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Primary and secondary shelf-life of bread as a function of formulation and MAP conditions: Focus on physical-chemical and sensory markers

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ABSTRACT

Bread is one of the most widely consumed foods worldwide, and extending its shelf-life is a key concern for reducing waste, especially in light of the expected increase in the world population. This study aimed to assess how the bread formulation (flour, leavening agent) or storage conditions (modified atmosphere packaging (Air or Ar)) could influence its primary shelf-life (PSL) and secondary shelf-life (SSL), also determining possible physical, chemical and sensory markers. The results revealed that the choice of leavening agent had a significant effect on the PSL of bread, especially when combined with the gas used in the packaging. Compared to Air, Ar combined with sourdough slowed down weight loss and the staling process and allows bread to have a longer shelf-life, preserving its initial characteristics. The same synergistic effect was not observed for bread made with baker's yeast, suggesting the potential need of employing a different storage gas in the packaging. Indeed, for the SSL, the only effects detected are related to the leavening agents, where the sourdough exhibits a longer shelf-life compared to the baker's yeast. These findings lead us to conclude that easily and quickly measurable parameters such as weight loss and water activity decrease, together with sensory analysis, can be used as markers to assess the PSL and SSL of bread.

1. Introduction

Common wheat (*Triticum aestivum*) is one of the most important cereal crops worldwide. It is rich in calories, dietary fiber, vitamins, minerals, amino acids and bioactive compounds (Bianchi et al., 2023c; Taglieri et al., 2021a), therefore the wheat-based products are fundamental in human nutrition (Dapčević-Hadnađev et al., 2022).

In the European area breadmaking is an ancient and deeply rooted cultural tradition, especially in Italy where approximately 3.2 million tons of bread are annually consumed (Taglieri et al., 2021a). In the different Italian regions bread has evolved along with local history and customs, adapting its recipe to specific flour and dough characteristics influenced by local varieties and environmental conditions (Sacchi et al., 2019). For this reason, many traditional breads obtained the European Protected Geographical Indication (PGI) or even the Protected Designation of Origin (PDO) product certifications, such as the PDO Tuscan bread produced with local varieties of common wheat and the use of sourdough for the fermentation.

Especially today, consumers ask for high quality bakery products from both nutritional and sensory perspective (Bianchi et al., 2023c, 2022b; Taglieri et al., 2021a; Venturi et al., 2016). In this context, ancient and local wheat varieties are the focus of an increasing attention supported by many studies that show their higher and healthier profile in terms of phytochemical lipids, soluble dietary fiber, and minerals (Angioloni & Collar, 2011; Dapčević-Hadnađev et al., 2022; Dinu et al., 2018; Sacchi et al., 2019; Shewry & Hey, 2015; Suchowilska et al., 2012); however the scientific literature is lacking in understanding wheat variety role and effect on the bakery product shelf-life.

Besides, the increase in the world population and the global challenge of providing food for all push us to try to improve not only the quantity of production but, above all, the nutritional quality and shelf-life of bread (Bianchi et al., 2022b; Taglieri et al., 2021a).

There are two types of shelf-life for food products: primary shelf-life (PSL), which is the time after packaging during which the food maintains a predetermined level of quality under specified storage conditions, and secondary shelf-life (SSL), which is the length of time during

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which a food product retains its characteristics after opening the package (Bianchi et al., 2022a; Nicoli & Calligaris, 2018; Torrieri, 2016).

Many research papers in literature are based on the study of kinetics of PSL of bread and other food matrix (Bianchi et al., 2022b; Caballero et al., 2007; Jensen et al., 2011; Taglieri et al., 2021b), but few of them focus on SSL (Anese et al., 2006; Bianchi et al., 2023b; Lacivita et al., 2023). This is due to the difficulty of defining the experimental conditions as the package may be opened at different moments thorough the primary shelf-life; in addition, it causes a sudden change in environmental conditions (presence of oxygen, composition of the atmosphere, temperature fluctuations, humidity, etc.), determining a strong acceleration in the rate of product quality decay (Calligaris et al., 2016; Manzocco et al., 2010; Nicoli & Calligaris, 2018; Robertson, 2009; Taglieri et al., 2021a), thus making it really difficult to predict the final SSL (Bianchi et al., 2023b; Corradini, 2018).

Bread is a dynamic system undergoing physical-chemical, sensory and microbiological changes which limit its shelf-life. Physicochemical and sensory changes determine loss of freshness, in terms of desirable texture and taste, and lead to the progressive firming-up of the crumb (Taglieri et al., 2021a). Microbiological spoilage by yeasts, bacteria and molds consists of visible mold growth and invisible production of mycotoxins and formation of off-flavors (Galić et al., 2009).

The bakery industry and the scientific community have long been working to identify and implement strategies and methods which allow a longer bread shelf-life, the lowest number of changes in bread organoleptic quality and also bread safety.

Physical methods like infrared, ultraviolet light, microwave heating, ultra-high pressure treatments are used to destroy post-baking contaminants (Melini & Melini, 2018; Smith et al., 2004). Chemical preservatives, such as acetic acid, potassium acetate, sodium acetate, and others are applied in accordance with the limits laid down on food additives by the European Regulation (EC 1333/2008) (Melini & Melini, 2018).

The difficulty in clearly defining what shelf-life is has had repercussions on the methodologies developed to carry out its evaluation. This is especially true for the food industry, where companies create commonly their own shelf-life assessment methods with sometimes dubious scientific basis, as they lack a comprehensive and unified perspective on the issue of shelf-life, as well as accessible approaches economically advantageous (Calligaris et al., 2012; Nicoli & Calligaris, 2018). Nevertheless, a definition of the food SSL and the best operating conditions to extend it to the maximum, could provide a viable approach for reducing food waste not only at the industrial level but also in household use (Bianchi et al., 2023b; Corradini, 2018; Wood & Neal, 2009).

Sourdough has also recently become an established form of food biopreservation, thanks to its low pH and the high concentration of lactic and acetic acid, which help to reduce spoilage by molds and slow the staling process (Bianchi et al., 2022b; Taglieri et al., 2021a). Moreover, several studies (Bianchi et al., 2023c; Colosimo et al., 2020; Pejcz et al., 2021) have confirmed how the sourdough fermentation increases the availability of phenolic compounds and the consequent antioxidant capacity of the flour used. Its use also influences the sensory profile of bread, which exhibits more complex flavor and higher sapidity than industrial bread made with a different leavening agent (Ma et al., 2021; Venturi et al., 2016). Conversely, the use of baker's yeast is preferred for industrial application thanks to its technological properties (Struyf et al., 2017), as it simplifies the production process and involves lower costs (Gélinas, 2014).

Another possible widespread strategy to extend the shelf-life of bread, avoiding the addition of preservatives, is the use of appropriate modified atmosphere packaging (MAP) (Kotsianis et al., 2002). Generally, when MAP was utilized to store bakery products, the gas mixture consists of 60% or more CO_2 with N_2 (Axel et al., 2017; Fernandez et al., 2006). Due to the high water content of bread, CO_2 can dissolve in water of products to form carbonic acid, leading to a lowering of the pH (Sanguinetti et al., 2016). Specifically, a crucial requirement for preventing mold growth was to keep the oxygen (O_2) level below 0.4%. This observation aligns with existing research demonstrating the correlation between O_2 content and fungal growth in other products subjected to MAP condition (Sanguinetti et al., 2016; Stamatis & Arkoudelos, 2007; Taniwaki et al., 2001). Nevertheless, the conclusive impact of CO_2 in the MAP on the bread quality appears still contradictory (Upasen & Wattanachai, 2018) because the high concentration of CO_2 may determine an increase in perceived acidity to the taste (Fik et al., 2012; Stamatis & Arkoudelos, 2007; Suppakul et al., 2016).

In this regard, as recently showed (Bianchi et al., 2022b), the use of 100% Ar as filler gas may be a viable approach to preserving sourdough bread, showing good results both from a chemical-physical and especially sensory point of view; the use of CO_2 as a gas is not practicable for sourdough bread because it increases the perception of acidity in the product.

On the basis of previous studies, especially considering the lack in literature about this topic, this research aims to evaluate how different formulation (flour and leavening agent) can affect the Primary Shelf-life (PSL) and Secondary shelf-life (SSL) of bread stored in modified atmosphere (Air or Ar) packaging, focusing on the determination of possible its physical-chemical and sensory markers for their rapid evaluation.

2. Materials and methods

2.1. Raw materials and breadmaking process

A mix of four varieties of common wheat (*Triticum aestivum*), namely Bolero, Verna, Pandas, and Bologna, provided by the University of Florence, was milled to obtain a strong wheat flour type 0, generally used for PDO Tuscan bread and used as Control (C) in this research. Mix (M) was obtained from a combination of two flours: the Control one and an old variety ("Avanzi 3-Grano 23″) of common wheat (*Triticum aestivum*), provided by the University of Pisa, mixed in 1:1 ratio (w/w). These flours were used in a previous work evaluating their properties and exploring the potentiality of the old variety for breadmaking (Bianchi et al., 2023c). Chemical and technological features of both flours (C and M) are shown in Table S1.

The sourdough used was stored at the Food Technologies laboratory of the University of Pisa, daily back slopped and periodically controlled, as previously described (Bianchi et al., 2023c). Baker's yeast was a compressed yeast (Zeus Iba s.r.l., Firenze, Italy) available on the market.

The breadmaking was performed using the "biga" pre-ferment method (sourdough (S) or baker's yeast (Y)); the recipe was defined in previous studies (Bianchi et al., 2023b; Taglieri et al., 2021b). The characterization of the two biga (S-biga and Y-biga) is reported in Table S2.

The formulation and procedure for the preparation of the biga, the dough and bread (SB-M, SB-C and YB-M, YB-C) using M and C flours are reported in the Fig. 1.

2.2. Chemical characterization of samples

The characterization of flours (humidity (% w/w), proteins (% w/w), ashes (% w/w), total dietary fiber (% w/w), total fats (% w/w), sugars (maltose, glucose, fructose, sucrose) (% w/w),total starch (% w/w), falling number (seconds), dry gluten (% w/w), wet gluten (% w/w) and Chopin alveogram value) was performed as previously reported (Bianchi et al., 2022b).

pH, total titratable acidity (TTA) (meq lactic acid/g dw), acetic acid (mmol/kg dw), lactic acid (mmol/kg dw), ethanol(mmol/kg dw) and dry weight (dw) of samples (biga or bread) were assessed as previously reported (Bianchi et al., 2022b).

Moreover, the total polyphenols (mg gallic acid equivalents (GAE)/kg dw) (Macaluso et al., 2021), total flavonoids (mg catechin equivalents (CE)/kg dw), and anti-radical activity (DPPH, ABTS and FRAP)

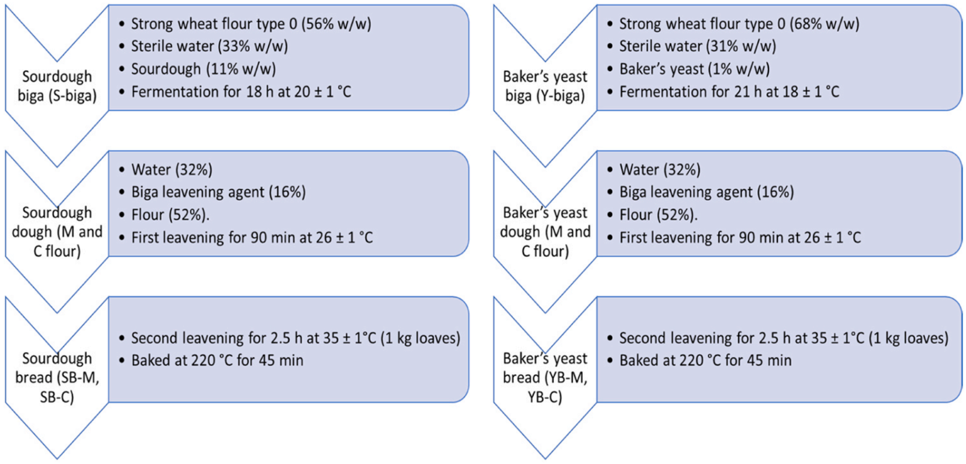

Fig. 1. The formulation and procedure for the preparation of the biga, the dough and bread.

(μmol TE/g dw) (Bianchi et al., 2023c) of samples (flour, biga, breads) were determined.

2.3. PSL and SSL assessment

40 loaves (1 kg each) were baked, cooled for 2 h at room temperature (22 ± 1 °C), then sliced by an automatic slicing machine (20 mm thickness).

For the PSL, plastic bags (outer nylon layer, two plastic layers, Food Saver, Moncalieri, Torino, Italy; the characteristics of the plastic film are provided in Table S3) were used to pack each slice individually in 2 different MAPs (Air (100%) or Ar (100%)), by 450 GAS packaging machine (Lavezzini, Fiorenzuola d'Arda, Piacenza, Italy). Single slices were used to evaluate the process in an accelerated PSL, due to their wider exposed surface, which determines a faster evolution of parameters, as reported in previous study (Bianchi et al., 2022b). A total of 100 packages for each combination of flour (C and M), leavening agent (S and Y) and MAP (Air and Ar)) were stored at T = 22 ± 1 °C during the whole observation period. Four packages were daily opened and examined for weight loss, water activity (a_w), softness of the crumb and the results were expressed as a percentage compared to the starting value as previously described (Bianchi et al., 2022b). All the samples were checked daily for the presence of mold; each experimental run was stopped when 3% of the samples showed mold spoilage (Fig. S1).

For SSL assessment, 4 whole sliced loaves of each leavening agent (S and Y), for each flour (C and M) and in the two different MAPs (Ar and Air) were packaged in a plastic bag. To simulate the SSL of bread, the packages were stored at a controlled temperature (22 ± 1 °C) and after 24 h were opened. The opening times were scheduled considering the average SSL of an additive-free bread (Taglieri et al., 2021a). 4 slices were used for the sensory analysis, then the package was manually closed to simulate the domestic use. The process was repeated daily, until the appearance of molds on the surface of bread.

The sensory bread profile was evaluated by a panel of 10 trained judges (aged between 23 and 60 years) of the Department of Agriculture, Food, and Environment Sciences of the University of Pisa.

The tasting was carried out according to the protocol previously developed (Taglieri et al., 2021b) and using a sensory sheet composed by 7 parameters (visual attractiveness, olfactory pleasantness, texture, tasting pleasantness, aftertaste, acceptability, global pleasantness).

Finally, the overall hedonic index (HI) of bread, representing the overall acceptability of the product, was calculated starting from the mean of the parameters converted on a scale from 0 to 10, as previously reported (Bianchi et al., 2022b).

The evolution of this parameter was used as a marker of the acceptability of the product in the evaluation of PSL and SSL, as reported and validated in other works (Bianchi et al., 2023b, 2022b; Taglieri et al., 2021b). Moreover, the value of HI = 6 was used as a limit of acceptability to define the end of sensory shelf-life of the bread samples.

2.4. Statistical analysis

CoStat v. 6.451 software (CoHort Software, Birmingham, UK) were used to apply one-way ANOVA and Tukey's HSD test ($p < 0.05$) for the significance separation of the samples. The JMP v.17 software (SAS Institute, Cary, NC, USA) was used to describe the trend of the shelf-life parameters (weight, water activity, and softness).

Finally, the Big Sensory Soft 2.0 software (v. 2018) was utilized on the sensory data using two-way ANOVA with panelists and samples taken as main factors.

3. Results and discussion

3.1. PSL evaluation

Water migration, which is one of the main causes of the staling phenomenon, is the relocation of free water molecules as a result of the gradient that exists between various parts of the product, primarily from the crumb to the crust (Taglieri et al., 2021a; Wang et al., 2015). Due to the process of starch recrystallization, the water contained in the gel portions slowly leaks out during storage, causing the characteristic loss of softness of the crumb in bread staling (Monteau et al., 2017; Ottenhof & Farhat, 2004).

The decrease in weight and water activity can be correlated to the decrease in softness (Bianchi et al., 2023a, 2022b), an useful marker for assessing the bread PSL. Therefore, the linear regressions of the decrease in weight (Fig. S2) along with the decrease of water activity (Fig. S3) were calculated. The slopes of the regression line and the R^2 are reported

Table 1
Linear regression parameters (slope and coefficient of determination (R^2)) for the decrease of weight and water activity (a_w) of the bread samples during storage.

Sample	Slope (weight)	R^2	Slope (a_w)	R^2
YB-C-Air	0.756^a	0.996	0.474^a	0.969
YB-M-Air	0.725^a	0.993	0.489^a	0.964
YB-C-Ar	0.593^b	0.999	0.421^b	0.995
YB-M-Ar	0.524^c	0.992	0.371^{bc}	0.974
SB-C-Air	0.580^b	0.998	0.409^b	0.983
SB-M-Air	0.526^c	0.995	0.360^c	0.991
SB-C-Ar	0.458^d	0.998	0.290^d	0.985
SB-M-Ar	0.457^d	0.997	0.293^d	0.984

In the column, different letters indicate a statistically different value ($p < 0.05$).

in Table 1.

By analyzing the slopes of the lines which are an expression of the speed of the process, it is possible to observe that the leavening agents and atmosphere of storage are key factors in determining the rate of bread dehydration. In particular, M and C flours slow down the rate of water loss, when sourdough as leavening agent and Argon as filling gas are used. Furthermore, as shown in Table 1, the leavening agents have the major effect on the PSL: in fact, the slope values obtained for the SB-C-Air and SB-M-Air breads were statistically the same of the respective samples made with baker's yeast and preserved in Ar (YB-C-Ar and YB-M-Ar).

Weight and water activity decreases were faster when bread was stored in Air (Table 1) and produced with baker's yeast. This was due to the production of lactic and acetic acid in sourdough breads compared to baker's yeast ones (Bianchi et al., 2022b; Taglieri et al., 2021a) and resulted in highest TTA and lowest pH (Table S4). No significant difference was found between the two flours.

PSL time was affected by phytochemical characteristics among other parameters. Interestingly, C breads were generally richer in polyphenols, resulting in higher antioxidant activity (Table S3), also due to the phytochemical profile of C flour (Table S4). However, it is noteworthy that the M flour and sourdough combination (SB-M) seems to determine a significant increase in phytochemical compounds than its starting value (Table S4), which was not detected for the baker's yeast breads (YB-C and YB-M) (Table S3). According to the literature (Bianchi et al., 2023c; Colosimo et al., 2020; Dapčević-Hadnađev et al., 2022; Pejcz et al., 2021), the lower pH of sourdough breads can actually improve the bioavailability of phytochemical compounds. This confirmed the results obtained in a previous research work (Bianchi et al., 2023c), although no significant difference in bread shelf-life trend (Table 1) seems to be related with the two flours (C and M), probably because the high level of phytochemical compound has a sufficient effect against mold in both types of flours.

The rate of decrease of water activity is generally lower than that of weight (Table 1). This could be due to the equilibrium between these two opposite effects in bread over time. In fact, during storage, the water tends to evaporate, resulting in a decrease of a_w that varies according to relative humidity outside. Moreover, a part of the water immobilized during starch gelatinization is released during the recrystallization of starch granules, thus increasing a_w (Brancoli et al., 2019; Curti et al., 2016; Rasmussen & Hansen, 2001). Besides, the softness of bread slices declines over time as a result of the staling process associated with the starch retrogradation.

The same trend of weight loss was observed for the decrease in softness (Fig. 2), and the lowest rate was measured for breads produced with sourdough and preserved in Ar (SB-C-Ar and SB-M-Ar); conversely, the use of baker's yeast and Air as filler gas (YB-C-Air and YB-M-Air) significantly reduced the PSL of bread.

One of the main causes of the organoleptic quality decay of bread is the formation of molds, as they lead to the development of off-flavors (mainly alcohols and esters) and abnormal color spots on the product surface.

As shown in Fig. 3, mold formation occurred later in slices stored in Ar compared to those stored in Air. In addition, the development of mold seems to have been slowed down using sourdough, regardless of the atmosphere adopted during storage. These results confirm how the combined effect of the absence of oxygen, due to the storage in Ar, and the antimicrobial effect of sourdough can significantly extend bread shelf-life, by slowing the development of mold.

The same synergistic effect with the leavening agent, was not observed in bread produced with the baker's yeast, due to its higher pH; this is the reason why the use of a different MAP such as CO_2 or a mix with other gas (Ar o N_2) might be more effective, as reported in literature (Bianchi et al., 2022b; Fernandez et al., 2006; Khoshakhlagh et al., 2014; Rasmussen & Hansen, 2001). This highlights that the leavening agent has the greatest effect and that a combination of different strategies is required to prolong bread shelf-life.

Samples were also subjected to daily panel tests during the whole observation period. As expected, staling caused a decline in overall pleasantness during storage; however, breads produced with sourdough were still acceptable (HI > 6) at the end of trials, irrespective of the gas used for storage (Fig. 5), thus confirming the main influence of the leavening agent used, as discussed above.

Assuming gas atmosphere as the main effect (Figs. 4 and 5), bread stored in 100% Ar exhibited the best sensory profile; in particular, the slowest sensory decay was observed for the sourdough breads stored in argon (SB-C-Ar and SB-M-Ar) which better maintain their aromatic profiles.

Figs. 4 and 5 shows how the trend of HI could overlap with the trend of weight loss and a_w decrease: in fact, the slope for breads produced with sourdough and stored in Air (SB-C-Air and SB-M-Air) was the same of breads produced with baker's yeast but preserved in Ar (YB-C-Ar and

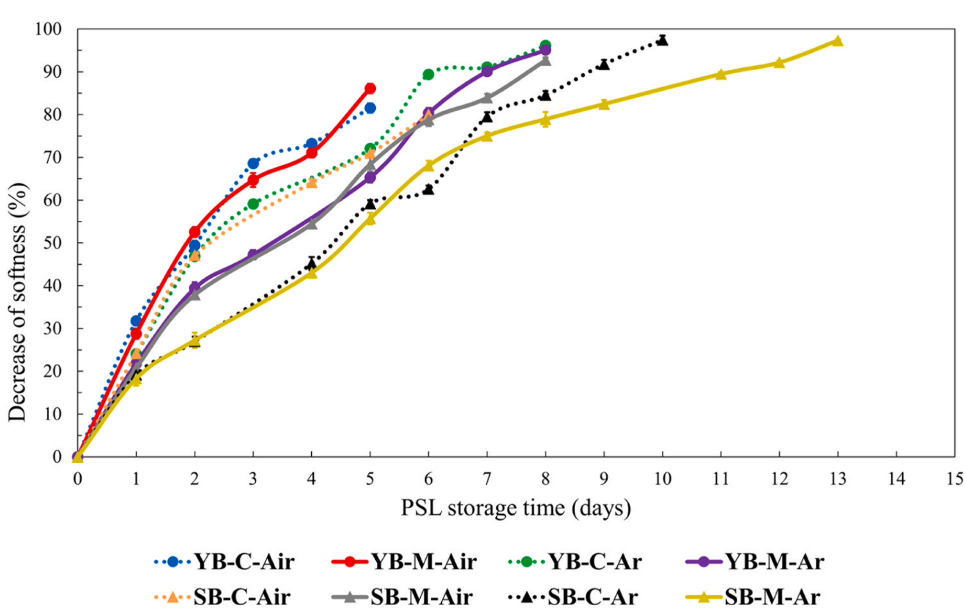

Fig. 2. Reduction of bread softness (%) as a function of PSL storage time (days). Results are expressed as mean ± SD ($n = 4$).

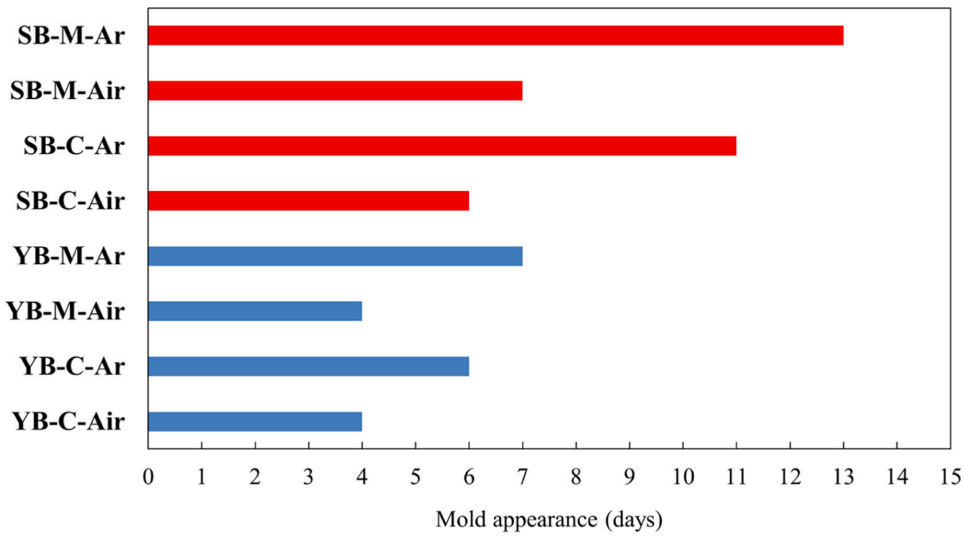

Fig. 3. Mold appearance on the surface of bread (days).

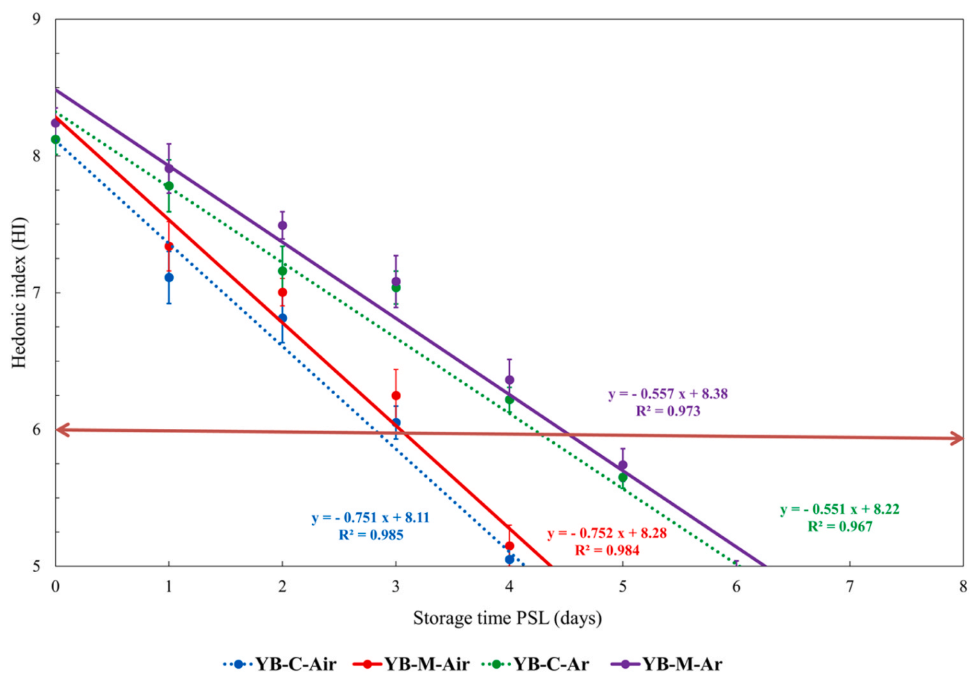

Fig. 4. Hedonic index of the different baker's yeast bread in the 2 system of MAP during PSL storage time.

YB-M-Ar), confirming how the leavening agent has a stronger effect compared to the MAP used.

3.2. SSL evaluation

SSL is more difficult to predict since it is strongly influenced by the opening time of the package. In this work, the samples were opened one day after packaging (t = 0) to simulate the domestic use.

Fig. 6 shows the evolution of HI, used as marker of SSL. No statistically difference was detected between the slope of bread stored in Ar and Air for the same leavening system; the only difference being in the value of intercept indicating that the Ar-packed breads, upon opening, reached a higher HI than bread with the same formulation but stored in Air. This is because, after opening (t = 0), the atmosphere inside the package becomes the same for all the samples, since Air moves inside the package and removes the Argon. Fig. 6 shows that the only difference was found in breads made with sourdough, with a lower slope than breads made with baker's yeast.

It is interesting to note that, regardless the gas used, the slope of SSL of the breads produced with sourdough (SB-C and SB-M) was the same of the slope of PSL of the breads YB-C-Air and YB-M-Air. This is related to the change in the environmental conditions of the package after opening, which results in a significant increase of the rate of quality decay.

4. Conclusions

The study of the shelf-life of food products is very complex because each product has a different degradation rate correlated to its formulation, storage conditions, packaging atmosphere, etc. and each product has also a specific acceptability limit, defined according to its peculiar qualities. Moreover the main limit is the difficulty of comparing data with literature on this topic, due to the lack of homogeneity in bread

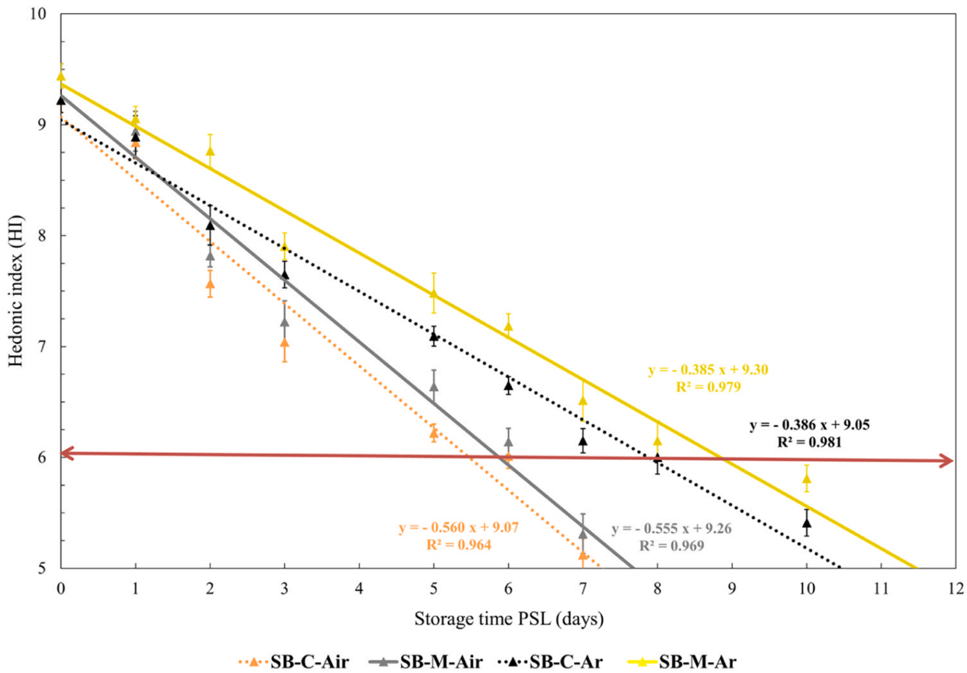

Fig. 5. Hedonic index of the different sourdough bread in the 2 system of MAP during PSL storage time.

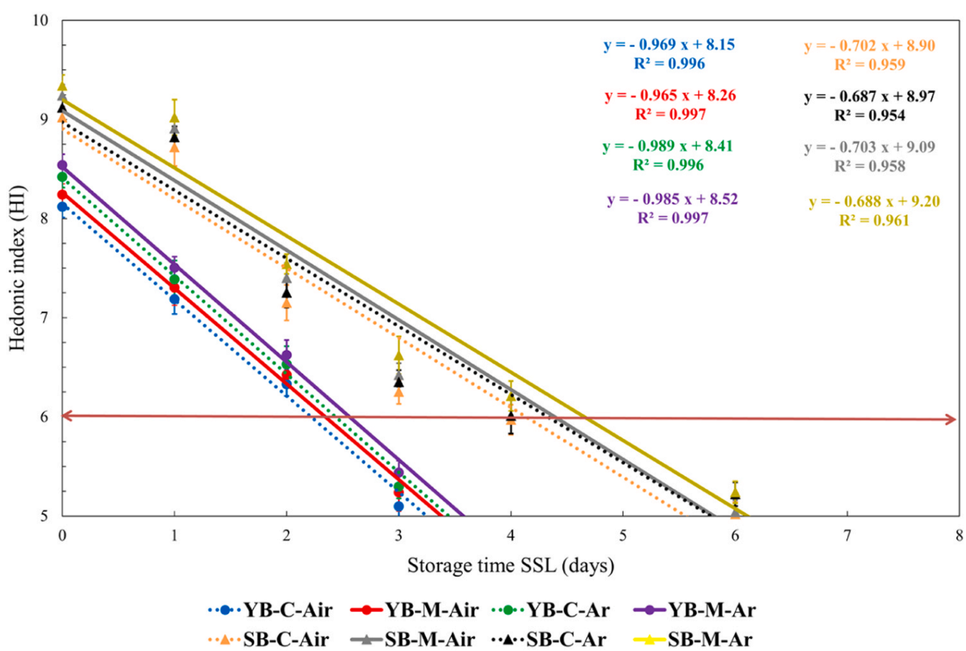

Fig. 6. Evolution of Hedonic index (HI) during SSL storage time (days) of the different bread stored in the 2 system of MAP.

shelf-life assessment methods and the limited availability of similar studies, especially on the secondary shelf-life. According to our results and in the experimental conditions adopted, regardless of the two leavening agents and MAP used, although there were significant differences in some of the chemical parameters of the breads produced, no evident effect of flour on PSL and SSL was observed. Besides, a very important effect on PSL is related to the leavening agent used, as sourdough bread showed a lower decrease of physical-chemical and sensory parameters compared to the baker's yeast one. This completely agrees with what has been reported in a previous work (Bianchi et al., 2023a, 2022b), which showed the same trends for the decrease in weight and water activity. The results of PSL confirmed also that Ar, in comparison with Air, can reduce the water loss, slowing down the staling process and allowing the sourdough bread to have a longer shelf-life, preserving its initial characteristics regardless the mix of flour utilized. Based on these findings, it can be concluded that the decrease in weight and water activity, which are easily and quickly measurable, can be utilized to evaluate bread PSL, along with sensory analysis.

Regarding the SSL results, significant differences could be related only to the leavening agent. No significant difference could be associated to the use of the two different gases (Ar and Air) in defining SSL of bread and this could be linked to the simulation test adopted. However, as shown in the results and in previous work (Bianchi et al., 2023b, 2022a; Nicoli & Calligaris, 2018), the trend of acceptability (Hedonic

Index (HI)) during time can be profitably used as a marker for the evaluation of the SSL of the product. In conclusion, a combined approach based both on physical-chemical and sensory markers can be a valid tool to define the primary or secondary shelf-life of bread. Future trials will be carried out to confirm and validate the markers proposed for PLS and SSL assessment of whole loaves and larger volumes of bread.

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CRediT authorship contribution statement

Venturi Francesca: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Bianchi Alessandro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sanmartin Chiara:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Conceptualization. **Tavarini Silvia:** Writing – review & editing, Visualization, Methodology. **Taglieri Isabella:** Writing – original draft, Resources, Investigation, Formal analysis. **Palermo Carmelo:** Investigation, Formal analysis. **Angelini Luciana Gabriella:** Writing – review & editing, Resources, Methodology.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2024.101241.

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Article

Effect of Fertilization Regime of Common Wheat (*Triticum aestivum*) on Flour Quality and Shelf-Life of PDO Tuscan Bread

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Abstract: The shelf-life of bread is influenced by flour components, such as starch, composed of amylose and amylopectin. The aim was to test the effect of different balances of N (45, 90, 135 kg/ha) and P (48, 96 kg/ha) fertilizers on the flour characteristics and consequently the shelf-life of PDO Tuscan bread, stored in different modified atmosphere packaging (Ar, N_2, Air). The amylose and phytochemical compounds were increased by N and decreased by the addition of P, but excessive doses of N (135 kg/ha) had a negative effect on flour quality. In the bread, the study highlighted the tendency of N_2 and Ar, as storage filler gases, to reduce water loss, slow down the staling process, and prolong shelf-life. However, the most significant influence on shelf-life was related to the different fertilizations of wheat. In fact, when N was present in equal dose to P (90/96 or 45/48 kg/ha) or slightly higher (90/48 kg/ha), the bread tended to last longer over time. Instead, when these ratios were unbalanced in favor of N (135/48 or 135/96 kg/ha) and in favor of P (45/96 kg/ha), the shelf-life decreased considerably.

Keywords: phosphorus; nitrogen; amylose; amylopectin; phytochemical; modified atmosphere packaging; sourdough; sensory analysis; breadmaking

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1. Introduction

Wheat-based products are the most consumed foods all over the world, especially in the Mediterranean area [1]. In particular, the cultural tradition of breadmaking found its origins in Italy, where around 3.2 million tons of bread are produced and consumed per year [2]. The expected world population increase by 2050 and the economic globalization lay the foundations for reinvesting in bread production, looking not only to enhance quality and quantity production, but also the shelf-life of bread [3,4].

Based on the history and culture of the area, bread in Tuscany has developed into a staple food, like in many other Italian regions [5]. In 2013, Tuscan bread received the protected denomination of origin (PDO) by the Commission of the European Community [4]. The product specification requires specific flour and dough characteristics that are the sum of local varieties and environmental conditions of the origin area [5]. PDO Tuscan bread exhibits a considerably higher level of taste complexity than common commercial white bread because sourdough is used as a leavening agent [6,7], even without any salt added in the formulation. Additionally, the low pH and high levels of lactic and acetic acids in the crumb might also explain the longer shelf-life, which is mostly related to decreased mold spoilage and slowed staling process [1,4].

Bread composition is the result of a wide interaction of factors, including wheat genotypes, agronomic management, environmental conditions, flour composition, bread-making conditions, and product storage [8,9]. The nutritional value and shelf-life of the final product are mainly influenced by starch composition [10] and in particular the amylose/amylopectin ratio [11,12]. Starch is present as granules and is the most important carbohydrate in wheat flour due to its water-absorbing capacity [13]. During bread storage, starch retrogradation is accompanied and driven by a complex process of moisture redistribution across the loaf, followed by moisture loss [14]. Furthermore, starch recrystallization can be significantly reduced also by the interaction between the gluten network and starch granules via hydrogen bonds [13,15]. As reported in the literature, moisture retention and water mobility play a vital role in the shelf-life of bread, especially during storage [1,4]. In this context, carbohydrates can slow down starch retrogradation, as they interfere in the interaction between water and starch [14,16].

Furthermore, macronutrients' supply is one of the most important factors influencing wheat production, especially the application of nitrogen and phosphorus [17]. Both elements have to be managed following different application times and rates, and their application influences different aspects of crop production [18]. Nitrogen is an essential macroelement for wheat production, able to enhance grain yield, protein storage, starch composition, and, as a consequence, flour quality [19,20].

Nevertheless, the application of N is highly dependent on environmental conditions, such as soil structure and water availability [21–23]. Different authors [24–28] have shown that increasing N application in wheat is able to increase protein concentration, in particular, a different modality of N application modifies the protein composition (gliadin and glutenin proportions) as well as the quality of cooking [29]. Zhou et al. (2020) [30] suggest that increasing N from 0 to 100 kg/ha improves amylose and amylopectin contents, but that excess nitrogen decreases starch content. Xue et al. (2016) [31] found that splitting N application influences the composition of the grain, influencing the wheat flour quality, and that delaying the N supply is able to influence breadmaking quality, favoring protein build-up.

Furthermore, phosphorus supply is important to ensuring the production of energy from photosynthesis and transportation of carbohydrates, root growth, and increasing yield [32]. However, soils are usually P deficient due to rapid element immobilization, highly dependent on soil pH and organic matter content [33]. Zhang et al. (2017) [34] found that applying P to wheat from 0 to 400 kg/ha increases yield by up to 50 kg ha, but the protein concentration in grain and flour decreases. Indeed, Guerrini et al. (2020) [27] showed that P fertilization is able to increase starch content, but significantly reduces the ratio of amylose to amylopectin.

To strengthen the impact of the final product on the market and increase the sustainability of the bread supply chain, knowing the effect of management practices on wheat varieties and dough quality and quantity characteristics would be useful.

This is relevant for storage bread, as refrigerating storage of freshly baked bread is not applicable because its texture and taste are negatively affected by low temperatures [35]. To preserve both the sensory qualities and nutritional content of PDO Tuscan bread while extending its shelf-life without using preservatives, whose use is prohibited by the traditional recipe, proper modified atmosphere packaging (MAP) appears to be the most effective strategy [36,37].

Bianchi et al. (2022) [4] recently showed that the use of 100% Ar or 100% N_2 for the MAP can be the best solution to preserve PDO Tuscan bread and a good compromise from a chemical–physical point of view, but above all, on a sensory level, because the use of CO_2 is not recommended with the high level of acidity of this type of sourdough bread [7].

For this reason, the aim of this work was to evaluate how different nitrogen and phosphorus rates applied to the wheat genotypes allowed for PDO Tuscan bread production influenced the flour features and consequently the shelf-life of the bread stored in modified atmosphere packaging (Ar, N_2, air).

2. Materials and Methods

2.1. Experimental Field

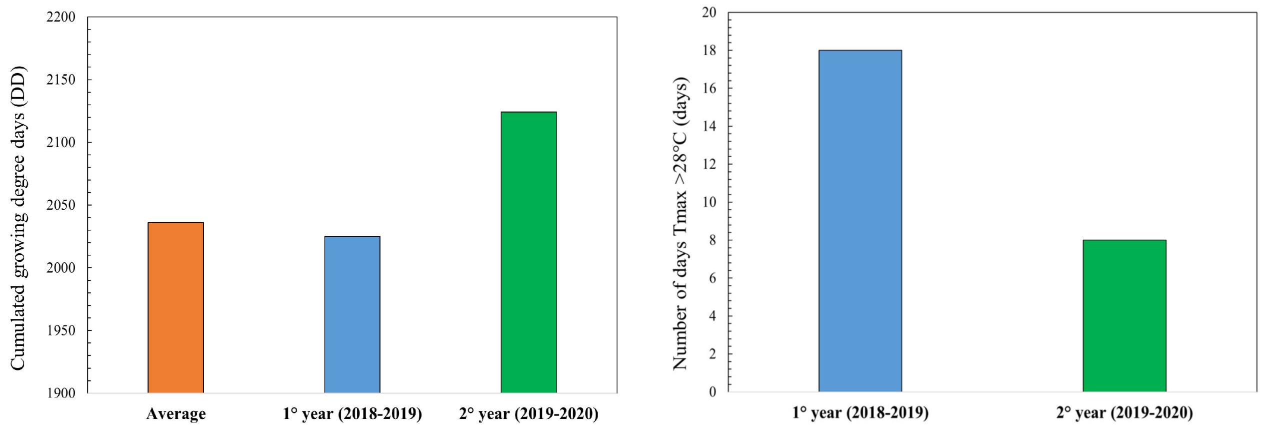
Field experiments were carried out for two consecutive growing seasons from September 2018 to August 2020 under rainfed conditions in Pienza, Tuscany, Italy (42.986569° N, 11.763888° E, 330 m a.s.l.). The 0–0.3 m soil layer was silty clay loam (Aquic Haplustepts, fine, mixed, mesic), sub-alkaline (pH 8.1), and contained 13.8 g/kg of total organic carbon, 1.2 g/kg of total nitrogen, and 7.6 mg/kg of available phosphorus.

The treatments consisted of factorial combinations of two phosphorus (P) levels (48 and 96 kg/ha), three nitrogen (N) levels (45, 90 and 135 kg N/ha), and four common wheat varieties (*Triticum aestivum*) allowed in the mix for PDO Tuscan bread production, for a total of 24 treatments. The four bread varieties comprised three registered dwarf varieties (namely Panda, Bolero, and Bologna) and one old, non-dwarf landraces (namely Verna). The experiment field was arranged in a strip-plot design with three replicate blocks per year (Figure S1). Egyptian clover (*Trifolium alexandrinum*, L.) was the previous crop in both growing seasons. In both seasons, the fields were plowed and then disk harrowed in late October. Then, phosphorous (triple superphosphate; P_2O_5: 46%) was distributed homogeneously on the soil surface and incorporated by disk harrowing at a 5 cm depth. Common wheat seeds were sown in December 2018 and 2019 with an inter row distance of 13 cm. The total dose of nitrogen was distributed in three applications: 20% at sowing by broadcasting urea (N: 46%), 50% by broadcasting ammonium nitrate (N: 26%) at tillering, and 50% by broadcasting urea (N: 46%) at stem elongation. Common wheat from each treatment was harvested separately using a plot combine-harvester equipped with Trimble GPS sensors. For each treatment, 5 kg of harvested wheat kernel were sampled for preparing the flour mix to be used for the analyses and the breadmaking trials. Weather conditions were monitored by consulting data acquired from a weather station located in the field where the experiment was being conducted. During the growing and production seasons (November–June), cumulative rainfall (mm) and cumulative growing degree days (DD) were calculated and analyzed. The cumulative degree days value was calculated daily as the difference between the average daily temperature and the base temperature considered useful for growth and development of wheat. For the characterization of the entire crop cycle, a thermal threshold of 4 °C was taken into consideration as reported by Saiyed et al. (2009) [38]. The analysis of the thermometric trend showed that compared to an average thermal of 2036 DD, the first year of tests (2018–2019) was substantially in line, while in the second year (2019–2020), there was a positive anomaly of 76 DD mainly due to a mild winter (Figure 1a). Conversely, in the maturation phase, there were more days with maximum temperatures above 28 °C in the 2018–2019 season, with June 2019 recording 18 hot days, compared to 8 days in the second year (Figure 1b).

Rainfall during the 2018–2019 production season was 431 mm, and 515 mm in the 2019–2020 season (Figure 2a). Analyzing the distribution of rainfall, the first year showed a much higher number of rainy days than the second did (Figure 2b). However, a monthly analysis showed a slight winter drought in the first year of trials (2018–2019) and excessive rainfall in June 2020 that affected the final stage of wheat ripening. The final stage of cereal ripening plays a key role in starch accumulation and protein translocation. In fact, very high temperatures in June are often the cause of a sudden senescence of the plant with interruption of starch synthesis and accumulation [38].

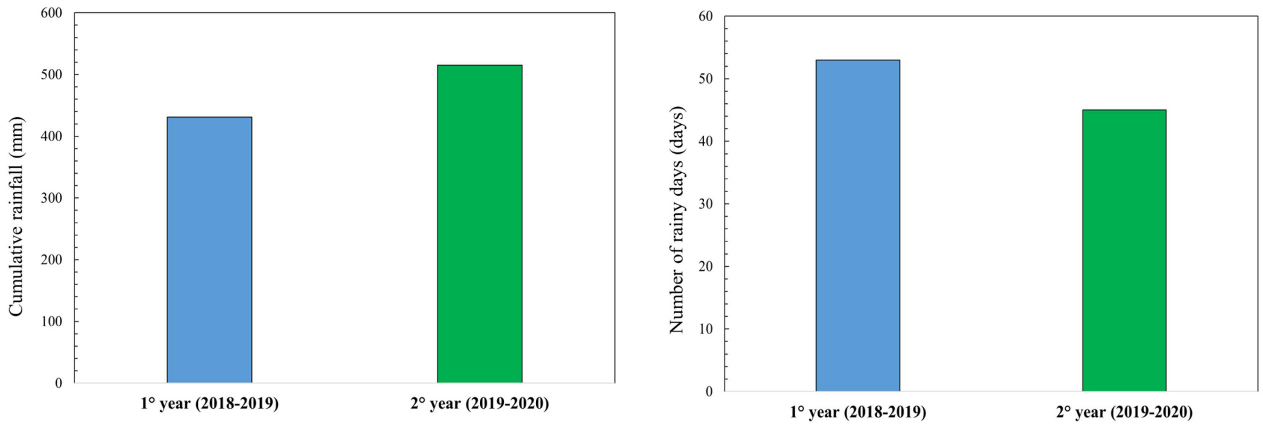
2.2. Characterizzation of Flours

According to the specifications for PDO Tuscan bread production, the flour was obtained by a mix of four varieties of common wheat divided for the six combination of N/P (kg/ha) treatments (M1 = 45/48, M2 = 45/96, M3 = 90/48, M4 = 90/96, M5 = 135/48, M6 = 135/96) produced in the two years (2019 and 2020 crop seasons).



(**a**) (**b**)

Figure 1. Climatic parameters in the two years during the growing season of November–June: (**a**) cumulated growing degree days (DD); (**b**) number of days with maximum temperatures above 28 °C.



(**a**) (**b**)

Figure 2. Climatic parameters in the two years during the growing season of November–June: (**a**) cumulated rainfall (mm); (**b**) number of rainy days.

A commercial mill (Industry-Combi, Waldner Biotech, Lienz, Austria) was used for the milling process at the Department of Agricultural, Food, and Environment (DAFE) at the University of Pisa.

The chemical composition and the technological features of flours were determined as previously reported [4] according to the methods accepted by the International Organization for Standardization (ISO) and by the Association of Official Analytical Chemists International (AOAC): humidity (ISO 712:2009); ashes (ISO 2171:2007); proteins (ISO 20483:2013); total fats (ISO 11085:2015); falling number (ISO 3093:2009); wet gluten and gluten index (ISO 21415-2:2015); dry gluten (ISO 21415-3:2006); total dietary fiber (AOAC 2011.25-2012); sugars (AOAC 982.14-1983); amylose and amylopectin (ISO 6647-1:2020); total starch (AOAC 996.11-2005); Chopin alveogram (W, P/L, P, L, G) (ISO 27971:2015); Brabender farinogram (water absorption corrected to 14% humidity, dough time, stability, softening degree (E10: degree of softening after 10 min; E(ICC): softening degree 12 min, after max), and FQN: number of farinographic quality) (ISO 5530-1:2013).

Moreover, the flour was characterized from a phytochemical point of view (total polyphenols, total flavonoids, and anti-radical activity). In particular, an 80% methanol solution was used to perform a solid/liquid extraction (ratio 1/20 *w*/*v*) from 0.5 g of a fresh flour sample, sonicating the mixture for 30 min. All the extracts were subsequently centrifuged (15 min, 3500 rpm), filtered on a syringe filter (0.45 µm), and stored at 4 °C for the immediate analysis.

The Folin–Ciocalteu colorimetric method was applied for total polyphenols spectrophotometry as previously reported [39], expressing the results as milligrams of gallic acid equivalents (GAE) per kilogram of dry matter (dm). The total flavonoids were estimated according to the procedure reported by Bianchi et al. (2023) [40], comparing the measures to a standard curve of catechin, and the results were reported as milligrams of catechin equivalents (CE) per kilogram of dm. Using the free radical methods (FRAP [40], ABTS [41], and DPPH [40]), the anti-radical activity of the extracts was determined. According to different standard curves of Trolox (range: 0–2.0 mM for the FRAP H, 0.2–1.5 mM range for ABTS, and 0–200 µmol/L for the DPP), the results were expressed as micromoles of Trolox equivalents (TE) per gram of dm.

2.3. Breadmaking Process

The Consortium for the protection of PDO Tuscan Bread procured the sourdough used in the study. Consecutive back slopping was used for preserving the sourdough in order to maintain its acidifying and leavening properties [42]. Breadmaking was carried out from a pre-ferment leavening agent, according to the method of "biga". According to the production specification for PDO Tuscan bread, for all the experimental runs, sourdough biga was prepared by mixing a strong wheat flour type 0 (56% *w*/*w*) and sterile water (33% *w*/*w*), and (11% *w*/*w*) then leaving to ferment for 18 h at 20 °C. For each experimental run, the specific formulation was produced with 32% of water, 16% of leavening agent (biga), and 52% of flour selected among the six different treatments (M1–M6) described in Section 2.2. The first leavening lasted for 90 min at 26 ± 1 °C, then, the dough was broken and shaped and left for a further 2.5 h at 35 ± 1 °C (second leavening). Finally, the loaves were baked at 220 °C for 45 min.

2.4. Bread Shelf-Life Assessment

After baking, the bread loaves were cooled for 2 h at room temperature (23 ± 1 °C), then sliced with an automatic slicing machine to a 20 mm thickness. Each slice was packed individually in plastic bags (two plastic layers, outer nylon layer, Food Saver, Moncalieri, Torino, Italy), by an industrial packaging machine (Lavezzini 450 GAS, Fiorenzuola d'Arda, Piacenza, Italy). Twenty loaves (1 kg each) for the six treatments were prepared, 500 slices each were packed separately in three different MAPs (Ar (100%), N_2 (100%), air (100%)). Each pack was stored at a controlled temperature (23 °C) during the whole observation period.

In this study, the samples are represented by bread cut into slices, not by the whole loaf, as the result is a much larger exposed surface that determines a more rapid evolution of the parameters. The choice of slices was made in order to be able to study the process in an accelerated-shelf-life way, as reported in a previous study [4].

Four sliced samples of each storage condition were opened each day and subjected to the following analysis in order to evaluate the bread shelf-life as a function of the flours and storage conditions.

Slices were weighed daily for each experimental run to determine the weight loss brought on by the evaporation of water from the slices during storage; the value was shown as a percentage decrease from the starting value.

Water activity (a_w) was measured by a HygroPalm HP23-AW-A equipment (Rotronic AG, Bassersdorf, Switzerland). The results were calculated as a percentage reduction in water activity compared to the initial value.

As reported by Bianchi et al. (2022) [4], the softness of the crumb was determined by a penetrometer PNR-12 (Anton Paar, Rivoli, Italy), and the results were expressed as a percentage reduction in softness compared to the initial value.

All the samples were checked daily for the presence of mold; each experimental run was stopped when 3% of the samples showed mold spoilage.

Finally, the sensory profiles of the bread samples were evaluated by a panel of eight trained judges (aged between 23 and 60 years) of the Department of Agriculture, Food, and Environment Sciences of the University of Pisa. The tasting was carried out according to a previously developed protocol [43], and the overall hedonic index of bread was calculated as reported by Bianchi et al. (2022) [4]. The research obtained the approval of the Ethics Committee of the University of Pisa (protocol no. 0088081/2020).

2.5. Statistical Analysis

All the physical chemical parameters were evaluated in quadruplicate.

One-way ANOVA (CoStat, Version 6.451, CoHort Software, Pacific Grove, CA, USA) was used to assess the significance of the difference between the samples, and Tukey's HSD test ($p \leq 0.05$) was used for the separation of the samples.

On the parameters of flour quality, two-way ANOVA was also used to determine the effect of the two different factors (year or treatments or the combination) on the parameters of flour quality.

The trend of the shelf-life parameters over time (decrease of weight, water activity, and softness) and the linear regression were elaborated with the JMP software package version 17 (SAS Institute, Cary, NC, USA).

Big Sensory Soft 2.0 software (ver. 2018) analyzed the findings of the sensory analysis. Panelists and samples were the factors in the two-way ANOVA used to assess the sensory data [4].

3. Results and Discussion

3.1. Flour Quality Characterization

Among the analyses carried out on the various types of flours related to two successive years (2019 and 2020), the most significant parameters related to further bread spoilage attitude, mainly related to the staling process and mold development, are reported below and further discussed (Tables 1 and 2). All data showed almost the same trend as a function of fertilization rate, regardless of the crop season analyzed.

Table 1. Chemical and technological parameters of the six flours used in the breadmaking trial (year 2019).

			Year 2019					
Parameters	**Units**	[1]	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
Chemical								
Humidity	% *w/w*	ns	11.13	10.92	11.28	11.00	10.05	10.82
Ashes	% *w/w*	ns	1.24	1.17	1.26	1.08	1.09	1.13
Proteins	% *w/w*	**	13.34 c	13.42 c	13.69 b	13.98 ab	14.26 a	14.51 a
Total fats	% *w/w*	ns	2.05	2.18	2.14	1.98	2.06	2.15
Total dietary fiber	% *w/w*	**	5.02 c	6.83 a	5.38 c	5.31 c	6.12 b	6.21 b
Sucrose	% *w/w*	*	0.79 ab	0.68 c	0.87 a	0.86 a	0.71 bc	0.72 bc
Glucose	% *w/w*	*	0.293 ab	0.22 b	0.30 a	0.33 a	0.22 b	0.24 b
Fructose	% *w/w*	ns	0.10	0.08	0.12	0.10	0.09	0.11
Maltose	% *w/w*	***	5.02 ab	4.42 d	5.21 a	5.28 a	4.90 b	4.70 c
Wet gluten	% *w/w*	**	38.72 b	36.12 c	38.65 b	38.14 b	41.22 a	41.86 a
Dry gluten	% *w/w*	**	11.51 c	10.43 d	11.54 c	11.70 c	11.94 b	12.84 a
Gluten index	% *w/w*	**	67.64 c	63.21 d	70.74 b	67.33 c	69.12 b	71.92 a
Total Starch	% *w/w*	**	83.64 cd	83.02 d	84.85 ab	84.03 b	85.09 a	85.71 a
Amylose	% *w/w*	***	22.82 c	24.64 b	22.64 c	22.72 c	25.73 a	25.42 a
Amylopectin	% *w/w*	***	77.21 c	75.44 b	77.48 c	77.31 c	74.32 a	74.68 a

Table 1. *Cont.*

Parameters	Units	[1]	M1	M2	M3	M4	M5	M6
			Year 2019					
Falling number	seconds	ns	303	298	300	310	312	316
Total polyphenol	mg GAE/kg dm	***	635 [a]	449 [c]	617 [b]	642 [a]	440 [c]	416 [d]
Total flavonoids	mg CE/kg dm	**	55.82 [ab]	46.43 [c]	53.45 [b]	57.42 [a]	40.44 [d]	48.42 [c]
ABTS	µmol TE/g dm	***	0.79 [a]	0.53 [c]	0.68 [b]	0.82 [a]	0.50 [c]	0.41 [d]
DPPH	µmol TE/g dm	**	0.40 [ab]	0.32 [c]	0.38 [b]	0.45 [a]	0.31 [c]	0.35 [c]
FRAP	µmol TE/g dm	***	0.79 [a]	0.56 [c]	0.81 [a]	0.84 [a]	0.55 [c]	0.48 [d]
Technological								
W	10^{-4} joules	*	229 [c]	248 [bc]	237 [b]	240 [b]	258 [a]	262 [a]
P/L		*	1.62 [b]	1.92 [a]	1.54 [b]	1.67 [b]	1.98 [a]	2.06 [a]
P	mm	ns	106	112	105	112	113	114
L	mm	*	48 [a]	40 [b]	49 [a]	48 [a]	41 [b]	39 [b]
G		*	13.5 [a]	12.6 [b]	13.6 [a]	13.4 [a]	12.7 [b]	12.7 [b]
Water absorption	%	**	67.4 [c]	73.1 [a]	67.4 [c]	67.2 [c]	72.0 [b]	71.5 [b]
Dough time	Minutes	ns	5.6	5.2	5.8	5.3	5.5	5.3
Stability	Minutes	ns	5.1	4.6	4.9	4.8	4.6	4.9
E10	UF	ns	41	50	52	50	48	42
E(ICC)	UF	ns	74	72	74	80	79	81
FQN		ns	75	64	70	72	68	65

[1] Significance level: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant ($p > 0.05$). In the same row, different letters indicate significant differences among samples.

Table 2. Chemical and technological parameters of the six flours used in the breadmaking trial (year 2020).

Parameters	Units	[1]	M1	M2	M3	M4	M5	M6
			Year 2020					
Chemical								
Humidity	% *w*/*w*	ns	11.08	11.22	10.85	10.90	11.05	11.22
Ashes	% *w*/*w*	ns	1.34	1.37	1.36	1.37	1.30	1.33
Proteins	% *w*/*w*	**	12.24 [c]	12.52 [bc]	12.64 [b]	12.90 [b]	13.46 [a]	13.50 [a]
Total fats	% *w*/*w*	ns	2.45	2.52	2.44	2.50	2.46	2.45
Total dietary fiber	% *w*/*w*	*	5.82 [d]	8.83 [a]	6.58 [c]	6.70 [bc]	7.32 [b]	7.41 [b]
Sucrose	% *w*/*w*	*	0.94 [ab]	0.80 [c]	0.97 [a]	0.96 [a]	0.91 [b]	0.89 [b]
Glucose	% *w*/*w*	**	0.43 [ab]	0.31 [c]	0.46 [a]	0.45 [a]	0.39 [b]	0.38 [b]
Fructose	% *w*/*w*	ns	0.12	0.13	0.11	0.14	0.13	0.13
Maltose	% *w*/*w*	**	7.22 [a]	6.52 [c]	7.21 [a]	7.28 [a]	6.75 [b]	6.70 [b]
Wet gluten	% *w*/*w*	**	39.72 [c]	36.22 [d]	39.65 [c]	39.14 [c]	41.22 [b]	42.86 [a]
Dry gluten	% *w*/*w*	**	12.51 [c]	11.63 [d]	12.54 [c]	12.90 [c]	12.94 [b]	13.84 [a]
Gluten index	% *w*/*w*	*	67.60 [d]	69.21 [cd]	73.71 [b]	75.32 [a]	72.14 [bc]	73.92 [b]
Total Starch	% *w*/*w*	**	86.29 [b]	85.03 [c]	86.65 [b]	86.71 [b]	88.54 [a]	88.32 [a]
Amylose	% *w*/*w*	***	20.72 [b]	23.64 [a]	21.04 [b]	20.82 [b]	23.73 [a]	23.42 [a]
Amylopectin	% *w*/*w*	***	79.31 [b]	76.44 [a]	79.03 [b]	79.22 [b]	76.32 [a]	76.68 [a]
Falling number	seconds	ns	333	332	340	327	318	326
Total polyphenol	mg GAE/kg dm	**	835 [a]	749 [b]	827 [a]	842 [a]	719 [c]	716 [c]
Total flavonoids	mg CE/kg dm	***	75.82 [a]	63.43 [c]	73.45 [b]	77.42 [a]	60.44 [d]	58.42 [e]
ABTS	µmol TE/g dm	**	1.15 [ab]	0.83 [c]	1.09 [b]	1.23 [a]	0.79 [c]	0.66 [d]
DPPH	µmol TE/g dm	**	0.70 [ab]	0.52 [c]	0.65 [b]	0.75 [a]	0.51 [cd]	0.45 [d]
FRAP	µmol TE/g dm	***	1.50 [a]	1.22 [c]	1.41 [b]	1.54 [a]	1.16 [c]	1.01 [d]
Technological								
W	10^{-4} joules	*	245 [d]	262 [bc]	258 [c]	259 [c]	273 [ab]	282 [a]

Table 2. *Cont.*

			Year 2020					
Parameters	**Units**	[1]	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
P/L		*	2.62 [b]	2.92 [a]	2.54 [b]	2.67 [b]	2.98 [a]	3.06 [a]
P	mm	ns	156	162	150	152	160	160
L	mm	*	63 [a]	56 [b]	61 [a]	68 [a]	51 [b]	56 [b]
G		*	15.5 [a]	14.1 [b]	15.6 [a]	15.4 [a]	14.7 [b]	14.8 [ab]
Water absorption	%	**	68.4 [b]	69.7 [a]	68.4 [b]	68.2 [b]	69.9 [a]	69.5 [a]
Dough time	Minutes	ns	4.6	4.2	4.8	4.3	4.5	4.3
Stability	Minutes	ns	6.2	5.5	5.9	5.4	6.4	6.2
E10	UF	ns	46	59	48	46	58	52
E(ICC)	UF	ns	81	92	81	90	87	87
FQN		ns	85	73	80	81	74	75

[1] Significance level: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant ($p > 0.05$). In the same row, different letters indicate significant differences among samples.

Further, as reported in Table S1, the most significant differences among the flour samples were determined by treatment, followed by year crop season, while only a few parameters were significantly affected by the combination "treatment × year".

According to Rekowski et al. (2019) [25], higher protein content in flour determines a reduction in the loss of free water during staling, because protein's water retention power allows it to gradually counteract the starch recrystallization process. A greater amount of protein and starch were induced by the highest nitrogen fertilization rate (flours M5 and M6), followed by that with M3 and M4 flours, while the lowest values were reported for M1 and M2 flours.

As the sugar content increases, the gelatinization temperature increases too, while the rate of the starch retrogradation decreases [16]. In the experimental conditions, flours M1, M3, and M4 had the highest sum of total sugars compared to that of the other flours. The concentration of gluten, both dry and wet, is fundamental to evaluating the breadmaking properties of flour [10]. The higher its value, the more the flour is suitable for this purpose. M5 and M6 flours showed the highest gluten concentration among others, while M2 flour showed the lowest one, thus suggesting that the increase in N fertilization had a positive effect on this parameter [30].

According to Schirmer et al. (2013) [11], amylose/amylopectin content is directly proportional to starch retrogradation rate, as amylose tends to recrystallize much faster than amylopectin. Looking at the results obtained in the two years (Tables 1 and 2), it is clear that the flours M1, M3, and M4 contained statistically significantly lower amylose content than the others and therefore could show a reduced tendency of staling [10,11].

Furthermore, phenolic compounds have an inhibitory activity towards the development of microorganisms; therefore, a high concentration of them determines a delayed appearance of fungal bodies on the bread. In addition, as reported in [40], the combination with sourdough leavening allows for their increase in the produced bread, thanks to the ability to increased availability and bio-accessibility of this phytochemical compound. Flours M1, M3, and M4 had a statistically higher quantity of phenolic compounds (both total polyphenols and total flavonoids) and higher antioxidant power compared with those of the other flours (Tables 1 and 2).

Taken together, the data showed that the six flour mixes could be divided into two main groups showing both a different chemical composition and potentially different technological features mainly related to bread spoilage, with flours M5 and M6 being clearly different from flours M1 and M2, regardless of the crop season analyzed.

3.2. Trend of Weight Loss, Water Activity, and Softness

Water migration consists of the redistribution of free water molecules due to the moisture gradient between different areas of the product, mainly from the crumb to the crust, and this migration contributes strongly to the phenomenon of staling. During storage, the water initially included in the gel fraction of starch is gradually released because of the starch recrystallization process, thus driving further crumb softness loss typical of bread staling.

To evaluate the shelf-life trend during the storage period as a function of storage atmosphere composition, the linear regression of weight loss (Figure S2a,b) together with the water activity decrease (Figure S3a,b) was calculated. The slopes of the regression lines and R^2 are reported in Table 3 for the two-year observation period.

Table 3. Slope and the coefficient of determination (R^2) for the linear regression of the decrease of weight and the decrease of water activity (a_w) in the two years (2019 and 2020).

Sample	Year 2019				Year 2020			
	Slope (Weight)	R^2	Slope (a_w)	R^2	Slope (Weight)	R^2	Slope (a_w)	R^2
M1-Air	0.448 c	0.987	0.361 c	0.972	0.473 b	0.986	0.261 b	0.962
M1-Ar	0.333 d	0.993	0.236 d	0.996	0.321 c	0.984	0.135 c	0.973
M1-N_2	0.344 d	0.995	0.255 d	0.994	0.352 c	0.986	0.155 c	0.968
M2-Air	0.643 a	0.970	0.509 a	0.964	0.571 a	0.990	0.376 a	0.960
M2-Ar	0.466 bc	0.980	0.380 bc	0.982	0.478 b	0.991	0.303 b	0.976
M2-N_2	0.500 b	0.979	0.420 b	0.974	0.473 b	0.986	0.306 b	0.959
M3-Air	0.441 c	0.988	0.359 c	0.984	0.445 b	0.992	0.265 b	0.982
M3-Ar	0.329 d	0.995	0.236 d	0.994	0.347 c	0.998	0.156 c	0.978
M3-N_2	0.341 d	0.978	0.250 d	0.985	0.341 c	0.983	0.158 c	0.968
M4-Air	0.440 c	0.991	0.353 c	0.991	0.455 b	0.992	0.273 b	0.963
M4-Ar	0.321 d	0.994	0.238 d	0.993	0.336 c	0.985	0.132 c	0.972
M4-N_2	0.327 d	0.997	0.249 d	0.998	0.345 c	0.993	0.149 c	0.965
M5-Air	0.625 a	0.969	0.506 a	0.967	0.548 a	0.988	0.388 a	0.966
M5-Ar	0.534 b	0.991	0.388 b	0.963	0.471 b	0.992	0.275 b	0.960
M5-N_2	0.527 b	0.992	0.396 b	0.976	0.459 b	0.990	0.298 b	0.973
M6-Air	0.643 a	0.994	0.505 a	0.966	0.556 a	0.985	0.413 a	0.976
M6-Ar	0.467 bc	0.994	0.390 b	0.987	0.436 b	0.986	0.274 b	0.966
M6-N_2	0.483 b	0.992	0.415 b	0.992	0.437 b	0.992	0.298 b	0.970

Different letters in the column indicate a statistically different value ($p \leq 0.05$).

According to what was reported about the flour's features as a function of fertilization rate, both weight loss and water activity decrease showed the same trend in all the experimental conditions tested, regardless of the crop season analyzed.

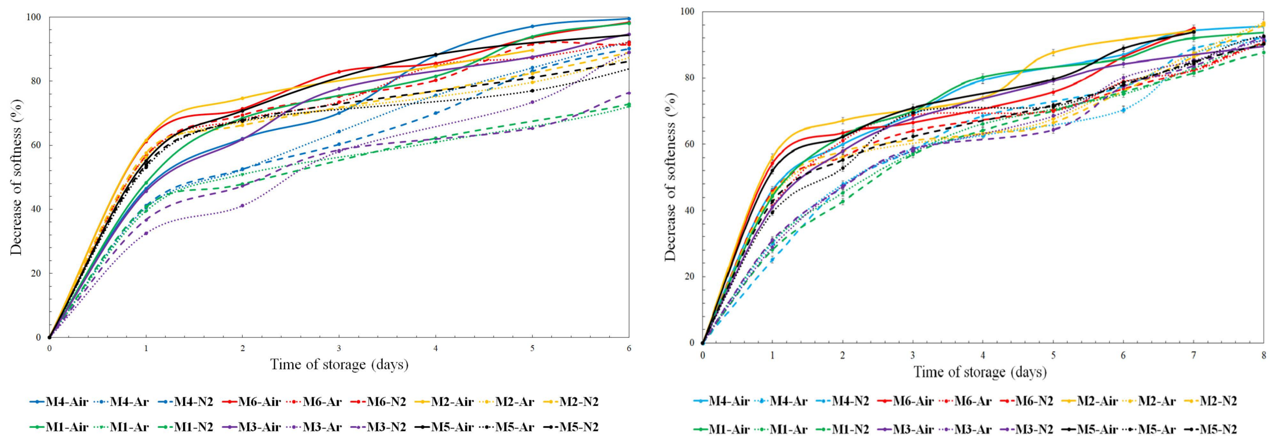
As expected [4], given the flour mix, significantly faster water loss was detected when the bread was stored in air (Table 3).

Furthermore, given the storage atmosphere, the flour composition represented the main factor in determining the crumb softness reduction rate (Figure 3a,b), with a lower one measured for breads produced with flours M1, M3, and M4, while flours M2, M5, and M6 significantly reduced the bread's shelf-life. Furthermore, the technological characteristics of the flour induced by the different fertilization regimes appeared to be more important than the storage atmosphere in determining the bread's shelf-life. Indeed, breads produced with flours M1, M3, and M4 stored in air showed the same trend as when breads produced with flours M2, M5, and M6 were stored in an inert atmosphere (100% Ar or 100% N_2).

3.3. Mold Appearance

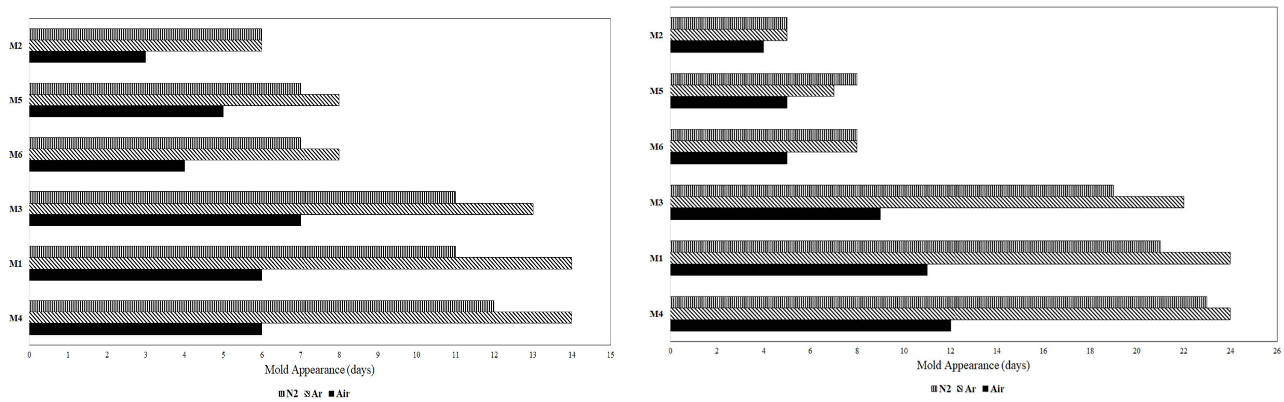
Molds determine the decay of the organoleptic quality that derives from the production of off-flavors in terms of alcohols and esters, which cause unpleasant hints and the appearance of spots with an abnormal color on the surface of the product.

As shown in Figure 4a,b, flours M1, M3, and M4 significantly delayed mold development, thus improving the bread's shelf-life regardless of the storage atmosphere or the crop season. These results can be explained well by the high phenolic content detected in flours M1, M3, and M4. Among the different gas compositions of storage atmospheres, as expected, air induced the fastest mold development.



(**a**) (**b**)

Figure 3. Trend of the decrease of softness (%) as a function of storage days: (**a**) year 2019; (**b**) year 2020.



(**a**) (**b**)

Figure 4. Mold appearance on the surface of bread: (**a**) year 2019; (**b**) year 2020.

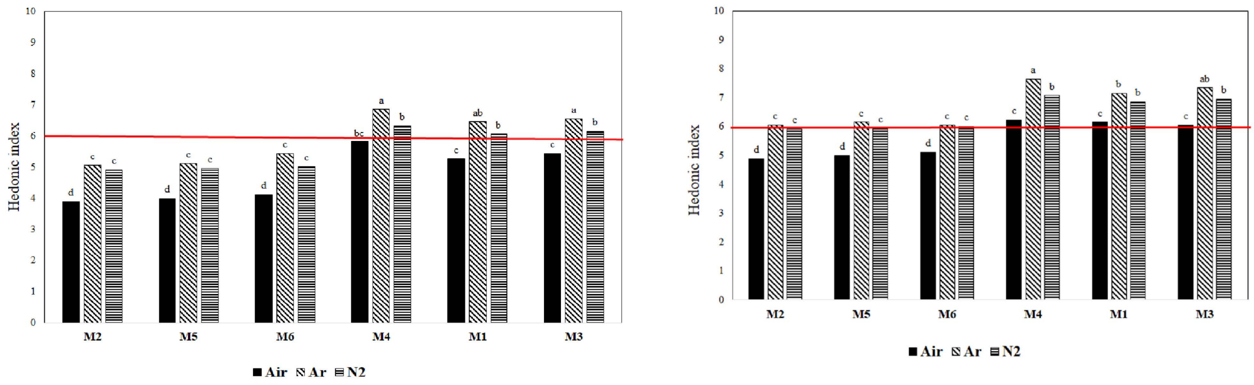
3.4. Sensory Evaluation of Bread

Breads produced with the different flours were assessed before packaging to determine their sensory profile at t = 0 (Figure S4a,b), and flours M1, M3, and M4 produced the breads with the best sensory profiles.

During the whole observation period, further panel tests were performed daily on the breads stored in different gas atmospheres until the first molds were observed on the slice surface of the sample stored in the worst conditions.

As expected, a decrease in overall pleasantness during storage was observed due to the staling phenomenon, where the breads produced with flours M1, M3, and M4 were still acceptable (HI > 6) at the end of the trials regardless of the storage atmosphere, thus confirming the main effect of flour composition discussed above.

When gas composition was assumed as the main effect (Figure 5a,b), the best sensory profile was observed for breads stored in 100% Ar, probably due to its superior antioxidant power that better preserved aromatic molecules.



(**a**) (**b**)

Figure 5. Hedonic index (HI) of the different breads in the three systems of MAP at the end of the storage period: (**a**) year 2019; (**b**) year 2020. The red line indicates the HI reference limit of shelf-life. Different lowercase letters indicate significant differences at $p < 0.05$.

4. Conclusions

The study confirmed that N_2 and Ar could reduce water loss, slow down the staling process, and allowed the bread to not only last longer but also to better maintain its initial characteristics, compared to those with air. However, a more important effect is linked to differences in fertilizations of the wheat flour. In fact, the breads using the flours M1, M3, and M4 in air had the same results as those of the flours M2, M5, and M6 in a protective atmosphere of Ar and N_2.

During two different crop seasons with different climatic behaviors, the main parameters related to flour quality and composition were mainly affected by the fertilization approach, followed by crop season, while only a few parameters were significantly affected by the combination "treatment × year".

In fact, in N/P the fertilization, when N was present in a dose equal to that of P (90/96, 45/48 and 90/48 kg/ha) or in a higher but not excessive dose (90/48 kg/ha), the bread obtained tended to have better characteristics and to last longer over time. When the N/P ratios were unbalanced in favor of nitrogen (135/48 and 135/96 kg/ha) or in favor of phosphorus (45/96 kg/ha), the shelf-life and also the chemical–physical and sensory characteristics strongly decreased.

In conclusion, the flours obtained with less abundant fertilizations of N and P had better results, representing an opportunity for producers to save by administering lower doses of fertilizer. At the same time, there would be an environmental benefit, as less energy and chemical inputs would be used.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12142672/s1, Figure S1: Strip-plot design of the experimental field in the two years. P arranged in vertical strips as the main plot (P1 = 46 kg/ha, P2 = 98 kg/ha), N was assigned to the vertical sub-plots (N1 = 45 kg/ha, N2 = 90 kg/ha, N3 = 135 kg/ha), and varieties were applied horizontally in sub-sub-plots (Bologna, Bolero, Pandas, Verna) with three replicate blocks per year. Each sub-sub-subplot was 1800 m^2; Figure S2: Linear regression of the decrease of weight (%): (a) year 2019; (b) year 2020; Figure S3: Linear regression of the decrease of water activity (%): (a) year 2019; (b) year 2020; Figure S4: Hedonic index (HI) of the bread after baking (t = 0): (a) year 2019; (b) year 2020. The red line indicates the HI reference limit of shelf-life. Different lowercase letters indicate significant differences at $p < 0.05$; Table S1. Average of the chemical and technological parameters for the 6 treatments (T) and for the years 2019 and 2020 (Y).

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Effect of Argon as Filling Gas of the Storage Atmosphere on the Shelf-Life of Sourdough Bread—Case Study on PDO Tuscan Bread

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Abstract: The short shelf-life of PDO Tuscan bread limits its distribution to markets close to the production area, affecting its commercial success and the economic return by supply chain operators. While the application of MAP to store bread is widely accepted, the suitability of this technique to extend the shelf life of the PDO Tuscan bread is still to be explored. Furthermore, to the best of our knowledge no data are available in the literature about the use of argon as filling gas neither in pure atmosphere nor in combination with CO_2. In this context, the aim of this study was to evaluate the effect of different modified packaging atmospheres on the shelf-life of sourdough bread. Slices of bread were stored individually in plastic bags at 23 °C in five different atmospheres (Ar (100%), N_2 (100%), CO_2 (100%), Mix CO_2/N_2 (70% CO_2, 30% N_2), Mix CO_2/Ar (70% CO_2, 30% Ar)), and Air was selected as a control. To select the best storage conditions, both chemical-physical, rheological, and organoleptic features were evaluated. Results showed that pure gases (CO_2, N_2, Ar) displayed good qualities as storage atmospheres compared to Air. In contrast, both Mix CO_2/N_2 and Mix CO_2/Ar were the best in slowing down the staling process, thus doubling the shelf-life of bread, compared to other atmospheres. In conclusion, argon, as a preservation atmosphere, seems to be the best solution to extend the shelf-life of PDO Tuscan bread.

Keywords: modified atmosphere packaging; shelf-life; sourdough bread; staling; PDO Tuscan bread

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1. Introduction

Over the last decades, European consumer behavior in the baked goods category has changed and an increasing demand for products with reduced environmental impact together with symbolic features with ethical values such as naturalness, healthiness, distinctiveness, and consistency has been observed [1]. These new consumer concerns, together with the instability of the European/Italian wheat market, has improved the likelihood of success of rural systems and production strategies associated with traditional local productions with a cultural identity (i.e., traditional food products, geographical indications, etc.) [2].

Across Italy, in the last years, a re-localization process based on the establishment of Protected Designations of Origin (PDO) and Protected Geographical Indications (PGI) for bread was aimed at closing the gap between producers, processors, and consumers. The actuation of innovative, localized bread supply chains based on different assumptions such as variety, production method, baking process, zero-miles consumption models, etc. represents a possible strategy to sustain the cereal sector giving an economically viable alternative for Italian smallholders to compete on the market [1,3].

As in many other Italian regions, in Tuscany, bread has evolved as a basic consumption item in relation to the local history and culture. In order to foster and protect Tuscan bread, the process for the recognition of the "PDO Tuscan Bread" was promoted from 2002 by baker and farmer associations, the milling industry, and other local stakeholders [3]. The original recipe and the related product specification have been codified, starting from the specific varieties of wheat traditionally cultivated in Tuscany and to be used for the milling of wheat.

Some crucial aspects differentiate PDO Tuscan bread supply chains from conventional ones: (a) wheat must belong to a set of soft wheat varieties cultivated in Tuscany; (b) sourdough leavening is compulsory; (c) no salt can be added to the recipe; (d) flour must include wheat germ; (e) the final weight must range between 0.45 and 1.10 kg; and (f) the Consortium label is compulsory.

Thanks to the use of sourdough as leavening agent [4], the PDO Tuscan bread shows a significantly enhanced flavor complexity and higher savories than a widespread industrial white bread [5,6], even without any salt added in the formulation. Furthermore, the low pH together with high concentrations of lactic and acetic acid in the crumb can also explain the extended shelf life observed, mainly linked to the reduced mold spoilage as well as the slowing of the staling process [3,7–10].

Nevertheless, despite the improved shelf-life shown by PDO Tuscan bread, the geographic area of its supply chain appears still limited and a huge quantity of waste is generated with a consequent loss of economic resources together with a significant environmental impact [11,12].

As the refrigeration of freshly baked bread is not applicable as its texture and taste are negatively affected by low temperatures [13], the use of a proper modified atmosphere packaging (MAP) appears the best choice to extend the shelf life of the PDO Tuscan bread in order to maintain both sensorial features and nutritional value without using any preservatives [14,15], the use of which is not allowed by the traditional recipe.

Generally, when MAP is utilized to store bakery products, the gas mixture consists of 60% or more carbon dioxide (CO_2) with nitrogen (N_2) acting as a filler gas [16,17]. Similar to high-water content foods, CO_2 can dissolve in the water of the bakery products to form carbonic acid, leading to a lowering of the pH. As reported by Smith et al. 1986 [18], the mold growth could only be delayed up to 5 and 10 days, but not prevented by N_2 and/or CO_2. In particular, a necessary condition to prevent mold growth was to maintain the level of O_2 below 0.4%, in accordance with the results showing the reliance between O_2 content and fungi growth on other types of products under MAP [19–22]. Nevertheless, the high concentration of CO_2 may determine an increase in the perceived acidity to the taste [19,23–26]. Furthermore, the conclusive impact of CO_2 in the MAP on the bread quality appears still contradictory [13].

In this context, the aim of this study was to evaluate the effect of different MAP on the shelf-life of the PDO Tuscan bread. Slices of bread were stored individually in plastic bags at 23 °C in five different atmospheres (Ar (100%), N_2 (100%), CO_2 (100%), Mix CO_2/N_2 (70% CO_2; 30% N_2), Mix CO_2/Ar (70% CO_2; 30% Ar)) and Air was utilized as control. To the best of our knowledge, no data are yet available in the literature concerning the feasibility to use Argon (Ar) as filling gas in MAP for sourdough bread storage. To select the best storage conditions, chemical-physical, rheological, and organoleptic features were evaluated.

2. Materials and Methods

2.1. Raw Materials

The flour (type 0) was obtained by a mix of four varieties (Bolero, Bologna, Verna, and Pandas) of common wheat (*Triticum aestivum*) produced by the Department of Agriculture, Food, Environment, and Forestry of the University of Florence during the 2021 crop season. The milling process was carried out at the Department of Agricultural, Food, and Environment (DAFE) of the University of Pisa using a commercial mill (Industry-Combi, Waldner Biotech, Lienz, Austria).

The chemical composition and the technological features of flour (Table 1) were determined according to the methods accepted by the International Organization for Standardization (ISO): humidity [27]; ashes [28]; proteins [29]; total fats [30]; falling number [31]; wet gluten and gluten index [32]; dry gluten [33]; total dietary fiber and sugars (sucrose, glucose, fructose, maltose) [34]; amylose and amylopectin [35]; total starch [36]; total polyphenols [37]; Chopin alveogram (W, P/L, P, L, G) [38]; Brabender farinogram (water absorption corrected to 14% humidity, dough time, stability, softening degree (E10: degree of softening after 10 min; E(ICC): softening degree 12 min, after max); and FQN: number of farinographic quality) [39].

Table 1. Chemical composition and technological features of flour. Results are expressed as mean ± SD (n = 4).

Parameters	Units	Flour
Chemical		
Humidity	% w/w	10.90 ± 0.30
Ashes	% w/w	1.37 ± 0.05
Proteins	% w/w	12.90 ± 0.31
Total fats	% w/w	2.53 ± 0.53
Total dietary fiber	% w/w	6.72 ± 0.22
Sucrose	% w/w	0.96 ± 0.05
Glucose	% w/w	0.43 ± 0.02
Fructose	% w/w	0.14 ± 0.01
Maltose	% w/w	6.28 ± 0.26
Wet gluten	% w/w	34.12 ± 2.02
Dry gluten	% w/w	10.94 ± 1.64
Gluten index	% w/w	75.32 ± 10.01
Amylose	% w/w	20.83 ± 0.23
Amylopectin	% w/w	79.23 ± 0.23
Total Starch	% w/w	83.72 ± 0.52
Total polyphenols	mg gallic acid/kg	833 ± 17
Technological		
W	10^{-4} joules	255 ± 29
P/L		3.3 ± 0.8
P	mm	152 ± 13
L	mm	48 ± 9
G		15.0 ± 1.6
Falling number	Seconds	327 ± 26
Water absorption	%	68.9 ± 0.7
Dough time	Minutes	4.3 ± 1.5
Stability	Minute	5.4 ± 2.7
E10	UF	58 ± 5
E(ICC)	UF	90 ± 23
FQN		75± 2

E10 = degree of softening after 10 min; E(ICC) = softening degree 12 min after max, FQN = number of farinographic quality; UF = Farinographic unit.

2.2. Breadmaking Process

The sourdough utilized during the study was obtained by the Consortium for the protection of PDO Tuscan bread. The maintenance of sourdough was performed through consecutive back slopping in order to preserve the sourdough's acidifying and leavening performances. Starter dough maintenance, back slopping, and baking were carried out under controlled operating conditions (time and temperature); bread making was carried out from a pre-ferment leavening agent, according to the two-step method of "biga" [8], as reported in Figure S1.

All the tests were conducted at the Food Technology laboratory of the Department of Agriculture Food and Environment of Pisa University; moreover, for each formulation, three replications were performed.

2.3. Bread Packaging and Storage

After baking, bread loaves were cooled for 2 h at room temperature, then sliced with an automatic slicing machine to 20 mm thickness. Each slice was packed individually in plastic bags (two plastic layers, outer nylon layer, Food Saver, Moncalieri, Torino, Italy), by an industrial packing machine (Lavezzini 450 GAS, Fiorenzuola d'Arda, Piacenza, Italy). During packaging, the composition of the internal atmosphere was modified to obtain 5 different MAP conditions (Ar (100%); N_2 (100%), CO_2 (100%), Mix CO_2/N_2 (70% CO_2; 30% N_2), Mix CO_2/Ar (70% CO_2; 30% Ar)); storage atmosphere composed by 100% Air was utilized as control.

Each pack was stored at a controlled temperature (T = 23 °C) during the whole observation period.

2.4. Bread Shelf-Life Assessment

To determine the bread shelf-life as a function of storage conditions, a total of 50 loaves (1 kg each) were prepared, 1500 slices were packed separately as described above, and divided into 6 groups as a function of MAP composition (5 different MAPs and Air 100% as control). To follow both the chemical-physical and sensory evolution of the packed bread during storage, as well as visible mold appearance on bread surface, four sliced samples for each storage condition were opened daily and analyzed as described below.

2.4.1. Control of the Gaseous Atmosphere Inside the Packages

A Dansensor® CheckPoint 3 CO_2 (infrared sensor) and O_2 (electrochemical sensor) (Ametek Mocon, Brooklyn Park, MN, USA) was used to measure the gas composition inside each pack during storage. The handheld non-destructive gas analyzer is easy to use, fast at processing data, and uses an optical sensor to provide the highest level of accuracy among similar products.

2.4.2. Chemical Characterization of Bread

The moisture content of bread crumbs, taken from the center of each slice, was determined on approximately 5 g sample drying at 105 °C until constant weight, while the pH value was measured according to the AACC (American Association of Cereal Chemists) standard method, as previously reported [8]. Total titratable acidity (TTA) was determined following Gélinas et al. 1995 [40] The concentration of the main fermentative metabolites was investigated using specific enzymatic kits (Megazyme Ltd., Wicklow, Ireland), as described in Taglieri et al. 2020 [8].

2.4.3. Weight Loss, Crumb Softness, and Water Activity (a_w)

For each experimental run, the slices were weighed daily to assess the weight loss associated to water evaporation from the slices during storage; the value was expressed as a percentage reduction compared to the starting value.

a_w was measured by a HygroPalm HP23-AW-A equipment (Rotronic AG, Bassersdorf, Switzerland). The results were expressed as a percentage reduction in water activity compared to the starting value [41].

To measure the softness of the crumb, its compressibility was determined by a penetrometer PNR-12 (Anton Paar, Rivoli (TO), Italy) as described by Al Omari et al. (2016) [42] with some modifications: each sample was compressed in five spots by a weight of 90 g for 10 s. The compression spots were identified by holes, on the four corners and in the center, on a cardboard template, placed on the surface of each sample. The compressibility was measured in mm of penetration (0.1 mm = 1 penetration unit) and results were expressed as a percentage reduction in softness compared to the initial value.

2.4.4. Mold Appearance

All the samples were checked daily for the presence of mold; each experimental run was stopped when 5% of the samples showed mold spoilage.

2.4.5. Sensory Analyses

The sensory profiles of the bread samples were evaluated by a panel of 8 trained judges (aged between 23 and 60 years). All the people involved were members of the "Committee of Experts" of the Department of Agriculture, Food, and Environment Sciences of the University of Pisa. The tasting was carried out according to the previously developed protocol [8,43]. Before the tasting sections, a consensus panel was carried out to set up a sensory card specific for the shelf-life assessment. A final sensory sheet, including quantitative (visual aspect, olfaction, texture, taste, acidity, evolutionary state) together with hedonic ones (global pleasantness, overall acceptability), was individuated by agreement among panelists. Therefore, the overall hedonic index of bread was calculated [8], starting from the mean of the hedonic indices converted on a scale from 0 to 10, according to the following equation:

$$\text{Overall hedonic index} = \text{Mean [Hedonic indexes]} \times 1.11 \quad (1)$$

The research obtained the approval of the ethical committee of the University of Pisa (Comitato Bioetico dell'Università di Pisa, protocol n. 0088081/2020).

2.5. Statistical Analysis

The data obtained were processed by statistical analysis and the significance of differences among means was determined by one-way ANOVA (CoStat, Version 6.451, CoHort Software, Pacific Grove, CA, USA).

Chemical evaluations were performed at least in triplicate and the data are reported as average values. Tukey's HSD test at $p \leq 0.05$ significance was used for the separation of the samples.

The trend of the parameters over time was elaborated with the JMP software package version 17 (SAS Institute, Cary, NC, USA).

The results of the sensory analysis were processed by the Big Sensory Soft 2.0 software (version 2018). Sensory data were analyzed by two-way ANOVA with panelists and samples taken as main factors [44].

3. Results and Discussion

3.1. Weight Loss, Softness of the Crumb, and Water Activity Trend

After baking, the moisture redistribution from the crumb to the crust and the water loss that causes the decrease of softness of the crumb represent two critical issues deeply affecting bread shelf-life [7].

As reported in Figures 1 and 2, the decrease of both the weight loss and the softness of the crumb of the slices during storage showed the same trend as a function of MAP composition. When Air was used as storage atmosphere, the fastest decay rate was observed, while the lowest ones were observed when Mix CO_2/N_2 as well as Mix CO_2/Ar were used, regardless of MAP composition.

Bread preserved with the three pure gas MAPs (Ar, N_2, and CO_2) exhibited an intermediate quality decay rate when compared to Air and both Mix, and very similar performances were observed regardless of the pure gas used for storage.

During storage, the free water moves inside the bread, with a flow going from the crumb to the crust. In the latter, the water tends to evaporate reducing the a_w with a variable intensity depending on the relative humidity present outside. In addition, the retrogradation of starch is one of the main issues of the staling process. Accordingly, a part of the water immobilized during the gelatinization of the starch becomes free during the recrystallization of the starch granules, thus increasing a_w [7,10,41]. Finally, in any time the a_w value of stored bread (Figure 3) gives a measure of the balance between these two opposite phenomena.

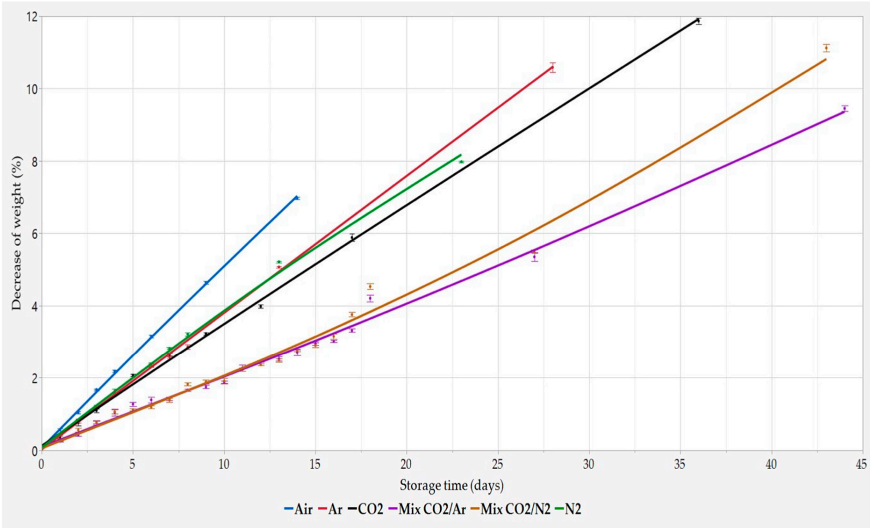


Figure 1. Percentage decrease in weight during storage time. Results are expressed as mean ± SD (n = 4).

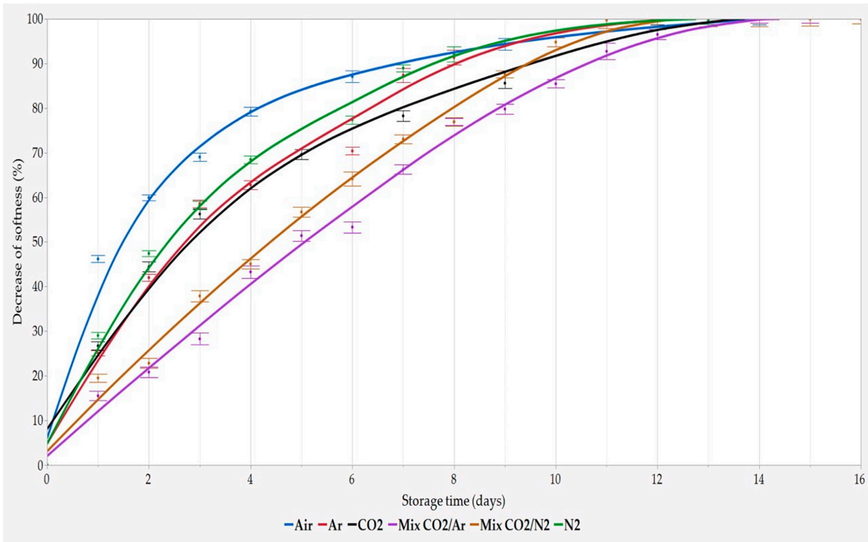


Figure 2. Percentage decrease of softness during storage time. Results are expressed as mean ± SD (n = 4).

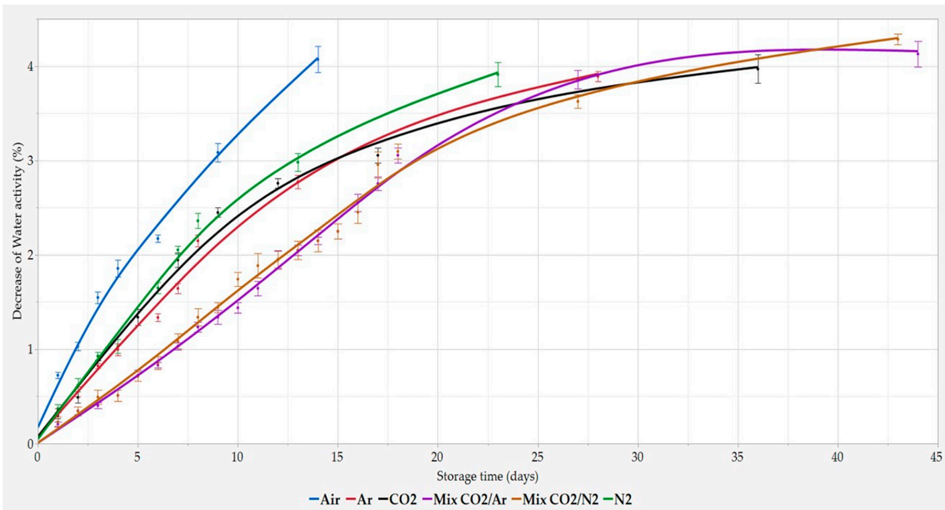


Figure 3. Percentage decrease in water activity during storage time. Results are expressed as mean ± SD (n = 4).

The a_w evolution followed the same trend previously reported for weight loss and crumb softness, with the best storage conditions obtained with both Mix CO_2/N_2 and Mix CO_2/Ar, followed by the three pure gas MAPs, while Air was confirmed as the worst preserving atmosphere.

3.2. Mold Appearence

As mold spoilage on the surface of bread deeply affects its organoleptic quality decay during storage, this parameter (Figure 4) can be used as marker of the acceptability limit as a function of the storage conditions.

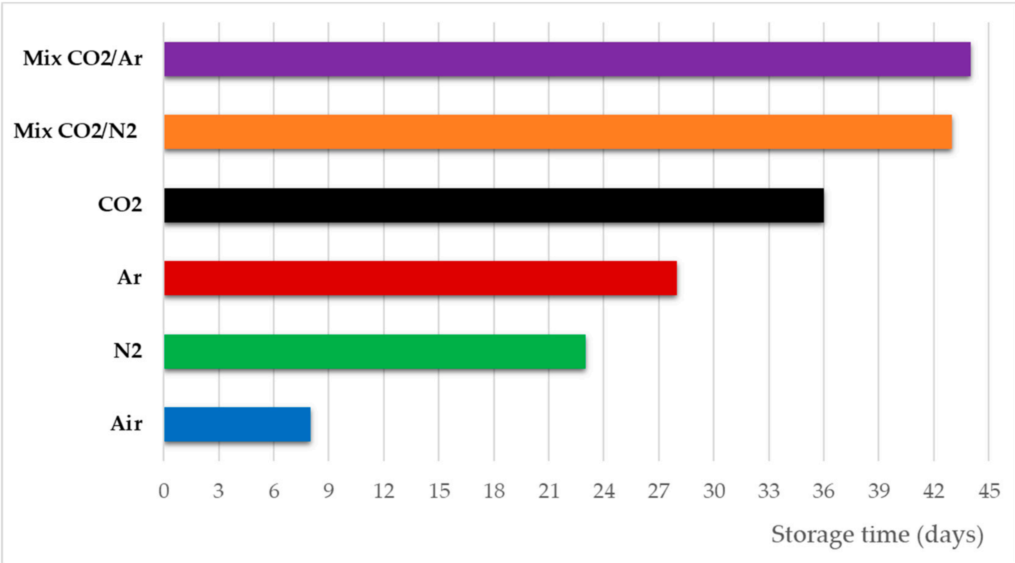


Figure 4. Period of time (days from packaging) during storage before the appearance of fungal bodies on 5% of bread samples, for each experimental condition.

As expected, mold spoilage was firstly evident when Air was used as storage atmosphere, thanks to the aerobic conditions provided in this experimental run.

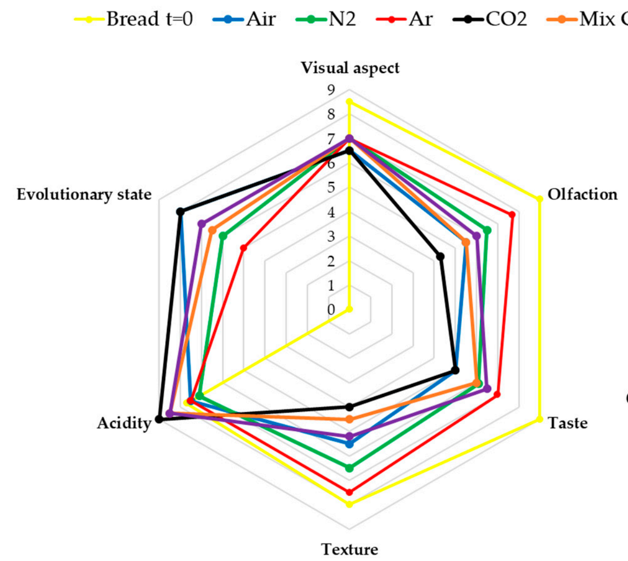
Among the five different MAPs, the use of Mix CO_2/Ar delayed the appearance of the molds, closely followed by Mix CO_2/N_2. Given the antimicrobial activity showed by CO_2, MAP obtained by pure CO_2 was the best one to counteract mold spoilage when compared with pure Ar and pure N_2.

Furthermore, as the packaging utilized for storage did not allow water evaporation, the higher the water loss from the bread slice, the higher the relative humidity (RH) increasing inside the bag with consequent higher opportunity for mold development.

3.3. Sensory Evaluation

Panel tests were carried out on the slices of bread before packaging (t = 0) and daily, to evaluate the impact of different MAPs on the organoleptic profile of stored bread.

Given that the experimental run with Air as preserving atmosphere was stopped at day 8 because of mold development, data related to the panel test made at day 8 for all the storage conditions were reported in Figure 5 and further discussed.



Qualitative parameters	T
Visual aspect	20.10
Olfaction	20.89
Taste	20.07
Texture	20.72
Acidity	19.03
Evolutionary state	20.66

Figure 5. Median of significant qualitative parameters. Friedman's ANOVA analysis (T > χ^2, χ^2 = 14.07).

After 8 days of storage, the organoleptic profile of bread was significantly affected by the different MAPs, with the worst results observed when pure CO_2 was utilized as preserving atmosphere, closely followed by Air. These results are consistent with what was previously observed [23,26]: the presence of CO_2 in the storage atmosphere can significantly increase the sourness of the stored food with a negative impact on its taste. Furthermore, in all the other conditions (Air, Ar, N_2), the value of the acid taste did not increase if compared to the value of bread at t = 0, regardless of the composition of filling atmosphere. In the case of sliced bread, the olfactory parameters were also affected, showing the highest decay. At last, the bread stored with pure Ar best retained its organoleptic profile when compared with bread at t = 0.

The hedonic quality level of a product is fundamental in determining its acceptability and overall pleasantness of the product. A value of 6 was taken as a reference point for the acceptability limit.

The hedonic indexes of bread samples, at time zero and after 8 days of storage in the different experimental conditions, are reported in Figure 6.

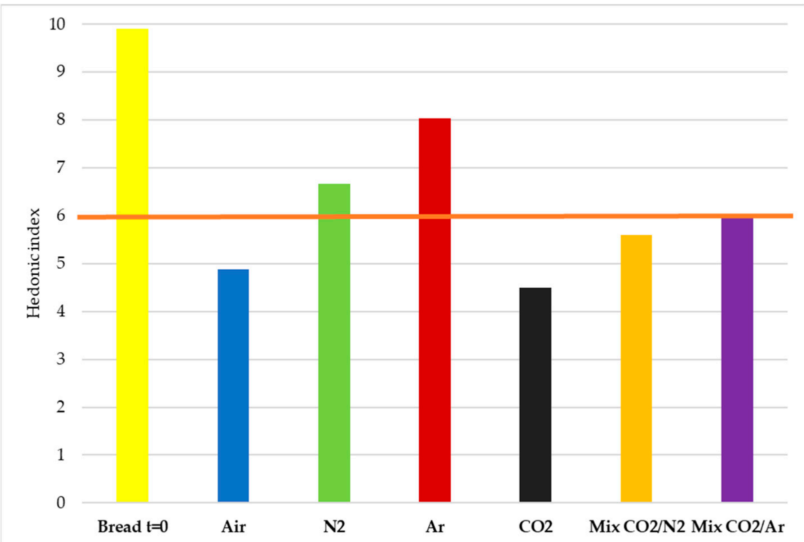


Figure 6. Hedonic index of the bread at time zero and after 8 days of storage in the different MAPs. The orange line indicates the acceptability limit value taken as a reference for this parameter.

The slices of bread preserved in Air and CO_2 were clearly the least appreciated, while those preserved in pure argon were described as the most pleasant, closely followed by samples stored in pure N_2. Furthermore, the presence of CO_2 in the storage atmosphere, even in mix with N_2 and Ar, reduced the pleasantness of the stored bread.

3.4. Chemical Characterization

Similar to what was reported for the sensory characterization of stored bread, the chemical composition of the bread samples was reported and discussed at time zero and after 8 days of storage under different MAPs (Table 2).

Table 2. Chemical composition of the sourdough bread produced at time zero and after 8 days of storage under different MAPs. Results are expressed as mean ± SD (n = 4).

Parameters	*p*-Value [1]	t = 0	Air	CO_2	N_2	Ar	Mix CO_2/Ar	Mix CO_2/N_2
% of dry matter (% dm)	***	61.63 ^{d}	64.23 ^{a}	63.48 ^{bc}	63.62 ^{b}	63.68 ^{b}	63.34 ^{c}	63.28 ^{c}
pH	**	3.89 ^{a}	3.81 ^{ab}	3.76 ^{c}	3.84 ^{ab}	3.85 ^{ab}	3.78 ^{bc}	3.81 ^{abc}
Total titratable acidity (meq lactic acid/g dm)	*	0.032 ^{b}	0.034 ^{ab}	0.036 ^{a}	0.035 ^{ab}	0.035 ^{ab}	0.038 ^{a}	0.036 ^{a}
Acetic acid (mmol/g dm)	*	0.062 ^{b}	0.076 ^{ab}	0.086 ^{a}	0.073 ^{ab}	0.072 ^{ab}	0.091 ^{a}	0.093 ^{a}
Lactic acid (mmol/g dm)	*	0.052 ^{b}	0.057 ^{ab}	0.060 ^{a}	0.056 ^{ab}	0.057 ^{ab}	0.061 ^{a}	0.059 ^{a}
Ethanol (mmol/g dm)	n.s.	0.087	0.087	0.088	0.087	0.085	0.082	0.088

In the same row, different letters indicate significant difference among values. [1] Significance level: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; n.s. = not significant ($p > 0.05$).

The time evolution of the chemical-physical parameters of the stored bread was significantly affected by the composition of the atmosphere inside the pack. In fact, as seen in the Table 2, pH, titratable acidity, and the other metabolites are statistically higher in the slices of bread stored in atmosphere containing CO_2. Therefore, we can assume a dissolution of CO_2 in the matrix, that, on a sourdough bread where the acidity is high results in a sum effect and determines an unacceptability from the sensory point of view. No significant differences were detected between N_2 and Ar. Furthermore, after 8 days of storage, the evolution over time of the main chemical-physical parameters fully confirmed what was highlighted by the sensory analysis.

4. Conclusions

While the application of MAP to store bread is widely accepted, the suitability of this technique to extend the shelf life of PDO Tuscan bread is yet to be explored. Furthermore, to the best of our knowledge, no data are available in the literature on the use of argon as filling gas nor in pure atmosphere nor in mixture with other filling gas (i.e., CO_2 or N_2).

Based on the merging of the chemical-physical, rheological, and organoleptic data collected daily during this storage trial, it was possible to confirm that MAP is a proficiency strategy to significantly extend the shelf-life of this product, the worst results being observed in the control packaging (100% Air as filling gas).

According to the literature [19,23–26], in the storage conditions of this study, the presence of CO_2 inside the package significantly affected the taste of the stored bread by increasing the perceived acidity. Given that the PDO Tuscan bread is a sourdough bread, the above-mentioned increase was even more evident due to the high acidity that characterized the bread before packaging.

All in all, the slowest quality decay rate was observed when 100% argon was utilized as filling gas. Thanks to the promising results obtained with pure argon, subsequent industrial-scale experiments will be carried out with different mixtures of Ar/N_2, to identify also the best economic solution.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11213470/s1, Figure S1: Baking protocol and operating conditions adopted for the experimental trials.

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