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Light and storage time influence the microbial quality of minimally processed rocket

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Abstract

Ready-to-eat minimally processed fruits and vegetables are an ideal substrate for the growth of microorganisms, including human pathogens and mycotoxin-producing species, which question their quality and safety for customers. While we are aware of the importance of production workflows in structuring the products' microbial communities, we still know little about the factors that shape microbiomes during the timeframe products are available to customers, and beyond this timeframe. Here, we study the influence of storage light condition (light or dark) on microbiological and physico-chemical parameters of minimally processed rocket leaves at different shelf life timepoints (the day the product becomes available to consumers, expiration date, 3 days after the expiration date). Our results suggest that the total microbial load increases from the day the product becomes available to consumers, to the expiry date and after the product's expiration. However, when studying the composition of the fungal microbiome, we did not observe significant changes in its structure as the effect of product shelf life or storage light condition. We also found that products stored under light condition had a higher total bacterial load compared to those stored in darkness. Our results might be helpful in crafting improved workflows for product's storage during its shelf life, which might ultimately lead to a re-evaluation of storage times resulting in reduced food waste due to product spoilage or expiration.

KEYWORDS
amplicon sequencing, arugula, bacteria, fungi, plant pathogens, ready-to-eat

1 | INTRODUCTION

The prevalence of ready-to-eat minimally processed fruits and vegetables is steadily increasing in the markets of several countries (Singla et al., 2020). These products are perceived as healthy and convenient by consumers (Sillani & Nassivera, 2015), and their success is also driven by the shift to vegetarian or vegan diet. However, minimally processed products have a reduced shelf life, mainly limited by microbial growth and contamination that can severely affect products' characteristics and safety (Gullino et al., 2019). Indeed, minimally processed vegetables are a substrate for microbial growth, including both plant pathogens, human pathogens and fungi able to produce mycotoxins (Gullino et al., 2019; Singla et al., 2020), causing million cases of food-borne illnesses every year (De Corato, 2020). Microbial

growth on minimally processed fruits and vegetables has also the effect of reducing products' shelf life, which contributes to the increase in food waste if products are not timely sold. Despite these concerns, we still know little about the factors that influence the development of microbiomes associated with minimally processed fruits and vegetables, and which strategies we can use to limit their growth.

Previous studies investigated the microbiological quality of different minimally processed vegetable products, including rocket, spring onion, chicory, broccoli, cabbage, kale, lettuce, mushrooms, watercress and many others (De Oliveira et al., 2011; Jeddi et al., 2014; Jiang et al., 2018; Maistro et al., 2012; Meireles et al., 2017; Piscopo et al., 2019; Sant'Ana et al., 2011; Seo et al., 2010). Often, the outcome of these investigations shows contamination by microorganisms that can potentially cause harm to human health (Sant'Anna et al., 2020). Thus, current workflows are still not able to provide a product that is completely safe for consumers, although in the last years the research has tested some different technological applications to reduce this risk (Galgano et al., 2015; Pinto et al., 2015; Piscopo et al., 2019; Romeo et al., 2010). In addition to looking for new strategies to control the spread of contaminants during production, and new ways to process the product after harvest, it is also important to understand the factors that can influence the microbial development after the vegetables have been packed and delivered to grocery stores.

Once minimally processed products are packaged, they are usually available to consumers within 1 day, and they expire within 7–10 days, depending on the country's regulations. During this timeframe, microbial communities have the chance to develop and influence the products' quality and safety (Gullino et al., 2019; Sant'Anna et al., 2020; Singla et al., 2020). A previous work compared fresh minimally processed vegetables to those at expiry date, and found that the increase of microbial load depended on the producer (Xylia et al., 2021). Minimally processed products are also stored in refrigerators, and the package is usually transparent, allowing the consumers to see the product they are buying. Previous studies (Olarte et al., 2009; Sanz Cervera et al., 2009; Zappia et al., 2019) suggest that light might influence the microbial quality of minimally processed vegetables. While there is evidence suggesting that both storage time and light conditions might influence the development of microbial communities associated with minimally processed products, we still know little about the interaction of these two factors on the microbiome of minimally processed vegetables and their magnitude of change beyond the expiration date.

In this study, we test the influence of storage time and light condition on the microbial communities associated with minimally processed rocket. Specifically, we tested the influence of two light conditions (light or darkness) on a series of microbiological and physico-chemical parameters in samples collected when products become available to consumers, at the expiration date, and beyond the expiration date. We also characterized the rocket fungal microbiome using high-throughput amplicon sequencing. This information is pivotal to understand which factors contribute to the development of microbial communities during storage, potentially leading to alternative strategies to prevent microbial growth, and to test whether there are significant changes beyond the official product's expiration date, which might help reducing waste if it could be safely extended.

2 | METHODS

2.1 | Sampling

Minimally processed rocket (*Eruca vesicaria*) from three different producers (here named R1, R2 and R3) was purchased at grocery stores on the same day it was delivered (which is the same day it becomes available to consumers). Each sample was represented by a single product item as purchased by consumers, each containing ~125 g of product and packaged in PP bags (25 cm × 9 cm × 20 cm of size, 35 μm of thickness). Analyses were performed from samples collected on the purchase day (1 day after harvest, T0), on the expiration day (7 days after harvest, T7) and 3 days beyond the expiration date (10 days after harvest, T10). Throughout the experiment, samples were stored at 4°C and 95% R.H. either in the dark or exposed to light (8 W/430 lm fluorescent lamps). Each group included three replicated samples, yielding three (producers) × three (timepoints) × two (light conditions) × three (replicates) = 54 samples.

2.2 | Microbiological and physico-chemical analyses

For each sample, we first characterized the culturable portion of the microbiome. From each individual bag, we collected 10 g of sample, and homogenized it with 10 ml of sterile Ringer's solution using a Stomacher (BagMixer interscience). Each sample was then serially diluted to a $1:10^4$ ratio, and we used it as inoculum for a series of microbiological tests. Total culturable bacteria were counted on Plate Count Agar (PCA) plates inoculated with 1 ml of sample and after incubation at 26°C for 48 h. Total culturable yeasts and moulds were counted from Dichloran Rose-Bengal Chloramphenicol (DRBC) Agar plates (VWR) inoculated with 1 ml of sample and after incubation at 27°C for 72 h. Total *Pseudomonas* count was obtained by inoculating Pseudomonas Agar Base plates (Biolife Italiana) with 100 μL of sample and counting colonies after incubation at 26°C for 48 h. Finally, total coliforms were counted from Violet Red Bile Lactose Agar plates (Liofilchem) inoculated with 1 ml of sample and after incubation at 37°C for 24 h. For each test, microbial loads are expressed as $\log_{10}$ CFU/g of sample.

Then, each sample was processed to measure a series of physico-chemical properties. The dry matter percentage was measured by collecting a subsample from each bag, and then comparing its dry weight (samples kept at 70°C until constant weight) to the starting fresh weight. The water activity was measured using a Aqualab LITE hygrometer (Decagon Inc.), while the pH was measured using the AOAC method 942.15 (AOAC, 2000). Then, 5 g from each sample were processed to obtain an extract according to Zhan et al. (2012), and using a double-beam UV–visible spectrophotometer (Agilent

8453 UV–Vis, Germany) this extract was used to measure total phenolic content (Singleton & Rossi, 1965), total chlorophyll content (Lichtenthaler & Buschmann, 2001), and to perform the DPPH (Brand-Williams et al., 1995) and ABTS (Re et al., 1999) assays to estimate the total antioxidant activity.

Data was analysed using R v4.1.2 (R Core Team, 2020) and the package *lme4* (Bates et al., 2014), using two modelling strategies. First, for each parameter, we fit a linear model specifying producer, sampling timepoint, storage light conditions and their interactions as fixed factors. Second, for each parameter, we fit a linear-mixed effect model specifying storage timepoint, light condition, and their interactions as fixed factors, and producer as a random effect. The package *emmeans* was used to infer pairwise contrasts (corrected using false discovery rate, FDR).

2.3 | Metabarcoding characterization of fungal communities

We characterized the composition of the whole fungal community in our samples using metabarcoding. Samples were powdered using liquid nitrogen, and DNA was extracted from ~25 mg of sample using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and normalized to 50 ng/μl using ultrapure water. The ITS2 region from the fungal rRNA was amplified using the primers ITS3_KYO2 and ITS4 (Toju et al., 2012) modified to include Illumina overhang adaptors. PCRs were performed in 25 μl of reaction mix (~50 ng of DNA, 0.4 μM each primer, 1X KAPA Biosystems HiFi HotStart ReadyMix and nuclease-free water) using a Mastercycler Ep Gradient S (Eppendorf) set at 95°C for 3 min; 98°C for 30 s, 58°C for 15 s and 72°C for 30 s repeated 35 times; and ending with 5 min of extension at 72°C. Amplifications were performed in technical triplicate, in order to reduce the stochastic variability during amplification. A non-template control in which nuclease-free water (replacing target DNA) was included in all PCR assays, and all the reactions in that bulk were discarded if the no-template control showed amplification. PCR products were then purified with Agencourt Ampure XP SPRI beads (Beckman Coulter Inc.), and 1 μl of the purified amplicons was used for a second PCR to integrate Illumina adaptors using the Nextera XT index Kit (Illumina). Amplicons were purified a second time as reported above, quantified using a Qubit 3.0 Fluorometer (Thermo Fisher) and pooled at equimolar ratio. The pooled library was then sequenced on a Illumina MiSeq instrument using the 300PE chemistry.

Paired-end reads were processed using the DADA2 v1.22 (Callahan et al., 2016) pipeline implemented in R v4.1.2 (R Core Team, 2020) to remove low-quality data, identify ASVs and remove chimeras. Taxonomy was assigned using UNITE v8.3 database (Nilsson et al., 2019). Data was analysed using R v4.1.2 (R Core Team, 2020) with the packages *phyloseq* (McMurdie & Holmes, 2013), *vegan* (Dixon, 2003), *DESeq2* (Love et al., 2014) and *lme4* (Bates et al., 2014). The diversity of microbial communities was estimated for each sample using Faith's phylogenetic diversity index (Faith, 1992), and tests were performed by using two different modelling strategies. First, we fit a linear model specifying producer, sampling timepoint, storage light conditions and their interactions as fixed factors. Second, we fit a linear-mixed effect model specifying sampling timepoint, storage light conditions, and their interactions as fixed factors and producer as a random effect. The package *emmeans* was used to infer pairwise contrasts (corrected using false discovery rate, FDR). Similarly, we tested the influence of producer, sampling timepoint, and storage light conditions on the structure of fungal microbiomes in our system using a multivariate approach. Distances between pairs of samples, in terms of community composition, were calculated using an unweighted Unifrac matrix, then visualized using a CAP procedure. Differences between sample groups were inferred through permutational multivariate analysis of variance (PERMANOVA) (999 permutations), using two different strategies. As above, first we specified producer, sampling timepoint, storage light conditions, and their

TABLE 1 Results from the linear mixed-effect model testing the effect of light condition, storage time and their interactions (including producer as random effect) on several microbiological and physico-chemical parameters

	Light conditions (*L*)		Storage time (*S*)		*L* × *S*	
	χ^2	*p*	χ^2	*p*	χ^2	*p*
TBC	9.59	.002	61.47	<.001	5.14	.077
Yeasts and moulds	2.77	.096	10.94	.004	1.51	.471
Pseudomonas	3.82	.051	120.6	<.001	3.3	.192
Coliforms	2.83	.093	58.07	<.001	1.65	.439
ABTS assay	0.32	.571	4.69	.096	0.3	.859
DPPH assay	0.01	.937	1.17	.556	0.18	.914
Total phenolic content	1.36	.244	11.39	.003	0.97	.615
Total chlorophyll content	12.49	<.001	5.24	.073	7.28	.026
Dry weight	0.06	.802	23.28	<.001	1.56	.459
Total acidity	1.83	.176	18.75	<.001	0.96	.619
pH	0.72	.396	43.42	<.001	0.54	.765
Water activity	0.1	.758	41.45	<.001	1.7	.428

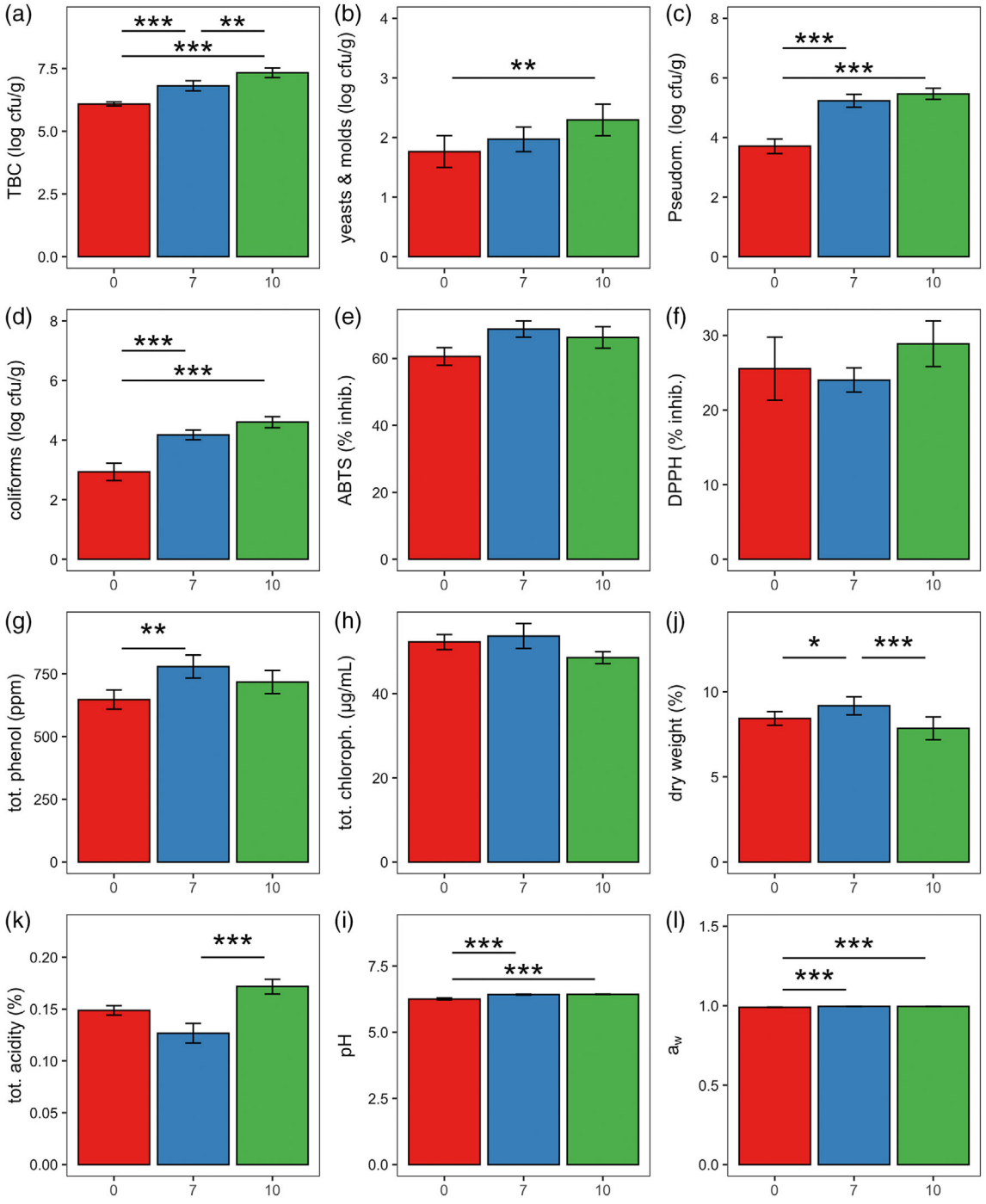

FIGURE 1 Microbiological and physico-chemical parameters measured on our samples at three different timepoints: T0 (sampling day), T7 (expiration day) and T10 (3 days after expiration). (a) Total bacterial count, (b) total mould count, (c) total *Pseudomonas* count, (d) total coliforms count, (e) % inhibition ABTS assay, (f) % inhibition DPPH assay, (g) total phenolic content, (h) total chlorophyll content, (j) percentage of dry weight over total weight, (k) total acidity, (i) pH and (l) water activity. Pairwise contrasts are shown only for significant comparisons ($p < .05$).

interactions as fixed factors. Then, we ran PERMANOVA using sampling timepoint, storage light conditions, and their interactions as fixed factors, and specifying *producer* to stratify permutations. We also tested which ASVs varied in relative abundance after 7 and 10 days of storage when compared to samples processed right after purchase. Using *DESeq2*, we built a model including storage time as fixed factor, extracting the appropriate contrasts (day 7 vs day 0 and day 10 vs day 0), and filtering ASVs with a FDR-corrected $p < .05$.

3 | RESULTS

3.1 | Microbiological and physico-chemical analyses

We tested the influence of light conditions and storage time on several microbiological (total bacteria, yeasts and moulds, *Pseudomonas*, coliforms) and physico-chemical (total phenolic content, dry weight,

total acidity, pH, water activity, total chlorophyll content) parameters. First, we included *producer* as fixed factor in our analysis, and we found that this influenced all the parameters above (Appendix S1). Given our interest in testing the effect of light condition and storage time on these parameters, we decided to use a linear mixed-effect model including *light condition* and *storage time* as fixed factors, and *producer* as random effect.

Results (Table 1) suggest that TBC ($\chi^2 = 9.59$, $p = .002$) and chlorophyll content ($\chi^2 = 12.49$, $p < .001$) were influenced by light conditions. Specifically, post-hoc tests suggest that total bacterial counts were higher when products were exposed to light compared to those kept in dark ($p = .003$), while chlorophyll content was higher when products were kept in dark conditions ($p < .001$).

Most of the parameters we measured were influenced by storage time (Table 1, Figure 1). Total bacterial counts (Figure 1A) significantly increased from the first sampling to the sampling at the expiration day ($p < .001$), to the one after the expiration day (T7–T10 $p = .006$; T0–T7 $p < .001$). Also, mould counts (Figure 1B) increased from the first sampling to the one after 10 days ($p = .005$), but no differences were found between T0 and T7, and T7 and T10 ($p > .05$). Counts of *Pseudomonas* and coliforms (Figure 1C,D) were both higher at T7 ($p < .001$) and T10 ($p < .001$) compared to T0, while no differences were found between T7 and T10 ($p > .05$). Similar patterns were found for pH and water activity (Figure 1I–L), with lower values at T0 compared to both T7 and T10 ($p < .001$), with no differences between T7 and T10 ($p > .05$). We also found differences in the total phenolic content, with higher values at T7 compared to T0 ($p = .004$, Figure 1G), and total acidity, with higher amounts at T10 compared to T7 ($p < .001$, Figure 1K). The percentage of dry weight was increased from T0 to T7 ($p = .02$) and then decreased at T10 ($p < .001$, Figure 1K). Storage time did not influence results from ABTS and DPPH assays (Table 1, Figure 1E,F), and chlorophyll content (Table 1, Figure 1H).

3.2 | Metabarcoding characterization of fungal communities

We used high-throughput amplicon sequencing targeting the ITS rRNA gene to characterize the fungal communities associated with our samples, which were classified in 340 ASVs. Results (Figure 2A) suggest a high abundance of the genus *Vishniacozyma* (14.64%),

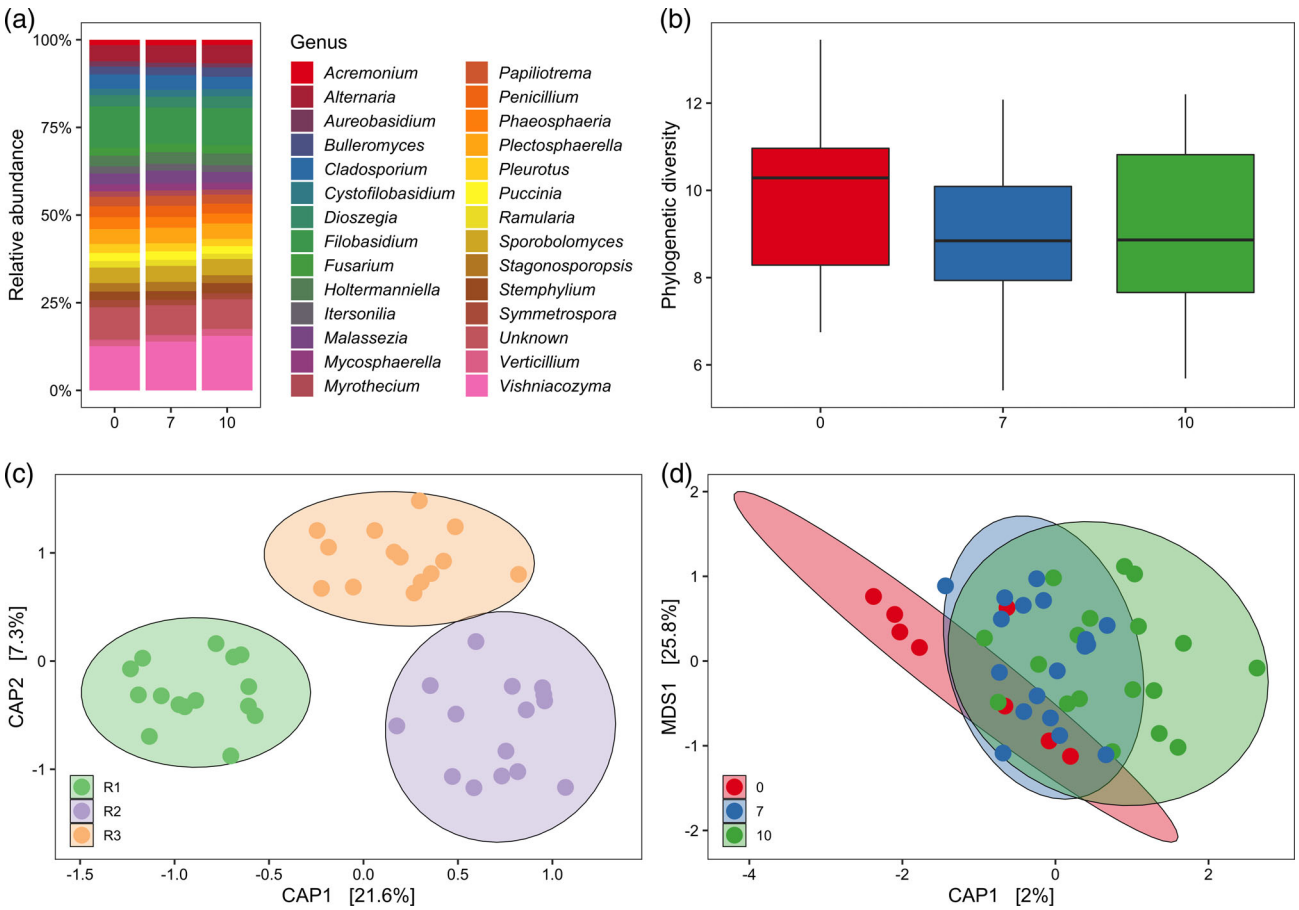

FIGURE 2 (a) Fungal community composition at different timepoints (genera with a relative abundance < 1% are not reported). (b) Comparison of Faith's phylogenetic diversity index between sampling timepoints. (c, d) Canonical analysis of principal (CAP) coordinates ordination using a Unifrac distance matrix, visualizing the effect of producer (c) and sampling timepoint (d) on the structure of fungal communities (percentages in parentheses report the variance explained by the respective axis).

followed by *Filobasidium* (10.72%), *Alternaria* (5.03%), *Sporobolomyces* and *Plectosphaerella* (both ~4%). We also found *Cladosporium*, *Malassezia*, *Holtermanniella*, *Dioszegia*, *Phaeosphaeria*, *Penicillium* (all ~3%). A minor portion was represented by members of *Papiliotrema*, *Stemphylium*, *Bulleromyces*, *Stagonosporopsis*, *Fusarium*, *Pleurotus*, *Puccinia*, *Cystofilobasidium*, *Mycosphaerella*, *Itersonilia*, *Verticillium*, *Symmetrospora*, *Ramularia*, *Acremonium*, *Myrothecium* and *Aureobasidium*, each representing ~1% to 2% of the fungal community. About 8.61% of the ASVs were not identified.

When testing the effects of producer, light conditions and storage time on the diversity of fungal communities, we found an effect driven by producer ($F = 47.23$; df = 2, 29; $p < .001$), while all the other factors and the interactions between them did not report significant effects ($p > .05$). When we included producer as random factor, neither light conditions, storage time, nor their interaction influenced the phylogenetic diversity index ($p > .05$; Figure 2B).

Similarly, when testing the influence of the same factors on the structure of fungal communities, we found a strong effect driven by producer ($F = 8.71$, df = 2, $p < .001$; Figure 2C) explaining 29.58% of the variation. All the other factors and their interaction did not have any effect on the structure of rocket-associated fungal communities ($p > .05$). Post-hoc contrasts confirmed pairwise differences between all the three producers ($p < .001$). When running the PERMANOVA using *producer* as random effect, we found that storage time influenced the structure of fungal communities ($F = 1.76$, df = 1, $p = .04$) but with a marginal effect ($R^2 = 0.04$), so that post-hoc contrasts were not able to identify differences between groups ($p > .05$; Figure 2D).

We also tested the influence of storage time on the relative abundance of single ASVs. Comparing T7 towards T0, we found an increase ($p < .001$) in the abundance of 3 ASVs at T7 (*Paramyrothecium*, *Pleurotus*, *Cystofilobasidium*), while comparing T10 towards T0 we found an increase ($p < .001$) in the abundance of 2 ASVs at T10 (*Pleurotus*, *Cystofilobasidium*), and a decrease ($p < .001$) in the abundance of a single ASV identified as *Filobasidium* (also found when comparing T10 towards T7).

4 | DISCUSSION

Here we tested the influence of product storage time and light condition on the microbiological and physico-chemical properties of minimally processed rocket. We found an increase in microbial load (both bacteria and fungi) over time from T0 to T10. In general, storage time influenced most of the microbiological and physico-chemical properties we measured, while storage light conditions influenced only total bacterial counts and total chlorophyll content. The analysis of the fungal microbiome using high-throughput amplicon sequencing showed a small effect driven by storage time, while no effect was driven by light condition. In general, producer identity was the major factor in driving differences across the variables we measured.

Previous studies on minimally processed rocket salad show similar results in terms of total bacterial counts (~6 to 7 log cfu/g), yeasts and moulds (~3 log cfu/g) and *Pseudomonas* (~4 log cfu/g) (Giannoglou et al., 2020; Xylia et al., 2019; Xylia et al., 2021; Yahya et al., 2019; Zappia et al., 2018). Our results show a progressive increase in microbial load from the first sampling to the expiration day, and after the product's expiration, including total bacteria and fungi. This effect is observed also when focusing on *Pseudomonas* spp. and total coliforms. Increases in microbial loads during storage time have also been previously reported in minimally processed rocket (Xylia et al., 2019, 2021) and other vegetables (Allende et al., 2004; Corbo et al., 2006). On the other hand, metabarcoding data did not show changes in fungal diversity as effect of sampling timepoint, while changes in fungal community structure were only marginal. Although our study lacks an in-depth characterization of the bacterial community throughout storage time, our results suggest that the structure of fungal microbiomes does not change within our sampling timeframe, but the whole microbial load increases. In addition, our results suggest that the major driver of the structure of these microbiomes is the producing company, and this is supported by previous studies (Xylia et al., 2019, 2021). Thus, the growing conditions and the handling procedures before packaging are the most important factors in establishing the microbiomes of minimally processed rocket, which is of primary importance for guaranteeing food safety. Results also suggest a higher total phenolic content after 7 days of storage and no changes in the antioxidant activity (ABTS and DPPH assays).

To the best of our knowledge, only few studies focused on the characterization of the microbiome associated with rocket leaves, and how different factors influence its structure. Cernava et al. (2019) characterized the bacterial microbial communities associated with rocket plants, and found that Enterobacteria are a major part of their core microbiota, while another study (Taffner et al., 2019) found that the archaeal community was dominated by the phylum Thaumarchaeota. Here, we described the fungal community associated with minimally processed rocket leaves. We found that it was mostly dominated by members of the genus *Vishniacozyma*, which is a yeast commonly reported associated with plants (Félix et al., 2020; Into et al., 2020; Kusstatscher et al., 2019; Vujanovic, 2021). We also found fungal genera that are widely known as endophytes (e.g., *Cladosporium*, *Malassezia*, *Aureobasidium*), but also several that could include plant pathogens (i.e., *Alternaria*, *Penicillium*, *Fusarium*, *Mycosphaerella*, *Verticillium*, *Plectosphaerella*, *Stemphylium*, *Stagonosporopsis*, *Puccinia*, *Itersonilia*, *Ramularia*, *Myrothecium*, *Paramyrothecium*), some of which are known to produce mycotoxins. As reported above, we found a weak signature driven by storage time on the composition of the fungal rocket microbiome and, indeed, the abundance of few ASVs (*Paramyrothecium*, *Pleurotus*, *Cystofilobasidium*, *Filobasidium*) was observed to change as response to storage time. While *Pleurotus* is likely to be an environmental contaminant, *Paramyrothecium* has been previously reported to cause leaf spots on different plant species (Haudenshield et al., 2018; Matić et al., 2019; Wu et al., 2021), while members of the genus *Cystofilobasidium* are known as biocontrol agents in post-harvest disease control (Liu et al., 2011; Spotts et al., 2009). Thus, the increase in abundance of *Cystofilobasidium* might represent a response of the microbiome in contrasting the development of potential disease agents. While this

needs to be further investigated, it might also represent a source of potential novel biocontrol agents that can be safely used to extend the shelf life of minimally processed vegetables.

Storage light conditions only influenced the total bacterial counts and chlorophyll content, while no effect was observed for any other parameter we measured or within the metabarcoding dataset. Our previous study (Zappia et al., 2019) investigated a similar question, suggesting also that storage light condition influenced the total bacterial count, with higher counts when the product was exposed to light. Similar results were observed also on asparagus (Sanz Cervera et al., 2009), cauliflower and broccoli (Olarte et al., 2009). The presence of light might trigger microbial growth through a series of mechanisms, but we speculate that this is driven by small increases in leaf's surface temperature, helping microorganisms to grow faster. We also observed a decrease in chlorophyll content in the group that was not exposed to light. While, to the best of our knowledge, no previous study focused on this parameter, other works report differences in the colour of minimally processed vegetables stored in light or dark condition (Olarte et al., 2009; Sanz Cervera et al., 2007; Sanz Cervera et al., 2009), an effect driven by light, which slows down chlorophyll degradation (Okada et al., 1992).

As minimally processed salads are steadily permeating the market, it is important to understand the factors that drive the post-harvest and post-packaging life of these products. Our results show a constant increase of the overall microbial load of minimally processed rocket leaves over 10-days post-packaging, which includes also increases in the load of *Pseudomonas* and coliforms, potential threats to human health. However, the changes in microbiological parameters observed between the expiry date shown on the label and the longer storage time did not appear significant. We also found that storage in dark conditions slows down the growth of most cultivable bacteria, which might be a strategy to use to extend the products' shelf life and safety for consumers. The characterization of the fungal microbiota did not highlight potential threats to human health, and we did not observe changes driven by storage time or light condition. While here we focused on the fungal microbial communities, results suggest that a broader analysis of other components of the microbiome, including bacteria, might be important to fully understand the microbial diversity of minimally processed salads and their dynamics over time. Overall, results show that the production facility and processes have the highest impact on rocket microbiome, thus changes in handling procedures might be key in structuring a microbiota that develops less rapidly, helping to extend the shelf life of minimally processed vegetables without harm to consumers. This will economically benefit both producers and retailers, and contribute to reduce food waste.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Raw data is available at the NCBI Sequence Read Archive database under the Bioproject number PRJNA848755.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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