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The aim of this paper is to provide information on raw equid cheese making that may be exploited an innovative dairy-production of equid cheese.Until this year scientific literature reported that equid milk is not suitable for cheese production because unlike other milk no curd is formed on addition of common rennet. Potentially before they could be produced dairy products from equid milk without rennet, but treated with thickened substances eg cottage cheese, but these products have different chemical, organoleptic and rheological characteristics of the cheese that is obtained with the use of coagulating enzymes.Preliminary investigations suggests that asinine milk under the action of bovine chymosin form a very weak gel compared to the gel formed from bovine milk, instead mare's milk with calf chymosindoes not show gel formation. However, the author has surprisingly found that pure camel chymosin is able to clot effectively equid milk with an appropriate technological process, which excludes thermal treatment of thermization or pasteurization of milk in order to not inhibit the subsequent enzymatic reaction. Equid cheese making using pure camel chymosin gave a curd with elasticity properties. More research is needed to study the mechanism of enzymatic coagulation in equid milk, to use the nutritious whey that is produced from cheese, and especially assess if cheese and derivatives from equid milk can be addressed, as alternative food for people suffering from food allergies.

Key words: Equid milk, camel chymosin, donkey cheese, equine cheese, milk clotting enzyme, Equid cheese manufacturing

INTRODUCTION

The nutritional and therapeutic properties of equine milk *(Equuscaballus)* have been known since ancient times according to the historian Erodoto (V century BC).Although horses are of minor importance for milk production in comparison with cows, buffalo, sheep and goats, they have been traditionally important dairy animals in Mongolia and in the southern states of the former Soviet Union, e.g., Kazakhstan, Kyrgyzstan and Tajikistan (Uniacke-Lowe et al, 2010).

Estimates suggest that more than 30million people world wide drink equine milk regularly, with that figure increasing significantly annually (Doreau and Martin-Rosset, 2002).

The use of dairy products from donkeys *(Equusasinus)* was known in the Roman era and for a long time donkey milk was recognized as a common remedy (Salimei and Fantuz, 2012).However, equid milk has always been regarded not suitable for cheese production. Although extensive reviews are available on horse and donkey milk properties, the aim of this paper is to provide information on raw equid cheese making that may be exploited an innovative dairy-production of equid cheese.

Current equid milk product

In the late nineteenth century, donkey milk was successfully used for feeding orphaned infants in France, as reported by D'Arval (1912); also recently asinine milk it has been used successfully as a substitute for human milk in Western Europe and to up regulate the immune response of healthy elderly humans (Salimei and Fantuz, 2012). Moreover, donkey milk whey proteins showed in vitro anti-proliferative and anti-tumor activity (Mao et al., 2009).

Horse and donkey milk has been used successfully as an alternative food for infants with food allergies, e.g., cows' milk protein allergy (CMPA) (Salimei and Fantuz, 2012; Polidori and Vincenzetti, 2013), a common food allergy in childhood with a prevalence of approximately 3% during the first 3 years of life; but tolerability must be tested first (Businco et al., 2000). Subsequent clinical studies showed interesting results on equid milk tolerability most likely related to horse and donkey phylogenetic differences with ruminants. (Restani et al., 2009; Salimei and Fantuz, 2012). However, results on equid milk tolerability cannot be considered conclusive (Iacono et al., 1992; Vita et al., 2007).

Unlike other milk, cheese is not produced from equid milk as no curd is formed on addition of common rennet. Equine milk will form a weak coagulum under acidic conditions and this is exploited in the production of yoghurt-type products, especially in the Netherlands, where it is generally flavoured with concentrated fruit extract, traditional fermented horse milk beverages (airag and koumiss) are very popular in Eurasian steppe areas (Uniacke-Lowe et al, 2010).

Koumiss (fermented mare's milk) is used in Russia and Mongolia for the management of digestive and cardiovascular diseases (Lozovich, 1995).

In health foods hops and some pharmacies inWesternEurope, equine milk is sold frozen or as capsules of lyophilised milk. Other products from equine milk include frozen or lyophilized colostrum which isused mostly in the high-value horse industry to feed orphaned foals.It is claimed that many of the products relieve metabolic and intestinal problems while having-cleansing effect coupled with 'repair' of intestinal flora. Relief from stomach ulcers, high blood pressure, high cholesterol and liver problems are also reported and equine milk is recommended as an aid in the treatment of cancer patients. There commended amount of equine milk is 250mL per day. The use of equine milk in the production of cosmetics is relatively new and includes soaps, creams and moisturisers (Uniacke-Lowe et al, 2010; Doreau and Martin-Rosset,2002).As sales of equine milk have increased considerably during recent years, research is now focused on the development of new products or new methods for extending the shelf-life, while maintaining some of the unique components of equid milk, however in scientific literature and in trade magazines are not reported methods of cheese making with 100% of equid milk.

Previous investigation on rennet-induced coagulation of equid milk in Encyclopedia of Dairy Sciences 2nd edn (Uniacke-Lowe T and Fox PF, 2011b) reported that horse milk does not form a gel during renneting, by bovine chymosin, and donkey milk, although coagulable forms a very weak gel and that equid milk is not suitable for cheese production. However, the author has surprisingly found that pure camel chymosin is able to curd effectively donkey milk, with an appropriate technological process (Iannella, 2014; Iannella,2015c), and more recently also mare's milk (Iannella, 2015b) as he was previously assumed, because these milks are very similar; therefore this discovery has opened definitively the possibility of using equid milk for cheese production (Iannella,2015d).

Applications of rennet enzymes on cheese production

Cheese production represents one of the earliest biotechnological applications of enzymes (Szecsi, 1992). The active ingredients in this process were identified as the proteolytic enzymes pepsin and chymosin, previously referred to as 'rennet' (Foltmann, 1966). Both chymosin and pepsin belong to the pepsin-like family of aspartic peptidases and their sequences are 55% identical (Langholm et al., 2013). Chymosin is prevalent in the stomach of newborn mammals unlike pepsin predominates in adult mammals.

The dairy industry characterizes rennet enzymes using two properties. The first is the milk-clotting activity (C) expressed in International Milk-Clotting Units (IMCU). It is determined by a standard method (International Dairy Federation, 2007) that describes the ability to aggregate milk by cleaving the Phe_{105}-Met_{106} bond or nearby bonds of κ-casein. The second property is the general proteolytic activity (P), which is the ability to cleave any bond in casein (Kappeler et al., 2006). The ratio between the two properties, the C/P ratio, captures the essential quality of a milk-clotting enzyme. The higher the value the better the rennet, and in this regard chymosin is superior to all other known rennet enzymes (Foltmann, 1992).

The C/P ratio of bovine chymosin towards bovine milk is higher than those of the chymosins from lamb, pig, cat and seal (Foltmann, 1970). Bovine chymosin is the preferred enzyme in the cheese making process, since specificity for K-casein is high, general proteolytic activity, especially with regard to milk proteins, is low, and optimal activity is achieved at mildly acidic conditions (Williams et al.,1997).

However, camel chymosin expressed in *Aspergillusniger* shows a sevenfold higher C/P ratio than bovine chymosin (70% higher clotting activity and only 25% of the general proteolytic activity; Kappeler *et al.,* 2006). Bovine and camel chymosin both consist of 323 residues and display high sequence identity 85% (Langholm et al., 2013). In contrast, bovine chymosin displays a very low milk-clotting activity towards camel milk (Farah and Bachmann, 1987;Kappeler et al., 2006); most likely due to major variations between the primary k-casein structures of the two species (Kappeler et al., 1998).

Other aspartic proteases, such as pepsin and microbial peptidases, exhibit a broader proteolytic activity and are less suitable for cheese production, since bitter, hydrophobic peptides, formed by undesirable proteolytic action, impair the sensory value of the cheese (Kappeler et al., 2006). In bovine milk with rennet, chymosin hydrolyses the Phe_{105}-Met_{106} bond of κ-casein or nearby bonds (Fox and McSweeney, 1998). As a result, the micelles lose steric stabilization and become susceptible to aggregation, particularly in the presence of Ca^{2+} (Walstra, 1990; Walstra et al., 2006), thereby leading to gel formation and phase separation of the milk into curds and whey.

Enzymatic coagulation of equid milk

Preliminary research, suggests that asinine milk under the action of bovine chymosin, forming a very weak gel compared to bovine milk coagulum treaty under similar conditions (Uniacke-Lowe and Fox, 2011a) and without curd formation.Equine milk (mare's milk) with calf chymosin is susceptible to hydrolysis at the Phe_{97}-Ile_{98} bond of equine κ- casein (Egito et al., 2001), but its hydrolysis is considerably slower than that of bovine κ-casein (Kotts and Jenness, 1976) and without gel formation (Uniacke-Lowe and Fox, 2011b). In addition Egito et al. (2001) have verified that also equine β-casein is hydrolysed by calf chymosin at Leu_{190}-Tyr_{191}, however this hydrolysis occurred at a much slower rate compared to that of bovine κ-casein (Uniacke-Lowe et al. 2013) (Figure 1).

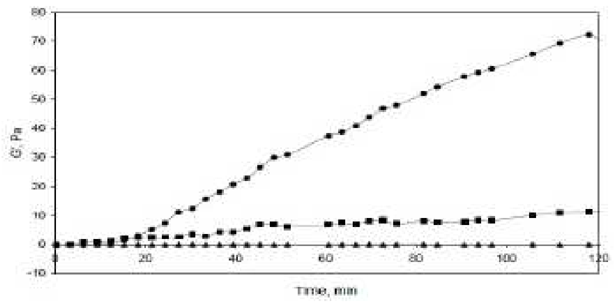

Figure.1 Effect of renneting with 6 µl mL-1 Maxiren 180 (bovine chymosin) on the storage modulus, G´ (Pa), of bovine (•), asinine (■) or equine (▲) milk at pH 6.6. (Uniacke-Lowe, 2011).

It is clearly seen in the graphs that no gel is formed from equine milk, as judged by lack of an increase in storage modulus, G'. Asinine milk seems to form a gel although it is very weak compared to the gel formed on bovine milk and without curd formation.

Therefore, it is not surprising that the majority of pastoral systems have produced at least one type of cheese, no traditional methods exist for making cheese from 100% of mare's milk.

However, until today, according to the author, besides fermented donkey (yogurt) and the traditional fermented horse milk beverages (airag and koumiss) only through lactic fermentation or thermo-acid coagulation could produced a dairy product from equid milk that is, dairy product with strains of lactic acid bacteria, mesophilic or thermophilic, optionally thickened with thickening agent, but these products have different chemical, organoleptic and rheological characteristics of the cheese that is obtained with the use of coagulating enzymes.

New knowledge of coagulation equid milk: Iannella finds

A decisive step in the possibility to use equid milk in the dairy sector, for cheese production, has been made thanks to the surprising finding of the italian food technologist, Iannella (2014), which has discovered that pure camel chymosin (FAR-M®), enzyme found in camel rennet, is able to clot effectively donkey milk with an appropriate technological process, which excludes thermal treatment of thermization or pasteurization of milk in order to not inhibit the subsequent enzymatic reaction (Iannella, 2014).

Discovery published by Iannella through the multinational biotechnology company Chr Hansen (Chr Hansen Co. and Iannella, 2015) and through scientific journal (Iannella 2015a, Iannella, 2015c).

More recently Iannella (2015b) has verified that also mare's milk are effectively coagulated by pure camel chymosin (FAR-M®), under the same operating conditions used by him to curdle donkey milk; hypothesis and found towards mare's milk published by Iannella in Dairy Reporter through the journalist Astley (2015a; 2015b).

For this reason pure camel chymosin now used to curdle camel milk (Bruntse, 2011), can also be used with Iannella method for donkey cheese production (Iannella, 2014; Chr. Hansen Co and Iannella, 2015; Iannella,2015a; Iannella,2015c), and now also for mare's cheese production (Iannella,2015b, Iannella,2015d).

Unfortunately, computational methods for proteins have not yet reached a state that enables the modelling of the interactions of an entire casein micelle with chymosin, in particular the pure camel chymosin with casein micelles of equid milk. However, without wishing to be bound to any theory empirically is contemplated that curd firmness is formed when raw mare's milk is treated with pure camel chymosin (FAR-M®), with a proper dairy technology process because Iannella (2015b) found that mare's milk subjected to a prior heat treatment, pasteurization or also slight thermization, did not give a clot, as he had previously seen in donkey's milk.

Iannella states that the heating of equid milk is the most critical point in the process of cheese production, that for this reason must take place in a "soft way" e.g. in a water bath and through its empirical testing has verified that the water temperature of the water bath should not exceed about 43°C (Iannella,2015c). Therefore using pure camel chymosin (FAR-M®) in an appropriate technological process, with Iannella method named by him "*Nativity-equid cheese making method ©",* one can now produce cheese with 100% of equid milk (donkey or mare's milk).

One must remember that the promising industrial applications of camel chymosin were first reported by Kappeler et al. (2006), who claim that recombinant camel chymosin expressed in *Aspergillusniger* reveals superior properties of bovine chymosin. In their study is reported that camel chymosin exhibits a 70% higher clotting activity for bovine milk and has only 20% of the unspecific protease activity for bovine chymosin. This results in a sevenfold higher ratio of clotting to general proteolytic activity. Kinetic analysis showed that half-saturation is achieved with less than 50% of the substrate required for bovine chymosin and turnover rates are lower (Kappeler et al., 2006). Langholm et al. (2013) in their work "Camel and bovine chymosin: the relationship between their structures and cheese-making properties" have reported that camel and bovine chymosin share the same overall fold, except for the antiparallel central β-sheet that connects the N-terminal and C-terminal domains. In bovine chymosin the N-terminus forms one of the strands which is lacking in camel chymosin. This difference leads to an increase in the flexibility of the relative orientation of the two domains in the camel enzyme. Variations in the amino acids delineating the substrate-binding cleft suggest a greater flexibility in the ability to accommodate the substrate in camel chymosin. Both enzymes possess local positively charged patches on their surface that can play a role in interactions with the overall negatively charged C-terminus of k-casein. Camel chymosin contains two additional positive patches that favour interaction with the substrate. The improved electrostatic interactions arising from variation in the surface charges and the greater malleability both in domain movements and substrate binding contribute to the better milk-clotting activity of camel chymosin towards bovine milk. (Langholm et al., 2013).

While raw camel milk cannot be clotted with bovine chymosin (FAO, 2001; Ramet, 2001), a high clotting activity was found with camel chymosin (Kappeler et al., 2006).

However the ability of pure camel chymosin (FAR-M®) to clot equid milk that is donkey and mare's milk (Iannella, 2015abcd) was a surprising found because as mentioned k-casein is present in trace amounts in casein micelles of asses' milk (Amadoro et al., 2011), and small quantities in horse (Malacarne et al., 2002); normally k-casein represents the key protein of coagulation process by chymosin in common milks. One must remember that preliminary research, suggests that asinine milk under the action of bovine chymosin, forming a very weak gel, compared to bovine milk under similar conditions (Uniacke-Lowe and Fox, 2011a) and without curd formation, indeed mare's milk with calf chymosin does not show gel formation (Uniacke-Lowe and Fox, 2011b).

This interesting result according Iannella should open a detailed study on the activity of camel chymosin in equid milk. Taking into account the complexity of casein micelles of equid milk and according to the studies described above, Iannella hypothesizes that the improved milk clotting activity of camel chymosin, compared to bovine chymosin, in equid milk can be attributed to variations on the surface charge, at the binding sites, that facilitate the association between camel chymosin and equid casein and to the improved flexibility of camel chymosin in the ability to accommodate the substrate.

The steric stabilization of casein micelles on mare's milk, in compensation to the absence of κ-casein, it is given by unphosphorylated β-casein on the micelle surface (Ochirkhuyag et al., 2000 ; Doreau and Martin-Rosset, 2002); and then Iannella hypothesizes that the key protein in coagulation process of mare's milk with pure camel chymosin is β –casein.

However in addition to k-casein other fractions of casein (αs1-, αs2-, and β-caseins) and also α-lactalbumin can be hydrolysed by chymosin in bovine milk (Carles and Ribadeau, 1985; Miranda et al., 1989) but at a much slower rate than κ-casein, then probably some of these fractions also present in equid milk, they can be hydrolysed by camel chymosin, although to a lesser degree, however contributing to the coagulation of equid milk according Iannella hypothesis.

Iannella hypothesizes the absence of clot in equid milk heat-treated (thermised , pasteurized or heated with the traditional method on the fire) is due to the change of spatial conformation of proteins (β –casein and others) in response to this process, that inhibits the interaction "key-lock" between camel chymosin with fractions of casein involved in the clotting process. For this reason to warm equid milk you have to use soft method (e.g. in a water bath at 43°C).

Equid cheese manufacturing

The processing of equid milk into cheese is technically more difficult than milk from other domestic dairy animals. This is mainly due to its low total solids content, and casein content of donkey and mare's milk. The flow steps for equid cheese manufacturing are summarized in Figure 2. (Iannella, 2015c; 2015d).

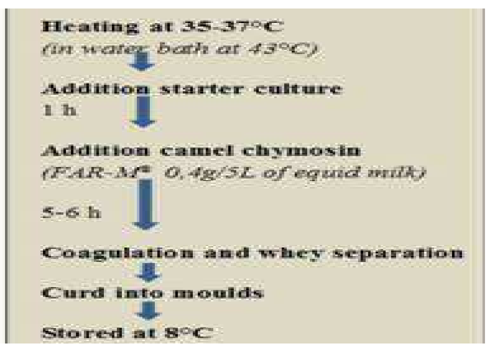

Figure 2. Flow diagram of Equidcheesemaking: Nativity-Equid cheese making method; by Giuseppe Iannella.

METHODOLOGY

Equid milk, kept at room temperature, were taken in a stainless steel container and heated to 37°C in a water bath at 43°C.Then the milk was inoculated with thermophilic starter cultures (L. delbrueckii ssp. bulgaricus and S. thermophilus), after about 1 h was added pure camel chymosin, FAR-M® (0.4 g /5 L of equine milk). The milk was incubated for about 6 h, at room room controlled temperature at 37°C. After the curd formation, most of the whey was removed, and the curd was cut and then the remaining whey was drained off. The curd was moulded and pressed at room temperature. Cheese was kept in the mould and stored at 8°C.

RESULTS AND DISCUSSION

The coagulum obtained in this process, after about 6 h as the addition of commercial pure camel chymosin (FAR-M®), in environment sub-acid product with starter cultures, was a precipitate in the form of clot, with elastic properties.

The average cheese yield obtained from equid milk, around 4% form mare' milk (Iannella 2015,d) and 3,2 % for donkey milk (Iannella,2015d) was lower than that reported in literature for cow milks (around 10-12%) because depending to the lower contents of casein and fat of equid milk however the result is promising.

Cheese curds obtained by these processes can be treated in different ways according to the type of cheese to be obtained or used as an ingredient in other foods. In any case these methodologies can be appropriately modified but respecting the use of camel chymosin as coagulant and no one heat treatment of thermization or pasteurization.

In equid cheese making process, the use of starter cultures is necessary because they are responsible of lactic acid production, which improve curd firmness and suppresses the growth of undesirable bacteria in the curd, obtained from raw milk. In addition the pure camel chymosin have optimum pH at 5.1 (Kappeler et al., 2006), therefore also for this reason the pH of the milk must be lowered. These data indicate that cheese making without use of starter cultures should be discouraged.

This application represents an informative steps for further trials and could be useful for industrial scale cheese processing of mare's milk.

Conclusion

More research is needed to study the mechanism of enzymatic coagulation in equid species, to improve cheese yield, to use the nutritious whey that is produced from cheese in addition to "ricotta" production, and especially assess if cheese and dairy product from equid milk can be addressed, as alternative food for people suffering from food allergies.

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