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The Influence of Heat Treatment on Antimicrobial Proteins in Milk

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Abstract**—**the obligatory step during immunoglobulin and lysozyme concentration process is thermal treatment. The combination of temperature and time used in processing can affect the structure of the proteins and involve unfolding and aggregation. The **aim** of the present study was to evaluate the heat stability of total Igs, the particular immunoglobulin classes and lysozyme in milk. Milk samples were obtained from conventional dairy herd in Latvia. Raw milk samples were pasteurized in different regimes: 63 °C 30 min, 72 °C 15-20 s, 78 °C 15-20 s, 85 °C 15-20 s, 95 °C 15-20 s. The concentrations of Igs (IgA, IgG, IgM) and lysozyme were determined by turbodimetric method. During research was established, that activity of antimicrobial proteins decreases differently. Less concentration reduce was established in a case of lysozyme.

Keywords**—**immunoglobulins, lysozyme, milk, pasteurization

I. Introduction

ALTHOUGH proteins including *lysozyme, lactoferrin, lactoperoxidase and immunoglobulins* represent only a minor fraction of milk proteins, they play an important role as first line defence due to their direct and indirect antimicrobial activity [1], [2], [3] and for other important physiological and health promoting functions [4].

Increasing antibiotic resistance among pathogens gives emphasis to develop new supplements to prevent diseases by nutritional intervention. Modulation of the gastrointestinal flora has turned out to be an integral part of health promotion [5]. It is suggested that colostral Igs and lysozyme would provide as one of the considerable prospects for consumers health promotion in the future [5], [6].

The immunoglobulins, or antibodies, found in colostrum or milk are the same as those found in the blood or mucosal secretions. They are a family of proteins with a range of protective bioactivities. Immunoglobulins are divided into several classes, the major immunoglobulin classes in mammary secretions are IgG, IgA and IgM [7]. IgM is the class that appears initially when an organism is exposed to an antigen for the first time (primary infection). IgM has a low specificity and hence a lower potency in defeating the infection. IgA is the major immunoglobulin class found in mucosal secretions and prevents mucosal infections by agglutination of microbes, whereas IgG is the primary immunoglobulin class found in bovine colostrum and milk. Several subclasses of IgG exist, with IgG1 and IgG2 being the major immunoglobulins in serum [8].

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Immunoglobulins (*Igs*), together with lactoferrin, lactoperoxidase and lysozyme form important antimicrobial system of bovine lacteal secretions. Bovine colostrum, which is a rich source of Igs, also confers passive immunity to the newborn during development of its own immune system [9]. Igs with specific antibody activities can be increased in lacteal secretions through targeted hyperimmunization protocols, using vaccines containing inactivated pathogenic microbial material against which the Igs activities are desired [10], [11], [9].

The concentration of the various bovine Igs in serum and in lacteal secretions varies according to the breed, age, health status, and stage of lactation of the animal [12], [13], [14].

Many milk processing protocols include heat treatment of the colostrum, milk or whey. Therefore it is necessary to know effect of pasteurization on different immunoglobulin classes and lysozyme. In literature there is contradictory information about thermostability of antimicrobial proteins.

According Chen and Chang (1998) research results immunoglobulins are thermolabile. Exposure to temperatures of 75 °C can reduce detectable isolated bovine IgG by 40% in 5 min, and by 100% at 95 °C for 15 s [15]. The explanation of it is conformational changes in the IgG molecule causes by heat exposure [16]. Antigen-binding activity of bovine IgG also is reduced after heat treatment [17], [18]. This is consistent with studies suggesting that the antigen-binding region of the immunoglobulin molecule is more thermolabile than the other regions of the molecule [18], [19]. Detectable IgG in colostrum or colostral whey also are reduced by heat treatment, however at a slower rate than for isolated IgG. Thermal protectants such as sugars or glycerol can increase the stability of isolated IgG to heat treatment [20], [21].

Although comparing different immunoglobulin classes, Mainer (1997) detected, that IgG is the most thermostable and IgM is the least thermostable [19].

Lysozyme is an antimicrobial enzyme that is found in a wide variety of organisms including birds, mammals, plants, insects, and bacteria [22]. The content of lysozyme is from 0 to 3 mg l^{-1} in cow's milk to 790 mg l^{-1} in mare's milk [23]. The enzyme is often used for lysing of peptidoglycan present in the bacterial cell walls. Gram-positive cells are quite susceptible to this hydrolysis as their cell walls have a high proportion of peptidoglycan. Gram-negative bacteria are less susceptible due to the presence of an outer membrane and a lower proportion of peptidoglycan [6].

Lysozyme protects against bacterial infection by breaking down the carbohydrates in bacterial cell walls, killing them [24], [6]. Hence, lysozyme is a part of the innate immune response in saliva it protects the oral cavity from pathogens. In times of increased inflammation, such as during inflammation of the salivary glands or after a tonsillectomy, there is an increase in lysozyme. This protein also works in synergy with

the bactericidal properties of lactoferrin, another common protein as immunoglobulins [25].

Lysozyme also has fungicidal properties, protecting mucosal areas from invasion by pathogenic yeast or fungi. Lysozyme has been shown to inhibit viral replication and infection such HIV [26].

In addition, the concentration of soluble lysozyme in milk varies considerably from one species to another and within the same species depending on various factors such as the breed, stage of lactation, parturition, nutrition, udder health and season of the year [27], [28].

Lysozyme is termostable, 75% of lysozyme activity mantains after milk heat treatment 75 °C 15 min or 80 °C 15 s [23].

The concentration of immunoglobulins and lysozyme is significantly higher in colostrum comparing with milk [29], [30]. This increased pool of antimicrobial components can be enriched further through concentration techniques, leading to production of products containing high immunoglobulin and lysozyme concentration. Such preparation may find beneficial application as in human healthcare and wellbeing by preventing infection and controlling microorganisms grow and diseases, as in a new functional food development [5]. Through such processing, immunoglobulins and lysozyme are exposed to a number of conditions that may alter the structure and function of the proteins. Some of methods used for concentration or isolation of immunoglobulins and lysozyme include steps that involve exposing the protein to *heat, acid or pressure* which may affect the conformation of the protein, and ultimately the immunological activity of it. Independent on method, which is used for concentration immunoglobulin and lysozyme, thermal treatment, is obligatory step. The combination of temperature and time used in processing can affect the structure of the proteins and involve unfolding and aggregation [31]. Therefore the *aim* of the present study was to evaluate the heat stability of total Igs, the particular immunoglobulin classes and lysozyme in milk.

II. Materials and Methods

A total 50 bulk milk samples were analyzed. Milk samples were collecting according to standard method LVS EN ISO 707:2011 “Milk and milk products. Guidance on sampling”.

Milk samples were obtained from conventional dairy herd in Latvia. Individual milk samples were taken from 15 healthy cows, pooled together, that was immediately cooled to 4–8 °C and transported to the laboratory, arriving at a temperature not exceeding 8 °C. At the same time milk samples were pasteurized in different regimes: 63 °C 30 min, 72 °C 15-20 s, 78 °C 15-20 s, 85 °C 15-20 s, 95 °C 15-20 s.

The concentrations of Igs (IgA, IgG, IgM) and lysozyme were determined by turbodimetric method [32], using pH-meter “Jenway 3520” and spectrophotometer “Jenway 6705 UV/VIS” (UK).

The principle of the immunoglobulin detection methods is following: anti-human Ig antibodies mixed with samples containing Igs form insoluble complexes. These complexes cause an absorbance change, dependent upon the Igs concentration of the analyzed sample, that can be quantified by comparison from a calibrator of know Igs concentration.

Determination of lysozyme activity is based on its lytic activity on the cell wall of *Micrococcus lysodeikticus*, by measuring the loss of intensity of light in the direction of propagation of the incident beam, with reference to a standard solution.

The factors as cow’s breed and age were not taken into consideration in this research.

Descriptitive statistics were carried out to determine the differences of IgA, IgG, IgM and lysozyme concentration in different milk samples by Microsoft Windows for SPSS software packages.

III. Results and Discussion

The mean concentration of total *immunoglobulins* in raw milk was 0.73 g/l (see Table 1), it was according to the data in literature – 0.15−0.80 g/l [33] and it was lower comparing with previous research done in Latvia [34], [35]. The proportion of immunoglobulins classes were following: IgG 76.7 %, IgA 15.2 % and IgM 8.1 %. Mehra (2006) reported, that the proportion of IgG in milk is ranged between 80–90 %; in current study the same results were obtained. Proportion of IgA was higher and IgM was lower, the same results were established during previous research [34], the proportion of IgA was higher (12.9 %), but IgM lower (11.8 %). Muller (1981) explains the differences of proportion with cows’ breeds [36].

The mean concentration of lysozyme in milk was controlled too. The mean concentration of lysozyme in raw milk was 0.60 mg/l. It ranged from 0.40 to 0.67 mg/l according to the literature data is from 0 to 2 mg/l [24].

TABLE I
The Effect of Thermal Treatment on Quantity of Immunoglobulins and Lysozyme in Milk

Milk samples	Quantity	
	Immunoglobulins, g/l	Lysozyme, mg/l
Raw milk	0.730 ± 0.036	0.600 ± 0.030
63 °C, 30 min	0.640 ± 0.030	0.590 ± 0.028
72 °C, 15-20 s	0.630 ± 0.030	0.580 ± 0.026
78 °C, 15-20 s	0.610 ± 0.029	0.550 ± 0.025
85 °C, 15-20 s	0.590 ± 0.025	0.520 ± 0.022
90 °C, 15-20 s	0.490 ± 0.024	0.510 ± 0.020

The stability of the immunoglobulins and lysozyme in milk is influenced by many factors. One of them is thermal treatment. The results obtained in current research given in Table I.

The concentration of *lysozyme* during thermal treatment was reduced from 0.60 g/l to 0.51 g/l, it was decreased for 15.0 %.

The decrease of mean concentration of *immunoglobulins* was determined already using pasteurization regime at 63 °C 30 min. Quantity of immunoglobulins was reduced for 12.3 ± 3 %. Similar, but not the same results, were obtained during another research. Elfstrand, Lindmark-Mansson, Paulsson, Nyberg, Akesson (2002) had done research with colostrum, which was treated at 60 °C, 30 min and likewise no

effect on the concentration of the Igs was observed [31]. These experiments show that Igs in colostrum and colostrum whey were more resistant to heat treatment than in colostrum concentrate. Therefore there is hypothesis, milk components, like fat, casein, salts and lactose may protect the immunoglobulins during heat treatment.

Increasing temperature up to 72 °C with holding time 15-20 s had similar influence on quantity of immunoglobulins, it was decreased for 13.7 ± 3 %.

Other authors have similar results, indicating that during high temperature/short time (HTST) pasteurisation at 72 °C 15 s only 10 ± 30 % of the Ig activity is lost, whereas ultrahigh temperature (UHT) treatment (138 °C 4 s) destroys the majority of the specific immune activity of milk [37], [38]. In a similar study, Mainer, Dominguez, Randrup, Sanchez, and Calvo (1999) also demonstrate the insignificant effect of pasteurization at 72 °C 15 s on Igs activity [39].

After extension increase of temperature, activity of immunoglobulins continued to decrease: pasteurization at 78 °C 15-20 s reduced for 16.4 ± 4.0 %, at 85 °C 15−20 s and 90 °C 15-20 s, respectively for 19.2 ± 4.0 % and 32.9 ± 6.0 %. The most significant influence ($p < 0.05$) on quantity of immunoglobulins in milk had pasteurization at 90 °C 15−20 s.

Previous mentioned research data shows that each immunoglobulin class changes differently during thermal treatment: IgG1 unfolded at 79.4 °C, IgG2 at 76.7 °C and IgM at 80.3 °C [40]. Authors also found that the unfolding of the individual Igs was irreversible and almost independent of pH [41]. In the current research the concentration of different immunoglobulin classes was determined too (see Table 2).

TABLE II
THE EFFECT OF THERMAL TREATMENT ON QUANTITY ON QUANTITY OF DIFFERENT IMMUNOGLOBULIN CLASSES IN MILK

Milk samples	Quantity, g/l		
	IgA	IgG	IgM
Raw milk	0.110±0.006	0.556±0.026	0.059±0.002
63 °C, 30 min	0.104±0.005	0.485±0.021	0.051±0.002
72 °C, 15-20 s	0.103±0.005	0.479±0.020	0.049±0.002
78 °C, 15-20 s	0.091±0.005	0.467±0.020	0.048±0.002
85 °C, 15-20 s	0.087±0.005	0.461±0.020	0.039±0.002
90 °C, 15-20 s	0.070±0.004	0.390±0.020	0.032±0.001

The mean concentration of *IgG* in raw milk samples was 0.56 ± 0.026 g/l, the wide range of IgG concentration in raw milk was not determined; it varied from 0.52 to 0.59 g/l. In all analyzed milk samples the concentrations of immunoglobulin was according to the literature data 0.00–0.76 g/l [14], [33]. The quantity of IgG decreased from 0.556 ± 0.026 g/l to 0.390 ± 0.020 g/l during thermal treatment.

The mean concentration of *IgA* in raw milk samples was 0.11±0.006 g/l in all analyzed milk samples, but the concentration of IgA was according to the literature data 0–0.13 g/l [14], [33]. The concentration of IgA in raw milk samples ranged between 0.097 to 0.130 g/l. The quantity of IgA decreased during thermal treatment from 0.110 ± 0.006 g/l to 0.070 ± 0.004 g/l.

The mean concentration of *IgM* in raw milk samples was 0.06±0.002 g/l, in all analyzed milk samples the concentrations of IgM was low, but it still was according to the literature data 0.00–1.00 g/l [14], [33] and ranged from 0.035 g/l to 0.065 g/l. During thermal treatment the quantity of IgM had decreased and ranged from 0.059 ± 0.002 g/l to 0.032 ± 0.001 g/l.

During research was determined, that antimicrobial proteins decrease differently. Less concentration reduce was established in a case of lysozyme, its activity decreased for 16% during the thermal treatment at 90 °C 15−20 s.

The percentages losses of activity of immunoglobulin classes and lysozyme during pasteurization are shown in Figure 1.

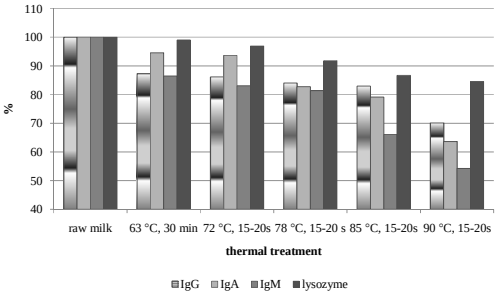

Fig. 1 The percentage of immunoglobulins and lysozyme losses in milk during thermal treatment

The decrease of *IgA* activity was established in the research: it was decreased for 5.5 ± 1.0 % during milk pasteurization at 63 °C 30 min, for 6.4±1% and 17.3 ± 4 %, at 72 °C 15−20 s and 78 °C 15−20 s correspondingly.

Significant decrease of IgA activity ($p < 0.05$) was determined during pasteurization at 85 °C 15−20 s and 90 °C 15−20 s, accordingly it was decreased for 20.9 ± 5.0 % and 36.4 ± 6.0 %.

The decrease of *IgG* activity at 63 °C 30 min was higher comparing to IgA, it decreased for 12.8 ± 3.0%. The activity of IgG continued to decrease for 13.8 ± 3.0% after milk pasteurization at 72 °C 15−20 s and for 16.0 ± 4.0 % at 78 °C 15−20 s. As in a case of IgA, significant decrease of activity of IgG ($p < 0.05$) was determined at 85 °C 15−20 s and at 90 °C 15−20 s, respectively, it was decreased for 17.1 ± 4.0 % and 29.9 ± 5.0 %, but reduction was not so high as it was in a case of IgA.

Mainer *et al* (1997) had different research results, high-temperature short-time pasteurization (72 °C for 15 s) led to 25–40 % loss of IgG concentration and a similar reduction in antigen-binding activities. In current research only 29.9 ± 5.0 % of mean IgG concentration had decreased at 90 °C 15−20 s.

It is not possible to determine accurate percentage of immunoglobulin losses during pasteurization; it ranged in different samples and was not the same in all analysed samples. It means, that there are others factors, as chemical composition, pH and others [24], effecting concentration of immunoglobulin during thermal treatment. Detectable IgG in

colostrum or colostral whey also are reduced by heat treatment, however at a slower rate than for isolated IgG.

Thermal protectants such as sugars or glycerol can increase the stability of isolated IgG to heat treatment [20], [21]. In current research such factors were not taken into consideration, therefore the research in this area should be continued in the future. The decrease of quantity of *IgM* was higher comparing with reduce of mean concentration of IgA. Decrease of IgM activity was determined already after pasteurization at 63 °C 30 min, it was reduced for 13.6 ± 4.0 %, at 72 °C 15−20 s, the activity of *IgM* was decreased for 16.9 ± 3.0 %, after extension pasteurization temperature, it continued to decrease: at 78 °C 15−20 s it was reduced for 18.6 ±3.0 %, at 85 °C 15−20 s and 90 °C 15−20 s, accordingly for 43.9 ± 6.0 % and 45.8 ± 6.0 %.

Mainer *et al* (1997) reported that bovine milk Igs could resist the HTST pasteurization treatment without affecting their structure. Only 1 % of the IgG, 2 % of the IgA, and 14 % of the IgM concentrations were denatured when milk samples were heated in glass capillaries and a temperature controlled water bath. In a similar further study, Mainer *et al* (1999) also showed that the HTST pasteurization had little effect on the activity of bovine colostral IgG [19]. In current research the similar results were obtained, IgG and IgA are more thermostable, but IgM is less stable.

One more research had been done in this area by Ustunol and Sypien (2006). Heat treatment at 80 °C for 25 min completely inactivated IgA, the higher pasteurization temperature 85 °C for 20 min inactivates IgM, and more thermostable was IgG for its inactivation at the same regime [42]. The activity of *lysozyme*, a very important antimicrobial protein, was controlled during research too. The decrease of lysozyme activity was established, it was decreased only for 3.1±1% after milk pasteurization at 72 °C 15−20 s, for 8.2 ± 2 % at 78 °C 15−20 s. After increasing the temperature activity of lysozyme continued to decrease, but not significant: at 85 °C 15−20 s and 90 °C 15−20 s, respectively it was decreased for 13.3 ± 3.0 % and 15.5 ± 3.0 %. Different results have showed other authors, it was mentioned, and that only 75 % of lysozyme activity maintains after milk pasteurization at 80 °C 15 s [23]. In current research lysozyme showed higher results and was more stable during heat treatment.

IV. Conclusions

The equal heat treatment regimes have different influence on IgA, IgG, IgM concentrations in milk. IgM was termolable after pasteurization at 90 °C 15−20 s, its activity decreased for 45.8 %.

More thermostable was lysozyme, during pasteurization at 90 °C 15−20 s, its activity decreased only for 15.5 %.

The research results show, that the necessary heat treatment regime should be used – 72 °C 15−20 s for promotion antimicrobial activity of Igs and lysozymes in milk and for antimicrobial proteins concentration techniques.

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