

THE *IN VITRO* EVALUATION OF ANTIBACTERIAL ACTIVITY OF A TOTAL CASEIN EXTRACT FROM GOAT MILK

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Abstract

The goat milk composition can be distinguished by significant proportions of biologically active proteins that can exert antimicrobial effects. In this context, the present work is focused on evaluating antimicrobial activity of total caseins in vitro, extracted from goat milk, concerning some reference bacterial strains such as Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and Staphylococcus aureus. The research was conducted on milk samples collected from two groups of clinically healthy goats, Carpathian (group I, n = 12) and Alpine (group II, n = 12) breeds. Milk samples were subjected to a procedure of total casein separation. In vitro test procedure consisted in evaluating the inhibitory effect of casein on the microbial strains listed, using the serial micro-dilution method in liquid broth, which led to the establishment of minimum inhibitory concentration (MIC), correlated with diffusion method. Thus, the concentrations tested were 10, 20, 30 and 40 mg casein / mL, and the interpretation was based on the measurement of the inhibition zones diameters. Our results indicate antimicrobial activity with variations dependent on the bacterial strain tested with no significant differences between the two goat breeds. Thereby, E. coli strain was proven to be very sensitive to all concentrations used, P. aeruginosa at the 20, 30 and 40 mg/mL concentrations, and the St. aureus to 30 and 40 mg casein/mL concentrations. Our results reveal the antimicrobial potential of milk proteins, analysing it in the current context of developing microbial resistance to antibiotics. In conclusion, caseins may be an alternative solution to diminish the consequences of the antibiotic resistance phenomenon, giving some dairy products the character of functional foods.

Key words: goat, milk, casein, antibacterial effect

INTRODUCTION

Goat milk is different from the milk of other ruminant species through richer content in proteins with intense functional activities, which also exercise a good antimicrobial activity (Korhonen and Pihlanto, 2001 și 2009; Roncada et. al., 2012; Murata et al., 2013). The main protein of milk is casein, a phosphoprotein with phosphate groups attached to the side chains of the amino acids, primarily to the hydroxyl groups of serine and threonine. Actually, the casein is a mixt between at least three similar proteins, which differ mainly in molecular weight and in the amount of phosphorus it contains (Daniel, 2000). Casein exists in milk in the form of the calcium salt, calcium caseinate (Minard, 2002) and it is considered an important source of antimicrobial

peptides (Lopez et al., 2006; Park et al., 2007; Eriksen et al., 2008). This salt has a complex structure, being composed of α , β and κ caseins, which form a mycelium. Neither α -casein, nor the β -casein is soluble in milk. κ casein has fewer phosphate groups and high carbohydrate content linked to it (Veloso et al., 2002; Miranda et al., 2004). It seems that all the κ -casein contains serine and threonine (which have hydroxyl groups) and also carbohydrates, linked to only one side of the outer surfaces. This portion of the outer surface, it is easily solubilized in water. Calcium caseinate, has the isoelectric point at pH 4.6, therefore, it is insoluble in solutions with a pH below 4.6 (McMaster, 1997; Pavia et al., 2000; Minard, 2002; Yeditepe, 2006). Besides the major milk proteins (caseins and whey proteins), there are also small amounts of

minor proteins and peptides in milk. These bioactive peptides are inactive in the sequence of the protein they come from and can only be released by enzymatic proteolysis. As soon as they are released in the body, they can act as compounds with antimicrobial activity (Bellamy, 1992, 1993; Atanasova and Ivanova, 2010). Thereby, these peptides represent a health potential with applications in the food and pharmaceutical industry (Ariyoshi, 1993). Milk quality, both in terms of health (Ognean, 2001), as well as nutritional value and biologically active, is considered essential for consumer welfare and safety (Michel, 2001, Yadav, et al., 2014). Unlike endogenous bioactive peptides, peptides derived from milk are characterized by multifunctional properties with specific sequences, which have two or more biological activities. The bioactivity of immuno-peptides present in the milk has been proven and is characterized both in vitro and in vivo, immuno-peptides being derived from the amino acid residues present in casein (Migliore et al., 1989; Coste et al., 1992; Assargird et al., 1994; Kayser, 1996). Bio-peptides were defined as specific protein fragments that have a functionally beneficial impact on the body and, consequently, on health status (Haque and Chand, 2001). They are encrypted in milk proteins, obtained only by enzymatic hydrolysis, in vivo, during gastrointestinal digestion. This process is dependent on food processing by microbial enzymes specific for fermented products. Thanks to their physiological and physicochemical diversity, lactic proteins are essential components of dairy products, which may also have applications in the pharmaceutical industry (De-Felice, 1995; Hans, 1999).

Taking everything into account, the aim of our study is to test the casein extracted from the milk of Alpine and Carpathian goats, for the evaluation of the antimicrobial activity on some reference pathogenic bacteria strains.

MATERIALS AND METHODS

The group composition and milk sampling. The research was conducted during July-August 2016, on milk samples (n=20) from a goat farm situated in the heart of Transylvania. For this purpose, from a total of 60 animals, clinically

healthy goats were selected and divided, by breed, into two groups: group I, including 10 Carpathian goats and group II, 10 Alpine goats. From the experimental groups of goats milk samples were collected, taking into consideration the hygienic conditions and the usual sanitation measures (Ognean, 2001). The collection procedure was finalized by reuniting the individual samples into an average sample per batch, which was the subject of the following investigation.

The casein separation from the milk. Immediately after collection, the samples were subjected to a procedure for the separation of casein, based on the initial acidification of milk. Thereby, a first separation of the milk was obtained, into its morphological components, represented by a solid fraction (curd) and a liquid fraction (whey), followed by the casein uptake from the solid component (Mohr and Wayne, 1994; Jahnvi et al, 2016; www.chemistry.mcmaster.ca).

Testing the antimicrobial effect of the casein extract. To evaluate the antimicrobial effect of the total casein extract, isolated from goat milk, reference microbial strains were used: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* isolated from clinical cases. Inoculum preparation was performed from 18-24 hours microbial strains. For this purpose 4-6 colonies were selected, which were diluted in saline solution (PBS, Sigma Aldrich) - representative inoculum. Microbial density was adjusted based on nephelometry, using the MacFarland scale 1, thus establishing a concentration of 5×10^8 CFU/ml (Oxoid).

The obtained inoculum was seeded on Muller Hinton agar, by making the wells to about 15 mm from the periphery of the plate and, respectively, 30 mm apart from each other (Andrews, 2001). In order to dissolve the total casein extract and to obtain the dilutions, a saline solution was used PBS and the testing was made for the 10, 20, 30 and 40 mg/mL concentrations. The dilutions were performed according to the method proposed by (Costa et al., 2014; López-Expósito et al., 2006). The obtained solutions were distributed in the previously prepared wells and the plates were incubated at 37°C. The results were evaluated after 24 hours of incubation. As a control

sample, micro tablets of 10 mg of Ampicillin (Amp, Oxoid) were used and, saline solution, as a negative control.

The interpretation of the results consisted in the measurement of the inhibition zones diameters, which are considered to be directly proportional to the sensitivity of the tested strain. Therefore, the more active the tested substance is the more extended the inhibition area of microbial growth will be.

The study was conducted using the serial micro-dilution method in liquid broth (Carson et al., 1995; Mann, 1998; Brut, 2004), method which led to the establishment of minimum inhibitory concentration (MIC) (Costa, 2014). The data were statistically interpreted using the GraphPad software and the "p" index value was calculated by ANOVA test.

RESULTS AND DISCUSSIONS

The inhibition of bacterial growth, evaluated by the micro dilution method in liquid medium and correlated with diffusion method used for this study, proved to be sufficiently relevant for evaluating the antibacterial action of goat milk casein. The results are comparable to those provided by conventional pathogen susceptibility (classical antibiogram - Kirby-Bauer method).

The measurement of the inhibition zones highlights the diameters size as directly proportional to the sensitivity of the bacterial strains tested. The diameter of the inhibition zones of *Echerichia coli*, strain showed multiple variations and the differences to standard antibiotic are statistically significant ($p \leq 0.005$). Zones of inhibition were seen in all casein concentrations tested. The larger diameters (15-16 mm) were recorded at a concentration of 40 mg/mL, followed by the 30 mg/mL (12-13 mm) and 20 mg / mL (Fig. 1). The data presented also shows very close degrees of antimicrobial activity concerning the concentrations of 30 and 40 mg casein/mL.

Based on the arithmetic averages obtained when testing the two casein samples we appreciated that they exhibit a proper antimicrobial activity towards the tested strains, with no significant differences between the two goat breeds. Thus, after the quantification of the values obtained evaluating the casein

antimicrobial activity from the two milk samples, different levels of activities were found: The *E. coli* strain presented the highest degree of sensitivity towards the concentration of 40 mg casein/mL, followed in descending order by 30, 20 and 10 mg / mL concentrations (Fig.1); *P. aeruginosa* strain proved to be sensitive to 20, 30, 40 mg/mL concentrations, without any inhibition area at other concentrations towards the positive control (Fig. 2); For *St. aureus* strain, the diameter of the inhibition zones varied between 16 and 17 mm to 30 and 40 mg/mL concentrations, without having inhibition zones for the other dilutions (Fig. 3).

Statistical analysis of recorded values for the inhibition zones of *E. coli* strain has emphasized significant variations, with statistically significant differences ($p \leq 0.005$), between the values registered for 40 mg casein/mL concentration and those recorded in the case of positive control antibiotic (Fig. 1).

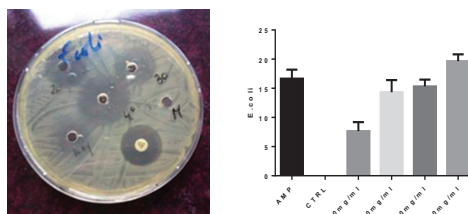


Figure 1. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *E.coli* and the graphical representation of the obtained values.

The statistical analysis of recorded values in the inhibition areas of *P. aeruginosa* strain revealed a slight sensitivity to dilutions of 20, 30 and 40 mg. casein / mL, statistically significant ($P < 0.0001$) (Fig. 2).

The statistical analysis of recorded values in the inhibition areas of *St. aureus* strain revealed significant variations only at dilutions of 30 and 40 mg casein/mL, statistically significant ($P < 0.0001$). They present an inhibition zone which is superior to the positive control (Fig. 3).

The results obtained show that the higher the inhibition zone of microbial growth is the higher the antimicrobial activity of the tested substance is.

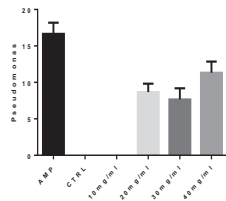
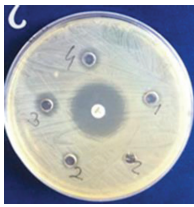


Figure 2. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *P. aeruginosa* and the graphical representation of the obtained values.

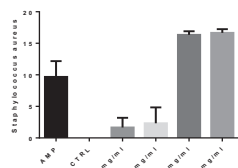
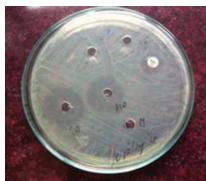


Figure 3. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *St. aureus* and the graphical representation of the obtained values.

General analysis of the recorded values when testing the inhibitory effect of casein on bacterial growth, have revealed that it is influenced by the bacterial strain introduced in testing and ranged from the minimum concentration of 20µg /mL and a maximum of 40µg/mL.

The results of our study bring new arguments in favour of supporting the complex functions exercised by milk proteins, which are essential not only in terms of nutrition or biologically active, but also in terms of antibacterial protection of new born and, by default, the consumer. According to the consulted bibliographic data, antibacterial action exerted by the lactic proteins, suggest the possibility of their use for therapeutic purposes, contribute to obtain functional foods and nutritional formulas for infants (Malkoski, 2001; Vajih, 2012).

In the context of the above, we should also mention other research, which proved that the antimicrobial activity can be attributed mainly to milk caseins which release the active peptides against various pathogens (McCann,

2006; Haque, 2008; Faiza et al., 2013). Thereby, we should mention that there are some of bioactive peptides which are not released *in vivo*. They are encrypted in major milk proteins and activated only by enzymatic proteolysis (De-Felice, 1995). They could receive commercial/trading forms assigned to the functional foods category for the prevention and therapy of diseases with microbial component (Hans, 1999; Schmidl, 1993). In this regard, we should remind the results of the preliminary study on the inhibitory effects of a β-casein hydrolysate towards *E. coli* (JM103), without the identification of the peptides responsible for this action (Gomez, 2005; 2006). Later on, four antibacterial peptides from a αs2-casein hydrolysate have been identified (Lopez, 2006). It is also known that antimicrobial peptides derived from milk proteins are generally active against Gram positive bacteria (Bellamy, 1992; Recio, 1999; Pellegrini, 1999; 2001; 2003; Faiza et al., 2013).

CONCLUSIONS

The results of our study revealed that total extracts of caseins from goat milk exert inhibition activity against *in vitro* development of various bacterial strains, such as *E. coli*, *P. aeruginosa* and *St. aureus*. Such extracts can also be an alternative solution for reducing the consequences of antibiotic resistance phenomenon and can assign a character of functional foods to dairy products.

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