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Biochemical bases of the activity of phytoextracts and use of microalgae in Veterinary Medicine

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SUMMARY

ABSTRACT

This research is a multidisciplinary exploration of phytoextracts, focusing on the medicinal properties of *Boswellia serrata* extracts and the nutritional efficacy of *Arthrospira platensis*, commonly known as spirulina. The study also evaluates Spirugrass®, a by-product derived from the extraction of phycocyanin from *A. platensis*, for its potential use in animal nutrition.

Through the use of analytical chemistry, statistical analysis and advanced techniques, the research provides a holistic view of the multiple applications of these natural phytoextracts.

The study has evaluated the effects of supplementing Leghorn laying hens with *B. serrata and S. alba* on productivity, welfare and biomarkers. The supplementation did not affect productivity or performance and the welfare status of the hens was maintained. Serum biochemistry analysis showed no significant differences between the control and supplemented groups during the early stages of laying, indicating the safety of supplementation within the posology used. In addition, there were no significant differences in egg white protein and lower cholesterol levels were measured in the treatment group.

The focus on *A. platensis* included biochemical characterisation and exploration of potential applications in veterinary medicine. *A. platensis*, which is rich in essential nutrients, particularly proteins, lipids, carbohydrates, vitamins and minerals, was investigated for iron bioaccumulation and speciation. The research also investigated the bioaccessible iron fraction in A. platensis using an in vitro canine digestion model, providing insights into potential applications in animal nutrition.

The characterisation of Spirugrass® included an assessment of its protein profile and iron content, and the effect of high pressure pasteurisation. Spirugrass® showed potential as a cost-effective ingredient in animal feed, suggesting wider implications for environmental sustainability in livestock production.

In summary, this multi-faceted research expands our understanding of phytoextract applications in veterinary medicine, ranging from animal nutrition to sustainable practices. The use of diverse methodologies and interdisciplinary studies enriches our knowledge of the potential impact of natural products in different areas.

1. INTRODUCTION

1.1 TRADITIONAL MEDICINE AND HERBAL MEDICINE

Plants have been used for therapeutic purposes since millennia by humans, which have developed traditional medical treatments based on phytoextracts. Particularly, traditional Chinese medicine (TCM) has an ancient history of thousands of years confirmed by the oldest herbal text written 2000 years ago. Additionally, other cultures developed a traditional use of medicinal plants based on empiric experiences, such as Ayurvedic medicine that was originally born in India [1, 2].

Phytotherapy is commonly defined as "*the study of the use of extracts of natural origin as medicines or health-promoting agents and it is an allopathic discipline that is focused on the effects aimed to countering symptoms of a specific disease using herbal medicine products*" [3]. Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or a combination of those [4]. Conventional medicines are not fully integrated into the dominant health-care system, including often a paired not-conventional approach as deep breathing exercise, meditation, chiropractic care, yoga and diet-based therapies [4, 5].

The World Health Organization (WHO) estimates that 80% of people worldwide rely on herbal medicines as part of their primary health care [6]. Therefore, the use of herbal medicine is not limited to Eastern countries but has become a worldwide practice, although in those countries a larger use than the rest of the world was present, likely due to economic and cultural reasons and to a more difficult access to classical pharmacology drugs. WHO estimated that in Africa up to 90% and in India up to 70% of the population depend on traditional medicine for their primary healthcare [6]. Hence, due to the above-mentioned reasons, studies investigating the use of herbal medicine has constantly increased in the last two decades: in 2000 the publications regarding this topic on PubMed were 3017 respect the 17282 of 2021, as shown in Figure 1 [7].

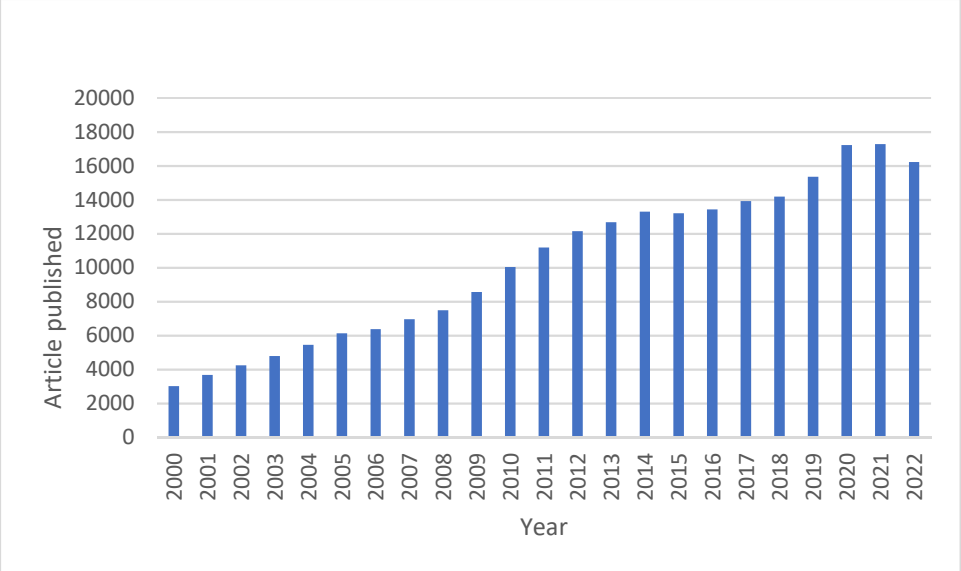

Figure 1. Articles published from 2000 to 2022 found on PubMed using the keyword "herbal medicine" (accessed on 31th of August 2023).

From the 19th century, with the discovery of the first bioactive molecules, a science-based approach started in the use of herbal remedies and this has led to a new era in drug discovery and pharmacological use of single natural compounds, such as morphine, atropine, acetylsalicylic acid used also as precursors of synthetic drugs [8]. The origin of research on the potential pharmacological activity of natural products can be traced to 1805, when the German pharmacist Serturner isolated morphine from opium latex and discovered the better therapeutic properties of the pure compound. Nowadays, plant-derived substances are still an indispensable and considerable source in drug discovery [9].

1.2 LIMITATIONS

However, a fundamental issue that the modern phytotherapy has to face is related to the high variability of the biologically active components in plants, due to different origin, growth conditions, date of harvest. Cultivating medicinal plants in a controlled environment can aid to reduce the variability and allows to monitor the content of secondary metabolites leading to a possible standardization. Nevertheless, the majority of medicinal plants are still harvested from the wild [3]. Moreover, scientific data on safety and mechanism of action are available for few medicinal plants. Although medicinal plants represent an unlimited and attractive source of new bioactive compounds, there is a lack of scientific evidence-based data. Thus, there is an absence of clarity in the herbal medicine market, which is based on products with wide variations in the content of bioactive molecules and pharmacological efficacy [10, 11].

There is a common belief that herbal products are safe, while taking herbal remedies can be harmful and consumers are often not aware of their potential adverse effects [12]. Furthermore, clinical investigation of the effects of plant extracts is often difficult due to the high heterogeneity of the commercial products, different administration protocols and duration of treatments. Therefore, there is a discrepancy between the wide availability of these products and the scientific information often characterized by poor methodology and unreliable clinical analysis [8, 13]. Another important aspect to consider regarding herbal remedies is the contamination due to the presence of pollutants. Of those, many studies report a considerable risk for health due to the presence of toxic metals and metalloids present at concentrations above acceptable regulatory standards [14, 15]. For instance, a review that investigated 88 medicinal plant species detected a Pb content beyond the permissible limits in 21 plant species, Cd in 44 species, and Hg in 10 [16].

1.3 REGULATION

The directive 2004/24/EC provides guidelines for the use of traditional herbal medicines based on an easier registration step called "traditional use registration" for herbal medicines. Particularly, a bibliographical or expert evidence on the effect of the medicinal

product, or a corresponding product which has been used during a period of at least 30 years preceding the date of application, including 15 years of use within the European Community is considered enough for the registration [17]. The above-mentioned directive also gives the task to the Committee on Herbal Medicinal Products (HMPC) to issue scientific opinion on herbal substances and preparations, along with information and recommended uses and safe conditions, on behalf of European Medicine Agency (EMA) and provides monographs to support the therapeutic use of the latter, in order to regularize the European market [17]. Despite many studies claim to solve the lack of evidence-based use of herbal medicine, the European framework has provided a powerful regulation model for the harmonization of scientific assessment and facilitation of product marketing. Furthermore, the use of EU monograph could bring benefits for pharmaceutical industries. Qu et al., (2018) suggest that the European model should be used as an example to establish the legislation in countries with strong traditional use of herbal remedies and can contribute to the safe use of them [18]. Eventually, in 2013 WHO released the WHO Traditional Medicine Strategy 2014-2023, a document reviewed by 20 WHO Collaborating Centres for Traditional Medicine and 22 members of WHO's Expert Advisory Panel on Traditional Medicine. WHO confirmed that many countries have gradually accepted the contribution of traditional and complementary medicine in the healthcare system, although pointed out that it is often underestimated and suggested that a global strategy to foster its integration, regulation and supervision can be useful to countries wishing to develop a policy towards this important part of healthcare [19].

1.4 ETHNOVETERINARY

As reported by Furst et al., 2015, and shown in Figure 2, animal self-medication is a known phenomenon, and it seems that in prehistoric times man has learnt to use certain plants through the observation of animal behaviour [8]. Afterwards, humans started to use herbal medicines for his own healthcare and for animals reared for human purpose. The application of herbal medicines in human and animal health care has a long history that can be traced back over millennia. Veterinary herbal medicines comprise plant-based medicines and their therapeutic, prophylactic, or diagnostic application in animal health care [20]. The application of veterinary herbal medicines is particularly recurrent in rural areas where small holders are unable to spend on health of their livestock, mainly for economic reasons.

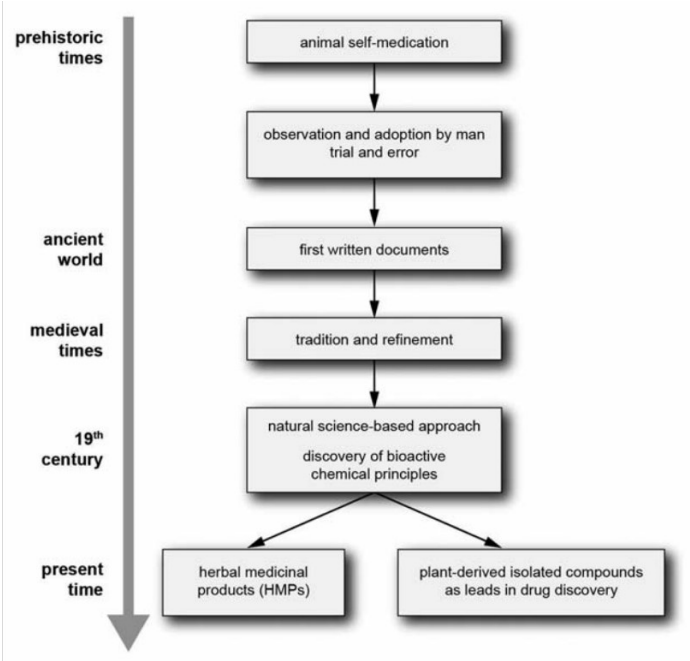

Figure 2. The history of herbal remedies adapted from Furst et al., 2015 [8].

Despite the traditional use of plants in animal care, the definition of ethnoveterinary (EVM) is referred to 1998 as "*the interdisciplinary study of local knowledge and its associated skills, practices, beliefs and social structures pertaining to the healthcare and healthful husbandry of food, work, and other income-producing animals, with an eye to practical development applications within livestock production and livelihood system and with the ultimate goal of increasing human well-being via increased benefits from stock raising*" [21]. Albeit in minor size respect to the herbal medicines for human use, the development of EVM is nowadays characterised by an increase of publications year over year, as reported in Figure 3: in 2000 the publications regarding this topic on PubMed were only two respect the 48 of 2022.

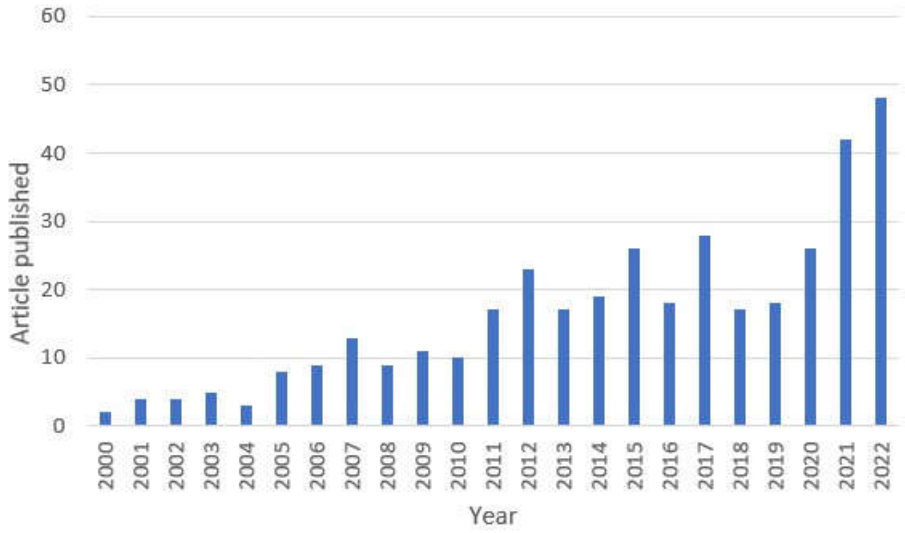

Figure 3. Articles published from 2000 to 2022 found on PubMed using the keyword "ethnoveterinary" (accessed on 5th of September 2023).

Particularly, most of the studies investigating ethnoveterinary are limited to countries where the use of synthetic drugs for livestock health care is unaffordable [22, 23, 24]. This situation led to important insight to face worldwide problems as antibiotic-resistance in livestock. For instance, in the case of bovine mastitis, an infectious disease of the udder tissue mainly caused by Gram-negative and Gram-positive bacteria, the use of plants is of particular importance in order to avoid the rising of antibiotic resistance phenomenon [25, 26]. WHO defined antibiotic resistance as one of the biggest threats to global health, food security and development due to the fact that a growing number of infections are becoming harder to treat as the antibiotic used becomes less effective. Antibiotic resistance occurs naturally, but misuse of antibiotics in humans and animals is accelerating the process [27].

Regarding Europe, the EC regulation for Organic Farming states that phytotherapeutic products should be used in preference to chemically synthesized allopathic veterinary treatment or antibiotics [28]. In Europe, a total of 590 plants species are reported to be used for animal treatment, although ethnoveterinary data are available just in few countries. Of those, the most comprehensive data were available from Italy, Spain and Turkey [29]. Moreover, very few phytotherapic products are currently registered for the treatment of livestock and scientific information regarding veterinary phytotherapy is rare [29]. Phytotherapic products are not authorised centrally in the European Union, but locally by the national institutions. Thus, one of the most significant issues that hampers the application of ethnoveterinary is due to legal uncertainties. Nevertheless, plant materials containing different formulation are allowed to be sold as feed additives with claims concerning optimisation of nutrition, support of physiological conditions, unless they don't contain a claim declaring that they prevent or treat a specific disease of the animals. Therefore, herbal remedies can be sold as feed additives, and they can be used as presumed products with presumed therapeutic properties without a scientific assessment of their efficacy.

The limitations in ethnoveterinary field are quite similar to those of herbal medicines for human use: poor study design, lack of reproducibility, poor standardisation of products, cost-benefit concerns, lack of veterinarian training and poor data availability. As reported by Blanca-Penedo et al., (2018), to overcome these obstacles there is a need to improve study the design in clinical trials and to implement a monitoring system to assess the efficacy of phytotherapic treatments in farm practice [30]. Eventually, the use of phytotherapic remedies in veterinary medicines can be fundamental in the prevention of many diseases and helpful to avoid antibiotic resistance.

1.5 FOOD SUPPLEMENT, NOVEL FOOD AND FEED ADDITIVES

The definition of food supplement was established in the Directive 2002/46/EC of the European Parliament and of the Council of 10th June 2002, recently consolidated in September 2022. Food supplement means "*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional*

or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms to be taken in small unit quantities" [31].

Due to the globalization, an increasing number of foodstuffs enters in the EU market offering to consumers new products, dietary alternatives, and environmentally sustainable sources: this phenomenon led to create the so-called "novel food" category, regulated by the 2015/2283 EU Regulation [32]. The European Food Safety Authority (EFSA) has been involved in the risk assessment of novel food since 2003; however, the implementation of the Regulation above-mentioned in 2018 declared that EFSA is the only EU institution responsible of risk assessment and authorisation to market access of novel foods in European Union [33].

Novel food has been defined as all kind of food that has not been used in an important measure for human consumption in the European Union before the 15th of May 1997, including foods originating from plants (including some algae species), animals, microorganisms, cell cultures, minerals and specific categories of food as insects, vitamins, food supplements, resulting from production processes, practices and state of art technologies intentionally modifies or new molecular structure, nanomaterials which were not produced or used before 1997 [32, 33]. Novel food can be newly developed innovative food, food produced using new technologies and production processes, as well as food which is or has been traditionally eaten outside of the EU. The underlying principles of EFSA evaluation of novel foods are that they have to be safe for consumers, properly labelled (as not to mislead consumers) and it must not differ in a way that the consumption would be nutritionally disadvantageous for the consumer if it is intended to replace another food. The maximum yearly number of novel food applications received by EFSA between 2003 and 2017 was 10 (in 2016), with an average of 5 applications per year; with the implementation of the new regulation, it peaked to 40 in 2018 and 39 in 2019 [33]. Furthermore, in accordance with the number of applications proposed to EFSA, there was an exponential increase of publication regarding Novel foods, as shown in Figure 4.

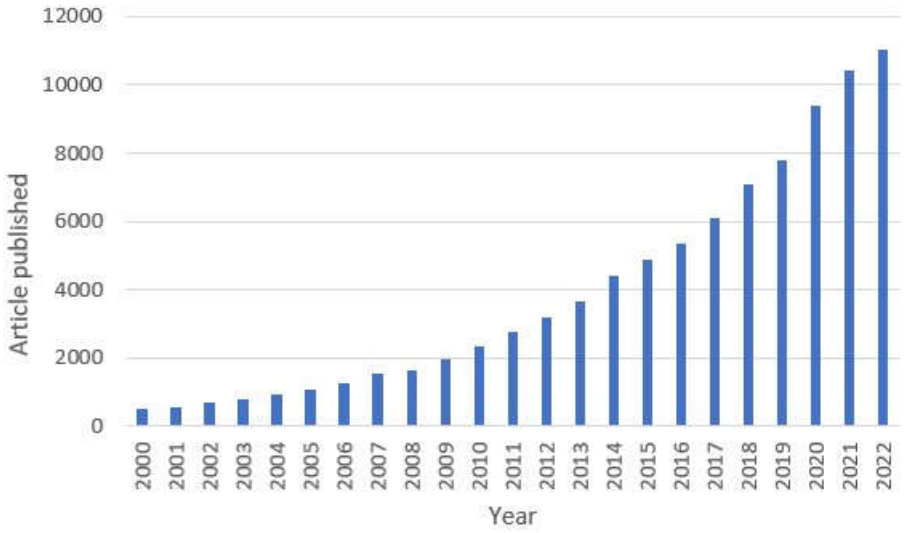

Figure 4. Articles published from 2000 to 2022 found on PubMed using the keyword "Novel food" (accessed on 7th of September 2023).

The Regulation 767/2009 (EC) defined feed additives as *"products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin or to improve the animals' performance and health"*, for example providing enhanced digestibility of the feed materials [34, 35]. Feed additives may not be put on the market unless authorisation has been given following a scientific evaluation demonstrating that the additive has no harmful effects on human and animal health and on the environment [35]. One of the pivotal procedures that allows to put feed additives on the market is the risk assessment conducted by EFSA and the risk management that falls under the responsibility of the EC. Risk assessment is the scientific evaluation of a feed additive, while risk management refers to the final decision on a request for authorisation taking in account the opinion delivered by EFSA. Another step mandatory for obtaining the authorisation is that the applicant must send samples of the additive to the European Union Reference Laboratory for analysis [36]. The Regulation 1831/2003 (EC) amended by the 767/2009 Regulation (EC) classified feed additives in 5 categories: technological, sensory, nutritional, zootechnical additives and coccidiostats or histomonostats [37]. Despite the Regulations for the authorisation process, there is a lack of specification in feed additives catalogue respect to the novel food catalogue used for human purpose that can arise safety concerns. Particularly, for novel food the species must be declared, while in the feed catalogue there is no need of doing it. For instance, in the feed catalogue it's allowed to use algae, dried algae, algae flours, oil and extracts of algae with no definition of the species. Hence, feed additives could present serious concern about animal and human health.

1.6 CHALLENGES

As reported above, plant remedies as herbal medicines have an ancient history of use that reports to ancient times. The first manuscripts about the use of plants and derivates as remedies for healthcare are dated of 2000 years ago, but it's reasonable to suppose that the

first use of medicinal plants begins thousands of years before based on observation of animal behaviour and subsequent adoption by human being. Furthermore, empirical errors and new discoveries increased the experience through time till achieving a wide knowledge of the use of plants as remedies in different cultures and countries. Nowadays, plants are still often used as remedies for health care in humans, as reported by WHO, and also in veterinary medicine. Despite the ancient history, herbal remedies still cause important challenges worldwide, as the need of a standardization of law regulation and analytical methods. Consequently, in order to achieve this goal, an approach based on scientific evidence is needed with emphasis on safety and biological activity of herbal remedies.

Also feed additives in animal nutrition cause challenges regarding the biological activity to promote healthcare and improve the life quality of animals, from livestock to pets. Although the regulation and classification of herbal remedies and feed additives are basically very different, both point out the need of more investigation aimed to produce scientific evidence.

A well-known herbal remedy claimed mainly for its anti-inflammatory properties and used also as feed additive in veterinary medicine is the oleogum resin obtained from *Boswellia* genus. Recent data provided by the Government of India Department of Commerce attest the export of the oleogum resin of *Boswellia serrata* to 177.36 metric tons for a value of half a million US dollars in the three-year period from 2015 to 2017 [38]. The five largest importers of this product from India were Trinidad and Tobago, Germany, Guatemala, Mexico and USA, but many European countries are included in the report as Belgium, France, UK, Italy, and Netherlands. Data regarding the worldwide market of *B. serrata* are difficult to obtain, but it is reasonable to suppose that the commercialisation is more consistent. It has been suggested that the worldwide *Boswellia* market is estimated to reach 86.4 million US dollar by 2027 [39].

Another feed additive of wide interest are microalgae. Microalgae are having a large impact on the worldwide market; the two most commercialised species are *Arthrospira platensis* and *Chlorella vulgaris.* In general, algae can produce several interesting biomolecules for both human and animal nutrition as astaxanthin, lutein, β-carotene, chlorophyll, phycobiliprotein, polyunsaturated fatty acids (PUFAs), beta-1,3-glucans; moreover, they contain high-quality protein, vitamins, and trace elements as zinc and iron. Thus, microalgae have a wide application potential beyond merely be a food source, such as animal feed, biofertilizers, bioremediation, or the extraction of added-value biochemical compounds. The global market of algae was valued at 594 million of euro and probably will reach 1131 million by 2027 [40]. Algae can be a good alternative to traditional crops as they do not require arable land and are able to grow on minimal nutrients.

1.7 AIM of the THESIS

The first aim of the thesis was to investigate the biochemical effects of integrating *Boswellia serrata* (*B. serrata*) and *Salix alba* (*S. alba*) in laying hens, with a primary focus on assessing

changes in the proteomic profiles of serum and egg albumen as potential indicators of treatment efficacy during the critical phase of the onset of animals' production cycle. Protein separation and quantification techniques such as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and agarose gel electrophoresis were used to analyze these proteomic changes. Additionally, the study aimed to evaluate the cholesterol-lowering effect in egg yolks between treated and control groups using colorimetric-enzymatic assays, which provided insights into the potential health benefits of B. serrata and S. alba integration in poultry production systems.

In parallel, research was carried out on algae as novel food sources and for animal supplementation, focusing on *Arthrospira platensis* (*A. platensis*) and *Chlorella vulgaris* (*C.vulgaris*), two dominant species in global algae production and markets. The research explored their role as a supplement for essential trace elements, notably iron (Fe). Iron uptake studies were carried out by cultivating *A. platensis* in media with different Fe concentrations, and comparisons of Fe concentrations among different market samples were made. In collaboration with Professor Giacomo Biagi, the bioaccessibility of Fe from these novel foods was assessed using an in vitro canine digestive simulation, providing valuable insights into their potential efficacy in pet supplementation. Moreover, as part of an international collaboration at IRTA in Monells (Catalunya, Girona), studies in the field of microalgae has been extended through a contribution to the PROFUTURE project led by Dr. Massimo Castellari. This project aimed to incorporate different algae into foods and beverages, evaluating their palatability and promoting algae as a sustainable protein source in the European market. Specifically, the aim of the work focused on the characterization of proteins and the evaluation of Fe content in a by-product derived from the extraction of phycocyanin from intact *A. platensis* 12 samples. This research aimed to explore potential applications in animal nutrition using advanced protein extraction techniques, such as HPLC, followed by Fe quantification using AAS (Atomic Absorption Spectroscopy).

Statistical studies and biostatistical analyses were an integral part of the doctoral research, particularly in the analysis of published and pending articles on the analysis of pyrrolizidine alkaloid in honey samples from different Italian regions. These analyses strictly followed the EFSA (European Food Safety Authority) guidelines, regarding the treatment of missing data and ensuring robust statistical conclusions. Furthermore, meta-analysis and meta-regression techniques were applied to consolidate the results of studies published on the anti-inflammatory properties of *B. serrata* in the treatment of knee osteoarthritis. The aim was to provide conclusive evidence, strengthened by internationally accepted statistical methods, with implications for advancing the understanding of the use of *B. serrata* phytoextracts, including their potential applications in ethnoveterinary research and practice.

2.1 *BOSWELLIA SERRATA*

Boswellia is a genus of plants endemic in Africa, Arabian Peninsula, India, and neighbouring countries; it has been suggested that formerly occurred in Sri Lanka, where it is presumed to be extinct [41]. *Boswellia* is a relatively small genus of 28 species primary used to obtain an oleogum resin known internationally as Indian frankincense or Indian olibanum [42]. The most interesting species are *Boswellia serrata* from India, *Boswellia carterii* and *Boswellia sacra* from Arabian Peninsula and East Africa countries.

*B. serrata (*Figure 5*)* grows at altitudes up to 1150m in regions with annual temperatures between 0 and 45°C and annual rainfall between 500 and 2000mm, in dry tropical deciduous forests, including teak forests, on slopes, ridges and flat terrain. Height is dependent on soil depth and soil fertility, in general the tree can grow up to 15 m. The fruits are three-valved drupes with compressed and pendulous seeds.

Figure 5. *Boswellia serrata tree*

The oleogum resin, an exudate obtained by the incision of bark, occurs in transparent, yellow brown fragrant tears around 5 cm long with a waxy surface. The resin burns easily and produces a characteristic, resinous balsamic smell and taste. The exudate contains 20-36% gum, 56-65% acid resin (up to 43% containing triterpene acids) and 4-8% essential oil and traces of other compounds as lacturonic acid, digitoxose, arabinose, galactose and xylose [42]. More than 200 phytochemicals have been identified from the oleogum resin and the composition depends upon age, quality, geographical distribution, harvestings time, and species. Particularly, of pivotal importance for pharmacological effects, the resin contains pentacyclic triterpenes called boswellic acids, of those Acetyl-11-keto-β-boswellic acid (AKBA) and 11-keto-β-boswellic acid (KBA) are the most relevant (Figure 6). The resin from different species contains different amounts of boswellic acids. An interesting difference was observed between extracts from *B. carterii* and *B. serrata*. Indian samples (*B.*

serrata) contained similar amounts of AKBA and KBA, while African samples (*B. carterii*) contained less KBA than AKBA [43]. Further compounds are tetracyclic triterpenes acids, among which tirucallic acids (Figure 7) have shown a biological effect consisting in 5-LO inhibition, although in less evidence.

Boswellic acids exerts a main anti-inflammatory effect through the inhibition of 5-lipoxygenase (5-LO), an enzyme that leads to the production of leukotrienes by leukocytes. Moreover, boswellic acids act through inhibition of serine protease cathepsin G, microsomal prostaglandin E synthase, cyclooxygenase-2 (COX-2) and nuclear factor-kB (NF-kB) activities. Al-Yasiri et al., (2016) reported that based on *in vitro* studies, *Boswellia* extracts are involved in the inhibition of activation, proliferation and differentiation of B- and T-lymphocytes, tissue destruction, action of natural killer (NK) cells, antibody production and fever, suggesting the immunomodulating and anti-inflammatory effects of boswellic acids [42].

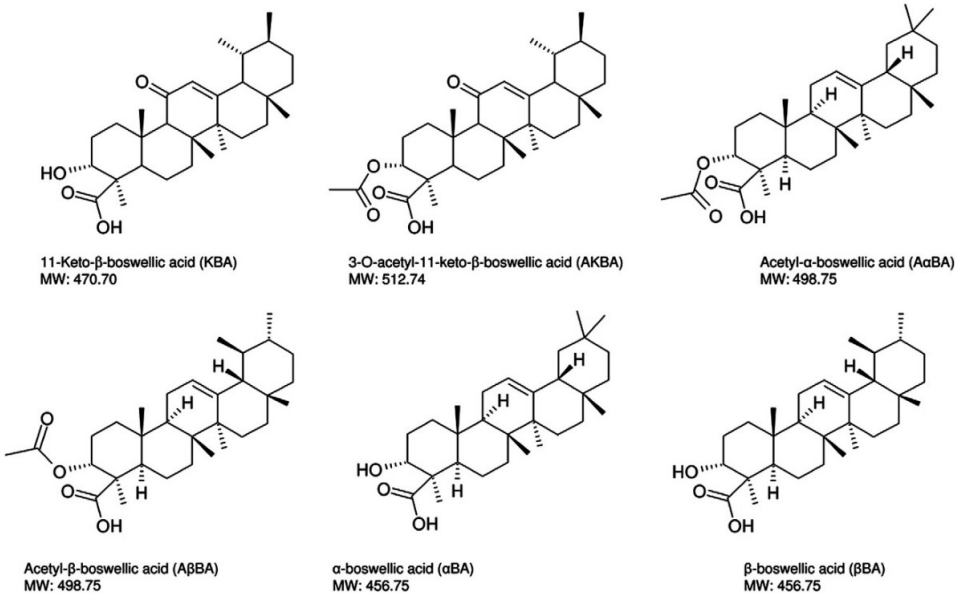

Figure 6. Structure of different triterpenic boswellic acids present in the oleogum resin of plants of genus *Boswellia*, adapted from Gerbeth, 2013 [44].

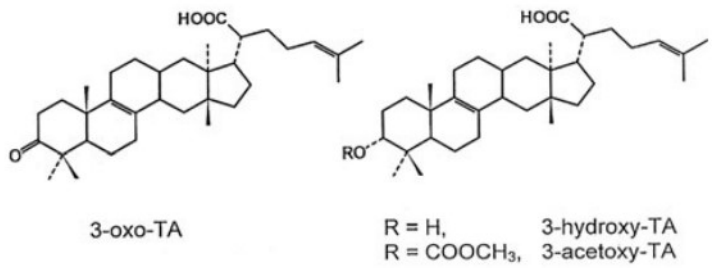

Figure 7. Chemical structure of tirucallic acids from *Boswellia serrata* oleogum resin, adapted from Ammon, 2006 [43].

The inhibition of 5-LO made *Boswellia* extracts useful in the treatment of diseases characterised by an inflammatory condition as ulcerative colitis, irritable bowel syndrome,

bronchitis, sinusitis, osteoarthritis. Extracts of *B. serrata* and *B. carterii* reduce the inflammatory condition of rheumatism trough the inhibition of human leukocyte elastase (HLE) and degrading glycosaminoglycans [43]. In addition, boswellic acids inhibit the proliferation of tumor cells of leukemia and glioblastoma; this anti-tumour effect is due to the inhibition of topoisomerase I, II-alpha and the promotion of apoptosis phenomenon [42].

Boswellia extracts have also been used as feed additives in animals with different aims. Supplementation of *B. serrata* extracts can improve productivity, chemical composition and nutritive value of poultry meat [45]. It has been found that dietary supplementation with *B. serrata* enhances growth, feed efficiency, antioxidant status and minimizes caecal pathogenic bacteria in rabbits [46]. Furthermore, the supplementation with *B. serrata* in dogs with osteoarthritis produced significant beneficial effects alleviating pain and reducing the clinical signs of the disease [47].

Regarding the regulation, *Boswellia* can be used as feed additive for improving welfare and productivity, based on the Regulation 1831/2003 (EC) [37]. Recently, in 2022, EFSA released a scientific opinion on the safety and efficacy of extract from *B. serrata* when used as additive in feed for dogs and horses. The calculated safe concentration was 100mg/kg and 330 mg/kg in complete feed for horses and dogs, respectively.

In this complex scenario, one of the main purposes during the PhD training was to investigate and evaluate the safety and beneficial effects of a supplementation with *B. serrata* extracts in laying hens. The effects of *B. serrata* extracts was studied in the critical production phase, when intestinal inflammation process can occur. Thus, *B. serrata* can represent an alternative valuable option to counteract the inflammation, due to the claimed biological activity of boswellic acids. A full-spectrum analysis was carried out including evaluation of main serum analytes and yield and feed consumption parameters. The research also aimed to evaluate how the supplementation with *B. serrata* could affect the nutritional profile of eggs. The investigation lasted after the ending of supplementation to evaluate possible long-terms effects.

Due to anti-inflammatory activity, *Boswellia* extracts are used in human phytotherapy to treat different disease, as knee osteoarthritis; however, with conflicting results. Hence, statistical analysis techniques as meta-analysis and meta-regression were applied to evaluate the efficacy of *B. serrata* extracts in human patients affected by knee-OA. Meta-analysis was applied in human health field due to the wide number of publications; the study lay the foundation to investigate the efficacy of anti-inflammatory *B. serrata* extracts effects in the treatments of the diseases also in animal health field, leading to possible further research in veterinary medicine.

Guerrini, A., Dalmonte, T., Lupin, C., Andreani, G., Salaroli, R., Quaglia, G., Zannoni, A., Scozzoli, M., Forni, M., Isani, G. Influence of dietary supplementation with *Boswellia serrata* and *Salix alba* on performance and blood biochemistry in free-range Leghorn laying hens. *Veterinary sciences*, **2022**, 9, 182.

In the first paper the safety and the beneficial effects of dietary supplementation with *B. serrata* and *Salix alba* in Leghorn laying hens was evaluated during the critical pre-laying and laying phases through monitoring productive performance, serum analytes, H/L ratio, IgA and anti-IBV antibodies. A total of 120 hens were assigned to two experimental groups, 60 for each group: one of them received a diet supplemented with 5% of *B. serrata* and *S. alba* extracts, the other one received the same feed without extracts and is considered as the control group. The treated group received the feed supplement for 12 weeks. The study lasted 19 weeks. In the last 6 weeks, the treated group received the same feed as the control group. This time point was included in the trial to observe potential long-term effects of the supplementation.

veterinary sciences

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Article

Influence of Dietary Supplementation with *Boswellia serrata* and *Salix alba* on Performance and Blood Biochemistry in Free-Range Leghorn Laying Hens

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Abstract: This study was conducted to evaluate the safety and the beneficial effects of dietary supplementation with *Boswellia serrata* (*Bs*) and *Salix alba* (*Sa*) in Leghorn hens during the critical pre-laying and laying phases. A total of 120 pullets, 17 weeks of age, were assigned to two groups (Control—C; Treated—T, $n = 60$ each). For 12 weeks, the T group received a diet supplemented with 0.3% of dry extracts of *Bs* (5%) and *Sa* (5%). The study lasted 19 weeks. Productive performance, serum analytes, H/L ratio, IgA and anti-IBV antibodies were investigated. Water intake was significantly higher, while body and egg weight was significantly lower for the T group ($p < 0.05$). No other differences were detected in performance parameters, serum analytes, IgA and H/L ratio excluding t0, with a significantly ($p < 0.05$) higher H/R ratio and higher titers of anti-IBV antibody for the T group. Overall, the data obtained in this study show that the supplementation with *Bs* and *Sa* was safe and resulted in an increase in water consumption, a decrease in egg weight, and a sedative effect in the hens. In the future, it would be interesting to test this supplement in hens reared on intensive farms.

Keywords: phytoextracts; Leghorn hen; *Boswellia serrata*; *Salix alba*; free-range

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1. Introduction

In recent years, attention to nutrition-based health strategies and the interest in alternative approaches to reduce antibiotic use in animal production have grown [1,2]. In fact, organic farming, in particular for laying hens, represents an attractive perspective due to the increasing environmental awareness worldwide and the increasing consumer attention to animal welfare [2]. Antibiotic growth promoters (AGP) are useful in enhancing the growth performance and improving the feed efficiency in livestock, including poultry, while at the same time reducing intestinal inflammation due to productive stress [3]. However, the excessive use of AGP in animal diets enhances the development of antibiotic-resistant bacterial strains, which in turn determines negative effects on animal and human health, including dysbiosis [4]. In this regard, phytoextracts are considered an attractive alternative option. Phytoextracts are widely used in modern poultry production to improve welfare and productivity [5]. They include, for example, feed additives also approved for use in poultry production, such as *Boswellia serrata* and *Salix alba* extracts [6].

The resin of *B. serrata* (*Bs*) (Fam. *Burseraceae*), also called Indian olibanum, is obtained from trees native to India and is widely used for the treatment of inflammatory diseases in animals, including those affecting the gastrointestinal tract (e.g., diarrhoea, dysentery, and inflammatory bowel disease), due to the bioactive compounds contained therein. Particularly, boswellic acids pentacyclic triterpenes with 3-acetyl-11-keto-β-boswellic acid (AKBA), 11-keto-β-boswellic acid (KBA) and β-boswellic acid (BA) characterised by the highest biological activities. These compounds are responsible for anti-inflammatory, antiseptic, analgesic, antibacterial, anticancer, hepatoprotective, hypolipidemic, hypocholesterolemic, immunomodulatory, and antiproliferative effects in vitro and in vivo [7–13]. The supplementation of this phytoextract in broiler diets improves the breeding performance and the digestibility of nutrients due to the microbiological stabilisation of the small intestine by the activity of boswellic acids [14,15]. Moreover, some studies have reported the hypolipidemic effects of *Bs* and an improvement in the quality of poultry meat [16–18].

The bark of *S. alba* (*Sa*), known as white willow, has been used since ancient times in folk medicine for the treatment of chronic and acute inflammation, infection, pain and fever. The phytochemical characterisation of the bark extract of this plant indicated that its main component is salicin, a precursor of the anti-inflammatory acetylsalicylic acid [19]. The bark extract also contains phenolic and flavonoid compounds with antioxidant activity [20–22]. In addition to antioxidant and anti-inflammatory properties, willow bark extract is used in weight loss supplements [23], and for its hypocholesterolemic activity [24,25]. Few data are available on the use of *Sa* in poultry species, despite acetylsalicylic acid and sodium salicylate being considered safe for poultry and used in avian medicine [26].

In this study, we evaluated the safety and the effects on performance, behaviour and blood biochemistry of the dietary supplementation with two anti-inflammatory phytoextracts, *Bs* and *Sa*, in white Leghorn laying hens reared with the rural free-range method. In particular, the effects of the two phytoextracts were studied during a critical period of the hens' lifecycle, the transition from pre-deposition to the start of egg deposition, monitoring the production parameters and selected serum analytes. This is the first study focused on the blood biochemistry of the Leghorn breed; therefore, the data obtained can be considered an attempt to collect preliminary information on this important Italian breed.

2. Materials and Methods

2.1. Animals, Study Design and Sampling

A total of 120 17-week-old (120-day-old) pure breed white Leghorn pullets certified *Salmonella*-free, vaccinated for Newcastle Disease and Infectious Bronchitis and non-beak trimmed, were randomly assigned to 2 experimental groups, a Control group (C) and a Treated group (T), of 60 pullets each, housed in 2 single hen houses.

The trial lasted 19 weeks, from mid-August to December and was conducted at a rural farm located in Tuscany, in Mugello (43°59′20.2″ N 11°27′58.1″ E, Florence, Italy). Based on the physiological phases of the hens, the study of the performance was divided into 3 different periods, reported as weeks of observation: (*i*) first phase (1° P—7 weeks), from the supplement administration (1st week) to the start of laying (7th week); (*ii*) second phase (2° P—5 weeks), from the start of laying until the end of the supplementation (8th to 12th week); (*iii*) third phase (3° P—7 weeks) from the stop of supplementation to the end of the trial (13th to 19th week), with hens in laying phase.

Regarding the biochemical analyses, including the evaluation of H/L ratio, 4 blood samplings were performed at different timepoints: at the start of the trial (t0), after 5 weeks of supplementation (t1), at the end of supplementation (t2—12 weeks of observation) and at the end of the trial (t3—19 weeks of observation). Blood samples (2.5 mL) were collected from brachial vein using sterile syringe with 23 G-0.60 mm needle, in clean centrifuge tubes. At each experimental timepoint, the same hens were sampled. Each blood sample was divided into 2 tubes (with and without EDTA as an anticoagulant). A minimum of 0.2–0.3 mL of whole blood was kept in EDTA tubes for the evaluation of Heterophil/Lymphocytes (H/L) ratio. The serum was obtained from the remaining blood

after centrifugation at 1500× *g* at 4 °C for 10 min and stored in 2 mL plastic vials at −80 °C until analysis. From the same birds, IgA were also detected in cloacal cotton swabs and in serum. The swabs were dispensed in 5 mL vials and maintained at −20 °C until analysis.

2.2. Management and Feeding

The 2 groups of animals were managed following the same procedures. Throughout the experiment, the natural photoperiod and temperature were maintained to permit the animals to continue their natural development until sexual maturity and laying. The average environmental temperatures were between 31.0 °C (mid-August) and 28.3 °C (September) and from 14.5–21.7 °C (October) to 5.4–6.5 °C (December). The hen houses were wooden, fenced and closed over the top with an anti-bird of prey net and located inside a wood. In each hen house, the nests for laying (1 nest/5 hens) and 20 cm/hen of perches were available. Each group was separated with a net and each hen house had an external paddock for scratching. The outside paddock consisted of an activity area without pasture or vegetation to eliminate interference with the normal feed intake. The hens had free daytime access to the paddock (from 07:00 a.m. to 16:30 p.m.) until they returned to the hen house at night.

Feeders and plastic water tanks (2 per type outdoor for each group then brought inside the hen house at night, for the first intake of feed and water in the morning) provided *ad libitum* feed and water. On rainy days, to prevent animals from drinking rainwater, they were closed inside hen houses.

For 1 week after housing, both groups received a commercial diet (*Cargill s.r.l.* feed, Table 1) gradually offered to get the animals used to the new diet and minimise diet change stress. Then the T group received a complementary feed for 12 weeks (0.3%) containing 5% of a standardised commercial dry extract of *Bs* (containing 24% of boswellic acids) and 5% of a standardised commercial dry extract of *Sa* (containing 43% of salicin). The combined use of the two extracts can be justified by the possible future use of the supplement as a commercial product due to the synergistic effect of the two phytocomplexes on the inflammatory response: *Bs* extract acts on lipoxygenase, while *Sa* extracts act on cyclooxygenase. Once the integration with phytoextracts was suspended, the feeders and water tanks were carefully washed and disinfected to avoid potential carry-over effects. From the 13th to the 19th week the T group received the commercial diet without supplementation. This period was included in the trial to observe potential long-term effects, including toxic effects, of the supplementation. The C group received the commercial diet without supplements throughout the trial. The animals were not subjected to any medical treatment plan.

2.3. Leghorn Hens' Performance

The performance data were recorded during all the trial periods. At t0, for each experimental group, 8 hens were randomly selected as a sample unit and identified with numbered irremovable rings.

For each group, feed and water consumption and egg production was recorded daily, as feed intake (FI-expressed as g/hen/day), water intake (WI-expressed as mL/hen/day), obtained based on the feed and water residue left and weighed at the end of each day. The number of eggs produced was expressed as *n*° egg produce daily (*n*° eggs/day per group), drawing up a production curve with deposition rate (DR%). Instead, body weight (BW) and egg weight (n = 20 per group, expressed in g) were recorded weekly. Egg mass (Em) was calculated as egg-laying rate × egg weight/100 and feed efficiency (FE) as FI/Em (g/g), whereas feed conversion ratio (FCR) was calculated every week throughout the whole experimental period, from when the laying phase started.

Table 1. *Cargill s.r.l.* feed formulation based on only the indications of the commercial tag and complementary feed composition.

Composition	Values of Nutrients (%/kg of Finisher Diet)	Additives (mg/kg; IU; OTU/kg)	Complementary Feed Composition (%)
Corn; Corn gluten flour; Soybeans meal (* CP 43%); Calcium carbonate; roasted Soybeans; Rice husk; Corn gluten; Wheat bran; Soybean oil	CP, 17%; ** CF, 5%; *** CF, 3.51%; [!] Cash, 13.27%; [ǂ] Ca, 4.02%; [ǂǂ] P, 0.58%; [≠] NaCl, 0.15%; [ˈ] Ly, 0.85%; [$] Met: 0.33%.	Vitamin A, 9950 IU; Vitamin D_3, 2701 IU; Vitamin E, 38 mg; Vitamin K3, 2 mg; Vitamin B1, 1.5 mg; Vitamin B2, 4.5 mg; Vitamin B6, 2.5 mg; Vitamin B12, 0.008 mg; niacin, 35 mg; Ca-D-pantotenate, 10 mg; folic acid, 1 mg; biotin, 0.1 mg; betaine hydrochloride, 250 mg; Cu, 5 mg; anhydrous calcium iodate, 0.50 mg; Mn, 50 mg; Se, 0.075 mg; Zn, 40 mg; Cantaxantine, 2 mg; promoters of digestion 6-phytase, 213 OTU; DL-Methionine, 627 mg	Calcium carbonate, 74.5%; Colloidal silica, 15%; *Salix* [&] DE, 5%; *Boswellia* [&] DE, 5%; Sodium chloride, 0.35%; Magnesium carbonate, 0.15%

* CP: Crude Protein; ** CF: Crude Fats; *** CF: Crude Fibre; [!] Cash: Crude ash; [ǂ] Ca: Calcium; [ǂǂ] P: Phosphorus; [≠] NaCl: Sodium chloride; [+] Ly: Lysine; [$] Met: Methionine; [&] DE: Dry Extract.

2.4. Mortality and Behaviour Observations

The mortality was recorded daily throughout the experiment for each group. During the experiment, specialised technicians controlled the animals several times during the day. Attention was also paid to the establishment of the social hierarchy (pecking order) and laying behaviour. Other observations, such as the attitude to exploit the external paddock and the resourcefulness to explore the external environment, were considered.

The percentage of prolapses was also calculated for each group.

2.5. Health Checks

At 8, 12, and 19 weeks, 10 eggs for each group were tested for *Salmonella* spp. on shell and yolk pool separately. The microbiological test in eggs was performed according to the UNI-EN-ISO 6579-1:2017 procedures. For each group, yolk and shell pools were adequately homogenised in a stomacher. Two hundred twenty-five millilitres of Buffered Peptone Water-BPW at room temperature were added to 25 g of matrix (yolk and shell separately) and incubated at 36 °C for 18 ± 2 h. Afterward, two selective-enrichment liquid media, Rappaport-Vassilliadis Soy-RVS (Oxoid Deutschiand GmbH, Wesel, Germany) and Muller Kaufmann Tetrathionate Novobiocin-MKTTn (Oxoid Deutschiand GmbH, Wesel, Germany) were inoculated with 100 µL and 1.0 mL of culture broth, respectively, and incubated at 41.5 °C and 37 °C for 24 ± 3 h, respectively. In duplicate, a loop-full of broth was streaked on Xylose Lysine Desoxycholate-XLD (Oxoid Deutschiand GmbH, Wesel, Germany) and Brillant Green Agar-BGA (MEUS s.r.l., Piove di Sacco, Padua, Italy) and incubated at 37 °C for 24 h. Colonies referable to *Salmonella* spp., appear pink with or without a black point in the middle of the colony on XLD or BGA medium. At least once a month and for the whole period of the trial, a parasitological exam from a pool of 250 g of faeces was carried out for each group to highlight any parasitic forms.

2.6. Serum Biochemistry

A profile of 13 analytes was chosen, including: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, glucose, cholesterol, triglycerides, total proteins (TP), albumin, globulins, uric acid, calcium, phosphorus. Analyses were performed using available commercial kits (Olympus Systems Reagents),

with an automated biochemical analyser (Olympus AU400, Mishima Olympus Co., Ltd., Shizuoka, Japan).

2.7. Serology for the Detection of Anti-Infectious Bronchitis Virus (IBV) Antibodies

Anti-IBV antibody titers were determined in serum samples using a commercial kit (IDEXX IBV Ab Test-IDEXX), following the manufacturer's instructions.

2.8. Heterophils and Lymphocytes (H/L ratio) Percentages Assessment by Flow Cytometry

Fixation of blood samples was obtained by the addition of IntraPrep Reagent 1 (Beckman Coulter, Life sciences, Indianapolis, IN, USA) according to the manufacturer's instruction (50 µL blood and 100 µL reagent 1). Blood samples were stored at +4 °C for 48 h. Two samples were obtained from each hen as a technical duplicate.

Heterophils and Lymphocytes percentage was assessed using a single staining CD45-APC (Invitrogen, Carlsbad, CA, USA), no-lyse no-wash method, as described by Seliger and colleagues [27]. Briefly, 20 µL of fixed-EDTA-blood were diluted with 980 µL of PBS 1X, then 100 µL of the diluted blood sample were mixed with 0.5 µL of CD45-APC antibody in a tube and incubated for 45 min in the dark at +4 °C. Negative controls, to evaluate inherent background or auto fluorescence, were obtained omitting primary antibodies. Subsequently, 400 µL of PBS 1X were added and samples were kept on ice and protected from light until analyses. From each sample 100 µL were analysed by MacsQUANT® Analyser 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analysed using the Flowlogic software (Inivai Technologies, Mentone Victoria, Australia). To start, not only debris but also artefacts (very high FSC-A) were excluded using forward (FSC-A) vs. side scatter (SSC-A). Doublets exclusion was achieved by plotting FSC-area vs. height (FSC-A/FSC-H). CD45-APC positive singlets population was gated and plotted on forward scatter (FSC) and side scatter characteristics (SSC) as described by Naghizaden and colleagues [28].

In the cytogram derived by this gating strategy, H and L area and percentage were defined, and H/L ratio was calculated.

2.9. IgA Determination from Cloacal Swab and Serum

The IgA extraction from cloacal swabs was performed as reported by Merino-Guzman [29]. Briefly, after thawing samples, 1 mL of PBS (Phosphate Buffered Saline) buffer containing Tween 20 (0.5%) was added to each sample without removing the swabs, vortexed for 30 s and centrifuged at 1500× *g* for 10 min at +4 °C. The supernatants were collected and analysed (dilution 1:100), together with serum sample, by a specific ELISA kit (Chicken IgA ELISA kit Cat. E33-103, Bethyl Laboratories Inc., Montgomery, TX, USA) to evaluate the IgA concentration according to the manufacturer's instructions. Total protein concentration in cloacal samples was evaluated using Total Protein kit (Total Protein kit Micro Lowry Peterson's Modification, Cat. TP0300, Sigma, St. Louis, MO, USA) to normalise the IgA content on protein concentration. Data are expressed as ng IgA/µg total proteins.

2.10. Statistical Analysis

For the evaluation of the performance, the Wilcoxon (Mann–Whitney) Rank Sum test was used to test if samples were drawn from populations with the same distributions, and the Kruskal–Wallis test (KWt) was applied to evaluate if samples were from the same population (as it is a multisampling generalisation of the two-sample Wilcoxon/Mann–Whitney Rank Sum test). The Kruskal–Wallis test was used to test the robustness of the results. The *p*-value obtained from the application of Wilcoxon (Mann–Whitney) Rank Sum test was omitted for the sake of brevity. Therefore, in Table 2, a single *p*-value derived from the application of KWt was reported because the differences obtained between the two experimental groups matched for both tests. The statistical analysis of performance data was performed for each phase (Section 2.1). The data obtained from the Flow Cytometry tests, serology and biochemistry of blood were analysed using the Mann–Whitney test. The IgA data were

analysed using the Kruskal–Wallis test. All tests were applied using STATA®—Statistical Software Package (StataCorp, College Station, TX, USA), version 16 (StataCorp, 2019) and the results were considered statistically significant at $p < 0.05$. The values are reported as mean ± SD (standard deviation).

Table 2. Summary of the evaluations of the performance parameters.

Parameters	T	C	*p*-Value (KWt)
	Mean ± DS	Mean ± DS	
Kg/BW 1°P (1–7 week)	1.0 ± 0.1	1.1 ± 0.1	$p > 0.749$
Kg/BW 2°P (8–12 week)	1.5 ± 0.1	1.5 ± 0.1	$p > 0.601$
Kg/BW 3°P (13–19 week) ″	1.7 ± 0.0	1.8 ± 0.0	* $p < 0.004$
FI 1°P (1–7 week) **	71.9 ± 18.5	70.2 ± 17.6	$p > 0.100$
FI 2°P (8–12 week)	115.8 ± 16.8	109.5 ± 16.9	$p > 0.077$
FI 3°P (13–19 week)	139.8 ± 8.3	139.4 ± 8.9	$p > 0.970$
FI (1–19 week)	108.3 ± 33.2	105.3 ± 33.4	$p > 0.516$
WI 1°P (1–7 week) ***	140.1 ± 35.5	125.6 ± 38.2	* $p < 0.013$
WI 2°P (8–12 week)	208.5 ± 46.3	163.0 ± 46.6	* $p < 0.000$
WI 3°P (13–19 week)	251.4 ± 32.0	251.2 ± 34.1	$p > 0.929$
WI (1–19 week)	198.7 ± 61.2	181.2 ± 67.7	* $p < 0.013$
FCR (1–7 week) [!]	1.7 ± 1.0	1.2 ± 0.4	$p > 0.609$
Feed Efficiency (8–19 week) [£]	2.8 ± 0.7	3.3 ± 1.7	$p > 0.508$
Egg mass (2–12 week/laying) [#]	50.1 ± 10.7	46.3 ± 15.0	$p > 0.921$
n° eggs/day/lay (1–12 week/laying)	46.6 ± 17.9	44.4 ± 18.0	$p > 0.246$
n° total egg/group (1–12 week/laying)	3924	3559	$p > 0.068$
Mean DR% (2–12 week/laying) [$]	83.4 ± 26.6	75.3 ± 31.8	$p > 0.418$
Egg weigth ($n = 20$/group) (3–12 week/laying) [&]	59.9 ± 2.5	61.0 ± 3.4	* $p < 0.000$

″: body weight expressed as kg/BW; **: Feed Intake, expressed as g/day/hen; ***: Water Intake, expressed as (mL/day/hen); [!]: Feed Convertion Ratio; [£]: Feed Efficiency = Feed Intake/Egg mass (g/g); [#]: Egg mass = (egg production × egg weight)/100; [$]: Deposition rate (DR%); [&]: weight expressed in g; *: statistically significant at $p < 0.05$.

3. Results

3.1. Leghorn Hens' Performance

Feed and water consumption (FI and WI)—The data on feed and water consumption show statistically significant differences in the different phases of the study and are reported in Table 2.

In the first phase (1° P—7 weeks), during the first 7 weeks of observation, from the start of the trial to the start of laying, a significantly higher water consumption ($p < 0.05$) was measured for the T group, with mean values of 140.1 ± 35.5 mL/hen/day and 125.6 ± 38.2 mL/hen/day for the T and C group, respectively. On the other hand, the feed consumption did not show statistically significant differences between the two groups ($p > 0.05$) with mean values of 71.9 ± 18.5 g/hen/day and 70.2 ± 17.6 g/hen/day for the T and C group, respectively.

In the second phase (2° P—5 weeks), from the start of laying to the end of supplementation, which coincided with the early stage of the laying period for both groups, for water consumption, statistically significant differences were found between the groups. For the T group, the consumption of water was statistically higher compared to the C group ($p < 0.05$), with mean values of 208.5 ± 46.3 mL/hen/day (Table 2). For feed consumption, however, no significant difference was found between the groups (Table 2).

In the third phase (3° P—7 weeks) from the end of supplementation to the end of the trial, no significant differences in water and feed consumption were found. Overall, during the whole period of the trial, independent of the physiological phase of the hens, the T group had a significantly higher water consumption than the C group ($p < 0.05$), but not for feed (Table 2). For both groups, the mean daily feed consumption was 108.3 ± 33.2 g/hen/day and 105.3 ± 33.4 g/hen/day for the T and C groups, while the water consumption was 198.7 ± 61.2 mL/hen/day and 181.2 ± 67.7 mL/hen/day, respectively.

Body weight (kg-BW), FCR and FE—The BW was not significantly different, with the exception of the values recorded in the third period, between the 12th and 19th week of observation (3° P—7 weeks), where a statistically significant difference was highlighted ($p < 0.05$), with a mean value of 1.7 ± 0.0 kg-BW and 1.8 ± 0.0 kg-BW for the T and C groups, respectively. The FCR in the pre-laying phase from the 1st to the 7th week of observation was 1.7 ± 1.06 and 1.2 ± 0.40 for T and C, respectively, and not statistically different (Table 2). From the 8th to the 19th week of the trial, the hens were in the laying phase and FE recorded mean values of 2.8 ± 0.7 g/g for the T group and 3.3 ± 1.7 g/g for the C group, but these were not statistically different ($p > 0.05$) (Table 2).

Egg production—The egg production curve is reported in Figure 1. The egg-laying started in the 8th week of the trial for the T group and 6 days later for the C group (Table 2). It can be noted that the T group, already showed an increase in weekly eggs laid from the 1st week and a relative DR% greater than the C group (Figure 1). Between the 13th and 14th week of observation, corresponding to the 5th and 6th week of laying, the T group reached its productive peak with a DR% between 95% and 98.3%, while the C group reached its highest rate only at the 7th week of laying, with a DR% of 98.3%. From the 6th week to the 12th week of laying, a >95% weekly DR% was recorded, and from the 1st to 12th week of laying there was a regular increase in egg mass (Em) equal to 50.1 ± 10.7 and 46.3 ± 15.07 for the T and C groups, respectively (Table 2). However, for Em and FE, no statistically significant differences were found between the groups ($p > 0.05$). The daily mean number of eggs produced per group was 46.6 ± 17.9 and 44.4 ± 18.0 for the T and C groups, respectively, while the total number of eggs produced was 3924 vs. 3559 for the T and C groups, respectively. Significant differences were detected in the egg weight. In fact, the weight of the eggs produced by the T group was statistically lower than the weight of the eggs of the C group ($p < 0.05$), with mean egg weight values of 59.9 ± 2.5 g and 61.0 ± 3.4 g, respectively. No cases of oviduct prolapse were detected in both groups.

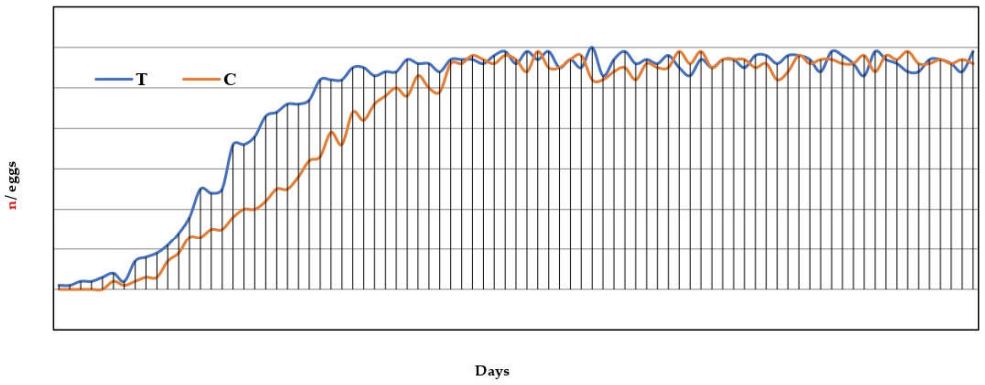

Figure 1. Egg production curve for a total of 12 weeks of laying, corresponding to the 7th to 19th week of the trial, expressed as day of laying. T = supplemented hens ($n = 60$); C = control hens ($n = 60$).

3.2. Mortality, Behaviour Observations and Health Checks

Throughout the trial, no mortality and cases of pica and cannibalism were recorded. In the T group, no abnormal behaviour was observed in feed intake, despite the perceptible and typical smell of phytoextracts. However, after 12 days of supplementation, a change in behaviour was observed in the hens of the T group, characterised by drowsiness, reduced reactivity and reduced restlessness. The peaks of inactivity were characterised by stopping on the perch or on the litter, in a position with the head under the wing, which was found at consistent times from approximately 9.30 a.m. to 15.00 p.m. However, this behaviour

did not affect FI and WI (Table 2). The behaviour was maintained until the integration was stopped. Six days after the suspension of the supplement, the hens assumed the typical normal behaviour of the breed. In the C group, no abnormal behaviour occurred, and the FI and WI were constant. Both groups were monitored once a month for the presence of intestinal parasitic forms, such as Salmonella spp. infection, always resulting in a negative and with an isolation rate of 0.00%.

3.3. Serum Biochemistry

The effect of dietary supplementation on selected serum analytes is reported in Table 3. No significant differences were determined for the analysed serum analytes between the T and C groups, with few exceptions. Albumin at t0 and t2 was significantly higher ($p < 0.05$) in the C group with respect to the T group, ALT at t3 was significantly higher ($p < 0.05$) in the T group than in the C group, and glucose at t0 was significantly higher ($p < 0.05$) in the T group than in the C group. On the other hand, significant differences ($p < 0.05$) were determined over time (t0 vs. t1 vs. t2 vs. t3) in both groups due to physiological development and laying activity: a significant decrease in hepatic enzyme activity as AST and ALP, also glucose concentration and a significant increase in triglycerides, total proteins, albumin, and Ca concentrations were observed. The analysis of these differences is out of the scope of the present research.

Table 3. Serum biochemical analytes in Leghorn hens. Data are reported as mean ± SD ($n = 8$ for each group and at each. Timepoint: at the start of the trial (t0), after 5 weeks of supplementation (t1), at the end of supplementation (t2—12 weeks) and at the end of the trial (t3—19 weeks).

Serum Analytes	t0		t1		t2		t3		From Reference [§]
	C	T	C	T	C	T	C	T	
AST (U/L)	270 ± 40.0	243 ± 21.94	216 ± 20.4	207 ± 12.5	221 ± 25.8	207 ± 17.7	214 ± 16.3	217 ± 25.1	118–298 [30]
ALT (U/L)	1.25 ± 0.46	1.71 ± 0.76	1.88 ± 0.64	2.50 ± 0.93	1.57 ± 0.53	1.38 ± 0.52	1.63 ± 0.74	2.88 ± 1.25	25.2 ± 7.77 [31]
ALP (U/L)	1159 ± 310	1510 ± 367	713 ± 182	673 ± 98.5	730 ± 167	595 ± 209	480 ± 206	552 ± 236	407 ± 39.84 [31]
Bilirubin (µmol/L)	0.17 ± 0.00	0.34 ± 0.17	0.88 ± 0.68	0.34 ± 0.17	0.34 ± 0.32	0.34 ± 0.17	0.51 ± 0.34	0.51 ± 0.51	–
Cholesterol (mmol/L)	3.42 ± 0.17	3.16 ± 0.35	2.98 ± 0.17	2.92 ± 0.31	3.19 ± 0.55	3.00 ± 0.71	2.98 ± 0.74	3.32 ± 0.62	3.37 ± 0.35 [31]
Triglycerides (g/L)	1.05 ± 0.49	0.90 ± 0.21	1.29 ± 0.19	1.15 ± 0.10	12.6 ± 3.38	12.6 ± 4.63	12.29 ± 5.94	17.36 ± 3.46	13.6 ± 3.56 [31]
Glucose (mmol/L)	12.9 ± 1.23 *	14.3 ± 0.74 *	8.63 ± 0.79	9.35 ± 1.21	10.78 ± 0.49	11.2 ± 1.32	7.20 ± 1.72	7.70 ± 1.96	9.41–13.6 [30]
Total proteins (g/L)	42.7 ± 3.13	39.3 ± 2.92	48.4 ± 3.62	46.2 ± 4.11	48.7 ± 4.85	46.8 ± 4.29	45.9 ± 3.40	48.3 ± 4.09	39–70 [30]
Albumin (g/L)	16.3 ± 1.03 *	15.0 ± 0.80 *	19.2 ± 1.19	18.4 ± 1.46	18.7 ± 1.10 *	17.5 ± 0.75 *	18.3 ± 0.82	18.8 ± 0.84	15.0–33.0 [30]
Globulins (g/L)	25.8 ± 1.85	26.2 ± 4.92	29.2 ± 2.58	27.8 ± 3.17	30.0 ± 4.01	29.2 ± 4.15	27.7 ± 2.70	29.5 ± 3.34	16.0–43.0 [30]
Albumin/ Globulins	0.65 ± 0.06	0.61 ± 0.05	0.66 ± 0.03	0.67 ± 0.07	0.63 ± 0.06	0.61 ± 0.07	0.67 ± 0.05	0.64 ± 0.05	–
P (mmol/L)	1.78 ± 0.32	1.53 ± 0.19	2.01 ± 0.18	1.92 ± 0.23	2.01 ± 0.17	2.07 ± 0.21	2.17 ± 0.37	2.33± 0.42	0.52- 2.33 [30]
Ca (mmol/L)	2.65 ± 0.34	2.98 ± 0.20	2.70 ± 0.21	2.46 ± 0.43	6.10 ± 0.41	6.40 ± 0.63	6.75 ± 0.10	7.25 ± 0.64	> 2.73 [30]
Uric acid (mmol/L)	0.30 ± 0.06	0.28 ± 0.06	0.42 ± 0.08	0.39 ± 0.08	0.37 ± 0.11	0.33 ± 0.09	0.39 ± 0.12	0.30 ± 0.12	0.05–0.53 [30]
IgA (g/L)	0.72 ± 0.22	0.83 ± 0.09	0.70 ± 0.01	0.71 ± 0.08	0.70 ± 0.00	0.65 ± 0.03	0.63 ± 0.00	0.51 ± 0.25	–

*: statistically significant at $p < 0.05$.; [§]:- when data in the reference were not expressed in SI unit, they were converted to SI unit.

3.4. Anti-IBV Antibody Titers

ELISA anti-IBV antibody titers detected in four samples per experimental group are reported in Figure 2. No significant differences were observed between the C and T groups ($p > 0.05$) with the sole exception of t0 when the hens of the T group presented significantly higher anti-IBV antibody titers ($p < 0.05$).

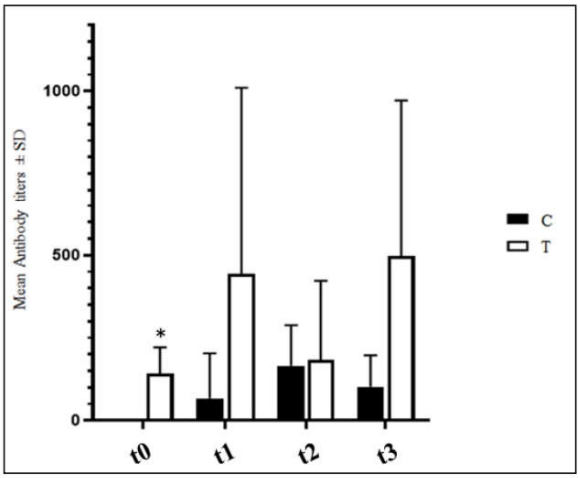

Figure 2. Mean IBV antibody titers ± SD detected in C and T groups. T = supplemented hens ($n = 60$); C = control hens ($n = 60$); t0 = start of the trial; t1 = after 5 weeks of supplementation; t2 = end of supplementation; t3 = end of the trial. *: statistically significant at $p < 0.05$.

3.5. Heterophils and Lymphocytes (H/L) Ratio

Cellular debris located at the lower-left corner of the cytogram (forward versus side scatter plot) was excluded from the analysis. Doublets have double the area values of single cells whilst the height is roughly the same and so disproportions between height and area were used to eliminate doublets. The singlets' population was evaluated for the expression of the CD45 pan-leucocytes antigen. It is important to point out that the nucleated erythrocytes appeared as a smear on the left and, as reported by Seliger and co-workers [27] on whole blood staining, the autofluorescence of erythrocytes leads to a relatively high background fluorescence of the CD45 negative population, which overlaid the signal of the CD45 low thrombocytes (Figure 3). By selecting the population with the highest APC fluorescence values and excluding, in this fluorescence range, the population with the lowest SSC values (low complexity cells) thrombocytes could be excluded. The positively selective population was evaluated based on the morphological parameters and lymphocytes (low FSC and SSC) and heterophils (high FSC and SSC) areas were identified (Figure 3). This gating strategy was applied to all samples analysed by Flow Cytometry, heterophils and lymphocytes percentages and the H/L ratios were calculated. At t0, control animals had an H/L ratio value of 0.4. No significant differences were detected between the T and C groups, with the sole exception of t0, when the T group presented an H/L ratio value significantly higher than that of the C group ($p < 0.05$) (Figure 4). At t1, the H/L ratio of the T group significantly decreased ($p < 0.05$) returning to control values (≤ 0.4).

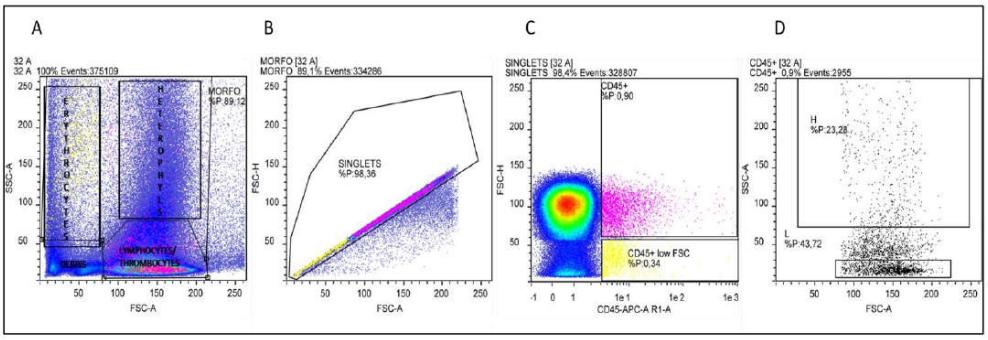

Figure 3. Results of gating strategy in Flow Cytometry analysis. (**A**) Representative cytogram (FSC-A versus SSC-A): debris is located at the lower-left corner of the cytogram, nucleated erythrocytes appear as a smear on the left, in the lower-right area lymphocytes and thrombocytes are placed, in the upper-right area heterophils are contained. (**B**) FSC-A/FSC-H plot for doublets exclusion: singlets have a proportion between area and height values while doublets have double the area values of single cells whilst the height is the same. (**C**) Singlets were analysed for CD45-APC fluorescence intensity. In the area with the highest APC fluorescence, two populations can be identified: a population with low SSC values (yellow) and a population with higher SSC values (pink) (**D**) CD45-positive cells cytogram in which lymphocytes and heterophils areas are selected.

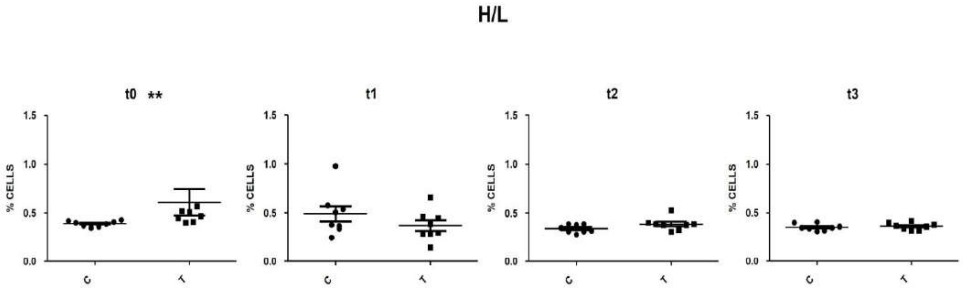

Figure 4. Scatter plot of H/L ratio in C and T group at t0, t1, t2 and t3. **: statistically significant ($p < 0.01$). T = supplemented hens ($n = 60$); C = control hens ($n = 60$); H/L = heterophils/ lymphocytes ratio; t0 = start of the trial; t1 = after 5 weeks of supplementation; t2 = end of supplementation; t3 = end of the trial.

3.6. IgA Concentration in Faecal Swabs and Serum Samples

In all samples, it was possible to detect the IgA concentration. The IgA faecal content showed a wide variability among animals and among timepoints (Figure 5). No significant differences were observed in faecal IgA between the C and T groups and during the timepoints ($p > 0.05$).

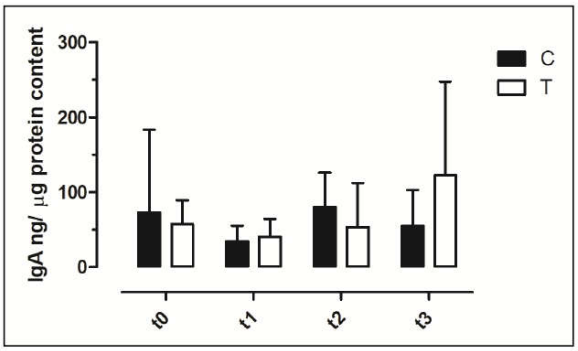

Figure 5. Concentration of Immunoglobulin A (IgA) in faecal swabs. The data are reported as mean ± SD. T = supplemented hens (n = 60); C = control hens (n = 60); t0 = start of the trial; t1 = after 5 weeks of supplementation; t2 = end of supplementation; t3 = end of the trial.

4. Discussion

Investigations carried out on a variety of domestic animals have confirmed the multiple effects of phytoextracts on nutrition, health improvement and productivity [5,14,32,33]. However, studies on the use of *Sa* and *Bs* extracts in poultry are scarce or even lacking in laying hens. This research can be considered the first attempt focused on the effects of the combination of the two phytoextracts in free-range Leghorn hens.

Leghorn Hen Performance and Behaviour—This study indicated a significant effect of supplementation on performance. Significant differences were found between the two experimental groups in the consumption of water, body weight and egg weight. These differences were evident particularly during the second period of the trial when supplementation overlapped with the start of laying. Hens from the T group showed significantly higher consumption of water and started the laying phase 6 days before the hens of the C group. During the laying phase, a greater consumption of water is considered normal, but nevertheless, the hens of the T group consumed more water compared to the C group. The presence of additional NaCl in the complementary feed, though in a very low percentage (0.35%), might have contributed to the increased water consumption in the T group. The production peak and the deposition rate of 100% were reached faster by the supplemented hens, despite the fact that they demonstrated slowed development of their secondary reproductive somatic characteristics (crest and wattle development) 15 days following the administration of the supplement. This observation in hens supplemented with *Bs* and *Sa* is not documented by other studies and remains unexplained.

The results of the present research indicated that the supplementation of *Bs* and *Sa* in the diet significantly improved egg production during the first 5 weeks of laying, and hens from the T group produced a higher number of eggs than those of the control group. Significant improvement in egg production was also reported in hens that were fed diets supplemented with pre/pro-synbiotics [31]. However, at the end of the trial, the eggs of the T group had a significantly lower weight than those of control hens.

In the 3° P, the BW was significantly lower for hens of the T group, possibly due to the effects of the supplement. Data on the effect of *Bs* extract on BW are not available in the literature, while willow bark extract is used in weight loss products for humans, frequently in association with other phytoextracts, such as extracts of green tea and ginger. Accordingly, Pozniak and a co-worker found that the BW gain decreased in broilers when the feed was supplemented with 0.04% of acetylsalicylic acid; this effect is presumably related to anti-inflammatory and analgesic activities of the phytocomplex [26]. However, a paucity of controlled clinical trials has been performed to evaluate the efficacy of willow

bark extract and the use in association with other extracts hampers the evaluation of its direct efficacy on weight loss [23].

The behavioural changes observed in the hens of the T group are interesting; in fact, the psychoactivity of *Boswellia* extract has been recognised since ancient times. Accordingly, sedative and hypnotic effects were recently reported in mice treated with silver nanoparticles loaded with AKBA, one of the most active terpenes isolated from the oleogum resin of different species of the genus *Boswellia* [34]. Moreover, Moussaieff and co-workers showed that incensole acetate, a diterpene present in *Boswellia* resin, is an agonist of the ion channel transient receptor potential vanilloid (TRPV) [35]. The activation of the channel determined anxiolytic-like and antidepressive-like behavioural effects in mice. Finally, Okano and colleagues reported that *Boswellia* essential oil counteracts the negative effects of stress by effectively relieving sleep debt in rats [36]. The increased drowsiness and the reduced reactivity observed in the hens of the T group during the supplementation might be due to the combined effects of the aforementioned active molecules and add additional in vivo evidence of the sedative effects of *Bs* extracts.

Blood Biochemistry, H/L ratio and Faecal IgA—To the author's knowledge, data on blood biochemistry in Leghorn pure breed are limited to AST, ALT, uric acid and creatinine [37]; therefore, the present results will be discussed in the framework of knowledge also obtained in other chicken breeds. Recently, Board and co-workers [30] reported the biochemical reference intervals for backyard hens (Table 3). The data obtained in this study fall in those reference intervals with the sole exception of glucose at t1 in the T group, which is higher than the upper limit reported by Board and colleagues [30], but it falls in the reference interval reported for Lohman silver laying hens by Ding and co-workers [38]. The activity of enzymes in the serum provides important information on the integrity and functionality of specific organs. In birds, the activity of serum AST is considered the most reliable biomarker of hepatic function [37]. However, serum AST can also originate from muscles; consequently, in this study, two other enzyme biomarkers of hepatic function, namely ALT and ALP, were also analysed. The supplementation did not negatively affect the activity of these enzymes, suggesting the absence of hepatotoxicity. Similar results were reported in Leghorn hens supplemented with silkworm pupae [39], and in Lohmann LSL-Lite and Lohmann Brown-Lite laying hens supplemented with *Chondrus crispus* and *Ascophyllum nodosum* [40]. In birds, uric acid, the major end product of nitrogen catabolism, is excreted by the kidneys and is considered a biomarker of renal function. The data obtained in this study fall within the interval reported for laying hens [30] and no significant variations were detected between the C and T groups, suggesting the absence of impairment of renal function. Despite the well-recognised hypoglycaemic and hypocholesterolemic effect of *Bs* extracts in mammals [41], in the present study, no significant reduction of glucose and cholesterol was observed in the serum of hens fed the supplemented diet. Accordingly, no effect of *Bs* on the serum lipid profile was noted by Kiczorowska and co-workers [18] in broilers, and the concentration of serum cholesterol did not show significant differences between broilers fed a control diet and broilers fed diets supplemented with 0.025% and 0.05% *Sa* extracts [42]. Regarding glucose, no significant differences were reported in broilers fed diets supplemented with 3, 4, and 5% *Bs* [5], whereas a significant decrease in serum glucose was observed in broilers fed diets supplemented with 0.025% and 0.05% *Sa* [42]. To the author's knowledge, no studies are reported for laying hens.

The H/L ratio has become widely accepted as an indicator of stress responses in poultry [43,44] since Gross and Siegel [45] first found decreasing numbers of lymphocytes and increasing number of heterophils in response to different physiological stressors. Gross and Siegel concluded that the H/L ratio was a more reliable parameter than plasma corticosteroids for the measurement of stress in poultry and that it was also less variable than total cell numbers. The use of haematology analysers in mammalian veterinary medicine has led to a considerable reduction of labour costs with more reliable results compared to traditional microscopic procedures. Haematological peculiarities of birds, in particular nucleated erythrocytes and thrombocytes, have precluded the successful analysis

of avian blood samples by current haematological analysers [27,46]. Flow Cytometry protocol utilised in this study proved to be effective as the H/L values obtained are comparable to those reported in the literature for healthy non-stressed chickens [47]. The random difference existing between the two groups at t0 showed a higher H/L ratio in the T than in the C group, with a value higher than the indicative value of 0.4 reported in the literature for non-stressed birds [45,48]. At t1, following the supplementation, in the hens from the T group, the H/L ratio significantly decreased, returning to values < 0.40. These observed changes could be suggestive of a potential beneficial effect from the *Bs* and *Sa* extracts.

Three classes of immunoglobulins (IgY, IgA and IgM) are expressed in chickens, and IgA play an essential role in mucosal defense [49]. As IgA are the major antibody component of the gastrointestinal mucosa, the IgA content in the faeces can be considered a marker of the local immune system and an increase could be due to enhanced protection against pathogens. However, increased IgA concentrations might also represent a response to increased antigenic stimulation without enhanced immunity or to the body's protective mechanism against a harmful stimulus [50]. Different papers reported the possibility to quantify IgA in the intestinal lumen [51], faecal samples [52] and cloacal swabs [29]. The concentrations of IgA measured in this research showed wide variations among animals and timepoints with a mean value at t0, if expressed in ng/mL, 10 times higher than the value reported in the study by Merino-Guzman [29]. The difference may be due to the different management of animals and the diet utilised. Due to the difference in cloacal swab sampling, in terms of the amount of faeces present on the swab, we decided to quantify the protein content of each sample and express the IgA data as ng/µg protein. As faecal IgA concentration was used to evaluate the effect of different dietary supplementation on mucosal defense in poultry [52,53], we investigated the IgA content in cloacal samplings and no significant change after dietary supplementation was observed.

5. Conclusions

Overall, the supplementation with *Bs* and *Sa* resulted in a significant decrease in egg weight accompanied by a reduction of BW. In addition, reduced activity occurred during the supplementation, suggesting a possible sedative effect of the supplement. The authors are aware that the use of a supplement containing two different phytoextracts has made the discussion of the results particularly complex because it was not possible to determine the specific contribution of the single extract. However, in the author's opinion, it is important to test the efficacy and safety of supplements that often contain mixtures of different phytoextracts. Nonetheless, the supplementation was safe, did not compromise the productivity and the performance of the treated group and maintained the welfare status. Finally, it should be evidenced that hens reared with a free-range method have a potentially better welfare status compared to the hens reared with traditional intensive methods. In the future, therefore, it would be interesting to also test this supplement in laying hens reared on intensive farms.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Trovato, G.M. Behavior, nutrition and lifestyle in a comprehensive health and disease paradigm: Skills and knowledge for a predictive, preventive and personalized medicine. *EPMA J.* **2012**, *3*, 8. [CrossRef] [PubMed]
2. Forte, C.; Acuti, G.; Manuali, E.; Proietti, P.C.; Pavone, S.; Trabalza-Marinucci, M.; Moscati, L.; Onofri, A.; Lorenzetti, C.; Franciosini, M.P. Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens. *Poult. Sci.* **2016**, *95*, 2528–2535. [CrossRef] [PubMed]
3. Dibner, J.J.; Richards, J.D. Antibiotic growth promoters in agriculture: History and mode of action. *Poult. Sci.* **2005**, *84*, 634–643. [CrossRef] [PubMed]
4. Castanon, J.I.R. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poult. Sci.* **2007**, *86*, 2466–2471. [CrossRef] [PubMed]
5. Al-Yasiry, A.R.M.; Kiczorowska, B.; Samolińska, W.; Kowalczuk-Vasilev, E.; Kowalczyk-Pecka, D. The effect of *Boswellia serrata* resin diet supplementation on production, hematological, biochemical and immunological parameters in broiler chickens. *Animals* **2017**, *11*, 1890–1898. [CrossRef] [PubMed]
6. Council of the European Union; European Parliament. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition (Text with EEA Relevance), CELEX1. Available online: http://op.europa.eu/en/publication-detail/-/publication/c746ceb6-1330-4b77-9a2e-5a7705b22c33 (accessed on 19 January 2022).
7. Ammon, H.P.T. Modulation of the immune system by *Boswellia serrata* extracts and boswellic acids. *Phytomedicine* **2010**, *17*, 862–867. [CrossRef]
8. Azemi, M.E.; Namjoyan, F.; Khodayar, M.J.; Ahmadpour, F.; Padok, A.D.; Panahi, M. The Antioxidant Capacity and Anti-diabetic Effect of *Boswellia serrata* Triana and Planch Aqueous Extract in Fertile Female Diabetic Rats and the Possible Effects on Reproduction and Histological Changes in the Liver and Kidneys. *Jundishapur J. Nat. Pharm. Prod.* **2012**, *7*, 168–175. [CrossRef]
9. Hartmann, R.M.; Fillmann, H.S.; Martins, M.I.M.; Meurer, L.; Marroni, N.P. *Boswellia serrata* has Beneficial Anti-Inflammatory and Antioxidant Properties in a Model of Experimental Colitis. *Phytother. Res.* **2014**, *28*, 1392–1398. [CrossRef]
10. Umar, S.; Umar, K.; Sarwar, A.H.M.G.; Khan, A.; Ahmad, N.; Ahmad, S.; Katiyar, C.K.; Husain, S.A.; Khan, H.A. *Boswellia serrata* extract attenuates inflammatory mediators and oxidative stress in collagen induced arthritis. *Phytomedicine* **2014**, *21*, 847–856. [CrossRef]
11. Al-Yasiry, A.R.M.; Kiczorowska, B.; Samolińska, W. Effect of *Boswellia serrata* Resin Supplementation on Basic Chemical and Mineral Element Composition in the Muscles and Liver of Broiler Chickens. *Biol. Trace Elem. Res.* **2017**, *179*, 294–303. [CrossRef]
12. Beghelli, D.; Isani, G.; Roncada, P.; Andreani, G.; Bistoni, O.; Bertocchi, M.; Lupidi, G.; Alunno, A. Antioxidant andEx VivoImmune System Regulatory Properties of *Boswellia serrata* Extracts. *Oxidative Med. Cell. Longev.* **2017**, *2017*, 7468064. [CrossRef] [PubMed]
13. Bertocchi, M.; Isani, G.; Medici, F.; Andreani, G.; Usca, I.T.; Roncada, P.; Forni, M.; Bernardini, C. Anti-Inflammatory Activity of *Boswellia serrata* Extracts: An In Vitro Study on Porcine Aortic Endothelial Cells. *Oxidative Med. Cell. Longev.* **2018**, *2018*, 2504305. [CrossRef] [PubMed]
14. Kiczorowska, B.; Al-Yasiry, A.; Samolińska, W.; Marek, A.; Pyzik, E. The effect of dietary supplementation of the broiler chicken diet with *Boswellia serrata* resin on growth performance, digestibility, and gastrointestinal characteristics, morphology, and microbiota. *Livest. Sci.* **2016**, *191*, 117–124. [CrossRef]
15. Tabatabaei, S.N. Effect of Olibanum (*Boswellia thurifera*) as a feed additive on performance, some blood biochemical and intestinal morphology in broiler chicks. *Res. Opin. Anim. Vet. Sci.* **2016**, *6*, 130–134. [CrossRef]
16. Apperson, K.D.; Cherian, G. Effect of whole flax seed and carbohydrase enzymes on gastrointestinal morphology, muscle fatty acids, and production performance in broiler chickens. *Poult. Sci.* **2017**, *96*, 1228–1234. [CrossRef]
17. Chowdhury, S.; Mandal, G.P.; Patra, A.K. Different essential oils in diets of chickens: 1. Growth performance, nutrient utilisation, nitrogen excretion, carcass traits and chemical composition of meat. *Anim. Feed Sci. Technol.* **2018**, *236*, 86–97. [CrossRef]
18. Kiczorowska, B.; Samolińska, W.; Al-Yasiry, A.; Zając, M. Immunomodulant feed supplement *Boswellia serrata* to support broiler chickens' health and dietary and technological meat quality. *Poult. Sci.* **2020**, *99*, 1052–1061. [CrossRef]
19. Csonka, C.; Sárközy, M.; Pipicz, M.; Dux, L.; Csont, T. Modulation of Hypercholesterolemia-Induced Oxidative/Nitrative Stress in the Heart. *Oxidative Med. Cell. Longev.* **2016**, *2016*, 3863726. [CrossRef]
20. Khayyal, M.T.; El-Ghazaly, M.A.; Abdallah, D.M.; Okpanyi, S.N.; Kelber, O.; Weiser, D. Mechanisms Involved in the Anti-inflammatory Effect of a Standardized Willow Bark Extract. *Arzneimittelforschung* **2005**, *55*, 677–687. [CrossRef]
21. Gawlik-Dziki, U.; Sugier, D.; Dziki, D.; Sugier, P. Bioaccessibility In Vitro of Nutraceuticals from Bark of Selected Salix Species. *Sci. World J.* **2014**, *2014*, 782763. [CrossRef]

22. Maistro, E.L.; Terrazzas, P.M.; Perazzo, F.F.; Gaivao, I.O.D.M.; Sawaya, A.C.H.F.; Rosa, P.C.P. *Salix alba* (white willow) medicinal plant presents genotoxic effects in human cultured leukocytes. *J. Toxicol. Environ. Health Part A* **2019**, *82*, 1223–1234. [CrossRef] [PubMed]
23. Shara, M.; Stohs, S.J. Efficacy and Safety of White Willow Bark (*Salix alba*) Extracts. *Phytother. Res.* **2015**, *29*, 1112–1116. [CrossRef] [PubMed]
24. Karimi, I.; Modaresi, M.; Cheshmekaboodi, F.; Miraghaee, S.S. The Effects of Aromatic Water of Salix aegyptiaca L. and its Major Component, 1,4-Dimethoxybenzene, on Lipid and Lipoprotein Profiles and Ethology of Normolipidemic Rabbits. *Int. J. Clin. Toxicol.* **2015**, *2*, 55–63. [CrossRef]
25. Zabihi, N.A.; Mahmoudabady, M.; Soukhtanloo, M.; Hayatdavoudi, P.; Beheshti, F.; Niazmand, S. *Salix alba* attenuated oxidative stress in the heart and kidney of hypercholesterolemic rabbits. *Avicenna J. Phytomed.* **2018**, *8*, 63–72.
26. Poźniak, B.; Świtała, M.; Bobrek, K.; Graczyk, S.; Dzimira, S. Adverse effects associated with high-dose acetylsalicylic acid and sodium salicylate treatment in broilers. *Br. Poult. Sci.* **2012**, *53*, 777–783. [CrossRef]
27. Seliger, C.; Schaerer, B.; Kohn, M.; Pendl, H.; Weigend, S.; Kaspers, B.; Härtle, S. A rapid high-precision flow cytometry based technique for total white blood cell counting in chickens. *Vet. Immunol. Immunopathol.* **2012**, *145*, 86–99. [CrossRef]
28. Naghizadeh, M.; Larsen, F.T.; Wattrang, E.; Norup, L.R.; Dalgaard, T.S. Rapid whole blood assay using flow cytometry for measuring phagocytic activity of chicken leukocytes. *Vet. Immunol. Immunopathol.* **2019**, *207*, 53–61. [CrossRef]
29. Merino-Guzmán, R.; Latorre, J.D.; Delgado, R.; Hernandez-Velasco, X.; Wolfenden, A.D.; Teague, K.D.; Graham, L.E.; Mahaffey, B.D.; Baxter, M.F.A.; Hargis, B.M.; et al. Comparison of total immunoglobulin A levels in different samples in Leghorn and broiler chickens. *Asian Pac. J. Trop. Biomed.* **2017**, *7*, 116–120. [CrossRef]
30. Board, M.M.; Crespo, R.; Shah, D.H.; Faux, C.M. Biochemical Reference Intervals for Backyard Hens. *J. Avian Med. Surg.* **2018**, *32*, 301–306. [CrossRef]
31. Tang, S.G.H.; Sieo, C.C.; Ramasamy, K.; Saad, W.Z.; Wong, H.K.; Ho, Y.W. Performance, biochemical and haematological responses, and relative organ weights of laying hens fed diets supplemented with prebiotic, probiotic and synbiotic. *BMC Vet. Res.* **2017**, *13*, 248. [CrossRef]
32. Tamminen, L.-M.; Emanuelson, U.; Blanco-Penedo, I. Systematic Review of Phytotherapeutic Treatments for Different Farm Animals Under European Conditions. *Front. Vet. Sci.* **2018**, *5*, 140. [CrossRef] [PubMed]
33. Al-Yasiry, A.R.M.; Kiczorowska, B.; Samolińska, W. Nutritional value and content of mineral elements in the meat of broiler chickens fed *Boswellia serrata* supplemented diets. *J. Elem.* **2017**, *22*, 1027–1037. [CrossRef]
34. Khan, A.; Al-Harrasi, A.; Rehman, N.U.; Sarwar, R.; Ahmad, T.; Ghaffar, R.; Khan, H.; Al-Amri, I.; Csuk, R.; Al-Rawahi, A. Loading AKBA on surface of silver nanoparticles to improve their sedative-hypnotic and anti-inflammatory efficacies. *Nanomedicine* **2019**, *14*, 2783–2798. [CrossRef] [PubMed]
35. Moussaieff, A.; Rimmerman, N.; Bregman, T.; Straiker, A.; Felder, C.C.; Shoham, S.; Kashman, Y.; Huang, S.M.; Lee, H.; Shohami, E.; et al. Incensole acetate, an incense component, elicits psychoactivity by activating TRPV3 channels in the brain. *FASEB J.* **2008**, *22*, 3024–3034. [CrossRef] [PubMed]
36. Okano, S.; Honda, Y.; Kodama, T.; Kimura, M. The Effects of Frankincense Essential Oil on Stress in Rats. *J. Oleo Sci.* **2019**, *68*, 1003–1009. [CrossRef]
37. Tully, T.N.; Dorrestein, G.M.; Jones, A.K.; Cooper, E.J. Handbook of Avian Medicine—University of Missouri Libraries. Available online: http://link.library.missouri.edu/portal/Handbook-of-avian-medicine-edited-by-Thomas-N./c65YkONeUPE/ (accessed on 7 March 2022).
38. Ding, H.; Yue, Q.; Chang, L.; Xi, J.; Chen, H.; Li, F.; Wang, D.; Zhou, R. Whole blood gas and biochemical reference intervals for Lohmann Silver layers. *Poult. Sci.* **2021**, *100*, 101368. [CrossRef]
39. Rafiullah; Khan, S.; Khan, R.U.; Ullah, Q. Does the gradual replacement of spent silkworm (*Bombyx mori*) pupae affect the performance, blood metabolites and gut functions in White Leghorn laying hens? *Res. Vet. Sci.* **2020**, *132*, 574–577. [CrossRef]
40. Borzouie, S.; Rathgeber, B.M.; Stupart, C.M.; MacIsaac, J.; MacLaren, L.A. Effects of Dietary Inclusion of Seaweed, Heat Stress and Genetic Strain on Performance, Plasma Biochemical and Hematological Parameters in Laying Hens. *Animals* **2020**, *10*, 1570. [CrossRef]
41. Ismail, I.E.; Abdelnour, S.A.; Shehata, S.A.; El-Hack, M.E.A.; El-Edel, M.A.; Taha, A.E.; Schiavitto, M.; Tufarelli, V. Effect of Dietary *Boswellia serrata* Resin on Growth Performance, Blood Biochemistry, and Cecal Microbiota of Growing Rabbits. *Front. Vet. Sci.* **2019**, *6*, 471. [CrossRef]
42. Panaite, T.D.; Saracila, M.; Papuc, C.P.; Predescu, C.N.; Soica, C. Influence of Dietary Supplementation of *Salix alba* Bark on Performance, Oxidative Stress Parameters in Liver and Gut Microflora of Broilers. *Animals* **2020**, *10*, 958. [CrossRef]
43. Davis, A.K. Effect of handling time and repeated sampling on avian white blood cell counts (wfecto del tiempo de manipulación en muestras repetidas en el conteo de celulas blancas en aves). *J. Field Ornithol.* **2005**, *76*, 334–338. [CrossRef]
44. Scanes, C.G. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. *Poult. Sci.* **2016**, *95*, 2208–2215. [CrossRef] [PubMed]
45. Gross, W.B.; Siegel, H.S. Evaluation of the Heterophil/Lymphocyte Ratio as a Measure of Stress in Chickens. *Avian Dis.* **1983**, *27*, 972. [CrossRef] [PubMed]
46. Campbell, T.W. *Avian Hematology and Cytology*, 2nd ed.; Iowa State University Press: Ames, IA, USA, 1995; ISBN 978-0-8138-2970-8.
47. Maxwell, M.H.; Robertson, G.W. The avian heterophil leucocyte: A review. *World's Poult. Sci. J.* **1998**, *54*, 155–178. [CrossRef]

48. Lentfer, T.L.; Pendl, H.; Gebhardt, S.; Fröhlich, E.K.F.; Von Borell, E. H/L ratio as a measurement of stress in laying hens - methodology and reliability. *Br. Poult. Sci.* **2015**, *56*, 157–163. [CrossRef] [PubMed]
49. Davison, F.; Kaspers, B.; Karel, A. Schat Avian Immunology, Free Online Publishing. Available online: https://authorzilla.com/lx8zn/avian-immunology.html (accessed on 26 January 2022).
50. Jugan, M.C.; Rudinsky, A.J.; Parker, V.J.; Gilor, C. Use of probiotics in small animal veterinary medicine. *J. Am. Vet. Med. Assoc.* **2017**, *250*, 519–528. [CrossRef] [PubMed]
51. Ren, Z.; Bütz, D.E.; Whelan, R.; Naranjo, V.; Arendt, M.K.; Ramuta, M.D.; Yang, X.; Crenshaw, T.D.; Cook, M.E. Effects of dietary methionine plus cysteine levels on growth performance and intestinal antibody production in broilers during Eimeria challenge. *Poult. Sci.* **2020**, *99*, 374–384. [CrossRef]
52. Bi, S.; Zhang, J.; Qu, Y.; Zhou, B.; He, X.; Ni, J. Yeast cell wall product enhanced intestinal IgA response and changed cecum microflora species after oral vaccination in chickens. *Poult. Sci.* **2020**, *99*, 6576–6585. [CrossRef]
53. Ricci, F.G.; Terkelli, L.R.; Venancio, E.J.; Justino, L.; Dos Santos, B.Q.; Baptista, A.A.S.; Oba, A.; de Oliveira Souza, B.D.; Bracarense, A.P.F.R.L.; Hirooka, E.Y.; et al. Tryptophan Attenuates the Effects of OTA on Intestinal Morphology and Local IgA/IgY Production in Broiler Chicks. *Toxins* **2020**, *13*, 5. [CrossRef]

Andreani, G., Dalmonte, T., Guerrini, A., Lupini, C., Fabbri, M., Ferlizza, E., Isani, G. Supplemention of *Boswellia serrata* and *Salix alba* extracts during early laying phase: effects on serum and albumen proteins, trace elements, and yolk cholesterol. *Animals*, **2022**, 12, 2014.

In the second paper, the profiles of serum and egg albumen proteins, serum iron and zinc concentration and egg cholesterol content were investigated in the same subjects of the previous study to assess the effects of the supplementation with *B. serrata* and *S. alba*. Serum protein profile was investigated using agarose gel electrophoresis, while both serum and egg albumen proteins were analysed using SDS-PAGE searching for possible changes in proteomes as markers of the effects of feed supplementation. Furthermore, iron and zinc concentration in serum was measured using atomic absorption spectrometry and yolk cholesterol was determined using an enzymatic colorimetric test.

animals

MDPI

Article

Supplementation of *Boswellia serrata* and *Salix alba* Extracts during the Early Laying Phase: Effects on Serum and Albumen Proteins, Trace Elements, and Yolk Cholesterol

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Simple Summary: Consumers' attention to sustainability and animal welfare has increased, strengthening the demand for eggs produced through alternative and extensive farming methods. At the same time, the need to reduce antibiotics has fostered the use of alternative dietary supplements. The aim of this research was to study the effects of botanical extracts from *Boswellia serrata* (*Bs*) and *Salix alba* (*Sa*) on serum and albumen proteins, serum iron and zinc, and egg cholesterol in Leghorn hens, during the critical phase of the onset of laying. The supplementation did not alter the protein profile of egg albumen and the cholesterol content of egg yolk. For the serum and albumen protein profile, no significant differences were observed between control and supplemented hens. Overall, this study confirms that the dietary supplementation with phytoextracts did not negatively affect the physiological variations in serum proteins and, therefore, can be safely used as a treatment to prevent inflammatory states during the early laying phase.

Abstract: Extracts from *Boswellia serrata* (*Bs*) and *Salix alba* (*Sa*) are used as supplements in poultry feed. The aims of this research were to study the possible effects of dietary supplementation with *Bs* and *Sa* extracts on serum and albumen proteins, zinc and iron, and yolk cholesterol content in Leghorn hens during the critical phase of the onset of laying. A total of 120 pullets, 17 weeks of age, were assigned to two groups (control (C) and treated (T), n = 60 each). The T group received a supplement containing *Bs* (5%) and *Sa* (5%) for 12 weeks. The study lasted 19 weeks. Serum proteins were fractionated using agarose gel electrophoresis (AGE) and SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Trace elements were determined in serum using atomic absorption spectrometry, and yolk cholesterol was determined using a colorimetric test. No significant differences were observed between control and supplemented hens for the analyzed biochemical indices. Moreover, the supplementation with phytoextracts did not negatively affect the physiological variations in serum proteins; therefore, it can be safely used as a treatment to prevent inflammatory states at onset and during the early laying phase.

Keywords: protein electrophoresis; SDS–PAGE; SPE–AGE; phytoextracts; albumen protein; chicken serum proteins; iron; zinc

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1. Introduction

Over the last few years, consumer demand has gradually focused on product quality, which includes both the intrinsic characteristics and procedures of the productive process.

Recently, consumers' concerns about sustainability and animal welfare have increased [1–3], strengthening the demand for eggs and meat produced through alternative and extensive farming methods [4]. Moreover, the shift away from antibiotic use has fostered research on alternative control methods and dietary solutions aiming to improve animal health and welfare.

A wide range of feed additives, including those obtained from plants, may fall under the category of phytogenic feed additives. The European Union Register of Feed Additives [5] reported that extracts of *Boswellia serrata* and *Salix alba* can be used as additives in animal diets. The resin of *B. serrata* (*Bs*) is widely used for the treatment of inflammatory diseases, including those affecting the gastrointestinal tract, due to the bioactive compounds contained therein, particularly boswellic acid [6,7]. The supplementation with *Bs* in broiler diets improves the digestibility of nutrients due to the microbiological stabilization of the small intestine [8,9] and leads to hypolipidemic effects, with an improvement in the quality of meat [10,11]. Like *Bs*, the bark of *S. alba* (*Sa*) is used for the treatment of chronic and acute inflammation, infection, pain, and fever. The pharmacological activity is attributed to its main component, salicin, a precursor of the anti-inflammatory acetylsalicylic acid [12]. In broilers, the salicylates derived from *Sa* have been used to reduce heat stress [13]. In hens, the use of these phytoextracts might be useful during the critical phase between the last vaccination during the pullet phase, usually carried out at the age of 13–16 weeks, and the production peak (about 30 weeks of age). In this phase, hens are subjected to high stress conditions, frequently leading to intestinal dysbiosis and inflammation. Recently, Guerrini et al. (2022) [14] reported that the use of a feed supplement containing extracts of *Bs* and *Sa* is safe and can have beneficial effects on Leghorn hens during the critical pre-laying and early laying phases. Therefore, in this research, we sought to further study the effects of supplementation on blood serum and egg albumen proteins using a proteomic approach.

Gel electrophoresis is widely used to analyze protein-rich samples, from agarose gel electrophoresis used in clinical settings to separate serum proteins (SPE–AGE) to the more sensitive mono- or bidimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) used to separate complex protein mixtures in different biological samples [15–17]. Despite its routine use in domestic and farm mammals and its increasing popularity in non-conventional avian species [18], SPE–AGE has rarely been applied to poultry. The lack of standardization of fraction separation and information regarding the protein composition makes the use of this diagnostic technique in poultry limited. To the best of the authors' knowledge, no data are present in the literature in terms of the use of this approach in laying hens. In these animals, the onset of productive phase is accompanied by major physiological changes, which determine the modifications of serum proteins in response to hormones. One of these proteins is vitellogenin, a complex phosphoglycolipoprotein produced by the liver following estrogen stimulus. Recently, Kaab et al. (2019) [19] used SDS–PAGE and reported an increase in vitellogenin and apolipoprotein-B in the sera of hens concomitant with the onset of laying.

The aims of this study were to: (*i*) evaluate the effects of a diet supplemented with botanical extracts of *Bs* and *Sa* on serum proteins and iron and zinc concentrations during the critical phases of pre-laying and early laying in Leghorn hens; for this purpose, samples collected during the research reported by Guerrini et al. (2022) [14] were used; (*ii*) evaluate if a diet supplemented with botanical extracts of *Bs* and *Sa* modifies the profile of albumen proteins and yolk cholesterol content.

2. Materials and Methods

2.1. Animals, Study Design, and Sampling

This study is part of a Research Project approved by the Italian Ministry of Health N° 602/2021-RP and by the Ethical Committee of the University of Bologna (Protocol N° 1139). In total, 120 pure-bred white Leghorn pullets (17 weeks of age/120 days old) were randomly assigned to 2 experimental groups, a control group (C) and a treated group

(T), with 60 pullets each. Pullets were certified *Salmonella*-free, vaccinated for Newcastle Disease and Infectious Bronchitis, and non-beak-trimmed. The animals were raised on a free-range farm located in Mugello (43°59′20.2″ N 11°27′58.1″ E, Florence, Italy) for 19 weeks of trial, from mid-August to December 2021. Supplementation with *Bs* and *Sa* was administered to the T group during the first 12 weeks of the trial. Four blood samplings were performed: at the start of the trial (t0), after 5 weeks of supplementation (t1), at the end of supplementation (t2, 12 weeks), and at the end of the trial (t3, 19 weeks). The hens of the T group started laying eggs at the 7th week of supplementation, and the C group started 6 days later. Blood samples (2.5 mL) were collected from the brachial vein of 8 identified hens, using a sterile syringe with a 23G-0.60 mm needle, in clean centrifuge tubes. At each experimental time point, the same hens were sampled. The serum was obtained after centrifugation at 1500× *g* at 4 °C for 10 min and stored in 2 mL plastic vials at −80 °C until analysis.

During the 10 weeks of laying, 5 eggs were collected each week from the T and C groups, separated in albumen and yolk, which were separately pooled and stored at −20 °C for protein analysis and cholesterol quantification, respectively.

2.2. Management and Diet

The two experimental groups were managed following Guerrini et al. (2022) [14], where detailed information on animal management and performance can be found. The animals were fed with a commercial diet (*Cargill* s.r.l. feed, Table 1) that was offered *ad libitum*, supplemented for 12 weeks for the T group, with 300 g of supplement/100 kg feed (0.3%). The supplement contained 5% of dry extract of *B. serrata* (*Bs*) and 5% of dry extract of *S. alba* (*Sa*). Water was provided *ad libitum*. The C group received the commercial diet without supplement throughout the trial, while from the 13th to 19th week (the end of the trial), the T group received the commercial diet without supplement. Animals were not subjected to medical treatment during the trial to eliminate interference with the normal feed intake. The outside paddock consisted of an activity area without pasture or vegetation.

Table 1. Feed formulation of the commercial diet (Cargill s.r.l) based on the indications of the commercial tag and composition of the complementary feed. The table was obtained from Guerrini et al. (2022) [14].

Composition	Values of Nutrients (%/kg of Finisher Diet)	Additives (mg/kg; IU; OTU/kg)	Complementary Feed Composition (%)
Corn; Corn gluten flour; Soybeans meal (* CP 43%); Calcium carbonate; Roasted soybeans; Rice husk; Corn gluten; Wheat bran; Soybean oil.	CP, 17%; ** CF, 5%; *** CF, 3.51%; ! Cash, 13.27%; ‡ Ca, 4.02%; ‡‡ P, 0.58%; ≠ NaCl, 0.15%; + Ly, 0.85%; $ Met: 0.33%.	Vitamin A, 9950 IU; Vitamin D_3, 2701 IU; Vitamin E, 38 mg; Vitamin K3, 2 mg; Vitamin B1, 1.5 mg; Vitamin B2, 4.5 mg; Vitamin B6, 2.5 mg; Vitamin B12, 0.008 mg; Niacin, 35 mg; Ca-D-pantothenate, 10 mg; Folic acid, 1 mg; Biotin, 0.1 mg; Betaine hydrochloride, 250 mg; Cu, 5 mg; Anhydrous calcium iodate, 0.50 mg; Mn, 50 mg; Se, 0.075 mg; Zn, 40 mg; Cantaxantine, 2 mg; Promoters of digestion 6-phytase, 213 OTU; DL-methionine, 627 mg.	Calcium carbonate, 74.5%; Colloidal silica, 15%; *Salix alba* & DE, 5%; *Boswellia serrata* & DE, 5%; Sodium chloride, 0.35%; Magnesium carbonate, 0.15%.

* CP: crude protein; ** CF: crude fats; *** CF: crude fiber; ! Cash: crude ash; ‡ Ca: calcium; ‡‡ P: phosphorus; ≠ NaCl: sodium chloride; + Ly: lysine; $ Met: methionine; & DE: dry extract.

2.3. Roche Scale for Yolk Color Evaluation

The Roche scale (Rs) Yolk Color Fan comprises a range of yolk colors from 1 to 15 color points. Five eggs/group were collected every week, for evaluation of the Roche scale. The yolk color score was individually attributed to each single egg, in natural laboratory light conditions.

2.4. Serum Protein Separation Using Agarose Gel Electrophoresis (SPE–AGE)

Serum samples (10 µL) were fractionated on 0.8% agarose gel (Hydragel, Sébia, Lisses, France). The electrophoresis was run on an automated system (Hydrasis, Sébia, Lisses, France) following the manufacturer's instructions. Gels were stained using amido-black. Stained gels were digitalized with a scanner yielding the densitometric profile (Phoresis 6.1.2 software, Sébia, Lisses, France) and the relative percentage of each protein fraction; then, the densitometer calculated the absolute value of the different protein fractions by multiplying the total protein concentration by the corresponding fractional percentage. For fraction identification, in the absence of reference data for hens, the profile of broiler serum proteins was used as a reference. Total protein concentration was determined using a commercial kit (Olympus Systems Reagents, Brea, CA, USA), with an automated biochemical analyzer (Olympus AU400, Mishima Olympus Co. Ltd., Shizuoka, Japan).

2.5. Serum and Albumen Protein Separation Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Samples (15 µg of serum protein and 10 µL of albumen previously diluted 1:100 (*v*:*v*) with bidistilled water) were loaded onto 4–12% polyacrylamide pre-cast gels (NuPage/Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis was carried out in a Novex Mini-Cell (Invitrogen) 4-(3-sulfonatopropyl) morpholin-4-ium buffer (MOPS; NuPage/Thermo Fisher Scientific, Waltham, MA, USA), containing lithium dodecyl sulfate (LDS). Each gel was also loaded with standard proteins of known molecular weight (See-Blue Pre-Stained Standard). The electrophoresis system was connected to a power supply (Power Pack Basic—Bio-Rad, Hercules, CA, USA) at a constant voltage of 200 V. The gels were stained with Quick Coomassie Stain (Protein Ark, Sheffield, UK). After destaining, each gel was digitalized using ChemiDocMP (Bio-Rad, Hercules, CA, USA), and the pherograms were obtained using the ImageLab 5.2.1 software (Bio-Rad, Hercules, CA, USA). The ImageLab software estimates the volume of protein bands based on pixel density. In serum samples, the quantification of the protein bands was performed, knowing the content of total proteins (15 µg) loaded on the gel for each sample and the percentage of the band determined after densitometric analysis, a process applied in clinical routine with agarose gel electrophoresis.

2.6. Iron and Zinc Determination in Sera

To avoid contamination, all the reagents were carefully handled, and polyethylene disposables were thoroughly washed with HCl 1 N under a fume hood. All the reagents were from Merck (Darmstadt, Germany); the acids were of Suprapur grade. Samples of sera were diluted 1:10 with bidistilled water and were directly analyzed using a flame atomic spectrophotometer (AAnalyst 100, PerkinElmer, Waltham, MA, USA). The accuracy of the method was evaluated by analyzing an international standard (ERM®-BB422 fish muscle). The concentrations found with the method used in this study fell into the certified uncertainty interval given by the ERM, corresponding to a 95% confidence level. The detection limits were 0.04 µg mL^{-1} and 0.01 µg mL^{-1} for iron and zinc, respectively. Element concentrations were reported as µg mL^{-1}.

2.7. Cholesterol Quantification in Egg Yolks

A commercial test kit (Cholesterol Quantification Assay Kit, Sigma-Aldrich®; Darmstadt, Germany) was used for the determination of total cholesterol in egg yolk. During the 10 weeks of laying, 5 eggs were collected from C and T groups. Yolks were separated

from albumens, pooled, and stored at −20 °C. Following Pasin et al. (1998) [20], 3 g of the liquid yolk were solubilized in 27 mL of 5% NaCl solution to obtain a 10-dilution factor. Afterwards, samples were homogenized with UltraTurax and gently stirred for 2 h. Subsequently, 100 µL of each sample were diluted with 900 µL of 5% NaCl solution to obtain a 1:100 final dilution factor and used as a working sample. Cholesterol was determined following the manufacturer's instructions, in a microplate reader (Tecan Trading, Männedorf, Switzerland).

2.8. Statistical Analysis

Shapiro–Wilk and Lilliefors statistical tests were used to determine the distribution of data. To assess the homoscedasticity, Levene and Brown–Forsythe tests were performed. The Friedman test was used to determine significant differences among time points within the same group for serum and albumen SDS–PAGE, serum AGE, and cholesterol quantification in yolk samples. The Nemenyi post hoc test was then performed to evaluate the significant difference when the Friedman test was significant. The comparison between the C and T groups at the same time point was performed using the Mann–Whitney test. All tests were conducted using a statistical software program (RStudio-1.2.1335 Statistical and R, R version 3.4), and the results were considered statistically significant at $p < 0.05$. Data are reported as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Roche Scale

Compared with the 1/15 scale, the eggs of the T group recorded an average score of 10.36 ± 0.32, while the eggs of the C group recorded a score of 10.5 ± 0.58 color scale points. No statistically significant differences were found.

3.2. SPE–AGE

In non-mammalian species, serum protein electrophoresis on agarose gel might be considered a useful clinical tool with a still underexploited diagnostic and prognostic potential. However, there are no defined criteria for the identification of different protein fractions in many species, including *Gallus gallus*. Recently, a study conducted by Tóthová et al. (2019) [21] reported the separation of agarose gel in broiler serum proteins using the same protocol and platform as that used in the present research. Therefore, three serum samples obtained from healthy broilers were analyzed before hen samples and pherograms were compared with those obtained by Tóthová et al. (2019) [21]. Similar pherograms were obtained, and protein fractions were separated accordingly. At t0, based on the profile obtained for broilers, five major fractions were also separated in hens, including albumin, the predominant, most anodic band, followed by α, β, and γ globulins. A faint band of prealbumin was also present before the albumin band in all the examined samples. The alpha zone was clearly divided into α1 and α2, while it was not possible to separate β1 and β2 subfractions (Figure 1, t0).

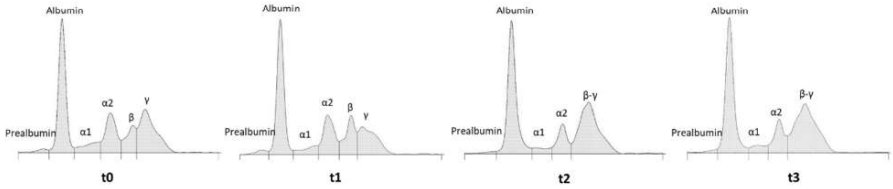


Figure 1. Representative electropherograms of serum proteins using agarose gel electrophoresis (AGE) obtained from the same control hen at different experimental time points: t0, start of the trial; t1 (5th week), before the start of laying; t2 (12th week), end of the supplementation; and t3 (19th week), end of the trial.

It is well-recognized that the AGE applied to serum and plasma samples suffers from differences due to commercial platforms and reagents [22]. However, despite subtle differences in electrophoretic profiles, Cray (2021) [18], using a different platform (Helena SPIFE 3000 system), reported the same fractions (prealbumin, albumin, α1, α2, β, and γ globulins) in the electropherograms of chicken sera that were found in this study. Therefore, taken together, these data can be considered a first attempt to define a standard profile for serum proteins in healthy chickens.

In the control hens, qualitative and quantitative variations were evident over time, from t0 to t3, due to the onset of the laying phase, which is accompanied by physiological changes and variations in serum proteins. As an example, the pherograms obtained from the same specimen at the different experimental time points are reported in Figure 1. The complete data and statistical analysis are reported in Table 2. Between t1 and t2, the hens started the laying phase, and a significant ($p < 0.05$) increase in albumin concentration from 15.0 ± 1.6 (t0) to 21.9 ± 0.5 g L^{-1} (t3) was measured in the control group. An increasing trend of serum albumin was also reported by Gyenis et al. (2006) [23] for Hy-Line W98 and Hy-Line Brown hens from the 6th to 31st week, during the critical phases including pre-laying, the onset of laying, and the phase reaching the production peak. In the control group, a significant decrease ($p < 0.05$) was detected for α2 globulins from 7.1 ± 0.9 to 5.2 ± 0.05 g L^{-1}. However, the most marked differences were present in β and γ globulins, with a fusion of these two zones (Figure 1, t2 and t3), likely due to an increase in the β zone. Therefore, starting from t2, it was not possible to separate the peaks of β and γ globulins. Hypotheses for these variations are discussed below.

Table 2. Concentrations of serum protein fractions determined after agarose gel electrophoresis in control (C) and supplemented (T) hens at different experimental time points: t0, start of the trial; t1 (5th week), before the start of laying; t2 (12th week), end of the supplementation; t3 (19th week), end of the trial. Data are reported as mean ± SD (n = 8 for each group).

Proteins	C				T			
	t0	t1	t2	t3	t0	t1	t2	t3
Total proteins (g L^{-1})	42.7 ± 3.13 $^{(1,2)}$	48.4 ± 3.62 $^{(1)}$	48.7 ± 4.85 $^{(2)}$	45.9 ± 3.40	39.3 ± 2.92 $^{(1,2,3)}$	46.2 ± 4.11 $^{(1)}$	46.8 ± 4.30 $^{(2)}$	48.3 ± 4.10 $^{(3)}$
Prealbumin (g L^{-1})	0.70 ± 0.20	0.80 ± 0.40	0.70 ± 0.20	0.80 ± 0.20	0.80 ± 0.20	0.80 ± 0.10	0.60 ± 0.20	0.80 ± 0.30
Albumin (g L^{-1})	15.0 ± 1.6 $^{(1,2)}$	19.5 ± 1.36 §	22.2 ± 1.40 $^{(1)}$	21.9 ± 0.50 $^{(2)}$	14.2 ± 3.00 $^{(1,2)}$	14.9 ± 1.69 $^{(3,4),\S}$	20.9 ± 1.70 $^{(1,3)}$	23.5 ± 1.10 $^{(2,4)}$
α1 Globulins (g L^{-1})	2.30 ± 0.30 $^{(1)}$	2.50 ± 0.70 $^{(2,3)}$	1.60 ± 0.30 $^{(1,2)}$	1.90 ± 0.30 $^{(3)}$	2.50 ± 0.70 $^{(1,2)}$	2.10 ± 0.20	1.80 ± 0.30 $^{(1)}$	1.50 ± 0.30 $^{(2)}$
α2 Globulins (g L^{-1})	7.10 ± 0.90 $^{(1,2)}$	8.40 ± 1.40 $^{(3,4)}$	5.00 ± 0.20 $^{(1,3)}$	5.20 ± 0.50 $^{(2,4)}$	6.40 ± 0.80 $^{(1)}$	7.80 ± 0.50 $^{(2,3)}$	4.80 ± 0.09 $^{(1,2)}$	5.30 ± 0.40 $^{(3)}$
β Globulins (g L^{-1})	4.80 ± 1.5	6.60 ± 1.60	-	-	3.40 ± 1.20	7.80 ± 1.10	-	-
γ Globulins (g L^{-1})	12.3 ± 2.2	11.5 ± 2.10	-	-	11.5 ± 2.10	12.9 ± 1.90	-	-
β-γ Globulins (g L^{-1})	-	-	18.1 ± 4.30	16.2 ± 3.50	-	-	18.6 ± 3.50	17.2 ± 3.70

$^{1–4}$ In the same row and for each group, the same superscript number indicates a significant difference among time points. § In the same row and for the same time point, this indicates significant difference between control and supplemented hens.

Supplemented hens presented the same variations over time. No significant differences were detected between the two experimental groups, with the sole exception of albumin at t1. At this time point, the control hens had a concentration of serum albumin significantly higher than the hens supplemented with the phytoextracts.

3.3. SDS–PAGE of Serum Proteins

Information on proteins present in the different globulin fractions is fragmentary in chickens, but we can hypothesize that they are the same as those in mammals due to their essential and shared physiological and biochemical role. To shed more light on these proteins and their possible changes during the laying phase, serum samples were further fractionated using SDS–PAGE. The representative gels of serum samples obtained from

the control hens at t0 (before laying) and at t2 (hens in the laying phase) are reported in Figure 2. The band profiles obtained in Leghorn hens are the same as those reported by Kaab et al. (2019) [19], who identified 11 abundant proteins via mass spectrometry in the sera of Lohmann Brown hens after SDS–PAGE fractionation. Therefore, the identity of some of the bands obtained in this research can be hypothesized based on the apparent molecular mass (MM) and comparing the data with those reported by Kaab et al. (2019) [19]. At t0, protein profiles revealed a common pattern in all the analyzed samples, characterized by the presence of a high-abundance band at an apparent MM of 55 kD; this band can be identified as putative albumin, in accordance with the data obtained using agarose gel electrophoresis (Figure 1). Over time, from t0 to t3, qualitative and quantitative differences were recorded, mirroring the onset of the laying phase.

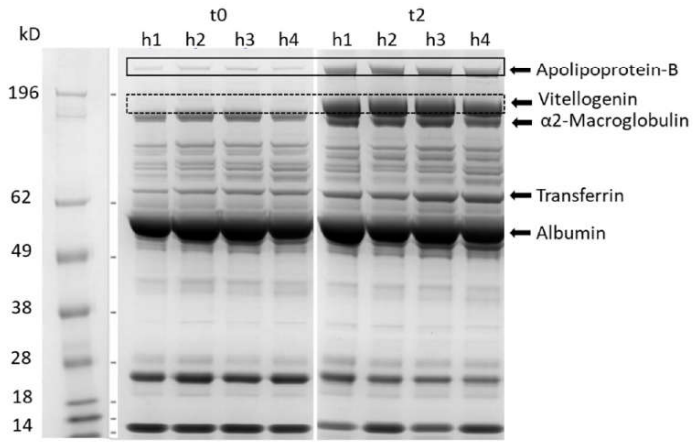


Figure 2. Representative gel of serum proteins using SDS–PAGE obtained from the same 4 control hens (h1–h4) at t0, start of the trial (before the laying phase) and at t2, 12 weeks later (hens in laying phase). The molecular mass (kD) of marker proteins is shown to the left of lane 1. Putative proteins are identified based on the MM and the position on the gel.

Besides albumin, at t0, one other high-abundance protein band had an apparent MM of 160 kD and can be identified as α2 macroglobulin (α2M) (Figures 2 and 3c). Vertebrate α2Ms are large tetrameric glycoproteins consisting of four identical subunits, the main function of which is to inhibit the circulating proteases resulting in different biological properties, such as antibacterial and anti-inflammatory activity [24]. In chickens, this protein, previously known as ovostatin, is present also in the egg albumen [25]. The intensity of this band significantly decreased ($p < 0.05$) over time from t1 to t3 (Figure 3c). Similarly, Kaab et al. (2019) [19] reported a decrease in α2M, starting from the onset of the laying period, suggesting an increased demand for this protein to be incorporated into the eggs. The decrease in the α2 zone observed using AGE might be in part due to the decrease in α2M, which is one of the main components of the α2 zone, together with haptoglobin.

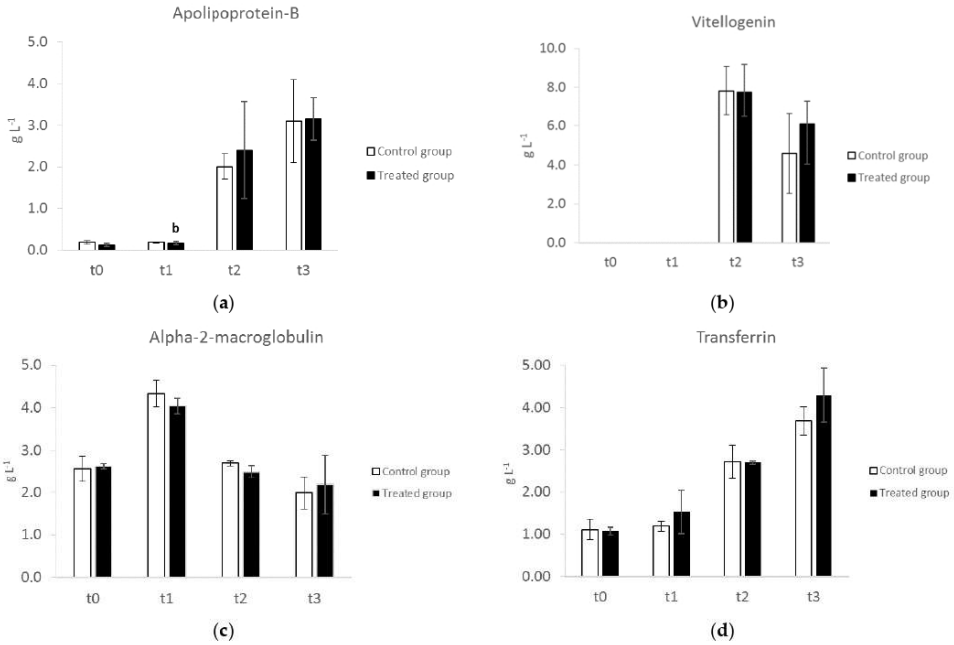


Figure 3. Variation in selected serum proteins in control and supplemented hens at different time points: t0, start of the trial; t1 (5th week), before the start of laying; t2 (12th week), end of the supplementation; and t3 (19th week), end of the trial. The concentrations were obtained after densitometric analysis of protein bands and are expressed as g L^{-1} (n = 4). Bars sharing the same symbol (control hens) or the same letter (treated hens) are significantly different ($p < 0.05$).

Starting from t2, in parallel with the onset of the laying phase, the upper part of the gel showed two additional high MM proteins at >200 and 175 kD, respectively (Figure 2). These two proteins can be identified as apolipoprotein-B (Apo-B) and vitellogenin (Vg), likely Vg2, which is the most abundant Vg isoform. Accordingly, Kaab et al. (2019) [19] reported in Lohmann Brown layers that these two proteins significantly increased by the 17th week and reached a peak by the 23rd week just before the production peak. Apo-B and Vg are essential for egg formation, contributing to the lipid and protein components of eggs. Apo-B is the main protein of LDLs and VLDLs and, in mammals pherograms, appears in the beta zone on agarose gels. In laying hens, VLDLs are devoted to the transport of triacylglycerols from the liver to the oocytes. With the onset of egg production, lipoprotein production in the liver is shifted from generic VLDL to VLDLy (yolk-targeted) because triacylglycerols, which are accumulated and stored in egg yolk, are needed to satisfy the embryo's energy requirement for hatching [26]. Vitellogenins are the most relevant source of nutrients for the developing embryo in non-mammalian vertebrates. These proteins are produced by the liver after estradiol stimulus, secreted into the bloodstream, and taken up in the oviduct by receptor-mediated uptake [27,28]. The location of Vg in the globulin zone of agarose gel pherograms is still unknown. However, it can be hypothesized that the fusion of beta and gamma zones at t2 and t3 might be in part due to the presence of Vg. The presence of vitellogenins in the egg-laying females of non-mammalian vertebrates should be considered when analyzing pherograms in clinical settings.

Based on the position on the gel and the MM, the band at 72 kD can be identified as transferrin. Transferrins are iron-binding glycoproteins that bind and transport ferric ions in biological fluids. Due to this binding specificity, transferrins have antibacterial activity, making iron unavailable for bacterial growth [29]; at the same time, in laying hens, they are essential for iron delivery to the eggs. Watanabe and Iwasaki (1985) reported an increase in transferrin concentrations in the sera of laying hens, suggesting an important function associated with iron metabolism in egg laying [30]. Accordingly, a significant increase ($p < 0.05$) in this protein was found from t0 to t3 (Figures 2 and 3d) in this study.

The supplemented hens presented the same profile and the same variations in serum proteins over time (Figure 3) as the control hens. No significant differences in serum proteins were detected between the control and supplemented hens, indicating that the supplementation with phytoextracts did not negatively affect the serum protein profiles.

3.4. Iron and Zinc Concentrations in Serum

In hens, the trace element dynamics in serum during the laying phase is still an underexplored field, and the reference intervals are also not defined. Therefore, iron and zinc concentrations were analyzed at t0 and at different time points to evaluate the possible variations related to egg laying and supplementation. Iron concentrations determined in this study in the control hens varied from 8.03 ± 2.75 (t0) to 9.59 ± 2.57 (t3) μg mL^{-1} (Figure 4) in accordance with the increase of transferrin. These concentrations are similar to those reported in turkey laying hens by Richards (1989) [31], while they are higher than the values reported by Sarlak et al. (2021) [32] in Shaver White laying hens.

The zinc concentrations determined in this study in the control hens varied from 1.95 ± 1.05 (t0) to 3.86 ± 1.85 (t3) μg mL^{-1}. In broilers, similar concentrations (1.68–2.07 μg mL^{-1}) (1.98 μg mL^{-1}) were reported by Bartlett and Smith (2003) [33] and by Olukosi et al. (2018) [34], respectively, while higher concentrations (10.53 ± 0.26 μg mL^{-1}) were reported by Sohail et al. (2011) [35].

No significant variations were detected among different time points, despite an increasing trend from t1 to t3 for iron and from t2 to t3 for zinc, probably associated with the onset of egg laying between t1 and t2. In fact, egg formation requires the mobilization of the stored iron from the liver and its transport to the eggs, which should contain an adequate amount of this essential element to ensure embryonic growth and development.

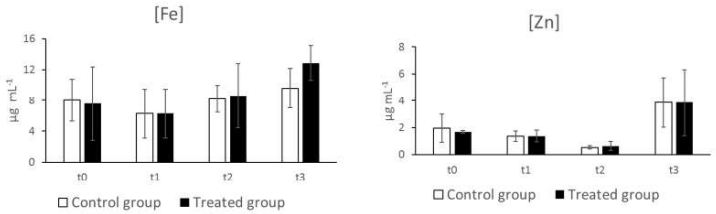


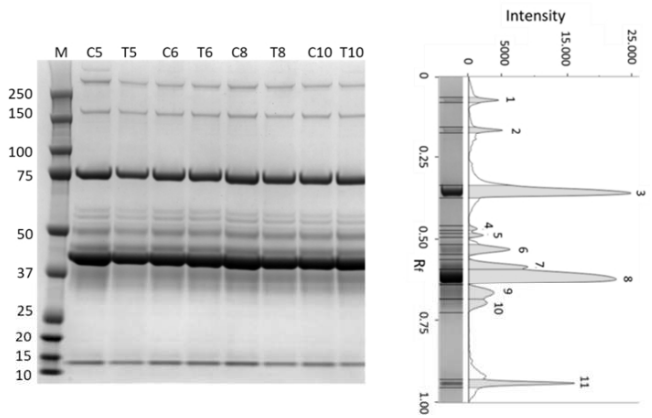
Figure 4. Iron (Fe) and zinc (Zn) concentrations in sera of control (C) and supplemented hens (T). Data are reported as mean ± SD (n = 8) and expressed as μg mL^{-1}.

Iron and zinc concentrations in sera did not significantly differ between the control and supplemented hens.

3.5. SDS–PAGE of Albumen Proteins

Egg albumen provides important nutrients for embryo development and contains proteins of high nutritional value. Figure 5 reports representative gel and pherograms of egg albumen proteins obtained from the control hens at different laying weeks. SDS–PAGE separation revealed 11 protein bands, ranging in the MM from >250 to 14 kD. The identity

of these proteins can be hypothesized based on the apparent MM, the position on the pherogram, and comparing the profiles with those reported in the literature.



(**a**) (**b**)

Figure 5. Representative gel and pherogram of egg albumen proteins separated using SDS–PAGE 4–12%: (**a**) lane 1: molecular mass marker (kD); lanes 2, 4, 6, and 8: albumens from control hens at 5th, 6th, 8th and 10th week (C5–C10); lanes 3, 5, 7, and 9: albumens from supplemented hens at 5th, 6th, 8th, and 10th week (T5–T10); (**b**) the pherogram obtained from lane 3 is reported as an example (right).

In fact, during the last two decades, studies reported a common protein profile after SDS–PAGE for the proteome of egg albumen in hens, indicating that the most abundant proteins are ovalbumin (45 kD MM), ovotransferrin (75 kD MM), ovomucoid (28 kD MM), lysozyme (14 kD MM), and ovomucin [36,37]. The profiles obtained in the samples analyzed in this study were characterized by two abundant protein bands at 43 (band 8) and 75 kD (band 3) that can be identified as ovalbumin and ovotransferrin. These data confirmed the findings of previous studies [25,38,39], with interesting differences regarding the abundance of ovalbumin and ovotransferrin. In all the analyzed samples, and in the optimized experimental conditions in this study, a less intense band of ovalbumin associated with an evident band of ovotransferrin was detected. Wang et al. (2012) [40] reported that the abundance of ovalbumin was significantly different ($p < 0.05$) among six egg varieties and ovalbumin spots after 2DE in brown-shelled eggs were 81% higher than those of white-shelled eggs. It could be hypothesized that white-shelled eggs, such as eggs from Leghorn hens, have a lower content of ovalbumin. If confirmed by future studies, the abundance of ovotransferrin in the albumen of Leghorn hen eggs could represent an interesting source of bioavailable iron for human nutrition.

A diffuse zone containing two bands was present between 38 (band 9) and 33 (band 10) kD. These bands could be attributed to ovomucoid and ovoflavoprotein, two highly glycosylated proteins [36]. The high glycosylation degree, estimated at 25% for ovomucoid [41], could be responsible for the low migration rate in SDS–electrophoresis and the difference between the theoretical (28 kD) and measured (35 kD) MM for ovomucoid [42]. Finally, the band at 14 kD (band 11) could be identified as lysozyme, a well-known antimicrobial protein of albumen.

Similar profiles of albumen proteins were detected in the control and supplemented hens (Figure 5).

3.6. Cholesterol Content in Egg Yolk

Cholesterol was measured in egg yolk at the 1st, 5th, 8th, and 10th laying week, corresponding to the start of laying, the end of supplementation (12th week of trial), 21 days from the end of supplementation (15th week of trial), and the end of the trial (19th week of trial), respectively (Table 2). The content of cholesterol detected in the eggs of the control hens, with a mean value over the 10 weeks of 12.8 mg g^{-1} of wet yolk, is in accordance with the mean value of 12.8 mg g^{-1} of yolk reported by Abou-Elkhair et al. (2018) [43] in Lohmann Brown Lite hens. A different value for cholesterol content in the eggs of Leghorn hens was reported by Panda et al. (2003) [44], with a mean value of 13.5 ± 0.15 mg g^{-1} of yolk. It is well-known that cholesterol content in yolk may be influenced by many variables, including breed, age, and housing system [45]; therefore, these differences were expected.

Regarding the effects of feed supplementation with *Bs* and *Sa* extracts, a lower, though not significant, value for yolk cholesterol content was measured in the T group (Table 3). It is well-recognized that the extracts of *Bs* have hypolipemic effects in rats [46], humans [47], and broilers [48], but there is little information regarding their possible effects on the content of egg yolk. Abdelli et al. (2021) [49] and Panda et al. (2003) [44] reported that phytogenic feed additives and probiotic supplementation reduced the yolk's cholesterol content. Further insights are needed to disclose the effects of *Bs* and *Sa* supplementation on the cholesterol content of eggs.

Table 3. Cholesterol content in yolk. Data are reported as mean ± SD (n = 3) and expressed as mg g^{-1}.

Laying Week	C (Control Hens)	T (Supplemented Hens)
1st	12.8 ± 1.49	10.8 ± 0.81
5th	11.9 ± 1.50	11.0 ± 1.30
8th	13.4 ± 2.12	10.8 ± 1.05
10th	13.2 ± 3.12	10.1 ± 1.38

4. Conclusions

No significant differences in serum proteins were detected between the control and supplemented hens, indicating that the supplementation with botanical extracts of *Bs* and *Sa* did not negatively affect these biomarkers during the pre-laying and early laying phases and, therefore, can be safely used as a treatment to prevent inflammatory states during these critical phases. No significant differences in egg albumen proteins and cholesterol content in yolk were observed between the two groups, indicating that phytoextracts did not influence the quality of eggs in Leghorn laying hens. A possible effect on the content of cholesterol in eggs needs further studies and could be interesting for future applications if the lower cholesterol content is confirmed in the yolk of hens fed a diet enriched with these phytoextracts.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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References

1. Leinonen, I.; Kyriazakis, I. How can we improve the environmental sustainability of poultry production? *Proc. Nutr. Soc.* **2016**, *75*, 265–273. [CrossRef] [PubMed]
2. Verbeke, W.; Scholderer, J.; Lähteenmäki, L. Consumer appeal of nutrition and health claims in three existing product concepts. *Appetite* **2009**, *52*, 684–692. [CrossRef]
3. Vanhonacker, F.; Verbeke, W. Buying higher welfare poultry products? Profiling flemish consumers who do and do not. *Poult. Sci.* **2009**, *88*, 2702–2711. [CrossRef]
4. Gangnat, I.D.M.; Mueller, S.; Kreuzer, M.; Messikommer, R.E.; Siegrist, M.; Visschers, V.H.M. Swiss consumers' willingness to pay and attitudes regarding dual-purpose poultry and eggs. *Poult. Sci.* **2018**, *97*, 1089–1098. [CrossRef] [PubMed]
5. European Commission. *European Union Register of Feed Additives—(EC) No 1831/2003. Appendixes 3e, 4*; European Commission: Luxembourg, 2016; ISBN 0195-3842.
6. Hartmann, R.M.; Fillmann, H.S.; Martins, M.I.M.; Meurer, L.; Marroni, N.P. *Boswellia serrata* has beneficial anti-inflammatory and antioxidant properties in a model of experimental colitis. *Phyther. Res.* **2014**, *28*, 1392–1398. [CrossRef] [PubMed]
7. Bertocchi, M.; Isani, G.; Medici, F.; Andreani, G.; Usca, I.T.; Roncada, P.; Forni, M.; Bernardini, C. Anti-inflammatory activity of *Boswellia serrata* extracts: An in vitro study on porcine aortic endothelial cells. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 2504305. [CrossRef]
8. Kiczorowska, B.; Al-Yasiry, A.R.M.; Samolińska, W.; Marek, A.; Pyzik, E. The effect of dietary supplementation of the broiler chicken diet with *Boswellia serrata* resin on growth performance, digestibility, and gastrointestinal characteristics, morphology, and microbiota. *Livest. Sci.* **2016**, *191*, 117–124. [CrossRef]
9. Tabatabaei, S.N. Effect of Olibanum (*Boswellia thurifera*) as a feed additive on performance, some blood biochemical and intestinal morphology in broiler chicks. *Res. Opin. Anim. Vet. Sci.* **2016**, *6*, 130–134.
10. Chowdhury, S.; Mandal, G.P.; Patra, A.K. Different essential oils in diets of chickens: Growth performance, nutrient utilisation, nitrogen excretion, carcass traits and chemical composition of meat. *Anim. Feed Sci. Technol.* **2018**, *236*, 86–97. [CrossRef]
11. Kiczorowska, B.; Samolińska, W.; Al-Yasiry, A.; Zając, M. Immunomodulant feed supplement *Boswellia serrata* to support broiler chickens' health and dietary and technological meat quality. *Poult. Sci.* **2020**, *99*, 1052–1061. [CrossRef] [PubMed]
12. Shara, M.; Stohs, S.J. Efficacy and safety of white willow bark (*Salix alba*) extracts. *Phyther. Res.* **2015**, *29*, 1112–1116. [CrossRef] [PubMed]
13. Saracila, M.; Panaite, T.D.; Soica, C.; Tabuc, C.; Olteanu, M.; Predescu, C.; Rotar, C.M.; Criste, R.D. Use of a hydroalcoholic extract of *Salix alba* L. bark powder in diets of broilers exposed to high heat stress. *S. Afr. J. Anim. Sci.* **2019**, *49*, 942–954. [CrossRef]
14. Guerrini, A.; Dalmonte, T.; Lupini, C.; Andreani, G.; Salaroli, R.; Quaglia, G.; Zannoni, A.; Scozzoli, M.; Forni, M.; Isani, G. Influence of dietary supplementation with *Boswellia serrata* and *Salix alba* on performance and blood biochemistry in free-range leghorn laying hens. *Vet. Sci.* **2022**, *9*, 182. [CrossRef]
15. Ferlizza, E.; Dondi, F.; Andreani, G.; Bucci, D.; Archer, J.; Isani, G. Validation of an electrophoretic method to detect albuminuria in cats. *J. Feline Med. Surg.* **2017**, *19*, 860–868. [CrossRef] [PubMed]
16. Crisi, P.E.; Dondi, F.; De Luca, E.; Di Tommaso, M.; Vasylyeva, K.; Ferlizza, E.; Savini, G.; Luciani, A.; Malatesta, D.; Lorusso, A.; et al. Early renal involvement in cats with natural feline morbillivirus infection. *Animals* **2020**, *10*, 828. [CrossRef] [PubMed]
17. Isani, G.; Ferlizza, E.; Cuoghi, A.; Bellei, E.; Monari, E.; Bianchin Butina, B.; Castagnetti, C. Identification of the most abundant proteins in equine amniotic fluid by a proteomic approach. *Anim. Reprod. Sci.* **2016**, *174*, 150–160. [CrossRef]
18. Cray, C. Protein electrophoresis of non-traditional species: A review. *Vet. Clin. Pathol.* **2021**, *50*, 478–494. [CrossRef] [PubMed]
19. Kaab, H.; Bain, M.M.; Bartley, K.; Turnbull, F.; Wright, H.W.; Nisbet, A.J.; Birchmore, R.; Eckersall, P.D. Serum and acute phase protein changes in laying hens, infested with poultry red mite. *Poult. Sci.* **2019**, *98*, 679–687. [CrossRef]
20. Pasin, G.; Smith, G.M.; O'Mahony, M. Rapid determination of total cholesterol in egg yolk using commercial diagnostic cholesterol reagent. *Food Chem.* **1998**, *61*, 255–259. [CrossRef]
21. Tóthová, C.; Sesztáková, E.; Bielik, B.; Nagy, O. Changes of total protein and protein fractions in broiler chickens during the fattening period. *Vet. World* **2019**, *12*, 598–604. [CrossRef]
22. Cray, C.; King, E.; Rodriguez, M.; Decker, L.S.; Arheart, K.L. Differences in protein fractions of avian plasma among three commercial electrophoresis systems. *J. Avian Med. Surg.* **2011**, *25*, 102–110. [CrossRef] [PubMed]
23. Gyenis, J.; Sütő, Z.; Romvári, R.; Horn, P. Tracking the development of serum biochemical parameters in two laying hen strains—A comparative study. *Arch. Tierz.* **2006**, *49*, 593–606. [CrossRef]
24. Rehman, A.; Ahsan, H.; Khan, F.H. α-2-Macroglobulin: A physiological guardian. *J. Cell. Physiol.* **2013**, *228*, 1665–1675. [CrossRef] [PubMed]

25. Mann, K. The chicken egg white proteome. *Proteomics* **2007**, *7*, 3558–3568. [CrossRef] [PubMed]
26. Walzem, R.L.; Hansen, R.J.; Williams, D.L.; Hamilton, R.L. Estrogen inducition of VLDL assembly in egg-layng hens. *J. Nutr.* **1999**, *129*, 467s–472s. [CrossRef] [PubMed]
27. Deeley, R.G.; Mullinix, K.P.; Wetekam, W.; Kronenberg, H.M.; Meyers, M.; Eldridge, J.D.; Goldberger, R.F. Vitellogenin synthesis in the avian liver. *J. Biol. Chem.* **1975**, *250*, 9060–9066. [CrossRef]
28. Li, J.; Leghari, I.H.; He, B.; Zeng, W.; Mi, Y.; Zhang, C. Estrogen stimulates expression of chicken hepatic vitellogenin II and very low-density apolipoprotein II through ER-α. *Theriogenology* **2014**, *82*, 517–524. [CrossRef]
29. Giansanti, F.; Leboffe, L.; Pitari, G.; Ippoliti, R.; Antonini, G. Physiological roles of ovotransferrin. *Biochim. Biophys. Acta* **2012**, *1820*, 218–225. [CrossRef]
30. Watanabe, S.; Iwasaki, S. Developmental changes in serum albumin, transferrin, iron, and iron binding capacity of growing chickens and egg-laying hens. *Jpn. J. Zootech. Sci.* **1985**, *56*, 221–225.
31. Richards, M.P. Influence of egg production on zinc, copper and iron metabolism in the turkey hen (*Meleagris gallopavo*). *Comp. Biochem. Physiol.—Part A Physiol.* **1989**, *93*, 811–817. [CrossRef]
32. Sarlak, S.; Tabeidian, S.A.; Toghyani, M.; Shahraki, A.D.F.; Goli, M.; Habibian, M. Effects of replacing inorganic with organic iron on performance, egg quality, serum and egg yolk lipids, antioxidant status, and iron accumulation in eggs of laying hens. *Biol. Trace Elem. Res.* **2021**, *199*, 1986–1999. [CrossRef]
33. Bartlett, J.R.; Smith, M.O. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. *Poult. Sci.* **2003**, *82*, 1580–1588. [CrossRef]
34. Olukosi, O.A.; Van Kuijk, S.; Han, Y. Copper and zinc sources and levels of zinc inclusion influence growth performance, tissue trace mineral content, and carcass yield of broiler chickens. *Poult. Sci.* **2018**, *97*, 3891–3898. [CrossRef] [PubMed]
35. Sohail, M.U.; Rahman, Z.U.; Ijaz, A.; Yousaf, M.S.; Ashraf, K.; Yaqub, T.; Zaneb, H.; Anwar, H.; Rehman, H. Single or combined effects of mannan-oligosaccharides and probiotic supplements on the total oxidants, total antioxidants, enzymatic antioxidants, liver enzymes, and serum trace minerals in cyclic heat-stressed broilers. *Poult. Sci.* **2011**, *90*, 2573–2577. [CrossRef]
36. Desert, C.; Guérin-Dubiard, C.; Nau, F.; Jan, G.; Val, F.; Mallard, J. Comparison of different electrophoretic separations of hen egg white proteins. *J. Agric. Food Chem.* **2001**, *49*, 4553–4561. [CrossRef]
37. Abeyrathne, E.D.N.S.; Lee, H.Y.; Ahn, D.U. Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents-A review. *Poult. Sci.* **2013**, *92*, 3292–3299. [CrossRef] [PubMed]
38. Uysal, R.S.; Sumnu, G.; Boyaci, I.H. Effects of heat-treated liquid whole egg on cake batter rheology and the quality of baked cake. *J. Food Process Eng.* **2019**, *42*, e12977. [CrossRef]
39. Mann, K.; Mann, M. In-depth analysis of the chicken egg white proteome using an LTQ Orbitrap Velos. *Proteome Sci.* **2011**, *9*, 7. [CrossRef] [PubMed]
40. Wang, J.; Liang, Y.; Omana, D.A.; Kav, N.N.V.; Wu, J. Proteomics analysis of egg white proteins from different egg varieties. *J. Agric. Food Chem.* **2012**, *60*, 272–282. [CrossRef] [PubMed]
41. Li-Chan, E.; Nakai, S. Biochemical basis for the properties of egg white. *Crit. Rev. Poult. Biol.* **1991**, *2*, 21–59.
42. Giosafto, C.V.L.; Rigby, N.; Sorrentino, A.; Mulholland, F.; Mills, C.; Mackie, A.R. Optimization of in vitro N-deglycosylation of ovomucoid protein. *MOJ Food Process. Technol.* **2016**, *2*, 205–212. [CrossRef]
43. Abou-Elkhair, R.; Selim, S.; Hussein, E. Effect of supplementing layer hen diet with phytogenic feed additives on laying performance, egg quality, egg lipid peroxidation and blood biochemical constituents. *Anim. Nutr.* **2018**, *4*, 394–400. [CrossRef] [PubMed]
44. Panda, A.K.; Reddy, M.R.; Rama Rao, S.V.; Praharaj, N.K. Production performance, serum/yolk cholesterol and immune competence of White Leghorn layers as influenced by dietary supplementation with probiotic. *Trop. Anim. Health Prod.* **2003**, *35*, 85–94. [CrossRef] [PubMed]
45. Kraus, A.; Zita, L.; Krunt, O.; Härtlová, H.; Chmelíková, E. Determination of selected biochemical parameters in blood serum and egg quality of Czech and Slovak native hens depending on the housing system and hen age. *Poult. Sci.* **2021**, *100*, 1142–1153. [CrossRef]
46. Pandey, R.S.; Singh, B.K.; Tripathi, Y.B. Extract of gum resins of *Boswellia serrata* L. inhibits lipopolysaccharide induced nitric oxide production in rat macrophages along with hypolipidemic property. *Indian J. Exp. Biol.* **2005**, *43*, 509–516.
47. Ahangarpour, A.; Heidari, H.; Fatemeh, R.A.A.; Pakmehr, M.; Shahbazian, H.; Ahmadi, I.; Mombeini, Z.; Mehrangiz, B.H. Effect of *Boswellia serrata* supplementation on blood lipid, hepatic enzymes and fructosamine levels in type2 diabetic patients. *J. Diabetes Metab. Disord.* **2014**, *13*, 29. [CrossRef]
48. Mohamed, S.H.; Attia, A.I.; Reda, F.M.; Abd El-Hack, M.E.; Ismail, I.E. Impacts of dietary supplementation of *Boswellia serrata* on growth, nutrients digestibility, immunity, antioxidant status, carcase traits and caecum microbiota of broilers. *Ital. J. Anim. Sci.* **2021**, *20*, 205–214. [CrossRef]
49. Abdelli, N.; Solà-Oriol, D.; Pérez, J.F. Phytogenic feed additives in poultry: Achievements, prospective and challenges. *Animals* **2021**, *11*, 3471. [CrossRef]

Dalmonte, T., Andreani, G., Rudelli, C., Isani, G. Efficacy of extracts of oleogum resin of *Boswellia* in the treatment of knee osteoarthritis: a systematic review and meta-analysis. **2024 (currently in minor revision on Phytotherapy research).**

In the third paper, a systematic review and meta-analysis was applied to evaluate the efficacy of phytoextracts from the oleogum resin of the *Boswellia* genus as a supplementation for patients affected by knee OA. Meta-analysis was developed including sub-group and meta-regression analysis, to point out the efficacy of *Boswellia* in a full scenario, taking in consideration different types of controls, supplementation, and duration of the studies. Four electronic databases were used and the PRISMA flowchart was used to select the studies to be included in the analysis. Publication bias through Egger test and heterogeneity through I^2 statistic has been evaluated to assess the parameters of the meta-analysis. The study has been submitted to Phytotherapy Research and is currently under revision.

Efficacy of extracts of oleogum resin of *Boswellia* in the treatment of knee osteoarthritis: a systematic review and meta-analysis

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Abstract

Background: Knee osteoarthritis (OA) has recently been ranked as the 11th highest contributor to global disability. More than 40% of the patients use complementary and alternative medicine including supplements containing phytoextracts with anti-inflammatory properties as those from *Boswellia* genus.

Objective: The aim of this meta-analysis was to evaluate the efficacy of phytoextracts from the oleogum resin of the *Boswellia* genus as a supplementation for patients affected by knee OA.

Search Methods: Four electronic databases were used for the research and PRISMA statements were followed throughout the study.

Selection Criteria: The following inclusion criteria were used: a) the subjects of the study were humans with a diagnosis of knee OA reported by medical staff; b) randomisation and the presence of a control (placebo, negative or positive control), and c) outcomes reported with WOMAC and/or VAS score.

Data collection and analysis: Publication bias was assessed with funnel plot and through the Egger test. Jadad scale was used in order to assess the quality of the studies included. The statistical heterogeneity was assessed using I^2 statistics. Results of meta-analysis and sub-group analysis were reported using forest plot.

Results: A total of 13 studies involving 850 (WOMAC) and 1185 (VAS) patients met the inclusion criteria. The meta-analysis did not detect a significant effect of the use of *Boswellia* extracts between the control and the treatment groups due to the high heterogeneity of the studies (p=0.0865 for WOMAC) and (p= 0.3966 VAS). However, the subsequent sub-group analysis demonstrated the significant beneficial effect of *Boswellia* extracts with respect to a placebo (lower WOMAC score in the treatment groups). This was also confirmed in the meta-regression applied to the WOMAC scores.

Conclusion: *Boswellia* extracts showed potential benefits in the treatment of knee OA respect to a placebo using the WOMAC score. This is an important finding as people exposed to NSAID-related adverse effects could benefit from the use of *Boswellia* extracts. However, further high-quality studies are needed to establish the clinical efficacy of extracts from genus *Boswellia.*

Keywords: *Boswellia* extract; frankincense; osteoarthritis; knee; anti-inflammatory phytoextracts; complementary and alternative medicine

1. INTRODUCTION

In 2019, 1.7 billion people worldwide lived with musculoskeletal pathologies, of these people, 528 million suffered from osteoarthritis (OA), leading to 19 million years lived with disability (YLD) [1, 2]. Osteoarthritis is a chronic inflammatory disease characterised by a reduction in mobility which involves the joints, causing pain and stiffness. It is a common disease in adult people over 55 years of age, with a higher prevalence in women, and it is related to other diseases, such as obesity and diabetes mellitus [3]. A prevalence of OA of 10% in men and 18% in woman has been estimated in adults over 60 years of age, and it is expected that, in 2050, 130 million people will suffer from musculoskeletal diseases [4]. Knee OA has recently been ranked as the 11th highest contributor to global disability [5].

As reported by Colletti et al., the approaches in the treatment of OA include pharmacological intervention with the use of non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitor drugs, lifestyle changes associated with physical activity, and the rehabilitation and application of a specific nutritional plan [6]. Nevertheless, if the above-mentioned approaches do not provide any benefit, the latest possibility in the treatment of this disease is surgery. The *Western Ontario and McMaster University (WOMAC) score is a recommended Patient-Recorder Outcome (PRO) used in the evaluation of treatment goals for hip and knee osteoarthritis [7]. The WOMAC score is composed of three items: pain (5 questions), stiffness (2 questions) and physical function (17 questions); each question offers a score from 0 to 4, with 0 scored as none and 4 as extreme. T*he subscale scores can vary, with pain ranging from 0 to 20 points; stiffness, from 0 to 8 points; and physical function, from 0 to 68 points for a total score which can range from 0 to 96 points. Higher scores represent worse pain, stiffness, and functional limitations [8]. *As reported by Woolacott et al. [9],* improved adherence to the standard use of the WOMAC scoring system, with clear reporting of it in trials of OA of the knee should be encouraged. *The Visual Analog Scale (VAS)* is a unidimensional measure of pain intensity. It is made up of a horizontal (HVAS) or vertical (VVAS) 10-centimetre (cm) scale which has been widely used in different adult populations, including those with rheumatic or musculoskeletal diseases [10]. The scale score can vary from 0 (no pain) to 100 (worst imaginable pain) and is usually reported in cm from 0 to 10. The WOMAC and the VAS scores are often used together for assessing the degree of knee OA and the likely efficacy of a treatment.

Due to the possible adverse effects on the cardiovascular system of the drugs used in the standard management and to the limited efficacy of the therapies available, more than 40% of the patients with knee OA use complementary and alternative medicine (CAM) which also includes supplements containing phytoextracts with anti-inflammatory properties [11, 12]. Extracts from the gum resin of plants of the genus *Boswellia* (family Burseraceae), also known as frankincense or guggal, have been used in traditional Ayurvedic medicine for the treatment of inflammation, including OA [13, 14, 15, 16]. These extracts contain a plethora of bioactive molecules, including boswellic acids. Of these, 3-acetyl-11-keto-β-boswellic acid (AKBA), 11-keto-β-boswellic acid (KBA) and β-boswellic acid (BA) are well known for their biological activity *in vitro* and *in vivo* [17, 16]. Boswellic acids act by inhibiting 5-lipoxigenase (5-LO) and are also involved in inhibiting the prostaglandins synthesis of COXs and modulating the

immune system [18]. Given this premise, there are suggestions to use extracts from the resin of the genus *Boswellia* in treating anti-inflammatory diseases, including knee OA [16].

A recent systematic review and meta-analysis carried out by Yu et al. (2020) analysed the effectiveness of *Boswellia* extracts for the treatment of OA in 7 randomised controlled trials. Based on the results of this meta-analysis, *Boswellia* and its extracts could be considered to be an effective option for patients affected by OA [19]. However, the authors did not consider that, of the 7 studies included, 3 did not use extracts but pure boswellic acids and 4 used a combination with other bioactive molecules. In fact, many studies investigating the beneficial effects of *Boswellia* extracts use a combination with other plant extracts having anti-inflammatory properties, such as *Curcuma longa, Zingiber officinalis, Witamnia somnifera,* and *Harpagophytum procumbens*, or are used in association with standard management treatments [20, 21, 22]. Finally, due to the aforementioned causes, the studies published so far were heterogeneous, and the scientific quality was often not satisfactory; therefore, data regarding the use of *Boswellia* extracts in the treatment of OA were contradictory.

The aim of this meta-analysis was to evaluate the efficacy of phytoextracts from the oleogum resin of the *Boswellia* genus as a supplementation for knee OA patients. Due to the heterogeneity of the studies in the literature, a subgroup analysis was carried out, and a meta-regression approach was used to investigate the role of exclusively using extracts from *Boswellia* or using them in combination with other phytoextracts, and whether the type of control (placebo, negative or positive control) and the duration of the treatment could affect the outcomes.

2. MATERIALS AND METHODS

2.1. Search strategy

The statements reported in Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 were followed throughout the study [23].

Four electronic databases were used for this research: PubMed, MEDLINE, Cochrane library and SCOPUS. The keywords used included (*Boswellia* OR Frankincense OR Salai guggal OR Shallaki) AND (Knee Osteoarthritis OR Knee OA OR Osteoarthritis OR OA OR Arthritis OR joint OR Skeletal OR Musculoskeletal OR Bones OR Joint). Furthermore, the search was widened, taking into consideration all the references of the articles selected. The search was carried out from December 2022 to February 2023. No registration number was provided for the review protocol.

2.2. Eligible criteria and study selection

Each article satisfying the following inclusion criteria was included in the meta-analysis: a) the subjects of the study were humans with a diagnosis of knee OA reported by medical staff; b) randomisation and the presence of a control (placebo, negative or positive control), c) outcomes reported with both WOMAC and VAS scores, or at least one of them, and d) *Boswellia* extracts, herbal formulation containing *Boswellia* and pure boswellic acids have been included in the analysis.

The exclusion criteria were: a) any study with subjects suffering from OA without indication of the specific region of the body; b) the presence of concomitant diseases; c) studies which used baseline (control group and treatment group was composed by the

same subjects at different time period, usually before the treatment and at the end of the study) as control; d) studies which did not report, or reported an incomplete, WOMAC or VAS score, and e) short papers, case reports, reviews and studies without English translation available.

Three authors (T.D., C.R., and G.A.) independently selected the studies by title and reviewed the abstracts of the articles selected. Studies which did not meet the pre-defined inclusion criteria were excluded. Any divergence in the study selection was dealt by a co-author (G.I.) and was subsequently resolved.

2.3. Data extraction

The data from each study were independently extracted by two authors (T.D. and C.R.) under the supervision of two co-authors (G.I. and G.A.). In the case of missing data, the corresponding author of each study was contacted by email, and no one replied. When the studies provided only the standard error mean (SEM), the standard deviation (SD) was calculated using the following equation:

$SD = SEM/\sqrt{n}$

The Jadad scale was used to evaluate the methodological quality of the studies selected. The Jadad scale is a 3 items assessment scale and the score is based on randomization (0 to 2 points), blinding (0 to 2 points) and account of all patients (0 to 1 point). Authors included in meta-analysis studies which showed at least 3 point judgment in Jadad scale. The score of the studies is reported in Table 1. When studies used more than a single control group, a different posology or different types of supplements in the groups treated, they were split and were treated as separate datasets in the meta-analysis. In the case of studies which had with more than one follow-up, they were split and treated as suggested by Dunlap et al. [24]. Additional information is reported in the 2.5 Statistical analysis section. Table of Jadad scale is reported as Supplementary Figure S1.

2.4. Risk of bias assessment

The risk of bias assessment was done through RoB 2 tool. As reported by Sterne et al. [25], assessment of risk of bias is considered as an essential component of a systematic review and the most commonly used tool for randomised trial is the Cochrane risk-of-bias (RoB) tool. The tool is composed considering biases that can arise at different stage of a trial. Thus, based on both empirical evidence and theoretical considerations, the tool identified 5 bias domains. Judgments in RoB 2 tool are provided by algorithms based on answers to the signalling questions of every domain of bias. Rob2 analysis of WOMAC and VAS score is reported below in Figure 1 and Figure 2, respectively.

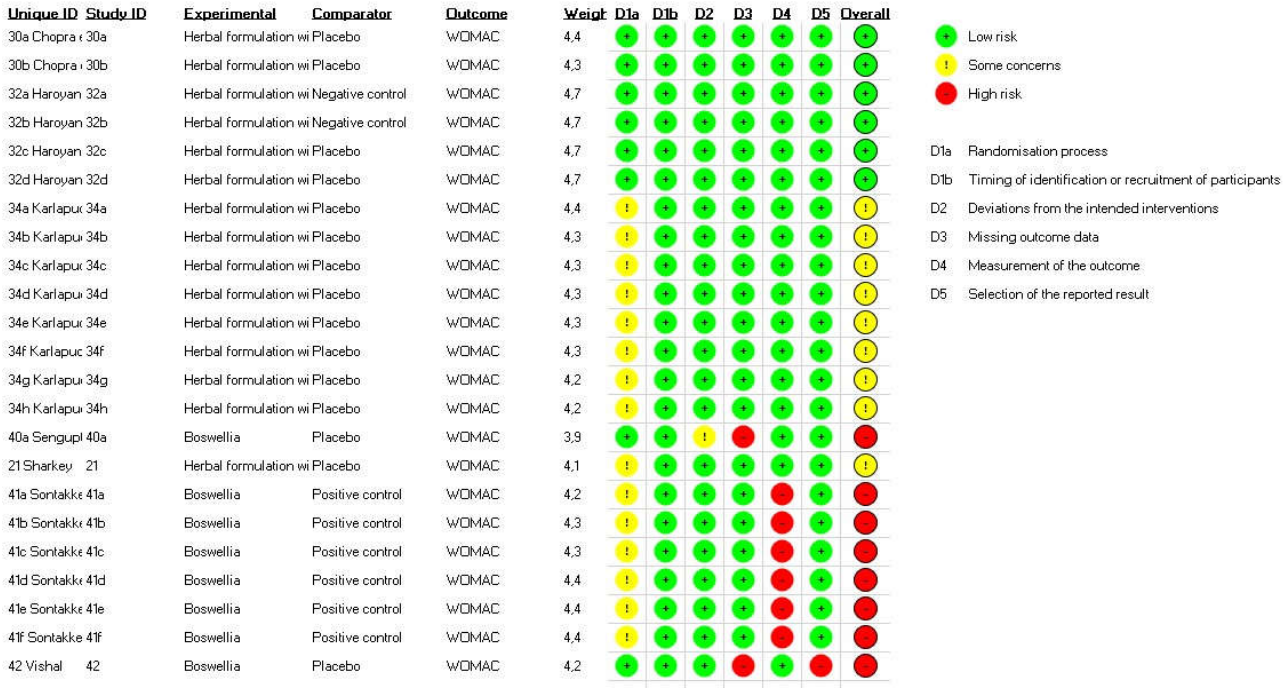

Figure 1. Rob 2 analysis of WOMAC score

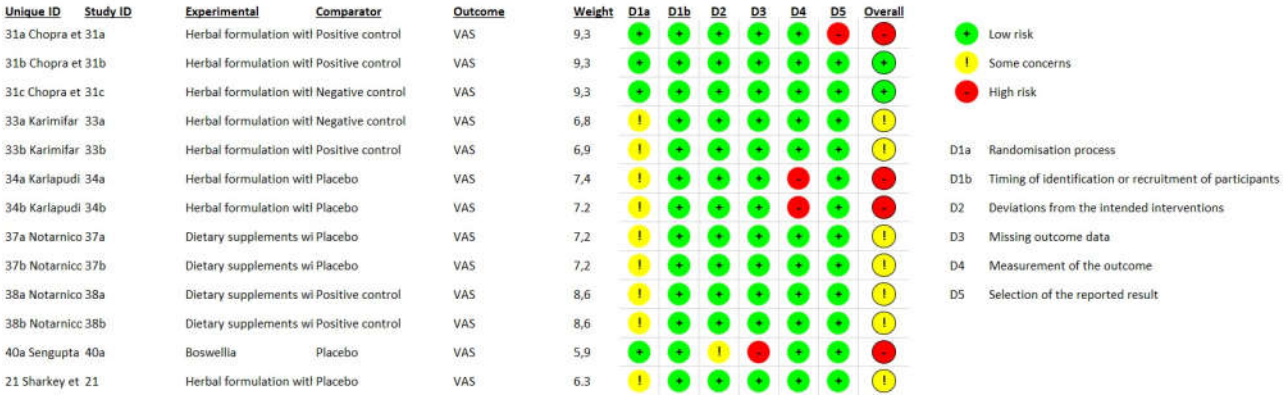

Figure 2. Rob 2 analysis of VAS score

2.5 **Statistical analysis**

An all-time point standard mean difference (SMD) meta-analysis was carried out to take into consideration likely outcomes in every time point of the studies. As reported by Dunlap et al. [24], if an effect size resulted after the statistical test without taking the correlation between the repeated measures into account, the effect size would be overestimated in studies with a repeated measures design. Furthermore, Dunlap et al. [24] suggested that, in a repeated measures design study, the effect size should be calculated using an equation which takes into consideration the correlation r between every follow-up [Equation 1]. Conversely, when the correlation between measures is not provided, the meta-analyst must use the means and standard deviations to estimate effect size directly with the equation provided using Cohen's method [Equation 2] [24, 26]. Moreover, in a Montecarlo simulation with 10000 iterations, it was shown that the differences in the effect size between the two equations were quite small, calculating with very similar outcomes as the sample size was bigger [24]. Due to the lack of the correlation r coefficient in the studies taken into consideration in the meta-analysis, the effect size was

calculated using Cohen's d method [26]. The two equations mentioned above are reported below.

D (effect size) = tc [2 (1 – r) / n] ^1/2

Equation 1. The equation suggested by Dunlap et al. for calculating effect size using a repeated measures study design. tc = t statistic for matched groups, r = correlation across pairs of measures, n = sample size [24].

D (effect size) = (Me – Mc) /SD

Equation 2. The equation suggested by Cohen et al. for calculating effect size. Me = mean of the experimental group, Mc = mean of control group, SD = common standard deviation [24, 26].

However, in all the studies with repeated measures which were taken into consideration, a correlation was not provided. Hence, the corresponding author of each study was contacted by email, and no one replied to give additional information regarding the data elaboration.

When a study presented more than one control, all the controls were considered separately in the meta-analysis. A control without any bioactive compounds was considered to be a "placebo", a formulation containing phytoextracts or bioactive compounds (except for those from *Boswellia*) was considered to be a "negative control", and a formulation used as standard management in the treatment of knee OA was considered to be a "positive control". In the presence of multiple treatments with *Boswellia* within a study, the different dosage groups were considered to be different treatment groups.

The presence of publication bias was assessed using the Egger test [27] and the evaluation of the funnel plots. As reported by Sterne et al., tests for funnel plot asymmetry should be used when there are more than 10 studies [28]. The statistical heterogeneity was assessed using I^2 statistics. Heterogeneity was classified across the studies as low (<30%), moderate (31 to 60%), substantial (61 to 74%) and considerable (>75%) [29]. Random models were used owing to the heterogeneity. Data previously normalised in a 0 to 100 scale were converted to WOMAC (0 to 96) and VAS score (0 to 10) ranges. Subsequently, subgroup analyses were carried out among the types of controls, the types of treatment and the duration of the studies (all the time points were taken into consideration) in order to minimise the impact of the heterogeneity.

A meta-regression approach using the controls, the types of treatment and the duration of the studies was used to evaluate whether the variables were statistically significant and whether they affected the outcome of the studies. Differences were considered to be statistically significant for $p < 0.05$.

The statistical analyses were carried out using R 4.2.2 (R foundation for statistical computing; Vienna, Austria; https://www.R-project.org/, accessed on 1st May 2023).

3. RESULTS AND DISCUSSION

3.1. Search results

Two hundred and twelve potential studies were found in the primary search. After a careful analysis based on the inclusion criteria, 159 studies were excluded, and 53 studies were

selected (Figure 1). Of these, 38 were excluded for the following reasons: concomitant presence of pathologies other than OA, data not extractable from the paper, lacking WOMAC or VAS scores.

A total of 15 studies met the inclusion criteria, 11 studies (30 separate datasets taking into consideration controls, treatments and time-points) reported the WOMAC score and 12 studies (27 separate datasets taking in consideration controls, treatments and time points) reported the VAS score. The search selection process is reported in Figure 1.

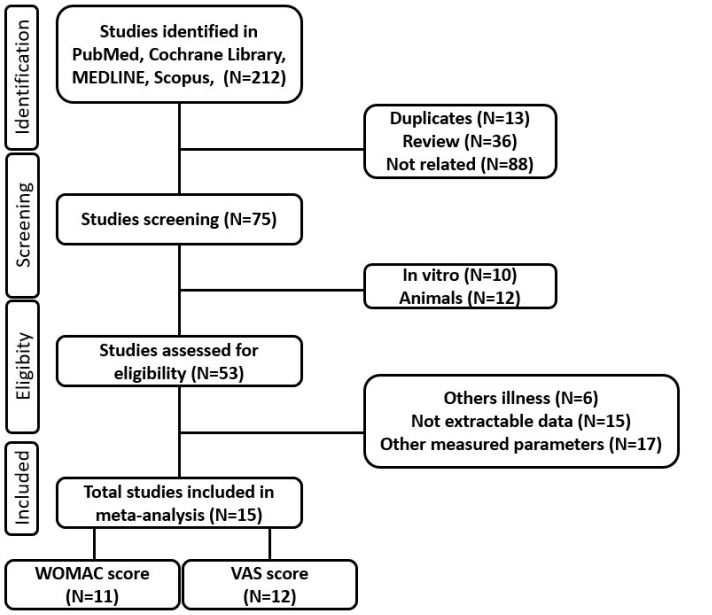

Figure 3. PRISMA flowchart of the study selection process

3.2. Characteristic of the studies

The characteristics of the studies included in the present meta-analysis are reported in Table 1.

The studies were published between 2004 and 2021. They were carried out in Italy, India, Armenia, Iran and the U.S.A.

To sum up, 820 patients were eligible when considering the inclusion criteria for the WOMAC score analysis and 1185 for the VAS score analysis.

In the WOMAC score analysis, 6 studies used supplements containing only extracts from *Boswellia,* 5 studies used an herbal formulation containing other extracts in addition to *Boswellia*, and of these, 1 used an herbal formulation containing *Boswellia* in association with a standard management drug. Of these 11 studies, 2 used a positive control, 8 used a placebo as a control and the remaining one used both a placebo and a negative control. Regarding the duration of the trial, 3 studies lasted up to a maximum of 2 months, 6 studies lasted 4 months, 1 lasted 6 months and the remaining one lasted 8 months. Seven studies were organised with more than one follow-up, or more than one treatment was investigated or tested versus different types of control groups, the other 4 did not include multiple follow-ups, different treatments or more than one control group.

In the VAS score analysis, 5 studies used supplements containing only extracts of *Boswellia,* while 5 studies used an herbal formulation containing extracts of other extracts in addition to *Boswellia* and two studies used a dietary supplement containing boswellic acids. Of these 12 studies, 1 used a positive control, 9 used a placebo and the other 2 studies also included a negative control. Three studies analysed for the VAS score lasted up to a maximum of 2 months, 5 studies lasted 4 months, 3 lasted 6 months and the remaining one lasted more than 6 months. Eight studies were organised with more than one follow-up, or more than one treatment was investigated or tested versus different type of control groups; the other 4 studies did not include multiple follow-ups, different treatments or more than one control group.

Table 1. Characteristics and Jadad scale score of the studies included in the meta-analysis based on design, treatment, posology, follow-up, type of control and score provided. The number of the reference is reported in the square brackets; the numbers of the split datasets re reported in parentheses.

Author	Year	N	Study design	Treatment	Posology	Time points	Control	Score	Jadad scale score
Chopra et al. [30] (30a, 30b)	2004	78 first time point 62 second time- point	Randomized double-blind comparative trial	Herbal formulation with *Boswellia*	2 tablets per day (dosage of *B. serrata* not provided)	4 months 8 months	placebo	WOMAC and VAS	4
Chopra et al. [31] (31a, 31b, 31c)	2013	418	Randomized double-blind comparative trial	Herbal formulation with *Boswellia*	6 tablets per day (*B. serrata* oleoresin hydroalcoholic extract 100 mg per tablet)	6 months	positive control negative control	VAS	5
Haroyan et al. [32] (32a, 32b, 32c, 32d)	2018	188 first time point 178 second time point	Randomized double-blind comparative trial	Herbal formulation with *Boswellia*	500 mg capsules 3 times per day (*B. serrata* 150 mg gum resin extract containing 75% of boswellic acids)	1 month 3 months	placebo negative control	WOMAC	5
Karimifar et al. [33] (33a, 33b)	2017	75	Randomized double-blind controlled trial	Herbal formulation with *Boswellia*	200 mg/day with negative control 400 mg/day with positive control (*B. thurifera* 100 mg oleo-gum resin hydroalcoholic extract containing 70% of boswellic acids)	1 month	positive control negative control	VAS	3
Karlapudi et al. [34] (34a, 34b, 34c, 34d, 34e, 34f, 34g, 34h)	2018	96	Randomized double-blind controlled trial	Herbal formulation with *Boswellia*	200 mg/day 400 mg/day (*B, serrata* extract with 0.6% of AKBA)	14 days 1 month 2 months 3 months	placebo	WOMAC and VAS	4
Karlapudi et al. [35]	2021	67	Randomized double-blind controlled trial	*Boswellia*	100 mg/day (*B. serrata* oleo-gum resin extract containing 20% of AKBA)	1 month	placebo	WOMAC and VAS	5

Majeed et al. [36]	2019	42	Randomized double-blind controlled trial	*Boswellia*	2 tablets/day (*B. serrata* extract 169.33 mg containing 87.3 mg of β-boswellic acids)	4 months	placebo	WOMAC and VAS	5
Notarnicola et al. [37] (37a, 37b)	2011	60	Randomized trial	Dietary supplement with boswellic acids	2 sachets/day (5g MSM 7.2 mg boswellic acids per sachets)	2 months 6 months	placebo	VAS	4
Notarnicola et al. [38] (38a, 38b)	2016	120	Randomized trial	Dietary supplement with boswellic acids	2 sachets/day (5g MSM 7.2mg boswellic acids per sachets)	2 months 6 months	postive control (glucosamine sulfate)	VAS	4
Sengupta et al. [39] (39a, 39b)	2008	70	Randomized double-blind controlled trial	*Boswellia*	100 mg/day 250 mg/day (*B. serrata* extract enriched with 30% AKBA)	3 months	placebo	WOMAC and VAS	5
Sengupta et al. [40] (40a, 40b)	2010	57	Randomized double-blind controlled trial	*Boswellia*	100 mg/day (*B. serrata* extract enriched with 30% AKBA) 100 mg/day (formulation 2: (*B. serrata* extract enriched with 20% AKBA	3 months	placebo	WOMAC and VAS	5
Sharkey et al. [21]	2021	43	Randomized double-blind trial	Herbal formulation with *Boswellia*	4 tablets/day (not provided the amount of *B. serrata)*	3 months	placebo	WOMAC and VAS	3
Sontakke et al. [41] (41a, 41b, 41c, 41d, 41e, 41f)	2007	66	Randomized trial	*Boswellia*	3 capsules/day (333 mg of *Boswellia serrata* extract containing 40% of boswellic acids per capsule)	1 month 2 months 3 months 4 months 5 months 6 months	positive control (valdecoxib)	WOMAC	3
Vishal et al. [42]	2011	59	Randomized double-blind controlled trial	*Boswellia*	100 mg/day (*B. serrata* extract containing 20% of boswellic acids)	1 month	placebo	WOMAC and VAS	5

3.3 Publication bias and heterogeneity percentage

The publication bias was measured using the Egger test for linear regression of funnel plot asymmetry. A significant publication bias was found in both the WOMAC ($p<0.0001$) and the VAS score ($p<0.0001$) groups. Thus, the presence of publication bias was resolved by removing the datasets one by one and repeating the Egger test at each step and by means of the visualisation of the funnel plot. The datasets responsible for the asymmetry were: 4a, 4b, 30a, 30b, 35, 36, 39a, 39b, 40b for the WOMAC score, and 30a, 30b, 34c, 34d, 34e, 34f, 34g, 34h, 35, 36, 39a, 39b, 40b, 35 for the VAS score. These datasets were not included in the meta-analysis. The funnel plots for the WOMAC and the VAS scores are reported in Figures 4 and 5, respectively.

Consequently, the meta-analysis was carried out on 6 studies (split into 20 datasets, 32a, 32b, 32c, 32d, 34a, 34b, 34c, 34d, 34f, 34g, 34h, 40a, 21, 41a, 41b, 41c, 41d, 41e, 41f, 42) and 7 studies (split into 13 datasets, 31a, 31b, 31c, 33a, 33b, 34a, 34b, 37a, 37b, 38a, 38b, 40a, 21) with 568 and 869 patients for the WOMAC and the VAS scores, respectively ($p=0.0603$, $p=0.0589$).

The heterogeneity was checked using the I^2 test. A heterogeneity percentage of 85.0% for the WOMAC score and 75.5% for the VAS score were detected; therefore, the use of random models was justified [28]. The heterogeneity of studies can be traced basically to three main causes: 1) different control types, 2) different supplement types, and 3) different duration of the trials; this was analysed through subgroup analysis.

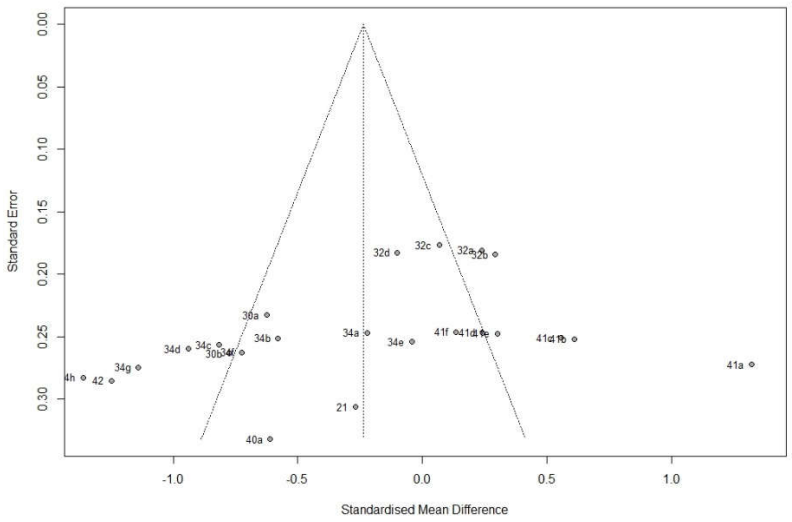

Figure 4. A funnel plot for the WOMAC score (Egger test $p=0.0603$). The numbers refer to the respective references.

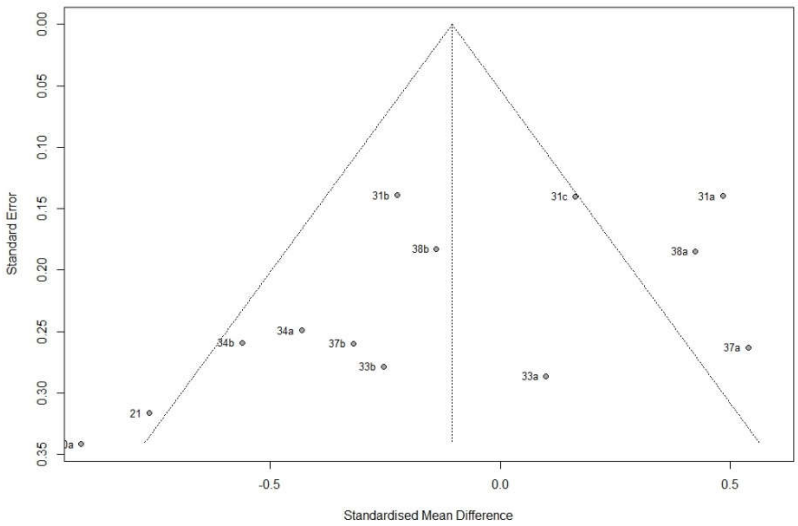

Figure 5. A funnel plot for the VAS score (Egger test p=0.0589). The numbers refer to the respective references.

3.4 WOMAC score meta-analysis

The meta-analysis random model did not detect a significant effect of the use of *Boswellia* extracts between the control and the treatment groups (p=0.0865, SMD=-0.2370, CI = -0.580 to 0.0340). The forest plot of the outcome of the meta-analysis for the WOMAC score is reported in Figure 6.

In particular, 11 datasets (32a, 32b, 32c, 32d, 34a, 34e, 40a, 21, 41d, 41e, 41f) showed no significant effect between the treatment and the control groups. Conversely, 12 datasets (30a, 30b, 34b, 34c, 34d, 34f, 34g, 34h, 41a, 41b, 41c, 42) showed a significant effect. Of those, 9 datasets (30a, 30b, 34b, 34c, 34d, 34f, 34g, 34h, 42) showed a lower WOMAC score in the treatment group, suggesting that the supplementation with *Boswellia* extracts might have produced beneficial effects in patients with knee OA. The last 3 datasets (41a, 41b and 41c) showed a higher WOMAC score in the treatment group, suggesting a significant positive effect of the valdecoxib used in the positive control group [41].

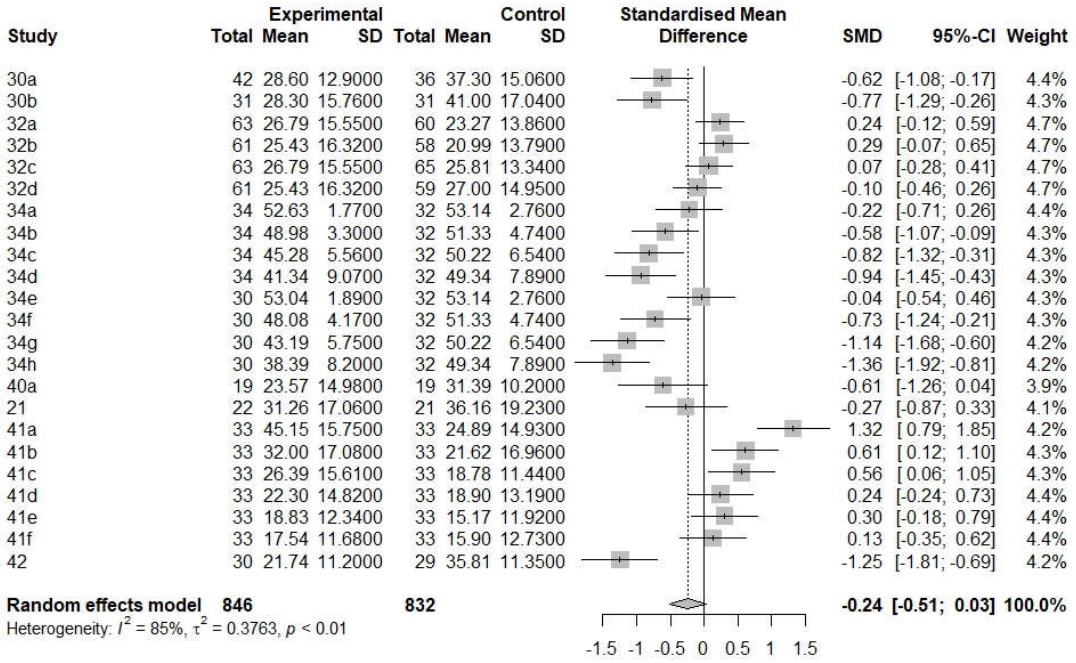

Figure 6. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the outcome of the meta-analysis for WOMAC score between control and treatment groups in a standardised mean difference meta-analysis (SMD=-0.24, 95%-CI=-0.51 to 0.03).

3.5 WOMAC score subgroup analysis

The differences in the control groups, treatments, and time points were present in the studies used for the meta-analysis. In this case, a sub-group analysis should be used as a supportive and exploratory approach, as reported by Moyé et al. [43], in order to understand which factors could influence the meta-analysis. Therefore, the subgroup analysis was carried by splitting the studies based on the control type, treatment formulation and duration of the study.

3.5.1 WOMAC score subgroup analysis based on control type

In the subgroup analysis based on different types of controls, 3 groups were identified: "positive control" indicating the use of a drug usually administered in the management of knee OA (41a, 41b, 41c, 41d, 41e, 41f), "placebo" indicating a control group which took a similar drug/formulation of the treatment group without any bioactive compounds (30a, 30b, 32c, 32d, 34a, 34b, 34c, 34d, 34e, 34f, 34g, 34h, 40a, 21, 42), and finally, "negative control" indicating the administration of the same formulation containing phytoextracts or bioactive compounds except those from *Boswellia* (32a, 32b). The outcome of the WOMAC score subgroup analysis based on control type is reported in Figure 7.

Significant differences ($p<0.0001$) were detected using the random effects model, showing a clear effect of the type of control used in the trial. A placebo was used in 15 datasets and

showed a medium effect size (SMD=0.5954), suggesting that the use of *Boswellia* produces an improvement in the treatment of knee OA with respect to the administration of no supplementation. Furthermore, studies which used a placebo in the control group make up 64.6% of the random model applied in the meta-analysis of the WOMAC score. The use of a negative control (SMD=0.2638) provided a small effect size, and no significant effect was detected, likely due to the fact that only one study (two different time points 32a, 32b) compared the effect of extracts of *Boswellia* and the administration of a formulation without it [32]. Finally, the use of a positive control reported by Sontakke et al. [41] showed a medium effect size (SMD=0.5111), suggesting that the drugs usually administered in the treatment of knee OA determined a lower WOMAC score with respect to the use of formulations containing extracts from the resin of *Boswellia.* In this study, the patients were followed at monthly time intervals for up to six months. Interestingly, the supplement containing the extract of *Boswellia* showed an onset slower than that of the conventional drug (valdecoxib); consequently, a significantly lower WOMAC score in the positive control group was measured during the first three months (41a, 41b and 41c), while during the following three months (41d, 41e, 41f), no significant differences were recorded between the two groups.

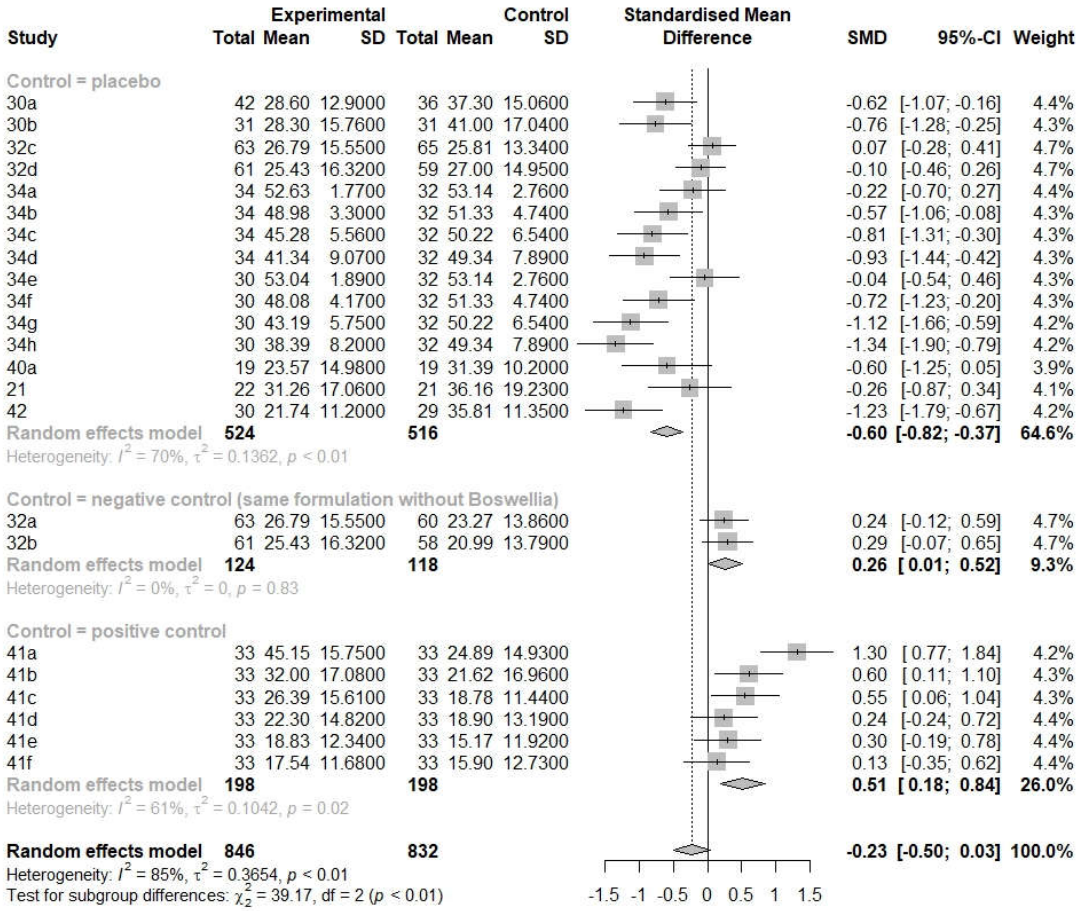

Figure 7. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the WOMAC score subgroup analysis based on control type.

3.5.2 WOMAC score subgroup analysis based on supplement type

In the subgroup analysis based on treatment type, 2 groups were detected: the use of mixed herbal formulations with *Boswellia* (30a, 30b, 32a, 32b, 32c, 32d, 34a, 34b, 34c, 34d, 34e, 34f, 34g, 34h, 21) and the use of a formulation exclusively containing an extract of *Boswellia* (40a, 41a, 41b, 41c, 41d, 41e, 41f, 42). The outcome of the WOMAC score subgroup analysis based on treatment types is reported in Figure 8.

Significant differences between the two groups were detected by the random effects model ($p=0.0423$), showing a possible influence of the type of formulation used as a supplement in the trials. In particular, the use of an herbal formulation with *Boswellia* or the use of an extract of *Boswellia* exclusively showed a small (SMD= -0.4348) and a very small (SMD = 0.1725) effect size, respectively, suggesting that the exclusive presence of extracts from the gum resin of *Boswellia* produced just a mild improvement in the treatment of knee OA. Concerning the administration of an herbal formulation with *Boswellia*, 8 of the 15 datasets considered showed a significant difference between the control and the treatment groups, with the latter showing a lower WOMAC score. In contrast, regarding the exclusive use of an extract of *Boswellia*, only one study (42) showed a significantly lower WOMAC score in the treatment group, while another study (41, split in three datasets 41a, 41b, 41c) showed a significant lower WOMAC score in the control group which, however, received NSAID as positive control. These results suggested that other plants contributed to the beneficial effects or, alternatively, that a synergic effect between different phytoextracts could be hypothesised.

The Authors emphasise that the result should be interpreted carefully; of note is that the only study (41) which compared the treatment groups with a positive control was in the subgroup of those using only *Boswellia*. Thus, it was not possible to evaluate and include in the meta-analysis studies comparing the administration of herbal formulations containing *Boswellia* bioactive compounds with a positive control. Furthermore, the use of an herbal formulation with *Boswellia* accounts for the 65.9% of the random model applied in the meta-analysis of the WOMAC score.

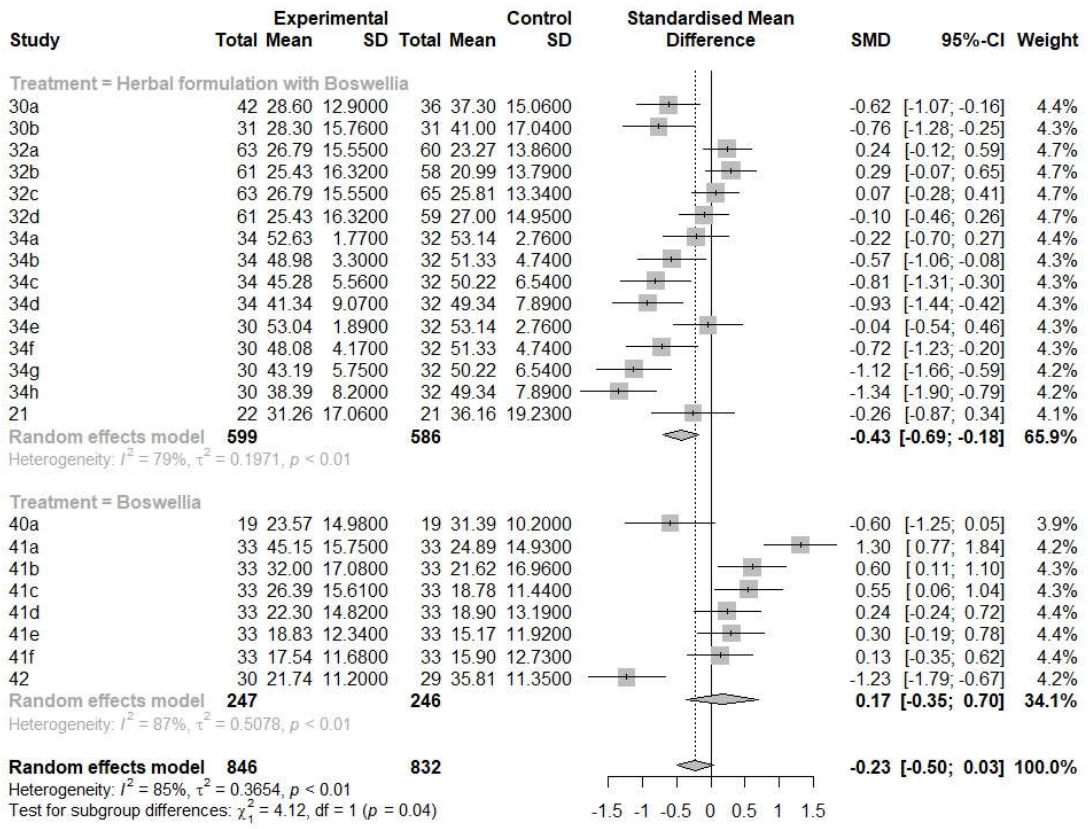

Figure 8. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the WOMAC score subgroup analysis based on treatment type.

3.5.3 WOMAC score subgroup analysis based on the duration of the trial

In the subgroup analysis based on the duration of the trial, 4 groups were selected: 0-2 months (32a, 32c, 34a, 34b, 34c, 34e, 34f, 34g, 41a, 41b, 42), 3-4 months (30a, 32b, 32d, 34d, 34h, 40a, 21, 41c, 41d), 5-6 months (41e, 41f) and >6 months (30b). The outcome of the WOMAC score subgroup analysis based on the duration of the studies is reported in Figure 9.

Significant differences were detected by the random effects model among the four groups (p= 0.0159), highlighting the fact that the duration of the studies and the outcomes of each time point could influence the efficacy of the extracts of *Boswellia* in the treatment of knee OA. Except the >6 -month time period, all the studies showed a small size effect (SMD= -0.2208 in the 0-2 months period, 0.2921 in the 3-4 months period, and 0.2150 in the 5-6 months period, respectively). To the best of the Authors' knowledge, Chopra et al. published the only study lasting more than 6 months and showed a medium size effect (SMD=-0.7641) (30b). Due to the different outcomes shown in the studies which provided more than one time point, it is difficult to understand how time could affect the treatment of the disease when using *Boswellia*.

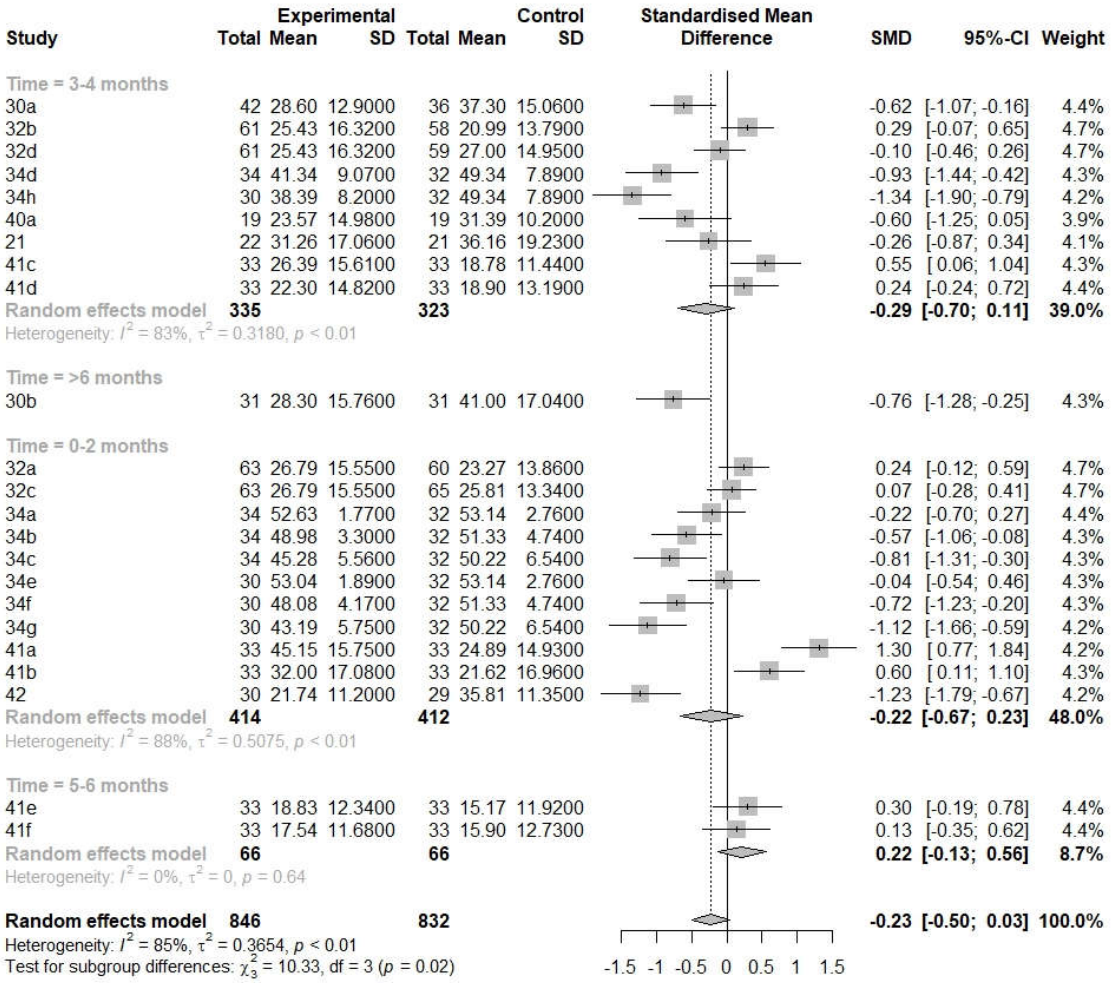

Figure 9. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the WOMAC score subgroup analysis based on duration of the studies including different time-points.

3.6 VAS score meta-analysis

The meta-analysis random model did not detect significant differences between the control and the treatment groups (p= 0.3966, SMD= -0.1049, 95%-CI= -0.3473 to 0.1376). The forest plot of the VAS score meta-analysis is reported in Figure 10.

Seven datasets (31b, 31c, 33a, 33b, 34a, 37b, 38b) did not show significant differences. On the other hand, six datasets showed a significant effect (31a, 34b, 37a, 38a, 40a, 21). Of those, 3 showed a higher VAS score in the treatment group (31a, 37a, 38a); however, 37a and 38a did not use an extract of *Boswellia* but a supplement containing pure boswellic acids. The other 3 datasets (34b, 40a, 21) showed a higher VAS score in the control group demonstrating that, in the treatment of knee OA, the effects of extracts of *Boswellia* are contradictory.

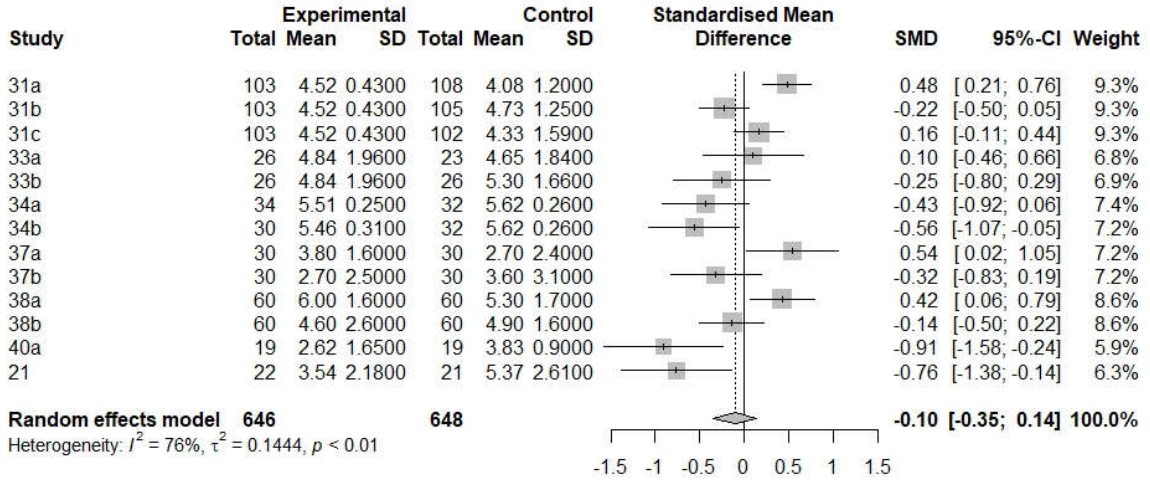

Figure 10. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the VAS score between the control and the treatment groups in a standard mean difference meta-analysis.

3.7 VAS score subgroup analysis

As previously reported for the WOMAC score, a subgroup analysis was carried out, splitting the studies based on control types, treatment formulation and duration of the study.

3.7.1 VAS score subgroup analysis based on control type

In the subgroup analysis based on different types of controls, 3 groups were identified: positive control (31a, 31b, 33b, 38a, 38b), placebo (34a, 34b, 37a, 37b, 40a, 21), and finally, negative control (31c, 33a). The outcome of the VAS score subgroup analysis is reported in Figure 11.

Significant differences among the groups were not detected by the random effects model (p=0.0817), suggesting that different outcomes regarding the efficacy of *Boswellia* did not depend on the type of control. It should be noted that 2 datasets (31a, 38a) reported a lower VAS score in the positive control group, while another 3 datasets did not show significant differences between the control and the treatment groups. A negative control group was used in two studies, and no significant differences were detected in the VAS score with respect to the treatment group. Furthermore, the trials which used a placebo as a control group had contrasting outcomes with 3 studies which detected a lower VAS score in the treatment group (34a, 40a, 21), while another study reported a higher VAS score in the treatment group (37a) at the first follow-up but not at the second [34, 40, 21, 37].

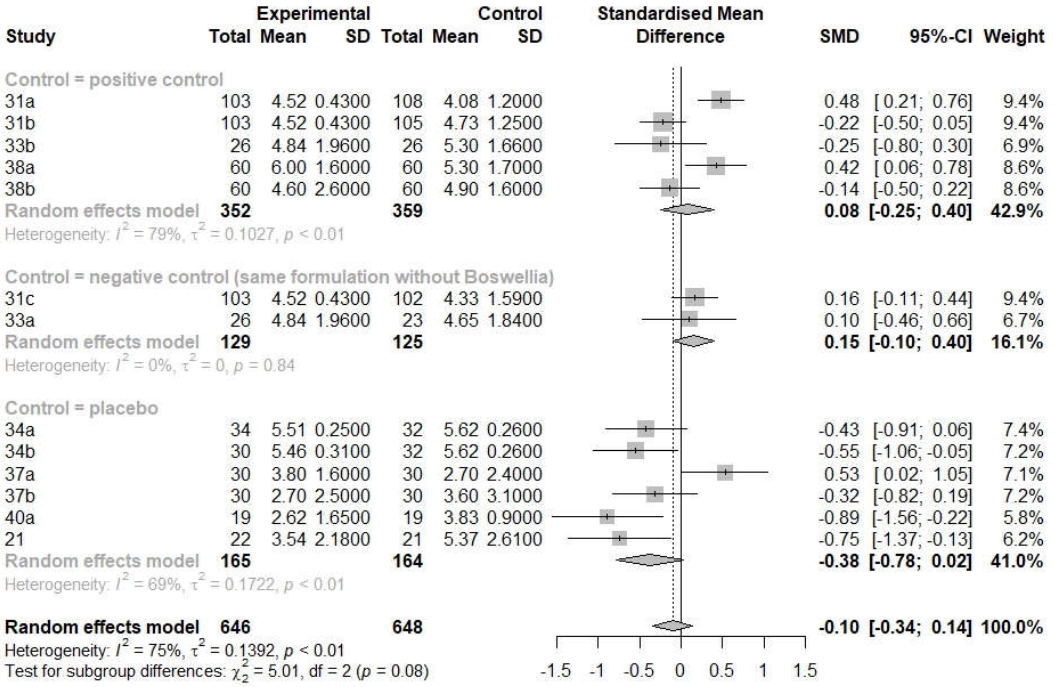

Figure 11. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the VAS score subgroup analysis based on control type.

3.7.2 VAS score subgroup analysis based on the supplement type

A subgroup analysis was carried out splitting the studies into 3 different groups: the use of herbal formulation with *Boswellia* (31a, 31b, 31c, 33a, 33b, 34a, 34b, 21), the use of a formulation exclusively containing extract of *Boswellia* (40a) and the use of a dietary supplement containing pure boswellic acids (37a, 37b, 38a, 38b). The outcome of the VAS score subgroup analysis based on treatment type is reported in Figure 12.

Only one study used a formulation exclusively containing a *Boswellia* extract [40]. Significant differences among the three subgroups were detected (p= 0.0371); in fact, patients supplemented with the extract of *Boswellia* showed a significant lower VAS score in the treatment group respect to the placebo, suggesting that this type of formulation could be helpful in the treatment of patients with knee OA [40].

Of the studies which used an herbal formulation also containing *Boswellia* in association with other phytoextracts, 5 datasets did not show a significant difference among the groups (31b, 31c, 33a, 33b, 34a) and only 2 datasets showed a significantly lower VAS score in the treatment group (34b, 21), while only one dataset showed a significantly higher score than the control group (31a). However, a small effect size was calculated regarding the use of an herbal formulation containing *Boswellia* (SMD= -0.1384). Notarnicola et al. [37, 38] were the only authors who investigated the use of dietary supplements containing pure boswellic acids; at the first time point of both studies (2 months) a significantly lower VAS score was determined in the control group (37a, 38a), while at the second time point (6 months), there was no significant difference in the VAS score between the treatment and the control groups

(37b, 38b). A small effect size was detected in these studies which had investigated the use of dietary supplements containing pure boswellic acids (SMD=0.1256).

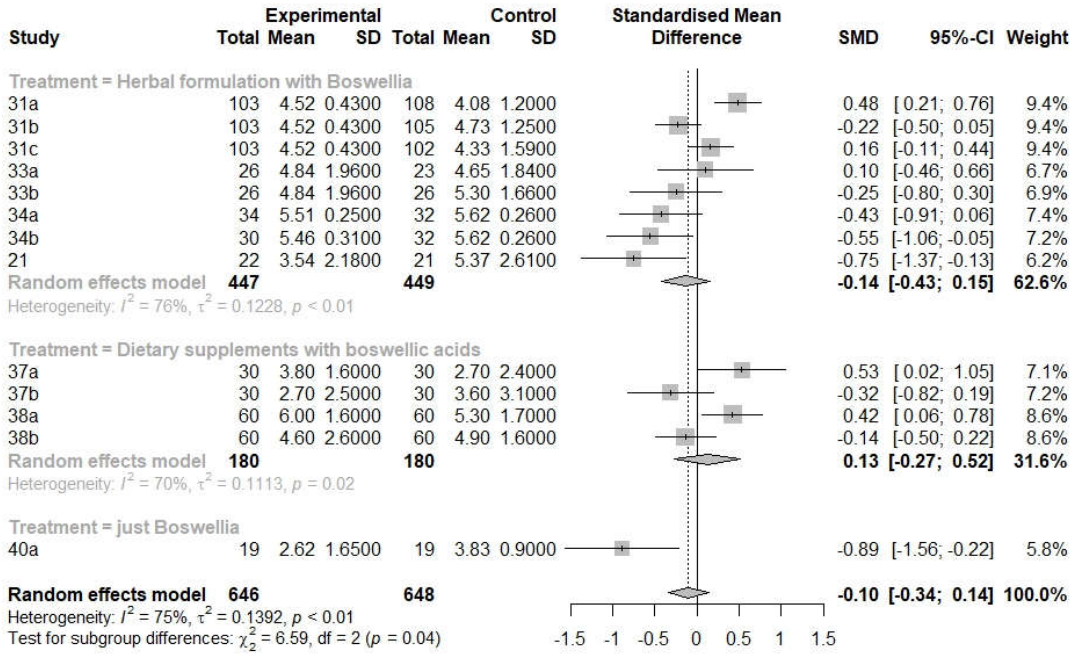

Figure 12. The efficacy of *Boswellia* extracts in the management of knee OA: a forest plot of the VAS score subgroup analysis based on treatment type.

3.7.3. VAS score subgroup analysis based on the duration of the trial

The subgroups were split as reported in 3.6.3, with the exception of the last group (>6 months). To the best of the Authors' knowledge, no research regarding the efficacy of *Boswellia* in the treatment of knee OA using VAS score has lasted longer than 6 months. Four studies (six datasets) lasted 0-2 months (33a, 33b, 34a, 34b, 37a, 38a), 2 lasted 3-4 months (40a, 21) and 3 (5 datasets) lasted 5-6 months (31a, 31b, 31c, 37b, 38b). The forest plot of the VAS score subgroup analysis based on the duration of the trial is reported in Figure 13.

A significant difference between the groups was detected (p=0.0069). Of the studies which lasted 0-2 months, 3 datasets (33a, 33b, 34a) did not show significant differences between the control and the treatment groups, while 2 (37a, 38a) reported a higher VAS score in the treatment group, and the remaining one (34b) a lower score with respect to the control group. Only the studies of Sengupta et al. (2010) [40] and Sharkey et al. (2021) [21] lasted 3-4 months and reported a significant difference between the control and the treatment groups, with the latter showing a lower VAS score, suggesting a beneficial effect of the supplementation. Despite the positive outcomes reported in studies lasting 3-4 months, of

the 5 studies lasting 5-6 months, only one study showed significant differences between the groups, with a lower VAS in the positive control group [31].

Of note, in the studies lasting 0-2 months (37a, 38a), Notarnicola et al. reported a significant difference in the control group which had a lower VAS score than the control group; however, at the last time point (5-6 months), no significant difference was detected (37b, 38b).

The studies lasting 0-2 and 5-6 months showed a very small effect size (SMD= -0.0183 and SMD= 0.0190, respectively), while the studies included in the 3-4 months group showed a large effect size (SMD= -0.8145).

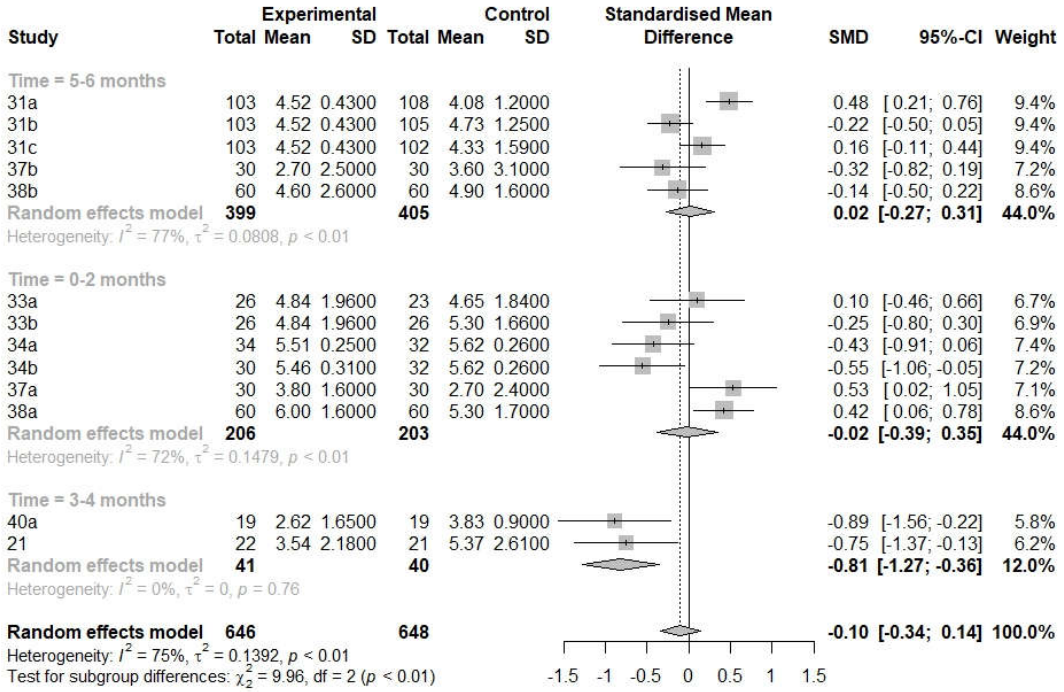

Figure 13. The efficacy of *Boswellia* extracts in the management of knee OA: **a** forest plot of the VAS score subgroup analysis based on duration of the studies.

3.8 Meta-regression approach

To identify which factors had a significant influence on the meta-analysis effect size, meta-regression was carried out for type of control, type of treatment and time points for both the WOMAC and the VAS scores, respectively. The WOMAC score meta-regression outcome is reported in Supplementary Figure S2.

The residual heterogeneity detected was 67.64%% and R^2 was 67.29%; this percentage satisfactorily explained the I^2 residual. No significant association was detected concerning the time points and the different formulations used as supplements, suggesting that the duration of the study, the follow-ups, and different types of formulation with *Boswellia* (mixed with other extracts or used alone) did not influence the effect size. On the other

hand, the use of a placebo as a control was significantly and positively associated with the outcome, indicating for the WOMAC score a higher effect size in comparison with a negative control ($p=0.0365$, estimate coefficient=0.8168) or a positive control ($p<0.001$, estimate coefficient=1.6282). Therefore, the meta-regression showed that the use of a placebo in the control group is associated with a significant decrease in the WOMAC score in the treatment group which received the supplementation with extracts of *Boswellia*. This could be of particular interest for those people who cannot tolerate the standard management (NSAIDS) due to concomitant pathologies.

By contrast, the outcomes obtained for the VAS score meta-regression detected a residual heterogeneity of 75.73%; however, the R^2 of 11.84% was too low to explain the substantial heterogeneity. Therefore, the results of the meta-regression of the VAS score did not show any significant association. The VAS score meta-regression outcome is reported in Supplementary Figure S3.

4. Limitations

On the market there are different products to alleviate OA symptoms based on plant extracts with anti-inflammatory activities as *C. longa, Z. officinalis, W. somnifera,* and *H. procumbens. Boswellia* extracts have been tested in different clinical studies often producing contradictory results due to differences in formulations, types of control, and finally, time points. Therefore, the meta-analysis was limited by the heterogeneity of the studies considered, but this weakness has been overcome using subgroup analysis and meta-regression. The authors also point out that WOMAC and VAS score are self-reported questionnaires, although they are commonly accepted and encouraged in the evaluation of knee OA treatment.

Extracts of the oleogum resin obtained from the *Boswellia* genus have been used for centuries as traditional treatment in Indian Ayurvedic medicine [15]. Accordingly, the majority of the studies included in this meta-analysis were carried out in Eastern populations, with the exception of some studies carried out in Italy and in the U.S.A. This evidence could limit the generalisation of the results obtained.

The variability and the insufficient characterisation of the supplements deserved special attention. It is well known that *Boswellia* extracts show different composition and quality, due to factors which include the use of different species of the genus *Boswellia*, different environmental conditions, and different extraction procedures. A prime example concerns the content of boswellic acids which is generally reported to be 65%. This value is often unrealistic as boswellic acids represent a percentage of the organic acids present in the phytoextract, the content of which is generally determined using unspecific titration methods which quantify all the organic acids present in the extract. This inconsistency is well known and has previously been highlighted by various authors [17, 44, 45].

Finally, the authors stress the fact that meta-analysis is a statistical and scientific technique which attempts to point out evidence in areas in which there are papers which report divergent outcomes. However, it cannot resolve a lack of evidence [45]. The presence of possible effects deriving from not measured or incompletely measured factors cannot be excluded. Moreover, as reported by Spector et al. (1991), publication bias and search bias

are potential problems in all meta-analyses as unpublished studies may be in contrast with published results [46].

5. Strengths and Additional Research Needs

Recently, three meta-analyses on the efficacy of the use of *Boswellia* extracts on OA have been published. Yu et al. (2020) analysed the effectiveness of *Boswellia* extracts for the treatment of OA in 7 randomised controlled trials [19], while Bannuru et al. (2018) evaluated the effects of curcumin and *Boswellia* [47], and Smedslund et al. (2022) evaluated different available treatment options for OA, including the use of *Boswellia* extracts [48]. This study represents an expansion from what Yu et al. [19] reported as it included analysis of 15 studies; in addition, unlike the other two studies, it is focused only on *Boswellia* extracts.

The strengths of the present meta-analysis are related to the inclusion of prospective studies with long-term follow-ups, up to 8 months, and the analysis of the possible sources of heterogeneity. In particular, the subgroup analysis and the meta-regression approach have clarified which factors, of those analysed, were involved and could significantly change the effect size and, consequently, the outcomes of the studies.

Additional studies including a positive control and analysing the possible effects of *Boswellia* alone or in combination with other phytoextracts are needed. Moreover, this meta-analysis showed a lack of studies carried out in Europe, with the exception of Italy.

6. Conclusions

The anti-inflammatory activity of extracts from the oleogum resin of the *Boswellia* species has been addressed by many studies, including reviews, systematic reviews, and meta-analyses without, however, coming to any definitive conclusions regarding the actual efficacy of this phytoextract [16, 19, 49]. The present study provided additional evidence regarding the efficacy of extracts or bioactive molecules obtained from species of the *Boswellia* genus as CAM in knee OA management. In particular, the subgroup analysis demonstrated the significant beneficial effect of *Boswellia* extracts with respect to a placebo (lower WOMAC score in the treatment groups). This was also confirmed in the meta-regression applied to the WOMAC score. This is an important finding as people exposed to NSAID-related adverse effects could benefit from the use of *Boswellia* extracts.

However, data regarding adverse effects and toxicity of these supplements are still incomplete, albeit extracts of *Boswellia spp*. haven't exhibited toxicity in *in vivo* animal models [50]. There is a common sense that herbal products are safe, whilst taking herbal remedies can be harmful and consumers are often not aware of their potential adverse effects [51]. Therefore, there is a discrepancy between the availability of these products on the market and the paucity of scientific information often characterized by poor methodology and unreliable clinical analysis [52, 53]. Further high-quality studies are needed to establish the clinical efficacy of *Boswellia* oleogum extracts.

Finally, another item worth to be considered is the chemical composition of supplements. The variability and the inaccurate supplement characterisation deserve special attention, due to the lack of unbending guidelines regarding safety and quality of phytoextracts; therefore, some marketed products might be absolutely ineffective. A formulation containing amounts of boswellic acids determined using a specific, accurate, and possibly

standardised HPLC method is a prerequisite for evaluating any beneficial effects of a *Boswellia* extract.

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Conflict of interests

The authors declare that they have no competing interest.

References

1. *Cieza, A., Causey, K., Kamenov, K., Hanson, S.W., Chatterji, S., Vos, T. Global estimates of the need for rehabilitation based on the Global Burden of Disease study 2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet.* ***2019****, 396(10267), 2006-2007.*
2. *Williams, A., Kamper, S.J., Wiggers, J.H., O'Brien, K.M., Lee, H., Wolfenden, L., Yoong, S.L., Robson, E., McAuley, J.H., Hatvigsen, J., Williams, C.M. Musculoskeletal conditions may increase the risk of chronic disease: a systematic review and meta-analysis of cohort studies. BMC Med.,* ***2018****, 16, 167.*
3. Paul, A.K., Jahan, R., Paul, A., Mahboob, T., Bondhon, T.A., Jannat, K., Hasan, A., Nissapatorn, V., Wilairatana, P., Pereira, M.L., Wiart, C., Rahmatullah, M. The role of medicinal and aromatic plants against obesity and arthritis: a review. *Nutrients*, **2022**, 14, 985.
4. Bolognesi, G., Belcaro, G., Feragalli, B., Cornelli, U., Cotellese, R., Hu, S., Dugall, M. Movardol® (N-acetylglucosamine, *Boswellia serrata*, ginger) supplementation in the management of knee osteoarthritis: preliminary results from a 6-month registry study. *Eur. Rev. Med. Pharmacol. Sci.*, **2016**, 20, 5198-5204.
5. Cross, M., Smith, E., Hoy, D., Nolte, S., Ackerman, I., Fransen, M., Bridgett, L., Williams, S., Guillemin, F., Hill, C.L., Laslett, L.L., Jones, G., Cicuttini, F., Osborne, R., Vos, T., Buchbinder, R., Woolf, A., March, L. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. *Ann. Rheum. Dis.*, **2014**, 73(7), 1323-1330.
6. Colletti, A., Cicero, A.F.G. Nutraceutical approach to chronic osteoarthritis: from molecular research to clinical evidence. *Int. J. Mol. Sci.*, **2021**, 22, 12920.
7. Peter, W.F., Jansen, M.J., Hurkmans, E.J., Bloo, H., Dekker, J., Dilling, R.G., Hilberdink, W., Smit, C.K., Rooij, M., Veenhof, C., Vermeulen, H.M., Vos, R.J., Schoones, J.W., Vlieland, T.P.V. Physiotherapy in hip and knee osteoarthritis: development of a practice guideline concerning initial assessment, treatment and evaluation. *Acta. Reumatol. Port.*, **2011**, 36(3), 268-281.
8. Barber-Westin, S.D., Noyes, F.R. Noyes' knee disorders: surgery, rehabilitation, clinical outcomes. *Elsevier,* **2017,** 2nd edition, 1211-1221.
9. Woolacott, N.F., Corbett, M.S., Rice, J.C. The use and reporting of WOMAC in the assessment of the benefit of physical therapies for the pain of osteoarthritis of the knee: findings from a systematic review of clinicals trials. *Rheumatology*, **2012**, 51, 1440-1446.
10. Hawker, G.A., Mian, S., Kendzerska, T., French, M. Measures of adult pain. *Arthritis Care and Res.*, **2011**, 63, 11, 240-252.
11. Vina, E.R., Youk, A.O., Quinones, C., Kwoh, C.K., Ibrahim, S.A., Hausmann, L.R.M. Use of complementary and alternative therapy for knee osteoarthritis: race and gender variations. *ACR open rheumatology*, **2021**, 3, 9, 581-667.
12. Basedow, M., Runciman, W.B., March, L. Australians with osteoarthritis: the use of and beliefs about complementary and alternative medicines. *Complement. Ther. Clin. Pract.*, **2014**, 20, 237-242.
13. Abdel-Tawab, M., Werz, O., Schubert-Zsilavecz, M. *Boswellia serrata*: an overall assessment of in vitro, preclinical, pharmacokineticand clinical data. *Clin. Pharmacokinet.*, **2011**, 50 (6), 349–369.
14. Ammon, H.P.T. Boswellic acids in chronic inflammatory diseases. *Planta Med.*, **2006**, 72(12), 1100–1116.

15. Ammon, H.P.T. Boswellic acids and their role in chronic inflammatory diseases. In: Anti-Inflammatory Nutraceuticals and Chronic Diseases, S. C. Gupta, S. Prasad, and B. B. Aggarwal, **2016**, Eds., 291–327, Cham: Springer International Publishing, Switzerland
16. Efferth, T., Oesch, F. Anti-inflammatory and anti-cancer activities of frankincense: targets, treatments and toxicities. *Semin. Cancer Biol.*, **2022**, 80, 39-57.
17. Bertocchi, M., Isani, G., Medici, F., Andreani, G., Usca, I.T., Roncada, P., Forni, M., Bernardini, C. Anti-inflammatory activity of *Boswellia serrata* extracts: an *in vitro* study on porcine aortic endothelial cells. *Oxid. Med. Cell. Longev.*, **2018**, 25, 2504305.
18. Ammon, H.P.T. Modulation of the immune system by *Boswellia serrata* extracts and boswellic acids. *Phytomedicine*, **2010**, 17, 862-867.
19. Yu, G., Xiang, W., Zhang, T., Zeng, L., Yang, K., Li, J. Effectiveness of *Boswellia* and *Boswellia* extract for osteoarthritis patients: a systematic review and meta-analysis. *BMC Complement. Med. Ther.*, **2020**, 20, 225.
20. Belcaro, G., Dugall, M., Luzzi, R., Hosoi, M., Ledda, A., Feragalli, B., Hu, S., Ganguly, A., Eggenhoffner, R., Corti, A., Giacomelli, L. Phytoproflex®: supplementary management of osteoarthritis: a supplement registry. *Minerva Med.*, **2018**, 109 (2), 88-94.
21. Sharkey, P., Shah, Z., Gross, M., Meade, T., Davidoff, S., Shohat, N., Parvizi, J. The effect of a natural oral nutritional supplement on the level of intra-articular inflammatory mediators in patients with osteoarthritis of the knee. *J. Orthop. Exp. Innov.*, **2021**.
22. Dragos, D., Gilca, M., Gaman, L., Vlad, A., Iosif, L., Stoian, I., Lupescu, O. Phytomedicine in joint disorder. *Nutrients*, **2017**, 9(1), 70.
23. Hoffmann, T.C., Mulrow., C.D., Shamseer., L., Tezlaff., J.M., Akl, E.A., Brennan, S.E., Chou., R., Glanville, J., Grimshaw., J.M., Hrobjartsson., A., Lalu, M.M., Li., T., Loder., E.W., Mayo-Wilson., E., McDonald., S., McGuinness., L.A., Stewart., L.A., Thomas., J., Tricco., A.C., Welch., V.A., Whiting., P., Moher., D. The PRISMA 2020 statement: an update guideline for reporting systematic reviews. *B. M. J.*, **2021**, 372.
24. Dunlap, W.P., Cortina, J.M., Vaslow, J.B., Burke, M.J. Meta-analysis of experiments with matched groups or repeated measures design. *Psychol. Methods*, **1996**, 1, 170-177.
25. Sterne, J.A.C, Savocic, J., Elbers, R.G., Blencowe N.S., Boutron, I., Cates, C.J., Cheng, H.Y., Corbett, M.S., Eldridge, S.M., Hernan, M.A., Hopewell, S., Hrobjartsson, A., Junqueira, D.R., Juni, P., Kirkham, J.J, Lasserson, T., Li, T., McAleenan, A., Reeves, B.C., Shepperd, S., Shrier, I., Stewart, L.A., Tilling, K., White, I.R., Whiting, P.F., Higgins, J.P.T. RoB 2: a revised tool for assessing risk of bias in randomised trials. *BMJ*, **2019**, 366, 14898
26. Cohen, J. Satistical power analysis for the behavioral sciences. New York: Academic Press.
27. Egger, M., Smith, G.D. Bias in meta-analysis detected by a simple, graphical test. *BMJ*, **1997**, 315-629
28. Sterne, J.A.C., Sutton, A.J., Terrin, N., Jones, D.R., Lau, J., Carpenter, J., Rucker, G., Harbord, R.M., Schmid, C.H., Tetzlaff, J., Deeks, J.J., Peters, J., Macaskill, P., Schwarzer, G., Duval, S., Altman, D.G., Moher, D., Higgins, J.P.T. Recommendations for examining and interpreting funnel plot asymmetry in meta-analyses of randomised controlled trials. *B.M.J.*, **2011**, 343.
29. Higgins, J.P.T., Thomas, J., Chandler, J., Cumpston, M., Li, T., Page, M.J., Welch, V.A. *Cochrane handbook for systematic reviews of interventions*, **2019**.
30. Chopra, A., Lavin, P., Patwardhan, B., Chitre, D. A 32-week randomized, placebo-controlled clinical evaluation of RA-11, an ayurvedic drug, on osteoarthritis of the knees. *J. Clin. Rheumatol.*, **2004**, 10, 236-245.
31. Chopra, A., Saluja, M., Tillu, G., Sarmukkaddam, S., Venugopalan, A., Narsimulu, G., Handa, R., Sumantran, V., Raut, A., Bichille, L., Joshi, K., Patwardhan, B. Ayurvedic medicine offers a good alternative to glucosamine and celecoxib in the treatment of symptomatic knee osteoarthritis: a randomized, double-blind, controlled equivalence drug trial. *Rheumatology*, **2013**, 52, 1408-1417.

32. Haroyan, A., Mukuchyan, V., Mkrtchan, N., Minasyan, N., Gaspraryan, S., Sargsyan, A., Narimanyan, M., Hovhannisyan, A. Efficacy and safety of curcumin and its combination with boswellic acid in osteoarthritis: a comparative, randomized, double-blind, placebo-controlled study. *BMC Complement. Altern. Med.*, **2018**, 18, 7.
33. Karimifar, M., Soltani, R., Hajhashemi, V., Sarrafchi, S. Evaluation of the effect of *Elaeagnus angustifolia* alone and combined with *Boswellia thurifera* compared with ibuprofen in patients with knee osteoarthritis: a randomized double-blind controlled clinical trial. *Clin. Rheumatol,* **2017**, 36, 1849-1853.
34. Karlapudi, V., Mungara, A.V.V.P., Sengupta, K., Davis, B.A., Raychaudhuri, S.P. A placebo-controlled double-blind study demonstrates the clinical efficacy of a novel herbal formulation for relieving joint discomfort in human subjets with osteoarthritis of knee. *J. Med. Food*, **2018**, 21, 5, 1-10.
35. Karlapudi, V., Sunkara, K.B., Konda, P.R., Sarma, K.V., Rokkam, M.P. Efficacy and safety of Aflapin®, a novel *Boswellia serrata* extract, in the treatment of osteoarthritis of the knee: a short-term 30-day randomized, double-blind, placebo-controlled clinical study. *J. Am. Coll. Nutr.*, **2021**, 42, 2, 159-168.
36. Majeed, M., Majeed, S., Narayanan, N.K., Nagabhushanam, K. A pilot, randomized, double-blind, placebo-controlled trial to assess the safety and efficacy of a novel *Boswellia serrata* extract in the management of osteoarthritis of the knee. *Phytother. Res.*, **2019**, 33, 1457-1468.
37. Notarnicola, A., Tafuri, S., Fusaro, L., Moretti, L., Pesce, V., Moretti, B. The "MESACA" study: methylsulfonylmethane and boswellic acids in the treatment of gonarthrosis. *Adv. Ther.*, **2011**, 28, 10, 894-906.
38. Notarnicola, A., Maccagnano, G., Moretti, L., Pesce, V., Tafuri, S., Fiore, A., Moretti, B. Methylsulfonylmethane and boswellic acids versus glucosamine sulfate in the treatment of knee arthritis: randomized trial. *Int. J. Immunopathol. Pharmacol.*, **2016**, 29, 1, 140-146.
39. Sengupta, K. Alluri, K.V., Satish, A.R., Mishra, S., Golakoti, T., Sarma, K.V.S., Dey, D., Raychaudhuri, S.P. A double-blind, randomized, placebo-controlled study of the efficacy and safety of 5-Loxin® for treatment of osteoarthritis of the knee. *Arthritis Res. Ther.*, **2008**, 10, 85.
40. Sengupta, K., Krishnaraju, A.V., Vishal, A.A., Mishra, A., Trimurtulu, G., Sarma, K.V.S., Raychaushuri, S.K., Raychaudhuri, S.P. Comparative efficacy and tolerability of 5-Loxin® and Aflapin® against osteoarthritis of the knee: a double-blind, randomized, placebo controlled clinical study. *Int. J. Med. Sci.*, **2010**, 7, 6, 366-377.
41. Sontakke, S., Thawani, V., Pimpalkhute, S., Kabra, P., Babhulkar, S., Hingorani, L. Open, randomized, controlled clinical trial of *Boswellia serrata* extract as compared to valdecoxib in osteoarthritis of knee. *Indian J. Pharmacol.*, **2007**, 39, 1, 27-29.
42. Vishal, A.A., Mishra, A., Raychaudhuri, S.P. A double blind, randomized, placebo controlled clinical study evaluates the early efficacy of Aflapin® in subjects with osteoarthritis of knee. *Int. J. Med. Sci.*, **2011**, 8, 7, 615-622.
43. Moyé, L.A., Deswal, A. Trials within trials: confirmatory subgroup analyses in controlled clinical experiments. *Control Clin. Trials*, **2001**, 22, 605-619.
44. Meins, J., Artaria, C., Riva, A., Morazzoni, P., SchubertZsilavecz, M., Abdel-Tawab, M. Survey on the quality of the top-selling European and American botanical dietary supplements containing boswellic acids. *Planta Med.,* **2016**, 82, 6, 573–579.
45. Mannino, G., Occhipinti, A., Maffei, M. Quantitative determination of 3-O-acetyl-11-keto-β-boswellic acid (AKBA) and other boswellic acids in *Boswellia sacra* Flueck (syn. *B. carteri* Birdw) and *Boswellia serrata* Roxb. *Molecules*, 2016, 21, 10, 1329-2016.
46. Gurevitch, J., Koricheva, J., Nakagawa, S., Stewart, G. Meta-analysis and the science of research synthesis. *Nature*, **2018**, 555, 175-182.

47. Spector, T.D; Thompson, S.G. The potential and limitations of meta-analysis. *J. Epidemiology Community Health*, **1991**, 49, 85-92.
48. Bannuru, R.R., Osani, M.C., Al-Eid, F., Wang, C. Efficacy of curcumin and *Boswellia* for knee osteoarthritis: systematic review and meta-analysis. *Seminars in arthritis and rheumatism*, **2018**, 48, 416-429.
49. Smedslund, G., Kjeken, I., Musial, F., Sexton, J., Osteras, N. Interventions for osteoarthritis pain: a systematic review with network meta-analysis of existing Cochrane reviews. *Osteoarthritis and cartilage open*, **2022**, 4, 100242.
50. Di Lorenzo, C., Dall'Agli, M., Badea, M., Dima, L., Colombo, E., Sangiovanni, E., Restani, P., Bosisio, E. Plant food supplements with anti-inflammatory properties: a systematic review (II). *Crit. Rev. Food. Sci. Nutr.*, **2013**, 53, 5, 507-516.
51. Dodda, S., Madireddy, R.K., Alluri, V.K., Golakoti, T., Sengupta,K. Safety assessment of a novel water-soluble extract of *Boswellia serrata* gum resin: acute toxicity, 90-day sub-chronic toxicity, Ames' bacterial reverse mutation, and *in vivo* micronucleus assays. *Toxicol. Mech. Methods*, **2022**, 32, 5, 362-372.
52. Furst, R., Zundorf, I. Evidence-based phytotherapy in Europe: where do we stand? *Planta medica*, **2015**, 81, 962-967.
53. Izzo, A.A., Hoon-Kim, S., Radhakrishnan, R., Williamson, E.M. A critical approach to evaluating clinical efficacy, adverse events and drug interactions of herbal remedies. *Phytotherapy research*, **2016**, 30, 691-700.

Supplementary material

ID	PAPER	Was the study described as randomized?	Was the study described as double blind?	Was there a description of withdrawals and dropouts?	The method of randomisation was described in the paper, and that method was appropriate	The method of blinding was described, and it was appropriate.	Jadad score total
30	Chopra et al.	1	1	1	1	1	5
31	Chopra et al.	1	1	1	1	0	4
32	Harovan et al.	1	1	1	1	1	5
33	Karimifar et al.	1	1	1	0	0	3
34	Karlapudi et al.	1	1	1	0	0	3
35	Karlapudi et al.	1	1	1	1	1	5
36	Majeed et al.	1	1	1	1	1	5
37	Notarnicola et al.	1	1	1	0	0	3
38	Notarnicola et al.	1	1	1	0	0	3
39	Sengupta et al.	1	1	1	1	1	5
40	Sengupta et al.	1	1	1	1	1	5
21	Sharkey et al.	1	1	1	0	0	3
41	Sontakke et al.	1	0	1	1	0	3
42	Vishal et al.	1	1	1	1	1	5

Supplementary Figure S1. Jadad score of the studies included in meta-analysis and meta-regression.

```
Mixed-Effects Model (k = 23; tau^2 estimator: REML)

  logLik  deviance       AIC       BIC      AICc
 -9.2963   18.5926   34.5926   40.7733   55.1640

tau^2 (estimated amount of residual heterogeneity):     0.1231 (SE = 0.0653)
tau (square root of estimated tau^2 value):             0.3509
I^2 (residual heterogeneity / unaccounted variability): 67.64%
H^2 (unaccounted variability / sampling variability):   3.09
R^2 (amount of heterogeneity accounted for):            67.29%

Test for Residual Heterogeneity:
QE(df = 16) = 49.7488, p-val < .0001

Test of Moderators (coefficients 2:7):
QM(df = 6) = 35.6238, p-val < .0001

Model Results:

                                                    estimate      se     zval    pval    ci.lb    ci.ub
intrcpt                                              -1.1776  0.5632  -2.0908  0.0365  -2.2815  -0.0737    *
Controlnegative control (same formulation without Boswellia)  0.8168  0.3065   2.6654  0.0077   0.2162   1.4175   **
Controlpositive control                               1.6282  0.3953   4.1190  <.0001   0.8534   2.4029  ***
TreatmentHerbal formulation with Boswellia            0.4038  0.3530   1.1440  0.2526  -0.2880   1.0956
Time0-2 months                                        0.2965  0.4631   0.6403  0.5220  -0.6111   1.2041
Time3-4 months                                        0.1485  0.4701   0.3159  0.7521  -0.7729   1.0700
Time5-6 months                                       -0.2328  0.5895  -0.3948  0.6930  -1.3882   0.9227

---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Supplementary Figure S2. Meta-regression of the WOMAC score for control, treatment types and time points of the studies.

```
Mixed-Effects Model (k = 13; tau^2 estimator: REML)

  logLik  deviance       AIC       BIC      AICc
 -3.1891    6.3782   22.3782   20.7123  166.3782

tau^2 (estimated amount of residual heterogeneity):     0.1283 (SE = 0.1012)
tau (square root of estimated tau^2 value):             0.3582
I^2 (residual heterogeneity / unaccounted variability): 75.73%
H^2 (unaccounted variability / sampling variability):   4.12
R^2 (amount of heterogeneity accounted for):            11.14%

Test for Residual Heterogeneity:
QE(df = 6) = 27.6622, p-val = 0.0001

Test of Moderators (coefficients 2:7):
QM(df = 6) = 7.7294, p-val = 0.2586

Model Results:

                                                    estimate      se     zval    pval    ci.lb   ci.ub
intrcpt                                              -0.4797  0.7384  -0.6497  0.5159  -1.9271  0.9676
Controlnegative control (same formulation without Boswellia)  0.5243  0.4065   1.2897  0.1972  -0.2725  1.3210
Controlpositive control                               0.3376  0.3060   1.1033  0.2699  -0.2621  0.9372
TreatmentDietary supplements with boswellic acids     0.4684  0.7442   0.6294  0.5291  -0.9902  1.9270
TreatmentHerbal formulation with Boswellia            0.1478  0.6882   0.2147  0.8300  -1.2011  1.4966
Time3-4 months                                       -0.4307  0.5479  -0.7862  0.4318  -1.5045  0.6431
Time5-6 months                                       -0.0938  0.2708  -0.3466  0.7289  -0.6245  0.4368
```

Supplementary Figure S3. Meta-regression of the VAS score for controls, treatment types and time points of the studies.

2.2 MICROALGAE

Algae are a heterogeneous group of organisms that range in size from tiny cells to giant seaweeds; they are mostly photosynthetic organisms including either eukaryotes or prokaryotic Cyanobacteria. Algae can be classified based on size and structure in macroalgae, which are large enough to be seen with unaided eye, and microalgae with algal bodies that need microscope to be observed [48]. Microalgae capture sunlight and perform photosynthesis by producing half of atmospheric oxygen on earth and absorbing massive amounts of carbone dioxide as a major feed. Furthermore, microalgae can grow in both fresh and marine water as well as in almost every environmental condition on earth from frozen lands of Scandinavia to hot desert of Sahara. First attempts of microalgae culturing begin in 1910 by cultivating *Chlorella* sp. in Germany [48].

Biochemical composition of microalgae and Cyanobacteria is well documented; they can be a source of proteins, carbohydrates, lipids and important trace nutrients as vitamins, antioxidants and trace elements as Fe, Zn and Cu. Furthermore, they can produce compounds with antibiotic and anticancer activity. For instance, a wide variety of Cyanobacteria produce compounds including sulfolipids that can be active against herpes virus, pneumonia virus and HIV [49]. Thus, their nutritional and protective potential is gaining importance as a renewable source to substitute the conventional ingredients in the field of animal feed [48]; in fact, approximately 30% of microalgae production worldwide is sold in the animal feed market [50].

Some microalgae are also used in the skin care market, the main ones are *Arthrospira* sp. and *Chlorella* sp. commercialised for contrasting skin aging, stimulating collagen synthesis, supporting tissue regeneration and prevenings stria formation [48]. The two above-mentioned microalgae are considered as safe (GRAS) by Food and Drug Administration (FDA).

Arthrospira sp., known with the popular name of Spirulina, is a genus of blue-green microalgae characterised by multicellular and filamentous trichomes belonging to the phylum of Cyanobacteria; it has gained considerable popularity in the health and food industry and as a protein and vitamin supplement [51]. According to the cylindrical arrangement of the multicellular trichomes which is the main morphological hallmark of the genus, there are two main species of *Arthrospira*: *Arthrospira maxima* and *Arthrospira platensis* [52]. The latter is the most cultivated photosynthetic prokaryote. Spirulina can be found in soil, seawater, freshwater, brackish water, marshes and thermal springs and it has long been used as a dietary supplement by dwellers living close tropical lakes characterised by the presence of alkaline water (pH 11) where the species grows naturally, as lake Chad in Africa and lake Texcoco in Mexico. Of note, many studies have hypothesized therapeutic properties for *A. platensis* including immunomodulating, immunostimulating, biomodulating, antitumor, regulatory effects in neurodegenerative disorders, antidiabetic, anti-obesity, anti-hypertensive and hepatoprotective activity [52]. Furthermore, *A.platensis* has a high mean protein content of more than 60% of the dry mass, is a source of essential

amino acids, provides appreciable amount of lipids (7.2% w/w), carbohydrates (10.3% w/w), crude fibres (8.5 % w/w) and minerals (6.9% w/w), and is considered an interesting source of vitamin of B groups (B_3, B_6 and B_{12}), making it of particular interest in food and feed field.

Figure 8. Commercial samples of *A.platensis* sold in form of tablets or powder.

Despite the ancient and consolidated use of this species as food supplement in the countries of origin, Spirulina was rediscovered and became of interest by the food and feed industries only from the 20th century. Due to the above-mentioned properties, the Food and Agriculture Organisation defined *Arthrospira* sp. as a wonderful future source [51]. Furthermore, Spirulina had a role in MELISSA (Micro-Ecological Life Support System Alternative) project with the aim of being a likely food supplement with long-term storage for astronauts in space and in the PROFUTURE project, a European funded research project focused on boosting the production and use of protein-rich ingredients from microalgae in food and feed [53, 54]. Nowadays, Spirulina is usually grown in photobioreactors where the growing conditions can be subjected to controlled conditions improving the quality of the product. *A. platensis* is an attractive food and feed supplement also due to the high content of essential trace elements. On the top of that, it has been reported that *A. platensis* has a relevant iron content [55].

Phycocyanin is a multimeric blue phycobiliprotein and is the most abundant protein present in *A. platensis* with a pivotal role as light-capturing antenna for photosystem II. *A. platensis* hold two distinct photosynthetic reaction centres, included in photosystem I and photosystem II, respectively. Each reaction centre is associated with an antenna belonging to a light-harvesting complex (LHC) that consist of phycobilisomes which in turn contain photosynthetic phycobiliproteins, including phycocyanin, phycoerythrin and allophycocyanin [56]. These phycobyliproteins are organized as supramolecular structures that assemble forming the phycobilisome as reported in Figure 9.

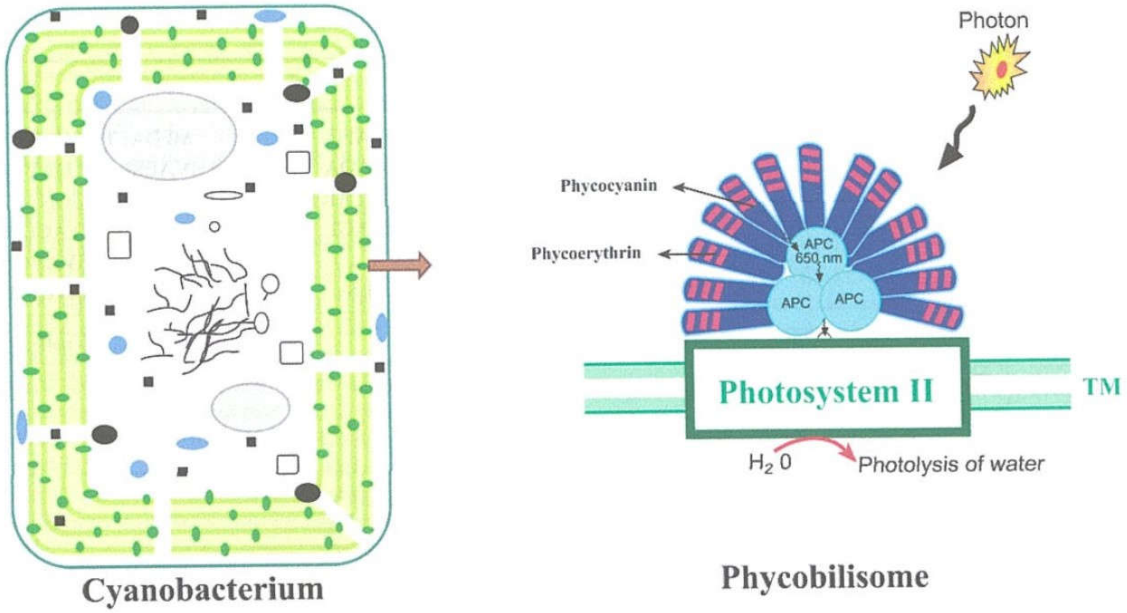

Figure 9. Structure of phycobilisome along with different phycobiliproteins in cyanobacterial cell, adapted from Bannu et al., 2019 [56].

Phycocyanin is a very complex molecule: it contains α and β-subunits, with a molecular mass of 16.3 and 18.9 kDa respectively, and the chromophore phycobilin (Figure 10). Alpha and β subunits form the $\alpha\beta$ monomer, which further aggregates in trimer or hexamer [56, 57]. In addition to its essential role as a light capturing antenna, phycocyanin is also studied for its medical applications with immunity booster, antioxidant, detoxifier, neuroprotective and hepatoprotective activity and it can also be used as a natural blue food colouring. It has been reported that in the decade from 2021 to 2031 phycocyanin bioactive compounds will represent an estimated global market of 250 million of US$ [58]. Furthermore, the molecular structure of this proteins makes it as the main candidate for binding iron in *A. platensis*, as recently suggested by authors that investigated the iron-chelating properties of phycocyanin, at the level of the chain tetrapyrrole chromophore [56, 59].

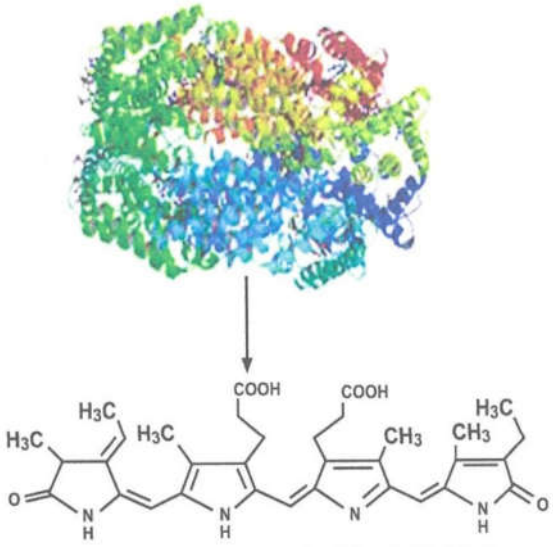

Figure 10. 3D structure of phycocyanin and chemical structure of the chromophore tetrapyrrole chain, adapted from Bannu et al., 2019 [56].

Currently, *A. platensis* found applications as feed in aquaculture for many species (*Rhabdosargus sarba*, *Macrobrachium rosenergii*, *Oreochromis niloticus*), poultry, cattle, and domestic animals [51].

Part of the research carried out during the PhD training was focused on *A. platensis*, with with the aim to investigate iron-chelating ability, of bioaccesibility, and speciation of iron, with special attention to the proteins that can bind this trace element. In addition, research aimed at detecting iron amounts in different samples of *A. platensis,* assessing variability among commercial products. Afterwards, the iron bioaccumulation based on the availability of this trace element in the environment of culture was investigated to evaluate how it can affect the quality of biomasses of *A. platensis.* Through chromatographic and electrophoretic techniques, proteins of *A. platensis* were studied, highlighting the pivotal role of phycocyanin. Furthermore, the bioaccessibility of iron from *A. platensis* and others algal species was using an *in vitro* canine digestion model. This latter research shed light on this pivotal issue because iron bioaccessibility is fundamental for animal and human health, considering the essential biochemical role of this trace element.

Isani, G., Ferlizza, E., Bertocchi, M., Dalmonte, T., Menotta, S., Fedrizzi, G., Andreani, G. Iron content, iron speciation and phycocyanin in commercial samples of *Arthrospira* spp. *International journal of molecular sciences*, **2022**, 23, 13949.

In the fourth paper ten commercial samples of *A.platensis* sold as food supplement have been collected in order to evaluate the iron content using atomic absorption spectrometry (AAS). Furthermore, in order to assess the distribution of iron between the soluble and insoluble fractions of spirulina extracts through exclusion chromatography, SDS-PAGE for protein separation and AAS for measuring the iron content were performed. Phycocyanin was also investigated as iron-binding protein, and in six of the ten samples, 14 essential and non-essential trace elements were analysed through inductively coupled plasma mass spectrometry (ICP-MS).

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Iron Content, Iron Speciation and Phycocyanin in Commercial Samples of *Arthrospira* spp.

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Abstract: Cyanobacteria are characterized by high iron content. In this research, we collected ten commercial samples of *Arthrospira* spp. sold as food supplement to determine iron content and assess whether iron speciation showed variability among samples and changed respect to *A. platensis* grown in controlled conditions. Particular attention was also paid to phycocyanin, as an iron-binding protein. In six of the ten samples, 14 essential and non-essential trace elements were analysed using ICP-MS. Iron content measured in samples using atomic absorption spectrometry (AAS) varied from 353 (sample S5) to 1459 (sample S7) μg g^{-1} dry weight and was in the range of those reported by other authors in commercial supplements. Iron speciation was studied using size exclusion chromatography followed by the analysis of the collected fraction for the determination of iron by AAS and for protein separation using SDS-PAGE. Overlapping chromatographic profiles were obtained for total proteins, phycocyanin and iron, although quantitative differences were evidenced among the samples analysed. In most samples, iron was mainly bound to ligands with high molecular mass; however, in four samples iron was also bound to ligands with low molecular mass. In fractions containing the most relevant iron burden, the principal protein was phycocyanin, confirming its role as an iron-binding protein in commercial samples.

Keywords: *Arthrospira*; biochemical parameters; cyanobacteria; phycocyanin; trace elements

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1. Introduction

Arthrospira spp. are photosynthetic cyanobacteria used as a dietary supplement (spirulina) due to their high content of high-quality proteins, including phycocyanin, pigments, essential trace elements, particularly iron, and other bioactive molecules [1]. This unique nutritional profile makes spirulina an attractive alternative and novel food [2].

Iron is an essential trace element for all living organisms. However, its redox activity poses significant challenges to cells: the reactivity of Fe^{2+} toward H_2O_2 determines the formation of the hydroxyl radical (HO•). For this reason, in biological systems iron is not found in the state of free ion but always bound to proteins involved in sophisticated homeostatic control mechanisms [3]. Iron is of special relevance for photosynthetic cyanobacteria, which have evolved complex uptake mechanisms [4] to cope with the low concentrations of this essential metal in their environments and are able to bioaccumulate iron concentrations of four to six orders of magnitude higher than those of the aquatic environment in which they grow [5]. Bacteria utilizes two types of ferritin-like molecules

to store iron in the trivalent state as ferric ions, namely bacterial ferritin (Ftn) and bacterioferritin (Bfr) [6]. Alternatively, iron can be stored as inorganic iron. It has been reported that *Arthrospira platensis* trichomes accumulate iron mostly in the form of Fe^{3+} as ferrihydrite [7]. In cyanobacteria, due to these different storage possibilities, iron can be stored in high concentrations, at the same time protecting cell from potentially harmful Fe^{2+}/Fe^{3+} redox cycling.

Different growing conditions and environmental parameters dramatically impact the biochemical composition of the biomass, determining wide variations among commercial products based on *Arthrospira* [8,9]. Therefore, in addition to research performed in controlled laboratory conditions or pilot studies, there is a need for analyses and comparisons among commercial samples of different origins and manufacturers. Moreover, commercial products based on *Arthrospira* are subjected to processing and packaging which can modify the biochemical composition, further affecting the nutritional quality. In previous research using *A. platensis* F&M-C256 strain reared in optimal and controlled conditions, we focused on the effects of varying iron concentrations in the culture medium on iron speciation and iron-binding proteins [10]. In this research, we collected ten commercial samples of *Arthrospira* spp. sold as food supplements to determine iron content and to assess whether iron speciation showed variability among samples or changed respect to *A. platensis* F&M-C256, using different molecular approaches. Particular attention was paid to phycocyanin, the most abundant protein of *Arthrospira*, as a candidate iron-binding protein. Finally, 14 essential and non-essential trace elements were measured in samples from Italy and China.

2. Results and Discussion

2.1. Iron Content in Commercial Samples

Iron content is reported in Table 1. The obtained values varied between 353 (sample S5) and 1459 μg g^{-1} dry weight (dw) (sample S7), with a mean value (±SD) of 608 (±611). The high standard deviation among samples is due to the high iron content detected in sample S7 from China, which has an iron content two to four times higher than the other samples. A similar outcome was reported by Kejžar et al. (2021) [9] for a spirulina-based supplement from Hawaii, which had an iron content as high as 3.29 ± 0.27 mg g^{-1}dw. Nevertheless, the iron content falls within the range of those reported by other authors. Principe et al. (2020) [8] reported values from 63 to 1066 μg g^{-1} dw in seven dietary supplements sold in Argentina. Kejžar et al. (2021) [9] found a mean iron content of 1360 ± 1330 μg g^{-1} in dietary supplements sold in Slovenia. In 46 spirulina supplements available on the Slovenian market, an iron content from 370 to 3480 μg g^{-1} dw was reported by Rutar et al. (2022) [11]. These data highlight the high variability of iron in supplements based on *Arthrospira* spp. and *Spirulina* spp. It is well known that the iron concentrations in the medium used for the cultivation of cyanobacteria is able to determine wide variations of the metal content in the biomass [10,12].

2.2. Protein Fractionation and Iron Speciation

The ten samples were extracted to analyse the distribution of iron between the soluble and insoluble fractions. The percentages are reported in Table 1. In all the samples, iron was more present in the pellet than in the soluble fraction. The percentage of iron in the soluble fraction varied from 30% in sample S1 to 7% in sample S7, with most samples presenting a percentage between 14 and 18%. Low percentages of iron from 6 to 10% were also detected in soluble fractions of extracts from *A. platensis* F&M-C256 strain reared in controlled laboratory conditions [10]. Taken together, these data argue in favour of the suggestion reported by Perfiliev et al. (2018) [7] that *A. platensis* trichomes accumulate iron mostly in an inorganic form as ferrihydrite. This compound could be responsible of the high iron percentages determined in the pellet fraction of all samples examined.

Table 1. Iron content in ten commercial samples (S1–S10) of *Arthrospira* spp. sold as dietary supplement. Data are expressed as μg g^{-1} dw and are reported as mean ± SD (n = 3). The distribution of iron between the pellet and the soluble fraction (sf) is reported as percentage.

	[Fe] (μg g^{-1} dw)	Fe % pellet	Fe % sf
S1	467 ± 15	70	30
S2	782 ± 27	84	16
S3	440 ± 30	84	16
S4	540 ± 22	83	17
S5	353 ± 18	86	14
S6	474 ± 2	86	14
S7	1459 ± 17	93	7
S8	538 ± 19	82	18
S9	387 ± 20	78	22
S10	461 ± 16	82	18

In addition to inorganic compounds, iron can bind to biomolecules, of which proteins, amino acids, and heme are of particular interest. All these molecules are present mostly in the soluble fraction of extracts. The concentration of total proteins varied from 36.3 mg mL^{-1} in sample S8 to 66.2 mg mL^{-1} in sample S1 (Figure 1a), while the concentration of phycocyanin varied from 1.77 mg mL^{-1} in sample S6 to 3.21 mg mL^{-1} in sample S7 (Figure 1b).

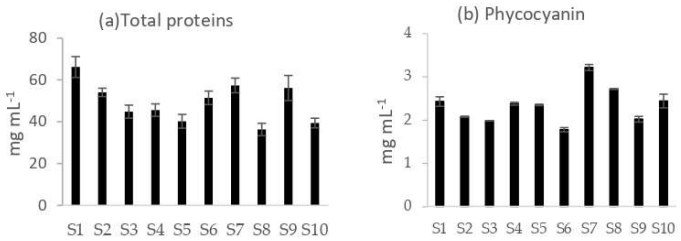

Figure 1. Concentration of total proteins (**a**) and phycocyanin (**b**) in *Arthrospira* spp. soluble fraction of commercial samples. Data are expressed in mg mL^{-1} and are reported as mean ± SD (n = 3).

To obtain a general insight on the quality of the extracts, total proteins present in soluble fraction were separated using SDS-PAGE (Figure 2). Despite the different origin and formulation of the samples, the profiles obtained were similar and indicative of a good quality, without sign of protein degradation, despite the storage of commercial samples at room temperature. The quality of these extracts was higher than that reported by Jin et al. (2020) [13] for spirulina powder extracts. Those authors obtained only three diffuse bands at 42, 37, and <25 kDa after SDS-PAGE, while in the samples analysed in the present research different common bands ranging from 250 to <15 kDa were evidenced, comparable to those present in extracts from *A. platensis* F&M-C256 strain reared in controlled laboratory conditions [10]. Most of the protein bands were in the ranges between 35–50 and 15–20 kDa; similar ranges were reported by Ismaiel et al. (2018) [14].The most abundant band at 18 kDa is the subunit of phycocyanin. The intensity of the band shows wide variations among the samples, from samples S1 and S7, which present the most intense bands, to sample S3, which shows only a faint band. On the other hand, this sample (S3) presents a band at 20 kDa, more evident than that of all the other samples. The electrophoretic profiles can be considered an interesting molecular fingerprint to evaluate the quality and integrity of proteins in supplements based on *Arthrospira* spp. and its use should be encouraged.

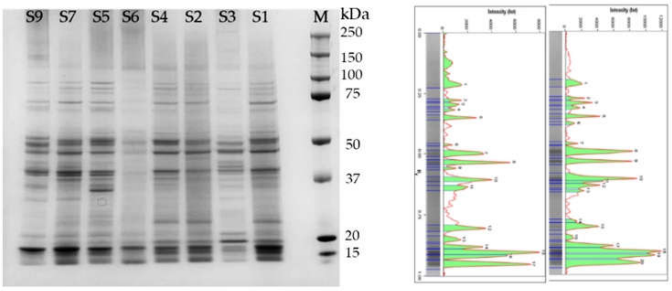

Figure 2. The 1D proteomics analysis of *Arthrospira* spp. soluble fractions from commercial samples. Proteins (3 µg) were separated using SDS-PAGE (12%) and stained with Coomassie® Brilliant Blue R-250. Left: representative gel; lanes 1–8: soluble extracts of samples S1, S2, S3, S4, S5, S6, S7 and S9; lane 10 (M): molecular mass marker (Precision Plus Protein Standard, Biorad); right: representative pherograms obtained from samples S4 and S2.

The study of iron speciation was addressed by combining a chromatographic fractionation of proteins using size-exclusion chromatography (SEC) associated with sensitive metal detection in the fractions using AAS, a hyphenated approach which is commonly used in metallomics studies [15]. Total proteins and phycocyanin in fractions obtained after SEC are reported in Figure 3. Overlapping chromatographic profiles were obtained in all the samples analysed, although differences were evidenced.

Regarding proteins, the first peak is present between fractions 10 and 15, with maximum absorbance at 280 nm measured in fraction 11, correlated with the concentration of total proteins measured in the soluble extracts (Figure 1) ($p < 0.05$). This peak contains proteins with high molecular mass (MM) between >75 to 40 kDa. A second peak of absorbance is present in fractions 29–34. In these fractions, small peptides and free amino acids elute, including the so-called mycosporine-like amino acids (MAAs). These secondary metabolites are low molecular mass (<400 Da) molecules produced by a wide range of organisms and are characterized by strong UV absorption maxima between 310 and 362 nm. Mycosporine-like amino acids have the function of absorbing ultraviolet radiation, protecting organisms from the possible damage caused by exposure to the sun [16]. The presence of these amino acids in the peak was verified by measuring the absorbance at different wavelengths between 310 and 360 nm. The data are reported in Supplementary Figure S1. The chromatographic profiles obtained at 280 nm are comparable to those reported for *A. platensis* F&M-C256 grown in optimal laboratory conditions [10].

Iron profiles after SEC are reported in Figure 4. In all the samples, two main peaks were present, namely in fractions 10–13 and in fractions 15–18, except for sample S6, which presented only the first peak. Iron distributed differently between these two peaks, depending on the sample analysed. In most samples, iron was mainly bound to ligands with high molecular mass (HMM) between >75 and 60 kDa (fractions 10–12). Differently, in samples S3 and S7, a consistent percentage of the metal (34% and 40%, respectively) was bound to ligands with intermediate molecular mass (IMM) between 40 and 20 kDa (fractions 15–18). In samples S4, S7, S9, and S10, iron was also bound to ligands with low molecular mass (LMM) (fractions 28–32). Particularly, in sample S10, a consistent percentage of iron (32%) was detected in these last fractions. This peak was overlapping with the peak of MAAs (Figure 2a and Figure S1). Recently, Varnali et al. (2022) [17] reported the computational analysis of stable iron-complexed MAA structures, supporting previous suggestions made in the literature on the potential iron-chelating ability of MAAs. The iron peak present in fractions 28–32 could be considered the first evidence of the iron-binding capacity of MAAs in *Arthrospira*. However, the presence in these fractions of other LMM iron-binding molecules, as other free amino acids, cannot be excluded.

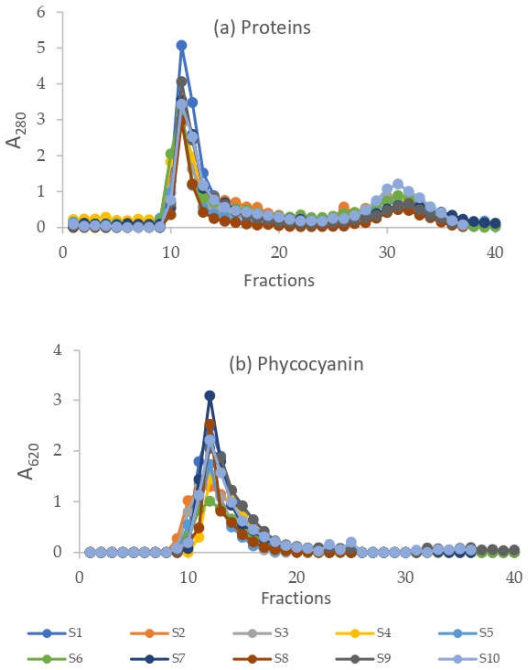

Figure 3. Chromatographic pattern after the size exclusion chromatography of extracts obtained from samples S1–S10. Total proteins were detected at 280 nm (**a**); phycocyanin was detected at 620 nm (**b**).

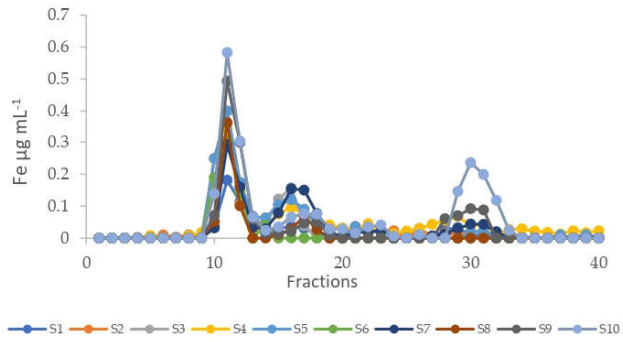

Figure 4. Iron chromatographic profiles after size exclusion chromatography of extracts from samples S1–S10. Iron concentration is expressed as µg mL^{-1}.

Contrasting results have been reported on the molecular ligands of iron in cyanobacteria. Cepoi et al. (2018) [18] reported that in *A. platensis* grown in a medium containing Fe_3O-glycin (50 mg L^{-1}), high percentages of iron were bound to organic ligands: 43% was bound to proteins and 24% to free amino acids. The presence of an iron peak in the fractions of LMM ligands found in this study could be indicative of iron bound to amino acids

or small peptides, as discussed above. On the contrary, Perfiliev et al. [7] reported that *A. platensis* accumulates iron mainly as Fe^{3+} in an inorganic form, such as ferrihydrite. Accordingly, Rutar et al. (2022) [11] showed that most iron (82–92%) found in spirulina dietary supplements was present as Fe^{3+}. The authors concluded that the bioavailability of iron from the supplements is low, as only a small amount of the metal is present as a more bioavailable ferrous ion, Fe^{2+}. The partitioning of iron between the pellet and the soluble fraction found in most of the samples analysed in this study, except for sample S1, showed that percentages of the metal between 78 and 93% are present in the pellet, presumably as inorganic iron. These data are in accordance with those reported by Rutar et al. (2022) [11]. However, different suggestions were made by Puyfoulhoux et al. (2001) [19], who compared the iron bioavailability of iron-fortified spirulina to different iron sources. Those authors measured ferritin formation in Caco-2 cells exposed to digests obtained from meat, yeast, spirulina, and wheat. The results obtained were indicative of a metal bioavailability for spirulina not significantly different from that exhibited by the meat digest, despite the different chemical form of iron.

2.3. SDS-PAGE of Fractions after SEC

To shed more light on iron-binding proteins, fraction 11, which contains the most relevant metal burden, underwent SDS-PAGE on 4–12% gels. A representative gel is reported in Figure 5. Fraction 11, which had the highest absorbance at 280 nm and the highest iron concentration, showed different protein bands, with an apparent molecular mass ranging from 75 to 10 kDa. The most represented bands are at 18 and 17 kDa (Figure 5, lanes 3–7). These bands contain C-phycocyanin (C-PC) [10]. This multimeric blue protein belonging to the phycobiliprotein family presents two subunits, which bind a particular prosthetic group, phycocyanobilin, similar to bilverdin and bilirubin. In addition to functioning as a light-capturing antenna for photosystem II, this protein is able to bind iron in vitro [20] and in vivo [10]. The data of our study indicate that iron is bound to C-PC in commercial products sold as nutritional supplements, despite harvesting, processing, and packaging process that could modify iron speciation and the biochemical profile of cyanobacteria.

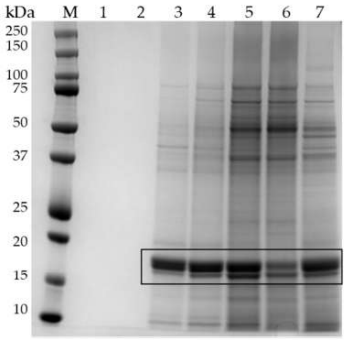

Figure 5. Representative SDS-PAGE gel (4–12%, Coomassie staining) of proteins contained in fractions obtained after size exclusion chromatography of cellular extracts. M: molecular mass marker; lanes 1–2: fraction 31 of samples S9 and S10; lanes 3–7: fraction 11 of samples S8, S4, S1, S9, and S7, respectively. Rectangle indicates the bands at 18 and 17 kDa containing the subunits of the phycocyanin.

Fraction 31 from samples S9 and S10, despite a significant iron burden, did not contain proteins (Figure 5 lanes 1–2); other small molecules are responsible for iron binding in this fraction (Figure 4), presumably amino acids, as discussed above.

2.4. Essential and Non-Essential Trace Elements Content

Commercial supplements based on *Arthospira* are interesting sources of macro and trace elements. However, many producers are located in Asia, particularly in India and China, and use cultivation practices not subjected to strict regulation. High contents of aluminium, lead, and inorganic arsenic due to environmental contamination or harvesting systems have been reported [21,22]. For this reason, we decided to compare three samples of *Arthospira* spp. grown in Italy (S3, S5, and S6) with three samples from China (S1, S4, and S7).

The mean content of essential trace elements presented significant differences between samples from Italy and China (Table 2). This decreasing order was present in Italian samples: Fe > Zn > Cu > Mn > Ni > Cr > Mo > Co > Se, while in Chinese samples elements followed this order: Fe > Mn > Zn > Ni > Cu > Cr > Co > Se > Mo and were characterized by a significantly higher content of manganese and a significantly lower content of copper and cobalt (Table 2). High levels of manganese were previously reported by Rzymski et al. (2019) [22] and Rutar et al. (2022) [11], while Sandgruber et al. (2021) [23] found in some commercial samples of *A. platensis* a manganese content < LOQ. The ability of cyanobacteria to bioaccumulate metals is well known and the variability is strictly related to the metal concentration and bioavailability in the water. Ghanbarzadeh et al. (2022) [24] reported that *A. platensis* MGH-1 is able to accumulate manganese levels as high as 3 g kg^{-1} when exposed to a manganese concentration of 40 mg L^{-1} in the culture medium.

Table 2. Trace element content in six selected commercial samples. Samples S1, S4, and S7 were produced in China (CN), while samples S3, S5, and S6 were produced Italy (IT). Data are expressed as μg g^{-1}dw. The *p*-value for comparison between Italian and Chinese samples is reported; a *p* -value < 0.05 was considered significant.

Sample	Fe	Zn	Cu	Mn	Ni	Cr	Mo	Co	Se	Al	Pb	As	Cd	Hg
S3 (IT)	432	65.3	10.3	10.1	8.17	0.99	0.09	0.06	0.04	3.83	0.13	0.12	0.01	0.01
S5 (IT)	367	28.9	7.23	7.19	6.06	0.24	0.09	0.06	nd	1.46	0.04	0.01	0.01	nd
S6 (IT)	448	34.3	9.92	7.27	2.52	0.94	0.09	0.07	0.02	8.94	0.17	0.08	0.02	nd
mean	416	42.8	9.16	8.22	5.58	0.72	0.09	0.06	0.03	4.74	0.11	0.07	0.02	-
SD	43.1	19.7	1.68	1.70	2.86	0.42	0.00	0.01	0.01	3.82	0.07	0.06	0.00	-
S1 (CN)	517	16.8	0.65	21.7	0.61	0.21	0.10	0.20	0.10	21.2	0.13	0.46	0.01	nd
S4 (CN)	576	10.5	2.13	34.7	5.83	0.41	0.11	0.45	0.14	176	0.47	1.46	0.11	0.02
S7 (CN)	1518	11.4	1.89	36.8	0.68	1.36	0.14	0.38	0.71	117	0.55	0.36	0.02	0.01
mean	870	12.9	1.56	31.1	2.37	0.66	0.11	0.34	0.32	105	0.38	0.76	0.05	0.01
SD	561	3.41	0.79	8.20	2.99	0.61	0.02	0.13	0.34	77.9	0.22	0.61	0.06	0.01
p-value	0.234	0.060	*0.002*	*0.009*	0.250	0.892	0.133	*0.020*	0.336	0.091	0.115	0.122	0.384	-

nd: not detected.

Some peculiarities emerge from the analysis of other trace elements. For example, a mean selenium content of 0.34 μg g^{-1} dw was detected in samples from China, with sample S7 with a content as high as 0.71 μg g^{-1}dw. These values are in the range of those reported for grains and cereals [25] and spirulina could be considered an interesting integrative source of selenium for vegan individuals. However, besides the total intake of dietary selenium, the element speciation may also be important and requires future studies.

Regarding non-essential trace elements, aluminium content with a mean value of 104.7 μg g^{-1} was higher in samples from China compared to samples from Italy. This difference could be related to cultivation and harvesting systems. Adding chemicals to induce flocculation is a method widely used to harvest microalgal biomass [26] and aluminium chloride salts are the most efficient for this purpose [27]. The aluminium content found in two samples from China is of particular concern because this metal has been recently classified by IARC as a carcinogen for humans. The discussion of possible health risks posed by the consumption of these supplements is out of the scope of the present research. However, to avoid biomass contamination, alternative physical or biological harvesting methods are suggested.

The content of cadmium and lead in samples was low, below the maximum level (1.0 mg kg^{-1}) set by the European Commission [28], and indicative of low environmental contamination. The data obtained in the supplements analysed in this research are in accordance with those recently reported by other authors [22,29].

3. Materials and Methods

3.1. Commercial Samples of Arthrospira and Sample Preparation

Commercial samples from different companies were purchased in Italy. A total of ten samples (S1–S10) were analysed (Table 3). The tablets were ground using a mortar and pestle. Meanwhile, the capsules were opened and powders were directly used for analysis.

Table 3. Origin and form of analysed samples.

Sample	Species	Origin	Form	
S1	*Arthrospira maxima*	China	powder	Non-organic
S2	*Arthrospira maxima*	Italy	tablet	Non-organic
S3	*Arthrospira platensis*	Italy	powder	Non-organic
S4	*Arthrospira maxima*	China	powder	Non-organic
S5	*Arthrospira platensis*	Italy	pellet	Non-organic
S6	*Arthrospira platensis*	Italy	powder	Non-organic
S7	*Arthrospira platensis*	China	powder	Organic
S8	*Arthrospira platensis*	Italy	powder	Organic
S9	*Arthrospira platensis*	Italy	powder	Non-organic
S10	*Arthrospira platensis*	India	capsule	Organic

3.2. Iron Analysis

To avoid contamination, all the reagents were handled carefully; polyethylene disposables were thoroughly washed with HCl 1 N under a fume hood. All the reagents were from Merck (Darmstadt, Germany); the acids were of Suprapur grade. Commercial samples (400 mg) and pellets (200 mg) obtained as described in Section 3.3 were placed in individual acid-washed Teflon jars and were digested with the protocol optimized for metals [30]. Briefly, samples were added with 1–2 mL 65% HNO_3 and 0.25–0.5 mL 30% H_2O_2, digested in a microwave oven, transferred into 5–10 mL polyethylene volumetric flasks, and analysed using a flame atomic spectrophotometer equipped with a deuterium lamp background correction (AAnalyst 100, Perkin Elmer, Waltham, MA, USA). Supernatants obtained as described in Section 3.3 were diluted 1:10 with MilliQ water and directly analysed without any further treatment. The accuracy of the method was evaluated with ERM®–BB422 fish muscle. The concentrations found with the method used in this study fell into the certified uncertainty interval given by ERM, corresponding to a 95% confidence level. The iron detection limit was 0.04 μg mL^{-1}. Iron concentrations were reported as μg mL^{-1} or μg g^{-1} dw depending on the sample analysed.

3.3. Iron Speciation and Size Exclusion Chromatography (SEC)

One hundred milligrams of commercial samples were crushed in a mortar with pestle under liquid nitrogen and homogenized in 30 volumes (w/v) of Tris–HCl 20 mM and 10 mM mercaptoethanol, pH 8.6, using an Ultraturrax (IKA, Staufen, Germany) homogenizer. The homogenate was sonicated for 10 min at 38 kHz and then centrifuged at 20,000× g for 40 min at 4 °C, obtaining the separation between supernatant (soluble fraction) and pellet. For each extract, a volume of 0.8 mL supernatant was applied to a Sephadex G-75 chromatography column (0.9 × 90 cm). The column was calibrated using a commercial kit (GF70-1KT, Sigma-Aldrich, St Louis, MO, USA). Fractions of 1.5 mL were collected and analysed for iron concentration using direct aspiration of the solution into the flame of an atomic absorption spectrophotometer, as described above (Section 3.2). The pellets obtained from the centrifugation of the homogenate were digested in a microwave oven, as reported above (Section 3.2), and iron concentration was determined using a flame

atomic absorption spectrophotometer (AAnalyst 100, Perkin Elmer, Waltham, MA, USA) as reported in Section 3.2, "Iron analysis".

The concentration of total proteins in soluble fractions was determined using Lowry assay (DC Protein Assay, Biorad, Hercules, CA, USA) following the manufacturer's instruction, while phycocyanin concentration was calculated after reading the absorbance at 615 and 652 nm using the following formula [31]:

$$(A_{615} - 0.474 \times A_{652})/5.34$$

In fractions obtained from size exclusion chromatography, total proteins, phycocyanin, and mycosporine-like amino acids were determined by direct measurement of the absorbance at 280, 615, and 310–360 nm, respectively (DeNovix DS-11 Series Spectrophotometer, Wilmington, DE, USA).

3.4. SDS-PAGE

Three µg of proteins were loaded onto 4–12% Bis-Tris polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, MA, USA), and electrophoresis (PAGE) was carried out in an Xcell SureLock Mini-Cell with 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific, Waltham, MA, USA), containing sodium dodecyl sulphate (SDS). Each gel was also loaded with standard proteins of known molecular weight (Precision Plus Protein™ Dual Color Standard, Bio-Rad, Hercules, CA, USA). The electrophoresis was performed following the procedure reported by Isani et al. (2022) [10].

3.5. Trace Elements Analysis

The samples were digested using a wet procedure in polypropylene tubes (Digi TUBES SCP Science, QC, Canada). Ten mL of 70% nitric acid (J.T. Baker Instra-Analyzed™) were added, and the tubes were placed in Digi-Prep graphite Digestion Blocks (SCP Science, Baie-D'Urfé, QC, Canada) at 75 ± 5 °C overnight. After cooling, the clear solutions were diluted to 20 mL with high purity deionized water (Evoqua Water Technologies, Günzburg, Germany). The samples were additionally diluted with a solution of 2% nitric acid and 0.5% hydrochloric acid (Sigma, Suprapur, St Louis, MO, USA). The analyses were carried out using inductively coupled plasma mass spectrometry (ICP-MS 7700 Series Agilent Technologies Inc., Santa Clara, CA, USA) with an ASX-500 CETAC Autosampler (Cetac Technologies, Omaha, NE, USA) following the procedure reported by Andreani et al. (2019) [32].

The accuracy of the method was determined by analysing certified reference material (Joint Research Centre BCR-185R Bovine Liver) in each batch. The concentration values of the reference materials fell within the confidence interval given by the Joint Research Centre (Brussels). For each series of analyses, a white sample (acid used for sample mineralization) was mineralized and treated as described above; the limit of quantification (LOQ) was 0.005 µg/g and the limit of detection (LOD) was 0.003 µg/g for each analysed element. The results were expressed in µg/g dry weight (dw).

3.6. Statistical Analysis

Statistical analysis was carried out using statistical software (RStudio-1.2.1335 Statistical and R, R version 4.2.1, Vienna, Austria). All data were evaluated using standard descriptive statistics and are reported as mean ± standard deviation (SD). The comparison between Italian and Chinese samples was performed with *t*-test, and a p-value < 0.05 was considered significant.

4. Conclusions

The results obtained indicate that the commercial samples maintained a good biochemical profile, despite harvesting, processing, packaging processes, and storage at room temperature. The electrophoretic pattern of proteins from soluble extracts and the chromatographic profiles are comparable to those obtained from biomass grown in optimal laboratory conditions. The molecular approach based on different and hyphenated tech-

niques allowed the production of a comprehensive fingerprint useful to compare the supplements analysed.

The high variability of iron in supplements based on *Arthrospira* spp. is confirmatory of data previously reported by other authors. The nature of iron-binding ligands and the bioaccessibility of the metal is still an open question, and further research is needed to address this important issue and shed more light on the complex relation between iron and iron-binding molecules in cyanobacteria. Of particular interest is the confirmation that phycocyanin maintain the ability to bind iron also in commercial products, opening new perspectives to the use of this protein in supplements. However, other low molecular weight ligands, such as mycosporine-like amino acids, could be involved in iron-binding and should be considered in future studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232213949/s1.

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References

1. Lafarga, T.; Fernández-Sevilla, J.M.; González-López, C.; Acién-Fernández, F.G. Spirulina for the food and functional food industries. *Food Res. Int.* **2020**, *137*, 109356. [CrossRef]
2. Niccolai, A.; Chini Zittelli, G.; Rodolfi, L.; Biondi, N.; Tredici, M.R. Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Res.* **2019**, *42*, 101617. [CrossRef]
3. Andrews, N.C. Forging a field: The golden age of iron biology. *Blood* **2008**, *112*, 219–230. [CrossRef]
4. Qiu, G.W.; Jiang, H.B.; Lis, H.; Li, Z.K.; Deng, B.; Shang, J.L.; Sun, C.Y.; Keren, N.; Qiu, B.S. A unique porin meditates iron-selective transport through cyanobacterial outer membranes. *Environ. Microbiol.* **2021**, *23*, 376–390. [CrossRef]
5. Morel, F.M.M.; Price, N.M. The biogeochemical cycles of trace metals in the oceans. *Science* **2003**, *300*, 944–947. [CrossRef] [PubMed]
6. Rivera, M. Bacterioferritin: Structure, dynamics, and protein-protein interactions at play in iron storage and mobilization. *Acc. Chem. Res.* **2017**, *50*, 331–340. [CrossRef]
7. Perfiliev, Y.D.; Tambiev, A.K.; Konnychev, M.A.; Skalny, A.V.; Lobakova, E.S.; Kirpichnikov, M.P. Mössbauer spectroscopic study of transformations of iron species by the cyanobacterium *Arthrospira platensis* (formerly *Spirulina platensis*). *J. Trace Elem. Med. Biol.* **2018**, *48*, 105–110. [CrossRef] [PubMed]
8. Principe, M.V.; Permigiani, I.S.; Della Vedova, M.C.; Petenatti, E.; Pacheco, P.; Gil, R.A. Bioaccessibility studies of Fe, Cu and Zn from Spirulina dietary supplements with different excipient composition and dosage form. *J. Pharm. Pharmacogn. Res.* **2020**, *8*, 422–433.
9. Kejžar, J.; Jagodic Hudobivnik, M.; Nečemer, M.; Ogrinc, N.; Masten Rutar, J.; Poklar Ulrih, N. Characterization of algae dietary supplements using antioxidative potential, elemental composition, and stable isotopes approach. *Front. Nutr.* **2021**, *7*, 618503. [CrossRef]
10. Isani, G.; Niccolai, A.; Andreani, G.; Dalmonte, T.; Bellei, E.; Bertocchi, M.; Tredici, M.R.; Rodolfi, L. Iron speciation and iron binding proteins in *Arthrospira platensis* grown in media containing different iron concentrations. *Int. J. Mol. Sci.* **2022**, *23*, 6283. [CrossRef]
11. Rutar, J.M.; Hudobivnik, M.J.; Nečemer, M.; Mikuš, K.V.; Arčon, I.; Ogrinc, N. Nutritional quality and safety of the spirulina dietary supplements sold on the Slovenian market. *Foods* **2022**, *11*, 849. [CrossRef] [PubMed]
12. Ermis, H.; Guven-Gulhan, U.; Cakir, T.; Altinbas, M. Effect of iron and magnesium addition on population dynamics and high value product of microalgae grown in anaerobic liquid digestate. *Sci. Rep.* **2020**, *10*, 3510. [CrossRef]
13. Jin, S.-E.; Lee, S.J.; Park, C.-Y. Mass-production and biomarker-based characterization of high-value Spirulina powder for nutritional supplements. *Food Chem.* **2020**, *325*, 126751. [CrossRef] [PubMed]

14. Ismaiel, M.M.S.; Piercey-Normore, M.D.; Rampitsch, C. Proteomic analyses of the cyanobacterium *Arthrospira* (Spirulina) *platensis* under iron and salinity stress. *Environ. Exp. Bot.* **2018**, *147*, 63–74. [CrossRef]
15. Mounicou, S.; Szpunar, J.; Lobinski, R. Metallomics: The concept and methodology. *Chem. Soc. Rev.* **2009**, *38*, 1119–1138. [CrossRef]
16. Chrapusta, E.; Kaminski, A.; Duchnik, K.; Bober, B.; Adamski, M.; Bialczyk, J. Mycosporine-like amino acids: Potential health and beauty ingredients. *Mar. Drugs* **2017**, *15*, 326. [CrossRef]
17. Varnali, T.; Bozoflu, M.; Şengönül, H.; Kurt, S.I. Potential metal chelating ability of mycosporine-like amino acids: A computational research. *Chem. Pap.* **2022**, *76*, 2279–2291. [CrossRef]
18. Cepoi, L.; Chiriac, T.; Rudi, L.; Djur, S.; Zosim, L.; Bulimaga, V.; Batir, L.; Elenciuc, D.; Rudic, V. Spirulina as a raw material for products containing trace elements. In *Recent Advances in Trace Elements*; Chojnacka, K., Saeid, A., Eds.; John Wiley & Sons Ltd.: Hoboken, NJ, USA, 2018; pp. 403–420. ISBN 9781119133780.
19. Puyfoulhoux, G.; Rouanet, J.M.; Besançon, P.; Baroux, B.; Baccou, J.C.; Caporiccio, B. Iron availability from iron-fortified spirulina by an in vitro digestion/Caco-2 cell culture model. *J. Agric. Food Chem.* **2001**, *49*, 1625–1629. [CrossRef]
20. Bermejo, P.; Piñero, E.; Villar, Á.M. Iron-chelating ability and antioxidant properties of phycocyanin isolated from a protean extract of *Spirulina platensis*. *Food Chem.* **2008**, *110*, 436–445. [CrossRef]
21. Hedegaard, R.V.; Rokkjær, I.; Sloth, J.J. Total and inorganic arsenic in dietary supplements based on herbs, other botanicals and algae-a possible contributor to inorganic arsenic exposure. *Anal. Bioanal. Chem.* **2013**, *405*, 4429–4435. [CrossRef]
22. Rzymski, P.; Budzulak, J.; Niedzielski, P.; Klimaszyk, P.; Proch, J.; Kozak, L.; Poniedziałek, B. Essential and toxic elements in commercial microalgal food supplements. *J. Appl. Phycol.* **2019**, *31*, 3567–3579. [CrossRef]
23. Sandgruber, F.; Gielsdorf, A.; Baur, A.C.; Schenz, B.; Müller, S.M.; Schwerdtle, T.; Stangl, G.I.; Griehl, C.; Lorkowski, S.; Dawczynski, C. Variability in macro-and micronutrients of 15 commercially available microalgae powders. *Mar. Drugs* **2021**, *19*, 310. [CrossRef] [PubMed]
24. Ghanbarzadeh, M.; Moazami, N.; Shahavi, M.H.; Mirdamadi, S. Study of bioactive compounds in *Arthrospira platensis* MGH-1 fortified with micronutrients of iron, zinc, and manganese. *J. Appl. Phycol.* **2022**, *34*, 2449–2462. [CrossRef]
25. Rayman, M.P. Selenium and human health. *Lancet* **2012**, *379*, 1256–1268. [CrossRef]
26. Lee, S.J.; Kim, S.B.; Kim, J.E.; Kwon, G.S.; Yoon, B.D.; Oh, H.M. Effects of harvesting method and growth stage on the flocculation of the green alga *Botryococcus braunii*. *Lett. Appl. Microbiol.* **1998**, *27*, 14–18. [CrossRef]
27. Papazi, A.; Makridis, P.; Divanach, P. Harvesting *Chlorella minutissima* using cell coagulants. *J. Appl. Phycol.* **2010**, *22*, 349–355. [CrossRef]
28. EU Commission. COMMISSION REGULATION (EC) No 629/2008 of 2 July 2008 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuff. *Off. J. Eur. Union* **2008**, *173*, 6–9.
29. Desideri, D.; Cantaluppi, C.; Ceccotto, F.; Meli, M.A.; Roselli, C.; Feduzi, L. Essential and toxic elements in seaweeds for human consumption. *J. Toxicol. Environ. Health Part A* **2016**, *79*, 112–122. [CrossRef]
30. Orlando, P.; Silvestri, S.; Ferlizza, E.; Andreani, G.; Carpenè, E.; Falcioni, G.; Tiano, L.; Isani, G. Biochemical responses to cadmium exposure in *Oncorhynchus mykiss* erythrocytes. *Ecotoxicol. Environ. Saf.* **2017**, *145*, 476–482. [CrossRef]
31. Bennett, A.; Bogobad, L. Complementary chromatic adaptation in a filamentous blue-green alga. *J. Cell Biol.* **1973**, *58*, 419–435. [CrossRef]
32. Andreani, G.; Cannavacciuolo, A.; Menotta, S.; Spallucci, V.; Fedrizzi, G.; Carpenè, E.; Isani, G. Environmental exposure to non-essential trace elements in two bat species from urbanised (*Tadarida teniotis*) and open land (*Miniopterus schreibersii*) areas in Italy. *Environ. Pollut.* **2019**, *254*, 113034. [CrossRef] [PubMed]

Isani, G., Niccolai, A., Andreani, G., Dalmonte, T., Bellei, E., Bertocchi, M., Tredici, M.R., Rodolfi, L. Iron speciation and iron binding proteins in *Arthrospira platensis* grown in media containing different iron concentrations. *International journal of molecular sciences*, **2022**, 23, 6283.

In the fifth paper iron bioaccumulation and speciation was investigated in biomasses of *A.platensis* grown in culture media with different iron concentration. Iron content in biomasses was measured using AAS, while the iron speciation was investigated through size exclusion chromatography followed by proteomic analysis using SDS-PAGE and mass spectrometry to identify the most abundant proteins of the biomasses. This research provided scientific evidence of the role of phycocyanin as the main iron-binding protein in *A.platensis* and pointed out that *A.platensis* can accumulate iron depending on the concentration present in the culture media. These biomasses can be considered of interest for the production of spirulina-based supplements with low and high iron content.

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Iron Speciation and Iron Binding Proteins in *Arthrospira platensis* Grown in Media Containing Different Iron Concentrations

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Abstract: Cyanobacteria are characterized by high iron content. This study investigated the effects of varying iron concentrations (1, 5, and 10 mg L^{-1}) in the culture media on the biochemical composition and the iron bioaccumulation and speciation in *Arthrospira platensis* F&M-C256. Iron content measured in biomasses varied from 0.35 to 2.34 mg g^{-1} dry weight depending on the iron concentration in the culture media. These biomasses can be considered of interest for the production of spirulina-based supplements with low and high iron content. Iron speciation was studied using size exclusion chromatography followed by atomic absorption spectrometry and proteomic analysis. The role of C-phycocyanin as an iron binding protein was also investigated. Overall, the present results provide a better understanding of iron metabolism in cyanobacteria and a foundation for further studies.

Keywords: *Arthrospira platensis*; cyanobacteria; culture media; iron bioaccumulation; iron speciation

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1. Introduction

The cyanobacteria of the genus *Arthrospira*, commonly known as spirulina, are characterized by an excellent nutritional profile with high digestible protein content (60–70%) [1,2]. They are also considered an interesting source of macro (Ca, Mg, P) and trace (Fe, Zn, Cu, Se) elements, vitamins, γ-linolenic acid, sulfated polysaccharides, phycocyanin, and bioactive molecules [3]. The possibility of modifying the biochemical composition of algal biomass by varying the growing conditions is an attractive opportunity to obtain products with enhanced nutraceutical properties. In particular, the presence of high concentrations of iron could be an interesting opportunity for people subjected to an inadequate dietary iron intake.

Iron performs important functions in biological systems; however, its redox activity, essential for many biochemical reactions, can be dangerous as a possible source of radicals, and, consequently, iron concentrations are tightly regulated by complex homeostatic mechanisms [4]. For this reason, iron is not found in the state of free ions, such as calcium and magnesium, but is always bound to proteins. Generally, iron is present in the trivalent state in the storage molecule ferritin and during transport and in the bivalent state when it performs its biochemical functions [4]. Once transported into the cell, iron can be used as a

cofactor for many enzymes, for the assembly of heme, or it can be used to form Fe–S clusters. Excess iron is stored in an inorganic form complexed with phosphate in specific storage molecules, such as ferritins in multicellular organisms and bacterioferritins in bacteria, which differ from ferritins in the presence of a heme molecule [5].

In addition, photosynthetic organisms, including cyanobacteria, have particularly high iron requirements due to Fe-rich photosynthetic machinery. Iron is found within the reaction centers of both photosystems, with 2–3 Fe atoms in photosystem II (PSII) and 12 iron atoms in photosystem I (PSI) [6,7]. Consequently, in cyanobacteria, the intracellular iron concentrations are 4–6 orders of magnitude higher than those of the aquatic environment in which they grow [8]. However, in these organisms, information on the molecular ligands of iron is scarce. Regarding bioaccessibility and bioavailability, it has been well recognized that chemical speciation is more important than the absolute metal concentration [9]. The study of iron speciation could be addressed by hyphenated techniques, including chromatographic fractionation of metal-binding molecules associated with sensitive metal detection in the fractions obtained by atomic absorption spectrometry (AAS) [10].

This research is devoted to study the effects of varying iron concentrations in the growth media on the biochemical composition and the iron bioaccumulation and speciation in *Arthrospira platensis* F&M-C256. Zarrouk medium containing 25 mg L^{-1} of $FeSO_4 \cdot 7H_2O$ was considered as the reference. Two other concentrations of $FeSO_4 \cdot 7H_2O$ were chosen, namely 5 mg L^{-1} and 50 mg L^{-1}, to explore the possibility of obtaining biomasses with low and high iron content, respectively. Particular attention is paid to the relationship between iron speciation and the most abundant proteins. Finally, the possible contribution to human nutritional requirements of iron obtained from *A. platensis* F&M-C256 is discussed.

2. Results

2.1. Production of Arthrospira Platensis F&M-C256 Biomass and Biochemical Composition

Figure 1 shows the growth curves of *A. platensis* F&M-C256 cultivated at three different iron concentrations: 1, 5, and 10 mg L^{-1}. The biomass concentration at day 0 was set between 0.3 and 0.4 g L^{-1} in all the cultures. Overall, no statistical difference in growth among the cultures grown at different iron concentrations was observed. *A. platensis* F&M-C256 cultures grown at different iron concentrations showed no morphological difference in terms of filaments length and shape during cultivation.

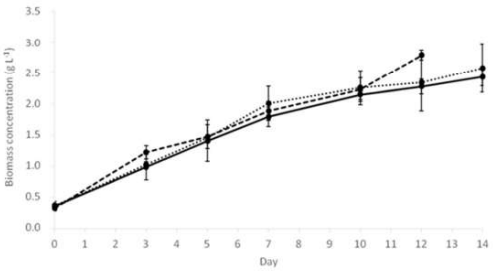

Figure 1. Growth curves of *A. platensis* F&M-C256 grown at three different iron concentrations: 1 mg L^{-1} (continuous line), 5 mg L^{-1} (line with dots), and 10 mg L^{-1} (line with dashes). Values are expressed in g L^{-1} and are reported as means ± SD (n = 2).

No statistical difference for carotenoids (0.09% dry biomass weight on average) and phenolic content (21.6 mg GAE g^{-1} on average) among *A. platensis* F&M-C256 biomasses grown at different iron concentrations was observed (Table 1). Statistically significant differences were found for chlorophyll *a*, phycocyanin, and antioxidant activity. Chlorophyll *a* content followed the iron concentration in the culture medium with the highest value

of 0.9% at the highest iron concentration. Biomass grown at 5 mg L^{-1} of iron contained the highest phycocyanin content (7.8%). Concerning the antioxidant activity, the biomass grown at the lowest iron concentration (1 mg L^{-1}) showed the strongest radical scavenging capacity (82.3%).

Table 1. Iron concentration in the culture medium at time zero and chlorophyll *a*, carotenoids, phycocyanin, antioxidant activity, phenolic content, iron concentration in the biomass, algal pellet, and cytosolic extract at the end of cultivation of *A. platensis* F&M-C256 are reported for each iron nominal concentration of 1, 5, and 10 mg L^{-1}. Values are reported as means ± SD ($n = 3$). Different letters in a column indicate a significant difference ($p < 0.05$).

[Fe] Nominal Concentration (mg L^{-1})	[Fe] Culture Medium at Time Zero (mg L^{-1})	Chlorophyll *a* (% d.w.)	Carotenoids (% d.w.)	Phycocyanin (% d.w.)	Antioxidant Activity (% RSA)	Phenolics (mg GAE g^{-1})	[Fe] Biomass (mg g^{-1} dw)	[Fe] Algal Pellet (mg g^{-1} dw)	[Fe] Algal Cytosolic Extract (μg mL^{-1})
[Fe] 1	$0.84^{c} \pm 0.04$	$0.50^{c} \pm 0.05$	$0.08^{a} \pm 0.03$	$5.16^{c} \pm 0.14$	$82.29^{a} \pm 0.57$	$23.93^{a} \pm 1.22$	$0.35^{c} \pm 0.02$	$0.056^{c} \pm 0.003$	$1.53^{b} \pm 0.11$
[Fe] 5	$3.44^{b} \pm 0.18$	$0.66^{b} \pm 0.10$	$0.08^{a} \pm 0.01$	$7.81^{a} \pm 0.48$	$73.83^{b} \pm 1.57$	$20.26^{a} \pm 0.77$	$1.28^{b} \pm 0.03$	$0.160^{b} \pm 0.020$	$3.68^{a} \pm 0.42$
[Fe] 10	$6.90^{a} \pm 0.42$	$0.90^{a} \pm 0.16$	$0.11^{a} \pm 0.04$	$6.10^{b} \pm 0.90$	$72.69^{b} \pm 0.57$	$20.49^{a} \pm 1.14$	$2.34^{a} \pm 0.10$	$0.248^{a} \pm 0.021$	$4.23^{a} \pm 0.16$

dw: dry weight, RSA: radical scavenging activity, GAE: gallic acid equivalent.

2.2. Iron Bioaccumulation

Iron concentrations in culture media at time zero and in biomasses at the end of cultivation are reported in Table 1. The concentrations measured in culture media were lower than the corresponding nominal concentration. Iron content in the algal biomass varied from 0.35 to 2.34 mg g^{-1} dw and presented a positive and significant correlation with the metal concentrations measured in the culture media ($r = 0.999$, $p < 0.05$) (Figure 2). To better understand the dynamics of iron accumulation, the metal concentration was also determined at defined time intervals (0, 3, 7, 11, and 14 days) in culture medium and algal biomass (Figure 2) of cultures with an initial iron concentration of 5 mg L^{-1}. On day 3, an increase of iron content in algal biomass corresponded to a concomitant decrease in the metal in the culture medium. In the following days (7, 11, and 14), iron content in the biomass varied between 1.5 and 2.5 mg g^{-1}. On the same days, decreasing iron concentrations in the culture medium were observed, up to a complete depletion of iron on the last day of the trial, in accordance with an increase in the biomass.

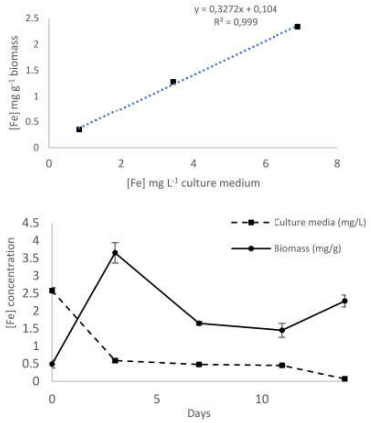

Figure 2. (**Top**) Iron accumulation in algal biomass as a function of iron concentration in the culture medium. (**Bottom**) Iron concentration in culture medium (dotted line reported as mg L^{-1}) and iron content in algal biomass (solid line reported as mg g^{-1} dry wt) of cultures with an initial iron concentration of 5 mg L^{-1} during a period of 14 days. Data are reported as mean±SD ($n = 3$).

2.3. Size Exclusion Chromatography (SEC) and Iron Speciation

The iron distribution between the pellet and the soluble fraction obtained after centrifugation of the algal samples was determined and is reported in Table 1. In all the samples analyzed, iron was more concentrated in the pellets than in the soluble extracts, and the iron percentage in the pellets significantly increased with increasing iron in the culture media. The percentage of iron in the soluble fraction decreased from 10.5% in the biomass grown at 1 mg Fe L^{-1} to 6.04% in the biomass grown at 10 mg Fe L^{-1}.

Chlorophyll *a*, total proteins, phycocyanin, and A620/A280 ratio in fractions obtained from SEC are reported in Figure 3A–D. The bulk of proteins eluted in fractions of molecular mass >75 kDa (highest absorbance at 280 nm in fraction 11), while fractions over 22 no longer contained proteins but only small peptides and free amino acids. The absorbance of soluble proteins in fraction 11 and that of phycocyanin in fraction 12 followed this decreasing order: extract C> extract B> extract A (Figure 3B,C). The maximum absorbance of phycocyanin was detected in fraction 12, while the maximum A620/A280 ratio was in fraction 13 for extracts A and C and in fraction 14 for extract B (Figure 3C,D).

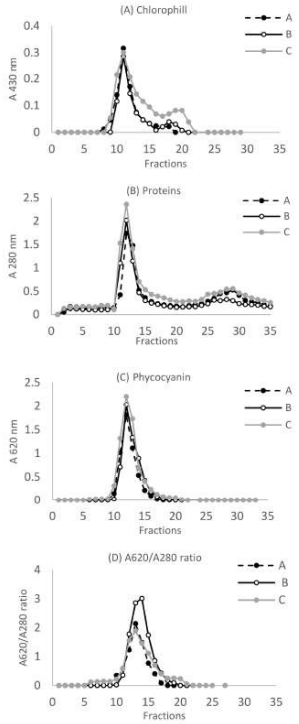

Figure 3. Chromatographic pattern after size exclusion chromatography of extracts A, B, and C obtained from samples grown at 1, 5, and 10 mg Fe L^{-1}, respectively. (**A**) Chlorophyll *a* was detected at 430 nm; (**B**) total proteins were measured at 280 nm; (**C**) phycocyanin was measured at 620 nm; (**D**) the purity of phycocyanin was determined by the ratio absorbance at 620 nm/absorbance at 280 nm. Each chromatogram is the mean of two independent chromatographies.

Iron profiles after SEC are reported in Figure 4. Two main peaks were present, namely in fractions 10–12 and in fractions 13–16. However, iron is distributed differently between

these two peaks depending on the concentration of the metal in the culture medium. In extract A, iron was mainly bound to ligands with molecular mass >75 kDa (high molecular mass, HMM,) (fractions 10–12). Differently, in extract C, a consistent percentage of the metal (37%) was bound to ligands with intermediate molecular mass (IMM) (fractions 13–16). In all the samples analyzed, low concentrations of iron were bound to ligands with low molecular mass (LMM) (fractions 17–25). An intermediate pattern was present in extract B.

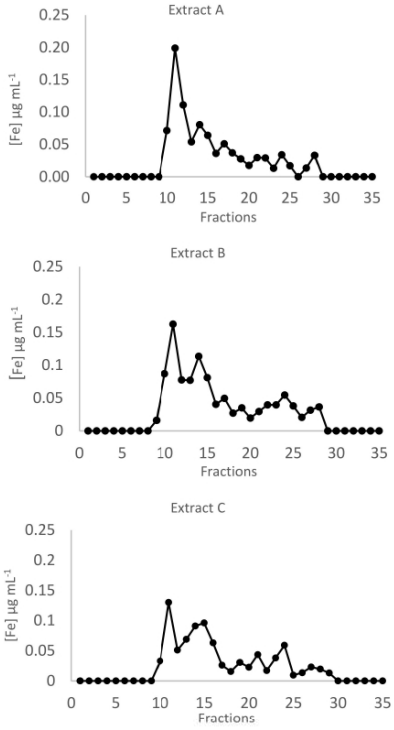

Figure 4. Iron chromatographic profiles after size exclusion chromatography of extracts **A**–**C** obtained from samples grown at 1, 5, and 10 mg Fe L^{-1}, respectively. Each chromatogram is the mean of two independent chromatographies. Iron concentration is expressed as µg mL^{-1}.

2.4. SDS-PAGE and Protein Identification Using Mass Spectrometry

The fractions containing the most relevant metal burden, namely fractions 11 and 14, underwent SDS-PAGE on 4–12% gels. A representative gel is reported in Figure 5. Fractions 11, which had the highest absorbance at 280 nm and the highest iron concentration, showed many different protein bands, with apparent molecular mass ranging from 150 to <14 kDa. Fractions 11 from extracts A, B, and C showed quite similar pherograms, with the sole exception of the central zone of the gel in extract C (Figure 6). Fractions 14 showed a major band with an apparent MM of 17–18 kDa.

The most represented bands were cut from the gel and further processed for LC-ESI-QO-MS/MS analysis. Ten proteins were successfully identified from the 12 bands excised from the gel (Table 2). The identity of proteins contained in three bands (Figure 5) could not be found, probably because the protein concentration in the bands was very low, hampering the identification. The biological processes and molecular functions related

to these proteins are reported according to gene ontology (GO) and UniProt in Table 2. The proteins identified were involved in relevant biological processes, including energy metabolism (glycolysis), photosynthesis, sulfate assimilation, one-carbon metabolism, carbohydrate transport, and cell redox homeostasis.

Table 2. Protein identification in fractions from cytosolic extracts of *A. platensis* using mass spectrometry.

Band [a]	Entry Name [b]	Protein Full Name	MM (Da) [c]	Organism	Score [d]	Sign Pept [e]	Sign Seq [f]	Molecular Function	Biological Process
1	A0A015IXE4_9GLOM	J-domain containing protein	33,255	*Arthrospira platensis*	21	1	1	DNA binding	
2	WP_035760125.1	Iron-uptake porin	67,484	*Arthrospira platensis*	59	6	6	Porin activity	Carbohydrate transport
3	K1VSN7_ARTPT	Carbohydrate-selective porin OprB	68,358	*Arthrospira platensis*	119	13	12	Porin activity	Carbohydrate transport
4	H1WGM5_9CYAN	Glutathione oxidoreductase	48,615	*Arthrospira platensis*	155	10	8	Oxidoreductase	Cell redox homeostasis—Cell oxidant detox
5	H1WMK8_9CYAN	Transketolase	72,899	*Arthrospira platensis*	67	3	2	Transferase, metal ion-binding	Pentose shunt
6	H1W8T2_9CYAN	Phosphoglycerate kinase	42,306	*Arthrospira platensis*	213	12	8	Transferase	Glycolysis
	H1WJF3_9CYAN	Adenosylhomocysteinase	46,765	*Arthrospira platensis*	124	7	7	Hydrolase	One-carbon metabolism
	H1WJ30_9CYAN	Sulfate adenylyltransferase	44,594	*Arthrospira platensis*	92	6	6	Transferase	Sulfate assimilation
7	H1WF80_9CYAN	Ferredoxin NADP reductase	45,396	*Arthrospira platensis*	74	7	6	Oxidoreductase	Photosynthesis
8–9	A0A0C4VZ70_ARTPT	CpcA protein	17,703	*Arthrospira platensis*	682	34	8	Chromophore	Photosynthesis

[a] Number of the identified band as marked in Figure 5. [b] Protein entry name from the UniProt knowledge database. [c] Theoretical protein molecular mass. [d] The highest scores obtained with the Mascot search engine. [e] Significant peptides: total number of significant peptides matching the identified proteins. [f] Significant sequences: total number of significant distinct sequences matching the identified proteins.

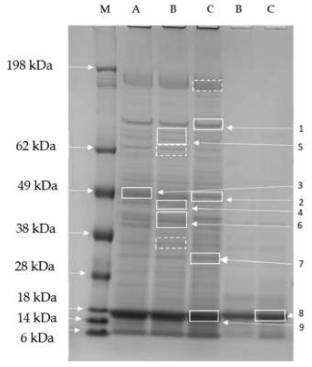

Figure 5. Representative SDS-PAGE (4–12%, Coomassie staining) of fractions obtained after size exclusion chromatography of cellular extracts of samples A, B, and C. Right: Lane 1, molecular mass marker (M) (kDa); lanes 2–4 fraction 11 from samples A (A), B (B), and C (C), respectively; lanes 5–6, fraction 14 from samples B (B) and C (C), respectively. Rectangles indicate bands excised and analyzed by mass spectrometry: continuous line rectangles indicate bands that provided protein identifications (Table 2), while dotted line rectangles indicate bands excised and analyzed by mass spectrometry that did not result in protein identification.

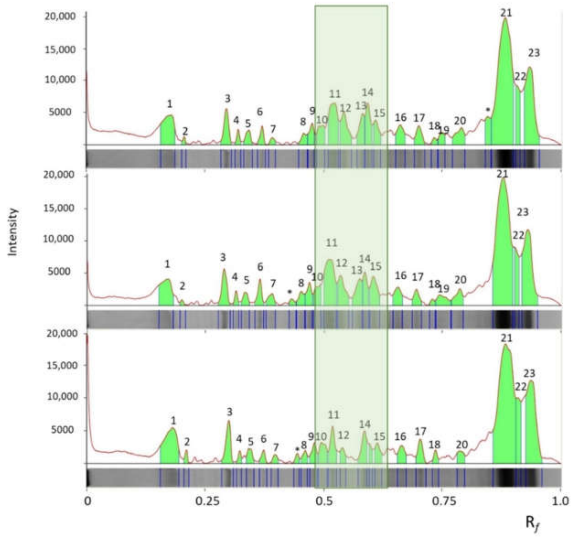

Figure 6. Pherograms obtained from the SDS-PAGE gel reported in Figure 5: lanes 2 (fraction 11, sample A), lane 3 (fraction 11, sample B), and lane 4 (fraction 11, sample C) from the top to the bottom of the figure. In the rectangle, the central zone of the pherogram, containing the excised bands 2, 3, 4, and 6, is outlined, highlighting the different intensities of the protein bands. In the three pherograms, the same peaks are marked by the same number. The peaks present only in one or two pherograms are marked with an asterisk. R_f is the migration distance of the protein band through the gel.

3. Discussion

3.1. Production of A. platensis F&M-C256 Biomass and Biochemical Composition

Iron is a trace element essential for the survival of all organisms, including cyanobacteria. In the present study, no statistical difference in growth among *A. platensis* F&M-C256 cultivated at different iron concentrations was observed, indicating that the lowest iron concentration is able to satisfy the requirements for this essential trace element. Accordingly, Delrue et al. (2017) found that modified Zarrouk's medium could be diluted up to five times without affecting the growth rates in 28-days batch cultivation [11]. Ismaiel et al. (2018) studied the effects of increasing iron concentration in culture media and reported that concentrations of iron ranging from 0.035 to 0.18 mM (from 1.9 to 10 mg L^{-1}) had no significant effect on growth [12].

In photosynthetic organisms, iron is essential for chlorophyll biosynthesis. Accordingly, in the present research, a significant increase in chlorophyll *a* was found in *A. platensis* F&M-C256 with the increase of iron concentration in the growth medium. Ismaiel et al. (2018) found a similar effect in *A. platensis* for chlorophyll *a* and phycocyanin [12]. In our research, biomass grown at the standard iron concentration of Zarrouk medium (5 mg L^{-1}) contained the highest phycocyanin content (about 8%). Xing et al. (2007) found that iron limitation induces phycobiliprotein degradation in cyanobacteria, which could explain the lower phycocyanin amount found for *A. platensis* F&M-C256 grown at 1 mg Fe L^{-1} [13]. In our study, as the iron concentration increased, a reduction in antioxidant capacity was observed. It is worth pointing out that in the sample of *A. platensis* F&M-C256 grown at the highest iron concentration, the bands containing glutathione oxidoreductase and adenosylhomocysteinase, two antioxidant enzymes, are actually less intense (bands 4 and 6, Figures 5 and 6), suggesting lower amounts of these proteins and less effective antioxidant defenses.

3.2. Iron Bioaccumulation

The data obtained in the present research showed iron concentrations in the culture media lower than the nominal ones. The fate of iron in the alkaline medium used for *A. platensis* cultures is still poorly explored. It has been hypothesized that iron precipitates as $FePO_4$, determining the decrease in the metal concentration available for cell growth [14]. Moreover, it has been reported by Perfiliev et al. (2018) that iron concentrations in the culture medium higher than 5 μg L^{-1} causes sedimentation of the metal, determining a lower bioaccessibility of iron [15]. In the present study, the discrepancy between the iron nominal concentration and the concentration determined in culture media by AAS suggests iron precipitation.

Iron concentrations measured in *A. platensis* F&M-C256 biomass varied from 352 to 2342 μg g^{-1} dw. These data are in accordance with concentrations from 300 to 2160 μg g^{-1} reported in the literature for iron in different *A. platensis* samples [16,17]. It is known that the wide variability of iron concentrations in *Arthrospira* is due to the metal bioavailability in the water, which, in turn, is dependent upon environmental parameters, such as salinity, pH, light intensity, and nutrients [18].

The ability of cyanobacteria to bioaccumulate metals is an interesting opportunity to obtain biomasses enriched with specific trace elements. This behavior can be exploited in different ways, from bioremediation of polluted environments to the production of high-value food supplements. On average, for biomass obtained at the highest iron concentration in this research, daily supplementation with the commonly recommended dietary intake of 3.0 g of spirulina would constitute 64% of the population reference intake (PRI) of 11 mg day^{-1} reported by EFSA for iron [19]. It has been reported that *A. platensis* trichomes accumulate iron mostly in the form of Fe^{3+} as ferrihydrite [15]. Therefore, the bulk of iron present in the pellet of all the examined samples should be stored in this inorganic form. The chemical form of iron, in particular, the presence of heme or non-heme iron, is critical in determining the bioavailability of this essential element. Puyfoulhoux et al. (2001) compared the iron bioavailability of iron-fortified spirulina with different iron sources by measuring ferritin formation in Caco-2 cells exposed to digests obtained from meat, yeast, and wheat [20]. The results obtained by these authors with spirulina were indicative of a metal bioavailability not significantly different from that exhibited by the meat digest, despite the different chemical forms of iron. Moreover, the effect of spirulina supplementation has been tested on anemia in senior citizens. Over the 12-weeks study period, an increase in mean corpuscular hemoglobin was detected in both sexes [21], indicating a possible application as a high-value supplement to improve anemia. However, the biochemical basis of this effect is still to be discovered, and future studies should be focused on large clinical trials.

On the other hand, the biomass with the lowest iron concentration associated with a higher antioxidant activity could be an interesting supplement for people subjected to a reduced iron dietary intake due to cardiovascular risk. In fact, it is well recognized that high iron intake and stores are associated with an increased risk of iron-induced oxidative stress, inflammation, and cardiovascular diseases [22].

3.3. Iron Speciation and Protein Identification

In addition to inorganic iron, a percentage of the metal varying from 6% (extract C) to 10% (extract A) was present in the soluble fraction, likely bound to proteins. Studies on trace element speciation, i.e., the distribution of the element among defined chemical species in biological systems, are rare in cyanobacteria. In this research, non-denaturing SEC followed by iron analysis of the chromatographic fractions using AAS allowed the separation of soluble proteins. To further fractionate these proteins, before identification with mass spectrometry, an additional separation step using SDS-PAGE electrophoresis was applied to chromatographic fractions 11 and 14, which contained the highest concentrations of iron.

A total of 3 of the 10 proteins identified by mass spectrometry are at the crossroad between photosynthesis and iron metabolism. In all the samples, the most abundant protein in fractions 11 and 14 identified in bands 8 and 9 is C-phycocyanin (C-PC), a multimeric blue protein belonging to the phycobiliprotein family. In cyanobacteria, C-PC is an essential pigment of the phycobilisome and contains a prosthetic group similar to bilverdin and bilirubin, known as phycocyanobilin (PCB). This protein consists of two subunits, which bind one and two PCB, respectively. The molecular mass (MM) of α and β subunits varies depending on the species, from 11 to 24.4 [23]. The MM obtained in this study for the α subunit is like that reported for spirulina by Patel et al. (2005) [24]. In addition to its essential function as a light-capturing antenna for photosystem II, C-PC is a potent scavenger of hydroxyl and peroxyl radicals and can bind iron in vitro [25]. Bermejo et al. concluded that the antioxidant capacity of *Spirulina platensis* protein extract arises from both radical scavenging and Fe^{2+} chelation activity. Other evidence was previously reported by Bhat and Madyastha (2000) [26], who found that phycocyanin interacts with iron with an association constant of $1.11 + 0.06 \times 10^5$ M, and recently by Kim et al. (2014), who purified an iron-chelating peptide from spirulina protein hydrolysates [27]. In our study, the abundance of C-PC in fractions 11 and 14, which contain the bulk of iron, adds evidence to the role of C-PC as a potential iron-binding protein also in vivo.

One other protein related to photosynthesis is ferredoxin NADP(H) oxidoreductase (EC 1.18.1.2) (FNR), identified in band 8 [28]. This enzyme receives electrons from ferredoxin and reduces NADP+ to NADPH [29]. Therefore, its main function is to ensure the final step of photosynthetic electron transport, providing NADPH for CO_2 assimilation. In cyanobacteria, FNR has been found to be associated mostly with phycobilisomes, and two isoforms have been reported: a short isoform of 33 kDa, similar to plant stromal FNR, and a long isoform of 45 kDa [29]. The FNR identified in this study had a molecular weight of 34 kDa, suggesting the presence of the short isoform in *A. platensis*. The concomitant identification of C-PC and FNR in fraction 11 is suggestive of the presence of phycobilisomes, if not intact in their complex molecular architecture, at least as sub-complexes, including some of the proteins that take part in the structure. It is important to point out that the extracts prepared in this study are obtained in non-denaturing conditions, which preserve the functional structure of proteins and protein complexes. However, during the subsequent step of SDS-PAGE, denaturing conditions are applied, resulting in protein denaturation, including dissociation of protein subunits, which appear after electrophoresis with MM lower than the active polymers.

Iron-uptake porin identified in band 2 is another interesting protein involved in iron metabolism. Cyanobacteria require a large amount of iron to maintain the photosynthetic machinery. Consequently, iron availability is a key factor in controlling cyanobacteria populations and frequently is a limiting factor for biomass growth. In *Synechocystis* sp, complex membrane pathways are present to cope with the intake of iron, including the presence of the TonB-dependent transport system, which transports the ferri-siderophore complexes, especially in the situation of iron depletion [30]. However, siderophore-mediated iron uptake is functioning at a low rate in iron-replete environments, suggesting the existence of alternative systems for iron uptake. Recently, Qiu et al. (2021) reported the presence of a specific iron-selective porin in *Synechocystis* sp [6]. The identification of this protein in the extracts of the samples analyzed in this research adds in vivo evidence of the presence of this protein also in *A. platensis*. Interestingly, the intensity of this band decreases as the iron concentration in the culture medium increases (Figures 5 and 6), suggesting the existence of a regulatory mechanism controlling intracellular iron content.

Iron challenge followed by increased intracellular metal concentrations exposes organisms to an increased risk of oxidative stress. Two of the identified proteins have recognized antioxidant activity: glutathione oxidoreductase identified in band 4 and adenosyl-homocysteinase identified in band 6. Particularly, the first enzyme is a key player in the antioxidant defense system by reducing glutathione (GSH) from its oxidized

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many); the acids were of Suprapur grade. Samples of lyophilized biomasses (400 mg) and pellets obtained as described in Section 4 (200 mg) were placed in individual acid-washed Teflon jars and were digested with 1–2 mL 65% HNO_3 and 0.25–0.5 mL 30% H_2O_2 in a microwave oven for 5 min at 250 W, 5 min at 400 W, 5 min at 500 W and 1 min at 600 W. The cooled samples were transferred into 5–10 mL polyethylene volumetric flasks and were directly analyzed using a flame atomic spectrophotometer equipped with a deuterium lamp background correction (AAnalyst 100, Perkin Elmer, Waltham, MA, USA) for iron determination. Supernatants obtained as described in Section 4.4 were directly analyzed without any further treatment. All the samples were run in batches, which included blanks; there was no evidence of any contamination in these blanks. The accuracy of the method was evaluated using the analysis of international standards (ERM®-BB422 fish muscle). The concentrations found with the method used in this study fell into the certified uncertainty interval given by ERM, corresponding to a 95% confidence level. The iron detection limit for flame atomic spectrophotometry was 0.04 μg mL^{-1}. Iron concentrations were reported as μg $mI.^{-1}$ or μg g^{-1} depending on the sample analyzed.

4.4. Iron Speciation and Size Exclusion Chromatography (SEC)

Two hundred milligrams of lyophilized biomass from *A. platensis* cultivated in the presence of nominal Fe concentrations of 1, 5, and 10 mg L^{-1} were crushed in a mortar with a pestle under liquid nitrogen and homogenized in 3 volumes (*v*/*w*) of Tris–HCl 20 mM and 10 mM mercaptoethanol, pH 8.6, using an Ultraturrax (IKA, Staufen, Germany) homogenizer. The homogenate was sonicated for 10 min at 38 kHz and then centrifuged at 24,000× *g* for 40 min at 4 °C, obtaining the separation between supernatant and pellet. The supernatant obtained from *A. platensis* cultivated in the presence of nominal iron concentrations of 1 mg L^{-1} is indicated as extract A, while the supernatants obtained in the presence of nominal iron concentrations of 5 and 10 mg L^{-1} are indicated as extract B and C, respectively. For each extract, a volume of 0.8 mL supernatant was applied to a Sephadex G-75 chromatography column (0.9 × 90 cm). The column was calibrated using a commercial kit (GF70-1KT, Sigma-Aldrich, St Louis, MO, USA). Fractions of 1.5 mL were collected and analyzed for iron concentration using the direct aspiration of the solution into the flame of an atomic absorption spectrophotometer as described above. The pellets obtained from the centrifugation of the homogenate were digested in a microwave oven, as reported above, and iron concentration was determined using a flame atomic absorption spectrophotometer (AAnalyst 100, Perkin Elmer, Waltham, MA, USA) as reported in "Metal analysis". In the supernatants and fractions obtained from size exclusion chromatography, total proteins, phycocyanin, and chlorophyll were determined by direct measurement of the absorbance at 280, 615, and 430 nm, respectively (DeNovix DS-11 Series Spectrophotometer, Wilmington, DE, USA). The purity of phycocyanin was determined by the ratio absorbance at 620/absorbance at 280.

4.5. SDS-PAGE

Five μg of protein were loaded onto 4–12% Bis-Tris polyacrylamide gels (NuPage/Thermo Fisher Scientific, Waltham, MA, USA), and electrophoresis (PAGE) was carried out in an Xcell SureLock Mini-Cell with 2-(N-morpholino) ethanesulfonic acid buffer (MES; NuPage/Thermo Fisher Scientific, Waltham, Massachusetts, MA, USA), containing sodium dodecyl sulfate (SDS). Each gel was also loaded with standard proteins of known molecular weight (SeeBlue™ Plus2 Pre-stained Protein Standard, Thermo Fisher Scientific, Waltham, MA, USA). The electrophoresis system was connected to a power supply (Power Pack Basic—Bio-Rad, Hercules, CA, USA) with a constant voltage of 200 V for 40 min. The gels were stained with Coomassie G250 compatible with mass spectrometry analysis. After staining, each gel was digitalized by ChemiDocMP (BioRad, Hercules, CA, USA), and pherograms were obtained using ImageLab 5.2.1 software (BioRad, Hercules, CA, USA).

4.6. Protein Identification Using Mass Spectrometry

The most represented bands were manually cut from the gel and underwent "in-gel" tryptic digestion. The bands were first destained with acetonitrile (ACN); the proteins were then reduced by 10 mM Dithiothreitol (DTT) at 56 °C for 30 min and were subsequently alkylated using 55 mM iodoacetamide for 20 min in the dark. After drying, the proteins were digested with 12.5 ng μL^{-1} Trypsin (Promega, Madison, WI, USA) by incubation overnight at 37 °C. Subsequently, the peptides were extracted using a solution composed of 1% trifluoroacetic acid/50% ACN and were finally concentrated in a vacuum dryer (Eppendorf Concentrator Plus).

The dried digested samples were resuspended in 95% water/3% ACN/2% formic acid and analyzed using a UHPLC-MS QExactive™ system (Thermo Fisher Scientific, Reinach, Switzerland), composed of a UHPLC 3000 Ultimate System coupled to an ESI-QExactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (LC-ESI-QO-MS/MS System). The separations were carried out on a ZORBAX RRHD Eclipse Plus C18 column (50 × 2.1 mm ID, 1.8 µm particle size, Agilent Technologies, Santa Clara, CA, USA) with a mobile phase composed of 0.1% aqueous formic acid solution (A) and ACN (B), using the following gradient elution at a flow rate of 0.3 mL/min: 0 to 3 min: isocratic at 2% (B); 3 to 21 min: linear gradient from 2% to 27% (B); 21 to 25 min: linear gradient from 27% to 90% (B); 25 to 28 min: isocratic at 90% (B); 28 to 28.1 min: linear gradient from 90% to 2% (B). An equilibration period of 6.9 min was interposed between each run. Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell, and as damping gas in the C-trap. The ESI source operated in positive ionization mode, with a capillary voltage of 3.5 kV. The analyses were checked using Xcalibur™ software (version 29 build 2926).

The raw mass data acquired, converted into Mascot generic format using MsConvert (version 3.0.10730, ProteoWizard tools), were searched using the MASCOT search engine (version 2.7, http://mascot.cigs.unimo.it/mascot, accessed on 30 May 2022). Trembl and SwissProt, together with C-RAP protein databases, were selected for peptide sequence and contaminant searching, respectively, setting the following restriction parameters: trypsin as a proteolytic enzyme, max two missed cleavages; peptide mass tolerance ± 10 ppm (for precursor ions) and fragment mass tolerance ± 0.02 Da (for product ions); unrestricted protein mass; carbamidomethylation to cysteine residues as fixed modification, and deamidated (NQ) and oxidated (M) methionine as variable modifications.

4.7. Statistical Analysis

Part of the statistical analysis was carried out using statistical software (RStudio-1.2.1335 Statistical and R, R v3.4.3, Development Core Team, Vienna, Austria) and part using Statgraphics Centurion XV (StatPoint Technologies Inc., Washington, DC, USA). Statistical differences between values of chlorophyll a, carotenoids, phycocyanin, antioxidant activity, and phenolic content were determined using ANOVA followed by Duncan's multiple range tests (MRT) to determine the least significant difference (LSD). All data were evaluated using standard descriptive statistics and reported as mean ± standard deviation (SD). A p-value < 0.05 was considered significant.

5. Conclusions

This research explored the possibility of obtaining cyanobacterial biomasses with different iron concentrations by varying the metal concentration in the culture media. *A. platensis* F&M-C256 was demonstrated to be a suitable model organism for the above purpose. The study of iron speciation using hyphenated techniques represents an innovation opening new scenarios in the field of iron metabolism in cyanobacteria. The abundance of C-PC in fractions 11 and 14, which contain the bulk of soluble iron, adds evidence to the role of C-PC as an Fe-binding protein in vivo. Finally, the iron concentrations measured in the three *A. platensis* F&M-C256 biomasses (from 0.35 to 2.34 mg Fe g^{-1} dry weight) can

be considered of interest to produce spirulina-based supplements with low and high iron content to satisfy different groups of consumers.

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References

1. Lafarga, T.; Fernández-Sevilla, J.M.; González-López, C.; Acién-Fernández, F.G. Spirulina for the food and functional food industries. *Food Res. Int.* **2020**, *137*, 109356. [CrossRef] [PubMed]
2. Niccolai, A.; Chini Zittelli, G.; Rodolfi, L.; Biondi, N.; Tredici, M.R. Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Res.* **2019**, *42*, 101617. [CrossRef]
3. Kulshreshtha, A.; Anish, J.Z.; Jarouliya, U.; Bhadauriya, P.; Prasad, G.B.K.S.; Bisen, P.S. Spirulina in health care management. *Curr. Pharm. Biotechmol.* **2008**, *9*, 400–405. [CrossRef] [PubMed]
4. Andrews, N.C. Forging a field: The golden age of iron biology. *Blood* **2008**, *112*, 219–230. [CrossRef] [PubMed]
5. Kranzler, C.; Rudolf, M.; Keren, N.; Schleiff, E. Iron in Cyanobacteria. *Adv. Bot. Res.* **2013**, *65*, 57–105. [CrossRef]
6. Qiu, G.W.; Jiang, H.B.; Lis, H.; Li, Z.K.; Deng, B.; Shang, J.L.; Sun, C.Y.; Keren, N.; Qiu, B.S. A unique porin meditates iron-selective transport through cyanobacterial outer membranes. *Environ. Microbiol.* **2021**, *23*, 376–390. [CrossRef]
7. Raven, J.A.; Evans, M.C.W.; Korb, R.E. The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. *Photosyn. Res.* **1999**, *60*, 111–150. [CrossRef]
8. Morel, F.M.M.; Price, N.M. The Biogeochemical Cycles of Trace Metals in the Oceans. *Environ. Sci.* **2003**, *300*, 944–947. [CrossRef]
9. Beck, K.L.; Conlon, C.A.; Kruger, R.; Coad, J. Dietary determinants of and possible solutions to iron deficiency for young women living in industrialized countries: A review. *Nutrients* **2014**, *6*, 3747–3776. [CrossRef]
10. Mounicou, S.; Szpunar, J.; Lobinski, R. Metallomics: The concept and methodology. *Chem. Soc. Rev.* **2009**, *38*, 1119–1138. [CrossRef]
11. Delrue, F.; Alaux, E.; Moudjaoui, L.; Gaignard, C.; Fleury, G.; Perilhou, A.; Richaud, P.; Petitjean, M.; Sassi, J.F. Optimization of *Arthrospira platensis* (Spirulina) Growth: From Laboratory Scale to Pilot Scale. *Fermentation* **2017**, *3*, 59. [CrossRef]
12. Ismaiel, M.M.; Piercey-Normore, M.D.; Rampitsch, C. Proteomic analyses of the cyanobacterium *Arthrospira* (*Spirulina*) *platensis* under iron and salinity stress. *Environ. Exp. Bot.* **2018**, *147*, 63–74. [CrossRef]
13. Xing, W.; Huang, W.M.; Li, D.H.; Liu, Y.D. Effect of iron on growth, pigment content, photosystem II efficiency, and siderophores production of *Microcystis aeruginosa* and *Microcystis wesenbergii*. *Curr. Microbiol.* **2007**, *55*, 94–98. [CrossRef]
14. Belay, A. Mass Culture of Spirulina Outdoors—The Earthrise Farms Experience (Chapter 8). In *Spirulina Platensis Arthrospira: Physiology, Cell-Biology and Biotechnology*, 1st ed.; CRC Press: Boca Raton, FL, USA, 1997. [CrossRef]
15. Perfiliev, Y.D.; Tambiev, K.; Konnychev, M.A.; Skalny, A.V.; Lobakova, E.S.; Kirpichnikov, M.P. Mössbauer spectroscopic study of transformations of iron species by the cyanobacterium *Arthrospira platensis* (formerly *Spirulina platensis*). *J. Trace Elem. Med. Biol.* **2018**, *48*, 105–110. [CrossRef]
16. Cepoi, L.; Chiriac, T.; Rudi, L.; Djur, S.; Zosim, L.; Bulimaga, V.; Batir, L.; Elenciuc, D.; Rudic, V. Spirulina as a Raw Material for Products Containing Trace Elements (Chapter 19). In *Recent Advances in Trace Elements*, 1st ed.; Wiley-Blackwell: Hoboken, NJ, USA, 2017. [CrossRef]
17. Rzymski, P.; Budzulak, J.; Niedzielski, P.; Klimaszyk, P.; Proch, J.; Kozak, L.; Poniedziałek, B. Essential and toxic elements in commercial microalgal food supplements. *J. Appl. Phycol.* **2019**, *31*, 3567–3579. [CrossRef]
18. Wild, K.J.; Steinga, H.; Rodehutscord, M. Variability in nutrient composition and in vitro crude protein digestibility of 16 microalgae products. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 1306–1319. [CrossRef]
19. European Food Safety Authority (EFSA). Scientific Opinion on Dietary Reference Values for iron. *EFSA J.* **2015**, *13*, 4254. [CrossRef]
20. Puyfoulhoux, G.; Rouanet, J.M.; Besançon, P.; Baroux, B.; Baccou, J.C.; Caporiccio, B. Iron Availability from Iron-Fortified Spirulina by an in Vitro Digestion/Caco-2 Cell Culture Model. *J. Agri. Food Chem.* **2001**, *49*, 1625–1629. [CrossRef]

21. Selmi, C.; Leung, P.S.C.; Fischer, L.; German, B.; Yang, C.Y.; Kenny, T.P.; Cysewski, G.R.; Gershwin, M.E. The effects of Spirulina on anemia and immune function in senior citizens. *Cell. Mol. Immunol.* **2011**, *8*, 248–254. [CrossRef]
22. Hunnicutt, J.; He, K.; Xun, P. Dietary iron intake and body iron stores are associated with risk of coronary heart disease in a meta-analysis of prospective cohort studies. *J. Nutr.* **2014**, *144*, 359–366. [CrossRef]
23. Fernández-Rojas, B.; Hernández-Juárez, J.; Pedraza-Chaverri, J. Nutraceutical properties of phycocyanin. *J. Funct. Foods.* **2014**, *11*, 375–392. [CrossRef]
24. Patel, A.; Mishra, S.; Pawar, R.; Ghosh, P.K. Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expr. Purif.* **2005**, *40*, 248–255. [CrossRef]
25. Bermejo, P.; Piñero, E.; Villar, Á.M. Iron-chelating ability and antioxidant properties of phycocyanin isolated from a protean extract of *Spirulina platensis*. *Food Chem.* **2008**, *110*, 436–445. [CrossRef]
26. Bhat, V.B.; Madyastha, K.M. C-Phycocyanin: A potent peroxyl radical scavenger in vivo and in vitro. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 20–25. [CrossRef] [PubMed]
27. Kim, K.; Choi, J.; Ji, Y.; Park, S.; Do, H.; Hwang, C.; Lee, B.; Holzapfel, W. Impact of bubble size on growth and CO_2 uptake of *Arthrospira* (*Spirulina*) *platensis* KMMCC CY-007. *Bioresour. Technol.* **2014**, *170*, 310–315. [CrossRef]
28. Vorphal, M.A.; Bruna, C.; Wandersleben, T.; Dagnino-Leone, J.; Lobos-González, F.; Uribe, E.; Martìnez-Oyanedel, J.; Bunster, M. Molecular and functional characterization of ferredoxin NADP(H) oxidoreductase from *Gracilaria chilensis* and its complex with ferredoxin. *Biol. Res.* **2017**, *50*, 39. [CrossRef]
29. Morsy, F.M.; Nakajima, M.; Yoshida, T.; Fujiwara, T.; Sakamoto, T.; Wada, K. Subcellular localization of ferredoxin-NADP+ oxidoreductase in phycobilisome retaining oxygenic photosysnthetic organisms. *Photosyn. Res.* **2008**, *95*, 73–85. [CrossRef]
30. Qiu, G.W.; Lou, W.J.; Sun, C.Y.; Yang, N.; Li, Z.K.; Li, D.L.; Zang, S.S.; Fu, F.X.; Hutchins, D.A.; Jiang, H.B.; et al. Outer membrane iron uptake pathways in the model cyanobacterium *Synechocystis* sp. strain PCC 6803. *Appl. Environ. Microbiol.* **2018**, *84*, e01512-18. [CrossRef] [PubMed]
31. Sannasimuthu, A.; Kumaresan, V.; Anilkumar, S.; Pasupuleti, M.; Ganesh, M.R.; Mala, K.; Paray, B.A.; Al-Sadoon, M.K.; Albeshr, M.F.; Arockiaraj, J. Design and characterization of a novel *Arthrospira platensis* glutathione oxido-reductase-derived antioxidant peptide GM15 and its potent anti-cancer activity via caspase-9 mediated apoptosis in oral cancer cells. *Free Radic. Biol. Med.* **2019**, *135*, 198–209. [CrossRef]
32. Rendón, J.L.; Calcagno, M.; Mendoza-Hernández, G.; Ondarza, R.N. Purification, properties, and oligomeric structure of glutathione reductase from the cyanobacterium *Spirulina maxima*. *Arch. Biochem. Biophys.* **1986**, *248*, 215–223. [CrossRef]
33. Wang, H.; Yang, Y.; Chen, W.; Li, D.; Zhao, X.; Wang, X.; Li, A.; Bao, Q. Identification of differentially expressed proteins of *Arthrospira* (*Spirulina*) *plantensis*-YZ under salt-stress conditions by proteomics and qRT-PCR analysis. *Proteome Sci.* **2013**, *11*, 6. [CrossRef] [PubMed]
34. Herrera, A.; Boussiba, S.; Napoleone, V.; Hohlberg, A. Recovery of c-phycocyanin from the cyanobacterium *Spirulina maxima*. *J. Appl. Phycol.* **1989**, *1*, 325–331. [CrossRef]
35. Bennett, A.; Bogobad, L. Complementary chromatic adaptation in a filamentous blue-green alga. *J. Cell Biol.* **1973**, *58*, 419–435. [CrossRef] [PubMed]
36. Jeffrey, S.W.; Humphrey, G.F. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.* **1973**, *167*, 191–194. [CrossRef]
37. Parsons, T.R.; Strickland, J.D.H. Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. *J. Mar. Res.* **1963**, *21*, 155–163. [CrossRef]
38. Rajauria, G.; Jaiswal, A.K.; Abu-Gannam, N.; Gupta, S. Antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland. *J. Food Biochem.* **2013**, *37*, 322–335. [CrossRef]
39. Ganesan, A. The impact of natural products upon modern drug discovery. *Curr. Opin. Chem. Biol.* **2008**, *12*, 306–317. [CrossRef]

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In the sixth paper, in collaboration with the research team headed by Prof. Biagi, the effect of four microalgae (*A.platensis, Chlorella vulgaris, Haematococcus pluvialis, and Phaeodactylum tricornutum)* was evaluated on faecal bacterial population in an *in vitro* canine gut model. The microalgae were subjected to an *in vitro* digestion procedure, composed in a 2 hours part representing the gastric phase followed by a 4 hours part simulating the intestinal phase. Then, the digested biomasses underwent to a colonic *in vitro* fermentation. The study was a preliminary assessment of the role of microalgae in canine nutrition and their effects on the microbiota.

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Assessment of the Effects of Edible Microalgae in a Canine Gut Model

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Simple Summary: The gut microbiota plays a crucial role in maintaining host health and is considered a potential target of novel therapeutics. Microalgae represent an interesting source of bioactive compounds for nutritional supplementation in humans and animals. However, there is a lack of information on the effects of microalgae on canine gut microbiota. The present study investigated for the first time the effects of four microalgae (Arthrospira platensis, Haematococcus pluvialis, Phaeodactylum tricornutum, Chlorella vulgaris) on some fecal bacterial populations and metabolites, such as SCFA, BCFA, ammonia and biogenic amines, in an in vitro canine gut model. Following the in vitro fermentation, chemical and microbiological analysis displayed significant differences between the control and microalgae groups, particularly the Phaeodactylum tricornutum group. Nonetheless, further investigations are needed to better understand the potential inclusion of these notable natural resources in pet nutrition.

Abstract: Microalgae are a source of bioactive compounds having recently been studied for their possible application as health-promoting ingredients. The aim of the study was to evaluate in an in vitro canine gut model the effects of four microalgae, Arthrospira platensis (AP), Haematococcus pluvialis (HP), Phaeodactylum tricornutum (PT) and Chlorella vulgaris (CV), on some fecal microbial populations and metabolites. The four microalgae were subjected to an in vitro digestion procedure, and subsequently, the digested biomass underwent colonic in vitro fermentation. After 6 h of incubation, PT increased propionate (+36%) and butyrate (+24%), and decreased total BCFA (−47%), isobutyrate (−52%) and isovalerate (−43%) and C. hiranonis (−0.46 log10 copies/75 ng DNA). After 24 h, PT increased propionate (+21%) and isovalerate (+10%), and decreased the abundance of Turicibacter spp. (7.18 vs. 6.69 and 6.56 log10 copies/75 ng DNA for CTRL vs. PT, respectively); moreover, after 24 h, CV decreased C. coccoides (−1.12 log10 copies/75 ng DNA) and Enterococcus spp. (−0.37 log10 copies/75 ng DNA). In conclusion, the microbial saccharolytic activities and the shift in fecal bacterial composition were less pronounced than expected, based on current literature. This study should be considered as a preliminary assessment, and future investigations are required to better understand the role of microalgae in canine nutrition.

Keywords: dog intestinal microbiota; microalgae; canine nutrition; in vitro fermentation

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1. Introduction

The gastrointestinal tract of mammals harbors one of the largest and most complex ecosystems known, including numerous bacterial species, along with fungi, protozoa and viruses. Over the past decades, there has been a growing understanding that the gut microbiota profoundly contributes to improve host health [1]. For example, intestinal bacteria either generate or turn dietary molecules into bacteria-derived metabolites, and the gut microbiome is considered an important metabolic organ. A well-balanced gut microbiome positively affects host health by modulating immune system, exerting protective

action against intestinal pathogens, and providing some nutrients [2]. Among the bacterial metabolites, straight short-chain fatty acids (SCFA) acetate, propionate and butyrate and branched-chain fatty acids (BCFA) isobutyrate and isovalerate [3] exercise very different but important activities in the host body [4]. In particular, SCFA from microbial metabolism enhance the function of the intestinal barrier, regulating the luminal pH and the mucus secretion, modulating the mucosal immune function and providing a source of energy for colonocytes [5].

In this vein, modulation of gut microbiota should be taken into consideration as a possible novel therapeutic [6].

The diet plays an important role in shaping the composition of intestinal microbiota and its relation with the host [6]. In the last decades, scientific research has widely investigated different nutritional strategies with the purpose of positively affecting the microbial ecosystem of canine gastrointestinal tract [7]. Among the several dietary components investigated in this context, edible microalgae, also called microphytes, represent an interesting source of bioactive compounds including protein, polysaccharides, polyunsaturated fatty acids, pigments, minerals, phenolic compounds, vitamins, volatile compounds and sterols, hence offering several possible health benefits [8]. Microalgae are ancient living organisms belonging to a taxonomically heterogeneous group, which includes organisms from a series of diverse classes and phyla [9]; some cyanobacteria belong to microalgae as well [8]. Among the high number of microalgae species in nature, Arthrospira and Chlorella are the algal strains more presented on the market worldwide [10]. Arthrospira has a global annual production of 12,000 tons, followed by Chlorella with about 5000 tons per year [11], and both genera are commercially available for human consumption, in accordance with the Food and Drug Administration [12], being found on the market as functional foods [13]. Furthermore, dried algae, algal oil, algae meal and extract are listed in the European catalogue of feed materials [14]. Arthrospira and Chlorella are rich in unsaturated fatty acids, proteins, various minerals, group B vitamins and pigments with relevant antioxidant characteristics, and thus, they may be used in foods [15,16]. Apart from the interest for the whole dried algae, currently, specific high-value constituents from microphytes are also being investigated. In this domain, Haematococcus pluvialis and Phaeodactylum tricornutum are two microphyte species with relevant bioactive compounds [13]. The first is cultured to obtain astaxanthin (a carotenoid with antioxidant properties) [17], while the second is a marine diatom contemplated as a significant potential source of eicosapentanoic acid (EPA) and carotenoids [18].

For their properties, microphytes have been suggested to be a promising sustainable alternative to traditional animal feed resources and a possible health-promoting ingredient both in human diet [15,17] and animal feeds [19], particularly in livestock and poultry feed industry [20,21], and in the aquaculture field [22,23]. However, microalgae supplementation requires a clear understanding of their effects on the intestinal microbiota and bacterial metabolome of the host.

In recent years, studies have identified many positive benefits of microalgae, including immunomodulatory [24,25], antioxidant [26], anti-inflammatory [27,28] and anti-bacterial effects [29]. In addition, some microalgae are also known to have prebiotic properties [30], thus modulating the gastrointestinal microbiota. Recently, De Medeiros et al. [31] investigated the prebiotic properties of some digested microalgae biomass, including Chlorella vulgaris and Arthrospira platensis, on human colonic microbiota during in vitro fermentation. Microalgae biomass showed a selective stimulation of beneficial microorganisms and inhibition of undesired bacteria on colonic microbiota. A previous study conducted by Jin et al. [32] reported that supplementation with Chlorella vulgaris and other microalgae increased propionate-producing bacteria in an in vitro human gut fermentation model. According to the previously cited studies, in order to evaluate the effects of microalgae on intestinal microbiota, it is appropriate to use an in vitro digestion procedure and subsequently undergo the digested biomass to colonic in vitro fermentation, with the aim to examine the potential microbiota modulation activities of microalgae [31,32].

It must be emphasized that very few studies have investigated the use of microphytes in dogs and they were mainly focused on anti-inflammatory and immunomodulating activities of whole microalgae or specific components. Cortese et al. [33] investigated the immune-modulating effect of a specific diet in canine Leishmaniosis. Dogs were given an anti-Leishmania drug therapy combined with a standard diet or with the specific immune-modulating diet containing some nutraceutical substances including Astaxanthin from Haematococcus pluvialis. The immune-modulating diet appeared to control the immune response in canine Leishmaniosis during the standard pharmacological therapy. In fact, the immune-modulating diet decreased T-helper 1 cells inflammatory response and increased regulatory T cells in diseased dogs. It must be underlined that the dosage of astaxanthin and other individual nutraceutical substances was not reported in the study. For that reason, it is not possible to discriminate their effect separately. More recently, Satyaraj et al. [25] evaluated the immunomodulatory effect of dietary supplementation with 0.2% spray-dried Arthrospira platensis in dogs. Dogs fed diets supplemented with Arthrospira showed enhanced immune status and higher levels of fecal IgA as compared to the control group. It has been shown that fecal concentration of IgA decreases in dogs with chronic enteropathies, and thus, fecal IgA level in dogs may be considered as an indicator of intestinal immune health [34,35]. Furthermore, this investigation demonstrated that supplementing diets with Spirulina also resulted in significantly increased gut microbiota stability [25].

However, to our knowledge, there are no studies investigating the effects of microphytes on canine gut microbiota and concentrations of bacterial metabolites such as SCFA, BCFA, ammonia and biogenic amines, which are known to be of fundamental relevance in host–microbial interactions [36].

The aim of the present study was to evaluate in an in vitro canine gut model the effects of four microalgae (Micoperi Blue Growth, Ravenna, Italy) Arthrospira platensis (AP), Haematococcus pluvialis (HP), Phaeodactylum tricornutum (PT) and Chlorella vulgaris (CV) on some fecal microbial populations and metabolites. We selected these four species because they currently represent the microalgae of greatest interest in the food and feed sector [8,13]. We hypothesized that composition and metabolism of canine fecal microbiota would be influenced by microalgae supplementation; particularly, we expected an increase concentration of the three major SCFA (acetate, propionate and butyrate), in accordance with previously in vitro cited studies [31,32], and higher concentration of microbial species known to be SCFA producers (i.e., Ruminococcaceae, Clostridium cluster XIV, Turicibacter spp., C. leptum, C. coccoides) [37]; in addition, we assumed that microbial species thought to have undesired influences on canine gut health would decrease. Lastly, we hypothesized a decrease of metabolites deriving from bacterial proteolysis (ammonia, BCFA, biogenic amines) due to the enhanced saccharolytic activities of bacterial populations. To the authors' knowledge, this is the first study considering the effects of microalgae on proteolytic microbial activities.

2. Materials and Methods

2.1. Experimental Set-Up

Preliminarily, the microalgae were subjected to in vitro digestion, in order to simulate the digestion processes that take place in the stomach and small intestine of dogs, according to the method proposed by Biagi et al. [38]. Briefly, the samples undergo two incubation phases, a first lasting 2 h and taking place in the presence of pepsin, gastric lipase and HCl (gastric phase) and a second 4 h one with phosphate-bicarbonate buffer, pancreatin and bile salts (intestinal phase). Proximate analysis of the four microalgae, their undigested fraction and the digestibility coefficients are reported in Table 1. In this experiment, the undigested fraction of each microalga was tested as a fermentation substrate.

Five healthy adult dogs (mixed breed; average body weight of 21 kg; age 3.6 years), privately owned, were fed the same commercial dry diet for adult dogs (Agras Delic Spa, Genova, Italy) for 28 d. The diet contained the following ingredients: corn, barley, dehydrated venison, potato protein, purified pork fat, dried beet pulp, sunflower oil,

brewer's yeast, dried chicory pulp, FOS, cod liver oil, dicalcium phosphate, potassium chloride, sodium chloride, herbs (dog rose, bearberry, blackcurrant, taraxacum and thistle) and Yucca schidigera. The proximate composition of the diet (on dry matter basis) was the following: crude protein (CP) 23.6%, ether extract (EE) 12.5%, crude ash (ash) 5.71%, starch 38.9% and crude fiber (CF) 2.08%.

Table 1. Proximate analysis of four microalgae and their undigested fraction, and digestibility coefficients of microalgae subjected to in vitro digestion. Values are reported as % on dry matter basis.

Item	Crude Protein	Crude Ash	Crude Fibre	Total Digestibility
Microalgae				
Arthrospira platensis	70.9	5.03	0.70	86.2
Haematococcus pluvialis	10.4	4.03	15.67	7.87
Phaeodactylum tricornutum	39.6	22.4	0.40	67.5
Chlorella vulgaris	31.1	9.97	11.8	55.3
Microalgae, undigested fraction				
Arthrospira platensis	55.7	4.04	13.7	
Haematococcus pluvialis	10.2	1.53	9.84	
Phaeodactylum tricornutum	18.6	27.0	0.56	
Chlorella vulgaris	18.0	10.7	16.8	

The same dry food that was fed to the dogs used as fecal donors was subjected to in vitro digestion using the same procedure used for the microalgae. After in vitro digestion, the recovery rate of the diet was 18.5% (on dry matter basis) and the chemical composition of the undigested residue (on dry matter basis) was the following: CP 17.3%, EE 2.43%, starch 3.87%, ash 14.6% and CF 9.94%.

After the 28-day feeding period, a sample of fresh feces was collected from each dog immediately after defecation and delivered to the laboratory. Within one hour from the excretion, the feces were pooled and suspended at 10 g/L in prereduced Wilkins Chalgren anaerobe broth. The fecal suspension was used to inoculate (100 mL/L) a previously warmed (39 °C) and prereduced medium prepared according to Sunvold et al. [39]. Five vessels (30 mL) were arranged per treatment.

Five treatments were arranged: (1) control diet with no addition of experimental substrates and control diet with (2) Arthrospira platensis, (3) Haematococcus pluvialis, (4) Phaeodactylum tricornutum, or (5) Chlorella vulgaris. All vessels contained the undigested residue of the commercial dry food for dogs at 10 g/L. The amount of microalgae that was added to the inocula is reported in Table 2. Amounts were calculated based on the different in vitro digestibility coefficients of microalgae (Table 1). The dose that was used should reflect the amount of microalgae that reach the hindgut when they are included in a commercial extruded food for dogs (with a digestibility of approximately 90%) at a concentration of 40 g/kg.

The pH of fecal cultures was adjusted to 6.7; bottles were sealed and incubated for 24 h at 39 °C under an 85% N_2, 10% CO_2 and 5% H_2 atmosphere. Samples of inocula were collected from each vessel at 6 and 24 h for the determination of pH, ammonia, biogenic amines, SCFA and for microbial analysis.

2.2. Chemical Analyses

The commercial dry food, the algae and their undigested residue were analyzed according to the AOAC International standard methods (method 950.46 for water, method 954.01 for CP, method 920.39 for EE, method 920.40 for starch, method 942.05 for ash and method 962.09 for CF) [40]. Ammonia was determined by an enzymatic colorimetric procedure (Urea/BUN—Color; BioSystems S.A., Barcelona, Spain) [41]. The SCFA and

BCFA were separated using 10% SP-1000 + 1% H_3PO_4, 100/120 mesh Chromosorb W AW in a 1.8-m by 2-mm (internal diameter) glass column, with nitrogen as the carrier [42]. The chromatograph was a Fisons HRGC MEGA 2 series 8560 with a flame ionization detector. The temperatures of the injector and detector were 200 °C, and the oven temperature was 155 °C. 2-ethylbutyric acid was used as the internal standard. For the determination of biogenic amines, samples were diluted 1:5 w/v with perchloric acid (0.3 M); biogenic amines were later separated by HPLC and quantified through fluorimetry [43].

Table 2. Amount of undigested fraction of the commercial dry food and microalgae that were added to each bottle. Each bottle contained 21 mL of fecal culture.

Treatment	Commercial Dry Food, Undigested Fraction (mg)	Algae, Undigested Fraction (mg)
Control (CTRL)	210	-
Arthrospira platensis (AP)	210	11.6
Haematococcus pluvialis (HP)	210	77.4
Phaeodactylum tricornutum (PT)	210	37.5
Chlorella vulgaris (CV)	210	27.3

2.3. Microbial Analysis

At each sampling time, a 1 mL portion of fermentation fluid was collected from each vessel and centrifuged at 4 °C for 5 min, at 18,000× *g*. The supernatant was removed and immediately frozen at −80 °C for further analysis. Bacterial genomic DNA was extracted from remaining pellet using the Stool DNA isolation kit (Norgen Biotek Co., Thorold, ON, Canada). Isolated DNA concentration (ng/µL) and purity were measured using a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Template DNA was diluted to 50 ng/µL and stored at −20 °C until further analysis. Turicibacter, Ruminococcaceae, Blautia, *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Clostridium* cluster XIV, *Clostridium coccoides*, *Clostridium leptum* and *Clostridium hiranonis* were quantified via quantitative polymerase chain reaction (qPCR) using specific primers. Detailed information of primers is presented in Table 3.

Reaction mixtures were prepared in 15-µL volumes containing 7.5 µL 2XSensiFAST No-ROX PCR MasterMix (Meridian Bioscience Inc., Cincinnati, OH, USA), 4.8 µL of nuclease-free water, 0.6 µL of each 10 pmol primer and 1.5 µL of sample DNA. The assay consisted of a 2-min denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 s, primer annealing at 56–66 °C for 10 s and 72 °C for 8 s. The cycle was repeated 40 times. A negative control (without the DNA template) was also run for each primer pair. The qPCR assay was performed using a CFX96 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification was performed in duplicate for each bacterial group within each sample, while standard curves were run in triplicate. Standard curves were constructed from eight tenfold dilutions for each target. Cycle threshold values were plotted against standard curves for the quantification of the target bacterial DNA from fecal inoculum. Melting curves were checked after amplification to ensure the single product amplification of a consistent melting temperature.

2.4. Statistical Analyses

Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons were performed for data with unequal variances, while normally distributed data were compared using a one-way ANOVA with Dunnett's multiple comparison test. Differences between groups were considered significant for $p < 0.05$. Each vial represented a single experimental unit. Significance and tendency for statistical tests were set at $p < 0.05$ and $0.05 < p < 0.1$, respectively. Statistical analyses were performed using Statistica 10.0 software (Stat Soft Italia, Padua, Italy).

Table 3. Primers used in the qPCR assay.

Target	Primer	Sequence (5′→3′)	Annealing Temperature (°C)	Reference
Blautia spp.	Blautia_F Blautia_R	TCTGATGTGAAAGGCTGGGGCTTA GGCTTAGCCACCCGACACCTA	62.0	[44]
Turicibacter spp.	Turicibacter_F Turicibacter_R	CAGACGGGGACAACGATTGGA TACGCATCGTCGCCTTGGTA	59.3	[44]
Ruminococcaceae	Ruminococcaceae_F Ruminococcaceae_R	ACTGAGAGGTTGAACGGCCA CCTTTACACCCAGTAAWTCCGGA	64.2	[45]
Bifidobacterium spp.	Bif_F Bif_R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	62.0	[46]
Lactobacillus spp.	Lac_F Lac_R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	64.2	[47]
Clostridium leptum	sg-Clept-F sg-Clept-R	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	59.3	[48]
Clostridium coccoides	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	64.2	[49]
Clostridium hiranonis	C.hiranonis_F C.hiranonis_R	AGTAAGCTCCTGATACTGTCT AGGGAAAGAGGAGATTAGTCC	65.4	[50]
Escherichia coli	Coli_F Coli_R	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	62.0	[51]
Enterococcus spp.	Ent_F Ent_R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	59.3	[46]
Clostridium cluster XIV	CloXIV-F CloXIV-R	GAWGAAGTATYTCGGTATGT CTACGCWCCCTTTACAC	57.2	[52]

3. Results

The chemical parameters evaluated on samples of fermentation fluid collected after 6 and 24 h of incubation are shown in Tables 4 and 5, respectively. After 6 h of incubation, pH was decreased by HP, PT and CV compared to CTRL (6.58, 6.63 and 6.56 vs. 6.71, respectively; $p < 0.05$). Conversely, after 24 h of incubation, pH values were not affected by treatments ($p > 0.05$). Moreover, the concentration of ammonia did not change after 6 and 24 h of incubation. Total concentrations of SCFA were not influenced by treatments after 6 and 24 h. On the contrary, total BCFA were decreased in flasks containing PT and CV (−46% for both; $p < 0.05$) at 6 h; however, this effect was no longer present after 24 h. At 6 h, flasks with PT contained higher concentration of propionate (+36%; $p < 0.05$) and butyrate (+24%; $p < 0.05$). Additionally, propionate proportions were higher in all the flasks treated with microalgae, both at 6h (+2.6% for AP; +2.8% for HP; +6.2% for PT; +3.5% for CV; $p < 0.05$) and 24 h (+1.1% for AP; +1.4% for HP; +3.9% for PT; +1.9% for CV; $p < 0.05$). After 6 h of incubation, isobutyrate was reduced by PT (−52%; $p < 0.05$) and isovalerate was decreased by all treatments except HP (−43% for AP, PT, CV; $p < 0.05$). At 24 h, propionate was still higher in vessels containing PT (+21%; $p < 0.05$), while BCFA were not affected by microalgae with the exception of isovalerate concentration that was higher in PT (+10%; $p < 0.05$). In addition, no significant effects were observed in regard to biogenic amines both at 6 and 24 h, as reported in Table 6.

The data relating to the composition of the fecal microbiota evaluated at 6 and 24 h of incubation are presented in Figures 1 and 2, respectively. After 6 h, treatments containing PT decreased the abundance of *C. hiranonis* (6.88 vs. 7.34 $\log_{10}$ copies/75 ng DNA; $p < 0.05$). Microphyte treatments decreased the DNA concentration of some bacterial populations after 24 h. In particular, *Turicibacter* spp. was reduced by HP and PT (6.69 and 6.56 vs. 7.18 $\log_{10}$ copies/75 ng DNA, respectively; $p < 0.05$). Finally, *C. coccoides*

(8.26 vs. 9.38 $\log_{10}$ copies/75 ng DNA; $p < 0.05$) and *Enterococcus* spp. (6.99 vs. 7.36 $\log_{10}$ copies/75 ng DNA; $p < 0.05$) were less abundant in flasks containing CV.

Table 4. pH values, ammonia and short-chain fatty acids concentrations after 6 h of an in vitro incubation of canine fecal inoculum supplemented with microalgae [1].

Item	CTRL	AP	HP	PT	CV	Pooled SEM	Anova *p*-Value
pH	6.71	6.63	6.58 *	6.63 *	6.56 *	0.03	0.005
Ammonia, mmol/L	30.2	32.2	31.4	29.6	31.9	1.62	0.586
Straight-chain SCFA, mmol/L							
Acetate	8.62	8.66	8.97	8.85	8.57	0.42	0.954
Propionate	4.54	4.92	5.13	6.19 *	5.14	0.23	0.001
Butyrate	2.55	2.58	2.62	3.16 *	2.69	0.12	0.013
Total SCFA	15.7	16.2	16.7	18.2	16.4	0.78	0.232
BCFA, mmol/L							
Isobutyrate	0.27	0.15	0.15	0.13 *	0.13	0.03	0.022
Isovalerate	0.46	0.26 *	0.30	0.26 *	0.26 *	0.03	0.009
Total BCFA	0.73	0.41	0.45	0.39 *	0.39 *	0.08	0.006
Individual SCFA proportions, %							
Acetate	51.5	52.2	52.1	47.6 *	51.0	0.44	<0.001
Propionate	27.1	29.7 *	29.9 *	33.3 *	30.6 *	0.23	<0.001
Butyrate	15.2	15.5	15.2	17.0 *	16.0 *	0.15	<0.001
Isobutyrate	1.54	0.88	0.90	0.71 *	0.80 *	0.13	0.001
Isovalerate	2.70	1.59	1.74	1.42 *	1.56 *	0.13	<0.001

[1] Values are the means of five bottles per treatment. * Significantly different from CTRL, $p < 0.05$. CTRL, control; AP, *Arthrospira platensis*; HP, *Haematococcus pluvialis*; PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris*; SCFA, short-chain fatty acid; BCFA, branched-chain fatty acid.

Table 5. pH values, ammonia and short-chain fatty acids concentrations after 24 h of an in vitro incubation of canine fecal inoculum supplemented with microalgae [1].

Item	CTRL	AP	HP	PT	CV	Pooled SEM	Anova *p*-Value
pH	5.84	5.84	5.81	5.95	5.81	0.01	0.004
Ammonia, mmol/L	39.6	39.9	36.0	38.0	35.7	1.29	0.065
Straight-chain SCFA, mmol/L							
Acetate	16.7	16.9	16.6	16.5	16.5	0.48	0.960
Propionate	9.68	10.5	10.3	11.7 *	10.7	0.28	0.001
Butyrate	5.43	5.73	5.31	5.65	5.61	0.14	0.271
Total SCFA	31.8	33.1	32.2	33.8	32.8	0.89	0.536
BCFA, mmol/L							
Isobutyrate	0.60	0.64	0.60	0.64	0.62	0.02	0.289
Isovalerate	0.92	0.95	0.90	1.01 *	0.94	0.02	0.041
Total BCFA	1.52	1.59	1.50	1.65	1.56	0.04	0.086
Individual SCFA proportions, %							
Acetate	48.7	47.3 *	47.9 *	45.2 *	46.7 *	0.20	<0.001
Propionate	28.3	29.4 *	29.7 *	32.2 *	30.2 *	0.09	<0.001
Butyrate	15.9	16.0	15.4 *	15.6	15.9	0.10	0.001
Isobutyrate	1.76	1.80	1.74	1.76	1.75	0.01	0.041
Isovalerate	2.68	2.66	2.59	2.78	2.65	0.05	0.254

[1] Values are the means of five bottles per treatment. * Significantly different from CTRL, $p < 0.05$. CTRL, control; AP, *Arthrospira platensis*; HP, *Haematococcus pluvialis*; PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris*; SCFA, short-chain fatty acid; BCFA, branched-chain fatty acid.

Table 6. Biogenic amines concentrations (nmol/mL) 6 h and 24 h of an in vitro incubation of canine fecal inoculum with a control diet supplemented with microalgae [1].

Item	CTRL	AP	HP	PT	CV	Pooled SEM	Anova *p*-Value
6 h							
Putrescine	177.4	186.6	175.6	169.2	179.0	4.87	0.241
Cadaverine	101.0	124.6	132.4	87.4	96.4	15.1	0.371
Spermidine	24.4	68.8	36.4	21.6	23.6	9.55	0.043
Spermine	3.80	3.70	5.02	0.98	1.28	6.85	0.041
24 h							
Putrescine	166.4	174.0	111.4	140.4	107.8	10.2	0.007
Cadaverine	129.8	154.4	72.4	97.4	113.8	25.6	0.223
Spermidine	22.0	21.8	18.2	22.6	24.6	3.00	0.669
Spermine	1.32	1.16	1.04	0.58	2.12	0.53	0.414

[1] Values are the means of five bottles per treatment. CTRL, control; AP, *Arthrospira platensis*; HP, *Haematococcus pluvialis*; PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris*.

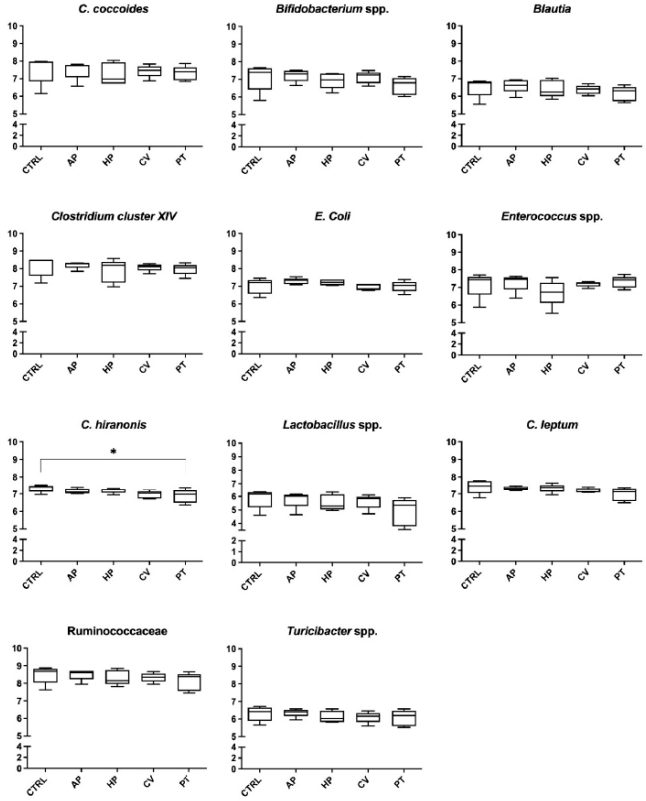

Figure 1. Microbial analysis (log_{10} copies DNA/75 ng DNA) after 6 h of an in vitro incubation of canine fecal inoculum with a control diet supplemented with microalgae. Values are the means of five bottles per treatment. CTRL, control; AP, *Arthrospira platensis*; HP, *Haematococcus pluvialis*; CV, *Chlorella vulgaris*; PT, *Phaeodactylum tricornutum*. * $p < 0.05$.

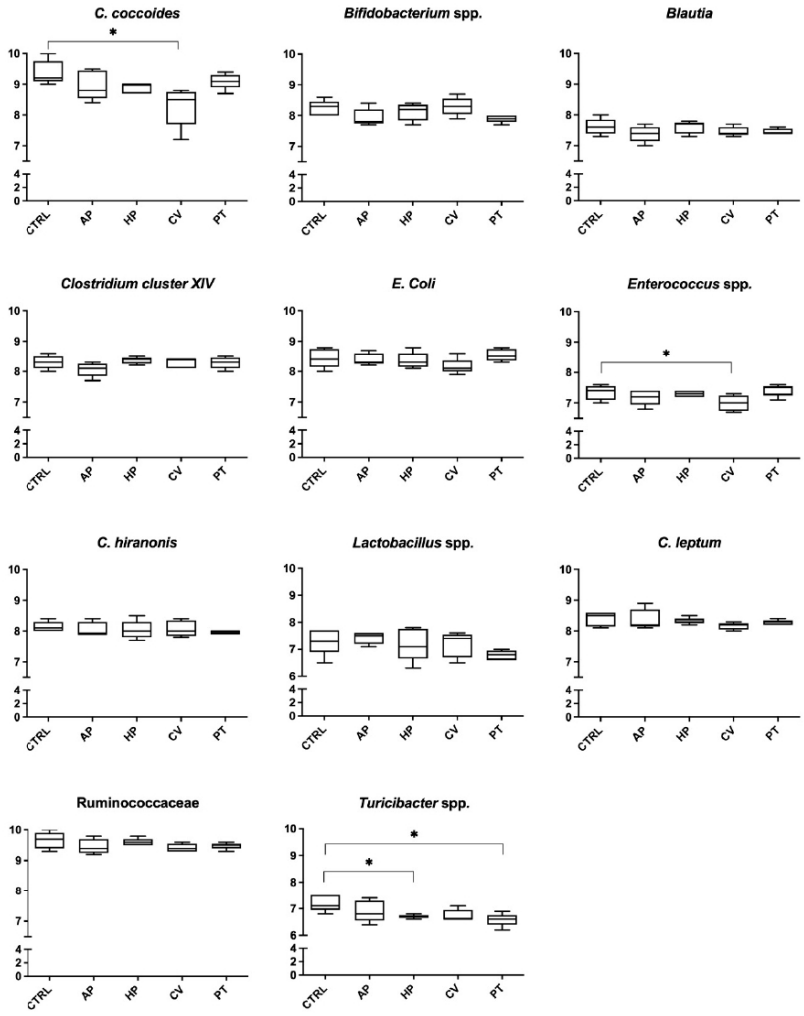

Figure 2. Microbial analysis ($\log_{10}$ copies DNA/75 ng DNA) after 24 h of an in vitro incubation of canine fecal inoculum with a control diet supplemented with microalgae. Values are the means of five bottles per treatment. CTRL, control; AP, *Arthrospira platensis*; HP, *Haematococcus pluvialis*; CV, *Chlorella vulgaris*; PT, *Phaeodactylum tricornutum*. * $p < 0.05$.

4. Discussion

The purpose of this investigation was to assess the in vitro effects of four microalgae on some canine fecal microbial populations and metabolites.

In this study, supplementation with microalgae partially affected the gut ecology. In particular, pH was decreased after 6 h in three out of four microalgae groups (HP, CV, PT). Changes in pH can affect microbial communities, thus impacting the concentration and

profile of fermentation products [53]. Particularly, the reduction of intestinal pH could be a desirable effect, as the acidification of the environment has a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria. At the same time, it is known how the colonic pH is influenced by fermentation processes of bacterial populations, in particular in the proximal colon, where the pH is lower due to the production of SCFA that mainly derive from the fermentation of carbohydrates [54,55]. However, in the present investigation, total concentration of SCFA was not affected by the treatments.

After 6 h of incubation, the concentration of propionate and butyrate was increased by PT. A previous study conducted in an in vitro human gut model demonstrated that supplementation with digested microphytes (2.5 g/dL) could affect both intestinal microbiota composition and metabolites [32]. In that study, Jin et al. [32] investigated the effects of three edible microalgae (*Chlorella vulgaris*, *Chlorella protothecoides* and *Schizochytrium* sp.) on human gut microbiota, showing that microalgae supplementation increased the proportion of propionate in the colonic culture together with the relative abundance of some bacterial populations involved in propionate metabolism (genera *Bacteroides* spp. and *Dialister* spp.). Moreover, total SCFA were significantly increased by *C. vulgaris*. Similar effects, although to a minor extent, were observed in the present study regarding the higher concentration and proportions of propionate in PT group, both after 6 and 24 h. Intestinal SCFA are linked with some health-promoting effects, such as anti-inflammatory, immune-regulatory and anticarcinogenic functions [56]. Specifically, propionate is metabolized in the liver and plays a role in reducing the concentration of blood sugar and serum cholesterol, while butyrate is an important source of energy for colonocytes [57]. In addition, butyrate is known to be responsible for functions associated with the gut microbiota and physiology, maintaining cell growth and differentiation. It also plays a role in the prevention of colonic cancer and in the reduction of inflammation [58].

Nevertheless, in the present trial higher concentration of some SCFA did not reflect a change in microbial populations known as SCFA producers. Moreover, after 24 h of fermentation, lower abundance of *Enterococcus* spp. and *C. coccoides* (in CV vessels) and *Turicibacter* spp. (in PT and HP vessels) were detected in three of the groups to which microalgae were added. These last outcomes are in contrast with the results recently obtained by Wan et al. [59], who studied the effect of a bioactive polysaccharide from microalga *Chlorella pyrenoidosa* (CPP) at a dosage of 150 and 300 mg/kg, on gut microbiota of mice fed a high-fat diet. The authors pointed out that the growth of some bacterial genera, including *Turicibacter*, and the concentrations of acetate, propionate and butyrate were drastically increased in both CPP treatments. *Turicibacter* spp., belonging to the Firmicutes phylum, is considered an important producer of SCFA [60], suggesting an important role of *Turicibacter* spp. in promoting gut health.

In relation to the decrease of *C. coccoides* in the CV group observed in the present study after 24 h, existing outcomes regarding the role of this bacterial species in host physiology appear to be controversial. *C. coccoides* is a member of the Firmicutes phylum, one of the most preponderant bacterial populations in the human intestine, and many species in this group, such as *Eubacterium* spp., *Roseburia* spp., *Subdoligranulum variabile* and *Faecalibacterium prausnitzii*, directly produce butyrate from dietary polysaccharides and other substrates [61]; moreover, changes in relative abundance of the Firmicutes are also associated with an increased ability to harvest energy from diet [62]. In the literature, the decrease in abundance of the *E. rectale-C. coccoides* group (Clostridium cluster XIVa and XIVb) has also been correlated with the presence of phenolic compounds with inhibitory effects on the growth of potentially adverse bacteria [63]. Phenolic compounds are a wide group of secondary metabolites encompassing phenolic acids, flavonoids, isoflavonoids, stilbenes, lignans and phenolic polymers. These molecules show a broad spectrum of biological activities comprising antioxidant activities as well as anti-inflammatory, anti-cancer, anti-allergic, anti-diabetes, anti-aging and antimicrobial properties [64]. High levels of phenolic compounds with antioxidant activity are present in microalgae, and like other active biological compounds, both the composition and content of polyphenol

from microalgae are species-dependent [11]. According to Goiris et al. [65], *Phaeodactylum tricornutum* is the microalga that contains the greatest number of polyphenols [375 mg Gallic Acid Equivalent (GAE)/100 g DW, against the 54 mg (GAE)/100 g DW from *Haematococcus pluvialis*], while *Chlorella vulgaris* exhibits 221 mg GAE/100 g DW. Microalgae polyphenols content could be linked with the lower abundances of *C. coccoides* in CV group after 24 h of fermentation observed in the present study. However, in the present investigation, the phenolic compounds content of the four microalgae was not evaluated, and for that reason, it is not possible to discuss its role in modulating microbial population.

The decrease in *E. rectale-C. coccoides* group relative abundances were also observed by de Medeiros et al. [31] during their in vitro study with a human colonic model. The 48 h fermentation was performed in batches composed of 40% of a fermentation medium, 40% of pooled human fecal inoculum and 20% of a digested microalgae biomass, including *Chlorella vulgaris* and *Arthrospira Platensis*. In the same experiment, digested microalgae biomass increased the *Lactobacillus-Enterococcus* and *Bifidobacterium* spp. relative abundance. On the contrary, in the present investigation, microbial analysis showed that, at 24 h, CV treatment decreased enterococci, considered as members of the healthy intestinal microbiota and potentially probiotic. Enterococci have different features such as auto- and co-aggregation ability, tolerance to low pH and bile salts, adherence to hydrocarbons, sensibility to some antibiotics, as well as exo-polysaccharide synthesis [66].

After 6 h of incubation, PT resulted in decreased abundance of *C. hiranonis*. *C. hiranonis* is a bacterial species of interest, as it shows bile acid 7 alpha-dehydroxylating activity, and a decrease in *C. hiranonis* may suggest bile acid dysmetabolism [50]. These findings are apparently in contrast with previously mentioned studies [31,32], in which microphytes seemed to enhance intestinal health by stimulating the growth of beneficial bacterial population, such as SCFA-producing bacteria. Certainly, it must be underlined that, in the present study, only a few of the main populations of canine microbiota have been evaluated by quantitative PCR. This fact represents a limitation, as we cannot exclude that any changes regarding other bacteria could not have been detected.

One of the main reasons for considering microalgae as an interesting source of food is their high protein content (e.g., 55–70% for *S. platensis* and 42–55% for *C. vulgaris* on a dry matter basis [67]). In this study, microalgae were preliminarily subjected to in vitro digestion and the undigested fraction was used as the fermentation substrate. Proteins were highly represented in the undigested fraction of AP (55.7%), CV (18.0%) and PT (18.6%). Interestingly, the presence of microalgae, despite the increased presence of protein, decreased BCFA after 6 h and did not result in higher concentrations of ammonia and biogenic amines, metabolites deriving from bacterial proteolysis [36,68]. In particular, PT seemed to have the greatest effect on BCFA by decreasing both the concentration of isobutyrate and isovalerate. BCFA are synthetized in the colon from bacterial metabolism of branched-chain amino acids valine, leucine and isoleucine [69]. According to Safi et al. [70], HP resulted higher in branched-chain amino acids when compared to AP. Therefore, despite the higher amount of crude protein in AP vessels, we speculate that differences in isovalerate concentrations could be associated to the different amino acids' composition of the substrates. The biological significance of BCFA and biogenic amines is still poorly understood. It has been hypothesized that BCFA may have a role in the regulation of ionic exchanges in colonic mucosa [71] and that isobutyrate may act as a potential source of energy for colonocytes after exhaustion of butyrate [72]. Similarly, biogenic amines seem to have a beneficial influence on the intestinal mucosa [73]; however, on the other hand, they could act as precursors in the formation of nitrosamines, known as carcinogens in humans [74]. The decrease of BCFA that we observed could indicate a reduction of proteolytic activities operated by some bacterial populations. However, other parameters, including concentration of ammonia and biogenic amines, did not reflect this trend. In this regard, the effects of microalgae supplementation on metabolites deriving from bacterial proteolysis are still poorly investigated, and hence, it could represent an interesting aspect to be explored.

5. Conclusions

During the present in vitro study, microalgae partially affected canine fecal microbiota. Among the four microphytes, PT showed the greatest effect on microbial metabolites after 6 h of incubation by increasing propionate, butyrate and decreasing BCFA. These outcomes suggest that microalgae, especially PT, could have a potential modulatory effect on the metabolic activities of canine intestinal microorganisms. However, PT led to a reduction of *C. hiranonis* at 6 h, while after 24 h, HP, CV and PT resulted in a decrease of some beneficial bacterial populations belonging to Firmicutes, known to be butyrate-producing bacteria.

The present study has led to unexpected results, as we would have presumed a more pronounced microbial saccharolytic activities and a greater shift in fecal bacterial composition. Therefore, recommendation of microalgae as food supplements required more research on the effects of their dietary inclusion. Thus, our work should be considered as a preliminary study for future investigations.

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Institutional Review Board Statement: Since the dogs were fed with the same commercial extruded dog food with the aim to standardize their diet for 4 weeks before fecal samples collection and no dog's habits had been modified, approval by a research ethics committee was not required for this study. All samples were provided by private dog owners who consented to our use for research purposes.

Informed Consent Statement: Written informed consent authorizing the participation of dogs in this study was obtained from all dog owners.

Data Availability Statement: All data are available in the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tuddenham, S.; Sears, C.L. The intestinal microbiome and health. *Curr. Opin. Infect. Dis.* **2015**, *28*, 464–470. [CrossRef] [PubMed]
2. Suchodolski, J.S. Analysis of the gut microbiome in dogs and cats. *Vet. Clin. Pathol.* **2021**, *50*, 6–17. [CrossRef] [PubMed]
3. Mondo, E.; Marliani, G.; Accorsi, P.A.; Cocchi, M.; Di Leone, A. Role of gut microbiota in dog and cat's health and diseases. *Open Vet. J.* **2019**, *9*, 253–258. [CrossRef] [PubMed]
4. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut microbiota functions: Metabolism of nutrients and other food components. *Eur. J. Nutr.* **2018**, *57*, 1–24. [CrossRef]
5. Blaak, E.E.; Canfora, E.E.; Theis, S.; Frost, G.; Groen, A.K.; Mithieux, G.; Nauta, A.; Scott, K.; Stahl, B.; van Harsselaar, J.; et al. Short chain fatty acids in human gut and metabolic health. *Benef. Microbes* **2020**, *11*, 411–455. [CrossRef]
6. Ercolini, D.; Fogliano, V. Food Design to Feed the Human Gut Microbiota. *J. Agric. Food Chem.* **2018**, *66*, 3754–3758. [CrossRef]
7. Huang, Z.; Pan, Z.; Yang, R.; Bi, Y.; Xiong, X. The canine gastrointestinal microbiota: Early studies and research frontiers. *Gut Microbes* **2020**, *11*, 635–654. [CrossRef]
8. Camacho, F.; Macedo, A.; Malcata, F. Potential industrial applications and commercialization of microalgae in the functional food and feed industries: A short review. *Mar. Drugs* **2019**, *17*, 312. [CrossRef]
9. Kay, R.A.; Barton, L.L. Microalgae as Food and Supplement. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 555–573. [CrossRef]
10. Abu-Ghosh, S.; Dubinsky, Z.; Verdelho, V.; Iluz, D. Unconventional high-value products from microalgae: A review. *Bioresour. Technol.* **2021**, *329*, 124895. [CrossRef]
11. Levasseur, W.; Perré, P.; Pozzobon, V. A review of high value-added molecules production by microalgae in light of the classification. *Biotechnol. Adv.* **2020**, *41*, 107545. [CrossRef]
12. GRAS Notices. Available online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices (accessed on 27 June 2022).

13. Joint Research Centre, Institute for Prospective Technological Studies; Barbosa, M.; Enzing, C.; Ploeg, M.; Sijtsma, L. *Microalgae-Based Products for the Food and Feed Sector: An Outlook for Europe*; Vigani, M., Parisi, C., Rodríguez Cerezo, E., Eds.; Publications Office: Luxembourg, 2016; pp. 19–37.
14. Commission European Commission Regulation (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials. *Off. J. Eur. Union* **2017**, *159*, 48–119.
15. Da Silva Vaz, B.; Moreira, J.B.; de Morais, M.G.; Costa, J.A.V. Microalgae as a new source of bioactive compounds in food supplements. *Curr. Opin. Food Sci.* **2016**, *7*, 73–77. [CrossRef]
16. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278. [CrossRef]
17. Guerin, M.; Huntley, M.E.; Olaizola, M. Haematococcus astaxanthin: Applications for human health and nutrition. *Trends Biotechnol.* **2003**, *21*, 210–216. [CrossRef]
18. Silva Benavides, A.M.; Torzillo, G.; Kopecký, J.; Masojídek, J. Productivity and biochemical composition of Phaeodactylum tricornutum (Bacillariophyceae) cultures grown outdoors in tubular photobioreactors and open ponds. *Biomass Bioenergy* **2013**, *54*, 115–122. [CrossRef]
19. Yaakob, Z.; Ali, E.; Zainal, A.; Mohamad, M.; Takriff, M.S. An overview: Biomolecules from microalgae for animal feed and aquaculture. *J. Biol. Res.* **2014**, *21*, 1–10. [CrossRef]
20. De Medeiros, V.P.B.; Pimentel, T.C.; Sant'Ana, A.S.; Magnani, M. Microalgae in the meat processing chain: Feed for animal production or source of techno-functional ingredients. *Curr. Opin. Food Sci.* **2021**, *37*, 125–134. [CrossRef]
21. Kang, H.K.; Salim, H.M.; Akter, N.; Kim, D.W.; Kim, J.H.; Bang, H.T.; Kim, M.J.; Na, J.C.; Hwangbo, J.; Choi, H.C.; et al. Effect of various forms of dietary Chlorella supplementation on growth performance, immune characteristics, and intestinal microflora population of broiler chickens. *J. Appl. Poult. Res.* **2013**, *22*, 100–108. [CrossRef]
22. Charoonnart, P.; Purton, S.; Saksmerprome, V. Applications of Microalgal Biotechnology for Disease Control in Aquaculture. *Biology* **2018**, *7*, 24. [CrossRef]
23. Sagaram, U.S.; Gaikwad, M.S.; Nandru, R.; Dasgupta, S. Microalgae as feed ingredients: Recent developments on their role in immunomodulation and gut microbiota of aquaculture species. *FEMS Microbiol. Lett.* **2021**, *368*, fnab071. [CrossRef] [PubMed]
24. Manzo, E.; Cutignano, A.; Pagano, D.; Gallo, C.; Barra, G.; Nuzzo, G.; Sansone, C.; Ianora, A.; Urbanek, K.; Fenoglio, D.; et al. A new marine-derived sulfoglycolipid triggers dendritic cell activation and immune adjuvant response. *Sci. Rep.* **2017**, *7*, 6286. [CrossRef] [PubMed]
25. Satyaraj, E.; Reynolds, A.; Engler, R.; Labuda, J.; Sun, P. Supplementation of Diets With Spirulina Influences Immune and Gut Function in Dogs. *Front. Nutr.* **2021**, *8*, 667072. [CrossRef] [PubMed]
26. Guzmán-Gómez, O.; García-Rodríguez, R.V.; Quevedo-Corona, L.; Pérez-Pastén-Borja, R.; Rivero-Ramírez, N.L.; Ríos-Castro, E.; Pérez-Gutiérrez, S.; Pérez-Ramos, J.; Chamorro-Cevallos, G.A. Amelioration of ethanol-induced gastric ulcers in rats pretreated with phycobiliproteins of Arthrospira (Spirulina) maxima. *Nutrients* **2018**, *10*, 763. [CrossRef]
27. Montero-Lobato, Z.; Vázquez, M.; Navarro, F.; Fuentes, J.L.; Bermejo, E.; Garbayo, I.; Vílchez, C.; Cuaresma, M. Chemically-induced production of anti-inflammatory molecules in microalgae. *Mar. Drugs* **2018**, *16*, 478. [CrossRef]
28. Rodríguez-Luna, A.; Ávila-Román, J.; González-Rodríguez, M.L.; Cózar, M.J.; Rabasco, A.M.; Motilva, V.; Talero, E. Fucoxanthin-containing cream prevents epidermal hyperplasia and UVB-induced skin erythema in mice. *Mar. Drugs* **2018**, *16*, 378. [CrossRef]
29. Martínez, K.A.; Lauritano, C.; Druka, D.; Romano, G.; Grohmann, T.; Jaspars, M.; Martín, J.; Díaz, C.; Cautain, B.; de la Cruz, M.; et al. Amphidinol 22, a New Cytotoxic and Antifungal Amphidinol from the Dinoflagellate Amphidinium carterae. *Mar. Drugs* **2019**, *17*, 385. [CrossRef]
30. Patel, A.K.; Singhania, R.R.; Awasthi, M.K.; Varjani, S.; Bhatia, S.K.; Tsai, M.-L.; Hsieh, S.-L.; Chen, C.-W.; Dong, C.-D. Emerging prospects of macro- and microalgae as prebiotic. *Microb. Cell Factories* **2021**, *20*, 112. [CrossRef]
31. De Medeiros, V.P.B.; de Souza, E.L.; de Albuquerque, T.M.R.; da Costa Sassi, C.F.; dos Santos Lima, M.; Sivieri, K.; Pimentel, T.C.; Magnani, M. Freshwater microalgae biomasses exert a prebiotic effect on human colonic microbiota. *Algal Res.* **2021**, *60*, 102547. [CrossRef]
32. Jin, J.B.; Cha, J.W.; Shin, I.S.; Jeon, J.Y.; Cha, K.H.; Pan, C.H. Supplementation with *Chlorella vulgaris*, *Chlorella protothecoides*, and *Schizochytrium* sp. increases propionate-producing bacteria in in vitro human gut fermentation. *J. Sci. Food Agric.* **2020**, *100*, 2938–2945. [CrossRef]
33. Cortese, L.; Annunziatella, M.; Palatucci, A.T.; Lanzilli, S.; Rubino, V.; Di Cerbo, A.; Centenaro, S.; Guidetti, G.; Canello, S.; Terrazzano, G. An immune-modulating diet increases the regulatory T cells and reduces T helper 1 inflammatory response in Leishmaniosis affected dogs treated with standard therapy. *BMC Vet. Res.* **2015**, *11*, 295. [CrossRef] [PubMed]
34. Maeda, S.; Ohno, K.; Uchida, K.; Nakashima, K.; Fukushima, K.; Tsukamoto, A.; Nakajima, M.; Fujino, Y.; Tsujimoto, H. Decreased Immunoglobulin A Concentrations in Feces, Duodenum, and Peripheral Blood Mononuclear Cells of Dogs with Inflammatory Bowel Disease. *J. Vet. Intern. Med.* **2013**, *27*, 47–55. [CrossRef] [PubMed]
35. Littler, R.M.; Batt, R.M.; Lloyd, D.H. Total and relative deficiency of gut mucosal IgA in German shepherd dogs demonstrated by faecal analysis. *Vet. Rec.* **2006**, *158*, 334–341. [CrossRef] [PubMed]
36. McCarville, J.L.; Chen, G.Y.; Cuevas, V.D.; Troha, K.; Ayres, J.S. Microbiota Metabolites in Health and Disease. *Annu. Rev. Immunol.* **2020**, *38*, 147–170. [CrossRef] [PubMed]

37. Minamoto, Y.; Minamoto, T.; Isaiah, A.; Sattasathuchana, P.; Buono, A.; Rangachari, V.R.; McNeely, I.H.; Lidbury, J.; Steiner, J.M.; Suchodolski, J.S. Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. *J. Vet. Intern. Med.* **2019**, *33*, 1608–1618. [CrossRef]
38. Biagi, G.; Cipollini, I.; Grandi, M.; Pinna, C.; Vecchiato, C.G.; Zaghini, G. A new in vitro method to evaluate digestibility of commercial diets for dogs. *Ital. J. Anim. Sci.* **2016**, *15*, 617–625. [CrossRef]
39. Sunvold, G.D.; Hussein, H.S.; Fahey, G.C.; Merchen, N.R.; Reinhart, G.A. In vitro fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *J. Anim. Sci.* **1995**, *73*, 3639–3648. [CrossRef]
40. AOAC (Association of Official Analytical Chemists). *Official Methods of Analysis*, 17th ed.; AOAC: Washington, DC, USA, 2000.
41. Pinna, C.; Vecchiato, C.G.; Bolduan, C.; Grandi, M.; Stefanelli, C.; Windisch, W.; Zaghini, G.; Biagi, G. Influence of dietary protein and fructooligosaccharides on fecal fermentative end-products, fecal bacterial populations and apparent total tract digestibility in dogs. *BMC Vet. Res.* **2018**, *14*, 106. [CrossRef]
42. Pinna, C.; Giuditta Vecchiato, C.; Grandi, M.; Maria Eugenia Mammi, L.; Stefanelli, C.; Biagi, G. In vitro evaluation of the effects of tylosin on the composition and metabolism of canine fecal microbiota. *Animals* **2020**, *10*, 98. [CrossRef]
43. Stefanelli, C.; Carati, D.; Rossoni, C. Separation of N1- and N8-acetylspermidine isomers by reversed-phase column liquid chromatography after derivatization with dansyl chloride. *J. Chromatogr. B Biomed. Sci. Appl.* **1986**, *375*, 49–55. [CrossRef]
44. Suchodolski, J.S.; Markel, M.E.; Garcia-Mazcorro, J.F.; Unterer, S.; Heilmann, R.M.; Dowd, S.E.; Kachroo, P.; Ivanov, I.; Minamoto, Y.; Dillman, E.M.; et al. The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease. *PLoS ONE* **2012**, *7*, e51907. [CrossRef] [PubMed]
45. Garcia-Mazcorro, J.F.; Suchodolski, J.S.; Jones, K.R.; Clark-Price, S.C.; Dowd, S.E.; Minamoto, Y.; Markel, M.; Steiner, J.M.; Dossin, O. Effect of the proton pump inhibitor omeprazole on the gastrointestinal bacterial microbiota of healthy dogs. *FEMS Microbiol. Ecol.* **2012**, *80*, 624–636. [CrossRef]
46. Rinttilä, T.; Kassinen, A.; Malinen, E.; Krogius, L.; Palva, A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J. Appl. Microbiol.* **2004**, *97*, 1166–1177. [CrossRef]
47. Malinen, E.; Rinttilä, T.; Kajander, K.; Mättö, J.; Kassinen, A.; Krogius, L.; Saarela, M.; Korpela, R.; Palva, A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am. J. Gastroenterol.* **2005**, *100*, 373–382. [CrossRef]
48. Matsuki, T.; Watanabe, K.; Fujimoto, J.; Takada, T.; Tanaka, R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl. Environ. Microbiol.* **2004**, *70*, 7220–7228. [CrossRef]
49. Matsuki, T.; Watanabe, K.; Fujimoto, J.; Miyamoto, Y.; Takada, T.; Matsumoto, K.; Oyaizu, H.; Tanaka, R. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl. Environ. Microbiol.* **2002**, *68*, 5445–5451. [CrossRef]
50. Alshawaqfeh, M.K.; Wajid, B.; Minamoto, Y.; Markel, M.; Lidbury, J.A.; Steiner, J.M.; Serpedin, E.; Suchodolski, J.S. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. *FEMS Microbiol. Ecol.* **2017**, *93*, 136. [CrossRef]
51. Malinen, E.; Kassinen, A.; Rinttilä, T.; Palva, A. Comparison of real-time PCR with SYBR Green I or 5′-nuclease assaysand dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantificationof selected faecal bacteria. *Microbiology* **2003**, *149*, 269–277. [CrossRef]
52. Song, Y.; Liu, C.; Finegold, S.M. Real-time PCR quantitation of clostridia in feces of autistic children. *Appl. Environ. Microbiol.* **2004**, *70*, 6459–6465. [CrossRef]
53. Ilhan, Z.E.; Marcus, A.K.; Kang, D.-W.; Rittmann, B.E.; Krajmalnik-Brown, R. pH-Mediated Microbial and Metabolic Interactions in Fecal Enrichment Cultures. *mSphere* **2017**, *2*, e00047-17. [CrossRef]
54. Hamer, H.M.; de Preter, V.; Windey, K.; Verbeke, K. Functional analysis of colonic bacterial metabolism: Relevant to health? *Am. J. Physiol. Gastrointest. Liver Physiol.* **2012**, *302*, G1–G9. [CrossRef] [PubMed]
55. Zhao, R.; Cheng, N.; Nakata, P.A.; Zhao, L.; Hu, Q. Consumption of polysaccharides from Auricularia auricular modulates the intestinal microbiota in mice. *Food Res. Int.* **2019**, *123*, 383–392. [CrossRef]
56. O'Keefe, S.J.D. Diet, microorganisms and their metabolites, and colon cancer. *Nat. Rev. Gastroenterol. Hepatol.* **2016**, *13*, 691–706. [CrossRef] [PubMed]
57. Guarner, F.; Malagelada, J.R. Gut flora in health and disease. *Lancet* **2003**, *361*, 512–519. [CrossRef]
58. McNabney, S.M.; Henagan, T.M. Short chain fatty acids in the colon and peripheral tissues: A focus on butyrate, colon cancer, obesity and insulin resistance. *Nutrients* **2017**, *9*, 1348. [CrossRef]
59. Wan, X.; Ai, C.; Chen, Y.; Gao, X.; Zhong, R.; Liu, B.; Chen, X.; Zhao, C. Physicochemical Characterization of a Polysaccharide from Green Microalga Chlorella pyrenoidosa and Its Hypolipidemic Activity via Gut Microbiota Regulation in Rats. *J. Agric. Food Chem.* **2019**, *68*, 1186–1197. [CrossRef]
60. Sivaprakasam, S.; Prasad, P.D.; Singh, N. Benefits of short-chain fatty acids and their receptors in inflammation and carcinogenesis. *Pharmacol. Ther.* **2016**, *164*, 144–151. [CrossRef]
61. Jamar, G.; Santamarina, A.B.; Dias, G.C.; Masquio, D.C.L.; de Rosso, V.V.; Pisani, L.P. Relationship between fatty acids intake and Clostridium coccoides in obese individuals with metabolic syndrome. *Food Res. Int.* **2018**, *113*, 86–92. [CrossRef]

62. Turnbaugh, P.J.; Ley, R.E.; Mahowald, M.A.; Magrini, V.; Mardis, E.R.; Gordon, J.I. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **2006**, *444*, 1027–1031. [CrossRef]
63. Menezes, F.N.D.D.; da Cruz Almeida, É.T.; da Silva Vieira, A.R.; de Souza Aquino, J.; dos Santos Lima, M.; Magnani, M.; de Souza, E.L. Impact of Cashew (*Anacardium occidentale* L.) by-Product on Composition and Metabolic Activity of Human Colonic Microbiota In Vitro Indicates Prebiotic Properties. *Curr. Microbiol.* **2021**, *78*, 2264–2274. [CrossRef]
64. Galasso, C.; Gentile, A.; Orefice, I.; Ianora, A.; Bruno, A.; Noonan, D.M.; Sansone, C.; Albini, A.; Brunet, C. Microalgal derivatives as potential nutraceutical and food supplements for human health: A focus on cancer prevention and interception. *Nutrients* **2019**, *11*, 1226. [CrossRef] [PubMed]
65. Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* **2012**, *24*, 1477–1486. [CrossRef]
66. Nami, Y.; Bakhshayesh, R.V.; Jalaly, H.M.; Lotfi, H.; Eslami, S.; Hejazi, M.A. Probiotic properties of enterococcus isolated from artisanal dairy products. *Front. Microbiol.* **2019**, *10*, 300. [CrossRef]
67. Matos, Â.P. Microalgae as a Potential Source of Proteins. In *Proteins: Sustainable Source, Processing and Applications*; Academic Press: Cambridge, MA, USA, 2019; pp. 63–96. [CrossRef]
68. Blachier, F.; Mariotti, F.; Huneau, J.F.; Tomé, D. Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amin. Acids* **2006**, *33*, 547–562. [CrossRef]
69. Kaneda, T. Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* **1991**, *55*, 288–302. [CrossRef]
70. Safi, C.; Charton, M.; Pignolet, O.; Silvestre, F.; Vaca-Garcia, C.; Pontalier, P.Y. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. *J. Appl. Phycol.* **2013**, *25*, 523–529. [CrossRef]
71. Musch, M.W.; Bookstein, C.; Xie, Y.; Sellin, J.H.; Chang, E.B. SCFA increase intestinal Na absorption by induction of NHE3 in rat colon and human intestinal C2/bbe cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2001**, *280*, G687–G693. [CrossRef]
72. Jaskiewicz, J.; Zhao, Y.; Hawes, J.W.; Shimomura, Y.; Crabb, D.W.; Harris, R.A. Catabolism of isobutyrate by colonocytes. *Arch. Biochem. Biophys.* **1996**, *327*, 265–270. [CrossRef]
73. Heby, O. Role of Polyamines in the Control of Cell Proliferation and Differentiation. *Differentiation* **1981**, *19*, 1–20. [CrossRef]
74. Smith, E.A.; Macfarlane, G.T. Studies on amine production in the human colon: Enumeration of amine forming bacteria and physiological effects of carbohydrate and pH. *Anaerobe* **1996**, *2*, 285–297. [CrossRef]

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Finally, the aim of the seventh paper was to investigate the iron content in *A. platensis, C. vulgaris, H. pluvialis, and P. tricornutum*, with emphasis on iron bioaccessibility assessed using an *in vitro* digestion model. It is of vital importance to study how different species of microalgae can be used in animal nutrition, assessing how they are digested and what amount of nutrients can be absorbed. After the *in vitro* digestion process carried out in the previous study with the collaboration of the research team headed by Professor Biagi, iron quantification was measured using atomic absorption spectrometry (AAS) determining how much iron was bioaccesible. Moreover, extraction of soluble proteins and size exclusion chromatography were applied to investigate iron speciation in the four microalgae species investigated. The study suggested preliminary information of the use of microalgae in animal nutrition to improve feed iron intake.

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Iron Bioaccessibility and Speciation in Microalgae Used as a Dog Nutrition Supplement

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Simple Summary: In recent years, the microalga market has grown due to its interesting nutritional profile, characterised by high quality proteins, bioactive molecules, and essential trace elements. Therefore, it is of vital importance to study how different species of microalgae can be used in animal nutrition, assessing how they are digested and what percentage of valuable nutrients is really absorbed. Iron is an essential trace element and the availability of natural sources of this nutrient may be useful in dog nutrition. This study investigated the iron content in four microalgae (*Chlorella vulgaris*, *Arthrospira platensis*, *Haematoccocus pluvialis*, and *Phaeodactylum tricornutum*) with emphasis on the iron bioaccessibility in dogs. The present results show that, in *C. vulgaris*, a high percentage (30%) of iron was available to be absorbed by the canine gastrointestinal system, suggesting that this microalga could be used as valuable iron supplementation in dog nutrition.

Abstract: *Chlorella vulgaris*, *Arthrospira platensis*, *Haematoccocus pluvialis*, and *Phaeodactylum tricornutum* are species of interest for commercial purposes due to their valuable nutritional profile. The aim of this study was to investigate the iron content in these four microalgae, with emphasis on their iron bioaccessibility assessed using an in vitro digestion system to simulate the process which takes place in the stomach and small intestine of dogs, followed by iron quantification using atomic absorption spectrometry. Furthermore, the extraction of soluble proteins was carried out and size exclusion chromatography was applied to investigate iron speciation. Significant differences ($p < 0.004$) in iron content were found between *C. vulgaris*, which had the highest (1347 ± 93 μg g^{-1}), and *H. pluvialis*, which had the lowest (216 ± 59 μg g^{-1}) iron content. *C. vulgaris*, *A. platensis*, and *H. pluvialis* showed an iron bioaccessibility of 30, 31, and 30%, respectively, while *P. tricornutum* showed the lowest bioaccessibility (11%). The four species analysed presented soluble iron mainly bound to proteins with high molecular mass ranging from >75 to 40 kDa. *C. vulgaris* showed the highest iron content associated with good bioaccessibility; therefore, it could be considered to be an interesting natural source of organic iron in dog nutrition.

Keywords: *Arthrospira platensis*; *Chlorella vulgaris*; *Haematococcus pluvialis*; *Phaedactylum tricornutum*; in vitro digestion; biochemical characterisation

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1. Introduction

Approximately 30% of microalgae production worldwide is sold in the animal feed market [1]. Together, *Chlorella vulgaris* and *Arthrospira platensis* represent the most popular microalgae produced for commercial purposes [2]. They are known and appreciated for their nutritional profile, including highly digestible proteins with an elevated content of essential amino acids [3], dietary fibres, n3-PUFAs, macro (Ca, Mg, P) and trace (Fe, Zn, Cu, Se) elements, and other essential molecules, in particular vitamins D and E. Other interesting microalgae are *Haematococcus pluvialis* which, in the red phase, is considered the best natural source of astaxanthin [4] and the diatom *Phaeodactylum tricornutum* as a

sustainable source of nutrients, especially eicosapentaenoic acid, fucoxanthin, and chrysolaminarin [5]. Exhaustive data regarding the complete biochemical and nutritional profile of these microalgae are scarce. Sandgruber et al. (2021) have recently reported the nutrient composition of 15 microalgae, including *A. platensis*, *C. vulgaris*, and *H. pluvialis*, concluding that, despite wide variations among their biochemical composition, they can be considered an interesting source of nutrients for human nutrition [6]. Actually, *A. platensis* is a cyanobacterium. However, it is usually included with the microalgae; therefore, the term microalgae will be used to include all four species studied.

To obtain beneficial effects, algal nutrients must be digested and absorbed by the intestinal epithelium (bioaccessibility). They finally enter the circulatory system, becoming accessible to the organism (bioavailability). Many studies have investigated the bioaccessibility of nutrients from microalgae, focusing mainly on phenolic compounds, chlorophylls, and carotenoids [7]. Far less research has dealt with the bioaccessibility of trace elements, especially iron. Iron performs essential functions in biological systems as a cofactor for many enzymes and is essential for the assembly of heme and ferrous sulfide FeS clusters. These enzymes are involved in energy generation, free radical detoxification, synthesis of prostaglandins, DNA, fatty acid synthesis, and signal transduction [8]. Contrasting results have been reported regarding iron bioaccessibility in microalgae. In vitro [9,10] and in vivo [11] studies have suggested that *A. platensis* and *C. vulgaris* could be considered interesting sources of bioaccessible iron. On the other hand, Muszynska et al. (2017) [12] investigated iron bioaccessibility from commercial supplements based on *C. vulgaris* used as a human dietary supplement and concluded that they are not a good source of this essential element.

In vitro digestion models are widely used to study digestibility of individual ingredients as well as complete diets in both human and animal nutrition. Moreover, these in vitro digestion models have already been adopted to evaluate the digestibility of algal biomasses in both humans [13,14] and dogs [15], with the final purpose of investigating the effects which microalgae may exert on the intestinal microbiota in vitro.

To the best of the authors' knowledge, studies investigating iron bioaccessibility in dog feed and supplements are lacking. The majority of commercial pet foods contain different sources of iron in order to help meet dietary requirements; meat meals, meat and bone meals, dicalcium phosphate, and ferrous sulfate are rich in iron while milk is not. Therefore, growing dogs are very susceptible to dietary iron deficiency, while inadequate dietary iron intake is rare in adult dogs that are fed balanced diets [16,17]. The main cause of iron deficiency in dogs is chronic blood loss [18]. However, iron deficiency (and severe anaemia) has occasionally been described in intestinal bowel disease in dogs, following chronic intestinal blood loss [19]. Highly available sources of iron could therefore be useful to supplement this trace metal in dogs.

The aim of this study was to determine the iron content of *A. platensis*, *C. vulgaris*, *H. pluvialis*, and *P. tricornutum* before and after incubation with artificial digestive juices under conditions which simulate the canine gastrointestinal tract to determine its bioavailability. Moreover, extracts of these four microalgae were obtained to investigate iron speciation.

2. Materials and Methods

2.1. In Vitro Digestion

Four commercial samples of microalgae, *Arthrospira platensis* (AP), *Chlorella vulgaris* (CV), *Haematococcus pluvialis* (HP), and *Phaeodactylum tricornutum* (PT) kindly offered by Micoperi Blue Growth (Ravenna, Italy) were submitted to a digestion in vitro to simulate the process that takes place in the stomach and small intestine of dogs as described by Biagi et al. (2016) [20]. Briefly, the samples were subjected to two phases of incubation, which occurred under controlled temperature (39 °C). The first one lasted two hours in the presence of pepsin, gastric lipase, and 0.075 N HCl followed by a second four-hour phase with phosphate-bicarbonate buffer pH 7.5, pancreatin, and bile salts. At the end, a supernatant representative of the digested bioaccessible fraction and a pellet representative

of the undigested fraction were obtained. The pellets were dried at 65 °C until a constant dry weight was obtained. The supernatants were frozen and stored at −20 °C for further analyses. Microalgae samples were digested in triplicates. These supernatants and pellets were obtained from our previous study [15].

2.2. Iron Detection and Bioaccessibility

Algal biomasses, supernatants, and pellets obtained from in vitro digestion were analysed to determine iron content, using flame atomic absorption spectrometry (AAS) following the mineralization and analysis protocol reported by Isani et al. (2022a) [21]. The iron detection limit was 0.04 μg mL^{-1}. The iron concentration is reported as μg g^{-1} dry weight (dw) or as μg mL^{-1} depending on the sample analysed.

In this research, the term bioaccessibility is used to define the fraction of iron released from the algal biomass during the in vitro digestibility analysis performed by Delsante et al. (2022) [15]. To determine the bioaccessible iron fraction, the following equation was used:

$$([Fe] \text{ supernatant}/[Fe] \text{ algal biomass}) \times 100 \quad (1)$$

2.3. Extraction of Soluble Proteins

The extraction of soluble proteins was carried out starting from microalgal biomass. The alga biomass (100 mg) was pulverised and extracted following the protocol reported by Isani et al. (2022a) [21]. Iron concentration was measured as reported in Section 2.2. Extractions were performed in duplicate.

2.4. Iron Speciation after Size Exclusion Chromatography (SEC)

A volume of 0.8 mL of supernatant obtained from each algal extract was applied to a Sephadex G-75 chromatography column (0.9 × 90 cm). A gel filtration marker kit (MWGF70-1KT, Sigma-Aldrich, St Louis, MO, USA) was used to calibrate the column. Fractions of 1.5 mL were collected and analysed for iron concentration as described in Section 2.2. In the supernatants and fractions obtained from SEC, total proteins, chlorophyll a, and phycocyanin were determined by measuring the absorbance at 280, 430, 662, and 620 nm, respectively (DeNovix DS-11 Series Spectrophotometer, Wilmington, DE, USA). In addition, in the samples from *A. platensis*, phycocyanin was detected at 620.

Furthermore, in the samples of *H. pluvialis*, astaxanthin was determined at 477 nm.

2.5. Statistical Analysis

The central limit theorem was applied in order to choose the correct statistic test [22]. Thus, taking into consideration the number of samples for each microalga (n = 3), the Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons post-hoc test was performed to determine significant differences of iron among the four microalgae, pellets, supernatants and bioaccessible fraction obtained by in vitro digestion, pellets, and supernatants obtained by soluble proteins extraction. The p-value obtained underwent to Bonferroni's correction and differences between groups were considered significant for $p < 0.05$.

Statistical analyses were performed using R 4.2.1 (R foundation for statistical computing; Vienna, Austria; https://www.R-project.org/, accessed on 1 October 2022). Data are reported as median, mean ± SD (standard deviation). All graphs were made using GraphPad Prism version 9.2 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. Iron Content in Microalgae

The iron content in microalgae follows this decreasing order: *C. vulgaris* > *P. tricorutum* > *A. platensis* > *H. pluvialis*, and is reported in Figure 1. Significant differences were found between *C. vulgaris* and *H. pluvialis*.

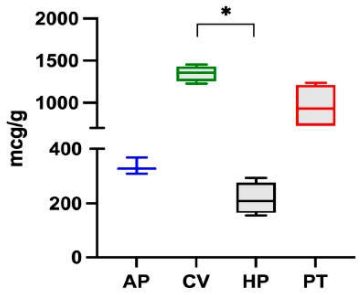

Figure 1. Iron content in the four microalgae (*A. platensis*, *C. vulgaris*, *H. pluvialis*, *P. tricornutum*) analysed. The data are expressed as micrograms per gram of dry algae biomass and are reported as medians and interquartile ranges. * Significantly different for the Dunn post-hoc test ($p < 0.05$).

Of the four species examined, *C. vulgaris* had the highest iron content (1347 ± 93 μg g^{-1}). The value was in the range of those reported by other authors. Muszynska et al. (2017) [12] analysed different commercial samples of *C. vulgaris* and found iron contents ranging from 880 to 2112 μg g^{-1} while Kejzar et al. (2021) [23] found a value of 1000 ± 520 μg g^{-1} in *Chlorella* spp. and Rzymski et al. (2019) [24] found values from 438 to 1661 μg g^{-1}. On the other hand, Sandgruber et al. (2021) [6] reported an average iron content of 536 μg g^{-1} in two *C. vulgaris* samples while Maruyama et al. (1997) [25], Tokosoglu et al. (2003), [26] and Panahi et al. (2012) [27] reported iron contents equal to 2000, 2600, and 6800 μg g^{-1} in *C. vulgaris*, respectively.

In the present study, *A. platensis* presented an iron content of 335 μg g^{-1}, a value which was lower than those reported in the literature. Isani et al. (2022b) [28] reported that ten commercial samples of *A. platensis* had an iron content between 353 and 1459 μg g^{-1}. Principe et al. (2020) [10] detected values between 63 and 1066 μg g^{-1} in samples of *A. platensis* from the Argentinian market. Kejzar et al. (2021) [23] reported iron values of 1360 ± 1330 μg g^{-1} and Rutar et al. (2022) [29] reported values from 370 to 3480 μg g^{-1}. Finally, Sandgruber et al. (2021) [6] and Rzymski et al. (2019) [24] reported iron contents in *A. platensis* ranging from 11.6 to 835 μg g^{-1} and from 369 to 2287 μg g^{-1}, respectively.

Data regarding the iron content of the other two microalgae are limited. In the present study, *H. pluvialis* presented an iron content of 216 μg g^{-1}, lower than the value of 972 μg g^{-1} reported by Sandgruber et al. (2021) [6] while *P. tricornutum* had a higher iron content of 956 ± 267 μg g^{-1}. Although various studies [30,31] have investigated the response of this microalga grown in media with varying iron concentrations, to the authors' knowledge, there are no studies reporting its iron content, likely because *P. tricornutum* cannot be marketed for human use.

3.2. Iron Bioaccessibility

The amount of iron released into the soluble fraction after gastrointestinal digestion is the bioaccessible fraction which has been calculated using Equation (1). Therefore, the bioaccessibility of iron in the four microalgae was determined using the iron content measured in the digested fractions obtained in an in vitro system simulating the process which takes place in the stomach and small intestine of dogs.

The values obtained demonstrated a similar iron bioaccessibility of 31% for *A. platensis* and 30% for *C. vulgaris* and *H. pluvialis*, while *P. tricornutum* had the lowest bioaccessibility, namely 11% (Table 1). The interaction of different factors impacts iron bioaccessibility; these include the cellular organisation of the microalgae and the biochemical nature of iron ligands. In *A. platensis*, the high bioaccessibility associated with the highest digestibility could be due to the structure of Gram-negative bacterial cells which are surrounded by a wall of peptidoglycans, while the other eucaryotic algae have a cellulose cell wall which

opposes more resistance to digestive enzymes. In particular, *H. pluvialis*, in the aplanospore form or the red phase, presents a rigid cell wall which can hinder the action of digestive enzymes, limiting access to the intracellular molecules [4,32]. Despite its low digestibility, *H. pluvialis* presented an iron bioaccessibility similar to *A. platensis* and *C. vulgaris*; however, the low iron content in the sample analysed (216 μg g^{-1} dw) resulted in the lowest iron bioaccessibility of the four microalgae (Table 1). On the contrary, despite its relatively high digestibility, this study disclosed that the diatom *P. tricornutum* had the lowest iron bioaccessibility of the four microalgae analysed. However, due to the high iron content in the algal sample (956 μg g^{-1} dw), one gram of this microalga contained 105 μg of bioaccessible iron.

Table 1. The iron content in algal biomass, digestibility and bioaccessibility in four microalgae (*A. platensis*, *C. vulgaris*, *H. pluvialis*, *P. tricornutum*) undergoing an in vitro digestion process.

Algae	Digestibility § (%)	Iron DF * μg mL ^{1}	Iron UF ° μg g ^{1} dw	Bioaccessible Fraction %	Bioaccessible Iron μg g ^{1} alga
A. platensis	86	1.30 ± 0.43	1955 ± 352 ^{b}	31 ± 12	104 ± 9
C. vulgaris	55	5.04 ± 2.15 ^{a}	1861 ± 267	30 ± 12	404 ± 28 ^{c}
H. pluvialis	8	0.80 ± 0.15 ^{a}	226 ± 49 ^{b}	30 ± 9	64 ± 18 ^{c}
P. tricornutum	68	1.31 ± 0.29	1690 ± 70	11 ± 0.6	105 ± 29

§ The data were obtained from Delsante et al. (2022) [15]. * DF: digested fractions (supernatants after in vitro digestion); ° UF: undigested fractions (pellets after in vitro digestion). In the same column, the same superscript letters indicate significant differences between the microalgae ($p < 0.05$). Data are expressed as mean ± standard deviation.

The minimum iron requirements of adult dogs according to the European Pet Food Industry federation (FEDIAF) (2021) [33] are 9 mg per 1000 kcal of metabolisable energy. Accordingly, considering that *C. vulgaris* provides 404 μg g^{-1} of bioaccessible iron, the daily iron requirement of an adult dog with a body weight of 10 kg (approximately 5.6 mg per day) could be covered by 14 g of *C. vulgaris*.

Contrasting results have been reported in the literature regarding the bioaccessibility of iron from microalgae; this important issue is still a matter of debate. Puyfoulhoux et al. (2001) [9] examined the iron availability in *A. platensis* using an in vitro digestion/Caco-2 cell culture system, concluding that this microalga could represent an adequate source of iron. Principe et al. (2020) [10] reported an iron bioaccessibility ranging from 2.8 to 27% under oral conditions, 3 to 60% under gastric conditions, and 3 to 40% under intestinal conditions for *A. platensis* in different human digestion phases while Rutar et al. (2022) [29] reported that the iron bioavailability in *A. platensis* was low, due to the small amount of iron in the more bioavailable ferrous ion Fe^{2+}. Uribe-Wandurraga et al. (2020) [34] reported an iron bioaccessibility from 40 to 50% in cookies enriched with 1.5 and 2% *C. vulgaris* and *A. platensis*, using an in vitro system which simulated the human digestive process. In 32 pregnant women (second and third trimester), the oral supplementation with *Chlorella pyrenoidosa* for 12–18 weeks decreased markers of anaemia as compared to the control group [11], suggesting that *C. pyrenoidosa* contained bioaccessible and bioavailable iron. On the contrary, Muszynska et al. (2017) [12] reported that the content of iron in commercial preparations of *C. vulgaris* was negligible after incubation with artificial digestive juices, indicating that the preparations examined were not a good source of this essential element.

3.3. Iron Speciation and Protein Fractioning Using Size Exclusion Chromatography (SEC)

Th various bioaccessibilities determined from the four microalgae analysed could also be related to the molecular characteristics of iron ligands. In biological systems, iron can bind to different ligands, either inorganic, such as ferrihydrite, or organic, such as iron-binding proteins. The speciation of the element, namely its distribution among these molecules, is an important issue when studying its bioaccessibility. Therefore, to shed more light on this important topic, the four microalgae were extracted to analyse the distribution of iron between the insoluble fraction (pellets), which, for the most part,

contains inorganic iron, and the soluble fraction (supernatant), which contains iron bound to hydrophilic molecules, for the most part proteins, peptides, and amino acids. In all the samples analysed, the iron content was higher in the pellet than in the soluble fraction (Figure 2). The percentage of the iron content determined in *A. platensis* was in the range of data reported by Isani et al. (2022 b) [28] in the same species. The data in the present study provided additional evidence as to what had been hypothesised by Perfiliev et al. (2018) [35] that *A. platensis* accumulated iron, for the most part in an inorganic form such as ferrihydrite. An even higher iron content in pellets was found in *C. vulgaris*, *H. pluvialis*, and *P. tricornutum*, with percentages from 92 to 97%. Ferrihydrite could be responsible for the high iron percentages determined in the pellet fraction of these three microalgae.

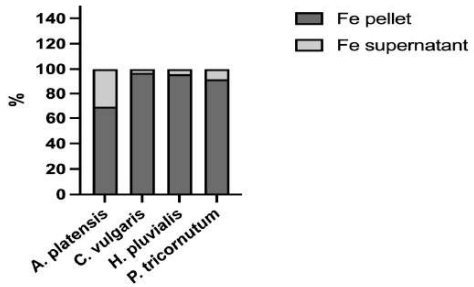

Figure 2. Iron distribution between the supernatants (soluble fractions) and pellets (insoluble fractions) obtained after the extraction of *A. platensis*, *C. vulgaris*, *H. pluvialis*, and *P. tricornutum*. The data are expressed as percentages.

Subsequently, to investigate the iron speciation in the soluble fractions, supernatants underwent SEC associated with sensitive metal detection in the fractions using atomic absorption spectroscopy (AAS), a hyphenated approach which is commonly used in metallomic studies [36] and had previously been applied to *A. platensis* [21,28]. Total proteins and iron measured in fractions after SEC are reported in Figures 3 and 4. Phycocyanin in *A. platensis*; chlorophyll a in *A. platensis*, *C. vulgaris*, and *P. tricornutum*; and astaxanthin in *H. pluvialis* are reported in Figures 5–7.

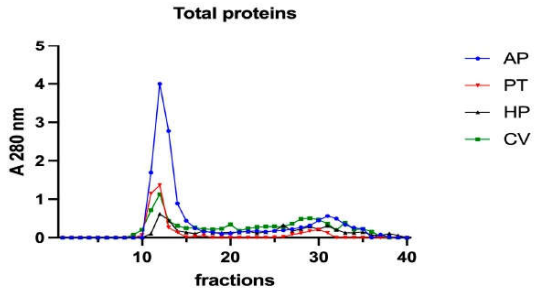

Figure 3. Chromatographic profiles of total proteins after SEC of extracts from algal samples. Proteins were detected at 280 nm. Each chromatographic profile is the mean of two chromatographies. AP = *A. platensis*; PT = *P. tricornutum*; HP = *H. pluvialis*; CV = *C. vulgaris*.

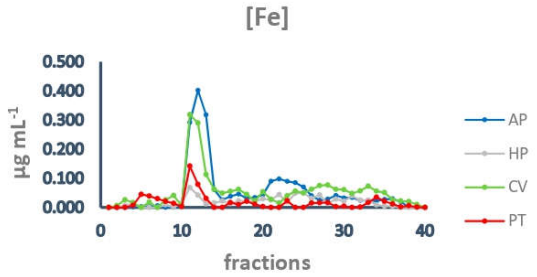

Figure 4. Chromatographic profiles of iron after SEC of extracts from algal samples. Iron concentration is expressed as µg mL^{-1}. Each chromatographic profile is the mean of two chromatographies. AP – *A. platensis*; PT – *P. tricornutum*; HP – *H. pluvialis*; CV – *C. vulgaris*.

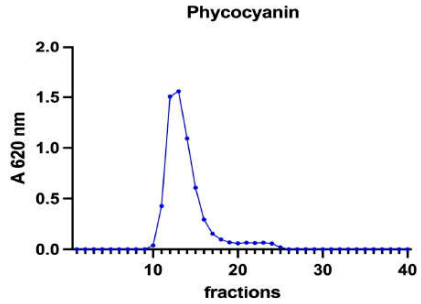

Figure 5. A chromatographic profile of phycocyanin after SEC of the *A. platensis* extract. Phycocyanin concentration is reported as mg mL^{-1}. The chromatographic profile is the mean of two chromatographies.

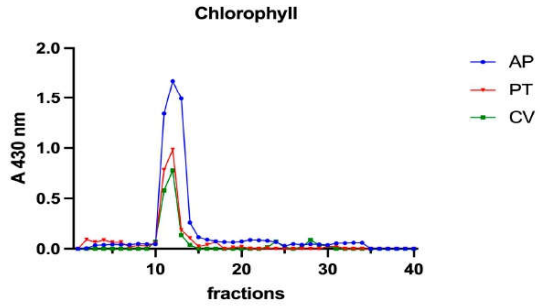

Figure 6. Chromatographic profiles of chlorophyll a after SEC of extracts from algal biomass. Chlorophyll a was detected at 430 nm. Each chromatographic profile is the mean of two chromatographies.

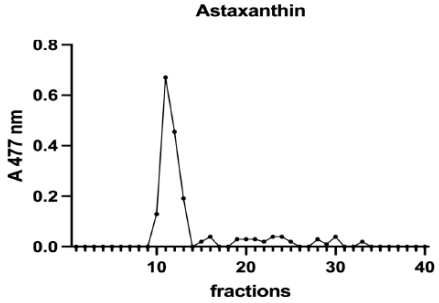

Figure 7. A chromatographic profile of astaxanthin after SEC of an *H. pluvialis* extract. Astaxanthin was detected at 477 nm. The chromatographic profile is the mean of two chromatographies.

In all the samples analysed, a major protein peak was eluted between fractions 11 and 15 (Figure 3). This peak contains proteins with a high molecular mass (HMM), ranging from >75 to 40 kDa. *A. platensis* showed a higher content of soluble proteins than the other microalgae in accordance with its high digestibility. A second peak of absorbance is present between fractions 29 and 34. Small peptides, amino acids and, finally, free ions (starting from fraction 32) elute in these chromatographic fractions. In *A. platensis*, it has been hypothesised that mycosporine-like amino acids (MAAs) may be present among these low molecular mass molecules [28]. The presence of a second peak is also detectable in *C. vulgaris*, *H. pluvialis*, and *P. tricornutum*, suggesting the presence of small peptides and free amino acids in these species too. Accordingly, Ba et al. (2016) [37] reported the presence of low molecular mass proteins (<10 kDa) in *H. pluvialis* in the vegetative phase after SEC protein separation.

In all the samples analysed, the elution profile of iron after SEC presented a major peak between fractions 11 and 14 (Figure 4), overlapping the main peak of proteins at HMM (Figure 3), indicating that the most relevant metal burden is bound to proteins. *A. platensis* shows the highest amount of iron, followed by *C. vulgaris*, while *H. pluvialis* and *P. tricornutum* are characterised by a far lower iron concentration. Other minor peaks are present in fractions containing intermediate MM molecules from 40 to 20 kDa (fractions 15–18) and low MM molecules (fractions 28–32). In *A. platensis*, iron was also bound to ligands with an MM of 12–10 kDa (fractions 21–25).

The nature of these ligands is still an open question. In *A. platensis*, the elution profile of phycocyanin (Figure 5) overlaps both the total proteins and the iron peaks, with a maximum at fraction 12 (Figures 3 and 4). Phycocianin is a multimeric blue phycobiliprotein containing α and β subunits. Phycocianin is involved in the photosynthetic machinery of *A. platensis*, functioning as a light-capturing antenna in photosystem II. In addition to this essential role, it has been reported by Bermejo et al. (2008) [38] that this protein was able to bind iron in vitro. The data in the present study add additional evidence regarding the role of phycocyanin as an iron binding protein in vivo as recently suggested by Isani et al. (2022a and b) [21,28]. Regarding *P. tricornutum*, Sutak et al. (2012) [39] reported the presence of ISIP1 (iron starvation-induced protein 1), suggesting a role of this protein in iron uptake. The ISIP1 protein has a molecular mass of 61 kDa, in the range of those eluted after SEC in fractions 10–15 in which a peak of proteins in the *P. tricornutum* supernatant and a peak of iron were found. Behnke et al. (2020) [40] confirmed that ISIP1 was an additional protein involved in the utilisation of diverse iron pools, thereby securing the success of *P. tricornutum* in iron-poor environments.

A. platensis showed the highest chlorophyll a content while *C. vulgaris* and *P. tricornutum* showed a similar but lower content. These three microalgae showed a unique peak between fractions 11 and 14. In *H. pluvialis*, no chlorophyll was detected. These data

add additional proof that chlorophyll decreases when *H. pluvialis* starts to accumulate astaxanthin as reported by Fang et al. (2019) [41]. In *H. pluvialis*, astaxanthin shows a peak between fractions 11 and 14 (Figure 7). Despite its lipophylic nature, astaxanthin has been extracted, at least in part, in the soluble hydrophilic extract and, after SEC, appears in HMM chromatographic fractions, likely bound to proteins.

4. Conclusions

To the best knowledge of the authors, this is the first study which investigated iron bioaccessibility from *A. platensis*, *C. vulgaris*, *P. tricornutum*, and *H. pluvialis* using a validated in vitro canine digestion simulation system. Of the four algal samples analysed, *C. vulgaris* contained the highest iron content associated with high bioaccessibility. Therefore, this microalga provided the highest amounts of iron available to be absorbed across the intestinal epithelium of dogs and could be considered as an interesting iron supplement in dog nutrition.

Taken together, the data in the present study suggested that the four species analysed presented soluble iron mainly bound to proteins with an HMM ranging from >75 kDa to 40 kDa, with emphasis on phycocyanin in *A. platensis*.

This study has provided a promising foundation for investigating the supplementation of microalgae in dog nutrition with the possibility of obtaining health benefits. Nevertheless, additional studies are needed to increase knowledge regarding the effects of microalgae supplementation in dogs and the pathways involved in iron speciation in microalgae.

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References

1. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278. [CrossRef]
2. Mendes, M.C.; Navalho, S.; Ferreira, A.; Paulino, C.; Figueiredo, D.; Silva, D.; Gao, F.; Gama, F.; Bombo, G.; Jacinto, R.; et al. Algae as food in europe: An overview of species diversity and their applications. *Foods* **2022**, *11*, 1871. [CrossRef]
3. Niccolai, A.; Zittelli, G.C.; Rodolfi, L.; Biondi, N.; Tredici, M.R. Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Res.* **2019**, *42*, 101617. [CrossRef]
4. Wang, G.; Shen, S.; Niu, J.; Zhu, D.; Li, J. An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis*. *Biotechnol. Adv.* **2011**, *29*, 568–574. [CrossRef]
5. Yang, R.; Wei, D.; Xie, J. Diatoms as cell factories for high-value products: Chrysolaminarin, eicosapentaenoic acid, and fucoxanthin. *Crit. Rev. Biotechnol.* **2020**, *40*, 993–1009. [CrossRef]
6. Sandgruber, F.; Gielsdorf, A.; Baur, A.C.; Schenz, B.; Muller, S.M.; Schwerdtle, T.; Stang, G.I.; Griehl, C.; Lorkowski, S.; Dawczynski, C. Variability in macro- and micronutrients of 15 commercially avaiable microalgae powders. *Mar. Drugs* **2021**, *19*, 310. [CrossRef]
7. Nass, P.P.; Nascimento, T.C.; Fernandes, A.S.; Caetano, P.A.; de Rosso, V.V.; Jacob-Lopes, E.; Zepka, L.Q. Guidance for formulating ingredients/products from *Chlorella vulgaris* and *Arthrospira platensis* considering carotenoid and chlorophyll bioaccessibility and cellular uptake. *Food. Res. Int.* **2022**, *157*, 111469. [CrossRef]
8. Pauline, T.; Lieu, M.H.; Peterson, A.; Yang, Y. The roles of iron in health and disease. *Mol. Asp. Med.* **2001**, *22*, 1–87. [CrossRef]
9. Puyfoulhoux, G.; Rouanet, J.M.; Besancon, P.; Baroux, B.; Baccou, J.C.; Caporiccio, B. Iron availability from iron-fortified spirulina by an in vitro digestion/Caco-2 cell culture model. *J. Agric. Food Chem.* **2001**, *49*, 1625–1629. [CrossRef]

10. Principe, M.V.; Permigiani, I.S.; Della Vedova, M.C.; Petenatti, E.; Pacheco, P.; Gil, R.A. Bioaccessibility studies of Fe, Cu and Zn from *Spirulina* dietary supplements with different excipient composition and dosage form. *J. Pharm. Pharmacogen. Res.* **2020**, *8*, 422–433.
11. Nakano, S.; Takekoshi, H.; Nakano, M. *Chlorella pyrenoidosa* supplementation reduces the risk of anemia, proteinuria and edema in pregnant women. *Plant. Food. Hum. Nutr.* **2010**, *65*, 25–30. [CrossRef] [PubMed]
12. Muszynska, B.; Krakowska, A.; Lazur, J.; Jekot, B.; Zimmer, L.; Szewczyk, A.; Sulkowska-Ziaja, K.; Poleszak, E.; Opoka, W. Bioaccessibility of phenolic compounds, lutein, and bioelements of preparations containing *Chlorella vulgaris* in artificial digestive juices. *J. Appl. Phycol.* **2017**, *30*, 1629–1640. [CrossRef] [PubMed]
13. De Medeiros, V.P.B.; de Souza, E.L.; de Albuquerque, T.M.R.; da Costa Sassi, C.F.; dos Santos Lima, M.; Sivieri, K.; Pimentel, T.C.; Magnani, M. Freshwater microalgae biomasses exert a prebiotic effect on human colonic microbiota. *Algal Res.* **2021**, *60*, 102547. [CrossRef]
14. Jin, J.B.; Cha, J.W.; Shin, I.S.; Jeon, J.Y.; Cha, K.H.; Pan, C.H. Supplementation with *Chlorella vulgaris*, *Chlorella protothecoides*, and *Schizochytrium* sp. increases propionate-producing bacteria in in vitro human gut fermentation. *J. Sci. Food Agric.* **2020**, *100*, 2938–2945. [CrossRef]
15. Delsante, C.; Pinna, C.; Sportelli, F.; Dalmonte, T.; Stefanelli, C.; Vecchiato, C.G.; Biagi, G. Assessment of the effects of edible microalgae in a canine gut model. *Animals* **2022**, *12*, 2100. [CrossRef]
16. Case, L.P.; Daristotle, L.; Hayek, M.G.; Raasch, M. *Canine and Feline Nutrition: A Resource for Companion Animal Professionals*, 3rd ed.; Elsevier Health Sciences: Amsterdam, The Netherlands, 2010.
17. McCown, J.L.; Specht, A.J. Iron homeostasis and disorders in dogs and cats: A review. *J. Am. Anim. Hosp. Assoc.* **2011**, *47*, 151–160. [CrossRef]
18. Geiger, U. Regenerative anemias caused by blood loss or hemolysis. In *Textbook of Veterinary Internal Medicine*, 6th ed.; Elsevier: Amsterdam, The Netherlands, 2005; pp. 1886–1907.
19. Ristic, J.M.; Stidworthy, M.F. Two cases of severe iron-deficiencyanaemia due to inflammatory bowel disease in the dog. *J. Small Anim. Pract.* **2002**, *43*, 80–83. [CrossRef]
20. Biagi, G.; Cipollini, I.; Grandi, M.; Pinna, C.; Vecchiato, C.G.; Zaghini, G. A new in vitro method to evaluate digestibility of commercial diets for dogs. *Ital. J. Anim. Sci.* **2016**, *15*, 617–625. [CrossRef]
21. Isani, G.; Niccolai, A.; Andreani, A.; Dalmonte, T.; Bellei, E.; Bertocchi, M.; Tredici, M.R.; Rodolfi, L. Iron speciation and iron binding proteins in *Arthrospira platensis* grown in media containing different iron concentrations. *Int. J. Mol. Sci.* **2022**, *23*, 6283. [CrossRef]
22. Masha, E.J.; Vetter, T.R. Significance, errors, power, and the sample size: The blocking and tackling of statistics. *Anesth. Analg.* **2018**, *126*, 691–698. [CrossRef]
23. Kejzar, J.; Jagodic, H.M.; Ogrinic, N.; Masten, R.J.; Poklar, U.N. Characterization of algae dietary supplements using antioxidative potential, elemental composition, and stable isotopes approach. *Front. Nutr.* **2021**, *5*, 618503. [CrossRef] [PubMed]
24. Rzymski, P.; Budzulak, J.; Niedzielski, P.; Klimaszyk, P.; Proch, J.; Kozak, L.; Poniedzialek, B. Essential and toxic elements in commercial microalgal food supplements. *J. Appl. Phycol.* **2019**, *31*, 3567–3579. [CrossRef]
25. Maruyama, I.; Nakao, T.; Shigeno, I.; Ando, Y.; Hirayama, K. Application of unicellular algae *Chlorella vulgaris* for the mass-culture of marine rotifer *Brachionus*. *Hydrobiologia* **1997**, *358*, 133–138. [CrossRef]
26. Tokosoglu, O.; Unal, M.K. Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrisis galbana*. *J. Food Sci.* **2003**, *68*, 1144–1148. [CrossRef]
27. Panahi, Y.; Pishgoo, B.; Jalalian, H.R.; Mohammadi, E.; Taghipour, H.R.; Sahebkar, A. Investigation of the effects of *Chlorella vulgaris* as an adjunctive therapy for dyslipidemia: Results of a randomised open-label clinical trial. *Nutr. Diet.* **2012**, *69*, 13–19. [CrossRef]
28. Isani, G.; Bertocchi, M.; Ferlizza, E.; Dalmonte, T.; Fedrizzi, G.; Andreani, G. Iron content, iron speciation and phycocyanin in commercial samples of *Arthrospira platensis*. *Int. J. Mol. Sci.* **2022**, *23*, 13949. [CrossRef]
29. Rutar, J.M.; Hudobivnik, M.J.; Necemer, M.; Mikus, K.V.; Arcon, I.; Ogrinc, N. Nutritional quality and safety of the *Spirulina* dietary supplements sold on the slovenian market. *Foods* **2022**, *11*, 849. [CrossRef]
30. Zhao, P.; Gu, W.; Huang, A.; Wu, S.; Liu, C.; Huan, L.; Gao, S.; Xie, X.; Wang, G. Effect of iron on the growth of *Phaeodactylum tricornutum* via photosynthesis. *J. Phycol.* **2017**, *54*, 34–43. [CrossRef]
31. Castell, C.; Bernal-Bayard, B.; Ortega, J.M.; Roncel, M.; Hervas, M.; Navarro, A.J. The heterologous expression of a plastocyanin in the diatom *Phaeodactylum tricornutum* improves cell growth under iron-deficient conditions. *Physiol. Plant* **2020**, *171*, 277–290. [CrossRef]
32. Demarco, M.; Moraes, J.O.; Matos, A.P.; Derner, R.B.; Neves, F.F.; Tribuzi, G. Digestibility, bioaccessibility and bioactivity of compounds from algae. *Trends Food Sci. Technol.* **2022**, *121*, 114–128. [CrossRef]
33. The European Pet Food Industry Federation—FEDIAF. *Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs*; FEDIAF: Brussels, Belgium, 2021.
34. Uribe-Wandurraga, Z.N.; Igual, M.; Garcia-Segovia, P.; Martinez-Monzo, J. In vitro bioaccessibility of minerals from microalgae-enriched cookies. *Food Funct.* **2020**, *11*, 2186–2194. [CrossRef] [PubMed]

35. Perfiliev, Y.D.; Tambiev, K.; Konnychev, M.A.; Skalny, A.V.; Lobakova, E.S.; Kirpichnikov, M.P. Mössbauer spectroscopic study of transformations of iron species by the cyanobacterium *Arthrospira platensis* (formerly *Spirulina platensis*). *J. Trace Elem. Med. Biol.* **2018**, *48*, 105–110. [CrossRef] [PubMed]
36. Mounicou, S.; Szpunar, J.; Lobinski, R. Mettalomics: The concept and methodology. *Chem. Soc. Rev.* **2009**, *38*, 1119–1138. [CrossRef]
37. Ba, F.; Ursu, A.V.; Laroche, C.; Djelveh, G. *Haematococcus pluvialis* soluble proteins: Extraction, characterization, concentration/fractioning and emulsifying properties. *Bioresour. Technol.* **2016**, *200*, 147–152. [CrossRef] [PubMed]
38. Bermejo, P.; Piñero, E.; Villar, Á.M. Iron-chelating ability and antioxidant properties of phycocyanin isolated from a proteanextract of *Spirulina platensis*. *Food Chem.* **2008**, *110*, 436–444. [CrossRef]
39. Sutak, R.; Botebol, H.; Blaiseau, P.L.; Léger, T.; Bouget, F.Y.; Camadro, J.M.; Lesuisse, E. A comparative study of iron uptake mechanism in marine microalgae: Iron binding at the cell surface is a critical step. *Plant Physiol.* **2012**, *160*, 2271–2284. [CrossRef]
40. Behnke, J.; Laroche, J. Iron uptake proteins in algae and the role of iron starvation-induced proteins (ISIPs). *Eur. J. Phycol.* **2020**, *55*, 339–360. [CrossRef]
41. Fang, L.; Zhang, J.; Fei, Z.; Wan, M. Chlorophyll as key indicator to evaluate astaxanthin accumulation ability of *Haematococcus pluvialis*. *Bioresour. Bioprocess.* **2019**, *6*, 52. [CrossRef]

ONGOING RESEARCH

1. Introduction

Regarding the research in microalgae field, a further study was carried out in collaboration with IRTA (Institute of Agrifood Research and Technology) in Monells (Girona, Spain) under the supervision of Dr. Massimo Castellari, senior coordinator of the European project PROFUTURE. This project is aimed at investigating microalgae as a source of high biological value proteins with a reduced environmental impact. Particularly, during the period at IRTA from 1st of May to 30th of September 2022 and from 1th of May to 31th of July 2023 the research activity was focused on the characterization of Spirugrass®, a by-product derived from phycocyanin extraction of biomasses of *Arthrospira platensis*.

Microalgae are going to be exploited as alternative sources in animal nutrition, gaining space in commercial products due to their nutritional characteristics, including the high content of ω-3 fatty acids, proteins, vitamins and other compounds as carotenoids. Currently, microalgae have been added to animal feed for dogs, cats, poultry, swine, rabbits and in aquaculture field [1]. However, the production costs of microalgae are still too high to be competitive with other sources, such as soybean. Therefore, reuse of by-production can be attractive from a perspective of circular economy. In this case Spirugrass® can be considered an interesting ingredient for feed integration.

This research was organized in two steps. In the first phase Spirugrass® obtained from AlgoSource (Saint-Nazaire, France) was extracted and characterized. Different protocols of extraction of soluble proteins were tested to find a method that didn't affect the stability of phycocyanin and the yield of the process. Unlike the usual process applied in the extraction of soluble proteins from *A. platensis*, which is based on disrupting procedures as sonication to break the cell wall of cyanobacteria, a lighter extraction was chosen due to the fact that Spirugrass® is a residual of biomasses which have been previously extracted to obtain phycocyanin. Then the soluble fraction was subjected to gelfiltration chromatography. The iron content was measured using atomic absorption spectrometry (AAS).

During the second phase the effect of high-pressure pasteurization (HPP) was studied. Samples of Spirugrass® from three different batches underwent HPP at 6000 bar for 5 minutes, while an aliquot of the same samples was preserved without the above-mentioned treatment. Proteins were separated using high pressure liquid chromatography (HPLC). Furthermore, proteomic profiles of the fractions were investigated using SDS-PAGE.

The aims of this research are to characterize the Spirugrass® and to evaluate if HPP affects proteomic profile and quality of the product.

2. Material and Methods

2.1. Soluble protein extraction

The extraction of soluble proteins was performed starting from *A. platensis* dried biomass and Spirugrass®. One-hundred milligrams of the dried biomass of Spirugrass® or *A. platensis* were diluted 1:30 w/v in Tris-HCl 20 mM and 5 mM 2-mercaptoethanol. The

samples were vortexed 3 times for 10 seconds and then incubated for 3 hours at 4°C. Afterwards, samples were homogenized with Ultra-turrax T25 (Janke & Kunkel; IKA®-Labortechnik) at 20,500 rpm 3 times for 10 seconds and then centrifuged at 18,000xg for 40 minutes at 4°C, obtaining the separation between supernatant and pellet. Samples were stored at -20°C until analysis. Each extraction was done in triplicate.

2.2. SDS-PAGE

Supernatant from previous extractions, SEC and HPLC fractions representative of the major peak of iron detected in AAS were loaded onto 4-12% polyacrylamide pre-cast gels (NuPage, Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was previously quantified using the Lowry method and phycocyanin concentration was quantified using the Bennet & Bogorad equation, as reported below [2].

Phycocyanin concentration (mg/mL) = [A620 - 0.474(A652)]/5.34

Equation 1. Phycocyanin concentration calculation (Bennet & Bogorad, 1973).

Samples were treated with lithium dodecyl sulfate (LDS; Thermo Fisher Scientific, Waltham, MA, USA) and reducing agent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and denatured at 70°C for 10 minutes. SDS-Page was carried out in Novex Mini-Cell (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and the running buffer used was 2-morpholinoethanesulfonic acid (MES) (Nupage, Thermo Fisher Scientific, Waltham, MA, USA). Each gel was also loaded with standard proteins of known molecular weight (SeeBlue Pre-Stained Standard). The electrophoresis system was connected to a power supply (Power Pack Basic—Bio-Rad, Hercules, CA, USA) at a constant voltage of 200 V. The gels were stained with Quick Coomassie Stain (Protein Ark, Sheffield, UK). After destaining, each gel was digitalized using ChemiDocMP (Bio-Rad, Hercules, CA, USA), and the pherograms were obtained using the ImageLab 5.2.1 software (Bio-Rad, Hercules, CA, USA).

2.3. Size exclusion chromatography (SEC)

For each extract, a volume of 0.8 mL supernatant was applied to a Sephadex G-75 column (0.9 × 90 cm). The column was calibrated using a commercial kit (GF70-1KT, Sigma-Aldrich, St Louis, MO, USA) and the buffer used was Tris-HCl 20 mM and 5 mM 2-mercaptoethanol. Fractions of 1.5 mL were collected and analysed for iron concentration using direct aspiration of the solution into the flame of an atomic absorption spectrophotometer (AAnalyst 100, Perkin Elmer, Waltham, MA, USA). As for proteomic analysis, SDS-PAGE of fractions was performed.

2.4. Iron analysis using atomic absorption spectrometry

To avoid contamination all the reagents were handled carefully and polyethylene equipment were washed with 1 N HCl under a fume hood. The pellet obtained from soluble proteins extraction process was placed in Teflon jars and was digested in a microwave oven according to the following method: 1-2 mL of 65% HNO_3 and 0.25-0.5 mL of 30% H_2O_2 were

added to the samples and then analysed using a flame atomic spectrophotometer equipped with a deuterium lamp background correction (AAnalyst 100, Perkin Elmer, Waltham, MA, USA). Supernatants obtained from soluble proteins extraction were diluited 1:10 with MilliQ water and directly analysed. Fractions obtained from HPLC were directly analyses without any further treatment. The accuracy of the method was evaluated with ERM®–BB422 fish muscle. The concentrations found with the method used in this study fell into the certified uncertainty interval given by ERM, corresponding to a 95% confidence level. The iron detection limit was 0.04 μg mL^{-1}. Iron concentrations were reported as μg mL^{-1} or μg g^{-1} dw depending on the sample analysed. All the reagents were from Merck (Darmstadt, Germany) and the acids were of Suprapur grade.

2.5. High pressure liquid chromatography (HPLC)

The mobile phase for HPLC was Tris-HCl 20 mM and NaCl 0.15 M; 37% HCl was added drop by drop until a pH 8.0 was obtained and then filtered with 0.2μm 47mm GHP membrane (Pall Corporation; 600 South Wagner Road; Ann Arbor, Michigan 48103-9019). HiPrep 16/60 Sephacryl S-300 High Resolution column (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used as stationary phase. The column was washed with 60 mL of bi-distilled water at 500 μL/min for 2 hours, then with buffer at 500 μL/min overnight. One millilitre of the supernatant previously obtained by soluble proteins extraction was injected in HPLC system with a Hamilton glass syringe. Chromatography was carried out with the following parameters: AT=1024, CS=0.1, Flow rate 1.000 (1mL/min). The system was plugged in with a 280 nm detector. Sixty fractions of 1.5 mL were collected and stored at -20°C.

3. Preliminary results and discussion

3.1 Characterization of Spirugrass®

A preliminary SDS-PAGE of supernatants obtained from the extraction of soluble proteins of *A. platensis* and Spirugrass® was performed to check the quality and the integrity of the proteomes (figure 1). In the third and fourth wells, the supernatants obtained from intact *A. platensis* biomass are shown, while the supernatant of Spirugrass® is shown in the fifth well. As a comparison, in the first 2 wells two samples of Spirulysat®, a phycocyanin extract from *A. platensis* biomass produced by Algosource (Saint Nazaire, France), are reported. In all samples, including Spirugrass®, the most represented bands are at 18 and 17 kDa. These bands contain C-phycocyanin (C-PC) [3]. No intense protein degradation was evidenced. However, Spirugrass® extract has an electrophoretic profile characterized by lower concentration of phycocyanin and lower molecular mass bands, suggesting possible partial fragmentation of proteins of high molecular mass, or, alternatively disaggregation of complex molecular structures.

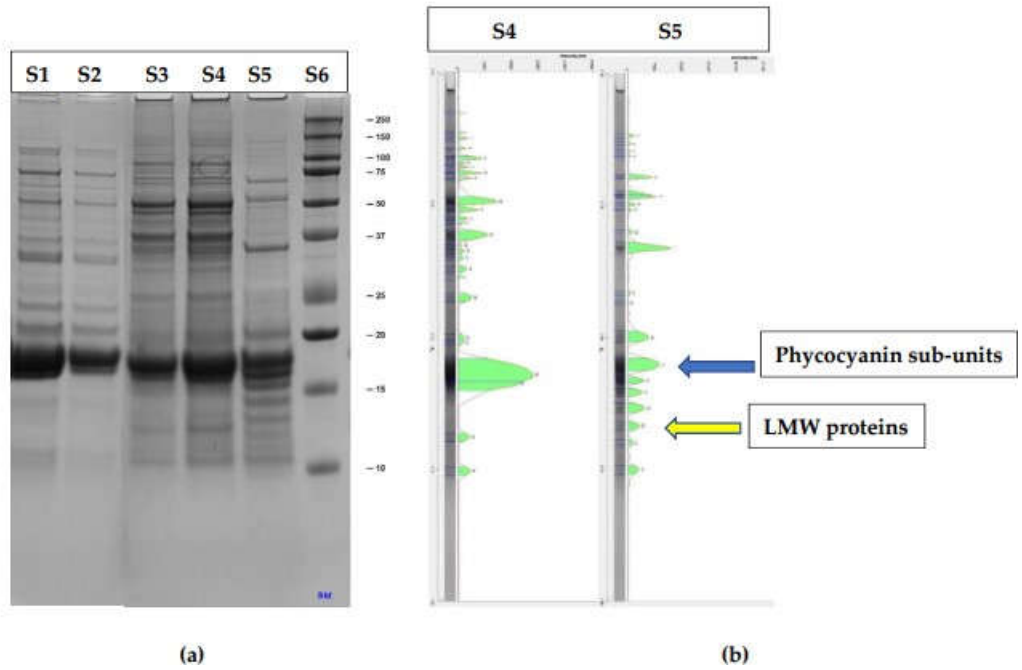

Figure 1. Representative SDS-PAGE gel (4-12%, Comassie staining): **(a)** from left to right, Spirulysat® diluted 1:5 and 1:10 (S1, S2), two *A. platensis* supernatants diluted 1:5 (S4) and 1:10 (S3,) and one Spirugrass® supernatant diluted 1:5 (S5); molecular mass marker (S6); **(b**) pherograms obtainend from S4 (*A. platensis* diluted 1:5) and from S5 (Spirugrass® diluted 1:5) are reported as examples highlighting the differences between the two matrices.

The next step involved the analysis of different batches of Spirugrass® as shown in figure 2 where an SDS-PAGE of three different supernatants is reported. In all the samples, bands of low molecular mass between 17 and 18 kDa are present, likely due to the presence of phycocyanin α and β-subunits, while differences in high and medium molecular mass proteins were evident in extracts from batch B4. Attention should be paid to these differences among batches. This variability is not unusual, in fact differences of SDS-PAGE profiles in extracts obtained from different commercial samples *A. platensis* have been previously reported by Isani et al., (2022b), suggesting that what we have found in Spirugrass® extracts might be due differences of the starting biomasses [4].

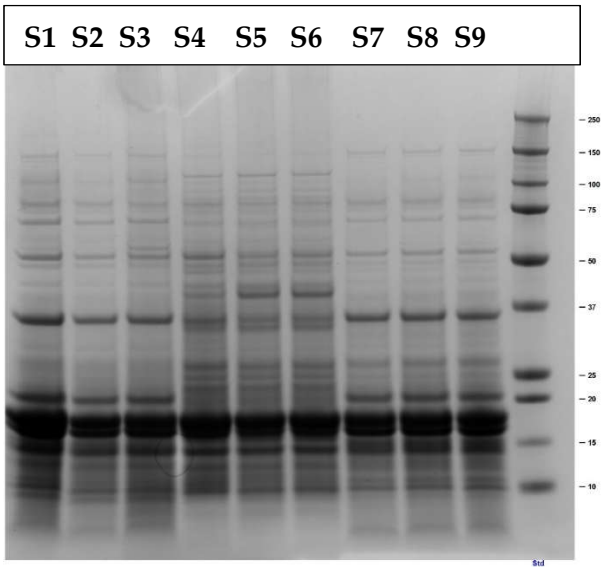

Figure 2. Representative SDS-PAGE gel (4-12%, Comassie staining): Spirugrass® of three different batches B2 (S1, S2, S3), B4 (S4, S5, S6), B1 (S7, S8, S9). Each supernatant was analyzed in triplicate.

Non-denaturing size exclusion chromatography (SEC) allowed the separation of soluble proteins followed by phycocyanin analysis reading the absorbance at 620 nm and iron determination using AAS. The presence of phycocyanin was confirmed after the analysis of fractions. In supernatants of *A. platensis* phycocyanin was present between fractions 10 and 15 which contain proteins of molecular mass between >75 and 60 kDa, in accordance with previous findings (figure 3) [4]. The amount of phycocyanin in Spirugrass® was lower and distributed in two peaks of different molecular mass, the first one at >75 kDa and the second one at 35-40 kDa, suggesting the presence of different aggregation states of the protein.

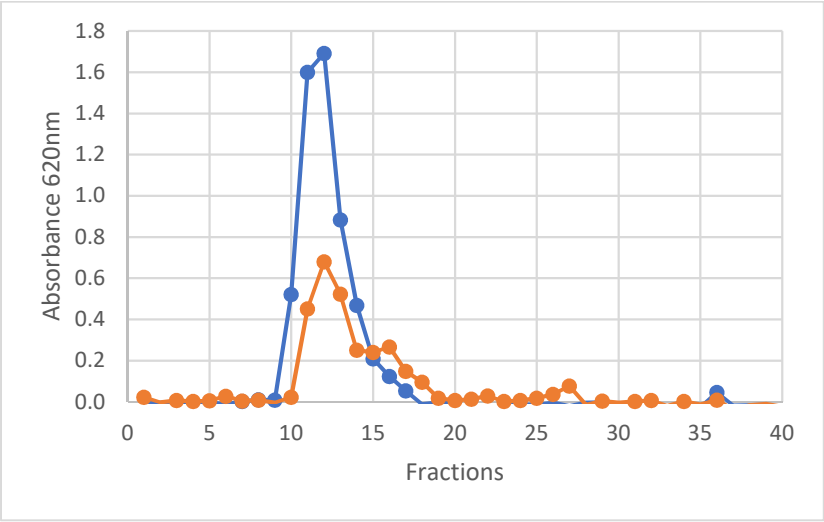

Figure 3. Chromatographic pattern after the size exclusion chromatography of supernatants obtained from *A. platensis* (blue) and Spirugrass® (orange). Phycocyanin was detected at 620 nm.

3.2 Iron content and speciation

A. platensis is characterized by a high iron content. Nevertheless, it has been reported a wide variation in iron content of different samples of *A. platensis* [4]. For this reason, a preliminary analysis iron content of *A. platensis*, Spirugrass® and Spirulysat® was measured using AAS. Data are reported in Table 1. Iron content measured in *A. platensis* biomass (374±94 μg g^{-1}) was in line with those reported by Isani et al. (2022b) in commercial samples of *A. platensis*. Interestingly, a consistent iron content was still present in Spirugrass® (261±15 μg g^{-1}), suggesting a possible use in the field of animal supplementation [4].

Table 1. Iron content of *A. platensis*, Spirugrass® and Spirulysat® measured using AAS. Data are reported as mean ± standard deviation (n=3).

Matrix	Fe μg g^{-1}	Fe μg mL^{-1}
Platensis biomass	374.33 ± 94.04	
Spirugrass®	261.15 ± 15.01	
Spirulysat®		5.87 ± 3.37

Iron speciation was studied by combining a chromatographic fractionation of proteins using SEC associated with sensitive metal detection in fractions using AAS, a hyphenated approach which is commonly used in metallomics studies. Iron content was therefore measured in all the fractions obtained from SEC of *A. platensis* and Spirugrass® supernatants (figure 4). The two samples showed a major peak of iron detected between fractions 10 and 13, indicating that the element is bound to high molecular mass proteins between >75kDa and 60 kDa; the profile obtained after SEC is in line with those reported by Isani et al. (2022b) [4]. Moreover, the major peak of iron is overlapping to the peak of phycocyanin, confirming that this protein can bind iron also in Spirugrass®. A second lower peak of iron was detected between fractions 14-17, as reported by Isani et al. (2022b) who found in commercial samples of *A. platensis* iron also bound to ligands with intermediate molecular mass [4].

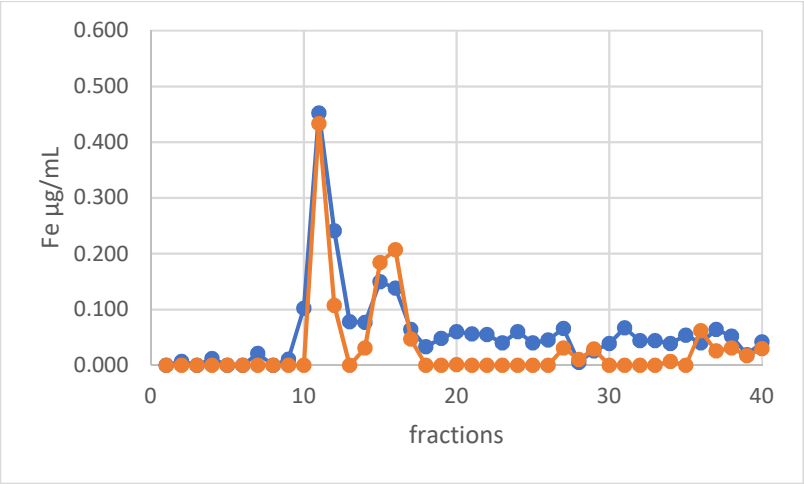

Figure 4. Iron concentrations in SEC fractions of *A. platensis* (blue) and Spirugrass® (orange) supernantants.

3.3 Effect of HPP on Spirugrass® proteins

HPLC analysis was applied to search for differences in proteomic profiles due to the HPP treatment. Representative chromatograms of one batch of Spirugrass® before and after treatment are reported in figure 5.

Before HPP, the chromatogram at 280 nm showed four peaks (figure 5). After HPP treatment, the chromatogram showed remarkable changes. In particular, the first peak, containing high molecular mass proteins (≥160 kDa), turned in a lower and wider peak. The second and third peaks, representative of the blue fractions traceable to phycocyanin, showed differences between the chromatograms. Particularly, after HPP, the two peaks were less clearly separated and the third peak resulted higher than the second one. It has been reported that phycocyanin is very sensible to structure modification, due to temperature, pH, solvent used and techniques of extractions as high-pressure methods [5]. Noteworthy, pressure of 600 MPa (equal to 6000 bar used in HPP) led to 50% of colour losses, and seemed to be likely related to modifications of phycocyanin structure [5]. Furthermore, the hypothesis of structure modification or partial degradation of high molecular mass proteins and phycocyanin might be confirmed by the increase of the fourth peak of the chromatogram which contains lower molecular mass proteins and peptides.

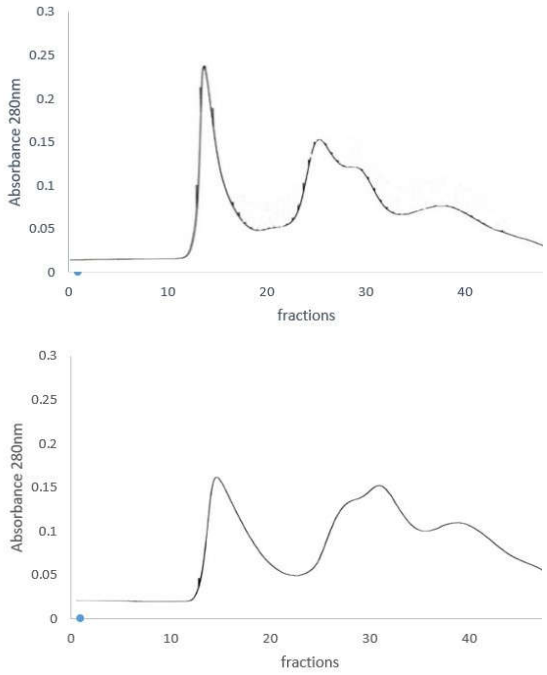

Figure 5. HPLC chromatograms of batch 4 of Spirugrass® before HPP (above) and after HPP (below).

Afterwards, absorbance at 620 nm was read in fractions from 20th to 35th, to detect changes in phycocyanin due to the HPP treatment. Absorbance values of fractions of interest are reported in figure 6. Before treatment, two peaks of absorbance were measured in fractions 23 and 31, while after HPP the peaks were less evident and a shift towards low molecular mass molecules was detected.

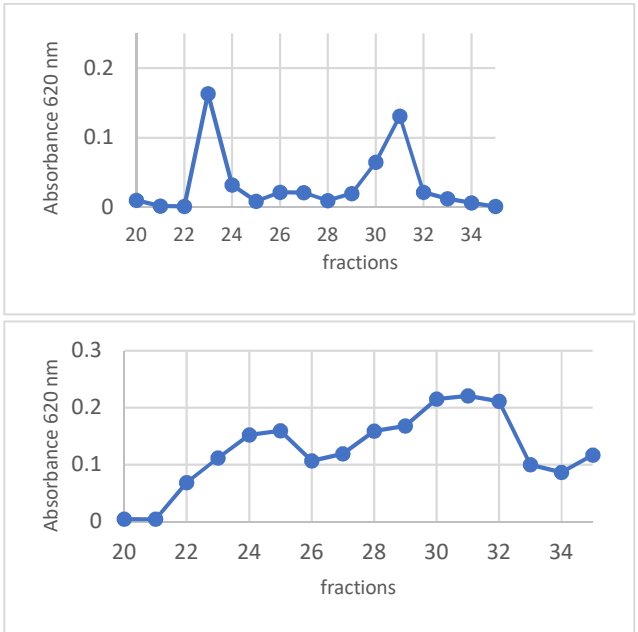

Figure 6. Chromatograms of fractions of interest of batch 4 of Spirugrass® before HPP (above) and after HPP (below). Phycocyanin was detected at 620nm.

Finally, electrophoresis was applied to evaluate and separate the proteome of Spirugrass® before and after HPP. Representative SDS-PAGE gel is reported in figure 6. Phycocyanin is

represented by the two sub-unit α and β at 18 and 17 kDa. After the treatment only slight differences in protein profile were detected, while an evident decrease of band intensity was noted. As reported in bibliography, phycocyanin is a very sensible molecule to structure modifications [5].

SDS-PAGE confirmed that no intense protein degradation was present after HPP, suggesting a potential use of Spirugrass® as feed supplement after HPP treatment.

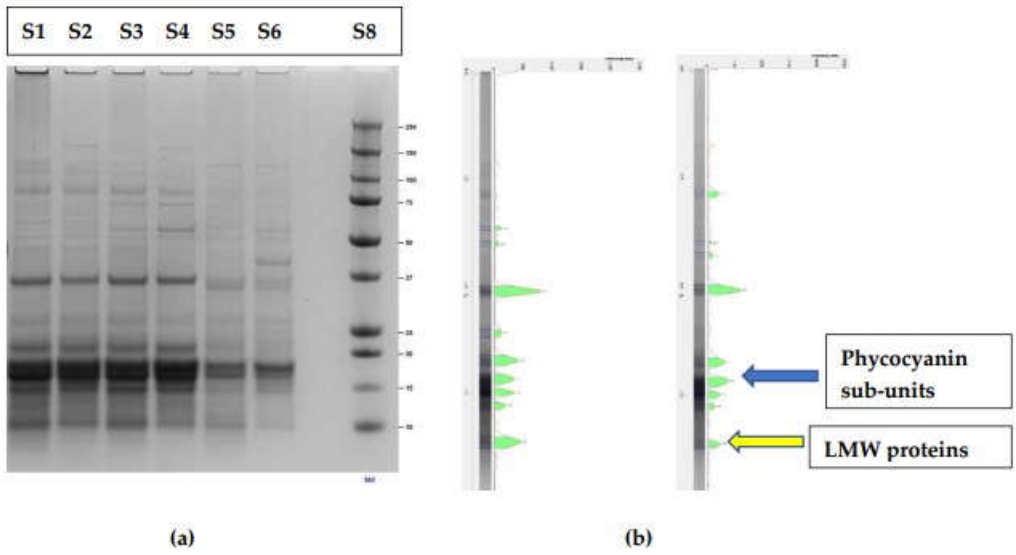

Figure 7. Representative SDS-Page gel (4-12%, Comassie staining): **(a)** Spirugrass® supernatants after (S1(B1), S3 (B2), S5 (B4)) and before (S2 (B1), S4 (B2), S6 (B4)) HPP. Std (S8): molecular mass marker; **(b)** pherograms obtained from S1 (Spirugrass® of B1 after HPP treatment) and from S2 (Spirugrass® of B1 before HPP treatment) are reported as examples highlighting the differences between Spirugrass® before and after HPP treatment.

4. Conclusions

After SDS-PAGE, the intensity of bands of phycocyanin subunits suggests that a considerable amount of proteins, albeit lower than in *A. platensis*, is still present in Spirugrass®. The SDS-PAGE analysis also highlighted batch-to-batch variations in Spirugrass®, underscoring the need for quality control before utilizing Spirugrass® as a feed ingredient.

The investigation of iron speciation using SEC and AAS showed that iron is predominantly bound to high molecular mass proteins, including phycocyanin, in both *A. platensis* and Spirugrass®. The presence of a consistent amount of iron in Spirugrass® suggests its potential use as a source of this essential trace element in feed supplementation.

The application of high-pressure pasteurization (HPP) on Spirugrass® biomass derived from *A. platensis* revealed alterations of the proteomic profile. The observed reduction in the first peak containing high molecular mass proteins after HPP suggests a potential degradation or modification of these proteins. Notably, also the second and third peaks,

indicative of the blue fractions containing phycocyanin, showed changes after HPP. The sensitivity of phycocyanin to degradation processes, as reported in the literature, further supports the hypothesis that the applied pressure during HPP might influence protein structure leading to changes of phycocyanin aggregation state. The increase of the fourth peak in the chromatograms, representing lower molecular mass proteins and peptides, adds evidence supporting the hypothesis of possible modifications of high molecular mass proteins and phycocyanin.

In conclusion, this research on Spirugrass® and its response to HPP has provided valuable insights into its potential utilization in animal nutrition. These findings have implications for the potential utilization of microalgae by-products in a circular economy, particularly in the field of animal feed supplementation. Moreover, understanding the effects of HPP contributes valuable knowledge for optimizing extraction processes and ensuring the quality of bioactive compounds. Further investigations are needed to explore the specific mechanisms by which HPP influences protein structures and to optimize conditions for preserving the integrity of valuable components of *A. platensis*.

5. References

1. Souza, C.M.M, Bastos, T.S., Santos, M.C. Microalgae use in animal nutrition. *Research society and development*, **2021**, 10, 16.
2. Bennet, A., Bogorad, L. Complementary chromatic adaptation in a filamentous blue-green alga. *Journal of cell biology*, **1973**, 58, 2, 419-435.
3. Isani, G., Niccolai, A., Andreani, G., Dalmonte, T., Bellei, E., Bertocchi, M., Tredici, M.R., Rodolfi, L. Iron speciation and iron binding proteins in *Arthrospira platensis* grown in media containing different iron concentrations. *International journal of molecular sciences*, **2022**, 23, 6283.
4. Isani, G., Ferlizza, E., Bertocchi, M., Dalmonte, T., Menotta, S., Fedrizzi, G., Andreani, G. Iron content, iron speciation and phycocyanin in commercial samples of *Arthrospira* spp. *International journal of molecular sciences*, **2022**, 23, 13949.
5. Jaeschke, D.P., Teixera, I.R., Marczak, L.D.F., Mercali, G.D. Phycocyanin from Spirulina: a review of extraction methods and stability. *Food research international*, **2021**, 143, 110314.

3. CONCLUSIONS

Supplementation of *B. serrata* and *S. alba* in laying hens has shown that these two phytoextracts can be considered safe and didn't affect productivity performance and blood biochemical parameters in the subjects of the study. Moreover, no negative effects have been detected during pre-laying and early laying phases in serum proteins, egg albumen proteins and cholesterol content in yolk between treated and control group. Moreover, in eggs of treated group a lower cholesterol content was detected, suggesting an hypocholesterolemic effect of the supplementation. This research provides fundamental for further research to explore the supplementation efficacy in poultry field, especially in laying hens reared on intensive farms and to deepen the potential effects on cholesterol content in eggs.

Furthermore, the meta-analysis study offered significant insights into the potential efficacy of *Boswellia* extracts in knee OA management, particularly in alleviating symptoms as reflected by lower WOMAC scores. The findings suggest a promising alternative for individuals that suffered the NSAIDs side effects. However, the paucity of complete data highlighted the need of additional high-quality research to establish a more comprehensive understanding of the clinical benefits and safety profile of *Boswellia* species in knee OA management. This research boost further studies of the use of *Boswellia* extracts in the management of osteoarticular diseases also in animals. However, there is a lack of studies regarding the treatment of OA in animals, although OA is a common disease especially in dogs.

Commercial samples of *A. platensis*-based supplements showed a high variability in iron content, confirming data reported in bibliography where processing, storage and growing conditions affect the biochemical profile of the microalgae. Moreover, the use of atomic absorption spectrometry provided a fingerprint in trace elements field between different supplements of Spirulina sold in the market. The authors demonstrated that the content of iron in *A. platensis* can be improved through varying iron concentrations in media of cultivation. *A. platensis* showed that its iron content could be improved increasing iron concentration in medium. Furthermore, iron speciation was studied using SEC followed by spectrometry techniques, SDS-PAGE, AAS, suggesting the potential role of phycocyanin as the main iron binding protein in *A. platensis*. The studies hold promise for producing spirulina-based supplements with different iron content based on the control of the concentration of this essential trace element in the culture medium. However, future researches are needed on iron-binding ligands in cyanobacteria.

Afterwards, research on *A. platensis* continued comparing its supplementation with other microalgae used as feed additives in animal nutrition (*C.vulgaris, P.tricornutum* and *H.pluvialis*) through an *in vitro* canine digestion system. The digestion simulation allowed to investigate the bioaccessibility of iron. *C. vulgaris* was found to have the highest bioaccessible iron content, making it a potentially interesting iron supplement in dog nutrition. Spirulina showed also a high iron bioaccessible fraction and high digestibility

percentage of 31 and 86%, respectively. *A. platensis* and *C. vulgaris* can be both useful as feed additives in animal nutrition field, due to the content of iron and proteins. The study established promising foundation for further investigation into microalgae used as supplementation, emphasizing implication for animal nutrition.

The study explored the potential use of Spirugrass®, a residual of phycocyanin extraction from biomasses of *Arthrospira platensis*, in animal nutrition field with a reduced environmental impact. High-pressure pasteurization (HPP) altered proteomic profiles, notably reducing high molecular mass proteins and inducing possible changes in phycocyanin aggregation state. However, further investigations are needed to understand the mechanisms and optimize conditions for preserving valuable components.

4. REFERENCES

1. Xutian, S., Zhang, J., Louise, W. New exploration and understanding of traditional Chinese medicine. *American journal of Chinese medicine*, **2009**, 37, 411-426.
2. Andrade, F.A., Portella, C.F.S. Research methods in complementary and alternative medicine: an integrative review. *Journal of integrative medicine*, **2018**, 16, 6-13.
3. Ghosh, D. Seed to patient in clinically proven natural medicines. *Nutraceuticals*, **2014**, 64, 925-930.
4. WHO. Traditional, complementary and integrative medicine. https://www.who.int/health-topics/traditional-complementary-and-integrative-medicine#tab=tab_1
5. Barnes, P.M., Bloom, B., Nahin, R.L. Complementary and alternative medicine use among adults and children: United States, 2007. *National health statistics reports*, **2008**, 12, 1-24.
6. WHO. National policy on traditional medicine and regulation of herbal medicine: report of a WHO global survey. *WHO press*, **2005**, Geneva, Switzerland.
7. https://pubmed.ncbi.nlm.nih.gov/?term=herbal+medicine+
8. Furst, R., Zundorf, I. Evidence-based phytotherapy in Europe: where do we stand?. *Planta medica*, **2015**, 81, 962-967.
9. Potterat, O., Hamburger, M. Drug discovery and development with plant-derived compounds. *Progress in drug research*, **2008**, 65, 47-118.
10. Varoni, E.M., Lodi, G., Iriti, M. Efficacy behind activity – phytotherapeutics are not different from pharmaceuticals. *Pharmaceutical biology*, **2015**, 53, 404-406.
11. Colalto, C. What phytotherapy needs: evidence-based guidelines for better clinical practice. *Phytotherapy research*, **2018**, 32, 413-425.
12. Capasso, R., Izzo, A.A., Pinto, L., Bifulco, T., Vitobello, C., Mascolo, N. Phytotherapy and quality of herbal medicines. *Fitoterapia*, **2000**, 71, 858-865.
13. Izzo, A.A., Hoon-Kim, S., Radhakrishnan, R., Williamson, E.M. A critical approach to evaluating clinical efficacy, adverse events and drug interactions of herbal remedies. *Phytotherapy research*, **2016**, 30, 691-700.
14. Mikulski, M.A., Wichman, M.D., Simmons, D.L., Pham, A.N., Clottey, V., Fourtes, L.J. Toxic metals in ayurvedic preparations from a public health lead poisoning cluster investigation. *International journal of occupational and environmental health*, **2017**, 23, 3, 187-192.
15. Gyamfi, E.T. Metals and metalloids in traditional medicines (Ayurvedic medicines, nutraceuticals and traditional Chinese medicines). *Environmental science and pollution research*, **2019**, 26, 15767-15778.
16. Sarma, H., Deka, S., Deka, H., Saikia, R.R. Accumulation of heavy metals in selected medicinal plants. *Reviews of environmental contamination and toxicology*, **2011**, 214, 63-86.
17. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32004L0024 (accessed on 4th of Semptember 2023).
18. https://www.ema.europa.eu/en/human-regulatory/herbal-medicinal-products (accessed on 4th of Semptember 2023).

19. Qu, L., Zou, W., Wang, Y., Wang, M. European regulation model for herbal medicine: the assessment of the EU monograph and the safety and efficacy evaluation in marketing authorization or registration in Member state. *Phytomedicine*, **2018**, 42, 219-225.
20. WHO. WHO traditional medicines strategy 2014-2023. *WHO*, **2013**, Geneva, Switzerland, https://www.who.int/publications/i/item/9789241506096
21. Rastogi, S., Pandey, M.K., Prakash, J., Sharma, A., Singh, G.N. Veterinary herbal medicines in India. *Pharmacognosy reviews*, **2015**, 9, 18, 155-163.
22. McCorkle, M., Martin, M. Parallels and potentials in animal and human ethnomedical technique. *Agriculture and human values*, **1998**, 15, 139-144.
23. Alasbahi, R., Groot, M.J. Ethnoveterinary uses of certain Yemeni plants: a review of the scientific evidence. *Planta medica*, **2022**, 88, 237-253.
24. Assefa, A., Bahiri, A. Ethnoveterinary botanical survey of medicinal plants in Abergelle, Sekota and Lalibela districts of Amhara region, northern Ethiopia. *Journal of ethnopharmacology*, **2018**, 213, 340-349.
25. Uprety, Y., Karki, S., Poudel, R.C., Kunwar, R.M. Ethnoveterinary use of plants and its implication for sustainable livestock management in Nepal. *Veterinary science*, **2022**, 9, 1-11.
26. EFSA. Scientific opinion on the overall effects of farming system on dairy cow welfare and disease. *EFSA journal*, **2009**, 1143, 1-38.
27. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed on 5th of Semptember 2023).
28. Commision implementing regulation (EU) No 505/2012 of 14 June 2012. *Official journal of the European Union*, **2012**.
29. Mayer, M., Vogl, C.R., Amorena, M., Hamburger, M., Walkenhorst, M. Treatment of organic livestock with medicinal plants: a systematic review of european ethnoveterinary research. *Forschende Komplementarmedizin*, **2014**, 21, 375-386.
30. Blanco-Penedo, I., Gonzalez, C.F., Tamminen, L.M., Sundrum, A., Emanuelson, U. Priorities and future actions for an effective use of phytotherapy in livestock – outputs from an expert workshop. *Frontiers in veterinary science*, **2018**, 4, 248, 1-7.
31. European Parliament, Council of Europen Union of 10th of June 2002. Directive 2002/46/EC on the approximation of the laws of the Member States relating to food supplements. *Official journal of European Union*, **2002**, L183, 51. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02002L0046-20220930 (accessed on 7th of Semptember 2023).
32. European Parliament; Council of the European Union. Regulation (EU) 2015/2283. *Official journal of European Union*, **2015**, L327/1, 1-22. https://food.ec.europa.eu/safety/novel-food/legislation_en (accessed on 7th of Semptember 2023).
33. Ververis, E., Ackler, R., Azzollini, D., Colombo, P.A., Sesmaisons, A., Dumas, C., Fernandez-Dumont, A., Costa, L.F., Germini, A., Goumperis, T., Kouloura, E., Matijevic, L., Precup, G., Roldan-Torres, R., Rossi, A., Svejstil, R., Turla, E., Gelbmann, W. Novel foods in the European Union: scientific requirements and challenges of the risk assessment process by the European Food Safety Authority. *Food research journal*, **2020**, 137, 109515.

34. European Union. Regulation (EC) 767/2009 of the European Parliament and the Council of 13th July 2009. *Offical journal of European Union*, **2009**, L229, 1-28.
35. https://food.ec.europa.eu/safety/animal-feed/feed-additives_en
36. Von Holst, C., Robouch, P., Bellorini, S., Huebra, M.J.G., Ezerskis, Z. The work of the European Union Reference Laboratory for food additives (EURL) and its support for the authorisation process of feed additives in the European Union: a review. *Food additives & contaminants*, **2016**, 33, 1, 66-77.
37. European Union. Regulation (EC) 1831/2003 of the European Parliament and the Council of 22th September 2003. *Offical journal of European Union*, **2003**, L268, 1-29.
38. Brendler, T., Brinckmann, J.A., Schippmann, U. Sustainable supply, a foundation for natural product development: the case of Indian frankincense (*Boswellia serrata* Roxb. Ex Colebr.). *Journal of ethnopharmacology*, **2018**, 225, 279-286.
39. https://www.industryarc.com/Research/Boswellia-Market-Research-511116
40. Mendes, M.C., Navalho, S., Ferreira, A., Paulino, C., Figueiredo, D., Silva, D., Gao, F., Gama, F., Bombo, G., Jacinto, R., et al. Algae as food in Europe: an overview of species diversity and their application. *Foods*, **2002**, 11, 1871.
41. MOE. The national red list 2012 of Sri Lanka; conservation status of the fauna and flora. *Ministry of environment (Colombo, Sri Lanka)*, **2012**.
42. Al-Yasiry, A.R.M., Kiczorowska, B. Frankincense – therapeutic properties. *Postepy higieny i medycyny doswiadczalnej*, **2016**, 70, 380-391.
43. Ammon, H.P.T. Boswellic acids in chronic inflammatory diseases. *Planta medica*, **2006**, 72, 12, 1100-1116.
44. Gerbeth, K., Husch, J., Fricker, G., Werz, O., Schubert-Zsilavecz, M., Abdel-Tawab, M. *In vitro* metabolism, permeation, and brain availability of six major boswellic acids from *Boswellia serrata* gum resins. *Fitoterapia*, **2013**, 84, 99-106.
45. Al-Yasiry, A.R.M., Kiczorowska, B., Samolinska, W. Effect of *Boswellia serrata* resin supplementation on basic chemical and mineral element composition in the muscles and liver of broiler chickens. *Biological trace element research*, **2017**, 179, 294-303.
46. Ismail, I.E., Abdelnour, S.A., Shehata, S.A., El-Hack, M.E.A., El-Edel, M.A., Taha, A.E., Schiavitto, M., Tufarelli, V. Effect of dietary *Boswellia serrata* resin on growth performance, blood biochemistry, and cecal microbiota of growing rabbits. *Frontiers in veterinary science*, **2019**, 6, 471.
47. Martello, E., Bigliati, M., Adami, R., Biasibetti, E., Bisanzio, D., Meineri, G., Bruni, N. Efficacy of a dietary supplement in dogs with osteoarthritis: a randomized placebo-controlled, double-blind clinical trial. *PLOS ONE*, **2022**, 17, 2.
48. Yakoob, Z., Ali, E., Zainal, A., Mohamad, M., Takriff, M.S. An overview: biomolecules from microalgae for animal feed and aquaculture. *Journal of biological research*, **2014**, 21, 6.
49. Gustafson, K.R., Cardellina, J.H., Fuller, R.W., Weisiow, O.S., Kiser, R.F., Snader, K.M., Patterson, G.M., Boyd, M.R. AIDS-antiviral sulfolipids from cyanobacteria (blue-green algae). *Journal of the national cancer institute*, **1989**, 81, 16, 1254-1258.
50. Safi, C., Zebib, B., Merah, O., Pontalier, P., Garcia, C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review. *Renewable and sustainable energy reviews*, **2014**, 35, 265-278.

51. Habib, M.A.B., Parvin, M., Huntington, T.C., Hasan, M.R. A review on culture, production and use of spirulina as food for humans and feeds for domestic animals and fish. *FAO fisheries and aquaculture circular*, **2008**, 1034, 33.
52. Gentscheva, G., Nikolova, K., Panayotova, V., Peycheva, K., Makedonski, L., Slavov, P., Radusheva, P., Petrova, P., Yotkovska, I. Application of *Arthrospira platensis* for medicinal purposes and the food industry: a review of the literature. *Life*, **2023**, 13, 845.
53. https://www.melissafoundation.org/
54. https://www.pro-future.eu/
55. Morel, F.M.M, Price, N.M. The biogeochemical cycles of trace metals in the oceans. *Science*, **2003**, 300, 944-947.
56. Bannu, S., Lomada, D., Gulla, S., Chandrasekhar, T., Reddanna, P., Reddy, M.C. Potential therapeutic applications of C-Phycocianin. *Current drug metabolism*, **2019**, 20, 967-976.
57. Yu, P., Wu, Y., Wang, G., Jia, T., Zhang, Y. Purification and bioactivities of phycocyanin. *Critical reviews in food science and nutrition*, **2016**, 21, 14.
58. Ashaolu, T.J., Samborska, K., Lee, C.C., Tomas, M., Capanoglu, E., Tarhan, O., Taze, B., Jafari, S.M. Phycocyanin, a super functional ingredient from algae; properties, purification characterization, and applications. *International journal of biological macromolecules*, **2021**, 193, 2320-2331.
59. Bermejo, P., Pinero, E., Villar, A.M. Iron-chelating ability and antioxidant properties of phycocyanin isolated from a protean extracts of *Spirulina platensis*. *Food chemistry*, **2008**, 110, 436-445.

5. APPENDIX

5.1 ADDITIONAL PAPER

Roncada, P., Isani, G., Peloso, M., Dalmonte, T., Bonan, S., Caprai, E. Pyrrolizidine alkaloids from monofloral and multifloral italian honey. *International journal of environmental research and public health*, **2023**, 20, 5410.

In this paper the PhD student took care of the statistical analysis regarding the presence of pyrrolizidine alkaloids (PA) in the honey of three different Italian regions following the rules suggested by EFSA for the analysis of this type of compounds. Particularly, the lower-bound approach was used to substitute with zero the values below the LOQ, as indicated by EFSA. The central limit theorem was taken into consideration to assess the normality of distribution. Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons post-hoc test was performed to determine significant differences among the honey types in the same region of origin and among the honey types without taking into consideration the origin. The *p*-value obtained underwent Bonferroni's correction. The Levene test was performed to assess the homoscedasticity and consequently the robust one-way ANOVA Tahmane post-hoc test was applied to detect significant differences of the PA/PANO content among the three different regions. The presence/absence of PAs was used to create a dichotomous variable and X2 test was performed to detect significant association between the above-mentioned variable, the honey types and the region of origin. The type and the strength of association were assessed by calculating the Cramer V value.

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Article

Pyrrolizidine Alkaloids from Monofloral and Multifloral Italian Honey

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Abstract: Pyrrolizidine alkaloids (PAs) are secondary metabolites produced by plants as a self-defense against insects. After bioactivation in the liver, some PAs can cause acute or chronic toxicity in humans. The aim of this study was to determine the presence of PAs in 121 samples of monofloral and multifloral honey from three different Italian regions (Friuli-Venezia Giulia, Marche and Calabria) to meet the European Food Safety Authority (EFSA) suggestion. An in-house liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was validated according to European Union Reference Laboratory (EURL) performance criteria. This method allowed the detection and quantification of 35 PAs. Of the 121 honey samples, 38 (31%), mostly from Calabria, contained PAs. The total content of the PAs ranged from 0.9 µg/kg to 33.1 µg/kg. In particular, echimidine was the most prevalent PA. A rapid human exposure assessment to PAs in honey and a risk characterization was performed using the EFSA RACE tool. The assessment highlighted a potential health concern only for toddlers who frequently consume elevated quantities of honey. This study showed a low presence of PAs in Italian honey; however, the importance of continuously monitoring these compounds is stressed, along with the suggestion that the relevant authorities establish maximum limits to guarantee support for producers and consumer safety.

Keywords: honey; Boraginaceae; pyrrolizidine alkaloids; echimidine; health risk assessment

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1. Introduction

Pyrrolizidine alkaloids (PAs) and their N-oxides (PANOs) are secondary metabolites derived from a necine base produced by plants as a self-defense against insects. Pyrrolizidine alcaloids have received increasing attention due to their toxicity as well as their presence in several plant species relevant to human and animal nutrition [1]. More than 660 different PAs have been identified [2]. They have been detected in more than 6000 plant species [2,3], mainly *Senecio* spp. and *Eupatorium* spp. (Asteraceae), *Echium* spp. (Boraginaceae) and *Crotalaria* spp. (Fabaceae) [4]. The PA content can vary depending on the plant species, site of accumulation, harvest time and climatic conditions. In general, they are found in greater quantities in flowers and seeds and to a lesser extent in leaves, stamens, and roots. The possible routes of human dietary exposure to PAs occur through the ingestion of plants and herbal products (drugs, herbal teas, dietary supplements) [5–8] as well as animal products such as honey [4]. Honey and other hive products can be contaminated with PAs as a results of bees foraging on alkaloid-producing plants [9]. Recent studies have shown that PAs can also be found in water and soil [10,11].

The toxicity of PAs has been widely documented, being almost exclusively associated with their metabolites [1]. Pyrrolizidine alkaloids themselves are pro-toxins, biologically and toxicologically inactive, and need to be metabolically activated in order to exert toxicity;

consequently, if not activated, they do not develop toxicity [5]. These compounds are mainly bioactivated in the liver by CYP450 monooxygenases [1,2,4,12] producing 6,7-dihydro-7-hydroxy-1-(hydroxymethyl)-5H-pyrrolizine (ester pyrrolyl), a strong electrophile that can rapidly bind to nucleophilic centers such as nucleic acids, proteins and amino acids, forming pyrrole complexes that can persist in tissues and generate toxicity, especially in the liver [2,9,13–15]. These compounds have been shown to be hepatotoxic, pneumotoxic, genotoxic and carcinogenic and exhibit developmental toxicity [5,7]. An important detoxification pathway is by conjugation with glutathione, forming soluble compounds that are much less toxic and more easily eliminated [16].

Pyrrolizidine alkaloids are hepatotoxic to animals and humans; they can cause acute toxicity and have chronic effects [9,12,17]. Chronic exposure to low levels of PAs can cause liver cirrhosis and cancer as metabolic activation produces genotoxic and carcinogenic reactive pyrrolic forms [9]. The International Agency for Research on Cancer (IARC) has evaluated several PAs and has classified lasiocarpine, monocrotaline and riddelliine as Group 2B (possibly carcinogenic to humans), while hydroxysenkirkine, isatidine, jacobine, retrorsine, seneciphylline, senkirkine and symphytine were included in Group 3 (not classifiable as to their carcinogenicity to humans) [15,18,19]. As a consequence, the EFSA, the European Food Safety Authority, has repeatedly considered the issue of the presence of pyrrolizidine alkaloids in food with the aim of establishing the level of risk to public health [12,13].

In fact, maximum levels in bee pollen have been set by the Commission Regulation (EU) 2020/2040 [1,20], while there is still no regulation for the presence of these alkaloids in honey and limits to establish criteria for acceptance or rejection in the marketing of this food have not yet been set [21]. For this reason, the EFSA has suggested collecting data regarding the content of PAs/PANOs in honey of different geographical and botanical origins. Exhaustive data regarding PA/PANO content in honey from Italy are scarce. Lucatello et al. (2021) recently found 17 PAs/PANOs in honey samples from the Veneto region and showed that 45% of the samples analyzed contained at least one PA. However, the consumption of this honey did not seem to represent a risk for adult consumers [22].

To meet the EFSA suggestion, the aim of this research was to determine the presence of PAs in Italian honey. Honey samples from three Italian regions (Friuli-Venezia Giulia, Marche, and Calabria) were analyzed for their PA/PANO content using an in-house liquid chromatography with tandem mass spectrometry (LC-MS/MS) method, developed and validated by the National Reference Laboratory for Plant Toxins in Food (LNR-TVN) of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) in Bologna. This method is capable of detecting up to 35 analytes, according to Commission Regulation (EU) 2020/2040 [20]. The assessment of human exposure to PAs in honey has been estimated on the basis of the results of the analysis in order to characterize the health risk for all age groups of the population.

2. Materials and Methods

2.1. Sampling

A total of 121 different types of honey were collected from several Italian beekeepers in three Italian regions: Friuli-Venezia Giulia (n = 37), Marche (n = 39) and Calabria (n = 45). The province of origin of the honey is reported in Table 1. Samples included both multifloral (n = 34) and monofloral (n = 87) honey. Monofloral honey was the most representative and the plant species are reported in Table 2.

Table 1. Botanical characteristics and origin of honey samples.

Honey Types	Origin
Multifloral, Winter heath, Common whitebeam, Dandelion, Acacia, Chestnut and Linden	Udine (Friuli-Venezia Giulia)
Multifloral, Rapeseed, Linden, Acacia, Bastard Indigobush and Chestnut	Pordenone (Friuli-Venezia Giulia)
Multifloral and Mahaleb Cherry	Trieste (Friuli-Venezia Giulia)
Multifloral, Sunflower, Rapeseed, Honeydew, Mustard, Clover and Betony	Ancona (Marche)
Multifloral, Chestnut and Acacia	Fermo (Marche)
Multifloral and Sunflower	Macerata (Marche)
Multifloral, Chestnut, Honeydew and Acacia	Ascoli Piceno (Marche)
Multifloral, Acacia, Sulla, Citrus Fruits and Chestnut	Vibo Valentia (Calabria)
Acacia, Citrus Fruits and Chestnut	Cosenza (Calabria)
Multifloral, Acacia, Sulla, Citrus Fruits, Eucalyptus and Chestnut	Catanzaro (Calabria)
Chestnut and Winter heath	Crotone (Calabria)
Acacia and Citrus Fruits	Reggio Calabria (Calabria)

Table 2. Botanical origin of monofloral honey samples.

Monofloral Honey Types	Total Samples
Acacia or robinia (*Robinia pseudoacacia*)	25
Chestnut (*Castanea sativa*)	15
Sunflower (*Heliantus annuus L.*)	14
Citrus fruits	7
Linden (*Tilia L.*)	6
Rapeseed (*Brassica napus*)	4
Honeydew	3
Winter heath (*Erica carnea*)	2
Sulla (*Sulla coronaria*)	2
Eucalyptus (*E. camaldulensis, E. occidentalis*)	2
Mustard (*Sinapis arvensis*)	1
Clover (*Trifolium pratense*)	1
Betony (*Stachys officinalis*)	1
Common whitebeam (*Sorbus aria*)	1
Bastard indigobush (*Amorpha fruticosa*)	1
Dandelion (*Taraxacum officinale*)	1
Mahaleb cherry (*Prunus mahaleb*)	1

2.2. Chemicals and Standards

Analytical standards of all pyrrolizidine alkaloids and their N-oxide were purchased from Phytolab (Vestenbergsgreuth, Germany): Echimidine (Em), Echimidine-N-oxide (EmNO), Echinatine (En), Echinatine-N-oxide (EnNO), Erucifoline-N-oxide (ErNO), Europine (Eu), Europine-N-oxide (EuNO), Heliosupine (Hs), Heliosupine-N-oxide (HsNO), Heliotrine (Ht), Heliotrine-N-oxide (HtNO), Indicine (Id), Indicine-N-oxide (IdNO), Integerrimine (Ir), Integerrimine-N-oxide (IrNO), Intermedine (Im), Intermedine-N-oxide (ImNO), Lasiocarpine (Lc), Lycopsamine (Ly), Lycopsamine-N-oxide (LyNO), Retrorsine (Rt), Retrorsine-N-oxide (RtNO), Rinderine (Rn), Rinderine-N-oxide (RnNO), Senecionine (Sn), Senecionine-N-oxide (SnNO), Seneciphylline (Sp), Seneciphylline-N-oxide (SpNO), Senecivernine (Sv), Senecivernine-N-oxide (SvNO), Senkirkine (Sk), Spartioidine (St), Spartioidine-N-oxide (StNO), Usaramine (Us) and Usaramine-N-oxide (UsNO). Methanol (LC-MS grade) was from VWR Chemicals (Rosny-sous-Bois-cedex, France), sulphuric acid (96%) and acetonitrile (LC-MS grade) were from Carlo Erba Reagents (Val de Reuil Cedex, France), formic acid was from Carlo Erba Reagents (Milan, Italy) and ammonium formate (analytical grade) was from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water used

throughout the experiments was produced by an EvoQua Water Technologies system (Diessechem, Milan, Italy).

2.3. Materials

QuEChERS reagents (magnesium sulphate 4 g, sodium chloride 1 g, sodium citrate 1 g, disodium hydrogen citrate sesquihydrate 0.5 g) were from Agilent Technologies (Santa Clara, CA, USA).

2.4. Working Solutions

Stock standard solutions of each compound were prepared by dissolving suitable quantities of reference material in methanol to obtain a concentration of 1000 µg/mL. Working standard solutions containing all PAs—native and N-oxide—were prepared in water/methanol (95:5 *v*/*v*) for spiking purposes. All solutions were stored at −20 °C [23].

2.5. Sample Preparation

A 2.5 ± 0.1 g aliquot of homogenized sample was weighed into a 50 mL Falcon tube.

Samples were treated as follows: addition of 15 mL of sulphuric acid 0.1 M, vortex and horizontal shaker for 45 min; addition of 15 mL of acetonitrile and QuEChERS extraction reagents, horizontal shaker for 30 min and centrifuged at 3000 rpm at room temperature for 10 min. An aliquot of supernatant extract was dried with a gentle flow of nitrogen in a water bath at 40 °C. The dry extract was dissolved in 1 mL of water/methanol (95:5 *v*/*v*) and transferred into vial for LC-MS/MS analysis. A quality control sample (i.e., spiked sample at LOQ) was assessed at every batch analysis.

2.6. Melissopalynological Analysis

To identify the botanical and geographical origin of honey, a qualitative-quantitative melissopalynological analysis was carried out using the microscopic method UNI 11299:2008. The analysis was performed on four honey samples showing higher quantities of PAs: two multifloral (27.1–33.1 µg/kg) and one chestnut (30.6 µg/kg) from the Calabria and one Stachys honey (9.2 µg/kg) from the Marche. All honey samples were diluted in ultrapure water and centrifuged. The sediment was transferred onto the microscope slide to be examined. For the estimation of the relative frequencies of pollen types, a minimum of 300 pollen grains were counted at 400x magnification using light microscope Axiolab 5 (Carl Zeiss, Jena, Germany) [24].

2.7. Instrumentation

The LC-MS/MS system used was an Acquity ultra-performance liquid chromatograph (UPLC) coupled to Quattro Premiere XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The system was computer-controlled and data acquisition, peak integration and calibration were performed using TargetLynx software v.4.1. The chromatographic column was an Acquity UPLC C8 100 cm × 2.1 mm, 1.7 µm (Water Corporation, Milford, CT, USA). The mobile phase consisted of 5 mM ammonium formate and 0.1% formic acid in water (A) and in methanol (B) [25]. The flow rate was set at 0.3 mL/min; 10 µL was injection volume. The mobile phase gradient was set as follows: from 5% to 20% of B for 10 min, from 20% to 50% for 5 min and return to initial condition for 0.5 min and hold for 1.5 min. Total run time was 17 min. The ESI source was in positive ionization mode with a capillary voltage of 1.0 kV, a cone voltage of 40 V, a source temperature of 120 °C and a desolvation temperature of 450 °C. Ionization and fragmentation conditions for PAs were identified by using a continuous infusion of the tuning solutions and gradually changing the parameters.

2.8. Quantification

Pyrrolizidine alkaloids were identified and quantified on the basis of retention time, ion fragments produced and ion ratio. The retention time had to be ± 0.2 min compared to

reference peaks. In Figures 1 and 2 are shown the chromatograms of a standard mixture of PAs and a standard mixture of PANOs, respectively, at 5 ng/mL concentration.

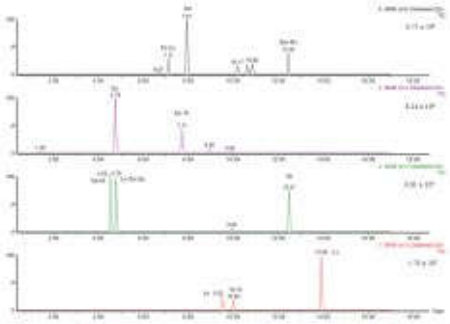

Figure 1. Chromatogram of a standard mixture of PAs at 5 ng/mL concentration.

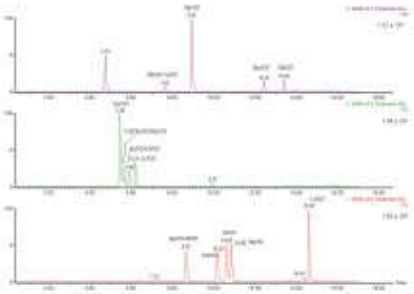

Figure 2. Chromatogram of a standard mixture of PANOs at 5 ng/mL concentration.

A honey matrix-matched calibration curve was prepared to quantify PA content of unknown samples. Pyrrolizidine alkaloid concentration was extrapolated by means of the least squares regression method. Calibration curve concentration levels were 0.5–1–2.5–5–10–25 ng/mL.

2.9. Performance Evaluation

The method was validated according to the EURL-MP guidance document plant toxins performance criteria [26] and Regulation 401/2006/EC [27]. Specificity, recovery rates, linearity, repeatability, within-laboratory reproducibility, limit of detection (LOD) and limit of quantification (LOQ) were evaluated. Specificity was verified and the presence of interference was checked by analyzing 20 honey samples of different species. Correlation coefficients (R^2) of the matrix-matched calibration curve had to be ≥ 0.99 for all analytes. The LOD at 0.5 µg/kg and LOQ at 1 µg/kg were established on the basis of a signal-to-noise ratio, S/N = 3 (LOD) and S/N $\geq$ 5 (LOQ). Repeatability and overall recovery were assessed by analyzing blank samples fortified with PAs at concentrations of 1–10–25 µg/kg in six replicates per level. The same experiment was carried out in two additional sessions to determine within-laboratory reproducibility. The selectivity of the LC–MS/MS method is obtained by acquiring the data in MRM mode and monitoring one precursor ion and two daughter ions for each molecule according to SANTE/12089/2016 [28]. The LC-MS/MS parameters are reported in Table 3. Of the 35 analytes, 14 were isomers and were classified into five groups: Sn-group (Sn, Sv, Ir), Ly-group (Ly, Im, Id, En, Rn), Sp-group (Sp, St), Em-group (Em, Hs) and Rt-group (Rt, Us); the same applied to the N-oxides.

Table 3. The LC-MS/MS parameters for PAs and PANOs (CE: collision energy, Q: Quantifier ion, q: qualifier ion).

Pyrrolizidine Alkaloids	MH+	CE	mb	Q, q
Ne-group	336.2	35 35	120.1 138.0	Q q
Ly-group	299.7	30 35	138.0 156.0	Q q
Ht	314.1	30 35	138.0 156.0	Q q
Es	330	30 35	138.0 156.0	Q q
Sk	366.1	30 25	122.0 167.0	Q q
EsN	366.1	35 25	94.1 109.1	Q q
LyNi-group	316.1	25 25	172.0 136.0	Q q
HtN	330.2	35 35	172.0 111.0	Q q
EsN	346.2	35 30	172.0 326.1	Q q
Lc	412.1	35 30	138.1 220.0	Q q
Sp-group	334	35 35	138.1 138.0	Q q
Em-group	398.6	30 35	119.9 220.4	Q q
Rt-group	352.1	35 35	120.0 136.3	Q q
SnNi-group	352.1	35 30	94.0 138.0	Q q
LcN	428.1	30 25	254.0 94.0	Q q
SpNi-group	350.1	30 25	94.0 120.0	Q q
EmNi-group	414.2	30 25	254.0 220.0	Q q
RtNi-group	368.3	30 30	94.0 120.0	Q q

2.10. Statistical Analysis

The lower-bound approach was used to substitute with zero the values below the LOQ, as indicated by EFSA [12]. The central limit theorem was taken into consideration to assess the normality of distribution [29]. Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons post-hoc test was performed to determine significant differences among the honey types in the same region of origin and among the honey types without taking into consideration the origin. The *p*-value obtained underwent Bonferroni's correction. The Levene test was performed to assess the homoscedasticity and consequently the robust one-way ANOVA Tahmane post-hoc test was applied to detect significant differences of the PA/PANO content among the three different regions.

The presence/absence of PAs was used to create a dichotomous variable and X^2 test was performed to detect significant association between the above-mentioned variable, the honey types and the region of origin. The type and the strength of association were assessed by calculating the Cramer V value [30]. Statistical analyses were performed using R 4.2.1 (R foundation for statistical computing; Vienna, Austria; https://www.R-project.org/ accessed on 15 January 2023). Data are reported as median, mean ± SD (standard deviation). A *p*-value < 0.05 was considered statistically significant.

2.11. Exposure Assessment and Risk Characterization

Human exposure assessment and risk characterization were performed using the EFSA RACE tool [31]. It is a spreadsheet that calculates human intake of food contaminants (e.g., PAs) for all member state subgroups of population taking into account: (1) food consumption data [32]; (2) detailed food description based on FoodEx2 food classification; (3) experimental occurrence of selected substances in the food commodity. In order to evaluate the health risk, exposure is therefore compared with the relevant toxicological reference points. The first is the Acute Reference Dose (ARfD), which is an estimate of

the quantity of a substance in food and/or drinking-water, normally expressed on a body-weight basis, that can be ingested in a period of 24 h or less without appreciable health risk to the consumer on the basis of all the known facts at the time of the evaluation [33]. The Acute Reference Dose is a reference point for short-term exposure. The second is the benchmark dose lower confidence limit for a 10% excess cancer risk ($BMDL_{10}$), a reference value for long-term exposure that corresponds to a specific change in an adverse response compared to the response in unexposed subjects [34].

For contaminants such as PAs, these two toxicological values for both acute and chronic exposure were considered. The acute human health risk was characterized in terms of percentage of ARfD ingested while chronic risk was described with the Margin of Exposure (MoE) approach. The MoE is the ratio between the dose associated with a small increase in adverse effect ($BMDL_{10}$) and the level of human exposure calculated by RACE.

For RACE exposure assessment, the selected food item was "honey" while the highest PA content detected in the samples was entered as our worst-case occurrence data. The ARfD was 2 mg/kg bw per day and $BMDL_{10}$ was 237 µg/kg bw, as updated reference points for the sum of 1,2-unsaturated PAs assuming equal potency [13].

A percentage of ARfD ingested lower than 100% and an MoE of 10,000 or higher would be of low concern from a public health point of view for acute and chronic risk, respectively. The RACE tool analyzed every population subgroup in terms of consumption pattern. This approach results in outcomes referencing the whole population or consumers only depending on the answers given within the food consumption survey. For all population groups, mean, median and 95th percentile output values were calculated. The 95th percentile was considered the relevant value for a high honey consumption pattern.

3. Results and Discussion

3.1. LC-MS/MS Method Validation

Pyrrolizidine alkaloids (n = 18) and their related PANOs (n = 17) were detected and quantified. In the validation phase, the possible coelution of alkaloid isomers was assessed and the following co-elutions were found according to Commission Regulation (EU) 2020/2040: lycopsamine/indicine, renderine/echinatine, intermedine-N-oxide/indicine-N-oxide, senecivernine/integerrimine, echimidine/heliosupine, seneciphylline/spartioidine, seneciphylline-N-oxide/spartioidine-N-oxide, retrosine/usaramine and retrosine-N-oxide/usaramine-N-oxide. In chromatograms of blank honey extracts no significant interfering peaks were detected at the retention time of all 35 PAs/PANOs. The method exhibited linearity for PA concentrations in the 1 to 50 µg/kg range; R squared (R^2) was $\geq$ 0.99 for all the PAs in honey. In accordance with the EURL-MP guidance document for plant toxin performance criteria [35], a recovery range of 70–120%, a relative standard deviation (RSD_r %) of repeatability $\leq$ 20% and an RSD_R% of within laboratory reproducibility $\leq$ 25% were required. The experimental values of all the substances are reported in Table 4 and all the performance criteria were met. Therefore, the method can be considered fit for purpose. The limit of quantification (LOQ) for all PAs and PANOs was 1 µg/kg.

Table 4. Validation parameters for all 35 PAs and PANOs (RSD_r: repeatability, RSD_R: within-laboratory reproducibility).

Analyte	Conc (µg/kg)	Recovery %	RSD_r	RSD_R
Ene-4/6	1	94.5	13.53	14.81
	10	85.3	13.21	23.08
	25	76.9	4.52	6.28
EneN	1	82.9	10.79	11.43
	10	75.4	10.84	10.75
	25	72.9	9.61	11.21
Ha	1	77.5	14.83	17.97
	10	68.3	4.47	16.61
	25	84.8	8.96	23.87
HaN	1	83.6	13.24	15.28
	10	79.3	4.19	16.96
	25	71.9	5.33	10.59
Ht	1	98.6	11.86	13.52
	10	75.7	8.91	14.42
	25	78.4	5.91	7.13
HtN	1	91.3	11.45	12.67
	10	98.6	4.48	5.36
	25	71.8	8.19	9.81
Ins	1	79.9	13.17	20.19
	10	78.1	2.95	19.66
	25	62.6	5.28	7.50
InsN	1	74.3	8.69	15.41
	10	71.1	7.12	9.81
	25	67.1	12.75	9.66
Lc	1	93.7	7.01	15.43
	10	94.3	11.45	23.03
	25	76.2	4.91	7.64
LcN	1	74.8	17.86	20.46
	10	68.3	12.96	13.77
	25	67.6	4.51	9.67
Lg, Ld, Ls, Rs	1	94.9	7.72	20.84
	10	72.5	6.81	26.21
	25	65.6	3.64	15.78
LgN, LdN	1	74.8	9.56	9.99
	10	68.3	5.54	8.86
	25	67.6	7.52	11.24
Rt, Us	1	75.8	11.34	16.64
	10	71.5	7.89	19.31
	25	64.7	7.28	7.63
RtN, UsN	1	87.6	9.92	21.43
	10	74.7	8.36	18.76
	25	66.7	6.95	11.48
Sp, Sr	1	98.3	15.31	19.67
	10	87.6	7.01	23.97
	25	79.6	4	9.28
SpN, SrN	1	79.7	11.9	19.64
	10	78.1	4.51	16.11
	25	65.2	6.81	11.89
Sq, Is	1	96.4	10.37	11.41
	10	94.9	6.45	16.22
	25	91	5.14	5.87
SqN	1	85.1	11.37	13.61
	10	78.5	5.71	12.32
	25	74.2	2.98	12.05
Sv	1	88.1	12.1	22.98
	10	71.9	8.39	20.51
	25	72	4.6	20.14
SvN	1	97.9	9.38	10.21
	10	85.8	8.14	11.68
	25	84.5	7.63	10.81
Vl	1	87.4	12.85	16.87
	10	76	11.11	17.55
	25	77.5	2.58	16.36
VlN	1	92.1	13.85	20.76
	10	75.3	11.11	9.22
	25	61.9	2.58	10.03
RuN	1	76.1	14.45	15.31
	10	78.5	5.96	17.63
	25	68.9	8.37	13.12
LiN	1	88.4	6.27	12.48
	10	81.6	4.28	7.22
	25	77.2	10.13	13.36
HeN	1	95.1	8.64	10.21
	10	86.4	7.7	9.88
	25	87.7	3.69	11.66

3.2. PA/PANO Content in Honey Samples

The method was used to determine 35 PAs in 121 honey samples from three different Italian regions. The samples were analyzed in six analytical sessions and a quality control (QC) sample was evaluated simultaneously, having a recovery in the 70–120% range. The percentage of PA-positive samples is reported in Figure 3. Figure 4 shows the compounds

detected in the honey samples and their sum when more than one PA was present while, in Figure 5, the mean content of the PAs is reported.

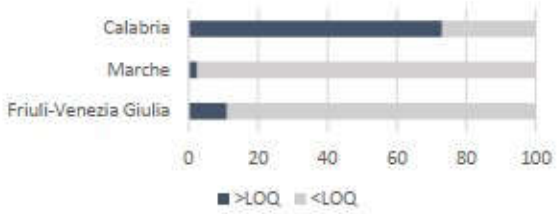

Figure 3. The percentage of honey samples with PA content >LOQ and <LOQ is reported to highlight the differences among regions.

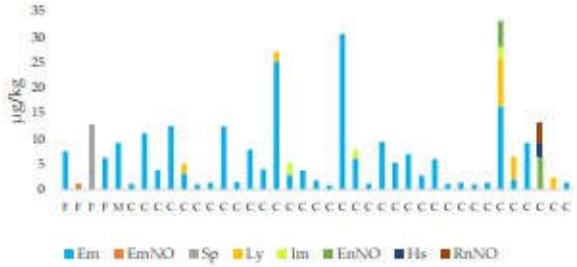

Figure 4. Pyrrolizidine alkaloids (PAs) in honey samples from different Italian regions (F = Friuli-Venezia Giulia; M = Marche; C = Calabria). Data expressed in µg/kg, Em = echimidine; EmNO = echimidine N-oxide; Sp = seneciphylline; Ly = lycopsamine; Im = intermedine; EnNO = echinatine N-oxide; Hs = heliosupine; RnNO = rinderine N-oxide.

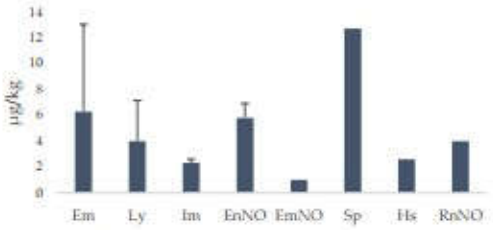

Figure 5. Mean content of pyrrolizidine alkaloids (PAs) in honey samples from Friuli-Venezia Giulia, Marche and Calabria Italian regions. Data are expressed in µg/kg and reported as mean ± SD, with the exception of EmNO, Sp, Hs and RnNO, which were found in only one sample. Em = echimidine (n = 35); EmNO = echimidine N-oxide (n = 1); Sp = seneciphylline (n = 1); Ly = lycopsamine (n = 5); Im = intermedine (n = 3); EnNO = echinatine n-oxide (n = 2); Hs = heliosupine (n = 1); RnNO = rinderine n-oxide (n = 1).

Of the 37 honey samples analyzed from Friuli-Venezia Giulia, PAs were found in concentrations above the LOQ in only 4 multifloral samples (11%). The predominant PAs found in three samples (8%) were echimidine (mean ± SD 4.94 ± 3.47 µg/kg) followed by seneciphylline in one sample (3%, 12.7 µg/kg) while in the Marche only one sample of monofloral honey from *Stachys officinalis* contained echimidine above the LOQ

(9.2 µg/kg) (Figure 6). *S. officinalis* can be considered a typical crop of the Marche. It belongs to the family of Lamiaceae and does not belong to one of the major PA-producing families; therefore, the presence of an echimidine content above the LOQ was probably due to pollen contamination by the PA-producing plant species, as confirmed by melissopalynological analysis. Thirty-three samples from Calabria contained PAs above the LOQ. In particular, all 9 multifloral samples were contaminated, while 24 samples of the 36 monofloral honey samples were contaminated. The predominant PAs were echimidine (31 samples) followed by lycopsamine (5 samples), intermedine (3 samples), echinatine N-oxide (2 samples), rinderine N-oxide (1 sample) and heliosupine N-oxide (1 sample) (Figure 4).

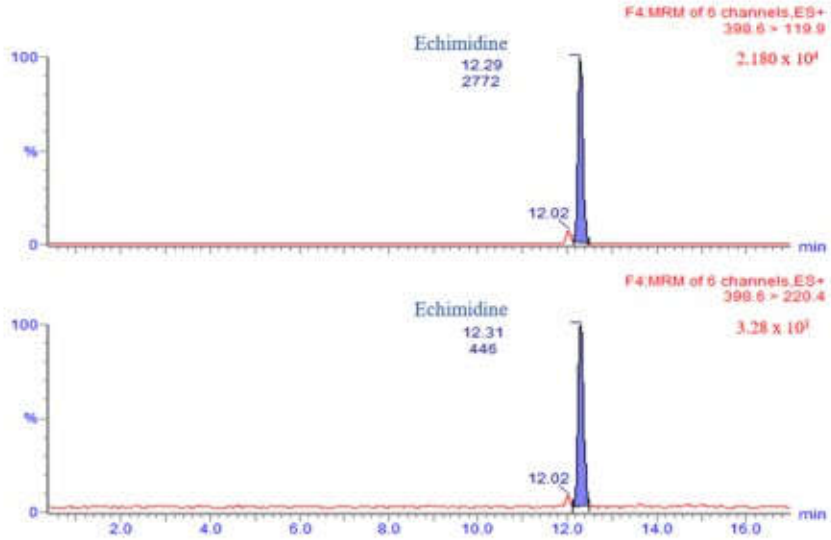

Figure 6. Chromatograms of echimidine (9.2 µg/kg) present in a *Stachys officinalis* honey sample.

Overall, the total content of PAs ranged from 0.9 µg/kg in a chestnut honey from Calabria to 33.1 µg/kg in a multifloral sample from the same region (Figure 4). The mean content (±SD) of the PAs detected was 7.11 ± 8.25 µg/kg, of the same order of magnitude, albeit higher, than the mean value of 4.7 ± 11.1 µg/kg reported by Lucatello et al. (2021) for honey samples from local producers in the Veneto [22].

Of the 35 PAs analyzed, 8 showed values higher than the LOQ (Figure 5). The most abundant and most variable PA was echimidine, detected in 35 samples (27%) with a mean content (±SD) of 6.28 ± 6.76 µg/kg, followed by lycopsamine, detected in 5 samples (4%), intermedine in 3 samples (2.5%), echinatine N-oxide in 2 samples (1.7%) and finally seneciphylline, echimidine N-oxide, rinderine N-oxide and heliosupine N-oxide detected in 1 sample each (0.8%). One sample from Calabria contained four different PAs: echimidine, lycopsamine, intermedine and echinatine N-oxide. In particular, echimidine, lycopsamine and intermedine were the most prevalent PAs. This finding was in agreement with previous publications [36–42]. Table 5 summarizes the PA/PANO data present in the scientific literature.

Table 5. Content of PAs/PANOs detected in honey in different studies. Maximum levels or, when possible, range or average values have been reported. The PAs/PANOs covered by this work are listed (n.i.: not investigated).

	Kowalczyk et al., 2022		Picron et al., 2020		Martinello et al., 2017	Lucatello et al., 2016	Griffin et al., 2014	Martinello et al., 2014	Orantes-Bermejo et al., 2013		This Research
PA/PANO (ug/kg)	Polish Honey	Foreign Honey	Belgian Honey	Foreign Honey	Retail Honey	Italian Beekeepers	Retail Honey	Retail Honey	Spanish Honey by Beekeepers		Italian Honey
Echimidine	7.3	120.0	5.91	8.84	0.4–3.3	0.3–1.0	545.5	169	36.9 ± 44.36	237	1.0–30.6
Echinatine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Europine	n.i.	n.i.	0.009	134.85	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Heliosupine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	LOQ–2.6
Heliotrine	n.i.	n.i.	<LOQ	39.44	<LOQ	<LOQ	<LOQ	<LOQ	n.i.	n.i.	<LOQ
Indicine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Integerrimine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Intermedine	9.2	23.3	n.i.	n.i.	<LOQ	<LOQ	n.i.	31	n.i.	n.i.	1.9–2.4
Lasiocarpine	n.i.	n.i.	<LOQ	5.77	<LOQ	<LOQ	n.i.	<LOQ	n.i.	n.i.	<LOQ
Lycopsamine	14.1	22.5	n.i.	n.i.	0.2–74.7	<LOQ	392.6	42	5.7 ± 4.28	18	1.9–9.3
Retrorsine	4.3	4.3	5.82	5.32	<LOQ	0.9–14.5	<LOQ	<LOQ	n.i.	n.i.	<LOQ
Rinderine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Senecionine	2.2	2.7	9.67	1.46	<LOQ	0.8–2.1	8.4	<LOQ	n.i.	n.i.	<LOQ
Seneciphylline	4.1	4.0	7.35	4.04	<LOQ	0.6–1.1	5.7	<LOQ	4.1 ± 4.79	20	LOQ–12.71
Senecivernine	<LOQ	3.0	4.15	0.41	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Senkirkine	n.i.	n.i.	42.44	1.15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Spartioidine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Usaramine	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Echimidine-N-oxide	n.i.	n.i.	8.24	0.17	n.i.	n.i.	n.i.	n.i.	21.4 ± 23.09	70	LOQ–1.01
Echinatine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	5–6.6
Erucifoline-N-oxide	n.i.	n.i.	0.09	0.14	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Europine-N-oxide	n.i.	n.i.	0.54	1.30	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Heliosupine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Heliotrine-N-oxide	n.i.	n.i.	<LOQ	0.37	n.i.	n.i.	n.i.	n.i.	2.3 ± 0.58	4	<LOQ
Indicine-N-oxide	n.i.	n.i.	0.47	0.18	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Intermedine-N-oxide	n.i.	n.i.	0.26	0.21	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Integerrimine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Lycopsamine-N-oxide	n.i.	n.i.	0.29	0.09	n.i.	n.i.	n.i.	n.i.	4.0 ± 2.79	8	<LOQ
Retrorsine-N-oxide	n.i.	n.i.	0.86	0.90	n.i.	n.i.	<LOQ	n.i.	<LOQ	<LOQ	<LOQ
Rinderine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	LOQ–4
Senecionine-N-oxide	n.i.	n.i.	0.54	0.26	n.i.	n.i.	<LOQ	n.i.	<LOQ	<LOQ	<LOQ
Seneciphylline-N-oxide	n.i.	n.i.	0.16	0.28	n.i.	n.i.	<LOQ	n.i.	<LOQ	<LOQ	<LOQ
Senecivernine-N-oxide	n.i.	n.i.	0.67	<LOQ	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Spartioidine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ
Usaramine-N-oxide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	<LOQ

Pollen composition was investigated in order to understand the origin of PAs in the most contaminated Calabria honey and in the *Stachys officinalis* honey sample from the Marche. Melissopalynological analysis revealed the presence of pollen from the genus belonging to the Boraginaceae family: *Echium*, *Cerinthe* and *Cynoglossum*. Echimidine is a typical alkaloid of the genus *Echium*, in particular of *Echium plantagineum*, a plant widespread throughout Italy (Portal to the Flora of Italy, https://dryades.units.it/floritaly/index.php?procedure=taxon_page&tipo=all&id=4297 (accessed on 7 February 2023)). The elevated levels of echimidine and its N-oxide derivative, which were higher than the LOQ in the honey from all three regions, could be explained by the notable presence of this species in Italy. Lycopsamine is another alkaloid that was present in the samples, although in lower quantities. This alkaloid is produced by a number of plants, including *Echium vulgaris*, belonging to the Boraginaceae family and widespread in Italy (Portal to the Flora of Italy, https://dryades.units.it/floritaly/index.php?procedure=taxon_page&tipo=all&id=4297 (accessed on 7 February 2023)).

The results obtained in the present study regarding Italian honey were consistent with the results published by other authors who have reported the concentration of PAs in European honey. A study of 40 samples of Polish multifloral honey collected directly from beekeepers showed an alkaloid content ranging from 1.0 to 20.2 µg/kg, with an average content of 2.9 µg/kg. In the same study, the analysis of 14 honey samples of Asian origin showed a much higher content of PAs [43]. In a more recent study [44], the PA content in the Polish honey ranged from 2.2 to 31.6 µg/kg while the total PA content monitored in foreign honey ranged from 5.8 to 147.0 µg/kg.

Bodi et al. (2014) published the results of PA content in honey sampled from German and Austrian beekeepers. These samples showed a significantly lower rate of positive samples than those bought at the supermarket and from other sources. The mean total PA content ranged from 6.1 µg/kg of honey in beekeeper samples to 14 µg/kg in discount products and 15 µg/kg in branded honey [45]. Dübecke et al. (2011) observed substantial differences in the quantity of PAs found in honey depending on the country of origin. The mean concentration of PAs in 381 European honey samples was 17 µg/kg, including negative samples [37]. These data were very similar to the present data. Honey from Germany, Bulgaria and Romania showed lower levels of PAs (1–43 µg/kg) than honey from Italy and Spain (concentrations up to 225 µg/kg) as honey from these regions often contained an elevated number of *Echium* pollen grains (18 PAs and N-oxides monitored) [46].

Martinello et al. (2014) reported a higher content of PAs (nine monitored alkaloids) in honey samples that were blends of EU and non-EU honey for which the mean content determined was 17.5 µg/kg. The mean PA content in the EU honey was 3.1 µg/kg [41]. All the results reported, however, could have been underestimated due to the limited number of PAs monitored.

Honey samples produced in Belgium were less contaminated and presented a different contamination profile; PAs were found in 67% of the samples examined, with maximum and average concentrations of 60.53 µg/kg and 1.20 µg/kg, respectively. The majority of samples (49%) contained from 0.05 to 0.99 µg/kg of contaminants [47].

The analysis of 103 Spanish honey samples (*Echium* spp. honey) showed the presence of PAs in 97 samples with a content ranging from 1 to 237 µg/kg. The mean PA concentration of the PA-positive samples was 48 ± 55.5 µg/kg. The PA pattern was clearly dominated by echimidine, lycopsamine and their N-oxides, which accounted for 97.8% of the total ΣPA, while seneciphyilline and heliothrine N-oxide were detected at a much lower incidence [42].

3.3. Variables Affecting the Content of PAs/PANOs in Honey Samples

Qualitative variables: a significant, albeit moderate (Cramér's V = 0.483), association ($p = 1.56 \times 10^{-13}$) was detected between the presence/absence of PAs and the specific region using the Chi-square test. In Calabria, 73% of the samples were found to contain PAs.

Quantitative variables: significant differences among honey types were detected within the same region. In the Marche, the sum of the PAs and echimidine were significantly higher in Stachys honey ($p = 7.53 \times 10^{-06}$), while in honey samples from Friuli-Venezia Giulia no significant differences were recorded. A more complex pattern was evidenced in honey from Calabria due to the presence of a high percentage of samples having a PA content > the LOQ; this made multiple comparisons among the different types of honey possible. In particular, the multifloral honey samples showed a significantly higher content of echimidine (10.28 ± 10.69 µg/kg) than citrus fruit honey (1.7 ± 1.25 µg/kg) ($p = 0.0261$) (Figure 7) and a significantly higher content of intermedine than the acacia honey ($p = 0.0253$). Finally, one monofloral Sulla honey from the province of Catanzaro was characterized by the exclusive presence of N-oxide derivatives of three PAs, echinatine, rinderine and heliosupine, which were not present in any other honey sample (Figure 4).

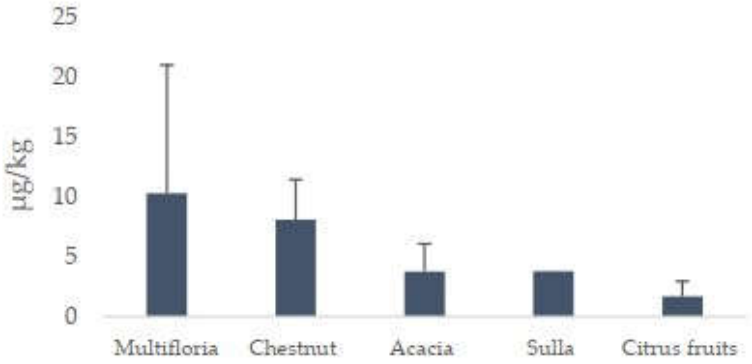

Figure 7. Mean content of echimidine in samples from Calabria region. Data are expressed in µg/kg and reported as mean ± SD.

3.4. Exposure Assessment and Health Risk Characterization

Data entries on the RACE spreadsheet are summarized in Table 6 while the outcomes of the calculation are reported in Tables 7 and 8 for acute and chronic exposure to PAs, respectively.

Table 6. Summary of RACE parameters for exposure assessment and risk characterization.

RACE Parameters	
Contaminant	Pyrrolizidine alkaloids (sum of 1,2-unsaturated)
Food description	Honey
Analytical result	33.1 µg/kg
Reference value acute (ARfD)	2 mg/kg bw
Reference value chronic ($BMDL_{10}$)	237 µg/kg bw
Survey country	Italy [32]

Table 7. Summary of RACE outputs for acute health risk characterization.

Output (% ARfD)			
Acute Consumers Only	Mean	Median	95th Percentile
Toddlers	0.0022	0.0024	0.0047 [1]
Other children	0.0012	0.0008	0.0024 [1]
Adolescents	0.0007	0.0005	0.0016 [1]
Adults	0.0005	0.0003	0.0011
Elderly	0.0004	0.0003	0.0010
Very elderly	0.0006	0.0005	0.0009 [1]
Acute whole population	Mean	Median	95th percentile
Toddlers	0.00023	- [2]	0.00236
Other children	0.00008	- [2]	0.00072
Adolescents	0.00001	- [2]	-
Adults	0.00003	- [2]	0.00018
Elderly	0.00003	- [2]	0.00026
Very elderly	0.00004	- [2]	0.00031

[1] Number of observations lower than 60; the 95th percentile may not be statistically robust. [2] Values not given by the model.

Table 8. Summary of RACE outputs for chronic health risk characterization.

Output (MoE)			
Chronic Consumers Only	Mean	Median	95th Percentile
Toddlers	7303	7876	3759 [1]
Other children	12,496	15,752	5783 [1]
Adolescents	24,134	24,702	17,184 [1]
Adults	36,740	50,476	12,411
Elderly	44,500	42,961	20,764 [1]
Very elderly	27,294	31,147	7279 [1]
Chronic whole population	Mean	Median	95th percentile
Toddlers	52,581	- [2]	4654
Other children	141,868	- [2]	16,468
Adolescents	851,578	- [2]	- [2]
Adults	456,875	- [2]	62,293
Elderly	391,059	- [2]	39,953
Very elderly	327,532	- [2]	37,591

[1] Number of observations lower than 60; the 95th percentile may not be statistically robust. [2] Values not given by the model.

For acute exposure assessment, all values were well below the ARfD. On the other hand, for chronic assessment the RACE tool calculated a MoE lower than 10,000 (i.e., mean 7302, median 7876) for toddler consumers. The number of observations (< 60) was not sufficient for an accurate MoE calculation for the 95th percentile output for toddlers, both as consumers and as total population. However, these values were below the 10,000 threshold (3759 and 4654, respectively). This means that the daily consumption of elevated quantities of honey containing PAs at the highest concentration detected could probably pose a health risk for toddlers and children. Unfortunately, the RACE tool does not give specific information regarding food consumption; however, looking at the Italian food consumption survey, which is the RACE reference for Italy, it can be seen that means of 16.6 g/day for the total population and 19.6 g/day for consumers are data referring to the category "sugar, fructose, honey and other nutritious sweeteners". For consumers of large

quantities, these values can double [32]. Furthermore, this survey is quite outdated as it was carried out in 2005–2006 and consumption habits have changed over time.

The health concerns for toddlers and children, who are frequent consumers of large quantities of honey, were also highlighted by the 2011 EFSA opinion regarding PAs [12]; however, it should be noted that a previous $BMDL_{10}$ of 70 μg/kg bw was used for the risk characterization while the mean occurrence in honey could be considered in line with the highest findings of the authors. The exposure assessment and risk characterization were carried out according to the worst-case scenario, having used a single occurrence datum of the PAs detected in a single sample. In order to better understand whether there is a real risk associated with the consumption of honey, additional PA monitoring should be carried out. The margins of exposure for all other population groups were greater than 10,000, signifying a negligible risk for those age groups.

4. Conclusions

This study, along with others carried out in different countries, could be very useful for both Food Safety Authorities and beekeepers in identifying, classifying and creating a map of the distribution of geographical areas at risk for the presence of PA-producing flora. In addition, health authorities need to develop better traceability of the origin of honey, together with the integration of data on nomadism practices, so that geographic areas at risk for the presence of PA-containing plants may easily be identified. This identification could be very useful in improving the safety and quality of honey.

While a sum of 33.1 μg PAs/kg has been associated with negligible health risks related to the consumption of honey, chronic exposure assessment and risk characterization have highlighted a potential health concern only for toddlers who frequently consume elevated quantities of honey. This finding could be influenced by uncertainties deriving from real honey consumption habits and a "worst-case" PA occurrence. In order to better characterize the risk, additional monitoring studies regarding PAs should be implemented. It is worth pointing out that the ingestion of honey could be associated with infant botulism as honey is a dietary reservoir of *Clostridium botulinum* spores [48]. This possibility is well known to pediatricians who should not recommend honey-containing supplements or the use of honey as a flavoring agent for infants, in particular those younger than 12 months [48,49].

Despite the possible health risks for specific population subgroups, honey is a very rich food, possessing health and therapeutic properties that vary, similarly to its aroma, depending on the flowers from which the bees have extracted the nectar. In addition to glucose and fructose, honey contains polyphenols, flavonoids, alkaloids, glycosides and volatile compounds with proven antioxidant, anti-microbial and anti-inflammatory effects and with potential neuroprotective effects [50–53].

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References

1. Moreira, R.; Pereira, D.; Valentão, P.; Andrade, P. Pyrrolizidine Alkaloids: Chemistry, Pharmacology, Toxicology and Food Safety. *Int. J. Mol. Sci.* **2018**, *19*, 1668. [CrossRef] [PubMed]
2. Ebmeyer, J.; Braeuning, A.; Glatt, H.; These, A.; Hessel-Pras, S.; Lampen, A. Human CYP3A4-Mediated Toxification of the Pyrrolizidine Alkaloid Lasiocarpine. *Food Chem. Toxicol.* **2019**, *130*, 79–88. [CrossRef] [PubMed]
3. Stegemann, T.; Kruse, L.H.; Brütt, M.; Ober, D. Specific Distribution of Pyrrolizidine Alkaloids in Floral Parts of Comfrey (*Symphytum officinale*) and Its Implications for Flower Ecology. *J. Chem. Ecol.* **2019**, *45*, 128–135. [CrossRef] [PubMed]
4. Brugnerotto, P.; Seraglio, S.K.T.; Schulz, M.; Gonzaga, L.V.; Fett, R.; Costa, A.C.O. Pyrrolizidine Alkaloids and Beehive Products: A Review. *Food Chem.* **2021**, *342*, 128384. [CrossRef]
5. Casado, N.; Morante-Zarcero, S.; Sierra, I. The Concerning Food Safety Issue of Pyrrolizidine Alkaloids: An Overview. *Trends Food Sci. Technol.* **2022**, *120*, 123–139. [CrossRef]
6. Chen, L.; Zhang, Q.; Yi, Z.; Chen, Y.; Xiao, W.; Su, D.; Shi, W. Risk Assessment of (Herbal) Teas Containing Pyrrolizidine Alkaloids (PAs) Based on Margin of Exposure Approach and Relative Potency (REP) Factors. *Foods* **2022**, *11*, 2946. [CrossRef]
7. Dusemund, B.; Nowak, N.; Sommerfeld, C.; Lindtner, O.; Schäfer, B.; Lampen, A. Risk Assessment of Pyrrolizidine Alkaloids in Food of Plant and Animal Origin. *Food Chem. Toxicol.* **2018**, *115*, 63–72. [CrossRef]
8. Habs, M.; Binder, K.; Krauss, S.; Müller, K.; Ernst, B.; Valentini, L.; Koller, M. A Balanced Risk–Benefit Analysis to Determine Human Risks Associated with Pyrrolizidine Alkaloids (PA)—The Case of Tea and Herbal Infusions. *Nutrients* **2017**, *9*, 717. [CrossRef]
9. Edgar, J.A.; Colegate, S.M.; Boppré, M.; Molyneux, R.J. Pyrrolizidine Alkaloids in Food: A Spectrum of Potential Health Consequences. *Food Addit. Contam. Part A* **2011**, *28*, 308–324. [CrossRef]
10. Hama, J.R.; Strobel, B.W. Pyrrolizidine Alkaloids Quantified in Soil and Water Using UPLC-MS/MS. *RSC Adv.* **2019**, *9*, 30350–30357. [CrossRef]
11. Hama, J.R.; Strobel, B.W. Occurrence of Pyrrolizidine Alkaloids in Ragwort Plants, Soils and Surface Waters at the Field Scale in Grassland. *Sci. Total Environ.* **2021**, *755*, 142822. [CrossRef]
12. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on Pyrrolizidine Alkaloids in Food and Feed. *EFSA J.* **2011**, *9*, 2406. [CrossRef]
13. EFSA Panel on Contaminants in the Food Chain (CONTAM); Knutsen, H.K.; Alexander, J.; Barregård, L.; Bignami, M.; Brüschweiler, B.; Ceccatelli, S.; Cottrill, B.; Dinovi, M.; Edler, L.; et al. Risks for Human Health Related to the Presence of Pyrrolizidine Alkaloids in Honey, Tea, Herbal Infusions and Food Supplements. *EFSA J.* **2017**, *15*, e04908. [CrossRef]
14. Ruan, J.; Yang, M.; Fu, P.; Ye, Y.; Lin, G. Metabolic Activation of Pyrrolizidine Alkaloids: Insights into the Structural and Enzymatic Basis. *Chem. Res. Toxicol.* **2014**, *27*, 1030–1039. [CrossRef]
15. Xia, Q.; He, X.; Shi, Q.; Lin, G.; Fu, P.P. Quantitation of DNA Reactive Pyrrolic Metabolites of Senecionine—A Carcinogenic Pyrrolizidine Alkaloid by LC/MS/MS Analysis. *J. Food Drug Anal.* **2020**, *28*, 167–174. [CrossRef]
16. Xu, J.; Wang, W.; Yang, X.; Xiong, A.; Yang, L.; Wang, Z. Pyrrolizidine Alkaloids: An Update on Their Metabolism and Hepatotoxicity Mechanism. *Liver Res.* **2019**, *3*, 176–184. [CrossRef]
17. Kakar, F.; Akbarian, Z.; Leslie, T.; Mustafa, M.L.; Watson, J.; van Egmond, H.P.; Omar, M.F.; Mofleh, J. An Outbreak of Hepatic Veno-Occlusive Disease in Western Afghanistan Associated with Exposure to Wheat Flour Contaminated with Pyrrolizidine Alkaloids. *J. Toxicol.* **2010**, *2010*, 313280. [CrossRef]
18. IARC (International Agency for Research on Cancer). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, 1st ed.; World Health Organization: Lyon, France, 1987; ISBN 978-92-832-1411-3.
19. IARC (International Agency for Research on Cancer). Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; IARC: Lyon, France, 2002; ISBN 978-92-832-1282-9.
20. European Commission. Commission Regulation (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs. *Off. J. Eur. Union* **2020**, 1–5.
21. Alvarado-Avila, L.Y.; Moguel-Ordóñez, Y.B.; García-Figueroa, C.; Ramírez-Ramírez, F.J.; Arechavaleta-Velasco, M.E. Presencia de Alcaloides Pirrolizidínicos En Miel y Los Efectos de Su Consumo En Humanos y Abejas. Revisión. *Rev. Mex. Cienc. Pecu.* **2022**, *13*, 787–802. [CrossRef]
22. Lucatello, L.; Merlanti, R.; De Jesus Inacio, L.; Bisutti, V.; Montanucci, L.; Capolongo, F. Pyrrolizidine Alkaloid Concentrations in Local Italian and Retail Honeys of Different Origin: A Scenario of Human Exposure. *J. Food Compos. Anal.* **2021**, *104*, 104182. [CrossRef]
23. Griffin, C.T.; Danaher, M.; Elliott, C.T.; Glenn Kennedy, D.; Furey, A. Detection of Pyrrolizidine Alkaloids in Commercial Honey Using Liquid Chromatography–Ion Trap Mass Spectrometry. *Food Chem.* **2013**, *136*, 1577–1583. [CrossRef] [PubMed]
24. Von Der Ohe, W.; Persano Oddo, L.; Piana, M.L.; Morlot, M.; Martin, P. Harmonized Methods of Melissopalynology. *Apidologie* **2004**, *35*, S18–S25. [CrossRef]
25. Bundesinstitut für Risikobewertung. *Determination of Pyrrolizidine Alkaloids (PA) in Honey by SPE-LC-MS/MS Method Protocol BfR-PA-Honey-1.0/2013*; Bundesinstitut für Risikobewertung: Berlin, Germany, 2013; pp. 1–17.
26. *EURL-MP-Method_002 (Version 2) Determination of Pyrrolizidine Alkaloids in Plant-Based Food and Feed Materials, Including (Herbal) Teas, Herbal Food Supplements, Fodder and Feedstuffs by LC-MS/MS*; RIKILT Wageningen University & Research: Wageningen, The Netherlands, 2019; pp. 1–26.

27. European Commissions. Commission Regulation (EC) No 401/2006 of 23 February 2006 Laying down the Methods of Sampling and Analysis for the Official Control of the Levels of Mycotoxins in Foodstuffs. *Off. J. Eur. Union.* **2006**, 12–34.
28. European Commission. *Health and Food Safety Directorate-General (DG SANTE). Guidance Document on Identification of Mycotoxin in Food and Feed. SANTE/12089/2016*; European Commission DG SANTE: Bruxelles, Belgium, 2016.
29. Mascha, E.J.; Vetter, T.R. Significance, Errors, Power, and Sample Size: The Blocking and Tackling of Statistics. *Anesth. Analg.* **2018**, *126*, 691–698. [CrossRef] [PubMed]
30. Cramér, H. The Two-Dimensional Case. In *Mathematical Methods of Statistics*; Princeton University Press: Princeton, NJ, USA, 1946; p. 282. ISBN 0-691-08004-6.
31. Fürst, P.; Milana, M.R.; Pfaff, K.; Tlustos, C.; Vleminckx, C.; Arcella, D.; Barthélémy, E.; Colombo, P.; Goumperis, T.; Pasinato, L.; et al. Risk Evaluation of Chemical Contaminants in Food in the Context of RASFF Notifications. *EFSA Support. Publ.* **2019**, *16*, 1625E. [CrossRef]
32. Leclercq, C.; Arcella, D.; Piccinelli, R.; Sette, S.; Le Donne, C.; Turrini, A.; INRAN-SCAI 2005–06 Study Group. The Italian National Food Consumption Survey INRAN-SCAI 2005–06: Main Results in Terms of Food Consumption. *Public Health Nutr.* **2009**, *12*, 2504–2532. [CrossRef]
33. FAO-WHO. Pesticide residues in food. In Proceedings of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues, Rome, Italy, 20–29 September 2004.
34. Dakeishi, M.; Murata, K.; Tamura, A.; Iwata, T. Relation Between Benchmark Dose and No-Observed-Adverse-Effect Level in Clinical Research: Effects of Daily Alcohol Intake on Blood Pressure in Japanese Salesmen. *Risk Anal.* **2006**, *26*, 115–123. [CrossRef]
35. European Union Reference Laboratory (EURL). EURLMP-Background doc_003 Guidance Document Performance Criteria v1.1 draft of 17 September 2021. Available online: https://www.wur.nl/en/research-results/research-institutes/food-safety-research/reference-laboratory/european-union-reference-laboratory/eurl-mycotoxins-plant-toxins/library-eurl-mp.htm (accessed on 16 January 2023).
36. Cao, X.; Deng, W.; Fu, M.; Zhu, Y.; Liu, H.; Wang, L.; Zeng, J.; Wei, Y.; Xu, X.; Yu, J. Seventy-Two-Hour Release Formulation of the Poorly Soluble Drug Silybin Based on Porous Silica Nanoparticles: In Vitro Release Kinetics and In Vitro/In Vivo Correlations in Beagle Dogs. *Eur. J. Pharm. Sci.* **2013**, *48*, 64–71. [CrossRef]
37. Dübecke, A.; Beckh, G.; Lüllmann, C. Pyrrolizidine Alkaloids in Honey and Bee Pollen. *Food Addit. Contam. Part A* **2011**, *28*, 348–358. [CrossRef]
38. Griffin, C.T.; O'Mahony, J.; Danaher, M.; Furey, A. Liquid Chromatography Tandem Mass Spectrometry Detection of Targeted Pyrrolizidine Alkaloids in Honeys Purchased within Ireland. *Food Anal. Methods* **2015**, *8*, 18–31. [CrossRef]
39. Huybrechts, B.; Callebaut, A. Pyrrolizidine Alkaloids in Food and Feed on the Belgian Market. *Food Addit. Contam. Part A* **2015**, *32*, 1939–1951. [CrossRef]
40. Kempf, M.; Wittig, M.; Schönfeld, K.; Cramer, L.; Schreier, P.; Beuerle, T. Pyrrolizidine Alkaloids in Food: Downstream Contamination in the Food Chain Caused by Honey and Pollen. *Food Addit. Contam. Part A* **2011**, *28*, 325–331. [CrossRef]
41. Martinello, M.; Cristofoli, C.; Gallina, A.; Mutinelli, F. Easy and Rapid Method for the Quantitative Determination of Pyrrolizidine Alkaloids in Honey by Ultra Performance Liquid Chromatography-Mass Spectrometry: An Evaluation in Commercial Honey. *Food Control* **2014**, *37*, 146–152. [CrossRef]
42. Orantes-Bermejo, F.J.; Serra Bonvehí, J.; Gómez-Pajuelo, A.; Megías, M.; Torres, C. Pyrrolizidine Alkaloids: Their Occurrence in Spanish Honey Collected from Purple Viper's Bugloss (*Echium* spp.). *Food Addit. Contam. Part A* **2013**, *30*, 1799–1806. [CrossRef]
43. Kowalczyk, E.; Sieradzki, Z.; Kwiatek, K. Determination of Pyrrolizidine Alkaloids in Honey with Sensitive Gas Chromatography-Mass Spectrometry Method. *Food Anal. Methods* **2018**, *11*, 1345–1355. [CrossRef]
44. Kowalczyk, E.; Kwiatek, K. Simultaneous Determination of Pyrrolizidine and Tropane Alkaloids in Honey by Liquid Chromatography–Mass Spectrometry. *J. Vet. Res.* **2022**, *66*, 235–243. [CrossRef]
45. Bodi, D.; Ronczka, S.; Gottschalk, C.; Behr, N.; Skibba, A.; Wagner, M.; Lahrssen-Wiederholt, M.; Preiss-Weigert, A.; These, A. Determination of Pyrrolizidine Alkaloids in Tea, Herbal Drugs and Honey. *Food Addit. Contam. Part A* **2014**, *31*, 1886–1895. [CrossRef]
46. Kast, C.; Dübecke, A.; Kilchenmann, V.; Bieri, K.; Böhlen, M.; Zoller, O.; Beckh, G.; Lüllmann, C. Analysis of Swiss Honeys for Pyrrolizidine Alkaloids. *J. Apic. Res.* **2014**, *53*, 75–83. [CrossRef]
47. Picron, J.-F.; Herman, M.; Van Hoeck, E.; Goscinny, S. Monitoring of Pyrrolizidine Alkaloids in Beehive Products and Derivatives on the Belgian Market. *Environ. Sci. Pollut. Res.* **2020**, *27*, 5693–5708. [CrossRef]
48. Abdulla, C.O.; Ayubi, A.; Zulfiquer, F.; Santhanam, G.; Ahmed, M.A.S.; Deeb, J. Infant Botulism Following Honey Ingestion. *Case Rep.* **2012**, *2012*, bcr1120115153. [CrossRef]
49. Tanzi, M.G.; Gabay, M.P. Association Between Honey Consumption and Infant Botulism. *Pharmacotherapy* **2002**, *22*, 1479–1483. [CrossRef] [PubMed]
50. Vică, M.L.; Glevitzky, M.; Dumitrel, G.-A.; Bostan, R.; Matei, H.V.; Kartalska, Y.; Popa, M. Qualitative Characterization and Antifungal Activity of Romanian Honey and Propolis. *Antibiotics* **2022**, *11*, 1552. [CrossRef] [PubMed]
51. Fratianni, F.; Amato, G.; d'Acierno, A.; Ombra, M.N.; De Feo, V.; Coppola, R.; Nazzaro, F. In Vitro Prospective Healthy and Nutritional Benefits of Different Citrus Monofloral Honeys. *Sci. Rep.* **2023**, *13*, 1088. [CrossRef] [PubMed]