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Dr. Jagus contributed on the study design, data interpretation and revising the article critically for important intellectual content

Dr. Agüero contributed on the study design, data interpretation and revising the article critically for important intellectual content

Dr. Fernandez contributed on study design, data analysis and interpretation, and drafting the article.

Fruit and vegetable smoothies preservation with natural antimicrobials for the assurance of safety and ~~integral~~ quality

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Abstract

Current consumption trends indicate a clear increased interest in more natural, nutritious and healthier foods. Accordingly, natural fruits and vegetables (F&V) based beverages (juices, smoothies) companies showed great growth, since perceived as a practical way of ingesting the F&V nutrients and bioactives. However, when untreated, these products have a short shelf-life, mainly due to microbial spoilage. The combination of natural antimicrobials for their preservation constitutes an option in line with consumers' requirements. This study aims to evaluate different combinations of natural antimicrobials, nisin, natamycin, green tea extract (GTE) and citric acid, to preserve the ~~integral~~ quality of a mixed F&V smoothie, extending their shelf-life and ensuring their safety. The results obtained suggest that a treatment with nisin 12.5 mg/kg ~~500 UI/mL,~~ natamycin 200 mg/kg and citric acid (until pH 3.5) could achieve a shelf-life extension of 14 d, a product ~~sensory acceptable and of~~ with great nutritional and microbiological quality until 28 d of storage at 5 ºC. Moreover, this treatment would allow controlling a 6 log CFU/mL *Listeria monocytogenes* contamination. Furthermore, if GTE (0.2%) is added to that combination, a product with fortified antioxidant properties (more than 10 times higher than control) is achieved, fulfilling the requirements of the most demanding natural products consumers.

Keywords: Beverages, antioxidants, shelf-life, green tea

1. Introduction

In recent years, the consumption of mixed beverages (juices, smoothies) based on fruits and vegetables (F&V) has increased significantly, making this sector of the food industry one of the highest growth worldwide (Grand View Research, 2018; Morales-de la Peña Welti-chanes, & Martín-belloso, 2016). Indeed, awareness of consumers for healthier foods and the fact that mixed beverages combine nutrients and bioactive compounds from different F&V with significant health-related benefits (Nunes et al., 2016; Formica-Oliveira et al., 2017) along with their attractive sensory properties and the fact that they are ready to drink are the main reasons for their success (Bevilacqua et al., 2018).

However, “natural” (untreated) beverages have a short shelf-life, mainly due to spoilage associated with microbial growth (Bevilacqua et al., 2018). Although they are usually highly acidic products (pH <4.6), some acid-tolerant microorganisms can survive and grow (Gram et al., 2002). Moreover, there has been an increased incidence of foodborne disease outbreaks associated with the consumption of F&V beverages, mainly caused by *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella*, especially recurrent in untreated juices (Callejón et al. 2015). Traditional heat treatments, applied to achieve preservation and safety on these products, lead to chemical and physical changes that affect sensory properties and reduce nutrients and bioactives contents or bioavailability, modifying their natural attributes and their so wished benefits (Bevilacqua et al., 2018; Morales-de la Peña et al., 2016). Hence, they are not an option for this product and the targeted consumer niche.

A practical alternative highly in line with consumers´ requirements, is the use of natural antimicrobials. They are easy to incorporate into production lines, not requiring large investments in equipment for their implementation. Moreover incorporation of these compounds to the smoothie would not represent a significant increase in the cost of the product since the amounts to be incorporated are usually very low. Additionally, the combination of antimicrobials with different mechanisms of action is recommended to increase inhibition or inactivation of targeted microorganisms by a “hurdle technology” approach (Khan, Tango, Miskeen, Lee, & Oh, 2017), reducing the effective doses, and consequently treatment cost. In this sense, nisin, a bacteriocin produced by *Lactococcus lactis* is a Generally Recognized as Safe (GRAS) compound (FDA, 1988), highly effective in the inactivation of a wide range of Gram-positive bacteria and spores resistant to high temperatures. Natamycin (pyramycin) is an antifungal produced by *Streptomyces natalensis* (Delves-Broughton & Weber, 2011) designed as a natural preservative by the European Union (EEC N° 235). Citric acid, an organic acid naturally present in F&V or synthesized by microorganisms, has demonstrated antimicrobial activity against a wide spectrum of bacteria, such as *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*, among others (Kim & Rhee, 2015). Lastly, green tea extracts (GTE) have demonstrated antibacterial, antiviral, antifungal and antioxidant activity and promote numerous health benefits, particularly the prevention of various types of cancer and cardiovascular diseases (Perumalla & Hettiarachchy, 2011). Since mixed F&V smoothies have gained strength in the market very recently, as part of the trend towards healthy eating habits, the effect of preservation treatments on this type of matrix, more complex than juices, remains largely unexplored and is an issue that needs to be properly addressed. Considering the above, the objective of this study was to select an adequate combination of natural antimicrobials (nisin, natamycin, GTE and citric acid) to preserve the microbiological and nutritional ~~and sensory~~ quality of a mixed F&V smoothie, extending its shelf-life and ensuring its safety.

2. Materials and Methods

2.1. Smoothie preparation

The technological scheme used for the smoothie preparation is presented in Figure 1. The raw material was purchased in a local market in Buenos Aires, Argentina. Once in the laboratory, the selected raw material was washed and disinfected by immersion in chlorinated water (200 mg/kg) for 5 min and dried. The composition (by weight) of the ingredients was: orange juice 59%, apples 15%, carrots 15%, beet greens 6% and beet stems 5%. Orange juice was extracted using a home squeezer (Oster, USA), carrots and apples were peeled and chopped into small pieces. Then all ingredients were mixed in a homogenizer (JTC OmniBlend, Guangdong, China) for 60 s. For packaging, polyethylene terephthalate flasks (33mL) were used.

2.2. Antimicrobial preparation

Commercial natural antimicrobials were used: nisin (Nisin®, DSM), natamycin (Delvocid® Salt, DSM), citric acid (Anedra, Research Ag., Argentina) and green tea extract (GTE) powder from Taiyo International, Inc. (Minneapolis, Minnesota) containing >90% total polyphenols, >80% total catechins, >40% Epigallocatechin gallate (EGCG) and <1% caffeine. Concentrated antimicrobial solutions were prepared immediately before use and dosed according to the desired concentration in the product.

2.3. Impact of antimicrobial treatments on native microflora

In a first assay, samples were prepared as previously detailed ~~on 2.1~~ and before closing each flask, the antimicrobials were applied in the corresponding doses (Table 1), and the flask was then shaken vigorously. Treatment with hydrochloric acid (Anedra, Research Ag., Argentina) was carried out to estimate the antimicrobial effect of pH reduction alone, being a control of citric acid treatment ~~to demonstrate the antimicrobial activity of citric acid beyond its acidifying effect~~. This is very common practice when antimicrobial effects of organic acids are studied (Buchanan et al., 1993; Eswaranandam et al., 2004; Lehrke et al. 2011). This is because while hydrochloric acid is completely dissociated into protons (H+) and anions, the mechanism of action of organic acids is based on their capacity to reduce the pH of the medium and the ability of the undissociated forms to penetrate through the cell membranes. Sterile water in similar amounts than used for samples with antimicrobial treatments was dispensed into control (untreated) samples. Treatments doses were selected taking into account the results of preliminary studies, bibliographical references, and the limits established by Argentinean legislation (CAA, 1996). The samples were stored at 5±1 °C and periodically (0, 3, 7, 14, 21, 28 d) triplicates of each treatment were taken for analysis. Mesophilic aerobic bacteria (MAB), Enterobacteriaceae (EB) and molds and yeasts (M&Y) counts were determined according to the described by Fernandez et al. (2018b). The detection limit (DL) of the method was 2.00 log CFU/mL , and the end of microbiological shelf-life was settled when 6.0 log CFU/mL for MAB or M&Y were achieved (Fernandez, Denoya, Jagus, Vaudagna, & Agüero, 2019b).

2.4. Effectiveness of treatments against *Listeria innocua* and *Escherichia coli*

In a second assay, samples prepared as previously described ~~in 2.1~~, were inoculated with a mixed culture of *Listeria innocua* (CIP 80.11 and ATTC 33090) and *E. coli* (ATCC 3526 and ATCC 8739), prepared as described by Fernandez, Denoya, Agüero, Vaudagna, & Jagus (2019a), to achieve an initial bacterial count of ~10^6 CFU/mL , simulating contamination during the process. The selected strains are commonly used as *L. monocytogenes* and *E. coli* 0157: H7 surrogates, respectively, since they have shown similar behavior and resistance (Evrendilek, Zhang, & Richter, 1999; Omac, Moreira, Castillo, & Castell-Perez, 2015). Inoculation was conducted in each sample before the incorporation of natural antimicrobials, and then each flask was shaken vigorously, closed and stored at 5±1 °C. Treatments were those selected in the previous study based on the improvements achieved in shelf-life. Periodically, *Listeria* spp. and *E. coli* counts were determined according to the described by Fernandez et al. (2019a). Results were expressed as log CFU/mL and DL was 2.00 log CFU/mL .

2.5. Physical-chemical and nutritional ~~and sensory~~ quality of treated smoothies

In a third assay, samples prepared as previously described ~~in 2.1~~ and treated with the antimicrobials selected in the previous stages, were stored at 5±1 °C and analyzed each sampling day, for the following quality indicators:

2.5.1. ***Total soluble solids (TSS) and pH:*** The TSS were determined with a Milwaukee MA871 Refractometer (Milwaukee Instrument, Rocky Mount, USA) and the results were expressed as ~~°Brix~~ the percentage of soluble solids on the solution (%); the pH was measured with a digital pH-meter (Hanna, HI99163, Romania, with FC232D electrode, Italy).

2.5.2. ***Total phenolic content (TPC) and antioxidant capacity:*** The extraction and determination of total phenolic compounds by Folin-Ciocalteau methodology, and of antioxidant capacity by FRAP and DPPH assays, were carried out according to Fernandez, Denoya, Agüero, Jagus, & Vaudagna (2018a) with modifications, as informed in the supplementary material section S.2.5.a. TPC results were expressed on milligrams of gallic acid equivalents per kilogram of smoothie (mg GAE/kg), FRAP and DPPH results were expressed on trolox equivalent antioxidant capacity per kilogram of smoothie (TEAC/kg).

2.5.3. ***Betaxanthins and betacyanins:*** Their determination was carried out according to the method described by Fernandez et al. (2018a) with some modifications, as described in supplementary material section S.2.5.b. The results were expressed as milligrams of Bx or Bc per liter of fresh smoothie.

~~***Sensory quality:*** The samples were subjected to sensory quality evaluation by eight trained panelists, following the methodology described by Tomadoni, Cassani, Ponce, Moreira, & Agüero (2016). The attributes evaluated were color, aroma, texture, flavor and overall liking using a descriptive scale of 1-9, where 9: like extremely 5: neither like nor dislike, the limit of acceptance from the consumers' point of view; 1: dislike extremely.~~

2.6. Statistical analysis

Results were analyzed using Origin®8 statistical software (OriginLab®, USA). Two-way analysis of variance (ANOVA) was performed using as sources of variation: TREAT (treatment according to Table 1), TIME (storage time, day of sampling) and TREAT-TIME interaction. Differences were determined using the Tukey test ($p < 0.05$).

3. Results and discussion

3.1. Impact of antimicrobial treatments on native microflora

The most relevant results regarding changes in native microflora on samples of smoothies with different treatments during storage at 5±1 ºC are presented in Figure 2. Additionally, complete results are shown in the supplementary material section (Table S1). In regards to MAB counts, only samples containing citric acid showed significant differences from control (C) at day 0, with reductions between 1-1.65 log CFU/mL, with treatment containing combined green tea extract, nisin, natamicyn and citric acid (TNiNaCi) presenting the best initial results. In the case of C samples, they remained at values between 5.3-5.6 during the first wk of storage, then increased exceeding the limit of 6 log CFU/mL (Fernandez et al., 2019a; Formica-Oliveira et al., 2017) on day 14 of refrigerated storage. Samples acidified with hydrochloric acid (CH) as well as those treated individually with nisin (Ni), natamicyn (Na) and green tea extract (T), exceeded the limit after 21 d. On the other hand, treatment containing citric acid (Ci), green tea extract combined with citric acid (TCi), treatment containing combined nisin, natamicyn and citric acid (NiNaCi) and TNiNaCi treatments kept the BAM counts below the limit during the 28 days of storage.

Regarding EB counts, only samples T, TCi, NiNaCi and TNiNaCi showed significant initial reductions, between 0.64-1.23 log CFU/mL from C values. Although there is no limit value established for EB in this type of product, they are a key indicator of safety and quality, mostly related to agricultural practices and the efficiency of sanitation procedures (Tortorello, 2003). C samples remained in values among 5-6 log CFU/mL during all storage, probably due to the low pH of smoothies that impeded their development. CH showed similar behavior to control during the first days of storage, then a reduction was observed until values around 3 log CFU/mL, remaining on those values from day 7 onwards. Ni, Na and T treatments presented reductions between 1-2 log during the first wk of storage, then Ni and Na showed an increase presenting similar values to control from day 14 onwards, while T maintained lower values (1-2 log lower than C) until the end of storage. Treatments containing citric acid presented remarkable results, while Ci samples exhibited a sustained decrease of EB values with time, from around 4.5 at day 0 to 2.5 log CFU/mL at the end of storage, TCi showed counts below 3 log CFU/mL from day 3 onwards and both NiNaCi and TNiNaCi below DL from day 3 onwards.

In the case of M&Y, all treatments except CH presented significant reductions from C at day 0, highlighting Na and combined treatments TCi, NiNaCi and TNiNaCi that showed reductions of more than 1.67 log (counts under DL). None of the treatments exceeded the limit (6 log CFU/mL) during storage. Control samples showed a slow but constant increase from values around 3.7 log CFU/mL at day 0 to 5.6 log CFU/mL at day 28. Samples CH presented similar behavior, showing slightly lower counts than control throughout storage. Ni and T treatments exhibited similar behavior among them, with values between 1-1.5 log beneath control during the whole storage. Among individual treatments Na, as expected, showed the better performance exhibiting counts under 3 log CFU/mL during all storage. Regarding samples containing citric acid, Ci exhibited similar behavior than C with slightly lower counts, while TCi showed counts 1-2 log below control throughout storage. Remarkable results were observed in samples NiNaCi and TNiNaCi with values close or under DL during the whole storage.

These results showed that four of the proposed treatments extended the microbial shelf-life for at least two additional wk compared to the untreated samples. Therefore, these samples were selected for the next stage. Moreover, the antimicrobial effect of citric acid, beyond the effect generated by acidulation was probed since significant differences were observed between treatment CH and Ci. As previously mentioned, while CH is completely dissociated into protons (H+) and anions, and cell membrane has a very low permeability to protons, the undissociated forms of Ci penetrate through the cell membranes. Once inside, the higher intracellular pH produces the dissociation of these molecules, releasing protons and acidifying the cytoplasm. This affects normal activity of the cell since proton gradient and transport systems are affected. This is why weak organic acids such as acetic, lactic, citric, and malic acids have a better antimicrobial action than strong inorganic acids such as hydrochloric acids, at the same external pH (Lehrke et al, 2011;Tewari & Juneja, 2012). Additionally, while with antimicrobials applied individually the reductions achieved were low, the combined treatments showed significant reductions, many times to levels below DL, demonstrating that microbial control can be achieved by the combination of different antimicrobials.

3.2. Effectiveness of treatments against *Listeria innocua* and *Escherichia coli*

Changes in *E. coli* and *L. innocua* counts in control samples and treated with the treatments selected in the previous study during storage at 5±1 ºC are presented in Figure 3.

Regarding *E. coli*, control samples presented an initial count of 6.29±0.17 log CFU/mL and remain in counts between 6 and 5 log CFU/mL throughout storage. Indeed, their development was not expected since the minimum temperature for their growth is 7 ºC and the minimum pH ~~for their growth~~ is around 4.4 (Adams & Moss, 2008). However, it is well known that some strains of this microorganism can be acid-resistant, surviving for long periods in acidic foods (Fernandez et al., 2019a; Foster, 2004) as in this study. In fact, Foster (2004) highlight the ability of *E. coli* to survive in environments that are more suited to acidophiles than enterics, ensuring that *E. coli* cells have at least three systems that can use to survive acid stress and that both pathogenic and non-pathogenic strains have equally remarkable levels of acid resistance. The most effective treatments for *E. coli* control were those containing GTE (TCi and TNiNaCi), with no significant differences between them. Their effect was not immediate and only reductions < 1 log were observed at day 0, then further inactivation was showed with time, reaching in both cases values below DL at day 21 of refrigerated storage. The samples corresponding to Ci and NiNaCi treatments presented similar counts to C during the first 14 days of storage, then decreasing significantly, to values 2-3 log below C from day 21 onwards. ~~Regarding these results,~~ Although as expected neither nisin nor natamycin affected Gram-negative microorganisms, both citric acid and GTE played a role in the inactivation of *E. coli*, which is consistent with the observed by other authors (Fernandez, Jagus & Agüero, 2018b; Kim & Rhee, 2015; Perumalla & Hettiarachchy, 2011). Moreover, it must be considered that real contaminations are usually much lower than the one simulated here, and the effect of the antimicrobial treatments is usually greater in that case. Hence, probably better performance of the treatments can be expected in a real contamination scenario.

Concerning *L. innocua*, C smoothies presented an initial count of 6.42±0.31 log CFU/mL showing a reduction over time to values below DL (at day 28). This behavior of *L. innocua* was previously observed in this matrix (Fernandez et al., 2019a), and it was attributed to the low pH of the smoothie which is below the reported minimum necessary for their growth (4.5). Moreover, since in this case storage temperature was higher than the minimum for their growth (-1 °C) (Adams & Moss, 2008), a stronger pH effect is evidenced. Nevertheless, it is well known that 100 bacterial cells of *L. monocytogenes* are sufficient to develop listeriosis (McLaughlin, Mitchell, Sinerdon, & Jewell, 2004). Hence, it should be noted that starting from high levels of contamination, in the C smoothie *Listeria* persists at high-risk values for at least two wk at 5±1 ºC, evidencing the importance of applying a treatment for *Listeria* control. The treatments Ci and TCi presented initial reductions of 0.4 and 0.9 log, respectively, then counts were reduced with time, showing significant differences with control until day 21 when all treatments (including C) presented values close to DL. The NiNaCi and TNiNaCi treatments were highly effective for *L. innocua* control since reduced their counts to values below DL from day 0 until the end of storage.

Considering their potential to control interest microorganisms, especially by their performance against *Listeria innocua,* treatments NiNaCi and TNiNaCi were selected for the next stages of the study. Moreover, in the following assay also C and Ci treatments were followed as controls.

3.3. Physical-chemical and nutritional ~~and~~ ~~sensory~~ quality of treated smoothies

3.3.1. Total soluble solids and pH

In the case of control samples, the initial pH was 3.93±0.02, while the ones containing citric acid (Ci, NiNaCi, TNiNaCi) presented values of 3.54±0.04. No variations were observed in the pH of C samples during storage, while samples containing citric acid presented variations between values 3.50-3.64, although statistical analysis established as significant, these variations have little practical relevance. Accordingly, the stability of the pH of this smoothie has already been observed in previous works (Fernandez, Bengardino, Jagus, & Agüero, 2020; Fernandez et al., 2019a).

Concerning TSS, samples without GTE (C, Ci and NiNaCi) presented an average value of 10.60±0.15 ~~ºBrix~~, while samples containing GTE (TNiNaCi) stood out with a value of 11.33±0.12 ~~ºBrix~~. During storage, C and Ci samples showed a slight reduction, probably due to microbial metabolic activities resulting in the conversion of the sugars naturally present in the samples (Kaddumukasa, Imathiu, Mathara, & Nakavuma, 2017). On the other hand, NiNaCi and TNiNaCi kept the soluble solids values stable over time, which is consistent with the enhanced microbial control. Moreover, the increased initial TSS value in TNiNaCi samples could be related to the fact that the main components of GTE are water-soluble compounds (Perumalla & Hettiarachchy, 2011). Additionally, GTE contains ~~epigallocatechin gallate (~~EGCG~~)~~ which is a natural inhibitor of PME (Lewis et al., 2008). PME activity generates the crosslinking of free carboxylic groups belonging to pectin chains giving insoluble macropolymers that entrap other components of the cloud, including soluble solids and other compounds related to flavor, texture and color of juices

(Carbonell, Contreras, Carbonell, & Navarro, 2006). Hence, PME inactivation by GTE could also explain the higher ~~ºBrix~~ TSS values and the better stability ~~of TSS~~ on TNiNaCi samples.

3.3.2. Total phenolic content (TPC) and antioxidant capacity

Changes in nutritional indicators during refrigerated storage of the smoothies with different treatments are presented in Table 2. For TPC treatments C, Ci and NiNaCi presented at day 0 values between 534.1 and 613.6 mg GAE/kg without significant differences between them. During storage of these samples, significant decreases were observed showing, on day 21, reductions between 35-40% of initial values. This indicates that treatments Ci and NiNaCi did not affect smoothies´ TPC and stability. The treatment containing GTE presented significant differences from the rest, showing at day 0 TPC of about 9 times higher. This result is attributed to the high phenolics compounds content of GTE (Perumalla & Hettiarachchy, 2011). Additionally, this treatment showed a more stable behavior over time, with a reduction of only 17% on day 28. Hence, the addition of GTE not only fortified the product in its phenolic content but, furthermore, these added polyphenols were more stable during storage than the native polyphenols of the product. Certainly, it is well known that tea catechins in aqueous solutions are very stable during storage when pH is below 4 and refrigeration temperatures are employed (Ananingsih Sharma, & Zhou, 2013).

In regards to antioxidant capacity, results were as expected considering that phenolic compounds present in GTE have demonstrated great antioxidant properties due to their redox potential; that enables them to act in various forms such as hydrogen donors, reducing agents, nascent oxygen quenchers, and/chelating metal ions (Perumalla & Hettiarachchy, 2011). For DPPH antiradical activity, the treatment containing GTE showed significantly higher DPPH values (around 13 times) than the rest of the treatments. For all treatments, significant reductions were observed over time. On day 21 reductions on DPPH values were 54, 48, 45 y 22% for C, Ci, NiNaCi, and TNiNaCi, respectively. Concerning FRAP**,** treatment with GTE also stood out, with values of around 35 times higher than C. Although in all samples decreases in FRAP values were observed over time (about 25% of the initial value at day 21), these were not statistically significant. Differences observed between FRAP and DPPH results, as observed in many other studies (Fernandez et al., 2020; Nunes et al., 2016), may be related to the fact that they are indicators of different antioxidant mechanisms that depend on different compounds and the interaction among them (Huang, Ou, & Prior, 2005). Undoubtedly, tea catechins have an important role in both of them. Indeed, results are a clear indicator of the antioxidant benefit of fortifying the product with GTE, as observed by many other authors (Fernandez et al. 2018b; Jeong et al. 2018; Pourashouri, Shabanpour, Kordjazi, & Jamshidi, 2020; Tappi et al., 2017). Moreover, if the total antioxidant capacity (DPPH + FRAP) of the product is settled as a biomarker for shelf-life, and the usual limit of a 50% loss is considered (Fernandez et al., 2019b) all treatments, including control, met this criterion during storage at 5±1 ºC.

3.3.3. Betaxanthins and betacyanins:

Changes in **betacyanins (Bc) and betaxanthins (Bx)** contents on smoothies with different treatments are presented in **Table 2**. C samples presented at day 0 values of 11.50±0.50 and 6.08±0.54 mg/L of Bc and Bx, respectively, and significant reductions were observed during storage. Ci samples presented similar behavior to the control. NiNaCi treatment showed a higher initial value (6%) of Bc, and values of Bx similar to C, while changes during storage were also similar to C. The sample containing GTE presented higher initial values (17.7%) of Bc and registered much higher Bc retention over time (~65% vs ~14% at day 21) than the other treatments. Similar results were observed for Bx, which although initially presented similar values to control, towards the end of storage showed significantly higher retentions (~71% vs ~45% at day 21). Concerning these results, antimicrobial agents act as protective agents in the prevention of oxidative processes that leads to betalains deterioration. Degradation of betalains is usually related to the activities of glycosidases, polyphenoloxidases (PPO) and peroxidases (Strack, Vogt, & Schliemann, 2003), all oxygen-dependent actions. In this sense, GTE have shown inhibitory effects on glycosidases and PPO (Chen, Qu, Fu, Dong, & Zhang, 2009; Klimczak & Gliszczynska-Swigło, 2017). This could explain why in samples containing GTE the initial value of Bc was higher as well as the better retention with time observed for both compounds (Bc and Bx). On the other hand, differences in the behavior of Bc and Bx are common because of their different chemical structure. In this way, Bx was found to be more prone to oxidation and less stable than Bc at acidic pH (Herbach, Stintzing, & Carle, 2006). In any case, the results of this study demonstrate the protective effect of green tea on these compounds during refrigerated storage.

~~3.3.4. Sensory quality~~

~~Changes over time on the sensory indicators of smoothies treated with antimicrobials and stored at 5±1ºC are presented in **Table 3**. For **aroma and texture** parameter, no changes were recorded either by the addition of antimicrobials or during storage. Regarding **color**, it was not affected by treatments although there were changes during storage, related to advancement in shelf-life, consistent with the natural degradation of the compounds that generate this attribute. Although there were no significant differences between treatments for the previously mentioned parameters, samples C and Ci presented averages scores equal to or below the acceptable limit for color and/or aroma at day 21 of storage. NiNaCi and TNiNaCi presented higher average scores, probably due to the effect of antimicrobials on the native microflora, which inhibits the development of deterioration processes associated with their activity and/or oxidative processes that can affect these attributes. In the case of **flavor**, samples containing 1% of GTE presented a taste described as astringent, bitter, showing scores lower than the limit from day 0. On day 3 results were similar, hence, it was decided to discard this treatment and was not further rehearsed. **The overall liking** parameter is an indicator~~

~~that integrates different sensory aspects and the observed results, as expected, reflect the observations made for the other analyzed sensory parameters. Interestingly, treatment NiNaCi presented good sensory attributes until day 28 when a score of 5 on overall liking determined the end of their sensory shelf-life.~~

3.4. Final considerations

Considering the results of the three trials, it was concluded that the best treatments from a ~~both~~ microbiological ~~and sensory~~ point~~s~~ of view were NiNaCi and TNiNaCi, ~~From sensory quality analysis, it was evident that the GTE dose used in this study, which was selected based on previous studies to achieve a microbiological effect, was not sensory viable. Additionally, there were~~ with no major microbiological differences between them ~~NiNaCi and TNiNaCi treatments~~. Thus, the use of GTE as an antimicrobial in this product was no longer considered ~~was dismissed. Considering~~. Taking into account that the objective of this study was the integral quality improvement of the product, and to take advantage of the nutritional benefits of green tea, a final trial was carried out to determine if significant fortification of the product could be achieved by using lower doses of GTE. When half of original dose was tested (0.5% GTE) values 6.2, 12.7 and 26 times higher than the control were observed for TPC, DPPH and FRAP. While, when only 20% of the original dose was considered (0.2% GTE) values 4.2, 10.3 and 10.7 times higher than the control were observed for TPC, DPPH and FRAP. These results indicate that the use of very small doses of GTE, in spite of not showing antimicrobial effects, leads anyway to significant increases in the polyphenol content and antioxidant activity of the samples, fortifying the product. Moreover, to reduce the used dose has the advantage of diminish possible sensory impacts associated with the bitter taste of green tea, as well as minimizing costs of treatment. ~~–the maximum dose of GTE that allows obtaining a sensory acceptable smoothie and evaluate the nutritional benefits of the addition of GTE in that dose. Six doses were considered (0.1, 0.2, 0.25, 0.3, 0.4, 0.5%) and results indicated that while from 0.3% onwards the flavor became unacceptable, at 0.25% the~~

sensory acceptability was good (6.3±1.0) and at 0.2% very good (7.7±0.6). The samples containing 0.20% of GTE presented TPC values 4.2 times higher than the control and those containing 0.25% 5.1 times higher than the control. Likewise, important increases in antioxidant capacity of 10.7 and 14.3, respectively, were observed for FRAP and 10.3 and 11.6, respectively, for DPPH.

Conclusions

The results obtained in this study suggest that the treatment of fruit and vegetable smoothies with nisin 500 UI/mL, natamycin 200 mg/kg and citric acid (up to pH 3.5) will lead to obtaining a stable product from a microbiological point of view and sensory of great nutritional quality, that can to be stored for 28 days at 5±1 ºC. These results are of great commercial relevance since this treatment increases its shelf-life by 14 days compared to an untreated smoothie. Moreover, this treatment would allow controlling a 6 log CFU/mL *L. monocytogenes* contamination. Furthermore, if GTE (0.2%) is added to that combination, a product with fortified antioxidant properties (more than 10 times higher than control) is achieved, fulfilling the requirements of the most demanding natural products consumers.

Ethics declaration

The authors confirm that they have no conflicts of interest concerning the work described in this manuscript.

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Table 1- Treatments schedule

Treatment	Compound and dose				
	Nisin	Natamicyn	GTE	Citric acid	Hydrochloric acid
	12.5 mg/kg	200 mg/kg	1% ~~v/v~~	to pH 3,5	to pH 3,5
C					
Ci				x	
CH					x
Ni	x				
Na		x			
T			x		
TCi			x	x	
NiNaCi	x	x		x	
TNiNaCi	x	x	x	x	

Table 2- Changes on nutritional indicators in smoothies with different treatments during storage at 5 ± 1ºC.

Nutricional indicator	Sample	Day 0	3	7	14	21	28
	C	534.1±21.9^{a,A}	398.1±38.0^{a,B}	389.0±33.6^{a,B,C}	291.1±43.7^{a,C}	351.2±43.7^{a,B,C}	*
	Ci	613.6±6.7^{a,A}	358.2±45.7^{a,B}	378.1±9.6^{a,B}	326.4±23.3^{a,B}	366.2±19.5^{a,B}	*
TPC	NiNaCi	564.3±34.7^{a,A}	420.6±22.6^{a,B}	320.0±9.6^{a,B}	335.7±69.3^{a,B}	333.2±20.1^{a,B}	328.3±20.9^{a,B}
mg GAE/kg	TNiNaCi	5246±460^{b,A}	5317±128^{b,A}	4675±224^{b,A}	4845±582^{b,A}	4983±328^{b,A}	4334±553^{b,A}
	C	2447.0±313.4^{a,A}	1744.7±96.2^{a,B,C}	1808.7±133.1^{a,B}	1193.4±326.9^{a,C,D}	1124.4±86.8^{a,D}	*
	Ci	2434.4±322.5^{a,A}	1605.0±163.4^{a,B}	1656.6±41.4^{a,B}	1254.0±378.4^{a,B}	1263.5±42.1^{a,B}	*
DPPH	NiNaCi	2338.5±126.8^{a,A}	1803.9±179.9^{a,B}	1665.2±159.2^{a,B,C}	1532.9±212.8^{a,B,C}	1271.1±47.0^{a,C}	721.3±225.8^{a,D}
TEAC/kg	TNiNaCi	32145±3371^{b,A}	35045±872^{b,A}	29488±951^{b,A,C}	22439±1848^{b,B}	25045±249^{b,B,C}	22748±2697^{b,C}
	C	2100.3±160.0^{a,A}	2116.6±79.2^{a,A}	2263.4±263.6^{a,A}	1657.0±139.7^{a,A}	1383.7±246.7^{a,A}	*
	Ci	2028.2±260.2^{a,A}	1726.3±95.3^{a,A}	1882.7±264.8^{a,A}	1618.9±178.1^{a,A}	1652.9±225.3^{a,A}	*
FRAP	NiNaCi	2075.8±175.8^{a,A}	1927.6±311.1^{a,A}	1682.8±315.0^{a,A}	1383.7±246.7^{a,A}	1345.6±328.2^{a,A}	1353.8±324.9^{a,A}
TEAC/kg	TNiNaCi	71467±14828^{b,A}	66487±9105^{b,A}	64211±2315^{b,A}	52522±4182^{b,A}	50577±7221^{b,A}	53133±10615^{b,A}
	C	11.50±0.50^{a,A}	8.89±1.81^{a,B}	6.71±1.22^{a,C}	2.10±0.24^{a,D}	1.75±0.10^{a,D}	*
	Ci	11.21±0.26^{a,b,A}	6.60±0.97^{a,B}	5.06±0.94^{a,C}	1.96±0.10^{a,D}	1.60±0.10^{a,D}	*
	NiNaCi	12.18±0.47^{b,A}	7.22±1.23^{a,B}	3.35±0.20^{a,C}	1.93±0.20^{a,D}	1.44±0.14^{a,D}	1.18±0.10^{a,D}
Bc mg/L	TNiNaCi	13.54±0.32^{c,A}	12.21±0.34^{b,B}	11.38±0.61^{b,B,C}	10.85±0.44^{b,C}	8.87±0.58^{b,D}	9.14±0.49^{b,D}
	C	6.08±0.54^{a,A}	5.19±0.53^{a,B}	4.86±0.53^{a,B}	2.76±0.14^{a,C}	2.70±0.10^{a,C}	*
	Ci	5.80±0.21^{a,A}	5.86±0.94^{a,A}	4.45±0.38^{a,b,A,B}	3.20±0.22^{a,b,B}	2.73±0.18^{a,B}	*
	NiNaCi	5.91±0.20^{a,A}	4.92±0.19^{a,b,B}	4.12±0.16^{b,C}	3.30±0.29^{b,D}	2.61±0.17^{a,E}	2.26±0.13^{a,F}
Bx mg/L	TNiNaCi	5.83±0.18^{a,A}	4.87±0.07^{b,B}	3.91±0.17^{b,B}	4.23±0.50^{c,B}	4.30±0.28^{b,C}	4.14±0.34^{b,B}

* Samples C and Ci were followed only until day 21 since visible molds were observed in some samples.

Different lower case letters indicate significant differences between treatments (comparison within each column) Different capital letters mean differences over time (comparison within each row).

TPC: Total phenolic content; DPPH: radical scavenning activity; FRAP: ferric reducing activity; Bc: Betacyanin content; Bx: Betaxanthins content.

Figure 1- Technological scheme used for the smoothie and samples preparation

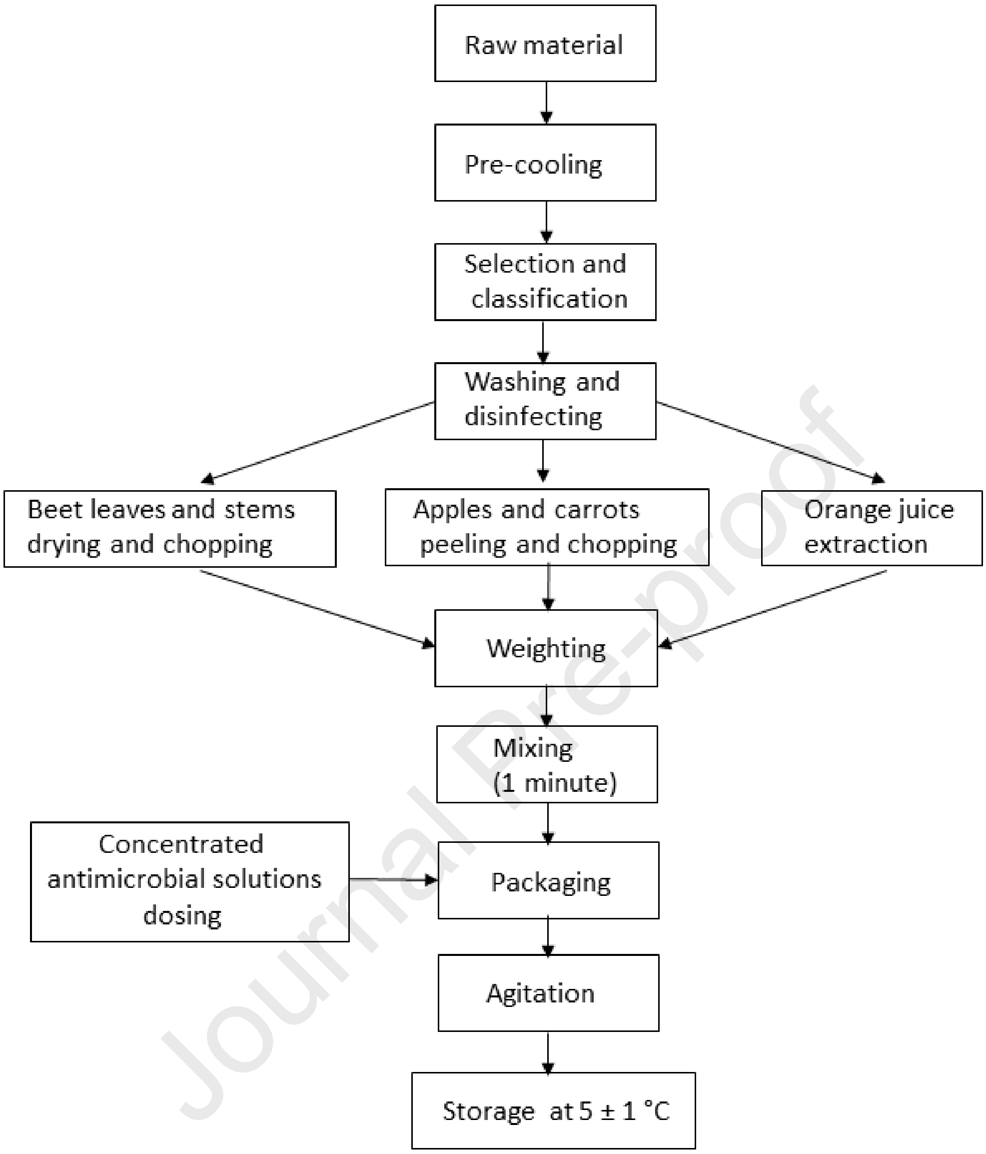


Figure 2- Counts of mesophilic aerobic barcteria (a), enterobacteriae (b) and molds and yeast (c) in smoothie samples with different treatments (–●–:control; -■-:Ci; -▲-:TeCi; -▼-: NiNaCi; -◆-: TNiNaCi) during storage at 5 ± 1 °C.

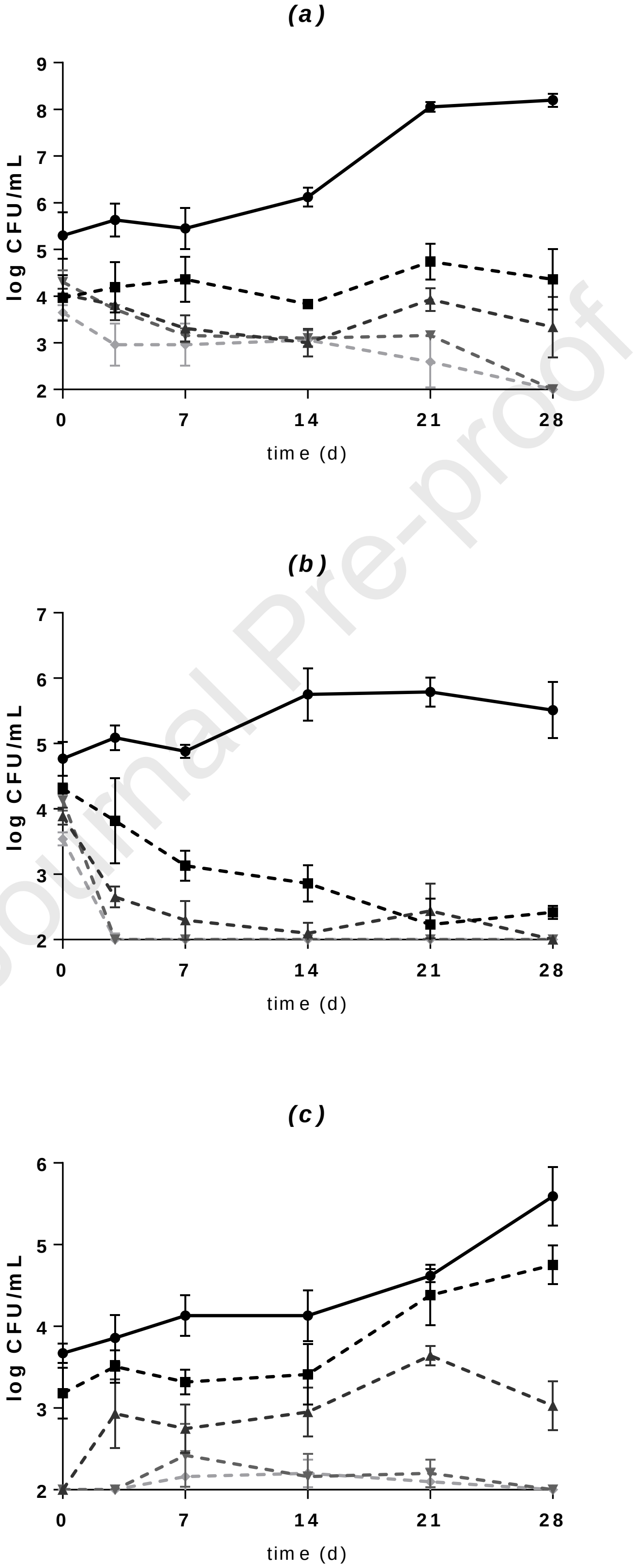
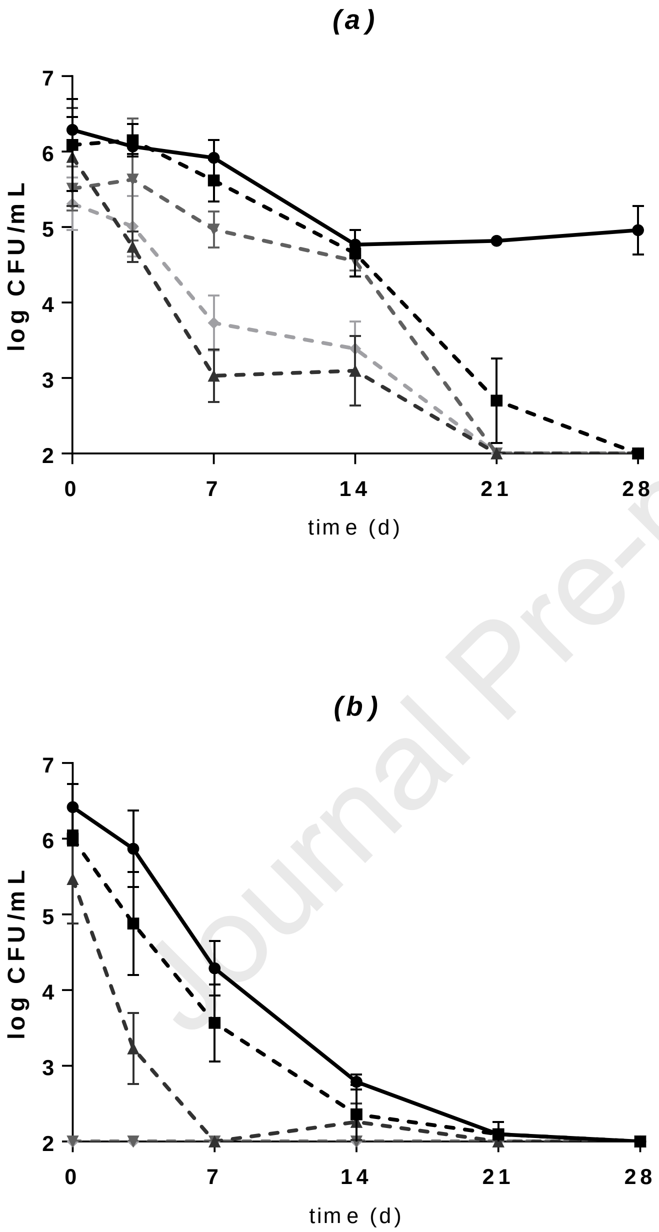


Figure 3- Counts of *E. coli* (A) and *L. innocua* (B) in smoothie samples with different treatments (-•-:control; -■-:Ci; -▲-:TeCi; -▼-: NiNaCi; -◆-: TNiNaCi) during storage at 5 ± 1 °C.



Highlights

- Nisin, natamycin & citric acid combined treatment extend product´s shelf-life 14 days
- Selected treatment preserve smoothie´s integral quality during 28 days at 5 ºC
- Selected treatment allow to control a 6 log CFU/mL *L. monocytogenes* contamination
- With green tea (0.2%) addition, antioxidant content are 10 times higher than control