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PhD Thesis

“Valorisation of agro-industrial residues in animal nutrition, a sustainable approach”

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La più grande eresia scientifica è che l’applicazione non richieda la comprensione
P.J. Van Soest

Alla mia famiglia

Index

List of abbreviation

IVGPT:	In vitro gas production
GHG:	greenhouse gas
DM:	dry matter
PUFA:	polyunsaturated fatty acid
FAO:	Food and Agriculture Organisation of the United Nation
SDO:	Sustainable Development Objectives
NSC:	no structural carbohydrates
NDF:	neuter detergent fiber
ADF:	acid detergent fiber
ADL:	acid detergent lignin
EE:	ether extract
CP:	crude protein
VFA:	volatile fatty acid
PPS:	pomegranate pomace silage
APS:	apple pomace silage
PAPS:	pomegranate apple pomace silage
TMS:	total mix ratio
Δ-9 THCA:	tetrahydrocannabinol acid
uNDF:	undigested neuter detergent fiber organic matter
DIVDM:	*in vitro* dry matter digestibility
WL:	wine less
NDFom:	neuter detergent fiber
DPPH:	1,1-diphenyl-2-picrylhydrazyl
OM:	organic matter
ME:	metabolizable energy
R_{max}:	maximum fermentation rate
$T_{1/2}$:	time which maximum fermentation rate occur
CLA:	conjugated linoleic acid
HC:	hemp cake
SFA:	short chain fatty acid
MUFA:	monounsaturated fatty acid
PSCFA:	putrefactive short-chain fatty acid
LC:	lignocellulose
SBP:	sugar beet pulp
EU:	European Union
GOP:	dried golden orange pulp
ROP:	dried red orange pulp
LP:	dried lemon pulp

List of abbreviation

LM:	concentrated lemon molasses
COM:	concentrated orange molasses
DPN:	dried olive cake variety Nocellara
DPB:	dried olive cake variety Biancolilla
DPC:	dried olive cake variety Cerasuola
CF:	crude fiber
AOAC:	Association Of Official Analytical Collaboration
T_{max}:	Time wich R_{max} occur
SCFA:	short-chain fatty acid
BCFA:	branched chain fatty acid
OMCV:	organic matter cumulative volume
OMD.	organic matter degradability
RMSE:	root mean square error
HSD:	honestly significant difference
Ace:	acetate
Prop:	propionate
Iso-but:	Iso-butyrate
But:	butyrate
Iso-val:	Iso-valerate
Val:	valerate
PPB:	prickly pear by-products
OPA:	polyamide bioriented
PP:	polypropylene
ISTAT:	Istituto Nazionale di Statistica
CDA:	canonical discriminant analysis
CAN:	canonical function
CC:	canonical coefficients
SAS:	statistical analysis system
TN:	total nitrogen
CSIRO:	Commonwealth Scientific and Industrial Research Organisation
CBDA:	cannabidiolic acid
dOM:	degraded organic matter
FT:	thin refusal of trimming flower
FR:	rough refusal of trimming flower
SF:	refusal of flour production
SO:	refusal of oil extraction
UAM:	ultrasound assisted maceration
ANOVA:	Analysis of Variance
IDA:	information dependent acquisition

List of abbreviation

CE: collision energy
CBDA-C4: cannabidiolic acid C4
CBFA: cannabifuranic acid
CBNA: cannabinolic acid
CBNDA: cannabinodiolic acid
CBEA: cannabielsoic acid
CBGA: cannabigerolic acid
pCH4: methane production as percentage of total gas
OMD24h: organic matter degradability after 24 h of incubation
dCH4: methane production related to degraded organic matter
iCH4: methane production related to incubated organic matter
VFA24 h: total volatile fatty acids after 24 h of incubation
iOM: incubated organic matter
T-diet: tallow diet
H-diet: hempseed cake diet
T-group: group fed tallow supplementation
H-group: group fed hempseed cake supplementation
LA: linoleic acid
ALA: α-linoleic acid
EFSA: European Food Safety Authority
BCS: body condition score
FEDIAF: The European Pet Food Industry
FAMEs: methyl esters
FID: ionization detector
PI: peroxidation index
T0: Time 0
T30: Time 30
RBC: red blood cell count
HCT: haematocrit
Hb: haemoglobin
MCV: mean corpuscular volume
MCH: mean corpuscular haemoglobin
MCHC: mean corpuscular haemoglobin concentration
RDW: red cell distribution width
Ret: reticulocytes
CHr: reticulocyte haemoglobin content
Leu: Leukocytes
Lymphs: lymphocytes
Mono: monocytes

List of abbreviation

Eos:	eosinophils
Baso:	basophils
PLT:	platelets
TP:	total protein
Alb:	albumin
AP:	alkaline phosphatase
GPT:	glutamic pyruvic transaminase
ALT:	alanine transaminase
GGT:	gamma-glutamyl transferase
AST:	aspartate transferase
GLDH:	glutamate dehydrogenase
Fr:	fructosamine
GLU:	glucose
LP:	lipase
CHOL:	cholesterol
Tri:	triglycerides
CRA:	creatinine
BUN:	urea
CK:	creatine kinase
Cort:	cortisol
T14:	Time 14
EPG:	eggs per gram
OPG:	oocyst per gram
CPG:	cist per gram
LPG:	larvae per gram
Tmin:	minimum temperature
Tmax:	maximum temperature
NFE:	nitrogen free extract
TVA:	trans vaccenic acid
GLA:	α-linolenic acid
AA:	arachidonic acid
EPA:	eicosapentenoic acid
TFA:	total fatty acid
SFA:	saturated fatty acid
NRC:	National Research Council
DHA:	docosahexaenoic acid
GFR:	glomerular filtration rate
ESCCAP:	.European Scientific Counsel Companion Animal Parasites

List of abbreviation

List of tables

Riassunto

La valorizzazione dei residui agro-industriali in alimentazione animale, un approccio sostenibile

L'impiego degli scarti agricoli ed industriali per alimentare gli animali è una pratica invalsa per limitare i costi di allevamento e ridurre gli sprechi. Tuttavia, appare evidente come la sporadica integrazione della dieta con sottoprodotti, spesso caratterizzati da composizione chimica eterogenea, stretta stagionalità e scarsa conservabilità, possa risultare poco efficace se non addirittura dannosa per le produzioni e la salute degli animali. Lo scopo del presente progetto di tesi è stata la valutazione delle caratteristiche chimico nutrizionali di alcuni sottoprodotti diffusi nell'area mediterranea, attraverso studi effettuati in vivo ed in vitro, al fine di individuarne il corretto impiego in alimentazione animale. La tesi è una raccolta di cinque articoli pubblicati su riviste internazionali, un'introduzione generale e un capitolo di discussione dei risultati ottenuti. In particolare, nel capitolo due è presentata un'ampia disamina della letteratura scientifica pubblicata a riguardo nell'ultimo decennio. Nei capitoli tre, quattro e cinque è stata utilizzata la tecnica della produzione cumulativa di gas in vitro (IVGPT) per valutare le caratteristiche di fermentazione di alcuni sottoprodotti derivati dalla lavorazione della frutta e dalla produzione dell'olio di oliva e di canapa. Il primo di questi studi, effettuato usando il contenuto ciecale di suini come inoculo, ha evidenziato come i sottoprodotti della lavorazione degli agrumi (pastazzo e melasso), nonostante una certa disomogeneità di composizione chimica, possano rappresentare fonti di energia e di carboidrati fermentescibili utili per la salute dei suini in ogni fase di allevamento. Più difficile è, invece l'impiego delle sanse di oliva in quanto pur essendo ricche di grassi ed energia, presentano contenuti elevati in lignina, che ne limitano la digeribilità per cui potrebbero essere usati solo nella fase di ingrasso, quando i fabbisogni energetici sono molto elevati e la capacità di utilizzazione digestiva è massima.

Nel capitolo quattro è stata affrontata la problematica della limitata conservabilità di ingredienti particolarmente fermentescibili, come i derivati della lavorazione dei fichi d'india. Usando un inoculo a base di liquido ruminale bovino si è evidenziato che l'insilamento della biomassa con l'aggiunta di paglia di frumento in ragione del cinque percento, consente di preservare le caratteristiche nutrizionali del substrato. Ulteriori indagini da effettuare in vivo consentiranno di valutare aspetti quali l'appetibilità del prodotto e gli effetti delle sostanze antiossidanti in esso contenute sulla produzione quanti-qualitativa di latte.

Riassunto

Gli ultimi due contributi sperimentali hanno avuto lo scopo di valutare le caratteristiche chimico nutrizionali dei residui di lavorazione della canapa (Cannabis sativa L). Questa coltura erbacea, dopo anni di disuso, dovuto prevalentemente all'equivoca confusione con la Cannabis indica L., ricca in tretaidrocannabinolo (Δ9-THC) ad azione stupefacente, nell'ultimo decennio è tornata a riscuotere interesse in tutta Europa. Alcuni aspetti agronomici, la rivalutazione delle fibre tessili naturali e i potenziali impieghi innovativi della biomassa, nonché i potenziali effetti benefici per la salute dell'uomo connessi all'assunzione di olio e farina di canapa sarebbero alla base del ritrovato interesse verso la coltivazione della canapa. Da una primaria indagine è emersa l'estrema eterogeneità dei residui della coltivazione e della lavorazione dei semi e delle infiorescenze della canapa in termini di tenore lipidico e in carboidrati di struttura, mentre tutti hanno mostrato livelli proteici e in ceneri grezze piuttosto rilevanti. Si è ritenuto, pertanto opportuno valutare in vitro, con liquido ruminale bufalino, le caratteristiche di fermentazione dei sottoprodotti più fibrosi (capitolo 5), mentre gli effetti dell'integrazione di sansa di semi di canapa, ricca in acidi grassi polinsaturi sono stati valutati in cani adulti sani (capitolo 6).

Lo studio delle caratteristiche di fermentazione dei sottoprodotti della canapa, ha confermato le buone caratteristiche chimiche dei sottoprodotti della produzione dell'olio, mentre quelli derivati dalla lavorazione delle infiorescenze, più ricchi in carboidrati di struttura, lignina e fitocannabinoidi sembrano essere poco utilizzati dai microrganismi presenti nel rumine, come dimostra la ridotta digeribilità e la scarsa produzione di gas e in particolare di metano. Quest'ultima osservazione suggerirebbe un loro potenziale impiego per limitare le emissioni di questo gas serra negli allevamenti di ruminanti.

Lo studio effettuato sui cani adulti sani, confrontando gli effetti dell'integrazione di una dieta del commercio con due fonti lipidiche differenti (strutto vs residuo grezzo della premitura dei semi di canapa), ha evidenziato che l'integrazione con il sottoprodotto della canapa consente in cani sani un significativo miglioramento di alcuni parametri biochimici quali i livelli di colesterolo, e di alcuni marcatori delle funzionalità renale ed epatica. Tali effetti sarebbero da ascriversi prevalentemente agli elevati tenori in acidi grassi polinsaturi del sottoprodotto della canapa, mentre sono necessarie ulteriori indagini per stabilire l'eventuale ruolo giocato dalle molecole bioattive in esso contenute. Tuttavia, occorre evidenziare che il profilo acidico della dieta contenete il sottoprodotto della canapa ha *influenzato negativamente l’indice di perossidazione della dieta per cui prima dell’impiego industriale sarà necessario valutare le strategie per preservare i mangimi così integrati dai rischi di ossidazione*

Abstract

Using agricultural and industrial residues is an established practice to feed animals, limiting farming costs, and reduce waste. However, by-products showed a heterogeneous chemical composition, strict seasonality, and short shelf life. Consequently, diets supplementation with by-products can be ineffective if not harmful for animal production and health. The doctoral project aimed to evaluate the chemical and nutritional characteristics of some by-products of the Mediterranean area. *In vivo* and *in vitro* studies were carried out to identify the correct use of by-products in animal nutrition. In particular, the thesis contains five manuscripts published in international journals, a general introduction, a discussion, and conclusion chapter. In this regard, chapter two presents an extensive review of the scientific literature published in the last decade. In chapters three, four, and five *in vitro* technique of cumulative gas production (IVGPT) has been used to evaluate the fermentation characteristics of some by-products derived from fruit processing, olive, and hemp oil production. The first study was carried out using the caecal content of pigs as *inoculum* highlighting how the by-products of citrus fruit processing (pulps and molasses) can be applied as energy sources in every phase of swine rearing. Indeed, these kinds of by-products are rich in fermentable carbohydrates, which are useful for pig health, despite inhomogeneity of chemical composition. Although this olive pomace has a high content of lipids and energy, it is difficult to use due to the high lignin content that limits its digestibility. However, it could be used only in the fattening phase when energy requirements and digestive utilization are high.

The fermentable ingredients' short shelf-life issue, such as by-products of prickly pears’ processing, was addressed in chapter four. The ensiling of biomass with the addition of wheat straw in the ratio of five percent allows preserving the substrate's fermentable carbohydrates using bovine rumen fluid as *inoculum*. However, further investigations must be carried out *in vivo* to evaluate other aspects, such as the palatability and the effects of the antioxidant molecules on milk yield and quality.

The chemical and nutritional characteristics of hemp (*Cannabis sativa* L.) processing residues have been assessed in the last two studies. This herbaceous crop was abandoned for years mainly due to the equivocal confusion with *Cannabis indica* L., which is rich in tetrahydrocannabinol (Δ9-THC) with psychoactive action. This plant has been returned to arousing interest throughout Europe in the last decade. In this regard, several aspects, such as agronomic advantages and the potential innovative uses of biomass, would be at the basis of the newfound interest in the cultivation of hemp. In particular, hemp oil and flour, being rich in polyunsaturated fatty acids and bioactive molecules, could have beneficial effects on human health. A primary investigation revealed the extreme heterogeneity of the residues from the cultivation and processing of hemp seeds and inflorescences in terms of lipid content and structural carbohydrates. Moreover, all substrates showed quite significant levels of protein and crude ash. Therefore, the fermentation characteristics of the most fibrous by-products have been evaluated *in vitro* with ruminal buffalo fluid (chapter 5). Whilst, the effects of hempseed cake supplementation, being rich in polyunsaturated fatty acids, were tested in healthy adult dogs (chapter 6).

The study regarding hempseed by-products’ fermentation characteristics showed that residues of oil and flour have an interesting chemical composition. Furthermore, the limited digestibility and the low gas production of hemp inflorescence by-products indicated these substrates are a little used by ruminal microorganisms. These results could be due to the high content of lignified structural carbohydrates and phytocannabinoids, which are little used by ruminal microorganisms. The poor methane production resulting in fermentation of these by-products would suggest that they could have the potential to limit the greenhouse emissions in ruminant farms.

The last study was carried out on healthy adult dogs, comparing the effects of commercial diet supplementation with two different lipid sources (tallow vs. hempseed cake). Significant improvements in some biochemical parameters, such as cholesterol levels and some markers of renal and hepatic function, were observed when dogs feed a diet supplemented with hempseed cake. These effects would be mainly attributable to the high levels of polyunsaturated fatty acids of the hempseed cake, while further investigations are needed to establish the possible role played by the bioactive molecules contained therein. However, it should be noted that the fatty acidic profile of the diet containing hempseed cake has negatively affected the peroxidation index of the diet, so before industrial use, it will be necessary to evaluate the strategies to preserve the feed thus integrate from the risk of oxidation

Chapter 1

General Introduction

1.1. Background

Advanced economies are causing an increase of food losses (Porter et al., 2016). During food processing and preparation, a high quantity of waste material is produced (Omre et al., 2018), which is enhancing environmental impact, in terms of air and water pollution, greenhouse gas emissions, and health problems for human and animals. Globally, high food losses (about 1/3 of the produced ones) globally occur along the food chain, i.e., during harvesting, cultivation, processing, and consumption (Bedoić et al., 2019). Forwood et al. (2021) reported as approximately 31% of food waste is produced through primary production whose main part (24%) during manufacturing. Since ever, many agricultural wastes are used. For cereals, during harvesting the huge amounts of residues generated, especially straw, is mostly used in livestock farms providing clean and thermically insulated area for animals. From flour production by several cereals (e.g., wheat, oat, barely, etc.) the bran derived by a multi-stage process is used in animal and human nutrition; while husks and cobs derived from corn, are often end up as burning material. Also, the processing of fruits and vegetables produce residues as well: for fruits different products can result in function of the process type and purpose (i.e., juice or jelly preparation). Similarly, several vegetables are used as raw material in the production of different commodities and semi-products, such as sauces, preserved and frozen products. Consequently, some residues (e.g., twigs, leaves, and woody branches from olives or sugar beet leaves and stones) appear during the cultivation stage and some (damaged vegetables) during the harvesting period (Bedoić et al., 2019).

However, the valorisation and use of all these kinds of residues needs to be increased, as also request by Agenda 2030 (Target 12.5). In recent years, there has been a transition from the well-established concept of by-product to the new term of co-product. The first refers to a residue, inevitably produced through different processing methods, usually characterized by a lower nutritional value than the product which derived. On the other hand, the co-products are still processing residues, which nevertheless retain, or in some cases improve, the nutritional composition of products which derived. In any case, both could still represent important resources of nutrients. For example, the residues of agro-industrial processing could be an interesting source of second metabolites with important benefits on human and animals’ health (due to antioxidant, carbohydrates, and fatty acids content), and environmental impact (for the reduction of methane production).

1.2 Objectives of the Thesis

The use of agro-industrial co-products in animals' diets could reduce the environmental impact of food processing and improve the valorisation of the residues as feed. By- and co-products could be useful to satisfy the nutritional requirement of the different animal species in function of their chemical and nutritional characteristics. The main objective of this research project was to characterise some agro-industrial by-products through *in vivo* and *in vitro* trials. In the attempt to identify the animal species that may more benefit from the intake of specific by-products.

For this purpose, in the present PhD project different kinds of agricultural and industrial by- and co-products have been studied for their possible use in different animal species, monogastric and ruminant. The trials reported in chapter three, four, and five have been performed by *in vitro* cumulative gas production technique (IVGPT) according to the procedure described by Calabrò et al. (2005).

The IVGPT method is ethically advantageous, faster, and less expensive than *in vivo* techniques. Indeed, the benefits of the *in vitro* procedures include being able to run large batches simultaneously, the ability to measure fermentation kinetics of a single feedstuff, making comparisons among different feedstuffs. The IVGPT is essentially based on anaerobic digestion of fermentable carbohydrates by microorganisms. On this basis, it is possible to obtain information on fermentation kinetics, organic matter degradability, and fermentation end-products (e.g., volatile fatty acids, ammonia). In the last decade, the IVGPT was also suggested to measure the methane production (Kumar et al., 2014); in this regard, it can represent a valid instrument to preliminary test nutritional strategies to reduce greenhouse gas (GHG) emissions in animal production. For instance, evaluating feedstuff or bioactive molecules able to limit the action of methanogens bacteria. The application of IVGPT could be useful to determine the influence of methane inhibitors on fermentation patterns and methane as a new criterion in diet formulation.

Although the IVGPT has been developed for ruminants, it can be also applied to monogastric animals to evaluate the nutritive value of feeds and test potential prebiotics (Musco et al., 2015). Considering the different fermentative activity and role of fermentable carbohydrates in monogastric species, faecal or caecal *inoculum* is used, and the incubation period needs to be reduced (48 - 96h).

The association between chemical composition data and IVGPT parameters represents one of the most complete methods to evaluate feedstuffs' nutritive value before their use, especially when novel feedstuffs, such as by-products are studied.

To achieve the above objectives, in the PhD thesis, citrus and olive oil processing by-products were evaluated for their use in pigs' nutrition. The efficacy of ensilage as a preservation method for prickly pears co-products comparing different inclusion of wheat straw were also verified using bovine rumen liquor as *inoculum*. The fermentation characteristics of co-products rich in carbohydrates and protein (seeds and flowers processing residues) have been tested with buffalo rumen liquor.

In chapters five and six were evaluated different hemp co-products. In particular, the fermentation characteristics of co-products rich in carbohydrates and protein (seeds and flowers processing residues) have been tested with buffalo rumen liquor. Whilst hemp seedcake that was characterized of high ether extract amount (>10% DM) and fatty acid profile particularly rich in polyunsaturated fatty acid (PUFA) was tested (chapter six) *in vivo* on healthy adult dogs. This study is aimed to evaluate the effect of polyunsaturated fatty acids present in hempseed cake on animal health.

1.3 Layout of the Thesis

As reported in the following scheme (Figure 1.1), the thesis is structured in seven chapters. First a general overview of the issue treated (Chapter 1) is presented. Then, a review has reported which analyses scientific articles published between 2010 and 2021 on the evaluation of by-/co-products in different animal species using *in vitro* and *in vivo* methods (Chapter 2). After, four experimental contributes, each complete of introduction, materials and methods, results and discussion, conclusions, and references, are reported (Chapters 3-6). Lastly some final considerations are added (Chapter 7). The thesis also includes a list of abbreviations.

Chapter 2

- A review on the use of agro-industrial co-products in animals ‘diets

Chapter 3

- *In vitro* fermentation and chemical characteristics of Mediterranean by-products for swine nutrition

Chapter 4

- Silage of prickly pears (*Opuntia* spp.) juice by-products

Chapter 5

- Chemical and nutritional characteristics of *Cannabis sativa* L. co-products

Chapter 6

- Hemp seed cake as a novel ingredient for dog’s diet

Chapter 7

- General conclusion

Fig. 1.1. Structure of chapters within the thesis

1.4 References

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Chapter 2

A Review On The Use Of Agro-Industrial Co-Products In Animals' Diets

Review accepted by Italian Journal of Animal Science (in press)

Chapter 2 A Review On The Use Of Agro-Industrial Co-products in Animals’ Diets

2.1 Abstract

The use of agro-industrial co-products in animal nutrition could represent an opportunity to reduce the environmental impact of the food production chain. Co-products can decrease the feeding cost and improve animal products in terms of quality and sustainability. To evaluate the use of co-products as animal feed 57 studies published in the last 11 years on agro-industrial residues were considered.

In vitro trials demonstrated that some co-products, such as ginseng meal, grape pomace, and olive cake, in animal diets could affect fermentation parameters decreasing the gas production, particularly the methane emission. Indeed, thanks to their chemical composition and the presence of some bioactive compounds, such as tannins, these co-products seemed able to modify the ruminal and the intestinal environment and consequently fermentation kinetics and end-products. Furthermore, fruits, vegetables, and oil extraction co-products could be valid sources of energy, fiber, and protein, respectively.

The remaining studies, conducted *in vivo* on different animal species, evidenced as some fruits and oil extraction co-products such as prickly pears, olive, and hemp cake could modify the quality of milk and/or meat, improving their fatty acid profile. Moreover, the antioxidant compounds of these co-products could have beneficial effects on gut microbiota and animal health *status*.

The replacement of traditional feedstuffs with agricultural or industrial co-products could represent an interesting prospect for animal production. However, it is important to individuate the right dosage of supplementation in the animal diet, considering that all fruit and vegetable residues showed high variability in chemical composition.

2.2 Introduction

The increase in food waste is a relevant problem of these last decades. On a global level, 1.3 billion tons of food are lost or wasted each year (FAO, 2011). Reducing the environmental impact of human activities is one of the most difficult challenges. In this scenario, in 2015, 193 member states of the United Nations (UN) signed the Agenda 2030 with 17 Sustainable Development Objectives (SDO) and 169 targets which reflect the necessity of world leaders to improve policies and plans to preserve the natural resources to guarantee environmental sustainability (Dunque-Acevedo et al., 2020). In particular, objectives #2 and #9 focus on sustainability in agriculture and industry and provide a foundation for concerns surrounding waste disposal and food waste over the years (FAO, 2019). For this purpose, the European Commission (EC) set the "Farm to Fork Strategy - for a fair, healthy and environmentally-friendly food system" (European Union, 2020) to reduce the losses along the food chain and guarantee the sustainability of food's production, processing, and consumption. To achieve these goals, the huge quantities of non-human-edible biomass waste generated along the food chain can be valorised as co-products (Rakita et al., 2021). In this regard, to reduce the environmental impact of waste, agro-industrial co-products, or former foods could represent a valid element like feed ingredients in animal nutrition. The co-products are obtained from different agro-industrial processes such as the production of oil, sugar, fruit juice, and canned or frozen vegetables, root, and tuber (Pinotti et al., 2020). While former foods are foods manufactured for human consumption that despite their important nutritional characteristics are no longer suitable for human consumption (Luciano et al., 2020). In this regard, former food could be useful in animal nutrition (Pinotti et al., 2020).

The co-products are already included in the animals' diet several times thanks to their interesting nutritional characteristics. Some co-products such as beet pulp, corn gluten feed, soybean (hulls, meal, and molasses), and sunflower meal are largely used as animal feedstuff like sources of fiber, protein, and sugar (Rakita et al., 2021; Serrapica et al., 2019). However, new agro-industrial crops such as cardoon (*Cynara cardunculus* L.) and hemp (*Cannabis sativa* L.) are emerging within the last few years (Serrapica et al., 2019; Bailoni et al., 2021). Moreover, the co-products derived from fruit and vegetable processes seem to have applications in animal nutrition because of their considerable amounts of bioactive components (i.e., polyphenol, flavonoids, and tannins) (Correddu et al., 2020; Branciari et al., 2021). Indeed, co-products can bestow several advantages when included in animal diet, such as reducing the feeding cost for farmers, conferring added value to animal products, and improving animal health status (Correddu et al., 2020). Moreover, some co-products such as olive mill vegetation water could be used to extract bioactive compounds (e.g., phenol metabolites) able to improve microbial quality of meat (Branciari et al., 2021) or to increase the presence of bioactive molecules with antioxidant effects in milk and dairy products (Branciari et al., 2020).

Additionally, the inclusion of agro-industrial residues in the animals' diet represents a major opportunity for the development of a circular economy, improving economic and environmental sustainability. Indeed, the traditional production models are based on a linear economy, where the natural resources are converted to useful products, and unusable waste (Murray et al., 2017) represents a growing disposal problem. Today, the attention to limit the impacts of wastes is increased, so it is necessary to develop new production systems. The new economy, based on a circular model, aims to develop a more efficient system. This last ensures a reduction in natural resource use and wastes products as well, effectively reducing the wastes to be processed and re-used as valuable co-products (Toop et al., 2017).

Otherwise, co-products show some limits, such as the high variability in nutrients' composition due to the different processing methods to which they are submitted. Moreover, the co-products require preservation treatments that are essential for product stabilization and attenuation of seasonal availability, and increase the shelf-life, particularly for the co-products which show high moisture and lipids values (Halmemies-Beauchet-Filleau et al., 2018; Salami et al., 2019).

Industrial processes are the main cause of residues development whose fruit and vegetable represented the most abundant waste, such as pulps, skins, pomace, roots, and tubers, with a percentage of the residues around 40-50% of the total discards. In this regard, grape and olive pomace derived from wine and oil production, other fruit co-products (i.e., apples, pears, peaches, citrus fruits) coming from juice, jelly, and jam industries as well as all waste from the processing of vegetables such as potatoes, tomatoes, fennels, artichoke, and carrots (Dilucia et al., 2020).

The review aims to emphasize the importance of introducing the co-products derived from agro-industrial processing as ingredients in animal diets.

2.3 Methodology

Dunque-Acevedo et al. (2020) reported that nearly 60% (1888 on 3148) of articles about agriculture waste were published between 2009 and 2018. Moreover, the articles, which tested either former foods or extract obtained by co-products were excluded. The main database Scopus has been used to perform this review. Overall, 1637 articles were found and analysed for this study to guarantee the criteria of inclusion and exclusion. A total of 57 *in vitro* and *in vivo* studies on co-products derived from agriculture and industrial processing were selected and described Figure 2.1. shows the different stages of research and selection of the articles described in the following manuscript.

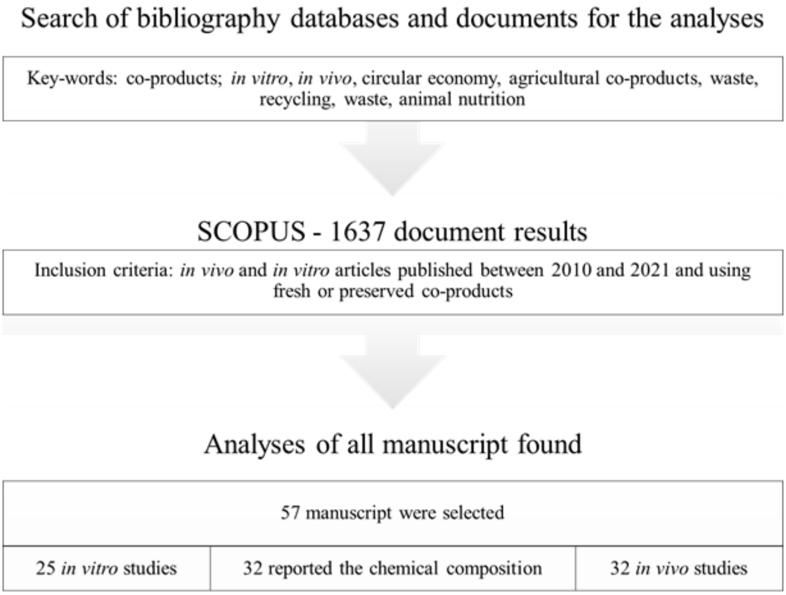

Fig 2.1. Stages of the bibliography research analysis process

2.4 Co-products characteristics and chemical composition

Among the fifty-seven selected manuscripts, thirty-seven (65% of all described articles) reported the chemical composition of tested co-products. The studies that reported only a few values of the main nutrient parameters (i.e., crude protein, structural carbohydrates; ether extract) were excluded from the chemical composition tables. The main part of described co-products derives from fruit juice, jam, and wine production (*citrus* spp., apple, pomegranate, prickly pears) (Table 2.1). Several authors (Garcìa-Rodrìguez et al., 2019; Lashkari and Taghizadeh, 2013; Paya et al., 2012; De Blas et al., 2018; Santos et al., 2014; Abdel-Gawad et al., 2020; Brambillasca et al., 2013; Olivo et al., 2017) reported the chemical composition of citrus pulp obtained by different citrus fruits (orange, lemon, tangerine citrus). Indeed, all these co-products showed low levels of protein, a moderate amount of fiber (varying from 11 to 47% dry matter DM), and a high percentage of non-structural carbohydrates (NSC: from 50 to 68% DM). The chemical composition of these co-products seemed affected more by the production method and by the process carried to increase the co-product shelf-life (drying, pelleting, or addition of salt) than fruit species. In any case, citrus pulp seemed a useful source of energy for ruminates, swine, and source of soluble dietary fiber for companion animals.

The residues of grape, apple, and prickly pear process could be considered sources of structural carbohydrates, with a high amount of NDF (Vastolo et al., 2020; Todaro et al., 2020;Steyn et al., 2018; Chedea et al., 2017; Atalay, 2020; Sato et al., 2020; Marcos et al., 2019; Amer et al, 2019) only partially lignified. Cilev et al. (2016) and Olivo et al. (2017) evidenced in grape residues values of ether extract particularly high (from 10 to 21 % DM). Similarly, Garcìa-Rodrìguez et al. (2019) reported a level of lipids about double in apple pomace compared to the data reported by other authors (Brambillasca et al., 2013; Abdollahzadeh et al., 2010; Paya et al., 2012). In both cases, the authors considered that the higher content of lipids could be related to the higher incidence of seeds into the residual matter.

Mirzaei-Aghsaghali et al. (2011) reported the chemical composition of pomegranate seed and peel. The seed co-product showed a high value of fiber (68.0% DM) and a moderate level of crude protein (15.4% DM). Otherwise, the peel had a high percentage of NSC (69.6% DM). Also, Serrapica et al. (2019) reported similar values for pomegranate seed cake (71.6% of NDF and 14.9% of crude proteins)

Tab 2.1 Fruit co-products chemical composition (% DM)

Co-products	CP	NDF	ADF	ADL	EE	Ash	NSC	References
Citrus *spp.* pulp								
dried orange*	7.95	12.3	10.4	-	3.04	5.22	-	[1]
dried lemon*	8.65	17.7	15.1	-	2.87	5.90	-	[1]
dried grapefruit	9.14	16.7	13.1	-	2.38	5.87	-	[1]
dried lime	8.75	17.5	14.5	-	2.74	8.12	-	[1]
dried tangerine	6.64	11.4	8.48	-	2.64	5.57	-	[1]
dried orange	7.90	22.4	15.3	-	1.80	4.7	-	[2]
dried lemon	6.60	20.9	16.4	-	3.2	5.1	-	[2]
dried lemon	7.60	24.7	17.1	0.30	7.70	-	55.8	[3]
dried orange*	9.50	26.5	17.9	0.80	2.55	-	56.8	[3]
dried clementine	7.30	13.9	9.60	0.20	2.00	-	74.0	[3]
dried citrus	7.12	22.6	13.9	1.21	3.50	4.90	-	[4]
dried citrus	6.00	19.1	14.6	0.80	1.90	-	67.7	[5]
dried citrus	13.3	47.3	30.8	6.53	3.89	14.8	54.2	[6]
fresh citrus	6.10	22.0	16.3	-	-	3.52	-	[7]
pellet citrus	1.47	19.1	14.6	6.84	-		68.2	[8]
Apple								
dried pomace	5.10	67.2	46.0	15.0	6.00	-	20.1	[3]
dried pomace	6.74	44.2	35.4	13.4	3.71	-	43.5	[8]
dried pomace	2.65	34.0	23.8	-	-	1.75	-	[6]
fresh pomace	5.60	45.3	38.0	-	4.70	-	-	[9]

fresh pomace	7.20	43.3	32.3	-	2.90	3.00	-	[2]
				Grape				
fresh residues	12.7	-	-	-	10.6	4.36	-	[10]
residues	9.18	65.2	55.5	13.0	21.5	2.98	-	[7]
dried pomace*	9.93	46.7	41.1	-	5.71	6.69	-	[11]
dried pomace	12.8	-	50.2	-	5.50	6.8	-	[12]
dried pomace	12.4	38.8	37.5	-	4.02	11.8	-	[13]
dried pomace	9.40	28.4	25.0	-	-	-	-	[14]
fresh wine-less	9.30	20.9	9.60	-	0.40	32.2	37.3	[15]
				Pomegranate				
seed	15.4	68.0	49.0	-	0.60	2.40	13.5	[16]
peel	3.6	28.0	15.1	-	0.60	5.40	69.6	[16]
peel	8.4	-	-	-	0.40	4.30	55.4	[17]
pomace	9.2	35.3	30.6	12.3	6.3	3.20	49.9	[18]
seed cake	14.9	71.6	50.5	10.9	0.95	4.10	-	[19]
				Prickly pear				
fruit	7.60	24.70	13.70	11.20	3.50	9.45	-	[20]
peel	7.20	22.10	13.80	8.90	1.90	9.61	-	[20]

*Data reported as mean value. DM: dry matter; CP: crude protein; CF: crude fiber; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; EE: ether extract; NSC. non-structural carbohydrate. [1] (Lashkari & Taghizadeh, 2013); [2]: (Paya et al., 2012); [3]: (García-Rodríguez et al., 2019); [4]: (De Blas et al., 2018); [5]: (Santos et al., 2014); [6]: (Brambillasca et al., 2013); [7]: (Olivo et al., 2017); [8]: (Steyn et al., 2018); [9]: (Abdollahzadeh et al., 2010); [10]: (Cilev et al., 2016); [11]: (Atalay, 2020); [12]: (Chedea et al., 2017); [13]: (Ianni et al., 2019); [14]: (Kolláthová et al., 2020); [15]: (Sato et al., 2020); [16]:]:(Mirzaei-Aghsaghali et al., 2011); [17]: (Kara, 2016); [18]:(Kara et al., 2018); [19]: (Serrapica et al., 2019); [20]: (Amer et al., 2019); [21]: (Ersahince & Kara, 2017); [22]: (Meneses et al., 2020); [23]: (Panwar et al., 2017); [24]: (Aghajanzad-Golshami et al., 2010); [25]: (Abdel-Gawad et al., 2020); [26]: (Klir et al., 2017); [27]: (Castellani et al., 2017); [28]: (Chiofalo et al., 2020); [29]: (Liotta et al., 2019); [30]: (Marcos et al., 2019); [31]: (Woyengo et al., 2016); [32]: (Kroger et al., 2017); [33]: (Kleinhenz et al., 2020); [34]:(Mierliţă, 2018); [35]:(Karlsson et al., 2010); [36]: (Hamid et al., 2021)

Furthermore, the refusals of vegetable (tomato, pepper, broccoli, artichoke, Jerusalem artichoke) transformation were studied by several authors (Ersahince and Kara, 2017; Meneses et al., 2020; Garcìa-Rodrìguez et al., 2019; Abdel-Gawad et al., 2020; Panwar et al., 2017; Aghajanzad-Golshami et al., 2010; Cilev et al., 2016; Abdollahzadeh et al., 2010) and the results of chemical composition are reported in Table 2.2. The chemical composition of this co-product varied considerably in function of the species and the portion of vegetable used, particularly in terms of carbohydrates fractions, crude protein, and lipids contents. Artichoke and pepper skin showed a high percentage of NDF (>20 and 70% DM, respectively). While broccoli and pepper (core, and fresh residue) are mainly rich in protein. Compared to the fruit co-products the vegetable ones are richer in crude protein and NDF, but in some cases (e.g., artichoke, Jerusalem artichoke) also the ADL content is quite high. This last characteristic could negatively affect their digestibility.

Tab 2.2 Vegetables refusal chemical composition (% DM)

Co-products	**CP**	**NDF**	**ADF**	**ADL**	**EE**	**Ash**	**NSC**	**References**
Jerusalem artichoke (*Helianthus tuberosus* L.)								
vegetative	16.36	28.79	27.37	5.65	0.94	13.64	40.25	[21]
early flowering	7.37	39.03	31.70	6.78	1.70	11.7	40.15	[21]
full flowering	7.14	40.63	33.36	7.39	1.77	11.2	39.28	[21]
early seeding	6.59	44.74	36.69	8.82	2.19	8.90	37.56	[21]
Cynara spp								
raw	11.5	43.1	31.1	10.5	2.10	0.57	-	[22]
hay	17.7	67.8	46.7	7.60	4.10	5.20	5.1	[3]
Cardoon cake	21.1	46.8	36.0	6.43	7.72	5.57	-	[19]
Broccoli								
boiled	31.1	20.4	13.1	1.8	3.1	0.632	-	[22]
fresh chaffed	27.20	24.3	21.9	3.30	5.12	5.78	-	[23]
stalk hay	15.5	55.6	34.9	5.4	6.6	12.2	-	[3]

				Pepper				
core	19.2	31.1	22.2	5.50	6.70	-	32.3	[3]
skin	9.90	75.3	64.1	38.3	3.30	5.10	6.40	[3]
fresh residue	18.8	-	-	-	8.18	-	-	[10]
				Tomato				
dried pulp	19.0	55.7	42.7	26.0	5.10	-	16.7	[3]
dried pomace	13.7	-	-	-	0.90	11.1	55.2	[18]
dried pomace	22.2	49.2	32.6	-	15.0	-	6.63	[24]
dried pomace	18.9	45.2	13.4	5.30	4.14	7.56	41.6	[25]
fresh residues	21.1	-	-	-	13.2	3.38	-	[26]
fresh pomace	21.7	57.4	50.7	-	13.4	-	-	[9]

*Data reported as mean value. DM: dry matter; CP: crude protein; CF: crude fiber; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; EE: ether extract; NSC. No-structural carbohydrate. [1]: (Lashkari & Taghizadeh, 2013); [2]: (Paya et al., 2012); [3]: (García-Rodríguez et al., 2019); [4]: (De Blas et al., 2018); [5]: (Santos et al., 2014); [6]: Brambillasca et al., 2013); [7]: (Olivo et al., 2017); [8]: (Steyn et al., 2018); [9]: (Abdollahzadeh et al., 2010); [10]: (Cilev et al., 2016); [11]: (Atalay, 2020); [12]: (Chedea et al., 2017); [13]: (Ianni et al., 2019); [14]: (Kolláthová et al., 2020); [15]: (Sato et al., 2020); [16]:(Mirzaei-Aghsaghali et al., 2011); [17]: (Kara, 2016); [18]:(Kara et al., 2018); [19]: (Serrapica et al., 2019); [20]: (Amer et al., 2019); [21]: (Ersahince & Kara, 2017); [22]: (Meneses et al., 2020); [23]: (Panwar et al., 2017); [24]: (Aghajanzad-Golshami et al., 2010); [25]: (Abdel-Gawad et al., 2020); [26]: (Klir et al., 2017); [27]: (Castellani et al., 2017); [28]: (Chiofalo et al., 2020); [29]: (Liotta et al., 2019); [30]: (Marcos et al., 2019); [31]: (Woyengo et al., 2016); [32]: (Kröger et al., 2017); [33]: (Kleinhenz et al., 2020); [34]:(Mierliţă, 2018); [35]:(Karlsson et al., 2010); [36]: (Hamid et al., 2021)

In Table 2.3 the chemical composition of different refusal of oil, beer, and flour production were reported. The examined data confirmed that the co-products often utilized as ingredients in animal diets, such as soybean meal, beet pulp, are sources of protein and fiber (Santos et al., 2014; Abdel-Gawad et al., 2020; Paya et al., 2012). Additionally, olive cake showed lipid and lignin content higher than 10% DM (Castellani et al., 2017; Chiofalo et al., 2020; Liotta et al., 2019).

Recently, new co-products have been evaluated, such as hemp seed cake, red ginseng and canola meal, cassava foliage, and coffee hulls (Mierliţă et al., 2018, Hamid et al., 2021; Woyengo et al., 2016; Olivo et al., 2017). Hemp seed cake and canola meal had a high content of protein, while cassava foliage and coffee hulls showed a high percentage of NDF (>50% DM). Kleinhenz et al. (2020) reported the chemical composition of extracted flower, leaves, and chaff of hemp. The authors observed in all samples high percentage of protein, fiber, and ash. Hamid et al. (2021) reported that red ginseng meal showed a moderate amount of protein (>15% DM) and NDF (>38% DM).

Serrapica et al. (2019) evaluating the nutritional characteristics of sunflower, tobacco co-products suggested as these residues could be sources of protein. Indeed, all tested co-products reach protein level of 20% DM, except for the pomegranate seed cake that had less than 15% DM of crude protein.

Tab 2.3 Oil, sugar, beer, and flour production refusal and novel co-product chemical composition (% DM)

Co-products	**CP**	**NDF**	**ADF**	**ADL**	**EE**	**Ash**	**NSC**	**References**
				Olive				
trees leave	9.8	52.9	34.5	23.1	4.7	-	16.8	[3]
dried pomace	7.65	58.3	-	-	15.2	-	-	[27]
cake	8.63	49.4	39.4	23.2	30.3	4.09	-	[28]
cake	7.86	41.3	32.5	15.6	27.7	4.25	-	[29]
cake	1.58	56.1	39.9	29.5	10.91	-	-	[30]
cake	1.96	64.7	45.9	22.9	1.80	-	-	[30]
cake	1.73	44.7	31.5	15.6	15.1	-	-	[30]
				Pumpkin				
seed cake	52.9	-	-	-	16.3	8.51	-	[26]
				Sunflower				
cake	19.9	41.1	32.6	10.7	14.0	6.57	-	[19]
				Canola				
meal	36.5	22.82	16.3	-	9.7	7.12	-	[31]
				Soybean				

meal	50.7	13.9	39.6	5.82	13.5	5.03	16.2	[7]
hulls	2.28	63.1	8.20	0.80	2.20	-	26.6	[5]
Brewer's grain								
dried	19.8	55.1	25.2	NA	8.0	-	13.1	[24]
fresh	19.5	21.9	12.9	-	7.8	4.9	-	[2]
Beet								
dried pulp	9.65	41.7	27.9	0.91	2.57	3.74	66.4	[25]
pulp	27.4	13.5	4.00	1.50	11.1	5.20	43.2	[32]
Hemp								
extracted flower	24.5	30.9	18.1	-	3.2	25.7	4.7	[33]
leaves	13.0	44.7	20.8	-	8.9	21.2	5.9	[33]
chaff	21.2	27.9	18.0	-	4.6	24.9	6.3	[33]
seed cake	33.4	43.6	36.2	-	11.7	-	-	[34]
seed cake	34.4	39.3	32.1	-	12.4	6.7	-	[35]
seed cake	30.0	51.9	39.5	6.31	10.2	7.7	-	[19]
Coffee								
hulls	0.78	36.9	31.9	10.4	14.4	6.2	41.9	[7]
Cassava								
foliage	2.13	57.2	48.4	14.4	7.88	6.65	21.4	[7]
Red ginseng								
meal	15.0	38.6	31.6	-	1.22	3.11	42.0	[36]
Tobacco								
cake	37.0	46.7	34.7	10.9	12.0	5.65	-	[19]

*Data reported as mean value. DM: dry matter; CP: crude protein; CF: crude fiber; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; EE: ether extract; NSC. No-structural carbohydrate. [1]: (Lashkari & Taghizadeh, 2013); [2]: (Paya et al., 2012); [3]: (García-Rodríguez et al., 2019); [4]: (De Blas et al., 2018); [5]: (Santos et al., 2014); [6]: Brambillasca et al., 2013); [7]: (Olivo et al., 2017); [8]: (Steyn et al., 2018); [9]: (Abdollahzadeh et al., 2010); [10]: (Cilev et al., 2016); [11]: (Atalay, 2020); [12]: (Chedea et al., 2017); [13]: (Ianni et al., 2019); [14]: (Kolláthová et al., 2020); [15]: (Sato et al., 2020); [16]:(Mirzaei-Aghsaghali et al., 2011); [17]: (Kara, 2016); [18]:(Kara et al., 2018); [19]: (Serrapica et al., 2019); [20]: (Amer et al., 2019); [21]: (Ersahince and Kara, 2017); [22]: (Meneses et al., 2020); [23]: (Panwar et al., 2017); [24]: (Aghajanzad-Golshami et al., 2010); [25]: (Abdel-Gawad et al., 2020); [26]: (Klir et al., 2017); [27]: (Castellani et al., 2017); [28]: (Chiofalo et al., 2020); [29]: (Liotta et al., 2019); [30]: (Marcos et al., 2019); [31]: (Woyengo et al., 2016); [32]: (Kröger et al., 2017); [33]: (Kleinhenz et al., 2020); [34]: (Mierliţă, 2018); [35]: (Karlsson et al., 2010); [36]: (Hamid et al., 2021)

The results demonstrate that the co-products could be useful sources of different nutrients. Furthermore, different parts of the co-product can provide different elements. For instance, as in the case of hemp, whose plant areal part residues are sources of structural carbohydrates while the residues from the seeds could be sources of protein. In any case high variability has been observed in several samples, which could be due to different reason such as botanical variety, processing, and preservation methods.

2.5 *In vitro* studies

Totally twenty-five *in vitro* manuscripts described the *in vitro* fermentation parameters of co-products that have potential use in large and small ruminant, and monogastric animal nutrition (Tables 2.4, 2.5, and 2.6, respectively).

The *in vitro* research performed using ruminal liquor of large ruminant (Table 2.4) were carried out according to several methods. The most used protocols were the two-step system for digestibility suggested by Tilley and Terry (1963) and the method to measure gas production with glass syringes described by Menke and Steingass (1988). In general, total gas production, and volatile fatty acids (VFA) were reported and, sometimes, methane production has been evaluated.

In particular, Mirzaei-Aghsaghali et al. (2011) studied *in vitro* parameters (organic matter digestibility, total gas production, and volatile fatty acids), and estimated the nutritive value of pomegranate (*Punica granatum* L.) seeds and peel. The latter showed higher nutritive value, and total gas production compared to seeds. Similarly, Kara et al. (2018) incubated a total mix ratio (TMR: maize silage, wheat straw, alfalfa hay, and concentrate) supplemented with 10 and 20% of pomegranate and apple pomaces silage (PPS and APS) and their mix (PAPS, 50 and 50%, respectively) to evaluate silage quality and *in vitro* parameters. Regarding the silage quality, PPS was significantly lower than APS for dry matter losses (19.78 vs. 27.37% DM, respectively), while the lactic acid concentration and pH value in APS and PAPS were significantly higher than PPS. Concerning *in vitro* parameters, both concentrations (10 vs. 20%) of PPS in the total mix ratio caused a reduction of total gas production, while did not affect methane production. Substitution of APS or PAPS in dairy cow TMR instead of other forages did not negatively affect *in vitro* digestibility. In this regard, the results obtained by both studies demonstrate as pomegranate co-products fresh or ensiled could be good resources for ruminant nutrition.

Ersahince and Kara (2017) studied the nutrient composition and *in vitro* digestion parameters of Jerusalem artichoke (*Helianthus tuberosus* L.) herbage at different maturity stages (vegetative, early flowering, full flowering, and early seeding) to evaluate its potential use as forage. In this regard, a significant difference between the vegetative and the early seed stages in terms of gas production was observed. The *in vitro* ruminal methane production at 24 h was significantly higher for the most precocious stages (vegetative: 39.82 vs. early flowering: 30.31, ml g^{-1} DM). Moreover, *in vitro* dry, and organic matter disappearance and metabolizable energy values significantly decreased with plant maturation. The *in vitro* microbial crude proteins produced at 24 h of incubation in early and full flowering stages were higher than those of vegetative and early seeding stages (122 and 116 vs. 72 and 95 mg g^{-1} DM, respectively). The amount of acetate, propionate, butyrate, and total VFA decreased with plant maturation. The authors concluded that artichoke co-products, especially at the vegetative stage (stem length <100 cm; no buds or flowers; green leaves) could be suitable alternative forage in terms of high nutrient composition and satisfactory digestion values in ruminants.

Kleinhenz et al. (2020) characterized the nutrient concentration, digestibility, and cannabinoid concentration of hemp (*Cannabis sativa* containing <0.2% Δ-9 THCA) plant and co-products (stalks remaining after seed harvesting, unprocessed female flowers intended for cannabinoid extraction, whole seed heads for seed production, leaves obtained from the drying process, chaff obtained after seed harvesting and cleaning, and processed female flowers after cannabinoid extraction). From the results emerged as the nutrient concentration and fiber digestibility varied in function of the tested plant portion. Regarding the NDF digestibility and *in vitro* rumen undigestible NDF at 240 h (uNDF), whole plant and plant parts are relatively indigestible while the seed heads, chaff, and leaves had the lowest uNDF. Besides, these last showed higher digestibility amounts comparable to corn stalks, oat, or barley straw. The authors concluded that the hemp plant, despite the great fat concentration, could be an interesting source of fiber for ruminants.

Hamid et al. (2021) suggested red ginseng (*Panax ginseng* C.A. Meyer) co-products as protein resource in ruminants. The authors investigated *in vitro* effects incubation on rumen fermentation characteristics, microflora population, CO_2, and CH_4 production after 48 h of. Indeed, after the soluble substances have been extracted with water or alcoholic solution (70–75%), a residue, known as ginseng meal, is formed. The authors compared the red ginseng meal with three conventional co-products (corn gluten feed, wheat gluten, and corn germ meal). The results obtained evidenced as red ginseng residue can be used as an alternative to conventional protein co-products in ruminants' diets. However, this co-product showed the lowest *in vitro* crude protein digestibility compared to other co-products (83.72 vs. 94.55, 93.57, 90.20 % CP, respectively). Additionally, ginseng co-product resulted in the lowest NH_3-N production (15.54 vs. 36.05, 34.09, and 22.58 mg/100 ml, respectively). Moreover, it is interesting the ginseng co-product ability in decreasing CH_4 emissions without affecting the rumen fermentation characteristics thanks to its bioactive phenolic compounds (i.e., saponins, phenols, peptides, polysaccharides, alkaloids, lignans, and polyacetylenes) able to modulate microbial behaviour. In this regard, Serrapica et al. (2019) characterized oilseed cakes from cardoon, hemp, tobacco, and pomegranate by chemical composition and *in vitro* digestibility of dry matter, neutral detergent fiber, and crude protein. The authors observed that the cakes residual after oil extraction from cardoon, hemp, and tobacco seeds may be potentially used as protein feeds for ruminants. Concerning the *in vitro* digestibility, the highest value of rumen undegradable protein has been observed for pomegranate and hemp. Moreover, hempseed cake showed the highest value of intestinal digested protein. The rumen degradability of dry matter and especially of NDF of samples were quite low, particularly for pomegranate cake. The highest value of *in vitro* NDF degradability was detected for the tobacco cake samples. In the same way, the study proposed by Olivo et al. (2017) determined the chemical composition, *in vitro* digestibility of dry matter, crude protein, and NDF, and gas production of eight different types of agro-industrial co-products and conventional feed (coffee hulls, pelleted citrus pulp, grape residue, soybean hulls, cottonseed, cassava foliage, corn silage, and ground corn concentrate).

Pelleted citrus pulp and ground corn grain presented the highest levels of *in vitro* dry matter digestibility (DIVDM: 95.33 and 94.76% DM, respectively). These values were higher than the soybean hulls and the coffee hulls (83.44 and 80.73% DM, respectively), followed by corn silage (72.67% DM). The lowest values of DIVDM were found for samples of cassava foliage and grape residue (53.17 and 51.24 % DM, respectively). Corn silage produced a larger volume of gas from the fast degradation fraction compared to the co-products and corn concentrate. The chemical composition, mainly lipid, pectin, and NDF content affected *in vitro* characteristics. The authors concluded that compared to common feeds, it is possible to use these co-products as a substitute for feed as a source of energy in the ruminants' diets.

Sato et al. (2020) investigated the usability of wine lees as feed for ruminants in fattening conditions by *in vitro* trials. Four treatments were prepared: 100% DM rolled barley as a control (WL0) and replaced 7.5, 15.0, and 22.5% DM of wine lees as three treatments (WL7.5, WL15, WL22.5, respectively). A linear decrease in gas production was observed at 48 h of incubation with an increase in the percentage of wine lees in the substrate. The dry matter and crude protein digestibility were linearly decreased with WL inclusion, while NDF expressed exclusive of residual ash (NDFom) digestibility was linearly increased. The polyphenolic contents and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability of fermented residue were linearly increased with WL inclusion. Furthermore, the proportion of α-linolenic acid and total polyunsaturated fatty acids (PUFA) in the residues after incubation was linearly increased with wine lees inclusion. The authors observed as the wine lees substituted for the fattening ration up to 20% DM had no adverse effects on apparent digestibility and ruminal fermentation. Although the gas production, dry matter, and crude protein digestibility were decreased, wine lees inclusion protected PUFA from ruminal biohydrogenation during ruminal fermentation. Thus, WL has the potential to be an important alternative as a partial substitute for antioxidant supplements.

Tab 2.4. In vitro studies with ruminal liquor of large ruminants

Co-products	**Method**	**Species**	**Authors**
pomegranate	(Menke and Steingass, 1988)	dairy cows	(Kara et al., 2018)
pomace	(Menke and Steingass, 1988)	dairy cows	(Kara et al., 2018)
apple pomace	(Menke and Steingass, 1988)	dairy cows	(Kara et al., 2018)
pomegranate	(Menke and Steingass, 1988)	steers	(Mirzaei-Aghsaghali et al., 2011)
Jerusalem artichoke	(Menke and Steingass, 1988)	beef cattle	(Ersahince & Kara, 2017)
hemp co-products	(Goeser et al., 2009)	ruminant	(Kleinhenz et al., 2020)
red ginseng co-product	(Theodorou et al., 1994)	cattle	(Hamid et al., 2021)

coffee hulls	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
pelleted citrus pulp	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
grape residue	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
soybean hulls	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
cottonseed	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
cassava foliage	(Tilley and Terry, 1963)	dairy cow	(Olivo et al., 2017)
wine lees	(Tilley and Terry, 1963)	beef cattle	(Sato et al., 2020)
pomegranate cake	(Robinson et al., 1999)	bulls	(Serrapica et al., 2019)
tobacco cake	(Robinson et al., 1999)	bulls	(Serrapica et al., 2019)
cardoon cake	(Robinson et al., 1999)	bulls	(Serrapica et al., 2019)
hemp cake	(Robinson et al., 1999)	bulls	(Serrapica et al., 2019)

Table 2.5 showed a selection of *in vitro* studies carried out with ruminal liquor of small ruminants. Lashkari and Taghizadeh (2013) measured the proportion of protein and carbohydrate fractionations, ruminal, post-ruminal, and total tract protein disappearance rates, *in vitro* dry matter digestibility, apparent and true rumen digestibility, metabolizable energy, and digestible organic matter of citrus co-products (pulps from fresh orange, lime, lemon, grapefruit, sweet lemon, bitter lemon, bergamot orange, and tangerine) using a modified three-step method (Holden, 1999). Protein fractions and acid detergent insoluble nitrogen were the highest in grapefruit pulp; ruminal crude protein disappearance was the highest in orange pulp

(71.89 % CP); the level of post-ruminal crude protein disappearance was the highest for lemon pulp (45.95% CP). The highest *in vitro* dry matter digestibility was found for tangerine pulp followed by that one of bergamot pulp (80.44 and 78.38 % OM, respectively). The estimated metabolizable energy (MJ/kg DM) varied from 9.77 for lime pulp to 12.91 for bergamot pulp. This study showed that the crude protein content of citrus co-products is potentially highly digestible. Additionally, it can be concluded that citrus co-products can be introduced as non-forage fiber sources that can release high metabolizable energy and high digestible organic matter. The same authors (Lashkari and Taghizadeh, 2015) determined digestibility and fermentation characteristics of whole, NDF, and ADF fractions of four citrus co-products using *in vitro* gas production. Pulps from orange, lime, lemon, and grapefruit presented a potential appropriate as source of degradable carbohydrate fractions which may be suitable as the energy source for ruminant nutrition. Similarly, Paya et al. (2012) compared fresh brewers' grain, apple pomace and dried orange, and lemon pulps co-product using *in vitro* e *in situ* technique. Potential gas production and rates of gas production differed among feedstuffs. Compared to the other feeds, apple pomace showed the highest potential gas production (364 ml g^{-1} DM), whereas lemon pulp had the lowest (220 ml g^{-1} DM), while orange pulp had a higher fermentation rate. The metabolizable energy (ME) values ranged from 7.66 to 10.83 MJ kg^{-1} DM in lemon pulp and apple pomace. Regarding *in situ* technique, orange pulp had higher soluble dry matter, while lemon pulp had higher insoluble potentially degradable dry matter (89.8% DM), and orange pulp presented a higher degradation rate than other feeds (36.9%). The authors concluded that all tested co-product can be economically employed as potential fibrous and energy sources in ruminant nutrition, even if, apple pomace seemed the best one.

Atalay (2020) evaluated the chemical composition and anti-methanogenic potential of eight different samples of grape pomace deriving from wine production around the world, which differ in the function of grape type, wine production method, and ratios of pomace components. The results showed that there is a considerable amount of variation among the grape pomace samples in terms of chemical composition, *in vitro* gas production, CH_4 production, ME, and organic matter digestibility. However, many samples had CH_4 mitigation potential possibly associated with the EE, condensed tannins, lignin, and tartaric acid contents and could be included in ruminant diets to mitigate the environmental impact.

Comparing brewers grain and tomato pomace (*Lycopersicon esculentum* Mill.) by *in vitro* technique at 24 h of incubation, Aghajanzad-Golshami et al. (2010) observed as the estimated organic matter digestibility (64.4 vs. 52.7 % OM, respectively), VFA (0.86 vs. 0.69 mmol, respectively), ME (11.8 vs. 9.05 MJ kg^{-1}DM) were higher for tomato pomace than brewers grain. Overall, the authors did not exclude the use of both co-products as fibrous and protein sources, respectively, in animal diets. On this subject, Romero-Huelva et al. (2013) evaluated the *in vitro* inclusion of two agricultural co-products typical of the Mediterranean area, tomato, and cucumber, in a ruminant diet. The results obtained by the authors showed that both co-products and their mix could represent an interesting low-cost strategy to replace concentrate in the diet. Moreover, it was observed as tomato and cucumber could have the potential to reduce some methanogen activities, unaffecting VFA production.

Regarding, co-products of oil extraction, Marcos et al. (2019) determined the variability in the chemical composition and *in vitro* ruminal fermentation deriving from oil production. Forty-two olive cake samples with different storage times (1-14 months) and processing (crude, exhausted, and cyclone) were evaluated. The exhausted ones had the greatest average gas production rate, whereas the greatest fermented organic matter was obtained for exhausted and cyclone. In this regard, Pallara et al. (2014) evidenced as supplementation of diets with stone olive pomace alters the rumen bacterial community, modifying the fatty acids profile of the rumen liquor. Hence, the authors suggested that use of olive co-products aimed to produce meat or dairy products enriched in functional lipids (essential fatty acids) can be hypothesised.

Concerning vegetable co-products, Meneses et al. (2020) evaluated chemical composition, nutritive characteristics, *in vitro* rumen degradability, *in vivo* digestibility, and phytosanitary residue contents of raw artichoke (*Cynara scolymus* L.), which correspond to whole outside bracts and stems leftover in the first stage of industrial process, and boiled broccoli (*Brassica oleracea*, var. *Italica*) that do not pass the quality control after washing and scalding at 90°C for 20 min. The authors evidenced that both ensiled wastes of processed artichoke and broccoli were adequate for feeding ruminant animals thanks to their nutritive value. The *in vitro* rumen dry matter disappearance at 72 h was high, although it was higher in the broccoli silage. The authors concluded that silage could be a suitable method to preserve both co-products.

García-Rodríguez et al. (2019) determined the chemical composition, *in vitro* digestibility, and fermentation kinetics of a wide range of agro-industrial co-products as an indicator of their potential use as feedstuffs for ruminants: dehydrated or ensiled sugar beet pulp, sugar beet tops (including leaves), beetroot leftovers (including rootlets, hairs, root tips, and beet tails), grape seeds, olive tree leaves, almond hulls, broccoli stalk (hay), lettuce leaves, asparagus rinds, green bean haulms, artichoke co-products, peas haulms, broad beans haulms, dried tomato pulp, pepper cores and skin, apple pomace, citrus pulps (lemon, clementine, and orange). As expected, chemical composition, *in vitro* digestibility, and fermentation kinetics varied largely among the co-products. Olive tree leaves, pepper skins, and grape seeds were less degradable, whereas sugar beet, orange, lemon, and clementine pulps were more degradable. Considering the large variability among co-products, most of them can be deemed as potential ingredients in ruminant diets. Depending on the characteristic nutritive value of each co-product, these feedstuffs can provide alternative sources of energy (e.g., citrus pulps), protein (e.g., asparagus rinds), soluble fiber (e.g., sugar beet pulp), or less digestible roughage (e.g., grape seeds or pepper skin).

Tab 2.5. In vitro studies with small ruminant liquor

Co-products	**Method**	**Species**	**Authors**
citrus pulps	(Holden, 1999)	sheep	(Lashkari & Taghizadeh, 2013)
citrus co-products	(Fedorak and Hurdy, 1983)	sheep	(Lashkari & Taghizadeh, 2015)
brewers' grain	(Orskov and Mcdonald, 1979; Fedorak and Hurdy, 1983)	sheep	(Paya et al., 2012)

apple pomace	(Orskov and Mcdonald, 1979; Fedorak and Hurdy, 1983)	sheep	(Paya et al., 2012)
orange pulp	(Orskov and Mcdonald, 1979; Fedorak and Hurdy, 1983)	sheep	(Paya et al., 2012)
lemon pulp	(Orskov and Mcdonald, 1979; Fedorak and Hurdy, 1983)	sheep	(Paya et al., 2012)
olive cake	(Goering and Van Soest, 1975)	sheep	(Marcos et al., 2019)
olive cake	(Pell and Schofield, 1993)	sheep	(Pallara et al., 2014)
artichoke	(Goering and Van Soest, 1975)	goat	(Meneses et al., 2020)
broccoli	(Goering and Van Soest, 1975)	goat	(Meneses et al., 2020)
grape pomace	(Menke et al., 1979)	sheep	(Atalay, 2020)
tomato pomace	(Menke et al., 1979)	rams	(Aghajanzad-Golshami et al., 2010)
brewers grain	(Menke et al., 1979)	rams	(Aghajanzad-Golshami et al., 2010)
sugar beet	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
grape	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
olive tree	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)

almond	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
broccoli	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
lettuce	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
asparagus	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
green bean	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
artichoke	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
peas	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
broad beans	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
tomato	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
pepper	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
apple pomace	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
citrus pomace	(Goering and Van Soest, 1975)	sheep	(García-Rodríguez et al., 2019)
tomato	(Tilley and Terry, 1963)	goat	(Romero-Huelva et al., 2013)
cucumber	(Tilley and Terry, 1963)	goat	(Romero-Huelva et al., 2013)

Table 2.6 showed the *in vitro* studies on co-products that referred to monogastric species. De Blas et al. (2018) evaluated the use of citrus co-products in diets for rabbits as a source of energy. The authors analysed 33 samples of citrus pulp obtained from commercial industries using different manufacturing procedures for chemical composition (including dietary fiber), *in vitro* dry matter, and crude protein digestibility by two-step method (Ramos et al., 1992). Overall, citrus pulps can be considered a good source of energy and soluble fiber for rabbits, according to their analytical composition; in particular, the low supply of indigestible fiber and crude protein and the high level of calcium ($Ca(OH)_2$). Moreover, Kara (2016) studied both common (sugar beet pulp, wheat bran, lucerne meal) and uncommon (tomato pomace, maize bran, rice bran, lentil bran, and pomegranate peel) feed comparing nutrients, condensed tannins, fiber, and *in vitro* fermentation parameters using rabbit faecal *inoculum*. The author observed that tomato pomace, maize bran, and rice bran could be recommended for use as alternative dietary fiber sources for post-weaning, young, and breeding rabbits. Although tomato pomace showed excessive fermentation capacity, its dietary fiber content was low. On the other hand, pomegranate pomace and lentil bran can be recommended for the growing rabbit due to their high fiber content and low fermentation capacity.

Regarding the use of co-products in companion animal diets, Brambillasca et al. (2013) studied *in vitro* fermentation characteristics kinetics, and *in vivo* nutrient digestion, faecal characteristics, and bacterial populations of dogs food mixed with citrus pulp and apple pomace. In the trial, a pre-digested dog food supplemented with both fiber source in four different inclusion levels (0, 30, 50, and 70 g/kg on DM basis) were tested. As result, citrus pulp and apple pomace included in a dog diet enhanced the fermentation activity in the hindgut. Apple pomace showed the highest maximal fermentation rate of gas production (R_{max}:14.08 ml/h) reached in the shortest time ($T_{1/2}$: 5.46 h). Furthermore, gas production improved linearly and quadratically as fiber levels increased. The results evidenced as citrus pulp and apple pomace included in a dog diet could enhance the fermentation activity in the hindgut. Calabrò et al. (2013) tested *in vitro* several samples including some co-products (sugar-cane fiber, beet pulp, wheat bran, and pea hulls) to evaluate their inclusion in dogs' diets. The samples were incubated with the dog's faecal *inoculum* for 48h at 39°C under anaerobic conditions testing the gas production and fermentation kinetics (Theodorou et al., 1994). Moreover, VFA have been determined at the end of *in vitro* trial. Considering the tested co-products, the results showed as pea hull and beet pulps produced a moderate amount of organic matter disappearance and VFA. While wheat bran had a rapid fermentation and produced a high proportion of butyrate. Otherwise, the sugar cane fiber produced a low quantity of gas and VFA. The authors observed that the tested co-products could be included in dogs' diets as sources of soluble and insoluble dietary fiber. On this basis, Panasevich et al. (2013) indicated potato fiber, which is a co-product of potato starch manufacture, as a high-quality fiber source in dog foods. The authors evaluated this co-product for chemical composition, *in vitro* digestion and fermentation characteristics, and *in vivo* responses. For the *in vitro* digestion, the substrates were first subjected to hydrolytic-enzymatic digestion to determine organic matter disappearance and subsequently fermented using dog faecal *inoculum*, measuring the fermentation after 0, 3, 6, 9, and 12 h. When tested *in vitro*, the potato fiber resulted in high fermentability with a favourable proportion of insoluble to soluble fiber. In particular, acetate, propionate, butyrate, and total VFA concentrations increased over time.

As sources of amino-acids and energy, Woyengo et al. (2016) determined in the *in vitro* fermentation characteristics of the canola (*Brassica juncea* and *napus*) co-products (solvent-extracted meal, expeller-pressed meal, cold-pressed cake) in comparison with soybean meal in pig feeds. The authors observed as canola co-products can replace the soybean meal as a source of protein in pigs' diet. However, differences in fermentability among *B. napus* co-products indicate that fat may limit their fermentability in the hindgut of pigs. Hence, fermentation characteristics can vary depending on the efficiency of oil extraction.

Ersahince and Kara (2017), as previously illustrated for beef cattle, demonstrated that also in horses Jerusalem artichoke (*Helianthus tuberosus* L.) herbage, especially at the vegetative stage, could be useful as forage with high/moderate nutrient characteristics and satisfactory digestion values.

All presented *in vitro* studies demonstrate as laboratory techniques highlight the main nutritional characteristics of numerous co-products and suggest their potential use for animal nutrition as fiber, protein, or lipids sources. That evidence showed as co-products can improve the fermentative characteristics such as dry matter digestibility and can reduce methane emission. Moreover, *in vitro* experiments are cost-effective and short time-consuming methods that allow to verify the characteristics of new co-products or test the quality of conservation methods on nutritional characteristics

Tab 2.6. In vitro studies with monogastric faecal inoculum

Co-products	**Method**	**Species**	**Authors**
citrus pulp	(Ramos et al., 1992)	rabbit	(De Blas et al., 2018)
tomato pomace	(Menke et al., 1979)	rabbit	(Kara, 2016
maize bran	(Menke et al., 1979)	rabbit	(Kara, 2016
rice bran	(Menke et al., 1979)	rabbit	(Kara, 2016
lentil bran	(Menke et al., 1979)	rabbit	(Kara, 2016
pomegranate peel	(Menke et al., 1979)	rabbit	(Kara, 2016)
citrus pulp apple pomace	(Theodorou et al., 1994).	dog	(Brambillasca et al., 2013)
canola co-products	(Menke and Steingass, 1988)	pig	(Woyengo et al., 2016)
Jerusalem artichoke	(Menke and Steingass, 1988)	horse	(Ersahince & Kara, 2017)
Beet pulp	(Theodorou et al., 1994)	dog	(Calabrò et al., 2013)
Sugar cane fiber	(Theodorou et al., 1994)	dog	(Calabrò et al., 2013)
Potato fiber	(Bourquin et al., 1991)	dog	(Panasevich et al., 2013)

2.6 *In vivo* studies

For this review, a total of 32 *in vivo* studies were selected so as to evaluate the potential use of some agricultural and industrial co-products; most of them evaluated the effect on animal health and the quality of animal products.

Table 2.7 reports the studies about large ruminant species. Many of the research concerns the integration of co-products into the diet of dairy cows. Chedea et al. (2017) evaluated the effect of grape pomace supplementation (3 kg head^{-1} day^{-1}) on general health status and the milk quality of dairy cows. The authors observed as dietary replacement of cereal with grape pomace did not affect dairy cows' plasma biochemistry parameters, except for urea concentration, which increased significantly with grape pomace (7.00 vs. 8.20 mg dl^{-1}, respectively). However, the inclusion of grape pomace did not affect milk fat and protein levels. Moreover, the significantly higher values of ß-lactoglobulin and lactose give the milk the properties of a functional food, particularly with respect to the biological activities of ß-lactoglobulin. Similarly, Steyn et al. (2018) studied the effect of replacing ground maize with dried apple pomace on milk yield and rumen health of Jersey cows, evaluating four levels of inclusion (0, 25, 50, and 75%). As in the previous study, the authors did not observe any changes in milk composition in terms of protein and fat. On the contrary, as demonstrated by Castellani et al. (2017), the utilization of dried olive pomace in the diet of lactating dairy cows may modify the quality of dairy products. Indeed, olive co-products could increase unsaturated fatty acid (oleic acid, vaccenic acid, and conjugated linoleic acid CLA) and decrease saturated fatty acid (short- and medium-chain fatty acids until palmitic acid) suggesting a positive role of dried olive pomace to improve the nutritional and nutraceutical properties of milk and corresponding cheese. Additionally, Santos et al. (2014) observed as pelleted citrus pulp can be used as a corn grain substitute in dairy cows' diet without negative effect on the nutritional value of total mixed ratios, milk yield, and quality. Indeed, the addition of pelleted citrus pulp (9 and 18%) to standard diets increased the total polyphenol and flavonoid concentration, and ferric reduction antioxidant power in milk from cows fed high PUFA.

Abdollahzadeh et al. (2010) have used an ensiled mix of tomato and apple pomace (in ratio of 50:50) replacing alfalfa hay to evaluate Holstein dairy cows' performance. Three diets with different levels of replacement (0, 15, and 30% DM) were used. The results showed significant differences between diets in terms of milk yield (19.9, 21.9, 20.4 kg/d, for 0, 15, and 30%, respectively), dry matter intake (21.3, 23.7, 24.5 kg/d, respectively); feed efficiency (0.93, 0.92, 0.82, respectively). In this regard, the nutritional value of tomato and apple pomace improved when the co-products were used together; moreover, a mix of tomato and apple pomace silage can be substituted efficiently up to 30% of the diet without any adverse effect on dairy cows' performance.

Karlsson et al. (2010) studied the use of hemp seed cake as protein supplement in dairy cow diets, evaluating the effects on milk yield, and composition. Four experimental diets formulated with different inclusion of hemp seed cake (HC0: 0, HC14: 143, HC23: 233, or HC32: 318 g/kg of DM) were tested. No effects in dry matter intake but significant linear increases in crude protein, fat, and NDF intakes were observed with the increase of the proportion of hemp seed cake in the diets. Milk yield and energy-corrected milk were affected by the proportion of dietary levels, with the highest value for the lowest inclusion (28.7 kg/d and 1.13 yield/intake, respectively, with 143g/kg of DM of hemp seed cake). Moreover, with the increasing of hemp seed cake level the milk protein (HC0: 3.63, HC14: 3.61, HC23: 3.49, HC32: 3.40%) and fat (HC0: 4.31, HC14: 4.21, HC23: 4.07, HC32: 3.89%) linearly decreased. Furthermore, due to the increase of crude protein intake, a significant linear increase in milk urea concentrations (HC0: 2.7, HC14: 3.7, HC23: 4.4, HC32: 5.1%) occurs with the enhancement of hemp seed cake. In conclusion, the optimal and maximum level of hemp seed cake inclusion suggested in this experiment was 143 g/kg DM.

Regarding buffalo's species, Abdel Gawad et al. (2020) observed that the use of tomato pomace, citrus, and beet pulp to replace wheat bran as a concentrate in animal diet had a positive effect on animal performance and fat milk. Moreover, tomato pomace silage in lactating Egyptian buffalos' diet could be substitute clover as forage having a positive effect on nutrients digestibility; besides, milk production and its fat percentage increased (Klir, 2014).

Only two studies that evaluated the effect of co-products on calves' performance and meat quality were found. In particular, Ianni et al. (2019) demonstrated that grape pomace inclusion in calves diet resulted in a significant increase of linoleic acid concentration in meat, a condition predisposing to a positive increase in the polyunsaturated to saturated fattyacids ratio (PUFA/SFA). Furthermore, an interesting improvement in the oxidative stability of meat samples was evidenced.

Chiofalo et al. (2020) evaluated the inclusion of olive cake in partial substitution of cereals in the beef cattle diet. The levels of 7 and 15% on dry matter basis increased the body weight, average daily gain, slaughter traits, and intramuscular fat content probably due to lipids amount, and quality, coming from the olive cake.

Tab 2.7. In vivo studies on large ruminant

Co-products	**Species**	**Authors**
grape pomace	dairy cow	(Chedea et al., 2017)
apple pomace	dairy cow	(Steyn et al., 2018)
olive pomace	dairy cow	(Castellani et al., 2017)
citrus pulp	dairy cow	(Santos et al., 2014)
tomato and apple	dairy cow	(Abdollahzadeh et al., 2010)
hemp seed cake	dairy cow	(Karlsson et al., 2010)
grape pomace	beef cattle	(Ianni et al., 2019)
olive cake	beef cattle	(Chiofalo et al., 2020)
tomato pomace	buffaloes	(Abdel Gawad et al., 2020)
beet pulp	buffaloes	(Abdel Gawad et al., 2020)
citrus pulp	buffaloes	(Abdel Gawad et al., 2020)
tomato pomace	buffaloes	(Ebeid et al., 2014)

Regarding *in vivo* studies on small ruminants (Table 2.8) Antunović et al. (2018) and Klir et al. (2017) evaluated the use of pumpkin seed cake in lamb and goat, respectively. In both studies, the authors aimed to replace soybean meal with pumpkin seed cake. Antunović et al. (2018) concluded that the partial replacement of soybean meal with pumpkin seed cake promotes adequate carcass characteristics, and it is feasible to changes in haemato-chemical parameters of Merinolandschaf lambs’ blood in organic farming. Klir et al. (2017) suggested substituting completely soybean meal with pumpkin seed cake for dairy Alpine goats because no decrease in milk yield or sharp changes in fatty acid profile were observed. Meneses et al. (2020), in the same study above mentioned as *in vitro* trial, evidenced *in vivo* that ensiled raw artichoke (*Cynara scolymus* L.) and boiled broccoli (*Brassica oleracea*, var. *Italica*) co-products were suitable for ruminant diet. No differences were observed i*n vivo* for DM digestibility in both silages. However, crude protein digestibility was higher in the boiled broccoli silage than raw artichoke (83.0 vs. 55.1% DM, respectively). A similar result was observed for NDF digestibility (88.2 and 78.8% DM, for broccoli and artichoke, respectively). Furthermore, broccoli crop residue can successfully sustain the growth of small ruminants, especially goats without supplementing concentrate mix and in general can replace some of the soybean and corn in the ratio of animal diets (Panwar et al., 2017).

Nudda et al. (2015) and Sato et al. (2020) tested the use of grape co-products in ewes, and wethers, respectively. In both studies, the authors concluded that grape co-products can be included in the animals’ diet without affecting production or nutrient apparent digestibility, ruminal fermentation, and nitrogen balance. Buffa et al. (2020) evaluated the use of small quantities of dried co-products (e.g., tomato pomace, grape marc, and exhausted myrtle berries) on rumen fermentation parameters, and microbiota in dairy ewes. Definitively, the use of these co-products at low dosage did not evidence any negative effects on rumen bacteria or animal performance.

The use of a mixture of different co-products in the goat diet could be also useful for environmental sustainability, as demonstrated by Romero-Huelva et al. (2013). Indeed, the authors verified as the replacement of 47% of conventional ingredients in a concentrate for lactating goats with a mixture of tomato fruits, citrus pulp, brewer’s grain, and brewer’s yeast reduced animal feeding costs and methane emissions. Furthermore, the quality of milk in terms of fatty acids profile improved whereas ruminal fermentation, nutrient digestibility, and milk yield were not compromised. Besides, Arco-Pérez et al. (2017) noted as replacing oats hay in the diet of lactating goats with silages made of tomatoes or olive oil co-products together with sunflower oil supplementation (20 g/kg of DM) improved milk quality without affecting animal efficiency. Additionally, the long-term use of tomato silage in dairy goats fostered voluntary intake, which resulted in higher body weight gain, without compromising the milk production and composition.

Mierliţă (2018) studied the effect of dietary intake of hemp seeds or cake on milk production, fatty acid profile, and milk oxidative stability. In particular, the author carried out an experiment using 30 Turcana dairy sheep divided into three groups. The three diets consisting of a control one based on hay and mixed concentrates and two experimental diets designed to provide the same amount of fat using hemp seed (180 g/d) or cake (480 g/d). Both hemp co-products compared to the control diet increased milk yield and ewes' milk fat content, and decreased milk lactose; moreover, the PUFA content (especially n-3 fatty acids) increased, and the n-6/n-3 ratio improved. Total CLA content doubled in the milk of the ewes that received hemp seed and increased over 2.4 times with the hemp cake inclusion. The alpha-tocopherol and antioxidant activity increased using hemp seed in the diets, reducing the risk of lipid oxidation in raw milk.

Concerning the *in vivo* studies using co-products in monogastric species (Table 2.9), Amer et al. (2019) tested prickly pears (*Opuntia ficus-Indica* L. Mill.) co-products in rabbits'diets. In particular, the study was carried out to evaluate the effects of different levels (25 and 50% of DM) of dietary substitution of barley by prickly pear fruits and peel as alternative feed resources and antioxidants on growth performance, carcass traits, and antioxidant status of weaned male New Zealand White rabbits. As result, the inclusion of both co-products in the two concentrations had positive effects on rabbits' performance. Prickly pear fruits and peel are excellent sources of dietary antioxidants components that may have beneficial effects on rabbits' health.

Tab 2.8 In vivo studies on small ruminant

Co-products	**Species**	**Authors**
pumpkin seed cake	lamb	(Antunović et al., 2018)
pumpkin seed cake	goat	(Klir et al., 2017)
artichoke and broccoli	goat	(Meneses et al., 2020)
broccoli	goat	(Panwar et al., 2017)
grape seed	ewes	(Nudda et al., 2015)
wine lees	wethers	(Sato et al., 2020)

tomato pomace	dairy ewes	(Buffa et al., 2020)
grape marc	dairy ewes	(Buffa et al., 2020)
exhausted myrtle berries	dairy ewes	(Buffa et al., 2020)
tomato fruits	goat	(Romero-Huelva et al., 2017)
citrus pulp	goat	(Romero-Huelva et al., 2017)
brewer’s grain	goat	(Romero-Huelva et al., 2017)
tomato	goat	(Arco-Pérez et al., 2017)
olive	goat	(Arco-Pérez et al., 2017)
hemp seed cake	sheep	(Mierliţă, 2018)

Peiretti et al. (2013) observed as rabbits’ diet integrated with 6% of tomato pomace could positively influence the quality of meat. Indeed, the authors dived 144 weaned crossbred rabbits into three groups. The first group was fed a basal diet without tomato pomace, while the other two groups were fed the basal diet after replacing 3 or 6% of DM with tomato pomace, respectively. They observed a significant difference between the experimental groups in terms of live and carcass weights. The meat of rabbits fed a 6% DM tomato pomace diet exhibited higher yellowness and chroma values when compared to others. Moreover, the saturated fatty acids (SFA) content in the *Longissimus dorsi* muscle and perirenal fat decreased significantly with increasing tomato inclusion, while PUFA increased.
Similarly in swine, Liotta et al. (2019) studied the inclusion of olive cake to evaluate the meat quality. Seventy-two Pietrain pigs, during the growing-finishing period, were fed with three dietary treatments: 0 (as control), 5, and 10% of the olive cake. The results showed as the inclusion of the lowest level of olive cake in the diet significantly improved body weight (112, 117, and 113 kg, for 0, 5, and 10% of olive cake inclusion), while the highest level of inclusion increased feed conversion (3.70, 3.28, and 4.13 kg/kg of weight gain, for 0, 5, and 10%, respectively). Both levels of inclusion significantly reduced backfat thickness and intramuscular fat and modified their fatty acid composition, particularly, the inclusion of 5 and 10%

increased the concentration of PUFA. Whereas 10% of inclusion increased monounsaturated fatty acids (MUFA) amount.

Cerisuelo et al. (2010) supplemented ensiled citrus pulp in diets for growing pigs to evaluate the effect on growth performance, gut microbiology, and meat quality. Three dietary treatments were formulated to contain 0, 50, and 100 g of ensiled citrus pulp per kg on DM basis. The inclusion of citrus pulp significantly reduced *Enterobacteriaceae* in faeces and did not affect the *Lactobacillus* population. Moreover, no differences were found in backfat thickness at *Gluteus medius* and meat colour, but carcass yield tended to decrease, and oleic acid percentage in subcutaneous fat increased. This study evidenced like growing pigs can use the citrus pulp as a source of high-fermentable carbohydrates without detrimental effects on growth performance and meat quality, and potential benefits on gut microbiology.

On the same species, Cilev et al. (2016) examined the possibilities of the maize's substitution as an energetic nutrient with co-products obtained by manufacturing tomatoes, peppers, and grapes in the nutrition of weaned piglets. The piglets from the control group were fed with a mixture without a share from the examined co-products, whereas the experimental groups were fed a diet with the substitution of maize with different qualities of the above-mentioned co-products. No significant differences were observed between the groups. Maize's partial substitution with a co-product (3% DM) has any negative effects on a weaned piglet performance.

Colombino et al. (2020) studied whether apple, blackcurrant, or strawberry pomaces could be suitable ingredients in broiler diets and their effect on gut health. A total of 480 male broilers were randomly allotted to 8 dietary treatments with lower or higher level of dietary fiber (3 and 6%, respectively). The results of the study suggest that fruit pomaces could be a new, low-cost fiber source in poultry nutrition. Indeed, they did not impair growth performance or gut morphometry/histopathology, they improved the production of VFA, and they reduce the production of putrefactive short-chain fatty acid (PSCFAs) both in the small intestine and in the ceca. However, they showed a potential negative modulation of gut microbiota with a decrease of *Lactobacillus* spp. and an increase of *Enterobacteriaceae* and *Enterococcaceae*.

Kolláthová et al. (2020) aimed to investigate the effects of dietary inclusion of dried grape pomace on biochemical blood serum indicators and digestibility of nutrients in horses. Animals were divided into three groups: control group, experimental group 1 (feed rations + 200 g of dried grape pomace), and experimental group 2 (feed rations + 400 g of dried grape pomace). The concentrations of potassium increased in experimental group 2 compared to control and experimental group 1 and alanine aminotransferase decreased in experimental group 2 in comparison with experimental group 1 and control. No statistical differences emerged between the experimental groups compared to control one for digestibility coefficients (dry matter, organic matter, and crude protein). Nevertheless, in experimental group 2 a lower digestibility of all the studied nutrients were found in comparison with the control group and experimental group 1. These results suggest that dried grape pomace could be used in horse diets up to 200 g without negative effect on their health and for a possible digestibility improvement of some nutrients.

According to *in vitro* study, as discussed previously, Brambillasca et al. (2013) conducted an *in vivo* trial to evaluate the digestibility of nutrients, stool characteristics, and faecal microbial populations of dogs. Animals were fed two fiber sources (citrus pulp and apple pomace) added at different levels in pre-digested dog food. The inclusion of fiber sources in the diets resulted in higher faecal output and defecation frequency, and lower faecal pH and digestibility values. Faecal consistencies and microbial populations did not differ among treatments. Citrus pulp and apple pomace included in a dog diet led to well-formed faeces with small reductions in nutrient digestion. Similarly, Panasevich et al (2013) performed *in vitro* and *in vivo* trials to evaluate the inclusion of potato fiber in dogs' diets as a carbohydrates source. Regarding the *in vivo* experiment, the authors provided different concentrations (0, 1.5, 3, 4.5, or 6%) of potato fiber to 10 female mixed-breed dogs by 5x5 Latin square design. The results showed that the physiological outcomes measured (faecal branched-chain fatty acids, ammonia, phenol, and indole concentrations) were mostly unaffected by dietary potato fiber concentrations. Additionally, faecal spermidine, which is considered a beneficial biogenic amine, and its concentration were increased with graded concentrations of that co-products. Moreover, an excellent stool consistency, without negative effects on nutrient digestibility, has been observed. The authors indicated that potato fiber has the potential to compare favourably with most dietary fibres found in commercial foods. Kröger et al. (2017) compared the effect of lignocellulose (LC) and sugar beet pulp (SBP) on apparent nutrient digestibility, microbial composition, metabolites, and faecal parameters. The authors tested three diets: high SBP (12% SBP and 3.1% crude fiber), low SBP (2.7% SBP and

0.96% crude fiber), and LC (2.7% LC and 2.4% crude fiber) on eight Beagles divided into two groups. The diets were formulated with the same concentration of fiber sources or a similar percentage of crude fiber. The authors observed as the daily faeces amount was lower and the faecal dry matter was higher when dogs were fed the LC diet and the low SBP diet compared with the high SBP diet. Moreover, apparent digestibility of CP, Na, and K was highest with the low SBP diet. Considering the bacterial cell counts of *Lactobacillus* spp., *Bifidobacterium* spp., and the *Clostridium leptum* cluster were lower when dogs received LC diet compared to faeces obtained with diets high SBP and low SBP. The bacterial cell count of the *Clostridium coccoides* cluster was lower in LC and low SBP compared with high SBP. The faeces of dogs fed LC and low SBP had lower concentrations of acetate, propionate, *n*-butyrate, total fatty acids, and L-lactate compared with dogs fed high SBP. The administration of LC diet increased the levels of both butyrate and valeriate compared to high and low sugar beet pulp diets. The lignocellulose and sugar beet pulp affected differently the faecal microbiota and the apparent digestibility of nutrients. However, both fiber sources could be used in dog diets.

Tab 2.9 *In vivo* studies on monogastric animals

Co-products	**Species**	**Authors**
prickly pears	rabbit	(Amer et al., 2019)
tomato pomace	rabbit	(Peiretti et al., 2013)
olive cake	pig	(Liotta et al., 2019)
citrus pulp	pig	(Cerisuelo et al., 2010)
tomatoes	piglet	(Ciliev et al., 2016)
peppers	piglet	(Cilev et al., 2016)
grapes	Piglet	(Cilev et al., 2016)
strawberry pomace	poultry	(Colombino et al., 2020)
apple pomace	poultry	(Colombino et al., 2020
blackcurrant pomace	poultry	(Colombino et al., 2020)

grape pomace	horses	(Kolláthová et al., 2020)
citrus pulp	dog	(Brambillasca et al., 2013)
apple pomace	dog	(Brambillasca et al., 2013)
potato fiber	dog	(Panasevich et al., 2013)
beet pulp	dog	(Kröger et al., 2017)

In vivo studies evidenced as the inclusion of agro-industrial co-products in animal diets could improve the performance and sometimes animal health, increasing technological and nutritional characteristics of meat, and milk. In the case of the inclusion of grape pomace in horses diet, and beet pulp in companion animal diet, the residues improved nutrient digestibility and did not negatively affect the animal health status. Furthermore, these studies allow us to understand the right level of inclusion of co-products into the diets to avoid negative effects.

2.7 Conclusion

In conclusion, all evaluated studies proved that co-products could be a useful source of several nutrients in animal diets. In this regard, *in vitro* trials demonstrated like the supplementation of co-products can influence the fermentative parameters. Indeed, most of the tested co-products showed high digestibility and degradability comparable to the conventional feed. Moreover, these studies demonstrated that the co-products could decrease the gas production (i.e., pomegranate peel) or additionally could decrease the methane emission like in the case of red ginseng meal. While *in vivo* studies showed that the co-products do not negatively affect the production of farm animals. Still, in some cases, they could improve the nutritional characteristics and microbiological quality of milk, cheese, and meat. Moreover, in such cases, particularly for horses could improve the health status of the animals. Regarding the companion animals, several studies described as the co-products could be useful sources of fiber in alternative to conventional ingredient. Indeed, the inclusion of agro-industrial such as potato residues into the diet seems not to influence nutrient digestibility.

Furthermore, some authors tested the co-products with ensilage storage technique, testifying as this approach could be a valid method to guarantee the presence of fresh co-products during the year and reduce the seasonality. Nevertheless, fruit and vegetable processing co-products still showed high variability in chemical composition, which might influence their digestibility either in the rumen or in the intestine. Commercial application of fruit and vegetable industry co-products as functional feed ingredients provide challenges and opportunities. Indeed, investing in the development of these co-products could guarantee the implementation of the circular economy, towards greater sustainability, with a reduction of waste along the production chain.

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Chapter 3

In Vitro Fermentation and Chemical Characteristics of Mediterranean By-Products for Swine Nutrition

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Chapter 3 *In Vitro* Fermentation and Chemical Characteristics of Mediterranean By-Products for Swine Nutrition

3.1 Abstract

The purpose of the study is to determine the nutritional characteristics of some by-products derived from fruit juice and olive oil production to evaluate their use in pig nutrition. Five by-products of citrus fruit (three citrus fruit pulp and two molasses) and three by-products of olive oil (olive cake) obtained by different varieties are analysed for chemical composition. The fermentation characteristics are evaluated *in vitro* using the gas production technique with swine faecal *inoculum*. All the citrus by-products are highly fermentable, producing gas and a high amount of short-chain fatty acids. The fermentation kinetics vary when comparing pulps and molasses. Citrus fruit pulps show lower and slower fermentation rates than molasses. The olive oil by-products, compared to citrus fruits ones, are richer in NDF and ADL. These characteristics negatively affect all the fermentation parameters. Therefore, the high concentration of fiber and lipids represents a key aspect in the nutrition of fattening pigs. The preliminary results obtained in this study confirm that the use of by-products in pig nutrition could represent a valid opportunity the reduce the livestock economic cost and environmental impact.

3.2 Introduction

The EU is the largest pig meat producer and exporter in the world (FAO, 2018). Nevertheless, the increasing intensification of pig production raises concerns about its sustainability, especially in terms of nutrient sources. Nearly two thirds of the EU's cereals are used in animal feeds (European Commission, EC, 2019), but only an average of 25-30% of global animal dietary gross energy is retained in meat and milk products. Consequently, the relevant proportion of nitrogen and organic matter intake excreted leads to a potentially important environmental impact. According to the European Environment Agency (2018), pig slurries in the EU are responsible for about 15% of ammonia ($N\text{-}NH_3$) and 4% of total methane (CH_4) emissions. In the future, the feed industry will need to find alternative feedstuffs and minimize their eco-footprint (Ferrer et al., 2018).

In the last few years, there has been widespread social and environmental pressure for the efficient reutilization of agricultural industry residues (Santana-Meridas et al., 2012) due to the global intensification of food production, which creates large quantities of food co-products and wastes (Waldron, 2007). Utilization of agro-industrial by-products in farm animal nutrition reduces feeding cost as well as the environmental impact of the food production. Moreover, this kind of recycling improves agriculture profitability, turning low-quality materials into high-quality foods (Elferink et al., 2008).

By-products are not to be considered waste, but raw material obtained from agriculture and/or industrial process (Afshar and Naser, 2008), and this is compliant with current legislation that strongly encourages the food industry to find new end-uses for refusals (European Commission, EC, 2012). In recent years, European legislators have enacted rules to distinguish waste from by-products (EU, 2015; EU, 2017). In addition, in the last decade, industrial by-products, co-products, insect materials, seaweed ingredients (Spranghers et al., 2016; Woyengo et al., 2016), and ex-food or former food products have been proposed as categories with great potential as alternative ingredients for animal diets (Giromini et al., 2017; Tretola et al., 2017a; Tretola et al., 2017b). Furthermore, these agricultural industry residues pose increasing disposal and potentially severe pollution problems and represent a loss of valuable biomass and nutrients (Laufenberg et al., 2003). In addition, industrial ecology and circular economy are considered the leading principles for eco-innovation focusing on a ‘zero waste’ society (Mirabella et al., 2014). The main parameters affecting the extensive application of byproducts such as functional feed ingredients in livestock nutrition are related to animal factors, logistics, and their commercial value (Kasapidou et al., 2015). The by-products are considered valid instruments to reduce dietary costs, since they contain the feed price variation and benefit from their specific nutritional characteristics. Different studies have underlined the advantage in using residues in poly- and monogastric animal nutrition. Several studies (Chiofalo et al., 2004; Polyorach et al., 2015; Volpato et al., 2015; Liotta et al., 2019) have shown that by-products administration of livestock could improve meat, milk, and cheese nutritional quality. In addition, several by-products contain bioactive substances, called nutraceuticals (Ferrie, 2007). Given this, the functional properties of several plant extracts have been investigated for their potential use as novel nutraceuticals; these biological substances are ‘pharmacological multitaskers’ (Bizzarri and Dinicola, 2011). Research into the use of natural antioxidant and health-promoting compounds from plant sources has resulted in experimental feeding trials, which have examined the effects of plant extracts/nutraceuticals in the diet of dairy and meat-producing animal (Nieto et al., 2010; Sedighi-Vesagh et al., 2015; Faixovà and Faix, 2008), derived from their capacity to improve animal health and the quality and nutritional value of food due to the content of bioactive compounds, which may be considered ‘natural functional ingredients’. The by-products have high potential for feeding, though their use has different limits due to the large variability in their chemical composition and physical status (Afshar and Naser, 2008), due to their seasonal production and to their short shelf-life, due to their high moisture and fat levels. To increase the by-products’ shelf-life, some precautions could be adopted such as treating by-products rich in moisture with a hygroscopic substance (e.g., lime) or adding anti-oxidants to the by-products rich in fats. The production of olive oil and citrus fruits has been widespread in the Mediterranean area from ancient times. As reported by Azbar et al. (2004), olives have been cultivated in this area for more than 7000 years, whereas citrus fruits were first imported to the Mediterranean Countries during the tenth century. Both crops play important social and economic roles in Italy, particularly in the south. Italy is the second largest olive oil producing country (Salomone et al., 2012) in the world, producing more than 429,000 tons per year (Sarnari, 2018), whereas it is the eighth biggest producer of citrus fruits, producing 3.2 million tons per year (Pergola et al., 2013). This production is generally concentrated in several medium-large cooperatives. Olives and citrus fruits are processed to obtain oil and juice, with the consequent production of enormous quantities of by-products from the industrial process. These by-products represent important nutritional resources (Villarreal et al., 2006) thanks to their high nutritional value and bioactive compound levels (Volpato et al., 2015; Marin et al., 2002; Rigane et al., 2012; Varricchio et al., 2019; De Blas et al., 2018).

Many agro-industrial residues, such as citrus pulps, citrus molasses, and olive dry pomace, have the potential to be used to feed livestock animals (ruminants, swine), and several studies have highlighted the presence of nutraceutical components (Manthey and Grohmann, 2001; Olivo et al., 2017; Chiofalo et al., 2015; Chiofalo et al., 2016).

The catalogue of raw materials for feeds (EU, 2017) includes the ‘dried citrus pulp’ as ‘product obtained from the citrus fruits processing and subsequently dried’. Its use in animal nutrition has long been known (Kasapidou et al., 2015), and several studies have confirmed its potential usefulness, mainly concerning its integration in lambs (Lanza et al., 2001; Lashkari et al., 2017) and pigs (Watanabe et al., 2010) diets.

The purpose of the present study is to characterize juice and oil industry by-products, in terms of their chemical composition and *in vitro* fermentation characteristics, for their possible use in pigs’ diets. With this aim in mind, the *in vitro* gas production technique is used with pig faecal *inoculum.*

3.3 Materials and methods

Eight by-products derived from citrus fruit or olive oil processing were evaluated, i.e., dried golden orange pulp (GOP), dried red orange pulp (ROP), dried lemon pulp (LP), concentrated lemon molasses (LM), concentrated orange molasses (COM), dried olive cake variety *Nocellara* (DPN), dried olive cake variety *Biancolilla* (DPB), and dried olive cake variety *Cerasuola* (DPC). All citrus fruit were treated with lime, to reduce the moisture and increase their shelf-life during the storage. On the other hand, olive oil by-products were stabilized by the addition of citric acid. Each by-product was sampled by pooling three different lots of production.

3.3.1 Chemical Composition

At the laboratory of the Consortium of Meat Research (CoRFilCarni, Messina, Italy), each sample was milled (1.1 mm) and analysed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), ash, and starch contents according to AOAC (2015) procedures (ID number: 2001.12, 978.04, 920.39, 978.10, 930.05, and 996.11, respectively). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were also determined according to Van Soest et al. (1991).

The analysis of each parameter was replicated three times for each by-product, and the results were reported as a mean of the three replications.

3.3.2 In Vitro Gas Production

The *in vitro* gas production technique proposed by Theodorou et al. (1994) was used to evaluate the fermentation kinetics in a pig’s large intestine. This technique, originally designed to evaluate feedstuffs for ruminant species, was subsequently also utilized in monogastric species using faecal *inoculum* (Bauer et al., 2003; Cutrignelli, 2007; Calabrò et al., 2012; Musco et al., 2015). At the laboratory of Feedstuffs Evaluation (University of Napoli Federico II, Italy), *in vitro* gas production trial was performed. Each sample was weighed in triplicate (0.5068 ± 0.0045 g), and 82 mL of anaerobic medium was added into serum flasks. In order to correct fermentation parameters, three bottles were incubated without substrate and were used as blank. As *inoculum*, faecal samples were collected *per rectum* from three adult neutered Large White pigs (mean age: 350 ± 10 days; mean live weight:159.8 ± 5.2 kg) fed a commercial diet (CP: 14.8%; CF: 4.0%). The faecal pool was filtered through a double thickness of cheesecloth, diluted (1:6) in NaCl solution, homogenized, and added to each bottle (5 mL) under anaerobic conditions. The flasks were incubated at 39°C for 72 h. During the incubation, the volume and pressure of gas produced were measured every 2-4 h (totally 26 times) using a manual pressure transducer (Cole and Parmer Instrument Co., Vernon Hills, IL, USA). The cumulative gas volume (OMCV, mL/g) was related to the incubated organic matter, and the gas profiles were fitted to the equation described by Groot et al. (1996) as follows:

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

where G is the total gas produced (mL/g of incubated OM) at t (h) time, A is the asymptotic gas production (mL/g of incubated OM), B is the time at which half of the asymptote is reached (h), and C is the switching characteristic of the curve.

The maximum fermentation rate (R_{max}, mL/h) and time at which it occurred (T_{max}, h) were also calculated (Bauer et al., 2001) as follows:

$$T_{max} = C * [\frac{(B-1)^{\frac{1}{B}}}{(B+1)}]$$

$$R_{max} = \frac{A * B^{C} * B * T_{max}{}^{(B-1)}}{[1 + \left(C^{B} * T_{max}{}^{-B}\right)]^{2}}$$

The fermentation was stopped at 4°C, and the pH of each flask was measured using a pH-meter (model 720A+ Thermo Fisher Scientific, Rodano, MI, Italy). The organic matter disappearance (OMD, %) was determined by filtering the residues under vacuum using pre-weigh sintered glass crucibles (Scott Duran, porosity#2).

3.3.4 End Products

Fermentation liquor was collected from each flask after 72 h of incubation in order to determine the ammonia and short-chain fatty acid production. The ammonia (NH_3, mmol/g) was measured using a spectrophotometer (model Helios gamma UV/VIS Thermo Fisher Scentific, Rodano, MI, Italy), at a wavelength of 623 nm.

The short-chain fatty acids (SCFA, mmol/g) were determined by centrifuging the samples twice at 12000 × g for 10 min at 4 ◦C, then 1 mL of supernatant was diluted in 1 mL of oxalic acid 0.06 M. The obtained samples were injected into a gas chromatograph (Thermo Fisher Scientfic, Rodano, MI, Italy; model trace 1310) equipped with a fused silica capillary column (Supelco, 30 m × 0.25 mm × 0.25 µm film thickness), and each fatty acid (acetate, propionate, iso-butyrate, butyrate, valerate and iso-valerate) concentration was measured, comparing the sample peak areas of each SCFA with that of an external standard (Cutrignelli et al., 2009). Branched-chain fatty acids proportion (BCFA) were also calculated as follows: [(iso-valerate + iso-butyrate)/SCFA].

3.3.5 Statical Analysis

Fermentation characteristics (OMCV, OMD, pH), model parameters (T_{max}, R_{max}), and the final products (SCFA, BCFA, NH_3) were statistically analysed to detect the differences between substrates by ANOVA for one-way (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989–2019) according to the following model:

$$y_{ij=\mu+Sub_i+\varepsilon_{ij}}$$

where y is the experimental data, µ represents the general mean, Sub is the substrates (i = 1, 2, ... 8), and ε is the error term. The significance level was verified using HSD Tukey test at $p < 0.01$ and $p < 0.05$.

3.4 Results

In Table 3.1, the chemical composition of the tested by-products is reported. Most samples had high level of dry matter (around 96.0%), except orange and lemon molasses, where low DM levels were found (52.43 and 44.49%, respectively). The tested by-products had low protein content (varying from 5.36 to 9.14% DM, in OM and DPN, respectively). Regarding lipid content, the three olive oil by-products had more than 29% DM, while the orange fruit by-products were characterized by low fat values, except for lemon pulp (4.89% DM). The structural and reserve carbohydrates content was almost variable among the analysed by-products. Both molasses samples had very low structural carbohydrates values and an untraceable content of starch; consequently, they were composed of 60% soluble sugars, together with organic acids (6-17% DM), pectin (6-10% DM), and flavonoids (0.5-1.7% DM). The NDF level of citrus pulp by-products was higher than 30% DM, and mainly constituted by cellulose and hemicelluloses, while the three dried pomaces were highly lignified. Very low content of starch was detected in all the by-products, while the highest percentage was found in ROP (2.91% DM). The three citrus pulp samples had a particularly high ash content (mean 17.77% DM ± 0.6), as was the case in lemon molasses. The ash content of the other by-products varied from 3.67 to 4.43% DM.

Tab 3.1 Chemical composition.

Samples	**DM**	**CP**	**EE**	**NDF**	**ADF**	**ADL**	**Ash**	**Starch**
	%				**% DM**			
GOP	95.75	5.82	1.10	31.80	23.35	3.74	18.07	2.11
ROP	96.58	6.02	0.91	33.05	25.72	3.69	17.08	2.91
LP	96.90	7.42	4.89	37.42	28.37	4.23	18.16	1.91
LM	44.49	6.74	0.43	1.37	0.36	0.20	15.01	-
COM	52.43	5.36	0.40	0.92	0.27	0.11	7.15	-
DPN	95.63	9.14	29.51	50.19	38.83	27.19	4.43	1.76
DPB	95.66	8.67	30.04	53.88	39.69	21.49	3.67	1.11
DPC	95.55	8.07	31.46	44.05	39.65	20.50	4.17	1.57

GOP: dried golden orange pulp; ROP: dried red orange pulp; LP: dried lemon pulp; LM: concentrated lemon molasses; COM: concentrated orange molasses; DPN: dried olive cake variety Nocellara; DPB: dried olive cake variety Biancolilla; DPC: dried olive cake variety Cerasuola. DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin

The *in vitro* fermentation characteristics are reported in Table 3.2. For all the parameters, statistical and significant differences ($p < 0.01$) emerged. Citrus fruit by-products were significantly higher fermentable than the olive oil by-products, as demonstrated by the highest OMD and OMCV values (>70% and >200 mL/g, respectively).

Tab 3.2 In vitro fermentation characteristics.

Samples	**OMD**	**OMCV**	**T_{max}**	**R_{max}**
	%	**mL/g**	**h**	**mL/h**
GOP	81.23^{B}	222.2^{A}	16.9^{A}	6.27^{C}
ROP	83.91^{B}	218.9^{A}	17.3^{A}	5.91^{C}
LP	72.67^{C}	212.6^{A}	18.1^{A}	5.30^{C}
LM	91.82^{A}	211.8^{A}	4.08^{C}	13.0^{A}
COM	93.63^{A}	167.3^{B}	4.06^{C}	10.7^{B}
DPN	23.47^{E}	77.66^{C}	8.90^{B}	2.21^{D}
DPB	24.17^{E}	78.78^{C}	9.30^{B}	2.20^{D}
DPC	31.74^{D}	89.11^{C}	11.1^{B}	2.44^{D}
RMSE	1.52	9.67	0.73	0.61

OMD: organic matter disappearance; OMVC: cumulative volume of gas related to incubated organic matter; R_{max}: maximum fermentation rate; T_{max}: time at which R_{max} occurs; RMSE: root mean square error. Along the column, different letters indicate difference for $p < 0.01$.

On the other hand, the incubation of dried pomace substrates showed low fermentability and produced a low gas amount. As regards the kinetics parameters, the by-products have been significantly ($p < 0.01$) differentiated into three categories, i.e., the citrus pulp, that showed the intermediate R_{max}, and the highest T_{max}, the molasses, that presented the highest fermentation rate that was reached in the lowest time, and the olive oil by-products, which showed the lowest rate of fermentation and needed more than eight hours to reach it. To better highlight the differences between the by-product categories and to understand the curve trends, the fermentation rates are depicted in Figure 3.1

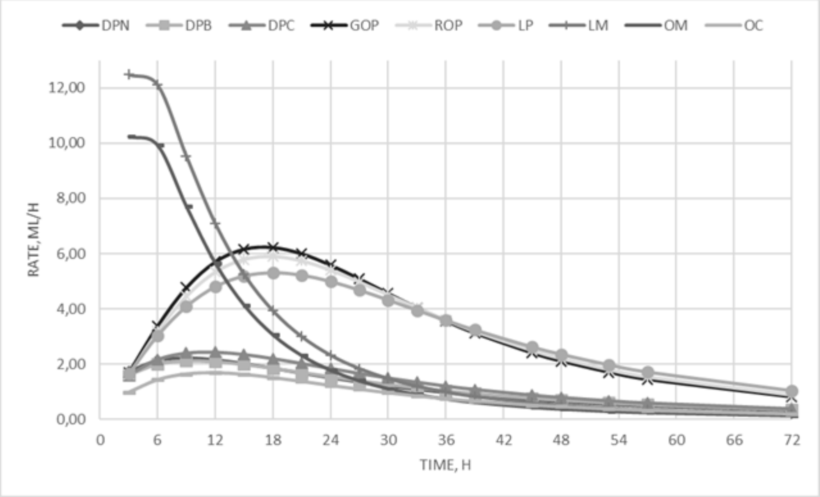

Fig 3.1. In vitro fermentation rate over time.

In vitro fermentation end-products of fermenting liquor are reported in Table 3.3. Significant ($p < 0.01$) differences were observed for pH values registered at the end of fermentation; DPB had the highest value and ROP the lowest. The molasses samples showed the highest ($p < 0.01$) SCFA production (mean values:165.5 mmol/g ± 14.1), followed by both the orange fruit pulps (mean values: 107.2 mmol/g ± 9.2); very low SCFA production was detected in the three pomaces (mean values: 43.11 mmol/g ± 2.9). Acetate and propionate were the most representative fatty acids of all the substrates; they represented more than 75% of the total SCFA. The fermentation of all the citrus fruit by-products and, in particular, of both molasses resulted in there being high proportion of butyrate (18 and 13% SCFA, for molasses and pulps, respectively). On the other hand, BCFA production was higher for oil olive by-products than all the citrus fruit by-products. Ammonia production was significantly higher for both molasses samples, while the DPN showed the significantly lowest values.

Table 3.3 *In vitro* fermentation end products (mmol/L).

	Samples								
	GOP	ROP	LP	LM	COM	DPN	DPB	DPC	RMSE
pH	6.71^{ab}	6.61^{b}	6.65^{ab}	6.70^{ab}	6.68^{ab}	6.75^{a}	6.76^{a}	6.74^{a}	0.018
Ace	68.95^{C}	80.53^{BCbc}	56.73^{Cd}	117.0^{Aa}	95.87^{ABb}	27.49^{D}	23.35^{D}	27.20^{D}	7.34
Prop	17.37^{A}	17.70^{A}	15.84^{A}	19.77^{A}	18.59^{A}	7.96^{B}	7.32^{B}	7.92^{B}	1.51
Iso-But	1.50^{BCDab}	1.70^{B}	1.57^{BCa}	2.70^{A}	2.53^{A}	1.06^{Dc}	$1.09C^{Dc}$	1.12^{CDbc}	0.13
But	12.60^{DCa}	13.22^{C}	9.48^{Db}	23.51^{B}	27.95^{A}	4.65^{E}	4.57^{E}	5.26^{E}	1.06
Iso-Val	2.65^{C}	2.47^{C}	2.53^{C}	4.93^{A}	3.85^{B}	2.12^{C}	2.20^{C}	2.33^{C}	0.27
Val	1.71^{BC}	1.75^{BC}	2.18^{BCa}	3.78^{A}	3.42^{A}	1.27^{Cb}	1.18^{Cb}	1.25^{Cb}	0.32
SCFA	104.8^{BC}	117.4^{BCa}	88.35^{Cb}	171.7^{A}	152.2^{A}	44.55^{D}	39.71^{D}	45.08^{D}	8.28
BCFA	0.030^{B}	0.030^{B}	0.040^{B}	0.030^{B}	0.037^{B}	0.063^{A}	0.070^{A}	0.063^{A}	0.006
NH_3	19.44^{BC}	21.87^{BC}	22.68^{BCa}	43.64^{A}	42.92^{A}	14.96^{Cb}	22.87^{BCa}	25.68^{B}	2.47

Ace: acetate; Prop: propionate; Iso-But: Iso-Butyrate; But: butyrate; iso-Val: iso-Valerate; Val: valerate; SCFA: total short-chain fatty acids; BCFA: branched-chain fatty acids; NH_3: ammonia. RMSE: root mean square error. Along the column, different lowercase letters indicate difference for $p < 0.05$ and different capital letters indicate difference for $p < 0.1$.

3.5 Discussion

3.5.1 Citrus Fruit By-Products

Overall, the chemical composition data of citrus fruit by-products were in agreement with those reported in literature (Villarreal et al., 2006; Watanabe et al., 2010), considering the differences in terms of production process as well as botanical varieties. As expected, pulp and molasses, coming from the same production process, were consistently different due to their physical and chemical characteristics. The pulps, which made up the waste solid part, were rich in dry matter and fermentable carbohydrates, represented by soluble dietary fiber, such as pectin, an interesting alternative, and a low-priced source of fiber in animal feed. Citrus pectin has multiple biological activities, including glycaemic and cholesterol level control (Baker, 1994; Musco et al., 2017). Conversely, molasses is the liquid waste of juice production process and, consequently, compared to the pulps presented lower concentration of all the nutrients. The chemical composition of these by-products was reflected in their specific fermentation characteristics. In fact, all the citrus by-products were highly fermentable, producing a high amount of gas and short-chain fatty acids, particularly acetate, propionate, and butyrate. Despite this, the specific fermentation kinetics varied when comparing pulps and molasses. The citrus fruits pulp, richer in complex fermentable carbohydrates, showed significantly lower and slower fermentation rates than molasses, suggesting that they are more fermented into the distal part of gastrointestinal tract (Calabrò et al., 2012) and could be considered as pre-biotics. Instead, the two molasses were richer in sugars and showed a shorter and more intense fermentation process; thus, they are mainly solubilised into the stomach and small intestine and may represent a useful energy source. In any case, the pH values respected the physiological range (5.5-7.5) reported by Younes et al. (2001). The production of short-chain fatty acids was significantly high for all the citrus fruit by-products. In particular, the high butyrate production indicates a potential pre-biotic rule of these substrates (Musco et al., 2018). The fermentable carbohydrates represented an optimal pabulum for microbiota growth, and the high proportion of butyrate could be useful for the colonic epithelium as a main energy source for cell growth and differentiation (Musco et al., 2018). In pulps, the high concentration of ash, due to the lime treatment, reduced the fermentation processes. As demonstrated by previous *in vivo* studies (Chiofalo et al., 2007), the use of citrus fruit by-products in partial substitution of hay to feed Nero Siciliano pigs did not affect swine performance and meat quality in terms of oxidative stability, protein, and fat content. Moreover, they improved the fatty acid profile, reducing significantly saturated fatty acids amount and increasing polyunsaturated fatty acids of n-3 and n-6 series levels. Lemon by-product administration also affected positively the sensorial characteristics of Nebrodi cured sausages due to the volatile fraction of citrus essential oils, which were transferred from citrus to adipose tissue (Chiofalo et al., 2013; Muriel et al., 2004).

3.5.2 Olive Oil By-Products

The chemical composition of olive cake varies widely depending on the olive variety, the proportion of its main components (skin, pulp, and stone), and the oil extraction process (Marcos et al., 2019). For this reason, it was considered important to characterize the individual by-product, differentiating it according to the most represented varieties in Sicily (*Nocellara*, *Biancolilla*, and *Cerasuola*). As reported by Molina-Alcaide et al. (2008), all pomace samples, residues obtained after the pressure extraction of oil from the entire olive fruit, showed a high percentage of ether extract. These substrates were also characterized by high levels of NDF, which had high lignin content, whereas the level of crude protein was quite low. As a consequence, the substrates were less fermentable, as demonstrated by all the fermentation parameters and kinetics profile. Despite that, the good proportion of butyrate on the total SCFA (always higher than 10%) was indicative of a partial bacterial utilization (Cutrignelli et al., 2009). As regards the fiber levels, during the last 15 years, efforts have been made to formulate diets that better meet the pig's requirements to improve performance and to contribute to reducing cost, odour, and pollutant excretion. Dietary fiber in pig reduced ammonia emission; improved gut health; and, consequently, the pig welfare (Aarnink and Verstegen, 2007; Courboulay et al., 2001). The dietary fiber escaping digestion in the upper part of the gastro-intestinal tract was potentially available for bacterial fermentation in the large intestine. This means that the gut microflora of healthy animals could be modified in relation to the presence of fiber in the diet (Awati et al., 2005). Dietary fiber significantly modifies the microbial equilibrium with a positive or a detrimental impact on animal health, according to the dietary fiber source and the physiological status of the pig. Chiofalo et al. (2013) reported that the high-fiber diet caused a negative effect on some pig performances, even if the technical and nutritional characteristics of the meat were not influenced by the dietary treatment. Despite the mentioned disadvantages, there have been several important advantages that are inexpensive protein sources and can be grown at small-farm level. Often, they are by-products or co-products of multipurpose crops. Further studies devoted to the relationship between dietary fiber content and its functionalities are necessary to identify an appropriate fiber level that reduces ammonia emission, promotes intestinal health, and still allows acceptable pig growth rates.

The digestive utilization of these by-products is probably reduced by high lignin concentration, albeit by the fact that the high percentage of fat that these by-products could represent an interesting characteristic. Lipids, in fact, supply a greater energy yield compared to carbohydrates and proteins. From a biochemical point of view, fats contain carbon and hydrogen in a more reduced state; therefore, these elements can potentially be much more oxidised (Giromini et al., 2017). For this reason, the supplementation of fat in swine nutrition is a common practice. In addition, the proportion in monounsaturated fatty acids (MUFA), especially in oleic acid, could turn it into an interesting dietary ingredient because it might modify the fatty acid profile of the pig fat tissues (Mas et al., 2010; Joven et al., 2014). The increasing demand from modern society for healthy meat has to be taken into account. A meta-analysis of epidemiological studies has cast doubt on the relationship between long chain SFA and cardiovascular diseases (Siri-Tarino et al., 2010). Instead, the increase of the MUFA percentage in relation to the olive oil by-products intake modifies the unsaturation degree and the atherogenic index. It has been shown that oleic acid (cis-9-octadecanoic acid) has beneficial effects on blood cholesterol and other health related outcomes in humans. Moreover, despite the fact that the cholesterol-lowering response to PUFA is greater than that to MUFA, the potentially adverse health effects of lipoperoxidation products are higher for PUFA in relation to the higher presence of double bonds.

3.6 Conclusion

The preliminary results obtained in this study confirm that the use of local by-products in pig nutrition could represent a valid system to reduce the animal production cost and limit the environmental impact of some production systems typical of the Mediterranean area.

Chemical data, associated with the supplementary information obtained from the *in vitro* gas fermentation technique (fermentation characteristics, kinetics, and end-products), allow us to estimate the potential utilization along the digestive tube.

Results allow one to consider the by-products analysed in relation to their specific nutritional characteristic for different uses in swine nutrition. Whereas citrus fruit pulp could improve microbiota and enterocytes homeostasis, citrus fruit molasses could probably increase the diet nutritional values due to its high concentration in soluble sugars. A different approach is necessary in the evaluation of dried olive cake, which has interesting nutritional characteristics, but its utilization is prevented by its high lignin concentration.

Further studies could be performed focusing on the effect of raw material processing conditions on the composition (characterization and quantification of the active/target compounds) in the produced by-products, as well as evaluating the *in vitro* enzymatic digestibility that occurs in the proximal tract of the digestive tract and identifying the bioactive compounds and/or the toxic factors for the better exploitation of these residual biomasses in the agroindustry. This will allow us to take into consideration the citrus fruit and olive oil by-products as a food resource and not as a waste, whose correct disposal will always be an added cost for the food companies, and to evaluate the functionality of new types of feed ingredients in large-scale (commercial) feeding trials.

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Chapter 4

Silage of Prickly Pears (*Opuntia* spp.) Juice By-Products

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4.1 Abstract

Cactus pear cladodes are used as forage in the most arid regions. In Italy, the human consumption of prickly pear fruits and juice is gradually increasing for their numerous health benefits. In manufacturing plants that produce prickly pear juice, several by-products (prickly pear by-products PPB) are obtained. Despite their interesting nutritional characteristics, PPB are not very usable because of their poor shelf-life which is related to their high moisture and sugar content. The aim of this study was to verify the efficacy of ensilage to preserve PPB and to compare different inclusion levels (0, 5, and 10% as fed) of wheat straw. For each treatment, four under vacuum micro-silos were prepared and, after 35 days of storage, the state of preservation was evaluated. Subsequently, the aliquots were analysed for chemical composition and incubated with bovine rumen fluid to evaluate the fermentation kinetics. The PPB 5% of straw showed significant lower pH and ammonia nitrogen concentration, indicating a better preservation process. Moreover, PPB 5% of straw showed better nutritional parameters (higher crude protein and lower Neutral Detergent Fibre) and fermentation characteristics (higher degradability and volatile fatty acids VFA production) when compared with the other PPB silages. Ensilage with straw represents a suitable storage technique to preserve the nutritional characteristics of PPB

4.2 Introduction

The cactus pear plant (*Opuntia* spp.), which belongs to the *Cactaceae* family, originates from the Americas and it is cultivated in many countries around the world (Miranda-Romero et al., 2018). Cactus pear is considered an excellent and resistant plant, well adapted to arid and hot environments. Furthermore, the adaptation over millennia to these adverse environments determined the efficiency of the cactus pear in converting water to dry matter (Nobel et al., 2002). Thanks to its morphology, this species is considered a highly valuable source of nutrients in many of the world's environments and is considered as valuable feedstuff in regions where other plants are not able to survive due to extreme environmental conditions (FAO, 2013). Recently, many studies have been carried out to evaluate the effect of *Opuntia* cladodes as forage on livestock performance and rumen physiology (FAO, 2017; De Oliveira et al., 2017; Dubeux, 2016; Cardoso et al., 2019).

Italy is the third-largest producer in the world of the *Opuntia* fruits, commonly named prickly pears, after Mexico and the United States. In Italy, the prickly pear fruits have always been consumed but in recent years, thanks to the highlighting of its numerous health benefits, the human consumption of the fruit and its juice has gradually increased. Prickly pear fruit is considered a functional food with recognized antioxidant properties (Amer et al., 2019), mainly due to the ascorbic acid, polyphenols, and flavonoids compounds which it contains (Sumaya-Martinez et al., 2011; Zenteno-Ramirez et al., 2018). Sicily is the Italian region which produces most prickly pear fruits (85% of national production), with a total production of 146,987 tons/year (ISTAT, 2020). In this region, there are several manufacturing plants where the fruits are processed in order to extract the juice, and as a consequence, various by-products (i.e., peel, pulp, and seeds) are obtained. Some authors have evaluated the nutritional characteristics of this by-product, underlining the moderately high ether extract value, amounts of crude protein, fiber, and sugar (Todaro et al., 2020). However, the poor shelf-life of these products due to their high level of moisture and fermentable carbohydrates has also been evidenced. In view of the environmental changes being experienced, the use of some of these by-products could be a way to satisfy animal needs, while, at the same time, making their production more sustainable and reducing waste production (Vastolo et al., 2019; Serrapica et al., 2019; Castrica et al., 2019). The by-products of prickly pears (PPB) are mainly managed in a fresh state, and outdoor storage is only possible for a few days due to bacterial fermentations (Todaro et al., 2020).

Ensiling could be a valid way of conserving these by-products for a long period due to the anaerobic fermentation process which it involves. This storage method has also been suggested as a way of preserving *Opuntia* cladodes and fruit (Hiriart, 2008; Chendly and Lee, 1999). However, since PPB show high levels of moisture, it is advisable to ensile them with dry forages or mature crop residues, such as wheat straw, in order to partially absorb the water and to balance water-soluble carbohydrates and nitrogen fractions (Sahoo, 2018). There is no research information on PPB silage referring to nutritional aspects and *in vitro* fermentation characteristics.

In order to study feedstuff preservation in laboratory-scale silos, various systems have been developed (McDonald et al., 1991; Seale et al., 1986; Johnson et al., 2005). The use of vacuum-packed polyethylene bags suggested by Johnson et al. (2005) is particularly flexible, repeatable, and cheap. Moreover, this method allows different aliquots to be obtained and the comparison of different treatments, such as the inclusion of additives or other feedstuffs.

The evaluation of fermentation characteristics with *in vitro* cumulative gas production technique proposed by Theodorou et al. (1994), in addition to the chemical evaluation, allows the nutritional characteristics of a novel feedstuff to be defined. More particularly, the evaluation of fermentation kinetics and the determination of the end-products (i.e., ammonia, volatile fatty acids) are useful in order to predict the rumen fermentation pathway.

The aim of this study was to verify the efficacy of ensilage as a conservation method for PPB comparing different inclusion levels (0, 5, and 10% as fed) of wheat straw. For this purpose, the nutritional characteristics and the *in vitro* fermentation characteristics and kinetics parameters of the silage have been studied.

4.3 Materials and Methods

4.3.1 Micro Silos Preparation

The ensiling process at laboratory scale was employed in this study, in accordance with Johnson et al. (2005). A commercial chamber vacuum-packing machine (Lavezzini device; Fiorenzuola d'Arda, Piacenza, Italy) was used to remove air from the bag equipped with an automatic heat-sealing mechanism that seals the bag after air extraction.

At the end of August 2019, 45 kg of PPB was taken directly from a prickly pear juice extraction factory in the province of Palermo (Sicily, Italy) and transferred in the experimental laboratories of the Department of Agricultural, Food and Forest Science, University of Palermo, Italy, where three samples were prepared: adding to PPB chopped (2 cm) wheat straw in ratio of 0, 5, and 10% on a fresh weight basis. For each treatment, four polyethylene bags were filled with 500 g of PPB and/or straw. The bags (400 x 500 mm) were made of polyamide bioriented (OPA) and polypropylene (PP) (15 µm OPA/75 µm PP) and were characterized by an oxygen permeability of 30 cm^3; the air vacuum pump drawed air at 10 m^3 h^{-1} at 25°C (Alpak srl, Taurisano, Italy). The vacuum bag silos were stored in a conditioned room (at 18°C) for 35 days. Subsequently each bag was opened, and chemical, physical, and *in vitro* gas production analysis were performed.

4.3.2 Evaluation of Chemical Composition and Silage Quality

The 12 silage samples together with straw were analysed according to the procedures of the Association of Official Agricultural Chemists (AOAC, 2005) to determine dry matter (DM, 934.01), ether extract (EE, 920.39), crude protein (CP, 2001.11) and ash (942.05). The fiber fractions Neutral Detergent Fiber NDF on organic matter basis (NDFom, 2002.04), Acid Detergent Fiber on organic matter basis (ADFom, 973.18) and Acid Detergent Lignin (973.18) were determined in accordance with AOAC (2005) and Van Soest et al. (1991) and expressed exclusive of residual ash. To study the micro-silos quality, ammonia nitrogen ($N\text{-}NH_3$) was determined in the silage juice following the procedure of the official method of analysis (AOAC, 1990) and pH was measured directly using a pH-meter (HI 9025 142) equipped with a spear electrode FC 200 (Hanna Instruments Inc., Woonsocket, RI, USA)

4.3.3 In Vitro Gas Production

The fermentation characteristics and kinetics were studied using the *in vitro* gas production technique as proposed by Theodorou et al. (1994). All samples were weighted (1.0005 g ± 0.0003) in three replications by two gas-runs and were incubated at 39°C under anaerobic conditions in 120 mL serum bottles, to which 74 mL of anaerobic buffer were added with rumen fluid (Calabrò et al., 2005b). The rumen fluid was collected in a pre-warmed thermos at a slaughterhouse authorized according to EU legislation (EU, 2004) from six fasting bovine (*Bos taurus*) young bulls fed a standard diet (% DM: NDF 45.5 and CP 12.0). The collected rumen content was rapidly transported to the laboratory of the Department of Veterinary Medicine and Animal Production (University of Napoli, Italy), where it was pooled, flushed with CO_2, filtered through a cheesecloth, and added to each bottle in ratio of 10 mL. For each gas-run three bottles without substrate were incubated as blanks to correct for the disappearance of organic matter (OM) and the production of gas and end-products. Gas production of the fermenting cultures was recorded 21 times (from 2 to 24 h intervals) during the period of incubation using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA). The fermentation was stopped at 120 h by cooling at 4°C and the fermentation liquor was analysed for pH using a pH-meter (ThermoOrion 720 A+, Fort Collins, CO, USA) and sampled for end-product analysis. The extent of sample disappearance, expressed as organic matter degradability (OMD, %), was determined by weight difference of the incubated OM and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550°C for 3 h. The cumulative volume of gas produced after 120 h of incubation was related to incubated OM (OMCV, mL/g).

Regarding the determination of volatile fatty acids (VFA), the fermentation liquor was centrifuged at 12,000 g for 10 min at 4°C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Melle-Neuenkirchen, Germany) and 1 mL of supernatant was then mixed with 1 mL of oxalic acid (0.06 mol). The VFA were measured by gas chromatography (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy; fused silica capillary column 30 m, 0.25 mm ID, 0.25 µm film thickness), using an external standard solution composed of acetic, propionic, butyric, iso-butyric, valeric and isovaleric acids (Calabrò et al., 2005b).

4.3.4 Statistical Analysis

For each bottle, the gas production profiles were processed with a sigmoid model described by Groot et al. (1996):

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

where G is the total gas produced (mL/g of OM) at time t (h), A is the asymptotic gas production (mL/g of OM), B (h) is the time at which one-half of the asymptote is reached, and C is the switching characteristic of the curve. Maximum fermentation rate (R_{max}, mL/h) and the time at which it occurred (T_{max}, h) were also calculated according to the following formulas (Bauer et al., 2001):

$$T_{max} = C * [\frac{(B-1)^{\frac{1}{B}}}{(B+1)}]$$

$$R_{max} = \frac{A * B^{C} * T_{max}{}^{(B-1)}}{[1 + \left(C^{B} * T_{max}{}^{-B}\right)]^{2}}$$

The GLM and CANDISC procedures of the SAS software package version 9.2 (SAS, 2010) were used for the statistical analysis. Chemical characteristics, silage quality, and *in vitro* fermentation data were analysed by GLM procedure for repeated measures, with the effect of substrate (PPB with 0, 5, and 10% of straw added; only straw) as the principal factor. When a significant effect ($p < 0.05$) was detected, Tukey's test was used for means comparisons. A multivariate statistical approach was performed by a canonical discriminant analysis according to the CANDISC procedure, in order to ascertain the ability of chemical characteristics, silage quality and *in vitro* fermentation parameters to discriminate between the different samples. The general objective of Canonical Discriminant Analysis (CDA) is to distinguish among different populations using a particular set of variables (Mardia et al., 2000). Unlike cluster analysis, in CDA, the group to which each individual belongs is known. In this study, CDA was applied to discriminate feed substrate using chemical, silage quality and *in vitro* gas production parameters. Given the classification criterion (the substrate), CDA derives a new set of variables, the canonical functions (CAN), which are linear combinations of the original markers. The coefficients of the linea combinations are the canonical coefficients (CC), which indicate the partial contribution of each original variable. In this study, two canonical functions (CAN1 and CAN2) were derived. The statistical significance in group separation can be expressed by means of the Mahalanobis distance and the corresponding Hotelling's T-square test. Groups are declared significantly separated if the Hotelling's test shows a p-value < 0.05. This test can be developed only if the pooled (co)variance matrix of data is not singular. However, visual inspection of the CAN1 vs. CAN2 scatter plot and the values of distances among groups can be useful in assessing if groups are separated. CDA and the related tests were developed using the CANDISC procedure implemented in SAS software (2010)

4.4 Results

4.4.1 Micro Silos Quality and Composition

In Table 4.1 the parameters to evaluate the silage quality (pH, DM, N-NH_3) and the chemical composition of different substrates, including wheat straw are reported. The silage dry matter content varied from 26.46 to 28.42%, in PPB 5% and PPB 10%, respectively. The values of pH registered at the end of storage period varied from 3.85 to 3.99 in silages at 5 and 0% of straw inclusion, respectively. The ammonia nitrogen of residual fluid was below 16% in all the theses. The effect of substrate resulted significant ($p < 0.001$) for all chemical parameters considered. In particular, the straw showed mean values significantly ($p < 0.01$) different compared to all PPB silage, with the exception of ether extract. In particular, crude protein and ash contents were lower in straw, while among the structural carbohydrates, straw cellulose, and hemicellulose values resulted higher than silage samples, and an opposite result was shown for lignin. Observing PPB silage parameters, few significant differences appeared: only PPB silage with 5% of straw inclusion showed the lowest ($p < 0.01$) value for NDFom while the lignin content significantly ($p < 0.01$) decreased as the percentage of added straw increased.

Tab 4.1 Micro silos evaluation and chemical composition of by-products of prickly pears (PPB) silages and wheat straw.

		PPB Silage: Straw Percentages				
Parameters	**Units**	**0%**	**5%**	**10%**	**Straw**	**Substrate *p*-Value**
DM	%	27.68±0.42^{B}	26.46 ±0.27^{B}	28.42± 0.27^{B}	91.95 ±0.38^{A}	<0.001
pH		3.99±0.01^{A}	3.85 ± 0.01^{C}	3.96 ± 0.01^{B}	Nd	<0.001
$N\text{-}NH_3$	% TN	14.10 ±0.18^{B}	13.29±0.12^{C}	15.24±0.12^{A}	Nd	<0.001
CP	% DM	6.91±0.14^{Aa}	6.68±0.09^{Aab}	6.40±0.09^{Bb}	2.85±0.13^{C}	<0.001
Ether extract	“	6.13±0.39^{AB}	6.97±0.25^{A}	5.30±0.25^{B}	7.01±0.35^{A}	<0.001
NDFom		61.12±0.38^{B}	59.50±0.24^{C}	60.69±0.24^{B}	82.13±0.34^{A}	<0.001
ADFom	“	48.39±0.51^{B}	48.57±0.33^{B}	49.34±0.33^{B}	54.66±0.47^{A}	<0.001
Hemicellulose	“	12.73±0.44^{B}	10.94±0.29^{C}	11.34±0.29^{C}	27.48±0.40^{A}	<0.001
ADL	“	14.68±0.21^{A}	13.86±0.14^{B}	12.98±0.14^{C}	9.46±0.19^{D}	<0.001
Cellulose	“	33.71±0.40^{Cd}	34.70±0.26^{Cc}	36.36±0.26^{B}	45.19±0.37^{A}	<0.001
Ash	“	10.26±0.22^{A}	10.20±0.14^{A}	10.19±0.14^{A}	7.83±0.20^{B}	<0.001

PPB: Prickly pear by-products; DM: Dry matter; NH3-N (% TN): Ammonia nitrogen expressed as Total Nitrogen; CP: Crude Protein; NDFom: Neutral Detergent Fibre on organic matter basis; ADFom: Acid detergent fibre on organic matter basis; ADL: Acid Detergent Lignin; Nd: Not determined. Along the row different capital superscript letters indicate difference for $p < 0.01$; different lowercase superscript letters indicate difference for $p < 0.05$.

4.4.2 In Vitro Fermentation Characteristics

In Table 4.2, the *in vitro* fermentation characteristics are reported. The OMD and gas produced after 120 h of incubation (OMCV and A) were significantly ($p < 0.01$) lower for all silage samples compared to straw; for all these parameters, the lowest value ($p < 0.01$) was observed in PPB silage without straw added. Regarding the *in vitro* fermentation kinetics, PPB silage without straw presented the highest values ($p < 0.01$) for R_{max} and the lowest values for B and T_{max}, whereas these last parameters resulted higher for straw ($p < 0.01$). The differences between substrates in the fermentation process are clearer in Figures 4.1 and 4.2, where gas production rate and *in vitro* fermentation rate over time are shown. The curve of PPB 0% of straw reached the asymptote immediately (T_{max}: 1.92 and R_{max} 7.02; $p < 0.01$) and the fermentation process rapidly decreased after only 24 h of incubation. On the contrary, the curve related to straw fermentation was completely different; it reached half of the asymptote later (B: 19.60 h; $p < 0.01$) and the fermentation process continued for the 120 h of incubation. The gas production kinetics obtained incubating the PPB silages with 5 and 10% of straw showed a similar profile and, after 12 h of incubation, the process was in between the other two substrates.

Tab 4.2 Cumulative gas production, organic matter degradability and fermentation kinetics parameters of PPB silages and wheat straw

Parameters		**PPB Silage: Straw Percentages**			**Straw**	**Substrate**
		0%	5%	10%		*p*-Value
OMD	%	45.09 ± 1.00^{D}	56.10 ± 0.65^{B}	50.36 ± 0.65^{C}	60.36 ± 0.91^{A}	<0.001
OMCV	mL/g	132.68 ± 5.28^{C}	206.53 ± 3.41^{B}	211.93 ± 3.41^{B}	252.33 ± 4.82^{A}	<0.001
A	mL/g	120.92 ± 4.09^{D}	184.52 ± 2.64^{B}	163.57 ± 2.64^{C}	227.78 ± 3.73^{A}	<0.001
B	h	12.35 ± 0.47^{C}	18.59 ± 0.31^{B}	29.24 ± 0.43^{B}	19.60 ± 0.31^{A}	<0.001
C		1.25 ± 0.06^{C}	1.61 ± 0.04^{B}	1.53 ± 0.04^{B}	2.00 ± 0.06^{A}	<0.001
R_{max}	mL/h	7.32 ± 0.30^{A}	5.80 ± 0.23^{B}	5.38 ± 0.23^{B}	5.07 ± 0.33^{B}	<0.001
T_{max}	h	1.92 ± 0.55^{D}	7.91 ± 0.35^{B}	6.32 ± 0.35^{C}	16.84 ± 0.50^{A}	<0.001

PPB: Prickly pear by-products. OMD: Organic matter disappearance; OMVC: Cumulative volume of gas related to incubated organic matter; A: asymptotic gas production, B: is the time at which one-half of the asymptote is reached; C: switching characteristic of the curve; R_{max}: maximum fermentation rate; T_{max}: time at which R_{max} occurs. Along the row different capital superscript letters indicate difference for $p < 0.01$; different lowercase superscript letters indicate difference for $p < 0.05$

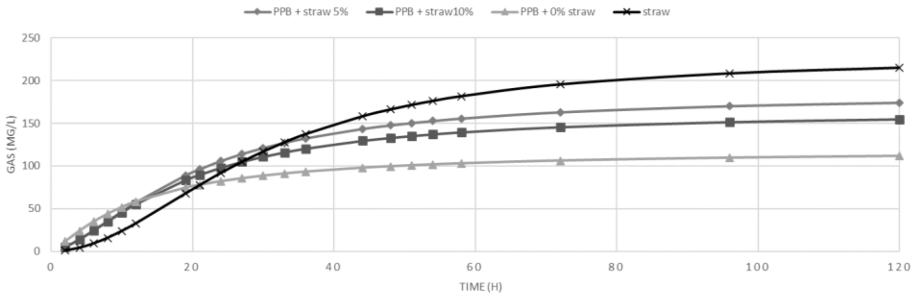

Fig 4.1 In vitro gas production over time of prickly pear by-products (PPB) silages and wheat straw.

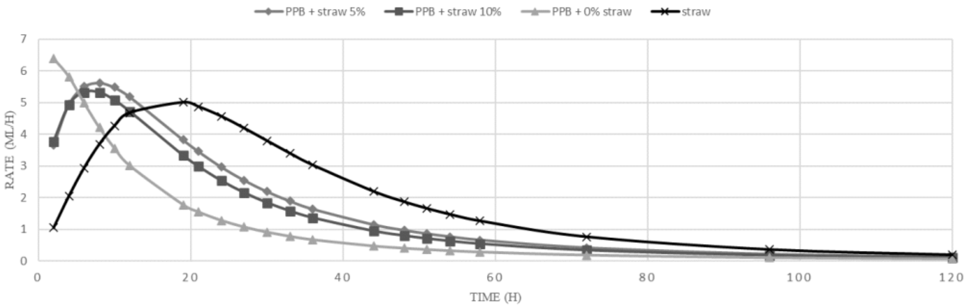

Fig 4.2 In vitro fermentation rate of prickly pear by-products (PPB)silages and wheat straw

Table 4.3 reports pH and end-products of fermentation registered after 120 h of incubation. For all PPB substrates, pH values were statistically higher ($p < 0.01$) than straw, with values higher than 6.60. For all incubated substrates, the VFA production was mainly due to the sum of acetate and propionate. The incubation of straw, compared to all the tested silages, produced the highest ($p < 0.01$) concentration of all VFA except for valerate, which was higher for PPB 5%. Comparing the silages, PPB with 5% of straw showed significantly higher production of all VFA ($p < 0.01$) except for butyrate. The PPB silage without straw showed the highest proportion of BCFA.

Tab 4.3 pH and in vitro fermentation end-products after 120 h of incubation of PPB silages and wheat straw

Parameters		**PPB Silage: Straw Percentages**			**Straw**	**Substrate**
		0%	5%	10%		*p*-Value
pH		6.86±0.07^{A}	6.75±0.04^{A}	6.87±0.04^{A}	6.63±0.06^{B}	<0.001
Acetate	mmol/g OM	34.75±1.60^{D}	46.17±1.04^{B}	42.51±1.00^{C}	59.64±1.47^{A}	<0.001
Propionate	“	11.24±0.51^{D}	17.52±0.33^{B}	15.24±0.33^{C}	21.90±0.47^{A}	<0.001
iso-Butyrate	“	0.35±0.01^{C}	0.39±0.01^{B}	0.32±0.01^{C}	0.49±0.01^{A}	<0.001
Butyrate	“	3.98±0.28^{Bc}	4.60±0.18^{Bb}	3.95±0.18^{Bc}	6.50±0.25^{Aa}	<0.001
iso-Valerate	“	0.49±0.03^{B}	0.67±0.02^{A}	0.48±0.02^{B}	0.75±0.03^{A}	<0.001
Valerate	“	0.76±0.05^{B}	1.03±0.03^{A}	0.75±0.03^{B}	0.54±0.05^{C}	<0.001
VFA	“	52.78±2.19^{D}	70.38±1.4^{B}	63.75±1.41^{C}	89.80±2.00^{A}	<0.001
BCFA	%VFA	1.62±0.06^{Aa}	1.52±0.04A^{Ba}	1.28±0.04^{Cc}	1.39±0.05B^{Cb}	<0.001

PPB: Prickly pear by-products. VFA: Volatile fatty acids; BCFA: Branched-chain fatty acids (iso-valerate + iso-butyrate/VFA × 100); OM: Organic Matter. Along the row different capital superscript letters indicate difference for $p < 0.01$; different lowercase superscript letters indicate difference for $p < 0.05$.

The multivariate statistical analysis results are shown in Tables 4.4 and 4.5 and Figure 4.3. Multivariate analysis confirms results of univariate analysis described. Moreover, these results allow a clear discrimination of different substrates in function of their chemical characteristics and *in vitro* fermentation parameters (Figure 4.3). Mahalanobis distances were statistically different between all centroids, separating the straw from the silage samples in a more marked way (Table 4.4).

Tab 4.4 Canonical discriminant analysis: Mahalanobis quadratic distances.

Substrate	**PPB 0% Straw**	**PPB 5% Straw**	**PPB 10% Straw**	**Straw**
PPB 0% straw	0	49 ($p < 0.001$)	69 ($p < 0.001$)	729 ($p < 0.001$)
PPB 5% straw		0	15 ($p < 0.001$)	955 ($p < 0.001$)
PPB 10% straw			0	956 ($p < 0.001$)
Straw				0

PPB: Prickly pear by-products.

Tab 4.5 Total canonical structure: Correlations between canonicals and original variables.

Variables	**1st Canonical Variable**	**2nd Canonical Variable**
Ash	0.890	−0.007
CP	0.976	0.069
Ether extract	−0.293	0.168
Cellulose	−0.954	−0.164
Hemicellulose	−0.981	0.115
Lignin	0.908	0.321
pH	0.419	0.011
OMD	−0.623	−0.375
OMCV	−0.622	−0.712
T_{max}	−0.888	−0.310
R_{max}	0.321	0.594
Acetate	−0.805	−0.319
Propionate	−0.746	−0.418
Butyrate	−0.795	0.004
Iso-butyrate	−0.778	0.081
Valerate	0.585	−0.089
Iso-valerate	−0.556	−0.060
BCFA	0.123	0.563
Explained variance (%)	93.8	5.3

PPB: Prickly pear by-products; CP: Crude Protein; OMD: Organic matter disappearance; OMVC: Cumulative volume of gas related to incubated organic matter; R_{max}: Maximum fermentation rate; T_{max}: Time at which R_{max} occurs, BCFA: Branched-chain fatty acids (iso-valerate + iso-butyrate/VFA × 100). In bold the heavier correlation coefficients are reported.

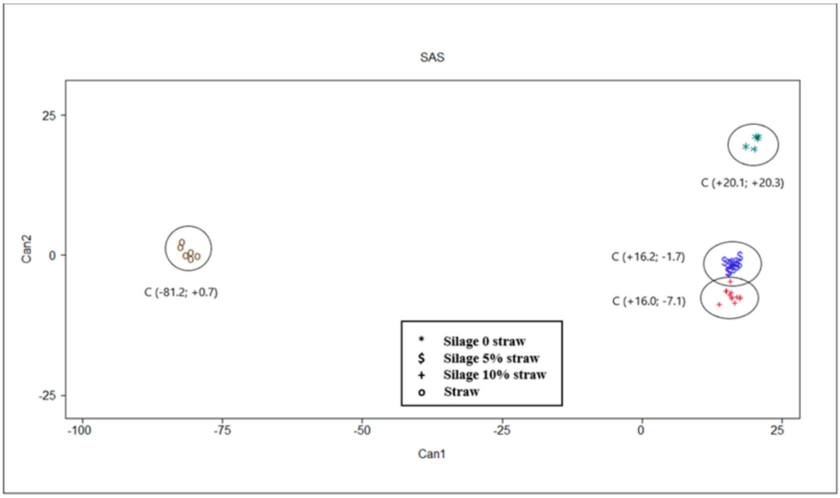

Fig 4.3 Plot from canonical discriminant analysis in which different feeds are distributed in function of canonical variables 1 and 2 based on chemical, silage quality and in vitro gas production parameters. Centroid coordinates in parentheses are reported

In Table 4.5, the correlations between canonicals and original variables are reported. About 94% of the total variance was explained by the first canonical variable (CAN 1), which was positively correlated with ash, CP, lignin and valerate, while it was negatively correlated with cellulose, hemicellulose, OMD, T_{max} and all VFA with the exception of valerate. The second canonical variable (CAN 2) explain 5% of total variance and it was positively correlated with R_{max} and BCFA and negatively correlated with OMCV. Only ether extract and pH after incubation parameters presented very low correlation coefficients with canonicals

4.5 Discussion

Regarding chemical composition, data related to straw are in line with data present in literature (Calabrò et al., 2005a). For prickly pears silage no data exist, but, in any case, our data were in line with those regarding fresh prickly pears by-products observed in a previous study (Todaro et al., 2020). The addition of straw guaranteed the preservation of higher soluble carbohydrates and CP in PPB silages, indicating a potential reduction in losses due to leachate. As regards the parameters considered to test the silage quality (DM and N-NH_3), the results showed good silage processing (Fantini, 2014) for PPB, especially when 5% of straw is added (significantly lower pH and ammonia nitrogen concentration).

Only a few data are present in literature on *in vitro* gas production for *Opuntia* spp. Batista et al. (2003) compared three cactus varieties (Gigante, IPA-20 and Miùda) using the *in situ* degradability and *in vitro* gas production techniques. They observed differences among species for chemical composition (CP and NDF; $p < 0.05$) and potential gas production after 48 h of incubation which was significantly higher for Gigante than for the Miuda or IPA-20 varieties, despite a similar lag-phase in gas production. They reported R_{max} and a potential gas production values close to that registered in this study. In our study, comparing the three PPB silages for the *in vitro* fermentation characteristics, the inclusion of 5% of straw seems to guarantee suitable chemical parameters (higher CP and ether extract content; lower NDF and ADF values) which contribute to the higher digestibility and VFA production.

The results of the multivariate statistical analysis confirmed that of univariate analysis. Indeed, the canonical discriminant analysis ascertained the ability to discriminate the substrates ($p < 0.001$). Particularly interesting were the correlation coefficients shown between canonicals and original variables. The first canonical variable, that explained most of the variability, seems to be linked to fermentation of the substrate into the rumen. The straw alone is placed on the left of the plot (Figure 4.3) contrary to the ensiled substrates which have higher values on CAN 1 (on the right of the plot). The latter seem to be less fermentable than straw, as they are associated with higher contents of ash (r = +0.890), lignin (r = +0.908) and proteins (r = +0.976) and consequently determine a lower quantity of VFA, correlated almost all negatively with CAN 1. The positive correlation between CP and CAN 1, together with lignin and ash, could be explained by a possible interference of some bioactive molecules, such as condensed tannins in chemical measurement of lignin using the conventional gravimetric method (Guglielmelli et al., 2011; Marles et al., 2008). Indeed, Marles et al. (2008) observed that in presence of condensed tannins the ADF-ADL technique overestimate the lignin content in comparison to alternative techniques such as thioglycolic acid lignin assay. On the other hand, the possible presence of condensed tannins in PPB (Sumaya-Martinez et al., 2011; Castrica et al., 2019) could reduce the rumen fermentability. As regards the gas production parameters, OMD and T_{max} resulted negatively correlated with the first canonical, and this fact showed that straw fermentation is slower than silages. The second canonical variable is able to discriminate between the silage with straw (5 and 10%) from that without straw, which ranks higher in the plot among the canonical discriminants (Figure 4.3) and is associated with the highest values of CAN 2. The gas production parameters R_{max} ($r = +0.594$) and OMCV ($r = -0.712$) are more capable of discriminating PPB silage. The presence of straw in the silage, especially in the amount of 5%, determines a more intense fermentative process, in terms of higher values of OMD, volume of gas, and VFA production (mainly acetate and propionate) but slowed down the fermentation kinetics (lower R_{max} and higher B and T_{max} values for PPB 5% compared to PPB 0% and 10%). These results could be due to a ‘dilution effect’ of the components (ether extract, lignin, ash) that can reduce the extent of the *in vitro* fermentations. On the other hand, a major presence of fermentable carbohydrates in silage without straw may have favoured a quick start of the fermentation process. Moreover, the absence of straw in PPB silage determines a higher BCFA ($r = +0.563$) production probably due to a major degradation of protein, and consequently a higher production of BCFA deriving from some amino acids (Macfarlane and Gibson, 2004) during the ensilage process.

4.6 Conclusion

From these preliminary results it is possible to demonstrate that ensilage is a suitable storage technique to preserve the nutritional characteristics of PPB. In particular, the addition of wheat straw to PPB seems useful as a way of reducing nutrient losses during ensiling. Both chemical parameters and *in vitro* fermentation data indicated the silage obtained with 5% of straw as the best preserved. This observation could also denote an economical advantage for the lower level of straw inclusion. In order to evaluate the palatability of PPB silages and the effects of their administration to dairy cows or dairy ewes on milk yield and quality, further studies are necessary

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Chapter 5

Chemical and nutritional characteristics of *Cannabis sativa* L. co-products

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Chapter 5 Chemical and nutritional characteristics of *Cannabis sativa* L. co-products

5.1 Abstract

Cannabis sativa L. is an annual herbaceous plant. It was used for centuries to obtain different products. In the last century, hemp cultivation was forbidden due to the psychoactive effects of tetrahydrocannabinol acid (THCA). In the last years, new strains, characterized by high cannabidiolic acid (CBDA) and low THCA level, were developed renewing the interest in hemp cultivation to obtain food or to extract essential oils from flowers. All these processes produce many residues with different chemical-physical characteristics. In order to evaluate their potential use also in animal nutrition, some hemp co-products were evaluated. Two different co-products of seed processes (flour and oil) and two co-products obtained trimming the flowers, differing in granulometry were used. The samples were analysed for chemical composition and evaluated *in vitro* using the gas production technique with buffaloes' ruminal *inoculum*. All hemp co-products showed interesting nutritional characteristics, such as crude protein content always higher than 20% on a dry matter basis, and high neutral detergent fibre concentration partially lignified. The *in vitro* gas production parameters at 120 h of incubation showed quite low fermentability testified by the low organic matter degradability and cumulative gas volume (OMD from 28.09 to 45.64% and OMCV from 110 to 164 ml/g, respectively). Also, the methane produced after 24 h of incubation was particularly low (from 1.78 to 11.73 ml/g dOM). These results could be due to the high lipid and ash amounts or to the CBDA content that probably affected the CH_4 formation processes. According to preliminary results obtained by this study, it is possible to hypothesize that these co-products could be useful to mitigate the methane production into the rumen. Further studies are necessary in order to evaluate the correct inclusion into the diet for ruminants.

5.2 Introduction

Hemp is a dicotyledonous plant from the family of *Cannabaceae*, genus Cannabis, and is considered one of the oldest domesticated crops. For several years, this plant was used for different purposes, such as clothing and shoes, cordages, carpets and tarps, maritime ropes, sails and nets, and paper production (Crini et al., 2020). However, in the last century, hemp cultivation was decreased due to several reasons. First, the competition with other natural fibres such as cotton and jute for textile applications, and the intensive development of synthetic fibres. Moreover, *Cannabis sativa* L. was confused with *Cannabis indica* L., known for its high level of Δ9-tetrahydrocannabinol acid (THCA), a chemical compound responsible for some psychoactive effects. This relation is, nevertheless, incorrect, indeed *Cannabis sativa* L. has <0.2-0.3% of TCHA (Milanovic et al., 2012; Żuk-Gołaszewska and Gołaszewski, 2018). Moreover, cultivars of *Cannabis sativa* L. are rich in cannabidiolic acid (CBDA), the main phytocannabinoid of this plant particularly concentrated in its products (fibre and seeds) (Fiorini et al., 2019).

Nowadays, the renewal of hemp cultivation was increased throughout the world. In Europe, the cultivation areas for hemp increased over 50% (from 12,232 to 24,939 ha) from 2008 to 2018 (FAOSTAT, 2020). Indeed, hemp could be considered a valid alternative to the conventional crops produced in excess due to its limited environmental impact (less use of water and land exploitation). Hemp provides many agricultural benefits, such as weed control, pest and disease resistance, fast growth, capacity to remove significant quantities of heavy metals from the soil (bioremediation), and to provide high biomass production with low inputs. These characteristics make *C. sativa* an adaptable, affordable, and ecological crop (Ranelli and Venturi, 2004).

The cultivation and processing of hemp have many purposes, especially in food production to obtain many products, such as decorticated seeds, flour, and oil. These products are considered sources of essential fatty acids and dietary fibre and are used as ingredients for several food preparations such as biscuits, bread, and protein powder. The seeds of *C. sativa* present on average 25-35% of lipids and 20-25% of proteins. The fatty acid profile of hemp seeds includes n-3 and n-6 essential fatty acids; in particular, the oil shows an abundance of α-linolenic acid providing a n-6/n-3 ratio nearly to

2.5- 3.0/1.0, within the range indicated as optimal for human dietary recommendations (Faugno et al., 2019). Additionally, hemp is also rich in natural antioxidants and other bioactive components (peptides, phenolic compounds, tocopherols, carotenoids and phytosterols) (Farinon et al., 2020). During the flowers and seeds processing to obtain hemp prepared food, various residues are produced. We have hypothesized that these co-products could be used also for ruminant nutrition considering their nutritional properties. The study aimed to characterize hemp co-products in terms of chemical composition and *in vitro* fermentation characteristics. The amounts of phytocannabinoids were also measured.

5.3 Material and methods

5.3.1 Sampling

All samples were collected in a storage shed, sited in the province of Caserta (South Italy). Totally four different hemp co-products were evaluated. All evaluated substrates derive from the same cultivar (*Futura 75*). This cultivar, particularly widespread in South Italy, is usually sowed, for seed production, in March-April and mechanically harvested in September-October. While the flowers were obtained from previous harvesting (July-September), immediately after, the apical portion of the plant was dried at 21-23°C for 10 days, subsequently, the flowers were mechanically separated (trimmed) and a lot of biomasses were discarded. Two co-products were obtained after trimming the flowers and sieving at different granulometry (rough_ FR and thin_FT). This biomass was partially chopped, and, for the evaluation, we have separated by sieving the chopped biomass into two portions, higher or lower than 4 mm. Considering one kilo of biomass 565 and 435 g of FR and FT were respectively obtained.

Two co-products were obtained from the seed processing, which was made within 20 days from the harvesting. SF was the hemp oil meal obtained by cold mechanical extraction while, SO was the flour of the seeds discarded because they were too small or too light.

5.3.2 Chemical composition

The samples were ground with a 1 mm screen and analysed for chemical composition (dry matter, crude protein, ether extract and ash) as reported by AOAC (2015) procedures (ID number: 2001.12, 978.04, 920.39 and 930.05 for DM, CP, EE, and ash, respectively). Neutral detergent fibre, acid detergent fibre and free ash acid detergent lignin were also determined as indicated by Van Soest et al. (1991).

5.3.3 Phytocannabinoid content assessment

The phytocannabinoid contents were detected at the University of Campania, Luigi Vanvitelli (Caserta, Italy), where the substrates were first ground using mortar and pestle in liquid nitrogen, and then extracted through an ultrasound-assisted maceration (UAM). For this purpose, an ultrasonic bath system was used (Branson UltrasonicsTM BransonicTM M3800-E, Danbury, CT, USA), with n-hexane as the extracting solvent. Three ultrasound cycles of 30 min each were carried out, with operating frequency set at 40 kHz, and solid to solvent ratio equal to 1:5. The obtained extracts were dried using a rotary evaporator (Heidolph Hei-Vap Advantage, Schwabach, Germany), and reconstituted in n-hexane for the UHPLC-ESI-QqTOF-MS and MS/MS analyses. Thus, the apolar hemp by-products extracts (10 mg/ml) were investigated using a Shimadzu NEXERA UHPLC system (Shimadzu, Tokyo, Japan) equipped with Luna Omega C18 column (50 × 2.1 mm i.d., 1.6 µm particle size). The mobile phase consisted of a binary solution composed of water (solvent A) and acetonitrile (solvent B), both acidified with formic acid (0.1% v/v). A linear gradient was used as follows: 0-1 min, 38% B; 1-5 min, 38→55% B; 5-10 min, 55% B; 10-12 min, 55→75% B; 12-14 min, 75→95% B; 14-15 min, 95% B. The total run time was 17 min with a flow rate of 0.5 ml/min; the injection volume was 2.0 µl. The AB SCIEX TripleTOF 4600 (AB Sciex, Concord, ON, Canada) system, equipped with a DuoSprayTM ion source, was combined with the UHPLC and was operated in the negative ESI mode. Data were collected by information dependent acquisition (IDA) using a TOF-MS survey scan of 100-1,000 Da (250 ms accumulation time) and eight dependent TOF-MS/MS scans of 80-800 Da (250 ms accumulation time). The MS parameters were as follows: curtain gas (CUR) 35 psi, nebulizer gas (GS 1) 60 psi, heated gas (GS 2) 60 psi, ion spray voltage (ISVF) 4.5 kV, interface heater temperature (TEM) 600° C and declustering potential (DP) -70 V. In TOF-MS/MS experiments, collision energy (CE) applied was -45 kV with a collision energy spread (CES) of 25 kV. The instrument was controlled by Analyst® TF 1.7 software (AB Sciex, Concord, ON, Canada, 2016), while data processing was carried out using PeakView® software version 2.2 (AB Sciex, Concord, ON, Canada, 2016). For quantitation purposes, the calibration curves of cannabidiolic acid (CBDA) and Δ9-tetrahydrocannabinolic acid (THCA), were constructed. Both the compounds were previously isolated and fully characterized by means of spectroscopic and spectrometric techniques (Formato et al., 2020). Thus, working solutions of each standard, prepared by dilution from a stock solution, were injected into the UHPLC- ESI-QqTOF MS system under the same conditions as the samples (Piccolella et al., 2020).

5.3.4 In vitro fermentation

All samples were incubated in a serum flask (seven replications per substrate ±1 g for each replication) with buffalo rumen fluid (10 ml) at 39° C under anaerobic conditions as indicated by Theodorou et al. (1994). The rumen liquor was collected, at slaughterhouse, from four buffaloes according to EU legislation (EU Council, 2004). The buffaloes fed a total mixed ration containing corn silage, oat hay and concentrate. All procedures involving animals were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II (Prot. 2019/0013729 of 08/02/2019). The collected rumen fluids were placed inside to pre-heated thermos and transported within 2 h to the laboratory of Feed Evaluation of University of Napoli, Federico II. The rumen fluid was mixed and strained through four layers of cheese cloths and diluted in a buffered medium (75 ml), successively, the reducing agent (4 ml) was added into the flasks (Vastolo et al., 2020). On seven replication, three bottles for each substrate were utilized for cumulative gas production measurement (120 h of incubation), the remaining were used for methane production evaluation (24 h of incubation).

The gas produced during 120 h of incubation, into the fermenting cultures, was recorded 21 times (from 2 to 24 h of intervals) using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA). The cumulative volume of gas produced after 120 h of incubation was related to incubated OM (OMCV, ml/g). At the end of the incubation period, the fermentation liquor was analysed for pH using a pH meter (ThermoOrion 720 A+, Fort Collins, CO, USA). The organic matter degradability (OMD, %) was determined by weight difference of the incubated OM and the undegraded filtered (sintered glass crucibles; Schott Duran, Mainz, Germany, porosity # 2) residue burned at 550° C for 3 h.

5.3.5 End-products measurement

In order to determine the volatile fatty acids (VFA), the fermentation liquor was cooled at 4° C and, before analyses, centrifuged at 12,000 g for 10 min at 4° C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Melle-Neuenkirchen, Germany); the supernatant (1 ml) was then mixed with 1 ml of 0.06 mol oxalic acid. The VFA was measured by gas chromatography (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy) equipped with a fused silica capillary column (30 m, 0.25 mm ID, 0.25 μm film thickness), using an external standard solution composed of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids. The percentage of branched-chain fatty acids were calculated as: (iso-butyric acid + iso-valeric acid/VFA)/100. The ammonia nitrogen ($N\text{-}NH_3$) production was assessed according to the colorimetric method proposed by Searle (1984).

5.3.6 Methane production evaluation

For each substrate, four flasks of seven were stopped at 24 h to measure the methane (CH_4) production as described by Guglielmelli et al. (2011); the relative end-products were also determined. The gas-phase from each flask was sampled (3 ml) in duplicate with a gastight syringe and injected into a gas chromatograph (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy), equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16-inch, 80/100 mesh).

5.3.7 Data processing

To estimate the fermentation kinetics, for each bottle stopped at 120 h, the gas production profiles were fitted to the sigmoidal model (Groot et al., 1996):

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

where G is the total gas produced (ml per g of incubated OM) at time t (h), A is the asymptotic gas production (ml/g), B is the time at which one-half of A is reached (h), and C is the curve switch. Maximum fermentation rate (R_{max}, ml/h) and the time at which it occurs (T_{max}, h) were calculated utilizing model parameters (Bauer et al., 2001):

$$\mathrm{T_{max}} = \mathrm{C} * [\frac{(\mathrm{B}-1)^{\frac{1}{\mathrm{B}}}}{(\mathrm{B}+1)}]$$

$$R_{max} = \frac{A * B^{C} * T_{max}{}^{(B-1)}}{[1 + \left(C^{B} * T_{max}{}^{-B}\right)]^{2}}$$

Statical analyses were performed by ANOVA for one-way (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989-2019) to evaluate the substrate effect.

In particular, the *in vitro* parameters concerning 120 h of incubation (OMCV, OMD, T_{max}, R_{max}) and the data related to the end products (pH, VFA, BCFA, N-NH_3) were statically analysed, as well as the results of the methane analysis (methane production as percentage of total gas, related to incubated organic matter, and related to degraded organic matter, respectively). The significance level was verified using HSD Tukey's test at $p < 0.01$ and $p < 0.05$.

The correlations between chemical composition values and fer- mentation parameters and between chemical compound and methane production were also evaluated (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989-2019

5.6 Results

5.6.1 Chemical composition

Table 5.1 shows the chemical composition of the tested samples. The co-products of hemp seeds presented a significantly higher ($p < 0.01$) level of crude protein (>30% DM) than flower co-products. The structural carbohydrates were quite high and variable for all the tested samples. Indeed, NDF values were higher ($p < 0.01$) in seeds' samples (>38% DM) than in flowers co-products (37 and 31% DM); lignin content was always higher than 6% DM. Moreover, both seeds co-products showed significantly higher ($p < 0.01$) values of all structural carbohydrates' fractions. While the ether extract content of flowers co-products was significantly ($p < 0.05$) higher than seed co-products. Ash content of both seeds co-products was significantly ($p < 0.01$) lower compared to flower ones.

Tab 5.1 Chemical composition in hemp co-products (n = 4)

	DM	**CP**	**NDF**	**ADF**	**ADL**	**EE**	**Ash**
Substrate	**%**			**% DM**			
SF	88.64^{B}	34.13^{A}	39.47^{BC}	28.83^{A}	11.41^{AB}	6.71^{b}	9.16^{A}
SO	91.11^{A}	32.09^{A}	39.86^{A}	29.81^{A}	11.93^{A}	8.79^{b}	6.98^{B}
FT	88.24^{B}	21.16^{B}	31.29^{C}	17.02^{C}	6.85^{C}	14.78^{a}	22.91^{A}
FR	88.01^{B}	20.23^{B}	36.85^{B}	21.76^{B}	7.41^{BC}	17.80^{a}	23.28^{A}
MSE	0.12	3.44	5.65	8.74	2.51	8.01	7.50

FR, refusal of trimming of flower (rough); FT, refusal of trimming of flower (thin); SF, refusal of flour production; SO, refusal of oil extraction. Note: Along the column, A, B, C: $p < 0.01$; a, b: $p < 0.05$. Abbreviations: NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; EE, ether extract; FR, refusal of trimming of flower (rough); FT, refusal of trimming of flower (thin); SF, refusal of flour production; SO, refusal of oil extraction; MSE: mean square error.

5.6.2 Phytocannabinoid content

UHPLC-HRMS analysis on a polar extract from the hemp co-products matrices provided qualitative information on their phytocannabinoids' content, whereas the main acidic phytocannabinoids, namely CBDA and Δ9-THCA, were quantized thanks to calibration curves available from the pure isolated reference compounds (Piccolella et al., 2020). CBDA and Δ9-THCA metabolites showed the deprotonated molecular ion ($[M-H]^-$) ion at m/z 357.21 according to the molecular formula $C_{22}H_{30}O_5$. The constitutional isomers were identified based on their TOF-MS/MS fragmentation pattern and their relative retention time (Formato et al., 2020; Piccolella et al., 2020). In particular, FR and FT extracts contained a greater amount of the two compounds with respect to SO and SF samples (Table 5.2). This could be due to the plant part origin, which was the aerial part for both FR and FT samples, and the fruit (also known as hemp seed) for SO and SF samples. The lowest content of both phytocannabinoid was detected in the flours obtained after the oil extraction from the hemp seed. However, FR and FT extracts showed a high diversity in phytocannabinoids. Indeed, it was observed that FR and FT extracts contained, beyond CBDA and Δ9-THCA, other acidic phytocannabinoids such as CBDA-C4 and cannabivarinic acid with respective [M-H]− ion at m/z 343.1906 and 329.1755. Moreover, cannabifuranic acid (CBFA), cannabinolic acid (CBNA) and cannabinodiolic acid (CBNDA), which share the [M-H]− ion at m/z 353.17 ($C_{22}H_{26}O_4$), were also identified. Furthermore, TOF-MS experiment detected eleven compounds with the [M-H]− ion at m/z 373.20 ($C_{22}H_{30}O_5$), among which TOF-MS/MS data allowed us to recognize cannabielsoic acids (CBEA-C5 A and B) and epoxy-phytocannabinoid acids. Considering the relative areas, these metabolites represented the 57.94% in FR and 49.28% in FT. This latter sample also contained cannabigerolic acid (CBGA), 6,7-epoxycannabigerolic acid ($C_{22}H_{32}O_5$) and its neutral form, 6,7-epoxycannabigerol ($C_{21}H_{32}O_3$) in a relative percentage equal to 2.8%. Finally, five metabolites with [M-H]− ion at m/z 389.19, which could be O6-type phytocannabinoids, were three-fold higher in FR than in FT extract.

Tab 5.2 Cannabidiolic and Δ9 -tetrahydrocannabinol acids amount (average ± SD) in hemp co-products extract (n = 4)

	Compounds	
Extract	**CBDA mg/g**	**Δ^9 -THCA mg/g**
SF	2.80 ± 0.21	0.011 ± 0.0003
SO	4.45 ± 0.76	0.089 ± 0.004
FT	19.00 ± 1.7	1.850 ± 0.090
FR	16.50 ± 1.3	1.250 ± 0.050

Abbreviations: FR, refusal of trimming of flower (rough); FT, refusal of trimming of flower (thin); SF, refusal of flour production; SO, refusal of oil extraction; THCA, Δ^9 -tetrahydrocannabinol acid; CBDA, Cannabidiolic acid.

5.6.3 In vitro fermentation characteristics

The *in vitro* fermentation characteristics and kinetics are reported in Table 5.3. The refusal of flour production (SF) showed OMD values significant higher ($p < 0.05$) compared to FR. The cumulative gas production parameter showed highly significant differences between substrates ($p < 0.01$). Thin refusal of trimming flower (FT) resulted in the highest ($p < 0.01$) OMCV values and SO the lowest. Regarding the kinetic parameters (T_{max} and R_{max}), the *in vitro* fermentation rate of all hemp co-products was lower than 7 ml/h and the maximum rate was reached at the beginning of the incubation, within 5 h. In particular, FT presented the highest fermentation rate ($p < 0.01$) compared to SO and FR. Whilst SO showed a significantly high ($p < 0.01$) T_{max} values compared to the other substrates. All these findings were better represented in Figures 5.1 and 5.2.

Tab 5.3 In vitro fermentation characteristics in hemp coproducts (n = 3)

Substrate	**OMD** **%**	**OMCV** **ml/g**	**R_{max}** **ml/h**	**T_{max}** **h**
SF	45.65^{a}	149.91^{B}	6.33^{A}	3.60^{B}
SO	35.63^{ab}	110.11^{C}	4.66^{B}	4.95^{A}
FT	30.08^{b}	164.13^{A}	4.86^{AB}	3.00^{B}
FR	28.09^{b}	136.37^{B}	3.21^{C}	2.35^{B}
MSE	4.97	6.24	0.54	0.52

Note: Along the column, different capital superscript letters indicate difference for $p < 0.01$; different lowercase superscript letters indicate difference for $p < 0.05$. MSE: mean square error. Abbreviations: SF, refusal of flour production; SO, refusal of oil extraction; FR, refusal of trimming of flower (rough); FT, refusal of trimming of flower (thin); OMD, organic matter degradability; OMVC, cumulative volume of gas related to incubate organic matter; R_{max}, maximum fermentation rate; T_{max}, time at which R_{max} occurs.

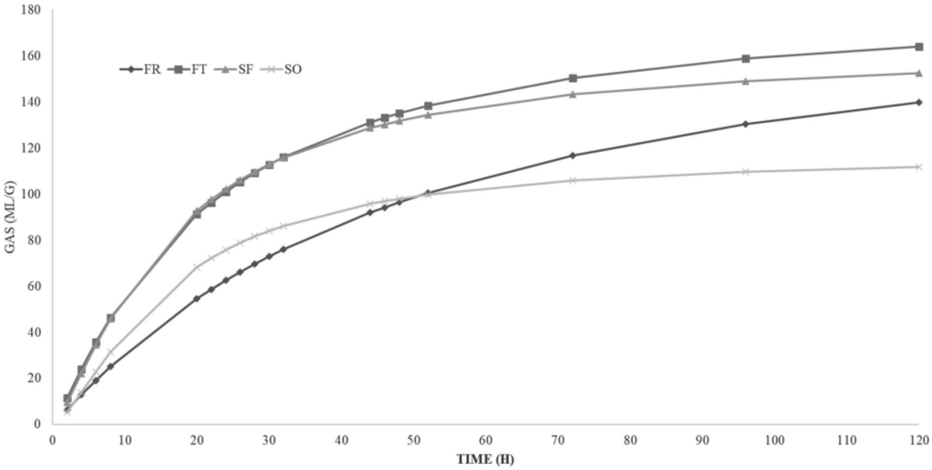

Fig 5.1 In vitro gas production over time in hemp co-products

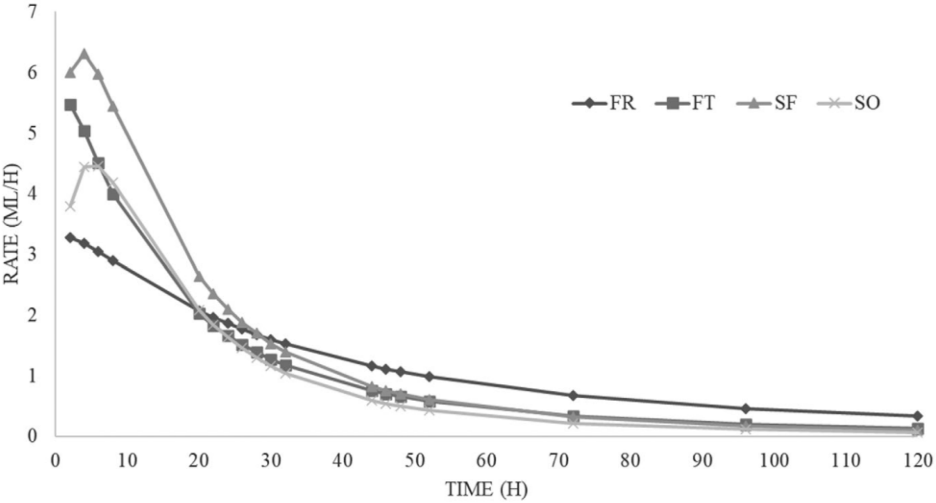

Fig 5.2 In vitro fermentation rate in hemp co-products

5.6.4 End-products parameters after 120h of incubation

In Table 5.4 the *in vitro* end-products parameters are reported. No statically differences between the tested substrates for pH values emerged. Volatile fatty acid production differs between substrates. Particularly, SO showed the lowest VFA value (75.58 Mmol/g OM; $p < 0.01$) and highest values of BCFA (9.60% VFA; $p < 0.01$) and N-NH_3 (62.69 mg/g OM; $p < 0.01$). Acetate and propionate were the most representative VFA of all the substrates; they represented 80% of the total. SO showed the lowest proportion of acetate (56.71% VFA) while SF had the lowest proportion of propionate (18.47% VFA). The flower co-products showed significantly higher ($p < 0.01$) acetate and propionate production compared to seeds co-products. The ratio acetate/propionate was significantly higher for SF compared to the other samples. Regarding butyrate production, the seeds co-products showed the highest production compared to flowers co-products.

Tab 5.4 In vitro fermentation end products evaluated after 120 h of incubation (n = 6)

Substrate	**pH**	**VFA**	**NH_3**	**Ace**	**Prop**	**iso-But**	**But**	**iso-Val**	**Val**	**BCFA**	**A/P**
		Mmol/gOM					**%VFA**				
SF	6.47	92.05^{A}	54.4^{B}	67.36^{A}	18.47^{B}	1.86^{B}	7.12^{AB}	3.45^{B}	1.74^{B}	5.31^{B}	3.64^{A}
SO	6.44	75.58^{B}	62.69^{A}	56.71^{B}	22.59^{A}	3.49^{A}	7.27^{A}	6.16^{A}	3.82^{A}	9.60^{A}	2.56^{B}
FR	6.44	99.60^{A}	50.55^{B}	64.27^{A}	23.57^{A}	1.72^{B}	6.15^{BC}	2.62^{BC}	1.81^{B}	3.42^{C}	2.75^{B}
FT	6.47	98.04^{A}	51.97^{B}	65.16^{A}	24.65^{A}	1.19^{C}	5.31^{BC}	2.21^{C}	1.22^{C}	5.12^{B}	2.64^{B}
MSE	0.002	36.47	3.88	3.52	5.04	0.08	0.51	0.28	0.05	0.0027	0.10

Note: Along the column, different capital letters indicate difference for $p < 0.1$. Abbreviations: SF, refusal of flour production; SO, refusal of oil extraction; VFA, total volatile fatty acids; Ace, acetate; A/P, ratio between acetate and propionate; FR, refusal of trimming of flower (rough); Prop, propionate; iso-But, isobutyrate; But, butyrate; iso-val, iso-valerate; BCFA, branched-chain fatty acids; FT, refusal of trimming of flower (thin); N-NH3, ammonia; VFA, total volatile fatty acids; Val, valerate; MSE, mean square error.

5.6.5 Methane production

The methane production registered after 24 h of incubation, the organic matter degradability and relative VFA values are reported in Table 5.5. The refusal of flour production resulted in the highest ($p < 0.01$) CH_4 values when reported as a percentage of total gas and related to incubated organic matter. If methane production was related to OMD, FT and SF showed significantly higher methane production. On contrary, FR showed the lowest ($p < 0.01$) CH_4 production to all parameters. After 24 h of incubation, FR and SF showed the highest ($p < 0.01$) levels of OMD, while FT and SF showed the highest ($p < 0.01$) levels of total VFA. The refusal of oil extraction showed the lowest ($p < 0.01$) value for both parameters.

Tab 5.5 In vitro methane production and main volatile fatty acids values obtained after 24 h incubation (n = 4)

Substrate	**pCH_4 %Total gas h**	**iCH_4 ml/g iOM**	**dCH_4 ml/g OMD_{24}**	**$OMD_{24\ h}$ %**	**$VFA_{24\ h}$ Mmol/g OM**
SF	8.24^{A}	3.88^{A}	10.39^{A}	37.7^{A}	40.47^{AB}
SO	6.43^{AB}	1.34^{C}	5.80^{B}	23.22^{B}	34.87^{B}
FT	5.56^{B}	2.00^{B}	11.73^{A}	27.18^{AB}	53.24^{A}
FR	4.80^{B}	0.56^{D}	1.78^{B}	31.46^{A}	38.76^{B}
MSE	0.11	0.08	0.75	3.32	5.20

Note: Along the column, different capital letters indicate difference for $p < 0.01$. SF, refusal of flour production; SO, refusal of oil extraction; FT, refusal of trimming of flower (thin); FR, refusal of trimming of flower (rough); pCH_4, methane production as percentage of total gas; $OMD_{24\ h}$, organic matter degradability after 24 h of incubation; dCH_4, methane production related to degraded organic matter; iCH_4, methane production related to incubated organic matter; $VFA_{24\ h}$, total volatile fatty acids after 24 h of incubation; MSE, mean square error.

5.7 Discussion

Regarding chemical composition, the data of hemp co-products of this study agreed to that reported in literature (Alaru et al., 2011; Gibb et al., 2005; Vonapartis et al., 2015) for flower and seeds co-products. In this regard, hemp co-products chemical composition, particularly of seeds, could be comparable to soybean meal ones (Semwogerere et al., 2020). Indeed, taking into count the high protein content and considering the high amount of essential amino acids reported by House et al. (2010) for similar substrates, all tested co-products could be evaluated as a source of high-quality protein. Moreover, the crude protein amount of hemp co-products is higher than the endorsed ruminant dietary requirement for maintenance and growth (CSIRO, 2007). In particular, these co-products seem to be able to satisfy the nutritional requirements for buffaloes in early lactation as indicating by Bartocci et al. (2002). As reported by Mustafa et al. (1999) the hemp oil meal obtained by mechanical oil extraction could be considered excellent natural sources of rumen undegradable protein. These authors included hemp oil meal in the diet for sheep (20% DM) and did not observe a detrimental effect. No data in the literature were present about protein degradability into the rumen of hemp discarded seeds and flower co-products. In particular, these last showed interesting protein and NDF contents comparable to legume forages usually utilized in ruminant nutrition such as berseem clover (Sabia et al., 2015; Tsiobani et al., 2019). The high content of structural carbohydrates could make available a high energy level in these substrates. Nevertheless, the particularly high amount of lignin could limit the availability of these resources (House et al., 2010). However, the active molecules present in the hemp might cause an overestimation of lignin, as suggested by Marles et al. (2008) for substrates rich in condensed tannins. Similarly, the ether extract values could be influenced by the high content of resins in hemp flowers (Formato et al., 2020). Both flowers' co-products showed higher amounts of acidic phytocannabinoids. CBDA and THCA were the most abundant, whereas some other derivatives with higher oxygenation degrees or with different alkyl chains were detected thanks to the sensitivity of high-resolution spectrometric tools. These results are according to the observation of Kleinhenz et al. (2020) which detected six different cannabinoids in a different portion of the hemp plant such as stalks, flowers, leaves and seeds. These authors indicated that phytocannabinoids were present in all plant fractions and that CBDA and THCA were always the most concentrated. It is fair to mention that the determination of the qualitative profile in phytocannabinoids and the quantization of the individual substances in the raw materials selected for feed (panel cake, oil, flour and hemp fibre) is mandatory according to EU Reg. 1017/2017. The compositional analyses of cannabinoid levels will be useful to define the admissibility of raw materials based on hemp for animal feed, since the compliance of the raw material, already defined in Reg. (EC) 767/09, takes into account a maximum content of Δ9-THCA equal to 0.2%. The ash amount registered in the study for all samples was higher compared to the literature data (Callaway, 2004; Semwogerere et al., 2020) in particular for FR and FT. A lot of factors such as botanical species, type of soil, fertilization, harvesting and processing methods could affect the mineral profile of samples (McDonald et al., 2011).

Regarding the *in vitro* gas production, unlike the chemical composition, there are no more studies about hemp (*Cannabis sativa* L.) plants and/or co-products. Only Kleinhenz et al. (2020) studied the *in vitro* degradability of different portions of the hemp plant (whole plants, stalks, unprocessed female flowers, whole seed heads, dried leaves, chaff and processed female flowers). They concluded that seed heads, chaff, and leaves had the lowest undegradable NDF amount after 240 h of incubation and observed a percentage of degradability similar to that registered in this study. In particular, the higher acetate and propionate production registered in rumen fermentation liquor of flower co-products could indicate that the structural carbohydrates of these substrates were better fermented compared to seeds co-products ones. Concerning of the kinetics of fermentation, all substrates showed the maximum fermentation rate (ml/h) in the first 5 h of incubation and after it decreased rapidly within 20 h of incubation. The higher concentration of fat of both flower co-products could have negatively affected their fermentation processes.

The substrates SO and SF showed the highest OMD and the lowest OMCV values. The most intense fermentation could be due to the high crude protein concentration probably, considering that protein fermentation did not produce gas (Abreu and Bruno Soares, 1998). When the microorganisms are not able to ferment the carbohydrates, they must use other nutrients such as protein, in order to survive. Also, the highest BCFA proportion an N-NH_3 production registered by these substrates after 120 h of incubation could indicate a protein fermentation. Moreover, the higher values of BCFA and ammonia were significantly related to the protein content (BCFA vs. CP: r = 0.813, p < 0.05 and N-NH_3 vs. CP: r = 0.727, p < 0.05); indeed, nocorrelation was found between BCFA and N-NH_3 production, as described by Davila et al. (2013) this could be due to the specific amino acids profile. In this regard, iso-butyrate and iso-valerate production are the result of branched-chains amino acids metabolism, such as valine, leucine, and isoleucine; they can be hydrolysed and fermented to phenols, and biogenic amines (i.e., indole, skatole, 4-ethylphenol, p-cresol) (Musco et al., 2018). Semwogerere et al. (2020) reported the amino acid profile of seeds and different co-products indicating that leucine and valine were among the main represented amino acids in hemp.

The shape representing the fermentation rate of the hemp co-products indicates that FR is a quite slow substrate, probably due to some component that does not facilitate the enzymatic attack by microorganisms. On the contrary, FT and SF show a rapid fermentation process, due to an easily fermentable component. About the SO, the curve is in an intermediate position.

Regarding the methane production, the data of this study seem particularly low compared to that one obtained in previous studies (Calabrò et al., 2012; Guglielmelli et al., 2011) and reported by other authors (Tuyen et al., 2013; Elghandour et al., 2016), probably due to the different experimental condition in terms of hours of incubation. The limited methane production could be ascribed to specific nutritional characteristics of hemp co-products or to the phytocannabinoids presence. The negative relation (EE vs. CH_4: r = -8792; $p < 0.05$) between ether extract content and CH_4 (% total gas) production observed in this study, suggest an inhibiting role of the lipid fraction on methanogenesis. In literature, only a few studies are referred to these substrates. Particularly, Wang et al. (2017) measured the *in vitro* methane production of diets characterized by different lipids sources (seeds of safflower, poppy, hemp and camelina vs. coconut and linseed). These authors demonstrated that the use of safflower and hemp seeds was more efficient than linseed in abating the level of methanogenesis. They suggested that the results were not affected by n-6/n-3 fatty acids or C18:2/C18:3 ratios. Moreover, they suggest a possible role of uncommon fatty acids (C18:3, n-6 and C18:4, n-3) present in hemp and camelina seeds on methanogens microbes. Shibata and Terada (2010) indicated that the supplementation of unsaturated fatty acids (linoleic, α-linolenic, and oleic acids) in the ruminant’s diet inhibits CH_4 production into the rumen.

A significant correlation between OMCV and both phytocannabinoids concentrations (CBDA vs. OMCV, r = 0.548; $p < 0.01$; TCHA vs. OMCV, r = 0.617; $p < 0.01$) could indicate a positive interference between *in vitro* fermentation and these compounds. No correlations were observed between OMD and cannabinoids or methane production and cannabinoids. All these results suggest that acidic phytocannabinoids, mainly CBDA and TCHA could affect the fermentation pathways without limiting the organic matter degradability.

5.8 Conclusion

These preliminary results underline how the use of residues of hemp processing could be a valid nutrient resource in the diet of ruminants. Indeed, hemp co-products showed interesting nutritive values as demonstrated by the results of the chemical composition. However, *in vitro* data (i.e., OMD and OMCV) indicate as these substrates are quite low utilized by the rumen microbes. However, the low methane values suggest as these samples could be used in the ruminant diets to contain gas emission, and therefore, the environmental impact. Further studies are needed to evaluate if it is possible to improve the hemp structural carbohydrates utilization fermentability and to investigate the mechanisms that determine the trend registered *in vitro*. Moreover, could be interesting to conduce *in vivo* studies to understand in what doses these co-products could be included in ruminants' diet and if they could be useful to mitigate the environmental impact of intensive farm.

5.9 References

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Chapter 6

Hemp Seed Cake as Novel Ingredient for Dog's Diet

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6.1 Abstract

In the last few years, the popularity of industrial hemp and its products is increased. From a nutritional point of view, hemp and its products are rich in protein, polyunsaturated fatty acids, vitamins, and useful minerals. Nowadays, the European Commission authorizes the use of hempseed and hempseed oil co-products in animal nutrition. This study is aimed to evaluate the use of hempseed cake in dogs' nutrition, comparing the effect of the supplementation of two lipid sources: swine tallow (T-diet) and hempseed cake (H-diet). A double-blind nutritional trial was performed at a municipal kennel located in Naples. Eight crossbreed neutered dogs recognized in good health were recruited and divided into two homogeneous groups (T- vs. H-group). Both diets were analysed for chemical composition and fatty acid profile. Blood count and biochemical profile were evaluated at recruitment (T0) and the end of the trial (T30). Oleic, palmitic, and stearic acids were the most representative fatty acids in both diets; however, the H-diet contains more than double concentration of linoleic and α-linoleic acids compared to the T-diet ($p < 0.01$). The H-diet has shown significantly ($p < 0.01$) higher peroxidation index as the only negative aspect, which could compromise its shelf-life. After 30 days of administration, the H-group has shown a significant ($p < 0.01$ and $p < 0.05$) reduction of liver and renal markers [aspartate transferase (AST), alanine transaminase (ALT), and creatinine] and cholesterol, due to the healthier fatty acid profile. Hempseed cake seems a suggestable source of polyunsaturated fatty acids for dogs considering these preliminary results.

6.2 Introduction

In the middle of the 20th century, the *Cannabis sativa* L. hemp variety has been unfairly abandoned because of its similarity to *Cannabis indica* L., which was illegal. However, the popularity of industrial hemp and its products increased in the last few years (Aladic et al., 2016). Indeed, nowadays, it can be possible to cultivate hemp varieties containing <0.2% Δ-9-tetrahydrocannabinol (THCA) (EU, 2013). In this scenario, food production is the main purpose of cultivation (Crimaldi et al., 2017). Hemp and its products are rich sources of protein, polyunsaturated fatty acids (PUFA), vitamins, and useful minerals as evidenced by Callaway (2004). Hemp seeds contain 25-35% of oil (della Rocca and Di Salvo, 2020), which have shown a favourable fatty acid profile. Indeed, it contains linoleic acid (LA, C18:2 n-6) and α-linolenic acid (ALA, C18:3 n-3) and most of n-6 and n-3 PUFA (Da Porto et al., 2012). Regarding the use of hemp products in animal nutrition, the European Food Safety Authority (EFSA) allows the administration of hempseed and hempseed co-products as feed ingredients for all animal species with species-specific differences in diet’s rate inclusion (EFSA, 2011). Moreover, hemp oil can be used as a supplement in feed mixtures for animals as a rich source of essential fatty acids, while hempseed and hempseed cakes can be used as fat and protein sources in animals’ diets (della Rocca and Di Salvo, 2020).

The study aims to evaluate the use of hempseed cake as a source of lipids in dogs’ diets. For this purpose, the effect of diets supplemented with two lipid sources (swine tallow vs. hempseed cake) was compared.

6.3 Materials and methods

6.3.1 Animals and Diets

A double-blind nutritional trial has been performed at a municipal kennel located in Naples. The kennel was affiliated with the Department of Veterinary Medicine and Animal Production, University of Naples, Federico II, and with the National Health Service (ASL NA1 Nord). Eight crossbreed neutered dogs [age 5 ± 1.77 years, weight 16.66 ± 6.38 kg; body condition score (BCS) 4.38 ± 0.18 on a nine-point scale] were recognized in good health and recruited after clinical evaluation and haematological, biochemical, and parasitological tests. The dogs were divided into two groups [swine tallow (T-group) and hempseed cake (H-group)] homogeneous for gender, body weight, and BCS. Each dog was housed in an individual 12 m^2 box (3 × 4) divided between a closed rest section (1 × 2) and an open area. A canned commercial diet was supplemented with the same amount (3.5% as feed) of swine tallow or hempseed cake. The latter was collected in a storage shed, which is sited in the province of Caserta (South Italy). The experimental period has lasted 40 days (10 days of adaptation and 30 days of trial) from June 8, 2021. Each diet [swine tallow (T-diet) and hempseed cake (H-diet)] was administered twice daily in a ratio of 110 kcal/$kg^{0.75}$ of metabolizable energy (ME) (FEDIAF, 2020).

6.3.2 Diet Chemical Composition and Fatty Acid Profile

An aliquot of 500 g for each diet was collected twice for chemical composition (AOAC, 2005) and fatty acid profile assessment. Total fat was previously extracted (Folch et al., 1957) from each sample and subsequently turned into methyl esters (FAMEs) by direct transesterification (Christie, 1993) to analyse fatty acid profiles. FAMEs were dissolved in n-hexane, filtered, and injected into a gas chromatographic system (Focus GC, Thermo Electron Corporation, Waltham, Massachusetts, USA) with a flame ionization detector (FID) for FAMEs assessment according to Oteri et al. (2021). Fatty acid peaks were identified and quantified by comparison with an external standard composed of pure FAMEs mixture (Larodan Fine Chemicals, AB, Limhamnsgardens Malmo, Sweden). The peroxidation index (PI) was calculated using an equation (Luciano et al., 2013), where the singular acids are reported as percentage (%): PI = (dienoic × 1) + (trienoic × 2) + (tetraenoic × 3) + (pentaenoic × 4) + (hexaenoic × 5).

6.3.3 Clinical Findings

A blood sample (±10 ml) was collected to determine blood count and biochemical profile at recruitment (T0) and the end of the trial (T30), after an overnight fasting period. Blood count samples were refrigerated and quickly transported to the clinical analysis laboratory of the Department of Veterinary Medicine and Animal Production of the Federico II-University of Naples. Each blood sample was analysed using an impedance device to perform an instrumental count (HeCo 5 Vet C, Real Time Diagnostic Systems: San Giovanni a Valdarno, Italy) after slow and constant mixing for 20 min. The following parameters were analysed: red blood cell (RBC) count, haematocrit (HCT), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MHC), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), reticulocytes (Ret), reticulocyte haemoglobin content (CHr), leukocytes (Leu), lymphocytes (Lymphs), monocytes (Mono), eosinophils (Eos), basophils (Baso), and platelets (PLT).

The gel separator tubes were left at room temperature for about 15 min until a clot formed. After that, the samples were centrifuged for 10 min at a speed of 1,500 × g to obtain the serum. All procedures were performed at the kennel. The serum was stored at -80°C and sent with dry ice to a reference laboratory (Kornwestheim, Germany). There, the following parameters were determined using a Beckman biochemical analyser (Beckman Coulter AU5400; Olympus America, Melville, NY, USA): globulin, total protein (TP), albumin (Alb), alkaline phosphatase (AP), glutamic pyruvic transaminase (GPT), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), aspartate transferase (AST), glutamate dehydrogenase (GLDH), fructosamine (Fr), glucose (GLU), α-amylase, lipase (LP), sodium (Na), potassium (K), calcium (Ca), chloride (Cl), phosphorus (P), magnesium (Mg), cholesterol (CHOL), triglycerides (Tri), creatinine (CREA), urea (BUN), creatine kinase (CK), and cortisol (Cort).

6.3.4 Parasitological Examination

All dogs, upon their arrival in the kennel, were screened for intestinal helminths and protozoa and, if positive, were treated with antiprotozoal and/or anthelmintic drugs, before the beginning of the study. Then, stoolsamples from all dogs included in the study were collected at the beginning of the study (T0), after 14 days (T14), and at the end of the trial (T30) for copromicroscopical examinations. All samples were analysed using the FLOTAC dual technique (Cringoli et al., 2010) with sodium chloride (specific gravity, s.g. = 1.20) and zinc sulphate (s.g. = 1.20) as flotation solutions and an analytic sensitivity of 2 eggs/oocysts/cysts/larvae per grams of faeces (EPG/OPG/CPG/LPG).

6.3.5 Statistical Analysis

All statistical analyses were performed using the software JMP 14 (SAS Institute, NC, USA). T-test was used to analyse the differences between the fatty acid profiles of the two tested diets, while Wilcoxon non-parametric test was used to analyse the effect of the diets and time on blood count and biochemical profile due to the low number of subjects. The level of significance was $\alpha = 0.05$; for all variables, mean values and standard deviation of multiple analyses are reported.

6.4 Results

No significant differences have been observed for body weight and BCS between groups and/or periods after 1 month of diet administration. Daily feed intake for all dogs corresponded to the administered ratio (110 kcal ME/kg$^{0.75}$). Only two dogs during the second week of the trial left about 30% of daily ratio. This variation could probably be due to the sudden change in climatic conditions (during the week: mean T_{min} = 22.00 ± 0.53° C; T_{max} = 29.57 ± 1.18° C, and humidity = 64.85% ± 3.23%) registered in the week, because, subsequently, these dogs also consumed all the administered ratio.

6.4.1 Chemical Composition and Fatty Acid Profile

The H-diet has shown higher crude protein and crude fiber content and lower ether extract amount compared with the T-diet (Table 6.1).

Tab 6.1 Chemical composition (mean and standard deviation) of administered diets.

Nutrients (% as feed)	Diet* (n = 4)	T-diet (n = 4)	H-diet (n = 4)
Moisture	75.02±0.25	67.35±0.26	66.09±0.31
CP	11.81±0.18	10.54±0.73	11.53±0.02
CF	4.14±0.20	2.99±0.15	3.52±0.35
EE	5.55±0.56	8.91±0.60	7.09±0.02
Ash	3.25±0.21	3.15±0.07	3.70±0.14
NFE	13.29±0.36	6.95±1.11	8.00±0.22
ME (kcal/kg)#	1,350.3	1,286.4	1,369.4

*Commercial diet was reported as term of comparison, and it was not statistically analysed.
#Calculated according to modified Atwater's factors.
T-diet, tallow diet; H-diet, hemp diet; CP, crude protein; CF, crude fiber; EE, ether extract; NFE, nitrogen free extract.

In Table 6.2, the fatty acid profiles of the administered diets and commercial diet are shown. In the H- and T-diets, oleic acid was the most representative fatty acid (C18:1 cis 9: 28.44% vs. 35.72% TFAs, respectively). Swine tallow shows higher ($p < 0.01$) values of saturated fatty acids (palmitic C16:0, margaric C17:0, myristic C14:0, pentadecanoic C15:0, and behenic C22:0). Moreover, γ-linolenic (GLA C18:3 n-6) and docosadienoic (C22:2 n-6) acids were higher ($p < 0.01$ and $p < 0.05$, respectively) in the diet supplemented with swine tallow than that supplemented with hempseed cake. The percentage of LA was higher in the H-diet than the T-diet ($p <$

0.01). Regarding the other fatty acids recommended for dog nutrition, α-linolenic (ALA C18:3 n-3) resulted in more than double amounts in the H-diet compared to the T-diet ($p < 0.01$). Moreover, the H-diet had higher amount of trans-vaccenic (TVA C18:1 trans 11), butyric (C4:0) ($p < 0.01$), and caproic (C6:0) ($p < 0.05$) acids than the T-diet.

Tab 4.2 Fatty acid profiles (% of total fatty acids) of tested diets.

Fatty acids	**Diet***	**T-diet**	**H-diet**	***p*-value**
C4:0	3.23±0.03	2.11±0.07	2.68±0.02	0.0082
C6:0	0.69±0.05	1.61±0.03	2.37±0.07	0.0246
C8:0	0.05±0.007	0.04±0.004	0.15±0.003	0.0005
C14:0	1.97±0.09	1.77±0.01	0.79±0.09	<0.0001
C15:0	0.17±0.009	0.18±0.008	0.06±0.001	0.0066
C16:0	21.3±0.53	23.6±0.15	17.7±0.04	0.0003
C17:0	3.46±0.18	2.31±0.08	1.71±0.07	0.0069
C17:1	0.46±0.02	0.46±0.01	0.69±0.09	0.0675
C18:1 cis6	0.22±0.015	0.25±0.02	0.09± 0.07	0.0125
C18:0	8.6±0.57	15.7±0.09	15.4±0.10	0.0816
C18:1 trans 11 (TVA)	0.95±0.06	1.23±0.01	1.90±0.04	0.0019
C18:1 cis 9	35.5±0.033	35.7±0.17	28.4±0.56	0.0032
C18:1 cis 10	0.16±0.03	0.29±0.02	0.38±0.05	0.0277
C18:1 cis 11	0.01±0.007	0.02±0.002	0.01±0.005	0.1641
C18:2 cis n-6 (LA)	18.4±0.34	9.96±0.06	20.9±0.09	<0.0001
C20:0	0.15±0.01	0.19±0.05	0.37±0.03	0.0141
C18:3 n-6 (GLA)	0.04±0.001	0.49±0.03	0.23±0.01	0.0093
C18:3 n-3 (ALA)	1.79±0.01	1.30±0.02	3.53±0.02	<0.0001
C20:2 n-6	0.09±0.07	0.32±0.01	0.15±0.02	0.0064
C22:0	0.27±0.02	1.12±0.01	0.16±0.01	0.0001
C20:3 n-6	0.07±0.05	0.04±0.01	0.04±0.02	0.9357
C20:3 n-3	0.20±0.02	0.22±0.01	0.61±0.03	0.0026
C20:4 n-6 (AA)	0.28±0.004	0.18±0.02	0.12±0.02	0.0691
C22:2 n-6	0.36±0.01	0.29±0.01	0.08±0.03	0.0107
C24:0	0.06±0.003	0.09±0.03	0.06±0.01	0.0695
C20:5 n-3 (EPA)	0.12±0.002	0.15±0.006	0.18±0.004	0.1214
C24:1	0.02±0.003	0.02±0.005	0.15±0.005	0.0006

*Commercial diet was reported as term of comparison, and it was not statistically analyzed. T-diet, tallow diet; H-diet, hemp diet; C4:0, butyric acid; C6:0, caproic acid; C8:0, caprylic acid; C14:0,myristic acid; C15:0, pentadecylic acid; C16:0, palmitic acid; C17:0,margaric acid; C17:1, heptadecenoic acid; C18:1 cis6, petroselinic acid; C18:0, stearic acid; C18:1 trans 11, trans vaccenic acid (TVA); C18:1 cis 9, oleic acid; C18:2 cis n-6, linoleic acid (LA); C20:0, arachidic acid; C18:3 n-6, α -linolenic acid (GLA); C18:3 n-3, α-linoleic acid (ALA); C20:2 n-6; C22:0, behenic acid; C20:3 n-6; C20:3 n-3, dihomo α-linolenic; C20:4 n-6, arachidonic acid(AA); C22:2 n-6, docosadienoic acid; C24:0, lignoceric acid; C20:5 n-3, eicosapentenoic (EPA).

The differences between fatty acid profiles of the diets have been shown in Table 6.3. As observed previously, the T-diet had higher levels of saturated (SFA) and monounsaturated (MUFA) fatty acids ($p < 0.01$), while the H-diet had higher ($p < 0.01$) levels of PUFA of both categories (n-6 and n-3). Moreover, the H-diet resulted in significantly lower n-6/n-3, LA/ALA, and AA/eicosapentenoic acid (EPA) ratios ($p < 0.01$ and $p < 0.05$, respectively). Otherwise, the T-diet had a lower ($p < 0.01$) PUFA/SFA ratio. The PI was significantly ($p < 0.01$) higher in the H-diet compared to the T-diet.

Tab 6.3 Categories of fatty acids (% of total fatty acids) of the administered diets.

Categories	Diet*	T-diet	H-diet	p-value
SFA	40.5±0.53	48.9±0.19	41.5±0.10	0.0004
MUFA	37.8±0.42	38.3 0.18	31.9±0.60	0.0048
PUFA	21.2±0.38	12.8±0.09	25.9±0.01	<0.0001
n-6	19.2±0.36	11.3±0.09	21.6±0.002	<0.0001
n-3	2.01±0.01	1.57±0.01	4.32±0.01	<0.0001
PUFA/SFA	0.52±0.002	0.26±0.008	0.62±0.002	<0.0001
n-6/n-3	9.57±0.11	7.18±0.05	4.99±0.01	0.0003
LA/ALA	10.3±0.10	7.18±0.04	5.93±0.03	0.0004
AA/EPA	13.3±1.35	3.47±0.10	0.69±0.03	0.0418
PI	82.5±0.35	66.1±0.02	88.2±0.58	0.0004

*Commercial diet was reported only as data, and it was not statically analysed.
T-diet, tallow diet; H-diet, hemp diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LA/ALA, linoleic acid/α-linolenic acid; AA/EPA, arachidonic acid/eicosapentaenoic acid; PI, peroxidation index

6.4.2 Clinical Findings

All values of blood count (data not shown) fall in the physiological range for the species. No significant differences were observed between groups and sampling periods in blood count parameters. The number of reticulocytes was the only one that was significantly lower in the animals fed with the T-diet than in those fed with the H-diet (38.8 ± 1.15 vs. 70.1 ± 1.30 K/µl; $p < 0.05$).

Tab 6.4 Main biochemical parameters

Parameters	Units	T-group	H-group	T0	T30	*p*-value	
						Diet effect	Time effect
AP	U/l	35.3±4.0	25.6±2.0	51.0 ± 9.2	19.4±3.55	0.7144	0.0080
CREA	µmol/l	103±8.55	87.0±11.0	96.8±12.7	95.2±13.6	0.0270	0.8538
AST	U/l	49.6±6.66	30.7±3.01	40.6±11.0	38.2±11.8	0.0058	0.5200
ALT	U/l	43.3±4.50	29.0±3.56	38.2±6.98	37.0±10.4	0.0101	0.9163
BIL	µmol/l	3.12±0.28	2.19±0.83	1.70±0.92	3.19±1.09	0.1483	0.0136
CHOL	Mmol/l	5.59±0.45	4.41±0.63	4.82±0.77	5.14±0.85	0.0057	0.4747
GLU	Mmol/l	5.04±0.48	5.13±0.39	4.77±0.36	5.33±0.30	0.9489	0.0197
CK	Mmol/l	143±10.4	82.0±5.9	116±11.8	121±12.7	0.0105	0.9168
Cl	Mmol/l	113±3.25	112±3.27	110±2.41	114±2.54	0.6976	0.0177

AP, alkaline phosphatase; CREA, creatinine; AST, aspartate transaminase; ALT, alanine aminotransferase; BIL, bilirubin; CHOL, cholesterol; GLU, glucose; CK, creatine kinase; CL, chloride.

All biochemical parameters were within the normal reference ranges for dogs. For brevity, only statically significant results were reported (Table 6.4). Comparing the groups, animals fed with the T-diet showed higher levels of AST and CHOL ($p < 0.01$) and CREA, ALT, and CK ($p < 0.05$). Considering the time effect, AP significantly decreased during the trial ($p < 0.01$). Otherwise, after 30 days, bilirubin (BIL), glucose, and Cl were higher ($p < 0.05$) than at the beginning of the trial.

6.4.3 Parasitological Examination

All dogs tested negative for all endoparasites throughout the trial

6.5 Discussion

6.5.1 Chemical Composition and Fatty Acid Profile

Both diets guarantee minimal requirement of macronutrients suggested for adult dogs located in a kennel (FEDIAF, 2020). The registered value of feed intake seems to indicate that the diets were palatable. Indeed, the temporary reduction of feed intake shown by two dogs, one per group, during the first week of July seems related to the climatic condition variation.

Regarding the fatty acid profile of the two tested diets, hempseed cake supplementation seems to increase the percentage of PUFA into the commercial diet, especially linoleic (LA C18:2 n-6) and α-linoleic (ALA C18:3 n-3) acids. The increase could be due to the high percentage of polyunsaturated fatty acids in hempseed cake (Mierlietặ, 2018; Juodka et al., 2018). The swine tallow diet appears to be influenced by the chemical composition of the supplementation like the H-diet. The T-diet has shown a high percentage of SFA and MUFA. The saturated fat present in tallow could be useful for the companion animals as source of energy to work, regulate body temperature, growth, and reproduction. Moreover, these fats could be stored in adipose tissues for future mobilization and used for energy when needed (Bauer, 2008). Otherwise, hempseed cake is rich in LA and ALA (Baioloni et al., 2021; Klir et al., 2019), which are essential for dogs considering that this species cannot synthesize LA and ALA *ex novo*. Linoleic acid (LA 18:2 n-6) was the only essential fatty acid listed for dogs by the National Research Council until recently (NRC, 2006). Both types of omega fatty acids can be converted to longer chain polyunsaturated fatty acids that have additional essential functions (Bauer, 2008). Particularly, ALA could be converted to EPA and docosahexaenoic (DHA) acids, which are necessary for dog. Indeed, n-6 and n-3 fatty acids operate as precursors of the eicosanoids, which are important to the cell functions (Bauer, 2008). Consequently, it is necessary to include LA and ALA in dogs' diets (Orlandi et al., 2021). The differences between fatty acid categories affected the ratio between categories, and these changes could influence human (Luciano et al., 2013) and animal (NRC, 2006) health. The hempseed cake diet has shown an n-6/n-3 ratio lower than 5:1, which has been claimed as ideal for humans and dogs (Simopoulos, 2008). Polyunsaturated fatty acids are very important for the development of the nervous system, and an optimal dietary n-6/n-3 fatty acid ratio reduces the incidence of some diseases, such as cancer and sudden cardiac death (Biagi et al., 2004). Moreover, it is important to have adequate amounts of DHA and EPA, which are useful for neurologic development, particularly during the early stage of growth. In our case, the level of ALA in the hempseed cake diet is sufficient to allow the conversion to EPA and DHA. In this regard, Bauer (2007) observed that puppies that suckle ALA-rich milk appear to accumulate DHA in their plasma for a short time prior to weaning. Furthermore, the presence of PUFA in the diet influences growth and health status of dogs, helps to increase general metabolic rates, and may promote the burning of fat (Leizer et al., 2000).

The presence of n-3 PUFA in the diet allows obtaining other benefits such as anti-inflammatory and potentially anti-thrombotic properties (Ahlstrom et al., 2004). In particular, a higher amount of n-3 fatty acids could have a positive effect, especially in dogs with pruritus and other inflammatory diseases (Abba et al., 2004). Indeed, essential fatty acids from n-6 and n-3 families are proven to have anti-inflammatory effects and immunomodulating properties on skin diseases (Bosch et al., 2008). Furthermore, dietary PUFA seem to affect animal behavioural changes. Indeed, the dopaminergic and serotonergic systems in the brain are known to play important roles in learning, emotions, and impulse control. Both these systems are known to be influenced by PUFA; thus, it is necessary to feed animals with the right amount of polyunsaturated fatty acids in the diet (Bauer et al., 1994).

However, the higher percentage of PUFA could limit diet shelf-life as demonstrated by the increased peroxidation index of the H-diet with respect to the T-diet. Diets that contain a high amount of PUFA must be protected from light and high temperatures that could cause their oxidation during the production process and storage (Biagi et al., 2004).

6.5.2 Clinical Findings

All dogs were in good health at the end of the trial as evidenced by clinical evaluation (body weight and BCS) and blood parameters. The increase of chloride and AP could be difficult to explain as well as the rise in reticulocytes in the dogs fed with the H-diet.

The animals in the H-group have shown lower levels of cholesterol, AST, ALT, creatinine, and CK compared to those fed with the T-diet. These results could suggest a beneficial effect of diet supplemented with hempseed cake due to the high amount of PUFA, particularly the linoleic and α-linolenic acids. In this regard, dietary supplementation with PUFA, in particular ALA, may be a useful addition to treatment in renal disease, rheumatoid arthritis, cutaneous inflammatory disorders, thromboembolic disease, and autoimmune diseases (Bauer et al., 1994).

All liver markers (AST and ALT) and cholesterol decreased by the presence of both n-6 and n-3 PUFA. Furthermore, these results partially agreed with the one's obtained by Welch-White et al. (2013), who observed the reduction of several serum parameters, such as AST, ALT, GGT, cholesterol, and triglycerides, with the increasing level of PUFAs administered with the diets in rats. Moreover, Kaushal et al. (2020) evidenced the specific anti-hypercholesterolemic effects of hemp seed related to redox-sensitive modulation of inflammatory pathways in rats, which prevent fat deposition into the liver and arterial lumen.

The higher PUFA concentration of the H-diet could have also caused the significant reduction of creatinine, considered a biomarker of renal function. Brown et al. (1998) observed in short-term studies in dogs with naturally occurring renal disease that supplementation with n-6 PUFA led to an increased glomerular filtration rate (GFR). The same authors detected that dietary supplementation with n-3 PUFA decreased glomerular capillary pressure and seems to be renoprotective. On that basis, n-3 PUFA, particularly EPA and DHA, and their precursor (ALA) could be useful in the management of dogs with naturally occurring renal diseases.

It is possible to hypothesize that both lipidic supplementations of the diet had modified the dogs' serum lipidomic profile as suggested by Boretti et al. (2020), even if in this study the lipidomic serum was not analysed.

6.5.3 Parasitological Examination

All dogs screened tested negative for intestinal helminths and protozoa during the entire trial. That may be due to a regular monitoring and subsequent treatment of dogs as suggested by the guidelines of the European Scientific Counsel Companion Animal Parasites (ESCCAP, 2018; ESCCPA, 2021). Moreover, good management and hygiene practices (Fiechter et al., 2012; Ursache et al., 2019) were used as a complementary program to achieve effective control of endoparasitosis in dogs.

6.6 Conclusion

It seems possible to suggest the use of this co-product as a polyunsaturated fatty acid source for dog nutrition from these preliminary results, despite the lack of proximate analysis data of hempseed cake. Even if the increased peroxidation index of the diet supplemented with hemp co-product seems to indicate that, further studies are necessary to better identify the strategies to prevent the oxidation of hempseed cake during pet-food production and storage.

Despite the low number of recruited subjects and the lack of lipidomic serum analysis, the improvement of several haematological parameters, such as cholesterol, renal, and hepatic biomarkers, confirmed that the addition of hempseed cake and its enriched PUFA profile are safe for dogs to consume.

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Chapter 7

General Discussion and Conclusion

7.1 Use of by-products in swine nutrition

Chemical composition and *in vitro* gas production data allow to consider the by-products analysed in relation to their specific nutritional characteristic for different uses in swine nutrition, estimating the potential use along the digestive tube. Moreover, the results showed in chapter 3 indicate that citrus by-products (pulps and molasses) were highly fermentable. The pulps might improve microbiota and enterocytes homeostasis, whereas, the molasses, being rich in soluble sugars and could increase diet energy density. Whilst, olive oil by-products resulted in high amount structural carbohydrates, particularly in lignin, and lipids and could be useful in fattening pigs nutrition. All these results confirm that the use of by-product could be a strategy to reduce the environmental impacts of swine production as indicated by Elferink et al. (2008); Mackenzie et al. (2016); Meul et al. (2012); Van Zanten et al. (2015); Beshir et al. (2017).

7.2 Preservation method

The results obtained in chapter 4 demonstrate that ensilage is a suitable technique to preserve prickly pears by-products nutritional characteristics. Furthermore, fermentative process was improved with the presence of straw in the silage, especially in the amount of 5%, that which would appear to be able to limit leaching losses. As described by Monllor et al. (2020) ensiling is a way to preserve perishable products rich in lignocellulose. Choong et al. (2007) suggested that during ensiling, the bioactive components, such as polyphenols, could lose their beneficial effect on animal health (e.g., antioxidant potential) but could improve the nutritional value of animals' products (Łozicki et al., 2015).

7.4 Hemp co-products evaluation

The renewed interest in hemp (*Cannabis sativa* L.) cultivation in Europe was triggered by several developments (economic, environmental, agronomic, and social) (Struik et al., 1999). Hemp is rediscovered as an interesting 'new' crop with a large plasticity, which allows it to be grown under a wide variety of agro-ecological conditions (Van der Werf et al., 1996) that may contribute to the sustainability of arable farming. Moreover, hemp has been classified as an attractive crop, which produces a wide variety of renewable resources for the development of multi-output systems through stepwise breakdown of biomass into several useful components. Also relevant is the use of some hemp seed derivatives (e.g., oil and flour) as novel food with beneficial effects for humans and animals (Peiretti, 2009).

7.4.1 ruminants

The chemical composition obtained by hemp co-products resulted in interesting nutritive values indicating that the tested co-products seem to be able to satisfy buffaloes early lactation nutritional requirements. Hemp co-products could be considered as a source of high-quality protein as well as high energy source due to the high content of structural carbohydrates. However, *in vitro* data indicate as these substrates are quite low utilized by the rumen. However, a positive correlation between phytocannabinoids and the gas production parameters suggest how the former may affect the fermentation pathways without limiting the organic matter degradability.
Regarding the presence of phytocannabinoids, all substrates resulted in low level and the differences in CBDA and TCHA between flower and seeds could be ascribe to different plant part origin.

7.4.2 dogs

Regarding the use of hempseed cake in dogs' nutrition. It seems possible to suggest the use of this co-product as a source of polyunsaturated fatty acid. Indeed, when the hempseed cake is included in the diet the level of n-3 and n-6 fatty acids increase. In this regard, essential fatty acids from n-6 and n-3 families are proven to have anti-inflammatory effects and immunomodulating properties on skin diseases. Furthermore, dietary polyunsaturated fatty acids seem to affect the dopaminergic and serotonergic systems in the brain which have a role in learning, emotions, and impulse control consequently essential fatty acids seem influenced animal behavioural changes. Moreover, all values of blood count and biochemical parameters fall in the physiological range for the species.

The higher level registered in peroxidation index could suggested that high percentage of polyunsaturated fatty acids could limit diet shelf-life. In this context, it is worth considering that diets with high content of lipids must be protected from light and high temperatures that could cause their oxidation during the production process and storage.

7.5 Further studies

7.5.1 In vitro evaluation of bioactive extract

Some of the by-products that have been tested for this thesis contain bioactive molecules (e.g., phytocannabinoids from hemp, tannins, and antioxidants from prickly pears). The extraction of these molecules from by-products could help reduce waste. Indeed, the reuse of waste would limit the amount of material that has to be discarded, leaving the raw materials for their original use. It would be necessary to evaluate the effects of these molecules on animal health. Some extracts could have effects on ruminal fermentation, limiting the production of methane and carbon dioxide. Other molecules could have a bacteriostatic or bactericidal action and their use could limit the utilization of antibiotics in livestock and companion animal breeding. Lastly, other molecules, such as antioxidant, mono- and poli-unsaturated fatty acids could be used to improve the nutritional properties of animal products.

7.5.2 In vivo trials

Several studies have shown the reliability of *in vitro* results for estimating the nutritional value of various feedstuffs in different animal species. However, some aspects such as the palatability and the effects on animal products quality can be confirmed only through specific *in vivo* feeding trials. Also, the potential beneficial effects on animal welfare and health status should be confirmed by this kind of trial. For instance, several authors investigated on the use of hempseed meal or hempseed cake in ovine and bovine, but only few studies evaluated the effect of the use of hemp flowers co-products in animal nutrition.

7.5.3 Strategies for the conservation of co-products

Most of agri-food co-products are obtained in the harvest seasons of primary products. Their use in animal feed would require less limited availability. In chapter four ensiling with wheat straw supplementation has been proposed as useful method to preserve prickly pears co-products. However, for other co-products further methods of preservation, perhaps industrial, might be more appropriate.

Different studies evidenced for some by-products that the species and the processing methods could significantly affect the nutritional characteristics. The use of industrial techniques could limit the nutritional heterogeneity of by-products. Therefore, the effectiveness and sustainability of some industrial processes, such as pelleting, extrusion and dehydration, should be evaluated in order to preserve their nutritional characteristics of each by-product.

7.5.4 Assessment of ecological and economic advantages in co-products utilization.

Numerous studies emphasized that the use of by-products can be a strategy for limiting the environmental impact of production. The reuse of waste is target 12 in 2030 Agenda of Union Nations. In this regard, the use of co-products in animal nutrition is an opportunity of recycling. The livestock environmental impact may be reduced by feeding agricultural co-products to livestock, as this transforms inedible products for humans into edible products such as meat and milk. Alternative feed sources are required to improve the environmental and economic sustainability of current animal production systems and to reduce the competition for cropland between the feed and food sectors. Nevertheless, co-products could also have more applications, such as bioenergy production. Therefore, for each co-product should be necessary a specific ecological and economic assessment in order to evaluate the most adequate reuse strategy. For this purpose, several new biophysical methodologies have been developed and the most used are the measurement of benefits of CO_2 capture and utilization and the life cycle assessment that evaluate the physical or causal relationships between inputs and outputs (i.e., co-products). These aspects are particularly important when novel coproducts, such as that derived from hemp production, were evaluated. Indeed, the biomass derived from seeds and flower production could be useful for other applications (e.g., as renewable energy source in biogas production or thermal insulator materials in buildings) and it is necessary to understand which application is more sustainable in ecological and economic terms.

Moreover, a specific cost/benefit analysis would be necessary in order to evaluate the effectiveness in economic terms of the nutritional planes.

7.6 References

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