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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

Virginia Glicerina[a], Lorenzo Siroli[b*], Giada Canali[a], Fabio Chinnici[ab], Filippo Capelli[c], Rosalba Lanciotti[ab], Vittorio Colombo[cd] and Santina Romani[ab]

Alma Mater Studiorum - Università of Bologna:

[a] *Interdepartmental Centre for Agri-Food Industrial Research, Campus of Food Science, Via Quinto Bucci 336, Cesena (FC), Italy*

[b] *Department of Agricultural and Food Sciences, Campus of Food Science, Piazza Goidanich 60, Cesena (FC), Italy*

[c] *Department of Industrial Engineering, Via Terracini 28, Bologna (BO), 40131, Italy*

[d] *Advanced Mechanics and Materials, Interdepartmental Center for Industrial Research (AMM-ICIR), Via Terracini 28, Bologna (BO), 40131, Italy*

[*] Corresponding author: lorenzo.siroli2@unibo.it

Abstract

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

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

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

1. Introduction

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

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

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

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

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

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

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

2. Material and Methods

2.1 Materials

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

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

2.2 Activation of PLA film and packaging production

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

preliminary tests in which PVOH was activated with different concentrations of enzyme ranging from 1000 to 1500 mg/L and antimicrobial activity was assessed in-vitro. It was proven that the lysozyme concentration of 1250 mg/L was the best condition since increasing the concentration up to 1500 mg/L did not result in a significant raise of antimicrobial activity.

After drying, the activated film was folded and sealed by using a heat-sealer at 100 °C on the two main sides in sterile conditions. The dimension of the final pouch was 20x10 cm; totally a number of 72 pouches was obtained.

2.3 HPLC Lysozyme release kinetics

The method was performed according with Riponi, Natali & Chinnici, (2007) with some modifications. Two strips of PLA film (2.5 x 10 cm) activated with lysozyme, as previously described (section 2.2), were immersed into a flask containing 50 ml of a buffer acetate solution at pH 5.3, similar to that of smoothie sample, and stored for 204 hours respectively at two different temperature conditions: 4 and 10 °C under continuous shaking at 100 rpm. The temperatures of 4 and 10 °C were chosen in order to simulate an optimal refrigerated storage (4 ° C) and a condition of thermal abuse (10 ° C). For both temperatures, sampling was performed taking 500 µl of solution by using a micropipette (Gilson, Villiers le bel, France) at the following storage times: 0, 24, 36, 48, 60, 72, 96, 120, 132, 144, 156, 168, 180, 192 and 204 hours. The volume of the release medium was kept constant by replacing the collected samples volume with the same amount of buffer each time.

High-performance liquid chromatography analysis was conducted using a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20-µL loop, a Rheodyne valve (Cotati, CA), a photodiode detector (PU MD 910), a fluorometric detector (FP 2020), and a column oven. The column was a Toso Bioscience (Stuttgart, Germany) TSKgel Phenyl 5PW RP (7.5 cm x 4.6

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

2.4 *In vitro* antimicrobial activity of lysozyme activated film

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

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

2.5 Challenge test screening

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

For each sample at each storage time, the data were reported as the means of three independent repetitions. Analysis of variance (ANOVA) and the test of mean comparisons according to Fisher's least significant difference (LSD) with a 0.05 level of significance were applied to find out significant differences among samples. Moreover, a multiple linear regression was applied on lysozyme release data to estimate the kinetic rates. The statistical package STSG Statistica for Windows, 6.0 version (Statsoft Inc., Tulsa, USA) was used.

3. Results and Discussion

3.1 HPLC lysozyme release kinetics

Figure 1 shows the kinetics of lysozyme release from PLA activated film to the buffer solution during 204 hours of storage at 4 and 10 °C. In samples stored at 10 °C the lysozyme release was revealed after 168 h of storage, showing a fast increase during the subsequent time, while in samples stored at 4°C a slight lysozyme release was detected only after 192 h. As expected, the highest lysozyme release rate was observed from the activated packaging stored at 10 °C with a k value of $1.50E^{-3}$, while sample stored at 4°C showed a k of $9.00E^{-4}$; in both samples kinetic rates of enzyme release were evaluated on data obtained from 168 to 204 h. The different lysozyme rate release is due to the different storage temperatures. In fact, the highest temperature directly influences the swelling and thus a rearrangement of PVOH crosslinking network, allowing the molecules of lysozyme a greater diffusion (Corradini et al., 2013). At lower temperatures, the mobility of the polymeric chains is assumed to be slowed, making the network more rigid and entrapping lysozyme strongly (Corradini et al., 2013).

3.2 *In vitro* antimicrobial activity of lysozyme activated film

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

3.2 Water activity, pH and Microbiological analysis

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

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

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

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

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

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

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

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

3.3 Colour

As reported in Table 3 smoothie samples packed in AP showed the significantly highest lightness and hue angle values compared to control one during storage at 10 °C. In sample packed in CP a significant decrease of lightness and a reduction in hue angle were observed during storage at 10°C. In both samples the colour changes, which are reflected mainly by a browning increase (reduction of lightness), may be due to the PPO and POD enzymes' activities (Terefe, Tepper, Ullman, Knoerzer & Juliano, 2016). Moreover, the highest colour degradation due to enzymatic browning was more evident in the control sample (CP) because of its highest microbial growth, mainly in terms of *L. monocytogenes* growth, that induced a greater cell disruption (Zhou et al., 2014). Table 4 shows the lightness and hue angle colour parameters variations in AP and CP samples during storage at 4°C. 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. Also, in this case both samples showed a colour degradation during time, even if less intense than in samples stored at 10°C. This is due, as known, to the reduced enzymatic activity and microorganisms grow because of the lowest storage temperature (Martin-Diana, Rico, Barry-Ryan, Mulcahy, Frias & Henehan, 2005). The control sample CP underwent the significantly highest browning also during storage.

Conclusions

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

as expected, lysozyme activated pouches showed the best antimicrobial effect in samples stored at 10 °C compared to those stored at 4 °C. This difference is due to the fastest kinetic release of lysozyme, from the internal surface of packaging material, at the highest storage temperature, as also demonstrated by the lysozyme release kinetics. As a consequence, smoothie samples packed in lysozyme activated pouches showed a lower microbial grow and a better colour retention during storage than control samples.

Obtained results highlighted the potentiality of lysozyme activated biodegradable packaging to be applied successfully in food industry for the improvement of shelf-life and safety of minimally processed juices and smoothies. In addition, the combination of this active packaging with other non-thermal technologies such as pulsed electric field, high pressure homogenization, ultrasound or cold plasma can be considered as a strategy to further increase the preservation potential of this innovative packaging since it can further increase the antimicrobial activity of lysozyme.

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Figure Captions

Figure 1. Kinetics release of lysozyme from activated PLA film to buffer solution until 204 h of storage at 4 and 10 °C.

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

Figure 2a. The microbial cell loads of *Listeria monocytogenes* Scott A inoculated in the smoothie samples packed in lysozyme activated (AP) and not activated (CP) pouches, during 10 days of storage at 10 °C.

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

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

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

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

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

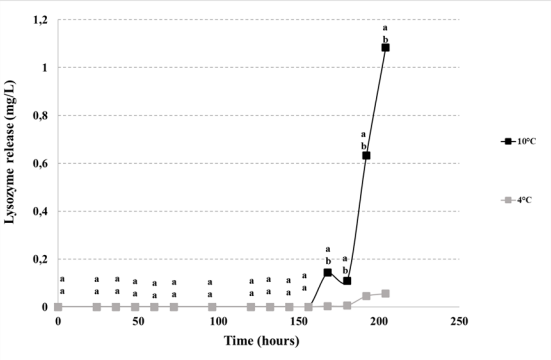
$^{A-F}$ Values followed by different letters differ significantly for each sample during 10 days of storage at P<0.05 level

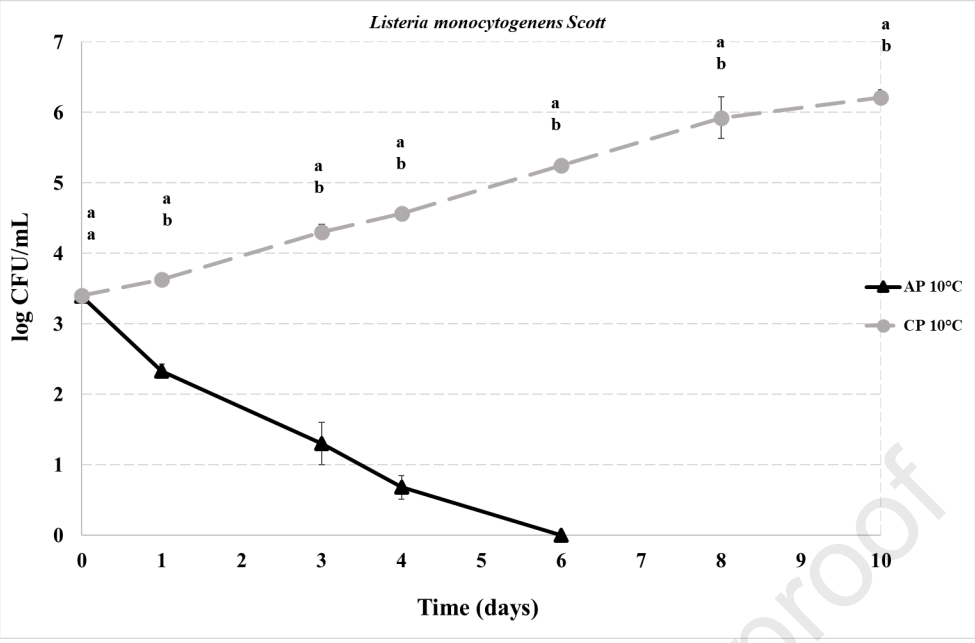
Table 4. Lightness (L*) and hue angle (h°) values of smoothie samples packed in lysozyme activated (AP) and not-activated (CP) pouches during storage at 4 °C.

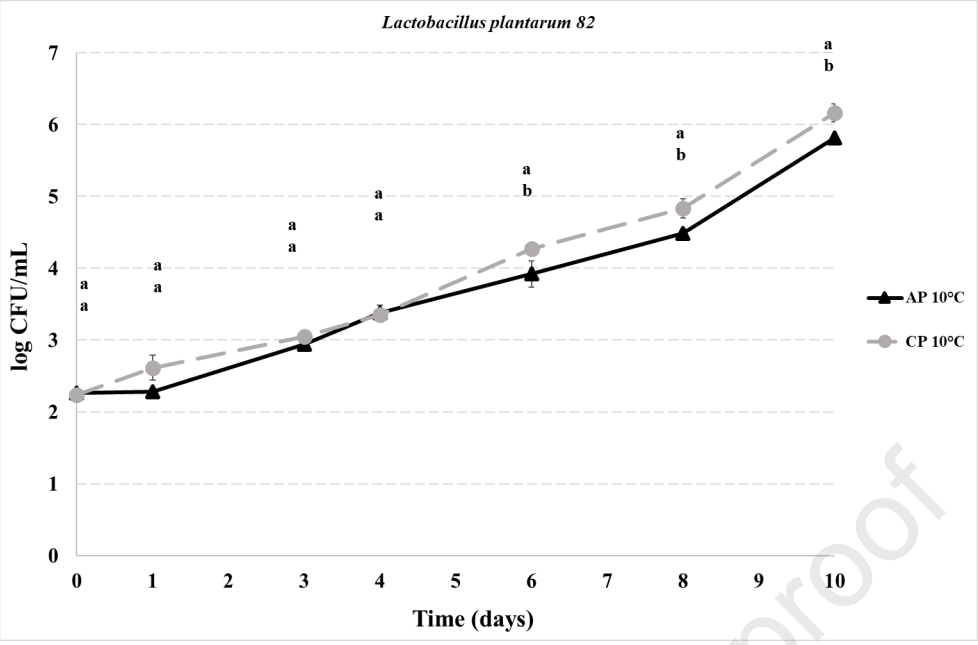
Lightness (L*)							
Days of storage	**0**	**2**	**4**	**7**	**10**	**13**	**16**
AP 4°C	56.53±0.01^{aA}	56.23±0.02^{aB}	56.08±0.7^{aC}	56.13±0.09^{aC}	56.11±0.12^{aC}	56.00±0.18^{aC}	55.01±0.14^{aD}
CP 4°C	56.53±0.01^{aA}	56.12±0.09^{aB}	56.00±0.3^{aB}	55.28±0.20^{bC}	55.47±0.09^{bC}	54.81±0.20^{bD}	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}

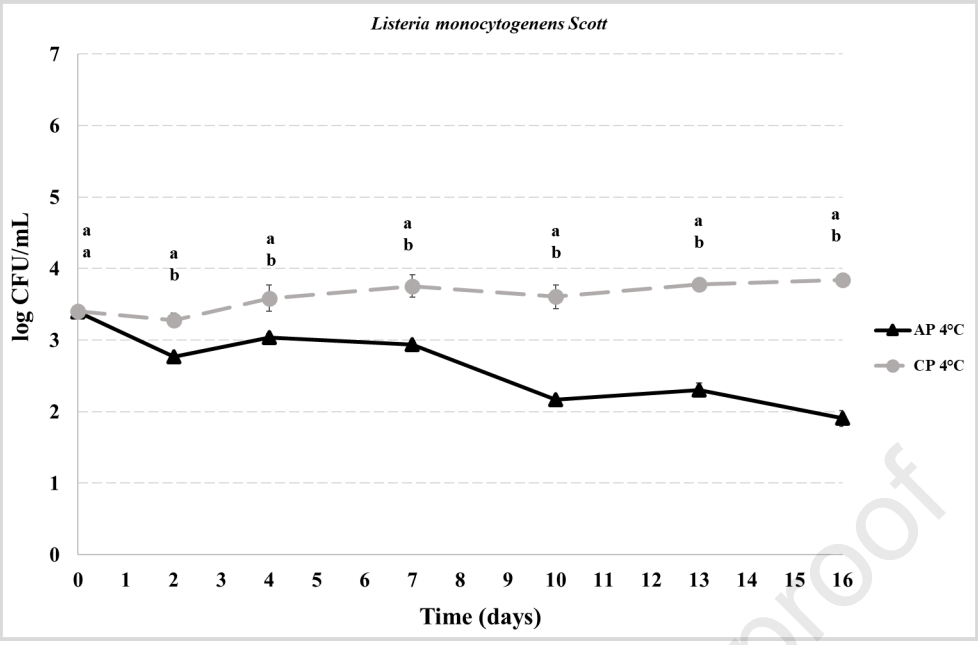
$^{a–b}$ Values followed by different letters differ significantly between samples at each time of storage at P<0.05 level

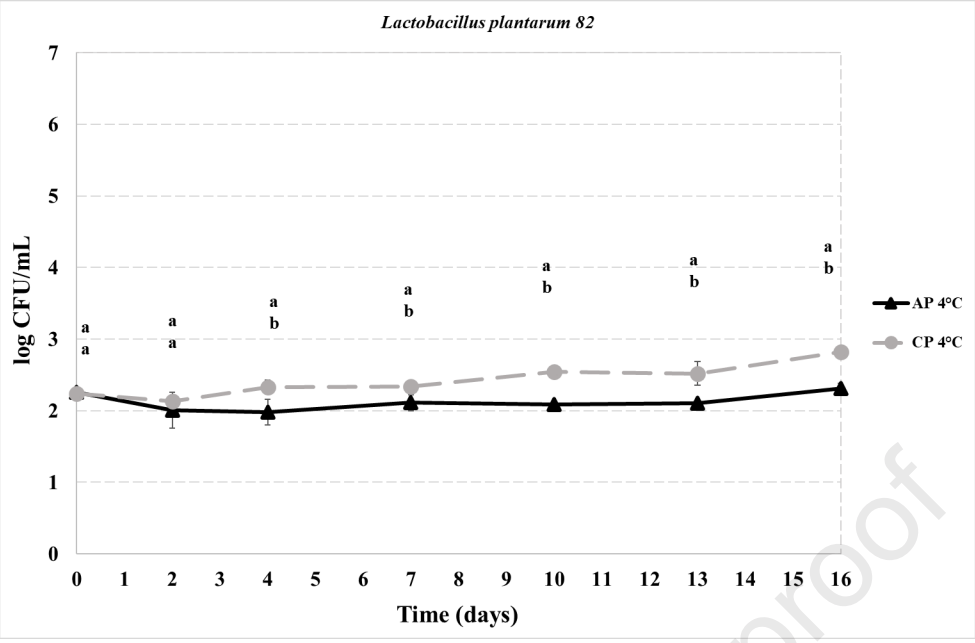
$^{A-D}$ Values followed by different letters differ significantly for each sample during 16 days of storage at P<0.05 level











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

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: