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ORIGINAL ARTICLE

Application of a combined treatment using natural antimicrobials and modified atmosphere packaging to enhance safety, quality, and shelf-life of fresh-cut beet leaves

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Abstract

Fresh-cut horticultural products have a very limited shelf-life and have been associated with several foodborne illnesses. In previous studies, natural antimicrobials green tea (GTE) and nisin have shown great effect on microbial control, while modified atmosphere packaging (MAP) with modified initial atmosphere (MIA) was great for nutritional preservation. The aim of this study was to select an adequate combination of those treatments to enhance safety, quality, and shelf-life of fresh-cut beet leaves. Results shows synergistic behavior with the combination of 2.5% GTE, 500 UI/g nisin and MAP with MIA for native microbiota, *Listeria*, and *Escherichia coli* control. During refrigerated storage, combined treatment showed a significant reduction in microbial counts and generate a "fortification" increasing greatly its total polyphenols content and antioxidant capacity. While control samples presented a shelf-life of less than 7 days, an adequate combination of technologies improved greatly the overall quality and prolonged shelf-life to at least 14 days.

Practical applications

In the context of a growing demand for healthy, nutritious, minimally processed, free of additives, and environmentally friendly products, this study proposes the use of the modified atmospheres packaging technology in combination with the application of natural antimicrobials to improve overall quality and enhance safety of minimally processed vegetable products. The combination of these two technologies for the preservation of minimally processed beet leaves has proven to be an alternative that not only allows microbiological control of the product, but also causes a significant improvement in nutritional and sensory quality. The results presented in this study have a clear practical application in the minimally processed vegetable industries. Indeed, developments as the one presented in this research are needed by the industry to reach the demanding requirements of consumers as well as the new regulatory requirements and to increase the added value of this kind of products.

1 | INTRODUCTION

Fresh horticultural products, especially minimally processed ones, provide a good substrate for the growth of microorganisms during production, commercialization and storage, limiting their shelf-life. Moreover, in the last two decades a marked increase in foodborne illnesses associated with the consumption of fresh or minimally processed vegetables (MPV) was observed (Mercanoglu Taban & Halkman, 2011). An interesting approach with great potential to overcome these drawbacks is biopreservation, by which MPV shelf-life and safety is increased through the use of natural compounds with antimicrobial properties.

In a previous study (Fernández, Agüero, & Jagus, 2017a), it has been demonstrated that green tea extract (GTE) is a very promising option for reducing and preventing the growth of pathogenic and spoilage microorganism of leafy vegetables, specifically beet leaves. Moreover, their combination with nisin, a bacteriocin with GRAS degree, presented an increased effect for *Listeria innocua* and native

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mesophilic aerobic bacteria. Nevertheless, beet leaves exposed to GTE treatments at effective concentrations (5%) presented some changes in their appearance, showing a more reddish tonality compared to control samples, which could be a drawback for their implementation.

Another technology that was tested for fresh-cut beet leaves preservation was modified atmosphere packaging (MAP). This technology is considered, after refrigerated storage, the most effective method to extend the shelf-life of minimally processed products (Oliveira et al., 2015). The reduction of oxygen and the increase of carbon dioxide levels in the atmosphere around fresh produce have several positive effects: reduce respiration rates, decrease softening rates, improve pigment retention, and reduce microbial growth, among others (Jung, 2005). Indeed, with application of MAP significant improvements in retentions of bioactive compounds such as betalains, carotenes, chlorophylls, and total polyphenols has been shown in fresh-cut beet greens (Fernández, Martínez Melo, Jagus, & Agüero, 2016), but the treatment was not enough to achieve an improvement in their microbiological quality.

It is well known that the application of combined preservation treatments (Hurdle technology) constitute an excellent approach to improve overall quality of products (Khan, Tango, Miskeen, Lee, & Oh, 2017), reducing nutritional and sensory losses while enhancing food safety, as it requires "softer" intensities than when preservation treatments are applied individually. Thus, combination of MAP with natural antimicrobials could result in a promising strategy to simultaneously achieve safety and quality for preservation of beet leaves. In fact, some studies have demonstrated good results of natural antimicrobials combined with MAP, but for meat products preservation. However, there are very few applied on horticultural products (Severino, Ferrari, Dang, & Donsì, 2015; Siroli et al., 2014).

Owing to all facts listed above, the aim of this study was to select an adequate combination of the natural antimicrobials GTE and nisin with MAP to control microbial growth (native microflora and contaminations with *L. innocua* and *Escherichia coli*) of fresh-cut beet leaves and determine the enhancement of microbiological, nutritional, and sensory quality during refrigerated storage.

2 | MATERIALS AND METHODS

2.1 | Plant material

Beets (*Beta vulgaris* L. Conditiva var.) were obtained in a local market in Buenos Aires, Argentina. The purchase took place on the first day of the study, and immediately afterwards beet plants were transported in refrigerated conditions to the laboratory. Once there, roots and stems were removed, and beet leaves were stored at 5 ± 1 °C until used.

2.2 | Study of the effect of modified atmospheres in combination with natural antimicrobials on microorganisms of interest: Treatment selection

In this first study, it was evaluated whether through the combination of technologies, improvements could be obtained in the control of microorganisms of interest with respect to individual treatments and if it is possible to reduce natural antimicrobial concentrations. The microorganisms selected were the native aerobic mesophilic bacteria as a representative group of the native microbiota; *L. innocua* (strain CIP8011), representing the group of gram-positive bacteria; *E. coli* (strain ATCC8739) representing the group of gram-negative bacteria. Moreover, the last two are surrogates of *Listeria monocytogenes* and *E. coli* O157:H7, respectively, two pathogens of great interest in MPV.

2.2.1 | Preparation of antimicrobials

For nisin treatments a 50,000 IU ml^{-1} stock solution was prepared dissolving 250 mg of commercial nisin (Delvo Plus, DSM) in 5 ml of sterile distilled water. For green tea extracts treatments, a 40% stock solution of Sunphenon 90LB (Taiyo International) were prepared adding 4 g of the product and carried to 10 ml with sterile distilled water.

2.2.2 | Cultures preparation

A mixed culture of *L. innocua* and *E. coli* was used, for which each of the microorganisms was cultivated separately in its corresponding trypticase soy broth enriched with 0.6% yeast extract (TSBYE, Biokar Diagnostics, France) as detailed by Fernández et al. (2017a). An aliquot of each broth was mixed together to obtain the mixed broth containing 10^8 cfu/ml of *L. innocua* and 10^8 cfu/ml of *E. coli*.

2.2.3 | Preparation of the samples

The beet leaves were washed with cold tap water and disinfected by immersion in a solution of sodium hypochlorite (200 ppm of free chlorine) for 5 min. They were dried for 1 min in a manual centrifugal dryer and then cut perpendicularly to the veins, obtaining 2–3 cm wide strips. Then, 40 g of fresh-cut beet leaves were placed in 20 x 25 cm plastic bags (Polyolefin PD960, with a permeability at 23 °C of 6,000–8,000 and 19,000–22,000 cm^3 m^{-2} 24 hr^{-1}, for O_2 and CO_2, respectively, and water vapor permeability of 15–18 g m^{-2} 24 hr^{-1}, CRYOVAC, Argentina). Before the addition of antimicrobial solutions, an aliquot of the mixed culture was added into all systems to obtain an initial bacterial count of approximately 10^5–10^6 cfu/g of *L. innocua* and *E. coli*. In Table 1, treatment scheme is presented. For each treatment, an aliquot of antimicrobial stock solution was added to the system to achieve the desired concentration. To match the water content in all the samples, the necessary amount of sterile distilled water was added in each case. The bags were closed, with or without initial atmosphere modification (Table 1), using a MULTIVAC C200 packer (MULTIVAC, Germany). Based on previous studies (Fernández et al., 2016) the gas mixture used was 5% O_2-10% CO_2-85% N_2, while for control samples air was used (21% O_2-0.03% CO_2). Samples were immediately stored in refrigeration until further analysis.

TABLE 1 Systems composition scheme

Treatment	Nisin (UI/g)	Green tea (%)	Initial atmosphere
C	No	No	Air
MIA	No	No	5% O_2-10% CO_2
T2.5N	500	2.5	Air
T2.5N + MIA	500	2.5	5% O_2-10% CO_2
T5N	500	5	Air
T5N + MIA	500	5	5% O_2-10% CO_2

2.2.4 | Atmosphere composition

Before opening the bags, a sample of internal gaseous atmosphere was taken. The concentration (%) of O_2 and CO_2 in the headspace of each packing unit was determined using the MAP-PAK Combi kit (AGC Instruments, Ireland).

2.2.5 | Inactivation and survival studies

In the food supply chain, temperature fluctuation during transport, storage, and home abuse is very common, which can result in the growth of surviving microbial cells and therefore in an increased health risk. In this study, a storage under an extreme abuse temperature (12 ± 2 °C) was allowed to analyze the treatment potential on the worst-case scenario. Periodically (0, 1, 2, 5, and 7 days) samples of 10 g were homogenized with 90 ml of sterile 0.1% peptone water (Biokar Diagnostics, France) in stomacher (Interscience Laboratories, Inc. BagMixer 400P, France) for 120 s. Decimal dilutions were prepared with sterile 0.1% peptone water and plated in the growth media for microbial counts. Mesophilic aerobic bacteria counts were determined in plate count agar (Biokar Diagnostics, France) after 48 hr at 37 °C, *Listeria* spp. counts were determined using Oxford Agar (Biokar Diagnostics, France) with *Listeria* Selective Supplement (Oxoid, SR140), incubated at 37 °C during 24–48 hr; and ***E. coli*** counts were performed using MacConkey agar (Biokar Dignostics, France) with the addition of the supplement 4-methylumbelliferyl-beta-D-glucuronide "MUG" (Biokar Dignostics), incubated at 37 °C during 24 hr. Results were expressed as the logarithm of colony forming units per gram (log cfu/g). The detection limit of the method (DL), in all cases was 1 log cfu/g.

2.3 | Stability study and determination of the shelf-life of minimally processed beet leaves stabilized with the selected combined treatment

Once the best combination of natural antimicrobials and MAP was selected, a second study was developed to evaluate its performance to enhance global quality of the product during refrigerated storage.

2.3.1 | Preparation of antimicrobials

Antimicrobials were prepared as detailed in Section 2.2.1.

2.3.2 | Sample preparation, storage, and sampling

Samples were prepared as detailed in Section 2.2.3 but without the inoculation step and stored at 5 ± 1 °C. Considering the results of the study detailed in Section 2.2, for this study only treatments C, MIA, T2.5N and T2.5N + MIA were prepared. Periodically, for 3 weeks, the quality indicators detailed in the following section were determined.

2.3.3 | Determination of quality indices

Atmosphere composition

Atmosphere composition was determined according with Section 2.2.4.

Microbiological quality

Preparation of samples for microbiological determinations was described in Section 2.2.5 as well as the mesophilic aerobic bacteria and *Listeria* spp. determination methodology. Moreover, native *Enterobacteriaceae* count was determined in MacConkey agar (Biokar Dignostics, France), incubated at 37 °C during 24 hr and yeast and molds counts were determined in yeast extract glucose chloramphenicol agar (YGC, Biokar Diagnostics, France) incubated at 28 °C during 48–72 hr. The results were expressed as the logarithm of colony forming units per gram of leaf (log cfu/g). The detection limit of methods was 1.00 log cfu/g.

Nutritional quality

The total phenolic content was determined using the Folin–Ciocalteu method, expressed as milligrams of Gallic acid equivalent per 100 g of fresh tissue (mg AG/100 g). The betaxanthines (Bx) and betacyanins (Bc) content was reported as mg/100 g of fresh tissue. All the nutritional determinations were carried out according to the methodologies described by Fernández, Agüero, & Jagus, 2017b. The antioxidant capacity was evaluated through the DPPH and FRAP assays, expressed as milligrams of ascorbic acid equivalents per 100 g of fresh tissue (mg/100 g) and micromole equivalent of trolox per gram of fresh tissue (μmol TX/g), respectively.

Sensory analysis

Sensory analysis was carried out by a trained panel composed of eight members, aged 24–62 years. The coded samples were randomly presented to the judges who evaluated the sensory parameters (odor, color, texture, and overall visual quality) using a 1–9 hedonic scale, where 9: excellent; 5: acceptance limit; 1: totally unacceptable.

2.4 | Statistical analysis

All the determinations were made in triplicate in two separate experimental treatments runs. Results were expressed as the mean of all repetitions together with the *SD*. The statistical analysis was performed using the Origin 8 software (OriginLab). The data were subjected to an analysis of variance (ANOVA) using as sources of variation: TIME (storage time, day of sampling), TRAT (treatment according to Table 1), and TIME-TRAT interaction. Differences between factors were determined by the Tukey test ($p < .05$).

3 | RESULTS AND DISCUSSION

3.1 | Study of the effect of modified atmospheres in combination with natural antimicrobials on microorganisms of interest: Treatment selection

3.1.1 | Atmosphere composition

Samples without modification of the initial atmosphere (C, T2.5N, and T5N) presented initial concentrations of around 20.2 ± 0.1% of O_2 and 1% ± 0.3 of CO_2 and all the samples packed with modification of the initial atmosphere (MIA, T2.5N + MIA, and T5N + MIA) presented concentrations of 5.1 ± 0.2% of O_2 and 10.5 ± 0.6% of CO_2.

Changes during storage in gasses concentration in samples with antimicrobials were similar to those registered in samples without these compounds. All samples reached dynamic equilibrium, with an atmosphere of 8.0% of O_2 and 3.5% of CO_2, at 24 hr in samples packed with MIA and at 6 days in samples without MIA stored at 12 °C. From these results, two important facts were observed. On the one hand, the addition of antimicrobials did not affect the atmospheres inside the packages, which means that did not affect significantly the respiratory rate of this product. On the other hand, samples packed with MIA reached the dynamic equilibrium significantly faster than the other ones.

Although there is not much information available about changes in gasses concentrations of vegetable samples treated with the combination of these technologies, similar results were found by Helland et al. (2016) working with fresh-cut turnips packaged with or without MIA (5% O_2), as observed that the equilibrium was reached almost immediately in MIA samples, while for controls it took 8 days (at 5 °C).

3.1.2 | Effect on microorganisms during storage

Mesophilic aerobic bacteria (MAB), *L. innocua* and *E. coli* counts in samples treated according to Table 1 during storage at 12 °C are presented in Figure 1. Statistical analysis indicated significant interaction between factors TREAT and TIME in all cases.

Regarding MAB counts (Figure 1a), control samples initially presented 6.2 ± 0.4 log cfu/g, showing a constant growth, reaching values of around 8.0 log cfu/g from 48 hr onwards. Samples packed with MIA (without antimicrobials) did not differ statistically ($p > .05$) from control. Conversely, samples treated only with antimicrobials showed significant initial reductions of around 2 and 3 log cycles for T2.5N and T5N, respectively. Regarding combined treatments, while T2.5N + MIA presented an additional initial reduction of 1 log with respect to the individual antimicrobial treatment, T5N + MIA samples did not differ significantly from the observed for T5N. During storage, MAB grew in all samples, highlighting that samples corresponding to treatments T5N, T2.5N + MIA, and T5N + MIA did not show significant differences among them. T2.5N treatment presented an intermediate behavior differing statistically from all the other treatments. It is interesting to note that the combined treatment T2.5N + MIA achieved significant improvements compared to its equivalent without MIA, which implies a synergistic effect between the applied preservation technologies.

Several researchers have demonstrated the effectiveness of the combination of MAP technology with natural antimicrobials for the native microbiota control of different products, mostly meats, considering generally a shelf-life limit of ≥7 log cfu/g of MAB counts. Working with minimally processed shrimp, Lu (2009) found that the combination of an antimicrobial preparation (1 g L^{-1} 4-hexylresorcinol, 500 IU ml^{-1} nisin and 5 g L^{-1} sodium dehydroacetate) with MAP managed to prolong the shelf-life of the shrimps 4 days (with 100% CO_2) or 8 days (with 40% CO_2-30% O_2-30% N_2). Schelegueda, Delcarlo, Gliemmo, and Campos (2016) working with hake burgers found even more remarkable effects, while control samples presented a shelf-life of less than 5 days, individual treatment with MAP (55% CO_2–45% N_2) keep the counts below the acceptability limit during

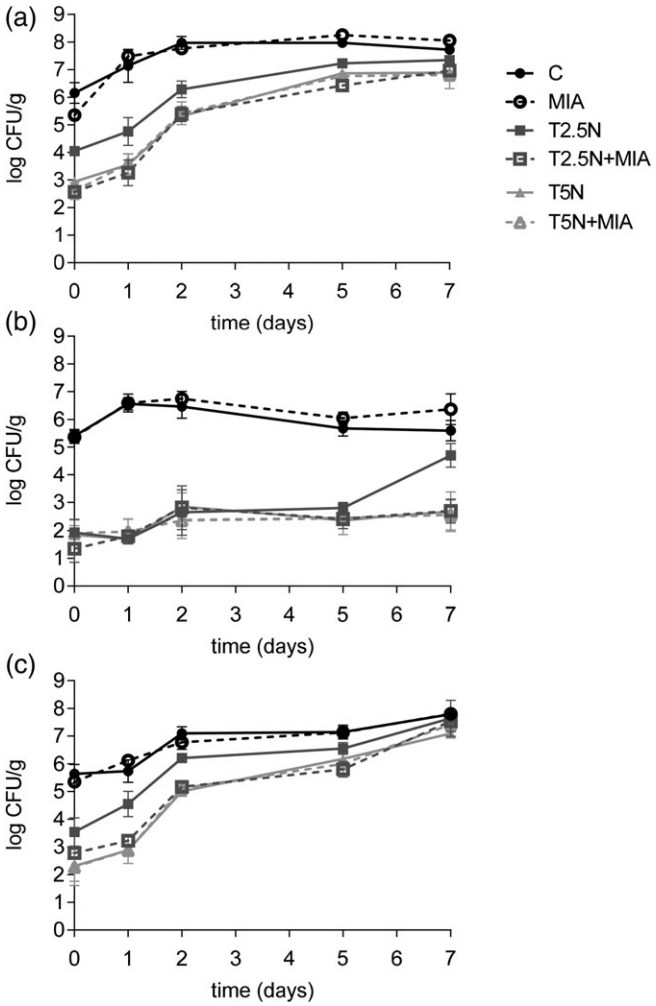

FIGURE 1 Mesophilic aerobic bacteria (a), *Listeria innocua* (b), and *E. coli* (c) counts in samples of minimally processed beet leaves with different treatments during storage at 12 °C. Error bars represent the *SD* of the mean

30 days of storage at 4 °C and the combination with antimicrobials (chitosan, nisin, and sodium lactate mixture) presented a bacteriostatic effect, keeping MAB counts at 3 log cfu/g until the end of storage. Similar results were obtained by Petrou, Tsiraki, Giatrakou, and Savvaidis (2012) who working with chicken meat treated with MAP (30% CO_2-70% N_2) and oregano oil or chitosan achieved a shelf-life extension of 6 or 15 days, respectively.

In the case of *L. innocua* (Figure 1b), the initial count in control samples was 5.40 ± 0.24 log cfu/g, maintaining values between 5.5 and 6.5 log cfu/g throughout storage. Individual treatment with MIA presented similar behavior to control, without significant differences ($p > .05$). Samples containing natural antimicrobials achieved initial reductions between 3.5 and 4 log cycles, presented a lag phase of 24 hr from which a slight growth was observed, remaining around 2.5 and 2.8 log cfu/g until the end of storage, except for samples of T2.5N treatment, which in the last 48 hr presented a notable regrowth, differing significantly from the rest of the treatments at that point. Hence, the combination of the lower concentration of green tea extracts with nisin and MIA managed to maintain lower *L. innocua* counts at the end of storage compared to the individual treatments. It is interesting to

note that through the combination of technologies it is possible to reduce the effective concentration of tea, achieving with tea 2.5% results similar to those observed with treatments containing 5%.

The results reported in bibliography regarding MAP effect on *Listeria* counts are usually not consistent. According to Oliveira et al. (2015) review, numerous studies have reported that the survival and growth of *L. monocytogenes* in fruits and vegetables is not reduced or affected with MAP. On the other hand, there is encouraging results regarding the combination of MAP with antimicrobials to achieve *Listeria* spp. control. In this sense, Schelegueda et al. (2016) found that MAP treatment with MAI (55% CO_2-45% N_2) was not able to control the development of a contamination of 3 log cfu/g of *L. innocua* in hake burgers. But when combined with a mixture of antimicrobials (chitosan, nisin, and sodium lactate), the counts were reduced to values below the limit of detection until the end of storage (30 days at 4 °C). similar results were presented by Paparella et al. (2016) who evaluated the potential of chitosan at 1% and oregano essential oil (OEO) at 2 and 4% against contamination with 4.5 log cfu/g of *L. monocytogenes* in fresh pork meat stored in MAP (70% O_2-20% CO_2-10% N_2). These authors observed that while treatment with MAP by itself did not reduce the growth of *L. monocytogenes*, the treatment with chitosan (alone or combined with OEO) achieved reduction of 2 log cycles and inhibit the growth until day 15 of refrigerated storage (4 °C).

Changes in *E. coli* counts along storage (Figure 1c) and their response to treatments were very similar to that observed for mesophilic aerobic bacteria. This may be related with the fact that native microbiota in this type of product is conformed mainly by enterobacteria (Fernández et al., 2017a). For *E. coli* control, again, the combined treatment T2.5N + MIA achieved significant improvements compared to its equivalent without MIA, implying a synergistic effect between preservation technologies.

In relation to the effect of MAP on the survival and growth of *E. coli* in minimally processed F&V products, there is no conclusive results reported in literature and in many cases it has been shown that the technology did not achieves significant effects on this microorganism (Oliveira et al., 2015). Regarding the use of combined methods (MAP and antimicrobials) also little literature is available. This could be related to the fact that it is very difficult to find antimicrobials that are effective against gram-negative microorganisms. The combined treatment proposed in this study manages to control this type of microorganism, which can be of great interest to the food industry.

Severino et al. (2015) studied the effect of a chitosan-based coating containing a carvacrol nanoemulsion for packaging in MAP (60% O_2-30% CO_2-10% N_2) against contamination with *E. coli* O157: H7 in green peas. MAP treatment alone did not produce any reduction but managed to avoid further growth during storage at 4 °C. The use of the bioactive coating reduced *E. coli* population to values of approximately 1.7 log below control, founding no detectable bacteria after 11 days of storage. In this study, combined treatment (MAP and bioactive coating) presented similar behavior to the individual treatment with bioactive coating.

From the above analysis of the three microbiological indicators behavior, it was concluded that T2.5N + MIA treatment has the same effect as T5N and T5N + MIA, due to a synergistic effect between antimicrobials at the lowest concentration and MAP technology with MIA. This is very encouraging result, because reducing the amount of antimicrobial not only represents an economic advantage, but also can reduce the previously mentioned negative sensory impact that GTE has on the product. Therefore, the combined treatment T2.5N + MIA was selected as the most appropriate to evaluate in further studies. Moreover, control (C) and individual treatments (T2.5 and MIA) samples were followed for comparison.

3.2 | Stability study and determination of the shelf-life of minimally processed beet leaves stabilized with the selected combined treatment

3.2.1 | Atmosphere composition

Figure 2 shows the O_2 (a) and CO_2 (b) composition in samples with the selected treatment (T2.5N + MIA), control (C) and individual treatments (AM and T2.5N) stored at 5 °C. Again, there were no significant differences in the atmosphere evolution of samples with and without the addition of natural antimicrobials. Comparing the results with those of the first study presented, which were carried out at 12 ± 2 °C, it was verified that the equilibrium concentrations continue to be the same, around 7–8% O_2 and 3–4% CO_2. The main difference is the time it takes to achieve equilibrium. According to Oliveira et al. (2015) is time depends on the requirements of the vegetable product and the permeability of the packaging, which are a function of the temperature and relative humidity of storage. Similar results to the observed in the present study were informed by Helland et al. (2016) who did not notice significant differences in the equilibrium atmospheres achieved during the storage of minimally processed turnips at 5 and 10 °C. Likewise, Jacxsens, Devlieghere, and Debevere (2002) working with a mixed lettuce product packed with MIA (3%

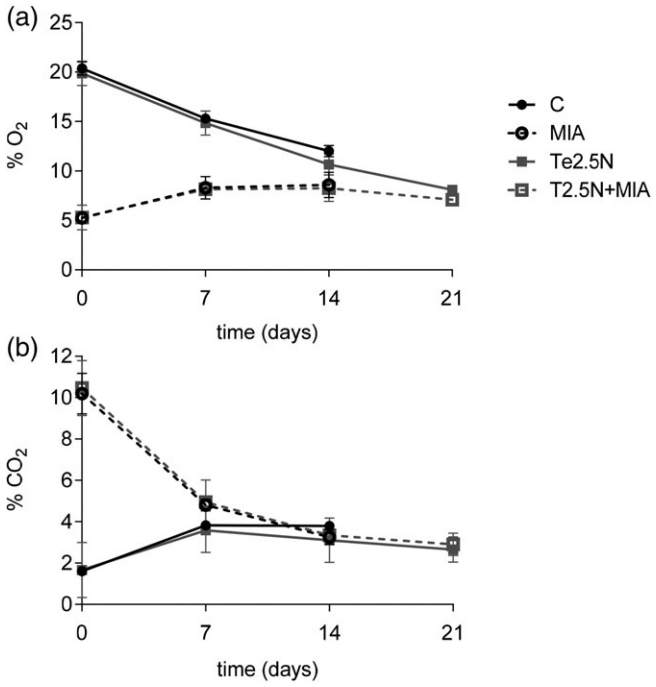

FIGURE 2 O_2 (a) and CO_2 (b) concentration in samples of minimally processed beet leaves with different treatments during storage at 5 °C. Error bars represent the *SD* of the mean

O_2-5% CO_2) and stored at 2, 4, 7, and 10 °C for 11 days observed that the changes in the concentrations of O_2 and CO_2 were similar for all temperatures evaluated.

3.2.2 | Microbiological quality

The mesophilic aerobic bacteria (a), enterobacteria (b) and molds and yeasts (c) counts in samples with different treatments stored at 5 °C are presented in Figure 3. For mesophilic bacteria and enterobacteria statistical analysis indicated significant interaction between factors TREAT and TIME, while in the case of M&L were the interaction was not significant and the effect of both individual factors TREAT and TIME was significant. Regarding MAB, control samples presented an initial load of 5.89 ± 0.14 log cfu/g, showing a constant growth during storage, exceeding the accepted limit for fresh vegetables (7 log cfu/g, according to Corbo, Del Nobile, and Sinigaglia (2006)) at day 7 of storage. Similar results were observed for MIA samples. On the other hand, T2.5N treatment achieved an initial reduction of 2.62 log, exceeding the microbiological limit between day 14 and 21, while treatment T2.5 + MIA presented an initial reduction of 3.7 log, also exceeding the microbiological limit between day 14 and 21. It is noteworthy that combined treatment differed statistically from the individual ones (T2.5N and MIA). Hence, the proposed treatment produces an extension of at least 15 days in the microbiological shelf-life of the product. Additionally, these results have the value of give information about the combination of these technologies in a horticultural product, a topic that until now has been very little studied.

For enterobacteria, the control started from values of 4.97 ± 0.25 log cfu/g, growing until reaching values of 6.98 ± 0.35 after 14 days of refrigerated storage. Treated samples behavior was similar to that observed for MAB probably since, as previously mentioned, enterobacteria is the predominant bacteria on this type of product. Again, samples of T2.5N + MIA treatment remained always below its pair without MIA, differing statistically during the first weeks, although finally similar values were observed from day 14 onwards.

Regarding M&Y, samples C and MIA started from initial values of 3.16–3.30 log cfu/g and samples T2.5N and T2.5N + MIA presented initial counts of 1.85–2.05 log cfu/g, maintaining their counts 1 log below C and MIA samples throughout storage. During storage an increase in M&Y counts was observed in all cases.

Although MAB behavior is the most commonly studied and adopted for microbiological shelf-life establishment as discussed in previous section, there are other microorganisms of deterioration with different levels of relevance in different products and several authors have included them in their studies. In this sense, Nirmal and Benjakul (2011) studied the effect of MAP (50% CO_2-5% O_2-45% N_2) with or without addition of GTE and ascorbic acid on the quality of white shrimp, founding that those treated with GTE and MIA were more stable microbiologically, achieving reductions of around 2 log in the count of enterobacteria, psychrotrophic, acid-lactic and H_2S-producing bacteria, with respect to the control throughout the storage. Moreover, Petrou et al. (2012) who studied the effect of the natural antimicrobials chitosan (CH), oregano (O), and its combination (CH-O) on the shelf-life of chicken meat packaged in MAP stored at 4 °C, found that in general both antimicrobial combined with MAP were effective to control the growth of enterobacteria and M&Y, highlighting the effect of treatment MIA-CH-O which maintained both microbial counts around 2 log cfu/g throughout storage (21 d).

Changes on native *Listeria* spp. counts in control and treated samples is presented in Figure 3d. In control samples an initial value of 4.11 ± 0.12 log cfu/g was observed, reaching a value of 5.15 ± 0.26 log cfu/g at day 14. No differences were observed between samples MIA and C. On the other hand, samples containing antimicrobials managed to reduce native *Listeria* spp. to values below the limit of detection up to day 14 of storage. Between day 14 and 21 a regrowth was observed, showing values of 4.39 ± 0.14 log for T2.5N and 3.09 ± 0.12 for T2.5N + MIA, with significant differences between

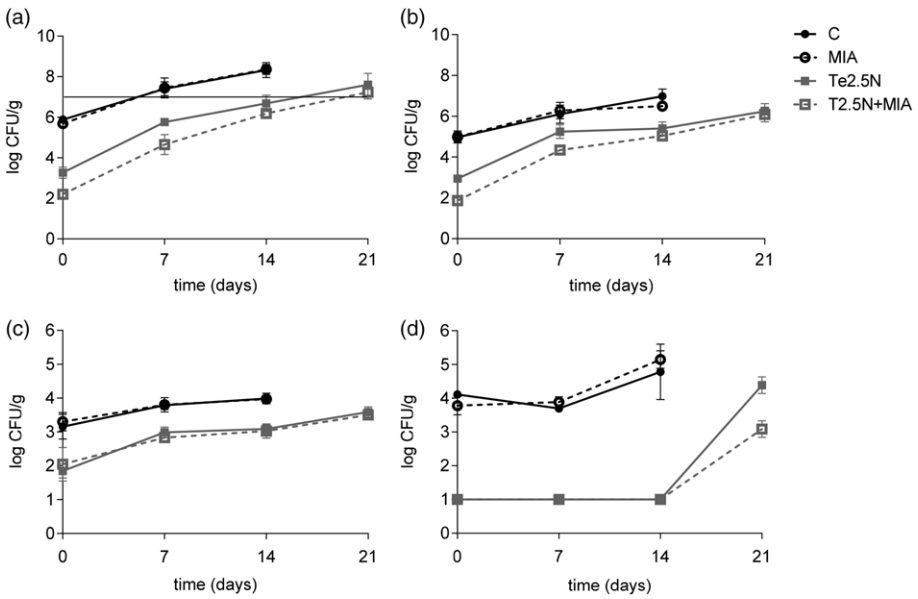

FIGURE 3 Aerobic mesophilic bacteria (a), enterobacteria (b), molds and yeasts (c), and *Listeria* spp. (d) Counts in samples of minimally processed beet leaves with different treatments during storage at 5 °C. Error bars represent the *SD* of the mean

TABLE 2 Total polyphenols (mg AG/100 g) content in samples of minimally processed beet leaves with different treatments during storage at 5 °C.[a] Mean and *SD*

	Time (days)			
Treatment	**0**	**7**	**14**	**21**
C	147.9 ± 4.0^{aA}	125.2 ± 21.2^{aA}	81.5 ± 18.8^{aB}	
MIA	178.6 ± 22.7^{aA}	148.8 ± 22.6^{aAB}	93.9 ± 6.8^{aB}	
T2.5N	1839.7 ± 230.0^{bA}	1929.2 ± 37.2^{bA}	1947.6 ± 307.1^{bA}	1,504.6 ± 56.7^{aA}
T2.5N + MIA	2,197.7 ± 60.1^{bA}	1943.7 ± 22.5^{bA}	1990.7 ± 296.0^{bA}	1953.2 ± 86.4^{bA}

[a] Different lowercase letters indicate differences between treatments (compare columns) and different capitals indicate differences over time (compare rows).

them at day 21. This is a very interesting result because unlike what was presented in the previous study, where a contamination with *L. innocua* had been simulated by inoculating the samples, this is a real case of contamination of the raw material with this bacteria and results indicate that the selected treatment was efficient to reduce the contamination to values below detection limit during at least 14 days of storage.

3.2.3 | Nutritional quality

Table 2 shows the total polyphenols content in the samples with different treatments. The control and MIA samples presented similar behaviors, without significant differences between them, although MIA maintained higher values than the control throughout storage. The tea-containing samples had total polyphenol contents about 12 times higher than the samples without tea. This result is attributed to the high content of phenolic compounds on GTE, which are associated with their antioxidant and antimicrobial capacity and with numerous health benefits. In fact, GTE have been used in various products to fortify them. In this sense, Tappi et al. (2017) used green tea extract (GTE 1%) to enrich minimally processed apples to obtain a nutritionally fortified product. While the apples without GTE presented a content of 30 mg AG/100 g, those impregnated with GTE presented values of around 300 mg AG/100 g that they maintained during 7 days of storage at 10 °C. In another recent work, Muniandy, Shori, and Baba (2016) used GTE 2% (2 g/100 ml) to fortify yogurt. While the control yogurt had a content of 118 μg AG/ml, the yogurt with added GTE presented a value of 479.5 μg AG/ml. Moreover, they reported that polyphenols content remained constant throughout storage, like what was observed in this investigation for beet leaves.

It is interesting to note that the combined treatment improves total polyphenols retention at the end of storage, hence, MAP technology has a positive effect on the stability of green tea polyphenols.

In regards to the betaxanthins (a) and betacyanins (b) content in the fresh-cut beet leaves with different treatments during storage at 5 °C, results are showed in Figure 4. For both pigments, ANOVA shows no interaction between TREATMENT and storage TIME and a significant effect of both individual factors. Initial content of betaxanthin was 6.0 ± 0.8 mg/100 g. Decreases in this pigment were detected in all samples during storage, but samples with initial atmosphere modification (MIA and T2.5N + MIA) showed lower losses (24% at the end of storage) than samples packaged in control atmospheres (C and T5N, higher than 50%). Initial content of betacyanines was of 3.80 ± 0.66 mg/100 g. All samples presented decreases in this pigment along refrigerated storage. However, the amount of this decreases were dependent on the treatment received by samples. In fact, a Bc loss of 40 and 24% were observed in samples without antimicrobials (C and MIA) at day 14 and a loss of 34% was observed in samples treated with T2.5N. The combined treatment presented a remarkable effect, since it managed to maintain the initial Bc contents during the 21 days of storage.

In agreement with what was observed in previous studies (Fernández et al., 2016), the MIA samples presented a better performance than controls. Moreover, for betacyanins the combination of MIA with antimicrobials achieved an even greater retention of these pigments along refrigerated storage. This "protective effect" of antimicrobials is probably related to the polyphenol activity of green tea. It is well known that the degradation of betalains during storage is usually related to glycosidases, polyphenoloxidases (PPO) and peroxidases activities (Strack, Vogt, & Schliemann, 2003), and in general these

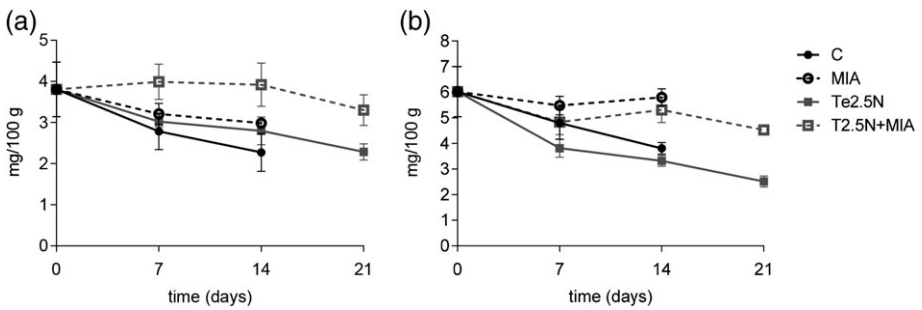

FIGURE 4 Betaxanthins (a) and betacyanins (b) content in samples of minimally processed beet leaves with different treatments during storage at 5 °C. Error bars represent the *SD* of the mean

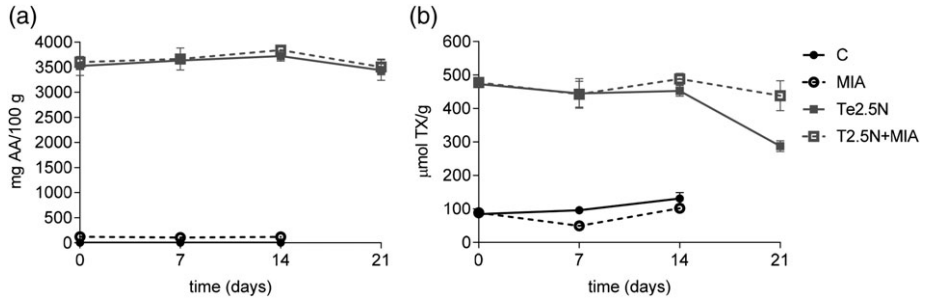

FIGURE 5 Antioxidant capacity by method DPPH (a) and FRAP (b) in samples of minimally processed beet leaves with different treatments during storage at 5 °C. Error bars represent the *SD* of the mean

reactions are oxygen-dependent, so it is logical to find smaller reductions in the samples with MIA. Additionally, recent studies have shown the ability of GTE to inactivate PPO (Klimczak & Gliszczynska-Swigło, 2017) which could explain the results obtained in the present study.

The results obtained for the determinations of antioxidant capacity by DPPH and FRAP in beet leaves samples with different treatments are presented in Figure 5a,b. Samples without antimicrobials showed initial DPPH values of 102.6 ± 18.6 (for C) and 123.1 ± 22.6 mg AA/100 g (for MIA), values that remained stable during the 14 days at 5 °C, probably because the low temperatures favored the retention of the different antioxidant compounds. Samples containing antimicrobials (T2.5N and T2.5N + MIA) showed values between 3,500 and 3,600 mg AA/100 g that were stable during 21 days of refrigerated storage, demonstrating a remarkable improvement in antioxidant capacity. Similar behavior was observed on FRAP test, although it is important to highlight that in this case significant differences were observed between the treatments containing antimicrobials with and without MIA at the end of storage, when those packaged with MIA managed to maintain 90% of their initial antioxidant capacity, while those packaged in air only maintained the 60%.

Other authors working with GTE or other compounds rich in polyphenols, either only for fortification or for take advantage of their antimicrobial activity, have also observed substantial improvements in the antioxidant capacity of the fortified products. In this sense, Siroli et al. (2014) studied the application of natural tea extracts (TE) and grape extract (GRA) in pork burgers stored in modified atmosphere (20 days at 2 °C), and evaluated the lipid oxidation (TBARS). While in control samples the oxidation increased markedly with the storage time (0.09–0.63 mg of MDA/kg), the samples treated with the extracts showed significantly lower values (0.2 for GTE and 0.4 for GRA). According to these authors, this result could be explained due to the ability of tea catechins to bind to the iron atom of myoglobin, thus delaying the oxidation of lipids by reaction with free radicals. Similar results regarding the antioxidant activity of GTE were reported by other authors (Muniandy et al., 2016; Nirmal & Benjakul, 2011; Siroli et al., 2014; Tappi et al., 2017, among others).

3.2.4 | Sensory analysis

Results of the sensory analysis on samples with different treatments are presented in Table 3. At the beginning of storage, all samples

TABLE 3 Sensory analysis performed on samples of minimally processed beet leaves whit different treatments during storage at 5 °C. Mean and *SD*

Day	Treatment	Odor	Color	Texture	OVQ
0	C	$8.0 \pm 1.1^{a.b.A}$	$8.0 \pm 0.8^{a.A}$	$8.2 \pm 0.9^{a.A}$	$8.1 \pm 1.0^{a.A}$
	MIA	$8.1 \pm 0.6^{a.A}$	$8.4 \pm 0.8^{a.A}$	$8.6 \pm 0.8^{a.A}$	$8.3 \pm 0.8^{a.A}$
	T2.5N	$7.5 \pm 0.9^{a.b.A}$	$7.7 \pm 1.3^{a.A}$	$7.7 \pm 1.2^{a.A}$	$8.0 \pm 0.6^{a.A}$
	T2.5N + MIA	$7.0 \pm 0.8^{b.A}$	$7.8 \pm 1.3^{a.A}$	$8.1 \pm 1.1^{a.A}$	$7.7 \pm 1.1^{a.A}$
7	C	$7.8 \pm 0.9^{a.b.A}$	$8.5 \pm 0.7^{a.A}$	$8.4 \pm 0.7^{a.A}$	$8.4 \pm 0.7^{a.A}$
	MIA	$8.1 \pm 0.9^{a.A}$	$8.4 \pm 0.5^{a.A}$	$8.4 \pm 0.5^{a.A}$	$8.4 \pm 0.6^{a.A}$
	T2.5N	$7.2 \pm 0.9^{a.b.A.B}$	$6.6 \pm 0.8^{b.A}$	$7.1 \pm 1.2^{b.A}$	$6.9 \pm 1.0^{b.A}$
	T2.5N + MIA	$6.9 \pm 0.7^{b.A}$	$6.9 \pm 0.7^{b.A}$	$7.6 \pm 0.9^{a.b.A}$	$6.9 \pm 0.7^{b.A}$
14	C	$4.7 \pm 1.1^{a.B}$	$6.8 \pm 1.0^{a.B}$	$6.3 \pm 1.2^{a.b.B}$	$5.6 \pm 1.3^{a.b.B}$
	MIA	$5.5 \pm 0.7^{a.b.B}$	$7.2 \pm 0.4^{a.B}$	$7.0 \pm 0.5^{a.B}$	$6.5 \pm 1.2^{a.B}$
	T2.5N	$6.1 \pm 0.8^{b.B}$	$4.4 \pm 1.2^{b.B}$	$5.2 \pm 1.3^{b.B}$	$4.8 \pm 1.4^{b.B}$
	T2.5N + MIA	$5.7 \pm 0.5^{b.B}$	$5.3 \pm 0.8^{b.B}$	$5.4 \pm 1.3^{b.B}$	$5.5 \pm 1.0^{a.b.B}$
21	C	$3.0 \pm 0.7^{a.C}$	$4.7 \pm 1.1^{a.C}$	$4.5 \pm 1.1^{a.C}$	$4.1 \pm 1.1^{a.C}$
	MIA	$2.4 \pm 1.1^{a.C}$	$5.1 \pm 1.0^{a.C}$	$5.3 \pm 0.5^{a.C}$	$4.6 \pm 1.2^{a.C}$
	T2.5N	$2.7 \pm 1.2^{a.C}$	$1.6 \pm 0.8^{b.C}$	$2.0 \pm 1.1^{b.C}$	$1.8 \pm 0.9^{b.C}$
	T2.5N + MIA	$2.6 \pm 1.2^{a.C}$	$2.1 \pm 1.2^{b.C}$	$2.7 \pm 1.3^{b.C}$	$2.0 \pm 1.2^{b.C}$

[a] Different lowercase letters indicate significant differences between treatments (compare columns). Different capital letters indicate differences in storage time.

presented scores between 7.0 and 8.6 for all quality attributes, without significant differences among treatments. Samples corresponding to MIA treatment presented the highest score in all the attributes, and samples containing antimicrobials the lowest, probably due to the color and aroma of the green tea that gave it a "different, but not unpleasant, appearance and aroma". It is important to note that even though the panelists detected a difference on samples containing antimicrobials, they did not reject it. After 7 days of storage, the trends were very similar to those of day 0. On day 14 of storage, an important reduction in all scores was show. It is interesting to note that while on the beginning of storage samples with antimicrobials showed the lowest "odor" scores, at day 14 the samples without antimicrobials showed the lowest values for this parameter, being the aroma one of the first indicators of the loss of freshness. At day 21, all treatments presented parameters with scores below 5, thus concluding the analysis. The results show the already mentioned positive effect of MIA treatment for sensory characteristics preservation. Moreover, the combined treatment (T2.5N + MIA) was also very effective, showing not only that green tea at the selected concentration did not generate changes that lead to its rejection, but also, that this treatment is effective to preserve the product´s organoleptic characteristics, achieving a sensory shelf-life of at least 14 days.

Several authors have evaluated the effect of MAP, the addition of natural antimicrobials and/or their combination on the sensory characteristics of food products. Petrou et al. (2012) working with chicken meat packed in modified atmosphere stored at 4 °C treated with chitosan, oregano, and their combination, found that the samples treated with chitosan or chitosan–oregano were sensory (smell and taste) acceptable during the entire refrigerated storage period of 21 days, showing improvements with respect to those treated only with MAP which were acceptable only for 11 days. Similar results were reported by Nirmal and Benjakul (2011) regarding the sensory acceptability of shrimp treated with the combination of green tea, ascorbic acid, and modified atmospheres.

4 | CONCLUSIONS

The combination of the natural antimicrobials 2.5% green tea extract and 500 UI/g nisin with the packaging technology with MIA has shown a great potential to control the native beet leaves microbiota and could also be effective against possible contamination with pathogenic microorganisms such as *L. monocytogenes* and/or *E. coli* O157: H7.

The selected treatment not only preserved the initial contents of betalain compounds but, by the addition of GTE, has the additional value of generating a "fortification" of the product, since greatly increased its total polyphenols content and, with this, its antioxidant capacity, with great stability of these nutritional indicators during storage. Moreover, this fortification entails enormous advantages for the consumer given the numerous health benefits recognized and associated with tea polyphenols.

Additionally, the selected technology manages to preserve the sensitive organoleptic attributes of the product, extending its acceptability until the 14th day of storage inclusive. While control samples presented a shelf-life of less than 7 days considering both microbiological and sensory acceptability, by an adequate combination of technologies, the shelf-life of the product was prolonged to at least 14 days.

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