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Development of Gluten-Free Functional Bread Adapted to the Nutritional Requirements of Celiac Patients

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Abstract: Celiac patients suffer from nutritional deficiencies before and during the maintenance of a gluten-free diet; this is due to non-fortified, mostly processed foods that are high in saturated fat and deficient in minerals typically present in wheat. A literature search was carried out to determine the deficiencies of these patients in calcium, iron, fiber, folic acid, omega-3, vitamin B12 and vitamin D. Different formulations of gluten-free bread enriched with olive extract (hydroxytyrosol of natural (HXT_O) and synthetic (HXT_S) origin), acerola extract, citrus extract, spinach extract, calcium, iron and linseed were used. Antioxidant capacity, nutritional composition, folates, minerals, color and pH were studied, and a microbiological study and sensory analysis were conducted to assess organoleptic quality. These studies were carried out on days 0, 4, 7 and 11 to study their evolution. The results of the HXT_S bread showed a higher antioxidant capacity, higher antimicrobial capacity and higher fiber content, as well as higher amounts of minerals. It also showed higher consumer acceptability, even relative to commercial gluten-free bread. The HXT_O bread showed higher antimicrobial capacity than the control (C), higher fiber content and higher mineral content, but had lower antimicrobial capacity than HTX_S bread. It also had better sensory acceptability than C but was worse than HXT_S bread. Taking into account the physicochemical and organoleptic characteristics, the HXT_S sample is the most suitable for enriching the diet of celiac patients.

Keywords: celiac disease; deficiencies; gluten-free bread; hydroxytyrosol; functional food

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1. Introduction

Celiac disease is an autoimmune disease in which patients' antibodies react against an enzyme after consuming gluten as part of the diet. This reaction mainly causes intestinal malabsorption problems that affect other areas of patients' health due to nutritional deficits. It is a disease highly influenced by both genetic and environmental factors, in addition to a wide range of clinical presentations. Its prevalence is approximately 1%, but more cases of celiac disease are diagnosed each year [1,2]. It increases morbidity and mortality in the people affected, but a strict gluten-free diet improves this, in addition to reducing the risk of complications such as gastrointestinal cancer [3].

Gluten is found mainly in wheat, rye and barley. Wheat is the largest cultivated cereal and the basis of many diets, including the Mediterranean diet. The average consumption rate of gluten is 5 to 20 g per day, and it is present in many of the fundamental foods of the diet, such as bread and pasta [4]. The current single treatment for this disease is following a gluten-free diet throughout life. According to a review by Gobbetti et al. (2018) [5], gluten-free products show a higher glycemic index, lower protein content and a higher percentage of fats, especially saturated, and a gluten-free diet results in a decreased intake of folic acid, vitamin B12, vitamin D, calcium, iron and other minerals. In addition, the gluten-free diet contains many processed products in order to replace gluten-based foods. All of these lead to an increased risk of metabolic syndrome and overweight and obesity.

For these reasons, there is a great need to develop products for people with gluten-related disorders. The protein content of wheat in different varieties ranges from 11–12% [6], and the complete elimination of wheat from the diet would mean the exclusion of a very good source of protein. Therefore, the protein content of wheat should be considered. So, the protein content of alternative sources, such as cereals (sorghum, rice, maize, teff, millet, Montina™, etc.), pseudo-cereals (buckwheat, quinoa, amaranth, etc.), legumes (carob germ, soybean meal, chickpea, lentils, peas, etc.), flour, seeds (chia, blackcurrant, strawberry, etc.), etc. [7–9], should be considered.

In this way, natural extracts rich in minerals and vitamins, such as spinach and citrus fruits, could be used to make bread since it is a main food in the Mediterranean diet, thus covering the nutritional recommendations and improving health. In addition to being useful for the celiac population, it could be useful for the rest of the population or for another type of population, such as the pregnant population, since it would help supplement the maternal diet by providing folic acid, which is currently causing concern in Europe due to the high number of cases of neural tube defects in newborn children [10]. This is a defect that is prevented by periconceptual folic acid supplementation, but since 40% of pregnancies are unplanned, it is likely that women are not taking supplemental folic acid [11]. Therefore, it would be a good alternative for pregnant women with celiac disease to consume foods fortified with folic acid. In fact, in some countries, for years, flours have been fortified with folic acid, producing good results [12–14]. Therefore, in this study, the main objective of the work was to design and make functional bread enriched in specific minerals and nutrients while taking into account the nutritional deficiencies of celiac patients.

2. Materials and Methods

2.1. Extracts

The green leafy vegetables used to prepare freeze-dried spinach extract were purchased fresh from a local supermarket (Hipercor, S. A.). The sample was chopped, eliminating the fibrous part, and crushed together with 150 mL of distilled water using a Thermomix® until a homogeneous mixture was obtained. The sample was frozen for 24 h at −18 °C and introduced into a Telstar freeze dryer (Cryodos-80 model), where it underwent a freeze-drying process for 4 days at a temperature of −70 °C and a pressure of 0.3 Pa. Acerola extract was purchased from the company Ferrer Alimentación, S.A. (Barcelona, Spain). The citrus extracts (CBCs) were obtained from the non-edible parts of a citric fruit (*Citrus sinensis* L.), with 55.11% carnosol and organic hydroxytyrosol (HTXo), obtained from vegetation waters from olive trees (*Olea europaea)* containing 7.26% pure bioactive compound, was supplied by the company Nutrafur-Frutarom, S. A. (Alcantarilla, Murcia, Spain). Synthetic hydroxytyrosol (HXTs) was obtained via acid hydrolysis of oleuropein, containing 99.2% HTX and 0.3% HTX acetate, and was supplied by Seprox Biotech, S.L. (Fuente Álamo, Murcia, Spain).

2.2. Preparation of the Bread

For the preparation of the enriched samples, a gluten-free bread flour mix was used. Mix B of the Schär brand was used, whose ingredients are corn starch, rice flour, vegetable fibers (psyllium, bamboo), brown rice flour 3.8%, lentil flour 3.6%, dextrose, hydroxypropyl methylcellulose (thickener) and salt. The flour was bought in a local supermarket (Hipercor S.A., Murcia). All samples were made and mixed in a Silvercrest "IAN 285058" automatic bakery with the "gluten free" program. The breads were made with 500 g of flour, with the final weight being approximately 800 g. The program for this size lasts 2 h and 14 min and includes 12 min of kneading, 10 min of rest, 16 min of kneading in three stages, 66 min of rest and 60 min of baking. Three different breads were developed. The control sample (C) was made following the recipe indicated on the package: 500 g of mix, 10 g of dry yeast, 400 mL of warm water, 20 g of oil and 5 g of salt. To the other two loaves, we added 200 ppm of hydroxytyrosol, one with synthetic (HXTs) and one with organic (HXTo), citrus

extract (CBC), 5 g of lyophilized spinach per 100 g of flour based on [15], 160 mg of acerola extract, 220 mg of calcium carbonate ($CaCO_3$) per 100 g of flour [16] and 40 g of ground flax (Table 1).

Table 1. Formulations of the samples.

Sample	MixB Schär (g)	Water (mL)	Oil (g)	Dry Yeast (g)	Salt (g)	CBC (mg)	Acerola (mg)	$CaCO_3$ (g)	Freeze-Dried Spinach (g)	Ground Flax (g)	HXT Org. (mg)	HXT Sint. (mg)
C	500	400	20	10	5	-	-	-	-	-	-	-
HXT_O	500	500	20	10	5	160	160	1.1	5	40	160	-
HXT_S	500	500	20	10	5	160	160	1.1	5	40	-	160

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; CBC: citrus extract; HXT: hydroxytyrosol.

For the experiments, samples C, HXT_O, HXT_S and a commercial bread were used, the ingredients of which included water, corn starch, rice flour, vegetable fibers (psyllium, citrus), hydroxypropyl methylcellulose (thickener), sunflower oil, soya protein, yeast, salt, sugar, citric acid (acidifier) and lactose-free (lactose < 0.007 g/100 g), in order to compare the functional breads with a commercial supermarket bread.

For the first sample (HXT_{OES}), 5 g of spinach was added per 100 g of flour based on the article by [15]. The spinach flavor was very intense, which made it intolerable to taste and completely different from the normal taste of bread. From this sample, it was decided to reduce the amount of lyophilized spinach to 1 g/100 g flour. The remaining samples contain the quoted quantity.

In the next two, the addition of flaxseed and iron sulfate ($FeSO_4$) served as an experiment to create an iron-enriched product [17]. It started with a concentration of 8 g of ground flax and 220 mg of $FeSO_4$ per 100 g of flour and the same concentration of CBC, acerola, HXT and $CaCO_3$ as in HXT_{OES} (HXT_{OFE1}), and after a bibliographic search, the measure was adjusted, and 3.2 mg of FeSO4 was added per 100 g flour with the same amount of flax and natural extracts (HXT_{OFE2}) [18,19]. In both cases, the taste of iron was intense, making it unacceptable organoleptically, so it was concluded that it would not be enriched with iron sulfate.

All 4 samples under study were run. Therefore, they were all subjected to the same production and storage conditions in order to reduce these external factors. The three samples and the commercial one were stored for 11 days in the refrigerator at 4 °C covered by a film; the representation of these conditions and ingredients of the different breads can be seen in Figure 1. Tests were carried out during this period (on days 0, 4, 7 and 11) to check the variations undergone by the time.

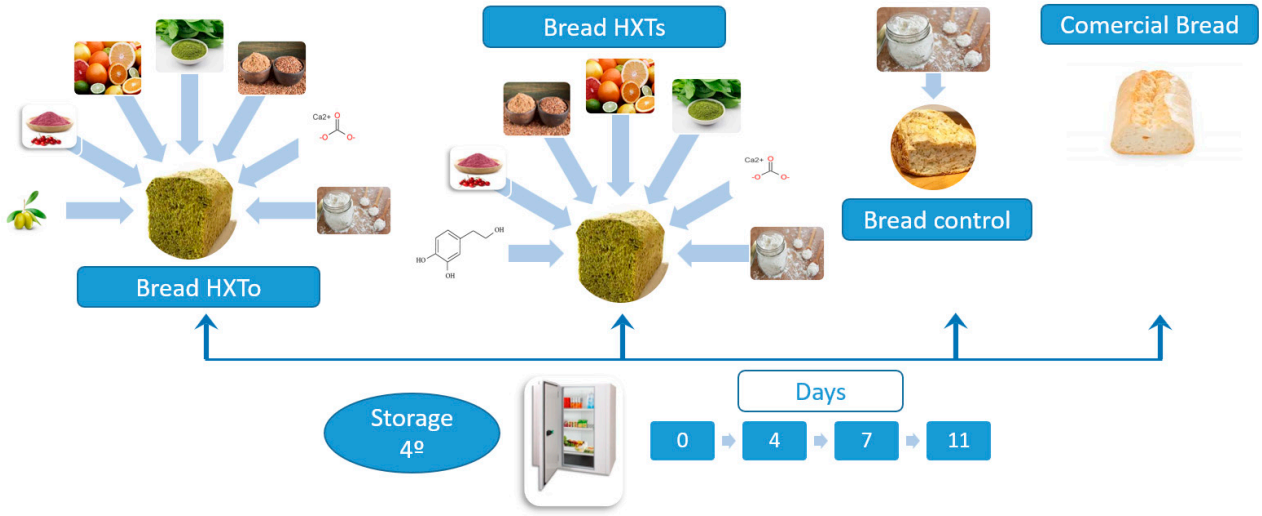


Figure 1. Summary representation of the different breads in the study and storage conditions during the different days.

2.3. Physical-Chemical Analysis

2.3.1. Macronutrient Determination

Moisture Determination

The sample drying method (AOAC, 1995) was used to determine the humidity. About 3 g of sample were weighed and allowed to dry at 110 °C in a forced air oven for 24 h.

Determination of Inorganic Matter

The technique and procedure of the AOAC were used [20], based on the complete incineration of the organic matter of the sample in a muffle furnace at 525 °C, leaving only the residue of inorganic matter. The empty porcelain crucible and 1 g of fresh sample are weighed and placed on a heating plate that reaches 525 °C. Leave approximately 24 h.

Determination of Total Nitrogen and Protein

The Kjeldahl procedure was followed for the determination [20]. The basis of the method is the destruction of organic matter with sulfuric acid that results in ammonium sulfate; adding an excess of sodium hydroxide releases ammonia that is distilled over boric acid, forming ammonium borate. After this, it is titrated with 0.1 N hydrochloric acid. About 2 g of fresh sample were weighed, and 7 g of catalytic mixture and 15 mL of concentrated sulfuric acid were added. The tubes were placed in a heating block reaching 450 °C and maintained for 1 h at that temperature. Once the time had elapsed, they were allowed to cool, and the distillation was carried out with the addition of 32% NaOH until it underwent a color change (light blue to brown) and was distilled for 4 min. It was collected in a flask with 25 mL of 4% boric acid and a protein indicator. The distillation product was titrated until the indicator was turned with 0.1 N hydrochloric acid.

Determination of Total Fat

For the determination of total fat, a Soxhlet-type Tecator extractor was used [20]. The dried empty aluminum cups and about 1 g of dried sample are weighed in a cellulose cartridge. After introducing the samples into the extractor, 50 mL of ether is added to each sample and the extraction process that lasts about 3 h with different steps is started. The ether heated to 80 °C extracts the fat from the sample, which fell on the aluminum vessel that was previously weighed. At the end of the process, they are put in the stove to remove the excess ether and the glass is reweighed with the residue of the fat removed.

Determination of Dietary Fiber

For the determination of the fiber, procedure 985.29 of the AOAC, described by Prosky et al. (1985) [21], was used. Then, 1 g of the sample was weighed, and 50 mL of phosphate buffer 0.05 M pH 6 was added. After this, the sample was taken to a bath at 95 °C, and the α-amylase was added. After 30 min, adjust pH 4.5, and add amyloglucosidase at 60 °C for 30 min. After finishing the digestion, add ethanol to precipitate the fiber; once precipitated, filter it. At the end of the filtration, the residue is left in the oven, and once dried, the proteins and ashes of this residue are determined.

Determination of Carbohydrates

The determination of carbohydrates is carried out by difference according to the recommendations of the FAO and WHO [22] (1982); based on the results, the determinations of fat (F), ash (A), protein (P), moisture (M) and dietary fiber (DF) determinations are obtained so that

$$\text{Carbohydrates (\%)} = 100 - (F + A + P + M + FD).$$

Determination of the Energy Value

The energy value of the samples is obtained by summing the energy value of the protein, carbohydrate and fat of each sample using the energy conversion factors of Atwater [23].

Determination of Folate

Folate standards: Trihydrochloride tetrahydrofolic acid (H4), 5-Methyltetrahydrofolic Acid,5-formyltetrahydrofolic acid. Folic acids were obtained from Dr. Schirck's Laboratory (Jona, Switzerland).

Extraction and deconjugation of folates from samples: Folates were extracted from the sample following the procedure described by Konings et al. (1999) and Pfeiffer et al. (1997) [24,25]. One gram of sample was mixed with 25 mL of extraction buffer (50 mmol/L, 50 mmol/L HEPES, containing 2 g sodium ascorbate/100 mL and 10 mmol/L 2-mercaptoethanol, pH 7.85) under a nitrogen atmosphere. The extraction mixtures, in screw-capped tubes, were placed in a boiling water bath for 10 min, cooled on ice and homogenized. Then, the pH was adjusted to 4.9 with 60 mmol/L HCl. Enzymatic deconjugation and purification of samples were carried out following the methodology described by Vahteristo et al. (1996) [26]. An aliquot of 5 mL was incubated for 3 h at 37 °C under a nitrogen atmosphere with 1 mL of hog kidney conjugase prepared from fresh pig kidneys, as described by Gregory, Sartain and Day (1984) [27], and 1 mL of α-amylase preparation (20 mg/mL in 1 g Na ascorbate/100 mL). To inactivate the enzymes, the samples were boiled at 100 °C for 5 min and then cooled on ice. The samples were filtered through 0.45 μm pore size and purified and strong anion-exchange (SAX) cartridges connected to a Supelco 12-port vacuum manifold. First, the cartridges were conditioned with 3 mL of n-hexane, methanol and Mili-Q water and then equilibrated with 3 mL of purification buffer (10 mmol/L dipotassium hydrogen phosphate, 2-mercaptoethanol (*v*/*v*), pH 7.0). Second, the sample was slowly loaded and eluted with 2 mL of elution buffer (10 g sodium chloride/100 mL, 10 mmol/L sodium acetate, 1 g ascorbic acid/100 mL) at a flow rate of <0.5 mL/min. The eluted sample was weighed.

HPLC analysis of folates: The separation and analysis of samples were performed with an HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Automated Multisampler module and a High-Speed Binary Pump and connected to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Experimental parameters for HPLC and Q-TOF were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). Standards or samples (20 μL) were thermostatted at 4 °C and injected onto a Zorbax Eclipse Plus C18 (2.1 × 100 mm, 1.8 μm) HPLC column at a flow rate of 0.4 mL/min. Column was equilibrated at 30 °C. Solvents A (MilliQ water with 0.1% formic acid) and B (acetonitrile) were used for the compound separation.

The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 40 psi, whereas the drying gas flow was set to 16 L/min at a temperature of 150 °C, and the sheath gas flow was set to 12 L/min at a temperature of 300 °C. The capillary spray, nozzle, fragmentor and octopole 1 RF Vpp voltages were 4000 V, 1000 V, 350 V and 750 V, respectively. Profile data in the 100–600 *m/z* range were acquired for MS scans in 2 GHz extended dynamic range mode with 3 spectra/s, 333.3 ms/spectrum and 1999 transients/spectrum. Reference mass at 121.0509 was used for mass correction during the analysis. Data analysis was performed with MassHunter Qualitative Analysis Navigator software (Agilent Technologies, Rev. B.08.00).

2.3.2. Mineral Determination

Prior to mineral analysis, digestion was performed using a microwave laboratory station (Ethos D) type Ethos plus 1 purchased from Milestone Inc. (USA). For each sample, 5 g of dry matter and 10 mL of soluble and dialyzable fractions resulting from digestion were weighted in a Teflon digestion vessel with 7 mL of concentrated (65%) nitric acid (HNO_3) and 1 mL of 30% hydrogen peroxide (H_2O_2). Then, the sample was subjected to a microwave program as follows: step 1, 25–200 °C for 10 min at 1000 W; step 2, 200 °C for 10 min at 1000 W. Digests were finally made up with deionized water to 25 mL in acid-washed standard flasks. The following elements were measured using inductively

coupled plasma mass spectrometry (ICP-MS) (Thermo electron X7 inductively coupled plasma mass spectrometry, model X series, UK): sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), phosphorus (P), iron (Fe), zinc (Zn), selenium (Se), and copper (Cu). ICP-MS operating conditions were the following: nebulizer gas flow, 0.91 L min^{-1}; radio frequency (RF), 1200 W; lens voltage, 1.6 V; cool gas, 13.0 L min^{-1}; auxiliary gas, 0.70 L min^{-1}.

2.4. Shelf-Life Study

pH was measured at days 0, 4, 7 and 11 in triplicate and was determined with the help of a pH meter (Crison GLP21) after mixing 5 g of fresh sample with the same amount of distilled water at room temperature [20].

Color was measured using a Konica Minolta CR-410 chromameter (Minolta Camera Co., Osaka, Japan), and the DP-400 data processor of "AQ instruments" was used to measure crust color (CIE Lab* values). CIE L* values (lightness), CIE C* values (saturation), a* values (red-green) and b* values (yellow-blue) were measured. The color coordinates were analyzed at 0, 4, 7 and 11 in triplicate.

2.4.1. Determination of Antioxidant Capacity

Antioxidants are compounds that inhibit the formation or spread of free radicals. They achieve this by donating a hydrogen atom or by transferring electrons [28,29]. In this study, four methods were used for the measurement of the antioxidant activity of breads, namely, oxygen radical scavenging capacity, DPPH radial scavenging activity, ABTS radial scavenging activity and antioxidant capacity to reduce ferric ions [30].

Absobality of Oxygen Radicals (ORAC)

AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) is used, which, upon decomposition, produces peroxyl radicals used and oxidized to fluorescein protein that reduces its emission light at 528 nm. If the radicals are reduced, the light emission will remain high, and, therefore, the antioxidant power is high [29,31]. A straight Trolox standard (vitamin E analogue, antioxidant) is used, which is used as a reference [32].

It was performed following the protocol previously described by Ehlenfeldt and Prior (2001) [33]. A 96-well black plate was used, the edges of which were filled with 200 µL of distilled water; the blanks consisted of 20 µL of phosphate buffer (used for dilution of samples) and 20 µL of samples and Trolox standards. After the preparation of the plate, the process is started in the Synergy HT plate reader after a purge with water and the corresponding reagents. The GEN 5 program was used in which the protocol was stipulated (200 µL of fluorescein in each well; after 15 min, 20 µL of AAPH (0.216 g in 10 mL of phosphate buffer) was added in each well and then measured with an excitation of 485 nm and an emission of 528 nm every minute for an hour and a half). The whole process is carried out at 37 °C. With the support software, the area under the curve was obtained for all measures, and the data were extrapolated by Trolox standard curves (having known concentration).

Iron Reducing Antioxidant Power (FRAP)

This technique is based on the reduction of the complex of TPTZ (2,4,6-tripyridyl-s-triazine) and ferric iron (Fe^{+3}) by antioxidants, reaction after which iron converts to ferrous iron (Fe^{+2}), which produces a blue color. Variations are detected by spectrophotometry that is measured at 593 nm wavelength [34].

The technique was performed based on the test by Benzie and Strain (1999) [34]. The reagents are TPTZ (10 mM), hydrochloric acid (40 mM HCl), sexahhydrate ferric chloride ($FeCl_3 \bullet 6H_2O$ 20 mM), as well as 300 mM acetate buffer with pH 3.6). FRAP solution with 20 mL of acetate buffer, 2 mL TPTZ and 2 mL of FeCl was performed $3 \bullet 6H_2O$. In the cuvette, 1 mL of reagent FRAP and 100 µL samples or standards were added, and

absorbance at 593 nm at 4 min was measured. Blank is the FRAP solution. To compare the results, a Trolox standard curve was performed.

DPPH Technique

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical with a high absorbance at 515 nm; when interaction with an antioxidant compound, DPPH no longer has an unpaired electron and thereby loses the absorbance in direct proportion to the amount of antioxidant [35].

The technique was performed based on the methodology described by Brand-Williams et al. (1995) and Sánchez-Moreno et al. (1998) [36,37]. The DPPH reagent was made with 0.0063 g of DPPH and 250 mL of ethanol, keeping it protected from light. 3.9 mL of DPPH reagent were mixed and 100 µL samples and standards by measuring absorbance at 515 nm after 30 min mixing (kept protected from light). Zero was measured with methanol, and the blank was DPPH reagent without sample.

ABTS Technique

The ability of antioxidant compounds to capture the radical cation that is generated after the reaction of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) with MnO_2 is evaluated. The radical generated absorbs at a wavelength of 734 nm, but after being neutralized by the antioxidants, it decreases its absorbance directly proportionally [29,38].

The method described by Re et al. (1999) [39]. The necessary reagents were a phosphate-buffered saline at pH 7.4; ABTS (7 mM) activated by passing through a filter with manganese oxide II (MnO_2). Then, 1 mL ABTS solution was used and 100 µL of sample or standard. Water was used for zeroing and for blank the ABTS solution with an absorbance of approximately 0.700 (adjustment with water and MnO_2). It was stirred for 30 s, and the absorbance was measured after 2 min at 734 nm.

2.4.2. Microbiological Analysis

Total microbiological growth of total coliform count (TCC), *Escherichia coli*, total vial count (TVC) and molds and yeasts (TMY) were determined at days 0, 4, 7 and 11 from elaboration. Mass seeding was performed on PCA (to determine mesophilic aerobes), rapid E. coli (to determine coliforms and *E. coli*) and oxytetracycline-glucose yeast extract (OGYE) agar medium. Peptone water was used to make dilutions. Analyses were performed in a laminar flow hood (Telstar, BIO-II-A, Madrid, Spain). After seeding, plates were incubated for 24 h at 37 °C for E. coli and TCC, 48 h at 37 °C for TVC and 96 h at room temperature for TMY. Analyses were performed in triplicate and expressed in cfu/g.

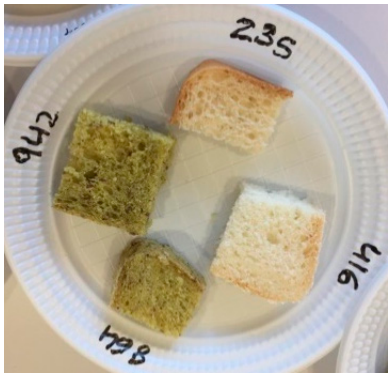
2.5. Sensory Analysis

The tasting room for the sensory evaluation was air-conditioned and free of influencing factors.

It was conducted with 30 untrained panelists aged between 20 and 58 years. Samples were coded with four random digits and presented individually to the panelists on a plate, as shown in Scheme 1. Mineral water was provided for mouthwash between samples. The attributes measured were appearance, aroma, texture, taste, flavor, color, juiciness, purchase intention and overall acceptability. A five-point hedonic scale was used to evaluate the attributes of the breads; panelists scored on a scale of 1 (a little) to 5 (a lot).

2.6. Statistical Analysis

Data were analyzed with the statistical package SPSS 23.0 (Statistical Package for the Social Science for Window (IBM, Armonk, New York, NY, USA). A descriptive statistical analysis of the results was performed, as well as ANOVA test using pairs of factors and Pearson's correlation of the data over time. A ratio of $p < 0.05$ was considered significant, while $p < 0.01$ was considered highly significant.



Scheme 1. Dish prepared for sensory analysis with the 4 coded samples. 235: commercial bread; 416: control bread; 864: organic hydroxytyrosol bread (HXT_S); 942: organic hydroxytyrosol bread (HXT_S).

3. Results and Discussion

3.1. Proximal Composition

3.1.1. Macronutrients of Flaxseed and Spinach Powder

Table 2 shows the approximate composition of the different extracts that provide macronutrients to the gluten-free bread in the study. High values of all macronutrients were observed in both samples, highlighting the quantity of all macronutrients analyzed in spinach compared to flaxseed.

Table 2. Nutritional composition of flaxseed and spinach power (g/100 g) (mean ± standard deviation values) (n = 3).

Parameters	Sample	
	Flaxseed	**Spinach Power**
Proteins	21.99 ± 0.12 [a]	29.36 ± 0.06 [b]
Moisture	5.03 ± 0.09 [a]	8.36 ± 0.36 [b]
Ash	6.96 ± 0.03 [a]	16.98 ± 0.21 [b]
Fat	40.89 ± 0.74 [a]	6.03 ± 0.14 [b]
Fiber	20.85 ± 0.49 [a]	32.01 ± 0.69 [b]
Carbohydrates	28.89 ± 0.25 [a]	32.36 ± 0.49 [b]
Energy	532.32 ± 3.15 [a]	305.01 ± 2.01 [b]

[a,b] Means in the same row not followed by a common superscript letter are significantly different ($p < 0.05$; HSD Tukey test).

The high protein content of spinach (29.36 g/100 g) observed in this study is consistent with previous studies, representing 28.05 g/100 g [40] and 27.8 g/100 g [41]. On the other hand, a slightly higher carbohydrate content was observed for spinach (32.36 g/100 g) than in a previous study which obtained 30.28 g/100 g [41]. However, with respect to fiber, a slightly lower content of 32.01 g/100 g was found than in a previous study where 35.21 g/100 g [41] was obtained and contrary to what Junejo et al. (2021) [40] found where 8.82 g/100 g was obtained, a lower value of fiber compared to that obtained in the current study.

Flaxseed, on the other hand, was found to have a higher proportion of fat (40.89 g/100 g), followed by carbohydrates (28.89 g/100 g) and protein (21.99 g/100 g), which was comparable to some recent studies [42–44].

3.1.2. Macronutrients of the Samples

Nutritional differences were obtained between the different breads, as can be seen in the following table (Table 3).

Table 3. Nutritional composition of four bread (g/100 g) (mean ± standard deviation values) (n = 4).

Parameters	Sample			
	Control	Com	HTX_O	HTX_s
Proteins	1.78 ± 0.69 ^{b}	3.03 ± 0.09 ^{a}	2.98 ± 0.06 ^{a}	3.28 ± 0.11 ^{a}
Moisture	47.10 ± 0.27 ^{c}	37.73 ± 0.03 ^{a}	45.41 ± 0.83 ^{a}	44.70 ± 0.37 ^{a}
Ash	0.61 ± 0.09 ^{a}	0.79 ± 0.19 ^{a}	2.10 ± 0.05 ^{b}	1.25 ± 0.01 ^{c}
Fiber	11.36 ± 1.53 ^{a}	6.29 ± 0.43 ^{b}	16.44 ± 1.7 ^{c}	19.89 ± 0.55 ^{d}
Fat	2.40 ± 0.18 ^{a}	3.16 ± 0.11 ^{a}	5.94 ± 0.09 ^{b}	5.97 ± 0.49 ^{b}
Carbohydrates	36.75 ± 0.60 ^{a}	49.00 ± 0.43 ^{b}	27.13 ± 0.84 ^{c}	24.91 ± 0.31 ^{c}
Energy	175.72 ± 1.61 ^{a}	236.56 ± 0.29 ^{b}	173.90 ± 0.32 ^{a}	166.49 ± 0.17 ^{c}

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial. $^{a–d}$ Means in the same row not followed by a common superscript letter are significantly different ($p < 0.05$; HSD Tukey test).

The proteins in Com, HXT_O and HXT_S samples had similar values and showed no significant differences, while C is the one with the least amount of protein and is highly significant ($p < 0.01$). These findings were also observed by Galla et al. (2017) [41] in their study, where they found that the addition of thorn in the biscuits increases the amount of protein compared to the control biscuit. Other studies, such as Khemiri et al. (2020) [45] and Krupa-Kozak et al. (2021) [46], observed that the addition of green leafy vegetables or seaweed increases the percentage of protein in gluten-free bread compared to the control. Also, the fat in HXT_O and HXT_S is significantly higher than Com and C ($p < 0.01$) due to the addition of flaxseed, which provides them with a higher percentage of fat, providing omega-3 [47]. Flaxseeds are rich in α-linolenic acid [48], which is especially important for good cardiovascular health. The amount of protein in this study is lower than those obtained by Dur et al. (2019) [49], who developed a gluten bread enriched with spinach powder and ferrous sulphate ($FeSO_4$). The results for fat may be due to the fact that, compared to other studies, spinach contains a significant level of fat between 3–6 g/100 g [40,41]. It may also be due to the addition of hydroxytyrosol, as Martínez-Zamora et al. (2020) [50] obtained a higher amount of fat in a fish stick compared to the control.

Carbohydrates depend on the amount of moisture, fat, fiber and protein. The highest carbohydrate content was obtained in the Com, and the bread with the lowest level was HTX_S, with significant differences between all samples ($p < 0.01$). Carbohydrates were slightly lower in HXT_O and HXT_S because of the amount of flaxseed added, which lowered the proportion of carbohydrates, as well as the addition of spinach, as observed by Galla, who found that, with the addition of spinach, the biscuits decreased their carbohydrate content. Filip and Vidrih (2015) [51] produced protein-rich pasta using 40% pea protein isolate and 3% dry spinach powder and obtained a carbohydrate reduction (44.6, 40.4%) of 77% in wheat pasta.

The sample with the highest energy value was Com, and the one with the lowest energy value was HTX_S; in all of them, significant differences were obtained ($p < 0.01$), except in the Control sample compared with HXT_O—their energy value is very similar. There were no significant differences in moisture between the samples; the only sample where a lower value than the others was observed was the Com. Very similar results were obtained as was found in the study of Dur et al. (2019) [49], where their bread contained about 50% of carbohydrates.

The ashes were higher in HXT_O and HXT_S, being highly significant in the case of HXT_O ($p < 0.01$) and less significant in the case of HXT_S ($p < 0.05$). This is due to the addition of spinach and flaxseeds, which provide a large number of minerals that are very important in celiac patients when they are recovering from the disease. Very similar results were obtained to the study of Dur et al., 2019 [49], where their bread contained about 2% of ashes. In addition, Galla et al. (2017) [41] found the same finding in biscuits, where incorporating spinach increases the ash content compared to the control.

The highest fiber content was obtained in HTXs and the lowest in Com, and there were significant differences between all samples ($p < 0.05$). As shown in Table 2, it can

be observed that the enriched breads have a higher fiber content; this could be due to the acerola since, according to the study of Carmo et al. (2018) [52], the fiber content of acerola is approximately 70–80% on a dry basis. Also, flax and spinach because, according to Bekhit et al. (2018) and El-Sayed (2020) [53,54], flax contains about 20–35% and linseed 20%. In addition, the flour used has a high content of corn starch and rice flour, which contain vegetable fibers that, apart from helping the cohesion of the bread, as well as the agglutination of the dough, which is essential for achieving the correct texture, also provides fiber [55]. The results obtained for fiber, where a higher content was observed in the enriched breads, were also obtained by Galla et al. (2017) [41] in the spinach-enriched biscuits compared to the control. The increase in fiber, along with the increase in the amount of healthy fat, helps the enriched samples have a lower glycemic index, according to the literature [56–58]; this reduces the risk of metabolic syndrome and associated diseases.

3.2. Folate Content

Table 4 shows the concentration of each of the four folate monoglutamates, as well as the total folate monoglutamates and the total folate concentration in the three developed breads and commercial bread. Total folate concentration was highest in the HXT_O bread (2879.31 µg folic acid equivalents/100 g FW) followed by bread HXT_S (2112.12 µg folic acid equivalents/100 g FW), C (1330.13 µg folic acid equivalents/100 g FW), and COM (839.55 µg folic acid equivalents/100 g FW), with significant differences of $p < 0.01$. In the folic acid (FA) content, significant differences of $p < 0.01$ were observed between the different breads, except for the HXT_S bread with the Com bread and the control. Regarding the tetrahydrofolate (THF) content, significant differences ($p < 0.05$) were found between the different breads, except for the HXT_O bread with Com and HXT_S with Control. In the 5M-THF (5-methyltetrahydrofolate) composite, significant differences of $p < 0.05$ were observed in all breads. No significant differences were observed between samples regarding 5F-THF (5-formyltetrahydrofolate) content, but it could be found that the one with the highest 5F THF content was HXT_O, and the one with the lowest 5F-THF content was COM.

Table 4. Folate vitamers (FA, THF, 5M-THF, 5F-THF expressed as µg/100 g FW) and total folate (expressed as µg folic acid equivalents/100 g FW). ($n = 4$).

Sample	Folic Acid	THF	5M-THF	5F-THF	Total
HXTO	1669.79 ± 0.49 ^{a}	432.14 ± 0.27 ^{a}	549.19 ± 0.43 ^{a}	228.19 ± 0.33 ^{a}	2879.31 ± 0.55 ^{a}
HXTS	744.36 ± 0.32 ^{b}	348.56 ± 0.34 ^{a}	745.23 ± 0.20 ^{a}	273.97 ± 0.10 ^{a}	2112.12 ± 0.42 ^{a}
COM	496.58 ± 0.74 ^{b}	192.36 ± 0.34 ^{b}	65.25 ± 0.14 ^{c}	85.36 ± 0.28 ^{a}	839.55 ± 0.15 ^{c}
C	654.23 ± 0.16 ^{b}	295.12 ± 0.22 ^{b}	185.37 ± 0.65 ^{d}	195.41 ± 0.11 ^{a}	1330.13 ± 0.36 ^{d}

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial; FA: folic acid; THF: tetrahydrofolate; 5-MTHF: 5-methyltetrahydrofolate; 5-FTHF: 5-formyltetrahydrofolate. $^{a–d}$ Means in the same row not followed by a common superscript letter are significantly different ($p < 0.05$).

The main form of natural folate found in all our samples was folic acid, which is consistent with the results of studies where FA was found to be the most predominant natural form of folate in legumes [59]. However, López-Nicolás et al. (2014) [60] observed that the main abundant natural form of folate was 5M-THF in their gluten breads enriched with spinach and chard, but this is perhaps because they only analyzed two natural forms of folate, 5M-THF and THF.

According to market consultant Mintel, Spain ranks as the third country in the world that has launched the most gluten-free products, after Brazil and the United States (*AECOC*, 2016) [61]. Furthermore, according to the food consumption report, Spain increased its consumption of gluten-free products by 3.08% in 2020 [62]. Spanish citizens consumed 8.5 g of gluten-free bread per day. Taking these data into account, the results obtained in the present study showed that consumption of studio bread could reach 48–80% RDI of folate (400 µg), respectively, with the highest HXT_O and the lowest HXT_S. Brevik et al. (2005) [63] reported that bread is one of the foods that contribute the most to total folate intake, even more than other folate-containing foods, such as vegetables and fruits. Vegetables, as rich sources of folate and other vitamins, are widely recommended for balanced diets; therefore,

breads enriched with spinach and chard would be a good choice to provide an additional source of folates [60]. In addition, besides celiac patients, it would also be a good option for pregnant women since they would supplement their diet and thus prevent neural tube defects in newborns; this would thus assist 40% of those who experience unplanned pregnancies through the inclusion of folic acid fortification in the bread as the base food of the Mediterranean diet [10]. It would also be a good alternative because celiac patients have deficiencies in the intake of micronutrients, including iron; folic acid; vitamins A, B6, B12, D, E and K; copper; and zinc. Due to malabsorption [64], gluten-free products also contain significantly lower levels of vitamins D, E and B_{12}; iron; folate; magnesium; potassium; and sodium than foods containing gluten [65]. It has also been shown that only 5% of gluten-free breads contain the four mandatory fortification nutrients (calcium, iron, niacin and thiamine), while 28% are fortified only with calcium and iron. However, it should be noted that not all countries require fortification of foods. Some countries only require wheat-based products to be fortified, and these rules do not apply to "dietetic or special foods", such as gluten-free products. This lack of fortification may increase the risk of micronutrient deficiency in subjects with celiac disease who are following what appears to be an adequate gluten-free diet [65].

3.3. Minerals

The results of the minerals are shown in Table 5. No arsenic, beryllium, bismuth, lanthanum, selenium or vanadium were found in any of the samples, and no differences were seen between the samples of the amount of cadmium, cobalt, potassium, lithium, molybdenum, sodium, nickel, rubidium, antimony, titanium and zinc.

Table 5. Results of mineral determination in functional samples at day 0. (n = 4).

Mineral	Sample			
	HXT_O	**HXT_S**	**C**	**Com**
Al (mg/Kg)	8.61 ± 0.01 ^{a}	7.30 ± 0.01 ^{b}	11.39 ± 0.02 ^{c}	9.26 ± 0.01 ^{d}
As (mg/Kg)	Nd ^{a}	Nd ^{a}	0.07 ± 0.01 ^{b}	0.03 ± 0.01 ^{c}
B (mg/Kg)	1.58 ± 0.01 ^{a}	1.69 ± 0.01 ^{b}	0.46 ± 0.01 ^{c}	0.34 ± 0.02 ^{d}
Ca (g/100 g)	0.11 ± 0.01 ^{a}	0.11 ± 0.01 ^{a}	0.01 ± 0.01 ^{b}	0.03 ± 0.01 ^{b}
Cr (mg/Kg)	0.13 ± 0.01 ^{a}	0.14 ± 0.01 ^{a}	0.03 ± 0.01 ^{b}	0.02 ± 0.01 ^{b}
Cu (mg/Kg)	1.51 ± 0.01 ^{a}	1.63 ± 0.01 ^{b}	1.01 ± 0.01 ^{c}	1.07 ± 0.01 ^{d}
Fe (mg/Kg)	9.99 ± 0.02 ^{a}	10.16 ± 0.04 ^{b}	9.67 ± 0.01 ^{c}	7.33 ± 0.01 ^{d}
Mg (g/100 g)	0.06 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	0.02 ± 0.01 ^{b}	0.03 ± 0.01 ^{b}
Mn (mg/Kg)	7.18 ± 0.08 ^{a}	7.34 ± 0.06 ^{a}	3.88 ± 0.16 ^{b}	2.12 ± 0.12 ^{b}
P (g/100 g)	0.10 ± 0.04 ^{a}	0.10 ± 0.01 ^{ab}	0.07 ± 0.01 ^{ab}	0.05 ± 0.01 ^{b}
Si (mg/kg)	31.99 ± 0.01 ^{a}	24.91 ± 0.01 ^{b}	29.41 ± 1.39 ^{c}	17.67 ± 1.46 ^{d}
Sr (mg/Kg)	3.46 ± 0.04 ^{a}	3.26 ± 0.08 ^{b}	0.81 ± 0.06 ^{c}	0.25 ± 0.02 ^{d}
Tl (mg/Kg)	0.41 ± 0.03 ^{a}	0.35 ± 0.01 ^{a}	1.37 ± 0.06 ^{b}	0.22 ± 0.06 ^{c}
Zn (mg/Kg)	7.07 ± 0.01 ^{a}	7.01 ± 0.01 ^{a}	8.16 ± 0.02 ^{b}	3.65 ± 0.01 ^{c}
Se (mg/kg)	nd	nd	nd	nd

Nd: not identified; C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial. $^{a–d}$ Means in the same row not followed by a common superscript letter are significantly different ($p < 0.05$).

High aluminum content was observed in the C and in Com, being the lowest in the HXT_S ($p < 0.01$). Arsenic was only detected in sample C (0.07 mg/Kg) and in the Com (0.03 mg/Kg). In boron, copper and iron, the highest content of these minerals was found in HXT_S (1.69 mg/kg; 1.63 mg/kg; 10.16 mg/kg) ($p < 0.05$). However, higher contents of silicon and strontium were observed in the HXT_O (31.99 mg/Kg; 3.46 mg/Kg) ($p < 0.05$). In the case of thallium and zinc, the content of C is greater than that of the other samples ($p < 0.05$). It was also observed that in the case of zinc, it was higher in the developed samples than in the Com; this may be due to the fact that flaxseeds (5.5 mg/100 g) provide a large amount of zinc [47]. Regarding the iron content, this is higher in the samples that had spinach added ($p < 0.05$). With respect to calcium, chromium, magnesium and manganese,

significantly higher contents were observed in the fortified breads than in the control and commercial breads. For phosphorus, a small significant difference was found between the control (0.05 g/100 g) and the formulated breads. The same finding was obtained by [41], who observed an increase in phosphorus when spinach was added to biscuits.

The minerals which are increased in C can be distinguished between two main groups because the majority of them are found in cereals and minerals which increase in all samples enriched or some of them due to the various ingredients added.

According to authors such as Pennington and Schoen (1995) and Soni et al. (2001) [66,67], cereals are the main source of aluminum through food. The main ingredients in sample C were corn starch and rice flour. It was noted that enrichment in other extracts caused a lower concentration of aluminum since the percentage of cereals was reduced. Spinach is also a food rich in aluminum [68], and the higher spinach content in this sample compensates for the lower percentage of cereals. Arsenic is present in corn starch and rice, so something similar to aluminum occurs [69]. Thallium was mostly seen in sample C; this is because cereals are rich in thallium [70].

Boron increased proportionally to the amount of spinach added, being very low in sample C and quite high in the HXT_S sample. In the rest of the samples, the value is similar and intermediate. Based on the literature, spinach is rich in boron and is the ingredient used with a higher concentration of this mineral [71,72]. Chromium was quite increased in the enriched samples; green leafy vegetables such as spinach are sources of chromium [73]. Copper was similarly increased in all enriched breads. The main source of copper is the flax and light contribution of spinach; in the same way, it occurs with magnesium (380 mg/100 g) and phosphorus (470 mg/100 g) [47], very abundant minerals in flaxseeds and somewhat less in spinach [47]. Manganese was especially increased in the enriched samples with respect to C and Com, reaching double the amount of this. Manganese is especially abundant in spinach and somewhat less in flaxseeds [47,74]. The increase in silicon, especially in fortified breads, suggests that the main source is spinach enrichment. Even more obviously, it occurs with the strontium mineral; in the fortified breads sample, an increase of more than 3 times compared to the rest of the bread samples was observed and more than 13 times compared to C [74].

The minerals that stand out the most are calcium and iron, as these are common deficiencies in celiac patients, and this fortification was specifically sought. In calcium, an increase of 10% was observed in the enriched breads with respect to C and 3% in Com compared to the enriched ones, which contain spinach and flaxseeds (170 mg/100 g) [47], ingredients in which this mineral is present. In iron, which is found naturally in spinach, flaxseed (7.06 mg/100 g) [47] and lentil flour [47,74–76], an increase of 36–39% was observed for reformulated breads compared to Com and 3–5% compared to C. This finding was also found by Galla et al. (2017) [41], who observed an increase in calcium and iron by adding spinach to the biscuits, but the observed increase was much higher, with calcium increasing by 100–305.62% and iron by 31.23–176.84%.

3.4. pH and Color during Different Days of Storage

The pH results can be seen in Table 6; it was observed that HXT_O has the highest. A significant increase ($p < 0.05$) in pH from D0 to D11 was observed in the HXT_O, HXT_S and control loaves, but a decrease in pH was observed in the control loaf. On days 0 and 4, the HXT_O had the highest pH (5.88;5.90), and the control had the lowest pH (4.85;5.08), but on day 7, the HXT_S had the highest pH (5.90), and the control had the lowest pH (5.11). On day 11, a higher pH was observed in HTX_O (5.90) and a lower one in control (5.13).

In the study by Moore et al. (2008) [77], no significant differences were seen in the pH of the sample over time, but it was only studied at 24 and 48 h with sourdough bread. The pH value is affected by many conditions, such as the amount of CO_2 formed by fermentation and other ingredients, such as calcium carbonate and spinach. The absence of variation of the HXT_O and HXT_S samples can be associated with greater stability provided by the number of natural antioxidants.

Table 6. Evolution of the pH of bread samples over different days.

	Samples			
Days of Storage	**Control**	**HTX_O**	**HTX_S**	**Com**
Day 0	$4.85 \pm 0.02^{a\,v}$	$5.88 \pm 0.01^{d\,w}$	$5.82 \pm 0.01^{c\,w}$	$5.15 \pm 0.02^{b\,x}$
Day 4	$5.08 \pm 0.01^{b\,w}$	$5.90 \pm 0.01^{c\,w}$	$5.80 \pm 0.02^{a\,wx}$	$5.21 \pm 0.01^{d\,w}$
Day 7	$5.11 \pm 0.01^{c\,w}$	$5.82 \pm 0.01^{b\,v}$	$5.90 \pm 0.02^{d\,v}$	$5.17 \pm 0.01^{a\,x}$
Day 11	$5.17 \pm 0.01^{d\,x}$	$5.90 \pm 0.01^{a\,w}$	$5.77 \pm 0.01^{b\,x}$	$5.13 \pm 0.01^{c\,x}$

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial. $^{a–d}$ Means in the same row not followed by a common superscript letter are significantly different between samples ($p < 0.05$). $^{v–x}$ Means in the same column that are not followed by a common letter in superscript are significantly different between days of analysis ($p < 0.05$).

The color measurements of the different breads can be seen in Table 7. A significantly higher difference ($p < 0.05$) in brightness (L*) was observed in the C and Com compared to the enriched breads (HTX_O and HXT_S) on all days. The variable a* was observed to be significantly lower in the enriched breads representing the green shades compared to the C and Com breads, which is found on day 0, day 4, day 7 and day 11. This coincidence was also observed in the variable b* (yellow), where it was found to be significantly higher in the enriched breads than in the C and Com bread on all days. The hue angle (h) also follows the same pattern of measurements, being higher in the HXT_S and HXT_O and lowest in the C and Com. These findings of how the inclusion of spinach affects bread variables, increasing the b* variable and decreasing L* and a*, were also found by Junejoet al. (2017) [40] in gluten breads. All measurements (L*, a*, b*, C*, h) of the C and Com were found to be highly significant ($p < 0.01$) with respect to the HXT_O and HXT_S breads; however, there is no significant difference between the HXT_O and HXT_S breads. Regarding the color variables in the same samples during the storage time, no major changes were found, the brightness was not affected during storage; however, the a*, b*, C* showed a tendency to decrease from day 0 to day 11 in all the breads, finding a significant difference ($p < 0.01$) in the C* measure with respect to the days of storage, but only with a significant difference ($p < 0.01$) in the b* in the control, Hxts, commercial and control breads.

The differences in color are due to the addition of lyophilized spinach, which causes darker dough with a more greenish and yellowish tone. The color differences are evident to the naked eye, being imperceptible between the HXT_O and HXT_S (there is no significant difference). The C increased its luminosity with the passing of days, which is an unfavorable characteristic since gluten-free breads already have a lighter color due to their high content of starches and rice flour [78]. The absence of correlation of brightness change of HXT_O and HXT_S can also be associated with greater stability of the sample and, therefore, better maintenance of color and conditions during storage.

Table 7. Color development during refrigerated storage of bread samples.

		Control	**HXT_O**	**HXT_S**	**Com**
Day 0	L*	$79.21 \pm 0.19^{a,\,v}$	$58.78 \pm 0.83^{b,\,v}$	$62.29 \pm 0.64^{c,\,v}$	$69.27 \pm 2.22^{d,\,v}$
	a*	$-2.03 \pm 0.84^{a,\,v}$	$-6.15 \pm 0.99^{b,\,w}$	$-5.53 \pm 0.13^{b,\,v}$	$5.62 \pm 0.17^{c,\,v}$
	b*	$20.61 \pm 0.42^{a,\,w}$	$30.91 \pm 0.04^{b,\,v}$	$32.70 \pm 2.08^{b,\,v}$	$19.13 \pm 1.16^{a,\,v}$
	C*	$20.28 \pm 0.08^{a,\,x}$	$31.73 \pm 0.48^{b,\,w}$	$31.68 \pm 0.30^{b,\,w}$	$19.93 \pm 1.15^{a,\,w}$
	h	$93.29 \pm 0.07^{a,\,w}$	$99.57 \pm 0.39^{b,\,w}$	$100.18 \pm 0.22^{b,\,w}$	$73.60 \pm 0.49^{c,\,vw}$
Day 4	L*	$78.91 \pm 0.05^{a,\,v}$	$57.76 \pm 0.76^{b,\,v}$	$59.24 \pm 0.93^{b,\,w}$	$70.29 \pm 0.17^{c,\,v}$
	a*	$-1.50 \pm 0.44^{a,\,v}$	$-3.17 \pm 0.52^{b,\,v}$	$-5.16 \pm 0.08^{c,\,v}$	$5.60 \pm 0.04^{d,\,v}$
	b*	$15.65 \pm 0.03^{a,\,v}$	$25.38 \pm 0.64^{b,\,w}$	$31.04 \pm 0.20^{c,\,v}$	$19.44 \pm 0.04^{d,\,v}$
	C*	$15.79 \pm 0.16^{a,\,w}$	$25.44 \pm 0.22^{b,\,v}$	$31.53 \pm 0.18^{c,\,w}$	$20.23 \pm 0.04^{d,\,w}$
	h	$95.13 \pm 0.20^{a,\,v}$	$98.64 \pm 0.34^{b,\,w}$	$99.42 \pm 0.12^{c,\,w}$	$73.92 \pm 0.09^{d,\,v}$

Table 7. *Cont.*

		Control	HXT_O	HXT_S	Com
Day 7	L*	81.57 ± 0.69 $^{a, v}$	59.95 ± 0.57 $^{b, v}$	60.56 ± 0.51 $^{b, vw}$	71.18 ± 2.23 $^{c, v}$
	a*	−0.87 ± 0.08 $^{a, v}$	−5.11 ± 0.23 $^{b, vw}$	−5.42 ± 0.37 $^{b, v}$	5.70 ± 0.21 $^{c, v}$
	b*	9.15 ± 0.05 $^{a, x}$	23.83 ± 0.29 $^{b, w}$	25.34 ± 0.98 $^{b, w}$	18.86 ± 0.76 $^{c, v}$
	C*	9.32 ± 0.21 $^{a, v}$	24.18 ± 0.03 $^{b, v}$	25.22 ± 0.37 $^{b, x}$	19.47 ± 1.47 $^{c, w}$
	h	96.01 ± 0.53 $^{a, v}$	102.40 ± 0.06 $^{b, v}$	102.88 ± 0.03 $^{b, v}$	73.27 ± 0.11 $^{c, vw}$
Day 11	L*	81.46 ± 0.61 $^{a, v}$	59.17 ± 0.75 $^{b, v}$	59.11 ± 0.13 $^{b, w}$	69.96 ± 0.61 $^{c, v}$
	a*	−1.50 ± 0.22 $^{a, v}$	−4.37 ± 0.14 $^{b, v}$	−4.73 ± 0.11 $^{b, v}$	5.70 ± 0.21 $^{c, v}$
	b*	17.46 ± 0.49 $^{a, v}$	25.67 ± 0.47 $^{b, w}$	28.17 ± 0.91 $^{b, x}$	18.62 ± 1.48 $^{a, v}$
	C*	17.67 ± 0.53 $^{a, y}$	25.87 ± 0.08 $^{b, v}$	28.06 ± 0.36 $^{c, v}$	19.47 ± 1.47 $^{d, w}$
	h	95.31 ± 0.22 $^{a, v}$	99.42 ± 0.20 $^{b, w}$	99.54 ± 0.24 $^{b, w}$	72.94 ± 0.78 $^{c, w}$

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial. $^{a–d}$ different letters in the same row indicate significant differences among samples ($p < 0.05$). $^{v–y}$ Means in the same column that are not followed by a common letter in superscript are significantly different between days of analysis ($p < 0.05$).

3.5. Antioxidant Capacity

3.5.1. Characterisation of Preservative Extracts

The knowledge of the antioxidant activity of antioxidant ingredients allows a comparative evaluation between the preservative extracts analyzed. The results obtained from each method are shown in Table 8.

Table 8. Antioxidant activity of natural extracts by measuring their ABTS and DPPH represented in the percentage of chelating activity (%) and antioxidant activity (µmol TE/g) of the samples ($n = 4$).

Samples	Chelating Activity Percent (%)		Antioxidant Activity (µmol TE/g)	
	ABTS	DPPH	ORAC	FRAP
Sp	22.02 ± 0.12 ^{a}	44.06 ± 0.32 ^{a}	1467.20 ± 38.24 ^{a}	1980.80 ± 6.13 ^{a}
CBC	16.42 ± 0.21 ^{b}	9.55 ± 0.42 ^{b}	7719.20 ± 56.87 ^{b}	8858.90 ± 17.68 ^{b}
A	47.92 ± 0.26 ^{c}	79.25 ± 0.52 ^{c}	19,684 ± 56.406 ^{c}	1899.02 ± 25.478 ^{a}
HXTo	83.32 ± 3.62 ^{d}	84.16 ± 2.35 ^{d}	41,326 ± 236.65 ^{d}	61,326 ± 526.36 ^{d}
HXTs	94.32 ± 5.32 ^{e}	89.89 ± 3.92 ^{e}	71,326 ± 278.32 ^{e}	65,326 ± 1362.52 ^{e}

CBC: citrus extracts; Sp: spinach; A: acerola; control, HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; FRAP: iron reducing antioxidant power; ORAC: oxygen radical absorption capacity. $^{a–e}$ different letters in the same row indicate significant differences among samples ($p < 0.05$).

Firstly, considering the results presented in Table 8, it can be observed that HXTs, HXTo and acerola showed the highest chelating capacity against the radical cations DPPH and ABTS. At the same time, the lowest values were presented by CBC with 9.55% and spinach with 44.06% of chelation in DPPH.

On the other hand, the scavenging activity against the hydrophilic radical ABTS is generally lower than against DPPH. In both methods, the hydroxytyrosol stands out, especially the hydroxytyrosol from synthetic sources. As a result, the oxygen radical scavenging capacity of HXTs was 5% higher than that of HXTo, 10% higher than that of extract A, 45% higher than that of sp and 79% higher than that of CBC. For the ferric-reducing extract, the antioxidant power of HXTs was 72% higher than that of HXTo. Furthermore, the percentage chelating activity of HXTs against ABTS radicals was only 11% compared to HXTo. This was due to the purity of the HXT (99.2%) compared to the 7.26% bioactive compound content in HXTo. HXT is known for its antioxidant potential, which is ten times higher than green tea and twice as high as coenzyme Q10 [79]. It also has a major role as a free radical scavenger and outstanding efficacy under stressful conditions. This antioxidant activity is based on the chemical structure of this phytochemical compound: a phenolic ring consisting of a catechol group and three hydroxyl groups [80].

3.5.2. Antioxidant Capacity during the Shelf-Life Study

The results of the antioxidant capacity of the different samples in different samples are shown in Table 9. The methods were chosen based on the bibliography [75,81,82]. It is important to highlight the importance of carrying out more than one study of antioxidant capacity in order to understand the behavior of antioxidants [83].

Table 9. Chelating activity percent (%) and antioxidant activity (μmol TE/g) of the samples of bread (n = 4).

		Control	HXT_O	HXT_S	Com
Day 0	FRAP	102.46 ± 0.82 $^{a, w}$	208.42 ± 0.54 $^{b, w}$	245.17 ± 3.95 $^{c, w}$	12.33 ± 1.82 $^{d, w}$
	ORAC	467.39 ± 0.55 $^{a, w}$	754.75 ± 1.39 $^{b, w}$	674.68 ± 1.53 $^{c, x}$	111.09 ± 6.02 $^{d, w}$
	ABTS	22.24 ± 0.24 $^{a, w}$	43.82 ± 1.32 $^{b, w}$	60.58 ± 0.65 $^{c, w}$	8.97 ± 0.60 $^{d, w}$
	DPPH	13.96 ± 1.52 $^{a, w}$	33.53 ± 1.61 $^{b, w}$	48.26 ± 0.66 $^{c, w}$	3.40 ± 0.84 $^{d, w}$
Day 4	FRAP	66.47 ± 1.37 $^{a, x}$	107.85 ± 0.50 $^{b, x}$	186.91 ± 1.41 $^{c, x}$	9.78 ± 0.71 $^{d, x}$
	ORAC	402.15 ± 0.24 $^{a, x}$	469.09 ± 0.24 $^{b, x}$	672.57 ± 1.05 $^{c, x}$	105.34 ± 4.98 $^{d, w}$
	ABTS	18.71 ± 0.44 $^{a, x}$	37.98 ± 1.22 $^{b, x}$	45.37 ± 1.35 $^{c, x}$	5.56 ± 0.52 $^{d, x}$
	DPPH	2.29 ± 0.32 $^{a, x}$	28.80 ± 0.56 $^{b, x}$	35.70 ± 0.62 $^{c, x}$	2.21 ± 0.49 $^{a, w}$
Day 7	FRAP	59.26 ± 1.19 $^{a, y}$	92.99 ± 1.75 $^{b, y}$	181.14 ± 1.20 $^{c, y}$	5.42 ± 2.25 $^{a, x}$
	ORAC	221.18 ± 1.05 $^{a, y}$	459.82 ± 1.12 $^{b, y}$	665.98 ± 1.00 $^{c, x}$	103.05 ± 7.64 $^{d, w}$
	ABTS	17.35 ± 1.16 $^{a, x}$	36.65 ± 1.15 $^{b, x}$	39.38 ± 0.88 $^{b, y}$	4.44 ± 0.21 $^{c, y}$
	DPPH	1.66 ± 0.31 $^{a, x}$	17.99 ± 0.55 $^{b, y}$	33.11 ± 1.16 $^{c, y}$	1.68 ± 0.02 $^{a, w}$
Day 11	FRAP	50.09 ± 1.63 $^{a, z}$	89.37 ± 1.61 $^{b, y}$	176.68 ± 1.75 $^{c, y}$	4.59 ± 1.23 $^{a, x}$
	ORAC	90.45 ± 0.53 $^{a, z}$	459.51 ± 1.27 $^{b, y}$	647.17 ± 2.38 $^{c, y}$	86.67 ± 1.97 $^{a, x}$
	ABTS	10.77 ± 1.14 $^{a, y}$	35.05 ± 1.82 $^{b, x}$	31.62 ± 2.23 $^{c, z}$	2.69 ± 0.47 $^{d, z}$
	DPPH	1.11 ± 0.11 $^{a, x}$	11.31 ± 0.87 $^{b, z}$	28.36 ± 0.98 $^{c, z}$	0.44 ± 0.08 $^{a, w}$

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; FRAP: iron-reducing antioxidant power; ORAC: oxygen radical absorption capacity. $^{a–d}$ different letters in the same row indicate significant differences among samples ($p < 0.05$). $^{w–z}$ Means in the same column that are not followed by a common letter in superscript are significantly different between days of analysis ($p < 0.05$).

Significantly ($p < 0.01$) higher antioxidant capacity was found in all methods in the fortified samples compared to the control and commercial samples. This finding was also reported by Junejo et al. (2021) [40], who observed that the addition of spinach improved antioxidant versus control activities; this could be due to the presence of phytochemicals and bioactive compounds of spinach such as polyphenols, lutein, lycopene, α-carotene,-and α-tocopherol [84].

FRAP and DPPH were significantly higher in HTX_S on all days of the analysis. However, in ORAC, the highest antioxidant capacity was found in HXT_O on days 0 (754.75 μmol TE/g) ($p < 0.01$). In ABTS, the highest concentration was obtained on days 0 (60.58%), 4 (45.37%) and 7 (39.38) in HXT_S ($p < 0.01$). It was observed that in all methods, commercial bread was significantly lower ($p < 0.05$).

The difference in the averages between HXT_O and HXT_S is especially interesting since the only difference between them is the origin of hydroxytyrosol. This demonstrates that the chemically synthesized hydroxytyrosol has a greater antioxidant capacity than the hydroxytyrosol extracted from the olive leaf. In all methods and samples, a decrease was observed as the days progressed, where a decrease was found with a great difference in the control bread and commercial bread; however, a low decrease was observed in the enriched breads, especially from day 4 onwards. In the case of HXT_S, there is not so much variation as the days go by. This may be because synthetic hydroxytyrosol is more stable, provides greater antioxidant protection and is more sustained over time (Table 9).

The HXT_O and HXT_S samples showed a higher antioxidant capacity consistent with the studies of Branciari et al. (2017) [85] on hydroxytyrosol enriched with meat and [86], who analyzed the antioxidant capacity in vitro of hydroxytyrosol and tyrosol. In both, a high antioxidant capacity of hydroxytyrosol was determined by ORAC, DPPH and

FRAP. Moreover, [87], in his gluten-free breads developed with chestnut flour at different concentrations, observed significant differences in the stability of the antioxidant capacity throughout the days. This also occurs in our results since HXT_S presents much greater stability of the antioxidant capacity with respect to HXT_O due to HTXs at antioxidant purity (99.2%) compared to 7.26% bioactive compound content in HXTo.

In the studies by Jensen, Oestdal, Clausen, Andersen and Skibsted (2011) and Jensen, Ostdal, Skibsted, and Thybo (2011) [88,89], it is revealed that the variation of antioxidant capacity over time depends on the bread formulation; this could explain the difference between control and commercial enriched breads. Paciulliet al. (2016) [87] observed that the antioxidant capacity decreased from day 1, results that coincide with those obtained. However, Jensen, Oestdal, Clausen, Andersen and Skibsted (2011) [88] observed a very low decrease in their samples of whole wheat bread compared to the results that we found, where a much higher decrease in antioxidant capacity is observed, but in bread with Hxts from day 4 onwards, the decrease is very low, and it could be said that it is almost stable.

3.6. Microbiological Analysis

The results of the microbiological study are shown in Table 10.

Table 10. Evolution of microbiological content (cfu/g) of bread for eleven days of refrigerated storage in aerobic conditions.

		Days of Storage			
Microorganism	**Samples**	**0**	**4**	**7**	**11**
TVC	Control	$1.6 \times 10^3 \pm 200$ $^{a, w}$	$5.13 \times 10^3 \pm 404.15$ $^{a, w}$	$6.03 \times 10^4 \pm 5033.22$ $^{a\,b, w}$	$1.37 \times 10^5 \pm 55{,}075$ $^{b, w}$
	HXTs	17.67 ± 6.81 $^{a, w}$	$1.57 \times 10^4 \pm 351.19$ $^{a, w}$	$1.32 \times 10^5 \pm 11{,}239.81$ $^{b, w}$	$7.87 \times 10^5 \pm 70{,}237.69$ $^{c, x}$
	HXTo	$3.57 \times 10^3 \pm 305.51$ $^{a, w}$	$1.7 \times 10^4 \pm 1000$ $^{a, w}$	$6.53 \times 10^4 \pm 5507.57$ $^{a, w}$	$7.63 \times 10^5 \pm 73{,}711.15$ $^{b, x}$
	Commercial	$1.81 \times 10^3 \pm 45.83$ $^{a, w}$	$2.27 \times 10^3 \pm 309.89$ $^{a, w}$	$1.12 \times 10^4 \pm 251.66$ $^{a, w}$	$1.26 \times 10^5 \pm 9073.77$ $^{a, w}$
TMY	Control	<10	$1.44 \times 10^3 \pm 45.09$ $^{a, w}$	$8.2 \times 10^4 \pm 3605$ $^{b, w}$	$1.5 \times 10^5 \pm 14{,}525$ $^{c, w}$
	HXTs	<10	$3.78 \times 10^3 \pm 196.55$ $^{a, w}$	$4.07 \times 10^4 \pm 2081.67$ $^{a, w, x}$	$3.90 \times 10^5 \pm 29{,}569.12$ $^{b, y}$
	HXTo	<10	$5.57 \times 10^3 \pm 602.77$ $^{a, w}$	$2.78 \times 10^4 \pm 1795.36$ $^{a, x}$	$6.83 \times 10^5 \pm 30{,}550$ $^{b, x}$
	Commercial	$1.7 \times 10^3 \pm 170.59$ ^{a}	$2.2 \times 10^3 \pm 67.35$ $^{a, w}$	$2.16 \times 10^4 \pm 602.77$ $^{a, x}$	$1.2 \times 10^5 \pm 3605.55$ $^{b, w, x, y}$
E. coli	Control HXTs HXTo Commercial	<10			
TCC	Control HXTs HXTo Commercial	<10			

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; TVC: total vial count; TCC: total coliform count; TMY: total molds and yeasts. $^{a–c}$ Different letters within the same row indicate significant differences between samples at different times of analysis ($p < 0.05$). $^{w–z}$ Different letters within the same column indicate significant differences between samples ($p < 0.05$).

HXT_S on the initial day presented the lowest number of total microorganisms, which shows that the synthetic source of HXT (99%purity) inhibits the total growth of microorganisms with respect to the control and commercial supermarket bread, but with respect to the passage of storage time a change can be observed in the bread with HXTs, as it increases the growth of mesophilic aerobic microorganisms; however, in molds and yeasts, it is in the bread with HXTo. With these results, it can be extrapolated that hydroxytyrosol has a higher shelf life and microbiological safety compared to the control, including a possible higher antimicrobial effect. This finding coincides with the results obtained in the study by Martínez-Zamora et al. (2020) [80], who developed burgers with different types of hydroxytyrosol and observed that HXTs has a high antimicrobial capacity against mesophilic aerobic microorganisms.

In other studies using fish patties, the authors observed the same antimicrobial reaction due to hydroxytyrosol and natural extracts (citrus extracts) [50,90]. Shapira and Mimran (2007) [91] suggested that their antimicrobial effect could be due to phenolic compounds.

This antimicrobial activity was also shown in pork sausages made with emmer wheat, almonds, hazelnuts and pomegranate and citrus extracts [92–94]. This behavior can also be compared with previous studies by Azaizeh et al. (2011) [95], which also demonstrated the antibacterial power of olive extracts (as sources of HXT) in lamb patties.

According to RM Law number 615-2003 SA/DM, (2003) [96], bakery and pastry products with or without filling that do not require refrigeration must include the following microorganisms: molds, E. coli, Staphylococcus aureus, Clostridium perfringens and Salmonella sp. In the case of bread without filling, only mold analysis is required, and the allowed limit is 100–1000 CFU/g. In the samples developed, it is fulfilled on day 0—less in the Com since it is above 103 CFU/G; from day 4, no bread falls within the rule.

3.7. Sensory Analysis

In the sensory analysis, with the samples prepared as in Scheme 1, parameters of the organoleptic quality of each piece of bread were measured, and the samples were ordered according to their taste, from highest to lowest. In Table 11, the difference between the averages received is shown.

Table 11. Descriptive statistics sensory analysis of the functional sample (n = 30).

Sample		Appearance	Aroma	Texture	Taste	Color	Juiciness	Purchase Intention	Global Acceptance	Order Received
Com	Average ± SD	4.37 ± 0.72	3.50 ± 1.04	3.80 ± 1.03	3.30 ± 1.18	4.47 ± 0.82	3.93 ± 1.02	3.43 ± 0.97	3.70 ± 0.70	2.23 ± 1.19
C		3.83 ± 1.02	3.40 ± 1.19	3.17 ± 1.15	3.17 ± 0.83	3.60 ± 1.07	3.17 ± 1.09	2.77 ± 1.10	3.27 ± 0.79	2.87 ± 1.04
HXT_O		3.77 ± 1.01	3.17 ± 1.32	4.07 ± 0.79	3.33 ± 1.30	3.37 ± 1.27	4.07 ± 0.70	3.17 ± 1.32	3.40 ± 1.00	2.53 ± 1.01
HXT_S		3.87 ± 0.90	3.43 ± 1.07	3.93 ± 0.79	3.17 ± 1.32	3.50 ± 1.33	3.87 ± 0.86	3.10 ± 1.32	3.30 ± 1.18	2.43 ± 1.17

C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol; Com: commercial.

Com stood out, and more than 50% of the tasters gave it a score of 5, and 40% placed it in the first place. C is the one with the least first places (only 10%) and earned the most last-place ratings (36.7%). HXT_O is the one with the most second places (33.3%), and the HXT_S sample had a great acceptability, having the second most first-place ratings (30%) after Com. Differences in score regarding the texture of C with HXT_O are highly significant ($p < 0.01$) and significant ($p < 0.05$) for C with the HXT_S, with C's texture being the lowest valued. For the color, highly significant differences were observed in Com with HXT_O and HXT_S and significant differences with C, with Com being better valued. Regarding juiciness, the difference between C and the rest of the samples is highly significant, with C having a worse evaluation in all cases. If the average score of the samples is compared, C was the one that received the highest score, followed by the HXT_S, HXT_O and finally C. The order of choice by the testers was Com> HXT_S > HXT_O > C, as shown in Figure 2. However, Zhou et al. (2023) [97] obtained different results, as they found that by supplementing vegetables in breads, they provide greater satisfactory acceptability on the part of the consumer.

The texture and juiciness (related qualities) of C were poor compared to the other samples, so the ingredients added to HXT_O and HXT_S improved these two characteristics, making the bread more enjoyable. However, the differences in color are significant, with those of HXT_O and HXT_S being worse valued, but sample C also scored low. The best value was obtained by the Com sample since it showed a darker color, which reinforces the study of Wronkowska et al. (2013) [78], in which it was observed that a darker color is more satisfactory in gluten-free bread for the tester.

Taking into account these results, HXT_S showed a good acceptability, despite having a very different appearance from "normal" bread. This finding was also obtained by Zamora-Martínez et al. (2020) [80], who found better near-commercial acceptability results in HXTs lamb burgers compared to HXTo. After analyzing the characteristics of the tasters, it is even more striking since the majority (63.3%) were people between 18 and 30 years who tend to be more reluctant to foods enriched for their health and to vegetables in general.

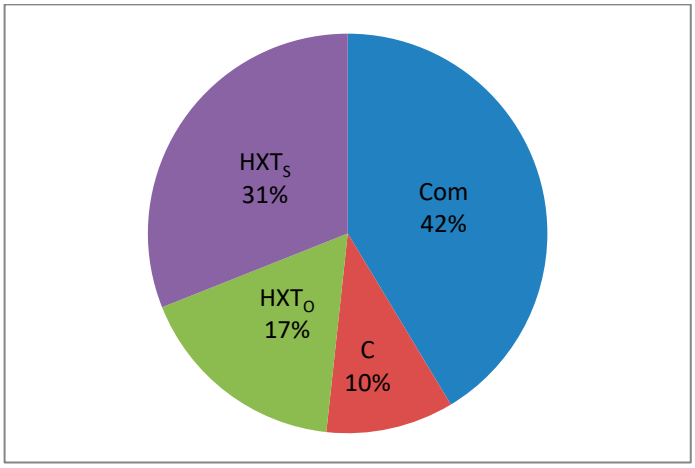


Figure 2. Graph of the frequency of the order received regarding the acceptability of the product. C: control; HXT_O: organic hydroxytyrosol; HXT_S: synthetic hydroxytyrosol.

4. Conclusions

The samples with HXT showed a greater antioxidant capacity, a more stable pH, a better nutritional value and a greater number of minerals, especially calcium, of folates (especially important due to the generalized deficiency in celiac patients), in addition to an antimicrobial activity compared with the control and less degradation and aging. In the sensory analysis, the samples with hydroxytyrosol showed a higher acceptability than the control; HXT_S was the one with the highest acceptability, reaching levels similar to those obtained by commercial gluten-free bread. With these results, it can be concluded that the fortified samples meet the proposed objectives since they are healthier and have a greater contribution of specific nutrients, and the acceptability of the product compared to the control was higher.

After analyzing the deficiencies of celiac patients before and after establishing a gluten-free diet, it can be determined that the HXT_O and HXT_S samples are a good source of calcium, omega-3, fiber and other minerals such as boron, chromium, copper, magnesium, manganese, phosphorus, silicon and strontium—elements that are usually deficient in gluten-free products and that are beneficial for the health of celiac patients. Taking into account the physicochemical and organoleptic characteristics, the HXT_S sample is the most appropriate to enrich the diet of celiac patients.

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Abbreviations

AAPH	2:2′-azobis(2-amidinopropane)-dihydrochloride
ABTS	2:2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
C	Control
CBC	Citrus extract
Com	Commercial
DPPH	2:2-difenil-1-picrilhidrazilo

FA	Folic acid
FRAP	Ferric Reducing Antioxidant Power
5-FTHF	5-formyltetrahydrofolate
GAE	gallic acid equivalents
HXT_S	hydroxytyrosol synthetic
HXT_O	hydroxytyrosol natural origin
5-MTHF	5-methyltetrahydrofolate
OGYE	oxytetracycline-glucose yeast extract
ORAC	oxygen radical absorbance capacity
PCA	Plate Count Agar
RDI	Reference Daily Intakes
SAX	Strong anion-exchange
SPSS	Statistical Package for the Social Science
THF	Tetrahydrofolate
TPC	Total phenolic content
TPTZ	2:4,6-tripyridyl-s-triazine

References

1. Lebwohl, B.; Sanders, D.S.; Green, P.H.R. Coeliac Disease. *Lancet* **2018**, *391*, 70–81. [CrossRef] [PubMed]
2. Leonard, M.M.; Cureton, P.A.; Fasano, A. Managing Coeliac Disease in Patients with Diabetes. *Diabetes Obes. Metab.* **2015**, *17*, 3–8. [CrossRef] [PubMed]
3. Trott, N.; Kurien, M.; Branchi, F.; Sanders, D. PTH-148 Comparing Dietitian-Led Group Clinics to Individual Appointments for Newly Diagnosed Patients with Coeliac Disease (CD). *Gut* **2016**, *65*, A292. [CrossRef]
4. Shewry, P. What Is Gluten—Why Is It Special? *Front. Nutr.* **2019**, *6*, 101. [CrossRef] [PubMed]
5. Gobbetti, M.; Pontonio, E.; Filannino, P.; Rizzello, C.G.; De Angelis, M.; Di Cagno, R. How to Improve the Gluten-Free Diet: The State of the Art from a Food Science Perspective. *Food Res. Int.* **2018**, *110*, 22–32. [CrossRef]
6. Žilić, S.; Barać, M.; Pešić, M.; Dodig, D.; Ignjatović-Micić, D. Characterization of Proteins from Grain of Different Bread and Durum Wheat Genotypes. *Int. J. Mol. Sci.* **2011**, *12*, 5878–5894. [CrossRef]
7. Jnawali, P.; Kumar, V.; Tanwar, B. Celiac Disease: Overview and Considerations for Development of Gluten-Free Foods. *Food Sci. Hum. Wellness* **2016**, *5*, 169–176. [CrossRef]
8. Wang, K.; Lu, F.; Li, Z.; Zhao, L.; Han, C. Recent Developments in Gluten-Free Bread Baking Approaches: A Review. *Food Sci. Technol.* **2017**, *37*, 1–9. [CrossRef]
9. Breshears, K.L.; Crowe, K.M. Sensory and Textural Evaluation of Gluten-Free Bread Substituted with Amaranth and Montina™ Flour. *J. Food Res.* **2013**, *2*, 1. [CrossRef]
10. Petch, S.; McAuliffe, F.; O'Reilly, S.; Murphy, C.; Coulter-Smith, S.; de Campos, D.A.; Khattak, H.; Mahmood, T. Folic Acid Fortification of Flour to Prevent Neural Tube Defects in Europe—A Position Statement by the European Board and College of Obstetrics and Gynaecology (EBCOG). *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2022**, *279*, 109–111. [CrossRef]
11. Cawley, S.; Mullaney, L.; McKeating, A.; Farren, M.; McCartney, D.; Turner, M.J. A Review of European Guidelines on Periconceptional Folic Acid Supplementation. *Eur. J. Clin. Nutr.* **2016**, *70*, 143–154. [CrossRef]
12. Bearak, J.M.; Popinchalk, A.; Beavin, C.; Ganatra, B.; Moller, A.B.; Tunçalp, Ö.; Alkema, L. Country-Specific Estimates of Unintended Pregnancy and Abortion Incidence: A Global Comparative Analysis of Levels in 2015-2019. *BMJ Glob. Health* **2022**, *7*, e007151. [CrossRef]
13. De Wals, P.; Tairou, F.; Van Allen, M.I.; Uh, S.-H.; Lowry, R.B.; Sibbald, B.; Evans, J.A.; Van den Hof, M.C.; Zimmer, P.; Crowley, M.; et al. Reduction in Neural-Tube Defects after Folic Acid Fortification in Canada. *N. Engl. J. Med.* **2007**, *357*, 135–142. [CrossRef] [PubMed]
14. Williams, L.J.; Mai, C.T.; Edmonds, L.D.; Shaw, G.M.; Kirby, R.S.; Hobbs, C.A.; Sever, L.E.; Miller, L.A.; John Meaney, F.; Levitt, M. Prevalence of Spina Bifida and Anencephaly during the Transition to Mandatory Folic Acid Fortification in the United States. *Teratology* **2002**, *66*, 33–39. [CrossRef]
15. Patring, J.; Wandel, M.; Jägerstad, M.; Frølich, W. Folate Content of Norwegian and Swedish Flours and Bread Analysed by Use of Liquid Chromatography-Mass Spectrometry. *J. Food Compos. Anal.* **2009**, *22*, 649–656. [CrossRef]
16. Salinas, M.V.; Puppo, M.C. Optimization of the Formulation of Nutritional Breads Based on Calcium Carbonate and Inulin. *LWT—Food Sci. Technol.* **2015**, *60*, 95–101. [CrossRef]
17. Hurrell, R.F. Efficacy and Safety of Iron Fortification. In *Food Fortification in a Globalized World*; Academic Press: Cambridge, MA, USA, 2018; pp. 195–212.
18. Cook, J.D.; Minnich, V.; Moore, C.V.; Rasmussen, A.; Bradley, W.B.; Finch, C.A. Absorption of Fortification Iron in Bread. *Am. J. Clin. Nutr.* **1973**, *26*, 861–872. [CrossRef] [PubMed]
19. Lopez De Romaña, D.; Brown, K.H.; Guinard, J.X. Sensory Trial to Assess the Acceptability of Zinc Fortificants Added to Iron-Fortified Wheat Products. *J. Food Sci.* **2002**, *67*, 461–465. [CrossRef]
20. AOAC. *Official Methods of Analysis of Official Analytical Chemist*; AOAC International: Rockville, MD, USA, 1995; p. 684.

21. Prosky, L.; Asp, N.G.; Furda, I.; DeVries, J.W.; Schweizer, T.F.; Harland, B.F. Determination of Total Dietary Fiber in Food and Food Products: Collaborative Study. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 677–679. [CrossRef]
22. FAO/OMS. *Carbohydrates in Human Nutrition: Report of a Joint FAO/OMS Expert Consultation, Roma, 14–18 April 1997*; Estudio FAO Alimentación y Nutrición 66; FAO: Roma, Italy, 1998.
23. Greenfield, H. *Datos de Composición de Alimentos*; FAO: Roma, Italy, 2006; ISBN 9789253049493.
24. Konings, E.J.M. A Validated Liquid Chromatographic Method for Determining Folates in Vegetables, Milk Powder, Liver, and Flour. *J. AOAC Int.* **1999**, *82*, 119–127. [CrossRef]
25. Pfeiffer, C.M.; Rogers, L.M.; Gregory, J.F. Determination of Folate in Cereal-Grain Food Products Using Trienzyme Extraction and Combined Affinity and Reversed-Phase Liquid Chromatography. *J. Agric. Food Chem.* **1997**, *45*, 407–413. [CrossRef]
26. Vahteristo, L.T.; Ollilainen, V.; Koivistoinen, P.E.; Varo, P. Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography. *J. Agric. Food Chem.* **1996**, *44*, 477–482. [CrossRef]
27. Gregory, J.F.; Sartain, D.B.; Day, B.P.F. Fluorometric Determination of Folacin in Biological Materials Using High Performance Liquid Chromatography. *J. Nutr.* **1984**, *114*, 341–353. [CrossRef]
28. Brewer, M.S. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Compr. Rev. Food Sci. Food Saf.* **2011**, *10*, 221–247. [CrossRef]
29. Pérez-Jiménez, J.; Serrano, J.; Tabernero, M.; Arranz, S.; Díaz-Rubio, M.E.; García-Diz, L.; Goñi, I.; Saura-Calixto, F. Effects of Grape Antioxidant Dietary Fiber in Cardiovascular Disease Risk Factors. *Nutrition* **2008**, *24*, 646–653. [CrossRef] [PubMed]
30. Song, G.; Liu, J.; Wang, Q.; Wang, D.; Chu, B.; Li, L.; Xiao, G.; Gong, J.; Zheng, F. Layer-by-Layer Self-Assembly of Hollow Dextran Sulfate/Chitosan-Coated Zein Nanoparticles Loaded with Crocin: Fabrication, Structural Characterization and Potential Biological Fate. *Food Hydrocoll.* **2022**, *125*, 107420. [CrossRef]
31. Niki, E. Assessment of Antioxidant Capacity in Vitro and in Vivo. *Free Radic. Biol. Med.* **2010**, *49*, 503–515. [CrossRef]
32. Shahidi, F.; Zhong, Y. Measurement of Antioxidant Activity. *J. Funct. Foods* **2015**, *18*, 757–781. [CrossRef]
33. Ehlenfeldt, M.K.; Prior, R.L. Oxygen Radical Absorbance Capacity (ORAC) and Phenolic and Anthocyanin Concentrations in Fruit and Leaf Tissues of Highbush Blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222–2227. [CrossRef]
34. Benzie, I.F.F.; Strain, J.J. Ferric Reducing/Antioxidant Power Assay: Direct Measure of Total Antioxidant Activity of Biological Fluids and Modified Version for Simultaneous Measurement of Total Antioxidant Power and Ascorbic Acid Concentration. *Methods Enzymol.* **1999**, *299*, 15–27. [CrossRef]
35. Nimse, S.B.; Pal, D. Free Radicals, Natural Antioxidants, and Their Reaction Mechanisms. *RSC Adv.* **2015**, *5*, 27986–28006. [CrossRef]
36. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT—Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
37. Sánchez-Moreno, C.; Larrauri, J.A.; Saura-Calixto, F. A Procedure to Measure the Antiradical Efficiency of Polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270–276. [CrossRef]
38. Shahidi, F.; Zhong, Y. *Measurement of Antioxidant Activity in Food and Biological Systems*; ACS Symposium Series; ACS Publications: Washington, DC, USA, 2007; Volume 956, pp. 36–66.
39. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef] [PubMed]
40. Junejo, S.A.; Rashid, A.; Yang, L.; Xu, Y.; Kraithong, S.; Zhou, Y. Effects of Spinach Powder on the Physicochemical and Antioxidant Properties of Durum Wheat Bread. *LWT* **2021**, *150*, 112058. [CrossRef]
41. Galla, N.R.; Pamidighantam, P.R.; Karakala, B.; Gurusiddaiah, M.R.; Akula, S. Nutritional, Textural and Sensory Quality of Biscuits Supplemented with Spinach (*Spinacia oleracea* L.). *Int. J. Gastron. Food Sci.* **2017**, *7*, 20–26. [CrossRef]
42. Adam Omer Ishag, O.; Khalid, A.A.; Abdi, A.; Yaagoub Erwa, I.; Babiker Omer, A.; Nour, A.H. Proximate Composition, Physicochemical Properties and Antioxidant Activity of Flaxseed. *Annu. Res. Rev. Biol.* **2020**, *34*, 1–10. [CrossRef]
43. Beheshti Moghadam, M.H.; Rezaei, M.; Behgar, M.; Kermanshahi, H. Effects of Gamma and Electron Radiation on Chemical Composition and Some Phyto-Chemical Properties of Whole Flaxseed. *J. Radioanal. Nucl. Chem.* **2019**, *321*, 1019–1025. [CrossRef]
44. Sibiya, H.; Bhagwat, P.; Amobonye, A.; Pillai, S. Effects of Flaxseed and Soybean Supplementation on the Nutritional and Antioxidant Properties of Mahewu—A South African Beverage. *S. Afr. J. Bot.* **2022**, *150*, 275–284. [CrossRef]
45. Khemiri, S.; Khelifi, N.; Nunes, M.C.; Ferreira, A.; Gouveia, L.; Smaali, I.; Raymundo, A. Microalgae Biomass as an Additional Ingredient of Gluten-Free Bread: Dough Rheology, Texture Quality and Nutritional Properties. *Algal Res.* **2020**, *50*, 101998. [CrossRef]
46. Krupa-Kozak, U.; Drabińska, N.; Baczek, N.; Šimková, K.; Starowicz, M.; Jeliński, T. Application of Broccoli Leaf Powder in Gluten-Free Bread: An Innovative Approach to Improve Its Bioactive Potential and Technological Quality. *Foods* **2021**, *10*, 819. [CrossRef] [PubMed]
47. Bernacchia, R.; Preti, R.; Vinci, G. Chemical Composition and Health Benefits of Flaxseed. *Austin J. Nutr. Food Sci.* **2014**, *2*, 1045.
48. Cunnane, S.C.; Ganguli, S.; Menard, C.; Liede, A.C.; Hamadeh, M.J.; Chen, Z.-Y.; Wolever, T.M.S.; Jenkins, D.J.A. High α-Linolenic Acid Flaxseed (*Linum usitatissimum*): Some Nutritional Properties in Humans. *Br. J. Nutr.* **1993**, *69*, 443–453. [CrossRef] [PubMed]
49. Dur, S.; Allai, F.M.; Ganai, S.; Gul, K.; Majeed, D.; Jabeen, A. Effect of Fortification on Textural, Microbiological and Physico-Chemical Properties of Bread. *Carpathian J. Food Sci. Technol.* **2019**, *11*, 41–51.

50. Martínez-Zamora, L.; Ros, G.; Nieto, G. Designing a Clean Label Fish Patty with Olive, Citric, Pomegranate, or Rosemary Extracts. *Plants* **2020**, *9*, 659. [CrossRef]
51. Filip, S.; Vidrih, R. Amino Acid Composition of Protein-Enriched Dried Pasta: Is It Suitable for a Low-Carbohydrate Diet? *Food Technol. Biotechnol.* **2015**, *53*, 298–306. [CrossRef]
52. do Carmo, J.S.; Nazareno, L.S.Q.; Rufino, M.D.S.M. Characterization of the Acerola Industrial Residues and Prospection of Their Potential Application as Antioxidant Dietary Fiber Source. *Food Sci. Technol.* **2018**, *38*, 236–241. [CrossRef]
53. Bekhit, A.E.D.A.; Shavandi, A.; Jodjaja, T.; Birch, J.; Teh, S.; Mohamed Ahmed, I.A.; Al-Juhaimi, F.Y.; Saeedi, P.; Bekhit, A.A. Flaxseed: Composition, Detoxification, Utilization, and Opportunities. *Biocatal. Agric. Biotechnol.* **2018**, *13*, 129–152. [CrossRef]
54. El-Sayed, S.M. Use of Spinach Powder as Functional Ingredient in the Manufacture of UF-Soft Cheese. *Heliyon* **2020**, *6*, e03278. [CrossRef]
55. Collar, C. Gluten-Free Dough-Based Foods and Technologies. In *Sorghum and Millets: Chemistry, Technology, and Nutritional Attributes*; AACC International Press: Washington, DC, USA, 2018; pp. 331–354. ISBN 9780128115275.
56. Salmerón, J.; Ascherio, A.; Rimm, E.B.; Colditz, G.A.; Spiegelman, D.; Jenkins, D.J.; Stampfer, M.J.; Wing, A.L.; Willett, W.C. Dietary Fiber, Glycemic Load, and Risk of NIDDM in Men. *Diabetes Care* **1997**, *20*, 545–550. [CrossRef]
57. Schulze, M.B.; Liu, S.; Rimm, E.B.; Manson, J.A.E.; Willett, W.C.; Hu, F.B. Glycemic Index, Glycemic Load, and Dietary Fiber Intake and Incidence of Type 2 Diabetes in Younger and Middle-Aged Women. *Am. J. Clin. Nutr.* **2004**, *80*, 348–356. [CrossRef]
58. McLaren, D.S. Glycemic Index of Foods: A Physiological Basis for Carbohydrate Exchange—Not Fade Away—The Glycemic Index. *Nutrition* **2000**, *16*, 151–152. [CrossRef] [PubMed]
59. Jha, A.B.; Ashokkumar, K.; Diapari, M.; Ambrose, S.J.; Zhang, H.; Tar'an, B.; Bett, K.E.; Vandenberg, A.; Warkentin, T.D.; Purves, R.W. Genetic Diversity of Folate Profiles in Seeds of Common Bean, Lentil, Chickpea and Pea. *J. Food Compos. Anal.* **2015**, *42*, 134–140. [CrossRef]
60. López-Nicolás, R.; Frontela-Saseta, C.; González-Abellán, R.; Barado-Piqueras, A.; Perez-Conesa, D.; Ros-Berruezo, G. Folate Fortification of White and Whole-Grain Bread by Adding Swiss Chard and Spinach. Acceptability by Consumers. *LWT—Food Sci. Technol.* **2014**, *59*, 263–269. [CrossRef]
61. AECOC. Crece El Consumo de Productos Sin Gluten En España. Available online: https://www.aecoc.es/noticias/crece-el-consumo-de-productos-sin-gluten-en-espana/ (accessed on 9 March 2022).
62. MAGRAMA. *Informe Del Consumo Alimentario En España 2020*; Ministerio de Agricultura, Pesca y Alimentación: Madrid, India, 2021.
63. Brevik, A.; Vollset, S.E.; Tell, G.S.; Refsum, H.; Ueland, P.M.; Loeken, E.B.; Drevon, C.A.; Andersen, L.F. Plasma Concentration of Folate as a Biomarker for the Intake of Fruit and Vegetables: The Hordaland Homocysteine Study. *Am. J. Clin. Nutr.* **2005**, *81*, 434–439. [CrossRef] [PubMed]
64. Di Nardo, G.; Villa, M.P.; Conti, L.; Ranucci, G.; Pacchiarotti, C.; Principessa, L.; Raucci, U.; Parisi, P. Nutritional Deficiencies in Children with Celiac Disease Resulting from a Gluten-Free Diet: A Systematic Review. *Nutrients* **2019**, *11*, 1588. [CrossRef]
65. Vici, G.; Belli, L.; Biondi, M.; Polzonetti, V. Gluten Free Diet and Nutrient Deficiencies: A Review. *Clin. Nutr.* **2016**, *35*, 1236–1241. [CrossRef]
66. Pennington, J.A.T.; Schoen, S.A. Estimates of Dietary Exposure to Aluminium. *Food Addit. Contam.* **1995**, *12*, 119–128. [CrossRef]
67. Soni, M.G.; White, S.M.; Flamm, W.G.; Burdock, G.A. Safety Evaluation of Dietary Aluminum. *Regul. Toxicol. Pharmacol.* **2001**, *33*, 66–79. [CrossRef]
68. Jenkins, D.J.A.; Wolever, T.M.S.; Taylor, R.H.; Barker, H.; Fielden, H.; Baldwin, J.M.; Bowling, A.C.; Newman, H.C.; Goff, D.V. Glycemic Index of Foods: A Physiological Basis for Carbohydrate Exchange. *Am. J. Clin. Nutr.* **1981**, *34*, 362–366. [CrossRef]
69. Schoof, R.A.; Yost, L.J.; Eickhoff, J.; Crecelius, E.A.; Cragin, D.W.; Meacher, D.M.; Menzel, D.B. A Market Basket Survey of Inorganic Arsenic in Food. *Food Chem. Toxicol.* **1999**, *37*, 839–846. [CrossRef]
70. Sherlock, J.C.; Smart, G.A. Thallium in Foods and the Diet. *Food Addit. Contam.* **1986**, *3*, 363–370. [CrossRef]
71. Anderson, D.L.; Cunningham, W.C.; Mackey, E.A. Determination of Boron in Food and Biological Reference Materials by Neutron Capture Prompt-γ Activation. *Fresenius' J. Anal. Chem.* **1990**, *338*, 554–558. [CrossRef]
72. Salinas, F.; De La Peña, A.M.; Murillo, J.; Jiménez Sånchez, J.C. Spectrofluorimetric Determination of Boron in Plants with Quinizarin-2-Sulphonic Acid. *Analyst* **1987**, *112*, 913–915. [CrossRef]
73. Anderson, R.A.; Bryden, N.A.; Polansky, M.M. Dietary Chromium Intake—Freely Chosen Diets, Institutional Diets, and Indidual Foods. *Biol. Trace Elem. Res.* **1992**, *32*, 117–121. [CrossRef] [PubMed]
74. Bhattacharjee, S.; Dasgupta, P.; Paul, A.R.; Ghosal, S.; Padhi, K.K.; Pandey, L.P. Mineral Element Composition of Spinach. *J. Sci. Food Agric.* **1998**, *77*, 456–458. [CrossRef]
75. Rocchetti, G.; Lucini, L.; Rodriguez, J.M.L.; Barba, F.J.; Giuberti, G. Gluten-Free Flours from Cereals, Pseudocereals and Legumes: Phenolic Fingerprints and in Vitro Antioxidant Properties. *Food Chem.* **2019**, *271*, 157–164. [CrossRef] [PubMed]
76. Di Cairano, M.; Galgano, F.; Tolve, R.; Caruso, M.C.; Condelli, N. Focus on Gluten Free Biscuits: Ingredients and Issues. *Trends Food Sci. Technol.* **2018**, *81*, 203–212. [CrossRef]
77. Moore, M.M.; Dal Bello, F.; Arendt, E.K. Sourdough Fermented by Lactobacillus Plantarum FST 1.7 Improves the Quality and Shelf Life of Gluten-Free Bread. *Eur. Food Res. Technol.* **2008**, *226*, 1309–1316. [CrossRef]
78. Wronkowska, M.; Haros, M.; Soral-Śmietana, M. Effect of Starch Substitution by Buckwheat Flour on Gluten-Free Bread Quality. *Food Bioprocess Technol.* **2013**, *6*, 1820–1827. [CrossRef]

79. Lemonakis, N.; Poudyal, H.; Halabalaki, M.; Brown, L.; Tsarbopoulos, A.; Skaltsounis, A.L.; Gikas, E. The LC–MS-Based Metabolomics of Hydroxytyrosol Administration in Rats Reveals Amelioration of the Metabolic Syndrome. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2017**, *1041–1042*, 45–59. [CrossRef] [PubMed]
80. Martínez-zamora, L.; Ros, G.; Nieto, G. Synthetic vs. Natural Hydroxytyrosol for Clean Label Lamb Burgers. *Antioxidants* **2020**, *9*, 851. [CrossRef] [PubMed]
81. Drabińska, N.; Ciska, E.; Szmatowicz, B.; Krupa-Kozak, U. Broccoli By-Products Improve the Nutraceutical Potential of Gluten-Free Mini Sponge Cakes. *Food Chem.* **2018**, *267*, 170–177. [CrossRef] [PubMed]
82. Lin, J.; Zhou, W. Role of Quercetin in the Physicochemical Properties, Antioxidant and Antiglycation Activities of Bread. *J. Funct. Foods* **2018**, *40*, 299–306. [CrossRef]
83. Frankel, E.N.; Meyer, A.S. The Problems of Using One-Dimensional Methods to Evaluate Multifunctional Food and Biological Antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941. [CrossRef]
84. Duthie, G.; Campbell, F.; Bestwick, C.; Stephen, S.; Russell, W. Antioxidant Effectiveness of Vegetable Powders on the Lipid and Protein Oxidative Stability of Cooked Turkey Meat Patties: Implications for Health. *Nutrients* **2013**, *5*, 1241–1252. [CrossRef] [PubMed]
85. Branciari, R.; Galarini, R.; Giusepponi, D.; Trabalza-Marinucci, M.; Forte, C.; Roila, R.; Miraglia, D.; Servili, M.; Acuti, G.; Valiani, A. Oxidative Status and Presence of Bioactive Compounds in Meat from Chickens Fed Polyphenols Extracted from Olive Oil Industry Waste. *Sustainability* **2017**, *9*, 1566. [CrossRef]
86. Warleta, F.; Quesada, C.S.; Campos, M.; Allouche, Y.; Beltrán, G.; Gaforio, J.J. Hydroxytyrosol Protects against Oxidative DNA Damage in Human Breast Cells. *Nutrients* **2011**, *3*, 839–857. [CrossRef]
87. Paciulli, M.; Rinaldi, M.; Cirlini, M.; Scazzina, F.; Chiavaro, E. Chestnut Flour Addition in Commercial Gluten-Free Bread: A Shelf-Life Study. *LWT* **2016**, *70*, 88–95. [CrossRef]
88. Jensen, S.; Oestdal, H.; Clausen, M.R.; Andersen, M.L.; Skibsted, L.H. Oxidative Stability of Whole Wheat Bread during Storage. *LWT* **2011**, *44*, 637–642. [CrossRef]
89. Jensen, S.; Ostdal, H.; Skibsted, L.H.; Thybo, A.K. Antioxidants and Shelf Life of Whole Wheat Bread. *J. Cereal Sci.* **2011**, *53*, 291–297. [CrossRef]
90. Martínez, L.; Castillo, J.; Ros, G.; Nieto, G. Antioxidant and Antimicrobial Activity of Rosemary, Pomegranate and Olive Extracts in Fish Patties. *Antioxidants* **2019**, *8*, 86. [CrossRef] [PubMed]
91. Shapira, R.; Mimran, E. Isolation and Characterization of Escherichia Coli Mutants Exhibiting Altered Response to Thymol. *Microb. Drug Resist.* **2007**, *13*, 157–165. [CrossRef] [PubMed]
92. Ranucci, D.; Roila, R.; Andoni, E.; Braconi, P.; Branciari, R. Punica Granatum and Citrus Spp. Extract Mix Affects Spoilage Microorganisms Growth Rate in Vacuum-Packaged Cooked Sausages Made from Pork Meat, Emmer Wheat (*Triticum dicoccum* Schübler), Almond (*Prunus dulcis* Mill.) and Hazelnut (*Corylus avellana* L.). *Foods* **2019**, *8*, 664. [CrossRef] [PubMed]
93. Azaizeh, H.; Tafesh, A.; Najami, N.; Jadoun, J.; Halahlih, F.; Riepl, H. Synergistic Antibacterial Effects of Polyphenolic Compounds from Olive Mill Wastewater. *Evid.-Based Complement. Altern. Med.* **2011**, *2011*, 431021. [CrossRef]
94. Munekata, P.E.S.; Nieto, G.; Pateiro, M.; Lorenzo, J.M. Phenolic compounds obtained from olea europaea by-products and their use to improve the quality and shelf life of meat and meat products—A review. *Antioxidants* **2020**, *9*, 1061. [CrossRef] [PubMed]
95. Nieto, G.; Bañón, S.; Garrido, M. Administration of distillate thyme leaves into the diet of Segureña ewes: Effect on lamb meat quality. *Animal* **2012**, *6*, 2048–2056. [CrossRef] [PubMed]
96. Diario el Peruano. *Norma Sanitaria Que Establece Los Criterios Microbiologicos de Calidad Sanitaria e Inocuidad Para Los Alimentos y Bebidas de Consumo Humano*; Ministerio de Salud de la República del Perú: Lima, Peru, 2008.
97. Zhou, Q.; Liang, W.; Wan, J.; Wang, M. Spinach (*Spinacia oleracea*) Microgreen Prevents the Formation of Advanced Glycation End Products in Model Systems and Breads. *Curr. Res. Food Sci.* **2023**, *6*, 100490. [CrossRef] [PubMed]