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Safety and quality along the buffalo milk and cheese chain

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REGULATION (EC) No 178/2002 of 28 January 2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, provides the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, taking into account in particular the diversity in the supply of food, including traditional products, whilst ensuring the effective functioning of the internal market (U. J. EC L31/1).

This Regulation applies to all stages of production, processing and distribution of food and feed, with the exclusion of foods destined to private domestic consumption. It takes account of animal health, animal welfare, plant health and environment and aims as well at the protection of the interests of consumers, providing them a basis to make informed choices in relation to (a) fraudulent or deceptive practices;(b) the adulteration of food; and (c) any other misleading practice.

Food safety is of primary importance. REGULATION (EC) No 178/2002 requires that food shall not be placed on the market if it is unsafe and that food shall be deemed to be unsafe if it is considered to be (a) injurious to health or (b) unfit for human consumption.

In determining whether any food is unsafe, several aspects must be considered, in particular(a) the normal conditions of use of the food by the consumer, at each stage of production, processing and distribution, and (b) the information provided to the consumer, including information on the label, or other information generally available to the consumer concerning the avoidance of specific adverse health effects from a particular food or category of foods.

In determining whether any food is unfit for human consumption, it is necessary to evaluate whether the food is unacceptable for human consumption according to its intended use, for reasons of contamination, whether by extraneous matter or otherwise, or for putrefaction, deterioration or decay.

The traceability of food, feed, food-producing animals and any other substance intended to be, or expected to be incorporated into a food or feed must be established at all stages of production, processing and distribution. Recent food scares, such as BSE and the dioxin crisis, have demonstrated the need to be able to identify the origins of any food item to ensure consumers' protection.

Traceability has different objectives such as food safety, fair trading between operators and reliability of the information provided to consumers. The Regulation introduces the traceability requirement with in particular the objective to ensure food safety and to enable unsa-

fe food/feed to be removed from the market. For this reason food business operators have:
- to be able to identify from whom and to whom a product has been supplied, i.e. the "one step back"-"one step forward" approach;
- to establish a link "supplier-product";
- to arrange systems and procedures that allow for this information to be made available to the competent Authorities upon their request.

Process and production systems may be highly complex and require considerable technical input. It is therefore important to study and manage the different aspects of food production in relation to the total chain implications and not to single stages only.

The term "Food chain" is usually reserved to the total supply process, from primary production and/or manufacturing, to storage and distribution, at retail sale or for use in catering. Food business operators have to establish clear specifications for raw materials and ingredients so that they can meet the requirements for further processing. This relates to both food safety and quality attributes, as it is important to ensure keeping and eating qualities in developing final products (U.J. EC L31/1, U.J. EC L226/3, U.J. EC L226/22).

Food safety management systems are based on a detailed understanding of all variables of the manufacturing process. A food item supplied to the consumer may not be free from hazards. Knowing this, it is important that appropriate food safety measures are understood and put into practice to eliminate or reduce risks. An analysis of potential key weaknesses in the chain, the observation of trends as well as mechanisms to target research on the issues of particular interest may bring great benefit in the production of foods.

The internationally recognised approach for assuring food safety is HACCP. This requires an intimate understanding of the interaction between process and product and the identification of critical control points in the manufacturing process, including distribution, storage and consumer practices.

Food manufacturers apply the HACCP principles when they establish their food processing procedures. Good hygienic practices, i.e. adequate cooking, cooling and subsequent storage and avoidance of cross-contamination, are however essential also for the consumers to eliminate/reduce foodborne diseases ((U. J. EC L31/1, Reij *et al* 2004).

Traceability is essential to establish that procedures have been applied and are effective to reach the required targets.

Safety of buffalo milk and mozzarella cheese

The buffalo was introduced in Italy in the seventh century. It 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 date from the beginning of the twelfth century. At the beginning it was produced in small quantities but from the second half of the eighteenth century mozzarella became widespread throughout the South of Italy and its production increased more and more.

The buffalo mozzarella is an unripened "pasta filata" cheese which may be obtained from raw or pasteurized whole buffalo milk and is manufactured according traditional procedures to guarantee the consumer its unique sensory characteristics. The production of "Mozzarella di Bufala Campana" 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 DOP (Protected Denomination of Origin) quality brand (EC Reg. 1107/96 L148/1).

Milk contamination

Milk contaminations may be due to clinical or sub-clinical mastitis and/or environmental contaminations taking place at different steps of the milk chain. Milk is a natural medium for microorganism replication and for this reason specific and hygienic procedures are required, from milking to delivering to the final consumer, to control hazards (Jayarao *et al* 2001, Rueg P.L. 2003).

Milking hygiene is a very important step for milk and cheese production and many requirements for food business operators carrying out primary productions and the associated operations are included in Reg. (EC) 852/2004 on hygiene and Reg. (EC) 853/2004 on specific hygiene rules for food of animal origin (U.J. EC L31/1, U.J. EC L226/3). Milking must be carried out hygienically, ensuring in particular that milking equipment, and premises where milk is stored, handled or cooled must be located and constructed so as to limit the risk of contamination of milk. Milk-hazards should be identified at level of primary productions and adequately controlled and for this purpose guides to good hygiene practice should include appropriate information on hazards that may arise in primary production and associated operations and actions to control hazards, including relevant measures set out in Community. Microbiological criteria for raw buffalo milk destined to the production of foods made with raw milk establish that the total plate count at 30°C must be < 500 000/ mL (U.J. EC L31/1, U.J. EC L226/3).

No criteria are set for pathogens in milk at primary production. A study on 189 samples of milk obtained by single animals and 18 samples of bulk milk has been carried out in order to research Listeria monocytogenes and Salmonella spp. natural contamination in some buffalo farms in Campania. The results showed the absence of Salmonella (qualitative method) and the presence of L. monocytogenes in n.5 samples of milk from single animals and in 2 samples of bulk milk by qualitative method (Murru *et al*.,in press).These preliminary results may be considered satisfying also in consideration of other aspects such as the milking hygiene.

Microbiological hazards in mozzarella cheese

Cheese-associated food poisoning outbreaks have been reported worldwide but are not common if pasteurized milk is used and hygienic measures are applied during cheese processing. Particular attention has been given to some milk-borne pathogens such as Salmonella spp, Listeria monocytogenes and E.coli (Caprioli *et al* 2005, Roopnaire *et al* 2007, Spano *et al* 2003).

Salmonella spp. have been reported to be present in raw milk and raw cheese but no research has been done on growth and survival of this pathogen in buffalo mozzarella made from contaminated raw milk. Cortesi *et al*.(1996) manufactured buffalo mozzarella cheese, following the traditional procedures in use in Southern Italy, with raw milk artificially inoculated with different amounts of S. senftenberg (initial contamination levels approximately from 10^2 to 10^6 CFU·mL^{-1}). Results demonstrated that Salmonella counts increased, on average, 1.5 $\log_{10}$ in ripened curd before stretching and remained rather stable or showed a decrease in whey. Stretching and moulding strongly reduced (up to > 6 $\log_{10}$) the

number of salmonellae but S. senftenberg was detected in mozzarella cheese samples. Data on Salmonella natural contamination levels in buffalo raw milk are scarce but the lowest Salmonella inoculum tested in this research ($2x10^2$ CFU·mL^{-1}) may be considered a high contamination level for naturally contaminated cow bulk milk. The absence of S. senftenberg in the cheese made from the milk inoculated at this level may therefore be considered to be reassuring with regard to Salmonella contamination of buffalo mozzarella cheese during commercial practice.

Listeria spp.

The animals, the farm environment, the production facilities and the storage temperature may be the source of dairy products Listeria spp. Contamination (Fedio *et al* 1990).

Outbreaks of human listeriosis caused by milk and dairy products consumption have prompted an increasing surveillance on the incidence and/or the survival of Listeria monocytogenes in these foods.

The safety of fresh and soft cheeses made from raw milk and the consequences for public health deriving from the consumption of these foods have been discussed in recent years. In bulk milk the presence of Listeria monocytogenes from environmental contamination (silage quality, milking hygiene, etc.) can vary in relation to the season and the area. Listeria mastitis is rare but associated with a persistent infection and high concentration in milk. Many authors describe that one infected quarter can shed between 10^3 and 10^6 Listeria monocytogenes mL^{-1} in milk.

Kim *et al*. (1998) (Junghee *et al*. 1998) produced mozzarella cheese with pasteurized milk experimentally contaminated at levels of 10^3 CFU·mL^{-1} and observed that time and temperature of stretching can destroy high levels of Listeria monocytogenes in the curd.

Murru *et al*.(1999) (Murru *et al*. 1999) studied the behaviour of Listeria monocytogenes inoculated at different levels (from 10^3 to 10^4 CFU·mL^{-1}), into raw buffalo milk during the traditional manufacture of mozzarella cheese. Listeria monocytogenes numbers decreased on average 1.3 $\log_{10}$ in the brine but remained rather high in the curd. In the cheese Listeria monocytogenes was always recovered, at levels ranging from $5.0x10^3$ to $1.1x10^4$ CFU·g^{-1}, with one exception in 25g samples.

The microorganism survival was supposed to be due to the curd protein matrix protection, the high fat level and/or the non uniform temperature reached in the curd during stretching. According to the obtained data curd stretching cannot be considered a thermal treatment equivalent to pasteurization as the D-values necessary to kill pathogens are not reached in every point of the curd mass. However results showed that buffalo mozzarella cheese may be safely produced according to the traditional ways if raw milk of good hygienic quality is used and the established manufacturing procedures are followed regularly, in particularly those concerning stretching time and temperature.

REGULATION (EC) No 2073/2005 on microbiological criteria applicable to food, in Annex I, Chapter I, establishes that the acceptable maximum level of Listeria monocytogenes in ready-to-eat foods, such as cheeses, where pH and a_w allow the multiplication of this microorganism is 100 CFU·g^{-1}. This criteria must be guaranteed by the producer during all the commercialization phases. Moreover it is advisable to perform a good monitoring system for premises, production phases and seasoning places in order to carry on a correct risk management.

E. coli O:157 H:7

Stress needs to be given to another microorganism. Escherichia coli is part of the normal microflora of the gastrointestinal tract of mammals and birds but certain strains have been associated with gastrointestinal diseases in both humans and animals.

E. coli strains have been categorized into groups on the basis of virulence properties. One of these group - STEC - is characterized by the production of potent citotoxins that inhibit protein synthesis. It can be found in the gut of numerous animals species but ruminants have been identified as a major reservoir of strains that are highly virulent to humans, in particular EHEC O:157 H:7 (Roopnaire *et al*. 2007, Spano *et al*. 2003).

The water buffalo is a potential source of STEC infections. A recent survey conducted in Southern Italy showed that buffalo dairy herds were frequently colonised by EHEC O157, yet the bacteria was not found in a study conducted on mozzarella cheese prepared with non pasteurized buffalo milk (Caprioli *et al*. 2005).

Quality of mozzarella cheese

The coagulation of milk is a crucial phase during cheese making and slow acid production has an important negative effect on this production step. In buffalo mozzarella cheese the acid development 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.

The most common causes of slow acid production are natural inhibitors in milk, antibiotics and infection by bacteriophages (Fox *et al*. 2000).

It is well known that bacteriophage infection is quite common among LAB and represents a serious problem as it affects the manufacture of cheese. The observation of repeated defaults noticed in Southern Italy during buffalo milk acidification prompted an investigation on LAB phages (Aprea G. 2006).

LAB are a group of Gram-positive micro-organisms with 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 and produce lactic acid as the major end-product during the fermentation of carbohydrates (Bergey 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 comprises also the genera *Enterococcus* and *Streptococcus* (Coppola *et al* 1988, Coppola *et al*. 1990, Seppo *et al*. 1998).

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; the 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); the protein hydrolysis, that causes the texture and, partially, the taste of the product; the synthesis of inhibitory compounds, flavour compounds and texturing agents, which may influence the consistency of the product (Fox P.F. 1982).

Milk coagulation

Milk coagulation 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: a)the glycomacropeptide (caseinomacropeptide) which is hydrophilic and soluble, and will diffuse away from the micelle after k-casein splitting; b)the para-k-casein which 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 takes 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 *et al*. 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 *et al*. 1998).

Slow acid development during cheese making

Slow acid development during cheese making is an important cause of poor quality cheese. The most common causes of slow acid production are presence in milk of natural inhibitors and LAB infection by bacteriophages (phages) (Fox *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. Lysozime is 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 : 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 *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 *et al*. 2000).

Bacteriophage (phage) infection Today, one of the most important causes 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 *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, a 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 diversity of form greater 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 Group E large capsomers are absent and the viron presents a simple regular hexagonal outline. The core is made up of a single-RNA chain. The sixth 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 D. 1967).

Phages are very specifically related to their hosts. Different phages of LAB must be considered : lactic streptococcal phages, thermophilic streptococci phages, lactobacillus phages, leuconostoc phages (Jarvis A. W. 1989).

<According to Bradley (1967) (Bradley D. 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 *Strep-*

tococcus thermophilus have been reported to have isometric head morphology and to belong to Bradley's Group B.

Families of bacteriophages identified up to now

Among the Families of bacteriophages that have been identified up to now, 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: the "lytic cycle" and the "lysogenic cycle".

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 synthesis 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 *et al*. 2000) and coded by the phage genome.

In the "lysogenic cycle" the first step is characterized by adsorption and DNA injection that occurs as in the lytic cycle. Then, 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 *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. For this reason the two terms really apply to phage-host systems rather than to the bacteriophage alone (Bradley D. 1967).

No studies have been reported on bacteriophages from water buffalo milk and very little is known about lytic *Lactobacillus* spp. phages. A research is being carried out on identifications and characterizations of lytic *Lactobacillus brevis* phages from buffalo "wild" starter cultures (Aprea G. 2006).

RESULTS AND CONCLUSIONS - Experience has shown that it is necessary to adopt measures aimed at guaranteeing that unsafe food is not placed on the market and at ensuring that systems exist to identify and respond to food safety problems in order to ensure the proper functioning of the internal market and to protect human health. The Rapid Alert System for Food and Feed (RASFF) is a system which has been in place since 1979. The legal basis of the RASFF is Reg. EC 178/2002 and its purpose is to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety. A Community framework of control systems is supposed to improve the quality of official controls and is being established. Task of the competent authorities in the Member States is to organise official controls in order to verify and monitor the correct implementation of feed and food legislation by businesses at all stages of the feed and food chain. National control systems must improve the impartiality, effectiveness and quality of official controls, guarantee a sufficient number of suitably qualified and experienced staff and dispose of adequate facilities

and equipment to carry out their duties properly.The official controls must be carried out using the appropriate techniques developed for that purpose. These techniques include routine surveillance checks and more intensive controls such as inspections, verifications, audits, sampling and the testing of samples. The frequency of official controls must be regular and proportionate to the risk, taking into account the results of the own checks carried out by feed and food business operators under HACCP based control programmes or Quality Assurance Programmes, where these are designed to meet requirements of feed and food law. Official controls must take place on the basis of documented procedures so as to ensure that these controls are carried out uniformly and are constantly of a high level. Official controls and food business operators own checks must be carried out in a synergic way,to reduce costs and grant foods with the expected safety and sensory qualities.

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