Active coating for storage of *Mozzarella* cheese packaged under thermal abuse

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Abstract

A novel antimicrobial packaging system, as active coating on a commercial Poly(ethylene terephthalate) (PET) film, was formulated and tested in vitro against the spoilage bacteria of *Mozzarella* cheese. It is based on layered double hydroxide (LDHs) intercalated with salicylate and carbonate anions dispersed in a solvent-based heat seal. The population of spoilage microorganisms (total coliforms, *Pseudomonas*, fungi), along with the functional microbiota of *Mozzarella* cheese (lactic acid bacteria) was characterized. Microbial shelf life was evaluated at 18°C, to simulate thermal abuse. Experimental results show an increase in the microbial shelf life of the packaged *Mozzarella* cheese of about 20 days, confirming that the investigated active coating may exert an inhibitory effect on the microorganisms responsible for spoilage phenomena, without affecting the functional microbiota of the product**.**

Keywords: PET; active packaging, food preservation; microbial shelf life

1. Introduction

The research on the formulation and production of innovative materials for food applications, such as packaging and other food contact surfaces, is rapidly increasing in recent years thanks to the growing field of preparation of advanced structural and functional composites and nano-composites. A considerable effort to extend the shelf-life to retain the food quality has encouraged the exploration of new packaging materials, as well as innovative coatings (Ahmed and Alam, 2012; Azzi et al., 2012; Rhima et al., 2013 ; Silvestre et al., 2011). Among the challenging solutions in this field, antimicrobial packaging is gaining interest from researchers and industries due to its potential to provide quality and safety benefits (Appendini and

Hotchkiss, 2002; Joerger, 2007; Reig et al., 2014; Suppakul et al., 2003). The reason of antimicrobials incorporation into a food package is to prevent surface microbial growth. Great attention has recently emerged around the hybrid organic-inorganic systems and, in particular to those in which layered fillers are dispersed in a polymeric matrix. Such hybrid composites possess very unusual properties, very different from their microscale counterparts. In particular Layered Double Hydroxide (LDHs), or hydrotalcite like compounds, have received considerable attention as active molecular ions delivery vehicles due to their anion exchange properties. These compounds, also known as ‘‘anionic clays” have general formula $[M(II)_{(1-x)}M(III)_x(OH)_2](A_{x/n})^- \cdot mH_2O$ where M(II) is a divalent cation such as Mg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} or Co^{2+} and M(III) is a trivalent cation such as Al^{3+}, Cr^{3+}, Fe^{3+}, or Ga^{3+} with A^{n-} an anion of charge n such as CO_3^{2-}, Cl^-, NO_3^- or an organic anion. (Costantino et al., 1998; Tammaro et al., 2005). The new trend of the research is based on the fact that the active molecules, fixed by ionic bonds to the inorganic lamellae, not only can improve the compatibility with the polymer matrix but can exhibit the antimicrobial activity being anchored to the lamellae, or being slowly released in particular environments. The release occurs via a de-intercalation process, which consists in anion exchange displacement reactions. As a consequence, the release rate is also dependent on the rate of the de-intercalation process. This in turn, depends on the electronic and spatial structure of the guest species. In all these processes is of fundamental importance the transport of water to bring the counter ion and to allow the exchange and the release of the active molecule (Costantino et al., 2009). Fresh *Mozzarella* cheese is a typical “*pasta filata*” cheese from Southern Italy, with high moisture and a high fat content. It could be cut and manufactured in various shapes, and usually brined. It is characterized by a notable economic relevance because of the steady rise in its production and consumption. Traditional *Mozzarella* is packaged in a dilute solution of salts (NaCl and/or $CaCl_2$) called conditioning brine. The determining factors affecting preservation of the freshness of the *Mozzarella* are different: pH; hydrolysis of casein; calcium; ratio of calcium/sodium; acidity; presence of microorganisms. This last factor is crucial to maintain the freshness and the microbial shelf life of the *Mozzarella* (Baruzzi et al., 2012; Quintieri et al., 2012). Soft cheese products are excellent growth media for a wide range of microorganisms and, thus, display a short shelf life (Ruegg, 2003). Several studies have characterized the *Mozzarella* from a microbiological point of view (Costa Dias et al., 2012; Belli et al., 2013; Faccia et al., 2014; Caputo et al., 2015), recovering several microbial species: Lactic Acid bacteria (LAB) group, such as *Lactobacillus lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Streptococcus thermophilus*, enterococci such as *Enterococcus faecium* and *E. faecalis*, *Enterobacteriaceae* such as *Escherichia coli*, yeasts such as *Debaryomyces hansenii*

and *Kluyveromyces marxianus*, and various spoilage psychrophilic microflora (Cantoni et al., 2003a,b; Parisi, 2003a,b). Lactic acid bacteria constitute indigenous microflora of raw milk and represent the major component of starter cultures used for the production of dairy products. These microorganisms contribute to the quality by improving the taste and texture and inhibiting food spoilage bacteria by producing growth-inhibiting substances bacteriocins and large amounts of lactic acid (Jana and Mandal, 2011). Thus the evaluation of lactic acid bacteria concentration during production and microbial shelf life should be considered as an indicator of quality. Faecal coliforms live in the intestine of mammals and their presence in foods is an index of faecal contamination (Sherfi et al., 2006; Sinigaglia et al., 2008). According to the Italian legislation (Gazzetta Ufficiale della Repubblica Italiana, D.P.R. 14/1/1997), coliforms are the test microorganisms for evaluating the microbial shelf life of *Mozzarella* cheese. Another microbiological parameter for safety control is the Total Viable Count (TVC) that gives a quantitative idea of the presence of mesophilic aerobic microorganisms of animal origin and of the global contamination. The growth of *Pseudomonas* spp. on the cheese surface has also to be considered a consequence of contaminated water using during manufacture (Cantoni et al., 2003a,b). Presence of coliforms in cheese is an indication of poor sanitation Coliforms grow rapidly during the first days of storage, producing lactic acid, acetic acid, formic acid, succinic acid, ethanol, CO_2 and causing decrease in pH. The pH is also an important factor. It affects the casein proteolysis, the amount of calcium, the ratio Ca-Na-Casein and sensory characteristics of *Mozzarella* cheese, also during thermal abuse at 15°C and 18°C (Laurienzo et al., 2006; Conte et al., 2007). Extending the microbial shelf life of *Mozzarella* cheese is an important issue to the dairy industry due to the high interest in extending the distribution of the traditional product beyond the market borders. Currently, *Mozzarella* is packaged in rigid containers or flexible films of multilayer material, packages made of polyethylene/paper laminated films or tetrapack-type packages. None of these packages solves the problems of the limited shelf life of fresh *Mozzarella* cheese. Generally, at 4°C, the shelf life of *Mozzarella* is about 2-3 days. One approach for extending the microbial shelf life of *Mozzarella* cheese is to introduce antimicrobials. (Gill and Holley, 2000). The aim of our work was to develop an innovative packaging system for *Mozzarella* cheese that would be able to guarantee prolonged microbial shelf life, with no influence on the production methodology and no influence on the taste, mechanical properties, and nutritional properties of the product. We verified the effectiveness of a controlled-release active system containing an antimicrobial molecule incorporated in a LDH within the packaging material of fresh *Mozzarella*. We report on the main results of our research, which include evaluation of pH, microbiological analysis and microbial shelf life evaluation, as

function of storage time. It is worth noting that the temperature used in this study (i.e. 18°C) is not the storage temperature of *Mozzarella*. We operated in this extreme conditions in order to accelerate spoilage phenomena.

2. Materials and Methods

2.1 Materials

Poly(ethylene terephthalate) (PET) (CLEARTUF 8006, intrinsic viscosity 0.80 dL/g), was supplied by 2R Packaging Ltd, Naples, Italy, in film form 12µm thick.

The active filler, based on a Layered Double Hydroxide intercalated with antimicrobial salicylate anions (listed in EC-Directive 10/2011/EC of 14 January 2011) and carbonate anion, was produced by Nicefiller Ltd, an start up of the University of Salerno (Italy), accordingly to a previously reported procedure (Frunza et al., 2008). The glue used was a solvent-based paint normally used for packaging of dairy products (Novacote HS-8256, solid content 38 $\pm$ 2%, viscosity 300-600 mPa·s at 25°C) purchased from COIM S.p.A. (Italy). Its constituents are in accordance with the EC-Directive 2002/72/EC of 6 August 2002, including amendments.

Ethyl acetate (99% pure) was purchased from Sigma-Aldrich (Italy) and used as received.

2.2 Release kinetics

The release kinetic of the salicylate molecule in a fixed volume of a saline solution (0.9% w/v) was obtained by ultraviolet spectrometric measurement at ambient temperature, using a Spectrometer UV-2401 PC SHIMADZU.

2.3 Coatings preparation and processing

The active filler was dispersed in the solvent-based heat paint using ethyl acetate as solvent to obtain a relatively smooth and uniform coating (Vittoria et al., 2011). After the application of coating on PET film the solvent was evaporated at room temperature. The obtained active film, named PET_active, has 3.8 g/m^2 surface density and a coating thickness of 8-10 µm, measured respectively by weight difference on a known area, and a micrometer.

142 *2.4 Samples Packaging*

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144 *Mozzarella* cheese, made from cow milk, were purchased from a cheese factory located in
145 Campania (De Caro Factory, Fisciano (SA)), as soon as prepared, and brought to our laboratory
146 under refrigeration (4 °C). In Italy, *Mozzarella* is sold in small size pieces (cylindrical cheese
147 samples of 50g called *bocconcino*), usually 20 pieces for 1kg of mozzarella and packaged in
148 bags containing 1 liter of diluted brine solution (preservation solution).
149 For our experiment a single cylindrical cheese samples (*bocconcino*) (50 g of weight and 5-7 cm
150 of diameter) were removed from their commercial packages and packaged in individual pouches
151 (20 cm x 20 cm) of PET_active containing 50 mL of pristine preservation solution, in the same
152 ratio product/preservation solution of commercial mozzarella. *Mozzarella* cheeses packaged in
153 PET bags were used as control. The obtained packaged samples (in total 17 single *bocconcini*)
154 were stored at 18°C to simulate thermal abuse during storage and analyzed at different storage
155 times. All sample handling operations were done under aseptic conditions using gloves, sterile
156 forceps and laminar flow hoods. The analysis of the *Mozzarella* samples were run in triplicates,
157 analyzing tree pieces of the single *bocconcino* and the measurements were performed according
158 to the following time schedule (h): time zero (day of the packaging of samples), 8, 24, 48, 72,
159 144, 240, 360, 528 storage time.

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161 *2.5 Microbiological Analyses*

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163 For microorganisms isolation 10 g of *Mozzarella* cheese from each sample were aseptically
164 collected at storage time of 8, 24, 48, 72, 144, 240, 360, 528 hours and added to 90 ml of saline
165 peptone water. The mixture was homogenized for 1 min in a stomacher 400 (Lab Blender,
166 Seward Medical, London, UK) and 1ml of homogenate subjected to serial dilutions in the same
167 diluent. Aliquots of 0.1 ml of different dilutions were spread onto the culture media selective for
168 each type of organism (all from Oxoid). Microorganisms were enumerated with the method
169 based on count of Colonies Forming Units (CFU), by using 25-250 $CFUplates^{-1}$ as range of
170 countable colonies to limit the error due to variability (1, 2, 3). Total viable count (TVC) were
171 evaluated by unselective Plate count (PCA) agar incubating at 30 °C for 48 hours (h) (UNI EN
172 ISO 4833-1:2013 / UNI EN ISO 4833-2:2013/Cor.1:2014). Yeasts and moulds were growth on
173 yeast peptone dextrose agar (YEPD) (10 g/L yeast extract; 20 g/L peptone; 20 g/L dextrose; 20
174 g/L agar) at 30°C for 3 days (d) (ISO 21527-1:2008). Total coliforms were isolated on violet red
175 bile (VRB) agar, with a covering layer of the same medium, incubated at 30°C for 48 h (ISO

4832:2006). *Pseudomonas* spp were selected on *Pseudomonas* agar base supplemented with (with selective supplement, CFC) at 30°C for 72 h (ISO/TS 11059:2009 (IDF/RM 225:2009). Lactic acid streptococci were isolated on M17 agar after 48 h at 35°C (ISO 7889:2003 (IDF 117:2003)). De Man, Rogosa and Sharpe (MRS) agar was utilized in anaerobic conditions for mesophilic and thermophilic lactic acid bacilli (LAB), by incubating 48 h at 35 or 42 °C, respectively (ISO 15214:1998). Microbiological values reported are the average of three replicates.

2.6 pH evaluation

pH determinations were carried out on 20 ml of samples of homogeneized *Mozzarella* cheese stored in both packaging (PET and PET_active) by a pH-Burette 24 pH-meter equipped with a type 5014T electrode (Crison Instruments, Spain). Data are the average of three replicates.

3. Results and discussions

3.1 Release kinetic of salicylate

The release of salicylate molecule from the hybrid LDH-salicylate (8.2 micron) is shown in Figure 1. The same granulometry was used for the active coating. In the first times of contact with the solution it can be observed a fast release of the salicylate molecule (~65%) after ~4 h, followed by a slower release step (~4-48 hours) and a level off of release around 76% after about 72h of contact with solution.

3.2 Antimicrobial activity and microbial shelf life evaluation

3.2.1 pH measurements

The pH was measured on *Mozzarella* cheeses stored in both packaging (PET and PET_active) at the beginning of the tests and at regular intervals throughout. Figure 2 displays the pH evolution, as function of time (days) for the solution stored in PET and PET_Active film. The numerical values are reported in Table 1. The *Mozzarella* sample stored in the PET film (control) showed a gradual decrease of pH over the time and at 48 hour the reduction was almost complete (-0.71 vs -0.75 units). In PET active film this reduction was less rapid and regular and was characterize by

a slow phase in the first 48 hours (-0.35 units) and a faster reduction the next 24 hours. This different trend could be attributed to the presence of carbonate anions in the active filler dispersed on the PET_active film and to the action of active material on the microbial community evolution that could change the balance of organic acids. In many studies, correlations among pH, calcium content, and proteolysis of *Mozzarella* have been identified (Guinee et al., 2002; Kindstedt et al., 2001; Almena-Aliste et al., 2002; Jeremiah et al., 2003). In general, a pH reduction increases the ratio between soluble calcium and colloidal calcium, which, in turn, would increase the degree of casein hydration (Sood et al., 1979) and reduce its aggregation state, ultimately increasing the susceptibility of casein to hydrolysis (Feeney et al., 2002). It is interesting to observe that at 72h it is reached a level off of pH variation (5.08), in agreement with the salicylate release kinetic, observed in Figure 1.

3.2.2 Microbiological Analyses

Samples of *Mozzarella* cheese individually packaged in PET and PET active film were homogenized at indicated time and released microorganisms enumerated by growth on appropriate culture media. Figures 3 and 4 show the dynamic of community in the short (72 hours, Fig. 3) and long period of storage at 18 °C (up to 22 days, Fig. 4). The number of lactic acid bacteria (LAB) increased after only a few hours of storage at 18 °C, reached maximum level between 48 and 72 h and remained constant throughout the duration of the experiment. For the entire phase of storage, the number of lactobacilli and streptococci were respectively comparable between the control and the samples packged in PET_active film, indicating that the presence of the antimicrobial agent does not have a significative effect on the viability of lactic acid bacteria. The total viable bacteria count (TVC) show similar growth curves, however for the samples packged in PET their level was always higher than those stored in PET_active film. Significant differences were instead evidenced for other microbial groups, both in population successions and in the final biomass (at plateau phase). Small numbers of coliforms (1.6 10^3 CFU g^{-1}) were found in the cheese samples at the packaging time (time 0). In the first eight hours the levels of bacteria increased slightly (about of 0.1 log) in the packged PET sample and remained constant at least for the next 40 hours. Subsequently, when the growth of lactic acid bacteria stopped (between 48 and 72 hours), coliforms resumed their growth and between 6 and 10 days reaching a plateau value that was about 1.7 log greater than that a time 0. On the contrary, the PET_active film, completely blocked the growth of coliforms for at least the first 3 days. On the sixth day their number increased by 0.5 log and reached its maximum value of 1 log higher than the initial

number, between 10 and 22 day. Thus, the number of coliforms found to 72 hours from the packaging in control PET, were found only after many days of storage in PET_active film (> 6 days). Furthermore, in PET-active film up to 22 days this number remained well below that found in the control. A similar trend with slight differences was found for yeast populations. At the time of packaging these fungi were not detectable in our *Mozzarella* cheese. In the sample stored in PET, a few hours after packaging the population began to grow and reached almost the maximum value on the third day of Log 5.5 CFU g^{-1} (Log 6 CFU g^{-1} at sixth day). As for coliforms, storage in PET_active film completely blocked growth of the yeasts at least for 3 days, and their number was too low to be quantifiable. At sixth day the fungi number reached plateau also in PET-active film, but was > 0.5 log lower than in the control, corresponding to that we found at 48 h in PET stored samples. Therefore, as well as for coliforms, the growth of fungi population was shifted some days forward and greatly limited by the PET_active film. The numbers of such microorganisms found in PET_active film at 6 days were comparable for both population at those found in control cheese in the first storage hours in PET. *Pseusomonas* spp. were undetectable in all samples analysed for almost 10 days. At 15th day their number was 100 and 10 CFU/g respectively, in samples stored in PET and PET_active film. Such difference was costant until the 22th day, though was slightly reduced (0.7 log). Thus, our data indicate that the active PET film is able to exert a long-term inhibitory action on the main microorganisms responsible of spoilage of the cheeses (coliforms, yeasts and *Pseudomonas* spp.), while it does not interfere with the growth of lactic acid bacteria. It has not established a standard for this group of micro-organisms in *Mozzarella* cheese; however, it is known that yeasts and moulds have an important role in the spoilage of sliced cheese. (Dos Santos Pires et al., 2008). The PET_active film was able to inhibit yeast growth by approximately >0.5 log10 unit compared with that of the control over a 15 days of storage.

3.2.3 Microbial shelf life determination

To calculate the microbial shelf life (S.L.) of the packaged *Mozzarella* cheese, the approach proposed by Corbo et al., 2004, using the Gompertz equation (Eq. 1), was adopted:

$$Log\left(\frac{cfu}{g}\right) = \left|\log\left(\frac{cfu}{g}\right)\right|_{max} - A * \exp\left\{-\exp\left\{\left[\mu_{max} * 2{,}71\frac{\lambda - S.L.}{A}\right] + 1\right\}\right\} + \\ + A * \exp\left\{-\exp\left\{\left[\mu_{max} * 2{,}71\frac{\lambda - t}{A}\right] + 1\right\}\right\} \quad (1)$$

where $\left|\log\left(\frac{cfu}{g}\right)\right|_{max}$ is the decimal logarithm of the microbial acceptability limit, A is the maximum bacteria growth attained at the stationary phase, μ_{max} is the maximal specific growth rate, λ is the lag time (days) and t is the time (days).

All analyses were carried out in triplicate. The media and standard deviations were calculated. The data shown in the figures and tables are the average of all repetitions, whereas the error bars are the standard deviation. The confidence intervals of model's parameters were evaluated as follows: first, a fit was run with the original data; then, using the data points standard deviation, 100 additional fits were run on artificial data sets, which were generated by randomly varying the data around the fitted function. From these additional fits, a distribution of values for each parameter was obtained. The sets of data obtained for each parameter were statistically treated to obtain the 95% confidence interval. The results on microbial growth of *Mozzarella* cheese packaged in the two different systems, with and without active filler, were reported in Figure 5 for coliforms. The observed differences between the two packaging, suggest that salicylate molecule exerts a great influence on the maximum cell load reached at the stationary phase. Moreover, a prolongation of the lag time λ (days) was recorded. As matter of fact, the lag time increases by more than one day for the active film compared to the control system (Table 2). Because the microbial coliform group is one of the main responsible for quality decay during storage of *Mozzarella* cheese, cell load data were used to estimate the microbial shelf life of the investigated samples by fitting Equation 1 to the experimental data. According to the European Union (1997) the value of $\left|\log\left(\frac{cfu}{g}\right)\right|_{max}$ max was set to 4 for coliforms.

The curves shown in Figure 5 show the best fitting of the experimental data, whereas the model parameters obtained as well as the confidence intervals are reported in Table 2. As shown in the above table, the active packaging system slowed the growth of the spoilage microbiota during *Mozzarella* storage, leading to an increase in the microbial shelf life of the investigated *Mozzarella* cheese, which is approximately 6 days. Moreover the maximal specific growth rate (μ_{max}) becomes a third of the control value. All the investigated kinetic parameters show that the antimicrobial anions intercalated in the LDH exert an inhibitory effect on the growth of the coliforms microorganisms**.**

306 *3.2.4 Sensory Inspection*

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308 The analysis of the organoleptic properties (color, smell, texture) of *Mozzarella* samples stored
309 in the active packaging system were evaluated using the method MI 423 rev. 1 2015. The
310 differences between the active packaging and the control were already evident after 5 days of
311 storage. The test was extended to 22 days, and the differences between the active packaging and
312 the control cheeses were maintained (Figure 6). At 22 days of storage the colour and the texture
313 of *Mozzarella* cheese stored in PET_active packaging are quite unchanged. The cheese
314 appeared dense, elastic and the internal curd was granular and soft. The odour, although initially
315 less evident, became the typical odour of fresh cheese and the curd was still soft and elastic. In
316 the control, on the contrary, the degradation processes became more evident: the *Mozzarella*
317 cheese showed a rather translucent and slightly slimy surface. The internal pasta was buttery and
318 weak, with low persistency of the typical aroma.

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320 **4. Concluding remarks**

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322 In this paper we report an innovative active packaging based on an antimicrobial coating on
323 Poly(ethylene terephthalate) (PET), that would increase the microbial shelf life of *Mozzarella*.
324 The temperature used in this experimental study was 18°C, to simulate thermal abuse. The
325 antimicrobial molecule, anchored to a layered double hydroxide (LDH), was the salicylate anion.

326 - The release of the active molecule and the antimicrobial activity of the packaging were
327 tested on fresh *Mozzarella*, to demonstrate the effectiveness of controlled-release on
328 extending the microbial shelf life of food.
329 - The release of the salicylate is significant in the first 72 hours of contact with the
330 solutions, and in this period it was observed the main inhibitory effect on the Mozzarella
331 spoilage microorganisms.
332 - The best results were found with yeasts and coliform bacteria, for which it was calculated
333 also the microbial shelf life.
334 - Lactic bacteria, considered indicator of quality for the *Mozzarella* production, are
335 preserved in the whole investigated storage time.

336 Work is in progress to test the same material at storage temperature of *Mozzarella* (4°C) and to
337 on optimize the film properties through a correlation between physicochemical, biochemical, and
338 morphological properties. The final aim is to better understand the mechanisms that regulate the
339 interesting effects reported herein.

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