Effect of formulations and fermentation processes on volatile organic compounds and prebiotic potential of gluten-free bread fortified by spirulina (Arthrospira platensis)

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ARTICLE

Effect of formulation and fermentation process on volatile organic compounds and prebiotic potential of gluten-free bread fortified by spirulina (*Arthrospira platensis*)

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Gluten free (GF) foods, designed and marketed for the needs of people who are unable to metabolize gluten, in recent years have aroused a growing interest that has led to the conquest of important market segments, with a strongly growing trend. Given the low protein content of standard GF flours, it is particularly important to fortify GF foods, and to study the effect that this process determine on functional and sensorial characteristics. In this work, fortification of GF bakery goods was done with the addition of *Arthrospira platensis* (Spirulina) flour. Two different dough formulations (with and without fortification) were fermented by four different processes, including spontaneous, single strains and sourdough straters. The baked products were then subjected to "consumer's tests". During the process, fermentation performances, prebiotic activity, and the VOCs (Volaitle Organic Compounds) profiles were analyzed and compared through a robust multivariate statistics. The results obtained evidenced that fortification led to a product with more abundant (medium organic acids) and exclusive bioactives (Thymol, Borneol, and Nicotinic acid), that were correlated to the prebiotic activity of spirulina breads. This work, for the first time indicates that spirulina can be used to fortify GF bakery, improving also its functional potential.

1. Introduction

Celiac disease (CD) is an autoimmune disease triggered by the ingestion of gluten present in wheat, barley, and rye in genetically predisposed individuals. The prevalence of celiac disease in the general population is 1%, with regional differences[1], and its management requires exclusion of dietary gluten and the substitution of gluten-containing products with gluten-free (GF) products. The manufacturing of GF products is challenging not only from an organoleptic but also from a nutritional point of view. GF products are often nutritionally less adequate than standard products for the low protein and high fat, sugar, and salt content[2].

Gluten-free (GF) product development presents major challenges for the food industry in terms of organoleptic, technological, and nutritional characteristics. The GF food market is continuously growing, with estimated market share sales worldwide of 18% gluten-free pasta in 2022, with an annual growth rate of 7.4%[3]. Nowadays, target audience for GF foods stretches beyond coeliac sufferers. In 2015, only 9% of US gluten-free consumers followed a GF diet due to a celiac disease, while others were adopting a GF lifestyle because it made them feel healthier (12%) or wanted to lose weight (7%)[4].

In response to consumer's needs, more and more gluten-free products, such as bread, have appeared on the market. However, these products often do not satisfy the nutritional deficiencies of these consumers in terms of dietary fiber, vitamins (B12, D), and minerals (iron, calcium, zinc)[5]. Furthermore, consumers consider GF diet hard to follow due to low availability, lack of variety, texture problems, poor palatability, and high prices of the GF products[6]. Moreover, GF products, especially the bakery ones are poor in protein content and a protein implementation is common[7]. However, the effects of protein fortification by different flours as protein source on the food quality, need to be clarified.

Arthrospira platensis (Spirulina) powder/flour (FA) represents a potential ingredient for GF bakery products. This microalgae presents a high protein content, up to 70% dry weight[8]. Its aminoacid composition has a great interest, not only because *A. platensis* possesses all of the essential aminoacids, but also because these aminoacids have a great bioavailability[8]. The carbohydrates of *A. platensis* constitute approximately 15% of the dry matter. The major carbohydrate are polysaccharides. Among the monomeric forms, glucose, galactose, ribose, and mannose are preferentially found[8]. On the other hand, its lipid fraction accounts for about 5% of its dry weight[8]. *A. platensis* is rich in polyunsaturated fatty acids, carotenoids, vitamins, minerals, phenolic compounds, and bioactive molecules[8]. *A. platensis* also shows various activities of pharmacological interest, such as antioxidant, immunomodulatory, hypolipidemic and anti-inflammatory activity[9,10].

Fermentation with sourdough or with beneficial LAB (Lactic Acid Bacteria) strains could further increase the nutritional value of FA-

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enriched GF products since it provides health benefits to consumers, due to the ingestion of beneficial bacteria and microbial metabolite[11]. During fermentation, several VOCs (volatile organic compounds) are synthesized naturally by microorganisms (as secondary metabolites) interacting with the food matrix. VOCs are organic molecules that include esters, alcohols, aldehydes, ketones, phenols, organic acids, terpenes, etc. Beyond their flavoring properties, various reports have shown the potential role of VOCs in human health, including their antioxidant, anti-inflammatory, anti-microbial, and anti-obesity activities[11]. Lastly, certain VOCs among the aforementioned compounds have been reported to possess prebiotic activity, as organic acids, and terpenes[12,13]. When microalgae are integrated into food, aroma is an important aspect to consider. The presence of sulfuric compounds, diketones, α-ionone, and β-ionone in fresh microalgae biomass is explained by the mechanisms of aroma formation such as enzymatic oxidation of lipids, enzymatic and chemical degradation of dimethyl sulfoniopropionate (which generates dimethyl sulfide), phenylalanine (generation of benzaldehyde) and carotenoids (generation of ionones)[14]. Due to the presence of these unpleasant compounds, the volatilome analysis conducted in this work aimed to predict the aromatic properties of FA-enriched GF products, and to evaluate the impact of the fermentation process on the development of a characteristic flavor profile, in order to improve the aromatic properties of baked goods. Thus, given the common industrial knowledge gap about quality of protein fortified GF foods, our work aimed to consider the effect of fortification over the volatilome, in respect to VOCs with bioactivity and in respect to prebiotic activity.
The recipient study is based on a metabolomic approach to evaluate VOCs production in a sourdough-fermented GF bread enriched with *A. platensis* and to relate these compounds to flavoring and health properties of the final product. Besides, processing variables, consumer's palatability, and prebiotic activity were pondered and correlations among VOCs production were evaluated.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Microbial strains belonged to the microbial collection of the Department of Agricultural and Food Sciences, University of Bologna (Italy)[11,15]. *Lactobacillus plantarum* 98a, *Lb. sanfrancisciensis* Bb12 and *Saccharomyces cerevisiae* LBS were obtained from 30% (*v*/*v*) glycerol stocks stored at -80 °C. Bacteria were propagated in MRS (de Man–Rogosa–Sharpe) broth (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) (Dextrose 20%; Peptone; 10%; Beef Extract 8%; Sodium Acetate 5%; Yeast Extract 4%; Ammonium Citrate 2%; Dipotassium Phosphate 2%; Polysorbate80 1%; Magnesium Sulfate 0.2%; Manganese Sulfate 0.05%) at 37 °C for at least 48 h and yeasts in Sabouraud Dextrose broth (Oxoid, Thermo Fisher Scientific, USA) (Dextrose 20%; Peptone; 10%; Pancreatic digest of casein 5%; Pancreatic digest of animal tissue 5%; Chloramphenicol 0.05 g/L) at 30 °C for 24 h.

2.2. Doughs and Bread Preparation

Flours were commercial organic certified products (Table S1). Experimental doughs (approximately 700 g) were prepared according to the formulation reported in Table S2. Maize and rice were partially substituted with 5.3% (*w*/*w*) FA (Algae Flour) to obtain a GF formulation suitable to be considered as a protein source. The list of samples and their codes are described in Table 1. Two types of doughs formulations were used: a standard type (ST) including maize and rice flours and an algae type (AT) where FA (5.3% *w*/*w*) replaced standard flours. The percentage of FA to be used for enrichment was set to generate a dough with at least 12% of energy derived from protein that can be claimed "protein source" and was based on the sensory characteristics of the final products (data not shown), and on a similar approach adopted in formulation non-GF fortified bakery products[16]. Both types were used for direct fermentations and for sourdough fermentations. Direct fermentations were as follows: (i) not inoculated (X); (ii) inoculated with Log_{10} 6 CFU/mL of an equal LAB mix of *Lb. sanfrancisciensis* Bb12 and *Lb. plantarum* 98a (L); (iii) inoculated with Log_{10} 7 CFU/mL of *S. cerevisiae* LBS (Y), and were conducted for 18 h at 31 °C. The samples for sourdough fermentations, indicated by a "+" in the labels, were made by replacing 20% of AT and ST dough formulations with 140 g of direct LAB mix 18 h fermented doughs (L) and inoculated with Log_{10} 7 CFU/mL of *S. cerevisiae* LBS (Y). The complete sourdoughs were then fermented for 6 h at 31 °C. As an additional control, a direct fermentation with Y was conducted for 6 h at 31 °C. All fermented doughs were baked at 180 °C for 20 min to produce breads (B). All samples were produced in triplicates in two independent experiments. The doughs were prepared with a kneading machine (Bimby Tm31, Vorwerk, Wuppertal, Germany) setting the program for bread making, then were formed in single steel containers, fermented in a laboratory incubator (MPM Instruments, Srl, Bernareggio, Italy), and baked with an electric oven (Mod.KOABS31X, Electrolux, Stockholm, Sweden).

Table 1. Description of samples codes.

Sample	Description
FA	Algae Flour (*Arthrospira platensis*)
FM	Maize Flour
FR	Rice Flour
AX	Algae dough not inoculated (direct fermentation)
AL	Algae dough LAB inoculated (direct fermentation)
AY	Algae dough *S. cerevisiae* LBS inoculated (direct fermentation)
SX	Standard dough not inoculated (direct fermentation)
SL	Standard dough LAB inoculated (direct fermentation)
SY	Standard dough *S. cerevisiae* LBS inoculated (direct fermentation)
YA+	Algae dough added with sourdough
YS+	Standard dough added with sourdough
AX18	AX fermented 18 h
AL18	AL fermented 18 h
AY18	AY fermented 18 h
SX18	SX fermented 18 h

SL18	SL fermented 18 h
SY18	SY fermented 18 h
YA+6	YA+ fermented 6 h
YS+6	YS+ fermented 6 h
YA6	YA* fermented 6 h
YS6	YS* fermented 6 h
YA+B	Bread from YA+6
YS+B	Bread from YS+6
YAB	Bread from YA6
YSB	Bread from YS6
ALB	Bread from AL18
AYB	Bread from AY18
SLB	Bread from SL18
SYB	Bread from SY18

* same formulations of AY and SY, respectively.

2.3. Microbial Quantification during the Process

Microbial quantification was obtained by both culture-dependent and culture-independent protocols. The culture-dependent quantification was done by plating serial dilutions of the samples in sterile physiological solution (NaCl 0.9% w/v). LAB were plated on MRS (de Man–Rogosa–Sharpe) (Oxoid, Thermo Fisher Scientific, USA) agar and cycloheximide (0.1 g/L) (Sigma, Saint Louis, MO, USA) and incubated aerobically for 48 h at 37 °C. Yeasts were plated on Sabouraud Dextrose Agar (Oxoid, Thermo Fisher Scientific, USA) and chloramphenicol (0.05 g/L) (Sigma, USA) and incubated aerobically for 24 h at 30 °C. Quantification was calculated as Log_{10} CFU/mL (Colony Forming Units/mL). Culture independent protocol was performed by qPCR with the SYBR Green I chemistry, applying genus specific primers (Eurofins Genomics GmbH, Ebersberg, DE) as Lac1 for *Lactobacillus* spp., then named LAB, (forward: 5'-GCAGCAGTAGGGAATCTTCCA-3' and reverse: 5'-GCATTYCACCGCTACACATG-3')[17] and ITS 23S for *S. cerevisiae* LBS, then named yeasts, (forward: 5'-GTTTCCGTAGGTGAACCTGC-3' and reverse: 5'-ATATGCTTAAGTTCAGCGGGT-3')[18]. Extraction of bacterial DNA was obtained with Nucleo Spin Food DNA Extraction Kit (Macherey Naegel, Duren, Germany) prior a pre-treatment of 10 min at 20 Hz of ultra-pure water diluted doughs in a sonication bath. Genetic standards were prepared from relative PCR amplicons from pure cultures of the target bacterial species as described previously[11,15]. Templates for qPCR to generate standard curves were amplified by PCR using a ProFlex PCR System apparatus (Thermo Fisher Scientific, USA) with SuperFi Platinum Taq (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Amplicons were purified with GeneJet PCR Purification kit (Thermo Fisher Scientific, USA). For both the targets, qPCR reactions were performed with Power-Up Master Mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) on a RotorGene 6000 (Qiagen, Hilden, Germany) with the RotorGene Q Series Software 2.3.1 Release (Qiagen, Germany), set and analysed as previously described[11,15]. Sample reactions were conducted in triplicate, with positive, negative, and background controls. Quantification was calculated as GCN/mL (Gene Copy Number/mL) and the value divided by three (the presumptive copies of a ribosome per cells), expressed as Log_{10} cells/mL[11,15].

2.4. pH Changes during the Process

The pH was determined at 20 °C with a pH meter (Crison, Alella, Spain) appropriately calibrated with three standard buffer solutions at pH 9.21, pH 4.00, and pH 2.00. The pH values were measured in duplicate at the beginning and the end of fermentation (Table S3).

2.5 Prebiotic score

Stomacher-homogenized (3500 paddle blender, Seward Ltd., Worthing, UK) and sterile physiological solution (NaCl 0.9% w/v) 1:10 (w/v) diluted samples were employed to test for prebiotic potential with the prebiotic score method previously described by Fissore et al.[19] with modifications[12]. Briefly the method is based on selective growth of probiotics and enteropathogens on minimal broths enriched with 1% (w/v) of homogenized food products in comparison to control sugar (glucose 1% w/v) and prebiotic fructo-oligosaccharides (FOS 1% w/v) from chicory (Sigma, USA). The bacterial type strains *Lb. plantarum* 98b, *B. bifidum* 700795, and *E. coli* 25922 were used, and propagated as previously reported[19,12]. Bacterial loads of the inocula were adjusted with the aid of a spectrophotometer (Tecan M200 Plate Reader, Tecan Trading AG, CH) to obtain a final concentration of 6 Log_{10} CFU/mL, afterwards confirmed by culture dependent and independent quantifications. The prebiotic activity score was calculated with the related formula from two independent experiments and technical triplicates as previously described[19,20], including qPCR quantifications[12].

2.6. Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS)

Evaluation of volatile organic compounds (VOCs) was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV), equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME-GC-MS (solid phase micro-extraction gas chromatography–mass spectrometry) protocol and the identification of volatile compounds were done according to previous reports, with minor modifications[11,13,15]. Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 40 °C. The SPME fiber was exposed to each sample for 40 min and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 0 min, then programmed at 1.5 °C/min to 65 °C, and finally at 3.5 °C/min to 220 °C, which was maintained for 20 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in splitless mode, and helium (3 mL/min) was used as carrier gas. Identification

of molecules was carried out by comparing their retention times with those of pure compounds (Sigma, USA) and confirmed by searching mass spectra in the available databases (NIST version 2005 and Wiley version 1996) and literature[11,13,15]. Ethyl alcohol, 1,4-butandiol, 2-butanone-3-hydroxy and acetic acid were absolutely quantified in mg/Kg (Table 3), while all other VOCs were relatively quantified in percentage.

2.7 Sensory evaluation

The breads were evaluated after 3 h from baking by 20 untrained testers (consumers), that scored the produced breads according to a preference protocol with a scale from 0 (unacceptable) to 6 (excellent)[21]. Two independent consumers' tests were performed, and results were marked in a spider chart as average scores for color, aftertaste, smell, taste, crispiness, and overall appreciation of the breads.

2.8. Statistical Analyses

All statistical analyses were performed using TIBCO Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA). Normality was checked with the Shapiro–Wilk test, while homoscedasticity was evaluated with the Levene's test[22]. Differences between all samples were evaluated with untargeted Analysis of Variance (ANOVA) set at $P < 0.05$. Multivariate analysis was conducted with principal component analysis (PCA), K-mean clustering, and MANOVA ($P < 0.05$). Pearson correlations were used to generate the heatmap of VOCs prior fermentation. For post hoc test, a Tukey's HSD (honestly significant difference) test was employed ($P < 0.05$). Except for the quantification in mg/Kg of major metabolites, independently normalized data set was proposed for each chemical class of molecules. The data were normalized using the mean centering method. All results are expressed as mean values obtained at least from duplicate batches in two independent experiments[11,13,15]. A Student's T-test was used to compare values from the sensory evaluation test ($P < 0.05$).

3. Results

3.1. Microbial Quantification and pH Values during the Process

Quantification of LAB and yeasts obtained by plate count and qPCR are shown in Figure 1. Results are expressed as Log_{10} cell/g and represent the mean value of culture-dependent and a culture-independent data[13,15]. Accounting yeast top quantification was recorded by direct fermentation with yeast of FA (Algae Flour) enriched GF doughs after 18 h of fermentation (AY18) (8.62 ± 0.24 Log_{10} cell/g), that was slightly higher than in the standard dough (SY18). Oppositely, LAB top quantification was scored by standard sourdoughs (YS+6) (11.00 ± 0.14 Log_{10} cell/g) slightly higher than the relative FA-enriched GF dough (YA+6). In FA-enriched GF and standard sourdoughs, the microbial load resulted similar either for yeast or LAB quantifications ($P > 0.05$). In all conditions, direct inoculation of LAB was the starter that mostly reduced pH during fermentation (Table S3). In particular, the FA-enriched GF doughs (pH 4.04 ± 0.12) had an acidification milder than their relative standards (ST) ($P > 0.05$). After 24 h of sourdough fermentation, the most acidified dough was still the standard (YS+6), but with no difference to the YA+6 (4.31 ± 0.15).

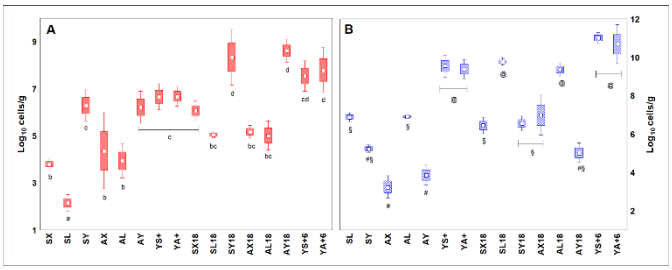

Figure 1. Mean values of absolute quantifications (Log_{10} cells/g) of A) yeasts and B) LAB (Lactic Acid Bacteria) prior baking. Each value is a mean derived from technical duplicates (Log_{10} CFU/mL) and triplicates Log_{10} GCN/mL) from two independent experiments (n = 20). Middle point = mean; box = mean ± standard deviation (SD): whiskers = mean ± SD*1.96. Different letters or symbols indicate statistical significance by Tukey's HSD test (at least $P < 0.05$). For samples abbreviations see Table 1.

3.2. Prebiotic activity

The prebiotic activity was determined according to Fissore et al.[18], with some modifications, as microbial quantification by qPCR and the use of FOS (Fructo-oligosaccharides from chicory) (Sigma, USA) as prebiotic positive control. Huebner et al.[20] established a quantitative score to easy describe the extent to which prebiotics foster the selective growth of probiotic species of lactobacilli and bifidobacteria. A given bioactive compound has a positive prebiotic activity score if is rather metabolized by probiotic bacteria and not by opportunistic intestinal ones[20]. The highest score of prebiotic activity towards *L. plantarum* was obtained with the not inoculated FA-enriched GF dough (AX), similar to FOS ($P > 0.05$) (Table 2). From this level, there was a reduction in the score of 1.70-fold following the baking step (YA+B) ($P < 0.05$). However, compared to FOS, YA+B did not produce high output ($P > 0.05$). The same results were observed when the prebiotic activity was assayed versus *B. bifidum*. Indeed, the best outcome was obtained by AX, with no significant differences from FOS ($P > 0.05$). In this case, however, a significant score decrease was observed following baking step ($P < 0.05$), compared to the score recorded for AX. Again, however, compared to FOS, YA+B did not produce high output ($P > 0.05$). In this work the average of the prebiotic activity versus *L. plantarum* (0.188 ± 0.10) was higher than that versus *B. bifidum* (0.143 ± 0.12), similarly to previous literature[12]. Anyhow, the two AT samples performed better than the standard samples. Indeed, these latter did not have any prebiotic activity, mainly because were not able to inhibit the growth of enteropathogens.

Table 2. Prebiotic score on doughs and breads.

Sample	*Lactobacillus plantarum**	*Bifidobacterium bifidum***

AX	0.344 ± 0.13[a]	0.342 ± 0.14[a]
SX	0.122 ± 0.16[c]	0.077 ± 0.07[b]
YA+B	0.202 ± 0.12[b]	0.134 ± 0.08[b]
YS+B	0.087 ± 0.09[c]	0.019 ± 0.04[b]
FOS	0.354 ± 0.15[a]	0.298 ± 0.11[a]

Values are means of two independent experiments, two replicates for plate counts and three for qPCR (n = 10). [abc]Different letters among a column indicate significative differences by Student's t-test (P < 0.05). **Lb. plantarum* 98b; *B. bifidum* NCIMB 800875. For samples abbreviations see Table 1.

3.3. Analysis of the Volatilome

Volatilome analysis identified more than 250 molecules and relatively quantified approximately 147. For a landscape description of the volatilome, two datasets normalized with the mean centering method were proposed: (i) one including quadruplicates of not fermented cases (n = 36) and 177 molecules (Section 3.3.1) (Figure S1) and (ii) one including quadruplicates of all experimental cases (n = 116) considering the sums of relative abundances of significant VOCs (ANOVA P < 0.05) grouped by chemical classes, employed to compare the not fermented cases to the means of fermented cases and breads cases (Section 3.3.2) (Figure 2). Afterward, for a more specific investigation and to generate robust data trainings for multivariate analysis, two other options were chosen: (iii) the most abundant VOCs (Ethyl alcohol, Acetic acid, 2-Butanone-3-hydroxy, and 1,4-Butandiol) were set apart and independently quantified in mg/Kg using an internal standard as described previously[12,23] (Section 3.3.3) (Table 3); (iv) all other 93 significant VOCs (ANOVA P < 0.05) were super-normalized in five distinct data sets organized by chemical classes (17 organic acids, 21 ketones, 20 aldehydes, 22 alcohols, 13 alkenes) to perform multivariate analyses (PCA, K-Means, and MANOVA) (Figures 3-7) (Section 3.3.4).

3.3.1. Quantification of VOCs before fermentation.

Considering flours and not fermented doughs, 128 VOCs were detected in ST and 167 in AT. The data set was clustered by Pearson analysis in two major groups, the first including ST not fermented doughs and FM and FR, while the second including AT not fermented doughs and FA (Figure S1). A higher quantity and a wider speciation of compounds was identified in AT samples. In most cases, Hexanal and Benzenamine N-ethyl were the two most abundant VOC, reaching the top level in FR and AX samples, but among the entire dataset 8-Heptadecene, (Z) of FA samples was the most abundant. In AT not fermented samples, Heptadecene, 3-Tetradecene, (E), and Butylated hydroxy toluene were the most abundant. Of note, these latter VOC was 1.96-folds higher than in ST samples. As well, Nicotinic acid, propyl ester, Borneol, and Phycocyanin were exclusive signature of AT samples.

3.3.2. Effect of Processing.

Either in AT or ST samples, fermentation caused a significant increase of VOCs related to alcohols, aldehydes, and organic acids, while a reduction in alkenes (P < 0.05). AT samples after fermentation had the highest load in organic acids, significantly higher than in ST samples. After baking, in FA-enriched GF bread the concentration of total VOCs significantly decreased in comparison to FA-enriched GF fermented doughs, but VOCs retained in bread were still significantly higher than in FA-enriched GF not fermented doughs (P < 0.05). FA-enriched GF breads in comparison to ST samples had higher abundance of ketones and organic acids, while less abundance of alkanes, amines, and esters (P < 0.05) (Figure 2).

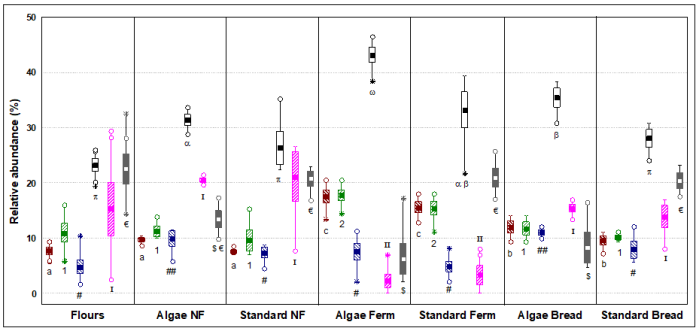

Figure 2. Relative quantification of total volatile organic compounds (VOCs) divided by chemical classes. Different letters indicate different significance values by Tukey's HSD (honestly significant difference) test (P < 0.05). Sample abbreviations: NF = not fermented; Ferm = fermented. Box = mean value; Rectangles = mean ± Standard Deviation (SD); Whiskers = min and max; circles = outliers; Asterisks = extremes. Red plots = Alcohols; Green plots = aldehydes; Blue plots = Ketones; Black and White plots = Organic acids; Fuchsia plots = Alkenes; Gray plot = others (amines, alkanes, and esters).

3.3.3. Quantifications of the Main Fermentation Metabolites.

Quantification of main fermentation metabolites (mg/Kg of fermented matrix) is reported in Table 3. Ethanol produced in FA-enriched GF yeast fermented dough (AY18) did not show a significantly different score from its control (SY18) (P > 0.05) (Table 3), and the same trend was observed when the fermentation process was performed by FA-enriched GF sourdough (YA6 and YS6) (P > 0.05). 1,4-Butanediol in FA-enriched doughs scored the greatly highest concentration when directly fermented with yeast for 18 h (AY18) while FA-enriched sourdoughs (YA+6) produced 1.24-folds more 1,4-Butanediol than the control (YS+6) (P > 0.05). In baked samples, the FA-enriched GF sourdough breads (YA+B) had 5.21-folds more 1,4-Butanediol than the control (YS+B) *(*P < 0.05). In comparison to the control, FA-enriched doughs directly fermented by LAB (AL18) produced 1.25-fold more 2-Butanone-3-hydroxy than the control (SL18) (P < 0.05), but in standard bread (SLB) this compound was retained 1.58-folds more (P > 0.05) than in FA-enriched bread (ALB). YS+6 produced 1.14-folds more Acetic acid than the FA-enriched dough (YA+6) (P > 0.05), and the same result was observed in baked samples. In fact, YS+B produced 1.69-folds more Acetic acid than YA+B (P < 0.05).

Table 3. Quantification (mg/Kg) of major fermentation compounds by SPME GC-MS with a close relative internal standard compound.

Sample	Ethyl alcohol	1,4-Butanediol	2-Butanone-3-hydroxy	Acetic acid

FR	n.d.	n.d.	n.d.	n.d.
FM	n.d.	n.d.	n.d.	n.d.
FA	n.d.	n.d.	n.d.	n.d.
AX	n.d.	n.d.	n.d.	0.20 ± 0.04^{a}
AL	n.d.	n.d.	n.d.	0.28 ± 0.09^{a}
AY	n.d.	n.d.	n.d.	n.d.
SX	tr.	n.d.	n.d.	n.d.
SL	0.23 ± 0.02^{a}	n.d.	n.d.	n.d.
SY	tr.	n.d.	n.d.	n.d.
YA+	5.55 ± 0.87^{c}	6.69 ± 1.16^{d}	0.51 ± 0.16^{a}	0.69 ± 0.42^{a}
YS+	4.03 ± 0.72^{c}	6.43 ± 1.32^{d}	0.37 ± 0.09^{a}	0.33 ± 0.11^{a}
AX18	14.69 ± 2.65	17.11 ± 2.12^{e}	8.44 ± 1.13^{bc}	2.95 ± 0.45^{bc}
AL18	22.57 ± 4.76^{e}	19.73 ± 0.97^{e}	11.09 ± 1.99^{d}	8.30 ± 0.99^{d}
AY18	26.45 ± 3.97^{e}	23.37 ± 3.01^{e}	13.88 ± 2.08^{d}	6.25 ± 1.56^{c}
YA+6	22.77 ± 2.78^{d}	26.56 ± 2.32^{e}	9.13 ± 1.09^{c}	7.46 ± 1.33^{cd}
YA6	16.53 ± 1.95^{d}	17.88 ± 1.45^{e}	8.02 ± 1.45^{bc}	5.99 ± 1.11^{c}
SX18	9.61 ± 2.69^{cd}	7.77 ± 0.34^{d}	7.11 ± 2.02^{bc}	3.46 ± 0.87^{bc}
SL18	19.12 ± 2.88^{d}	14.69 ± 1.49^{de}	8.84 ± 0.99^{c}	9.15 ± 1.57^{d}
SY18	28.02 ± 4.09^{e}	26.01 ± 1.17^{e}	9.08 ± 0.85^{c}	8.64 ± 1.69^{d}
YS+6	19.86 ± 3.78^{d}	28.75 ± 2.21^{e}	7.11 ± 2.02^{bc}	8.54 ± 2.41^{d}
YS6	15.77 ± 2.78^{d}	22.11 ± 2.03^{e}	6.12 ± 0.55^{b}	6.76 ± 0.55^{c}
ALB	0.71 ± 0.08^{b}	0.24 ± 0.02^{a}	6.78 ± 0.89^{b}	1.78 ± 0.65^{b}
AYB	0.54 ± 0.11^{ab}	0.34 ± 0.06^{a}	7.53 ± 1.96^{bc}	0.44 ± 0.09^{a}
SLB	0.83 ± 0.06^{b}	0.31 ± 0.01^{a}	4.45 ± 0.44^{b}	1.99 ± 0.78^{b}
SYB	0.45 ± 0.09^{ab}	1.02 ± 0.17^{b}	6.77 ± 0.99^{b}	tr.
YAB	0.55 ± 0.06^{ab}	1.76 ± 0.35^{b}	6.00 ± 0.78^{b}	0.30 ± 0.06^{a}
YSB	tr.	1.45 ± 0.78^{b}	5.45 ± 1.30^{b}	tr.
YA+B	0.69 ± 0.08^{ab}	0.47 ± 0.09^{a}	7.54 ± 1.76^{bc}	2.49 ± 0.21^{b}
YS+B	1.47 ± 0.34^{b}	2.45 ± 0.43^{c}	6.89 ± 1.45^{bc}	4.22 ± 1.04^{c}

Values are means of two replicates and two different batches (n = 4). ^{abc}Different letters among a column indicate significative differences by Tukey's HSD test (p < 0.05). n.d. = not detected (< 0.1 mg/Kg); tr. = traces (0.1 – 0.2 mg/Kg). For samples abbreviations see Table 1.

3.3.4. Multivariate Analysis of VOCs Organized by Different Chemical Classes

Organic Acids. From analysis of variance including all samples (n = 58), 16 organic acids resulted significantly different (P < 0.05) and on PCA their loadings on independent variables (Figure 3A) were clustered in four sets by K-means analysis (Figure S2A). AT samples were grouped in two clusters: in Cluster 1 collecting the fermented doughs and in Cluster 4 the breads. Cluster 1 was mainly described by six significant (P < 0.05) VOCs, as: Propanoic acid hydroxy, Nicotinic acid, Ethyl acetate, Propanoic acid, Nonanoic acid, and Thiophene acetic acid. By K-means clustering analysis (Figure S2A), these six VOCs accounted to be produced for averagely the 50% of total cases by the members of this cluster. In particular, Cluster 1 was addressed responsible of around 66% of Propanoic acid and 56% of Nonanoic acid productions. Cluster 4 consisted of FA-enriched GF breads, and was described by seven significant (P < 0.05) organic acids, namely: Dehydroacetic acid, Butanoic acid, Pentanoic acid, Hexanoic acid, Heptanoic acid, Octanoic acid, and n-Hexadecanoic acid. In particular, the contribution of FA-enriched GF breads on the production of these VOCs among all cases was pretty large, comprised from the 89% of Butanoic acid to the 66% of Octanoic acid. Lastly, the abundances of Heptanoic acid and Octanoic acid found in this cluster were the two top of the dataset of organic acid (Figure S2A). With MANOVA (P < 0.05) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on the production of these VOCs was evaluated. Considering the matrix (Figure 3b), Heptanoic acids was found to be a rich and exclusive signature of AT samples, as well as minor Nicotinic acid, while Propanoic acid hydroxy was almost 2-folds higher in AT samples. Considering the starters (Figure 3c), Butanoic and Hexanoic acids were mainly produced by LAB, while a higher proportion of Propanoic acid hydroxy was made by sourdoughs. This latter VOC was quadrupedally produced after 24 h of fermentation (Figure 3d), but was subjected to a baking loss by almost 8-folds (Figure 3e). After the baking stage, an increase in the production of n-Decanoic acid was observed (Figure 3e), which is a MCFA (medium-chain fatty acid) with bioactive characteristic.

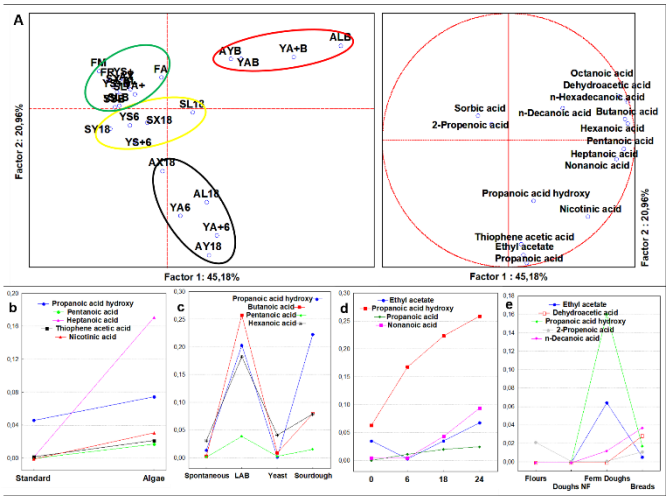

Figure 3. (**A**) Principle component analysis (PCA) of cases and variables on organic acids (ANOVA P < 0.05); (**b**) MANOVA categorized for the matrix (at least P < 0.05); (**c**) MANOVA categorized for the starters (at least P < 0.05), LAB = lactic acid bacteria; (**d**) MANOVA categorized for the time of fermentation (at least P < 0.05), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (**e**) MANOVA categorized for the process stages (at least P < 0.05); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Ketones. From analysis of variance including all samples (n = 58), 19 ketones resulted significantly different (P < 0.05) and on PCA their loadings on independent variables (Figure 4A) were clustered in three sets by K-means analysis (Figure S2B). AT samples were grouped in two clusters: in Cluster 2 the breads and the algae flour and in Cluster 3 the doughs. Cluster 2 was mainly described by six significant (P < 0.05) VOCs, as: 2-Pentanone, 2-Heptanone, 7-Octen-

2-one, 2,7-Octadienone, 3-Nonen-2-one and 2,(3H)-Furanone,5-ethyldihydro. By K-means clustering analysis (Figure S2B), these six VOCs accounted to be produced for averagely the 90% of total cases by the members of this cluster. In particular, Cluster 2 was addressed responsible of around 95% of 3-Nonen-2-one and 100% of 7-Octen-2-one productions. Cluster 3 consisted of FA-enriched GF doughs, and was described by three significant ($P < 0.05$) ketones, namely: Benzofuranone, Mint furanone and Nepetalactone. In particular, the contribution of FA-enriched GF doughs on the production of these VOCs among all cases was quite variable, ranging from 45% of Benzofuranone and Mintfuranone to 90% of Nepetalactone. With MANOVA ($P < 0.05$) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on the production of these VOCs was evaluated. The matrix (Figure 4b) confirmed what was highlighted by the K-means, Nepetalactone was associated to AT samples. Considering the starters (Figure 4c), Spontaneous fermentation produced 2-Undecanone 25-folder higher than LAB and sourdough, while Nepetalactone was produced mainly by yeasts after fermentation, however, decreased after baking (Figure 4e). In contrast to Nepetalactone, 2-Pentanone and 2-Decanone increased after baking.

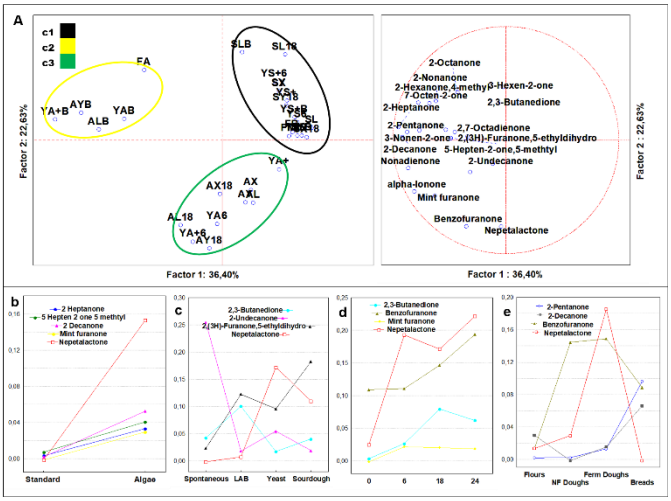

Figure 4. (**A**) Principle component analysis (PCA) of cases and variables on ketones (ANOVA $P < 0.05$); (**b**) MANOVA categorized for the matrix (at least $P < 0.05$); (**c**) MANOVA categorized for the starters (at least $P < 0.05$), LAB = lactic acid bacteria; (**d**) MANOVA categorized for the time of fermentation (at least $P < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (**e**) MANOVA categorized for the process stages (at least $P < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Aldehydes. From analysis of variance including all samples (n = 58), 20 aldehydes resulted significantly different ($P < 0.05$) and on PCA their loadings on independent variables (Figure 5A) were clustered in four sets by K-means analysis (Figure S2C). AT samples were grouped in two specific clusters: in Cluster 3 the FA-enriched GF breads and in Cluster 4 the not fermented doughs. On the other hand, FA-enriched GF fermented doughs were grouped in Cluster 1 together with ST doughs. Cluster 3 (FA-enriched GF breads) was positioned in quadrant II and was mainly described by five significant ($P < 0.05$) VOCs, as: Pentanal, 2-Hexenal (E), 2,4-Heptadienal (E,E), Dodecanal and 9-Octadecenal, for averagely the 80% of total cases. In particular, around 95% of Heptadienal (E,E) and 90% of Hexenal (E) productions (K-means clustering, Figure S2C). Cluster 4 consisted of FA-enriched GF not fermented doughs, and was described by three significant ($P < 0.05$) aldehydes, namely: 2-Butenal,2-methyl (E), Benzaldehyde and Hexanal, for the 85%, 70%, and 85% of total cases (K-means clustering, Figure S2A). With MANOVA ($P < 0.05$) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on the production of these VOCs was evaluated. Considering the matrix (Figure 5b), Furfural was found to be an exclusive signature of ST samples, as opposed to 2,4-Heptadienal (E,E), 2,6-Nonadienal (E,E), 2,4-Decadienal and Dodecanal, which were characteristic of AT samples. Considering the starters (Figure 5c), Furfural was mainly associated to LAB, while a higher proportion of 2,4-Decadienal and 2,6-Nonadienal (E,E) was made by Yeast. Sourdough produced an amount of 2-Hexenal (E) comparable to that produced by yeast. While the production of Hexanal, Nonanal and Benzaldehyde decreased from 0 to 6 h of fermentation, the production of Acetaldehyde showed an opposite trend, increased approximately 20-folds from after 6 h of fermentation (Figure 5d). Hexanal was an exclusive signature of NF doughs, while Furfural, Heptadienal, (E,E), and Butanal methyl were characteristic of baking stage (Figure 5e).

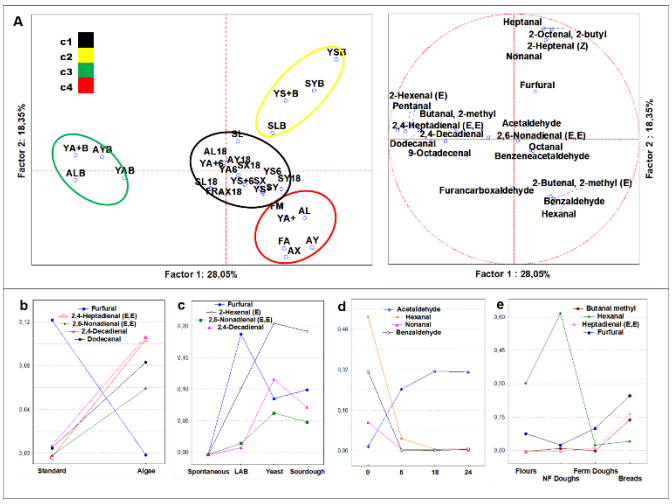

Figure 5. (**A**) Principle component analysis (PCA) of cases and variables on aldehydes (ANOVA $P < 0.05$); (**b**) MANOVA categorized for the matrix (at least $P < 0.05$); (**c**) MANOVA categorized for the starters (at least $P < 0.05$), LAB = lactic acid bacteria; (**d**) MANOVA categorized for the time of fermentation (at least $P < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (**e**) MANOVA categorized for the process stages (at least $P < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Alcohols. From analysis of variance including all samples (n = 58), 22 alcohols resulted significant ($P < 0.05$) and on PCA their loadings on independent variables (Figure 6A) were clustered in four sets by K-means analysis (Figure S2A). AT samples were grouped in three clusters: in Cluster 1 the fermented doughs, in Cluster 3 the not fermented doughs and in Cluster 4 the breads. Cluster 1 was mainly

described by five significant (P < 0.05) VOCs, as: 1-Pentanol, 3-Cyclohexen-1-ol, 3-methyl, 1-Heptanol, 2,4-Decadien-1-ol, (E,E) and Borneol. By K-means clustering analysis (Figure S2D), Cluster 1 (FA-enriched GF fermented doughs) was responsible for the 96% of 1-Pentanol and of 2,4-Decadien-1-ol, (E,E) productions as well as the 92% of 1-Heptanol and of Borneol productions. Cluster 3 (FA-enriched GF not fermented doughs) was characterized by lower speciation made by just 11 alcohols and by a typical signature made by Phenylethyl alcohol. Lastly, Cluster 4 (FA-enriched GF breads) was the exclusive producer of Mequinol and Thymol. With MANOVA (p < 0.05) on the matrix (Figure 6b), 2-Octen-1-ol, (E) was found to be an exclusive signature of AT samples, while Phenylethyl alcohol was almost 2-folds higher in ST samples. Considering the starter (Figure 6c), 2-Octen-1-ol, (E) and 2,4-Decadien-1-ol, (E,E) were mainly produced in the presence of yeasts and after 24 hours of fermentation, while 2-Furanmethanol and Phenylethyl decreased after 6 h of fermentation (Figure 6d), while 2-Decanol appeared to be a characteristic signature of baking stage (Figure 6e).

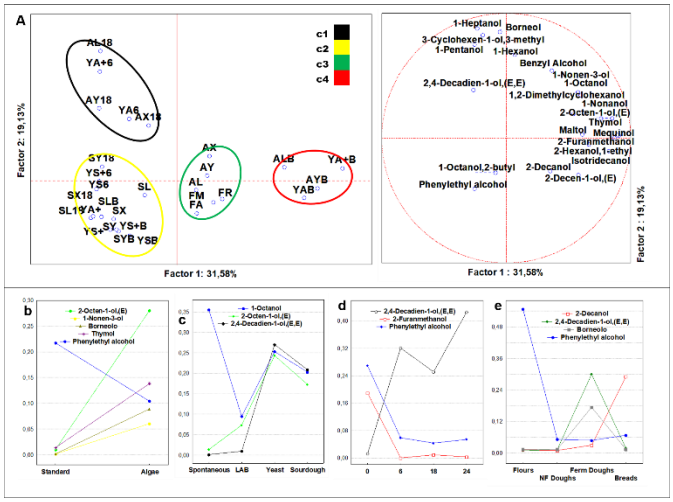

Figure 6. (**A**) Principle component analysis (PCA) of cases and variables on alcohols (ANOVA P < 0.05); (**b**) MANOVA categorized for the matrix (at least P < 0.05); (**c**) MANOVA categorized for the starters (at least P < 0.05), LAB = lactic acid bacteria; (**d**) MANOVA categorized for the time of fermentation (at least P < 0.05), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (**e**) MANOVA categorized for the process stages (at least P < 0.05); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Alkenes. From analysis of variance including all samples (n=58), 13 alkenes resulted significantly different (p < 0.05) and on PCA their loadings on independent variables (Figure 7A) were clustered in four sets by K-means analysis (Figure S2E). AT samples were grouped in two clusters: in Cluster 1 the breads and in Cluster 4 the fermented and not fermented doughs. Cluster 1, consisted of FA-enriched GF breads, was mainly described by five significant (P < 0.05) VOCs, as: Azulene, 1-Heptadecene, (Z), 5-Octadecene, (E), Naphthalene and Phycocyanin. By K-means clustering analysis (Figure S2E), these five VOCs accounted to be produced for averagely the 55% of total cases by the members of this cluster. In particular, Cluster 1 (FA-enriched GF breads) was addressed responsible of around 85% of Azulene and 92% of 5-Octadecene, (E) productions. Cluster 4 (FA-enriched GF doughs) was described by almost any aldehydes, and it was addressed by higher concentrations of 7-Hexadecene, (Z) and Phycocyanin, in particular of 90% and 95%, respectively (Figure S2E). With MANOVA (P < 0.05) on matrix (Figure 7b), Azulene, 1-Heptadecene, (Z), 5-Octadecene, (E) and Phycocyanin were found to be a particular characteristic of AT samples, while 3-Undecene,6-methyl, (E) was characteristic of ST samples. This latter VOC was mainly produced by yeast, while a higher proportion of Napthalene was made by sourdough. LAB, on the other hand, were the main producers of Thiophene, 2-decyl (Figure 7c). Considering the time of fermentation (Figure 7d), Phycocyanin was highly delivered after 24 h of fermentation but it was not retained after baking (Figure 7e). 4-Undecene, 3-methyl, (Z) was a compound characteristic of NF doughs, which decreased with fermentation and baking stages (Figure 7e).

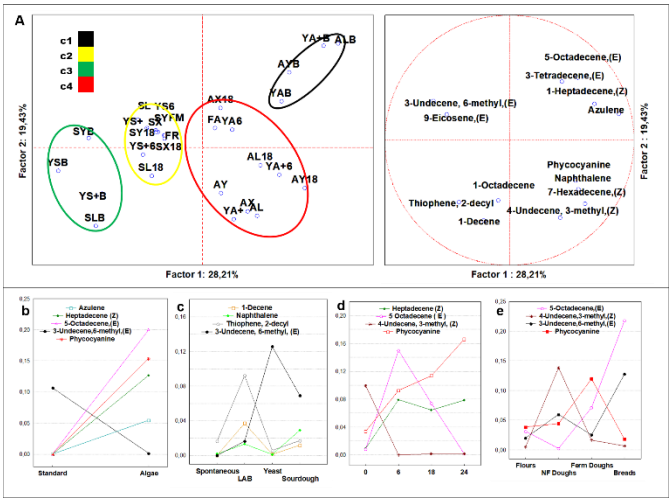

Figure 7. (**A**) Principle component analysis (PCA) of cases and variables on alkenes (ANOVA P < 0.05); (**b**) MANOVA categorized for the matrix (at least P < 0.05); (**c**) MANOVA categorized for the starters (at least P < 0.05), LAB = lactic acid bacteria; (**d**) MANOVA categorized for the time of fermentation (at least P < 0.05), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (**e**) MANOVA categorized for the process stages (at least P < 0.05); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

3.4. Sensory Evaluation

The breads were evaluated after 3 h from baking by 20 semi-trained testers (consumers) (Figure S3). Generally, the standard breads were more appreciated than the FA-enriched GF, and this difference was more evident on features such as “color” or “smell” (P < 0.05), rather than on “taste” or “crispiness” (P > 0.05). The FA-enriched GF breads showed no significant differences among the starters (P > 0.05).

4. Discussion

In all algae types (AT) fermented samples, microbial growth was similar to their standards (ST). This result was confirmed also by the acidification observed in this study, that was similar for AT and ST

samples. Acidification is a fundamental parameter for evaluating the effectiveness of fermentation. Dough acidifications seemed slightly but significantly different that may be done by the higher buffering activity of standard doughs partially replaced with FA in AT samples. Similar trends of bacterial growth and pH value were obtained by other authors in a non-GF bakery formulation obtained after sourdough fermentation and containing the 6% of spirulina. The authors reached pH 4.89 after 2 h of fermentation with a single LAB specie and a yeast[16]. Instead, from our result, a lower pH value (4.31) was obtained after 6 h of fermentation with two LAB species and a yeast. Moreover, FA addition to the dough directly fermented with LAB (AL18), with respect to the standard (SL18), provided a higher production of Ethanol, the most important descriptor for an efficient leavening process. Although the content of 2-Butanone-3-hydroxy, which is essential for the structure and a pleasant aroma of the baked product, decreased in the ALB bread due to the baking loss, it was still higher than that of standard breads.

4.1. Prebiotic potential

The prebiotic activity on FA-enriched GF dough and bread was much more effective than the respective standard samples. This result is consistent with previous studies, according to which Spirulina biomass supported the growth of *Lb. casei, Lb. acidophilus*, *Streptococcus thermophilus* and other beneficial bacteria, such as *Bifidobacterium* spp., while inhibiting the growth of harmful bacteria, such as *Proteus vulgaris*, *Bacillus subtilis* and *B. pumulis*[24-26] .This can be attributed to the high content of polysaccharides (PS) or their derivatives, namely oligosaccharides or low-molecular-weight (LMW)-PS which also includes the so-called dietary fibers. In fact, several studies indicate that some of these compounds, also called non-digestible oligosaccharides (NDOs), offer a significant advantage to the host health by stimulating the growth of beneficial bacteria and modulating the composition of the colon microbiota, thus satisfying the criterion of prebiotics[27]. For example, alginate oligosaccharide (AlgO) is enzymatically hydrolyzed from alginate and possesses prebiotic properties, which have been shown to stimulate the growth of bifidobacteria, both in *in vitro* and *in vivo*[28.] Agarose oligosaccharides (AO) from agarose enzymatic hydrolysis also exhibit prebiotic effects, stimulating the growth of bifidobacteria and lactobacilli and escaping digestion in the upper gastrointestinal tract[28]. Part of the strong prebiotic activity score of the FA-enriched GF samples may be due to the higher level of propionate and butyrate in the AT samples, particularly on the FA-enriched GF breads for butyrate and the FA-enriched GF doughs for propionate (Figure S2A). Indeed, these compounds are known to promote the selective microbial growth of probiotics and beneficial microbes in the intestine[29], stimulate epithelial immune function[30], and modulate the inflammatory response to pathogens[31]. The present work is the first describing the prebiotic potential of a bakery product containing spirulina for human consumption. A recent research addressed the impact of spirulina supplementation on broiler chickens, concluding that the prebiotic effect was relative to enhancement of epithelial morphology in the small intestine[32].

4.2. Multivariate Analysis of VOCs Sorted by Chemical Class

4.2.1. Organic Acids

In our study, the organic acids profile of the AT samples was superior to the standard ones. Principally, a higher speciation and greater abundance of organic acids was found after fermentation of AT samples (*e.g.* Lactic acid and Propanoic acid) and was kept up to the final product (*e.g.* Butanoic acid and MCFAs). According to the previous study[23,33], the increased concentration of short-chain or medium-chain organic acids depended mainly on the fermentation process. Propanoic and Lactic acid are flavoring compounds, determining typical sharp, acid, vinegar taste, with a buttery nuance given by Lactic acid[34], but are also involved in the quality and safety of fermented foods due to their antimicrobial activity in baked goods, inhibiting ubiquitous bacilli, deteriorating microbes and food-borne pathogens[35]. Butanoic acid production in our study was mainly derived by LAB fermentation, as reported by several authors[36-38]. This compound, along with Propanoic acid, fits the new definition of prebiotics[39], according to which a prebiotic is a compound that selectively stimulates the growth and/or activity of gut bacteria associated with health and to well-being, thus excluding opportunists or pathogens. In particular, Butanoic acid is known to form the main energy source for intestinal epithelial cells and affects a wide range of cellular functions that affect colon health[40], while Propanoic acid is known to promote the growth of probiotic commensals of bifidobacteria[41]. Hexanoic acid is a volatile compound resulting from the fermentation carried out by lactobacilli or yeast and responsible for the inhibition of molds in bread[34]. However, our results show an increase in Hexanoic acid following the baking phase and this may have been caused by the splitting of hydroperoxides[42] generated by lipoxygenases during the fermentation phase. Hexanoic acid and Nonanoic acid are medium-chain fatty acids (MCFA) known for their effectiveness in the excessive consumption of calories, inducing weight loss[43]. MCFAs, in fact, are considered health-related compounds as they protect against insulin resistance during calorie excess[44]. However, Hexanoic acid and Nonanoic acid have a cheese, waxy, fatty, goat scent that is sensorially unpleasant and the modulation of their content should be expected for commercial development. We have to consider that the high amount detected in the breads could have impacted the low score of the sensory test (Figure S3).

4.2.2. Ketones

AT samples were described by a larger speciation and abundance than ST samples. In particular, within the AT samples, the greatest abundance was found in FA-enriched GF breads, probably indicating the incidence of baking step in ketones production. In FA-enriched GF breads were founded ketones with a pleasure aroma, such as 2-Pentanone and 2-Heptanone. 2-Pentanone is described as sweet,

fruity aroma and is found both in sourdough and yeast bread[34]. On the other hand, 2-Heptanone is found in wheat or rye sourdough and confers a typical aroma described as fruity, spicy, sweet, coconut[45]. Contrariwise, these two aromatic VOCs were found only to a much lesser extent in ST samples. FA-enriched GF doughs were almost exclusively responsible for the Nepetalactone production which, however, was lost, even if not totally, during the baking step. Nepetalactone has been found in the essential oils of several *Nepeta* species (*Lamiaceae/Labiatae*), which bacteriostatic, fungistatic and antiviral activities have been attributed to nepetalactones[46]. In a recent paper describing the enrichment of pasta with spirulina, 2-Heptanone was found to be typical compound of the volatilome of pasta with 2.5% of spirulina[47].

4.2.3. Aldehydes

For the dataset of aldehydes distinctions based on the process have occurred, but not based on the matrix. FA-enriched GF breads was described by Pentanal, 2-Hexenal, (E), 2,4-Heptadienal, (E,E). This latter, a derivative of sorbic acid, has pleasant, green, floral flavor[34] and is found in camelina oil, raw adzuki beans[48], and perilla seeds oil[49] with an antifungal efficacy[50]. Fermented doughs, both AT and ST samples, were almost exclusively characterized by Acetaldehyde production. Our results showed that Acetaldehyde was present in small quantity in not fermented doughs and that it increased during the fermentation. Indeed, this compound is derived, besides by Maillard reaction, by lipid oxidation and yeast fermentation and was described to have a pungent, ethereal, fruity, floreal, green, roasted, malty odor[34]. It was found in fermented sourdough and in breads directly fermented with yeast[34]. Lastly, Furfural, that has a leading role in the aroma of bakery products[34], was absent in the AT samples but was present only in the ST samples. This result represents an additional value because Furfural was recently investigated as a potential carcinogen[51,52].

4.2.4. Alcohols

The profile of alcohols of AT samples was characterized by a higher speciation and a greater abundance compared to ST samples, especially for bread. The specificity of AT samples was related to process effects. In particular, the main descriptors of fermented doughs were 1-Pentanol, 1-Heptanol, 2,4-Decadien-1-ol, (E,E), and Borneol. 1-Heptanol was reported to be present in rice, soybean, rye and wheat flours or products, and is used as a flavoring agent conferring a typical olfactory issue described as musty, pungent, leafy, green[53]. 1-Heptanol instead is still associated to sourdough, but from our results was not retained in breads[34]. Borneol is considered a bioactive molecule and is reported to modulate beneficially the gut microbiome[54-56] and to possess anti-inflammatory and antioxidant activity[57]. The exclusive presence in AT samples can be considered an added value. However, only a small amount was retained after the cooking phase (about 10% retained). Thymol, which from our results proved to be an exclusive feature of FA-enriched GF doughs, is a monoterpene phenol that has the same bioactive characteristics as borneol. The presence of this alcohol in the finished product can have a positive effect on conservation thanks to its ability to inhibit spoilage microbes[58]. In particular, this volatile compound has strong antifungal activity including *Aspergillus* spp. and *Penicillium* spp.[59]. A similar profile of alcohols, in particular the presence of 1-Pentanol and 1-Heptanol, were previously described in pasta enriched with 2.5% of spirulina[47].

4.2.5. Alkenes

In our study, the alkenes profile of the AT samples was superior to the standard ones. In particular, AT samples appeared to be the main responsible for the production of Phycocyanin, which appear to decrease upon baking, as well as Naphthalene. Conversely, FA-enriched GF breads were responsible for the increased delivery of Azulene and 5-Octadecene, (E). Phycocyanin, a blue photosynthetic pigment widely used in foods and cosmetics[60], occurs naturally in the cyanobacterium *A. platensis*[61] and *Geitlerinema* spp.[62], the eukaryotic algae *Rhodophytes* and *Cryptophytes*[63]. This photosynthetic pigment has a peptide nature and has a low thermal stability[64,65], in fact our results show a high quantity of it associated with AT doughs, while only a small part is associated to AT breads. Phycocyanin is often used as nutritional supplement and has great potential benefits for human nutrition and health, as it contains all the essential amino acids[66]. Phycocyanin has significant anti-oxidative, anti-inflammatory, hepatoprotective, and radical scavenging properties[67]. Azulene is a blue organic chromophore, found in nature, having two aromatic rings. It is an isomer of naphthalene and has a similar odor, but the color of the crystal is dark blue[68]. In a recent work the enrichment of a low-fat yogurt with spirulina generates a final product with higher content of similar VOCs, *e.g.* phycocyanin[69].

5. Conclusions

In this work, FA was used to formulate a GF bread enriched in proteins. The bread was quantitatively and qualitatively analyzed throughout the process to evaluate fermentation performances and volatilome composition. The metabolomic profiles of FA-enriched GF breads were considered to investigate the potential of *A. platensis* as a vehicle for the addition of flavoring and bioactive compounds in bakery products. Multivariate analysis on VOCs provided a deeper description of the effects of *A. platensis* addition and sourdough fermentation process on flavoring and bioactive compounds, mainly evidencing an increased concentration of antimicrobial compounds, a larger spectrum of bioactive VOCs, and a typical flavoring profile. The addition of FA and the use of different fermentation types gave rise to specific VOCs profile predicting the organoleptic characteristics of bread. AT breads were characterized by green floral nuances derived from the aldehyde content, as well by musty and pungent traits ascribed by that of alcohols, or by sweet and fruity recall given by the ketones profile. In contrast, the higher presence of hexanoic and nonanoic acids could have contributed to the

unpleasant sensorial evaluation scored in this study. Besides, the blue pigment Phycocyanin was maintained after baking that was responsible for low scores recorded for the attribute "color". Marketing strategies or slight formulation changes by camouflaging ingredients may offer many solutions to easily bypass that limit at industrial scale. Considering the bioactivity of the compounds found in AT samples, the presence of Thymol and Borneol, as well as that of Phycocyanin or SCFAs and MCFAs, as well as Nicotinic acid, represents an important nutritional and functional added value. Even if the content of some of these compounds should be controlled during the process, as some of them are not retained in baked breads, *e.g*. Phycocyanin and Nicotinic acid. The enrichment with *A. platensis* could be indicated even as a solution to reduce harmful Furfural in the final product.

It is important to mention that due to high baking loss the estimation of the health potential of bioactive compounds delivered in the experimental breads towards humans is difficult. Moreover, further studies coupling volatilome analysis to sensorial assessment are needed to understand how the food processing may influence consumers' acceptance. Finally, the evaluation of the shift of VOCs could represent a comprehensive, sensitive, and reliable method guiding the formulation of innovative food with enhanced nutritional value.

Author Contributions

Conceptualization: A.G., L.N.; methodology: A.G., L.N.; software: F.C. and L.N.; validation: A.G., L.N; investigation: A.G., L.N, and F.C.; resources: A.G.; data curation: A.G., L.N, and F.C.; writing—original draft preparation: F.C. and L.N.; writing—review and editing: A.G., L.N, and F.C.; supervision: A.G. and L.N.; funding acquisition: A.G.

Conflicts of interest

The authors declare no conflict of interest.

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