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Combined use of natural antimicrobial based nanoemulsions and ultra high pressure homogenization to increase safety and shelf-life of apple juice

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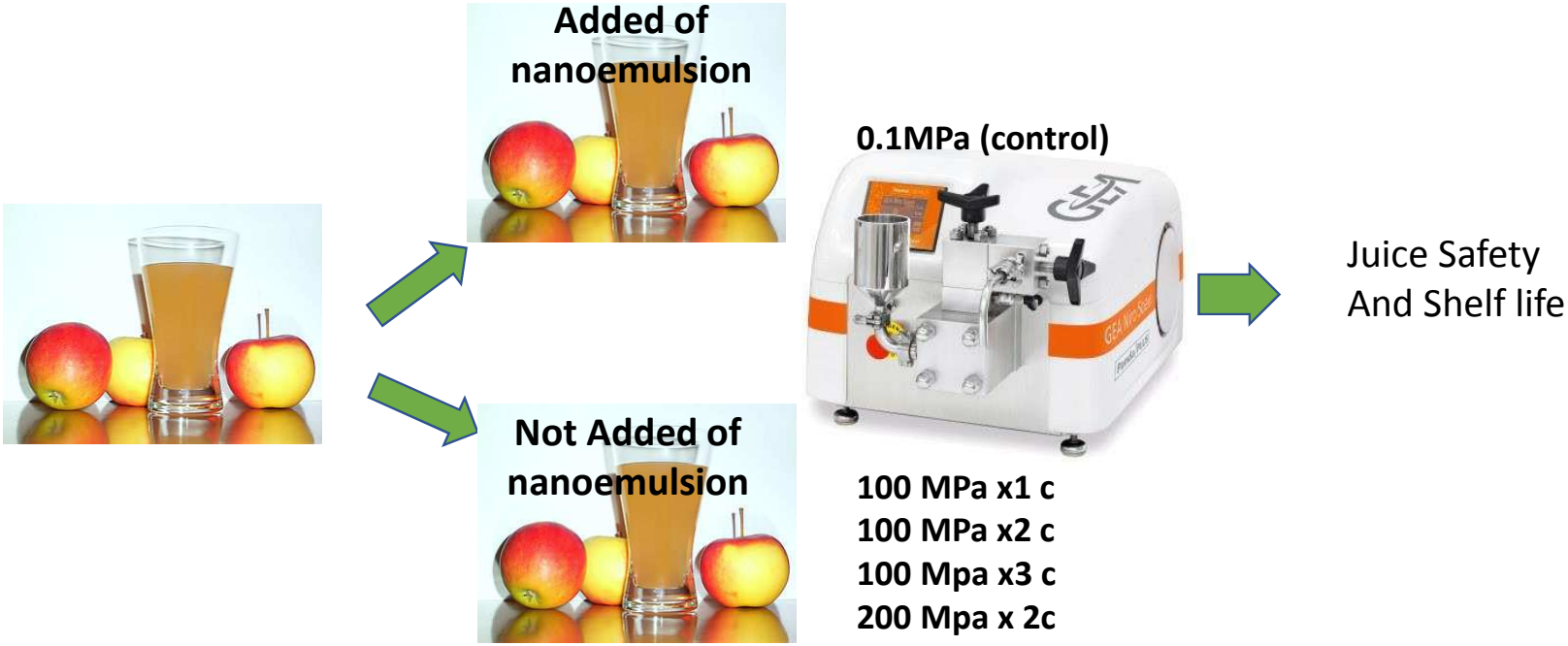
Added of nanoemulsion
Not Added of nanoemulsion
0.1MPa (control)
100 MPa x1 c
100 MPa x2 c
100 Mpa x3 c
200 Mpa x 2c
Juice Safety
And Shelf life

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Combined use of natural antimicrobial based nanoemulsions and ultra high pressure homogenization to increase safety and shelf-life of apple juice

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Abstract

The present research was aimed to investigate the potentialities of ultra high pressure homogenization (UHPH) to produce stable natural antimicrobial based nanoemulsions. Initially, the nanoemulsions were characterized for their size, stability over time and antimicrobial properties against several pathogenic and spoilage microorganisms. After that nanoemulsions were tested to increase the safety and shelf-life of apple juice deliberately inoculated with pathogenic (*Listeria monocytogenes Scott A*, *Staph. aureus* SR231, *Escherichia coli* 555) and spoilage microorganisms (*Saccharomyces cerevisiae SPA*, *Lactobacillus plantarum* 82) and treated with different high pressure homogenization treatments. The analyses performed by dynamic laser light scattering showed that the hexanal and trans-2-hexenal based nanoemulsions were characterized by an average size of 86 and 100 nm, respectively while they were characterized by a stability, over time, of 14 months without separation. Moreover, the nanoemulsions resulted, after the UHPH treatment, colourless. The pathogenic species deliberately inoculated in apple juice decreased their cell loads with different kinetics in relation to the use of hexanal and trans-2-hexenal and high pressure homogenization treatment applied. Regarding spoilage microorganisms, *S. cerevisiae* cell loads decreased under detection limit (1 log CFU/mL) in juice containing nanoemulsions and treated at 200MPa for 2 cycles. The data of the present research contribute to support the application of natural antimicrobial based nanoemulsions into complex food system, enlarging the experimental evidence for their use in food sector. In particular, the obtained hexanal and tran-2-hexenal based nanoemulsions have demonstrated great application, due also to their organoleptic compatibility, to the fruit juice sector, promoting also an increase of quality of apple juice.

Keywords: nanoemulsion, natural antimicrobials, ultra high pressure homogenization, apple juice, safety.

1. Introduction

Natural antimicrobials and essential oils (EO) are of great appeal in the global market because they are perceived by the consumers as natural and green alternatives to traditional industrial preservatives. The analysis of the literature has pointed out that they are gifted of several properties such antimicrobial, antioxidant and anticancer activities (Patrignani, Siroli, Serrazanetti, Gardini, & Lanciotti, 2015). Despite their high antimicrobial activity against pathogenic and spoilage microorganisms, their practical application in food is currently limited due to several factors. In fact, their strong flavour impact, their great variability in composition, depending in its turn by the plant geographic origin, agricultural techniques, seasonality, and methods of extraction, and, finally, the scarce knowledge on their interactions with the food matrix macromolecules are the principal factors limiting their use in foods (Gutierrez et al., 2008). In fact, antimicrobial agents are chemically reactive species, which can cause considerable problems when embedded into a complex food system, such as negative effects on the physical stability or integrity of the food chemistry as well as the degradation of the biological activity of bioactive compounds (Donsì & Ferrari, 2016, Kumar et al., 2019). These drawbacks result into the need to use concentrations able to inhibit the microbial growth within the limits imposed by food regulations and the product shelf-life span time and, at the same time, without modification on the food qualitative properties (Weiss, Gaysinksy, Davidson, & McClements, 2009). However, the use of natural antimicrobials as preservatives is reduced by their fast degradation and the lacking of appropriate carriers to vehiculate and protect them in foods against oxidation. In fact, according to their lipophylic nature, they need to be conveyed by a right carrier to be dissolved in the matrix such as ethanol. However, the recent literature has proposed also the nanoencapsulation of bioactive compounds or EOs as viable and efficient approach to increase their physical stability, to facilitate their vehiculation and protecting them also from the interactions with the food ingredients. A huge literature on the encapsulation of essential oils or natural antimicrobials, such as hexanal, carvacrol, terpenic molecules, deals with the use of micrometric size capsules, used to protect the active compounds

against oxygen, light, moisture and low pH values. For example, β-cyclodextrin–pectin blends have been proposed for the microencapsulation of hexanal by radiant energy vacuum drying to inhibit *Penicillium expansum* in apple tissue (Sáenz-Garza, Delaquis, & Durance, 2013). On the other side, several biopolymers have been widely used as wall materials of microparticles in the protection of essential oils, both in aqueous phase and/or in spray-dried powders. Among them chitosan (Pedro, Cabral-Albuquerque, Ferreira, & Sarmento, 2009), Ca-alginate (Wang, Gong, Huang, Yu, & Xue, 2009), modified starch (mainly for agrochemical applications of pest control) (Glenn et al., 2010; Varona, Martin, & Cocero, 2009), milk proteins (Baranauskiene, Venskutonis, Dewettinck, & Verhe, 2006) and different polysaccharides (Adamiec & Kalemba, 2006; Krishnan, Bhosale, & Singhal, 2005) are the most important and widely studied. While microcapsules may guarantee excellent protection of essential oils against degradation or evaporation, they do not affect antimicrobial activity. In contrast, nanometric size delivery systems, due to the subcellular size, may increase the passive cellular absorption mechanisms, thus reducing mass transfer resistances and increasing antimicrobial activity (Donsì et al. (2011, 2012a, 2012b). The increase of bioactivity due to the reduction to sub-cellular size allow their usage at levels effective against the spoilage microorganisms while maintaining the sensory properties of foods and avoiding the use of carrier such as ethanol. Among the nanometric encapsulation systems currently being used for the delivery of bioactive compounds, nanoemulsions are particularly suitable for food applications (Donsì & Ferrari, 2016; Chaudhari et al., 2019; Kumar et al., 2019; Liu et al 2020), owing to the possibility of formulation with natural ingredients and the easy industrial scalability of the production process. The literature has pointed out the use of Ultra High Pressure up to 350 MPa to obtain nanoemulsions of a mixture of terpenes using natural ingredients such as lecithin and sun flower oil as carriers (Donsì et al., 2011)

Thus, in this contest, first aim of the research was to assess the capacity of Ultra High Pressure Homogeneization (UHPH) at 300 MPa to produce stable hexanal and trans 2-hexenal based nanoemulsions. Moreover, the second aim of this research was to evaluate the antimicrobial activity

of the obtained nanoemulsions comparing their activity to those of the same compounds carried on in ethanol. Finally, the obtained nanoemulsions were tested in order to evaluate their potential to increase safety and shelf-life of apple juice, deliberately inoculated with pathogenic and spoilage microorganisms, treated at different levels of pressure (100 MPa x1,2,3 cycles and 200 MPa for 2 cycles) stored at 10 °C

2. Materials and methods

2.1. Preparation of nanoemulsions

Nanoemulsions were prepared at Gea (Parma, Italy) according to the method reported by Donsì et al. (2011) with some modification. Nanoemulsions of hexanal and trans-2-hexenal were prepared separately. Briefly, each compound (50 g/kg) (Sigma Aldrich, Milan, Italy) was primarily mixed with commercial soy lecithin (10 g/kg) by Ultra Turrax at 10000 rpm for 10 min and then added with water (940 g/kg). The emulsions were treated by Ultra High Pressure Homogenization (Atena model, Gea, Italy) at 300 MPa for 14 passes up to the reduction to nanoparticles.

2.2. Nanoemulsion physical characterization

The nanoemulsions were characterized by Gea for their size by Laser Particle size analyzer type Coulter LS13320 (Beckman Coulter, Brea, CA). Each measurement was repeated three times. The samples were prepared by mixing them gently and adding to the vessel. The optical model used is Fraunhofer with Mie correction.

The stability of nanoemulsions were tested by Gea by the High-end Dispersion Analyzer Lumisizer® (Lumisizer, Berlin, Germany). The stability testing can be accelerated by up to 2147X compared to traditional test tube tests

2.3. Determination of nanoemulsion Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC)

The determination of MIC and MBC of the two obtained nanoemulsions was performed on five different microbial strains such as *L. monocytogenes* Scott A, *E. coli* 555, *Staph. aureus* SR231, *L. plantarum* 82 and *S. cerevisiae* SPA. The bacterial species were cultured two times in Brain Heart Infusion (Oxoid, Basigstone, UK) at 37 °C for overnight while the yeast strain was propagated in Sabouraud broth (Oxoid, Basigstone, UK) at 25 °C for 24h. The assay was performed in tubes according to the method proposed by Siroli et al. (2014) with some modifications. For each nanoemulsion, the tested concentrations ranged between 100 to 10000 ppm. For each microorganism, three different inoculum levels were tested (2, 4 and 6 log CFU/mL). In addition, the MIC and MBC of the nanoemulsions were compared with the MIC and MBC of the two aldehydes carried on in ethanol (<1%). The MIC and MBC were evaluated after 24 and 48 h at the microorganism optimal growth temperature.

2.4. Safety and shelf-life of apple juice in relation to the presence of nanoemulsions

2.4.1. Preparation of juice samples and Challenge test

The apple juice was obtained by apples, var. *Golden delicious*, purchased at local supermarket. The juice was obtained by a lab extractor (Russel, Hobbs, 27700-56). The obtained juice was divided in two batches. One batch (B1) was inoculated with selected microorganisms (see below) and treated by high pressure homogenization (HPH) at different pressure levels and cycles, the other batch (B2) was represented by inoculated apple juice, added of nanoemulsions (35 ppm for E-2-Hexenal and 70 ppm for Hexanal) and treated by HPH at the same pressure levels and cycles of B1.

The two batches were subjected to HPH treatments, performed at 0.1 MPa, 100 MPa for 1, 2 and 3 cycles and at 200 MPa for 2 cycles. The resulting samples are reported in Table 1.

In all the cases, the HPH treatments were performed using a continuous high pressure homogenizer PANDA (Gea, Parma, Italy). The machine was supplied with a homogenizing R type valve with a flow rate of 10 l/ h; the valve assembly includes a ball-type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten-carbide passage head. The inlet

temperature of the untreated apple juices was 4 °C and it increased of about 1.5 °C/ 10 MPa. After each pass at 100 MPa and 200 MPa, apple juices were cooled by using a thermal exchanger (Geai, Parma, Italy). The maximum temperature reached by the samples did not exceed 40 °C.

All the obtained juices were incubated at 10 °C and analysed immediately after treatment and over storage for microbial cell loads, pH.

The samples from B2 were also characterized for colour and volatile molecule profiles.

2.4.2. Microorganisms used in the Challenge test and microbial sampling

L. monocytogenes Scott A, *E. coli* 555, *Staph. aureus* SR231, *L. plantarum* 82 and *S. cerevisiae* SPA, belonging to the Department of Agricultural and Food Sciences (DISTAL, University of Bologna) collection, were used for the Challenge test. The bacterial species were cultured two times in Brain Heart Infusion (Oxoid, Basigstone, UK) at 37 °C for overnight while the yeast strain was propagated in YPD broth (Oxoid, Basigstone, UK) at 25 °C for 24h. The microbial species were inoculated in mix the two apple juice batches at level of 3-4 log CFU/mL. Immediately after the application of the decontamination treatments and during the product storage, the microbial cell loads were determined by sampling on different selective media such as Listeria Selective Agar (Oxford formulation) added of Listeria Supplement, Violet Red Bile Aagar added of MUG, Baird Parker, MRS and Sabouraud (Oxoid, Basigstone, UK) for *L. monocytogenes*, *E. coli*, *Staph. aureus*, *Lb. plantarum* and *S. cerevisiae*, respectively. The plates were incubated at 37 °C for 24-48 h for the bacterial species and 25 °C for 48 h for *S. cerevisiae*.

2.4.3. Colour analysis and molecule volatile profiles

Colour was measured using the CIELab scale and Illuminant D65. The instrument was calibrated with a white tile (L* 98.03, a* _0.23, b* 2.05) before the measurements. Results were expressed as L*, a* and b*. The molecule volatile profiles were detected by using the GC/MS/SPME technique as described by Patrignani et al. (2013).

2.5. Statistical analysis

Microbial cell loads, colour and volatile profile data are means of three independent experiments. For colour, three different measures were taken for each sample considered. The coefficients of variability for microbiological data, expressed as the percentage ratios between the standard deviations and the mean values, ranged between 2 and 5%. The significance of colour parameters was evaluated using ANOVA followed by LSD test at $p<0.05$. The volatile molecule profiles were analyzed using a principal component analysis (PCA) performed by Statistica software (STAT soft tools).

3. Results

3.1. Formulation, dimension and stabilization of nanoemulsions

The hexanal and trans-2-hexenal based nanoemulsions were obtained using soy lecithin as carrier and following the treatment by Ultra High Pressure at 300 MPa for 14 cycles. The dimensions and stabilization parameters of the obtained nanoemulsions are reported in Table 1. The analyses performed by dynamic laser light scattering showed that the hexanal and trans-2-hexenal based nanoemulsions were characterized by an average size of 86±23 and 100±31 nm, respectively. The results obtained by Lumisizer showed for these two samples stability profiles of almost 14 months avoiding phase separation. Moreover, the nanoemulsions resulted, after the performed UHPH treatment, colourless.

3.2. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the natural antimicrobial based nanoemulsions

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the two natural antimicrobial based nanoemulsions were studied against pathogenic and spoilage microorganisms and in comparison with the pure hexanal and trans-2-hexenal carried in 1%

ethanol. The results are reported in Tables 2. The MICs were evaluated after 24 and 48 h of incubation and in relation to different inoculum levels of the pathogenic and spoilage species considered while, the MBCs were detected after 24 h spotting 10µl of the cellular suspension in Petri dishes. The results obtained highlighted the major efficacy of trans-2- hexenal compared to hexanal, independently on the carrier (lecithin or ethanol) and the considered microorganisms. The MIC values, after 24 h and using an initial inoculum of level of 2 log CFU/mL, were always lower than 700 ppm. *Saccharomyces cerevisiae*, used at level of 2 log CFU/mL, resulted more sensitive to trans-2-hexenal, showing a MIC value of 100 ppm both in ethanol and in nanoemulsion. On the other side, *L. plantarum*, at the same operative conditions, resulted the most resistant to trans-2-hexenal, exhibiting MIC values of 700 and 500 ppm when carried out in lecithin and ethanol, respectively. By contrast, hexanal showed a lower antimicrobial efficacy against all the considered microorganisms. In general, hexanal, when delivered as nanoemulsion, exhibited higher MIC values at 24 h, compared to hexanal in ethanol; however, the differences among the two, at 32 and 48 h, decreased showing a slow release of this aldehyde when delivered as nanoemulsion. Thus, the results obtained showed a fast release of nanoencapulated trans-2-hexenal and a slow release of hexanal when nanoencapsulated, suggesting a proper combined use of the two nanoemulsion.

3.3. Safety and shelf-life of apple juice: challenge test

In Figures 1-3, the evolution of *L. monocytogenes*, *E. coli* and *Staph. aureus* cell loads at 10 °C is, respectively, reported in relation to the high pressure homogenization (HPH) treatment alone or in presence of hexanal (70 ppm) and trans-2-hexenal (35 ppm) nanoemulsions. The concentration of each nanoemulsion to employ in the experiments were selected on the basis of previous results performed using the same food matrix. The pathogenic species considered decreased their cell loads with different kinetics in relation to the hyperbaric treatment considered, the combination of the two hurdles (HPH treatment and the presence of nanoemulsions) and as consequence of the low pH of the juice. The inactivation kinetic of *L. monocytogenes* was increased after 2 days of storage in the

presence of nanoemulsions and without application of HPH. After 6 days of product storage, *L. monocytogenes* cell load was under the detection limit. The combined use of HPH and nanoemulsions increased the inactivation kinetics of this pathogenic species. The combination of the level of pressure at 200 MPa for 3 cycles and the nanoemulsion determined an instantaneous inactivation of 3 log order.

Staph. aureus inactivation kinetic was initially more affected by the pressure (200 MPa x 2 cycles) than the presence of nanoemulsion. However, after 8 days of storage at 10 °C, *Staph. aureus* cell loads were under the detection limit in samples treated at 200 MPa for 2 cycles and in the presence of nanoemulsion while it was still detectable in the conditions where only the pressure was applied.

E. coli was very sensitive both to the pressure and to the use of nanoemulsions, alone or in combination.

The inactivation kinetics of *S. cerevisiae* is reported in Fig. 4. The inactivation kinetics were different in relation to the pressure applied. When *S. cerevisiae* was treated at 200 MPa x 2 cycles, in the presence or not of nanoemulsions, its cell loads decreased of almost 3 log cycles. However, during the first 14 days at 10°C, *S. cerevisiae* was able to recover in the juice initially treated only by hyperbaric process and reach the spoilage threshold fixed at 6 log CFU/mL in 12-14 days. On the other hand, *S. cerevisiae* cell loads decrease under detection limit (1 log CFU/mL) in juice treated at 200 MPa for 2 cycles and added of nanoemulsions. *Lactobacillus plantarum* showed barotollerance to the HPH treatments applied (data not showed). However, its cell loads decreased over time. The presence of nanoemulsions further increased this behaviour.

3.4. Colour and volatile profiles of the apple juices added of nanoemulsions

The juice colour data in relation to the addition of nanoemulsions and HPH treatment applied are reported in Table 3. The L* parameter increased initially in relation to the pressure applied but not in significant way among samples. In general, this parameter increased over time and after 1d of storage, all the samples added of nanoemulsions before pressure treatment were characterized by a

significant increase of this parameter with respect to the control not added of nanoemulsion and not HPH treated. On the other hand, L* parameter decreased over time in the control (juice treated at 0.1 MPa). At the end of the storage, it reached in the samples values ranging between 44.05 and 50.59 in relation to the level of pressure applied. The highest significant value was detected in juice added of nanoemulsions and treated at 200 MPa for 2 cycles. The highest a* values, that for positive values tends toward red, were found at level of 8.48, 8,81, 8.06 in samples added of nanoemulsions and treated at 100 MPa for 1, 2, 3 cycles, respectively. However, they were not significant different from the remaining samples. Overtime, this parameter decreased in all the samples, without any significant change at the end of the storage.

About b*parameter, which for positive values tends toward yellow, it was found at highest values, but not in significant way, in samples treated at 100 MPa for 1,2, and 3 cycles. At the end of the juice storage, the highest significant values were detected in samples added of nanoemulsions and in samples added of nanoemulsion and treated at 100 MPa for 1 and 2 cucles and 200 MPa for 2 cycles.

The apple juices added of nanoemulsions and treated by HPH were analysed, over time, for their molecule volatile profiles by GC/MS-SPME. Also, a control sample (juice only treated at 0.1 MPa) was included. The analysis permitted to identify about 40 molecules belonging to different classes of compounds (Table 4). It is clear that the most present compounds belonged to aldehydes and alcohols. The major peak areas for aldehydes in the samples were detected for the treatments performed at 100 MPa x 3 and 200MPa x 2 after 8 days of sample storage. However, after 22 days of storage at 10 °C, the total areas of hexanal and trans-2-hexenal decreased, while the principal detoxification product of these two aldehydes, hexanol, increased in relation to the HPH treatments applied. To better highlight the effects of the combined use of nanoemulsions and HPH treatments, the GC/MS/SPME data were analyzed by Principal Component Analysis (PCA). The projection of the samples is reported in Fig. 5 (5a) where it is clear that PC1 and PC2 are able to explain the 55% of the total variance among the samples. The first cluster included, with the exception of the control

(C, Juice treated at 0.1 MPa and not added of nanoemulsions), all the samples added of nanoemulsions immediately after the HPH treatment, irrespective of the level of application. The second cluster, separated by the first along the PC2, was represented, with the exception of the control sample which clustered in group 3, by the samples after 8 days of storage at 10 °C. In the cluster 3 grouped all the samples after 22 days of storage and the control sample after 8 days. The sample added of nanoemulsion and treated at 0.1 MPa was separated from all the other ones. In Fig.5b, the molecules which permitted the clusterization of the samples are reported. Hexanol, considered a detoxification product, affected particularly the grouping of cluster 3 where samples after 22 days of storage were included.

4. Discussion

The increase of fruit juice safety and shelf-life by using innovative technologies, alternative to thermal treatment, is a great industrial challenge. The successful results obtained on cell disruption of dense microbial cultures stimulated researches on the application of ultra high pressure homogenization (UHPH) for food safety and shelf-life improvement showing a maximal retention of physico-chemical product properties, as well as nutritional and sensory features. Recently, UHPH was indicated by several authors as a useful approach, together with colloid milling and ultrasonication, to obtain natural antimicrobial based nanoemulsions to be used, instead of thermal treatment or in combination with innovative technologies, to increase fruit juice safety and shelf-life (Donsì et al., 2011; Donsì and Ferrari, 2016; Fathi, Martín, & McClements, 2014). On the other hand, also Pucek et al. (2016) have exploited the HPH technology to produces multiple drug based nanocarriers. Due to reached nanometric size, nanoemulsions have unique properties. First of all, differently from emulsions, thermodynamically instable and tending to separate, they are very stable (McClements and Rao, 2011). Our results demonstrated that the hexanal and tran-2-hexenal based nanoemulsions were stable for more than 1 year when treated at 300 MPa for 14 passes. These findings are in agreement also with the results of Donsì et al. (2011) who found for terpenic

molecule based nanoemulsions a comparable stability. Secondly, since the nanoemulsion droplet diameters are much smaller than the wavelength of light, they form transparent or only slightly turbid systems, which are suitable also for addition to clear beverages, sauces, soups, and syrups (Salvia-Trujillo, Rojas-Grau, Soliva-Fortuny, & Martin-Belloso, 2014). The antimicrobial based nanoemulsions obtained in this research work appeared colourless and showed high solubility in water, passing up the problem of the delivery in food systems of these lipophilic compounds through ethanol solution which represent for food industry an additional cost. Moreover, nanoemulsions, due to their chemico-physical properties, did not affect the palatability of the tested apple juice.

Thus, for these important features, and because compatible from a sensory point of view with vegetable food matrixes, the obtained nanoformulations showed a great potential application in the field of juice and beverage as reported also by several Authors (Salvia-Trujillo, Rojas-Grau, Soliva-Fortuny, & Martin-Belloso, 2015; Silva, Cerqueira, & Vicente, 2012).

Although, in general, the reduction to nanoparticle size should increase the transport mechanisms through the cell membrane of spoilage and pathogenic microorganisms (Donsì et al., 2011), the data of the present research showed MIC and MBC values not different, in the first 24 h of analysis, from the compounds vehiculated in ethanol. This could be due to the strong interaction between the compounds and the material used for nanoencapsulation which delayed the released of the compounds in the first 24 h of analysis. In fact, the results obtained showed that the kinetics of release of the two antimicrobials in nanoemulsion were different overtime suggesting a potential use in combination in real system. In fact, the microbiological results achieved in apple juice, regarding the nanoemulsion alone or in combination with high pressure homogenization, showed their great potential to inactivate the target pathogenic bacteria and *S. cerevisiae*, deliberately inoculated in the juice. On the other hand, *S. cerevisiae* represents, for this kind of products, characterized by low pH and high level of sugar, the principal spoiling agent (Patrignani et al., 2013). The data resulting from the Challenge test showed that also *E. coli* dynamic inactivation was

fastened by the presence of nanoencapsulated antimicrobials, also in the absence of the application of high pressure homogenization. Nanoemulsion droplets, thanks to their size and to the exposition of the hydrophilic groups of the emulsifying molecules, can be efficiently transported through the porin proteins of the outer-membrane, generally impermeable to free essential oil or natural antimicrobials, enabling an effective delivery of EOs also to the cell membrane of Gram negative bacteria (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). From a technological point of view, the combined use of high pressure homogenization and nanoemulsions increased the lightness of the apple treated juice, due both to the effects of HPH and to the antioxidant activity of the natural antimicrobial (Patrignani, & Lanciotti, 2016; Prakash et al., 2018). On the other hand, the HPH treatment is reported to increase L* parameter due to the higher light scattering properties of smaller size particles (Calligaris et al., 2012, Patrignani et al 2019). A similar result was also observed by Yi et al. (2017) on apple juice with 50% of kiwifruit addition upon the application of dynamic pressure. In addition, the use of natural antimicrobial based nanoemulsions can reduce the sensory impact that the free molecules generally have (Espina et al., 2014). Additionally, the use of suitable delivery systems can favour the dispersion of the natural antimicrobials into the food matrix, avoiding the use of huge amount of EOs which adversely affect the sensory properties of the product. Moreover, as reported by Donsì et al., (2012a) the choice of the good delivery system can prevent the EO evaporation rate, as well as the negative interaction with other food components (Shah, Davidson, & Zhong, 2012). The data from GC/MS-SPME analysis showed that the addition of the two natural antimicrobials, although as nanoemulsions, affected the apple juice molecule volatile profiles since hexanal and trans-2-hexenal was the most abundant identified compounds. However, their kinetic release in the product were different over time. In fact, the apple juice samples clusterized together dependently on the storage time. In addition, the presence of hexanol, as detossification product deriving from hexanal by microbial enzymatic activity and vegetable tissue one, was more pronounced in samples subjected to a faster microbial spoilage.

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5. Conclusion

The data of the present research contribute to support the application of natural antimicrobial based nanoemulsions into complex food system, enlarging the experimental evidence for their use in food sector. In particular, the obtained hexanal and tran-2-hexenal based nanoemulsions demonstrated great application potential, due also to their compatibility with the tested food matrix, promoting also an increase of quality of apple juice. The data reported in the present research also highlighted the potential use of nanoemulsions in combination with another hurdle, such as high pressure homogenization. However, for a further application in food industry, the action mechanisms of encapsulated antimicrobials need to be deeply understood and studied also in order to replace other carrier such as ethanol which can represent an additional cost for the food industry.

Figure caption

Figure 1) *L. monocytogenes* cell load (log CFU/mL) evolution detected in apple juice in relation to the initial inoculum level, treatment applied and time of storage at 10 °C.

Figure 2) *Staphylococcus aureus* cell load (log CFU/mL) evolution detected in apple juice in relation to the initial inoculum level, treatment applied and time of storage at 10 °C.

Figure 3) *Escherichia coli* cell load (log CFU/mL) evolution detected in apple juice in relation to the initial inoculum level, treatment applied and time of storage at 10 °C.

Figure 4) *Saccharomyces cerevisiae* cell load (log CFU/mL) evolution detected in apple juice in relation to the initial inoculum level, treatment applied and time of storage at 10 °C.

Figure 5) Projection on the factor plane (1x2) of variables (b) and apple juice samples (a) added (samples 1,2,3,4,5) or not (C) of nanoemulsions, treated at different pressures and stored at 10°C for several days (T0, T8, T22).

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Table 1. Samples obtained within the present research

Batch 1
Inoculated juice treated at 0.1 MPa (control)
Inoculated juice treated at 100MPa x 1cycle
Inoculated juice treated at 100MPa x 2cycles
Inoculated juice treated at 100MPa x 3cycles
Inoculated juice treated at 200MPa x 2cycles
Batch 2
Inoculated juice added of nanoemulsion (NE) and treated at 0.1 MPa
Inoculated juice added of nanoemulsion (NE) and treated at 100MPa x 1cycle
Inoculated juice added of nanoemulsion (NE) and treated at 100MPa x 2cycles
Inoculated juice added of nanoemulsion (NE) and treated at 100MPa x 3cycles
Inoculated juice added of nanoemulsion (NE) and treated at 200MPa x 2cycles.

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Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the natural antimicrobials carried in nanoemulsion or ethanol

		Staphylococcus aureus			*L.monocytogenes*			*E.coli*			*S.cerevisiae*			*Lb.plantarum*		
	Log cfu/ml	MIC 24h (ppm)	MIC 48h (ppm)	MBC 48h (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC 48h (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC 48h (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC 48h (ppm)	MIC 24h (ppm)	MIC 48h (ppm)	MBC 48h (ppm)
Hexanal nanobased emulsion	6	4000	5000	10000	3000	3000	5000	2000	3000	4000	800	3000	4000	5000	8000	>10000
	4	3000	5000	8000	3000	3000	5000	2000	2000	3000	800	2000	3000	5000	8000	>10000
	2	2000	4000	5000	1000	3000	5000	700	800	1000	800	1000	2000	4000	8000	>10000
Hexanal in ethanol	6	4000	4000	>10000	2000	2000	2000	2000	2000	3000	1000	2000	3000	5000	5000	>10000
	4	2000	3000	8000	1000	1000	2000	800	2000	2000	800	2000	2000	4000	5000	>10000
	2	2000	2000	5000	800	1000	2000	300	500	1000	500	800	2000	2000	5000	>10000
(E)-2-hexenal nanobased emulsion	6	700	3000	3000	500	800	1000	700	700	1000	500	700	700	700	1000	2000
	4	700	1000	2000	300	800	1000	500	700	700	300	500	500	700	1000	2000
	2	500	800	1000	300	500	800	500	500	500	100	100	300	700	1000	1000
(E)-2-hexenal in ethanol	6	1000	1000	2000	500	500	800	500	700	700	300	500	500	500	700	700
	4	500	700	800	300	500	800	500	700	700	300	500	500	500	700	700
	2	300	700	700	300	500	500	500	500	500	100	300	300	500	700	700

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Table 3. Apple juice colour parameters detected in different samples in relation to the presence of natural antimicrobial nano-based emulsions (NE), pressure applied and time of storage

	Time (d)								
L*	**0**	**1**	**4**	**6**	**8**	**12**	**15**	**19**	**22**
0.1 MPa (control)	35.09±3.18[a]	34.90±2.58[a]	33.35±1.21[a]	33.70±3.27[a]	33.10±3.11[a]	33.00±2.87[a]	29.70±3.79[a]	30.10±3.26[a]	28.75±3.22[a]
NE +0.1MPa	35.15±2.56[a]	36.57±3.05[b]	41.79±3.24[b]	38.23±2.89[ab]	41.88±2.28[b]	42.05±2.56[b]	42.56±2.25[b]	43.10±2.98[b]	48.05±1.55[c]
NE +100 MPa x1c	39.91±2.79[a]	41.08±1.89[b]	40.60±3.08[b]	38.48±3.02[ab]	42.42±3.02[b]	41.04±3.22[b]	41.89±3.06[b]	43.68±3.09[b]	45.75±3.27[bc]
NE +100 MPa x2c	39.66±3.03[a]	40.99±1.22[b]	36.77±1.79[ab]	42.02±1.89[bc]	42.02±2.74[b]	42.74±2.98[b]	42.66±2.88[b]	50.82±2.21[c]	46.15±2.54[bc]
NE +100 MPa x 3c	39.54±2.55[a]	41.38±2.05[b]	37.51±3.11[b]	46.69±2.02[c]	41.39±1.96[b]	40.96±3.18[b]	45.85±2.77[b]	42.26±2.57[b]	41.93±3.01[b]
NE +200 MPa x 2c	37.17±1.86[a]	40.27±2.36[b]	39.86±2.94[b]	41.05±2.02[b]	39.86±2.09[b]	41.45±2.22[b]	42.84±3.02[b]	50.32±2.64[c]	50.59±2.88[c]
a*	**0**	**1**	**4**	**6**	**8**	**12**	**15**	**19**	**22**
0.1 MPa (control)	6.90±0.96[ab]	6.30±0.78[a]	4.10±1.46[a]	4.50±1.50[a]	4.35±0.84[a]	3.89±1.25[a]	3.75±2.02[a]	3.70±0.58a	3.10±0.95[a]
NE +0.1MPa	6.78±1.07[ab]	7.94±0.95[ab]	5.44±1.55[a]	6.65±1.18[a]	4.82±1.05[a]	4.80±0.95[a]	4.85±1.34[a]	2.63±1.89a	4.26±0.79[a]
NE +100 MPa x1c	8.48±1.18[bc]	8.39±1.10[b]	6.63±1.37[a]	5.13±0.95[a]	5.58±1.18[a]	3.55±1.85[a]	5.58±1.08[a]	5.06±1.10a	5.02±1.05[a]
NE +100 MPa x2c	8.81±1.33[bc]	7.83±0.88[ab]	4.25±1.03[a]	6.40±1.22[a]	5.97±1.29[a]	5.25±1.16[a]	5.41±0.95[a]	4.22±0.85a	5.48±1.25[a]
NE +100 MPa x 3c	8.06±1.09[bc]	8.26±1.06[b]	5.15±1.18[a]	4.69±1.33[a]	5.36±0.85[a]	5.79±1.33[a]	5.79±1.45a	5.60±0.96a	4.53±0.86[a]
NE +200 MPa x 2c	7.58±0.74[b]	8.04±1.18[ab]	5.21±0.99[a]	6.23±1.56[a]	5.19±1.10[a]	5.62±1.05[a]	5.66±1.23[a]	5.84±0.88a	4.24±1.13[a]

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b*	0	1	4	6	8	12	15	19	22
0.1 MPa (control)	17.35±3.25[a]	16.10±1.85[a]	13.20±2.45[a]	13.00±2.20[a]	12.55±1.65[a]	12.00±2.01[a]	12.20±1.42[a]	10.50±3.03[a]	10.78±2.55[a]
NE +0.1MPa	17.25±2.89[a]	16.20±2.03[a]	22.66±1.54[b]	19.35±1.75[bc]	20.93±1.55[b]	31.47±3.22[c]	17.01±2.22[b]	29.65±3.89[d]	21.65±1.78[b]
NE +100 MPa x1c	21.62±2.55[a]	24.44±2.64[b]	21.57±1.88[b]	16.78±2.18[ab]	22.39±2.01[b]	17.32±1.18[b]	15.99±1.38[ab]	16.33±2.00[b]	19.26±2.08[b]
NE +100 MPa x2c	20.73±1.93[a]	21.01±1.52[b]	14.33±2.02[a]	21.87±1.61[cd]	20.72±1.64[b]	19.76±1.56[b]	17.87±1.77[b]	23.65±1.93[c]	20.24±1.35[b]
NE +100 MPa x 3c	21.12±1.85[a]	21.86±1.38[b]	16.47±1.85[a]	25.20±2.05[d]	19.41±1.09[b]	19.07±2.01[b]	22.07±2.09[c]	14.53±2.18[ab]	13.59±2.09[a]
NE +200 MPa x 2c	17.89±2.01[a]	24.47±1.86[b]	21.04±1.45[b]	20.86±2.04[bc]	18.40±0.98[b]	19.48±1.34[b]	17.21±1.32[b]	23.58±1.64[c]	21.88±1.96[b]

Data are reported as average values and standard deviations. Values with different letter within the column are significantly different ($p < 0.05$).

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Table 4. Apple juice GC/MS/SPME profiles (expressed as total area/10000) in relation to the presence of natural antimicrobial nano-based emulsions, pressure applied and time of storage

	1	2	3	4	5	C	1*	2*	3*	4*	5*	C*	1*	2*	3*	4*	5*
	0d						**8d**						**22d**				
Molecules																	
Ethanol	27	31	28	29	22	48	39	124	81	38	81	897	3307	2253	1870	2192	2778
1-propanol	-	-	-	-	-	-	-	-	-	-	-	40	73	92	50	56	85
1-propanol-2 methil	-	-	-	-	-	-	-	-	-	-	-	-	38	14	9	10	38
1-Butanol, 3-methyl-	-	-	-	-	-	-	-	-	-	-	-	53	331	169	50	74	154
1-Hexanol	111	61	83	83	79	92	3467	4683	2344	1116	1512	2827	4866	6166	6059	5976	4863
3-Hexen-1-ol	-	-	-	-	-	-	462	38	45	29	42	14	-	-	-	9	25
2-Methylene cyclopentanol	31	22	22	20	23	32	16	17	12	28	19	-	-	-	-	29	28
6-Tetradecanol	25	35	-	15	19	-	18	30	10	20	25	-	-	-	-	38	31
2-Butyl-2,7-octadien-1-ol	935	898	805	683	1022	995	888	925	818	1103	697	351	661	516	601	553	384
1-Octanol, 2-butyl-	-	-	-	-	-	-	-	53	23	25	33	-	14	99	86	282	96
Cyclohexanol, 1-(1-hexenyl)-, (E)-	237	125	213	258	208	234	196	169	184	92	80	-	68	194	30	169	106
Phenylethyl Alcohol	-	-	-	-	-	-	-	-	-	-	-	7	60	84	14	30	56
Totale Alcohols	**1366**	**1173**	**1150**	**1088**	**1373**	**1401**	**5087**	**6039**	**3517**	**2450**	**2488**	**4189**	**9420**	**9586**	**8768**	**9417**	**8643**
ethil acetato	-	-	-	-	-	-	-	-	-	-	-	31	176	153	36	75	121
Hexanoic acid, ethyl ester	-	-	-	-	-	-	-	-	-	-	-	7	528	34	-	13	27

Acetic acid, hexyl ester	104	47	32	8	21	62	11	19	35	23	7	7	381	203	172	475	582
Octanoic acid, ethyl ester	-	-	-	-	-	-	-	-	-	-	-	-	93	72	-	-	-
2-Propenoic acid, octyl ester	-	-	-	-	-	-	-	-	-	-	-	-	124	60	-	-	-
2-Thiopheneacetic acid, 2-methyloct-5-yn-4-yl ester	278	200	242	49	152	299	114	144	93	214	136	145	227	148	237	137	125
Decanoic acid, ethyl ester	-	-	-	-	-	-	-	-	-	-	-	-	331	30	25	-	-
Oxalic acid, cyclobutyl decyl ester	-	-	-	-	-	-	-	-	21	-	8	-	251	-	-	-	-
Oxalic acid, cyclohexylmethyl tetradecyl ester	208	163	164	167	156	204	85	167	97	116	142	20	96	107	155	168	153
Hexadecenoic acid, ethyl ester	116	33	24	31	36	89	53	45	56	46	36	-	-	38	8	11	-
Total Esters	**706**	**443**	**462**	**255**	**364**	**654**	**262**	**375**	**302**	**400**	**330**	**210**	**2205**	**845**	**633**	**878**	**1008**
2 Butanone	10	6	2	10	8	12	8	1	4	2	2	-	-	-	4	2	-
2-hexanone- 4 metil	15	38	6	45	11	16	8	15	12	13	11	38	21	8	19	4	50
3-Hexen-2-one	55	75	82	96	103	118	23	21	56	59	41	-	-	-	-	-	-
Bicyclo[3.2.0]heptan-2-one	-	25	-	23	15	10	-	4	4	7	11	-	39	9	37	16	51
Propanal, dipropylhydrazone	46	47	24	28	19	62	14	16	14	16	17	-	22	-	-	-	-
Total Ketones	**126**	**191**	**115**	**203**	**156**	**218**	**53**	**57**	**90**	**96**	**81**	**38**	**81**	**17**	**61**	**22**	**101**
2-Butenal, (Z)	7	13	25	15	12	25	7	7	7	8	11	-	8	6	9	8	9
Hexanal	7207	6885	6132	5542	5913	35	3014	3171	4350	4370	4382	25	24	15	31	56	51
2-Hexenal, (E)-	5314	6357	4785	5484	5621	12	3402	3042	4150	4266	4291	6	12	18	12	52	37
2-Heptenal	-	-	9	30	63	9	6	-	14	23	25	5	26	17	9	26	13
2-4-Hexadienal,(E,E)	227	226	60	107	68	128	17	26	78	61	72	-	-	46	64	-	-
2-Octenal,(E)	44	57	19	26	62	19	8	17	27	25	15	-	-	-	-	-	-

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2,4 Heptadienal, (E,E)	41	13	14	16	16	14	13	17	21	20	18	-	-	-	-	-	-
Benzaldehyde	23	62	22	55	33	22	21	22	48	40	34	33	31	43	21	22	33
Totale Aldehydes	**12862**	**13613**	**11067**	**11275**	**11788**	**264**	**6487**	**6302**	**8694**	**8813**	**8847**	**69**	**101**	**145**	**148**	**164**	**142**
Hexanoic acid	852	531	610	601	666	720	578	409	583	622	460	15	28	24	5	8	-
Octanoic Acid	-	-	-	-	-	-	-	-	-	-	-	-	100	24	62	89	52
Totale Acids	**852**	**531**	**610**	**601**	**666**	**720**	**578**	**409**	**583**	**622**	**460**	**15**	**129**	**48**	**66**	**97**	**52**
Total Molecules	**15912**	**15951**	**13403**	**13422**	**14347**	**3257**	**12467**	**13181**	**13186**	**12382**	**12207**	**4522**	**11936**	**10641**	**9676**	**10579**	**9947**

1: juice treated 0.1 MPa+nano emulsion (NE)
2: juice + nano emulsion (NE) and treated at 100 MPax1c
3: juice + nano emulsion (NE) treated at 100 MPa x 2c
4: juice + nano emulsion (NE) treated at 100 MPa x 3c
5: juice + nano emulsion (NE) treated at 200 MPa x 2c
C: juice treated at 0.1MPa (control)
-: Under the detection limit

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Figure 1.

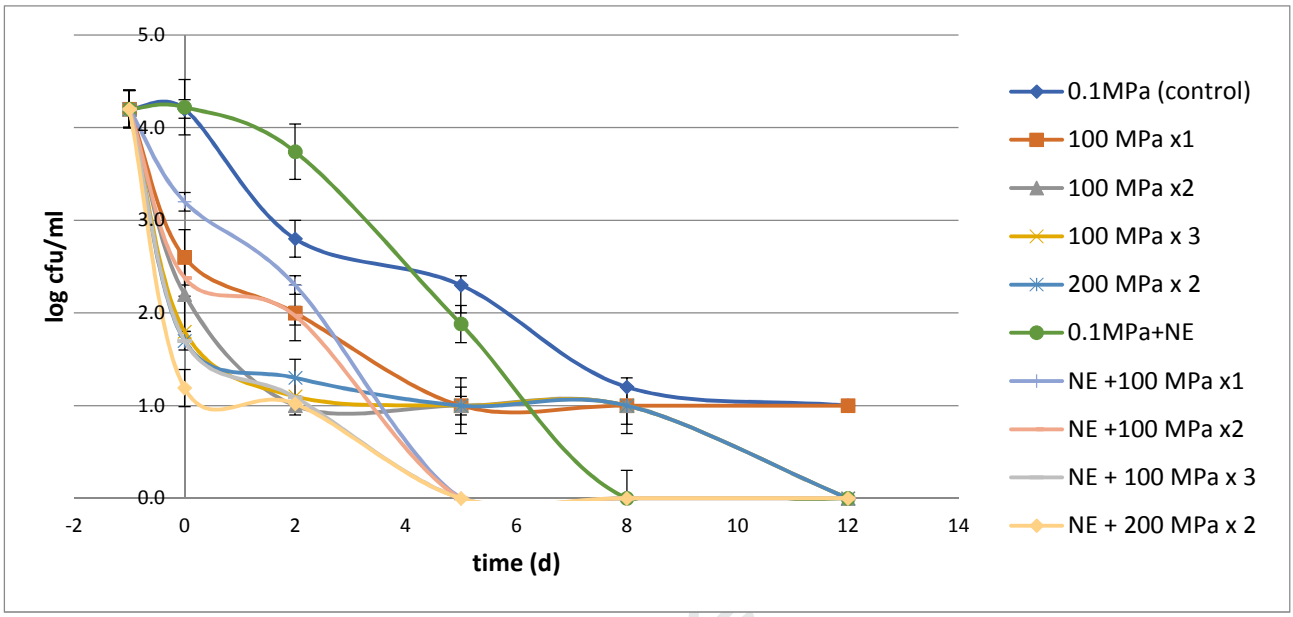

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Figure 2

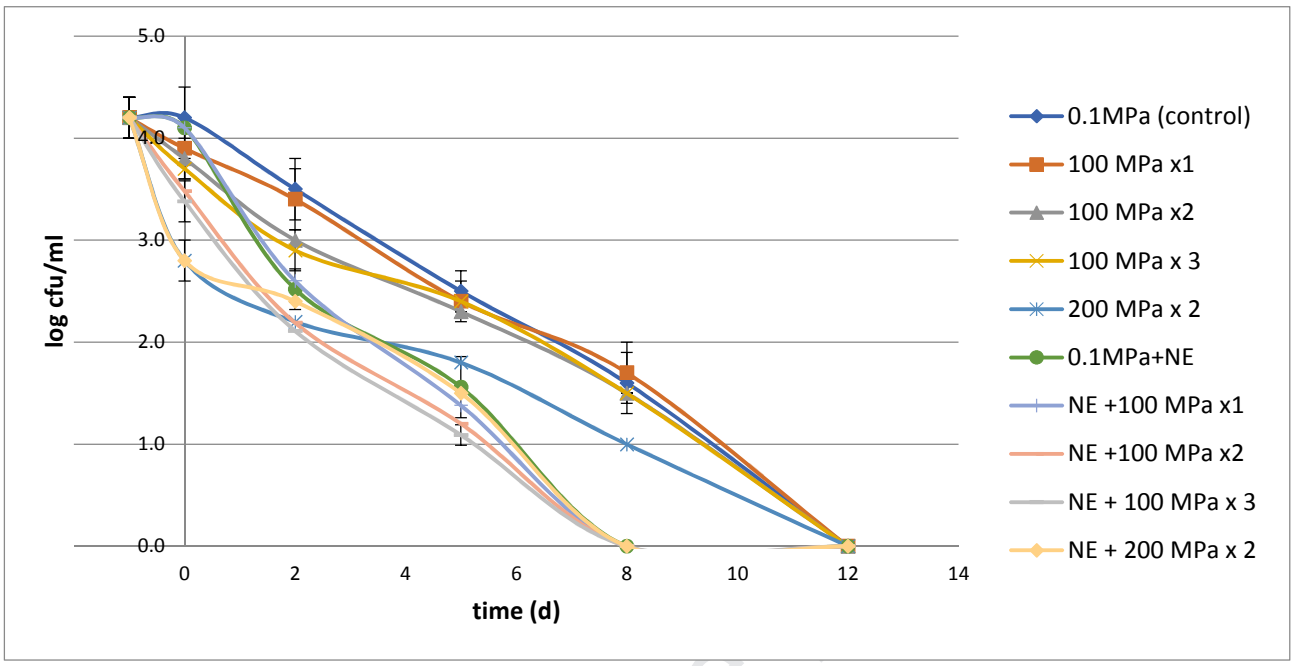

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Figure 3

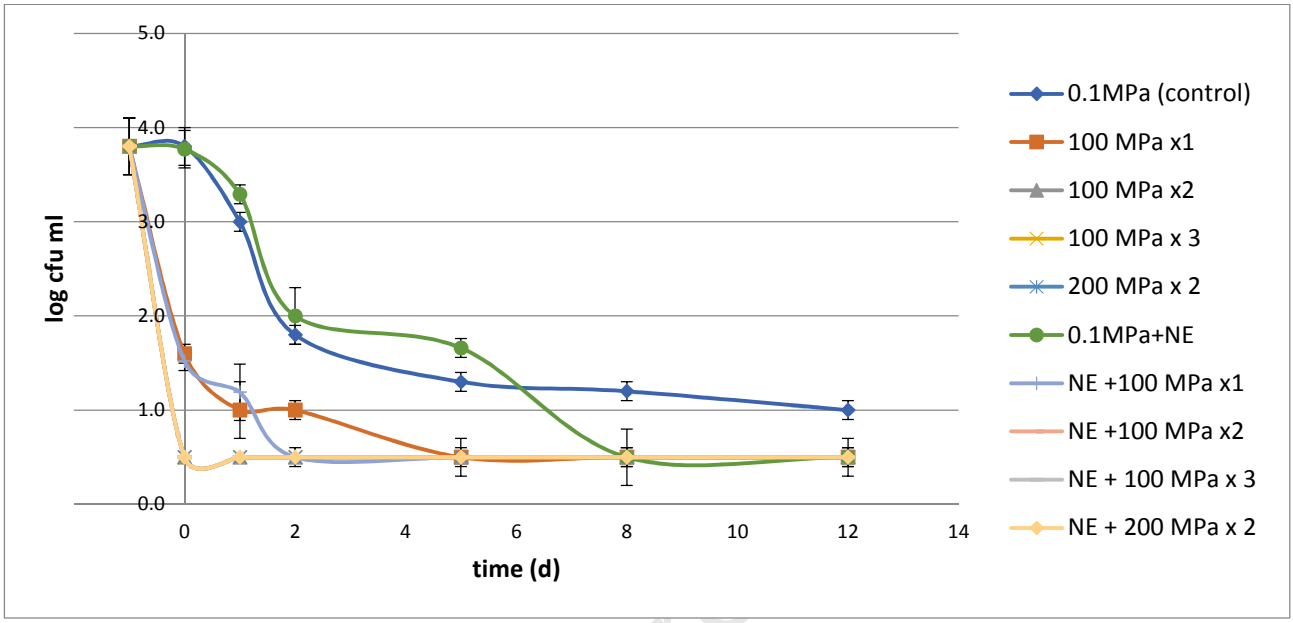

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Figure 4.

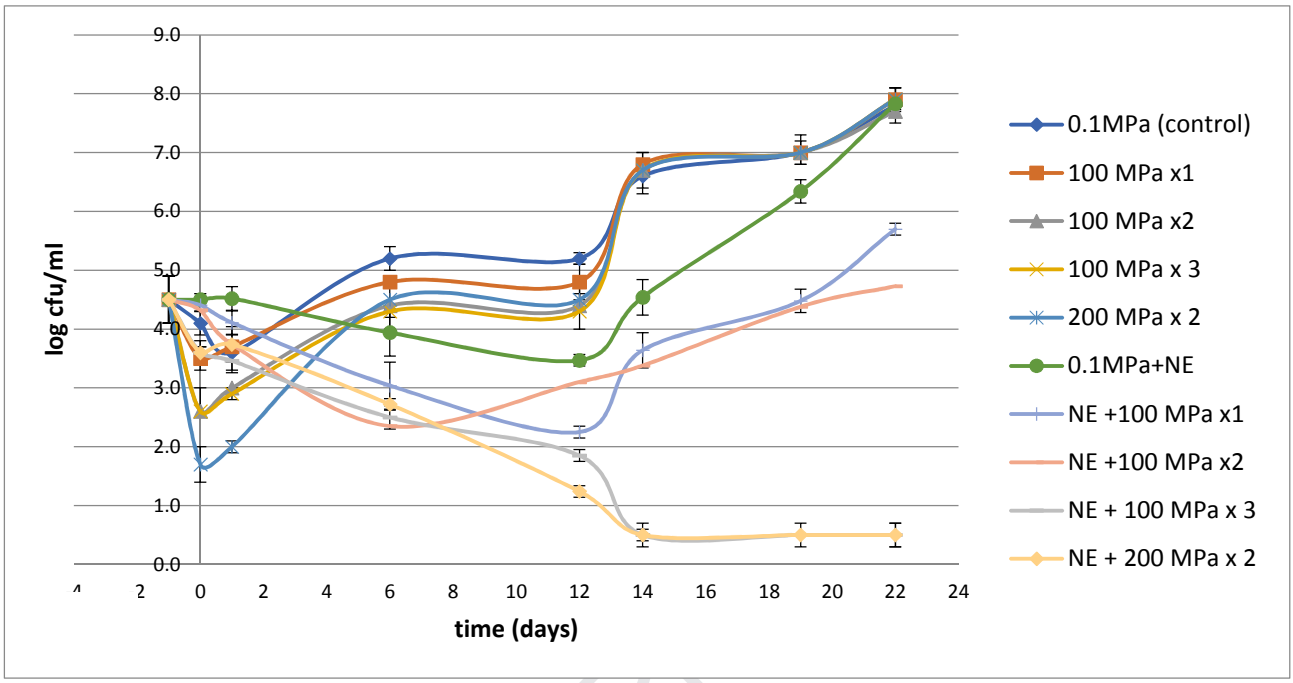

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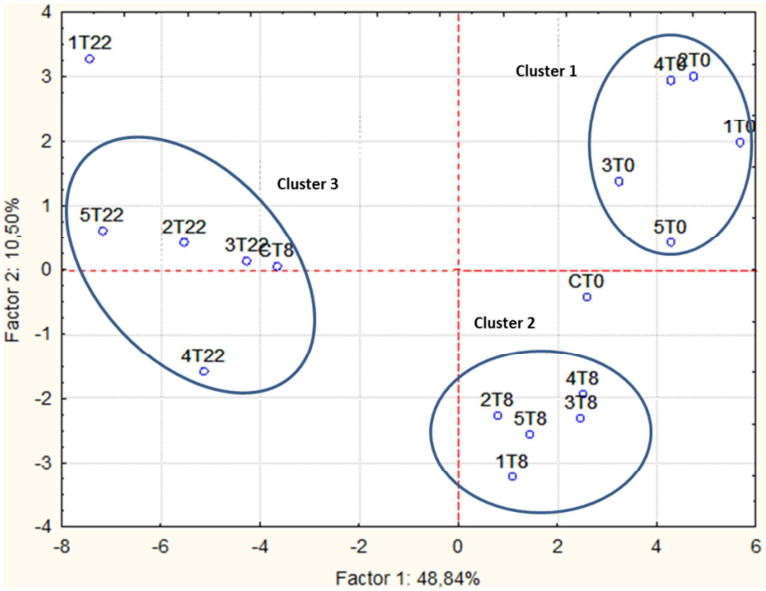
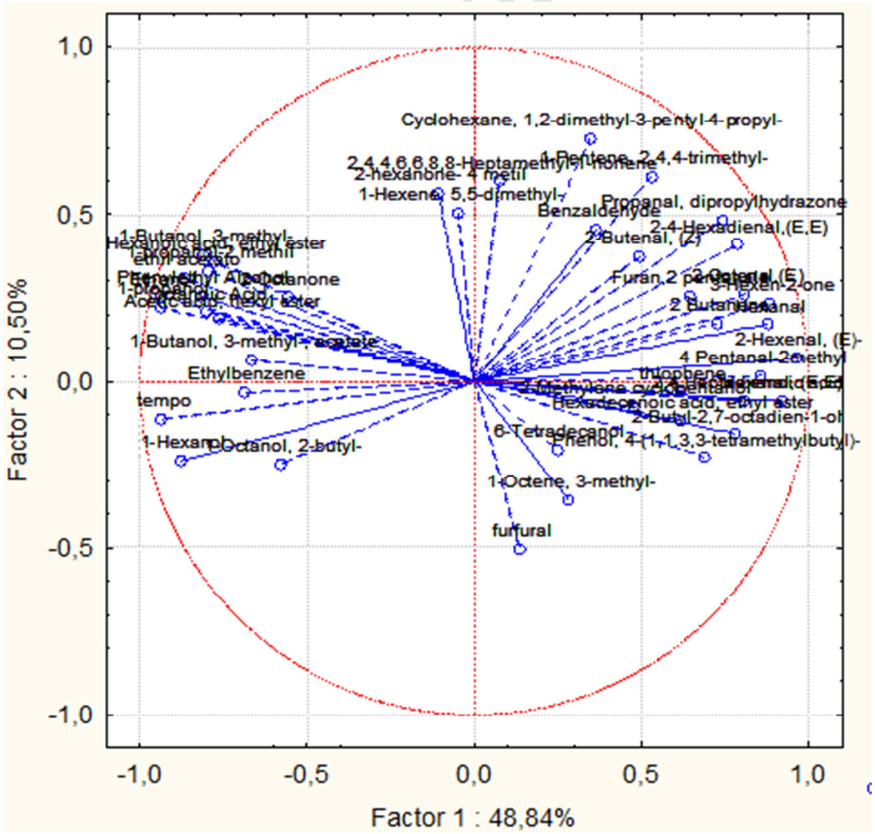
Figure 5.

a



C: 0.1 MPa (control); 1: sample treated at 0.1 MPa added of NE obtained after 14c at 300 MPa; 2: sample added of NE (obtained after 14c at 300 MPa) and treated at 100 MPa x 1; 3: sample added of NE (obtained after 14c at 300 MPa) and treated at 100 MPa x 2; 4: sample added of NE (obtained after 14 c at300 MPa) and treated at 100 MPa x 2; 5: sample added of NE (obtained after 14 c at300 MPa) and treated at 200 MPa x 2

b



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Highlights

Obtained Nanoemulsions were stable for more than one year

Obtained Nanoemulsions were colourless, water soluble, and gifted of antimicrobial properties

Obtained Nanoemulsions combined with high pressure homogenization increased juice shelf-life

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Dear Editor in Chief

Hereby, I declare that no conflict of interest exists for the publication of the present research.

Faithfully

Francesca Patrignani

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