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Evaluation of the causes implicated in fermentation delay and complete fermentation failure during the manufacture of "Mozzarella di Bufala"

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This Thesis is
dedicated to

My Mum Carla

Contents

ABSTRACT

The primary emphasis of this study was concerned with an investigation on some of the causes that could be responsible for the acidification problem during Buffalo Mozzarella cheese-making recently observed in Southern Italy.

Bacteriophage infection is still one of the main reasons leading to fermentation delay and complete fermentation failure and it was therefore the principle aim of this research.

156 strains of Lactic Acid Bacteria (LAB) (*Lactobacillus* spp, *Lactococcus* spp, *Streptococcus salivarius thermophilus*, *Pediococcus* spp and *Leuconostoc* spp) have been isolated and characterized to genus level and 44% of the colonies have been identified to species level. They have been randomly isolated from twelve wild whey starters used for the production of the local buffalo Mozzarella cheese manufactured in Salerno province in order to check their sensitivity to phage infections.

The first interesting founding derives from the microscopic examination of LAB: a large number of lactobacilli (60%) revealed the characteristic presence of inclusion bodies into their cytoplasms. Schaeffer-Fulton reagent, Nile Blue and Neisser staining demonstrated that the granules were belonging to polyphosphates aggregation. Because of the potential role of poly-P in intracellular pH control, the presence of this polymer in starter bacteria may be an environmental adaptation to the typical high acidity storage

conditions of natural starters. This result was never been described before.

Two bacteriophages *ΦApr-1* and *ΦApr-2* have been isolated from one sample of buffalo whey starter and their principle phenotypic and genotypic features have been investigated. Their host was identified as *Lactobacillus brevis.*

The lytic-cycle of the bacteriophages has been demonstrated by the over lysis of the host after three hours of incubation in PLGYG broth.

The genomes, approximately 31.4 kb and 31 kb long, were double-stranded linear DNA molecules with a *pac*-type system. SDS-PAGE showed a protein structure of respectively 1.152 KDa and 524,7 KDa.

After examination at electronic microscope, *ΦApr-1* and *ΦApr-2* showed a very similar morphology: many round and empty virus particles associated in clusters with isometric and hexagonal heads of about 45 nm in diameter and a short non-contractile tails of about 29 nm of length. They belonged to Podoviridae family and they have been ascribed to morphotype C1.

This is the first report of the isolation of virulent lytic *Lactobacillus brevis* phages isolated from buffalo whey starter cultures.

In order to prevent phage infection and propagation we tested the resistance of the two bacteriophages to pasteurization (63° C for 30 min) and high-pressure treatments up to 800 MPa. Phage sensitivity

for both heat and pressure treatments was tested in PLGYG broth and raw natural media (buffalo milk and whey).

The results showed a very high sensitivity of *ΦApr-1* and *ΦApr-2* to high temperatures and pressures and very interesting was the highest bacteriophage activity impairment when samples of whey starters were screened after infection. That founding in particular was completely unexpected.

CHAPTER I

"MOZZARELLA DI BUFALA CAMPANA"

"BUFFALO MOZZARELLA CHEESE"

1. HISTORY , LEGISLATION AND CHEESE MANUFACTURING

The buffalo was introduced in Italy in the seventh century and was a common animal among the families in the countryside and widely used as a draught animal in ploughing for watery terrains: its strength and the size of its hooves, which do not sink too deeply into puddles, were highly appreciated.

Buffalo Mozzarella cheese is not a recent product, the first references about cheese products made from buffalo milk dated from the beginning of the twelfth century and it was produced in small quantities. From the second half of the eighteenth century Mozzarella became widespread throughout the south of Italy and its production increased more and more.

Nowadays “Mozzarella di Bufala Campana” is an Italian particular unripened “pasta filata” cheese obtained from raw whole buffalo milk and traditionally manufactured to guarantee to the consumer its unique organoleptic characteristics. Its production is protected by a special Trade Organization that monitors the manufacturing process and marketing of the cheese in compliance with the production rules for the DOC (Certified Origin Brand) and DOP (Protected Denomination of Origin) quality brands.

All the phases of the manufacturing are regulated by the Presidential Decree 10/5/1993 (G.U. n. 219, 17/9/1993) and it defines the geographical area of production (Caserta and Salerno and part of Benevento, Naples, Frosinone, Latina, Rome, Foggia and

Isernia), milk characteristics, shape, weight and principal organoleptic qualities of the mozzarella itself, production phases and all the labelling legislation.

Always according to the Presidential Decree, the cheese is produced by using natural wild starter cultures that derive from the whey of a previous successful batch of production and it is left fermenting for 24 hours at room temperature (18°-22° C) prior to be used. Rennet and natural starter cultures are added to the raw milk. The milk coagulation occurs in 20 min and after about 40 min the curd is cut in small pieces and left under its whey until the stretching phase. This phase is generally carried on 4 hours after the addition of the rennet and by adding hot water (ca. 100° C) (D.P.C.M. 10/5/1993; G.U. n. 219, 17/9/1993). The micro flora of milk and whey is the main responsible for the acid production that is necessary for a successful milk coagulation. Lactic Acid Bacteria are also very important in the development of the peculiar organoleptic characteristics thanks to complex biochemical activities.

The DOP mark was conferred with the CE Regulation n.1107/1996 (G.U. n. L 148 del 21/06/1996 pag. 0001 – 0010).

2. MILK AND CHEESE CHARACTERISTICS

Buffalo milk is sweet and white/opaque because of absence of carotenoids, pH is 6,6-6,8 and fat content is 6-9% with prevalence of oleic acid (unsaturated acid) and palmitic acid (saturated acid). Nitrogen content is between 3,8-4%. Non protein nitrogen (NPN) values are between 0,20-0,30%. Lactose varies between 4,5 and 5%.

The main difference between cow and buffalo milk is represented by fat and protein content. The values are respectively of 3,3% and 2,7% in cow milk and 7,5% and 4,4% in buffalo milk.

Fat and protein are two fundamental elements in cheese making in fact the cheese yield to produce Mozzarella using Friesian milk is 9,5% against the 2,5% using buffalo milk (Zicarelli L., 2004).

Another important factor is that a Friesian can produce an average of 25/30 L of milk daily or more against the 9 L of a buffalo.

Also the microbiological composition is different: buffalo milk is characterized by an higher concentration of *Lactobacillus* spp. than cow milk.

"Mozzarella di Bufala Campana" is a fresh, stringy textured cheese with porcelain/white color, an extremely thin rind and delicate taste. When cut it releases a white watery fluid with the aroma of milk enzymes. It is highly nutritional because rich in calcium, high in protein, lactic flora substances, vitamin and mineral/salt content. Baffalo mozzarella cheese is also rich of live lactic ferments and therefore highly digestive.

100 gr of product contain: proteins 19%, fat 21%, vitamin A mg 0,15, vitamin B mg 0,003, vitamin B1 mg 0,3, calcium mg 510,

phosphorus mg 380, sodium mg 0,4, iron mg 0,7 for a total calories for 100 gr of 270 Kcal. The minimum fat content required by D.P.C.M. 10/5/1993 is 52% and maximum moisture allowed is 62%.

It is sold in a variety of shapes, small bites called “bocconcini”, bigger spheres of several size and weight and plaits. The weight can vary between 10 gr and 800 gr. (D.P.C.M. 10/5/1993; G.U. n. 219, 17/9/1993). It might be enjoyed soon after the production and it has to be kept at room temperature and always in its brine.

The best indication of freshness is granted by the following characteristics: elastic consistency, tight, smooth, and humid surface, neither too dry nor too wet. No yellowish marks or spots have to be present and, when pressed with a finger, the texture should be neither soft nor rubbery. Once sliced, mozzarella has to show a grainy surface and appear to be composed of many layers, like an onion, especially near the surface. Pearls of milky whey should seep out when cut and mozzarella’s bites should melt in mouth while enjoyed. When sold each package has to show the typical D.O.P. mark in green and red with the shape of the buffalo head (D.M. 7/4/1998).

3. LAB AND STARTER CULTURES

LAB are a group of Gram-positive micro-organisms united by common morphological, metabolic and physiological characteristics. They are non-sporing, non-respiring, cocci or rods, single or in chains of several length, oxidase-negative, catalase-negative, indole-negative. They produce lactic acids as the major end-product during the fermentation of carbohydrates (Begey *et al.*, 1985; Seppo *et al.*, 1998).

Up to 1992 *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus* were considered the most important core of the group (Billie *et al*., 1992). The description of new genera suggests that the LAB comprise also the genera *Enterococcus* and *Streptococcus* (Coppola S., Parente E. *et al*., 1988; Coppola S., Villani F. *et al*., 1990; Parente E., Villani F *et al*., 1989; Seppo *et al*., 1998; Cogan *et al.,* 1996).

The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and in different media, configuration of lactic acid produced, sugars from which they produce acids and gas, ability to growth at different salt concentration.

Some physiological functions of LAB are of great importance in dairy-production manufacturing and maturation, influencing the final organoleptic qualities of these products, fermentation of sugars that leads to a pH decrease that is important for the coagulation of milk and the reduction or prevention of adventitious micro flora, protein hydrolysis that causes the texture and, partially, the taste of

the product, synthesis of flavour compounds, synthesis of texturing agents, which may influence the consistency of the product, production of inhibitory compounds (P.F., 1982; Fox P.F., McSweeney P.L.H., 1998).

LAB grow very well in de Man Rogosa Sharpe (MRS) agar or broth, these media are not selective and contain several elements useful for the metabolism of lactic bacteria, they can be used also for maintenance. Another medium used for the isolation of some LAB is the Rogosa agar, a selective media that allows the growth of the genera *Leuconostoc, Lactobacillus* and *Pediococcus* but not of *Lactococcus* and *Sreptococcus* (Billie *et al.*, 1992; Harrigan; Roberts *et al.,* 1995). Mesophilic and thermophilic acid bacteria can be enumerated in Skim Milk (Oxoid) (Coppola S., Parente E. *et al.*, 1988). Mesophilic and termophilic lactobacilli can be counted and isolated in Acetate Agar (Coppola S., Parente E. *et al.*, 1988). Mesophilic and termophilic Streptococci grow in Yeast Glucose Lempco Agar (Coppola S., Parente E. *et al.*, 1988); they can also be very well isolated in Neutral Red Chalk Lactose Agar, Litmus Milk and B-Elliker agar (Harrigan W.F., McCance M.E., 1966).

Since media for lactobacilli and streptococci are not particularly selective other tests and microscopic examination (Coppola S., Parente E. *et al.*, 1988) are generally required. Enterococci can be isolated in Enterococci Presumptive broth (Difco) (Coppola S., Parente E. *et al.*, 1988) or on Yeast-Glucose-Lemco Agar and Barnes Agar (Coppola S., Parente E. *et al.*, 1988). *Leuconostoc* can be isolated on high-sucrose Mayeux Agar or on Yeast-Glucose-Citrate Agar (Coppola S., Parente E. *et al.*, 1988). The temperatures

of incubation vary according to the type of bacteria (22°, 30°, 37°, 45° C). The incubation period is of about 48 h and in microaerophilic condition.

4. MILK COAGULATION PHASE AND PRINCIPAL CAUSES IMPLICATED IN FERMENTATION FAILURE

Milk coagulation is the most important phase that occurs during cheese manufacture. It is a two-stage process. The primary phase involves the specific enzymatic modification of the k-casein micelles that are attacked by the proteases of the rennet, specifically in the region of the bond Phe_{105}-Met_{106} to yield two peptides: the glycomacropeptide (caseinomacropeptide) is hydrophilic and soluble, and will diffuse away from the micelle after k-casein splitting, the para-k-casein is instead strongly hydrophobic and remains on the micelle (Fox P.F., 1982). The progressive hydrolysis of k-casein during the primary stage leads to the alteration of the properties of the casein micelles so that they become capable of aggregation. This aggregative phase that occurs in presence of Ca2+ characterizes the second stage of the coagulation process that is the non-enzymatic phase (Fox P.F., 1982). K-casein is the only compound that is hydrolized by rennet. It tooks place on the surface of the casein molecules and it is hydrophobe.

When the 85% of the total k-casein has been hydrolized, the micelles begin to aggregate progressively into a gel network (Fox P.F., McSweeney P.L.H., 1998). The principal proteinase in rennet

is chymosin; about 10% of the milk-clotting activity of calf rennet is instead due to pepsin. The optimum of pH for their activities is about 4 and for this reason fundamental is the drop in pH that is caused by LAB growth and acid production (Fox P.F., McSweeney P.L.H., 1998).

For the reasons previously discussed, slow acid development during cheese making is an important cause of poor quality cheese. Between all the causes of slow acid production, the most common are presence in milk of natural inhibitors and LAB infection by bacteriophages (phages) (Fox P.F., Guinee T.P. *et al*., 2000).

Lactoperoxidase system is one of the natural inhibitors of the milk that plays an important role linked to its anti-microbial power. Peroxidase catalyses reactions in which hydrogen peroxide is reduced while other compounds are suitable electron donors and are subsequently oxidised. In milk the thiocyanate ion (SNC) is the electron donor in the enzymatic reaction leading to anti-microbial effects.

Compounds that have antibacterial effects are produced during the oxidation such as sulphurydicyanide ($S(CN)_2$) and oxyacids of thiocyanate, i.e. OSCN and O_2SCN. OSCN and O_2SCN cause oxidation of vital SH-groups showing inhibition in the growth of Gram-positive bacteria such as lactococci and lactobacilli.

Lysozime is another natural inhibitor in milk, it cleaves the glycosidic bond between N-acetylmuramic acid and N-acetyglucosamine of the bacterial peptidoglicans, which constitute the major part of the bacterial cell wall. Gram-positive bacteria are generally more susceptible because they have a simpler cell-wall

with a high percentage (90%) of peptidoglicans. Lysozimes are heat stable.

Lactoferrin is an iron chelating protein that could be present in milk. Its antibacterial mechanism is linked to its capability to chelate iron, preventing bacteria's growth. It is possible that lactoferrin plays an important role in the defence of mammary gland during lactation.

Immunoglobulins are transferred from the mother to the offspring firstly via placenta, in utero, and then after birth, via milk. There are five major classes of Ig as IgA, IgG, IgM, IgD and IgE. IgG can be divided into two subclasses: IgG1 and IgG2. They cause susceptible starter bacteria to aggregate with consequent localized acid production and precipitation. The starters still continue to grow but localized acid production is very strong and they inhibit themselves. Ig are inactivated by pasteurization (Fox P.F., Guinee T.P. *et al*., 2000).

Antibiotic residues occur in milk because of their use to control mastitis in the breeding. The concentration of the chemical substances, especially penicillin and its derivatives, decreases with each milking. Milk from animals treated with antibiotics should be withheld for the whole length of time prescribed for the specific antibiotic preparation. The most part of antibiotics kills starter cultures important for cheese manufacture (Fox P.F., Guinee T.P. *et al*., 2000).

Today, one of the most important cause of slow acid production in cheese plants is the **bacteriophage (phage)** infection. This can significantly upset manufacturing processes and, in extreme cases,

results in complete failure of acid production. Phages are viruses that can multiply only within a bacterial cell. They are ubiquitous in nature and can be seen only with electron microscope (Fox P.F., Guinee T.P. *et al*., 2000).

They are differentiated on the basis of morphology, serology, DNA characterization and way of infection (lytic/virulent and lysogenic/temperate bacteriophages). All the viruses are generally built on the same principles, nucleic acid called "core" surrounded by a protein coat called "capsid". The nucleic acid is a long filamentous molecule and may be DNA, in single or double chain, or RNA.

Phages exhibit a greater diversity of form than other groups. Six basic morphological types have been described by Bradley in 1967 and they are still valid. The first type (Group A) has a head with hexagonal outline which may or may not be elongated; a tail with a contractile sheath is attached to it. This is usually rigid and may have various appendages, such as fibres or terminal structures. The core is made up of 2-DNA chains. The second group (Group B) also has a six-sided head and a tail. The tail is relatively flexible, may or may not have terminal appendages and it is longer than the head diameter and has also no contractile apparatus. The core is made up of 2-DNA chains. The third type (Group C) also has a tail and a six-sided head, the tail is shorter than the head diameter or maximal dimension and may also have appendages attached to it and it is non-contractile. The core is made up of 2-DNA chains. The fourth group (Group D) has no tail. It is still six-sided in outline. Each apex of the hexagon has a knob or large capsomer on it. The core is made up of a single-

DNA chain. In the next group (Group E) large capsomers are absent and the viron present a simple regular hexagonal outline. The core is made up of a single-RNA chain. The final group (Group F) is quite unlike the others, the viron being in the form of a long flexible filament with no additional structures of any kind attached to it. The core is made up of a single-DNA chain. The kind of nucleic acids found in the members of each group has proved to be the same regardless of the genus of host bacterium (Bradley, 1967).

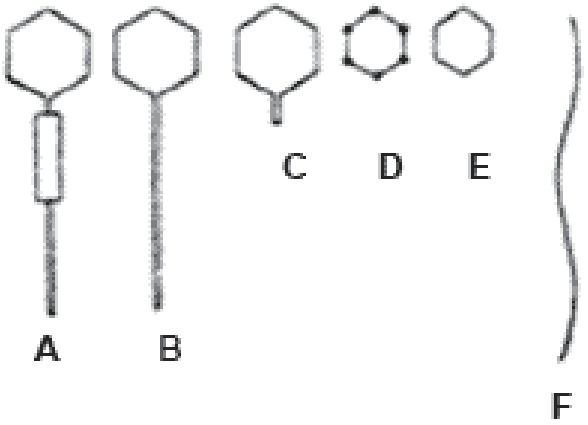


Figure 1. Basic morphological types of bacteriophages Bradley (1967)

Phages are very specifically related to their hosts. Among the phages of LAB we have to differentiate between lactic streptococcal phages, phages of thermophilic streptococci, lactobacillus phages, leuconostoc phages (Jarvis, 1989).

According to Bradley (1967), phages attacking LAB belong to Groups A, B and C. The majority of lactococcal phages belong to Group B and it has been reported that lactococcal isometric phages

have heads ranging from 45-65 nm and tails ranging from 100-250 nm. Prolate phages are generally smaller with head sizes ranging from 55-65 x 40-48 nm and tails ranging from 80-110 nm. Phages may possess complex tail appendages, collars and other structural components that require special staining techniques to be identified.

Streptococcus thermophilus, lactobacilli and leuconstocs phages have also been studied. Lactobacilli and leuconstocs are hosts to both Group A and B phages and phages for *Streptococcus thermophilus* have been reported to have isometric head morphology and to belong to Bradley's Group B.

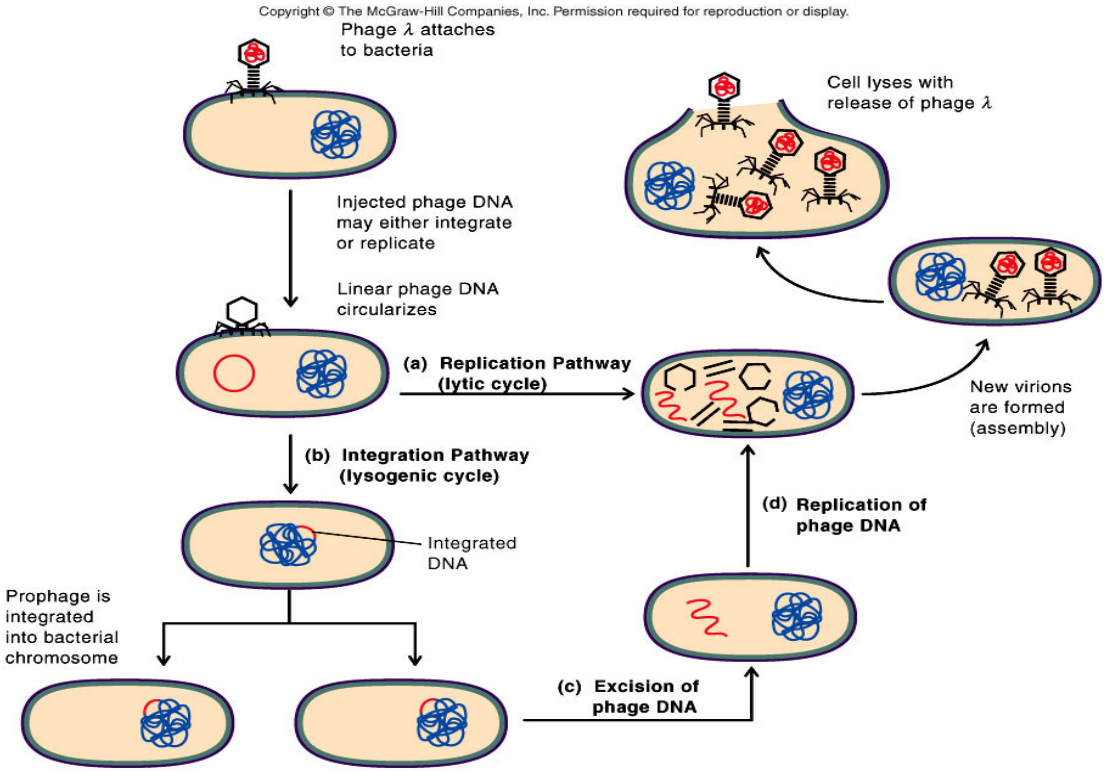
Among the Families of bacteriophages that have been up to now identified, LAB phage Families belong to **Myoviridae** A1, **Podoviridae** C1, C2 and C3 and **Siphoviridae** B1, B2 and B3.

Bacteriophage can have two different replicative cycles: in the "lytic cycle" the first step involves adsorption of the phage onto special attachment sites, called phage receptors, on the cell surface of the host. Phage adsorbs to the cell through its tail. Once a phage has attached to the receptors, it injects its DNA into the host cell. Immediately phage DNA and phage proteins are produced rather than host cell DNA and proteins. The phage DNA is packaged in the phage head and when the syntesis is completed, the cell lyses, releasing new phage active particles, which start the process again. Cell lysis is caused by a lytic enzyme called "lysine"(Fox P.F., Guinee T.P. *et al*., 2000) and coded by the phage genome.

The "lysogenic cycle" is the second way of multiplication. The first step is characterized by adsorption and DNA injection that occurs as in the lytic cycle. Than, instead of direct phage replication, the phage DNA is integrated into the genome of the bacteria by the

integrase enzyme. As the bacterial genome multiplies, the genome of the phage multiplies simultaneously without lysing the bacterial cell. The bacteriophage integrated into the bacterial genome is called "prophage". Bacteria containing prophages are called lysogens and are resistant to infections of bacteriophages that are genetically the same as the prophage (Fox P.F., Guinee T.P. *et al*., 2000). It is usual to call bacteriophages with the first type of infective cycle "virulent" and those with the second type "temperate". However many phages are capable of both replicative cycles, depending on the bacterial strains they infect; that is why the two terms really apply to phage-host systems rather than to the bacteriophage alone (Bradley, 1967).

The two bacteriophage lifecycles: the lytic lifecycle and the lysogenic lifecycle



5. THESIS OBJECTIVES

1) Isolation and characterization of LAB from buffalo whey starter cultures

2) Isolation and characterization of bacteriophages infecting the isolated LAB

3) Evaluation of phages' sensitivity to heat and high pressure treatments

CHAPTER II

Isolation of Lactic Acid Bacteria from natural whey starters utilized in the manufacture of "Mozzarella di bufala"

ABSTRACT

Since phage infection was suspected as the main cause of acidification problem in the manufacture of the water buffalo mozzarella cheese in some dairy farms in Southern Italy, 156 strains of LAB were detected in order to evaluate their bacteriophage sensitivity.

They have been isolated from twelve samples of "wild" whey starters by using selective and non-selective media and their characterization has been carried out by using biochemical and genetic tools.

INTRODUCTION

Buffalo mozzarella cheese is a fresh and stringy textured cheese with porcelain-white colour, with an extremely thin rind and delicate taste. It is enjoyed soon after the production and kept at room temperature in brine for few hours, depending on the size of the product.

The milk is curdled by using natural starter cultures that derive from the whey of a previous successful batch of production and stored at room temperature for 24 hours before being used.

A seasonal fermentation delay that leaded to a complete fermentation failure of the coagulation phase of the milk was observed in few factories in the South of Italy.

We focused the attention of this research on the presumptive phage infection of LAB, still being a major problem in the field of industrial fermentations.

Because of the high specificity between phage and its host, we isolated and characterized a large number of LAB from samples of whey starters in order to detect the phages implicated in the slowing down process of the acid development.

MATHERIAL AND METHODS

Characteristics of samples Twelve samples of water buffalo whey starters were collected from a pool of dairy factories of the South of Italy, transported to the Department of Zootechnical Sciences and Food Inspection, Faculty of Veterinary Medicine, Naples, Italy, to be frozen at -20°C and then transported to the North of Ireland for analysis (Microbiology laboratories of Food Technology Education Branch, College of Agriculture, Food and Rural Enterprise, Loughry Campus, Cookstown, Co Tyrone, Northern Ireland, BT80-9AQ, U.K.).

They were divided into two groups: group A included four whey starters that showed acidification problems and group B the eight samples not showing any anomalies during cheese making. They were frozen and refrigerated during the transport using solid carbon dioxide.

Samples of group B were treated with an equal volume of sterile buffalo milk (110°C, 10 minutes) or with 10% v/v sterile calcium carbonate prior freezing in order to reduce the adverse effects on starter cultures given from high acidity combined with frozen storage.

Isolation and identification of LAB The twelve whey starters were thawed at 37°C and serial, decimal dilutions were made using maximum recovery diluent (Straker *et al*., 1957).

Dilutions were plated onto MRS agar (De Man *et al.,* 1960) and incubated at 30° C for 72 h in microaerophilic conditions.

Colonies were randomly selected using Harrison disk (Harrigan *et al*., 1998), their colour, shape and size (diameter length) were determined and then each isolate was taken by sterile and disposable wire-loop and incubated in MRS broth (De Man *et al.,* 1960) at 30°C for 24-72 h, MRS plates were streaked with broth cultures to isolate pure colonies. 144 colonies have been isolated (67 from group A and 77 from group B).

For this research we updated the scheme devised by Billie *et al*. (1992) for their identification to genus level (table 1). Each strain was tested according to the analysis suggested by the scheme. Moreover size and shape of vegetative cells were observed under optic microscope and catalase and oxidase test were carried out.

An high percentage of colonies (44 %) was classified to the species level using API 50 CHL computer-assisted identification scheme (API Laboratory Products Ltd., Basinstoke, England). Two isolates were subjected to commercial 16S rRNA gene sequence analysis performed using Applied Biosystems MicroSeq™ microbial analysis software and database (MIDI Labs, 125 Sandy Drive, DE 19713, N.Y., U.S.A.).

In order to isolate *Streptococcus* spp. for testing them as host for the study of phage infection in wheys of group A, only whey samples of group B were streaked onto Neutral Red Chalk Lactose Agar and B-Elliker agar and incubated at 30° C for 72 h. The isolates were grown in Litmus Milk (Harrigan *et al.*, 1998) at 30° C for 24-72 h.

Strains were classified to species level using the API 50 CHL computer-assisted identification scheme (API Laboratory Products Ltd., Basinstoke, England).

The most common spoiling bacteria and pathogens titres were evaluated according to table 2.

The pH of the samples was tested by using electronic pH-controller.

Table 1: Scheme of Billie *et al* (1992) for identification of LAB to genus level updated by Aprea G. for this research
(updated part in red)

Test	*Lacto-coccus*	*Leuco-nostoc*	*Lacto-bacillus*	*Pedio coccus*	*Entero-coccus*	*Strepto-coccus*
Cell morf.	cocci	Cocci/ cocco-bacilli	Rods/ cocco-bacilli	Cocci pairs/ tetrad	Cocci/ chain	Cocci pairs/ chains
Gram	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
Sugar fermantation	Homo	Hetero	Homo/ Hetero		Homo	Homo
Ferm. Glu	+	+	+		+	+
Ferm.Gal.	+	+	+-		+	-
Ferm. Lact.	+	+	+		+	+
Ferm. Rhm.	-		+-			-
Ferm. Man.		+-	+-		+	+
Ferm. Raf.	-	+-	+-		+-	
Ferm. Mlt.		-	+-		+-	-
Gas from glucose	-	+	+ -	-	-	-
Gas from fructose	-	+	+ -	-		
Gas from trehalose	-	+ -	+ -	-		
Growth pH 4.4	+-	+-	+-	+	+	-
Growth pH 9.6	-	-	-	-	+	-
Growth T 10° C	+	+	+-	+-	+	-
Growth T 40° C	+-	-	+		+	+
Growth T 45° C	-	-	+-	+-	+	+
Growth 6.5 % NaCl	-	+-	+-	+-	+	-
Growth 18 % NaCl	-	-	-	-	-	-
Lactate isomer	L	D	DL, D, L	DL	L	L
Growth on Rogosa Agar	-	+	+	+		-
Growth on M 17	+	+		+-		-
NH_3 from arginine	+ -	-	+ -	+	+	-
Dextran production from sucrose	-	+	+ -	-		
Reduction of Litmus	+	-	-			-
Metab. of citrate	+-	+	-			-

Table 2: Media and techniques used in isolating vegetative cells

Germ	Medium	Days and T° of incubation	Technique
TVC	MPCA	30 C/3 dd	Pour plate
Psycotrofic count	MPCA	7 C/10 dd	Spread plate (0.1 ml)
Enterob.	VRBGA	37 C/24 h	Double pour plate
Coliforms	Coli ID	37 C/ 2dd	Double pour plate
Sth. aureus	Baid Parker + Rabbit Plasma Fibrogen (RPF)	37 C/2 dd	Spread plate (0.1 ml)
LAB	MRS agar Gogosa agar	30 C/3 dd	Pour plate; incubation in microaerofilic conditions
Yeast/ moulds	YGCA	25 C/ 5 dd	Pour plate

RESULTS

The totality of the isolates (144) resulted Gram positive and oxidase and catalase negative and they were characterized to genus level.

An overview of the genus composition of the two groups of whey starters is given in table 3. A lower concentration of Lactococci in the group A was noticed.

Among the colonies characterized to species level 40% were *Lactococcus lactis cremoris*, 20% *Lactococcus lactis lactis*, 17.1% *Lactobacillus paracasei*, 8.6% *Lactobacillus curvatus*, 8.6% *Lactobacillus delbrukii bulgaricus*, 5.7% *Lactobacillus fermentum.*

The commercial 16S rRNA gene sequence analysis (MIDI Labs, U.S.A.) performed on two isolates gave the following results: *Lactobacillus paracasei* and *Lactobacillus brevis*.

Classification of the colonies on the basis of their colour, shape and size (diameter length) are showed in Annex I, table 5 and table 6.

Details of shape and size of vegetative cells of LAB are described in Annex I, table 7 and table 8.

Twelve *Streptococcus salivarius thermophilus* strains were isolated in Neutral Red Chalk Lactose Agar, Litmus milk and B-Elliker agar and identified by API 50 CHL computer-assisted identification scheme (API Laboratory Products Ltd., Basinstoke, England).

The results of the microbiological analyses and pH are showed in table 4.1 and 4.2.

Table 3: Presumptive identification of LAB present in natural whey starters

Group	Lacto-coccus	Leuconostoc	Lacto-bacillus	Pedio coccus	Entero-coccus	Streptococcus	Genus not identified
A	2.4%	ND	78.6%	ND	ND	ND	19%
B	54.7%	ND	39%	ND	ND	ND	6.3%

Table 4.1: Evaluation of microflora and pH of whey starters of Group A

Group A	***Sample 1*** (ufc) *	***Sample 2*** (ufc)	***Sample 3*** (ufc)	***Sample 4*** (ufc)
Total Viable Count	8.4×10^6	1.6×10^6	2.2×10^5	5.1×10^3
MRS count	2.35×10^5	1.4×10^4	1.25×10^2	1.1×10^3
ROG count	2.9×10^4	1.1×10^3	$< 1.0 \times 10^1$	1.3×10^2
Enterobatteri	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Coliformi	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
E.coli	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
S.aureus	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
Psycotrophic count	2.8×10^4	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
pH	6.53	4.98	4.16	4.55

* Whey obtained from the curdle during the manufacturing

Table 4.2: Evaluation of microflora and pH of whey starters of Group B

Group B	***Sample 5*** (ufc)	***Sample 6*** (ufc)	***Sample 7*** (ufc)	***Sample 8*** (ufc)	***Sample 9*** (ufc) **	***Sample 10*** (ufc) **	***Samp. 11*** (ufc) ***	***Samp. 12*** (ufc) ***
Total Viable Count	3.0×10^6	2.1×10^7	1.0×10^7	1.7×10^7	6.2×10^7	1.6×10^7	8.9×10^7	2.0×10^7
MRS count	2.8×10^5	7.7×10^5	2.0×10^5	3.5×10^5	3.3×10^6	4.7×10^5	5.7×10^5	7.5×10^6
Enterob.	$<1.0 \times 10^0$	3.0×10^0	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	1.0×10^0	$<1.0 \times 10^0$
Coliformi	$<1.0 \times 10^0$	1.0×10^0	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$
E.coli	$<1.0 \times 10^0$	1.0×10^0	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$	$<1.0 \times 10^0$
S.aureus	$< 1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Psycotrop. count	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Yeast/ moulds	1.8×10^1	3.5×10^2	1.9×10^2	3.0×10^2	9.8×10^3	2.4×10^4	6.8×10^3	5.2×10^3
pH	4.14	5.21	4.05	4.17	5.24	5.09	5.42	5.53

** Whey starters treated with sterile buffalo milk (50%)
*** Whey starters treated with calcium carbonate (10%)

CONCLUSIONS AND CONSIDERATIONS

This report focused on the LAB isolation as first step in evaluating the phage infection of the starter cultures in buffalo mozzarella cheese.

Some problems occurred during the research: firstly no particularly selective media are available for many groups of LAB so that other tests and microscopic examination were carried out.

Moreover whey samples were frozen and this was another critical point since many LAB were killed by the treatment. For this reason more samples were analysed in a second moment and this time they were treated with 50% of sterile buffalo milk and 10% of calcium carbonate to reduce the negative effect of the combination of low temperature and low pH on LAB.

A significant difference was noticed between the total MRS count of samples of group A and the ones of group B. The percentage of Lactobacilli isolated from the samples of Group A is extremely higher then Lactococci. In group B the discrimination between the two groups is quite low but Lactococci prevail against the number of lactobacilli.

Contrary to what observed before from Coppola *et al.* (1988, 1989, 1990), *Lactobacillus fermentum* (group A) and *Lactobacillus para paracasei* (group B) were the most frequently isolated among Lactobacilli. According to the same Authors, *Lactococcus* was the most common LAB in the group B but *Lactococcus lactis cremoris*

was isolated the most, followed soon after by *Lactococcus lactis lactis.* Still according to Coppola *et al.* (1988, 1989, 1990) thermophilic LAB were identified more frequently than mesophilic ones.

ANNEX I

Table 5: Group A: colony description of shape, colour and size

SAMPLE	COLONY	SHAPE, COLOUR, SIZE
SB	SB 1	oval, brown, 1mm
SB	SB 2	round, white, 0.5mm
SB	SB 3	round, white, 1mm
SB	SB 4	oval, brown, 1.5mm
SB	SB 5	round, white, 0.5mm
SB	SB 6	round, white, 0.5mm
SB	SB 7	oval, white, 1mm
SB	SB 8	irregular, white, 0.5mm
SB	SB 9	round, white, 1mm
SB	SB10	oval, white, 1mm
SB	SB 11	round, white, 0.5mm
SB	SB 12	round, white, 0.5mm
SB	SB 13	round, white, 5mm
SB	SB14	round, white, 3mm
C	C1	round, white, 1mm
C	C2	oval, brown, 1mm
C	C3	round, white, 2mm
C	C4	round, white, 1mm
C	C5	oval, brown, 1mm
C	C6	round, brown, 1mm
C	C7	oval, white, 0.5mm
C	C8	round, white, 1mm
C	C9	oval, white, 0.5mm
C	C10	oval, brown, 1mm
C	C11	round, white, 1.5mm
C	C12	oval, brown, 1.5mm
C	C13	round, white, 2mm

C	C14	round, white, 0.5mm
C	C15	round, white, 1.5mm
C	C16	round, white, 1mm
C	C17	round, white, 0.5mm
C	C18	oval, brown, 1mm
C	C19	oval, brown, 1mm
W	W 1	Round, white, 1.5 mm
W	W 2	Round, white, 1.5 mm
W	W 3	Round, brown, 1 mm
W	W 4	Round, white, 0.5 mm
W	W 5	Round, white, 1 mm
W	W 6	Round, white, 1.5 mm
W	W 7	Round, white, 1 mm
W	W 8	Oval, brown, 1 mm
W	W 9	Round, brown, 0.5 mm
W	W 10	Round, white, 2 mm
W	W 11	Round, white, 1 mm
W	W 12	Round, white, 1 mm
G	G 1	Round, white, 1.5 mm
G	G 2	round, white, 1mm
G	G 3	round, white, 1mm
G	G 4	round, white, 1mm
G	G 5	round, brown, 2mm
G	G 6	round, white, 1mm
G	G7	Irregular, white, 2.5mm
G	G8	Oval, white, 1 mm
G	G9	Round, white, 1mm
G	G10	irregular, white, 1.5mm
G	G11	Irregular, white, 3mm
G	G12	Oval, white, 1mm
G	G13	oval, white, 1mm
G	G14	round, white, 1mm
G	G15	round, white, 0.1mm
G	G16	round, white, 0.1mm
G	G17	Round, white, 1mm
G	G18	oval, white, 1mm
G	G19	irregular, white, 2mm
G	G20	Round, white, 1mm
G	G21	round, white, 1mm
G	G22	round, white, 0.1mm

Table 6: Group B: colony description of shape, colour and size

SAMPLE	COLONY	SHAPE, COLOUR, SIZE
S	S 1	irregular, white/brown, 3mm
S	S 2	round, brown, 2mm
S	S 3	Oval, brown, 1mm
S	S 4	round, white, 1mm
S	S 5	irregular, white, 1.5mm
S	S 6	round, brown, 1mm
S	S 7	Oval, brown, 1mm
S	S 8	round, brown, 3mm
S	S 9	irregular, brown, 1mm
S	S10	irregular, white, 3.5mm
A	A1	round, brown, 1.5mm
A	A2	round, white, 1.5mm
A	A3	round, white, 2mm
A	A4	round, brown, 1.5mm
A	A5	Oval, brown, 1mm
A	A6	oval, brown, 1mm
A	A7	round, white, 2mm
A	A8	Oval, brown, 1mm
A	A9	oval, brown, 1mm
3S	3S 1	round, white, 0.5mm
3S	3S 2	round, brown, 1mm
3S	3S 3	oval, brown, 1mm
3S	3S 4	oval, brown, 1mm
3S	3S 5	oval, brown, 1mm
3S	3S 6	irregular, brown, 1mm
3S	3S 7	round, white, 0.5mm
3S	3S 8	round, brown, 1.5mm
4B	4B 1	oval, brown, 1mm
4B	4B 2	round, white, 3mm
4B	4B 3	round, white, 0.5mm
4B	4B 4	round, brown, 1mm
4B	4B 5	round, brown, 1.5mm
4B	4B 6	oval, brown, 1mm
4B	4B 7	Round, brown, 1 mm
4B	4B 8	Round, white, 1 mm
4B	4B 9	oval, brown, 1 mm
4B	4B 10	Round, white, 1.5 mm
5BL	5BL 1	round, brown, 1.5mm
5BL	5BL 2	Irregular, brown, 1.5mm
5BL	5BL 3	round, brown, 1mm
5BL	5BL 4	oval, brown, 1.5mm

5BL	5BL 5	round, brown, 1.5mm
5BL	5BL 6	round, brown, 0.5mm
5BL	5BL 7	Round, white, 1mm
6L	6L 1	oval, brown, 1mm
6L	6L 2	round, brown, 1.5 cm
6L	6L 3	round, brown, 1mm
6L	6L 4	round, brown, 1.5mm
6L	6L 5	round, brown, 1mm
6L	6L 6	round, white, 0.5mm
6L	6L 7	round, brown, 3mm
8VCC	8VCC 1	round, white, 2mm
8VCC	8VCC 2	oval, brown, 1mm
8VCC	8VCC 3	oval, brown, 1.5mm
8VCC	8VCC 4	oval, brown, 1mm
8VCC	8VCC 5	round, white, 0.3mm
8VCC	8VCC 6	round, white, 3mm
8VCC	8VCC 7	round, brown, 1mm
8VCC	8VCC 8	irregular, white, 1.5mm
9BCC	9BCC 1	oval, brown, 1mm
9BCC	9BCC 2	oval, brown, 1mm
9BCC	9BCC 3	round, white, 2mm
9BCC	9BCC 4	oval, brown, 1mm
9BCC	9BCC 5	irregular, white, 1.5mm
9BCC	9BCC 6	round, white, 1mm
9BCC	9BCC 7	oval, brown, 1mm
9BCC	9BCC 8	round, brown, 1mm
9BCC	9BCC 9	Irregular, white, 2mm
9BCC	9BCC10	oval, brown, 1mm

Table 7: Group A: isolate description of size and shape

COLONY	GRAM	SIZE (micron)	SHAPE
SB 1	+	5	Rods/chains (intracellular inclusion?)
SB 2	+	4	Rods
SB 3	+	3	Rods
SB 4	?	3.5	Rods/chains
SB 5	?	2	Rods/chains (intracellular inclusion)
SB 6	+	2.5	Rods (intracellular inclusion)
SB 7	+	4	Rods
SB 8	?	2	Rods (intracellular inclusion)
SB 9	?	2	Rods (single) (intracellular inclusion)
SB10	+	2	Rods in chains (intracellular inclusion)
SB 11	+	2.5	Rods
SB 12	+	2.5	Rods/chains
SB 13	-	-	-
SB 14	+	3	Rods (intracellular inclusion)
C1	+	3	Rods (intracellular inclusion)
C2	+	2	Rods (intracellular inclusion?)
C3	+	1.2	Coccus/chains
C4	?	2	Rods (intracellular inclusion)
C5	?	3	Rods (intracellular inclusion)
C6	+	2.5	Rods/chains (intracellular inclusion?)
C7	+	3.5	Rods (intracellular inclusion)
C8	?	3	Rods
C9	+	3	Rods (intracellular inclusion)
C10	+	4	Rods
C11	-	-	-
C12	+	2	Rods (intracellular inclusion)
C13	?		Rods (intracellular inclusion)
C14	?	2.5	Rods (intracellular inclusion)
C15	?	2	Rods (intracellular inclusion)
C16	?	2.5	Rods (intracellular inclusion)
C17	+		Tetrads
C18	-	-	-

C19	+	2	Rods
W 1	+	2	Rods in chains
W 2	+	2	Rods in chains
W 3	?	2	Rods (intracellular inclusion)
W 4	?	3	Rods (intracellular inclusion)
W 5	+	2	Rods (intracellular inclusion?)
W 6	?	2.5/4	Rods (intracellular inclusion)
W 7	?	3	Rods (intracellular inclusion)
W 8	?	2	Rods
W 9	-	-	-
W 10	?	1.5	Rods/chains (intracellular inclusion)
W 11	?	3	Rods (intracellular inclusion)
W 12	?	2	Rods (single) (intracellular inclusion?)
G 1	+	2	Rods in chains
G 2	+	1	Coccus
G 3	+	1	Coccus/chains
G 4	+	1	Coccus/chains
G 5	+	1	Coccus or small rods
G 6	+	1	Coccus/chains
G7	+	1	Coccus/chains
G8	+	1	Coccus
G9	-	-	-
G10	-	-	-
G11	-	-	-
G12	+	2	Rods
G13	?		Coccus or small rods
G14	+	1	Coccus/chains
G15	+	2-3	Rods
G16	+	1	Coccus/chains
G17	+	1	Coccus/chains
G18	+	1	Tetrads
G19	+	1	Coccus/chains
G20	+	1	Coccus/chains
G21	?	1	Coccus/chains

Table 8: Group B: isolate description of size and shape

COLONY	GRAM	SIZE (micron)	SHAPE
S 1	+	1	Rods/chains
S 2	+	2	Rods/chains (intracellular inclusion?)
S 3	+	1	Rods/chains
S 4	+	1	coccus
S 5	+	1	Rods/chains
S 6	+	2	Rods/chains
S 7	?	3	Rods/chains (intracellular inclusion)
S 8	?	3	Rods/chains (intracellular inclusion)
S 9	?	3	Rods/chains (intracellular inclusion?)
S10	+	1	Coccus
A1	?	2	Rods
A2	?	1	Coccus/chains
A3	+	1	Coccus/chains
A4	+	1	Coccus/chains
A5	+	1	Coccus/chains
A6	+	1	Coccus/chains
A7	+	1	Coccus/chains
A8	?	1	Coccus/chains
A9	+	1	Coccus/chains
3S 1	+	1	Coccus/chains
3S 2	+	1	coccus
3S 3	+	1	coccus
3S 4	+	1	coccus
3S 5	?	1	coccus
3S 6	+	1	Coccus/chains
3S 7	+	1	Coccus
3S 8	?	2	Rods
4B 1	+	3	Rods (intracellular inclusion?)
4B 2	+/?	2	Rods (intracellular inclusion)
4B 3	+	3.5	Rods
4B 4			
4B 5	+	2	Rods Filamentous colony (intracellular inclusion)
4B 6	+	2	Rods (intracellular inclusion)
4B 7	+	2	Rods (intracellular inclusion)
4B 8	?/+	2.5	Rods
4B 9	+		Tetrads
4B 10	?	3	Rods
5BL 1	?	2.5	Rods

			(intracellular inclusion)
5BL 2	+	1	Coccus/chains
5BL 3	+	1	Coccus/chains
5BL 4	?	1	Coccus/chains
5BL 5	?	1	Coccus/chains
5BL 6	+	2	Rods
5BL 7	+	2	Rods (intracellular inclusion)
6L 1	+	1	coccus
6L 2	+	1	Coccus/chains
6L 3	?	3	Easts
6L 4			Rods + Coccus
6L 5	+	1	Coccus/chains
6L 6	+	3	Rods
6L 7			
8VCC 1	+	2	Rods
8VCC 2	+	1	Coccus/chains
8VCC 3	+	2	Rods
8VCC 4	?	3	Rods
8VCC 5	?	3	Rods (intracellular inclusion?)
8VCC 6	?		Tetrads
8VCC 7	?	4	Rods (intracellular inclusion)
8VCC 8	?	4	Rods
9BCC 1	+	4	Rods
9BCC 2	?	1	Coccus/chains
9BCC 3	+	1	Coccus/chains
9BCC 4	+	1	Coccus/chains
9BCC 5	+	1	Coccus/chains
9BCC 6	?	1	Coccus
9BCC 7	+	1	Coccus/chains
9BCC 8	?	1	Coccus/chains
9BCC 9	+	1	Coccus/chains
9BCC10	+	1	Coccus/chains

CHAPTER III

Isolation of polyphosphate-accumulating Lactic Acid Bacteria from natural whey starters

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The isolation of LAB as described in Chapter II was the key factor in the phage infection study of this research.

In the same time the microscopic examination of vegetative cells as reported in the previous chapter showed the presence of intracellular inclusions in the most part of the isolated LAB.

Since inclusion bodies in LAB have never been described before, we also focused our attention on their characterisation and details and results are showed in this Chapter.

ABSTRACT

Natural water-buffalo whey starters are used in the manufacture of Mozzarella cheese in Southern Italy. Microscopic examination of twelve commercial whey starters revealed high concentrations of lactic acid bacteria, in particular lactobacilli, containing inclusion bodies.

Differential staining techniques revealed that the inclusion bodies contained high concentrations of polyphosphate (polyP). Because of the potential role of polyP in intracellular pH control, the presence of this polymer in starter bacteria may be an environmental adaptation to the typical high acidity storage conditions of natural starters.

INTRODUCTION

Natural whey starters, despite their unpredictable performance, are still used extensively in the manufacture of Mozzarella cheese using milk obtained from water buffaloes (*Bubalus arnee)* in Southern Italy.

Water-buffalo whey starters are derived from the whey of a previously successful batch of cheese and are generally stored at ambient temperature for 24 h prior to use. Relatively little research has been undertaken on these natural starters but they are known to contain leuconstocs, lactobacilli, lactococci and frequently streptococci (Coppola S., Parente, S. *et al*., 1988).

During a study to characterise the bacteriophage sensitivity of strains isolated from whey starters we observed large numbers of LAB containing inclusion bodies. Inclusion bodies are often found in bacteria grown under certain conditions and may be composed of the biopolymers poly-β-hydroxybutrate, polyphosphate, sulphur, lipid or polysaccharide. Large inclusion bodies may also be mistaken for endospores. Using specific staining techniques it is possible to distinguish and identify the inclusion bodies present.

In this communication we provide information that suggest that many lactobacilli, and perhaps lactococci, present in water-buffalo whey starters contain inclusion bodies of polyphosphate and speculate on the environmental significance of this finding.

MATERIALS AND METHODS

Source and treatment of whey starters: the details of this section have been described in Chapter II

Isolation and characterisation of LAB*:* the details of this section have been described in Chapter II

Staining methods The presence of endospores was examined by the Schaeffer-Fulton procedure, using hot malachite green staining with safranin counter stain (Doetsch *et al*, 1981). Poly-β-hydroxybutrate (PHB) was assessed under fluorescence by Nile Blue staining (Ostle *et al*, 1982).

Polyphosphate (polyP) was investigated using both Neisser staining (Gurr, 1965) and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), fluorescence (Streichan *et al*., 1990). DAPI stains DNA blue. Due to the similar properties of polyP, a yellow fluorescence of DAPI-polyP was detected by using DAPI at 50µg/ml. Fluorescent cells were visualised using a Leica DMR microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland) with a Leica DC 300F digital camera and Leica IM1000 Image Manager software.

RESULTS AND DISCUSSION

Microscopic examination of the Group A and Group B starters revealed that a large number of the lactobacilli (ca. 60%) had inclusion bodies. This finding was unexpected. Some of the lactococci also appeared to contain inclusion bodies but because of their small size, we could not confirm this unequivocally. The presence of inclusion bodies was not dependent on growth in water-buffalo milk, they were apparent in cultures propagated in MRS broth. This property appeared to be stable since inclusion bodies were apparent after several subcultures in MRS.

Preliminary experiments using Schaeffer-Fulton reagent confirmed that the inclusion bodies were not endospores. Staining with Nile Blue also gave a negative result, indicating that the inclusion bodies did not contain PHB.

Using Neisser staining, inclusions appeared purple/black-characteristic of polyP. Inclusions had a polar location and were often numerous in ZW-9 and 9BCC-4, a lactococcus. In the case of isolates W-4, 4B-10 and 5BL-6 single inclusion bodies, almost completely occupying the cell volume, were also obvious, again this is also consistent with polyP. Typical results are given in figure 1. These inclusions were subsequently confirmed as polyP by their unique yellow fluorescence under DAPI staining.

The occurrence of intracellular polyP in these isolates may be significant. PolyP is known to be involved in phosphate, ATP regulation and in adaptation to stress conditions in environmental

microorganisms (Kornberg *et al.,* 1999). The reason for such high polyP accumulation in these isolates, especially W-4, 4B-10 and 5BL-6 is unknown and will require further study. Due to the multifunctional nature, and complexity of polyP metabolism in microorganisms it is often difficult to attribute the accumulation of polyP to one specific role or function.

At a low environmental pH, polyP accumulation may function in several ways including intracellular pH regulation, energy conservation and/or regulation of key stress survival genes (Kornberg *et al.,* 1999).

PolyP may assist in regulation of pH in a low pH environment, countering a drop in cytoplasmic pH. During synthesis of negatively charged polyP by polyphosphate kinase, H^+ ions may be trapped as a counterion in polyP, balancing the negative charge of polyP, and preventing the drop or decrease in pH that would otherwise occur due to influx of H^+ ions. A role of polyP has been postulated for the maintenance of intracellular pH of *Saccaromyces cerevisiae* (Bental *et al,* 1991), *Dunaliella salina* (Castro *et al*, 1995) and *Burkholderia cepacia* AM19 (Mullan A., McGrath J.W. *et al*, 2002).

The detrimental effects of storing starter cultures under low pH and at elevated temperatures on their subsequent growth and acid-producing potential are well documented (Lawrence *et al*, 1976).

CONCLUSIONS AND CONSIDERATIONS

Since natural whey starters are subject to variable storage temperatures under high acid conditions for extended periods and generally function satisfactorily, it would appear that they have some resistance to the detrimental effects of high acidity. It is possible that the polyP metabolism of these starters may contribute, at least in part, to this resistance. Obviously further study is required to confirm this hypothesis.

Table 1: Reaction of typical isolates to stains

Isolate	Stain			
	Schaeffer-Fulton (Spore)	**Nile Blue (PHB)**	**Neisser (PolyP-)**	**DAPI (PolyP-)**
Lactobacillus (unclassified) ZW-9	-	-	+	+
Lactobacillus fermentum W-4	-	-	+++	+++
*Lactobacillus paracasei** 4B-10	-	-	+++	+++
Lactobacillus fermentum 5BL-6	-	-	+++	+++
Lactococcus lactis biovariant *cremoris* 9BCC-4	-	-	+	+

Notes

* Genus and species identity confirmed by commercial 16S rRNA gene sequence analysis

\- no reaction

\- + positive reaction, +++ high concentration detected

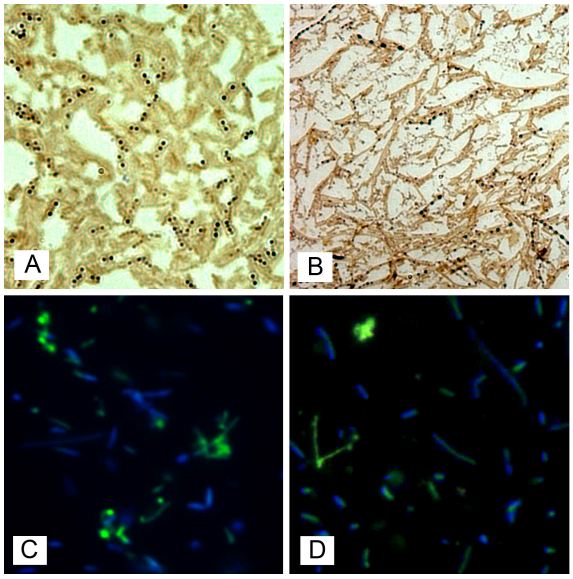


Fig 1: Detection and confirmation of polyphosphate inclusions in lactobacilli from whey starters. Purple/black inclusions of polyphosphate observed under Neisser stain A, *Lactobacillus paracasei* 4B-10, B, *Lactobacillus fermentum* 5BL6. Yellow fluorescence of polyphosphate under DAPI staining C, *Lactobacillus paracasei* 4B-10, D, *Lactobacillus fermentum* 5BL6.

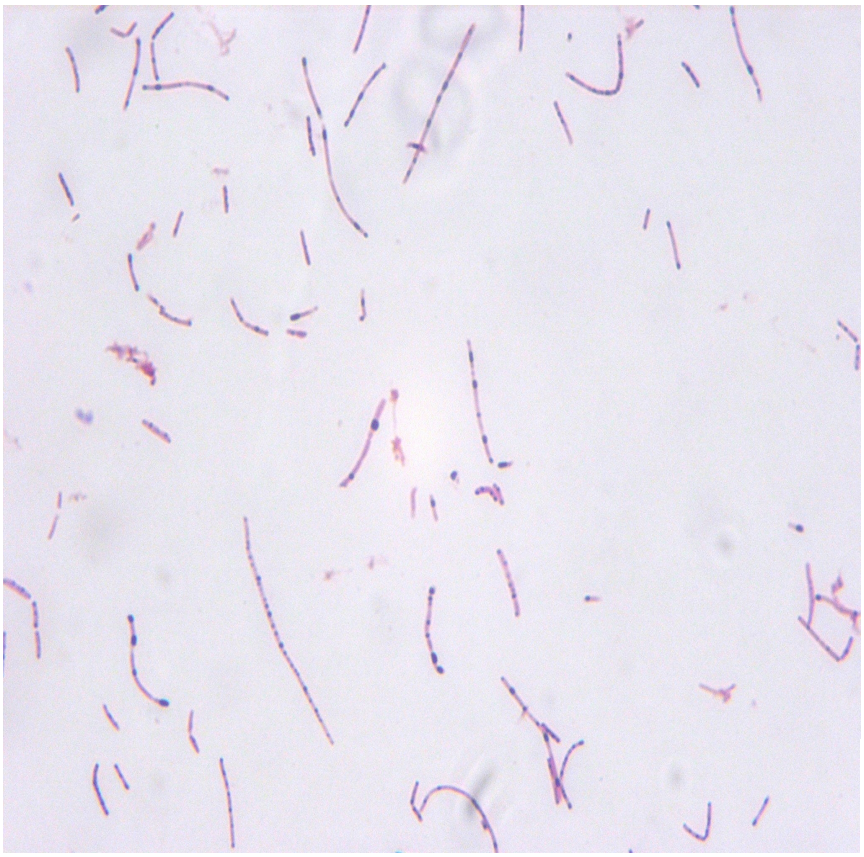


Fig. 2: Mix culture of LAB with inclusion bodies

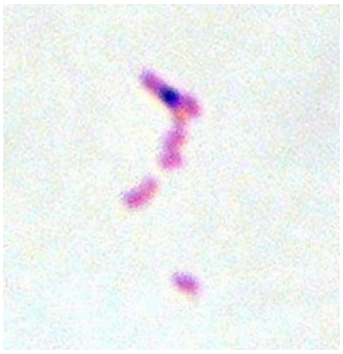


Fig. 3: *Lactobacillus fermentum* with inclusion body

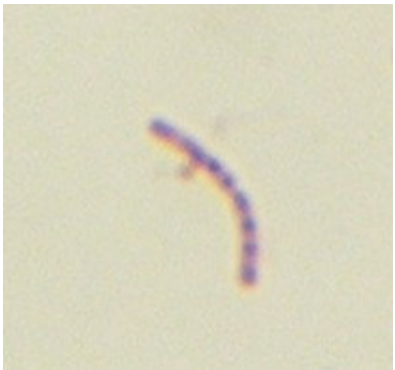


Fig. 4: *Lactobacillus para paracasei* with inclusion bodies

CHAPTER IV

Isolation and characterization of Two lytic bacteriophages *ΦApr-1* and *ΦApr-2* of *Lactobacillus brevis* from natural cultures utilized as starters in the manufacture of buffalo Mozzarella cheese

ABSTRACT

Twelve samples of wheys used as source of natural wild starter cultures for the manufacturing of the water buffalo Mozzarella cheese and implicated in fermentation delay and complete fermentation failure during the coagulation phase of the milk were screened for bacteriophage infection. Two phages active on *Lactobacillus brevis* were isolated from one sample and their phenotypic and genotypic features were investigated. Their life-cycles have been evaluated at 30 and 37° C.

The lytic-cycle of the bacteriophages has been demonstrated by the over lysis of the host after three hours of incubation in PLGYG broth.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed one major structural protein of approximately 32.5 kDa and several minor proteins. The genomes, approximately 31.4 kb and 31 kb long, were double-stranded linear DNA molecules with a *pac*-type system. Phages spreading into the wild whey cultures used as starter during the production of the cheese are responsible of the unsuccessful curdling of the milk and adversely affect the acidification phase. This is the first report of the isolation of virulent lytic *Lactobacillus brevis* phages isolated from buffalo whey starter cultures.

INTRODUCTION

Buffalo mozzarella cheese is a particular kind of Italian “niche product”, an unripened “pasta filata” cheese produced with raw and whole buffalo milk. It is traditionally manufactured in order to ensure its unique organoleptic characteristics. It gained the recognition of the D.O.P. mark (Protected Denomination of Origin) in 1996 and all the phases of the production are regulated by precise rules according to the D.P.C.M. 10/5/1993 (G.U. n. 219, 17/9/1993).

The coagulation of milk is the most important phase that occurs during the manufacture of cheese. In the Buffalo mozzarella cheese the acid development during cheese making is granted by natural starter cultures that derive from the whey of a previous successful batch of curdle, stored at ambient temperature for 24 h prior to be used.

Slow acid production has an important negative effect on the cheese making. Between all the causes of slow acid production, the most common are: natural inhibitors in milk, antibiotics and infection by bacteriophages (Fox P.F., Guinee T.P. *et al*, 2000).

It is well known that bacteriophage infection is quite common among LAB and it is a serious problem when it affects the coagulation phase of the cheese manufacturing.

MATHERIAL AND METHODS

Source and treatment of whey starters: the details of this section have been described in Chapter II.

Isolation and characterisation of LAB*:* the details of this section have been described in Chapter II.

All the 156 colonies of LAB isolated as reported in Chapter II have been screened for phage sensitivity by using the techniques described in the following paragraph.

Growth curve of the strain 8VCC-1 was carried out by testing its growth at 30°C in MRS broth in 24 h and periodically measuring the O.D. λ 600 by Thermo Spectronic.

Spot assay and plaque assay The two groups of whey samples were tested to evaluate bacteriophages presence ("*spot assay*"). They were thawed at 37°C, micro filtered (0.45 micron) and stored at 4°C prior to be tested. M17 soft agar (2.5 ml) constantly heated at the temperature of 46°C by heating blocks was inoculated with each one of the 144 colonies and with $CaCl_2$ 0.185 M. The media was poured onto M17 agar plates (Terzaghi *et al,* 1975). The plates were tested with a drop of each whey and incubated aerobically at 30° and 37° C for 24 hours.

Streptococcus salivarius thermophilus strains (12) were screened by using B-Elliker media.

The proof of bacterial sensitivity to phage infection is showed by an irregular zone of clearing on the plate where the whey has been spotted as consequence of phage activity against the strain.

"*Plaque assay*" was carried out in order to evaluate the original phage titre by plaque counting. A "plaque" is a visible circular area of clearing in the confluent bacterial growth caused by cellular death as consequence of virus replication inside a specific strain of bacteria called "host" and it can be or not surrounded by a zone of secondary lysis.

Dilutions of the whey from Group A that showed to infect and lyse one strain among the 156 isolates were prepared with MRD. M17 soft agar (2.5 ml) constantly heated at the temperate of 46°C by heating block was inoculated with the host (strain 8VCC-1 isolated from a whey sample of group B), $CaCl_2$ 0.185 M and with one ml of each dilution of the whey. The media was then poured onto M17 agar plates (Terzaghi *et al,* 1975) and incubated at 30° and 37°C for 24-48 h. Plaques were finally counted.

To screen the host specificity of the isolated phages, we tested them with a pool of fifteen lactobacilli of the U.C.C. private collection. Seven were strains of *Lactobacillus brevis* and the others were *Lactobacillus* spp. We chose also three strains of *Lactobacillus plantarium* since a cross activity between *Lactobacillus brevis*, *Lactobacillus plantarum* and their bacteriophages was described by Lu *et al.* (2003).

Phage propagation We used PLGYG broth in order to increase the phage titre of the whey that showed to inhibit the starter cultures'

growth. An aliquot of 10 ml of PLGYG broth was inoculated with the sensitive starter culture (strain 8VCC-1 grown in M17 broth for 14-18 h), whey sample and $CaCl_2$ 0.1 M. The broth was then incubated at 30° and 37° C for 6 hours. The media was micro-filtered (0.45 micron) and the titre was tested by *plaque assay*, following the procedure described before.

Analysis of phage genome More phage genetic features were investigated in the Microbiology Laboratories of the University College Cork in the Republic of Ireland.

Six plaques were randomly chosen and the six lysates were consequently tested by digestion of their DNA extractions with ten restriction enzymes: EcoRV, EcoRI, AluI, HinfI, HindIII, Pst I, Sau3AI, BamHI, Sal I and Kpn. DNA samples digested with each one of the restriction enzymes were, and not, subjected to a 10 min heating treatment at 65° C in order to evidence the presence of cohesive ends and consequently differentiate between *pac*-type or *cos*-type phages (Forseman *et al*, 1991).

DNA fragments were separated by agarose (1% w/v) gel electrophoresis in Tris-acetate-EDTA buffer and visualized by ultraviolet light after staining with ethidium bromide.

DNA standard fragments of DNA molecular weight marker X (Roche) were used to calculate the size of the fragments. Genome length was estimated by the sum of molecular weights of fragments generated by digestion.

Concentration and purification of phages Two of the lysates that showed different genetic profiles have been subjected to high scale purification.

High titre phage suspensions were obtained by using PLGYG broth up to 1.5 L of phage lysate. Final phage titres had values of 10^9 pfu/ml for both lysates.

NaCl was added to a final concentration of 0.5 M and centrifugation was used to remove cellular debris.

PEG 8000 was added to the lysates followed by centrifugations, TBT pellets reconstitutions and chloroform purification.

Ultracentrifugation tubes were progressively filled up with lysate suspention, 3M CsCl, 5M CsCl and TBT over the top, heat-sealed and followed by ultracentrifugation at 34000 *g* for about 150 min.

Upper and lower phage bands were successively extracted through the plastic tube by syringe and the phage lysates were consequently purified over night at 4°C in dialysis cellulose tubes.

Analysis of phage structural proteins Each one of the two purificated phage dilutions were subjected to SDS-PAGE (Laemmli, 1970). The bands were stained with Coomassie brilliant blue R250, followed by destaining with 10% glacial acetic acid and 10% isopropanol. Prestained molecular weight marker was used to calculate the size of the phages proteins.

Electronic microscopy Bacteriophage morphology was analyzed by transmission electron microscopy (Université du Québec-INRS Institut Armand-Frappier, Québec, CANADA).

RESULTS

Phage isolation and plaque formation The growth of one culture among 156 isolates was clearly inhibited when it was tested with one of the whey samples from Group A.

The host (8VCC-1) was isolated from one whey sample of group B and identified as *Lactobacillus brevis* by 16S rRNA gene sequence analysis performed by using Applied Biosystems MicroSeq™ microbial analysis software and database (MIDI Labs, 125 Sandy Drive, DE 19713, N.Y., U.S.A.).

In Fig. 5 we show the growth curve of the host *Lactobacillus brevis* 8VCC-1.

Plaque formation was observed when the plates were incubated at 30° and 37°C. The plaques were very small and without zones of secondary lysis (Fig. 6).

The results concerning the values of bacteriophage titre in the infected whey from group A are expressed in table 1.

Because of the low titre of bacteriophage revealed from the microbiological methods, we propagated the virus using PLGYG broth.

The results are showed in table 2. This technique determined the increasing value that is generally expected with these media. The activity of the two phages characterized by over lysis of the hosts in only three hours of incubation during the propagation was indicative of a lytic-cycle of replication.

Spot and plaque assays by using the strains of U.C.C. private collection as hosts for testing phage specificity were all negative.

Characterization of phage genome The digestions of the DNA extracted from six lysates with the ten restriction enzymes did not give results when the enzymes Sau3AI, BamHI, Sal I and Kpn were tested.

On the basis of the results of the genomic patterns related to the other seven restriction enzymes used (EcoRV, EcoRI, AluI, HinfI, HindIII, Pst I and BamHI) we grouped the six lysates into two groups, each one including three lysates. The lysates related with the same group had no genomic differences. The most significative difference between the two groups was showed by one band of 2700 bp obtained by digestion with enzyme EcoRV and that was revealed only in 3 lysates among the six tested.

From these results we can affirm that the whey contained a mixture of two different phages that had very little differences when their restrictions enzymes profiles were compared.

The two phages have been named *ΦApr-1* and *ΦApr-2*.

The genome size of *ΦApr-1* and *ΦApr-2* were estimated by the sum of molecular weights of fragments generated by digestion. The genome lengths were approximately and respectively of 31.4 kb and 31 kb. The most important difference between *ΦApr-1* and *ΦApr-2* was shown by EcoRV digestion and one band more was detected (2700 bp) for *ΦApr-1*.

The DNA of *ΦApr*-1 and *ΦApr-2* did not show any heat-inducible fragment-dissociation with any of the other seven tested endonucleases: this is indicative for *pac*-type phage genomes.

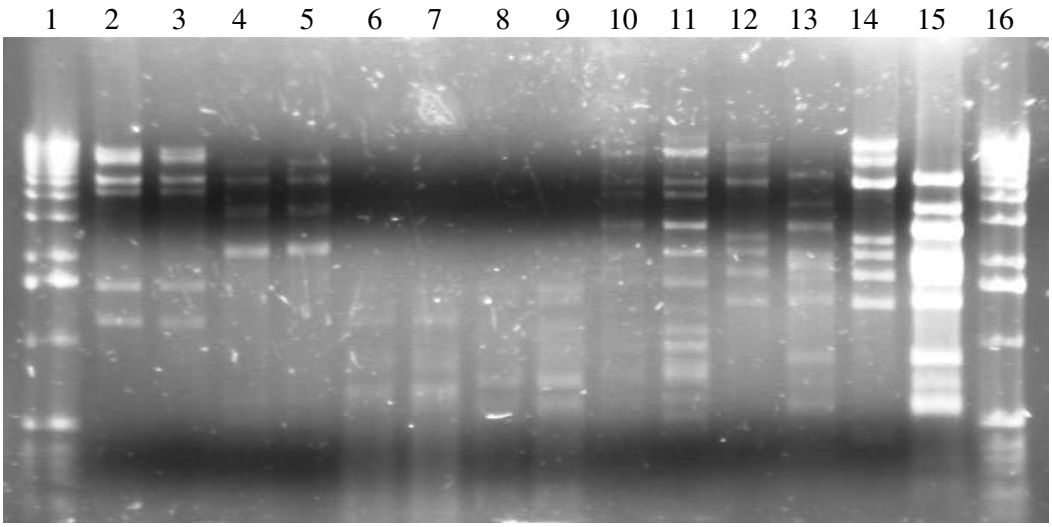


Fig. 1 Agarose gel electrophoresis of *ΦApr-2* DNA digested with different restriction enzymes. Lanes 1 and 16: Marker X; lanes 2 and 3: EcoRV and EcoRV 65°C treatment; lanes 4 and 5: EcoRI and EcoRI 65°C treatment; lanes 6 and 7: AluI and AluI 65°C treatment; lanes 8 and 9: HinfI and HinfI 65°C treatment; lanes 10 and 11: HindIII and HindIII 65°C treatment; lanes 12 and 14: PstI and PstI 65°C treatment; lanes 13 and 15: Sau3A and Sau3A 65°C treatment.

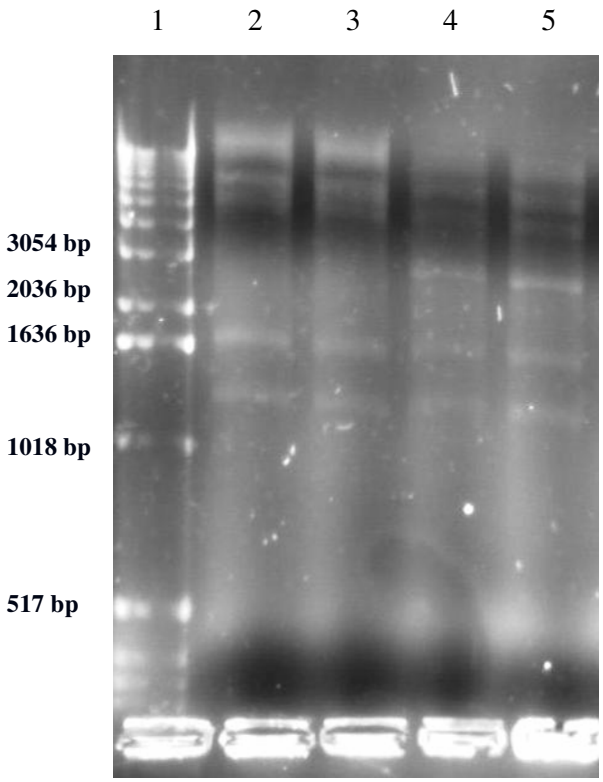


Fig. 2 Lane 1: Marker X; lanes 2 and 3: *Φ Apr-2* DNA tested with EcoRV and EcoRV 65°C treatment; lanes 4 and 5: *Φ Apr-1* DNA tested with EcoRV and EcoRV 65°C treatment

Protein composition of ΦApr-1 and ΦApr-2 The SDS-PAGE profiles of *ΦApr-1* and *ΦApr-2* contained one major protein with molecular weight of 32.5 kDa (Fig. 3).

ΦApr-1 viral particle had 23 other minor proteins of 155 ,145, 103, 78.8, 66.2, 57.2, 54.7, 50, 45, 43.7, 42.5, 35, 37.5, 30.5, 30, 28, 27.5, 25, 22.8, 13.5, 11.5, 10.5, 6.5 kDa for a total molecular weight of 1152 KDa.

ΦApr-2 protein profile contained other 14 proteins of 103, 54.7, 52, 50, 43.7, 38, 30.5, 28, 24, 22.8, 13, 12, 10.5, 10 kDa for a total molecular weight of 524.7 KDa.

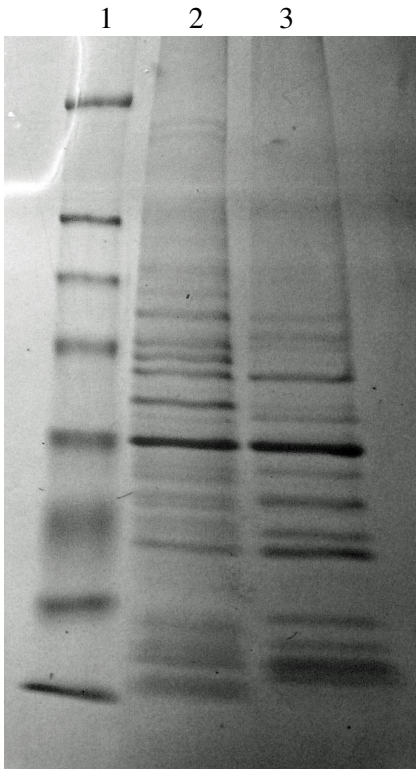


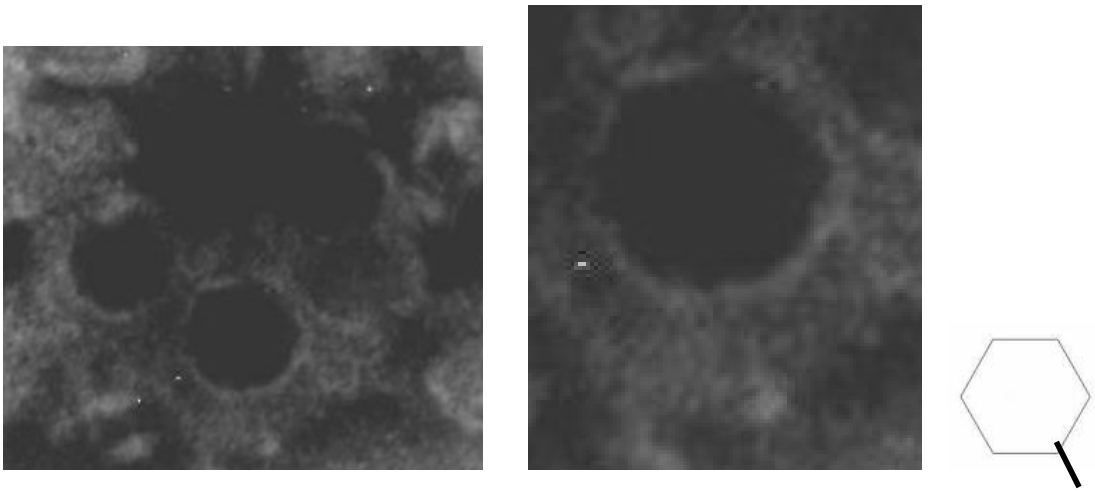
Fig. 3 Structural proteins. Lane 1: Molecular weight protein marker; lane 2: *ΦApr-1*; lane 3: *ΦApr-2*

Morphology After examination at electronic microscope, *ΦApr-1* and *ΦApr-2* showed a very similar morphology (Fig. 4).

Many round and empty virus particles were found in clusters associated with large proteinous aggregation.

They revealed and isometric and hexagonal head of about 45 nm in diameter and a short noncontractile tail of about 29 nm of length. A complex base plate on the tail was clearly visible.

The phages were ascribed to **morphotype C1** according to Ackermann and Du Bow (Ackermann *et al*, 1986) or to the **Podoviridae** family according to the International Committee on Taxonomy of Viruses (Matthews, 1982).



a) b) Particular from a)

Fig. 4 Electron micrograph of *Lactobacillus brevis* phage *Apr-1*

CONCLUSIONS AND CONSIDERATIONS

Mozzarella made from raw and whole water buffalo milk is one of the most appreciated and highly valued Italian local cheese.

We analysed some of the causes that could lead to a slow acid production as recently noticed in Southern Italy.

Bacteriophage infection is still one of the major causes of acidification defaults and it was investigated in this study.

The results were exhaustive and we observed the activity of two lytic bacteriophages against a particular strain of lactic acid bacteria: *Lactobacillus brevis*. No studies have been carried out before on bacteriophages from water buffalo milk and very little is known about lytic *Lactobacillus* spp. phages. This is the first report of the isolation of lytic *Lactobacillus brevis* phages from buffalo "wild" starter cultures and we provide in this study the first information about their identification and characterization.

Interesting is the relatively small size of the plaques and the absence of surrounding zones of secondary lysis; this pattern can be indicative of some phage typical characteristics that need to be confirmed.

Another interesting aspect of this founding is that the host Lactobacillus brevis (8VCC-1) was isolated from one sample of whey collected from the same dairy farm from where *Apr-1* and *Apr-2* phages have been detected. We need to underline that while *ΦApr-1* and *ΦApr-2* derive from one sample of whey pointed out as responsible of acidification failure during buffalo Mozzarella cheese making, the host, *Lactobacillus brevis*, was isolated from one

sample of whey coming from the same dairy farm but not showing any anomaly during the cheese production by the time the whey was collected.

On the basis of our results we can conclude that the phage infection is the main cause of the coagulation problem that some factories are recently reporting during the production of the buffalo Mozzarella cheese.

Currently more research is being carried out on *ΦApr-1* and *ΦApr-2* and their sensitivity to different environmental stress factors in order to prevent their activity and to minimize the risk of fermentation delay and complete fermentation failure that they can cause during the cheese manufacturing.

Table 1: Bacteriophage titre (*ΦApr-1 + Φ Apr-2*) in the infected whey of group A

T	**Host**
	8VCC-1 (ufp)
30°	$3 * 10^3$
37°	$6 * 10^4$

Table 2: Bacteriophage titre after four progressive propagations using PLGYG broth. The host is *Lactobacillus brevis*, the only strain that showed phage-sensitivity (8VCC-1)

T	2° P (ufp)	3° P (ufp)	4° P (ufp)
37°	$1.4 * 10^8$	$2.4 * 10^9$	$4.3 * 10^9$
30°	$6 * 10^5$	10^9	10^9

Fig. 5: Host's (8VCC-1) growth curve

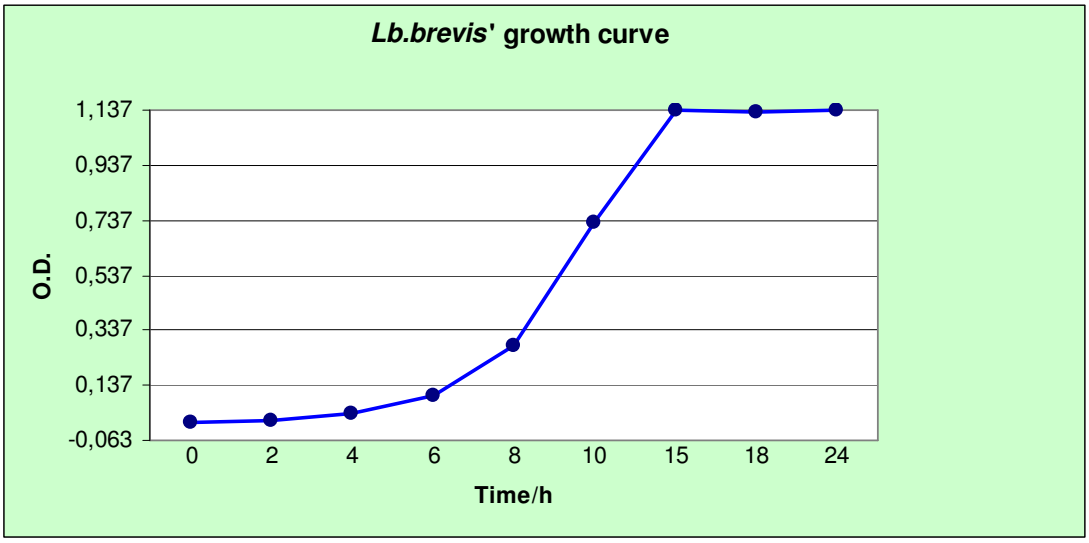
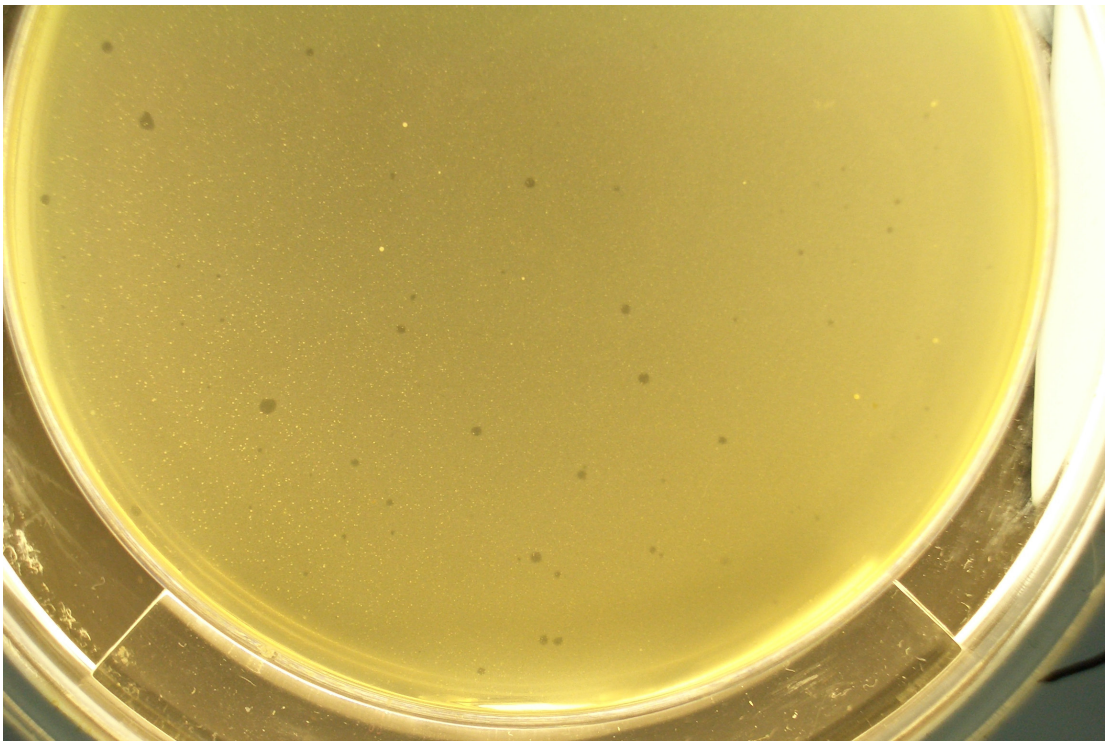


Fig. 6. Plaque assay: host 8VCC-1 tested with the whey from group A infected by bacteriophages



CHAPTER V

Inactivation of lytic bacteriophages *ΦApr-1* and *ΦApr-2* of *Lactobacillus brevis* isolated from natural whey of buffalo milk by pasteurization and high pressures

ABSTRACT

Phages *ΦApr-1* and *ΦApr-2* isolated from wild whey starter cultures are implicated in fermentation delay and complete fermentation failure during the production of the buffalo Mozzarella cheese.

In order to prevent this problem, we tested the resistance of the two bacteriophages to pasteurization (63° C for 30 min) and high-pressure treatments up to 800 MPa. Phage sensitivity for both heat and pressure treatments was tested in PLGYG broth, raw buffalo milk and raw buffalo whey, the latter gave an objective idea about phage behaviour in a natural environment.

The results of this research showed a very high sensitivity of *ΦApr-1* and *ΦApr-2* to both high temperatures and pressures.

Very interesting was the highest phage thermal and pressure-sensitivity when the bacteriophages were tested in samples of whey.

INTRODUCTION

In a previous study focusing on some of the causes that were implicated in the slowing down process of acid development during the production of the Italian "Mozzarella di Bufala", two phages *ΦApr-1* and *ΦApr-2* have been isolated and characterized as reported in Chapter IV.

They are two lytic phages infecting a strain of *Lactobacillus brevis*. The images at the electronic micrograph showed an isometric and hexagonal head of about 45 nm in diameter and a short non–contractile tail of about 29 nm of length. A complex base plate on the tail was clearly visible. They are ascribed to **morphotype C1** (Ackermann *et al*, 1986) and to the **Podoviridae** family (Matthews, 1982).

Several authors have experimented the heat inactivation of dairy phages (Yakivlev *et al*, 1941; Nichols *et al*, 1945; Daoust *et al*, 1965; Zottola *et al*, 1966; Koka *et al*, 1967; Koka *et al,* 1970; Quiberoni A., Suarez V.B. *et al*, 1999; Quiberoni A., Guglielmotti D.M. *et al*, 2002) and few communications have been published about high-pressure inducing inactivation of phages (Grox *et al*, 1992; Moroni *et al,* 2002; Chen *et al,* 2004; Muller *et al,* 2005; Avsaroglu *et al,* 2006; Smiddy *et al,* 2006;) but the most part of the researches were carried out using media different from natural whey and milk.

The cheese-makers producing Buffalo Mozzarella cheese prefer to use raw milk in order to preserve all the typical flavours of this

cheese. In fact the negative effects of the high temperature on milk properties have been extensively studied in the past (Dannenberg *et al*, 1986; Lucey *et al*, 1993; Walstra *et al,* 1999).

In contrast high pressure has very little effect on milk qualities (Knorr *et al,* 1993; Lopez *et al,* 1996; Patterson *et al*, 1998; Borst *et al*, 1999; Rademacher *et al*, 1999; Needs *et al*, 2000) and that is the reason why we also focused on these treatments, in order to find a solution to the phage infection still respecting all the cheese qualities that the consumer expects to enjoy when buying this product.

In this Chapter we analysed *ΦApr-1* and *ΦApr-2* sensitivity to pasteurization and to a large range of high pressures in order to suggest some techniques to minimize the phage infection in buffalo milk and whey. In this study we also used natural buffalo milk and whey samples in order to have objective data about phage and LAB resistance and activity when pasteurization and high pressures are applied to natural media.

Moreover this is the first report about sensitivity to high temperature and pressures of lytic *Lactobacillus* spp phages isolated from milk buffalos.

MATERIALS AND METHODS

Phages, host and media *ΦApr-1* and *ΦApr-2* were propagated in PLGYG broth. 10 ml of PLGYG broth was inoculated with 0.1 ml of overnight host (M17 broth), 0.5 ml of 0.1 M $CaCl_2$ and 0.1 ml of each bacteriophage previously propagated in PLGYG broth or eventually stored in M17 broth. The suspension was incubated for maximum 6 hours at 30° C. Generally overlysis and completely clearance of the media was occurring after only 3 hours of incubation. The lysates were micro filtered using 0.45 micron filters in order to remove cells and cell debris.

The titre was tested after each propagation and it was constantly 10^9 pfu/ml. These fresh phage dilutions were used to inoculate raw buffalo milk and raw buffalo whey prior testing for pasteurization and high-pressure treatments.

Pure lysates were stored at 4° or stocked at -20° and -80° C.

The host, *Lactobacillus brevis*, was isolated in MRS agar, GRAM stained and tested according to the scheme devised by Billie and Espie (1992) and updated for this study as reported in Chapter II. Moreover the host was identified by 16S rRNA gene sequence analysis performed using Applied Biosystems MicroSeq™ microbial analysis software and database (MIDI Labs, 125 Sandy Drive, DE 19713, N.Y.). The host was commonly grown in MRS broth at 30° or 37° C. Overnight cultures for spot assay and plaque assay were grown in M17 broth at 30° C for 14-16 hours. Pure colonies were stocked at -20° and -80° C in MRS or M17 broths.

The raw buffalo milk and the raw whey used to be inoculated with *ΦApr-1* and *ΦApr-2* before thermal and pressure tests were collected from a pool of dairy factories of the South of Italy, transported to the Department of Zootechnical Sciences and Food Inspection, Faculty of Veterinary Medicine, Naples, Italy, to be frozen at -20°C and then transported frozen and refrigerated using solid carbon dioxide to the Microbiology Department of University College Cork, Co. Cork, Cork, Republic of Ireland. These whey samples were different from the ones of the groups A and B mentioned in Chapter II.

In order to be inoculated with the phages *ΦApr-1* and *ΦApr-2*, frozen samples of milk and whey were thawed at 37° C in water bath and kept in ice until phage inoculation.

Thermal treatment Three different samples were tested for each phage: the phage lysate in PLGYG broth as from the propagation, raw buffalo milk and whey infected with phage. This was carried out for both *ΦApr-1* and *ΦApr-2* and the starting titre of bacteriophages chosen for all the samples before the treatment was 10^6 pfu/ml. The phage titre in the whey from where the phages were firstly isolated was 10^3 pfu/ml; we decided to use an higher starting-titre to have a better idea of the hypothetical phage sensitivity.

For each phage the three suspensions (lysate, milk and whey after infection) were filled into eppendorfs with an inner volume of 1.5 ml so that no head space was left. All the samples were heated at the temperature of 63°C for 30 min by using heating block. This treatment is the equivalent of the pasteurization process (@ 71.7° C for @ 15 sec) applied to milk during the production of buffalo Mozzarella even if this cheese is mostly produced from raw milk.

The temperature was constantly checked by thermometers, the test was carried out in duplicate. It took less than 1 min for each suspension inside the tubes to reach the 63° C. Accordingly after 1 min control tubes were taken out of the heating block and the titre N_0 was checked. Those were reference titres that represented the starting point for the isothermal inactivation (t = 0).

After the treatment all the samples were placed in ice in order to stop the effect of the high temperature.

Some eppendorfs were filled just with raw buffalo milk and whey in order to compare their Total Viable Count (TVC) titre and LAB titre before and after pasteurization.

High-pressure treatment High pressure treatments were applied to both *ΦApr-1* and *ΦApr-2* in three different suspending media, PLGYG broth (lysate), raw buffalo milk and whey. The initial titre of bacteriophages chosen for all the samples before the treatment was 10^6 pfu/ml. For each phage, 2 ml of each of the three suspensions (lysate, milk and whey after infection) were dispensed into sterile stomacher bags and heat sealed. These bags were subsequently double and triple packaged to prevent leaks. The samples were placed in a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power Ltd., Stansted, Essex, UK) and treated at pressures in the range 200 MPa - 800 MPa, with holding times of 10 min.

All HP treatments were performed at 20° C; adiabatic heating on compression resulted in an increase in the temperature of the processing fluid (by up to 15° C at 800 MPa). On depressurisation

samples were immediately assayed for phage titres using plaque assay.

Some stomacher bags were separately filled with raw buffalo milk and whey in order to compare TVC and LAB count before and after high pressure treatments.

Determination of phage titre Bacteriophages *ΦApr-1* and *ΦApr-2* titres tested after pasteurization and high pressure treatments (200, 400, 600 and 800 MPa) were determined according to the double-layer method (Terzaghi *et al*, 1975).

Determination of TVC and LAB The coagulation of milk is the most important phase that occurs during the manufacture of cheese. In Buffalo mozzarella cheese the acid development during cheese making is granted by natural starter cultures that derive from the whey of a previous successful batch of curdle, stored at ambient temperature for 24 h prior to use.

It is for this reason that we also tested some samples of buffalo milk and whey not infected from bacteriophages *ΦApr-1* and *ΦApr-2* in order to estimate the impairment that the thermal and pressure-treatments could bring to the useful starter cultures.

After pasteurization and pressure-treatments, milk and whey were diluted in Ringer and each dilution was pour-plated using PCA and MRS agar (De Man *et al*, 1960). The plates were incubated at 30° C for 3 days before counting, MRS plates were incubated in microaerophilic conditions.

RESULTS

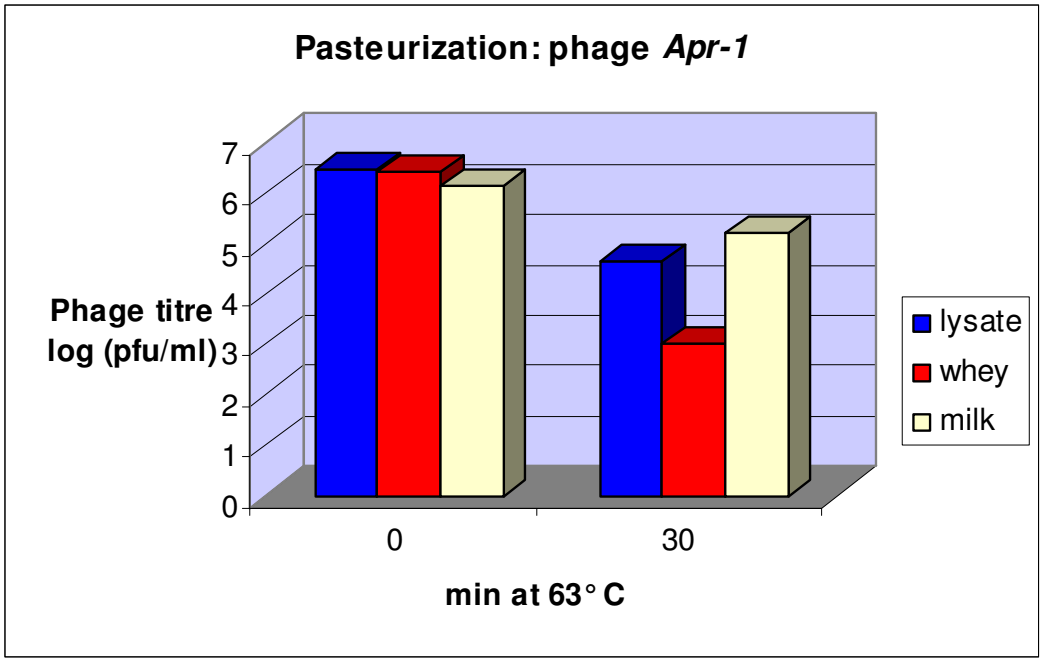
Phages inactivation by pasteurization Fig. 1 shows the effects of the inactivation of *ΦApr-1* and *ΦApr-2* after the pasteurization treatment (63°C/30 min).

ΦApr-1 titre after the treatment was reduced by 2, 3 and 1 log cycles when it was tested respectively in PLGYG broth, natural raw buffalo whey and natural raw buffalo milk.

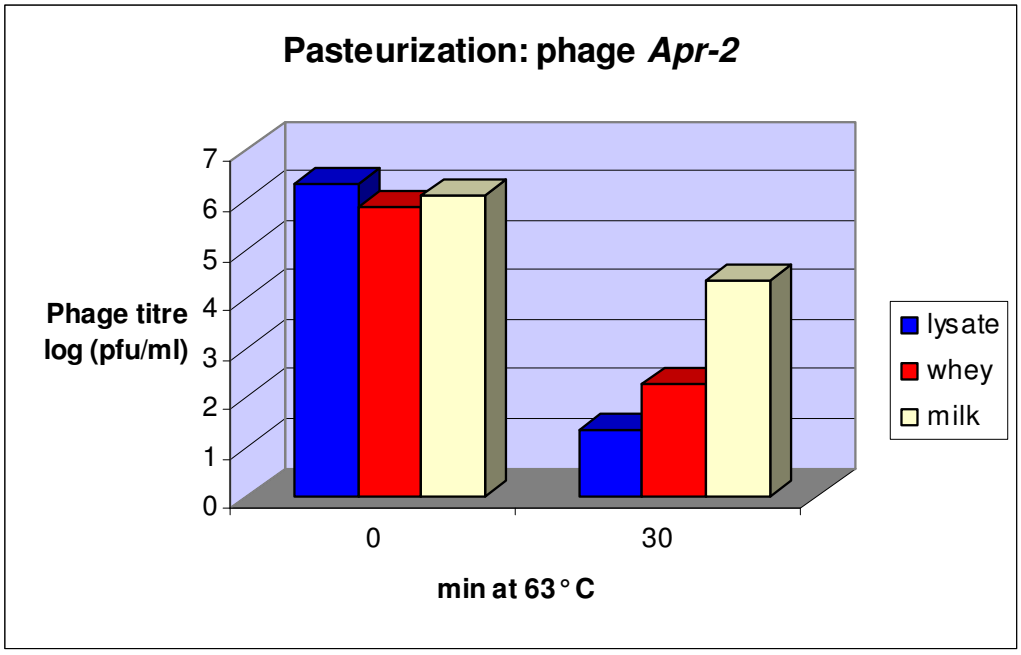
ΦApr-2 titre was reduced respectively by 5, 4 and 2 log cycles when tested with the same media.

Phage Apr-2 resulted more heat-sensitive than *ΦApr-1*.

In particular *Phage Apr-1* showed higher heat-sensitivity in infected whey than in the lysate in the same treatment conditions. These result was interesting and not expected.



a)

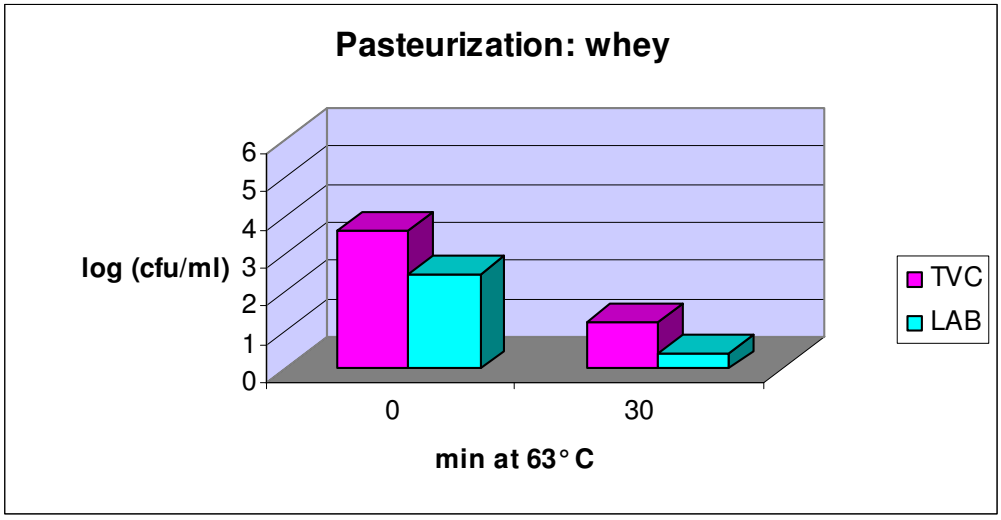


b)

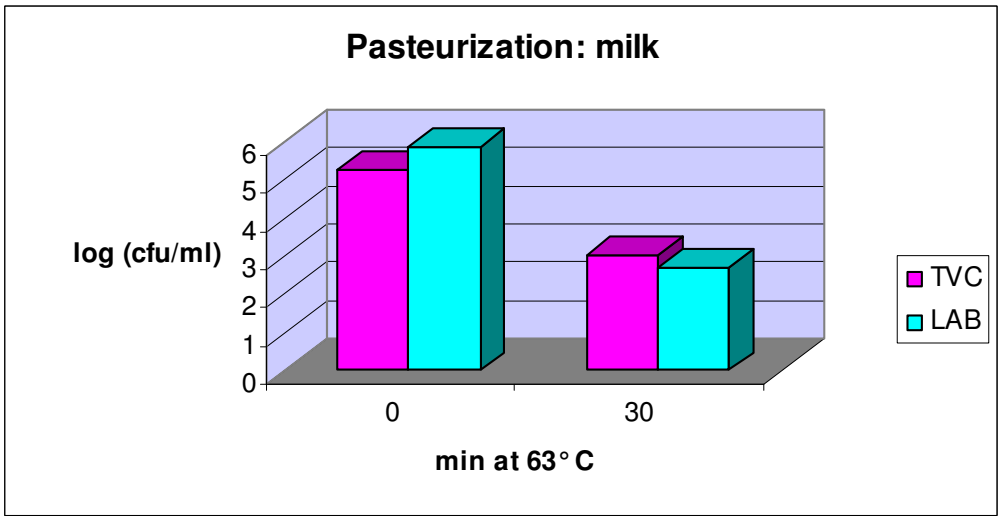
Fig 1. Pasteurization treatment effects (63° C/30 min) on *ΦApr-1*(a) and *ΦApr-2*(b)

TVC and LAB resistance to pasteurization The results in Fig. 3 show the reduction of TVC and LAB when pasteurization treatment was carried out on whey and milk.

Reduction of 2 and 1 log cycles occurred respectively for TVC and LAB in the whey while reduction of 2 log cycles occurred for both TVC and LAB when milk was tested.



a)



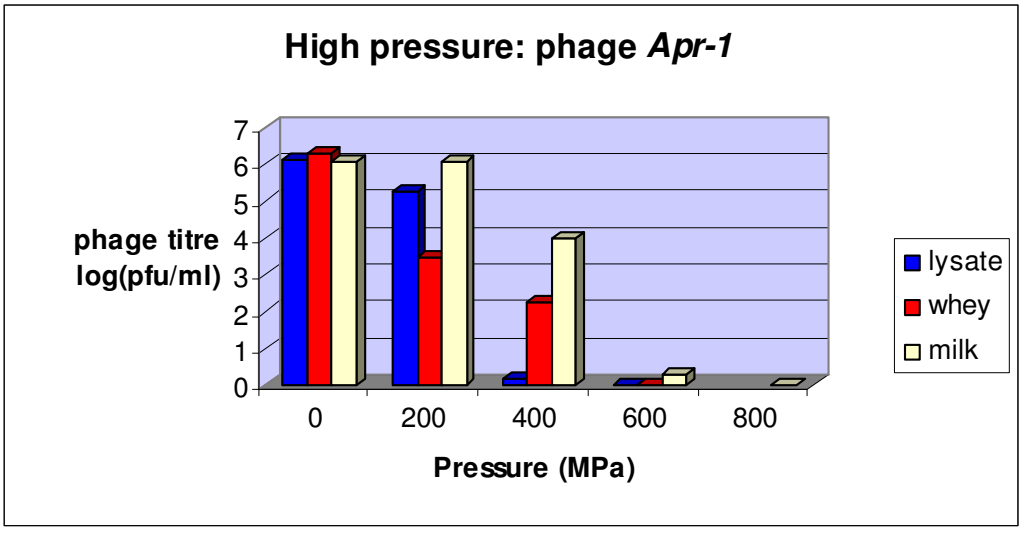
b)

Fig. 2: Pasteurization treatment effects (63°C/30 min) on TVC and LAB in buffalo whey (a) and buffalo milk (b)

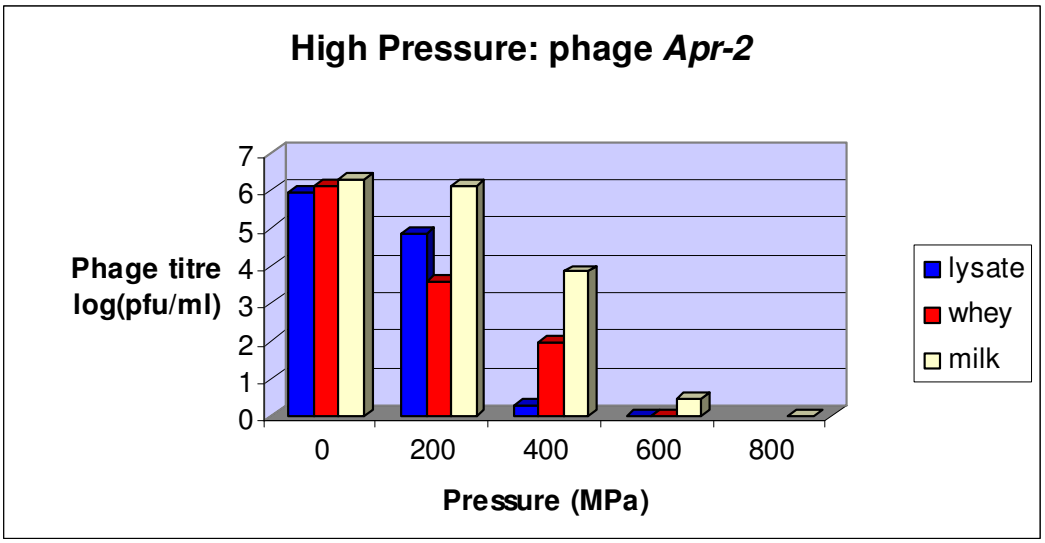
Phages inactivation by high pressures The results of the high pressure treatments on *ΦApr-1* and *ΦApr-2* are shown in Fig. 3. The pressures used were 200, 400 and 600 MPa. A pressure of 800 MPa was used only to treat milk infected with phage, since preliminary studies demonstrated a stronger phage-protection in milk compared to culture broth or whey.

The pressure sensitivities of both *ΦApr-1* and *ΦApr-2* were very similar. A difference can be observed between lysates treated at 200 MPa: *ΦApr-2* was one log cycle less pressure-stable than *ΦApr-1*.

As for the pasteurization treatment, the two phages appear more pressure-sensitive when tested in whey, especially at the lower pressure values of 200 MPa when a reduction of 3 log cycles was observed. This is again a very important and interesting characteristic that was not expected and whose significance will be better explained in the conclusions.



a)



b)

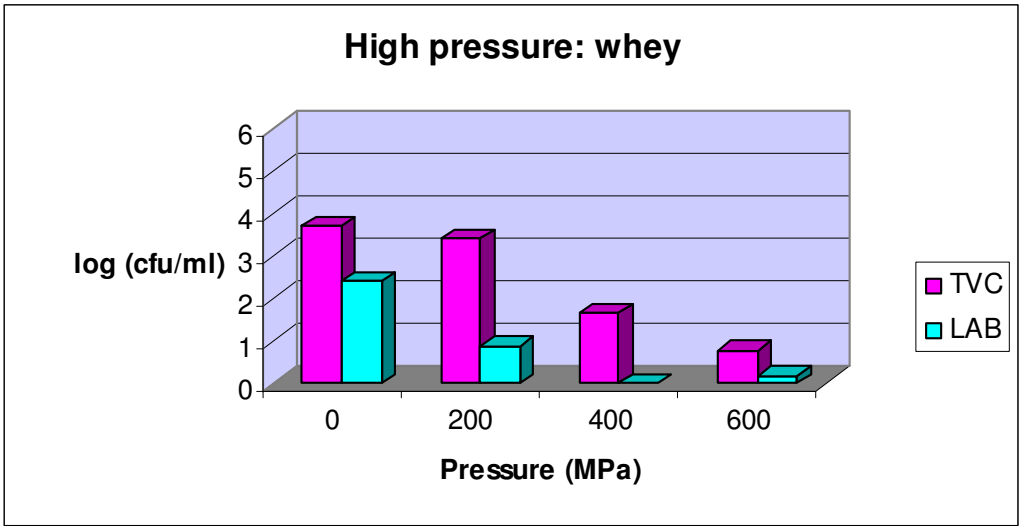
Fig. 3: High pressure treatment effects (200, 400, 600, 800 MPa) on *ΦApr-1* (a) and *ΦApr-2* (b)

TVC and LAB resistance to high pressure The resistance that TVC and LAB of whey and milk offered against High Pressure-treatments of 200, 400 and 600 MPa are shown in Fig 4. Again 800 MPa pressure was applied when the milk was tested since preliminary studies demonstrated a baroprotective effect of milk constituents on vegetative bacteria (Elaine P. Black *et al*, in press).

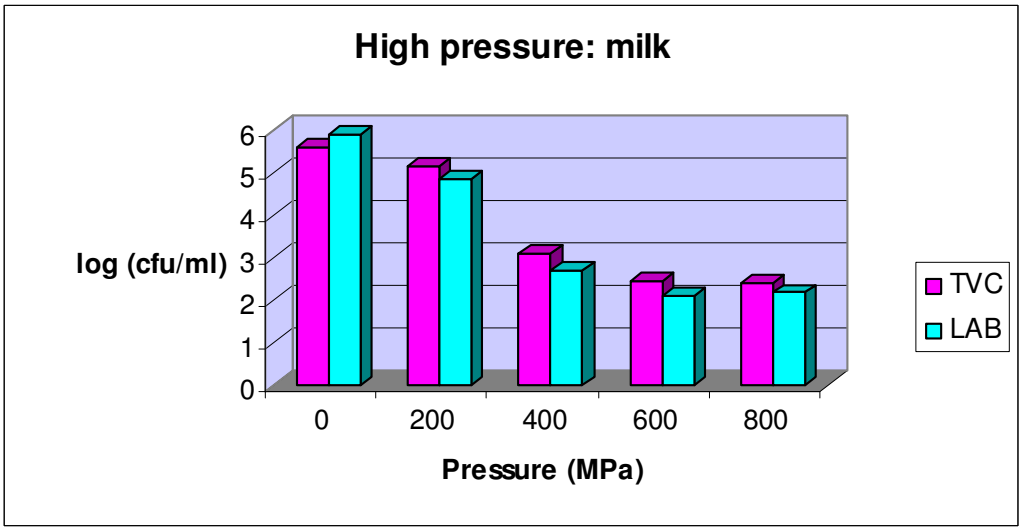
No reduction in the TVC of whey was observed following treatment at 200 MPa. A reduction of 2 log however was observed for LAB.

In milk, 1 log reduction of TVC and LAB was shown following treatment at 200 MPa. A correlation of about one log reduction every 200 MPa pressure was observed for milk-TVC. Unexpectedly a 2 log survival was observed in milk up to pressures of 800 MPa.

This pressure is known to kill most vegetative bacteria (Hupperts *et al*, 2006) suggesting that spore-forming contaminants may have been present in the raw milk tested.



a)



b)

Fig. 4: Effects of high pressures (200, 400, 600, 800 MPa) on TVC and LAB in buffalo whey (a) and buffalo milk (b)

CONCLUSIONS AND CONSIDERATIONS

The principle aim of this research was to investigate the effects that pasteurization and high pressure treatments have on the activity of *ΦApr-1* and *ΦApr-2* in order to prevent their negative effects during the manufacturing of buffalo Mozzarella cheese.

The results showed phages inactivation by pasteurization when infecting whey and milk and comparing the results of phage-sensitivity in PLGYG broth, whey and milk, we demonstrated that for *ΦApr-1* the highest reduction grade was noticed when whey was pasteurized, with a titre dropping from 10^6 to 10^3 pfu/ml, 4 log cycles of reduction were shown by pasteurizing whey infected with *ΦApr-2*.

Also the results of high pressure treatments were satisfactory. The highest titre-reduction at the high pressure of 200 MPa for both *ΦApr-1* and *ΦApr-2* was noticed when whey was tested and with values of 3 log cycles of reduction while *ΦApr-1* and *ΦApr-2* were only slightly inactivated when 200 MPa were applied to lysates and infected milk.

One more log cycle of reduction was noticed when 400 MPa treatment was applied to lysates. These results are very interesting since 10^3 was the initial infective titre of the phages in the natural whey from where they have been firstly isolated.

The titres of both the bacteriophages was reduced from 10^6 to 10^4 pfu/ml when 400 MPa high pressure was applied to the infected milk.

The total phage inactivation was noticed for the two phages when 600 MPa was tested on infected whey and milk and 800 MPa on milk.

Since acid development during cheese making and traditional local flavours are granted from natural starter cultures that derive from the whey of a previous successful batch of curdle, we also tested TVC and LAB titre in natural media to evaluate the grade of impairment that the same treatments of pasteurization and high pressure could eventually have on the survival of the useful starter cultures.

Comparing the results of pasteurization and high pressures with phages and starter culture sensitivities we can conclude that a preventive scheme to avoid the fermentation delay or the complete fermentation failure by *ΦApr-1* and *ΦApr-2* noticed during the buffalo mozzarella cheese-making could include:

a) Pasteurization of the whey used as starter culture soon after its separation from the curdle or the day after prior being used to inoculate the fresh milk or

b) High pressure treatment of 200 Mpa applied to the whey soon after being obtained from the curdle or after being stored 24 hours at room temperature or

c) High pressure of 400 MPa treatment applied to the milk used for the cheese production or

d) Combining a) (or b) and c)

We would recommend solution b) since high pressure is one of the best treatment to be applied in order to keep flavour characteristics unaltered and 200MPa could determine a total phage inactivation

associated with a slight reduction of LAB titre. Nevertheless the cost of high pressure machine and eventually of specific technical operators should be taken in account and it could be onerous for the small dairy farms where buffalo Mozzarella cheese is nowadays mostly produced.

Moreover all these suggestions come from the experimental results of this study and their final effect must be confirmed in the reality of the cheese-factory scenario.

The results concerning TVC and LAB count after the treatments a) b) and c) suggested as solution for the phage infection should not negatively affect the starter culture activity since the reduction of their titre was very low, up to a maximum of two log cycles. Moreover the starting titres of TVC and LAB for whey and milk in this report are underestimate, since the samples were kept frozen for few months before being tested; freezing has a negative effect on bacteria.

However if a relevant impairment of the starter culture activity would be noticed during the cheese making, these procedures could also be associated with a starter culture concentration in the whey. More studies must be carried out on some of the techniques that can be used at this purpose such as a) continue centrifugation, b) reverse osmosis (R.O.), c) dialysis d) starter culture propagation on pH control. These procedures are based on simple principals and are easy to be applied to large scale factory cheese production.

CHAPTER VI

GENERAL DISCUSSION

In this research we analysed the principal causes responsible of fermentation problems during cheese making of the Italian "Buffalo Mozzarella".

Particular emphasis was given to phage infection since this is still one of the main reasons leading to acidification failures during cheese production.

Since phage infection is host specifically related, we started our investigation from isolating LAB from samples of wheys and testing the lactic bacteria from wheys that did not show any acidification problems (group B) with the ones that had shown failures during cheese making (group A) in order to evaluate LAB-phage-sensitivity and consequently isolate the active phages. 156 LAB pure colonies were isolated and characterized to genus level and 44% to species level.

We noticed a difference in titre between the total MRS count of samples of group A and the ones of group B and the percentage of Lactobacilli isolated from the samples of Group A was extremely higher then Lactococci. In group B Lactococci prevailed against Lactobacilli but the discrimination between the two groups was not very important.

Moreover *Lactobacillus fermentum* (group A) and *Lactobacillus para paracasei* (group B) were the most frequently isolated among Lactobacilli. *Lactococcus* was the most common LAB in the group B and the species most commonly detected was *Lactococcus lactis cremoris* followed by *Lactococcus lactis lactis.*

Spot assays revealed phage-sensitivity of one strain of *Lactobacillus brevis* to two lytic bacteriophages that we named *ΦApr-1* and *ΦApr-2*.

The two phages were isolated from one sample of whey starter pointed out as responsible of acidification failure during buffalo Mozzarella cheese making and the host, *Lactobacillus brevis*, was isolated from one sample of whey deriving from the same dairy farm from where the phages were detected but from a whey sample not showing any anomaly during the cheese production by the time the whey was collected.

These two phages showed a very little difference in their genomic patterns and that suggests they could be the result of DNA modifications of the same virus.

Plaques from *ΦApr-1* and *ΦApr-2* were relatively small in size and the absence of surrounding zones of secondary lysis could be indicative of some bacteriophage typical characteristics that need to be confirmed.

On the basis of our results we can affirm that the phage infection can be considered as the main cause of the coagulation problem that some factories are recently reporting in Southern Italy during the production of the buffalo Mozzarella cheese.

We also investigated the effects that pasteurization and high pressure treatments have on the activity of *ΦApr-1* and *ΦApr-2* in order to suggest a solution when the problem arises.

The results showed phages inactivation by pasteurization when infecting whey and milk and comparing the results of phage-sensitivity of the lysate, whey and milk, we demonstrated that for *ΦApr-1* the highest reduction grade was noticed when whey was pasteurized, with a titre dropping from 10^6 to 10^3 pfu/ml, 4 log cycles of reduction were shown by pasteurizing whey infected with *ΦApr-2*.

High pressure treatments up to 800 MPa were applied to phage lysates, infected whey and milk. We noticed the highest titre-reduction (3 log cycles) at the high pressure of 200 MPa for both *ΦApr-1* and *ΦApr-2* when infected whey was tested.

These results are interesting since 10^3 was the initial infective titre of the phages in the natural whey from where they have been firstly isolated.

The titres of both the bacteriophages was reduced from 10^6 to 10^4 pfu/ml when 400 MPa high pressure was applied to the infected milk.

We also tested TVC and LAB titre in natural media to evaluate the grade of impairment that the same treatments of pasteurization and high pressure could eventually have on the survival of the useful starter cultures from which acid development during cheese making and traditional local flavours derive.

Comparing the results of pasteurization and high pressures with phages and starter culture sensitivities we suggested some solutions when an *ΦApr-1* and *ΦApr-2* infection of LAB is noticed during buffalo mozzarella cheese-making:

a) Pasteurization of the whey used as starter culture soon after its separation from the curdle or the day after prior being used to inoculate the fresh milk or

b) High pressure treatment of 200 Mpa applied to the whey soon after being obtained from the curdle or after being stored 24 hours at room temperature or

c) High pressure of 400 MPa treatment applied to the milk used for the cheese production or

d) Combining a) (or b) and c)

We would recommend solution b) since high pressure is a treatment that does not alter flavour characteristics in whey and milk and 200MPa could determine a total phage inactivation associated with a slight reduction of LAB titre.

From a practical and economical side the cost of high pressure machine and eventually of specific technical staff should be taken in account and it could be a financial problem for the small dairy farms where buffalo Mozzarella cheese is still mostly produced.

From this point of view whey pasteurization could be recommended as an inexpensive solution. Moreover all these observations derive from experimental results and their final effect must be confirmed in the cheese-factory scenario.

The results concerning TVC and LAB count after the treatments a) b) and c) suggested as solution for the phage infection should not negatively affect the starter culture activity since the

reduction of their titre was very low, up to a maximum of two log cycles. Moreover the starting titres of TVC and LAB for whey and milk in this report are underestimate, since the samples were kept frozen for few months before being tested and freezing has a negative effect on bacteria. More studies are currently being carried out by evaluating TVC and LAB titres in fresh buffalo samples of whey and milk and some preliminary results confirmed this hypothesis, being TVC and LAB titres in fresh samples much higher than the ones derived from the frozen samples tested in this research.

However if a relevant impairment of the starter culture activity would be noticed during the cheese making, the procedures a) b) and c) could also be associated with a starter culture concentration in the whey.

More studies must be carried out on some of the techniques that can be used at this purpose such as a) continue centrifugation, b) reverse osmosis (R.O.), c) dialysis d) starter culture propagation on pH control.

These procedures are based on simple principals and are easy to be applied to large scale factory cheese production.

An easy solution that can be also suggested and that is currently being applied by the dairy farms when an anomaly in the activity of the whey starter they use is noticed is represented by changing the whey with another from farms whose milk is not infected.

The guide-lines proposed in this study completely respect the D.P.C.M. 10/5/1993 that regulates the buffalo Mozzarella cheese production and could help in the future if the problem of

fermentation failure by an *Apr-1* and *Apr-2* phage infection should arise also during the D.O.P. buffalo Mozzarella cheese-making.

For future work we can suggest more investigation of *ΦApr-1* and *ΦApr-2* genomes and the partial or total DNA sequencing. This analysis in particular could give confirmation about the lytic-cycle pattern of the phages since too little is actually known about lytic *Lactobacillus phages* and could also confirm the hypothetical mutation that presumptively leaded to the genetic feature differences between *ΦApr-1* and *ΦApr-2* described in this study.

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……..SARA’ MICA
TUTTA UNA BUFALA ! ! !