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The chemistry and bioactive properties behind microalgae-enriched gluten-free breads

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Summary This work aims to: (i) assess volatiles composition of microalgae-enriched gluten-free breads (prepared with *Tetraselmis chuii, Chlorella vulgaris* and *Microchloropsis gaditana*); (ii) report the bioaccessibility of protein and minerals after a static *in vitro* digestion model; (iii) understand how it affects the bioactive properties of gluten-free breads in regard to antioxidant capacity and the α-amylase inhibitory potential. Therefore, nutritional composition and bioactive properties of gluten-free breads before and after *in vitro* digestion model were examined. Identification of compounds responsible for the overall aroma of microalgae using GC–MS was done. There was a considerable decrease in protein and minerals available after *in vitro* digestion, as well as in the antioxidant potential. The overall aroma of microalgae-enriched breads was mainly due to the presence of alcohols, representing the major class of volatiles present in breads (3% to 59%), being lower for *T. chuii*-enriched bread. Terpenes also existed in considerable amounts especially in *M. gaditana* (24%) where γ-terpinene was the most abundant. Alkanoic acids were the most abundant lipophilic bioactive compounds (25%–68%), and 9,12-octadecadienoic acid was the major identified compound (13%–51%). Phytic acid is also present in all microalgae-enriched breads and may contribute to the decrease in the bioaccessibility of nutrients.

Keywords Bioaccessibility, gluten-free bread, *in vitro* digestibility, lipophilics, microalgae, volatiles composition.

Introduction

When it comes to treating celiac disease or other related conditions, despite extensive research that has been directed towards the development of alternative therapies, the fact is that adopting a lifelong gluten-free (GF) diet remains the only effective solution (Wungjiranirun *et al.*, 2016). Also, the growing consumer demand for dietetic products 'free from' certain components has provided a great incentive for product innovation and may drive the GF market in years to come (GFFMSG, Gluten Free Food Market Size, & Growth, 2021–2028).

Due to deficiencies of macro and micronutrients in patients with gluten-associated pathologies (Caio *et al.*, 2019) is essential to develop fortified products that help replace these nutrients. Therefore, the focus on this staple food is based on the reality of the market for GF products – usually full of synthetic additives and unhealthy amounts of added sugars and saturated fats (Penagini *et al.*, 2013). Reference should also be made to the misinformation surrounding gluten: The increased demand for these products by the non-celiac population (mainly due to unfounded beliefs), causes market to grow sharply with high prices (Lis *et al.*, 2015). Therefore, the market for GF products, as it stands, only presents one advantage for celiac patients: The elimination of gluten from food formulations. Disadvantages? Many. Going through the price, until you get to the poor nutritional value and sensory drawbacks (Penagini *et al.*, 2013) of the vast majority of products on the market, GF foods represent a real problem for those who need them. This is precisely where microalgae come into play. Able to grow in extreme conditions, present both in terrestrial and aquatic environments, microalgae are the most common organisms to inhabit the Earth (Ścieszka & Klewicka, 2019). Today, market begins to contemplate several products, from nutraceuticals to functional ingredients and functional foods manufactured from microalgae (Batista *et al.*, 2017). Representing an excellent and sustainable food source, the balanced chemical composition of microalgae, rich in proteins, profile of polyunsaturated fatty acids

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(PUFAs), polyphenols, carotenoids, vitamins (A, B1, B2, B3, B5, B9, B12, C, D and E), minerals and other bioactive molecules makes them the perfect vehicle for food biofortification (Khan *et al*., 2018). The richness of these organisms in terms of antioxidants and antimicrobial agents means that their addition as a new food ingredient makes it possible not only to improve the nutritional quality of products but also to reduce the use of artificial preservatives, in addition to offering a wide range of benefits to the health of those who include them in their diet (Lopes *et al*., 2020) – especially in the case of celiac patients, who have limitations in the intestinal absorption of nutrients, due to the characteristic flattening of the mucosal surface (Ciarán, 2020).

Several products have already been developed incorporating microalgae, such as breads (Khemiri *et al*., 2020), pasta (Fradique *et al*., 2013), biscuits (Batista *et al*., 2017), cereal bars or cheeses. More recently, within the scope of the 'A2F - Algae To Future' (https://www.algae2future.no) and 'AlgaeHealthyBread' projects, GF bread enriched with *Tetraselmis chuii*, *Chlorella vulgaris* and *Microchloropsis gaditana* were developed. The addition of these microalgae to the GF bread recipe has been shown to effectively improve their nutritional value, significantly increasing protein, lipid and mineral (Ca, Mg, K, P, S, Fe, Cu, Zn, Mn) contents (Qazi *et al*., 2022).

GF breads incorporated with microalgae were prepared based on a previously developed and optimised formulation (Qazi *et al*., 2022), and the impact of the microalgae in the nutritional and technological aptitude of the breads was reported (Qazi *et al*., 2022).This work aimed to: (i) assess the volatile compounds composition; (ii) evaluate the bioaccessibility of protein and minerals; (iii) understand the functional properties of the prepared GF breads enriched with microalgae. The different microalgae biomasses were first subjected to ethanolic bleaching (Qazi *et al*., 2022) to address sensory issues – since their use in raw had been shown to affect the colour, taste and odour of the baked goods in which they were included, contributing to a lower acceptance by consumers.

The microalgae *Tetraselmis chuii* (TcR), *Chlorella vulgaris* (CvR), *Microchloropsis gaditana* (MgR), and the corresponding ethanol-treated biomasses (*Tetraselmis chuii*-treated, TcT, *Chlorella vulgaris*-treated, CvT and *Microchloropsis gaditana*-treated, MgT) were incorporated in GF bread recipe. The objective was to evaluate the different baked products in terms of protein and mineral bioaccessibility through an *in vitro* digestive assay – as well as to evaluate their bioactivity, namely through the assessment of a possible antidiabetic potential based on the inhibition of α-amylase. The flavour profile of these breads (in terms of volatile and non-volatile compounds) was also scrutinously evaluated. For all cases, the possible influence of the ethanolic treatment on the bread properties, as well as the impact of the microalgae, were discussed.

Results and discussion

Bioaccessible protein content

Before this work, protein quantification of the various loaves of bread under study was performed and studied by Qazi *et al*. (2022), as can be seen in Materials and Methods section. In the same publication, the authors reported that the incorporation (4% w/w) of different microalgal biomasses, managed to significantly increase the protein content of GF bread, which otherwise would have a low nutritional value. In this sense, in trying to understand what portion of this protein is for intestinal absorption, a comparative study was carried out based on an *in vitro* digestive test, where protein characterisation of *digesta* was assessed. New protein quantification of GF bread was also carried out before digestion. Bread results were compared with *digesta* results (Fig. 1).

With this test, it is possible to verify that the amount of protein available for absorption after an *in vitro* digestion process is considerably reduced for the various samples enriched with microalgal biomass (μsamples = 3.60 g Protein/100 g DW) when compared to the results for non-digested GF bread, either in this work (μsamples = 8.35 g Protein/100 g DW) or

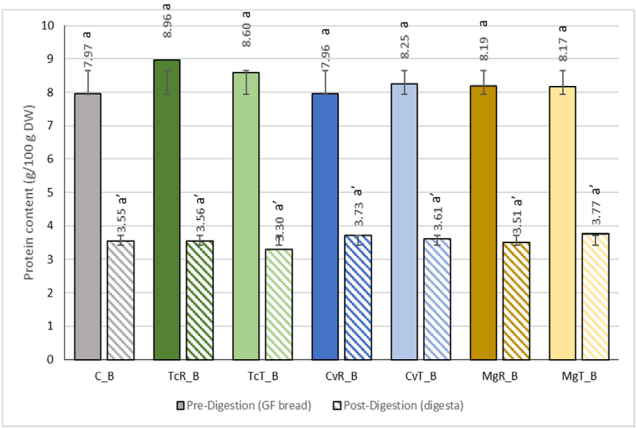

Figure 1 Measurement of Protein content (g Protein/100 g DW) before and after *in vitro* digestion of the various GF breads enriched with *Tetraselmis chuii* (TcR_B), *Tetraselmis chuii* ethanol-treated (TcT_B), *Chlorella vulgaris* (CvR_B), *Chlorella vulgaris* ethanol-treated (CvT_B), *Microchloropsis gaditana* (MgR_B) and *Microchloropsis gaditana* ethanol-treated (MgT_B). Values represent the mean ($n = 3$). No significant differences ($P < 0.05$) using Tuckey's HSD test were observed between samples for the same evaluated parameter.

previously by Qazi and co-workers (μsamples = 7.40 g Protein/100 g DW) (Qazi *et al*., 2022). This represents an approximate loss of 55% of protein content with digestion. Likewise, this assay shows that the proportion of ingested protein that is broken down into absorbable constituents by the gastrointestinal tract represents about 45% of the total protein value of bread. This is in congruent with other studies done with *Microchloropsis sp*. and *Chlorella sp*., which showed similar protein digestibility (50% and 55%, respectively) (Niccolai *et al*., 2019). But when gathering the published values for the protein availability of *Chlorella sp*., Tibbetts and co-workers found that these vary between 27% and 97% (Tibbetts *et al*., 2015). This variability between studies makes their comparison frivolous, as it makes it difficult to isolate the determining factors in the digestibility of microalgal protein. Generally, these differences can be attributed to a wide range of methodological differences existing in literature, including the microalgae cellular disruption applied methods and the use of different enzyme mixtures, assay conditions and sample processing and cellular disruption applied methods (Wild *et al*., 2018). This may eventually explain the discrepancy between these results and others with higher values.

Nevertheless, this loss of protein by 55% is not surprising, taking into account some of the factors that affect the bioaccessibility of nutrients, namely their composition and the food matrix in which they are inserted. Consumption of certain protein sources, especially in combination with other macronutrients, can delay the assimilation of protein constituents (Schoenfeld & Aragon, 2018). Considering GF bread as a vehicle for microalgae compounds, a food matrix that contains not only protein but also carbohydrates (including fibre) and fats, it is logical to speculate that protein digestibility might be affected by some of these molecules (Schoenfeld & Aragon, 2018), either by the inhibitory action of digestive enzymes or by the inability of said enzymes to break microalgal proteins into assimilable portions.

In this regard, the digestibility of microalgal protein has already been shown to be affected by other compounds that inhibit the activity of digestive enzymes, despite cellular disruption application (Annamalai *et al*., 2021). For example, a study demonstrated that the low digestibility of *Microchloropsis sp*. may be associated with its high levels of trypsin inhibitor, an enzyme that prevents the proteolytic activity of other enzymes (Valente *et al*., 2019). A relationship between fibre content and the digestibility of organic matter, protein and polysaccharides has also been established, whereby fibre and other anti-nutritional components have been shown to negatively affect the activities of digestive enzymes, including proteolytic enzymes (Annamalai *et al*., 2021). Thus, the high fibre content of microalgae may be contributing to the values presented here for protein availability (Zanella & Vianello, 2020). Lastly, the phenolic content of the evaluated breads (1.93–5.47 mg GA g^{-1} DW) (Qazi *et al*., 2022) may affect protein bioavailability, as phenolic compounds have been shown to cause protein precipitation (Annamalai *et al*., 2021). This decrease in the amount of peptides available for assimilation was demonstrated even at very low levels of phenolic content (0–20 mg GA g^{-1} DW) (Tibbetts *et al*., 2015). Together, these factors justify the decrease observed in the protein content of GF breads after *in vitro* digestion. The combined action of these agents may be such a determining factor as to why no significant differences were observed between the microalgae-enriched breads and the control after digestion – even when significantly higher values had been determined in the same breads before digestion (Qazi *et al*., 2022).

Overall, most studies published to date report an increase in the protein content of loaves of bread after incorporation with microalgal biomass in their formulations(Qazi *et al*., 2022). However, these mostly concern crude protein from bread that contains gluten. Protein bioaccessibility from GF bread continues to be under-researched in this field. Replacing the gluten network with other ingredients, such as dry microalgal biomass, affects the rheological and physicochemical characteristics of the dough and the resulting product (Qazi *et al*., 2022). This study suggests that the interaction of some microalgae constituents, together with the GF matrix, can result in a considerable decrease in protein bioavailability, while the lack of standardised methodologies makes it difficult to interpret results. In the future resolution of this problem, studies are needed that consider the various agents involved in the protein accessibility of microalgae to identify and quantify them – so that eventually the cultivation of microalgae with greater protein digestibility can be optimised.

Bioaccessible mineral content

The raw mineral content of the various loaves of bread enriched with microalgal biomass was evaluated beforehand and is already published (Qazi *et al*., 2022). An adaptation of these results is also available in the Materials and Methods section. Overall, the incorporation of algal biomass was shown to improve the mineral profile of GF bread. Of all of them, bread enriched with *Tetraselmis chuii* (both raw and ethanol-treated) was particularly high in Ca and Fe (Qazi *et al*., 2022).

To better determine which portion of this mineral content is bioavailable for intestinal absorption, mineral quantification was performed by ICP-OES after

an *in vitro* digestion procedure. The results are shown in Table 1.

According to this test, the soluble mineral content of breads after *in vitro* digestion is low for all cases. In physiological terms, this may mean that the assimilable mineral portion after digestion of GF breads is minimal. However, Qazi and co-workers (2022) showed that the mineral content in the same loaves of bread evaluated here (but not digested) was significantly ($P < 0.05$) improved with the inclusion of microalgae in the formulation (4% w/w). Namely, it was possible to reach the recommended daily value (RDV) status for Mg, P, Fe and Mn, for all combinations of GF breads. According to the European Union Regulation N-1924/2006, Directive N-9090/494 (CE), when a food has 15% RDV in its composition, it is considered a source of the corresponding minerals. In cases where it has at least twice the content required for this nutritional declaration, that is, 30% of the RDV, it is then considered to have a high content of that mineral (Regulation (EC) (2022) No 1924/2006). The work developed here suggests that, despite reaching the RDV in legislative terms before (Qazi *et al*., 2022), the mineral content of the microalgae is not so bioaccessible. For example, of the total Fe content presented generically by GF breads (2.6–5.5 mg/100 g) (Qazi *et al*., 2022), up to 88% is shown to be insoluble after *in vitro* digestion. Thus, regarding this mineral, of its total content, only 12–26% is soluble in *digesta* (0.7–0.7 mg/100 g), suggesting low assimilation by the intestine and consequent loss of bioaccessibility. Similar results happen with Mn. With the quantification of the remaining minerals in the *digesta*, such as P and Zn, it was possible to observe a higher bioaccessible mineral content, despite the marked decrease when compared to undigested breads.

When determining the bioaccessible mineral content of Ca and Cu, some samples showed higher values than those of bread before digestion. It should be noted that the percentages of mineral content presented here take as maximum those obtained by Qazi *et al*. (2022), directly from bread. Therefore, some percentages that were found to be greater than 100% for Ca and Cu can be explained by methodological differences that may have affected the determination of minerals, namely the abnormal values obtained for *Ca*. The *in vitro* digestion simulation included the use of a $CaCl_2$ solution as an integral part of the simulated gastrointestinal fluids which, when not considered, could be behind such false positives. Finally, it is worth mentioning that both Ca and Cu function as enzymatic co-factors that are normally bound to proteins (Mahfouz, 2014). Thus, the enzymatic digestion carried out in this work may have contributed to greater solubilisation of these minerals, resulting in the values presented.

Several reasons can explain the generalised low mineral bioaccessibility of the samples. Although high temperatures can help to control anti-nutritional factors, a study that looked into the *in vitro* mineral bioaccessibility of microalgae-enriched cookies suggested that heat treatment (baking) could influence it (Uribe-Wandurraga *et al*., 2020). Not by the destruction of minerals, given their stability, but by protein denaturation and consequent enzymatic inactivation of crucial enzymes in mineral bioaccessibility. Another study established a negative correlation between the bioavailable percentages of metals (Fe, Cu, Zn) and proteins. The reason is that proteins are hydrolysed to amino acids during *in vitro* digestion and most of the positively or negatively charged soluble amino acids, at physiological pH (simulated in the work developed here), increase the ionic strength of the aqueous phase, resulting in a low solubility of metals, due to salting-out effects. The marked decrease in the bioaccessibility of these metals may still be related to the lipid content

Table 1 Mineral composition (mg/100 g) of the *in vitro* digested control (C_B) and GF loaves of bread with 4% microalgal biomass incorporation (*Tetraselmis chuii* (TcR_B), *Tetraselmis chuii* ethanol-treated (TcT_B), *Chlorella vulgaris* (CvR_B), *Chlorella vulgaris* ethanol-treated (CvT_B), *Microchloropsis gaditana* (MgR_B) and *Microchloropsis gaditana* ethanol-treated (MgT_B)) ($n = 3$, wet basis)

Digesta	K	Ca	Mg	P	S	Fe	Cu	Zn	Mn
C_B	76.6 [38%]†	11.9 [>100%]†	18.5 [33%]†	74.5 [53%]†	54.1 [77%]†	0.5 [20%]†	0.1 [>100%]†	0.7 [77%]†	0.1 [26%]†
TcR_B	81.3 [36%]†	17.5 [26%]†	22.2 [32%]†	74.6 [45%]†	64.7 [62%]†	0.6 [13%]†	0.1 [49%]†	0.5 [46%]†	0.1 [12%]†
TcT_B	82.9 [41%]†	18.8 [21%]†	21.5 [34%]†	75.6 [44%]†	64.1 [62%]†	0.7 [12%]†	0.1 [100%]†	0.5 [48%]†	0.1 [12%]†
CvR_B	83.8 [38%]†	19.9 [>100%]†	20.6 [33%]†	76.3 [46%]†	63.4 [67%]†	0.6 [22%]†	0.1 [52%]†	0.5 [54%]†	0.1 [17%]†
CvT_B	83.5 [38%]†	20.5 [>100%]†	19.5 [28%]†	75.8 [41%]†	61.2 [56%]†	0.6 [17%]†	0.1 [53%]†	0.5 [49%]†	0.1 [15%]†
MgR_B	89.6 [44%]†	17.3 [>100%]†	20.2 [32%]†	79.3 [49%]†	60.7 [65%]†	0.7 [21%]†	0.1 [>100%]†	0.6 [59%]†	0.1 [21%]†
MgT_B	96.1 [47%]†	14.3 [>100%]†	21.1 [31%]†	82.2 [47%]†	59.5 [60%]†	0.7 [26%]†	0.1 [>100%]†	0.7 [69%]†	0.1 [25%]†
15% RDV (mg)	300.0	120.0	56.3	105.0	NM	2.1	0.2	1.5	0.3

15% of the Recommended daily value (RDV) per European Community Regulation N-1924/2006, Directive N-9090/494 (CE) is listed below. NM is not mentioned.

†Soluble/bioaccessible mineral content (%) of the sample, taking the pre-digestion values obtained by Qazi *et al*. (2022) as maximum.

of microalgae. A negative correlation between metals and lipids has already been established, attributed to the fact that the metals are not emulsified by the bile salts and are brought into the liquid phase before they can be assimilated (Demarco *et al.*, 2022). Thus, the high lipid content previously found in the TcR, CvR and MgR biomasses, and the high protein content found in TcT, CvT and MgT (Qazi *et al.*, 2022) may explain the observed values for Fe, for example.

It has been stated before that algae are known to be rich in dietary fibre and phenolic compounds, which can affect mineral bioavailability. However, the same is true for phytic acid. Fibre can form insoluble complexes with minerals, reducing their bioaccessibility (Demarco *et al.*, 2022). Phytic acid (or inositol hexakisphosphate) is the main way plants and algae store phosphorus. When phytic acid is consumed, it binds to other compounds and creates phytates (Demarco *et al.*, 2022). The latter are considered anti-nutritional factors as they can affect the digestibility and bioavailability of various nutrients, including proteins and the minerals analysed here. It is also necessary to consider the food matrix where the microalgae were included. Cereals such as rice, and seeds such as buckwheat (the main ingredients of these GF breads), have already been shown to have 69%–90% of their phosphorus stored as part of phytic acid which can bind to cations such as K2+, Ca2+, Mg2+, Fe2+, Zn2+ and Mn2+, making them unavailable for absorption (Verspreet *et al.*, 2021).

Ultimately, bioaccessibility can be affected by different chemical forms of minerals and their interaction with other plant components used in the making of GF breads (Silva *et al.*, 2020). It has been shown several times that microalgae have an enormous supply of nutritionally rich compounds, but the presence of anti-nutritional compounds has been scantly considered and investigated. Thus, concerning the inclusion of microalgae in foods, the study of the interaction between bioactive compounds of interest with others such as phytic acid should be of primary interest to effectively develop fortified products.

Antidiabetic capacity

α-Amylase is one of the enzymes that regulate the metabolism of dietary carbohydrates. According to previous studies, the inhibition of this enzyme promotes a delay in the absorption of glucose, preventing post-meal spikes in blood glucose that would eventually stimulate the development of diabetes (Kifle & Enyew, 2020). To determine a possible antidiabetic capacity in the developed GF breads enriched with microalgae, an α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method. The results obtained are shown in Fig. 2.

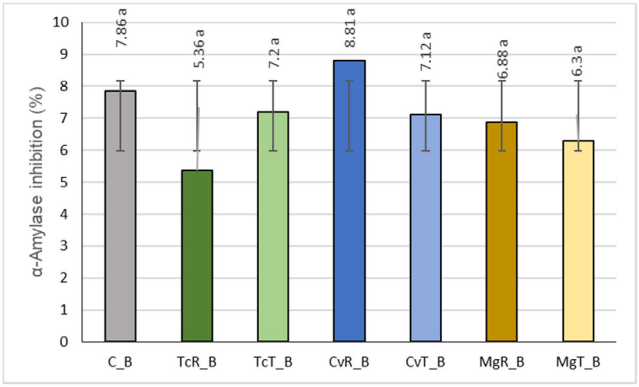

Figure 2 α-Amylase inhibition (%): GF breads enriched with *T. chuii* (TcR_B), *T. chuii* ethanol-treated (TcT_B), *C. vulgaris* (CvR_B), *C. vulgaris* ethanol-treated (CvT_B), *M. gaditana* (MgR_B) and *M. gaditana* ethanol-treated (MgT_B). Values represent the mean ($n = 3$). Different letters for a given parameter indicate a significant difference ($P < 0.05$) using Tuckey's HSD test.

Here, the mean values obtained through a one-way ANOVA analysis, when compared according to a *post hoc* test, are not significantly different from each other ($P < 0.05$). Thus, this trial failed to demonstrate that the enrichment of the various microalgae under study in the bread formulation promotes an increase in the inhibition of α-amylase.

From the results, little can be inferred regarding the antidiabetic capacity of the samples. In the future, analysing the different GF loaves of bread regarding their influence on the activity of other enzymes with antidiabetic effects (such as α- and β-glucosidases) (Kifle & Enyew, 2020) may clarify this hypothesis. With some studies reporting antidiabetic effects resulting from microalgae supplementation in model organisms (namely, the reduction in circulating glucose and glycosylated haemoglobin levels) (Nacer *et al.*, 2020), rare are those that focus on the physiological effects in humans resulting from its incorporation in baked products. With such scarce literature, it becomes relevant to evaluate higher levels of incorporation of different microalgae in bread and verify their influence on these enzymes, but such proportions would cause a negative impact on dough rheology and sensory attributes, compromising consumer receptiveness (Qazi *et al.*, 2022).

Lipophilic bioactive compounds

Lipophilic bioactives are important for human health and have different roles in the body. Usually include compounds such as carotenoids, tocopherols and fatty acids. These compounds are known to have antioxidant properties and play important roles in maintaining cellular and tissue health.

Microalgae have been identified as a potential source of several lipophilic compounds such as fatty acids, alcohols and aldehydes and nitrogen-containing compounds. Several alcohols can be produced by fermenting the biomass of microalgae (Wang *et al.*, 2019). *C. vulgaris* has been shown to produce ethanol and propanol, while *M. oceanica* can produce ethanol, butanol and propanol (Melo *et al.*, 2021). Microalgae are also a potential source of aldehydes, including short-chain aldehydes such as acetaldehyde, propanal and butanal, that can be produced by microalgae as part of their metabolic processes or in response to environmental stresses (Moran *et al.*, 2022). The different metabolic pathways of microalgae species have been shown to produce pyridine derivatives. For instance, *Arthrospira platensis* (Spirulina) have been shown to produce pyridoxine, which is a vitamin B6 compound that contains a pyridine ring, important for the synthesis of neurotransmitters, such as serotonin and dopamine, and also plays a role in the metabolism of amino acids and glycogen (Gentscheva *et al.*, 2023).

To understand the advantages of incorporating microalgae in GF breads or human health, the composition of dichloromethane extracts of the breads (enriched with lipophilic compounds) was determined (Table 2).

The total identified lipophilic compounds ranged between 26% and 7%, for ethanol-treated *T. chuii* bread and the control bread, respectively. Alkanoic acids were the major identified compounds in all the breads under evaluation, ranging from 25% to 69%, for ethanol-treated *T. chuii* bread and the ethanol-treated *M. gaditana* bread, correspondingly, whereas 9,12-octadecadienoic acid was the most abundant compound, especially for the raw *T. chuii* and *M. gaditana* breads. Hexadecanoic acid was the second most abundant alkanoic acid identified, ranging from 1% and 11% in raw *C. vulgaris* and *M. gaditana* breads. *N*-derivative compounds were also identified in control, raw *T. chuii* and raw and ethanol-treated *M. gaditana* breads. Alcohols were identified in much smaller amounts in raw and ethanol-treated *T. chuii* and

Table 2 Composition of dichloromethane extracts of control bread (C_B) and breads enriched with *T. chuii*, *M. gaditana* and *C. vulgaris*, before (raw, R) and after (ethanol-treated, T) ethanol treatment, in % of the normalised peak areas in TIC

		Raw			Ethanol-treated		
Compound	**C_B***	**TcR_B***	**MgR_B***	**CvR_B***	**TcT_B***	**MgT_B***	**CvT_B***
Alcohol	3.2 ± 0.31^{a}	0.4 ± 0.00^{b}	0.6 ± 0.34^{b}	–	0.5 ± 0.01^{b}	1.8 ± 0.17^{c}	–
Cyclohexan-1-ol	1.8 ± 0.00	–	0.1 ± 0.00	–	–	1.0 ± 0.00	–
2,4-hexadien-1-ol	1.4 ± 0.01	0.4 ± 0.00	0.5 ± 0.00	–	0.5 ± 0.01	0.8 ± 0.02	–
Alkane	2.1 ± 0.07^{d}	1.4 ± 0.00^{e}	2.6 ± 0.05^{f}	–	–	1.5 ± 0.09^{e}	0.3 ± 0.00^{f}
Docosane	2.1 ± 0.07	1.4 ± 0.00	2.6 ± 0.05	–	–	1.5 ± 0.09	0.3 ± 0.00
Alkanoic acids	65.2 ± 19.01^{a}	56.2 ± 17.79^{a}	65.3 ± 17.48^{a}	59.0 ± 16.40^{a}	25.4 ± 4.86^{b}	68.75 ± 19.40^{a}	41.4 ± 7.90^{c}
Hexadecanoic acid	9.4 ± 0.40	10.7 ± 1.25	10.8 ± 0.04	1.2 ± 0.01	1.7 ± 0.01	9.79 ± 0.11	1.1 ± 0.00
Linoleic acid	2.9 ± 0.11	–	3.6 ± 0.10	–	1.1 ± 0.01	3.41 ± 0.00	0.2 ± 0.00
9,12-octadecadienoic acid	49.1 ± 4.0	42.2 ± 2.00	45.8 ± 0.45	15.0 ± 1.01	13.1 ± 0.04	50.53 ± 1.24	19.5 ± 1.02
Trans-9-octadecenoic acid	1.3 ± 0.07	1.4 ± 0.52	3.7 ± 0.13	5.1 ± 0.06	5.8 ± 0.01	1.56 ± 0.02	7.8 ± 0.02
11-eicosaenoic acid	1.0 ± 0.00	1.4 ± 0.00	1.1 ± 0.00	37.7 ± 0.14	3.6 ± 0.01	1.67 ± 0.00	12.5 ± 0.01
9,12-octadienoic acid	1.5 ± 0.04	–	–	–	–	1.79 ± 0.00	–
Arachidonic acid	–	0.3 ± 0.07	0.3 ± 0.00	–	–	–	0.2 ± 0.00
N-derivatives	7.0 ± 0.92^{a}	1.8 ± 0.00^{b}	2.4 ± 0.32^{c}	–	–	3.5 ± 0.35^{d}	–
2-ethyl-5-pentyl-1-pyrrolidine	1.1 ± 0.00	0.9 ± 0.00	1.0 ± 0.01	–	–	0.8 ± 0.07	–
Brassilexin	2.2 ± 0.00	0.8 ± 0.00	1.0 ± 0.01	–	–	1.4 ± 0.0	–
4-methoxyphenyl-amino-phenol	0.9 ± 0.00	–	–	–	–	–	–
2,6-bis(hydroxyethynyl)pyridine	2.5 ± 0.02	–	–	–	–	1.3 ± 0.01	–
1-hydroxy-2-methoxy-1,2,3,4-dihydrocacalone	0.6 ± 0.00	0.2 ± 0.00	0.4 ± 0.01	–	–	–	–
Glycerol derivatives	1.8 ± 0.00^{a}	2.8 ± 0.00^{b}	2.1 ± 0.00^{c}	0.4 ± 0.00^{d}	–	2.2 ± 0.01^{e}	–
Monopalmitin	1.8 ± 0.00	2.8 ± 0.00	2.1 ± 0.00	0.4 ± 0.00	–	2.2 ± 0.01	–
Myo-inositol	3.4 ± 0.25^{a}	21.2 ± 4.28^{b}	17.2 ± 2.24^{b}	14.2 ± 4.76^{b}	18.7 ± 5.23^{b}	13.3 ± 2.44^{b}	13.2 ± 5.02^{b}
Myo-inositol	3.4 ± 0.25	21.2 ± 4.28	17.2 ± 2.24	14.2 ± 4.76	18.7 ± 5.23	13.3 ± 2.44	13.2 ± 5.02
Identified compounds	82.6 ± 25.29^{a}	83.9 ± 22.13^{a}	90.1 ± 25.39^{a}	73.6 ± 23.59^{a}	44.6 ± 11.51^{a}	91.0 ± 26.63^{a}	54.8 ± 16.64^{a}
Non-identified compounds	17.4	16.1	9.9	26.4	55.4	9.0	45.2
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Letters in superscript are from the statistical analysis: different letters correspond to significant ($P < 0.05$) different results between the same chemical families of compounds, using Tuckey's HSD test.

M. gaditana breads (from 1% to 2%). Docosane (less than 1% to 3% of all compounds) and monopalmitin (less than 1% to 3% of all compounds) were identified in low amounts. Comparing the raw microalgae breads with the ethanol-treated microalgae breads, it is interesting to notice that there was an increase in alcohols and alkanes contents in ethanol-treated *T. chuii* and *M. gaditana* breads, except for *C. vulgaris* breads where no alcohol compounds were identified. It was also observed a 2-fold decrease in alkanoic acids content in ethanol-treated *T. chuii* breads when compared to the raw *T. chuii* breads, which is more noticeable in hexadecanoic and 9,12-octadecadienoic acids (11% to 2% of all compounds and 42% to 13% of all compounds, respectively). The same trend was observed for *C. vulgaris* bread: a decrease in the alkanoic acids content is evident in the ethanol-treated bread, namely for 11-eicosaenoic acid (from around 38% to 12%). However, an increase in 9-octadecenoic and 9,12-octadecadienoic acids is observed. Control bread shows an interesting composition in terms of lipophilic compounds, whereas 65% are alkanoic acids, being the 9,12-octadecadienoic acid the most abundant (around 75% of all alkanoic acids). When compared to this control bread, raw *M. gaditana* bread has a similar content (around 65%), with 9,12-octadecadienoic acid being the most abundant (45% of all compounds). However, the opposite is observed for the raw *T. chuii* and *C. vulgaris* breads, where the alkanoic acid content is lower (around 56% and 59%, respectively). Except for ethanol-treated *M. gaditana* bread, there was a decrease in the alkanoic acids contents for ethanol-treated breads. *Myo*-inositol, which decreases the absorption of nutrients and their bioavailability (Bohn *et al.*, 2008), was found in all microalgae-enriched GF breads, ranging around 13% to 21% and also in control bread at lower content (around 3%).

Volatile organic compounds

Microalgae, like other types of algae, produce volatile organic compounds (VOCs) that contribute to their characteristic odour and flavour. These compounds may include terpenes, aldehydes, ketones and other organic molecules. Microalgae are known to produce a wide variety of volatile alcohols, including ethanol, propanol, butanol and hexanol, as part of their metabolic processes (Achyuthan *et al.*, 2017). These alcohols are typically produced through the fermentation of sugars and other organic compounds and can accumulate in microalgal cultures under certain conditions, such as nutrient limitation or high cell densities (Gentscheva *et al.*, 2023). Volatile aldehydes and ketones are typically produced as by-products of lipid metabolism or as part of the response to environmental stress (Hosoglu, 2018). While acid halides are not commonly reported as being produced by microalgae, some studies have reported the production of halogenated organic compounds by certain species of marine microalgae (Paul & Pohnert, 2011). For example, one study found that the marine microalga *P. tricornutum* produced the halogenated compound 2-chlorohexadecanoic acid under high light stress conditions.

The specific volatile profile of microalgae can vary depending on the species and growth conditions. Research on microalgae volatiles has suggested that they may have potential applications in the food, fragrance, and pharmaceutical industries (Nunes *et al.*, 2023). Volatiles in food refers to the compounds that are responsible for the aroma and flavour of food. These compounds are typically small, volatile molecules that are released into the air and detected by the olfactory system when we eat or smell food. Therefore, they are extremely important for the overall acceptance of microalgae-based food, such as bread.

Table 3 summarises the results on the volatile composition of the prepared GF breads.

Microalgae-enriched GF breads have a complex matrix of volatiles, where alcohols are the most abundant, ranging between 13% and 44% in the raw *T. chuii* and *M. gaditana* breads, respectively, and between 3% and 35% in ethanol-treated *T. chuii* and *M. gaditana* breads. Among alcohols, 2-octen-1-ol was the only compound identified in raw *C. vulgaris* bread (40% of all compounds) and ethanol-treated *C. vulgaris* bread (less than 1% of all compounds). 1-Methylcyclohexan-1-ol was the most abundant alcohol found in both raw- and ethanol-treated *M. gaditana* breads (91% and 41% of alcohols) and in ethanol-treated *C. vulgaris* bread (93% of all alcohols). Isoamylalcohol was the most abundant alcohol identified in raw *T. chuii* bread, but not identified in the correspondent ethanol-treated bread. In the control bread, 1-methylcyclohexan-1-ol is the most abundant compound, representing 57% of all compounds and 96% of the identified alcohols. Terpenes were the second most abundant class of compounds in the raw *M. gaditana* GF bread, representing 24% of all compounds, where γ-terpinene was the most abundant compound (76% of all terpenes). N-based compounds were the second most abundant class of compounds in raw *C. vulgaris* and the 3,3-dimethyl-4-(ethyl-1-amino)-azetidin-2-one was the most representative compound (88% of all nitrogen compounds). In raw *T. chuii* breads, 4-dimethyltridecane is the major alkane identified (95% of all alkanes, 7% of all compounds). For ethanol-treated microalgae-based GF breads, the second most abundant class of volatiles identified is the class of terpenes, ranging from 8 to 10% in *M. gaditana* and *T. chuii*, respectively. Among

Table 3 Volatile composition control bread (C_B) and breads enriched with *T. chuii*, *M. gaditana* and *C. vulgaris*, before (raw, R) and after (ethanol-treated, T) ethanol treatment, in % of the normalised peak areas in TIC

Compound	C_B	TcR_B	MgR_B	CvR_B	TcT_B	MgT_B	CvT_B
Hydrocarbons	0.1 ± 0.04[a]	0.1 ± 0.04[a]	0.1 ± 0.00[a]	0.1 ± 0.01[a]	7.4 ± 1.96[b]	0.1 ± 0.05[a]	–
1,2,3,4-tetramethylbenzene	0.03 ± 0.000	0.02 ± 0.000	0.03 ± 0.000	–	4.74 ± 0.004	0.0 ± 0.00	–
3,7-dimethyl-1,3,6-octatriene	0.08 ± 0.001	–	0.03 ± 0.000	–	1.35 ± 0.000	0.1 ± 0.00	–
Tetradecane	0.02 ± 0.000	0.07 ± 0.000	0.01 ± 0.000	0.07 ± 0.000	1.35 ± 0.001	–	–
Alcohols	59.8 ± 23.26[a]	13.2 ± 9.31[b]	43.6 ± 16.05[a]	40.2 ± 3.55[a]	3.4 ± 0.07[c]	35.3 ± 9.57[a]	32.6 ± 0.20[a]
1-methylcyclohexan-1-ol	57.4 ± 2.24	–	40.0 ± 1.19	–	–	14.4 ± 0.01	30.3 ± 0.41
3-methylbutan-1-ol	–	–	–	–	–	19.8 ± 0.04	–
3-amino-propan-1-ol	0.1 ± 0.0	0.0 ± 0.00	0.0 ± 0.00	–	3.4 ± 0.07	–	–
Isoamylalcohol	0.0 ± 0.00	13.2 ± 0.04	0.0 ± 0.00	–	–	–	0.0 ± 0.00
1-methylcyclohexanol	0.2 ± 0.00	–	0.2 ± 0.00	–	–	–	–
2-amino-1-phenylpropan-1-ol	1.9 ± 0.01	–	3.0 ± 0.00	–	–	1.2 ± 0.00	2.1 ± 0.01
2-octen-1-ol	0.2 ± 0.00	–	0.4 ± 0.00	40.2 ± 3.55	–	–	0.2 ± 0.00
Aldehydes	0.7 ± 0.39[a]	0.3 ± 0.00[a]	1.2 ± 0.636[a]	–	4.7 ± 0.01[b]	0.4 ± 0.00[a]	–
Penta-2,4-dienal	0.1 ± 0.00	–	0.2 ± 0.00	–	4.7 ± 0.01	–	–
Octan-1-al	0.6 ± 0.0	0.3 ± 0.00	1.1 ± 0.00	–	–	0.4 ± 0.00	–
Alkane	0.3 ± 0.121[a]	7.7 ± 4.93[b]	0.2 ± 0.00[a]	–	–	0.5 ± 0.05[a]	0.9 ± 0.049[c]
4-dimehtyltridecane	0.0 ± 0.00	7.3 ± 0.58	–	–	–	0.0 ± 0.00	0.0 ± 0.00
6-dimethyloxytetradecane	0.2 ± 0.01	0.4 ± 0.0	–	–	–	0.4 ± 0.07	0.9 ± 0.01
6-dimethyl(hydroxy)tetradecane	0.1 ± 0.0	–	0.2 ± 0.00	–	–	–	–
Ketones	3.3 ± 0.10[a]	–	1.1 ± 0.01[b]	–	–	1.2 ± 0.00[c]	–
Bicyclo[3.3.1]non-6-en-2-one	3.3 ± 0.14	–	1.1 ± 0.01	–	–	1.2 ± 0.00	–
5-methyl-2-(1-methylethyl) cyclohexanone	0.0 ± 0.00	–	–	–	–	–	–
Alkanoic acids	2.7 ± 0.37[a]	–	–	–	0.3 ± 0.00[c]	2.1 ± 0.73[a]	1.8 ± 0.00[a]
9,12,15-octadecatrienoic acid, 2-hydroxymethoxy, methyl ethyl ester	1.6 ± 0.04	–	–	–	–	1.6 ± 0.02	1.8 ± 0.00
2-pyridinepropanoic acid, α-methyl, β-oxo, ethyl ester	1.1 ± 0.00	–	–	–	0.3 ± 0.00	0.5 ± 0.01	–
Terpenes/terpenoids	8.0 ± 1.81[a]	2.4 ± 0.74[b]	24.2 ± 6.35[c]	6.1 ± 3.4[a]	10.6 ± 1.26[d]	8.4 ± 5.94[b,d]	8.8 ± 4.070b,d
Γ-terpinene	5.2 ± 0.10	0.2 ± 0.07	18.3 ± 2.42	6.0 ± 0.48	–	8.4 ± 0.01	7.6 ± 0.50
B-pinene	–	–	3.5 ± 0.64	–	0.3 ± 0.03	–	–
Limonene	2.6 ± 0.02	1.9 ± 0.01	1.3 ± 0.03	–	–	–	1.2 ± 0.01
P-cymene	–	–	–	–	–	–	–
D-carene	–	–	–	–	1.5 ± 0.20	–	–
B-myrcene	–	0.1 ± 0.01	–	–	2.4 ± 0.04	–	–
Sabinene	–	0.1 ± 0.00	–	–	3.4 ± 0.08	–	–
Limonene	0.0 ± 0.01	0.1 ± 0.01	0.0 ± 0.01	–	2.7 ± 0.12	–	–
A-humelene	0.2 ± 0.01	–	–	0.1 ± 0.00	0.3 ± 0.01	–	–
Acid halides	21.0 ± 4.56[a]	1.4 ± 0.41[b]	6.6 ± 0.46[c]	–	1.0 ± 0.01[b]	8.7 ± 2.74[d]	–
Dichloroacetic acid	21.0 ± 4.56	1.4 ± 0.41	6.6 ± 0.46	–	1.0 ± 0.01	8.7 ± 2.74	–
N-based compounds	3.0 ± 1.94[a]	4.4 ± 0.70[a]	3.8 ± 2.11[a]	9.2 ± 4.94[a]	0.3 ± 0.02[b]	12.3 ± 5.51[a]	12.9 ± 5.70[a]
1,2-dihydro-1,4-diphenyl phthalazine	2.8 ± 0.11	2.7 ± 0.04	3.4 ± 0.85	1.1 ± 0.41	–	1.9 ± 0.47	1.9 ± 0.46
Azetidin-2-one, 3,3-dimethyl-4-(ethyl-1-amino)	–	1.7 ± 0.11	–	8.1 ± 0.13	0.3 ± 0.02	10.4 ± 2.86	10.8 ± 2.22
2,5-dihydro-1-nitro-1*h*-pyrrole	0.2 ± 0.10	–	0.4 ± 0.08	–	–	0.1 ± 0.01	0.2 ± 0.05
S-based compounds	0.1 ± 0.007[a]	–	–	–	–	–	–
N-ethyl-1,3-dithioisoidoline	0.1 ± 0.007	–	–	–	–	–	–
Other	0.6 ± 0.318[a]	0.7 ± 0.12[a]	–	–	3.7 ± 0.41[b]	–	0.3 ± 0.01[a]
2-butyl-3-methyloxirane	0.5 ± 0.047	–	–	–	3.7 ± 0.41	–	0.3 ± 0.01
2,2-bioxirane	0.1 ± 0.003	0.7 ± 0.12	–	–	–	–	–
Total identified compounds	99.7 ± 17.911[a]	30.4 ± 4.22[b]	80.9 ± 12.87[a]	55.6 ± 12.07[c]	33.0 ± 3.56[b]	69.0 ± 10.58[c]	57.1 ± 11.84[c]
Non-identified compounds	0.3	69.6	19.1	44.4	67.0	31.0	42.9
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

these, γ-terpinene is the major identified terpene in ethanol-treated *M. gaditana* and *C. vulgaris* breads (99% and 86% of all terpenes, correspondingly), and sabinene is the major identified terpene in ethanol-treated *T. chuii* breads (31% of all terpenes identified). 3,3-Dimethyl-4-(ethyl-1-amino)-azetidin-2-one is the most abundant *N*-based compound identified in ethanol-treated *M. gaditana* and *C. vulgaris* breads (84% of all identified *N*-based compounds), and in the raw *C. vulgaris* bread (88% of all identified N-based compounds). Aldehydes, hydrocarbons, ketones, alkanoic acids and acid halides were also identified in the microalgae-based GF breads, although in smaller amounts (under 8%). Control bread was enriched in 1-methylcyclohexan-1-ol and dichloroacetic acid, although in higher amounts when compared to the microalgae-based GF breads (almost 60% and 21%, correspondingly). Research on volatiles from microalgae is still limited.

Materials and methods

Gluten-free breads

Bread samples were prepared with buckwheat flour (Próvida, Mem Martins, Portugal), rice flour (Espiga, Portugal), potato starch (Globo, Portugal), dried yeast (Fermipan, Portugal), hydroxipropylmethylcellulose (HPMC, WellenceTM 321, Dow, Germany) and commercially available sugar, sunflower oil and salt.

Three microalgal biomasses *Tetraselmis chuii*, *Chlorella vulgaris* and *Microchloropsis gaditana* (TcR, CvR, MgR) used in bread preparation were produced and collected by A2F partners at NORCE/UiB (Bergen, Norway), who provided the information about protein and ash contents of the macroalgal biomass (Qazi et al., 2021).

Microalgae cultivation, harvesting and cell disruption

The freshwater green microalga *Chlorella vulgaris* SAG 211-11b (Cv) was obtained from the SAG Culture Collection of Algae (Göttingen, Germany) (Harris, 2009), and the marine microalgae specie *M. gaditana* CCMP526 and. *T. chuii* were obtained from Bigelow NCMA and from the UTEX culture collection, respectively (Cultivation and harvesting conditions were described by (Qazi et al., 2021).

To increase the nutrient availability of the microalgae, all three species were subjected to bead milling for cell wall disruption. The bead milling conditions were chosen based on optimisation trials for Tc, Mg and Cv performed by our project partners in Norway (Qazi et al., 2021). For Cv approximately 92% cell wall disruption, 97% for Tc and ~84% disruption for Mg.

Drying was performed at Nofima in Bergen, Norway. Cell wall disrupted biomass of Cv was spray-dried and dried biomass of the three different algae species were termed TcR, CvR and MgR (Qazi et al., 2022).

Ethanol treatment

The raw biomasses of the three microalgae species (TcR, CvR, MgR) were treated with 96% ethanol using a Soxhlet extractor apparatus (Adams & Chittenden Scientific Glass, Berkeley, CA, USA). TcR and CvR, which consisted of small particles (<1 mm), were placed in a cellulose thimble (~50–70 g), MgR, which consisted of larger particles (>1 mm), was placed directly in a glass thimble with a glass sinter filter in the base (80–100 g). The thimbles were placed inside the extraction chamber. To condense the evaporated ethanol, a condenser was attached to the top of the extraction chamber. Water held at 9 °C was circulated through the condenser. A volume of 500 mL ethanol was placed in the boiling flask and heated with a stirred silicon oil bath set at 150 °C. Each batch was continuously extracted until the ethanol in the extraction chamber was colourless, which took approximately 65 h for Tc, 54 h for Cv and 43 h for Mg. The extracted biomasses were spread on a large tray and placed at 75 °C for 6 h in a ventilated oven (Termaks, Bergen, Norway) to remove traces of remaining ethanol and were termed TcT, CvT and MgT. The yields of extraction ranged between 76% and 83% (for TcR and CvR, respectively) (Qazi et al., 2022).

Bread preparation

The gluten-free bread used in this investigation was previously crafted by Qazi et al. (2021) using the same optimised formulation method as outlined by Wang *et al.* (2019). The formulation is primarily based on a combination of buckwheat, rice flour and potato starch. Table 4 provides a comprehensive overview of the total ingredient composition for both the control bread and the variations with a 4% incorporation of microalgae (w/w, based on total flour). The optimal water absorption capacity (at 14% moisture basis) for each formulation was determined using the MicroDoughLab 2800 from Perten Instruments, located in Sidney, Australia. General Flour Testing Method protocol was used, mixing the samples at a constant 63 r.p.m., at 30 °C for 20 min. The peak value of torque of the optimised control formulation was used as a reference to assess the optimum water absorption for each GF bread formulation with microalgal biomass addition (Qazi et al., 2021).

Table 5 shows the mineral composition of the control and GF loaves of bread with 4% microalgal

Table 4 Total ingredient composition of GF control bread (Control) and GF bread formulations with the incorporation of 4% microalgal biomass: *T. chuii* enriched bread (TcR_B), *T. chuii* ethanol-treated enriched bread (TcT_B), *C. vulgaris* enriched bread (CvR_B), *C. vulgaris* ethanol-treated enriched bread (CvT_B), *M. gaditana* enriched bread (MgR_B) and *M. gaditana* ethanol-treated enriched bread (MgT_B). Adapted from: (M. W. Qazi *et al.*, 2022; Qazi *et al.*, 2021)

Bread ingredients (g/100 g)	Control	TcR_B	TcT_B	CvR_B	CvT_B	MgR_B	MgT_B
Buckwheat flour	46.0	44.2	44.2	44.2	44.2	44.2	44.2
Rice flour	31.0	29.8	29.8	29.8	29.8	29.8	29.8
Potato starch	23.0	22.1	22.1	22.1	22.1	22.1	22.1
Microalgae biomass replacement	0.0	4.0	4.0	4.0	4.0	4.0	4.0
Sunflower oil (in relation to flour)	5.5	5.5	5.5	5.5	5.5	5.5	5.5
HPMC (in relation to flour)	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Instant dried yeast (in relation to flour)	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Commercial salt (in relation to flour)	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Commercial sugar (in relation to flour)	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Water absorption (%)	75.0	74.5	75.5	75.5	75.3	75.5	75.3

biomass incorporation prepared as described by Qazi *et al.* (2021).

In vitro digestion simulation

To evaluate the bioaccessibility of nutrients and bioactive compounds in control and digested bread, samples were first subjected to *in vitro* static digestion method as developed by Lopes *et al.* (2020) based on the protocol already established by Brodkorb and co-workers (Brodkorb *et al.*, 2019). This method consists of simulating the three sequential digestive phases: oral, gastric and intestinal.

In short, 4 g of each bread sample was diluted 1:1 (v/v) into a simulated salivary fluid (SSF) with alpha-amylase from Bacillus *sp.* (75 IU/mL), and incubated for 2 min at 37 °C, under continuous agitation in an overhead rotator (Reax 2, model 444–1113, Heidolph Instruments, Germany). The volume of simulated gastric fluid (SGF) with porcine pepsin (2000 IU/mL) to dilute the oral bolus was 1:1 (v/v) and the pH was adjusted to 3 with 10 M HCl. The mixture was incubated for 2 h, at 37 °C, with constant stirring. Gastric chyme was diluted 1:1 (v/v) with simulated intestinal fluid (SIF), and bile salts (10 mM) and pancreatin (100 U/mL) were added followed by adjustment to pH 7 (with 1 M NaOH). Incubation at 37 °C, with shaking, was stopped after 2 h with 320 μL of Pefabloc (5 mM) and stored at −80 °C. A sample-free tube was included in these trials, where the 4 g of bread was replaced with demineralised water.

All the *digesta* were centrifuged at 8000 r.p.m. for 10 min at 4 °C, and the supernatant was collected.

Nutritional evaluation

The estimation of the crude protein content was carried out by an automatic protein analyzer (NDA 702 Dumas Nitrogen Analyzer) following the CEREAL MEAL 1 method within the DUMASoftTM software. To determine the mineral content of the ash, acid digestion of the supernatant was carried out in *aqua regia* (a mixture of hydrochloric acid and nitric acid in the proportion of 3:1) at 105 °C for 90 min, with subsequent quantification by ICP-OES.

Table 5 Mineral composition (mg/ 100 g) of the control and GF loaves of bread with 4% microalgal biomass incorporation ($n = 2$, wet basis)

GF Bread	K	Ca	Mg	P	S	Fe	Cu	Zn	Mn
Control	199.2 ± 2.7	6.4 ± 0.1	56.4 ± 0.6	140.0 ± 1.6	70.0 ± 0.2	2.6 ± 0.1	0.1 ± 0.0	0.9 ± 0.0	0.5 ± 0.0
TcR_B	223.7 ± 1.8	67.5 ± 1.6	68.6 ± 1.2	164.6 ± 0.3	104.3 ± 0.4	4.8 ± 0.4	0.2 ± 0.0	1.0 ± 0.0	0.7 ± 0.0
TcT_B	201.6 ± 0.5	88.7 ± 0.3	64.1 ± 0.1	173.3 ± 0.4	103.8 ± 0.8	5.5 ± 0.2	0.1 ± 0.0	1.0 ± 0.0	0.8 ± 0.0
CvR_B	218.6 ± 1.2	8.0 ± 0.2	62.6 ± 0.1	166.5 ± 1.3	94.7 ± 0.3	2.9 ± 0.1	0.2 ± 0.0	0.9 ± 0.0	0.6 ± 0.0
CvT_B	220.4 ± 2.2	9.2 ± 0.2	69.8 ± 0.8	183.4 ± 2.7	108.7 ± 1.0	3.8 ± 0.2	0.2 ± 0.0	1.0 ± 0.0	0.7 ± 0.0
MgR_B	202.4 ± 2.8	10.1 ± 0.1	63.1 ± 0.2	162.1 ± 2.6	94.0 ± 2.0	3.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0	0.6 ± 0.0
MgT_B	202.3 ± 3.2	14.0 ± 0.1	68.7 ± 0.4	173.8 ± 0.6	99.8 ± 0.4	2.8 ± 0.0	0.1 ± 0.0	1.1 ± 0.0	0.7 ± 0.0
15% RDV (mg)	300.0	120.0	56.3	105.0	NM	2.1	0.2	1.5	0.3

15% of the recommended daily value (RDV) per European Union Regulation N.1924/2006, Directive N-9090/494 (CE) is listed below. NM; not mentioned. Adapted from (M. W. Qazi *et al.*, 2022; Qazi *et al.*, 2022)

Extraction and preparation of samples for bioactivity evaluation

The undigested bread samples (Control, TcR_B, TcT_B, CvR_B, CvT_B, MgR_B and MgT_B) were grounded into small crumbs in a thermo-processor (Bimby-Vorwerk, Cloyes-sur-le-Loir, France), suspended in methanol with a solvent ratio of 1:10 (w/v) and placed in a shaking water bath (Thermo Scientific Precision 2864 Water Bath) for 90 min at 40 °C. After standing and cooling to room temperature, samples were centrifuged, the liquid phases collected, and the solid phases were allowed to dry at 60 °C overnight for further analysis.

Antidiabetic activity assessment

The study of the antidiabetic capacity of the different bread samples was estimated through *in vitro* inhibition of the digestive enzyme α-amylase, based on the 3,5-dinitrosalicylic acid (DNSA) method with some modifications (Kifle & Enyew, 2020). α-Amylase inhibition was evaluated by adding 200 μL of the substrate starch (1%, MilliporeSigma) to 200 μL of the sample extract followed by 10 min incubation at 30 °C, and the addition of 20 μL of the enzyme (0.25 mg/mL, thermostable, from *Bacillus sp.*, 3000 U/mL, E-BSTAA, Megazyme) followed by a new 3 min of incubation at 37 °C. Then, 400 μL of DNS colour reagent (DNS 96 mM, potassium sodium tartrate 5.31 M in NaOH 2 M) were added and the mixture was incubated at 85 °C for 5 min in a shaking water bath (Thermo Scientific Precision 2864 Water Bath). The reaction stopped with the addition of 900 μL of cold water and then diluted with 15 mL of Milli-Q water, and the absorbance of sample extracts (As) was read at 540 nm using a UNICAM UV4 UV/Vis Spectrometer.

A blank with 100% enzyme activity (Ac+) was prepared with 200 μL of buffer in place of the extract, and a blank with 0% activity (Ab) with buffer in place of the enzyme.

The inhibition of α-amylase was expressed as a percentage of inhibition and calculated using the following equation:

$$\text{Inhibition}(\%) = [(\text{Ac}+)-(\text{Ac}-)]-[(\text{As}-\text{Ab})/((\text{Ac}+)-(\text{Ac}-))] \quad (1)$$

Lipophilic bioactive compounds

The lipophilic composition was performed using the methodology previously reported (Pérez *et al.*, 2021). Aliquots of the dichloromethane extracts (5 mL) were evaporated under N_2 flow and dried under vacuum at room temperature. Sample derivatisation was made before analysis. Residues were dissolved in 150 μL of pyridine and the compounds with hydroxyl and carboxyl groups were trimethylsilylated into trimethylsilyl (TMS) ethers and esters, respectively, by adding 100 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The reaction mixture was heated at 60 °C for 3 h in an oven. The derivatised extracts (1 μL) were immediately analysed by GC–MS (Thermo Scientific Trace 1300 gas chromatograph coupled with a mass spectrometer XX (Massachusetts, EUA), ionisation energy of 70 eV and MS source were kept at 250 °C under the following GC conditions: DB-1 capillary column (30 m × 0.25 mm × 0.25 μm film thickness), injector 250 °C. The column was initially held at 100 °C for 2 min, raised to 250 °C at a rate of 15 °C min^{-1}, then to 270 °C at 5 °C min^{-1} followed by an isothermal period of 2 min. Compounds were identified as TMS derivatives by comparing their mass spectra with a GC–MS spectral library (Wiley, NIST) and by comparing their fragmentation profiles with published data. The peak areas in the TIC were determined and expressed as normalised relative percentages. The calculated composition was semi-quantitative/qualitative since no standards for each chemical family were co-injected nor were their response factors determined. Each aliquot was injected in triplicate.

Volatile organic compounds

Volatile organic compounds composition was determined using a previously reported methodology with some modifications (de Alencar *et al.*, 2017). Static headspace sampling was performed with the headspace autosampler, TriPlus RSH System (Thermo Finnigan, San Francisco, USA). A 2.5 mL headspace syringe for PAL System was used for the injection of 2 mL from the 20 mL headspace vials with 1 g of measured dry sample. The autosampler conditions were set as follows: incubation temperature, 80 °C; incubation time, 10 min; syringe temperature, 100 °C; agitator speed, 500 r.p.m.; fill speed, 100 μL/s; pull-up delay, 1 s; injection speed, 500 μL/s; pre- and post-injection delay, 500 ms; flush time, 10 s. After each injection, carryover in the syringe was eliminated by an automatic flush of the syringe with carrier gas. Chromatographic separation was achieved by Thermo Scientific TRACE 1300 gas chromatograph coupled to a Thermo ISQ mass selective detector. A DB-1 (30 m × 0.25 mm i.d.) (Agilent J & W Scientific, USA) fused silica capillary column with a 0.25 μm film thickness was used with helium as carrier gas (purity >99.9997% and flow rate = 1.0 mL min^{-1}). The oven temperature programme was started at 60 °C (not held), and a linear temperature gradient was applied at a rate of 3 °C min^{-1} to a final temperature of 260 °C and held for 5 min (total run time: 65 min). The ion source temperature was kept at

230 °C, the transfer line was at 150 °C, and the mass spectra were obtained in the 50 to 500 m/z range, at an electron energy of 70 eV.

Compounds were identified as TMS derivatives by comparing their mass spectra with a GC–MS spectral library (Wiley, NIST) and by comparing their fragmentation profiles with published data. The peak areas in the TIC were determined and expressed as normalised relative percentages. The calculated composition was semi-quantitative/qualitative since no standards for each chemical family were co-injected nor were their response factors determined. Each aliquot was injected in triplicate.

Statistical analysis

The experimental data were duly submitted and analysed using Microsoft Excel, where a single-factor analysis of variance (ANOVA) was performed for all experiments. *Post hoc* analysis was performed using Tukey's honestly significant difference (HSD) test for a significance level of 95% ($P < 0.05$).

Conclusions

When considering innovative food fortified with microalgae, the intense green colour and the fish-like aroma are major drawbacks when it comes to including them in our daily food routine.

In the present work, we reported for the first time the lipophilic composition and the volatiles present in the GF breads prepared with three different strains of microalgae: *M. gaditana, C. vulgaris* and *T. chuii*. Alcohols and terpenes/terpenoids were identified as the major compounds responsible for the aroma, and also 9,12-octadecadienoic acid was the main lipophilic constituent of the prepared GF breads. Phytic acid was also present in all microalgae-enriched GF breads at considerable amounts and may contribute to the decrease observed in the bioaccessibility of nutrients such as protein and minerals. Therefore, the incorporation of microalgae into new food products has incredible advantages, nutrition and health wise, but also some weaknesses concerning the sensory acceptance by consumers.

Ongoing and future research are focused on have a full characterisation of the compounds responsible for the overall aroma and perception of aromas of microalgae and microalgae-based food products, find mitigation strategies that will allow to obtain more sustainable food products with high nutritional and sensory scores and with a positive impact on health.

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Author contributions

Marco A. Freitas: Investigation (equal); writing – original draft (equal). **Joana Ferreira:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (supporting); methodology (lead); supervision (equal); validation (lead); visualization (lead); writing – original draft (equal); writing – review and editing (lead). **Maria Cristiana Nunes:** Conceptualization (equal); formal analysis (equal); supervision (equal). **Anabela Raymundo:** Conceptualization (equal); funding acquisition (lead); project administration (lead); supervision (equal); visualization (equal).

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

The research did not include any human subjects or animal experiments.

Peer review

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Data availability statement

Data supporting the findings of this study are available upon request from the corresponding author.

References

Achyuthan, K.E., Harper, J.C., Manginell, R.P. & Moorman, M.W. (2017). Volatile metabolites emission by in vivo microalgae—an overlooked opportunity? *Metabolites*, **7**, 39.

Annamalai, S.N., Das, P., Thaher, M.I.A. *et al.* (2021). Nutrients and energy digestibility of microalgal biomass for fish feed applications. *Sustainability*, **13**, 13211.

Batista, A.P., Niccolai, A., Fradinho, P. *et al.* (2017). Microalgae biomass as an alternative ingredient in cookies: sensory, physical

and chemical properties, antioxidant activity and *in vitro* digestibility. *Algal Research*, **26**, 161–171.
Bohn, T. (2008). Dietary factors influencing magnesium absorption in humans. *Current Nutrition & Food Science*, **4**, 53–72.
Brodkorb, A., Egger, L., Alminger, M. *et al.* (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols*, **14**, 991–1014.
This is a very relevant publication were it is described the latest INFOGEST Protocol for the static *in vitro* simulation of gastrointestinal food digestion. It has a fully detailed description on the protocol, on the preparation of the digestive fluids.
Caio, G., Volta, U., Sapone, A. *et al.* (2019). Celiac disease: a comprehensive current review. *BMC Medicine*, **17**, 142.
Ciarán, P.K. (2020). Celiac disease. In Sleisenger and Fordtran's gastrointestinal and liver disease. In: (edited by B.H.R. Lebwohl & P. Green). Pp. 1849–1872, Vol. **2**. Elsevier.
de Alencar, D.B., Diniz, J.C., Rocha, S.A.S. *et al.* (2016). Chemical composition of volatile compounds in two red seaweeds, Pterocladiella capillacea and Osmundaria obtusiloba, using static headspace gas chromatography mass spectrometry. *Journal of Applied Phycology*, **29**(3), 1571–1576.
Demarco, M., Oliveira De Moraes, J., Matos, A.P., Derner, R.B., de Farias Neves, F. & Tribuzi, G. (2022). Digestibility, bioaccessibility and bioactivity of compounds from algae. *Trends in Food Science and Technology*, **121**, 114–128.
This review provides an overview of existing information of ongoing studies on the digestibility and bioavailability of algae derived compounds. A special attention is also given to the influence of cell wall disruption techniques on the bioaccessibility of algal bioactive compounds, show casing carefully the *in vitro* bioaccessible nutrients of foods enriched with algal biomass.
Fradique, M., Batista, A.P., Nunes, M.C., Gouveia, L., Bandarra, N.M. & Raymundo, A. (2013). *Isochrysis Galbana* and *Diacronema Vlkianum* biomass incorporation in pasta products as PUFA's source. *LWT – Food Science and Technology*, **50**, 312–319.
Gentscheva, G., Nikolova, K., Panayotova, V. *et al.* (2023). Application of *Arthrospira platensis* for medicinal purposes and the food industry: a review of the literature. *Life*, **13**, 845.
GFFMSG, Gluten Free Food Market Size & Growth. (2021–2028). Forecast. Available from: https://www.fortunebusinessinsights.com/industry-reports/gluten-free-food-market-100188 [Accessed 19th July 2022].
Harris, E.H. (2009). *Edited by: Elizabeth H. Harris, David B. stern and George B. Witman. The Chlamydomonas Sourcebook: Introduction to Chlamydomonas and Its Laboratory Use*. Pp. 1–480, 2nd edn. San Diego, CA: Academic Press.
Hosoglu, M.I. (2018). Aroma characterization of five microalgae species using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. *Food Chemistry*, **240**, 1210–1218.
Khan, M.I., Shin, J.H. & Kim, J.D. (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial Cell Factories*, **17**, 1–21.
Khemiri, S., Khelifi, N., Nunes, M.C. *et al.* (2020). Microalgae biomass as an additional ingredient of gluten-free bread: Dough rheology, texture quality and nutritional properties. *Algal Research*, **50**, 101998.
Kifle, Z.D. & Enyew, E.F. (2020). Evaluation of *in vivo* antidiabetic, *in vitro* α-amylase inhibitory, and *in vitro* antioxidant activity of leaves crude extract and solvent fractions of *Bersama Abyssinica Fresen* (*Melianthaceae*). *Journal of Evidence-Based Integrative Medicine*, **25**, 2515690X2093582.
Lis, D.M., Stellingwerff, T., Shing, C.M., Ahuja, K.D.K. & Fell, J.W. (2015). Exploring the popularity, experiences, and beliefs surrounding gluten-free diets in nonceliac athletes. *International Journal of Sport Nutrition and Exercise Metabolism*, **25**, 37–45.
Lopes, M., Pierrepont, C., Duarte, C.M., Filipe, A., Medronho, B. & Sousa, I. (2020). Legume beverages from kpea and Lupin, as new milk alternatives. *Food*, **9**, 1458.
Mahfouz, G.N. (2014). Mineral uptake rates and yield coefficients of the Green microalgae *Scenedesmus Dimorphus*. ETD Archive. 356. https://engagedscholarship.csuohio.edu/etdarchive/356
Melo, T., Figueiredo, A.R.P., da Costa, E. *et al.* (2021). Ethanol extraction of polar lipids from *Nannochloropsis oceanica* for food, feed, and biotechnology applications evaluated using Lipidomic approaches. *Marine Drugs*, **19**, 593.
Moran, L., Bou, G., Aldai, N. *et al.* (2022). Characterisation of the volatile profile of microalgae and cyanobacteria using solid-phase microextraction followed by gas chromatography coupled to mass spectrometry. *Science Reports*, **12**, 3661.
Nacer, W., Baba Ahmed, F.Z., Merzouk, H., Benyagoub, O. & Bouanane, S. (2020). Evaluation of the anti-inflammatory and antioxidant effects of the microalgae *Nannochloropsis Gaditana* in Streptozotocin-induced diabetic rats. *Journal of Diabetes and Metabolic Disorders*, **19**, 1483–1490.
Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N. & Tredici, M.R. (2019). Microalgae of interest as food source: biochemical composition and digestibility. *Algal Research*, **42**, 101617.
Nunes, M.C., Ferreira, J. & Raymundo, A. (2023). Volatile fingerprint impact on the sensory properties of microalgae and development of mitigation strategies. *Current Opinion in Food Science*, **51**, 101040.
This manuscript provides an overview of existing information of the volatile composition of the different microalgae specie. It also suggest some strategies to improve the overall sensory analysis of microalgae and be able to increase the level of incorporation of microalgae into novel foods.
Paul, C. & Pohnert, G. (2011). Production and role of volatile halogenated compounds from marine algae. *Natural Products Reports*, **28**, 186–195.
Penagini, F., Dilillo, D., Meneghin, F., Mameli, C., Fabiano, V. & Zuccotti, G.V. (2013). Gluten-free diet in children: an approach to a nutritionally adequate and balanced diet. *Nutrients*, **5**, 4553–4565.
Pérez, J.P., Muñoz, A.A., Figueroa, C.P. & Agurto-Muñoz, C. (2021). Current analytical techniques for the characterization of lipophilic bioactive compounds from microalgae extracts. *Biomass and Bioenergy*, **149**, 106078.
Qazi, W.M., Ballance, S., Kousoulaki, K. *et al.* (2021). Protein enrichment of wheat bread with microalgae: *Microchloropsis gaditana*, *Tetraselmis chui* and *Chlorella vulgaris*. *Foods*, **10**, 3078.
Qazi, M.W., de Sousa, I.G., Nunes, M.C. & Raymundo, A. (2022). Improving the nutritional, structural, and sensory properties of gluten-free bread with different species of microalgae. *Food*, **11**, 397.
This manuscript is a valuable basis for the development of gluten-free breads incorporated with microalgae, focusing on their preparation, technological properties and sensory profile.
Regulation (EC). (2022). No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on Nutrition and Health Claims Made on Foods. Available from: https://www.legislation.gov.uk/eur/2006/1924/contents [Accessed 2nd October 2022]
Schoenfeld, B.J. & Aragon, A.A. (2018). How much protein can the body use in a single meal for muscle-building? Implications for daily protein distribution. *Journal of the International Society of Sports Nutrition*, **15**, 10.
Ścieszka, S. & Klewicka, E. (2019). Algae in food: a general review. *Critical Reviews in Food Science and Nutrition*, **59**, 3538–3547.
Silva, J.G.S., Rebellato, A.P., Caramês, E.T.D.S., Greiner, R. & Pallone, J.A.L. (2020). *In vitro* digestion effect on mineral bioaccessibility and antioxidant bioactive compounds of plant-based beverages. *Food Research International*, **130**, 108993.
Tibbetts, S.M., Milley, J.E. & Lall, S.P. (2015). Chemical composition and nutritional properties of freshwater and marine microalgal

biomass cultured in photobioreactors. *Journal of Applied Phycology*, **27**, 1109–1119.
Uribe-Wandurraga, Z.N., Igual, M., García-Segovia, P. & Martínez-Monzó, J. (2020). *In vitro* bioaccessibility of minerals from microalgae-enriched cookies. *Food & Function*, **11**, 2186–2194.
Valente, L.M.P., Custódio, M., Batista, S., Fernandes, H. & Kiron, V. (2019). Defatted microalgae (*Nannochloropsis* Sp.) from biorefinery as a potential feed protein source to replace fishmeal in European Sea bass diets. *Fish Physiology and Biochemistry*, **45**, 1067–1081.
Verspreet, J., Soetemans, L., Gargan, C., Hayes, M., Bastiaens, L. & Pereira, L. (2021). Nutritional profiling and preliminary bioactivity screening of five micro-algae strains cultivated in Northwest Europe. *Foods*, **10**, 1516.
Wang, S., Shang, H., Abomohra, A.E.-F. & Wang, Q. (2019). One-step conversion of microalgae to alcohols and esters through co-pyrolysis with biodiesel-derived glycerol. *Energy Conversion and Management*, **198**, 111792.
Wild, K.J., Steingaß, H. & Rodehutscord, M. (2018). Variability in nutrient composition and *in vitro* crude protein digestibility of 16 microalgae products. *Journal of Animal Physiology and Animal Nutrition*, **102**, 1306–1319.
Wungjiranirun, M., Kelly, C.P. & Leffler, D.A. (2016). Current status of celiac disease drug development. *The American Journal of Gastroenterology*, **111**, 779–786.
Zanella, L. & Vianello, F. (2020). Microalgae of the genus Nannochloropsis: Chemical composition and functional implications for human nutrition. *Journal of Functional Foods*, **68**, 103919.