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ORIGINAL ARTICLE

Natural antimicrobials for beet leaves preservation: in vitro and in vivo determination of effectiveness

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Abstract Nisin (Ni), natamycin (Na), green tea extract (GTE) and their combinations were evaluated for controlling beet leaves' native microbiota as well as *Listeria innocua* and *Escherichia coli* external contaminations. Antimicrobial effectiveness was evaluated through in vitro and in vivo studies. In the in vitro studies, GTE treatment (0.85%) completely eliminated growth of native microbiota, reduced *L. innocua* from values of 8.5–3.5 log from 24 h onwards and reduced *E. coli* below detection limit (DL) after 72 h. Ni (500 IU/mL) was the most effective against *L. innocua* (7 log CFU/mL reduction) and its combination with GTE presented significant interactions for mesophilic aerobic bacteria (MAB) and *L. innocua* control. Na (200 ppm) alone or in combination with GTE did not show antimicrobial activity against microorganisms under study. Additionally in vivo evaluation showed that 2.5–5% GTE concentrations are needed to achieve significant inhibitory effects on MAB, *L. innocua* and *E. coli*. Furthermore, the best results for MAB and *L. innocua* control were obtained with the GTE5 + Ni treatment. This study revealed that GTE, either alone or combined with nisin, is a highly promising option with potential for reducing or preventing the growth of pathogenic and spoilage microorganisms present in leafy vegetables, specifically in beet leaves.

Keywords Nisin · Natamycin · Green tea · Biopreservation · Beet leaves

Introduction

Consumption trends show a growing interest towards more natural, nutritious, safe and chemical additives-free foods. The current lifestyle has led to an increase in the demand for foods that are easy to prepare or ready for consumption stimulating the expansion of minimally processed fruit and vegetable market (Wiley and Yildiz 2017). Moreover, "gourmet salads" with novel ingredients like leaves of several vegetables (radishes, beets, endive, among others) have had an important growth. In particular, beet leaves are a rich source of nutrients, fiber and phytochemicals (Fernandez et al. 2017) and its visual appearance with green leaves and red veins is very attractive.

The intrinsic characteristics of minimally processed fruits and vegetables, such as their low acidity and high humidity, together with the high number of cut surfaces, increase their susceptibility to microbial growth (Wiley and Yildiz 2017). In fact, these products have been implicated in numerous foodborne illness outbreaks during the last two decades. Etiologic factors in most of the cases were contaminations with pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enteritidis* (Castro-Ibáñez et al. 2017).

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The exploration of natural antimicrobials for food preservation is intended to cover not only consumers' demand for healthier foodstuffs, but also, the growing concern of microbial resistance towards conventional preservatives (Ponce et al. 2003). Among natural antimicrobials, nisin and natamycin are widely known and have "Generally Recognized As Safe" (GRAS) degree for foodstuff applications (Jagus et al. 2016). Nisin is a bacteriocin produced by strains of *Lactococcus lactis* subsp. Lactis and exhibits antimicrobial activity towards a wide range of Gram positive bacteria, including *L. monocytogenes* (Cui et al. 2017); 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. 2010). Other antimicrobial that have gained much attention in recent years is green tea (*Camellia sinensis* L.) extract. Many studies have shown that they have antibacterial, antiviral, antifungal and radical scavenging activities, which were associated with the polyphenols present therein (Bansal et al. 2013). The effectiveness of these three antimicrobials has been demonstrated in several food products, mainly meat and dairy ones (Fernandez et al. 2014; Ollé Resa et al. 2014; Özvural et al. 2016). However, scarce information can be found on their application on vegetable products.

Considering the foregoing, the aim of this study was to evaluate the potential of the natural antimicrobials nisin, natamycin, green tea extract and their combinations for controlling native microbiota growth on beet leaves, as well as for preventing *Listeria innocua* and *E. coli* development when an external contamination occurs.

Materials and methods

Plant material

The beets (*Beta vulgaris* L. var. Conditiva) were obtained from a local market in Buenos Aires (Argentina) and immediately transported in refrigerated conditions to the laboratory. Once there, roots and stems were removed, and beet leaves were stored at 5 °C until treatments were applied.

Natural antimicrobials

Antimicrobial solutions were prepared as follows: For nisin treatments a 50,000 IU/mL stock solution was prepared dissolving 250 mg of commercial nisin (Delvo®Plus, DSM) in 5 mL of sterile distilled water. For natamycin treatments a 24 mg mL^{-1} stock solution was prepared dissolving 240 mg of commercial natamycin (Delvocid®, DSM) in 10 mL of sterile distilled water. For green tea extracts treatments, 20 and 40% stock solutions of Sunphenon 90LB (Taiyo International) were prepared dissolving 2 and 4 g of the product respectively, and carried to 10 mL with sterile distilled water.

Culture preparation

In this study, *L. innocua* (CIP 8011, CCMA 29, Facultad de Farmacia y Bioquímica, UBA, Argentina) and *E. coli* ATCC 8739 were used. 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 (Evrendilek et al. 1999; Omac et al. 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, 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*.

In vitro assay

The in vitro assays are often carried out to determine the sensitivity of several microorganisms to different antimicrobial agents, allowing the selection of the most promising ones.

Beet leaves' native microbiota culture

Native microbiota from beet leaves was extracted from 10 g of raw material macerated in 90 mL of peptone water, using a Stomacher (Interscience Laboratories Inc. Bagmixer® 400 P, France) and incubated overnight at 37 °C, to obtain the final desired concentration of cells (approximately 1.0×10^8 CFU/mL) determined by optical density when achieved an absorbance of 0.14 at 615 nm.

Preparation of broth model systems based on beet leaves

The model systems were prepared in sterile Falcon tubes containing 20 mL of fresh TSBYE broth. For broth model systems "A", each falcon tube was inoculated with native microbiota culture to achieve an initial microbial load of approximately 10^5 CFU/mL. The broth model systems "B" and "C" were prepared inoculating the microorganism under evaluation (*L. innocua* or *E. coli*, respectively), with approximately 10^6 CFU/mL load, in addition to beet

leave native microbiota at a load of 10^2 CFU/mL. Native microbiota was incorporated in "B" and "C" these systems as its presence may significantly influence the growth and death of microorganisms under study (Omac et al. 2015), simulating a more real situation.

The antimicrobials were applied in the "A", "B" and "C" broth model systems, according to the scheme presented in Table 1. The concentrations were chosen taking into account results obtained in previous studies (data not shown) in which several concentrations and combinations were tested. For the three broth model systems, tubes without antimicrobial were considered as control (CO). For antimicrobial treatments aliquots of the stock solution were added to the model systems so as to achieve the corresponding concentration.

In vivo assay

It is well known that the effectiveness of antimicrobials decreases when passing from model systems to real one. The presence of carbohydrates, proteins, fats, salts and pH strongly influence the activity of these agents (Busatta et al. 2008; Ponce et al. 2011). Moreover, in in vitro tests, the pool of microorganisms present in the model systems are available to be targeted by the antimicrobials, while in in vivo assays, the microorganisms could be internalized or forming biofilms hindering antimicrobial action (Ponce et al. 2003). Thus, in vivo assays are necessary to evaluate the performance of antimicrobials in real situations.

Table 1 Treatments application scheme

Treatments[a]	Broth model system			Beet leaves system		
	A	B	C	D	E	F
CO	x	x	x	x	x	x
Ni250	–	x	–	–	–	–
Ni500	x	x	–	x	x	–
Na200	x	x	–	–	–	–
GTE0.425	x	x	x	–	–	–
GTE0.85	x	x	x	x	x	x
GTE1.25	–	–	–	x	x	x
GTE2.5	–	–	–	x	x	x
GTE3.75	–	–	–	x	x	x
GTE5	–	–	–	x	x	x
GTE0.425 + Ni500	x	x	x	–	–	–
GTE0.425 + Na200	x	x	–	–	–	–
GTE0.85 + Ni500	x	–	x	x	x	x
GTE0.85 + Na200	x	–	–	–	–	–
GTE1.25 + Ni500	–	–	–	x	x	–
GTE2.5 + Ni500	–	–	–	x	x	–
GTE3.75 + Ni500	–	–	–	x	x	–
GTE5 + Ni500	–	–	–	x	x	–

The "X" indicates the selected treatments

[a]The water content was matched in all systems by the addition of an adequate amount of sterile water

Preparation of beet leaves systems and sampling procedure

The beet leaves were washed with cold tap water and disinfected by immersion in a cooled sodium hypochlorite solution (200 ppm free chlorine) for 5 min. Then, they were dried for 1 min in a manual centrifugal dryer and cut, perpendicularly to the veins, to obtain strips of 2–3 cm wide. Samples of 10 g were weighed and placed in stomacher bags (Nasco Whirl-Pack®, USA). Three systems were considered. The "D" beet leaves system was used to study the effectiveness of natural antimicrobials against native microbiota. The "E" and "F" beet leaves systems were used for the evaluation of effectiveness against *L. innocua or E. coli* contamination, for which inoculations were performed with *L. innocua* or *E. coli*, respectively, with approximately 10^6 CFU/mL load in both cases. From in vitro results, the most promising antimicrobials were selected (Ni and GTE at the highest concentrations) and their effects on the real system were evaluated. Moreover, considering that the effectiveness of antimicrobial decreases when passing from model systems to real systems, higher concentration of GTE were also evaluated. Antimicrobials were applied in the beet leaves systems, according to the scheme presented in Table 1. Afterward all samples were gently massaged, according to the methodology proposed by Tsiraki and Savvaidis (2014) in order to obtain a homogeneous distribution of the antimicrobials in the system. Samples without antimicrobial were considered as a control (CO).

Storage and sampling procedure

In both studies, samples were stored at 15 ± 1 °C. These were accelerated tests but, additionally, the selected temperature would correspond to a typical commercial thermal abuse. In in vitro studies samples were taken periodically at 0 (within the first hour after adding the antimicrobials in the corresponding systems), 6, 24, 48, 72, 132 h for analysis. For a first approach towards its application in real systems (in vivo studies), initial effects (0 h) are crucial, moreover, information after 24 and 72 h of storage was provided to complete these studies.

Microbiological studies

To evaluate native microbiota ("A" broth model systems and "D" beet leaves systems), mesophilic aerobic bacteria (MAB) counts were performed using plate count agar

(PCA, Biokar Diagnostics, France) incubated at 37 °C for 24–48 h. Also, for the in vitro test molds and yeasts (M&Y) counts were determined in yeast extract glucose chloramphenicol agar (YGC, Biokar Diagnostics, France) incubated at 28 °C for 48–72 h and *Enterobacteriaceae* (EB) were determined in Mac Conkey agar (Biokar Diagnostics, France) incubated at 37 °C for 24 h. *L. innocua* evaluation, *Listeria* spp. counts ("B" broth model systems and "E" beet leaves systems) were performed using Oxford Agar (Biokar Diagnostics, France) incubated at 37 °C for 48 h. Finally, *E. coli* counts ("C" model systems and "F" beet leaves systems) were performed using Mac Conkey agar (Biokar Dignostics, France) with the addition of the supplement 4-methylumbelliferyl-beta-D-glucuronide "MUG" (Biokar Dignostics, France), incubated at 37 °C for 24 h. 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 milliliter (log CFU/mL) for in vitro results and in logarithm of colony forming units per gram (log CFU/g) for in vivo results.

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®, USA). The factors used as sources of variation were TREAT (treatment, different antimicrobial tested or control), TIME (storage time) and TREAT and TIME interaction. Differences among samples were determined by the Tukey–Kramer multiple comparison test. Wherever differences were reported as significant, a 95% confidence level was used.

Results and discussion

In vitro assay

Effectiveness against native microbiota

The changes of mesophilic aerobic bacteria (MAB) during storage at 15 °C are shown in Fig. 1a. Control samples presented an initial MAB load of 5.1 log CFU/mL that rapidly increased to a maximum population density of 10.2 log CFU/mL after 72 h, value that was maintained until the end of storage. Individual treatment with Na200 did not produce any significant ($p > 0.05$) inhibitory effect on MAB counts, hence presented a behavior similar to control. Indeed, Te Welscher et al. (2010) revealed that natamycin blocks fungal growth by binding specifically to ergosterol, present almost exclusively in the fungi plasma membranes. The treatment with Ni500 presented the typical initial reduction that characterizes nisin activity (Fernandez et al. 2014); however, within the first 6 h MAB count increased reaching values similar to control samples. This low effectiveness of nisin against MAB could be associated with a high prevalence of Gram negative bacteria in the native microbiota of beet leaves. Certainly, according to Leff and Fierer (2013), *Enterobacteriaceae* is the most abundant bacterial family in produce that are grown closer to the soil surface. Furthermore, it is well known the low effectiveness of nisin to control Gram-negative bacteria (Helander and Mattila-Sandholm 2000).

Regarding the samples containing tea, an initial reduction of around 1 log cycle was obtained with GTE0.425 treatment. These samples presented a lag phase of 48 h, and after that growth was resumed. Higher initial reductions were obtained in samples treated with GTE0.85. Moreover, their MAB counts continued to decrease for 48 h, reaching at that moment values below detection limit (DL: 1 log CFU/mL) and maintaining those values until the end of storage, differing significantly ($p < 0.0001$) from CO. These results indicate that tea presents an antibacterial effect similar to nisin as it generates initial reductions that are in the same order. Additionally, tea effectiveness increases with higher concentrations. Indeed, it is well known that their effectiveness is directly proportional to tea polyphenol content (von Staszewski et al. 2011). Effectiveness of tea was also observed by Chiu and Lai (2010), who found initial reductions of around 1.5 log cycles in the MAB counts of fruit-based salads treated with coatings with different concentrations of GTE.

No significant differences ($p > 0.05$) were observed in the changes of MAB during storage between treatments GTE0.425 and GTE0.425 + Na200, neither between treatments GTE0.85 and GTE0.85 + Na200. These indicate, as expected, that natamycin does not introduce any improvement in GTE antimicrobial action. The combined treatment of tea and nisin, GTE0.425 + Ni500 proved to be more effective ($p < 0.0001$) than individual applications. At the same time, GTE0.85 + Ni500 presented a powerful initial bactericidal effect (5 log cycles reduction) reaching values below DL, differing significantly from the individual treatments Ni and GTE0.85 ($p = 0.0002$ and $p = 0.0008$, respectively). Furthermore, this treatment completely suppressed the growth of MAB until the end of storage. These results show a clear enhancement when nisin and GTE were combined. The particular mechanisms of action of nisin and catechins present in green tea could explain this phenomenon. Nisin binds electrostatically to the negatively charged phospholipids (Jagus et al. 2016) and increases the permeability of the membrane by pore

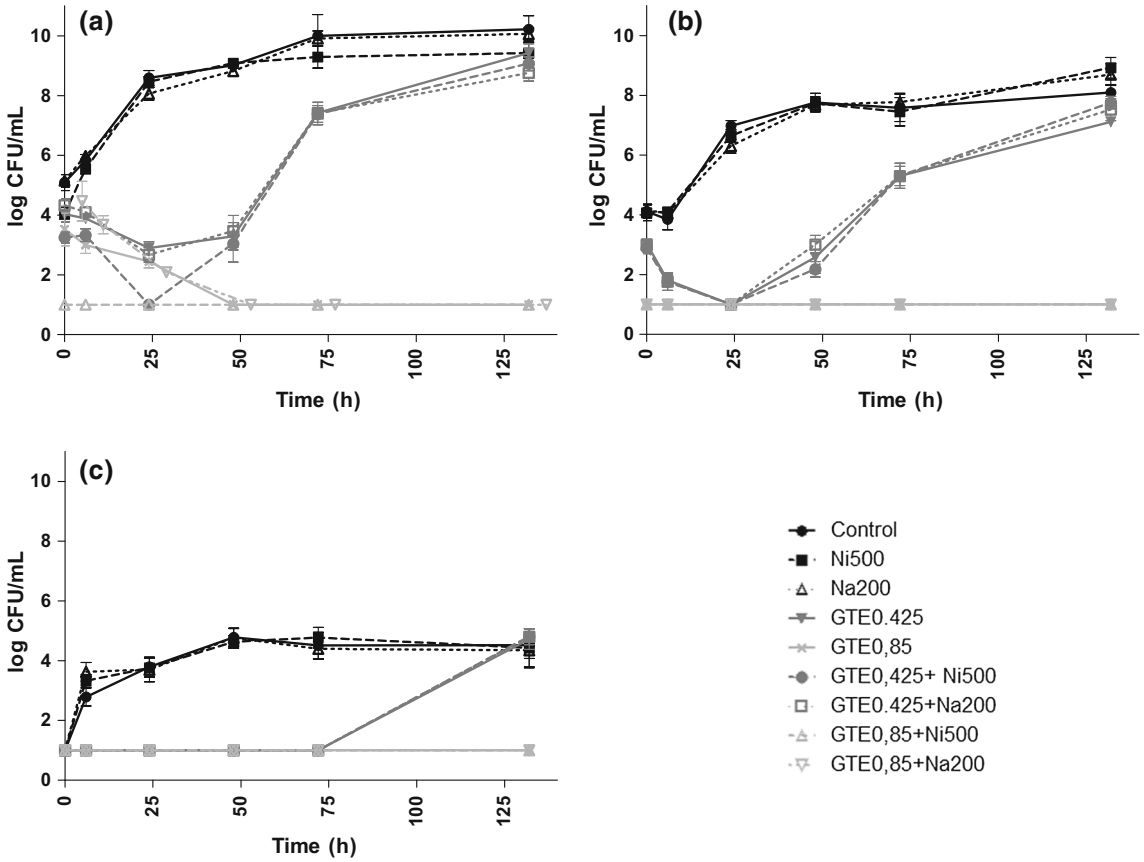

Fig. 1 Effect of nisin, natamycin, green tea extract, and their combinations on mesophilic bacteria (**a**), *Enterobacteriaceae* (**b**) and Molds and yeasts (**c**) in model systems stored at 15 °C

formation, resulting in rapid efflux of essential intracellular small molecules, and interferes with cell wall biosynthesis. On the other hand, epigallocatechin gallate (EGCG), the main catechin present in green tea, can directly bind to peptidoglycans and incite its precipitation (Bansal et al. 2013), inducing damage in the cell wall and interfering with its biosynthesis. Therefore, using antimicrobial compounds that have similar action on the bacterial cell wall, through multiple hurdle approach, could yield synergistic effects. According to Theivendran et al. (2006) these synergistic activities may be due to the facilitated diffusion of major phenolic compounds of green tea through the pores formed by the activity of nisin in the microbial cell membrane. To the best of our knowledge, there is no published literature documenting the combined effect of tea and nisin on native MAB in vegetable products.

In the case of *Enterobacteriaceae* (EB), samples treated with Ni500 and Na200 presented a behavior similar to control (Fig. 1b). These results are in accordance with previous studies that did not find any effectiveness of natamicyn or nisin against Gram-negative bacteria (Helander and Mattila-Sandholm 2000).

Samples treated with GTE0.425, alone or in combination with Ni500 or Na200 had similar behavior, presenting a significant initial decrease ($p < 0.001$) of about 1 log cycle, reaching undetectable values at 24 h. After that, the growth was resumed. When the green tea concentration was doubled (GTE0.85 alone or in combination with Ni500 or Na200), a significant improvement was observed ($p < 0.0001$), since EB counts were initially reduced to undetectable values and the growth was suppressed throughout the storage. Similar results were found by Kumudavally et al. (2008), who observed reductions of about 5 log cycles in EB counts of fresh mutton sprayed with GTE (5%) after 2 days of storage at 25 °C. Also, Özvural et al. (2016) achieved reductions of only 0.5 log cycles in the EB counts of hamburgers elaborated with green tea extract (5%) when stored at 4 °C. It should be noted that the differences found in literature may be due to the diverse food matrices as well as the different techniques used for the application of the antimicrobial, factors that can greatly affect the results (Ollé Resa et al. 2014; Özvural et al. 2016, Ponce et al. 2011; von Staszewski et al. 2011).

Regarding antimicrobials effect on EB, the combination of GTE with nisin or natamycin failed to improve the effectiveness. Hence, GTE was the only effective treatment against *Enterobacteriaceae*, with an effect proportional to the antimicrobial concentration.

The changes on molds and yeast (M&Y) during storage at 15 °C are presented in Fig. 1c. Although control samples presented low initial counts (< DL) a significant ($p < 0.05$) increase during the first 6 h were observed, achieving values of about 4–4.5 log CFU/mL. M&Y are usually associated with food spoilage, but also it must be considered that high counts may be a health hazard because of the mycotoxins produced by molds (Cabañes and Bragulat 2018). Behavior of samples treated with Ni and Na did not differ significantly ($p > 0.05$) from control. For nisin treatment this result was expected, but not for natamycin, known for its action against molds and yeasts. Nevertheless, similar results were found by Tsiraki and Savvaidis (2014) who observed very slight reductions (of at most 0.6 log cycles) in the M&Y of 'Tzatziki', a traditional Greek salad, treated with natamycin (20 ppm) and stored at 4 °C. In contrast, many studies have documented significant effectiveness of Na for controlling M&Y on the surface of many types of cheeses (Ollé Resa et al. 2014). However, when working with native microbiota, it should be considered that there may be a large variability with respect to the type of microorganism present in the product and hence, with their sensitivity to the antimicrobial (Ollé Resa et al. 2014).

Conversely, the presence of GTE0.425 produced a lag phase of 72 h, after which growth took place. Greater effects were observed in samples treated with GTE0.85, as the M&Y growth was completely suppressed differing significantly ($p < 0.001$) from the other treatments. In the same way, Chiu and Lai (2010) observed reductions of approximately 2 log cycles on native M&Y of fruit-based salads treated with tapioca coatings containing GTE (430 mg GAE/g) along storage at 4 °C.

Again, no significant differences were observed when GTE was combined with nisin or natamycin. Hence, among the tested antimicrobials, only GTE was effective for controlling native Y&M.

Effectiveness against Listeria innocua contamination

The changes in *L. innocua* in the model systems during storage at 15 °C are presented in Fig. 2. In relation to the individual treatments it was found, as expected, that the addition of natamycin did not cause any significant reduction of *L. innocua* counts. On the other hand, individual treatments with nisin were the most effective against *L. innocua.* Indeed, both Ni250 and Ni500 presented significant initial reductions ($p < 0.0001$) and remained

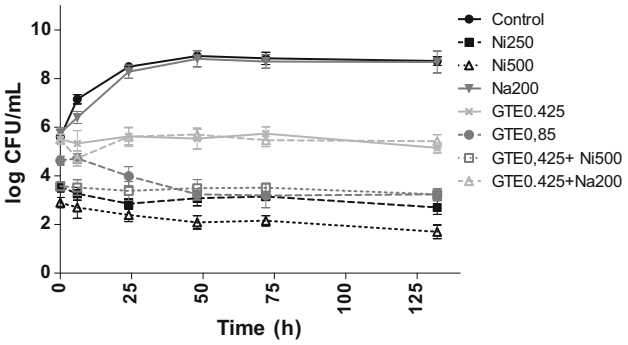

Fig. 2 Effect of nisin, natamycin, green tea extract, and their combinations on *Listeria innocua*, in model systems stored at 15 °C

stable over time, hovering values around 3 and 2 log CFU/mL, respectively.

Effectiveness of nisin against *L. innocua* was widely probed in both model systems (Lehrke et al. 2011) and foodstuffs, mainly in cheeses (Fernandez et al. 2014). Nevertheless, scarce literature can be found on its application on vegetable products. In this sense, Randazzo et al. (2009) found reductions of 1 log cycle on *L. monocytogenes* counts of minimally processed iceberg lettuce treated with a commercial nisin (2500 IU/mL) spray after 7 d of storage at 4 °C.

Treatments GTE0.425 and GTE0.425 + Na200 presented similar behavior between them. They did not presented initial reduction in the *L. innocua* counts but managed to suppress its growth, remaining in counts around 5.5 log CFU/mL along storage, differing statistically to control ($p < 0.0001$). As expected, GTE0.85 treatment was significantly ($p = 0.014$) more effective than GTE0.425. Interestingly, no significant differences were detected between treatments GTE0.85 and Ni250 after 48 h, so we can consider that both treatments are analogous for *L. innocua* control. Several studies have been carried out to evaluate the effect of green tea extracts against *Listeria* spp. In this sense, Chiu and Lai (2010) found reductions of about 6 log cycles after 48 h of refrigerated storage in romaine lettuce hearts treated with coatings containing GTE (430–500 mg GAE/g). Moreover, von Staszewski et al. (2011) observed a bacteriostatic effect of green tea infusions (3%) against *L. innocua,* during storage of a food model system containing WPC35 (8% w/v solids) stored at 20 °C.

It is interesting to note that samples treated with GTE0.425 + Ni500 presented an intermediate behavior between individual treatments (GTE0.425 and Ni500). That is, the presence of nisin improves the effect of green tea alone; however, green tea seems to weaken the effect of nisin alone as reductions were lower in the combined treatment than in the simple one. Similar results regarding initial effects were observed by Theivendran et al. (2006)

working with nisin (10,000 IU/mL) and green tea (1%) applied on PBS medium inoculated with *L. monocytogenes* (9 log CFU/mL). They found that while nisin individual treatment achieved a large initial reduction (4–5 log cycles), when it is combined with tea, lower initial reductions (2–3 log cycles) were observed. However, after 6 h of storage at 37 °C, samples treated with nisin presented the typical regrowth of resistant survivors, while samples treated with the combination of antimicrobials remained stable and close to the initial values. This difference between treatments was not observed in the present study, as no regrowth was observed in any treatment.

Effectiveness against Escherichia coli contamination

Samples inoculated with *E. coli* presented an initial count of 6 log CFU/mL (Fig. 3). The control samples showed a constant growth reaching values of 10 log CFU/mL at the end of storage. All the tested antimicrobial treatments achieved a significant ($p < 0.0001$) initial reduction of 6 log cycles, presenting values below DL. Samples with green tea extract at 0.425% resumes growth after 6 h, achieving similar values to control at the end of storage. On the other hand, samples treated with green tea at 0.85% showed a longer lag phase (72 h) and then presented a regrowth, achieving values 6 log cycles significantly ($p < 0.001$) lower than control at the end of storage.

In the same way, Neyestani et al. (2007) found that tea extracts (25 mg/mL) completely inhibited *E. coli* growth in brain heart broth inoculated with 5 log CFU/mL after 5 and 7 h when evaluated the in vitro microbiologic effects of green and black tea, respectively.

Some applied studies can be found in literature, but they are mostly limited to meat products (Kumudavally et al.,2008; Hong et al. 2009; Over et al. 2009). Among them, Kumudavally et al. (2008) observed a reduction of *E. coli* from an initial load of 2.6 log CFU g^{-1} to undetectable levels during the first 4 days of storage at 25 °C of fresh mutton treated with GTE (5%). Cho et al. (2007) carried out proteomic analysis to investigate the cellular responses of *E. coli* exposed to green tea polyphenols extracted from Korean green tea in order to elucidate the specific mechanisms of action. These authors (Cho et al. 2007) observed changes in cell-membrane fatty acids, presence of perforations, and irregular rod forms with wrinkled surfaces after exposure of *E. coli* to different concentrations of tea polyphenols (TPP). Also, the expression of eight proteins was down regulated, including proteins involved in carbon and energy metabolism and in amino-acid biosynthesis. The presence of perforations could favored the access of other antimicrobials, such as nisin, which generally is unable to penetrate the outer membrane of Gram-negative bacteria due to its large size (1.8–4.6 kDa) (Helander and Mattila-Sandholm 2000). However, in the present study no synergisms were detected. Certainly, in the combined treatments (GTE0.425 + Ni500; GTE0.85 + Ni500) nisin did not introduce any significant ($p > 0.05$) improvement in *E. coli* reductions in comparison with GTE individual treatments.

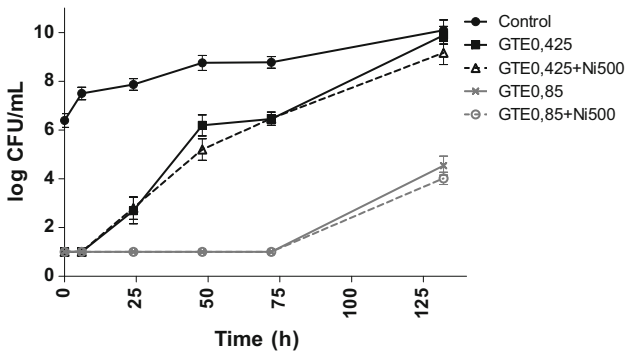

Fig. 3 Effect of nisin, natamycin, green tea extract, and their combinations on *Escherichia coli*, in model systems stored at 15 °C

In vivo assay

In the in vivo assay the GTE concentration applied was up to five times the one selected in in vitro assay (0.85,1.25, 2.5, 3.75 and 5%) as suggested by Busatta et al. (2008). The nisin concentration was 500 IU/mL, since this value is the maximum allowed by the Argentinean Alimentary Code (1996). It is worth mentioning that for GTE no legal limits are set in Argentinean legislation. Since natamycin was not effective when individually applied nor in combination with GTE during in vitro tests, even having tested the maximum concentration allowed, it was not evaluated in vivo. For MAB and *L. innocua,* GTE and nisin combinations were assessed since the in vitro results suggested possible interaction; this was not the case for *E. coli*.

Results of in vivo assays are presented in Table 2. In the case of MAB, significant initial reductions ($p < 0.05$) were achieved in presence of GTE with concentration of 1.25% or higher. Interestingly, no significant differences ($p > 0.05$) were observed in GTE2.5, GTE3.75 and GTE5 initial reductions, but in accordance with that observed on in vitro results, with higher GTE concentrations better performances over time were achieved. Certainly, GTE5 treatment stood out for presenting a lag phase of 24 h and a final count 2.6 log below control.

Individual treatment with nisin did not produce any significant inhibitory effect. On the other hand, combinations of nisin with GTE achieved better results than individual GTE treatments, presenting significant differences between the ones containing GTE 3.75 and 5% and their combinations with nisin. The most effective treatment for

Table 2 Microbiological counts in beet leaves systems

Beet leaves system	D (MAB counts)			E (*L. innocua* counts)			F (*E. coli* counts)		
Treatment/time (h)	0	24	72	0	24	72	0	24	72
CO	$5.98 \pm 0.42^{a,A}$	$8.02 \pm 0.38^{a,B}$	$9.18 \pm 0.30^{a,C}$	$6.29 \pm 0.37^{a,A}$	$7.40 \pm 0.32^{a,B}$	$8.15 \pm 0.38^{a,C}$	$6.31 \pm 0.28^{a,A}$	$7.05 \pm 0.27^{a,B}$	$7.69 \pm 0.42^{a,B}$
Ni500	$5.83 \pm 0.25^{a,b,A}$	$8.06 \pm 0.40^{a,B}$	$8.89 \pm 0.32^{a,C}$	$3.04 \pm 0.29^{b,A}$	$3.61 \pm 0.43^{b,A,B}$	$4.04 \pm 0.39^{b,B}$	–	–	–
GTE0.85	$5.54 \pm 0.44^{a,b,A}$	$7.88 \pm 0.30^{a,b,B}$	$8.90 \pm 0.24^{a,C}$	$6.13 \pm 0.36^{a,A}$	$7.10 \pm 0.36^{a,B}$	$8.05 \pm 0.34^{a,C}$	$5.76 \pm 0.49^{a,A}$	$6.96 \pm 0.32^{a,B}$	$7.59 \pm 0.34^{a,B}$
GTE1.25	$5.01 \pm 0.47^{b,A}$	$7.75 \pm 0.34^{a,b,B}$	$8.56 \pm 0.44^{a,b,B}$	$5.92 \pm 0.17^{a,A}$	$7.30 \pm 0.28^{a,B}$	$8.22 \pm 0.45^{a,C}$	$5.78 \pm 0.27^{a,A}$	$6.55 \pm 0.32^{a,B}$	$7.53 \pm 0.38^{a,C}$
GTE2.5	$3.88 \pm 0.31^{c,A}$	$7.08 \pm 0.25^{b,e,B}$	$7.87 \pm 0.42^{b,c,C}$	$5.48 \pm 0.42^{a,c,A}$	$5.33 \pm 0.41^{c,A}$	$5.73 \pm 0.38^{c,A}$	$4.02 \pm 0.38^{b,A}$	$4.72 \pm 0.32^{b,B}$	$5.56 \pm 0.28^{b,C}$
GTE3.75	$3.67 \pm 0.40^{c,A}$	$5.24 \pm 0.40^{c,B}$	$7.11 \pm 0.29^{c,d,C}$	$4.87 \pm 0.19^{c,A}$	$4.92 \pm 0.28^{c,A}$	$5.04 \pm 0.25^{c,A}$	$3.03 \pm 0.25^{c,A}$	$3.90 \pm 0.39^{c,B}$	$4.52 \pm 0.31^{c,B}$
GTE5	$3.36 \pm 0.28^{c,A}$	$3.87 \pm 0.30^{d,A}$	$6.58 \pm 0.49^{d,B}$	$3.25 \pm 0.39^{b,A}$	$3.67 \pm 0.41^{b,A}$	$3.83 \pm 0.34^{b,d,A}$	$< 1.00^{*d,A}$	$< 1.00^{*d,A}$	$3.13 \pm 0.46^{d,B}$
GTE0.85 + Ni500	$5.61 \pm 0.25^{a,b,A}$	$7.99 \pm 0.50^{a,B}$	$8.84 \pm 0.25^{a,C}$	$3.05 \pm 0.41^{b,A}$	$3.59 \pm 0.26^{b,A,B}$	$4.02 \pm 0.28^{b,B}$	–	–	–
GTE1.25 + Ni500	$3.87 \pm 0.20^{c,A}$	$7.63 \pm 0.40^{a,b,B}$	$8.48 \pm 0.39^{a,b,C}$	$2.82 \pm 0.35^{b,A}$	$3.51 \pm 0.27^{b,B}$	$4.09 \pm 0.21^{b,C}$	–	–	–
GTE2.5 + Ni500	$3.52 \pm 0.29^{c,A}$	$6.74 \pm 0.38^{e,B}$	$7.67 \pm 0.28^{b,c,C}$	$2.63 \pm 0.35^{b,A}$	$3.50 \pm 0.38^{b,B}$	$3.71 \pm 0.37^{b,d,B}$	–	–	–
GTE3.75 + NI	$2.33 \pm 0.39^{d,A}$	$4.14 \pm 0.46^{d,B}$	$6.97 \pm 0.45^{c,d,C}$	$2.53 \pm 0.38^{b,A}$	$3.01 \pm 0.29^{b,d,A}$	$3.69 \pm 0.33^{b,d,B}$	–	–	–
GTE5 + Ni500	$< 1.00^{*e,A}$	$< 1.00^{*f,A}$	$5.23 \pm 0.47^{e,B}$	$< 1.00^{*d,A}$	$2.25 \pm 0.24^{d,B}$	$3.15 \pm 0.23^{d,C}$	–	–	–

Mean values log CFU/g ± SD

Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$). Means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$)

*Below detection limits

MAB proved to be the combination GTE5 + Ni500 which managed to reduce the initial counts to values below DL, presenting a final count (76 h) 4 log below control.

In the case of *L. innocua*, from GTE 2.5% onwards significant differences from control were observed. Nisin treatment was significantly effective, reducing more than 3 log cycles the initial load ($p < 0.05$) and keeping the counts 4 log cycles below CO at the end of storage (72 h). No significant differences were observed between GTE5% and Ni500, so these treatments can be considerate analogous for controlling *L. innocua*. The combined treatments did not produce any significant improvement in comparison to individual ones with nisin, except for GTE5 + Ni500, which initial counts was reduced to values below the DL, differing significantly ($p < 0.0001$) from individual treatments GTE5% and Ni500. Moreover, its behavior over time stood out for having the lowest values among treatments under study. That is, contrary to that suggested in in vitro tests, when working with higher concentrations of GTE in combination with nisin, the combined treatments were more effective than the individual ones. This probably was not seen in the in vitro assays because we worked with very low concentrations of GTE.

In regard to the response of *E. coli* to GTE treatment, as well as on in vitro test, strong concentration dependence was observed. Significant differences with respect to control were observed from treatment GTE2.5 onwards. The most effective treatment was GTE 5%, which exhibited an initial reduction to values below DL, a lag phase of at least 24 h and a final count 4.5 log below control.

These results would indicate that treatments with GTE 2.5–5% are promising for the control of beet leaves' native microbiota as well as for the reduction and control of external contaminations with *L. innocua* and *E. coli*. It is relevant to emphasize the importance of finding antimicrobials effective against Gram-negative bacteria, which are particularly difficult to control because of the presence of the protective outer membrane of lipopolysaccharides, main characteristic of these microorganisms.

Furthermore, this study showed that with the combination of GTE and nisin better results can be achieved for native MAB as well as for *L. innocua* control, standing out the GTE5 + Ni500 treatment, which proved to be the most effective in both cases. In this sense, the application of the combined treatment would allow to apply lower concentration of each antimicrobial as well as to extend the activity spectrum.

It is interesting to mention that no noticeable differences were observed in the appearance of the beet leaves among treatments. Although further studies are needed, this observation is in accordance with the information by others authors (Kumudavally et al. 2008; Randazzo et al. 2009; Siripatrawan and Noipha 2012) who have applied nisin and GTE in food systems.

Future research will focus on the application of the best results of this development in beet leaves stored at refrigeration temperature to evaluate their impact on quality attributes and to determine the shelf life prolongation that can be achieved.

Conclusion

The in vitro tests showed that the antimicrobial with the greatest potential for controlling beet leaves' native microbiota as well as *L. innocua* and *E. coli* growth was green tea extract. Moreover, nisin was very effective against *L. innocua* and its combination with tea resulted in significant interactions for controlling native MAB and *L. innocua*. The results of the in vivo tests indicated that nisin treatment, widely used in dairy products, could be also highly effective against *Listeria* spp. in leafy vegetables. In the real system, higher GTE concentrations (2.5–5%) were needed in order to achieve significant inhibitory effects on MAB, *L. innocua* and *E. coli*. Furthermore, the best results for mesophilic aerobic bacteria and *L. innocua* control were obtained with the combination of GTE 5% and Nisin 500 IU/mL. Summarizing, among the tested antimicrobials, green tea extract stands out since, either alone or combined with nisin, is highly effective against a wide range of microorganisms. Hence, it is a highly promising option with an interesting potential for reducing or preventing the growth of pathogenic and spoilage microorganisms present in leafy vegetables, specifically for beet leaves.

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References

Argentinean Alimentary Code (1996) Food additives. In: De la Canal y Asociados SRL (ed) Argentinean alimentary code. Buenos Aires, Argentina. Cap XVIII. Article 1398

Bansal S, Choudhary S, Sharma M, Kumar SS, Lohan S, Bhardwaj V, Jyoti S (2013) Tea: a native source of antimicrobial agents. Food Res Int 53(2):568–584

Busatta C, Vidal RS, Popiolski AS, Mossi AJ, Dariva C, Rodrigues MRA, Cansian RL (2008) Application of *Origanum majorana* L. essential oil as an antimicrobial agent in sausage. Food Microbiol 25:207–211

Cabañes FJ, Bragulat MR (2018) Black aspergilli and ochratoxin A-producing species in foods. Curr Opin Food Sci 23:1–10

Castro-Ibáñez I, Gil MI, Allende A (2017) Ready-to-eat vegetables: current problems and potential solutions to reduce microbial risk in the production chain. LWT Food Sci Technol 85:284–292

Chiu PE, Lai LS (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. Int J Food Microbiol 139:23–30

Cho YS, Schiller NL, Kahng HY, Oh KH (2007) Cellular responses and proteomic analysis of *Escherichia coli* exposed to green tea polyphenols. Curr Microbiol 55:501–506

Cui H, Wu J, Li C, Lin L (2017) Improving anti-listeria activity of cheese packaging via nanofiber containing nisin-loaded nanoparticles. LWT Food Sci Technol 81:233–242

Evrendilek GA, Zhang QH, Richter ER (1999) Inactivation of *Escherichia coli* O157: H7 and *Escherichia coli* 8739 in apple juice by pulsed electric fields. J Food Prot 62:793–796

Fernandez MV, Jagus RJ, Mugliaroli SL (2014) Effect of combined natural antimicrobials on spoilage microorganisms and *Listeria innocua* in a whey cheese "Ricotta". Food Bioprocess Technol 7:2528–2537

Fernandez MV, Jagus RJ, Agüero MV (2017) Evaluation and characterization of nutritional, microbiological and sensory properties of beet greens (2017). Acta Sci Nutr Health 1(3):37–45

Helander IM, Mattila-Sandholm T (2000) Permeability barrier of the Gram-negative bacterial outer membrane with special reference to nisin. Int J Food Microbiol 60:153–161

Hong YH, Lim GO, Song KB (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. J Food Sci 74:6–10

Jagus RJ, Gerschenson LN, Resa CO (2016). Combinational approaches for antimicrobial packaging: natamycin and nisin. In: Antimicrobial food packaging, pp 599–608

Kumudavally KV, Phanindrakumar HS, Tabassum A, Radhakrishna K, Bawa AS (2008) Green tea—a potential preservative for extending the shelf life of fresh mutton at ambient temperature (25 ± 2 °C). Food Chem 107:426–433

Leff JW, Fierer N (2013) Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS ONE 8:e59310

Lehrke G, Hernaez L, Mugliaroli SL, von Staszewski M, Jagus RJ (2011) Sensitization of *Listeria innocua* to inorganic and organic acids by natural antimicrobials. LWT Food Sci Technol 44:984–991

Neyestani TR, Khalaji N, Gharavi AA (2007) Selective microbiologic effects of tea extract on certain antibiotics against *Escherichia coli* in vitro. J Altern Complement Med 13:1119–1124

Ollé Resa CP, Jagus RJ, Gerschenson LN (2014) Natamycin efficiency for controlling yeast growth in models systems and on cheese surfaces. Food Control 35:101–108

Omac B, Moreira RG, Castillo A, Castell-Perez E (2015) Growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves: effect of storage temperature and natural microbiota. Postharvest Biol Technol 100:41–51

Over KF, Hettiarachchy N, Johnson MG, 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. J Food Sci 74:515–521

Özvural EB, Huang Q, Chikindas ML (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 Sci Technol 68:385–390

Ponce AG, Fritz R, Del Valle C, Roura SI (2003) Antimicrobial activity of essential oils on the native microbiota of organic Swiss chard. LWT Food Sci Technol 36:679–684

Ponce A, Roura SI, Moreira MR (2011) Essential oils as biopreservatives: different methods for the technological application in lettuce leaves. J Food Sci 76:34–40

Randazzo CL, Pitino I, Scifò GO, Caggia C (2009) Biopreservation of minimally processed iceberg lettuces using a bacteriocin produced by *Lactococcus lactis* wild strain. Food Control 20:756–763

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

Te Welscher YM, Jones L, van Leeuwen MR, Dijksterhuis J, de Kruijff B, Eitzen G, Breukink E (2010) Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. Antimicrob Agents Chemother 54(6):2618–2625

Theivendran S, Hettiarachchy NS, Johnson MG (2006) Inhibition of *Listeria monocytogenes* by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. J Food Sci 71:39–44

Tsiraki MI, Savvaidis IN (2014) Citrus extract or natamycin treatments on "Tzatziki"—a traditional Greek salad. Food Chem 142:416–422

von Staszewski M, Pilosof AMR, Jagus RJ (2011) Antioxidant and antimicrobial performance of different Argentinean green tea varieties as affected by whey proteins. Food Chem 125:186–192

Wiley RC, Yildiz F (2017) Introduction to minimally processed refrigerated (MPR) fruits and vegetables. In: Yildiz F, Wiley R (eds) Minimally processed refrigerated fruits and vegetables. Food Engineering Series. Springer, Boston