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**Thesis title**

# SHELF-LIFE EXTENSION OF LACTO-FERMENTED MOZZARELLA THROUGH SUSTAINABLE PROCESS OPERATIONS

*AGR/15*

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**LIST OF ABBREVIATIONS**

AP aerobic packaging

BJ concentrated bergamot juice or bergamot extract

CL calcium lactate

GL governing liquid

HMMC High-Moisture Mozzarella Cheese

KDPC Khartoum Dairy Products Company

LAB lactic acid bacteria

LMMC Low-Moisture Mozzarella Cheese

MAP modified atmosphere packaging

M-BJ experimental trial code

MC Lacto-Fermented Mozzarella Cheese, with a high moisture content

M-PLA experimental trial code

M-SALTS experimental trial code

M-SW experimental trial code

NaCl sodium chloride

NPN not protein nitrogen

PDO Protected Designation of Origin

PLA Poly Lactic Acid (polylactic acid or polylactide)

PP Poly Propylene

SN soluble nitrogen

SN-TCA soluble nitrogen in Trichloroacetic Acid

SW stretching water

TN total nitrogen

VP vacuum packaging

**Short abstract**

The principle objective of this research was to identify the best operation to preserve and improve the quality of  fresh Calabrian cheese through the use of alternative governing liquids and a choice of eco-friendly biopolymers with low environmental impact. The production cycle of lacto-fermented mozzarella was analysed while experimental trials were conducted to extend its shelf-life, by using different salts and concentrated bergamot juice (BJ), which is a natural source of antimicrobial and antioxidant compounds.

This experimentation demonstrates that by adding calcium lactate, stretching water and using BJ can satisfactorily prolong the shelf-life of samples of mozzarella cheese stored at 5 °C.

**Keywords**

Lacto-fermented mozzarella cheese, concentrated bergamot juice, calcium lactate, alternative governing liquid, stretching water, shelf-life.

## Extended abstract

Scientific research in Food Science is oriented towards the study of unconventional methods for application in the food industry in order to discover alternatives to conventional approaches and strategies. New and emerging technological processes often accommodate the consumers' needs, ensuring safe, high quality nutritional and sensory properties, minimally processed foods, sustainability and respect for the environment.

When focusing on the dairy sector, it is economically important to extend the shelf-life of perishable fresh cheeses, while at the same time preserving the quality of such products.

The lacto-fermented mozzarella cheese with high-moisture has a limited shelf-life. It is strictly linked to the quality of the milk used (cow or buffalo) and to the technology of processing (production and packaging methods).

The short shelf-life of traditional mozzarella cheese has been mainly attributed to microbiological spoilage. The traditional lacto-fermented mozzarella cheese, obtained with pasteurized milk and selected starter, can maintain a prolonged shelf-life of up to 7 days at 5 °C. Only products obtained with direct acidification have a shelf-life of approximately 20 days, however their organoleptic characteristics are not comparable with those of traditional artisan mozzarella cheese.

The aim of this study was to extend the shelf-life of lacto-fermented mozzarella cheese using different strategies. In particular, this research has evaluated the impact on the shelf-life of cheese-making practices commonly used in the dairy processing industry compared to the strategies based on the use of biodegradable packaging (Poly Lactic Acid) and different governing liquid containing calcium lactate (CL), bergamot juice concentrate (BJ) and stretching water (SW).

The samples were characterised in terms of their microbiological and physico-chemical properties. The results showed that an inhibition on Pseudomonas *spp.* growth and a lower total bacteria count were observed on CL and BJ samples.

The biodegradable material, the packaging, and the stretching water as governing liquid represent promising opportunities for extending the shelf-life of mozzarella cheese and, furthermore, in reducing the environmental impact of this form of manufacturing. In the polypropylene packaged samples adding CL and BJ demonstrated an inhibition of Pseudomonas *spp*. growth, especially in SW, although this was less in tap water , as compared to the control samples.

Moreover, the application of concentrated bergamot juice used in association with calcium lactate, at both 0.2% and 0.6% concentrations has allowed an increase in the bacteriostatic effects of calcium lactate and offers an alternative to local products. This last aspect could assist the promotion, understanding and spread of "bergamot lacto-fermented mozzarella" across the borders

of the local Calabrian market. The increase in shelf-life was extended to over 9 days, when stored at 5 ° C.

This testing validated the hypothesis that simple cheese-making strategies relating to the composition of governing liquids can be used to optimize mozzarella shelf-life.

Future research could investigate the physico-chemical and microbiological parameters with the aim of extending the shelf-life of mozzarella cheese stored in stretching water when adopting bergamot essential oil, rather than bergamot juice concentrate.

# Chapter 1

## 1. Introduction to Thesis

The scientific panorama over the past few years has seen an enrichment in research and innovative experimentation aimed at improving the storage of mozzarella cheese, and/or highly perishable fresh foods. Mozzarella cheese is made from buffalo or cow milk. Mozzarella cheese is a soft white cheese obtained after the coagulation cow milk by rennet and/or coagulant enzymes with the addition of Lactic Acid Bacteria (LAB) by stretching acidified curd.

This research gauges the extension of the shelf-life of this typology of lacto-fermented mozzarella cheese (MC), with high moisture content (50-60%).

The shelf-life of mozzarella cheese depends on various factors; such as different production processes, the microbiological quality of the milk, physico-chemical properties and packaging; for these reasons the shelf-life of mozzarella is variable: ranging from 3-4 days (Cantoni *et al.*, 2003) to 5-7 days (Altieri *et al.*, 2005). Mastromatteo *et al.* (2015) demonstrated a shelf-life of about 10 days was achievable through the use of active coating and MAP (Modified Atmosphere Packaging) composed of 50% $CO_2$ and 50% $N_2$.

Although mozzarella receives heat treatment, post-processing contamination by spoilage microorganisms (Pseudomonas spp. and coliforms) may occur (Spano *et al.*, 2003) and it contributes to reducing its shelf-life. These microorganisms can cause proteolysis, discolorations, pigmentation and the development of off-flavour (Cantoni and Bersani, 2010).

Furthermore, light, oxygen and the level of lipid and protein oxidation can also reduce the shelf-life of mozzarella during storage. Lipid oxidation starts with the production of free radicals, inducing the synthesis of hydroperoxides and then the production of volatile carbonyl compounds which are responsible for off-flavour (Kristensen and Skibsted, 1999).

Lactobacillus species produce metabolites with antioxidant activity (Osuntoki and Korie, 2010) and degrade the superoxide anion and hydrogen peroxide. LAB strains can bio-preserve the product due to the fermentation, production of acids and reduction of pH and improve the rheological properties (viscosity and texture) of fermented milk products (Favaro *et al.,* 2015).

The shelf-life of mozzarella is also affected by packaging and the “governing liquid” (water or diluted brine), which are microbiological and chemical critical points.

There has also been lengthy research into the composition of the governing liquid and the impact of NaCl or KCl on the production and quality of mozzarella cheese (Thibaudeau *et al.*, 2015).

Some substances have been used to prolong the shelf-life of dairy products; such as potassium sorbate, sodium benzoate, calcium lactate, calcium ascorbate (Lucera *et al.*, 2014), sodium alginate coating containing Lactobacillus *reuteri* (Angiolillo *et al.*, 2014), nisin (Amir *et al.*, 2013), chitosan (Altieri *et al.*, 2005) and some essential plant oils such as lemon extract.

The development of new methodologies (so called mild-technologies) and the use of innovative materials such as the biopolymers (e.g. polylactic acid or PLA) from renewable and compostable resources facilitates recycling and generates reusable waste at the point of production, whilst also ensuring compliance with the requirements for food contact. The trend towards sustainability in packaging remains a major factor in improving the environmental performance of products without impacting on their quality.

As regards to the contents of this thesis; *Chapter 2* contains reviews of that research that affects this specific field of research.

*Chapter 3* reports the materials and methods used in this research.

*Chapter 4* focuses on the effect of using different governing liquids and packaging material on the quality and shelf-life of MC, in terms of results and discussions.

*Chapter 5* summarizes the conclusions of the research and bibliographic references are listed in *Chapter 6*.

## 1.1 Objectives

In order to extend the shelf-life of MC and therefore also expand the distribution channel over the local market, the specific aims of this project were the following:

- The development of possible alternatives to the common governing liquid for lacto-fermented mozzarella cheese to extend its shelf-life, using salts and extract of bergamot juice (BJ) obtained from the endocarp of the fruit and considered a secondary product of the essential oil industry. BJ has been actually recognized as a source of beneficial effects.
- The employment of eco-friendly packaging in responding environmentally to reducing the use of plastic.

# Chapter 2

## 2. State of the art

This chapter, initially, considers the dairy market, pasta filata cheese, mozzarella cheese news, and discusses the factors that influence both the production and methods of production.
The final part of this chapter focuses on shelf-life issues and offers solutions to dairy concerns related to lacto-fermented mozzarella cheese.

### 2.1 Status of cheeses production in Italy

In generally the dairy industry is based on the production of cheese (fresh, seasoned, cooked, etc.), butter, cream and pasteurized, sterilized, condensed, concentrated and fermented milk. Cheese-making began about 8000 years ago, and today there are more than 1000 cheese varieties worldwide, each unique with respect to flavour and form (Beresford *et al.*, 2001; Sandine and Elliker, 1970).
From the technological point of view, the production of dairy products results from either the destabilization of fat droplets present in the emulsion phase (as in butter, cream etc.) or the destabilization of the proteins in the colloidal suspension phase (as in cheese) in milk (Euston, 2008).
In Italy, the nation with the largest number of locally-made cheeses, approximately 400 products, 55 % of total milk consumption is used for 'Protected Designation of Origin' cheeses (Cassandro, 2003).
A typical definition of cheese is 'the fresh or matured product obtained after the coagulation of casein of milk, cream, skimmed or partly skimmed milk, buttermilk or a combination thereof' (FAO, 2004) through lactose fermentation, reduction of water activity, addition of salt and starter (Fox and McSweeney, 2004).
The peculiarity to Italian Milk Production is the destination of milk for the production of typical and quality cheeses at PDO (Protected Designation of Origin), such as parmigiano, provolone, mozzarella (*https://www.clal.it*).
The quality of milk and the use of the starter culture are at the central point for the protection and valourisation of typical dairy products.
In particular, mozzarella cheese is now produced all over the world and has become common to all.

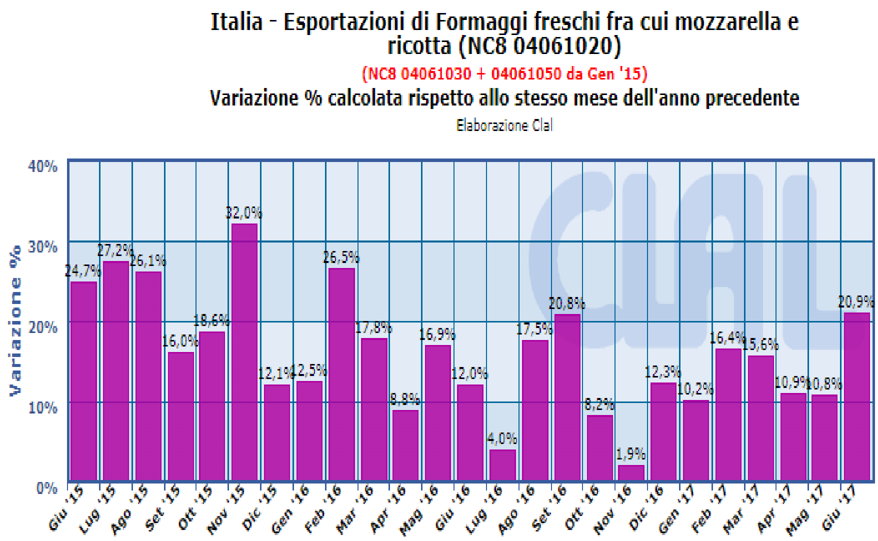
The popularity of this cheese is attributed to its great taste, convenience, versatility of use and its nutritional value. The protection of the name is only recognized to some specific varieties, such as the Mozzarella di Bufala Campana DOP or Fior di Latte Southern Apennines of Italy (National transient protection MiPAAF by the decree of 1 March 2002; Mucchetti and Neviani, 2006).

The names and the related operational protection are received and collected in an EC Regulation, valid for the whole of Europe.

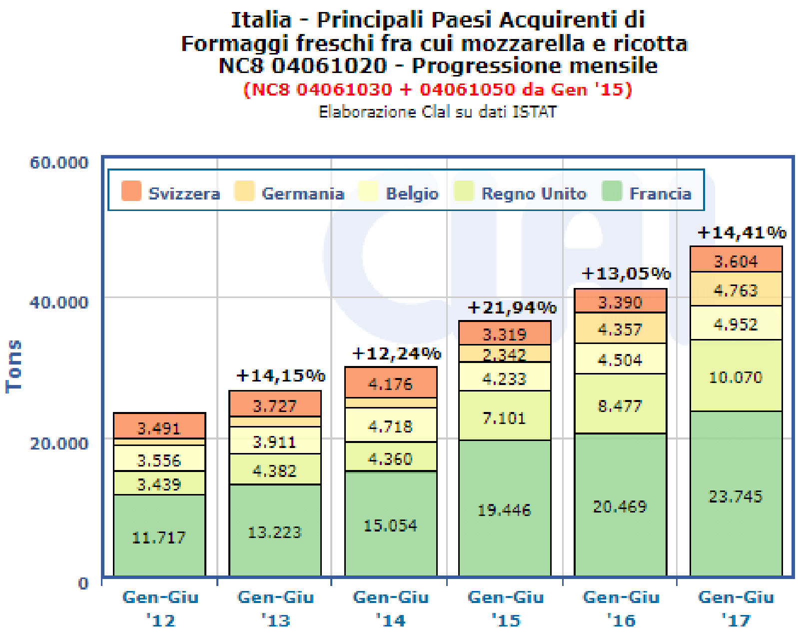
According to the Food and Agricultural Policy Research Institute (FAPRI, 2009), the expected trend in cheese production in Europe from 2008 to 2018 will be +11 %. Within the same period, the consumption of milk is expected to have an increase (+12 %) while its production +2%.

The quota system imposes  limits of production in the EU of milk for the Italian dairy industry.

Nevertheless, future market liberalization is expected: recent policy developments including the reduction of intervention prices and an increase of quotas by 1% annually from 2009 to 2013 and the consequent expiry of the quota system in 2015 (Kempen *et al.*, 2011). In this situation the dairy industry is expected to maintain its economic importance for the Italian agriculture sector and the production of milk which is suitable for cheese processing is becoming more and more important for increasing the efficiency of the dairy chain. Figure 1 a, b show the percentage change in cheese exports (including mozzarella cheese) compared to the same month in the previous year. The period analyzed is between June 2015 and 2017. From an analysis of the same figure cheese exports are shown to be growing. Over a period of months, including November 2015 and February 2016, there were significant increases in exports. This positive trend is confirmed in Figure 1b and can be explained in part by the increase in cheese purchases by France and the United Kingdom.




a




b

**Figure 1** Export of fresh cheeses (a) and buyers countries (b) including "Mozzarella" and "Ricotta" cheese. *Source: https://www.clal.it/*

## *2.2 Mozzarella Cheese*

Among the cheeses produced around the world, a special category is that of the fresh cheeses and of the "paste filate group", including the mozzarella cheese. The term "mozzarella" derives from the cheese-maker manually 'cropping' the curd during the stretching, which forms the typical oval shape of the cheese. It follows a dual process: the coagulation of casein achieved iso-electrically (acid) or enzymatically (rennet) and the stretching of the curd in hot water (Salvadori Del Prato, 2005). Biochemical reactions and textural changes occur during these process steps.

Traditionally, Italian mozzarella cheese is made from both buffalo and cow milk (in the second case also referred to as fiordilatte cheese) with or without the addition of bacterial cultures. It is manufactured in a large number of forms and sizes (Scott, 1986).

Buffalo milk has a specific characteristic aroma and physical attributes which a distinct and different from cow milk mozzarella cheese (El-Koussy *et al.*, 1995). In fact the types of milk (cow, buffalo or their blend) used for the production of Mozzarella cheese influence the cheese composition.

Buffalo mozzarella cheese has a superior nutritional value (Sameen *et al.*, 2008) and the use of admixture milk rather than only one type of milk boosts the organoleptic quality (e.g. meltability).

Buffalo mozzarella must be kept in whey, brine or water solution.

It has a short shelf life, less than 7 days (Romano, 2001), if stored at a temperature of between 4-10°C with no loss to its characteristics (translucent external skin, soft and elastic curd, white colour and wild aroma).
Instead, cow milk Mozzarella, whose composition is reported in Table 1 (Kosikowski, 1997; Mucchetti and Neviani, 2006) has a quite different texture and flavour from buffalo Mozzarella; it is harder, drier and less flavourful, with a rubbery texture and a longer shelf life.

Furthermore, it melts easily and has great stretchability. It is also used as an ingredient in baked dishes, especially for pizza (Ma, 2013).

**Table 1** *Indicative Composition of Italian Mozzarella Cheese*

| Cheese | Fat | Total protein | Moisture | Lactose | NaCl | Calcium | Ash |
|---|---|---|---|---|---|---|---|
| | % | % | % | % | % | mg/100g | % |
| Mozzarella from whole milk | 18 | 22.1 | > 50 | 0.3 | 0.5-1.0 | 160-350 | 1.6-1.8 |

On the basis of moisture content and fat in dry matter, mozzarella cheese is divided into four categories as indicated in Table 2 (Kindstedt,1993).

**Table 2** *Classification of Mozzarella Cheese*

| Type of cheese | Moisture (%) | Fat-on-dry matter (%) |
|---|---|---|
| Mozzarella (Traditional) | 52-60 | Min. 45 |
| Mozzarella (Part skim) | 52-60 | 30-45 |
| Mozzarella (Low moisture) | 45-52 | Min. 45 |
| Mozzarella (Low moisture, part skim) | 45-52 | 30-45 |

In particular traditional mozzarella and part-skim Mozzarella have a high moisture content (> 50%); these typologies are defined High-Moisture Mozzarella Cheese (HMMC) and often consumed fresh. Instead, low-moisture and low-moisture part skim milk mozzarella cheese have lower water content (< 50%), good shredding properties and are therefore used primarily as ingredients for pizza and related foods.
This last typology is defined Low-Moisture Mozzarella Cheese or LMMC (Kindstedt *et al*., 2004).

### 2.2.1 Methods of production

The mozzarella production methods can vary considerably depending on the company type (artisanal or industrial), the market to which it is destined (local or foreign) and the place of production.

In the ***STARTER CULTURE METHOD*** mozzarella is made from pasteurized milk, full or skimmed, with chymosin, enzyme contained in the rennet (Vitagliano, 1976) or similar enzymes, acidified with lactic acid bacterial cultures (Streptococcus *spp*. and Lactobacillus *spp*.) in order to form curd and whey. After separation from the whey the separated curd (pH 5.2) is traditionally stretched utilizing water at 80 °C and mechanically worked into shapes of 150-250 g, which are cooled in running water (about 10-12 °C for 30 min), followed by immersion in chilled brine (5°C), (Weckx and Delbeke, 1971).
This traditional method produces an excellent finished product, but it involves the loss of valuable milk proteins in the whey and in spinning liquid of mozzarella cheese.
Moreover, there are significant financial and logistical costs associated with the use of fresh milk since large quantities of milk must be shipped and stored under refrigerated conditions.

Another method is the ***DIRECT ACIDIFICATION***: Mozzarella cheese is obtained by acidifying the milk with lactic, malic or citric acids to pH of 5.2 or 5.6 before rennet coagulation, formation and the stretching of the curd. It is a relatively short process (about 2 h from addition of coagulant) while the use of milk with a fat content of 2% is preferable.
There are two types of direct acidification, generally used to produce pizza mozzarella cheese:
a) quiescent storage method (Singh and Ladkani, 1984);
b) continuous agitation method (Kim and Yu, 1988).
Low-moisture mozzarella cheese (LMMC; Kindstedt *et al.*, 2004) can be produced using a direct acidification by addition of citric acid.
There is a wealth of research concerning the direct effects of acidification on the rheological properties of the mozzarella (Keller *et al.,* 1974; Micketts and Olson, 1974; Schafer and Olson, 1975; Dave *et al.*, 2003).
McMahon *et al.* (2005) studied the influence of calcium, pH and moisture on the structure of nonfat direct-acid Mozzarella Cheese, in particular in the low-calcium cheese where the protein matrix appeared less dense, thus indicating the proteins were more hydrated.

***MOZZARELLA MADE FROM STORED CURD***. According to actual European Union (EU) regulations the use of curd (semi-finished product) instead of milk cannot be indicated in the attached label. It would be regarded as misinformation toward the consumers and unfair competition to the disadvantage of the traditional dairies.

Faccia *et al*. (2014) found a molecular marker present in very small amounts of cheese made from fresh curd and at high levels in cheese made from stored curd. This approach was used to identify “Mozzarella or Fiordilatte cheese” produced with or without the employment of stored curd.

***MOZZARELLA CHEESE FROM TREATED MILK:*** recombined milk (Lelievre *et al*., 1990), ultra-filtrated milk (Mann, 1982; Pal and Cheryan, 1987; Ardisson-Korat and Rizvi, 2004). In particular, mozzarella cheese made from recombined milk does not show the organoleptic and rheological property characteristics of fresh milk cheeses.

***ALTERNATIVE PATENTED METHODS.*** One method of making processed mozzarella to obtain a longer shelf-life and storage without refrigeration is achieved by applying a suitable time-temperature combination to inactivate proteolytic enzymes and microorganisms (Rizvi *et al.*, 1999). This discovery by Silver *et al*. (2002) demonstrates the production natural mozzarella cheese using dry dairy ingredients.

Dry dairy ingredients comprise blends of milk protein concentrates, whey protein isolate, calcium caseinate, sodium caseinate, rennet casein, acid casein, non-fat dry milk and mixtures thereof.

However, dried whey ingredients ideally should have a bland, delicate flavour (Morr and Ha, 1993; Wright *et al*., 2009), as off-flavors in whey products can limit their use (Quach *et al*., 1999; Childs *et al*., 2007; Drake *et al*., 2009; Wright *et al*., 2009).

Moreover, mozzarella cheeses were also made according to the methods shown in the study by Owni *et al.* (2009) that are reported below:

- method described by Kosikowski (1982), Figure 2a;
- method modified by Khartoum Dairy Products Company (KDPC), Figure 2b.

Mozzarella cheese made by the KDPC method has a better protein (23.33±2.12) and total solids content, while the protein content by the Kosikowski (1982) method was lower (20.06±0.82) when compared to that obtained by the KDPC method.

However, the fat content of the cheese made by the Kosikowski (1982) method was higher than the cheese made by the KDPC method.

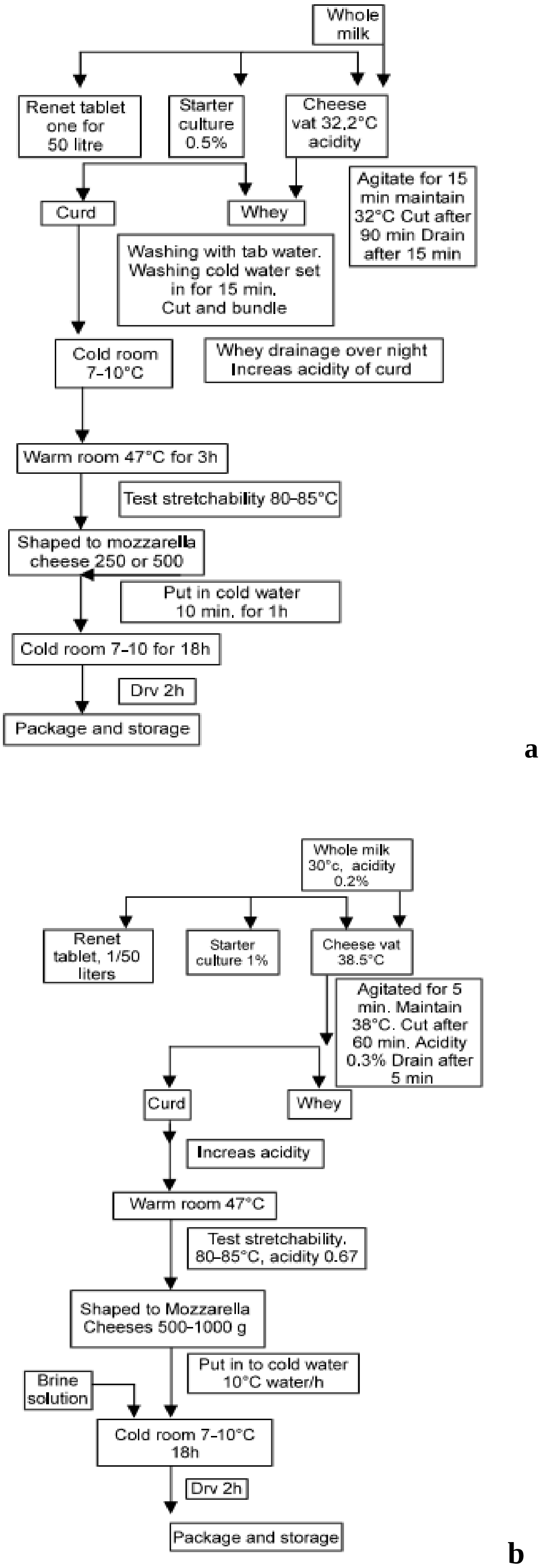



**Figure 2** Flow sheet diagram for the manufacture of Mozzarella cheese according to Kosikowski (1982) (a) and to modified method used in KDPC (b)

### 2.2.2 Manufacture of lacto-fermented mozzarella cheese

Lacto-fermented mozzarella cheese, with high moisture content (50-60%), is made from full milk or partially skimmed milk, acidified by bacterial cultures. This method traditionally uses microbial starters and  is based on the steps reported in Figure 3 (Jana and Mandal, 2011).

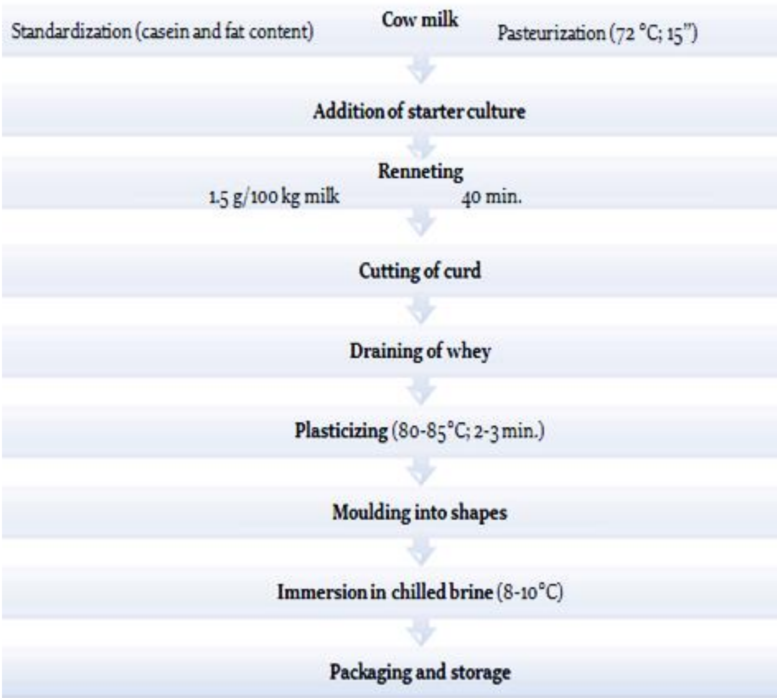



**Figure 3** Flow chart of Mozzarella cheese

*Microbiological quality of cow milk.* The manufacture of lacto-fermented mozzarella starts with an assessment of the microbiological quality of the milk. In fact cheese milk must be free of pathogens, spoilage microorganisms and antibiotics (DPR 54/97).

Spoilage is often caused by coliforms (Cantoni *et al*., 2006), by proteolytic psychrotrophs (Baruzzi, *et al*., 2012), by discoloration (Andreani *et al*., 2014) or by Pseudomonas *spp.* In fact, Pseudomonas counts exceeding $10^6$ cfu/g have been associated with a reduced acceptability of mozzarella (Lucera *et al*., 2014).

*Pasteurization and pre-treatment of cow milk.* Cow milk is pasteurized at a temperature of 63°C for 30 min. or 72°C for 15 sec., so that the enzyme phosphatase test on milk results negative (DPR 54/97).

Moreover, pasteurization can be defined as a thermic process of 70°C/2 min. or equivalent (*z* value of 7.5 °C).

The *z* value is the number of degrees Celsius which will result in a 10-fold change in the *D* value given to chilled food products.

The decimal reduction time (*D*) value is the time required at a given temperature for the surviving population to be reduced on a log cycle or 90%.

Under this process a six-log reduction (*6D*) in *Listeria monocytogenes* and other vegetative pathogens can be achieved. It is also achieves sufficient inactivation of most spoilage bacteria such as Enterobacteriaceae, Pseudomonas *spp.* and yeasts.

The milk can be subjected to fat standardization and to the homogenization of fat globules in order to distribute the fat evenly in the milk; to achieve a perfect relationship between fat content and protein thereby increasing the rate of coagulation and cheese yield (Fox and McSweeney, 2004). A milk fat level of 2.5% is optimal for pizza cheese making (Valle *et al*., 2004).

The removal of carotenoids (chlorophyll) in cow milk is necessary to maintain the white color of the final product; for example by use of benzoyl peroxide and titanium dioxide (Kosikowski, 1975).

*Addition of starter cultures*, which start the acidification at a temperature of about 37°C.

For this reason the bacterial cultures are known as *"starter" (= those which trigger)*. They are present in the market in various forms of commercial preparations (lyophilized, frozen or liquid) and constituted by pure species, associations or mixtures of strains and species.

One of the primary events in the manufacture of most, if not all, cheese varieties is the fermentation of lactose to lactic acid by selected lactic acid bacteria or, in traditional cheese-making, by the indigenous microflora.

The most commonly used starter bacteria for lacto-fermented mozzarella cheese include the mesophilic and thermophilic starter bacteria. Traditionally, mesophilic cultures have an optimum temperature of ~ 30°C (Streptococcus *cremoris*, Streptococcus *lactis*, S. *diacetylactis*, and/or Leuconostoc *spp.*). S. *lactis* and S. *cremoris* are responsible for acid production, while S. *diacetylactis* and Leuconostoc *spp.* are required for flavour development (mainly diacetyl, acetate) and $CO_2$ production.

Thermophilic cultures, with an optimum of ~ 45°C, are usually composed of S. thermophilus and Lactobacillus (Lb. *acidophilus*, *helveticus*, delbrueckii *subsp. lactic*, and delbrueckii *subsp.*) (Fox *et al*., 1990; Christensen, 1966).

The secondary microflora is composed of non-starter lactic acid bacteria (NSLAB) such as other bacteria, yeasts and/or moulds which grow internally or externally and usually in specific cheese varieties. This flora may be intentionally added or develop spontaneously through contamination from the surrounding environment (Beresford *et al.*, 2001). Secondary microorganisms do not contribute to acid production during production, but generally play a significant role during ripening.

The reduced pH of cheese curd, which reaches variable values from 4.5 to 5.2, depending on the variety, affects at least the following characteristics of curd and cheese: syneresis (and hence cheese composition), retention of calcium (which affects cheese texture), retention and activity of coagulant (which influences the extent and type of proteolysis during ripening), the growth of contaminating bacteria.

Fermentation of lactose to lactic acid by lactic acid bacteria is an essential primary reaction in the production of all cheese varieties.

Most (98 %) of the lactose in milk is removed from the whey during production, either as lactose or lactic acid.

The residual lactose in cheese curd is metabolized during the early stages of ripening. During ripening lactic acid is also altered, mainly through the action of non starter bacteria.

Since lactose is present in the aqueous phase of cheese curd, a decrease in the water content of curd is paralleled by a decrease in lactose.

Conversely, curds with a high moisture content are likely to contain a high concentration of lactose, which can, eventually, be converted to lactic acid. A rennet coagulum undergoes considerable syneresis when cut or broken, and this is the main factor responsible for the transfer of solutes (lactose, lactic acid, etc.) from the aqueous phase of curd to whey.

*Renneting.* Caseins account that 76% to 86 % of the total milk proteins and are composed of αs1-, αs2, β- and κ- caseins at a ratio of 3: 0.8: 3: 1 (Updhyay *et al*., 2004).

In the coagulation process the role of kappa-casein micelles is important.

Under normal conditions, this milk casein stabilizes the micelles dispersed in solution (sol) and it prevents their aggregation (gel).

However, with the coagulation process, under the influence of calcium (at least 80 mg/L of soluble calcium phosphate in the milk), rennet, temperature and pH, casein form gel.

The milk clotting starts with liquid rennet (deriving from the stomachs of ruminants, calf and adult bovine), after acidification (about pH 6.3) at 30-32°C.

The aggregation of micelles starts with the hydrolysis of Phe105 – Met106 bond of *κ*-casein (Crabbe, 2004); it causes the incorporation of fat globules within the matrix (Gunasekaran and Ak, 2003) and the loss of "hydrophilic colloidal-protector peptide residues of κ-casein".

*Cutting of curd* starts after about 20-30 minutes to facilitate the separation of the whey (syneresis). The cut gentle process to avoid the loss of fat.

The long coagulation gives softer clots, while curdling curds are obtained in larger cheeses. Moreover, a low cooking temperature (37°C) and short cooking time (40 min) decrease syneresis and can contribute to the high-moisture content in mozzarella cheese.

*Draining of whey*. The whey is drained normally at pH of 6.1-6.2 and the curds are subjected to a process called 'cheddaring'. During the cheddaring lactic acid increases rapidly (pH 5.2) to a point where coliform bacteria are killed by the free hydrogen ions.

During the time, the curd blocks ripened improve their structure, plastic properties, flavour and texture (Lawrence *et al*., 2004).

A series of chemical and biochemical reactions occur during lacto-fermented mozzarella cheese ripening including glycolysis, lipolysis and most importantly proteolysis (Fox et al., 1993).

*Plasticizing and addition of salts. C*orrect ripening of the curd at the time of spinning is essential to obtain good mozzarella cheese.

An excessive acidification of the curd may cause fat and protein loss in the water spinning and reduction of shelf-life of the product, due to increased proteolysis and a shift from insoluble calcium to soluble calcium (Guo and Kindstedt, 1995); instead in the presence of low acidity the dough is difficult to spin for the hard and fibrous structure.

The stretching takes place in hot water (80°C-90°C), generally mechanically, through spinners-machines.

Following the stretching stage, there appears the formation of oval shapes.

After being extruded, the mozzarella cheese is cooled in water prior to being placed into its cold governing liquid (or brine). The governing liquid quality is vital to the prevention of flavour defects in mozzarella cheese, since any off-flavours present in the brine will be readily transmitted to the cheese.

Sodium chloride (NaCl) can be added to the milled curd, by cooking and stretching in hot brine.

The different roles of salt in cheese are reported in Figure 4.

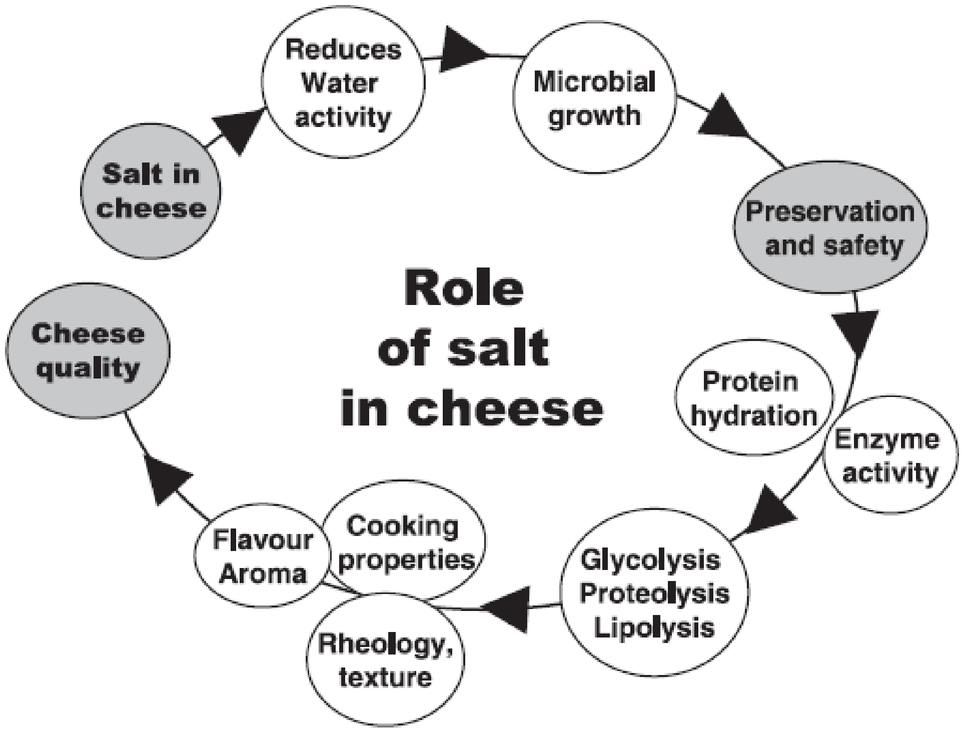



**Figure 4** Different roles of salt in cheese (Guinee, 2004)

Traditionally, some cheese factories dip freshly made cheese in a concentrated cold brine solution. Brine salted cheese has a NaCl gradient from the surface to the centre of the block that is brought about by the slow diffusion of salt to the centre (Farkye *et al.,* 1991).

There are several equations to predict the amount of salt absorbed as a function of the conditions of curing. One of these is based on Fick's law on the diffusion transport substances (Chamba, 1988):

$$\mathbf{S = 2Cs\ A/V\ (D\ t/\pi)^{1/2}}$$

where:

S = percentage of salt absorbed in the aqueous fraction of the cheese;

Cs = percentage of salt of the brine;

A = cheese surface;

V = cheese volume;

D = salt diffusion coefficient ($cm^2$ / day);

t = time expressed in days.

Geurts *et al*. (1974) concluded that the penetration of salt into cheese and the concomitant outward migration of water during brining could be described as an impeded diffusion process.

Therefore NaCl and $H_2O$ move in response to their respective concentration gradients but their diffusion rates are much lower than those in pure solution due to a variety of impeding factors.

The diffusion coefficient for NaCl in cheese moisture (*D**) is typically 0.2 $cm^2$/day, although it varies from about 0.1 to 0.45 $cm^2$ /day with cheese composition and brining conditions (Geurts *et al*., 1974; Guinee and Fox, 1983; Guinee, 1985; Turhan and Kaletunç, 1992; Turhan and Gunasekaran, 1999; Simal *et al*., 2001), compared to 1.0 $cm^2$/day for the diffusion coefficient of NaCl in pure water (*D*) at 12.5°C.

The lower diffusion rate of NaCl in cheese moisture compared to water is due to the fact that cheese moisture is held within a protein matrix (which occludes fat globules and dissolved solids), while not all moisture in cheese, some of which is bound by the protein, is free and available for diffusion.

There is little data available on the traditional brine-salting of mozzarella with a mixture of NaCl and KCl.

It is possible to reduce $Na^+$ concentration in mozzarella cheese by adding mixtures of NaCl and KCl to the milled curd after the cheddaring step and achieve its plasticization with 4% NaCl/KCl brine solutions at 80°C for 5 min. (Ayyash and Shah 2011 a,b; Ayyash *et al*. 2013).

In the study by Thibaudeau *et al*. (2015) mozzarella cheeses were brine-salted by the traditional method both with NaCl rather than with mixtures of NaCl and KCl.

They also obtained similar results to those observed in mozzarella cheeses for pizza that were salted by adding dry NaCl/KCl salt to the milled curd.

There were some changes both in the pH value of mozzarella cheese, which was higher with KCl than in cheese salted only with NaCl or in sensorial parameters. In fact a metallic taste was detected by judges of cheeses with a high KCl concentration, therefore the substitution of KCl for NaCl should not exceed a ratio of 25%.

Initially, cheese moisture and calcium content were lower at the external surface than in the centre, whereas $Na^+$ and $K^+$ were higher. This gradient between edge and centre was different for brine solutions with KCl. After 28 d, equilibrium between the external surface and the centre was obtained for moisture, but not for $Na^+$ or $K^+$.

Hardness at the surface of unmelted cheeses seemed to be influenced more by the moisture content than by the $K^+$ concentration, but an opposite trend was observed for the hardness of melted cheese (Thibaudeau *et al.,* 2015).

Moreover, to monitor the effect of sodium content in different regions of mozzarella cheese during brining, Lou *et al.* (2013) measured calcium, sodium and water contents, as reported in figure 5. The sodium content of mozzarella cheese differed by region for the cheese and brining time.

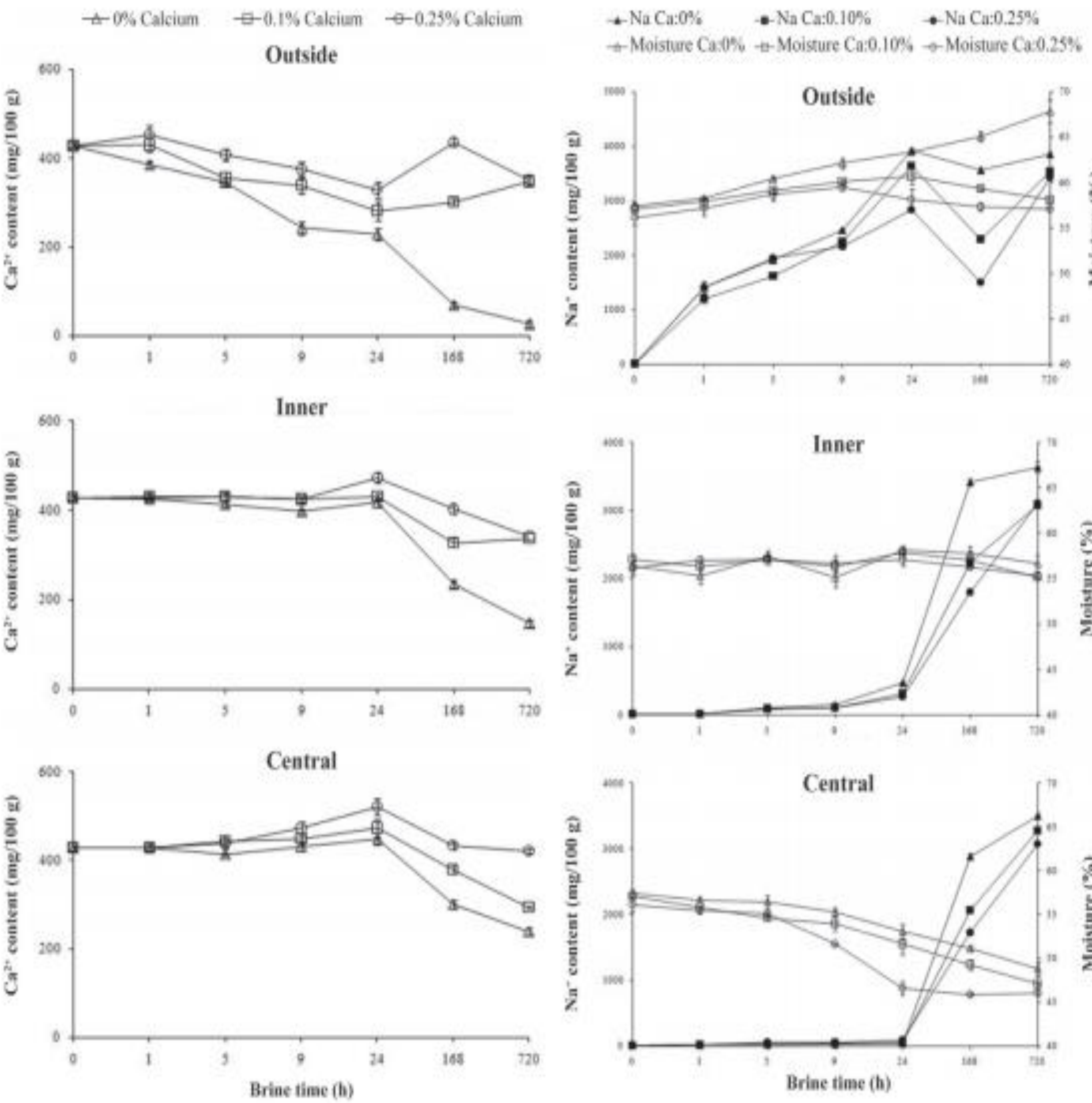



**Figure 5** Diffusion properties of mozzarella cheese solutes (Lou *et al.,* 2013)

*Packaging in governing liquid and storage under refrigeration (5 °C).*

Currently, the packaging of mozzarella cheese consists of flexible multilayer materials, trays made of polyethylene/paper laminated films and tetrapack-type packages.

It is usually a composite item which meets several different needs (Cruz *et al.,* 2007). Principally it acts as a physical barrier between the food and the external environment (oxygen, moisture, volatile chemical compounds and microorganisms).

### 2.2.3 The factors that influence the production of mozzarella cheese

The quality of the milk and cheese yield are influenced by several factors: the animals' age (Schaar, 1984), health, breed (De Marchi *et al*., 2007) and stage of lactation (Ostersen *et al*., 1997), composition of feeding rations (Macheboeuf *et al*., 1993), season (Okigbo *et al*., 1985) and hygienic condition.

These factors modify milk composition (in term of fat, protein, and lactose content and microbiological parameters) and as a consequence the productive process of cheese.

In particular, fat content can influence mozzarella free oil formation which is considered a defect in melted cheese on pizzas and other foods. The aggregation of the fat globules increases the formation of free oil; instead the homogenization, which reduces the size of the fat globules, decreases this phenomenon (Rudan *et al*. 1998; Tunick, 1994).

Kindstedt and Rippe (1990) found that increased fat content in cheese can result in excessive oiling off when melted, while a low level of fat can produce tough and rubbery melted cheeses (McMahon *et al*., 1993).

Furthermore, milk protein quality and content of casein types (Auldist *et al*., 2002; Wedholm *et al*., 2006), pH (Ikonen *et al*., 2004) and the proportion of undissolved milk salts, specifically calcium (Ostersen *et al*., 1997) and sodium content (Guo and Kindstedt, 1995), have important effects on the cheese production process, yield and the texture of fresh cheese.

In fact, during clotting, the presence of sodium increases the water-binding capacity and the solubilization of caseins in the protein matrix (Guo and Kindstedt, 1995). Colloidal calcium phosphate offers an important role in buffering during the acidification of milk and cheese. The addition of calcium (Ca) reduces the rennet coagulation time of milk due to the neutralization of negatively charged residue on casein, which increases the aggregation of renneted micelles. The addition of low concentrations of Ca also increases gel firmness.

Moreover, it is known that the absorption of salt at the surface of cheese increases with greater brine concentration (Fox *et al*., 2000 a,b).

The rate of acid production and the pH of the whey at draining are the critical factors that determine the mineral content of cheese.

Therefore, pH variations at different stages of production also affect the overall product quality (Guinee *et al*., 2002; Feeney *et al*., 2002) by disrupting binding sites on the casein molecule and ultimately altering the structure of the matrix, destabilizing the integrity of the colloidal calcium phosphate and casein micelles (Guinee *et al*., 2002; Feeney *et al*., 2002).

## 2.3 Shelf-life of lacto-fermented mozzarella cheese

### 2.3.1 Definition

Despite its importance, there is no simple, generally accepted definition of shelf-life in food technology research.

The Institute of Food Science and Technology defines it as «*the period of time during which the food product will remain safe be certain to retain its desired sensory, chemical, physical, microbiological and functional characteristics; where appropriate, comply with any label declaration of nutrition data, when stored under recommended conditions*» (IFST, 1993).

Consumers of dairy products must have some indication of the expected or potential shelf-life of the products they buy. Therefore, to allow consumers to assess the age of products at the time of purchase, a date is placed on the container that indicates either the date of packaging or the last date that the product may be sold or offered for sale.

The consumer then expects that a product purchased at any time up to that date is of an acceptable quality. In addition, if properly treated, it should remain acceptable beyond the last date of sale (Hankin *et al*., 1977). The consolidated EU Directive on food labelling (2000/13/EEC) requires pre-packaged foods to bear a date of 'minimum durability' or, in the case of foods that from the microbiological point of view are highly perishable, the 'use by' date.

The date of minimum durability is defined as the 'date until which a foodstuff retains its specific properties when properly store and any special storage conditions (e.g. temperature not to exceed 7°C) must be specified'.

Furthermore, shelf-life was defined in EU legislation under Commission Regulation (EC) N. 2073/2005 thus: "shelf-life means either the period corresponding to the period preceding the 'use by' or the minimum durability date, as defined respectively in Articles 9 and 10 of Directive 2000/13/EC' (EC, 2000)'. This date helps the consumer to decide how long the product may be stored prior to consumption and also helps with stock rotation in food stores.

Lacto-fermented mozzarella cheese with high-moisture (MC) has a limited shelf-life.

The shelf-life of lacto-fermented mozzarella cheese is a finite interval of time, after manufacture and packaging, during which this product retains the required level of quality acceptable for consumption.

It is strictly linked to the type of milk (unpasteurized or pasteurized) used and to the technology of processing (production and packaging methods).

Some indications of shelf-life of mozzarella cheese are given. For example, buffalo mozzarella obtained from unpasteurized milk and immersed in mother solution can be stored for 3-4 days at 4-10°C. After such a period, the external surface peels off and it loses consistency, becomes buttery following the destruction of the structure obtained during high temperature stretching of the curd. Cow mozzarella cheese, obtained with pasteurized milk and selected starter can maintain a prolonged shelf-life of up to 7 days (Altieri *et al*., 2005). Only industrial products, obtained with direct acidification maintain a shelf-life of about 20 days (Ricciardi *et al.*, 2015) at 5 °C, but they are unsatisfactory when compared to traditional artisan mozzarella cheese, for same standard parameters.

There are microbiological standards that identify both the Traditional Speciality Guaranteed cow milk Mozzarella cheese (De Angelis *et al*., 2011) used for traditional Mozzarella cheese production (De Candia *et al*., 2007; Parente *et al*., 1997) and the Protected Designation of Origin Water-Buffalo Mozzarella cheese (De Filippis *et al*., 2014; Ercolini *et al*., 2004).

### 2.3.2 Factors affecting shelf-life of mozzarella cheese

The factors influencing the shelf-life of cheeses are:

- The product’s characteristics including the description in subparagraph 2.3.2.1 (intrinsic factors);
- The environment to which the product is exposed during distribution and storage (estrinsic factors);
- The properties of the packaging.

Generally, the stability of short shelf-life dairy products depends on the growth and subsequent degradation by spoilage micro-organisms. In contrast, the shelf-life of intermediate and long life dairy products is essentially determined by enzymatic or chemical deterioration.

#### 2.3.2.1 Intrinsic factors

*Biotic factors*

Generally, high densities of microorganisms are present in cheese (Table 3) throughout storage and they play a significant role in the maturation process (Cogan, 2000). Several research studies have characterized the microbial biodiversity which occurs in natural whey starter cultures for cow Mozzarella cheese (De Angelis *et al*., 2008; De Candia *et al*., 2007, Speranza *et al*., 2015), mesophilic (Lactobacillus *plantarum* and/or Lactobacillus *casei subsp. casei*) and thermophilic bacteria (Lactobacillus *fermentum,* L. *helveticus,* L. *delbrueckii subsp. lactis,* L. *delbrueckii subsp. bulgaricus* and streptococci such as S. *thermophilus*).

Microbiology of mozzarella cheese is a complex and dynamic equilibrium between lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB); it results in an appreciated product when well balanced, but it is responsible for product failure in terms of sensory characteristics and safety issues if unbalanced.

**Table 3** *Microbial association in dairy products*

| Products | Conditions | Microorganisms |
|---|---|---|
| Milk | raw | P.fragi, P. aeruginosa, Staphylococcus *spp.,* Micrococcus *spp.* |
| | raw, refrigerated | Bacillus *spp.,* Paenibacillus *spp.* |
| | pasteurised | B. cereus, B. circulans, B. mycoides, B. licheniformis |
| | bulk tank sampling | Streptococcus uberis |
| | from mastitis infected animal | Streptococcus agalactiae, S. uberis, S. aphaureus |
| | | Alcaligenes *spp.,* Acinetobacter spp., Aeromonas *spp.,* |
| Cream | pasteurised | Enterobacteriaceae |
| Butter and reduced fat dairy spreads | | P. fragi, P. putrefaciens |
| Dairy products | | P. fragi, P. fluorescens,P. hundensis |
| Cheese | brine salted, hard and semi-hard | Clostridium tyrobutyricum |
| | hard cheese | Clostridium *spp.* |
| | cheese rind | P. aeruginosa |
| Dairy products, butter, European cheeses, cheese, yogurts | | Saccharomyces cerevisiae, S. dairenensis, S. exiguus, S. kluyver, Rhodotorula, Cryptococcus, Candida, Penicillium commune, Mucor racemosus, Mucor circinelloides, Pennicillium solitum, Mucor plumbeus, Torulopsis candida, Kluyveromyces fragilis, Mucor hiemalis |

***Source:*** *Kilcast and Subramaniam (2011)*

Cold-tolerant Enterobacteriaceae also occur in chilled fresh cheese storage.

Moreover, a vast number of studies of fresh cheese microbiology have established that spoilage can be attributed to a consortium of bacteria, commonly dominated by Pseudomonas *spp.*

Pseudomonas *spp.* is in most cases responsible for spoilage during the aerobic storage of these products at different temperatures (-1 to 25 °C) producing coloured or fluorescent pigments, which cause food discoloration.

In particular, three species of Pseudomonas *spp.*, namely P. *fragi,* P. f*luorescens* and P. *hundensis,* (Table 3), are the most serious in producing negative colour and odour as the main signs of spoilage (Stanbridge and Davies, 1998).

There was a heightened interest in P. *fluorescens* as a spoiler of dairy products following the increase of cases of “blue mozzarella” that occurred in 2010, in which some European consumers noted discolouration in some mozzarella products. The microbiological analyses on the cheese samples showed high concentrations of P. *fluorescens,* up to $10^6$ CFU per gram of cheese (Bogdanova *et al.,* 2010).

Little is known about the nature of the blue pigment that was observed in the mozzarella cheese, and the characteristics must be understood, in contrast with the thorough studies of pigments, such as pyocyanine, pyoverdine, fluorescein, pyorubin and pyomelanin, that are typically produced by some P. *fluorescens* strains (Andreani *et al.,* 2014).

The Pseudomonas *spp.* are able to produce extracellular enzymes, such as differing proteases, lipases and lecithinases, which are often heat resistant, responsible for spoilage and instability problems in food, and their production increases in suboptimal storage conditions (Marchand *et al.,* 2009; De Jonghe *et al.,* 2011).

*Enzymes during storage*

Several reactions occur during cheese storage including glycolysis, lipolysis and proteolysis (Fox *et al.,* 1993).

In particular, the most complex biochemical reaction (proteolysis) which occurs during mozzarella cheese storage, necessarily non-ripened, is caused by agents from a number of sources: residual coagulants (usually chymosin), indigenous milk enzymes, starters, NSLAB, and in many varieties, enzymes from secondary flora:

- coagulants such as chymosin and pepsin, which are present in the traditional calf rennet;
- proteinases from the milk including cathepsins B, D, G, H, L, elastase derived from lysosomes of the somatic cells (Keely and McSweeney, 2003) and plasmin, stable trypsin-like serine proteinase with optimum activity at pH 7.5 and 37°C.
- starters, containing peptidases, which are responsible for the hydrolysis of short peptides and the liberation of amino acids.
- NSLAB and probiotic additions.

The proteolytic system of other LAB such as NSLAB and probiotics are generally similar (Kunji *et al*., 1996). In addition to NSLAB as an adjunct to the normal starter to improve proteolysis, casein hydrolysis increases and enhances flavour development.

Probiotic bacteria also possess proteolytic system that may contribute to the release of small peptides and free amino acids in cheese (Shihata and Shah, 2000).

Proteolysis contributes to textural changes of the cheese matrix due to a break-down of the protein network, decrease in the $a_w$ value through water binding by liberated carboxyl and amino groups, and increase the pH value, which facilitates the release of sapid compounds during mastication.
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It contributes directly to flavour and off-flavour (e.g. bitterness) of cheese through the formation of peptides and free amino acids (Sousa *et al.*, 2001).
Lipolysis is also an important biochemical event during storage, for both soft and hard Italian cheeses.
However, in the case of other cheeses, in which the extent of lipolysis is only moderate, the contribution of lipolytic products to cheese quality and flavour is less important.
In regard to sugar metabolism and glycolysis different isomers of lactate are formed. The mechanisms of sugar transport in LAB have been identified and characterized (Table 4, Fox *et al.*, 1990).

**Table 4** *Salient features of lactose metabolism of the Lactic Acid Bacteria found in starter cultures*

| Organisms | Transport | Pathway | Products (mol/mol used) | Isomer of lactate |
|---|---|---|---|---|
| Streptococci Group | PEP/PTS | GLY | 4 lactate | L |
| S. thermophilus | ATP | GLY | 4 lactate | L |
| Thermophilic lactobacilli | ATP | GLY | 4 lactate | L or D |

Note: PEP/PTS = phosphoenolpyruvate-phosphotransferase system; GLY = glycolysis

Intentional alteration of the lactose content of curd to produce low-lactose (LL) or high-lactose (HL) cheeses (Huffman and Kristoffersen, 1984) should help to clarify the functions and importance of lactose in mozzarella cheese-making.

*Moisture and water activity*

Water is the central component in food systems, influencing processing, product quality, safety and stability. Water can exist in ‘free’ or ‘bound’ states depending on its interaction with other components, such as proteins, carbohydrates and salts, within the cheese. Water activity describes the level of *boundness* of the water within the food, and thus its availability governs physical, chemical and microbiological reactions (Duggan *et al.*, 2008). Moisture and water activity ($a_w$) are correlated through sorption isotherms.

The adsorption and desorption isotherms (Figure 6) are the key to understanding and controlling product stability, moisture sensitivity, temperature effects and drying characteristics (Simatos, 2002).

Brunauer *et al.* (1940) classified the types of isotherm. Through this classification, isotherms for food products are typically Type II and are sigmoid shaped.

These isotherms may be divided into three regions: the first (at low aw and moisture content) corresponds to the hydration monolayer where water molecules are tightly bound to the product; the second region, which is linear, corresponds to multilayered water bound to the monolayer or capillary water. The third region relates to free water, which is available for chemical reactions and microbial growth (Mathlouthi, 2001).

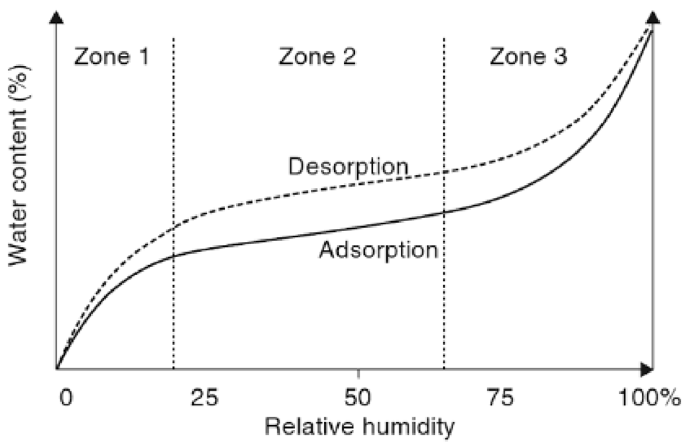



**Figure 6** Typical food adsorption and desorption isotherms (*Kilcast and Subramaniam, 2011)*

To date, water activity and isotherms have been used as a controlling factor for the ripening process of various cheeses (Mathlouthi *et al*., 1980; Ruegg and Blanc, 1981; Saurel *et al*., 2004). Others have related aw to the chemical composition of the cheese, such as water content, NaCl content or pH (Esteban and Marcos, 1990; Marcos *et al*., 1981).

In the work of Hwang *et al*. (2015) moisture isotherms were plotted using the equilibrium moisture value *vs.* the water activity at 20, 25, and 30 °C (Figure 7, 8 a).

The effect of temperature on the water activity was investigated by the Clausius-Clapeyron equation (Figure 8 b):

$$\mathbf{ln a_2/a_1 = (Qs/R)\ [(1/T_1) - (1/T_2)]}$$

Where:

$a_2$= water activity at temperature $T_2$

$a_1$= water activity at temperature $T_1$

Qs= heat of sorption (cal/mole), and

R= the gas constant.

The heat of sorption (Qs) was calculated from the slope of the Clausius-Clapeyron relationship.

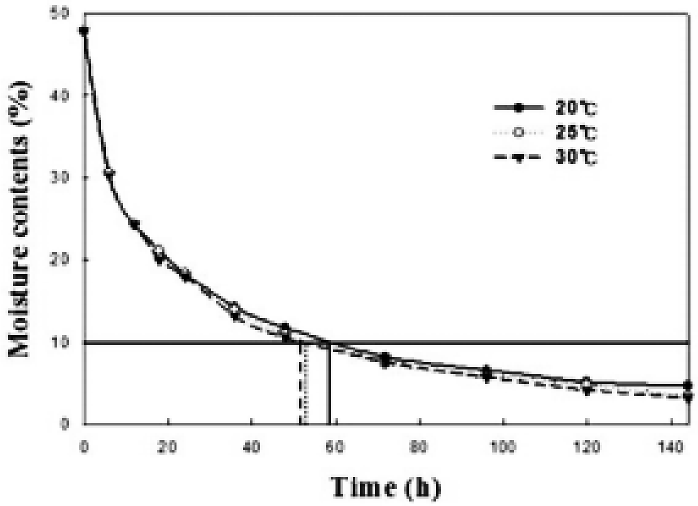
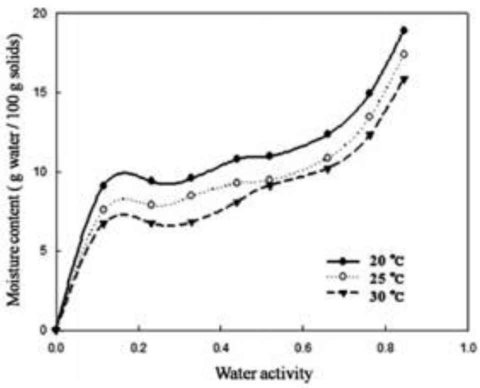
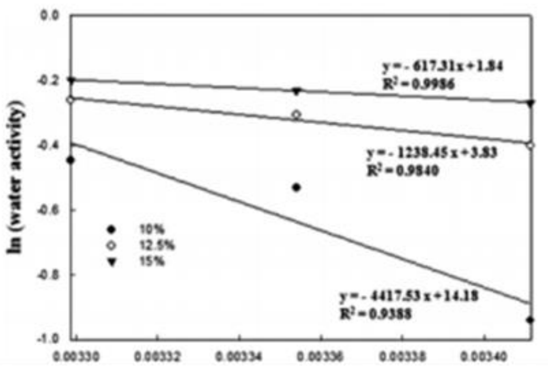



**Figure 7** Changes in moisture contents of 1 cm mozzarella cheese cubes under different drying temperatures. Times of 58 h at 20 °C, 53 h at 25 °C and 51 h at 30 °C were required for the moisture content to reach 10± 0.5% (Hwang *et al*., 2015)




a




b

**Figure 8** Sorption isotherms of dried mozzarella cheese at different temperatures a) and Clausius- Clayperon plot of water activity vs. the reciprocal of the absolute temperature for dried Mozzarella cheese b), (Hwang *et al*., 2015)

### *Total acidity and pH*

The pH of fresh cheese strongly affects protein solubility and functionality. Variations in the pH of cheese also change both the shape and properties of proteins, thus significantly affecting cheese storage stability. Feeney *et al*. (2002) investigated the effects of pH and Ca content and their interaction on proteolysis in low-fat Mozzarella cheese.

The pH strongly affects enzymatic activity and each enzyme has a region of pH for optimal stability. Enzymes can completely lose their activity under extremely high or low pH environments.

*Oxygen*

The presence of oxygen in a package not only facilitates the growth of aerobic microbes and molds, but also triggers or accelerates oxidative reactions that result in cheese deterioration, developing off-odours, off-flavours, undesirable colour changes, and reduced nutritional quality.

In experiments performed by Rodriguez-Aguilera *et al.* (2011) mould ripened cheese packed under anaerobic conditions MAP (0% $O_2$) showed a decrease in its shelf-life.

Therefore, storing the ripened cheeses under low levels of $O_2$ (2%), could elongate the shelf-life of the cheese by over 20%.

### 2.3.2.2 Estrinsic factors

*Temperature*

Temperature is an important factor in influencing spoilage as well as the safety of fresh cheeses.

The greater the temperature, generally, the greater the rate of chemical reactions, resulting in faster deterioration.

**Table 5** *Typical activation energies for reactions important in food deterioration*

| **Reaction** | **Activation Energy ($E_A$) (kJ mol$^{-1}$)** |
|---|---|
| Diffusion- controlled reaction | 8 - 40 |
| Lipid oxidation | 40 - 105 |
| Flavor degradation in dry vegetables | 40 - 105 |
| Enzymic reactions | 40 - 130 |
| Hydrolysis | 60 - 110 |
| Vitamin degradation | 85 - 130 |
| Colour degradation in dry vegetables | 65 - 150 |
| Nonenzymic browning | 105 - 210 |
| Microbial growth | 85 - 250 |
| Protein denaturation | 350 - 700 |

***Source:*** *Kilcast and Subramaniam (2011).*

The dependence of reaction rate on temperature is described by the Arrhenius equation (Kilcast and Subramaniam, 2011):

$$\mathbf{K = Ae^{-Ea/RT}}$$

Where:

$A$ = "pre-exponential factor"

$E_a$ = the activation energy (Table 5)

$R$ = the gas constant

$T$ = the absolute temperature.

Pastorino *et al.* (2002) demonstrated that applied heat in mozzarella cheese would favour hydrophobic interactions, and possibly, re-association of β-casein and calcium with the protein matrix, promoting protein-to-protein interactions. Thus, the protein matrix contracts, occupy less area in the cheese matrix, and microphase separation occurs, causing serum pockets to grow in size, and micro-structural heterogeneity to increase.

This suggests that the increased size of aggregates and heterogeneity of the cheese at 50°C promotes light reflection, thus increasing cheese opacity.

Mozzarella cheese is generally stored at temperatures below 8 °C. Storage at a chilled temperature reduces the growth rates of micro-organisms, but many spoilage organisms and/or pathogenic bacteria are able to grow at refrigeration temperature. Additional factors (low pH, water activity, etc.) may be applied to control the activity of these organisms. When dairy products leave the plant and are displayed on supermarket shelves and, finally, reach the home refrigerator, where low temperature refrigeration is necessary (Overcast, 1968). Temperatures below 7°C are one of the key factors to extending shelf-life (Barnard, 1974).

It has been generally accepted that an increase in storage temperature of 5 °F decreases the shelf-life by approximately one-half (Janzen *et al*., 1981). Mozzarella cheese can be frozen to stop ripening and prolong shelf-life during storage (Diefes *et al*., 1993).

The alterations produced by freezing depend on the operating variables of the process and on the ripening time of the product before freezing and/or after thawing.

Dahlstrom (1978) observed that storage at 4°C after frozen storage improved the functional properties (cohesiveness and meltability) of Mozzarella cheese. Cervantes *et al*. (1983) showed that the textural changes of Mozzarella cheese that occur as a result of a freeze–thaw cycle can be controlled to a certain extent by a suitable combination of salt concentration and aging time. Stress relaxation and dynamic profiles have been performed to study the rheological behaviour of frozen and thawed low-moisture, part skimmed Mozzarella cheese (Diefes *et al*., 1993).

*Relative humidity*

Relative air humidity is defined as the ratio of the vapour pressure of air to its saturation vapour pressure. The equilibrium relative humidity (ERH) of fresh cheese is defined as the relative humidity of the air surrounding the cheese that is in equilibrium with its environment.

When the equilibrium is obtained, the ERH (in percent) is equal to the water activity ($a_w$) multiplied by 100, i.e. ERH (%) = $a_w$ x 100. Water activity is variable in different foods (Table 6).

When a cheese is exposed to constant humidity, the product will gain or lose moisture until the ERH is reached. The moisture migration significantly affects the physical and chemical properties of the cheese; it can cause deterioration in texture, promote chemical deterioration reaction, and change molecular stability, thus limiting the shelf-life of fresh cheeses.

**Table 6** *Summary of the water activity of some common foods*

| $a_w$ | Typical foods |
|---|---|
| 1–0.95 | Fresh and canned fruits, meat, milk, breads, fish, cooked sausages |
| 0.95–0.9 | Cheese, cured meat |
| 0.95–0.85 | Margarine, fermented sausage, sponge cakes |
| 0.85–0.8 | Salted meats, syrup, flour |
| 0.8–0.75 | Jam, glace fruits |
| 0.75–0.65 | Nuts, jelly, molasses |
| 0.65–0.6 | Honey, caramel, toffee |
| 0.5 | Pasta |
| 0.3 | Cookies, crackers |
| 0.2 | Dried vegetable |

***Source:*** *Kilcast and Subramaniam (2011)*

*Light*

Many of the deteriorative changes in the quality of foods are initiated by light (Andersen and Skibsted, 2010). Fresh cheeses are exposed to daylight (or artificial light) at various points in the supply chain. Light accelerates the oxidation process and therefore the rate at which rancidity develops. Light induced oxidative processes occur when light from the sun and particularly from illumination on retail shelves passes through the packaging material and reaches the cheese surface, and when simultaneously there is $O_2$ in the headspace of the package (Mortensen *et al*., 2004).

The sensitivity of dairy products to light depends mainly on the presence of $O_2$ and the photosensitizing agent riboflavin (vitamin $B_2$). The latter is capable of absorbing energy and initiating an oxidative chain reaction that can lead to the development of flavour defects, the loss of nutrients such as vitamins and amino acids, and the discolouration of pigments (Alves *et al*., 2007).

However, transparent packaging materials are generally favoured by consumers.

Light is essentially an electromagnetic vibration (in visible spectrum between 400 and 700 nm). Each colour is represented by a specific wavelength: violet is in the area of 400 nm, blue and green are in the middle of the visible spectrum, and red is in the area of 700 nm.

The wavelength of UV spectrum light ranges between 200 and 400nm. The catalytic effects of light are most pronounced in the lower wavelength of the visible spectrum and in the UV spectrum. The intensity of light and the length of exposure are significant factors in the production of discolouration and flavour defects in packaged fresh foods.

The total amount of light absorbed by a packaged food can be calculated using the following formula (Fellows, 2009):

$$\mathbf{I_a = I_i\, T_p\, (1\text{-}R_f)/[(1\text{-}\ R_f)\ R_p]}$$

Where:

$I_a$ is the intensity of light adsorbed by the food

$I_i$ is the intensity of incident light

$T_p$ is the fractional transmission by the packaging material

$R_f$ is the fraction reflected by the food

$R_p$ is the fraction reflected by the packaging

Thus, two main factors influence the process: the amount of light and the amount of $O_2$ present in the package headspace. The first is obviously determined by the light source and by the barrier the packaging may impose. Incidental light is partially absorbed and partially reflected by the package, and only a portion of it is transmitted to the cheese.

The light transmitted through a given packaging material will have characteristics which are determined by the spectrum of the incidental light and the modification determined by the absorption characteristics of the packaging material (Mortensen *et al.*, 2004).

*Packaging*

The use of appropriate packaging is important in maintaining the quality of fresh cheese and achieving the required shelf-life. The principal function of packaging is to protect cheese from light, oxygen, temperature, moisture, and micro-organisms.

The protection offered by a package is determined by the nature of the packaging material and the format or type of package construction (REG. CE N.1935/2004 and REG. CE N.450/2009). A wide variety of materials is used in packaging and primary packaging materials consist of one or more of the following materials: paper and plastic polymers (Robertson, 2006, 2009; Yam, 2009). Typical permeability coefficients of food packaging are briefly described below (Table 7).

**Table 7** *Typical permeability coefficients of food packaging polymers and permeants at 25 °C*

| | p x 10 [11] [mL (STP) cm cm$^{-2}$ S$^{-1}$ (cm Hg)$^{-1}$] | | | | |
|---|---|---|---|---|---|
| **Polymer** | **$O_2$** | **$CO_2$** | **$N_2$** | **$SO_2$** | **$H_2O$** |
| Low density polyethylene | 15-30 | | | | 800 |
| Linear low density polyethylene | 31-36 | | | | |
| High density polyethylene | 6.0-12 | 45 | 3.3 | 57 | 180 |
| Ethylene vinyl acetate (12% VA) | 27-54 | 170 | | | |
| Polypropylene | 9.0-16 | 92 | 4.4 | 7 | 680 |
| Poly(vynyl chloride) | 0.3-1.2 | 1.2-3.0 | 0.0093 | 1.2 | 93 |
| Polystyrene (high impact) | 15-27 | 60-150 | 2.4-7.8 | 220 | 12-18.000 |
| Nylon 6 (0% RH) | 0.09-0.11 | 0.2-0.3 | 0.015-0.05 | 11 | 7.000 |
| Nylon MXD6 | 0.01 | | | | |
| Poly(ethylene terephthlate | 0.3-0.75 | 0.7-1.2 | 0.02-0.06 | | 1.300 |
| Polycarbonate | 10.0-15 | 47-66 | 1.7 | | |
| PVdC/PVC copolymer | 0.005-0.07 | 0.23-0.48 | 0.006-0.012 | | 14 |
| EVOH copolymer (0% RH) | | | | | |
| 27 mol% ethylene | 0.0018 | 0.024 | | | |
| 44 mol % ethylene | 0.0033 | 0.012 | 0.0005 | | |

***Source:*** *Kilcast and Subramaniam (2011)*

Typical packages are plastic trays of high density polyethylene (HDPE) or PP, which provide a good moisture barrier, and polystyrene (PS).

Sealed lids for integrity and snap-on outer lids are common. The use of high barrier materials such as polyamides (PA)/low density polyethylene (LDPE) laminates is essential (Roberson, 2009) to provide a higher barrier to $O_2$ and maintain a modified atmosphere with correct $CO_2$ level in the headspace to cause its dissolution into the product. Packaging also shields cheese from mechanical damage, shock and vibration encountered during distribution; additionally, it serves to communicate information to the consumer about the food rather like a marketing tool, and provides consumers with details of use and convenience.

Packaging under various atmospheres (vacuum, carbon dioxide, modified atmosphere packaging, etc.) and storage at low temperatures can have a major influence on the shelf-life.

The potential of modified-atmosphere packaging (MAP) and active packaging for extending the shelf-life of dairy products, including cheese, has been demonstrated (Floros *et al*., 2000).

The potential of MAP to extend the commercial shelf-life of cheese has been clearly demonstrated, although cheese packaging depends on the type of cheese, the starter used during manufacturing and storage condition, which are very important parameters (Gammariello *et al*., 2009).

The migration of components from packaging materials must also be considered.

The migration of monomers such as styrene from polystyrene (PS) packages (Bendall, 2007) and additives such as plasticizers from films (Goulas *et al*., 2000; Grob *et al.,* 2007) has been recorded in cheese packaged in plastic polymers.

Most of the migration studies are performed in food stimulants, because of standardization, analytical determinations, and compliance with legislation.

For a given migrant, the rate of migration and the amount of migrant transferred from the material into the food depend on the contact material and on the nature of the food, in this case cheese.

Polyolefins, and in particular polyethylene, are among those plastics which show the highest migration rates for most additives. Studies on the migration of diphenylbutadiene, triclosan, and butylated hydroxytoluene (BHT), from low density polyethylene (LDPE) film into cheeses containing different amounts of fat and water, showed that not only does the fat content influence the migration of lipophilic migrants, but also that the ratio of fat to water and the consistency of the cheese play an important role in the whole process (Cruz *et al*., 2008).

Moreover, antimicrobial and antioxidant substances and other different packaging materials such as polyamide, polypropylene, natural hydrogel (in place of brine) or bio-based coating (Del Nobile *et al.,* 2010) may be used to allow better preservation of fresh dairy products.

The use of biodegradable packaging materials could be a key to limiting both the risk of contamination, due to the passing of toxic monomers to the food, and also environmental pollution. According to Huang *et al.* (1990), the first generation of biodegradable materials consisted of blends of polymers with natural food sources such as starch.

The second generation concentrated on the insertion of functional groups such as ester linkages on the polymeric backbone. The third stage is the development of materials such as polylactic acid (PLA) that are naturally synthesized by bacteria which can be considered as truly biodegradable and eco-friendly following their composting (Bhatia *et al.*, 2007; Huang *et al.*, 1990). PLA is a thermoplastic aliphatic polyester derived from renewable resources such as corn starch, tapioca products (roots, chips or starch) or sugarcane. It has been studied studied for particular use in cheese packaging.

PLA with low barrier properties has been approved as a good packaging material for foods with relatively short shelf-lives (Dukalska *et al*., 2011).

By applying the metalizing process to the PLA film, the barrier properties can be optimised (Dimitrov *et al*., 2011).

In the research by Conte *et al.*, (2013) copper nano-particles were satisfactorily incorporated into the biodegradable polymer matrix.

PLA has negligible solubility in water and has a melting point of 170°C.

The migration of volatile compounds from PLA into the cheese was minute (Holm *et al.,* 2006) and well below any critical levels (Table 8).

**Table 8** *Volatile compounds exhibiting significant migration from poly-lactic acid packaging to oil or water when exposed for 12 days at 5°C (Holm et al., 2006)*

| | **Migration to water** | | | **Migration to oil** | | | **Maximal amounts allowed in drinking water (µg L$^{-1}$)** |
|---|---|---|---|---|---|---|---|
| | Sample µg L$^{-1}$ | Blank µg L$^{-1}$ | *p* | Sample µg L$^{-1}$ | Blank µg L$^{-1}$ | p[b] | |
| Ethyl acetate | 2 | 0.2 | 0.009 | 0.66 | 0.11 | 0.0012 | |
| 2-Butanone | 2.3 | 0.1 | 0.03 | 0.93 | 0.77 | 0.012 | |
| Benzene | 0.07 | 0.05 | 0.11 | 0.31 | 0.19 | 0.008 | 10 |
| Pentanal | 0.24 | 0.1 | 0.11 | _c | _ | _ | |
| Chloroform | 2 | 0.2 | 0.09 | 5.7 | 0 | 1.9E-05 | 200 |
| Toluene | 0.03 | 0.01 | 0.011 | 0.83 | 0.27 | 0.016 | 700 |
| Hexanal | 0.35 | 0.23 | 0.44 | _ | _ | _ | |
| 2-Methyl-1-propanol | _ | _ | _ | 1.8 | 0 | 0.0006 | |
| Styrene | 0.02 | 0 | 0.014 | 0.96 | 0.2 | 0.06 | 20 |

[a]WHO, 2004.
[b]*p*: *p*-values for the difference between sample and blank. *p*-values above 0.05 are not significant.
[c] Not possible to quantify.

Other attributes such as flexural properties, heat distortion temperature (HDT), gas permeability, impact strength, and viscosity for processing are considered extraneous for packaging (Ogata *et al.,* 1997).

Additionally, the high price and brittleness of PLA reduces interest in its commercialisation. Therefore, mixing PLA with other suitable biodegradable polymer (such as polyDL-lactic acid, polyethylene oxide -PEO; polyvinyl acetate-PVA; polyethylene glycol-PEG; PBS,trade name ‘BIONOLLE’, biodegradable aliphatic polyester produced by the polycondensation reaction of 1, 4-butanediol with succinic acid) which has comparably better properties, will improve its characteristics and also contribute towards low overall material costs (Bhatia *et al.,* 2007).

## 2.4 Keeping quality of MC and shelf-life determination

Lacto-fermented mozzarella cheese is a complex food. This complexity is due to a microbial community, which exerts synergistic actions (producing useful metabolites, antimicrobial substances, etc.), but also antagonistic actions (group of micro-organisms can compete for nutrients, develop undesirable odours, flavours and colours), which are a major cause of deterioration over time of the cheese and cannot be completely stopped.

Mozzarella cheese spoilage is determined to microbiological, physicochemical, chemical and biochemical changes: microbiological changes caused by a great variety of bacteria, moulds and yeasts; physicochemical changes such as creaming of fat, gelation of protein solution, syneresis of curds and crystallization of minerals; chemical reactions such as non-enzymatic browning and oxidation of fat; biochemical transformations such as growth of micro-organisms, enzymatic degradation, ripening of cheese and fermentation (Kilcast and Subramaniam, 2011).

The relationship between microbiological growth and the bio-chemical changes occurring during mozzarella cheese storage has been recognised as a potential indicator which may be useful for monitoring freshness and safety. Mozzarella cheese is considered spoiled when it is no longer acceptable to the consumer and when it becomes a food safety issue, threatening health.

Generally, to evaluate the quality of cheeses, food scientists use microbiological tests and objective chemical or physical analytical methods, which must ultimately be correlated with organoleptic data to establish their reliability as a quality index.

The shelf-life of most fresh cheese has only been evaluated by observing the time taken to arrive at undesirable sensory or microbiological changes.

In particular, many tests and assays have been developed to estimate the quality and potential shelf-life of dairy products:

*Sensory evaluation* infers measurement, analysis and interpretation of characteristics of food as they are perceived by the senses of sight, smell, taste, touch. It is the most comprehensive way of assessing the quality of food (O'Mahony, 1979). Traditional sensory methods are:

-*texture evaluation and grading by 'expert' tasters,* which assign quality scores on the appearance, flavour and texture of the products based on the presence or absence of predetermined defects. The shortcomings of this method include the inability to predict consumer acceptance and the lack of objectivity in quality assessment. Two products with different relative intensities of sensory characteristics may receive similar quality scores based on defects detected by the 'experts' (Claassen and Lawless, 1992).

-*Descriptive analysis* is commonly used to deal with the total profile of food products. It requires at least three evaluative processes: discrimination of the trait; description of the trait and quantitation of the trait. External standards are commonly used to define the attributes and standardize the scale for each assessor. Developing and refining a vocabulary is an essential part of sensory profile work. Panel training is then performed to increase panelist sensitivity and memory and to help panelists to make valid, reliable judgments which are independent of personal preferences.

Sample testing is usually carried out in replicated (commonly three) sessions, using experimental designs that can minimize any bias. Descriptive analysis results are subjected to univariate statistics (e.g. multi-way analysis of variance) or multivariate statistics (e.g. principal component analysis) (Hugi and Voirol, 2010, Borgognone *et al.*, 2001).

These limitations make instrumental methods important in evaluating food quality changes during storage (Stone and Sidel, 2004). Compared with sensory analysis, instrumental methods usually have improved accuracy and reproducibility (Gordon, 2004).

-*Coupling sensory analysis with chemical and physical measurements* can provide more insights than using either techniques alone. A reliable instrumental technique should be well correlated with relevant sensory attributes.

*Chemical measurements*

A chemical analysis is used to measure the final points of chemical reactions occurring in MC during storage, or to confirm the results obtained by the sensory panels (De Angelis *et al.,* 2008; Del Nobile *et al.,* 2009; Lucera *et al.,* 2014, Ricciardi *et al.,* 2015). The level of rancidity in lipids is often measured using the peroxide value and the free fatty acid content (Singh and Anderson, 2004). A recent development in detecting odours and aromas is the electronic nose, based on the gas chromatography (GC) volatile methods. It is capable of determining the odour intensity of mixtures of a variety of volatile oil degradation compounds, due to its special detection system which consists of an array of gas sensors (mainly semiconductors).

It may function as a rapid and not destructive tool for on-line flavour characterization, especially for rancidity analysis for foods during storage.

*Physical measurements:*

-*Colour* is the first sensory attribute of most foods that customers can appraise. It often degrades during storage as a result of enzymatic and non-enzymatic reactions, oxidation, and physical reactions. Colour is commonly measured using a tristimulus colourimeter or a reflectance spectrophotometer. The colour data can be obtained in terms of tristimulus values, chromaticity coordinates, hue, and chroma (Clydesdale, 1998). Research shows good correlation between colour and food quality. For mozzarella cheese the colour (Andreani *et al*., 2014), and sensory properties have also been studied (De Angelis *et al*., 2008; Del Nobile *et al*., 2009; Lucera *et al*., 2014; Kamleh *et al*., 2012, Ricciardi *et al*., 2015).

-*Methods of measuring moisture content* fall into two categories: direct measurements and indirect measurements. The direct measurements, including the oven-drying method, mostly involves weighing the sample before and after removing the water. The indirect methods measure a property of the food that is itself related to moisture content, for example, the electrical resistance and the dielectric constant of a sample. Water activity values are often obtained by either a capacitance or a dew point hygrometer (Mathlouthi, 2001).

The differences in water activity resulted mainly from different salt-to-moisture ratios in mozzarella samples (Imm *et al.*, 2003)

-*Texture* is one of the most commonly used physical indicators of food quality. The texture of a food is often defined by the stress/strain or force/deformation relationship obtained when food is subjected to an instrumental determination.

Most of the instrumental texture measurements involve mechanical tests which quantify the resistance of the food to applied forces, from which quality attributes such as hardness, crispness, and cohesiveness are derived.

A large number of instruments are available for testing food texture, and the most popular ones include the Instron universal testing machine (Yuan and Chang, 2007), and texture technologies TA.XT2 Universal texture Analyzer (Kong *et al*., 2007).

More sophisticated methods are also available, such as the acoustic method (not destructive test), X-ray diffraction, microscopy, and dilatometry to study crystalline structure and glass transition (Farhat, 2004), or nuclear magnetic resonance spectroscopy to monitor the molecular mobility.

Texture degradation occurs due to moisture migration, enzymatic hydrolysis, and other physical or chemical deterioration.

*Mathematical models*

An indirect measurement of spoilage and shelf-life, may be quantitatively predicted using mathematical models (Fu and Labuza,1993).

Mathematical modelling of quality deterioration is commonly conduced to describe the fate of quality indicators as a function of intrinsic and extrinsic factors in the fresh cheese chain.

Examples of such indicators could be colour, presence or absence of certain flavour compounds, presence or absence of certain microorganisms, texture, vitamins, protein composition, and so on (Van Boekel, 2008). Building mathematical models with which we can simulate such quality indicators requires knowledge of food science and nutrition, as well as of modelling:

- *modelling chemical reactions* (zero-order reaction, first-order reactions and second-order reactions);
- *modelling temperature dependence of chemical reactions* (Arrhenius equation);
- *modelling enzymatic reactions* (Michaelis–Menten equation);
- *modelling physical reactions* (equation derived by Einstein (1906) for dilute dispersions and Eilers equation; Anema *et al*. (2004) model for skimmed milk samples, Manski *et al.*, (2007) model to predict the rheological properties, etc.);
- *modelling microbial changes* (the modified Gompertz model; Bigelow model, etc.) used in several researches (e.g. Altieri *et al*., 2005; Baruzzi *et al*., 2012; Conte *et al*., 2007; Del Nobile *et al*., 2009; Gammariello *et al*., 2008; Losito *et al*., 2014; Sinigaglia *et al*., 2008).

### 2.4.1 Shelf -life measurements by microbial models

The entire concept of the shelf-life of mozzarella cheese is based on the well documented supposition that the primary cause of shelf-life deterioration is due to the growth of gram-negative psychrotrophic bacteria. Most of these bacteria are members of the genus Pseudomonas. Consequently, most of the tests which have been designed to predict or estimate the shelf-life of fresh dairy products are geared to measure this genus.

Microbiological methods have been almost exclusively used in the actual evaluation of spoilage (European Commission, 2005).

Initially for the enumeration of microbial populations, the actual number of colonies grown on a Petri dish has played a critical role in the evaluation of food spoilage.

This evaluation has been based on the existing knowledge of the microbial process which contributes to spoilage in genus (specific spoilage) or species (ephemeral spoilage) level.

Several predictive models for spoilage microorganism and food-borne pathogens are available on the internet.

Some useful sites include the following.

The Food Spoilage Predictor (FSP), which includes the Pseudomonas model of Neumeyer *et al*. (1997), can be found at http://www.hdl.com.au/html.body_fsp.htm.

The basic free web-based database of food microbiology data is ComBase: www.combase.cc.

Many EU-funded projects deal with predictive modelling:

-SYMBIOSIS-EU (www.symbiosis-eu.net)

-SMAS (http://smas.chemeng.ntua.gr/start.php?module=overview)

-FAIR CT98-4083 project (www.londonmet.ac.uk).

Predictive, or alternatively quantitative, food microbiology (McMeekin *et al.,* 1993) involves knowledge of microbial growth responses to environmental factors expressed in quantitative terms through mathematical equations (models).

Data and models can be stored in databases and used to interpret the effect of processing, distribution and storage conditions on microbial growth (McMeekin *et al.*, 1993).

This approach offers precision in estimating the shelf-life of foods. In addition, the combination of data on the environmental history of the product with mathematical models may lead to 'intelligent' product management systems for the optimisation of food quality and safety at the time of consumption (Koutsoumanis *et al.,* 2002, 2003; Giannakourou *et al.*, 2001).

Most developed models have focused on the effect of environmental factors on the maximum specific growth rate of a microorganism without taking into account the lag phase.

Koutsoumanis (2001) demonstrated that lag phase duration can be a significant part of the total shelf-life of foods. Ignoring the lag phase may lead to an underestimation of the shelf-life, with significant economic losses for the food industry.

The development of most models is based on observations in a well-controlled laboratory environment, using microbiological media. Examples of tests are (Bishope and White, 1986):

1. Use of preincubation to build up relatively large populations, which are easier to enumerate;
2. Use of selective media that permit growth of contaminants but inhibit growth of other bacteria;
3. Testing for certain final products of microbial metabolism or changes in the substrate;
4. Applying the sample on the surface of agar media to accelerate the growth of aerobes.

## 2.5 Extension of shelf-life of mozzarella cheese

According to research (Jalilzadeh *et al.,* 2015), the principle methods for mozzarella cheese shelf-life extension are shown in Table 9:

**Table 9** ***References relating to shelf-life extension of mozzarella cheese***

| Methods | Results | Author |
|---|---|---|
| Addition of chitosan | Inhibited the growth of some spoilage microorganisms such as coliforms. | Altieri *et al*., 2005 |
| Use of lysozyme and Na2-EDTA | Control of listeria monocytogenes. | Sinigaglia *et al*., 2008 |
| Ozone | Reduction microbial mozzarella cheese contamination in water treated with ozone. | Segat *et al*., 2014 |
| Active compounds (potassium sorbate PS, sodium benzoate, calcium lactate and calcium ascorbate) dispersed in a sodium alginic acid solution | The coating with PS (3%) showed a certain inhibition on microbial proliferation and samples remained acceptable for 8 days with respect to the control that was refused after about 4 days. | Lucera *et al*., 2014 |
| Gas mixture of equal parts of $CO_2$ and $N_2$ and vacuum packaging effect | Storage methods are appropriate to preserve Mozzarella cheese during the studied storage period. | Olivares *et al*., 2012 |
| Gas-barrier bags under three different atmospheres (100% $N_2$, 100% $CO_2$, and 50% $CO_2$/50% $N_2$) | The mozzarella cheese did not show any physical or chemical alteration in all packaging treatments. | Alves *et al*., 1996 |
| Modified atmospheres (air, vacuum and mixtures carbon dioxide/nitrogen) | Levels of 75% CO2 were optimal to repress undesirable organisms and reduce gas formation. | Eliot, *et al*.,, 1998 |
| Sodium alginate coating containing Lactobacillus reuteri | A strategic solution to prolong the shelf life | Angiolillo *et al*., 2014 |
| PLA–copper films | Antimicrobial actions | Conte *et al*., 2013 |
| Chitosan, sodium alginate, and soy protein isolate | Preservation effect | Zhong *et al*., 2014 |
| Antimicrobial packaging system | An increase in the microbial shelf-life of the packaged Mozzarella cheese | Gorrasi *et al*., 2016 |
| Antimicrobial active compounds (chitosan, lemon and sage extract) | Addition of lemon extract and chitosan increased cheese shelf life. In particular, the highest increase (129%) was recorded by adding 1000 mg⁄kg of lemon | Gammariello *et al*., 2010 |

| | | |
|---|---|---|
| Bio-based coating containing silver-montmorillonite nanoparticles combined with modified-atmosphere packaging (MAP) | The developed nanocomposite system allowed a significant prolongation of shelf-life to more than 5 days | Gammariello *et al*., 2011 |
| Effect of calcium lactate (CL) | A modest inhibition effect of CL on the microbial proliferation and substitution of calcium chloride by CL improved the acceptability of the product, thus extended the shelf-life | Faccia *et al*., 2013 |
| The addition of chitosan into cheese making, combined with either coating or active coating (lysozyme and ethylenediamine tetraacetic acid, disodium salt) and MAP (modified atmosphere packaging) | Fior di latte" cheese preservation by increasing the shelf-life | Del Nobile *et al*., 2009 |
| Active coating (sodium alginate, Lysozyme, EDTA, disodium Salt) and MAP | Fior di Latte cheese preservation, increasing the shelf-life to more than 3 days. | Conte *et al*., 2009 |
| Lemon extract, at 3 different concentrations in combination with brine and with a gel solution made of sodium alginate | A double advantage: both preserving the product quality and reducing the cost of its distribution, due to the lower weight of the package. | Conte *et al*., 2007 |
| Innovative mild approach | For counteracting blue Mozzarella event | Caputo *et al*., 2015 |
| Silver nanoparticles (Ag-NP) incorporated into a bio based coating in combination with MAP (50% $CO_2$, 50% $N_2$) | A valid preservation strategy to boost the diffusion of this dairy product beyond the local market. | Mastromatteo *et al*., 2015 |
| Sustainable Process Operations | A new liquid formulation containing antimicrobials, that extends the shelf-life of mozzarella, was tested as governing liquid to contrast the product degradation due to the genus Pseudomonas.<br>Life Cycle Assessment and Life Cycle Costing methodologies were applied, taking also into account the potential reduction of food loss. | Falcone *et al*., 2017 |

# Chapter 3

# Materials and Methods

## 3.1. Lacto-fermented Mozzarella processing

High moisture lacto-fermented mozzarella cheese (125 g of weight, moisture >55%) was used beginning with cow milk by commercial starter, according to the flow chart shown in Figure 9:

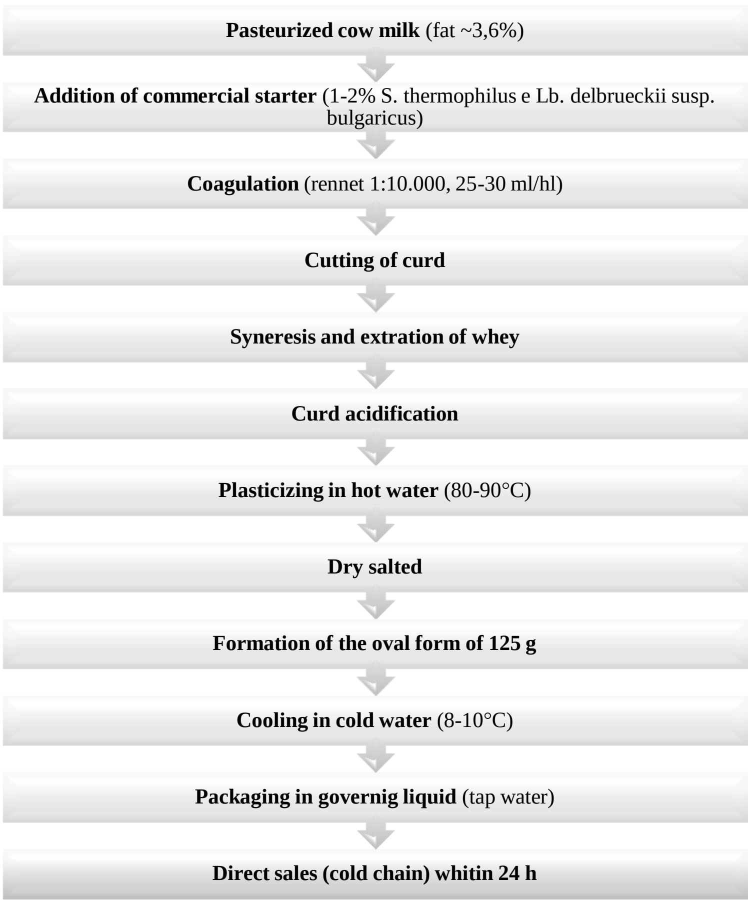



**Figure 9** Flow chart of lacto-fermented mozzarella

Pasteurized cow's milk was acidified with lactic acid cultures and liquid rennet was added. When the curd pH reached a value of about 5.80, the whey was removed; the curd was cut and stretched. After cooling in cold water, the mozzarella cheese samples were directly packaged in trays containing tap water or other governing liquids, as reported in paragraph 3.2, immediately transported to the laboratory and stored at 5°C.

The productive process following the flow chart of production of mozzarella cheese it was manufactured by “Delizie della Natura” (Figure 10), a cheese factory located in Reggio Calabria (Southern Italy).


**Figure 10** “Delizie della Natura” cheese factory

### 3.2 Lacto-fermented Mozzarella storage

The objective of PhD thesis is related to the shelf life extension of the lacto-fermented mozzarella by using different approaches such as changing of governing liquids and use of bio-based materials. Samples of lacto-fermented mozzarella cheese (125 g) were stored in tap water or stretching water added to sodium chloride (NaCl) and/or calcium lactate (CL), also in consociation with concentrated bergamot juice (BJ), a natural source of antimicrobial compounds.

Table 10 shows several experimental trials tested to extend the shelf-life of cow milk mozzarella.

**Table 9** *Experimental trials relating at the use of different governing liquid composition*

| PRODUCTION LOT | CODE SAMPLES | GOVERNING LIQUID COMPOSITION | PACKAGING MATERIAL |
|---|---|---|---|
| M-SALTS | 0.2% CL | 0.2 % calcium lactate in tap water | Polypropylene (PP) |
| | 0.6% CL | 0.6 % calcium lactate in tap water | Polypropylene (PP) |
| | 0.2% NaCl | 0.2 % sodium chloride in tap water | Polypropylene (PP) |
| | 0.6% NaCl | 0.6 % sodium chloride in tap water | Polypropylene (PP) |
| | 0.4% NaCl + 0.2% CL | 0.4 % sodium chloride and 0.2 % calcium lactate in tap water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |
| M-BJ | 0.10 % BJ | 0.10 % bergamot juice in tap water | Polypropylene (PP) |
| | 0.2 % CL+BJ | 0.2 % calcium lactate and 0.05% bergamot juice in tap water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |
| M-PLA | 0.6 % CL PLA | 0.6 % calcium lactate in tap water | Polylactide (PLA) |
| | 0.6 % CL+ BJ PLA | 0.6 % calcium lactate and 0.05 % bergamot juice in tap water | Polylactide (PLA) |
| | Control PLA | tap water in PLA trays | Polylactide (PLA) |
| | Control PP | tap water in PP trays | Polypropylene (PP) |
| M-SW | 0.6 % CL | calcium lactate at 0.6 % in tap water | Polypropylene (PP) |
| | 0.6 % CL+ BJ | calcium lactate at 0.6 % and 0.05 % bergamot juice in tap water | Polypropylene (PP) |
| | SW 0.6 % CL | calcium lactate at 0.6% in stretching water | Polypropylene (PP) |
| | SW 0.6 % CL+ BJ | calcium lactate at 0.6% and 0.05 % bergamot juice in stretching water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |

In particular in Table 10 the following acronyms of the experimental procedures are reported:

- M-SALTS is referred to samples of 125 g of lacto-fermented mozzarella cheeses packaged in PP trays, stored at 5 °C in different governing liquid based on salts, such as sodium chloride and calcium lactate and analysed at day 0, 6, 12 and 18;
- M-BJ is referred to samples of 125 g of lacto-fermented mozzarella cheese packaged in PP trays, stored at 5 °C in different bergamot juice concentrations and analysed at day 0, 5, 7, 13 and 20;
- M-PLA is referred to samples of 125 g of lacto-fermented mozzarella cheese packaged in PLA trays, stored at 5 °C in different bergamot juice and calcium lactate concentrations and analysed at day 0, 2, 7, 9, 14 and 19;
- M-SW is referred to samples of 125 g of lacto-fermented mozzarella cheese packaged in PP trays, stored at 5° C in different governing liquid using as solvent (water or stretching water) and analysed at day 0, 1, 6, 14 and 21.

**Calcium lactate** pentahydrate (98%), a white crystalline salt and a food additive (Figure 11), identified with the code **E327**, was supplied by Carlo Erba Reagents S.r.l., Italy.

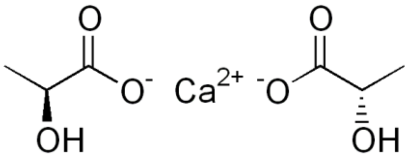



**Figure 11** Calcium lactate chemical formula

**Sodium chloride** also known as salt, an ionic compound with the chemical formula NaCl, was supplied by Carlo Erba Reagents S.r.l., Italy.

**Concentrated Bergamot juice** (Figure 12) was obtained from Nava Domenico factory, located in Gallico Superiore (Reggio Calabria, Italy). BJ was collected, transported in containers that were certified microbiologically safe, and stored at 4° C in dark conditions for 24 h before their use and analysis. Acidity, total sugars, volatile compounds of concentrated BJ were reported in the findings (Moufida and Marzouk, 2003).

**Figure 12** Concentrated Bergamot Juice (BJ) used

**Packaging materials**

Relating to trays TB 750 CC (143 x 174 x 60 mm) in Polylactic acid by Coopbox Group Spa possessed an OTR of <50 $cm^3/m^2$ x day x bar (at 23 °C, RH 85%) and $CO_2$TR of < 200 $cm^3/m^2$x day x bar (at 23 °C, RH 85%). PLA was obtained from annually renewable sources. It is a lactic acid polymer, a natural product found in different sources such as milk and its by-products, or plant processing waste (*http://www.coopbox.it*).

Poly Propylene (PP) trays (Figure 13) were provided by "Delizie della Natura" factory, also responsible for the packaging of the Lactofermented Mozzarella samples (Figure 14).

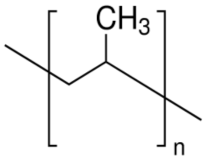



**Figure 13** Polypropilene chemical formula

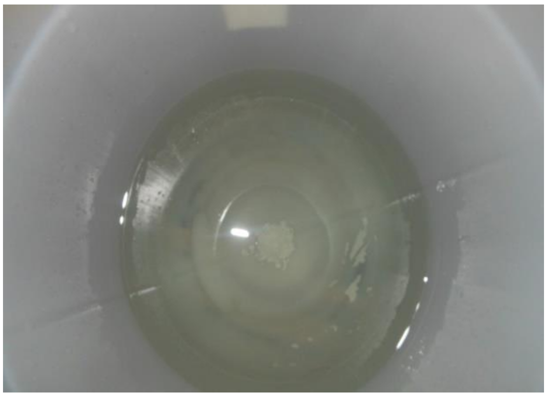


**Figure 14** Calcium lactate dissolution (0.6 % w/v) in tap water

## 3.3 Microbiological analyses of mozzarella cheeses

Two mozzarella cheese samples from each tray (Figure 15 a, b) at each storing time were analysed. Approximately ten grams of mozzarella cheese, for each sample, were diluted with a sterile Ringer's solution in a stomacher bag filter and homogenised with a Bag Mixer (Interscience, France) for 3 min. Decimal dilutions ranging from $10^{-1}$ to $10^{-7}$ were prepared and plated on Petri dishes.

Subsequently, mesophiles counts or total bacterial count (TBC), total lactic acid bacteria and Pseudomonas *spp.* count were determined. Moreover, TBC and Lactic acid bacteria (LAB) were enumerated on Plant Count Agar (PCA-Oxoid), after incubation at 26 °C for 48 h and on MRS Agar (Oxoid), after an anaerobic incubation at 32°C for 48 h, respectively. Instead for the Pseudomonas spp. count the dilutions of 0.1 mL were plated adding Pseudomonas Agar Base with

CFC supplement at 25 °C for 48 h. After incubation, colonies were counted and the results were expressed as $Log_{10}$ cfu/g of cheese. All samples were analysed in triplicate.

a


b

**Figure 15** Lacto-fermented Mozzarella samples packaged in PP (a) and PLA (b) ready to the microbiological analyse

### 3.3.1 Microbiological analyses of governing liquid

Decimal dilutions of each governing liquid were taken, and TBC, LAB and Pseudomonas spp. counts were determined in Petri dishes (Figure 16). Moreover, TBC and Lactic acid bacteria (LAB) were enumerated on Plant Count Agar (PCA-Oxoid), after incubation at 26 °C for 48 h and on MRS Agar (Oxoid), after an anaerobic incubation at 32°C for 48 h, respectively.

Instead for Pseudomonas spp. Pseudomonas Agar Base was added with CFC supplement at 25 °C for 48 h. After the incubation, colonies were counted and the results were expressed as $Log_{10}$ cfu/g of governing liquid. Each governing liquid sample was evaluated 3 times.

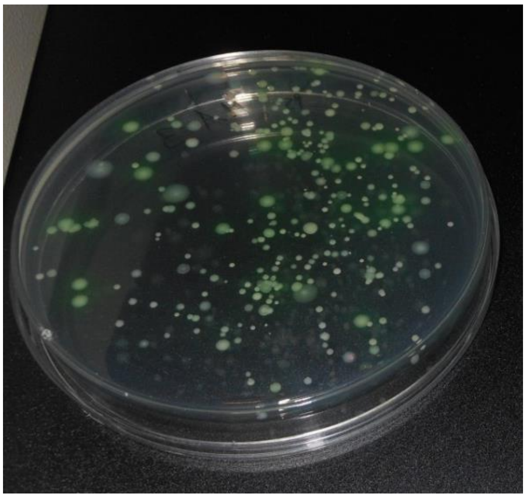


**Figure 16** Growth of Pseudomonas *spp.* in Petri dish

### 3.4 Physicochemical analyses

Physicochemical analyses were conducted both on the governing liquid and on the water extract obtained from two mozzarella cheese samples, containing each tray at each storage time.

#### 3.4.1 Water extract preparation

The water extract was obtained by homogenizing 10 grams for each mozzarella cheese with 40 mL of deionized water, using Ultra-Turrax T 25 basic (at 11.000 rpm for 1 min). The homogenate was centrifuged with centrifuge (manufacturer ALC; model 4236-V1D) at 5000 rpm for 5 min, and the supernatant was filtered through filter paper. The filtrate (Figure 17) was the water-soluble extract of the cheese. It was collected in a calibrated flask (50 mL) and brought to volume with deionized water.

**Figure 17** Filtered water extract

### 3.4.2 pH determination

After calibrating with pH 4.0 and 7.0 standard buffers, for pH measurement, the water extract (40 mL undiluted) and the governing liquid (40 mL undiluted) were measured using a Crison instrument (Crison BasiC 20, Barcelona, Spain).

### 3.4.3 Total acidity

Total acidity was determined according to the AOAC method (1980a) expressed as g/100 ml of lactic acid.

Governing liquid (10 mL) and water extract (10 mL) were transferred in conical flask and added to 50 mL of deionized water and 3-4 drops of phenolphthalein indicator (1%). The content was titled with 0.1 NaOH solution until a constant change (for 10 to 15 seconds) to a pink colour occurred.

The total acidity was calculated using the following formula expressing grams as a percentage of lactic acid:

$$\textbf{\% Lactic acid} = (V*M*d*PE*100/1000)/w$$

Where:

V = the number, in milliliters (mL), of sodium hydroxide solution used during titration; M = the molar concentration of sodium hydroxide solution in theoretical definition of acidity, mol/L (0.1); d = sample dilution; EW = Equivalent Weight (90 g/mol); w = the weight (grams) of the test sample (sample test)

### 3.4.4 Chlorides analyses

Chlorides were determined (Figure 18) by the Mohr method according to the AOAC method (1980b) and expressed as NaCl %. Chloride ions are present as a different form of salts, which are extremely soluble in water. The most common salts are NaCl, KCl, $MgCl_2$ and $CaCl_2$. Water sample (1mL) was titrated against standard silver nitrate solution ($AgNO_3$) using potassium chromate ($K_2CrO_4$) as indicator.

$$\textbf{\% Chloride = (VSN} \times \textbf{ NSN } \times \textbf{5.845)}$$

Where:

VSN = volume of titrant used

NSN = normality of silver nitrate

Vw = volume of water sample used (mL)

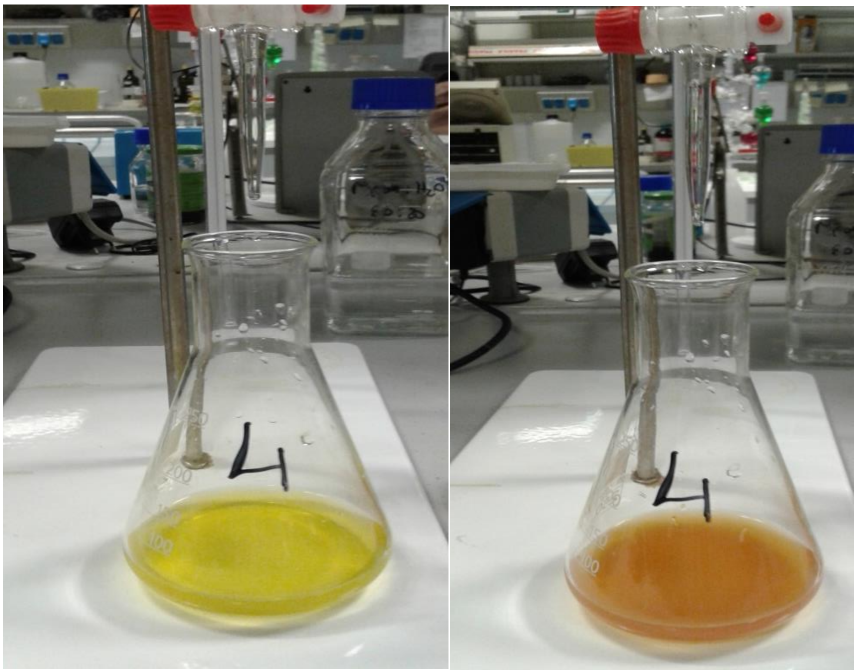


**Figure 18** Chlorides determination using silver nitrate solution. Colour changing of solution before (yellow colour) and after (orange colour) the use of potassium chromate

### 3.4.5 Moisture

The percentage of moisture content was determined by measuring a known mass of mozzarella cheese samples (about 5 grams, Figure 19) placed in evaporating dishes (silica, porcelain or equivalent), before and after drying. The weighed samples were placed in an oven at a constant temperature of 105°C ± 5 °C until constant weight.

**Figure 19** Dried sample of lacto-fermented mozzarella cheese

This is the formula to determine the % of moisture:

$$\textbf{\%Moisture} = [(M_{INITIAL} - M_{DRIED}) / M_{INITIAL}] \times 100$$

Where, $M_{\mathrm{INITIAL}}$ and $M_{\mathrm{DRIED}}$ are the mass of the sample before and after drying, respectively. The basic principle of this technique is that water has a lower boiling point than the other major components within foods, *e.g.,* lipids, proteins, carbohydrates and minerals. Sometimes a related parameter, known as the *total solids,* is reported as a measure of the moisture content (% Total solids = 100 - % Moisture or ($M_{\mathrm{DRIED}}$ / $M_{\mathrm{INITIAL}}$) x 100). The total solids content is a measure of the amount of material remaining after all the water has evaporated:

$$\textbf{\% Total Solids} = (M_{DRIED} / M_{INITIAL}) \times 100$$

### 3.4.6 Water activity ($a_w$)

The water activity was measured by a LabMaster-$a_w$ (Novasina) instrument (Figure 20).


**Figure 20** LabMaster-$a_w$ (Novasina) instrument during the analyses

The water activity ($a_w$) represents the ratio of the water vapour pressure of the mozzarella cheese to the water vapour pressure of pure water under the same conditions and it is expressed as a fraction. It ranges from 0.0 $a_w$ to 1.0 $a_w$ (pure water).

$$\mathbf{a_w = p/p_o = ERH\ (\%) / 100}$$

As described by the above equation, the multiplication of water activity by 100 gives the equilibrium relative humidity (ERH) as a percentage.

### 3.4.7 Colour determination

The colour determination (figure 21) both on inner and outer layers of mozzarella cheese and on governing liquid was performed by a Konica Minolta model CM-700d tristimulus colorimeter (Japan) basing on the CieLab (L*, a*, b*) system (figure 22).
In the CieLab colour space L* axis represents lightness (L* = 0 yields black and L* = 100 indicates diffuse white), a* axis is the position between red/magenta and green (negative values indicate green and positive values indicate magenta), and b* axis is the position between yellow and blue (negative values indicate blue and positive values indicate yellow).

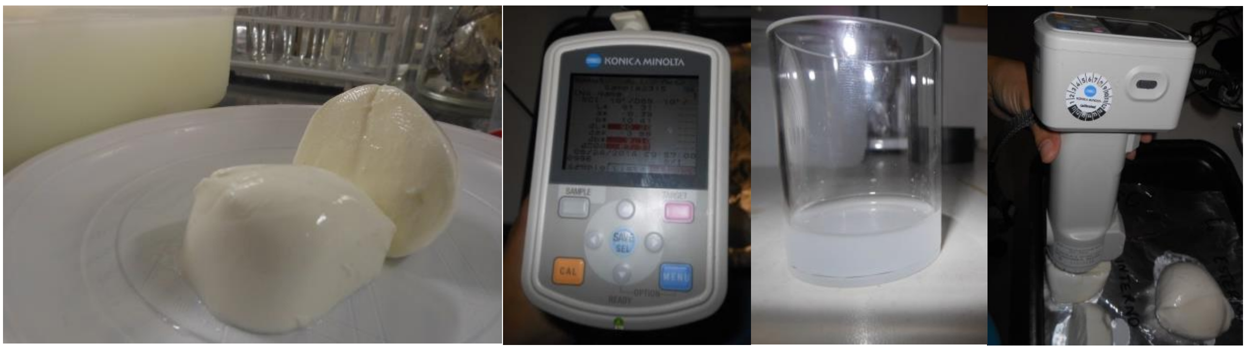



**Figure 21** Colour determination both on inner and outer layers of mozzarella cheese and on governing liquid by Minolta CR-300

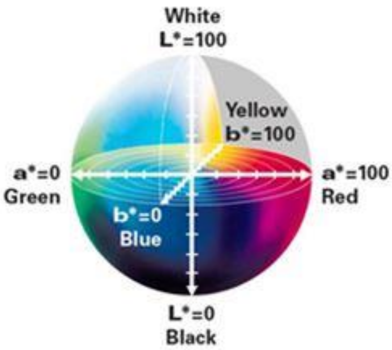



**Figure 22** CIE-LAB colour space

### 3.4.8 Rheological Analyses

The textural properties of mozzarella cheese samples were determined by performing the texture profile analysis (TPA) using a TA-XT Plus Texture Analyzer (Stable Micro Systems, Godalming, Surrey, UK, figure 23) at room temperature (22 °C).

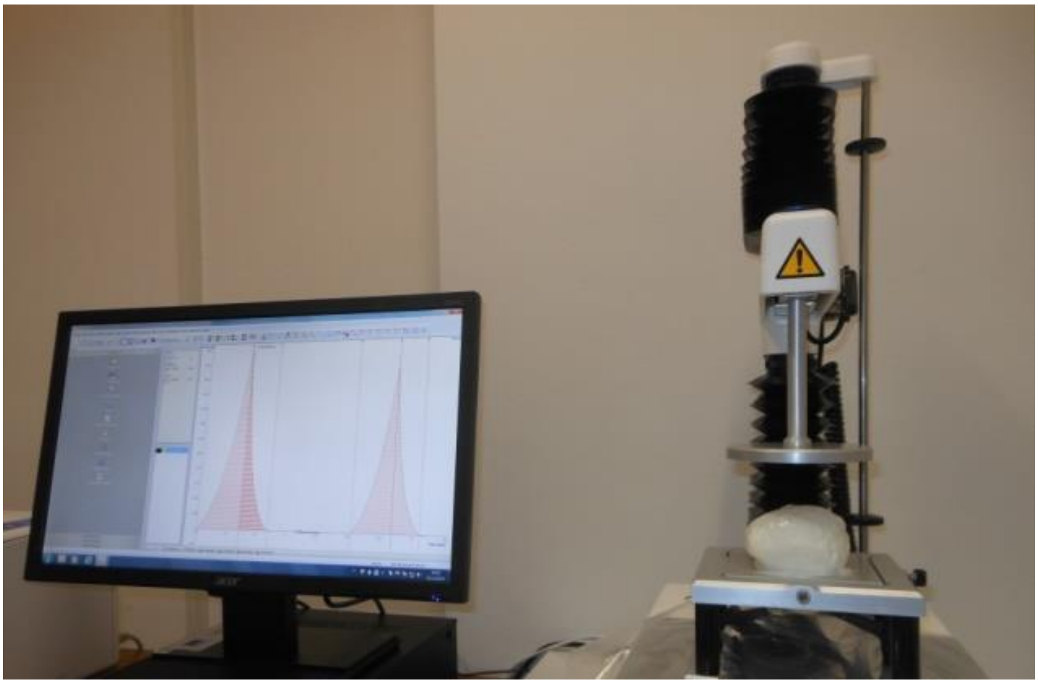


**Figure 23** TA-XT Plus Texture Analyzer

The compression test was performed in two successive cycles and hardness, cohesiveness, springiness, gumminess, adhesiveness, chewiness and resilience parameters were calculated as shown in the research (Pons and Fiszman 1996; Bourne, 1978).

The operating conditions of the texture analyser used to measure cheese samples texture are given in Table 11 and figure 24.

**Table 10** *TA settings parameters*

| Test-Mode and Option | Value/Units |
|---|---|
| Test speed | 5 mm $sec^{-1}$ |
| Trigger type | Auto |
| Target Mode: | |
| Distance | 18 mm |
| Time | 5 sec |
| Force | Grams (g) |
| Compression probe (platens) | 100 mm Ø (P/100 aluminium) |

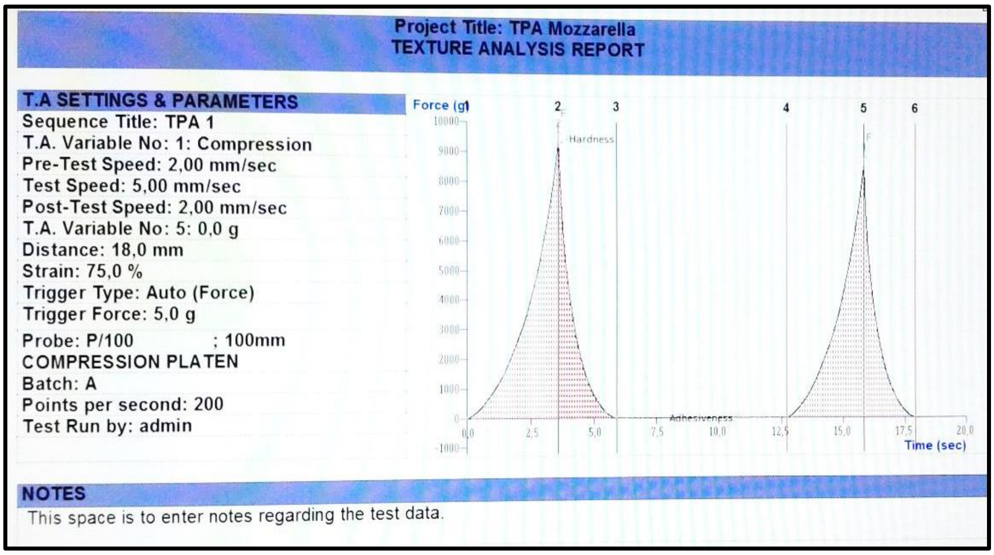



**Figure 24** TA settings parameters

In Figure 25 Hardness is “the peak force during the first compression cycle” (“first bite”).
Cohesiveness is represented by “the ratio of the positive force area during the second compression portion to that during the first compression (Area 2l Area l), excluding the areas under the decompression portion in each cycle.”
Adhesiveness is “the negative force area for the first bite, representing the work necessary to pull the plunger away from the food sample.”
Springiness (originally called elasticity) is “the height that the food recovers during the time that elapses between the end of the first bite and the start of the second bite.”

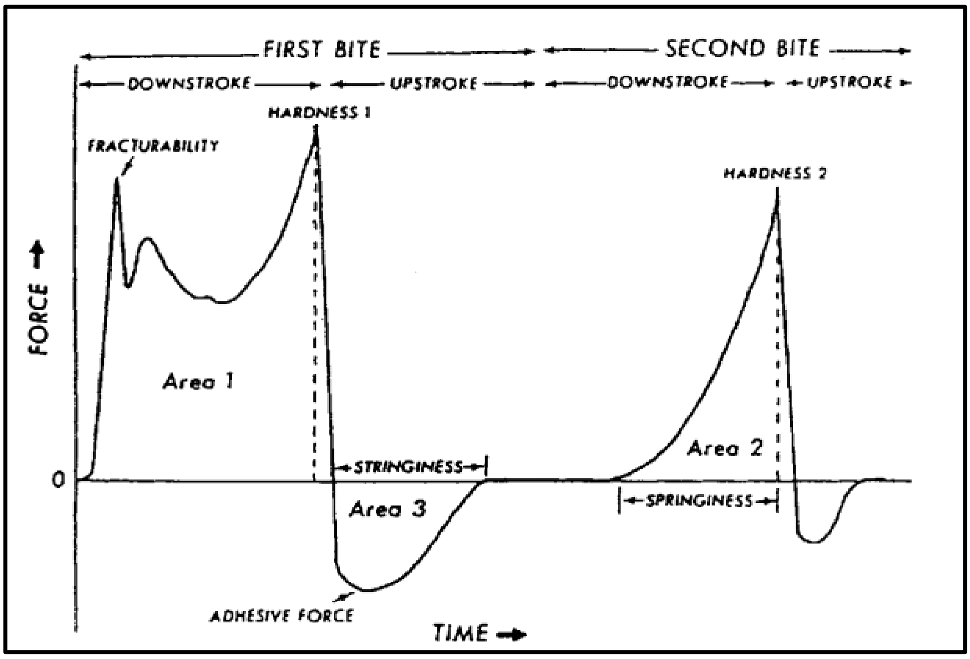



**Figure 25** A generalized TPA curve from the Instron universal Testing machine (Bourne 1978)

Gumminess is defined as “the product of hardness x cohesiveness”.
Chewiness is defined as “the product of gumminess x springiness”.
Resilience, defined as “a measure of how well a product fights to regain its original position” is a parameter similar to elasticity.

### 3.4.9 Proteolysis assessment

The mozzarella cheese samples subjected to two different storage periods (initial and final storage) were performed by means of the Kjeldahl method (IDF, 1993, figure 26) with Foss equipment (Tecator™ and Kjeltec™ systems, Figure 27) to determine nitrogen.
For total nitrogen (TN), about 0.5 grams of mozzarella cheese was mixed with concentrated sulfuric acid and Kjeltabs catalyst (SIGMA-ALDRICH Kjeldahl Catalyst according to Wieninger) and analysis was carried out by means of the Kjeldahl method.
Nitrogen values were converted to protein equivalents using the 6.38 conversion factor (Nitrogen x 6.38). The nitrogen conversion factors given in the table below (table 12) are taken from *Food and Agriculture Organization (1970) Amino acid content of foods and biological data on proteins, Rome (FAO Nutritional Studies, N° 24).*

**Table 11** *The nitrogen conversion factors*

| Foodstuff | Conversion factor for protein content as reported in food composition tables | Correction factor for conversion of reported protein to “crude protein” |
|---|---|---|
| Milk, all species, fresh or dry cheese, hard or soft, whey cheese | 6.38 | 0.98 |

Proteolysis was assessed. Water-soluble nitrogen (SN) and non-protein nitrogen content (NPN) were determinated. Ten grams of cheese was homogenised in 100g of distilled water and homogenised at 11.000 rpm for 1 min using Ultra-Turrax T 25 basic. After 60 min at 40 °C, the cheese dispersion was re-homogenised under the same conditions.
The homogenate was centrifuged at 3000 rpm for 30 min at 6 °C, and the supernatant was filtered through filter paper. The filtrate, that is; the water-soluble extract of the cheese, was used for the determination of water-soluble nitrogen (SN) of cheese; ten grams from the extract were analysed in triplicate by means of the Kjeldahl method.
Furthermore, 25 mL of the water-soluble extract were mixed with an equal quantity of TCA or trichloroacetic acid (24% w/w), remaining overnight at 4°C after which the mixture was filtered through filter paper (Zoidou *et al.,* 2015). Fifteen grams of the supernatant were used for the

determination of 12% TCA-soluble N of the cheese (SN-TCA), and analyses was carried out by means of the Kjeldahl method.

The amount of NPN, expressed as a SN/TN% and SN-TCA/TN%, was determined as it represents those parameters necessary to indicate the extent of proteolysis. Primary proteolysis in cheese may be defined as those changes in beta-, gamma-, alphas-caseins, peptides, and other minor bands. Secondary proteolysis products could include those peptides, proteins, and amino acids which are soluble in the aqueous phase of cheese and are extractable as the water-soluble fraction (Rank *et al.*, 1985).


**Figure 26** Phases of Kjeldahl method (AOAC, 1990)

**Figure 27** Tecator™ and Kjeltec™ systems

### 3.5 Statistical analysis

All the analyses were repeated three times except for colour determination, which was repeated ten times. The results were expressed as mean ± Standard Deviation (SD).

The data was subjected to analysis of variance (one-way ANOVA) and post-hoc Tukey test using SPSS Statistics 21.0 software (Figure 28).

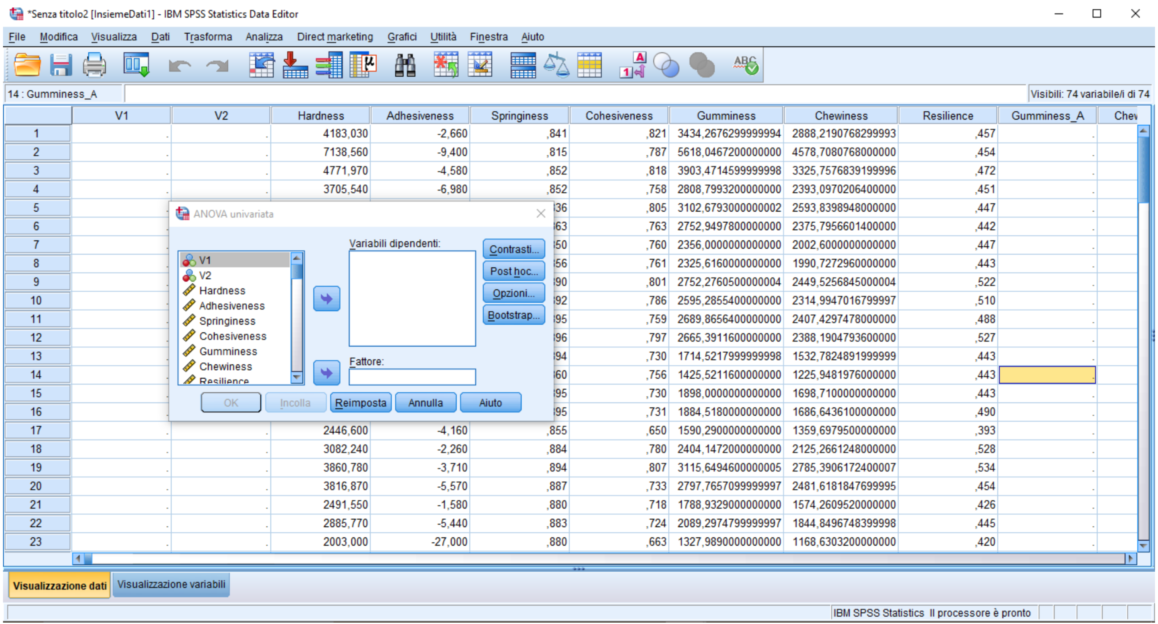



**Figure 28** SPSS Statistics 21.0 software

# Chapter 4

## Monitoring of lacto-fermented mozzarella cheese stored in different governing liquid

Lacto-fermented mozzarella is usually stored in governing liquid until used and then the effect of the governing liquid on the properties of cheese should be considered during preparation. Accordingly, the effects of governing liquid on the physicochemical and microbiological properties of lacto-fermented mozzarella cheese during storage are examined in this chapter.

The results, for each trial followed the order reported in chapter 3, and demonstrated that changes in the combination and concentrations of compounds dissolved in the governing liquid significantly influence the shelf-life of mozzarella cheese.

Thus, the selection of governing liquid could be one of the important factors determining product quality.

### 4.1 M-SALTS Results and Discussions

In this first experimental trial, the effect of calcium lactate (CL) and sodium chloride (NaCl) on both physical properties and microbiological quality of lacto-fermented mozzarella cheese (Table 13) was evaluated.

**Table 12** *Experimental trial M-Salts*

| PRODUCTION LOT | CODE SAMPLES | GOVERNING LIQUID COMPOSITION | PACKAGING MATERIAL |
|---|---|---|---|
| M-SALTS | 0.2% CL | 0.2 % calcium lactate in tap water | Polypropylene (PP) |
| | 0.6% CL | 0.6 % calcium lactate in tap water | Polypropylene (PP) |
| | 0.2% NaCl | 0.2 % sodium choride in tap water | Polypropylene (PP) |
| | 0.6% NaCl | 0.6 % sodium chloride in tap water | Polypropylene (PP) |
| | 0.4% NaCl + 0.2% CL | 0.4 % sodium choride and 0.2 % calcium lactate in tap water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |

The peculiarity of this experimental phase is the addition of salt to the tap water, prior to packaging, instead of adding calcium during processing. The mozzarella cheese quality was monitored for 18 days.

As shown in Figure 29, all the mozzarella cheese samples were acceptable microbiologically in terms of Pseudomonas *spp.* below the limit reported even at the eighteenth day ($10^6$ cfu/g may represent the contamination level at which the alterations of the product start to appear; Bishop and White, 1986).

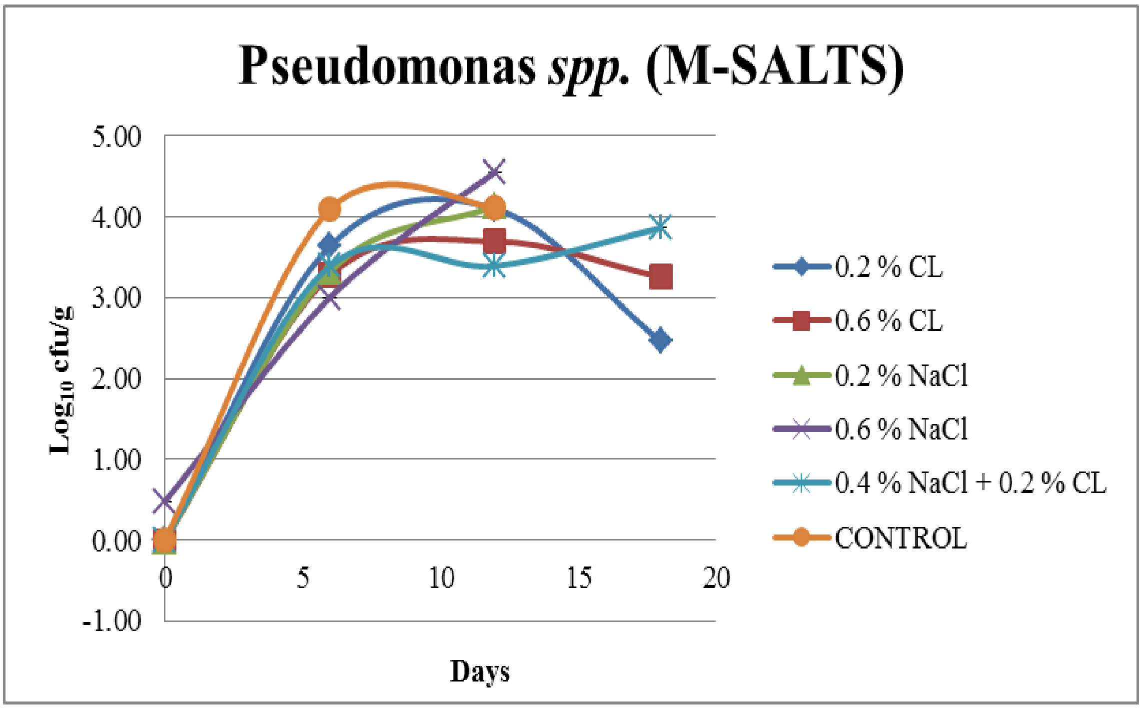



**Figure 29** Pseudomonas *spp*. evolution on mozzarella samples during storage

Salt concentrations used in this experimental trial did not inhibit the Lactic Acid Bacteria growth that increased during the time.

Calcium lactate contributed to slowing the growth of Pseudomonas spp. on day 12 of storage.

Sodium chloride did not have an inhibitory effect on Pseudomonas spp. except in the concentrated state with CL (Table 14).

There are statistically significant differences ($P$<0.01) between the control samples and the samples stored in alternative governing liquids.

The use of calcium lactate did not inhibit the growth of lactic acid bacteria (LAB).

On each day calcium lactate samples showed the highest LAB content.

In particular, at the end of storage (18 days) 0.6% CL samples reported the highest $\log_{10}$ cfu/g value (7.86 ± 0.00). On the contrary, Akarca *et al.* (2015) reported that LAB count of mozzarella cheese decreased during storage, using different packaging techniques: aerobic packaging (AP), vacuum packaging (VP) and modified atmosphere packaging (MAP).

0.6% CL samples showed the lowest TBC value at 18 days ($P$< 0.01), although the same value was the highest at the beginning of monitoring (day 0; $P$ <0.01).

Probably, the highest value in terms of TBC was also linked at the growth of LAB.

**Table 13** *Microbiological analysis ($log_{10}$ cfu/g ± SD=3) of lacto-fermented mozzarella cheese during the refrigerated storage at 5°C*

| *TIME* | *M-SALTS* | *TBC* | *Pseudomonas spp.* | *LAB* |
|---|---|---|---|---|
| 0 | **0.2 % CL** | 6.03 ± 0.00 b | 0.00 ± 0.00 b | 5.95 ± 0.00 b |
| | **0.6 % CL** | 6.98 ± 0.00 a | -0.01 ± 0.00 b | 6.88 ± 0.00 a |
| | **0.2 % NaCl** | 4.69 ± 0.00 d | -0.01 ± 0.00 b | 4.61 ± 0.00 d |
| | **0.6 % NaCl** | 5.93 ± 0.00 c | 0.47 ± 0.00 a | 5.86 ± 0.00 c |
| | **0.4 % NaCl + 0.2 % CL** | 4.38 ± 0.00 f | 0.00 ± 0.00 b | 4.28 ± 0.00 e |
| | **CONTROL** | 4.61 ± 0.00 e | 0.00 ± 0.00 b | 4.21 ± 0.01 f |
| | **Significance** | ** | ** | ** |
| 6 | **0.2 % CL** | 6.40 ± 0.00 | 3.64 ± 0.00 b | 6.19 ± 0.00 b |
| | **0.6 % CL** | 6.51 ± 0.00 | 3.28 ± 0.00 e | 6.30 ± 0.00 b |
| | **0.2 % NaCl** | 6.59 ± 0.00 | 3.32 ± 0.00 d | 6.31 ± 0.00 b |
| | **0.6 % NaCl** | 6.44 ± 0.00 | 3.00 ± 0.00 f | 6.14 ± 0.00 b |
| | **0.4 % NaCl + 0.2 % CL** | 6.68 ± 0.00 | 3.39 ± 0.00 c | 6.68 ± 0.00 a |
| | **CONTROL** | 6.03 ± 0.71 | 4.09 ± 0.01 a | 5.18 ± 0.11 c |
| | **Significance** | n.s. | ** | ** |
| 12 | **0.2 % CL** | 6.75 ± 0.00 d | 4.10 ± 0.00 c | 6.82 ± 0.00 c |
| | **0.6 % CL** | 6.92 ± 0.00 bc | 3.70 ± 0.00 d | 7.07 ± 0.00 b |
| | **0.2 % NaCl** | 6.90 ± 0.00 c | 4.14 ± 0.00 b | 6.79 ± 0.00 d |
| | **0.6 % NaCl** | 7.25 ± 0.00 a | 4.55 ± 0.00 a | 6.53 ± 0.00 e |
| | **0.4 % NaCl + 0.2 % CL** | 6.95 ± 0.00 b | 3.39 ± 0.00 e | 7.12 ± 0.00 a |
| | **CONTROL** | 6.66 ± 0.03 e | 4.11 ± 0.02 bc | 6.34 ± 0.02 f |
| | **Significance** | ** | ** | ** |
| 18 | **0.2 % CL** | 7.03 ± 0.00 a | 2.46 ± 0.00 c | 7.00 ± 0.00 b |
| | **0.6 % CL** | 6.72 ± 0.00 c | 3.26 ± 0.00 b | 7.86 ± 0.00 a |
| | **0.2 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.6 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.4 % NaCl + 0.2 % CL** | 6.97 ± 0.00 b | 3.86 ± 0.00 a | 6.87 ± 0.00 c |
| | **CONTROL** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **Significance** | ** | ** | ** |

**Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*

In relation to physico-chemical analysis (Table 15 a, b), all the mozzarella cheese samples at day 0 presented a higher percentage of NaCl (from 1.53% to 2.13%) compared to governing liquid (from 0.15% to 0.46%).

The % NaCl value (day 0) in mozzarella are similar to those reported by Guinee (2004) in contributing to protein hydration. Moreover, the % NaCl content increased during the storage time in the governing liquid, probably for a redistribution of the same salt.

Conversely, moisture increased in all the mozzarella samples (for a water diffusion phenomenon), tending to enhance $a_w$ (from a range of 0.969- 0.972 at day 0 to 0.974-0.976 at day 18) and protein hydration according to research that reported an enhancing of protein hydration of cheese in the presence of salt up to 1.4% (Guinee, 2004). Lacto-fermented mozzarella exhibited a high moisture content, which is recognized as an important factor in the evolution of cheese texture. Ahmed *et al.* (2005) reported that high moisture content cheeses produce a fragile protein network that results in less-firm cheeses.

Lacto-fermented mozzarella cheese and governing liquids pH values decreased during storage as shown in table 15 a, b.

These changes of values of pH could be responsible for changes in the rheological characteristic of lacto-fermented mozzarella as indicated in published works (Laurenzio *et al.*, 2008).

The published works provide little information on the effects of pH and Ca content, and of their interaction, during the storage of mozzarella cheese in governing liquid.

The lacto-fermented mozzarella stored in 0.6% CL, despite the higher pH (6.01± 0.01) at time 0 (*P* <0.01) than the other theses, was the sample that suffered the fastest reduction in pH. In fact, at day 12 and 18, it recorded pH values of 5.63 ± 0.02 and 5.70± 0.01 respectively. These data were significantly lower than the other theses (*P* <0.01).

Conversely, the thesis with 0.2% NaCl was that with the highest pH until the 12$^{th}$. The pH value of mozzarella control samples at day 12 (the final day of shelf-life; tab. x a) was the highest, while $a_w$ and % NaCl values were the lowest among the examined samples.

Furthermore, the $a_w$ values incremented in all samples with significant variations between the theses (*P* <0.01). The % NaCl was significantly lower (*P* <0.01) at time 0 in 0.2 % CL (1.56 ± 0.04) and in control sample (1.53 ± 0.10). At day 12 the control sample had a significantly lower NaCl content (*P* <0.01) than the other theses, while the highest data was recorded for the thesis with 0.6% NaCl.

In governing liquid, salt concentration tends to become homogeneous in all mozzarella cheese; in fact, from external zones of mozzarella cheese, salt migrates toward the inner layers, replacing itself with Ca (Laurenzio *et al.*, 2008); NaCl promotes micro-structural swelling, a concomitant increase in water holding capacity (Guo *et al.*, 1997).

**Table 14** *M-Salts trial: physico-chemical analysis (Means ± SD=3) of lacto-fermented mozzarella cheese (a) and governing liquid (b) during the refrigerated storage at 5°C*

| TIME | M-SALTS | $a_w$ | % NaCl | % Moisture | pH | % Lactic Acid |
|---|---|---|---|---|---|---|
| 0 | **0.2 % CL** | 0.971 ± 0.000 $^{ab}$ | 1.56 ± 0.04 $^{b}$ | 64 ± 0.69 $^{ab}$ | 5.94 ± 0.06 $^{a}$ | 0.20 ± 0.00 $^{b}$ |
| | **0.6 % CL** | 0.971 ± 0.000 $^{ab}$ | 1.96 ± 0.11 $^{a}$ | 64 ± 1.61 $^{a}$ | 6.01 ± 0.01 $^{a}$ | 0.16 ± 0.00 $^{c}$ |
| | **0.2 % NaCl** | 0.972 ± 0.000 $^{b}$ | 1.87 ± 0.02 $^{a}$ | 66 ± 1.22 $^{a}$ | 5.90 ± 0.04 $^{ab}$ | 0.18 ± 0.00 $^{bc}$ |
| | **0.6 % NaCl** | 0.971 ± 0.000 $^{ab}$ | 1.86 ± 0.04 $^{a}$ | 63 ± 1.53 $^{ab}$ | 6.00 ± 0.01 $^{a}$ | 0.16 ± 0.00 $^{c}$ |
| | **0.4 % NaCl + 0.2 % CL** | 0.969 ± 0.000 $^{c}$ | 2.13 ± 0.06 $^{a}$ | 64 ± 0.80 $^{a}$ | 5.75 ± 0.06 $^{b}$ | 0.24 ± 0.01 $^{a}$ |
| | **CONTROL** | 0.971 ± 0.000 $^{b}$ | 1.53 ± 0.10 $^{b}$ | 59 ± 1.38 $^{b}$ | 5.98 ± 0.02 $^{a}$ | 0.17 ± 0.02 $^{c}$ |
| | **Significance** | ** | ** | * | ** | ** |
| 6 | **0.2 % CL** | 0.977 ± 0.000 $^{a}$ | 1.73 ± 0.10 $^{ab}$ | 65 ± 0.37 $^{c}$ | 5.94 ± 0.01 $^{b}$ | 0.18 ± 0.01 $^{bc}$ |
| | **0.6 % CL** | 0.976 ± 0.000 $^{a}$ | 1.19 ± 0.10 $^{c}$ | 65 ± 0.97 $^{c}$ | 5.86 ± 0.01 $^{c}$ | 0.11 ± 0.00 $^{d}$ |
| | **0.2 % NaCl** | 0.977 ± 0.000 $^{a}$ | 1.56 ± 0.10 $^{b}$ | 69 ± 0.29 $^{b}$ | 6.02 ± 0.02 $^{a}$ | 0.14 ± 0.01 $^{bc}$ |
| | **0.6 % NaCl** | 0.976 ± 0.000 $^{a}$ | 1.66 ± 0.10 $^{ab}$ | 74 ± 0.04 $^{a}$ | 6.02 ± 0.01 $^{a}$ | 0.13 ± 0.01 $^{cd}$ |
| | **0.4 % NaCl + 0.2 % CL** | 0.976 ± 0.000 $^{a}$ | 1.95 ± 0.00 $^{a}$ | 69 ± 0.09 $^{b}$ | 5.87 ± 0.02 $^{c}$ | 0.19 ± 0.00 $^{ab}$ |
| | **CONTROL** | 0.971 ± 0.001 $^{b}$ | 1.65 ± 0.01 $^{ab}$ | 64 ± 0.00 $^{c}$ | 5.88 ± 0.00 $^{bc}$ | 0.23 ± 0.02 $^{a}$ |
| | **Significance** | ** | ** | ** | ** | ** |
| 12 | **0.2 % CL** | 0.977 ± 0.000 $^{a}$ | 1.72 ± 0.10 $^{c}$ | 63 ± 0.20 $^{c}$ | 5.61 ± 0.01 $^{c}$ | 0.14 ± 0.02 $^{bc}$ |
| | **0.6 % CL** | 0.975 ± 0.000 $^{b}$ | 1.80 ± 0.11 $^{bc}$ | 64 ± 0.45 $^{c}$ | 5.63 ± 0.02 $^{cd}$ | 0.17 ± 0.02 $^{bc}$ |
| | **0.2 % NaCl** | 0.975 ± 0.000 $^{b}$ | 1.81 ± 0.00 $^{bc}$ | 68 ± 0.09 $^{b}$ | 5.74 ± 0.01 $^{a}$ | 0.18 ± 0.01 $^{ab}$ |
| | **0.6 % NaCl** | 0.975 ± 0.000 $^{b}$ | 2.19 ± 0.10 $^{a}$ | 71 ± 0.57 $^{a}$ | 5.67 ± 0.00 $^{b}$ | 0.23 ± 0.02 $^{a}$ |
| | **0.4 % NaCl + 0.2 % CL** | 0.976 ± 0.000 $^{ab}$ | 2.11 ± 0.11 $^{ab}$ | 68 ± 0.36 $^{b}$ | 5.65 ± 0.00 $^{bc}$ | 0.19 ± 0.02 $^{ab}$ |
| | **CONTROL** | 0.974 ± 0.001 $^{c}$ | 1.16 ± 0.01 $^{d}$ | 64 ± 0.05 $^{c}$ | 5.76 ± 0.00 $^{a}$ | 0.19 ± 0.02 $^{ab}$ |
| | **Significance** | ** | ** | ** | ** | ** |
| 18 | **0.2 % CL** | 0.976 ± 0.000 | 1.66 ± 0.13 | 68 ± 0.05 $^{b}$ | 5.78 ± 0.02 $^{a}$ | 0.21 ± 0.02 |
| | **0.6 % CL** | 0.976 ± 0.000 | 1.51 ± 0.11 | 65 ± 0.90 $^{c}$ | 5.70 ± 0.01 $^{b}$ | 0.21 ± 0.02 |
| | **0.2 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.6 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.4 % NaCl + 0.2 % CL** | 0.974 ± 0.000 | 1.86 ± 0.01 | 71 ± 0.08 $^{a}$ | 5.75 ± 0.00 $^{a}$ | 0.22 ± 0.00 |
| | **CONTROL** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **Significance** | n.s. | n.s. | * | * | n.s |

**a)**

| TIME | *GL (M-SALTS)* | pH | % Lactic acid | % NaCl | L* | a* | b* |
|---|---|---|---|---|---|---|---|
| 0 | **0.2 % CL** | 6.36 ± 0.04 $^{bc}$ | 0.03 ± 0.00 | 0.18 ± 0.00 $^{de}$ | 31.20 ± 0.06 $^{c}$ | -0.02 ± 0.04 $^{b}$ | -0.13 ± 0.03 $^{c}$ |
| | **0.6 % CL** | 6.28 ± 0.01 $^{c}$ | 0.03 ± 0.00 | 0.21 ± 0.01 $^{d}$ | 31.09 ± 0.13 $^{c}$ | 0.01 ± 0.05 $^{b}$ | -0.15 ± 0.06 $^{c}$ |
| | **0.2 % NaCl** | 6.40 ± 0.01 $^{b}$ | 0.02 ± 0.00 | 0.30 ± 0.01 $^{c}$ | 31.02 ± 0.06 $^{c}$ | -0.04 ± 0.04 $^{b}$ | -0.13 ± 0.07 $^{c}$ |
| | **0.6 % NaCl** | 6.41 ± 0.04 $^{b}$ | 0.02 ± 0.00 | 0.46 ± 0.01 $^{a}$ | 32.22 ± 0.13 $^{a}$ | 0.00 ± 0.05 $^{b}$ | -0.49 ± 0.06 $^{d}$ |
| | **0.4 % NaCl + 0.2 % CL** | 6.43 ± 0.02 $^{b}$ | 0.02 ± 0.00 | 0.36 ± 0.01 $^{b}$ | 30.56 ± 0.20 $^{d}$ | -0.01 ± 0.05 $^{b}$ | 0.12 ± 0.09 $^{b}$ |
| | **CONTROL** | 6.92 ± 0.02 $^{a}$ | 0.01 ± 0.00 | 0.15 ± 0.00 $^{e}$ | 32.00 ± 0.06 $^{b}$ | 0.17 ± 0.01 $^{a}$ | 1.36 ± 0.07 $^{a}$ |
| | ***Significance*** | ** | n.s. | ** | ** | ** | ** |
| 6 | **0.2 % CL** | 5.73 ± 0.01 $^{bc}$ | 0.12 ± 0.00 $^{a}$ | 0.30 ± 0.01 $^{d}$ | 30.88 ± 0.07 $^{b}$ | -0.05 ± 0.04 $^{c}$ | 0.07 ± 0.03 $^{d}$ |
| | **0.6 % CL** | 5.72 ± 0.01 $^{c}$ | 0.12 ± 0.00 $^{a}$ | 0.27 ± 0.01 $^{d}$ | 30.62 ± 0.17 $^{b}$ | -0.02 ± 0.02 $^{bc}$ | 0.36 ± 0.08 $^{b}$ |
| | **0.2 % NaCl** | 5.80 ± 0.03 $^{ab}$ | 0.08 ± 0.00 $^{b}$ | 0.38 ± 0.00 $^{c}$ | 29.56 ± 0.63 $^{cd}$ | -0.01 ± 0.04 $^{bc}$ | 0.17 ± 0.09 $^{cd}$ |
| | **0.6 % NaCl** | 5.80 ± 0.04 $^{ab}$ | 0.07 ± 0.00 $^{c}$ | 0.52 ± 0.01 $^{a}$ | 29.03 ± 0.49 $^{c}$ | 0.08 ± 0.04 $^{b}$ | 0.39 ± 0.01 $^{bc}$ |
| | **0.4 % NaCl + 0.2 % CL** | 5.70 ± 0.00 $^{c}$ | 0.11 ± 0.00 $^{a}$ | 0.45 ± 0.01 $^{b}$ | 29.04 ± 0.40 $^{d}$ | 0.01 ± 0.01 $^{bc}$ | 0.27 ± 0.06 $^{bc}$ |
| | **CONTROL** | 5.85 ± 0.01 $^{a}$ | 0.09 ± 0.00 $^{b}$ | 0.15 ± 0.00 $^{e}$ | 31.71 ± 0.04 $^{a}$ | 0.10 ± 0.01 $^{a}$ | 1.36 ± 0.06 $^{a}$ |
| | ***Significance*** | ** | ** | ** | ** | ** | ** |
| 12 | **0.2 % CL** | 5.03 ± 0.00 $^{bc}$ | 0.20 ± 0.00 $^{ab}$ | 0.60 ± 0.02 $^{d}$ | 31.00 ± 0.08 $^{c}$ | -0.03 ± 0.03 $^{b}$ | 0.05 ± 0.03 $^{b}$ |
| | **0.6 % CL** | 5.08 ± 0.01 $^{a}$ | 0.21 ± 0.00 $^{a}$ | 0.60 ± 0.02 $^{d}$ | 30.97 ± 0.06 $^{c}$ | -0.03 ± 0.02 $^{b}$ | -0.08 ± 0.03 $^{c}$ |
| | **0.2 % NaCl** | 5.03 ± 0.01 $^{bc}$ | 0.17 ± 0.00 $^{c}$ | 0.83 ± 0.02 $^{c}$ | 30.86 ± 0.25 $^{c}$ | 0.01 ± 0.01 $^{b}$ | -0.06 ± 0.01 $^{c}$ |
| | **0.6 % NaCl** | 5.00 ± 0.01 $^{c}$ | 0.18 ± 0.01 $^{bc}$ | 1.07 ± 0.02 $^{a}$ | 33.46 ± 0.08 $^{a}$ | -0.01 ± 0.03 $^{b}$ | 0.01 ± 0.02 $^{b}$ |
| | **0.4 % NaCl + 0.2 % CL** | 5.00 ± 0.01 $^{c}$ | 0.18 ± 0.00 $^{bc}$ | 0.94 ± 0.00 $^{b}$ | 30.95 ± 0.18 $^{c}$ | 0.01 ± 0.04 $^{b}$ | 0.03 ± 0.06 $^{b}$ |
| | **CONTROL** | 5.06 ± 0.01 $^{ab}$ | 0.20 ± 0.00 $^{a}$ | 0.42 ± 0.01 $^{e}$ | 31.83 ± 0.07 $^{b}$ | 0.09 ± 0.01 $^{a}$ | 1.41 ± 0.01 $^{a}$ |
| | ***Significance*** | * | ** | ** | ** | ** | ** |
| 18 | **0.2 % CL** | 5.19 ± 0.02 | 0.25 ± 0.00 | 0.54 ± 0.02 $^{c}$ | 30.99 ± 0.00 $^{b}$ | 0.00 ± 0.02 | 0.04 ± 0.00 $^{c}$ |
| | **0.6 % CL** | 5.20 ± 0.01 | 0.24 ± 0.00 | 0.61 ± 0.00 $^{b}$ | 30.85 ± 0.01 $^{c}$ | -0.02 ± 0.01 | 0.19 ± 0.01 $^{a}$ |
| | **0.2 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.6 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.4 % NaCl + 0.2 % CL** | 5.21 ± 0.01 | 0.24 ± 0.01 | 0.95 ± 0.02 $^{a}$ | 31.36 ± 0.01 $^{a}$ | -0.01 ± 0.01 | 0.07 ± 0.01 $^{b}$ |
| | **CONTROL** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | ***Significance*** | n.s. | n.s. | ** | ** | ** | ** |

**b)**

**Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*

With regards to TPA (table 16), when considering the hardness parameter, it can be seen that sodium chloride determined a reduction in the hardness values (*P* <0.05) during the time of storage. It determined a loss of integrity in the outer layer of the product making it unacceptable after 12 days of storage, such as with the control samples.

The 0.6% CL sample, despite at day 0 detecting the lowest hardness values, at day 12 it shows significantly higher value (P< 0.01). The thesis with 0.2% CL showed 12 significantly higher values (2666 g) than the other theses, but lower than 0.6% CL.

While the samples stored with 0.6% NaCl (with the highest values at day 0) and 0.2% NaCl recorded the lowest values at 12, respectively of 1562 g and 2125 g. It would therefore seem that the calcium lactate had a positive effect on maintaining the hardness, whereas NaCl has a negative effect.

The study by Luo *et al*. (2013) elucidated details of how calcium added to brine affects salt diffusion and reduces the risk of the soft defect for mozzarella cheese.

In all theses the adhesiveness values decreased from day 0 to day 12. There were significant differences (*P* <0.01) between the theses during this period. At day 12 the theses showed high negative values with respect to control (-6.23) with the highest and lowest relative value of CL 0.6% (-7.18) and NaCl 0.6% (-55.62), respectively. At day 18, no data was recorded for the samples referred to as control, 0.2 % NaCl and 0.6% NaCl because they were visibly unacceptable.

Springiness, cohesiveness, gumminess, chewiness and resilience diminished over time in all theses.

There were changes in the colour parameters (Table 17).

There was an increase in outer luminosity, producing a whiter cheese and recording significant differences (*P*<0.05) between treatments at day 6 and 12.

The increase in L* values of samples could be caused by not losing water in the package during the storage period and therefore the conservation of the initial white colour during the storage period, in line with the study by Akarca *et al.* (2015).

For those samples stored in sodium chloride, used alone or concentrated, the decrease of inner luminosity was very evident at day 12, a result which was due to integrity loss on the surface.

However, there was an increase in the a* value, both in the outer and inner of all products, during storage.

The b* values (yellow component) were lower in 0.6% NaCl (*P*<0.01) both in both outer and in inner samples at day 12.

**Table 15** *TPA (Means ± SD=3) of lacto-fermented mozzarella cheese during the refrigerated storage at 5°C*

| *TIME* | *M-SALTS* | Hardness (g) | Adhesiveness (g*sec) | Springiness | Cohesiveness | Gumminess | Chewiness | Resilience |
|---|---|---|---|---|---|---|---|---|
| | **0.2 % CL** | 6423.64 ± 33.23 b | -6.12 ± 0.02 c | 0.820 ± 0.028 ab | 0.778 ± 0.021 c | 5007.00 ± 0.52 c | 4202.59 ± 3.51 c | 0.446 ± 0.001 c |
| | **0.6 % CL** | 5309.05 ± 0.21 d | -0.75 ± 0.00 a | 0.866 ± 0.001 a | 0.807 ± 0.000 a | 4287.00 ± 0.71 e | 3711.56 ± 2.14 f | 0.481 ± 0.001 a |
| | **0.2 % NaCl** | 6002.01 ± 0.13 c | -6.31 ± 0.01 c | 0.855 ± 0.001 a | 0.793 ± 0.007 b | 4752.54 ± 2.83 d | 4057.92 ± 0.73 d | 0.457 ± 0.001 b |
| **0** | **0.6 % NaCl** | 7509.55 ± 13.42 a | -10.00 ± 0.01 d | 0.838 ± 0.001 ab | 0.815 ± 0.007 a | 6091.56 ± 0.76 a | 5106.47 ± 1.59 a | 0.488 ± 0.002 a |
| | **0.4 % NaCl + 0.2 % CL** | 6445.82 ± 6.20 b | -10.62 ± 0.10 d | 0.854 ± 0.001 a | 0.786 ± 0.099 bc | 5063.27 ± 0.52 b | 4327.73 ± 7.71 b | 0.481 ± 0.001 a |
| | **CONTROL** | 6315.80 ± 152.87 b | -4.24 ± 0.58 b | 0.802 ± 0.008 b | 0.812 ± 0.583 a | 5026.39 ± 14.60 c | 4008.09 ± 1.38 e | 0.436 ± 0.006 c |
| | ***Significance*** | ** | ** | * | ** | ** | ** | ** |
| | **0.2 % CL** | 2768.80 ± 6.50 b | -5.13 ± 0.01 d | 0.861 ± 0.007 b | 0.731 ± 0.014 b | 2026.38 ± 0.08 c | 1753.91 ± 1.46 d | 0.470 ± 0.001 b |
| | **0.6 % CL** | 2620.82 ± 15.97 d | -2.77 ± 0.01 b | 0.894 ± 0.001 a | 0.771 ± 0.007 a | 2006.30 ± 0.56 d | 1792.69 ± 0.83 c | 0.469 ± 0.001 b |
| | **0.2 % NaCl** | 3189.14 ± 7.10 a | -12.33 ± 0.07 e | 0.826 ± 0.000 d | 0.734 ± 0.071 b | 2341.42 ± 7.09 a | 1933.04 ± 7.11 a | 0.434 ± 0.001 e |
| **6** | **0.6 % NaCl** | 1829.76 ± 9.97 f | -30.30 ± 0.01 f | 0.830 ± 0.014 cd | 0.740 ± 0.014 b | 1364.69 ± 0.81 f | 1145.46 ± 1.48 f | 0.444 ± 0.001 d |
| | **0.4 % NaCl + 0.2 % CL** | 1912.83 ± 3.67 e | -4.89 ± 0.01 c | 0.858 ± 0.002 bc | 0.764 ± 0.014 a | 1462.42 ± 1.45 e | 1256.22 ± 1.48 e | 0.476 ± 0.001 a |
| | **CONTROL** | 2687.06 ± 0.71 c | -2.20 ± 0.01 a | 0.885 ± 0.007 ab | 0.765 ± 0.014 a | 2075.07 ± 0.01 b | 1825.16 ± 0.79 b | 0.462 ± 0.001 c |
| | ***Significance*** | ** | ** | ** | ** | ** | ** | ** |
| | **0.2 % CL** | 2666.10 ± 0.64 b | -12.90 ± 0.01 c | 0.803 ± 0.001 b | 0.708 ± 0.014 c | 1885.77 ± 4.10 c | 1513.09 ± 0.70 c | 0.409 ± 0.001 c |
| | **0.6 % CL** | 2879.39 ± 0.13 a | -7.18 ± 0.07 b | 0.855 ± 0.002 a | 0.752 ± 0.071 b | 2162.84 ± 7.11 b | 1857.67 ± 1.70 b | 0.466 ± 0.001 b |
| | **0.2 % NaCl** | 2125.29 ± 2.14 e | -14.29 ± 0.11 d | 0.761 ± 0.001 d | 0.648 ± 0.113 e | 1368.43 ± 10.54 e | 1052.52 ± 4.96 e | 0.382 ± 0.001 e |
| **12** | **0.6 % NaCl** | 1562.01 ± 2.24 f | -55.62 ± 0.03 e | 0.681 ± 0.001 e | 0.561 ± 0.028 f | 871.75 ± 8.47 f | 594.41 ± 6.36 f | 0.303 ± 0.001 f |
| | **0.4 % NaCl + 0.2 % CL** | 2196.30 ± 0.70 d | -14.42 ± 0.03 d | 0.803 ± 0.001 b | 0.677 ± 0.028 d | 1474.41 ± 21.34 d | 1196.37 ± 1.40 d | 0.398 ± 0.001 d |
| | **CONTROL** | 2473.21 ± 0.71 c | -6.23 ± 0.00 a | 0.790 ± 0.001 c | 0.792 ± 0.001 a | 2518.08 ± 28.25 a | 2067.41 ± 2.12 a | 0.476 ± 0.001 a |
| | ***Significance*** | ** | ** | ** | ** | ** | ** | ** |
| | **0.2 % CL** | 1978.46 ± 0.71 | -11.96 ± 0.01 a | 0.768 ± 0.002 | 0.658 ± 0.007 a | 1304.69 ± 6.38 c | 1000.65 ± 0.52 c | 0.357 ± 0.001 a |
| | **0.6 % CL** | 2606.61 ± 707.81 | -23.04 ± 0.01 b | 0.726 ± 0.004 | 0.568 ± 0.014 b | 1766.73 ± 2.22 a | 1284.16 ± 1.00 a | 0.295 ± 0.005 c |
| | **0.2 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| **18** | **0.6 % NaCl** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **0.4 % NaCl + 0.2 % CL** | 2106.68 ± 0.62 | -13.55 ± 0.71 a | 0.739 ± 0.040 | 0.514 ± 0.707 c | 1478.30 ± 14.14 b | 1191.45 ± 11.31 b | 0.325 ± 0.006 b |
| | **CONTROL** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | ***Significance*** | n.s. | ** | n.s. | ** | ** | ** | * |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

**Table 16** *Colour analysis results of lacto-fermented mozzarella cheese*

| | | OUTER | | | | | | INNER | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***TIME*** | ***M-SALTS*** | **L*** | | **a*** | | **b*** | | **L*** | | **a*** | | **b*** | |
| 0 | **0.2 % CL** | 94.49 ± 0.21 | | -0.39 ± 0.05 | | 5.31 ± 0.21 | ab | 91.36 ± 0.58 | b | -0.62 ± 0.11 | bc | 8.38 ± 0.52 | |
| | **0.6 % CL** | 94.48 ± 0.75 | | -0.38 ± 0.06 | | 5.10 ± 0.08 | ab | 91.64 ± 0.81 | ab | -0.70 ± 0.04 | c | 8.70 ± 0.43 | |
| | **0.2 % NaCl** | 94.34 ± 0.69 | | -0.32 ± 0.05 | | 5.13 ± 0.33 | ab | 92.13 ± 2.34 | ab | -0.55 ± 0.07 | bc | 7.84 ± 1.09 | |
| | **0.6 % NaCl** | 94.13 ± 0.30 | | -0.35 ± 0.06 | | 5.19 ± 0.37 | ab | 91.22 ± 2.43 | b | -0.55 ± 0.15 | bc | 7.73 ± 1.30 | |
| | **0.4 % NaCl + 0.2 % CL** | 94.05 ± 0.72 | | -0.35 ± 0.03 | | 4.90 ± 0.34 | b | 92.22 ± 0.94 | ab | -0.50 ± 0.08 | b | 7.56 ± 0.50 | |
| | **CONTROL** | 95.11 ± 0.56 | | -0.33 ± 0.04 | | 5.54 ± 0.33 | a | 94.69 ± 1.93 | a | -0.31 ± 0.04 | a | 6.45 ± 1.11 | |
| | ***Significance*** | **n.s.** | | **n.s.** | | ***** | | ***** | | ****** | | ***** | |
| 6 | **0.2 % CL** | 94.31 ± 0.86 | ab | -0.34 ± 0.13 | | 5.14 ± 1.05 | | 92.39 ± 1.44 | ab | -0.61 ± 0.04 | | 7.85 ± 0.75 | a |
| | **0.6 % CL** | 93.96 ± 0.63 | b | -0.29 ± 0.07 | | 5.31 ± 0.29 | | 93.17 ± 1.13 | ab | -0.50 ± 0.05 | | 6.27 ± 0.39 | bc |
| | **0.2 % NaCl** | 94.03 ± 1.17 | b | -0.32 ± 0.09 | | 4.84 ± 1.19 | | 90.84 ± 3.27 | ab | -0.63 ± 0.24 | | 7.44 ± 0.96 | ab |
| | **0.6 % NaCl** | 94.71 ± 1.44 | ab | -0.36 ± 0.09 | | 4.34 ± 1.37 | | 83.18 ± 14.29 | b | -0.53 ± 0.43 | | 5.93 ± 0.54 | c |
| | **0.4 % NaCl + 0.2 % CL** | 94.30 ± 1.05 | ab | -0.31 ± 0.10 | | 4.91 ± 1.11 | | 92.08 ± 0.49 | ab | -0.64 ± 0.11 | | 7.65 ± 0.53 | a |
| | **CONTROL** | 96.07 ± 0.72 | a | -0.25 ± 0.05 | | 4.66 ± 0.55 | | 95.77 ± 0.90 | a | -0.44 ± 0.12 | | 6.08 ± 0.44 | c |
| | ***Significance*** | ***** | | **n.s.** | | **n.s.** | | ***** | | **n.s.** | | ****** | |
| 12 | **0.2 % CL** | 95.18 ± 0.41 | ab | -0.25 ± 0.03 | a | 5.22 ± 0.28 | ab | 91.73 ± 1.53 | a | -0.51 ± 0.08 | b | 7.88 ± 0.33 | a |
| | **0.6 % CL** | 94.47 ± 1.03 | ab | -0.37 ± 0.09 | bc | 5.84 ± 0.93 | a | 91.13 ± 1.56 | a | -0.57 ± 0.09 | b | 8.17 ± 0.21 | a |
| | **0.2 % NaCl** | 95.29 ± 0.60 | ab | -0.29 ± 0.03 | ab | 5.59 ± 0.28 | a | 79.96 ± 8.09 | b | -0.03 ± 0.47 | a | 7.93 ± 1.13 | a |
| | **0.6 % NaCl** | 94.32 ± 1.37 | b | -0.47 ± 0.05 | d | 3.79 ± 0.55 | c | 85.05 ± 5.41 | ab | -0.70 ± 0.24 | b | 5.63 ± 0.54 | b |
| | **0.4 % NaCl + 0.2 % CL** | 95.88 ± 0.18 | a | -0.31 ± 0.02 | ab | 4.37 ± 0.17 | bc | 90.39 ± 1.54 | a | -0.61 ± 0.07 | b | 8.04 ± 0.12 | a |
| | **CONTROL** | 95.71 ± 0.42 | ab | -0.44 ± 0.04 | cd | 5.49 ± 0.13 | a | 92.92 ± 1.19 | a | -0.45 ± 0.20 | ab | 8.42 ± 1.52 | a |
| | ***Significance*** | ***** | | ****** | | ****** | | ****** | | ***** | | ****** | |
| 18 | **0.2 % CL** | 95.17 ± 0.90 | | -0.01 ± 0.10 | a | 5.65 ± 1.15 | | 92.65 ± 0.56 | ab | -0.15 ± 0.11 | a | 7.85 ± 0.59 | |
| | **0.6 % CL** | 95.83 ± 0.64 | | -0.17 ± 0.12 | ab | 5.26 ± 0.48 | | 93.61 ± 1.09 | a | -0.41 ± 0.04 | b | 7.50 ± 0.74 | |
| | **0.2 % NaCl** | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | |
| | **0.6 % NaCl** | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | |
| | **0.4 % NaCl + 0.2 % CL** | 95.04 ± 0.88 | | -0.30 ± 0.06 | b | 4.63 ± 0.22 | | 91.98 ± 1.09 | b | -0.44 ± 0.10 | b | 7.82 ± 0.21 | |
| | **CONTROL** | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | | n.d. ± n.d. | |
| | ***Significance*** | **n.s.** | | ***** | | **n.s.** | | ***** | | ****** | | **n.s.** | |

**Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test*

### 4.2 M-BJ Results and Discussions

Bergamot extract is a natural compound from citrus fruits, which is typical to Calabria and well-known for its high content in flavonoid compounds and vitamin C. Moreover, it is appreciated for its antiseptic and antibacterial action.

There are some interesting documented applications of lemon extracts improving the acceptability of dairy products (Fernandez-Lopez *et al.*, 2005; Conte *et al.*, 2007; Gammariello *et al.*, 2008).

In particular the work of Gammariello *et al.*, (2008) reported the screening relating to several essential oils by a panel test for suitability for dairy applications. Among the selected natural compounds, the panelists selected the following: *Salvia officinalis* (sage), *Thymus vulgaris* (thyme), *Rosmarinus officinalis* (rosemary), *Citrus aurantium* (sour orange), *Citrus sinensis* (sweet orange), *Citrus paradisii macf* (grapefruit), *Citrus limonum* (lemon essential oil), and hydroalcoholic extracts such as citral 95%, thymol, and nonanoic acid 96%. Natural compounds such as *Mentha piperita* (mint) were discarded because the panelists disliked their smell when applied to dairy products. These selected compounds were directly dissolved into FiordiLatte brine.

Due to the potential use of lemon extract in food preservation, the experimental trial M-BJ (table 18) allowed for an analysis of the effect of a similar citrus extract in conjunction with the natural preservative calcium lactate. In particular, calcium lactate and concentrated bergamot juice were used to extend the shelf-life of lacto-fermented mozzarella cheese. Another aim of this trial was to evaluate, collaborating with Falcone *et al.* (2017), both environmental and economic perspective of this governing liquid composition, in order to reduce the cheese losses.

**Table 17** *M-BJ trial: samples legend*

| PRODUCTION LOT | CODE SAMPLES | GOVERNING LIQUID COMPOSITION | PACKAGING MATERIAL |
|---|---|---|---|
| M-BJ | 0.10 % BJ | 0.10 % bergamot juice in tap water | Polypropylene (PP) |
| | 0.2 % CL+BJ | 0.2 % calcium lactate and 0.05% bergamot juice in tap water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |

This trial was preceded by the evaluation in vitro of the potentially microbicide action of BJ on Pseudomonas *spp*., as shown in the figure 30.

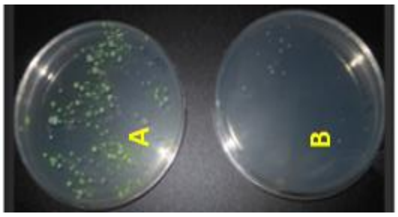



**Figure 30** Pseudomonas *spp.* growth in A (homogenate of lacto-fermented mozzarella) and B (homogenate A added with 0.1 mL BJ), in petri dishes

The counts for mesophiles are shown in table 19. The growth of Pseudomonas *spp.* was kept below 5 log10 cfu/g cycles in lacto-fermented mozzarella cheese and below 6 cycles in governing liquid at the end of shelf-life (13 days for 0.10 % BJ and control samples and 20 for 0.2% CL+BJ samples). The substitution of NaCl (as used in M-SALTS trial) with BJ and its association with calcium lactate (0.2 %) was sufficient to delay the growth of Pseudomonas *spp.* during storage, even after 13 days of storage, with growth at less than 4 log10 cfu/g. The initial load of Pseudomonas *spp.* was higher in 0.10% BJ sample compared to other theses, but at day 13 it was significantly lower ($P <$ 0.05) compared to control. 0.2% CL+BJ samples, with a higher degree of TBC and LAB at day 13, showed low pH values (table 20) at the end of storage. From this data it can be deduced that the concentrated bergamot juice at this concentration could have an inhibitory effect on Pseudomonas spp. and also on LAB. The cell numbers of the different microbial groups of the governing liquids are shown in Table 19 under the code *GL M-BJ*. Generally, the microbiological analyses on the governing liquid represented a process hygiene index during storage, with respect to the trend of mesophilic microorganisms. In table 19, some of the evaluated physicochemical properties are shown for the 3 types of mozzarella samples.

The change in pH was significantly different ($P$<0.05) after 7 days in all samples accordingly on table 19. For the acidity values no statistical differences were found among mozzarella cheese samples; however, there was an increase of this parameter in particular, at seven days as reported by Akarca and Çag˘lar (2013) in their study of mozzarella cheese stored for 28 days. In governing liquid (table 20) titratable acidity increased during the storage period with significant statistical differences at day 0, 5, 7 ($P$<0.05). This may be the result of the absence of oxygen in the packaging, producing suitable conditions for the growth of lactic acid bacteria (LAB) with a consequential increase of lactic acid and pH reduction.

The moisture content was significantly different ($P$<0.01), decreasing in time, in all samples. The higher moisture content at day 0 suggests a salting-in of the casein in the cheese and a concomitant increase in the degree of para-casein hydration during plasticization (Guinee, 2004).

Moreover, this trial revealed that changes in concentration of BJ dissolved in a governing liquid had a measurable impact on the physicochemical properties of mozzarella cheese.

In particular, the addition of 0.10% BJ determined the highest values in terms of moisture content ($P$<0.01) up to day 7. The limited changes in the colour parameters of the mozzarella samples are given in Table 19.

At the end of shelf-life, as observed in governing liquid, an increase of b* value was noted on the inner layers of all mozzarella samples, that is a sign of a gradual yellowing of the product as confirmed by Multivariate statistical analysis with a significance for time variable of ($P$<0.01). The data of the physicochemical analysis of governing liquids are summarized in Table 21. Changes in the $b^*$ values (*yellowness*) of the governing liquid samples were highlighted with significant differences among treatments ($P < 0.01$) by Anova statistical analysis.

As regards the textural parameters (Table 22), there were significant differences between the three cheese types.

The time significantly affected hardness, gumminess and resilience. Since gumminess expresses the energy required to masticate the cheese and its value is dependent on hardness and cohesiveness, it obviously should be greater in fresh cheese (Raphaelides *et al.,* 1995).

Mizuno *et al.* (2016) demonstrated that the addition of citric acid influenced the hardness of the mozzarella cheese and increasing the amount of citric acid added resulted in softer cheese.

These conclusions are concordant with the results of this trial, in which the changes in the physicochemical properties of mozzarella cheese stored in governing liquid composed of 0.10% BJ were caused by the action of the acidifier BJ.

If the application of the 0.2 % CL+ BJ obviously contributed to the increasing costs, the decrease of economic performances was less than proportionate with shelf-life extension. This aspect was treated in a study conducted in collaboration with Falcone *et al.* (2017) in which we hypothesized that the extension of 50 % of shelf-life could correspond to a reduction of 50 % of unsold produce (Figure 31).

As for the control sample, at the end of shelf-life (day 13), it presented hardness values similar to the investigated theses at previous time, but it was flattened and this could influence the hardness when maintaining this parameter at a constant value after the first period of time.

The hardness and gumminess parameters showed higher values at day 0 than at day 5. After this, the casein which is largely responsible for forming the cheese structure underwent proteolysis resulting in a weakening of the structure and softening of the cheese.

The extent of proteolysis between the three ripening periods (time 0, 13, 20) can be seen in Table 23.

When the moisture content decreased and the protein content increased, fat did not replace the moisture on an equal basis so the total filler volume was decreased (Madadlou *et al*., 2007).

Fat and moisture act as the filler in the casein matrix of cheese texture (Kahyaoglu and Kaya, 2003; Madadlou *et al.*, 2005), giving it lubricity and softness. The casein matrix provides the elastic character of cheese texture (Khosrowshahi *et al.,* 2006). The % TN was significantly influenced (P<0.05) by the treatments investigated in this trial (table 24).

**Table 18** *Microbiological analysis ($log_{10}$ cfu/g ± SD=3) of lacto-fermented mozzarella cheese and governing liquid during the refrigerated storage*

| *TIME* | *M-BJ* | *TBC* | *Pseudomonas spp.* | *LAB* |
|---|---|---|---|---|
| 0 | **0.10 % BJ** | 3.68 ± 0.71 | 1.50 ± 0.01 $^{a}$ | 2.81 ± 0.00 $^{a}$ |
| | **0.2% CL + BJ** | 3.33 ± 0.70 | 0.97 ± 0.03 $^{b}$ | 2.86 ± 0.00 $^{a}$ |
| | **CONTROL** | 3.08 ± 0.74 | 0.12 ± 0.22 $^{c}$ | 2.21 ± 0.01 $^{b}$ |
| | ***Significance*** | n.s. | ** | ** |
| 5 | **0.10 % BJ** | 5.30 ± 0.71 | 4.08 ± 0.07 $^{a}$ | 4.88 ± 0.04 $^{b}$ |
| | **0.2% CL + BJ** | 5.60 ± 0.72 | 3.37 ± 0.12 $^{b}$ | 5.66 ± 0.07 $^{a}$ |
| | **CONTROL** | 6.03 ± 0.71 | 4.09 ± 0.01 $^{a}$ | 5.18 ± 0.11 $^{b}$ |
| | ***Significance*** | n.s. | ** | ** |
| 7 | **0.10 % BJ** | 6.45 ± 0.00 $^{b}$ | 3.67 ± 0.00 | 5.99 ± 0.00 $^{b}$ |
| | **0.2% CL + BJ** | 6.75 ± 0.01 $^{a}$ | 3.62 ± 0.07 | 6.13 ± 0.03 $^{a}$ |
| | **CONTROL** | 6.31 ± 0.02 $^{c}$ | 3.60 ± 0.02 | 5.29 ± 0.04 $^{c}$ |
| | ***Significance*** | ** | n.s. | ** |
| 13 | **0.10 % BJ** | 6.20 ± 0.01 $^{c}$ | 3.96 ± 0.03 $^{b}$ | 6.00 ± 0.03 $^{c}$ |
| | **0.2% CL + BJ** | 6.98 ± 0.05 $^{a}$ | 4.05 ± 0.03 $^{ab}$ | 6.94 ± 0.01 $^{a}$ |
| | **CONTROL** | 6.66 ± 0.03 $^{b}$ | 4.11 ± 0.02 $^{a}$ | 6.34 ± 0.02 $^{b}$ |
| | ***Significance*** | ** | * | ** |
| 20 | **0.2% CL + BJ** | 6.63 ± 0.01 | 3.87 ± 0.02 | 6.48 ± 0.00 |

| *TIME* | *GL (M-BJ)* | *TBC* | *Pseudomonas spp.* | *LAB* |
|---|---|---|---|---|
| 0 | **0.10 % BJ** | 4.06 ± 0.00 $^{c}$ | 1.39 ± 0.12 $^{c}$ | 4.02 ± 0.00 $^{a}$ |
| | **0.2% CL + BJ** | 4.23 ± 0.02 $^{b}$ | 1.74 ± 0.06 $^{b}$ | 3.68 ± 0.03 $^{b}$ |
| | **CONTROL** | 4.56 ± 0.01 $^{a}$ | 2.39 ± 0.01 $^{a}$ | 3.44 ± 0.06 $^{c}$ |
| | ***Significance*** | ** | ** | ** |
| 5 | **0.10 % BJ** | 5.72 ± 0.02 $^{c}$ | 5.23 ± 0.00 $^{c}$ | 5.37 ± 0.01 $^{c}$ |
| | **0.2% CL + BJ** | 6.51 ± 0.01 $^{b}$ | 5.37 ± 0.01 $^{b}$ | 6.24 ± 0.09 $^{a}$ |
| | **CONTROL** | 6.77 ± 0.00 $^{a}$ | 5.91 ± 0.00 $^{a}$ | 5.66 ± 0.01 $^{b}$ |
| | ***Significance*** | ** | ** | ** |
| 7 | **0.10 % BJ** | 7.04 ± 0.01 $^{b}$ | 5.63 ± 0.01 $^{b}$ | 6.74 ± 0.01 $^{a}$ |
| | **0.2% CL + BJ** | 6.72 ± 0.01 $^{c}$ | 5.52 ± 0.01 $^{c}$ | 6.67 ± 0.01 $^{b}$ |
| | **CONTROL** | 7.08 ± 0.01 $^{a}$ | 6.31 ± 0.01 $^{a}$ | 6.46 ± 0.02 $^{c}$ |
| | ***Significance*** | ** | ** | ** |
| 13 | **0.10 % BJ** | 7.60 ± 0.02 $^{b}$ | 5.82 ± 0.01 $^{b}$ | 7.32 ± 0.03 $^{b}$ |
| | **0.2% CL + BJ** | 7.65 ± 0.01 $^{a}$ | 5.69 ± 0.01 $^{c}$ | 7.76 ± 0.01 $^{a}$ |
| | **CONTROL** | 7.68 ± 0.01 $^{a}$ | 6.09 ± 0.02 $^{a}$ | 7.06 ± 0.03 $^{c}$ |
| | ***Significance*** | * | ** | ** |
| 20 | **0.2% CL + BJ** | 7.49 ± 0.02 | 5.29 ± 0.02 | 7.35 ± 0.01 |

**Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*

**Table 19** *Physicochemical analysis results of lacto-fermented mozzarella cheese*

| *TIME* | *M-BJ* | $a_w$ | *pH* | *% LACTIC ACID* | *% NaCl* | *Moisture %* |
|---|---|---|---|---|---|---|
| 0 | **0.10 % BJ** | 0.963 ± 0.001 | 5.95 ± 0.03 | 0.15 ± 0.02 | 2.40 ± 0.10 $^{b}$ | 82.13 ± 0.00 $^{c}$ |
| | **0.2% CL+BJ** | 0.961 ± 0.000 | 5.99 ± 0.01 | 0.11 ± 0.00 | 2.69 ± 0.10 $^{b}$ | 76.96 ± 0.00 $^{b}$ |
| | **CONTROL** | 0.962 ± 0.000 | 5.98 ± 0.02 | 0.17 ± 0.02 | 3.41 ± 0.10 $^{a}$ | 79.62 ± 0.00 $^{a}$ |
| | ***Significance*** | n.s. | n.s. | n.s. | ** | ** |
| 5 | **0.10 % BJ** | 0.973 ± 0.001 | 5.99 ± 0.02 $^{a}$ | 0.17 ± 0.02 | 1.96 ± 0.10 $^{b}$ | 66.89 ± 0.03 $^{a}$ |
| | **0.2% CL+BJ** | 0.972 ± 0.000 | 5.83 ± 0.01 $^{c}$ | 0.19 ± 0.02 | 1.38 ± 0.10 $^{b}$ | 65.88 ± 0.05 $^{b}$ |
| | **CONTROL** | 0.971 ± 0.001 | 5.88 ± 0.00 $^{b}$ | 0.23 ± 0.02 | 3.62 ± 0.21 $^{a}$ | 64.34 ± 0.00 $^{c}$ |
| | ***Significance*** | n.s. | ** | n.s. | ** | ** |
| 7 | **0.10 % BJ** | 0.973 ± 0.001 $^{a}$ | 5.81 ± 0.01 $^{a}$ | 0.23 ± 0.02 | 7.68 ± 0.11 $^{a}$ | 67.70 ± 0.01 $^{a}$ |
| | **0.2% CL+BJ** | 0.973 ± 0.000 $^{a}$ | 5.76 ± 0.01 $^{b}$ | 0.20 ± 0.00 | 2.83 ± 0.10 $^{c}$ | 63.44 ± 0.02 $^{b}$ |
| | **CONTROL** | 0.970 ± 0.000 $^{b}$ | 5.77 ± 0.01 $^{b}$ | 0.24 ± 0.01 | 6.39 ± 0.20 $^{b}$ | 63.22 ± 0.01 $^{c}$ |
| | ***Significance*** | ** | * | n.s. | ** | ** |
| 13 | **0.10 % BJ** | 0.974 ± 0.000 | 5.82 ± 0.03 $^{a}$ | 0.13 ± 0.00 $^{b}$ | 10.79 ± 0.02 $^{a}$ | 62.90 ± 0.00 $^{b}$ |
| | **0.2% CL+BJ** | 0.973 ± 0.001 | 5.71 ± 0.01 $^{b}$ | 0.14 ± 0.02 $^{b}$ | 3.52 ± 0.10 $^{c}$ | 62.13 ± 0.00 $^{c}$ |
| | **CONTROL** | 0.974 ± 0.001 | 5.76 ± 0.00 $^{ab}$ | 0.19 ± 0.02 $^{a}$ | 8.47 ± 0.08 $^{b}$ | 64.12 ± 0.05 $^{a}$ |
| | ***Significance*** | n.s. | * | * | ** | ** |
| 20 | **0.2% CL+BJ** | 0.967 ± 0.001 | 5.56 ± 0.01 | 0.31 ± 0.00 | 0.92 ± 0.10 | 64.05 ± 0.00 |

| | | *Outer* | | | *Inner* | | |
|---|---|---|---|---|---|---|---|
| ***TIME*** | ***M-BJ*** | **L*** | **a*** | **b*** | **L*** | **a*** | **b*** |
| **0** | **0.10 % BJ** | 93.99 ± 1.10 | -0.41 ± 0.08 | 6.56 ± 0.36 | 93.41 ± 1.49 $^{ab}$ | -0.50 ± 0.22 | 7.26 ± 0.96 |
| | **0.2% CL+BJ** | 93.40 ± 1.95 | -0.34 ± 0.16 | 6.38 ± 0.47 | 95.47 ± 1.15 $^{a}$ | -0.28 ± 0.04 | 6.25 ± 0.32 |
| | **CONTROL** | 94.18 ± 0.96 | -0.35 ± 0.08 | 6.00 ± 0.72 | 93.02 ± 1.57 $^{b}$ | -0.41 ± 0.08 | 7.20 ± 1.43 |
| | ***Significance*** | n.s. | n.s. | n.s. | * | n.s. | n.s. |
| **5** | **0.10 % BJ** | 94.67 ± 0.81 | -0.30 ± 0.22 | 5.51 ± 1.28 | 93.61 ± 1.43 | -0.23 ± 0.20 | 6.95 ± 1.03 |
| | **0.2% CL+BJ** | 94.89 ± 0.28 | -0.30 ± 0.16 | 5.76 ± 0.42 | 93.11 ± 1.82 | -0.44 ± 0.18 | 8.01 ± 1.32 |
| | **CONTROL** | 93.68 ± 1.21 | -0.06 ± 0.25 | 6.21 ± 1.16 | 93.21 ± 2.59 | -0.38 ± 0.05 | 6.97 ± 1.19 |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| **7** | **0.10 % BJ** | 94.21 ± 3.25 | -0.42 ± 0.16 | 2.82 ± 5.43 | 92.51 ± 1.66 $^{b}$ | -0.48 ± 0.11 $^{b}$ | 8.85 ± 1.33 $^{a}$ |
| | **0.2% CL+BJ** | 94.84 ± 0.35 | -0.32 ± 0.04 | 5.68 ± 0.21 | 94.83 ± 0.11 $^{a}$ | -0.25 ± 0.01 $^{a}$ | 6.47 ± 0.23 $^{b}$ |
| | **CONTROL** | 94.44 ± 1.09 | -0.36 ± 0.08 | 6.06 ± 0.96 | 94.15 ± 1.35 $^{ab}$ | -0.29 ± 0.08 $^{a}$ | 6.99 ± 1.12 $^{b}$ |
| | ***Significance*** | n.s. | n.s. | n.s. | * | ** | ** |
| **13** | **0.10 % BJ** | 95.75 ± 1.66 | -0.58 ± 0.10 | 6.39 ± 1.63 | 93.65 ± 1.18 | -0.59 ± 0.20 | 7.85 ± 1.05 $^{b}$ |
| | **0.2% CL+BJ** | 94.89 ± 0.65 | -0.44 ± 0.06 | 6.53 ± 0.33 | 92.29 ± 1.13 | -0.49 ± 0.14 | 9.11 ± 0.46 $^{a}$ |
| | **CONTROL** | 94.29 ± 1.45 | -0.46 ± 0.13 | 6.26 ± 0.74 | 92.49 ± 1.43 | -0.63 ± 0.19 | 8.40 ± 0.53 $^{ab}$ |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. | * |
| **20** | **0.2% CL+BJ** | 95.66 ± 0.36 | -0.42 ± 0.02 | 5.87 ± 0.62 | 94.98 ± 1.30 | -0.50 ± 0.06 | 7.45 ± 0.81 |

**Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*

***Table 20*** *Physicochemical properties of governing liquid in trial M-BJ*

| *TIME* | *GL (M-BJ)* | *pH* | *% LACTIC ACID* | *% NaCl* | *L** | *a** | *b** |
|---|---|---|---|---|---|---|---|
| 0 | **0.10 % BJ** | 4.86 ± 0.17 c | 0.03 ± 0.00 a | 0.26 ± 0.01 a | 32.86 ± 0.05 b | -0.02 ± 0.02 ab | 0.40 ± 0.03 b |
| | **0.2% CL + BJ** | 5.89 ± 0.06 b | 0.01 ± 0.00 b | 0.13 ± 0.00 b | 32.87 ± 0.11 b | -0.01 ± 0.01 ab | 0.45 ± 0.02 a |
| | **CONTROL** | 6.68 ± 0.10 a | 0.02 ± 0.00 ab | 0.14 ± 0.01 b | 33.48 ± 0.08 a | -0.03 ± 0.01 b | -0.07 ± 0.03 c |
| | ***Significance*** | ** | * | ** | ** | * | ** |
| 5 | **0.10 % BJ** | 5.43 ± 0.19 | 0.08 ± 0.00 b | 0.28 ± 0.01 c | 34.84 ± 0.05 a | -0.09 ± 0.04 | 0.20 ± 0.02 b |
| | **0.2% CL + BJ** | 5.49 ± 0.02 | 0.10 ± 0.00 ab | 0.23 ± 0.01 b | 33.91 ± 0.06 b | -0.04 ± 0.02 | 0.54 ± 0.01 a |
| | **CONTROL** | 5.64 ± 0.04 | 0.11 ± 0.00 a | 0.39 ± 0.01 a | 34.88 ± 0.14 a | -0.06 ± 0.03 | 0.02 ± 0.05 c |
| | ***Significance*** | n.s. | * | ** | ** | n.s. | ** |
| 7 | **0.10 % BJ** | 5.14 ± 0.01 c | 0.12 ± 0.00 b | 0.27 ± 0.01 | 32.19 ± 0.92 b | 0.06 ± 0.02 ab | 1.10 ± 0.05 a |
| | **0.2% CL + BJ** | 5.27 ± 0.01 a | 0.15 ± 0.00 a | 0.28 ± 0.00 | 32.73 ± 0.40 b | 0.07 ± 0.02 ab | 0.84 ± 0.11 b |
| | **CONTROL** | 5.19 ± 0.01 b | 0.16 ± 0.00 a | 0.28 ± 0.01 | 34.40 ± 0.15 a | -0.01 ± 0.01 b | 0.13 ± 0.06 c |
| | ***Significance*** | ** | * | n.s. | ** | ** | ** |
| 13 | **0.10 % BJ** | 5.02 ± 0.01 c | 0.20 ± 0.00 | 0.31 ± 0.01 c | 31.45 ± 0.14 b | -0.02 ± 0.02 | 0.46 ± 0.04 a |
| | **0.2% CL + BJ** | 5.11 ± 0.01 a | 0.20 ± 0.00 | 0.36 ± 0.01 b | 31.23 ± 0.20 b | -0.01 ± 0.04 | 0.29 ± 0.05 b |
| | **CONTROL** | 5.06 ± 0.01 b | 0.20 ± 0.00 | 0.42 ± 0.01 a | 31.93 ± 0.18 a | -0.02 ± 0.03 | 0.27 ± 0.03 b |
| | ***Significance*** | ** | n.s. | ** | ** | n.s. | ** |
| 20 | **0.2% CL + BJ** | 5.06 ± 0.04 | 0.23 ± 0.00 | 0.62 ± 0.01 | 32.81 ± 0.02 | -0.09 ± 0.01 | -0.44 ± 0.03 |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

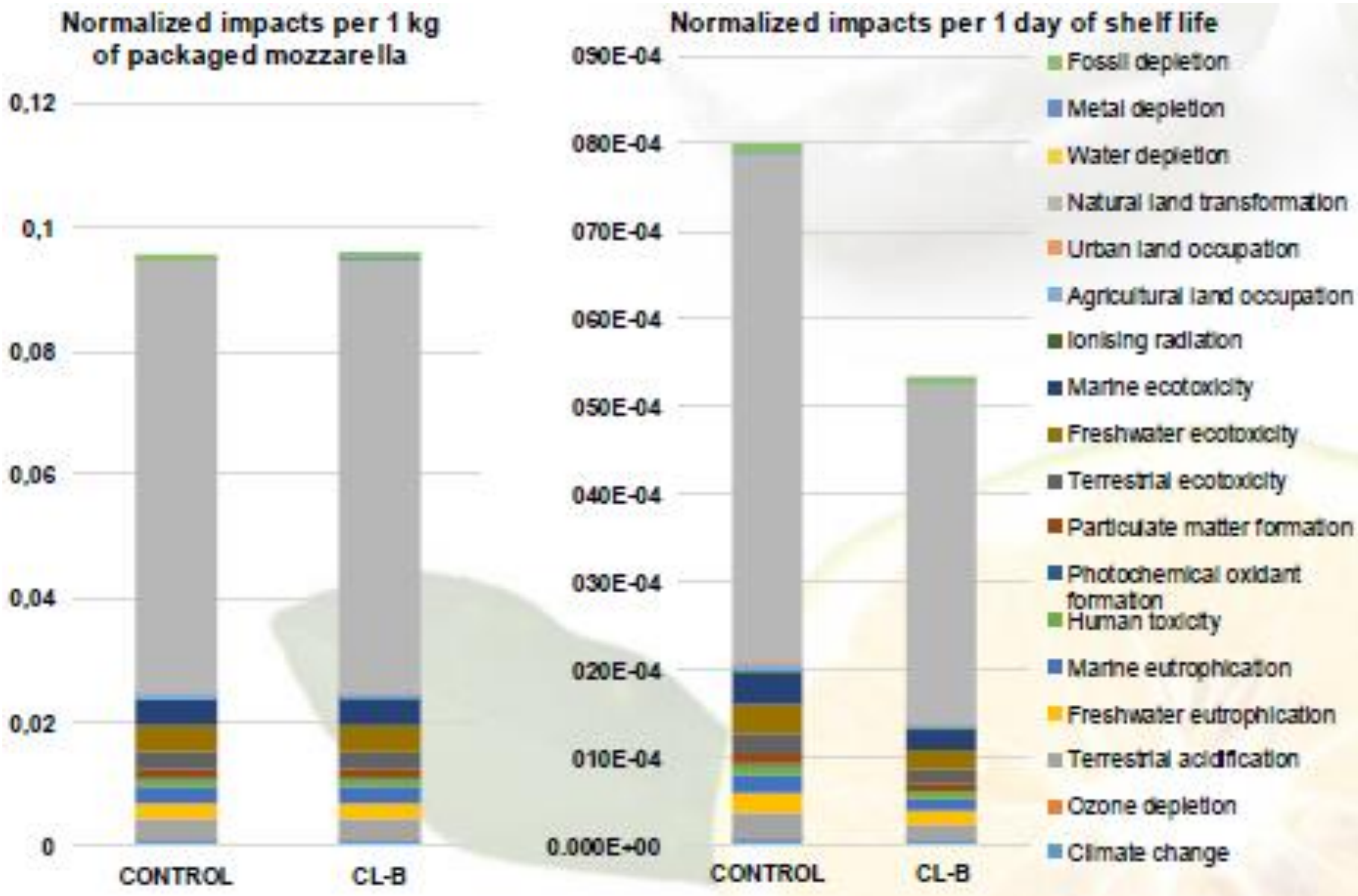



**Figure 31** Normalized Life Cycle Impact Assessment results (Falcone *et al.*, 2017)

**Table 21** *TPA of lacto-fermented mozzarella cheese*

| TIME | M-BJ | Hardness (g) | Adhesiveness (g*sec.) | Springiness | Cohesiveness | Gumminess | Chewiness | Resilience |
|---|---|---|---|---|---|---|---|---|
| 0 | **0.10 % BJ** | 7071.93 ± 0.04 c | -6.37 ± 0.01 b | 0.86 ± 0.00 a | 0.81 ± 0.00 c | 5721.72 ± 0.73 c | 4947.17 ± 0.71 c | 0.47 ± 0.00 b |
| | **0.2% CL + BJ** | 8842.32 ± 2.99 b | -15.53 ± 0.11 c | 0.85 ± 0.00 b | 0.77 ± 0.00 b | 6843.95 ± 14.82 b | 5783.16 ± 31.88 a | 0.46 ± 0.00 a |
| | **CONTROL** | 9271.54 ± 2.14 a | -1.65 ± 0.21 a | 0.86 ± 0.00 a | 0.82 ± 0.00 a | 7575.50 ± 1.43 a | 6494.15 ± 6.36 b | 0.48 ± 0.00 c |
| | ***Significance*** | ** | ** | ** | ** | ** | ** | ** |
| 5 | **0.10 % BJ** | 4295.31 ± 2.13 b | -6.13 ± 0.03 c | 0.89 ± 0.01 a | 0.78 ± 0.01 a | 3365.33 ± 7.14 b | 2997.39 ± 0.81 b | 0.48 ± 0.00 a |
| | **0.2% CL + BJ** | 6352.31 ± 0.70 a | -5.07 ± 0.06 b | 0.86 ± 0.01 b | 0.75 ± 0.01 b | 4762.55 ± 0.71 a | 4123.85 ± 1.48 a | 0.45 ± 0.00 c |
| | **CONTROL** | 2687.06 ± 0.71 c | -2.20 ± 0.01 a | 0.89 ± 0.01 a | 0.77 ± 0.01 ab | 2075.07 ± 0.01 c | 1825.16 ± 0.79 c | 0.46 ± 0.00 b |
| | ***Significance*** | ** | ** | * | * | ** | ** | ** |
| 7 | **0.10 % BJ** | 4524.65 ± 2.90 a | -6.98 ± 0.01 | 0.87 ± 0.01 | 0.77 ± 0.00 b | 3271.19 ± 1.42 a | 3021.38 ± 0.01 | 0.47 ± 0.00 ab |
| | **0.2% CL + BJ** | 3459.29 ± 286.83 b | -4.21 ± 4.64 | 0.93 ± 0.04 | 0.81 ± 0.00 a | 2805.02 ± 216.86 a | 2607.79 ± 310.87 | 0.49 ± 0.01 a |
| | **CONTROL** | 3412.11 ± 140.71 b | -15.84 ± 10.61 | 0.92 ± 0.01 | 0.74 ± 0.00 c | 2100.09 ± 0.59 b | 2722.63 ± 77.84 | 0.45 ± 0.00 b |
| | ***Significance*** | * | n.s. | n.s. | ** | ** | n.s. | * |
| 13 | **0.10 % BJ** | 1664.54 ± 0.71 c | -2.79 ± 0.00 | 0.88 ± 0.00 a | 0.77 ± 0.00 | 1276.00 ± 0.71 c | 1123.39 ± 7.07 c | 0.42 ± 0.00 b |
| | **0.2% CL + BJ** | 4309.27 ± 14.66 a | -6.50 ± 2.45 | 0.85 ± 0.02 a | 0.77 ± 0.01 | 3307.89 ± 66.11 a | 2822.88 ± 14.65 a | 0.45 ± 0.02 ab |
| | **CONTROL** | 3473.21 ± 0.71 b | -6.23 ± 0.00 | 0.79 ± 0.00 b | 0.79 ± 0.00 | 2518.08 ± 28.25 b | 2067.41 ± 2.12 b | 0.48 ± 0.00 a |
| | ***Significance*** | ** | n.s. | * | n.s. | ** | ** | * |
| 20 | **0.2% CL + BJ** | 4741.87 ± 59.70 | -5.84 ± 0.93 | 0.86 ± 0.00 | 0.77 ± 0.00 | 3662.58 ± 45.76 | 3156.61 ± 25.13 | 0.45 ± 0.00 |

**Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*

**Table 22** *Nitrogen determination between the three ripening periods (time 0, 13, 20)*

| *M-BJ* | Time | 0.10 % BJ | 0.2 % CL+ BJ | CONTROL |
|---|---|---|---|---|
| **Protein ($g*100g^{-1}$)** | $t_0$ | 15.02 ± 0.05 | 17.35 ± 0.90 | 15.15 ± 0.05 |
| | $t_{13}$ | 15.63 ± 0.18 | 16.08 ± 0.63 | 15.95 ± 0.45 |
| | $t_{20}$ | n.d. | 15.89 ± 0.59 | n.d. |
| ***Significance*** | | * | n.s. | n.s. |
| **% TN** | $t_0$ | 2.36 ± 0.01 | 2.72 ± 0.14 | 2.38 ± 0.01 |
| | $t_{13}$ | 2.46 ± 0.03 | 2.49 ± 0.10 | 2.43 ± 0.07 |
| | $t_{20}$ | n.d. | 2.56 ± 0.09 | n.d. |
| ***Significance*** | | * | n.s. | n.s. |
| **% SN/TN** | $t_0$ | 0.47 ± 0.00 | 0.34 ± 0.01 b | 0.55 ± 0.01 |
| | $t_{13}$ | 0.50 ± 0.04 | 0.41 ± 0.00 a | 0.57 ± 0.05 |
| | $t_{20}$ | n.d. | 0.45 ± 0.02 a | n.d. |
| ***Significance*** | | n.s. | ** | n.s. |
| **% SN-TCA/TN** | $t_0$ | 0.12 ± 0.00 | 0.11 ± 0.01 | 0.13 ± 0.00 |
| | $t_{13}$ | 0.13 ± 0.00 | 0.13 ± 0.00 | 0.13 ± 0.01 |
| | $t_{20}$ | n.d. | 0.12 ± 0.00 | n.d. |
| ***Significance*** | | n.s. | n.s. | n.s. |

**Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

**Table 23** *Multivariate analysis results for Total Nitrogen (TN) and Not Protein Nitrogen content (P<0.05) related to the effect of alternative treatments and storage time*

| *M-BJ TRIAL* | Treatment | Time | Treatment *x* Time |
|---|---|---|---|
| **% TN** | 0.014 | 0.689 | 0.042 |
| **% SN/TN** | 0.000 | 0.015 | 0.479 |
| **% SN-TCA/TN** | 0.059 | 0.120 | 0.217 |

### 4.3 M-PLA Results and Discussions

The results of TBC in M-PLA experimental trial (storage time: from 0 to 19 days, table 25) showed highly significant differences between mozzarella samples with the proposed governing liquids and the control at 9 days (P<0.01) with the following values: 6.55 ± 0.02 $\log_{10}$ cfu/g (0.6% CL) and 6.67 ± 0.01 $\log_{10}$ cfu/g (0.6% CL + BJ) 7.31 ± 0.00 $\log_{10}$ cfu/g (control samples in PLA) as reported in Table 26.

**Table 24** *Experimental trial M-PLA*

| PRODUCTION LOT | CODE SAMPLES | GOVERNING LIQUID COMPOSITION | PACKAGING MATERIAL |
|---|---|---|---|
| M-PLA | 0.6 % CL PLA | 0.6 % calcium lactate in tap water | Polylactide (PLA) |
| | 0.6 % CL+ BJ PLA | 0.6 % calcium lactate and 0.05 % bergamot juice in tap water | Polylactide (PLA) |
| | Control PLA | tap water in PLA trays | Polylactide (PLA) |

Instead, statistical differences were not found for TBC (*P*>0.05) in the governing liquids (table 26). For the lactic bacteria growth statistically significant differences were observed only after 7 days of storage in both mozzarella and governing liquid samples: 6.17 ± 0.01 $\log_{10}$ cfu/g (0.6% CL) and 6.61 ± 0.02 $\log_{10}$ cfu/g (0.6% CL + BJ) and the 6.44 ± 0.10 $\log_{10}$ cfu / g (control sample PLA); moreover, in governing liquid, 5.37 ± 0.04 $\log_{10}$ cfu/g (0.6% CL), 5.46 ± 0.04 $\log_{10}$ cfu/g (0.6% CL + BJ) and 5.52 ± 0.03 $\log_{10}$ cfu/g (control PLA).

At 9 days highly significant differences (*P*<0.01) were observed in the Pseudomonas *spp.* growth with the lowest value in CL 0.6%+BJ mozzarella cheese (4.34 ± 0.06 $\log_{10}$ cfu/g).

This sample obtained a reduction of about one $\log_{10}$ cfu/g in the microbial load compared to others.

It also evaluated the lacto-fermented mozzarella stored in tap water and packaged in Polypropylene (PP), only at day 0 and 14.

TBC values of the control sample packaged in PP were 3.65 ± 0.05 at initial time (0 day) and 7.98 ± 0.33 $\log_{10}$ cfu/g at the end (14 days).

The LAB values of the samples control in PP of governing liquid and mozzarella cheese at day 0 were respectively 5.28 ± 0.01 and 3.14.± 0.01 $\log_{10}$ cfu/g, at the end (14 days) were respectively 8.32 ± 0.27 and 6.70.± 0.15 $\log_{10}$ cfu/g.

In this trial one of the most important structural modifications was the softening of the samples control PP (figure 32) that can be related after production.

**Table 25** *Microbiological analysis ($log_{10}$ cfu/g ± SD=3) of lacto-fermented mozzarella cheese and governing liquid during the refrigerated storage at 5°C*

| *TIME* | *M-PLA* | *TBC* | *Pseudomonas spp.* | *LAB* |
|---|---|---|---|---|
| **0** | **Mozzarella cheese** | **3.65 ± 0.05** | **1.08 ± 0.82** | **3.14 ± 0.01** |
| **2** | **0.6 % CL** | 4.07 ± 0.71 | 2.31 ± 0.16 | 3.81 ± 0.02 |
| | **0.6% CL + BJ** | 4.07 ± 0.63 | 2.29 ± 0.17 | 3.99 ± 0.19 |
| | **Control PLA** | 4.14 ± 0.75 | 2.37 ± 0.17 | 4.23 ± 0.18 |
| | ***Significance*** | n.s. | n.s. | n.s. |
| **7** | **0.6 % CL** | 4.67 ± 0.71 | 4.33 ± 0.03 $^{b}$ | 6.17 ± 0.01 $^{b}$ |
| | **0.6% CL + BJ** | 4.67 ± 0.73 | 4.45 ± 0.00 $^{b}$ | 6.61 ± 0.02 $^{a}$ |
| | **Control PLA** | 4.87 ± 0.73 | 4.82 ± 0.08 $^{a}$ | 6.44 ± 0.10 $^{a}$ |
| | ***Significance*** | n.s. | ** | * |
| **9** | **0.6 % CL** | 6.55 ± 0.02 | 5.30 ± 0.03 $^{a}$ | 6.36 ± 0.02 |
| | **0.6% CL + BJ** | 6.67 ± 0.01 | 4.34 ± 0.06 $^{b}$ | 6.26 ± 0.01 |
| | **Control PLA** | 7.31 ± 0.00 | 5.28 ± 0.05 $^{a}$ | 6.47 ± 0.10 |
| | ***Significance*** | ** | ** | n.s. |
| **14** | **0.6 % CL** | 7.61 ± 0.72 | 4.97 ± 0.09 | 6.65 ± 0.21 |
| | **0.6% CL + BJ** | 7.62 ± 1.02 | 4.99 ± 0.24 | 6.85 ± 0.23 |
| | **Control PLA** | 7.90 ± 0.53 | 5.23 ± 0.04 | 7.32 ± 0.08 |
| | ***Significance*** | n.s. | n.s. | n.s. |
| **19** | **0.6 % CL** | 8.30 ± 0.71 | 6.00 ± 0.01 | 7.33 ± 0.01 |
| | **0.6% CL + BJ** | 8.19 ± 0.71 | 5.89 ± 0.00 | 7.33 ± 0.01 |
| | **Control PLA** | n.d. | n.d. | n.d. |
| | ***Significance*** | n.s. | ** | n.s. |

**M-PLA**

| *TIME* | *GL (M-PLA)* | *TBC* | *Pseudomonas spp.* | *LAB* |
|---|---|---|---|---|
| **0** | **Governing liquid** | **6.30 ± 0.06** | **3.20 ± 0.03** | **5.28 ± 0.01** |
| **2** | **0.6 % CL** | 4.19 ± 0.19 | 2.90 ± 0.06 | 4.59 ± 0.04 |
| | **0.6% CL + BJ** | 3.93 ± 0.64 | 2.85 ± 0.43 | 4.51 ± 0.23 |
| | **Control PLA** | 3.96 ± 0.20 | 2.85 ± 0.41 | 4.54 ± 0.08 |
| | ***Significance*** | n.s. | n.s. | n.s. |
| **7** | **0.6 % CL** | 6.56 ± 0.04 | 5.24 ± 0.02 $^{b}$ | 5.37 ± 0.01 $^{b}$ |
| | **0.6% CL + BJ** | 6.62 ± 0.04 | 5.32 ± 0.02 $^{b}$ | 5.46 ± 0.01 $^{ab}$ |
| | **Control PLA** | 6.71 ± 0.03 | 5.58 ± 0.01 $^{a}$ | 5.52 ± 0.04 $^{a}$ |
| | ***Significance*** | n.s. | ** | * |
| **9** | **0.6 % CL** | 8.35 ± 0.06 | 7.20 ± 0.07 $^{b}$ | 5.89 ± 0.37 |
| | **0.6% CL + BJ** | 8.34 ± 0.01 | 6.94 ± 0.02 $^{a}$ | 6.14 ± 0.03 |
| | **Control PLA** | 8.44 ± 0.01 | 7.30 ± 0.03 $^{a}$ | 6.04 ± 0.00 |
| | ***Significance*** | n.s. | * | n.s. |
| **14** | **0.6 % CL** | 8.35 ± 0.24 | 7.11 ± 0.09 $^{a}$ | 8.06 ± 0.02 |
| | **0.6% CL + BJ** | 7.85 ± 0.57 | 6.58 ± 0.03 $^{b}$ | 7.91 ± 0.17 |
| | **Control PLA** | 8.13 ± 1.11 | 7.10 ± 0.06 $^{a}$ | 7.47 ± 0.29 |
| | ***Significance*** | n.s. | * | n.s. |
| **19** | **0.6 % CL** | 8.26 ± 0.00 | 7.66 ± 0.01 | 8.15 ± 0.00 |
| | **0.6% CL + BJ** | 8.19 ± 0.02 | 7.45 ± 0.01 | 8.17 ± 0.01 |
| | **Control PLA** | n.d. | n.d. | n.d. |
| | ***Significance*** | * | ** | n.s. |

**Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test.*


**Figure 32** TPA analysis (14 days)

The control samples in PP reported pH values at day 1 of 5.79 ±0.01 and at the end of shelf-life (14 days) of 5.88 ± 0.02; while % moisture was of 57.81 ± 0.13 (time 1) and 67.38 ± 2.19 (time 14). More proteolysis occurred in mozzarella cheeses with higher moisture content, in line with Zisu and Shah (2005). Proteolysis evolved similarly in the 4 samples, showing a moderate increase of % SN/TN (table 27).

**Table 26** *Results of Protein, %TN,% SN/TN and %SN-TCA/TN parameters*

| **M-PLA** | **Time** | **0.6 % CL** | **0.6 % CL+ BJ** | **CONTROL PLA** | **CONTROL PP** |
|---|---|---|---|---|---|
| **Protein (g*100g $^{-1}$)** | $t_1$ | 16.11 ± 0.04 | 15.76 ± 0.98 | 15.64 ±0.89 | 15.95 ± 0.44 |
| | $t_{14}$ | 15.54 ± 0.49 | 15.73 ± 0.40 | 15.86 ±0.31 | 16.01 ± 0.44 |
| ***Significance*** | | n.s. | n.s. | n.s. | n.s. |
| **% TN** | $t_1$ | 2.55 ± 0.04 | 2.51 ± 0.16 | 2.49 ± 0.14 | 2.54 ± 0.07 |
| | $t_{14}$ | 2.48 ± 0.08 | 2.51 ± 0.06 | 2.53 ± 0.05 | 2.55 ± 0.07 |
| ***Significance*** | | n.s. | n.s. | n.s. | n.s. |
| **% SN/TN** | $t_1$ | 0.12 ± 0.00 | 0.10 ± 0.02 | 0.14 ± 0.04 | 0.18 ± 0.02 |
| | $t_{14}$ | 0.24 ± 0.01 | 0.30 ± 0.02 | 0.32 ± 0.06 | 0.57 ± 0.04 |
| ***Significance*** | | ** | * | n.s. | ** |
| **% SN-TCA/TN** | $t_1$ | 0.08 ± 0.00 | 0.09 ± 0.00 | 0.11 ± 0.01 | 0.11 ± 0.01 |
| | $t_{14}$ | 0.11 ± 0.01 | 0.11 ± 0.00 | 0.12 ± 0.01 | 0.18 ± 0.02 |
| ***Significance*** | | * | * | n.s. | n.s. |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

Multivariate analysis (table 28) shows that treatments and time significantly influenced (*P*<0.01) non-protein nitrogen content indexes.

**Table 27** *P-values of the percentage Total Nitrogen (TN) and Not Protein Nitrogen content (expressed as SN/TN and SN-TCA/TN) from multivariate analysis (P<0.05) related to the effect of alternative treatments and storage time*

| ***M-PLA TRIAL*** | **Treatment** | **Time** | **Treatment *x* Time** |
|---|---|---|---|
| **% TN** | 0.921 | 0.875 | 0.869 |
| **% SN/TN** | 0.000 | 0.000 | 0.002 |
| **% SN-TCA/TN** | 0.000 | 0.000 | 0.019 |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

The physicochemical properties of mozzarella cheese is given in Table 29.

The % of NaCl is linked to changes in the water content of cheese (Payne and Morison, 1999). The distribution of water in lacto-fermented mozzarella is determined by the parallel protein fibers resulting from the stretching process (Kuo *et al.,* 2003) and water undergoes a continuous rearrangement during the storage period (McMahon *et al.,* 1999).

The moisture range of lacto-fermented mozzarella cheese is consistent of 56.4 - 67.4%, as documented (Nguyena *et al.,* 2017).

Only the pH value varied with any significance (*P* <0.05) among samples at 14 days. The addition of calcium probably better maintained pH values during the storage compared to the control samples in PLA. In fact, as reported by Luo *et al.* (2013), the addition of calcium restores the chemical equilibrium.

The adhesiveness parameter varied with significance (*P*<0.01) at 14 days. The adhesiveness parameter varied during the time because the adhesion forces of samples control become larger than the cohesion forces, as studied by Sherman (1970).

The decline of TPA parameters (Table 30) with increasing proteolysis is certainly a reflection of casein network loosening. The relationship among some chemical and textural parameters at the end (14 days) were established by Pearson's correlation coefficients: among the several parameters the hardness had a high positive correlation with dry matter (r = +0.996 P <0.01).

The mechanical behaviour of mozzarella cheese samples depends mainly on the resistance to the deformation of the casein (Bertola *et al.,* 1996).

**Table 28** *Physicochemical analysis results of lacto-fermented mozzarella and governing liquid in M- PLA trial*

| *TIME* | *M-PLA* | *$a_w$* | *pH* | *% Lactic Acid* | *% NaCl* | *Moisture %* |
|---|---|---|---|---|---|---|
| **0** | **Mozzarella cheese** | **0.977 ± 0.001** | **5.79 ± 0.01** | **0.25 ± 0.03** | **1.02 ± 0.21** | **57.81 ± 0.13** |
| **2** | **CL 0.6 %** | 0.978 ± 0.000 | 5.57 ± 0.13 | 0.25 ± 0.03 | 1.02 ± 0.21 | 61.78 ± 0.49 |
| | **CL 0.6 % + BJ** | 0.978 ± 0.001 | 5.73 ± 0.09 | 0.29 ± 0.03 | 0.87 ± 0.41 | 61.79 ± 1.43 |
| | **Control PLA** | 0.978 ± 0.000 | 5.78 ± 0.01 | 0.27 ± 0.06 | 0.87 ± 0.00 | 57.54 ± 8.85 |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. |
| **7** | **CL 0.6 %** | 0.979 ± 0.001 | 5.66 ± 0.05 [b] | 0.27 ± 0.00 | 1.15 ± 0.41 | 60.22 ± 0.80 |
| | **CL 0.6 % + BJ** | 0.973 ± 0.007 | 5.68 ± 0.01 [b] | 0.22 ± 0.00 | 0.85 ± 0.00 | 60.29 ± 0.26 |
| | **Control PLA** | 0.979 ± 0.001 | 5.84 ± 0.01 [a] | 0.22 ± 0.00 | 0.72 ± 0.20 | 61.35 ± 0.74 |
| | ***Significance*** | n.s. | * | n.s. | n.s. | n.s. |
| **9** | **CL 0.6 %** | 0.980 ± 0.000 | 5.93 ± 0.11 | 0.18 ± 0.00 | 0.72 ± 0.20 | 64.89 ± 0.73 |
| | **CL 0.6 % + BJ** | 0.979 ± 0.001 | 5.78 ± 0.06 | 0.17 ± 0.06 | 1.13 ± 0.40 | 63.67 ± 0.74 |
| | **Control PLA** | 0.981 ± 0.001 | 5.86 ± 0.04 | 0.22 ± 0.06 | 1.87 ± 1.43 | 63.41 ± 0.55 |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. |
| **14** | **CL 0.6 %** | 0.979 ± 0.001 | 5.73 ± 0.04 [b] | 0.17 ± 0.00 | 0.83 ± 0.39 | 62.93 ± 0.64 |
| | **CL 0.6 % + BJ** | 0.978 ± 0.000 | 5.79 ± 0.01 [b] | 0.20 ± 0.03 | 0.71 ± 0.20 | 64.52 ± 1.56 |
| | **Control PLA** | 0.979 ± 0.000 | 5.99 ± 0.01 [a] | 0.18 ± 0.00 | 1.31 ± 0.62 | 62.81 ± 1.19 |
| | ***Significance*** | n.s. | * | n.s. | n.s. | n.s. |
| **19** | **CL 0.6 %** | 0.976 ± 0.000 | 5.65 ± 0.04 | 0.22 ± 0.00 | 0.85 ± 0.00 | 61.63 ± 0.22 |
| | **CL 0.6 % + BJ** | 0.976 ± 0.000 | 5.71 ± 0.01 | 0.22 ± 0.00 | 0.86 ± 0.00 | 61.83 ± 0.18 |
| | **Control PLA** | n.d. | n.d. | n.d. | n.d. | n.d. |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. |

| *TIME* | *GL (M-PLA)* | *pH* | *% LACTIC ACID* | *% NaCl* |
|---|---|---|---|---|
| **0** | **Governing liquid** | **6.24 ± 0.02** | **0.02 ± 0.01** | **0.01 ± 0.02** |
| **2** | **CL 0.6 %** | 5.70 ± 0.01 [b] | 0.09 ± 0.01 | 0.12 ± 0.00 |
| | **CL 0.6 % + BJ** | 5.53 ± 0.01 [c] | 0.09 ± 0.03 | 0.12 ± 0.04 |
| | **Control PLA** | 5.89 ± 0.04 [a] | 0.05 ± 0.01 | 0.13 ± 0.02 |
| | ***Significance*** | ** | n.s. | n.s. |
| **7** | **CL 0.6 %** | 5.62 ± 0.03 [c] | 0.14 ± 0.01 | 0.23 ± 0.08 |
| | **CL 0.6 % + BJ** | 5.51 ± 0.01 [b] | 0.14 ± 0.00 | 0.15 ± 0.00 |
| | **Control PLA** | 5.72 ± 0.02 [a] | 0.14 ± 0.03 | 0.20 ± 0.04 |
| | ***Significance*** | ** | n.s. | n.s. |
| **9** | **CL 0.6 %** | 5.77 ± 0.13 | 0.17 ± 0.01 | 0.32 ± 0.04 [ab] |
| | **CL 0.6 % + BJ** | 5.56 ± 0.05 | 0.16 ± 0.01 | 0.29 ± 0.08 [b] |
| | **Control PLA** | 5.73 ± 0.04 | 0.14 ± 0.00 | 0.53 ± 0.00 [a] |
| | ***Significance*** | n.s. | n.s. | * |
| **14** | **CL 0.6 %** | 5.44 ± 0.06 | 0.21 ± 0.01 | 0.35 ± 0.00 |
| | **CL 0.6 % + BJ** | 5.41 ± 0.04 | 0.21 ± 0.01 | 0.32 ± 0.04 |
| | **Control PLA** | 5.43 ± 0.07 | 0.20 ± 0.00 | 0.38 ± 0.04 |
| | ***Significance*** | n.s. | n.s. | n.s. |
| **19** | **CL 0.6 %** | 5.28 ± 0.01 | 0.18 ± 0.00 | 0.35 ± 0.00 |
| | **CL 0.6 % + BJ** | 5.36 ± 0.01 | 0.26 ± 0.01 | 0.38 ± 0.04 |
| | **Control PLA** | | | n.s. |
| | ***Significance*** | n.s. | ** | |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

**Table 29** *TPA results of lacto-fermented mozzarella*

| TIME | M-PLA | Hardness (g) | Adhesiveness (g*sec.) | Springiness | Cohesiveness | Gumminess | Chewiness | Resilience |
|---|---|---|---|---|---|---|---|---|
| ***0*** | ***Mozzarella cheese*** | *5741.45 ± 322.24* | *-3.37 ± 1.00* | *0.847 ± 0.01* | *0.818 ± 0.01* | *4696.00 ± 314.80* | *3977.56 ± 312.40* | *0.478 ± 0.01* |
| | **0.6% CL** | 5660.80 ± 2089.88 | -6.03 ± 4.77 | 0.828 ± 0.02 | 0.804 ± 0.02 | 4526.16 ± 1544.17 | 3733.46 ± 1195.36 | 0.456 ± 0.00 |
| | **0.6% CL + BJ** | 4238.76 ± 754.08 | -5.78 ± 1.70 | 0.852 ± 0.00 | 0.788 ± 0.04 | 3356.14 ± 774.05 | 2859.43 ± 659.49 | 0.462 ± 0.01 |
| **2** | **CONTROL IN PLA** | 3731.16 ± 174.09 | -5.80 ± 3.00 | 0.850 ± 0.02 | 0.784 ± 0.03 | 2927.81 ± 247.30 | 2484.82 ± 154.18 | 0.445 ± 0.00 |
| | **CONTROL IN PP** | 3100.00 ± 0.00 | -7.06 ± 4.27 | 0.853 ± 0.02 | 0.761 ± 0.02 | 2357.55 ± 204.35 | 2110.01 ± 216.96 | 0.445 ± 0.00 |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | **0.6% CL** | 3368.97 ± 94.87 [a] | -2.41 ± 0.58 [a] | 0.891 ± 0.00 | 0.794 ± 0.01 | 2673.78 ± 111.01 [a] | 2382.26 ± 95.13 [a] | 0.507 ± 0.01 |
| | **0.6% CL + BJ** | 3444.12 ± 141.20 [a] | -3.49 ± 0.78 [a] | 0.896 ± 0.00 | 0.778 ± 0.03 | 2677.63 ± 17.31 [a] | 2397.81 ± 13.60 [a] | 0.508 ± 0.03 |
| **7** | **CONTROL IN PLA** | 2117.14 ± 327.43 [b] | -2.23 ± 0.19 [a] | 0.877 ± 0.02 | 0.743 ± 0.02 | 1570.02 ± 204.35 [b] | 1379.37 ± 216.96 [b] | 0.443 ± 0.00 |
| | **CONTROL IN PP** | 2600.00 ± 0.00 [b] | -12.53 ± 3.50 [b] | 0.895 ± 0.00 | 0.731 ± 0.00 | 1899.30 ± 2.19 [b] | 1699.87 ± 1.96 [b] | 0.467 ± 0.00 |
| | ***Significance*** | ** | * | n.s. | n.s. | ** | * | n.s. |
| | **0.6% CL** | 2764.42 ± 449.47 [ab] | -3.79 ± 1.34 [a] | 0.870 ± 0.02 | 0.715 ± 0.09 | 1997.22 ± 575.48 [ab] | 1742.48 ± 541.34 [ab] | 0.461 ± 0.10 |
| | **0.6% CL + BJ** | 3838.83 ± 31.05 [a] | -4.64 ± 1.32 [ab] | 0.887 ± 0.00 | 0.770 ± 0.05 | 2956.71 ± 224.78 [a] | 2633.50 ± 214.80 [a] | 0.494 ± 0.06 |
| **9** | **CONTROL IN PLA** | 2688.66 ± 278.76 [b] | -3.51 ± 2.73 [a] | 0.882 ± 0.00 | 0.721 ± 0.00 | 1939.12 ± 212.39 [ab] | 1709.56 ± 191.34 [ab] | 0.436 ± 0.01 |
| | **CONTROL IN PP** | 2003.00 ± 0.00 [b] | -21.50 ± 7.78 [b] | 0.880 ± 0.00 | 0.718 ± 0.08 | 1438.15 ± 155.80 [b] | 1265.58 ± 137.10 [b] | 0.457 ± 0.05 |
| | ***Significance*** | ** | * | n.s. | n.s. | * | * | n.s. |
| | **0.6% CL** | 5473.53 ± 749.88 | -5.65 ± 0.57 [a] | 0.893 ± 0.01 [a] | 0.763 ± 0.02 | 4167.82 ± 448.31 | 3717.08 ± 350.01 [a] | 0.499 ± 0.01 [a] |
| | **0.6% CL + BJ** | 3906.33 ± 1193.27 | -3.91 ± 1.37 [a] | 0.896 ± 0.00 [a] | 0.769 ± 0.03 | 3022.69 ± 1052.38 | 2707.19 ± 944.54 [ab] | 0.499 ± 0.03 [a] |
| **14** | **CONTROL IN PLA** | 5561.80 ± 2028.77 | -6.31 ± 4.07 [a] | 0.870 ± 0.02 [ab] | 0.718 ± 0.08 | 3914.47 ± 1024.05 | 3393.13 ± 810.14 [ab] | 0.457 ± 0.05 [ab] |
| | **CONTROL IN PP** | 1907.47 ± 707.11 | -63.71 ± 7.06 [b] | 0.824 ± 0.00 [b] | 0.613 ± 0.00 | 1170.78 ± 441.55 | 964.60 ± 366.10 [b] | 0.360 ± 0.00 [b] |
| | ***Significance*** | n.s. | ** | * | n.s. | n.s. | * | * |
| **19** | **0.6% CL** | 2232.965 ± 124.40 | -17.05 ± 1.43 | 0.810 ± 0.01 | 0.726 ± 0.01 | 1620.41 ± 104.46 | 1312.50 ± 108.63 | 0.425 ± 0.04 |
| | **0.6% CL + BJ** | 2405.94 ± 6.99 | -11.53 ± 0.71 | 0.820 ± 0.00 | 0.736 ± 0.01 | 1770.79 ± 18.75 | 1452.06 ± 17.88 | 0.395 ± 0.01 |
| | ***Significance*** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

*Significance at P<0.05;**Significance at P<0.01; n.s. not significant, n.d. not disponible. Data followed by different letters are significantly different by Tukey's multiple range test during the time.*

Table 31 shows the CIE-LAB colour parameters for cheese grouped by mode of treatment.

No significant differences among products obtained with different treatments mode were found for outer and inner colour parameters; only the a* inner (P<0.01) was significantly influenced by time variable.

**Table 30** *P-values by colour analysis of lacto-fermented mozzarella and governing liquid from multivariate analysis (P <0.05) related to the effect of alternative treatments and storage time*

***M-PLA TRIAL***

| Layer | Colour Parameters | Treatment | Time | Treatment *x* Time |
|---|---|---|---|---|
| *OUTER* | L* | 0.045 | 0.530 | 0.133 |
| *OUTER* | a* | 0.519 | 0.421 | 0.520 |
| *OUTER* | b* | 0.090 | 0.103 | 0.412 |
| *INNER* | L* | 0.633 | 0.415 | 0.040 |
| *INNER* | a* | 0.866 | 0.000 | 0.086 |
| *INNER* | b* | 0.742 | 0.445 | 0.066 |

***GL (M-PLA TRIAL)***

| Colour Parameters | Treatment | Time | Treatment *x* Time |
|---|---|---|---|
| L* | 0.000 | 0.000 | 0.000 |
| a* | 0.351 | 0.000 | 0.041 |
| b* | 0.001 | 0.000 | 0.002 |

While, in governing liquid samples, all parameters varied significantly ($P < 0.01$) with time. The governing liquid concentration had a significant impact on the L* and b* parameters and not on a* index ($P>0.05$).

As is reported by Gammariello *et al.* (2009), also in this experimental trial M-PLA, the success in cheese packaging is dependent on a number of important parameters, such as the use of starter cultures in cheese production, the type of cheese such as stabilized (cream, Feta), active (semi-soft, hard), ripened (Brie, Blue), its initial microbial contamination, and storage conditions.

### 4.4 M-SW Results and Discussions

The effect of calcium lactate (CL), concentrated bergamot juice (BJ) and stretching water (SW) on both the physical properties and microbiological quality of lacto-fermented mozzarella cheese (Table 32) was evaluated.

**Table 31** *Experimental trial M-SW: samples legend*

| PRODUCTION LOT | CODE SAMPLES | GOVERNING LIQUID COMPOSITION | PACKAGING MATERIAL |
|---|---|---|---|
| M-SW | 0.6 % CL | calcium lactate at 0.6 % in tap water | Polypropylene (PP) |
| | 0.6 % CL+ BJ | calcium lactate at 0.6 % and 0.05 % bergamot juice in tap water | Polypropylene (PP) |
| | SW 0.6 % CL | calcium lactate at 0.6% in stretching water | Polypropylene (PP) |
| | SW 0.6 % CL+ BJ | calcium lactate at 0.6% and 0.05 % bergamot juice in stretching water | Polypropylene (PP) |
| | Control | tap water | Polypropylene (PP) |

The peculiarity of this experimental phase is the addition of CL and BJ to the tap water or the stretching water, prior to packaging. Lacto-fermented mozzarella cheese quality was monitored for 21 days.

The microbiological analyses on mozzarella cheese after manufacturing revealed the following counts: 2.87 ± 0.00 $Log_{10}$ cfu/g of TBC and 2.65 ± 0.00 $Log_{10}$ cfu/g of LAB were observed in mozzarella cheese samples. In the governing liquid counts 2.56 ± 0.00 $Log_{10}$ cfu/g of TBC and 1.70 ± 0.00 $Log_{10}$ cfu/g of LAB were counted, whereas the Pseudomonas *spp.* growth was absent (as indicated in table 33).

TBC and Pseudomonas *spp.* growth were lower in treated mozzarella cheese samples then to the control one, in particular the lowest values of TBC were found in the mozzarella samples stored in filtered and pasteurized stretching water (SW) with calcium lactate and bergamot juice addition.

As regards Pseudomonas *spp.,* Bishop and White (1986) stated that a Pseudomonas *spp.* load equal to $10^6$ cfu/g may represent the contamination level at which the alterations of the product start to appear.

The microbiological acceptability of mozzarella based on Pseudomonas *spp.* count was up to 21 days for the samples stored with the experimental solutions (as indicated in table 32).

From the 6$^{th}$ day the stretching water and the added compounds promote the spoilage control against Pseudomonas *spp.*; it was also found in the sample stored in tap water and calcium lactate with BJ, but in a lesser extent (Figure 33).

Even if the content in Lactic Acid Bacteria increased in time in all samples, relative to the samples stored in the alternative solutions (as indicated in table 32) this growth was slower, probably due to the bacteriostatic action of calcium lactate and concentrated bergamot juice. In the samples control such Pseudomonas growth was fast.

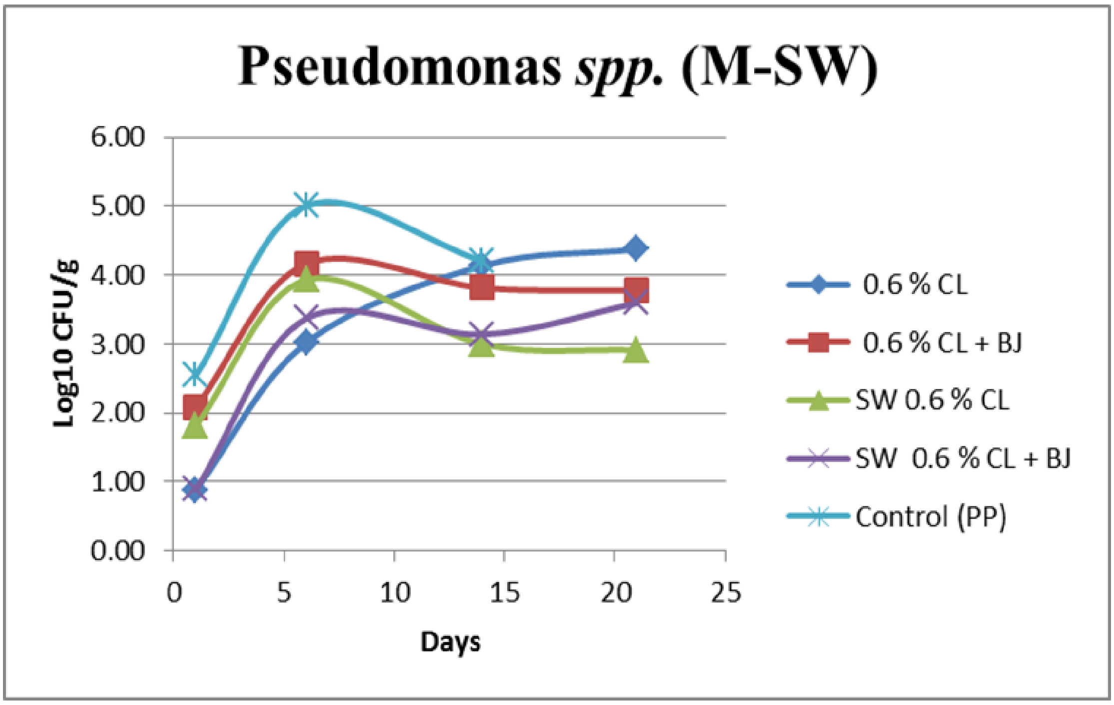



**Figure 33** Pseudomonas *spp*. growth: samples comparison

The cell numbers of the different microbial groups measured in the governing liquids of the samples stored at different times are shown in Table 33 (GL M-SW).

The microbiological analyses on the governing liquid are considered to be the index of hygiene of the production process, in particular with regard to the trend of mesophilic microorganisms.

It is important to initiate good hygiene practice (GHP) applications in farms to produce safe dairy products (Temelli *et al.*, 2006).

Under the experimental conditions of storage, the activity of LAB in MC might have been directly responsible for the increased growth and survival of mesophilic microorganisms in the governing liquids. For the Pseudomonas *spp.* parameter, significant differences were not found among the samples of governing liquid at 21 days.

The physicochemical results are shown in Table 34.

It is know that $a_w$ contributes to the control of the metabolic activity and multiplication of microorganisms (Brown, 1976).

LAB generally have higher $a_w$ than other cheese bacteria: e.g. the minimum $a_w$ for Streptococcus thermophiles is > 0.98 (Weber and Ramet, 1987).

Moreover as previously described, there was a growth of LAB over time and it is possible, that they may contribute to acid production lowing the pH.

**Table 32** *Microbiological analysis ($log_{10}$ cfu/g ± SD=3) results of mozzarella cheese (M-SW) and governing liquid (GL M-SW) during the refrigerated storage at 5°C*

| TIME | GL (M-SW) | TBC | *Pseudomonas spp.* | LAB |
|---|---|---|---|---|
| 1 | **0.6 % CL** | 4.71 ± 0.00 c | 1.00 ± 1.41 b | 3.51 ± 0.05 c |
| | **0.6 % CL+ BJ** | 4.81 ± 0.00 b | 3.73 ± 0.07 a | 4.24 ± 0.02 a |
| | **SW 0.6 % CL** | 4.85 ± 0.01 a | 3.31 ± 0.01 ab | 4.36 ± 0.08 a |
| | **SW 0.6 % CL+ BJ** | 4.45 ± 0.01 e | 2.00 ± 0.00 ab | 3.93 ± 0.04 b |
| | **Control PP** | 4.57 ± 0.01 a | 3.31 ± 0.01 ab | 4.01 ± 0.01 b |
| | ***Significance*** | ** | * | ** |
| 6 | **0.6 % CL** | 6.65 ± 0.07 e | 4.75 ± 0.01 e | 5.60 ± 0.00 e |
| | **0.6 % CL+ BJ** | 7.54 ± 0.01 d | 6.51 ± 0.01 a | 5.87 ± 0.04 d |
| | **SW 0.6 % CL** | 8.37 ± 0.00 a | 6.17 ± 0.01 c | 6.61 ± 0.01 a |
| | **SW 0.6 % CL+ BJ** | 8.09 ± 0.00 b | 6.04 ± 0.03 d | 6.30 ± 0.03 b |
| | **Control PP** | 7.88 ± 0.01 c | 6.36 ± 0.02 b | 6.16 ± 0.02 c |
| | ***Significance*** | ** | ** | ** |
| 14 | **0.6 % CL** | 8.22 ± 0.02 a | 6.27 ± 0.13 a | 7.05 ± 0.01 b |
| | **0.6 % CL+ BJ** | 7.82 ± 0.01 c | 5.98 ± 0.00 ab | 7.17 ± 0.04 b |
| | **SW 0.6 % CL** | 8.05 ± 0.10 ab | 5.69 ± 0.05 b | 6.97 ± 0.01 b |
| | **SW 0.6 % CL+ BJ** | 6.86 ± 0.02 d | 5.27 ± 0.16 c | 7.05 ± 0.24 b |
| | **Control PP** | 7.87 ± 0.04 bc | 5.80 ± 0.05 b | 7.68 ± 0.10 a |
| | ***Significance*** | ** | ** | * |
| 21 | **0.6 % CL** | 7.60 ± 0.01 a | 5.76 ± 0.22 | 7.08 ± 0.05 c |
| | **0.6 % CL+ BJ** | 6.93 ± 0.04 c | 5.66 ± 0.26 | 7.02 ± 0.03 c |
| | **SW 0.6 % CL** | 7.14 ± 0.04 b | 5.64 ± 0.07 | 7.79 ± 0.06 a |
| | **SW 0.6 % CL+ BJ** | 7.26 ± 0.05 b | 5.60 ± 0.02 | 7.45 ± 0.09 b |
| | **Control PP** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | ***Significance*** | ** | n.s. | ** |

| TIME | M-SW | TBC | *Pseudomonas spp.* | LAB |
|---|---|---|---|---|
| **1** | **0.6 % CL** | 3.62 ± 0.00 b | 0.88 ± 1.25 | 3.13 ± 0.00 c |
| | **0.6 % CL+ BJ** | 3.64 ± 0.01 b | 2.08 ± 0.12 | 3.32 ± 0.02 ab |
| | **SW 0.6 % CL** | 3.64 ± 0.01 b | 1.82 ± 0.21 | 3.30 ± 0.01 b |
| | **SW 0.6 % CL+ BJ** | 3.60 ± 0.01 b | 0.89 ± 1.26 | 3.34 ± 0.02 ab |
| | **Control PP** | 3.97 ± 0.03 a | 2.55 ± 0.07 | 3.37 ± 0.00 a |
| | **Significance** | ** | n.s. | ** |
| **6** | **0.6 % CL** | 6.26 ± 0.02 a | 3.02 ± 0.03 e | 5.61 ± 0.01 c |
| | **0.6 % CL+ BJ** | 4.87 ± 0.04 c | 4.16 ± 0.03 b | 5.69 ± 0.01 b |
| | **SW 0.6 % CL** | 4.73 ± 0.06 c | 3.93 ± 0.00 c | 5.79 ± 0.00 a |
| | **SW 0.6 % CL+ BJ** | 4.68 ± 0.17 c | 3.37 ± 0.01 d | 5.56 ± 0.01 c |
| | **Control PP** | 5.43 ± 0.01 b | 5.00 ± 0.00 a | 4.86 ± 0.04 d |
| | **Significance** | ** | ** | ** |
| **14** | **0.6 % CL** | 6.35 ± 0.00 b | 4.13 ± 0.03 a | 6.06 ± 0.00 b |
| | **0.6 % CL+ BJ** | 6.02 ± 0.01 c | 3.81 ± 0.01 b | 5.62 ± 0.05 c |
| | **SW 0.6 % CL** | 6.05 ± 0.01 c | 3.00 ± 0.06 c | 6.04 ± 0.00 b |
| | **SW 0.6 % CL+ BJ** | 5.74 ± 0.03 d | 3.14 ± 0.02 c | 5.40 ± 0.01 d |
| | **Control PP** | 7.37 ± 0.00 a | 4.20 ± 0.02 a | 7.00 ± 0.02 a |
| | **Significance** | ** | ** | ** |
| **21** | **0.6 % CL** | 6.02 ± 0.01 a | 4.37 ± 0.03 a | 6.26 ± 0.02 a |
| | **0.6 % CL+ BJ** | 5.37 ± 0.01 b | 3.77 ± 0.02 b | 5.85 ± 0.04 b |
| | **SW 0.6 % CL** | 5.29 ± 0.01 c | 2.91 ± 0.11 c | 5.42 ± 0.00 c |
| | **SW 0.6 % CL+ BJ** | 5.20 ± 0.01 d | 3.59 ± 0.02 b | 6.25 ± 0.01 a |
| | **Control PP** | n.d. ± n.d. | ± n.d. | n.d. ± n.d. |
| | **Significance** | ** | ** | ** |

**Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

In fact, significant differences in pH trend were found among the samples of lacto-fermented mozzarella; these values were lower in the samples stored in alternative governing liquids, after one day, compared to the control sample. Many of the thermophilic cultures are good acid producers (Beresford *et al.,* 2001).
The increased moisture content indicated that curd syneresis is retarded during cheese making. Probably, as suggested by McMahon *et al.* (1996), the fat interferes with the shrinkage of the casein matrix, lowering the driving force involved in expelling water from the curd particles.
Generally, for the brightness attribute (table 35), the outer surface of the mozzarella was not affected either by the presence of stretching water or concentrated bergamot juice.
At day 14, relating to outer brightness in lacto-fermented mozzarella stored in stretching water, calcium lactate with BJ and in control sample PP was significantly high ($P$ <0.05) compared to the other samples; instead the lowest value was in the samples 0.6 % CL.
Moreover, inner brightness decreased over time in all samples except in the sample called SW 0.6% CL +BJ, showing significant values ($P$ <0.05) higher at 21 days (93.93± 1.25).
At day 21, the lowest value in terms of inner brightness was in the sample 0.6 % CL + BJ (88.78 ± 3.34) suggesting better usage of stretching water compared to tap water.
At day 21, the brightest mozzarella sample both internally and externally was stored in stretching water and calcium lactate with BJ.
Moreover, at 21 days the SW 0.6% CL sample recorded the highest values (1.09 ± 1.02 ) of the red index ($P$ <0.01); the b* value (*yellowness*) increases in time as well as internally, without significant differences after day 1, and in sample SW 0.6% CL (at 21 days of storage) is higher than all others (11.10 ± 3.10).
The results of the b* parameter showed similarities with the results of Akarca *et al.* (2015) in which the increase in the *yellowness* of aerobic packaging samples was thought to be caused by microorganism growth during storage.
The values for the texture parameters as obtained by the texture profile analysis (TPA) for the lacto-fermented mozzarella after 0 ,1 ,6 ,14 and 21 days of storage are presented in Table 36.
The instrumental assessment of texture has indeed revealed subtle differences between the product stored in alternative governing liquids and the control sample.
The hardness mean values gradually decrease during storage for all lacto-fermented mozzarella cheese, which reflects the continuing break down of their protein matrix over time.

**Table 33** *Physico-chemical analysis results (Means ± SD=3) of lacto-fermented mozzarella cheese during the refrigerated storage at 5°C*

| TIME | M-SW | $a_w$ | pH | % Lactic Acid | % NaCl | % Moisture |
|---|---|---|---|---|---|---|
| **0** | **Mozzarella cheese** | **0.976 ± 0.000** | **5.49 ± 0.01** | **0.36 ± 0.04** | **1.53 ± 0.10** | **58.87 ± 1.38** |
| 1 | **0.6 % CL** | 0.977 ± 0.001 | 5.84 ± 0.02 $^{a}$ | 0.22 ± 0.00 | 2.44 ± 0.20 $^{ab}$ | 60.24 ± 0.90 |
| | **0.6 % CL+ BJ** | 0.977 ± 0.001 | 5.62 ± 0.00 $^{cd}$ | 0.24 ± 0.03 | 1.13 ± 0.00 $^{b}$ | 60.60 ± 0.85 |
| | **SW 0.6 % CL** | 0.980 ± 0.004 | 5.73 ± 0.05 $^{b}$ | 0.28 ± 0.03 | 4.67 ± 0.22 $^{a}$ | 60.76 ± 1.19 |
| | **SW 0.6 % CL+ BJ** | 0.981 ± 0.006 | 5.54 ± 0.01 $^{c}$ | 0.27 ± 0.00 | 1.60 ± 0.62 $^{b}$ | 60.97 ± 0.81 |
| | **Control PP** | 0.982 ± 0.006 | 5.70 ± 0.01 $^{bc}$ | 0.23 ± 0.00 | 1.46 ± 1.24 $^{b}$ | 60.26 ± 1.98 |
| | ***Significance*** | n.s. | ** | n.s. | * | n.s. |
| 6 | **0.6 % CL** | 0.983 ± 0.002 | 5.56 ± 0.04 $^{b}$ | 0.20 ± 0.03 | 1.01 ± 0.20 | 61.04 ± 0.81 $^{b}$ |
| | **0.6 % CL+ BJ** | 0.979 ± 0.001 | 5.59 ± 0.01 $^{b}$ | 0.22 ± 0.00 | 0.58 ± 0.00 | 60.87 ± 0.33 $^{a}$ |
| | **SW 0.6 % CL** | 0.982 ± 0.001 | 5.58 ± 0.01 $^{b}$ | 0.18 ± 0.00 | 0.58 ± 0.00 | 61.82 ± 2.35 $^{a}$ |
| | **SW 0.6 % CL+ BJ** | 0.981 ± 0.003 | 5.54 ± 0.01 $^{b}$ | 0.24 ± 0.03 | 0.57 ± 0.00 | 61.31 ± 1.11 $^{a}$ |
| | **Control PP** | 0.982 ± 0.001 | 5.66 ± 0.00 $^{a}$ | 0.21 ± 0.00 | 1.16 ± 0.68 | 62.02 ± 0.95 $^{ab}$ |
| | ***Significance*** | n.s. | ** | n.s. | n.s. | * |
| 14 | **0.6 % CL** | 0.987 ± 0.007 | 5.70 ± 0.06 $^{ab}$ | 0.22 ± 0.00 $^{a}$ | 0.85 ± 0.40 | 62.27 ± 0.52 |
| | **0.6 % CL+ BJ** | 0.985 ± 0.002 | 5.58 ± 0.01 $^{b}$ | 0.20 ± 0.03 $^{a}$ | 0.43 ± 0.20 | 63.20 ± 0.18 |
| | **SW 0.6 % CL** | 0.986 ± 0.005 | 5.72 ± 0.06 $^{ab}$ | 0.27 ± 0.00 $^{a}$ | 2.04 ± 1.24 | 62.92 ± 0.52 |
| | **SW 0.6 % CL+ BJ** | 0.987 ± 0.001 | 5.68 ± 0.02 $^{ab}$ | 0.22 ± 0.00 $^{a}$ | 0.57 ± 0.00 | 63.16 ± 1.75 |
| | **Control PP** | 0.987 ± 0.000 | 5.82 ± 0.04 $^{a}$ | 0.11 ± 0.03 $^{b}$ | 0.56 ± 0.00 | 62.00 ± 0.40 |
| | ***Significance*** | n.s. | * | ** | n.s. | n.s. |
| 21 | **0.6 % CL** | 0.979 ± 0.001 | 5.52 ± 0.01 $^{a}$ | 0.16 ± 0.03 | 0.73 ± 0.20 | 62.36 ± 0.06 |
| | **0.6 % CL+ BJ** | 0.981 ± 0.002 | 5.44 ± 0.01 $^{b}$ | 0.24 ± 0.09 | 0.70 ± 0.20 | 62.09 ± 0.18 |
| | **SW 0.6 % CL** | 0.984 ± 0.007 | 5.42 ± 0.01 $^{bc}$ | 0.18 ± 0.00 | 0.58 ± 0.00 | 64.24 ± 0.10 |
| | **SW 0.6 % CL+ BJ** | 0.982 ± 0.002 | 5.39 ± 0.00 $^{c}$ | 0.19 ± 0.03 | 0.42 ± 0.20 | 63.13 ± 2.25 |
| | **Control PP** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | ***Significance*** | n.s. | ** | n.s. | n.s. | n.s. |

*Values are mean ± SD (n=3). *Significance at $P<0.05$;**Significance at $P<0.01$; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

**Table 34** *Colour analysis results of lacto-fermented mozzarella in experimental procedure M-SW*

| | | OUTER | | | INNER | | |
|---|---|---|---|---|---|---|---|
| TIME | M-SW | L* | a* | b* | L* | a* | b* |
| 0 | **Mozzarella cheese** | 95.11 ± 0.56 | -0.33 ± 0.04 | 5.54 ± 0.33 | 94.69 1.93 | -0.31 0.04 | 6.45 1.11 |
| 1 | **0.6 % CL** | 94.78 ± 0.61 | -0.30 ± 0.11 a | 5.06 ± 0.55 | 94.38 ± 1.21 | -0.27 ± 0.06 | 6.08 ± 0.53 b |
| | **0.6 % CL+ BJ** | 95.27 ± 0.33 | -0.29 ± 0.08 a | 5.67 ± 0.22 | 92.82 ± 1.57 | -0.28 ± 0.14 | 7.59 ± 0.88 a |
| | **SW 0.6 % CL** | 95.31 ± 0.33 | -0.35 ± 0.05 ab | 5.18 ± 0.23 | 93.16 ± 1.99 | -0.24 ± 0.09 | 6.25 ± 0.82 ab |
| | **SW 0.6 % CL+ BJ** | 95.23 ± 0.29 | -0.44 ± 0.03 b | 5.69 ± 0.17 | 92.98 ± 0.98 | -0.27 ± 0.09 | 6.94 ± 1.05 ab |
| | **Control PP** | 95.37 ± 0.50 | -0.32 ± 0.05 ab | 4.96 ± 0.57 | 92.50 ± 0.84 | -0.23 ± 0.42 | 7.71 ± 0.46 a |
| | **Significance** | n.s. | * | n.s. | n.s. | n.s. | * |
| 6 | **0.6 % CL** | 95.62 ± 0.21 | -0.33 ± 0.01 ab | 5.17 ± 0.19 | 94.21 ± 1.65 | -0.54 ± 0.12 | 7.37 ± 1.05 |
| | **0.6 % CL+ BJ** | 95.42 ± 0.15 | -0.40 ± 0.03 b | 5.62 ± 0.15 | 94.52 ± 1.34 | -0.49 ± 0.10 | 6.72 ± 1.05 |
| | **SW 0.6 % CL** | 95.44 ± 0.35 | -0.34 ± 0.04 b | 5.26 ± 0.10 | 94.66 ± 1.20 | -0.41 ± 0.11 | 6.28 ± 0.67 |
| | **SW 0.6 % CL+ BJ** | 95.62 ± 0.10 | -0.41 ± 0.07 b | 5.53 ± 0.15 | 95.05 ± 1.59 | -0.46 ± 0.10 | 6.77 ± 1.26 |
| | **Control PP** | 96.07 ± 0.72 | -0.25 ± 0.05 a | 4.66 ± 0.55 | 95.77 ± 0.90 | -0.44 ± 0.12 | 6.08 ± 0.44 |
| | **Significance** | n.s. | ** | ** | n.s. | n.s. | n.s. |
| 14 | **0.6 % CL** | 92.84 ± 2.98 b | -0.22 ± 0.15 a | 5.56 ± 0.37 | 94.15 ± 0.64 | -0.47 ± 0.07 | 6.86 ± 0.70 |
| | **0.6 % CL+ BJ** | 95.40 ± 0.07 ab | -0.49 ± 0.02 b | 5.77 ± 0.22 | 93.72 ± 0.77 | -0.59 ± 0.05 | 7.41 ± 1.05 |
| | **SW 0.6 % CL** | 94.99 ± 0.76 ab | -0.39 ± 0.04 b | 5.52 ± 0.28 | 93.78 ± 1.09 | -0.61 ± 0.12 | 7.60 ± 1.29 |
| | **SW 0.6 % CL+ BJ** | 95.60 ± 0.34 a | -0.52 ± 0.06 b | 5.28 ± 0.11 | 93.83 ± 0.36 | -0.51 ± 0.03 | 6.79 ± 0.31 |
| | **Control PP** | 95.71 ± 0.42 a | -0.44 ± 0.04 b | 5.49 ± 0.13 | 92.92 ± 1.19 | -0.45 ± 0.20 | 8.42 ± 1.52 |
| | **Significance** | * | ** | n.s. | n.s. | n.s. | n.s. |
| 21 | **0.6 % CL** | 95.12 ± 0.99 | -0.41 ± 0.00 b | 6.25 ± 0.71 b | 93.37 ± 0.85 ab | -0.75 ± 0.07 b | 8.37 ± 0.77 |
| | **0.6 % CL+ BJ** | 94.40 ± 0.84 | -0.50 ± 0.02 b | 6.43 ± 0.08 b | 88.78 ± 3.34 b | 1.65 ± 2.10 a | 16.26 ± 7.81 |
| | **SW 0.6 % CL** | 91.63 ± 1.88 | 1.09 ± 1.02 a | 11.10 ± 3.10 a | 91.86 ± 3.82 ab | 0.05 ± 1.26 ab | 10.42 ± 5.47 |
| | **SW 0.6 % CL+ BJ** | 95.26 ± 0.34 | -0.46 ± 0.09 b | 6.32 ± 0.46 b | 93.93 ± 1.25 a | -0.68 ± 0.08 b | 7.79 ± 0.78 |
| | **Control PP** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | **Significance** | n.s. | ** | ** | * | * | n.s. |

*Values are mean ± SD (n=10). *Significance at P<0.05;**Significance at P<0.01; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

**Table 35** *TPA results of lacto-fermented mozzarella in experimental trial M-SW*

| TIME | M-SW | Hardness (g) | Adhesiveness (g*sec.) | Springiness | Cohesiveness | Gumminess | Chewiness | Resilience |
|---|---|---|---|---|---|---|---|---|
| 0 | **Mozzarella cheese** | 6315.80 ± 152.87 | -4.24 ± 0.58 | 0.80 ± 0.01 | 0.81 ± 0.00 | 5026.393 ± 14.60 | 4008.087 ± 1.38 | 0.436 ± 0.01 |
| 1 | **0.6 % CL** | 3908.57 ± 52.26 | -2.79 ± 1.18 | 0.86 ± 0.00 | 0.83 ± 0.00 | 2832.88 ± 556.80 | 2441.60 ± 479.89 | 0.48 ± 0.00 |
| | **0.6 % CL+ BJ** | 2584.67 ± 344.47 | -0.90 ± 0.02 | 0.88 ± 0.03 | 0.81 ± 0.02 | 2095.98 ± 231.74 | 1856.20 ± 266.27 | 0.46 ± 0.02 |
| | **SW 0.6 % CL** | 3803.95 ± 1813.18 | -2.48 ± 1.42 | 0.87 ± 0.02 | 0.84 ± 0.02 | 3174.17 ± 1459.54 | 2756.51 ± 1220.36 | 0.48 ± 0.02 |
| | **SW 0.6 % CL+ BJ** | 4234.27 ± 595.56 | -2.96 ± 0.66 | 0.86 ± 0.00 | 0.82 ± 0.01 | 3468.13 ± 549.83 | 2999.23 ± 482.18 | 0.49 ± 0.03 |
| | **Control PP** | 3807.50 ± 1231.05 | -1.61 ± 1.79 | 0.87 ± 0.02 | 0.80 ± 0.00 | 3062.72 ± 987.66 | 2670.78 ± 932.16 | 0.46 ± 0.00 |
| | Significance | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| 6 | **0.6 % CL** | 2536.45 ± 809.58 | -2.26 ± 0.14 | 0.88 ± 0.01 | 0.81 ± 0.01 | 2055.31 ± 681.50 | 1814.50 ± 620.62 | 0.48 ± 0.03 |
| | **0.6 % CL+ BJ** | 3133.02 ± 213.45 | -4.26 ± 3.25 | 0.85 ± 0.03 | 0.79 ± 0.06 | 2478.16 ± 18.21 | 2103.66 ± 100.16 | 0.46 ± 0.06 |
| | **SW 0.6 % CL** | 1468.98 ± 385.48 | -1.54 ± 0.05 | 0.87 ± 0.01 | 0.78 ± 0.02 | 1153.61 ± 333.36 | 1004.50 ± 299.99 | 0.46 ± 0.04 |
| | **SW 0.6 % CL+ BJ** | 2123.02 ± 474.42 | -3.43 ± 1.04 | 0.87 ± 0.01 | 0.78 ± 0.03 | 1645.28 ± 306.81 | 1423.35 ± 249.64 | 0.45 ± 0.01 |
| | **Control PP** | 3647.08 ± 659.95 | -8.20 ± 4.10 | 0.82 ± 0.00 | 0.79 ± 0.01 | 2878.58 ± 558.61 | 2358.90 ± 449.49 | 0.42 ± 0.01 |
| | Significance | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| 14 | **0.6 % CL** | 3245.29 ± 185.78 | -9.69 ± 2.76 | 0.86 ± 0.01 | 0.76 ± 0.02 | 2477.10 ± 200.23 | 2120.69 ± 156.95 | 0.45 ± 0.01 |
| | **0.6 % CL+ BJ** | 2435.02 ± 698.91 | -4.34 ± 2.57 | 0.87 ± 0.03 | 0.78 ± 0.01 | 1897.23 ± 512.55 | 1905.06 ± 458.51 | 0.46 ± 0.04 |
| | **SW 0.6 % CL** | 2813.40 ± 939.37 | -7.15 ± 7.16 | 0.88 ± 0.03 | 0.78 ± 0.05 | 2171.12 ± 599.25 | 1834.28 ± 1012.17 | 0.43 ± 0.07 |
| | **SW 0.6 % CL+ BJ** | 2785.97 ± 1215.16 | -5.78 ± 0.03 | 0.81 ± 0.00 | 0.76 ± 0.06 | 2164.42 ± 1103.19 | 4272.96 ± 1787.30 | 0.37 ± 0.05 |
| | **Control PP** | 4208.27 ± 698.71 | -30.11 ± 23.84 | 0.82 ± 0.03 | 0.74 ± 0.03 | 5280.52 ± 2408.61 | 1441.83 ± 125.37 | 0.50 ± 0.01 |
| | Significance | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| 21 | **0.6 % CL** | 1954.62 ± 226.54 | -1.13 ± 0.95 | 0.90 ± 0.01 | 0.82 ± 0.01 | 1607.02 ± 160.63 | 1641.78 ± 397.12 | 0.46 ± 0.03 |
| | **0.6 % CL+ BJ** | 2346.21 ± 1839.37 | -9.73 ± 10.31 | 0.86 ± 0.02 | 0.73 ± 0.05 | 1668.05 ± 1227.35 | 1415.81 ± 1014.42 | 0.42 ± 0.02 |
| | **SW 0.6 % CL** | 2777.41 ± 440.33 | -12.08 ± 7.54 | 0.85 ± 0.00 | 0.74 ± 0.05 | 2031.33 ± 176.67 | 1715.43 ± 143.26 | 0.45 ± 0.02 |
| | **SW 0.6 % CL+ BJ** | 2730.59 ± 328.00 | -14.72 ± 9.96 | 0.81 ± 0.08 | 0.71 ± 0.07 | 1934.45 ± 51.95 | 1555.58 ± 116.87 | 0.42 ± 0.06 |
| | **Control PP** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | Significance | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

*Values are mean ± SD (n=3). *Significance at P<0.05;**Significance at P<0.01; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

Both hardness and adhesiveness showed higher values at day 14 in the control samples. However, the instrumental hardness parameter had direct relations with the flattening of the product (figure 34), while relating to the adhesiveness parameter, it shows loss of integrity on the surface. Probably, when the moisture content decreased and the protein content increased, fat did not replace the moisture on an equal basis, so the total filler volume was decreased.

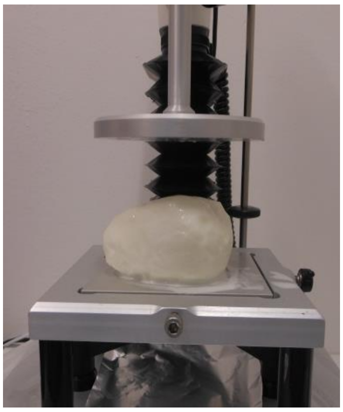


**Figure 34** Control samples at time 14 during TPA

Between day 1 and day 14, proteolysis (Table 37) remained stable in all samples however, a significant level of proteolysis occurred at day 21 ($P < 0.05$) in samples stored in alternative governing liquids. Higher moisture content may have enhanced accessibility to proteolytic enzyme resulting in accelerated proteolysis (Zisu and Shah, 2005).

There are different points of view about the impact of proteolysis on the textural and functional properties of mozzarella cheese. (Costabel *et al.*, 2006).

In fresh dairy food as *fiordilatte* free amino-acids and short chain peptides contribute to the basic flavour that is perceived (Costabel *et al.*, 2006). The release of amino groups as a consequence of proteolysis has been reported as a requisite to ensure browning by the Maillard reaction when the cheese is heated, regardless of the amount of residual sugars present in the cheese (Barbano *et al.*, 1993).

Ratio SN/TN was significantly ($P<0.01$) increased at the end of shelf-life (21 days of storage) for the samples 0.6% CL+BJ and SW 0.6% CL+BJ.

The protein content was similar among the products during the storage, but at time 14 for the samples stored with BJ was greater.

Multivariate analysis on soluble peptide profiles detected that storage time had more influence on proteolysis of Mozzarella cheeses than any other assayed variable.

*Time* and *treatment vs time* variables influenced significantly the NPN content (Table 38).

**Table 36** *Values of protein, percentage of Total Nitrogen, Soluble Nitrogen in trichloroacetic acid (SN- TCA) expressed as percentage of Total Nitrogen (TN), percentage ratio of these fractions (SN/TN) in lacto-fermented mozzarella cheeses samples at time 1, 14, 21*

| *M-SW* | Time | 0.6 % CL | 0.6 % CL+ BJ | SW 0.6 % CL | SW 0.6% CL+ BJ | CONTROL PP |
|---|---|---|---|---|---|---|
| **Protein (g*100g -1)** | $t_1$ | 15.63 ± 0.18 | 15.66 ± 1.49 | 16.08 ± 1.26 | 16.11 ± 0.50 | 16.43 ± 1.04 |
| | $t_{14}$ | 15.66 ± 1.04 | 16.94 ± 0.05 | 15.98 ± 0.59 | 16.94 ± 0.14 | 16.65 ± 0.36 |
| | $t_{21}$ | 15.66 ± 0.95 | 16.94 ± 0.14 | 15.95 ± 0.36 | 17.26 ± 0.68 | n.d. |
| *Significance* | | n.s. | n.s. | n.s. | n.s. | n.s. |
| **% TN** | $t_1$ | 2.45 ± 0.03 | 2.46 ± 0.23 | 2.52 ± 0.20 | 2.53 ± 0.08 | 2.58 ± 0.16 |
| | $t_{14}$ | 2.46 ± 0.16 | 2.66 ± 0.01 | 2.51 ± 0.09 | 2.66 ± 0.02 | 2.61 ± 0.06 |
| | $t_{21}$ | 2.46 ± 0.15 | 2.66 ± 0.02 | 2.50 ± 0.06 | 2.71 ± 0.11 | n.d. |
| *Significance* | | n.s. | n.s. | n.s. | n.s. | n.s. |
| **% SN/TN** | $t_1$ | 0.28 ± 0.05 b | 0.26 ± 0.04 b | 0.23 ± 0.04 b | 0.30 ± 0.02 b | 0.32 ± 0.03 |
| | $t_{14}$ | 0.30 ± 0.01 b | 0.26 ± 0.01 b | 0.27 ± 0.04 b | 0.29 ± 0.02 b | 0.36 ± 0.04 |
| | $t_{21}$ | 0.49 ± 0.06 a | 0.47 ± 0.02 a | 0.50 ± 0.06 a | 0.49 ± 0.00 a | n.d. |
| *Significance* | | * | ** | * | ** | n.s. |
| **% SN-TCA/TN** | $t_1$ | 0.11 ± 0.00 | 0.13 ± 0.01 | 0.09 ± 0.01 b | 0.11 ± 0.01 | 0.10 ± 0.01 |
| | $t_{14}$ | 0.11 ± 0.01 | 0.10 ± 0.00 | 0.11 ± 0.00 a | 0.11 ± 0.01 | 0.10 ± 0.00 |
| | $t_{21}$ | 0.11 ± 0.01 | 0.10 ± 0.00 | 0.11 ± 0.01 a | 0.11 ± 0.01 | n.d. |
| *Significance* | | n.s. | n.s. | * | n.s. | n.s. |

*Values are mean ± SD (n=3). *Significance at P<0.05;**Significance at P<0.01; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

**Table 37** *P-values of Total Nitrogen (TN) percentage and Not Protein Nitrogen content (expressed as SN/TN and SN-TCA/TN) by multivariate analysis (p<0.05) related to the effect of alternative treatments and storage time*

| *M-SW TRIAL* | Treatment | Time | Treatment *x* Time |
|---|---|---|---|
| **% TN** | 0.127 | 0.290 | 0.821 |
| **% SN/TN** | 0.055 | 0.000 | 0.905 |
| **% SN-TCA/TN** | 0.089 | 0.663 | 0.027 |

The data from the physicochemical analysis of governing liquids are summarized in Table 39.

The increase in terms of acidity in all governing liquid samples during the storage period was a result of the lactic acid content produced by the activities of starter and nonstarter bacteria found in the cheese which have the ability to ferment lactose (Akarca *et al.*, 2015).

The relatively to colour indexes of governing liquids: a* and b* index over time were lower in governing liquids composed from stretching water.

The brightness in the governing liquid called SW 0.6% CL and SW 0.6% CL + BJ is significantly higher ($P< 0.01$) at all times than the governing liquids composed from tap water. In particular, the governing liquid called SW 0.6% CL without bergamot has significantly high brightness.

**Table 38** *Physico-chemical analysis results of governing liquid*

| TIME | GL (M-SW) | pH | % LACTIC ACID | % NaCl | L* | a* | b* |
|---|---|---|---|---|---|---|---|
| **0** | **GOVERNING LIQUID** | **6.92 ± 0.02** | **0.01 ± 0.00** | **0.15 ± 0.00** | **31.94 ±0.06** | **0.16 ± 0.01** | **1.34 ± 0.01** |
| | **0.6 % CL** | 5.86 ± 0.01 b | 0.05 ± 0.00 b | 0.12 ± 0.00 | 31.79 ±0.20 c | 0.12 ± 0.02 a | 1.28 ± 0.05 a |
| | **0.6 % CL+ BJ** | 5.66 ± 0.00 c | 0.05 ± 0.00 b | 0.12 ± 0.04 | 31.32 ±0.69 c | 0.12 ± 0.01 a | 1.36 ± 0.10 a |
| **1** | **SW 0.6 % CL** | 5.59 ± 0.01 d | 0.09 ± 0.00 a | 0.12 ± 0.00 | 41.58 ±0.09 a | 0.05 ± 0.01 b | 0.05 ± 0.02 b |
| | **SW 0.6 % CL+ BJ** | 5.48 ± 0.00 e | 0.09 ± 0.01 a | 0.10 ± 0.02 | 37.77 ±0.09 b | 0.04 ± 0.01 b | 0.09 ± 0.02 b |
| | **Control PP** | 6.13 ± 0.01 a | 0.02 ± 0.01 c | 0.16 ± 0.02 | 31.69 ±0.08 c | 0.13 ± 0.01 a | 1.30 ± 0.03 a |
| | Significance | ** | ** | n.s. | ** | ** | ** |
| | **0.6 % CL** | 5.67 ± 0.03 b | 0.14 ± 0.00 b | 0.16 ± 0.02 | 31.70 ±0.02 d | 0.10 ± 0.02 a | 1.41 ± 0.02 ab |
| | **0.6 % CL+ BJ** | 5.57 ± 0.02 b | 0.15 ± 0.01 ab | 0.15 ± 0.00 | 32.07 ±0.39 c | 0.12 ± 0.02 a | 1.47 ± 0.11 a |
| **6** | **SW 0.6 % CL** | 5.42 ± 0.06 c | 0.16 ± 0.01 a | 0.15 ± 0.00 | 42.13 ±0.02 a | -0.03 ± 0.01 b | 0.15 ± 0.01 c |
| | **SW 0.6 % CL+ BJ** | 5.41 ± 0.01 c | 0.16 ± 0.00 a | 0.15 ± 0.00 | 38.48 ±0.10 b | -0.04 ± 0.01 b | 0.06 ± 0.02 c |
| | **Control PP** | 5.85 ± 0.01 a | 0.09 ± 0.00 c | 0.15 ± 0.00 | 31.76 ±0.04 cd | 0.12 ± 0.01 a | 1.33 ± 0.06 b |
| | Significance | ** | ** | n.s. | ** | ** | ** |
| | **0.6 % CL** | 5.67 ± 0.03 a | 0.18 ± 0.00 c | 0.16 ± 0.02 | 31.73 ±0.10 c | 0.09 ± 0.02 a | 1.31 ± 0.07 b |
| | **0.6 % CL+ BJ** | 5.57 ± 0.06 ab | 0.19 ± 0.00 c | 0.16 ± 0.02 | 31.69 ±0.07 c | 0.08 ± 0.01 a | 1.24 ± 0.04 b |
| **14** | **SW 0.6 % CL** | 5.36 ± 0.04 cd | 0.22 ± 0.01 a | 0.15 ± 0.00 | 40.29 ±0.11 a | 0.01 ± 0.00 c | 0.59 ± 0.02 c |
| | **SW 0.6 % CL+ BJ** | 5.48 ± 0.00 bc | 0.21 ± 0.01 ab | 0.16 ± 0.02 | 35.03 ±0.02 b | 0.04 ± 0.02 b | 0.61 ± 0.02 c |
| | **Control PP** | 5.27 ± 0.03 d | 0.19 ± 0.01 bc | 0.16 ± 0.02 | 31.74 ±0.07 c | 0.09 ± 0.01 a | 1.41 ± 0.01 a |
| | Significance | ** | ** | n.s. | ** | ** | ** |
| | **0.6 % CL** | 5.52 ± 0.01 a | 0.22 ± 0.01 c | 0.22 ± 0.02 | 31.08 ±1.08 c | 0.11 ± 0.04 c | 1.83 ± 0.67 a |
| | **0.6 % CL+ BJ** | 5.44 ± 0.01 b | 0.24 ± 0.00 b | 0.18 ± 0.08 | 31.44 ±1.12 c | 0.07 ± 0.02 c | 1.26 ± 0.13 ab |
| **21** | **SW 0.6 % CL** | 5.42 ± 0.01 bc | 0.27 ± 0.01 a | 0.23 ± 0.04 | 43.55 ±0.06 a | -0.19 ± 0.28 a | 0.22 ± 0.04 bc |
| | **SW 0.6 % CL+ BJ** | 5.39 ± 0.00 c | 0.26 ± 0.00 ab | 0.16 ± 0.06 | 37.43 ±0.89 b | -0.17 ± 0.02 b | 0.09 ± 0.10 c |
| | **Control PP** | n.d. ± n.d. | n.d. ± n.d. | n.d. ± n.d. | n.d. ±n.d. | n.d. ± n.d. | n.d. ± n.d. |
| | Significance | ** | ** | n.s. | ** | ** | ** |

**Significance at P<0.05;**Significance at P<0.01; n.s. not significant. Data followed by different letters are significantly different by Tukey's multiple range test.*

# Chapter 5

# Conclusions

This thesis verified the effectiveness of innovative solutions to avoid large changes of microbiological and physico-chemical parameters in Mozzarella cheese during preservation for up to 3 weeks, compared with the Mozzarella cheese control stored in tap water.

It explains the shelf-life of MC with calcium lactate and BJ extension, preserving the microbiological acceptability.

On the governing liquid, the microbiological analyses are considered to be the index for hygiene of the production process, in particular, with regard to the trend of mesophilic microorganisms, while the physicochemical properties are important in understanding the interactions within the product.

The growth of Pseudomonas *spp.* was kept below 5 cycles in lacto-fermented mozzarella samples and below 6 cycles in governing liquid at the end of shelf-life.

In fact, the inhibitory effects on Pseudomonas spp. were determined using:

- concentrations of 0.2% and 0.6% of calcium lactate;
- calcium lactate with BJ both in Polylactide (trial M-PLA) and Polypropylene (PP) packaging;
- Stretching water in substitution of tap water.

Moreover, the shelf-life of lacto-fermented mozzarella stored at 5 °C was extended with respect to control samples using:

- Calcium Lactate (trial M-SALTS);
- Stretching water in substitution of tap water (relating to trials M-SW). Stretching water with calcium lactate and / or BJ solutes has ensured a higher brightness of the product compared to other samples. The outer surface of the mozzarella samples was not affected either by the presence of stretching water or concentrated bergamot juice.
- Calcium Lactate is associated with 0.05 % of Bergamot juice concentrate (trial M-BJ; M-PLA; M-SW). Concentration of 0.10% BJ dissolved in tap water had a measurable impact on the rheological properties of mozzarella cheese probably due to the action of the acidifier BJ. For this reason the 0.05% value of BJ was used in addition with calcium lactate, thus reducing the risk of the softness defect for mozzarella cheese.

The results showed an extension of 50% of the shelf-life almost without environmental consequences.

The results were useful in highlighting the main hotspots in mozzarella cheese production and to suggest improvements for a more sustainable management.
The use of low concentrations of bergamot juice has not affected the sensorial characteristics of the product in terms of colour and texture.
The control sample became unacceptable after just 12 days of storage, due to the development of a bad odour and aspect. Moreover, a loss of integrity in the outer layer of the control samples made it unacceptable.
The hardness mean values gradually decrease during storage for all lacto-fermented mozzarella cheese, which reflects the continuing break down of their protein matrix during the time.
NaCl concentration in governing liquid increased the deterioration in the outer layer of the product making it unacceptable after two weeks.
The proteolysis index increased over time, but there were no appreciated statistically significant differences among the samples over the same time.
The PLA packaging can be an alternative to polypropylene, although the appearance of this material and the closure system should be improved.
A rigorous assessment of the sensory attributes of lacto-fermented mozzarella stored in alternative governing liquids would be required in order to couple the sensory data with those from chemical analysis or mechanical tests.
Finally, a direct comparison with other published work is difficult because a variety of aspects and on the contrary little information on the governing liquid are available in most papers on mozzarella cheese shelf-life.

# Chapter 6

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