Enhancing the nutritional value of olive cake for livestock: effects of soybean meal, barley, molasses, and lactic acid bacteria supplementation

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Index

List of figures

List of tables

List of Acronyms

ADF	Acid detergent fiber
ADL	Acid detergent lignin
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BA	Barley
BSM	BioStabil Mays
CF	Crude fat
COC	Crude olive cake
CP	Crude protein
DM	Dry matter
EE	Ether extract
EU	European Union
hoLAB	Homofermentative Lactic Acid Bacteria
IOC	International Olive Council
ISO	International Organization for Standardization
LAB	Lactic Acid Bacteria
LA	Lactic acid
ME	Metabolizable Energy
MO	Molasses
MRS	Man, Rogosa and Sharpe
NDF	Neutral Detergent Fiber
NPN	Non-protein Nitrogen
NS	Not significant
OC	Olive cake
OL	Olive leaves
OM	Organic matter
RSM	Response Surface Methodology
SO	Soybean meal
SW	Sample weight
TDN	Total digestible nutrient
WSC	Whole soluble carbohydrates

Abstract

Sheep farming represents a key sector in Portuguese agriculture, with 2.208 million animals across 16 indigenous breeds yet faces rising feed costs and resource efficiency challenges. Olive cake, a by-product of Portugal's annual olive oil production (180,000–200,000 tons), offers a sustainable feed alternative, though limited by high fiber content and low protein. This study aimed to optimize olive cake's nutritional quality through biotechnological enhancement using Response Surface Methodology (RSM) with a Box-Behnken design. Thirteen formulations combining soybean meal, barley, and molasses (2%, 4%, 6%) with lactic acid bacteria (LAB) inoculation were tested across 117 samples, analyzing dry matter, organic matter, ash, neutral detergent fiber (NDF), crude protein (CP), and crude fat. The optimal formulation (6% soybean meal, 4% barley, 6% molasses) achieved 15.7% CP, a 124% increase over untreated olive cake, with 91.2% organic matter and 53.6% NDF. Soybean meal significantly enhanced CP ($p < 0.001$), while barley and molasses increased organic matter and reduced ash ($p < 0.001$); NDF remained stable (~60%), indicating supplementation limitations on fiber modification. These results validate olive cake as a protein-enriched, sustainable feed for Portuguese sheep, leveraging local resources. Future research should explore in vivo digestibility, animal performance, economic analysis, and advanced microbial strategies to address fiber content.

Keywords: olive cake, sheep nutrition, alternative feed, response surface methodology, Box-Behnken design, lactic acid bacteria, soybean meal, barley, molasses, crude protein, neutral detergent fiber, agro-industrial by-products.

Resumo

A ovinocultura representa um setor chave na agricultura portuguesa, com 2,208 milhões de animais de 16 raças autóctones, enfrentando elevados custos de alimentação e desafios de eficiência de recursos. O bagaço de azeitona, subproduto da produção anual de azeite de Portugal (180.000–200.000 toneladas), oferece uma alternativa alimentar sustentável, embora limitado pelo alto teor de fibra e baixa proteína. Este estudo visou otimizar a qualidade nutricional do bagaço de azeitona através de aprimoramento biotecnológico utilizando Metodologia de Superfície de Resposta (RSM) com delineamento Box-Behnken. Treze formulações combinando farelo de soja, cevada e melaço (2%, 4%, 6%) com inoculação de bactérias ácido-láticas (BAL) foram testadas em 117 amostras, analisando matéria seca, matéria orgânica, cinzas, fibra em detergente neutro (FDN), proteína bruta (PB) e gordura bruta. A formulação ótima (6% farelo de soja, 4% cevada, 6% melaço) alcançou 15,7% PB, um aumento de 124% sobre o bagaço não tratado, com 91,2% de matéria orgânica e 53,6% FDN. O farelo de soja aumentou significativamente a PB ($p < 0,001$), enquanto cevada e melaço elevaram a matéria orgânica e reduziram cinzas ($p < 0,001$); a FDN permaneceu estável (~60%), indicando limitações da suplementação na modificação da fibra. Estes resultados validam o bagaço de azeitona como alimentação sustentável enriquecida em proteína para ovinos portugueses, aproveitando recursos locais. Pesquisas futuras devem explorar digestibilidade in vivo, desempenho animal, análise econômica e estratégias microbianas/enzimáticas avançadas para reduzir fibra.

Palavras-chave: bagaço de azeitona, nutrição ovina, alimentação alternativa, metodologia de superfície de resposta, delineamento Box-Behnken, bactérias ácido-láticas, farelo de soja, cevada, melaço, proteína bruta, fibra em detergente neutro, subprodutos agroindustriais.

I. INTRODUCTION

The shortage of animal feed represents a significant limitation to increasing sheep production in many regions. However, large quantities of underexploited agricultural by-products, such as olive cake, are generated annually. Incorporating cost-effective, unconventional feed resources, specifically agro-industrial by-products, into animal diets may help address the feed deficit, reduce feeding expenses, and mitigate environmental pollution (Moustafa *et al*., 2008). The use of such by-products in animal nutrition can also decrease the demand for conventional feed ingredients, thereby supporting global food security (Nasopoulou & Zabetakis, 2013).

Olive cultivation (*Olea europaea*) is well adapted to Mediterranean climates, which are characterised by hot, dry summers, mild winters with rare frosts, and an average annual rainfall of approximately 800 mm (Abazi *et al*., 2013). The two-stage olive oil extraction process generates substantial by-products; notably, for every 1,000 kg of olives processed, approximately 800 kg of semi-solid waste is produced (Molina-Alcaide & Yáñez-Ruiz, 2008). While olive cake has been used in ruminant nutrition, its broader application is limited by its low nutritional value, high levels of neutral detergent fiber (NDF) and acid detergent fiber (ADF), the presence of condensed tannins, seasonal availability, and the poor degradability of its cell wall components.

Barley (*Hordeum vulgare L.*) is another important crop in many arid and semi-arid regions, widely cultivated for both its grain and straw, which serve as feed for ruminants, including sheep, particularly during winter ((Nefzaoui, 1983; Martín García *et al*., 2003; Teimouri Yansari *et al*., 2007). Barley is also used as a base for malt in brewing and the production of distilled beverages (van Hao & Ledin, 2001). While it has a higher crude protein content than maize, its protein quality and digestibility are lower than that of wheat, and its energy content is about 10% less than that of maize, largely due to its higher fiber content (Zardo & Lima, 1999). Despite this, the primary component of barley is starch, and it contains between 7.5 - 18% protein on a dry matter basis, with total digestible nutrient (TDN) values ranging from 80 - 84% (Boyles *et al*., 2001).

Soybean meal is the principal protein source used in animal feed worldwide, accounting for approximately two-thirds of global protein feed production (Oil World, 2015). Owing to its high protein concentration and favourable amino acid profile, it is regarded as the benchmark for evaluating other protein sources (Cromwell, 1999). Soybean meal, a by-product of oil extraction, varies in protein and fiber content depending on the processing method; the high-protein meal (47 - 49% protein, 3% fiber) is derived from dehulled seeds (Cromwell, 2012).

One of the main strategies for upgrading the nutritional value of crude olive cake is supplementation with essential nutrients, especially protein and energy sources. Soybean meal provides high levels of digestible protein and essential amino acids, such as lysine and methionine (Guo *et al*., 2022), which are important for supporting growth, immune function, and overall productivity in sheep (Wu, 2013). Barley serves as an energy source, supplying easily digestible carbohydrates that help meet the energy requirements of sheep and improve feed conversion efficiency (Nikkhah, 2012). Additionally, the fiber in barley is more fermentable than that in olive cake, supporting better gastrointestinal function in ruminants (Sansoucy, 1985).

Cane molasses, with a dry matter content of 735 g/kg and high concentrations of sugar, crude protein, and ash (O'Grady, 1996), is commonly used as a silage additive. Its role is to increase the availability of fermentable energy and to promote the growth of lactic acid bacteria, which is particularly beneficial for ensiling materials low in soluble carbohydrates.

Lactic acid bacteria inoculants are vital in the fermentation of agro-industrial by-products, such as olive cake, by converting soluble carbohydrates into lactic acid and lowering the pH to stabilise the silage. LAB, mainly gram-positive microorganisms, are widely used in industrial lactic acid production. Recent studies have demonstrated that sequential inoculation with yeast (e.g., *Saccharomyces cerevisiae*) and LAB strains (e.g., *Leuconostoc* *mesenteroides*) can accelerate fermentation and improve the biochemical transformation of olive cake (Foti *et al*., 2023).

The purpose of this multi-substrate supplementation strategy lies in improving both the fermentability and the nutritional value of olive cake. Soybean meal contributes high levels of digestible protein and essential amino acids, supporting microbial growth and enhancing crude protein content through microbial synthesis (Cromwell, 2012; Guo *et al.*, 2022). Barley supplies fermentable starches that stimulate microbial fermentation, potentially reducing fiber content by activating cellulolytic enzymes (Nikkhah, 2012; Sansoucy, 1985). While molasses provide readily available sugars, enhancing the rapid growth of LAB and accelerating acidification, which helps preserve nutrients and improve palatability (Kung *et al.*, 1999; O'Grady, 1996). LAB inoculation itself promotes the production of lactic acid, lowers pH, and suppresses spoilage microorganisms, enhancing the safety and shelf-life of the treated feed (Foti *et al.*, 2023; McDonald *et al.*, 1991). Together, these components are expected to create a complementary effect that enhance fermentation efficiency, nutrient bioavailability, and digestibility.

Based on the known properties of these additives, we hypothesise that their combined use in controlled fermentation will significantly improve the nutritional quality of olive cake. Specifically, we expect to observe increases in crude protein and organic matter content, improved preservation through acidification, and potential reductions in fiber levels, thereby improving overall feed value. Furthermore, we anticipate interactive effects between the additives that could outperform their individual contributions.

Despite increasing interest in valorising agro-industrial by-products, few studies have explored the systematic optimisation of olive cake fermentation using a combined approach involving multiple nutrient additives (barley, soybean meal, molasses) and microbial inoculants with statistical experimental designs.

The objectives of this study are to evaluate and optimise treated olive cake as a sustainable and nutritionally improved feed resource for sheep through biotechnological enhancement; to enhance the nutritional quality of olive cake by means of controlled fermentation with lactic acid bacteria in combination with strategic supplementation using barley, soybean meal, and molasses; to systematically assess both the individual and interactive effects of these additives, applied at inclusion levels of 2%, 4%, and 6%, on key nutritional parameters including dry matter (DM), organic matter (OM),), ash content, crude fat (CF), neutral detergent fiber (NDF)and crude protein (CP), in order to identify the most effective treatment combinations; to develop a practical, cost-effective, and environmentally sustainable alternative feed solution that can be readily adopted by sheep producers; and ultimately, to contribute to improved animal nutrition and the promotion of circular agricultural practices through the efficient valorisation of locally available agro-industrial by-products.

To achieve these objectives, a Box–Behnken experimental design is employed to systematically evaluate the effects of barley, soybean meal, and molasses at three inclusion levels (2%, 4%, and 6%) during the fermentation of olive cake inoculated with lactic acid bacteria. Thirteen experimental formulations are developed and analysed in triplicate. Following fermentation, samples are subjected to chemical analyses to determine dry matter, organic matter, crude protein, crude fat, ash, and neutral detergent fiber contents. Statistical evaluation is performed using analysis of variance and Response Surface Methodology to quantify main and interaction effects and to identify optimal treatment conditions.

This thesis is organised into five main chapters. Chapter I provides a general introduction to the research topic, outlining the background, objectives, and scientific rationale of the study. Chapter II presents a comprehensive literature review, beginning with an overview of sheep farming and nutritional feed resources in Portugal, followed by a detailed discussion of olive cake, including its availability, production, and chemical composition, and concluding with a review of the main treatments used to improve its nutritional value, such as silage, fermentation processes, nutrient supplementation, and lactic acid bacteria inoculation. Chapter III describes the materials and methods employed in the study, including the characteristics of olive cake, the applied treatments with soybean meal, barley, molasses, and lactic acid bacteria, the experimental design, sample preparation procedures, analytical methods used to determine chemical composition, and the statistical analyses performed. Chapter IV presents and discusses the results, with particular emphasis on the effects of individual additives, the response surface methodology applied to key nutritional parameters, and the identification of optimal formulations through multi-objective optimisation. Finally, Chapter V summarises the main conclusions of the research and outlines future perspectives and recommendations for further studies and practical applications.

II. LITERATRE REVIEW

1. Chapter 1: Sheep farming in Portugal

1.1. A statistical overview

Sheep farming plays a prominent role in Portuguese agriculture, with over 50,000 farms and approximately 2 million sheep. Meat production is predominant, accounting for nearly 80% of the sheep population. Although dairy sheep farming holds a smaller share, it remains significant in the regions of Beira Interior and Beira Litoral. The Alentejo region is preeminent in terms of sheep numbers, possessing nearly half of the country's total sheep population (*Statistics – DGAV*, 2022).

Sheep milk production is largely concentrated in Beira Interior, which contributes an impressive 53% of the national dairy sheep flock. The Alentejo region also boasts a substantial dairy sheep population, though it represents only a small fraction (5%) of the region's total sheep. Sheep milk production is also present in Beira Litoral and Ribatejo Oeste. Unfortunately, sheep production in Portugal has experienced a decline, with a 27% reduction in the number of farms and a 24% decrease in sheep numbers between 1999 and 2009. More recent data indicate a continuing downward trend, with the total sheep population reaching 2.208 million animals by 2018, representing a 40% decrease since 1998 (Costa Freitas *et al.*, 2020).

Portugal is home to sixteen indigenous sheep breeds, each with unique characteristics. Some breeds are raised primarily for meat production, while others are specialised in milk. A few breeds exhibit dual-purpose aptitude, combining high fecundity with notable milk production. Additionally, certain breeds possess triple-purpose capabilities, suitable for meat, milk, and wool production (Tiberio & Diniz, 2014).

As shown in Figure 1, sheep production levels in Portugal remained relatively high and stable between 2014 and 2023, with the live sheep population consistently exceeding 2 million head annually (FAOSTAT, 2023). This highlights the continued importance of sheep farming within the Portuguese agricultural sector and its contribution to rural economies. Maintaining this production level is essential for ensuring sectoral stability and meeting market demand.

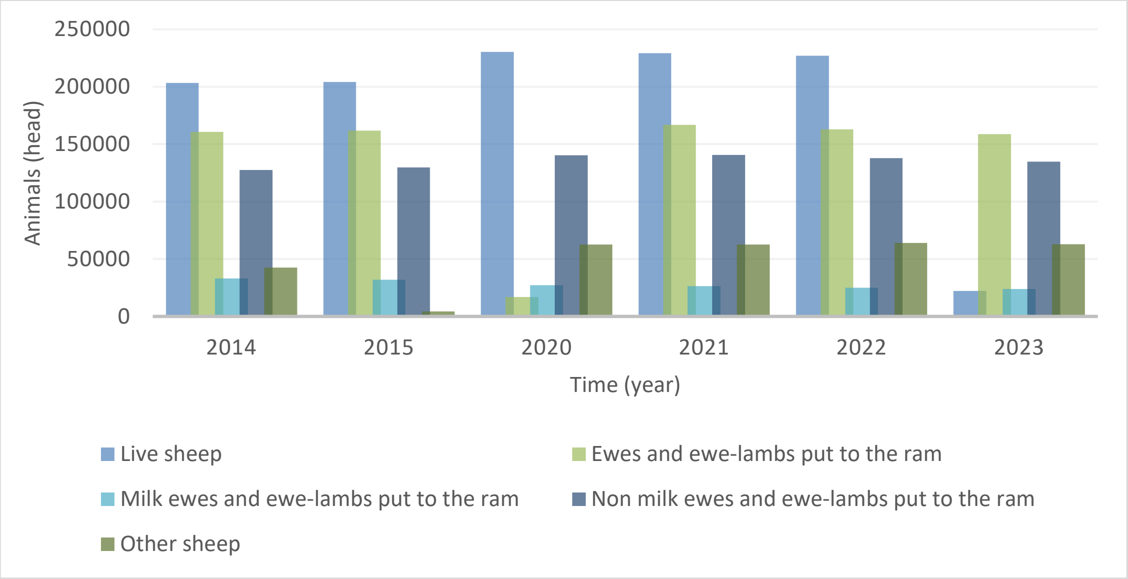


Figure 1 - Sheep population statistics in Portugal by category, 2014-2023 (FAOSTAT, 2023).

Despite this apparent stability, sheep farming in Portugal faces significant structural challenges. Several studies have reported a long-term decline in the number of sheep farms and increasing pressures on the sector due to rising labour costs, higher production expenses, and ongoing mechanisation (Costa Freitas et al., 2020). These challenges are further exacerbated by the emigration of young people from rural areas, internal migration toward coastal and urban regions, and the resulting shortage of young shepherds willing to enter or continue in the sector. Additionally, political and policy changes have influenced traditional production systems, particularly within the Montado management framework, affecting land use, farm viability, and generational renewal (Pinto-Correia & Godinho, 2013). Together, these factors contribute to a structurally vulnerable sector, despite the maintenance of overall sheep numbers.

Alves (2021) highlighted a challenging scenario in the Portuguese animal feed sector. The industry has struggled with a substantial surge in raw material prices, adding strain to both livestock producers and the sector as a whole. Contributing factors include the lingering impact of COVID-19, reduced production in the Black Sea region, and robust demand from China. Together, these elements have driven a notable increase in the costs of essential raw materials, such as maize and soybeans.

This price surge has not only posed profitability challenges for livestock producers but has also placed pressure on the margins of the feed industry. The interplay of these factors underscores the vulnerabilities and complexities of the Portuguese animal feed sector during this period.

The primary ingredients supplying essential nutrients, including protein, fiber, and energy, to various livestock species are soybean meal, maize, barley, and wheat bran (Aloueedat *et al.,* 2019). Escalating global demand for these staple feeds has led to significant increases in market prices, further straining livestock producers. Moreover, the availability of these key ingredients is subject to both seasonal and annual fluctuations, creating additional production challenges.

To address feed shortages without compromising the nutritional quality of animal diets, the utilisation of alternative resources or by-products is increasingly necessary. In this context, olive cake (OC) emerges as a promising candidate for alleviating deficiencies in feed supply (Obeidat, 2017). This agricultural by-product offers a viable solution, with the potential to mitigate the adverse effects of feed scarcity on animal nutrition and husbandry.

As illustrated in Figure 2, the major olive oil producing regions in Portugal are concentrated in specific areas, which reflects the sector's growing economic relevance. Over the past decade, olive oil production in Portugal has increased dramatically, while the prices of key livestock feed crops such as corn, rye, and wheat have also risen. This situation presents an opportunity to utilise olive oil by-products as substitutes for conventional feed crops, thereby reducing feed costs and providing a valuable outlet for these by-products.

Figure 2 - Major olive oil producing regions in Portugal
(O Azeite | Lisboa, 2024).

Between 2011 and 2021, Portugal's olive oil production more than tripled, rising from 76,203 metric tons to 228,954 metric tons (FAOSTAT, 2023). During the same period, the cost of essential livestock feed ingredients increased substantially: the price of corn rose from 238 euros per metric ton in 2012 to 334.9 euros per metric ton, and wheat increased from 238 euros to 404.3 euros per metric ton between 2012 and 2022. These rising feed costs exert growing pressure on livestock producers and highlight the importance of finding cost-effective alternatives.

Olive oil by-products, such as olive pomace and olive cake, are promising substitutes for corn, rye, and wheat in livestock diets. These by-products are valuable sources of nutrients for livestock and can be used to produce nutritious and affordable feed, helping to mitigate rising feed expenses while promoting sustainable waste management in the olive oil industry.

1.2. Sheep nutritional feed resources in Portugal

Portugal's diverse landscape and Mediterranean climate create a unique environment for sheep production, shaping both the availability and quality of nutritional feed resources. Natural grazing remains fundamental to sheep nutrition, particularly in extensive and semi-intensive production systems (Tiberio & Diniz, 2014). Native grasslands, primarily found in central and northern regions, supply a significant portion of the sheep diet, although their nutritional value fluctuates seasonally. In more arid areas, especially in the south, Mediterranean shrublands are vital for forage availability, with species such as *Cistus ladanifer*, *Erica arborea*, and *Ulex europaeus* serving as essential feed sources during periods of scarcity. The Montado system, characteristic of southern Portugal, especially the Alentejo region, is dominated by cork and holm oaks and provides a mix of grasses, shrub understory, and acorns that are valuable feed resources during autumn and winter (Castro *et al.*, 2016 ; Tiberio & Diniz, 2014).

In addition to natural grazing, cultivated feed resources play a significant role in sheep nutrition. Forage crops, including legumes such as alfalfa and clovers, and grasses like Italian ryegrass and tall fescue, are cultivated for their high nutritional value. Cereal crops such as barley, oats, and wheat contribute both grain and grazing opportunities, while crop residues provide essential feed during the summer months (Castro *et al.*, 2016). Despite these resources, supplementary feeding is often necessary, especially during critical physiological stages such as pregnancy and lactation or within intensive production systems. Commercial feed mixes, which provide balanced sources of energy and protein, are widely available, while mineral supplements help address common deficiencies in selenium, copper, and zinc (Tiberio & Diniz, 2014).

Beyond conventional feeds, alternative options also contribute to sheep nutrition in Portugal. Agro-industrial by-products, including olive leaves, olive cake, and grape pomace from the olive oil and wine industries, offer valuable feed alternatives. Additionally, tree fodder, such as oak acorns from Montado systems and carob pods from the Algarve region, enhances the sustainability of sheep production (Castro et al., 2016). These diverse feeding strategies reflect the adaptability of sheep production systems in Portugal and ensure that nutritional requirements are met across varied environmental conditions.

2. Chapter 2: Olive Cake

2.1. Olive oil production in Portugal and estimated availability of olive cake.

Table 1 highlights that in the 2020/21 crop year, International Olive Council (IOC) member countries collectively produced 2,809,500 metric tons of olive oil, accounting for 93.3% of global production. Within the European Union (EU), olive oil output reached 2,051,200 metric tons, reflecting a 6.8% increase compared to the previous crop year.

Within the EU, Spain led with a production of 1,389,000 metric tons, representing an increase of 23.4%. Italy produced 273,500 metric tons, a decrease of 25.4%, while Greece's production remained stable at 275,000 metric tons. Portugal produced 100,000 metric tons of olive oil, which marked a significant decrease of 28.8% compared to the previous crop year, although there was an overall variation rate of +20% from 2016 to 2022 (IOC, 2022).

Table 1 - Olive oil production (*1000 tons) in the European Union (EU) IOC member countries, other IOC member countries, and non-IOC member countries from 2016 to 2022.

Region/ Country	**2016/ 2017**	**2017 /2018**	**2018 /2019**	**2019 /2020**	**2020 /2021**	**Average (2016-2021)**	**2021/ 2022**	**Rate change : 2016-2021 avg vs. 2021-2022.**
EU IOC country members	1762	2188	2264	1920	2051	2035	1974	-3.8%
Spain	1291	1262	1790	1125	1389	1371	300	-6.4%
Greece	195	346	185	275	275	255	225	-18.2%

Italy	182	529	174	366	274	285	315	+15.2%
Portugal	69	135	100	140	100	109	120	+20%
Other IOC country	620	1007	808	1158	758	870	936	+23.5%
Tunisia	100	325	140	440	140	229	240	+71.4%
Turkey	178	263	194	230	210	215	228	+8.3%
Morocco	110	140	200	145	160	151	200	+25%
Algeria	63	82	97	126	70	88	96	+39%
Egypt	30	40	41	40	30	36	20	-
Argentina	24	45	28	30	30	31	30	0.0%
Non-IOC country	190	184	233	188	200	199	188	-6.2%
TOTAL	**2561**	**3379**	**3305**	**3266**	**3010**	**3104**	**3099**	**+2.9%**

Source: IOC 2022.

It is noteworthy that Mediterranean countries account for nearly 98% of the world's olive oil production (Manzanares *et al*., 2020). Given the substantial quantities of olive cake generated during olive oil extraction, incorporating this by-product into animal feed represents an efficient and sustainable utilisation strategy.

In the livestock industry, animal feed constitutes a predominant cost. Numerous studies highlight the strategic use of agro-food industry by-products, such as olive cake, as dietary supplements to reduce feed costs. Incorporating olive cake into animal diets not only helps lower production expenses but also promotes the valorisation of agro-food by-products, thereby enhancing the economic efficiency of livestock systems.

Olive cake is the main solid by-product of olive oil extraction. It is produced after pressing and decanting processes that remove the oil from the olive pomace. Different types of olive cake exist depending on the processing method:

- Crude olive cake: refers to the initial residue after mechanical extraction and contains relatively high levels of moisture (~24%) and oil (~9%).
- Exhausted olive cake: results from additional solvent extraction (commonly using hexane) and has a reduced oil content (Durante *et al.*, 2020).
- Partly destoned olive cake: is produced through partial separation of stones and pulp; it can be categorized as "fatty" if not solvent-extracted, or as "defatted" or "exhausted" when solvents are applied (Gullón *et al.*, 2020).

While olive cake has traditionally been used for energy production due to its calorific value (17.6 MJ/kg) (Gálvez-Pérez *et al.*, 2021), recent studies such as (Amato et al., 2024) and (Chiofalo et al., 2020) have emphasized its potential as a cost-effective feed ingredient in ruminant diets. Its inclusion has shown positive effects on animal welfare, productivity, and the quality of animal products.

2.2. Chemical composition of olive cake

Olive cake is a by-product of the second extraction of olive oil. The first extraction is performed in olive oil factories through the mechanical pressing of olives, resulting in virgin olive oil. The remaining brown mass, known as pomace, has a moisture content of about 60% and an oil content of approximately 7-8%. After the initial extraction, pomace is transported to specialised factories for a second extraction using hexane. This process yields second-extraction oil and a residue consisting of olive flesh, skin, and whole or crushed stones, with a final moisture content of around 13-14%. (Quality Management Guide for the Olive-Pomace Oil Extraction Industry, 2006)

The incorporation of non-conventional feedstuffs, such as olive pomace, into the diets of small ruminants requires comprehensive research to evaluate their effects on animal performance and health. Important considerations include palatability, intake, inclusion rate, digestibility, and performance, as well as other relevant parameters in response to agro-industrial by-products. Due to the inherent variability of these materials, it is essential to assess their nutrient composition, including crude protein (CP), neutral detergent fiber

(NDF), acid detergent fiber (ADF), and crude fat (CF) content, prior to their incorporation into ruminant diets (Obeidat, 2017).

According to the literature, the chemical composition of olive cake has been extensively studied, particularly in the context of its use as a feed resource for livestock.

Table 2 summaries the chemical composition of olive cake, highlighting key constituents such as fiber and polysaccharides. This provides valuable insight into the nutritional potential and limitations of olive cake for animal feeding.

It is evident that the chemical composition of olive cake varies considerably depending on its form and processing methods. For example, dry matter (DM) content ranges from 410 to 956 g/kg, while organic matter (OM) content varies from 86 to 973.7 g/kg. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) also show significant variability, reflecting the diverse nutritional characteristics of olive cake. These variations are crucial for understanding its potential impact on animal nutrition and performance. The data also highlight the influence of processing techniques on the chemical composition of olive cake, underscoring the need to consider these factors in practical livestock feeding.

Table 2 – Chemical composition of olive cake under different treatments.

Olive Cake	DM	OM	Ash	CF	NDF	CP	ADF	ADL	N	EE	sugar	Reference
Ensiled	410	-	130	-	630	60	550	-	-	-	-	**Abo Omar *et al.* (2012)**
Crude	900	-	123	-	601	52	500	-	-	-	-	**Abo Omar *et al.* (2012)**
Alkali treated	880	-	140	-	470	58	400	-	-	-	-	**Abo Omar *et al.* (2012)**
Fermented at day 0	648	-	45	433	-	74	-	-	-	113	349	**Alhamad *et al.* (2017)**
Fermented at day 60	649	-	41	438	-	75	-	-	-	117	313	**Alhamad *et al.* (2017)**
Crude	834	849	-	-	686	836	670	-	-	-	-	**AlJassim *et al.* (1997)**
Fresh	515	953	-	-	541	66	371	-	-	227	-	**Awawdeh & Obeidat, (2013)**

Olive cake	887	-	73	-	239.4	170	131.8	48.3	-	44.2	403.8	**B. Chiofalo *et al.* (2020)**
Dried	956	-	40.9	-	493.7	86.3	393.9	230.6	-	303.4	14.8	**V. Chiofalo *et al*. (2020)**
Crude	893	86	-	-	266	404	123	139	-	29.1	-	**García-Rodríguez *et al*. (2020)**
Crude silage	450.6	973.7	26.3	-	710.4	51.6	548.7	-	-	147.9	-	**Hadhoud *et al.* (2020)**
Crude	470	-	13	443	691	48	551	278	-	104	-	**Hadjipanayiotou, (1994)**
Dried	951.4	-	42.5	-	413.3	78.6	325.3	156.8	-	277.2	-	**Liotta *et al.* (2019)**
Crude	862- 946	873-948	-	-	402-721	-	248-501	170-250	13.1-18.6	73.3-14.5	7.-202	**Marcos *et al.* (2019)**
Exhausted	860-908	894-935	-	-	528-766	-	385-533	197-261	15.8-23.5	4-32	8-197	**Marcos *et al.* (2019)**
Cyclone	914-952	885-910	-	-	367-528	-	256-374	123-190	12.5-22.2	108-195	6.5-149	**Marcos *et al.* (2019)**

Two stages dried	871	850	-	1.3	624	788	540	328	-	-	-	**Martín-García *et al.* (2004)**
Crude silage	-	97.37	2.63	-	71.5	5.16	53.7	-	-	14.79	-	**Shaaban *et al.* (2021)**
silage	-	-	25.1	568.8	784.5	58.2	662.1	-	-	108.4	-	**Symeou *et al.* (2021)**

DM - Dry Matter, OM - Organic Matter, NDF - Neutral Detergent Fiber, ADF - Acid Detergent Fiber, ADL - Acid Detergent Lignin, CP - Crude Protein (Nitrogen * 6.25), CF -Crude Fiber, N - Nitrogen, EE - Ether Extract. All values, except for Dry Matter (DM), were calculated based on g per 1 kg of Dry Matter; Crude samples were dried and pelletize. Exhausted samples were dried and subjected to extraction using hexane; Cyclone samples were obtained from a cyclone separator after drying the crude samples. Values are presented as g/kg dry matter (DM), unless otherwise indicated.

3. Chapter 3: Treatments to improve the nutritional value of olive cake

3.1. Treatments to improve the nutritional value of olive cake.

Olive cake is considered a low-quality feedstuff due to its high lignocellulosic content and low levels of protein and energy. To address these limitations, various approaches have been explored to enhance the nutritive value of olive cake. Among these, chemical treatments and silage have emerged as practical avenues for improving its nutritional profile. Notably, the application of molasses, barley, soybean meal, and lactic acid bacteria has shown promise as an effective treatment strategy.

3.2. Silage and fermentation

Ensiling olive cake has emerged as an effective preservation strategy that not only stabilizes the material but also improves its nutritional value and digestibility. The ensiling process relies on the creation of anaerobic conditions that favor microbial fermentation, particularly by lactic acid bacteria. LAB convert water-soluble carbohydrates into organic acids, primarily lactic acid, lowering pH, suppressing spoilage organisms such as yeasts, molds, and pathogenic bacteria, and preserving valuable nutrients (Carvalho *et al*., 2021). This bioconversion process improves the palatability and digestibility of olive cake, transforming it into a more suitable and sustainable feed source for livestock (Basso *et al.*, 2014).

LAB are considered the cornerstone microorganisms in silage fermentation due to their metabolic efficiency in producing organic acids that inhibit undesirable microbes. According to a review by Okoye *et al.* (2023), among LAB, homofermentative strains such as *Lactobacillus plantarum* and *Pediococcus pentosaceus* are known for their ability to rapidly convert sugars into lactic acid through the Embden-Meyerhof pathway, driving swift acidification and nutrient preservation. However, homofermentative LAB alone may not ensure long-term silage stability, especially during aerobic exposure at feed-out, when spoilage organisms can metabolize lactic acid, leading to nutrient loss and deterioration.

To overcome this limitation, heterofermentative LAB such as *Lactobacillus buchneri* are often used in combination with homofermentative strains. These bacteria convert lactic acid into acetic acid and other metabolites via the phosphoketolase pathway, enhancing aerobic stability by inhibiting the growth of yeasts and molds. Although this pathway results in a slower pH drop, it contributes significantly to the silage's shelf life (Borreani *et al*., 2018; Kim *et al*., 2021).

Moreover, certain LAB strains can produce bacteriocins, exopolysaccharides, and lignocellulolytic enzymes, providing antimicrobial, biopreservative, and fiber-degrading functions (Sharma *et al.*, 2022), these are particularly beneficial for fibrous substrates such as olive cake.

3.3. Silage additives

Cecava (1995) report that additives for silages is a common procedure, it can be categorized as follows: bacterial inoculants, acids, nonprotein nitrogen sources, enzymes and other feeds. Some of these additives have proven efficacious in reducing nutrient loss and improving the feeding value of ensiled crops, Others have shown inconsistent response and may not be cost effective for routine use.

3.3.1. Molasses treatment

If the sugar content of green forage is low, there may be advantages to adding molasses during ensiling. This is particularly true for legumes and certain grasses, where a source of readily available carbohydrates can enhance the production of acetic and lactic acids, thereby improving the quality of the ensiled material. The addition of molasses can also increase feed consumption by improving the palatability of silage. Molasses may be added in either liquid or dehydrated form, as both are effective in supplying sugars (Cecava, 1995).

According to Abarghoei *et al.* (2011), chemical analysis of olive cake (OC) revealed notable effects from molasses treatment. The dry matter (DM) content of whole OC exceeded that of partly stoned cake due to a higher proportion of stones. Silage DM content increased with molasses addition, attributable to the high DM content of molasses. Furthermore, the addition of molasses reduced the organic matter content of partly stoned silage.

Crude protein (CP) content varied among OC types, with molasses supplementation resulting in more stable fermentation and higher CP levels in partly stoned OC silage. Fiber analysis showed high cell wall constituents in OC, with lower neutral detergent fiber (NDF) in ensiled OC, possibly due to degradation during fermentation. Additionally, silages mixed with molasses had lower lignin, likely due to the lower lignin content of molasses itself.

Ensiling caused a significant decrease in pH, which was further reduced by molasses addition, indicating improved fermentation quality. Ammonia-N levels remained within desirable limits (less than 50 g/kg total N), indicating good fermentation quality, with molasses-treated silages producing less ammonia-N and thus suggesting enhanced fermentation.

The in vitro organic matter digestibility (OMDiv) of OC, though generally low as a ruminant feed, improved with molasses treatment. OMDiv and metabolizable energy (ME) values were higher than previously reported, likely due to the lower lignin content. Partly stoned OC showed improved OMD and ME compared to whole OC. However, ensiling generally reduced OMD and ME by decreasing water-soluble carbohydrates (WSC), a crucial energy source for microorganisms. Molasses supplementation further enhanced OMD, ME, and fermentation parameters by stimulating microbial activity.

A study by Weinberg *et al*. (2008) found that the initial analysis of fresh olive cake revealed a content of 335 ± 6 g/kg DM, 72 ± 4 g/kg DM of water-soluble carbohydrates, and 13.1 ± 2.0 g/kg of polyphenols. During ensiling, the addition of molasses influenced the rate of pH reduction, with higher rates of molasses leading to faster decreases.

Chemical analysis of olive cake silages at day 38 indicated that higher molasses rates resulted in increased lactic acid production, although concentrations (0–28 g/kg DM) remained lower than typical values for conventional forage crops. Ethanol and acetic acid were present at low concentrations, with higher ethanol content observed in silages treated with 4% molasses, correlating with elevated yeast numbers. Dry matter digestibility was low and decreased further during ensiling due to fermentation losses. Microbiological analysis revealed substantial increases in lactic acid bacteria and yeast populations, particularly in silages treated with 4% and 6% molasses.

3.3.2. Barley and soybean meal treatment

Treating animal feed with soybean meal and barley is a targeted approach designed to enhance the nutritional and functional qualities of livestock diets. Grains and other concentrated feeds may be added to silage, and in some cases, silage made from legumes and grasses can be improved by the addition of grains such as ground corn, wheat, or barley. The added grain supplies carbohydrates for lactic acid-producing bacteria, improving the fermentation process. Furthermore, grain inclusion can raise the total digestible nutrient (TDN) content of silage, enhancing its feeding value (Cecava, 1995).

The inclusion of barley in animal diets, particularly for ruminants, has been extensively studied and shown to improve the nutritional value of various feed formulations. Nefzaoui (1985) noted similarities in amino acid composition between barley and olive cake, although olive cake was deficient in glutamic acid, proline, and lysine, which are critical for protein synthesis. Yáñez Ruiz *et al.* (2004) observed that supplementation of olive leaves with barley grain and faba beans enhanced microbial activity in goats and sheep, as indicated by urinary allantoin levels. Additionally, these researchers found that replacing alfalfa hay with a concentrate of barley and dried two-phase olive cake produced a medium-quality diet, as reflected by total volatile fatty acids, ammonia nitrogen, pH, and total protozoa counts. Muñoz *et al.* (1983) demonstrated that lambs fed a mixture of dried olive leaves ad libitum, along with barley and fishmeal, gained more weight compared to those supplemented solely with urea, and control lambs receiving lucerne hay and barley achieved the highest gains. Collectively, these studies confirm the value of barley as a component in ruminant diets, enhancing intake, digestibility, and growth performance, especially when combined with other feed sources.

The primary goal of this treatment is to address the limitations of crude olive cake, particularly its low protein content and high fiber, by enriching it with essential nutrients. Soybeans serve as a key protein source, rich in essential amino acids such as lysine and methionine. Soybean supplementation increases the protein content of olive cake, making it more suitable for supporting livestock growth and development. These amino acids are vital for various physiological functions, including muscle development, immune function, and overall animal productivity. Moreover, the protein from soybean meal is highly digestible, ensuring that treated olive cake delivers nutrients that animals can efficiently absorb and utilise.

Soybeans also contribute beneficial fatty acids and micronutrients, further enriching the nutritional profile of the treated feed. These components enhance the energy density and palatability of the feed, making it more attractive to livestock and potentially increasing feed intake and weight gain. This improvement in feed quality leads to enhanced animal performance, as livestock can consume a more balanced and digestible diet, supporting their health and growth (Alvaro, 2023).

Barley complements soybean meal by providing an abundant source of easily digestible carbohydrates, which are essential for meeting the energy requirements of livestock, particularly those raised for meat production. The carbohydrates in barley support better feed conversion efficiency and more effective weight gain in animals. In addition, the fiber in barley is more fermentable than that in olive cake, promoting better gastrointestinal function in ruminants. This enhances digestion and nutrient uptake, further contributing to the efficiency of treated olive cake as a feed ingredient.

The combination of soybean meal and barley not only improves the protein and energy content of olive cake but also creates a more balanced and digestible feed. By addressing key nutritional deficiencies, this treatment allows livestock to extract more nutrients efficiently from their diet.

3.3.3. Lactic acid bacteria Inoculant

Worldwide, many agro-industrial wastes and by-products of low economic value can serve as substrates for fermentation by various microorganisms. Among these, agricultural residues such as olive cake are abundant in mono- and disaccharides, which may require hydrolysis by pectinases to enhance lactic acid production.

Microbial inoculants are among the most widely used silage additives. Most inoculants contain live strains of bacteria that ferment soluble carbohydrates and produce lactic acid, thereby reducing the pH of the silage and resulting in a stable product. The effectiveness of silage fermentation depends on the number of viable organisms present in the ensiled material and the availability of carbohydrates for microbial metabolism (Cecava, 1995).

Predominantly, lactic acid bacteria are gram-positive microorganisms and are recognised as the main industrial-scale producers of lactic acid. They serve as the primary microorganisms employed in this process (Abedi & Hashemi, 2020).

As outlined by Tufariello *et al.* (2019), a sequential inoculation method involving yeast and LAB strains, mainly *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*, has been used in the fermentation of pâté olive cake. This approach has not only standardised the fermentation process but also resulted in rapid production of the final product within a notably short period of 50 days.

During fermentations initiated by starter cultures, the patterns of sugar consumption and the dynamics of organic acids (including lactic, citric, tartaric, and acetic acids) closely resemble those observed in green and black olive fermentations. A notable outcome of the fermentation process is the increased presence of hydroxytyrosol in the fermented olive cake samples, likely attributed to the enzymatic activities of microbial glucosidase and esterase.

LAB are responsible for conducting lactic fermentation in the latter stages of the process. Interestingly, fermented olive cake exhibits specific compounds, such as higher alcohols, terpenes, esters, and acetate esters, which indicate the completion of the fermentation process.

In a separate investigation by Foti *et al.* (2023), the use of LAB and yeast strains, *Candida boidinii*, *Wickerhamomyces anomalus*, and *Lactiplantibacillus plantarum*, in pâté olive cake fermentation proved instrumental in standardising the procedure. The data showed that samples treated with these inoculants exhibited higher hydroxytyrosol content compared to untreated controls. Specifically, throughout fermentation, samples inoculated with *C. boidinii*, either alone or co-cultured with *L. plantarum*, showed a substantial increase in hydroxytyrosol content, with increases of 275 mg/L and 261 mg/L, respectively, after 8 days, reaching their peak by the end of fermentation.

Recent research highlights the synergistic benefits of combining LAB species with complementary metabolic traits. As shown in these two studies (Tian *et al.*, 2017; Kim *et al.*, 2021), silage quality improves when multiple LAB strains are used together. For example, co-inoculating *L. plantarum* with *L. buchneri* ensures both a rapid initial acidification and aerobic stability during storage and feed-out. Other effective combinations include *P. pentosaceus* with *L. plantarum*, *L. casei*, and *Enterococcus faecium*, each contributing unique metabolic activities that enhance fermentation dynamics, pathogen inhibition, and nutrient preservation.

Innovative pairings of lactic acid bacteria with functional traits have shown promising results in enhancing silage quality. For instance, *Bacillus subtilis* and *B. pumilus*, known for their cellulase production, have been successfully combined with LAB to break down fibrous material and improve digestibility (Li *et al*., 2018; Zhang *et al*., 2019). The combination of *B. subtilis* and *L. plantarum* has also been shown to control yeast growth, maintain low pH, and enhance aerobic stability in alfalfa silages (Basso *et al*., 2014; Lara *et al*., 2018). Similarly, pairing heterofermentative LAB such as *L. hilgardii* and *L. buchneri* significantly improved aerobic stability in corn silage compared to more conventional inoculant pairings like *P. pentosaceus* and *L. buchneri* (Benjamim da Silva *et al*., 2021). Beyond these, bacteriocin-producing strains (e.g., *L. delbrueckii*) have been effectively combined with homofermentative *L. plantarum* to suppress yeast and mold growth in alfalfa silages (H. Li *et al.*, 2020).

The involvement of LAB in olive cake fermentation not only promoted the growth of these bacteria but also contributed to the degradation of phenolic compounds, thereby influencing the overall fermentation process. Application of LAB generally resulted in a decrease in total phenol concentration in most samples; however, exceptions were noted in samples inoculated with specific LAB strains, where an increase in total phenol content was observed. This nuanced response highlights the strain-dependent effects of LAB on the phenolic composition during olive cake fermentation.

III. MATERIALS AND METHODS

1. Materials

1.1. Olive cake (OC)

Olive cake (OC) was obtained from olive oil production facilities in the vicinity of Bragança, Portugal. As presented in figure 3, two types of olive cake were initially available: extracted and non-extracted olive cake. For this experiment, only extracted olive cake was used. Extracted OC refers to destoned olive cake that has been subjected to two rounds of milling, with the oil fully extracted, resulting in a dry, low-moisture by-product.

Figure 3 - Extracted (left) and non-extracted (right) Olive cake.

Extracted OC was selected for this study due to its low moisture content and nutrient density, making it suitable for controlled fermentation processes. Furthermore, its abundant availability in the region supports the sustainable use of local resources. Prior to use, the olive cake was milled to pass through a sieve with 1 mm diameter openings, ensuring a uniform particle size and consistent experimental results.

2. Treatment

The experimental material preparation and processing steps are summarised in Figure 4. This workflow details the procedures applied to the raw materials prior to and during fermentation, ensuring standardisation across all treatments.

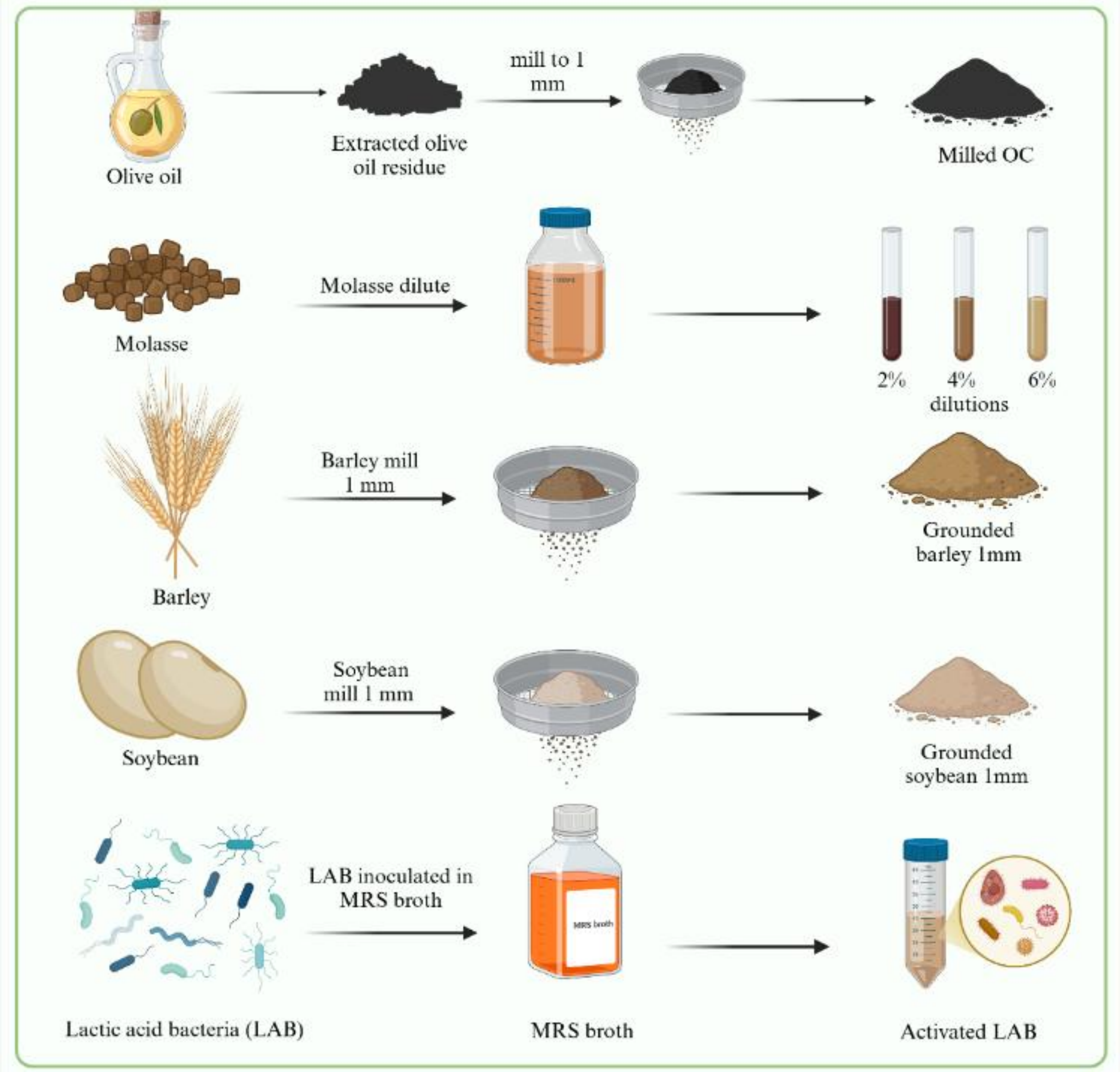


Figure 4 - Material preparation and processing workflow for the experimental setup

2.1. Soybean meal and barley

Both soybean meal (*Glycine max*) and barley (*Hordeum vulgare* L.) were sourced from animal feed suppliers in Bragança, Portugal. These materials were selected for their complementary nutritional profiles to olive cake. Soybean meal was chosen primarily for its high protein content (40-50%), as well as its lipids (20-30%) and carbohydrates (26-30%).

Approximately 35% of the carbohydrates are found in the seed, and 40% in soybean meal, comprising digestible sugars, starch, and non-digestible oligosaccharides (Karr-Lilienthal *et al*., 2005). The protein content in soybeans is higher than in other legumes (20-30%) and much higher than in cereals (8-15%). Thus, soy protein products are considered excellent substitutes for animal-based foods, providing a nearly complete protein profile with lower total and saturated fat than animal products (Qin *et al*., 2022).

Barley is primarily composed of 60-70% starch, mainly located in the endosperm, and contains 11-34% total fiber, of which 3-20% is soluble dietary fiber, including 5-10% β-glucan (βG), and 10-20% protein. Other components include 2–3% lipids and 1.5-2.5% minerals (Aldughpassi *et al.,* 2016). This composition makes the combination of soybean meal and barley well-suited to support the growth of fermentative microorganisms.

Both soybean meal and barley were ground to a 1 mm particle size to match the consistency of the olive cake. This uniform particle size ensured homogeneity in the mixtures and improved the consistency of the fermentation process.

2.2. Molasses

Molasses, a by-product of sugar refining, was obtained in its raw form from a local retail store. It was selected for its high carbohydrate content, which serves as an energy source for lactic acid bacteria during fermentation. Prior to mixing, the molasses was diluted in water to facilitate its distribution and reduce viscosity. Three different molasses concentrations were prepared: 2%, 4%, and 6% (w/w), with water making up 30% of the total mixture by weight in each case. These concentrations were used to evaluate the impact of varying sugar availability on the fermentation process and the final nutritional quality of the product.

After dilution, the molasses solutions were thoroughly blended with the dry ingredients to ensure an even coating and uniform distribution of sugar throughout the olive cake mixture.

2.3. Lactic acid bacteria (LAB)

Lactic acid bacteria were used to initiate the fermentation process. The starter culture was sourced from the stock collection of the Polytechnic Institute of Bragança. For initial activation, approximately 2.5 mg of the starter culture was inoculated into 200 mL of sterilised de Man, Rogosa, and Sharpe (MRS) broth, which had been autoclaved for 15 minutes at 121°C. This medium provides essential nutrients necessary for bacterial growth (Corry *et al*., 2003) and was incubated at 25°C for 24 hours. Subsequently, 500 µL of the activated culture was transferred into 250 mL of MRS broth and incubated again at 25°C for 48 hours.

Once the inoculum was prepared, it was thoroughly homogenised to ensure even distribution of LAB. Finally, 1.5 mL of the inoculum was added to each fermentation bag, and the contents were mixed thoroughly to ensure uniform dispersion of LAB throughout the olive cake mixture.

3. Methods

3.1. Experimental design

The experiment was conducted using a Box-Behnken design with three factors: soybean meal (SO), barley (BA), and molasses (MO), each tested at three levels (2%, 4%, and 6%). The Box-Behnken design is a three-level, fractional factorial design introduced by Box and Behnken (1960). It combines the principles of a two-level factorial design with those of an incomplete block design. In this approach, a subset of factors is fully varied across all possible combinations within each block, while the remaining factors are held constant at their central levels. This method efficiently explores the interactions between variables using fewer experimental runs than a full factorial design.

The Box-Behnken design was selected for its efficiency in investigating the interactions among factors while minimising the number of experimental runs required. It also allows for the exploration of non-linear relationships, making it an optimal choice for evaluating complex formulations such as those in this study.

Thirteen experimental formulations were evaluated using a nested design with three independent trials per formulation, each analysed in triplicate. This yielded 117 experimental samples alongside 9 control samples of untreated olive cake, totaling 126 samples for analysis. Complete experimental results are detailed in Annex 1.

The coded levels for each factor were calculated using the following formulas:

x_1 (Soybean meal): (SO-4)/2
x_2 (Barley): (BA-4)/2
x_3 (Molasses): (MO-4)/2

Table 3 presents the run order and standard order, along with the corresponding levels of the three selected factors: soybean meal (SO), barley (BA), and molasses (MO), as defined in the experimental matrix. These combinations were established based on a Box-Behnken design, which allows for the efficient evaluation of the interactions and quadratic effects of the three variables with a reduced number of experimental runs. Each row in the table represents a unique experimental mix, where the levels of the factors were varied systematically to optimize the conditions for the fermentation process.

Table 3 - Experimental Box-Behnken design for the factors: Soybean meal (SO), Barley (BA) and Molasses (MO).

Run order	Standard order	SO (%)	BA (%)	MO (%)
1	5	2	4	2
2	1	2	2	4
3	7	2	4	6
4	4	6	6	4
5	12	4	6	6
6	10	4	6	2
7	8	6	4	6

8	13	4	4	4
9	2	6	2	4
10	6	6	4	2
11	3	2	6	4
12	11	4	2	6
13	9	4	2	2

3.2. Sample preparation

Figure 5 presents the sample preparation and fermentation procedure used in this study. Sample preparation followed the experimental design, with a total of 126 sample mixtures prepared, strictly adhering to the specified weights of each component in every trial.

Initially, the dry matter content of each component, olive cake, soybean meal, and barley, was determined. These dry ingredients were then combined and thoroughly mixed to ensure homogeneity. Subsequently, a solution of molasses diluted in water was uniformly incorporated into the mixture. Finally, the lactic acid bacteria inoculum was applied using a fine spray to ensure even distribution across all samples.

The mixtures were immediately vacuum-sealed in polyethylene bags to establish anaerobic conditions and were incubated at 25°C in a dark, temperature-controlled environment for 30 days. At the end of the fermentation period, the silage bags were opened, and the contents were dried in a forced-air oven at 30°C until a constant weight was achieved. This drying process was critical for stabilizing the samples and ensuring the accuracy of subsequent chemical analyses.

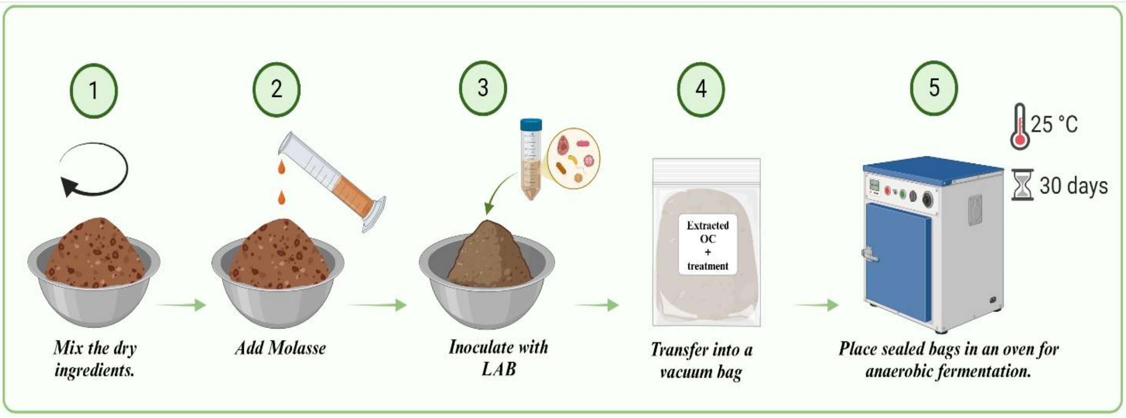


Figure 5 - Sample preprocessing and preparation for analysis

3.3. Chemical composition of treated olive cake

After drying, a comprehensive set of analyses was conducted on all samples to determine their chemical composition. All analyses followed the methods recommended by AOAC (1984). The assessments included determination of moisture content (dry matter), organic matter and ash content, crude content using the Soxhlet extraction method, fiber content using the Van Soest method, and crude protein content by Kjeldahl.

3.3.1. Dry matter

Figure 6 presents the analytical method used to determine the dry matter content of each sample. Moisture content was measured to determine the percentage of dry matter in each sample. Assessing dry matter is crucial for evaluating the storage stability and feed efficiency of the treated olive cake. Ensuring a low moisture content minimises the risk of spoilage, which is particularly important for the potential use of this product as a high-quality animal feed.

Briefly, 2.5 g of sample (SW) was placed into previously incinerated, cooled, and weighed porcelain crucibles (C_1). The crucibles containing the samples were then placed in an oven at 105°C for eight hours. After this period, the crucibles were cooled in a desiccator before being reweighed (C_2).

The following formula was used to determine the sample DM content:

$$\%\boldsymbol{DM} = \left(\frac{\boldsymbol{C2 - C1}}{\boldsymbol{SW}}\right)\mathbf{100}$$

Where :

- **C1** = Weight of the empty crucible.
- **C2** = Weight of the crucible with the dried sample (after drying and cooling).
- **SW** = Initial weight of the sample.
- **%DM** = Percentage of dry matter in the sample.

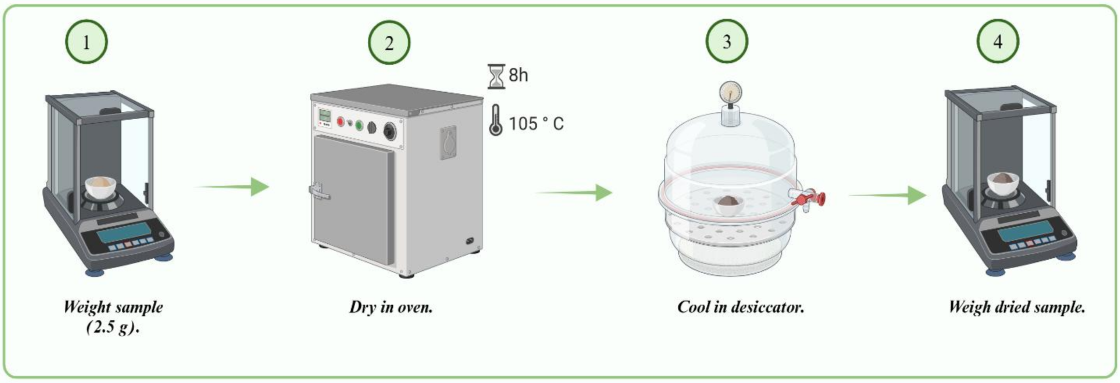


Figure 6 - Dry matter (DM) determination process

3.3.2. Organic matter and Ash

After the initial weighing, the samples were placed in a preheated furnace at 550°C for three hours (Figure 7). Following ashing, the crucibles were transferred to an oven at 105°C for 30 minutes and then cooled in a desiccator before the final weighing (C_3).

Organic matter (OM) was calculated using the following formula:

$$\%\boldsymbol{OM} = \left(\frac{\boldsymbol{C2 - C3}}{\boldsymbol{C2 - C1}}\right)\mathbf{100}$$

Since ash is the remaining mineral residue after organic matter combustion, the ash content (**%**Ash) is determined using the following formula:

$$\%\boldsymbol{Ash} = \frac{(\boldsymbol{C3} - \boldsymbol{C1})}{(\boldsymbol{SW}\%\boldsymbol{DM})}$$

Where:

- **C1** = Weight of the empty crucible.
- **C2** = Weight of the crucible with the dried sample (after drying and cooling).
- **C3** = Weight of the crucible with the sample after incineration at 550ºC and cooling.
- **SW**= Initial weight of the sample.
- **%DM** = Percentage of dry matter in the sample.
- **%OM** = Percentage of organic matter in the sample.
- **%Ash** = Percentage of ash content in the sample.

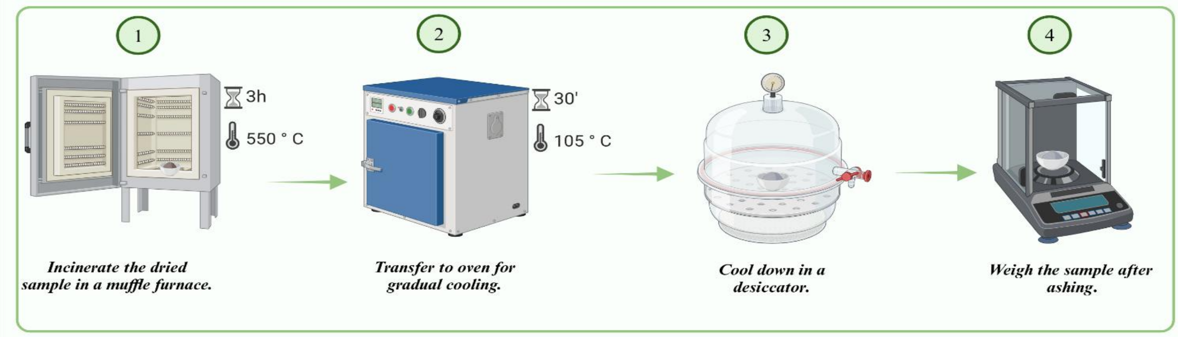


Figure 7 - Organic Matter (OM) and Ashe Determination Process

3.3.3. Crude fat

An overview of the Soxhlet-based crude fat analysis is provided in Figure 8, which illustrates the equipment and steps involved. This process involves extracting crude fat from the sample by boiling it in a solvent and then quantifying the extracted fat.

An automated Soxhlet system (Tecator Soxtec System HT 1043 extraction unit) was used for the analysis. First, aluminium cups were placed in an oven at 105°C for four hours, then cooled in a desiccator and weighed (F_0). Next, 2.5 g of each sample (SW) was weighed into a cellulose Soxhlet extraction thimble and covered with cotton wool. The thimbles were positioned in the device columns, while the aluminium cups were placed in the adapters and filled with 50 mL of petroleum ether as the solvent. The system was sealed, and the extraction process included boiling for 25 minutes, rinsing for 30 minutes, and evaporating for 5 minutes.

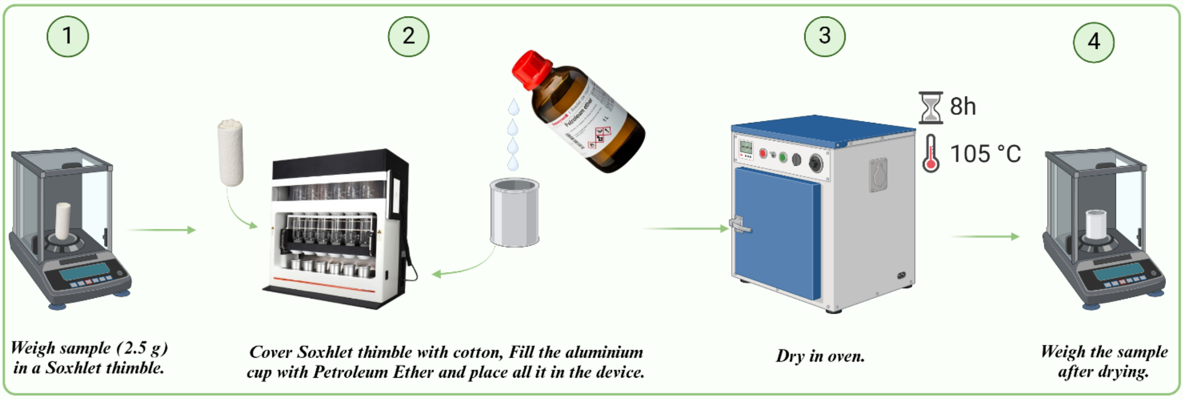
After extraction, the aluminium cups were again placed in the oven at 105°C for eight hours, cooled in a desiccator, and reweighed (F_1).

We calculated the crude fat percentage by the following equation:

$$\%\boldsymbol{FC} = \left(\frac{\boldsymbol{F1 - F0}}{\boldsymbol{SW - DM}}\right)\mathbf{100}$$

Where:

- **F0** = Initial weight of the empty aluminium cup before extraction.
- **F1** = Final weight of the aluminium cup with the extracted fat after drying.
- **SW** = Initial weight of the sample.
- **DM** = Weight of the dry matter in the sample.
- **%Fat** = Percentage of crude fat in the sample.



Weigh sample (2.5 g) in a Soxhlet thimble.

Cover Soxhlet thimble with cotton, Fill the aluminium cup with Petroleum Ether and place all it in the device.

Dry in oven.

Weigh the sample after drying.

Figure 8 - Crude fat determination by Soxhlet extraction

3.3.4. Neutral Detergent Fiber

The neutral detergent fiber (NDF) content was determined using the Van Soest method, the standard procedure for analysing cell wall components in animal feed (Robertson & Van Soest, 1981), as shown in Figure 9. Filtration crucibles with porosity 1 were first incinerated, cooled, and weighed (P_1). Then, 0.5 g of sample (P) was placed in a 600 mL Berzelius beaker, and 50 mL of neutral detergent solution was added at room temperature.

The mixture was rapidly brought to a boil within 5 to 6 minutes and then maintained at boiling with constant stirring for 60 minutes.

After the boiling period, the beakers were removed from the heat. The sample was filtered through the pre-weighed crucibles under gentle suction. The beakers were rinsed twice with boiling distilled water and once with acetone, ensuring complete transfer of the sample and washings to the filtration crucibles.

The crucibles were then placed in an oven at 105°C overnight, cooled to room temperature for 20 minutes, and weighed (P_2). Next, they were placed in a furnace at 550°C for 3 hours, transferred to an oven at 105°C for 1 hour, cooled again for 20 minutes, and weighed (P_3).

The following formula was used to calculate the neutral detergent fiber content:

$$\%\boldsymbol{NDF} = \left(\frac{\boldsymbol{P2 - P3}}{\boldsymbol{P1DM}}\right)\mathbf{100}$$

Where:

- **P1** = Initial weight of the sample.
- **P2** = Weight of the filtration crucible with the residue after drying at 105ºC.
- **P3** = Weight of the filtration crucible with the residue after ashing at 550ºC.
- **DM** = Weight of the dry matter in the sample.
- **%NDF** = Percentage of neutral detergent fiber in the sample.

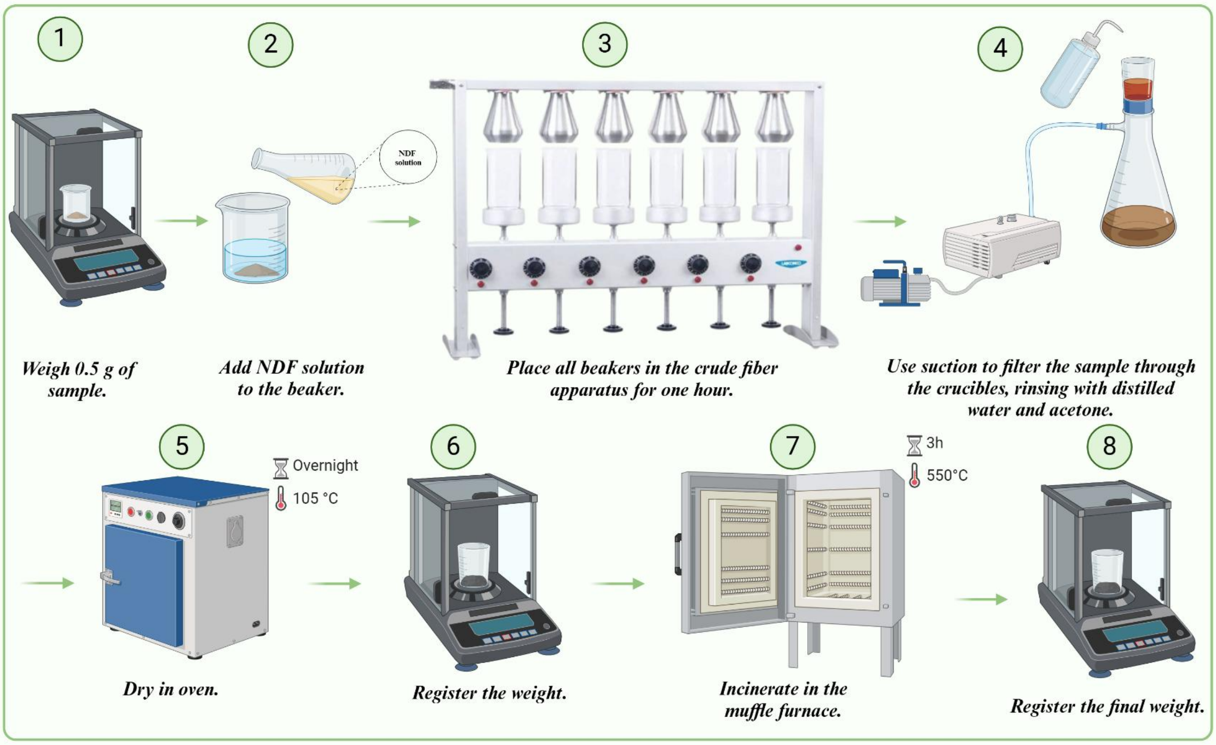
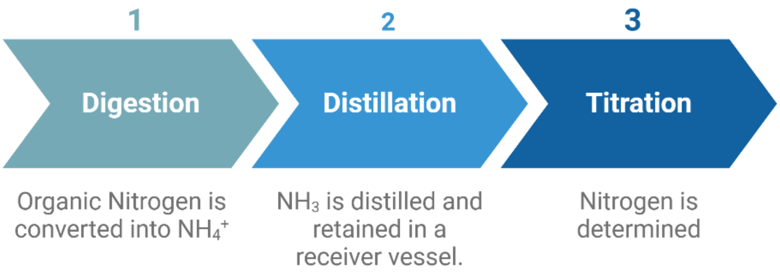


Figure 9 - Neutral detergent fiber (NDF) determination process

3.3.5. Crude protein

As demonstrated in Figure 10, the international reference method for determining the protein content of treated olive cake is the Kjeldahl method (Kjeldahl, 1883). Recognised as an official method, it is included in various standards and regulatory guidelines, including those from AOAC, ISO, and several European Directives. The Kjeldahl procedure consists of three main steps:



1) **Digestion**

   Approximately 0.2 g of dry, ground sample was weighed and placed into a digestion flask. A Kjeldahl catalyst tablet and 20 mL of 98% sulphuric acid (H_2SO_4) were added to facilitate the breakdown of organic material and convert nitrogen into ammonium ions (NH_4^+). Digestion was performed for three and a half hours at a controlled temperature between 350 and 380°C.

   Reaction: $CHNO + H_2SO_4 \rightarrow (NH_4)_2SO_4 + CO_2$

2) **Distillation**:

   The digested sample was neutralised by adding 50 mL of 50% NaOH, converting ammonium (NH_4^+) to ammonia (NH_3) under alkaline conditions. A stream of water vapour was introduced to carry the released ammonia into a 50 mL solution of 4% boric acid containing methyl red indicator, forming ammonium borate. This resulted in a colour change from red-violet to green (pH 4.4–5.8). Approximately 150 mL of condensate was collected in the boric acid solution for subsequent titration.

   Reaction: $(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3\ (gas) + Na_2SO_4 + 2H_2O$

3) **Titration**:

   The boric acid solution containing the captured ammonia was titrated with 0.25 mol/L HCl until a slight violet colour change was observed.

   Reaction: $B(OH)_4^- + HCl \rightarrow Cl^- + B(OH)_3 + H_2O$

   The volume and concentration of the hydrochloric acid consumed were used to determine the nitrogen content, which was subsequently used to calculate the percentage of protein in the sample using the following formula:

$$\%\boldsymbol{Nitrogen} = \frac{\boldsymbol{(mL\ standard\ acid - mL\ blank)N\ of\ acid\ 1.4007}}{\boldsymbol{sample\ weight(g)}}$$

Where:

- *mL standard acid is the volume of titrant used for the sample,*
- *mL blank* is the volume of titrant used for the blank sample,
- *N of acid* is the normality of the hydrochloric acid,
- *1.4007* is the nitrogen factor (which converts the amount of acid to nitrogen content),
- *Weight of sample* is the weight of the sample in grams.

The total nitrogen content obtained from the Kjeldahl analysis was converted to crude protein using the conventional nitrogen-to-protein conversion factor of 6.25, which assumes that proteins contain approximately 16% nitrogen. Accordingly, crude protein content was calculated as follow:

$$\%\boldsymbol{Crude\ protein} = \%\boldsymbol{Nitrogen} \times \mathbf{6.25}$$

This conversion is widely applied in nutritional analyses of agricultural and food products and is in line with AOAC and ISO standard methodologies.

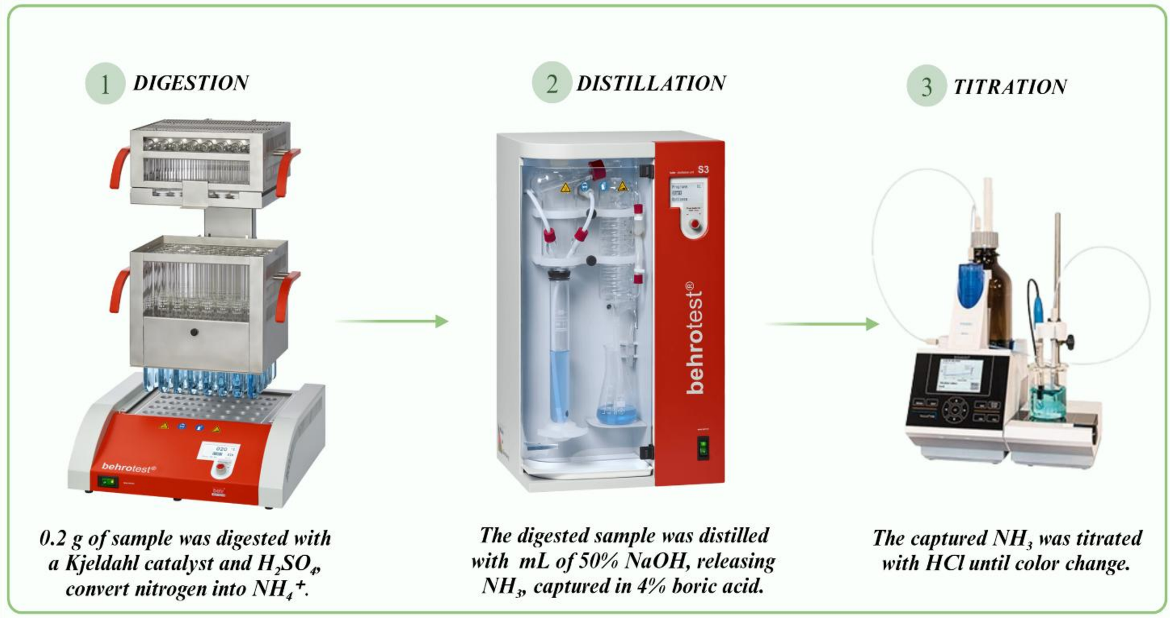


Figure 10 - Crude protein determination process.

3.4. Data analysis

All statistical analyses were performed using RStudio statistical software (version 4.2.2). Initial descriptive statistics - mean, standard deviation, and coefficient of variation - were computed for all measured variables.

Preliminary Analysis

One-way analysis of variance (ANOVA) was conducted to assess the individual effects of soybean meal, barley, and molasses supplementation on six nutritional parameters: dry matter (DM), organic matter (OM), ash content, crude fat (CF), neutral detergent fiber (NDF) and crude protein (CP) at three different levels (2%, 4% and 6%). Post-hoc comparisons were performed using Tukey's Honest Significant Difference test to identify specific treatment differences, with statistical significance set at $\alpha = 0.05$.

Response Surface Methodology

The main analytical approach was response surface methodology (RSM), used to model the relationships between ingredient combinations and nutritional outcomes. The analysis was conducted using the rsm package in R, which fits a second-order (quadratic) model that captures both linear effects and curvature in the response. This approach is particularly useful for identifying optimal conditions and exploring interactions between variables. Only treatment formulations were included in the model; the control group was excluded to prevent potential confounding from baseline compositional differences.

Experimental Design and Coding

Table 4 shows the three dietary ingredients served as predictor variables: soybean meal (SO), barley (BA) and molasses (MO). Each ingredient was tested at three proportional levels (2%, 4%, and 6% of dry matter), which were transformed to coded values to standardize effect magnitudes and facilitate model convergence.

Table 4 - Coding scheme for ingredient proportions in response surface methodology

Ingredient	Low Level (-1)	Center Point (0)	High Level (+1)
Soybean meal	2%	4%	6%
Barley	2%	4%	6%
Molasses	2%	4%	6%

Model Development Strategy

A hierarchical modeling approach was implemented for each response variable, testing three increasingly complex polynomial models:

1. **First-order (FO) model**: Linear main effects only

$$Y = \beta_0 + \beta_1 S + \beta_2 B + \beta_3 M + \varepsilon$$

2. **Two-way interaction (TWI) model**: Main effects plus all pairwise interactions

$$Y = \beta_0 + \beta_1 S + \beta_2 B + \beta_3 M + \beta_{12} SB + \beta_{13} SM + \beta_{23} BM + \varepsilon$$

3. **Second-order (SO) model**: Full quadratic response surface

$$Y = \beta_0 + \beta_1 S + \beta_2 B + \beta_3 M + \beta_{12} SB + \beta_{13} SM + \beta_{23} BM + \beta_{11} S^2 + \beta_{22} B^2 + \beta_{33} M^2 + \varepsilon$$

Model Selection Criteria

Sequential F-tests were employed to compare nested models and determine the minimum complexity required for adequate fit. Terms were retained only if they significantly improved model performance ($p < 0.05$) compared to the simpler alternative. This approach balanced model parsimony with explanatory power, ensuring that each response variable was described by the most appropriate level of complexity.

Model adequacy was evaluated through examination of coefficient significance, overall model F-statistics, and coefficient of determination (R^2) values.

The final model selection for each nutritional parameter was determined by statistical significance testing, retaining only those terms that contributed meaningfully to the prediction of compositional outcomes. And contour plots were generated to visualize the relationship between pairs of ingredients and their influence on the six nutritional parameters studied. Statistical significance was set at $p < 0.05$ throughout the analysis.

IV. RESULTS AND DISCUSSION

1. General results

Table 5 presents the mean and standard deviation for the effects of soybean meal, barley, and molasses supplementation on the chemical composition of LAB-treated olive cake, covering key nutritional parameters such as DM, OM and ash, fat, NDF, and CP. The table also highlights statistical significance levels for each main effect and interaction.

Data from the above-mentioned table reveal that supplementation with soybean meal, barley, and molasses led to significant changes in the chemical composition of the LAB-treated olive cake. Notably, crude protein content increased significantly with higher levels of soybean meal, reaching the highest value (16.2%) observed at the 6% supplementation level. Barley and molasses also contributed to improvements in crude protein, although the effect was most pronounced for soybean meal.

Ash content was significantly affected by both barley and molasses supplementation, with the lowest ash levels found at the highest barley and molasses inclusion rates. Additionally, barley and molasses were associated with significant increases in organic matter, with OM generally increasing at higher inclusion levels. However, supplementation with these ingredients did not significantly alter dry matter, fat, or NDF content in most cases, as indicated by non-significant (NS) results in the respective columns.

Interaction effects between soybean meal and molasses were significant for ash, fat, and OM, suggesting possible synergistic or antagonistic effects when these two supplements are combined.

The results indicate that targeted supplementation with soybean meal, barley, and molasses significantly enhances the nutritional quality of olive cake, particularly in terms of protein content and mineral composition. These improvements highlight the potential of such treatments to optimize olive cake as a valuable feed resource for livestock.

Table 5 - Mean and standard deviation of the effect of soybean, barley and molasses on the dry matter, organic matter, ash, crude fat, NDF and crude protein of olive cake treated with Lactic acid bacteria.

	DM	OM	ASH	CF	NDF	CP
Crude OC	97±0.042	90.58±0.039	8.86±0.039	1.18±0.009	50.78±1.470	13.4±0.304
Soybean meal	***	*	NS	NS	NS	***
2	97.0±0.226^{a}	91.1±0.266^{a}	8.36±0.243^{a}	1.22±0.148^{a}	62.1±16.6^{a}	14.3±0.865^{c}
4	96.8±0.221^{b}	91.1±0.314^{ab}	8.38±0.291^{a}	1.26±0.140^{a}	61.9±14.9^{a}	14.9±0.730^{b}
6	96.8±0.240^{b}	91.0±0.284^{b}	8.45±0.257^{a}	1.20±0.153^{a}	57.1±7.23^{a}	16.2±0.544^{a}
Barley	NS	***	***	NS	NS	***
2	96.8±0.209^{a}	90.8±0.174^{c}	8.66±0.145^{a}	1.25±0.125^{a}	62.09±16.8^{a}	15.5±0.888^{a}
4	96.9±0.255^{a}	91.1±0.215^{b}	8.35±0.195^{b}	1.20±0.161^{a}	61.3±6.906^{a}	15.1±1.10^{b}
6	96.8±0.264^{a}	91.3±0.242^{a}	8.20±0.230^{c}	1.24±0.148^{a}	58.5±16.5^{a}	14.9±1.01^{b}

Molasses	NS	***	***	NS	NS	***
2	96.8±0.227^{a}	91.0±0.235^{b}	8.47±0.212^{a}	1.26±0.168^{a}	60.8±2.83^{a}	15.4±0.928^{a}
4	96.9±0.207^{a}	91.0±0.292^{b}	8.44±0.260^{a}	1.23±0.131^{a}	60.6±14.9^{a}	15.2±1.08^{a}
6	96.8±0.304^{a}	91.2±0.308^{a}	8.27±0.284^{b}	1.19±0.137^{a}	59.9±18.3^{a}	14.8±0.972^{b}
Two-ways interactions						
Soybean×Barley	NS	NS	NS	NS	NS	NS
Soybean×Molasses	NS	*	*	*	NS	NS
Barley×Molasses	NS	NS	NS	NS	NS	NS

Significance codes: * p<0.05, ** p< 0.01, *** p<0.001 and NS p > 0.05

In the same column, values assigned the same letter are not significantly different at the α = 5% threshold.

Values in the same column that are assigned different letters are significantly different at the α = 5% threshold (p < 0.05).

DM = Dry matter; OM = Organic matter; CF = Crude fat; NDF = Neutral detergent fiber; CP = Crude protein.

Supplementation with soybean meal, barley, and molasses significantly influenced the nutritional profile of the olive cake, with specific components showing marked variations according to the type and inclusion level of each supplement. Crude protein content increased significantly with higher levels of soybean meal, while ash and organic matter content were notably affected by barley and molasses additions.

The combination of soybean meal providing essential nitrogen sources for LAB protein synthesis (Liu *et al*., 2017), barley supplying complex carbohydrates for sustained microbial energy (Agyekum *et al*., 2021), and molasses offering readily available simple sugars for rapid LAB growth initiation (Dumbrepatil *et al*., 2008), created optimal substrate conditions that enhanced LAB performance beyond what individual supplements could achieve alone.

This substrate diversity enabled LAB to achieve higher cell densities (Ni *et al*., 2017), produce a broader spectrum of beneficial metabolites including bioactive peptides and multiple organic acids (Kanmani *et al*., 2013), and facilitate the formation of chelated mineral complexes that improved nutrient bioavailability (Cotârleț et al., 2020) (García-Cano *et al.*, 2019). The resulting fermentation process generated nutritional improvements that exceeded the sum of individual supplement contributions, demonstrating true synergistic effects where the combined LAB fermentation system created enhanced feed quality benefits that neither the supplements alone nor LAB fermentation with single substrates could accomplish independently.

Comparing crude olive cake (non-treated) from our study with literature values further contextualizes these findings. The DM content of 96.96% is higher than the average values reported previously (around 80.5%; Al Jassim *et al.* , 1997; Al-Masri, 2001, 2003; Martín-García *et al*., 2003; Molina-Alcaide *et al.,* 2003; Yáñez-Ruiz *et al*., 2004), but aligns closely with values reported for dried olive cake (956 g/kg DM, V. Chiofalo *et al*., 2020; 946-952 g/kg DM, Marcos *et al*., 2019). This suggests that the olive cake used here underwent minimal moisture retention, comparable to more recently processed samples.

The OM content (90.58% or 905.8 g/kg DM) fits within the expected range, similar to those found by Marcos *et al*. (2019) for crude and exhausted olive cake, but is higher than some earlier studies and lower than values for fresh or ensiled olive cake (Awawdeh & Obeidat, 2013; Hadhoud *et al*., 2021). Such variability is likely due to differences in processing, extraction method, and storage.

Our crude protein value (13.4%) is within the range reported in the literature for untreated olive cake but is lower than values obtained for certain treated or fermented samples (Hadjipanayiotou, 1999; Al Jassim *et al*., 1997; García-Rodríguez *et al*., 2020). This variation likely reflects differences in nitrogen retention and microbial activity during processing.

The ash content of 8.86% is broadly consistent with previous findings, though slightly lower than some reports (Hadjipanayiotou, 1994) and slightly higher than others (B. Chiofalo et al., 2004; Shaaban *et al*., 2021; Hadhoud *et al*., 2021). Such differences are probably due to oil extraction efficiency, soil contamination, and the proportion of residual inorganic matter.

The crude fat content was very low (1.18%), substantially lower than values reported in most studies. This result supports the conclusion that the olive cake used here underwent efficient double extraction, leaving little residual oil, consistent with findings for intensively processed samples (Marcos *et al.,* 2019).

Regarding NDF, the value in our study (50.78%) is comparable to those reported for dried olive cake by V. Chiofalo *et al.* (2020) and for fresh olive cake by Awawdeh & Obeidat (2013), but higher or lower than some other literature reports (Abo Omar *et al*., 2012; Al Jassim *et al.,* 1997; B. Chiofalo *et al*., 2004; García-Rodríguez *et al*., 2020). These differences reflect the diversity of olive cake composition and processing across studies.

As shown in Table 4, supplementing olive cake, particularly with soybean meal, significantly improves its nutritional composition. This finding supports the potential of olive cake as a more effective and sustainable feed ingredient for livestock.

1.1. Effect of Soybean meal inclusion.

The inclusion of soybean meal increased ($p < 0.001$) the CP content, OM and NDF content ($p < 0.05$). In contrast, no significant differences were observed in ash and fat content ($p > 0.05$), indicating that these parameters remained relatively stable across soybean meal inclusion levels.

For LAB-treated olive cake, the DM content exhibited a marginal decline ($p < 0.001$) from 97.0% at 2% soybean inclusion to 96.8% at 4% and 6%. The DM content at higher soybean levels returned to values comparable to the control group. This slight reduction may be attributed to the moisture-retaining properties of soybean meal (Wang *et al.,* 1995). The variability of olive cake composition, as highlighted by Obeidat (2017), is influenced by factors such as seasonality, extraction method, fruit ripeness, and geographic origin. Similar trends in DM content variation following additive treatments have been reported by Abo Omar *et al*. (2012).

The application of fermentative bacteria in this study aimed to improve fermentation efficiency and the overall nutritive value of the silage. LAB inoculation can reduce aerobic deterioration and enhance silage hygiene. The observed improvement in DM content aligns with findings by Bolsen *et al*. (1992), who demonstrated that homofermentative LAB inoculants can enhance DM recovery by minimising fermentation losses and improving preservation efficiency. The ability of LAB to rapidly lower pH may have contributed to reduced nutrient degradation and improved DM retention in the silage.

CP content responded most markedly to soybean meal inclusion, increasing ($p < 0.001$) from 143 g/kg DM (14.3%) at 2% to 149 g/kg DM (14.9%) at 4%, and reaching 162 g/kg DM (16.2%) at 6% inclusion. This result demonstrates the effectiveness of soybean meal as a protein-rich supplement for livestock feeds. Other studies, such as Alhamad *et al.* (2017) and Abo Omar *et al*. (2012), have also reported CP increases in olive cake following fermentation or chemical treatment, albeit sometimes with more pronounced effects depending on treatment intensity and additive type. The current results, in combination with LAB inoculation, highlight the synergistic benefit of combining protein supplementation and microbial fermentation for enhancing feed quality.

Soybean meal supplementation also had decreased ($p < 0.05$) organic matter content. Across the inclusion levels, OM content ranged from 91.1% (911 g/kg DM) at 2% and 4% soybean to 91.0% (910 g/kg DM) at 6%. The narrow range and modest differences suggest that soybean meal's main impact is on protein enrichment rather than altering the overall organic matter or energy content of the feed. This is consistent with previous research on non-protein nitrogen (NPN) treatments, such as ammonia, which showed more substantial effects on OM content due to fiber breakdown (Nefzaoui, 1983).

Soybean meal had no effect ($p > 0.05$) on ash content. Given that ash and organic matter are complementary, the lack of change in ash further supports the conclusion that soybean meal serves primarily as a protein supplement, rather than altering mineral or structural composition.

Regarding NDF, soybean meal treatment did not produce statistically significant changes ($p > 0.05$), though a slight decreasing trend was noted, from 621 g/kg at 2% inclusion to 571 g/kg at 6%. This finding is consistent with the literature, which indicates that soybean meal and LAB inoculants generally have limited influence on fiber content, as most LAB strains lack the enzymatic capability to degrade plant cell walls (Yitbarek & Tamir, 2014).

1.2. Effect of Barley inclusion

Barley inclusion led to a decrease ($p < 0.01$) in ash content, an increase ($p < 0.001$) in organic matter, and a decrease ($p < 0.001$) in crude protein levels. No differences ($p > 0.05$) were observed for dry matter, fat, or neutral detergent fiber. The CP content steadily decreased from 15.5% at 2% barley inclusion to 15.1% at 4% and 14.9% at 6%. This decline aligns with previous findings indicating that barley's high starch and lower protein concentration can dilute overall dietary protein. OM content rose from 90.8% at 2% barley to 91.1% at 4% and 91.3% at 6%, while ash content dropped from 8.66% at 2% to 8.35% at 4% and 8.20% at 6%. This trend reflects a compositional shift, with the higher organic fraction introduced by barley and a reduction in mineral concentration. These changes are likely due to barley's nutritional composition and its impact on the overall mix. Other parameters, including DM, fat, and NDF, remained stable ($p > 0.05$) across barley inclusion levels, with no notable changes detected.

Crude protein content exhibited a consistent decrease ($p < 0.001$) as barley inclusion levels increased, declining from 15.5% at 2% inclusion to 15.1% at 4% and 14.9% at 6% inclusion. This trend is consistent with the findings of Nikkhah (2012), who reported that barley's relatively high starch and low protein content can produce a dilution effect on the overall protein concentration in ruminant diets. As barley replaces higher-protein ingredients in the ration, the total CP content decreases, underscoring the need for well-studied ration formulation to maintain adequate protein levels while using the energy benefits of barley. Typically, barley contains 8-13% CP, which is substantially lower than protein-rich feeds such as soybean meal (44-48% CP) (Senarathna *et al*., 2024). Therefore, increasing the proportion of barley in the mixture naturally reduces the overall protein content, a phenomenon particularly evident in olive cake-based diets, where baseline protein levels may already be modest.

Organic matter and ash contents were both highly significant ($p < 0.001$) and showed opposite trends. As the level of barley supplementation increased, OM content increased from 90.8% at 2% to 91.3% at 6%, whereas ash content decreased from 8.66% to 8.20% across the same range. This shift reflects the compositional characteristics of barley, which is rich in carbohydrates and organic material (Raj *et al*., 2023). The observed increase in OM likely results from the additional fermentable substrates contributed by barley, which, in combination with lactic acid bacteria (LAB) inoculation, may have enhanced microbial activity and organic acid production. Conversely, the reduction in ash content indicates a relative decrease in mineral concentration as the proportion of organic fraction increases. Similar patterns have been observed in silage studies, where microbial inoculation, particularly with species such as *Lactobacillus buchneri*, improves fermentation and aerobic stability (Kung *et al.,* 1999; Liang *et al*., 2008; Rice *et al*., 1990).

Barley supplementation did not alter ($p > 0.05$) dry matter, fat, or neutral detergent fiber content. DM values remained stable (96.8%-96.9%), indicating minimal impact on feed moisture. Fat content was unaffected (1.20%-1.25%), and while NDF content showed a slight numerical decline from 62.09 g/kg to 58.5 g/kg with increased barley inclusion, this change was not statistically relevant. These findings are consistent with evidence that most LAB strains lack the enzymatic capability to degrade plant cell walls (Liang *et al*., 2008), and barley, at the tested inclusion levels, does not substantially affect the fiber or lipid fractions of treated olive cake.

These results indicate that barley functions primarily as an energy source in olive cake-based rations, increasing the organic matter fraction but decreasing protein and mineral concentrations as its inclusion rises. Thus, careful consideration of barley's nutritional profile is essential for the formulation of balanced rations using olive cake as a feed component.

1.3. Effect of Molasse inclusion

The inclusion of molasses in LAB-treated olive cake produced effects like those observed with barley, likely due to their shared content of soluble carbohydrates, which influence the chemical composition of olive cake in comparable ways. Like barley, molasses inclusion led to an increase ($p < 0.001$) in organic matter, a decrease ($p < 0.001$) in crude protein, and a decrease ($p < 0.001$) in ash content. No differences ($p > 0.05$) were observed for dry matter, fat, or neutral detergent fiber. These effects are primarily attributable to the readily fermentable carbohydrates present in both molasses and barley, which enhance microbial activity and modify nutrient partitioning.

The observed impact of molasses inclusion is consistent with previous research on its role in silage fermentation. Weiss and Underwood (2009) reported that rapidly fermentable ingredients, such as molasses, stimulate fermentation in silages, especially those based on sugar-limited forages. The rapid availability of energy from molasses supports the activity of lactic acid bacteria, promoting fermentation and reducing silage pH (Henderson, 1993). However, the high viscosity of molasses requires dilution for uniform application, and in low dry matter forages, some loss of the additive may occur in the effluent during early ensiling.

The changes in OM, CP, and ash content observed with molasses supplementation are consistent with its known effects on microbial fermentation and nutrient dynamics. Yitbarek and Tamir (2014) found that the addition of molasses increased lactic acid production and reduced volatile nitrogen levels, indicating improved fermentation quality. These results support the findings of the present study, suggesting that the soluble carbohydrates in molasses enhance microbial fermentation in a manner comparable to barley. Nonetheless, as noted by Woolford (1984), the presence of fermentable sugars alone does not guarantee optimal lactic acid bacterial activity; other factors, including moisture content and microbial competition, also play crucial roles in determining fermentation success.

No significant differences were observed in NDF content ($p > 0.05$), with values remaining stable, ranging from 608 g/kg at 2% molasses inclusion to 599 g/kg at 6%. This outcome aligns with the findings of Tjandraatmadja *et al*. (1994), who demonstrated that 4% molasses was sufficient for effective silage preservation, with little impact on fiber content. Their work with Pangola grass silage similarly highlighted the stability of NDF levels across varying levels of molasses inclusion.

2. Response Surface methodology

Table 6 summarizes the results of the analysis of variance (ANOVA) performed on the response surface models fitted to the nutritional parameters of treated olive cake. Response Surface Methodology was employed to investigate the effects of the experimental variables and their interactions, using second-order polynomial models. This approach allowed for the evaluation of both linear and quadratic effects, as well as interaction terms, providing a comprehensive understanding of how the treatment factors influenced each nutritional outcome. To ensure model, only the treatment formulations were included in the analysis, excluding the control group.

The response surface analysis revealed that four nutritional parameters (DM, OM, Ash and CP) evaluated were significantly influenced by first-order effects ($p < 0.001$), for fat content and fiber content, they showed no significant response ($p > 0.05$). Significant quadratic effects ($p < 0.05$) were observed for organic matter, Ash, and crude protein content, while two-way interaction effects had no statistically significant impact on any of the responses ($p > 0.05$).

Lack of fit was found to be non-significant ($p > 0.05$) for all responses, indicating the adequacy of the fitted models. Among the models, crude protein content, organic matter, and Ash showed the best fit, with high adjusted R^2 values of 0.635, 0.603, and 0.603, respectively, and corresponding model p-values < 0.001. In contrast, fat content and neutral detergent fiber content showed low model fits (adjusted $R^2 = 0.060$ and 0.013), with no significant overall effects ($p > 0.05$). These results indicate differential sensitivity of the proximate composition traits to fermentation conditions, as detailed in Table 6.

Table 6: Analysis of variance of the response surface models fitted to the nutritional parameters of treated olive cake.

	DM		OM		Ash		CF		NDF		CP	
	Mean sq	**Pr(>F)**	**Mean sq**	**Pr(>F)**	**Mean sq**	**Pr(>F)**	**Mean sq**	**Pr(>F)**	**Mean sq**	**Pr(>F)**	**Mean sq**	**Pr(>F)**
First- order	0.316	p<0.001	1.922	p<0.001	1.573	p<0.001	0.037	p > 0.05	155.04	p > 0.05	25.102	p<0.001
Two-ways interactions	0.093	p > 0.05	0.080	p > 0.05	0.080	p<0.05	0.041	p > 0.05	212.97	p > 0.05	0.985	p > 0.05
Polynomial quadratic order	0.280	p > 0.05	3.165	p<0.05	0.094	p<0.05	0.033	p > 0.05	109.94	p > 0.05	1.169	p<0.05
Residuals	0.051	.	0.034	.	0.028	.	0.020	.	190.54	.	0.387	.

Pure error	0.053	.	0.034	.	0.028	.	0.020	.	188.46	.	0.372	.
Lack of fit	0.002	$p > 0.05$	0.036	$p > 0.05$	0.028	$p > 0.05$	0.045	$p > 0.05$	262.39	$p > 0.05$	0.928	$p > 0.05$
Stationary point (%)	S = 5.59 B = 4.18 M = 2.91		S = -0.21 B = 7.64 M = 4.032		S = 0.95 B = 7.49 M = 3.36		S = 4.22 B = 4.12 M = 3.86		S = -2.064 B = 5.202 M = 12.8		S = 0.89 B = 6.92 M = 3.33	
Adjusted R^2	0.149		0.603		0.603		0.060		-0.013		0.635	
p-value	$p<0.001$		$p<0.001$		$p > 0.05$		$p > 0.05$		$p > 0.05$		$p<0.001$	

2.1. Dry matter content

Figure 11 shows contour plots illustrating the effects of ingredient interactions on dry matter content: (**a**) soybean meal vs barley, with molasses fixed at its central level (Molasses = 0) ; (**b**) soybean meal vs molasses, with barley fixed at its central level (Barley = 0).

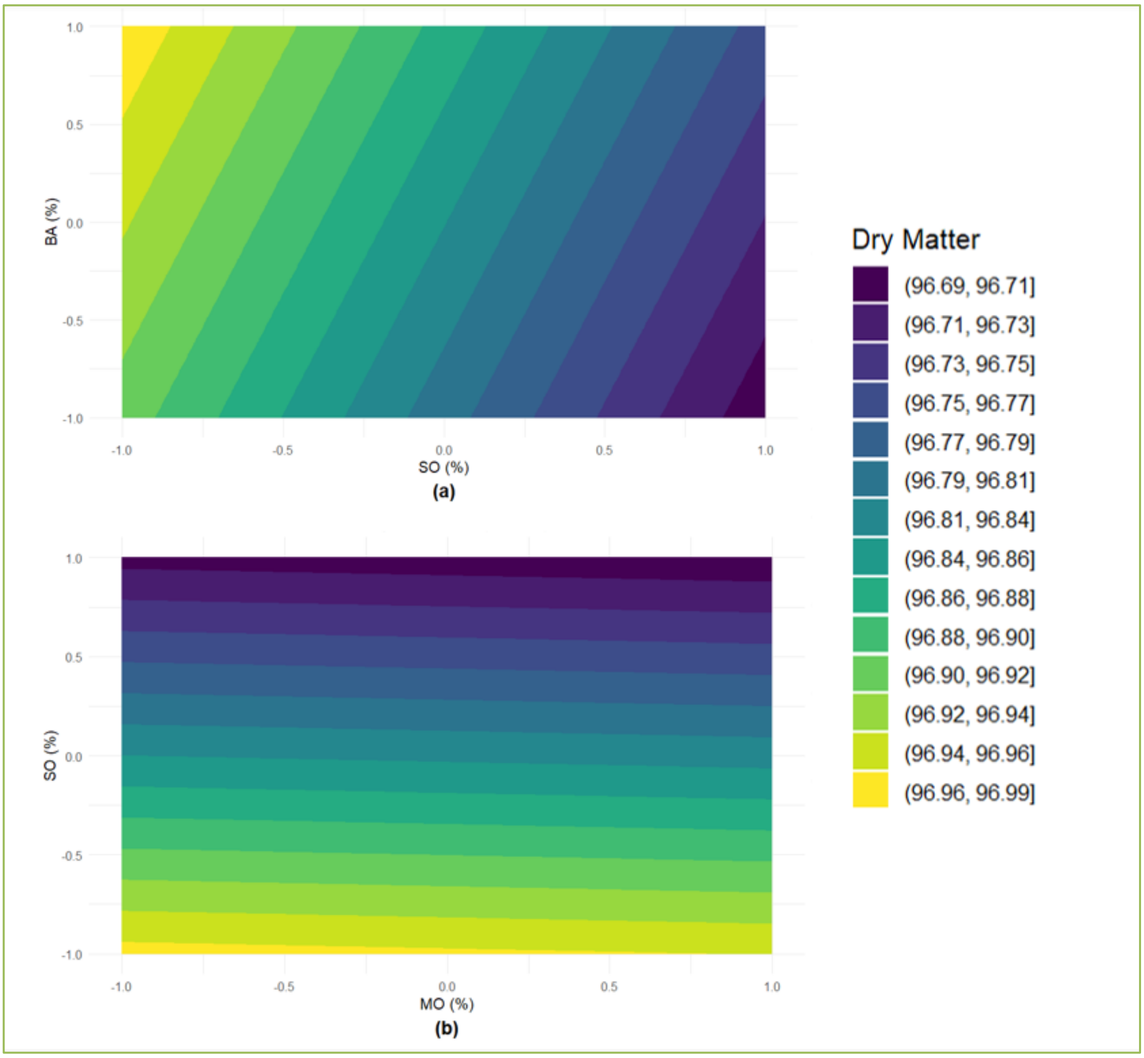


Figure 11 - Contour plots of ingredient interactions affecting dry matter content with third variable held at center point: **(a)** soybean meal vs. barley; **(b)** soybean meal vs. molasses.

RSM analysis of dry matter content, based on 117 observations, indicated that a first-order linear model provided the best fit. Although interaction ($p = 0.0015$) and quadratic ($p = 0.0016$) terms were statistically significant, they did not notably improve model performance over the linear model ($F_{3,113} = 5.883$, $p = 0.0009$), which explained 13.5% of the variance (adjusted $R^2 = 11.2\%$). Importantly, the lack-of-fit test was non-significant ($p = 0.31$), validating the model's adequacy for interpreting the experimental data.

As illustrated in Figure 11a, soybean meal had a clear and statistically significant negative effect on DM content ($p < 0.001$), with DM values consistently decreasing as soybean meal levels increased from –1 to +1. This decrease observed can be attributed to the high water-soluble fraction of soybean meal, including soluble sugars and nitrogenous compounds, which likely promoted water retention during silage fermentation. These observations agree with prior findings, which emphasized the moisture-retaining capacity of soybean meal as a key factor affecting the dry matter content (Wang *et al.,* 1995).

In figure 11b, barley exhibited a non-significant positive trend ($p > 0.05$) in its effect on DM. Despite being a fibrous and carbohydrate-rich cereal known to influence fermentation dynamics (Andrieu and Weiss, 2002), the concentration range employed in this study may not have been sufficient to elicit a measurable shift in moisture-related outcomes. Moreover, barley’s effects may have been partially masked by the dominant influence of soybean meal, especially in interactions where both ingredients were present at high levels.

Molasses, commonly incorporated as an energy-rich additive to enhance fermentability and support lactic acid production, showed no impact on DM within the tested range ($p > 0.05$). The nearly horizontal contour lines in the molasses-soybean meal interaction plot (Figure 11b) confirm that molasses did not meaningfully influence DM values. Contrary to previous findings by Abarghoei et al. (2011), who reported increased DM content with molasses addition in olive cake silage, our study showed no significant molasses effect. This discrepancy could be due to differences in molasses inclusion levels or olive cake composition. It’s also possible that the consistent use of LAB inoculants across all treatments in our study reduced the chance of detecting any independent effect of molasses on dry matter content.

Since all treatments in our study received LAB inoculation, the observed dry matter patterns likely reflect the combined effects of ingredient supplementation under optimized fermentation conditions. LAB strains such as *Lactobacillus plantarum* are known to accelerate pH decline, promote lactic acid accumulation, and inhibit spoilage microbes (Okoye *et al.,* 2023; Carvalho *et al.*, 2021), leading to enhanced nutrient conservation and reduced dry matter losses during fermentation (Cecava, 1995). Under these controlled conditions, the moisture-retaining properties of soybean meal appeared to play a more dominant role in influencing DM content, while the stabilizing effects of LAB may have minimized the typical DM-enhancing impact of molasses reported in non-inoculated systems (Abarghoei *et al*., 2011). This suggests that LAB inoculation creates a more regulated fermentation environment, where ingredient-specific effects on moisture dynamics become more pronounced. Furthermore, the synergy between LAB activity and proper silage management, such as rapid airtight sealing and moisture control, likely contributed to the effective preservation of DM, even in the presence of high-moisture ingredients like soybean meal.

The visual trends observed in the plots (Figure 11a and 11b) reinforce the statistical findings. In the soybean meal-molasses interaction plot (Fig. 14b), DM decreased progressively with increasing soybean meal, while molasses had little to no visible effect. In the soybean meal-barley plot (Fig. 14a), diagonal contours initially suggest an interaction; however, model output confirmed that only soybean meal was statistically significant, despite barley’s slight visual gradient.

From an optimization perspective, these findings point to soybean meal as the primary lever for modifying DM levels in treated olive cake. Given that higher levels of soybean meal consistently reduced DM, reducing its inclusion offers a practical approach to enhancing DM content in silage formulations. While barley and molasses may contribute under different conditions, their roles appear secondary under the tested parameters.

2.2. Organic matter content

Figure 12 shows contour plots of ingredient interactions affecting the organic matter content of LAB-treated olive cake, (a) soybean meal vs. barley (molasses at center point); (b) soybean meal vs. molasses (barley at center point); (c) molasses vs. barley (soybean meal at center point).

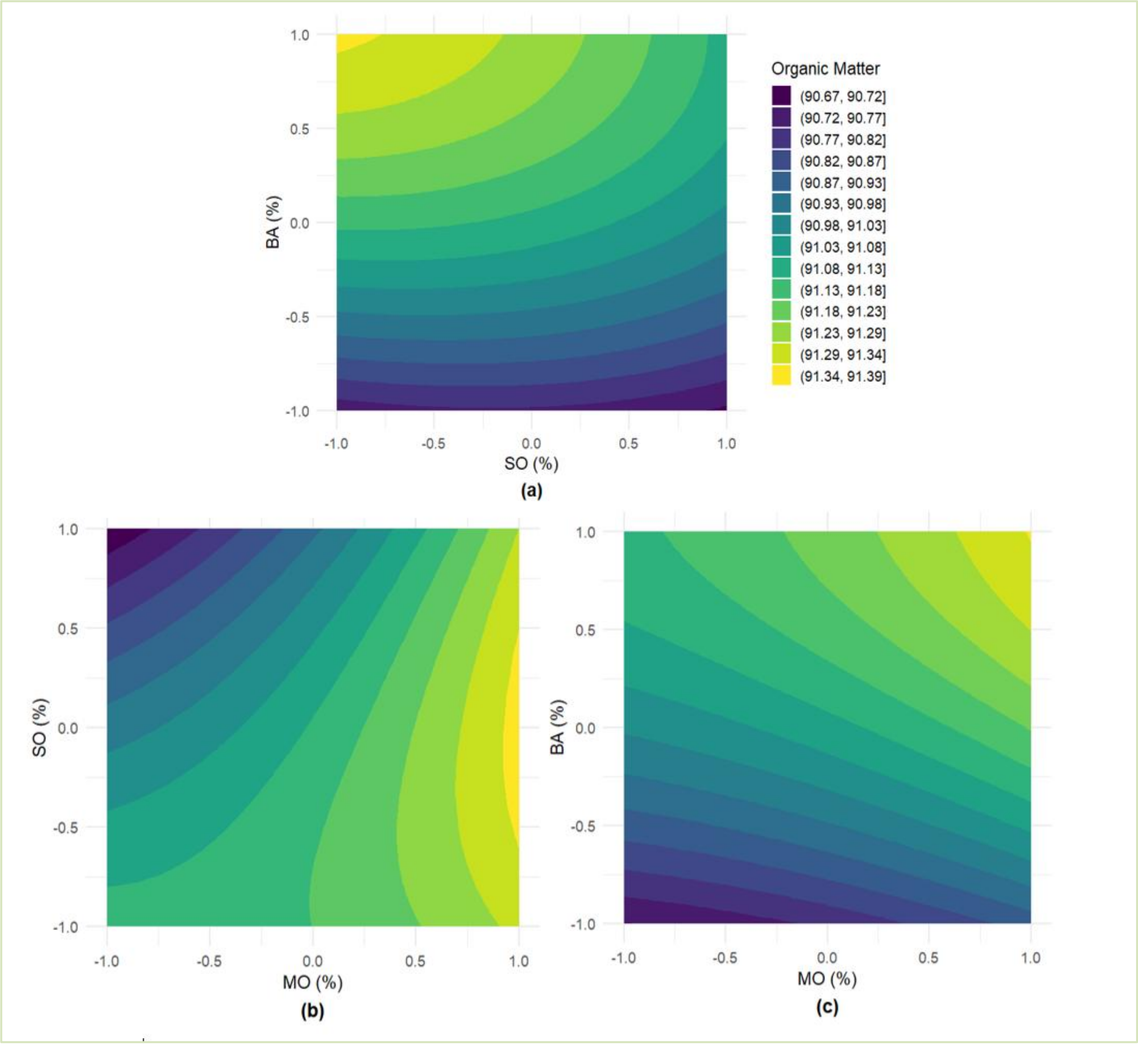


Figure 12 - Contour plots of ingredient interactions affecting organic matter content with third variable held at center point: **(a)** soybean meal vs. barley; **(b)** soybean meal vs. molasses; **(c)** molasses vs. barley.

A quadratic response surface model was selected to describe organic matter content, providing a statistically significant fit ($F_{9,107} = 20.6$, $p < 0.001$) with $R^2 = 63.4\%$ and adjusted $R^2 = 60.3\%$. Barley and molasses showed the strongest positive effects on OM ($p < 0.001$), followed by soybean meal ($p < 0.01$). Significant interaction between soybean meal and molasses ($p < 0.05$), and a quadratic effect of barley ($p < 0.05$), were also detected. The lack of fit was not significant ($p = 0.375$), confirming model adequacy.

Eigenvalues indicated a saddle point, suggesting no clear optimum within the studied space. These results imply that OM content could potentially be improved by increasing barley and reducing soybean meal beyond the tested levels.

Contour plots at figure 12 visually reinforce the experimental findings, demonstrating that organic matter content is maximized at high levels of both barley and molasses, especially when soybean meal is maintained at central or lower concentrations. In Figure 12a, barley exhibited the strongest positive effect on OM content ($p < 0.001$), with OM values increasing sharply as barley inclusion rose from -1 to +1. This effect aligns with barley's role as a highly fermentable carbohydrate source that supports LAB metabolism by providing essential substrates for rapid lactic acid production (Cecava, 1995). Meanwhile, Figure 12b reveals a notable curvature in the soybean meal versus molasses interaction, aligning with the statistically significant interaction term identified in the response surface analysis. These findings are supported by the studies of Yáñez-Ruiz *et al*. (2004) and Muñoz *et al*. (1983) demonstrated that barley supplementation improves rumen microbial activity, digestibility, and animal growth when combined with olive by-products. The significant quadratic effect ($p < 0.05$) in our model suggests a curved relationship between barley concentration and OM content, emphasizing the need to adjust its inclusion for maximum efficacy. The improvement in OM with barley addition reflects enhanced fermentation kinetics, LAB inoculum, converts soluble carbohydrates into lactic acid via the Embden-Meyerhof pathway, driving pH reduction and nutrient stabilization (Okoye *et al*., 2023).

Molasses also demonstrated a strong and significant positive effect on OM content ($p < 0.001$), as evidenced by the steep gradients in Figure 12c. The addition of molasses has long been recommended to enhance fermentation, especially when the forage has low levels of water-soluble carbohydrates (Cecava, 1995).

Our results echo the findings of Abarghoei *et al.* (2011) and Weinberg *et al.* (2008), who showed that molasses supplementation improves OM digestibility, fermentation stability, and microbial activity. The readily available sugars in molasses not only stimulate LAB growth but also reduce ammonia-N levels, improve acid production, and enhance overall fermentation quality.

In contrast, soybean meal exhibited a modest but statistically significant effect on OM content ($p < 0.01$), though far weaker than barley or molasses. This aligns with its role primarily as a protein and amino acid supplement, rather than a fermentation enhancer (Cecava, 1995).

All treatments received LAB inoculation, meaning the observed OM patterns reflect ingredient effects under optimized microbial conditions. Homofermentative strains, like *Lactobacillus plantarum*, rapidly lower pH to preserve nutrients, while heterofermentative strains, such as L. *buchneri,* ensure aerobic stability during storage (Kim *et al*., 2021). Studies such as Sharma *et al*. (2022) and Li *et al.* (2018) also indicate that certain LAB produce bacteriocins, fiber-degrading enzymes, and exopolysaccharides, further enhancing silage quality, especially when applied to lignocellulosic-rich substrates like olive cake.

While soybean meal improves the nutritional density of olive cake, supplying essential amino acids such as lysine and methionine (Nefzaoui, 1985), it lacks the fermentable carbohydrate content required to significantly boost LAB activity. This likely explains its limited contribution to OM preservation. However, its inclusion remains crucial to address the crude protein deficiency inherent to olive cake.

A notable interaction between soybean meal and molasses ($p < 0.05$), visualized in Figure 12b through curved contour lines, suggests that soybean meal's effect on OM is modulated by molasses concentration. At higher molasses levels, soybean inclusion may exert inhibitory effects, possibly due to nitrogen imbalance or microbial competition.

This highlights the importance of ingredient ratios, as the metabolic demands of LAB rely on a precise balance between readily fermentable energy and nitrogen sources. Excess protein without sufficient energy may lead to increased ammonia formation and reduced lactic acid yield (Borreani *et al.*, 2018).

These patterns collectively suggest that increasing barley and molasses concentrations can enhance OM preservation, particularly under conditions of reduced soybean meal. Eigenanalysis of the response surface indicated the presence of a saddle point, meaning that OM content can increase or decrease depending on the direction within the design space, without a single clear optimum.

This complexity highlights the importance of ingredient interactions: while higher barley levels likely promote OM preservation due to their fermentable carbohydrate content, which supports microbial activity during fermentation, excess soybean meal may disrupt this process, potentially by altering the balance of nutrients or affecting microbial dynamics (Borreani *et al.*, 2018) (Petrotos *et al.*, 2021). These findings are consistent with broader silage optimisation research, which emphasizes the role of carbohydrate-rich substrates in supporting favourable fermentation and nutrient preservation.

The RSM model confirmed that both barley and molasses had increased the organic matter content of the LAB-treated ($p < 0.001$), aligning with their known roles as fermentable carbohydrates sources (Raj *et al.*, 2023) that enhanced OM and decreased ash content at higher barley levels. The modest effect of soybean meal observed in the RSM model ($p < 0.05$) also align with previous results, which indicate that it has limited influence on the organic matter despite statistical significance that might be neglected in this case, enforcing its role in protein enhancement rather than fermentation (Kung *et al.*, 1999; Liang *et al.*, 2008; Rice *et al.*, 1990). Moreover, the identification of a saddle point in the RSM analysis supports the interpretation that the organic content of LAB-treated olive cake can be optimised along specific ingredient combinations, particularly high barley and molasses, rather than through uniform increase in any single input.

The saddle point configuration identified in eigenanalysis confirms that OM optimization cannot be achieved by simply maximizing each ingredient. Instead, it requires balancing energy and protein sources, with barley and molasses as the key levers. Increasing their concentrations slightly beyond the tested range, while maintaining moderate soybean levels, could enhance OM preservation by maximizing fermentable substrate availability for LAB.

2.3. Ash content

Figure 13 illustrates the contour plots showing the effects of two-factor ingredient combinations on ash content in LAB-inoculated olive cake silage, while holding the third variable at its central value: (a) soybean meal versus barley (molasses = 0); (b) soybean meal versus molasses (barley = 0); and (c) molasses versus barley (soybean meal = 0).

A quadratic model provided the best fit for ash content in LAB-inoculated olive cake silage ($F_{9,107} = 20.6$, $p < 0.001$), capturing 63.4 % of the total variation (adjusted $R^2 = 60.3$ %) with a non-significant lack of fit ($p = 0.395$), indicating suitability for interpretation.

Barley decreased ash content significantly ($p < 0.001$), as did molasses ($p < 0.001$), whereas soybean meal increased ash content, though to a lesser extent ($p < 0.05$). Among interaction terms, only the soybean meal × molasses interaction was significant ($p < 0.05$), suggesting that the influence of soybean meal on ash content depends on molasses level. A significant quadratic effect of barley ($p < 0.05$) indicated a curved response across barley additions. Eigenanalysis identified both positive and negative eigenvalues, confirming a saddle point configuration at SO = 0.95 %, BA = 7.49 %, MO = 3.36 %, which lies outside the experimental range. This indicates no single optimum, but rather that ash content can increase or decrease along different directions within the design space.

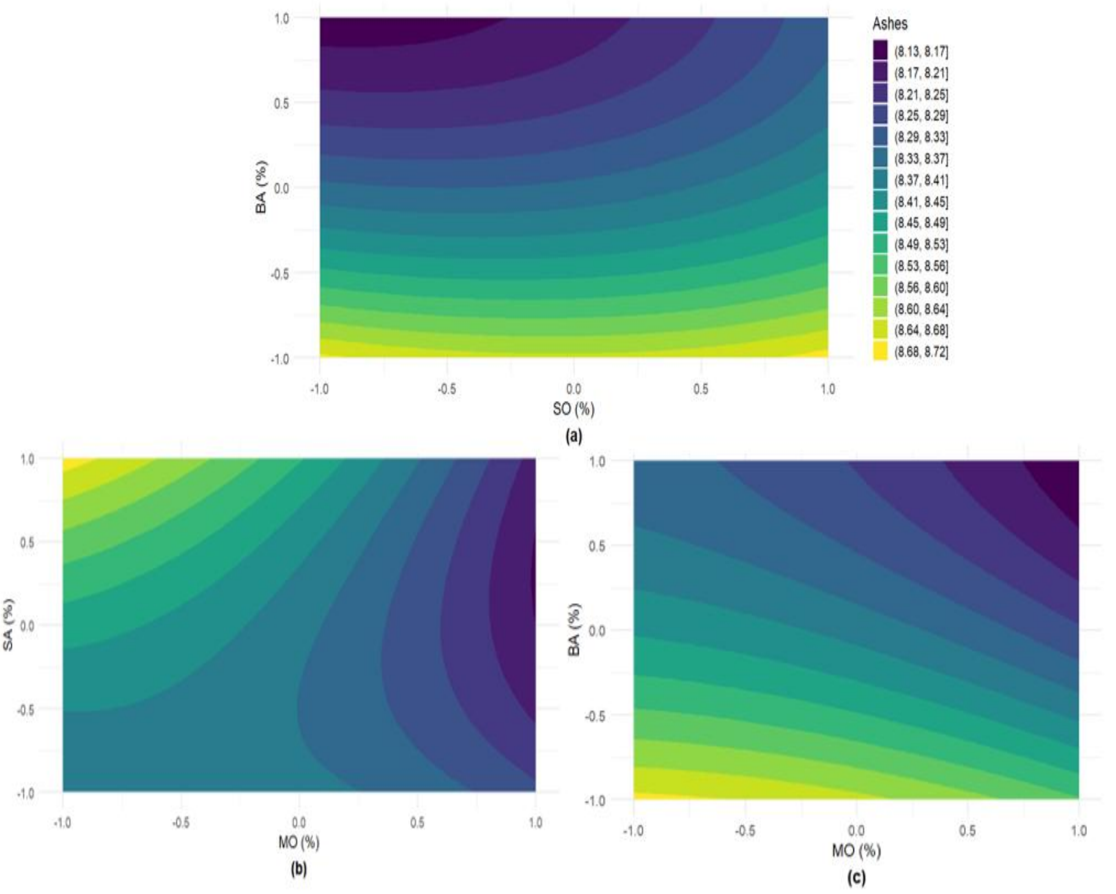


Figure 13 - Contour plots of ingredient interactions affecting ash content with third variable held at center point: (a) soybean meal vs. barley; (b) soybean meal vs. molasses; (c) molasses vs. barley.

The ash reducing effect of barley is consistent with its role as a rapidly fermentable carbohydrate source that supports lactic acid bacteria (LAB) proliferation, resulting in improved preservation of organic matter and a subsequent dilution of mineral concentration. Barley's capacity to enhance fermentation kinetics and nutrient retention has been reported to improve fiber digestibility and reduce ash content in treated lignocellulosic by-products (Muñoz *et al*., 1983; Yáñez-Ruiz *et al.,* 2004). Likewise, molasses, rich in soluble sugars, promotes rapid LAB growth and acidification (Weinberg *et al.,* 2008), facilitating efficient conversion of soluble substrates into fermentation products while minimizing leaching and oxidative mineral concentration. The present findings corroborate this behaviour, with higher molasses inclusion decreasing ash content, markedly reflecting enhanced nutrient stabilization through LAB fermentative efficiency.

In contrast, soybean meal, despite being an important protein source, exhibited a slight ash-increasing effect, likely due to its greater intrinsic mineral content (notably calcium, phosphorus, and potassium) and moisture-retaining character, which can elevate the proportion of residual inorganic matter upon drying (Nefzaoui, 1985; Wang *et al.,* 1995). Furthermore, the significant interaction between soybean meal and molasses suggests that, at higher molasses concentrations, the diluting effect on ash may partly counterbalance the mineral contribution of soybean meal. However, under conditions of moderate molasses input, soybean meal may contribute disproportionately to ash accumulation, probably due to inefficient use of nitrogen in the absence of sufficient fermentable energy, a known inhibitor of optimal LAB performance (Borreani *et al*., 2018).

These results imply that ash content may increase or decrease depending on the direction within the experimental space. There is potential to reduce ash content by increasing barley levels while reducing molasses and soybean meal within or slightly beyond the tested region. The response surface analysis revealed a saddle point, indicating that optimizing ash content is not achieved by maximizing or minimizing individual ingredients alone, but through targeted combinations, particularly those that boost fermentable carbohydrates (barley and molasses) while maintaining soybean meal at moderate levels.

The trends observed for organic matter (OM) and ash content followed a consistent pattern. Both models identified barley and molasses as key factors that increase OM while reducing ash content, reflecting their complementary effects on feed composition. The inverse relationship between OM and ash was clearly demonstrated: the reduction in ash content alongside the increase in OM suggests a relative decrease in mineral concentration as the proportion of organic material increases. Although soybean meal also enhanced OM, it slightly raised ash content, likely due to its own mineral contribution or its distinct influence on fermentation dynamics. The similar saddle-shaped response surfaces and alignment of stationary points indicate that adjusting barley and soybean meal levels could simultaneously improve both OM and ash content, thereby enhancing the nutritional and fermentative quality of olive cake silage under LAB-inoculated conditions.

Collectively, these findings highlight the critical importance of ingredient balance in modulating ash content. While barley and molasses act synergistically to promote microbial conversion of organic fractions and reduce relative mineral concentration, excess soybean meal can offset these benefits by adding mineral mass and impairing moisture dynamics. Optimizing feed formulations through strategic combinations of these additives improves nutrient retention and limits mineral concentration, ultimately enhancing the nutritive value and fermentation quality of olive cake silage.

2.4. Crude fat content

Figure 14 presents contour plots illustrating the effects of ingredient interactions on fat content: (a) soybean meal vs barley, with molasses fixed at its central level (Molasses = 0); (b) soybean meal vs molasses, with barley fixed at its central level (Barley = 0); (c) molasses vs barley, with soybean meal fixed at its central level (Soybean meal = 0).

Although a quadratic model was applied to analyse fat content, it explained only a small proportion of the variance ($R^2 = 13.3\%$, adjusted $R^2 = 6.0\%$), and the overall F-test was non-significant ($F = 1.82$, $p > 0.05$). Nevertheless, the lack-of-fit test was also non-significant ($p > 0.05$), confirming that the model adequately described the data. Among the predictors, molasses exerted a significant negative effect ($p < 0.05$), while the soybean meal × molasses interaction was positive and significant ($p < 0.05$). All other terms, including barley effects, were non-significant ($p > 0.05$). Eigenvalue analysis revealed a saddle point, indicating the absence of a clear optimum for fat content within the tested range. All other terms, including interactions and quadratic effects, were non-significant ($p > 0.05$).

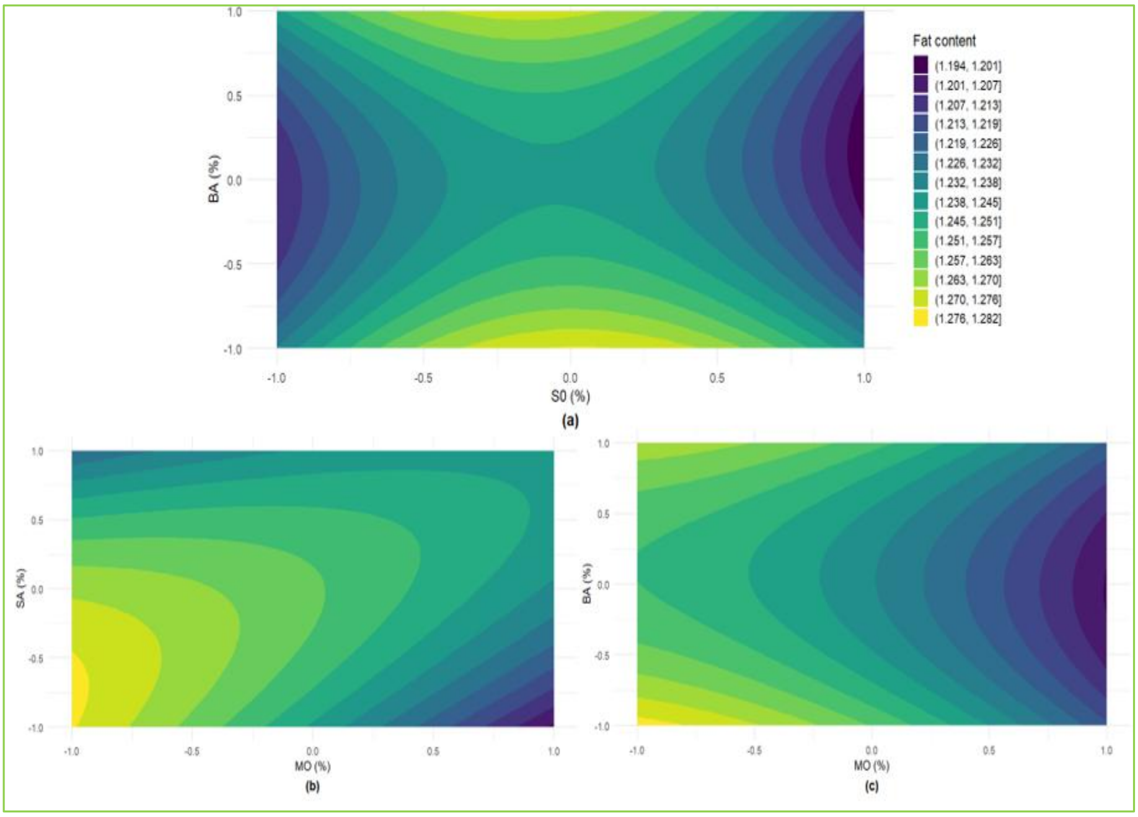


Figure 14 - Contour plots of ingredient interactions affecting crude fat content with third variable held at center point: (a) soybean meal vs. barley; (b) soybean meal vs. molasses; (c) molasses vs. barley.

As shown in Figure 14b, increasing molasses levels consistently reduced fat content. This trend can be attributed to a nutrient dilution effect, as molasses contributes primarily soluble carbohydrates and contains negligible lipid fractions (Cecava, 1995). By increasing the carbohydrate load of the silage, molasses lowers the relative proportion of residual olive oil present in olive cake. Abarghoei *et al.* (2011) also reported compositional shifts in olive cake silage after molasses addition, with notable changes in organic matter and fiber fractions driven both by dilution and fermentation activity. Furthermore, molasses stimulates LAB fermentation (Weinberg *et al.,* 2008), directing microbial metabolism toward lactic acid production and lowering pH (Carvalho *et al.,* 2021). This shift in metabolic priorities favors the preservation of proteins and carbohydrates but has little role in maintaining lipids, which may partly explain the decline in fat content with increasing molasses inclusion.

In contrast, soybean meal showed no significant main effect but displayed a positive interaction with molasses (Fig. 14b). Soybean meal is primarily valued as a protein source, yet it contains residual oil rich in polyunsaturated fatty acids, which may have contributed to stabilizing fat levels when combined with molasses. In addition, the protein fraction of soybean meal provides essential amino acids such as lysine and methionine (Nefzaoui, 1985), which improve microbial protein synthesis during fermentation. By enhancing nitrogen availability for microbial growth, soybean meal may reduce the extent to which lipids are mobilized as an energy source, thereby counteracting part of the dilution effect caused by molasses. Previous studies have similarly highlighted soybean meal's dual role as a protein and energy supplement, improving the nutritive balance of olive cake-based diets (Muñoz *et al*., 1983; Cecava, 1995).

Barley showed no statistically significant influence on fat content, either alone or in interaction terms. This is expected, as barley's contribution lies primarily in starch supplementation (Yáñez Ruiz *et al.,* 2004), which enhances fermentability and supports LAB activity but does not directly influence lipid fractions. Studies on barley supplementation to olive by-products have consistently emphasized improvements in digestibility, microbial activity, and growth performance in ruminants (Yáñez Ruiz et al., 2004; Muñoz et al., 1983), while reporting minimal effects on lipid composition. The nearly uniform contour lines in Figure 14a and 14c reinforce this finding, suggesting that barley's role in olive cake silage is linked more to carbohydrate supply than to fat modification.

The eigenvalue analysis indicating a saddle point confirms the absence of a clear optimum within the tested ranges. This reflects the fact that fat concentration in olive cake silage is largely a function of the original olive residues, rather than being substantially modified by additives such as molasses, barley, or soybean meal. As also discussed by Borreani *et al.* (2018) and Kim *et al.* (2021), the key benefits of these additives lie in fermentation quality, protein preservation, and aerobic stability, rather than altering lipid fractions.

From a nutritional standpoint, the observed reduction in fat content with molasses supplementation is unlikely to limit the feeding value of olive cake silage. In ruminant diets, energy and protein are generally the more limiting nutrients, while moderate reductions in fat are of minor practical importance. Thus, while soybean meal, barley, and molasses effectively enhance fermentability, protein balance, and digestibility (Okoye et al., 2023; Abarghoei *et al*., 2011), their collective influence on fat content remains modest, and fat should not be considered a target parameter for optimization in olive cake silage formulations.

2.5. Neutral Detergent Fiber content

Figure 15 presents contour plots illustrating the effects of ingredient interactions on neutral detergent fiber content: (a) soybean meal vs barley, with molasses fixed at its central level (Molasses = 0); (b) soybean meal vs molasses, with barley fixed at its central level (Barley = 0); (c) molasses vs barley, with soybean meal fixed at its central level (Soybean meal = 0).

The first-order linear model applied to NDF content failed to achieve statistical significance ($F = 0.82$, $p > 0.05$), explaining virtually no variance in the response ($R^2 = 2.1\%$, adjusted $R^2 = -0.5\%$). None of the tested ingredients (soybean meal, barley, or molasse) demonstrated significant effects on NDF content ($p > 0.05$), despite soybean meal showing the largest coefficient among predictors. The lack-of-fit test remained non-significant ($p > 0.05$), confirming model adequacy despite its poor predictive capability.

This remarkable stability in NDF content across all formulations reflects the dominant influence of the olive cake base material, which constitutes the primary fiber source in all treatments. Olive cake is inherently rich in lignocellulosic compounds, with high cell wall constituents (Sansoucy, 1985) that resist modification through simple ingredient supplementation. The supplementary ingredients tested contribute relatively minor fiber amounts compared to the baseline olive cake matrix, rendering their individual effects negligible within practical supplementation ranges.

The resistance of NDF to modification contrasts with findings from previous studies on olive cake ensiling. Abarghoei *et al*. (2011) reported an increased NDF values in ensiled and molasse treated olive cake compared to fresh material. However, our results suggest that while LAB fermentation may achieve modest NDF reductions through baseline degradation processes, further fiber modification through ingredient supplementation remains limited. This distinction highlights an important principle: fermentation can initiate fiber breakdown, but the extent of degradation depends more on fermentation duration, LAB strain characteristics, and environmental conditions than on the specific carbohydrate or protein supplements added (Soundharrajan *et al*., 2025).

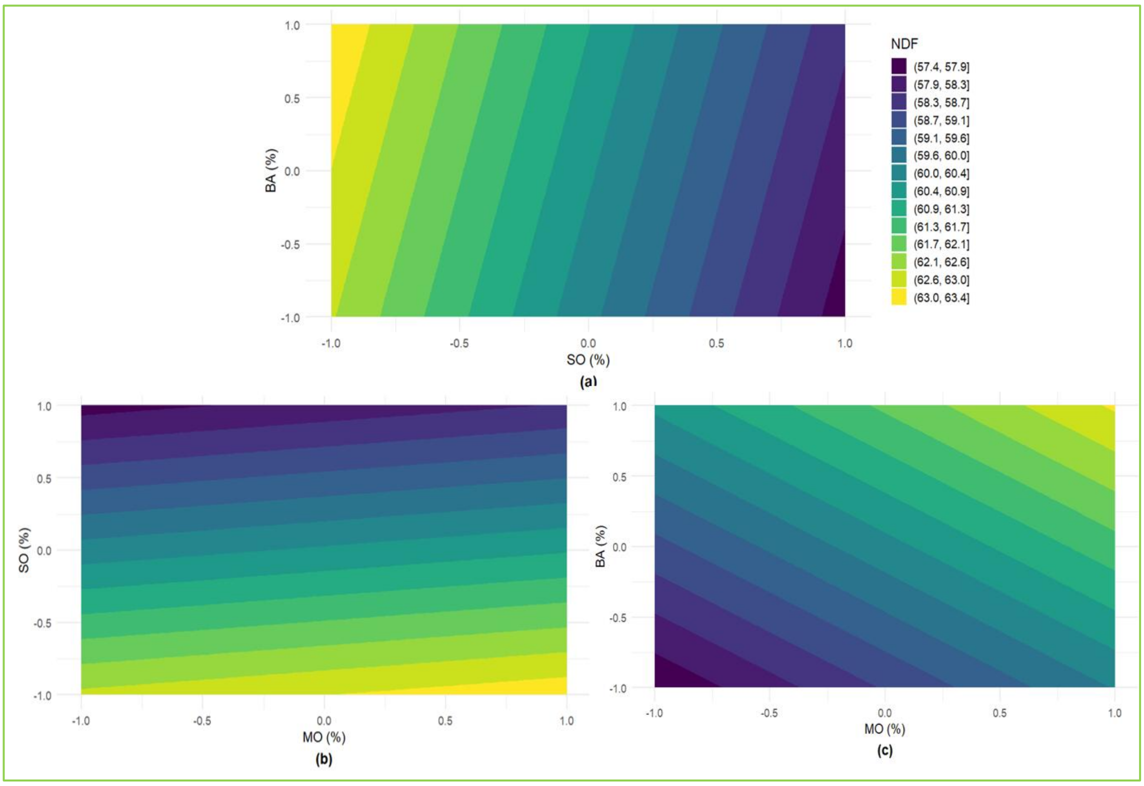


Figure 15 - Contour plots of ingredient interactions affecting the neutral detergent fiber content with third variable held at center point: (a) soybean meal vs. barley; (b) soybean meal vs. molasses; (c) molasses vs. barley.

The limited fiber-degrading capacity observed in this study aligns with the metabolic characteristics of typical LAB strains used in silage inoculation. While certain LAB can produce lignocellulolytic enzymes providing fiber-degrading functions (Sharma *et al.,* 2022), these capabilities vary substantially among strains and generally operate over extended fermentation periods. The homofermentative strains like *Lactobacillus plantarum* and *Pediococcus pentosaceus* prioritize rapid lactic acid production through the Embden-Meyerhof pathway (Okoye *et al.,* 2023), with fiber degradation representing a secondary metabolic activity that may not manifest significantly within typical ensiling timeframes.

More substantial NDF reduction might require innovative LAB combinations specifically selected for fiber-degrading capabilities. Studies have shown that pairing traditional LAB with *Bacillus subtilis* and *B. pumilus*, known for their cellulase production, can break down fibrous material and improve digestibility (Li *et al.*, 2018; Zhang *et al.,* 2019). Similarly, the combination of *B. subtilis* and *L. plantarum* has demonstrated enhanced fiber breakdown in alfalfa silages (Basso *et al.*, 2014; Lara *et al.*, 2018). These findings suggest that achieving meaningful NDF reduction in olive cake silage may require moving beyond conventional LAB monocultures toward strategic multi-species inoculation approaches that combine acidification capacity with enzymatic fiber degradation.

From a nutritional perspective, the consistency of NDF across formulations provides both challenges and opportunities. The persistently high fiber content (approximately 60.5%) confirms that olive cake remains a fibrous feedstuff regardless of supplementation strategy, necessitating careful diet formulation to balance fiber intake in livestock rations. However, this consistency also offers predictability: nutritionists can reliably estimate fiber contribution from olive cake-based feeds without concern for variation due to ingredient proportion adjustments within tested ranges.

The uniformity of contour lines across all three interaction plots (Figures 15a, 15b, and 15c) visually reinforces the statistical finding that NDF content remains essentially constant regardless of ingredient combinations. This pattern stands in marked contrast to the dynamic gradients observed for protein, ash, and organic matter, emphasizing that different nutritional parameters respond to supplementation through distinct mechanisms.

Alternative strategies for addressing olive cake's high fiber content might include enzymatic pretreatments with exogenous cellulases or xylanases prior to ensiling, chemical treatments that disrupt lignocellulosic structures (Abid *et al.*, 2020), or physical processing methods such as grinding or steam treatment that increase surface area for microbial attack. These approaches target fiber structure directly rather than relying on fermentation degradation, potentially achieving more substantial NDF reductions than ingredient supplementation alone can provide.

2.6. Crude protein content

Figure 16 presents contour plots illustrating the effects of ingredient interactions on crude protein content: (a) soybean meal vs barley, with molasses fixed at its central level (Molasses = 0); (b) soybean meal vs molasses, with barley fixed at its central level (Barley = 0); (c) molasses vs barley, with soybean meal fixed at its central level (Soybean meal = 0).

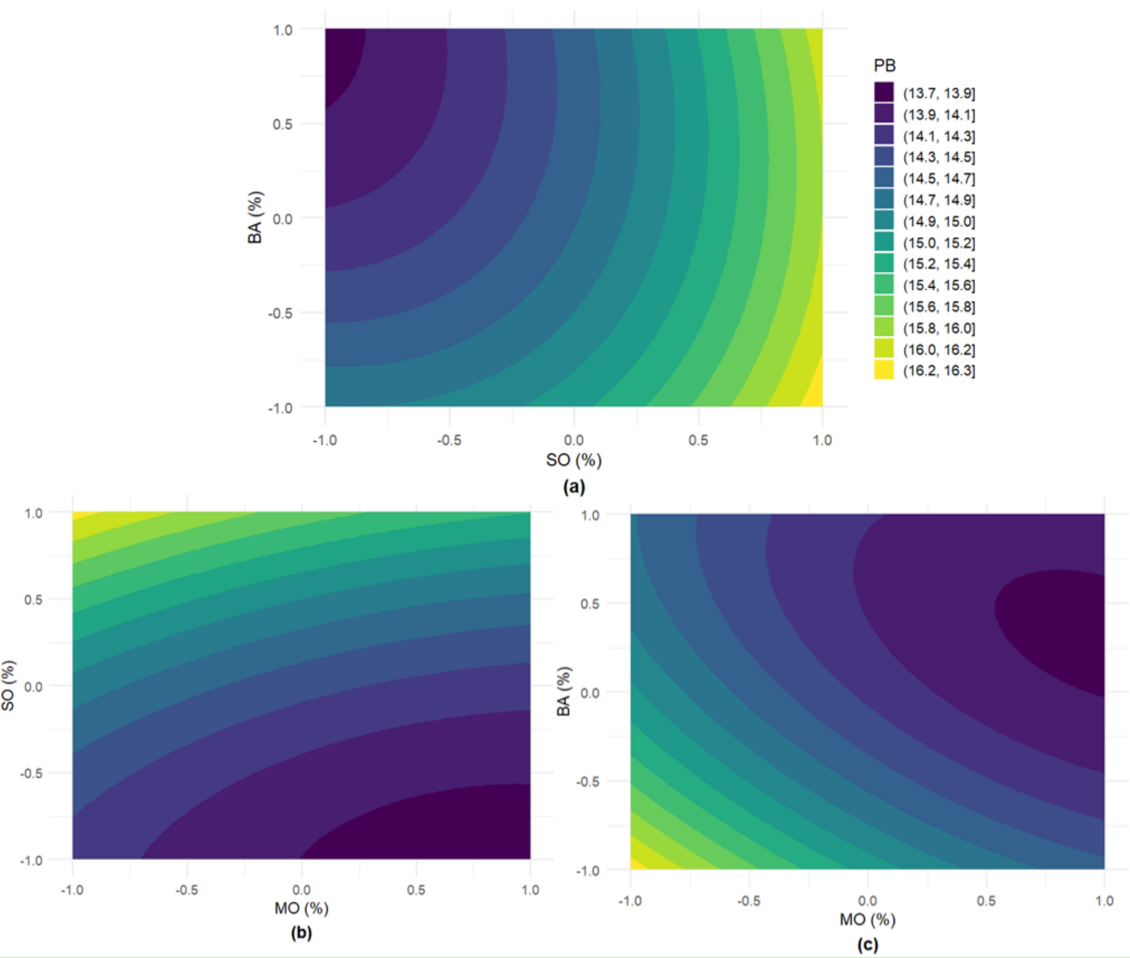


Figure 16 - Contour plots of ingredient interactions affecting the crude protein content with third variable held at center point: (a) soybean meal vs. barley; (b) soybean meal vs. molasses; (c) molasses vs. barley.

The quadratic response surface model developed for crude protein content achieved excellent statistical performance ($F_{9,107} = 23.47$, $p < 0.001$), explaining 66.4% of the total variance (adjusted $R^2 = 63.5\%$). This robust model fit, validated by a non-significant lack-of-fit test ($p > 0.05$), demonstrates that protein content is highly responsive to ingredient manipulation and follows predictable patterns within the experimental space.

All three main effects were highly significant. Soybean meal, as a high-protein supplement (44-48% CP), directly increased protein content (coefficient = 0.92, $p < 0.001$), while barley and molasses, with their lower protein concentrations (10-12% and 3-5%, respectively), decreased protein percentage through dilution ($p < 0.001$). A significant quadratic term for soybean meal ($p < 0.01$) indicated non-linear curvature in the response surface, suggesting that the effect of soybean meal intensifies at higher levels. The soybean meal-barley interaction approached significance ($p = 0.055$), indicating potential interactive effects between these ingredients.

Soybean meal emerged as the dominant factor influencing protein content, exhibiting the strongest positive effect (coefficient = 0.92, $p < 0.001$). This finding directly addresses one of olive cake's most critical nutritional limitations. As noted by Nefzaoui (1985), untreated olive cake is deficient in essential amino acids, particularly glutamic acid, proline, and lysine, which are vital for protein synthesis in livestock. Soybean meal, containing approximately 44-48% crude protein and rich in lysine and methionine, provides precisely the amino acid profile needed to complement olive cake's deficiencies.

The magnitude of soybean meal's protein-enhancing effect is clearly visible in Figures 16a and 16b, where protein content increases sharply along the soybean meal axis. This steep gradient reflects the substantial protein concentration difference between soybean meal and the base olive cake matrix (typically 6-8% protein), creating strong dilution and enrichment dynamics as supplementation levels change. The significant quadratic term for soybean meal ($p < 0.01$) indicates that its protein-enhancing effect intensifies at higher inclusion levels.

The practical significance of this protein enhancement is underscored by previous livestock feeding studies. Muñoz *et al.* (1983) demonstrated that lambs fed olive cake supplemented with protein sources achieved superior weight gains compared to those receiving urea supplementation or unsupplemented controls. Similarly, Yáñez Ruiz *et al.* (2004) showed that protein supplementation of olive by-products with barley and legumes enhanced microbial activity in goats and sheep, as indicated by urinary allantoin levels, a marker of microbial protein synthesis. These findings confirm that protein enrichment not only improves chemical composition but translates directly into enhanced animal performance.

Both barley and molasses demonstrated significant negative effects on crude protein content (both $p < 0.001$), operating primarily through nutrient dilution mechanisms rather than biological transformation. Barley, containing approximately 10-12% protein, dilutes overall protein concentration when added to olive cake, while molasses contributes minimal protein (typically 3-5%) alongside its concentrated carbohydrate load. These dilution effects, clearly visible in Figures 16b and 16c where increasing molasses or barley levels consistently shift contours toward lower protein zones, demonstrating that improving one nutrient can reduce another.

However, characterizing barley and molasses solely as protein diluents overlooks their complementary contributions to feed quality. Barley supplies readily fermentable carbohydrates that support LAB metabolism during ensiling (Cecava, 1995), while molasses provides soluble sugars that enhance fermentation kinetics and palatability. The challenge lies in balancing these ingredients to simultaneously achieve adequate protein levels, optimal fermentation conditions, and acceptable energy density - a multi-objective optimization problem that cannot be solved by maximizing any single component.

The near-significant soybean meal × barley interaction ($p = 0.055$) hints at potential synergistic or antagonistic effects warranting further investigation. This marginal interaction suggests that barley's diluting effect on protein may be moderated by soybean meal concentration, or that soybean meal's protein-enhancing capacity might be influenced by the fermentation dynamics that barley promotes. Studies by Yáñez Ruiz et al. (2004) demonstrated that combining barley with protein-rich supplements in olive by-product diets produced balanced fermentation parameters, suggesting that strategic ingredient ratios can optimize both protein content and fermentation quality simultaneously.

The combination of protein supplementation with LAB inoculation creates synergistic benefits beyond simple compositional changes. While soybean meal directly increases protein quantity, LAB fermentation enhances protein quality through proteolytic activity.

As described by Tufariello *et al*. (2019) and Foti *et al.* (2023), LAB strains produce enzymes including glucosidase and esterase that can modify protein structures, potentially generating bioactive peptides with enhanced digestibility and bioavailability. This dual enhancement, quantitative through supplementation and qualitative through fermentation, positions treated olive cake as a more complete protein source than either intervention could achieve independently.

Canonical analysis identified a stationary point at SO = 0.89%, BA = 6.92%, and MO = 3.33%, with all positive eigenvalues confirming this location represents a minimum point for crude protein content. This mathematical characteristic indicates that protein content increases by moving away from this point in any direction, with the most efficient path involving maximizing soybean meal while moderating barley and molasses to levels that support fermentation without excessive protein dilution. The location of this minimum provides practical formulation guidance: achieving target protein levels above 12-14% requires substantial soybean meal inclusion combined with generous use of carbohydrate supplements.

These results have important implications for olive cake use in livestock feeding systems. By elevating crude protein from typical baseline levels of 6-8% to potentially 12-16% or higher through strategic soybean meal supplementation, treated olive cake transitions from a low-quality fibrous filler to a viable protein-containing feedstuff suitable for incorporation into balanced ruminant diets. This transformation expands the potential applications of olive cake beyond maintenance rations toward productive feeding scenarios including growth, lactation, and reproduction.

2.7. Multi-Objective optimization and optimal formulation selection

Multi-objective optimization using response surface methodology, incorporating weighted desirability functions for crude protein (40%), organic matter (30%), ash content (20%), dry matter (5%), and fat content (5%), identified the optimal formulation among the thirteen experimentally tested combinations. Based on actual measured data, the formulation comprising 6% soybean meal, 4% barley, and 6% molasses, demonstrating superior nutritional balance across all evaluated parameters. This optimal formulation delivered 15.7% crude protein, representing a 124% increase over untreated olive cake, alongside 91.2% organic matter and 8.18% ash content. Notably, this combination also exhibited the lowest neutral detergent fiber content among all treatments (53.6%), suggesting enhanced digestibility potential compared to alternative formulations. While the formulation with 6% soybean meal, 6% barley, and 4% molasses produced marginally higher protein content (16.3%), its elevated ash (8.34%) and NDF levels (57.8%) resulted in lower overall nutritional quality.

Response surface model predictions suggest that a formulation with all three ingredients at maximum levels (6% soybean meal, 6% barley, 6% molasses) could potentially achieve 15.9% crude protein and 91.3% organic matter with 8.1% ash content, representing a marginal improvement over the experimentally validated 6-4-6 formulation. However, this predicted optimum lies at the boundary of the experimental design and would require validation through additional trials to confirm model accuracy in this region.

The optimization analysis confirmed that maximizing soybean meal and molasses inclusion while maintaining moderate barley levels effectively addresses olive cake's primary nutritional deficiencies, low protein and high fiber, thereby transforming it from a marginal feedstuff suitable only for maintenance into a viable protein-containing ingredient appropriate for productive ruminant feeding.

V. CONCLUSION AND FUTURE PERSPECTIVE

The escalating demand for livestock feed, coupled with the environmental burden of agro-industrial waste, necessitates innovative strategies to valorize underutilized by-products. This study addressed the challenge of transforming olive cake, a low-quality, fibrous by-product of olive oil extraction, into a nutritionally enhanced feed ingredient suitable for productive ruminant feeding through biotechnological treatment and strategic supplementation.

Employing a Box-Behnken response surface methodology design with 13 unique formulations tested across 117 samples, this research systematically evaluated the effects of soybean meal, barley, and molasses supplementation (at 2%, 4%, and 6% inclusion levels) combined with lactic acid bacteria inoculation on key nutritional parameters of olive cake. The comprehensive analytical approach, encompassing dry matter, organic matter, ash, fat, neutral detergent fiber, and crude protein determination, provided robust data for statistical modeling and optimization.

The results demonstrated that nutritional enhancement of olive cake is achievable through carefully balanced supplementation strategies. Multi-objective optimization, incorporating weighted desirability functions across all measured parameters, identified the formulation comprising 6% soybean meal, 4% barley, and 6% molasses with LAB inoculation as optimal among experimentally tested combinations. This formulation achieved 15.7% crude protein, a 124% increase over untreated olive cake, alongside 91.2% organic matter, 8.18% ash content, and notably, the lowest neutral detergent fiber content (53.6%) among all treatments. These improvements transform olive cake from a marginal feedstuff suitable only for maintenance rations into a viable protein-containing ingredient appropriate for productive ruminant feeding, including growing animals and lactating ewes.

The response surface models developed for individual nutritional parameters revealed distinct patterns of ingredient effects. Soybean meal emerged as the primary driver of protein enhancement, demonstrating strong positive effects ($p < 0.001$) with a quadratic response indicating accelerating returns at higher inclusion levels. Barley and molasses exhibited complementary roles, significantly increasing organic matter while reducing ash content (both $p < 0.001$), reflecting their function as fermentable carbohydrate sources that enhance LAB-mediated fermentation. The inverse relationship between organic matter and ash content confirmed the dilution effect of carbohydrate-rich ingredients on mineral concentration. Conversely, neutral detergent fiber proved resistant to modification through supplementation, maintaining stability around 60% across all treatments, a finding that highlight the inherent structural limitations of olive cake's lignocellulosic matrix and highlights the need for alternative fiber-reduction strategies such as enzymatic pretreatment or strategic multi-species LAB inoculation incorporating cellulolytic strains.

Response surface model predictions suggest that formulations at the boundary of the experimental design, particularly 6% soybean meal, 6% barley, and 6% molasses, could potentially yield marginal improvements (predicted 15.9% crude protein, 91.3% organic matter). However, these predictions represent extrapolations beyond the validated experimental range and require confirmation through additional trials to verify model accuracy in this region and assess potential non-linear effects at extreme inclusion levels.

From a practical standpoint, this research provides actionable guidance for olive oil-producing regions seeking sustainable feed solutions. The optimal 6-4-6 formulation offers a validated, implementable strategy that leverages locally available ingredients to convert olive cake from an environmental liability into a valuable feed resource. The methodology developed herein, combining biotechnological fermentation with response surface optimization, represents a transferable framework applicable to other agro-industrial byproducts, contributing to circular economy principles in agriculture.

Several limitations warrant acknowledgment. The unusually high dry matter values (96-97%) observed across treatments suggest either measurement artifacts or that the olive cake substrate was substantially dehydrated prior to treatment, differing from typical silage moisture conditions (30-40% DM). This discrepancy may affect the applicability of findings to traditional ensiling scenarios and should be investigated in future work. Additionally, the study focused exclusively on chemical composition without evaluating in vivo digestibility, animal performance, or palatability, critical parameters for practical feeding recommendations. Economic viability analysis, including cost-benefit assessment of supplementation strategies and comparison with conventional protein sources, remains unexplored.

Future research should focus on validating the predicted 6-6-6 formulation through targeted experimentation and conducting long-term feeding trials with sheep to evaluate intake, digestibility, growth performance, and product quality. Further studies could investigate the use of advanced LAB consortia incorporating cellulolytic strains (*Bacillus subtilis*, *B. pumilus*) to address the recalcitrant fiber fraction, as well as explore enzymatic pretreatments using cellulases and xylanases to enhance NDF degradation. Additional work is also needed to evaluate the storage stability and aerobic stability during feed-out; perform an economic analysis comparing treated olive cake to conventional protein supplements and undertake scaling studies to evaluate industrial feasibility and optimization of fermentation protocols for commercial production.

This study successfully demonstrates that systematic biotechnological enhancement of olive cake through LAB fermentation and strategic multi-substrate supplementation can substantially improve its nutritional profile, addressing critical deficiencies in protein content while maintaining acceptable fiber levels. The optimal formulation identified provides a scientifically validated, practical solution for incorporating olive cake into productive ruminant diets, contributing to sustainable livestock production, waste valorization, and circular agricultural systems. By converting an abundant agricultural by-product into a nutritionally balanced feed ingredient, this work advances both environmental sustainability and food security objectives in olive oil-producing regions.

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VII. ANNEX

Sample	SO	BA	MO	Trl	Rep	DM	Ash	OM	Fat	NDF	PB
Control	0	0	0	T1	R1	96.820	8.599	90.827	1.251	52.472	13.267
Control	0	0	0	T1	R2	96.754	8.563	90.853	1.125	49.851	13.461
Control	0	0	0	T1	R3	96.839	8.560	90.872	1.125	50.010	13.258
Control	0	0	0	T2	R1	96.995	8.844	90.599	1.319	59.622	12.923
Control	0	0	0	T2	R2	96.977	8.803	90.639	1.092	57.016	13.378
Control	0	0	0	T2	R3	96.944	8.813	90.622	1.119	57.584	13.244
Control	0	0	0	T3	R1	97.047	9.088	90.351	1.244	58.325	13.596
Control	0	0	0	T3	R2	97.157	9.203	90.250	1.045	56.274	13.904
Control	0	0	0	T3	R3	97.119	9.226	90.219	1.269	57.256	13.801
S2B2M4	2	2	4	T1	R1	97.125	8.467	91.024	1.350	60.236	13.509
S2B2M4	2	2	4	T1	R2	97.075	8.500	90.980	1.313	56.216	13.933
S2B2M4	2	2	4	T1	R3	97.065	8.455	91.026	1.250	72.581	13.660
S2B2M4	2	2	4	T2	R1	96.778	8.807	90.597	1.391	52.545	15.870
S2B2M4	2	2	4	T2	R2	96.764	8.777	90.626	1.176	52.572	15.311
S2B2M4	2	2	4	T2	R3	96.783	8.730	90.680	1.192	51.718	15.594
S2B2M4	2	2	4	T3	R1	96.797	8.800	90.607	1.449	60.370	14.883
S2B2M4	2	2	4	T3	R2	96.831	8.731	90.688	1.331	58.141	14.703
S2B2M4	2	2	4	T3	R3	96.887	8.789	90.637	1.016	61.788	14.769

S2B4M2	2	4	2	T1	R1	97.056	8.354	91.131	1.364	56.990	14.068
S2B4M2	2	4	2	T1	R2	96.935	8.449	91.009	1.194	59.805	14.072
S2B4M2	2	4	2	T1	R3	96.929	8.387	91.073	1.126	58.773	13.639
S2B4M2	2	4	2	T2	R1	96.644	8.282	91.133	1.551	56.540	14.681
S2B4M2	2	4	2	T2	R2	96.623	8.298	91.111	1.346	62.199	14.697
S2B4M2	2	4	2	T2	R3	96.609	8.253	91.158	1.030	61.742	14.682
S2B4M2	2	4	2	T3	R1	97.121	8.419	91.075	1.204	60.083	14.240
S2B4M2	2	4	2	T3	R2	97.131	8.456	91.037	1.068	60.898	14.342
S2B4M2	2	4	2	T3	R3	97.102	8.476	91.011	1.200	66.481	15.116
S2B4M6	2	4	6	T1	R1	97.292	8.426	91.098	1.029	58.917	13.702
S2B4M6	2	4	6	T1	R2	97.257	8.356	91.166	1.108	55.606	17.620
S2B4M6	2	4	6	T1	R3	97.226	8.289	91.231	1.046	71.519	14.108
S2B4M6	2	4	6	T2	R1	96.703	8.260	91.167	1.035	58.459	13.575
S2B4M6	2	4	6	T2	R2	96.650	8.047	91.385	1.132	57.369	13.360
S2B4M6	2	4	6	T2	R3	96.723	8.083	91.360	1.067	55.597	15.276
S2B4M6	2	4	6	T3	R1	97.239	8.247	91.278	1.321	56.773	13.699
S2B4M6	2	4	6	T3	R2	97.171	8.239	91.274	1.093	58.922	13.472
S2B4M6	2	4	6	T3	R3	97.166	8.286	91.224	1.042	62.080	13.628
S2B6M4	2	6	4	T1	R1	97.017	7.759	91.756	1.086	68.561	13.673
S2B6M4	2	6	4	T1	R2	96.996	7.998	91.499	1.133	60.478	13.409
S2B6M4	2	6	4	T1	R3	97.015	7.934	91.571	1.030	58.669	13.496

S2B6M4	2	6	4	T2	R1	96.821	8.322	91.122	1.224	56.486	14.526
S2B6M4	2	6	4	T2	R2	96.789	8.252	91.191	1.404	58.415	14.067
S2B6M4	2	6	4	T2	R3	96.827	8.240	91.212	1.347	155.222	14.259
S2B6M4	2	6	4	T3	R1	97.354	8.239	91.307	1.349	57.793	13.931
S2B6M4	2	6	4	T3	R2	97.306	8.308	91.226	1.433	59.854	13.957
S2B6M4	2	6	4	T3	R3	97.383	8.373	91.171	1.322	55.871	14.147
S4B2M2	4	2	2	T1	R1	96.764	8.715	90.693	1.309	56.030	16.343
S4B2M2	4	2	2	T1	R2	96.771	8.774	90.631	1.393	57.944	16.323
S4B2M2	4	2	2	T1	R3	96.723	8.756	90.641	1.289	59.053	16.395
S4B2M2	4	2	2	T2	R1	96.360	8.641	90.694	1.262	62.334	16.574
S4B2M2	4	2	2	T2	R2	96.431	8.696	90.648	1.369	58.556	16.256
S4B2M2	4	2	2	T2	R3	96.399	8.637	90.705	1.351	58.548	16.594
S4B2M2	4	2	2	T3	R1	96.983	8.650	90.803	1.360	59.664	15.155
S4B2M2	4	2	2	T3	R2	96.942	8.646	90.800	1.197	63.122	15.413
S4B2M2	4	2	2	T3	R3	96.973	8.667	90.784	1.380	59.797	15.674
S4B2M6	4	2	6	T1	R1	96.633	8.508	90.889	1.086	157.520	14.961
S4B2M6	4	2	6	T1	R2	96.661	8.481	90.923	1.224	60.237	15.238
S4B2M6	4	2	6	T1	R3	96.688	8.442	90.969	1.114	57.194	14.855
S4B2M6	4	2	6	T2	R1	97.161	8.466	91.032	1.037	57.804	14.681
S4B2M6	4	2	6	T2	R2	97.046	8.542	90.930	1.214	56.994	14.599
S4B2M6	4	2	6	T2	R3	97.063	8.447	91.034	1.432	57.800	14.690

S4B2M6	4	2	6	T3	R1	96.468	8.821	90.521	1.117	59.880	14.746
S4B2M6	4	2	6	T3	R2	96.458	8.716	90.632	1.160	58.047	14.759
S4B2M6	4	2	6	T3	R3	96.477	8.753	90.596	1.202	61.107	14.722
S4B4M4	4	4	4	T1	R1	96.761	8.399	91.029	1.083	58.920	14.229
S4B4M4	4	4	4	T1	R2	96.769	8.318	91.117	1.187	59.000	14.457
S4B4M4	4	4	4	T1	R3	96.837	8.306	91.143	1.101	57.940	14.794
S4B4M4	4	4	4	T2	R1	96.895	8.202	91.264	1.251	59.352	14.339
S4B4M4	4	4	4	T2	R2	96.930	8.197	91.275	1.303	63.286	14.362
S4B4M4	4	4	4	T2	R3	96.980	8.221	91.259	1.250	60.879	15.421
S4B4M4	4	4	4	T3	R1	96.906	8.397	91.058	1.465	62.525	15.212
S4B4M4	4	4	4	T3	R2	96.905	8.587	90.856	1.440	60.217	14.762
S4B4M4	4	4	4	T3	R3	96.903	8.419	91.034	1.112	57.924	14.310
S4B6M2	4	6	2	T1	R1	96.985	8.435	91.032	1.535	56.981	14.509
S4B6M2	4	6	2	T1	R2	97.065	8.433	91.050	1.549	61.283	15.046
S4B6M2	4	6	2	T1	R3	97.100	8.378	91.114	1.311	60.730	15.147
S4B6M2	4	6	2	T2	R1	96.703	8.051	91.390	1.338	63.842	14.614
S4B6M2	4	6	2	T2	R2	96.720	8.079	91.363	1.361	66.344	14.630
S4B6M2	4	6	2	T2	R3	96.651	8.061	91.371	1.013	61.271	14.654
S4B6M2	4	6	2	T3	R1	96.819	8.196	91.256	1.486	56.842	14.769
S4B6M2	4	6	2	T3	R2	96.791	8.231	91.214	1.375	60.906	15.377
S4B6M2	4	6	2	T3	R3	96.760	8.196	91.246	1.042	58.266	15.590

S4B6M6	4	6	6	T1	R1	96.448	7.645	91.782	1.306	60.805	13.682
S4B6M6	4	6	6	T1	R2	96.425	7.692	91.727	1.228	60.407	13.553
S4B6M6	4	6	6	T1	R3	96.467	7.688	91.738	1.052	61.089	13.830
S4B6M6	4	6	6	T2	R1	97.093	8.118	91.389	1.210	55.018	14.819
S4B6M6	4	6	6	T2	R2	97.029	8.054	91.445	1.073	55.028	14.651
S4B6M6	4	6	6	T2	R3	97.084	8.034	91.476	1.274	65.975	14.628
S4B6M6	4	6	6	T3	R1	96.767	8.456	90.969	1.181	52.296	14.609
S4B6M6	4	6	6	T3	R2	96.751	8.456	90.966	1.379	57.917	14.416
S4B6M6	4	6	6	T3	R3	96.817	8.375	91.065	1.164	68.442	14.305
S6B2M4	6	2	4	T1	R1	96.754	8.587	90.827	1.195	58.764	16.695
S6B2M4	6	2	4	T1	R2	96.710	8.570	90.837	1.078	58.140	16.124
S6B2M4	6	2	4	T1	R3	96.762	8.636	90.777	1.337	59.341	16.031
S6B2M4	6	2	4	T2	R1	96.656	9.087	90.274	1.166	55.407	16.315
S6B2M4	6	2	4	T2	R2	96.823	8.773	90.642	1.138	55.816	16.557
S6B2M4	6	2	4	T2	R3	96.781	8.874	90.526	1.128	56.650	16.916
S6B2M4	6	2	4	T3	R1	96.887	8.566	90.875	1.389	58.674	15.767
S6B2M4	6	2	4	T3	R2	96.761	8.572	90.845	1.470	59.028	16.015
S6B2M4	6	2	4	T3	R3	96.849	8.568	90.865	1.090	58.255	15.630
S6B4M2	6	4	2	T1	R1	97.184	8.528	90.971	1.065	63.934	16.138
S6B4M2	6	4	2	T1	R2	97.206	8.353	91.160	1.369	57.456	16.167
S6B4M2	6	4	2	T1	R3	97.043	8.435	91.043	1.050	59.541	15.987

S6B4M2	6	4	2	T2	R1	96.689	8.718	90.675	1.135	58.021	16.702
S6B4M2	6	4	2	T2	R2	96.751	8.712	90.693	1.559	56.691	16.619
S6B4M2	6	4	2	T2	R3	96.740	8.744	90.657	1.041	54.050	17.125
S6B4M2	6	4	2	T3	R1	96.652	8.673	90.716	1.124	63.625	16.195
S6B4M2	6	4	2	T3	R2	96.578	8.582	90.799	1.036	60.537	16.466
S6B4M2	6	4	2	T3	R3	96.590	8.564	90.820	1.092	59.705	16.122
S6B4M6	6	4	6	T1	R1	96.979	8.265	91.212	1.198	60.135	15.574
S6B4M6	6	4	6	T1	R2	96.980	8.239	91.240	1.215	54.402	15.799
S6B4M6	6	4	6	T1	R3	96.953	8.274	91.198	1.169	57.434	16.212
S6B4M6	6	4	6	T2	R1	96.817	8.092	91.367	1.035	58.419	15.472
S6B4M6	6	4	6	T2	R2	96.837	8.197	91.258	1.473	56.759	15.534
S6B4M6	6	4	6	T2	R3	96.851	7.646	91.849	1.043	55.798	15.213
S6B4M6	6	4	6	T3	R1	96.259	8.294	91.049	1.211	60.697	15.005
S6B4M6	6	4	6	T3	R2	96.290	8.313	91.034	1.476	60.697	16.932
S6B4M6	6	4	6	T3	R3	96.336	8.334	91.020	1.514	18.326	15.987
S6B6M4	6	6	4	T1	R1	96.956	8.381	91.085	1.236	59.027	17.146
S6B6M4	6	6	4	T1	R2	96.900	8.321	91.138	1.199	59.764	16.706
S6B6M4	6	6	4	T1	R3	96.898	8.376	91.079	1.303	65.040	15.849
S6B6M4	6	6	4	T2	R1	96.914	8.279	91.185	1.251	57.039	15.325
S6B6M4	6	6	4	T2	R2	96.963	8.218	91.259	1.264	59.275	15.800
S6B6M4	6	6	4	T2	R3	96.886	8.174	91.292	1.049	57.899	16.163

S6B6M4	6	6	4	T3	R1	96.403	8.465	90.891	1.077	49.774	17.085
S6B6M4	6	6	4	T3	R2	96.356	8.432	90.919	1.160	56.974	16.160
S6B6M4	6	6	4	T3	R3	96.329	8.380	90.969	1.039	55.685	16.299