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Abstract

In this study the effect of a high pressure homogenization (HPH) treatment, alone and in combination with a biodegradable polylactic acid (PLA) packaging activated with lysozyme on a smoothie made with apple, carrot and rice beverage was evaluated. In particular, the effects of the treatments on the microbiological and quality characteristics of the smoothies were assessed during storage at 4 and 10 °C in climatic chambers at RH of 50%. Obtained results showed the efficacy of HPH treatment at 100 MPa to reduce the initial cell load of mesophilic and psycothrophic aerobic bacteria, lactic acid bacteria, yeasts and aerobic spore-forming bacteria. Moreover, the combination of HPH and lysozyme activated packaging resulted in an increased antimicrobial effect against mesophilic, psychotropic and lactic acid bacteria during product storage at 10 °C. Yeast and spore forming bacteria showed, instead, lower cell loads independently from the samples. Samples packed in active packaging, both treated and not, showed also a better color retention in terms of lightness and red index, compared to the control (C) ones both when stored at 4 and 10 °C. The HPH treated smoothies, both activated and not, stored at 4 °C remained microbiologically stable for more than 20 days. However, the use of active packaging has also allowed to reduce the microbiological proliferation during storage at 4 °C. Moreover, few differences in color indices were highlighted between samples stored at 4 °C. Obtained results indicate that the combination of HPH treatment and active packaging may represent a useful strategy to increase safety and shelf-life of vegetable smoothies.

Keywords: High Pressure, lysozyme, active packaging, cold plasma, shelf- life, vegetable juices, combined treatments, quality, safety

1. Introduction

Vegetable smoothies are currently very popular beverages, which are consumed by people of all ages for their nutritional and sensory properties. They are perceived by consumers as "healthy" foods due to their low sodium, cholesterol, fat content and because they are rich in vitamin C, polyphenols and flavonoids. The latter compounds contribute to the good antioxidant properties of these products (Kumar et al. 2009; Patrignani, Vannini, Kamdem, Lanciotti, & Guerzoni, 2009; Patrignani et al. 2019). Furthermore, the current lifestyle has led consumers to look for more practical and ready-to-eat products (Andrés, Villanueva, & Tenorio, 2016). For these reasons, one of the fastest growing sectors in the fruit juice market in the last 10 years is represented by smoothies (Nieva, Jagus, Aguero & Fernandez, 2022). Smoothies are blended beverages that can contains fruit pulp, fruit juice, vegetables, yoghurt, milk or vegetable beverage (Nunes et al. 2016). The smoothies are generally minimally processed and not subjected to heat treatments compared to juices that are generally pasteurized this can favor the nutrients bioavailability and the presence of bioactive compounds (Bestwick et al., 2020). Furthermore, mixing multiple ingredients represents a good strategy to incorporate non-traditional and under-utilized vegetables, such as beet leaves and stems, increasing the added value of such products (Jayachandran, Chakraborty, & Rao, 2015). The growing variety of products launched on the market has amplified the issues concerning their safety and stability. Many microorganisms have been associated to these typologies of soft drinks as consequence of environmental or raw material contaminations (Siroli, Camprini, Pisano, Patrignani, & Lanciotti, 2019). In fact, smoothies are a suitable substrate for the growth of yeasts and lactic acid bacteria and if the final pH is not low enough, smoothies can favour the persistence of pathogenic microorganisms,

for this reason are increasingly associated to foodborne illnesses (Bevilacqua et al. 2018). Moreover, the overall quality of these products must remain unchanged over the whole shelf life (Palgan et al. 2012). Therefore, currently are being sought technological solutions aimed to guarantee the safety and stability of the product while preserving its nutritional and functional characteristics and consequently reducing the thermal damage associated with traditional heat treatments (Patrignani & Lanciotti, 2016; Gul, Saricaoglu, Mortas, Atalar, & Yazici, 2017; Yi et al., 2017). Among non-thermal technologies, high pressure homogenization (HPH) is widely reported as a very interesting alternative to traditional heat treatments for the stabilization, in terms of microbial inactivation and quality improvement, of fruit and vegetable beverages including juices based on tomato, apple, carrot, mango, orange, kiwi, sugarcane, apricot, and pomegranate (Donsì Esposito, Lenza, Senatore, & Ferrari, 2009; Patrignani et al., 2009; Patrignani, Tabanelli, Siroli, Gardini, & Lanciotti, 2013; Zhao et al., 2014; Betoret, Calabuig-Jiménez, Patrignani, Lanciotti, & Dalla Rosa, 2017; Patrignani et al., 2019; Singh et al., 2022). Due to the phenomena of cavitation, shear stress, turbulence, and impingement that take place during the food treatment with HPH, a strong antimicrobial activity (Zamora & Guamis, 2015; Patrignani & Lanciotti, 2016), a modulation of some enzyme activities and the maintenance of colour, flavour, and nutritional/functional properties can be observed in the treated matrices (Patrignani et al. 2013; Patrignani & Lanciotti, 2016; Błaszczak, Amarowicz, & Górecki, 2017). In addition, HPH is considered a green and mild technology with a lower impact on the environment, more sustainable, saving energy, time and additional costs since it can be used as a continuous processing treatment over a batch process such as high hydrostatic pressure (HHP) (Patrignani & Lanciotti, 2016; Singh et al., 2022). Several studies have reported that the efficacy of HPH treatments is increased when high-pressure is combined with other sublethal stresses. For example, the combination of HPH treatments with H_2O_2 or low pH is reported to increase the effectiveness against spore forming bacteria such as *Clostridium* spp. and *Bacillus* spp. (Patrignani & Lanciotti, 2016; Chaves-López et al. 2009). Other authors reported that the combined use of natural antimicrobial based nanoemulsions and HPH is able to increase safety and shelf-life of apple juice

due to the improved antimicrobial activity against spoiling *Saccharomyces cerevisiae* and *Lactiplantibacillus plantarum* and pathogens *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* (Patrignani, Siroli, Braschi & Lanciotti, 2020).

Another proposed solution to increase shelf-life and prevent microbial spoilage on food products is represented by active packaging (Mastromatteo, Mastromatteo, Conte, & Del Nobile, 2010). Different types of active packaging, based on the controlled release of the antimicrobials added either in the packaging itself or in additional elements positioned inside the package, are currently studied. The polymeric materials that can be used are different but, among the biodegradable polymers one of the most interesting is the polylactic acid (PLA), produced mainly from renewable agricultural resources, thanks to starch fermentation and the condensation of lactic acid (Krishnamurthy, Demirci, Puri, & Cutter, 2004). Recently, PLA activated with antimicrobial compounds has been proposed in food packaging (Jin & Zhang, 2008; Glicerina et al. 2021). Among antimicrobial molecules employed in active packaging, lysozyme showed interesting properties (Glicerina et al., 2021). In fact, lysozyme is a polypeptide with antimicrobial activity especially against Gram-positive bacteria (Iucci, Patrignani, Vallicelli, Guerzoni, & Lanciotti, 2007) and is one of the most studied natural antimicrobial agents for application in packaging to be used for food preservation (Barbiroli et al. 2012; Corradini et al. 2013; Ozer, Uz, Oymaci, & Altinkaya, 2016). It is classified as "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) and as a food additive by the European Union (E1105) with bacteriostatic, bacteriolytic and bactericidal activity (Muriel-Galet Talbert, Hernandez-Munoz, Gavara, & Goddard, 2013) currently it is widely used in the wine sector and in the production of Grana Padano (Wu et al. 2019). Recently, Glicerina et al. (2021) showed a strong *in-vitro* antimicrobial activity of PLA biodegradable packaging activated with lysozyme by cold plasma against several spoilage and pathogenic microorganisms associated to the food industry. The authors showed that this innovative packaging was able to prevent the growth of *Listeria monocytogenes* in rice based smoothie stored at 4 and 10 °C, allowing a better colour retention during storage compared to the controls.

In this context, the aim of this study was to evaluate the effect of an HPH treatment (100 MPa for 6 times), alone and in combination with the packaging in polylactic acid (PLA), activated with lysozyme by cold plasma, on the shelf-life of a smoothie consisting of a mixture of apple, carrot and rice based beverage, during storage at 4 °C and 10 °C, at 50% RH, in order to simulate real and accelerated storage conditions.

2. Material and methods

2.1. Smoothie preparation

The smoothie used in this study was made with apple, carrot and rice based beverage. Carrots and apples were purchased from a local market on the same day of the trial, sanitized in a solution of 120 ppm of sodium hypochlorite for two minutes and dried with a tissue paper; after cutting, their juice was extracted by a juice extractor (R.G.V. - Juice Art, Como, Italy). To avoid negative phenomena due to enzymatic browning, citric acid (Sigma Aldrich) was added at a concentration of 1 g/L. The rice-based drink used in the smoothie preparation was a commercial Ultra High Temperature (UHT) rice beverage (Alce nero, Bologna, Italy). Carrot extract, apple extract and rice beverage were finally mixed in a 3:1:1 ratio to obtain the final smoothie mixture. The final pH of the smoothie was 5.25.

2.2. Selection of the appropriate high-pressure homogenization treatment (HPH)

The prepared carrot/apple/rice milk smoothie was immediately subjected to HPH treatment by using a continuous high-pressure homogenizer PANDA (Gea, Parma, Italy) provided of a thermal exchanger and a PS-type valve. Prior to the use, the device was sterilized according to the manufacture's suggestions: 1% NaOH water solution, hot sterilized water, and finally refrigerated sterilized water. A total of 3 L of smoothie was prepared and treated by HPH at 100 MPa; after each passage at 100 MPa, an aliquot was collected. In fact, 150 mL were collected in sterile flask respectively after 1, 3, 5 and 6 passes at 100MPa. An aliquot of smoothie was treated at 0.1 MPa (control samples). Immediately after treatment, samples were microbiologically analysed for

153 mesophilic and psychotropic aerobic bacteria, yeasts, lactic acid bacteria, aerobic spore forming
154 bacteria and total coliforms according to the methodology reported below (2.5).
155

156 **2.3. PLA film activation and pouches realization**

157 The polymeric material and the supporting gel used for this study were respectively polylactic acid
158 (PLA) and polyvinyl alcohol (PVOH). PLA films were purchased by Taghleef Industries, S.p.A (San
159 Giorgio di Nogaro, Italy) while polyvinyl alcohol (PVOH) (MW = 88,000–97,000) was purchased
160 by Sigma–Aldrich (Gallarate, Italy). The active compound was lysozyme from hen egg white
161 (Sigma–Aldrich, Gallarate, Italy).
162 The PLA activation and packaging production were carried out according with the previous study of
163 Glicerina et al. (2021), by using a cold plasma treatment in order to activate one surface of the PLA
164 and promoting the adhesion between PLA and PVOH that forming a coating, entraps enzyme causing
165 its immobilization.
166

167 **2.4. High pressure homogenization (HPH) treatment and packaging**

168 Twelve litres of carrot/apple/rice smoothie were divided into two batches, one was subjected to 0.1
169 MPa treatment (used as control) and the other to 100 MPa for 6 cycles. This HPH treatment was
170 selected after preliminary trials aimed to evaluate the microbiological quality after different HPH
171 treatment cycles, as previous described in section 2.2. Half of each treated smoothie was used to fill
172 the PLA activated pouches and half was filled in PLA not activated pouches (control). A total of 204
173 pouches were obtained. Each pouch was filled with 50 mL of smoothie. The filling of the pouches
174 was performed in sterile conditions by using an automatic pipette, under a laminar flow cabinet, and
175 then sealed off on the upper part by using an automatic sealer according with the previous study of
176 Glicerina et al., (2021). Four different smoothie samples were obtained:
177 - Smoothies treated at 0.1 MPa and packed in not activated PLA pouches (C 0.1)
178 - Smoothies treated at 0.1 MPa and packed in lysozyme activated PLA pouches (L 0.1)

- Smoothies treated at 100 MPa for 6 cycles and packed in not activated PLA pouches (C 100)
- Smoothies treated at 100 MPa for 6 cycles and packed in lysozyme activated PLA pouches (L 100).

Obtained samples were stored at two different temperatures, 4 and 10 °C, in climatic chambers at 50% RH, for respectively 24 and 13 days, in order to simulate real and accelerated storage conditions. Smoothies stored at 4 °C were analysed in triplicate at 0, 2, 4, 6, 8, 10, 13, 15 and 24 days for pH and colour determinations and at 0, 4, 8, 13, 15 and 20 days for microbiological ones. At the same time, smoothies stored at 10 °C were analysed in triplicate at 0, 1, 2, 3, 4, 6, 8, 10 and 13 days for pH and colour parameters and at 0, 2, 4, 6, 8, 10 and 13 days for microbiological ones.

A total of 240 pouches were obtained.

In figure 1 a picture of an obtained pouch has been reported, by way of explanation.

Analytical determinations

2.5. Microbiological analysis

The cell loads of natural occurring mesophilic aerobic bacteria, psychotropic aerobic bacteria, lactic acid bacteria, yeasts, aerobic spore forming bacteria and total coliforms were detected by plate counting on Plate Count Agar (PCA) (Oxoid Ltd., Basingstoke, United Kingdom), de Man Rogosa and Sharpe (MRS) (Oxoid Ltd., Basingstoke, United Kingdom), Yeast Extract Peptone Dextrose (YPD) (Oxoid Ltd., Basingstoke, United Kingdom), PCA and Violet Red Bile Agar (VRBA) (Oxoid Ltd., Basingstoke, United Kingdom) according to Siroli et al. (2015). PCA and YPD plates were incubated at 30 °C for 48h. MRS and VRBA plates were incubated at 37 °C for 24h while PCA agar plates for psychotropic bacteria were stored at 10 °C for 7 days. The presence of *L. monocytogenes*, *Salmonella enteritidis*, and *E. coli* in all samples was investigated according to the ISO methods 6579-1:2017 (2017); ISO 11290-1:2017 (2017); and ISO 16649-3:2015 (2015), respectively. According to literature data, *L. monocytogenes*, *Salmonella enteritidis* and *E. coli* must be absent in 25g of product,

while the limit of acceptability of yeasts and mesophilic aerobic bacteria is reported to be 6.0 log CFU/g.

2.6. pH and colour

pH was measured immediately after treatments and at each storage time by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain).

Colour of samples treated at 0.1 and 100 MPa and packed in both activated (L) and not activated pouches (C) at 10 and 4 °C, was determined by using a tristimulus spectrophotocolorimeter (mod. A60-1010-615 ColorFlex, HunterLab, USA) equipped with a sample holder (12 mm diameter). Colour was measured using the CIE *L*a*b** colour space and illuminant D65, and expressed as lightness (L*), and red index (a*) values calculated as reported by Mc Guire, (1993).

2.7. Statistical analysis

At each time of storage, the data obtained represent the means of three independents replicates. Analysis of variance (ANOVA) and the test of mean comparisons according to Fisher's least significant difference (LSD) with a 0.05 level of significance were applied to find out significant differences among the different samples. The statistical package STSG Statistica for Windows, version 6.0 (Statsoft Inc., Tulsa, USA) was used.

3. Results and discussion

3.1. Effect of HPH treatments on microbiological quality of apple-carrot-rice smoothie

In table 1 the cell loads of mesophilic aerobic bacteria, psycothrophic aerobic bacteria, lactic acid bacteria, yeasts, aerobic spore forming bacteria and total coliforms, immediately after the HPH

228 treatments, are reported. In particular, the effect of the 100 MPa treatment, for a different number of
229 cycles (1, 3, 5 and 6), on the initial microbial population of the considered smoothies was evaluated.
230 As expected, the HPH treatment performed lead to a strong inactivation effect as the number of HPH
231 cycles increased. One HPH cycle at 100 MPa was enough to reduce the cell loads of lactic acid
232 bacteria (LAB) and yeasts below the detection limit. The increase of the number of HPH passes
233 significantly reduced the cell load of total mesophilic and psychotropic aerobic bacteria. One cycle
234 at 100 MPa reduced of more than 1.5 logarithmic cycles the total aerobic population, while 3 and 5
235 100 MPa cycles increased the inactivation to 3.06 and 3.23 logarithmic cycles, respectively. Finally,
236 6 passes at 100 MPa (C 100) reduced the mesophilic and psychotropic population of 3.74 and 3.93
237 logarithmic cycles, respectively. The observed microbial inactivation suggests an additive effect of
238 each HPH cycle but without a linearity in terms of reduction of the microbial load. Generally,
239 the effectiveness of HPH treatments is major against gram-negative bacteria and is also
240 dependent on the initial microbial load (Patrignani & Lanciotti, 2016; Patrignani et al., 2019).
241 Regarding the additive effect of HPH cycles on microbial deactivation, literature data are
242 contradictory. In fact, some authors indicate a non-additive behaviour of multiple HPH cycles
243 on microbial population, attributing this trend to the physiological diversity of microbial
244 populations and to the presence of resistant cells from the starting microbiota capable to survive
245 the cycles at the applied pressure (Donsì et al., 2009; Patrignani et al. 2013, Patrignani &
246 Lanciotti, 2016). On the basis of the results obtained, the treatment at 100 MPa for 6 cycles
247 was selected as the most suitable for the further shelf-life trials because it showed a
248 significant reduction of microbial population (mesophilic and psychotrophic aerobic bacteria),
249 compared to treatment for 5 cycles.

250

251 **3.2. Effect of HPH treatments combined or not with active packaging on smoothies**
252 **microbiological growth during storage**

253 The effect of the selected hyperbaric treatment (100 MPa for 6 cycles), combined or not
with lysozyme activated PLA packaging, on the microbiological shelf-life of apple, carrot
and rice

254 smoothie samples was evaluated. Several microbiological groups associated to the spoilage of this
255 kind of products were considered over storage at 4 and at 10 °C.

256 ***Storage at 10 °C***

257 Microbiological results obtained during storage at 10 °C indicate the mesophilic aerobic bacteria and
258 psychotropic aerobic bacteria as mainly responsible for the microbiological spoilage of the smoothies
259 (Table 2). On the contrary, yeasts and spore forming bacteria were not present at spoilage levels for
260 all the 13 days of storage considered. The pathogenic microorganisms *L. monocytogenes*, *Salmonella*
261 spp., and *E. coli* were never detected in the samples stored at 10 °C, independently on the treatment
262 applied (data not shown).

263 Moreover, the stabilizing treatment performed by HPH (6 steps at 100 MPa) allowed a significant
264 reduction of the initial microbial load of all the microbiological groups considered. In fact, the starting
265 load of yeasts, lactic bacteria and spore forming bacteria was below the detection limit (0.5 log
266 CFU/mL). Only total mesophilic aerobic bacteria and psychotropic ones were present in the HPH
267 treated samples respectively at 1.67 and 1.75 log CFU/mL, but with a cell load significantly lower
268 compared to the C.01 samples (total mesophilic and psychotropic ranged between 5.4 and 5.7 log
269 CFU/mL). LAB, yeasts and aerobic spore-forming bacteria which were present at a level ranging
270 between 1.1 and 1.9 log CFU/mL in untreated samples, were found to be below the detection limit
271 following hyperbaric treatment. On the other hand, HPH technology is widely reported to
272 significantly reduce the spoilage microbiota in fruit juices (Patrignani et al., 2013, 2019; Mesa et al.,
273 2020). For example, Patrignani et al. (2019) observed a reduction in yeast load of kiwi juice by more
274 than 2 logarithmic cycles following an HPH treatment at 200 MPa for two cycles. In addition,
275 Patrignani, Siroli, Braschi, & Lanciotti (2020) showed a decrease of the cell load of *S. cerevisiae*,
276 deliberately inoculated in apple juice, of almost 3 log cycles following an HPH treatment at 200 MPa
277 x 2 cycles.

278 During the storage, mesophilic and psychotropic bacteria quickly overcome the level of 7.0 log
279 CFU/mL within 2 days in smoothie samples not subjected to hyperbaric treatment and packed in not

activated pouches (C 0.1) and after 6 days in samples packed in activated pouches (L 0.1). Due to the rapid spoilage, the not HPH treated samples have been analysed until day 4 and day 6 when packed in traditional or active packaging respectively.

Regarding the HPH treated samples, mesophilic and psychotropic bacteria showed a slower growth kinetic in samples L 100 packed in activated pouches compared to the ones packed in not activated (C 100). In fact, the cell load of mesophilic bacteria, starting from the second day of storage, resulted significantly lower in L 100 samples respect to the C 100 samples. These differences were further increased in the following storage days and after 3 days, samples L 100 showed a mesophilic load lower of more than 2.0 log cycles compared to the controls (C 100). Also, LAB cell load was affected by the lysozyme activated packaging. On the other hand, lysozyme is an enzyme active mainly against Gram-positive bacteria because of its specific activity is to lyse the cell walls by hydrolyzing the linkage β (1-4) between the monomers of peptidoglycan (Iucci et al. 2007).

In fact, the samples packed in activated pouches (L 100), starting from the sixth day of storage, presented a significantly lower cell load compared to the C 100 samples. Yeasts and spore forming bacteria showed limited cell loads independently on the sample. However, in the case of yeasts, in samples L 100, they resulted below the detection limit (0.5 log CFU/mL) for the whole period of storage. On the contrary, starting from the eighth day of storage, the yeasts were found in the C 100 samples highlighting how the HPH treatment applied induced sub-lethal damages on the yeast population. In fact, as a result of HPH treatment part of the most resistant microbial population can be viable but not culturable (VBNC) and recovers their metabolic activity under favourable environmental conditions (Patrignani et al. 2019). Finally, no significant differences were found between the samples regarding spore forming bacteria that showed cell loads below 2.0 log CFU/mL for the whole period of storage independently to the sample considered.

As reported in literature, in vegetable matrices the threshold of total aerobic mesophilic bacteria which leads to spoilage of the product is generally considered 6.0 log CFU/g (Stannard, 1997; FSA of Ireland, 2016). As previous stated, in these trials, the not HPH treated samples (L 0.1 and C 0.1)

exceeded this limit after 2 days of storage at 10 °C, independently on the packaging type. However, L 0.1 samples resulted borderline showing a total mesophilic cell load of 6.11 log CFU/mL while C 0.1 samples showed a cell load of 7.46 log CFU/mL. Contrarily, in HPH treated samples, the packaging showed a higher effect, since the control samples (C100) exceeded the threshold limit of total aerobic mesophilic bacteria after 8 days of storage while the samples in active pouches, at the eighth day of storage still had a relatively low mesophilic load (3.49 log CFU/mL). In activated and treated pouches the limit of 6.0 log CFU/mL was exceeded only at the thirteenth day of storage. This represents a very interesting result, indicating the possibility of increasing the shelf-life of vegetable smoothies by combining active packaging and HPH technologies and preventing the negative effects of thermal abuse during the refrigerated storage. Other authors reported a microbial stabilization of mango, orange and apple juices after HPH treatments at 200-300 MPa for 1-3 cycles with inlet temperatures ranging between 20 and 60 °C in products stored at 12-20 °C up to 28 days (Suárez-Jacobo et al. 2010; McKay, Linton, Stirling, Mackle, & Patterson, 2011; Guan et al. 2016). However, all the reported authors have considered fruit juices with an extremely acidic pH ranging between 3.2 and 4.1, that, as known, represents a very strong hurdle for microbial growth. In this context, the microbial stabilization of a vegetable smoothie characterized by higher pH values as in the present study (5.35 for C100 and L 100 respectively at 8 and 13 days) represents a promising result. The use of the lysozyme activated packaging allowed an increase of 5 shelf-life days of the smoothie. On the other hand, lysozyme is effective against different Gram-positive bacteria including spoiling lactic acid bacteria in wine and beer fermentation (Silvetti, 2010; Azzolini, Tosi, Lorenzini, Finato, & Zapparoli, 2015). In addition, Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile (2006) showed that lysozyme immobilized on polyvinyl alcohol-based film was able to inhibit the growth of viable vegetative cells of *Alicyclobacillus acidoterrestris* including spores. Glicerina et al. (2021), showed that PLA biodegradable packaging activated with lysozyme was able to inhibit several spoiling and pathogenic microorganisms both *in-vitro* and in real systems and was characterized by a gradual release of lysozyme into a milk-based smoothie during refrigerated storage at 10 °C.

In addition, it is widely reported that microbial cells may be only partially damaged by HPH treatment, resulting viable but not culturable, and can easily recover their metabolic activity during the storage in particular in a nutrient-rich medium (Patrignani et al. 2009; Patrignani & Lanciotti, 2016; Patrignani et al. 2019). The combination of the HPH treatment with an active packaging that slowly releases lysozyme during the storage, may increase the antimicrobial activity of the peptide since the partially damaged microbial cells are more sensitive; the result is an effective control of microbial spoilage on the product during storage. On the other hand, extensive literature reports that the effects of combined stresses (chemical and physical) on microbial growth and survival may be synergistic (Patrignani & Lanciotti, 2016). In fact, even if the antimicrobial efficacy of lysozyme is almost exclusively against gram-positive bacteria, the damage of the cell wall of gram-negative bacteria caused by HPH treatment results in an increase in the antimicrobial activity of lysozyme also against these last microorganisms.

Storage at 4 °C

In tables 3 the cell loads of total mesophilic aerobic bacteria and psychotropic aerobic bacteria of samples stored at 4 °C are reported in relation to the treatment applied.

Microbiological results of smoothie samples stored at 4 °C highlighted the good efficacy of the HPH pre-treatment to stabilize the initial vegetable matrix. In fact, with the exception of mesophilic and psychotropic aerobic bacteria, present in C 100 and L 100 samples at a very low level (1.67 log CFU/mL and 1.75 CFU mL), all the other microbiological groups (lactic acid bacteria, yeasts, total coliforms and aerobic spore forming bacteria) were below the detection limit (data not shown). The not HPH treated samples reached the spoilage threshold within 8 days when packed in not activated pouches (C 0.1) and 13 days in activated pouches (L 0.1). On the contrary, HPH treated samples stored at 4 °C never exceeded the level of 3.0 log CFU/mL for total mesophilic and psychotropic bacteria over the 20th day of storage at 4 °C.

At the storage temperature of 4 °C, no particular differences were found between the HPH treated control samples and those packed in active pouches. In both cases, the microbiological groups were present at very low levels throughout the whole period of storage considered.

In HPH treated samples, the mesophilic aerobic bacteria did not reach the spoilage threshold of the product and never exceed the load of 2.7 log CFU/mL for all the storage periods. The pathogenic microorganisms *L. monocytogenes*, *Salmonella* spp., and *E. coli* were never detected in the samples stored at 4 °C independently to the treatment applied (data not shown).

The obtained results suggest that the HPH treatment may provide a microbial stabilization of vegetable smoothies at 4 °C. A comparable effect was observed by McKay et al. (2011) that reported a total aerobic count ranging between 2 and 3 log CFU/mL in HPH treated apple juice at 300 MPa for 1 cycle during a storage of 35 days at 4 °C. Other authors reported a microbial stabilization of apple juice treated at 200-300 MPa for 1-3 cycles and stored at 4 °C up to 28-35 days (Suárez-Jacobo et al. 2010; Maresca, Donsì, & Ferrari, 2011). Guan et al. (2016) showed that HPH treatments at 190 MPa for 1 and 3 cycles at an inlet temperature of 60 °C provided a long shelf-life up to 60 days of mango juice stored at 4 °C and no microorganisms increased during storage. Also, Patrignani et al. (2019), showed that the application of a treatment at 200 MPa for 3 cycles allowed to obtain a stable kiwifruit juice for more than 40 days under refrigerated storage and to extend the shelf-life of 1 week at room temperature with respect to the control, increasing at the same time the polyphenols availability and its antioxidant activity, and allowing to better retain the colour.

3.3. Effect of HPH treatments combined or not with active packaging on smoothies' pH and colour during storage

The pH values of smoothies treated with HPH and stored at 4 °C were 5.08±0.03 for both C and L samples and did not change during storage. On the contrary, in not HPH treated samples a rapid acidification was observed during the storage at 10 °C. In fact, the pH resulted below 5.0 after 2 and 4 days in samples packed in not activated and activated pouches respectively. In the not HPH treated

383 smoothies stored at 4 °C there was a pH decrease below 5.0 after 4 and 8 days of storage in C 0.1 and
384 L 0.1 samples, respectively.
385 As for colour of smoothies stored at 10 °C, it is possible to highlight that both C samples presented a
386 greater lightness decrease (Figure 2a) and red index increase (Figure 2b), than samples packed in
387 activated pouches, during all storage times, despite of the HPH treatment. This means that the colour
388 of smoothie samples packed in not activated pouches showed a greater and faster darkening during
389 storage, probably as a result of browning reactions due to oxidation phenomena of polyphenols,
390 carotenoids and other phytochemicals present in carrot and apple juice (Riganakos, Karabagias,
391 Gertzou, & Stahl, 2017). In both L samples, independently by HPH treatment, a general lower
392 browning was observed during storage compared to C ones. According with literature, these results
393 can be explained considering that the lysozyme tend to bind with phenolic compounds present into
394 the smoothie that probably, protecting phenols from oxidation, reduce browning phenomena (Green,
395 1995; Bartowsky, Costello, Villa, & Hensche, 2004; Rawduken, Suthiluk, Kamhangwong, &
396 Benjakul, 2012; Liburdi, Benucci & Esti, 2014). Moreover, in all four samples an increase in the lightness
397 was observed after 3 days of storage subsequently to its reduction, probably attributable to the presence of
398 unstable, suspended particles in juice that tends to settle during storage (Cortes et al., 2008; Cao et al., 2012).
399 Figure 3 (a, b) present the changes in lightness (L*) and red index (a*) colour values of all smoothie
400 samples during storage at 4 °C. Also, in this case, it was observed a similar trend as for samples stored
401 at 10 °C. Both L samples, packed in activated pouches and pre-treated at 0.1 and 100 MPa, presented
402 respectively higher lightness and lower red index values than C ones, corresponding to a general
403 greater colour maintenance. However, the colour values between L and C samples, mainly the L*
404 ones, did not always differ significantly during storage.
405

406 **4. Conclusions**

407 The results obtained in the present research highlighted the potential of HPH treatment combined to
408 a lysozyme active biodegradable packaging for the stabilization of a vegetable smoothie characterized

by a low-acidic pH (5.25). In fact, the applied HPH treatments allowed to reduce the initial microbial load of the smoothie thus slowing down the deterioration of the product stored at both 4 and 10 °C. The increased antimicrobial activity observed by the active packaging is related to the initial reduction of the load of microbial population that was more sensitive to the released lysozyme. The combination of these two technologies allowed the smoothie to reach a shelf-life of 13 days at 10 °C, compared to the only HPH treated product that showed a shelf-life of 8 days. Moreover, the HPH treated smoothies stored at 4 °C remained microbiologically stable for more than 20 days, independently from the packaging. However, the use of active packaging has also allowed to reduce the microbiological proliferation. In addition, L samples packed in active packaging (both HPH treated and not) showed a general better color retention, compared to C ones. In conclusion, the combination of the technologies used in this experiment could be an innovative strategy for increasing the safety and shelf-life of low-acid innovative drinks; the use of lysozyme active packaging could be also a solution to prevent the negative effects of thermal abuse during the refrigerated storage. However, further analyses are necessary to verify the perception of the consumer towards this type of minimally processed product and to quantify its nutritional and functional benefit.

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Declaration of Competing Interest

The authors report no declarations of interest.

References

Andrés, V., Villanueva, M. J., & Tenorio, M. D. (2016). Influence of high pressure processing on microbial shelf life, sensory profile, soluble sugars, organic acids, and mineral content of milk-and soy-smoothies. *LWT-Food Science and Technology*, *65*, 98-105. https://doi: 10.1016/j.lwt.2015.07.066.

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., & Zapparoli, G. (2015). Contribution to the aroma of white wines by controlled Torulaspora delbrueckii cultures in association with Saccharomyces cerevisiae. *World Journal of Microbiology and Biotechnology*, *31*(2), 277-293. https://doi: 10.1007/s11274-014-1774-1.

Barbiroli, A., Bonomi, F., Capretti, G., Iametti, S., Manzoni, M., Piergiovanni, L., & Rollini, M. (2012). Antimicrobial activity of lysozyme and lactoferrin incorporated in cellulose-based food packaging. *Food Control*, *26*(2), 387-392. https://doi.org/10.1016/j.foodcont.2012.01.046

Bartowsky, B, E. J., Costello, P. J., Villa, A., & Hensche, P. A. (2004). The chemical and sensorial effects of lysozyme addition to red and white wines over six months' cellar storage. *Australian*

Journal of Grape and Wine Research, *10*(2), 143-150. https://doi.org/10.1111/j.1755-0238.2004.tb00017.x

Bestwick, C., Scobbie, L., Milne, L., Duncan, G., Cantlay, L., & Russell, W. (2020). Fruit-based beverages contain a wide range of phytochemicals and intervention targets should account for the individual compounds present and their availability. *Foods, 9(7),* 891. doi: 10.3390/ foods9070891.

Betoret, E., Calabuig-Jiménez, L., A., Patrignani, F., Lanciotti, R., & Dalla Rosa, M. (2017). Effect of high pressure processing and trehalose addition on functional properties of mandarin juice enriched with probiotic microorganisms. *LWT-Food Science and Technology*, *85*, 418-422. https://doi: 10.1016/j.lwt.2016.10.036

Bevilacqua, A., Petruzzi, L., Perricone, M., Speranza, B., Campaniello, D., Sinigaglia, M., & Corbo, M. R. (2018). Nonthermal technologies for fruit and vegetable juices and beverages: overview and advances. *Comprehensive Reviews in Food Science and Food Safety*, *17*(1), 2-62. https:// doi: 10.1111/1541-4337.12299

Błaszczak, W., Amarowicz, R., & Górecki, A. R. (2017). Antioxidant capacity, phenolic composition and microbial stability of aronia juice subjected to high hydrostatic pressure processing. *Innovative Food Science & Emerging Technologies, 39*, 141-147. https://doi.org/10.1016/ j.ifset.2016.12.005Get rights and content

Cao, X., Bi, X., Huang, W., Wu, J., Hu, X., & Liao, X. (2012). Changes of quality of high hydrostatic pressure processed cloudy and clear strawberry juices during storage. *Innovative Food Science & Emerging Technologies*, *16*, 181-190.

Conte, A., Buonocore, G. G., Bevilacqua, A., Sinigaglia, M., & Del Nobile, M. A. (2006). Immobilization of lysozyme on polyvinylalcohol films for active packaging applications. *Journal of Food Protection*, *69*(4), 866-870. https:// doi: 10.4315/0362-028x-69.4.866

Corradini, C., Alfieri, I., Cavazza, A., Lantano, C., Lorenzi, A., Zucchetto, N., & Montenero, A. (2013). Antimicrobial films containing lysozyme for active packaging obtained by sol–gel technique. *Journal of Food Engineering*, *119*(3), 580-587. https://doi: 10.1016/j.jfoodeng.2013.05.046

Cortés, C., Esteve, M. J., & Frígola, A. (2008). Color of orange juice treated by high intensity pulsed electric fields during refrigerated storage and comparison with pasteurized juice. *Food control*, *19*(2), 151-158.

Donsì, F., Esposito, L., Lenza, E., Senatore, B., & Ferrari, G. (2009). Production of shelf-stable annurca apple juice with pulp by high pressure homogenization. *International Journal of Food Engineering*, *5*(4). https://doi: 10.2202/1556-3758.1602

Food Safety Authority of Ireland. (FSA) (2016). Monitoring & Surveillance Series, Chemical. Report on a total diet study carried out by the food safety authority of Ireland in the period 2012-2014. Dublin.

Glicerina, V., Siroli, L., Canali, G., Chinnici, F., Capelli, F., Lanciotti, R., Colombo, V., & Romani, S. (2021). Efficacy of biodegradable, antimicrobial packaging on safety and quality parameters maintenance of a pear juice and rice milk-based smoothie product. *Food Control*, *128*, 108170. https://doi.org/10.1016/j.foodcont.2021.108170

Green, J. L. (1995). The use of lysozyme in winemaking: the interaction of lysozyme with wine and efficacy in preventing malolactic fermentation in Oregon Pinot noir and Chardonnay. Oregon State University.

Guan, Y., Zhou, L., Bi, J., Yi, J., Liu, X., Chen, Q., Wu, X., & Zhou, M. (2016). Change of microbial and quality attributes of mango juice treated by high pressure homogenization combined with moderate inlet temperatures during storage. *Innovative Food Science and Emerging Technologies, 36*, 320-29. https://dx.doi.org/10.1016/j.ifset.2016.07.009

Gul, O., Saricaoglu, F. T., Mortas, M., Atalar, I., & Yazici, F. (2017). Effect of high pressure homogenization (HPH) on microstructure and rheological properties of hazelnut milk. *Innovative Food Science and Emerging Technologies, 41*, 411–420. doi: 10.1016/j.ifset.2017.05.002

Iucci, L., Patrignani, F., Vallicelli, M., Guerzoni, M. E., & Lanciotti, R. (2007). Effects of high pressure homogenization on the activity of lysozyme and lactoferrin against *Listeria monocytogenes*. *Food Control*, *18*, 558–565. https://doi: 10.1016/j.foodcont.2006.01.005

Jayachandran, L. E., Chakraborty, S., & Rao, P. S. (2015). Effect of high pressure processing on physicochemical properties and bioactive compounds in litchi based mixed fruit beverage. *Innovative Food Science & Emerging Technologies*, *28*, 1-9. https://doi.org/10.1016/j.ifset.2015.01.002

Jin, T., & Zhang, H. (2008). Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. *Journal of Food Science*, *73*(3), M127-M134. https://doi: 10.1111/j.1750-3841.2008.00681.x

Krishnamurthy, K., Demirci, A., Puri, V. M., & Cutter, C. N. (2004). Effect of packaging materials on inactivation of pathogenic microorganisms on meat during irradiation. *Transactions of the ASAE*, *47*(4), 1141. https://doi: 10.13031/2013.16546

Kumar, S., Thippareddi, H., Subbiah, J., Zivanovic, S., Davidson, P. M., & Harte, F. (2009). Inactivation of Escherichia coli K-12 in apple juice using combination of high-pressure homogenization and chitosan. *Journal of Food Science*, *74*(1), M8-M14. https://doi.org/10.1111/j.1750-3841.2008.00974.x

Liburdi, K., Benucci, I., & Esti, M. (2014). Lysozyme in wine: an overview of current and future applications. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 1062-1073. https://doi.org/10.1111/1541-4337.12102

546 Maresca, P., Donsì, F., & Ferrari, G. (2011). Application of a multi-pass high-pressure
547 homogenization treatment for the pasteurization of fruit juices. *Journal of Food Engineering, 104(3),*
548 364–372. https://doi: 10.1016/j.jfoodeng.2010.12.030

549

550 Mastromatteo, M., Mastromatteo, M., Conte, A. & Del Nobile, M.A., 2010. Advances in controlled
551 release devices for food packaging applications. *Trends in Food Science & Technology*, *21*(12),
552 pp.591-598. https://doi.org/10.1016/j.tifs.2010.07.010

553

554 McGuire, R. G. (1992). Reporting of objective color measurements. *HortScience*, *27*(12), 1254-1255.
555 https://doi.org/10.21273/HORTSCI.27.12.1254

556

557 Mesa, J., Hinestroza-Córdoba, L. I., Barrera, C., Seguí, L., Betoret, E., & Betoret, N. (2020). High
558 homogenization pressures to improve food quality, functionality and sustainability. *Molecules, 25,*
559 3305. https://doi: 10.3390/molecules25143305

560

561 McKay, A. M., Linton, M., Stirling, J., Mackle, A., & Patterson, M. F. (2011). A comparative study
562 of changes in the microbiota of apple juice treated by high hydrostatic pressure (HHP) or high
563 pressure homogenisation (HPH). *Food Microbiology, 28(8),* 1426–1431.
564 https://doi.org/10.1016/j.fm.2011.06.013

565

566 Muriel-Galet, V., Talbert, J. N., Hernandez-Munoz, P., Gavara, R., & Goddard, J. M. (2013).
567 Covalent immobilization of lysozyme on ethylene vinyl alcohol films for nonmigrating antimicrobial

packaging applications. *Journal of Agricultural and Food Chemistry, 61*(27), 6720-6727. https://doi: 10.1021/jf401818u

Nieva, S. G., Jagus, R. J., Agüero, M. V., & Fernandez, M. V. (2022). Fruit and vegetable smoothies preservation with natural antimicrobials for the assurance of safety and quality. *LWT-Food Science and Technology, 154,* 112663. https://doi.org/10.1016/j.lwt.2021.112663

Nunes, M. A., Barreira, J. C. M., Costa, A. S. G., Vinha, A. F., Alves, R. C., Rocha, A., & Oliveira, M. B. P. P. (2016). Influence of packing material on the quality parameters and bioactivity of smoothies. Livro de Atas do XIII Encontro de Química dos Alimentos, 170-173, Porto (Lisbon).

Ozer, B. B. P., Uz, M., Oymaci, P., & Altinkaya, S. A. (2016). Development of a novel strategy for controlled release of lysozyme from whey protein isolate based active food packaging films. *Food Hydrocolloids*, *61*, 877-886. https://doi.org/10.1016/j.foodhyd.2016.07.001

Palgan, I., Muñoz, A., Noci, F., Whyte, P., Morgan, D. J., Cronin, D. A., & Lyng, J. G. (2012). Effectiveness of combined Pulsed Electric Field (PEF) and Manothermosonication (MTS) for the control of Listeria innocua in a smoothie type beverage. *Food Control*, *25*(2), 621-625. https://doi.org/10.1016/j.foodcont.2011.11.009

Patrignani, F., Vannini, L., Kamdem, S. L. S., Lanciotti, R., & Guerzoni, M. E. (2009). Effect of high pressure homogenization on Saccharomyces cerevisiae inactivation and physico-chemical features in

apricot and carrot juices. *International Journal of Food Microbiology*, *136*(1), 26-31. https://doi: 10.1016/j.ijfoodmicro.2009.09.021

Patrignani, F., Tabanelli, G., Siroli, L., Gardini, F., & Lanciotti, R. (2013). Combined effects of high pressure homogenization treatment and citral on microbiological quality of apricot juice. *International Journal of Food Microbiology*, *160*(3), 273-281. https://doi.org/10.1016/j.ijfoodmicro.2012.10.021

Patrignani, F., & Lanciotti, R. (2016). Applications of high and ultra high pressure homogenization for food safety. *Frontiers in Microbiology, 7*, 1132. https://doi: 10.3389/fmicb.2016.01132

Patrignani, F., Siroli, L., Braschi, G., & Lanciotti, R. (2020). Combined use of natural antimicrobial based nanoemulsions and ultra high pressure homogenization to increase safety and shelf-life of apple juice. *Food Control, 111*, 107051. https://doi.org/10.1016/j.foodcont.2019.107051

Patrignani, F., Mannozzi, C., Tappi, S., Tylewicz, U., Pasini, F., Castellone, V., Riciputi, Y., Rocculi, P., Romani, S., Caboni, M.F., Gardini, F., Lanciotti, R., & Dalla Rosa, M. (2019). (Ultra) High pressure homogenization potential on the shelf-life and functionality of kiwifruit juice. *Frontiers in Microbiology*, *10*, 246. https://doi.org/10.3389/fmicb.2019.00246

Patrignani, F., Siroli, L., Braschi, G., & Lanciotti, R. (2020). Combined use of natural antimicrobial based nanoemulsions and ultra high pressure homogenization to increase safety and shelf-life of apple juice. *Food Control*, *111*, Article 107051. https://doi.org/10.1016/j.foodcont.2019.107051

Rawdkuen, S., Suthiluk, P., Kamhangwong, D., & Benjakul, S. (2012). Mechanical, physico-chemical, and antimicrobial properties of gelatin-based film incorporated with catechin-lysozyme. *Chemistry Central Journal*, *6*(1), 1-10. https://doi: 10.1186/1752-153X-6-131

Riganakos, K. A., Karabagias, I. K., Gertzou, I., & Stahl, M. (2017). Comparison of UV-C and thermal treatments for the preservation of carrot juice. *Innovative Food Science & Emerging Technologies*, *42*, 165-172. https://doi.org/10.1016/j.ifset.2017.06.015

Silvetti, T. (2010). Development of innovative techniques for studying microbial population in milk and dairy products. Doctoral Thesis.

Singh, S. V., Singh, R., Singh, A., Chinchkar, A. V., Kamble, M. G., Dutta, S. J., & Singh, S. B. (2022). A review on green pressure processing of fruit juices using microfluidization: Quality, safety and preservation. *Applied Food Research*, *2*, 100235. https://doi.org/10.1016/j.afres.2022.100235

Siroli, L., Camprini, L., Pisano, M. B., Patrignani, F., & Lanciotti, R. (2019). Volatile molecule profiles and anti-Listeria monocytogenes activity of nisin producers Lactococcus lactis strains in vegetable drinks. *Frontiers in Microbiology*, *10*, 563. https://doi.org/10.3389/fmicb.2019.00563

Stannard, C. (1997). Development and use of microbiological criteria for foods. *Food Science and Technology Today*, *11*(3), 137-177. https:// doi: 10.2903/j.efsa.2017.5052

635

636 Suárez-Jacobo, A., Gervilla, R., Guamis, B., Roig-Sagués, A. X., & Saldo, J. (2010). Effect of UHPH
637 on indigenous microbiota of apple juice: A preliminary study of microbial shelf-life. *International*
638 *Journal of Food Microbiology*, 136(3), 261–267. https://doi: 10.1016/j.ijfoodmicro.2009.11.011

639

640 Wu, T., Jiang, Q., Wu, D., Hu, Y., Chen, S., Ding, T., Ye, X., Liu, D., & Chen, J. (2019). What is
641 new in lysozyme research and its application in food industry? A review. *Food Chemistry*, 274, 698-
642 709. https://doi.org/10.1016/j.foodchem.2018.09.017

643 Yi, J., Kebede, B., Kristiani, K., Grauwet, T., Van Loey, A., & Hendrickx, M. (2017). Minimizing
644 quality changes of cloudy apple juice: the use of kiwifruit puree and high pressure homogenization.
645 *Food Chemistry, 249*, 202–212. https://doi:10.1016/j.foodchem.2017.12.088

646

647 Zamora, A. and Guamis, B., 2015. Opportunities for ultra-high-pressure homogenisation (UHPH) for
648 the food industry. *Food Engineering Reviews*, *7*(2), pp.130-142. https:// doi 10.1007/s12393-014-
649 9097-4

650

651 Zhao, L., Wang, Y., Wang, S., Li, H., Huang, W., and Liao, X. (2014). Inactivation of naturally
652 occurring microbiota in cucumber juice by pressure treatment. *International Journal of Food*
653 *Microbiology*, *174*, 12–18. https://doi: 10.1016/j.ijfoodmicro.2013.12.023

654

655 Zhou, C. L., Liu, W., Zhao, J., Yuan, C., Song, Y., Chen, D., Ni & Li, Q. H. (2014). The effect of
656 high hydrostatic pressure on the microbiological quality and physical–chemical characteristics of

Pumpkin (Cucurbita maxima Duch.) during refrigerated storage. *Innovative food science & Emerging Technologies*, *21*, 24-34. https://doi.org/10.1016/j.ifset.2013.11.002