ORIGINAL ARTICLE

Green tea extract: A natural antimicrobial with great potential for controlling native microbiota, *Listeria innocua* and *Escherichia coli* in fresh-cut beet leaves

María Verónica Fernández[1,2,3] | María Victoria Agüero[1,2]* | Rosa Juana Jagus[1,2]*

[1]Universidad de Buenos Aires (UBA), Consejo Nacional de Investigaciones Científica y Técnicas (CONICET), Instituto de Tecnologías y Ciencias de la Ingeniería (INTECIN), Facultad de Ingeniería, Buenos Aires, Argentina

[2]Departamento de Ingeniería Química, Laboratorio de Microbiología Industrial: Tecnología de alimentos, Buenos Aires, Argentina

[3]Peruilh Foundation, Facultad de Ingeniería, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence
María Verónica Fernández, Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires, Ciudad Universitaria, Intendente Güiraldes 2620, (C1428EGA), Buenos Aires, Argentina.
Email: fernandez.mveronica@gmail.com.ar

Abstract

Antimicrobial effect of green tea (GTE), nisin (Ni), natamycin (Na), and their combinations on native mesophilic bacteria (MAB), Enterobacteriaceae (EB), and molds and yeast (M&Y) of fresh-cut beet leaves and on inoculated *Listeria innocua* (LI) and *Escherichia coli* (EC) were determined during storage at 15 °C. Individual treatments with Ni and Na were not effective in reducing nor controlling native microbiota. Conversely, treatments containing GTE 5% reduced MAB and EB initial counts to values below detection limit (DL = 1 log UFC/g) for 24 hr. The most effective treatment for M&Y was the combination of GTE with Na, which maintained the counts in undetectable values (<DL) for 48 hr. Ni and GTE were very effective in controlling LI and their combination presented a significant improvement, it reduced by 4.4 log the initial count and maintained them around 4 log below control samples along storage. For *EC* control, only GTE treatments were effective presenting a concentration dependent behavior.

Practical applications

Consumers' demand for healthy, nutritious, minimally processed, free of additives and environmentally friendly products has markedly increased in recent years. In response to these demands, the application of green tea extract, nisin, natamycin, and their combinations, as natural antimicrobials against beet leaves native microbiota and inoculated *L. innocua* and *E. coli*, was evaluated in fresh-cut beet leaves; an underutilized by-product with high nutritional value. The results of this study showed that nisin, widely applied in dairy products, could be also highly effective against *Listeria* spp. on leafy vegetables. Moreover, green tea extract, either alone or combined, is a highly promising option with potential for reducing or preventing the growth of pathogenic and spoilage microorganisms (gram-negatives included) present in leafy vegetables, specifically in fresh-cut beet leaves.

1 | INTRODUCTION

It is well known that fresh horticultural products, especially minimally processed ones, provide a good substrate for the growth of microorganisms (spoilage or pathogenic) during production, commercialization, and storage. 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 (Taban & Halkman, 2011). Studies have shown that etiologic factors in most of the registered outbreak cases were contaminations with pathogens, including

Escherichia coli O157:H7, *Listeria monocytogenes*, *Salmonella* Enteritidis, *Shigella*, and *Staphylococcus aureus* (Sant'Ana, Franco, & Schaffner, 2014; Taban & Halkman, 2011). In the production chain of MPV, disinfection (usually with chlorine) is the only step where the number of microorganisms can be reduced (São José & Vanetti, 2012). However, this step does not guarantee that MPV are free of pathogens (Allende, Tomás-Barberán, & Gil, 2006). Therefore, it is important to develop and evaluate new preservation strategies for MPV to ensure food safety.

An interesting approach with great potential to overcome these drawbacks is the biopreservation, by which MPV's safety is increased through the use of natural compounds with antimicrobial properties

---

[†]These authors contributed equally to the manuscript.

(Tiwari et al., 2009). The exploration of naturally occurring antimicrobials for food preservation is intended to cover not only the consumer demand for healthier foodstuffs, but also, the growing concern about microbial resistance to conventional chemical preservatives (Ponce, Fritz, Del Valle, & Roura, 2003).

Among natural antimicrobials, green tea (*Camellia sinensis* L.) extracts have gained much attention in recent years. Many studies showed that these extracts have antibacterial (against gram-positive as well as gram-negative bacteria), antiviral, antifungal, and radical scavenging activity (Perumalla & Hettiarachchy, 2011) which was associated with the polyphenols present therein (Bansal et al., 2013). Other widely known antimicrobials are the bacteriocins nisin and natamycin, which present the GRAS status for their application in foodstuffs (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996; Koontz, Marcy, Barbeau, & Duncan, 2003). Nisin is produced by strains of *Lactococcus lactis* subsp. Lactis and exhibits antimicrobial activity toward a wide range of gram-positive bacteria, including *L. monocytogenes* (Martins, Cerqueira, Souza, do Carmo Avides, & Vicente, 2010); while natamycin is a natural antimycotic polyene, produced by *Streptomyces natalensis*, with antimicrobial activity against yeasts and molds but not against bacteria, viruses, and protozoa (Te Welscher et al., 2008). Studies of the effectiveness of these antimicrobials on microorganism populations are mostly limited to meat and dairy products (Delves-Broughton, 2011; Fernandez, Jagus, & Mugliaroli, 2014; Perumalla & Hettiarachchy, 2011). In fact, very little information can be found on its application in vegetable products. Thus, the objective of the present work was to determine the antimicrobial effect of green tea extract (GTE), nisin, natamycin, and their combinations against native microbiota (mesophilic, Enterobacteriaceae [EB] and molds and yeast [M&Y]) of fresh-cut beet leaves, as well as the effect on the survival and growth of inoculated *Listeria innocua* and *E. coli*.

2 | MATERIALS AND METHODS

2.1 | Culture preparation

In this study, *L. innocua* (CIP 8011, CCMA 29, Facultad de Farmacia y Bioquímica, UBA, Argentina) and *E. coli* (ATCC 25922) were employed. These strains have been widely considered as surrogates of *L. monocytogenes* and *E. coli 0157:H7*, respectively, since they have shown similar behavior and resistance as pathogenic strains (Kamat & Nair, 1996; Kim & Harrison, 2009; Omac, Moreira, Castillo, & Castell-Perez, 2015). In both cases, fresh cultures were obtained by inoculating 150 ml of fresh sterile trypticase soy broth enriched with 0.6% yeast extract (TSBYE, Biokar Diagnostics, Allonne, France), and incubating them in a continuously agitated temperature-controlled shaker at 28 °C overnight. Then, 3 ml of this culture were inoculated in 150 ml of fresh TSBYE, agitated until obtaining the final desired concentration of cells (approximately 1.0×10^8 CFU/ml) determined by optical density when achieved an absorbance of 0.05 at 540 nm for *L. innocua* and 0.14 at 630 nm for *E. coli*.

2.2 | Preparation of minimally processed beet leaves samples

The beets (*Beta vulgaris* L. var. Conditiva) were purchased in a local market in Buenos Aires (Argentina). Once in the laboratory, a cut was done 2 cm below the base of each leaf to separate them from roots and stems. Figure 1 presents the unit operations for processing of beet leaves. Briefly, the leaves were washed with cold tap water and disinfected by immersion in a cooled sodium hypochlorite solution (200 ppm free chlorine) for about 5 min. Then, disinfected leaves were dried for 1 min in a manual centrifugal dryer and cut using a sharp stainless steel knife, perpendicularly to the veins, to obtain strips of 2–3 cm wide. Samples of 10 g were weighed and placed in polyolefin bags (Cryovac PD960, Argentina) with an O_2 and CO_2 transmission rates of 6,000–8,000 and 19,000–22,000 $cm^3/m^2/24$ hr at 23 °C and 1 atm, respectively, and a water transmission rate of 0.90–1.10 $g/in^2/24$ hr[1] at 23 °C and 100% RH. Three systems were prepared to study the effectiveness of natural antimicrobials against native microbiota ("A" systems), as well as their performance against *L. innocua* ("B" systems) and *E. coli* ("C" systems), surrogates of two of the most conflictive pathogenic microorganisms in this type of product. For inoculations ("B" and "C" systems), an aliquot of the corresponding culture was directly added to the leaves inside the bags so as to achieve an initial bacterial inoculum of approximately 10^5 CFU/g. After that, antimicrobial treatments were performed, dosing directly on the leaves the corresponding quantity of antimicrobials according specification detailed in the next section. Afterward the bags were sealed and all samples were gently massaged, according to the methodology proposed by Tsiraki and Savvaidis (2014), to obtain a homogeneous distribution of the antimicrobials in the product. For each system, a batch without the addition of antimicrobials was maintained to evaluate the development of the population under study (control samples). Finally, all samples were stored in a 15 ± 1 °C chamber.

2.3 | Antimicrobial treatments

The antimicrobials used in this study were nisin (DelvoPlus, DSM, Delft, Netherlands), natamycin (Delvocid, DSM) and GTE (Sunphenon 90LB, Taiyo International, Minneapolis, USA). Their solutions were prepared as follows: for nisin treatments, a stock solution of 50,000 IU/ml was prepared dissolving 250 mg of commercial nisin in 5 ml of sterile distilled water. For natamycin treatments, a stock solution of 24 mg/ml was prepared dissolving 240 mg of commercial natamycin in 5 ml of sterile distilled water. For GTEs treatments, a stock solution of 20 and 40% of Sunphenon 90LB were prepared dissolving the correspondent amount of product in sterile distilled water. The antimicrobials were applied in the "A", "B," and "C" systems, according to the scheme presented in Table 1. In samples containing only nisin or natamycin, an amount of sterile water was added to match in all systems the water content within the package.

Treatments were selected taking into account results of preliminary studies (data not shown), where the initial effects of several concentrations and combinations of nisin (100–500 UI/g), natamycin (20–
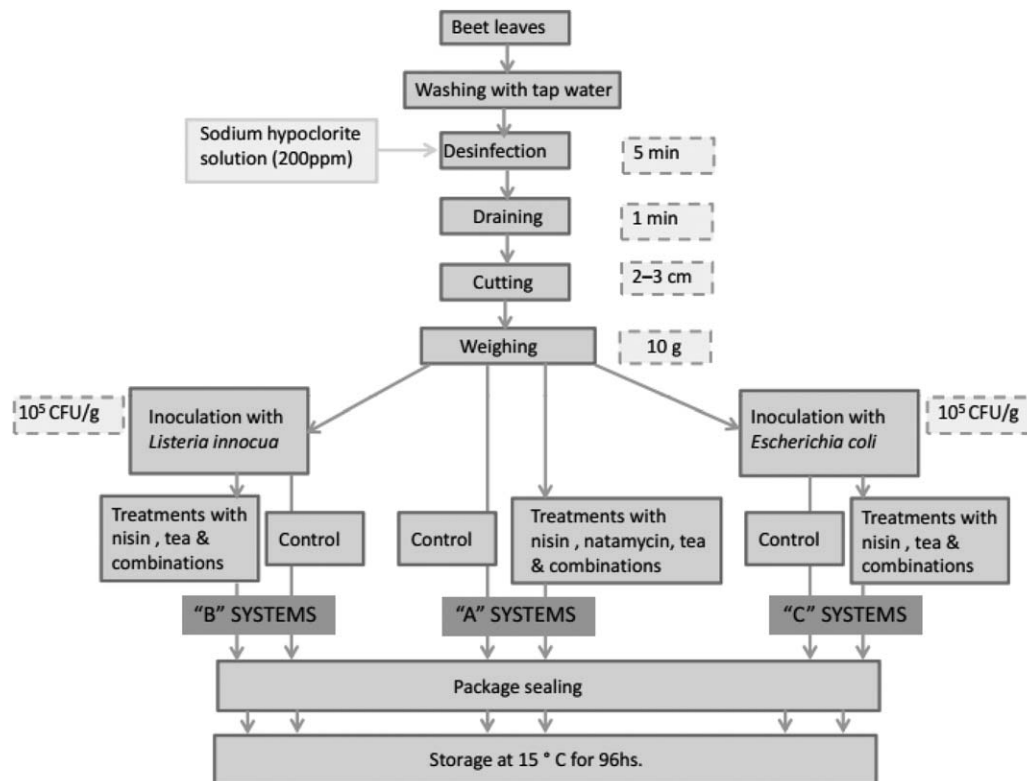
FIGURE 1 Experimental processes followed to obtain minimally processed beet leaves samples

200 mg/L), and GTE (0.5–5%) on fresh-cut beet leaves native microbiota, inoculated *L. innocua* and *E. coli* were tested. Also, the limits established by Argentinean legislation for nisin and natamycin were considered (Argentinean Alimentary Code, 1996). It is important to highlight, as indicate in Table 1, that natamycin was only applied in systems "A" since it is well known that this antimicrobial is not effective against bacteria (Te Welscher et al., 2008), as we have corraborated in preliminary studies. Conversely, although it is known that nisin is effective only against gram-positive bacteria, our previous studies has demonstrated that some interaction can occur when combined with GTE against gram-negative bacteria, thus this combination was studied in all systems.

2.4 | Sampling procedure

All systems were stored during 96 hr at $15 \pm 1 °C$. This temperature allows us to perform an accelerated testing, but as well, would correspond to a typical commercial thermal abuse. Samples were taken periodically at 0, 24, 48, 72, and 96 hr. For each time, beet leaves samples (10 g) were transferred aseptically to sterile stomacher pouches

TABLE 1 Treatment application scheme

Treatments	Systems in which treatments were applied	Solutions added				
		Nisin (50000 IU/ml)	Natamycin (24 mg/ml)	GTE 20%	GTE 40%	Sterile water
CO	A, B, & C					1.33 ml
Ni500	A & B	100 μL				1.23 ml
Na200	A		83 μL			1.24 ml
GTE2.5	A, B, & C			1.25 ml		100 μL
GTE5	A, B, & C				1.25 ml	100 μL
GTE2.5+Ni500	A, B, & C	100 μL		1.25 ml		
GTE2.5+Na200	A		83 μL	1.25 ml		17 μL
GTE5+Ni500	A, B, & C	100 μL			1.25 ml	
GTE5+Na200	A		83 μL		1.25 ml	17 μL

containing 90 ml solution of 0.1% (wt/vol) peptone water (Biokar Diagnostics), and homogenized in a stomacher (Interscience Laboratories, Inc., Woburn; Bagmixer 400 P, France) for 120 s. To determine the viable population of microorganisms, samples were serially diluted with 0.1% (wt/vol) peptone water. For "A" systems *mesophilic aerobic bacteria* (MAB) counts were performed using plate count agar (PCA, Biokar Diagnostics) incubated at 37 °C during 24–48 hr; EB counts were determined in Mac Conkey agar (Biokar Dignostics) incubated at 37 °C during 24 hr and M&Y counts were determined in yeast extract glucose chloramphenicol agar (YGC, Biokar Diagnostics) incubated at 28 °C during 48–72 hr. For "B" systems, *Listeria* spp. counts were performed using Oxford Agar (Biokar Diagnostics, France) with *Listeria* Selective Suplement (Oxoid, SR140), incubated at 37 °C during 24–48 hr. Finally, for "C" systems, *E. coli* counts were performed using Mac Conkey 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. For the detection of positive, fluorescent, *E. coli* colonies the examination took place under long-wave ultraviolet light (366 nm). 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.

2.5 | Statistical analyses

Microbiological determinations were made by triplicate in two separate experimental treatments runs and the mean of all repetitions together with the standard deviation were informed. Results were subjected to an Analysis of Variance (ANOVA) using the Origin 8 software (OriginLab, Massachusetts). The factors used as sources of variation were TREAT (treatment, different antimicrobial tested or control), TIME (storage time), and TREAT—TIME interaction. Differences were determined by the Tukey–Kramer multiple comparison test ($p < .05$).

3 | RESULTS

The statistical analysis of the experimental data yielded significant interactions among factors under study (TREAT and TIME, $p < .0001$) for all the analyzed microorganisms. This indicated that the evolution of microbial counts during storage was affected by the antimicrobial treatment applied to samples.

3.1 | Effectiveness against native microbiota

The changes of MAB in "A" systems during storage at 15 °C are presented in Figure 2a. Control samples (CO) presented an initial MAB load of 4.9 ± 0.2 log CFU/g. These counts rapidly increased in CO samples reaching values of 8.6 ± 0.3 log CFU/g at the end of storage (96 hr). Individual treatments with Na and Ni did not produce any significant inhibitory effect ($p > .05$) on MAB counts presenting similar behavior to control throughout storage. Taking this into account, and due to the high number of treatments, Ni and Na are not presented in Figure 2, to allow more clarity in the interpretation of it. While treatment GTE2.5 showed some effectiveness; counts remained about 1
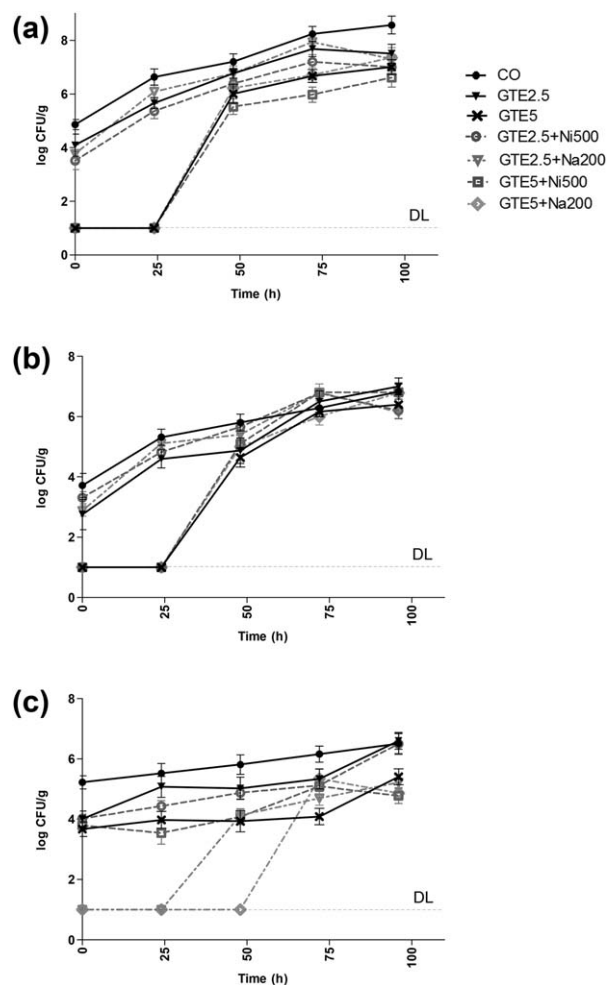

FIGURE 2 Effect of green tea, nisin, natamycin, and its combination on mesophilic bacteria (a), Enterobacericeae (b), and molds and yeast (c), in beet leaves stored at 15 °C. Error bars represent the standard deviation of the mean

log cycle under the control. Remarkable effects were observed with an increase in the tea concentration. Indeed, the treatment containing GTE at 5% (GTE5) presented a significant initial reduction ($p < .0001$), reaching values below the DL which remained for the first 24 hr. Then, they presented a regrowth, reaching values of around 1 log cycle below CO from the 48 hr onwards, differing significantly ($p = .021$) from control even at the end of storage.

In regards to the combined treatments, no significant differences ($p > .05$) were observed between treatments GTE2.5 and GTE2.5 + Na200, neither between treatments GTE5 and GTE5 + Na200, as they presented similar behavior during storage. On the contrary, significant differences ($p = .024$) were found between GTE2.5 + Ni500 and the individual treatment GTE2.5. At the same time, the combination of GTE 5% with nisin (GTE5 + Ni500) presented similar behavior than GTE5 during the first 48 hr and after that managed to stay in counts of about 0.5–0.7 log below GTE5 ($p = .106$). Even though in the last case the difference is not significant, the results showed that MAB were more sensitive to combined treatments.

EB behavior (Figure 2b) was quite similar to MAB. Only treatments containing GTE 5% (GTE5, GTE5 + Ni and GTE5 + Na) presented significant effects ($p < .0001$), being able to reduce EB counts to undetectable values during the first 24 hr. After that, the growth was resumed, reaching similar values to CO from 48 hr until the end of storage. For this microbial group no interaction was observed by combining GTE with nisin or natamycin since no significant differences ($p > .05$) were observed neither between treatments GTE2.5, GTE2.5 + Ni500 and GTE2.5 + Na200 nor GTE5, GTE5 + Ni500 and GTE5 + Na200.

The changes in M&Y during storage at 15 °C are presented in Figure 2c. Control samples showed initial values of 5.2 ± 0.2 log CFU/g and exhibited a gradual growth, reaching counts of 6.5 ± 0.3 log CFU/g at the end of storage (96 hr). Treatment with nisin did not produce any inhibitory effect ($p > .05$) on M&Y counts, since exhibited a behavior similar to control throughout storage. Surprisingly, the individual treatment with natamycin (Na200) did not show a significant inhibitory effect ($p > .05$) throughout storage either, although a reduction of 1 log cycle ($p = .068$) was observed at 96 hr. Regarding the treatments containing GTE, the M&Y counts of the GTE2.5 samples remained between 0.5 and 1.2 log below CO ($p < .0001$). Whereas those treated with GTE5 managed to stay between 1.1 and 2.1 log cycles below CO ($p < .0001$), during the evaluated period.

No significant differences ($p > .05$) were found between the combined treatments and the individual treatments, GTE2.5 + Ni500 with GTE2.5 and GTE5 + Ni500 with GTE5, respectively. However, it is important to highlight, that the combined treatments of natamycin and tea (GTE2.5 + Na200 and GTE5 + Na200) presented remarkable effects, since they maintained M&Y counts below the DL during 24 and 48 hr, respectively, differing significantly ($p < .0001$) from the individual treatments.

3.2 | Effectiveness against *L. innocua*

The behavior of *L. innocua* during storage at 15 °C is presented in Figure 3. Control samples showed an initial population of 5.4 ± 0.3 log CFU/g reaching a count of 7.4 ± 0.3 log CFU/g at the end of storage. All the tested treatments presented significant differences ($p < .0001$) from control. Individual treatment with nisin was highly effective for
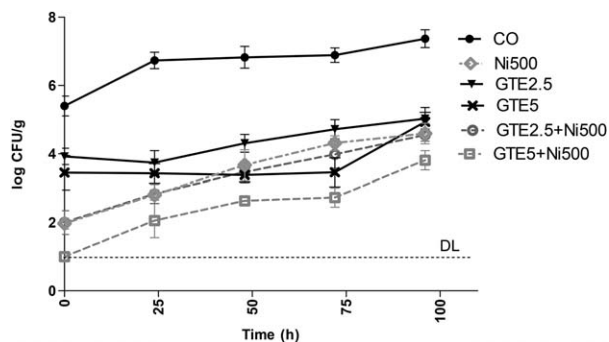
controlling *L. innocua* since it presented an initial reduction of 3.4 log cycles and managed to stay between 3.5 and 4.0 log cycles below control during the first 72 hr of storage and 2.8 log below at the end of storage (96 hr). GTE treatments were also very effective, with initial reductions of 1.5 and 1.8 log cycles for GTE2.5 and GTE5, respectively. During storage, GTE5 samples maintained greater stability, with counts around 3.5 log CFU/g during the first 72 hr. Nevertheless, by the end of storage (96 hr) no significant differences ($p > .05$) were observed between these two treatments, which in both cases remained about 2.4 log below CO.

Regarding the combined treatments, only the highest concentration of GTE with nisin showed a significant improvement ($p < .0001$) over individual nisin treatment. With this combination, a 4.4 log reduction was initially achieved, remaining in counts about 5–4 log orders below CO during the first 72 hr of storage and 3.6 log below CO at 96 hr of storage.

3.3 | Effectiveness against *E. coli*

The behavior of *E. coli* during storage at 15 °C is presented in Figure 4. The control samples (CO) showed an initial count of 4.9 ± 0.2 log CFU/g, then a constant growth was observed, reaching values of 6.8 ± 0.3 log CFU/g at the end of storage. In regard to the individual treatments with GTE, GTE2.5 presented significant effects ($p < .0001$) since it managed to stand *E. coli* counts about 2 log cycles below control during the first 72 hr of storage, but then achieved similar values. Treatment GTE5 was also highly effective ($p < .0001$) since it presented an initial reduction of 3.5 log cycles, achieving counts below the DL and maintaining those levels during the first 24 hr of storage. After that, the growth was resumed, reaching values similar to CO at the end of storage (96 hr). In the combined treatments (GTE2.5 + Ni500; GTE5 + Ni500), nisin did not introduce significant effects ($p = .132$ and $p = .243$, respectively) in *E. coli* reductions in comparison with treatments with green tea alone.

4 | DISCUSSION

Antimicrobial effects of GTEs, nisin, natamycin, and their combinations were evaluated in fresh-cut beet leaves. Individual treatment with
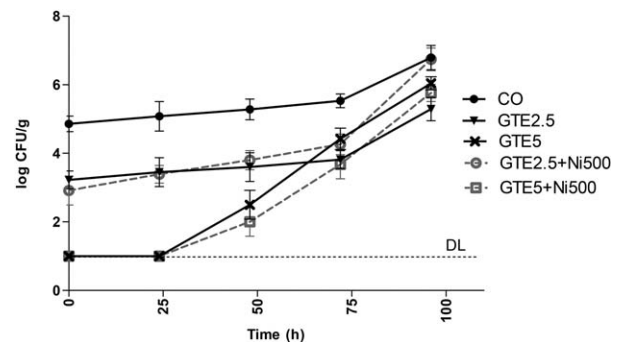

FIGURE 3 Effect of green tea, nisin, natamycin, and its combination on *Listeria innocua* in beet leaves stored at 15 °C. Error bars represent the standard deviation of the mean

FIGURE 4 Effect of green tea, nisin and its combination on *Escherichia coli*, in a beet leaf model system stored at 15 °C. Error bars represent the standard deviation of the mean

natamycin failed to reduce the counts and/or to control the growth of all bacterial groups tested, both native and inoculated ones, as expected; and surprisingly, also failed to control M&Y behavior. Indeed, it is well known that natamycin block fungal growth by binding specifically to ergosterol, present almost exclusively in the fungi plasma membranes (Te Welscher et al., 2008). Many studies have documented the effect of natamycin for the control of M&Y in foodstuff, especially in cheeses, with variable results (Kallinteri, Kostoula, & Savvaidis, 2013; Ollé Resa, Jagus, & Gerschenson, 2014; Tsiraki & Savvaidis, 2014). Among them, similar results to those obtained in this study were observed by Tsiraki and Savvaidis (2014), who only achieved very slight reductions (of at most 0.6 log cycles) in the M&Y population of "Tzatziki," a traditional Greek salad made of strained yogurt mixed with cucumber, garlic, salt, olive oil, and dill, treated with natamycin (20 ppm) and stored at 4 °C. On the contrary, Kallinteri et al. (2013) found that natamycin treatments (100 and 200 ppm) efficiently suppressed fungal growth in Galotyri cheese during 28 days at 4 °C. Certainly, when working with native microbiota, it should be considered the great variability with respect to the type of microorganism present in the product, and that not all M&Y have the same sensitivity to the antimicrobial (Ollé Resa et al., 2014). To the best of our knowledge there are no references of the effect of natamycin in leafy vegetables.

Furthermore, the results of this study indicate that individual treatment with nisin also failed to reducing counts and/or controlling the growth of native microbiota. According to Leff and Fierer (2013), EB is the most abundant bacterial family in produce that typically grown closer to the soil surface such as spinach, lettuce, tomatoes, and peppers. Furthermore, it is not surprising to find high relative abundances of EB in this kind of produce, since members of this family are known for colonize certain fruits and vegetables (Abadias, Usall, Anguera, Solsona, & Viñas, 2008). Moreover, it is well known that nisin is not effective for the control of gram-negative population (Helander & Mattila-Sandholm, 2000). Hence, in this research, the lack of effectiveness of nisin against MAB could be associated with a higher prevalence of gram-negative bacteria in beet leaves' native microbiota.

Conversely, nisin is recognized for being very effective against gram-positive bacteria. It has been proven that nisin acts by binding electrostatically to the negatively charged phospholipids (Bauer & Dicks, 2005) increasing the permeability of the membrane by pore formation, resulting in rapid efflux of essential intracellular small molecules (Breukink et al., 1997), and interfering with cell wall biosynthesis. In this study, individual treatment with nisin was very effective for *L. innocua* control, consistent with many other authors' observations. However, other studies have focused on their in vitro effectiveness and the few applied studies that can be found, are mainly in meat or dairy products (Fernandez et al., 2014; Nguyen, Gidley, & Dykes, 2008). Very little literature can be found on its application on vegetable products. In this sense, Randazzo, Pitino, Scifò, and Caggia (2009) found reductions of 1 log cycle on *L. monocytogenes* counts of minimally processed iceberg lettuce treated with commercial nisin (2,500 IU/ml) after 7 days of storage at 4 °C. Similarly, Cai, Ng, and Farber (1997) showed a reduction of 1.4 log cycles in *L. monocytogenes* counts of ready-to-eat Caesar

salad treated with nisin-producing *L. lactis* isolated from bean-sprouts, after 10 days of storage at 10 °C.

With respect to GTE performance, for all the populations under study, their activity was characterized by an initial count reduction followed by a regrowth. In all cases, a greater effectiveness was achieved for the highest GTE concentration (5%), indeed, it is well known that green tea effectiveness is directly proportional to tea polyphenol content, which increases with the concentration of tea (von Staszewski, Pilosof, & Jagus, 2011). In regard to *native microbiota*, as presented previously, GTE presented remarkable effects for MAB and EB control, while the reductions obtained for M&Y were lower, but yet significant. Only few authors have studied the effects of GTE on the native microbiota of food products and very variable results can be found. This may be related to the different composition and characteristics of the food system in which it is applied, due to the many interactions existing between antimicrobials and food components (von Staszewski et al., 2011). Additionally, the technique used for the application of the antimicrobial treatment (immersion, spray, incorporated in a superficial film or in formula, among others) can greatly affect the results (Özvural, Huang, & Chikindas, 2016). In this sense, we can mention the study of Kumudavally, Phanindrakumar, Tabassum, Radhakrishna, and Bawa (2008), who observed reductions of 4, 5, and 2 log cycles in MAB, EB, and M&Y counts, respectively, of fresh mutton sprayed with GTE (5%) after 2 days of storage at 25 °C. Chiu and Lai (2010), found initial reductions of roughly 1.5 and 2 log cycles in the MAB and M&Y counts, respectively, of fruit-based salads treated with coatings with various green tee extracts (6%, extracted with different methods). Also, Özvural et al. (2016) achieved reductions of only 0.5 log cycles or less in the MAB, EB, and M&Y counts of hamburgers elaborated with GTE (5%) in their formulation, when stored at 4 °C. Moreover, Siripatrawan and Noipha (2012), observed reductions of 6.5 and 2 log cycles in the MAB and M&Y counts, respectively, of pork sausages treated with a chitosan film containing a GTE (20% in film), at 20 days of storage at 4 °C. The accepted criterion for the limit of microbiological shelf life of fresh vegetables is equal to 7 log CFU/g for MAB counts (Corbo, Del Nobile, & Sinigaglia, 2006) and equal to 5 log CFU/g for M&Y counts (Fleet, 1992). Considering this, it is interesting to note that in this study under conditions of thermal abuse (15 °C) treatments containing GTE 5% managed to double the microbiological shelf life of the product.

In regard to pathogenic surrogate control, several in vitro studies have been carried out on the effect of GTE against *Listeria* spp and *E. coli* (Over, Hettiarachchy, Johnson, & Davis, 2009; von Staszewski & Jagus, 2008). Among them, only a few applied studies can be found, but they are mostly limited to meat or dairy products. In this sense, Hong, Lim, and Song (2009) found reductions of about 1 log in both, *L. monocytogenes* and *E. coli* counts of pork loins packed with a gelidium corneum-gelatin blend films containing GTE (4.2%) after 10 days of storage at 4 °C. Kumudavally et al. (2008) observed a reduction of *E. coli* from an initial load of 2.6 log CFU/g to undetectable levels during the first 4 days of storage at ambient temperature (25 °C) of fresh mutton treated with GTE (5%). In leafy vegetable products, Chiu and Lai (2010) found reductions of about 6 log cycles in the *L. monocytogenes*

counts of romaine lettuce hearts treated with tapioca starch coatings containing GTEs (430–500 mg GAE/g), after 48 hr of refrigerated storage.

According to Cui et al. (2012), Epigallocatechin gallate (EGCG), the main catechin present in green tea, affects gram-positive, and gram-negative bacteria differently. In gram-positive bacteria, EGCG directly bind to peptidoglycans and incite its precipitation (Shimamura, Zhao, & Hu, 2007), inducing damage in the cell wall and interfering with its biosynthesis. Conversely, EGCG also damage the cell wall of gram-negative bacteria, but this damage is induced mainly by H_2O_2 production. Hydroxy radicals generated from H_2O_2 are known to attack polyunsaturated fatty acid in membranes and initiate lipid peroxidation (Cabiscol et al., 2002). As a result, membrane properties such as fluidity can be changed (Cabiscol et al., 2002), eventually leading to membrane degradation (Yang et al., 2006). Moreover, Cui et al. (2012) observed that EGCG treatment of *E. coli* O157:H7 led to temporary changes of the cell walls, such as pore-like lesions.

In regard to *combined treatments*, combination of natamycin and GTE showed significant effects for M&Y control. This synergistic effect could be attributed to the fact that both, natamycin and tea catechins, act directly or indirectly on ergosterols of the membrane of M&Y cells, preventing its development. Indeed, Navarro-Martínez, García-Cánovas, and Rodríguez-López (2006) demonstrated that by disturbing the folate metabolism, EGCG could inhibit ergosterol production. While, as mentioned previously, Te Welscher et al. (2008) revealed that natamycin kills yeast by specifically binding to ergosterol. Considering the results of this study it is evident that through joint action of these antimicrobials, a synergistic effect in controlling beet leaves native M&Y is achieved.

To the best of our knowledge, this is the first study documenting the interactions or effects of GTE and nisin combination on native microbiota of a food product. Moreover, within our knowledge, no analysis has been made in model systems. The same applies to the case of *E. coli*. As previously mentioned, both antimicrobial have direct action in the bacterial cell wall, therefore it was not surprising that their combined action lead to improvements over individual treatments. In this study, it was not observed any improvement neither for EB nor for *E. coli* control. However, the combined treatment GTE5 + Ni500 presented a remarkable effect for *L. innocua* control, differing significantly from the individual treatments. The interactions between nisin and green tea were studied by Theivendran, Hettiarachchy, and Johnson (2006) who worked with nisin (10,000 IU/ml) and green tea (1%) applied, alone or in combination, on PBS medium inoculated with *L. monocytogenes* (9 CFU/ml). According to these authors, the incorporation of nisin with GTE dramatically enhanced the inhibitory effect against *L. monocytogenes*, as GTE prevented the growth of resistant survivors in the population when combined with nisin. According to Theivendran et al. (2006) this enhanced inhibitory effect may be due to the facilitated diffusion of major phenolic compounds of green tea (epicatechin, caffeic, benzoic, and syringic acid) through the pores formed in the microbial cell membrane caused by the activity of nisin. In this study, combined treatment did not prevent regrowth, but undoubtedly

a highly improved effect was achieved along storage, obtaining the greatest reductions and positioning this treatment as the most promising one to control *L. innocua*, among those tested.

Whether alone or combined, GTE treatments had a remarkable performance for the control of all the microbial groups tested. Nevertheless, it is important to mention that beet leaves exposed to tea treatments presented some changes in their appearance, especially toward the end of storage, showing a more reddish tonality compared to control samples and/or those treated only with Ni or Na. Considering this, future research should focus on the sensory evaluation of the samples treated to analyze whether the observed changes somehow affect consumer acceptance of the product.

Despite this, it is relevant to highlight the importance of finding antimicrobials that can be effective against gram-negative bacteria, a bacterial group that presents high difficulty to be controlled mainly due to the structural characteristics of their cell wall. In this study, the GTE treatment shows a remarkable activity against *E. coli*, a really important fact to emphasize since it is one of the main microorganisms associated with outbreaks caused by consumption of salads and MPV.

5 | CONCLUSION

Individual treatments with nisin or natamycin were not effective in controlling beet leaves' native microbiota. Treatments containing GTE 5% were very effective for the control of aerobic mesophilic bacteria and EB. The combination of GTE 5% with nisin was the most effective treatment for MAB, but did not introduce any improvement for EB control. The combined treatments of natamycin and GTE did not produce any additional effect to the obtained with GTE alone on MAB and EB. On the other hand, for controlling M&Y, while individual GTE treatment had a very limited effect, a remarkable synergistic behavior between natamycin and GTE (2.5 and 5%) was observed.

In regard to pathogenic surrogate control, both nisin and GTE were very effective against *Listeria*. Although nisin achieved better initial results, the long-term results were similar. The combination of these antimicrobials resulted in an outstanding improved effect, achieving the higher reductions throughout storage. Among the antimicrobial tested, the only effective one for *E. coli* control turned out to be GTE, which showed remarkable effects and a concentration dependent behavior.

According to these results, GTE is a very promising option, either alone or in combination with other antimicrobial (depending on the objective of treatment). It was shown that GTE has a great potential for reducing or preventing the growth of pathogenic and spoilage microorganism of leafy vegetables. Future research will focus on the sensory evaluation of the samples treated with GTE to analyze if their incorporation affects consumer acceptance of the product.

REFERENCES

Abadias, M., Usall, J., Anguera, M., Solsona, C., & Viñas, I. (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. _International Journal of Food Microbiology_, 123, 121–129.

Allende, A., Tomás-Barberán, A., & Gil, M. I. (2006). Minimal processing for healthy traditional foods. _Trends in Food Science & Technology_, 17, 513–519.

Argentinean Alimentary Code. (1996). Food additives. Cap XVIII. Article 1398. De la Canal y Asociados S.R.L., Buenos Aires, Argentina.

Bansal, S., Choudhary, S., Sharma, M., Kumar, S. S., Lohan, S., Bhardwaj, V., … Jyoti, S. (2013). Tea: A native source of antimicrobial agents. _Food Research International_, 53, 568–584.

Bauer, R., & Dicks, L. M. T. (2005). Mode of action of lipid II-targeting lantibiotics. _International Journal of Food Microbiology_, 101, 201–216.

Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P., & de Kruijff, B. (1997). The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. _Biochemistry_, 36, 6968–6976.

Cabiscol, E., Bellí, G., Tamarit, J., Echave, P., Herrero, E., & Ros, J. (2002). Mitochondrial Hsp60, resistance to oxidative stress, and the labile iron pool are closely connected in _Saccharomyces cerevisiae_. _Journal of Biological Chemistry_, 277, 44531–44538.

Cai, Y., Ng, L.-K., & Farber, J. M. (1997). Isolation and characterization of nisin-producing _Lactococcus lactis_ subsp. lactis from bean-sprouts. _Journal of Applied Microbiology_, 83, 499–507.

Chiu, P.-E., & Lai, L.-S. (2010). Antimicrobial activities of tapioca starch/decolorized hsian-tsao leaf gum coatings containing green tea extracts in fruit-based salads, romaine hearts and pork slices. _International Journal of Food Microbiology_, 139, 23–30.

Corbo, M. R., Del Nobile, M. A., & Sinigaglia, M. (2006). A novel approach for calculating shelf life of minimally processed vegetables. _International Journal of Food Microbiology_, 106, 69–73.

Cui, Y., Oh, Y. J., Lim, J., Youn, M., Lee, I., Pak, H. K., & Park, S. (2012). AFM study of the differential inhibitory effects of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria. _Food Microbiology_, 29, 80–87.

Delves-Broughton, J. (2011). Nisin, natamycin and other commercial fermentates used in food biopreservation. In C. Lacroix (Ed.), _Protective cultures, antimicrobial metabolites and for food and beverage biopreservation_ (pp. 63–99). Cambridge: Woodhead Publishing, Ltd.

Delves-Broughton, J., Blackburn, P., Evans, R. J., & Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. _Antonie Van Leeuwenhoek_, 69, 193–202.

Fernandez, M. V., Jagus, R. J., & Mugliaroli, S. L. (2014). Effect of combined natural antimicrobials on spoilage microorganisms and _Listeria innocua_ in a whey cheese "Ricotta". _Food and Bioprocess Technology_, 7, 2528–2537.

Fleet, G. (1992). Spoilage yeasts. _Critical Reviews in Biotechnology_, 12(1–2), 1–44.

Helander, I. M., & Mattila-Sandholm, T. (2000). Permeability barrier of the Gram-negative bacterial outer membrane with special reference to nisin. _International Journal of Food Microbiology_, 60, 153–161.

Hong, Y.-H., Lim, G.-O., & Song, K. B. (2009). Physical properties of _Gelidium corneum_–gelatin blend films containing grapefruit seed extract or green tea extract and its application in the packaging of pork loins. _Journal of Food Science_, 74, C6–C10.

Kallinteri, L. D., Kostoula, O. K., & Savvaidis, I. N. (2013). Efficacy of nisin and/or natamycin to improve the shelf-life of Galotyri cheese. _Food Microbiology_, 36, 176–181.

Kamat, A. S., & Nair, P. M. (1996). Identification of _Listeria innocua_ as a biological indicator for inactivation of _L. monocytogenes_ by some meat processing treatments. _LWT - Food Science and Technology_, 29, 714–720.

Kim, J. K., & Harrison, M. A. (2009). Surrogate selection for _Escherichia coli_ O157: H7 based on cryotolerance and attachment to romaine lettuce. _Journal of Food Protection_, 72, 1385–1391.

Koontz, J. L., Marcy, J. E., Barbeau, W. E., & Duncan, S. E. (2003). Stability of natamycin and its cyclodextrin inclusion complexes in aqueous solution. _Journal of Agricultural and Food Chemistry_, 51, 7111–7114.

Kumudavally, K. V., Phanindrakumar, H. S., Tabassum, A., Radhakrishna, K., & Bawa, A. S. (2008). Green tea – A potential preservative for extending the shelf life of fresh mutton at ambient temperature (25 ± 2 °C). _Food Chemistry_, 107, 426–433.

Leff, J. W., & Fierer, N. (2013). Bacterial communities associated with the surfaces of fresh fruits and vegetables. _PLoS One_, 8, e59310.

Martins, J. T., Cerqueira, M. A., Souza, B. W. S., do Carmo Avides, M., & Vicente, A. A. (2010). Shelf life extension of ricotta cheese using coatings of galactomannans from nonconventional sources incorporating nisin against _Listeria monocytogenes_. _Journal of Agricultural and Food Chemistry_, 58, 1884–1891.

Navarro-Martínez, M. D., García-Cánovas, F., & Rodríguez-López, J. N. (2006). Tea polyphenol epigallocatechin-3-gallate inhibits ergosterol synthesis by disturbing folic acid metabolism in _Candida albicans_. _Journal of Antimicrobial Chemotherapy_, 57, 1083–1092.

Nguyen, V. T., Gidley, M. J., & Dykes, G. A. (2008). Potential of a nisin-containing bacterial cellulose film to inhibit _Listeria monocytogenes_ on processed meats. _Food Microbiology_, 25, 471–478. ISSN 0740–0020

Ollé Resa, C. P., Jagus, R. J., & Gerschenson, L. N. (2014). Natamycin efficiency for controlling yeast growth in models systems and on cheese surfaces. _Food Control_, 35, 101–108.

Omac, B., Moreira, R. G., Castillo, A., & Castell-Perez, E. (2015). Growth of _Listeria monocytogenes_ and _Listeria innocua_ on fresh baby spinach leaves: Effect of storage temperature and natural microflora. _Postharvest Biology and Technology_, 100, 41–51.

Over, K. F., Hettiarachchy, N., Johnson, M. G., & Davis, B. (2009). Effect of organic acids and plant extracts on _Escherichia coli_ O157: H7, _Listeria monocytogenes_, and _Salmonella Typhimurium_ in broth culture model and chicken meat systems. _Journal of Food Science_, 74, M515–M521.

Özvural, E. B., Huang, Q., & Chikindas, M. L. (2016). The comparison of quality and microbiological characteristic of hamburger patties enriched with green tea extract using three techniques: Direct addition, edible coating and encapsulation. _LWT - Food Science and Technology_, 68, 385–390.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts—Potential applications in food safety and quality. _Food Research International_, 44, 827–839.

Ponce, A. G., Fritz, R., Del Valle, C., & Roura, S. I. (2003). Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. _LWT - Food Science and Technology_, 36, 679–684.

Randazzo, C. L., Pitino, L., Scifò, G. O., & Caggia, C. (2009). Biopreservation of minimally processed iceberg lettuces using a bacteriocin produced by _Lactococcus lactis_ wild strain. _Food Control_, 20, 756–763.

Sant'Ana, A. S., Franco, B. D. G. M., & Schaffner, D. W. (2014). Risk of infection with *Salmonella* and *Listeria monocytogenes* due to consumption of ready-to-eat leafy vegetables in Brazil. *Food Control*, *42*, 1–8.

São José, J. F. B., & Vanetti, M. C. D. (2012). Effect of ultrasound and commercial sanitizers in removing natural contaminants and *S. entericatyphimurium* on cherry tomatoes. *Food Control*, *24*, 95–99.

Shimamura, T., Zhao, W.-H., & Hu, Z.-Q. (2007). Mechanism of action and potential for use of tea catechin as an antiinfective agent. *Anti-Infective Agents in Medicinal Chemistry*, *6*, 57–62.

Siripatrawan, U., & Noipha, S. (2012). Active film from chitosan incorporating green tea extract for shelf life extension of pork sausages. *Food Hydrocolloids*, *27*, 102–108.

Taban, B. M., & Halkman, A. K. (2011). Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? *Anaerobe*, *17*, 286–287.

Te Welscher, Y. M., Hendrik, H., Balagué, M. M., Souza, C. M., Riezman, H., De Kruijff, B., & Breukink, E. (2008). Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal of Biological Chemistry*, *283*, 6393–6401.

Theivendran, S., Hettiarachchy, N. S., & Johnson, M. G. (2006). Inhibition of *Listeria monocytogenes* by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. *Journal of Food Science*, *71*, M39–M44.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, *57*, 5987–6000.

Tsiraki, M. I., & Savvaidis, I. N. (2014). Citrus extract or natamycin treatments on "Tzatziki"–a traditional Greek salad. *Food Chemistry*, *142*, 416–422.

von Staszewski, M., & Jagus, R. J. (2008). Natural antimicrobials: Effect of Microgard^TM and nisin against *Listeria innocua* in liquid cheese whey. *International Dairy Journal*, *18*, 255–259.

von Staszewski, M., Pilosof, A. M. R., & Jagus, R. J. (2011). Antioxidant and antimicrobial performance of different Argentinean green tea varieties as affected by whey proteins. *Food Chemistry*, *125*, 186–192.

Yang, L., Wang, K., Tan, W., He, X., Jin, R., Li, J., & Li, H. (2006). Atomic force microscopy study of different effects of natural and semisynthetic β-lactam on the cell envelope of *Escherichia coli*. *Analytical Chemistry*, *78*, 7341–7345.