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Influence of high-pressure homogenization treatments combined with lysozyme activated packaging on microbiological and technological quality of vegetable smoothie during shelf life

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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 psychotrophic 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.

49

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

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54 **1. Introduction**

55 Vegetable smoothies are currently very popular beverages, which are consumed by people of all ages
56 for their nutritional and sensory properties. They are perceived by consumers as "healthy" foods due
57 to their low sodium, cholesterol, fat content and because they are rich in vitamin C, polyphenols and
58 flavonoids. The latter compounds contribute to the good antioxidant properties of these products
59 (Kumar et al. 2009; Patrignani, Vannini, Kamdem, Lanciotti, & Guerzoni, 2009; Patrignani et al.
60 2019). Furthermore, the current lifestyle has led consumers to look for more practical and ready-to-
61 eat products (Andrés, Villanueva, & Tenorio, 2016). For these reasons, one of the fastest growing
62 sectors in the fruit juice market in the last 10 years is represented by smoothies (Nieva, Jagus,
63 Agüero & Fernandez, 2022). Smoothies are blended beverages that can contains fruit pulp,
64 fruit juice, vegetables, yoghurt, milk or vegetable beverage (Nunes et al. 2016). The smoothies
65 are generally minimally processed and not subjected to heat treatments compared to juices that
66 are generally pasteurized this can favor the nutrients bioavailability and the presence of
67 bioactive compounds (Bestwick et al., 2020). Furthermore, mixing multiple ingredients
68 represents a good strategy to incorporate non-traditional and under-utilized vegetables, such as
69 beet leaves and stems, increasing the added value of such products (Jayachandran, Chakraborty, &
70 Rao, 2015). The growing variety of products launched on the market has amplified the issues
71 concerning their safety and stability. Many microorganisms have been associated to these
72 typologies of soft drinks as consequence of environmental or raw material contaminations
73 (Siroli, Camprini, Pisano, Patrignani, & Lanciotti, 2019). In fact, smoothies are a suitable
74 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,

75 for this reason are increasingly associated to foodborne illnesses (Bevilacqua et al. 2018). Moreover,
76 the overall quality of these products must remain unchanged over the whole shelf life (Palgan et al.
77 2012). Therefore, currently are being sought technological solutions aimed to guarantee the safety
78 and stability of the product while preserving its nutritional and functional characteristics and
79 consequently reducing the thermal damage associated with traditional heat treatments (Patrignani &
80 Lanciotti, 2016; Gul, Saricaoglu, Mortas, Atalar, & Yazici, 2017; Yi et al., 2017). Among non-
81 thermal technologies, high pressure homogenization (HPH) is widely reported as a very interesting
82 alternative to traditional heat treatments for the stabilization, in terms of microbial inactivation and
83 quality improvement, of fruit and vegetable beverages including juices based on tomato, apple,
84 carrot, mango, orange, kiwi, sugarcane, apricot, and pomegranate (Donsì Esposito, Lenza, Senatore,
85 & Ferrari, 2009; Patrignani et al., 2009; Patrignani, Tabanelli, Siroli, Gardini, & Lanciotti, 2013;
86 Zhao et al., 2014; Betoret, Calabuig-Jiménez, Patrignani, Lanciotti, & Dalla Rosa, 2017; Patrignani
87 et al., 2019; Singh et al., 2022). Due to the phenomena of cavitation, shear stress, turbulence, and
88 impingement that take place during the food treatment with HPH, a strong antimicrobial activity
89 (Zamora & Guamis, 2015; Patrignani & Lanciotti, 2016), a modulation of some enzyme activities
90 and the maintenance of colour, flavour, and nutritional/functional properties can be observed in the
91 treated matrices (Patrignani et al. 2013; Patrignani & Lanciotti, 2016; Błaszczak, Amarowicz, &
92 Górecki, 2017). In addition, HPH is considered a green and mild technology with a lower impact on
93 the environment, more sustainable, saving energy, time and additional costs since it can be used as a
94 continuous processing treatment over a batch process such as high hydrostatic pressure
95 (HHP) (Patrignani & Lanciotti, 2016; Singh et al., 2022). Several studies have reported
96 that the efficacy of HPH treatments is increased when high-pressure is combined with
97 other sublethal stresses. For example, the combination of HPH treatments with H₂O₂ or low
98 pH is reported to increase the effectiveness against spore forming bacteria such as *Clostridium*
99 spp. and *Bacillus* spp. (Patrignani & Lanciotti, 2016; Chaves-López et al. 2009). Other authors
100 reported that the combined use of natural antimicrobial based nanoemulsions and HPH is able
to increase safety and shelf-life of apple juice

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

104 Another proposed solution to increase shelf-life and prevent microbial spoilage on food products is
105 represented by active packaging (Mastromatteo, Mastromatteo, Conte, & Del Nobile, 2010).
106 Different types of active packaging, based on the controlled release of the antimicrobials added either
107 in the packaging itself or in additional elements positioned inside the package, are currently studied.

108 The polymeric materials that can be used are different but, among the biodegradable polymers one of
109 the most interesting is the polylactic acid (PLA), produced mainly from renewable agricultural
110 resources, thanks to starch fermentation and the condensation of lactic acid (Krishnamurthy, Demirci,
111 Puri, & Cutter, 2004). Recently, PLA activated with antimicrobial compounds has been proposed in
112 food packaging (Jin & Zhang, 2008; Glicerina et al. 2021). Among antimicrobial molecules employed
113 in active packaging, lysozyme showed interesting properties (Glicerina et al., 2021). In fact, lysozyme
114 is a polypeptide with antimicrobial activity especially against Gram-positive bacteria (Iucci,
115 Patrignani, Vallicelli, Guerzoni, & Lanciotti, 2007) and is one of the most studied natural
116 antimicrobial agents for application in packaging to be used for food preservation (Barbiroli et al.
117 2012; Corradini et al. 2013; Ozer, Uz, Oymaci, & Altinkaya, 2016). It is classified as "generally
118 recognized as safe" (GRAS) by the Food and Drug Administration (FDA) and as a food additive by
119 the European Union (E1105) with bacteriostatic, bacteriolytic and bactericidal activity (Muriel-Galet
120 Talbert, Hernandez-Munoz, Gavara, & Goddard, 2013) currently it is widely used in the wine sector
121 and in the production of Grana Padano (Wu et al. 2019). Recently, Glicerina et al. (2021) showed a
122 strong *in-vitro* antimicrobial activity of PLA biodegradable packaging activated with lysozyme by
123 cold plasma against several spoilage and pathogenic microorganisms associated to the food industry.

124 The authors showed that this innovative packaging was able to prevent the growth of *Listeria*
125 *monocytogenes* in rice based smoothie stored at 4 and 10 °C, allowing a better colour retention during
126 storage compared to the controls.

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

132

133 **2. Material and methods**

134 **2.1. Smoothie preparation**

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

143

144 **2.2. Selection of the appropriate high-pressure homogenization treatment (HPH)**

145 The prepared carrot/apple/rice milk smoothie was immediately subjected to HPH treatment by using
146 a continuous high-pressure homogenizer PANDA (Gea, Parma, Italy) provided of a thermal
147 exchanger and a PS-type valve. Prior to the use, the device was sterilized according to the
148 manufacture's suggestions: 1% NaOH water solution, hot sterilized water, and finally refrigerated
149 sterilized water. A total of 3 L of smoothie was prepared and treated by HPH at 100 MPa; after each
150 passage at 100 MPa, an aliquot was collected. In fact, 150 mL were collected in sterile flask
151 respectively after 1, 3, 5 and 6 passes at 100MPa. An aliquot of smoothie was treated at 0.1 MPa
152 (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,

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

207

208 **2.6. pH and colour**

209 pH was measured immediately after treatments and at each storage time by using a pH-meter Basic
210 20 (Crison Instruments, Barcelona, Spain).

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

216

217 **2.7. Statistical analysis**

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

223

224 **3. Results and discussion**

225 **3.1. Effect of HPH treatments on microbiological quality of apple-carrot-rice smoothie**

226 In table 1 the cell loads of mesophilic aerobic bacteria, psychotrophic aerobic bacteria, lactic acid
227 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 (Donsi 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 psychotropic 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

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

Storage at 10 °C

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

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

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

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

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

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

303 As reported in literature, in vegetable matrices the threshold of total aerobic mesophilic bacteria
304 which leads to spoilage of the product is generally considered 6.0 log CFU/g (Stannard, 1997; FSA
305 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.

332 In addition, it is widely reported that microbial cells may be only partially damaged by HPH
333 treatment, resulting viable but not culturable, and can easily recover their metabolic activity during
334 the storage in particular in a nutrient-rich medium (Patrignani et al. 2009; Patrignani & Lanciotti,
335 2016; Patrignani et al. 2019). The combination of the HPH treatment with an active packaging that
336 slowly releases lysozyme during the storage, may increase the antimicrobial activity of the peptide
337 since the partially damaged microbial cells are more sensitive; the result is an effective control of
338 microbial spoilage on the product during storage. On the other hand, extensive literature reports that
339 the effects of combined stresses (chemical and physical) on microbial growth and survival may be
340 synergistic (Patrignani & Lanciotti, 2016). In fact, even if the antimicrobial efficacy of lysozyme is
341 almost exclusively against gram-positive bacteria, the damage of the cell wall of gram-negative
342 bacteria caused by HPH treatment results in an increase in the antimicrobial activity of lysozyme also
343 against these last microorganisms.

344

345 ***Storage at 4 °C***

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

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

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

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

364 The obtained results suggest that the HPH treatment may provide a microbial stabilization of
365 vegetable smoothies at 4 °C. A comparable effect was observed by McKay et al. (2011) that reported
366 a total aerobic count ranging between 2 and 3 log CFU/mL in HPH treated apple juice at 300 MPa
367 for 1 cycle during a storage of 35 days at 4 °C. Other authors reported a microbial stabilization of
368 apple juice treated at 200-300 MPa for 1-3 cycles and stored at 4 °C up to 28-35 days (Suárez-Jacobo
369 et al. 2010; Maresca, Donsì, & Ferrari, 2011). Guan et al. (2016) showed that HPH treatments at 190
370 MPa for 1 and 3 cycles at an inlet temperature of 60 °C provided a long shelf-life up to 60 days of
371 mango juice stored at 4 °C and no microorganisms increased during storage. Also, Patrignani et al.
372 (2019), showed that the application of a treatment at 200 MPa for 3 cycles allowed to obtain a stable
373 kiwifruit juice for more than 40 days under refrigerated storage and to extend the shelf-life of 1 week
374 at room temperature with respect to the control, increasing at the same time the polyphenols
375 availability and its antioxidant activity, and allowing to better retain the colour.

376

377 **3.3. Effect of HPH treatments combined or not with active packaging on smoothies' pH and** 378 **colour during storage**

379 The pH values of smoothies treated with HPH and stored at 4 °C were 5.08±0.03 for both C and L
380 samples and did not change during storage. On the contrary, in not HPH treated samples a rapid
381 acidification was observed during the storage at 10 °C. In fact, the pH resulted below 5.0 after 2 and
382 4 days in samples packed in not activated and activated pouches respectively. In the not HPH treated

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 L 0.1 samples, respectively.

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

4. Conclusions

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

409 by a low-acidic pH (5.25). In fact, the applied HPH treatments allowed to reduce the initial
410 microbial load of the smoothie thus slowing down the deterioration of the product stored at both
411 4 and 10 °C. The increased antimicrobial activity observed by the active packaging is related to
412 the initial reduction of the load of microbial population that was more sensitive to the released
413 lysozyme. The combination of these two technologies allowed the smoothie to reach a shelf-life of
414 13 days at 10 °C, compared to the only HPH treated product that showed a shelf-life of 8 days.
415 Moreover, the HPH treated smoothies stored at 4 °C remained microbiologically stable for more
416 than 20 days, independently from the packaging. However, the use of active packaging has
417 also allowed to reduce the microbiological proliferation. In addition, L samples packed in active
418 packaging (both HPH treated and not) showed a general better color retention, compared
419 to C ones. In conclusion, the combination of the technologies used in this experiment could
420 be an innovative strategy for increasing the safety and shelf-life of low-acid innovative drinks; the
421 use of lysozyme active packaging could be also a solution to prevent the negative effects of
422 thermal abuse during the refrigerated storage. However, further analyses are necessary to verify
423 the perception of the consumer towards this type of minimally processed product and to
424 quantify its nutritional and functional benefit.

425

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430

431 **Declaration of Competing Interest**

432 The authors report no declarations of interest.

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