LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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PROSPECTS OF *CLADOPHORA GLOMERATA* MACROALGAL BIOMASS THRIVING IN LITHUANIAN RIVERS FOR THE DEVELOPMENT OF A MORE SUSTAINABLE AND FUNCTIONAL RABBIT PRODUCTION CHAIN

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LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

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LIETUVOS UPĖSE KLESTINČIOS *CLADOPHORA GLOMERATA* MAKRODUMBLIŲ BIOMASĖS UTILIZAVIMO GALIMYBĖS, KURIANT TVARESNĘ IR FUNKCIONALESNĘ TRIUŠIENOS PRODUKCIJOS GRANDINĘ

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ABBREVIATIONS

a*	–	colour coordinate of redness
AA	–	amino acid
ABTS	–	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging
ADG	–	average daily gain
AI	–	atherogenic index
Ala	–	alanine
Arg	–	arginine
Asp	–	aspartic acid
AU	–	absorbance units
b*	–	colour coordinate of yellowness
B1	–	biomass from River Dubysa
B2	–	biomass from River Šventoji
B3	–	biomass from River Nevėžis
B4	–	biomass from River Jūra
BWG	–	body weight gain
C. glomerata	–	*Cladophora glomerata*
Ca	–	calcium
CA	–	crude ash
Cd	–	cadmium
CF	–	crude fat
CFB	–	crude fibre
CG4	–	standard compound diet supplemented with 4% *C. glomerata* biomass
CG8	–	standard compound diet supplemented with 8% *C. glomerata* biomass
CP	–	crude protein
Cr	–	chromium
Cu	–	copper
DFI	–	daily feed intake
DM	–	dry matter
DPPH	–	2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity
EU	–	European Union
FA	–	fatty acid
FCR	–	feed conversion ratio
FRAP	–	ferric ion-reducing antioxidant power
GAE	–	gallic acid equivalent
Glu	–	glutamic acid
Gly	–	glycine
h/H	–	hypocholesterolemic/hypercholesterolemic ratio
His	–	histidine
HL	–	hind leg
HL0	–	hind leg muscles 24 hours post-mortem stored at −80°C
HL3	–	hind leg muscles 24 hours post-mortem stored for 3 months at −18°C
Hyp	–	hydroxyproline
Ile	–	isoleucine
K	–	potassium
L*	–	colour coordinate of brightness

LD	–	*longissimus dorsi*
LD0	–	*longissimus dorsi* muscles 24 hours post-mortem stored at −80°C
LD3	–	*longissimus dorsi* muscles 24 hours post-mortem stored for 3 months at −18°C
Leu	–	leucine
Lys	–	lysine
LSMU	–	Lithuanian University of Health Sciences
MDA	–	malondialdehyde
Met	–	methionine
Mg	–	magnesium
MUFA	–	monounsaturated fatty acid
N	–	nitrogen
n.s.	–	not significant
Ni	–	nickel
P	–	phosphorus
Pb	–	lead
Phe	–	phenylalanine
Pro	–	proline
PUFA	–	polyunsaturated fatty acid
RP	–	reducing power
SCD	–	standard compound diet
SCFA	–	short-chain fatty acid
Ser	–	serine
SFA	–	saturated fatty acid
Thr	–	threonine
TI	–	thrombogenicity index
Trp	–	tryptophan
Tyr	–	tyrosine
USFA	–	unsaturated fatty acid
V/C	–	villus to crypt ratio
VA	–	Veterinary Academy
Val	–	valine
Zn	–	zinc

INTRODUCTION

The rise in the world population is directly correlated with rising consumption of animal products, signalling an impending limited availability of traditional protein feed ingredients [1]. Emphasising sustainable livestock production and exploring novel feed sources offer promising alternatives. Therefore, researching innovative feed materials addresses challenges while tapping into economic opportunities, demanding in-depth study for the development of appropriate handling and valorisation methods [2]. This comprehensive approach challenges researchers to discover alternatives to traditional feed materials while maintaining high standards for quality, safety, sustainability, and the mitigation of antinutritional aspects and potential toxicity. Finally, the goal is the development of functional feed and end animal-derived products. For instance, materials collected from aquatic environments, particularly algal biomass that contains protein and other essential nutrients, are currently being investigated as a more sustainable feed production option [3–5].

Algae, as a globally cultivated resource, are acknowledged for their biomass nutritional abundance in essential vitamins, minerals, proteins, polyunsaturated fatty acids (PUFAs), and antioxidants [6, 7]. The tremendous potential of algae begins with its ability to thrive in regions unsuited for conventional crops, with fast biomass development compared to typical agricultural plants [5]. By efficiently utilising solar energy, algae produce bioactive compound-enriched biomass, which has the potential to improve the overall nutritional value of food and feed [8]. In general, this kind of supplementation could contribute to addressing the scarcity of feed materials while simultaneously mitigating greenhouse gas emissions from livestock activities [9]. Recently, algae have been successfully employed as animal feed or functional feed ingredients globally [3, 4, 10, 11]. And according to the regulations, they are recognised within the European Union's regulatory framework for use as feed material [Commission Regulation (EU) No. 68/2013, January 16, 2013]. Algae's huge potential comes from its unique characteristics, such as resilience to harsh environments and production that exceeds that of higher plants. *Chlorophyceae*, such as green macroalgae, which are abundant in freshwater bodies, provide habitat structuring roles within benthic zones [12]. Particularly, opportunistic macroalgae like *Cladophora*, *Ulva*, and *Enteromorpha* thrive in certain environments, generating large agglomerations. Nevertheless, environmental conditions impact macroalgal populations, their life cycles, and the chemical structure of biomass. Freshwater macroalgae, though rich in diverse bioactive compounds, are

comparatively underexplored and underutilised as feedstock [13, 14], in contrast to extensively studied seaweed bio-compounds [15].

Algae, currently under scrutiny for their potential as feedstock or co-products in industrial extraction, face research challenges hindering their commercialization [16]. Generally, basic algae composition includes three major compounds: proteins, carbohydrates, and lipids. Additionally, algal biomass yields starch, pigments, antioxidants, vitamins, and phytohormones, which are applicable in pharmaceuticals, biofertilizers, natural colourants, or animal feed [17]. Algae species provide an abundance of biologically active substances, with distinct secondary metabolites sometimes exclusive to certain phylogenetic groupings, delivering diverse health advantages [18]. Furthermore, scientific data confirms the occurrence of phenolic compounds in algal species [18–22]. Macroalgae, characterised by different morphological and physiological properties, are abundant in bioactive chemicals such as antioxidants (phenols, pigments, etc.), demonstrating substantial therapeutic promise [20]. Defensive systems mainly rely on secondary metabolites, which are classified into important bioactive chemical types like alkaloids, flavonoids, phenolics, tannins, and terpenoids, all of which have notable pharmacological properties [23, 24]. Algae, particularly green macroalgae, serve as significant pigment sources, synthesising chlorophyll and carotenoids as photosynthetic organisms, emphasising their relevance in sustainable supply chain considerations for feedstock and other industrial opportunities [25].

Cladophora species, whether marine or freshwater, are ecologically and economically important as macroalgae, providing essential ecosystem benefits and finding adaptable applications in soil improvement, fertilisers, plant growth stimulants, food and animal feed, pharmaceuticals, cosmetics, treatment of wastewater, and renewable biofuel generation [26, 27]. *Cladophora glomerata* (*C. glomerata*), a green macroalgae, thrives in nutrient-rich water bodies, particularly slow-flowing rivers, where it forms large communities [28]. Despite its benefits, *C. glomerata* overpopulation reduces biodiversity, lowers recreational value in water bodies, and creates ecological and economic challenges. Notably, due to its high protein content, *C. glomerata* is suggested for inclusion in both human and animal diets, and it is utilised in the food and feed industries as a nutrient, vitamin, and fibre-rich raw material. [29]. Given current issues in animal husbandry, such as sustainability concerns, feed material shortages, and greenhouse gas emissions, integrating excess *C. glomerata* biomass into feed production provides a more sustainable alternative by utilising waste as a useful raw resource. In pursuit of sustainable and ecologically conscious raw materials, there is a rising interest in researching natural resources that may be used as

multifunctional raw materials in a sustainable manner. Within the context of the bioeconomy concept, biomass production must not only be environmentally beneficial but also involve a sustainable bioprocess that optimises raw material valorisation [30]. For instance, utilising macroalgal biomass like *C. glomerata* as a raw material not only provides multifunctional inputs for various industries but also contributes to water body clean-up and increased biodiversity. *C. glomerata*, a prevalent macroalga in aquatic ecosystems, exhibits rapid biomass accumulation and contains biologically active substances such as fatty acids, sterols, terpenoids, phenolic compounds, and pigments [6, 29]. While research on marine algae is extensive, limited information is available on freshwater *C. glomerata* from Lithuanian rivers. Past Lithuanian studies have explored the conversion of marine *C. glomerata* biomass to oil through pyrolysis and its potential as a feedstuff [31], as well as its application for biogas production in controlled bioreactors [32]. However, comprehensive research on freshwater *C. glomerata* from Lithuanian rivers is currently limited.

The human consumption of animal-derived products is increasing rapidly [33]. Nonetheless, to increase consumer acceptability and market appeal, it is critical to create novel strategies for positioning rabbit meat as a compelling alternative to regularly consumed pork, beef, and chicken. Introducing novel techniques not only raises the commercial appeal of rabbit meat but also matches customer needs, notably those for convenience [34]. Addressing sustainability issues and incorporating alternative raw materials, such as *C. glomerata* freshwater macroalgal biomass, into rabbit feed production is a sustainable option that reduces the ecological footprint associated with traditional feed production. As the world's population expands, so does the demand for meat [35], making sustainable field-to-table operations increasingly important. For example, recent scientific studies show the partial replacement of traditional feed materials with alternatives in animal diets [36-39], emphasising the growing interest in natural and renewable sources for developing sustainable livestock management practices. However, although attaining environmental and production sustainability is key to global food supply preservation, meat quality should not be compromised [36]. The constantly evolving concept of meat quality currently includes factors such as nutritional value, sensory characteristics, cooking convenience, and cost-effectiveness. As a result, knowing all the variables impacting meat quality is critical to successfully enhancing overall quality. Pethick et al. [40] emphasise key consumer-focused criteria, claiming that meat products possess health-enhancing properties by containing high-quality proteins and nutrients that align with a healthy diet. Furthermore, the inclusion of freshwater

C. glomerata macroalgal biomass offers both environmental benefits and increases water body diversity.

Specifically, *Cladophora* species have demonstrated potential in supplementing aquaculture diets [41–43]. For instance, research reveal potential advantages in reducing intestinal dysbiosis in pigs, suggesting a feasible alternative in rabbit husbandry [44]. Furthermore, incorporating algae into rabbit diets not only resonates with ecologically concerned customers, but it also has the potential to be a commercial advertising strategy for attracting individuals looking for novel, high-quality food. As previously stated, incorporating algae into animal diets directly improves meat quality [41, 43, 45, 46]. However, research on the effects of *Cladophora* biomass on rabbits is limited, and with the expanding demand for rabbit meat due to growing consumer awareness of healthy nutrition, there is a significant opportunity for deeper research [47]. For example, meat and its derivatives are considered functional foods due to their various beneficial components, presenting an opportunity to enhance production by incorporating qualitative or quantitative adjustments into meat production [48]. Given that meat production begins with animal husbandry, it is critical to thoroughly evaluate important requirements that extend from the agricultural field to the dining table. Despite the greater fibre content, adapting feeds formulated with *C. glomerata* biomass for rabbits, given their unique digestive physiology (caecotrophy), has promise for achieving high productivity and health status while providing functional meat [49]. While enhancing overall meat quality is vital, sensory factors like flavour, smell, and visual attractiveness continue to play an important role in determining product acceptability. Furthermore, customer expectations and preferences continue to play a crucial role in evaluating overall product appeal, highlighting their relevance in optimising meat quality, production, and profitability for meat producers and distributors [50]. Nevertheless, the value of meat products is a complicated interplay of customer demands impacting willingness to pay and the final choice when purchasing the product [51]. In the context of rabbit meat, paying attention to development, health, and growth performance is fundamental, and biomass supplementation, particularly with *C. glomerata* biomass, provides a long-term solution to enhance both production efficiency and product quality. Nevertheless, the supplementation of freshwater macroalgae *C. glomerata* biomass collected from Lithuanian rivers and the partial replacement of traditional raw materials in rabbit feed could contribute to more sustainable rabbit meat production from river to table, creating a final functional product. So, the primary essence of the dissertation was to modulate more sustainable novel feed formulations with supplemented Lithuanian river-sourced *C. glomerata* macroalgal biomass, reducing the dependence on traditional feed

materials and manipulating indicators of rabbit growth, development, overall health, and the functional qualities of rabbit meat without adversely impacting the meat's sensory profile.

The aim of the study

To evaluate the potential of utilising freshwater *Cladophora glomerata* macroalgal biomass and the influence of biomass-supplemented feed on rabbit productivity, physiological development, carcass quality, and rabbit meat biochemical and sensory feature profiles.

Objectives of the study

1. Thoroughly investigate the potential utilisation of *C. glomerata* by evaluating the safety and chemical composition of the biomass.
2. Thoroughly investigate the potential utilisation of *C. glomerata* by evaluating the antioxidant activity and bioactive components of the biomass.
3. Thoroughly investigate the impact of novel *C. glomerata* feed formulas on productivity, slaughter indicators, and the physiological development of rabbits.
4. Thoroughly investigate the impact of novel *C. glomerata* feed formulas on rabbit meat functionality.
5. Thoroughly investigate the impact of novel *C. glomerata* feed formulas on rabbit meat sensory features profile and general acceptance.

The scientific novelty and practical usefulness of the study

The development and maintenance of sustainability in the livestock industry is crucial, especially given its significant environmental effects. Recognising the sector's immediate impact on the environment demands the adoption of sustainable practices to prevent negative consequences. To promote these approaches, animal scientists are investigating an abundance of novel strategies for producing high-quality, environmentally conscious, and renewable feed components. As a result, the current study focused on assessing the possibilities for using natural resources to construct a more sustainable rabbit ecology while taking into consideration the circular economy and the green course intended in the livestock business, including the growing demand for rabbit meat. *C. glomerata*, a green algal biomass that thrives in Lithuania's freshwater bodies, particularly rivers, was chosen as an alternate beneficial feed raw material for rabbits for this purpose. In general, *C. glomerata* is a widespread macroalgae with a high rate of growth, and the excessive biomass produced immediately decreases the recreational value

and ecological variety of water bodies. These macroalgal agglomerations are frequently manually removed, and *C. glomerata* biomass is considered waste. Scientific studies demonstrate that such biomass may be employed as a multifunctional raw material in a variety of industries. Macroalgal biomass collected from Lithuanian rivers can replace certain traditional feed materials in the development of a more sustainable livestock industry. Since *C. glomerata* absorbs solar resources more efficiently, the biomass it generates has a unique chemical composition, making it potentially beneficial.

By utilising freshwater *C. glomerata* macroalgal biomass from Lithuanian rivers, two key advantages can be achieved: 1) reduced costs of macroalgae biomass cultivation and extraction (including energy resources), as specific conditions and equipment for biomass cultivation are not required; 2) removal of excess biomass from water bodies enhances biological diversity and recreational value in water ecosystems, resulting in the collection of a multifunctional raw material with real potential for usage. However, the primary challenge highlighted is that the chemical composition of biomass directly correlates to its environment.

1. MATERIALS AND METHODS

1.1. Investigation venue

The research was carried out over the period from 2020 to 2023 at the Lithuanian University of Health Sciences (LSMU) Veterinary Academy (VA) Faculty of Animal Sciences Institute of Animal Rearing Technologies (Kaunas, Lithuania); Faculty of Veterinary Medicine Department of Veterinary Pathobiology (LSMU VA; Kaunas, Lithuania); Institute of Animal Science Analytical Laboratory (LSMU; Baisogala, Lithuania); Nature Research Centre Laboratory of Algology and Microbial Ecology (Vilnius, Lithuania); Kaunas University of Technology Faculty of Chemical Technology Department of Organic Chemistry (Kaunas, Lithuania); Rabbit Breeding Farm (identification code 9447582; Šakiai district, Lithuania).

1.2. Biomass utilisation potential evaluation and alternative feed modulation

1.2.1. *C. glomerata* macroalgal biomass samples collection

The research involved the collection of freshwater *C. glomerata* biomass from four distinct Lithuanian rivers: Dubysa (N55°12’25.07”, E23°30’30.44”; **B1**), Šventoji (N55°39’20.14”, E25°10’18.39”; **B2**), Nevėžis (N55°5’46.52”, E23°46’55.57”; **B3**), and Jūra (N55°27’19.58”, E22°2’14.72”; **B4**) (Fig. 1.2.1.1 A). Selection criteria were based on the prevalence of dense *C. glomerata* agglomerations, covering over 50% of the riverbed area (Fig. 1.2.1.1 B). Despite this commonality, the rivers varied in catchment area and water chemistry, as reported by Nutautaitė et al. [52]. Manual harvesting of fresh macroalgal biomass was conducted from the rivers (Fig. 1.2.1.1 C).

Subsamples, each weighing approximately 1 kg of wet biomass, were amalgamated from up to six sites. Rigorous washing, aimed at eliminating sand and mud particles, was performed, followed by the manual removal of macrozoobenthos, macrophytes, and other debris from the biomass. The collected samples underwent overnight drying at 60°C in an oven and were subsequently stored in sealed plastic bags at room temperature for subsequent analysis (Fig. 1.2.1.1 D).

Fig. 1.2.1.1. *Biomass collection sites* ***(A)****; the presence of biomass agglomerations in the Nevėžis River* ***(B)****; manual collection of the biomass* ***(C)****; the dried state of the biomass after processing* ***(D)***

Principal scheme of *C. glomerata* macroalgal biomass utilisation potential evaluation is presented in Fig. 1.2.2.1.

1.2.2. *C. glomerata* macroalgal biomass chemical analysis

Grinded macroalgal biomass samples (n = 3 replicate samples/river) underwent analysis following the guidelines outlined in Commission Regulation (EU) No 691/2013 dated July 19, 2013, which amended Regulation (EC) No 152/2009 concerning sampling and analysis methodologies. Chemical assessment for the determination of elemental composition in the dried biomass and biomass safety evaluation were conducted within an accredited research facility, adhering to methodologies delineated in Table 1.2.2.1. The methodologies for analysing the remaining chemical indicators, including

crude components such as protein (CP), fat (CF), ash (CA), and fibre (CFB), as well as the profiles of amino and fatty acids, are described in detail by Nutautaitė et al. [52].

Table 1.2.2.1. *Determination methods for elemental composition in C. glomerata biomass*

Element	Method
N	The Commission Directive 72/199/EEC
P	Directive 71/393/EEC
K	Directive 71/250/EEC
Ca	
Mg	Directive 73/46/EEC
Zn	LST EN ISO 15510:2017
Cu	
Ni	LST EN ISO 15550:2017
Cd	
Pb	

To evaluate antioxidant activity of collected biomass, these indicators were determined: total phenolic content, phenolic acid profile including flavonoids and catechins, reducing power (RP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (ABTS), ferric ion-reducing antioxidant power (FRAP), and total content of pigments (chlorophyll *a*, *b*, carotenoids, and lutein). The methodologies employed for mentioned indicators are described by detail by Nutautaitė et al. [53].

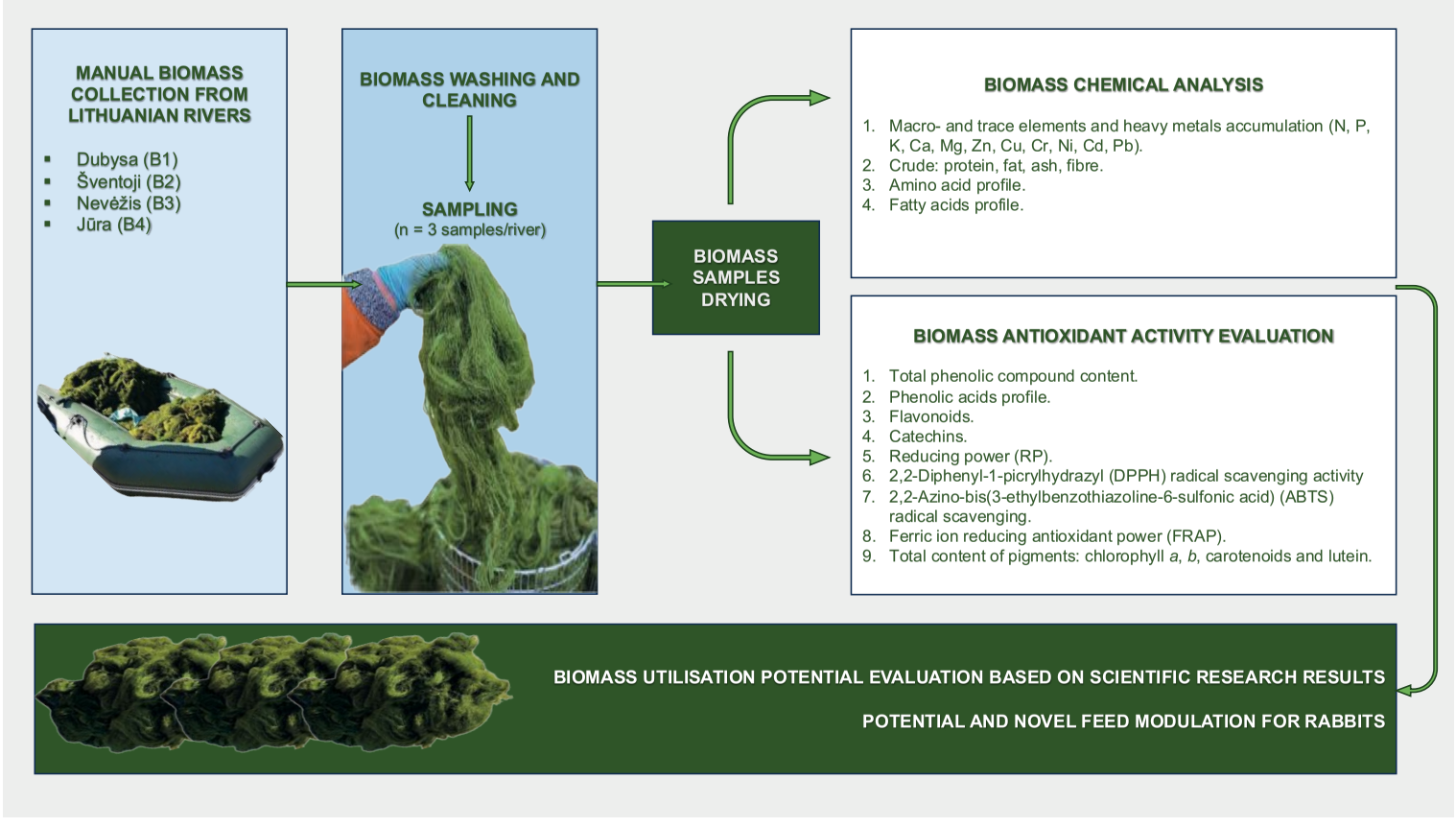


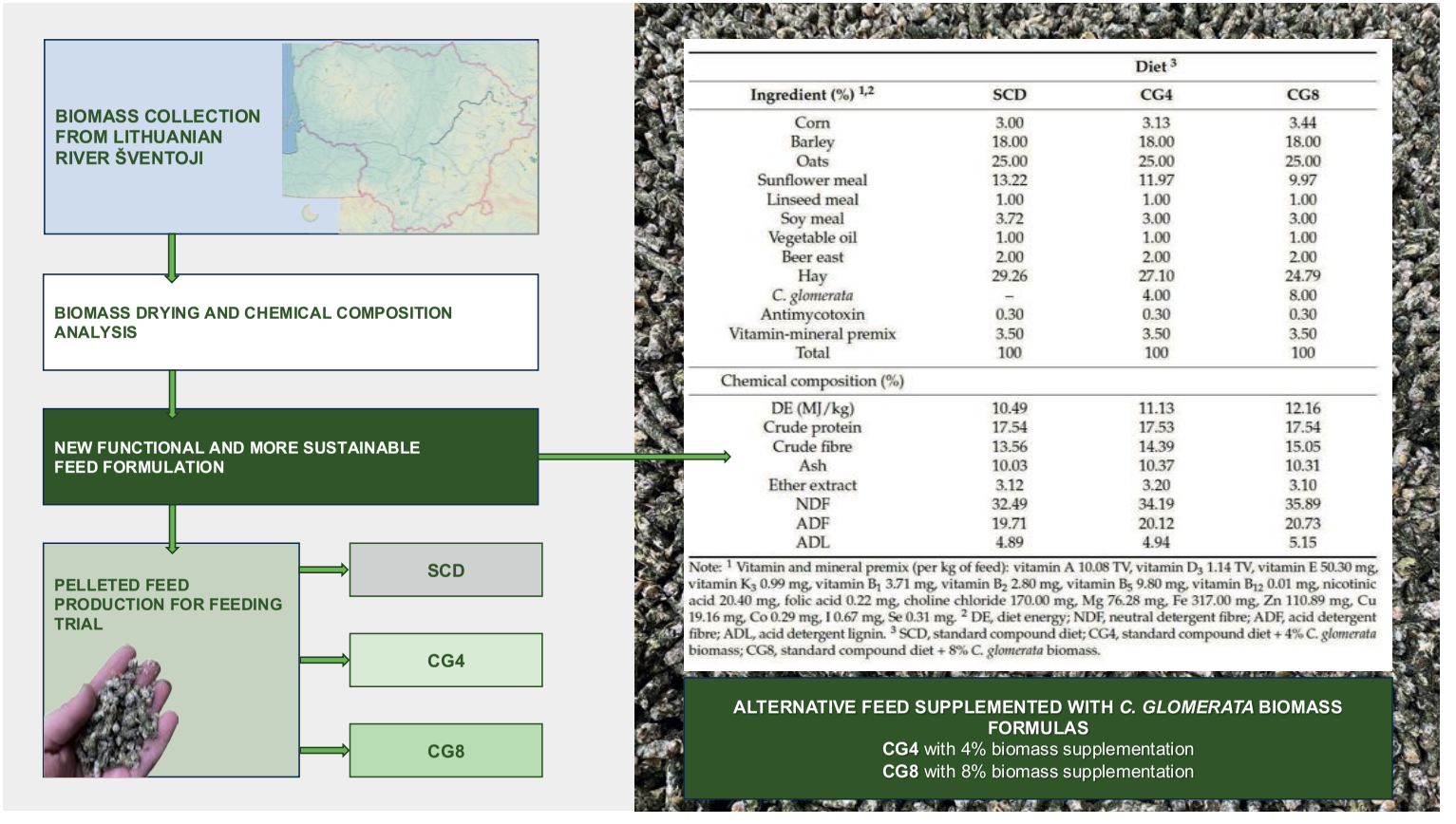
Fig. 1.2.2.1. *Principal scheme of C. glomerata macroalgal biomass utilisation potential evaluation*

1.2.3. *C. glomerata* macroalgal biomass supplemented feed formulas modulation and feed production

Following the assessment of biomass potential, a subsequent expedition to the Šventoji River (N55°39’20.14”, E25°10’18.39”) was conducted. The harvested biomass underwent analogous analyses, as outlined in Section 1.2.2. Based on the derived findings, two novel feed formulations were modulated, supplementing *C. glomerata* macroalgal biomass to substitute a portion of conventional feed materials. These feed formulations were modulated to align with the physiological parameters of rabbits. The standard compound diet was formulated and analysed to meet the nutrient requirements, including vitamins and minerals, of growing rabbits, as recommended by the National Research Council [54].

The realisation and production of the modulated feed formulas as well as the standard compound diet were carried out on the rabbit breeding farm (identification code 9447582; Šakiai district, Lithuania), employing feed production equipment: for weighting raw materials, digital scales SW1 S Plius (CAS Corp., East Rutherford, New Jersey, USA) and digital platform scales Ishida iGB (Ishida Co., Ltd., Kyoto, Japan); for chopping biomass and hay, farm machinery animal feed chopper 9ZT-0.6 (Mihao Electronic Factory, Ltd., Loudi, China); for mixing feed components together, electric feed mixing machine VTHS-100 (Zhengzhou Victor Machinery Co., Ltd., Zhengzhou, China); and for feed pelleting, granulator multifunctional feed pellet machine (Xiaoguang Reflective Material Co., Ltd., Shishi, China). Eventually, three different feeds were produced: the standard compound diet (**SCD**), SCD supplemented with 4% *C. glomerata* biomass (**CG4**), and SCD supplemented with 8% *C. glomerata* biomass (**CG8**). The produced feed in pellets was further used in the rabbit feeding trial.

Principal scheme of *C. glomerata* macroalgal biomass collection from Lithuanian River Šventoji and feed formulation including feed composition is presented in Fig. 1.2.3.1.



Ingredient (%) [1,2]	Diet [3] SCD	CG4	CG8
Corn	3.00	3.13	3.44
Barley	18.00	18.00	18.00
Oats	25.00	25.00	25.00
Sunflower meal	13.22	11.97	9.97
Linseed meal	1.00	1.00	1.00
Soy meal	3.72	3.00	3.00
Vegetable oil	1.00	1.00	1.00
Beer east	2.00	2.00	2.00
Hay	29.26	27.10	24.79
C. glomerata	–	4.00	8.00
Antimycotoxin	0.30	0.30	0.30
Vitamin-mineral premix	3.50	3.50	3.50
Total	100	100	100
Chemical composition (%)			
DE (MJ/kg)	10.49	11.13	12.16
Crude protein	17.54	17.53	17.54
Crude fibre	13.56	14.39	15.05
Ash	10.03	10.37	10.31
Ether extract	3.12	3.20	3.10
NDF	32.49	34.19	35.89
ADF	19.71	20.12	20.73
ADL	4.89	4.94	5.15

Note: [1] Vitamin and mineral premix (per kg of feed): vitamin A 10.08 TV, vitamin D_3 1.14 TV, vitamin E 50.30 mg, vitamin K_3 0.99 mg, vitamin B_1 3.71 mg, vitamin B_2 2.80 mg, vitamin B_5 9.80 mg, vitamin B_{12} 0.01 mg, nicotinic acid 20.40 mg, folic acid 0.22 mg, choline chloride 170.00 mg, Mg 76.28 mg, Fe 317.00 mg, Zn 110.89 mg, Cu 19.16 mg, Co 0.29 mg, I 0.67 mg, Se 0.31 mg. [2] DE, diet energy; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin. [3] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass.

Fig. 1.2.3.1. *Principal scheme of C. glomerata macroalgal biomass collection from Lithuanian River Šventoji and feed formulation*

1.3. C. glomerata supplemented feed utilisation in rabbit feeding trial

The feeding trial took place at a local rabbit breeding facility where rabbits were housed indoors in individual cages measuring 34 × 34 × 61 cm, with one rabbit per cage. Each rabbit had access to nipple drinkers for water and feed bowls to ensure optimal health and performance. The indoor temperature was maintained at 19 ± 2 °C. Throughout the trial, the photoperiod regimen consisted of 16 hours of illumination followed by an 8-hour period of darkness. The housing conditions complied with Council Directive 98/58/EC, which outlines standards for the welfare of farm animals.

Thirty male Californian breed rabbits, weaned at 52 days old, were selected based on similar weights and randomly divided into three dietary groups (n = 10 rabbits/diet). These groups were fed twice daily with three different types of feed: SCD, CG4, and CG8. Throughout the study, rabbits in all groups had *ad libitum* access to their respective feeds.

Principal scheme of *C. glomerata* supplemented feed utilisation in rabbit feeding trial is presented in Fig. 1.3.4.1.

1.3.1. Rabbits' growth performance analysis

Throughout the feeding trial extending from 52 to 122 days of age, the rabbits' individual weights including body weight gain (BWG), average daily gains (ADG), daily feed intake (DFI), and feed conversion ratios (FCR) were recorded periodically at intervals of 52–66, 66–80, 80–94, 94–108, and 108–122 days of age.

1.3.2. Rabbits' slaughter and physiological development assay

Upon completion of the feeding trial (122 days old), a total of 18 rabbits (n = 6 rabbits/diet) were randomly chosen, weighed, and subjected to an overnight fast. Following this procedure, the rabbits were euthanized and slaughtered in accordance with standard farming practices. The slaughtering procedures took place at a slaughterhouse on the rabbit breeding farm, following established protocols that align with the laws of the Republic of Lithuania, as outlined in Order No. B1-866 dated October 31, 2012, issued by the Director of the State Food and Veterinary Service. This order specifies the requirements for the care, housing, and use of animals for scientific and educational purposes. The ethical statement is provided, and the methods are extensively described by Nutautaitė et al. [55–57].

Carcass dissection procedures adhered to the guidelines outlined by the World Rabbit Science Association (WRSA) [58] and then chilled at 4°C for

24 hours in a well-ventilated room. A thorough examination of post-mortem carcass attributes, muscle, organ, and gut development was conducted. Specifically, segments of the *duodenum* and *ileum* from the middle part were collected for histomorphometric analysis (villus height and crypt depth), while the content of various intestinal segments (*duodenum*, small intestine, *caecum*, *ileum*, colon, and stomach) was collected for the determination of pH, dry matter (DM), and short-chain fatty acid (SCFA) profiles. The methodologies employed for mentioned indicators are described by detail by Nutautaitė et al. [57].

1.3.3. Rabbit meat functionality analysis

Following the slaughter performance assessment of the reference carcasses, the *Longissimus dorsi* (LD) and hind leg (HL) muscles were dissected. A total of 36 rabbit muscle samples were obtained 24 hours post-mortem, minced, and subsequently stored at −80 °C (referred to as fresh meat; LD0 and HL0, n = 6 samples per muscle type/diet) and at −18 °C for a duration of 3 months (referred to as stored meat; LD3 and HL3, n = 6 samples per muscle type/diet) for subsequent malondialdehyde (MDA) analysis. Other meat quality parameters, including chemical composition (protein, fat, ash, and dry matter), amino acid and fatty acid profiles, and cholesterol levels, were assessed using fresh meat samples (n = 6 samples per muscle type/diet). The methodologies employed for mentioned indicators are described by detail by Nutautaitė et al. [55].

1.3.4. Rabbit meat sensory profiles evaluation

Analogous schemes were employed for sampling the *Longissimus dorsi* (LD) and hind leg (HL) muscles to collect data on physical composition, muscle fibre area, sensory profile, and emotional response to samples (n = 6 samples per muscle type/diet).

The investigation covered an examination of multiple physical composition parameters, including moisture content, drip loss, water holding capacity, cooking loss, pH, and colour coordinates (coordinates L* indicate lightness, a* redness, and b* yellowness) in 24- to 48-hours after slaughter and cooked muscles, and muscle fibre area. Furthermore, sensory evaluations of cooked LD and HL rabbit muscles were conducted. These evaluations entailed the assessment of various attributes, such as overall odour intensity, rabbit odour intensity, non-typical odour, colour intensity, hardness, fibrines, juiciness, chewiness, crunchiness, mouthfeel, overall taste intensity, richness of taste, rabbit flavour intensity, non-typical taste, aftertaste, acceptability of odour, acceptability of taste, acceptability of texture, and overall acceptabi-

lity. Evaluation scores were assigned using a 7-point numerical scale, where 1 represented absence of the feature, 4 indicated moderate expression, and 7 signified strong expression.

Ten evaluators participated in another sensory assessment, employing FaceReader 8 software (Noldus Information Technology, Wageningen, The Netherlands) connected to a webcam (Microsoft Corporation, Redmond, WA, USA). This software facilitated the real-time assessment of evaluators' facial expressions while they viewed, smelled, and tasted the samples. It possessed the capability to recognise and record eight distinct emotional expressions, including neutral, happy, sad, angry, surprised, scared, disgusted, and contempt, while also computing valence to delineate the individual's positive or negative emotional state.

The more detailed sampling preparation and methodologies employed for mentioned indicators are described by detail by Nutautaitė et al. [56].

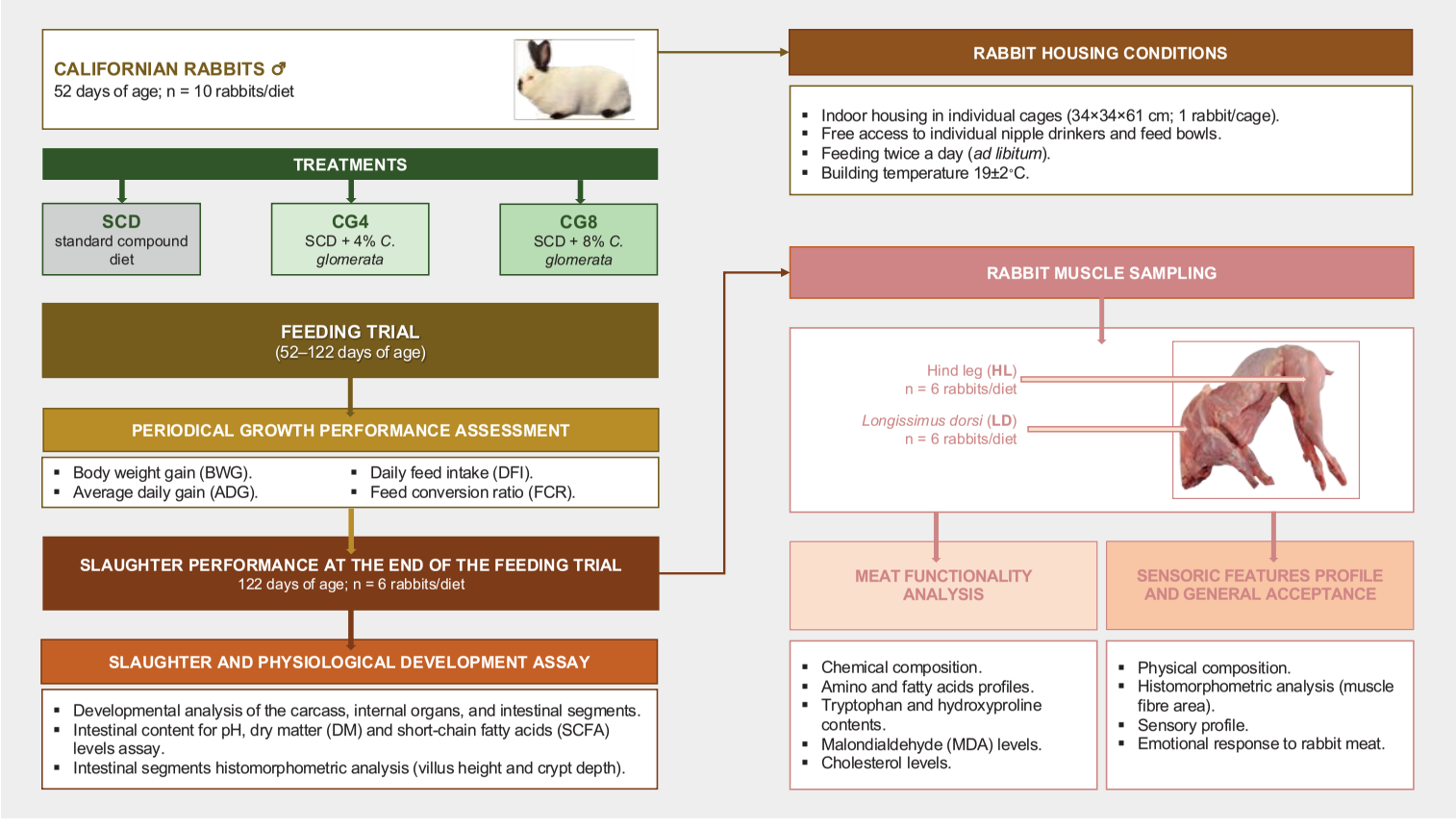


Fig. 1.3.4.1. *Principal scheme of C. glomerata macroalgal biomass supplemented feed utilisation in rabbit feeding trials*

1.4. Statistical analysis

1.4.1. Biomass analysis

The study followed a completely randomised design, consisting of four samples of *C. glomerata* macroalgal biomass (each from a different Lithuanian river) with three replicates each (n = 3 samples/river). Data analysis was conducted using SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). A one-way analysis of variance (ANOVA), followed by Fisher's least significant difference test as a post-hoc analysis, was employed to identify variations among samples. Statistical significance was determined with a calculated p-value of less than 0.05 ($p < 0.05$).

1.4.2. Rabbit growth, slaughter performance and meat quality, including sensory profiles, analysis

The feeding trial involved 10 rabbits per diet, while for slaughter performance, samples were taken from 3 groups of rabbits, each with 6 duplicates (n = 6 duplicate rabbits/diet). From each rabbit, intestinal samples, and samples from two types of muscles were collected for further analysis (n = 6 intestinal segments/diet; n = 6 LD/diet; n = 6 HL/diet). Data analysis was carried out analogously as presented in Section 1.4.1.

2. RESULTS AND DISCUSSION

2.1. Chemical composition of freshwater *C. glomerata* macroalgal biomass collected from Lithuanian rivers (https://doi.org/10.3390/agriculture11070582)

The incorporation of alternative protein sources into animal feed is critical for improving food supply chain sustainability and supporting ecological balance within farm animal ecosystems. The biomass of *C. glomerata* possesses a wide range of bioactive compounds, making it a promising material for feed additive integration [28]. However, extensive blooms of *C. glomerata* can cause ecological imbalances, reducing biodiversity and recreational value in impacted water bodies. So, the first step of this dissertation's experimental stage was to assess the viability of utilising *C. glomerata* biomass from Lithuanian rivers as a sustainable protein and other nutrient source in animal feed formulations through comprehensive chemical analysis and characterization of its amino and fatty acid profiles.

Compared to other feed materials, such as edible land plants, the content of essential elements in algae can be higher as much as 40% [59]. Recent findings suggest that *C. glomerata* can also serve as a carrier of various elements in animals' diets. The macronutrient distribution in algal biomass was as follows: Ca>K>N>P>Mg (Fig. 2.1.1 A), and for trace elements, Zn>Cu (Fig. 2.1.1 B). This distribution aligns with Messsyasz et al.'s [29] results, where Ca content in *C. glomerata* biomass from Oporzyn lake (Poland) was higher, similarly to our findings. Furthermore, Michalak et al. [28] demonstrated that *Cladophora* biomass enriched with microelement ions through biosorption can be a valuable feed additive for various animal breeds, partially substituting for inorganic salts.

Cladophora species are widely recognised as reliable indicators of nutrient and heavy metal pollution in aquatic environments [60]. Analysis of heavy metal concentrations in *C. glomerata* from Lithuanian rivers indicated levels within recommended thresholds (Cr>Ni>Pb>Cd; Fig. 2.1.1 B), thus demonstrating no apparent toxicity risk to animals consuming the algal biomass. These findings align with European Parliament and Council Directive 2002/32/EC guidelines on undesirable substances in animal feed.

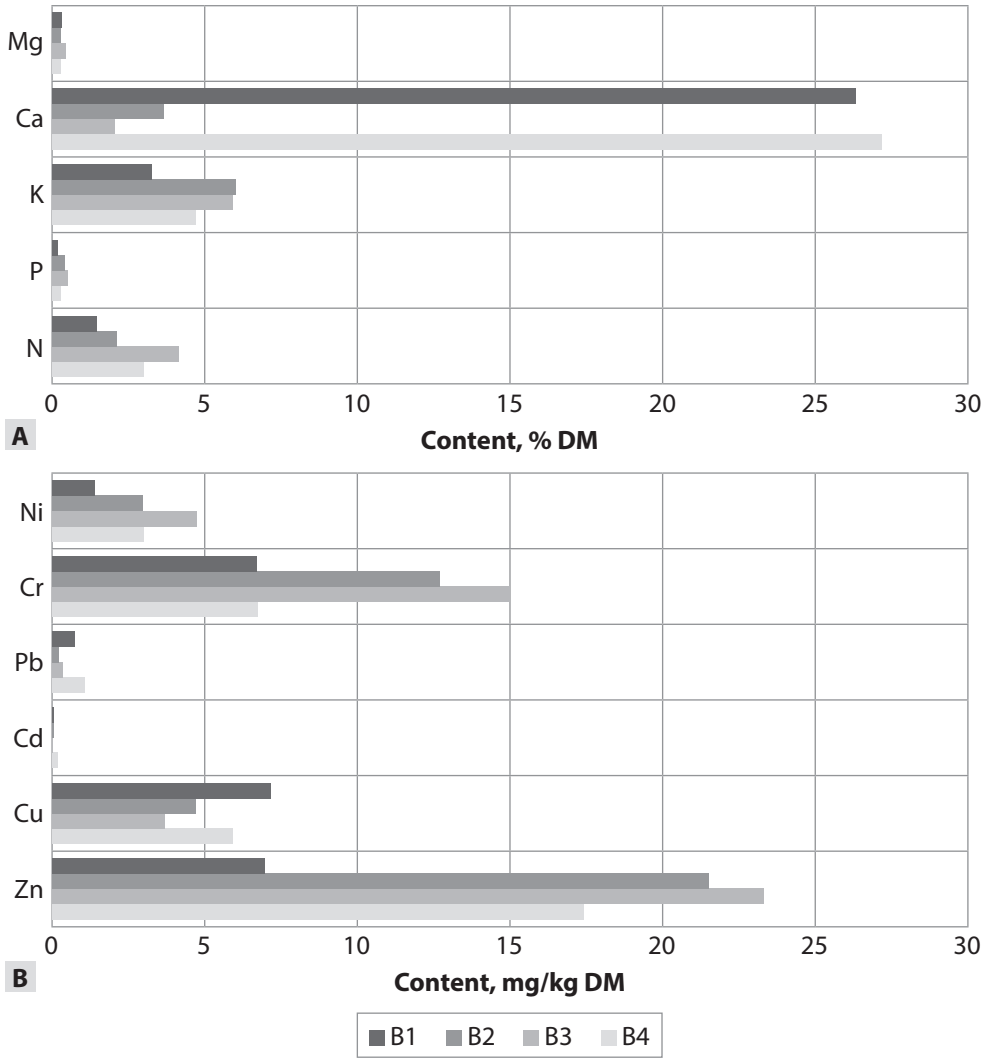


Fig. 2.1.1. *Macro elements* ***(A)****, trace elements and heavy metals* ***(B)*** *levels in freshwater C. glomerata biomass collected from Lithuanian Rivers: Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)*

Note: DM, dry matter.

Besides supplying crucial mineral elements, freshwater macroalgae also offer a valuable protein source for animal nutrition. *Cladophora* biomass, according to its protein levels, is comparable to other traditional feed materials since it typically ranges from 10% to 25% [61]. Similarly, our recent analysis of *C. glomerata* biomass from local rivers revealed a CP content ranging from 15.98% to 21.52% ($p < 0.05$; Fig. 2.1.2). To be more precise, B4 biomass samples for Jūra river exhibited significantly higher CP content compared to rivers Dubysa (B1), Šventoji (B2), and Nevėžis (B3) samples, with increases of 14.87%, 25.74%, and 15.52%, respectively ($p < 0.05$). Algal biomass holds potential as a feed ingredient and can be directly consumed by livestock species such as cattle, swine, and sheep [62]. Regarding the chemical composition of analysed biomass, the CF content is notably lower, as observed in our study (ranging 0.19–0.35%). Specifically, B1 samples exhibited the highest CF content, compared to samples from B2 and B4 ($p < 0.05$). Following CA determination, significant differences were observed among all biomass samples, with B3 showing the highest CA content and B4 the lowest ($p < 0.05$). Comparative analysis of Anh et al. [41] revealed varying CA levels in traditional and alternative protein feed materials, with fishmeal exhibiting the highest content (25.95%), followed by *Cladophora* meal (21.14%), rice bran (17.17%), soybean meal (8.26%), and cassava powder (1.97%). Our study discovered CA content in biomass ranged from 36.96% to 49.83%, while CFB content averaged approximately 13.46%. These findings align with previously conducted studies [29, 63], indicating potential utilisation of algal biomass with higher fibre content in rabbit diets due to their unique digestive physiology [64].

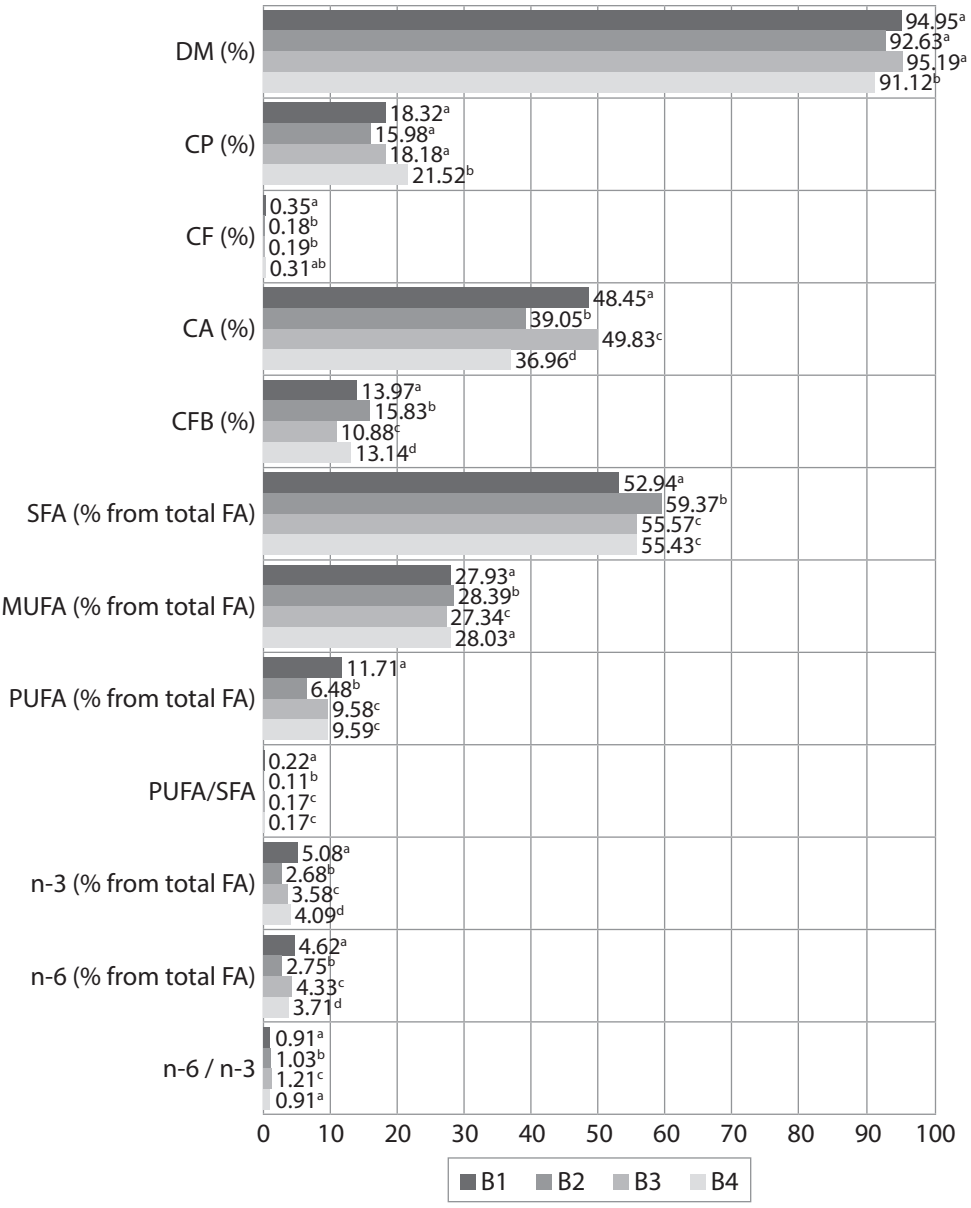


Fig. 2.1.2. *Main chemical composition indicators' contents in freshwater C. glomerata biomass collected from Lithuanian Rivers: Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)*

Note: DM, dry matter; CP, crude protein; CF, crude fat; CA, crude ash; CFB, crude fibre; FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acids. [a-d] means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$). [ab] means with ab superscript letters in a column of the same indicator did not have a significant difference ($p > 0.05$).

Various studies have highlighted the richness of saturated (SFA) and unsaturated fatty acids (USFA) in both marine and freshwater macroalgae [28, 65–67]. In recent investigation, total SFA in different algal biomass samples exceeded 50% of total fatty acids (FAs) ($p < 0.05$; Fig. 2.1.2), with biomass from Šventoji river (B2) displaying the highest content and from Dubysa river (B1) the lowest ($p < 0.05$). However, our findings contrast with those of Messyasz et al. [29], who reported lower SFA content in *C. glomerata* likewise. Total monounsaturated fatty acids (MUFA) accounted for 27.34–28.39% of total FAs, with B2 biomass exhibiting the highest content ($p < 0.05$). PUFA levels in freshwater *C. glomerata* biomass ranged 6.48–11.71% of total FAs, indicating their potential as functional feed and food elements [3]. Compared to marine biomass of *C. albida* analysed by Pereira et al. [68], our study discovered lower PUFA levels. In this case, marine and freshwater macroalgae, including *Cladophora* species, are being explored as alternative sources due to their scalability and cultivation feasibility [3, 5, 29, 68].

The PUFA/SFA ratio, an essential lipid index used to evaluate cardiovascular health impacts of diets [69], indicates a more favourable impact with higher ratios. In a recent study, the highest PUFA/SFA ratio observed was 0.22 in *C. glomerata* samples from Dubysa river (B1; Fig. 2.1.2). It was approximately 1.69 times higher than that of plant oils like palm stearin (PUFA/SFA = 0.13 [70]) usually used in feed production.

Long-chain n-3 and n-6 PUFAs, synthesised by the same enzymes, compete for metabolic pathways, affecting their balance [71]. Maintaining an optimal n-6 to n-3 ratio of 1 or 4:1 is crucial for health benefits [72]. *C. glomerata* biomass from rivers Dubysa (ratio: 1.30 (B1)) and Jūra (ratio: 1.35 (B4)) showed the lowest ratios, while Šventoji (ratio: 1.42 (B2)) and Nevėžis (ratio: 1.68 (B3)) had higher ratios within the ideal range (Fig. 2.1.2). These ratios make them appropriate as animal nutritional supplements or feed raw materials, potentially enhancing immune response, productivity, and nutritional value of final animal products [73].

Amino acids (AAs), particularly exogenous ones, found in various feed additives, enhance animals' nutrient digestibility, compensate for deficiencies, and improve feed quality and final animal production composition [5]. Therefore, both essential and non-essential AAs are vital for meeting animals' metabolic needs. In a recent study, comprehensive AA profiling of *C. glomerata* biomass revealed significant differences among collected samples, except for histidine content (Table 2.1.1). The highest total AA concentration identified in *C. glomerata* biomass from the Lithuanian river Jūra (B4), reaching 141 g/kg, with glutamic acid, aspartic acid, and leucine being the most prevalent AAs across all rivers. Likewise essential AAs, constituting

41.60–55.40 g/kg of total AAs, were most concentrated in Jūra samples (B4) compared to others ($p < 0.05$). Essential AAs like isoleucine, leucine, phenylalanine, and lysine also peaked in B4 biomass ($p < 0.05$). For example, methionine, essential for protein synthesis, exhibited concentrations comparable to *Spirulina platensis* and twice that of soybean meal [74]. Moreover, non-essential AAs also play crucial roles in metabolism and overall function [75–78]. A recent study revealed higher concentrations of several non-essential AAs in Jūra biomass (B4), all potentially valuable in mammalian diets. Our findings are in line with the observations of Messyasz et al. [29], suggesting that the AA composition of *Cladophora* biomass presents a promising new material with potential applications as an alternative feed supplement for animals.

Table 2.1.1. *Amino acid profile of freshwater C. glomerata biomass collected from Lithuanian Rivers*

AA (g/kg DM)	Biomass				p-value
	B1	B2	B3	B4	
Asp	10.90^{a}	13.63^{b}	14.18^{c}	15.97^{c}	0.001
Thr	3.49^{a}	6.52^{b}	6.28^{b}	6.77^{b}	0.001
Ser	4.14^{a}	6.21^{b}	6.29^{c}	7.25^{c}	0.001
Glu	14.16^{a}	16.90^{b}	17.53^{c}	19.50^{c}	0.000
Pro	5.98^{a}	5.92^{a}	6.40^{a}	7.22^{b}	0.006
Gly	8.06^{a}	9.23^{b}	9.54^{c}	11.46^{c}	0.000
Ala	8.49^{a}	8.12^{a}	10.09^{b}	10.73^{b}	0.000
Val	8.39^{a}	8.48^{a}	8.90^{a}	10.42^{b}	0.006
Met	1.94^{b}	4.14^{a}	2.24^{b}	2.08^{b}	0.000
Ile	5.94^{a}	6.17^{a}	6.80^{a}	7.69^{b}	0.004
Leu	9.71^{a}	9.64^{b}	10.81^{c}	12.01^{d}	0.002
Tyr	1.73^{a}	1.71^{a}	2.09^{b}	2.35^{b}	0.009
Phe	6.37^{a}	6.93^{a}	7.39^{a}	8.50^{b}	0.004
His	3.04	3.01	3.33	3.68	n.s.
Lys	5.76^{a}	5.85^{a}	6.46^{a}	7.88^{b}	0.001
Arg	5.25^{a}	5.79^{a}	6.31^{a}	7.47^{b}	0.000
Total	103.36^{a}	118.25^{b}	124.64^{c}	140.99^{d}	0.001

Note: AA, amino acid; DM, dry matter; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; His, histidine; Lys, lysine; Arg, arginine; n.s., not significant. Biomass collected from Lithuanian Rivers: B1, Dubysa; B2, Šventoji; B3, Nevėžis; B4, Jūra. $^{a-d}$ means with different superscript letters in a row were significantly different ($p < 0.05$).

2.2. Antioxidant activity and bioactive components of freshwater *C. glomerata* macroalgal biomass collected from Lithuanian rivers (https://doi.org/10.3390/w14071138)

Efforts are underway to extract high-quality, sustainable raw materials, such as macroalgal biomass like *C. glomerata*, which holds promise for various industries while contributing to environmental clean-up and biodiversity [6, 29–31, 79, 80]. Several factors influence the chemical composition of algal biomass and the presence of biologically active compounds, including species, habitat, climate, environmental stressors, and collection methods [81, 82]. Macroalgae's abundance of bioactive chemicals is due to its ability to adapt to adverse environmental circumstances. Algae are acknowledged as a natural source of phenolic compounds, which are recognised for their antioxidant qualities, which are principally due to their capacity to bind, stabilise, and neutralise free radicals [83, 84]. Thus, another step of this dissertation experimental stage was to analyse the phenolic compound profile, antioxidant activity, and pigment concentration of *C. glomerata* biomass from Lithuanian rivers as a potential raw material.

Only three phenolic acids (gallic, *p*-coumaric, and *p*-hydroxybenzoic) were detected in the macroalgal biomass during the study, out of the 13 assessed (Table 2.2.1). Gallic acid was mainly found in B3 biomass from Nevėžis river (35.13 μg/g DM) and *p*-hydroxybenzoic acid in B3 and B4 (29.05 μg/g DM and 28.31 μg/g DM, respectively; $p < 0.05$). *P*-coumaric acid was identified in three of the four biomass samples, with the highest concentration in B4 (6.46 μg/g DM). While our study identified fewer phenolic compounds compared to Korzeniowska et al. [83], authors confirmed the presence of gallic and *p*-coumaric acids in all biomass samples. These phenolic acids, along with *p*-hydroxybenzoic acid, offer health benefits, including antihyperlipidemic, antihyperglycemic, cardioprotective, and anti-cancer properties [85–88]. Generally, phenols exhibit various properties contributing to antioxidant activity and antibacterial and antiviral activities [89], although flavonoids were not detected in our study. The total phenolic compound content varied across biomass samples, with the highest concentration observed in B3 (1.32 mg GAE/g DM). However, differences in total phenolic content were significant between B1 and B3 (0.37 mg GAE/g DM lower in B1) and between B2 and B3 (0.22 mg GAE/g DM lower in B2; $p < 0.05$). Analysis for flavonoids (quercetin, myricetin, kaempferol, rutin, and xanthohumol) did not yield positive identifications.

Table 2.2.1. *Phenolic compounds profile of freshwater C. glomerata biomass collected from Lithuanian rivers*

Phenolic compound	Biomass				p-value
	B1	B2	B3	B4	
	Phenolic acids (µg/g DM)				
o-Coumaric	Not defined				
Cinnamic					
m-Coumaric					
Vanillic					
Caffeic					
Salicylic					
Ferulic					
Sinapic					
Chlorogenic					
3,4-Dihydroxybenzoic					
Gallic	12.94[a]	21.31[b]	35.13[c]	13.92[d]	0.000
p-Hydroxybenzoic	23.97[a]	25.43[b]	29.05[c]	28.31[c]	0.000
p-Coumaric	3.16[a]	1.79[b]	–	6.46[c]	0.000
	Flavonoids (µg/g DM)				
Quercetin	Not defined				
Myricetin					
Kaempferol					
Rutin					
Xantohumol					
	Catechins (µg/g DM)				
Catechin	Not defined				
Epicatechin					
Epigallocatechin gallate					
The total phenolic content (mg GAE/g DM)	0.95[a]	1.10[b]	1.32[c]	1.22[c]	0.003

Note: DM, dry matter; GAE, gallic acid equivalent. Biomass collected from Lithuanian Rivers: B1, Dubysa; B2, Šventoji; B3, Nevėžis; B4, Jūra. [a-d] means with different superscript letters in a row were significantly different ($p < 0.05$).

Previous studies have linked antioxidant activity with the RP of plant extracts [90, 91]. In this case, reductones, which break the free radical chain by donating a hydrogen atom, are associated with reducing abilities [91]. This method measures increased absorbance in reaction mixtures, indicating higher antioxidant activity. Our findings on the RP of freshwater *C. glomerata* biomass from Lithuanian rivers were distributed as follows: B3>B4>B1>B2 (Fig. 2.2.1). Specifically, the highest RP was observed in B3 biomass, reaching 0.737 absorption units (AU) at 700 nm ($p < 0.05$). Compared to other samples, B3 biomass exhibited 0.341, 0.436, and 0.152 AU higher RP compared to B1, B2, and B4 samples, respectively ($p < 0.05$).

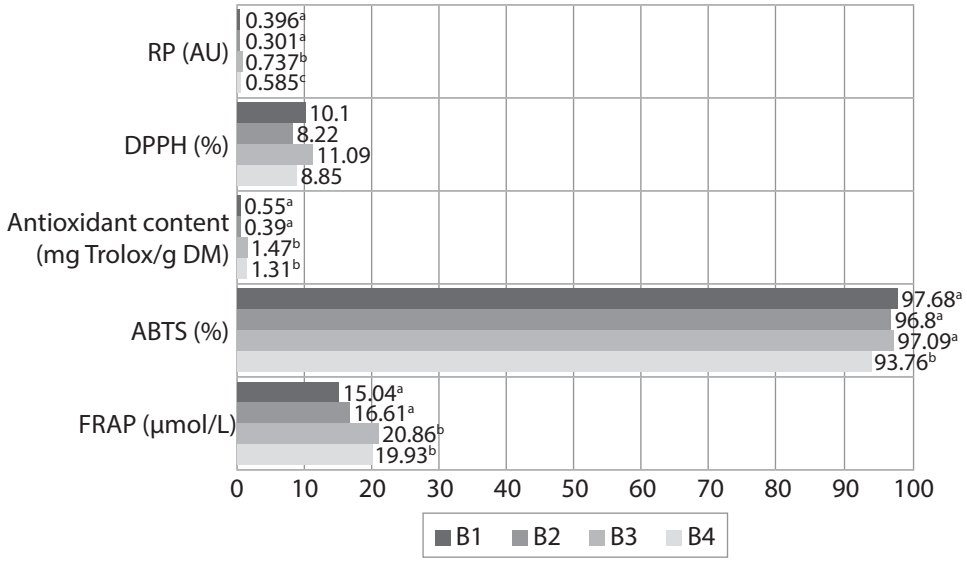


Fig. 2.2.1. *Reducing power (RP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), antioxidant content, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (ABTS) and ferric ion reducing-antioxidant power (FRAP) of freshwater C. glomerata biomass collected from Lithuanian Rivers: Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)*

Note: AU, absorbance units; DM, dry matter. [a-c] means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$).

The DPPH scavenging activity ranged from 8.22% to 11.09% across biomass samples, showing no significant differences ($p > 0.05$; Fig. 2.2.1). However, B3 and B4 exhibited higher antioxidant content levels (1.47 mg Trolox/g DM for B3 and 1.31 mg Trolox/g DM for B4) compared to B1 and B2 ($p < 0.05$). Specifically, B3 showed 2.7- and 3.8-times higher antioxidant,

and B4 showed 2.4- and 3.4-times higher content than B1 and B2, respectively ($p < 0.05$). In general, the antioxidant content of freshwater *C. glomerata* biomass varies with season and geographical location [92]. For instance, samples from Lithuanian rivers Dubysa (B1) and Šventoji (B2) exhibited lower antioxidant content (0.55 and 0.39 Trolox/g DM, respectively) compared to samples analogously analysed but collected from Polish water reservoirs (Wielkopolska region) [83], which had 2–3 times higher content. These variations may stem from differences in macroalgal habitats between the regions.

Following the ABTS assay, the highest scavenging activity was observed in B1 biomass samples (97.68%; Fig. 2.2.1), with no significant differences discovered between groups B1, B2, and B3 ($p > 0.05$). However, compared to B4, the ABTS scavenging activity of B1, B2, and B3 samples was 3.92%, 3.04%, and 3.33% higher, respectively ($p < 0.05$). The ABTS assay evaluates antioxidants' ability to scavenge ABTS*+ radicals, with results ranging from 93.76% to 97.68% across macroalgal biomass samples collected from Lithuanian rivers. This activity was notably higher compared to *C. glomerata* from Polish freshwaters [83]. Nevertheless, carotenoids derived from algae are known to exhibit better scavenging activity for ABTS radicals, albeit with lower reducing power [93], which aligns with our findings.

The FRAP method assesses antioxidants' ability to reduce ferric iron [94], with results measured by a shift in absorbance at 593 nm. FRAP activity indicates the reducing power of antioxidants and is more closely associated with phlorotannin content than radical scavenging capacity [95]. Recent FRAP analysis revealed activity ranging from 15.04 to 20.86 μmol/L across *C. glomerata* biomass samples (Fig. 2.2.1). Macroalgal samples B3 and B4 exhibited the highest FRAP activity, surpassing B1 and B2 by 5.82 and 4.25 μmol/L, respectively ($p < 0.05$).

The concentration of individual pigments was assessed in *C. glomerata* biomass (Fig. 2.2.2). The total chlorophyll *a* content ranged from 0.56 to 0.74 mg/g DM, with B3 exhibiting the highest concentration and B2 the lowest ($p < 0.05$). Significant differences in chlorophyll *b* concentrations were observed only between B1 and B4, with B1 having a higher concentration ($p < 0.05$). In general, chlorophyll is a vital pigment for photosynthesis in plants, algae, and cyanobacteria [96]. However, environmental factors such as water depth, nutrient levels, and light availability might affect chlorophyll concentration [6, 12]. A recent study revealed higher chlorophyll *a* content compared to previous scientific findings [67, 97], presumably due to differences in habitat conditions and biomass collection times. Additionally, discovered elevated levels of chlorophyll *b*, consistent with the species' adaptability to diverse environments [98].

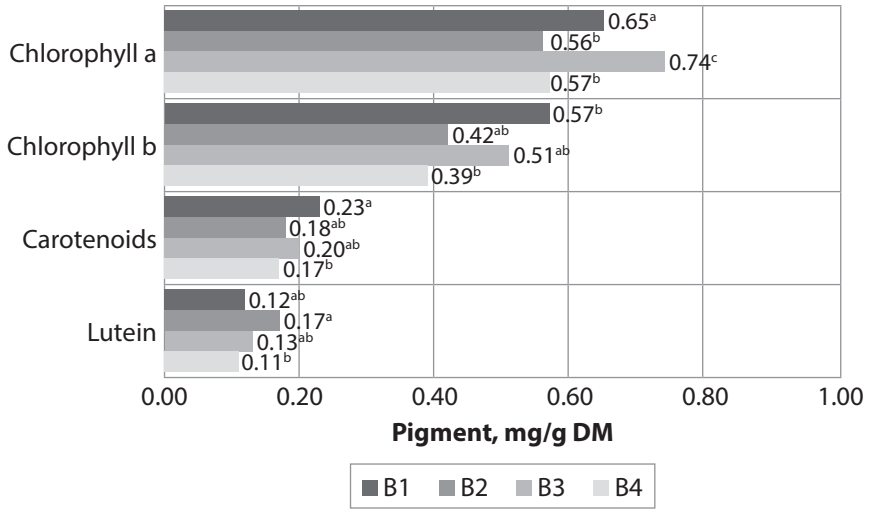


Fig. 2.2.2. *Concentration of various pigments in freshwater C. glomerata biomass collected from Lithuanian Rivers: Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4).*

Note: DM, dry matter. [a-c] means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$). [ab] means with ab superscript letters in a column of the same indicator did not have a significant difference ($p > 0.05$).

Carotenoids, a diverse group of compounds exclusive to algae, vary in chemical structure, colour, and antioxidant properties [25]. Nevertheless, similar to chlorophylls, the total carotenoid concentration exhibited a consistent trend, with B1 biomass containing 0.06 mg/g DM more carotenoids than in B4 ($p < 0.05$; Fig. 2.2.2). Generally, carotenoid levels in *C. glomerata* biomass ranged from 0.17 to 0.23 mg/g DM, with lower concentrations compared to previous scientific findings [99]. Moreover, variations in carotenoid levels among studies may result from biodiversity and environmental factors influencing algal metabolism and pigment production. For example, reduced temperatures and sunlight diminish metabolic rates in algae, leading to decreased chlorophyll production for photosynthesis and concurrent elevations in carotenoid synthesis [100].

Lutein, a vital xanthophyll carotenoid in green algae, aids in light absorption and shields against oxidative stress in photosynthesis [101]. Prazukin et al. [102] found a lutein content of 0.55 mg/g DM in *Cladophora* spp. from a Russian lake, suggesting at its potential in the pharmaceutical and food industries. Our study on Lithuanian rivers' *C. glomerata* revealed that lutein levels range from 0.11 mg/g in Jūra (B4) to 0.17 mg/g in Šventoji (B2), with significant differences between B2 and B4 ($p < 0.05$; Fig. 2.2.2). Lutein renders cognitive benefits, lowers cancer risk, and combats inflammation,

serving as a natural eye protector [103–106]. It's advised to consume 5 mg daily, obtained from the diet, due to its absence in the body. The lutein market spans pharmaceuticals, supplements, food, and animal feed [107].

2.3. Impact of *C. glomerata*-supplemented feed on productivity, slaughter indicators, and the physiological development of rabbits (https://doi.org/10.1080/1828051X.2024.2342380)

Rabbit growth performance significantly impacts production efficiency and overall enterprise profitability [108]. Meeting nutritional requirements is vital for optimal growth, and certain *Cladophora* species, with compositions akin to traditional feed plants, simplify this dependency [61]. Despite the prevalent use of macroalgae in aquaculture and poultry feed, research on *C. glomerata* application in rabbit nutrition is limited. Scientific evidence suggests that an optimal dosage ranging from 10% to 20% of *C. glomerata* can enhance productivity traits, such as FCR, in tiger shrimp [41]. Similarly, in studies on broiler chickens, a 15% supplementation of *C. glomerata* biomass in their feed demonstrated improved growth rates compared to standard feed [109]. Thus, a further step in this dissertation's experimental stage was to investigate the effects of novel *C. glomerat*a feed formulations on rabbit productivity, slaughter indicators, and physiological development.

A recent study examining rabbit growth performance, incorporating river-sourced *C. glomerata* biomass throughout a feeding trial spanning from 52 to 122 days of age, revealed no statistically significant impact on BWG and ADG across different dietary regimens ($p > 0.05$; Table 2.3.1). Therefore, achieving optimal growth requires a balance, as insufficient feed intake can lead to malnutrition, while excessive consumption may cause obesity [110]. In a recent study, rabbits fed with 4% biomass-supplemented feed (CG4) showed a higher DFI compared to a double dosage of biomass (CG8), suggesting a potential influence on palatability or satiety response to a CG4 diet. Observed significant differences between the CG4 and CG8 dietary treatments, with a 3.48 g increase noted in CG4 compared to CG8 ($p < 0.05$). This aligns with prior research indicating the palatability of diets containing macroalgae [111]. Moreover, improved potential palatability may be due to bioactive compounds in *C. glomerata*, such as polyphenols and secondary metabolites [4, 23, 53]. However, optimal biomass inclusion was determined to be 4%, as higher amounts reduced DFI in an 8%-enriched diet, suggesting a potential upper limit for acceptable inclusion.

Table 2.3.1. *Impact of produced C. glomerata-supplemented feed on rabbit growth performance*

Item	Trial period	Diet			p-value
		SCD	CG4	CG8	
BWG (g)	52–122 days of age	1633.00	1560.25	1492.75	n.s.
ADG (g)		22.59	23.74	22.04	n.s.
DFI (g)		71.55^{ab}	73.79^{a}	70.31^{b}	0.013
FCR (kg/kg)		3.71^{b}	4.23^{a}	2.93^{c}	< 0.001

Note: BWG, body weight gain; ADG, average daily gain; DFI, daily feed intake; FCR, feed conversion ratio; SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass; n.s., not significant. $^{a\text{-}c}$ means with different superscript letters in a row were significantly different ($p < 0.05$). ^{ab} means with ab superscript letters in a row did not have a significant difference ($p > 0.05$).

A lower FCR in rabbits indicates more efficient conversion of feed into body weight, impacting production costs and profitability in rabbit farming [112]. Variations in FCR patterns among dietary regimens may reflect differences in feed utilisation efficiency. In assessing FCR, CG4 showed a significantly higher indicator (4.23 kg/kg), followed by a lower one in the SCD (3.72 kg/kg), and the lowest in rabbits treated with CG8 (2.93 kg/kg) ($p < 0.05$; Table 2.3.1). The notably lower FCR in CG8 may be attributed to altered digestive processes or nutrient absorption influenced by components of *C. glomerata*, known for its reservoir of bioactive compounds such as polysaccharides and peptides, which can modulate nutrient utilisation and absorption in the gastrointestinal tract [113–116]. In our study, a higher dosage of river-sourced biomass reduced FCR, while a lower dosage increased it, suggesting that feed enriched with 8% biomass could lead to a more economical conversion of feed into rabbit body weight.

Rabbits' healthy growth and development require proper maturity of their internal organs and intestines, which supports proper nutrient absorption and a strong immune system [117]. However, no significant variations were identified across treatments, except for lung percentage based on pre-slaughter body weight. Rabbits on the SCD diet exhibited a significant increase in lung percentage compared to those in both *C. glomerata*-treated groups ($p < 0.05$). The observed influence on lung growth and respiratory performance reveals potential physiological processes of biomass, potentially due to the presence of antioxidants such as flavonoids and carotenoids [53]. These antioxidants can lower oxidative stress in the stomach, which promotes intestinal health and may influence respiratory processes such as lung formation. However, analogous lung percentages in *C. glomerata* treatments do not

show a significant dose-dependent association with the reported physiological result. Other slaughter performance parameters exhibit negligible differences, indicating that biomass has a minimal impact on factors such as meat water-holding capacity and live weight conversion efficiency ($p > 0.05$). This emphasises that the overall composition of meat-related anatomical structures remains largely unchanged with the inclusion of *C. glomerata* in rabbit diets.

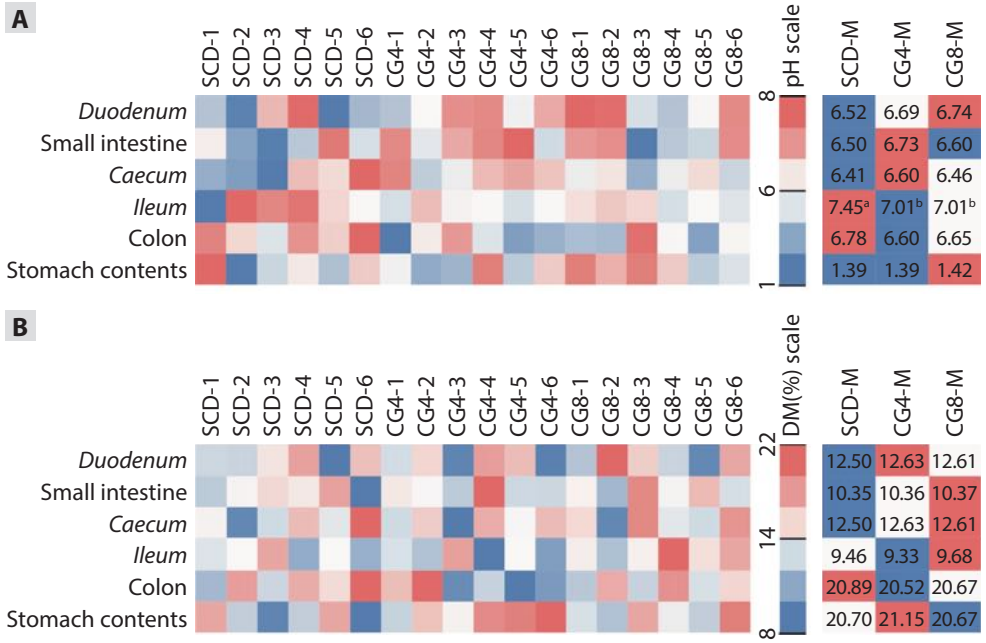


Fig. 2.3.1. *Impact of produced C. glomerata-supplemented feed on pH values* ***(A)*** *and dry matter (DM) content* ***(B)*** *in different intestinal segments content*

Note: DM, dry matter; SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass; Diet-number, means diet sample replicate number; Diet-M, represents the means of replicate samples. [a,b] means with different superscript letters in a row were significantly different ($p < 0.05$).

Initially, the impact of *C. glomerata*-enriched feed on rabbit digestive processes was assessed through pH and DM analysis of distinct intestinal contents (Fig. 2.3.1). Rabbits under the SCD treatment exhibited significantly higher pH values in the *ileum* content compared to those on *C. glomerata*-enriched diets ($p < 0.05$). Specifically, SCD-treated rabbits showed *ileum* content pH values surpassing those of the CG4 and CG8 diets by 0.44 and 0.38 units, respectively ($p < 0.05$; Fig. 2.3.1 A). The exact mechanisms governing biomass enrichment and its influence on pH and DM values in other intestinal segments remain elusive, as DM content was unaffected by the CG diets. Nevertheless, variations in *ileum* content pH may influence microbial ecology and fermentation processes, consistent with previous research indicating the prebiotic properties of macroalgae affecting gut microbiota composition [118–120].

Analysis of *duodenum* content across dietary treatments revealed significant differences in SCFA profiles, with acetic acid predominating, followed by propionic and lactic acids, consistent with established SCFA patterns associated with gastrointestinal microbial fermentation (Table 2.3.2). The highest acetic acid content was observed in CG4 (89.04%), followed by CG8 (87.53%), and the lowest in the SCD (83.23%; $p < 0.05$). Acetic acid's principal purpose as an energy substrate in rabbit tissues is to increase metabolic processes and impact the utilisation of dietary fat [121]. Furthermore, acetic acid may modulate rabbit appetite, influencing overall dietary intake. Elevated acetic acid in CG4 and CG8 indicates accelerated microbial fermentation, probably due to *C. glomerata* prebiotic characteristics, comparable with previous research on algae-based diets [122, 123]. Propionic acid, following acetic acid, contributes to hepatic glucose synthesis, which is critical for fulfilling energy requirements [124]. Moreover, CG8-treated rabbits exhibited 1.75 times more propionic acid compared to those on the SCD diet ($p < 0.05$), while no significant differences were noted in butyric acid levels, which were entirely absent in CG8-treated rabbits' *duodenum* content ($p > 0.05$). Interestingly, *C. glomerata* supplementation led to a significant reduction in lactic acid levels, with CG4 and CG8 showing lower content compared to SCD by 6.06% and 7.16%, respectively ($p < 0.05$). The reduction in lactic acid content with increasing *C. glomerata* biomass suggests prebiotic effects on gut microbiota composition, influencing fermentation efficiency along the gastrointestinal tract [125].

Table 2.3.2. *Impact of produced C. glomerata-supplemented feed on rabbit duodenum content, short-chain fatty acid profile, and histomorphological properties of duodenum and ileum*

Item	Segment	Diet			p-value
		SCD	CG4	CG8	
SCFA (%)	*Duodenum*				
Acetic		83.23^{c}	89.04^{a}	87.53^{b}	0.048
Propionic		6.18^{b}	7.19^{ab}	10.82^{a}	0.019
Butyric		1.78	1.03	n.d.	n.s.
Lactic		8.81^{a}	2.75^{b}	1.65^{b}	< 0.001
Villus height (V; µm)	*Duodenum*	664.21	606.40	623.74	n.s.
	Ileum	1215.36^{b}	1526.09^{a}	1079.12^{c}	< 0.001
Crypt depth (C; µm)	*Duodenum*	117.67^{b}	153.56^{ab}	191.97^{a}	0.002
	Ileum	217.29^{a}	142.42^{ab}	90.82^{b}	0.007
V/C	*Duodenum*	5.73^{a}	4.16^{b}	3.37^{b}	0.005
	Ileum	6.60^{a}	11.31^{b}	12.15^{b}	0.006

Note: SCFA, short-chain fatty acid; SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass; n.s., not significant. $^{a-c}$ means with different superscript letters in a row were significantly different ($p < 0.05$). ^{ab} means with ab superscript letters in a row did not have a significant difference ($p > 0.05$).

Histomorphometric analysis is vital for assessing the impact of alternative feed components on intestinal mucosa structure, particularly villus height and crypt depth, which are crucial for nutrient absorption and gut health. Understanding the adaptations of intestinal tissue is vital for improving dietary formulations to boost nutrient utilisation and increase animal well-being [126]. Furthermore, histomorphometric analyses assist in detecting possible benefits or concerns associated with alternative feed components, supporting the formulation of diets that improve digestive function and overall animal performance [127]. In a recent study, supplementing *C. glomerata* into rabbit diets significantly altered mucosal architecture (Table 2.3.2). While *duodenum* villus height remained consistent, the *ileum* showed variation, with CG4 exhibiting the highest height (1526.09 µm) and CG8 the lowest (1079.12 µm; $p < 0.05$). Comparing *C. glomerata*-enriched diets, the villus height in the CG4-treated *ileum* was significantly higher compared to CG8 and SCD ($p < 0.05$), indicating better mucosal morphogenesis and improved nutrient absorption [128].

Crypt depth increased with increasing *C. glomerata* biomass supplementation (CG8>CG4>SCD) in the *duodenum*, while in the *ileum*, it decreased with increased biomass (SCD>CG4>CG8; Table 2.3.2). Therefore, CG8-treated rabbits exhibited significantly greater *duodenum* crypt depth compared to SCD ($p < 0.05$), whereas CG8 *ileum* segment crypts were significantly shorter compared to SCD-treated rabbits ($p < 0.05$). Crypt depth reflects the structural dynamics of the intestinal mucosa, with deeper crypts in the *duodenum* indicating faster cell turnover and nutritional absorption capability, and shallower crypts in the *ileum* indicating a more mature mucosal architecture [129]. The segment-specific influence of *C. glomerata* on rabbit intestinal histomorphometry is evident from these observations. Reduced *duodenum* crypt depth suggests mature mucosal architecture, whereas increasing crypt depth demonstrates active mucosal development and potential nutritional absorption augmentation. Similarly, reduced crypt depth in the *ileum* indicates a stabilised mucosal architecture, while increasing crypt depth suggests active mucosal development and improved nutritional absorption [130].

Distinct villus-to-crypt (V/C) ratio patterns were observed in rabbit *duodenum* and *ileum* segments under *C. glomerata* treatments: SCD-treated rabbits had higher V/C in the *duodenum* compared to both CG4 and CG8 diets by 1.57 and 2.36 units, respectively ($p < 0.05$; Table 2.3.2). Conversely, biomass supplementation led to higher V/C in the *ileum*, significantly higher by 4.71 and 5.55 units for CG4 and CG8, respectively, compared to SCD ($p < 0.05$). These findings underscore the segment-specific effects of biomass on intestine histomorphometry, potentially influenced by biologically active substances in macroalgal biomass.

2.4. Impact of *C. glomerata*-supplemented feed on rabbit meat functionality (https://doi.org/10.3390/foods12040744)

Meat and its derivatives are considered functional foods due to their diverse beneficial components [48]. However, to enhance the functionality of rabbit meat, strategies involving the incorporation of *C. glomerata* biomass into rabbit diets should be explored. So, another step of this dissertation experimental stage was to replace conventional feed ingredients with different content of *C. glomerata* biomass collected from Lithuanian river Šventoji to enhance rabbit meat functionality and evaluate muscle protein and lipid profiles.

Rabbit meat is acknowledged for its high-quality protein content, containing around 22% protein and an optimal amino acid profile [44, 48]. Furthermore, *Cladophora* species, recognised for its protein content ranging

from 10% to 25%, has been investigated as a possible protein source in animal feeding [6]. In a recent study, we investigated the impact of different dosages of *C. glomerata* biomass in rabbit diets on muscle protein levels and amino acid composition. It was discovered that rabbits fed a 4% *C. glomerata* diet (CG4) exhibited the highest protein levels in the LD muscles (22.17%). However, doubling the biomass dosage to 8% (CG8) resulted in lower protein content compared to both the CG4 and SCD diets. Abu Hafsa et al. [131] also examined freshwater macroalgae inclusion at 4% in rabbit diets, yielding 18.63% protein in rabbit meat, slightly lower than our findings. Notably, while *C. aegagropila* from Egypt contained only 10.44% protein, our study's *C. glomerata* from Lithuanian Šventoji river contained 22.36% protein.

Animal husbandry is a key source of sustainable protein for human consumption, with rabbit meat standing out for its richness in essential AAs [132]. A balanced diet directly affects the quality of animal products, emphasising the significance of AAs in improving nutritional absorption, adjusting for shortages, and refining feed quality [5]. Recent research, including ours, has examined the AA content of *Cladophora* biomass, demonstrating its potential as a beneficial feed component [29, 52]. Furthermore, rabbit meat has greater quantities of important AAs than other meats, with significant effects reported when supplementing diets with 4% *C. glomerata* biomass (Table 2.4.1). This supplementation significantly raised levels of essential AAs in rabbit LD muscles ($p < 0.05$), improving their biological value and digestibility. The dietary pattern altered lysine levels in rabbit LD muscles, with significant modifications detected among treatments distributed as follows: CG4<SCD<CG8 ($p < 0.05$). *C. glomerata* did not significantly impact leucine-phenylalanine levels ($p > 0.05$), while 8% macroalgal biomass treatment resulted in greater concentrations of total essential AAs in rabbit HL muscles ($p < 0.05$). Overall, diets supplemented with 4% *C. glomerata* had the best nutritional value in terms of essential AA content, which is critical for addressing consumer demand for high-quality proteins [133].

A recent study found that glutamic acid, a conditionally essential AA, was the most abundant in both LD and HL muscles across all treatments, indicating its predominance in rabbit meat (Table 2.4.1). This finding is consistent with Morshdy et al. [134], who observed a similar dominance of glutamic acid in the LD muscles of New Zealand and Californian breed rabbits, akin to those in our study. However, the distribution of other conditionally essential AAs varied: glycine levels were higher in HL muscles with 8% macroalgae supplementation (CG8), while arginine content was elevated in SCD-LD muscles compared to CG8 ($p < 0.05$). Recent study found no significant impact of *C. glomerata* on serine and proline levels in different rabbit muscles ($p > 0.05$). Among nonessential AAs, CG8-LD

muscles exhibited lower alanine levels compared to other diets, while aspartic acid levels were highest in both LD and HL muscles under CG4 treatment ($p < 0.05$). Incorporating 4% *C. glomerata* in rabbit diets increased total AA content in LD muscles (192.16 g/kg), while doubling the dosage to 8% increased this indicator in HL muscles (176.13 g/kg). Moreover, the composition of AAs in meat is influenced by various biological factors and diet composition [135]. For instance, *C. glomerata* biomass from Lithuanian rivers can exhibit a total AA content ranging from 103.36 to 140.99 g/kg and essential AAs constitute about 40% of the total AA content in the biomass.

Table 2.4.1. *Impact of produced C. glomerata-supplemented feed on rabbit longissimus dorsi (LD) and hind leg (HL) muscle amino acid profiles*

AA (g/kg)	Muscle	Diet			p-value
		SCD	CG4	CG8	
Asp	LD	18.39^{a}	18.43^{a}	17.13^{b}	0.000
	HL	15.59^{a}	16.73^{b}	16.45^{ab}	0.016
Thr	LD	8.58^{a}	8.65^{a}	8.24^{b}	0.003
	HL	7.29	7.59	7.61	n.s.
Ser	LD	7.35	7.36	7.11	n.s.
	HL	6.32	6.83	6.89	n.s.
Glu	LD	31.65^{ab}	32.63^{a}	31.08^{b}	0.08
	HL	27.79^{a}	29.61^{b}	29.54^{b}	0.019
Pro	LD	7.26	7.40	7.14	n.s.
	HL	7.57	7.82	8.02	n.s.
Gly	LD	10.10	9.94	10.01	n.s.
	HL	11.05^{a}	11.65^{ab}	12.42^{b}	0.017
Ala	LD	10.27^{a}	10.31^{a}	9.29^{b}	0.001
	HL	8.87^{a}	9.89^{b}	10.14^{b}	0.001
Val	LD	10.40^{a}	10.42^{a}	9.56^{b}	0.001
	HL	8.80	9.07	9.16	n.s.
Met	LD	8.66^{a}	10.68^{b}	8.73^{a}	0.009
	HL	7.74	8.09	8.13	n.s.
Ile	LD	9.25^{a}	9.28^{a}	8.44^{b}	0.000
	HL	7.50	7.88	7.91	n.s.
Leu	LD	14.75^{a}	14.57^{a}	13.55^{b}	0.000
	HL	12.10^{a}	12.83^{b}	12.90^{b}	0.030
Tyr	LD	6.63^{ab}	6.69^{a}	6.13^{b}	0.039
	HL	5.25^{a}	6.65^{b}	5.70^{b}	0.030

Table 2.4.1. *Continued*

AA (g/kg)	Muscle	Diet			p-value
		SCD	CG4	CG8	
Phe	LD	7.47[a]	7.36[a]	6.89[b]	0.000
	HL	6.28[a]	6.60[ab]	6.68[b]	0.043
His	LD	8.78[a]	8.50[ab]	8.11[b]	0.003
	HL	7.46	7.63	7.98	n.s.
Lys	LD	15.86[a]	16.71[b]	15.60[a]	0.001
	HL	13.70	14.15	14.41	n.s.
Arg	LD	13.90[a]	13.23[ab]	12.67[b]	0.016
	HL	11.99	12.13	12.19	n.s.
Total	LD	189.30[a]	192.16[a]	179.74[b]	0.000
	HL	165.32[a]	174.33[b]	176.13[b]	0.021
Trp	LD	29.40[a]	30.84[b]	25.28[c]	0.000
	HL	26.27[a]	22.79[b]	24.02[c]	0.000
Hyp	LD	7.71[a]	6.30[b]	8.85[c]	0.000
	HL	18.71[a]	15.73[b]	16.70[ab]	0.049
Trp/Hyp	LD	3.82[a]	4.91[b]	2.86[c]	0.000
	HL	1.43	1.50	1.44	n.s.

Note: AA, amino acid; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; His, histidine; Lys, lysine; Arg, arginine; Trp, tryptophan; Hyp, hydroxyproline; SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass; n.s., not significant. [a-c] means with different superscript letters in a row were significantly different ($p < 0.05$). [ab] means with ab superscript letters in a row did not have a significant difference ($p > 0.05$).

Meat quality is multifaceted, with tenderness being a key concern for consumers, where connective tissue proteins play a pivotal role [136]. Hydroxyproline content serves as a marker for connective tissue, while tryptophan levels indicate muscle tissue [137]. Incorporating 4% *C. glomerata* in rabbit diets increased tryptophan levels in LD muscles but slightly decreased it at an 8% dosage ($p < 0.05$; Table 2.4.1). However, in HL muscles, tryptophan was most abundant in the SCD, followed by CG8, and then CG4 ($p < 0.05$). Hydroxyproline, predominantly found in connective and bone tissue, is another vital meat quality marker [138]. The lowest hydroxyproline levels were observed in the muscles of rabbits treated with 4% biomass diet, suggesting improved meat quality. A further crucial meat and protein quality indicator is the tryptophan-to-hydroxyproline ratio (Trp/Hyp) [139]. The

mentioned ratio was the highest in the LD muscles of rabbits under the CG4 diet, indicating enhanced nutritional value. However, in HL muscles, *C. glomerata* inclusion did not affect the Trp/Hyp ratio ($p > 0.05$).

Meat and meat-based products are often criticised for their high fat and calorie content, particularly SFAs and cholesterol, which are associated with cardiovascular disease, obesity, and diabetes [48]. However, rabbit meat stands out for its exceptional nutritional profile, being lower in fat and cholesterol compared to other meats, with rabbit meat containing 9.2 g/100 g of fat and 56.4 mg/100 g of cholesterol, making it a leaner option [140]. Notably, the inclusion of *C. glomerata* in rabbit feed further reduced fat accumulation in rabbit muscles, with higher doses of *C. glomerata* corresponding to lower fat content in LD and HL muscles ($p < 0.05$). This reduction in fat content aligns with findings from studies in aquaculture, where *C. glomerata* supplementation led to decreased fat accumulation in fish muscles [43]. Although dietary cholesterol's impact on plasma cholesterol is minor, it remains a concern for consumers. Rabbit meat has the lowest cholesterol content among common meats [48]. In our case, LD muscles contained 22.81 to 26.08 mg/100 g, and HL muscles contained 30.60 to 35.02 mg/100 g. However, while diet can influence cholesterol accumulation in rabbit tissues, *C. glomerata* supplementation did not significantly affect cholesterol levels in LD and HL rabbit muscles ($p > 0.05$).

Rabbit meat's favourable lipid profile, characterised by low cholesterol, SFAs, and high PUFAs, presents health benefits [141]. However, the higher PUFA content makes the meat susceptible to oxidative degradation, impacting its shelf life and sensory properties [44]. To mitigate lipid oxidation, enhancing animal feed with antioxidant-rich materials like *C. glomerata*, known for its antioxidant properties due to phenols and pigments, is effective [53]. Moreover, incorporating *C. glomerata* into rabbit diets lowered MDA levels in fresh LD and HL muscles, in addition to stored HL muscles ($p < 0.05$; Fig. 2.4.1); indicating improved meat quality and stability, particularly with higher biomass inclusion.

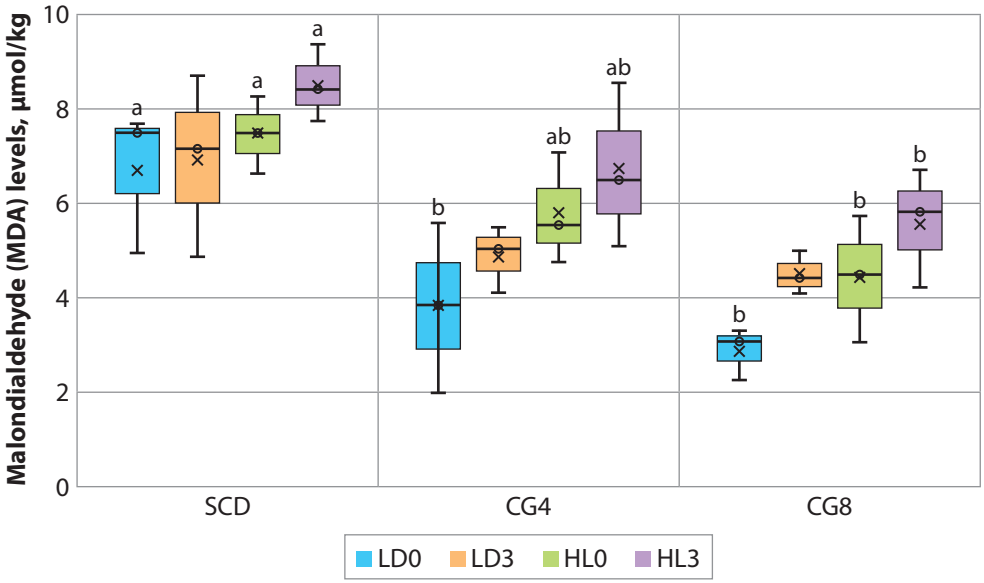


Fig. 2.4.1. *Impact of produced C. glomerata-supplemented feed on malondialdehyde (MDA) levels in fresh and stored for 3 months rabbit longissimus dorsi (LD0 and LD3) and hind leg (HL0 and HL3) muscle*

Note: SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass. [a-b] means with different superscript letters in a column with the same colour were significantly different ($p < 0.05$). [ab] means with ab superscript letters in a column of the same colour did not have a significant difference ($p > 0.05$).

Scientific evidence increasingly supports the notion that certain foods offer physiological and psychological benefits beyond basic nutrition [142–145]. FAs are essential for a well-balanced diet, with PUFAs being especially advantageous. Dietary factors can alter rabbit meat, which is noted for its high PUFA content. For instance, *C. glomerata*, which is rich in biologically active components, demonstrates a high proportion of SFAs, MUFAs, and PUFAs. In a recent study, rabbit muscles exhibited reduced SFA levels and increased PUFA levels with *C. glomerata* inclusion in the diet (Fig. 2.4.2); a trend supported by similar findings in lamb meat [146]. To be more precise, the FA composition of *C. glomerata* biomass was distributed as follows: SFA >50%, MUFA 27.34–28.39%, PUFA 6.48–11.71%. In rabbit muscles: SFA LD: SCD<CG4<CG8, HL: CG4<SCD<CG8; MUFA LD: SCD<CG4<CG8, HL: SCD<CG4<CG8; PUFA LD: CG8<CG4<SCD, HL: CG8<CG4<SCD.

Humans must obtain essential PUFAs from their diet, particularly long-chain n-3 FAs, known for various health benefits [3]. The PUFA profile in rabbit muscle increased with higher *C. glomerata* inclusion in the diet, with

linoleic acid being the predominant. While no significant impact was noted on linoleic acid levels ($p > 0.05$), other n-6 and n-3 fatty acids increased significantly with 8% *C. glomerata* inclusion ($p < 0.05$). Therefore, the inclusion of macroalgal biomass did not affect the total n-3 content in the rabbits' muscles ($p > 0.05$). However, the 8% dosage notably influenced individual n-6 FAs, resulting in a higher total amount of these FAs in the muscles collected from rabbits under the CG8 diet. Overall, supplementation with *C. glomerata* had no impact on the n-6/n-3 ratio ($p > 0.05$).

The PUFA/SFA ratio is a critical measure often used to assess the impact of diet on cardiovascular health [147]. It's theorised that higher PUFA/SFA correlate with lower LDL-C and serum cholesterol levels, promoting cardiovascular well-being [69]. A recent study observed a consistent trend: increasing doses of *C. glomerata* in rabbit diets led to higher PUFA/SFA in both LD and HL muscles (Fig. 2.4.2). Specifically, the ratio was the highest under the CG8 diet, followed by CG4 and then SCD ($p < 0.05$). So, these findings imply that incorporating *C. glomerata* into rabbit feed may improve the nutritional quality of rabbit meat, potentially benefiting cardiovascular health in consumers.

Another parameter assessed in rabbit muscles was the atherogenicity index (AI), reflecting the balance between total SFA and UFAs contents (Fig. 2.4.2). SFAs like lauric, myristic, palmitic, and octadecanoic are considered pro-atherogenic due to their role in promoting lipid adherence to circulatory and immune system cells [148, 149]. Consuming foods with lower AI values may decrease total cholesterol and LDL-C levels in human plasma, thus benefiting cardiovascular health [150]. In a recent study, the AI in LD muscles remained unaffected by *C. glomerata* diets ($p > 0.05$), while 8% macroalgal biomass inclusion reduced AI levels in HL muscles compared to other diets ($p < 0.05$). Similarly, the thrombogenicity index (TI), which assesses the balance between pro-thrombogenic and anti-thrombogenic FAs, is another crucial indicator for cardiovascular health [151]. However, in rabbit LD muscles, biomass supplementation had no significant impact on mentioned indicator ($p > 0.05$). Only CG4 increased TI in HL muscles, compared to the values of SCD and CG8 ($p < 0.05$).

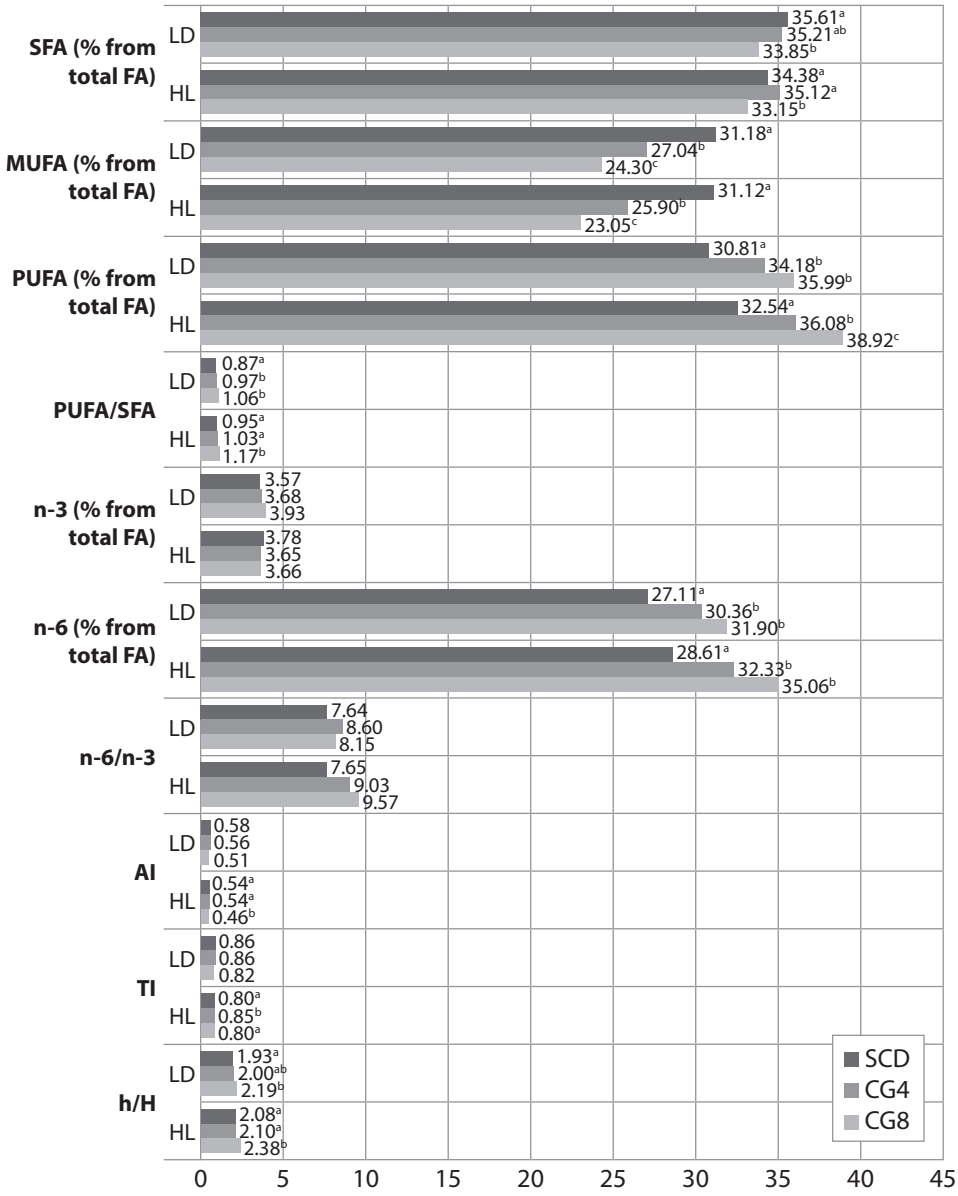


Fig. 2.4.2. *Impact of produced C. glomerata-supplemented feed on rabbit muscle longissimus dorsi (LD) and hind leg (HL) fatty acid profiles*

Note: FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; AI, atherogenic index; TI, thrombogenicity index; h/H, hypocholesterolemic/hypercholesterolemic ratio; SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass. $^{a\text{-}c}$ means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$). ^{ab} means with ab superscript letters in a column of the same indicator did not have a significant difference ($p > 0.05$).

Furthermore, the hypocholesterolemic/hypercholesterolemic ratio (h/H), a more precise measure than the PUFA/SFA, indicates the effect of FAs on cardiovascular disease risk likewise. So, foods with higher h/H are considered more nutritionally desirable [152]. Our results revealed that increasing biomass dosage corresponded to higher h/H, with 4% and 8% macroalgal inclusion resulting in 1.03- and 1.13-times higher ratios in LD muscles and 1.00- and 1.14-times higher ratios in HL muscles, respectively, compared to SCD ($p < 0.05$; Fig. 2.4.2).

2.5. Impact of *C. glomerata*-supplemented feed on rabbit meat sensory feature profiles (https://doi.org/10.3390/ani13132179)

Improving meat quality involves not just enhancing its chemical and physical qualities but also addressing sensory issues that may be caused by novel feed components. Despite health-conscious trends, sensory characteristics remain critical to consumer acceptance. Consequently, recognising customer preferences is critical to enhancing meat quality and production profitability [153]. So, the final step of this dissertation experimental stage was to assess the effect of feed supplemented with various dosages of *C. glomerata* on rabbit muscle physical properties and sensory profiles.

Rabbits exhibit efficient meat production, characterised by short gestation periods and high productivity [154]. However, sensory features, chemical composition, nutritional value, and safety are all important qualities of rabbit meat. Moreover, moisture content is a crucial parameter influencing meat quality, affecting its appearance, texture, flavour, and shelf life [155]. In a recent study, supplementing rabbit feed with *C. glomerata* increased muscle moisture content, with higher biomass dosages corresponding to greater moisture levels (SCD<CG4<CG8; $p < 0.05$; Fig. 2.5.1 A). However, seasonal variations may influence outcomes in macroalgae properties. Furthermore, moisture content influences water-holding capacity and drip loss, which are crucial quality factors determining meat's economic value [156]. *C. glomerata* inclusion had no significant impact on drip loss or water holding capacity in rabbit muscles ($p > 0.05$). However, cooking losses were notably higher in HL muscles with an 8% macroalgal biomass inclusion (CG8) compared to a lower dosage (CG4; Fig. 2.5.1 A). The results obtained may be attributable to protein denaturation during cooking [157].

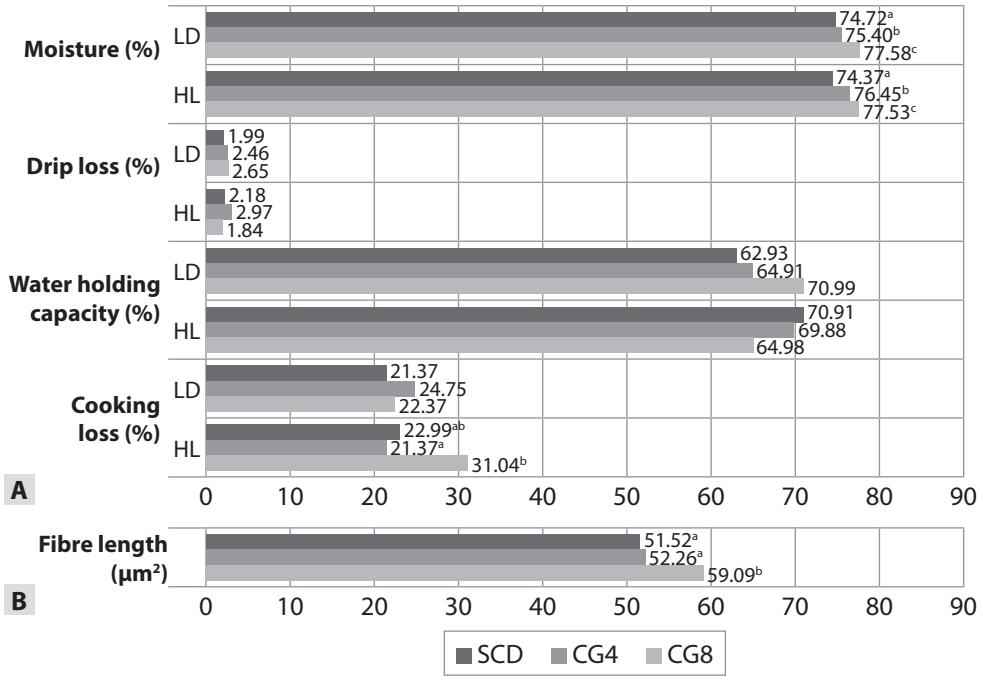


Fig. 2.5.1. *Impact of produced C. glomerata-supplemented feed on rabbit longissimus dorsi (LD) and hind leg (HL) muscle physical indicators* ***(A)*** *and fibre length in LD muscles* ***(B)***

Note: SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass. $^{a\text{-}c}$ means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$). ^{ab} means with ab superscript letters in a column of the same indicator did not have a significant difference ($p > 0.05$).

Muscle fibre length significantly affects meat yield and quality [131]. Recent research discovered that macroalgal biomass (CG8) increased LD muscle fibre length to 59.09 μm^2, compared to 51.52 μm^2 in the SCD ($p < 0.05$; Fig. 2.5.1 B). Nonetheless, fibre length and density directly influence meat tenderness [158]. Although the exact mechanism behind *C. glomerata* effect on fibre length is not fully understood, its nutritional properties likely play a role. Furthermore, the biomass is rich in proteins and minerals, potentially supporting muscle growth [52]. Thus, according to recent research, including 8% *C. glomerata* in rabbit diets stimulates LD muscle growth ($p < 0.05$).

Rabbit muscle pH decreased uniformly post-mortem, with CG8 showing a lower pH in LD muscles at 48 hours compared to SCD ($p > 0.05$). While pH decline affects water retention [159, 160], *C. glomerata* in rabbit feed didn't alter muscle water holding capacity, suggesting no clear pH-water retention relationship. Normal post-mortem muscle pH ranges from 7.00 to

5.50; in our study, pH ranged from 6.56 to 5.92 in LD and from 6.41 to 6.26 in HL due to myofilament lattice shrinkage and water loss [157].

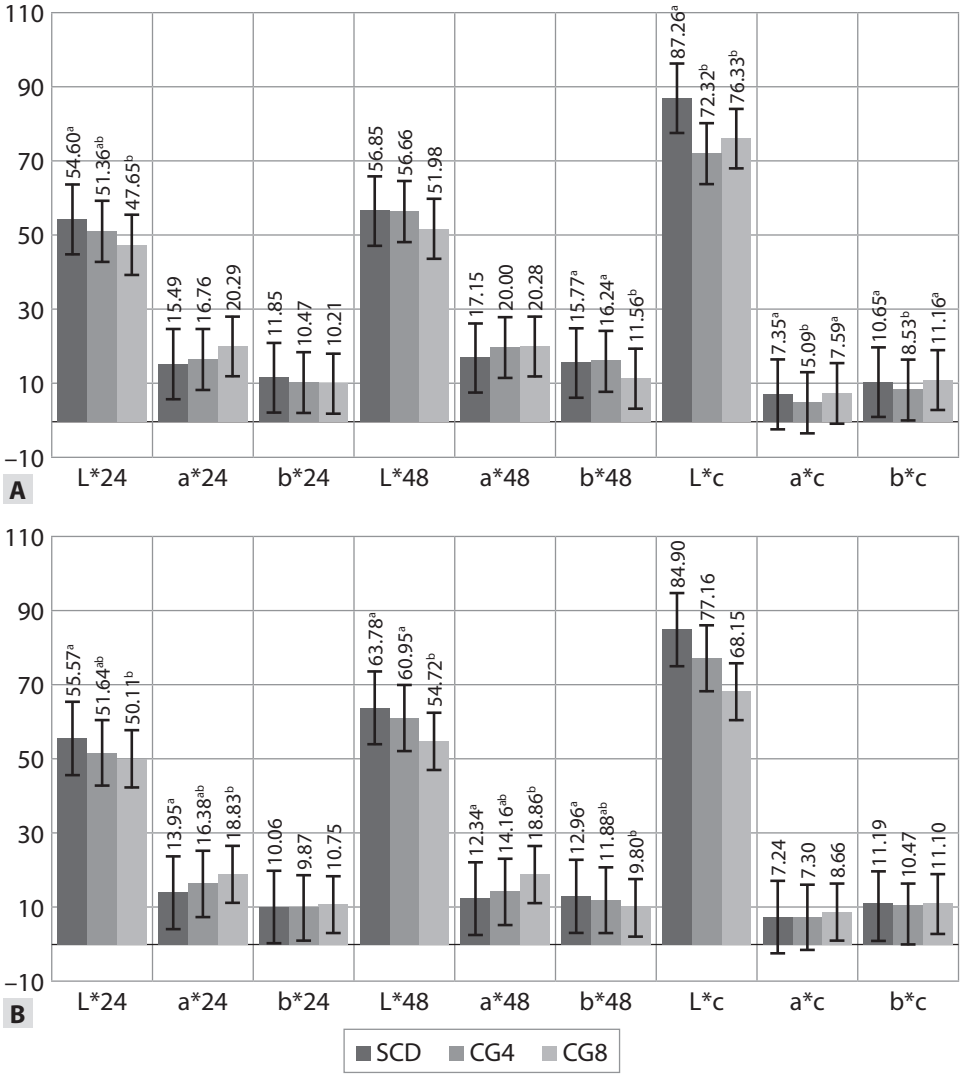


Fig. 2.5.2. *Impact of produced C. glomerata-supplemented feed on rabbit longissimus dorsi (LD; **A**) and hind leg (HL; **B**) muscle colour coordinates at 24 hours (L*24; a*24; b*24) and 48 hours (L*48; a*48; b*48) post-mortem and after cooking (L*c; a*c; b*c)*

Note: SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass. [a,b] means with different superscript letters in a column of the same indicator were significantly different ($p < 0.05$). [ab] means with ab superscript letters in a column of the same indicator did not have a significant difference ($p > 0.05$).

When purchasing meat, consumers prioritise colour, which is influenced by muscle tissue structure and histological pattern, alongside acidity, which impacts colour brightness and hue. Rabbit muscles under the CG8 diet initially appeared brighter and redder, correlating with a higher pH (Fig. 2.5.2). However, redness decreased slightly in LD muscles after 48 hours, with HL muscles becoming darker and less red ($p < 0.05$; Fig. 2.5.2 A). This relationship between pH and brightness aligns with previous scientists' research [161, 162]. Furthermore, antioxidant activity effects meat colour stability, with increased macroalgal biomass inclusion lowering redness post-mortem. Moreover, the appearance of cooked meat is altered by several elements, including muscle type, cooking, packaging, storage, fat content, taste, and preservation procedures [163]. Lastly, myoglobin plays a key role in colour changes. Inclusion of 4% *C. glomerata* significantly affected the cooked LD muscle colour to be less yellow and red compared to SCD and CG8 ($p < 0.05$).

Meat quality assessment traditionally considers sensory attributes like appearance, texture, odour, and flavour, with tenderness being an appreciable characteristic. However, in a recent study, rabbit muscles, known for their tenderness, exhibited increased hardness in HL muscles under *C. glomerata* treatments (Fig. 2.5.3 B). Sensory evaluation of rabbit LD and HL muscles, encompassing 19 criteria, revealed that *C. glomerata* supplementation affected HL muscle hardness, LD muscle odour and colour intensity, and HL muscle mouthfeel and taste richness (Fig. 2.5.3). Notably, 4% *C. glomerata* supplementation led to pronounced changes in LD muscle odour, while colour intensity increased with higher biomass dosage ($p < 0.05$). Rabbit HL muscles showed heightened mouthfeel under the CG8 diet but less richness in taste compared to other diets ($p < 0.05$). Despite these variances, the overall sensory evaluations of rabbit meat remained acceptable. Al-Soufi et al. [44] noted potential improvements in meat properties and stability with macroalgae supplementation in rabbit diets, aligning with our findings despite some differences between standard and *C. glomerata*-treated diets.

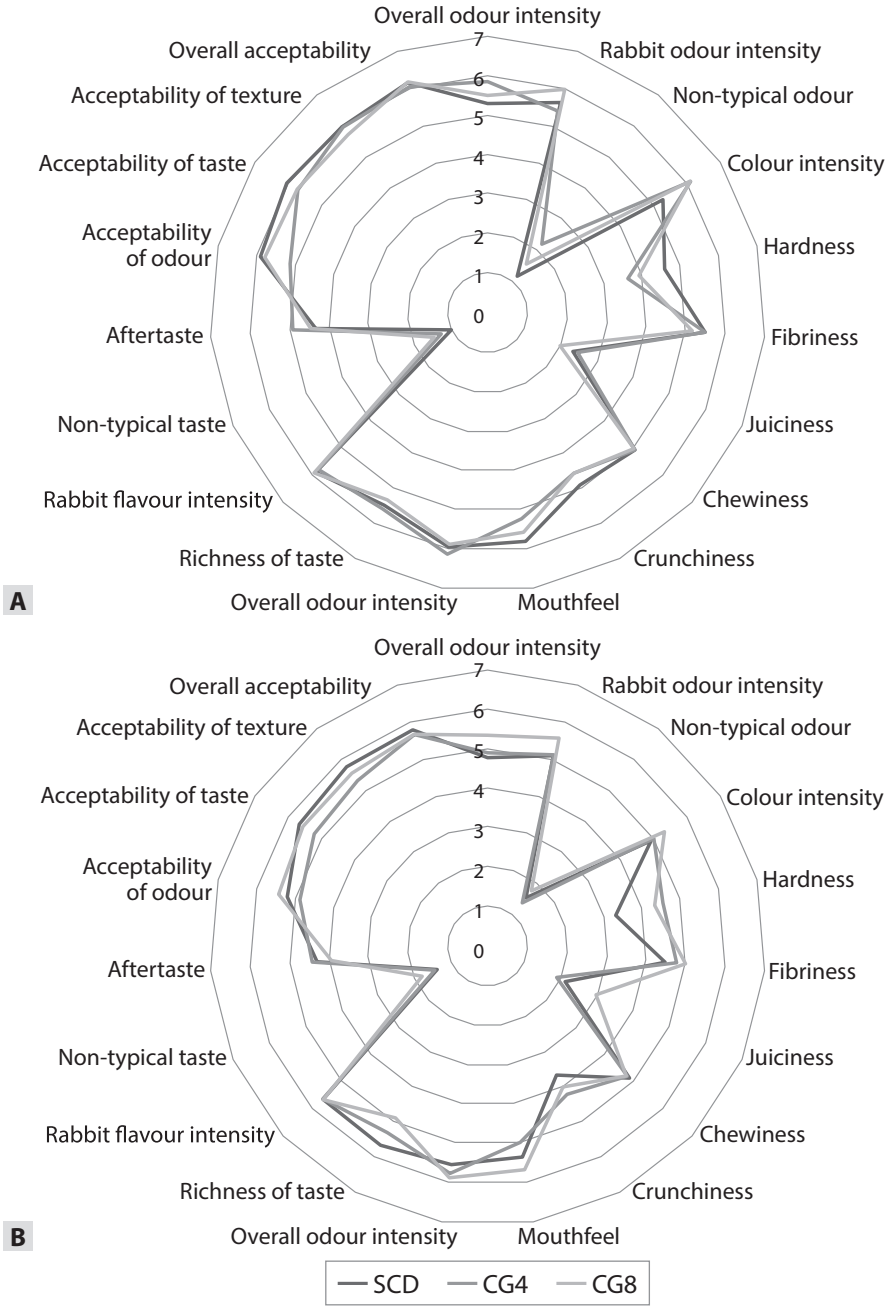


Fig. 2.5.3. *Impact of produced C glomerata-supplemented feed on cooked rabbit longissimus dorsi (LD;* ***A****) and hind leg (HL;* ***B****) muscle sensory profile*

Note: SCD, standard compound diet; CG4, SCD supplemented with 4% *C. glomerata* biomass; CG8, SCD supplemented with 8% *C. glomerata* biomass.

The relationship between consumption conditions and consumer feelings is critical in shaping food perception and satisfaction [164, 165]. Consumer evaluation, particularly in the context of meat, serves two main purposes: understanding preferences and purchase intent [166]. Evaluators' reactions to rabbit muscle smell samples fed *C. glomerata* diets were mostly neutral. However, certain dietary treatments altered emotional responses. For instance, smelling muscles from rabbits fed 4% biomass evoked less happiness and more sadness, while those from rabbits fed 8% biomass elicited less sadness and disgust ($p < 0.05$). SCD-treated muscles generally evoked more varied emotional responses compared to *C. glomerata* biomass-treated rabbit muscles. Taste evaluation revealed higher happiness levels with 8% *C. glomerata*-treated muscles but also increased disgust compared to 4% inclusion ($p < 0.05$). It is critical to comprehend that the rabbit muscles were cooked without seasoning, which may have an influence on the samples' acceptability to evaluators.

CONCLUSIONS

1. Chemical analysis of freshwater *C. glomerata* macroalgae from Lithuanian rivers (Dubysa, Šventoji, Nevėžis, and Jūra) demonstrates its potential commercial usage by meeting animal nutrition safety criteria with low heavy metal levels and balanced macro- and trace elements. However, prevalent chemical analyses are required post-collection to monitor possible toxicity from environmental conditions. Overall, the bioaccumulation capabilities of *C. glomerata* indicate that it could serve as a beneficial source of protein, essential AAs, and FAs for animal feed.
2. *C. glomerata* macroalgal biomass from Lithuanian rivers (Dubysa, Šventoji, Nevėžis, and Jūra) reveals potential utilisation as a functional raw material according to its antioxidant activity and bioactive element accumulation. The collected biomass contains bioactive substances such as phenols (gallic, *p*-coumaric, and *p*-hydroxybenzoic acids) and pigments. Additionally, it exhibits significant antioxidant activity, with high reducing power and total antioxidant content. Furthermore, the primary pigments, such as chlorophyll *a*, chlorophyll *b*, carotenoids, and lutein, exhibit significant potential for application in animal feed supplements and various other industries.
3. Supplementing rabbit diets with *C. glomerata* had no significant impact on BWG or ADG. However, including 8% of biomass in the rabbit diets reduces DFI and enhances FCR, which may be economically advantageous. Biomass-supplemented diets diminish lung percentage based on pre-slaughter body weight without significantly altering carcass components or slaughter performance, indicating a minor structural impact. Furthermore, *C. glomerata* increases acetic and propionic acid levels while decreasing lactic acid in the *duodenum*, potentially activating microbial fermentation. Histomorphometric analysis shows stable *duodenum* villus height and dose-dependent increases in *ileum* villus height with higher biomass dosage. Biomass supplementation increased *duodenum* crypt depth, suggesting effects on cell turnover and nutrient absorption, while decreasing crypt depth in the *ileum*, indicating a more mature mucosal architecture.
4. Utilising *C. glomerata* macroalgal biomass in rabbit diets at 4% and 8% levels can enhance the quality of rabbit meat and promote a more sustainable nutritional approach. At 4% inclusion, muscle protein and essential AAs such as threonine, valine, methionine, lysine, isoleucine, and tryptophan were significantly increased, whereas hydroxyproline

levels were reduced. This could lead to improved consumer digestion due to the higher biological value of muscle proteins. Increased biomass inclusion levels lowered fat content and lipid oxidation in fresh and stored rabbit muscles. Furthermore, both 4% and 8% inclusions reduced SFA and MUFA while increasing PUFA, enhancing muscle lipid quality. The *C. glomerata* dietary strategy potentially improved rabbit meat's cardiovascular preventive properties by significantly increasing PUFA/SFA and h/H levels while decreasing TI and AI.

5. Partly replacing traditional rabbit feed ingredients with freshwater *C. glomerata* macroalgal biomass provides a more sustainable and consumer-friendly approach to rabbit meat production. However, adding 4% or 8% biomass can significantly increase muscle moisture, with 8% resulting in greater cooking losses in HL muscles. Furthermore, *C. glomerata* can lighten and minimise redness in both fresh and cooked rabbit muscles, making them more appealing to customers. Recent research indicates that incorporating 8% biomass can also lengthen muscular fibres in LD muscles. Despite some differences between treatments, sensory evaluations indicate that rabbit meat from *C. glomerata*-fed rabbits is generally acceptable to evaluators. Notably, evaluators reported increased happiness after tasting CG8-HL rabbit muscles, despite variations in odour and appearance.

RECOMMENDATIONS

1. Freshwater *C. glomerata* macroalgal biomass, which thrives in Lithuanian rivers, is recommended for use only on small farms. It can be used to supplement the feed of rabbits aged 52–122 days, up to an 8% dosage. This limitation is due to the currently unoptimized productivity of biomass cultivation, which is necessary for the commercial production of large quantities of feed.
2. Freshwater *C. glomerata* macroalgal biomass collected each time must be consistently analysed for potential toxicity that may be influenced by biomass habitat factors.

SUMMARY IN LITHUANIAN

SANTRUMPOS

a*	–	rausvumo spalvos koordinatė
ABTS	–	2,2-azino-bis(3-etilbenzotiazolin-6-sulfonrūgštis) radikalų ir katijonų surišimo aktyvumas
AI	–	aterogeniškumo indeksas
AR	–	amino rūgštys
b*	–	gelsvumo spalvos koordinatė
B1	–	biomasė, surinktas iš Dubysos
B2	–	biomasė, surinktas iš Šventosios
B3	–	biomasė, surinktas iš Nevėžio
B4	–	biomasė, surinktas iš Jūros
C. glomerata	–	*Cladophora glomerata*
Ca	–	kalcis
Cd	–	kadmis
CG4	–	standartinis kombinuotasis pašaras, papildytas 4 proc. *C. glomerata*
CG8	–	standartinis kombinuotasis pašaras, papildytas 8 proc. *C. glomerata*
Cr	–	chromas
Cu	–	varis
DPPH	–	2,2-difenil-1-pikrilhidrazilo radikalų surišimo aktyvumas
FRAP	–	geležies jonų redukcijos antioksidacinė galia
G/K	–	gaurelių aukščio ir kriptų gylio santykis
GATI	–	Gyvūnų auginimo technologijų institutas
GRE	–	galo rūgšties ekvivalentas
h/H	–	hipocholesteroliškumo ir hipercholesteroliškumo santykis
HL	–	užpakalinės kojos
HL0	–	užpakalinių kojų raumenys po 24 valandų atvėsinimo, sandėliuoti esant –80 °C
HL3	–	užpakalinių kojų raumenys po 24 valandų atvėsinimo, sandėliuoti 3 mėnesius esant –18 °C
K	–	kalis
L*	–	šviesumo spalvos koordinatė
LD	–	ilgiausiasis nugaros raumuo (*longissimus dorsi*)
LD0	–	ilgiausiasis nugaros raumuo (*longissimus dorsi*) po 24 valandų atvėsinimo, sandėliuoti esant –80 °C
LD3	–	ilgiausiasis nugaros raumuo (*longissimus dorsi*) po 24 valandų atvėsinimo, sandėliuoti 3 mėnesius esant –18 °C
LSMU	–	Lietuvos sveikatos mokslų universitetas
MDA	–	malondialdehidas
Mg	–	magnis
MNRR	–	mononesočiosios riebalų rūgštys
N	–	natris
Ni	–	nikelis
NRR	–	nesočiosios riebalų rūgštys
P	–	fosforas
Pb	–	švinas

PKK	–	pašarų konversijos koeficientas
PNRR	–	polinesočiosios riebalų rūgštys
pvz.	–	pavyzdžiui
RG	–	redukcinė galia
RR	–	riebalų rūgštys
SCD	–	standartinis kombinuotasis pašaras
SM	–	sausosios medžiagos
SRR	–	sočiosios riebalų rūgštys
TGRR	–	trumpųjų grandinių riebalų rūgštys
TI	–	trombogeniškumo indeksas
VA	–	Veterinarijos akademija
vnt.	–	vienetas
Zn	–	cinkas

ĮVADAS

Greitai didėjanti gyventojų populiacija tiesiogiai susijusi ir lemia kartu didėjantį gyvūninės kilmės maisto produktų poreikį, kuris gali tiesiogiai prisidėti prie tradicinių pašarinių žaliavų stokos ateityje [1]. Todėl, atsižvelgiant į tvaresnio gyvulininkystės ūkio puoselėjimo modelį, ieškoma vis daugiau utilizavimo potencialą turinčių pašarinių žaliavų. Siekiant tikslingo tokių žaliavų komercializavimo, vertinant jų ekonominį naudingumą, pirmiausia reikalinga moksliniais tyrimais pagrįsta novatoriškų pašarinių žaliavų analizė [2]. Mokslininkų atrastos tradicinių pašarinių žaliavų alternatyvos kartu turi išlaikyti aukštus kokybės, saugos ir tvarumo standartus bei neviršyti antimitybinių veiksnių bei potencialaus toksiškumo. Galutinis tikslas yra sukurti ne tik funkcionaliuosius pašarus, bet ir išgauti aukštos kokybės gyvūninės kilmės produktus užtikrinant gyvūnų gerovę.

Iš vandens telkinių surinktos natūraliai juose esančios žaliavos, pvz., dumblių biomasė, turi konkurencingą baltymų ir kitų esminių maistinių medžiagų kiekį bei gali būti vertinamos kaip potenciali tvaresnio pašaro modeliavimo strategija [3–5]. Pasauliniu mastu kultivuojami dumbliai, kaip savaime atsinaujinantys ištekliai, atpažįstami dėl gaminamos biomasės maistinės vertės, į kurios sudėtį įeina vitaminai, mineralai, baltymai, polinesočiosios riebalų rūgštys ir antioksidantai [6, 7]. Fundamentalusis dumblių potencialas kyla dėl jų prisitaikymo prie įvairiausių aplinkos sąlygų bei klestėjimo vietose, kurios netinkamos įprastinėms žaliavoms, taip pat biomasės produktyvumas daug greitesnis, palyginti su kitais žemės ūkio augalais [5]. Efektyviai išnaudodami saulės energiją, dumbliai gamina biomasę, papildytą biologinį aktyvumą turinčiais junginiais, galinčiais tiesiogiai didinti maisto ir pašarų maistinę vertę [8]. Pašarų papildymas dumblių biomase, galėtų prisidėti prie pašarinių žaliavų trūkumo problemos sprendimo bei kartu sumažinti šiltnamio efektą sukeliančių dujų išsiskyrimą, atsiradusių dėl gyvulininkystės veiklos [9]. Pastaruoju metu

dumbliai integruojami į gyvūnų pašarus tiesiogiai arba kaip funkcinės pašarų sudedamosios dalys [3, 4, 10, 11].

Iššūkiai, kylantys mokslinių tyrimų metu siekiant nuoseklaus dumblių utilizavimo galimybių vertinimo, tiesiogiai veikia sklandžią jų komercializaciją pramonėje [16]. Įprastai pagrindinę dumblių sudėtį sudaro trys junginių grupės: baltymai, angliavandeniai ir lipidai. Be to, iš dumblių biomasės gaunamas krakmolas, pigmentai, antioksidantai, vitaminai ir fitohormonai, kurie naudojami farmacijos, biotrąšų, natūralių dažiklių ar gyvūnų pašarų gamybos pramonėse [17]. Į skirtingų dumblių rūšių sudėtį įeina daug biologiškai aktyvių junginių. Unikalūs antriniai metabolitai dažnai turi konkrečias filogenetines grupes, kurios turi tiesioginės įtakos vartotojo sveikatingumui [18]. Makrodumbliuose, turinčiuose įvairių morfologinių ir fiziologinių savybių, taip pat daug antioksidantų, kurie išskiriami dėl reikšmingos gydomosios gebos [20]. Žalieji makrodumbliai apibrėžiami kaip vertingi pigmento šaltiniai, sintetinantys chlorofilus, o karotinoidus – kaip fotosintetinius organizmus: pabrėžiama jų svarba tvarioje tiekimo grandinėje, pradedant žaliava bei baigiant plačiomis gamybos ir galutinių produktų sukūrimo perspektyvomis [25].

Cladophora rūšys – tiek jūrinės, tiek gėlavandenės – išskiriamos kaip ekologiškai ir ekonomiškai reikšmingi makrodumbliai, atliekantys esmines ekosistemos funkcijas, bei kaip įvairiapusiškai pritaikoma žaliava trąšų, maisto ir gyvūnų pašarų, vaistų, kosmetikos, nuotekų valymo bei atsinaujinančio biokuro pramonių srityse [26, 27]. Specifiškai, žalieji makrodumbliai *Cladophora glomerata* (*C. glomerata*) klesti vandens telkiniuose, kuriuose daug maistinių medžiagų, ypač lėtai tekančiose upėse, ir sudaro dideles biomasės aglomeracijas [28]. Neatsižvelgiant į vandens ekosistemose atliekamą makrodumblių funkciją, perteklinis *C. glomerata* biomasės dauginimasis mažina biologinę įvairovę bei rekreacinę vertę vandens telkiniuose bei kelia ekologinių ir ekonominių iššūkių. Tačiau *C. glomerata* biomasę dėl gana didelio baltymų kiekio bei kitų joje esančių esminių maistinių medžiagų, įskaitant vitaminus ir skaidulas, rekomenduojama įtraukti tiek į žmonių, tiek į gyvūnų mitybą [29]. Atsižvelgiant į dabartinius gyvulininkystės iššūkius, ypač tvarumo problemas, numatomą pašarinių žaliavų trūkumą ir šiltnamio efektą sukeliančių dujų išsiskyrimą gyvulininkystės veiklų metu, *C. glomerata* biomasės įtraukimas į pašarų gamybą padėtų spręsti išvardytas problemas – atliekas paversti vertinga žaliava. Pavyzdžiui, naudojant gėlavandenę *C. glomerata* makrodumblių biomasę, būtų išgaunama ne tik daugiafunkcė žaliava įvairioms pramonės šakoms, bet būtų prisidedama ir prie vandens telkinių valymo ir tiesioginio biologinės įvairovės didinimo. Pastebėta, kad vandens ekosistemose paplitę *C. glomerata* makrodumbliai labai greitai gamina biomasę bei biologiškai aktyvius junginius, pvz., riebalų rūgštis,

sterolius, terpenoidus, fenolinius junginius ir pigmentus [6, 29]. Atlikta daug jūrinių *C. glomerata* makrodumblių tyrimų, tačiau moksliniais tyrimais pagrįstos informacijos apie gėlavandenę *C. glomerata* iš Lietuvos upių stinga. Ankstesni Lietuvos mokslininkai tyrė jūrinės *C. glomerata* biomasės konversiją į aliejų pirolizės būdu ir jos, kaip pašaro, potencialą [31], taip pat biomasė panaudota biodujoms gaminti kontroliuojamuose bioreaktoriuose [32]. Tačiau išsamūs Lietuvos upėse klestinčių gėlavandenių *C. glomerata* makrodumblių tyrimai šiuo metu yra vis dar riboti.

Gyvūninės kilmės produktų suvartojimas ir toliau greitai didėja [33]. Populiariausi išlieka kiaulienos, jautienos bei paukštienos gaminiai, triušieną pradedama vertinti vis labiau dėl jos funkcionaliųjų savybių. Novatoriškų strategijų įgyvendinimas triušininkystėje didina tokios produkcijos patrauklumą rinkoje bei atitinka šiuolaikinio vartotojo poreikius [34]. Tačiau kaip ir visose gyvulininkystės šakose iškylantys tvarumo užtikrinimo iššūkiai turi būti sprendžiami. Vienas iš sprendimų – į triušių pašarų gamybą įtraukti gėlavandenių makrodumblių *C. glomerata* biomasę, kuri išvalius Lietuvos upes vertinama kaip atliekos. Toks sprendimas gali būti vertinamas kaip atsinaujinantis bei reikšmingai mažinantis ekologinį pėdsaką, susijusį su tradicinių pašarų gamyba. Labai svarbu užtikrinti tvaresnius gamybos procesus naudojant išteklius tausojančias priemones nuo lauko iki stalo. Naujausi moksliniai tyrimai rodo, kad gyvūnų mityboje tradicinės pašarinės žaliavos gali būti iš dalies pakeičiamos alternatyviomis [36–39], išskiriant didėjantį susidomėjimą natūraliais bei atsinaujinančiais šaltiniais ir kuriant tvarią gyvulininkystės valdymo praktiką. Šiuo metu aplinkos ir gamybos tvarumas yra vienas svarbesnių aspektų, tačiau, siekiant užtikrinti pasaulinį maisto tiekimą, tai neturėtų turėti įtakos mėsos kokybei [36]. Besivystanti mėsos kokybės koncepcija dabar apima išsamius aspektus, tokius kaip maistinė vertė, juslinės savybės, gaminimo patogumas ir ekonomiškumas. Todėl, norint veiksmingai pagerinti bendrą mėsos kokybę, būtina suprasti ir kompleksinius mėsos kokybę lemiančius veiksnius visoje gamybos grandinėje. Pethickas ir kt. [40] išskyrė pagrindinius į vartotoją orientuotus kriterijus, bei rekomenduoja, kad mėsos produktai turi turėti sveikatą gerinančių savybių, užtikrinti aukštą baltymų ir kitų maistinių medžiagų kokybę, atitinkančią sveikos mitybos standartus.

Dažniausiai *Cladophora* makrodumblių rūšys utilizuojamos papildant akvakultūros mitybą [41–43], tačiau eksperimentai rodo ir potencialią naudą žinduoliams, mažinant kiaulių žarnyno disbiozę [44]. Tokie moksliniais tyrimais pagrįsti rezultatai indikuoja *Cladophora* makrodumblių panaudojimo potencialą ir triušių mityboje. Be to, makrodumblių įtraukimas į triušių racioną ne tik orientuotas į aplinką tausojančius vartotojus, bet gali tapti ir komercine rinkodaros strategija, pritraukiančia tuos, kurie ieško novatoriško,

aukštos kokybės maisto. Mokslininkai išskiria ir skelbia, kad dumblių įtraukimas į gyvūnų racioną tiesiogiai pagerina mėsos kokybę [41, 43, 45, 46]. Tačiau *Cladophora* biomasės poveikio triušiams tyrimai yra itin riboti, o didėjant triušienos paklausai dėl didėjančios vartotojų sampratos apie sveiką mitybą, padeda puikų pagrindą atlikti gilesnius tyrimus [47]. Pavyzdžiui, mėsa ir jos gaminiai yra laikomi funkciniais maisto produktais dėl į jų sudėtį esančių naudingų komponentų ir kokybinių ir kiekybinių indikatorių manipuliavimo galimybių [48]. Taigi, atsižvelgiant į tai, kad mėsos gamybos grandinė prasideda nuo lauko ir tik tada nuo fermos, būtina kruopščiai apsvarstyti esminius kriterijus: pradedant nuo žemės ūkio lauko iki vartotojo valgomojo stalo.

C. glomerata makrodumblių biomasėje yra daug skaidulų, tačiau ja papildytų pašarų pritaikymas triušių mityboje, atsižvelgiant į unikalią jų virškinimo fiziologiją (cekotrofiją), teoriškai užtikrintų didelį produktyvumą ir sveikatingumą – padėtų išgauti funkcionalią triušieną [49]. Labai svarbu pagerinti bendrąsias mėsos kokybės charakteristikas, tačiau jusliniai aspektai, tokie kaip skonis, kvapas ir vizualinis patrauklumas, tebėra esminiai, lemiantys produkto priimtinumą. Be to, vartotojų lūkesčiai bei pageidavimai ir toliau svarbiausi vertinant bendrą produkto poreikį, pabrėžiant jų svarbą optimizuojant mėsos kokybę, gamybą ir mėsos gamintojų bei platintojų pelningumą [50]. Mėsos produktų vertė yra sudėtinga klientų lūkesčių sąveika, daranti įtaką norui mokėti už įsigyjamą produktą ir galutiniam sprendimui jį įsigyti [51]. Išskiriant triušienos gamybos grandinę, pagrindinis dėmesys skiriamas greitai plėtrai, sveikatingumui ir produktyvumui. Todėl gėlavandenės *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių, papildymas ir tradicinių žaliavų dalinis pakeitimas triušių pašaruose, prisidėtų prie tvaresnės triušienos gamybos nuo upės iki stalo, sukuriant galutinį funkcionalųjį produktą. Taigi pagrindinė disertacijos esmė buvo sumodeliuoti tvaresnes novatoriškas pašarų, papildytų *C. glomerata* makrodumblių biomase iš Lietuvos upių, formules mažinant priklausomybę nuo tradicinių pašarinių žaliavų ir manipuliuojant triušių augimo, vystymosi, sveikatingumo ir triušienos funkciniais kokybiniais rodikliais, nedarant neigiamo poveikio mėsos juslinių savybių profiliui.

Darbo tikslas

Įvertinti gėlavandenės *Cladophora glomerata* makrodumblių biomasės panaudojimo potencialą ir biomase papildytų pašarų įtaką triušių produktyvumui, fiziologiniams procesams, skerdenos kokybei, bei triušienos biocheminiams ir jusliniams savybių profiliams.

Uždaviniai

1. Išsamiai ištirti *C. glomerata* panaudojimo potencialą įvertinant biomasės saugą ir cheminę sudėtį.
2. Išsamiai ištirti *C. glomerata* panaudojimo potencialą įvertinant biomasės antioksidacinį aktyvumą ir biologiškai aktyvius junginius.
3. Išsamiai ištirti naujai sukurtų *C. glomerata* pašarų formulių įtaką triušių produktyvumo ir skerdimo rodikliams bei fiziologiniam išsivystymui.
4. Išsamiai ištirti naujai sukurtų *C. glomerata* pašarų formulių įtaką triušienos funkcionalumui.
5. Išsamiai ištirti naujai sukurtų *C. glomerata* pašarų formulių įtaką triušienos juslinių savybių profiliui ir bendram priimtinumui.

Darbo naujumas ir praktinis naudingumas

Tvarumo puoselėjimas ir užtikrinimas gyvulininkystės sektoriuje yra itin opus, pirmiausia atsižvelgiant į ekologinius aspektus ir tiesioginį poveikį gamtai. Svarbu, kad tvaraus gyvulininkystės ūkio vystymas prisidėtų prie maisto saugos, klimato kaitos mažinimo ir biologinės įvairovės išsaugojimo – iš esmės padėtų užtikrinti ilgalaikę planetos ir visuomenės gerovę. Šie iššūkiai skatina diegti bei ieškoti sprendimų ilgalaikei gyvulininkystės ūkio plėtrai, kuri remtų intensyvų, tačiau tvarų šio sektoriaus vystymą. Siekiant pagrįsti tokius sprendimus, gyvūnų mokslų srities mokslininkai ieško įvairiausių novatoriškų sprendimų, kuriais siekiama išgauti ne tik aukščiausios kokybės funkcionaliąsias pašarines žaliavas, bet ir žaliavas, kurios saugotų mūsų aplinką. Todėl, atsižvelgiant į žiedinę ekonomiką ir gyvulininkystės sektoriuje siekiamo žaliojo kurso bei didėjančio triušienos produkcijos poreikio, šios disertacijos pagrindas yra natūralių gamtos išteklių panaudojimo galimybių vertinimas, kuriant darnesnę triušių ekosistemą. Šiam tikslui kaip alternatyvi bei funkcionalioji pašarinė triušių mitybos žaliava buvo pasirinkta gėlavandenė makrodumblių biomasė *C. glomerata*, kuri puikiai klesti Lietuvoje esančiuose gėluose vandens telkiniuose, ypač upėse. *C. glomerata* yra viena iš labiausiai paplitusių makrodumblių rūšių, kurios dauginimosi našumas itin greitas, o pagamintos biomasės perteklius vandens telkiniuose tiesiogiai mažina telkinio rekreacinę vertę bei biologinę įvairovę. Šių makrodumblių suformuotos aglomeracijos dažnai šalinamos rankiniu būdu, o *C. glomerata* biomasė laikoma atliekomis. Moksliniais tyrimais paremtos analizės rodo, kad tokia biomasė gali būti utilizuojama kaip daugiafunkcė žaliava įvairiose pramonės šakose. Atsižvelgiant į tvaresnio gyvulininkystės sektoriaus plėtojimą, iš Lietuvos upių surinkta makrodumblių biomasė gali pakeisti dalį tradicinių pašarinių žaliavų. *C. glomerata* makrodumbliai efektyviau

naudoja saulės išteklius, todėl jų gaminamos biomasės cheminė sudėtis yra unikali – tokią žaliavą potencialiai galima laikyti netgi funkcionaliąja.

Išskirti du pagrindiniai pranašumai, utilizuojant gėlavandenę *C. glomerata* biomasę iš Lietuvos upių: 1) mažesnės dumblių biomasės auginimo ir išgavimo išlaidos (įskaitant energijos išteklius), nes nereikalingos specialios sąlygos bei įranga biomasei kultivuoti; 2) perteklinės biomasės šalinimas iš vandens telkinių didina biologinę įvairovę ir rekreacinę vertę vandens ekosistemose, išgaunant realų panaudojimo potencialą turinčią daugiafunkcę žaliavą. Išskirtas didžiausias iššūkis – biomasės cheminė sudėtis tiesiogiai koreliuoja su aplinkos veiksniais.

1. METODAI IR MEDŽIAGOS

1.1. Tyrimų vieta ir laikas

Eksperimentai atlikti 2020–2023 metais Lietuvos sveikatos mokslų universiteto (LSMU) Veterinarijos akademijos (VA) Gyvūnų mokslų fakulteto Gyvūnų auginimo technologijų institute (GATI; Kaunas, Lietuva); Veterinarijos fakulteto Veterinarinės patobiologijos katedroje (LSMU VA; Kaunas, Lietuva); Gyvulininkystės instituto Chemijos laboratorijoje (LSMU; Baisogala, Lietuva); Gamtos tyrimų centro Algologijos ir mikroorganizmų ekologijos laboratorijoje (Vilnius, Lietuva); Kauno technologijų universiteto Cheminės technologijos fakulteto Organinės chemijos katedroje (Kaunas, Lietuva); Veislinių triušių fermoje (identifikacijos kodas: 9447582; Šakių rajonas, Lietuva).

1.2. Biomasės panaudojimo potencialo vertinimas ir alternatyvaus pašaro modeliavimas

1.2.1. *C. glomerata* makrodumblių biomasės mėginių rinkimas

Moksliniams tyrimams atlikti gėlavandenė *C. glomerata* biomasė surinkta iš keturių skirtingų Lietuvos upių: Dubysos (N55°12’25.07”, E23°30’30.44”; **B1**), Šventosios (N55°39’20.14”, E25°10’18.39”; **B2**), Nevėžio (N55°5’46.52”, E23°46’55.57”; **B3**) ir Jūros (N55°27’19.58”, E22°2’14.72”; **B4**) (1.2.1.1 pav. A). Upių atrankos kriterijai buvo pagrįsti tankių *C. glomerata* aglomeracijų, apimančių daugiau nei 50 proc. upės vagos ploto, paplitimo (1.2.1.1 pav. B). Neatsižvelgiant į šį atitikimą, upių baseinas ir vandens chemija skyrėsi, kaip teigia Nutautaitė ir kt. [52].

Šviežia makrodumblių biomasė iš upių buvo renkama rankiniu būdu (1.2.1.1 pav. C). Kartotiniai mėginiai (1 mėginys = 1 kg drėgnos biomasės) buvo sujungti iš ėminių, paimtų iš šešių skirtingų upės vietų. Toliau atliktas kruopštus biomasės plovimas, siekiant pašalinti smėlio ir purvo daleles, po jo rankiniu būdu iš biomasės pašalintas makrozoobentosas, makrofitai ir kitos priemaišos. Surinkti mėginiai buvo džiovinami per naktį 60 °C temperatūroje džiovinimo spintoje, o vėliau laikomi sandariuose plastikiniuose maišeliuose kambario temperatūroje, kad būtų galima atlikti tolesnę analizę (1.2.1.1 pav. D). Pagrindinė *C. glomerata* makrodumblių biomasės utilizavimo potencialo schema pateikta 1.2.2.1 pav.

1.2.2. *C. glomerata* makrodumblių biomasės cheminė analizė

Susmulkintos makrodumblių biomasės mėginiai (n = 3 pakartotiniai mėginiai / upė) išanalizuoti pagal gaires, išdėstytas Komisijos reglamente (ES) Nr. 691/2013 (2013 m. liepos 19 d.), kuris iš dalies keičiamas Reglamento (EB) Nr. 152/2009 nuostatos dėl ėminių ėmimo ir analizės metodų. Cheminis džiovintos biomasės elementinės sudėties nustatymo ir biomasės saugos vertinimas atliktas akredituotoje tyrimų laboratorijoje laikantis atitinkamų metodikų (1.2.2.1 lentelė). Kitų cheminių rodiklių analizės metodikas, įskaitant žalius komponentus, tokius kaip baltymai, riebalai, pelenai ir ląsteliena, taip pat amino- ir riebalų rūgščių profilius, išsamiai aprašė Nutautaitė ir kt. [52].

Makrodumblių biomasės antioksidaciniam aktyvumui įvertinti nustatyti šie rodikliai: bendras fenolinių junginių ir antioksidantų kiekis, fenolio rūgščių profilis, įskaitant flavonoidus ir katechinus, redukcinė galia (RG), 2,2-difenil-1-pikrilhidrazilo radikalų surišimo aktyvumas (DPPH), 2,2-azinobis(3-etilbenzotiazolin-6-sulfonrūgštis) radikalų ir katijonų surišimo aktyvumas (ABTS), geležies jonų redukcijos antioksidacinė galia (FRAP) ir bendras pigmentų kiekis (chlorofilų *a* ir *b*, karotinoidų ir liuteino). Minėtiems rodikliams nustatyti taikytas metodikas detaliai aprašė Nutautaitė ir kt. [53].

1.2.3. *C. glomerata* makrodumblių biomase papildyto pašaro formulių modeliavimas ir pašaro gamyba

Atlikus nuoseklų makrodumblių biomasės potencialo vertinimą, įgyvendinta papildoma ekspedicija, skirta biomasei rinkti iš Šventosios (N55°39’20.14”, E25°10’18.39”). Surinkta biomasė analizuota analogiškai – kaip aprašyta 1.2.2 skyriuje. Remiantis gautais rezultatais, sumodeliuotos dvi naujos pašarų formulės, kurios papildytos gėlavandene *C. glomerata* makrodumblių biomase, siekiant pakeisti dalį įprastai naudojamų pašarinių žaliavų. Naujos pašarų formulės suformuluotos taip, kad atitiktų triušių fiziologinius poreikius. Standartinis kombinuotasis pašaras, kuris naudotas kaip kontroli-

nis, sumodeliuotas ir ištirtas, kad atitiktų augančių triušių maistinių medžiagų poreikius, įskaitant vitaminus ir mineralus, atsižvelgiant į Nacionalinės tyrimų tarybos rekomendacijas [54].

Naujai sukurtų pašaro formulių bei standartinio kombinuotojo pašaro realizavimas ir gamyba buvo vykdoma veislinių triušių fermoje (identifikacijos kodas: 99447582; Šakių rajonas, Lietuva), panaudojant pašarų gamybai skirtą įrangą. Šėrimo bandymui su triušiais atlikti pagaminti trys skirtingi granuliuoti pašarai: standartiniai kombinuotieji (**SCD**), SCD papildyti 4 proc. *C. glomerata* biomase (**CG4**) ir SCD papildyti 8 proc. *C. glomerata* biomase (**CG8**). Pagrindinė *C. glomerata* makrodumblių biomasės surinkimo iš Šventosios ir pašarų modeliavimo, įskaitant pašarų sudėtį, schema pateikta 1.2.3.1 pav.

1.3. *C. glomerata* makrodumblių biomase papildyto pašaro utilizavimas triušių šėrimo bandymo metu

Šėrimo bandymas vykdytas veislinių triušių fermoje, kur triušiai laikomi po vieną atskiruose narvuose, kurių matmenys atitiko šiuos matmenis: 34 × 34 × 61 cm. Siekiant užtikrinti triušių sveikatingumą ir produktyvumą, fermoje įrengta nipelinė girdykla, pašaras duodamas individualiai kiekvienam triušiui. Patalpų temperatūra palaikoma 19 ± 2 °C. Viso bandymo metu šviesos režimą sudarė 16 valandų apšvietimo, po kurio buvo 8 valandų tamsos laikotarpis. Laikymo sąlygos atitiko gaires, išdėstytas Tarybos direktyvoje 98/58/EB (1998 m. liepos 20 d.) dėl ūkinės paskirties gyvūnų apsaugos.

Trisdešimt (vnt.) Kalifornijos veislės triušių, nujunkytų 52 amžiaus dieną, kurie atrinkti pagal panašų svorį ir atsitiktinai suskirstyti į tris mitybos grupes (n = 10 triušių / grupė). Kiekviena grupė buvo šeriama du kartus per dieną skirtingais pašarais: SCD, CG4 ir CG8. Per visą šėrimo bandymo periodą triušiai turėjo *ad libitum* (iki soties) prieigą prie jiems pagal mitybos grupę paskirto pašaro. Pagrindinė *C. glomerata* papildyto pašaro utilizavimo triušių šėrimo bandymo schema pateikta 1.3.4.1 pav.

1.3.1. Triušių produktyvumo vertinimas

Viso šėrimo bandymo periodo metu, kuris truko 52–122 amžiaus dienas, periodiškai (52–66, 67–80, 81–94, 95–108, ir 109–122 amžiaus dienų intervalais) buvo registruojami individualūs triušių svoriai, įskaitant kūno svorio priesvorį, vidutinį kūno priesvorį per parą, pašarų suvartojimą per parą bei pašarų konversijos koeficientai (PKK).

1.3.2. Triušių skerdenų ir fiziologinio išsivystymo analizė

Šėrimo bandymo pabaigoje (122 amžiaus dieną) atsitiktine tvarka atrinkta 18 triušių (n = 6 triušiai / grupė), kurie individualiai pasverti ir 12 valandų iki skerdimo nešeriami. Po šios procedūros triušiams atlikta eutanazija, pagal standartinę ūkininkavimo praktiką atliktas skerdimas. Skerdimo procedūra atlikta veislinių triušių fermoje esančioje skerdykloje, laikantis nustatytų protokolų, atitinkančių Lietuvos Respublikos įstatymus, kaip nurodyta Valstybinės maisto ir veterinarijos tarnybos direktoriaus įsakyme Nr. B1-866 (2012 m. spalio 31 d.) dėl mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros, ir naudojimo reikalavimų patvirtinimo. Šėrimo bandymo metu taikyti etiniai aspektai detaliau išdėstyti Nutautaitės ir kt. [55-57].

Atliktos skerdenos išpjaustymo procedūros atitiko Pasaulinės triušių mokslo asociacijos pateiktas gaires [58]. Toliau skerdena 24 valandas 4 °C temperatūroje gerai ventiliuojamoje patalpoje atvėsinama. Atlikta išsami skerdenos po skerdimo, raumenų, organų ir žarnyno segmentų išsivystymo analizė. Histomorfometrinei analizei paimti dvylikapirštės ir klubinės žarnų segmentai iš vidurinės dalies (gaurelių aukščiui ir kriptų gyliui pamatuoti). Kitų skirtingų žarnyno segmentų (dvylikapirštės, plonosios, aklosios, klubinės ir storosios žarnų bei skrandžio) turiniai surinkti siekiant nustatyti pH ir sausųjų medžiagų (SM) kiekius bei trumpųjų grandinių riebalų rūgščių (TGRR) profilį. Išvardytų rodiklių nustatyto metodikas detaliai pateikia Nutautaitė ir kt. [57].

1.3.3. Triušienos kokybės analizė

Atlikus skerdenų išsivystymo vertinimą, atskirti ilgiausiojo nugaros (*longissimus dorsi* (LD)) ir užpakalinių kojų (HL) raumenys. Paimti 36 vnt. triušio raumenų mėginiai (n = 12 mėginiai / grupė) sumalti ir vėliau sandėliuoti –80 °C temperatūroje (šviežia mėsa; LD0 ir HL0; n = 6 kiekvieno raumens tipo mėginiai / grupė) ir sandėliuoti 3 mėnesius –18 °C temperatūroje (sandėliuota mėsa; LD3 ir HL3; n = 6 kiekvieno raumens tipo mėginiai / grupė). Šviežioje ir sandėliuotojo triušienoje nustatytas lipidų oksidacijos laipsnis (malondialdehido koncentracija (MDA)). Kiti mėsos kokybės parametrai (įskaitant cheminę sudėtį (baltymus, riebalus, pelenus), aminorūgščių ir riebalų rūgščių profilius bei cholesterolio kiekį) buvo įvertinti naudojant šviežios triušienos mėginius (n = 6 kiekvieno raumens tipo mėginiai / grupė). Išvardytų rodiklių analizei taikytas metodikas detaliai aprašo Nutautaitė ir kt. [55].

1.3.4. Triušienos juslinių savybių profilio vertinimas

Analogiškos mėginių ėmimo schemos buvo naudojamos paimant LD ir HL raumenis, siekiant išanalizuoti fizinius jų rodiklius, raumenų skaidulų plotą, juslinių savybių profilį ir emocinį atsaką į mėginius (n = 6 kiekvieno raumens tipo mėginiai / grupė). Triušienos fizinėms savybėms įvertinti atlikti tyrimai: drėgmės kiekio nustatymas, lašėjimo nuostoliai, vandens rišlumas, virimo nuostoliai, pamatuoti raumenų skaidulų ilgiai, nustatytas pH ir spalvų koordinatės (L*a*b*) praėjus 24 ir 48 valandų po skerdimo bei ją išvirus. Atliktas virtų LD ir HL triušių raumenų juslinių savybių vertinimas, kurio metu vertintojai vertino nustatytus galimus juslinius aspektus. Vertinimo balai skiriami vartojant septynbalę skaitinę skalę, kur 1 reiškė požymio nebuvimą, 4 – vidutinę išraišką, o 7 – stiprią išraišką. Dešimt vertintojų dalyvavo veido išraiškų (emocijų) vertinimo procedūroje, naudojant *FaceReader* 8 programinę įrangą (*Noldus Information Technology*, *Wageningen*, Nyderlandai), prijungtą prie internetinės kameros (*Microsoft Corporation, Redmond*, WA, JAV). Ši programinė įranga skenuoja vertintojų veido išraiškas realiuoju laiku, kai jie žiūrėjo, uostė ir testavo triušienos mėginius. Išsamesnės mėginių paruošimo ir vertinimo procedūras bei taikytas metodikas minėtiems rodikliams išsamiai aprašė Nutautaitė ir kt. [56].

1.4. Statistinė analizė

1.4.1. Biomasės analizė

Analizė atlikta lyginant *C. glomerata* makrodumblių biomasę iš keturių skirtingų Lietuvos upių, kai kiekvienas mėginys turėjo tris pakartojimus (n = 3 mėginiai / upė). Duomenų analizė atlikta vartojant programą SPSS, skirtą *Windows*, 25.0 versiją (IBM *Corp*., išleista 2017 m., *Armonk*, NY, JAV). Skirtumams tarp skirtingų grupių nustatyti atlikta vienpusė dispersinė analizė (ANOVA), naudotas testas Post-Hoc (Fišerio mažiausiai reikšmingo skirtumo testas). Statistiškai patikimi skirtumai laikyti, kai apskaičiuota p vertė buvo mažesnė nei 0,05 ($p < 0,05$).

1.4.2. Triušių augimo, skerdenų ir mėsos kokybės bei juslinių savybių profilio analizė

Šėrimo bandymas atliktas su dešimt triušių kiekvienoje mitybos grupėje, o skerdimo metu iš kiekvienos grupės paimta po šešis triušius. Iš kiekvieno analizuoto triušio paimti žarnyno segmentų mėginiai ir dviejų tipų raumenų mėginiai tolesnei analizei (n = 6 žarnyno segmentai / grupė; n = 6 LD / grupė; n = 6 HL / grupė). Duomenų analizė atlikta analogiškai, kaip nurodyta 1.4.1 skyriuje.

2. REZULTATAI IR DISKUSIJA

2.1. Gėlavandenės *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių, cheminė sudėtis (https://doi.org/10.3390/agriculture11070582)

Siekiant užtikrinti maisto grandinių tvarumą ir ekologinę pusiausvyrą gyvulininkystės ekosistemose į pašaro gamybos grandinę vis dažniau įtraukiamos alternatyvios, labiau aplinką tausojančios žaliavos. *C. glomerata* biomasės sudėtyje nustatoma įvairiausių biologiškai aktyvių junginių, kurie rodo šios žaliavos panaudojimo potencialą pašarų gamyboje [28]. Taigi pirmuoju šios disertacijos eksperimentinio etapo metu buvo siekiama įvertinti gėlavandenės *C. glomerata* makrodumblių biomasės, surinktos iš skirtingų Lietuvos upių, potencialą atliekant išsamią cheminę analizę, įskaitant amino- ir riebalų rūgščių profilius.

Palyginti su kitomis pašarinėmis žaliavomis, esminių elementų koncentracija dumbliuose gali siekti net iki 40 proc. [59]. Šio disertacinio darbo tyrimo rezultatai pirmiausia rodo, kad *C. glomerata* biomasė gali būti naudingas gyvūnų mitybos įvairių elementų šaltinis. Nustatytas makroelementų kiekio pasiskirstymas biomasėje: Ca>K>N>P>Mg (2.1.1 pav. A); mikroelementų: Zn>Cu (2.1.1 pav. B). Toks elementų pasiskirstymas sutampa su kitų mokslininkų gautais rezultatais. Pavyzdžiui, Messyaszas ir kt. [29] taip pat nustatė didesnę Ca koncentraciją *C. glomerata* biomasėje, surinktoje iš Oporzyn ežero Lenkijoje. *Cladophora* makrodumbliai dažnai atpažįstami kaip puikūs maistinių medžiagų ir sunkiųjų metalų vandenyje indikatoriai [60]. Nustatyta sunkiųjų metalų koncentracija *C. glomerata* biomasėje iš Lietuvos upių neviršijo reglamentuojamų normų, todėl pripažinta kaip saugi žaliava siekiant integruoti ją į gyvūnų mitybą.

Cladophora biomasėje esantis baltymų kiekis įprastai svyruoja tarp 10–25 proc. Šis kiekis yra konkurencingas, palyginti su tradicinėmis pašarinėmis žaliavomis [61]. Šio disertacinio darbo tyrimo metu nustatyta, kad *C. glomerata* biomasėje žalių baltymų kiekis kinta tarp 15,98–21,52 proc. (2.1.2 pav.). Palyginta iš skirtingų upių surinkta biomasė: iš Jūros (B4) – turėjo reikšmingai daugiau žalių baltymų, atitinkamai 14,87 proc., 25,74 proc. ir 15,52 proc., palyginti su biomase iš Dubysos (B1), Šventosios (B2) ir Nevėžio (B3) ($p < 0,05$). Analizuojant žalių riebalų kiekį biomasėje, šis rodiklis nustatytas daug mažesnis (0,19–0,35 proc.). Didžiausias žalių riebalų kiekis nustatytas B1 mėginiuose, palyginti su B2 ir B4 ($p < 0,05$). Ištyrus žalius pelenus, nustatyti reikšmingi skirtumai tarp visų biomasės mėginių: didžiausias žalių pelenų kiekis – B3 mėginiuose, o B4 – mažiausias ($p < 0,05$). Palyginamoji

žalių pelenų kiekio analizė tradicinėse ir alternatyviose pašarinėse žaliavose, atlikta Anho ir kt. [41] rodo, kad daugiausia žalių pelenų nustatyta žuvų miltuose (25,95 proc.), toliau – *Cladophora* miltuose (21,14 proc.), ryžių sėlenose (17,17 proc.), sojų miltuose (8,26 proc.) ir maniokų milteliuose (1,97 proc.). Šio disertacinio darbo metu nustatytas žalių pelenų kiekis kinta tarp 36,96–49,83 proc., o žalios ląstelienos kiekis siekė 13,46 proc. *C. glomerata* biomasėje. Gauti ląstelienos rezultatai sutampa su kitų mokslininkų atliktais tyrimų rezultatais [29, 63], kurie rodo didelį dumblių biomasės panaudojimo potencialą triušių mityboje, atsižvelgiant į šių žinduolių unikalią virškinimo organų sistemą, leidžiančią virškinti didesnius ląstelienos kiekius [64].

Moksliniais tyrimais pagrįsti rezultatai pažymi tiek jūrinių, tiek gėlavandenių makrodumblių sudėtyje esančių sočiųjų (SRR) ir nesočiųjų (NRR) riebalų rūgščių gausą [28, 65–67]. Šio disertacinio darbo metu nustatyta, kad SRR skirtinguose biomasės mėginiuose sudarė daugiau kaip 50 proc. nuo bendro riebalų rūgščių (RR) kiekio ($p < 0,05$; 2.1.2 pav.). Didžiausia bendra SRR koncentracija nustatyta B2 biomasėje, o mažiausia – B1 ($p < 0,05$). Messyaszo ir kt. [29] tyrimų rezultatai taip pat patvirtina mažesnes SRR koncentracijas analizuotuose *C. glomerata* mėginiuose. Mononesočiųjų riebalų rūgščių (MNRR) *C. glomerata* makrodumblių biomasėje iš Lietuvos upių nuo bendro RR kiekio kito tarp 27,39–28,39 proc., o didžiausia koncentracija nustatyta B2 biomasėje ($p < 0,05$). Polinesočiųjų riebalų rūgščių (PNRR) koncentracija kito tarp 6,48–11,71 proc. nuo bendro RR kiekio, tokia koncentracija rodo biomasės kaip funkcionaliojo ingrediento tiek maisto, tiek pašaro gamyboje potencialą [3]. Santykis tarp PNRR ir SRR (PNRR/SRR) apibūdinamas kaip esminis lipidų indeksas, naudojamas siekiant įvertinti mitybos poveikį širdies ir kraujagyslių sveikatingumui [69], didesnis santykis rodo didesnę naudą. Disertacinio tyrimo metu didžiausias PNRR/SRR nustatytas *C. glomerata* mėginiuose, surinktuose iš Dubysos (B1; 2.1.2 pav.). Palyginti su kitais augaliniais aliejais, pavyzdžiui, palmių stearinu (PNRR/SRR = 0,13 [70]), dažnai naudojamu pašarų gamyboje, šis santykis *C. glomerata* nustatytas net 1,69 karto didesnis. Norint užtikrinti sveikatingumą, itin svarbu išlaikyti optimalų n-6 ir n-3 santykį ties 1 arba 4 : 1 [72]. Analizuotos *C. glomerata* biomasės iš Dubysos (santykis: 1,30 (B1)) ir Jūros (santykis: 1,35 (B4)) upių rodė mažiausią minėtą santykį, o Šventosios (santykis: 1,42 (B2)) ir Nevėžio (santykis: 1,68 (B3)) – didesnius santykius apibrėžtame idealiame diapazone (2.1.2 pav.).

Aminorūgštys (AR), ypač egzogeninės, esančios įvairiuose pašarų prieduose, gerina gyvūnų maistinių medžiagų virškinamumą, kompensuoja jų trūkumus, gerina pašarų kokybę ir galutinę gyvūninės produkcijos sudėtį [5]. Atsižvelgiant į tai, AR yra esminės ar ne, abi grupės itin svarbios siekiant

patenkinti gyvūnų medžiagų apykaitos poreikius. Šio disertacinio darbo metu atliktas išsamus *C. glomerata* biomasės AR profiliavimas atskleidė reikšmingus skirtumus tarp surinktos biomasės, išskyrus histidino kiekį (2.1.1 lentelė). Didžiausias bendras AR kiekis nustatytas biomasėje iš Jūros (B4), kuris siekė 141 g/kg, o identifikuotos AR visose upėse buvo glutamo ir asparto rūgštys bei leucinas. Lyginant biomasę, surinktą iš kitų upių, esminių AR, sudarančių 41,60–55,40 g/kg nuo bendrojo AR kieko, daugiausia nustatyta Jūros mėginiuose (B4; $p < 0,05$). Esminių AR, izoleucino, leucino, fenilalanino ir lizino didžiausi kiekiai nustatyti B4 ($p < 0,05$). Šio disertacinio darbo tyrimo metu gauti rezultatai atitinka Messyaszo ir kt. [29] rezultatus, kurie rodo, kad *Cladophora* biomasės AR profilis atskleidžia novatoriškos žaliavos panaudojimo galimybes pašarų gamyboje.

2.2. Gėlavandenės *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių, antioksidacinis aktyvumas ir biologinį aktyvumą turintys junginiai (https://doi.org/10.3390/w14071138)

Veiksniai, pvz., dumblių rūšis, buveinė, klimatas, aplinkos veiksniai ir biomasės surinkimo metodai, turi įtakos dumblių biomasės cheminei sudėčiai ir biologiškai aktyvių junginių susidarymui bei susikaupimui [81, 82]. Bioaktyvių junginių gausa dumbliuose yra jų prisitaikymo prie įvairiausių aplinkos sąlygų rezultatas. Taigi kitas disertacinio darbo etapas buvo išanalizuoti *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių, fenolinių junginių profilį, antioksidacinį aktyvumą ir pigmentinių medžiagų koncentraciją.

Disertacinio darbo tyrimų metu makrodumblių biomasėje iš trylikos fenolio rūgščių aptiktos tik trys (galo, *p*-kumaro ir *p*-hidroksibenzenkarboksirūgštis; 2.2.1 lentelė). Didžiausia galo rūgšties koncentracija nustatyta B3 (35,13 μg/g SM), o *p*-hidroksibenzenkarboksirūgšties B3 ir B4 (atitinkamai 29,05 ir 28,31 μg/g SM; $p < 0,05$). Trijuose iš keturių biomasės mėginiuose aptikta *p*-kumaro rūgštis, kurios didžiausia koncentracija nustatyta B4 (6,46 μg/g SM). *C. glomerata* biomasės iš Lietuvos upių analizė identifikavo mažiau fenolinių junginių, palyginti su Korzeniowska ir kt. [83], tačiau autoriai patvirtino, kad visuose biomasės mėginiuose aptikta galo ir *p*-kumaro rūgščių. Dažniausiai fenoliniai junginiai turi įvairių savybių, kurios prisideda prie antioksidacinio ir antibakterinio bei antivirusinio aktyvumo [89]. Bendras fenolinių junginių kiekis skirtinguose biomasės mėginiuose kito, o didžiausia koncentracija nustatyta B3 (1,32 mg GRE/g SM). Reikšmingi skirtumai nustatyti tarp B1 ir B3 (0,37 mg GRE/g SM mažesnė koncentracija nei B1) ir tarp B2 ir B3 (0,22 mg GRE/g SM mažesnė koncentracija nei B2) ($p < 0,05$).

Tačiau atlikus flavonoidų (kvercetino, miricetino, kaempferolio, rutino ir ksantohumolio) analizę nenustatyta teigiamų rezultatų.

Moksliniais tyrimų rezultatais pagrįsta, kad antioksidacinis aktyvumas yra tiesiogiai susijęs su augalų ekstraktų RG [90, 91]. Disertacinio darbo metu nustatytas RG reikšmių pasiskirstymas gėlavandenėje *C. glomerata* biomasėje iš skirtingų upių: B3>B4>B1>B2 (2.2.1 pav.). DPPH aktyvumas biomasės mėginiuose kito tarp 8,22–11,09 proc., tačiau be nustatytų reikšmingų skirtumų ($p > 0,05$). Bendras antioksidantų kiekis B3 ir B4 biomasės mėginiuose nustatytas didesnis (atitinkamai 1,47 ir 1,31 mg Trolox/g SM), palyginti su B1 ir B2 ($p < 0,05$). *C. glomerata* biomasėje B3 bendras antioksidantų kiekis nustatytas net 2,7 ir 3,8 karto, o B4 – 2,4 ir 3,4 karto didesnis nei B1 ir B2 ($p < 0,05$). Dažniausiai antioksidantų kiekis gėlavandenėje *C. glomerata* biomasėje gali kisti, atsižvelgiant į sezoną ir geografinę padėtį [92]. Pavyzdžiui, mėginiuose iš Lietuvos upių Dubysos (B1) ir Šventosios (B2) nustatytas mažesnis antioksidantų kiekis (atitinkamai 0,55 ir 0,39 Trolox/g SM), palyginti su mėginiais, kurie analizuojami analogiškai, bet paimti iš Lenkijos vandens telkinių (Didžiosios Lenkijos regionas) [83].

Atlikus ABTS analizę, didžiausias radikalo skaldymo aktyvumas nustatytas B1 mėginiuose (97,68 proc.; 2.2.1 pav.), tačiau reikšmingų skirtumų tarp B1, B2 ir B3 nenustatyta ($p > 0,05$). B1, B2 ir B3 biomasės mėginiai, palyginti su B4, atitinkamai 3,92 proc., 3,04 proc. ir 3,33 proc. didesnis ABTS nustatytas B4 ($p < 0,05$). ABTS analizė įvertina antioksidantų gebą suskaldyti $ABTS^{*+}$ radikalus, kurių vertės kinta tarp 93,76–97,68 proc. makrodumblių biomasės mėginiuose, surinktuose iš Lietuvos upių. Nustatytas aktyvumas daug didesnis, palyginti su *C. glomerata,* surinkta iš Lenkijos gėlųjų vandenų [83]. FRAP aktyvumas rodo antioksidantų redukcinę galią ir yra labiau susijęs su florotanino kiekiu nei su radikalų skaldymu [95]. Atlikta FRAP analizė parodė, kad *C. glomerata* biomasės mėginiuose šis aktyvumas kinta tarp 15,04–20,86 µmol/l (2.2.1 pav.). Makrodumblių mėginiai B3 ir B4 turėjo didžiausią FRAP aktyvumą, atitinkamai 5,82 ir 4,25 µmol/l didesnį, palyginti su B1 ir B2 ($p < 0,05$).

Apskritai chlorofilas yra gyvybiškai svarbus augalų, dumblių bei cianobakterijų fotosintezės pigmentas [96]. Jo koncentracija tiesiogiai koreliuoja su daugeliu aplinkos veiksnių, tokių kaip vandens gylis, maistinių medžiagų koncentracija aplinkoje ir šviesos prieinamumas [6, 12]. Disertacinio darbo tyrimo metu analizuojamoje biomasėje chlorofilo *a* koncentracija nustatyta daug didesnė nei kitų mokslininkų atliktų tyrimų metu [67, 97]. Bendras chlorofilo *a* kiekis biomasėje kito tarp 0,56–0,74 mg/g SM, šio pigmento didžiausia koncentracija nustatyta B3, o mažiausia – B2 ($p < 0,05$; 2.2.2 pav.). Reikšmingi chlorofilo *b* koncentracijų skirtumai, palyginti su B1 ir B4, kai B1 koncentracija nustatyta didesnė ($p < 0,05$). Be to, nustatytas didesnis

chlorofilo *b* kiekis biomasėje atitinka rūšies prisitaikymą prie įvairios aplinkos [98]. Analogiška tendencija atsikartojo nustačius bendrą karotinoidų kiekį, kai 0,06 mg/g SM didesnė koncentracija nustatyta B1 biomasėje, palyginti su B4 ($p < 0,05$; 2.2.2 pav.). Bendras karotinoidų kiekis biomasėje kito tarp 0,17–0,23 mg/g SM, tačiau nustatytos koncentracijos buvo mažesnės nei kitų mokslininkų atliktuose tyrimuose [99]. Mokslininkai, analizavę makrodumblių biomasę, pateikia skirtingus karotinoidų kiekius, tai siejama su aplinkos ir biologinės įvairovės veiksniais, tokiais kaip mažesnė temperatūra ar saulės šviesa. Šie veiksniai tiesiogiai veikia medžiagų apykaitos greitį dumbliuose, taip mažėja chlorofilo gamyba fotosintezei, bet didėja karotinoidų sintezė [100]. Kitas gyvybiškai svarbus žaliųjų dumblių karotinoidas – liuteinas, padeda sugerti šviesą ir apsaugo nuo oksidacinio streso fotosintezės metu [101]. Prazukino ir kt. [102] nustatyta gėlavandenės *Cladophora* spp., surinktos iš Rusijoje esančio ežero, liuteino koncentracija 0,55 mg/g SM, kuri rodo biomasės panaudojimo potencialą farmacijos ir maisto gamyboje. Tačiau šio disertacinio darbo metu nustatytos liuteino koncentracijos beveik perpus mažesnės (0,11–0,17 mg/g SM; 2.2.2 pav.).

2.3. *C. glomerata* makrodumblių biomase papildyto pašaro įtaka triušių produktyvumui, skerdenos rodikliams ir fiziologiniam išsivystymui (https://doi.org/10.1080/1828051X.2024.2342380)

Triušių produktyvumas daro didžiulę įtaką triušienos gamybos efektyvumui ir bendram pelningumui [108]. Nors *C. glomerata* panaudojimo galimybės plačiai analizuotos paukščių ir akvakultūrų mityboje, tačiau mokslu pagrįstų tyrimų, panaudojant triušių racioną, trūksta. Pagal atliktus mokslinių tyrimų rezultatus galima teigti, kad *C. glomerata* gali pagerinti tigrinių krevečių produktyvumo rodiklius, kai biomasės kiekis siekia 10–20 proc. [41]. Panašūs tyrimai atlikti su viščiukais broileriais, kai jų lesalai buvo papildyti 15 proc. *C. glomerata* biomase. Jų metu nustatytas spartesnis viščiukų augimo greitis [109]. Kitas šio disertacinio darbo eksperimentinis etapas buvo ištirti naujų *C. glomerata* papildytų pašarų formulių poveikį triušių produktyvumui, skerdimo rodikliams ir fiziologiniam išsivystymui.

Disertacinio darbo metu ištyrus *C. glomerata* biomasės įtraukimo į triušių pašarus įtaką augimo efektyvumui tarp 52–122 amžiaus dienos, nepastebėta reikšmingų pokyčių tiek triušių priesvoriui per tiriamąjį laikotarpį, tiek vidutiniam kūno priesvoriui per parą ($p > 0,05$; 2.3.1 lentelė). Kitas svarbus aspektas – pašarų suvartojimas, kuris reikalingas, norint užtikrinti optimalų augimą [110]. Šėrimo bandymo rezultatai parodė, kad triušiai, šerti pašaru, papildytu 4 proc. *C. glomerata* (CG4), suvartojo daugiau pašarų per parą,

palyginti su pašarais, kuriuose biomasės kiekis buvo dvigubai didesnis (CG8; $p < 0,05$). Šie tyrimai gali būti siejami su biomasės įtaka pašaro skoninėms savybėms, o tai sutampa su ankstesniais mokslininkų tyrimų rezultatais, kurie rodo, kad pašarus papildžius makrodumbliais reikšmingai pagerėja pašaro skonis [111]. Tačiau svarbu pažymėti, kad nustatytas optimalus biomasės įtraukimas į triušių pašarus yra 4 proc., nes dvigubas jos kiekis reikšmingai sumažino pašarų sunaudojimą per parą, tai gali rodyti ribinį įterpimą, turintį įtakos bendram pašaro priimtinumui. PKK rodo pašarų pavertimą į kūno svorį, o tai įtakos turi ne tik gamybos sąnaudoms, bet ir bendram pelningumui [112]. Pirmiausia, PKK gali atspindėti pašarų panaudojimo efektyvumo skirtumus. Vertinant šį rodiklį, apskaičiuotą šio disertacinio tyrimo metu, CG4 pašarai PKK reikšmingai padidino iki 4,32 kg/kg, kai triušių, šertų SCD siekė 3,72 kg/kg, o CG8 tik 2,93 kg/kg ($p < 0,05$; 2.3.1 lentelė). Išryškėjo tendencija, kad didesnis biomasės kiekis reikšmingai mažina PKK, todėl galima daryti išvadą – pašarų papildymas 8 proc. *C. glomerata* biomase gali užtikrinti tausesnę pašarų konversiją į kūno svorį.

Tinkamas vidaus organų ir žarnyno išsivystymas yra vienas esminių aspektų, siekiant užauginti sveiką triušį, palaikant veiksmingą maistinių medžiagų pasisavinimą ir tvirtą imuninę sistemą [117]. Tačiau biomase papildytų pašarų reikšminga įtaka triušių skerdenų rodikliams nenustatyta. *C. glomerata* įterpimas į kombinuotuosius pašarus reikšmingai sumažino plaučių masės procentą nuo kūno svorio prieš skerdimą, palyginti su SCD ($p < 0,05$). Nors, lyginant minėtą rodiklį tik tarp biomase papildytų grupių, jis išliko identiškas ($p > 0,05$). Gauti rezultatai nerodo reikšmingos įtakos skerdenos rodikliams bei fiziologiniam išsivystymui, kurį lemtų priklausomybė nuo *C. glomerata* papildymo.

Išanalizuota pašarų, papildytų *C. glomerata* biomase, įtaka triušių virškinimo procesams, įvertinant skirtingų žarnyno segmentų turinio pH ir SM kiekio diapazonus (2.3.1 pav.). Triušių, šertų standartiniais kombinuotaisiais pašarais, klubinės žarnos pH vertės nustatytos didesnės, palyginti su *C. glomerata* papildytais pašarais. SCD šertų triušių klubinės žarnos pH vertės nustatytos atitinkamai 0,44 ir 0,38 vienetų didesnės, palyginti su CG4 ir CG8 ($p < 0,05$; 2.3.1 pav. A). Tačiau tikslus biomase papildytų pašarų veikimo mechanizmas neapibrėžtas, nes jų įtaka pH ir SM vertėms kituose žarnyno segmentų turiniuose nenustatyta. Tačiau svarbu pažymėti, kad klubinės žarnos turinio pH kitimai gali lemti mikroorganizmų ekologiją ir fermentacijos procesus, atitinkančius anksčiau atliktų tyrimų rezultatus, kurie atskleidžia makrodumblių prebiotines savybes, turinčias tiesioginės įtakos žarnyno mikrobiotai [118–120].

Šio disertacinio tyrimo metu atlikta triušių dvylikapirštės žarnos turinio TGRR profilio analizė. Tyrimų rezultatai parodė, kad vyraujanti TGRR buvo

acto rūgštis, kitos rūgštys – propiono ir pieno (2.3.2 lentelė). Didžiausia acto rūgšties koncentracija nustatyta CG4 (89,04 proc.), mažesnė CG8 (87,53 proc.) ir mažiausia SCD (82,23 proc.; $p < 0,05$). Pagrindinis acto rūgšties, kaip esminio energijos substrato, vaidmuo triušių audiniuose yra pagerinti medžiagų apykaitą ir riebalų panaudojimą [121]. Be to, acto rūgštis gali turėti įtakos triušių apetitui, kuris gali lemti pašarų suvartojimą. Padidėjusi acto rūgšties koncentracija CG4 ir CG8 rodo suintensyvėjusią mikroorganizmų fermentaciją, atitinkančią anksčiau atliktus tyrimus su dumbliais [122, 123]. Kita TGRR, propiono rūgštis, atlieka esminį vaidmenį gliukozės metabolizmo procese, prisidedant prie gliukozės gamybos kepenyse, kuri itin svarbi siekiant patenkinti energijos poreikius [124]. CG8 triušių dvylikapirštės žarnos turinyje nustatyta 1,75 karto daugiau propiono rūgšties, palyginti su SCD ($p <0,05$), o reikšmingų skirtumų tarp sviesto rūgšties koncentracijų nenustatyta ($p > 0,05$). *C. glomerata* papildyti pašarai (CG4 ir CG8) reikšmingai sumažino pieno rūgšties koncentraciją, atitinkamai 6,06 ir 7,16 proc., palyginti su SCD ($p < 0,05$). Pieno rūgšties dėsningas mažėjimas didėjant *C. glomerata* biomasės kiekiui pašaruose rodo prebiotinį poveikį žarnyno mikrobiotos sudėčiai, lemiantį fermentacijos efektyvumą virškinimo organų sistemoje [125].

Histomorfometrinė analizė itin svarbi siekiant įvertinti alternatyvių komponentų įtaką žarnyno gleivinės struktūrai, kurie tiesiogiai lemia maistinių medžiagų pasisavinamumą ir rodo žarnyno sveikatos būklę. Todėl žarnyno audinių adaptacijos supratimas yra būtinas siekiant optimizuoti gyvūno racioną [126]. Be to, histomorfometriniai tyrimai padeda nustatyti galimą naudą bei problemas, susijusias su alternatyviais pašarų komponentais – taip palengvinamas pašarų formulių, gerinančių virškinimo funkciją ir bendrą gyvūno produktyvumą, kūrimas [127]. Šio disertacinio darbo metu atlikta histomorfometrinė analizė parodė reikšmingą *C. glomerata* biomasės įtraukimo į triušių pašarus įtaką gleivinės sandarai (2.3.2 lentelė). Nors dvylikapirštės žarnos gaurelių aukštis nekito, klubinės žarnos gaureliai buvo paveikti: aukščiausi gaureliai nustatyti CG4 (1526,09 μm), o CG8, atvirkščiai, žemiausi (1079,12 μm; $p < 0,05$). Vertinant *C. glomerata* papildytų pašarų įtaką, gaurelių aukštis CG4 klubinėje žarnoje nustatytas reikšmingai didesnis, palyginti su CG8 ir SCD ($p < 0,05$). Šie rezultatai rodo biomasės įtaką geresniam gleivinės audinių išsivystymui, kuris lemia geresnį maistinių medžiagų pasisavinamumą [128]. Dvylikapirštėje žarnoje kriptos gylis didėjo didėjant *C. glomerata* kiekiui pašaruose (CG8>CG4>SCD), o klubinėje žarnoje – atvirkščiai, mažėjo biomasės kiekiui didėjant (SCD>CG4>CG8; 2.3.2 lentelė). Kriptų gylis atspindi žarnyno gleivinės struktūrinę dinamiką: gilesnės kriptos dvylikapirštėje žarnoje rodo greitesnę ląstelių apykaitą ir maistinių medžiagų pasisavinamumą, o mažiau gilios klubinės žarnos kriptos rodo

brandesnę gleivinės sandarą [129]. Nustatyta *C. glomerata* papildytų pašarų įtaka triušių dvylikapirštės ir klubinės žarnų segmentų pokyčiams vertinant gaurelių aukščio ir kriptų gylio santykį (G/K): SCD G/K nustatytas atitinkamai 1,57 ir 2,36 vienetų didesnis, palyginti su CG4 ir CG8 ($p < 0,05$). Tačiau biomasės papildymas 4 ir 8 proc. padidinimo G/K klubinėje žarnoje, atitinkamai 4,71 ir 5,55 vieneto, palyginti su SCD ($p < 0,05$). Gauti rezultatai parodo biomasės poveikį skirtingų žarnyno segmentų histomorformetrijai, kurią tiesiogiai gali paveikti makrodumblių biomasėje esančios biologiškai aktyvios medžiagos.

2.4. *C. glomerata* makrodumblių biomase papildyto pašaro įtaka triušienos funkcionalumui (https://doi.org/10.3390/foods12040744)

Dažnai mėsa ir jos gaminiai laikomi funkcionaliaisiais maisto produktais dėl į jų sudėtį įeinančių naudingų komponentų spektro [48]. Tačiau siekiant pagerinti triušienos funkcionalumą, reikėtų mokslinių tyrimų pagrindu pagrįsti strategijas, apimančias *C. glomerata* biomasės įtraukimą į triušių racioną. Todėl kitas disertacinio darbo eksperimentinis etapas buvo išanalizuoti naujų *C. glomerata* papildytų pašarų formulių poveikį triušienos funkcionalumui, ištiriant raumenų baltymų ir lipidų profilius.

Triušiena išskiriama dėl jos sudėtyje esančio aukštos kokybės baltymų profilio, kurį sudaro net apie 22 proc. baltymų [44, 48]. Atsižvelgiant į disertacinio darbo metu naudotą *C. glomerata* biomasę, joje esantis baltymų kiekis sudaro 10–25 proc., todėl tokia biomasė gyvūnų mityboje gali būti naudojama kaip potencialus baltymų šaltinis [6]. Nustatyta, kad 4 proc. biomasės papildyti pašarai turėjo didžiausią įtaką baltymų kiekio padidėjimui triušių LD raumenyse (22,17 proc.). Tačiau padvigubinus *C. glomerata* kiekį pašaruose, baltymų kiekis labai sumažėjo, palyginti tiek su CG4, tiek su SCD. Abu Hafsa ir kt. [131] taip pat išnagrinėjo gėlavandenių dumblių (*C. aegagropila*) 4 proc. įtraukimo į pašarą įtaką baltymų kaupimuisi, rezultatai parodė, kad baltymų kiekis triušienoje siekė tik 18,63 proc., tai, palyginti su šio disertacinio darbo rezultatais, yra mažiau.

Gyvulininkystės veiklos metu išgauti produktai yra vienas pagrindinių žmogaus mitybos baltymų šaltinių, o triušiena išsiskiria plačiu AR spektru [132]. Be to, triušienoje daug nepakeičiamųjų AR, palyginti su kitomis populiariomis mėsos rūšimis. Šio disertacinio darbo metu gauti rezultatai rodo, kad pašarai, papildyti 4 proc. *C. glomerata*, reikšmingai padidino AR kiekį triušių LD raumenyse ($p < 0,05$; 2.4.1 lentelė). Skirtingos pašaro formulės turėjo įtaką ir lizino kiekiui triušių LD raumenyse, kuris pasiskirstė taip: CG4<SCD<CG8 ($p < 0,05$). Biomasės papildymas neturėjo reikšmingos

įtakos leucino ir fenilalanino kiekiams ($p > 0,05$), tačiau didesnis nepakeičiamųjų AR kiekis buvo nustatytas triušių, kurie buvo šeriami CG8 ($p < 0,05$), HL raumenyse. Įvertintas ir pakeičiamųjų AR profilis, kuriame triušių LD ir HL raumenyse vyravo glutamo rūgštis. Gauti rezultatai sutampa su Morshdy'o ir kt. [134] tyrimų rezultatais, kai aptiktas glutamo rūgšties vyravimas Naujosios Zelandijos ir Kalifornijos veislių triušių LD raumenyse. Tačiau disertacinio darbo metu pakeičiamosios AR pasiskirstė netolygiai: didesnis glicino kiekis nustatytas HL raumenyse triušių, šertų CG8, o arginino kiekis HL raumenyse triušių, šertų SCD, palyginti su CG8 ($p < 0,05$). CG8-LD raumenyse nustatytas mažesnis alanino kiekis, palyginti su kitomis grupėmis, o asparto rūgšties daugiausia nustatyta tiek LD, tiek HL raumenyse šeriant CG4 ($p < 0,05$). Į pašarus įtraukus 4 proc. *C. glomerata,* reikšmingai padidėjo bendras AR kiekis LD raumenyse (192,16 g/kg), o biomasės kiekį padvigubinus iki 8 proc., šis rodiklis padidėjo HL raumenyse (176,13 g/kg). Mėsos švelnumas išskiriamas kaip vienas pagrindinių aspektų vartotojams, o šią savybę tiesiogiai veikia jungiamojo audinio baltymai [136]. Tiksliau hidroksiprolino kiekis mėsoje laikomas jungiamojo audinio indikatoriumi, o triptofano kiekis indikuoja raumeninį audinį [137]. Sukurti pašarai, papildyti 4 proc. *C. glomerata* biomase, padidino triptofano lygį LD raumenyse. Tačiau šis lygis sumažėjo, kai triušiai buvo šeriami CG8 ($p < 0,05$; 2.4.1 lentelė). Išanalizavus HL raumenis daugiausia triptofano nustatyta SCD grupėje, toliau – CG8 ir galiausiai – CG4 ($p < 0,05$). Kita vertus, mažiausios hidroksiprolino koncentracijos gautos triušių raumenyse, kurie buvo šerti CG4, tai rodo geresnę mėsos kokybę. Apskaičiuotas didžiausias triptofano ir hidroksiprolino santykis LD raumenyse triušių, šertų CG4 pašarais ($p < 0,05$). HL raumenų analizė reikšmingo biomasės poveikio minėtam santykiui neparodė ($p > 0,05$).

Pašarai, papildyti *C. glomerata* biomase, reikšmingai sumažino riebalų kaupimąsi triušių raumenyse. Pastebėta tendencija: didėjant biomasės kiekiui pašaruose, riebalų kaupimasis triušių LD ir HL raumenyse mažėja ($p < 0,05$). Panaši tendencija pastebėta atlikus tyrimus su žuvimis, kurių metu *C. glomerata* papildyti pašarai sumažino riebalų kaupimąsi žuvų raumenyse [43]. Su maistu gaunamo cholesterolio įtaka jo kaupimuisi plazmoje neryški, tačiau vartotojams tai kelia nerimą. Todėl būtina pažymėti, kad triušiena išskiriama dėl mažesnio cholesterolio kiekio, palyginti su kita dažnai vartojama mėsa [48]. Šio disertacinio darbo metu nustatytos cholesterolio kiekis LD raumenyse siekė 22,81–26,08 mg/100 g, o HL raumenyse – 30,60–35,02 mg/100 g. Tačiau reikšmingos pašaro papildymo biomase įtakos cholesterolio kaupimuisi nenustatyta ($p > 0,05$). Kita vertus, *C. glomerata* papildymas pašaruose reikšmingai sumažino lipidų oksidacijos laipsnį, šviežiuose LD bei šviežiuose ir 3 mėnesius sandėliuose HL raumenyse ($p < 0,05$; 2.4.1 pav.). Gauti

rezultatai rodo pagerėjusią mėsos kokybę ir stabilumą į pašarus įterpiant didesnius biomasės kiekius.

Triušiena išsiskiria dėl joje esančio PNRR kiekio, kurį manipuliuoti galima per mitybą. Disertacinio darbo metu atlikta triušienos analizė parodė, kad į triušių pašarus įterpus *C. glomerata*, triušienoje reikšmingai sumažėjo SRR ir padidėjo PNRR kiekiai (2.4.2 pav.). Ši tendencija paremta panašiais tyrimų rezultatais analizuojant ėrieną [146]. Šio disertacinio darbo metu triušių raumenyse skirtingos RR grupės pasiskirstė taip: SRR LD: SCD<CG4<CG8, HL: CG4<SCD<CG8; MNRR LD: SCD<CG4<CG8, HL: SCD<CG4<CG8; PNRR LD: CG8<CG4<SCD, HL: CG8<CG4<SCD. PNRR kiekis triušių raumenyse reikšmingai padidėjo, kai į pašarus buvo įtrauktas didesnis kiekis biomasės, PNRR profilyje vyravo linolo rūgštis. Biomasės papildymas reikšmingo poveikio linolo rūgšties kiekiui, palyginti tarp grupių, neturėjo ($p > 0,05$), tačiau kitų n-6 ir n-3 RR kiekiai labai padidėjo, kai į pašarus buvo įtraukta 8 proc. *C. glomerata* biomasės ($p < 0,05$). Makrodumblių biomasė pašaruose neturėjo įtakos bendram n-3 kiekiui triušių raumenyse ($p > 0,05$). Kita vertus, buvo paveiktos tam tikros n-6 RR, tai lėmė didesnį jų bendrą kaupimąsi CG8 triušių raumenyse. Gėlavandenės *C. glomerata* papildymas neturėjo įtakos n-6 ir n-3 santykiui ($p > 0,05$).

PNRR/SRR apibrėžiamas kaip vienas esminių indikatorių, siekiant įvertinti mitybos poveikį širdies ir kraujagyslių sveikatingumui [147]. Teoriškai teigiama, kad didesnis PNRR/SRR koreliuoja su mažesniu mažo tankumo lipoproteinų cholesterolio (MTL-C) ir bendro cholesterolio kiekiu serume, o tai gerina širdies ir kraujagyslių sistemos būklę [69]. Disertacinio darbo metu gauti rezultatai išskiria nuoseklią tendenciją: didinant *C. glomerata* kiekį pašaruose, didėja PNRR/SRR tiek LD, tiek HL raumenyse ($p < 0,05$). Kitas rodiklis, analizuotas triušių raumenyse, buvo aterogeniškumo indeksas (AI), kuris rodo pusiausvyrą tarp bendro SRR ir NRR kiekio (2.4.2 pav.). Maisto produktų, kurių AI vertės yra mažesnės, vartojimas gali sumažinti bendrojo cholesterolio ir MTL-C kiekį žmogaus plazmoje, o tai ypač naudinga atsižvelgiant į kraujotakos sveikatingumą [150]. Šio disertacinio darbo metu gauti rezultatai rodo, kad *C. glomerata* papildymas pašaruose nepaveikė AI LD raumenyse ($p > 0,05$), nors 8 proc. papildymas reikšmingai sumažino AI vertę HL raumenyse, palyginti su kitomis grupėmis ($p < 0,05$). Panašiai trombogeniškumo indeksas (TI), įvertinantis protrombogeninių ir antitrombogeninių RR pusiausvyrą, įvardijamas kaip esminis širdies bei kraujagyslių sveikatos rodiklis [151]. Tačiau šis rodiklis LD raumenyse, pašarus papildant biomase, paveiktas nebuvo ($p > 0,05$). Tik CG4 pašarai padidino TI HL raumenyse, palyginti su SCD ir CG8 ($p < 0,05$). Be to, hipocholesterolemijos ir hipercholesterolemijos santykis (h/H) yra tikslesnis rodiklis nei PNRR/SRR, taip pat indikuoja RR poveikį širdies ligų rizikai. Taigi maisto produktai, kurių h/H

yra didesnis, mitybos požiūriu laikomi labiau pageidautini vartotojams [152]. Šio disertacinio darbo metu nustatyta, kad didėjantis biomasės kiekis pašaruose didino h/H. Pašarus papildžius 4 ir 8 proc. makrodumblių biomase, apskaičiuotas santykis LD raumenyse nustatytas atitinkamai 1,03 ir 1,13 karto didesnis, o HL raumenyse – 1 ir 1,14 karto didesnis, palyginti su SCD ($p < 0,05$; 2.4.2 pav.).

2.5. *C. glomerata* makrodumblių biomase papildyto pašaro įtaka triušienos juslinių savybių profiliui (https://doi.org/10.3390/ani13132179)

Mėsos kokybės gerinimas apima ne tik cheminių ir fizinių rodiklių jos tobulinimą, bet ir juslinių aspektų, kuriuos gali veikti pašarų komponentai, gerinimą. Sveikatingumas yra vienas didžiausių aspektų pagal nūdienos tendencijas, tačiau juslinės savybės visada išliks esminėmis lemiant vartotojų priimtinumą. Todėl siekiant optimizuoti kokybinius mėsos rodiklius bei užtikrinti pelningumą, itin svarbu suprasti ir atsižvelgti į vartotojų pageidavimus [153]. Taigi galutinis disertacinio darbo eksperimentinis etapas buvo išanalizuoti naujų *C. glomerata* papildytų pašarų formulių poveikį triušienos raumenų fizinėms savybėms ir juslinių savybių profiliui.

Drėgmės kiekis – vienas esminių parametrų, turintis įtakos mėsos kokybei bei veikiantis jos išvaizdą, tekstūrą, skonį ir tinkamumo vartoti galiojimo laiką [155]. Nustatyta, kad didesnis *C. glomerata* kiekis pašaruose lėmė didesnę drėgmės koncentraciją, kuri mitybos grupėse pasiskirstė taip: SCD<CG4<CG8 (2.5.1 pav. A). Be to, drėgmės kiekis turi įtakos kokybiniams rodikliams – vandens rišlumui ir lašėjimo nuostoliams, kurie tiesiogiai lemia ekonominę mėsos vertę [156]. *C. glomerata* papildymas pašaruose reikšmingai nepaveikė nei lašėjimo nuostolių, nei vandens rišlumo triušių raumenyse ($p > 0,05$), tačiau virimo nuostoliai HL raumenyse nustatyti reikšmingai didesni, jei triušiai buvo šeriami CG8, palyginti su CG4 ($p < 0,05$; 2.5.1 pav. A). Gautus rezultatus galėjo lemti baltymų denatūracija virimo metu [157].

Raumenų skaidulų plotis tiesiogiai veikia mėsos išeigą ir bendrą kokybę [131]. Šio disertacinio darbo metu nustatyta, kad makrodumblių biomase papildyti pašarai CG8 padidino LD raumenų skaidulų ilgį iki 59,09 μm^2, kai SCD siekė tik 51,52 μm^2 ($p < 0,05$; 2.5.1 pav. B). Skaidulų ilgis ir plotis tiesiogiai veikia mėsos švelnumą [158]. Taigi, remiantis mokslinių tyrimų rezultatais, 8 proc. *C. glomerata* įtraukimas į triušių racioną skatina LD raumenų augimą bei gali pagerinti triušienos švelnumą.

Triušių raumenų pH po skerdimo mažėjo tolygiai, o jo mažėjimas turi įtakos vandens rišlumui [159, 160]. Tačiau *C. glomerata* įterpimas į triušių pašarus nepaveikė raumenų vandens rišlumo, vadinasi, nėra aiškios sąsajos tarp pH ir minėto rodiklio. Kitas aspektas, renkantis mėsą – vartotojai pirmenybę teikia spalvai, kuriai įtakos turi raumeninio audinio struktūra kartu su rūgštingumu, galinčiu lemti spalvos ryškumą ir atspalvį. Šio disertacinio darbo metu analizuotų triušių, šertų CG8, raumenų spalva praėjus 24 valandoms po skerdimo nustatyta ryškesnė ir raudonesnė, o tai gali koreliuoti su didesniu pH (2.5.2 pav.). Tačiau po 48 valandų LD raumenų paraudimas sumažėjo, o HL raumenys tapo tamsesni ir mažiau raudoni ($p < 0,05$). Nustatytas pH ir spalvos ryškumo ryšys sutampa su kitų mokslininkų atliktais tyrimais [161, 162]. Be to, antioksidacinis aktyvumas turi įtakos mėsos spalvos stabilumui, o didesnis makrodumblių biomasės įterpimas į pašarus sumažino paraudimą po skerdimo. Kaip ir šviežiai mėsai, virtos mėsos išvaizdai įtakos turi įvairūs veiksniai, pavyzdžiui, raumenų tipas, gaminimo parametrai, pakavimo būdas, sandėliavimas, mėsos riebumas, skonis ir konservavimo būdas [163]. Galiausiai mioglobinas atlieka pagrindinę funkciją keičiantis spalvai, o *C. glomerata* 4 proc. papildymas reikšmingai nulėmė virtų LD raumenų spalvą, kuri nustatyta mažiau gelsva ir raudona, palyginti su SCD ir CG8 ($p < 0,05$).

Vertinant mėsos kokybę tradiciškai atsižvelgiama į juslinius požymius, tokius kaip išvaizda, tekstūra, kvapas, skonis bei švelnumas. Triušių LD ir HL raumenų juslinių savybių vertinimas, apimantis devyniolika kriterijų, atskleidė, kad *C. glomerata* papildymas paveikė LD raumenų kvapą ir spalvos intensyvumą bei HL raumenų kietumą, pojūtį burnoje ir skonio sodrumą (2.5.3 pav.). Pažymėtina, kad 4 proc. biomasės papildymas sukėlė reikšmingus LD raumenų kvapo pokyčius, o pagal vertintojus spalvos intensyvumas didėjo, didėjant biomasės įterpimui ($p < 0,05$). Triušius šeriant CG8 ir vertinant jų HL raumenis buvo jaučiamas intensyvesnis pojūtis burnoje, tačiau mažiau sodrus, palyginti su kitomis grupėmis ($p < 0,05$). Neatsižvelgiant į nustatytus skirtumų tarp grupių, bendros juslinės triušienos savybės išliko priimtinos. Al-Soufis ir kt. [44] nustatė potencialius mėsos savybių ir stabilumo pagerėjimus panaudojus makrodumblius triušių racione. Tai sutampa su šio disertacinio darbo metu gautais rezultatais, nors buvo keletas skirtumų, nustatytų tarp standartinių kombinuotųjų ir *C. glomerata* papildytų pašarų.

Mėsos produktų vertinimo požiūriu išskiriami du pagrindiniai tiksliai: suprasti vartotojo pageidavimus bei įvertinti vartotojo ketinimą įsigyti konkretų produktą [166]. Šiais laikais inovatyviosios programinės įrangos leidžia analizuoti veido išraiškas bei atsaką į vertinamą maistą realiuoju laiku. Vertintojų emocinis atsakas į triušių raumenų mėginių kvapą, jei triušių pašarai

buvo papildyti *C. glomerata*, daugiausia sukėlė neutralių veido išraiškų. Triušių, šeriamų CG4, raumenų kvapas kėlė mažiau laimės ir daugiau liūdesio, o šeriamų CG8, atvirkščiai, – mažiau liūdesio ir pasibjaurėjimo ($p < 0,05$). Skonio vertinimas parodė reikšmingai padidėjusią laimę, bet padidėjusį pasibjaurėjimą, kai buvo ragaujami CG8 raumenys, palyginti su perpus mažesniu *C. glomerata* įterpimu (CG4; $p < 0,05$). Būtina pažymėti, kad triušienos mėginiai buvo išvirti be prieskonių ar druskos, tai galėjo turėti įtakos vertintojams.

IŠVADOS

1. Gėlavandenių *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių (Dubysos, Šventosios, Nevėžio ir Jūros), cheminės analizės rezultatai rodo jos komercinio panaudojimo galimybes, atsižvelgiant į gyvūnų mitybos saugos standartus, neviršijant sunkiųjų metalų koncentracijos, bei turi darnią makro- ir mikroelementų kompoziciją. Tačiau cheminė analizė po kiekvieno biomasės surinkimo būtina, siekiant išvengti galimos aplinkos įtakos biomasės toksiškumui ir kitoms cheminėms savybėms. Apskritai *C. glomerata* bioakumuliacinės savybės rodo šios biomasės panaudojimo galimybes baltymų, nepakeičiamųjų AR ir RR šaltinį pašarų gamyboje.
2. Gėlavandenės *C. glomerata* makrodumblių biomasės, surinktos iš Lietuvos upių (Dubysos, Šventosios, Nevėžio ir Jūros), analizės rezultatai rodo didelį jos kaip funkcionaliosios žaliavos panaudojimo potencialą atsižvelgiant į nustatytą antioksidacinį aktyvumą ir biologiškai aktyvių elementų akumuliaciją. Surinktoje biomasėje identifikuoti fenoliniai junginiai (galo, *p*-kumaro ir *p*-hidroksibenzenkarboksirūgštys) ir pigmentai. Biomasė pasižymi aukštu antioksidaciniu aktyvumu, aukšta redukcine galia bei bendru antioksidantų kiekiu. Be to, nustatyti pagrindiniai pigmentai, tokie kaip chlorofilai *a* ir *b*, karotinoidai ir liuteinas, rodo biomasės panaudojimo galimybes ne tik pašarų pramonėje, bet ir kitose pramonės šakose.
3. Pašarų papildymas *C. glomerata* biomase reikšmingos įtakos triušių kūno svoriui ir vidutiniam kūno priesvoriui per parą neturėjo. Tačiau papildžius pašarus 8 proc. *C. glomerata* reikšmingai sumažintas pašarų suvartojimas per parą bei pagerintas PKK – tai rodo tausesnį išteklių panaudojimą. Biomasės papildymo įtaka skerdenų rodikliams nenustatyta, išskyrus, reikšmingai sumažėjusį plaučių masės procentą nuo prieš skerdimą buvusio kūno svorio. *C. glomerata* papildymas padidino acto ir propiono rūgščių kiekius, kartu sumažino pieno rūgšties koncentraciją

dvylikapirštėje žarnoje. Histomorfometrinė analizė parodė, kad dvylikapirštės žarnos gaurelių aukštis išliko stabilus, o klubinėje žarnoje gaurelių aukštis didėjo didėjant biomasės kiekiui pašaruose. Biomasės papildymas pagilino dvylikapirštės žarnos kriptos gylį, rodantį poveikį ląstelių apykaitai ir maistinių medžiagų pasisavinamumui, bet patrumpino klubinės žarnos kriptos gylį, rodantį brandesnę gleivinės sandarą.

4. Utilizuojant *C. glomerata* biomasę triušių pašarų gamyboje tiek 4 proc., tiek 8 proc. įterpimu reikšmingai pagerinama triušienos kokybė ir skatinamas tvaresnių strategijų įgyvendinimas triušininkystėje. Į pašarus įtraukus 4 proc. biomasės, nustatytas reikšmingas raumenų baltymų ir nepakeičiamųjų AR, tokių kaip treoninas, valinas, metioninas, lizinas, izoleucinas ir triptofanas, padidėjimas, o hidroksiprolino sumažėjimas. Tai tiesiogiai didina raumenų baltymų biologinę vertę ir gali lemti dar geresnį triušienos virškinamumą. Didesnis biomasės įterpimas pašaruose sumažino riebalų kaupimąsi bei lipidų oksidacijos laipsnį tiek šviežiuose, tiek sandėliuotose triušių raumenyse. Skirtingi *C. glomerata* įterpimai pašaruose reikšmingai sumažino SRR ir MNRR, bet padidino PNRR – taip pagerino lipidų kokybę. Biomasės panaudojimas triušių mityboje padidino širdies ligų profilaktikos savybės, nes triušienoje nustatytos didesnės PNRR/SRR ir h/H vertės, bet mažesni TI ir AI.

5. Tradicinius pašarų ingredientus iš dalies pakeitus gėlavandene *C. glomerata* makrodumblių biomase yra kuriamas tvaresnis ir vartotojui palankesnis požiūris į triušienos gamybos grandinę. Į pašarus įtraukus 4 ir 8 proc. biomasės, drėgmės kiekis triušienoje padidėjo, o 8 proc. įterpimas padidino ir HL raumenų virimo nuostolius. Tačiau biomasės įterpimas gali padidinti patrauklumą potencialiems vartotojams: reikšmingai sumažintas šviežios ir virtos triušienos paraudimas, pasiektas pašviesėjimas. Papildant pašarus 8 proc. biomasės prailginamos LD raumenų skaidulos, tai gali lemti didesnį triušienos švelnumą. Neatsižvelgiant į tam tikrus skirtumus tarp mitybos grupių, juslinių savybių vertinimas rodo, kad *C. glomerata* šertų triušių mėsa yra priimtina vertintojams. Pažymėtina, kad vertintojai, ragaudami triušių, šertų 8 proc. *C. glomerata* papildytais pašarais, HL raumenis, neatsižvelgdami į kvapo ir išvaizdos skirtumus, išreiškė padidėjusį laimės emocinį atsaką.

REKOMENDACIJOS

1. Gėlavandenę *C. glomerata* makrodumblių biomasę, klestinčią Lietuvos upėse, rekomenduotina naudoti tik smulkiuose ūkiuose, papildant triušių nuo 52–122 amžiaus dienos pašarus iki 8 proc., dėl šiuo metu neoptimizuoto biomasės kultivavimo našumo, kuris būtų reikalingas komercinei didelio kiekio pašaro gamybai.
2. Kaskart surinkta gėlavandenė *C. glomerata* makrodumblių biomasė turi būti nuosekliai analizuojama – patikrinamas potencialus jos toksiškumas, kuriam įtakos gali turėti biomasės buveinės veiksniai.

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LIST OF PUBLICATIONS

The dissertation is based on the publications listed below.

1. **Nutautaitė, Monika**; Vilienė, Vilma; Racevičiūtė Stupelienė, Asta; Bliznikas, Saulius; Karosienė, Jūratė; Koreivienė, Judita. Freshwater *Cladophora glomerata* Biomass as Promising Protein and Other Essential Nutrients Source for High Quality and More Sustainable Feed Production // Agriculture. Basel: MDPI, 2021, vol. 11, no. 7, p.1-16, ISSN 2077-0472, 2077-0472. doi:10.3390/agriculture11070582. Link: https://hdl.handle.net/20.500.12512/111465, https://doi.org/10.3390/agriculture11070582. Science Citation Index Expanded; CAB Abstracts; VINITI; Scopus; Index Copernicus. [S1] [Field of science: A003] [Citav. rodiklis: 3.408, bendr. cit. rod.: 3.477, kvartilis: Q1 (20221. InCites JCR SCIE)]

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2. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Bliznikas, Saulius; Jonuškienė, Ilona; Karosienė, Jūratė; Koreivienė, Judita; Vilienė, Vilma. Evaluation of phenolic compounds and pigments in freshwater *Cladophora glomerata* biomass from various Lithuanian rivers as a potential future raw material for biotechnology // Water. Basel: MDPI, 2022, vol. 14, no. 7, p. 1-18, ISSN 2073-4441. Link: https://hdl.handle.net/20.500.12512/113653, https://doi.org/10.3390/w14071138. Science Citation Index Expanded. [S1a] [Field of science: A003, T005, N012] [Citav. rodiklis: 3.4, bendr. cit. rod.: 5.45, kvartilis: Q2 (2022. InCites JCR SCIE)]

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3. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Bliznikas, Saulius; Pockevičius, Alius; Vilienė, Vilma. River-sourced *Cladophora glomerata* macroalgal biomass as a more sustainable and functional feed raw material for growing rabbits // Italian Journal of Animal Science, 2024, t. 23, nr. 1, p. 607–617, ISSN 1828-051X, 1594-4077. Link: https://www.tandfonline.com/doi/full/10.1080/1828051X.2024.2342380, https://hdl.handle.net/20.500.12512/243045, https://doi.org/10.1080/1828051X.2024.2342380. Science Citation Index Expanded (Web of Science); Scopus. [S1] [Field of science: A003] [Citav. rodiklis: 2.5, bendr. cit. rod.: 2.4, kvartilis: Q1 (2022. InCites JCR SCIE)]

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4. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Bliznikas, Saulius; Vilienė, Vilma. Enhancement of Rabbit Meat Functionality by Replacing Traditional Feed Raw Materials with Alternative and More Sustainable Freshwater *Cladophora glomerata* Macroalgal Biomass in Their Diets // Foods. Basel: MDPI, 2023, vol. 12, no. 4, p. 1-23, ISSN 2304-8158,

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5. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Pockevičius, Alius; Vilienė, Vilma. Sensory Evaluation of Rabbit Meat from Individuals Fed Functional and More Sustainable Diets Enriched with Freshwater *Cladophora glomerata* Macroalgal Biomass // Animals, 2023, t. 13, nr. 13, p. 1-18, ISSN 2076-2615. Link: https://hdl.handle.net/20.500.12512/237881, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10339916/, https://doi.org/10.3390/ani13132179. Science Citation Index Expanded (Web of Science); Scopus; PubMed; Embase; PubMed Central. [S1] [Field of science: A003] [Citav. rodiklis: 3, bendr. cit. rod.: 2.4, kvartilis: Q1 (2022. InCites JCR SCIE)]

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1. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Vilienė, Vilma. Održivost i poboljšanje kvalitete hranidbe kunica korištenjem slatkovodne biomase makroalgi *Cladophora glomerata* // Zbornik Sažetaka 29. Međunarodnog Savjetovanja KRMIVA 2024 : Opatija, Hrvatska, 5.-7. lipnja 2024. = Book of Abstracts of the 29th International Conference KRMIVA 2024 : Croatia, June 5–7, 2024 / Glavni urednici: Zvonko Antunović, Zlatko Janječić, nr. 170, p. 51– 52, ISSN 1847-2370. Link: https://hdl.handle.net/20.500.12512/245669. [T1e] [Field of science: A003]
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1. **Nutautaitė, Monika**; Racevičiūtė Stupelienė, Asta; Jonuškienė, Ilona; Koreivienė, Judita; Karosienė, Jūratė; Vilienė, Vilma. Gėlavandenės makrodumblių cladophora glomerata biomasės, surinktos iš Lietuvoje esančių atsinaujinančių šaltinių, antioksidacinis aktyvumas // 11-osios jaunųjų mokslininkų konferencijos Jaunieji mokslininkai – žemės ūkio pažangai: pranešimų tezės: [2022 m. lapkričio 10 d.] / Lietuvos mokslų akademijos Žemės ūkio ir miškų mokslų skyrius; [Leidinį sudarė Reda Daukšienė]. Vilnius: Lietuvos mokslų akademija, 2022. ISBN 9789986080909, p. 12-12, ISBN 9789986080909. Link: https://hdl.handle.net/20.500.12512/116056. [T2] [Field of science: A003, N010, N003].

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Freshwater *Cladophora glomerata* Biomass as Promising Protein and Other Essential Nutrients Source for High Quality and More Sustainable Feed Production

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Abstract: A scientific justification, focused on the development of the sustainability of feed ingredients and farm animals' ecosystems, is required. Thus, it is crucial to search for alternative feed materials from natural sources for potential applications. The aim of this study was to evaluate the prospective utilization of freshwater *Cladophora glomerata* (*C. glomerata*) as an alternative source of protein and other essential nutrients in animals' feed. For this purpose, chemical analysis was performed on collected biomass samples from the Lithuanian rivers, Dubysa (B1), Šventoji (B2), Nevėžis (B3), and Jūra (B4). Microelements (Ca > K > N > P > Mg), trace elements (Zn > Cu), and heavy metals (Cr > Ni > Pb > Cd) have not exceeded permissible levels. The crude protein content of *C. glomerata* biomass ranged from 16% to 21.5% DM. The essential amino acid profile excelled, with the highest total amino acid amount of 140.99 g/kg in B4. The highest total presence of polyunsaturated fatty acids (11.71%) as well as the ratio of polyunsaturated to saturated fatty acids (0.22) was observed in B1. The lowest ratio of omega-6/omega-3 was in B1 (1.30). As a result of bioaccumulation, *C. glomerata* could serve as a source of proteins, as well as amino and fatty acids, implying that biomass could be an alternative and a beneficial component of animal feed.

Keywords: macroalgal biomass; *Cladophora glomerata*; amino acids; fatty acids; feed alternatives; sustainability

1. Introduction

The increasing population is directly correlated with the increasing consumption of animal products [1]. Therefore, a long-term strategy for the intensive, but sustainable, development of livestock farming is essential. Clearly, even according to optimistic predictions, a shortage of traditional protein feed components is inevitable. Intensified development of sustainable livestock farming, while applying the use of innovative or alternative feed additives, could contribute to the sustainability of any animals' ecosystem. For example, materials from aquatic ecosystems such as algal biomass as protein and other essential nutrients source could be applied in feed production [2–4]. This would help to alleviate the scarcity of fodder feed materials, and the incorporation of algal biomass into feed production would help to address another global issue: greenhouse gas emissions from livestock activities [5]. Since the cultivation of algae requires small areas and their reproduction is rapid, or they are simply found naturally in nature, the cultivation of fodder crops could be optimized.

It is necessary to search for and analyse the potential applications of alternative feed materials by ensuring their high quality, safety, and sustainability, taking into account anti-nutritional factors and potential toxicity. Algal biomass is characterized as a source of essential vitamins, minerals, proteins, polyunsaturated fatty acids, and antioxidants [6–8]. The high potential of algae derives from the fact that they are not as well-known as agricultural crops, that they can be grown in places where other plants cannot, and the productivity of algae exceeds higher plants many times. Since algae use solar resources more effectively, their ability to produce useful compounds and biomass is generally acknowledged, and they can be utilized to improve the nutritional value of food and feed [9]. Macroalgae, like microalgae, blend higher plant features with biotechnological properties unique to microbial cells. Namely, they can reproduce quickly in a liquid medium with ordinary nutritional requirements and still accumulate certain metabolites. *Cladophora* species, whether they are marine or freshwater, are ecologically and economically important macroalgae. These species provide essential ecosystem services, and their biomass is used for a variety of applications, including soil additives, fertilizers, plant growth biostimulants, food and animal feed, phycocolloids, nutraceuticals, pharmaceuticals, cosmetics, wastewater treatment, and renewable biofuel production [10,11]. *Cladophora* species are recommended as an important substitute for human food and animal feeding due to their high protein content [12]. Thus, in the food and feed industry, the species is used as a biomass with a low-calorie content and a high variety of nutrients, vitamins, and fibre [13].

The development of more sustainable feed materials and food supply chains and the sustainability of the farm animals' ecosystem require a scientific justification for the integration of alternative protein materials into new or existing animal feed. The specific *Cladophora glomerata* (*C. glomerata*) has a rich spectrum of bioactive components, which is reflected in its chemical composition. Therefore, such biomass would be adequate for use as a feed additive in the prism of today's issues. Filamentous green algae *C. glomerata* thrives and forms large communities in nutrient-rich water bodies, especially in slow-flowing rivers [14]. However, blooming caused by *C. glomerata* decreases biodiversity since the assemblage is mostly composed of only one green algae species, reducing the recreational value of water bodies, and creating a detrimental ecological and economic impact. Therefore, by harvesting excess macroalgal biomass from water bodies and integrating it into feed production, the waste would be adapted as a raw material, creating a sustainable production chain. Thus, the aim of this study was to evaluate the potential of freshwater *C. glomerata* biomass, from four different Lithuanian rivers, to be utilized as an alternative source of protein and other essential nutrients in animals' feed by conducting detailed biomass chemical analysis and defining profiles of amino and fatty acids.

2. Materials and Methods

2.1. C. glomerata Biomass Collection and Preparation

Freshwater *C. glomerata* biomass was collected in August–September 2019 from four Lithuanian rivers: Dubysa (N55°12′25.07″, E23°30′30.44″; B1), Šventoji (N55°39′20.14″, E25°10′18.39″; B2), Nevėžis (N55°5′46.52″, E23°46′55.57″; B3) and Jūra (N55°27′19.58″, E22°2′14.72″; B4) (Figure 1). The rivers were selected due to the presence of dense agglomerations of *C. glomerata*, usually covering over 50% of the river bottom area (Figure 1; Table 1). However, rivers differ by the catchment area and the water chemistry. Nutrient concentrations in the rivers were high: total nitrogen (TN) varied from 0.18 to 1.12 mg/L and total phosphorus (TP) from 0.012 to 0.073 mg/L (Table 1). The highest concentrations of nutrients and conductivity were determined in River Nevėžis, where *C. glomerata* was dominant among primary producers occupying a large area and building up a high quantity of excessive biomass. River Dubysa was distinguished by the lowest nutrient amount.

Figure 1. (**A**) The locations of *C. glomerata* biomass collection in the rivers of Lithuania; (**B**) Agglomerations of macroalgal *C. glomerata* biomass in River Nevėžis; (**C**) Collection of *C. glomerata* biomass; (**D**) Dried biomass of macroalgae.

Table 1. Characteristics of the rivers and the abundance of *Cladophora* macroalgae at the sampling sites.

River	* River length, km	* Catchment Area, km^2	* Average Annual Discharge at the Mouth, m^3/s	Current Velocity, m/s	TP, mg/L	TN, mg/L	Water Temperature, °C	pH	Conductivity, µS/cm	Bottom Coverage by *C. glomerata*, %
Dubysa (B1)	131	1972	14.2	0.11–0.57	0.012–0.035	0.18–0.40	16.4–20.1	8.41–8.80	535–581	40–90
Šventoji (B2)	246	6889	56.5	0.26–0.32	0.037–0.070	0.53–0.99	17.6–18.9	8.40–9.19	479–487	45–95
Jūra (B3)	172	3994	38	0.11–0.53	0.031–0.073	0.28–0.64	14.9–18.0	8.56–9.21	420–483	50–95
Nevėžis (B4)	209	6146	32.7	0.10–0.13	0.500–0.520	0.84–1.12	18.2–19.2	8.54–8.62	948–977	50–100

* Kilkus and Stonevičius [15].

Fresh macroalgal biomass was manually harvested from the river (Figure 1). Subsamples of about 1 kg of wet biomass were collected from up to 6 sites and intermixed altogether. Macroalgal biomass was several times carefully washed with tap water to remove sand and mud particles. Macrozoobenthos, macrophytes and other debris were

manually removed from the biomass. The samples were dried in an oven at 60 °C overnight and stored in closed plastic bags at room temperature until the analysis.

2.2. C. glomerata Biomass Chemical Analysis

Macroalgal biomass samples were analysed in accordance with the Commission regulation (EU) No 691/2013 of 19 July 2013 amending Regulation (EC) No 152/2009 as regards methods of sampling and analysis. Chemical analysis to determine individual elements in the dry biomass was performed in the accredited research laboratory following methods specified in Table 2.

Table 2. Methods by which each element was determined in *C. glomerata* biomass.

Element	Method	Element	Method
N	The Commission Directive 72/199/EEC	Zn Cu	LST EN ISO 15510:2017
P	Directive 71/393/EEC	Cr	
K Ca	Directive 71/250/EEC	Ni Cd	LST EN ISO 15550:2017
Mg	Directive 73/46/EEC	Pb	

C. glomerata samples from different Lithuanian rivers with each river's three replicates were grinded for further chemical analysis. The dry matter of algal biomass was determined by drying it in an oven at 105 °C until a constant weight and dry matter yield was calculated. Dried *C. glomerata* biomass samples were used for further laboratory analyses described below. Crude protein content was determined by the Kjeldahl method. A conversion factor of 6.25 was used to convert total nitrogen to crude protein [16]. Crude fat was extracted with petroleum ether (boiling range of 40–60 °C) by the Soxhlet extraction method. Crude ash was determined by incineration in a muffle furnace at 550 °C for 3 h (Commission Regulation (EC) No. 152/2009). Crude fibre was determined as the residue after sequential treatment with hot (100 °C) H_2SO_4 (conc. 1.25%) and hot (100 °C) NaOH (1.25%) according to the Weende method by the FiberCap system (Foss Tecator AB, Höganäs, Sweden).

2.3. C. glomerata Biomass Amino Acids Profile Analysis

The hydrolysis of algal biomass samples for amino acid analysis followed the procedures outlined in Commission Regulation (EC) No. 152/2009. The amino acid assay was performed by the AccQ Tag technology (Waters Corp., Milford, MA, USA). For amino acid analyses in samples, the Shimadzu low pressure gradient HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of solvent delivery module LC-10AT_{VP}, auto-injector SIL-10AD_{VP}, column oven CTO-10AC_{VP}, spectrofluorometric detector RF-10A_{XL}, system controller SCL-10A_{VP}, online degasser DGU-14A was used. For HPLC system control and data collection, the Workstation LC Solution (Shimadzu Corp., Kyoto, Japan) was used. Amino acid derivatives were separated on a Nova-Pak C18, 4 mm, 150 × 3.9 mm chromatography column (Waters Corp., Milford, MA, USA) at a temperature of 37 °C. For separation, 10 µL of each derivate was injected into the column. Separated derivatives were detected at the Ex 250 nm–Em 395 nm wavelength. A gradient flow was used for the separation of amino acid derivatives. The flow rate was set at 1 mL/min. The mobile phase consisted of eluent A (prepared from Waters AccQ Tag Eluent A concentrate by diluting 100 mL of concentrate to 1 L of ultrapure water), eluent B (acetonitrile) and eluent C (ultrapure water). Amino acids were identified by the retention times as compared to the retention times of the amino acids in the standard solution. The results were calculated by measuring the peak areas of the sample and the standard solution for each amino acid.

2.4. C. glomerata Biomass Fatty Acids Profile Analysis

Lipid extraction from biomass samples for further fatty acids analysis was performed with a chloroform-methanol (2:1) mixture as described by Folch et al. [17]. Exactly 0.5 g of algal biomass was taken, and 10 mL of extraction mixture was added. The extraction was carried out by leaving the mixture overnight at room temperature. The samples were filtered, then 20–40 mL of 0.74% KCl solution was added, shaken for 1–2 min, and left for 10–12 h to completely separate the layers. The lower layer was transferred via syringe to 20 mL tubes and evaporated at 50 °C on a vacuum thermostat. The resulting fat was methylated with freshly prepared 2% sodium methylate (NaOMe) solution according to Christopherson and Glass [18]. Then, 5 mL of NaOMe was added to the tube with fat, shaken and left for 1 h at room temperature. Subsequently, 7 mL of 1N HCl, 5 mL of hexane and 2 mL of H_2O were added. The tubes were stoppered and shaken for 1 min for better layer separation. Exactly 2 mL of the top layer was transferred to conical tubes and evaporated. The resulting mixtures of fatty acid methyl esters were analysed with a GC-2010 (Shimadzu Corp., Kyoto, Japan) gas chromatograph with FID detector (Shimadzu Corp., Kyoto, Japan). The Capillary column ATTM-FAME (30 m, ID: 0.25 mm) (Alltech Associates Inc., Deerfield, IL., USA) was used. The column temperature change was programmed from 150 °C to 240 °C. Inlet temperature—240 °C, detector temperature—240 °C. Carrier gas—nitrogen, flow rate—63.0 mL/min. Analysis time—60 min. Individual fatty acids were identified by retention times when compared to the retention times of fatty acids in a mixture of known composition. The fatty acid content (% of the total acid content) was determined using a chromatographic data processing program, GCsolution (Ver. 2.32; Shimadzu Corp., Kyoto, Japan).

The average amount of each fatty acid was used to calculate the total content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, PUFA/SFA, and omega-6/omega-3 (n6/n3) ratios.

2.5. Statistical Analysis

The research was a completely randomized design with 4 algal biomass samples with 3 replicates. Data analysis was performed by SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). One-way analysis of variance (ANOVA) test post-hoc (Fisher's least significant difference test) was conducted to detect differences among *C. glomerata* biomass from different rivers in Lithuania. A calculated p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Chemical Composition of C. glomerata Biomass

In our study, individual elements were identified to assess the overall chemical composition of freshwater *C. glomerata* biomass (Figures 2 and 3). The amount of macro elements varied from 1.46 to 4.15 mg/kg DM of nitrogen (N) and 0.16–0.49 mg/kg DM of phosphorus (P) in the *C. glomerata* biomass, with the highest values observed in B3 (Figure 2). Potassium (K) varied in the range of 3.25–6.00 mg/kg DM and the highest concentration was found in B2 biomass. Particularly high levels of up to 26.34 and 27.16 mg/kg DM of calcium (Ca) were found in biomasses B1 and B4 respectively, whereas the levels of magnesium macro-mineral (Mg) were very low (0.26–0.42 mg/kg DM) in all algal tested biomasses. For the other trace minerals tested, the highest concentration of zinc (Zn) was found in B3, while the highest concentration of copper (Cu) was found in B1 *C. glomerata* biomass (Figure 3).

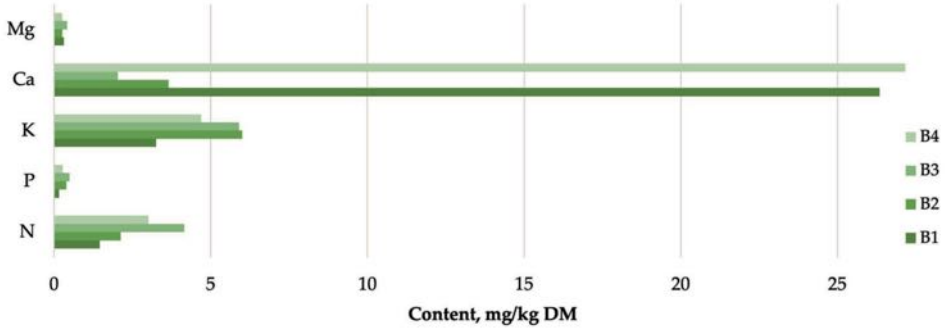

Figure 2. Separate macro elements content in *C. glomerata* biomass from different rivers in Lithuania (Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)).

To determine potential toxicity and to define if macroalgal biomass is safe to use for animals' feed, the most important heavy metals and their contents were determined (Figure 3). The highest amount in terms of biomass was chromium (Cr) followed by nickel (Ni), whereas percentages of lead (Pb) and cadmium (Cd) were more than ten times lower.

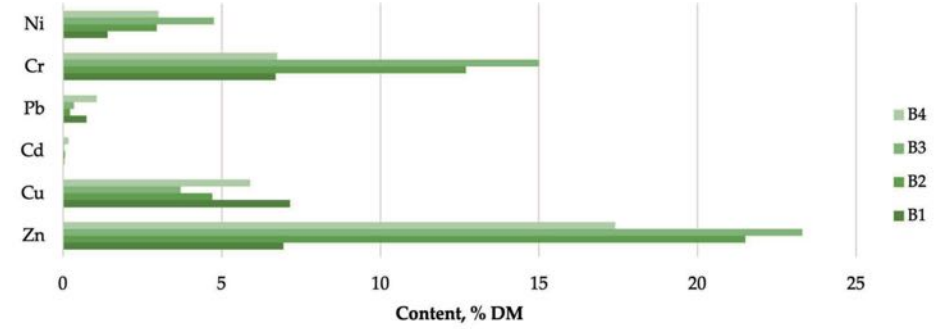

Figure 3. Separate trace elements and heavy metals content in *C. glomerata* biomass from different rivers in Lithuania (Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)).

The chemical composition of *C. glomerata* biomass from different rivers in Lithuania is presented in Table 3. Foremost, the biomass samples that had already been dried after their collection from the rivers, were dried again to determine the dry matter (DM) content again, due to the moisture that may have formed during storage. The highest crude protein (CP) content was determined in B4 *C. glomerata* biomass ($p < 0.05$). Compared to B1, B2, and B3 samples, B4 had 14.87%, 25.74%, and 15.52%, respectively, higher CP content ($p < 0.05$). The highest content of crude fat (CF) was observed in B1 samples, significantly higher than in B2 and B3 ($p < 0.05$). After crude ash (CA) determination, this item statistically differed between all biomass samples ($p < 0.05$). However, the highest amount of CA was observed in B3 biomass and the lowest in B4 ($p < 0.05$). Crude fibre (CFB) content between all biomass samples statistically differed likewise, while the biggest difference was observed between B2 and B3, when B2 CFB content was 15.83% DM and B3 only 10.88% DM ($p < 0.05$).

Table 3. Chemical composition of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [2,3,4]					
Item [1]	**B1**	**B2**	**B3**	**B4**	**SEM** [5]	***p* Value**
DM (% of dried samples)	94.95 [a]	92.63 [a]	95.19 [a]	91.12 [b]	0.35	0.000
			% DM			
CP	18.32 [a]	15.98 [a]	18.18 [a]	21.52 [b]	0.34	0.000
CF	0.35 [a]	0.18 [b]	0.19 [b]	0.31 [ab]	0.05	0.026
CA	48.45 [a]	39.05 [b]	49.83 [c]	36.96 [d]	0.23	0.000
CFB	13.97 [a]	15.83 [b]	10.88 [c]	13.14 [d]	0.19	0.000

Note: [1] DM, dry matter; CP, crude protein; CF, crude fat; CA, crude ash; CFB, crude fibre. [2] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [3] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [4] $n = 12$ (3 replicate samples from each river). [5] SEM, standard error of the means.

3.2. Amino Acids Profile of C. glomerata Biomass

In order to get a comprehensive profile of the amino acids in *C. glomerata*, the 16 most important and essential amino acids were identified (Table 4). In general, all data obtained after amino acids profiling of the collected macroalgal biomass significantly differed ($p < 0.05$). Except for one item, histidine content, where no significant differences between groups were found ($p > 0.05$). The total amount of amino acids was the highest in B4 (~141 g/kg) and was lower by 1/3 in B1. Glutamic acid, followed by aspartic acid and leucine, were the most prevalent amino acids in the *C. glomerata* profile from all rivers. In almost all cases, the highest concentration of a single amino acid was found in B4 biomass from the river Jūra (B4; $p < 0.05$). In terms of essential amino acids, the highest concentration of threonine was found in B4 biomass, half as large compared to B1 ($p < 0.05$). Valine content in B4 was higher than in B1, B2, and B3 biomass samples ($p < 0.05$). However, the highest concentration of the essential amino acid methionine was found in biomass B2 and compared to B4, this item was almost two times higher ($p < 0.05$). The highest concentration of the remaining essential amino acids, isoleucine, leucine, phenylalanine, and lysine, were also found in B4 *C. glomerata* biomass ($p < 0.05$), maintaining the trend. Regardless, amino acids which can be synthesized in the animal's body and are considered as "non-essential": the highest concentrations of aspartic acid, serine, glutamic acid, proline, glycine, alanine, tyrosine, and arginine, when comparing biomasses from the rivers, were determined in the B4 biomass samples again ($p < 0.05$). Overall, most of the amino acids were found in B4 biomass. Compared to the B1, B2, and B3 biomass profiles, the total amount of amino acids in B4 biomass was 37.63 g/kg, 22.74 g/kg, and 16.35 g/kg, respectively, higher ($p < 0.05$).

3.3. Fatty Acids Profile of C. glomerata Biomass

The fatty acids profile of *C. glomerata* biomass determined in our research is presented in Table 5. Palmitic (C16:0), elaidic (C18:1 n9), and myristic (C14:0) acids were dominant in the *Cladophora* biomass profile among the 33 fatty acids determined. Fatty acids with all or predominantly single bonds were calculated. In terms of total saturated fatty acids (SFA) in different algal biomass was above 50% of the total fatty acids content ($p < 0.05$). To be more precisely, the highest total SFA content was calculated in B2 biomass, lower, but slightly similar in B3 and B4, and the lowest amount in B1 *C. glomerata* samples ($p < 0.05$). After calculating the total monounsaturated fatty acids (MUFA) content, some significant differences between the analysed biomasses were obtained. The highest total MUFA content was found in B2 algal biomass. Compared to B1, B3 and B4, MUFA content was respectively higher by 0.46%, 1.05%, and 0.36% in B2 biomass ($p < 0.05$). Another calculated group of fatty acids is polyunsaturated fatty acids (PUFA). Most of these fatty acids were calculated from the B1 *C. glomerata* biomass. Comparing the total amount of PUFA among other algal biomasses, the lowest content was found in B2, slightly more, but very similarly between both in B3 and B4 biomasses ($p < 0.05$). An important lipid index PUFA/SFA

ratio was calculated. *C. glomerata* biomasses collected from different Lithuanian rivers and analysed during our study showed that the PUFA/SFA ratio significantly differs between all biomasses, except between B3 and B4 (the same ratio was obtained ($p > 0.05$)). After calculating the PUFA/SFA ratio in B1 and B2 algal biomasses, this indicator was found to be two times higher in B1 samples than in B2 ($p < 0.05$).

Table 4. Amino acids profile of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [1,2,3]					
Item (g/kg DM)	**B1**	**B2**	**B3**	**B4**	**SEM** [4]	***p* Value**
Aspartic acid	10.90 [a]	13.63 [b]	14.18 [c]	15.97 [c]	0.63	0.001
Threonine	3.49 [a]	6.52 [b]	6.28 [b]	6.77 [b]	0.42	0.001
Serine	4.14 [a]	6.21 [b]	6.29 [c]	7.25 [c]	0.34	0.001
Glutamic acid	14.16 [a]	16.90 [b]	17.53 [c]	19.50 [c]	0.48	0.000
Proline	5.98 [a]	5.92 [a]	6.40 [a]	7.22 [b]	0.25	0.006
Glycine	8.06 [a]	9.23 [b]	9.54 [c]	11.46 [c]	0.32	0.000
Alanine	8.49 [a]	8.12 [a]	10.09 [b]	10.73 [b]	0.24	0.000
Valine	8.39 [a]	8.48 [a]	8.90 [a]	10.42 [b]	0.39	0.006
Methionine	1.94 [b]	4.14 [a]	2.24 [b]	2.08 [b]	0.21	0.000
Isoleucine	5.94 [a]	6.17 [a]	6.80 [a]	7.69 [b]	0.30	0.004
Leucine	9.71 [a]	9.64 [b]	10.81 [c]	12.01 [d]	0.31	0.002
Tyrosine	1.73 [a]	1.71 [a]	2.09 [b]	2.35 [b]	0.14	0.009
Phenylalanine	6.37 [a]	6.93 [a]	7.39 [a]	8.50 [b]	0.35	0.004
Histidine	3.04	3.01	3.33	3.68	0.28	0.077
Lysine	5.76 [a]	5.85 [a]	6.46 [a]	7.88 [b]	0.26	0.001
Arginine	5.25 [a]	5.79 [a]	6.31 [a]	7.47 [b]	0.20	0.000
Total	103.36 [a]	118.25 [b]	124.64 [c]	140.99 [d]	3.88	0.001

Note: [1] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [2] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [3] $n = 12$ (3 replicate samples from each river). [4] SEM, standard error of the means.

Individual omega-3 (n3) fatty acids were identified and evaluated. The highest content of α-linolenic acid (C18:3 n3) was found in B1 algal biomass (4.29%), 2 times less in B2 (1.91%) ($p < 0.05$). In the remaining *C. glomerata* biomasses B3 and B4, this amount was observed to be very similar (2.90% and 2.96%, respectively). However, it was lower compared to B1 ($p < 0.05$), but higher compared to B2 ($p < 0.05$). Another n3 essential fatty acid identified in our study is eicosapentaenoic acid (C20:5 n3). The highest content of the mentioned fatty acid (C20:5 n3) was found in B4 macroalgal biomass. The content of the remaining biomasses (B1; B2; B3) was lower and differed statically from B4 ($p < 0.05$). However, the last identified n3 fatty acid, docosapentaenoic acid (C22: 5 n3), was observed only in B1 biomass. The maximum total amount of n3 fatty acids was calculated in B1 *C. glomerata* biomass. Compared to B1 and others, the total amount of n3 was 2.40%, 1.50%, and 0.99% lower in B2, B3, and B4, respectively ($p < 0.05$). The following omega-6 (n6) fatty acids were identified in our study: linoleic acid (C18:2 n6), arachidonic acid (C20:4 n6), docosadienoic acid (C22:2 n6). Linoleic acid content varied significantly across all algal biomasses ($p < 0.05$). However, the largest significant difference was found between B1 and B2 biomasses, when almost two times higher concentration of C18:2 n6 was observed in B1 compared to B2 ($p < 0.05$). Arachidonic acid (C20:4 n6) was defined in all biomass samples, except B2. However, other analysed biomasses revealed statistically significant differences, where most of C20:4 n6 was identified in B4 algal biomass, less in B3, and the least in B1 ($p < 0.05$). No statistically significant data were obtained after identifying docosadienoic acid (C22:2 n6), as in B2 and B4 samples, such an acid was not identified at all, and the content found in the remaining biomasses (B1; B3) was identical ($p > 0.05$). The total amount of n6 was calculated, and significant differences were obtained between all analysed biomasses. The largest difference was found between B1 and B2, where the total n6 content in B1 was 6.62% and only 3.80% in B2 ($p < 0.05$); lower amounts were found in

B3 and B4, which were 6.00% and 5.51%, respectively ($p < 0.05$). Ultimately, the n6/n3 ratio was calculated: in B3 biomass, this indicator was slightly higher compared to B1, B2 and B4 ($p < 0.05$), but comparing B1 and B4 this ratio was nearly the same ($p > 0.05$).

Table 5. Fatty acids profile of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [2,3,4,5]					
Item [1] **(% of the Total Fatty Acids Content)**	**B1**	**B2**	**B3**	**B4**	**SEM** [6]	***p* Value**
C10:0	n/d	0.37 [a]	n/d	0.24 [b]	0.02	0.000
X	0.75 [a]	0.56 [b]	0.88 [c]	0.69 [a]	0.02	0.000
C12:0	0.25 [a]	0.40 [b]	0.80 [c]	0.30 [d]	0.01	0.000
X	0.64 [ab]	0.50 [a]	n/d	0.61 [b]	0.03	0.000
X	5.03 [a]	3.59 [b]	5.58 [a]	5.03 [c]	0.04	0.000
X	0.19 [b]	0.35 [a]	0.22 [b]	n/d	0.02	0.002
C14:0	8.86 [a]	12.48 [b]	9.70 [c]	11.21 [d]	0.06	0.000
X	0.80 [a]	0.76 [a]	0.84 [a]	0.62 [b]	0.02	0.001
C15:0	0.99 [a]	0.18 [b]	0.30 [c]	0.82 [d]	0.04	0.000
iC15:0	5.95 [a]	4.83 [b]	7.65 [c]	5.60 [d]	0.07	0.000
C16:0	33.54 [a]	37.11 [b]	32.65 [c]	32.89 [d]	0.08	0.000
iC16:0	0.80 [b]	0.50 [a]	0.76 [b]	0.40 [c]	0.02	0.000
trans-C16:1 n7	1.13 [b]	0.81 [a]	1.18 [b]	1.03 [b]	0.05	0.002
C16:1 n9	1.71 [a]	1.00 [b]	1.88 [c]	1.17 [d]	0.06	0.000
C16:1 n7	5.31 [a]	6.89 [b]	6.63 [c]	7.88 [d]	0.03	0.000
C17:0	n/d	0.49 [a]	0.50 [a]	0.29 [b]	0.03	0.000
iC17:0	0.49 [a]	0.18 [b]	0.58 [a]	0.23 [c]	0.03	0.000
C18:0	2.07 [a]	2.52 [b]	2.39 [c]	3.25 [d]	0.04	0.000
C18:1 n9	12.72 [a]	14.53 [b]	11.16 [c]	12.33 [d]	0.05	0.000
C18:1 n7	6.06 [a]	5.16 [b]	6.12 [a]	5.16 [c]	0.05	0.000
trans-C18:2 n6	1.43 [a]	n/d	1.11 [b]	n/d	0.03	0.000
cis-trans-C18:2 n6	n/d	0.74 [a]	n/d	1.35 [b]	0.02	0.000
trans-cis-C18:2 n6	0.57 [b]	0.31 [a]	0.56 [b]	0.45 [c]	0.02	0.000
C18:2 n6	4.15 [a]	2.75 [b]	3.83 [c]	3.33 [d]	0.04	0.000
C18:3 n3	4.29 [a]	1.91 [b]	2.90 [c]	2.96 [c]	0.05	0.000
C20:1 n9	0.59 [a]	n/d	0.37 [b]	n/d	0.01	0.000
C20:4 n6	0.27 [a]	n/d	0.30 [a]	0.38 [b]	0.01	0.000
C20:5 n3	0.58 [a]	0.77 [a]	0.68 [a]	1.13 [b]	0.09	0.003
C22:1 n9	0.22	n/d	n/d	0.24	0.02	0.264
C22:2 n6	0.20	n/d	0.20	n/d	0.01	0.932
C22:5 n3	0.22	n/d	n/d	n/d	n/d	n/d
C24:0	n/d	0.30	0.24	0.21	0.04	0.067
C24:1	0.20	n/d	n/d	0.21	0.01	0.393
SFA	52.94 [a]	59.37 [b]	55.57 [c]	55.43 [c]	0.11	0.000
MUFA	27.93 [a]	28.39 [b]	27.34 [c]	28.03 [a]	0.10	0.000
PUFA	11.71 [a]	6.48 [b]	9.58 [c]	9.59 [c]	0.11	0.000
PUFA/SFA	0.22 [a]	0.11 [b]	0.17 [c]	0.17 [c]	0.00	0.000
Total n3	5.08 [a]	2.68 [b]	3.58 [c]	4.09 [d]	0.07	0.000
Total n6	6.62 [a]	3.80 [b]	6.00 [c]	5.51 [d]	0.04	0.000
n6/n3	1.30 [a]	1.42 [b]	1.68 [c]	1.35 [a]	0.02	0.000
Total X	7.42 [a]	5.76 [b]	7.51 [c]	6.95 [d]	0.02	0.000

Note: [1] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; X, unidentified fatty acids. [2] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [3] n/d, not defined. [4] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [5] $n = 12$ (3 replicate samples from each river). [6] SEM, standard error of the means.

During our study, "X" unidentified fatty acids were obtained. A total of five fatty acids were unidentified with a total of 7.42%, 5.76%, 7.51%, and 6.95% in B1, B2, B3, and B4, respectively.

4. Discussion

Algal biomass possesses biologically active ingredients which contain polysaccharides, proteins, polyphenols, pigments, mineral elements, and polyunsaturated fatty acids. Macroalgae are well-known for their high concentrations of biologically active compounds. Because of the need to rapidly adapt to changing environmental conditions, a variety of secondary metabolites, which are not found in other organisms, are produced [6]. The following compounds protect algal cells from stressful conditions such as UV radiation, sudden changes in temperature, or changes in nutrient concentrations [7]. Several studies have found a link between *C. glomerata* and high nutrient inputs [19]. Moreover, *Cladophora* species can survive in extremely saline environments (up to 100‰ of salinity) [20]. *Cladophora* macroalgae contains a high amount of carbohydrates, minerals, proteins and is featured by high moisture (around 90%) [21]. So, according to its nutritive value, theoretically, it could be used for animal nutrition. However, it should be taken into account that the composition of *Cladophora* macroalgal biomass is strongly dependent on environmental variables (weather conditions, season, method of algae cultivation, and collection). Because the content of compounds such as proteins, amino acids, lipids, and elements in *Cladophora* species is similar to that of plants used as feed materials, these algae could be used as a valuable feed additive [8].

4.1. Macro- and Micronutrients in Algal Biomass

Recent interdisciplinary studies of *C. glomerata* and *Ulva flexuosa* also suggest the suitability of freshwater macroscopic green algae as they are a rich source of macro- and micronutrients and other bioactive substances [12,22]. Mineral elements such as iodine, zinc, iron, copper, calcium, magnesium, sodium, and potassium are abundant in algal biomass. Compared to other feed materials, such as edible land plants, the content of these elements in algae can be found to be as much as 40% higher [23]. Therefore, given the potential mineral deficiency, traditional feed additives can be successfully replaced by algal culture additives [24,25]. Our results indicate that *C. glomerata* can also serve as a carrier of microelements for animals' diets. The macronutrient distribution in *C. glomerata* algal biomass from different Lithuanian rivers was as follows Ca > K > N > P > Mg and of the trace elements—Zn > Cu (Figure 2). This is in agreement with the results of Messsyasz et al. [12], where calcium content in collected freshwater *C. glomerata* biomass from Oporzyn lake in Poland was the highest as well. Moreover, Michalak et al. [26] demonstrated that *Cladophora* biomass enriched with microelement ions through biosorption can be used as a valuable feed additive for various animal breeds and can partially substitute for inorganic salts.

Green algae, especially *Cladophora* species, are widely regarded as the best bioindicators of nutrient and heavy metal toxicity in aquatic bodies. As a result, the concentrations of the most important heavy metals (Cr > Ni > Pb > Cd) were determined. The analysis revealed that the concentrations of the main heavy metals in *C. glomerata* did not exceed the recommended maximum levels. As a result, no potential toxicity could be observed for animals regarding heavy metal concentrations in algal biomass from different Lithuanian rivers, which was in line with the Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

Generally, macroalgae has a high ash content, as well as micro- and macro-elements, essential minerals, and trace elements that are required by all living organisms [6]. When comparing the amount of crude ash in traditional and alternative protein feed materials, fishmeal (25.95%) has the highest amount, followed by *Cladophora* meal (21.14%), rice bran (17.17%), soybean meal (8.26%), and cassava powder (1.97%) [27]. During our study, the crude ash content in *C. glomerata* from Lithuanian rivers varied from 36.96% to 49.83%, whereas the crude fibre content constituted about 13.46%. This result is similar to those of Messyasz et al. [12] and Michalak et al. [26], who found 39.25% of ash and 15.6–19.6% of crude fibre in *Cladophora* from lakes in Poland. Finally, algal biomass with a higher

fibre content could be adapted to rabbit diets because rabbits can achieve good growth performance on high-fibre diets due to their unique digestive physiology [28].

4.2. Proteins and Amino Acids in Algal Biomass

In addition to mineral elements, freshwater macroalgae can serve as a source of protein in animals' nutrition. In general, crude protein in algae dry matter can reach 44% but commonly does not exceed 5% [29]. Compared to other traditional protein sources used in feed production, the amount of crude protein found in soybean meal reaches from 40% to 49%. Considering the global challenges we are facing today with livestock and the predicted inevitable deficiency of traditional protein materials in animals' feed production in the future, freshwater macroalgae could be a solution as the protein content in *Cladophora* biomass ranges from 10% to 25% and it is comparable to other feed materials [8]. Similarly, crude protein in our analysed *C. glomerata* biomass from Lithuanian rivers ranged from 16% to 21.5%. However, *Cladophora* species are more commonly used as aquaculture feed additives than as livestock feed additives. It could be used as a fish meal replacement or as a protein source in aquaculture. For example, Anh et al. [27] indicated that replacement of fishmeal protein (60.08% crude protein in DM) with different levels of seaweed *Cladophora* meal from 10% to 50% in *Penaeus monodon* post larvae diets had significant effects on growth performance, feed efficiency and stress resistance. Thus, the use of *Cladophora* in animal feed production would contribute to the development of more sustainable livestock in the first place. Moreover, there are two advantages to using harvested wild freshwater *C. glomerata* biomass: (i) it will allow to save on expenses for algal biomass cultivation that requires special conditions and complex large pond systems; (ii) the removal of excessive waste biomass from natural water bodies allow to increase the biodiversity and the recreational value of aquatic ecosystems.

Amino acids, especially exogenous amino acids, used in various types of feed additives can increase animals' nutrient digestibility, compensate for nutrient deficiencies, and improve feed quality and final animal production composition [4]. Regardless of whether an amino acid is termed essential or non-essential, animals need sufficient amounts of all amino acids to meet their metabolic needs. Based on the total amount of amino acids, the highest total concentration was found in the *C. glomerata* biomass collected from the Lithuanian river Jūra (141 g/kg). Messyasz et al. [12] found that the amino acid content in *Cladophora* biomass indicates a very interesting new material which could be potentially used in animal feed as an alternative feed supplement.

Each amino acid's properties are determined by the structure of its chain, and thus, its carbon skeletons cannot be synthesized by higher animals. Eight of them (threonine, valine, isoleucine, leucine, phenylalanine, lysine, methionine) are considered nutritionally essential [30]. During our study, the essential amino acids totally accounted for 41.6–55.4 g/kg (~40% of the total amount of amino acid determined) in *C. glomerata* biomass and the concentration was the highest in samples from the River Jūra. The branched chain essential amino acids (isoleucine, leucine, and valine) act as tissue protein building blocks (accounting for 35% of the essential amino acids in muscle) and perform many indispensable metabolic functions [31]. Another essential amino acid, methionine, is one of the most limiting amino acids, playing a critical role in the body's protein synthesis [32]. Methyl groups of methionine are essential in animal nutrition and are involved in their metabolism. It should be noted that animals cannot synthesize them and must therefore obtain them from their daily ration [33]. The maximum amount of methionine in our study was observed in algal biomass collected from the Šventoji River (4.14 g/kg). Methionine constituted 3.5% of the total amino acid content in *Cladophora* biomass, like in the widely accepted additive *Spirulina platensis* [34], and was two times higher than in soybean meal. It is important to note that natural source macroalgal biomass is a promising source for replacing artificially grown *Spirulina* additives with a more sustainable animal feed material. Altogether, the amino acid arrangement in individual green algae is very similar: threonine improves plant

generative development; serine is required for chlorophyll synthesis. Proline improves plant generative development and regulates water management in the cell [6].

Research on protein nutrition has mainly focused on the dietary composition of essential amino acids which are not synthesized in animals' cells [35–37]. However, non-essential amino acids can be synthesized by the animal's body and do not need to be provided in the diet, but they still play an important role. Non-essential amino acids play an important and comprehensive role in whole-body metabolism and functions, according to scientific research and emerging evidence [38–41]. In our study, the highest concentrations of aspartic acid, serine, glutamic acid, proline, glycine, alanine, tyrosine, and arginine were determined in River Jūra macroalgal biomass samples. In terms of feed formation, all non-essential amino acids found in the algal biomass we studied would be useful in the mammalian diet, according to Elango et al. [35] and Wu [41]. However, higher concentrations of alanine and arginine are not recommended for carnivores, ferrets, minks, and young animals. The amino acid requirements of animals' diets are determined first by the animal's species, developmental stage, physiological status, small intestinal microbiota, general pathological state, and even environmental factors [31,42–44].

In general, according to the total amount of amino acids and according to the concentrations of individual amino acids, *C. glomerata* biomass can be used from any Lithuanian river we analysed. The majority of algae species contain all essential amino acids. However, one should take into account that their concentrations and mutual proportions are conditional on the season of occurrence [4].

4.3. Lipids and Fatty Acids in Algal Biomass

Lipids are high energy-dense compounds when compared to proteins, carbohydrates, or any other nutrient found in food and feed [2]. Algal biomass can potentially be used as a feed ingredient and can also be fed directly to livestock. For example, algal biomass can be digested by cattle, swine, and sheep [45]. Considering *C. glomerata* chemical composition indicators, the amount of crude fat is significantly lower, particularly in our study, where it varied from 0.19% to 0.35%. In this case, the amount of crude fat can be offset in feed production by supplementing the feed with other fat-rich additives, such as vegetable oils.

Fatty acids are considered one of the most important algae components, especially polyunsaturated fatty acids (PUFA), which are crucial for human and animal health. Overall, *Cladophora* species extracts can implement an antimicrobial function which may be attributed to the general presence of fatty acids [7,46]. Several research papers indicate that macroalgae (both marine and freshwater) are rich in saturated (SFA) and unsaturated fatty acids (UFA) [12,46–48]. During our study, the total SFA amount in different algal biomass was more than 50% of the total fatty acids. However, Messyasz et al. [12] found less SFA (32.70%) in *C. glomerata*. It is necessary to note that the lipid content in *Cladophora* species' biomass can vary depending on salinity—the higher the salinity, the lower the fat content is [20]. Since the absorption of SFA from the digestive system is hard, unsaturated fatty acids such as monounsaturated fatty acids (MUFA) and PUFA release more energy [49]. MUFA observed in *C. glomerata* biomass analysed by us accounted for 27.34–28.39% of the total fatty acids content.

Indispensable PUFA must be obtained from the diet since mammals are unable to synthesize them. PUFAs, or long-chain highly unsaturated fatty acids with an omega-3 configuration, in particular, are known as "functional feed and food" elements [2]. Given PUFAs' fundamental role in metabolism, it's no wonder that they are linked to a variety of health benefits, e.g., antibacterial [50,51], anti-inflammatory [52], antioxidant [53], prevention of cardiac diseases [54], and tumour progression inhibition [55]. In general, fish and their oils or meals are the main commercial sources of PUFAs, but their suitability for human or animal consumption has been questioned from a biosafety perspective. It raised the demand to investigate alternative sources of high quality PUFAs such as macroalgae. Consequently, marine and freshwater macroalgae such as *Cladophora* species have been studied as alternative potential sources, as many of them could easily be cultivated in

different water sources (sea, rivers, lakes etc.) on a large scale [2,4,12,51,56]. In our study of freshwater *C. glomerata* biomass from different rivers in Lithuania, PUFA levels ranged between 6.48% and 11.71% of the total fatty acid content. In comparison, Pereira et al. [56] detected almost two-fold more PUFAs in the marine biomass of *C. albida*.

An important lipid index, the PUFA/SFA ratio, is used to assess the impact of diet on cardio-vascular health [57]. It is hypothesized that all PUFAs in the diet will lower low-density lipoprotein cholesterol (LDL-C) and serum cholesterol levels, whereas all SFAs lead to high serum cholesterol levels [57]. As a result, the higher the ratio, the more advantageous the impact. The highest PUFA/SFA ratio observed in our studies for *C. glomerata* biomass was 0.22 and was about 1.69 times higher compared to plant oils such as palm stearin (PUFA/SFA = 0.13 [58]).

Although analysed *C. glomerata* macroalgae do not have as many lipids compared to microalgae or terrestrial plants (for example, linseed or rapeseed), they may have advantageous lipid quality, particularly fatty acids composition, that can compensate for the nutritional value. We calculated the essential omega-3 and omega-6 fatty acid content in the current study, which are two important components of PUFA. Docosapentaenoic acid, eicosapentaenoic acid, and -linolenic acid are omega-3 fatty acids that perform critical functions in both human and animal organisms [59]. In our study, the highest content of α-linolenic acid was found in *C. glomerata* biomass from the Lithuanian River Dubysa (4.29%). The highest content of eicosapentaenoic acid was in the River Jūra (1.13%). Docosapentaenoic acid was found only in Dubysa's *C. glomerata* biomass (0.22%). Other research has shown that the use of algal biomass in the diet of laying hens has a positive impact on the nutritional properties of the egg yolk by increasing the content of essential omega-3 fatty acids [3]. However, based on our research results and to improve the content of omega-3 fatty acids in animal nutrition and the final product, *C. glomerata* extracts could be combined with vegetable oils such as rapeseed oil, which is particularly high in omega-3. For instance, Fredriksson et al. [60] presented results showing that combining algae with rapeseed oil in laying chickens' diets increased omega-3 fatty acids by 15% and reduced omega-6 acids content by 8% in the egg yolk. Considering the omega-6 fatty acid content in the *C. glomerata* biomass studied in our experiment, the total omega-6 fatty acid content varied from 2.75% to 4.62%. Taking in conjunction the omega-3 and omega-6 results obtained from freshwater *C. glomerata* from different Lithuanian rivers, it can be assumed that use of algal biomass can be successfully applied as feed additive or raw material.

Since long chain omega-3 and omega-6 PUFAs are synthesized by the same enzymes, an increase in one of these essential fatty acids usually means a reduction in the other fatty acids due to competition for the same metabolic enzymes [61]. As a result, the health-promoting benefits of these essential fatty acids are dependent on maintaining a proper balance of omega-6 and omega-3 PUFAs [62]. However, deficits of essential unsaturated fatty acids, as well as an improper ratio of omega-6 to omega-3, have been linked to a variety of diseases. It is important to maintain a low omega-6 and omega-3 ratio in humans and animals' diets to reduce inflammation. Nowadays, the perfect ratio of omega-6 to omega-3 is considered to be 1 or 4:1 [63]. The lowest mentioned ratio in *C. glomerata* biomass analysed in our study was determined in these rivers, Dubysa (1.30) and Jūra (1.35). Higher ratios were found in the biomass of the Šventoji (1.42) and Nevėžis (1.68) rivers. These results fall within the limits of perfect omega-6 omega-3 and are therefore suitable for use as animal nutritional supplements. Based on research by other scientists, feed additives with such a ratio would play an important role in improving the immune response and productivity of animals, as well as the nutritional value of PUFA-enriched final animal production [64,65].

5. Conclusions

According to the determined chemical composition, freshwater *C. glomerata* microalgal biomass from the Lithuanian rivers Dubysa, Šventoji, Nevėžis, and Jūra constitute an important source for sustainable commercial applications. The biomass contains macro-

and trace elements within the recommended limits and is safe for applications in animal nutrition due to low levels of heavy metals. However, chemical analysis should be performed after each biomass collection, as toxicity can be induced by several environmental factors and temporary pollution. As a result of bioaccumulation, freshwater *C. glomerata* may potentially serve as a source of protein, essential amino acids, and fatty acids, implying that they could be a beneficial component of animal feed.

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Article

Evaluation of Phenolic Compounds and Pigments in Freshwater *Cladophora glomerata* Biomass from Various Lithuanian Rivers as a Potential Future Raw Material for Biotechnology

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Abstract: Freshwater macroalgae produces a wide range of bioactive compounds, and interest in utilizing its biomass is growing rapidly. Meanwhile, exploiting renewable sources for biomass collection could lead to more sustainable biotechnological chains. The aim of this study was to investigate *Cladophora glomerata* biomass from Lithuanian rivers as a potential raw material for biotechnology. For this reason, phenolic compound profiles, antioxidant activity and pigment concentrations were determined in macroalgal biomass samples collected from the following four Lithuanian rivers: Dubysa (B1), Šventoji (B2), Nevėžis (B3) and Jūra (B4). The highest total phenolic compound content was determined in B3 (1.32 mg GAE/g). Three phenolic acids were identified, namely gallic (12.94–35.13 μg/g), *p*-hydroxybenzoic (23.97–29.05 μg/g) and *p*-coumaric (1.79–6.46 μg/g). The results indicate significant *C. glomerata* antioxidant activity; the highest reducing power reached 0.737 AU (B3), the total antioxidant content was 1.47 mg Trolox/g (B3), DPPH and ABTS radical scavenging was 11.09% (B3) and 97.86% (B1) and FRAP activity 20.86 μmol/L (B3). The content of pigments ranged from 0.56-0.74, 0.39–0.57, 0.17–0.23 to 0.11–0.17 mg/g in chlorophyll *a*, *b*, carotenoids, and lutein, respectively. To conclude, *C. glomerata* macroalgal biomass may have the potential to act as a functional raw material, as several groups of bioactive compounds and antioxidant activities were observed.

Keywords: green macroalgae; bioactive compounds; antioxidant activity; sustainability; renewable sources

1. Introduction

Freshwater macroalgae are less diversified than marine seaweeds, yet they thrive in a variety of habitats and play an important ecological function in aquatic ecosystems. In freshwaters, macroalgal mats mainly consist of filamentous green algae species that may function as habitat-structuring elements within the benthic zone [1]. Cosmopolitan opportunistic macroalgae such as *Cladophora*, *Ulva* and *Enteromorpha* have rapid growth rates and form agglomerations under increased water fertility, reduced flow rates and good light conditions [1]. Additionally, environmental factors have an impact on the variation of macroalgal populations, the formation of their life strategies and the chemical composition of the biomass. Due to the synthesis of secondary metabolites (e.g., polyphenols, flavonoids, phenolic acids), algae are resistant to stress factors such as changes in environmental

variables [2,3]. Freshwater macroalgae produce a high variety of bioactive compounds; however, they are far less studied and underutilized as a feedstock [4–6] compared to the biocompounds of seaweeds [7].

Despite the fact that algae is being investigated as a potential feedstock or industrially relevant co-product extraction, there are numerous research barriers to the commercialization of algae-based products [8]. Regular algae are composed of three major compounds, namely proteins, carbohydrates, and lipids. Additionally, algal biomass also produces starch, pigments, antioxidants, vitamins and phytohormones, which can be used as pharmaceuticals, biofertilizers, natural colorants, or animal feed [9]. Biologically active compounds are prevalent in algae species and unique secondary metabolites are frequently confined to a small number of species within a phylogenetic group, which has a variety of health advantages [10]. Overall, there is scientific evidence that phenolic chemicals originate in algae species [10–14]. Macroalgae may be rich in bioactive compounds as well, such as antioxidants (phenols, pigments, etc.), which have significant therapeutic potential. Due to their various morphological and physiological characteristics, macroalgae synthesize these bioactive compounds, which have significant commercial relevance [12]. Secondary metabolites are critical components of defensive systems, and they are divided into several bioactive compound classes based on their structural arrangements [15]. Alkaloids, flavonoids, phenolics, tannins and terpenoids are the key classes, and they all have important pharmacological activities [16]. Pigments are organic compounds that preferentially absorb and reflect a particular spectrum of light. There are three major categories of pigments occurring in algae: chlorophylls, carotenoids and phycobiliproteins [17]. So, green macroalgae are also a valuable source of pigments since they can synthesize chlorophyll and carotenoids as photosynthetic organisms. These characteristics are critical when looking for feedstock and other manufacturing prospects that can be included into a sustainable supply chain.

Nowadays, a variety of strategies are being pursued to extract raw materials that not only exceed the highest quality requirements but that are also more sustainable and ecologically friendly. Therefore, there is a great deal of interest in considering the natural resources from which multifunctional raw materials can be extracted at a green rate. Considering the conception of the "bioeconomy" and its tactics today, not only must biomass production be environmentally beneficial, but the whole bioprocess must be sustainable, optimizing raw material valorisation [18]. For example, the macroalgal biomass *C. glomerata* could be used as a raw material and removing it from freshwater bodies would not only create a multifunctional raw material for various industries, but also clean up water bodies and increase biodiversity. Furthermore, *C. glomerata* is one of the most frequent macroalgae-forming mats in aquatic ecosystems and achieves high biomass in a short period of time [19]. Saturated and unsaturated fatty acids, sterols, terpenoids and phenolic compounds, pigments are among the physiologically active substances found in *C. glomerata* [19,20]. There has been a lot of study surrounding the subject of marine algae [21,22]. For example, with regard to research conducted in Lithuania, the biomass of marine *C. glomerata* collected at the northern beach of Klaipėda was converted to oil by pyrolysis as a potential feedstuff [23]. Other Lithuanian researchers, Baltrėnas and Misevičius [24], investigated the application of *C. glomerata* for biogas production, although these macroalgae were produced in bioreactors rather than being harvested from natural water sources. However, there is currently limited research available about freshwater *C. glomerata* from Lithuanian rivers. Our recently published research has provided a detailed analysis of this freshwater macroalgae's general nutritional value (chemical composition, element accumulation, fatty and amino acid profiles) [25]. Nevertheless, even more extensive and deeper research into chemical composition is required. As an outcome, the objective of this research was to investigate freshwater *C. glomerata* macroalgae biomass from various Lithuanian rivers as a potential raw material by analysing its comprehensive phenolic compound profile, antioxidant activity and most importantly, pigment concentration.

2. Materials and Methods

2.1. Macroalgal Biomass Collection

Freshwater *C. glomerata* biomass was collected in August–September 2019 from the following four Lithuanian rivers: Dubysa (N55°12′25.07″, E23°30′30.44″; B1), Šventoji (N55°39′20.14″, E25°10′18.39″; B2), Nevėžis (N55°5′46.52″, E23°46′55.57″; B3) and Jūra (N55°27′19.58″, E22°2′14.72″; B4). More detailed information about the tested rivers is provided in Nutautaitė et al. [25].

The rivers were selected because of the presence of dense agglomerations of *C. glomerata*, which typically cover more than half of the river bottom area (Figure 1A). The macroalgal biomass was harvested manually from the rivers (Figure 1B). Subsamples of up to 1 kg of wet biomass were collected and blended from up to six distinct locations. To remove sand and mud particles, macroalgal biomass was washed numerous times with tap water. The biomass was manually cleaned of macrozoobenthos, macrophytes and other debris (Figure 1C). The samples were dried overnight in a 60 °C oven and stored in darkness at room temperature in closed plastic bags until the analysis. To avoid access humidity forming during storage, biomass was dried again at 105 °C until constant weight before each analysis. Each river's three subsamples (n = 3 from each river) were evaluated for each analysis.

Figure 1. (**A**) Agglomerations of macroalgal *C. glomerata* biomass in River Šventoji; (**B**) Collected *C. glomerata* biomass; (**C**) Washed and cleaned biomass of macroalgae.

2.2. Macroalgal Biomass Phenolic Compounds Profile Analysis

All the chemicals were of analytical grade and were used as received. Solvents methanol HPLC Chromasolv gradient grade, acetonitrile Chromasolv (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and for high performance liquid chromatography (HPLC) gradient grade, ethanol absolute (Sigma Aldrich Chemie GmbH, Steinheim, Germany) were used. Acetic, formic, hydrochloric, orthophosphoric acids, sodium hydroxide, phenolic acid (*o*-coumaric, cinnamic, m-coumaric, vanillic, caffeic, salicylic, ferulic, sinapic, chlorogenic, 3,4-dihydroxybenzoic, gallic, *p*-hydroxybenzoic and *p*-coumaric) standards, flavonoid (quercetin, rutin trihydrate, kaempferol, myricetin, xantohumol) standards, cate-

chin (catechin hydrate, epicatechin, epigallocatechin gallate) standards and tertiary butylhydroxyquinoline (TBHQ) were purchased from Sigma-Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany). Ultrapure water (resistivity of 18.2 MΩ) was supplied by Elga purification system Purelab Ultra (Elga, Bucks, UK).

HPLC analyses were performed on a Shimadzu HPLC system (Shimadzu corp., Kyoto, Japan) consisting of system controller SCL-10A, solvent delivery module LC-10AT, auto injector SIL-10AD, UV-Vis detector SPD-10AV, column oven CTO-10AC and on-line degasser DGU-14A.

The retention periods of the test compounds were compared to the retention times of the corresponding standard solutions to identify each. Data collection and evaluation were performed by using the operating system Workstation LC solution (Shimadzu corp., Kyoto, Japan) and measuring the peak areas of the corresponding compounds in the sample and calculating their concentrations based on the calibration curves.

2.2.1. Phenolic Acids

The profile and content of phenolic acids was determined by extraction according to Kvasnička et al. [26]. One gram (±0.0001 g) of ground biomass sample was weighed and 25 mL of 0.1 M NaOH was added before shaking at 40 °C for 60 min in a water bath GFL 1083 (GFL GmbH, Burgwedel, Germany), cooling to room temperature, acidifying with 2 M HCl to pH 5–6and supplementing with 20 mL of methanol (99%). The flask was placed in an ultrasonic bath (Bandelin Electronic, Berlin, Germany) for 30 min, cooled to room temperature and made up to volume with methanol. The filtrate after filtration by a 0.22 µm membrane filter (Frisenette ApS, Knebel, Denmark) was analysed by HPLC. Phenolic acids in standard mixtures and extracts were separated by the method described by Amarowicz and Weidner [27] with some modifications (Table 1).

Table 1. High performance liquid chromatography (HPLC) conditions for phenolic compound determination.

Conditions	Phenolic Acids	Flavonoids	Catechins
Mobile phase (*v*/*v*/*v*)	A—methanol B—water/acetonitrile/acetic acid 88/10/2	A—methanol B—0.2% formic acid in water	A—0.1% orthophosphoric acid in water B—0.1% orthophosphoric acid in methanol
Elution	Gradient: 100% B (4 min), 0–100% A (15 min), holding 100% A 10 min, decrease to 0% A in 0.5 min holding 100% B 6.5 min	*Gradient:* 35–50% A at 0–25 min, 50–80% A at 25–30 min, 80–95% A at 30–35 min, 95-100% at 35–40 min, holding 100% A 5 min, decrease to 35% A in 5 min. After each run, the chromatographic system is set to 35% A for 5 min	*Gradient:* 0–5 min, 20% B; 5–7 min, linear gradient from 20 to 24% B; 7–10 min, holding 24% B; 10–20 min, linear gradient from 24 to 40% B; 20–25 min, linear gradient from 40 to 50% B; 25–30 min decrease to 20% B; holding 20% B 5 min
Flow rate (mL/min)	1.0	1.0	1.0
Column	LiChrospher 100 RP-18 250 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)	LiChrospher 100 RP-18 250 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)	LiChrospher 100 RP-18 150 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)
Guard column	LiChrospher 100 RP-18 7.5 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)	LiChrospher 100 RP-18 7.5 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)	LiChrospher 100 RP-18 7.5 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, IL, USA)
Column oven temperature (°C)	30	30	30
Injection volume (µL)	10	10	10
Detection (nm)	260 and 320	340 and 367	210 and 280

2.2.2. Flavonoids

Flavonoids were extracted according to Khuluk et al. [28]. One gram (±0.0001 g) of ground biomass was sonicated (Bandelin Electronic, Berlin, Germany) for one hour at room temperature in the presence of HCl 6M (5 mL) and TBHQ solutions (20 mL) in 62.5% methanol. The sample extracts obtained following sonication were filtered through a 0.22 µm membrane filter (Frisenette ApS, Knebel, Denmark) before being injected into the HPLC device. Flavonoids in standard mixtures and extracts were separated by the method described by Khuluk et al. [28] with some modifications (Table 1).

2.2.3. Catechins

The content of catechins was determined by the method described by Wang et al. [29]. About 0.5 g of ground biomass was accurately weighed (±0.0001 g) and extracted for 20 min using sonication (Bandelin Electronic, Berlin, Germany) with 40 mL of a solution of ethanol and water (10:90, v/v). The extraction solution was filtered into a 50 mL volumetric flask, the flask and filter were rinsed with the solution of ethanol and water (10:90, v/v) and then made to volume with the same solvent. For HPLC analysis, approximately 1 mL of the sample solution was centrifuged at 13,000× *g* (Sanyo MSE, London, UK) for 10 min prior to HPLC analysis. Catechins in standard mixtures and extracts were separated by the method described by Wang et al. [29] with some modifications (Table 1).

2.2.4. The Total Phenolic Compound Content

From each river, 0.25 g of dried algal biomass was mixed with 25 mL of methanol solvent (70%). The extraction was carried out for 2 h at 40 by shaking in the dark using a benchtop shaker Certomat (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The Folin–Ciocalteu method was used to determine the total phenolic content [30]. A total of 100 µL of *C. glomerata* extract, 1.8 mL of distilled water and 150 µL of the Folin–Ciocalteu reagent was added to the tubes. The tubes were mixed and 1.02 mL of 7.5% sodium carbonate (Na_2CO_3) was added after 1 min. The prepared samples were stored in the dark at 20 °C for 2 h. The absorbance of the samples was measured with a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 765 nm.

The total phenolic compound content was expressed as gallic acid equivalent (GAE) from the calibration curves of the standard gallic acid solutions per gram of dry mass of *C. glomerata*.

2.3. Macroalgal Biomass Antioxidant Activity Analysis

2.3.1. Biomass Extracts Preparation

The dried *C. glomerata* biomass (0.1 g) was extracted for 0.5 h in methanol (5 mL; 99%) at 45 °C. It was then centrifuged for 10 min at 9000× *g* (Hettich Universal 320Randreas Hettich GmbH & Co., Tuttlingen, Germany) and used for antioxidant activity analysis.

2.3.2. Reducing Power (RP) Bioassay

A total of 0.5 mL of algal methanol extract (0.02 g/mL) was mixed with phosphate buffer (1.25 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1.25 mL, 1%). For 20 min, the mixture was incubated at 50 °C. To stop the reaction, a portion (1.25 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 9000× *g* for 10 min (Hettich Universal 320Randreas Hettich GmbH & Co., Tuttlingen, Germany). The absorbance was measured with a UV/Visible spectrophotometer (UV-1280, Shimadzu, Japan) at a 700 nm wavelength after the upper layer of solution (1.25 mL) was mixed with distilled water (1.25 mL) and $FeCl_3$ (0.25 mL, 0.1%). A rise in the reaction mixture's absorbance indicated an increase in the reducing power.

2.3.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The antioxidant activity and content of antioxidants in *C. glomerata* extracts were determined using the spectrophotometric DPPH radical scavenging method according to

Korzeniowska et al. [6]. A solution of DPPH (6.5×10^{-5} M) in methanol was prepared. The test samples were prepared by mixing 0.1 mL of *C. glomerata* extract and 1 mL of prepared DPPH solution. For the preparation of the control sample, 1 mL of DPPH solution was mixed with 0.1 mL of methanol. The prepared samples were incubated in the dark at 20 °C for 30 min. The absorbance of the samples at 515 nm was measured by a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Additionally, the absorbance of the biomass extract without the reagent (background of the sample) was measured. Antioxidant activity is expressed as the radical scavenging activity calculated by the following formula:

$$\text{Radical scavenging activity } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is the absorbance of the control sample and A_s is the absorbance of the test sample (value after subtracting the sample background).

The antioxidant content was calculated according to the calibration curve of the standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solutions and expressed in Trolox equivalents (TE) per gram of dry mass of *C. glomerata*.

2.3.4. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) Radical Scavenging Assay

The ABTS stock solution (2 mM) was prepared by reacting ABTS with 0.17 mM potassium persulfate in 20 mM phosphate buffer (pH = 7.4) at room temperature under dark conditions for 12 h. A working ABTS solution was then prepared by diluting the stock solution with 20 mM phosphate buffer (pH = 7.4). The working reagent was adjusted to obtain an absorbance of 0.80 ± 0.05 at 734 nm. Then, 0.5 mL of the algal methanol extract (0.02 g/mL) was reacted with 0.3 mL of the working ABTS solution and 1.7 mL of 20 mM phosphate buffer (pH = 7.4). The ABTS radical scavenging ability was measured at 734 nm wavelengths using the UV/Visible spectrophotometer (UV-1280, Shimadzu, Japan). The radical scavenging activity (%) was calculated using the same formula presented in Section 2.3.3.

2.3.5. Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The FRAP assay is a method for determining the antioxidant activity of a sample to reduce Fe^{3+} to Fe^{2+}. The FRAP reagent was freshly prepared by mixing 300 mM of acetate buffer (pH = 3.6) with 10 mM of TPTZ solution and 20 mM of ferric chloride solution.

The dried biomass (0.1 g) was extracted for 0.5 h in methanol (5 mL; 99%) at 45 °C. It was then centrifuged for 10 min at 9000× *g* (Hettich Universal 320Randreas Hettich GmbH & Co., Tuttlingen, Germany). Then, the FRAP reagent (3 mL) was reacted with 100 µL of the algal methanol extract (0.02 g/mL) for 5 min before measurement with a UV/Visible spectrophotometer (UV-1280, Shimadzu, Japan) at 593 nm. A linear regression was generated using $FeSO_4 \cdot 7H_2O$ at a final concentration of 5–25 µmol/L. FRAP activity (µmol/L) was calculated and compared with the $FeSO_4 \cdot 7H_2O$ calibration curve.

2.4. Macroalgal Biomass Pigments Contents Analysis

2.4.1. Chlorophyll *a*, *b* and Carotenoids Contents

Dried algal material (0.1 g) was mixed with 10 mL of 96.3% ethanol for the determination of chlorophyll *a*, *b* and the total carotenoids amount [31]. The extract was centrifuged at 9000× *g* for 10 min (Hettich Universal 320Randreas Hettich GmbH & Co., Tuttlingen, Germany). Optical density readings were recorded at wavelengths of 662 nm (chlorophyll *a*), 644 nm (chlorophyll *b*) and 441 nm (carotenoids). Ethanol (96.3%) was used as a blank. The measurements of chlorophyll *a, b* and carotenoids were determined by the UV/Visible

spectrophotometer (UV-1280, Shimadzu, Kyoto, Japan). The concentrations of chlorophyll *a*, *b* and carotenoids were calculated by:

Chlorophyll *a* concentration (mg/L):

$$C_a = 9.784\ OD_{662} - 0.990\ D_{644}$$

Chlorophyll *b* concentration (mg/L):

$$C_b = 21.426\ OD_{644} - 4.650\ D_{622}$$

$$C_a + C_b = 5.134\ OD_{622} + 20.436\ OD_{644}$$

Carotenoid concentration (mg/L):

$$C_{car} = 4.695\ OD_{441} - 0.268\ (C_a + C_b)$$

The concentration of separate pigments per gram of DM (mg/g DM) was calculated by:

$$C_{pig} = \frac{C \times V \times V_2}{n \times V_1 \times 1000}$$

where:

OD—optical density according to the wavelengths of the pigments.
Coefficients—the absorption coefficients of the pigments according to the wavelengths.
C—chlorophyll *a*, *b*, carotenoids concentration, mg/L.
V—initial volume of the algal extract, mL.
V_1—initial volume of the algal extract for dilution, mL.
V_2—diluted volume of the algal extract, mL.
n—weight of the algal material, g.

2.4.2. Lutein Content

The lutein content, with a few modifications to the method, was determined according to Jajali Jivan and Abbasi [32]. In this analysis, 100 mg of dried algal biomass was mixed with 20 mL of acetone and left on the environmental shaker-incubator (ES-20, Grant Instruments, Shepreth, UK) for 3 h at 300× *g* at room temperature (25 °C) in dim light. The suspension was then allowed to stand for 5 min before we collected the supernatant, but the pellet was mixed (1 h) again with acetone (10 mL) under the same circumstances and the former process was repeated two more times until the pellet remained colorless. Finally, the supernatants were pooled and kept at 4 °C for lutein content analyses.

The molar extinction coefficient of lutein was used to quantify it [33]. The extract (2 mL) was centrifuged at 9000× *g* and 25 °C during 15 min (Hettich Universal 320Randreas Hettich GmbH & Co, Germany). Optical density (OD) was measured at 446 nm against acetone as a blank (UV/Visible Spectrophotometer UV-1280, Shimadzu, Japan), as the λmax of lutein had the least interference with other carotenoids. The lutein content in biomass samples was then calculated using the following formula:

$$C = A_{466}/(14.45 \times 10^4) \times (1/b) \times 568.88 \times V/M \times 1\ L/10^3\ mL \times 10^3\ mg/g \times Kg/10^3\ g$$

where C is the lutein content (mg/g), A_{446} is the absorbance wavelength, b is the path length (cm), 568.88 is the molecular weight of lutein (g mol^{-1}), V is the volume of algal extract (mL), M is the weight of the consumed algal biomass (kg)and 14.45×10^4 is the molar extinction coefficient of lutein in acetone (L mol^{-1} cm^{-1}).

2.5. Statistical Analysis

The study used four algal biomass samples, each with three duplicates (n = 3 duplicates/river). Data analysis was performed by SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). A one-way analysis of variance (ANOVA) test post

hoc (Fisher's least significant difference test) was conducted to detect differences among *C. glomerata* biomass from various Lithuanian rivers. A calculated p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Phenolic Compounds

In order to achieve a comprehensive profile of the phenolic compounds in biomass of *C. glomerata*, separate phenolic acids, flavonoids and catechins were identified (Table 2). The following three phenolic acids: gallic, *p*-hydroxybenzoic and *p*-coumaric of the thirteen analysed were identified. Gallic acid was mainly found in B3 biomass (35.13 μg/g DM) and was lower by 13.82, 21.39 and 22.19 μg/g DM, respectively in B2, B4and B1 ($p < 0.05$). Another phenolic acid, *p*-hydroxybenzoic, was also found in high amounts (29.05 μg/g DM) in B3 and B4 (28.31 μg/g DM) *C. glomerata* biomass ($p < 0.05$). However, in these cases, the differences between the various alga biomasses were found to be slightly smaller, as a difference of 5.08 and 3.62 μg/g DM was found in the biomass of B1 and B2 compared to the biomass of B3 ($p < 0.05$). *P*-coumaric acid was identified in only three out of the four biomasses analysed (unidentified in B3). The highest concentration of the mentioned phenolic acid was found in B4 biomass (6.46 μg/g DM; $p < 0.05$); compared to the concentrations found in B1 and B2, B4 biomass contained two and almost four times more p-coumaric acid than B1 and B2, respectively ($p < 0.05$). The highest total phenolic compounds content (1.32 mg GAE/g DM; $p < 0.05$) was determined for B3. The results obtained in B3 did not significantly differ from B4 as similar concentrations were found ($p > 0.05$). However, significant differences were obtained when the total phenolic content of B1 and B2 was 0.37 and 0.22 mg GAE/g DM lower than in B3, respectively ($p < 0.05$).

The analysis of phenolic compounds also sought to identify the following five flavonoids: quercetin, myricetin, kaempferol, rutin and xantohumol. However, when analysing the chromatograms and comparing the resulting peaks with standards of different flavonoids and catechins (catechin, epicatechin, epigallocatechin gallate), none of these phenolic compounds were identified.

Table 2. Phenolic compounds profile of *C. glomerata* biomass from various rivers in Lithuania.

Item [1,2]	*C. glomerata* Biomass [3,4,5]				SEM [6]	*p* Value
	B1	B2	B3	B4		
DM (% of dried samples)	94.95 [a]	92.63 [a]	95.19 [a]	91.12 [b]	0.35	0.000
	Phenolic acids (μg/g DM)					
o-Coumaric	n.d.	n.d.	n.d.	n.d.	-	-
Cinnamic	n.d.	n.d.	n.d.	n.d.	-	-
m-Coumaric	n.d.	n.d.	n.d.	n.d.	-	-
Vanillic	n.d.	n.d.	n.d.	n.d.	-	-
Caffeic	n.d.	n.d.	n.d.	n.d.	-	-
Salicylic	n.d.	n.d.	n.d.	n.d.	-	-
Ferulic	n.d.	n.d.	n.d.	n.d.	-	-
Sinapic	n.d.	n.d.	n.d.	n.d.	-	-
Chlorogenic	n.d.	n.d.	n.d.	n.d.	-	-
3,4-Dihydroxybenzoic	n.d.	n.d.	n.d.	n.d.	-	-
Gallic	12.94 [a]	21.31 [b]	35.13 [c]	13.92 [d]	0.16	0.000
p-Hydroxybenzoic	23.97 [a]	25.43 [b]	29.05 [c]	28.31 [c]	0.31	0.000
p-Coumaric	3.16 [a]	1.79 [b]	n.d.	6.46 [c]	1.71	0.000

Table 2. *Cont.*

Item [1,2]	*C. glomerata* Biomass [3,4,5]				SEM [6]	*p* Value
	B1	B2	B3	B4		
Flavonoids (μg/g DM)						
Quercetin	n.d	n.d	n.d	n.d	-	-
Myricetin	n.d.	n.d.	n.d.	n.d.	-	-
Kaempferol	n.d.	n.d.	n.d.	n.d.	-	-
Rutin	n.d.	n.d.	n.d.	n.d.	-	-
Xantohumol	n.d.	n.d.	n.d.	n.d.	-	-
Catechins (μg/g DM)						
Catechin	n.d.	n.d.	n.d.	n.d.	-	-
Epicatechin	n.d.	n.d.	n.d.	n.d.	-	-
Epigallocatechin gallate	n.d.	n.d.	n.d.	n.d.	-	-
The total phenolic content (mg GAE/g DM)	0.95 [a]	1.10 [b]	1.32 [c]	1.22 [c]	0.09	0.003

Note: [1] DM, dry matter. [2] GAE, gallic acid equivalent. [3] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [4] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [5] n.d., not defined. [6] SEM, standard error of the means.

3.2. Antioxidant Activity

The higher value of absorbance of the reaction mixture indicated a greater reducing power. Our research showed that the highest reducing power according to absorption units (AU) at 700 nm was in *C. glomerata* macroalgal biomass (B3) from the Nevėžis River; it reached 0.737 AU ($p < 0.05$; Table 3). Compared to the other groups, the B3 biomass reduction power was 0.341, 0.436and 0.152 AU higher, respectively, than in the B1, B2and B4 macroalgal biomass samples ($p < 0.05$).

Table 3. Reducing power (RP), DPPH scavenging activity, antioxidant content, the relative ability of antioxidants to scavenge (ABTS) and ferric reducing-antioxidant power (FRAP) of *C. glomerata* biomass from various rivers in Lithuania.

Item [1,2]	*C. glomerata* Biomass [3,4]				SEM [5]	*p*-Value [6]
	B1	B2	B3	B4		
RP (AU)	0.396 [a]	0.301 [a]	0.737 [b]	0.585 [c]	0.05	0.000
DPPH (%)	10.10	8.22	11.09	8.85	1.38	n.s.
Antioxidant content (mg Trolox/g DM)	0.55 [a]	0.39 [a]	1.47 [b]	1.31 [b]	0.11	0.000
ABTS (%)	97.68 [a]	96.80 [a]	97.09 [a]	93.76 [b]	1.18	0.011
FRAP (μmol/L)	15.04 [a]	16.61 [a]	20.86 [b]	19.93 [b]	1.54	0.005

Note: [1] AU, absorption units. [2] DM, dry matter. [3] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [4] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [5] SEM, standard error of the means. [6] n.s., not significant ($p > 0.05$).

The DPPH, ABTS and FRAP tests were used to assess freshwater *C. glomerata* macroalgal biomass biological activity (Table 3). The DPPH scavenging activity ranged from 8.22% to 11.09% and no significant differences between the *C. glomerata* biomasses collected from different rivers were observed ($p > 0.05$). The antioxidant content of macroalgae *C. glomerata* biomass from various Lithuanian rivers is presented in Table 3. Greater levels of antioxidants were found in B3 (1.47 mg Trolox/g DM; $p < 0.05$) and B4 (1.31 mg Trolox/g DM; $p < 0.05$). Compared to B1 and B2, the content of antioxidants was found to be higher by 2.7 and 3.8 times in B3 ($p < 0.05$) and in B4 by 2.4 and 3.4 times ($p < 0.05$), respectively.

Nevertheless, after ABTS assay, the highest scavenging was determined in B1 biomass samples (97.68%; Table 3). The ABTS assay also revealed no significant differences between groups B1, B2and B3, as the values were similar and differed minimally ($p > 0.05$). However, considering the significant differences between the mentioned groups and B4, the

scavenging of the biomass samples B1, B2 and B3 was 3.92%, 3.04% and 3.33% higher than in the B4 samples, respectively ($p < 0.05$).

The ferric reducing-antioxidant power (FRAP) analysis showed that the biomass of B3 and B4 macroalga had the highest power (20.86 and 19.93 μmol/L, respectively), with values calculated to be 5.82 and 4.25 μmol/L higher than B1 and 4.89 and 3.32 μmol/L higher than B2 ($p < 0.05$) (Table 3).

3.3. Pigments Content

The concentration of individual pigments was determined in *C. glomerata* biomass samples from various Lithuanian rivers (Table 4). In general, the total chlorophyll *a* content in the tested macroalgal biomass groups ranged from 0.56 mg/g to 0.74 mg/g DM and was distributed as follows: B2 (0.56 mg/g DM) < B4 (0.57 mg/g DM) < B1 (0.65 mg/g DM) < B3 (0.74 mg/g DM) ($p < 0.05$). Significant differences in chlorophyll *b* concentrations were obtained only between groups B1 and B4, when the biomass from the Dubysa River (B1) was found to be 0.18 mg/g DM higher in the mentioned pigment than the macroalgal biomass from the Jūra River (B4) ($p < 0.05$). The same trend was observed when calculating the total carotenoids concentration, when this pigments group was detected to be higher by 0.06 mg/g DM in B1 *C. glomerata* biomass compared to B4 ($p < 0.05$).

Table 4. Content of individual pigments (chlorophyll *a* and *b*, carotenoids, lutein) of *C. glomerata* biomass from various rivers in Lithuania.

	***C. glomerata* Biomass** [2,3,4]					
Pigment (mg/g DM) [1]	**B1**	**B2**	**B3**	**B4**	**SEM** [5]	***p*-Value**
Chlorophyll *a*	0.65 [a]	0.56 [b]	0.74 [c]	0.57 [b]	0.03	0.003
Chlorophyll *b*	0.57 [a]	0.42 [ab]	0.51 [ab]	0.39 [b]	0.06	0.042
Carotenoids	0.23 [a]	0.18 [ab]	0.20 [ab]	0.17 [b]	0.02	0.037
Lutein	0.12 [ab]	0.17 [a]	0.13 [ab]	0.11 [b]	0.03	0.044

Note: [1] DM, dry matter. [2] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [3] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [4] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [5] SEM, standard error of the means.

Lutein was calculated in the biomass of macroalgae *C. glomerata* from various Lithuanian rivers (Table 4). The most significant differences were found between the rivers Šventoji (B2) and Jūra (B4), with an almost 1.5 higher concentration of lutein obtained in B2 biomass (0.17 mg/g DM) compared to the results obtained in B4 samples (0.11 mg/g DM) ($p < 0.05$). No significant differences were found between the remaining groups ($p > 0.05$).

4. Discussion

4.1. Phenolic Compounds

Numerous factors determine the chemical composition of algal biomass and the level of biologically active compounds, including taxa, habitat, climatic conditions, environmental stressors, biomass collection time and techniques [34,35]. The quantity of bioactive compounds in macroalga is a result of the difficult, harsh, and competitive circumstances in which they thrive. In general, algae are a well-known natural raw material that contains phenolic compounds. One of the most significant and most discussed features of phenolic compounds is their antioxidant activity [6]. Its primary function is to bind, stabilize and inactivate free radicals [6,34,36].

Phenols, as bioactive components, possess a variety of chemical, physical and biological properties [6] that may contribute to the antioxidant activity of pharmaceutical and food products [12]. Many of them display not only antioxidant effects, but also antibacterial and antiviral activities, which are critical for protecting algal cells from stress. For example, one of the groups of phenolic compounds is flavonoids. Badshah et al. [37] recently demonstrated that flavonoids have strong antiviral activity against a variety of viruses,

with the tested flavonoids exhibiting strong antiviral activity, of more than 90% without impacting cell viability. However, we identified no flavonoids in freshwater *C. glomerata* biomass during a recent study. In terms of total phenolic compound content, the highest total phenolic compound content was determined in *C. glomerata* biomass from the Nevėžis River (1.32 mg GAE/g DM). While applying the same determination method and extractant on freshwater *C. glomerata* biomass, Korzeniowska et al. [6] discovered that the phenol content in biomass reaches only 0.27 mg GAE/g DM. Decreased or lower phenolic concentrations (0.30–20 mg GAE/g DM) in algal extracts may be linked to other chemicals, such as carotenoids, fatty acids, sterols, vitamins, and others that may be responsible for the indicated activities [6,38–40].

Only three phenolic acids (gallic, *p*-coumaric, *p*-hydroxybenzoic) were found in macroalgal biomass during the current study out of the 13 assessed. However, using HPLC qualitative examination, Korzeniowska et al. [6] were able to identify nine phenolic compounds (gallic, chlorogenic, syringic, *p*-coumaric, myricetin, 3,4-dihydroxybenzoic, vanillic, 4-hydroxybenzoicand rutin acids) in freshwater *C. glomerata* that belonged to both the phenolic acids and flavonoids [6]. These results are in line with our study as we identified gallic acid, which varied from 12.94 to 35.13% of DM and *p*-coumaric acid, which varied from 1.79 to 6.46% of DM, in all the analysed macroalgal biomass samples from various Lithuanian rivers. The other significant phenolic acid identified in our research was *p*-hydroxybenzoic, which varied from 23.97 to 29.05% of DM and prevailed in almost all biomass samples.

All three phenolic acids found in the recent study's macroalgal biomass present health benefits. Hydroxybenzoic acids, gallic and *p*-hydroxybenzoic, lead to dyslipidaemia treatment and glucose metabolism regulation [41]. They are considered as antihyperlipidemic, antihyperglycemic, cardioprotective [42] and anti-cancerous [43]. *P*-coumaric acid, a member of the hydroxycinnamic acid group, possesses anti-cancer properties as well [44].

4.2. Antioxidant Activity

The antioxidant activity of *C. glomerata* biomass is generally measured in terms of total phenolic and antioxidant content, reducing power, DPPH, ABTS radical scavenging activity and FRAP. Antioxidant activity is crucial in fighting free radicals and reactive oxygen species, which both cause oxidative stress. Several studies have found that it is the primary cause of several diseases, including human ailments [45–47]. In general, the accurate identification of antioxidant compounds can lead to their application in food, feed, cosmetics and medicine manufacturing.

In the reducing power assay, the antioxidant activity in the sample leads to the reduction of Fe^{3+} to Fe^{2+} by donating one electron [48]. Previously, researchers discovered a link between antioxidant activity and the reducing power of particular plant extracts [49,50]. Reductones, which have been shown to exert an antioxidant effect by breaking the free radical chain by donating a hydrogen atom, are frequently related to the occurrence of reducing abilities [50]. The basis of this method is an increase in the absorbance of the reaction mixtures. More absorption suggests increased antioxidant activity [51]. Our data on the reducing power of freshwater *C. glomerata* biomass from various Lithuanian rivers was found to be in the order of B3 (Nevėžis) > B4 (Jūra) > B1 (Dubysa) > B2 (Šventoji). Nonetheless, the highest reducing power observed in the River Nevėžis, considering all macroalgal biomass results, suggests that it is likely to contribute significantly towards the observed antioxidant effect.

Algae are a natural material with a diverse and complicated matrix constitution. As a result, the methods for assessing antioxidant activity have proven not only the existence of several phenolic compounds in *C. glomerata* biomass, but also the presence of additional antioxidants. In this case, DPPH, ABTS and FRAP procedures are rapid and easy ways by which to assess antioxidant activity [6]. The high quantity of phenols in algal extracts demonstrates substantial DPPH radical scavenging activity, allowing them to neutralize free radicals that can activate cancer cells [52]. The molecule of 2-diphenyl-1-picrylhydrazyl

(DPPH) is classified as a stable free radical due to the delocalization of the spare electron over the molecule, preventing dimerization, as most other free radicals do [51]. The deep violet colour is also induced by electron delocalization, which is described by an absorption band in methanol solution at around 517 nm. When a DPPH solution is treated with a substance capable of donating a hydrogen atom, a reduced form is created, but the violet colour disappears. During our study, the DPPH in different *C. glomerata* biomass varied from 8.22% to 11.09%. Compared to the results of other researchers [6,53], the DPPH activity obtained in our experiment is relatively low. By determining DPPH by the same method and using the same concentration of methanol for the preparation of extracts, Korzeniowska et al. [6] found this indicator as being almost three times higher ($32.67 \pm 0.40\%$). However, on the contrary and in line with our findings, is the research of Laungsuwon and Chulalaksananukul [54], who revealed that antioxidant activity measured by DPPH was 16.7% in freshwater *C. glomerata* biomass [54]. Nonetheless, the findings of the mentioned researchers were only slightly higher than ours (by approximately 5%). It should be noted that the biomass samples in their study were freeze-dried, which may have affected the final test substance. Furthermore, to explain the disparities in the researchers' findings, Korzeniowska et al. [6] reported that antioxidant activity in freshwater *C. glomerata* is dependent on extraction procedures and solvents. It is also important to note that there were no significant differences identified between Lithuanian rivers following a DPPH scavenging activity assessment of *C. glomerata* biomass in our study.

The total antioxidant content found in algae varies depending on the season and geographic region [53]. In the recent study, the total antioxidant content was calculated using the Trolox standard curve and expressed according to the DPPH method. The number of antioxidants in the macroalgal biomass from different Lithuanian rivers varied from 0.39 to 1.47 mg Trolox/g DM. Freshwater *C. glomerata* biomass from two water reservoirs in the Wielkopolska region (Poland) was tested using the same method [6]. It was in line with our findings when we assessed antioxidant content in biomass from the Nevėžis (B3; 1.47 mg Trolox/g DM) and Jūra (B4; 1.31 mg Trolox/g DM) rivers. However, the antioxidant content of biomass from the Lithuanian rivers Dubysa (B1) and Šventoji (B2) was only 0.55 and 0.39 Trolox/g DM, respectively, whereas the same index in biomass from Polish water reservoirs reached a content of 2–3 times higher. Differences between our and Polish scientists' findings could simply be due to differences in macroalgae habitats.

The antioxidant activity of four macroalgal extracts was determined using the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test, which is based on antioxidants' ability to scavenge the radical anion ABTS*+. The ABTS assay measures an antioxidant's capacity to prevent the absorption of ABTS radicals, which have a unique blue colour and maximum absorption of 734 nm [55]. The ABTS scavenging activity in macroalgal biomass collected from four different Lithuanian rivers ranged from 93.76 to 97.68% and was about 3 times higher compared with *C. glomerata* from freshwaters in Poland [6]. According to prior findings, carotenoids derived from algae often have a better scavenging activity for ABTS radicals, but with a lower reduction power [55], which in general explains our findings. In a recent study, the inhibitory activity against ABTS radicals was found to be greater than that against DPPH radicals in macroalgae biomass. This can be linked to the fact that ABTS is more sensitive in detecting antioxidant activity and has a stronger response to antioxidants due to faster reaction kinetics [56].

The ferric ion reducing antioxidant power (FRAP) method assesses antioxidants' capacity to decrease ferric iron [51]. The combination of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) is converted to ferrous form at low pH. This reduction is recognized by a shift in absorbance at 593 nm. The antioxidants in the biomass samples are treated as reductants in a redox-linked colorimetric process in the FRAP assay, and the value indicates the antioxidants' reducing power. Along with phenolic contents, molecular weight, and structural arrangement, FRAP activity is more strongly aligned to phlorotannin content than to radical scavenging capacity [57]. In our case, FRAP activity varied from 15.04 to 20.86 μmol/L.

The antioxidant activity of macroalgal extracts is mostly attributed to phenolic compounds. Carotenoids, on the other hand, assist in DPPH radical scavenging, ferric ion reduction antioxidant power (FRAP)and ABTS radical scavenging capacity activities [58]. The researchers furthermore provide data showing a strong link between phenolic compounds and FRAP with DPPH values in algal biomass [3,59]. As relatively few phenolic compounds were identified in our study and the levels of FRAP and DPPH were not high, we can assume and confirm that this dependence is also reflected in our results.

4.3. Pigments

Algae has been utilized as a food source for a long time due to its high nutritional value. Chlorophyll, a green pigment found in plants, algae and cyanobacteria that is necessary for photosynthesis, is one of its bioactive components. According to the correlation between habitat conditions and Cladophora species diversity, water depth, chloride, orthophosphate, nitrate, total dissolved salts, and chlorophyll *a* content in water are essential criteria that define it [19]. For example, Messyasz et al. [4] demonstrated that the chlorophyll *a* content in freshwater *C. glomerata* extract was 0.30 mg/g of dry algal biomass. In our study, *C. glomerata* biomass from various Lithuanian rivers contained 0.57 to 0.74 mg/g DM chlorophyll *a*, which is nearly two times higher than Messyasz et al. determined. This demonstrates that the content of this compound is influenced by the algae habitat. For example, disadvantageous environmental conditions trigger disruptions in the morphometric structure of thalli, the seasonality of their occurrence and the formation of vast quantities of filamentous algal biomass [1]. Even changes in biomass collection sites, such as the surface, middle and bottom habitats of a dense *C. glomerata* mat, impact nutrients, underwater light environment, and temperature. Green algae also contain chlorophyll *b*, in addition to chlorophyll *a*. In our study, this compound reached 0.57 mg/g DM in biomass from the river Dubysa. Pikosz et al. [60] conducted a study comparing two freshwater species of *Cladophora* (*C. glomerata* and *C. fracta*) from lakes in Poland. These researchers discovered that *C. glomerata* had larger quantities of pigments, especially chlorophyll *b* (6.36 ± 0.11 μg mL^{-1}), compared to *C. fracta* (2.95 ± 0.07 μg mL^{-1}). However, compared to our recent study, we obtained much higher concentrations of chlorophyll *b*. These results may be influenced primarily by the environmental conditions described above. Additionally, biomass from Poland was collected in June, from a lake, whereas our biomass was collected in August-September and from rivers.

Carotenoids are a rich category of compounds that include over a hundred compounds found exclusively in algae, and they exhibit a broad range of chemical structure by alterations of end groups or chains, as well as isomerization [17]. Carotenoids come in a variety of colours, ranging from yellow to red, and they are in a range of algal species. They have significant antioxidant properties due to their ability to quench singlet oxygen and scavenge free radicals [61]. In our study, carotenoids in *C. glomerata* biomass accounted for the smallest proportion of pigments tested, ranging from 0.17 to 0.23 mg/g DM. Khuantrairong and Traichaiyaporn [62] discovered higher but still relatively low concentrations of carotenoids in freshwater *C. glomerata* (0.89 mg/g DM). In contrast to our results, Fabrowska et al.'s [63] analysis of *C. glomerata* was considered to have significant total carotenoids and phenol content, as well as antioxidant activity. For example, the mentioned researchers reported exceedingly higher total carotenoid levels (16.59 mg fucoxanthin equivalents/g). Nevertheless, results obtained by Messyasz et al. [4] were found to be two times lower in total carotenoids (0.08 mg/g) in *C. glomerata* dry algal biomass compared to ours (0.17–0.23 mg/g DM). However, differences found between *C. glomerata* harvested and analysed in different experimental investigations may be due to biodiversity as well as the effect of growth and environmental circumstances. Lower temperatures and less sunlight, for example, slow down metabolic processes, so algae require fewer chlorophyll for photosynthesis. As a result, algae produce less chlorophyll while producing more carotenoids [30].

Lutein is a prominent xanthophyll carotenoid prevalent in green algae that is strongly associated with the light-harvesting complexes of the photosynthetic apparatus. In addition to capturing light and acting as a structural component in the photosynthetic apparatus, lutein defends the photosynthetic system from oxidative damage under high light conditions via a mechanism known as non-photochemical quenching [64]. Prazukin et al. [65] analysed filamentous green algae *Cladophora* spp. from a hypersaline lake in Crimea, Russia. Prazukin et al. results showed *Cladophora* spp. contained lutein 0.55 mg/g DM, which shows its mat's potential use as a cheap raw material for pharmaceutical and food production purposes. In our case, lutein content in freshwater *C. glomerata* biomass from Lithuanian rivers varied from 0.11 mg/g in biomass from the River Jūra to even 1.5 times higher (0.17 mg/g) in biomass from the Šventoji river. This pigment promotes cognitive performance, lowers the risk of cancer [66] and exhibits anti-inflammatory effects [67]. It is a great natural protector against retinal, macular, and crystalline eye lens damage due to its antioxidant characteristics [68,69]. Individuals must consume at least 5 mg of lutein daily [70]. Because it is not synthesized in the human body it must be consumed with food [69]. Overall, the lutein market is divided into the following four sectors: pharmaceuticals, dietary supplements, food and animal and fish feed [71].

Aside from limiting free radical production, pigments are utilized to improve the coloration of animal origin products, making them more appealing and desirable to customers. The inclusion of algae, which are a source of colour in animal feed, has positive benefits as well [72]. Natural pigments, such as polyphenols, can be used in the prevention and treatment of many diseases because they inhibit the formation of free radicals [73]. Algal dyes can also be used to enhance the colour of animal products, such as egg yolks. Consumers find these products more appealing and desirable [73]. Algal extracts used in animal feeding research could help to biofortify animal origin with biologically active compounds [74]. Shah et al. [75] specifically investigated and outlined the potential uses of freshwater *C. glomerata* for the following purposes: the development of traditional medicines, nutraceuticals, food, and agricultural industries; as separate compounds that can be used as pesticides and insecticides; and the production of biofuels. To summarize, macroalgae have numerous applications. It can be used as a natural colorant in food pigments, such as dairy products or beverages, as well as a feed supplement in aquaculture, livestock, and animals and as a component in cosmetics and pharmaceuticals. It is worth noting that macroalgae of *Cladophora* species are edible and can be found in many people's diets all around the world [76].

5. Conclusions

Among the materials of natural origin, the macroalgal biomass of *C. glomerata* from various Lithuanian rivers (Dubysa, Šventoji, Nevėžis, Jūra) may play an important role and has the potential as a functional raw material in biotechnology. In a recent study, several groups of bioactive compounds, such as phenols and pigments, were identified, as well as some antioxidant activities. To summarise:

- Identified phenolic acids (gallic, *p*-coumaric, *p*-hydroxybenzoic) have health benefits (antihyperlipidemic, antihyperglycemic, cardioprotective and anticancer features) which can be manipulated in the development of pharmaceuticals or functional foods and animal feed supplements.
- Almost all the macroalgal biomass results indicate that *C. glomerata* is likely to have a significant role in the observed antioxidant effect, and the highest reducing power reached 0.737 AU; the total antioxidant content reached 1.47 mg Trolox/g DM; DPPH and ABTS radical scavenging reached 11.09% and 97.86%, respectively; and FRAP activity reached 20.86 μmol/L.
- The observed content of main pigments (chlorophyll *a* and *b*, carotenoids, lutein) shows a wide range of applications for *C. glomerata*, including natural colorants used as food pigments, feed supplements and components of cosmetics and pharmaceuticals due to their potential health benefits.

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RESEARCH ARTICLE

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River-sourced *Cladophora glomerata* macroalgal biomass as a more sustainable and functional feed raw material for growing rabbits

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ABSTRACT

Animal scientists actively seek strategies for ensuring the sustainable viability of animal ecosystems, with a focus on exploring alternative feed raw materials to reduce reliance on traditional ones. So, this study aims to analyse the impact of river-sourced *Cladophora glomerata* macroalgal biomass on the growth, slaughter performance, and physiological development of rabbits. Thirty weaned (52 days of age) Californian rabbits were assigned to three dietary treatments: standard compound diet (SCD), SCD enriched with 4% *C. glomerata* (CG4), and SCD enriched with 8% *C. glomerata* (CG8). Growth performance was recorded throughout the feeding trial, and at its conclusion (122 days of age), rabbits were euthanized, slaughtered, and subjected to intestinal analysis. Incorporation of CG diets showed no significant impact on body weight or average daily gain ($p > 0.05$); however, CG8 significantly lowered daily feed intake and feed conversion ratio ($p < 0.05$). The SCD resulted in a significantly higher lung percentage ($p < 0.05$), while CG diets had minimal effects on remaining slaughter performance traits. In *duodenal* content, CG-enriched diets increased acetic and propionic acid levels but reduced lactic ($p < 0.05$). *Duodenal* villus height remained stable, while in the *ileum*, CG4 inclusion resulted in a significantly higher villus ($p < 0.05$). *Duodenal* crypt depth increased with biomass supplementation; conversely, in the *ileum*, increased biomass led to decreased crypt depth ($p < 0.05$). In general, recent research suggests that adding *C. glomerata* to rabbit diets can be an effective alternative without adverse effects on growth, slaughter performance, or physiologial development.

HIGHLIGHTS

- *C. glomerata* macroalgal biomass is usually considered waste.
- Collecting *C. glomerata* macroalgal biomass from natural water bodies not only enhances their biodiversity and recreational value but also yields valuable raw material for diverse applications in biotechnology.
- *C. glomerata* macroalgal biomass, with its high fibre content, aligns with the unique digestive physiology of rabbits, making it a suitable nutritional component for their dietary requirements.

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Introduction

Optimising growth performance in production animals is crucial for the economic viability and sustainability of livestock enterprises. Efficient development trajectories lead to faster weight gain, increased meat, milk, or egg production, and financial benefits (Fernandes et al. 2021). So, producers strive to enhance feed conversion ratios, improving the transformation of ingested grain into valuable products (Mayerfeld 2023). Enchanted growth rates directly boost livestock production, enabling a response approach to market demands. Furthermore, improved growth is also linked to better animal health, reducing susceptibility to illnesses, and minimising the need for veterinary interventions (Charlier et al. 2022). Strategic prioritisation and enhancement of animal growth not only increase farmers' profitability but also confer competitive advantages (Pomar et al. 2019), contributing to a more sustainable and resilient livestock sector.

Today, production-animal growers face a pressing challenge as they recognise the limitations of resources and grapple with the environmental impacts

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associated with intensive agricultural practices. Achieving a more sustainable livestock sector requires judicious management of natural resources, minimising ecological footprints, and addressing issues such as greenhouse gas emissions, biodiversity, and humane animal treatment (Banerjee et al. 2020). Growers are increasingly adopting circular economy principles, waste reduction strategies, and environmentally friendly technologies to enhance sustainable animal farming (Batra 2023). Striking a balance between meeting current production demands and ensuring long-term ecosystem prosperity is crucial for sustainability in the animal sector. As global concerns about climate change and resource depletion escalate, the imperative for sustainable practices in animal farming becomes more pronounced (Campos 2021).

Daily, animal scientists strive to develop strategies ensuring the sustainable viability of animal ecosystems. Exploring alternative feed additives to reduce dependency on traditional raw materials is crucial to this challenge. For instance, our prior research highlighted the significant potential of utilising freshwater *Cladophora glomerata* (*C. glomerata*) macroalgae biomass in rabbit nutrition, resulting in improved meat quality and consumer-accepted sensory properties (Nutautaitė et al. 2023; Nutautaitė et al. 2023). A comprehensive analysis of *C. glomerata's* chemical composition supports its viability as a protein and amino acid source in animal nutrition (Nutautaitė et al. 2021), substantiating its categorisation as functional (Nutautaitė et al. 2021; Nutautaitė et al. 2022a). Previous scientific investigations confirm that freshwater macroalgae offer elevated nutrient content and beneficial effects on animal health (Al-Soufi et al. 2022; Garcia-Vaquero and Hayes 2016; Silva et al. 2020; Wan et al. 2019). Notably, the abundance of this biomass in Lithuanian rivers, currently considered waste, presents an opportunity for waste-reduction strategies aligned with sustainable practices in animal husbandry and enhancing the biodiversity of local water bodies. However, despite obtaining a functional rabbit meat using *C. glomerata* macroalgal biomass, it is essential to delve into key indicators of animal husbandry. Therefore, the aim of this study was to analyse the impact of river-sourced *C. glomerata* macroalgal biomass on the growth, slaughter performance, and physiological development of rabbits.

Materials and methods

Animals and feeding trial scheme

The feeding trial involved thirty male Californian-breed rabbits that were weaned at 52 days of age. The rabbits for the study were selected based on comparable weights and then randomly allocated to one of three different dietary regimens ($n = 10$ rabbits/diet). The research was carried out at a local rabbit breeding facility, where the animals were housed in individual cages measuring $34 \times 34 \times 61$ cm, allowing for the housing of one rabbit per cage. Each rabbit was provided unrestricted access to dedicated nipple drinkers dispensing clean water and individual feed bowls to ensure optimal health conditions and performance. The facility was equipped with a heating system that maintained a temperature of $19 \pm 2\,^{\circ}C$. Throughout the trial, the photoperiod regimen consisted of 16 h of illumination followed by an 8-h period of darkness. The housing protocols conformed to the specifications delineated in Council Directive 98/58/EC of July 20, 1998, focusing on the welfare standards for animals in farming practices. The protocol structure of the rabbit feeding trail is displayed in Figure 1.

Rabbits were supplied with three distinct diets: a standard compound diet (SCD); SCD enriched with 4% biomass of freshwater *C. glomerata* (CG4); and SCD enriched with 8% biomass of *C. glomerata* (CG8). The feeding regimen was distributed twice daily, *ad libitum*. The formulation of the standard compound diet was meticulously designed to meet the nutritional requirements of growing rabbits by incorporating essential vitamins and minerals. The nutrient composition of the diet adhered to the recommendations provided by the National Research Council (NRC) (Arrington et al. 1977). Detailed information regarding the feed ingredients and their respective chemical compositions is presented in Table 1.

The biomass of *C. glomerata* utilised for feed formulation was manually collected from the Šventoji River in Lithuania, subjected to meticulous cleaning and drying processes, and subsequently incorporated into the production of CG-enriched feed. The chemical composition of the collected biomass was 22.36% crude protein, 2.35% crude fat, 20.80% crude ash, and 22.45% crude fibre. In previously reported research, biomass has been scrutinised for its more detailed chemical composition, including antioxidant properties (Nutautaitė et al. 2021; Nutautaitė et al. 2022a).

Growth and slaughter performance evaluation, sample collection

Over the period of the feeding trial (52–122 days) rabbits' ($n = 10$ rabbits/diet) individual weights including body weight gain (BWG), average daily gain (ADG),

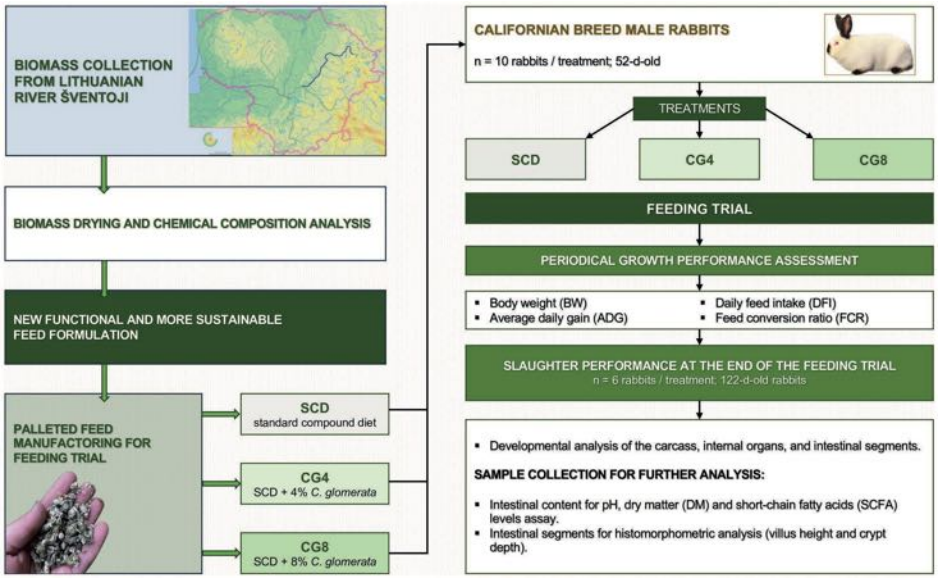

Figure 1. Protocol structure of the rabbit feeding trial.

Table 1. Ingredients in rabbit feed and chemical composition of a standard compound diet and diets supplemented with river-sourced *C. glomerata* biomass (52–122 days old).

	Diet		
Ingredients (g/kg as fed basis)	SCD	CG4	CG8
Corn	30.00	31.30	34.40
Barley	180.00	180.00	180.00
Oats	250.00	250.00	250.00
Sunflower meal	132.20	119.70	99.70
Linseed meal	10.00	10.00	10.00
Soy meal	37.20	30.00	30.00
Vegetable oil	10.00	10.00	10.00
Beer east	20.00	20.00	20.00
Hay	292.60	271.00	247.90
C. glomerata biomass	–	40.00	80.00
Antimycotoxin	3.00	3.00	3.00
Vitamin-mineral premix[a]	35.00	35.00	35.00
Chemical composition (g/kg as fed basis, unless stated otherwise)			
DE (MJ/kg)	10.49	11.13	12.16
Crude protein	175.40	175.30	175.40
Crude fibre	135.60	143.90	150.50
Ash	100.30	103.70	103.10
Ether extract	31.20	32.00	31.00
NDF	324.90	341.90	358.90
ADF	197.10	201.20	207.30
ADL	48.90	49.40	51.50

Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass.
DE: diet energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin.
[a]Vitamin and mineral premix (per kg of feed): vitamin A 10.08 TV, vitamin D3 1.14 TV, vitamin E 50.30 mg, vitamin K3 0.99 mg, vitamin B1 3.71 mg, vitamin B2 2.80 mg, vitamin B5 9.80 mg, vitamin B12 0.01 mg, nicotinic acid 20.40 mg, folic acid 0.22 mg, choline chloride 170.00 mg, Mg 76.28 mg, Fe 317.00 mg, Zn 110.89 mg, Cu 19.16 mg, Co 0.29 mg, I 0.67 mg, Se 0.31 mg.

daily feed intake (DFI), and feed conversion ratio (FCR) were systematically recorded and documented.

At the end of the feeding trial when the rabbits reached 122 days of age, a total of 18 rabbits ($n = 6$ rabbits/diet) were randomly selected, weighed, and subjected to an overnight fast. Subsequently, rabbits were humanely euthanized in accordance with established farming practices. The slaughter procedures were conducted at a rabbit breeding farm slaughterhouse, adhering to established protocols that align with the laws of the Republic of Lithuania, as specified in Order No. B1-866 of October 31, 2012, by the Director of the State Food and Veterinary Service. This order delineates the requirements for the care, keeping, and utilisation of animals for scientific and educational purposes.

The rabbit carcases underwent preparation as per the methods reported by Blasco and Ouhayoun (2010) and were then chilled at 4 °C for 24 h in a well-ventilated room. Dissection procedures of carcases followed the recommendations set forth by the World Rabbit Science Association (WRSA) (Blasco and Ouhayoun 2010). A comprehensive examination of post-mortem carcase attributes, muscle, organ, and gut development was carried out. For further analysis, *duodenum* and *ileum* segments from the middle part were specifically collected for histomorphometric assay, while the

content of distinct intestinal segments was collected for the determination of pH, dry matter (DM), and short-chain fatty acid (SCFA) profiles.

Intestinal content features

pH and dry matter assay

All intestinal content samples were collected post-mortem ($n = 6$ samples/diet). The pH of *duodenum*, small intestine, *caecum*, *ileum*, colon, and stomach contents chyme was determined using a pH metre InoLab pH 730 (WTW Electronic GmbH, Ihlow, Germany).

The dry matter (DM) content of the intestinal segment contents ($n = 6$ samples/diet) was assessed by subjecting them to drying in the Memmert UFE 400 drying cabinet (Memmert GmbH + Co, Schwabach, Germany), maintained at 105 °C. The samples underwent drying until reaching a consistent weight.

Short-chain fatty acid (SCFA) profile determination

The compositional analysis of short-chain fatty acids (SCFA) within *duodenal* content ($n = 6$ samples/diet) was conducted employing a gas chromatography system (Shimadzu GC-2010 Plus; Shimadzu Corp., Kyoto, Japan). A 2.5 mm x 2.6 mm glass tube filled with 10% SP-1200/1% HPO on 80/100 Chromosorb W AW was used, with the tube temperature set at 110 °C, the flame ionisation detector (FID) temperature at 108 °C, and the injector's temperature at 195 °C. SCFA accumulation values were derived by determining the concentration of individual SCFA within the digestive content, expressed as a percentage of the total SCFA, following the analytical procedures outlined by Zduńczyk et al. (2004).

Histomorphometrical analysis

For the assessment of histomorphometric properties, *duodenum,* and *ileum* samples from the middle part of the rabbit intestinal segment ($n = 6$ samples/diet) were fixed using a 10% neutral formalin solution. Subsequently, employing standard histologic procedures, the tissues were embedded in paraffin and sliced into 4 μm-thick tissue sections using a rotary microtome (Leica RM 2235; Leica Microsystems, Nussloch, Germany). These sections were stained with haematoxylin and eosin. Morphometric and microscopic measurements of villus heights and crypt depths for all diet samples were conducted. The prepared histological samples underwent examination using an Olympus BX63 microscope (Olympus Corp., Tokyo, Japan), an Olympus DP72 digital camera (Olympus Corp., Tokyo, Japan), and the Image-Pro Plus programme system for Windows, version 7.0 (Media Cybernetics, Inc., Bethesda, MD, USA, 2009).

Statistical analysis

The study utilised samples from three distinct dietary treatments of rabbits, with each treatment comprising ten duplicate rabbits for growth performance ($n = 10$ rabbits/diet) and six duplicate rabbits for slaughter and remained analysis ($n = 6$ rabbits/diet). Six samples per dietary treatment were collected for each intestinal segment assay ($n = 6$ samples/diet). The data analysis was conducted using SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). A one-way analysis of variance (ANOVA) test, followed by post-hoc analysis using Fisher's least significant difference test, was performed to identify differences among the diets. A calculated *p*-value of less than 0.05 ($p < 0.05$) was reconned statistically significant.

Results

Growth and slaughter performance

Growth performance features for the whole feeding trial period (52–122 days of age) are presented in Table 2. Rabbit feed enrichment with *C. glomerata* biomass did not yield a statistically significant effect on the body weight gain (BWG) or average daily gain (ADG) over the whole feeding trial period ($p > 0.05$). However, significant differences were discerned between the CG4 and CG8 dietary treatments upon examination of rabbits' daily feed intake (DFI). Specifically, a 3.48 g significant elevation in DFI was noted in CG4 in comparison to CG8 ($p < 0.05$). This trend persisted when evaluating the feed conversion ratio (FCR), with CG4, exhibiting a significantly higher FCR (4.23 kg/kg), followed by a notably

Table 2. Impact of river-sourced *C. glomerata* macroalgal biomass on rabbit growth performance.

		Diet				
Item	Trial period	SCD	CG4	CG8	SEM	*p*-value
BWG (g)	52–122	1633.00	1560.25	1492.75	240.65	n.s.
ADG (g)	days	22.59	23.74	22.04	1.27	n.s.
DFI (g)	of age	71.55^{ab}	73.79^{a}	70.31^{b}	1.13	0.013
FCR (kg/kg)		3.71^{b}	4.23^{a}	2.93^{c}	0.20	<0.001

Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass.
BWG: body weight gain; ADG: average daily gain; FCR: feed conversion ratio.
$^{a–c}$Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$).
^{ab}Means with ab superscript letters in a row were not significantly different ($p > 0.05$).
SEM: standard error of the means; n.s.: not significant ($p > 0.05$).

lower FCR in the SCD group (3.72 kg/kg), and the lowest FCR recorded in rabbits treated with CG8 (2.93 kg/kg) ($p < 0.05$).

At the end of the feeding trial, the slaughter performance was evaluated (Table 3). All anatomical components of rabbit carcases, inclusive of the intestinal organs and muscles, were extracted in percentage based on pre-slaughter weights. Subsequent analysis of these measurements revealed no statistically significant differences among the examined diets, with one exception: the lungs. A significantly higher percentage of lung persistence was discerned in rabbits treated with the SCD diet (0.57%) ($p < 0.05$), while under both CG4 and CG8 treatments, the corresponding lung percentages were 0.43%, exhibiting no significant disparity between the CG treatments ($p < 0.05$). Therefore, the examination of other distinct slaughter performance parameters resulted in no statistically significant differences among the diets in the assessment of drip loss, dressing-out percentage, carcase, leg muscle, and *longissimus dorsi* muscle yields ($p > 0.05$).

Features of intestinal segments

To assess the impact of *C. glomerata*-enriched feed on rabbit digestive processes, the pH and dry matter (DM) of the contents of distinct intestinal segments were analysed (Figure 2). Among all the analysed segments, significant differences were observed solely in the assessment of *ileum* content pH values. Rabbits treated with SCD exhibited a significantly elevated pH value in the *ileum* content, surpassing those of biomass-enriched diets CG4 and CG8 by 0.44 and 0.38 units, respectively ($p < 0.05$; Figure 2a). Nonetheless, the mechanistic underpinnings of *C. glomerata* biomass supplementation in rabbit feed and its impact on pH and DM values in other intestinal segments remain indeterminate.

The short-chain fatty acid profile of *duodenum* content is presented in Table 4. In general, acetic acid predominated across all diets, followed by propionic and lactic acids, with butyric acid notably absent in the *duodenal* contents of rabbits treated with CG8 diet. Excluding specific and significant differences between diets, SCD displayed the lowest acetic acid content (83.23%), with higher levels observed in CG8 (87.53%), and the highest content determined in CG4 (89.04%) ($p < 0.05$). After assessing propionic acid, CG8-treated rabbits exhibited 1.75 times more of this SCFA compared to those on the SCD diet ($p < 0.05$). On the contrary, no significant differences were noted in butyric acid levels, and it was entirely absent in CG8-treated rabbits' *duodenal* content ($p > 0.05$). Nevertheless, *C. glomerata* supplementation resulted in a notable reduction in lactic acid: the greater the biomass inclusion, the lower the lactic acid levels. In

Table 3. Impact of river-sourced *C. glomerata* macroalgal biomass on rabbit slaughter performance.

	Diet				
Item (%, unless stated otherwise)	SCD	CG4	CG8	SEM	*p*-value
Pre-slaughter weight (g)	2765.67	2753.00	2745.33	60.25	n.s.
Warm carcass	49.55	48.33	48.12	1.19	n.s.
Chilled carcass	47.67	46.49	46.33	1.30	n.s.
Carcass without muscles	11.10	11.49	11.40	0.79	n.s.
Head	8.12	8.12	7.91	0.33	n.s.
Intestine weight with chymus	13.41	12.18	12.45	1.75	n.s.
Stomach with content	4.31	4.48	5.20	0.97	n.s.
Stomach without content	1.63	1.45	1.38	0.14	n.s.
Heart	0.35	0.24	0.23	0.05	n.s.
Liver	4.51	6.16	4.42	0.89	n.s.
Pancreas	0.04	0.04	0.04	0.01	n.s.
Lungs	0.57[a]	0.43[b]	0.43[b]	0.04	0.017
Kidneys	0.76	0.65	0.62	0.06	n.s.
Perirenal fat	0.94	0.98	0.93	0.10	n.s.
Abdominal fat	4.13	4.19	4.22	0.33	n.s.
Fore part (without *longissimus dorsi* and legs)	5.25	4.84	5.12	0.23	n.s.
Hind part	19.33	19.17	19.03	0.80	n.s.
Longissimus dorsi muscle	7.28	7.31	7.27	0.31	n.s.
Thighs with bone	13.37	13.31	13.04	1.18	n.s.
Thighs without bone	11.14	11.28	11.07	1.24	n.s.
Shin with bone	4.60	4.63	4.31	0.37	n.s.
Shin without bone	3.50	3.54	3.47	0.33	n.s.
Longissimus dorsi yield	15.30	15.72	15.69	0.47	n.s.
Leg muscle yield	30.89	31.91	31.37	1.19	n.s.
Carcass yield	60.64	59.95	59.33	1.39	n.s.
Dressing-out percentage	49.55	48.33	48.12	3.36	n.s.
Drip loss	3.83	3.79	3.73	0.47	n.s.

Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass.
[a,b]Means with different superscript letters (a,b) in a row were significantly different ($p < 0.05$).
SEM: standard error of the means; n.s.: not significant ($p > 0.05$).

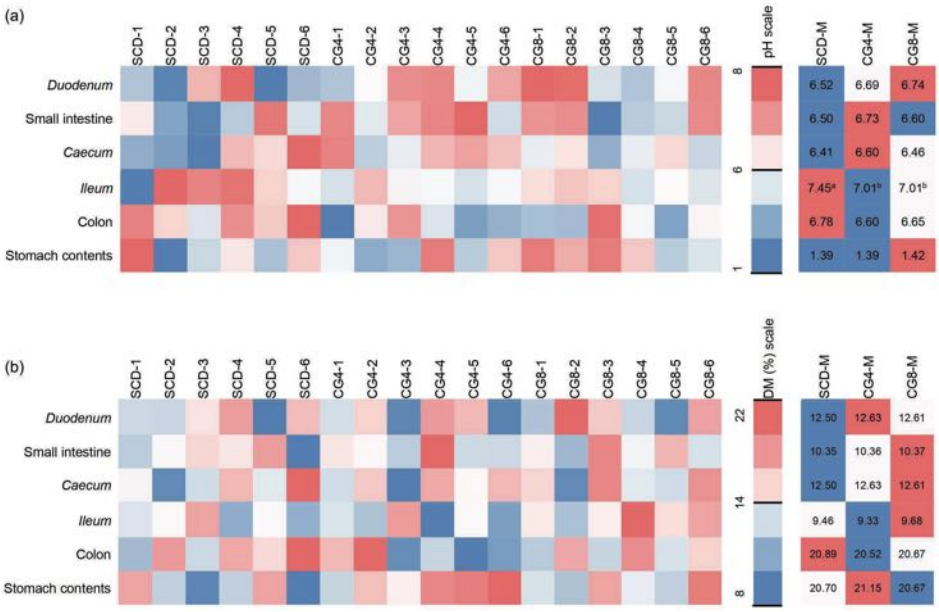

Figure 2. Impact of river-sourced *C. glomerata* macroalgal biomass on distinct intestinal segments content: (a) pH; (b) dry matter (DM).
Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass. Diet-number, means diet sample replicate number; Diet-M, represents the means of replicate samples.
[a,b]Means with different superscript letters (a,b) in a row were significantly different ($p < 0.05$).

Table 4. Impact of river-sourced *C. glomerata* macroalgal biomass short-chain fatty acids (SCFA) contents in rabbits' *duodenum*.

		Diet				
SCFA (%)	Segment	SCD	CG4	CG8	SEM	*p*-value
Acetic	*Duodenum*	83.23[c]	89.04[a]	87.53[b]	2.70	0.048
Propionic		6.18[b]	7.19[ab]	10.82[a]	1.76	0.019
Butyric		1.78	1.03	n.d.	1.28	n.s.
Lactic		8.81[a]	2.75[b]	1.65[b]	1.29	<0.001

Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass.
[a–c]Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$).
[ab]Means with ab superscript letters in a row were not significantly different ($p > 0.05$).
SEM: standard error of the means; n.d.: not defined; n.s.: not significant ($p > 0.05$).

Table 5. Impact of river-sourced *C. glomerata* macroalgal biomass on rabbits' *duodenum* and *ileum* histomorphometric measurements.

		Diet				
Item	Segment	SCD	CG4	CG8	SEM	*p*-value
Villus height (V; μm)						
	Duodenum	664.21	606.40	623.74	48.89	n.s.
	Ileum	1215.36[b]	1526.09[a]	1079.12[c]	57.11	<0.001
Crypt depth (C; μm)						
	Duodenum	117.57[b]	153.56[ab]	191.97[a]	18.41	0.002
	Ileum	217.29[a]	142.42[ab]	90.82[b]	38.70	0.007
V/C ratio						
	Duodenum	5.73[a]	4.16[b]	3.37[b]	0.69	0.005
	Ileum	6.60[a]	11.31[b]	12.15[b]	1.67	0.006

Diets: SCD, standard compound diet; CG4, SCD enriched with 4% macroalgal biomass; CG8, SCD enriched with 8% macroalgal biomass.
[a–c]Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$).
[ab]Means with ab superscript letters in a row were not significantly different ($p > 0.05$).
SEM: standard error of the means; n.s.: not significant ($p > 0.05$).

this context, compared to SCD, the lactic acid content in CG4 and CG8 was, respectively, lower by 6.06% and 7.16%, demonstrating a significant decrease ($p < 0.05$).

Histomorphometrical measurements of duodenal and ileum

Rabbit *duodenum* and *ileum* segments underwent histomorphometric evaluation (Table 5). However, no significant impact was observed on *duodenum* villus height, while in the *ileum*, the highest villus was discovered under CG4 treatment (1526.09 μm) and the lowest under CG8 (1079.12 μm). When comparing both *C. glomerata*-enriched diets, a significantly higher

446.97 μm villus was observed in the CG4 *ileum* compared to CG8; furthermore, in comparison to SCD, CG4 exhibited a significantly greater villus by 310.73 μm ($p < 0.05$).

The results of the crypt depth were distributed as follows: in the *duodenum*, the greater the biomass supplementation, the deeper the crypts (CG8 > CG4 > SCD); in the *ileum*, conversely, the greater the biomass supplementation, the lower the crypt depth (SCD > CG4 > CG8). CG8-treated rabbits exhibited 74.40 μm significantly greater *duodenal* crypt depth compared to SCD ($p < 0.05$). In contrast, CG8 *ileum* segment crypts were significantly shorter 2.39 times compared to SCD-treated rabbits ($p < 0.05$).

The villus-to-crypt ratio (V/C) exhibited a contrasting distribution: in the *duodenum*, the order was SCD > CG4 > CG8, while in the *ileum*, it was CG8 > CG4 > SCD (Table 4). In SCD-treated rabbits' *duodenum*, the V/C ratio was respectively higher by 1.57 and 2.36 units compared to both *C. glomerata* diets (CG4 and CG8, respectively) ($p < 0.05$). Conversely, biomass supplementation resulted in a higher V/C ratio in the *ileum*; it was significantly higher by 4.71 and 5.55 units in CG4 and CG8, respectively, compared to SCD ($p < 0.05$).

Discussion

Growth and slaughter performance

Rabbit growth performance significantly influences production efficiency and overall enterprise profitability (Mukaila 2023). Meeting the nutritional requirements is crucial for optimal growth, and certain *Cladophora* species, with comparable compositions to traditional plants used in feed production, simplify this principle (Heiba et al. 1997). Studies on *C. glomerata* biomass highlight its rich profile in essential fats and amino acids (Messyasz et al. 2015; Nutautaitė et al. 2021; Nutautaitė et al. 2022b). Notably, exogenous amino acids, known for enhancing nutrient digestibility and elevating quality, hold promise for more efficient growth performance in animals (Konkol et al. 2018). Despite macroalgal prevalent use in supplementing aquaculture and poultry feed, there is limited research on the application of *C. glomerata* in rabbit nutrition. Scientific evidence suggests that an optimal dosage ranging from 10% to 20% of *C. glomerata* can optimise productivity traits, including feed conversion ratio, in tiger shrimp (Anh et al. 2018). Similarly, in research on broiler chickens, a 15% supplementation of *C. glomerata* biomass in their feed demonstrated enhanced growth rates compared to standard feed (Abid and Abid 2006). Recent research into the growth performance of rabbits, incorporating river-sourced *C. glomerata* biomass throughout the entire feeding trial (52–122 days of age), indicates a lack of statistically significant impact on BWG and ADG across dietary treatments. However, notable differentials in DFI and FCR prompt a nuanced exploration of potential underlying mechanisms.

Daily feed intake, a crucial indicator of growth performance, signifies the essential quantity of feed required for the nutritional needs and well-being of animals (Kumar et al. 2022). So, achieving optimal growth entails a delicate balance, as insufficient intake may lead to malnutrition and poor growth, while excessive consumption can result in obesity and other health issues. In a recent study, a significant increase in DFI observed in CG4 compared to CG8 implies a potential impact on the palatability or satiety response to a diet enriched with 4% macroalgal biomass. This aligns with prior studies highlighting the palatability of diets incorporating macroalgae across various animal species (Øverland et al. 2019). The enhanced palatability may be attributed to bioactive compounds in *C. glomerata*, such as polyphenols and secondary metabolites, known to influence the taste and acceptability of feed ingredients (Bruneel et al. 2013; Nutautaitė et al. 2022a; Ramesh Kumar et al. 2019). Conversely, optimal biomass inclusion was determined to be 4%, as higher amounts led to reduced DFI in an 8%-enriched diet, indicating a potential upper limit for acceptable inclusion.

A lower FCR in rabbits indicates more efficient conversion of feed into body weight, directly affecting production costs and overall profitability in commercial rabbit farming operations (Exequiel et al. 2021). Furthermore, the distinct FCR patterns observed among the dietary regimens may indicate differences in feed utilisation efficiency. The notably lower FCR in CG8 observed in a recent study could potentially be linked to altered digestive processes or nutrient absorption influenced by the components of *C. glomerata*. For example, macroalgae are recognised reservoirs of bioactive compounds, including polysaccharides and peptides, which may modulate nutrient utilisation and absorption in the gastrointestinal tract (Beaulieu et al. 2015; Harnedy and FitzGerald 2011; O' Brien et al. 2022; Pimentel et al. 2019). In our case, the higher dosage of river-sourced biomass reduces FCR, while the lower dosage, on the contrary, increases this indicator. This means that feed enriched with 8% biomass could lead to more economical feed conversion into rabbit body weight.

The healthy growth and development of rabbits rely on the proper maturation of internal organs and

intestines, crucial for effective nutrient absorption and maintaining a robust immune system (Koletzko et al. 1998). Following the feeding trial, slaughter performance was assessed by dissecting all anatomical components of rabbit carcases. No significant differences were found among treatments, except for lung percentage. Rabbits on the SCD showed a notable increase in lung percentage, while both *C. glomerata*-treated groups had comparable, smaller percentages. The observed rise in lung percentage in SCD-treated rabbits, despite the consistent impact of biomass on carcase composition, prompts further consideration and raises questions about the potential physiological mechanisms of biomass. One possible explanation for the impact on lung development and respiratory function is the presence of antioxidants, such as flavonoids and carotenoids, in *C. glomerata* biomass (Nutautaitė et al. 2022a). These antioxidants can reduce oxidative stress in the gut, potentially promoting intestinal health and suggesting a connection to the modulation of respiratory processes and lung development. Similar lung percentages in *C. glomerata* treatments imply that changes in biomass levels may not significantly affect lung percentage, suggesting no dose-dependent relationship with the observed physiological outcome.

Moreover, the assessment of additional slaughter performance parameters revealed no significant differences among dietary regimens, whether incorporating *C. glomerata* or not. Biomass in rabbit diets had a minimal impact on specific aspects of slaughter performance, such as meat water-holding capacity and the efficiency of converting live weight into carcase weight. The similar yields obtained clearly emphasise that the overall composition of these meat-associated anatomical structures remains largely unchanged with the dietary inclusion of *C. glomerata*.

Features of intestinal segments

In assessing the impact of *C. glomerata*-enriched feed on rabbit digestive processes, the focus was initially on pH and DM analysis. Significantly elevated pH values were observed in the *ileum* content of rabbits under the SCD compared to CG diets. However, the mechanistic underpinnings of biomass enrichment and its effects on pH and DM values in other intestinal segments remain uncertain. Variations in *ileum* pH may implement potential modulation of microbial ecology and fermentation processes, consistent with previous research indicating macroalgal prebiotic properties influencing gut microbiota composition (Michalak and Chojnacka 2015; O'Sullivan et al. 2010; Shannon et al. 2021). Therefore, the absence of significant variations in other intestinal segments among dietary treatments prompts exploration into nuanced interactions between *C. glomerata* supplementation and digestive processes throughout the gastrointestinal tract.

In scrutinising dietary impacts on rabbit physiology, *duodenal* content analysis reveals a consistent SCFA profile. Acetic acid predominates, followed by propionic and lactic acids, aligning with established SCFA patterns linked to gastrointestinal microbial fermentation. Significantly, the predominant acetic acid serves as a vital energy substrate in rabbit tissues, enhancing metabolic processes and influencing dietary fat utilisation (He et al. 2020). Additionally, it may regulate appetite and satiety, impacting overall dietary intake in rabbits. The elevated acetic acid content in CG4 and CG8 shows a potential enhancement of microbial fermentation processes, possibly influenced by *C. glomerata's* prebiotic properties. This aligns with trends observed in prior studies on algae-based diets, promoting microbial activity and SCFA production in various animal species (Kulshreshtha et al. 2020; Patel et al. 2021). In contrast, following propionic acid in *duodenal* content, has been implicated in glucose metabolism (Lemosquet et al. 2004). It acts as a gluconeogenesis precursor, actively contributing to hepatic glucose production—a critical metabolic pathway for maintaining blood glucose levels and meeting energy requirements, especially during fasting or increased energy expenditure. The noteworthy decrease in lactic acid levels with increasing *C. glomerata* biomass highlights the potential prebiotic effects of specific macroalgal constituents, influencing gut microbiota composition. Moreover, the dose-dependent reduction in lactic acid content accentuates *C. glomerata's* capacity to induce shifts in microbial populations along the gastrointestinal tract. In rabbits, lactic acid, which is consistently present, shows a dose-dependent decrease, typically associated with microbial fermentation processes in the *caecum* and colon (Davies and Rees Davies 2003). However, the reduction in lactic acid content in *duodenum* in response to CG diets suggests potential alterations in microbial populations, with implications for overall gut health and fermentation efficiency. Butyric acid, on the other hand, emerges from dietary fibre fermentation and has been linked to a variety of health advantages (Wang et al. 2019). Nevertheless, the lack of butyric acid in CG8-treated rabbits' *duodenum* content demands a more in-depth investigation of the processes driving microbial metabolism and SCFA production in response to *C. glomerata*.

Histomorphomentrical properties of duodenum and ileum

Histomorphometric scrutiny is essential for comprehending the impact of alternative feed components on structural changes in the intestinal mucosa, including villus height and crypt depth, which directly influence nutrient absorption and overall gut health. Firstly, assessing the adaptability and response of intestinal tissue is crucial for optimising dietary formulations to enhance nutrient utilisation and promote animal well-being (Candebat et al. 2023). Secondly, histomorphometric assays are linked to the detection of possible issues or benefits connected with alternate feed components, directing towards the development of diets that improve digestive function and overall animal performance (der Poel et al. 2020). In a recent study, enriching rabbit diets with river-sourced *C. glomerata* significantly impacted mucosal architecture. While *duodenal* villus height remained stable, the *ileum* exhibited variation, being highest under CG4, lowest on CG8, and lower under SCD. In this case, increased villus height in the rabbit *ileal* segment indicates better mucosal morphogenesis, allowing for a larger surface area beneficial to improved nutrient absorption (Yu and Chiou 1997). In conclusion, the heightened villus in the rabbit *ileum* under a 4% biomass-enriched diet suggests improved digestion, better nutrient use, and potential benefits for optimal rabbit growth.

Crypt depth in the *duodenum* increased with increasing biomass supplementation (CG8 > CG4 > SCD), although in the *ileum* it decreased with increased biomass (SCD > CG4 > CG8). The importance of crypt depth stems from its ability to represent the structural dynamics of the intestinal mucosa. A deeper crypt in the *duodenum* signifies faster cell turnover and nutritional absorption capability, whereas a shallower crypt in the *ileum* indicates a more mature mucosal architecture (Modina et al. 2021). The observed differences highlight the segment-specific influence of *C. glomerata* on rabbit intestinal histomorphometry. Biomass impact on microbial fermentation, nutrient availability, and the modulation of specific signalling pathways involved in mucosal development are all potential mechanisms through which it may influence crypt depth. According to Al-Sagheer et al. (2023) reduced *duodenal* crypt depth in rabbits suggests mature mucosal architecture, whereas increasing crypt depth demonstrates active mucosal development as well as potential nutritional absorption augmentation. Reduced crypt depth in the *ileum* indicates a stabilised mucosal architecture, whereas increasing crypt depth suggests active mucosal development as well as improved nutritional absorption.

Distinct V/C ratio patterns were observed in rabbit *duodenum* and *ileum* segments under *C. glomerata* treatments. However, SCD-treated rabbits exhibited a higher V/C ratio in the *duodenum* than both *C. glomerata*-enriched diets, suggesting increased absorptive capacity. Conversely, the *ileum* displayed an improved ratio with higher *C. glomerata* enrichment, indicating a positive impact on mucosal architecture and nutrient absorption. These findings underscore the segment-specific effects of *C. glomerata* on intestine histomorphometry, potentially influenced by biologically active substances in macroalgal biomass. The observed variations in V/C ratios highlight the intricate balance between villi and crypts, reflecting the structural dynamics of the intestinal mucosa and providing nuanced insights into nutrient absorption efficiency and overall gastrointestinal health in rabbits.

Conclusions

Incorporating river-sourced *C. glomerata* in rabbit diets had no significant impact on body weight gain or average daily gain. However, the 8% biomass-enriched diet resulted in lower daily feed intake and feed conversion ratio, suggesting potential economic benefits. Both *C. glomerata*-enriched diets reduced lung percentage without significantly affecting remaining carcase anatomical components or overall slaughter performance, suggesting minimal structural alterations due to dietary biomass inclusion. Biomass-enriched diets increased acetic and propionic acid levels but reduced lactic acid in *duodenal* content, showing potential improvement in microbial fermentation. From a histomorphometric point of view, *duodenal* villus height remained stable, while the *ileum* exhibited dose-dependent variations, with a 4%-enriched diet resulting in greater villus height. *Duodenal* crypt depth increased with biomass supplementation, indicating potential effects on cell turnover and nutrient absorption. Conversely, in the *ileum*, increased biomass led to decreased crypt depth, suggesting a more mature mucosal architecture. Overall, recent research suggests that incorporating *C. glomerata* into rabbit diets has the potential to be a viable alternative to traditional feed materials with no adverse impacts on growth, slaughter performance, or physiological development.

Ethical approval

The research adhered to the guidelines outlined in Directive 2010/63/EU of the European Parliament and the Council (22 September 2010) on the protection of animals used for scientific purposes. Additionally, it followed the Commission's

recommendation (18 June 2007) for the welfare of animals in farming. Ethical approval (Bioethical permit No. PK042495) was obtained from the Lithuanian University of Health Sciences Centre of Postgraduate Studies on 7 November 2022, sanctioned by the State Food and Veterinary Service through official letters (No. B6-(1.9)-2625 dated 16 October 2013 and updated on 28 March 2017, No. B6-(1.9)-852).

Authors' contributions

Conceptualisation, MN, and VV; methodology, MN, SB, and AP; software, MN, and AP; data collection, MN, VV and ARS; investigation, MN, ARS, SB, AP and VV; resources, MN and VV; writing—original draft preparation, MN; writing—review and editing, VV and ARS; supervising, VV. All the authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

The data that supports the findings of this study are available on request from the corresponding author.

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Enhancement of Rabbit Meat Functionality by Replacing Traditional Feed Raw Materials with Alternative and More Sustainable Freshwater *Cladophora glomerata* Macroalgal Biomass in Their Diets

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Abstract: Today's challenges in the animal husbandry sector, with customers' demand for more beneficial products, encourage the development of strategies that not only provide more sustainable production from the field to the table but also ensure final product functionality. Thus, the current research was aimed at replacing some traditional feed raw materials in rabbit diets with *C. glomerata* biomass to improve the functionality of meat. For this purpose, thirty weaned (52-d-old) Californian rabbits were assigned to 3 dietary treatments: standard compound diet (SCD), SCD + 4% *C. glomerata* (CG4), and SCD + 8% *C. glomerata* (CG8). At the end of the feeding trial, 122-d-old rabbits were slaughtered, *longissimus dorsi* (LD) and hind leg (HL) muscles were dissected post-mortem, and moisture, protein, and lipid profiles were determined. Results revealed that CG4 treatment can increase protein (22.17 g/kg), total (192.16 g/kg) and essential (threonine, valine, methionine, lysine, and isoleucine) amino acid levels in rabbit muscles. Both inclusions gradually reduced fat accumulation in muscles (CG8 < CG4 < SCD) but improved the lipid profile's nutritional value by decreasing saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) and increasing polyunsaturated fatty acids (PUFA). As the dose of *C. glomerata* increased, the level of lipid oxidation decreased. Biomass supplementation enhanced PUFA/SFA and h/H levels while decreasing thrombogenicity index (TI) and atherogenic index (AI) levels in rabbit muscles, potentially contributing to the prevention of heart disease. Overall, dietary supplementation with *C. glomerata* biomass may be a more beneficial and sustainable nutritional approach to functionally enhancing rabbit meat.

Keywords: amino acids; fatty acids; lipid stability; functional food; bioactive compounds; alternative feedstuff; macroalgae

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1. Introduction

A long-term strategy for developing intensive but sustainable livestock farming is required, as a rapidly growing population directly correlates with steadily increasing demand for high-quality animal products. Considering the economic benefits and today's challenges, to at least partially replace traditional feed additives that are predicted to be in short supply, novel feed materials are being sought [1]. It is necessary not only to search for but also to analyse the potential uses of alternative feed materials to develop effective technological preparation and volarization methods, as well as to ensure high quality and safety, including sustainability, antinutritional factors, and potential toxicity, ultimately creating functional feed and a functional final product. Algae, for instance, are cultivated and used for nutritional or other purposes all over the world, and their biomass is recognised as a source of essential vitamins, minerals, proteins, polyunsaturated fatty acids, and antioxidants [2,3]. The great potential of algae comes from the fact that, compared to

conventional agricultural crops, they can be grown in places where other plants cannot grow, and their biomass is produced extremely quickly [4]. Due to the more efficient use of solar energy, algae can produce biomass enriched with bioactive compounds that can be used to improve the nutritional value of food and feed [5]. Algae is already successfully used as animal feed or as a functional feed additive in many parts of the globe [6–9]; and in the European Union it can be used as feed material according to Commission Regulation (EU) No. 68/2013 of 16 January 2013, regarding the catalogue of feed materials.

Specifically, *Cladophora* species, whether marine or freshwater, are distinguished as ecologically and economically important macroalgae. These species perform key ecosystem functions, and their biomass is used for a variety of purposes, such as soil improvement additives, fertilizers, plant growth stimulants, food and animal feed, pharmaceuticals, cosmetics, wastewater treatment, and renewable biofuels [10,11]. The green macroalgae *Cladophora glomerata* (*C. glomerata*) thrives and forms large communities in nutrient-rich bodies of water, especially slow-flowing rivers [12]. However, the blooms cause a reduction in biodiversity since the biomass assemblage consists mainly of only one species of green algae, reducing the recreational value of water bodies and creating harmful ecological and economic impacts. Frequently, due to its relatively high protein content, *C. glomerata* is recommended to be integrated into both human and animal diets [13]. Both the food and feed industries utilise this biomass as a raw material that contains few calories but many nutrients, vitamins, and fibres [14]; specifically, *C. glomerata* is characterised by a rich spectrum of bioactive components, which is reflected in the chemical composition, especially in the profiles of amino and fatty acids, phenolic compounds [3,8,14–16]. Thus, when viewed through the lens of today's animal husbandry problems (general concept of sustainability, lack of feed materials, and greenhouse gas emissions), the biomass of *C. glomerata* macroalgae would be eligible for use as a feed ingredient. Thus, harvesting excess biomass from water bodies and incorporating it into feed production would adapt waste as a raw material, resulting in a more sustainable production chain.

According to recent research, *Cladophora* species have been commonly used to supplement aquaculture diets [17–19]. Furthermore, the incorporation of algae into animal feed has been demonstrated in experiments to help reduce intestinal dysbiosis in pigs and could potentially be an alternative in rabbit farming [20]. Additionally, algae integration in rabbit diets has the potential to become a commercial marketing strategy for attracting new consumers who are conscious of environmental sustainability and seeking innovative, high-quality foods. In this regard, it has already been established that supplementing animals' feed with algae directly increases meat quality [17,19,21,22]. Moreover, there is limited research on the effects of *Cladophora* biomass on rabbits, and the demand for rabbit meat is expanding as the concept of healthy nutrition among consumers becomes more common [23]. Meat and its derivatives may be termed functional foods since they contain various beneficial components, and to create a more functional production, various strategies for combining qualitative or quantitative adjustments into meat occur [24]. However, functional feed must first be established in order to produce even more functional rabbit meat. Alternative feeds formulated with *C. glomerata* biomass, despite its higher fibre content, can be adapted to the diet of rabbits, considering their unique digestive physiology (caecotrophy) [25]. In this way, rabbits could achieve high productivity results and health status by eating high-fibre feed and, at the same time, produce functional meat production. Thus, the goal of this study was to replace some traditional feed raw materials in rabbit diets with different dosages of *C. glomerata* biomass to improve meat functionality and determine different muscle protein and lipid profiles.

2. Materials and Methods

2.1. Animal and Experimental Treatments

The research was conducted at a local rabbit breeding farm where animals were farmed indoors in individual cages (34 × 34 × 61 cm; 1 rabbit per cage) and had free access to individual nipple drinkers with clean drinking water and feed bowls to ensure

optimal health conditions and performance. The heating system in the building maintained a temperature of 19 ± 2 °C. Housing standards were in accordance with Council Directive 98/58/EC of 20 July 1998, concerning the protection of animals kept for farming purposes.

The trial was carried out with thirty weaned (52-d-old) Californian breed male rabbits. Selected by similar weight, rabbits were randomly assigned to 3 dietary treatments (n = 10 rabbits/diet) and were fed twice a day with a standard compound diet (SCD), SCD supplemented with 4% freshwater *C. glomerata* biomass (CG4), and SCD supplemented with 8% *C. glomerata* biomass (CG8). The standard compound diet was formulated and analysed to cover the nutrient requirements of growing rabbits, including vitamins and minerals (Table 1), as recommended by the National Research Council [26].

Table 1. Ingredients in rabbit feed and chemical composition of a standard compound diet and diets supplemented with different dosages of *C. glomerata* biomass (52–122 days old).

	Diet [3]		
Ingredient (%) [1,2]	**SCD**	**CG4**	**CG8**
Corn	3.00	3.13	3.44
Barley	18.00	18.00	18.00
Oats	25.00	25.00	25.00
Sunflower meal	13.22	11.97	9.97
Linseed meal	1.00	1.00	1.00
Soy meal	3.72	3.00	3.00
Vegetable oil	1.00	1.00	1.00
Beer east	2.00	2.00	2.00
Hay	29.26	27.10	24.79
C. glomerata	–	4.00	8.00
Antimycotoxin	0.30	0.30	0.30
Vitamin-mineral premix	3.50	3.50	3.50
Total	100	100	100
Chemical composition (%)			
DE (MJ/kg)	10.49	11.13	12.16
Crude protein	17.54	17.53	17.54
Crude fibre	13.56	14.39	15.05
Ash	10.03	10.37	10.31
Ether extract	3.12	3.20	3.10
NDF	32.49	34.19	35.89
ADF	19.71	20.12	20.73
ADL	4.89	4.94	5.15

Note: [1] Vitamin and mineral premix (per kg of feed): vitamin A 10.08 TV, vitamin D_3 1.14 TV, vitamin E 50.30 mg, vitamin K_3 0.99 mg, vitamin B_1 3.71 mg, vitamin B_2 2.80 mg, vitamin B_5 9.80 mg, vitamin B_{12} 0.01 mg, nicotinic acid 20.40 mg, folic acid 0.22 mg, choline chloride 170.00 mg, Mg 76.28 mg, Fe 317.00 mg, Zn 110.89 mg, Cu 19.16 mg, Co 0.29 mg, I 0.67 mg, Se 0.31 mg. [2] DE, diet energy; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin. [3] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass.

Throughout the study, all the groups received the rations twice a day with an *ad libitum* access to the feed, supplied in the form of pellets. The biomass used in the production of feed was collected from the Šventoji River in Lithuania. Previously examined and described parameters of biomass chemical indicators, nutritional value, and antioxidant activity are published in previous studies [15,16,27]. The protocol structure from river to muscle analysis is presented in Figure 1.

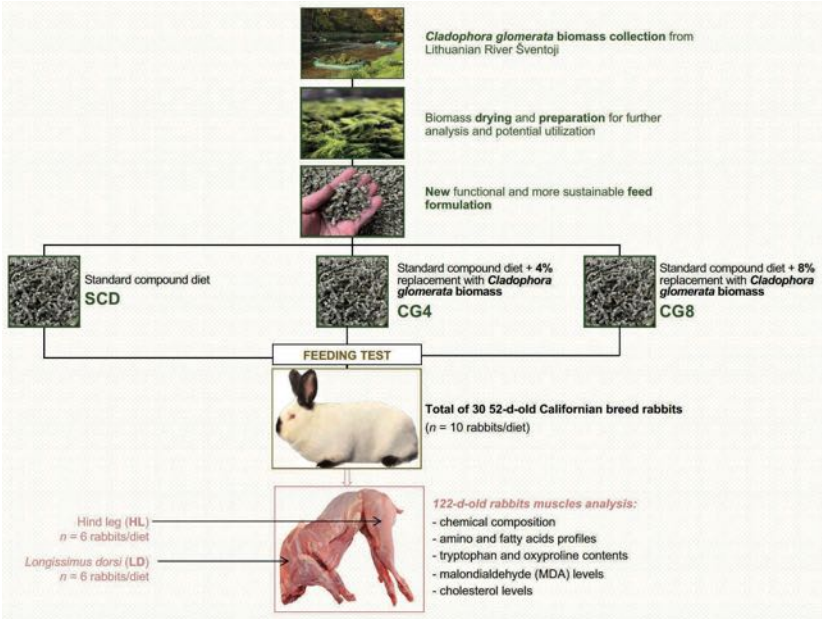

Figure 1. Protocol structure from the Šventoji River to rabbit muscle analysis.

2.2. Samples Collection

At the end of the feeding trial (122-d-old), 18 rabbits (n = 6 rabbits/diet) were randomly selected, weighed, starved overnight, and then euthanized in accordance with normal farming practice. The slaughter was carried out at a rabbit farm slaughterhouse in line with the established procedures which comply with the law of the Republic of Lithuania (Order No B1-866 of 31 October 2012 of the Director of the State Food and Veterinary Service on the approval of requirements for the keeping, care, and use of animals for scientific and educational purposes).

The carcasses of rabbits were prepared as reported by Blasco and Ouhayoun [28] and chilled at 4 °C for 24 h in a ventilated room. From the reference carcasses, the *Longissimus dorsi* (LD) and hind leg (HL) muscles were separated. The dissection procedures of warm and chilled carcasses followed the World Rabbit Science Association (WRSA) recommendations [28]. A total of 36 rabbit muscle samples were collected 24 h post-mortem, minced, and stored at −80 °C (as fresh meat; LD0 and HL0 (n = 6 LD/diet; n = 6 HL/diet)) and at −18 °C for 3 months (as stored meat; and LD3 and HL3 (n = 6 LD/diet; n = 6 HL/diet)) for later malondialdehyde (MDA) analysis. Other meat quality traits (chemical composition, amino and fatty acids profiles, and cholesterol levels) were analysed on fresh meat (n = 6 LD/diet; n = 6 HL/diet).

2.3. Reagents

Sulfuric acid, hydrochloric acid, formic acid, perchloric acid, sodium nitrate, sodium hydroxide, potassium hydroxide, potassium chloride, sodium sulfate anhydrous, potassium dihydrogen phosphate, chloramine T trihydrate, trichloroacetic acid, 2-thiobarbituric acid, ethylenediaminetetraacetic acid (EDTA), propylgallate, 4-(dimethylamino)benzaldehyde, petroleum ether (b.p. 40–60 °C), methanol, ethanol, hexane, acetonitrile, 2-propanol, chlo-

roform, sodium methoxide solution, amino acid standard, L-tryptophan, hydroxyproline, L-2-aminobutyric acid, and 1,1,3,3-tetraehoxypropane were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA).

Supelco 37 comp. FAME mix, linoleic acid methyl ester isomer mix were purchased from Supelco (Supelco Analytical, Bellefonte, PA, USA), trans FAME mix K110 from Grace (Grace, Deerfield, IL, USA).

AccQ Fluor reagent kit and AccQ Tag eluent A concentrate was purchased from Waters (Waters Corp., Miliford, MA, USA). Boric acid was purchased from AFT (Bratislava, Slovakia), Kjeltabs from Velf Scientifica (Velf Scientifica srl, Usmate, Italy).

2.4. Chemical Assay of Muscles

Total protein content was determined by the Kjeldahl method [29]. Intramuscular fat content was determined by the Soxleth extraction method [30] by extraction with petrol ether (b.p. 40–60 °C). The ash content was determined by incinerating the samples in a furnace at 550 °C [30]. The dry matter content of different rabbit muscles was determined by drying samples at 60 °C, after which they were equilibrated to room humidity overnight, milled and passed through a 1-mm sieve, and further dried at 105 °C to a constant weight.

2.5. Amino Acid Profile

Hydrolysis of rabbit muscle samples for amino acid analysis was proceeded as described in Commission regulation (EC) No 152/2009. The amino acid assay was performed by AccQ Tag technology (Waters Corp., Miliford, MA, USA). For amino acid analyses in samples, a Shimadzu low pressure gradient high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Kyoto, Japan) consisting of a solvent delivery module LC-10ATVP, auto injector SIL-10ADVP, column oven CTO-10ACVP, spectrofluorometric detector RF-10AXL, system controller SCL-10AVP, and online degasser DGU-14A was used. Workstation LC Solution (Shimadzu Corp., Kyoto, Japan) for HPLC system control and data collection was used. Amino acid derivatives were separated on Nova-Pak C18, 4 μm, 150 × 3.9 mm chromatography column (Waters Corp., Miliford, MA, USA) with a temperature of 37 °C. A total of 10 μL of derivatives was injected to separation. Separated derivatives were detected at E_x 250 nm–E_m 395 nm wavelengths. A gradient flow was used for separation of amino acids derivatives. Flow rate was set at 1 mL/min. Amino acids were identified by the retention times as compared to the retention times of the amino acid standard solution. The results were calculated by measuring the peak areas of the sample and standard solution for each amino acid.

The content of hydroxyproline in rabbit muscles samples was measured spectrophotometrically at 560 nm, as described by Stegemann and Stalder [31], and the content of tryptophan at 610 nm according to the procedure described by Miller [32]. Based on the obtained values, the tryptophan and hydroxyproline (T/H) ratio was calculated.

2.6. Fatty Acid Profile

Extraction of lipids for fatty acid analysis was performed with chloroform/methanol (2:1 *v/v*), as described by Folch et al. [33]. Fatty acid methyl esters (FAME) of the total lipids were prepared according to the procedure described by Christopherson and Glass [34].

The FAMEs were analysed using a gas chromatograph Shimadzu GC—2010 Plus (Shimadzu Corp., Kyoto, Japan) fitted with flame ionization detector. The separation of methyl esters of fatty acids was carried out on a capillary column Rt-2560 (100 m; 0.25 mm ID; 0.25 μm df) (Restek, Bellefonte, PA, USA) by temperature programming from 160 °C to 230 °C. The temperature of the injector was 240 °C, and the detector was 260 °C. The rate of flow of carrier gas (nitrogen)—0.79 mL/min. The injection volume was 1.0 μL.

The FAMEs were identified by comparing their retention times with those of the authentic standard mixtures: Supelco 37 comp. FAME mix; trans FAME mix K110; Linoleic acid methyl ester isomer mix. The relative content of each fatty acid in the sample was expressed as relative percentage of the sum of the fatty acids.

The average amount of each fatty acid was used to calculate the total of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. Lipid quality indices, atherogenic index (AI), and thrombogenicity index (TI), were calculated according to Ulbricht and Southgate [35]:

$$AI = [C12:0 + (4 \times C14:0) + C16:0] / [\Sigma PUFA\ n6 + \Sigma PUFA\ n3 + \Sigma MUFA]$$

$$TI = [C14:0 + C16:0 + C18:0] / [(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma PUFA\ n6) + (3 \times \Sigma PUFA\ n3) + (\Sigma PUFAn3 / \Sigma PUFAn6)]$$

The hypocholesterolemic/hypercholesterolemic (h/H) ratio was calculated according to Fernández et al. [36]:

$$h/H = (C18:1\ n9 + C18:2\ n6 + C20:4\ n6 + C18:3\ n3 + C20:5\ n3 + C22:5\ n3 + C22:6\ n3) / (C14:0 + C16:0)$$

2.7. Cholesterol and Lipid Oxidation Levels

The cholesterol content in rabbit muscles was determined according to the method described by Polak et al. [37]. A high-pressure gradient HPLC system Varian ProStar (Varian, Inc., Palo Alto, CA, USA) was used for the cholesterol content determination. Cholesterol separation was performed on a LiChrospher 100 RP-18e (150 × 4.6 mm, 5 µm) chromatography column (Alltech Associates Inc., Deerfield, IL, USA), and the chromatogram was processed at a wavelength of 210 nm. As the mobile phase, a mixture of acetonitrile and 2-propanol (55:45) at a flow rate of 1.0 mL min−1 was used. The chromatography data were summarized and averaged from 6 replicate rabbit muscle samples for each treatment.

An assay of lipid oxidation (malondialdehyde (MDA) content) levels in the muscle samples was tested at two intervals: 24 h post-mortem and after 3 months following the slaughter; the determinations were carried out by the high-performance liquid chromatography method described by Mendes [38]. For this purpose, a high-pressure gradient HPLC system Varian ProStar (Varian, Inc., Palo Alto, CA, USA) with a ProStar 363 fluorescence detector was used. The separation of malondialdehyde–2–thiobarbituric acid (MDA-TBA) was performed on a Gemini C18 (250 × 4.6 mm, 5 µm) chromatographic column (Phenomenex, Inc., Torrance, CA, USA). The mobile phase consisting of 50 mM KH2PO4, methanol, and acetonitrile at a ratio of 72:17:11 was supplied at a flow rate of 1.0 mL min^{-1}. The MDA-TBA compound was identified and quantified by measuring the fluorescence at E_x 525 nm–E_m 560 nm wavelengths. MDA-TBA compound was quantified by comparison between the peak area of the MDA-TBA compound in a sample and the peak area of this compound in standard solution.

2.8. Statistical Analysis

The study used samples from 3 groups of rabbits, each with 6 rabbit duplicates (n = 6 duplicate rabbits/diet); from one rabbit, two kinds of muscles were taken as samples for further analysis (n = 6 LD/diet; n = 6 HL/diet). Data analysis was performed by SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). A one-way analysis of variance (ANOVA) test post-hoc (Fisher's least significant difference test) was conducted to detect differences among treatments. A calculated p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Chemical Composition of Rabbit Muscles

After the inclusion of freshwater *C. glomerata* biomass in the rabbits' diet, the chemical composition of the different rabbit muscles was analysed (Table 2). According to recent findings, CG4 treatment resulted in the highest protein levels in LD muscles, reaching 22.17%. Compared to 8% biomass inclusion (CG8), protein levels in LD muscles from CG4 rabbits were higher by 1.81% ($p < 0.05$). However, after HL muscle analysis, the highest protein levels were discovered under SCD treatment. Therefore, the results were not significant ($p > 0.05$). The fat content of both muscles decreased as the biomass dosage in

the diet increased. Therefore, the highest amount of fat was found in SCD, with a fat content of 1.62% in LD and 2.73% in HL, respectively. Compared to *C. glomerata* experimental diets, CG4 and CG8 fat contents in LD were respectively lower by 0.47% and 1.08%, and in HL, respectively, lower by 1.29% and 1.90%, compared to SCD ($p < 0.05$).

Table 2. Impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on the chemical composition of different rabbit muscles.

		Diet [3,4,5]				
Item (%) [1]	**Muscle** [2]	**SCD**	**CG4**	**CG8**	**SEM** [6]	***p*-Value** [7]
Protein	LD	21.19 [ab]	22.17 [a]	20.36 [b]	0.51	0.003
	HL	20.65	19.43	19.69	0.69	n.s.
Fat	LD	1.62 [a]	1.15 [b]	0.54 [c]	0.11	0.000
	HL	2.73 [a]	1.44 [b]	0.83 [c]	0.28	0.000
Ash	LD	1.13 [a]	1.11 [ab]	1.06 [b]	0.03	0.026
	HL	1.12	1.10	1.06	0.03	n.s.
DM	LD	25.29 [a]	24.60 [b]	22.42 [c]	0.26	0.000
	HL	25.63 [a]	23.55 [b]	22.47 [c]	0.31	0.000

Note: [1] DM, dry matter. [2] LD, *longissimus dorsi*; HL, hind leg. [3] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [4] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [5] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [6] SEM, standard error of the means. [7] n.s., not significant ($p > 0.05$).

The highest ash content was determined in SCD-LD muscles, which was 0.07% greater than in CG8 ($p < 0.05$). In both LD and HL muscles, the dry matter (DM) levels differed significantly across all diets ($p < 0.05$). The highest DM was found in SCD, followed by CG4, and finally CG8. Both LD and HL muscles showed the same pattern of decreasing dry matter content with increasing dosages of *C. glomerata* ($p < 0.05$).

3.2. Amino Acid Profile of Rabbit Muscles

The profile of rabbit muscle amino acids is shown in Table 3. The predominant amino acid in both tested muscles from the identified 16 amino acids was glutamic acid. Its highest amount was determined in the muscles of CG4 rabbits, at 32.63 g/kg in LD and 29.61 g/kg in HL, respectively ($p < 0.05$).

The following essential amino acids were also identified: threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, and lysine. The highest levels of threonine, valine, methionine, and isoleucine were discovered in LD muscles under 4% inclusion of *C. glomerata* biomass (CG4). In CG4-LD muscles, threonine levels were higher by 0.41 g/kg than in CG8, valine levels were higher by 0.86 g/kg than in CG8, methionine levels were higher by 2.02 g/kg, and 1.95 g/kg than in SCD and CG8, respectively, and isoleucine levels were higher by 0.84 g/kg than in CG8 ($p < 0.05$). The highest levels of leucine and phenylalanine were found in SCD-LD muscles; when compared to CG8 levels, they were 1.20 g/kg and 0.58 g/kg higher, respectively ($p < 0.05$). However, the opposite trend was discovered after HL sample analysis: higher leucine and phenylalanine levels were observed in the CG8 diet by 0.80 g/kg and 0.40 g/kg compared to SCD, respectively ($p < 0.05$). The highest concentration of histidine was found in SCD LD muscles (8.78 g/kg), and the lowest concentration was found in the mentioned muscles from CG8 (8.11 g/kg) ($p < 0.05$). The distribution of lysine concentrations in LD muscles according to the diets of rabbits was as follows: CG4 (16.71 g/kg) < SCD (15.86 g/kg) < CG8 (15.60 g/kg) ($p < 0.05$).

Table 3. Impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on the amino acid profile of different rabbit muscles.

		Diet [2,3,4]				
Amino Acid (g/kg)	**Muscle [1]**	**SCD**	**CG4**	**CG8**	**SEM [5]**	***p*-Value [6]**
Aspartic	LD	18.39 [a]	18.43 [a]	17.13 [b]	0.22	0.000
	HL	15.59 [a]	16.73 [b]	16.45 [ab]	0.42	0.016
Threonine	LD	8.58 [a]	8.65 [a]	8.24[b]	0.12	0.003
	HL	7.29	7.59	7.61	0.19	n.s.
Serine	LD	7.35	7.36	7.11	0.17	n.s.
	HL	6.32	6.83	6.89	0.31	n.s.
Glutamic	LD	31.65 [ab]	32.63 [a]	31.08 [b]	0.51	0.08
	HL	27.79 [a]	29.61 [b]	29.54 [b]	0.69	0.019
Proline	LD	7.26	7.40	7.14	0.15	n.s.
	HL	7.57	7.82	8.02	0.31	n.s.
Glycine	LD	10.10	9.94	10.01	0.20	n.s.
	HL	11.05 [a]	11.65 [ab]	12.42 [b]	0.51	0.017
Alanine	LD	10.27 [a]	10.31 [a]	9.29 [b]	0.25	0.001
	HL	8.87 [a]	9.89 [b]	10.14 [b]	0.29	0.001
Valine	LD	10.40 [a]	10.42 [a]	9.56 [b]	0.20	0.001
	HL	8.80	9.07	9.16	0.24	n.s.
Methionine	LD	8.66 [a]	10.68 [b]	8.73 [a]	0.68	0.009
	HL	7.74	8.09	8.13	0.29	n.s.
Isoleucine	LD	9.25 [a]	9.28 [a]	8.44 [b]	0.14	0.000
	HL	7.50	7.88	7.91	0.23	n.s.
Leucine	LD	14.75 [a]	14.57 [a]	13.55 [b]	0.18	0.000
	HL	12.10 [a]	12.83 [b]	12.90 [b]	0.33	0.030
Tyrosine	LD	6.63 [ab]	6.69 [a]	6.13 [b]	0.25	0.039
	HL	5.25 [a]	6.65 [b]	5.70 [b]	0.19	0.030
Phenylalanine	LD	7.47 [a]	7.36 [a]	6.89 [b]	0.10	0.000
	HL	6.28 [a]	6.60 [ab]	6.68 [b]	0.18	0.043
Histidine	LD	8.78 [a]	8.50 [ab]	8.11 [b]	0.19	0.003
	HL	7.46	7.63	7.98	0.26	n.s.
Lysine	LD	15.86 [a]	16.71 [b]	15.60 [a]	0.28	0.001
	HL	13.70	14.15	14.41	0.37	n.s.
Arginine	LD	13.90 [a]	13.23 [ab]	12.67 [b]	0.45	0.016
	HL	11.99	12.13	12.19	0.40	n.s.
Total	LD	189.30 [a]	192.16 [a]	179.74 [b]	2.58	0.000
	HL	165.32 [a]	174.33 [b]	176.13 [b]	4.18	0.021

Note: [1] LD, *longissimus dorsi*; HL, hind leg. [2] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [3] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [4] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [5] SEM, standard error of the means. [6] n.s., not significant ($p > 0.05$).

After studying both rabbit muscles, the amount of the remaining identified aspartic and tyrosine amino acids was found to be highest under CG4 treatment: respectively 18.43 g/kg in LD and 16.73 g/kg in HL; 6.69 g/kg in LD and 6.65 g/kg in HL ($p < 0.05$). Glycine levels in HL muscles were higher by 1.37 g/kg in CG8 compared to SCD ($p < 0.05$). Compared to SCD and CG4 diets, lower alanine contents were discovered in CG8 LD muscles by 0.98 g/kg and 1.02 g/kg, respectively ($p < 0.05$). However, after HL muscle analysis, alanine levels in SCD treatment were lower (8.87 g/kg) than in the CG4 (9.89 g/kg) and CG8 (10.14 g/kg) experimental diets ($p < 0.05$). The SCD rabbits' LD muscles had the greatest amounts of arginine, which were 1.23 g/kg higher than those of the CG8 ($p < 0.05$). When comparing serine and proline in both analysed rabbit muscles, no significant differences between diets were found ($p > 0.05$).

The total amount of all amino acids in grammes per kilogramme of each muscle group was calculated (Table 3). The highest total amino acid amount in LD muscles was observed in the CG4 treatment (192.16 g/kg) and the lowest in the CG8 treatment (179.74 g/kg) ($p < 0.05$). Conversely, after HL muscle analysis, results showed that the highest total amino acid content was observed under CG8 treatment (176.13 g/kg), a comparably similar amount in CG4 (174.33 g/kg; $p > 0.05$), and the lowest amount in SCD (165.32 g/kg; $p < 0.05$). When comparing the total amount of amino acids between different muscles, a

higher amount of it was found in LD muscles, assuming these muscles are also characterised by a higher protein content.

Tryptophan, a marker for muscle tissue, and hydroxyproline, a marker for connective tissue, were discovered in the muscles of rabbits (Table 4). The highest tryptophan levels were discovered in rabbits' LD muscles under the CG4 diet (30.84 g/kg), followed by the SCD diet (29.40 g/kg), and the CG8 diet (25.28 g/kg) ($p < 0.05$). Tryptophan levels in HL muscles, in contrast, were higher in SCD by 3.48 g/kg and 2.25 g/kg compared to CG4 and CG8, respectively ($p < 0.05$). Hydroxyproline levels in LD muscles ranged from 6.30 g/kg in CG4 to 7.71 g/kg in SCD and 8.85 g/kg in CG8 ($p < 0.05$). Significant differences in hydroxyproline levels in HL muscles were found only between SCD and CG4 treatments, with its levels being 2.98 g/kg higher in SCD compared to CG4 ($p < 0.05$). The CG4 treatments' LD muscles exhibit the greatest tryptophan/hydroxyproline ratio (T/H). Compared to other diets, the LD muscle ratio of CG4 (4.91) was 1.3 times higher than in SCD and even 1.7 times higher than in CG8 ($p < 0.05$). When comparing T/H in HL rabbit muscles, no significant differences between diets were observed ($p > 0.05$).

Table 4. Impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on tryptophan and hydroxyproline levels and ratios in different rabbit muscles.

		Diet [2,3,4]				
Item (g/kg)	**Muscle [1]**	**SCD**	**CG4**	**CG8**	**SEM [5]**	***p*-Value [6]**
Tryptophan (T)	LD	29.40 [a]	30.84 [b]	25.28 [c]	0.35	0.000
	HL	26.27 [a]	22.79 [b]	24.02 [c]	0.37	0.000
Hydroxyproline (H)	LD	7.71 [a]	6.30 [b]	8.85 [c]	0.23	0.000
	HL	18.71 [a]	15.73 [b]	16.70 [ab]	1.39	0.049
T/H	LD	3.82 [a]	4.91 [b]	2.86 [c]	0.13	0.000
	HL	1.43	1.50	1.44	0.13	n.s.

Note: [1] LD, *longissimus dorsi*; HL, hind leg. [2] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [3] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [4] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [5] SEM, standard error of the means. [6] n.s., not significant ($p > 0.05$).

3.3. Lipids in Rabbit Muscles

3.3.1. Fatty Acid Profile

The fatty acid profiles of rabbit muscles under the influence of SCD, CG4, and CG8 diets are presented in Table 5. Overall, the most saturated fatty acids (SFA) were found in the SCD muscles (34.38–35.61%), while the CG4 and CG8 muscles were dominated by polyunsaturated fatty acids (PUFA) (34.18–38.92%). Comparing the total amount of SFA in LD muscles, SCD had 1.76% more SFA compared to CG8, and in HL muscles, the SFA amount was lower by 1.23% and 1.97%, respectively, in CG8 compared to SCD and CG4 ($p < 0.05$). The dominant individual SFA in all muscles was palmitic acid (C16:0); the highest amounts of this acid were observed in SCD-LD and HL muscles, at 26.81% and 25.21%, respectively. Higher concentrations of capric acid (C10:0) in LD muscles, lauric acid (C12:0) in LD and HL muscles, myristic acid (C14:0) in HL muscles, and pentadecylic acid (C15:0) in LD and HL muscles were found in SCD compared to CG8 ($p < 0.05$). Margaric acid (C17:0) was mostly found in SCD LD and HL muscles, with its levels being 0.06% equally higher in LD and 0.03% and 0.05% greater in HL compared to CG4 and CG8, respectively ($p < 0.05$). Stearic acid (C18:0) dominated in CG8 LD and HL muscles (7.57% and 8.25%, respectively). Greater amounts of arachidic acid (C20:0) were found in muscles under CG diets: 0.10% in the LD muscles of CG8 and 0.09% in the HL muscles of CG4. Behenic acid (C22:0) was dominant in CG8 HL muscles. Its concentration, compared to SCD and CG4 diets, was 0.09% and 0.08% higher in CG8-HL muscles, respectively ($p < 0.05$).

Table 5. Impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on the fatty acid profile of different rabbit muscles.

		Diet [3,4,5]				
Fatty Acid (%) [1]	**Muscle** [2]	**SCD**	**CG4**	**CG8**	**SEM** [6]	***p*-Value** [7]
C10:0	LD	0.09 [a]	0.06 [ab]	0.01 [b]	0.02	0.011
	HL	0.12	0.05	0.02	0.04	n.s.
C12:0	LD	0.11 [a]	0.09 [ab]	0.06 [b]	0.02	0.018
	HL	0.15 [a]	0.08 [ab]	0.05 [b]	0.03	0.023
C14:0	LD	2.20	2.06	1.76	0.25	n.s.
	HL	2.18 [a]	2.08 [ab]	1.58 [b]	0.22	0.032
C15:0	LD	0.44 [a]	0.36 [ab]	0.31 [b]	0.03	0.008
	HL	0.42 [a]	0.35 [ab]	0.30 [b]	0.03	0.008
C16:0	LD	26.81 [a]	26.03 [a]	23.54 [b]	0.55	0.001
	HL	25.21 [a]	25.00 [ab]	22.34 [b]	0.53	0.002
C17:0	LD	0.50 [a]	0.44 [b]	0.44 [b]	0.02	0.038
	HL	0.47 [a]	0.44 [b]	0.42 [b]	0.01	0.005
C18:0	LD	5.39 [a]	6.03 [a]	7.57 [b]	0.28	0.000
	HL	5.73 [a]	7.00 [b]	8.25 [c]	0.41	0.001
C20:0	LD	0.08 [a]	0.09 [ab]	0.10 [a]	0.01	0.017
	HL	0.06 [a]	0.09 [b]	0.07 [c]	0.00	0.001
C22:00	LD	n.d.	0.04	0.05	0.03	n.s.
	HL	0.04 [a]	0.05 [a]	0.13 [b]	0.03	0.016
Σ SFA	LD	35.61 [a]	35.21 [ab]	33.85 [b]	0.68	0.042
	HL	34.38 [a]	35.12 [a]	33.15 [b]	0.45	0.004
C14:1 n7	LD	0.14 [a]	0.08 [ab]	0.03 [b]	0.04	0.039
	HL	0.18 [a]	0.12 [ab]	0.07 [b]	0.04	0.041
C16:1 n9	LD	0.34 [a]	0.33 [ab]	0.32 [b]	0.01	0.032
	HL	0.36	0.34	0.33	0.02	n.s.
C16:1 n7	LD	2.81 [a]	2.09 [ab]	1.17 [b]	0.42	0.008
	HL	3.42 [a]	2.08 [b]	1.24 [b]	0.50	0.005
C17:1 n9	LD	0.25 [a]	0.18 [b]	0.19 [b]	0.02	0.018
	HL	0.26 [a]	0.17 [b]	0.14 [b]	0.03	0.010
C18:1 n9t	LD	0.30	0.31	0.41	0.06	n.s.
	HL	0.25 [a]	0.32 [b]	0.34 [b]	0.03	0.017
C18:1 n9	LD	25.96 [a]	22.82 [b]	20.82 [b]	0.82	0.001
	HL	25.34 [a]	21.65 [b]	19.61 [c]	0.68	0.000
C18:1 n7	LD	1.08 [a]	1.07 [a]	1.16 [b]	0.03	0.022
	HL	1.10 [ab]	1.03 [a]	1.16[b]	0.05	0.041
C20:1 n9	LD	0.30	0.18	0.20	0.07	n.s.
	HL	0.20 [a]	0.18 [ab]	0.16 [b]	0.01	0.028
Σ MUFA	LD	31.18 [a]	27.04 [b]	24.30 [c]	1.01	0.000
	HL	31.12 [a]	25.90 [b]	23.05 [c]	0.94	0.000
C18:2 n6t	LD	0.09	0.07	0.06	0.01	n.s.
	HL	0.07 [ab]	0.08 [a]	0.06 [b]	0.01	0.008
C18:2 n6ct	LD	0.04	0.06	0.10	0.05	n.s.
	HL	0.08	0.02	0.14	0.08	n.s.
C18:2 n6	LD	24.40	26.51	24.21	1.47	n.s.
	HL	25.46	28.26	27.59	1.37	n.s.
C18:3 n6	LD	0.06	0.07	0.08	0.01	n.s.
	HL	0.06	0.06	0.06	0.02	n.s.
C18:3 n3	LD	2.90 [a]	2.50 [ab]	1.80 [b]	0.37	0.025
	HL	3.01 [a]	2.44 [b]	1.69 [c]	0.24	0.001
C20:2 n6	LD	0.13 [a]	0.12 [a]	0.17 [b]	0.01	0.004
	HL	0.17 [a]	0.14 [b]	0.19 [a]	0.01	0.001
C20:3 n6	LD	0.15 [a]	0.21 [a]	0.39 [b]	0.04	0.002
	HL	0.21 [a]	0.27 [a]	0.48 [b]	0.04	0.001

Table 5. *Cont.*

		Diet [3,4,5]				
Fatty Acid (%) [1]	**Muscle** [2]	**SCD**	**CG4**	**CG8**	**SEM** [6]	***p*-Value** [7]
C20:3 n3	LD	0.03	0.01	n.d.	0.02	n.s.
	HL	0.05 [a]	0.01 [b]	0.02 [ab]	0.01	0.050
C20:4 n6	LD	1.96 [a]	3.05 [b]	6.40 [c]	0.84	0.002
	HL	2.23 [a]	3.17 [a]	6.01 [b]	0.67	0.001
C20:5 n3	LD	0.06 [a]	0.11 [b]	0.14 [c]	0.02	0.019
	HL	0.05 [a]	0.08 [a]	0.12 [b]	0.01	0.002
C22:4 n6	LD	0.41 [a]	0.39 [a]	0.64 [b]	0.09	0.029
	HL	0.47 [a]	0.42 [a]	0.72 [b]	0.08	0.009
C22:5 n3	LD	0.48 [a]	0.87 [b]	1.62 [c]	0.21	0.002
	HL	0.58 [a]	0.91 [b]	1.48 [c]	0.15	0.001
Σ PUFA	LD	30.81 [a]	34.18 [b]	35.99 [b]	0.92	0.001
	HL	32.54 [a]	36.08 [b]	38.92 [c]	0.93	0.000
Σ unidentified	LD	2.48 [a]	3.70 [a]	5.96 [b]	0.89	0.008
	HL	2.11 [a]	3.02 [a]	4.96 [b]	0.45	0.001
Σ PUFA/Σ SFA	LD	0.87 [a]	0.97 [b]	1.06 [b]	0.04	0.003
	HL	0.95 [a]	1.03 [a]	1.17 [b]	0.04	0.001
omega-3 (ω-3)	LD	3.57	3.68	3.93	0.44	n.s.
	HL	3.78	3.65	3.66	0.30	n.s.
omega-6 (ω-6)	LD	27.11 [a]	30.36 [b]	31.90 [b]	1.08	0.004
	HL	28.61 [a]	32.33 [b]	35.06 [b]	1.16	0.001
(ω-6)/(ω-3)	LD	7.64	8.60	8.15	1.16	n.s.
	HL	7.65	9.03	9.57	0.99	n.s.
AI	LD	0.58	0.56	0.51	0.03	n.s.
	HL	0.54 [a]	0.54 [a]	0.46 [b]	0.02	0.022
TI	LD	0.86	0.86	0.82	0.02	n.s.
	HL	0.80 [a]	0.85 [b]	0.80 [a]	0.01	0.002
h/H	LD	1.93 [a]	2.00 [ab]	2.19 [b]	0.08	0.017
	HL	2.08 [a]	2.10 [a]	2.38 [b]	0.08	0.011

Note: [1] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; AI, atherogenic index; TI, thrombogenicity index; h/H, hypocholesterolemic/hypercholesterolemic ratio. [2] LD, *longissimus dorsi*; HL, hind leg. [3] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [4] Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$). [5] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [6] SEM, standard error of the means. [7] n.s., not significant ($p > 0.05$).

The total amount of monounsaturated fatty acids (MUFA) revealed that the SCD diet's LD and HL muscles had most of these FAs (31.18% and 31.12%, respectively). Compared to other treatments, the MUFA content in SCD was higher by 4.14% and 6.88% in LD and by 5.22% and 8.07% in HL, compared to CG4 and CG8, respectively ($p < 0.05$). Myristoleic acid (C14:1 n7), hexadecanoic acid (C16:1 n9), and palmitoleic acid (C16:1 n7) concentrations were found to be higher in SCD LD and HL muscles compared to CG8 ($p < 0.05$). Margaric acid (C17:1 n9) was higher in SCD muscles as well, compared to both *C. glomerata*-based diets (CG4 and CG8) ($p < 0.05$). A lower amount of elaidic acid (C18:1 n9t) was discovered in SCD HL muscles; it was lower by 0.07% and 0.09% compared to CG4 and CG8, respectively ($p < 0.05$). According to the different diets, oleic acid (C18:1 n9) concentrations in both LD and HL rabbit muscles were distributed like this: SCD < CG4 < CG8. Under the influence of CG8, the same concentration (1.16%) of vaccenic acid (C18:1 n7) was found in the LD and HL muscles of rabbits. When *C. glomerata* inclusion increased, eicosenoic acid (C20:1 n9) decreased. However, only one significant difference was found between the mentioned acid concentration in SCD and CG8 HL muscles: it was higher by 0.04% in SCD compared to CG8 ($p < 0.05$).

Total PUFA content in muscle increased with increasing dosages of *C. glomerata* in the rabbit diet. PUFA content reached 35.99% and 38.92% in CG8 LD and HL muscles, respectively; 34.18% and 36.08% in CG4 muscles; and only 30.81% and 32.54% in SCD muscles ($p < 0.05$). However, a lower concentration of linolelaidic acid (C18:2 n6t) was obtained in CG8 HL muscles (0.06%), while in CG4 HL muscles it was higher by 0.02% (0.08%) ($p < 0.05$). The greatest content of α-linolenic (C18:3 n3) acid was discovered in both LD and HL muscles on the SCD diet. This fatty acid was observed in 2.90% and 3.01% of the samples, respectively. In comparison to CG4 and CG8, the α-linolenic acid content was found to be nearly 1.6 times lower in the LD muscles of CG8 and about 1.2 and 1.8 times lower in the HL muscles of CG4 and CG8, respectively ($p < 0.05$). Eicosatrienoic acid (C20:3 n3) was not defined at all in LD muscles under CG8 treatment. However, after HL muscle analysis, the concentration of the mentioned acid was the highest in SCD compared to diets supplemented with *C. glomerata* ($p < 0.05$). In contrast, remaining significant differences were found when individual PUFAs (eicosadienoic (C20:2 n6), dimoho-γ-linolenic (C20:3 n6), arachidonic (C20:4 n6), eicosapentaenoic (C20:5 n3), adrenic (C22:4 n6), and docosapentaenoic (C22:5 n3) acids) were higher in CG8 muscles compared to other diets ($p < 0.05$). Eicosadienoic acid levels were 0.04% and 0.05% greater in CG8 LD muscles than in SCD and CG4 muscles, as well as 0.05% higher than in CG4 HL muscles ($p < 0.05$). The dimoho-γ-linolenic acid concentrations in both LD and HL muscles were distributed descendingly, with the lowest concentrations in SCD muscles, higher in CG4, and the highest in CG8 ($p < 0.05$). Arachidonic acid was several times higher in CG8 muscles compared to SCD and CG4 diets: 3.3 and 2.1 times higher in LD muscles, respectively, and 2.7 and 1.9 times higher in HL muscles, respectively ($p < 0.05$). The lowest levels of eicosapentaenoic acid were determined in SCD muscles, which reached only 0.06% and 0.05% in LD and HL, while in CG4 it was 0.11% and 0.08%, and in CG8 even 0.14% and 0.12% ($p < 0.05$). Although adrenic acid was mainly detected in the muscles of CG8, the next highest level of this acid was discovered in SCD, followed by CG4 ($p < 0.05$). A greatly higher concentration of docosapentaenoic acid was found in CG8 muscles compared to SCD and CG4: 3.4 and 1.9 times higher of the mentioned fatty acid was discovered in LD muscles, and 2.6 and 1.6 times more in HL muscles, respectively ($p < 0.05$).

When evaluating the entire fatty acid profile, the most unidentified fatty acids were found in CG8 muscles. Such unidentified fatty acid levels in LD and HL muscles of CG8 reached 5.96% and 4.96% from all fatty acids, while lower levels were obtained in CG4 muscles (3.70% and 3.02%) and in SCD (2.48% and 2.11%) ($p < 0.05$).

The PUFA/SFA ratio was calculated (Table 5). This ratio proportionally increased when the *C. glomerata* biomass in the rabbit diet increased. The distribution in the LD and HL muscles was as follows: CG8 (1.06% and 1.17%) < CG4 (0.97% and 1.03%) < SCD (0.87% and 0.95%) ($p < 0.05$).

The addition of *C. glomerata* biomass to the diet had no influence on the level of omega-3 in the muscles of rabbits ($p > 0.05$). The omega-3/omega-6 ratio was not affected by CG diets as well ($p > 0.05$). However, the inclusion of biomass in the rabbit diet affected the omega-6 concentration in muscles, which was found to be 3.25% and 4.79% higher in CG4 and CG8 LD muscles and 3.72% and 6.45% higher in HL muscles compared to SCD, respectively ($p < 0.05$).

Atherogenic (AI) and thrombogenicity (TI) indexes, as well as the hypocholesterolemic/hypercholesterolemic ratio (h/H), were calculated. The AI index in LD muscles was not affected by CG experimental diets ($p > 0.05$). However, differences were obtained after evaluating the HL muscles, where a 0.08 lower index was obtained in CG8 compared to the same values obtained in SCD and CG4 (0.54) ($p < 0.05$). Analogous values of TI index were observed in SCD and CG4 LD muscles (0.86), so the means did not differ significantly between diets ($p > 0.05$). Therefore, a higher TI index was found in CG4 HL muscles (0.85), which was greater compared to the analogue values (0.80) of SCD and CG8 ($p < 0.05$). The highest h/H ratio was obtained in both LD and HL CG8 muscles. Compared to SCD, this

ratio was found to be 0.26 higher in CG8 LD muscles, and compared to SCD and CG4, it was 0.30 and 0.28 higher in CG8 HL muscles, respectively ($p < 0.05$).

3.3.2. Malondialdehyde (MDA) and Cholesterol Levels

Malondialdehyde (MDA) levels were examined in rabbit fresh muscles 24 h after slaughter and in rabbit muscles stored for 3 months at −18 °C in a freezer (Figure 2). The results were distributed proportionally: as the dose of *C. glomerata* increased, the level of lipid oxidation decreased. A lower level of MDA was obtained first when examining the LD fresh muscles (LD0) of CG8, then CG4. Compared with SCD, MDA levels in LD0 muscles of CG8 and CG4 were 3.83 µmol/kg and 2.90 µmol/kg lower, respectively ($p < 0.05$). However, *C. glomerata* biomass inclusion did not have any significant impact on lipid oxidation levels in stored LD (LD3) muscles ($p > 0.05$). The same trend was observed when MDA levels were higher in SCD diets and lower in CG diets after fresh (HL0) and stored (HL3) HL muscle evaluation. MDA levels were respectively higher by 3.04 µmol/kg and 2.94 µmol/kg in SCD HL0 and HL3 muscles compared to CG8 ($p < 0.05$).

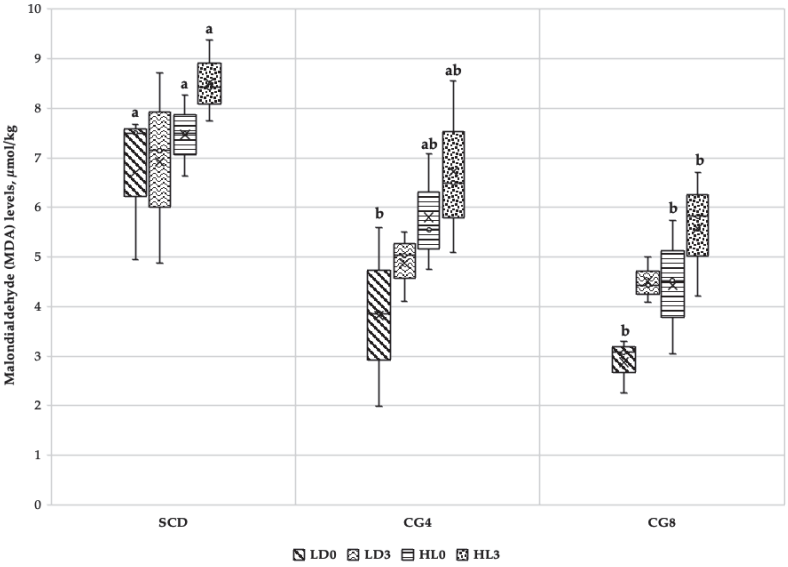

Figure 2. Impact of freshwater *C. glomerata* biomass inclusion in rabbit diets on malondialdehyde (MDA) levels (µmol/kg) in fresh (LD0 and HL0) and stored for 3 months (LD3 and HL3) rabbit muscles. SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. Columns with the same pattern but different superscript letters (a–b) differ significantly ($p < 0.05$).

The impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on cholesterol levels in different muscles is presented in Figure 3. Nevertheless, the CG diet had no significant impact on levels of cholesterol in the LD and HL rabbit muscles ($p > 0.05$).

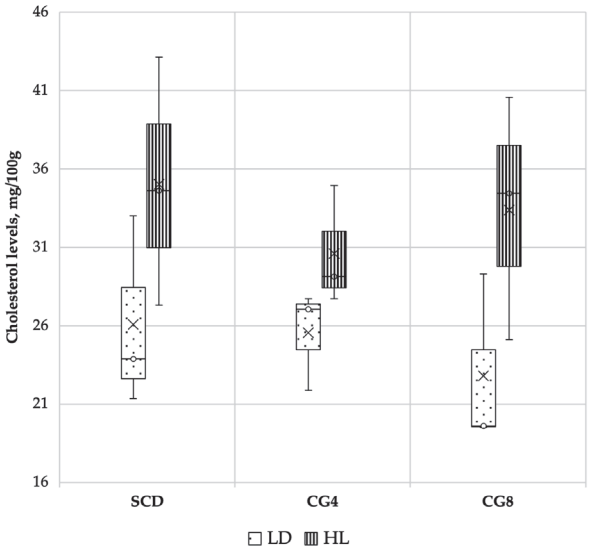

Figure 3. Impact of freshwater *C. glomerata* biomass inclusion in the diet of rabbits on cholesterol levels (mg/100 g) in different rabbit muscles (LD and HL). SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass.

4. Discussion

4.1. Dry Matter of Rabbit Muscles

Meat, in general, is exceedingly perishable due to its high moisture content, causing rapid quality deterioration and bacteria growth if not properly maintained [39,40]. However, *C. glomerata* inclusion in rabbits during our study significantly reduced the DM content of both tested rabbit muscles, thereby directly increasing muscle moisture. Hafsa et al. [41] obtained very comparable results to ours when they acquired rabbit meat with nearly 3% higher moisture in their test with the freshwater algae *C. aegagropila*, which belongs to *Cladophora* sp. Following ash determination in rabbit muscles, the same pattern was observed, with ash decreasing as *C. glomerata* inclusion in diet increased. Nevertheless, during LD muscle analysis, only significant results were observed. Given these indicators, it is to be assumed that meat has a high percentage of water, so it is critical to follow the instructions and properly store and handle meat-based foods to avoid deterioration. Thus, achieving functional food starts with the fundamentals. Food functional qualities, according to Fogliano and Vitaglione [42], can be included in a variety of ways: 1. by incorporating a functional component into a traditional food matrix to produce enriched food with a higher and unusual nutrient composition; 2. by manipulating food through technological processes, such as boosting the formation of compounds with specific biological activities or removing a potentially negative component; and 3. by enhancing functional nutrients or compounds through animal feeding, special growing conditions, or genetic manipulation. In our case, we use the third way by supplementing rabbits' feed with freshwater biomass, which contains a variety of biologically active compounds.

4.2. Proteins and Amino Acid Profiles of Rabbit Muscles

Based on its nutritional and dietetic features, rabbit meat is recognised as an exceptional quality protein in human diets [24]. It contains a significant amount of protein (approx-

imately 22%) and an excellent essential amino acid profile [20]. Considering *Cladophora* species, it can be utilised as a source of protein in animal nutrition since its biomass has a protein level ranging from 10 to 25%, which is comparable to other feed materials [2]. In a recent study, we evaluated how different dosages of *C. glomerata* biomass in rabbit diets can affect protein content and amino acid composition in their muscles. The highest protein levels (22.17%) were observed in the LD muscles of rabbits that were receiving 4% *C. glomerata* treatment in their diets. However, after doubling the macroalgal biomass dosage to 8% in rabbit diets, the protein content in LD muscles was discovered to be the lowest compared to both the 4% dosage of biomass and the standard compound feed. Abu Hafsa et al. [41] examined freshwater macroalgae from natural water resources in Egypt for inclusion at 4% in rabbit diets. They used algae similar to our study's *C. glomerata* freshwater macroalgae, *C. aegagropila*, and the results demonstrated that this type of inclusion resulted in 18.63% of protein in rabbit meat in general, which is slightly lower to our observations. Nevertheless, it is important to note that the protein content of *C. aegagropila* freshwater macroalgae from Egypt was only 10.44%, while *C. glomerata* collected from the Lithuanian river Šventoji that we used in our study contained 22.36% of protein [27]. According to our findings, freshwater macroalgae like *C. glomerata* can be utilised as a protein source in rabbit diets. Since rabbit meat is an excellent source of animal-derived protein that can partially meet human daily amino acid requirements [43], *C. glomerata* biomass in general can improve the functionality of meat even further. Therefore, given the global challenges we confront with livestock today, including the expected shortage of traditional protein sources in animal feed production, freshwater macroalgae could be one of the solutions for more sustainable, alternative, and functional feed materials [16].

Animal husbandry produces a large amount of sustainable protein for human consumption [44]. For example, rabbit meat is high in essential amino acids in addition to being high in protein [24]. Although animal production quality is directly related to the animal's diet, it is crucial to highlight that amino acids used in various types of feed materials can increase animals' nutrient digestibility, compensate for nutrient deficiencies, and improve feed quality and the final composition of animal production [4]. That implies that a healthy animal produces a high-quality product. Amino acids serve as protein building blocks, as an energy source, and as precursors for biologically active molecules [43]. Whether an amino acid is labelled as essential or non-essential, animals and humans require sufficient levels of all amino acids to meet their metabolic demands. Animals, for example, cannot synthesise essential amino acids and must consequently receive them from their diet [45]. Few scientists, including our prior study, examined the amino acid profile of *Cladophora* biomass, and the findings confirmed a highly remarkable new raw material that might potentially be utilized in animal feed [13,16]. Taking the rabbit's meat amino acid profile composition into account, it contains more lysine, threonine, valine, isoleucine, leucine, and phenylalanine compared to other meats [46]. The same essential amino acids—threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, and lysine—were identified in rabbit muscles during a recent study. In most cases, the diet supplemented with 4% *C. glomerata* biomass had the greatest impact on the levels of the aforementioned amino acids. After this treatment, rabbit LD muscles had the highest levels of threonine, valine, methionine, lysine, and isoleucine. In this way, rabbit LD muscle proteins have a high biological value due to their enhanced and balanced essential amino acid content and their simplicity of digestion, which was affected by *C. glomerata* biomass in the rabbit diet. Methionine, for example, is one of the most limiting amino acids and is essential for protein synthesis in the body, preserving its advantageous function as a methyl group donor [47,48]. The daily dietary intake of methionine is similar to that of other essential amino acids [49]; however, it can also be affected by human physiological phases such as pregnancy or infancy rather than only the availability of methyl donors or acceptors and cysteine [50]. The pattern of another essential amino acid lysine levels in rabbit LD muscles according to diet was as follows: CG4 < SCD < CG8. Lysine, along with leucine, is significant since it generates ketone bodies, which serve as an alternate energy source in

our bodies [51]. However, *C. glomerata* inclusions did not increase leucine-phenylalanine levels in LD muscles. Conversely, higher levels of the mentioned essential amino acids were found in the HL muscles of rabbits under 8% macroalgal biomass treatment. Histidine was slightly higher in LD muscles under standard compound diet treatment compared to 8% *C. glomerata* inclusion. However, in general, the nutritional value of meat proteins was demonstrated in most ways to be higher in rabbits treated with 4% *C. glomerata* inclusion, based on a higher proportion of essential amino acids in LD muscles. Considering essential amino acid concentrations are frequently used to estimate the biological value of proteins, the ability to meet customer demand for this kind of acid is crucial [52].

In recent research, conditionally essential glutamic acid was the most abundant amino acid in LD and HL muscles across all treatments. This amino acid is mostly prevalent in rabbit meat. Morshdy et al. [43] investigated the LD muscles of New Zealand and Californian breed rabbits (the same breed as in our study) and supported our results by discovering that the amino acid profile was dominated by the same glutamic acid. The remaining conditionally essential amino acids are unevenly distributed: higher levels of glycine in HL muscles were obtained when rabbits were supplemented with an 8% macroalgae biomass dose compared to standard feed; on the contrary, a higher amount of arginine was found in SCD LD muscles compared to CG8. *C. glomerata*, which had no effect on the levels of serine and proline in different rabbit muscles, according to our findings. Nonessential amino acids were distributed as follows: lower alanine levels were discovered in CG8 LD muscles compared to the remaining diets; aspartic acid levels were the highest in both analysed muscles under CG4 treatment.

The current study's findings, in general, point to the dietetic properties of rabbit meat, not only due to its high fraction of essential amino acid content but also due to its increased total amino acid content. A total of 4% inclusion of *C. glomerata* increased the total amino acid content in LD muscles (192.16 g/kg); a doubled dosage of 8% biomass in rabbit diets increased the same indicator in HL muscles (176.13 g/kg). First, amino acid compositions in meat are determined by diverse amino acid syntheses, which are associated with distinct biological stages of animals [53]. However, the feed can play a key role as well. For example, in *C. glomerata* biomass collected from Lithuanian rivers, the total amino acid content can vary from 103.36 to 140.99 g/kg [16]. Thus, only the essential amino acid content can vary from 41.60 to 55.40 g/kg, which is about 40% of the total amount of amino acids determined according to our previous study. Regardless of the ultimate result, the taste of the food will remain one of the primary factors shaping the consumer's daily choices and habits [54]. As a result, it is important to recognize that amino acids can lead to specific tastes in food, including meat [55]. So, the advantageous amino acid profile of *C. glomerata* macroalgal biomass could affect the taste of rabbit meat. Threonine, serine, proline, glycine, and alanine in biomass may activate a sweet flavour; valine, isoleucine, leucine, phenylalanine, histidine, lysine, and arginine may activate a bitter flavour; phenylalanine, tyrosine, and alanine may activate a sour flavour; and glutamic and aspartic acid may activate an umami flavour [56].

Meat quality is a complex notion; for example, one of the most crucial elements for consumers is meat tenderness. One of the variables that directly affect and decrease meat tenderness is connective tissue proteins [57]. The hydroxyproline content of muscles is commonly used as a marker of connective tissue, whereas tryptophan content is used as a marker of muscle tissue. Tryptophan levels in LD muscles were enhanced by the 4% inclusion of *C. glomerata* macroalgal biomass in rabbit diets. When the dosage of *C. glomerata* in the diet was increased twofold (8%), tryptophan levels were marginally decreased and were lower compared to the standard compound diet. However, after HL muscle analysis, the distribution of results differed from that of LD muscles, with tryptophan being the most abundant in SCD (26.27 g/kg), followed by CG8 (24.02 g/kg), and finally CG4 (22.79 g/kg). Another identified amino acid, hydroxyproline, is present primarily in connective and bone tissue and contributes up to 10% of collagen molecules [57]. So, hydroxyproline is considered to be an excellent marker for evaluating meat quality [58]. The lowest

hydroxyproline levels were observed in both the LD and HL muscles of rabbits fed a diet containing 4% *C. glomerata* biomass. It is significant since rabbit muscles from the CG4 diet had higher tryptophan and lower hydroxyproline levels, indicating that including 4% *C. glomerata* in rabbit diets can improve meat quality. To be more specific, the tryptophan-to-hydroxyproline ratio (T/H) is by far the most important criterion for meat quality and a protein quality indicator [58,59]. The higher this indicator, the greater the meats nutritional value, as well as the overall amount of muscle tissue and proteins, as well as essential amino acids. In our case, the CG4 diet enhanced the T/H ratio in LD muscles (4.91); thus, 8% biomass inclusion, on the contrary, decreased the T/H ratio and was the lowest (2.86) when compared to other treatments. *C. glomerata* inclusion by 4% in rabbit diets can increase the T/H ratio, which indicates the potential to enhance rabbit meat with a higher biological value. Therefore, according to the obtained results, any *C. glomerata* inclusion did not impact the T/H ratio in HL rabbit muscles.

4.3. Lipids and Fatty Acid Profile of Rabbit Muscles

Meat and meat-based products are rarely mentioned as unfavourable due to their high fat and calorie content, as well as higher SFA and cholesterol levels, which are usually linked to cardiovascular disease, obesity, and diabetes [24]. A number of these detrimental nutrients can be reduced by carefully selecting the meat parts eaten, manipulating productive factors, primarily feeding, and manipulating the carcass post-mortem. Furthermore, rabbit meat is highly regarded globally for its excellent nutritional features, with lower fat, fewer saturated fats, and lower cholesterol levels compared to other commonly consumed meats [24]. To be precise, rabbit meat is lower in fat (9.2 g/100 g) and cholesterol (56.4 mg/100 g) compared to chicken, beef, and pork [60]. Due to its low-fat content, rabbit meat has a lower energy value than red meats. According to other researchers, fat content can range from 0.6 to 14.4% depending on the carcass portion (average value of 6.8%), with the loin being the leanest part of the rabbit's carcass (1.2% lipids) [61]. In our case, synergism between alternative freshwater *C. glomerata* inclusion in rabbit feed and rabbit muscles, which are already considered functional foods, lowered the fat content in both LD and HL muscles. It was revealed that the greater the dosage of *C. glomerata* in the diet, the lower the fat accumulation in the rabbit's muscles. Fat distribution in rabbits' muscles: LD muscles SCD (1.62%) < CG4 (1.15%) < CG8 (0.54%); HL muscles SCD (2.73%) < CG4 (1.44%) < CG8 (0.83%). In aquaculture, more feeding studies with *C. glomerata* have been performed compared with other animal species. When we compared our findings to those of Promya and Chitmanat [19], who supplemented African sharptooth catfish (*Clarias gariepinus*) diet with a 5% dosage of *C. glomerata* biomass produced under artificial conditions, they discovered that the final fish muscles had less fat than the standard diet-fed ones. This demonstrates that not only our findings but also those of other researchers support the direct decrease of fat in muscles by supplementing feed with *C. glomerata* biomass. This is particularly crucial for individuals who prefer leaner meat in their diets. These preferences are mostly linked to the extraordinary leanness of the meat, the healthiness of the lipid profile, the micronutrient balance attributes, and the low level of cholesterol [62].

Rabbit meat has an excellent lipid profile since it is low in cholesterol and SFAs while being high in PUFAs, including a balanced ratio of essential omega fatty acids [63]. However, considering the higher PUFA content, the meat becomes more sensitive to oxidative degradation, which can directly affect not only the shelf life of the product but also its final sensory properties [20]. As a result, the degree of lipid oxidation in the final product is substantial, and to assure the stability of lipids in meat, we can first enhance animal feed with antioxidant-rich raw materials [63]. *C. glomerata* has been identified as having antioxidant properties due to the presence of specific phenols, pigments, and antioxidant activities [15]. It is reflected in our study, where we supplemented this type of biomass into rabbit diets and found a decrease in malondialdehyde (MDA) levels in fresh LD and HL muscles and stored HL muscles; the decrease was proportionally higher with higher biomass inclusion. However, *C. glomerata* biomass inclusion did not affect

MDA levels in stored (3-month) LD muscles. Overall, the findings suggest that including macroalgae in rabbit diets might improve the quality of the meat and enhance its stability.

The link between health and diet is becoming increasingly crucial in shaping consumer behaviours. Nonetheless, meat is commonly linked with cholesterol levels in it, and while it is now accepted that dietary cholesterol consumption has a minor impact on plasma cholesterol, this is another unfavourable factor in meat's nutritional perception for consumers [64]. Rabbit meat contains the least cholesterol of any common meat (47.0 mg/100 g in LD muscles and 61.2 mg/100 g in HL muscles) [24]. In our case, cholesterol levels in LD muscles varied from 22.81 to 26.08 mg/100 g and in HL from 30.60 to 35.02 mg/100 g. Up to a certain level, diet can influence the accumulation of cholesterol in rabbit tissues such as muscle. Nonetheless, since no significant differences between treatments were observed, the *C. glomerata* supplemented diet had no significant impact on cholesterol levels in the LD and HL rabbit muscles.

There is expanding scientific evidence that supports the concept that certain foods and dietary components provide physiological and psychological benefits along with basic nutrients [65–68]. In addition to these foods, new ones are being developed to improve or incorporate these health-promoting elements. Fatty acids (FAs), for example, are advantageous nutrients because their composition has a significant influence on a balanced, healthier diet because individual FA alter plasma lipids in different ways. The FA composition of rabbit meat is characterized by its high PUFA concentration; however, the FA profile may also be altered via diet. When it comes to algae, FAs are regarded as one of its most significant and biologically active components, particularly PUFAs, which are essential for human and animal health. The FA profile of different groups in the *C. glomerata* macroalgal biomass from natural sources used in our study was distributed as follows (% from the total FA content): saturated fatty acids (SFA) more than 50%, monounsaturated fatty acids (MUFA) 27.34–28.39%, and polyunsaturated fatty acids (PUFA) 6.48–11.71%. In the recent study, the accumulation of different FAs in rabbits' muscles was distributed as follows: SFA in LD muscles: SCD (35.61%) < CG4 (35.21%) < CG8 (33.85%), in HL muscles CG4 (35.12%) < SCD (34.38%) < CG8 (33.15%); MUFA in LD muscles: SCD (31.18%) < CG4 (27.04%) < CG8 (24.30 %), and in HL muscles SCD (31.12%) < CG4 (25.90%) < CG8 (23.05 %); PUFA in LD muscles: CG8 (35.99%) < CG4 (34.18%) < SCD (30.81%), in HL muscles CG8 (38.92%) < CG4 (36.08%) < SCD (32.54%). Fike et al. [69] found a partially similar effect of macroalgae on the FA composition of meat when they fed lambs *Ascophyllum nodosum* (brown macroalgae) extract at 3.0 kg/ha or 1% DM and discovered a decrease in total SFA and a non-significant increase in unsaturated FAs. In our case, *C. glomerata* inclusions in rabbit diets (4% and 8%) gradually decreased SFA and MUFA levels in rabbit muscles while significantly enhancing PUFA levels.

Since humans are unable to synthesize essential PUFAs, they must receive them through food. Functional food elements include PUFAs, especially long-chain, highly unsaturated FAs with an omega-3 structure [9]. Given their role in metabolism, it's not odd that they've been attributed to a variety of health advantages, including antibacterial, anti-inflammatory, antioxidant, cardiac disease prevention, and tumour growth suppression [70–73]. Looking deeper into the PUFA profile obtained when rabbits were fed different dosages of macroalgal biomass, the trend observed revealed that the higher the *C. glomerata* inclusion, the higher the PUFA deposition in rabbit muscle. The predominant fatty acid from all PUFAs was linoleic acid (C18:2 n6), but no significant impact was discovered when comparing its levels between diets. Individual omega-6 FAs (eicosadienoic (C20:2 n6), dimoho-γ-linolenic (C20:3 n6), arachidonic (C20:4 n6), and adrenic (C22:4 n6)), as well as some omega-3 FAs (eicosapentaenoic (C20:5 n3) and docosapentaenoic (C22:5 n3)), were significantly increased in both analysed rabbit muscles after treatment with 8% *C. glomerata* inclusion. It is commonly acknowledged that rabbits and other non-ruminants may directly convert dietary fatty acids into lipids in adipose and muscular tissues [74]. As a result, dietary lipid content has a substantial influence on fatty acid composition. Although the lipid concentration of *C. glomerata* macroalgae is lower compared to microalgae or oily

land plants like rapeseed or flaxseed, such biomass is distinguished by a more nutritionally advantageous quality of lipids, as evidenced by our findings and final rabbit meat production. Furthermore, since modification of the rabbit diet is notably effective in increasing levels of PUFA, this kind of functional rabbit meat intake might become a valuable source of bioactive compounds for consumers. FAs, for example, have a crucial role in human metabolism, health, and diseases as biological compounds [75].

The PUFA/SFA ratio is a key metric that is frequently used to determine the effect of diet on cardiovascular health (CVH). The following theory was proposed based on this indicator: Low-density lipoprotein cholesterol (LDL-C) and serum cholesterol levels can be reduced by consuming PUFAs, whereas all SFAs lead to high serum cholesterol levels [75]. As a result, the greater this ratio, the greater the beneficial effect. In our trial, the results again reflected the following trend: the higher the dosage of *C. glomerata*, the higher the value of the indicator. So, this ratio increased proportionally when the *C. glomerata* biomass in the rabbit diet increased by being distributed in the LD and HL muscles, as follows: CG8 (1.06% and 1.17%) < CG4 (0.97% and 1.03%) < SCD (0.87% and 0.95%). Furthermore, our findings suggest that including *C. glomerata* in feed might enhance rabbit meat, which has a stronger influence on humans' CVH when consumed.

Therefore, considering the final amount of omega-3 in the muscles, macroalgal biomass did not impact this indicator; however, since the 8% dosage directly affected most individual omega-6 FAs, a greater total amount of such FAs was obtained in the muscles of the CG8 diet. Overall, *C. glomerata* supplementation had no effect on the omega-3/omega-6 ratio.

Another metric evaluated in rabbit muscles was its atherogenicity index (AI), which reflects the correlation between total SFA and unsaturated FA contents. The primary classes of SFAs (lauric (C12:0), myristic (C14:0), palmitic (C16:0), and octadecanoic (C18:0)) are pro-atherogenic as they promote lipid adherence to cells of the circulatory and immune systems [76,77]. As a result, eating foods with lower amounts of AI can lower total cholesterol and LDL-C levels in human plasma [78]. The AI index in LD muscles was not affected by *C. glomerata* experimental diets, but on the contrary, 8% macroalgal biomass inclusion influenced HL muscles, which had the lowest AI levels compared to the remaining diets. As well as AI and PUFA/SFA ratio, another index, the thrombogenicity index (TI), should be lower to be beneficial to CVH. To be more explicit, TI defines the link between pro-thrombogenic FAs (C12:0, C14:0, and C16:0) and anti-thrombogenic FAs (MUFAs and omega 3, 6), which reflects the thrombogenic potential of FAs [35]. When comparing LD muscle TI values, nonetheless, *C. glomerata* had no significant impact on them. Only the inclusion of 4% biomass increased the TI value (0.85) in HL muscles; TI values in SCD and CG8 were analogues. Even though lower AI and TI values indicate greater nutritional quality and may reduce the risk of coronary heart disease (CVD), no organization has yet provided recommended values for these indicators. The hypocholesterolemic/hypercholesterolemic (h/H) ratio, which is more accurate than the PUFA/SFA ratio, also reflects FA effect on CVD. However, unlike AI and TI, foods with higher h/H indices are more nutritionally desirable [79]. According to our findings, the higher the *C. glomerata* inclusion, the higher the h/H obtained. The h/H ratio in LD muscles was 1.03 and 1.13 times higher in LD and 1.00 and 1.14 times higher in HL muscles with 4% and 8% macroalgal inclusion, respectively, as compared to SCD. Our findings suggest that supplementing the rabbit diet with *C. glomerata* biomass allows us to obtain rabbit meat, which can significantly reduce the risk of heart disease and is more nutritionally desirable.

5. Conclusions

In the production of enhanced functional rabbit meat, dietary supplementation with 4% and 8% inclusions of freshwater *C. glomerata* macroalgal biomass from natural resources might be considered not only beneficial but also a more sustainable nutritional strategy:

- A 4% inclusion can significantly increase protein and total amino acid levels in rabbits' muscles while also increasing levels of essential amino acids (threonine, valine, methionine, lysine, and isoleucine) and tryptophan but decreasing hydroxyproline. As

a result of their improved and balanced essential amino acid content, rabbit muscle proteins have a higher biological value, resulting in simpler digestion.
- Synergism between alternative freshwater *C. glomerata* inclusion in rabbit feed can lower the fat content of rabbits' muscles; the greater the dosage of biomass in the diet, the lower the fat accumulation. Since biomass has a reduced fat content, it can reduce lipid oxidation levels in both fresh and stored muscles.
- Inclusions of 4% and 8% can gradually decrease SFA and MUFA levels in rabbit muscles while significantly enhancing PUFA, which indicates a more nutritionally advantageous quality of lipids. Rabbit meat under both *C. glomerata* treatments can result in increased heart disease prevention abilities, as observed PUFA/SFA and h/H values were greater, and TI and AI were lower.

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Institutional Review Board Statement: The study was conducted in accordance with Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and in accordance with the Commission's recommendation of June 18, 2007, concerning the protection of animals kept for farming purposes. A bioethical permit to work with experimental animals (No. PK042495; date of issuing: 07/11/2022) has been issued by the Lithuanian University of Health Sciences Centre of Postgraduate Studies and approved by the State Food and Veterinary Service by official letter No. B6-(1.9)-2625 of 16 October 2013 and updated 28 March 2017, official letter No. B6-(1.9)-852.

Data Availability Statement: The data are available from the corresponding author.

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

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animals

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Article

Sensory Evaluation of Rabbit Meat from Individuals Fed Functional and More Sustainable Diets Enriched with Freshwater *Cladophora glomerata* Macroalgal Biomass

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Simple Summary: Researchers conducted a study to investigate the use of freshwater *Cladophora glomerata* macroalgal biomass as an alternative feed material for rabbits, with a focus on improving meat quality and sustainability while meeting consumer preferences. The rabbits were fed diets with varying amounts of biomass, and the resulting meat was evaluated for physical and sensory characteristics. The findings revealed that incorporating macroalgal biomass positively impacted meat production, making it more sustainable and appealing to consumers. However, the inclusion of biomass increased moisture content and cooking losses in hind leg muscles while reducing the darkness and redness of both fresh and cooked meat, enhancing its visual appeal. Moreover, using an 8% biomass inclusion led to longer muscle fibers. Notably, evaluators reported increased happiness after tasting hind leg muscles from the same diet, as evidenced by their emotional responses. Sensory evaluations further confirmed that the taste and overall quality of the rabbit meat were deemed acceptable.

Abstract: Maintaining meat quality is essential to sustainable livestock management. Therefore, identifying alternative feed materials while considering consumer acceptance is crucial. So, the aim of this study was to evaluate the effect of *C. glomerata*-biomass-supplemented feeds on rabbit muscles' physical properties, sensory profiles, and evaluators' emotional responses to them. A total of thirty 52-day-old weaned Californian breed rabbits were randomly allocated to one of three dietary treatments: standard compound diet (SCD), SCD supplemented with 4% *C. glomerata* (CG4), or SCD supplemented with 8% *C. glomerata* (CG8). After the 122-day-old rabbits were slaughtered, post-mortem dissection of the rabbit *Longissimus dorsi* (LD) and hind leg (HL) muscles was conducted. The physical and histomorphometric features, sensory analyses, and emotional responses to the rabbit's muscles were determined. Study results revealed CG4 and CG8 treatments significantly increased rabbit muscle moisture, while CG8 increased cooking losses in HL muscles ($p < 0.05$). Moreover, both CG treatments reduced the darkness and redness of fresh and cooked rabbit muscles compared to SCD ($p < 0.05$). CG8 treatment compared to SCD resulted in longer LD muscle fibers ($p < 0.05$). Evaluators discovered that the average scores for each sensory description of rabbit meat are acceptable and that consuming CG8-HL muscles can increase happiness based on emotional responses. Consequently, replacing traditional feed materials in rabbit feed with *C. glomerata* can lead to not only more sustainable production but also more consumer-acceptable rabbit meat.

Keywords: macroalgae; sustainability; muscle fiber length; histomorphometry; physical properties; emotional response

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1. Introduction

With a growing population that directly correlates with increasing demand for meat [1], field-to-table processes need to be ensured, and more sustainable solutions need to be sought. An increasing number of scientific studies are being published where traditional feed materials are partially replaced by alternative ones in animals' diets [2–5]. Scientists are increasingly turning to natural and renewable sources and trying to enhance or discover innovative strategies that would help develop more sustainable livestock management. For example, the freshwater *Cladophora glomerata* (*C. glomerata*) macroalgal biomass thrives in water bodies such as rivers. Collecting this kind of biomass has two huge benefits: firstly, it cleans up water bodies and thus increases their diversity; secondly, collected biomass can play a multifunctional role in many branches of biotechnology.

Although ensuring environmental and production sustainability is essential for preserving the global food supply, it should not be achieved at the cost of compromising quality [2]. Furthermore, the concept of meat quality is undergoing a paradigm shift, and contemporary consumers are increasingly concerned about the comprehensive aspects of meat, including its nutritional value, sensory attributes, cooking convenience, and cost-effectiveness. The sensory characteristics of meat, including its taste, odor, texture, and visual appearance, in conjunction with its nutrient composition, exert substantial influence on consumer preferences and purchasing decisions. Nerveless, it is critical to unravel the intricate interplay of multiple factors that govern meat quality and elucidate their underlying mechanisms in order to effectively enhance overall quality. Pethick et al. [6] highlighted several fundamental consumer-focused criteria for meat products' future value propositions. According to one of them, it is recommended that products possess health-enhancing properties by incorporating high-quality protein and nutrients, including fatty acids, minerals, and vitamins that align with the requirements of a healthy diet.

The development of innovative techniques is of utmost importance to attract consumers and encourage them to perceive rabbit meat as an enticing and viable alternative to the more commonly consumed pork, beef, and poultry products. By introducing novel approaches, it becomes possible to enhance the market appeal of rabbit meat and broaden its acceptance among consumers. Additionally, it is imperative to incorporate this approach into the development of novel technologies to expand market offerings and address specific consumer demands, including convenience [7]. Addressing consumer concerns about sustainability, the utilization of alternative raw materials such as the biomass of freshwater macroalgae, such as *C. glomerata*, in rabbit feed production offers a solution that is derived from renewable sources and reduces the ecological footprint of conventional feed production. In our previous studies, we presented the potential of *C. glomerata* biomass collected from Lithuanian rivers as a more sustainable and even functional feed material by analyzing its chemical composition, fatty and amino acid profiles, as well as antioxidant activity [8–10]. Furthermore, after conducting a feeding trial with rabbits and replacing traditional feed materials with different dosages of *C. glomerata* biomass, we discovered that this kind of alternative feed formulation can improve the functionality of rabbit meat even further [11].

While it is important to improve the general characteristics of meat quality, it is crucial not to neglect the sensory aspects, which newly introduced feed raw materials can directly affect. Despite the growing prevalence of health-conscious consumer trends, sensory attributes encompassing flavor, odor, and visual appeal continue to play a pivotal role as key determinants of product acceptance. These sensory characteristics remain crucial aspects influencing consumer perceptions and preferences, thereby underscoring their utmost importance in evaluating overall product desirability. In general, understanding what consumers demand in terms of meat, how they perceive it, and how they prefer it is therefore critical for optimizing meat quality and production, as well as maximizing profitability for meat producers and distributors [12]. Furthermore, the value of meat products is a complex amalgamation of customer expectations that directly influence both the willingness to pay and the ultimate decision to acquire these essential food sources in

the human diet [13]. As a result, the objective of this study was to assess the effect of feed supplemented with various dosages of *C. glomerata* on rabbit muscle physical properties, sensory characteristics, and the emotional responses of evaluators towards them.

2. Materials and Methods

2.1. Animals and Samples Collection

The study was carried out at a local rabbit breeding facility, where the animals were housed indoors in individual cages measuring 34 cm × 34 cm × 61 cm, accommodating one rabbit per cage. The rabbits had unrestricted access to individual nipple drinkers, providing clean drinking water as well as feed bowls, ensuring optimal health conditions and performance. The building was equipped with a heating system that maintained a temperature of 19 ± 2 °C. The housing conditions adhered to the standards outlined in Council Directive 98/58/EC of 20 July 1998, which focuses on the welfare of animals kept for farming purposes. The biomass of *C. glomerata* used for feed formulation was manually collected from the Šventoji River in Lithuania, cleaned, dried, and subsequently utilized in feed production. The chemical composition of the biomass has been previously examined and reported [8–10]. Protocol structure from the Šventoji River to rabbit muscle analysis is presented in Figure 1.

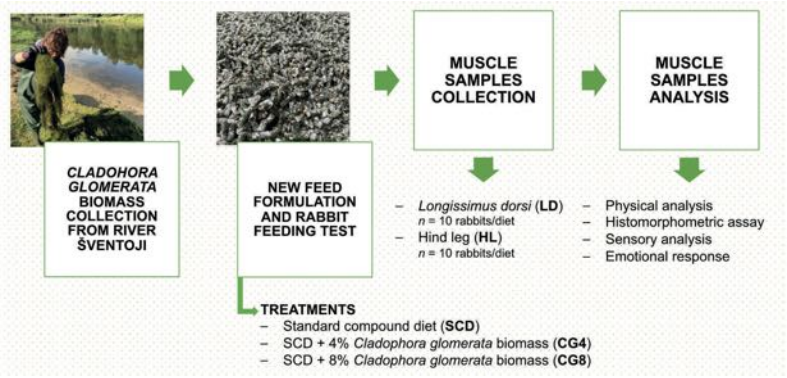


Figure 1. Protocol structure of the rabbit feeding trial.

The feeding trial was conducted using thirty male Californian breed rabbits that had been weaned at 52 days of age. The rabbits, selected based on having similar weight, were randomly allocated to three dietary treatments (n = 10 rabbits/diet). They were provided with a standard compound diet (SCD), SCD supplemented with 4% biomass of freshwater *C. glomerata* (CG4), and SCD supplemented with 8% biomass of *C. glomerata* (CG8), with feeding occurring twice a day *ad libitum*. The formulation of the standard compound diet was developed to fulfill the nutritional needs of growing rabbits by incorporating essential vitamins and minerals. The utilized feed ingredients and their corresponding chemical compositions can be found in the previously published research by Nutautaite et al. [11] (Section 2.1, Table S1). The nutrient composition of the diet was formulated based on the recommendations provided by the National Research Council [14].

Upon completion of the feeding trial, when the rabbits reached 122 days of age, a total of 30 rabbits (n = 10 rabbits/diet) were subjected to weighing, followed by an overnight period of fasting, and subsequently euthanized in accordance with standard practices. The slaughtering procedure was conducted at a rabbit farm slaughterhouse, following established protocols that align with the legal requirements set forth in the Republic of

Lithuania (Order No. B1-866 of 31 October 2012, issued by the Director of the State Food and Veterinary Service) outlining the approved regulations for the care, keeping, and utilization of animals for scientific and educational purposes.

The dissection procedures of warm and chilled carcasses followed the World Rabbit Science Association recommendations [15]. The rabbit carcasses were chilled at a temperature of 4 °C for 24 h in a well-ventilated room. Subsequently, the *Longissimus dorsi* (LD) and hind leg (HL) muscles were dissected from the reference carcasses (n = 10 LD muscles/diet; n = 10 HL muscles/diet).

2.2. Physical Analysis of Rabbit Muscles

The moisture content in samples was determined by drying 2 g minced muscle samples in an oven (105 °C) to a constant weight. The difference between before and after drying the sample was calculated and expressed in percentages (%).

The drip loss was determined according to the methods described by Honikel [16]. In total, 25 g of the studied muscles were placed in mesh bags to reduce evaporation and hung on hooks (4 °C). The samples were reweighed after 24 h to determine the weight change. Drip loss was expressed in percentages (%).

The pressing method was applied to determine the water-holding capacity [17]. Muscle samples were weighed (300 mg) on an analytical balance and placed on ash-free filter paper. On top, a plastic plate was placed and pressed down with a 1 kg weight. It was kept for 10 min, after which the weight was removed, and the boundaries of the compressed meat were defined on the filter paper with a graphite pencil. Using the DIGIPLAN Digital Planimeter 300 planimeter (Gebrüder Haff GmbH, Pfronten, Germany), the spot area was determined, and the difference between the inner and outer spot moisture areas was calculated. Water holding capacity was expressed as a percentage (%).

The cooking loss of the LD and HL muscles was determined by employing Honikel's method [18]. Meat samples were weighed (25 g), placed in polyethylene bags, and then cooked in a circulating water bath for 30 min at a temperature of 90 °C. The cooked samples were cooled to room temperature before being taken from the bags, drained, and weighed; the cooking loss was calculated and expressed in percentages (%) based on the change in mass before and after cooking.

At 24 and 48 h after slaughter, the pH of rabbit LD and HL muscles was determined using the Inolab 730 device (WTW GmbH, Weilheim, Germany).

Using the Chroma Meter CR-410 (Konica Minolta, Inc., Osaka, Japan), rabbits' raw muscles at 24 and 48 h post-mortem and cooked muscles (25 min in an 80 °C water bath) were examined. The same contrast color space was used to define the color coordinates. The light reflectance mode was used to estimate the coordinates L*, a*, and b* (brightness, redness, and yellowness coordinates, respectively, on the CIELAB scale). The measurements were performed using reference light source C, whose radiation is quite similar to that of typical daylight. The equipment was calibrated using a light trap and a white standard before each measurement.

2.3. Histomorphometric Assay of Rabbit LD Muscles

Histomorphometric properties of 30 LD muscles (n = 10 LD muscles/diet) were determined. Each 1 × 1 cm piece of muscle from the same location of the LD muscle's middle section was removed, and it was fixed in 10% neutral buffered formalin. Tissue sections of 4 mm thickness were cut using a rotary microtome Leica RM 2235 (Leica Microsystems, Nussloch, Germany) and stained with hematoxylin and eosin, followed by the standard paraffin embedding technique. Using an Olympus BX63 microscope (Olympus Corp., Tokyo, Japan), an Olympus DP72 digital camera (Olympus Corp., Tokyo, Japan), and a computer running the Image-Pro Plus application system for Windows, version 7.0 (Media Cybernetics, Inc., Bethesda, MD, USA, 2009), prepared *Longissimus dorsi* muscle histologic preparations were analyzed. LD muscle fibers' cross-sectional areas (fiber

length; 150 fibers were measured in each sample in three fields of view), as determined morphometrically, are expressed in micrometers squared (μm^2).

2.4. Sensory Analysis of Rabbit Muscles

A sensory profile test was used to evaluate the sensory properties. A group of ten trained evaluators aged between 34 and 42 years participated in the test and evaluated LD and HL muscles (n = 10 LD muscles/diet; n = 10 HL muscles/diet). Among the evaluators, 80% were female and 20% were male. Evaluators were selected and trained to work according to LST ISO 8586-1 (ISO). The evaluation was closed and performed according to the requirements of LST ISO 8589 (ISO). The evaluators assessed the samples in separate sensory booths. At the initial stage of research, sensory properties were selected, based on which the samples were analyzed and compared with each other.

2.4.1. The Preparation and Submission of Samples for Sensory Evaluation

Muscle samples of LD and HL were enclosed in a cooking bag and subsequently put into an HS-B20 water bath that has automatic temperature regulation (IKA Labortechnik, Staufen, Germany). The samples were cooked for 25 min at 80 °C according to the Martinez-Alvaro and Hernandez [19] method with some modifications. After that, the samples were removed from the bag, cooled to room temperature, and cut into 1.5 × 1.5 cm sample pieces. The samples prepared in this way were placed in plastic containers, covered with lids, coded, and immediately presented to the panel of evaluators. Tasteless, odorless water at room temperature was used to restore the taste receptors of the evaluators.

2.4.2. The Procedure for Submitting Samples to Evaluators and Evaluation

A fully balanced randomized sampling design was used for sensory profiling (n = 10 LD muscles/diet; n = 10 HL muscles/diet). Ten samples were presented in each session, and a panel of evaluators evaluated them for 10 min before and after the break. The intensity of each feature of the test samples was assessed using a 7-point numerical scale: 1 means the feature is not felt, 4 means it is moderately expressed, and 7 means it is very strongly expressed. The sensory profile in the results is presented as an average of all evaluators' responses.

2.5. Emotional Response Evaluation of Rabbit Muscles

Cooked (cooking conditions are described in Section 2.4.1) rabbit LD and HL samples were evaluated by the same 10 evaluators from a sensory assessment using FaceReader 8 software (Noldus Information Technology, Wageningen, The Netherlands) connected to a web camera (Microsoft Corporation, Redmond, WA, USA) to evaluate evaluators' expressions of emotion (by viewing, smelling, and tasting the samples). Participants evaluated LD and HL muscles (n = 10 LD muscles/diet; n = 10 HL muscles/diet). The program is capable of recognizing and capturing eight different models or emotions of facial expressions in real time, including neutral, happy, sad, angry, surprised, scared, disgusted, and contempt, as well as calculating the valence to describe the positive or negative emotional state of the individual. Before starting the assessment, the participants were familiarised with the assessment procedure. Meat samples from different rabbit muscles were cut into pieces, coded, and submitted to the evaluators. The evaluation sequence was view, smell, and taste. The software captures the evaluator's emotions. Between each sample, the evaluator rinsed the mouth with room-temperature water. The intensity of the evaluated emotions is measured on a numerical scale ranging from 0, indicating no expression of emotion, to 1, indicating the maximum value of the fitted model, while for valence, a scale of −1 to 1 was used. Emotional valence can be defined as a quantitative measure that assesses the emotion's polarity from positive to negative. The emotional response in the results is presented as an average of all evaluators' responses.

2.6. Statistical Analysis

SPSS for Windows, version 25.0 (IBM Corp., Armonk, NY, USA), was used to analyze the data. Before analyzing each data set, the Kolmogorov–Smirnov test was employed to determine normality. To identify any differences between treatments, a one-way analysis of variance (ANOVA) test was performed post hoc (Fisher's least significant difference test). A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Physical Features of Rabbits Muscles

The physical properties of different rabbit muscles are presented in Table 1. The inclusion of *C. glomerata* in the rabbit diet increased the moisture content of both muscles; the higher the *C. glomerata* dosage, the higher the moisture content observed ($p < 0.05$). The moisture content of the muscles was distributed as follows in both analyzed muscles: SCD < CG4 < CG8. Both 4% and 8% doses of *C. glomerata* had no significant impact on rabbit muscle drip loss or water-holding capacity ($p > 0.05$). However, CG8 treatment significantly increased cooking losses in HL muscles. Compared to the CG4 treatment, it was higher in HL muscles by 9.67% in CG8 ($p < 0.05$); LD muscles remained unaffected ($p > 0.05$).

Table 1. Effect of *C. glomerata* biomass supplementation in feed on the physical characteristics of rabbit muscles.

		Diet [2,3,4]				
Item (%)	**Muscle [1]**	**SCD**	**CG4**	**CG8**	**SEM [5]**	***p*-Value [6]**
Moisture	LD	74.72 [a]	75.40 [b]	77.58 [c]	0.26	0.000
	HL	74.37 [a]	76.45 [b]	77.53 [c]	0.31	0.000
Drip loss	LD	1.99	2.46	2.65	0.29	n.s.
	HL	2.18	2.97	1.84	0.51	n.s.
Water holding capacity	LD	62.93	64.91	70.99	3.32	n.s.
	HL	70.91	69.88	64.98	4.01	n.s.
Cooking loss	LD	21.37	24.75	22.37	2.81	n.s.
	HL	22.99 [ab]	21.37 [a]	31.04 [b]	3.74	0.041

Note: [1] LD, *Longissimus dorsi*; HL, hind leg. [2] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [3] The means with distinct superscript letters (a–c) in a row differ significantly ($p < 0.05$). [4] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [5] SEM, standard error of the means. [6] n.s., not significant ($p > 0.05$).

The pH of individual muscles was measured at 24 and 48 h after slaughter (Figure 2). The pH remained relatively constant throughout the study periods, and the values remained without significant fluctuations. Significant differences were found only after 48 h when the pH values of SCD and CG8 LD muscles differed significantly: the pH value for 8% *C. glomerata* inclusion was 0.43 units lower compared to SCD ($p < 0.05$). In other cases, no significant impact of biomass on pH values was observed ($p > 0.05$).

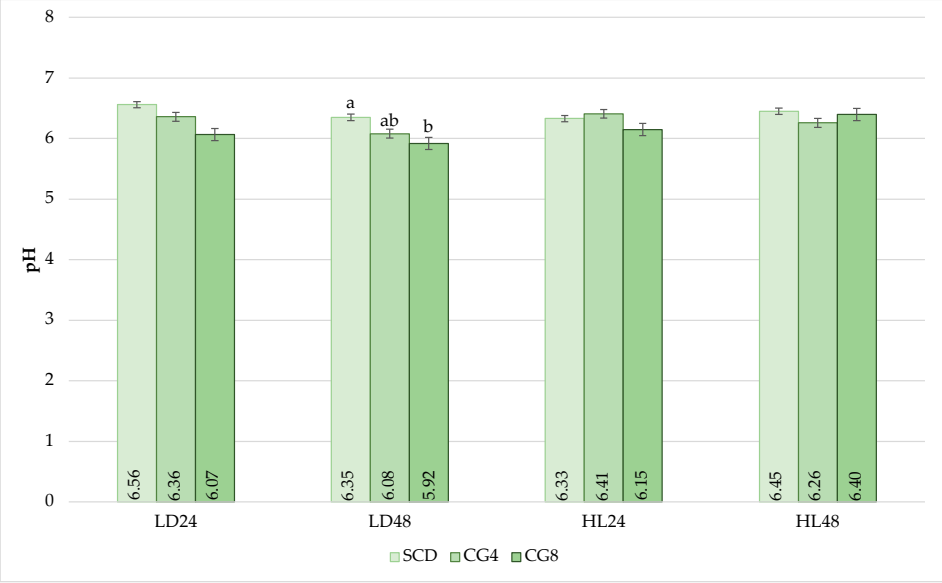
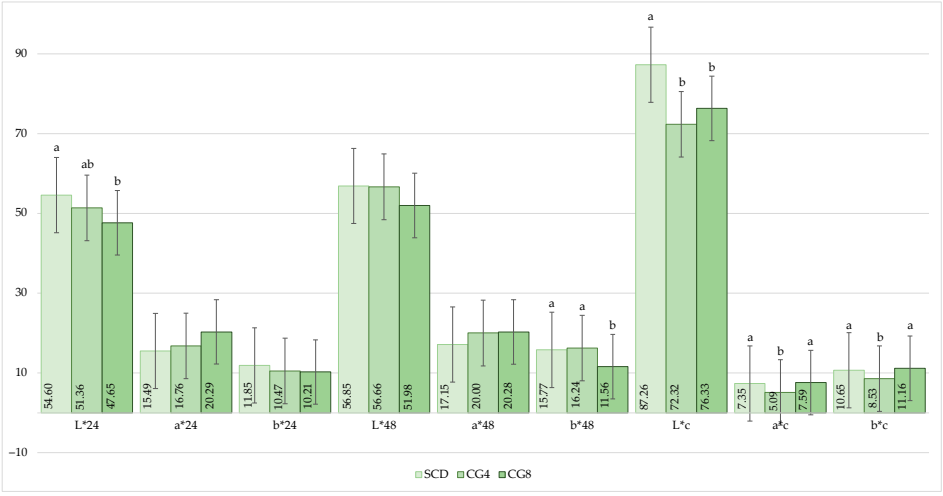
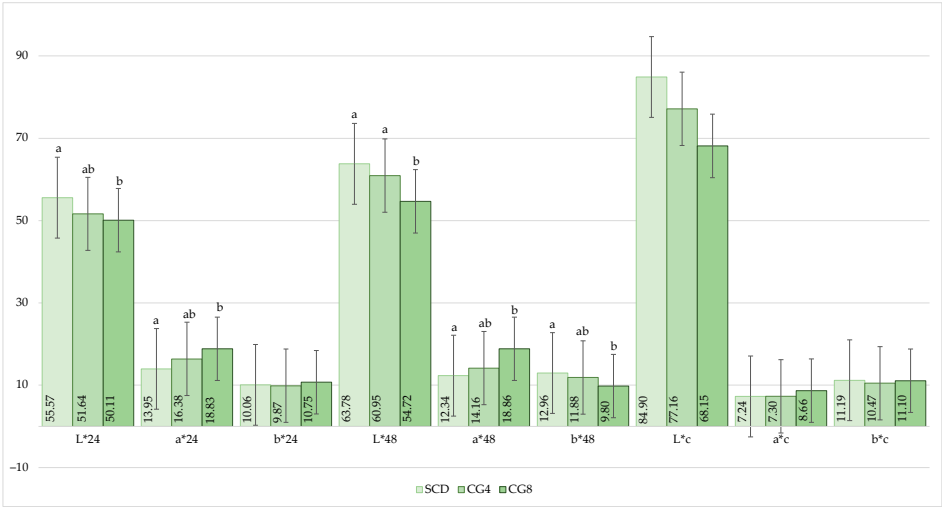


Figure 2. Effect of *C. glomerata* biomass supplementation in feed on pH values of rabbit muscles at 24 h (LD24 and HL24) and 48 h (LD48 and HL48) post-mortem. Note: SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. Columns of the same indicator but with different superscript letters (a,b) differ significantly ($p < 0.05$); those with ab superscript letters did not have significant differences between groups ($p > 0.05$).

The color coordinates of LD and HL muscles were measured at 24 h (L*24, a*24, b*24) and 48 h (L*48, a*48, b*48) after slaughter, as well as after cooking the rabbit meat (L*c, a*c, b*c). The results are shown in Figure 3a,b. At the starting point (24 h), the CG8 treatment had a significant impact on the L*24 coordinate of LD muscles, which was found to be 6.95 units lower compared to the SCD treatment ($p < 0.05$; Figure 3a). Similarly, at the same measuring point, the L*24 and a*24 coordinates of the HL muscles were significantly affected: L*24 decreased, and a*24 increased under the CG8 treatment compared to SCD ($p < 0.05$; Figure 3b). After 48 h, the b*48 coordinate was the lowest in the LD muscles treated with CG8; it was significantly lower by 4.21 and 4.68 units compared to SCD and CG4, respectively ($p < 0.05$). After analyzing the color coordinates of the HL muscles at 48 h, significant differences were observed among all the groups. Firstly, the L*48 coordinate was lower by 9.06 and 6.23 units under CG8 compared to SCD, while the a*48 coordinate was higher by 6.52 units compared to SCD. The b*48 coordinate was slightly lower by 3.16 units in CG8 compared to SCD ($p < 0.05$).



(**a**)



(**b**)

Figure 3. Effect of *C. glomerata* biomass supplementation in feed on the color coordinates at 24 h (L*24; a*24; b*24) and 48 h (L*48; a*48; b*48) post-mortem and after cooking (L*c; a*c; b*c): (**a**) LD muscles; (**b**) HL muscles; Note: SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. Columns of the same indicator but with different superscript letters (a,b) differ significantly ($p < 0.05$); those with ab superscript letters did not have significant differences between groups ($p > 0.05$).

After cooking the rabbit meat and measuring the color coordinates, *C. glomerata* macroalgal biomass inclusion significantly affected all the measured coordinates only in LD muscles ($p < 0.05$), while on the contrary, no impact was found on the color of cooked HL muscles ($p > 0.05$). The brightest cooked meat, according to L*c, was obtained in LD muscles under SCD treatment. The lowest a*c and b*c values were determined in LD muscles treated with 4% biomass inclusion. A*c was lower in CG4 by 2.26 and 2.50 units, and b*c was lower by 2.12 and 2.63 units compared to SCD and CG8, respectively ($p < 0.05$).

3.2. Histomorphometric Measurements of Rabbit LD Muscles

The fiber size of the rabbit *Longissimus dorsi* (LD) muscle was measured (Table 2). Macroalgal biomass inclusion had a positive impact on muscle fiber length by increasing it; under CG8 treatment, LD fiber length increased by 7.57 μm^2 compared to SCD and by 6.83 μm^2 compared to CG4 ($p < 0.05$).

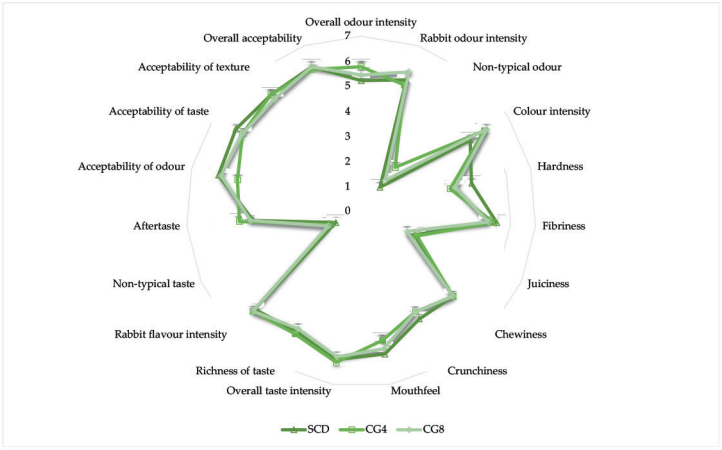
Table 2. Effect of *C. glomerata* biomass supplementation in feed on LD muscle fiber length.

		Diet [2,3]				
Item	**Muscle [1]**	**SCD**	**CG4**	**CG8**	**SEM [4]**	***p*-Value**
Fibre length (μm^2)	LD	51.52 [a]	52.26 [a]	59.09 [b]	2.38	0.002

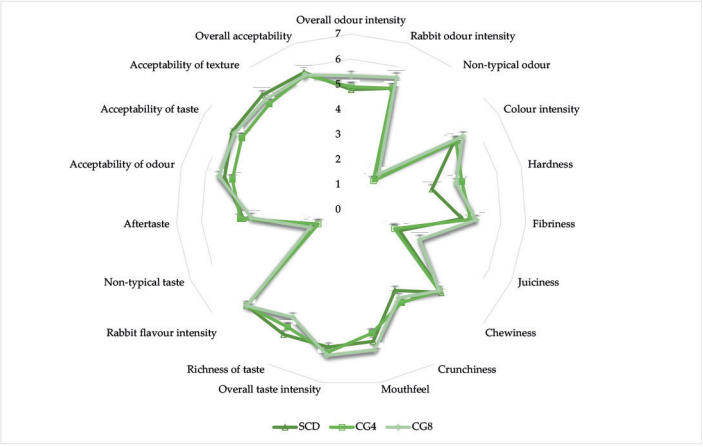
Note: [1] LD, *Longissimus dorsi*. [2] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [3] The means with distinct superscript letters (a,b) in a row differ significantly ($p < 0.05$) [4] SEM, standard error of the means.

3.3. Sensory Evaluation of Rabbit Muscles

The LD and HL muscles, which were cooked prior to analysis, were independently graded using a seven-point system based on 19 criteria (Figure 4a,b). Freshwater *C. glomerata* inclusion significantly affected only two criteria in LD muscles: non-typical odor and color intensity (Figure 4a). The LD-SCD had the lowest non-typical odor, whereas according to the evaluators, the CG4 and CG8 had 1 and 0.34 points higher non-typical odors compared to the SCD, respectively ($p < 0.05$). Therefore, more intense color was expressed in CG4 (5.89 points) and CG8 (6.00 points) LD muscles, whereas slightly less intensity was observed in SCD (5.22 points) ($p < 0.05$). After the sensory assessment of HL muscles, three criteria were significantly affected: hardness, mouthfeel, and richness of taste (Figure 4b). The HL muscles under the SCD treatment exhibited significantly lower hardness compared to the CG4 and CG8 treatments, with the SCD muscles being 1.23 and 1 point less hard, respectively ($p < 0.05$). The most expressed mouthfeel after tasting rabbit meat was evaluated in CG8, and when compared to CG4, the mouthfeel was expressed more by 0.67 points ($p < 0.05$). However, 8% *C. glomerata* inclusion decreased HL muscle richness of taste in comparison to SCD, which had 0.78 points more richness of taste ($p < 0.05$).



(**a**)



(**b**)

Figure 4. Effect of *C. glomerata* biomass supplementation in feed on sensory profile of cooked rabbit: (**a**) LD muscles; (**b**) HL muscles.

3.4. Emotional Response to Rabbit Muscles

The emotional response to the rabbit muscles was evaluated visually (response to the view), by smelling it (response to the odor), and by tasting it (response to the taste) (Table 3). When analysing evaluators' responses to the view of rabbit muscles, it mostly evoked neutral emotion. Compared to SCD, responses to the LD and HL muscles of CG4 and CG8 that evoked neutral emotion were, respectively, higher by 0.359 and 0.354 in the LD muscles and by 0.087 in the HL muscles of CG8 ($p < 0.05$). However, rabbit LD and HL muscles treated with CG4 and CG8 evoked less happy emotion compared to evaluators in SCD after viewing them ($p < 0.05$). Evaluators were sadder when viewing CG4 LD

muscles, but when responding to HL muscles, *C. glomerata*-treated muscles evoked less sad emotion in CG4 and CG8 compared to SCD ($p < 0.05$). CG8-HL muscles left evaluators less disgusted than after viewing SCD ($p < 0.05$). Contempt for evaluators was higher when evaluating CG4-LD muscles and, on the contrary, when responding to HL muscles, was higher in SCD ($p < 0.05$). A negative valence after viewing rabbit muscles was observed after evaluating CG8-LD muscles and SCD-HL muscles ($p < 0.05$). None of the evaluated views of the muscles evoked anger, surprise, or fear ($p > 0.05$).

Table 3. Effect of *C. glomerata* biomass supplementation in feed on the emotional response to cooked rabbit muscles.

		Diet [2,3,4,5]				
Evoked Emotion	**Muscle [1]**	**SCD**	**CG4**	**CG8**	**SEM [6]**	***p*-Value [7]**
		Response to the view Scale 0–1				
Neutral	LD	0.458 [a]	0.817 [b]	0.812 [b]	0.065	0.000
	HL	0.783 [a]	0.826 [ab]	0.870 [b]	0.035	0.017
Happy	LD	0.434 [a]	0.044 [b]	0.164 [b]	0.083	0.000
	HL	0.285 [a]	0.075 [b]	0.053 [b]	0.061	0.000
Sad	LD	0.022 [a]	0.063 [b]	0.043 [ab]	0.016	0.011
	HL	0.039 [a]	0.014 [b]	0.013 [b]	0.011	0.017
Angry	LD	0.041 [a]	0.015 [b]	0.024 [b]	0.009	0.004
	HL	0.027	0.014	0.015	0.009	n.s.
Surprised	LD	0.016	0.010	0.013	0.004	n.s.
	HL	0.008	0.009	0.009	0.003	n.s.
Scared	LD	0.002	0.003	0.003	0.001	n.s.
	HL	0.003	0.007	0.006	0.003	n.s.
Disgusted	LD	0.014	0.017	0.017	0.006	n.s.
	HL	0.023 [a]	0.011 [ab]	0.005 [b]	0.007	0.007
Contempt	LD	0.007 [a]	0.019 [b]	0.013 [ab]	0.005	0.009
	HL	0.023 [a]	0.011 [ab]	0.005 [b]	0.002	0.007
Valence	LD	0.079 [a]	0.014 [ab]	−0.042 [b]	0.042	0.005
	HL	−0.034 [a]	0.012 [ab]	0.021 [b]	0.024	0.024
		Response to the odor Scale 0–1				
Neutral	LD	0.024 [a]	0.870 [b]	0.817 [c]	0.019	0.000
	HL	0.811 [a]	0.661 [b]	0.767 [a]	0.047	0.002
Happy	LD	0.839 [a]	0.093 [b]	0.183 [b]	0.062	0.000
	HL	0.171 [a]	0.149 [ab]	0.061 [b]	0.049	0.027
Sad	LD	0.036	0.041	0.052	0.012	n.s.
	HL	0.025 [a]	0.043 [ab]	0.049 [b]	0.011	0.038
Angry	LD	0.024 [a]	0.011 [b]	0.019 [ab]	0.007	0.043
	HL	0.016 [a]	0.009 [ab]	0.004 [b]	0.004	0.007
Surprised	LD	0.014 [a]	0.011 [ab]	0.009 [b]	0.002	0.012
	HL	0.009	0.006	0.008	0.003	n.s.
Scared	LD	0.005 [a]	0.002 [b]	0.004 [ab]	0.002	0.023
	HL	0.003	0.005	0.005	0.002	n.s.
Disgusted	LD	0.006	0.049	0.018	0.025	n.s.
	HL	0.015 [a]	0.001 [b]	0.001 [b]	0.004	0.003
Contempt	LD	0.020	0.028	0.028	0.006	n.s.
	HL	0.009 [a]	0.004 [b]	0.002 [b]	0.001	0.000
Valence	LD	0.125 [a]	0.106 [a]	−0.059 [b]	0.033	0.000
	HL	−0.011 [a]	0.090 [b]	0.005 [a]	0.041	0.016

Table 3. *Cont.*

Evoked Emotion	Muscle [1]	Diet [2,3,4,5]				
		SCD	CG4	CG8	SEM [6]	*p*-Value [7]
		Response to the taste Scale 0–1				
Neutral	LD	0.806	0.843	0.821	0.028	n.s.
	HL	0.842	0.810	0.793	0.028	n.s.
Happy	LD	0.032	0.015	0.016	0.014	n.s.
	HL	0.006 [a]	0.033 [ab]	0.040 [b]	0.017	0.046
Sad	LD	0.023	0.018	0.024	0.007	n.s.
	HL	0.016	0.028	0.024	0.009	n.s.
Angry	LD	0.046	0.036	0.037	0.010	n.s.
	HL	0.041	0.039	0.034	0.012	n.s.
Surprised	LD	0.018	0.013	0.018	0.005	n.s.
	HL	0.012	0.015	0.019	0.005	n.s.
Scared	LD	0.002	0.003	0.004	0.001	n.s.
	HL	0.004	0.003	0.002	0.001	n.s.
Disgusted	LD	0.021 [ab]	0.015 [a]	0.025 [b]	0.050	0.045
	HL	0.023	0.025	0.024	0.007	n.s.
Contempt	LD	0.007	0.004	0.005	0.002	n.s.
	HL	0.004	0.009	0.010	0.005	n.s.
Valence	LD	−0.042	−0.045	−0.057	0.020	n.s.
	HL	−0.064	−0.049	−0.046	0.018	n.s.

Note: [1] LD, *Longissimus dorsi*; HL, hind leg. [2] SCD, standard compound diet; CG4, standard compound diet + 4% *C. glomerata* biomass; CG8, standard compound diet + 8% *C. glomerata* biomass. [3] The means with distinct superscript letters (a–c) in a row differ significantly ($p < 0.05$). [4] Means with ab superscript letters in a row did not have significant differences between groups ($p > 0.05$). [5] 0 means that no emotion is expressed at all, and 1 means the maximum value of the fitted model (for valence, a scale of −1 to 1). [6] SEM, standard error of the means. [7] n.s., not significant ($p > 0.05$).

The evaluator's reaction to the odor of rabbit meat mostly evoked a neutral emotion (Table 3). Evaluators remained more neutral after smelling CG4 and CG8 LD muscles compared to SCD ($p < 0.05$). Therefore, less neutral emotion was evoked after smelling the HL-muscles of CG4 compared to SCD and CG8 ($p < 0.05$). However, the happiest evaluators were those who evaluated the odor of SCD-treated LD and HL muscles. A sadder emotional response to odor was observed for CG8-HL muscles; it was almost two times higher compared to SCD ($p < 0.05$). Therefore, SCD LD and HL muscle odor evoked more angry emotions compared to *C. glomerata*-biomass-treated groups ($p < 0.05$). Similarly, SCD LD muscle odor elicited higher levels of surprise and fear, and HL muscle odor elicited higher levels of disgust and contempt ($p < 0.05$). A negative valence for the odor of rabbit muscles was observed after evaluating CG8-LD muscles and SCD-HL muscles ($p < 0.05$).

Evaluators also assessed one of the most important sensory properties of food: taste (Table 3). However, no significant response to the muscles of rabbits fed *C. glomerata* biomass was elicited in terms of the following emotions: neutral, sad, angry, surprised, scared, contempt, or valence ($p > 0.05$). However, evaluators expressed about seven times higher happiness levels after tasting 8% *C. glomerata*-treated rabbit HL muscles than when tasting SCD-HL muscles ($p < 0.05$). In comparison to both CG diets, CG8 left evaluators more disgusted than CG4 after tasting LD muscles ($p < 0.05$), whereas SCD-HL muscles had a nearly identical response to CG8 ($p > 0.05$).

4. Discussion

4.1. Physical Properties of Rabbit Muscles

Rabbits demonstrate exceptional attributes for meat production, characterized by a brief gestation period, abundant productivity, and remarkable feed conversion efficiency. Normally, the quality of rabbit meat is consistent [20]. The following and most important

meat quality traits are distinguished: the meat's sensory features, chemical and physicochemical composition, health-enhancing abilities, nutritional values, and safety. Moreover, the meat's moisture content is a significant quality parameter as well [21]; it can have a direct impact on a final food product's physical appearance (shape, color), texture, flavor, and weight, as well as aspects affecting shelf life, freshness, quality, and resistance to bacterial contamination. In our case, *C. glomerata* inclusion in rabbit feed significantly increased the moisture content of LD and HL muscles. The higher macroalgal biomass dosage in feed was determined by the higher moisture content in muscles (SCD < CG4 < CG8; $p < 0.05$). The moisture content of rabbit muscles under *C. glomerata* treatment ranged from 75.40% to 77.58% in LD muscles and from 76.45% to 77.53% in HL. Abu Hafsa et al. [22] utilized the freshwater macroalgae *Spirodela pollyrrhiza* and *Cladophora aegagropila*, which were collected from irrigation canals in Egypt, as dietary supplements for male New Zealand white rabbits. The reported results showed that the moisture content of rabbit meat in general ranged from 70.35% to 72.86%, which was slightly lower compared to our results. Therefore, it is important to note that the properties and nutritional value of macroalgae can be directly affected by seasonality, so the results compared to those of other scientists do not necessarily have to coincide. Dietary moisture content is also linked to water-holding capacity and drip loss [23]. Water holding capacity, for example, is an important quality indicator for determining meat's economic worth since it measures the meat's ability to retain the tissue water present in its structure [24]. Most water loss from meat is caused by structural factors such as shrinkage of myofibrils, breakdown of cell membrane structure, integrity of the cytoskeleton, progression of spaces between cells, and the development of a net between sarcoplasmic and myofibrillar proteins [25–27]. Nevertheless, *C. glomerata* inclusion had no significant impact on rabbits' LD and HL muscles' drip loss or water-holding capacity. Only one affected feature of muscles remained—cooking loss—which was significantly higher in HL muscles when dietary treatment contained 8% macroalgal biomass, compared to a two-times lower dosage (CG4). Water loss holds significant importance in the food industry as it influences the technological yield of the cooking process. The majority of water loss during cooking results from the expulsion of juices due to protein denaturation and muscle contraction, and it can be directly influenced by the cooking temperature [28]. Moreover, cooking causes an increase in the stiffness of the myofibrillar structure of meat due to protein denaturation, which is related to higher water loss [25], which can explain the results obtained in this study.

Meat pH has a substantial impact on meat quality since it affects water-holding capacity, color, tenderness, and even shelf life [29,30]. Rabbits' muscle pH fell uniformly after muscle maturation at 24 and 48 h post-mortem. Only one significant difference was detected between SCD and CG8 when comparing LD muscle pH values at 48 h; a significantly lower pH was obtained in CG8 compared to SCD. One of the major determinants of the water-holding capacity is the rate at which pH falls, which is usually connected with post-mortem anaerobic muscle glycolysis. However, *C. glomerata* biomass in rabbit feed had no effect on the water-holding capacity of muscles; hence, no concrete mechanism between pH and water-holding capacity could be found in our study. Generally, the drop in pH in normal muscle post-mortem ranges from 7.0 to 5.5 (in our case, pH ranged from 6.56 to 5.92 in LD and from 6.41 to 6.26 in HL) due to shrinkage in the myofilament lattice and water ejection, with the resultant loss of water from the meat via dripping, exudate, or purging [25].

When buying meat, consumers first pay attention to its color, which is an optical characteristic that is directly impacted by muscle tissue structure and histological pattern. Regardless of heme pigment content, meat color may be affected by the depth of light absorption and its reflection, as seen by the diversity of color brightness measurements [31]. Therefore, acidity can play a crucial role and directly impact meat color as well [32,33]. As well as the pH of the rabbits' different muscles, the color coordinates were determined 24 and 48 h after slaughter. When compared to standard compound diet-treated rabbit muscles 24 h after slaughter, LD and HL muscles treated with CG8 were significantly

brighter in the L* coordinate, and HL was also significantly redder in the a* coordinate. So according to our results, the higher the pH, the darker the muscles observed. Analyzing muscles after 48 h revealed that 8% *C. glomerata* inclusion slightly reduced redness in LD muscles compared to the remaining treatments. After 48 h, HL muscles became even darker under CG8 treatment; the muscles were more yellow but less red compared to SCD and CG4. The darker color was also reflected in pH, which increased by several units in SCD and CG8 after 48 h of maturation, but the difference was not significant. This mechanism of action, where pH can directly determine L* (brightness) parameters, was obtained not only during our study but also established and confirmed by other scientists [32,34]. Another factor that directly determines the color of the meat and its stability is the antioxidant activity or the increased number of antioxidants [35,36]. It is important to note that higher macroalgal biomass inclusion reduced the redness of the muscles at almost all points post-mortem. This could be based on our prior findings that freshwater *C. glomerata* macroalgal biomass collected from Lithuanian rivers plays a significant role in the identified antioxidant effect [9].

Cooked meat's appearance can be impacted not just by pH, as with fresh meat, but also by the source of the meat, packaging, storage conditions, fat accumulation, flavorings, and preservation methods [37]. Depending on the type of muscle, adequate cooking leads to color changes to off-white, grey, or brown tones. Myoglobin is the primary pigment responsible for color changes in meat, whereas the final color is determined by the degree of ferrihemochrome production, which is determined by the initial proportionality of the myoglobin as well as the final concentration of undenatured oxymyoglobin or deoxymyoglobin [38]. *C. glomerata* inclusion in rabbit feed had an impact only on cooked LD muscle color coordinates; HL muscle color coordinates were not affected. A biomass dosage of 4% in feed significantly brightened rabbits' cooked LD muscles; these kinds of muscles were significantly less yellow and red compared to SCD and CG8.

4.2. Fibre Length of Rabbit LD Muscles

Muscle fiber length is a valuable economic feature since it directly influences meat yield and quality [39]. In recent research, the macroalgal biomass of *C. glomerata* increased *Longissimus dorsi* muscle fiber length to 59.09 μm^2, whereas in SCD it was only 51.52 μm^2. In general, the number of muscle fibers remains steady during embryonic development, although muscle mass increases after birth due to skeletal muscle fiber hypertrophy [40]. This suggests that increasing muscle fiber width or length has a significant impact on muscle yield. According to Sarsenbek et al. [41], the length and density of muscle fibers can have a direct impact on the tenderness of meat tissue, which is also an important factor in evaluating muscle tenderness. While the exact mechanism behind the observed effect of *C. glomerata* biomass on muscle fiber length is not completely understood, it is possible that the nutritional properties of the biomass are involved. The macroalgal biomass may contain specific nutrients or bioactive compounds that play a role in the development and maintenance of muscle fibers. For instance, the freshwater *C. glomerata* biomass used in the study was found to be rich in proteins and minerals, which could potentially support muscle growth and development [10]. So, according to histomorphometric measurements of rabbit LD muscles, we can state that 8% *C. glomerata* inclusion stimulates muscle growth and development by increasing the fiber length of the *Longissimus dorsi*.

4.3. Sensory Profile of Rabbit Muscles

Meat quality has typically been evaluated by sensory factors such as appearance, texture, odor, and flavor, and humans cook meat to increase both its digestibility and sensory properties. Tenderness, on the other hand, remains one of the most highly valued features of cooked meat [42–45]. Rabbit meat is described as exceptionally tender by consumers. However, evaluators found HL muscles under *C. glomerata* treatments to be significantly harder. The LD and HL muscles of rabbits were sensory evaluated according to 19 criteria. Algal biomass treatment had an effect not only on HL muscle hardness but

also on the non-typical odor and color intensity in LD muscles and in HL muscles, and *C. glomerata* altered mouthfeel and richness of taste. The greatest impact on non-typical odor in LD muscles was caused by feed supplemented with 4% *C. glomerata*, and the intensity of the color rose was uniformly affected with increasing biomass dose in the diet. Rabbits' HL muscles had a much more expressed mouthfeel after tasting muscles under the CG8 diet but were evaluated as having a less rich taste compared to those under the remaining diets. The remaining sensory aspects were not affected by the addition of *C. glomerata* at 4% or 8% in rabbit feed. In their review of macroalgae in rabbit nutrition, Al-Soufi et al. [42] stated that available scientific research suggests that a macroalgae-supplemented rabbit diet could further improve the intrinsic properties of meat and enhance its stability. Although there were some substantial discrepancies between the standard compound diet and the *C. glomerata* treatments in our study, the findings indicate that the average scores for each sensory descriptor are adequate for the sensory profile of rabbit meat.

4.4. Emotional Response to Rabbit Muscles

It has been proposed that there is a close relationship between consumption conditions and how consumers feel [46]. Expressed feelings are regarded as fundamental modulators of food perception, food liking, and overall satisfaction with human eating experiences [47]. Consumer evaluation has been broadly applied to assess food and its products' acceptability and quality, especially meat. Torrico et al. [48] highlighted two key objectives of consumer food testing: (1) understanding the consumer's acceptability and liking preferences, and (2) understanding the consumer's intent to buy and pay for the product. A FaceReader software gadget was used to measure facial expression while viewing, smelling, and tasting different rabbit muscles. This type of innovative technology enables the analysis of facial expressions over time by recording the physical responses of the eyes, mouth, brows, and head. When analyzing evaluators' responses to the view, odor, and taste of rabbit muscles that were fed experimental *C. glomerata* diets, they mostly evoked a neutral emotion, which was the predominant expression among all treatments. Therefore, evaluators were significantly less happy and even sadder after viewing LD muscles when rabbits were fed 4% biomass in their feed. On the other hand, HL muscles evoked less sadness and less disgust after viewing samples from rabbits treated with 8% *C. glomerata*. The contempt of evaluators was higher when evaluating CG4-LD muscles, and, on the contrary, when responding to HL muscles, it was higher in the standard compound diet. None of the evaluated views of the muscles significantly evoked anger, surprise, or fear. The evaluator's reaction to the odor of rabbit meat mostly evoked a neutral emotion, similar to that after viewing them. Therefore, standard compound diet-treated LD and HL muscles evoked more happy and angry emotions at the same time compared to *C. glomerata*-biomass-treated groups. Similarly, SCD-LD muscle odor elicited higher levels of surprise and fear, and SCD-HL muscle odor elicited higher levels of disgust and contempt. Evaluators also assessed one of the most important sensory properties of food: taste. However, no significant response to the muscles of rabbits fed *C. glomerata* biomass was elicited in terms of the following emotions: neutral, sad, angry, surprised, scared, contempt, and valence. Therefore, evaluators expressed about seven times higher happiness levels after tasting 8% *C. glomerata*-treated rabbit HL muscles. In comparison to both *C. glomerata*-supplemented diets, 8% inclusion left evaluators more disgusted than 4% after tasting LD muscles. Organoleptic characteristics heavily influence consumer purchasing decisions and meat acceptance. As a result, unconscious consumer responses via biometrics combined with self-reported responses provide a deeper understanding of the acceptability of meat products. Despite the reactions to the odor and viewing samples, the evaluators were several times happier when tasting the HL muscles of rabbits that were fed with an 8% biomass dose in feed, which is a significantly positive result of this study. However, it is important to note that the rabbit's muscles were only cooked without any additional seasoning, which may have influenced the acceptability of the samples among evaluators.

5. Conclusions

Altering traditional feed materials with alternative freshwater *C. glomerata* macroalgal biomass in rabbit feed production can result in not only more sustainable production but also more consumer-acceptable rabbit meat. However, inclusions of 4% and 8% biomass can significantly increase rabbit muscle moisture, and 8% in the feed can significantly increase cooking losses in HL muscles. Moreover, *C. glomerata* can reduce the darkness and redness of fresh (24 and 48 h post-mortem) and cooked rabbit muscles, which can be more appealing to consumers. According to the research findings, including 8% of *C. glomerata* biomass in rabbit feed can lead to a significant increase in the length of muscle fibers in LD muscle. Despite several substantial discrepancies between SCD and CG treatments, evaluators claim that the average scores for each sensory descriptor are appropriate and acceptable for the sensory profile of rabbit meat. Notwithstanding their reactions to the odor and viewing the samples, CG8-HL rabbit muscles can increase happiness, according to the evaluators' emotional responses after tasting them.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13132179/s1, Table S1: Ingredients in rabbit feed and chemical composition of a standard compound diet and diets supplemented with different dosages of *C. glomerata* biomass (52–122 days old).

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Nuoširdžiai dėkoju visiems, kurie mane palaikė per visą doktorantūros kelionę. Be jūsų padrąsinimų ir pagalbos, šis darbas nebūtų buvęs įmanomas.

Pirmiausia, esu nepaprastai dėkinga savo mokslinei vadovei prof. dr. Vilmai Vilienei, kurios mokslingumas, kantrybė ir vadovavimas buvo neįkainojami. Jos įžvalgūs atsiliepimai ir nuolatinis palaikymas buvo labai svarbus rengiant disertacinį darbą. Taip pat noriu nuoširdžiai padėkoti prof. dr. Astai Racevičiūtei-Stupelienei už ypatingą pagalbą. Jos įžvalgos ir padrąsinimai reikšmingai prisidėjo prie šio disertacinio darbo progreso ir sėkmingo užbaigimo.

Nuoširdžiai dėkoju savo šeimai ir draugams. Jų nuolatiniai padrąsinimai ir tikėjimas manimi suteikė jėgų ir motyvacijos įveikti šio akademinio darbo iššūkius. Visada būsiu dėkinga už nesibaigiantį palaikymą savo mamai Ilonai ir močiutei Danguolei, taip pat tėčiui Sauliui už pagalbą nuo pirmųjų disertacijos darbo etapų. Taip pat esu be galo dėkinga savo partneriui Donatui už jo nuolatinį palaikymą, supratimą ir kantrybę per visą šią kelionę.