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Efficacy of biodegradable, antimicrobial packaging on safety and quality parameters maintenance of a pear juice and rice milk-based smoothie product

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1 **Efficacy of biodegradable, antimicrobial packaging on safety and quality**
2 **parameters maintenance of a pear juice and rice milk-based smoothie product**

3

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21 **Abstract**

22 In this study, the effect of a Polylactic acid (PLA) antimicrobial biodegradable packaging
23 activated with lysozyme by cold plasma on a pear juice and rice milk-based smoothie was
24 investigated. The antimicrobial effect of the active innovative packaging was evaluated *In-vitro*
25 and on the smoothie inoculated with *Listeria monocytogenens* and *Lactobacillus plantarum*.
26 After a preliminary evaluation of the lysozyme release kinetics in different conditions, its
27 influence on some smoothie quality parameters (water activity, pH, colour and microbial growth)
28 was evaluated. *In-vitro* trials showed an antimicrobial activity of the activated film against
29 different microorganisms. Inoculated smoothies packed in activated and not materials were stored
30 at 10 and 4 °C and analysed overtime. Results showed the capability of the activated package to
31 inhibit *Listeria monocytogenes* and to maintain a better and a more stable colour compared to
32 control ones. Activated pouches showed the best antimicrobial effect on samples stored at 10 °C
33 compared to 4 °C, difference due to the faster lysozyme release kinetic from the packaging
34 material at the highest storage temperature.

35 Obtained results highlight the potentiality of the biodegradable packaging activated with
36 lysozyme to be applied successfully in food industry, to improve safety and extend shelf-life of
37 juice-based product.

38

39 **Keywords:** Active Packaging, lysozyme, biodegradable packaging, cold plasma

40

41

42 1. Introduction

43 Food safety and quality are the main goals of the food industry (Santos, et al., 2018). In
44 particular, the prevention of food spoilage by inhibiting or destroying microorganisms is the basis

45 of food preservation (Cha & Chinnan, 2010; Janjarasskul & Suppakul, 2018). In the last years
46 innovative food preservation methods and technologies were studied including: high hydrostatic
47 pressure, high pressure homogenization, pulsed electric fields, high voltage arc discharge, cold
48 plasma, as well as pulsed light, ultraviolet short wavelength treatments (UV-C) and the use of
49 lytic bacteriophages to specifically control pathogens or antibiotic resistant opportunistic
50 pathogens (Patrignani & Lanciotti, 2016; Zhang, Wang, Zeng, Han & Brennan 2019; Marteens,
51 Klein, Barnes, Trejo-Sanchez, Roth & Ibey, 2020). Moreover, one of the most promising and
52 efficient technology to protect packed food from microbial proliferation during storage is the use
53 of active packaging (Cooksey, 2005; Yildirim et al., 2018). Active packaging is as a system in
54 which the package, the product and the environment actively interact prolonging shelf life and/or
55 enhancing safety and sensory properties of food products during storage (Prasad & Kochlar,
56 2014; Biji, Ravishankar, Mohan & Gopal, 2015).

57 Antimicrobial packing is a form of active packaging in which the activated material acts to
58 reduce, inhibit or retard the growth of microorganisms that may be present in the food or
59 packaging material itself (Appendini & Hotchkiss, 2002; Huang, Qian, Wei & Zhou, 2019). To
60 control undesirable microorganisms in foods, antimicrobial substances can be incorporated in or
61 coated onto food packaging materials (Biji et al., 2015). In fact, the active packaging systems can
62 be classified into two main groups: non-migratory active packaging, in which the food system
63 response can be obtained without the active component migration from the packaging into the
64 food (i.e. oxygen, ethylene absorbers, etc.) and active releasing packaging which permits a
65 controlled migration of non-volatile compounds or a release of volatile agents into the
66 atmosphere surrounding the food product (i.e. carbon dioxide, antioxidant, etc.) (Hosseinnejad,
67 2014).

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68 Several methods have been used to develop efficient antimicrobial packaging systems, such as
69 the inclusion of sachets or pads containing volatile antimicrobial compounds or the incorporation
70 of volatile and non-volatile compounds directly in the polymer matrix, through their
71 immobilization (Irkin & Esmer, 2015). This last method can be realized by activating the
72 polymer surface, with physical or chemical methods, to promote the adhesion of the active agents
73 (Limbo & Khaneghah, 2015). Among the possible methods, one of the most promising and
74 innovative seems to be cold plasma treatment. The plasma treatment induces polymer material
75 surface ionization, formation of free radicals and new functional groups which increase the
76 bonding capacity between film and active agent, thus overcoming also the problem related to the
77 use of primers and synthetic adhesives (Vartiainen, Rättö & Paulussen, 2005; Farghal,
78 Karabagias, El Sayed & Kontominas, 2017). Synthetic polymer materials are widely used in the
79 food packaging because of their low production costs and high technological performances (Pan,
80 Farmahini-Farahani, O'Hernd, Xiao & Ocampo, 2016). However, in the last decades because of
81 the environmental damage due to synthetic polymer materials (Park et al., 2012; Gómez-Estaca,
82 Gimenez, Montero & Gomez-Guillèn, 2016; Yang, Lee, Won, & Song, 2016; Han, Yu, & Wang,
83 2018) the consumer demand shifted to more sustainable bio-materials, from renewable
84 agricultural sources or food industry wastes and by-products (Jridi et al., 2017; Benbettaieb,
85 Tanner, Cayot, Karbowiak & Debeaufort, 2018). Nevertheless, it is known that most of
86 biopolymers are characterized by some limitations, including high moisture affinity, low thermal
87 stability and poor barrier and mechanical properties. Among the various biopolymers investigated
88 for their possible applications in food packaging, polylactic acid (PLA), recognized as safe
89 (GRAS), proved to be one of the most suitable biopolymer owing to its biodegradability,
90 renewability and superior technological properties (Farah, Anderson & Langer, 2016; Swaroop &
91 Shukla, 2018).

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92 The demand of safe product, obtained from natural sources, concerns also the substances that can
93 be employed to activate packaging materials. Among those with antimicrobial effect, it is well
94 recognised that essential oils, biomacromolecules as peptides, proteins (nisin and lactoferrin in
95 particular), enzymes (e.g., lysozyme) and some polysaccharides (e.g. chitosan) represent
96 excellent alternative solutions to the use of synthetic antimicrobial compounds (Vannini,
97 Lanciotti, Baldi & Guerzoni, 2004; Lucera, Costa, Conte & Del Nobile, 2012; Patrignani, Siroli,
98 Serrazanetti, Gardini & Lanciotti, 2015; Vilela et al., 2018). In particular, lysozyme, a
99 peptidoglycan *N*-acetyl-muramoylhydrolase, is one of the most commonly used natural proteins
100 and has a great potential to be used as antimicrobial in active packaging due to its stability over a
101 wide range of temperature and pH values, and thanks to its activity against numerous pathogens
102 (Aziz & Karboune, 2018). The antimicrobial activity of lysozyme is based on the hydrolysis of
103 the 1-4 β-linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine of peptidoglycan
104 that represent 90% of the cell wall of gram-positive bacteria (Iucci, Patrignani, Vallicelli,
105 Guerzoni & Lanciotti, 2007; Barbiroli et al., 2012). Anti-listeria effect of edible coatings
106 containing lysozyme are reported for different food matrices such as cheese and smoked salmon
107 (Costa, Maciel, Teixteira, Vicente & Cerqueira, 2018; Mehyar, Al Nabulsi, Saleh, Olaimat, &
108 Holley 2018; Neetoo, Ye & Chen, 2018). Different studies are present in the scientific literature
109 on the activation methods and release rate of lysozyme in packaging materials. Most of the
110 studied strategies are focused on physical blending or chemical bonding and in specific on the
111 changes of packaging material morphology by polymer concentration, additive concentration and
112 of type and degree of crosslinking (Ma, Tang, Yin, Yang, & Qi, 2013; Huang, Qian, Wei &
113 Zhou, 2019). In other studies, the release rate of lysozyme from films at different pH values in a
114 buffer solution (Fajardo, Balaguer, Gomez-Estaca, Gavara & Hernandez-Munoz2014), its release
115 from zein capsules incorporated in hydrophilic food packaging materials (Li et al., 2012) and the

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116 activity of the enzyme and lactoferrin incorporated in cellulose-based film were evaluated
117 (Barbiroli et al., 2012). Moreover, to our knowledge only one work (Mastromatteo, Lecce, De
118 Vietro, Favia & Del Nobile, 2011) deal with the use of a cold plasma treatment to immobilize
119 lysozyme preliminary dissolved in a solution of acrylic/methane and natural fibres; however, in
120 this study the authors did not evaluate the application and performances of activated packaging
121 on food.

122 The aim of this research was to investigate the antimicrobial effect of lysozyme, immobilized in a
123 biodegradable polylactic acid (PLA) packaging material by cold plasma, both *In-vitro* and on a
124 smoothie product (pear juice and rice milk mix), previously inoculated with *Listeria*
125 *monocytogenens* and *Lactobacillus plantarum*. The effect of activated packaging on some quality
126 parameters of smoothie samples during storage, was also evaluated.

127

128 **2. Material and Methods**

129 **2.1 Materials**

130 Polylactic acid (PLA) was used as polymeric material to be activated. Polyvinyl alcohol (PVOH)
131 was used as lysozyme supporting gel, after preliminary tests, related to its viscosity and inhibition
132 effect, and considering its biodegradability as reported by Chiellini et al., 2003; Mastromatteo et
133 al., 2011 and Da Silva et al., 2020 in their previous studies. Moreover, this polymer is approved
134 by the European Medicine Agency (EMA) and the United States Food and Drug Administration
135 (FDA) for human use. It can be used as a component of coatings and packaging in food
136 applications (Curley et al., 2014; Gómez-Aldapa et al., 2020). PLA films having a thickness of 30
137 µm and an oxygen transmission rate (OTR) of $500m^{-2}d^{-1}/bar^{-1}$ were supplied by IMA S.p.a
138 (Ozzano dell'Emilia, BO, Italy); polyvinyl alcohol (PVOH) (MW = 88.000–97.000) was
139 purchased by Sigma-Aldrich (Gallarate, MI, Italy). The active compound lysozyme, from hen

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140 egg white, was also purchased by Sigma–Aldrich (Gallarate, MI, Italy). The smoothie was
141 obtained by mixing two commercial products, UHT pear nectar (Alce Nero S.p.a, Bologna, Italy)
142 with UHT rice milk (Alce Nero, S.p.a , Bologna, Italy) in proportion of 2:1 by using an
143 household mixer (Bimby robot—Vorwerk, Germany). The stability of smoothies was maintained
144 by working under sterile conditions, during all steps. The mixture was realised after preliminary
145 trials in order to obtain a organoleptic good smoothie beverage, with a final pH of 5.3, that, as
146 known by literature (Nyhan, Begley, Mutel, Qu, Johnson & Callanan, 2018), is unable to inhibit
147 several pathogenic and spoilage microorganisms. The initial pH values of both ingredients were
148 3.8 and 7.3 respectively for pear nectar and rice milk.

149

150 **2.2 Activation of PLA film and packaging production**

151 The activation of a PLA monolayer film was realized at laboratory scale. PLA film was
152 previously subjected to a cold plasma treatment by using an open-air Dielectric Barrier Discharge
153 (DBD)source, operating at atmospheric pressure, with a peak voltage of 20 kV and 20 kHz of
154 frequency. The purpose of this treatment was to activate one surface of the PLA promoting the
155 adhesion between PLA and PVOH that forming a coating, entraps enzyme causing its
156 immobilization. Moreover, according with literature it is noteworthy that in absence of any
157 activation, no adhesion could be achieved between coating and substrate (Boselli et al., 2012;
158 Mericer et al., 2016) .Subsequently, working under a laminar flow cabinet, lysozyme was
159 dissolved into PVOH at a concentration of 1250 mg/L and the solution stirred by vortex for 5
160 min; then 1.5 ml of this gel-solution was withdrawn by sterile syringe and spread on 20 x 20 cm
161 size PLA film samples by using a sterile spatula. After deposition, the obtained films were dried
162 in an oven at 40 °C for 10 min. The lysozyme concentration was selected on the basis of

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163 preliminary tests in which PVOH was activated with different concentrations of enzyme ranging
164 from 1000 to 1500 mg/L and antimicrobial activity was assessed in-vitro. It was proven that the
165 lysozyme concentration of 1250 mg/L was the best condition since increasing the concentration
166 up to 1500 mg/L did not result in a significant raise of antimicrobial activity.
167 After drying, the activated film was folded and sealed by using a heat-sealer at 100 °C on the two
168 main sides in sterile conditions. The dimension of the final pouch was 20x10 cm; totally a
169 number of 72 pouches was obtained.
170

171 **2.3 HPLC Lysozyme release kinetics**

172 The method was performed according with Riponi, Natali & Chinnici, (2007) with some
173 modifications. Two strips of PLA film (2.5 x 10 cm) activated with lysozyme, as previously
174 described (section 2.2), were immersed into a flask containing 50 ml of a buffer acetate solution
175 at pH 5.3, similar to that of smoothie sample, and stored for 204 hours respectively at two
176 different temperature conditions: 4 and 10 °C under continuous shaking at 100 rpm. The
177 temperatures of 4 and 10 °C were chosen in order to simulate an optimal refrigerated storage (4 °
178 C) and a condition of thermal abuse (10 ° C). For both temperatures, sampling was performed
179 taking 500 µl of solution by using a micropipette (Gilson, Villiers le bel, France) at the following
180 storage times: 0, 24, 36, 48, 60, 72, 96, 120, 132, 144, 156, 168, 180, 192 and 204 hours. The
181 volume of the release medium was kept constant by replacing the collected samples volume with
182 the same amount of buffer each time.
183 High-performance liquid chromatography analysis was conducted using a Jasco apparatus
184 (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20-µL loop, a Rheodyne valve (Cotati,
185 CA), a photodiode detector (PU MD 910), a fluorometric detector (FP 2020), and a column oven.
186 The column was a Toso Bioscience (Stuttgart, Germany) TSKgel Phenyl 5PW RP (7.5 cm x 4.6

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187 mm i.d.) protected with a guard column, filled with the same resin. All runs were acquired and
188 processed using Borwin 5.0 software (JMBS Developments, Grenoble, France). UV detection
189 was performed at 280 and 225 nm. The fluorometric detector was set at λex 276 nm and λem 345
190 nm (gain 10, spectrum bandwidth 18 nm). The elution solvents were 1% CH_3CN, 0.2% TFA,
191 98.8% H_2O (solvent A), and 70% CH_3CN, 0.2% TFA, 29.8% H_2O (solvent B). Gradient elution
192 was as follows: 100% A for 3 min, then to 65.0% A in 7 min, maintained for 5 min, then to
193 40.5% A in 12 min, then to 0% A in 2 min, maintained 5 min, then to 100% A in 2 min, followed
194 by 10 min of re-equilibration at the initial conditions. The column operating conditions were at
195 30°C and with a flow of 1 mL/min. The identification of lysozyme in the samples was carried out
196 by comparing its retention time and UV-spectra to those of standard solutions. Quantification was
197 performed using an external standard; peak areas of standard lysozyme solutions at two different
198 temperatures within the chosen range were determined in triplicate.

199

200 **2.4 *In vitro* antimicrobial activity of lysozyme activated film**

201 The *In vitro* antimicrobial activity of lysozyme activated film was assessed against several
202 microbial strains of food interest such as: *Listeria monocytogenes* Scott A, *Listeria*
203 *monocytogenes* ATCC13932, *Listeria innocua* ATCC51742, *Listeria innocua* DSM2029Y,
204 *Lactobacillus plantarum* 82, *Staphylococcus aureus* SR41, *Enterococcus faecium* t2 e
205 *Pediococcus damnosus* 11. All the strains belong to the Department of Agricultural and Food
206 Sciences of Bologna University. Stocks of the strains were kept at - 80 °C in Brain Heart Infusion
207 (BHI) broth containing 20% (v/v) glycerol. The strains were preliminary grown (1% v/v) in BHI
208 and then inoculated at a level of 6.0 log CFU/mL in a pH 7.0 phosphate buffer soft agar (0.8%)
209 and poured in Petri plates. Lysozyme activated PLA film disks of 12 mm diameter were placed in

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210 the center of the agar plates. The inoculated plates were incubated at 37 °C for 24 h and then the
211 diameters of the halos were measured using a Vernier caliper.
212

213 **2.5 Challenge test screening**

214 Three litres of the previous described smoothie (section 2.1) were inoculated with the pathogenic
215 microorganism *Listeria monocytogenes* Scott A and the spoiling bacteria *Lactobacillus*
216 *plantarum* 82. The two strains were selected as representative of both pathogenic (*Listeria*
217 *monocytogenes*) and spoiling (*Lactobacillus plantarum*) microorganisms, frequently associated
218 with fresh fruit juices and similar products, such as smoothie. *Listeria monocytogenes* and
219 *Lactobacillus plantarum* were routinely grown (1% v/v) respectively in Brain Heart Infusion
220 (BHI) broth (Oxoid Ltd., Basingstoke, United Kingdom) and in the Man, Rogosa and Sharpe
221 (MRS) broth (Oxoid Ltd., Basingstoke, United Kingdom) at 37 °C for 24 h. *Listeria*
222 *monocytogenes* was inoculated at a level of 3.4 log CFU/mL while *Lactobacillus plantarum* at a
223 level of 2.3 log CFU/mL. The inoculum levels between 10^2 and 10^3 cells/mL of product was used
224 since it is reported (Wu et al., 2019) to be appropriate to ascertain the microbiological stability of
225 a formulation. Thirty six lysozymes activated (AP) and 36 not activated (control) PLA pouches
226 (CP) were filled with 20 ml of the inoculated smoothie, by using an automatic pipette, under a
227 laminar flow cabinet, and then sealed off on the upper part. 18 AP and 18 CP samples were
228 stored at 4 °C and the others 18 AP and 18 CP at 10 °C in climatic chambers at RH of 50% for
229 respectively 16 and 10 days. The temperatures of 4 and 10 °C were chosen in order to simulate
230 the optimal refrigerated storage (4 ° C) and a condition of thermal abuse (10 ° C). Packed
231 smoothies stored at 4 °C were analysed in triplicate respectively at 0, 2, 4, 7, 10, 13, 16 days;
232 samples stored at 10°C were analysed, in triplicate, at 0, 1, 3, 4, 6, 8, 10 days. The initial
233 sampling (T0) was performed from the initial inoculated batch before the filling of the pouches.

234

235 **2.6 Water activity**

236 The water activity (a_w) of smoothies was measured by using a dew point hygrometer, AcquaLab-
237 Water Activity Meter (mod. SERIES 3TE. Decagon Device, Inc., Nelson Court, NE).

238

239 **2.7 pH and Microbiological analysis**

240 The pH of samples was measured immediately after treatments and at each storage time by using
241 a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain).
242 The cell loads over time of the samples inoculated with *Listeria monocytogenes* and *Lb.*
243 *plantarum* were monitored by plate counting respectively on Listeria Selective Agar Base (Oxoid
244 Ltd., Basingstoke, United Kingdom) with selective listeria supplement (Oxoid Ltd.) and on
245 DeMan, Rogosa, and Sharpe (MRS) (Oxoid Ltd., Basingstoke, United Kingdom) according to
246 Siroli et al. (2015). The agar plates of both the considered strains were then incubated for 48 h at
247 37 °C at each time of storage.

248

249 **2.8 Colour**

250 Colour of smoothie samples was determined by using a tristimulus spectrophotocolorimeter
251 (mod. A60-1010-615 ColorFlex, HunterLab, USA) equipped with a sample holder (12 mm
252 diameter). Colour was measured using the CIE *L*a*b** colour space and illuminant D65 and was
253 expressed as lightness value (L*) and hue angle (h°), calculated as reported by Mc Guire (1992).

254

255 **2.9 Statistical analyses**

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256 For each sample at each storage time, the data were reported as the means of three independent
257 repetitions. Analysis of variance (ANOVA) and the test of mean comparisons according to
258 Fisher's least significant difference (LSD) with a 0.05 level of significance were applied to find
259 out significant differences among samples. Moreover, a multiple linear regression was applied on
260 lysozyme release data to estimate the kinetic rates. The statistical package STSG Statistica for
261 Windows, 6.0 version (Statsoft Inc., Tulsa, USA) was used.
262
263 **3. Results and Discussion**
264
265 **3.1 HPLC lysozyme release kinetics**
266 Figure 1 shows the kinetics of lysozyme release from PLA activated film to the buffer solution
267 during 204 hours of storage at 4 and 10 °C. In samples stored at 10 °C the lysozyme release was
268 revealed after 168 h of storage, showing a fast increase during the subsequent time, while in
269 samples stored at 4°C a slight lysozyme release was detected only after 192 h. As expected, the
270 highest lysozyme release rate was observed from the activated packaging stored at 10 °C with a k
271 value of $1.50E^{-3}$, while sample stored at 4°C showed a k of $9.00E^{-4}$; in both samples kinetic rates
272 of enzyme release were evaluated on data obtained from 168 to 204 h. The different lysozyme
273 rate release is due to the different storage temperatures. In fact, the highest temperature directly
274 influences the swelling and thus a rearrangement of PVOH crosslinking network, allowing the
275 molecules of lysozyme a greater diffusion (Corradini et al., 2013). At lower temperatures, the
276 mobility of the polymeric chains is assumed to be slowed, making the network more rigid and
277 entrapping lysozyme strongly (Corradini et al., 2013).
278
279 **3.2 *In vitro* antimicrobial activity of lysozyme activated film**

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280 The *In vitro* antimicrobial activity on activated packaging material was evaluated on a buffer to
281 assess the effect of lysozyme on the cell wall lysis of the target microorganisms. In Table 1 the
282 diameter of the lysis halos in phosphate buffer are reported. The results obtained show a good
283 efficacy of the lysozyme on the lysis of the cell wall, mainly against *Enterococcus faecium,*
284 *Listeria monocytogenes Scott A, Pediococcus damnosus* and *Lactobacillus plantarum*, with
285 diameter halos ranging between 22.34 and 18.34 mm depending on the microorganism. On the
286 contrary the cell wall lysis was limited for *Listeria innocua* and *Listeria monocytogenes*
287 ATCC13932; the halos did not exceed the diameter of the PLA disk sample for *Staphylococcus*
288 *aureus*. The difference in lysozyme resistance among *L. monocytogenes* and *L. innocua* strains
289 can be due to intrinsic factors, linked to genes that determine a strain-dependent response (Burke,
290 Loukitcheva, Zemansky, Wheeler, Bonecac Ivo & Portnoy, 2014). Moreover, it is known that
291 Staphylococci are one of the few gram positive species that are completely resistant to lysozyme;
292 this resistance contributes to their survival and colonization of skin and mucous membranes
293 (Bera, Herbert, Jakob, Vollmer & Gotz, 2005).

294

295 **3.2 Water activity, pH and Microbiological analysis**

296 All smoothie samples packed in lysozyme activated pouches (AP) and in control pouches (CP)
297 showed a water activity constant trend during storage without significant differences between
298 them. On the average the water activity values were respectively 0.984±0.001 for both AP and
299 CP samples stored at 10°C and 0.985±0.001, for both samples (AP and CP) stored at 4°C. In
300 Figures 2a and 2b the microbial cell loads of *Listeria monocytogenes* Scott A and *Lactobacillus*
301 *plantarum* 82, inoculated in the packed AP and CP smoothie samples and detected during 10

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302 days of storage at 10 °C, are respectively reported. Results showed a strong effect of the
303 lysozyme activated PLA packaging on the deactivation of *Listeria monocytogenes* (Figure 2a). In
304 fact, AP samples showed a decrease of the pathogen cell load starting from the first day of
305 storage, at the sixth day *L. monocytogenes* was below the detection limit. On the contrary, control
306 samples (CP) showed an increase in *Listeria* load from 3.4 log CFU/mL (zero time) up to over
307 6.0 log CFU/mL after 8 days of storage at 10 °C. For what concern *Lb. plantarum*, at 10 °C, the
308 microorganism showed similar growth kinetics in AP and CP samples (Figure 2b), without a
309 significative inhibitory effect of lysozyme activated pouches.

310 In Figures 3a and 3b the cell loads of *Listeria monocytogenes* Scott A and *Lactobacillus*
311 *plantarum* 82, inoculated in AP and CP samples during 16 days of storage at 4 °C, are
312 respectively reported.

313 Data obtained from the challenge tests carried out on samples stored at 4 °C indicate that the
314 lowest storage temperature reduced the release of lysozyme by the activated packaging material.
315 In fact, as showed in Figure 3a, it was not possible to achieve the complete deactivation of
316 *Listeria* by lysozyme in AP samples, contrary to what observed at 10 °C. However, starting from
317 the second day of storage, significant lower values of the pathogen cell load over time was
318 observed in AP samples compared to CP ones. In control samples the *Listeria* cell load was
319 always above 3.5 log CFU/mL and a slight increase over time of its load was detected. Also, in
320 this case, as observed for samples stored at 10 °C (Figures 2a and 2b), the antimicrobial effect of
321 lysozyme on *Lb. plantarum* was lower compared to *Listeria*. However, starting from the fourth
322 day of storage AP samples showed a lower cell loads of *Lb. plantarum* compared to control ones
323 (Figure 3b); the differences ranged between 0.23 and 0.51 log CFU/mL.

324 In Table 2, the pH values of AP and CP packed smoothie samples stored at 10 °C are reported.
325 AP samples packed in activated package maintained similar pH values during storage. CP

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326 samples showed a significant acidification after 10 days of storage. The acidification observed in
327 CP samples can be ascribed to the higher cell load detected for *Lb. plantarum* in these samples
328 (which exceeded 6.0 log CFU/mL). In fact, this strain has a strong acidifying capacity due to the
329 production of high amount of organic acids, mainly lactic acid, dependent on growth conditions
330 and substrates (Behera, Ray & Zdolec2018). On the contrary, the pH data of both samples stored
331 at 4 °C did not show significative deviations from the initial values over time. In fact, in both
332 types of sample, the pH values that initially were 5.30±0.01, never resulted below 5.20 ± 0.05 for
333 the entire storage period (data not reported).

334 The results obtained suggest a faster release of lysozyme at 10 °C as shown also by the lysozyme
335 release trial. In fact, at 10 °C the antimicrobial activity against *L. monocytogenes* was observed
336 already after 24h in AP smoothies and the complete deactivation of the pathogen was reached
337 within 6 days of storage.

338 In addition, as reported by several authors, lysozyme antimicrobial activity is affected by the
339 environmental conditions such as temperature, water activity, pH, medium composition, and
340 cation concentration (Iucci, Patrignani, Vallicelli, Guerzoni & Lanciotti et al., 2007). Barbiroli et
341 al. (2012) tested the antimicrobial activity of a cellulose material activated with lysozyme and
342 lactoferrin against *Listeria innocua* in a broth media, showing that the activated packaging
343 prolonged the lag phase of the microorganism but was not able to fully inhibit the growth of the
344 microorganism. Vannini, Lanciotti, Baldi & Guerzoni (2004) and Iucci et al (2007), showed that
345 the addition of lysozyme induced a viability loss and an extension of lag phases of *L.*
346 *monocytogenes* inoculated in skim milk, bovine milk and ewe milk at 37°C, this anti-listeria
347 effect was strongly incremented by combining lysozyme with high-pressure homogenization
348 treatments. Other authors showed a strong inhibition activity of calcium alginate coatings
349 containing lysozyme against the growth of *Listeria monocytogenes* and *Salmonella anatum* in the

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350 surface of smoked salmon stored at refrigerated temperatures (Datta, Janes, Xue, Losso & 351 Peyre,2008).

352 Concerning the effect of active packaging against *Lb. plantarum*, at both the considered 353 temperatures, only a limited inhibitory effect was observed contrary to what has been observed in 354 the preliminary *in vitro* trials (Table 1). This different inhibitory activity by the activated film can 355 be attributed to the different conditions of the trials. In fact, the *in vitro* antimicrobial activity was 356 assessed by measuring the cell wall lysis at 37 °C in a buffer with limited nutritional factors. In 357 addition, as showed by several authors, the response of bacteria to lysozyme is strongly strain 358 dependent and associated to the food matrix composition and environmental conditions (Dias, 359 Vilas-Boas; Campos, Hogg & Couto, 2015). Vannini et al. (2004), showed a limited 360 antimicrobial activity of the native form of lysozyme against *L. plantarum* in skim milk. 361 However, the activity against *L. plantarum* was strongly incremented by a pressurized treatment 362 of lysozyme, suggesting that the highest effect of high homogenization pressure on the lysozyme 363 activity is associated to an increased exposure of the microbial cells to the enzyme or to 364 conformational modification of the antimicrobial enzymes.

365 Both the microorganisms tested in these trials, *Listeria monocytogenes* Scott A and *Lactobacillus* 366 *plantarum* 82, are reported as extremely resistant and in many cases capable to grow even under 367 refrigerated conditions in a wide range of foods (Andreevskaya et al., 2018; Bucur, Grigore-368 Gurgu, Crawwels, Riedel & Nicolau, 2018; Ricci et al., 2018). For these reasons, the significant 369 reduction in *Listeria* cell load and the inhibition of *L. plantarum* growth observed, demonstrate a 370 good antimicrobial effect of the lysozyme activated film, even at the storage temperature of 4 °C.

371

372 **3.3 Colour**

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373 As reported in Table 3 smoothie samples packed in AP showed the significantly highest lightness
374 and hue angle values compared to control one during storage at 10 °C. In sample packed in CP a
375 significant decrease of lightness and a reduction in hue angle were observed during storage at
376 10°C. In both samples the colour changes, which are reflected mainly by a browning increase
377 (reduction of lightness), may be due to the PPO and POD enzymes' activities (Terefe, Tepper,
378 Ullman, Knoerzer & Juliano, 2016). Moreover, the highest colour degradation due to enzymatic
379 browning was more evident in the control sample (CP) because of its highest microbial growth,
380 mainly in terms of *L. monocytogenes* growth, that induced a greater cell disruption (Zhou et al.,
381 2014). Table 4 shows the lightness and hue angle colour parameters variations in AP and CP
382 samples during storage at 4°C. Lightness (L*) and hue angle (h°) values of smoothie samples
383 packed in lysozyme activated (AP) and not-activated (CP) pouches during storage at 4 °C. Also,
384 in this case both samples showed a colour degradation during time, even if less intense than in
385 samples stored at 10°C. This is due, as known, to the reduced enzymatic activity and
386 microorganisms grow because of the lowest storage temperature (Martin-Diana, Rico, Barry-
387 Ryan, Mulcahy, Frias & Henehan, 2005). The control sample CP underwent the significantly
388 highest browning also during storage.

389

390 **Conclusions**

391 Obtained results showed an *In-vitro* antimicrobial activity of the lysozyme activated film against
392 several spoilage and pathogenic microorganisms associated to the food industry and in particular
393 a greater efficacy of the biodegradable active packaging to prevent *Listeria monocytogenes*
394 growth. In fact, the antimicrobial effect of the tested activated material was higher against
395 *Listeria monocytogenens* than *Lactobacillus plantarum*, in rice milk-based smoothie. Moreover,

396 as expected, lysozyme activated pouches showed the best antimicrobial effect in samples stored
397 at 10 °C compared to those stored at 4 °C. This difference is due to the fastest kinetic release of
398 lysozyme, from the internal surface of packaging material, at the highest storage temperature, as
399 also demonstrated by the lysozyme release kinetics. As a consequence, smoothie samples packed
400 in lysozyme activated pouches showed a lower microbial grow and a better colour retention
401 during storage than control samples.
402 Obtained results highlighted the potentiality of lysozyme activated biodegradable packaging to be
403 applied successfully in food industry for the improvement of shelf-life and safety of minimally
404 processed juices and smoothies. In addition, the combination of this active packaging with other
405 non-thermal technologies such as pulsed electric field, high pressure homogenization, ultrasound
406 or cold plasma can be considered as a strategy to further increase the preservation potential of
407 this innovative packaging since it can further increase the antimicrobial activity of lysozyme.

408

409

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668

669 **Figure Captions**

670

671 **Figure 1**. Kinetics release of lysozyme from activated PLA film to buffer solution until 204 h of
672 storage at 4 and 10 °C.
673 [a–b] Values followed by different letters differ significantly at $P<0.05$ level.
674

675 **Figure 2a.** The microbial cell loads of *Listeria monocytogenes* Scott A inoculated in the
676 smoothie samples packed in lysozyme activated (AP) and not activated (CP) pouches, during 10
677 days of storage at 10 °C.
678 [a–b] Values followed by different letters differ significantly at $P<0.05$ level
679

680 **Figure 2b.** The microbial cell loads of *Lactobacillus plantarum* inoculated in the smoothie
681 samples packed in lysozyme activated (AP) and not activated (CP) pouches, during 10 days of
682 storage at 10 °C.

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683 [a–b] Values followed by different letters differ significantly at $P<0.05$ level

684

685 **Figure 3a.** Microbial cell loads of *Listeria monocytogenes* Scott A inoculated in the smoothie
686 samples packed in activated (AP) and not activated (CP) pouches detected during 16 days of
687 storage at 4 °C.
688 [a–b] Values followed by different letters differ significantly at $P<0.05$ level

689

690 **Figure 3b**. Microbial cell loads of *Lactobacillus plantarum* 82 inoculated in the smoothie samples
691 packed in activated (AP) and not activated (CP) pouches and detected during 16 days of storage at 4
692 °C.
693 [a–b] Values followed by different letters differ significantly at $P<0.05$ level

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Table 1. Halos of inhibition in agar plates of lysozyme activated PLA against different microorganisms in PBS buffer

	PBS pH 7.0 (mm)
Lactobacillus plantarum 82	18.34±1.17^{b}
Pediococcus damnosus 11	19.00±1.05^{b}
Listeria monocytogenes Scott A	19.34±0.82^{b}
Staphylococcus aureus SR41	12.00±0.00^{d}
Listeria innocua dsm2029y	15.28±0.88^{c}
Listeria monocytogenes atcc13932	15.89±0.56^{c}
Enterococcus faecium t2	22.34±0.41^{a}
Listeria innocua atcc 51742	14.27±0.54^{c}

$^{a-c}$ Values followed by different letters differ significantly $P<0.05$ level

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Table 2. pH values of the smoothie samples packed in lysozyme activated (AP) and not-activated (CP) pouches during 10 days of storage at 10 °C.

	pH	
Time (d)	**AP 10°C**	**CP 10°C**
0	5.31±0.03[a]	5.31±0.03 [a]
3	5.34±0.02 [a]	5.33±0.01 [a]
4	5.38±0.03 [a]	5.35±0.02 [a]
6	5.32±0.03 [a]	5.27±0.02 [a]
8	5.29±0.04 [a]	5.04±0.28 [a]
10	5.26±0.14 [a]	4.43±0.47 [b]

[a-b] Values followed by different letters differ significantly P<0.05 level at each time of storage

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Table 3. Lightness (L*) and hue angle (h°) values of smoothie samples packed in lysozyme activated (AP) and not-activated (CP) pouches during storage at 10 °C.

Lightness (L*)							
Days of storage	**0**	**1**	**3**	**4**	**6**	**8**	**10**
AP 10°C	56.53±0.01^{aA}	56.28±0.02^{aB}	56.05±0.9^{aC}	56.39±0.03^{aAB}	55.98±0.25^{aC}	55.39±0.22^{aD}	55.38±0.02^{aD}
CP 10°C	56.53±0.01^{aA}	55.12±0.17^{bB}	54.01±0.3^{bC}	53.70±0.20^{bD}	51.28±0.09^{bE}	51.13±0.20^{bE}	50.45±0.20^{bF}
Hue angle (h°)							
Days of storage	**0**	**1**	**3**	**4**	**6**	**8**	**10**
AP 10°C	88.01±0.02^{aA}	87.97±0.31^{aAB}	87.92±0.57^{aAB}	87.29±0.39^{aB}	86.23±0.41^{aC}	85.93±0.18^{aC}	85.39±0.34^{aC}
CP 10°C	88.01±0.02^{aA}	87.57±0.17^{aB}	85.82±0.30^{bC}	85.28±0.32^{bC}	84.11±0.22^{bD}	83.90±0.50^{bD}	82.44±0.20^{bE}

$^{a–b}$ Values followed by different letters differ significantly between samples at each time of storage at P<0.05 level

$^{A-F}$ Values followed by different letters differ significantly for each sample during 10 days of storage at P<0.05 level

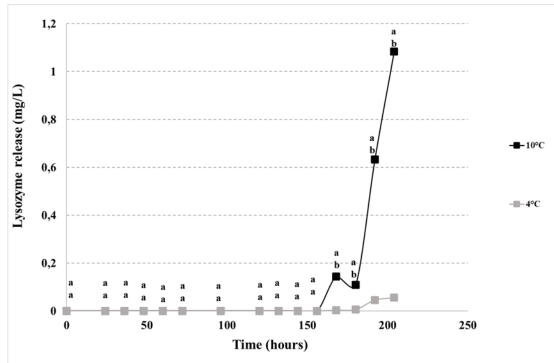
Table 4. Lightness (L*) and hue angle (h°) values of smoothie samples packed in lysozyme activated (AP) and not-activated (CP) pouches during storage at 4 °C.

Lightness (L*)							
Days of storage	**0**	**2**	**4**	**7**	**10**	**13**	**16**
AP 4°C	56.53±0.01^{aA}	56.23±0.02^{aB}	56.08±0.7^{aC}	56.13±0.09^{aC}	56.11±0.12^{aC}	56.00±0.18^{aC}	55.01±0.14^{aD}
CP 4°C	56.53±0.01^{aA}	56.12±0.09^{aB}	56.00±0.3^{aB}	55.28±0.20^{bC}	55.47±0.09^{bC}	54.81±0.20^{bD}	54.28±0.19^{bD}
Hue angle (h°)							
Days of storage	**0**	**2**	**4**	**7**	**10**	**13**	**16**
AP 4°C	88.01±0.02^{aA}	88.09±0.27^{aA}	87.95±0.57^{aAB}	87.39±0.39^{aB}	86.95±0.21^{aB}	86.89±0.18^{aB}	86.10±0.34^{aC}
CP 4°C	88.01±0.02^{aA}	88.08±0.17^{aAB}	87.53±0.30^{aB}	86.10±0.32^{bC}	85.96±0.22^{bC}	85.69±0.50^{bC}	84.36±0.20^{bD}

$^{a–b}$ Values followed by different letters differ significantly between samples at each time of storage at P<0.05 level

$^{A-D}$ Values followed by different letters differ significantly for each sample during 16 days of storage at P<0.05 level

Journal Pre-proof



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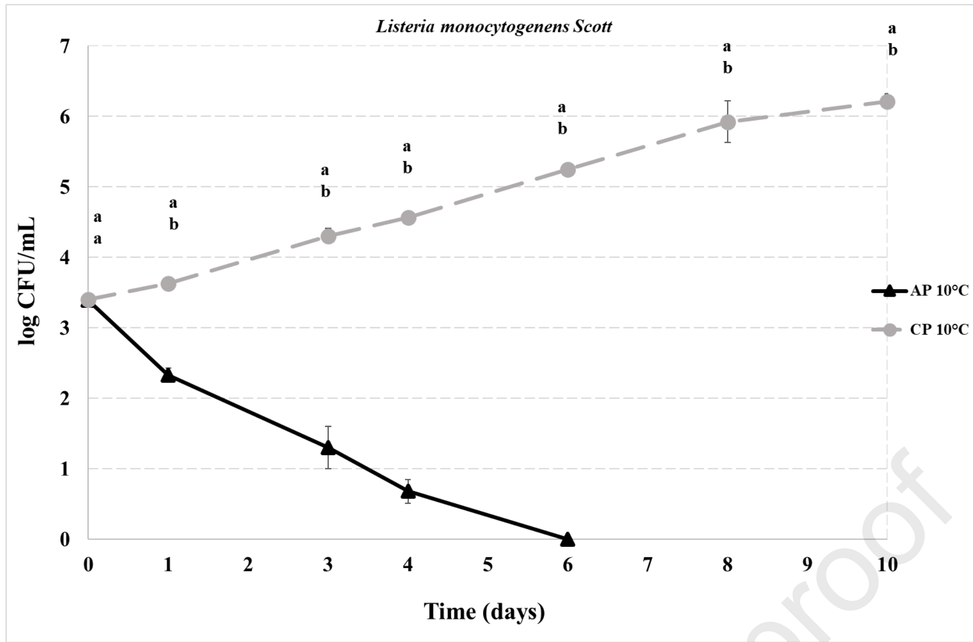

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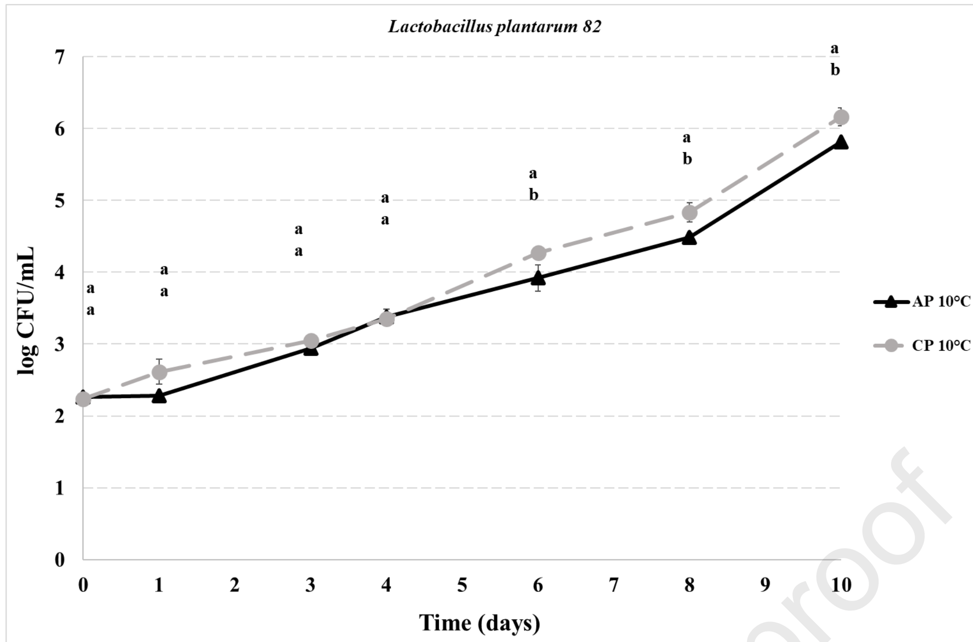

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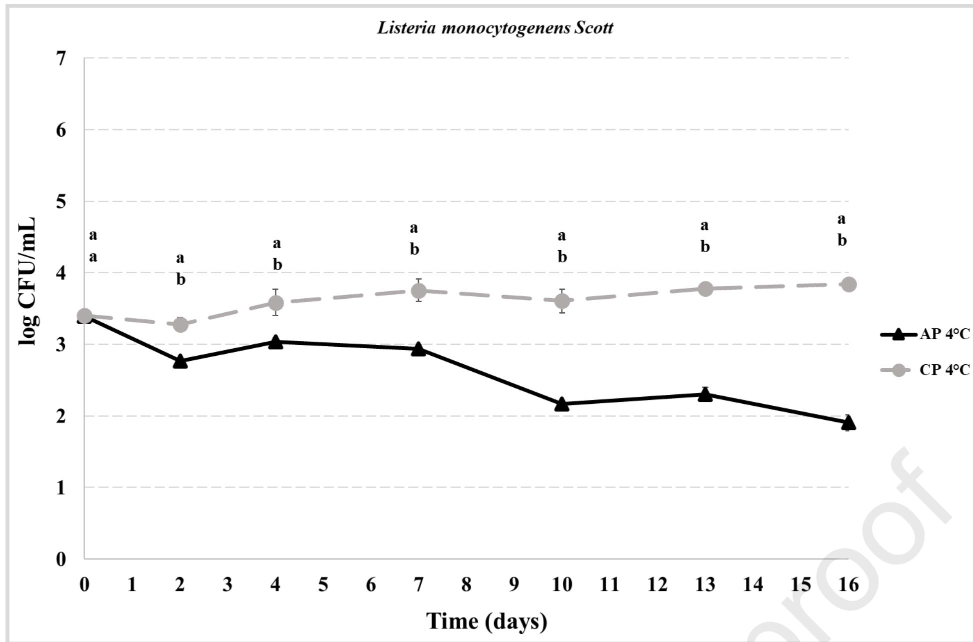

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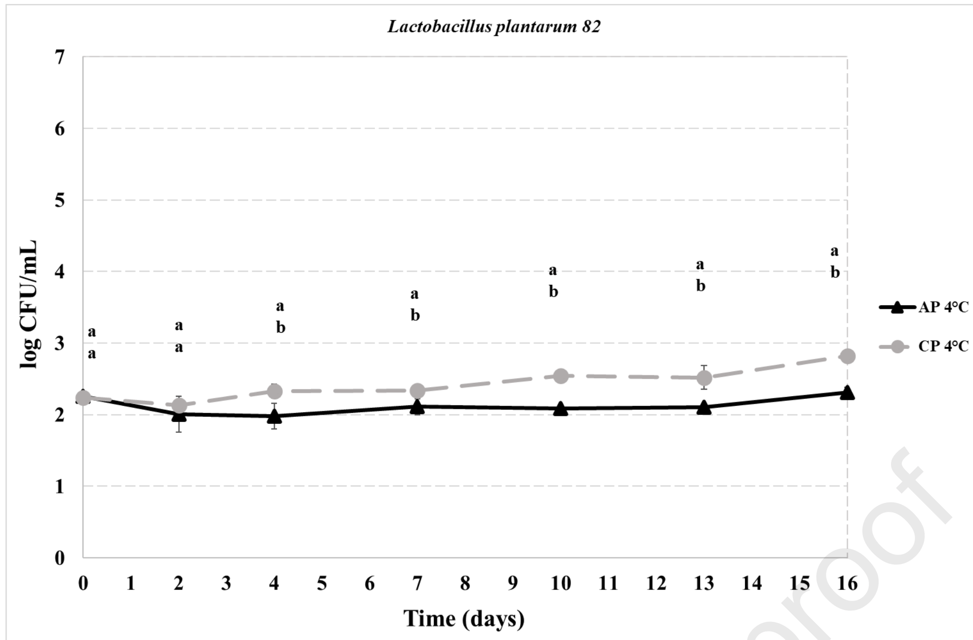

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Highlights

-Lysozyme active packaging showed great efficacy to inhibit *Listeria monocytogenens*

-Activated pouches maintained a better and a more stable colour during storage

- Shelf-life of smoothies was improved during storage

-Lysozyme activated pouches showed best antimicrobial effect at 10°C than 4°C

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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