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New strategy to improve animal welfare and product quality using different feed supplementation

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Preface

The aim of this thesis is to examine how incorporating alternative sources in animal nutrition affects the quality of animal products, such as milk, meat, and eggs.
The first chapter of the present work is concerned with the analysis of the available literature focusing on the problem of the introduction of alternative feed sources in animal nutrition and their potential impact on animal performances and product quality. In particular, an in-depth investigation was carried out on the use of microalgae in ruminant diets and its impact on the quality of milk and dairy products. The effects of feeding poultry with hemp seeds co- and by-products on animal performance, behaviour, and egg and meat quality were also reviewed. In the following chapters three experimental works are presented in which different feeding strategies and different feed ingredients on ruminant and non-ruminant animals. In particular, in the second chapter the effects of the cyanobacterium Spirulina (*Arthrospira platensis*) added to buffalo diet on mozzarella cheese chemical composition, nutritional quality, sensory profile and consumers acceptability with their willingness to pay were discussed. In the third chapter is presented how the inclusion of two different levels of feed-grade hemp seed in the diet of organically reared broilers affect carcass traits, chemical and nutritional characteristics of breast fillets, and their sensorial proprieties. In the fourth chapter the partial substitution of laying hens diet with hemp seed cake is evaluated on hens' egg production performance, and the physico-chemical and sensorial characteristics of eggs during 28 days of storage time. Finally, a general conclusion on the whole experimental works is provided.

Chapter 1

Alternative and novel ingredients for animal feeding: opportunities and challenges

Rapid global population growth and rising incomes will double the total demand for animal products by 2050 (FAO, 2007). Such an increase in demand will be particularly critical for livestock production (FAO, 2011). In this scenario, there is a lot of discussion about two major feed crops in terms of their sustainability in animal diets, which is closely linked to issues of land use, water footprint, climate change, and lastly, competition between food and feed (Madeira et al. 2017; Govoni et al. 2021). Seeking alternative feed ingredients is, therefore, of interest to address the challenges in livestock production systems. The feasibility of using alternative feeds for ruminants depends, among other things, on the nutritional value of novel feeds, animal production responses, and feed costs compared to conventional feeds (Serrapica et al., 2019; Vastolo et al., 2022). Moreover, while improving the sustainability of animal products by using alternative feed ingredients, their impact on product quality should also be considered (Govoni et al., 2021).

Animal product quality is a complex topic, generally based on a combination of chemical characteristics and sensory perceptions, which determine the suitability of animal-derived food for human consumption (Hartung et al., 2009). From a consumer's perspective, some of these parameters are objective and extrinsic, and others are subjective and intrinsic (Joo et al., 2013). The extrinsic parameters refer to those that cannot immediately be detected by physical or sensory examination of the products themselves, but which are associated with the way that the animal products are produced. These parameters focus more on animal welfare and ecological sustainability of the production systems (Salami et al., 2019; Beauchemin et al., 2022). On the other hand, the intrinsic parameters associated with sensory perception such as appearance, colour, flavour, texture, tenderness, juiciness, and aroma are the most important factors used to judge animal products quality and largely influence consumer's purchase decision (Joo et al., 2013; Liu et al., 2022). For example, meat colour is affected by the level of myoglobin in muscle fibre and its oxidative state (Miller, 2002). Depending on different cultural backgrounds, meat colour is judged diversely but is always associated with freshness. The "freshness" and "wholesomeness" of meat refer to the perception that meat is safe for human consumption and free from pathogens, parasites, infectious agents, and toxins (Purslow, 2022). Meat tenderness and juiciness are a result of the muscle structural

integrity and the ability of muscle proteins to bind water (Guerrero et al., 2013). Meat is essentially defined by its muscle composition, such as lean, fat, and connective tissue (Costa et al., 2021). Fat can be deposited intramuscularly as marbling or externally as subcutaneous fat. Intramuscular fat content has been shown to influence flavour, tenderness, juiciness, and visual characteristics of meat. Although a higher fat content is associated with increased palatability, the acceptable range is between 3 and 7.3% in ruminant (Miller, 2002; Vasta et al., 2008). In addition, too much visible fat in meat products is not appreciated by consumers due to health concerns and negative associations with increased risk of cardiovascular disease, obesity, and cancer (Miller, 2002). However, the optimal marbling level of meat depends on cultural tradition and individual preference (Ngapo et al., 2007; Font-i-Furnols et al., 2013; Cheng et al., 2015).

Consumer criticism of animal production has led to the introduction of quality standards, codes of practice and certification schemes aimed at ensuring safe and good quality animal products based on ethically acceptable production practices (Webb and Webb, 2022). Today, ethical animal production should ideally take place without causing suffering to animals (Webb and Webb, 2022). As animal-friendly and sustainably produced meat is well accepted by consumers (Alonso et al., 2020; Edenbrandt and Lagerkvist, 2021), such a change in the production system could drive individual decisions to purchase and consumption of animal products with improved attributes linked to the production system (e.g. environmentally sustainable).

Animal nutrition plays a central role in the production of high-quality animal products. Therefore, it is not surprising that the role of nutrition on product quality has been extensively addressed in different species and for different animal-derived food chains. Several studies have described the effects of providing alternative feed ingredients to animals on production efficiency and quality parameters of derived products, such as fat content, fatty acid (FA) composition, mineral content, and amino acid profile, among others (Resconi et al., 2009; Eiras et al., 2014).

This chapter summarizes the recent findings on the potential use of some selected alternative feeds to obtain high-quality animal products. The first part provides an overview of the dietary use of microalgae in dairy ruminants, emphasizing their effects on animal performance and milk and dairy product quality. Following this, a snapshot of the current knowledge on the potential use of industrial hemp products in feeding broilers and laying hens is presented.

1. Use of microalgae in dairy ruminant nutrition

In the past two decades, several studies have focused on developing nutritional strategies for dairy animals to enhance the quality of milk and derived products.

Improving milk composition is also a priority for producers, as dairy industries worldwide have implemented penalty and premium schemes to encourage dairy producers to enhance milk composition and quality (Draaiyer et al., 2009). In particular, there has been a growing interest in finding nutritional strategies to decrease the amount of saturated fatty acids (SFA) and increase the amount of polyunsaturated fatty acids (PUFA) in milk (Correddu et al., 2020; Serrapica et al., 2020). One effective strategy for increasing PUFA content in ruminant milk is dietary supplementation with PUFA-rich products such as oilseeds, vegetable, and fish oils (Samková and Kalač, 2021). Microalgae have recently gained attention as a dietary source of PUFAs. They are unicellular or multicellular photoautotrophic microorganisms that are smaller than 400 μm. Microalgae are an economical and unconventional source of animal feed due to their high efficiency in converting solar energy and their ability to produce more per unit area than conventional crops (Priyadarshani and Rath, 2012). Moreover, they can contribute to reducing the exploitation of natural resources (Holman and Malau-Aduli 2013). Certain species can also be cultivated for biodiesel production (Kovač et al., 2013), while the residual algal mass, partially or completely defatted, can be used as animal feed (Lum et al., 2013, Drewery et al., 2014). Microalgae are rich in macro-components, with their chemical composition varying widely depending on the genus, species, and growing conditions (Spolaore et al., 2006; Venckus et al., 2017). Typically, microalgae contain, on dry matter (DM), 39-71% of protein, 10-57% of carbohydrates (mainly polysaccharides, cellulose, and starches), and 6-86% of lipids, especially sterols and long chain PUFAs, mainly eicosapentaenoic and docosahexaenoic fatty acids (Spolaore et al., 2006; Chen et al., 2013; Ryckebosch et al., 2014). The number and location of double bonds in PUFA make them susceptible to enzymatic and non-enzymatic oxidation, including auto-oxidation and photo-oxidation, which produce reactive oxygen species and lead to oxidative stress (Tao, 2015; Nussey et al., 2009). The oxidative stress occurs when there is an imbalance between the levels of antioxidant and oxidant compounds in a biological system (Celi, 2010). This imbalance is associated with cellular and tissutal damage (Nussey et al., 2009), reduced productivity (Celi, 2010), and a decrease in the nutritional value and quality of animal products (Zisis et al., 2022). However, microalgae are rich in antioxidant compounds, such as carotenoids and flavonoids (Saadaoui et al., 2021), which can eliminate reactive oxygen species and protect biomolecules from oxidative stress (Yaakob et al., 2014).

As reported in the Feed Materials Register, Spirulina platensis is one of the microalgae approved as animal feed ingredients in Europe (www.feedmaterialsregister.eu, accessed on 8 January 2024). It is important to note that microalgae are commonly used in feeding aquatic animals, while their use in terrestrial species is a relatively new application. Lum et al. (2013) suggest that ruminants may also benefit from this

new feedstuff, as they can digest the cell walls of algal organisms and utilize the non-protein nitrogen compounds present in algae. Although microalgae have potential as a feed for dairy animals, their applications are currently limited compared to non-ruminant species.

1.1. Supplementing diet with microalgae: effects on feed intake, milk production and composition

The impact of adding microalgae to the diet on feed palatability and daily intake has been reported to be conflicting. Altomonte et al. (2018) reported that excessive supplementation of algal products can lead to a decrease in feed intake of dairy ruminants. However, a recent meta-analysis by Orzuna-Orzuna et al. (2023) showed an overall increase in dry matter intake (DMI) of small ruminants fed microalgae-supplemented diets. In cows fed total mixed ration, intake can decrease by 7 to 45% (Boeckaert et al., 2008; Moate et al., 2013). Some authors have reported qualitative changes in intake, where a reduction in intake of microalgae-containing concentrates was compensated by an increased intake of silage (Lamminen et al., 2017). Several studies have shown that the maximum level of microalgae that can be ingested by cows without affecting their feed intake is in the range of 4 to 79 g of microalgae per kg of DM in their diet (Altomonte et al., 2018). The extent of the decrease in feed intake depends on the type of feedstuff used. For instance, dairy cows can tolerate diets based on algal meal with inclusions levels of up to 10-11 g/kg of DMI (Boeckart et al., 2008; Moate et al., 2011). On the other hand, meal made up of defatted microalgae and soyhulls appears to be better tolerated, with up to 92 g/kg of DMI (Da Silva et al., 2016). Studies have shown that algal oil supplementation does not affect cow intake when integrated up to 194 g/day per head (Stamey et al., 2012). In sheep, a reduction in concentrate intake was observed with an algal biomass supplementation of about 12 g/kg of the ration (Papadopoulos et al., 2002). Three hypotheses have been proposed to explain the changes in feed intake associated with the administration of microalgae. One hypothesis attributes the poor palatability of microalgae to their taste and odour, as well as their physical structure, particularly if they are dry and finely powdered (Franklin et al., 1999; Papadopoulos et al., 2002; Lamminen et al., 2017). Pelleting the ration could improve palatability (Lamminen et al., 2017). The decrease in fibre digestibility may be due to the amount of fermentable carbohydrates in the algae and small particle size, which could negatively affect rumen pH (Stokes et al., 2015). Additionally, the PUFAs contained in the algae may have toxic effects on the rumen microflora, disturbing rumen fermentation (Boeckart et al., 2008). However, Christodoulou et al. (2023a) found that lactating ewes supplemented with 15 g/kg DM of Spirulina had a higher ruminal abundance of *Ruminococcus* albus bacteria. Similarly, Tsiplakou et al. (2017) observed a higher proportion of *Ruminococcus flavefaciens* bacteria in the ruminal fluid of lactating goats that were given microalgae

supplements. According to Koike and Kobayashi (2009), *Ruminococcus* albus and *Ruminococcus flavefaciens* are highly effective at digesting feed cellulose. This can increase the passage rate of fibre particles in the rumen and lead to higher dry matter intake (DMI). Rabee et al. (2022) found that lambs supplemented with microalgae had a lower relative abundance of bacteria from the genus *Succiniclasticum* in their ruminal fluid. The abundance of *Succiniclasticum* bacteria in growing lambs was negatively correlated with DMI (Li et al., 2022). Therefore, we can conclude that the effect of adding microalgae to the diet on DMI is dose-dependent.

Regarding the effects of microalgae supplementation on milk yield, most studies have found no effect in cows or small ruminants, even when reductions or changes in intake were observed (Franklin et al., 1999; Papadopoulos et al., 2002; Moate et al., 2013; Tsiplakou et al., 2017; Tsiplakou et al., 2018; Lamminen et al., 2017). Therefore, although the supplementation reduces feed intake, it does not affect milk yield. This is likely due to increased feed efficiency, as reported by Franklin et al. (1999) and Papadopulos et al. (2002). The increased feed efficiency is thought to be a result of the direct incorporation of fatty acids from algae into milk fat (Saadaoui et al., 2021). However, it should be noted that there have been reported cases of production losses. Boeckaert et al. (2008) found that cows fed algae at a rate of 43.0 g/kg of DM of the ration through the rumen fistula produced 45% less milk. Similarly, Reynolds et al. (2006) observed production decreases in sheep fed 25 g/kg of algal biomass of DM of the diet, along with corn silage and alfalfa hay silage. However, supplementing Spirulina at a rate of 200 g per day (equivalent to 10-14 g/kg of DM) resulted in a significant increase in milk yield in cows. During the 90-day experimental period, daily production increased by up to 25% (Kulpys et al., 2009). The authors attributed this improvement to the chemical composition of *Spirulina platensis*, which affects both the biological activity of the ruminal flora and the physiological status of the animal. Furthermore, studies have shown that steers receiving *Spirulina platensis* had a higher daily water intake (Panjaitan et al., 2010a; Panjaitan et al., 2010b). This aspect should be further investigated in dairy cows, as increased water intake may affect both milk yield and quality. In general, the increase in milk yield or greater dairy efficiency observed in response to microalgae supplementation may be related to the higher microalgae nutritional profile. Indeed, microalgae contain essential amino acids, fatty acids, and minerals that can improve animals' nutritional status, leading to higher milk yield and feed efficiency (Madeira et al., 2017; Valente et al., 2021; Costa et al., 2022). Furthermore, microalgae are a significant source of B vitamins, including B9 and B12, as demonstrated by Madeira et al. (2017) and Saadaoui et al. (2021). These vitamins have been proven to enhance energy metabolism efficiency, resulting in increased milk yield (MY) and feed efficiency (FE) in lactating ruminants (Duplessis et al., 2022). Similarly, adding microalgae to ruminant diets can improve nutrient

absorption (Kholif et al., 2020a; Kholif et al., 2020b), animal immune response and DNA integrity (El-Hawy et al., 2022), and blood plasma oxidative status (Christodoulou et al., 2023b). Regarding this issue, Tsiplakou et al. (2018) observed a higher levels of superoxide dismutase activity in the blood and milk, as well as higher levels of catalase activity in the blood plasma of goats fed *Chlorella vulgaris*. Superoxide dismutase and catalase are components of intracellular antioxidant defence mechanisms that regulate the accumulation of reactive oxygen species within tissues. Additionally, the enzyme lactoperoxidase in milk is related to the oxidation of lipids. The same authors also found a decrease in a biomarker of oxidative stress (protein carbonyls) in milk. As an overall result, the positive impact of microalgae ingestion on these physiological aspects also leads to higher productivity and feed efficiency of ruminants (Madeira et al., 2017).

Different results have been reported on the effects of microalgal supplementation on milk component synthesis, depending on the species, diet, ingestion, and milk yield. Some studies (Bichi et al., 2013; Moate et al., 2013; da Silva et al., 2016; Tsiplakou et al., 2017; Tsiplakou et al., 2018) reported that the dietary addition of microalgae did not change protein content in cows, sheep, and goat milk. However, other studies have reported a decrease in protein yield in cows, which was mainly related to a decrease in feed intake and milk yield (Boeckaert et al., 2008). It has been reported that milk protein tends to decrease, although this is not related to decreased intake or milk yield (Lamminen et al., 2017). According to Lamminen et al. (2017), the decrease in milk protein may be due to the low presence of histidine in microalgae. This amino acid limits milk production and may become suboptimal in the case of algal supplementation. In sheep, a decrease in the percentage of proteins has been found (Papadopulos et al., 2002; Toral et al., 2010). In contrast the findings of Boeckaert et al. (2008) in cows, these differences did not affect feed intake or have negative effects on rumen microflora. Reynolds et al. (2006) observed increased daily protein yield in sheep fed a diet of pelleted alfalfa hay and algae compared to a diet of corn silage and algae. The authors attributed the increase to the higher protein intake from the alfalfa hay. The study found that animals fed a diet based on alfalfa hay-silage supplemented with microalgae had a decrease in daily protein yield and an increase in percentages compared to those fed corn silage. The decrease in protein was linked to a concentration effect caused by the decrease in milk yield. Conflicting results have also been reported regarding the effects of algal supplementation on milk lactose. Some authors have found that the addition of microalgae to cows' feed leads to a decrease in lactose, which is mainly linked to a decrease in milk yield (Boeckaert et al., 2008). Similarly, decreases in lactose percentages have been observed in sheep (Papadopulos, 2002, Reynolds et al., 2006). In contrast, some authors have reported an increase in lactose (Moate et al., 2013), while others have reported no change (Kulpys et al., 2009;

Bichi et al., 2013; Póti et al., 2015; da Silva et al., 2016; Tsiplakou et al., 2017; Tsiplakou et al., 2018).

In line with studies on supplementing marine products such as fish oil, fish meal, or marine algae, dairy animals receiving microalgae supplementation usually experience milk fat yield losses, which ranging from 22% to 59% (Altomonte et al., 2018; Orzuna-Orzuna et al., 2023). The decrease in milk fat could be due to a higher fat content in the microalgae supplemented diets, negative energy balance resulting from low feed intake, or low-fat syndrome caused by the accumulation of trans FAs in the rumen and the formation of C18:2 isomer inhibitors of lipid synthesis (Boeckaert et al., 2008; Moate et al., 2013). The rise in inhibitors of fat synthesis may be linked to harmful effects on the ruminal microbiota, which hardly adapting to the dietary intake of very long chain n-3 PUFAs (Bichi et al., 2013). When diets supplemented with vegetable oils are considered, the most involved milk fat inhibitor isomers are known to be mainly trans-10, cis-12 C18:2 and trans-9, cis-11 C18:2, in both dairy cows and small ruminants (Altomonte et al., 2018, Orzuna-Orzuna et al., 2023). However, the inhibitor isomers in microalgae are not yet fully understood (Altomonte et al., 2018). Toral et al. (2010) hypothesized that the joint action of trans-9, cis-11 C18:2 and trans-10 C18:1, along with other unidentified intermediates, could be responsible. Boeckaert et al. (2008) suggest that the low-fat syndrome may be caused by a reduction in the synthesis of cis-9 C18:1, an essential FA for maintaining milk fat fluidity. Gama et al. (2008) propose that the secretion of cis-9 C18:1 may be inhibited, leading to a reduction in milk fat synthesis. Additionally, the decrease in milk fat content could be associated with the increase in milk production, as milk fat content decreases proportionally with milk yield (Craig et al., 2022).

On the other hand, the literature results on milk fat also vary. Some studies have reported positive increments (Papadopulos et al., 2002; Reynolds et al., 2006; Póti et al., 2015), while others have found no significant changes (Stamey et al., 2012; da Silva et al., 2016; Lamminen et al., 2017; Tsiplakou et al., 2017; Tsiplakou et al., 2018) in this milk component. According to some authors (Papadopulos et al., 2002, Reynolds et al., 2006, Póti et al., 2015), milk fat percentage can increase from 13% to 20% in small ruminants fed microalgae. The increase in milk fat content can be attributed to several factors. In some cases, the increase in fat percentage was linked to a concentration effect resulting from a decrease in milk yield (Reynolds et al., 2006). Griinari et al. (1998) and Papadopulos et al. (2002) suggest that increasing the forage to concentrate ratio in microalgae supplemented diet or reducing the synthesis of trans C18:1 (n-7) can lead to an increment in milk fat content. Poti et al. (2015) also suggest that certain algal species can have positive effects on ruminal fermentations. Stamm (2015) also supports this hypothesis, reporting a 9% increase in milk fat percentage in cows, which is linked to the beneficial effects of Spirulina sp. on rumen microbiota.

1.2. Milk fatty acids composition

The inclusion of microalgae in ruminant diets in order to modify positively the FA profile of milk is a recent nutritional strategy (Altomonte et al., 2018, Orzuna-Orzuna et al., 2023). Ruminal infusion of microalgae, as well as dietary administration, has led to a reduction in SFAs and an increase in PUFAs in ruminant milk (from increases of 54% to more than 100%) (Franklin et al., 1999, Boeckaert et al., 2008, Moate et al., 2013, Poti et al., 2015). These changes have also been found in dairy products derived from PUFA-enriched milk (Papadopoulos et al., 2002). Some authors have also observed an increase in milk MUFAs of goats and cows (+12% and +4%, respectively) (Póti et al., 2015, Boeckaert et al., 2008) and an increase in total de novo synthesized FAs with a chain length up to C16: 0 (Poti et al., 2015, Moate et al., 2013).

Although the content of C18:3 n-3 and C18:3 n-6 in microalgae is low (Lang et al., 2011; Saadaoui et al., 2021), their transfer to milk is high (Hagemeister et al., 1991; Bainbridge and Kraft, 2016). In humans, high intakes of C18:3 n-3 and C18:3 n-6 can reduce the symptoms and severity of various inflammatory diseases (Calder, 2012; Sergeant et al., 2016). In addition, C18:3 n-3 is a precursor of C20:5 n-3 and C22:6 n-3 (Calder, 2012; Póti et al., 2015), which are required for various metabolic processes in humans (Pereira, 2014). Studies in cows show that the transfer efficiency n-3 FAs in milk is greater in the case of ruminal infusions (+161%) (Boeckaert et al., 2008) and lower, but still significant, when microalgae are added to the ration (from +19% to increases of more than 100%) (Stamey et al., 2012; Moate et al., 2013; Póti et al., 2015). Increases have also been observed in goat's milk (+19%) (Póti et al., 2015). These results are significant because as attempts to increase the n-3 PUFAs content of milk by feeding have so far had limited success due to the massive biohydrogenation of PUFA in the rumen (Lock and Bauman, 2004). Of the n-3 FAs found in milk, studies have almost unequivocally reported an increase in C22:6 (DHA) as a result of microalgae supplementation. DHA is an essential FA that may improve cognitive function, lipid metabolism and prevent cardiovascular disease in humans. An increase in DHA has been observed in microalgae supplemented cows (Boeckaert et al., 2008; Moate et al., 2013; Póti et al., 2015), goats (Póti et al., 2015) and sheep (Papadopoulos et al., 2002; Reynolds et al., 2006; Bichi et al., 2013), with positive variation ranging from 100 to 1000% or more in cows (Boeckaert et al., 2008; Moate et al., 2013; Poti et al., 2015), + 660% in sheep (Bichi et al., 2013) and + 100% in goats (Poti et al., 2015). However, Weatherly (2015) reported that DHA enrichment occurs at inclusion levels in milk (15 g/kg DMI) that lead to sub-acidosis in cows with reduced intake and low milk fat secretion. In addition, the percentage of DHA in the milk fat of algae-fed cows decreased over time. Although this hypothesis has not been confirmed by other studies, Franklin et al. (1999) suggested that rumen microorganisms may become accustomed to the presence of non-rumen protected algae in the diet over time,

resulting in greater biohydrogenation of DHA with less incorporation of DHA into milk fat. C20:5 (EPA), another n-3 FA beneficial to human health, has been found to increase from +17 to +112% in cows (Stamey et al., 2012; Moate et al., 2013; Vahmani et al., 2013) and +133% in goats (Póti et al., 2015) and from 50 to 100% or more in sheep (Papadopoulos et al., 2000; Toral et al., 2010; Bichi et al., 2013) with a microalgae-supplemented diet. In addition, some studies have shown that unsaturated fatty acids with an 18-carbon chain such as linolenic acid (Franklin et al., 1999), linoleic acid (Franklin et al., 1999; Boeckaert et al., 2008), oleic acid and stearic acid decrease with supplementation in both cows and sheep (Papadopoulos et al., 2002; Reynolds et al., 2006; Toral et al., 2010; Moate et al., 2013). The exception is goat's milk, where linoleic acid increases (Kouřimská et al., 2014; Póti et al., 2015). The CLA fatty acids and their main isomer C18:2 cis-9, trans-11, whose beneficial effects on metabolism and anticancer activity have been demonstrated in animal models, increase in milk of cows (from +13 to +108%) (Boeckaert et al, 2008; Stamey et al., 2012; Moate et al., 2013; Póti et al., 2015), goats (+28%) (Póti et al., 2015) and ewes (+39%) (Reynolds et al., 2006; Bichi et al., 2013) fed microalgae supplemented diets. Similarly, increases in vaccenic acid (C18:1 trans 11) were observed in cow's milk (from +11 to +203%) (Boeckaert et al., 2008; Stamey et al., 2012; Moate et al., 2013; Póti et al., 2015) and goat's milk (+151%, Póti et al., 2015). The increase in C18:2 cis-9, trans-11 associated with the dietary inclusion of algae meal was probably due to the inhibitory effect of algae on rumen biohydrogenation and to the increased ruminal production of the C18:1 trans-11 substrate. The shift in the ruminal beta-hydroxybutyrate pathway towards the formation of trans-C18:1 fatty acid was also observed by Tsiplakou et al. (2017) in goats fed Chlorella vulgaris. This effect was associated with changes in the *Butyrivibrio fibrisolvens* population in their rumen fluid. On the other hand, direct effects of algae on animal metabolism, such as on the activity of the Δ9-desaturase enzyme, have been excluded (Boeckaert et al., 2008; Moate et al., 2013).

Dietary supplementation with microalgae decreased the content of C15:0 and C17:0 in milk, probably because of the content of B complex vitamins in microalgae (Madeira et al., 2017; Saadaoui et al., 2021). In ruminants, a deficiency of B-complex vitamins (e.g. B8 and B12) reduces the processes that convert propionic acid to succinyl-CoA for its subsequent entry into the Krebs cycle (Duplessis et al., 2022). This effect leads to the accumulation of propionic acid (Pećina and Ivanković, 2021) and increases the concentrations of C15:0 and C17:0 in different tissues (Pfeuffer and Jaudszus, 2016). Furthermore, a high intake of C15:0 and C17:0 can affect the function of the human nervous system (Jenkins et al., 2015; Pećina and Ivanković, 2021) and inhibit the hepatic oxidation of short-chain FAs (Jenkins et al., 2015). Therefore, reducing the levels of C15:0 and C17:0 in milk could have a positive impact on consumer health.

1.3. Milk and cheese sensory properties

Sensory analysis is the ultimate measure of product quality and success, and is often the final step in many experiments or applications (Drake, 2007). The sensory quality of cheese is mainly dependent on the technological process (e.g., manufacturing, ripening time, and cheese factory conditions) as well as the chemical and microbiological characteristics of the raw milk (Fox et al., 2017), which in turn are influenced by several upstream factors including the feeding of dairy animals (Martin et al., 2005). To the best of our knowledge, there are only a few studies available on the impact of feeding microalgae on sensory characteristics, as well as the overall quality of dairy products. In Cheddar cheese, made with milk from cows fed microalgae *(Schizochytrium limancinum*), Till et al. (2019) found that 20 of the 32 sensory attributes investigated were altered by microalgae supplementation , with a linear increase in cheese air holes, nutty flavour, and dry mouth aftertaste. Cheese creaminess decreased with microalgae inclusion rate and was positively correlated with saturated fatty acid content, which was lower in microalgae-produced milk. A quadratic effect was also observed for fruity odour, which was highest in cheese from cows fed high levels of microalgae (150 g/head per day), and for firmness and crumbliness of cheese texture. However, Manzocchi et al. (2020) showed no difference in the sensory profile of pasteurized milk obtained by replacing approximately 6% of the soybean meal with dehydrated spirulina (*Arthrospira platensis*) in the cow's diet. Similarly, *Schizochytrium* sp. supplementation (from 5 to 35 g/head per day), did not affect the aroma and sensory profile of raw goat's milk and cheese (Gebereyowhans et al., 2023; Pajor et al., 2023).

2. Use of hemp products in poultry feeding

Sustainable agriculture is the production of agricultural products at an environmental cost that does not compromise food/feed security and animal health. Scientific research is therefore investigating new alternatives to traditional protein sources to ensure these principles. This objective is strongly pursued by the European Union through the One Health approach and subsequently with the Green Deal of 1 December 2019, which recognizes that human, animal, and environmental health are interconnected and promotes the efficient use of resources through a circular and clean economy (Wolf et al., 2021). Among the possible alternatives, *Cannabis sativa* L., commonly known as hemp, is gaining increasing curiosity in the feed/food sector, not only because of the high nutritional and functional properties of hemp seeds, but also because of the role it plays in environmental protection. In addition to its ability to grow rapidly in different agro-ecological conditions with limited use of water and herbicides, it is an excellent candidate for carbon sequestration (nine to thirteen

tons/ha). In addition, its deep roots prevent soil erosion, and take up nutrients and metals from deeper soil layers (Rupasinghe et al., 2020; Rehman et al., 2021).

In particular, interest in hemp has increased following European Regulation 1251/1999/EC, which established a support system for all producers of certain types of seeds, including industrial hemp, and European Regulation 1370/2013, which included hemp cultivation among those eligible for Common Agricultural Policy payments, with the only condition that the seeds used for cultivation must be of varieties registered in the European catalogue with a Δ9-tetrahydrocannabinol content of less than 0.2-0.3% (Sorrentino, 2021). To ensure sustainable agriculture, one possible approach is to recycle nutrients from residues and unusable crop by-products and feed them to livestock, among which hemp by-products are of great interest, in particular the whole plant, leaves and flowers (obtained before/after the cannabinoid extraction process), stems, stalks, whole seed heads and seed shells (Kleinhenz et al., 2020; Vastolo et al., 2022; Ely and Fike, 2022).

However, the most recent report by the European Food Safety Authority (EFSA) on the use of hemp-based products in the feed sector dates from 2011 (EFSA, 2011). In this report, EFSA does not provide precise guidance on the levels that should be included in animal diets and recommends that, for the purpose of rational use of hemp derivatives, what is suggested in the literature should be considered. Therefore, there has been a significant increase in the number of studies on the use of hemp-based feed since 2011, particularly in monogastric animals. The main findings from the literature on the production performance and health of hemp-fed poultry are summarized below.

2.1. Laying hens

Hemp-based products have been extensively studied for use in the laying hen industry. The main focus of these studies has been on the functional and nutritional characteristics of eggs, as well as the evaluation of qualitative and quantitative parameters. Production performance and animal welfare have also been investigated. In terms of the functional aspect of eggs, Mierliță (2019) found no difference in the total cholesterol content of egg yolk from hens treated with different hemp seeds level (80 g/kg and 200 g/kg). In contrast, Shahid et al. (2015) found that by increasing the amount of hemp seeds in the diet (150 g/kg, 200 g/kg, and 250 g/kg) cholesterol levels were significantly reduced (16.91±0.01 mg/g, 14.29±0.01 mg/g, 11.65±0.01 mg/g) compared to the control diet (corn and soybean meal) (19.27±0.01 mg/g). Skřivan et al. (2019) observed similar trends even with lower hemp seeds inclusions (30 g/kg, 60 g/kg, and 90 g/kg). This is consistent with the findings of Mahmoudi et al. (2015) and Vispute et al. (2019). The positive effect is likely due to the presence of phytosterols, specifically β-sitosterol, which can reduce hypercholesterolemia by blocking

cholesterol absorption through coprecipitation and crystallization. Additionally, phytosterols, which have lower water solubility than cholesterol, can displace cholesterol from intestinal micelles (Shahid et al., 2015). Functionality also depends on the presence of antioxidant compounds. Skřivan et al. (2019) demonstrated that including hemp seeds increased tocopherol levels, an important antioxidant that prevents lipid peroxidation during storage. This finding was also confirmed by Mierliță (2019). The author reported that eggs from animals treated with hemp seeds had higher levels of α-tocopherol and lower levels of malondialdehyde (a marker of lipid peroxidation) compared to the control group.

In terms of nutritional characteristics, eggs from animals treated with hemp-based products demonstrated a superior lipid profile. Specifically, Shahid et al. (2015) reported that hemp-based products decrease the levels of SFAs and MUFAs in the yolk in favour of PUFAs. The high levels of n-3 PUFAs in the treatments with 150 g/kg (7.79±0.13 mg/g), 200 g/kg (10.29±0.09 mg/g), and 250 g/kg (15.11±0.81 mg/g) of hemp seeds, compared to the control (2.66±0.17 mg/g), resulted in an improved n-6/n-3 ratio (Shahid et al., 2015). Similar results were observed by Jing et al. (2017) when included hemp seeds in diets at levels of 40 g/kg and 80 g/kg, and by Neijat et al. (2016). Gakhar et al. (2012) found that including up to 300 g/kg of hemp seeds and up to 90 g/kg of hemp seeds oil did not significantly affect the total SFAs content, but it did decrease the MUFAs content while increasing the PUFAs content, particularly the n-3 PUFAs, thereby improving the n-6/n-3 ratio. According to Gakhar et al. (2012), α-linolenic acid (ALA) is the main n-3 fatty acid that experiences an increase. The study demonstrated that treatments with 100 g/kg and 200 g/kg hemp seeds resulted in higher levels of the fatty acid, with 51.7±3.23 mg/g and 91.3±3.23 mg/g, respectively, compared to the control group with 15.8±3.23 mg/g. The results were also confirmed in diets with 40 g/kg, 80 g/kg, and 120 g/kg hemp seeds oil. The high content of ALA plays a key role, as it can be converted to docosahexaenoic acid by the metabolism of chickens, which has been explained previously. Docosahexaenoic acid is derived from ALA through a sequential reaction of enzymes including desaturase, elongase, and β-oxidation, with eicosapentaenoic acid and docosapentaenoic acid as intermediates. This explains the high levels of these fatty acids found in the eggs of these animals, despite their lack in hemp seeds. Gakhar et al. (2012) demonstrated that treatments with hemp seeds (100 g/kg and 200 g/kg) increased EPA (0.9±0.08 mg/g, 1.2±0.08 mg/g, respectively) and DHA (39.2±1.64 mg/g, 47.4±1.64 mg/g) compared to the control (diet based on soybean meal and corn oil) (0.2±0.08 mg/g and 17.1±1.64 mg/g, respectively). These findings were also supported by Goldberg et al. (2012) and Kasula et al. (2021).

Research has shown that including hemp products in the chicken's diet can affect the physical characteristics of the egg. Specifically, the yolk colour of eggs was found to change when hemp-based products were added to the chicken's diet (Goldberg et al.,

2012; Park et al., 2014; Skřivan et al., 2019; Konca et al., 2019;). According to Goldberg et al. (2012), the intensity of yolk colour is primarily due to the presence of yellow and red oxy-carotenoids or xanthophyll pigments in the hens' diet. The yolk colour can range from almost absent to intense orange depending on the level of these compounds in the diet. However, it is important to emphasize that although changes in yolk colour may be statistically significant, they may not be noticeable to the human eye, and therefore may not reflect consumer preferences. Egg weight and eggshell thickness are key quality parameters of eggs. Generally, increase in egg weight is due to the increase in yolk weight, at the expense of other egg components. In the study conducted by Mierliță (2019), supplementation with 80 g/kg hemp seeds resulted in higher egg weights (61.7±0.937 g) compared to animals treated with 200 g/kg hempseed cake (58.3±0.937 g) and control animals. Similar results were observed by Gakhar et al. (2012), where animals treated with up to 200 g/kg hemp seeds produced eggs of 60.57±1.14 g, heavier than the control group (56.2±1.14 g). Regarding the eggshell, it is important to note that plasma concentration of Ca and P in laying hens are strictly related. The content of Ca and P in the diet affects the body's ability to regulate blood pH, which in turn affects the acid-base balance (Keshavarz, 1994). Maintaining an optimal acid-base balance is necessary for bicarbonate homeostasis and eggshell formation in laying hens. Changes in eggshell quality have been linked to variations in the mineral profile of hens' blood plasma (Keshavarz, 1994). However, according to Neijat et al. (2014), establishing a direct correlation between dietary treatment and mineral content in the blood is challenging. The authors suggest that the levels of these minerals likely reflect changes in demand as the hens age. During egg production peak, calcium requirements are at their highest, and this can be measured by serum/plasma calcium levels. The increase in Ca and P intake over metabolic requirements may be responsible for the increase over time, regardless of diet, as the hens exceed peak production. On eggshell thickness, the use of hemp seed products yielded conflicting results. Park et al. (2014) and Neijat et al. (2014) found no significant difference compared to the control groups, while Skřivan et al. (2019) reported a negative correlation with increasing hemp seeds inclusion. Specifically, the eggshell thickness of the control group was 363±1.5 μm, which was similar to the diets containing 30 g/kg (357±1.5 μm) and 60 g/kg (357±1.5 μm) of hemp seeds, but greater than the diet with 90 g/kg of hemp seeds (350±1.5 μm). The same minerals also play a crucial role in bone health. Skřivan et al. (2019) observed that the higher tibial strength agrees with the higher calcium level in groups fed diets enriched with hemp seeds, up to 90 g/kg. Additionally, positive results were also produced by cannabidiol and α-tocopherol. As previously reported, the former improves fracture healing by acting on collagen cross-linking, while the latter tends to increase osteogenic bone mass in vertebral secondary cancellous bone, where active bone remodelling occurs (Skřivan et al., 2019).

In regard to production parameters, the inclusion of hemp-based products has generally allowed production rates to be maintained or improved, with no negative trends. However, additional studies are required to confirm the observed responses due to conflicting results. Indeed, while Jing et al. (2017), Neijat et al. (2014), Gakhar et al. (2012), Mierliță (2019), Konca et al. (2019), and Kasula et al. (2021) did not observe any differences compared to the control, a significant egg production increments were recorded by Skřivan et al. (2019), Park et al. (2014), and Halle and Shone (2013). More precisely as reported by Skřivan et al. (2019), the laying rate improved with the 30 g/kg hemp seed treatment (93.6±0.59%) compared to the control group and the 60 g/kg and 90 g/kg treatments (88.7±0.59%, 86.4±0.59%, 89.49±0.59%, respectively). These results were also confirmed by Halle and Shone (2013) with a supplementation of 100 g/kg and 150 g/kg hemp seeds cake compared to flaxseeds cake. Park et al. (2014) showed that 16.8 g/kg hemp seeds oil increased the deposition rate (97.86±0.250%) compared to the control group (96.07±0.250%). Although it is difficult to hypothesize the explanation for this effect, it is very likely that hemp-based products have nutrigenomic value by modulating the genes responsible for oviposition rates.

2.2. Broilers

Promising results have been obtained with broilers fed diets supplemented with hemp seed. More specifically, as observed in the study by Khan et al. (2010), the inclusion of 20 g/kg of hemp seeds resulted in a better feed conversion ratio with a higher slaughter weight compared to the control group (2087.2±10.25 g/kg and 1861.4±32.2 g/kg). As explained by the authors, this trend is due to the high lipid and aminoacidic profile of hemp seeds, confirming the results reported by Parr et al. (2020), where the inclusion of 20 g/kg proved to be better than 10 g/kg, 30 g/kg, and 40 g/kg to modulate the growth performance of broilers. However, Skřivan et al. (2020) reported that including 40 g/kg of hemp seeds resulted in comparable performance and product quality to flaxseeds, even when added at 60 g/kg. This confirms the high nutritional profile of hemp seeds. The same trend was observed for bone health, which is a key parameter for animal welfare, especially under intensive livestock conditions. According to Skřivan et al. (2020), this result is likely linked to the higher presence of α-tocopherol in the diet and meat of animals that received the hemp seed diet. Supplementation with α-tocopherol has been shown to increase osteogenic bone mass in the vertebral secondary cancellous bone, where active bone remodelling occurs (Skřivan et al., 2020). However, while the results indicate that including up to 40 g/kg of hemp seeds in broiler diets has a positive effect, it is important to consider the potential impact of the high fibre and mineral content on animal health during the first weeks of the production cycle. Vispute et al. (2019) reported that at this stage, chickens have underdeveloped mucosa and are unable to produce sufficient enzymes to digest high cellulose content. Similar results were obtained using different concentrations of hemp

seed oil. Jing et al. (2017) demonstrated that adding 30 g/kg and 60 g/kg of hemp seed oil to a control diet based on corn oil did not affect the performance (body weight, feed intake, and feed conversion ratio) of the treated animals. However, it did increase the n-3 PUFAs content in the meat to 71.4(±14.8) g/100 g and 148.9(±14.8) g/100 g, respectively. This result was partially confirmed by Kanbur (2022). The author strongly advises against using hemp seed oil, especially in the first three weeks of rearing, due to recorded negative effects on growth performance. While Vispute et al. (2019) reported that high fibre content affected growth performance, in this case, it is difficult to speculate on the causes due to the complexity of dealing with a lipid matrix. According to Fouad and El-Senousey's (2014) report, a diet high in PUFAs for broilers can reduce fat deposition by promoting fatty acid β-oxidation.

There is growing interest in using hemp co-products in broiler chicken diets (Vastolo et al., 2022). However, there is limited literature on this topic. Stastnik et al. (2016) conducted a study on the effects of incorporating 25 g/kg of hemp seed expellers and 10 g/kg of hemp plant tops (flowers, seeds, and a small level of shives) in the diet of broilers. The study found no significant difference in carcass yield at slaughter when compared to the control diet (maize diet). However, the European Union has not yet authorized the use of these hemp-based products, indicating the need for further studies on their valorisation. On the other hand, the European Union has approved the use of hemp seeds cake. However, conflicting results have been observed for broilers. Eriksson and Wall (2012) showed that including 200 g/kg of hemp seed cake in diets resulted in growth performance parameters (feed intake, feed conversion ratio, and final weight) and mortality rates that were highly comparable to those of diets based on soybean cakes, rapeseed cakes, and peas. Although these results suggest that hemp seeds cake could be used as an alternative to soybean cakes, Stastnik et al. (2015) reported opposite results when including 50 g/kg and 150 g/kg, which caused a decrease in final weight compared to the control diet (soybean cake). This is likely due to the presence of anti-nutritional factors, particularly non-starch polysaccharides, which increase in content at the fibrous level when the lipid fraction is removed. More recently, Tufarelli et al. (2023) found no differences in growth performance and meat quality traits when supplementing broilers' diets with up to 10% hemp seeds cake. However, histomorphometric analysis showed a significant increase in villus height, surface area, and villus/crypt ratio, with a decrease in crypt depth. This suggests that dietary supplementation with hemp seeds cake may improve intestinal health in poultry.

3. Remarkable results and conclusion

The review of the impact of microalgae on milk production revealed significant heterogeneity in the available literature. This heterogeneity is due to variations in the

kinds and amounts of supplementation, the type of feed and diet composition, the different nutrient profiles among algae feedstuffs, and the length of the experimental period. Remarkable improvements were observed in the milk fatty acid profile, primarily due to an increase in long chain fatty acids and omega 3 fatty acids, particularly DHA and EPA. However, it is important to note that excessive algae supplementation may have negative effects on palatability, feed intake, ruminal metabolism, milk production, and fat. Therefore, caution should be exercised when supplementing with algae. It is important to deepen our understanding of the quality and sensory characteristics of dairy products from animals that are fed microalgae.

The effects of including hemp-based products in poultry diets vary depending on the species. Further investigation is needed regarding the use of these products in broilers, as there have been limited studies. The inclusion of hemp seeds improved chicken growth performance, as demonstrated by the final body weight, when included at a rate of 20 g/kg. Studies have shown that including oil in broiler diets, up to a maximum of 60 g/kg, can significantly improve the PUFAs profile in meat. However, this improvement does not always affect or may even reduce growth performance. Similar results have been observed with hemp co-products. The effects of seed cake inclusion at 50 g/kg, 150 g/kg, and 200 g/kg on feed conversion ratio and final body weight have been inconsistent. It is worth noting that there is currently no data available on feed-grade seeds.

For laying hens, on the other hand, there is a large body of scientific evidence confirming that hemp-based products are effective and safe matrices, able to positively modulate animal health and performance, while improving the nutritional and functional profile of eggs. These positive effects were recorded with different levels of hemp seeds inclusion, up to 250 g/kg. In addition, hemp seed oil was found to increase the nutritional value of eggs by raising the levels of PUFAs when included at a rate of up to 300 g/kg. Similarly, hemp seeds cake (up to 150 g/kg) was found to improve laying hens' production. However, determining the optimal inclusion rate of hemp-based products requires further evaluation.

To our knowledge, no studies have been conducted on the sensory characteristics of eggs and chicken meat obtained by feeding poultry with hemp-based diets.

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Chapter 2

Use of Cyanobacterium Spirulina (*Arthrospira platensis*) in Buffalo Feeding: Effect on Mozzarella Cheese Quality

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Abstract

The high demand for PDO buffalo mozzarella cheese is leading to the use of new strategies for feeding supplementation. Spirulina is acknowledged as a valuable source of protein with antioxidant and immune-modulatory effects in humans and animals. This investigation aimed to examine the effect of Spirulina integration in buffalo diets on mozzarella cheese quality, sensory profile, consumer acceptability, and willingness to pay (WTP). The trial was carried out on two groups of 12 buffaloes that differed in Spirulina integration: 50 g/head/d before calving (1 month) and 100 g/head/d after calving (2 months). Both the bulk milk and mozzarella cheese samples from the two groups did not differ in chemical composition. However, Spirulina inclusion influenced the sensory quality of mozzarella cheese, which resulted it being externally brighter, with a higher butter odour and whey flavour and greater sweetness, bitterness, juiciness, tenderness, oiliness, and buttermilk release than the control. The consumer test showed that information about Spirulina affected consumer liking, causing them to be in favour of the Spirulina group, leading to a higher price for it. In conclusion, Spirulina inclusion in buffalo diets affected the sensory quality of mozzarella cheese. The provision of product information to consumers can be a crucial factor in determining their liking and WTP.

Keywords: Arthrospira platensis, dairy buffaloes, PDO mozzarella cheese, sensory properties, consumer acceptability, willingness to pay

1. Introduction

In recent decades, buffalo breeding and milk production in Italy has been increasing, while in most other European countries, the number of buffaloes has been decreasing due to their use as draught animals, which has been replaced by mechanization (Borghese, 2013; Mota-Rojas et al., 2021). The growing progress of the Italian buffalo population, the nutritional and reproductive techniques, and the high quality of milk and meat products and markets have reached the top level in Europe. Many of the appreciated buffalo products come from milk, such as ricotta, provola, scamorza, and other cheeses, and from meat, such as steaks, roasts, ham, bresaola, and salami, but mozzarella cheese is the most important buffalo product on the Italian and international market (82% of consumption in Italy, and 18% for export, especially in Germany, France, UK, and USA) (Borghese, 2013).

Almost all of the buffalo milk produced in Italy (mainly in the southern regions) is transformed into mozzarella cheese, a product of some specific areas of the Campania and Lazio regions that have been endowed with the Protected Designation of Origin (PDO) (Commission Regulation EC 103/2008) (Sacchi et al., 2020; Zicarelli, 2020). Buffalo mozzarella is a "pasta fila-ta cheese", which is sold very fresh (a few hours

after the cheese is made) and is characterized by a soft, moist, and elastic texture, a bright and white porcelain colour, and a fresh flavour. Its sensory profile is influenced by the starter used in the cheesemaking process (Coppola et al., 1990), the technological process (Pizzolongo et al., 2007), and most importantly, the milk composition, which is closely linked to animal feeding (Napolitano et al., 2021). Although many authors have carried out studies on how a different diet can modify the chemical composition, sensory profile, or aromatic compounds of buffalo mozzarella cheese (Claps et al., 2007; Cifuni et al., 2007; Sabia et al., 2020; Uzun et al., 2018), none of them have used a "non-conventional" feed supplement. Recently, as reported in some reviews (Holman et al., 2013; Lamminen et al., 2021; Orzuna-Orzuna et al., 2023; Altomonte et al., 2018), there has been an increased focus on the impact of microalgae supplementation on the quality and production of milk in ruminants, as well as on the quality of eggs and chicken meat.

Microalgae are prokaryotic and eukaryotic organisms with a rapid growth rate, and they have the ability to grow in challenging environments, such as non-arable or marginal lands, and under different environmental conditions. They can be grown in either open or closed systems, under autotrophic or heterotrophic conditions, and their chemical compositions can be manipulated by changing the growth medium and/or the growth conditions. The species of microalgae with high concentrations of protein can partially replace conventional protein-based feeds, while those with high carbohydrate or lipid concentrations can be used as energy sources (Lamminen et al., 2021). To date, Arthrospira maxima and Arthrospira platensis, both commonly known as Spirulina, are two of the microalgae registered under EU Regulation 767/2009 as feeding ingredients or animal feed (Altomonte et al., 2018). Spirulina is considered one of the most nutritious foods due to its high concentrations of high-quality proteins (55–70% in dry weight, i.e., containing all essential amino acids) and vitamins and minerals such as B vitamins, iron, potassium, and many others. Currently, the effects of Spirulina as a feed supplement on animal health and/or production have been studied in different species, such as swine, poultry, and ruminants (Holman et al., 2013). Although most ruminants have been considered in many studies (Orzuna-Orzuna et al., 2023; Altomonte et al., 2018), little is currently known regarding the performance and production of buffaloes when microalgae are added to their diets (Ragab et al., 2023).

The objective of this study was to examine the impact of adding freeze-dried *Arthrospira platensis* biomass to the diets of dairy buffaloes on the chemical composition and sensory attributes of PDO mozzarella cheese. Furthermore, we assessed the influence of information regarding Spirulina's health advantages on consumer acceptability and willingness to pay.

2. Materials and Methods

2.1. Animals, Diets, and Mozzarella Cheese Production

All cows enrolled in this study were handled in compliance with the Italian legislation concerning the protection of animals used for scientific purposes (DL n. 26, 4 March 2014), and all procedures involving animals used in this study were reviewed and approved by the Ethical Animal Care and Use Committee of the University of Na-poli Federico II (Prot. 2020/007143 of 8/1/2020).

The trial was conducted on a buffalo dairy farm in the Southern Italian region of Campania (40°48′ N 15°01′ E, 14 m a.s.l.) between March 2021 and July 2021. Twenty-four late pregnant dairy buffaloes (*Bubalus bubalis*) were blocked for expected calving date and assigned to either a control or experimental group (hereafter referred to as C and S, respectively) balanced for cows' number (n = 12), parity (3 secondiparous and 9 pluriparous), and milk yield from the previous lactation (2642 ± 503 kg/head and 2729 ± 492 kg/head for C and S, respectively).

The two groups were housed in 2 adjacent free-stall barns (13 × 10 m each) equipped with feed bunks, water troughs, and an outdoor exercise area. The experimental period lasted 120 days in total, including an adaptation period of 30 days before calving and a sampling period covering the early 90 days of lactation. Across the adaptation and sampling periods, all cows were fed the same standard total mixed ration (TMR) formulated to meet or exceed the recommended nutritional requirements for dairy buffaloes in the near dry period and early lactation, as recommended by Bartocci et al. and Campanile et al., respectively. As for the farm routine, both TMRs were offered once a day at 08:00 h, allowing for approximately 10% feed refusal. In the Spirulina group, the freeze-dried Spirulina was top-dressed just after the TMR delivery at a daily dose of 50 g/head until calving and 100 g/head during lactation. Table 1 lists the detailed ingredients and chemical compositions of the experimental TMRs (dry and lactation diets).

Table 1. Ingredients (kg/head/day) and chemical composition (% of dry matter, DM, if not otherwise stated) of the experimental total mixed ration.

Item	Dry ration		Lactation ration	
	C	S	C	S
Ingredients				
Maize silage	6.0	6.0	18.0	18.0
Ryegrass hay	-	-	-	-
Wheat straw	7.0	7.0	3.2	3.2
Alfalfa hay	-	-	2.0	2.0
Ryegrass haylage	5.0	5.0	1.8	1.8
Concentrate mixture	2.0	2.0	8.0	8.0

Spirulina platensis	-	0.05	-	0.1
Chemical composition				
DM, % as fed	57.47	57.55	50.96	51.24
Ash	4.73	4.74	6.54	6.53
CP	9.16	9.35	16.91	17.17
EE	2.48	2.49	5.12	5.12
NDF	60.0	59.76	41.09	40.87
ADF	39.40	39.24	25.15	25.02
ADL	6.06	6.07	4.15	4.17
NFC	23.67	23.65	30.34	30.28
NEL (MJ/kg DM)	4.70	4.68	6.57	6.53

C, control group, S, Spirulina group, DM, dry matter, CP, crude protein, EE, ether extract, NDF, neutral detergent fibre, ADF, acid detergent fibre, ADL, acid detergent lignin, NFC, non-fibrous carbohydrate, NEL, net energy of lactation.

The used Spirulina biomass (Table 2) was provided by ATI Bio-tech s.r.l. (Castel Baronia, Avellino, Italy). Cultivation was performed through industrial-scale outdoor open raceway ponds by using standard Zarrouk's culture medium (20 °C, pH 10.25).

Table 2. Chemical composition of freeze-dried Spirulina.

Proximate Composition	***%***	***Fatty Acid Profile***	***%***	***Amino Acid Profile***	***mg/100 g***	***Minerals***	***mg/100 g***
Moisture	8.29	SFA	40.11	His (E)	1108.26	Ca	83.01
Ash	8.78	C 16:0	38.33	Arg (E)	4105.04	Fe	25.76
Fat	5.84	C 18:0	1.06	Thr (E)	3094.66	K	1879.29
Protein	64.95	MUFA	10.07	Val (E)	4163.97	Mg	178.44
		C 16:1 n-7	7.28	Met (E)	975.55	Mn	2118.37
		C 18:1 n-9	1.41	Lys (E)	2819.49	Na	1442.96
		PUFA	49.82	Ile (E)	3701.69	P	933.98
		C 18:2 n-6	23.89	Leu (E)	5527.34	Zn	0.75
		C 18:3 n-6	23.50	Phe (E)	2918.31	Cu	0.28
		C 20:3 n-6	1.40	Asp	5675.80		
				Ser	3206.42		
				Glu	9685.14		
				Gly	3055.51		
				Ala	4447.83		
				Pro	2381.84		
				Cys	458.28		
				Tyr	2616.80		

SFA, Saturated Fatty Acid, C 16:0, Palmitic Acid, C 18:0, Stearic Acid, MUFA, Monounsaturated Fatty Acid, C 16:1 n-7, Palmitoleic Acid, C 18:1 n-9, Oleic Acid, PUFA, Polyunsaturated Fatty Acid, C 18:2 n-6, Linoleic Acid, C 18:3 n-6, γ-Linolenic Acid, C 20:3 n-6, Dihomo-γ-Linolenic Acid, (E): Essential Amino Acid, His, Histidine, Arg, Arginine, Thr, Threonine, Val, Valine, Met, Methionine, Lys, Lysine, Ile,

Isoleucine, Leu, Leucine, Phe, Phenylalanine, Asp, Aspartic Acid, Ser, Serine, Glu, Glutamic acid, Gly, Glycine, Ala, Alanine, Pro, Proline, Cys, Cysteine, Tyr, Tyrosine, Ca, Calcium, Fe, Iron, K, Potassium, Mg, Magnesium, Mn, Manganese, Na, Sodium, P, Phosphorus, Zn, Zinc, Cu, Copper.

From 3 (±1) days of lactation onwards, buffaloes were milked two times a day (05:00 and 17:00 h) in the auto-tandem milking parlour equipped with a pipeline milking system that transports milk to a refrigerated (4 °C) tank.

After the first 30 days of milking, four mozzarella cheese-making sessions between the 2nd and 3rd month of lactation, with 2 sessions per month, were carried out by using refrigerated (4 °C) bulk tank milk that was separately collected at the evening milking session from each group (approximately 60 kg/group per milking). Bulk milk from each group was processed at the dairy farm, in separate vats, following the traditional procedure adopted for the PDO mark (Protected Designation of Origin) detailed by Sacchi et al. (2020). In brief, the raw milk was heated to 37 °C, and natural starter cultures and liquid calf rennet were added. The curd was then broken into small particles (2–3 cm) and left under whey until the pH reached 4.85. At this pH, the curd was manually stretched in water kept at 90–95 °C. Finally, 50 g of mozzarella (spherical shape) were cut mechanically, cooled in water, and left in brine (10% NaCl).

2.2. Measurements, Sampling Procedure, and Analytical Methods

2.2.1. Feeds and Diets

Feed intake was monitored weekly on a group basis along the lactation period by subtracting feed refusals from the TMR offered. On the same days, individual feed and TMR samples were collected, dried in a forced air oven at 65° until a constant weight was reached, and ground to pass through a 1 mm screen to be analysed for the proximate composition according to the standard procedures described elsewhere Serrapica et al., 2020, Serrapica et al., 2020).

2.2.2. Milk and Mozzarella Chemical Compositions

Triplicate bulk milk samples (150 mL/group) were collected immediately after milking before each cheese-making session and analysed on the same days of collection in duplicate for protein, fat, and lactose using mid-infrared method (MilkoScan Minor Type 78100, Foss Electric, Hillerød, Denmark). Three mozzarella cheese samples were taken from each production batch the day after manufacturing. Samples were cut in slices and then minced by using a blender (LB20ES, Waring Commercial Blender, New Hartford, CT, USA), and they were separately ana-lysed in duplicate (100 g per replication) for moisture, protein, fat, lactose, and salt contents using FoodScan Lab Analyzer (Foss Electric A/S, Hillerød, Denmark) operating between 2 scanning frequencies (850 and 1048 nm).

2.2.3. Fatty Acids Composition

Fatty acid (FA) profiles of mozzarella cheese (10 g) were performed according to Vargas-Ramella et al. (2020). Fatty acids were transesterified and detected using a gas chromatograph (GC-Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID), a PAL RTC-120 autosampler (maintained at 250 °C and 64.2 mL/min of total flow rate), and a DB-23 fused silica capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies) for the separation of Fatty Acid Methyl Esters (FAMEs). As standard, we used Supelco 37 Com-ponent FAME Mix (Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as a percentage of total methylated FA. Values for individual FA < 0.1 were not reported. Atherogenic index (AI), thrombogenic index (TI), and hypocholesterolemic/hypercholesterolemic ratio (h/H) were calculated according to Chen and Liu (2020).

2.2.4. Amino Acid Determination

Amino acid (AA) profiles were determined according to the procedure described by Vargas-Ramella et al. (2021). The HPLC system used was an Alliance 2695 model equipped with a 2475 scanning fluorescence detector (Waters, Milford, MA, USA). To control the system operation and results management, an Empower 2™ advanced software (Version 1.04.1037, Waters, Milford, MA, USA) was used. Separations were carried out using a Waters AccQ-Tag column (3.9 × 150 mm, with a 4 mm particle size) with a flow rate of 1.0 mL min−1 at 37 °C. Detection was carried out via fluorescence with excitation at 250 nm and emission at 395 nm. The AAs were quantified using the external standard method using an AA standard (Amino Acid Standard H, Thermo, Rockford, IL, USA) and identified via the retention time. The amount of each AA that was detected was expressed as mg/100 g sample.

2.2.5. Minerals Determination

Initially, a 3 g sample was subjected to incineration at a temperature of 600 °C using a muffle furnace (Carbolite RWF 1200, Hope Valley, UK). Afterwards, 10 mL of 1 M HNO3 was added, and the sample was further incinerated at 600 °C. Standards were diluted and used to calibrate the IPC-MS for mineral analysis. The incinerated samples were analysed for mineral contents (Ca, Fe, K, Mg, Mn, Na, P, Zn, and Cu) by using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo electron X7 inductively coupled plasma mass spectrometry, Model X series, Oxford, UK). For the calibration of the IPC-MS for mineral analysis, standards were diluted and used. The ICP-MS operating conditions were as follows: nebulizer gas flow, 0.91 L/min, radio frequency (RF), 1200 W, lens voltage, 1.6 V, cold gas, 13.0 L/min, auxiliary gas, 0.70 L/min (Peñalver et al., 2022). Results were expressed as mg/100 g of sample.

2.2.6. Volatile Organic Compound Determination

The volatile organic compound (VOC) extraction was carried out using a solid phase microextraction (SPME) device (Supelco, Bellefonte, PA, USA), including a 10 μm length fused silica fibre coated with a layer (50/30 μm thickness) of divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS), following the procedure described by Echegaray et al. (2020). Briefly, 1 g of minced mozzarella cheese was weighted in a 24 mL glass vial closed with a screw cap equipped with a laminated Teflon–rubber disc. The samples were kept at 35 °C for 15 min, allowing for the equilibration of VOC in the headspace. Then, the fibre, already conditioned at 270 °C for 60 min via heating in a gas chromatograph injection part, was inserted in the headspace of the vials through the septum and exposed to the headspace for 30 min at 35 °C. The splitless mode was used to inject the samples. Helium was used as carrier gas , with a linear velocity of 40 cm s−1. The total run time of 49 min and 30 s was divided as follows: 10 min at 40 °C (isothermal), and then increased to 20 min at 0°C (5 °C/min), and finally, 250 °C (20 °C/min), maintained for 5 min. Both the injector and detector temperatures were set at 260 °C. The gas chromatograph used for the VOC detection was 6890 N (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-624 capillary column (30 m, 0.25 mm i.d., 1.4 μm film thickness, J&W Scientific, Folsom, CA, USA) and coupled to a mass selective detector, 5973 N (Agilent Technologies). VOCs (expressed as area units (AUs) × 104 per g of product) were identified by comparing the detected mass spectra with those reported in the NIST05 library (National Institute of Standards and Technology, Gaithersburg, MD, USA) (matching was considered when coincidence was greater than 80%), and/or by comparing the mass spectra and retention times with the authentic standards (Supelco, Bellefonte, PA, USA), and/or by calculating the retention index against a set of standard alkanes (C5-C14) (for calculating Kovats indices, Supelco 44585-U, Bellefonte, PA, USA) and by comparing them with the data reported in the literature.

2.2.7. Colour Evaluation

The colour analysis of mozzarella cheese was performed by implementing a Computer Vision System (CVS), following the method by Girolami et al. (2013, 2014), on the same batch of samples used for the sensory analysis (QDA) before tasting and for each cheese-making session. Briefly, each mozzarella slice was cut in two, one half was used in order to evaluate the inner colour, and the other half was used to evaluate the outer colour of the sample. The images were captured with a digital cam-era (CANON EOS 450D, 12.2 Megapixel, Canon Inc., Tokyo, Japan) at a distance of 30 cm, in a black wooden box, under four fluorescent lamps at 50 cm from the sample and angled at 45° to ensure uniform light intensity on the sample. The camera was connected to an NEC MultySync LCD monitor with sRGB colour space (standard

RGB) and a resolution of 1600 × 1200 pixels. The monitor was calibrated, and the ICC monitor profile was created using the Eye-One Match 3.2 software. The rendering intent used was of perceptive type. Colour evaluation was performed using Adobe Photoshop CS3 software. RGB images were transformed into L*, a*, and b* values after acquisition via RAW photographs. Particularly, three areas of the digital im-age of each sample were spotted by the cursor, and the colorimetric characteristics were measured.

2.3. Sensory Analyses

Sensory analyses included sensory profile, assessed via quantitative descriptive analysis (QDA) according to Murray et al. (2001), consumer liking, and willingness to pay (WTP).

2.3.1. Quantitative Descriptive Analysis (QDA)

To perform QDA, fifteen regular eaters of buffalo mozzarella cheese (i.e., consuming it at least once a week) were enrolled. Among them, ten panellists (5 males and 5 females between 24 and 32 years of age) were chosen according to their capacity to identify the 4 basic tastes (Albenzio et al., 2013), as recommended by ISO recommendations (ISO 8586, 2012). A labelled scale with intervals of intensity was used to train panellists on the use of the 100 mm unstructured intensity linear scale (0 = absent, 100 = very high) provided for QDA (Stone and Sidel, 2004). In a subsequent session, the assessors tasted the mozzarella cheese samples and, based on specific works in the literature (Uzun et al., 2018a, Uzun et al., 2018b), they developed and agreed on a vocabulary of attributes (about appearance, odour, taste, flavour, and texture) and their definitions (Table 3). Their subsequent training was per-formed in individual sensory booths (ISO 8589, 2007) by using a specific frame of reference (Braghieri et al., 2018) to identify the intensity ranges for low and high intensity for each attribute. Then, panellists tasted the samples again with the two preferences of intensity for each attribute in blind conditions. This training phase was essential to calibrate the performance of the panellists in terms of repeatability, discrimination, and agreement. For the test, two 50 g mozzarella cheese samples for C and S were administered in a randomized order at a temperature of 13 °C. Each sample was identified by a three-digit number code. To avoid the effect of appearance on the perception of odour/flavour, taste, and texture attributes, the assessors evaluated the intensities of these attributes of the first cheese under red light, while the intensities of the appearance parameters were assessed on the second cheese under white, fluorescent lighting. Each product was evaluated in 3 replications. The interval between consecutive samples was roughly 10 min for each product, assessors were recommended to drink a sip of water and to eat a piece of smith apple between two

consecutive tastings to reset the effect of the previous sample. The Smart Sensory box 2.3.5 platform was used to man-age the QDA sessions (Smart Sensory Solution, Sassari, Italy).

Table 3. List of attributes used by the 10-member trained panel for buffalo mozzarella cheese sensory profiling.

Attribute	Definition
Appearance	
Colour uniformity	Overall colour uniformity
Surface uniformity	Product surface free of holes and granules
Inner colour	Intensity of the cheese's inner colour (from white to ivory)
Brightness	Shininess or glossiness of the surface
Number of eyes	Average number of eyes in the cheese mass
Uniformity after cutting	Cut surface free of holes and granules
Buttermilk release	Amount of buttermilk released after cutting
Odor	
Milk	Odor arising from milk at room temperature
Butter	Odor arising from butter at room temperature
Yoghurt	Characteristic odour of plain whole yoghurt
Taste	
Saltiness	Fundamental taste associated with sodium chloride
Sweetness	Fundamental taste associated with sucrose
Bitterness	Fundamental taste associated with quinine
Sourness	Fundamental taste associated with citric acid
Flavour	
Fruity	Odor associated with fruits such as pineapple
Whey	Characteristic whey flavour
Texture	
Tenderness	Minimum force required to chew cheese sample, the lower the force, the greater the tenderness
Oiliness	Amount of oily/fatty feeling in the mouth during chewing
Moisture	Moisture released by the cheese in the mouth during early mastication
Graininess	Perception of particles (grains) in the mouth
Cohesiveness	Degree to which a cheese sample holds together or adheres itself after chewing
Screechiness	Friction of the product against the teeth
Shear consistency	Consistency of the sample during cutting

2.3.2. Consumer Test and Willingness to Pay

A total of 68 consumers (42 females and 26 males, from 18 to 60 years old) participated in the test. The reduced number of participants in the consumer trial (68 versus 100) was a consequence of the COVID-19 pandemic, which posed a challenge to recruitment. Consumers among regular eaters of buffalo mozzarella cheese (i.e., consuming this product at least once a week) and who were available over the study peri-od were recruited by phone. The study was conducted in agreement with the guide-lines of the Declaration of Helsinki and the Italian ethical requirements on research activities and personal data protection (D.L. 30.6.03 n. 196).

Firstly, they were asked to taste 50 g of mozzarella cheese from both groups (C and S) and rate their liking in blind conditions, that is, they did not receive information on the products (perceived liking, B). For each sample, consumers rated their overall liking and their liking for the appearance, taste/flavour, and texture using a 9-point hedonic scale, where the 9 categories ranged from "dislike extremely" to "like extremely", with a central point (5) corresponding to "neither liked or disliked" (Markey et al., 2017; Peryam et al., 1957).

On the same day, in the second test, the subjects received two sheets with information concerning the nutritional quality of mozzarella cheese, and they were asked to carefully read the information and give their liking expectation for that product without tasting the samples (expected liking, E). In detail, the information about the two products was as follows:

- Information for sample 1 —Mozzarella di bufala Campana produced with milk from conventional breeding. "Mozzarella di bufala Campana" is produced exclusively with fresh whole buffalo milk according to the specific disciplinary published in the Italian Official Gazette n. 258 of 6.11.2003. Mozzarella di bufala Campana is porcelain white in colour, has a characteristic and delicate flavour, a fat content of at least 52% (on the dry matter), and a maximum moisture content of 65%.
- Information for sample 2—Buffalo mozzarella produced with milk from conventional breeding and Spirulina dietary supplement. "Mozzarella di Bufala Campana" is produced exclusively with fresh whole buffalo milk according to the specific disciplinary published in the Italian Official Gazzette n. 258 of 6.11.2003. Mozzarella di bufala campana is porcelain white in colour, has a characteristic and delicate flavour, a fat content of at least 52% (on the dry matter), and a maximum moisture content of 65%. Dietary supplementation with Spirulina (a natural product arising from the freeze-drying of microalgae) makes it possible to obtain a mozzarella with antioxidant, anti-cholesterol, and immune-stimulating properties.

The third test was performed the day after. Consumers received both products (C and S), each accompanied by the corresponding information. They had to read the in-formation before tasting the sample and reporting their liking scores (actual liking, A).

For the QDA, tests were performed in a controlled sensory analysis laboratory (Serrapica et al., 2022) equipped with individual booths under white fluorescent lighting. Consumers were asked to clean their mouths with some water and to eat a piece of smith apple between each sample evaluation to try to make the palate conditions similar for each sample. Samples, identified by a 3-digit number code, were served in random order. The Smart Sensory box 2.3.5 platform was used to manage the consumer test sessions (Smart Sensory Solution, Sassari, Italy).

2.3.3. Vickrey Auction

After the third test, two 250 g packs of mozzarella (C and S) were auctioned off. A second-price Vickrey auction (Lange et al., 2002) was performed to assess consumers' WTP mozzarella cheese produced from buffalo milk fed with Spirulina supplementation, according to Napolitano et al. (2008). A short explanation of the procedure for the auction was given to the participants, and it was clarified that the proposed offers involved a commitment to purchase the product. Participants signed a consent agreement to the procedure and received EUR 10 (in cash). Then, they were formally trained on the use of Vickrey's second-price auction. Each consumer separately had to write, on a paper, the maximum price to pay for one unit of product evaluated. The winner (i.e., the consumer submitting the highest value) had to purchase the product not at the proposed price, but at the second highest offer submitted. Thus, one of the participants was allowed to purchase a product at an equal or lower price than what they would normally have agreed to pay. In the case of more consumers offering the same highest offer, only one of them, randomly chosen by another consumer, would be selected as the winner. It was also clarified that the aim of the auction was to understand the value that the product had for consumers, and not its commercial value. To verify that all participants correctly understood the procedure, some practice with snacks was carried out. At this point, the test was performed. To prevent the participants from becoming less motivated in winning more products, it was explained that the mozzarella cheese pack would be evaluated under different information conditions, and only one condition, randomly chosen by a consumer, would be used for the actual auction.

2.4. Statistical Analyses

All the data collected were subjected to a Kolmogorov–Smirnov normality test. The chemical composition (i.e., fat, protein, lactose, fatty acid profile, amino acid pro-file, minerals for both milk and mozzarella, and moisture, salt, VOC, and colour for mozzarella only) was analysed via one-way analysis of variance (ANOVA) (GLM, general linear model), with group as a factor.

To verify panel performance, sensory data from the QDA test were subjected to a "Fixed" ANOVA model with 2 levels as products, 10 levels as assessors, and 3 levels

as replications and their interaction (products x assessors, products x replications, and assessors x replications). To evaluate panel performance, we analysed the significance of replication effects and the assessors x products and assessors x replications interactions. The analysis of the product effect enabled us to evaluate the significant differences between the products in terms of the perceived intensity for each attribute listed in Table 3 , and the perceived attributes' intensity differences. Consumers' liking data were analysed via one-way ANOVA (GLM) to identify the most liked product. Student's paired t-test was used to evaluate differences between mean scores obtained for the two products and for the same products under different conditions (tasting only, information only, and tasting with information). Data collected for the validation of information were subjected to analysis of variance with type of information as a factor. All the statistical analyses were performed by using SAS software (2009).

3. Results and Discussion

3.1. Spirulina Effects on Mozzarella Cheese's Chemical Composition

The milk composition was affected by Spirulina supplementation only in terms of protein, lowering the percentage from 4.59 to 4.31 (SEM 0.07, $p < 0.05$), while the fat and lactose contents were not influenced by Spirulina inclusion in the buffalo diets. The same trend was observed in the mozzarella cheese's chemical composition, the fat, lactose, and moisture contents were not different among the two groups, while the protein amount was tendentially higher in the C group ((18.01 vs. 17.12 ± 0.33%, $p < 0.10$ (mean ± SEM)) than the S group. Our results do not agree with the increases in protein, fat, and lactose found by Šimkus et al. (2007) as effects of Spirulina supplementation in cows, and the findings of Boeckaert et al. (2008) and Stamey et al. (2012), who registered an increase in milk fat. Despite the small differences in the protein content, the AA profiles of the mozzarella cheese samples did not change with Spirulina supplementation (Table 4). Among the EAAs, LEU and LYS are the most abundant in both products, tending to be higher in the S samples, which could be due to the AA profile of the added Spirulina. Microalgae in general are good sources of EAAs. The milk from the S group contained 27% more EAAs and 23% more NEAAs than the C group (data not shown). The presence of EAAs in milk and dairy products, especially branched-chain amino acids, is important in terms of their benefits for human health (Nie et al., 2022). Recently, Cacciola et al. (2022, 2023) reported interesting results about a possible beneficial effect of delactosed buffalo milk whey by-product on colorectal human carcinogenesis. It is well known that primary proteolysis occurs during mozzarella processing (Petrella et al., 2015), and the effect of the composition of milk protein fractions (relative contents of alpha S1 casein and beta casein) on milk coagulation properties has been widely studied (Bonfanti et al., 2013a, Bonfanti et al., 2013b).

Table 4. Amino acid profiles of mozzarella cheese from control (C) and Spirulina (S) groups.

Amino Acid (mg/100 g of Sample)		Group		SEM
Abbreviation	*Common Name*	C	S	
Total EAA		6031.10	7040.75	736.86
His	Histidine	388.00	448.76	46.68
Arg	Arginine	552.39	633.09	55.73
Thr	Threonine	520.98	608.85	60.38
Val	Valine	793.33	931.31	103.68
Met	Methionine	278.41	333.20	48.91
Lys	Lysine	1039.48	1226.28	134.79
Ile	Isoleucine	645.86	753.29	83.53
Leu	Leucine	1176.33	1373.75	155.64
Phe	Phenylalanine	636.33	732.23	79.97
Total NEAA		7150.25	8360.97	930.26
Asp	Aspartic acid	857.74	1015.54	114.24
Ser	Serine	742.36	873.18	94.15
Glu	Glutamic acid	2868.14	3361.24	389.88
Gly	Glycine	255.70	297.84	33.40
Ala	Alanine	364.21	424.53	43.46
Pro	Proline	1305.20	1520.22	171.65
Tyr	Tyrosine	733.81	844.68	85.86
Cys	Cysteine	23.09	23.74	6.92

SEM, Standard Error of the Mean, EAA, Essential Amino Acid, NEAA, Not Essential Amino Acid.

Table 5 shows no significant differences in the FA profiles of the mozzarella cheese samples between the C and S groups, nor in the calculated nutritional index. Manzocchi et al. (2020) did not observe any significant differences in the dairy cow milk FA content when substituting 5% of soybean meal with Spirulina in their diets, except for the total n-6 FA, which was higher in the control milk samples. This highlights that the diet supplemented with Spirulina lowered the apparent transfer efficiency of the total n-6 FA. In contrast, numerous authors have reported that replacing feed with Spirulina yields a significant improvement in the FA profile of ruminants' milk. Christodoulou et al. (2023) examined the effects of three levels of Spirulina substitution (i.e., 5%, 10%, and 15%) in the diets of ewes. Their findings revealed that only the highest level of substitution increased the PUFA and n-3 PUFA contents, while a mere 5% of substitution was adequate to decrease the AI. Christaki et al. (2012) added 40 g of powdered Spirulina to cow diets and observed a decrease in the SFA amount, while the MUFA and PUFA contents in the milk increased at the 45th day of experimentation. As stated by many authors, species and breeds strongly affect the animals' responses to diets (Orzuna-Orzuna et al., 2023, Altomonte et al., 2018). This might partially explain why the buffalo mozzarella cheese chemical

compositions did not differ among the two groups in our study. Moreover, the amount of Spirulina included in the S diets for the buffaloes could be too low to see changes/improvements in the milk and cheese compositions. To date, most of the studies in which microalgae were tested for animal feeding showed inconsistent and contradictory effects, probably due to the high variability among the studies, the doses of microalgae and species, the experimental period, and the percentage of forage in the diet (Orzuna-Orzuna et al., 2023). However, microalgae use in animal feeding originated with the objective of improving the nutritional quality, particularly in terms of the FA profile, of milk and dairy products (Altomonte et al., 2018).

Table 5. Fatty acid (FA) profiles (% on total FAs) and nutritional index of mozzarella cheese from control (C) and Spirulina (S) groups.

Fatty Acids		**Group**		**SEM**
Abbreviation	***Common Name***	**C**	**S**	
C4:0	Butiric acid	2.74	2.76	0.034
C6:0	Caproic acid	1.77	1.79	0.024
C8:0	Caprylic acid	1.05	1.05	0.016
C10:0	Capric acid	2.09	2.10	0.029
C12:0	Lauric acid	2.60	2.61	0.035
C14:0	Myristic acid	11.50	11.63	0.138
C15:0	Pentadecylic acid	1.07	1.04	0.019
C16:0	Palmitic acid	35.02	35.31	0.397
C16:1 n-7	Palmitoleic acid	1.72	1.65	0.062
C18:0	Stearic acid	12.76	12.59	0.169
C18:1 trans 9	Elaidic acid	0.63	0.64	0.008
C18:1 trans 11	Transvaccenic trans-11-Octadecenoic acid	1.62	1.59	0.037
C18:1 n-9	Oleic acid	19.77	19.61	0.236
C18:2 n-6	Linoleic acid	1.85	1.90	0.058
C18:3 n-6	γ-Linolenic acid	0.22	0.23	0.014
C18:2 c9-t11 CLA	Linolenic acid isomer	0.66	0.64	0.015
C20:0	Eicosanoic acid	0.23	0.22	0.004
oth	others	0.14	0.14	
SFA	Saturated FA	71.66	71.92	0.313
MUFA	Monounsaturated FA	25.09	24.80	0.266
PUFA	Polyunsaturated FA	3.25	3.29	0.102
n-3		0.448	0.445	0.023
n-6		2.15	2.21	0.069
Nutritional Index				
UFA/SFA		0.395	0.390	0.007
PUFA/SFA		0.046	0.046	0.002

n-6/n-3		4.840	4.985	0.161
AI	Atherogenic index	2.953	3.010	0.058
TI	Thrombogenic index	3.910	3.965	0.069
h/H	Hipocholesterolemic/hypercholesterolemic ratio	0.468	0.463	0.010

SEM, Standard Error of the Mean.

The mozzarella cheese's mineral content was not affected by the diets, as shown in Table 6. As it is well known, the major mineral that is present in buffalo milk, and thus in buffalo mozzarella cheese, is calcium, while the second most predominant mineral is phosphorus. Gulzar et al. (2019) reported that the mineral content is influenced by the cheese process, with the ash content reducing proportionally as the milling pH decreases. They explained that acid development solubilizes micellar calcium phosphate, leading to mineral expulsion in whey. Specifically, the calcium levels decrease in line with the milling pH reduction, although there is uncertainty about the influence of this cheese-making step on the potassium and sodium levels. The buffalo milk mineral content is subject to variation due to several factors, including the season, environment, diet, stage of lactation, animal breed, and genetics (Garau et al., 2021). The Mediterranean buffalo has been reported to possess the highest magnesium content in milk, whilst the greatest amount of minerals found in buffalo milk occurred during the summer season (Patiño et al., 2007). In our study, it was found that the mineral content was consistent across the samples because both groups were homogeneous, consisting of the same breed, and were fed the same diet. Therefore, the inclusion of Spirulina powder did not affect the mineral percentage in the buffalo mozzarella cheese.

Table 6. Mineral contents of mozzarella cheese from control (C) and Spirulina (S) groups.

Mineral (mg/100 g of Sample)		**Group**		**SEM**
Abbreviation	***Common Name***	**C**	**S**	
Ca	Calcium	248.093	248.228	25.388
Fe	Iron	0.323	0.285	0.037
K	Potassium	32.435	25.570	5.371
Mg	Magnesium	13.933	12.558	0.788
Mn	Manganese	19.880	16.365	4.724
Na	Sodium	93.263	101.718	10.848
P	Phosphorus	188.253	185.245	14.608
Zn	Zinc	1.773	1.615	0.224
Cu	Copper	0.143	0.095	0.020

SEM, Standard Error of the Mean.

The results of the analysis on VOCs can be found in Table 7. In total, 94 compounds were identified, and the most prevalent class in both groups is "others". This group

covers all of the individual VOCs that do not belong to the main classes considered in this study. Although the less abundant classes are the same in both samples, including esters, aldehydes, halogenated hydrocarbons, sulphur, and nitrogen compounds (from highest to lowest), the most abundant VOC classes are different between the two groups. In the C samples, the most and least frequent classes are alcohols, ketones, hydrocarbons, and aromatic hydrocarbons, in that order. In contrast, in the S samples, the most frequent classes are hydrocarbons, alcohols, aromatic hydrocarbons, and ketones, in that order. The only class that significantly differs from the others is aromatic hydrocarbons, which are higher in the C samples than the S samples, despite being ranked fifth on the list of VOC classes for the C group (in descending order) and fourth for the S group. However, the individual VOCs that were found to be significantly different (only 4% of the VOCs detected) do not belong to this class. Specifically, toluene ($p < 0.05$), isobutyl acetate ($p < 0.05$), and acetic acid 2-phenylethyl ester ($p < 0.01$) were found at higher levels in product C, while 1 Pentanol ($p < 0.01$) was detected at lower levels ($p < 0.01$) (Table 7). Unlike the study by Sabia et al. (2020), where the most prevalent VOC class was ketones, particularly in the cheese from buffalos that were fed wrapped ryegrass silage, our study found that ketones ranked third and fifth in the VOC lists of the C and S groups, respectively. Moreover, the level of ketones was higher in the C samples compared to the S samples (though not significantly). This is similar to the study by Sacchi et al. (2020), where ketones were more abundant in the control sample than the experimental ones. Ketones are common in many dairy products, and their origin can be attributed to SFA β-oxidation. Some studies suggest that these compounds are derived from animal feeds, and silage is the primary source of ketones (Sacchi et al., 2020). Further, animals consuming silage-based diets may result in the production of alcohols (Stefanon et al., 2004). There are numerous metabolic pathways implicated in the alcohol biosynthesis found in cheese. These pathways include the reduction of methyl ketones and aldehydes, the degradation of linoleic and linolenic acids, as well as AA and lactose metabolism. Table 7 shows that the alcohol class is higher in the C samples in comparison to the S samples, although not significantly different, and they are the same as the ketones levels. Our study involved two groups of animals that were fed the same diet, with the same amount of silage. Therefore, the variations in the levels of ketones and alcohols could be attributed to the addition of Spirulina, even in small quantities. Although there is no significant difference in the levels of halogenated hydrocarbons between the two groups, it should be noted that the level of this class is four times greater in the C samples than in the S samples. The mozzarella cheese production process impacts both the quantity and quality of the VOC composition. High temperatures during stretching can cause compound loss, while microflora activity during curd ripening can generate new compounds. Additionally, enzymes and milk bacteria are involved in the development of the cheese's flavour. The aroma

of cheese is also the result of the interaction of volatile and non-volatile chemical compounds, mainly concentrated in the water-soluble fraction, resulting from the transformation of the primary components (fat, proteins, and carbohydrates) and the action of bacteria present during milk processing. Therefore, understanding the production process is crucial to regulate the VOC composition of mozzarella cheese (Sacchi et al., 2020). However, the flavour of mozzarella cheese is not determined by the quantity of a particular compound/VOC, but by the equilibrium of all of the VOCs present.

Table 7. Individual VOCs and class levels expressed as area unit ((AU) × 10^4/g of product) in mozzarella cheese from control (C) and Spirulina (S) groups.

VOCs	RT	Match Factor	MZ	Group		SEM	*p*
				C	S		
Aldehyde				201.38	128.30	44.87	0.2934
Propanal, 2-methyl-	4.4	93.6	72	4.44	1.17	1.98	0.2872
Pentanal	12.1	95.2	58	9.59	2.58	2.23	0.0684
Hexanal	18.5	98.5	56	61.62	53.06	19.68	0.7690
Benzeneacetaldehyde	30.2	85.4	91	17.49	0.71	10.77	0.3130
Heptanal	23.5	96.5	70	5.27	6.61	2.18	0.6784
Benzaldehyde	26.7	98.7	106	44.87	59.03	21.52	0.6581
Butanal, 3-methyl-	8.9	97.2	58	44.37	2.30	18.28	0.1547
Butanal, 2-methyl-	9.5	94.0	58	13.73	2.84	8.11	0.3791
Ketone				451.35	239.79	169.09	0.4104
2,3-Pentanedione	12.5	90.9	100	3.94	4.78	1.59	0.7216
Cyclobutanone, 2,2,3-trimethyl-	14.7	92.1	55	3.33	1.50	1.10	0.2816
2-Heptanone	23.2	97.9	58	24.23	57.56	26.07	0.4008
2-Butanone	6.0	91.5	72	2.22	4.11	1.00	0.2296
Acetoin	14.9	96.2	45	406.57	158.01	155.26	0.3008
2-Pentanone	11.8	95.1	86	3.82	4.36	1.49	0.8083
Cyclohexanone	23.9	87.7	98	0.47	0.40	0.18	0.8010
2-Hydroxy-3-pentanone	20.3	90.4	45	3.93	2.99	1.09	0.5653
2-Octanone	27.4	90.6	58	0.19	0.48	0.16	0.2730
2-Nonanone	31.2	95.8	58	3.66	5.60	1.51	0.3988
Alcohol				498.54	303.11	232.63	0.5742
1-Propanol, 2-methyl-	8.4	96.2	43	14.25	6.73	8.70	0.5634
1-Pentanol	17.6	97.9	55	12.16	22.64	1.76	0.0057
1-Hexanol	22.6	98.1	56	15.31	39.64	12.49	0.2174
2-Heptanol	23.7	88.8	45	2.75	2.34	1.46	0.8489
Phenylethyl Alcohol	33.1	86.3	91	81.30	1.66	45.33	0.2702
Isopropyl Alcohol	10.8	86.1	45	10.58	32.60	16.28	0.3758
1-Hexanol, 4-methyl-	26.8	93.4	70	0.76	1.76	0.30	0.0563

2-Ethyl-1-hexanol	29.0	87.9	57	2.10	2.02	0.31	0.8595
Cyclobutanol	1.9	85.8	44	96.12	124.01	51.81	0.7166
1-Propanol	5.0	94.3	59	0.86	0.82	0.29	0.9331
1-Butanol, 3-methyl-	15.8	99.0	55	249.04	49.89	108.72	0.2428
1-Butanol, 2-methyl-	16.0	98.3	57	33.08	17.84	16.38	0.5351
1-Heptanol	26.8	89.8	55	0.59	1.17	0.22	0.1058
Ester				243.88	157.26	57.08	0.3245
Butanoic acid, ethyl ester	18.0	96.6	88	18.17	7.25	10.01	0.4699
Acetic acid, butyl ester	18.8	88.2	56	45.54	36.38	22.32	0.7815
1-Butanol, 3-methyl-, acetate	21.9	97.1	55	11.87	10.54	8.25	0.9129
Hexanoic acid, ethyl ester	27.0	96.1	88	15.86	4.17	11.11	0.4846
Sulphuric acid dibutyl ester	4.1	88.2	56	42.08	48.27	15.07	0.7813
$CH_3C(O)OCH(CH_3)C(O)CH_3$ (Acetoin acetate)	24.1	90.5	87	0.31	0.52	0.30	0.6337
n-Caproic acid vinyl ester	27.0	87.3	99	10.96	5.03	6.35	0.5337
Propanoic acid, pentyl ester	30.7	85.9	70	0.43	0.42	0.10	0.9127
Acetic acid ethenyl ester	5.8	96.3	86	35.55	21.50	11.36	0.4156
Ethyl Acetate	6.2	98.1	70	39.18	11.96	21.20	0.3987
Propanoic acid, ethyl ester	12.5	97.7	57	17.04	7.96	7.80	0.4419
n-Propyl acetate	12.8	97.4	61	1.31	1.86	0.86	0.6696
Isobutyl acetate	16.6	93.8	73	2.31	0.42	0.51	0.0471
1-Butanol, 2-methyl-, acetate	22.0	89.0	70	2.93	0.87	1.63	0.4077
Acetic acid, 2-phenylethyl ester	36.9	90.2	104	3.71	0.15	0.17	0.0048
Aromatic hydrocarbons				308.86	244.99	17.46	0.0414
D-Limonene	27.7	98.2	93	28.32	25.51	4.65	0.6841
Styrene	22.5	92.1	104	5.86	5.28	0.79	0.6187
Toluene	15.6	99.0	91	253.91	195.22	16.29	0.0436
Ethylbenzene	20.8	98.6	91	6.71	6.15	0.85	0.6537
Benzene, 1,3-dimethyl-	21.2	99.2	91	14.05	12.84	1.60	0.6118
Hydrocarbons				426.30	330.39	73.77	0.3934
Hexane, 2,2-dimethyl-	8.7	97.1	57	16.22	15.32	2.38	0.7976
Dodecane	33.3	98.5	57	56.37	32.22	7.55	0.0644
Cyclopentane	3.8	92.3	55	2.24	2.43	0.49	0.8032
Heptane	9.5	97.4	100	7.28	10.65	4.79	0.6361
Undecane	29.8	97.3	85	6.47	3.98	1.26	0.2131
n-Hexane	4.5	98.8	57	80.41	101.17	25.07	0.5796
Heptane, 2,2-dimethyl-	25.9	91.2	57	6.21	3.16	1.95	0.3107
Undecane, 2,8-dimethyl-	29.6	87.6	71	0.50	1.63	0.64	0.2670
Pentane, 3,3-diethyl-	30.7	86.6	57	0.48	0.53	0.18	0.8647
Tridecane	36.5	98.3	57	31.39	18.24	4.34	0.0761
Heptane, 4-methyl-	14.4	96.6	70	5.36	5.10	3.19	0.9554

Cyclopentane, 1-ethyl-2-methyl-	16.1	89.2	55	106.15	7.85	65.74	0.3311
Octane	16.4	95.7	85	6.89	7.10	1.20	0.9030
Heptane, 2,3-dimethyl-	19.6	92.1	84	0.55	0.81	0.21	0.4062
Octane, 4-methyl-	19.9	97.3	85	3.29	3.45	1.99	0.9558
Decane	26.0	94.3	57	5.52	4.34	1.35	0.5606
Decane, 4-methyl-	26.4	91.9	71	2.19	2.38	1.25	0.9184
Nonane, 2,6-dimethyl-	26.6	91.0	71	2.26	2.66	1.38	0.8474
Decane, 2,4-dimethyl-	28.3	91.6	85	1.36	1.90	0.38	0.3632
3-Ethyl-3-methylheptane	28.6	90.0	71	0.41	0.62	0.28	0.6143
Undecane, 4,7-dimethyl-	30.0	89.3	71	1.87	2.72	0.62	0.3740
Pentane, 2-methyl-	3.7	91.5	71	2.77	2.29	0.27	0.2552
Pentane, 3-methyl-	4.1	96.2	57	80.10	100.40	24.93	0.5856
Halogenated hydrocarbons				141.86	32.90	81.34	0.3801
Trichloromethane	6.8	97.8	83	141.58	32.17	81.24	0.3777
Pentane, 3-bromo-	29.1	87.6	71	0.29	0.73	0.43	0.4955
Nitrogen compounds				10.50	8.41	1.74	0.4297
2-Propen-1-amine	25.9	91.2	57	6.21	3.29	1.94	0.3288
Diazene, dimethyl-	3.1	95.7	58	4.29	5.12	1.43	0.6931
Sulphur compounds				21.77	17.44	9.23	0.7515
Disulfide, dimethyl	14.5	96.2	94	18.89	15.26	8.44	0.7711
Dimethyl trisulfide	26.2	93.2	126	2.08	1.78	0.78	0.7956
Dimethyl sulfide	3.2	97.8	62	0.80	0.40	0.51	0.6018
Others				529.14	571.37	159.96	0.8581
Dimethyl ether	2.7	92.4	45	157.64	131.45	92.96	0.8487
Methylene chloride	3.7	93.7	84	3.82	3.54	0.31	0.5382
1,3-Dioxolane, 2,4,5-trimethyl-	13.1	91.6	101	2.59	3.60	0.63	0.2991
Dimethylphosphinic fluoride	10.9	85.4	81	2.12	3.27	0.37	0.0665
Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	23.6	93.0	93	11.40	11.13	1.17	0.8762
Methane, trimethoxy-	6.0	87.4	75	22.01	20.55	3.09	0.7500
1-Hexene	4.4	92.0	56	42.20	52.79	13.13	0.5891
2,4-Dimethyl-1-heptene	19.0	97.6	70	8.14	7.37	4.59	0.9091
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	23.6	90.6	93	11.40	11.13	1.17	0.8762
Ethoxyacetylene	33.7	95.9	70	1.07	0.81	0.18	0.3384
Silanediol, dimethyl-	17.4	98.6	77	260.02	320.82	109.84	0.7090
tert-Butyl Hydroperoxide	19.9	93.1	59	6.04	4.40	1.90	0.5656
Butanal, 3,3-dimethyl-2-oxo-, hemihydrate	34.0	91.5	85	0.69	0.51	0.12	0.3245

SEM, Standard Error of the Mean.

The results about the instrumental colour of mozzarella showed that Spirulina integration was responsible of the higher values of the L*, a*, and b* indices in both the external and internal surfaces (Table 8). It is likely that the blue phycocyanin, one of the two pigmented antioxidants found in Spirulina, may have contributed to an increased lightness with a blue tint. However, it is worth noting that Park et al. (2018) did not find a significant correlation between the L* value, pigment content, or antioxidant activity of Spirulina. If this finding is confirmed in future studies, it would be a favourable outcome, considering the high demand for porcelain white mozzarella among consumers.

A correct instrumental measurement of colour is useful at the stage of analysing consumer preferences for the research, development, and improvement of cheese-making methods. When observed under a light source, PDO buffalo mozzarella cheese should have a porcelain white colour and a glowing appearance. The white colour is given by casein micelles, while the yellowish hues are imparted by carotenoids in green fodder, which are not absorbed in the cattle species and are therefore released in the milk. In contrast, buffaloes, sheep, and goats are able to assimilate and transform these compounds, so the colour of their milk tends to remain white.

Table 8. Values (mean ± S.E.) of CIE L*, a*, and b* of the external and internal surfaces of mozzarella cheese from control (C) and Spirulina (S) groups.

	External Surface			**Internal Surface**		
	Group		***p***	**Group**		***p***
	S	**C**		**S**	**C**	
L*	93.43 ± 0.02	92.91 ± 0.04	<0.0001	93.13 ± 0.06	92.80 ± 0.15	0.0410
a*	−2.26 ± 0.03	−2.13 ± 0.03	<0.0001	−3.42 ± 0.03	−3.13 ± 0.02	<0.0001
b*	0.62 ± 0.07	0.41 ± 0.01	0.004	4.14 ± 0.05	3.39 ± 0.05	<0.0001

3.2. Spirulina Effects on Mozzarella Cheese's Sensory Quality

As for the QDA, no significant product x replication or product x assessor interactions were observed, suggesting that the training program and the reference frame used in this study were efficacious in ensuring the high reliability of the panel (i.e., products were not evaluated differently in different replications or by different assessors).

Figure 1 shows a significant diet effect on the sensory profiles of mozzarella cheeses. The samples from the S group were evaluated as being brighter ($p < 0.05$) with a lower white inner colour ($p < 0.01$) than the C group. The S mozzarella cheeses were perceived with a higher butter odour ($p < 0.01$), a higher whey flavour, and a sweeter and more bitter taste than the samples from the C group ($p < 0.05$). Furthermore, the panellists perceived higher oiliness ($p < 0.01$) and moisture ($p < 0.0001$) intensities in the S samples with a greater milk release when cutting ($p < 0.001$) and a tendentially

higher tenderness ($p < 0.10$) than in the mozzarella cheeses from the C group, which, on the contrary, were perceived as being more grainy, cohesive, screechy, and having a greater consistency when cutting than the S mozzarella cheeses ($p < 0.01$).

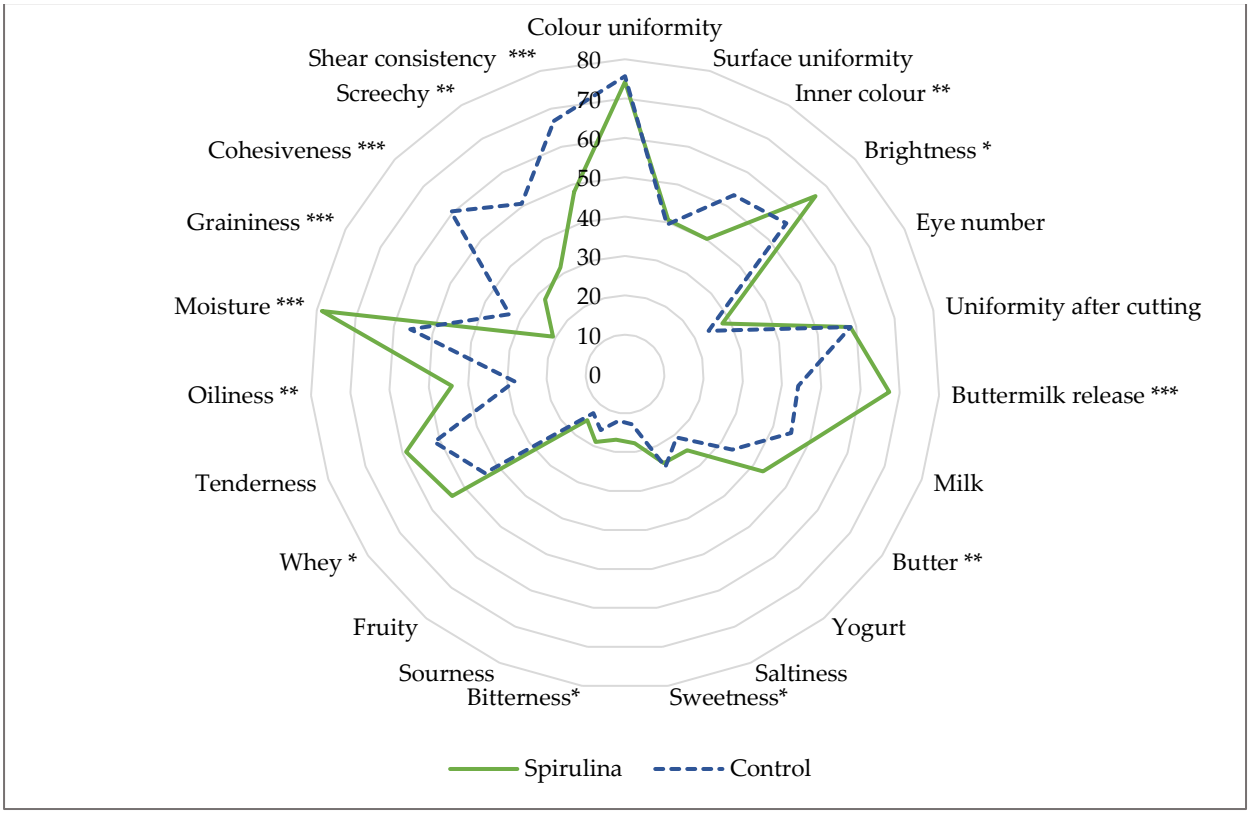

Figure 1. Spider plot of the sensory profile (QDA) of mozzarella cheese from Control (C) and Spirulina (S) groups (*p <0.05, **p <0.01, ***p <0.001).

In a recent study on buffalo mozzarella cheese (Uzun et al., 2018a), changes in texture were attributed, at least in part, to the corresponding fatty acid composition, which was lower in saturated fatty acids, such as C14:0, and richer in unsaturated fatty acids, such as C18:1, in products obtained from animals that were fed fresh fodder. The lower melting point of unsaturated fatty acids can produce softer cheeses. Furthermore, some attributes (e.g., bitter taste, flakiness, and grainy texture) may be influenced by more intense proteolysis that occurs in the summer, which, in turn, may contribute to the softening of the mozzarella and may have a direct effect on the flavour through the production of short-chain peptides and amino acids (McSweeney, 2004).

The greater oiliness could also be due to a different acid composition in the fat. For example, in Friesian cattle, Christaki et al. (2012), following the administration of 40 g/head/d of Spirulina, observed a reduction in the content of saturated fatty acids in milk, with a consequent increase in MUFA and PUFA compared to the control. The sensory characteristics of fresh mozzarella largely rely on the raw materials and production techniques used. Further investigation is required to evaluate the influence

of diverse components, including VOCs, AAs, FAs, and peptides, on each sensory attribute.

The consumers in blind conditions (P) evaluated both samples from the C and S groups to be greater than the middle value (i.e., 5) (Table 9), meaning there were satisfactory sensory properties in both products. Moreover, the blind evaluation was not influenced by the animals' diet, nor by the ages and sexes of the consumers. No difference was observed between the two groups for expected (E) liking. Likewise, the actual (A) liking was only influenced by diet, with a higher rate for the S group samples (7.29) than the C group samples (6.85). Table 10 shows that for both groups, the expected liking was significantly higher than the blind evaluation ($p < 0.01$), which means there was a negative disconfirmation: consumers evaluate the mozzarella cheese to be lower than their expectations. There was no difference between the actual and perceived liking of the C group samples, which means that the information does not increase the product evaluation, on the contrary, for the S mozzarella cheese, the information strongly influenced the actual liking ($p < 0.001$), as it was close to the expected acceptability. This means that there was a complete assimilation, because the information given to the consumers brought them to similarly evaluate the samples in both the actual and expected conditions (i.e., with and without tasting the mozzarella cheese).

Table 9. Mean rating (given by the consumers) during the three hedonic tests of mozzarella cheese from control (C) and Spirulina (S) groups.

Acceptability	Group		*p*
	C	S	
Perceived (P)	6.81	6.43	0.0903
Expected (E)	7.44	7.07	0.1034
Actual (A)	6.85	7.29	0.0508
P-E	−0.63 *	−0.64 *	
A-P	0.04	0.86 **	
A-E	−0.59	0.22	

* $p < 0.05$, ** $p < 0.01$.

Many aspects of the product can be used by consumers to make their food choices. Grunert et al. (2000) identified four main dimensions of quality for dairy products: hedonism, health, convenience, and process. A few of them can be experienced before purchase (e.g., colour), while most of them can be perceived after purchase (e.g., sensory properties), or may never be perceived (e.g., healthiness and appearance ethical). The latter must be communicated to the consumer, as they are characteristics that cannot be perceived before nor after the purchase (Grunert et al., 2004). Therefore, regarding these characteristics, consumers are forced to develop expectations to guide their food choices. In particular, to develop expectations on product quality attributes,

consumers can use both intrinsic (e.g., holes in the cheese, colour of the external rind, etc.) and extrinsic (e.g., price) characteristics. However, consumers tend to rely mostly on extrinsic characteristics provided to them in the form of product information (Banović et al., 2009). In particular, ethical concerns, such as environmental pollution and animal welfare, are becoming increasingly important in the hierarchy of purchasing motivations for animal products. Blockhuis et al. (2003) highlighted that animal welfare is increasingly recognized as an important component of quality for consumers of animal products. Numerous studies have been conducted on the effect of information on food liking (Daillant et al., 1993; Aaron et al., 1994). For example, the effect of information relating to animal welfare on lamb (Napolitano et al., 2007a) and beef (Napolitano et al., 2007b) liking has been studied, as well as the effect of information relating to organic production on the acceptability of foods and beverages (Caporale and Monteleone, 2004; Kihlberg et al., 2005). All of these studies have shown that information-induced expectations can influence the perception of quality. Therefore, if expectations receive a positive disconfirmation (when the satisfaction score of the product tasted without external information is higher than expected) or a negative one (when the product is worse than expected), the assimilation model is generally applicable. According to this model, when external information is provided, the hedonic tests highlight a shift in acceptability towards expectations and reach different values from those obtained by tasting the same food without external information (Cardello and Sawyer, 1992). In both groups, the expected liking was significantly higher than the liking expressed in the blind conditions ($p < 0.01$), thus indicating that a negative disconfirmation occurred: the consumers found the mozzarella to be less pleasant than expected. In these conditions, generally, actual liking moves in the direction of expected liking for group C, no significant difference was observed between the actual and perceived liking. Thus, in this case, there was no assimilation, as the information did not improve the acceptability of the product (Table 10). Conversely, regarding the mozzarella cheese obtained from the S group, the information greatly improved the actual liking ($p < 0.001$), which moved in the direction of the expectations. According to Blokhuis et al. (2003), the perception of food quality is determined by the welfare of the animals producing that food together with the overall nature and safety of the final product. In particular, the assimilation in this case was complete, since no difference was observed between the actual liking (expressed by the consumers with both sensory stimuli and information available) and expected liking (expressed by the consumers with only the information available). The complete assimilation observed for the S product is probably due to the important role played by information in determining the actual liking of mozzarella with Spirulina. This information is able to respond to some of the main and most current consumer concerns, such as the nutraceutical properties of products and animal welfare.

Napolitano et al. (2007a) showed that consumers are influenced by animal welfare information and shift their WTP in the direction of their expectations. In particular, the discrepancy between the expected liking and the actual willingness to pay was not fully assimilated, indicating that it was also expressed in relation to other aspects (for example, the sensorial properties of the products). Experimental auctions are able to place consumers in real situations where they can show their true preferences. In particular, the second-price Vickrey's auction has been widely used to assess consumers' WTP for real goods, including food (Lange et al., 2002), and the values that consumers place on food safety (Hayes et al., 1995) and animal welfare (Napolitano et al., 2008). Under this specific type of auction, consumers are individually asked to submit a sealed bid corresponding to the highest price they would agree to pay for a particular product. The highest bidder (i.e., the winner), by paying the second highest price, has the opportunity to purchase a product at a price that is equal to or, more often, lower than the value they attribute to the product (Vickrey, 1961). Currently, consumers are not looking for the cheapest food, but the best quality/price ratio, i.e., the maximum benefit for what they are willing to spend (McInerney, 2004). In our study, it was observed that gender had no influence on the overall offer, while it was found that the consumers offered EUR 4.05 (16.20 EUR/kg) for a 250 g pack of Spirulina mozzarella cheese compared to EUR 3.52 (14.08 EUR/kg) for the control mozzarella cheese ($F1,128 = 8.73$, $p = 0.0074$).

4. Conclusions

The inclusion of 100 g of freeze-dried Spirulina per day in the diets of buffaloes does not alter the chemical composition of mozzarella cheese or the characteristics identified using chromatographic instruments, including the fatty acid profile or the classes of volatile organic compounds. Nevertheless, this small amount of supplementation affects the sensory characteristics of mozzarella cheese, as assessed by the panel of experts. The experimental mozzarella cheese was found to be brighter, sweeter, more bitter, juicier, more tender, and oilier, with higher buttermilk release, butter odour, and whey flavour than traditional buffalo mozzarella cheese. In addition, providing information about the nutraceutical properties of Spirulina and its possible beneficial effect on animal health to consumers can have a strong effect on their liking, as they rated the experimental mozzarella greater than the conventional one, and expressed a higher willingness to pay for the mozzarella cheese produced by the buffalo fed with Spirulina. Further studies with a higher level of Spirulina are necessary in order to confirm these results.

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Chapter 3

Nutritional quality and sensorial properties of breast meat from organically reared broilers fed with feed-grade hemp seed

Poster presented at 69th International Congress of Meat Science and Technology

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Abstract

The aim of this study was to investigate the effects of including hemp seed feed grade in the grower-finisher diet of organically reared broilers on carcass parameters, meat quality traits and sensory profile of breast fillets. One hundred and fifty 30-days-old male Kabir chickens were equally and randomly housed in 6 adjacent fences (10 m^2/bird) and, then, assigned to 3 dietary treatments (2 pens of 25 birds *per* treatment) consisting of a standard commercial grower-finisher diet (Control), and 85% and 70% of the same diet integrated with 15 (Hemp15) and 30% (Hemp30) of hemp seed, respectively. At slaughter, the chickens were individually weighed and 3 birds per pen were randomly selected, tagged, and used to determine carcass characteristics and meat quality. Proximate composition, Warner-Bratzler Shear Force (WBSF), pH, amino acid, fatty acid profiles, and sensory analysis were performed on breast fillets. Compared to the Control, the inclusion of the hemp seeds increased the live body weight of birds, but the differences reached the threshold of significance only for the highest level of inclusion. A similar trend was observed for the eviscerated carcass weight, although only a statistical tendency was observed. The dressing percentage and breast and thigh + drumstick yields were not different among treatments, suggesting a higher incidence of inner organs and inedible parts (e.g., head, neck, shanks, and abdominal fat) on carcass weight of the hemp fed birds. As for meat quality, drip and cooking loss, pH, proximate composition, amino acids profile and WBSF of breast meat were not different among the groups. By contrast, fatty acids profile was affected by the highest level of inclusion. The amount of SFA in H30 was significantly lower than in both Control and H15, especially palmitic and stearic acids, while the n-3 and n-6 PUFAs were significantly higher in H30 than Control group. The MUFA levels were not affected by the treatments. Also the sensory profile resulted affected by 30% hemp seed inclusion, presenting a higher overall flavour intensity, a lower fibrousness, gumminess and with lower amounts of residue in the mouth after swallowing. We conclude that dietary inclusion of HSC may represent a sustainable strategy to improve healthiness and hedonistic properties of broiler meat under organic farming conditions with no detrimental effect on yield and commercial carcass parameters.

Keywords: organic agriculture, medium-growing chickens, hemp, meat quality, sensory profile

1. Introduction

Currently, there is a growing consumers interest in meat products, with chicken being the most widely consumed type of meat globally. This is likely due to its mild taste, favourable texture, and overall nutritional profile, including its low-fat content.

Moreover, chicken consumption is not hindered by cultural prejudices, making it a popular choice. Therefore, the broiler chicken sector has become one of the most intensive farming systems globally, characterized by high stocking densities, indoor rearing, and fast-growing hybrids obtained through genetic selection that allow them to be slaughtered within 40 days of age. On the other hand, in the last decade many countries have experienced a rapid increase in organic animal production. This development is a response to the growing demand for food with specific quality attributes, such as nutritional value, product safety (free of hormones, antibiotics, and harmful chemicals), and environmentally sustainable farming practices that prioritize high animal welfare standards (Pellattiero et al., 2020).

Organic poultry production differs from conventional production as it aims to integrate livestock and crop production, creating a symbiotic relationship of recyclable and renewable resources within the farm system. In addition to livestock production, organic poultry producers must consider several other factors. Optimizing the health and performance of organic poultry requires careful attention to basic husbandry principles. This includes selecting appropriate breeds and strains, implementing proper management practices and nutrition, and avoiding overstocking (Blair, 2018). In particular, the organic system regulation recommends using indigenous breeds or strains with a slow or medium-growing profile. This takes into account the birds' vitality, resistance to diseases, and adaptability to local outdoor conditions (Sirri et al., 2011). Regulation EC 889/2008 sets the minimum age for organic farming at 81 days for fast-growing birds and 96 days for slow-growing birds in order to achieve a market live weight typical for this type of bird in Italy (Napolitano et al., 2013). This is approximately twice the age required for conventional meat birds.

To the organic poultry production is also required certification and verification of the production system, which requires the organic producer to keep sufficient records in order to maintain the identity of all organically raised poultry. As a result, in the eyes of consumers, organic food has a very strong brand image and should command a higher price in the marketplace than conventionally produced food (Blair, 2018).

In addition, all feed, including pasture and forage, must be organically produced and health treatments must be within the range of accepted organic practices. In this context, diets are designed to ensure quality production of the birds rather than maximize production, while meeting the nutritional needs of the flock at different stages of development. Like all animals, poultry require five components in their diets as sources of nutrients: energy, protein, minerals, vitamins and water. A deficiency or imbalance in any one nutrient will adversely affect performance. Poultry require a well-balanced and easily digestible diet for optimal egg and meat production and are very sensitive to diet quality because they grow rapidly and make relatively little use of fibrous, bulky feeds such as alfalfa hay or pasture because they are birds.

Cereal grains such as maize, barley, wheat and sorghum are the main components of poultry diets and typically provide 30-60% of the total AA requirement. Other protein sources, such as soybean meal and canola meal, must be provided to ensure adequate levels and balance of essential AA. The level of protein required to provide an adequate intake of essential AA depends on the used feeds (Blair, 2018).

Industrial hemp, also known as *Cannabis sativa L.*, is a versatile crop with potential uses in food, feed, fibre, bioenergy, medicine, and phytoremediation. The nutritional properties of hemp seeds may vary depending on factors such as cultivar, growing conditions, and geography. However, whole hemp seeds generally contain 20-30% protein, 25-35% oil, and 25-35% carbohydrates, as well as beneficial vitamins and minerals (Callaway, 2004, Ely and Fike, 2022).

Many authors (Tufarelli et al., 2023, Klir et al., 2019) reported that since 2011, the European Food Safety Authority (EFSA) panel on Additives and Products or Substances used in Animal Feed has approved the use of hempseed and hempseed cake as feed ingredients for all animal species, including poultry. However, the dietary inclusion rates vary among animal species.

To date, the maximum level of hemp seed or hemp by-product included in broilers diet is 20% with different results among the studies (Khan et al. 2009, Stastnik et al., 2015, Tufarelli et al., 2023). Additionally, in recent years, the use of industrial hemp as an alternative crop has rapidly expanded in hilly areas of southern Italy to mitigate the effects of cereal monoculture and differentiate farm income (Satriani et al., 2021). Accordingly, the availability of insect- and weather-damaged, culls, broken, and odd-size hemp seed lots has increased, providing local livestock producers the opportunity to exploit these poor-quality seeds for feed purpose.

This study examined the impact of two different levels of feed-grade hemp seeds inclusion (15% and 30%) in grower-finisher diets of organically medium-growing broilers on carcass parameters, as well as the quality and sensory profile of breast fillets.

2. Materials and methods

The study took place on a private organic poultry farm situated in the south of Italy (41°03’63’’ N, 14°42’14’’ E, 430 m a.s.l.) where medium-growing broilers were raised using a free-range system between May to September 2022. All the animals involved in our study were humanely treated according to the principles stated by the EC Directive 86/609/EEC (Council Directive, 2008) regarding the protection of animals used for experimental and other scientific purposes.

2.1. Experimental Design and Data Collection

One hundred and fifty 30-day-old male Kabir chickens were weighed individually (initial body weight of 0.75 ± 0.15 kg). The birds were then randomly housed in six adjacent pens (1 m^2 per bird) divided by mesh transects. Each pen had a concrete floor indoor area (0.15 m^2/bird) with free access to wire fenced open-air runs (3 m^2/bird). The indoor areas were equipped with circular galvanized feeders, plastic drinkers, perches (15 cm *per* bird). Upon immediate housing, the birds were assigned to three dietary treatments (two pens of 25 birds *per* treatment). The treatments consisted of a standard commercial grower-finisher diet (Control diet), and 85% and 70% of the same diet, respectively, integrated with 15% (Hemp15) and 30% (Hemp30) of feed-grade hemp seeds. Diets' ingredients and chemical composition are shown in Table 1. The diets, manually distributed once a day (08:00 a.m.), were presented in crumble form and provided for ad libitum consumption throughout the feeding trial. At 120 days of age, the chickens were individually weighed and 3 birds per pen were randomly selected, labelled, and slaughtered in an accredited abattoir by manual exsanguination, and then plucked, eviscerated and weighted again. After refrigeration at 4°C for 24 hours, cold carcasses were weighted and the drip loss was calculated as follow:

$$drip\ loss\ (\%) = \frac{hot\ carcass\ weight\ (g) - cold\ carcass\ weight(g)}{hot\ carcass\ weight(g)} * 100$$

while the dressing percentage were calculated after the head and feet remotion as follow:

$$dressing\ percentage\ (\%) = \frac{ready\ to\ cook\ carcass\ (g)}{live\ body\ weight\ (g)} * 100$$

Then breast fillets and thighs with drumsticks were removed from carcasses and individually weighted in order to calculate commercial cut yields. Afterwards, left breast fillets (*pectoralis major)* were used for pH, proximate composition, water holding capacity (WHC), Warner-Bratzler Shear Force (WBSF) amino acid and fatty acid profiles determination. The right breast fillets were used for sensory analysis.

Table 1. Ingredients and chemical compositions of the three experimental diets.

Ingredient, g/kg	*Diet*		
	C	H15	H30
Cracked corn	565.0	480.25	395.5
Soybean meal (48% CP)	175.0	148.75	122.5
Sunflower meal (38% CP)	75.0	63.75	52.5
Wheat grain	75.0	63.75	52.5
Wheat middling	60.0	51.0	42

Dicalcium phosphate	20.0	17.0	14.0
Soybean oil	10.0	8.5	7.0
Hemp seeds	-	150.0	300.0
Calcium carbonate	10.0	8.5	7.0
L-Lysine	2.0	1.7	1.4
Sodium chloride	2.0	1.7	1.4
Sodium bicarbonate	2.0	1.7	1.4
Mineral-Vitamin premix[2]	2.0	1.7	1.4
L-Threonine	1.0	0.85	0.70
DL-Methionine	1.0	0.85	0.70
Chemical composition (% DM)			
Dry matter	89.90	90.20	90.49
Crude protein	19.90	21.26	22.62
Ether extract	4.90	5.70	6.49
Ash	6.50	6.59	6.67
Metabolizable energy (MJ/kg)	12.0	12.15	12.3

C, Control diet, H15, diet integrated with 15% of hemp seed, H30, diet integrated with 30%. of hemp seed, [2]Supplementation per Kg: vitamin A 12.000 UI, vitamin E 10 mg, vitamin D 2.200 UI, niacin 35.0 mg, d-pantothenic acid 12 mg, riboflavin 3.63 mg, piroxydine 3.5 mg, thiamine 2.4 mg, folic acid 1.4 mg, biotin 0.15 mg, vitamin B 0.03 mg, Mn 60 mg, Zn 40 mg, Fe 1.280 mg, Cu 8 mg, I 0.3 mg, Se 0.2 mg.

2.2. Chemical analysis

Proximate composition of chicken breast fillets was assessed according to Echegaray et al. (2020). Before determining the chemical composition of the breast fillets, the pH was measured manually by using a portable pH-meter (Hanna Instruments, Eibar, Spain) equipped with a penetration electrode. Moisture content was determined following the ISO 1442(1997) recommended standard by recording the weight loss of 5 g of minced meat maintained in the oven (Memmert UFP 600, Schwabach, Germany) at 105 °C, until constant weight. Similarly, ash content was determined by weight loss of 3 g of minced breast meat maintained in a muffle furnace (Carbolite® RWF 12–13, Hope Valley, England) at 600° C until constant weight, according to ISO standards recommendation 936 (1998). Protein content was calculated following Kjeldahl method, that multiplicate 6.25 to the total nitrogen content of samples. Briefly, according to ISO 937 (1978) standard recommendation, 1 g of minced breast meat reacted with H_2SO_4 (sulfuric acid) in a digester (Gerhardt Kjeldahl KB20 Vapodest®, Königswinter, Germany) using cuprum sulphate as a catalyst. Then, in a distillation apparatus (Gerhardt® Vapodest 50 Carrousel, Königswinter, Germany) in alkaline conditions, the organic nitrogen was distilled into ammonium sulphate. Finally the freed ammonium was trapped in a boric acid solution and titrated by using an HCl

1N. The analysis of intramuscular fat (IFM) was performed according to the Approved Procedure Am 5-04 (AOCS, 2005). The IFM percentage was calculated by gravimetric difference, performing a liquid-solid extraction on minced breast meat samples (1 g per sample) by using an extractor apparatus (AnkomHCI Hydrolysis System XT10, Macedon NY, USA) and petroleum ether as solvent.

2.3. Water holding capacity and Warner-Bratzler Shear Force

The WHC was measured as cooking loss following the formula:

$$WHC\ (\%) = \frac{raw\ meat\ weight\ (g) - cooked\ meat\ weight\ (g)}{raw\ meat\ weight\ (g)} * 100$$

Briefly, breast cuts were weighted and individually packaged in plastic bags, then they were cooked in a water bath (JP Selecta, Precisdg, Barcelona,Spain) until the core of samples reached the temperature of 70° C. Afterwards, the samples were cooled at room temperature and weighted again. To test the shear force of breast fillets, the cooked samples were cut into seven pieces measuring 1 × 1 × 2.5 cm (height × width × length), parallel to the muscle fibre orientation, according to Marrone et al. (2020). The WBSF test was performed by using a dynamometer (Instron Mod. 5565) equipped with a V-shaped blade (load 500 kg, head speed 200 mm/min) and the shearing force was applied perpendicular to the muscle fibre direction. For each sample, the measured WBSF was expressed in Newton as mean value of the seven cuts.

2.4. Amino acid profile determination

The amino acid (AA) profiles of chicken breast meat were determined according to Vargas-Ramella et al. (2021). An HPLC system (Alliance 2695 model) equipped with a 2475 scanning fluorescence detector (Waters, Milford, MA, USA) was used. The system operation and results management were controlled by an Empower 2™ advanced software (Version 1.04.1037, Waters, Milford, MA, USA). The separations were performed using a Waters AccQ-Tag column (3.9 × 150 mm, with a 4 mm particle size) with a flow rate of 1.0 mL/min at 37 °C, while the detection was performed via fluorescence with excitation at 250 nm and emission at 395 nm. An external standard method employing an AA standard (Amino Acid Standard H, Thermo, Rockford, IL, USA) was used to quantify and identify (via retention time) the AAs. The chicken breast fillets AAs content were expressed as g/100 g of sample.

2.5. Fatty acid profile determination and nutritional indices calculation

The fatty acid (FA) profiles of chicken breast meat (10 g) were performed according to the procedure described by Vargas-Ramella et al. (2022). The FAs were transesterified and detected using a gas chromatograph Agilent 7890B (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID), a PAL RTC-120

autosampler (maintained at 250 °C and 64.2 mL/min of total flow rate), and a DB-23 fused silica capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies) for the separation of FAMEs (Fatty Acid Methyl Esters). A Supelco 37 Component FAME Mix (Sigma-Aldrich, St. Louis, MO, USA) was used as standard. The FAs content was expressed as a percentage of total methylated FA. The nutritional indices, i.e., Atherogenic index (AI), thrombogenic index (TI), and hypocholesterolemic/hypercholesterolemic ratio (h/H) were calculated as reported by Chen and Liu (2020).

2.6. Quantitative Descriptive Sensory analysis

As said previously, the right breast fillet of each slaughtered bird was used for the sensorial quality analysis. The breast fillets were cut in slices of approximately 1 cm thick and 80 g each, than grilled on an open electric griddle (Sirman PDR3000, Eurodib, USA) at 300° C for about 7 min. The meat was considered cooked when the core of the meat slice reached 72° C, assessed by inserting a thermocouple probe (Testo735-2, SE & Co. KGaA, Titisee-Neustadt, Germany) into the centre of each sample. The 3 products (80 g/ sample) were offered to 8 trained panellists (4 males, 4 females, aged between 25 and 45 years) immediately after cooking. The panellists were recruited to perform a quantitative descriptive analysis, following the ISO 11035 (1994) recommendations. The evaluations were performed in individual booths under red lighting, in a controlled laboratory of sensory analysis according to ISO 8589 (1988) recommendations. Firstly, panellists were asked to develop and then to agree to a consensus list of attributes and the respective definitions according to the previous literature (Napolitano et al, 2013, Semwogerere et al., 2019), as shown in Table 2. Panellists were also trained on the use of the unstructured scale, a 100 mm line anchored at the ends (0: absent and 100: very strong intensity). At each session, 3 samples were presented, coded with a 3-digit randomized number and randomly served according to sample, replicate, and panelist, for a total of 3 sessions.

Table 2. List of attributes used by the 8 trained panellists for chicken breast fillets sensory evaluation.

Attribute	Definition
Overall odour	Overall intensity of the odour
Overall flavour	Overall intensity of the flavour
Umami	Fundamental taste elicited by certain peptides and nucleotides
Bitter	Fundamental taste associated with quinine
Sweet	Fundamental taste associated with sucrose
Salty	Fundamental taste associated with sodium chloride
Chewiness	Easiness to chew the meat sample for swallowing

Juiciness	Amount of juice released in the mouth during mastication (small to high amount)
Fibrousness	Extent to which fibres strands are perceived on chewing (not to very fibrous)
Gumminess	Energy required to disintegrate a semi-solid food to a state ready for swallowing.
Residue	Amount of residue remaining in the mouth after swallowing (small to high amount)

2.7. Statistical analysis

All the parameters measured and the chemical composition of the chicken meat were previously subjected to Kolmogorov-Smirnov test for normal distribution of data, and then to an analysis of variance (ANOVA) using a mixed procedure of the SAS software (2009, SAS Institute Inc., Cary, NC), with the pen as a random factor. A Tukey test was used to compare the least square means. Significance and tendencies were discussed respectively at $P < 0.05$ and $P < 0.10$. Data from the sensory evaluation (QDA) of chicken breast fillets were subjected to an ANOVA using a GLM (general linear model) procedure of SAS, with product, replication, and their interaction as factors. To evaluate differences among mean scores obtained for the three treatments, a Student's paired t-test was used. Significance was discussed at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

The live body weight of chickens receiving a diet with a 30% substitution of feed grade hemp seeds tended to increase compared to the Control group ($P< 0.10$) as shown in Table 3, while the substitution of 15% did not affect the live body weight of birds, that did not differ from the Control group and neither from Hemp30 group. As the live body weight, the carcass weight of chicken shows the same results, with the carcass weight of chickens from Hemp30 group tendentially higher than Control group ($P<0.10$). Although the weight of Hemp30 group chickens tended to be higher than the Control group, no differences were observed among the feeding treatment in term of dressing percentage, drip loss and yields of the main commercial cuts (i.e., breast, and thigh with drumstick). Likewise, the WHC and the shear force of the breast fillets did not differ among the three groups, showing that the inclusion of feed grade hemp seeds did not affect the rheological properties of chicken breast meat.

Table 3. Effect of diet on live body weight, carcass weight and commercial cuts yield, and physical parameters of chicken breast fillets.

	Diets			SEM	P value		
	Control	Hemp15	Hemp30		C vs H15	C vs H30	H15 vs H30
Live body weight, g	3871.58	4057.50	4464.17	117.7	ns	0.0750	ns

Carcass weight, g	3109.90	3260.93	3612.07	105.93	ns	0.0868	ns
Dressing percentage, %	68.03	67.82	68.98	0.81	ns	ns	ns
Drip loss, %	0.97	1.18	1.61	0.25	ns	ns	ns
Breast yield, %	16.31	15.51	16.54	0.52	ns	ns	ns
Thigh + Drumstick yield, %	24.71	25.90	26.52	0.57	ns	ns	ns
WHC, %	17.89	17.21	16.17	0.65	ns	ns	ns
WBSF, N	46.13	32.30	34.16	7.57	ns	ns	ns

WHC, Water Holding Capacity, WBSF, Warner-Bratzler Shear Force, ns, not significant.

The chemical composition of chicken breast meat was not affected by the diets, showing similar percentage of ash, moisture, IFM and protein among the three groups (Table 4). Similarly, the pH value of the chicken breast fillet was not significantly different among the groups, all falling within the normal pH range.

Table 4. Proximate composition (% on fresh meat) of breast chicken fillets.

	Diets			SEM	P value		
	Control	Hemp15	Hemp30		C vs H15	C vs H30	H15 vs H30
Ash	1.14	1.16	1.16	0.01	ns	ns	ns
Moisture	74.48	74.74	74.27	0.32	ns	ns	ns
IFM	0.51	0.58	0.78	0.21	ns	ns	ns
Protein	24.54	24.05	24.09	0.18	ns	ns	ns
pH	6.06	5.85	6.10	0.08	ns	ns	ns

IFM, intramuscular fat, ns, not significant.

As the protein content of breast fillets was similar among the groups, also the individual AA was not affected by feeding treatments, for both essential and not essential AAs, as shown in Table 5.

Table 5. Amino acid composition (g/100 g of product) of chicken breast fillets.

	Diets			SEM	P value		
	Control	Hemp15	Hemp30		C vs H15	C vs H30	H15 vs H30
EAAs	9.90	9.47	9.96	0.94	ns	ns	ns
Histidine	0.72	0.79	0.78	0.09	ns	ns	ns
Arginine	1.38	1.28	1.37	0.13	ns	ns	ns
Threonine	0.95	0.90	0.97	0.09	ns	ns	ns
Valine	1.04	0.98	1.05	0.10	ns	ns	ns
Methionine	0.57	0.56	0.58	0.06	ns	ns	ns
Lysine	1.73	1.65	1.67	0.16	ns	ns	ns
Isoleucine	0.99	0.94	1.00	0.09	ns	ns	ns
Leucine	1.66	1.58	1.66	0.16	ns	ns	ns
Phenylalanine	0.85	0.80	0.87	0.08	ns	ns	ns
NEAAs	9.54	8.84	9.46	0.87	ns	ns	ns

Aspartic acid	1.88	1.77	1.85	0.18	ns	ns	ns
Serine	0.83	0.78	0.86	0.08	ns	ns	ns
Glutamic acid	3.04	2.89	2.99	0.30	ns	ns	ns
Glycine	0.85	0.66	0.86	0.12	ns	ns	ns
Taurine	0.01	0.01	0.01	0.002	ns	ns	ns
Alanine	1.13	1.07	1.14	0.11	ns	ns	ns
Proline	0.70	0.67	0.69	0.07	ns	ns	ns
Cysteine	0.27	0.21	0.22	0.03	ns	ns	ns
Tyrosine	0.82	0.78	0.84	0.08	ns	ns	ns

EAAs, Essential Amino Acids, NEAAs, Not Essential Amino Acids, ns, not significant.

As shown in Table 6, dietary treatment significantly influence the FA profile of breast fillets. The main differences were registered between the Hemp30 and Control groups. In particular, the amount of saturated fatty acids (SFA) decreased with the 30% inclusion of hemp seeds in the diet compared to Control and Hemp15 treatments, indicating that a 15% of hemp seeds substitution to commercial feed was not enough to lowering SFAs content in chicken breast meat. Among the SFAs, the palmitic (C 16:0) and the stearic acid (C 18:0) follow the same trend registered for total SFA, decreasing its content as the inclusion of hemp seeds in diet reach the 30%. The content of heneicosanoic acid (C21:0) in Hemp30 was only significantly lower than in the Control group ($P<0.05$), suggesting that the higher is the hemp inclusion the lower is the amount of C 21:0 in the breast fillets. Conversely, the eicosanoic acid (C 20:0) showed an opposite trend, increasing its content with the 30% inclusion of hemp in the chickens diet. Although the total monounsaturated fatty acid (MUFA) content was not affected by dietary treatment, the cis-vaccenic acid (C 18:1 n-7) of breast fillets from Hemp15 and Hemp30 groups was significantly lower than Control. Conversely, the cis-11-eicosenoic acid (C 20:1 n-9) content of Hemp30 breast meat was significantly higher than Control and Hemp15 samples, suggesting that the inclusion of hemp seeds in the diet of broilers differently affect the pathway of individual MUFAs. Total polyunsaturated fatty acids (PUFA) and total n-3 PUFA were significantly higher in the Hemp30 group compared to the Control ($P<0.05$), whereas a tendency was found for total n-6 PUFA ($P<0.10$), suggesting that inclusion of less than 30% hemp seed in the broiler diet did not affect the PUFA content of breast fillet. The same as for total PUPA, also linoleic acid (C 18:2 n-6) was found significantly higher in Hemp30 than Control. Instead, differently from the total PUFA trend, the dihomo-γ-linolenic (C 20:3 n-6) and the arachidonic acid (C 20:4 n-6) content was lower in Hemp30 breast fillets than Hemp15 and Control samples. As well as for MUFAs, also individual PUFAs do not all follow the same trend of total PUFAs. For what concern the nutritional indices, the 30% inclusion of hemp seeds in the broilers diet significantly increased the UFA/SFA and PUFA/SFA ratios, and tendentially improved n-6/n-3 ratio. Moreover, table D shows that a 15% inclusion of hemp seeds did not improve UFA/SFA and

PUFA/SFA ratios, significantly lower in Control and Hemp15 compared to Hemp30. Instead, when AI, TI, and h/H ratio are considered, the lower hemp seeds inclusion of 15% in the diet of broilers, resulted sufficient to determine an improvement of these parameters, that increased (h/H) and decreased (AI and TI) proportionally with the increase of hemp seeds inclusion in the broilers diet.

Table 6. Fatty acids composition (% of total fatty acids) and nutritional parameters of chicken breast fillets.

	Diets			SEM	P value		
	Control	Hemp15	Hemp30		C vs H15	C vs H30	H15 vs H30
C 4:0	0.16	0.13	0.11	0.030	ns	ns	ns
C 6:0	0.07	0.05	0.04	0.008	ns	0.0810	ns
C 8:0	0.11	0.08	0.06	0.014	ns	ns	ns
C 10:0	0.08	0.06	0.04	0.009	ns	ns	ns
C 12:0	0.02	0.02	0.03	0.004	ns	ns	ns
C 14:0	0.21	0.23	0.27	0.019	ns	ns	ns
C 14:1 n-5	0.01	0.002	0.01	0.003	ns	ns	ns
C 15:0	0.06	0.06	0.07	0.005	ns	ns	ns
C 16:0	19.82	17.98	15.88	0.463	ns	0.0186	0.0960
C 16:1 n-7	0.56	0.30	0.39	0.071	ns	ns	ns
C 17:0	0.08	0.12	0.13	0.022	ns	ns	ns
C 18:0	10.79	10.96	9.05	0.265	ns	0.0374	0.0296
9t-C 18:1	0.08	0.07	0.05	0.005	ns	0.0822	ns
C 18:1 n-9	16.29	15.24	17.92	0.649	ns	ns	ns
C 18:1 n-7	1.95	1.57	1.52	0.065	0.0505	0.0369	ns
C 18:2 n-6	20.27	26.18	33.82	2.019	ns	0.0358	ns
C 18:3 n-6	7.48	5.88	4.90	1.319	ns	ns	ns
C 18:3 n-3	0.74	1.63	3.15	0.469	ns	0.0710	ns
9c,11t-C18:2 (CLA)	0.20	0.17	0.20	0.016	ns	ns	ns
C 20:0	0.12	0.18	0.25	0.014	ns	0.0140	0.0636
C 20:1 n-9	0.17	0.22	0.33	0.019	ns	0.0184	0.0528
C 20:2 n-6	0.46	0.43	0.38	0.022	ns	ns	ns
C 21:0	0.17	0.13	0.08	0.012	ns	0.0295	ns
C 20:3 n-6	0.74	0.69	0.48	0.046	ns	0.0532	0.0908
C 20:4 n-6	13.68	11.60	6.73	1.099	ns	0.0419	0.1022
C 20:3 n-3	0.03	0.04	0.04	0.002	0.0888	0.0264	ns
C 22:0	0.34	0.31	0.28	0.065	ns	ns	ns
C 20:5 n-3 (EPA)	0.07	0.07	0.08	0.011	ns	ns	ns
C 22:1 n-9	0.06	0.06	0.06	0.011	ns	ns	ns
C 22:2 n-6	0.08	0.08	0.05	0.009	ns	ns	ns
C 22:5 n-3 (DPA)	2.80	2.71	1.86	0.234	ns	ns	ns
C 24:1 n-9	2.31	2.77	1.69	0.440	ns	ns	ns
SFA	32.04	30.30	26.33	0.442	ns	0.0057	0.0160
MUFA	21.42	20.23	21.99	0.563	ns	ns	ns

PUFA	46.55	49.47	51.68	0.746	ns	0.0334	ns
n-3	3.64	4.45	5.13	0.226	ns	0.0373	ns
n-6	42.71	45.03	46.44	0.747	ns	0.0764	ns
UFA/SFA	2.13	2.30	2.80	0.051	ns	0.0052	0.0124
PUFA/SFA	1.46	1.64	1.97	0.048	ns	0.0099	0.0331
n-6/n-3	11.80	10.15	9.17	0.518	ns	0.0729	ns
AI	0.31	0.27	0.23	0.007	0.0764	0.0098	0.0546
TI	0.72	0.63	0.51	0.014	0.0456	0.0035	0.0154
h/H	2.69	3.16	3.94	0.091	0.0710	0.0048	0.0184

SFA, Saturated Fatty Acids, MUFA, Monounsaturated Fatty Acids, PUFA, Polyunsaturated Fatty Acids, AI, Atherogenic Index, TI, Thrombogenic Index, h/H, hypocholesterolemic/Hypercholesterolemic ratio, nd, not detected, ns, not significant.

The organoleptic profile of the breast fillets of chickens receiving three different treatment are depicted in the Figure 1. In general the taste, i.e., umami, bitter, sweet, and salty attributes, of chicken breast fillets were not affected by treatment, as well as, the overall odour intensity, and the chewiness and juiciness attributes. On the contrary, the overall flavour intensity was perceived by panelist as significantly different ($P<0.05$), in particular the flavour intensity of Hemp30 was rated higher than Control sample, while Hemp15 reached a mean value between them, as well as for juiciness, but in this case only a tendency was noted, Hemp30 higher than Control ($p<0.10$). Different is the trend of the other attributes perceived by the assessors as significant different. For the fibrousness, gumminess, and residue attributes, Hemp30 sample was rated as significantly lower than the other two group, i.e., Control and Hemp15, respectively at $P<0.001$, $P<0.01$, and $P<0.05$, indicating that when the hemp seed inclusion reaches the 30%, the texture quality of chicken breast fillet increase.

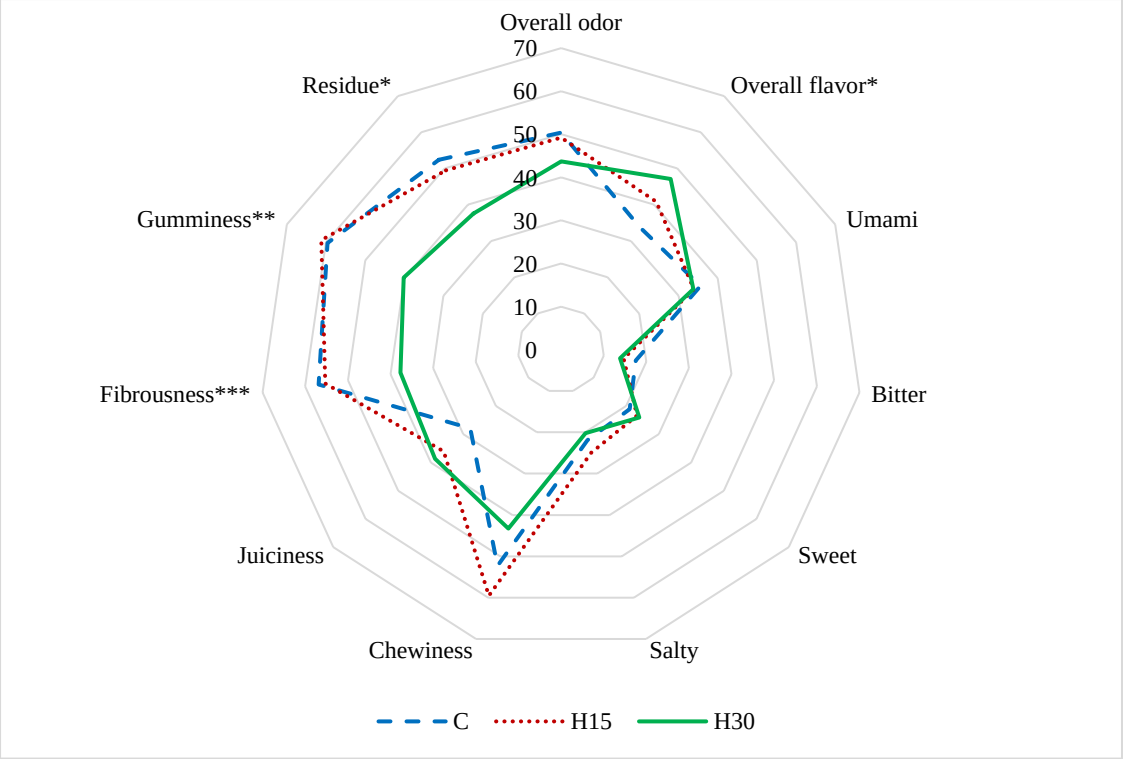

Figure 1. Spider plot of chicken breast fillet sensory profiles (QDA) from Control (C), Hemp15 (H15), and Hemp30 (H30) diets (*:P<0.05, **:P<0.01, ***P<0.001).

4. Discussion

Consumers' choices of meat and meat products are mainly influenced by hedonic, health, convenience and process aspects. Some aspects, such as the colour or texture of meat, can be perceived before the purchase, while sensory attributes can only be perceived after the purchase. There are other aspects that need to be communicated to the consumer before the purchase to be perceived, such as the process characteristic like the organic farming, the animal welfare, the non-GMO or the environmentally friendly system. In particular, the purchase of food produced according to organic rules is becoming one of the main concerns of consumers (Napolitano et al., 2013). Moreover, in the last decade, consumers, who are increasingly health conscious, are becoming more aware of the nutritional value of the food they eat (Mir et al., 2017). In this context, our findings suggest that the inclusion of feed-grade hemp seed up to 30% in the diet of organically reared broilers improved the nutritional quality and the sensory profile of chicken breast fillets.

As stated by many authors (Callaway, 2004; Klir et al., 2019; Ely and Fike, 2022), hemp seed products are a great source of PUFA and essential FA like linoleic acid (LA) and α-linolenic acid (ALA), 50% and 20% of total PUFA, respectively. It is also well known that LA and ALA cannot be synthesized in mammals, thus they must be assumed with the diet as they served as precursors to eicosanoids, in particular LA is the precursor of the arachidonic acid (AA), the FA responsible for inflammatory metabolites

production, while ALA is responsible for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) synthesis, precursor of anti-inflammatory metabolites (Ely and Fike, 2022). In our study, the hemp seed inclusion, increased the LA content in breast fillets compared to control samples proportionally with the increase of hemp inclusion, while the ALA content increased only with the higher percentage of inclusion in broilers diet. Similarly, Yalcin et al. (2016) observed the increase of LA and ALA content in the breast of Japanese quail fed with 20% hemp seed inclusion in the diet, and in particular the LA and ALA content was higher in males than females. Conversely, other study (Tufarelli et al., 2023), did not observed any difference of ALA content of chicken breast meat, and surprisingly the LA content decreased with the inclusion of 5% and 10% hemp seed cake in broilers diet.

The total SFA and PUFA content in the Hemp30 breast fillets of our study were significantly lower and higher, respectively, compared to control and Hemp15 samples, while the MUFA content was not affected by hemp inclusion in the diet. Differently, Tufarelli et al. (2023), did not recorded any differences in SFA, MUFA, and PUFA content in broiler breast fillet fed with 5% and 10% hemp seed cake inclusion in the diets, whereas Jing et al. (2017), who included 3% and 6% of hemp oil in broilers and hens diet, found higher amount of LC-n3-PUFA in breast meat. All these different findings suggested that the different source of inclusion, i.e., feed-grade hemp seed, hemp seed cake, and hemp oil, differently affect fatty acid profile of chicken breast fillets.

According to Tufarelli et al. (2023), the inclusion of 5% and 10% of hemp seed cakes in the broilers diet improved the n-6/n-3 ratio (2.29 and 2.85, respectively), halving its value compared to the control (6.43); in fact with the inclusion of hemp by-products, the authors observed an increase of n-3 content and a decrease of n-6 content in breast fillets. In our study an improvement of n-6/n-3 ratio in breast fillets was also observed with the inclusion of hemp seed in the diet, but the value was higher than the threshold of 5 suggested by the WHO guidelines (2003), in fact the n-3 and n-6 amount in the breast meat increased both proportionally with the inclusion of hemp seeds. Probably the different value of n-6/n3 ratio is linked to the different slaughtered age and genetic (Dal Bosco et al., 2012; Dal Bosco et al.,2014; Suliman et al., 2023), medium-growing chickens slaughtered at 120 days in our study, whereas in the other study were slow-growing birds slaughtered at 49 day of age. On the contrary, Skˇrivan et al. (2020) found that the inclusion of hemp seed in the diet of commercial hybrid chickens increased the n-6/n-3 ratio compared to the control and the other experimental diets with the inclusion of extruded flaxseed alone or combined with hemp seed. Also in this case, the different response to the inclusion of hemp seed in the diet, can be due to the different slaughter age (i.e., 35 days) and genetics (i.e., fast growing) of the birds. An important thing to consider is that in our study the average fat content in chicken breast fillets is lower than 1% in all sample, thus the high percentage on n-6 PUFA

compared to the n-3 is relative to the little content of fat of the samples. Although in our study the value of n-6/n-3 ratio exceed the threshold of 5, on the other hand the PUFA/SFA ratio in all sample was higher than 0.4, as recommended by WHO guidelines (2008). In particular this ratio was found to be greater with the inclusion of 30% of hemp seed compared to 15% inclusion and the control diet, indicating that the ratio between the n-6 and n-3 PUFAs in breast fillet is not well balanced, whereas the PUFA/SFA ratio follow the nutritional recommendation improving breast fillets quality. For what concern the nutritional indices AI, TI, and h/H ratio, in our study the inclusion of 15% of hemp seed was found to be already sufficient to improve the value of these parameters proportionally with hemp inclusion. Conversely, Tufarelli et al. (2023), with inclusion of lower percentage of hemp seed cake i.e., 5% and 10%, did not registered any difference in AI and TI values among the dietary treatments, indicating that probably these levels of inclusion are not enough to enhance the nutritional quality of chicken breast meat.

Hemp seeds are recognised as a source of high-quality protein, with a protein content in the whole seed between the 25% and 30%, percentage that can reach the 50% in the hemp seed cake after the oil extraction. Similarly to soybean, hemp seeds contain all essential AAs, although the amount of the first limiting AA, i.e., the lysine, in hemp seed is lower than in soybean (Ely and Fike, 2022). However, in our study, the protein content and the relative AA profile of broiler breast fillets of were similar among the three groups indicating that hemp seeds inclusion did not affect AA profile and content of broiler breast meat. Our results are similar to the AA profile of the leg meat of the Polverara genotype reported by Dalle Zotte et al. (2020) in which the meat AA profile of a commercial hybrid chicken was compared to those of a slow-growing indigenous chicken (i.e. Polverara). In particular, the content of EAAs such as isoleucine, leucine, phenylalanine, threonine, and valine, and NEAAs such as tyrosine of our study was similar to leg meat of the Polverara genotype, which in turn was significantly higher than the AAs content of the commercial hybrid. This similarity may be due to the similar rearing conditions and slaughter age of the birds, in fact indigenous genotype was usually reared by small local farmers under condition very similar to the organic rules and characterized by a slow-growing rate that lead to a higher slaughter age of the birds compared to the commercial hybrid, in order to reach the minimum commercial slaughter weight.

Although in our experiment an increase in live body weight and in carcass weight of Hemp30 chicken was observed, no difference in dressing percentage and yield of the main commercial cuts (i.e., breast and thigh with drumstick) was recorded, consistent with Khan et al. (2009) and Eriksson and Wall (2011) who did not observe any difference in dressing percentage among the animals fed without or with a 20% of hemp seed in the diet. In particular Khan et al. (2009) recorded an increase of live body weight of birds proportional to the adding of 5, 10 and 20% of grounded hemp seeds

that probably is the reason why the animals gained more weight than birds fed only the basal diet. Indeed, Šťastník et al. (2015) reported that the inclusion of 15% of hemp seed cake in commercial broilers diet, although did not affect carcass, breast and leg yield, but decreased broilers final live body weight (37 days of age). Vispute et al. (2019) suggest that the reduced body weight gain in chickens may be attributed to the high crude ash and cellulose content of hemp seeds. In particular, during the early stages of a broiler's life, the gut mucosa is not well developed, resulting in suboptimal digestive activity. Moreover, the high fibre content of hemp seed exacerbates this issue due to the absence of cellulase enzyme in birds.

As stated before, the sensory characteristics of meat play an important role in consumer's choice and purchase. Many authors (Mir et al., 2017, Pellattiero et al., 2020) discussed about the pre slaughter factors influencing meat quality. They reported that exposing birds to stress shortly before or during slaughter can result in an increased rate of post-mortem metabolism, accelerated glycolysis, and premature onset of rigor mortis. These pre-slaughter conditions often lead to the decrease of meat pH and the production of PSE (pale, soft, and exudative) meat, which in turn results in lower processing yields, increased WHC, and reduced juiciness. Moreover, Mir et al. (2017) explained that the WHC and the pH of meat are closely related between them and to meat texture. In our study, no differences in WHO and pH among the groups were observed, and following the proposed classification by Zhang et al. (2005) of breast chicken meat where a PSE meat occurs when the pH is lower than 5.7, normal when the pH is between 5.7 and 6.1, and DFD (dark, firm and dry) when the pH is higher than 6.1, all our samples fall into the normal category. Additionally, Zhang et al. (2005) observed that the PSE meat lost more moisture during cooking than normal meat, making it drier, whereas the DFD meat lost less moisture during cooking resulting more springy and chewy. In our case, panellists evaluate breast meat from Hemp30 group as less fibrous, gummy and with a lower residue remaining after the swallow compared to Control and Hemp15 samples, as depicted in Figure 1. Although the three groups belonging to the normal pH range and the pH of Hemp30 samples was found on the threshold between the normal and DFD meat, they showed a different behavior compared to DFD meat and the other two groups. Probably the higher content of PUFA in Hemp30 breast fillets determined changes in breast texture properties, making them less fibrous and gummy. Moreover, the panellists evaluated with a higher score the overall flavour of Hemp30 samples, score that decrease for Hemp15 samples and reach the lowest score with Control samples. This evaluation may be due to the different diet, and particularly, the different hemp seeds level of inclusion. In accordance with our finding, Barbut and Leishman (2022) explained that the flavour and aroma of meat developed during the cooking process in which the elevated temperature starts chemical reactions among different components that break down and becoming volatile, and then reacting with others small molecules.

This volatilization can be affected by diet and the kind of dietary supplement which can determine changes in lipid content and meat composition.

5. Conclusions

The inclusion of feed-grade hemp seed in organic broiler diets at two different levels, i.e., 15% and 30%, does not alter carcass traits and the yield of the main commercial cuts (i.e., breast and thigh with drumstick). As well as, the rheological, the chemical composition and the AA profile of broiler breast fillets are not affected by the three different dietary treatments. Instead, the FA profile of breast fillets resulted to be affected by hemp seed inclusion, in particular the higher level of inclusion improved SFA and PUFA content, decreasing and increasing respectively, whereas all the nutritional indices improved already with the lower level of hemp seed inclusion. Additionally, the sensory profile of chicken breast fillet described by experienced panellists shows that the inclusion of 30% hemp seed in broiler diet affects the sensorial properties of breast meat, in particular with a higher overall flavour intensity, and resulting less fibrous, gummy and with lower amounts of residue in the mouth after swallowing. Further studies with higher levels of feed-grade hemp seed are necessary in order to confirm these results.

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Chapter 4

Hemp seed cake as feed supplement for laying hens: laying performance, egg quality and sensory properties during shelf-life

Oral comunication at 25th congress of the Animal Science and Production Association

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Abstract

Hemp cake, a by-product of hemp seeds oil extraction, is a valuable protein, fibre, and fat source for animal feeding. It is used as an alternative feedstuff to replace soybean meal partially or totally. This study assessed the effects of including hemp seed cakes in laying hens diet on their production performance, eggs commercial quality, sensory properties, and yolk fatty acid profile over the shelf life. One hundred 18-week-old hens were assigned to two treatments (2 pens of 25 birds/treatment), consisting of a commercial standard diet (Control), and a diet containing 70% of the same basal diet integrated with 30% hemp seed cake (Hemp). Eggs production was recorded daily, while quality parameters and fatty acid profile of yolk were assessed at.0, 14 and 28 days of storage. A QDA and a consumer test on boiled eggs at 0 and 28 days of storage were also conducted. Eggs production was higher in Hemp group, while no differences were detected for eggs weight, eggs chemical composition, and eggs commercial quality. The percentage of SFA did not differ between the two diets and increased during the shelf life. MUFA profile was significantly higher in the Control group, in particular the oleic acid. Total and individual PUFA were significantly affected by the inclusion of hemp seed cakes, showing a higher percentage of PUFA and linoleic, α-linolenic and docosahexaenoic acids. Shelf-life differently influenced the amount of PUFA, decreasing in the Hemp and staying constant in the Control eggs. Eggs' sensory quality was not influenced by the diet and neither by the storage time, only pastiness was perceived as lower in Hemp than in Control by QDA and consumers test, due to the higher content of PUFA. The inclusion of hemp seed cake in laying hen diet improves the FA profile of eggs, especially in terms of total, n-6, and n-3 PUFA, without adverse effects on chemical composition, and commercial and sensory quality.

Key words: hemp seed cakes, laying hens, production efficiency, egg quality, sensory profile, shelf-life

1. Introduction

Soybean meal is commonly used as the primary protein source in poultry feed due to its high protein content, balanced amino acid profile, and essential fatty acid content (Hammershøj and Steenfeldt, 2005). The rise in poultry production has resulted in an increased demand for soybean meal worldwide, leading to reduced availability and higher prices (Laudadio and Tufarelli, 2011). The high and variable price of soybeans has led to their partial or total replacement as a protein source for livestock with alternative feedstuffs (Klir et al., 2019).

By-products created during commodity processing are important components of livestock feed and feed products. Upcycling these nutrients creates a market for by-products, turning waste into valuable feed ingredients. This benefits commodity producers and suppliers by generating additional profits and provides economical feed ingredients for livestock growers (Ely and Fike, 2022). In this context, hemp by-

products represent a valuable source of protein, fibre, and fat for all animal species. Cannabis sativa L., commonly known as hemp, is an herbaceous plant widely distributed throughout the world. It is used by humans as a textile plant for its cortical fibres and as an oil, extracted from the seeds, for therapeutic purposes or in pharmaceuticals and chemistry (Klir et al., 2019). Hemp seeds and cakes can be used in animal nutrition for all species, and the entire hemp plant, including stalk and leaves, may be suitable for ruminants. Hemp hurds can also be used for bedding (EFSA, 2011).

Previous research has shown that hemp oil can be used as a supplement in animal feed mixtures as a rich source of essential fatty acids (Cozma et al., 2015).The crude protein (CP) content in hemp by-products varied from 5.3% to 24.5%, while the average for the whole plant was 6.9%. The hemp neutral detergent fibre (NDF) content ranged from 27.9% to 84.4% for individual parts, with a whole plant NDF of 81.6%. By-products that were rich in leaf or flower components generally had lower NDF levels (Ely and Fike, 2022). Hempseed oil is rich in polyunsaturated fatty acids (PUFA), containing up to 80%. Specifically, it has high levels of linoleic acid (LA, C18:2 n-6) and α-linolenic acid (ALA, C18:3 n-3), with contents as high as 60% and 19%, respectively. This makes it comparable to other vegetable oils, except for linseed oil (Klir et al., 2019). Current research indicates that hemp and hemp by-products could serve as a valuable source of protein, fat, and fibre for livestock diets. This could potentially enhance the quality of animal-based food products such as meat, eggs, and milk (Ely and Fike, 2022). The role of essential fatty acids (EFAs), specifically linoleic and α-linolenic acid, and their metabolites in human and animal health is currently a subject of scientific inquiry. Due to insufficient intake of ω-3 PUFAs in the human diet, research has focused on producing animal food products that are enriched with a healthy fatty acid profile. Among the various foodstuffs evaluated for ω-3 FA enhancement, hen eggs have been reported as the easiest and most successful means of adding n-3 FAs to the human diet (Raza et al., 2016). Fabro et al. (2021) reported that changing the lipid source in the diet of laying hens can improve the fatty acid (FA) profile of their eggs, making them more appealing to consumers. Specifically, the inclusion of oils rich in n-3 polyunsaturated fatty acids (PUFA) in the hens' diets increases the deposition of these nutrients in the egg yolk. Additionally, hens are more efficient at converting α -linolenic acid to long-chain PUFA than humans. There is a growing interest in utilizing hempseed as a feed source for laying hens due to its higher levels of α-linolenic acid (ALA) and γ-linolenic acid (GLA) compared to other feed sources. Specifically, vegetable oils derived from soybean, maize, and rapeseed have lower levels of n-3 PUFA than hempseed oil. Currently, there are few studies that have examined the response of laying hens to feeding on hemp seed cakes. In most of these studies, the hemp used to feed the laying hens was in the form of seeds or oil due to its high unsaturated fatty acid content (Fabro et al., 2021). Silversides and

LefranÇois (2005) carried out the maximum integration of hemp by-product into the laying hens diet by substituting 20% of the standard diet with hemp seed cakes. Little is known about the quality and sensory profile evolution of eggs during storage. Therefore, our study aimed to investigate the effects of including 30% hemp seed cake in laying hens' diets on egg production and quality during shelf-life. We also aimed to determine the fatty acid profile of egg yolk at 0, 14, and 28 days of storage to verify if hemp seed cake can improve the n-3 fatty acid content of eggs. Additionally, we conducted a quantitative descriptive analysis and a consumer test with boiled eggs.

2. Materials and methods

All the animals involved in our study were humanely treated according to the principles stated by the EC Directive 86/609/EEC (Council Directive, 2008) regarding the protection of animals used for experimental and other scientific purposes.

2.1. Experimental design and sampling

The trial was carried out on an organic laying hens farm located in southern Italy (Roccabascerana, Avellino, Italy), from February to April 2020. A total of 100 18-week-old Lohmann White laying hens (average live weight 1.55 kg±0.12 standard deviation) were equally divided into 2 groups of 50 hens each. Along the experimental period of 10 weeks, hens were housed in two adjacent pens, each divided by mesh transects in two equal areas to obtain two replicates of 25 hens per group. Each pen had a concrete floor indoor area (0.15 m^2/hens) with free access to wire fenced open-air runs (3 m^2/hens). The indoor areas were equipped with circular galvanized feeders, plastic drinkers, perches (15 cm per hen) and nest boxes (4 hens per nest). The groups were fed two experimental diets, consisting of a standard soybean-maize meal-based diet (Control) and the same basal diet integrated with 30% hemp seed cakes (Hemp). Both diets were formulated to meet the nutrition requirements (17% of crude protein and 2.750 kcal/kg of metabolizable energy, NRC, 1994) of early-phase laying hens (18 to 40 weeks) consuming 120 g of feed/day and were presented in meshed form to reduce differences in feed physical form, to ensure the same quality, and to avoid feed selection by hens. Diets were manually distributed once a day (08:00 a.m.). Diet ingredients and chemical composition are listed in Table 1.

After 2 weeks of adaptation to the new diets (starting from 20 week of age) the collection of data was started. Feed intake was daily measured per replicate, weighing the amount of feed distributed and refused and was expressed as a group feed intake by day. To assess that nutritional requirements were met, all dietary ingredients were sampled weekly and analysed (Table 1), in duplicate, for dry matter (DM), ash, crude protein (CP), and ether extract (EE) according to AOAC International (2000). Neutral detergent fibre (NDF), were performed according to Van Soest methods (Van Soest et

al., 1991) using the sequential procedure and the filter bag system (Ankom Technology, New York, NY). Heat resistant α-amylase and sodium sulphite were added to NDF solution, all fibre fractions were expressed as inclusive residue ash. Eggs were collected daily, and egg production was calculated on a pen-day basis. Thirty eggs per treatment (15 eggs/pen) were sampled every week,7 were analysed no later a day, 7 after 14 days and other 7 after 28 days of storage at 4°C for physical and chemical characteristics, and 9 were left for the sensory analysis.

Table 1. List of ingredients of the two experimental diets with the respective chemical compositions and fatty acid profiles.

Ingredient, g/kg	*Diet*	
	Control	**Hemp**
Hemp seed cake	-	300
Maize meal	320	224
Wheat bran	310	217
Soybean cake	110	77
Roasted soy	50	35
Barley and Wheat flour	60	42
Dried alfalfa meal	50	35
Calcium carbonate	80	56
Minerals supplement	20	14
Chemical composition (% DM)		
Ash	16.40	13.73
Crude protein	15.80	20.48
Fat	5.40	7.08
NDF	23.30	23.06
Metabolizable energy (kcal/kg DM)	2884	3087
Fatty acid profile (%)		
SFA	15.18	10.16
Palmitic acid	10.85	6.91
MUFA	24.30	14.14
Oleic acid	23.75	13.53
PUFA	61.28	75.77
Linoleic acid	55.19	56.97
α- linolenic acid	5.59	14.44

2.2. Physico-chemical analysis

Each egg was weighted (W) fresh and after 14 and 28 days of storage, in order to get weight losses, and measured the height (H) (distance between the two poles) and length (L) at equator by micrometre calliper to calculate shape index (SI) according to Anderson et al. (2004) formula: SI=(H/L) *100. Shell surface was calculated according to Paganelli et al. (1974) formula: $Ps=4.4835*W^{0.662}$. Eggs were examined for shell

quality by specific gravity (SG). Shell thickness (with shell membrane) of the eggs was measured by micrometre. Shell thickness was a mean value of measurements at 3 locations on the eggs (air cell, equator, and sharp end). The Haugh Unit (HU) was calculated as Haugh Units (%) =100*log (H_a−1.7*$W^{0.37}$+7.57), where H_a is the height of the albumen, according to the formula proposed by Card and Nesheim (1972). Yolk colour was determined by the Roche yolk colour fan (Hoffman-La Roche Ltd., Basel, Switzerland, colour scale from 15, dark orange, to 1, light pale).

The contents of DM, ash, and CP of albumen and yolk were assayed according to Secci et al. (2018). The organic matter (OM) content was calculated as the difference between DM and ash contents. The pH was measured at room temperature using a portable digital pH meter (206-pH2, Testo AG., Limburg an der Lahn, Germany). A benchtop water activity meter (AquaLab, Steroglass, Perugia, Italy) was used to measure the water activity (a_w).

For the determination of fat content, about 5 g of each sample was extracted five times with a mixture of 5 ml chloroform and methanol (2:1 ratio), along with 4 ml of a saturated NaCl solution. The mixture was stirred for about 1 minute using a Vortex and then centrifuged for 10 minutes at 6500 rpm. The resulting subnatants were collected in glass flasks and dried using a rotavapor at a temperature of 30°C. The fat was dissolved in 10 ml of hexane and transferred to 15 ml centrifuge tubes. Then, 3 ml of saturated NaCl solution was added to each tube. After centrifuging the fat samples for 10 min at 6500 rpm, the supernatant was transferred to glass tubes and filtered with anhydrous sodium sulphate. The filtrate was dried under nitrogen flow to remove the solvent. The fat yield was calculated as a percentage (grams of fat/100 grams of sample).

Determination of yolks FA profile was performed by analysis of the fatty acid methyl esters (FAME) following Romano et al. (2011) method. The procedure involved the preparation of a 1% fat solution in hexane to which was added 300 µl of methanolic potash 2N. After stirring the solution for about 1 min, 1µl of supernatant was withdrawn with a syringe for gas chromatographic analysis. The equipment used for egg yolks FA determination consists of an Agilent Technologies 6890N gas chromatograph equipped with a programmed temperature vaporizer (PTV) and flame ionization detector (FID). A 100m×0.25mm inner diameter capillary column, 0.20µm film thickness, stationary phase 90% biscyanopropyl/10% cyanopropylphenyl Siloxane (Supelco Bellofonte, USA). The operating conditions were as follows: helium as carrier gas (flow rate of 2 ml/min), hydrogen and chromatographic air as auxiliary gases, PTV (Programmed Temperature Vaporizer) was 60°C for 0.1 min, increment 500°C/min to 260°C and dwell for 5 min, the initial temperature of OVEN (Chamber) was 140°C for 5 min, increment of 4°C/min up to 175°C and dwell for 20 min, increment of 3°C/min up to 240°C for 20 min, split ratio was 15:1, FID (15:1 air/H mixture) detector temperature was 260°C, injector temperature was 120°C. Fatty acid

identification was performed by comparison with a mixture of standards: FAME C4-C24, Sigma-Aldrich (37 components). The results were expressed as percentage of fatty acids. The nutritional indices, i.e., Atherogenic index (AI), thrombogenic index (TI), and hypocholesterolemic/hypercholesterolemic ratio (h/H) were calculated as reported by Chen and Liu (2020).

2.3. Sensory profile

A quantitative descriptive analysis (QDA) was conducted on boiled eggs, at 0 and 28 days of shelf-life stored at 4°C, by a panel of 11 judges (7 females and 4 males, aged between 20 and 60 years), selected following ISO 8586, (2012) recommendation. The judges, under the guidance of the panel leader, identified descriptors and shared common attribute vocabulary, listed in Table 2 (Drażbo et al., 2014, Hayat et al., 2014). These, subsequently were trained with specific reference standards. For the evaluations, eggs were cooked in already boiling water for 15 min, cooled under cold running water, shelled, split in two and immediately served. Each judge evaluated the two halves of each product, i.e., fresh and stored eggs, in individual booths under red light for descriptors related to flavour, taste and texture, while appearance was evaluated under white light. Three replicates per sample were performed. The Smart Sensory box platform version 2.3.5 (Smart Sensory Solution, Italy) was used to manage the QDA sessions.

Table 2. List of hedonistic attributes identified by trained panellists.

Attribute	Definition
Yellow	Yolk colour intensity (0 light yellow/ 100 deep yellow)
Opacity	Visual characteristic that describes the product's inability to reflect light
White	Albumen colour intensity (0 chalk white/100 off-white)
Odor	Overall odour intensity of overboiled egg
Flavour	Overall flavour intensity of overboiled egg
Bitter	Fundamental taste associated with quinine
Sweet	Fundamental taste associated with sucrose
Salty	Fundamental taste associated with sodium chloride
Friability	Easiness of a sample to break into fragments upon fork compression
Pastiness	Sample's tendency to form dense, compact mass when chewed without melting

In response to the Covid-19 emergency situation, a home-use test was conducted with 100 consumers divided into three age groups (18-40, 41-60, and >60). Each consumer received a kit containing two samples of fresh eggs (Hemp and Control) identified by a random 3-digit numerical code. The kit also included a liking questionnaire and instructions for cooking the samples (15 minutes in already boiling water). For each sample, consumers provided ratings for overall liking, appearance, taste/flavour, and texture using a 9-point hedonic scale ranging from “extremely unpleasant” (i.e., 1) to “extremely pleasant” (i.e., 9). The study was conducted in compliance with the

guidelines of the Declaration of Helsinki and Italian ethical requirements for research activities and personal data protection (D.L. 30.6.03 No. 196). Participants in the study signed an informed consent form.

2.4. Statistical analysis

All data were subjected to the Kolmogorov-Smirnov test to verify their normal distribution. Eggs commercial quality and chemical composition data were subjected to an analysis of variance (ANOVA) using a mixed procedure of the SAS software (2009, SAS Institute Inc., Cary, NC), with the pen as a random factor. Data from the sensory evaluation (QDA) of fresh and stored eggs were subjected to one-way ANOVA using a general linear model (GLM) procedure from SAS, with product, replicate, and their interaction as factors. Consumer test data were subjected to an ANOVA using a GLM procedure with diet as factor. Student's paired t-test was used to evaluate the differences between the means obtained for the two treatments. The significance level was discussed at $P < 0.05$, and $P < 0.01$, while tendences were discussed at $P < 0.10$.

3. Results

The daily ration (120 g/day per hens) was entirely consumed by the hens during the experimental period and, therefore, no difference in feed intake between groups was recorded. Since chemical composition of dietary ingredients did not vary across the sampling dates (data not shown), the hens' nutritional requirements over the experimental period were met. Similarly, no mortality was recorded during the trial.
The daily production of eggs resulted to be affected by dietary treatment with Hemp group producing an average of 17 eggs per day compared to 14 eggs of Control group (SEM 1.29, $P<0.05$). The physical characteristics of the eggs obtained by the two groups are listed in Table 3. Although the inclusion of hemp seed cake resulted in an improvement of hens production performance, their egg weight was not affected by diet. During the storage period, both Control and Hemp group recorded an egg weight loss, with the Control group tending to lose more weight than Hemp group after 14 days of storage. However, after 28 days, both groups lost a similar amount of weight, with the Hemp group showing a higher weight loss than the Control group between 14 and 28 days of shelf-life. Similarly to the weight of whole egg, the yolk weight was also comparable between the samples, losing weight during the storage period in both groups, whereas the albumen and shell of Hemp eggs tended to be heavier than Control, as showed in Table 3. Regarding egg physical traits, the Hemp group showed a slightly higher shell surface ($P <0.10$), while no significant differences were found in terms of yolk colour score, shell thickness, shell density, specific gravity, shape index, yolk ratio, albumen ratio, shell ratio, yolk/albumen, and Haugh Units

(Table 3). However, the yolk index ratio decreased at 28 days of shelf-life in both groups (Table 3).

Table 3. Eggs quality parameters as affected by diet (D), storage time (ST), and their interaction (D*ST).

	Storage time (days)	*Diet*		SEM	*P*		
		Control	Hemp		D	ST	D*ST
Egg weight, g	0	63.5400	65.2670				
	14	62.3940	65.1255	0.8513	ns	0.0882	ns
	28	61.7975	63.7360				
Weight loss, g	14	0.5725^{b}	0.3600^{B}	0.0382	ns	0.0067	0.0429
	28	0.8200^{a}	0.9165^{A}				
Yolk colour	0	6.0800	5.7830				
score	14	6.6990	6.2775	0.1879	ns	0.0004	ns
	28	7.7915	7.3865				
Yolk weight, g	0	16.4205	17.5995^{a}				
	14	16.0945	17.0790^{a}	0.3240	ns	0.0850	ns
	28	15.8045	16.3330^{b}				
Shell weight, g	0	6.4505	6.6450				
	14	6.3180	6.7170	0.1241	0.0696	ns	ns
	28	6.4375	6.9355				
Albumen	0	39.4415	41.6295				
weight, g	14	39.3370	41.5430	0.6547	0.0972	ns	ns
	28	37.5240	40.5350				
Shell thickness,	0	0.3690	0.3760				
mm	14	0.3790	0.3895	0.0066	ns	ns	ns
	28	0.3715	0.3840				
Shell surface,	0	67.6200	69.6835				
cm^2	14	67.1030	69.5665	0.5002	0.0555	ns	ns
	28	66.1180	70.1220				
Shell density,	0	95.3970	95.3885				
mg/cm^2	14	94.4585	98.2410	1.6893	ns	ns	ns
	28	97.9805	98.8070				
Specific	0	1.0925	1.0990				
Gravity, g/cm^3	14	1.0880	1.0695	0.0128	ns	ns	ns
	28	1.0740	1.0835				
Shape Index, %	0	74.7725	75.0355				
	14	76.7875	74.5860	1.3897	ns	ns	ns
	28	72.9195	74.2425				
Yolk ratio, %	0	26.4955	26.4060				
	14	26.4125	26.1820	0.6286	ns	ns	ns
	28	26.2500	25.5540				
Albumen ratio,	0	63.3665	63.6110				
%	14	63.4375	63.8415	0.6913	ns	ns	ns
	28	62.7080	63.0565				
Shell ratio, %	0	10.3585	10.1975				

	14	10.3120	10.6045	0.1845	ns	ns	ns
	28	10.7965	10.5375				
Yolk/Albumen,	0	42.4270	42.1195				
%	14	41.9690	41.2735	1.4764	ns	ns	ns
	28	41.5890	40.1245				
Haugh Units	0	80.4890	79.3910				
	14	81.6440	80.4295	2.2133	ns	ns	ns
	28	78.3170	77.4620				
Yolk Index, %	0	48.0840	47.0140				
	14	47.9110	47.0265	0.6195	ns	0.0003	ns
	28	46.0880	44.9660				

SEM, Standard Error of the Mean; ns, not significant; mean values in the column with different superscript letters significantly differ at the P<0.05 level if lowercase and at the P<0.01 level if uppercase.

As shown in Table 4, there was no significant difference in DM content of the yolk and albumen between the treatments. Similarly, the content of ash, CP, and OM, expressed as percentage of dry matter, and the a_w were unaffected by the diets, for both yolk and albumen (Table 4). Instead, fat content and pH of yolks changed during the storage period, in particular the fat amount started to decrease after 14 days of shelf-life, while the pH increased during the storage period, in both groups. The interaction between the diet and storage time only slightly affected the ash content and consequently the organic matter of the albumen. Table 4 shows that the ash content in Control albumen increased at 28 days of storage compared to the period before, while the ash content in Hemp albumen remained stable during the storage period. Furthermore, the pH of the fresh albumen (i.e. 0 days) differed between the groups, with the Hemp having a higher pH than the Control. The pH of albumen resulted to be affected by dietary treatment. Table 4 shows that the pH of the Control tended to be higher than that of Hemp albumen, in particular this difference is visible at 28 days of shelf life, as the pH of the Control increased (P<0.05) and that of the Hemp decreased (not significantly) over the storage period.

Table 4. Yolk and albumen chemical composition, pH and a_w as affected by diet (D), storage time (ST), and their interaction (D*ST), expressed as % of dry matter.

	Storage time (days)	*Diet*		SEM	*P*		
		Hemp	Control		D	ST	D*ST
Yolk							
Dry	0	50.9380	51.5420				
matter	14	50.2340	51.2730	0.3969	ns	ns	ns
	28	51.0345	51.3035				
Ash	0	3.6065	4.1360				
	14	4.0535	3.8765	0.4092	ns	ns	ns
	28	4.6690	3.1960				
Protein	0	32.3845	32.1785				

	14	32.2750	31.4040	0.3866	ns	ns	ns
	28	32.7615	31.5675				
Fat	0	29.1360	29.0455				
	14	29.1410	29.1195	0.4226	ns	0.0260	ns
	28	27.6380	27.2445				
Organic	0	96.3935	95.8640				
matter	14	95.9465	96.1235	0.4092	ns	ns	ns
	28	95.3310	96.8040				
pH	0	6.0645	6.0290				
	14	6.0630	6.0505	0.0223	ns	0.0006	ns
	28	6.3115	6.2385				
a_w	0	0.9925	0.9940				
	14	0.9925	0.9925	0.0010	ns	ns	ns
	28	0.9910	0.9915				
Albumen							
Dry	0	12.0040	12.3965				
matter	14	12.5925	12.8885	0.2036	ns	ns	ns
	28	12.7105	13.3580				
Ash	0	5.6645^{aby}	7.2410^{x}				
	14	5.2990^{b}	6.0760	0.3658	ns	ns	0.0741
	28	6.7310^{a}	5.9545				
Protein	0	88.8635	88.6405				
	14	92.3525	89.5940	1.6881	ns	ns	ns
	28	87.7855	85.4925				
Organic	0	94.3355^{axy}	92.7590^{y}				
matter	14	94.7010^{a}	93.9240	0.3658	ns	ns	0.0741
	28	93.2690^{b}	94.0455				
pH	0	9.0385^{b}	9.0510				
	14	9.0540^{b}	9.0330	0.0186	0.0824	ns	0.0312
	28	9.1335^{aX}	8.9935^{Y}				
a_w	0	0.9965	0.9940				
	14	0.9950	0.9965	0.0009	ns	ns	ns
	28	0.9940	0.9950				

SEM, Standard Error of the Mean; ns, not significant; mean values in the column with different superscript letters significantly differ at the P<0.05 level if lowercase and at the P<0.01 level if uppercase; mean values in the row with different superscript letters significantly differ at the P<0.05 level if lowercase and at the P<0.01 level if uppercase.

Table 5 shows the FA profile of egg yolk of both groups. The SFA content was not affected by the diet or the storage time, unlike MUFA which was higher in the Control group, and PUFA content which was higher in the Hemp group. These content changed differently during the shelf-life due to the interaction between the diet and

the storage time. In fact, MUFA content decreased in Control eggs during the shelf-life with the lowest content at 28 days of shelf-life, whereas in Hemp eggs the MUFA content increased during the shelf-life with the highest amount at 28 days of storage, time in which the MUFA content of both group was similar, i.e., 35%, while at 0 and 14 days of storage MUFA content resulted higher in Control eggs. Among the MUFAs, the oleic acid partially showed a similar trend of MUFA, presenting a higher content in the Control group compared to the Hemp. Although the oleic acid content decreased in both groups during the storage period, it decreased significantly in Control eggs at 28 days of shelf-life, whereas in the Hemp eggs it decreased already after 14 days of storage.

Conversely, the PUFA content was higher in the Hemp eggs than Control, particularly at 0 and 14 days of shelf-life, while after 28 days of storage the PUFA content of both eggs groups was similar. The PUFA content of Control eggs was similar over the storage period, decreasing with time but not significantly. In contrast, the PUFA content of fresh Hemp eggs was significantly higher than that of the Control, presenting a similar amount after 14 days of storage but decreased drastically after 28 day of shelf-life, almost reaching the PUFA content of the Control eggs at the same storage time. Among the PUFAs, LA, ALA, arachidonic acid and DHA were significantly affected by the dietary treatment. All these FAs were present at higher percentage in Hemp eggs compared to Control, except for arachidonic acid content, higher in Control group. In particular LA was higher in Hemp eggs than Control at 0 and 14 days of shelf-life, while at 28 days in both group the content of LA was about 21%, but while in Hemp eggs the LA content decreased after 28 days of storage, in Control eggs it slightly increased after the same period of storage, albeit the difference was not significant. For what concern the ALA content, it was significantly higher in Hemp egg at each time of storage, doubled its content compared to Control eggs, but as well as for LA, also ALA content in Control eggs was similar over the storage period, while in Hemp eggs ALA content decreased during the shelf-life. Similarly, DHA content was higher in Hemp at each time of storage compared to Control eggs, but in this case DHA content over the storage period decreased in both groups, with the lowest DHA content at 28 days of shelf-life. On the other hand, arachidonic acid content was higher in Control eggs at 0 and 14 days of storage, while at 28 days it was only tendentially higher compared to Hemp eggs. In both groups the arachidonic acid content decreased over the time, with the lowest amount at 28 days of shelf-life. As well as for PUFA content, also the n-3 and n-6 PUFAs were higher in Hemp eggs than Control. The n-3 PUFA content in Hemp group almost doubled the content of Control eggs at each storage time, in both groups decreasing during the storage period, with the lowest amount at 28 days of shelf-life. The n-6 PUFA instead, similarly to LA behaviour, presenting the same amount of n-6 PUFA over the time in Control eggs while decreased in Hemp group at 28 days of shelf-life.

For what concern the nutritional indices, both PUFA/SFA and n-6/n-3 ratio were in favour of Hemp group. During the storage period the PUFA/SFA ratio remained unchanged in the Control eggs, whereas decreased in Hemp group with the lowest value at 28 days of shelf-life. The n-6/n-3 ratio, instead, increased in the Control samples during the storage time, while was stable in Hemp eggs. The AI, TI, and h/H ratio were not affected by the diet, only TI was tendentially higher in Control eggs compared to Hemp, whereas were affected by the storage time, increasing their value of AI and TI, and decreasing the h/H ratio over the time.

Table 5. Yolk fatty acid profile and nutritional indices as affected by diet (D), storage time (ST), and their interaction (D*ST), expressed as % of dry matter.

	Storage time (days)	*Diet*		SEM	*P*		
		Control	Hemp		D	ST	D*ST
Myristic acid	0	0.22	0.19				
	14	0.24	0.21	0.03	ns	ns	ns
	28	0.27	0.25				
Palmitic acid	0	23.69	25.03				
	14	25.00	24.79	0.64	ns	ns	ns
	28	24.26	25.97				
Palmitoleic acid	0	1.24	1.40				
	14	1.21	1.38	0.27	ns	ns	ns
	28	1.47	1.73				
Eptadecanoic	0	0.18	0.19				
	14	0.22	0.21	0.05	ns	ns	ns
	28	0.27	0.28				
Stearic acid	0	10.72	10.33				
	14	10.98	11.03	0.38	ns	ns	ns
	28	11.08	10.78				
Oleic acid	0	38.22^{AX}	33.22^{aY}				
	14	37.41^{AX}	30.50^{bY}	0.46	0.0058	0.0038	0.0400
	28	33.85^{BX}	30.54^{bY}				
Linoleic acid (LA)	0	19.52^{Y}	24.31^{aX}				
	14	19.62^{Y}	24.18^{aX}	0.76	0.0596	ns	0.0278
	28	21.69	21.18^{b}				
α linolenic acid (ALA)	0	0.81^{Y}	1.80^{aAX}				
	14	0.77^{Y}	1.44^{bX}	0.08	0.0085	0.0822	0.0459
	28	0.89^{Y}	1.28^{bBX}				
Eicosadienoic acid	0	0.16	0.42				
	14	0.20	0.26	0.07	ns	ns	ns
	28	0.18	0.18				
Eicosatrienoic acid	0	0.28	0.25				
	14	0.19	0.18	0.04	ns	0.0452	ns
	28	0.10	0.11				
Arachidonic acid	0	2.02^{Ax}	1.87^{Ay}				
	14	1.98^{A}	1.77^{aY}	0.03	0.0251	0.0013	ns

	28	1.69^{B}	1.57^{Bb}				
Docosahexaenoic	0	1.63^{AY}	2.23^{AX}				
acid (DHA)	14	1.39^{aY}	2.05^{aX}	0.07	0.0074	0.0026	ns
	28	1.04^{BbY}	1.68^{BbX}				
SFA	0	34.80	35.77				
	14	36.44	35.36	0.71	ns	ns	ns
	28	36.58	36.94				
MUFA	0	39.83^{AX}	32.14^{bY}				
	14	38.86^{aX}	32.14^{bY}	0.54	0.0081	ns	0.0047
	28	35.34^{Bb}	35.01^{a}				
PUFA	0	26.24^{Y}	30.87^{AX}				
	14	24.21^{Y}	29.86^{AX}	0.78	0.0361	ns	0.0105
	28	23.79	25.99^{B}				
n-6 PUFA	0	22.01^{Y}	26.84^{AX}				
	14	22.07^{Y}	26.37^{AX}	0.77	0.0718	ns	0.0232
	28	23.28	23.04^{B}				
n-3 PUFA	0	2.58^{AY}	4.03^{aAX}				
	14	2.20^{aY}	3.49^{bX}	0.11	0.0044	0.0023	ns
	28	1.73^{BbY}	2.96^{CX}				
UFA/SFA	0	1.84	1.77				
	14	1.72	1.78	0.05	ns	ns	ns
	28	1.69	1.67				
PUFA/SFA	0	0.70^{y}	0.87^{ax}				
	14	0.66^{y}	0.86^{ax}	0.03	0.0480	ns	0.0661
	28	0.72	0.71^{b}				
n-6/n-3	0	9.26^{BX}	7.03^{Y}				
	14	10.64^{ABX}	7.78^{Y}	0.45	0.0154	0.0115	0.0378
	28	13.18^{AX}	7.81^{Y}				
AI	0	0.39	0.41				
	14	0.42	0.42	0.01	ns	0.0075	ns
	28	0.43	0.44				
TI	0	0.92	0.85				
	14	1.02	0.88	0.03	0.0777	0.0842	ns
	28	0.97	0.96				
h/H	0	2.60	2.41				
	14	2.42	2.40	0.06	ns	0.0270	ns
	28	2.33	2.25				

SEM, Standard Error of the Mean; ns, not significant; SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, Polyunsaturated Fatty Acids; AI, Atherogenic Index; TI, Thrombogenic Index; h/H, hypocholesterolemic/Hypercholesterolemic ratio; mean values in the column with different superscript letters significantly differ at the $P<0.05$ level if lowercase and at the $P<0.01$ level if uppercase; mean values in the row with different superscript letters significantly differ at the $P<0.05$ level if lowercase and at the $P<0.01$ level if uppercase.

The results of the QDA analysis (Figure 1) indicate that there is no significant effect of either diet or storage time on the sensory profile of the eggs, except for pastiness, which was less intense in eggs from the Hemp group (P<0.01). Storage time had a significant effect on opacity (P<0.01), which decreased after 28 days, and flavour (P<0.001), which became more intense with storage. Regarding odour, there is evidence of different behaviour based on diet (P<0.05). Specifically, odour intensity increased at 28 days in the Hemp group, while in Control eggs, less intensity was perceived after 28 days of storage.

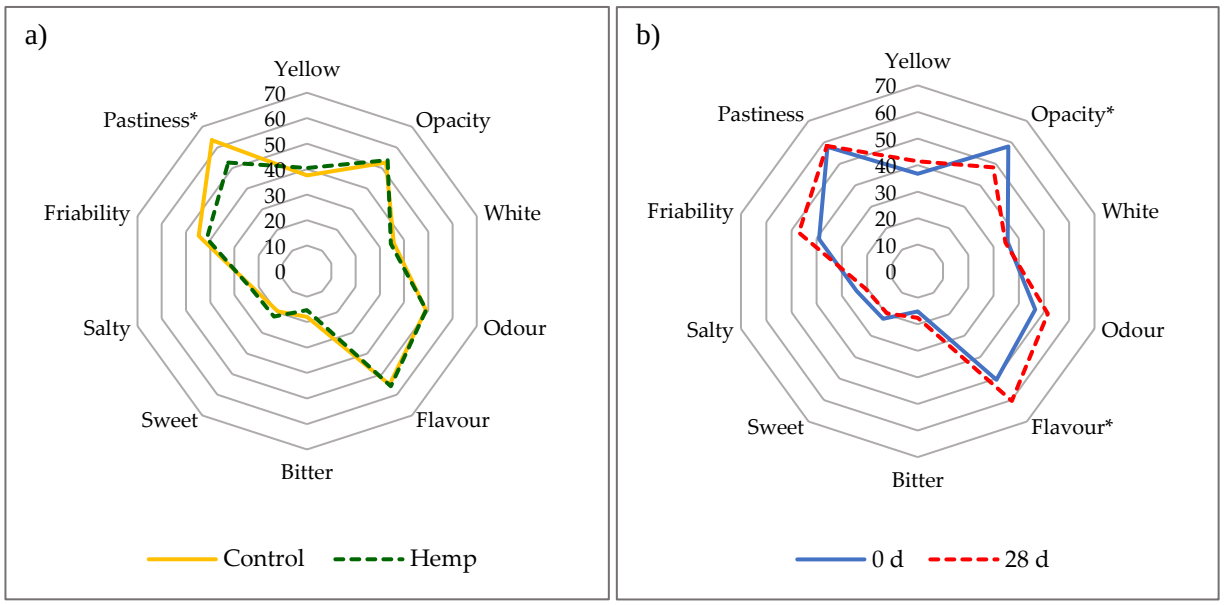

Figure 1. (a) Spider plot of the sensory profile (QDA) of eggs as affected by diet (*P <0.05), (b) Spider plot of the sensory profile (QDA) of eggs as affected by storage time (*P <0.05).

No significant differences were found between the experimental and control samples for acceptability, except for consistency, which tended to be more preferred (P<0.10) in eggs from the Hemp group. This suggests that the difference in texture identified by the panel was also perceived by consumers. For both products, consumers gave satisfactory ratings for all aspects considered, including overall likeability, taste/flavour, appearance, and texture.

4. Discussion

The aim of this study was to verify if the use up to 30% of hemp seed cakes, a by-product of hemp oil extraction, as animal feed, would have an impact on the production efficiency of laying hens and the commercial, nutritional, and sensory quality of egg.

Our results show that a 30% standard diet substitution with hemp seed cakes affected laying performance of hens without impairing eggs quality parameters.

Most of the published literature on this subject and the related areas is in other species and with using whole hemp seed or hemp oil or other by-products (see the review of Fabro et al., 2021). Given the limited published research on feeding hemp seed cake to laying hens, we are constrained with few supporting references to quote the findings. In attempts to stretch test hemp products feeding safety in laying hens, a daily intake reduction has been reported by Gakhar et al. (2012) and Yalcin et al. (2018) in hemp seeds fed laying hens and quails, respectively. Although the studied hens were fed restricted amount of balanced diet, there was no evidence of refusals or reduced consumption in our study. Similar findings were reported by Silversides and Lefrançois (2005) with dietary inclusion of hemp cake up to 20%, when the diet did not significantly affect feed intake, whereas a significant reduction of feed intake was observed by Halle and Schöne (2013) when hemp cake was supplemented at 15% versus 5% and 10%. In our study, the lack of differences between groups in feed intake could be explained by the long adaptation period to diet (2 weeks), that helped the hens adjust to the new ingredient.

Regarding the egg weight, previous studies based on the use of hemp extraction cake have shown conflicting results. According to Silversides and Lefrançois (2005), increasing levels of hemp cakes (0, 5, 10, and 20%) in the diets of laying hens did not change laying intensity, egg weight, or feed conversion efficiency, by contrast, a positive effect on eggs weight was observed by Halle and Schöne (2013), who concluded that the introduction of hemp cakes (10%) into hens diet did not negatively affect the laying performance of the hens. Egg weight can be influenced by both the content and the type of lipid matrix of feed gave to laying hens (Balevi and Coskun, 2000). According to Balevi and Coskun (2000), feeding diets containing corn and soybean oils, both the major ingredients of the control diet in our study, may lead to egg weight reduction, due to a low PUFAs intake. High ingestion rate of PUFAs and LA, which account of 17% of hemp total fatty acids, can promote egg weight and size (Mazalli et al., 2004). Conversely, an egg weight increase was observed by Beynen (2004) by feeding laying hens with linseed, a rich LA source. A different trend was reported by Rowghani et al. (2007) who showed a slight increase in egg weight after the use of rapeseed oil, lacking of LA.

All quality parameters were not affected by dietary treatment, included the HU. Following the classification reported by Oliveira et al. (2020), eggs can be considered of AA quality when HU ≥ 72, A quality when 72 > HU ≥ 60, B quality when 60 > HU ≥ 31, and C quality when HU < 30, in our study the eggs from both groups and at each time of storage were of AA quality.

On the other hand, the FA profile of eggs was affected by the inclusion of hemp seed in laying hens diet, by decreasing MUFA content and increasing both n-3 and n-6 PUFA content of Hemp eggs. Konca et al. (2014) reported that the n-3 FA content of eggs increased linearly with increasing dietary hemp seed content in the diet.

Similarly to our findings, Shahid et al. (2015) showed that adding 25% hemp seed to the laying diet decreased egg yolk total cholesterol and total MUFA, while significantly increased total, n-3, and n-6 PUFAs. Moreover, Raza et al. (2016) found that adding 25% hemp seed to hens' diets improved the n-6/n-3 ratio in egg yolks. Although the works cited before were about the effects of adding the whole hemp seed in laying hens diet, several studies have also investigated the effect of hemp seed cake. Silversides and Lefrançois (2005) reported that including hemp seed cake in the diet of hens increased the content of LA and ALA and decreased the concentration of palmitic acid in hen eggs. Goldberg et al. (2012) found that the 12% hemp oil group had the highest total n-3 PUFA content compared to the control group. Halle and Schöne (2013) reported that hens fed hemp seed cake produced eggs with lower LA and higher ALA compared to those fed linseed cake. The main reason for this outcome is the lower ALA and higher LA content of hemp seed compared to linseed. Neijat et al. (2016) indicated that laying hen diets containing any hemp seed product can effectively manipulate the total lipid and FA profile, reducing the n-6/n-3 ratio of yolk. It was reported that a benefit could be achieved within four weeks of feeding hens diets containing either hemp seed or hemp seed oil. Similarly, Raza et al. (2016) found that adding 25% hemp seed to the diet of hens improved the n-6/n-3 ratio in the yolk of eggs. In addition, Fabro et al. (2021) in their meta-analysis concluded that diets for laying hens containing hemp seed products resulted in eggs with increased PUFA content and a reduction in the n-6/n-3 ratio. In our work, by including 30 % of hemp seed cake in laying hens diet, we observed an increase of PUFA content that led in an increase of the n-3 and n-6 PUFAs amount with a great improvement of the n-6/n-3 ratio and also PUFA/SFA ratio of the egg yolks. Moreover, we observed that while in Control eggs the PUFA amount stayed stable over the storage time, in Hemp eggs the higher PUFA content was stable until 14 days of shelf-life and decrease at Control eggs levels of PUFA at 28 days of shelf-life suggesting that the antioxidant activity of hemp seed may contribute to the stability of the improved PUFA content in Hemp eggs until 14 days of storage.

Regarding the sensory profile of eggs, our study found that only egg texture was affected by diet. Specifically, the attribute of pastiness was evaluated by trained panellists as less intense in the Hemp eggs compared to Control eggs. This difference may be related to the composition and microstructure of the egg, which in turn may depend on the diet of the laying hens. For instance, Zhu et al. (2019) discovered a distinct microstructure in the yolks of eggs from laying hens that were given two different dietary supplements (oil vs. cottonseed meal). They observed an increase in yolk texture in eggs obtained with cottonseed oil supplementation, which was positively correlated with the increase in SFA. In contrast, Goldberg et al. (2012) found that hemp seeds could increase the amount of PUFA in the yolk, resulting in a softer

yolk texture during chewing. From the analysis of FA profile of eggs (Table 5), in fact, the amount of PUFA was higher in eggs obtained from hens fed Hemp ($P<0.05$).

5. Conclusions

Our results showed that the inclusion of hemp seed cakes in diet of laying hens up to 30% improved their production performance without affecting the chemical composition and the commercial quality of eggs. The fatty acid profile of eggs was also improved not only in term of total, n-3, and n-6 PUFAs amount, but also nutritionally, decreasing n-6/n-3 ratio and increasing PUFA/SFA ratio. Additionally, the higher content of PUFAs in Hemp eggs remains stable until 14 days of shelf-life, after which it decreases to level of commercial eggs.

Moreover, hemp seed cake supplementation did not cause any particular changes in the sensory characteristics of the eggs nor it did not compromise their acceptability.

These results suggest that supplementing laying hens diets with the residual product of hemp seed oil extraction could a new strategy to improve hen performance and the nutritional profile of eggs without detriment to their commercial and sensory quality. However, further studies are necessary to confirm these findings and to understand whether a greater percentage of hemp seed cakes inclusion in laying hens diet can extend the nutritional quality improvement of eggs for up to 28 days of shelf-life.

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General conclusions

As it is well known, diet plays a central role in animal welfare and animal product quality. Nowadays, both of these aspects are strongly considered by consumers.

In the first study, the use of freeze-dried Spirulina as integration in Mediterranean buffalo diet was investigated. Twenty-four lactating buffaloes, divided in two equal groups, were fed the same basal diet and to one group 50 g and 100 g per day of freeze-dried Spirulina was added respectively for 30 days before and 90 days after calving. We studied the effects of Spirulina supplementation on the quality and sensory properties of buffalo mozzarella cheese. As expected, no differences in the chemical composition and quality of mozzarella cheese were detected. The amount of freeze-dried Spirulina was very small, it corresponds to about 1% of the daily feed intake (on dry matter basis). Only a few volatile organic compounds (VOC) were detected in different amount in the samples, but this difference could not only be related to the diet, there is also the cheese making phase that can affects the VOC profile. On the other hand, the sensory profile of buffalo mozzarella cheese was perceived by experienced panellists as different. The experimental mozzarella cheese was found to be brighter, sweeter, more bitter, juicier, more tender, and oilier, with higher buttermilk release, butter odour, and whey flavour than traditional mozzarella cheese. All these differences mean that, although the amount of Spirulina added to the buffaloes diet was very small, it was enough to affect the sensory profile of buffalo mozzarella cheese. These findings are particularly important considering that sensory quality is one of the main drivers for consumers choice in purchasing. Another aspect influencing consumer choice is the nutritional quality of a product. This may explain why consumers expressed their major preference for the experimental mozzarella when provided with information about the nutraceutical properties of Spirulina and its potential beneficial effects on animal health. Furthermore, the consumer test revealed that participants were willing to pay a higher price for the experimental mozzarella cheese in comparison to the traditional one. This confirms the significant impact of information on their expressed preference. Of course, further studies with a higher level of Spirulina are necessary in order to confirm these results and verify if the nutritional quality of buffalo milk and mozzarella cheese can also improve.

In the second study, the use of hemp seed by-products in poultry diet was investigated. Firstly, grade hemp seeds were included in the diet of organically reared broilers. One hundred and fifty medium-growing broilers were divided into three dietary treatments, all receiving the same basal diet formulated in order to contain 0, 15, and 30% of feed-grade hemp seed, for 90 days. The carcass traits and yields, and

breast fillets chemical composition, nutritional and sensory quality of the three groups were evaluated. The diet with 30% of hemp seeds tendentially increased live body weight and carcass weight (not eviscerated) of birds compared to control group, while the 15% inclusion did not affect these traits. Although this difference was detected between the highest level of inclusion and the control group, no difference was detected in terms of dressing percentage or breast and legs yield, indicating that probably the increase in weight can be addressed to inner organs and inedible parts (e.g., head, neck, shanks, and abdominal fat) weights. Considering the chicken breast meat, no difference was recorded among the groups for physico-chemical composition, excepted for the fatty acid profile. The SFA and PUFA content in chicken breast fillets were improved by the higher hemp seed inclusion in broilers diet compared to the control, respectively decreasing and increasing their contents. This finding suggests that 15% of hemp seed inclusion is not enough to improve fatty acid profile of chicken breast fillets, whereas it resulted sufficient to improve the nutritional parameters of breast meat. Thus, the total SFA and PUFA content apparently do not change in breast meat of broilers fed diet with 15% hemp seed inclusion, but actually the individual fatty acid content already improves when the lower level of hemp seed was included, with the consequent improvement of nutritional quality of breast fillets. Additionally, the sensory profile of chicken breast fillet assessed by experienced panellists shows that the inclusion of 30% hemp seed in broiler diet affects the sensory characteristics of breast meat, in particular with a higher overall flavour intensity, and resulting less fibrous, gummy and with lower amounts of residue in the mouth after swallowing. The enhanced nutritional and sensorial quality of broiler breast fillet when fed diet included with 30% of hemp seed suggests that the use of feed-grade hemp seed in organic poultry farm may be a great strategy in order to reduce the amount of highly used and requested feed ingredients (e.g., soybean) and at the same time improve breast meat quality. Further studies with different levels of feed-grade hemp seed inclusion are necessary in order to confirm these results.

Finally, the effects of 30% partially substitution of laying hens diet with hemp seed cakes was investigated on hens laying performance and eggs properties (commercial, nutritional and sensory quality) at 0, 14, and 28 days of shelf-life. As expected, the daily eggs production was higher in hemp group due to the slightly higher total energy content of the experimental diet, whereas the weight and commercial parameters of eggs did not differ between the groups and over the storage time. Instead, the fatty acid profile of eggs yolk resulted affected by the diet, particularly in MUFA and PUFA content, respectively lower and higher in hemp group. In particular, among the PUFAs, α linolenic acid content in hemp group was two times higher than in control eggs, reflecting the fatty acid profile of hens diet. This result confirms the important role of diet directly connected to animal product quality. Additionally, the

PUFA content in hemp group eggs was higher in both n-3 and n-6 PUFAs compared to control group leading in an improvement of the nutritional quality of experimental eggs. These nutritional enhancements lasting over the storage time excepted for total PUFA content that at 28 day of shelf-life decreased to PUFA content of control eggs suggesting that the antioxidant compounds of hemp seed cakes absorbed by hens, and presumably present in eggs yolk, lose their antioxidant activity after two weeks of storage. For what concern the sensory profile of eggs, the inclusion of hemp seed cake did not result in any particular changes and without compromising their acceptability. Only the texture properties were perceived as different by both trained panellists and consumers, with a lower rate of pastiness for experimental eggs and more preferred by consumers. All these findings suggest that the residual product of hemp seed oil extraction is a valuable ingredient for laying hens, improving their laying performance and the nutritional quality of the eggs for at least 14 days of storage without worsening the chemical composition and the commercial quality of the eggs. It might be of interest to investigate with further studies whether increasing the inclusion of hemp seed cakes in the laying hens diet, the nutritional quality of the eggs remains stable throughout the shelf-life.