

CAN FRESHWATER *CLADOPHORA GLOMERATA* MACROALGAL BIOMASS BE COMBINED IN RABBIT DIETS TO ENHANCE NOT ONLY RABBIT MEAT QUALITY BUT ALSO RABBIT HEALTH?

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Abstract

Despite numerous challenges and rising consumer demands, strategies for growing healthy animals and improving sustainability are being pursued, such as using natural, renewable sources to replace traditional feed materials. Thus, the aim of this study was to analyse the impact of macroalgal *C. glomerata* biomass on rabbit physiological features. The feeding trial (52-122 days) was carried out with twenty male Californian rabbits assigned to two dietary treatments ($n=10$ rabbits/treatment) and fed standard compound diet (SCD) and SCD + 4% *C. glomerata* (CG4). Macroalgal biomass was collected from the Lithuanian River Šventoji. At the end of the feeding trial, twelve rabbits ($n=6$ rabbits/treatment) were randomly selected and euthanized; samples were collected post-mortem. A comprehensive analysis of rabbit organ development, histomorphometry, short-chain fatty acids (SCFA), and ammoniacal nitrogen ($\text{NH}_3\text{-N}$) accumulation in intestinal contents was performed. The CG4 treatment had no impact on organ or intestinal development or the concentration of $\text{NH}_3\text{-N}$ in the duodenum ($P>0.05$). However, the crypt depth was significantly lower in the duodenum of CG4 compared to SCD, but $14.40\ \mu\text{m}$ deeper in the ileum ($P<0.05$). Although CG4 had no impact on villus height, it had an impact on the villus/crypt ratio (V/C), which was higher in the duodenum in CG4 but lower in the ileum in SCD ($P<0.05$). After examining the gut contents from the duodenum, CG4 significantly reduced the lactic acid concentration, which was almost four times lower compared to SCD ($P<0.05$). Overall, *C. glomerata* in rabbit diets can improve the crypt depth in the ileum and therefore the absorption of nutrients that remain digested; nevertheless, it can reduce the crypt depth in the duodenum and thus the digestive area required to carry out the primary nutrient absorption. Moreover, such inclusion can reduce the concentration of lactic acid, whose decrease can affect weaker peristalsis and bowel movements.

Keywords: alternative feedstuff, freshwater macroalgae, SCFA, histomorphometry, villus height, crypt depth

1. INTRODUCTION

Sustainability and animal health are crucial concerns for the animal feed industry. With increasing demands for healthy animal products, there is a growing need to find alternative feed sources that are natural, renewable, and eco-friendly. Macroalgae are considered as one such potential feed source, which can substitute traditional feed materials and improve the overall sustainability of the animal feed industry. For example, *Cladophora glomerata* (*C. glomerata*) is a freshwater macroalga that has been reported to have high nutrient content and beneficial effects on animal health (Garcia-Vaquero and Hayes, 2016; Wan *et al.*, 2019; Silva *et al.*, 2020; Al-Soufi *et al.*, 2022).

The *Cladophora* species, whether found in freshwater or marine environments, are widely recognized as macroalgae of ecological and economic importance. These species perform crucial ecosystem functions and their biomass is utilized for various purposes, including as soil improvers, fertilizers, plant

growth biostimulants, food and animal feed, pharmaceuticals, cosmetics, wastewater treatment, and renewable biofuel production (Mihranyan, 2011; Zulkifly *et al.*, 2013). *C. glomerata* macroalgae form large communities in nutrient-rich water bodies, especially slow-flowing rivers (Pikosz, Messyasz and Gąbka, 2017). Their blooms can have harmful ecological and economic impacts and reduce the recreational value of water bodies by decreasing biodiversity, as they mostly comprise only one species of algae. Despite this, *C. glomerata* is often recommended for integration into human and animal diets due to its relatively high protein content (Messyasz *et al.*, 2015). In the food and feed industry, this biomass is utilized as a raw material that is low in calories but rich in nutrients, vitamins, and fibers (Akköz *et al.*, 2011). Specifically, *C. glomerata* contains a rich spectrum of bioactive components that are reflected in its chemical composition, particularly in its amino and fatty acid and phenolic compound profiles (Nutautaitė *et al.*, 2021; Nutautaitė *et al.*, 2022; Nutautaitė, Vilienė, Racevičiūtė-Stupelienė, Bliznikas, Karosienė and Koreivienė, 2022). Therefore, *C. glomerata* macroalgae biomass is convenient for use as a feed supplement in the context of the problems encountered in today's livestock sector, such as the overall sustainability concept, shortage of traditional feed raw materials, and reduction of greenhouse gas emissions during livestock activities. Moreover, by collecting excess macroalgae biomass from water bodies and integrating the biomass into feed production, waste can be repurposed as a raw material, creating a more sustainable production chain.

Previous studies have demonstrated the potential of *C. glomerata* as a feed source for various animals, including fish, poultry, and ruminants (Abid and Abid, 2006; Mukherjee *et al.*, 2020; Sirakaya, 2023). However, limited research has been conducted on the use of *C. glomerata* in rabbit diets. In recent research, we have demonstrated that the supplementation of rabbit feed with freshwater *C. glomerata* macroalgal biomass collected from the Lithuanian River Šventoji can enhance the functionality of rabbit meat. However, the safety and health of the animal are of paramount importance in ensuring a wholesome and healthy product. Therefore, it is crucial to investigate the potential effects of *C. glomerata* supplementation on the physiological health and well-being of the rabbits in order to ensure that the final product meets the necessary standards for consumption. So, the aim of this study was to investigate the effect of *C. glomerata* biomass on rabbit physiological features. In particular, the study analyzed the impact of *C. glomerata* on rabbit organ development, histomorphometry, short-chain fatty acids (SCFA), and ammoniacal nitrogen (NH₃-N) accumulation in intestinal contents.

2. MATERIALS AND METHODS

2.1. Animals and dietary treatments

The research was carried out at a local rabbit breeding facility, where the animals were reared indoors in separate cages (n=1 rabbit/cage). The rabbits were provided with unrestricted access to individual nipple drinkers that delivered clean drinking water and feed bowls to maintain their optimal health conditions and performance. The housing practises adhered to the guidelines established by Council Directive 98/58/EC of 20 July 1998, which concerns the welfare of animals kept for farming purposes.

Twenty weaned male rabbits of the Californian breed, aged 52 days, were used for the experiment. The rabbits were selected based on similar weight and then randomly assigned to one of two dietary treatments, with each treatment group containing ten rabbits (n=10 rabbits/diet). The rabbits were fed a standard compound diet (SCD) and a standard compound diet enriched with 4% freshwater *C. glomerata* biomass (CG4). The standard compound diet was developed and analysed to meet the nutrient requirements of growing rabbits, including vitamins and minerals, as described in our previously published research (Nutautaitė *et al.*, 2023). During the feeding trial, all groups were provided with feed twice a day and had ad libitum access to pellets. The biomass used in the production of feed was collected from the Šventoji River in Lithuania. The chemical parameters, nutritional value, and antioxidant activity of the biomass were previously examined and described in published studies (Nutautaitė, Vilienė, Racevičiūtė-Stupelienė, Bliznikas, Karosienė and Koreivienė, 2022; Nutautaitė *et al.*, 2022; Nutautaitė *et al.*, 2021).

2.2. Slaughter performance and samples collection

At the end of the feeding trial when the rabbits were 122 days old, 12 rabbits (n=6 rabbits/diet) were randomly chosen, weighed, and then subjected to an overnight fast. Subsequently, they were euthanized in accordance with standard farming practices. The slaughter was carried out at a rabbit farm slaughterhouse using established procedures that conform to the laws of the Republic of Lithuania, as outlined in Order No B1-866 of 31 October 2012 by the Director of the State Food and Veterinary Service, which sets forth the requirements for keeping, caring for, and using animals for scientific and educational purposes.

Post-mortem organ and gut development were investigated; duodenum and ileum segments from the middle were taken for further histomorphometric analysis; content from the duodenum was taken to determine the short-chain fatty acid profile and ammoniacal nitrogen content. The digestive tract was removed and weighed post-mortem. Each intestinal segment was weighed as well. The length of every intestinal segment was measured with flexible tape from Hoechstmass (Hoechstmass Balzen GmbH, Sulzbach, Germany) on a glass surface. The intestinal walls were washed with physiological solution and dried up with filter paper (Lentle et al., 1998).

2.3. Histomorphometrical assay

To determine histomorphometric properties, rabbits' duodenum and ileum samples from the middle (n=6 samples/diet) were fixed with 10% neutral formalin solution, and using standard procedures for histologic evaluation, the tissues were embedded in paraffin and cut with a rotary microtome (Leica RM 2235; Leica Microsystems, Nussloch, Germany) into 4 µm thick tissue sections, which were painted with haematoxylin and eosin. Both treatment samples villus heights and crypt depths were morphometrically and microscopically measured. Prepared histological samples were examined using an Olympus BX63 microscope (Olympus Corp., Tokyo, Japan), an Olympus DP72 digital camera (Olympus Corp., Tokyo, Japan), and the computer Image-Pro Plus programme system for Windows, version 7.0 (Media Cybernetics, Inc., Bethesda, MD, USA, 2009).

2.4. Short-chain fatty acids (SCFA) analysis

The profile of SCFA in duodenum content (n=6 samples/diet) was determined by using a gas chromatography system (Shimadzu GC-2010 Plus; Shimadzu Corp., Kyoto, Japan) with a 2.5 mm x 2.6 mm glass tube, filled in with 10% of SP-1200/1% HPO on 80/100 Chromosorb W AW, tube temperature 110°C, detector's FID temperature 108°C, injector's temperature 195°C. The value of the SCFA accumulation calculated as a concentration of separate SCFA in a digestive content (Zduńczyk et al., 2004).

2.5. Ammoniacal nitrogen analysis

The quantities of ammonia nitrogen (NH₃-N) in duodenum content (n=6 samples/diet) were determined with a gas chromatography system (Shimadzu GC-14A; Shimadzu Ko, Tokyo, Japan) according to Foss-Tecator method ASN 3302.

2.6. Statistical analysis

The data obtained during the study were analyzed using SPSS for Windows, version 25.0 (IBM Corp., released in 2017; Armonk, NY, USA). Prior to data analysis, the normality of the data was determined using the Kolmogorov-Smirnov test. For normally distributed data, a parametric independent T-test was performed on all data obtained during each trial period to identify any differences among the different treatments. Statistical significance was defined as a P value less than 0.05 (P<0.05).

3. RESULTS

Table 1 shows the impact of freshwater *C. glomerata* macroalgal biomass on rabbit organ and intestinal development. Supplementation of rabbit feed with *C. glomerata* was found to decrease the weight of the intestine with chymus, stomach weight with content, liver weight, and lung weight, while increasing the length of the intestine, weight of the stomach without content, and size of the kidneys. The weight of

the pancreas remained consistent between treatments. However, all data pertaining to organ and intestinal development were determined to be statistically insignificant ($P>0.05$).

Item	SCD	CG4	P-value ¹
Intestine weight with chymus (g)	370.33	336.00	N/S
Intestine length (cm)	545.33	586.00	N/S
Stomach with content (g)	9.67	6.67	N/S
Stomach without content (g)	124.33	169.67	N/S
Liver (g)	15.67	12.00	N/S
Pancreas (g)	1.00	1.00	N/S
Lungs (g)	21.00	18.00	N/S
Kidneys (g)	118.67	123.33	N/S

Note, ¹ N/S, not significant ($P>0.05$).

Table 1. Freshwater *C. glomerata* macroalgal biomass impact on rabbit internal organ and intestinal development

A histomorphometric analysis was performed, the results of which are presented in Table 2. The addition of macroalgal biomass to the feed did not influence the *villus* height of the *duodenum* and *ileum* intestinal segments ($P>0.05$). As a result, the dosage of 4% *C. glomerata* significantly decreased the crypt depth of the *duodenum* by 57.72 μm , while increasing the crypt depth of the *ileum* by 19.65 μm compared to the standard compound diet ($P<0.05$). Due to the increase in crypt depth observed in the study, the V/C ratio was significantly different between the two diets. In the *duodenum*, the ratio was higher under the CG4 diet, while in the *ileum*, it was higher under the standard compound diet ($P<0.05$).

Item	SCD	CG4 ¹	P-value ²
Villus height (V; μm) Segment			
<i>Duodenum</i>	1256.63	1346.97	N/S
<i>Ileum</i>	669.76	655.00	N/S
Crypt depth (C; μm)			
<i>Duodenum</i>	191.82 ^a	134.10 ^b	0.002
<i>Ileum</i>	128.85 ^a	148.50 ^b	0.041
V/C ratio			
<i>Duodenum</i>	6.11 ^a	9.64 ^b	0.000
<i>Ileum</i>	5.63 ^a	4.65 ^b	0.017

Note, ¹ Means with different superscript letters (a–b) in a row were significantly different ($P<0.05$).² N/S, not significant ($P>0.05$).

Table 2. Freshwater *C. glomerata* macroalgal biomass impact on rabbit *duodenum* and *ileum* histomorphometrical features

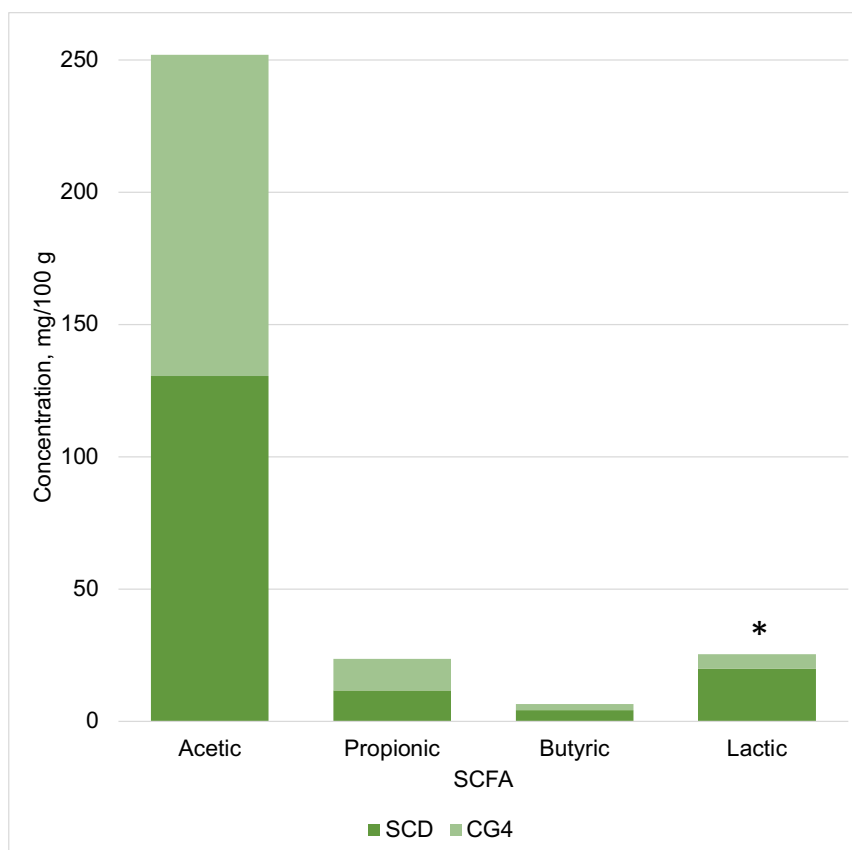


Fig. 1. Freshwater *C. glomerata* macroalgal biomass impact on short-chain fatty acid (SCFA) profile in rabbit *duodenum* content

Note, means with * over the column were significantly different ($P < 0.05$).

The study found that macroalgal biomass supplementation had minimal impact on the short-chain fatty acid (SCFA) profile, except for one indicator shown in Figure 1. Specifically, the CG4 treatment resulted in a significant reduction in the concentration of lactic acid in the *duodenum*, with levels nearly four times lower than those observed in SCD ($P < 0.05$).

Item	SCD	CG4	<i>P</i> -value ¹
NH ₃ -N (mg/100 ml)	136.17	126.49	N/S

Note, ¹ N/S, not significant ($P > 0.05$).

Table 3. Freshwater *C. glomerata* macroalgal biomass impact on ammoniacal nitrogen (NH₃-N) level in rabbit *duodenum* content

Table 3 demonstrates the impact of freshwater *C. glomerata* macroalgal biomass on the level of ammoniacal nitrogen (NH₃-N) in the *duodenum* content of rabbits. However, no significant effect on ammoniacal nitrogen levels was observed between the treatments ($P > 0.05$).

4. DISCUSSION

Proper development of internal organs and intestines is crucial for the healthy growth and development of rabbits. It also plays a vital role in their ability to efficiently absorb nutrients and maintain a strong immune system (Koletzko *et al.*, 1998). The present study examined the impact of freshwater *C. glomerata* macroalgal biomass on organ and intestinal development in rabbits, and the results showed minimal impact on their development. Although theoretically, the inclusion of *C. glomerata* in feed could affect internal organ and intestinal development in rabbits due to the presence of various bioactive compounds in the algae. The bioactive compounds present in *C. glomerata* can have either beneficial or adverse effects, depending on their concentration and duration of exposure. For example, the high dietary fiber content in *C. glomerata* can enhance gut motility and reduce digestive disorders by stimulating intestinal peristalsis and increasing fecal bulk. On the other hand, *C. glomerata* contains antioxidants such as flavonoids and carotenoids that can scavenge free radicals and reduce oxidative stress in the gut, promoting intestinal health (Nutaŭtaitė, Racevičiūtė-Stupelienė *et al.*, 2022). Overall, the findings suggest that the inclusion of *C. glomerata* in rabbit feed does not significantly affect the development of internal organs and the intestine. Although some differences were observed in weight and size, these differences were not statistically significant, and they are unlikely to have a significant impact on rabbit health or productivity. Further studies are necessary to explore the potential long-term effects of *C. glomerata* supplementation on rabbit organ and intestinal development.

Following the histomorphometric assay, it was found that the addition of *C. glomerata* did not produce any significant effect on the villus height of the duodenum and ileum segments. This result indicates that the structural integrity of the intestinal lining remained unaltered by the inclusion of *C. glomerata* in the rabbit diet since the maintenance of a stable villus height is important for preserving the absorptive and secretory functions of the small intestine (Yang and Liao, 2019). However, the observed changes in crypt depth and V/C ratio following the addition of 4% biomass to the feed may have important implications for gut health and nutrient absorption in rabbits. Crypts are responsible for the production of new cells that replenish the epithelium, and the depth of the crypt is an indicator of the rate of cell proliferation and differentiation (Dolan *et al.*, 2022). So, the observed decrease in crypt depth in the duodenum and increase in the ileum suggest that the addition of *C. glomerata* may impact the rate of cell proliferation and differentiation in different segments of the intestine. The observed decrease in crypt depth in the duodenum and increase in the ileum suggest that the macroalgal biomass may impact the nutrient absorption process. Specifically, the reduction in duodenum crypt depth implies that *C. glomerata* may reduce the efficiency of primary nutrient absorption, resulting in a reduced digestive area requirement. Meanwhile, the increased ileum crypt depth suggests an improvement in the absorption of nutrients that remain digested. These findings provide valuable insights into the potential impact of *C. glomerata* on nutrient uptake in rabbits and highlight the need for further research to elucidate the underlying mechanisms. The calculated V/C ratio is an important indicator of the surface area available for nutrient absorption and the number of cells responsible for nutrient transport. The higher V/C ratio observed under the CG4 treatment in the duodenum may suggest an increased capacity for nutrient absorption in this segment of the intestine, which could be beneficial for overall nutrient uptake. However, the opposite effect was observed in the ileum, with a higher V/C ratio under the standard compound diet. This suggests that the addition of macroalgal biomass to the feed may not have a consistent effect on nutrient absorption throughout the intestine.

Conversely, excessive ingestion of some bioactive compounds in *C. glomerata* such as phycotoxins, can cause adverse effects on the digestive system. Moreover, the inclusion of macroalgae in feed could impact the ratio of different bacteria in the gut, leading to changes in the production of short-chain fatty acids (SCFAs) and the absorption of nutrients. These changes in the gut microbiota composition and function can affect the development and function of internal organs such as the liver, pancreas, and intestine (Cani and Jordan, 2018).

However, our findings revealed that the addition of macroalgal biomass had minimal impact on the SCFA profile, except for a significant reduction in the concentration of lactic acid in the duodenum of rabbits receiving the CG4 treatment, with levels nearly four times lower than those observed in the SCD. These results suggest that the addition of macroalgal biomass to rabbit feed may affect microbial

fermentation in the intestine, resulting in a lower concentration of lactic acid. Lactic acid is an important metabolic product of bacterial fermentation and can contribute to maintaining the acidic pH of the intestine, which is necessary for optimal nutrient absorption and immune function (Pessione, 2012). It is produced by beneficial gut bacteria during fermentation of dietary fiber, and helps to create an acidic environment that suppresses the growth of harmful bacteria. The reduction in lactic acid concentration in the *duodenum* of rabbits fed with the CG4 treatment could have a negative impact on their gut health, which may result in a disruption of the gut microbial community and a decrease in nutrient absorption. This can lead to weaker peristalsis and bowel movements, which could ultimately affect the overall health and growth of the rabbits. It is worth noting that the specific effects of a reduction in lactic acid concentration may vary depending on the balance of other SCFAs and gut microorganisms present in the gut, as well as other factors such as diet and stress.

Ammoniacal nitrogen ($\text{NH}_3\text{-N}$) is an important indicator of gut health in rabbits. High levels of $\text{NH}_3\text{-N}$ in the gut can indicate poor feed quality, incomplete digestion, and impaired nutrient absorption, leading to reduced growth rates and poor overall health (Jha and Berrocoso, 2016). On the other hand, low levels of $\text{NH}_3\text{-N}$ may indicate a healthy gut environment with efficient nutrient absorption and proper feed digestion. Therefore, the evaluation of this indicator levels in rabbit gut content is an essential aspect of assessing their gut health status. In this study, while the addition of *C. glomerata* did not significantly impact $\text{NH}_3\text{-N}$ levels in rabbit *duodenum* content, further studies are necessary to investigate other potential changes in gut health parameters resulting from the addition of *C. glomerata* to the diet.

5. CONCLUSIONS

Supplementation of rabbit feed with 4% *C. glomerata* macroalgal biomass resulted in significant changes in crypt depth and V/C ratio, with a decrease in the duodenal crypt depth and an increase in the ileal crypt depth. This suggests that *C. glomerata* may improve the absorption of nutrients that remain digested but reduce the digestive area required for primary nutrient absorption. Additionally, the decrease in lactic acid concentration in the *duodenum* under the CG4 treatment warrants further investigation, as its reduction can affect weaker peristalsis and bowel movements. Overall, while further studies are needed to determine its potential benefits and drawbacks, the findings suggest that *C. glomerata* may have potential as a feed supplement for rabbits, with minimal effects on organ and intestinal development, as well as on the SCFA profile and $\text{NH}_3\text{-N}$ levels.

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