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Shelf Life Assessment of Fresh Poultry Meat Packaged in Novel Bionanocomposite of Chitosan Incorporated with ZnO nanoparticles synthesized using food industry by-products

Tesi in

Microbiologia Diagnostica E Predittiva

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Indice

Introduction

The aim of the work is to assess the stability of poultry meat wrapped with bio packaging that contains active compounds.

Poultry meat is the meat most consumed in the world, its consumption increased through the years, indeed its world production in 2003 was 77.335.000 tons and in 2018 reached 123.208.000 tons; in particular in the European Union the production in 2017 was 14.669.000 tons and forecasted to be 15.340.000 in 2030 (WATT Global Media, PoultryTrends, 2018).

This high consumption is due to many factors, like the nutritional values (low fat and high protein content) and according to Magdelain et al. (2008) is due to the absence of religious obstacles, but mostly thanks to the competitiveness of this meat respect the other ones, indeed these low prices will keep the poultry meat the most or one of the most consumed in the world for years.

Obviously, poultry meat, being a high perishable food, requires packaging and considering the production data reported before, we can imagine how big is the amount of plastic used to package this food. From few years traditional polymers started to be not well accepted and currently a lot of companies and institutions, like also European Union, are trying to reduce the use of the plastic through the sensitization of the costumers and adopting policies with this aim. Reduce the use and find alternatives is necessary, considering the high biological impact that synthetic polymers have on the environment, both for the CO_2 emission and for the presence of plastics and micro plastics in seas and oceans.

A possible alternative to the current polymers is the use of biopolymers, which term is referred to naturally occurring long-chain molecules but also materials which have been derived from these or bio-based monomers (Song et al., 2009). Many of them are also biodegradable, depending on the biopolymers used.

A bio packaging with the right formulation could well replace the traditional one, allowing the right conservation of fresh foods, in this case poultry meat, and keeping it acceptable until the end of the shelf life.

Chitosan is a good example of possible alternative polymeric material to replace petroleum-based plastics. This biopolymer is the second most abundant polysaccharide found in the nature after cellulose and due to its unique biological characteristics, including biodegradability and nontoxicity, many applications have been found either alone or blended with other natural polymers (starch, gelatin, alginates) in the food, pharmaceutical,

textile, agriculture, water treatment and cosmetics industries (Arvanitoyannis et al., 1998; Arvanitoyannis, 1999; Haque et al., 2005; Kim et al., 2005; Roberts, 1992; Yamada et al., 2005). Furthermore, antimicrobial activity of chitosan has been demonstrated against many bacteria, filamentous fungi and yeasts (Hirano and Nagao 1989; Kendra and Hadwiser, 1984; Uchida et al., 1989; Ueno et al., 1997).

Yet, the use of pristine chitosan is being hindered by the weak mechanical properties and water vapor barrier, but through the incorporation of nanofiller, like montmorillonite, this limitation can be overcome (Ferreira et al, 2016; Souza and Fernando, 2016; Pires et al, 2018).

Montmorillonite nanoparticles have shown improvements in the mechanical characteristics of certain biopolymers. In fact, it is reported that the addition of this compound to a film based on chitosan, even in small doses (1-5%w/w), resulted in increased resistance and a decrease in permeability (Souza et al, 2019a; Giannakas et al., 2014; Lavorgna et al., 2010; Xu et al., 2006).

Moreover, the incorporation of active compounds, with recognized antimicrobial and antioxidant activities, could be effective against microbial growth and lipid oxidation, extending the shelf life of food matrices (Pires et al, 2018; Souza et al, 2018c). Indeed the use of rosemary essential oil and zinc oxide nanoparticles can be an option. Antibacterial and antioxidant activities of essential oils (EOs) have long been acknowledged, and the food industry has recently been paying more attention to their application as natural antimicrobials and antioxidants, inside composite films (Du Plooy et al., 2009; Sánchez-González et al., 2010a). Among essential oils the largest number of reports on the effective antioxidant properties pertains to extracts from plants belonging to the family of *Labiatae*, particularly rosemary (*Rosmarinus officinalis* L.) (Waszkowiak, 2008).

According to Youssef et al. (2015) and Tian et al. (2019), the incorporation of nanometal oxides into biopolymers leads to an improvement in antimicrobial properties and increased UV filtration, as well as contributing to the reinforcement of the material. In particular, the incorporation of ZnO nanoparticles can lead to an improvement in the antimicrobial activity of the material, together with the improvement of packaging characteristics in terms of mechanical resistance, barrier effect and thermal stability (Espitia et al, 2012).

ZnO NPs obtained through chemical synthesis from natural sources is the cheapest and safest method (Agarwal et al, 2017). The ZnO NPs incorporated in our film are “eco-

friendly" as they were synthesized according to Alves et al. (2019) using apple peels, a by-product from the food industry. Through this innovative technique, which perfectly includes the concept of circular economy, it is possible to exploit a waste of the food industry, encouraging the use of natural resources (Souza et al, 2020; Fidelis et al, 2019; Homrich et al, 2018).

Therefore, the aim of this work was to create a bio composite based on chitosan incorporated with rosemary essential oil and different nanoparticles, namely ZnO NPs or MMT, alone or combined, to assess it potential to extend the shelf life of fresh poultry meat.

1. Poultry meat shelf life

1.1 Microbial growth

Poultry meat is a highly perishable product and according to Mead (2004), this kind of meat, like all types of meat, is an excellent medium for microbial growth.

The use of an appropriate cold chain throughout the production chain up to consumption ensures microbiological safety and the acceptability of the final product by the consumer. Indeed, shelf life depends directly on the storage temperature and on various intrinsic and extrinsic factors, which influence the degradation process and the microflora associated (Mead, 2004).

1.2 Spoilage

Microbial spoilage is an "ecological phenomenon", as it includes variation of different substrates availability during microbial proliferation that characterizes microbial association of meat during storage (Nychas et al, 2008).

Prevalence of a given microbial association is due to several factors (implicit, extrinsic, intrinsic, and process) related to processing, transport and preservation stages of meat market. Besides influencing degradative microflora, these factors also determine the speed with which is reached the final microbial population, so called "Ephemeral/specific spoilage microorganism" (ESO), able to develop through adaptation strategies (Koutsoumanis & Nychas, 2000; Nychas et al, 2007).

Poultry meat, like other types of meat, has a high-water content and a high nutrient content that provides carbon and energy resources needed for microbial development (Mead, 2004). According to Gill (1982), it is just after muscle transformation into meat, called "rigor mortis", which happens a change in concentration of certain water-soluble compounds. In fact, during this process there is a depletion of energy reserves, represented by adenosine triphosphate (ATP) and creatine phosphate, and conversion of glycogen into lactic acid. As a result, there is a typical decrease in pH, which promotes protein denaturation and associated with enzymatic proteolysis leads to release of peptides and aminoacids.

According to Gill (1986) and Nychas et al (1988,1998), substances that are catabolized by almost all microorganisms present in the meat, are subdivided into three classes:

- Compounds contributing to glycolysis (e.g., glucose, glucose 6 phosphate);

- Metabolic compounds (e.g., gluconate, gluconate 6 phosphate, pyruvate, lactate, etc.);
- Nitrogen compounds (e.g., urea, amino acids, water-soluble proteins, etc.).

In Table 1.1 we can see what are substrates utilized by different bacteria and preference of consumption.

Concentration of these compounds has been shown to influence: microbial association, type of deterioration (saccharolytic, proteolytic), rate of degradation, and main precursors of microbial metabolites that allow us to understand when the product has deteriorated (Koutsoumanis & Nychas, 1999; Nychas et al., 1998; Skandamis & Nychas, 2002; Tsigarida & Nychas, 2001).

Table 1.1 Order of substrate utilization during growth of major muscle spoilage bacteria[a] Nychas et al 2007

Substrate	Aerobic					Anaerobic[b]				
	A	B	C	D	E	A	B	C	D	E
Glucose/glucose-6-Ph	1	1	1	1	1	1	1	1	1	1
Lactate	2	2		2						
Pyruvate	3	3				2[c]				
Gluconate/Gl-6-Ph	4	4				2[c]				
Proprionate		5								
Formate							1[c]			
Ethanol		6								
Acetate		7				2[c]				
Amino acids	5	8	2	3		2[c]	1[c]		2	2
Ribose			3							
Glycerol			4							

Modified from Nychas et al. (2007).

[a] A: *Pseudomonas* spp.; B: *Shewanella putrefaciens;* C: *Brochothrix thermosphacta;* D: *Enterobacter* spp.; E; lactic acid bacteria.

[b] Under oxygen limitation and/or CO_2 inhibition.

[c] No specific order is given.

In fact, glucose has been found to be precursor of many compounds held responsible for off-odors detected during meat storage, while its limitation/exhaustion could be cause of metabolism change from a saccharolytic ones to an aminoacidic ones (Nychas et al, 1998, 2007).

According to Rouger et al (2017), storage temperature as well as nature and concentration of gases that make up atmosphere inside packaging, are selective factors for microbial populations that arise. In most studies, bacteria most present in spoiled product were

considered to be responsible for degradation, so a maximum value of 7 CFU/g for total viable counts was considered as a microbiological acceptability criterion (Höll et al, 2016; Zhang et al, 2012).

Microorganism considered as potential spoilers in poultry meat are:

- *Pseudomonas spp.*
- *Enterobacteriaceae*
- LAB
- *Brochothrix termosphacta*

When meat is stored under aerobic conditions and at different temperatures (-1 to 25°C), spoilage microflora is usually predominantly by *Pseudomonas* spp (Koutsoumanis et al, 2006; Koutsoumanis et al, 2006; Stanbridge & Davis, 1998). According to Nychas et al (2008), species belonging to genus *Pseudomonas*, *Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis*, are those most present in products stored under aerobic conditions, and a population of $10^7/10^8$ CFU/g is associated with slime and off odors' formation.

Appearance of these compounds occurs when pseudomonads have exhausted glucose and lactate's reserves, and pass to an aminoacidic metabolism. Other Gram-negative bacteria that can sometimes be isolated in poultry meat are *Shewanella putrefaciens* and some tolerant cold species of *Enterobacteriaceae* family (Mead, 2004). Among *Enterobacteriaceae*, main genera found in this products' class are *Hafnia* (*Hafnia alvei* and *Hafnia paralvei*), Serratia (*Serratia fonticola, Serratia grimesii, Serratia liquefaciens, Serratia proteamaculans* and *Serratia quinivorans*), *Rahnella*, *Yersinia* and *Buttiauxella* (Rouger et al, 2017).

Lactic acid bacteria and *Brochothrix thermosphacta* have been identified as a cause of deterioration due to product's acidification, rather than putrefaction and this type of spoilage is commonly associated with meat products packed in vacuum or modified atmosphere (Table 1.2) (Nychas et al, 2008).

Table 1.2 Spoilage association dominating on fresh meat stored at 0-4 °C under different gas atmosphere (Nychas et al, 2008)

Gas composition	Meat and poultry
Air	*Pseudomonas* spp.
>50% CO_2 with O_2	*Brochothrix thermosphacta*
50% CO_2	*Enterobacteriaceae*, lactic acid bacteria
<50% CO_2 with O_2	*B. thermosphacta*, lactic acid bacteria
100% CO_2	Lactic acid bacteria
Vacuum packaged	*Pseudomonas* spp., *B. thermosphacta*, *Sh. putrefaciens*

According to Mead (2008), preferential substrate for pseudomonads is glucose or glucose 6 phosphate, and its consumption determines a gradient of concentration with consequent outcrop of other glucose on meat surface where bacterial multiplication is taking place. Glucose can therefore be converted to 2-oxo-gluconate or gluconate, but since the latter are not readily assimilated by other organisms, this can lead to a competitive advantage (Nychas et al, 1988). During logarithmic development phase, Dainty (1996) detected a non-malodorous compounds production, such as short-chain fatty acids, alcohols and ketones.

Once glucose reserves are over, lactate and amino acids begin to be catabolized resulting in maleodorant compounds formation, in particular following sulfuric aminoacids use (cysteine, methionine, cystine), and when aminoacids source is over and microbial development enters a stationary phase, there may still be proteolytic and lipolytic activity (Mead, 2008). When product is stored in presence of carbon dioxide, some CO_2-tolerant lactic acid bacteria develop by consuming glucose and producing organic acids that produce sour/cheesy flavors, and despite pseudomonads may still develop, under these conditions are no longer decisive for deterioration (Mead, 2008).

In conclusion, spoilage is a result of environmental conditions applied and microorganisms interactions that make up the microflora (Nychas et al, 1998; Tsigarida et al, 2003). This interaction requires more in-depth studies as nutrient content can foster synergy between microorganisms or lead to competition for nutrients, and it is also necessary to evaluate how metabiosis phenomena and quorum sensing affect certain physiological attributes (Nychas et al, 2006; Nychas et al, 2007).

1.3 Pathogens

According to Rouger et al (2017), considering growth in poultry meat consumption, ensuring the market entry of microbiologically safe products remains a primary aspect. Before or after slaughter, it is possible that bacteria present in animal microbiota, those present in slaughterhouse and/or those present on working gear, may contaminate carcasses and consequently meat cuts. Among these microorganisms, pathogenic bacteria may also be present, such as species belonging to genera *Salmonella* and *Campylobacter*, which are mainly responsible for gastroenteritis occurrence following poultry meat consumption (EFSA, 2016). Also, according to EFSA (2016), *Campylobacter* has been the pathogen responsible for the largest gastroenteritis number in humans since 2005 and in particular 229,213 campylobacteriosis and 94,625 salmonellosis were recorded in 2005. According to Chai et al (2017), poultry meat was the first foodborne outbreaks cause in United States between 1998 and 2012.

In smaller measure, pathogens responsible for human disease after poultry meat consumption are also *Clostridium perfrigens*, *Escherichia coli* O157 and *Listeria monocytogens* (Corry and Atabay, 2001).

Campylobacter species most frequently isolated from poultry meat are *Campylobacter jejuni* and *coli* (Hue et al, 2011), and in particular *jejuni* is the main human campylobacteriosis cause (Mead, 2004). While *Salmonella enterica* is the most present pathogen in human intestine and in particular serovar *Enteritidis* is the one most associated with poultry meat and outbreaks (Jackson et al, 2013).

According to Mead (2004), *Campylobacter* has a greater ability to colonize birds' gastrointestinal tract than *Salmonella*. Indeed, Hue et al (2011) for *Campylobacte*r detected an average faecal contamination of 8.05 CFU/g log and an average carcass contamination of 2.39 CFU/g log, while according to Mead (2004) in case of *Salmonella* there is a high variability in the incidence of contaminated carcasses and would rarely exceed 100 CFU/g, remaining well below the level of contamination associated with food-borne disease.

These bacteria are very invasive in poultry and can penetrate into organs and deep into tissues, making their destruction by cooking less immediate; moreover, *Campylobacter's* surface contamination tends to be rather high, reaching 9 log CFU/g per carcass (Mead, 2004). Considering that both microorganisms types are thermophilic, cold chain use can

significantly reduce their growth. However, health precautions should be taken at all supply chain stages to avoid an excessive initial contamination.

In general, according to Mead (2004), contamination level that plagues these types of products is commonly accepted and it is widespread knowledge that an adequate cooking allows to reduce infection risk.

1.4 Sources of contamination

Modern intensive poultry farming often see a high number of animals kept together and, in order to achieve high production efficiency, carcasses are located close to breeding. In these conditions, it is easy to spread pathogens that are able to access breeding facilities (Mead, 2004). Microorganisms carried by live animals after slaughter may contaminate slaughterhouse and cause cross-contamination (Rouger et al, 2017), as we can see in Table 1.3. Compared to mammal slaughter process, poultry slaughter process has some differences which it is important to note:

- Water baths (cold or hot) at different points of process;
- Mechanical removal of feathers;
- Reduced carcass size, which facilitates management process and mechanization (Rouger et al, 2017).

Water baths can carry out a washing action promoting a bacterial loads reduction, but can also promote cross contamination (Göskoy et al, 2004; Russel, 2008). In particular, hot water use (50-60 °C) promotes a skin contamination reduction , but on the other hand causes skin relaxation and pores dilation with possible entry of bacteria in subsequent stages (Rouger et al, 2017). Evisceration phase is a critical carcass contamination point, because birds gastrointestinal tract hosts several bacteria and Hue et al (2011) and Pacholewicz et al (2016) have demonstrated a correlation between faecal contamination level and carcass contamination level. Psychotropic degradative bacteria contamination may also occur at this stage (Lahellec et al, 1993) and pseudomonads are often present on rubber gloves, so is best to avoid excessive carcass handling (Mead, 2004).

Table 1.3 Carcass contamination after each slaughter process stage (Mead 2004; Berrang & Dickens, 2000)

Processing stage	Aerobic plate count, 37ºC	Coliform bacteria*	*Campylobacter* spp.*
After:			
bleeding	6.8 ± 0.1**	5.0 ± 0.2	4.7 ± 0.5
scalding	5.0 ± 0.2	2.9 ± 0.2	1.8 ± 0.3
plucking	5.0 ± 0.1	3.4 ± 0.3	3.7 ± 0.3
evisceration	4.5 ± 0.1	3.1 ± 0.1	3.4 ± 0.3
washing	3.6 ± 0.2	2.2 ± 0.1	2.3 ± 0.3
chilling	2.9 ± 0.3	1.9 ± 0.2	1.5 ± 0.4

* Mean values from positive samples only (30 samples tested in each case). Results expressed as $\log_{10}$ cfu/ml of rinse fluid.
** ±95% confidence interval.

1.5 Lipid Stability

Lipid oxidation is one of the main problems in meat industry , as it leads to off flavor formation and nutritional value deterioration (Ahn et al, 1992). According to Baèza (2004) meat susceptibility to lipid oxidation depends on fatty acids unsaturation degree, pro-oxidizing agents content (iron-containing compounds) and antioxidants presence (vitamin A, C, E and enzymes containing selenium). Fatty acid composition of lipid fraction is strongly influenced by animal's diet during rearing (Jung et al, 2010; Dal Bosco et al, 2012). Lipid oxidation is a complex process whereby unsaturated fatty acids react with oxygen because of radicals action or ferrous compounds, determine peroxides formation or other primary oxidation products; secondary oxidation products, as aldehydes, ketones and esters, determine rancid aroma formation (Pèrez-Chabela, 2012).

The most common method to evaluate lipid oxidation degree in meat products is TBARS test, which involves spectrophotometric measurement at 532-535 nm of thiobarbituric acid reacting substances.

Chmiel et al (2019) have carried out a research to assess chicken breast lipid oxidation over time, with different packaging types. It has been found that cuts packaged under aerobic conditions (O_2 permeable film) and with MAP (75% O_2 and 25% CO_2) there has been a progressive increase in oxidation, the highest value was recorded for product packed in MAP and malondialdehyde concentration was 0.52 MDA/kg product; while for vacuum packed meat there was no significant increase in oxidation products during storage with an average of 0.25 MDA/kg product.

2. Meat preservation

2.1 Storage conditions

The main factor influencing fresh products shelf-life is storage temperature, as spoilage is directly related to microbial growth (Mead, 2004).

Indeed, fresh meat preservation is typically done in chilling conditions, as according to Smolander (2004) when temperature is below the optimum, duplication time and lag phase duration increase.

European Directive 71/118/EEC implies that poultry meat is kept at a temperature not exceeding 4°C, although according to Mead (2004) in commercial practice storage temperature may fluctuate during different points in supply chain, whose effects will then influence real shelf life.

Many of microorganisms causing spoilage are cold-adapted psychotropic bacteria that can develop at low temperatures, even near 0°C (Russel, 2002). For this reason, use cold chain may not be sufficient and it is necessary to supplement storage with a modified oxygen-free atmosphere packaging (Mead, 2004).

Smolander et al (2004) investigated temperature effect on bacterial populations development in MAP-packed chicken meat (80% CO_2 and 20% N_2) for a 12 days period; samples were stored at different temperatures, sometimes with a constant and sometimes variable thermal profile to simulate fluctuations that occur during distribution.

Initial total mesophilic aerobic count was about 4 log CFU/g (Fig. 2.1) in all tests performed and, according to criteria defined by Nordisk Ministerråd, was considered a maximum contamination of 7 log CFU/g as acceptable limit. Most bacteria contributing to aerobic count on plate were psychotropic.

Considering this limit, samples kept at a constant temperature of 6,1°C and at variable temperatures (average temperature of 7,4°C and 8,3°C) were already unacceptable after 5 days of storage. However, at most of other storage temperatures, samples exceeded acceptance limit at 9 days of storage, except sample kept at a constant temperature of 3,4°C which exceeded limit after 12 days of storage. Then there was a 7 day difference in shelf life when average temperature varied by 5°C.

Table 2.3 Mean temperatures for poultry meat samples stored in different conditions (constant or variable temperatures) (Smolander et al, 2004)

Test run/sample	Mean temperature, °C (0–12 d)	Remarks
A1	5.4	Constant temperature below statutory (in Finland)
A2	7.7	Constant temperature above statutory (in Finland)
A3	6.6	Variable temperature profile representing typical conditions in distribution chain from the producer to the consumer
B1	6.1	Constant temperature corresponding the highest statutory temperature (in Finland)
B2	2.9	Variable temperature profile representing ideal conditions in distribution chain from the producer to the consumer
B3	7.4	Variable temperature profile representing non-ideal conditions in real distribution chain from the producer to the consumer
C1	3.4	Constant temperature well below statutory, often recommended by the manufacturer
C2	6.5	Variable temperature profile representing typical conditions in real distribution chain from the producer to the consumer
C3	8.3	Variable temperature profile representing distribution chain with clearly broken cold chain

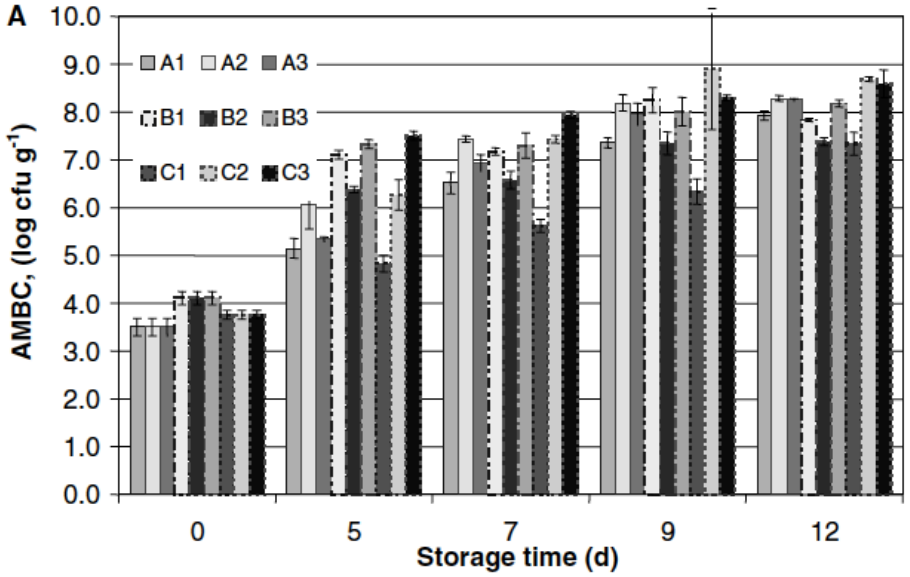

Figure 2.1 Aerobic mesophilic bacteria count for poultry meat samples stored at different temperatures (check table 2.1) (Smolander et al, 2004)

In lactic acid bacteria case (Fig. 2.2), their development has been hampered in samples stored below 3,4°C; while for temperature profiles above 6°C and in samples with high initial contamination, after 7 days the load has increased from 3,8 log to 7 log CFU/g, indicating that these microorganisms were a significant proportion of microbial degradation population.

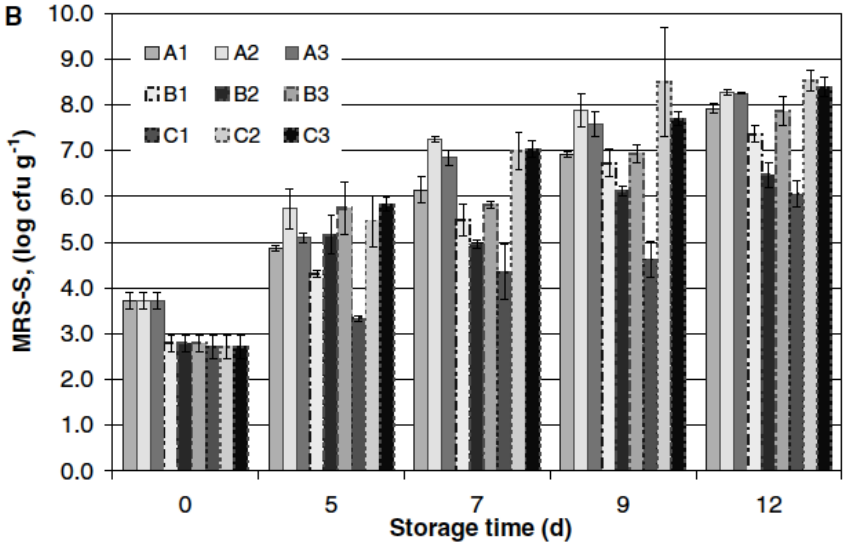

Figure 2.2 Lactic acid bacteria count for poultry meat samples stored at different temperatures (check table 2.1) (Smolander et al, 2004)

On the other hand, for *Enterobacteriaceae,* storage temperature had a more pronounced effect than for mesophilic and psychotropic counts (Fig 2.3). Initial contamination was about 2 CFU/g logs in all samples and sample kept at a constant temperature of 3,4°C, after 12 days had an increase of only 1 log CFU/g; while it reached 7-8 log CFU/g in all samples stored at an average temperature of more than 5,4°C.

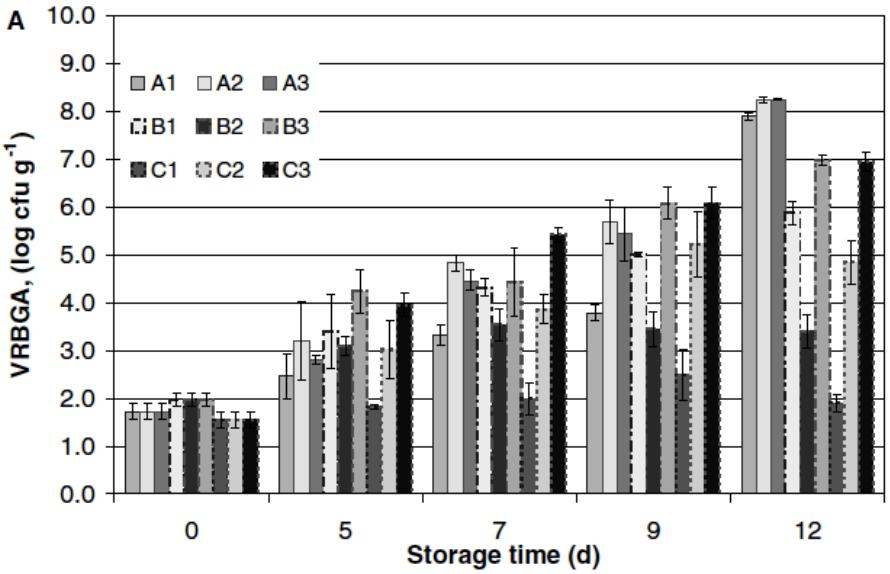

Figure 2.3 Enterobacteriaceae count for poultry meat samples stored at different temperatures (check table 2.1) (Smolander et al, 2004)

2.2 Traditional packaging

Traditional food packaging has four primary functions, namely: containment, information, convenience and protection (Dawson and Stephens, 2004).

In particular, according to Zhou et al (2010), packaging protects products from deterioration, whose factors are discoloration, off-flavor and off-odor development, nutrients loss, texture changes, increased pathogenicity and other measurable factors.

Indeed, variables influencing packaged meat shelf life are: product's type, gas mixture composition, packaging material, storage temperature and headspace (Zhou et al, 2010).

Poultry meat, compared to other animal species meat, is characterized by a high content in unsaturated fatty acids (UFA), very susceptible to oxidation, and by presence of specific microorganisms capable to propagate also in typical refrigeration conditions (4°C) (Kozačinski et al, 2012).

For these reasons, according to Marcinkowska-Lesiak et al (2015), packaging methods commonly applied to extend the shelf life of these products are modified atmosphere (MAP) and vacuum packaging (VP).

In particular, poultry meat is often packaged in a CO_2/N_2 atmosphere (with low O_2 residues), as these atmospheres effectively inhibit bacterial proliferation and, being a "white meat", coloring problem given by oxymyoglobin absence is only marginal (McKee, 2007; Sante et al, 1994).

At commercial level, packaging takes place using synthetic polymers and, according to Dawson and Stephens (2004), depending on material nature you will have different properties, such as barrier effect, mechanical resistance and sealing capacity. Permeability to oxygen and humidity are very important characteristics for meat packaging, indeed water vapor transmission rate (WVTR) and oxygen transmission rate (OTR) affect poultry meat quality.

Using vacuum packaging, retail meat cuts are usually packaged with vacuum skin packaging systems (VSP) where cut is placed in a polystyrene or polypropylene tray and sealed by vacuum with a shrink barrier film that fits product (Belcher, 2006).

Generally, film is made up of nylon, a barrier polymer (PVdC, EVOH, LDPE), tie layers and ionomers (Zhou et al, 2010).

According to Jenkins and Harrington (1991), nylon provides bulk, toughness and low melting points, while barrier films reduce permeability and ionomers provide seal for emetic closure.

Adopting modified atmosphere packaging with low O_2 content is necessary to use a packaging with high barrier effect for both water vapor and oxygen (Zhou et al, 2010).
This MAP type provides substitution of oxygen by N_2 and CO_2, divided in different percentages. Nitrogen is an inert gas, thus it is not absorbed by meat and does not react with pigments, but allows to maintain package integrity forming a headspace; while carbon dioxide dissolves in product and over a certain amount has an antimicrobial effect (Zhou et al, 2010).
Indeed, in CO_2-enriched atmospheres, carbon dioxide influences both lag phase duration and microorganisms duplication time, in particular when 80% CO_2 content is reached, aerobic bacteria growth rate can be slowed (Dawson and Stephens, 2004).
Although under these conditions some lactic acid bacteria species are not inhibited and are even involved in final spoilage (Sander and Soo, 1978).
As shown by Rouger et al (2017), using CO_2-enriched atmosphere you can prolong product shelf life respect the one stored with permeable film.

2.3 New techniques

Last twenty years research had the trend to improve or replace traditional conservation techniques. In particular studies were focused on natural substances with antimicrobial activity, mild technologies and active packaging (often biobased and/or biodegradable polymers).

Some natural compounds, such as essential oils, chitosan, nisin and lysozyme, have been designed to replace traditional preservative in order to obtain “green label” products (Zhou et al, 2010).

Treatment at high hydrostatic pressures (100-1000 MPa) is a non-thermal mild technology that, according to Patterson (2005), allows to obtain a more stable product thanks to its ability to reduce degradative microorganisms and block enzyme activity, without affecting nutritional characteristics (Zhou et al, 2010). In addition, according to Hugas et al (2002), HHP is an effective tool to control risk associated with *Salmonella spp* and *Lysteria monocytogenes* in fresh meat.

HHP at low temperatures could affect meat color, due to globin denaturation, but this is limited in poultry considering low myoglobin content (Hansen et al, 2003).

In United States, several manufacturers have started using this technology to prolong meat products shelf life (Hugas et al, 2002).

Active packaging consists in specific compounds incorporation into packaging that interact with product or environment, with purpose to maintain or extend product quality over time and consequently its shelf life (Kerry et al, 2006).

Antimicrobial substances incorporation able to contain undesirable microorganisms growth on product surface, has taken great attention (Zhou et al, 2010).

According to Cooksey (2005), antimicrobial films can be divided into four categories:

- Antimicrobial substances contained in a sachet and released into packaging during storage;
- Antimicrobial substances directly incorporated into film;
- Packaging covered with a substances carrier;
- Macromolecules forming biopolymer with antimicrobial activity.

3. Bioactive packaging

3.1 Biopolymers

Biobased polymers are compounds derived from renewable sources, present in nature already in polymeric form or as monomers that can be polymerized to obtain desired bioplastics (Figure 3.1).

It is important to note that bio-based polymers are not necessarily also biodegradable, for example there are bioplastics such as bio-polyethylene (PE) and bio-polyethylene terephthalate (PET) derived from renewable sources, such as sugar cane and ethanol, but not biodegradable.

A bio-based polymer, to be defined as biodegradable, must be able to decompose thanks microorganisms and enzymes action present in nature, until it is degraded to CO_2, H_2O and mineral (Souza and Fernando, 2016).

Renewable resources examples, from which biodegradable polymers can be obtained, include polysaccharides (e.g., starch, alginates, pectins, carrageenas, chitosan), proteins (e.g., caseins, serum, collagen, gelatin, plant proteins) and lipids (e.g., fats, waxes, oil) (Comstock et al, 2004; Cutter and Sumner, 2002).

There are also biodegradable polymers synthesized from monomers, such as polylactic acid (PLA) obtained from lactic acid produced by sugars fermentation from vegetable sources.

Finally, there are polymers synthesized by some microorganisms, this is polyhydroxyalkanoates (PHA) case.

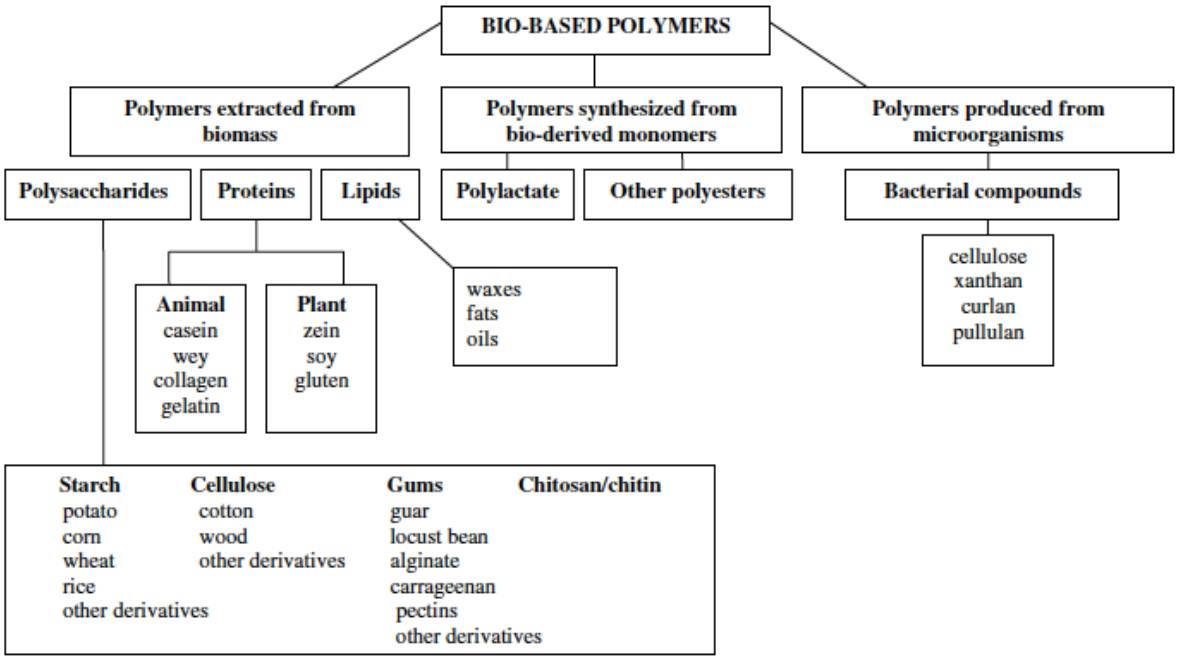

Figure 3.1 Bio-based polymers derived from different sources (Weber et al, 2002)

According to Comstock et al (2004), recent technological innovations have made possible to process these biopolymers, similar to petroleum-based plastics, that is, sheets, extrusion, spinning, injection molding and thermoforming.

Films obtained from these biopolymers and used in food packaging must meet requirements, such as: high barrier and mechanical resistance, biochemical, chemical-physical and microbiological stability, non-toxic, non-polluting and low-cost (Debeaufort et al, 1998).

Often, characteristics of these films are influenced by compounds addition, including plasticizers, cross-linking agents, antimicrobials, antioxidants (Cutter and Summer, 2002).

3.1.2 Chitosan: antimicrobial biopolymer

Chitosan is a biopolymer and is the second most abundant polysaccharide on our planet, after cellulose (Zivanovic et al, 2005).

The term “chitosan” (poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose) describe partially or totally deacetylate chitin compounds (Figure 3.2) (Tikhonov et al, 2006).

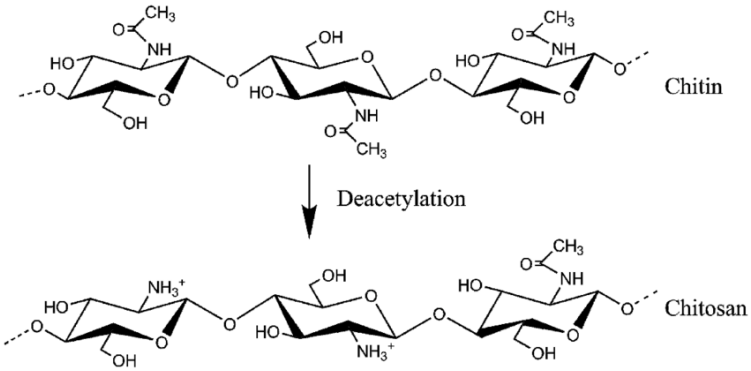

Figure 3.2 Chitin deacetylation representation (Wikipedia)

This polymer has the advantage to derive from renewable sources and we can find it in crustaceans exoskeleton and in microorganisms cell wall. Many researches are driving this polymer possible use in traditional plastics substitution, as in addition to be biodegradable, biocompatible and non-toxic, it is able to form films and membranes and also possesses intrinsic antimicrobial properties (Dutta et al, 2009; Darder et al, 2003).

Polysaccharides, including chitosan, thanks to well-ordered bound hydrogens network, are equipped with low permeability against oxygen, thus limiting food oxidation (Hassan et al, 2018; Ferreira et al, 2016; Souza et al, 2019a).

According to Cerisuelo et al (2012), this biopolymer has a permeability comparable to plastic obtained with the synthetic polymer EVOH, considered the least permeable to oxygen.

However, being a hydrophilic compound, it is characterized by high permeability to water (Srinivasa et al, 2007) and compared to traditional plastics has poorer mechanical characteristics (Azeredo et al, 2009).

According to Souza et al (2018a), bioplastic obtained with this polymer is characterized by a high barrier effect to UV lights, helping to limit lipid oxidation.

Chitosan antimicrobial action is influenced by several factors, indeed depends on microorganism target type, by intrinsic factors (e.g., positive charges density, concentration, chelating capacity etc.), by physical state (liquid or solid) and environmental factors (e.g., pH, ionic force).

According to Kong et al (2010), chitosan has a broad spectrum of action, but interaction mechanism is very complex and varies depending on Gram-negative and Gram-positive

bacteria. Studies are contradictory in assessing with which it is most effective, but many works did not reveal differences in its efficacy against bacteria (Wang et al, 2004).

Chitosan antimicrobial activity is pH-dependent, as this compound is soluble in an acidic environment and becomes polycationic at a pH lower than pKa (6,3-6,5) (Lim and Hudson, 2004).

When chitosan is in solid state, with a pH lower than pKa, surface molecules interact with surrounding environment as if they were in soluble state, and with anionic bacterial superficies create electrostatic interactions on which antibacterial activity depends (Kong et al, 2010).

3.2 Active packaging based on essential oils incorporated

Essential oils (EOs) extracted from plants and spices are endowed with antioxidant and antimicrobial activity, making their use interesting in food industry (Viuda-Martos et al, 2010).

Many of them have also been recognized as “GRAS”, but their use in food is limited due to strong flavor, to avoid this problem it is possible to incorporate them into packaging materials (Ruiz-Navajas et al, 2013).

Indeed, in recent years, EOs have been extensively studied as possible additives to add to biodegradable polymers, emulsifying them to obtain active packaging (Atarès and Chiralt, 2016).

These lipid compounds incorporation occurs through emulsifiers addition and/or using homogenizing techniques, and when the film has dried, lipid droplets remain trapped in the polymeric matrix (Atarès and Chiralt, 2016), as can be seen with electron microscope in Fig. 3.3 (Souza et al. 2019a).

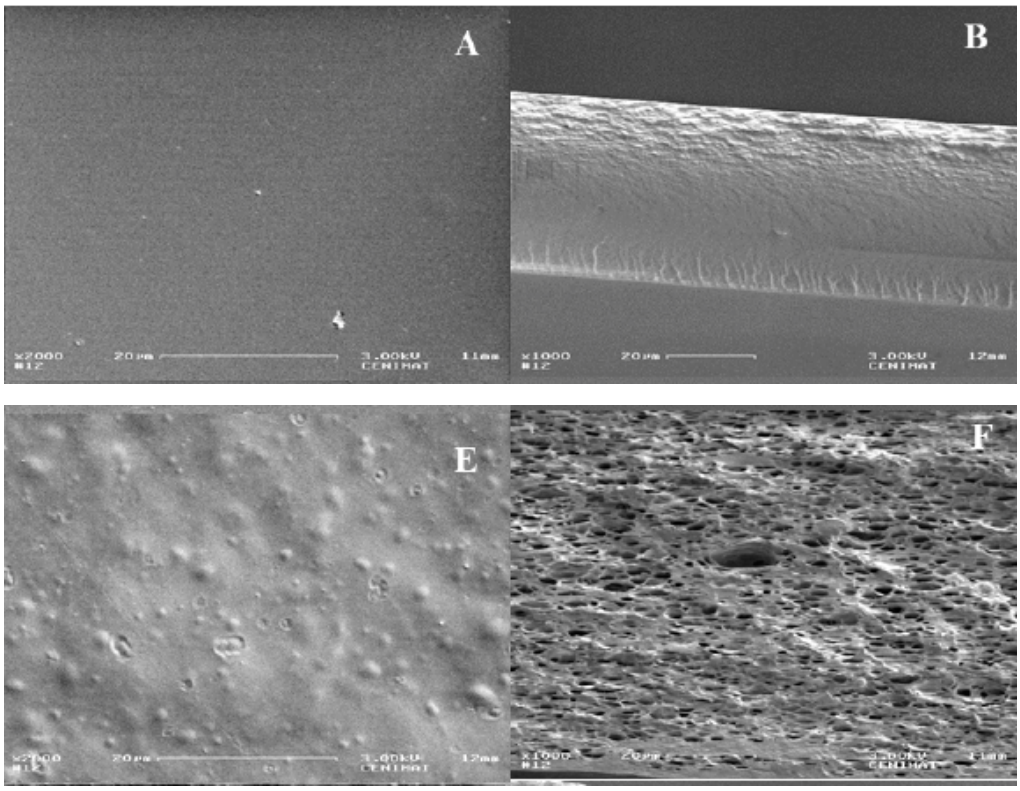

Figure 3.3 Scanning electron microscopy micrographs of surface and cross-section, respectively: chitosan film (A and B), chitosan film + 2% GEO (E and I) (Souza et al, 2019a)

Incorporation of 2% ginger essential oil (GEO) in chitosan film resulted in a spongy-like structure formation , and this is probably due to internal presence of oil drops that increased surface roughness (Souza et al, 2019a; Acevedo-Fani et al, 2015).

As far as permeability to oxygen is concerned, essential oils incorporation, in particular ginger and rosemary, leads to decrease barrier effect, as they act as plasticizers resulting in an increase in material elongation capacity (Souza et al, 2018a, 2019a).

As essential oils are rich in terpenes and phenolic acids, they have long been recognized as having antioxidant properties (Alves-Silva et al, 2013; Ruiz-Navajas et al, 2013). When oils are added to packaging, they serve as oxygen scooping or their compounds are transferred to food explaining antioxidant action on it (Bonilla et al, 2013; Atarès and Chiralt, 2016).

Pires et al (2019) found that MDA concentration during storage of a chicken meat sample packed with chitosan and rosemary essential oil did not vary significantly.

Bioactive compounds that characterize these essential oils have also been recognized as antimicrobial agents by Ruiz-Navajas et al (2013).

According to Arques et al (2008) and Burt et al (2007), essential oils attack microbial cells with different mechanisms: destabilizing the double phospholipid layer, destroying enzymatic systems, compromising the genetic material and forming fatty acid hydroperoxides.

Antimicrobial action depends on essential oil's type, Pires et al (2018) found a reduction in contamination of chicken packed with chitosan and ginger essential oil, while it did not detect antibacterial action for film with rosemary essential oil. Zivanovic et al (2005), obtained a reduction in contamination of meat packed with chitosan and oregano essential oil.

3.3 Reinforcement with nanoparticles

Biopolymers have excellent characteristics, as they are low permeable to oxygen, environment friendly, biocompatible and present abundantly in nature, but their use is limited by poor mechanical properties and barrier to water (Souza and Fernando et al, 2014).

Nanoparticles can interact chemically and/or physically with polymeric chain, making the gaps between filaments more tortuous and strengthening the structure, resulting in nanoreinforced materials with improved mechanical characteristics (Mihindukulasuriya & Lim, 2014; Souza and Fernando et al, 2016).

3.3.1 Montmorillonite (MMT)

Montmorillonite is a mineral clay belonging to phyllosilicates, a stratified clays family with a 2:1 ratio, two tetrahedral sheets formed by silica separated by an octahedral alumina sheet, and Van der Waals forces act between layers, creating spacing and where are present cations (Tang et al, 2012).

Chemically, can be defined as a hydrated hydroxide silicate containing sodium, calcium, aluminum and magnesium, $(Na,Ca)_{0,33}(Al,Mg)_2Si_4O_{10}(OH)_2 \cdot n(H_2O)$.

Attention is focused on its possible use as a bioplastics reinforcement material, as it has a plastic action and provides mechanical resistance, in addition to its high availability and low cost (Coelho et al, 2007).

Souza et al (2019a), have produced a biofilm based on chitosan and have assessed how 2.5% of MMT addition modifies polymer chemical, physical and morphological characteristics.

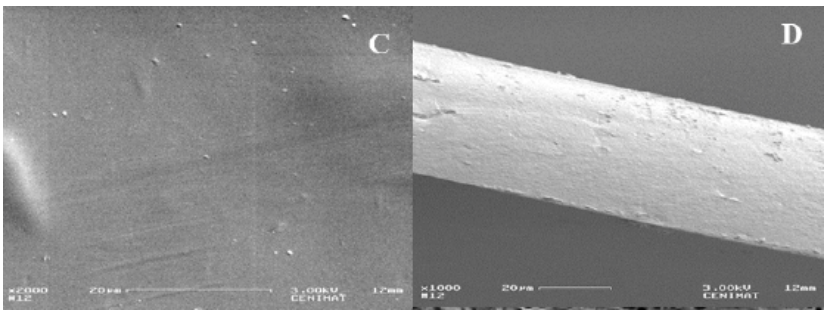

Figure 3.4 Scanning electron microscopy micrographs of surface and cross-section of chitosan film + 2,5% MMT (Souza et al, 2019a)

Cross-section of film incorporate with MMT shows a polymer high homogeneity, with a high interaction between nanoclay and chitosan determining an even more compact network (Figure 3.4) (Souza et al, 2019a).

Also, according to Souza et al (2019a), the high interaction degree between nanoclay and chitosan, allows to obtain a film with greater tensile strength and plasticity, without interfering with rigidity.

3.3.2 ZnO Nanoparticles

Nanometal oxides are interesting to researchers, as these compounds incorporation into biofilms contributes to improve antimicrobial, UV filtration and magnetic biopolymers properties (Youssef et al, 2015; Tian et al, 2019).

Nanoparticles antimicrobial action may depend on several mechanisms (Figure 3.5):

• Antimicrobial ions release (Kasemets et al, 2009);

• Electrostatic interactions between nanoparticles and bacterial membrane (Zhang et al, 2008);

• ROS formation due to light radiation (Jalal et al, 2010).

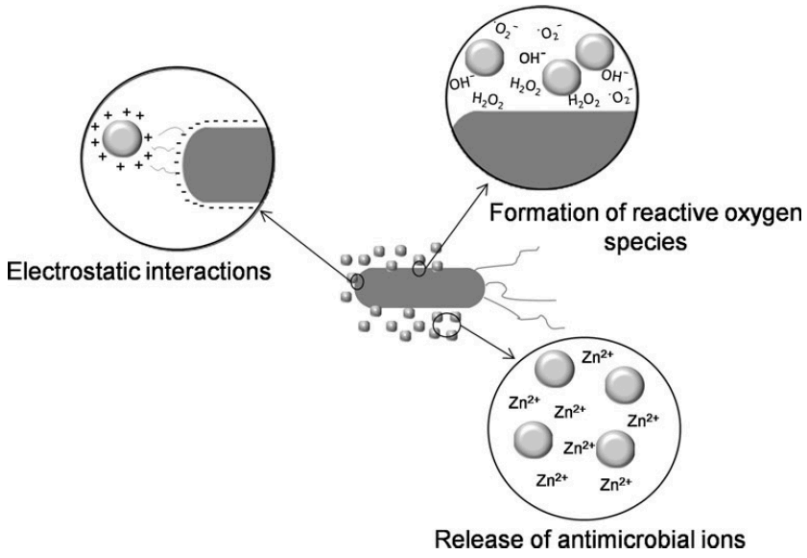

Figure 3.5 Different mechanism of antimicrobial activity of ZnO nanoparticles (Espitia et al, 2012)

In addition, according to Espitia et al (2012), these nanoparticles lead to an improvement in mechanical resistance, barrier properties and polymer stability.
In particular, zinc oxide is considered a very valid compound to be incorporated in food packaging, as it has antimicrobial and antifungal activity, and because it has been recognized as GRAS by the FDA. EFSA, assessed ZnO nanoparticles safety in food contact materials, recommending a maximum 25 mg zinc per person daily intake . Indeed, it noted that nanoform migration does not occur and therefore the safety assessment should focus on zinc soluble ions migration (EFSA, 2016).

These components cost is very low and ZnO nanoparticles production for marketing is mainly done by two methods: mechanochemical processing (MCP) and physical vapor synthesis (PVS).
Alves et al (2019) demonstrated that it is possible to produce ZnO nanoparticles from apples, adding zinc nitrate to extracts deriving from peel or flesh, or to solutions containing pure phytochemical compounds, such as sucrose and quercetin. In this way, you can valorize food waste and re-enter the green economy.

Souza et al (2020) incorporated ZnO NPs, made from apple peel, into a biofilm based on chitosan in order to package chicken meat. They found that film antioxidant capacity has

improved, probably thanks to phenolic compounds presence derived from apple skins used to obtain nanoparticles (Vrhovsek et al, 2004; Riaz et al, 2018)

Zinc oxide nanoparticles antimicrobial activity was tested on Gram-positive bacteria and some sensitivity to these compounds was found (Adams et al, 2006; Gordon et al, 2011; Reddy et al, 2007). ZnO has also shown some activity against Gram-negative bacteria such as *Pseudomonas aeuroginosa*, *Campylobacter jejuni* and *Escherichia coli* (Brayner et al, 2006; Ohira t al, 2008; Premanathan et al, 2011; Sawai et al, 2003; Xie et al, 2011).
Similar results were obtained from Souza et al (2020), which assessed nanoparticles incorporation effectiveness in a biofilm containing chitosan against *Staphylococcus aureus* and *Escherichia coli*.
On the other hand, no difference was found in total mesophilic count between only chitosan film and the one with ZnO, and indeed worse results were found at the higher ZnO concentration (2%) in comparison to film without nanoparticles. This could be due to the high interaction between chitosan and nanoparticles, with the latter limiting chitosan amino groups action against bacterial membrane.

Moreover, according to Espitia et al (2012), several factors can change zinc oxide antimicrobial activity, starting from particles size, surface and interaction with other antimicrobial substances.

4. Material and methods

4.1 Materials and reagents

All experiments were conducted using a completely randomized design with two replications. Different films were tested, namely: pristine chitosan (CH) film; CH + 2.5% montmorillonite (MMT); CH + 1% zinc oxide nanoparticles (ZnO NP); CH + 2.5% MMT + 1% ZnO NP; CH + 1% ZnO NP + 0.5% rosemary essential oil (REO) and CH + 2.5% MMT + 1% ZnO NP + 0.5% REO. Unwrapped meat was used as control. Results are the average ± standard deviation of the two replicates analyzed.

4.2 Sample preparation

4.2.1 Films production

Bio nanocomposite films were produced according to Souza et al (2017) and Dias et al (2014). Chitosan film forming solution was prepared by dissolving chitosan 1,5% (w/v) in glacial acetic acid solution 1% (v/v) with constant agitation using a magnetic stir plate overnight.

Glycerol was added as plasticizer in 30 % (w/w of chitosan) proportion and system was agitated for 5 minutes with ultraturrax at 15,000 rpm (IKA® T18, Staufen, Germany), followed by 15 minutes (360 W) in an ultrasound bath (Selecta, Barcelona, Spain), in order to obtain a complete homogenization.

When films were added by one or more composites, production process changed.

Bio-based films containing ZnO, were obtained by composite addition in 1% (w/w of chitosan) proportion prior to agitation process.

Bio-based films containing MMT, were obtained adding nanoclay in 1,5 % (w/w of chitosan) proportion, followed by three agitation process cycles and ultrasound treatments.

At last, in bio-based films containing rosemary essential oil (REO), REO in 0,5% (v/v of film forming dispersion) proportion and emulsifier tween 80 (0,2% v/v of REO) were added before agitation process and ultrasound treatment.

When MMT and REO were both in the film, EO and emulsifier were added before the last cycle of agitation.

Each homogenized dispersion (140 mL) was casted in glass molds (18 cm x 25 cm) and dried for 72h at room temperature.

Dried films were peeled and stored protected from light at 25°C until application on the meat samples.

4.2.2 Fresh poultry meat preparation

Chicken breast was bought at the local supermarket. The meat was subdivided into 4 sets of 14 samples (30g each), in order to wrap the meat with the six different films obtained plus an unwrapped sample as control, experience was done in duplicate.

Poultry meat samples (wrapped and unwrapped) were stored inside sterile plastic boxes with screw cap, under refrigeration (5°C ± 2°C). Each set was randomly collected and characterized at 3, 7, 10 and 15 storage days. Unwrapped meat was also characterized at the initial time (0 days of storage). In total, 58 poultry meat samples were evaluated.

4.3 Microbiological characterization

The two analyses types carried out are based on a horizontal method for microorganisms' enumeration. Such strategy choice is based on its applicability in food chain products, as is intended to be developed in this work, following ISO 4833-1 (2013) guidelines. Total mesophilic aerobic microorganism (TMAM) and *Enterobacteriaceae* microorganisms were chosen as they are parameters that are legislated in Regulation (EC) No 1441/2007.

4.3.1 Total mesophilic aerobic microorganism

The first method to assess microbiological quality is based on total mesophilic microorganisms count, following ISO 4833-1:2013. For meat, new collectors have been used in sterile conditions, the remaining material and media being autoclaved at 121 ºC for 15 minutes. The procedure is initiated by diluting 1 g of food in 9 ml of water with tryptone (0.1% m/v) and sodium chloride (0.85% m/v). From the dispersion obtained a small portion was removed, and decimal dilutions were made, and this step was repeated until the desired concentration was obtained. Inoculation was made in Petri dishes (85 mm in diameter) with 1 mL of sample, adding to the posterior culture medium (Plate Count Agar (PCA), 2.05% m/v). All the above techniques occurred under aseptic conditions in a horizontal laminar

flow chamber (Steril Helios 42, Italy). The plates were turned over and placed in oven (Memmert, Germany) at 30°C ± 1°C for 72 h, under aerobic conditions.

4.3.2 *Enterobacteriaceae*

In addition, food product *Enterobacteriaceae* determination was carried out, and the method shown in ISO 21528-2:2017 was executed for this purpose. Again, strategy matches chapter 4.3.1, distinguishing itself by the culture medium used (Violet Red bile glucose (VRBG), 3.95% m/v) and incubation time (only 24h). Enumeration is usually done at 37 ºC as an indicator of hygiene, however, in this work a 30 ºC temperature was chosen in order to include *Enterobacteriaceae* that can develop at lower temperatures. This makes it possible to highlight microorganisms that grow preferentially at meat storing temperature (ideally, in present study, it should not exceed 4 ºC).

4.4 Chemical characterization

4.4.1 Volatile Basic Nitrogen

Nitrogen determination was performed directly by an internal method (validated in the FCT/UNL DCTB laboratory). That resembles Kjeldahl method, but the acid digestion process is avoided (James et al, 1995). Initially, 10 g was taken from each sample, to which 100 ml of water previously heated at 50 ºC was added. This mixture was shaken for 15 minutes and then filtered with qualitative filter paper. Half of this filtrate (50 mL) was diluted in 50 mL of water. 3 drops of 0.1% phenolphthalein have been added to the mixture and neutralized with 3 NaOH 6N drops and is now ready to be placed in the distiller (Foss Tecator Kjeltec System 1002 Distilling Unit, Sweden). To receive distillate, a solution with 50 mL of boric acid 2% (m/v) plus 1 mL of boric acid indicator (methylene blue 0,067 % (m/v) and methyl red 0,13 % (m/v) was prepared in ethanol). Distillation was stopped when the distillate collection reached the volume of 200 mL to ensure that all volatile basic nitrogen had already been distilled. When it is found that the distillate collection solution does not change its colour from purple to greenish tones, this means that the sample has no volatile basic nitrogen which can be quantified. Using HCl 0,02 N, analysis was finished with a titration, stopping the leakage when a pale purple color was reached. Nitrogen content was calculated by Equation (4.1) (Xia et al, 2016).

$$ABV = \frac{V\ x\ N\ x\ Ma}{ma/2}\ x\ 100$$

(4.1)

ABV = ammoniacal nitrogen, mg (N)/100g;

V = titrant volume, mL;

N = titrant normality, N;

Ma = nitrogen molar mass, 14 g/mol;

ma = sample weight, g.

4.4.2 Color

Color grading was performed using a colorimeter (Konika Minolta CR-410, Japan), with a D 65 light source and 10 º visual angle, as defined by CIE-LAB system (Hernàndez et al, 2016). Meat was contained in a glass 50 mm in diameter on a white reference background and measured at 3 different points to determine its coordinates L*a*b*. For these parameters, L*values may vary between 0 (black) and 100 (white); a* results may range from -60 (green) to +60 (red); b* measurements fall between -60 (blue) and +60 (yellow) (Barbera et al, 2018; Souza et al, 2018c) .Hue angle was calculated through Equations (4.2) to (4.4) (Abril et al, 2001; Souza et al, 2018c).

$$Hue^* = arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi}, para\ a^* > 0\ e\ b^* > 0$$

(4.2)

$$Hue^* = arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi} + 180, para\ a^* < 0$$

(4.3)

$$Hue^* = arctan\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi} + 360, para\ a^* > 0\ e\ b^* < 0$$

(4.4)

Hue* = Hue angle, degrees.

4.4.3 Moisture

For moisture content determination was followed AOAC protocol, with some adaptations (AOAC, 2016). In an analytical balance (accuracy 0,0001g) were measured 2 g of sample for properly identified weights, which were heading for a drying oven (Memmert 854 Schwabach, Germany). Have remained in this equipment for 2 hours at 105 ºC, considering meat as dried at the end of this period. The test object was then packed in a desiccator, waiting for it to cool to room temperature. This method is completed by measuring the mass of each unit, with the content in water and volatile substances as expressed by Equation (4.5) as a percentage (Chen et al, 2018).

$$H = \frac{m_2 - m_3}{m_2 - m_1} \times 100 \tag{4.5}$$

H = moisture, %;

m1 = weight of weighing bottle alone, g;

m2 = weight of weighing bottle with meat (before), g;

m3 = weight of weighing bottle with meat (after), g.

4.4.4 Quantification of thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was measured by measuring thiobarbituric acid reactive substances concentration (TBARS) expressed as malondialdehyde amount (MDA) in mg per kg of meat (Kerth et al, 2016). An7.5% trichloroacetic acid (TCA) (m/v) aqueous solution, 0.1% ethylenediamine tetracetic acid (EDTA) (m/v) and 0.1% propyl gallate (GP) (m/v) was prepared for extraction, the latter found as antioxidant dissolved in ethanol (5% m/v). 10 mL of extracting mixture was stirred with 5 g of meat for 1 hour and the liquid passed through a qualitative filter paper. 5 mL of the filtrate was removed and supplemented with 5 mL of thiobarbituric acid (TBA) 0,02 M. These samples were introduced in a water bath (Memmert, Germany) and exposed to a temperature of 95 ºC for 30 minutes. When the cooling is complete, absorbance is read at a wavelength of 530 nm in a UV/VIS spectrophotometer (Biochrom Libra S4, United Kingdom). MDA values were determined using a calibration line. For such comparison, 1, 2, 3, 4 and 5 mL of 1,1,3,3-tetraethotoxipropane (TEP) 10-5 M were used (Souza et al, 2018c).

$$TBARS = \frac{M_{MDA} \times Abs}{y \times m_a \times 5} \times (10 + m_a \times \frac{H}{100})$$

(4.6)

TBARS = thiobarbituric acid reactive substances, mg MDA/kg meat;

M_{MDA} = malondialdehyde molecular mass, 72,06 g/mol;

Abs = Absorbance;

y = slope of calibration line, μmol^{-1};

m_a = sample weight, g;

H = humidity, %.

4.4.5 pH and Total Titrable Acidity

The two studied properties defining acidity were performed according to AOAC method. Initially, 5 g of meat was isolated, to which 50 ml of water was added at 40 ºC. This mixture was subsequently shaken for 15 minutes and filtered on qualitative paper. The solution obtained was subsequently analyzed by dipping into this dispersion pH meter electrode (Crison micropH 2001, Spain), previously calibrated at two points (pH buffer solution equal to 4 and 7). After this evaluation, three 0.1% phenolphthalein drops were added to the mixture, acting as an indicator. The characterization was completed, titrating with NaOH 0,1 N until a pink coloring was achieved. Total titratable acidity is defined by Equation (4.7) (Tyl et al, 2017).

$$ATT = \frac{N \times V \times Eq}{m_a \times 1000} \times 100$$

(4.7)

ATT = total titratable acidity (g oleic acid / 100g meat);

N = titrating solution normality, 0,1 mEq/mL;

V = titrating solution volume, mL;

Eq = equivalent of predominant acid (oleic acid), 282,47 mg/mEq;

m_a = sample weight, g.

4.5 Statistical analysis

Data statistical analysis was performed through a one-way variance analysis (ANOVA) using Software OriginLab (version 8.5), and when ANOVA was significant ($p < 0.05$) differences among mean values were processed by Tukey test.

Significance was defined at $p < 0.05$.

5. Results and discussion

5.1 Microbial growth

5.1.1 Total mesophilic aerobic microorganism (TMAM)

Aerobic mesophilic microorganisms count was carried out according to ISO 4833-1:2013, incubating the samples at 30°C for 72 hours. Results are depicted in Figure 5.1 and Table 5.1.

According to Economou et al (2009), fresh poultry meat is no longer considered acceptable when bacterial count exceeds 7 CFU/g log, due to organoleptic degradation caused by spoilage.

In our product, total aerobic mesophilic bacterial count at time 0 was 6,64 log CFU/g, therefore, microbial contamination was high and according to data obtained by Smolander et al (2004), the product was at an advanced shelf-life period.

During storage period, there was an increase in bacterial count in all samples ($p<0,05$), reaching in unwrapped product a TMAM of 9,94 log CFU/g on the tenth storage day. While in packaged samples, at the end of the storage period, microbial load was lower than unwrapped sample and the best result showed a 1.24 CFU/g log reduction in comparison with unwrapped meat.

Already on the third storage day, a significant difference was found between unwrapped sample and biofilm wrapped samples microbial count ($p<0,05$). Although all samples already exceeded acceptance limit of 7 log CFU/g, probably due to high initial contamination, in unwrapped sample TMAM was 8,67 log CFU/g, while in wrapped samples microbial count was from 7,36 CFU log/g meat to 7,83 CFU log/g meat. Among biofilm wrapped samples there was no significant difference ($p>0.05$). Therefore, it can be said that all biofilms have been equally effective in delaying TMAM growth. Similar results were obtained from Souza et al (2018c, 2020) and Pires et al (2018), which found no differences between biofilms with only chitosan and biofilms with added MMT, ZnO NPs and REO, in different combinations.

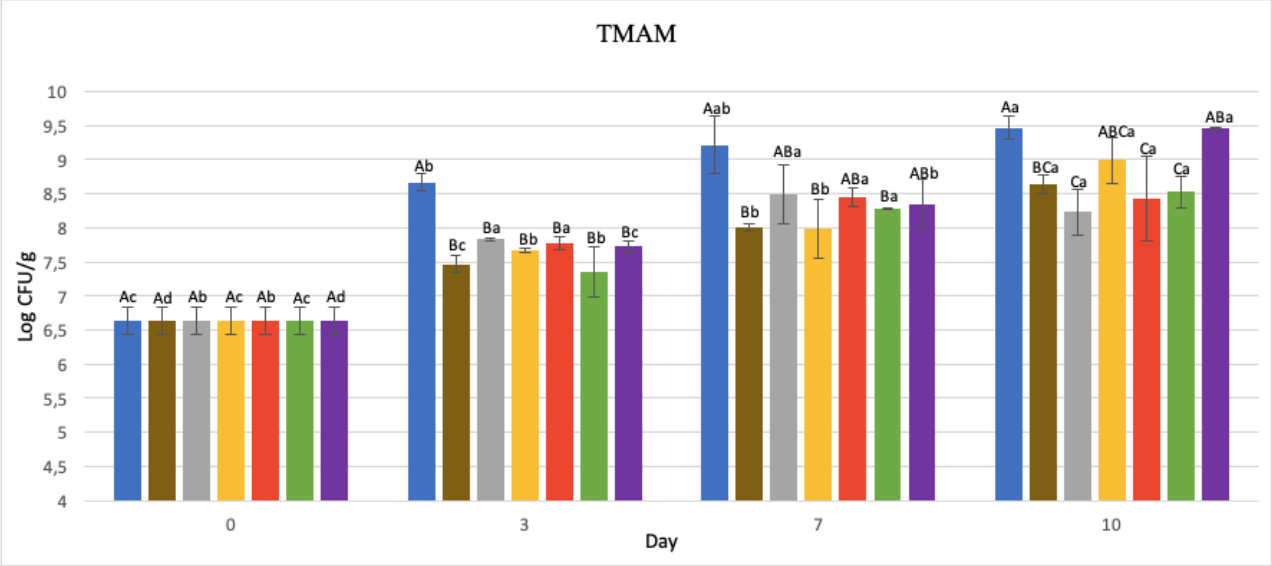

Figure 5.1 Total mesophilic aerobic counts at day 0, 3, 7, 10. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-C): Within each day, values not sharing upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each type of film, values not sharing lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

On the seventh storage day, a significant difference ($p<0,05$) was found between unwrapped sample and CH; CH + ZnO NPs; CH + ZnO NPs + REO biofilm wrapped samples, confirming antimicrobial action shown on the third day. Other samples, all containing MMT, showed no significant difference ($p>0,05$) either with unwrapped sample or with the three samples mentioned above, which means that their microbial action was not as significant as in other films' case, but a reduction in microbial charge has occurred.

Thus, even at day 7 there was no significant difference ($p>0,05$) in antimicrobial action between CH biofilm and those containing nanocompounds. Souza et al (2019b, 2020) also did not found significant differences due to nanocompounds addition on TMAM. On the other hand, Pires et al (2018), found a significant reduction in microbial counts when MMT and REO was added, either alone or in combination. At day 7, unwrapped sample had a microbial count of 9,22 CFU/g log, while other samples all had a lower value. Best results were obtained with films showing significant difference with unwrapped sample ($p<0,05$), in particular CH + ZnO NPs wrapped sample showed the highest reduction with a 7,99 log CFU/g count, although characterized by high variability. Then chitosan alone and CH +

ZnO NPs + REO film wrapped samples, had 8.01 log CFU/g and 8.29 log CFU/g respectively.

At the last storage day, differences between films observed on the seventh day were intensified. In unwrapped sample, TMAM count was 9.47 log CFU/g, and among samples showing significant difference with the later ($p<0,05$), the best result was CH + MMT film, with 8.23 log CFU/g. Curious result, considering that on the seventh day this film had a higher count, equal to 8,5 log CFU/g, and showed no significant difference with unwrapped sample ($p>0,05$).

Same situation was found in CH + MMT + ZnO NPs wrapped sample, which on the tenth day showed a statistical difference with unwrapped sample ($p<0,05$), but on the seventh day it did not show it ($p>0,05$). Both samples mentioned above showed no significant difference ($p>0,05$) with same films wrapped samples on the seventh day, so we could assume that the film has succeeded in retarding microbial growth.

On the tenth day, sample wrapped in CH + ZnO NPs film, showed no significant difference either with unwrapped sample or with other biofilms ($p>0,05$), but, although not significant, this type of biopolymer resulted in a lower microbial count than unwrapped sample. Also, biofilm containing chitosan and all compounds proves to be less active in terms of antimicrobial activity, being also not significantly different ($p>0,05$) when compared to unwrapped sample.

Again, compounds added to biofilm did not result in a significantly higher antimicrobial action ($p>0,05$), comparing with biofilm containing only chitosan. Similar results were also obtained by Souza et al (2018c, 2019b, 2020) and Pires et al (2018).

It should be noted that films containing CH + MMT, CH + MMT + ZnO NPs, CH + ZnO NPs + REO, showed a significant reduction ($p<0,05$) compared to biofilm containing CH, ZNO NPs, MMT and REO.

Analyzing storage period data from the beginning to the end, we can say that films have carried out discreet antimicrobial activity showing, with a few exceptions, a significant microbial count reduction ($p<0,05$).

Between CH biofilm and other biofilms there has never been a significant difference ($p>0.05$), sometimes presenting a higher contamination and sometimes a lower contamination.

These slight differences may therefore be due to samples natural variability, but also to different interaction between compounds over time.

In fact, it is reported in literature that compounds can interfere with chitosan antimicrobial action, acting as plasticizers, resulting in increased permeability to oxygen and water vapor, which can result in a better environment for the microbial growth. Sanchèz– Gonzalez et al (2011), have assumed that interaction between essential oils and chitosan networks (loaded polymeric chains), determine a limitation on the antimicrobial activity when both compounds are merged.

Also, Souza et al (2020), has hypothesized that excellent interaction between ZnO nanoparticles and chitosan can reduce available amino groups number in chitosan, reducing its antimicrobial activity.

In fact, biofilm containing all compounds exhibits the less antimicrobial activity at the storage end, confirming negative interaction between different compounds.

Table 5.1 Total mesophilic aerobic counts at day 0, 3, 7, 10 and standard deviation. Within each day, values not sharing upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each type of film, values not sharing lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10
Total Mesophilic Aerobial Microorganism (log CFU/g)	**Unwrapped**	6,64±0,2Ac	8,67 ± 0,12 Ab	9,22 ± 0,42 Aab	9,47 ± 0,175 Aa
	Chitosan	6,64 ± 0,2 Ad	7,47 ± 0,12 Bc	8,01 ± 0,06 Bb	8,64 ± 0,14 BCa
	Chitosan + Montmorillonite	6,64 ± 0,2 Ab	7,83 ± 0,16 Ba	8,5 ± 0,5 ABa	8,23 ± 0,025 Ca
	Chitosan + ZnO	6,64 ± 0,2 Ac	7,67 ± 0,025 Bb	7,99 ± 0,43 Bb	8,99 ± 0,34 ABCa
	Chitosan + Montmorillonite + ZnO	6,64 ± 0,2 Ab	7,77 ± 0,095 Ba	8,45 ± 0,13 ABa	8,43 ± 0,63 Ca
	Chitosan + ZnO + REO	6,64 ± 0,2 Ac	7,36 ± 0,365 Bb	8,29 ± 0,01 Ba	8,53 ± 0,235 Ca
	Chitosan + Montmorillonite + ZnO + REO	6,64 ± 0,2 Ad	7,73 ± 0,085 Bc	8,35 ± 0,355 ABb	9,46 ± 0,02 ABa

5.1.2 *Enterobacteriaceae*

EFSA recommends *Enterobacteriaceae* monitoring and detection in both processing and finished products. In fact, *Enterobacteriaceae*, apart from some pathogenic species, includes bacteria derived from environment that do not pose a risk to consumer health.

Enterobacteriaceae were determined according to ISO 21528-2:2017; enumeration is usually done at 37 ºC as an hygiene indicator, however, in this work was chosen a 30 ºC temperature in order to include *Enterobacteriaceae* that can develop at lower temperatures. This makes it possible to highlight microorganisms that grow preferentially in typical meat storage condition (ideally, in the present study, it should not exceed 4 ºC).

Initial contamination was rather high, equal to 4,13 log CFU/g, and compared to data obtained from Smolander et al (2004), it can be hypothesized that this product is at an advanced shelf-life state.

At day 10, unwrapped sample had a contamination of 8,16 log CFU/g, having a significant increase ($p<0,05$). Results are showed in Figure 5.2 and Table 5.2.

On the third day of storage, unwrapped sample had a rapid development, reaching 7,5 log CFU/g, while all film wrapped samples showed a good microbial development containment, in fact all are significantly different from unwrapped sample ($p<0,05$).

In particular, most effective samples were those based on:

- CH + MMT + ZnO NPs;
- CH + ZnO NPs + REO;
- CH + MMT + ZnO NPs + REO.

These three films are significantly different not only from unwrapped sample, but also from other films ($p<0,05$), and have shown effective bacterial development containment, reducing initial contamination. Therefore, on the third day, biofilms containing CH + MMT and CH + ZnO NPs were not significantly different from biofilm with only CH ($p>0,05$). While other biofilms, in which combined action of these compound took place, with or without essential oil, a significant reduction of *Enterobacteriaceae* was achieved compared to biofilm with only chitosan.

Even Souza et al (2020), at the same storage time, had not found a significant difference between biofilms with only chitosan and those with CH + ZnO NPs. Not even Pires et al (2018) and Souza et al (2018c), at the same storage time, had found a significant difference between film with only chitosan and the one with MMT.

As far as we know, association between ZnO NPs and MMT improves compounds effectiveness respect used individually.

Rosemary essential oil addition also seems to improve biofilm effectiveness, in contrast to what was found by Souza et al (2019b) which had found a lower effectiveness by MMT and REO joint action. Pires et al (2018) found no significant difference between biofilms with only chitosan and biofilms with MMT + REO.

However, there are contradictory studies regarding rosemary essential oil antimicrobial action, in fact Abdollahi et al (2012b) found a greater effectiveness in vitro of biofilm containing also MMT + REO, compared to the chitosan alone, against *Escherichia coli*.

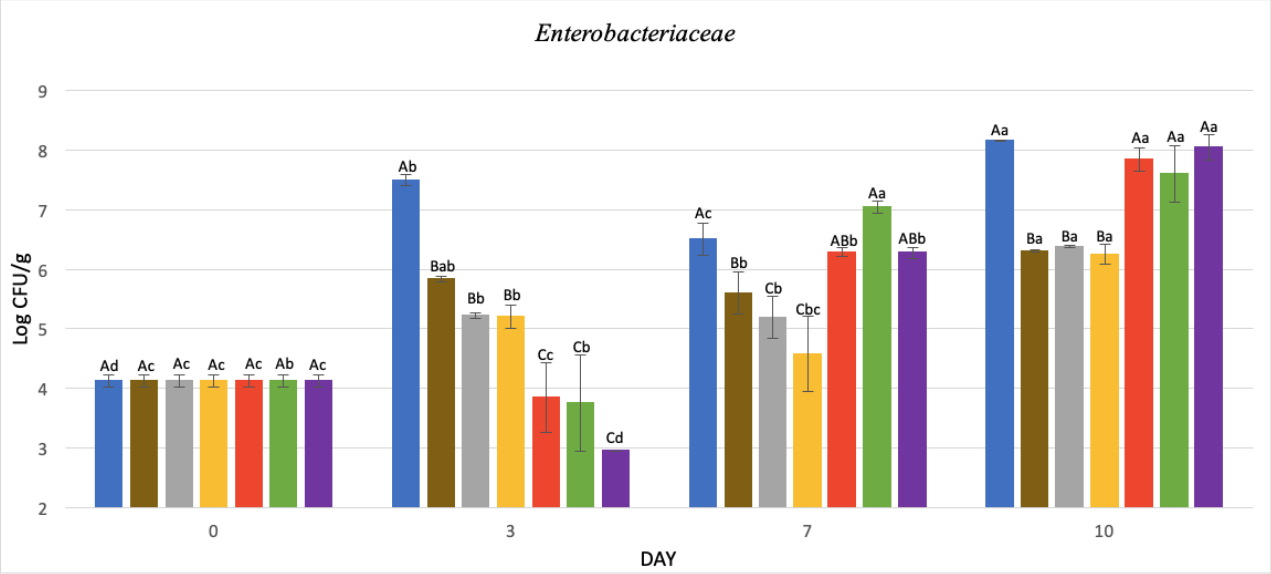

Figure 5.2 Enterobacteriaceae counts at day 0, 3 ,7, 10. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-E): Within each parameter day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

At the seventh storage day, unwrapped sample contamination is significantly reduced ($p<0,05$), with a 6,5 log CFU/g count, compared with day 3.

In addition, the three samples which had previously shown greater efficacy become less effective by achieving contamination similar to unwrapped sample.

While wrapped films samples containing:

• CH + MMT,

• CH + ZnO NPs,

remain stable compared to the third day, continuing to show significant difference with unwrapped sample ($p<0,05$) and achieving a significant reduction compared to biofilm with only chitosan ($p<0,05$).

On the same storage day, Souza et al (2020) had not found any significant difference with ZnO NPs addition from biofilm with only chitosan.

As for MMT addition, Souza et al (2018c, 2019b) found contrasting results at same storage period, once found a significant reduction by biofilm with only chitosan, and by adding MMT no additional antimicrobial activity was observed, in agreement also with Pires et al (2018).

Instead, biofilms containing also REO, in one case are not significantly different with the biofilm with only chitosan ($p>0,05$), while in the other are not significantly different with unwrapped sample ($p>0,05$). Similar results were also obtained from Pires et al (2018) and Souza et al (2019b).

On the tenth day, previous day trend seems to continue, with samples containing REO and those containing both MMT and ZnO NPs not significantly different from unwrapped sample ($p>0,05$), denoting a worsening antimicrobial efficacy. This is maybe caused by the higher permeability to O_2 of the biofilm caused by REO, which allowed the growth of *Enterobacteriaceae.* Other biofilms instead are significantly different from unwrapped sample ($p<0,05$), but biofilm with CH + MMT and the one with CH + ZnO NPs are no longer significantly different from film with only chitosan ($p>0,05$), according to what was found by Souza et al (2018c, 2019b, 2020). Results obtained are significantly different from those recorded for total mesophilic count ($p<0,05$), except for sample containing all compounds which gradually worsens by confirming that high interaction between different compounds limits their antimicrobial action.

Table 5.2 Enterobacteriaceae counts at day 0, 3 ,7, 10 and standard deviation. (A-C): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$).

	FILM	0	3	7	10
Enterobacteriaceae (log CFU/g)	**Unwrapped**	4,13±0,1Ad	7,5±0,09Ab	6,5±0,27Ac	8,16±0,01Aa
	Chitosan	4,13±0,1Ac	5,84±0,05Bab	5,6±0,35Bb	6,31±0,02Ba
	Chitosan + Montmorillonite	4,13±0,1Ac	5,22±0,65Bb	5,19±0,23Cb	6,38±0,08Ba
	Chitosan + ZnO	4,13±0,1Ac	5,20±0,19Bb	4,58±0,63Cbc	6,25±0,16Ba
	Chitosan + Montmorillonite + ZnO	4,13±0,1Ac	3,84±0,58Cc	6,29±0,07ABb	7,84±0,2Aa
	Chitosan + ZnO + REO	4,13±0,1Ab	3,75±0,8Cb	7,05±0,1Aa	7,6±0,47Aa
	Chitosan + Montmorillonite + ZnO + REO	4,13±0,1Ac	2,95±0,05Cd	6,28±0,09ABb	8,05±0,21Aa

Results obtained for *Enterobacteriaceae* and TMAM reaffirm that the main antimicrobial action is effectively made by the chitosan as also stated in several works, such as the work of Souza et al. (2020).

5.2 Chemical-physical analysis

5.2.1 Thiobarbituric Acid Reactive Substances Index (TBARS)

Product lipid oxidation state was assessed with TBARS test, quantifying secondary oxidation products production, expressed in mg of malondialdehyde (MDA) per kg of product and presented in Figure 5.3 and Table 5.3.

Meat presented an initial oxidation equal to 0.016 mg MDA/kg, which is rather low compared to what was found by Pires et al (2018) and Souza et al (2018c), even if it increases through time ($p<0,05$), it remains rather low over time.

MDA concentration does not have a linear increase in time, in fact a decrease happens between day 10 and day 15, a similar behavior is found by Souza et al (2020).

According to Remya et al (2016), concentration limit of MDA is 0.5 mg/kg, above this threshold off-odor production becomes unacceptable to consumer.

On the third storage day, unwrapped sample oxidation remained almost stable, in fact it is not significantly different from the beginning ($p>0,05$). Biofilms are not particularly effective, because when they are significantly different from unwrapped sample ($p<0,05$), it is because they have given worse results. Sample wrapped in biofilm containing CH + MMT has been slightly reduced compared to day 0, but not significantly different ($p>0,05$).

On the seventh day, oxidation is still quite contained in unwrapped sample and is not statistically different with any of the biofilms wrapped samples ($p>0,05$), and all samples are not statistically different from the third day ($p>0,05$), thus showing a general oxidation containment.

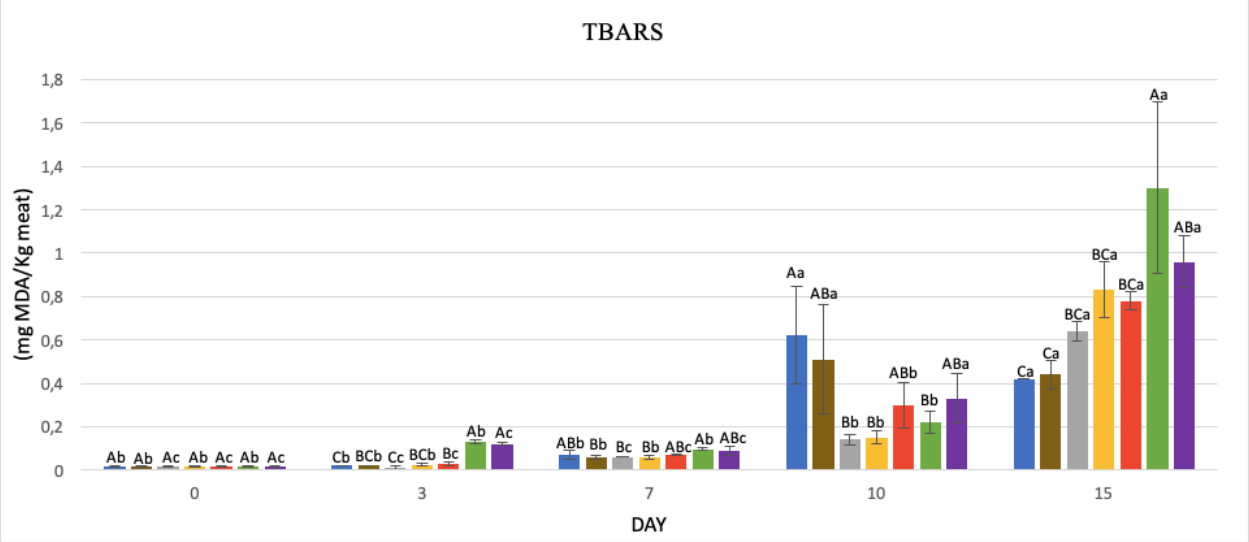

Figure 5.3 Thiobarbituric acid reactive substances at day 0, 3 ,7, 10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-C): Within each parameter day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

On the tenth day, there was a significant increase in oxidation products in unwrapped sample, reaching 0,62 mg MDA/kg meat, exceeding the limit value of 0,5 mg/kg. Except chitosan, which is placed on threshold value with a concentration of 0,51 mg/kg, all other biofilms have a lower concentration.

However, only biofilms with CH + MMT and CH + ZnO NPs are statistically different from unwrapped sample ($p<0,05$), other biofilms are not statistically different from either unwrapped sample or biofilms mentioned above ($p>0,05$). Apparently, the incorporation of ZnO NPs into the polymeric matrix enhanced the film's antioxidant ability, probably due to the presence of phenolic compounds from the apple peel used in the synthesis of the nanoparticles (Souza et al., 2020), and the incorporation of MMT may delay oxidative rancidity in meat due to its good barrier properties against oxygen and light (Souza et al., 2019b).

Among biofilms, the best is still the one containing CH + MMT with an oxidation level of 0.14 mg MDA/kg product, a similar result is obtained from film containing CH + ZnO NPs which is not significantly different from same film on the seventh day ($p>0,05$).

At the last storage day, oxidation products amount decreases in unwrapped sample, though not significantly ($p>0,05$).

Biofilms based on: CH + ZnO NPs + REO, CH + MMT + ZnO NPs + REO, have significantly higher MDA content than unwrapped sample.

While other biofilms are not significantly different from unwrapped sample or even each other ($p>0,05$). Therefore, rosemary essential oil seems to worsen biofilm antioxidant effectiveness. This result could however be due to an MDA level overestimation through TBARS test, as other volatile compounds could interfere with spectrophotometric reading (Papastergiardis et al, 2012) or the high content may be due to oxidation products formed by essential oil itself.

Another possible negative effect resulting explanation from essential oil addition could be its interaction with the matrix and its contained substances, such as tocopherols (Yen et al, 1997; Huang and Frankel, 1997). Indeed, according to Wang et al (1995) and Fang and Wada (1993), phenolic compounds and tocopherols can interact with each other. This leads to a possible pro-oxidant activity.

However, Souza et al (2018c, 2019b) and Pires et al (2018) achieved very different results, with positive feedback from essential oils addition.

At day 15, contrary to day 10, MMT and ZnO NPs addition seems to have provided no added value to chitosan, in fact there has never been a significant difference ($p>0,05$). This

contrasts with what Souza et al (2020) found for ZnO NPs addition, which had found a significant reduction. As for MMT addition are in line with what was found by Pires et al (2018), which had not found a significant difference.

Table 5.3 Thiobarbituric acid reactive substances at day 0, 3 ,7, 10, 15 and standard deviation (A-C): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
TBARS (mg MDA/kg)	**Unwrapped**	0,016±0,005 Ab	0,02±0,001Cb	0,07±0,02 ABb	0,62±0,22 Aa	0,42±0,001Ca
	Chitosan	0,016±0,005Ab	0,02±0,001BCb	0,06±0,01Bb	0,51±0,25ABa	0,44±0,07Ca
	Chitosan + Montmorillonite	0,016±0,005Ac	0,01±0,01Cc	0,06±0,001Bc	0,14±0,025Bb	0,64±0,05 BCa
	Chitosan + ZnO	0,016±0,005Ab	0,023±0,01BCb	0,06±0,01Bb	0,15±0,03Bb	0,83±0,13 BCa
	Chitosan + Montmorillonite + ZnO	0,016±0,005Ac	0,03±0,01Bc	0,07±0,001ABc	0,3±0,16ABb	0,78±0,04BCa
	Chitosan + ZnO + REO	0,016±0,005 Ab	0,13±0,01Ab	0,096±0,01Ab	0,22±0,05Bb	1,3±0,4Aa
	Chitosan + Montmorillonite + ZnO + REO	0,016±0,005 Ac	0,12±0,01Ac	0,09±0,02ABc	0,33±0,12ABb	0,96±0,12ABa

5.2.2 Total Volatile Basic Nitrogen

Total volatile basic nitrogen (TVBN) was detected according to AVOC method, using Kjeldahl method, without acid digestion. Results are presented in Figure 5.4 and Table 5.4.

During storage, protein degrades due to microbial development and enzymatic action, releasing alkaline substances containing nitrogen (TVB-N), which includes ammonia and amino substances (Your et al, 2016).

According to Khulal et al (2017), this technique provides an important physical-chemical index to measure meat freshness and wholesomeness.

At the beginning, TVB-N content was 0,42 mg/g meat.

During storage, content did increase significantly in unwrapped sample ($p<0,05$), as observed by Souza et el (2020), while in biofilm wrapped samples the same happened also but at a lower rate ($p<0,05$).

On the third day, content increased significantly in all samples ($p<0,05$), and unwrapped sample had a TVB-N content of 1,18 mg/g, while all other samples had a lower content.

Among biofilm wrapped samples, those significantly different from unwrapped sample, were:

• CH + MMT;

• CH + ZnO NPs;

• CH + MMT + ZnO NPs;

with a content ranging from 0.38 to 0.62 mg/g, it can therefore be assumed that protein degradation has been limited by montmorillonite and ZnO nanoparticles presence, which also limited microbial contamination.

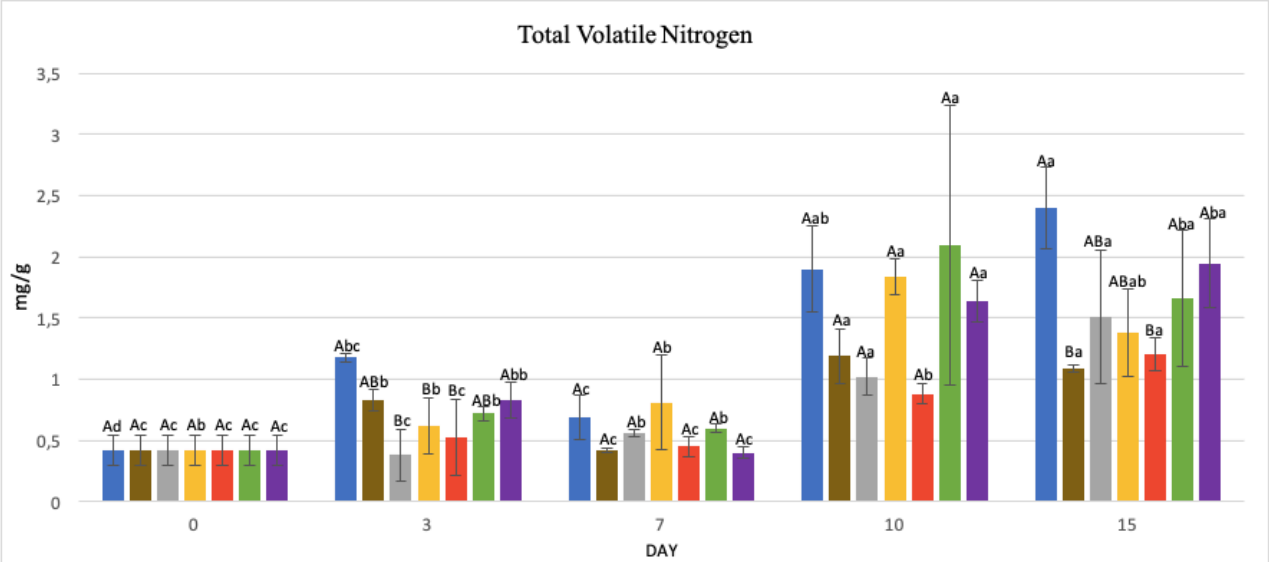

Figure 5.4 Total volatile nitrogan at day 0, 3, 7 ,10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-B): Within each day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

On the seventh day, unwrapped sample contents did vary significantly ($p<0,05$). Also, in other samples the same was observed.

In any case, between biofilm-wrapped samples and unwrapped sample there is no significant difference; unwrapped sample had a 0,69 mg/g content and other samples had a lower content, except biofilm containing CH + ZnO NPs which had a 0.81 mg/g content.

On the tenth day, an increase in TVB-N content was found in all samples compared to days 3 and 7, sometimes significantly ($p<0,05$) and sometimes not ($p>0,05$). Unwrapped sample

increased significantly to 1,9 mg/g ($p<0,05$). None of the biofilm wrapped samples is significantly different from unwrapped sample, although the trend observed was to a lower TVB-N.

On the 15th day, TVB-N content in unwrapped sample had increased to 2,4 mg/g, but not significantly since the 10th day ($p>0,05$). Among biofilm wrapped samples, those that showed significant difference with unwrapped sample, were chitosan-based biofilm with a 1.09 mg/g content and biofilm with CH + MMT + ZnO NPs with 1.21 mg/g ($p<0,05$). These results are in line with microbial spoilage. The higher the contamination the higher the TVB-N.

Table 5.4 Total volatile nitrogen at day 0, 3, 7 ,10, 15 and standard deviation. (A-B): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
Total volatile basic nitrogen (mg/g of meat)	**Unwrapped**	0,42±0,12 Ad	1,18±0,03Abc	0,69±0,18Ac	1,9±0,35Aab	2,4±0,33Aa
	Chitosan	0,42±0,12 Ac	0,831±0,09ABb	0,42±0,02Ac	1,19±0,22Aa	1,09±0,03Ba
	Chitosan + Montmorillonite	0,42±0,12 Ac	0,38±0,21Bc	0,56±0,03Ab	1,02±0,15Aa	1,51±0,55ABa
	Chitosan + ZnO	0,42±0,12 Ab	0,62±0,23Bb	0,81±0,39Ab	1,84±0,15Aa	1,38±0,35ABab
	Chitosan + Montmorillonite + ZnO	0,42±0,12 Ac	0,53±0,31Bc	0,45±0,09Ac	0,88±0,08Ab	1,21±0,14Ba
	Chitosan + ZnO + REO	0,42±0,12 Ac	0,72±0,05ABb	0,6±0,03Ab	2,1±1,14Aa	1,66±0,55Aba
	Chitosan + Montmorillonite + ZnO + REO	0,42±0,12 Ac	0,83±0,15ABb	0,4±0,05Ac	1,64±0,17Aa	1,95±0,36Aba

5.2.3 Total Titrable Acidity and pH

pH value in poultry meat generally varies between 5,2 and 7 (Pires et al, 2018; Emamifar et al, 2010). In our product, initial value was 6.1 and increased significantly during shelf life ($p<0,05$).

According to Georgantelis et al (2007), pH increase over time may be due to bacterial growth, such as *Enterobacteriaceae* and *Pseudomonas* that degrade proteins and aminoacids releasing volatile substances such as ammonium ion that causes pH elevation.

On the third day, pH value was slightly increased in all samples, without taking a significant difference from day 0 ($p>0,05$), but all biofilm wrapped samples are significantly different

from unwrapped sample (p<0,05). pH growth containment is in line with microbiological results, confirming a microbial development limitation. However, this increase in pH is in contradiction with total volatile nitrogen results analysis, which on day 3 decreases.

On the seventh day, in all samples we have seen a decrease in pH, significantly different for all biofilm wrapped samples (p<0,05) but not for unwrapped sample (p>0,05), which shows a certain stability. This behavior, never found in studies carried out by Souza et al and Pires et al, could be due to the high initial product spoilage in which there are also lactic acid bacteria colonies, according to Smolander et al (2004). Biofilms are characterized by low permeability to oxygen (Souza et al, 2019b), so it could have happened an oxygen consumption by aerobic bacteria growth. Then, anaerobic environment created could have promoted lactic acid bacteria growth, releasing organic acids that cause pH fall.

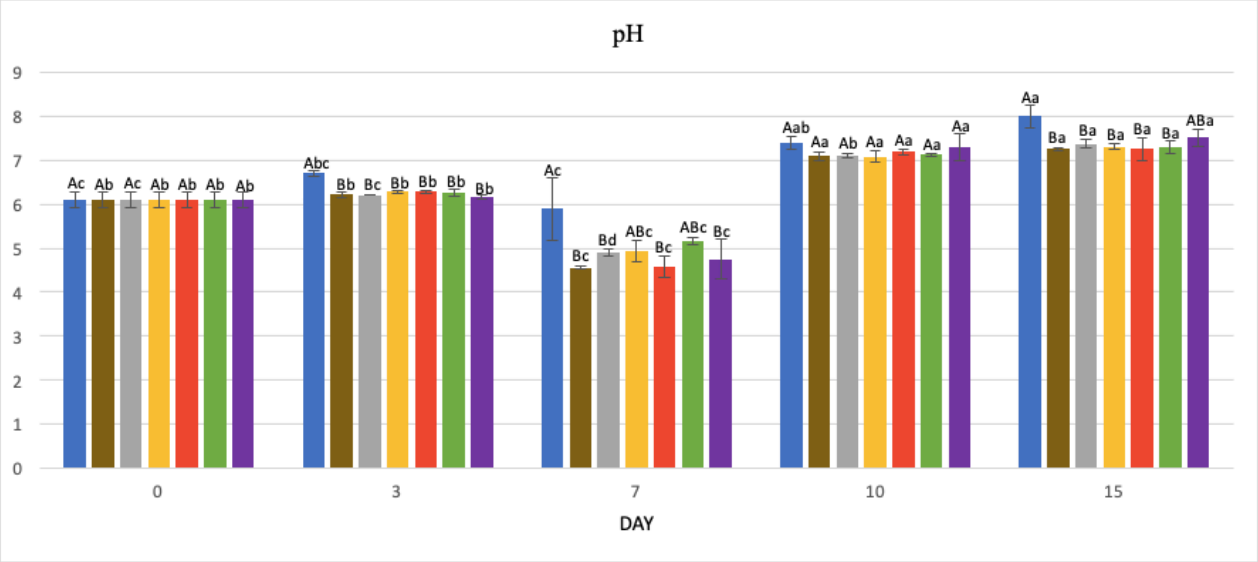

Figure 5.5 pH at day 0, 3, 7 ,10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-B): Within each day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations (p < 0.05). (a–d): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days (p < 0.05)

On the tenth day, there was a significantly high increase in pH in all samples (p<0,05). Unwrapped sample reached a 7,4 pH value, while biofilm wrapped samples had a slightly lower pH, but always above 7 and never significantly different from unwrapped sample (p>0,05). In some cases, in line with microbiological analyses findings. Total volatile

nitrogen content had also risen to the tenth day, but compared to the initial time it was still lower, so it does not seem to be in line with the most pronounced pH growth.
Similar results were found from Pires et al (2018) on the same storage day, with regard to pH.

At day 15, pH values increased slightly in all samples, never significantly ($p>0,05$), except in CH + MMT biofilm wrapped sample ($p<0,05$).
However, there was a significant difference between unwrapped sample, which had a 7.99 pH, and those biofilm wrapped ($p<0,05$), which had a 7.25 to 7.37 pH; excluding chitosan, MMT, ZnO NPs and REO biofilm, showing ($p>0,05$) no significant difference with unwrapped sample. So, we can say that almost all biofilms have contained pH rise, in line with results obtained by Pires et al (2018) and Souza et al (2018c, 2019b).

Table 5.5 pH at day 0, 3, 7 ,10, 15 and standard deviation. (A-B): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–d): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
pH	**Unwrapped**	6,1±0,16Ac	6,7±0,06Abc	5,89±0,7Ac	7,4±0,15Aab	7,99±0,24Aa
	Chitosan	6,1±0,16Ab	6,22±0,06Bb	4,56±0,04Bc	7,09±0,1Aa	7,25±0,04Ba
	Chitosan + Montmorillonite	6,1±0,16Ac	6,21±0,01Bc	4,91±0,08Bd	7,1±0,05Ab	7,37±0,095Ba
	Chitosan + ZnO	6,1±0,16Ab	6,29±0,03Bb	4,94±0,24ABc	7,08±0,13Aa	7,3±0,06Ba
	Chitosan + Montmorillonite + ZnO	6,1±0,16Ab	6,28±0,04Bb	4,59±0,24Bc	7,19±0,07Aa	7,25±0,27Ba
	Chitosan + ZnO + REO	6,1±0,16Ab	6,26±0,07Bb	5,16±0,07ABc	7,12±0,04Aa	7,29±0,145Ba
	Chitosan + Montmorillonite + ZnO + REO	6,1±0,16Ab	6,16±0,04Bb	4,75±0,45Bc	7,29±0,3Aa	7,52±A0,19Ba

Titratable acidity measures food total acidity and for some types of products is a microbial development indicator better than pH (IFT, 2001).
Titratable acidity, as expected, varies with a trend opposite the pH.
In fact, on the third day there was a slight decrease in acidity, but there was no significant difference between unwrapped sample and biofilms ($p>0,05$).

On the seventh day, it occurred in a significant acidity increase in unwrapped sample ($p<0,05$), returning to a level similar to the initial one. Among biofilms, only ones to show significant difference with unwrapped sample, were the one with only chitosan (higher) and the one with also ZnO NPs + REO (lower) ($p<0,05$).

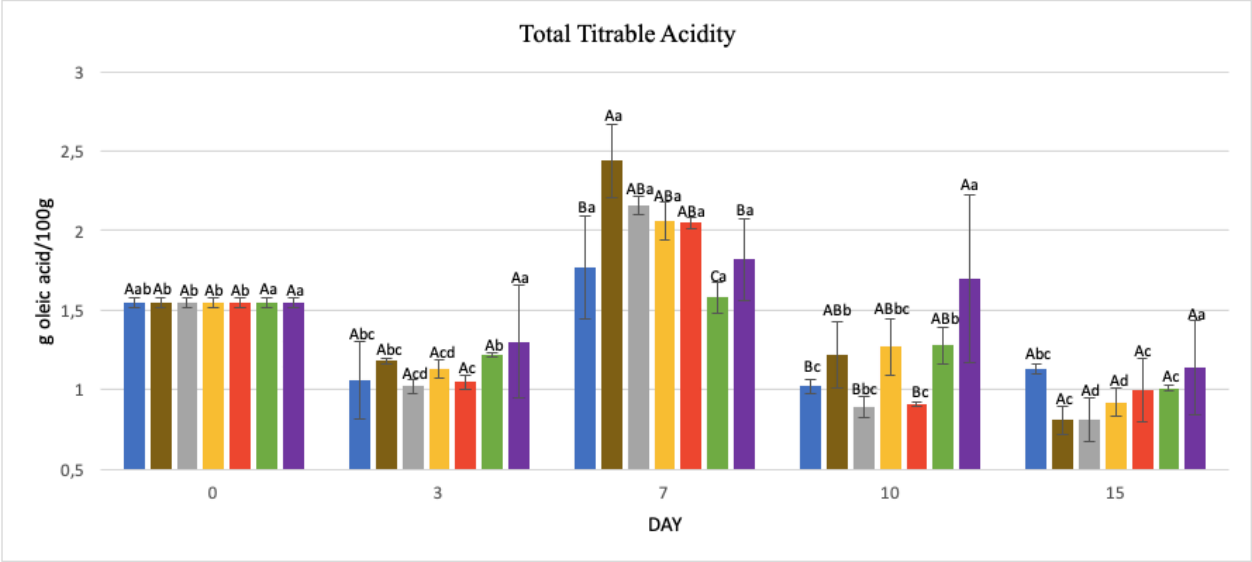

Figure 5.6 Total Titrable Acidity at day 0, 3, 7 ,10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-C): Within each day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–d): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

On the tenth day, there was a significant decrease in acidity in unwrapped sample and among biofilms ($p<0,05$), only biofilm with all bio components showed significantly difference to unwrapped sample ($p<0,05$), the others did not ($p>0,05$).

Finally, at the last day, acidity did not vary significantly in unwrapped sample ($p>0,05$) and none of biofilms shows significant difference with unwrapped sample ($p>0,05$).

Table 5.6 Total Titrable Acidity at day 0, 3, 7 ,10, 15 and standard deviation. (A-C): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–d): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
Total titrable acidity (g oleic acid/100g)	**Unwrapped**	1,55±0,03Aab	1,06±0,24Abc	1,77±0,32Ba	1,02±0,04Bc	1,13±A0,03bc
	Chitosan	1,55±0,03Ab	1,18±0,03Abc	2,44±0,23Aa	1,22±0,21ABb	0,81±0,09Ac
	Chitosan + Montmorillonite	1,55±0,03Ab	1,02±0,04Acd	2,16±0,06ABa	0,89±0,06Bbc	0,81±0,14Ad
	Chitosan + ZnO	1,55±0,03Ab	1,13±0,05Acd	2,06±0,12ABa	1,27±0,17ABbc	0,92±0,09Ad
	Chitosan + Montmorillonite + ZnO	1,55±0,03Ab	1,05±0,04Ac	2,05±0,03ABa	0,91±0,01Bc	1±0,2Ac
	Chitosan + ZnO + REO	1,55±0,03Aa	1,22±0,01Ab	1,58±0,09Ca	1,28±0,11ABb	1,01±0,01Ac
	Chitosan + Montmorillonite + ZnO + REO	1,55±0,03Aa	1,3±0,35Aa	1,82±0,25Ba	1,7±0,53Aa	1,14±0,29Aa

5.2.4 Color

Meat color detection was done by colorimeter, obtaining CIE-Lab coordinates, then transformed by equation into Hue angle.

Initial Hue angle was 56.6°, while at conservation end it had increased significantly to 66.16°. Results are presented in Figure 5.7 and Table 5.7.

The smaller the Hue angle, the greater the tendency to red colour, the more the value tends to 90°; and the more the color tends to yellow, so when Hue angle increases, there is a flesh discoloration.

Biofilms limited meat color loss, in fact at conservation end, Hue values were often not significantly different to initial value, and when they were significantly different it was because color was improved.

This is a very important result, as appearance is one of the primary consumer acceptability product factors (Fletcher, 2002).

By the third day Hue value in unwrapped sample had increased, although slightly and not significantly to 58,72°. For some biofilms Hue value on the third day was significantly lower than the initial one and for all it was significantly different from unwrapped sample.

Biofilm with chitosan, MMT and ZnO NPs has also shown itself to be significantly different from other biofilms, reducing Hue angle to 50.35°.

The interaction between these compounds seems positive, as taken individually do not differ significantly from the chitosan alone, as also noted by Pires et al (2018) and Souza et al (2020).

On the seventh day, Hue's value continues to increase, although not significantly, reaching 62.36°. Except films with only chitosan and CH + MMT, the others are significantly different from unwrapped sample, with a value ranging between 52.6 and 55.09°, thus showing a certain ability to limit color loss.

Meat packaged in biofilm with CH + ZnO NPs is significantly different from those protected with biofim with only chitosan but not from other biofilms. Better result than that obtained by Souza et al (2020) at the same storage period.

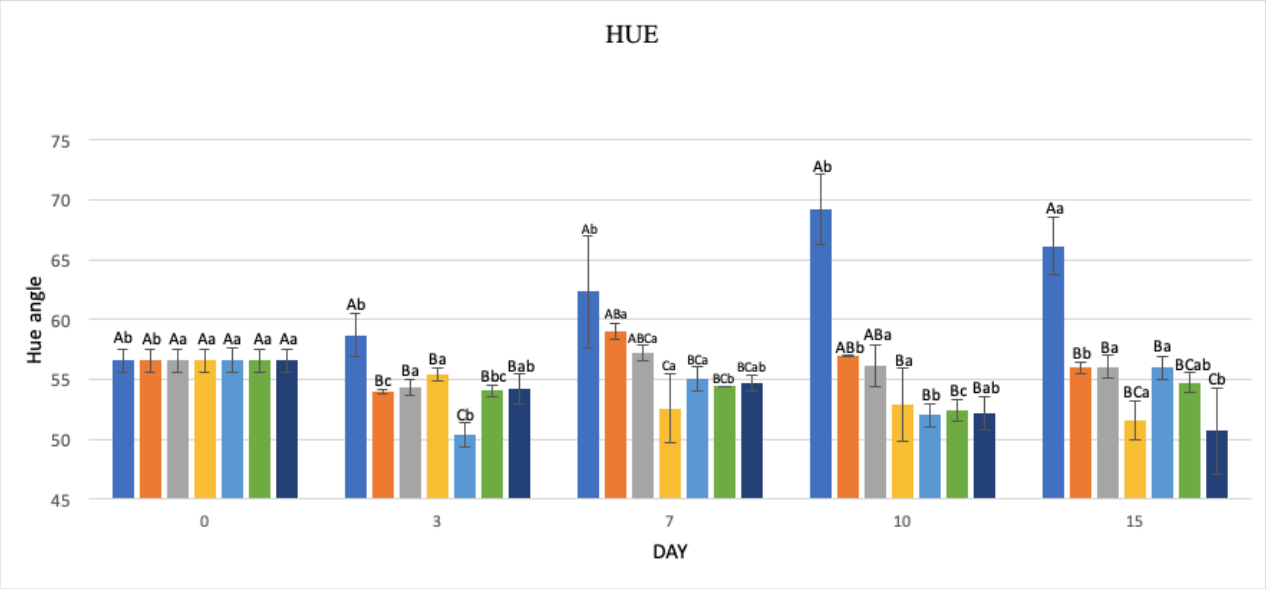

Figure 5.7 Hue angle at day 0, 3, 7 ,10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-C): Within each day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

On the tenth day, Hue angle continues to increase reaching 69.22°, while in samples wrapped by biofilm the trend is quite different, in fact the values remain stable and sometimes decrease compared to the seventh day. In fact, with chitosan biofilm exception, in all other wrapped samples Hue angle is still lower than initial one, demonstrating biofilm is performing an effective action on color maintenance.
Although none of biofilms with nanocompounds was significantly different from biofilm with only chitosan ($p>0,05$), while the latter and CH + MMT biofilm did not show significantly difference from unwrapped sample ($p>0,05$), other biofilms were all significantly different from unwrapped sample ($p<0,05$).

At the last day, there was an atypical Hue angle reduction in unwrapped sample, with a 66.16°value. Biofilms continue to carry out their action, in fact values are significantly lower than unwrapped sample ($p<0,05$) and all have a lower value than the initial one. Therefore, biofilm has maintained with remarkable results product's color, showing sometimes also an improvement.
Sample containing all components showed significant difference ($p<0,05$) with biofilm with only chitosan.
According to Pires et al (2018), chitosan color retention mechanism is not yet fully explained, it seems to be due to its ability to chelate iron. In fact, free iron seems to be absorbed by chitosan and this interaction would determine meat surface color stabilization over time (Park et al, 2010).
According to Souza et al (2018a), MMT and/or REO addition to chitosan biofilm, increases barrier effect to light radiation, in particular UV radiation, responsible for oxidation and color changes.
Finally, according to Youssef et al (2015) and Tian et al (2019), ZnO nanoparticles have filtering capacity against UV light.

Table 5.7 Hue angle at day 0, 3, 7 ,10, 15 and standard deviation. (A-C): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–c): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
HUE angle	**Unwrapped**	56,60±0,94Ab	58,72±1,84Ab	62,36±4,7Ab	69,22±2,93Ab	66,16±2,43Aa
	Chitosan	56,60±0,94Ab	53,97±0,13Bc	59,03±0,67ABa	56,96±0,03ABb	55,97±0,53Bb
	Chitosan + Montmorillonite	56,60±0,94Aa	54,32±0,62Ba	57,22±0,61ABCa	56,13±1,77ABa	56,06±0,95Ba
	Chitosan + ZnO	56,60±0,94Aa	55,46±0,55Ba	52,6±2,91Ca	52,9±3,03Ba	51,56±1,61BCa
	Chitosan + Montmorillonite + ZnO	56,60±0,94Aa	50,35±0,87Cb	55,09±1,11BCa	52,02±1,2Bb	55,97±0,81Ba
	Chitosan + ZnO + REO	56,60±0,94Aa	54,06±0,46Bbc	54,43±0,02BCb	52,38±0,9Bc	54,75±0,84BCab
	Chitosan + Montmorillonite + ZnO + REO	56,60±0,94Aa	54,18±1,27Bab	54,70±0,68BCab	52,23±1,37Bab	50,72±3,59Cb

5.2.5 Moisture

Initially, meat moisture content was 73.75%, and during storage unwrapped sample experienced a significant increase ($p<0,05$) in water content (Figure 5.8, Table 5.8), although in a non-linear way, since on the third day moisture content fell and then gradually got up. Samples wrapped in biofilm have also undergone the same trend, but reaching storage end in some cases without being changed significantly ($p>0,05$) from beginning, while in others with significant decrease ($p<0,05$).

Souza et al and Pires et al also found a decrease in moisture biofilm wrapped samples content. This is because chitosan is a hydrophilic polysaccharide, able to absorb high water amounts (Souza et al, 2017). Also, according to Souza et al (2019a), water vapor transmission rate (WVTR) is rather high for chitosan and after montmorillonite and essential oils addition increases.

On the third day, moisture decreased in all samples, in unwrapped sample to 69.89%, while in biofilm wrapped samples water loss was higher, often significantly ($p<0,05$). Biofilm containing only chitosan was the only one that did not differ significantly from unwrapped

sample (p>0,05), confirming the plasticizing effect and barrier effect decrease resulting in bio components addition.

On the seventh day, all samples recovered water, but did not return to initial level. The difference in moisture between unwrapped sample and biofilm samples decreases, and although biofilms still had a lower content, there was no significant difference (p>0,05).

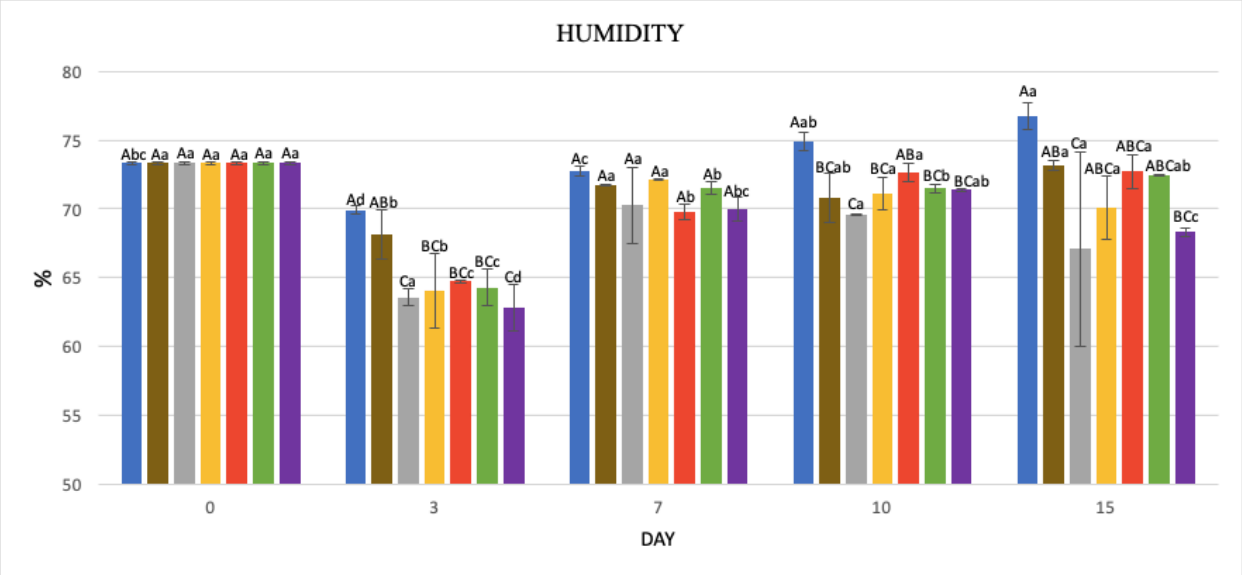

Figure 5.8 Moisture at day 0, 3, 7 ,10, 15. Samples: Control (•); CH (•); CH + 2.5 % MMT (•); CH + 1% ZnO NPs (•); CH + 2.5% MMT + 1% ZnO NPs (•); CH + 1% ZnO NPs + 0.5% REO (•); CH + 2.5% MMT + 1% ZnO NPs + 0.5% REO (•). (A-C): Within each day, values in the same line not sharing lower upper case superscript letters indicate statistically significant differences among formulations (p < 0.05). (a–d): Within each parameter type of film, values in the same column not sharing upper lower case superscript letters indicate statistically significant differences among days (p < 0.05)

On the tenth day, unwrapped sample showed a significant increase (p<0,05) up to 74.91% in moisture, while in other samples level remained more or less stable, sometimes showing a significant change. Among biofilm wrapped samples the only one to show no significant difference (p>0,05) was the one containing CH + MMT +ZnO NPs.

At the last day, in unwrapped sample moisture is increased, not significantly to 76.75% (p>0,05). Biofilm wrapped samples have always lower moisture than unwrapped sample, and compared to day 10 some samples have decreased and others have dropped.

Generally, compared to initial moisture no biofilm showed significant difference at the storage end ($p>0,05$), except the one containing all the bio components that suffered a significant decrease in moisture ($p<0,05$).

Table 5.8 Moisture at day 0, 3, 7 ,10, 15 and standard deviation. (A-C): Within each day, values in the same line column not sharing lower upper case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). (a–d): Within each parameter type of film, values in the same column line not sharing upper lower case superscript letters indicate statistically significant differences among days ($p < 0.05$)

	FILM	0	3	7	10	15
Humidity %	**Unwrapped**	73,35±0,11Abc	69,89±0,3Ad	72,73±0,37Ac	74,91±0,7Aab	76,75±1Aa
	Chitosan	73,35±0,11Aa	68,09±1,7ABb	71,69±0,04Aa	70,80±1,7BCab	73,13±0,36ABa
	Chitosan + Montmorillonite	73,35±0,11Aa	63,56±0,64Ca	70,24±2,8Aa	69,55±0,06Ca	67,10±7Ca
	Chitosan + ZnO	73,35±0,11Aa	64,05±2,75BCb	72,11±0,08Aa	71,12±1,14BCa	70,12±2,31ABCa
	Chitosan + Montmorillonite + ZnO	73,35±0,11Aa	64,73±0,1BCc	69,76±0,55Ab	72,65±0,67ABa	72,73±1,22ABCa
	Chitosan + ZnO + REO	73,35±0,11Aa	64,27±1,35BCc	71,52±0,42Ab	71,47±0,35BCb	72,42±0,06ABCab
	Chitosan + Montmorillonite + ZnO + REO	73,35±0,11Aa	62,79±1,69Cd	69,99±0,83Abc	71,39±0,08BCab	68,32±0,31BCc

6. Conclusions

Films obtained showed good mechanical resistance, remaining intact during the storage period, confirming the good interaction between different components. Considering results obtained from microbiological and chemical-physical analyses, it is not possible to indicate the best film in absolute terms. As in some cases the different components combined use has provided better results than chitosan alone use, while in others it has not.

Meat microbiological contamination was quite high from the beginning, both as regards total mesophilic aerobic microorganisms count and *Enterobacteriaceae* count. TMAM acceptability limit is generally around 7 logs CFU/g, and considering our product contamination it is plausible to say that it was already in an advanced shelf life state. During storage, microbial load increased linearly and significantly ($p<0.05$), both for TMAM and *Enterobacteriaceae*.

Films showed the ability to reduce microbial load, with a contamination level often significantly different ($p<0,05$) from control, although on the third day all samples exceeded acceptance limit, but this is due to the high initial contamination.

Some of films containing multiple compounds have shown a reduction in effectiveness over time, without being significantly different from the control ($p>0,05$). This is due to the high interaction between compounds, so these bonded each other, limiting each antimicrobial action.

pH didn't increase constantly through time, firstly there was a decreasing, this may be due to the very low permeability of these films to oxygen and once aerobic bacteria have consumed the remaining one, an anaerobic environment has been created that has favored LAB development, resulting in organic acids production. During storage then pH increased significantly ($p<0.05$) in all samples, although samples in film had a significantly lower pH ($p<0.05$) than control, confirming film's effectiveness in microbiological development containment.

Chitosan saccharide nature causes high permeability to water vapor, therefore it is inevitable that water exchanges take place between product and environment. However, most films have shown a good water vapor exchanges containment, as humidity has not changed significantly ($p>0.05$) unlike control which has undergone a significant increase ($p<0.05$).

Meat was initially in a rather low oxidation state, and MDA concentration increased significantly over time ($p<0.05$) but did not reach particularly high levels. During storage first days, films kept the product in a low oxidation state. Contrary to expectations, films

containing rosemary essential oil did not contain oxidation and even deteriorated during storage. This could be due to an oxidation of same essential oils, which have released compounds on the product.

As far as color is concerned, films use has yielded good results, which is very important as it is a rather relevant characteristic of the product. In film wrapped samples, there was a maintenance and sometimes a significant improvement ($p<0.05$) in color, compared to storage beginning. In particular films containing compounds showed better results than film with only chitosan, in fact sample containing all compounds together provided the best results.

In conclusion, as mentioned above, finding the best biofilm is not possible. Surely, we can say that film with only chitosan has shown a certain constancy, always providing good results, even if they haven't always been the best. This is probably due to chitosan solitary action as, the high interaction between compounds and between compounds and chitosan, sometimes provides better and sometimes worse results.

In general, we can say that from microbial point of view, more or less all films have shown themselves to be effective, as far as chemical analysis is concerned, more differences have been found between films, in particular as regards product's oxidation containment. So, we can say that from a technological point of view it is a good alternative to conventional polymers, but we need further analysis to understand how these compounds interact with product.

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