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Chlorella vulgaris-enriched plant-based meat analogue: nutritional, vitamin B12 and techno-functional properties

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ABSTRACT

Microalgae have emerged as promising alternative protein sources owing to their high nutritional value and abundance of essential micronutrients. In this study, *Chlorella vulgaris* biomasses produced under varied cultivation conditions were incorporated into plant-based meat (PBM) formulations through the partial replacement of egg white powder, starch, and psyllium. The aim was to evaluate the effects of *Chlorella* incorporation on the nutritional, digestive, and techno-functional properties of the resulting formulations.

Chlorella significantly increased protein, mineral, and vitamin B12 contents. All biomasses and PBM formulations contained substantial amounts of methylcobalamin and cyanocobalamin, with HPLC-DAD analysis identifying methylcobalamin as the predominant cobalamin form. In vitro digestion demonstrated improved protein and dry matter digestibility, particularly in heterotrophically–cultivated *Chlorella* formulations. Rheological and textural analyses showed that microalgal incorporation increased firmness and viscoelastic behavior, whereas cohesiveness remained unaffected. The differences observed among formulations reflected the compositional characteristics of the respective Chlorella biomasses.

Overall, these findings demonstrate the potential of Chlorella vulgaris biomass as a multifunctional ingredient for improving the nutritional quality and techno-functional properties of plant-based meat analogues.

1. Introduction

Meat remains a staple element in the human diet across the globe, valued for its robust nutritional profile, both as a primary source of high-quality protein, essential amino acids, vitamins, and minerals (Boukid, 2021). Globally, per capita meat consumption and overall meat intake remains on the rise, driven by population growth and affordable market options. According to the Food Balance Sheet data, the average global meat consumption is approximately 122 *g* per day. One-third of this amount consists of pork and chicken, one-fifth of beef, and the remainder of sheep, goats, and other animal sources (FAO, 2023).

However, as global consumption continues to rise due to population growth and economic development, the sustainability of large-scale meat production has come under increasing scrutiny. Key sustainability challenges include greenhouse gas emissions, deforestation, high water consumption, and ethical issues related to animal welfare (Imran & Liyan, 2023).

These environmental and ethical challenges, together with growing awareness of public health issues such as antibiotic resistance and zoonotic diseases associated with intensive animal farming, have intensified the global scientific, economic, and societal debate on diversifying protein sources (Ismail et al., 2020; van der Weele et al., 2019). Consequently, the popularity of plant-based dietary patterns, including vegan and flexitarian diets, has increased, stimulating policy initiatives and industrial investment in plant-based alternatives (Apostolidis & McLeay, 2016).

Efforts to develop meat alternatives, also referred to as meat substitutes, mock meat, or faux meat, began with early attempts to replace processed meat products using soy, wheat gluten, and other plant-based proteins. Bakhsh et al. (Bakhsh et al., 2021) provided a comprehensive timeline describing the development of plant-based meat analogues from CE 620 to 2020. Despite significant advances, attributes such as taste, texture, and the overall sensory experience often fall short of replicating the complexity of conventional meat. Consumer preference studies in Western countries indicate that meat-eaters are more likely to adopt plant-based meat analogues when these products closely replicate

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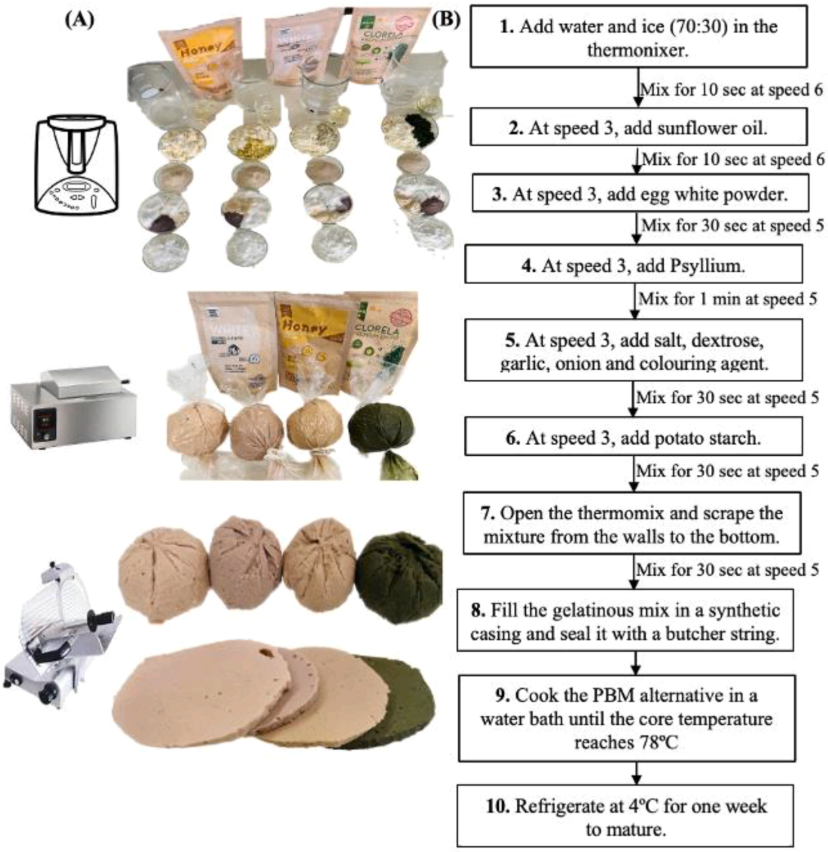

Fig. 1. Photographs (**A**) and schematic representation (**B**) of plant-based meat analogue manufacturing/cooking.

the texture and sensory characteristics of conventional meat. Furthermore, reproducing a meat-like eating experience facilitates the integration of these products into familiar dietary habits (Elzerman et al., 2011). Consequently, the food industry has increasingly focused on developing products that mimic the appearance, texture, and sensory properties of conventional meat.

Maintaining the nutritional value of conventional meat remains a major challenge for plant-based alternatives. Animal-derived proteins provide highly bioavailable vitamins and minerals that are difficult to reproduce using plant-derived ingredients alone.

Microalgae, particularly *Chlorella vulgaris*, have attracted considerable interest as food ingredients because of their high protein content and rich composition of vitamins, minerals, pigments, and bioactive compounds (Williamson et al., 2024; Fatima et al., 2023; Son & Kim, 2023; Khemiri et al., 2021; Ali et al., 2025). Previous studies have explored the incorporation of Chlorella into products such as pasta and couscous (Fradinho et al., 2020; Zouari et al., 2011; Khemiri et al., 2022), bakery products (Raymundo & Fradinho, 2023; Mahmoud et al., 2024; Khemiri et al., 2020; Nunes et al., 2020), mayonnaise (Coelho et al., 2026), and cheese (Hernández et al., 2025), reporting improvements in nutritional composition and functional properties.

Previous studies have also highlighted the techno-functional potential of *Chlorella* and other microalgae in meat analogue applications, particularly with respect to protein functionality, emulsifying properties, extrusion behavior, and texture formation (Zhu et al., 2024). More recent studies have compared *Chlorella* proteins with conventional plant proteins such as soy and pea (Akhter et al., 2026). Nevertheless, most of these studies have focused either on isolated protein comparisons or on processing characteristics, with limited attention to the combined nutritional, functional, and technological effects of *Chlorella* incorporation into final food products.

Among the nutritional limitations associated with plant-based diets, vitamin B12 deficiency remains a major concern because this vitamin is naturally found primarily in foods of animal origin. Although *Chlorella* has been incorporated into various food products, limited information is available regarding the vitamin B12 content of microalgae-enriched meat analogues. Most previous studies have focused on the characterization of cobalamin in the microalgal biomass itself or on the influence of cultivation conditions on vitamin B12 content (Santos et al., 2024; Bito et al., 2016).

The 4% *Chlorella* incorporation level was selected based on previous studies and preliminary formulation trials demonstrating its suitability for balancing nutritional enhancement, product acceptability, and techno-functional performance (Khemiri et al., 2021; Khemiri et al., 2020; Zhu et al., 2024; Khemiri et al., 2025; Oliveira et al., 2023). Recent studies suggest that incorporation levels below 10% help minimize undesirable sensory and textural changes while maintaining nutritional benefits (Zhu et al., 2024).

To the best of our knowledge, few studies have simultaneously evaluated the nutritional and techno-functional effects of *Chlorella* incorporation into plant-based meat (PBM) formulations, particularly with respect to the characterization of vitamin B12 forms in the final product. Therefore, the present study aimed to evaluate the effects of incorporating three distinct *Chlorella vulgaris* biomasses into PBM analogues on their nutritional composition, vitamin B12 content, digestibility, textural and rheological properties, and antimicrobial activity.

2. Materials and methods

2.1. Base formula and key ingredients

The ingredients used in the experiment included distilled water, ice, sunflower oil, egg white powder, dextrose, salt, garlic powder, onion powder, and potato starch. All ingredients, as well as the synthetic casing, were commercially available in the Portuguese market. The egg white powder contained 81% (w/w) protein, while the potato starch comprised 83.3% (w/w) starch.

Three microalgal biomasses, identified as White *Chlorella*, Honey *Chlorella*, and Organic *Chlorella*, were supplied by Allmicroalgae – Natural Products S.A. (Pataias, Portugal). Organic *Chlorella* corresponds to autotrophically cultivated *Chlorella* vulgaris, whereas White and Honey *Chlorella* are derived from heterotrophic cultures, representing strains obtained through mutagenesis that led to altered pigmentation profiles.

Post cultivation, the biomasses were harvested by centrifugation, spray-dried, and milled into powder prior to use. No additional mechanical cell disruption treatment was applied before incorporation. Further details regarding cultivation conditions and strain development are available in Khemiri et al. (2021).

2.2. Plant-based meat analogue preparation

PBM analogues were prepared using a formulation adapted from (Carvalho et al., 2025), with the ingredients listed above and the procedure outlined in Fig. 1. Four distinct formulations underwent to post analysis. The control product consisted of: 61.5% ice water, 13% oil, 12% egg white powder, 4% psyllium, 4% potato starch, 1.1% flavorant, 1% dextrose, 1% salt, 1% colorant, 0.5% garlic, 0.5% onion, and 0.4% vinegar, yielding a total of 100 g. *Chlorella* biomass was incorporated at 4% (w/w) of the total formulation. This was achieved through proportional substitution within the main functional fraction of the formulation composed of egg white powder, psyllium, and potato starch (20% of the total formulation). The reduction of each component was performed proportionally to its initial contribution within this fraction (12:4:4), resulting in decreases of 2.4%, 0.8%, and 0.8% of the total formulation weight, respectively.

2.3. Plant-based meat analogue quality characterization: color, pH, and water activity

The color determination of the samples was conducted using the CIELAB scale, with direct readings obtained from a Chroma Meter CR 400 Series by Konica Minolta (Osaka, Japan). Three coordinates were assessed: L* representing the luminosity of the sample between black (0) and white (100); a* indicating redness (+60) and greenness (−60); and b* indicating yellowness (+60) and blueness (−60). Prior to measurement, the colorimeter was calibrated using a standard white plate (L*=94.68; a*=−0.52; b*=3.47). Each sample was measured at six distinct areas of a slice under identical size and shape conditions.

The pH was measured using a BASIC 20+ pH meter by Crison Instruments (Spain). pH readings were obtained in triplicate from three different areas of a stack of slices with specific height.

Water activity (aw) was assessed using the HygroPalm HP23-AW-A apparatus manufactured by Rotronic (Switzerland), coupled with a GH refrigerated water bath by Haake (Fisons, Germany).

2.4. Nutritional composition

The moisture and ash content of the PBMs were determined in accordance with ICC methods 110/1 and 104/1, respectively. To measure the total fat content, acidic hydrolysis was performed, followed by centrifugation and extraction with n-hexane (Khemiri et al., 2021). The resulting crude fat residue was then determined gravimetrically following rotary evaporation. For the assessment of the mineral profile, inductively coupled plasma optical emission spectrometry (ICP-AES) was utilized, employing a Thermo System ICAP-7000 instrument (Waltham, MA, USA). Crude protein content was determined using the Dumas method with a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Intersscience, Breda, the Netherlands), applying a nitrogen-to-protein conversion factor of 6.25. Carbohydrate content was calculated by difference, subtracting the protein, lipid, ash, and moisture contents from the total.

The nutritional composition of the *Chlorella* biomasses (White, Honey, and Organic) was determined using the same analytical procedures described for the plant-based meat analogue samples. Moisture, crude protein, lipid, and ash contents were determined as described above, while crude fiber was analyzed using the Weende method (AOAC 978.10). Chlorophyll content was determined spectrophotometrically after extraction of the algal biomass. Extract aliquots were prepared at a concentration of 1 mg/mL in ethanol, and absorbance was measured at 470, 648, and 664 nm. Chlorophyll content was calculated using the Lichtenthaler equations. Carbohydrate content was calculated by difference, and vitamin B12 (methylcobalamin and cyanocobalamin) was quantified by HPLC as described in Section 2.5.

2.5. Vitamin B12 determination

Vitamin B12 was quantified as methylcobalamin and cyanocobalamin using high-performance liquid chromatography (HPLC). Both *Chlorella* biomasses and plant-based meat analogue (PBM) samples were analyzed.

Samples were extracted with water and purified with Amberlite® resin columns prior to analysis.

Chromatographic analysis was performed by HPLC system (Chromaster, Hitachi, VWR) equipped with a diode-array detector (5430 DAD, Hitachi, VWR) and a C18 column (150 × 4.6 mm, 5 μm). Separation was achieved under isocratic conditions using a methanol-based mobile phase adjusted to pH 4 with trifluoroacetic acid (TFA), at a flow rate of 0.6 mL/min. The injection volume was 50 μL, and detection was carried out at 230 nm. Retention times were approximately 3.2 min for cyanocobalamin and 3.8 min for methylcobalamin.

Quantification was performed using external calibration curves prepared with pharmacopeia-grade standards of methylcobalamin and cyanocobalamin over a concentration range of 0–1000 μg/100 g. The method showed good linearity, with coefficients of determination (R^2) of 0.996 for methylcobalamin and 0.995 for cyanocobalamin.

Method precision was evaluated through repeatability tests ($n = 10$), measured by coefficients of variation (CV) below 2%. Accuracy was assessed through recovery tests in both standards and sample matrices with recovery values exceeding 90%.

Limits of detection (LOD) and quantification (LOQ) were 0.082 μg/100 g and 0.25 μg/100 g, respectively. Compound identification was confirmed by comparing retention times with reference standards.

Samples (PBMs and *Chlorella* biomasses) were analyzed as single measurements, and analytical reliability was ensured through method validation.

2.6. Antimicrobial analysis

The in vitro antimicrobial activity of the PBM formulations was based on the drop test method applied by (Khemiri et al., 2025). The tested microorganisms included *Listeria innocua* (BISA 3008; NCTC11288), *Staphylococcus aureus* (BISA 3966; ATCC 6538), *Escherichia coli* (BISA 3967; ATCC 25,922), *Salmonella typhimurium* (BISA 3969; ATCC 14,028), and *Bacillus cereus* (BISA 4043; ATCC 11,778), obtained from the ISA (Instituto Superior de Agronomia) bacterial collection.

Bacterial strains were cultivated in Brain Heart Infusion (BHI) medium at 37 °C, except *Bacillus cereus*, which was incubated at 28 °C. Cultures were grown to the mid-exponential phase and serially diluted from 10^{-1} to 10^{-5} prior to analysis. Aliquots (3 μL) of each bacterial dilution were directly inoculated onto 2 cm slices of PBM analogues under aseptic conditions and incubated for 48 h. BHI plates inoculated under the same conditions were used as controls. Images were recorded after 48 h of incubation. The entire experimental procedure was conducted in duplicate.

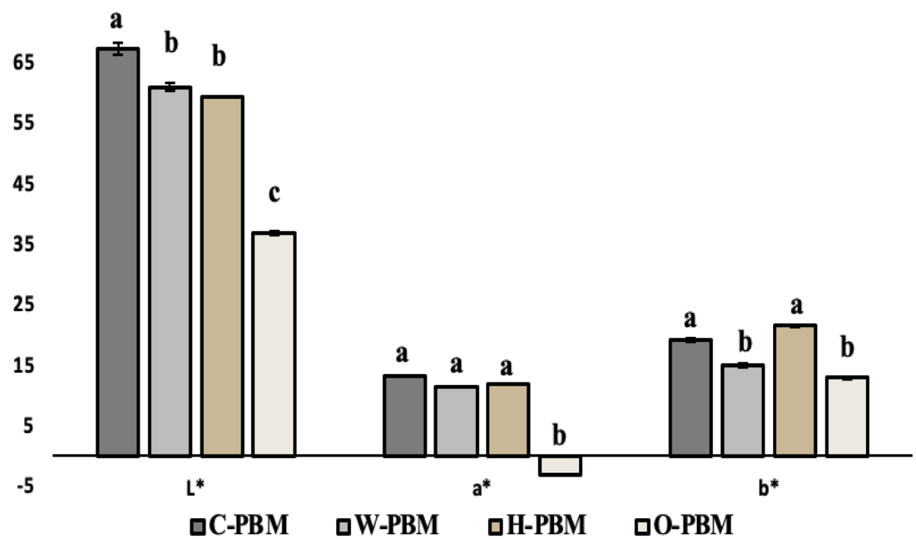

Fig. 2. Color attributes (L*, a*, b*) of meat-alternative product with 4% *Chlorella vulgaris* addition. C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. Different letters in each bar indicate significant differences ($p < 0.05$).

2.7. In vitro digestibility tests: infogest static model

The in vitro digestibility of the cooked PBM analogue samples was evaluated according to the standardized INFOGEST protocol. Prior to digestion, enzyme activities and bile concentration were determined following the procedure described by Minekus et al. (2014). Each sample was sequentially mixed with simulated salivary, gastric, and intestinal fluids, as described in detail by Khemiri et al. (2021). Digestion was performed at 37 °C under continuous gentle mixing using a rotating wheel. The intestinal phase was terminated by adding the protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; Pefabloc®, 500 mmol/L; Roche, Basel, Switzerland). The digestibility of the *Chlorella* biomasses was evaluated using the same protocol. For each digestion, 1 g of microalgal powder was mixed with distilled water to obtain a final sample weight of 2.5 g (sample-to-water ratio of 2:3, w/w). PBM analogues and microalgal samples were digested in triplicate, together with triplicate blank controls containing only simulated digestive fluids and reagents. Following digestion, all samples were centrifuged at 18,000 × g and 4 °C. The undigested residues were collected and dried at 80 °C for 6 h, followed by drying at 45 °C until constant weight. The in vitro dry matter and crude protein digestibility (%) of the PBM analogues were calculated from the difference between the initial dry weight and the weight of the undigested residue, expressed as a percentage of the initial dry weight (Khemiri et al., 2021).

2.8. Texture evaluation

Texture Profile Analysis (TPA) was performed on 2 cm-thick PBM analogue slices using a texture analyzer equipped with a black Delrin cylindrical probe (P/10: DIA Cylinder Delrin; 10 mm diameter, 35 mm height, contact area 78.54 mm^2). The prototypes were sliced using a SAS 120 C1 electric multi-purpose slicer (SilverCrest, Germany), and slice thickness was verified using a digital caliper (PowerFix Profi, South Africa). The TPA test was conducted in penetration mode at a test speed of 1 mm/s, with a 5 s interval between compression cycles and a penetration distance of 5 mm. The following texture parameters were obtained: firmness (N), defined as the maximum force required during the first compression; adhesiveness (−N·s), defined as the work required to overcome the attractive forces between the food surface and the probe; and cohesiveness, which reflects the strength of the internal structural bonds of the sample.

2.9. Rheology measurements

Rheological measurements were performed using a controlled-stress rheometer (Haake MARS III, Thermo Scientific, Germany) equipped

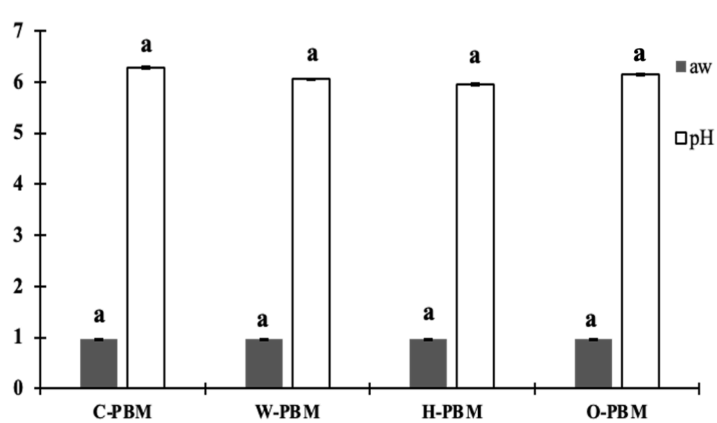

Fig. 3. Basic properties of meat-alternative product with 4% *Chlorella vulgaris* addition. C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. Different letters in each bar indicate significant differences ($p < 0.05$).

Table 1
Nutritional profile of *Chlorella* biomass.

	White chlorella	*Honey chlorella*	*Organic Chlorella*
Moisture (%W/W)	6.57±0.30[b]	4.72±0.13[c]	8.03±0.22[a]
Protein g/100 *g*	28.77±2.69[a]	25.70±0.08[a]	26.86±0.22[a]
Carbohydrate g/100 *g*	29.20	45.37	32.05
Lipid g/100 *g*	9.80±0.10[b]	11.48±0.17[a]	9.50±0.21[b]
Ash g/100 *g*	4.02±0.08[b]	2.48±0.46[c]	8.50±0.21[a]
Fiber g/100 *g*	21.64±0.12[a]	10.25±0.09[c]	15.06±0.13[b]
Chlorophyll mg/100 *g*	3.55±0.06[c]	65.22±0.60[b]	879.76±33.83[a]
Vitamin B12 µg/100 *g*			
Cyanocobalamin	287.34	278.54	265.23
Methylcobalamin	477.12	467.21	456.01

* Carbohydrate content was determined by difference.
Data shown are mean ± SD, $n = 3$. Different superscripts within the same line indicate significant difference ($p < 0.05$).

with an air compression system (Eheim Professional 3) and a Peltier temperature control unit. A serrated parallel-plate geometry (PP20, 20 mm diameter) was used to analyze 2 cm-thick PBM analogue slices. The measuring gap was adjusted according to the sample thickness, which was determined using a digital caliper.

Prior to the frequency sweep measurements, a stress sweep test was performed at an oscillation frequency of 1 Hz to determine the linear viscoelastic region (LVR). Frequency sweep tests were subsequently carried out within the LVR over a frequency range of 0.01–100 Hz (0.0628–628 rad/s) to determine the storage modulus (G′) and loss modulus (G″). All measurements were performed in a temperature-controlled room.

The storage modulus (G′) and loss modulus (G″), expressed in Pa, were plotted as a function of angular frequency (ω, rad/s) and fitted to the following power-law equations, where α′ and α″ are the scaling coefficients and b′ and b″ are the fitted exponents (Khemiri et al., 2021; Khemiri et al., 2020):

$$G'(\omega) = \alpha' \omega^{b'} \quad (1)$$

$$G''(\omega) = \alpha'' \omega^{b''} \quad (2)$$

2.10. Statistics

Statistical analyses were performed using OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA). Differences among samples were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. All experiments were performed in at least triplicate, and the results are expressed as mean ± standard deviation (SD). Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Quality properties

The incorporation of *Chlorella* biomass did not significantly affect the pH or water activity of the PBM analogues (Figs. 2 and 3), with average values of 6.20 and 0.97, respectively. Maintaining comparable pH and water activity across formulations is advantageous because these parameters strongly influence microbial growth, product stability, and shelf life. The similar values observed among treatments indicate that replacing part of the formulation with *Chlorella* biomass did not alter the physicochemical environment of the products.

Unlike conventional PBMs, which frequently rely on color additives such as annatto extract (E160b), lycopene, beetroot extract, leghemoglobin, or titanium dioxide to mimic the appearance of meat (Oreopoulou & Tzia, 2007; Schreuders et al., 2019), the PBM analogues developed in this study derived their color from the natural pigments present in the *Chlorella* biomasses. Consequently, significant differences

Table 2
Nutritional profile and energy value of plant-based meat analogue with 4% *Chlorella vulgaris* incorporation.

	C-PBM	W-PBM	H-PBM	O-PBM
Moisture (% W/W)	63.79 ±0.26[a]	63.83 ±0.26[a]	63.82 ±0.14[a]	64.28 ±0.18[a]
Protein (g/100 g)	9.54±0.27[b]	**10.34 ±0.26[a]**	9.67±0.15[b]	**10.31 ±0.27[a]**
Fat (g/100 g)	14.57 ±1.68[a]	13.75 ±0.72 [a]	13.71 ±0.42 [a]	13.76 ±0.35 [a]
Ash (g/100 g)	1.79±0.30[a]	1.87±0.05[a]	2.00±0.05[a]	2.07 ±0.20[a]
Total carbohydrate*(g/100 g)	10.30 ±1.34	10.21 ±1.23	10.81 ±0.70	9.57 ±0.56
Energy value (Kcal/100 g)	210.55 ±10.58[a]	205.97 ±2.53[a]	205.27 ±1.60[a]	203.37 ±1.89[a]
Minerals mg/100 *g***				
K (15% RDV= 300)	134.43 ±6.52[a]	136.23 ±11.11[a]	145.32 ±15.20[a]	122.91 ±5.96[a]
P (15% RDV= 120)	20.66 ±1.30[c]	56.63 ±4.25[a]	57.55 ±5.96[a]	63.92 ±3.94[a]
Mg (15% RDV= 45)	7.90±0.51[b]	11.31 ±0.89[a]	10.21 ±1.14[a]	12.18 ±0.81[a]
Ca (15% RDV= 120)	18.54 ±1.72[c]	30.02 ±2.61[b]	24.21 ±2.54[b]	45.43 ±3.04[a]
Fe (15% RDV= 2.1)	0.85±0.06[c]	0.94 ±0.06[bc]	1.41±0.34[b]	**3.38 ±0.24[a]**
Zn (15% RDV= 2.3)	0.18±0.08[b]	1.22±0.08[a]	0.68±0.02[a]	0.92 ±0.06[a]
Vitamin B12 µg/100 *g*				
Cyanocobalamin	84.62	105.35	106.58	89.63
Methylcobalamin	460.34	470.87	478.9	482.23

C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. Data shown are mean ± SD, $n = 3$. Different superscripts within the same line indicate significant difference ($p < 0.05$).
* Carbohydrate content was determined by difference.
** According to the recommended daily values (RDV) established by Regulation (European Community) No 1924/2006; Directive No 90/494 (EC).

in color parameters were observed among the formulations. In particular, the O-PBM sample exhibited the lowest a* value (highest greenness), which is consistent with the higher chlorophyll content measured in the Organic *Chlorella* biomass (Table 1). Previous studies have shown that thermal processing can influence the color of both plant-based and meat products. For example, (Yuliarti et al., 2021) reported decreased L* values in plant-based nuggets following deep frying, while (Ngadi et al., 2007) observed a similar reduction in lightness with increasing frying time in chicken nuggets. In the present study, however, all PBM analogues were processed under identical cooking conditions; therefore, the observed color differences can be primarily attributed to differences in the pigment composition of the *Chlorella* biomasses rather than to processing effects

3.2. Proximate nutritional composition, energy, and references values

The nutritional composition of the *Chlorella* biomasses and the formulated PBM products is presented in Tables 1 and 2, respectively. The three *Chlorella* biomasses showed distinct compositional profiles. Moisture content ranged from 4.72% to 8.03%, with Organic *Chlorella* exhibiting the highest value ($p < 0.05$). Protein content ranged between 25.70 and 28.77 g/100 g ($p > 0.05$) across the samples. However, significant differences were observed in lipid, ash, and fiber contents. Honey *Chlorella* presented the highest lipid content (11.48 g/100 g, $p < 0.05$), whereas Organic *Chlorella* showed the highest ash content (8.50 g/100 g, $p < 0.05$). Fiber content also varied markedly, with White *Chlorella* exhibiting the highest value (21.64 g/100 g), followed by Organic (15.06 g/100 g) and Honey *Chlorella* (10.25 g/100 g) (p <

0.05). Chlorophyll content also differed substantially among the biomasses, with Organic *Chlorella* exhibiting the highest concentration (879.76 mg/100 g) ($p < 0.05$).

Although protein content is often reported to be higher in autotrophically cultivated *Chlorella* (Sajadian et al., 2018), no significant differences were observed among the biomasses in the present study, possibly due to batch-to-batch variability and minor differences in processing conditions. Such variability is consistent with previous reports showing that the biochemical composition of microalgae depends on factors such as cultivation mode (autotrophic vs. heterotrophic), nutrient availability, and harvesting conditions (Khemiri et al., 2025; Sajadian et al., 2018; Khemiri et al., 2022). These compositional differences may influence the functional behavior of the biomasses when incorporated into food matrices.

In terms of the PBM formulations, the analysis of the biochemical composition revealed no significant differences in moisture, fat, ash, or energy content ($p > 0.05$). Protein content was significantly higher in W-PBM and O-PBM than in C-PBM ($p < 0.05$), whereas H-PBM did not differ from the control (Table 2). Higher mineral contents were also observed in the *Chlorella*-enriched PBMs, particularly for phosphorus, magnesium, calcium, iron, and zinc. Overall, O-PBM presented the highest mineral contents (Table 2).

Based on the contribution of protein to the total energy value, the control PBM formulation already met the requirements for the *source of protein* nutrition claim, with >12% of the total energy derived from protein, primarily provided by egg white powder. Nevertheless, W-PBM and O-PBM fulfilled the criteria for a *high in protein* claim, as >20% of their total energy was derived from protein. Total carbohydrate content remained comparable among all formulations.

Egg white protein is commonly incorporated into meat alternative products because of its high nutritional value and excellent functional properties, including foaming, gelation, and emulsification (Mirarab-Razi et al., 2024). In the present formulations, it served as the primary protein source, while the incorporation of *Chlorella* biomass provided an additional source of protein together with essential minerals, thereby improving the overall nutritional profile of the PBM matrix. Besides egg white, plant proteins such as soy, wheat gluten, legumes, pulses, beans, and nuts have also been explored in meat alternatives because of their ability to contribute to a meat-like texture and mouthfeel (van der Weele et al., 2019; Boukid & Gagaoua, 2022). Nevertheless, current product development extends beyond meeting protein requirements and increasingly aims to improve the overall nutritional quality of meat alternatives while promoting more sustainable food systems.

Apart from protein, the iron content of the *Chlorella*-enriched PBMs reached levels that would allow the products to be classified as a *source of iron* according to current regulatory criteria, thereby contributing to dietary iron intake. However, it should be noted that mineral content does not necessarily reflect bioavailability, as nutrient release and absorption may be influenced by the microalgal cell wall structure and interactions within the food matrix. Recent studies have demonstrated that the bioaccessibility and bioavailability of microalgae-derived minerals can vary considerably depending on the species, structural characteristics, and processing conditions. For instance, iron bioaccessibility in different microalgae has been reported to range from <1% to >80%, highlighting the strong influence of cellular structure and mineral speciation on nutrient release during digestion (Gao et al., 2026). Therefore, although the increased mineral content observed suggests a potential nutritional contribution, further studies are required to evaluate the bioaccessible and bioavailable fractions under gastrointestinal conditions.

Although the calcium content did not meet the threshold required for a nutritional claim, the incorporation of 4% *Chlorella* approximately doubled the calcium content compared with the control PBM (18.54 mg/100 g). Overall, the iron and calcium contents of the developed PBMs were notably higher than those reported for several meat products, including chicken and pork, by Yang et al. (2023). For instance, the calcium content of these meats ranged from 4.25 to 7.26 mg/100 g, substantially lower than the values observed in the present study (Table 2). Furthermore, the iron content of the *Chlorella*-enriched PBMs was approximately threefold higher than that reported for pork meat.

Additionally, similar nutritional improvements were previously reported using the same *Chlorella* biomasses incorporated into a different food matrix (Khemiri et al., 2021). In that study, the higher incorporation level (6%) resulted in more pronounced improvements in the nutritional profile. Moreover, the authors reported significant enhancements in the fatty acid composition, particularly with respect to omega-3 and omega-6 fatty acid contents.

Vitamin B12 (Cbl) content, determined as cyanocobalamin (CN—Cbl) and methylcobalamin (Me-Cbl), is presented in Tables 1 and 2 for the *Chlorella* biomasses and PBM formulations, respectively. In the *Chlorella* biomasses, total vitamin B12 content (sum of CN—Cbl and Me-Cbl) ranged from 721.24 to 764.46 μg/100 g, with White *Chlorella* exhibiting the highest concentration, followed by Honey and Organic *Chlorella* (Table 1). Methylcobalamin was the predominant form in all biomasses.

In the PBM formulations, both forms of vitamin B12 were detected in all samples, with consistently higher concentrations in the *Chlorella*-enriched PBMs than in the control. Total vitamin B12 content ranged from 544.96 μg/100 g in the control formulation to 576.22, 585.48, and 571.86 μg/100 g in W-PBM, H-PBM, and O-PBM, respectively. The vitamin B12 detected in the control formulation is likely attributable to the egg white powder used in the formulation, as eggs are recognized natural sources of cobalamin. The relative distribution of cyanocobalamin and methylcobalamin remained consistent across all formulations, with methylcobalamin representing the predominant form (Table 2).

The increase in vitamin B12 content observed in the *Chlorella*-enriched PBMs reflects the intrinsic composition of the microalgal biomasses. As shown in Table 1, all three *Chlorella* biomasses contained substantial amounts of both cyanocobalamin and methylcobalamin, which were subsequently incorporated into the PBM formulations. The differences observed among W-, H-, and O-PBM are consistent with the vitamin B12 contents of the respective biomasses, indicating that the vitamin B12 content of the final products was primarily determined by the composition of the added *Chlorella* biomasses.

Although microalgae are not generally considered primary producers of vitamin B12, the presence of cobalamin in *Chlorella* biomass has been widely attributed to its association with vitamin B12-producing microorganisms present during cultivation (Watanabe et al., 1997; Watanabe & Bito, 2018; van den Oever & McDougall, 2022). Microalgae themselves lack the metabolic capacity to biosynthesize cobalamin, as this ability is restricted to specific prokaryotes (van den Oever & McDougall, 2022; Rizzo et al., 2016). Instead, microalgae can take up exogenous cobalamin produced by bacteria, accumulate it intracellularly, and, in some cases, convert it into biologically active coenzyme forms (Bito et al., 2016; Edelmann et al., 2019). This symbiotic or commensal interaction between microalgae and bacteria provides a plausible mechanistic explanation for the presence of vitamin B12 in *Chlorella* biomass.

The variability observed in vitamin B12 content among the different *Chlorella* biomasses may therefore be related to differences in cultivation conditions, including the abundance of vitamin B12-producing bacteria, the nutrient composition of the culture medium, and environmental factors influencing microalgal–bacterial interactions. Additionally, the structural characteristics of the microalgal cell wall may influence cobalamin uptake, as biomasses with more complex or less permeable cell walls may exhibit reduced absorption capacities (Edelmann et al., 2019).

Previous studies using advanced chromatographic techniques have reported substantial variability in cobalamin content among *Chlorella* products, with values ranging from 9 to 211 μg/100 g depending on the

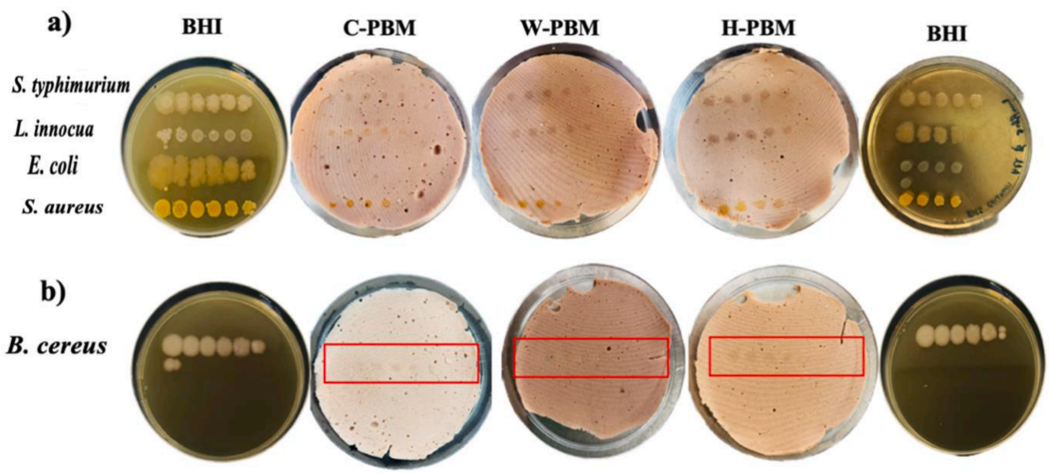

Fig. 4. Plates resulting from in vitro antimicrobial activity determination for pathogenic microorganisms of meat-alternative product with 4% *Chlorella vulgaris*. C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*. The growth control is BHI medium. From left to right, drops from 10^{-1} to 10^{-5} dilutions.

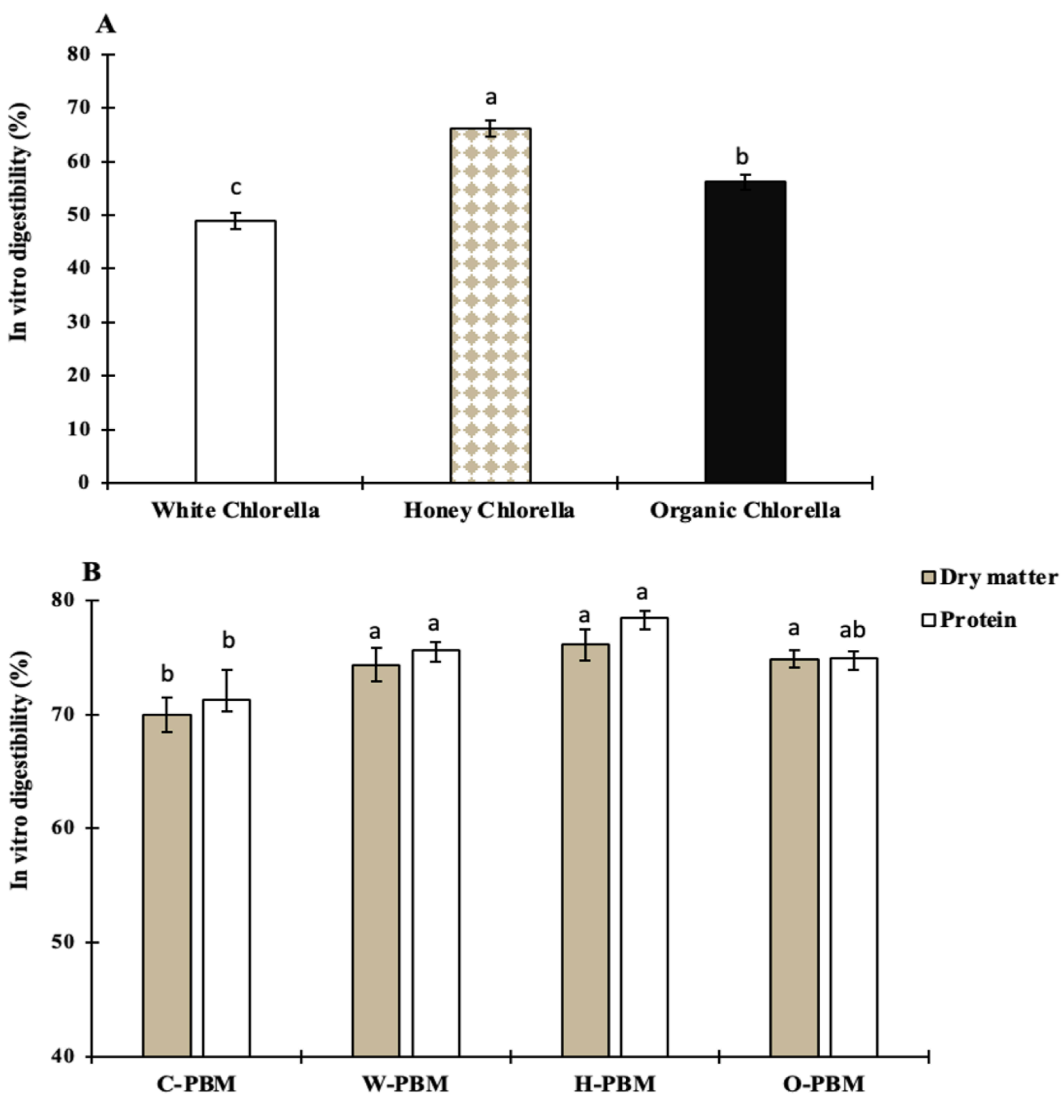

Fig. 5. (A) In vitro digestibility (%) of *Chlorella* biomasses and (B) in vitro digestibility (%) of plant-based meat (PBM) products formulated with 4% *Chlorella vulgaris*. C-PBM represents the control formulation without *Chlorella* addition; W-PBM, H-PBM, and O-PBM correspond to formulations containing white, honey, and organic *Chlorella*, respectively. Data are presented as mean ± standard deviation (n = 3). Different letters above bars within the same parameter indicate statistically significant differences ($p < 0.05$).

strain, cultivation conditions, and analytical methodology (Edelmann et al., 2019). In these studies, cobalamin was predominantly detected in its biologically active forms, supporting the potential nutritional relevance of *Chlorella* as a source of vitamin B12.

The relatively high vitamin B12 contents observed in both the *Chlorella* biomasses and the PBM formulations are consistent with previous reports using HPLC-based quantification methods, including studies on similar *Chlorella* biomasses, which have shown that cobalamin concentrations may exceed those stated on commercial labels (Santos et al., 2024). Such discrepancies are likely attributable to differences in analytical methodologies, as vitamin B12 quantification is known to vary depending on the detection technique employed (e.g., microbiological, radioisotope dilution, or chromatographic methods), as well as differences in extraction procedures and the specific cobalamin forms quantified (Santos et al., 2024; Santos et al., 2023). In addition, commercial labels often do not specify which cobalamin forms were measured, which may further contribute to variability in the reported values.

Variability in vitamin B12 content has also been reported for other microalgal species, such as *Limnospira (Spirulina) platensis*, where cobalamin has been detected at relatively high concentrations in some studies, whereas in others it was either not detected or found predominantly in biologically inactive forms (Koyande et al., 2019; Watanabe, 2007). These discrepancies highlight the complexity of vitamin B12 determination in microalgae and reinforce the importance of applying analytical approaches capable of distinguishing between different cobalamin forms.

As far as vitamin B12 is concerned, the recommended daily intake for adults is approximately 5 μg/day. Although the vitamin B12 concentrations measured in the present study exceed this value when expressed per 100 g of product, this does not diminish the nutritional relevance of the developed PBMs. Vitamin B12 absorption in humans is a tightly regulated and complex process involving specific transport mechanisms that limit the fraction of ingested cobalamin effectively absorbed and retained by the body (Stabler, 2013). Moreover, vitamin B12 deficiency remains prevalent among populations with low consumption of animal-derived foods, particularly individuals following vegetarian and vegan diets (Said, 2004), as well as older adults with impaired absorption capacity (Gironés-Vilaplana et al., 2021).

Overall, the observed nutritional enhancement reflects the biological complexity of *Chlorella* biomass, which acts as a natural source of organically bound minerals and vitamins embedded within a protein-rich cellular matrix. This differs from conventional fortification strategies based on isolated micronutrients, as the microalgal matrix may influence nutrient retention and bioaccessibility through physical entrapment and gradual release during digestion.

3.3. In vitro antimicrobial assessment

After 48 h of incubation under conditions favorable for bacterial growth, all bacterial strains exhibited substantial growth in the control rich medium (BHI). However, bacterial growth was less pronounced in both the control PBM (without *Chlorella*) and the *Chlorella*-enriched PBMs. *E. coli* did not grow on any of the PBM formulations (Fig. 4). Likewise, B. cereus exhibited only limited growth on the PBM samples, with colonies observed only at the three highest inoculum concentrations, in contrast to the robust growth observed in the BHI medium.

Furthermore, no visible differences in bacterial growth were observed between the control PBM and the *Chlorella*-enriched PBMs, suggesting that the PBM matrix itself may limit bacterial proliferation under the tested conditions. The incorporation of *Chlorella* did not substantially alter the microbial behavior observed in the PBMs. Nevertheless, further studies are required to better understand the factors influencing microbial growth in these formulations.

A previous study evaluating the microbiological spoilage of plant-based meat analogues reported that microbial proliferation depends on both formulation and storage conditions (Tóth et al., 2021). These findings highlight the importance of assessing microbial behavior in PBM products, particularly when incorporating novel ingredients such as microalgae.

3.4. In vitro digestibility

The in vitro digestibility (IVD) of food matrices is an important parameter for evaluating nutrient bioaccessibility under simulated gastrointestinal conditions. In recent years, increasing attention has been given to laboratory-scale studies evaluating the IVD of microalgae and microalgae-based foods, driven by their nutritional potential and their role as sustainable protein sources (Paterson et al., 2024; Machado et al., 2022). However, to the best of our knowledge, no studies have specifically addressed the IVD of *Chlorella*-based meat analogues.

Fig. 5 presents the in vitro digestibility of the *Chlorella* biomasses and PBM formulations. Significant differences were observed among the three *Chlorella* biomasses ($p < 0.05$). Honey *Chlorella* showed the highest IVD (67%), followed by Organic *Chlorella* (57%), while White *Chlorella* presented the lowest digestibility (49%).

Regarding the PBM formulations, both dry matter and protein digestibility were influenced by the incorporation of *Chlorella* biomass. For dry matter digestibility, all *Chlorella*-enriched PBMs showed significantly higher values compared to the control formulation (C-PBM), where H-PBM presented the highest digestibility (76%). W-PBM and O-PBM showed intermediate values, with no significant differences among the enriched formulations (Fig. 5A).

A similar trend was observed for protein digestibility. W-PBM and H-PBM showed significantly higher protein digestibility compared to C-PBM ($p < 0.05$), whereas O-PBM presented intermediate values and did not differ significantly from either the control or the other *Chlorella*-enriched formulations.

The differences observed among the *Chlorella* biomasses suggest that the intrinsic characteristics of the microalgae may influence their digestibility. Honey *Chlorella*, which showed the highest IVD, was characterized by lower fiber content compared to White and Organic *Chlorella* (Table 1). Since microalgal cell wall composition and structural organization are known to influence the accessibility of digestive enzymes (Paterson et al., 2024), these compositional differences may partially contribute to the variability observed among biomasses. However, no direct microstructural analyses were performed; therefore, causal relationships cannot be established.

Consistent with the biomass digestibility results, H-PBM showed the highest digestibility values among the PBM formulations. This consistency suggests that the intrinsic characteristics of the *Chlorella* biomass contribute to the digestibility of the final products. Nevertheless, the digestibility ranking of the *Chlorella*-enriched PBMs did not fully mirror that of the isolated biomasses, indicating that interactions within the food matrix also play an important role in determining digestibility.

Similar variability has been reported in previous studies evaluating the incorporation of microalgae into food products. For example, the addition of 3% *Limnospira platensis* to gluten-free pasta was associated with reduced digestibility (Fradinho et al., 2020), whereas our previous study evaluating the same *Chlorella* biomasses in couscous formulations reported increased IVD, particularly in products enriched with Honey and Organic *Chlorella* (Khemiri et al., 2021). These variations suggest that the IVD of microalgae-enriched foods may depend not only on the composition and structural properties of the microalgae, but also on processing conditions and the type of food matrix.

Previous studies comparing plant-based and animal-based meat products have also reported differences in protein digestibility, concluding that both the protein source and the structural organization of the food matrix influence digestive behavior (Xie et al., 2022). Similar observations were reported in that study, where differences in digestibility were attributed to variations in protein composition and food matrix structure (Xie et al., 2022).

Table 3
Structural properties of meat-alternative product with 4% *Chlorella vulgaris*.

	C-PBM	W-PBM	H-PBM	O-PBM
Firmness (N)	4.65±0.23[d]	5.21±0.21[c]	4.98±021[b]	6.79±0.13[a]
Adhesiveness (—N.s)	0.17±0.03[c]	0.48±0.09[b]	0.52±0.13[b]	0.71±0.13[a]
Cohesiveness	0.71±0.01[a]	0.65±0.01[a]	0.65±0.01[a]	0.66±0.03[a]

C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. Data shown are mean ± SD, $n = 3$. Different superscripts within the same line indicate significant difference ($p < 0.05$).

Overall, the present findings highlight the importance of both the characteristics of the microalgal biomass and the interactions within the food matrix in determining digestibility outcomes. Further studies combining microstructural characterization with in vivo approaches would contribute to a better understanding of nutrient bioaccessibility in *Chlorella*-enriched PBMs.

3.5. Mechanical properties

3.5.1. Texture properties

Texture is an essential quality attribute of plant-based meat analogues. Instrumental texture analysis provides objective measurements that are commonly associated with sensory characteristics (Steele et al.,

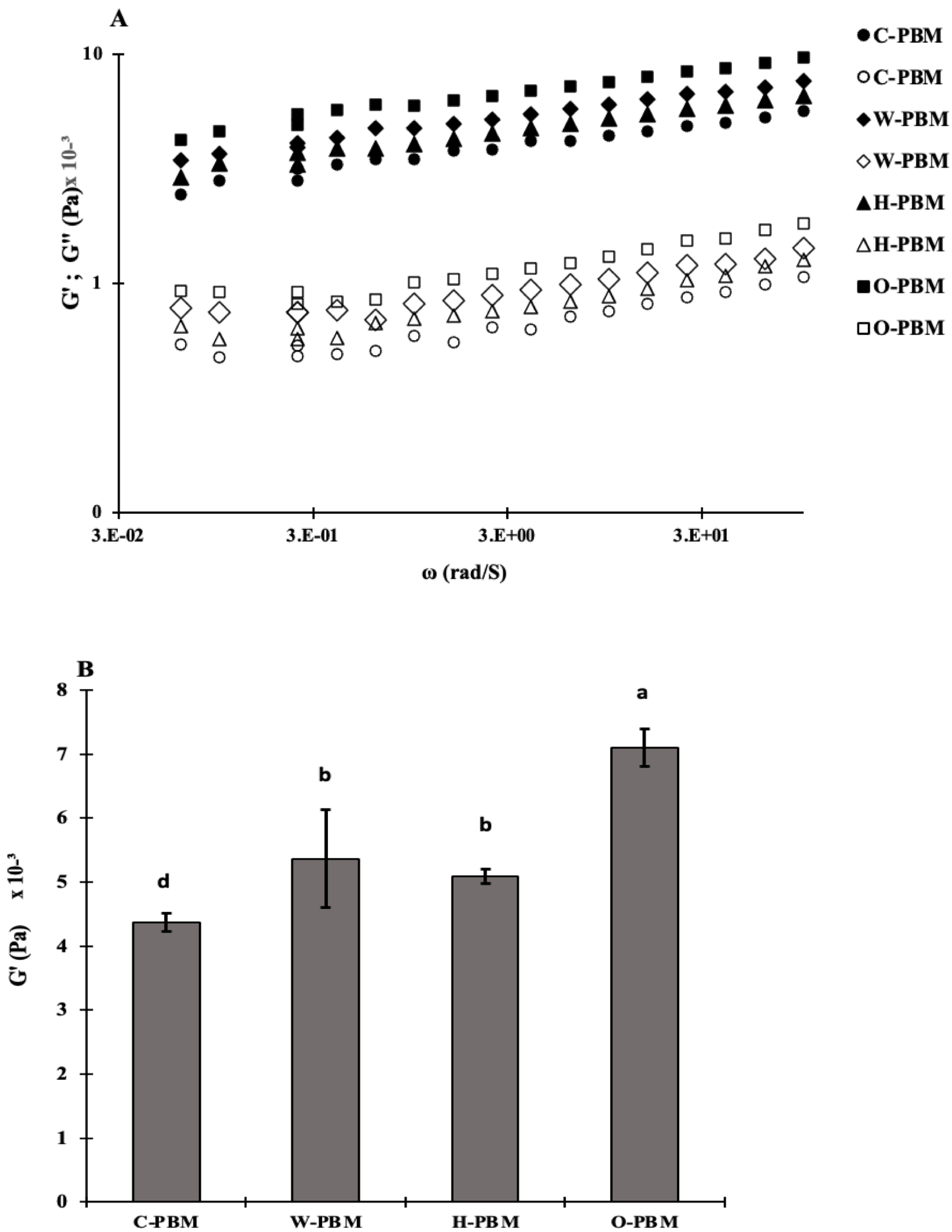

Fig. 6. Mechanical spectra of meat-alternative product with 4% *Chlorella vulgaris* (A) and values of G′ at 6.283 rad/s (1 Hz) (B). C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. Different letters in each bar indicate significant differences ($p < 0.05$).

Table 4
Power law parameters (α′, α", b′ and b") of meat-alternative product with 4% *Chlorella vulgaris*.

	G'		G"	
	α'	b'	α"	b"
C-PBM	43,611±291[d]	0.103 ±0.003[a]	7431±111[d]	0.112 ±0.007[a]
W-PBM	58,172±482[b]	0.103 ±0.002[a]	10,165 ±163[b]	0.111 ±0.013[a]
H-PBM	51,218±840[c]	0.105 ±0.004[a]	9079±269[c]	0.111 ±0.005[a]
O-PBM	72,143 ±1038[a]	0.105 ±0.001[a]	12,278 ±406[a]	0.113 ±0.004[a]

C-PBM is the product without *Chlorella* addition; W-PBM is the product with white *Chlorella*; H-PBM is the product with honey *Chlorella*; and O-PBM is the product with organic *Chlorella*. The goodness of fit (R2) ranged from 0.906 to 0.997. Data shown are mean ± SD, $n = 3$. Means with different superscripts within the same column differ significantly ($p < 0.05$).

2015). The textural properties of the *Chlorella*-based PBM formulations are presented in Table 3. The incorporation of *C. vulgaris* into the PBM formulations resulted in significant changes in texture, particularly in terms of firmness and adhesiveness ($p < 0.05$). Specifically, the formulation containing Organic *Chlorella* exhibited the highest firmness value (6.79 N, $p < 0.05$). This increase may be attributed to compositional differences associated with the incorporation of the *Chlorella* biomass (Khemiri et al., 2025). Adhesiveness was also affected, with the control formulation exhibiting the lowest value (0.17 N·s, $p < 0.05$), whereas all *Chlorella*-enriched formulations showed increased adhesiveness.

Although significant differences were observed for firmness and adhesiveness, cohesiveness remained relatively constant, with no significant differences detected between the control and the *Chlorella*-enriched formulations ($p > 0.05$) (Table 3).

Similar effects have been reported in previous studies evaluating different levels of microalgal incorporation. For instance, (Atitallah et al., 2019) investigated the incorporation of different microalgae into canned fish burgers and found that 1% *Picochlorum* sp. and 1% *Isochrysis galbana* significantly increased hardness ($p < 0.05$), whereas 0.5% *Chlorella minutissima* reduced hardness ($p < 0.05$). These findings suggest that the effect of microalgae on textural properties depends on both the species and the incorporation level. Likewise, (Marti-Quijal et al., 2018) reported that cooked turkey breast samples formulated with 1% *Chlorella* and *Spirulina* proteins exhibited lower hardness than samples prepared with soy protein, indicating that the impact of microalgal ingredients on texture is also influenced by the protein source.

3.5.2. Rheological behavior

Small-amplitude oscillatory shear measurements were performed to evaluate the viscoelastic behavior of the PBM formulations. Each sample was analyzed in triplicate to ensure measurement consistency and obtain representative rheological profiles for each formulation (Fig. 6A). All formulations exhibited typical viscoelastic solid-like behavior, with the storage modulus (G′) consistently exceeding the loss modulus (G″) over the entire frequency range evaluated. The incorporation of *Chlorella* modified the viscoelastic behavior of the PBMs, indicating that the microalgal biomass influenced the structural properties of the formulations (Fig. 6A). In particular, the formulation containing 4% Organic *Chlorella* exhibited significantly higher storage modulus values (G′ at 6.283 rad/s; Fig. 6B) than the control and the other formulations ($p < 0.05$). The higher G′ values observed for the White and Organic *Chlorella* formulations were consistent with their relatively higher protein contents (Table 2). Likewise, the α and b parameters, obtained from the linear regression of log G′ and log G″ versus log frequency, followed trends similar to those observed for G′. As shown in Table 4, higher α′ values were particularly evident for the White and Organic *Chlorella* formulations. Similar behavior has been reported for other plant-based products. For example, in pea protein-based nuggets, increasing the pea protein concentration resulted in higher G′ and G″ values, reflecting an increase in the viscoelastic properties of the system (Yuliarti et al., 2021).

4. Conclusions

The incorporation of *Chlorella vulgaris* into plant-based meat (PBM) formulations improved the nutritional composition of the products, particularly with respect to protein, mineral, and vitamin B12 contents. Significant differences were observed among the formulations, reflecting the compositional characteristics of the original *Chlorella* biomasses used as ingredients. Both methylcobalamin and cyanocobalamin were detected in the biomasses and the corresponding PBM formulations, highlighting the potential of *Chlorella* as a natural source of vitamin B12 for plant-based foods. *Chlorella* incorporation also influenced digestibility and techno-functional properties, including texture and rheological behavior, while no substantial differences in microbial behavior were observed compared with the control formulation. Overall, these findings demonstrate the potential of *Chlorella vulgaris* biomass as a multifunctional ingredient capable of enhancing both the nutritional quality and techno-functional properties of plant-based meat analogues. Future studies should focus on sensory evaluation, large-scale processing feasibility, and further optimization of formulation characteristics.

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Availability of data and material

The data supporting reported results can be accessed via email for the author (Sheymak@isa.ulisboa.pt).

Statement for studies in humans and animals

No studies involving humans or animals were conducted in this work.

CRediT authorship contribution statement

Sheyma Khemiri: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sara Ferreira:** Writing – review & editing. **Hugo Pereira:** Writing – review & editing. **Anabela Raymundo:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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