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

3  
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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 & Fernández, 2022). Smoothies are blended beverages that can contain 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 H<sub>2</sub>O<sub>2</sub> 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)



- 179 - Smoothies treated at 100 MPa for 6 cycles and packed in not activated PLA pouches (C 100)  
180 - Smoothies treated at 100 MPa for 6 cycles and packed in lysozyme activated PLA pouches (L  
181 100).

182 Obtained samples were stored at two different temperatures, 4 and 10 °C, in climatic chambers at  
183 50% RH, for respectively 24 and 13 days, in order to simulate real and accelerated storage conditions.

184 Smoothies stored at 4 °C were analysed in triplicate at 0, 2, 4, 6, 8, 10, 13, 15 and 24 days for pH and  
185 colour determinations and at 0, 4, 8, 13, 15 and 20 days for microbiological ones. At the same time,  
186 smoothies stored at 10 °C were analysed in triplicate at 0, 1, 2, 3, 4, 6, 8, 10 and 13 days for pH and  
187 colour parameters and at 0, 2, 4, 6, 8, 10 and 13 days for microbiological ones.

188 A total of 240 pouches were obtained.

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

190

191

## 192 **Analytical determinations**

### 193 **2.5. Microbiological analysis**

194 The cell loads of natural occurring mesophilic aerobic bacteria, psychotropic aerobic bacteria, lactic  
195 acid bacteria, yeasts, aerobic spore forming bacteria and total coliforms were detected by plate  
196 counting on Plate Count Agar (PCA) (Oxoid Ltd., Basingstoke, United Kingdom), de Man Rogosa  
197 and Sharpe (MRS) (Oxoid Ltd., Basingstoke, United Kingdom), Yeast Extract Peptone Dextrose  
198 (YPD) (Oxoid Ltd., Basingstoke, United Kingdom), PCA and Violet Red Bile Agar (VRBA) (Oxoid  
199 Ltd., Basingstoke, United Kingdom) according to Siroli et al. (2015). PCA and YPD plates were  
200 incubated at 30 °C for 48h. MRS and VRBA plates were incubated at 37 °C for 24h while PCA agar  
201 plates for psychotropic bacteria were stored at 10 °C for 7 days. The presence of *L. monocytogenes*,  
202 *Salmonella enteritidis*, and *E. coli* in all samples was investigated according to the ISO methods  
203 6579-1:2017 (2017); ISO 11290-1:2017 (2017); and ISO 16649-3:2015 (2015), respectively.

204 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

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

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  $\beta$  (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)

306 exceeded this limit after 2 days of storage at 10 °C, independently on the packaging type. However,  
307 L 0.1 samples resulted borderline showing a total mesophilic cell load of 6.11 log CFU/mL while C  
308 0.1 samples showed a cell load of 7.46 log CFU/mL. Contrarily, in HPH treated samples, the  
309 packaging showed a higher effect, since the control samples (C100) exceeded the threshold limit of  
310 total aerobic mesophilic bacteria after 8 days of storage while the samples in active pouches, at the  
311 eighth day of storage still had a relatively low mesophilic load (3.49 log CFU/mL). In activated and  
312 treated pouches the limit of 6.0 log CFU/mL was exceeded only at the thirteenth day of storage. This  
313 represents a very interesting result, indicating the possibility of increasing the shelf-life of vegetable  
314 smoothies by combining active packaging and HPH technologies and preventing the negative effects  
315 of thermal abuse during the refrigerated storage. Other authors reported a microbial stabilization of  
316 mango, orange and apple juices after HPH treatments at 200-300 MPa for 1-3 cycles with inlet  
317 temperatures ranging between 20 and 60 °C in products stored at 12-20 °C up to 28 days (Suárez-  
318 Jacobo et al. 2010; McKay, Linton, Stirling, Mackle, & Patterson, 2011; Guan et al. 2016). However,  
319 all the reported authors have considered fruit juices with an extremely acidic pH ranging between 3.2  
320 and 4.1, that, as known, represents a very strong hurdle for microbial growth. In this context, the  
321 microbial stabilization of a vegetable smoothie characterized by higher pH values as in the present  
322 study (5.35 for C100 and L 100 respectively at 8 and 13 days) represents a promising result. The use  
323 of the lysozyme activated packaging allowed an increase of 5 shelf-life days of the smoothie. On the  
324 other hand, lysozyme is effective against different Gram-positive bacteria including spoiling lactic  
325 acid bacteria in wine and beer fermentation (Silvetti, 2010; Azzolini, Tosi, Lorenzini, Finato, &  
326 Zapparoli, 2015). In addition, Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile (2006)  
327 showed that lysozyme immobilized on polyvinyl alcohol-based film was able to inhibit the growth of  
328 viable vegetative cells of *Alicyclobacillus acidoterrestris* including spores. Glicerina et al. (2021),  
329 showed that PLA biodegradable packaging activated with lysozyme was able to inhibit  
330 several spoiling and pathogenic microorganisms both *in-vitro* and in real systems and was  
331 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



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

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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