still the predominant cell type (Wood and Quiroz-Rocha, 2010). Red blood count and total leucocytes are characterized by minor changes during the pregnancy period, but several haematology parameters are reported to be influenced by seasonal and environmental factors, as seasonal parasite (Wood and Quiroz-Rocha, 2010).

Aim

As a further contribute to new advances in knowledge on the emerging *Nicotiana tabacum* L., cv. *Solaris* plant, the present study has been addressed to its ensiled biomass in terms of composition and possible use in ruminant nutrition.

The composition of ensiled cv. *Solaris* green biomass has been studied aiming to evaluate the potential storage attitude of this plant, as well as the possible growth of mycotoxigenic fungi. The use of the innovative tobacco silage was then tested in Holstein heifer diet and the effects on growth, welfare, nutritional and haematologic profile, and follow-up period were studied.

Materials and methods

The present on-field trial was performed in a private dairy farm located in Molise region (41°35'05.3"N 14°35'17.6"E). The experimental protocol, approved by the University of Molise Bioethics Committee (No. II/23-8354), was in accordance with the European Commission guidelines (2010/63/EU) concerning the protection of animals used for experimental and other scientific purposes.

Ensiling biomass and sampling

Nicotiana tabacum cv. *Solaris* green biomass (25 % dry matter, DM) was harvested when plants were in waxy ripening phase and chopped to a 4 cm particle size by a corn shredder. Briefly, cv. *Solaris* plants were transplanted in May, seed harvesting occurred in early summer, *i.e.*, about 60 days after transplanting and the green biomass has been harvested about 90 days later, *i.e.*, 150 days after transplanting. The green chopped biomass (11 t) of cv. *Solaris* was ensiled in a bunker-silo (6.5 x 3 x 1.1 m, max height) made of reinforced concrete walls on three sides and straw on the fourth side. Since *Nicotiana tabacum* cv. *Solaris* has never been subjected to ensiling practice, for a better performing of ensiling process of *Solaris* whole plant, characterized by high protein content (Table 2.1 - Chapter 2), a specific alfalfa silage microbiological additive containing different strains of *Lactobacillus plantarum* (Pioneer 11F79) was sprayed on the green biomass after diluting 10 g of product in approximately 35 L of water. At this stage, samples (n = 15, approximately 1 kg each) of biomass were collected in plastic bags (30 x 40 cm, as mini-silos, Figure 3.1) and stored under vacuum conditions at 8 - 9 °C. The biomass was appropriately pressed in the bunker-silo with a tractor to foster the best anaerobic

environment for microbial activity. For the same reason, the ensiled biomass was then covered with black and white PVC cloth on which wood and metal weights were placed to ensure the adhesion of the cloth to the biomass surface (Borreani *et al.*, 2018).
At d 0, 20, 35, 61 and 84 of ensiling, samples from mini-silos were analysed (in triplicate) to monitor their chemical and physico-chemical characteristics.

Figure 3.1 - Example of mini-silo sample stored in plastic bag under vacuum conditions. Courtesy Antonella Fatica.

Following the ensiling process in mini-silos, after eighty-eight days of ensiling, in February, the bunker-silo was opened and the ensiled biomass of cv. *Solaris* (SiloSolaris) was cleaned by removing the silage surface in contact with PVC cloth, where moulds developed down to 5 cm depth (Figure 3.2).
To investigate the potential growth of mycotoxigenic fungi on cv. *Solaris* silage, aliquots of moulds (n = 9) from different *Solaris* silage samples (n = 3) were sampled and analysed at the Plant Pathology laboratory of the University of Molise following the experimental protocol, applied with appropriate changes, used for *Fusarium* spp. isolation (De Curtis *et al.*, 2014; Vitullo *et al.*, 2014). More in detail, mould samples were vortexed with sterilized water for 5 min at 2000 x g, and diluted at 10^{-2}, 10^{-3}, and 10^{-4} respectively. Consequently, for each dilution, 10 μL of sample were inoculated in a Petri dish used Potato Dextrose Agar (PDA, n = 12) and Nutrient Yeast Dextrose Agar (NYDA, n = 32). Both substrates were treated with antibiotics, *i.e.*, ampicillin and streptomycin (n = 6 for PDA, and n = 16 for NYDA), characterized by two different mechanisms of action which allow only fungi growth, limiting bacteria. Particularly, on PDA were inoculated 90 μL of ampicillin and 90 μL of streptomycin, while on NYDA were inoculated 145 μL of

ampicillin and 145 μL of streptomycin. After incubation at 25° C for 4 days, aliquots of fungal colonies were re-inoculated on new PDA and NYDA substrates. The isolated pure colonies were used for the preparation of fresh slides observed by the optical microscope (Olympus BM-2) at 10x and 20x magnification, to perform the fungal genus identification.

Figure 3.2 - Bunker-silo section in which the grow of fungi colonies was evident. Courtesy Antonella Fatica.

Samples of SiloSolaris from bunker-silo (n = 8) were also subjected to chemical and physico-chemical analyses as mini-silo samples. The moisture, pH, crude protein (CP), ether extract (EE), ash, NDF, ADF, ADL, ammonia nitrogen, lactic, acetic and butyric acids, and total alkaloid (expressed as nicotine) contents were determined for all samples (n = 23) by an accredited laboratory (Mérieux NutriSciences Corporation, Resana, TV, Italy), according to official methods (AOAC, 1995, 2000; UNI EN ISO 2881/1992). The ammonia nitrogen content was also calculated as a percentage of the total nitrogen.

Feeding trial - Experimental design

Sixteen growing Holstein heifers were selected and divided into two groups, SiloSolaris (SS) and Control (CTR) group, homogeneous for age and body weight (BW). Average age of heifers was 359 (± 56.4) d and 349 (± 57.3) d, for SS and CTR group respectively, and BW was 298 (± 47.9) kg and 289 (± 43.5) kg, for SS and CTR group.

The grouping procedure occurred few days before the beginning of the trial to allow the animals adaptation to the environment. The two groups were kept in separate and adjacent pens on a concrete floor. Each group had unlimited access to fresh water by means of two different automatic drinkers positioned in each box.

For both groups, isonitrogenous and isoenergetic diets (Table 3.1) were formulated according to the nutritional needs of the animals (Van Amburgh *et al.*, 1998; NRC, 2001).

Heifers were group fed experimental diets characterized by a 70:30 forage to concentrate ratio on a DM basis. Both groups received daily an average of 16.2 kg DM of concentrate mixture, including corn meal (43.48%), wheat middlings (43.48%) and soybean meal (44% CP, 13.04%). CTR group received daily an average of 39.4 kg DM of hay (consisting of a prevalence of *Trifolium* spp. and *Lolium* spp.), and SS group received an average of 23.0 kg DM of the same hay and 12.7 kg DM of SiloSolaris blended with the concentrate mixture. Silage in SS diet accounted for 55% of the DM from forages.

Table 3.1 - Total group (n = 8 heads) DM administered, chemical components (g/kg DM) and calculated energy content (UFL/kg DM) of experimental diets for growing Holstein heifers.

	SS	**CTR**
Total group DM administered, kg DM per d	51.53	55.67
Chemical composition (g/kg DM) **and energy content** (UFL/kg DM)		
CP	150	140
EE	50	30
Ash	90	90
NDF	390	420
ADF	250	270
ADL	50	30
UFL	0.81	0.80

SS = SiloSolaris group; CTR = Control group; DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; UFL = Forage unit for lactation (1 UFL = 7.12 MJ NEI/ kg, Sauvant *et al.*, 2004).

Both groups received the daily ration divided into two meals at 07:00 and 17:30 h. For the first thirty-six days of the trial, all heifers were allowed to metabolically adapt to the diet, especially SiloSolaris heifers, which underwent a gradual daily increase of the ensiled biomass since they had never eaten any kind of silage before. Starting from the second week of the trial, the mixture of silage and feeds was offered in the manger separated from the hay to allow SS heifers to consume either SiloSolaris and concentrates or hay, based on their preferences. Including the adaptation phase (36 days), the feeding trial lasted eighty-one days. Data from forty-five days of experimental diet administration are reported and discussed.

Feeding trial - Measurements and recordings

Before each meal, residues left in SS and CTR mangers were weighed and recorded, as well as all feeds administered to both groups. Feedstuffs and residues were sampled weekly and analysed for their DM contents (AOAC, 2000). The daily group DM intake was calculated as the difference between feedstuffs administered and refusals collected for each group. Furthermore, at d 0, 36 and 81, all heifers were weighed and scored for body condition (BCS), fecal consistency (FS) and deambulatory capacity (LM) according to the literature (Edmonson *et al.*, 1989; Sprecher *et al.*, 1997; Stallings, 1998; Hall, 2002). For each group also average daily gain (ADG) and feed conversion rate (FCR) were calculated. Particularly, group FCR was calculated as the ratio between estimated average group DM intake and group ADG. At d 81, all experimental animals underwent blood sampling by jugular venipuncture using EDTA-treated vacuum tubes (BV Vacutainer® Collection Tubes) approximately 3 hours after the morning feeding. Samples were cooled on ice, centrifuged for 10 min at 1880 x g, frozen in liquid nitrogen and stored at - 80 °C. Analyses of plasma samples, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), glucose, cholesterol, triglycerides, total protein, albumin, creatinine, plasma urea nitrogen (PUN), uric acid, calcium, inorganic phosphorus, magnesium, and iron contents, and of the haematologic profile, *i.e.*, haematocrit (HCT), haemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), red blood cell (RBC), red cell distribution width (RDW), total leucocyte (WBC), mean platelet volume (MPV), neutrophils, lymphocytes, monocytes, eosinophils and basophils were performed by Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (Campobasso, Italy), according to literature methods (IZSAM, 2018). After the feeding trial, heifers were raised to the usual farm management and were monitored during the follow-up period, lasting up to 1 year after calving, for age at first insemination, age at first calving and average daily milk yield (305 days of lactation). One CTR heifer was lost during the follow-up period because of a uterine malformation.

Statistical analyses of results

Descriptive statistics were calculated for each variable (Microsoft© Excel© for Office 365 ProPlus Version 1903 -11425.202429). Data on the chemical components and pH of the silage from mini-silo were processed by analysis of variance (GLM). In case of significant effects ($P < 0.05$), differences among means were analysed by least significant difference.

Because there was only one pen per treatment, only descriptive statistics are reported for data on group DM intake, refusal and FCR.
As animals were group-fed with no replications of pens within treatments, following the assumptions that there was no pen effect, and that errors within pens were independent, the heifer was considered as experimental unit. Therefore, individual BW, BCS, ADG, FS and LM were processed by repeated measures analysis of variance, also considering the covariate effect of "age at d 0". Data on plasma and blood constituents were processed by analysis of variance considering the covariate effect of "age at d 81" (GLM, ANCOVA). Follow-up data were processed by analysis of variance considering the covariate effect of "age at d 0" (GLM, ANCOVA).
All statistical analyses were conducted with IBM SPSS Data Editor ver. 25.

Results and Discussion

Chemical composition of Nicotiana tabacum cv. Solaris silage (SiloSolaris)

The chemical components of SiloSolaris samples from bunker-silo are shown for the first time in Table 3.2, also reporting literature data for corn (*Zea mais* L.) and sorghum (*Sorghum vulgare* Pers.) silages (Crovetto, 2006; Colombini *et al*., 2010) for comparison as major silages used in dairy farms of Centre-South Italy.

Table 3.2 - Chemical components (g/100 g DM unless otherwise indicated) of SiloSolaris from bunker-silo (n = 8) administered to experimental heifers. Literature data $^{\alpha, \beta}$ on corn and sorghum silages are displayed for comparison. All values expressed as mean ± SD.

	SiloSolaris	Corn silage $^{\alpha, \beta}$	Sorghum silage$^{\alpha, \beta}$
DM, g/100g	24.1 (± 1.84)	34.4 (± 3.60)	24.9 (± 10.0)
OM	87.5 (± 0.63)	95.8 (± 0.50)	89.0 (± 4.50)
CP	16.4 (± 1.54)	8.20 (± 0.60)	10.0 (± 2.10)
EE	11.2 (± 0.90)	2.50 (± 0.04)	2.00 (± 0.40)
Ash	12.5 (± 0.63)	4.8	9.4
NDF	42.6 (± 2.01)	39.5 (± 5.00)	60.5 (± 4.80)
ADF	32.3 (± 1.58)	22.7 (± 2.90)	37.9 (± 4.10)
ADL	9.95 (± 0.79)	2.10 (± 0.70)	3.20 (± 2.40)
Starch	4.89 (± 0.64)	32.3 (± 4.60)	1.50 (± 2.40)

$^{\alpha}$ Crovetto, 2006; $^{\beta}$ Colombini *et al.*, 2010.
DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin.

Most likely because of the adverse climatic conditions during late harvesting (beginning of November), the *Solaris* silage average DM content was consistent with values observed in ensiled sorghum but lower than values reported for corn silage (Table 3.2), and for alfalfa (*Medicago sativa* L.) silage (61.0 g/100 g) as described by Vanhatalo *et al.* (1999). SiloSolaris OM content was close to values reported for alfalfa silage (87.7 g/100 g DM) (Crovetto *et al.*, 2006), but lower than those reported for sorghum and corn (Table 3.2). As reported in Table 3.2, the concentration of proteins in SiloSolaris was intermediate between those for sorghum and alfalfa silages (10.0 g/100g DM and 21.0 g/100g DM, respectively) (Vanhatalo *et al.*, 1999), and almost double the values reported for corn silage (Crovetto *et al*., 2006).

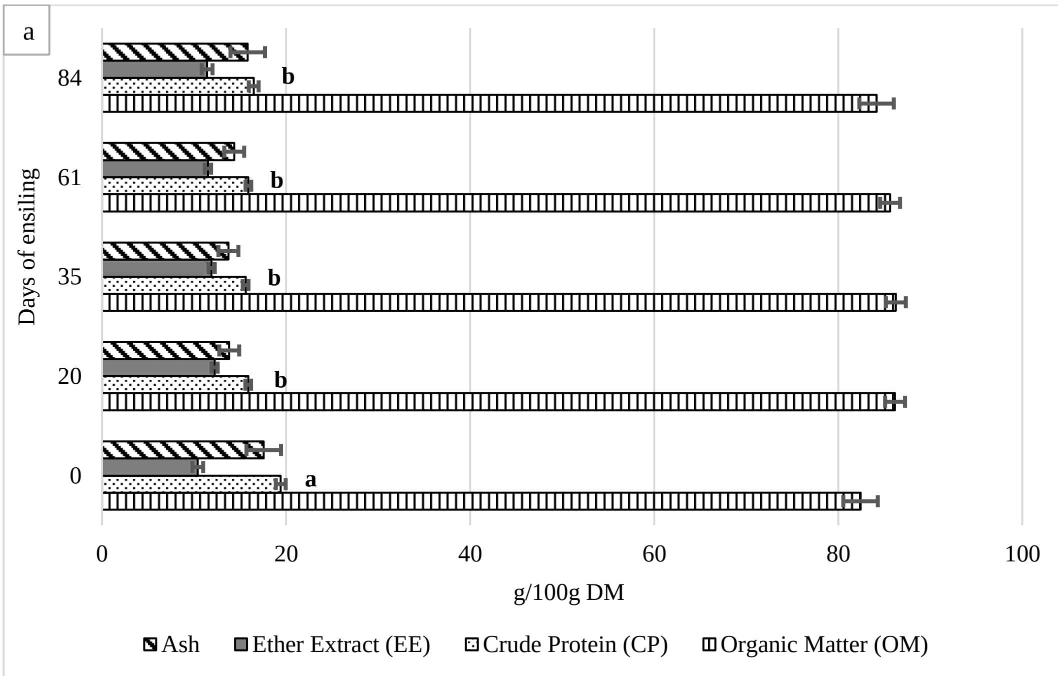
Bearing in mind the main characteristic of cv. *Solaris* as an energy plant, it is not surprising the higher SiloSolaris content of ether extract compared to the values reported for sorghum and corn silages (Table 3.2) and alfalfa silages (7.7 g/100 g DM) (Vanhatalo *et al.*, 1999). The starch content observed in SiloSolaris samples was approximately 5 times lower than that in corn silage, while the ash content was remarkably higher than that of corn and sorghum silages (Table 3.2) and alfalfa silage (7.5 g/100g DM) (Vanhatalo *et al.*, 1999).

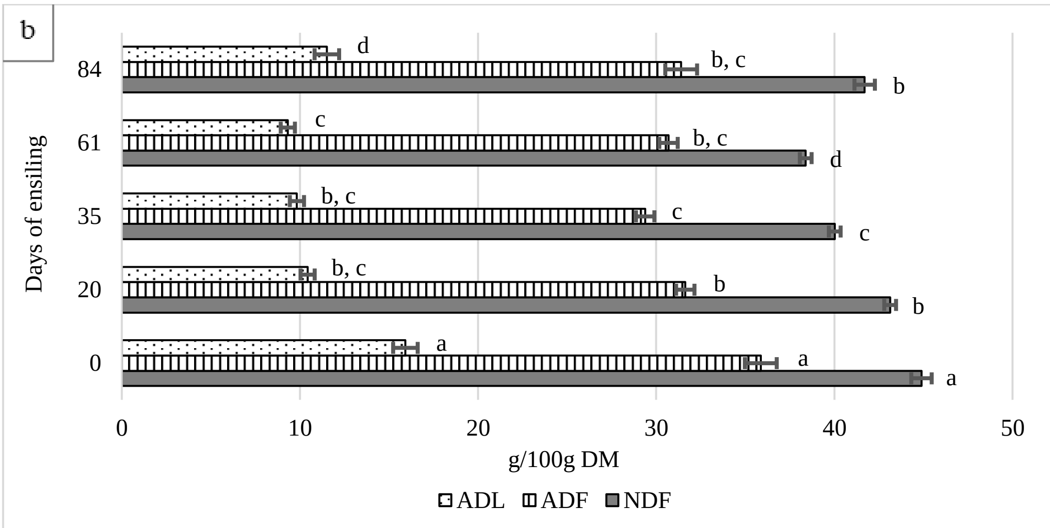
Among the fiber fractions of bunker-silo, SiloSolaris displayed higher values of ADL, possibly related to the late harvest of the biomass, but average NDF and ADF contents consistent with the values for corn and sorghum silages (Table 3.2), as well as for alfalfa silage (42.7 g/100 g DM and 33.5 g/100 g DM, respectively for NDF and ADF) (Crovetto *et al*., 2006). As far as the fermentation end products are concerned, in *Solaris* bunker-silo samples butyric acid was never detected, while lactic and acetic acids averaged 9.00 (± 7.52) g/100g DM and 4.27 (± 0.59) g/100g DM, respectively. It is worth noting that the observed lactic acid content was consistent with values reported for both legume and grass silages with a low DM content (Kung *et al.*, 2018); however, the ample variability among samples should be highlighted. Relatively stable values were found for the acetic acid content, whose concentration was found higher than that reported on a DM basis for corn sorghum and alfalfa silages (Crovetto, 2006). According to Kung *et al.* (2018), a high acetic acid concentration can be observed in wet silages but also when the ash content is high. The ammonia nitrogen content was 0.41 (± 0.12) g/100g DM and accounted for 15.7 (± 5.25) % of the total N, consistent with values reported for both sorghum (Crovetto, 2006) and high-moisture silages (Kung *et al.*, 2018). The mean pH value of bunker silo samples, in average 5.02 (± 0.23), were intermediate between sorghum and alfalfa silages (Crovetto, 2006). The previously mentioned SiloSolaris high protein and ash contents can be responsible for its high buffering capacity, according to Kung *et al.* (2018).

Furthermore, in the same samples the total alkaloid content was on average 1.35 (± 0.24) g nicotine/100 g DM, being lower than values reported for some traditional smoking tobacco cultivars (Wang *et al.*, 2008; Tassew and Chandravanshi, 2015; Fatica *et al.*, 2019).

The chemical changes monitored during the 84 days of ensiling process in mini-silo showed a DM content ranging (minimum and maximum value) from 23.1 g/100 g to 27.6 g/100 g. As reported in Figure 3.3 a, the organic matter content varied from 82.4 g/100 g DM to 86.3 g/100 g DM (Figure 3.3 a). CP content, ranging from 15.6 g/100 g DM to 19.4 g/100 g DM, showed a significative decline ($P < 0.01$) at the beginning of the ensiling process (Figure 3.3 a).

The ether extract content varied from 10.4 g/100 g DM to 12.2 g/100 g DM, while the observed values of ash content ranged between 13.7 g/100 g DM and 17.6 g/100 g DM (Fig. 3.3 a). Figure 3.3 b displays the average values of the fiber fractions observed during the ensiling period, showing significant reduction for NDF ($P < 0.001$), ADF and ADL ($P < 0.01$) contents. Observed minimum and maximum values were 38.4 g/100 g DM and 44.9 g/100 g DM, 29.4 g/100 g DM and 35.9 g/100 g DM, 9.3 g/100 g DM and 15.9 g/100 g DM, for NDF, ADF and ADL, respectively.





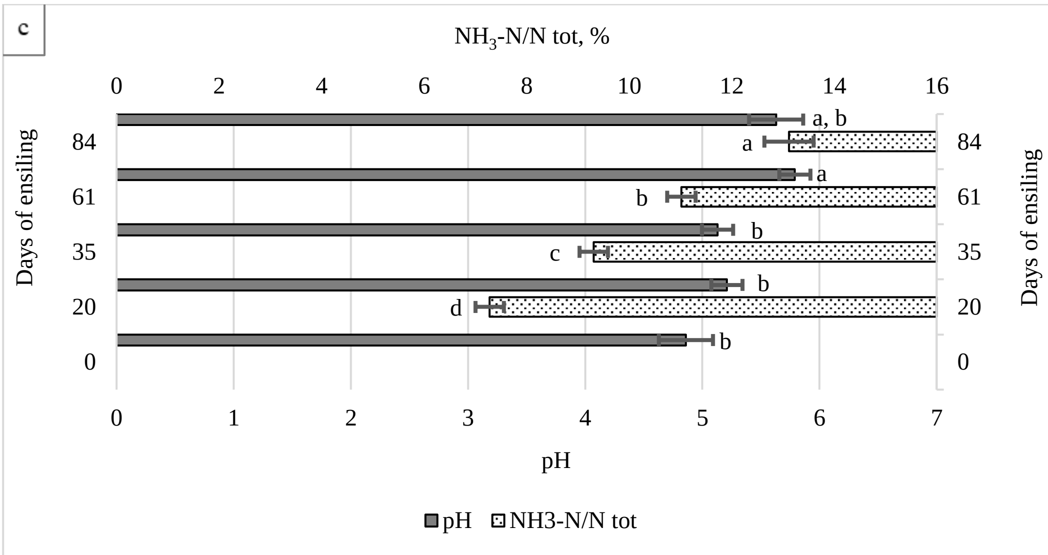


Figure 3.3 - Chemical components and pH (mean ± SEM) of *Solaris* silage during the ensiling process studied in mini-silo (n = 15): **a**) organic matter, crude protein, ether extract and ash contents; **b**) NDF, ADF and ADL contents; **c**) ammonia nitrogen and pH. (a, b, c, d means with different superscripts differ; $P < 0.05$).

Data on NH_3-N from the mini-silo study, not available for d 0, varied between 7.3 % of total nitrogen (d 20) and 13.1 % of total nitrogen (d 84), while pH values increased from 4.9 (d 0) to 5.8 (d 84). Significant differences during ensiling were found for both NH_3-N ($P < 0.001$) and pH ($P < 0.05$) (Figure 3.3 c).

As expected, the observed chemical components of SiloSolaris from mini-silo can be considered at d 84 consistent with values observed in bunker silo and the results provide the first evidence of the evolution in the ensiling process.

Fungi isolation from Solaris bunker-silo biomass

No fungal growth was observed in 10^{-2} diluted samples either PDA or NYDA, treated or not with antibiotics, probably because of the high total fungi load. About 10^{-3} and 10^{-4} sample dilutions, fungal colonies with spheric shape and white colour, and fungal colonies characterized by regular shape and white-light blue colour were observed (Figure 3.4). At the optical microscope, all the identified fungi appeared to belong to the genus *Penicillium* since they were characterised by basal structure on which conidiophores branches with spheric conidia were developed, according to literature (Jard *et al.*, 2011; Vandicke *et al.*, 2021).

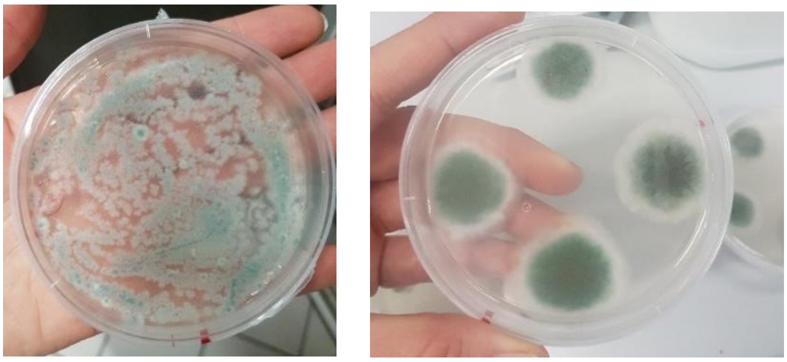


Figure 3.4 - Example of Petri dishes with fungi colonies characterized by regular shape and white-light blue colour at 10^{-3} dilution (left), and after pure colony inoculation (right). Courtesy Antonella Fatica.

Ensiled feed commonly used as ingredient of animal diets could be contaminated by mycotoxins, secondary metabolites produced by a variety of fungi which can cause severe toxic effects in consumers, both animals and humans (Vandicke *et al.*, 2021). *Penicillium* spp., *Aspergillus* spp. and *Fusarium* spp. are considered the major mycotoxigenic fungi as they can survive at low oxygen and pH conditions (Storm *et al.*, 2014). It is therefore essential to ensure the correct management of the plants from the field and then during the ensiling process. Following a poor ensiling procedure, oxygen could remain entrapped in the biomass allowing unwanted microbial activities. For instance, spores could germinate and colonize the ensiled biomass possibly producing mycotoxins (Richards *et al.*, 2007; Vandicke *et al.*, 2021).

Based on these first results, further studies are needed for a genetic identification by Polymerase Reaction Chain (PCR) analysis to investigate the possible development of toxigenic fungi in *Solaris* ensiled biomass.

Effects of dietary treatments on growth, welfare and follow up

During the 45 days of experimental phase, the daily group DM administered, DM intake and DM refusals were reported in Figure 3.5 and in Figure 3.6, respectively for SS and CTR group. SS during the first week of the feeding trial showed a group DM intake lower than CTR group. It is interesting to note that, at d 13 - 15, 23 - 25, and 39 - 41, both groups displayed an increase of DM refusal, probably related to environmental and management conditions (Figure 3.5 and Figure 3.6).

After 36 days of feeding and environmental adaptation phase, group DM intake in SS thesis averaged 48.5 (± 0.31 SEM) kg/d, ranging from 43.5 kg/d to 51.9 kg/d, while the observed group DM refusals ranging from 0.00 kg/d to 5.82 kg/d, was in average 2.22 (± 0.22 SEM) kg/d (Figure 3.5).

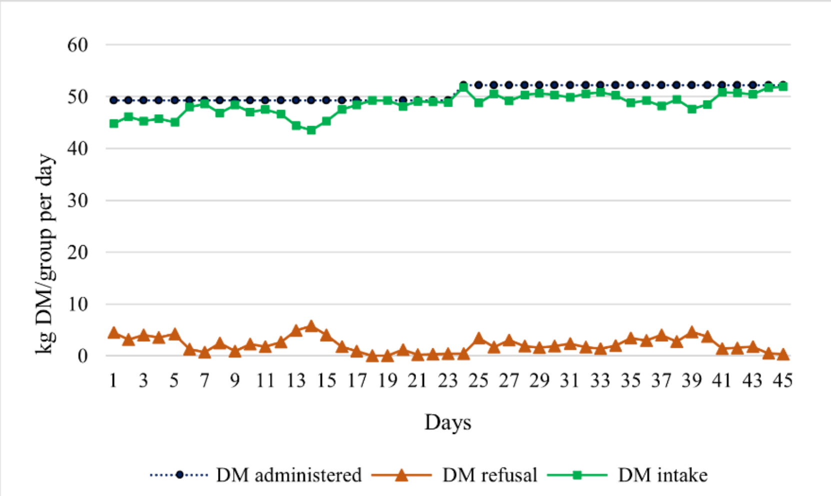


Figure 3.5 - SiloSolaris daily group DM administered, DM refusal and DM intake during 45 days of feeding trial.

The daily DM consumption of CTR group averaged to 54.9 (± 0.12 SEM) kg, ranging from 52.5 kg to 55.7 kg, with an average group DM refusal of 0.74 (± 0.11 SEM) kg/d, within a range 0.00 - 3.14 kg/d (Figure 3.6).

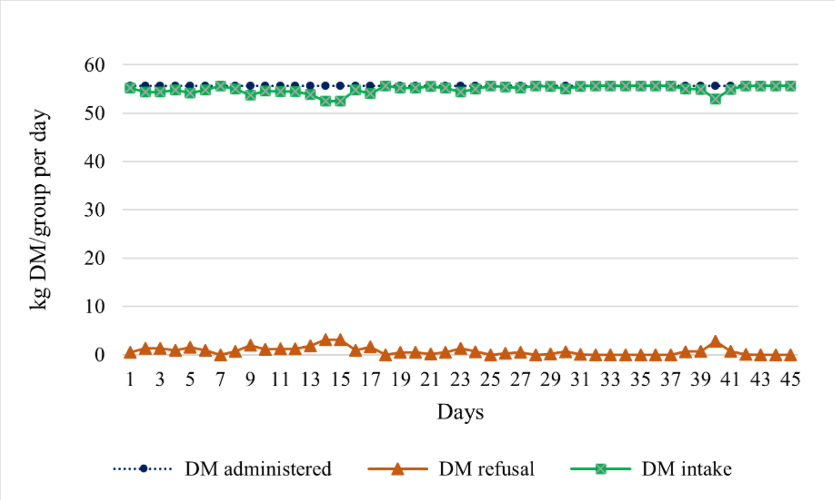


Figure 3.6 - Control daily group DM administered, DM refusal and DM intake during 45 days of feeding trial.

For purely descriptive purposes, the individual DM intake was calculated at 6.1 kg/d and 6.9 kg/d for SS and CTR diet, respectively.

The investigated individual growth performance indices, BW and ADG, were not affected by the dietary treatments (P > 0.05) (Table 3.3). The observed values were consistent with the optimal ADG before puberty as reviewed by Heinrichs *et al.* (2017). Sejrsen and Purup (1997) reported that an ADG of 0.9 kg/d could reduce the age at first calving to eighteen months even though they suggest an ADG lower than 0.7 kg/d during growth (from 90 to 300 kg BW) for optimal mammary gland development. Furthermore, Wattiaux (1997) recommended the first calving at twenty-four months with an average ADG close to 0.75 kg/d, but when the first insemination is carried out at twelve months, the ADG should be approximately 0.95 kg/d. Based on a group average daily bodyweight gain of 7.88 (± 0.20, SD) kg for SS diet and 5.91 (± 0.24, SD) kg for CTR diet, the estimated FCR was 6.16 kg DM/kg ADG for SS group and 9.29 kg DM/kg ADG for CTR group. However, as already mentioned, data on DM intake and FCR were not subjected to statistical analysis because heifers were group-fed, so that the reported descriptive data need further investigation and cannot be considered conclusive.

Regarding the welfare indices reported in Table 3.3, the dietary treatment did not significantly affect (P > 0.05) the heifers' body condition. Lean *et al.* (2015) reported that in cows the optimal BCS at calving should be 3.25 - 3.5, while Petrovski (2015) suggested a score of 4 as optimal for dry cows and heifers. According to El-Kasrawy (2020), the best BCS of heifers at first artificial insemination should range between 2.5 and 4, for a positive effect on productive efficiency during lactation.

As Table 3.3 shows, the fecal consistency of heifers was not affected by the dietary treatment: in both groups of heifers, manure in fact appeared thick and formed a solid pat two-three cm in height (Stallings, 1998), with scores ranging from 3 - 3.5 (on a 1 - 4 scale). The evaluation of fecal consistency may provide important information on ruminants' digestion and can allow for the diagnosis of early fermentation problems (Stallings, 1998). In this trial, the higher lipid intake by the SS group (5 % DM) *vs.* the CTR group (3 % DM) does not seem to have affected the rumen fermentations. Furthermore, in both experimental groups the locomotion scores were always 1 (in a 1 - 5 scale) meaning that the deambulatory capacity of the heifers was normal, as all animals stood and walked with a level back posture (Sprecher *et al.*, 1997). According to the tendentially better conversion rate and the observed welfare indices, results could suggest a better utilization of the diet in the SS group, but this hypothesis should be more in depth investigated. As far as the follow-up period is concerned, the investigated reproductive traits, age at first insemination and at calving, did not significantly differ (P > 0.05) between the two groups as well as the average daily milk yield (Table 3.3).

Table 3.3 - Experimental heifers body weight, growth, welfare, and follow-up parameters.

	SS	**CTR**	**SEM**	**P-value**
BW (kg), d 36	337.4	332.1	11.8	ns
BW (kg), d 81	374.1	354.1	11.6	ns
ADG (kg/d), d 36 – 81	0.94	0.80	0.07	ns
BCS, d 36	3.65	3.54	0.10	ns
BCS, d 81	3.87	3.54	0.09	ns
FS, d 36	3.18	3.23	0.08	ns
FS, d 81	3.12	3.38	0.08	ns
LM, d 36	1	1	-	-
LM, d 81	1	1	-	-
Age at first insemination, d	627.3	630.5	37.2	ns
Age at first calving, d	863.1	846.3	6.96	ns
Average milk yield, kg per d	27.0	26.2	0.96	ns

SS = SiloSolaris group; CTR = Control group; BW = body weight; ADG = average daily gain; BCS = body condition score, FS = fecal score; LM = locomotion score; ns = not significant. All values are expressed as the means and pooled SEM.

The average age at first calving observed in both experimental groups, reflecting a nonsignificant difference in age at first insemination, was higher than values generally reported in the literature for primiparous cows with a BW between 544 kg and 567 kg (Keown and Everett, 1986). According to Krpalkova *et al.* (2017), the optimal age for the first calving in Holstein-Friesian heifers should be twenty-four months to maximize production and minimize rearing costs. However, the optimal age for first calving is always a specific trait of each animal population and management strategy (Cooke *et al.*, 2013; Heinrichs *et al.*, 2017). The average daily milk yield did not significantly differ ($P > 0.05$) between the two groups (Table 3.3).

Nutritional and haematologic profile of heifers

As far as the nutritional profile of experimental heifers is concerned, the majority of the investigated plasma constituents were within or very close to the range reported in the literature for adult bovine (Kaneko *et al.*, 2008) and for heifers in the winter season (Stefani *et al.*, 2011). However, significant differences between the dietary treatments were observed for albumin ($P < 0.05$), triglycerides and cholesterol ($P < 0.01$), and magnesium ($P < 0.001$) contents (Table 3.4). More in detail, plasma constituent intervals (minimum - maximum) observed respectively for SS and CTR diet were 34 - 36 g/L and 28 - 34 g/L for albumin, 0.18 - 0.31 mmol/L and 0.14 - 0.21 mmol/L for triglycerides, 3.89 - 5.08 mmol/L and 2.20 - 4.35 mmol/L for cholesterol, and 0.99 - 1.05 mmol/L and 0.88 - 0.99 mmol/L for Mg.

The observed nutritional profile related to lipid metabolism could be associated with a higher lipid content in the SS diet. Lipids have many functions, among which the most important are considered energy storage and cell membrane structure. Cholesterol, besides being a precursor of steroid hormones, vitamin D, and bile acids, is a constituent of cell membranes and bile micelles (Kaneko *et al.*, 2008). Triglycerides, instead, are the most important lipids involved in energy storage, while phospholipids are important cell membrane constituents (Kaneko *et al.*, 2008). Regarding the cholesterol plasma concentration, it is worth noting that observed values were within the range 1.68 - 6.55 mmol/L reported by Van Saun (2008).

Plasma albumin concentration is determined by the hepatic synthesis rate, which normally is in equilibrium with its degradation. However, the investigated indices for hepatic disorders, *i.e.*, ALT and AST, as well as for the status of the biliary system, *i.e.*, ALP and GGT, suggest the integrity of the hepatocytes in both groups (Kaneko *et al.*, 2008).

Hepatic function could be assessed also by the urea plasma level, as further index of liver syntheses, or by the plasma bilirubin, as index of liver excretory capacity (Kaneko *et al.*, 2008). Bilirubin, yellow pigment insoluble in water produced by enzymatic degradation of heme, is transported by plasma albumin, from synthesis sites to the liver (Ryter *et al.*, 2006). In cattle with terminal hepatic insufficiency, a significant biochemical increase of plasma bilirubin is observed, in fact, clinical icterus in ruminants is often associated to an excess of the excretory bilirubin capacity (Kaneko *et al.*, 2008). Values of total bilirubin reported for SS and CTR heifers were found lower than the range of values (1.7 μmol/L - 6.8 μmol/L) reported for Holstein heifers between six months and two years old (Lumsden *et al.*, 1980), but within the range of 0.0 μmol/L and 8.6 μmol/L reported for Holstein heifers over two years of age (Lumsden *et al.*, 1980). Amylase, like lipase, is a low-molecular-weight enzyme that cleaves the glycan linkage of starch and glycogen used as diagnostic enzyme longer than any other enzyme (Kaneko *et al.*, 2008). Amylase, produced by the small intestine and liver, contributes to the normal serum amylase activity which is commonly used as a screening factor for both renal and pancreatic diseases (Kaneko *et al.*, 2008). The amylase levels observed for the experimental heifers were within the range 20 - 770 U/L reported for Holstein heifers from six months to two years of age (Lumsden *et al.*, 1980).

Regarding the Mg plasma content, founded higher in SS group than in CTR group, it must be considered that the normal range varies from 0.9 to 1.2 mmol/L, provided that the influx into the cellular space is larger than the efflux (Martens *et al.*, 2018). Mg is involved in enzymatic reactions after combining with enzyme/substrate systems, and plasma Mg is influenced in a nonspecific manner by catecholamines, insulin and the parathyroid hormone (Martens *et al.*, 2018). The inorganic phosphorus plasma levels although unaffected by the dietary treatment, were found slightly higher than values reported in Table 3.4 for comparison, although this, Van Saun (2008) reported that plasma P ranges from 1.45 to 2.58 mmol/L. Generally, inorganic phosphorus is excreted from the body via the kidneys and may also be elevated when the glomerular filtration rate is decreased by whatever causes (Kessell, 2015).

Table 3.4 - Plasma constituents of experimental heifers compared to literature [α, β] values.

Plasma parameters	Unit	SS	CTR	SEM	P-value	Reference intervals	
						2008 [α]	2011 [β]
AST	U/L	88.0	88.5	3.35	ns	78 - 132	51 - 89
ALT	U/L	34.4	34.6	1.51	ns	11 - 40	13 - 35
ALP	U/L	182.9	184.3	15.3	ns	0 - 488	113 - 268
GGT	U/L	11.8	13.4	1.72	ns	6.10 - 17.4	10 - 23
Glucose	mmol/L	3.17	3.11	0.15	ns	2.50 - 4.16	3.10 - 4.00
Cholesterol	mmol/L	4.51	3.64	0.16	**	2.07 - 3.11	1.93 - 4.33
Triglycerides	mmol/L	0.24	0.19	0.01	**	0 - 0.20	0.11 - 0.44
Total protein	g/L	68.0	68.0	1.14	ns	67.4 - 74.6	61 - 81
Amylase	U/L	21.00	28.25	2.66	ns	na	na
Albumin	g/L	34.7	32.4	0.55	*	30.3 - 35.5	31 - 40
Creatinine	mmol/L	108.3	103.9	4.17	ns	88.4 - 177	72 - 119
PUN	mmol/L	10.3	9.54	0.42	ns	7.10 - 10.6	na
Uric acid	mmol/L	58.5	57.5	2.05	ns	0 - 119	na
Total bilirubin	µmol/L	0.002	0.21	0.16	ns	na	na
Ca	mmol/L	2.58	2.55	0.02	ns	2.43 - 3.10	2.27 - 2.67
P	mmol/L	2.68	2.54	0.07	ns	1.81 - 2.10	1.53 - 2.44
Mg	mmol/L	1.03	0.93	0.01	***	0.74 - 0.95	0.71 - 0.99
Fe	µmol/L	26.0	24.1	0.87	ns	10.2 - 29.0	14.5 - 32.4

[α] Kaneko *et al.*, 2008; [β] Stefani *et al.*, 2011.
SS = SiloSolaris group; CTR = Control group; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; GGT = gamma-glutamyl transferase; PUN = plasma urea nitrogen; Ca = calcium; P = inorganic phosphorus; Mg = magnesium; Fe = iron; * P < 0.05; ** P < 0.01; *** P < 0.001*;* ns = not significant; na = not available. All values are expressed as the means and pooled SEM.

Haematologic profile of SS and CTR heifers is showed in Table 3.5 along with reference interval for Holstein cows in mid-lactation and bovine with an average age of 1.6 years (George *et al.*, 2010; Hermann *et al.*, 2018) reported for comparisons.

Table 3.5 - Haematologic profile of experimental heifers compared to literature $^{\alpha, \beta}$ values.

Blood Parameters	Unit	SS	CTR	SEM	P-value	Reference interval $^{\alpha, \beta}$
HCT	L/L	0.27	0.27	0.004	ns	0.24 - 0.39
HGB	g/dL	9.87	10.1	1.72	ns	8.20 - 13.0
MCH	pg	14.9	15.0	0.44	ns	14.3 - 19.6
MCHC	g/L	360.7	370.2	1.12	***	360 - 390
MCV	fl	41.4	40.5	1.21	ns	41.2 - 58.7
RBC	10^{12}/L	6.64	6.78	0.22	ns	4.80 - 7.60
RDW	%	19.8	19.9	0.36	ns	15.5 - 19.7
WBC	10^{9}/L	10.7	11.4	0.63	ns	4.90 - 12.0
Platelets	10^{9}/L	349.4	345.8	32.1	ns	193 - 637
MPV	fl	7.22	6.88	0.49	ns	4.50 - 7.50
Neutrophils	g/L	30.6	29.1	2.11	ns	18 - 63
Lymphocytes	%	59.8	58.8	2.36	ns	16 - 56
Monocytes	%	5.22	6.79	0.54	ns	0.0 - 8.00
Eosinophils	%	2.30	2.91	0.50	ns	0.0 - 9.00
Basophils	%	1.44	1.50	0.07	ns	0.0 - 3.00

$^{\alpha}$ George *et al.*, 2010; $^{\beta}$ Herman *et al.*, 2018.
SS = SiloSolaris group; CTR = Control group; HCT = haematocrit; HGB = haemoglobin; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; RBC = red blood cell; RDW = red cell distribution width; WBC = total leucocyte; MPV = mean platelet volume; *** P < 0.001; ns = not significant.

The investigated haematologic parameters were always within the range reported in literature (Table 3.5) however a significant difference (P < 0.001) between the dietary treatments was observed for mean corpuscular haemoglobin concentration (MCHC), accounting for 360.7 g/L and 370.2 g/L, respectively for SS and CTR group. The observed MCHC values were within the range also reported by George *et al.* (2010) and Herman *et al.* (2018). It is worth noting that both the red cell distribution width (RDW) and the lymphocyte count showed average values slightly higher (P > 0.05) than those reported in Table 3.5 for comparisons. However, Wood and Quiroz-Rocha (2010) reported for 99 % clinically healthy cows' values ranging 16 - 20 % and 18 - 81 %, for RDW and lymphocyte concentration, respectively.

Age, physiological stage, health, milk yield, and season period could affect bovine haematologic and biochemical parameters, whose analyses are relevant for the diagnosis of haematologic disorders as well as of many organs and systemic diseases (Kovačević *et al.*, 2021). Bovine erythrocytes or RBC, anucleate cells with a dimension of 6 μm and characterized to a lifetime of 130 - 160 days, are involved in the transport of oxygen through the bound with haemoglobin structure maintaining iron in the functional ferrous state (Brockus, 2011). Red blood cells, as response of erythropoietin produced by kidneys, originate in bone marrow and are responsible of gas exchange by carrying oxygen and carbon dioxide into the heme structure; for RBC complete description also the evaluation of HCT, HGB, MCV, MCH, and MCHC are needed (Jones and Allison, 2007; Roland *et al.*, 2014). In calves a tendential decrease is observed in HGB, MCH and MCHC values during the first month of age, followed by an increase during the first three months of age (Mohri *et al.*, 2007), nevertheless RBC count could be higher while MCV and MCHC lower in young cattle than in adult animals (Roland *et al.*, 2014).

Conclusions

In this chapter, the chemical components of *Nicotiana tabacum* L., cv. *Solaris* silage biomass, presented for the first time, show ensiling as valid storage process for this innovative forage. The *Solaris* silage pathology initial investigation allowed to identify fungal species potentially mycotoxigenic, that require more in-depth studies about plant cultivation, as well as storage processing in order to prevent mycotoxins development. Dietary treatment with *Solaris* silage did not affect heifers' growth and welfare as well as the productive and reproductive traits investigated in the follow-up period. Furthermore, the observed plasma and blood constituents were found always within or very close to the normal ranges, even though the high lipid content of *Solaris* silage could be responsible for the higher plasma triglycerides and cholesterol values observed in SiloSolaris than in Control group.

Although the presented results need to be confirmed, they suggest that the inclusion of *Nicotiana tabacum* cv. *Solaris* ensiled biomass as valuable forage in the diet of growing heifers may be interesting as further co-product of this versatile energy plant.

Due to the generalized crisis of smoking tobacco sector, occurred during the last decades, the valorisation of products obtained from this new cultivar could help revitalising those local economies of marginal areas traditionally devoted to the tobacco cultivation, as an innovative and crucial step towards sustainable and green agriculture, based on a circular economy approach that combines energy and feed sectors while reducing the competition between food and feed products.

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Chapter 4

Nicotiana tabacum cv. *Solaris* seed cake as dietary ingredient for growing lambs raised in inner areas of Molise region: first results

Introduction

Sheep farming has always been considered strategic especially for Mediterranean inner areas, also due to its important role about animal and vegetal diversity conservation and promotion. In most European countries, animals are raised according to extensive systems based on the use of grasslands and pasture for a large part of the year (Porqueddu *et al.*, 2017), thanks to the ability of these animals, which supply primary products as milk, meat, and wool, to have been adapted to strong climatic and land conditions (Idda *et al.*, 2010). Nowadays, mountainous areas are characterized by new economic, commercial, and social relationship and approaches which have determined important changes in land use and in the organization of livestock farming systems (Riedel *et al.*, 2007; Geß *et al.*, 2020) also including few changes in the selection of low-input feeds. Reducing both feeding costs and environmental impacts of the livestock production systems can be possible by using agro-industrial by-products without affecting animal health and welfare status, or growth performances (Tufarelli *et al.*, 2013; Monteiro *et al.*, 2020). On this regard, also considering the need of renewable energy sources (fuel) alternative to food plants, in order to decrease the competition between food and feed products (Rossi *et al.*, 2013), once again reference is made to *Nicotiana tabacum* L., cv. *Solaris*, innovative energy plant, characterized by high production of inflorescences and seeds suitable both for energetic purposes and innovative feedstuffs, as reported in literature (Fogher, 2008; Fatica *et al.*, 2019) and in current thesis. Each gram of tobacco seeds contains around 10 000 - 18 000 seeds (Mohammad and Tahir, 2014) so that, they require greenhouse for germination and plants are transplanted in soil only when they are around 25 - 30 cm height (MIPAAF, 2005). *Solaris* seeds, besides a low alkaloids level, are characterized by low moisture level that makes possible a long-time storage (Rossi *et al.*, 2013), and by a great content of unsaturated fatty acid, *i.e.*, oleic (18:1) and linoleic (18:2) acids (70 % and 14.5 %, respectively), able to enhance the nutritional and organoleptic meat characteristics when introduced in the diet (Stanisavljevic´ *et al.*, 2009).

Aim

As a further contribute to knowledge advances about the multifunctional potentialities of *Nicotiana tabacum* L., cv. *Solaris*, the present on-field trial aimed to study the seed cake chemical components, as well as the effects on diet palatability and growth performances when used as alternative ingredient in the diet of lambs raised in inner area of Centre-South Italy, according to the traditional farming system.

Materials and Methods

The present on-field trial was performed in a private small farm and the research protocol was in accordance with the European Commission guidelines (2010/63/EU) on protection of animals used for experimental and other scientific purposes.

Solaris seed cake chemical components

Seeds of *Nicotiana tabacum* L., cv. *Solaris* in waxy ripening phase with a dimension of 0.5 mm, were collected in August about 150 d after transplanting in Val di Sangro area (Chieti province, 42°10′ N 14°20′ E). After harvesting, carried out by cutting the floral apexes, seeds were dried and were subjected to cold mechanical pressing to obtain bio-jetfuel (oil) and seed cake, the co-product used as innovative dietary ingredient for animal feeding.

Seed cake (around 0.1 t) were pelleted (Figure 4.1) and stored in plastic bags. Moisture, crude protein (CP), KOH soluble protein, crude fiber (CF), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), glucose, fructose, saccharose, total sugars and starch contents, glyceridic composition, free fatty acid composition, free acidity as linoleic acid, total peroxide number, and total amino acid contents after protein hydrolysis, were determined on *Solaris* seed cake samples (n = 3) by Food Science laboratory of the University of Molise, and by an external accredited laboratories (Mérieux NutriSciences Corporation - Italy and Amino Lab Urban Farmer - Mozambique), according to official methods (AOAC, 1995, 2000; ISO 9001, 2015).

Figure 4.1 - Pelleted *Solaris* seed cake. Courtesy Antonella Fatica.

The chemical and amino acid composition of *Solaris* seed cake was discussed compared to the chemical characteristics of traditional seed cake used in a large part of lamb commercial feedings.

Farm description

The trial was carried out in a farm located in Roccamandolfi municipality (Isernia province, 41°30′ N 14°21′ E), in a mountainous area of Molise region at about 900 m a.s.l., during the spring period from March to May 2019. Because of its location, the farm is reached only by off-road vehicle when the weather allowed it. As typical of inner areas of Centre-South Apennines, the farm was characterized by a familiar management, and it was organized in a single building, where ewes and lambs were raised together. The total number of sheep was around 250 heads and, during the trial, around 120 lambs were raised. Generally, ewes grazed in natural pastures during daylight, while lambs remained in the stable. At evening ewes were brought into the farm in order to nurse lambs until the following morning. In winter-spring period, during days with bad weather conditions, ewes and lambs remained in stable and were fed *ad libitum* hay produced from farmlands and different mixture of concentrates, and *ad libitum* milk, according to nutritional requirements related to their physiological phase.

Due to the peculiar geographical and managerial characteristics of the selected farm, the trial was carried out in real and often difficult conditions. On this regard, climatic characterization of the trial area was reported. The thermo-pluviometric data for year

2019, through the analyses of monthly average values of temperature (°C) and total rainfalls (mm), have been collected for Roccamandolfi weather station from both the historical official archives of Dipartimento IV Servizio di Protezione Civile (Regione Molise, 2021), and the online weather site (3BMeteo, 2020).

Animals, diets, and experimental design

Thirty-two growing crossbred lambs (Ile-de-France x Merinizzata Italiana), sixteen males and sixteen females, average live weight 15.5 (± 3.01 SD) kg and age ranging between two and three months, were distributed in a completely randomized design into two dietary treatments: Control (CTR; n = 16, half males and half females) and *Solaris* seed cake (S; n = 16, half males and half females). Each dietary treatment was replicated four times: lambs, homogeneous for sex and age, were housed in 8 pens (4 animals per pen or subgroup) with a dimension of 1.60 m x 1.60 m x 0.8 m (maximum height) made by wood on three sides and by farm wall on one side. Daily, on the concrete floor, straw was added. Each pen was equipped with a drinker and a manger which allowed the contemporary access of all animals to feed. During the feeding trial, lambs were housed indoor in similar spatial and environmental conditions and were identified by different colors sprayed.

Two calculated isonitrogenous and isoenergetic diets were administered to the lambs, according to their nutritional needs (NRC, 2007). More in detail, *Nicotiana tabacum* cv. *Solaris* seeds cake has been introduced in the S sub-groups diet by replacing part of soybean meal (*Glicine max* L.) and other ingredients which are traditionally used into the selected farm, as reported in Table 4.1. The experimental diets have been offered to lambs in addition to a mixture of concentrate, *ad libitum* hay, and *ad libitum* milk suckled from mothers (Table 4.2).

Table 4.1 - Composition of experimental diets for growing crossbred lambs (n = 4/sub-group). Calculated chemical components (g/kg DM) and energy content (UFV/kg DM) of experimental diets.

	S	CTR
Ingredients, kg DM/sub-group per d		
Solaris seed cake, 35 % CP	0.03	-
Soybean meal, 46 % CP	0.04	0.05
Wheat middlings	0.04	0.06
Corn grain	0.09	0.11
Dietary chemical composition (g/kg DM) **and energy content** (UFV/kg DM)		
DM	8.8	8.7
CP	223	221
EE	66.8	43.1
Ash	49.7	36.5
NDF	217	185
ADF	108	73.3
Starch	421	472
Lys	11.3	9.9
UFV/kg DM	2.6	2.5

S = *Solaris* diet; CTR = control diet; DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; Lys = lysine; UFV = forage unit for meat production (UFV = 7.62 MJ NE/kg, Sauvant *et al.*, 2004).

More in detail, to CTR sub-groups were administered daily 0.22 kg DM/sub-group of mixture of concentrate made by soybean meal, 46 % CP (22.73 %), wheat middlings (27.27 %) and corn (50 %). A daily amount of 0.20 kg DM/sub-group of mixture of concentrate made by *Solaris* seed cake, 35 % CP (15 %), soybean meal, 46 % CP (20 %), wheat middlings (20 %) and corn (45 %) was administered to S sub-groups.

Diets also included a mix of commercial feeds, 0.45 kg DM/sub-group per day, composed by flacked mixed feed (51.11 %), weaning mixed feed (24.44 %) and growth mixed feed (24.44 %), as reported in Table 4.2.

Table 4.2 - Common diets daily administered to S and CTR sub-groups of growing crossbred lambs (n = 4) and dietary chemical components (g/kg DM).

	S	**CTR**
Ingredients, kg DM/sub-group per d		
Commercial flaked feed [a]	0.23	0.23
Commercial weaning feed [b]	0.11	0.11
Commercial growing feed [c]	0.11	0.11
Chemical composition (g/kg DM)		
DM	885.2	885.2
CP	133.9	133.9
CF	55.5	55.5
EE	27.6	27.6
Ash	52.2	52.2
Hay (alfalfa and meadow hay)	*ad libitum*	*ad libitum*
Chemical composition (g/kg DM) [d]		
DM	887.0	887.0
CP	74.0	74.0
CF	385.0	385.0
NDF	630.0	630
ADF	428.0	428.0
Ash	95.0	95.0
Milk (nursed)	*ad libitum*	*ad libitum*

S = *Solaris* diet; CTR = control diet; DM = dry matter; CP = crude protein; CF = crude fiber; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber.

[a] Labelled ingredients: flaked maize, flaked barley, flaked fava bean, carob. Composition of compound feed (percentage, as fed): Protein: 12.0, Fat: 2.0, Crude Fibre: 3.8, Ash: 2.3.

[b] Labelled ingredients: ground maize, toasted soybean meal, wheat groats, wheat middlings, fava beans, calcium carbonate, sugar beet molasses, dicalcium phosphate, soybean oil, sodium chloride, sodium bicarbonate. Composition of compound feed (percentage, as fed): Protein: 17.5, Fat: 4.4, Cellulose: 5.6, Ash: 7.5, Sodium: 0.4. Nutritional additive (per kg feed): Vitamin A: 16000UI, Vitamin D3: 3200UI, Vitamin E: 48 mg, Vitamin B1: 4.0 mg, Niacin: 160 mg, Choline chloride: 17.3 mg, Niacinamide: 240 mg, Ferrous carbonate: 166 mg, Manganous oxide: 52 mg, Zinc oxide: 198 mg, Sodium selenite: 0.35 mg, Potassium iodide: 5.2 mg.

[c] Labelled ingredients: maize, wheat middlings, wheat groats, sunflowers seed meal, fava beans, dried beet pulp, distillers' dried grains, barley, sugarcane molasses, oat, calcium carbonate, alfalfa meal, soybean meal, sodium chloride, dicalcium phosphate, sodium bicarbonate. Composition of compound feed (percentage, as fed): Protein: 16.0, Fat: 3.0, Crude Fibre: 9.5, Ash: 8, Sodium: 0.31. Nutritional additive (per kg feed): Vitamin A: 30000UI, Vitamin D3: 4000UI, Vitamin E: 145 mg, Co: 0.30 mg, I: 2 mg, Mn: 100 mg, Se: 0.30 mg, Zn: 100 mg, Mixture of flavouring compounds: 291 mg, Sepiolite: 130mg.

[d] Nir analyses on three samples.

For all sub-groups, diets were administered into two daily meals at 08:00 and at 18:00. At night, lambs were allowed to reach their mothers until the subsequently morning.

The feeding trial lasted forty-three days, including the environmental and dietary adaptation phase lasted seven days during which animals of S sub-groups were subjected to a gradual administration of *Solaris* seed cake since they had never eaten the *Solaris* co-product. Data from thirty-six days of dietary treatments about the palatability of *Solaris* seed cake and the relatives' effect of the inclusion as ingredient in lamb diet about sub-group dry matter (DM) intake, sub-group DM refusal and about their growing performances have been investigated and discussed.

Measurements and samplings

Before each meal, all feeds administered to sub-groups were daily weighted as all refusals collected from the manger of each pen. Feedstuffs and residues were sampled twice during the feeding trial and analysed for their DM contents (AOAC, 2000). The daily sub-group DM intake was calculated as difference between DM feedstuffs administered and DM refusals recorded for each S and CTR sub-group, respectively. At d 8, 25 and 32 all sub-groups were weighted, and sub-groups' body weight (BW) recorded. Furthermore, sub-groups average daily gain (ADG) was calculated as ratio between final BW of each sub-group and 36 days of dietary treatment. Sub-groups feed conversion rate (FCR) was calculated as ratio between estimated average sub-groups DM intake and relative ADG. All data about sub-group DM intake and DM refusal, and growing performances have been reported for S sub-groups (n = 8 female and n = 8 male) and CTR sub-groups (n = 8 female and n = 8 male), respectively.

Statistical analyses

All data were subjected to descriptive statistic (Microsoft© Excel© for Office 365 ProPlus Version 1903 -11425.202429). Data about sub-groups DM intake and refusals were processed by analyses of variance, considering the covariate effect of "sub-group daily DM administered" (GLM, ANCOVA). Groups data about lamb's body weight were processed by repeated measures analyses of variance. Data were presented as mean and standard error (SEM); differences were considered significant at $P < 0.05$. All statistical analyses were conducted with IBM SPSS Statistic Data Editor ver. 25.

Results and Discussion

Solaris seed cake chemical composition and amino acid content

Results on analytical components, on amino acid profile, and on oil fatty acid composition of *Solaris* seed cake are reported in Table 4.3, Table 4.4, and Table 4.5, respectively.

Table 4.3 - Chemical components of *Solaris* seed cake (g/100 g DM unless otherwise indicated) (mean values ± SD) [a].

	***Solaris* seed cake**
DM, g/100 g	92.7 (± 0.66)
CP	34.7 (± 0.25)
KOH soluble protein	19.0 (± 0.22)
CF	24.2 (± 0.97)
EE	16.3 (± 2.94)
NDF	41.8 (± 2.35)
ADF	30.9 (± 2.15)
ADL	19.4 (± 1.66)
Glucose	0.07 (± 0.01)
Fructose	0.05 (± 0.00)
Saccharose	1.74 (± 0.26)
∑ Sugars	1.87 (± 0.26)
Starch	2.17 (± 0.40)
Glyceridic composition, %	
Monoglycerides	1.24
1, 2 C34 Diglyceride	0.85
1, 3 C34 Diglyceride	1.65
1, 2 C36 Diglyceride	5.98
1, 2 C36 Diglyceride	9.08
Total peroxide number, meq O_2/kg	9.40
Free acidity as Linoleic acid, %	20.2

[a] n = 3, three replicates.
DM = dry matter; CP = crude protein; CF = crude fiber EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin.

Observing the chemical composition of *Solaris* seed cake (Table 4.3), it is interesting to noting as the *Solaris* seed cake EE, NDF, and starch contents were very close to values (14.2 g/100 g DM, 45.9 g/100 g DM, 2.30 g/100 g DM, respectively) reported in Chapter 2 for *Solaris* inflorescence (Table 2.1). Notwithstanding, DM, CF, and EE contents were higher compared to 90.3 g/100 g, 21.8 g/100 g DM, 10.4 g/100 g DM reported respectively for DM, CF and EE contents of *Solaris* seed cake samples investigated by Rossi *et al.* (2013). Although, crude protein content (Table 4.3) was lower compared to the value (36.6 g/100 g DM) reported in literature (Rossi *et al.*, 2013). Total peroxide

number was close to the maximum values (10 meq O_2/kg) reported for corn, soybean, and sunflower (*Helianthus annuus* L.) (Laboratorio Analisi Zootecniche, 2017).
Since in S diet *Solaris* seed cake replaced part of the traditional ingredients used in the selected farm (Table 4.1), the main chemical components of soybean meal (46 % CP), wheat middlings, and corn are reported according to Sauvant *et al.* (2004). Soybean meal (46 % CP) with an average DM content of 87.6 g/100 g, was characterized by an higher CP content (49.4 g/100 g DM), and by a lower CF, EE (6.96 g/100 g DM and 1.94 g/100 g DM, respectively) and fiber components contents (respectively 14.2 g/100 g DM, 8.45 g/100 g DM, 0.46 g/100 g DM, for NDF, ADF, ADL) compared to *Solaris* seed cake (Table 4.3). DM, CP, CF, and EE of wheat middlings, respectively equal to 88.1 g/100 g, 15.5 g/100 g DM, 7.00 g/100 g DM, 3.60 g/100 g DM (Sauvant *et al.*, 2004), were lower than values reported for *Solaris* seed cake (Table 4.3). Starch content of wheat middlings (31.4 g/100 g DM) is more than ten times higher compared to *Solaris* seed cake (Sauvant *et al.*, 2004). As wheat middlings, also the DM, CP, CF, and EE contents of corn, respectively equal to 86.4 g/100 g, 9.38 g/100 g DM, 2.55 g/100 g DM, 4.28 g/100 g DM (Sauvant *et al.*, 2004) were lower compared to *Solaris* values reported in Table 4.3. The average corn starch content equal to 74.2 g/100 g DM (Sauvant *et al.*, 2004) was higher than that reported for *Solaris* seed cake (Table 4.3).
To better describe *Solaris* seed cake as innovative feed, its chemical composition was compared to two co-products of oil manufacture frequently used as ingredients of commercial lamb feeds, such as partially dehulled cottonseed (*Gossypium* spp.) seed cake (oil 5 - 20 % and CF 15 - 20 %), and non-dehulled sunflower seed cake (oil 5 - 20 %) both obtained after mechanical oil pressing process (INRAE, 2021).
Focusing on components reported in Table 4.3, *Solaris* seed cake DM content was in the range of DM reported for sunflower (92.3 g/100 g) and for cottonseed (92.9 g/100 g) (INRAE, 2021). CP content was higher than 25.8 g/100 g DM indicated for sunflower, but close to 36.6 g/100 g DM reported for cottonseed, while an opposite tendency was observed for *Solaris* seed cake CF content found close to that of sunflower (27.5 g/100 g DM), but higher than that of cottonseed (19.3 g/100 g DM) (INRAE, 2021). The EE content reported for *Solaris* seed cake (Table 4.3) was higher than both cottonseed (8.1 g/100 g DM) and sunflower (15.8 g/100 g DM) (INRAE, 2021). As far as the cell wall components, NDF value was intermediate between values reported for cottonseed (36.1 g/100 g DM) and sunflower (44.4 g/100 g DM), ADF was very close to 31.5 g/100 g DM reported for sunflower, while ADL was higher than values reported for cottonseed and

sunflower (8.00 g/100 g DM and 10.6 g/100 g, respectively). The total sugars content of *Solaris* seed cake was lower than 5.00 g/100 g DM and 6.30 g/100 g DM reported for cottonseed and sunflower respectively, while starch content ranging between values reported for the cottonseed (1.90 g/100 g DM) and sunflower (3.90 g/100 g DM).

Considering protein fraction, the essential amino acid profile, *i.e.*, methionine, lysine, histidine, leucine, isoleucine, phenylalanine, threonine, and valine, was reported along with the non-essential amino acids profile, *i.e.*, alanine, arginine, glycine, serine, proline, tyrosine, aspartic acid, and glutamic acid (Table 4.4). Particularly, lysine, alanine and aspartic acid contents were close to the values reported in Table 2.2 (Chapter 2) for *Solaris* inflorescence (0.83 g/100 g DM, 1.13 g/100 g DM and 2.95 g/100 g DM, respectively). Methionine and cysteine contents were investigated only on one sample of *Solaris* seed cake (Table 4.4).

Table 4.4 - Amino acid content (g/100 g DM) of *Solaris* seed cake (mean values ± SD) [a].

	***Solaris* seed cake**
Methionine	0.53 (± 0.00)
Cysteine	0.44 (± 0.00)
Methionine + Cysteine	0.97 (± 0.00)
Lysine	0.77 (± 0.02)
Histidine	0.65 (± 0.06)
Leucine	2.02 (± 0.33)
Isoleucine	1.17 (± 0.07)
Phenylalanine	1.38 (± 0.08)
Threonine	1.17 (± 0.17)
Valine	1.31 (± 0.06)
Alanine	1.27 (± 0.12)
Arginine	3.47 (± 0.29)
Glycine	1.44 (± 0.12)
Serine	1.32 (± 0.20)
Proline	1.12 (± 0.20)
Tyrosine	0.76 (± 0.09)
Aspartic acid	2.95 (± 0.31)
Glutamic acid	5.72 (± 0.48)

[a] n = 3, three replicates.

Solaris seed cake methionine content (Table 4.4) was close to 0.50 g/100 g DM reported by Rossi ad colleagues (2013) and was in the range of 0.30 g/100 g DM - 0.70 g/100 g DM reported for wheat middlings and soybean meal (46 % CP) (Sauvant *et al.*, 2004). The sum of methionine and cysteine was within 0.60 g/100 g DM reported for wheat middlings and 1.40 g/100 g DM reported for soybean meal (46 % CP) (Sauvant *et al.*, 2004). The lysine content (Table 4.4) was lower compared 0.80 g/100 g DM reported for *Solaris* seed cake in previously study (Rossi *et al.*, 2013), and compared to the content of soybean meal (2.66 g/100 g DM) (Sauvant *et al.*, 2004). Valine was found lower to 1.41 g/100 g DM reported for *Solaris* seed cake (Rossi *et al.*, 2013) and to 2.40 g/100 g DM reported for soybean meal (46 % CP), but higher to 0.80 g/100 g DM reported for wheat middlings (Sauvant *et al.*, 2004). Aspartic acid (Table 4.4) was higher compared to 2.66 g/100 g DM reported for *Solaris* seed cake (Rossi *et al.*, 2013), but lower to 5.60 g/100 g DM reported for soybean meal (46 % CP) (Sauvant *et al.*, 2004). Glutamic acid (Table 4.4) was lower compared to data reported by Rossi *et al.* (2013) (6.12 g/100 g DM, but higher compared to corn and wheat middlings (1.80 g/100 g DM and 3.50 g/100 g DM, respectively) (Sauvant *et al.*, 2004).

Comparing the *Solaris* seed cake amino acid profile to cottonseed and sunflower seed cake, it is also worth noting that the *Solaris* seed cake methionine content (Table 4.4) was close to values reported for cottonseed and sunflower (0.55 g/100 g DM and 0.58 g/100 g DM respectively; INRAE, 2021). Moreover, the sum of methionine and cysteine contents was similar to sunflower (1.02 g/100 g DM) but lower than cottonseed (1.15 g/100 g DM), while the lysine content was lower compared to sunflower and cottonseed (0.97 g/100 g DM and 1.52 g/100 g DM, respectively; INRAE, 2021). *Solaris* seed cake leucine content was close to value reported for cottonseed (2.11 g/100 g DM), valine content was slightly lower compared to cottonseed (1.61 g/100 g DM), while alanine content was higher than values reported for sunflower (1.12 g/100 g DM; INRAE, 2021). The aspartic acid level ranged between sunflower (2.20 g/100 g DM) and cottonseed (3.41 g/100 g DM), as well as glutamic acid, 4.71 g/100 g DM and 6.90 g/100 g DM for sunflower and cottonseed, respectively (INRAE, 2021).

Fatty acid composition of *Solaris* seed cake (Table 4.5), consistent with data reported in literature (Rossi *et al.*, 2013), showed 88.1 % UFA content and a 12.8 % SFA/UFA ratio.

Table 4.5 - *Solaris* seed cake fatty acid composition (%) (n = 3) compared to literature data [α].

Fatty acid composition, % of fatty acid	***Solaris* seed cake**	**Reference data [α]**
Palmitic acid, C16:0	8.32	8.26
Palmitoleic acid, C16:1	0.14	0.11
Heptadecanoic acid, C17:0	0.21	na
Stearic acid, C18:0	3.06	2.85
Oleic acid, C18:1	11.0	11.0
Linoleic acid, C18:2	75.7	76.6
Linolenic acid, C18:3	1.09	0.91
Arachidonic acid, C20:0	0.25	0.16
Eicosenoic acid, C20:1	0.09	0.10
Behenico acid, C22:0	0.11	na
Saturated fatty acids	11.9	na
Monounsaturated fatty acids	11.3	na
Polyunsaturated fatty acids	76.8	na

[α] Rossi *et al.*, 2013.
na = not available.

The introduction of seed cake, co-product of mechanical oil pressing for biodiesel production, as ingredient for animal diets could reduce feeding costs also thanks to the oil residual content and the interesting fatty acid profile of this promising energy plant (Goiri *et al.*, 2019). On this regard, *Solaris* seed cake palmitic acid content was lower compared to soybean meal (46 % CP), corn, and wheat middlings (10.5 g/100 g DM, 11.1 g/100 g DM, 17.8 g/100 g DM, respectively), as oleic acid content, *i.e.*, 15.2 g/100 g DM, 21.7 g/100 g DM and 26.9 g/100 g DM, respectively in wheat middlings, soybean meal (46 % CP) and corn (Sauvant *et al.*, 2004). Linoleic acid content of *Solaris* seed cake (Table 4.5) was higher compared to data reported by Sauvant *et al.* (2004), *i.e.*, 53.1 g/100 g DM for soybean meal (46 % CP), 56.4 g/100 g DM for wheat middlings, and 56.5 g/100 g DM for corn.

Climatic characterization of study area

As previously reported, sometimes the on-field trial activities are strongly affected by extreme weather conditions. As located in Apennine mountains, the studied area was generally characterized by a cold-humid weather. During the 2019 the average temperature ranged from the minimum value of 0.0 °C recorded in January, to the maximum value of 23 °C recorded in July (Figure 4.2). May (231 mm), October (257 mm) and November (233 mm) were the rainiest months however the snow precipitations, exceptionally low for this area, occurred especially in March and April, when the trial was carried out. In more detail, during the trial period the average temperature ranged from 7.6 °C (March) to 10.8 °C (May) and the precipitations accounted for 53 mm, 135 mm and 231 mm, respectively for March, April and May (Figure 4.2). The snowy days were in total five (between March and April) and were characterized by 10 - 15 cm of snow per day.

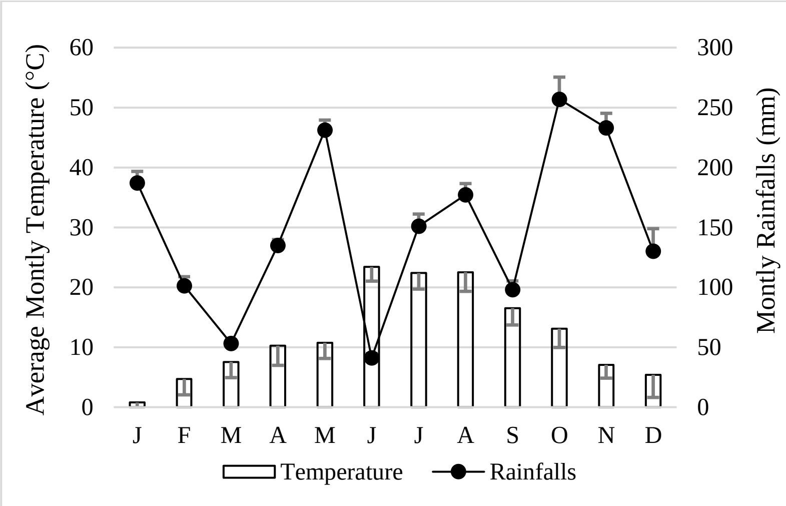

Figure 4.2 - Thermo-pluviometric characterization of Roccamandolfi area for year 2019.

Dietary treatments and lambs' feed intake

During the feeding trial the calculated daily DM intake was in average 2.15 (± 0.37 SD) kg and 2.18 (± 0.35 SD) kg, respectively for S and CTR group (n = 16). Group DM refusal averaged 0.98 (± 0.55 SD) kg and 1.00 (± 0.46 SD) kg, respectively for CTR and S.

Solaris seed cake, tested for the first time on growing lambs, did not significantly affect (P > 0.05) values of daily sub-group DM intake and daily sub-group DM refusal (Table 4.6). Furthermore, even if sex did significant affect (P < 0.001) the daily sub-group DM intake and sub-group DM refusal, the interaction diet x sex was not significant (P > 0.05) (Table 4.6).

Table 4.6 - Effects of dietary treatment, sex, and their interaction on total daily group DM intake and DM refusal for S and CTR sub-groups of growing crossbred lambs (n = 4).

	S		**CTR**		**SEM**	**P-value**		
	Female	**Male**	**Female**	**Male**		**D**	**S**	**D x S**
Total DM intake, kg	2.06	2.25	2.06	2.29	0.31	ns	***	ns
Total DM refusal, kg	1.10	0.91	1.09	0.87	0.31	ns	***	ns

S = *Solaris* diet; CTR = control diet; DM = dry matter; D = diet; S = sex; SEM = standard error of mean; *** P < 0.001; ns = not significant.

The observed values of sub-group DM intake and DM refusal suggested that, during the feeding trial, feed intake has been lowered in female sub-groups than in male sub-groups, confirming the significative effect of sex on intake levels. However, it was interesting noting how the dietary treatment did not significantly affect the DM intake of S and CTR sub-groups, as reply to the similar energy and protein contents of the two diets. Furthermore, the reported sub-group DM intake were consistent with the values reported by Sadeghi *et al.* (2009) for lambs 60 - 90 d old fed olive cake by-product, as well as with values reported for lambs fed either pea or faba bean (Lanza *et al.*, 2011). Six months-old lambs fed diets containing 10 % and 15 % of sunflower seed cake had almost double intake than those observed in this trial (Monteiro *et al.*, 2020).

Generally, the voluntary feed intake is affected by the energy content of the diet (Allen *et al.*, 2009), and it is positively correlated with the dietary crude protein content, but negatively related with fiber and tannin contents (Tseu *et al.*, 2020). On this regard, feeds having tannin concentration of about 50 g/kg DM are responsible for salivary glycol-proteins precipitation which can negatively affect the total DM intake capacity (Patra and Saxena, 2011). Nevertheless, the DM intake, and related DM refusal, could be defined as a multifactorial process strongly affected by animal genotypes, diet characteristics and environmental conditions (Pulina *et al.*, 2013). Estimating the real voluntary feed intake is important for animal productions especially when a supplementation of nutrient is needed by grazing animals or when appropriate economic evaluation of the productive system is carried out (Pulina *et al.*, 2013). As small ruminants, sheep intake is characterized by a behaviour linked to some anatomic aspects, *i.e.*, great ability than large ruminants to select the ingested feed as part of plant rich in nutrients but with a lower lignin, in ruminating so that digestion and the adsorption of nutrients are facilitated, by decreased particle size that increases the rumen turnover (Gallo *et al.*, 2021).

Dietary treatment and lambs' growth performances

At the end of trial, the average BW was 20.8 (± 4.23 SD) kg/head and 23.2 (± 2.88 SD) kg/head, respectively for S and CTR group (n = 16). At the beginning of the trial (d 8) CTR male sub-groups were characterized by the lower average BW (14.7 kg), while S female sub-groups were characterized by higher BW, averaging 17.7 kg (Figure 4.3). Nevertheless, at d 32 the sub-group with the highest average live weight was CTR male (23.9 kg), while the lowest average BW was observed in the S female sub-group (Figure 4.3).

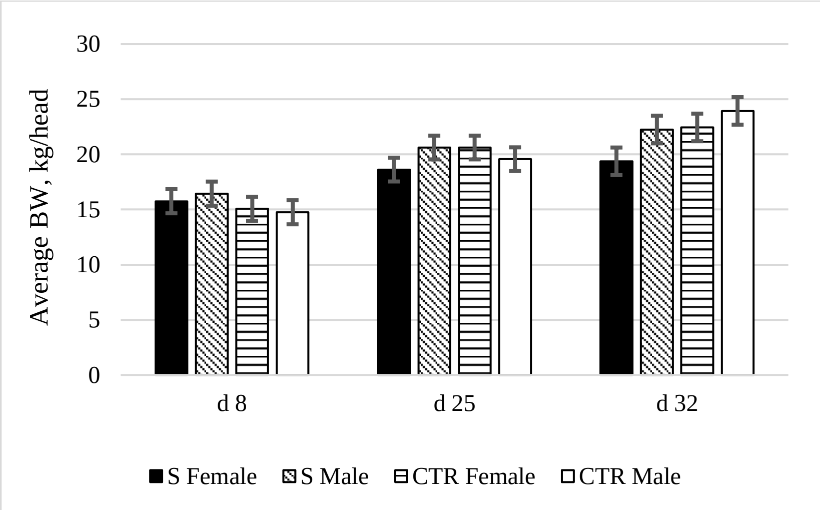

Figure 4.3 - Average lambs body weight (mean values ± SEM) changes observed during the trial period in female and male sub-groups of growing crossbred lambs (n =4).

On the statistical point of view (Table 4.7) both diet and sex, did not significantly affect (P > 0.05) BW of S and CTR sub-groups studied at d 8, 25 and 32 of trial, as well as the interaction diet x sex was not significant (P > 0.05), indicating that the effects of the investigated factors were additive.

Table 4.7 - Effects of dietary treatment, sex, and their interaction on the average individual body weight of S and CTR sub-groups of growing crossbred lambs (n = 4).

	S		**CTR**		**SEM**	**P-value**		
	Female	**Male**	**Female**	**Male**		**D**	**S**	**D x S**
BW 8 d, kg	15.7	16.4	15.06	14.75	1.09	ns	ns	ns
BW 25 d, kg	18.62	20.62	20.62	19.56	1.077	ns	ns	ns
BW 32 d, kg	19.37	22.25	22.44	23.94	1.251	ns	ns	ns

S = *Solaris* diet; CTR = control diet; BW = body weight; D = diet; S = sex; SEM = standard error of mean; ns = not significant.

The calculated sub-group ADG was 0.54 kg/d for S female *vs.* 0.62 kg/d for S male, and 0.62 kg/d *vs.* 0.66 kg/d, for CTR female and CTR male sub-groups, respectively. Consequently, the calculated sub-group FCRs were 3.81 kg DM intake/kg gain and 3.63 kg DM intake/kg gain, for S female sub-group and for S male sub-group respectively, while in CTR sub-groups an average 3.32 kg DM intake/kg gain was observed in females and 3.50 kg DM intake/kg gain in males.

Based on these results, the sub-group with the best growth rate was CTR female, while the S female sub-group showed the lowest growth rate. On this regard, it has to be considered that the variability of *in vivo* performances was high and not negligible. Moreover, these results could suggest that S growing lambs need a prolonged adaptation to the diet including *Solaris* seed cake, being this a completely new feed.

According to NRC (2007), the weight gain of lambs weighting 30 kg and raised in optimal environmental condition should be 0.20 kg/d, which is higher than values observed at the end of feeding trial. However, data from this trial suggest that both ADG and FCR values were consistent with the range of values reported by both Lanza *et al.* (2003, 2011) and Sadeghi *et al.* (2009), but lower compared to those observed in lambs fed cottonseed meal and soybean meal (Chegini *et al.*, 2019). Furthermore, ADG and FCR were respectively higher and lower than to values reported for lambs fed with 10 % and 15 % of sunflower seed cake (Monteiro *et al.*, 2020). Estimating animal body weight and related ADG and FCR represent reference parameters in defining the best time for slaughtering or breeding events, as indices of the health status of the animals (Mahmud *et al.*, 2014; Sabbioni *et al.*, 2020).

Conclusions

The *Nicotiana tabacum* cv. *Solaris* seed cake showed interesting components in terms of gross chemical composition, as well as nitrogenous and lipid profile. The use of *Solaris* seed cake represents a promising alternative ingredient in growing lambs' diet, even in farming conditions considered extreme. For the first time was successfully tested the palatability of *Solaris* seed cake in growing lamb diet as substitute of raw materials commonly used in feed formulations, such as corn grain, wheat by-products, and soybean meal. The dietary treatment with *Solaris* seed cake in fact did not influence the total sub-group DM intake and DM refusal, as well as the body weight of the experimental crossbred subjects that, on the other hand, showed high variability of *in vivo* performances, likely influenced by farm location, environment, and management, as factors which could not be directly controlled, managed, and modified during the experiment. Further studies will determine the effect of the dietary treatment with *Nicotiana tabacum* cv. *Solaris* seed cake on lambs' nutritional profile and welfare, and carcass quality and composition.

Based on the preliminary not conclusive results obtained in this on-field trial, the innovative co-product of this tobacco energy variety could represent a further opportunity to re-evaluate the tobacco cultivation know-how in the marginal area of inner Centre-South Italy, traditionally suited to its cultivation. At the same time, it could also represent an interesting co-product able to contribute to the reduction of feeding costs, but more in-depth studies should be addressed to the effects on meat quality besides animal welfare.

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Chapter 5

Effects diet/genotype on productive performances of broilers raised under semi-intensive conditions

Introduction

During 2018 the global chicken population was over 23 billion of birds and, in developing country, was raised for 80 % in rural households (FAO, 2020 a). United States of America is the world's largest poultry meat producer with 18 % of global output, followed by China, Brazil, and Russian Federation (FAO, 2020 b). The reported data suggest how poultry products' demand has been expanded and globalised during the last twenty-years so that, poultry sector is the fastest growing and the most flexible among livestock productions (FAO, 2020 b). Rural poultry farming has always been essential for many poor farmers in food-deficit status as it is able to contribute to human nutrition improvement, is characterized by small increase of incomes, and provides manure for crop production (FAO, 2013). Because of that, advances in poultry breeding technologies for more productive and fast-growing meat genotypes are challenging to overcome the growing global demand of animals, as food with high nutritional value. This implies and requires an intensive nutritional and health management to promote the correct expression of the genetic potential of selected birds (FAO, 2020 b) while considering the consumers' demand. In other words, when birds are raised for meat production, *i.e.*, broiler chickens, the goal will be maximizing body weight gain while minimizing both meat fat content and costs. For this reason, for instance, the age of broilers at 2 kg of commercial weight has decreased from 63 days in 1976 to 35 days in 2009 (Ravindran, 2013).

In terms of costs, feeding is the most important input for intensive poultry production (FAO, 2020 a), but dietary requirements range according to the nutritional needs of the animals, including genotype, productive attitude (meat or egg), farming system and management. In last decades, crossbred broilers have been selected for both rapid growth rate and efficient utilization of feeds. *Ad libitum* diets are therefore usually administered to ensure rapid growth, reducing the excessive deposition of fat which could affect meat quality and consumer's demand (NRC, 1994; Ahiwe *et al.*, 2018). However, the bird's feed intake could be controlled by balancing the nutrient content of the diet or manipulating the lighting program through increasing or decreasing the duration of darkness (Ahiwe *et al.*, 2018).

With the restrictions on the use of protein sources following the European Commission Decision No. 98/272/CE and No. 2000/374/CE, soybean (*Glicine max* L.) became the most common protein source suitable and used in poultry diet (Laudadio and Tufarelli, 2010; Dotas *et al.*, 2014). The large part of soybean meal (44 % CP) available in Europe is imported from non-European countries, often derived from genetically modified organisms (Dotas *et al.*, 2014). Also due to the high soybean meal price ranging from 410 to 710 euro/t (AGER, 2021), the costs of broiler chicken production increase, and feeding may easily reach as much as 70 % of the total costs (Kuźniacka *et al.*, 2020). For these reasons, in last years, alternative protein and energy sources and agroindustry by-products are investigated as cheaper choice for poultry diet ingredients (Diaz *et al.*, 2006; Palander *et al.*, 2006; Ciurescu *et al.*, 2019). Pea bean (*Pisum sativum* L.) fast-growing herbaceous plant largely cultivated in the Mediterranean area, is a legume crops used in human and animal nutrition (Muehlbauer and Tullu, 1997). Despite its good nutritive value (Ravindran and Blair, 1992; Castell *et al.*, 1996) and its high content of starch, different from that of commercial corn (*Zea mais* L.) and tapioca (*Manihot esculenta* Crantz) starches (Li *et al.*, 2019), the use of pea bean in poultry nutrition is today limited due to the low susceptibility of starch to hydrolytic enzymes, and due to the presence of antinutritional factors, *i.e.*, condensed tannins and protease inhibitors (Castell *et al.*, 1996; Dotas *et al.*, 2014). Moreover, because consumers increasingly consider ethic issues related to meat production besides safety and quality (Kuźniacka *et al.*, 2020), the inclusion of pea bean and/or other legumes, *i.e.*, faba bean (*Vicia faba* L.) and lupin seeds (*Lupinus albus* L.) appears more widespread in broiler diets (Diaz *et al.*, 2006; Palander *et al.*, 2006). Diet, together with genotype, age and sex of birds, and their management systems, is one of the most important factors involved into the quality of poultry meat (Nalle *et al.*, 2010; Osek *et al.*, 2013), also associated to the welfare of the animals. In this context, it should be considered that in Italy some agricultural products, as for example corn and soy products, are mainly used by feed industry (ISMEA, 2020 a).

However, the Italian supply of corn and soy products has contracted in last years, leading to a disequilibrium between demand and feed supply, resulting in an increase of import and price of these products (ISMEA, 2020 a).
In addition to the economic concerns, also the environmental issue should be considered. Nowadays, consumers are increasingly interested to the sustainability, health, and nutritional aspects of meat production, and they are also available to spend more for animal products perceived as natural, respectful of animal welfare, and environment friendly (Cygan-Szczegielniak *et al.*, 2019). The tendency is therefore oriented towards more renewable resources (Notarnicola *et al.*, 2015; Palmieri *et al.*, 2017). For instance, according to Palmieri *et al.* (2017), food and beverage sectors account for about 30 % of the environmental impacts of European private consumption, so that agriculture is faced with the challenge of producing foods and feeds with low environmental pressure.
For the 2010 reference period, greenhouse gas (GHG) emissions from livestock supply chains are estimated at 8.1 Gt CO_2-equivalent/year (FAO, 2020 b), consequently the improved global sustainability of feed production processes encouraged poultry sector to limit its environmental load, expressed in terms of GHG (Tallentire *et al.*, 2017). Environmental impacts associated to the broiler production, recently founded relatively low (Tellentire *et al.*, 2017), are derived mainly from the raw materials so that, it is increasingly important to pay attention to the diet formulation (Leinonen *et al.*, 2013), besides energy use and efficiency, growth rate of the selected genotypes, and animal and manure management practices.
According to Luciani *et al.* (2011), carbon footprint is an indicator utilized for measuring the impact of human activities on the climate, by quantifying the accumulation in the atmosphere of GHGs generated by the individual or collective activities. On this regard, the concept of carbon footprint has been used as an indicator of environmental sustainability, referring to the total amount of GHG emissions produced and/or accumulated during the life cycle of specific product or service, that contribute to the global warming potential (GWP) and to the climate change within 100-year period (Notarnicola *et al.*, 2015; Scrucca *et al.*, 2021).
GHGs are represented by natural and anthropogenic gaseous constituents of the atmosphere able to absorb and emit radiation with wavelengths near to the spectrum of terrestrial radiation emitted by Earth surface, atmosphere, and clouds (Intergovernmental Panel on Climate Change - IPCC, 2018). The main GHGs are water vapour (H_2O), carbon dioxide (CO_2), nitrous oxide (N_2O), methane (CH_4), ozone (O_3), and other human-made

gases, *i.e.*, halocarbons and other chlorine/bromine-containing substances, sulphur hexafluoride (SF6), hydrofluorocarbons (HFCs) and perfluorocarbons (PFCs) (IPCC, 2018). The GWP of these gases is calculated mathematically and expressed in units of carbon dioxide equivalent (CO_2-eq), equal to 1 CO_2-eq, 25 CO_2-eq, and 298 CO_2-eq, respectively for one kg of CO_2, CH_4 and N_2O (IPCC, 2007; Pandey *et al.*, 2011; IPCC, 2018). Furthermore, considering the whole life cycle of a product, *i.e.*, from cradle to grave, Life Cycle Assessment (LCA) represent an internationally standardized method (ISO 14040:2006) quantifying the environmental pressure related to the production of goods and products, whose use can lead to improved production process, as strategic management, and decision-making tool in the frame of a more sustainable and efficient society (Baldini *et al.*, 2017; Palmieri *et al.*, 2017).

Aim

The present on-field trial aimed to assess the effects of two different dietary protein sources (soybean *vs*. pea bean), and two different genotypes (Kabir Rosso Plus *vs*. New Red) on performances of broiler chickens raised according to the traditional schemes of semi-intensive system. Besides growth performances, the study was also extended to economic and environmental aspects, evaluated on a year basis.

Materials and Methods

The trial was performed applied a research protocol in accordance with the European Commission guidelines (2010/63/EU), concerning the protection of animals used for experimental and other scientific purposes.

Farm description

The on-field trial was carried out in a private small farm located in Benevento municipality (Benevento province, 41°07'12.8" N 14°43'34.8" E), in a hilly area at about 300 m of altitude. The experiment was conducted from October to December 2019, under semi-intensive rearing system conditions.

Chickens were housed in similar spatial and environmental conditions in pens with a dimension of 1.60 m x 0.80 m x 1.50 m (maximum height) made by wire mesh screen on four sides and dirt floor where straw was added weekly to create a permanent litter on which manure was removed only at the end of the trial. Each pen, representative of experimental unit, was equipped with a single manger and bottle-drinker to ensure free and simultaneously access to both feed and water. Pens were positioned under a structure characterized by two open sides, where environmental conditions, such as temperature, humidity, and lightness, were not controlled. For this reason, data on thermo-pluviometric conditions, *i.e.*, temperature (°C), rainfalls (mm), and humidity (%), were collected for year 2019 by the weather station close to the selected farm (Regione Campania, 2020).

Animals, diets, and experimental design

For the experiment, 120 male chickens, *i.e.*, Kabir Rosso Plus (n = 60) and New Red (n = 60) fast-growth broiler strains (Table 5.1) were studied from 47 to 83 d of age.

Table 5.1 - Commercial information about selected genotypes, reported both for male and female broiler chickens (Avizoo, 2020).

	Kabir Rosso Plus	**New Red**
Genetic	Kabir	Avizoo
Colour		
Male	Red Striped	Multicolor
Female	Red	Multicolor
Characteristics	Excellent resistance, fast growth, high meat quality	Fast growth, excellent conformation, and meat yield
Body weight, kg		
Male		
35 d	1.16	1.36
63 d	2.52	2.92
84 d	3.36	3.96
Female		
35 d	0.92	1.16
63 d	1.95	2.56
84 d	2.92	3.48

Chicks, born in a commercial hatchery (Gruppo Pollinari, Longiano, FC), were housed at 20 days of age, randomly divided in 20 homogeneous sub-groups on genotype and diet basis. Dietary treatment and genotype were replicated in 5 sub-groups with 6 birds per pen, *i.e.*, Kabir fed soybean diet (K-SOY, n = 30), New Red fed soybean diet (NR-SOY, n = 30), Kabir fed pea bean diet (K-PEA, n = 30), New Red fed pea bean diet (NR-PEA, n = 30).

During the first 15 days, all animals were fed *ad libitum* with a commercial complete weaning mixed feed (24 % CP, 16.33 MJ/kg dry matter (DM) of apparent metabolizable energy, aME) (Sibbald and Slinger, 1963; NRC, 1994) including coccidiostat additive.
After then, started a gradual weaning period divided in two phases in order to promote the gradual adaptation of the animals to the adult experimental diets. In the first phase, lasted 6 days, 15 % adult diet and 85 % weaning diet was administered the first 3 days, and 30 % adult diet and 70 % weaning diet was offered the last 3 days, while in the second phase, lasted 4 days, 60 % adult diet and 40% weaning diet was distributed.
Two isonitrogenous and isoenergetic diets were formulated according to birds' nutritional needs (NRC, 1994), including either flacked soybean (SOY) or pea bean (PEA) (Table 5.2). Both diets also included faba bean (*Vicia faba* L.), as common protein source (Table 5.2).

Table 5.2 - Ingredients, chemical composition and energy content of experimental diets daily administered to SOY and PEA sub-groups [a] of broiler chickens.

Feed administered	SOY	PEA
Ingredients, kg DM/sub-group per d		
Wheat bran	0.58	0.56
Durum wheat	0.30	0.31
Corn meal	0.18	0.15
Faba bean	0.08	0.08
Pea bean	-	0.08
Soybean flacked, 37 % CP	0.04	-
Chemical composition (g/kg DM) and energy content (MJ/kg DM)		
DM	889.0	892.0
CP	186.0	186.0
CF	81.6	81.4
EE	48.2	44.5
Ash	49.7	48.3
Lys	6.7	6.9
Met	2.5	2.4
aME	13.3	13.2

[a] Experimental unit = 5 pens per treatment; pen = 6 broiler chickens.
SOY = SOY diet; PEA = PEA diet; DM = dry matter; CP = crude protein; CF = crude fiber; EE = ether extract; Lys = lysine; Met = methionine; aME = apparent metabolizable energy.

SOY sub-groups received daily an average of 1.18 kg DM diet including wheat bran (49.15%), durum wheat (25.42%), corn meal (15.25%), faba bean (6.78%) and soybean flacked (3.39%), while PEA sub-groups received 1.18 kg DM diet including wheat bran (47.46%), durum wheat (26.27%), corn meal (12.71%), faba bean (6.78%) and pea bean

(6.78%). For all sub-groups, rations were administered into two daily meals, in the morning (08:00) and in the afternoon (17:00). Both diets, grinded by a mechanical mill (1 mm), were administered after the addition of fresh water (1 kg dry feed : 2 L water).
The feeding trial lasted thirty-six days and data about this period are reported and discussed.

Measurements and recordings

Before each meal, all feeds administered to each sub-group were weighted, as well as refusal collected from the manger of each pen at the end of the day. Every ten days, residuals were sampled and analysed for DM content, according to official methods (AOAC, 2000).
The sub-group DM intake was calculated as the difference between feedstuffs offered (kg DM per d) and refusal (kg DM per d). At 0, 14, 28, and 36 days of the trial all sub-groups were weighted and body weight (BW) of each sub-group was recorded. Sub-group average daily gain (ADG) was calculated by dividing the final weight of each sub-group by 36 days of feeding trial, while sub-group feed conversion rate (FCR) was calculated as ratio between estimated average sub-group DM intake and its relative ADG. During the feeding trial, also mortality within each sub-group was recorded.

Cost analyses of feeding treatments

Economic cost analyses of experimental diets were carried out according to Bologna Exchange Commodity weekly reports (Exchange Commodity, 2019, 2020). Despite the feeding trial lasted 36 days, dietary ingredient prices were collected and analysed as related to the last three months of 2019, in order to consider the fluctuations of markets over a medium period, as suggested by Taylor (2003). For comparisons, prices of the same ingredients, reported for the last three months of 2020, were also considered. So that the feeding costs were evaluated for 2019 and 2020 and their variation (%) was calculated.

Environmental impact of farming

The carbon footprint of the reported case study was studied by the Global Livestock Environmental Assessment Model - Interactive (GLEAM-i, version 1.8, http://gleami.org/). The model GLEAM-i (FAO, 2021), based on Life Cycle Assessment (LCA) and Geographic Information System (GIS) methodologies, was developed to perform and estimate the annual environmental impacts of several livestock production activities from different regions of the world. Particularly, it could be used as outcome of mitigation and adaptation strategies at local or global scale, also supporting stakeholders in the decision-making process towards more sustainable livestock sector (FAO, 2021). GLEAM-i allows the environmental impacts analyses for four ruminant species, *i.e.*, cattle, buffalo, sheep, goat, and two monogastric species, *i.e.*, pigs and chicken, which may be raised in the following production systems: grassland-based and mixed for ruminants, backyard and industrial for pigs, and backyard, broiler, and layers for chicken (FAO, 2017; MacLeod *et al.*, 2018; FAO, 2020 c). The functional unit used by the interactive software to define the emission intensity produced per kg of product is the equivalent of carbon dioxide, *i.e.*, emission per kg of protein was expressed as CO_2-eq/Prot (FAO, 2020 c).

The GLEAM-i programme procedure was based on the following steps:

1. Region and country selection (Figure 5.1).
2. Production systems setting and orientation for considered animal species. For instance, selections for chicken are backyard, broiler, and/or layers production system.

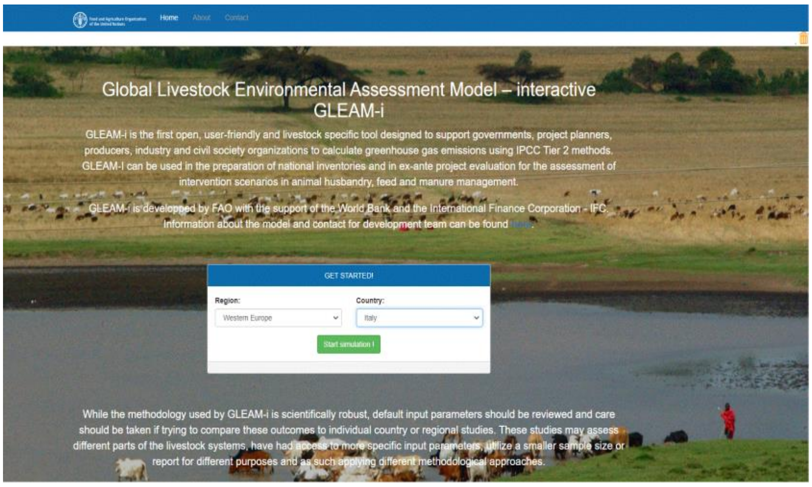


Figure 5.1 - GLEAM-i screen where the selection on region area is allowed (Source: FAO, 2021).

3. Definition of three main modules and related parameters (Figure 5.2):

   - *herd* (animal numbers, live weights, mortality rate, fertility, and production),

   - *feed* (dietary ingredients selected for broiler and/or layers farming systems),

   - *manure* (manure management strategies).

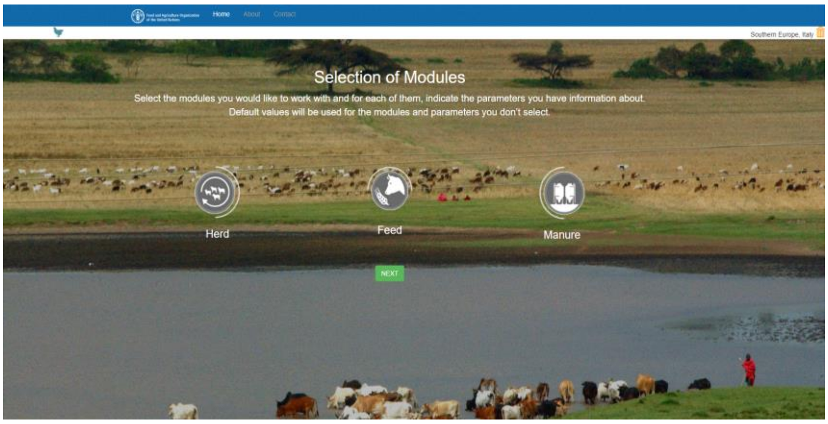
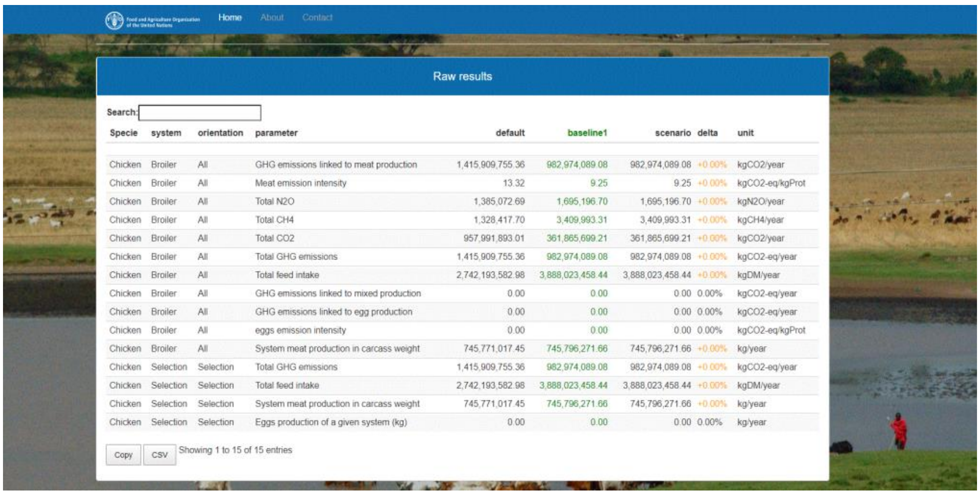


Figure 5.2 - GLEAM-i screen for modules selection (Source: FAO, 2021).

4. Identification of the case as *baseline*, benchmark from suggested default values possibly modified for a more accurate information, and *scenario* or study case, reproducing data on herd, activities and/or farming systems, diet ingredients, and manure management, whose impact on GHG emissions is compared to baseline system.
5. Description of results in three main sections devoted to data on absolute GHG emissions, protein production, and feed intake (part I), figures on emission intensity, protein production, total emission, and breakdown of emission by GHG and by source of feed (part II), and raw results referring to the breakdown of emission, the emissions intensity and details on relative sources (part III) (Figure 5.3). Furthermore, changes between scenario and baseline are also highlighted in a delta column (+/- %), reporting the ratio between the difference (scenario – baseline) and baseline value. All of results could be saved on the operator device.



Specie	system	orientation	parameter	default	baseline1	scenario	delta	unit
Chicken	Broiler	All	GHG emissions linked to meat production	1,415,909,755.36	982,974,089.08	982,974,089.08	+0.00%	kgCO2/year
Chicken	Broiler	All	Meat emission intensity	13.32	9.25	9.25	+0.00%	kgCO2-eq/kgProt
Chicken	Broiler	All	Total N2O	1,385,072.69	1,695,196.70	1,695,196.70	+0.00%	kgN2O/year
Chicken	Broiler	All	Total CH4	1,328,417.70	3,409,993.31	3,409,993.31	+0.00%	kgCH4/year
Chicken	Broiler	All	Total CO2	957,991,893.01	361,865,699.21	361,865,699.21	+0.00%	kgCO2/year
Chicken	Broiler	All	Total GHG emissions	1,415,909,755.36	982,974,089.08	982,974,089.08	+0.00%	kgCO2-eq/year
Chicken	Broiler	All	Total feed intake	2,742,193,582.98	3,888,023,458.44	3,888,023,458.44	+0.00%	kgDM/year
Chicken	Broiler	All	GHG emissions linked to mixed production	0.00	0.00	0.00	0.00%	kgCO2-eq/year
Chicken	Broiler	All	GHG emissions linked to egg production	0.00	0.00	0.00	0.00%	kgCO2-eq/year
Chicken	Broiler	All	eggs emission intensity	0.00	0.00	0.00	0.00%	kgCO2-eq/kgProt
Chicken	Broiler	All	System meat production in carcass weight	745,771,017.45	745,796,271.66	745,796,271.66	+0.00%	kg/year
Chicken	Selection	Selection	Total GHG emissions	1,415,909,755.36	982,974,089.08	982,974,089.08	+0.00%	kgCO2-eq/year
Chicken	Selection	Selection	Total feed intake	2,742,193,582.98	3,888,023,458.44	3,888,023,458.44	+0.00%	kgDM/year
Chicken	Selection	Selection	System meat production in carcass weight	745,771,017.45	745,796,271.66	745,796,271.66	+0.00%	kg/year
Chicken	Selection	Selection	Eggs production of a given system (kg)	0.00	0.00	0.00	0.00%	kg/year

Figure 5.3 - GLEAM-i raw results screen (Source: FAO, 2021).

To estimate the environmental impacts of the two selected diet SOY *vs.* PEA, following the main guidelines of GLEAM-i, SOY diet was defined as baseline case study while PEA diet was defined as scenario case study due to its possible contribute to the environmental mitigation. Details on data entered for herd, feed, and manure modules, are summarised in Table 5.3.

Table 5.3 - Gleam-i module parameters described for SOY and PEA diets.

Parameter	Unit	SOY (baseline)	PEA (scenario)
Herd module			
Number of animals	#	60	60
Live weight at slaughter	kg	2.61	2.58
Death rate of adult broilers	%	1.7	1.7
Feed module			
Dried grain by-products	%	49.15	47.46
Grains from wheat	%	25.42	26.27
Grains from maize	%	15.25	12.71
Leguminous beans	%	6.78	13.56
Beans from soy	%	3.39	-
Manure module			
Poultry manure with litter	%	100	100

It is worth noting that due to software restrictions the analysis process underwent some adaptations in feed data, summarised as follows:

- “grains from wheat” was referred only to *Triticum aestivum* L.,
- legume bean sources were not differentiated except for soy, reported separately.

In order to overcome these two limits, in the present work the dietary wheat inclusion was expressed as *Triticum aestivum* L. instead of *Triticum durum* Desf. and both pea bean and faba bean ingredients were reported as “Leguminous beans source”, by adding up the two protein source percentages.

Statistical analyses of results

All data were subjected to descriptive statistic (Microsoft© Excel© for Office 365 ProPlus Version 1903 -11425.202429). Sub-groups data about daily DM intake and DM refusals were processed by analyses of variance followed by Student-Newman-Keuls (SNK) post-hoc test. Sub-groups data on BW, ADG and FCR were processed by analyses of variance according to general linear model (GLM) procedure using a full factorial design, including diet treatment, genotype, and their interaction. The covariate effect of "body weight at d 0" was also included in both analyses. Data were presented as mean and standard error (SEM) and differences were considered significant for P < 0.05. Statistical analyses were conducted by IBM SPSS Statistic Data Editor ver. 25.

Results and Discussion

Climatic characterization of study area

To define the climatic characterization of the farm area, in Figure 5.4 were reported the 2019 monthly average values of temperature (°C) and rainfalls (mm). Particularly, observing the experimental period (October - December), temperature tendentially decreased from 15.8 °C to 9.0 °C (Figure 5.4). The average rainfalls ranged from the minimum value of October (48.4 mm of rain) to the maximum value of November (369.6 mm of rain), while in December the precipitations were in average of 208.2 mm (Figure 5.4). During the on-field experiment, relative humidity averaged 80.4 %, and the medium photoperiod was around 10 h light /14 h dark.

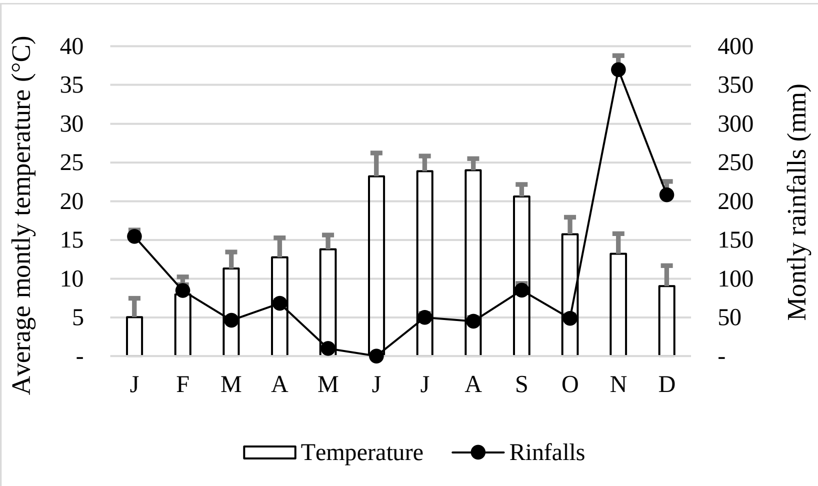


Figure 5.4 - Climatic characterization of studied area during 2019.

Dietary treatments and genotypes: effects on DM consumption and DM refusal

The lower daily value of total DM consumption was registered for K-PEA sub-group (1.10 kg/sub-group), while the higher was reported for NR-SOY sub-group (1.14 kg/sub-group) (Table 5.4). K-PEA and NR-PEA sub-groups were respectively characterized by the higher and the lower total daily dry matter refusal (Table 5.4).

Table 5.4 - Average values (± SEM) of total DM intake and total DM refusal for each group (n = 5 sub-groups, 6 individuals each).

	K-SOY	NR-SOY	K-PEA	NR-PEA
Total DM intake, kg	1.11 (± 0.08)	1.12 (± 0.10)	1.10 (± 0.09)	1.14 (± 0.06)
Total DM refusals, kg	0.05 (± 0.08)	0.03 (± 0.09)	0.05 (± 0.09)	0.02 (± 0.04)

K-SOY = Kabir fed soybean diet; NR-SOY = New Red fed soybean diet; K-PEA = Kabir fed pea bean diet; NR-PEA = New Red fed pea bean diet; DM = dry matter.

Moving to the results of statistical analyses (Table 5.5), the dietary treatment did not significantly affect ($P > 0.05$) the daily DM intake (1.11 kg *vs*. 1.12 kg for SOY and PEA sub-groups, respectively) as well as the daily DM refusal ($P > 0.05$) (Table 5.5). At the experimental conditions, neither genotype affects ($P > 0.05$) total daily sub-groups DM intake and DM refusal (Table 5.5). As the interaction was found significant ($P < 0.05$) (Table 5.5), the effects of diet and genotype on DM intake and DM refusals were not considered additive as displayed in Figure 5 (a, b).

Table 5.5 - Effect of diet treatment, genotype, and their interaction on daily sub-group DM intake and DM refusal (n = 5 sub-groups, 6 individuals each).

	Diet		SEM	Genotype		SEM	P-value		
	SOY	PEA		KABIR	NEW RED		D	G	D x G
Total DM intake, kg	1.11	1.12	0.04	1.13	1.10	0.009	ns	ns	*
Total DM refusal, kg	0.04	0.04	0.04	0.02	0.05	0.008	ns	ns	*

DM = dry matter; SEM = standard error of mean; D = diet; G = genotype; SEM = standard error of mean; * $P < 0.05$; ns = not significant. All values are express as means and pooled SEM.

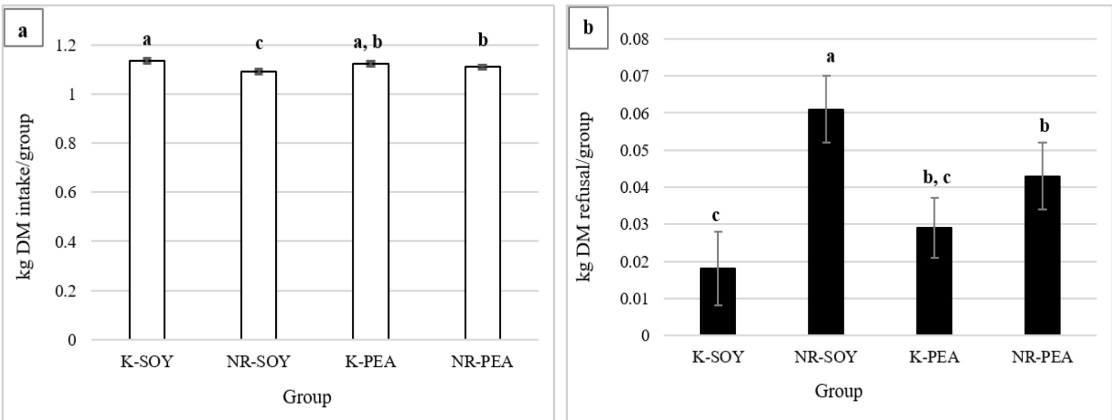


Figure 5.5 - Effect of diet and genotype interaction on daily group DM intake (**a**) and group DM refusal (**b**) (n = 5 sub-groups, 6 individuals each).

Several factors could affect poultry growth performances, *i.e.*, age, sex, diet, genotype, housing system, environmental factors, and number of animals raised per m^2. Especially in growing birds, any change in nutrition is reported to suddenly reflect in chicken performance due to peculiar gastrointestinal conformation (Ravindran, 2013). Generally, during growing period, poultry feed intake is affected by both chemical components of diet and daily intake, as related to chickens' physiological needs (NRC, 1994). A strong correlation among dietary chemical and nutritional components and potential growth response of chickens at different physiologic levels has been also reported in literature (Classen, 2017).

The average values of total daily DM intake (Table 5.5) were about twice than those reported in literature for slow-growing chicks aging 61 to 80 d (Dal Bosco *et al.*, 2013), and for Ross 308 male fast-growth broiler at 42 d of age (Dotas *et al.*, 2014). Nonetheless, Dal Bosco *et al.* (2013) referred that the general lower performances of birds could be mainly attributed to the introduction in starter diets of raw faba bean, which is characterized by both low essential amino acid (methionine, cysteine, threonine, tryptophan) contents and presence of several anti-nutritional factors. However, Dotas *et al.* (2014) showed that the supplementation of broiler chicken diet with raw field pea (*Pisum sativum* L.) had effects on feed intake similar to those observed following dietary treatments with no, low, medium, and high levels of raw field pea inclusion. Also analysing the average values of feed intake reported by Fanatico *et al.* (2008) for slow-growth poultry raised with outdoor access and fed a low nutrient diet (13.9 % CP, 12.4 MJ/kg DM) until 13 weeks of age, they turned out to be around 0.11 kg DM/chickens per d, slightly more than half of the quantities shown in Table 5.5. Similar average daily feed intake (about 0.12 kg DM/chickens at 49 d of age) were reported also when slow-growth broilers were fed a diet containing high-protein micronized peas as a substitute of soybean meal (Laudadio and Tufarelli, 2010). Furthermore, values of daily DM intake reported in literature for fast-growth chickens average 0.094 kg DM/chicken at 42 d of age (Maiorano *et al.*, 2017), 0.08 kg DM/chickens at 84 d of age (Mueller *et al.*, 2019), and 0.15 kg DM/chicken at 61 d of age (Cartoni Mancinelli *et al.*, 2020).

Dietary treatments and genotypes: effects on growth performances

At the beginning of the trial (d 0), the average sub-group BW range from (minimum - maximum value) 8.70 kg/K-SOY sub-group to 10.5 kg/K-PEA sub-group (Table 5.6). As reported in Table 5.6, at the end of the feeding trail, the lower and the higher sub-group

BW were recorded respectively in K-SOY (14.1 kg/sub-group) and in K-PEA (17.0 kg/sub-group). At 14 d of feeding trail, K-SOY and NR-SOY showed the same sub-groups BW, while at 28 d and 36 d, the K-SOY and NR-SOY sub-groups BW was very close (Table 5.6). K-PEA sub-group did not show an increase of BW (17.0 kg/sub-group) between 28 d and 36 d, while the sub-group BW of NR-PEA increased (Table 5.6).

According to data reported in Table 5.6, at the end of feeding trial, NR-SOY and K-PEA sub-group showed respectively the lower (0.15 kg/d) and the higher (0.18 kg/d) ADG. Consequently, K-PEA showed the best FCR (6.54 kg DM intake/kg gain), while NR-SOY showed the higher FCR (7.45 kg DM intake/kg gain) (Table 5.6).

Table 5.6 - Average values (± SD) of growth performance of each group (n = 5 sub-groups, 6 individuals each).

	K-SOY	NR-SOY	K-PEA	NR-PEA
BW, kg/sub-group				
0 d	8.70 (± 0.19)	8.92 (± 0.38)	10.5 (± 0.48)	10.4 (± 0.38)
14 d	11.8 (± 0.37)	11.8 (± 0.22)	13.8 (± 0.49)	13.3 (± 0.60)
28 d	14.1 (± 1.83)	14.3 (± 0.47)	17.0 (± 0.72)	16.3 (± 1.63)
36 d	14.3 (± 1.38)	14.4 (± 0.92)	17.0 (± 1.72)	16.6 (± 1.74)
ADG, kg/d	0.16 (± 0.04)	0.15 (± 0.03)	0.18 (± 0.04)	0.17 (± 0.04)
FCR, kg DM intake/kg gain	7.42 (± 2.13)	7.45 (± 1.23)	6.54 (± 1.57)	6.99 (± 1.97)

K-SOY = Kabir fed soybean diet; NR-SOY = New Red fed soybean diet; K-PEA = Kabir fed pea bean diet; NR-PEA = New Red fed pea bean diet; DM = dry matter; BW = body weight; ADG = average daily gain; FCR = feed conversion rate.

The observed growth performances showed BW values lower than those reported by commercial information about the selected genotypes (Table 5.1). More in detail, all sub-groups have been characterised by an average BW at 84 d close to that reported at 63 d of age for the same genotypes raised under controlled light, temperature, and humidity conditions (Avizoo, 2020).

BW reported in Table 5.6 for NR-SOY sub-group was close to the final BW (2.44 kg/bird), reported by Dotas *et al.* (2014) for 42 d old broiler chickens fed with 80 g/kg DM, 120 g/kg DM, and 240 g/kg DM of raw field pea respectively during starter, grower, and finished period a medium field peas content. Final BW of each sub-group was higher compared to the values of 2.40 kg/bird reported for fast-growing broilers 42 d old fed with olive pulp added to starter, grower, and finisher diet respectively at a level of 0 %, 2.5 % and 5 % (Pappas *et al.*, 2019). Moreover, NR-PEA, NR-SOY AND K-PEA sub-groups BW at d 36 were observed higher to 2.53 kg/bird reported in literature for fast-growing broilers 49 d old fed with a diet containing micronized and dehulled peas (Laudadio and Tufarelli, 2010).

Notwithstanding the descriptive data presented in Table 5.6, the effects of the different dietary protein sources and genotypes on productive performances of the broilers were reported in Table 5.7. The dietary treatment and genotype did not affect (P > 0.05) poultry BW at different times (Table 5.7), as well as ADG and FCR. No interactions diet x genotype was observed (P > 0.05) on poultry growth performances (Table 5.7).

Table 5.7 - Effect of dietary protein source, genotype, and their interaction on growth performances for each sub-group (n = 5 sub-groups, 6 individuals each).

	Diet		**SEM**	**Genotype**		**SEM**	**P-value**		
	SOY	**PEA**		**KABIR**	**NEW RED**		**D**	**G**	**D x G**
BW, kg/group									
14 d	12.8	12.5	0.11	14.4	12.9	0.23	ns	ns	ns
28 d	15.6	15.2	0.37	15.6	15.2	0.74	ns	ns	ns
36 d	15.7	15.4	0.40	16.2	15.0	0.82	ns	ns	ns
ADG, kg/d	0.17	0.16	0.01	0.18	0.15	0.02	ns	ns	ns
FCR, kg DM intake/kg gain	6.95	7.25	0.56	6.4	7.76	1.13	ns	ns	ns

BW = body weight; ADG = average daily gain; FCR = feed conversion rate; SEM = standard error of mean; D = diet, G = genotype; SEM = standard error of mean; ns = not significant. All values are express as means and pooled SEM.

Numerous references reported that the final body weight of poultry could be influenced by several key factors, including the rearing system (Cygan-Szczegielniak *et al.*, 2019). Generally, outdoor chickens had a lower body weight when compared to the indoor animals (Połtowicz and Doktor, 2011; Kuźniacka *et al.*, 2014), due to the effects of both variations in temperatures and physical exercise done by the animals, resulting in increased energy expenditure and higher feed conversion rate (Tong *et al.*, 2014), *i.e.*, cold temperatures are also known to increase feed intake but decrease the conversion efficiency (Laudadio and Tufarelli, 2010). Furthermore, a negative correlation between birds' BW and active behaviours was also reported in literature (Cartoni Mancinelli *et al.*, 2020). Comparing the physical activity of fast-growing and slow-growing chickens under the same farm and feed conditions, slow-growth birds start to walk quickly and with a higher speed compared with fast-growth genotypes (Bokkers and Koene, 2004), notwithstanding in fast-growing strains speed decreases with age and with the progressive increase of body weight (Cartoni Mancinelli *et al.*, 2020).

Experimental sub-group ADG (Table 5.7) were lower than values reported by Cygan-Szczegielniak *et al.* (2019) for fast-growing chickens raised for 56 d in semi-intensive system but were close to the ADG reported for broilers 61 - 80 d old fed soybean diet (Dal Bosco *et al.*, 2013). Data about the calculated FCR (Table 5.7) showed a better feeding efficiency in all sub-groups than FCR values reported for broiler fed soybean diet between 61 and 80 d of age (Dal Bosco *et al.*, 2013), and close to FCR values reported for broiler chickens 42 d old fed a diet with moderate inclusion of field peas (Dotas *et al.*, 2014), or after *in ovo* prebiotic inoculation (Maiorano *et al.*, 2017). However, FCR is a function of animal genetics and age, quality of dietary ingredients and environmental conditions (NRC, 1981; NRC, 1994; Arthur *et al.*, 2014).

The mortality rate recorded during the on-field trial (1.7 %) was higher than the range 0.6 % - 1.3 % reported for broilers 84 d old (Mueller *et al.*, 2019), but consistent with the value of 1.7 %, reported for crossbred chickens at 91 d of age (Nolte *et al.*, 2020).

Feeding costs: results and considerations

During the investigated period (last three months of 2019 and the same period of 2020), the price of raw materials for animal feeding has been characterized by a generalized increase, accounting for a minimum of + 3.7 % for faba bean (278.6 €/t *vs*. 298.0 €/t, respectively for 2019 and 2020) and a maximum of + 22.5 % for wheat bran (161.6 €/t *vs*. 197.9 €/t, respectively reported for 2019 and 2020) (Table 5.8). Soybean price varied from 367.5 €/t in 2019 up to 433.9 €/t in 2020 (+ 18.1 %), while pea bean price increase of 6.97 %, from 234.4 €/t in 2019 to 250.7 €/t in 2020 (Table 5.8).

Table 5.8 - Ingredients' prices average values (± SD) for the investigated period.

Feed ingredients	**Price [a]** (€/t)		**Change [b]** (%)
	2019	**2020**	
Wheat bran	161.6 (± 13.5)	197.9 (± 18.5)	+ 22.5
Durum Wheat	266.7 (± 8.31)	297.8 (± 7.97)	+ 11.7
Corn meal	174.8 (± 0.39)	195.3 (± 5.82)	+ 11.7
Faba Bean	278.6 (± 1.92)	289.0 (± 3.77)	+ 3.71
Pea Bean	234.4 (± 8.41)	250.7 (± 13.9)	+ 6.97
Soybean flacked, 37% CP	367.5 (± 3.78)	433.9 (± 20.1)	+ 18.1

[a] Based on the average price of the last three months of 2019 and of 2020 from Bologna Exchange Commodity (AGER, 2021).

[b] Change was calculated by the ratio between 2020 and 2019 prices (%).

As reported in Table 5.9, in both investigated periods (2019 - 2020) PEA diet resulted cheaper than SOY diet. More in detail, the introduction of pea bean in PEA diet allowed in 2019 to reduce feeding cost, - 0.40 % for 100 kg of raw feed (20.22 €/100 kg raw feed *vs.* 20.14 €/100 kg raw feed, respectively for SOY and PEA diet) and - 4.76 % for 1 kg DM of feed (0.21 €/kg DM *vs.* 0.20 €/kg DM, respectively for SOY and PEA diet) (Table 5.9). During last three months of 2020, although was observed a tendential increase of total feed costs, PEA diet still resulted cheaper than SOY diet, *i.e.*, - 1.15 % for 100 kg of raw feed and - 4.17 % for 1 kg DM of feed (Table 5.9). Total diet costs were 23.43 €/100 kg raw feed *vs.* 23.162 €/100 kg raw feed, respectively for SOY and PEA diet, and 0.24 €/kg DM *vs.* 0.23 €/kg DM, respectively for SOY and PEA diet (Table 5.9).

Table 5.9 - Calculated diet costs for each experimental treatment for last three months of 2019 and the same period of 2020.

	Diet cost (€/100 kg as fed)		**Diet cost** (€/kg DM)	
	SOY	**PEA**	**SOY**	**PEA**
2019				
Wheat bran	8.42	8.08	0.08	0.08
Durum wheat	6.40	6.67	0.07	0.07
Corn meal	2.56	2.18	0.03	0.02
Fava bean	1.74	1.74	0.02	0.2
Pea bean	-	1.46	-	0.2
Soybean flacked, 37 % CP	1.10	-	0.01	-
Total cost	**20.22**	**20.14**	**0.21**	**0.20**
Variation, %		**- 0.40**		**- 4.76**
2020				
Wheat bran	10.31	9.89	0.10	0.09
Durum Wheat	7.15	7.45	0.08	0.08
Corn meal	2.86	2.44	0.03	0.03
Faba Bean	1.81	1.81	0.02	0.02
Pea Bean	-	1.57	-	0.02
Soybean flacked, 37 % CP	1.30	-	0.02	-
Total cost	**23.43**	**23.16**	**0.24**	**0.23**
Variation, %		**- 1.15**		**- 4.17**

CP = crude protein.

The changes of ingredient prices recorded during the 2020 could be attributable to a disequilibrium between demand and supply for raw materials (ISMEA, 2020 b). Due to COVID-19 pandemic, the global demand for raw materials from feed industries was affected by a declining trend. For instance, farmers have been retaining their stocks longer, as result of both limited access to markets and slaughterhouses (FAO, 2020 d) and contraction of the animal products demand especially from the HoReCa (Hotellerie, Restaurant and Cafè) channels (ISMEA, 2020 b). Furthermore, in this context, also the reduced request to the animal feed industries, inputs and services, and from market have played important roles (FAO, 2020 d). According to ISMEA (2020 b), the increasing price trend of raw materials has been affected not only by the global reduction of the ingredients availability closely linked to SARS-CoV-2 disease, but also by a speculative factor possibly driven by an increased demand for cereals and oil seeds from China, which aimed to supply its stocks for a quick economy and productive recovery after the deep crisis due to both COVID-19 pandemic and African swine fever outbreak.

GLEAM-i elaboration: results and considerations

Based on GLEAM-i elaboration, data about environmental impacts calculated on a year basis period for SOY and PEA diet, and their variations (+/- delta, %) were reported in Table 5.10. Total GHG emissions, expressed as kg CO_2-eq/year, resulted higher for SOY than for PEA diet. More in detail, compared to SOY diet, PEA diet allowed the reduction of total GHG (- 8.22 %) and total annual CO_2 (- 66.2 %), and meat emission intensity (- 8.22 %) (Table 5.10). However, an increase of total CH_4 (+ 1.81 %), N_2O (+ 9.01 %) emissions has been calculated along with an increase of total DM consumption (+ 1.32 %). Furthermore, it should be noted that, notwithstanding an equal carcass weight production (kg/year), a reduction in GHG emission linked to meat production accounting for about 8 %, was observed for PEA diet (Table 5.10).

Table 5.10 - Raw results about the environmental impacts of default and selected diets (n = 60).

Parameters	Unit	Baseline SOY	Scenario PEA	Delta
Total GHG emissions	kg CO_2-eq/year	585.3	537.2	- 8.22
Total CO_2	kg CO_2/year	126.2	42.7	- 66.2
Total CH_4	kg CH_4/year	2.43	2.47	+ 1.81
Total N_2O	kg NO_2/year	1.26	1.38	+ 9.01
Total feed intake	kg DM/year	2 270.1	2 300.0	+ 1.32
System meat production in carcass weight	kg/year	87.8	87.8	+ 0.00
GHG emissions linked to meat production	kg CO_2/year	585.3	537.2	- 8.22
Meat emission intensity	kg CO_2-eq/kg Prot	46.8	42.9	- 8.22

Baseline SOY = SOY diet; Scenario PEA = PEA diet; DM = dry matter; GHG = greenhouse gas; Prot = protein. Delta = ((Scenario-Baseline)/Baseline) * 100 %.

The most common greenhouse gas emitted in the atmosphere from poultry sector, which negatively affect animals and workers, are CO_2, N_2O, and CH_4, closely related to dietary components and energy content, and by the growing energy input needed to produce mineral fertilizers, essential for crops and food intensive production systems (Gerber *et al.*, 2008; FAO, 2017). CO_2 is largely produced by burning biomass and fossil fuels, *i.e.*, coal, oil, gas, during the fertilizer manufacturing, and as consequence of land use changes and industrial processes (IPCC, 2018). CH_4 is the major component of natural gases whose production is associated to animal husbandry, for the emissions closely related to the diet composition and its digestibility in ruminant species, besides agriculture activities (IPCC, 2018). Agriculture practices, as soil and manure management, sewage treatment, chemical industrial processes, and fossil fuel combustion, are the main sources of N_2O (IPCC, 2018). In poultry sector, the production of N_2O is firstly associated to the high nutrient-feed requirements, but it is also linked to the emissions from arable lands and fertilizers production as well as it is produced by nitrification and denitrification processes (Gerber *et al.*, 2008; Calvet *et al.*, 2011; Sousa *et al.*, 2017).
Focusing on results from the on-field trial, the comparison with current experimental data to those reported as default by GLEAM-i for the Italian poultry population, should consider that the total Italian environmental impact of system meat production in carcass weight was around 745 Gt/year, while SOY and PEA diet data provided only 87 kg of carcass weight/year (Table 5.10). Therefore, the total GHG emission liked to the meat production for the Italian poultry sector was 1 415 Gt CO_2-eq/year, while for the presented case study the emission of approximately 550 kg CO_2-eq/year were estimated for both diets. Meat emission intensity for the default data was equal to 13.3 kg CO_2-eq/kg protein, while for the two selected diet treatments was about three time higher (46.8 CO_2-eq/kg protein *vs.* 42.9 CO_2-eq/kg protein respectively for SOY and PEA diet), likely related to the different farming system and growth rate of the case study (where birds were reared under semi-intensive conditions, in winter) compared to the default case, where birds were reared under intensive and controlled conditions for meat production, according to FAO guidelines (2020 c). However, it should be noted that meat emission intensity (CO_2-eq/kg protein) of the two dietary treatments (SOY *vs.* PEA) were very close, consistent with the average live weight (2.61 kg/head for SOY *vs.* 2.58 kg/head for PEA diet). Nevertheless, the obtained data 6.66 kg CO_2/kg BW (SOY) *vs*. 6.11 kg CO_2/kg BW (PEA), were higher than the GWP derived from feed and manure modules for small scale broiler (five annual cycles) where, for an average carcass weight of 1.20 kg, was estimated

the production of 3.68 kg CO_2-eq (Espino and Bellotindos, 2020). Furthermore, according to Espino and Bellotindos (2020), environmental impact from PEA diet was slightly above to 5.97 kg CO_2-eq/carcass weight reported for Malaysia case study.
Moreover, according to Zervas and Tsiplakou (2016), for chickens raised under conventional system, the global warming potential ranged from 1.4 CO_2-eq/kg BW to 2.30 CO_2-eq/kg BW, however it must be considered that each productive system is characterized not only by specific management and feeding organization, but also by quality of the final product (not presented here). In-depth study should therefore investigate comparable and alternative feed sources with an approach "from crop to table" of each food chain.

Conclusions

Dietary treatment with soybean or pea bean as well as chicken genotype did not affect dry matter intake and growing performances of broilers raised under semi-intensive condition, although a significant interaction diet x genotype was observed. Taken together the results suggest that the total replacement of soybean with pea bean can be an interesting feeding strategy in marginal areas of Centre-South Italy.
From an economic point of view, in the investigated medium period for 2019 and 2020, diet containing soybean was more expensive compared to diet including pea bean.
From an environmental point of view, as the impact intensity is strongly connected to the feed production practices and diet composition, PEA diet slightly reduced the total annual GHG emissions and those linked to meat production, as well as the CO_2 emissions. However, the introduction in the diet of pea bean resulted in increased feed intake and annual emissions of N_2O and CH_4. As a consequence, the enhancement of traditional farming system through formulating diets that match the nutritional animal requirements, improving feed digestibility and availability, introducing alternative protein source and/or by-product, implementing the good and green agricultural practices, and improving manure and animal waste processing, could help to limit the negative environmental impacts of poultry sector, allowing a more sustainable use of natural resources without negative effects on farm activities and remunerability.
In conclusion, even though the poultry chain has been able to adapt quickly to the social and economic changes imposed especially by the current global situation linked to the COVID-19 pandemic, it seems essential to consider new and alternative protein sources

in order to decrease the disequilibrium of market demand and supply, also combined to the global goods exchange, in order to limit negative effects or perceptions and externalities that food chains could have on environment preservation.

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Final considerations

Research activities described in these chapters are discussed with a multidisciplinary and transversal approach. The issues on animal nutrition have been investigated and, in some cases, stressed in view of contributing to advances of knowledge for a future prospective certainly marked as “green”, “sustainable”, “eco-compatible” and “renewable”.
On this regard, the first raised point concerned the evolution of Molise pasture areas over a long period suggests the enhancement of the link between animals and territory as a part of ecosystem services provided by the territory to the communities, when managed in the respect of its unique plant and animal resources. On the other hand, as further contribute to the advances of knowledge in animal nutrition, the chemical composition of an innovative energy tobacco plant (*Nicotiana tabacum* L., cv. *Solaris*) provides evidence on the possible use of its co-products as feedstuffs and encourages sustainable and environment friendly dietary and livestock farming practices especially in Centre-South inner areas, threatened by marginalization. The multiple utilizations of the new cultivar suggested by the presented results could indeed help the recovery of the traditional tobacco cultivation know-how of Central Apennine areas.
Finally, the study on one of the worldwide livestock productions, as broiler chickens, allowed to investigate the effects of different dietary protein sources on feed intake, growth performances and feeding costs also considering the environmental load of nutrition and breeding activities, as next frontiers for a modern livestock farming.

Because the innovative trait of the addressed topics, results need further in-depth studies to assess sustainable new farming practices respectful of health and welfare status of agricultural entrepreneurs, animals, and consumers, with a synergic and multidisciplinary approach in full compliance of the concept “from soil to the table”.

List of scientific contributions

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- Fatica A*, Fantuz F, Di Lucia F, Zuin M, Borrelli L, Salimei E (2021) - Ensiled biomass of *Solaris* tobacco variety used as forage: chemical characteristics and effects on growth, welfare, and follow-up of Holstein heifers. *Animal: The International Journal of Animal Biosciences*, 15(7). doi: 10.1016/j.animal.2021.100235. ID SCOPUS: 2-s2.0-85107162976.

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Speaker in International Scientific Meeting (Theatre presentation)

- Fatica A*, Di Lucia F, Fantuz F, De Feijter HF, Brandt BP, Salimei E (2019) - *Nicotiana tabacum* L. cv. *Solaris*: an innovative forage for dairy heifers. 70th Annual Meeting of the European Federation of Animal Science. 26 - 30 August 2019, Ghent (Belgium).

Contributions in International Scientific Meetings

- Fantuz F, Ferraro S, Todini L, Spurio R, Fatica A*, Marcantoni F, Salimei E (2021) - Distribution of minor trace elements in different fractions of donkey milk. 72nd Annual Meeting of the European Federation of Animal Science. 30 August - 03 September 2021, Davos (Switzerland). Accepted.

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