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Bioactive proteins against pathogenic and spoilage bacteria

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ABSTRACT

Background: It is likely that both human nutrition and the nutrition of livestock are benefited by the presence of bioactive proteins within their respective diet regimes. Bioactive proteins have been defined as specific protein fragments that positively impact bodily functions or conditions and may, ultimately, influence overall human health. The ingestion of bioactive proteins may have an effect on the major body systems—namely, the cardiovascular, digestive, immune and nervous systems. According to their functional properties, bioactive proteins may be classified as antimicrobial, antithrombotic, antihypertensive, opioid, immune-modulatory, mineral binding and anti-oxidative. There are many examples of biologically active food proteins and active peptides that can be obtained from various food protein sources. They have a physiological significance beyond the pure nutritional requirements; in other wordsthey have the acquisition of nitrogen for normal growth and maintenance.

Objective: This study aims to specify and characterize the extent and mode of action of bioactive proteins in their native form, (glycinin, glycinin basic sub-unit and β-conglycinin) against specific main pathogens (*Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella enterica serovar Enteritidis*). We will be using standard media while identifying the main constituents responsible for this action.

Methods: Glycinin, basic sub-unit and β-conglycinin were isolated from soybean protein and tested for their antimicrobial action against pathogenic and spoilage bacteria, They were thencompared to the properties of penicillin. Methylated soybean protein and also methylated chickpea protein (MSP and MCP), with isoelectric points around pI 8, were prepared by esterifying. 83 % of their free carboxyl groups and their interactions with Gram positive and Gram negative bacteria were examined.

Results: The three divisions of cationic proteins exhibited antibacterial activities equivalent to or higher than the activity of penicillin, with the basic sub-unit exhibiting the highest activity,

followed by glycinin.; β-conglycinin exhibited the lowest level of activity with a MIC of 50, 100 and 1000 μg/mL, respectively. The $IC_{50\%}$ values of the basic subunit, glycinin and β-conglycinin, against *Listeria monocytogenes,* were 15, 16 and 695 μg/mL; against *Bacillus subtilis* the values were 17, 20, and 612 μg/mL; and against *Salmonella Enteritidis* the values were 18, 21 and 526 μg/mL, respectively. Transmission electron microscopy images of *L. monocytogenes* and *S. Enteritidis* exhibited an increase in cell size and a separation of the cell wall from the cell membrane when treated with glycinin or basic sub-unit. The scanning electron microscopy of *B. subtilis* indicated signs of an irregular, wrinkled outer surface as well as the fragmentation, adhesion, and aggregation of damaged cells or cellular debris when treated with glycinin or the basic subunits; however not with penicillin. The proliferation of *L. monocytogenes*, *S. Enteritidis* and *Escherichia coli* O157:H7-when artificially inoculated in raw milk ,stored at 4 or 25 °C) was significantly ($P<0·05$) reduced by the glycinin sub-unit and nisin (0·5% w/v); but they were only slightly reduced by β-conglycinin and moderately reduced by lysozyme. The two substances (MSP and MCP) exhibited a concentration-dependent inhibitory action against two of the studied bacteria with a minimum inhibitory concentration of approximately 100 μg/mL. The supplementation of raw milk with esterified legume proteins (MSP and MCP) has significantly ($p < 0.05$) reduced the levels of TBC, PBC and PSC in raw milk stored at a temperature of 4 °C. This potentially will delaythe onset of spoilage of by four days.

Conclusion: Both glycinin and the basic sub-unit have a more swift antimicrobial action than that of penicillin. Basic sub-units exhibited the highest efficiency at killing bacterial cells, followed by glycinin, penicillin and β-conglycinin-with the lowest effect; while the bacteria most susceptible to the antimicrobial agents were shown to be *L. monocytogenes*, followed by *B. Subtilis* and *S. Enteritidis*- with the lowest susceptibility. The antibacterial action of glycinin was similar to the effects exerted by nisin, and was much more effective than lysozyme. The modified legume proteins have general antibacterial properties against both spoilage and pathogenic bacteria in raw milk preserved under refrigeration or at room temperature.

Keywords: bioactive proteins, pathogenic and spoilage bacteria,

1. INTRODUCTION

Globulins represent the majority of seed soybean proteins and can be subdivided into two main types according to their sedimentation coefficients: glycinin (11S) and β-conglycinin (7S). Glycinin has a molecular mass of 360 KD and is composed of six constituent subunits, each of which consists of an acidic and a basic polypeptide. These polypeptides are linked together by a disulfide bond [1], [2].(The relative molecular masses of basic and acidic sub-units are 20 and 34 KD, respectively [3]. β-Conglycinin is a trimeric glycosylated protein with a molecular mass of 150–200 KD [4; 5]. The esterification reaction is an important tool for modifying food proteins. Esterification with different alcohols leads to the blocking of free carboxyl groups raising the net positive charge; this makes the modified proteins more basic[6-8] The glycinin and its basic subunit are both hydrophobic and cationic and may be able to react with the

bacterial cell wall and membrane in spite of its attachment to the acidic sub-units. Previous reports indicated that basic proteins or peptides can have antimicrobial activity [9]. Consequently, the isolation and purification of soy bean proteins, e.g. glycinin (11S), basic sub-unit and β-conglycinin, (7S) or chickpea proteins (e.g. 11S legumin and the 7S vicilin) was of major interest. Alternatively, the esterification of legume proteins may impart them with cationic character associated with antibacterial activity against pathogenic and spoilage bacteria. [10-17] (;. Hence, the objective of the current work was to specify and characterize the extent and mode of action of these antimicrobial cationic proteins (native and esterified) against pathogenic and spoilage bacteria.

2. METHODS

2.1. Legume Proteins: Isolation and Characterization

Soybean and chickpea seeds were ground to pass through a 1 mm2 sieve and the resulting powder was defatted using a mixed solvent of chloroform: methanol (3:1 v/v) for 8 h. Soybean protein isolate and chickpea protein isolate were separated using the procedure of Johnson and Brekke [18]. Soybean protein isolate was used for the isolation of glycinin and β-conglycinin, according to Nagano et al. [19]. Basic sub-units were separated from the glycinin according to methods described by Damodaran and Kinsella [20]; some modifications were used. Glycinin was dissolved in a 30 mM Tris buffer (pH 8.0) containing 15 mM β-mercaptoethanol (at 0.5% w/v). The protein solution was heated to 90 °C for 30 min and then centrifuged at 10000 x g at 4 °C for 20 min. The precipitate (basic subunit) was washed twice with 30 mM Tris buffer (pH 8.0), suspended in distilled water, and freeze-dried. Protein samples were analyzed by SDS-PAGE according to Laemmli [21].

2.2. Chemical modification of proteins

Protein was esterified with methanol according to the procedure of Sitohy et al. [22], and the esterification extent was quantified by the color reaction with hydroxylamine hydrochloride [23]. The resultant modified proteins were denoted as MSP (methylated soybean protein) and MCP (methylated chickpea protein)

Esterified proteins were analyzed by different methods such as SDS-PAGE, native PAGE according to Laemmli [21], and Urea-PAGE according to Williams and Evans [24].

2.3. Antibacterial action evaluation

2.3.1. *In vitro*

Different methods were used to evaluate the antibacterial activity against pathogenic bacteria (*Listeria monocytogenes* and *Salmonell*a *enterica* subsp *enterica* serovar Enteritidis) and spoilage bacteria (*Bacillus subtilis*). It was done *in vitro* as following: Minimum inhibitory concentration (MIC) was evaluated using standard inoculums of 1×10^5 CFU/ mL [25; 26], scanning electron microscopy (SEM) analysis was performed [27] The SEM was performed to further explore the mode of action of the studied proteins on *B. subtilis* cell morphology and transmission electron microscopy (TEM**)**

2.3.2. *In situ*

The activity of native and esterified legume proteins in controlling the growth of pathogenic and spoilage bacteria contaminating raw milk kept at 4 °C or 24-h room temperature storage (25 °C) as well as milk quality was assessed [10-12; 15; 17].

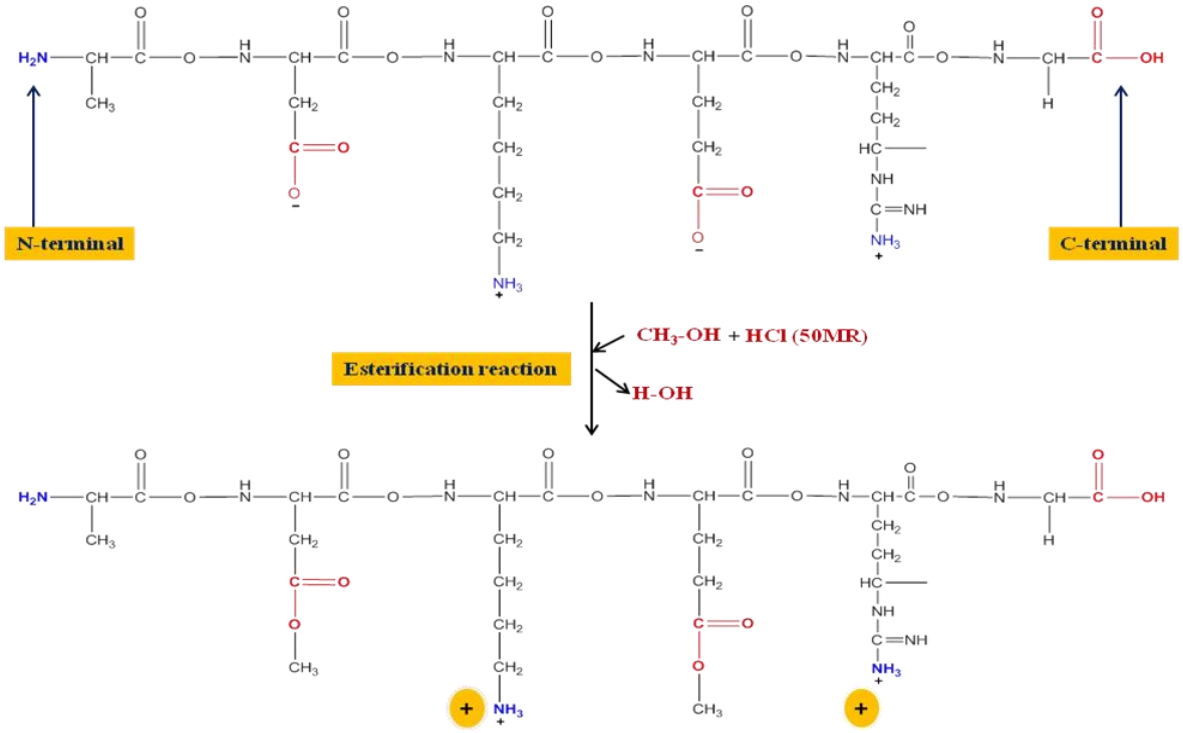

Figure 1: Esterification of legume protein by methanol

3. RESULTS

3. 1. Antibacterial Activity of Native Protein Fractions

A soybean protein isolate was fractionated into β-conglycinin, glycinin and its basic unit (Figure 2). All fractions were tested for their antimicrobial action against pathogenic (*Listeria monocytogenes* and *Salmonell*a *enterica* subsp *enterica* serovar Enteritidis) and spoilage bacteria (*Bacillus subtilis*), as compared to penicillin. The three fractions exhibited antibacterial activities equivalent to or higher than penicillin with the basic subunit exhibiting the greatest amount of activity.The subunit wasfollowed by glycinin and thenβ-conglycinin with a MIC of 50, 100 and 1000 µg/ml respectively. The $IC_{50\%}$ values of the basic subunit, glycinin and β -conglycinin against *L. monocytogenes* were 15, 16 and 695 µg/ mL; against *B. subtilis* the values were 17, 20, and 612 µg/mL; and against *S. Enteritidis* the values were 18, 21 and 526 µg/mL, respectively. The antibacterial action starts at an early stage of bacterial life cycle, i.e. maximum effect after 6 h of incubation at 37 ˚C. Glycinin and basic subunits were significantly more effective against *S. Enteritidis* than penicillin.

TEM images of *L. monocytogenes* and *S. Enteritidis* exhibited an increase in cell size and a separation of the cell wall from the cell membrane when treated with glycinin or a basic subunit. Cells treated with β-conglycinin were least affected while cells treated with penicillin showed fewer signs of deformation. SEM examination of *B. subtilis* (Figure 5) indicated signs of an

irregular, wrinkled outer surface.The SEM examination of B. subtilis also exhibited fragmentation, adhesion and aggregation of damaged cells or cellular debris. This occurred when treated with glycinin or the basic subunits; however it did not occur with penicillin.

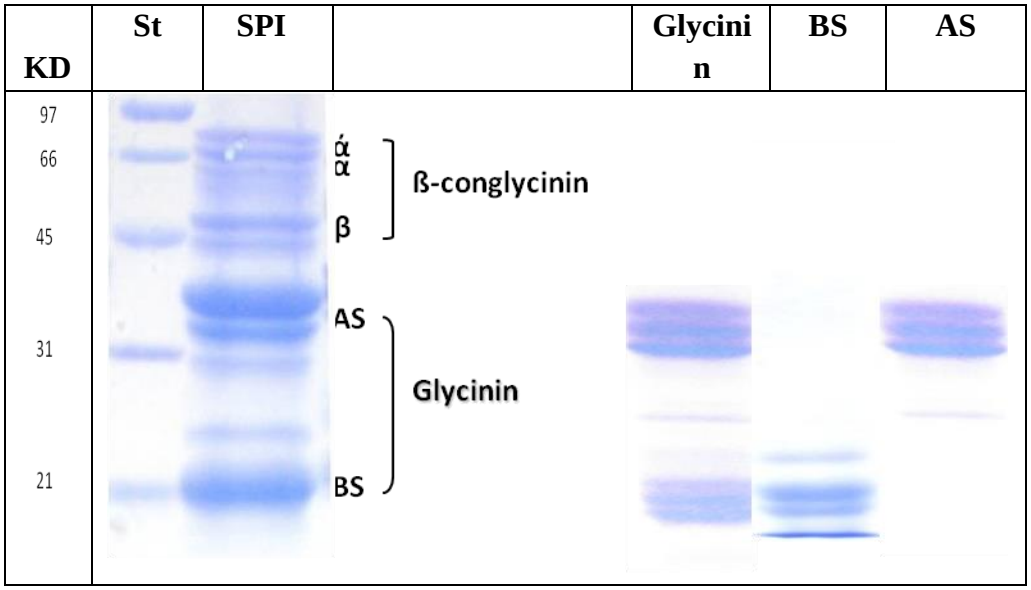

Figure 2. SDS-PAGE Electrophoretic patterns of soybean protein isolate (SPI), and its fractions; glycinin, basic and acidic subunits (BS and AS).

All tested substances showed increased concentration-dependent cell permeation, assessed by crystal violet uptakeThe basic subunit was the most active, followed by glycinin, and then penicillin. The kinetics of cell permeation were linked to the kinetics of cell lysis and the action on bacterial proteins. The antimicrobial action of glycinin and basic subunit was quicker than that of penicillin. The basic subunit exhibited the greatest efficiency at killing the bacterial cell, followed by glycinin, penicillin andβ–conglycinin (β–conglycinin with the lowest efficiency). The bacteria that was most susceptible to the antimicrobial agents was shown to be *L. monocytogenes*, followed by *B. Subtilis* and finally *S. Enteritidis*, the lowest susceptible one-. Adding glycinin and the basic subunit to pasteurized milk inoculated with the three bacteriae, *L. monocytogenes*, *B. Subtilis* and *S.* Enteritidis (ca. 5 log CFU/mL), could inhibit their propagation after 16-20 days of storage at 4 ˚C by 2.42-2.98, 4.25-4.77 and 2.57-3.01 log and by 3.22-3.78, 5.65-6.27 and 3.35-3.72 log CFU/mL, respectively [13].

Considerable inhibitory antibacterial action, comparable to the properties of nisin, was exerted by the soybean glycinin on the proliferation of total viable count, Pseudomonas count and Enterobacteriaceae count. This was done in bovine milk stored at 4 or 25 °C for 30 d and 48 h.; however 7S and lysozyme were much less effective. The maximum magnitudes of bacterial reduction by glycinin and nisin were in the range 2– 4 log CFU/ml. The proliferation of 3 pathogenic bacteria (*Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7) artificially inoculated into raw milk stored at 4 or 25 °C was significantly ($P<0{\cdot}05$) reduced by glycinin, subunit, and nisin (0·5% w/v). However, it was only slightly reduced by β–conglycinin and moderately by lysozyme. Lactose consumption, acidity development and casein

degradation during storage of bovine raw milk were attenuated during storage at 4 or 25 °C and sensorial traits were better maintained by supplementation with glycinin (0·5% w/v). As a result, glycinin may be recommended for use as a safe food preservative, if officially authorized [16].

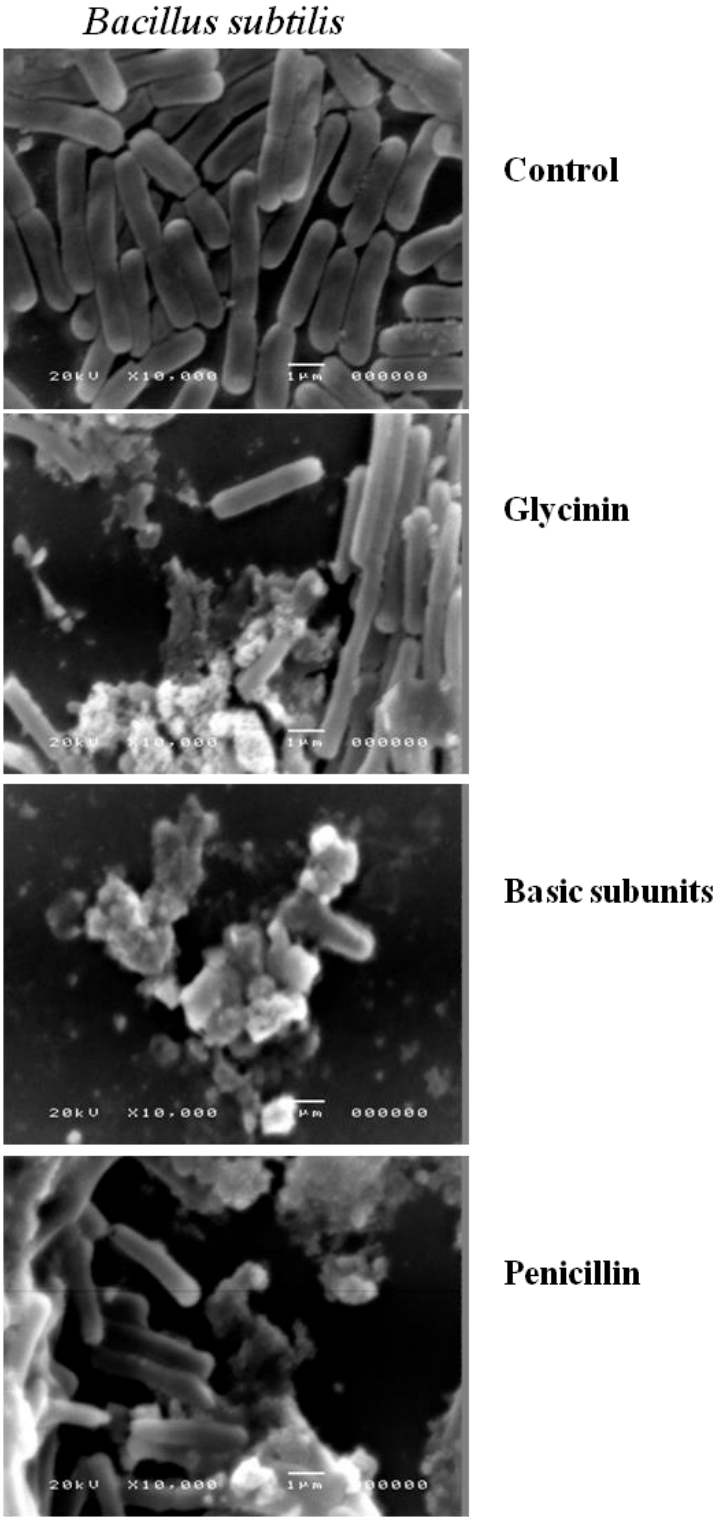

Figure 3. Scanning Electron microscopy (SEM) of B. subtilis treated with 100 µg/mL of glycinin, β-conglycinin, the basic subunit, and penicillin for 4 hrs. at room temperature

3.2. Antibacterial activity of chemically modified legume proteins

3.2.1. Protein modification

Although some protein subunit fractions are biologically active against bacteria, the whole mixture of legume proteins (e.g. soy protein isolate) is nearly free of significant antibacterial activity [13]. This is due to the fact that some active fractions are neutralized by other fractions. For example, the basic subunits are normally neutralized by the acidic subunits. Moreover, the antibacterial activities of the isolated subunits are sometime moderate. Alternatively, the fractionation of protein isolates into active subunits is costly and time consuming. Inactive legume protein isolates (soy protein isolate and chickpea protein isolate) were transformed into biologically active forms by esterification and tested against different bacteria as it will follow. Esterification is supposed to endow these modified proteins with positive charges and basic characters. Esterification can neutralize the negatively charged carboxyl groups of the aspartyl and glutamyl residues on protein molecules, transforming their net charge into positive. The obtained positively charged proteins were proved antimicrobially active [28] as it will be detailed. The methylated soybean protein and methylated chickpea protein (MSP and MCP) with isoelectric points around pI 8 were prepared by esterifying 83 % of their free carboxyl groups (Figure 4).

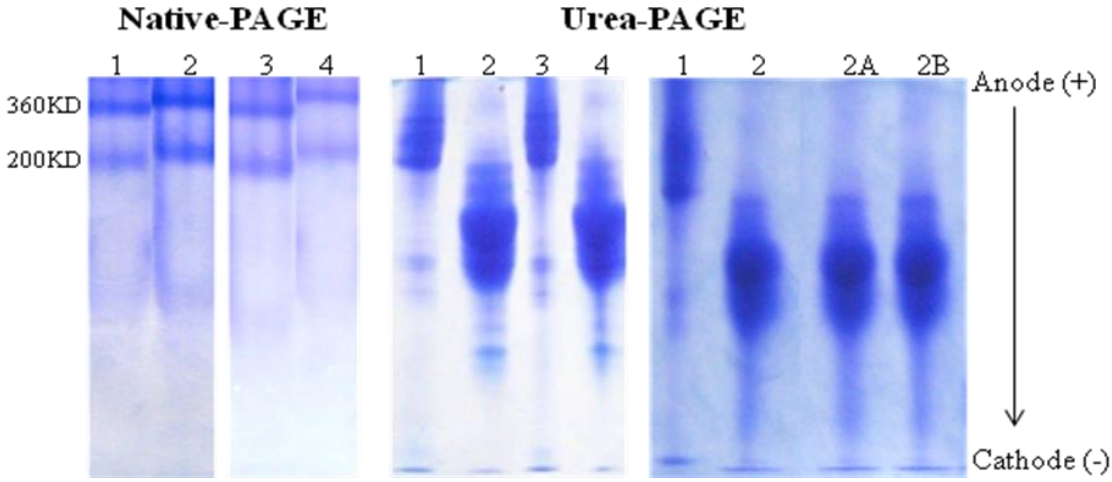

Figure 4. Native and Urea-PAGE of soybean and chickpea proteins before (1&3) and after (2&4) methylation as well as Urea-PAGE of the fractionated methylated.

3.2.2. *In vitro*

The interactions of MSP and MCP with *Listeria monocytogenes* and *Salmonella* Enteritidis were closely examined. The two substances exhibited a concentration-dependent inhibitory action against the two studied bacteria with a minimum inhibitory concentration of approximately 100 µg/mL. The IC_{50} % of the two proteins was comparable to penicillin against *L. monocytogenes* (17 µg/mL), but was also comparatively much lower (15 µlg/mL) than that of penicillin (85 µg/mL) against *S.* Enteritidis. The two proteins could inhibit the growth of *L.* monocytogenes and *S.* Enteritidis by about 97 and 91 %, respectively, after 6–12 h of incubation at 37 °C. The constituting subunits of MSP (methylated glycinin and methylated □-conglycinin) were both

responsible for its antimicrobial action. Transmission Electron Microscopy of the protein-treated bacteria showed various signs of cellular deformation (Figure 5). Cationic proteins can interact with the cell wall and the cell membrane by virtue of their positive electrostatic charges and hydrophobic character. This produces large pores and pore channels thatlead to the disintegration of the cell wall and cell membrane and enhance cell permeability. This will in turn lead to cell emptiness, lysis and death [14].

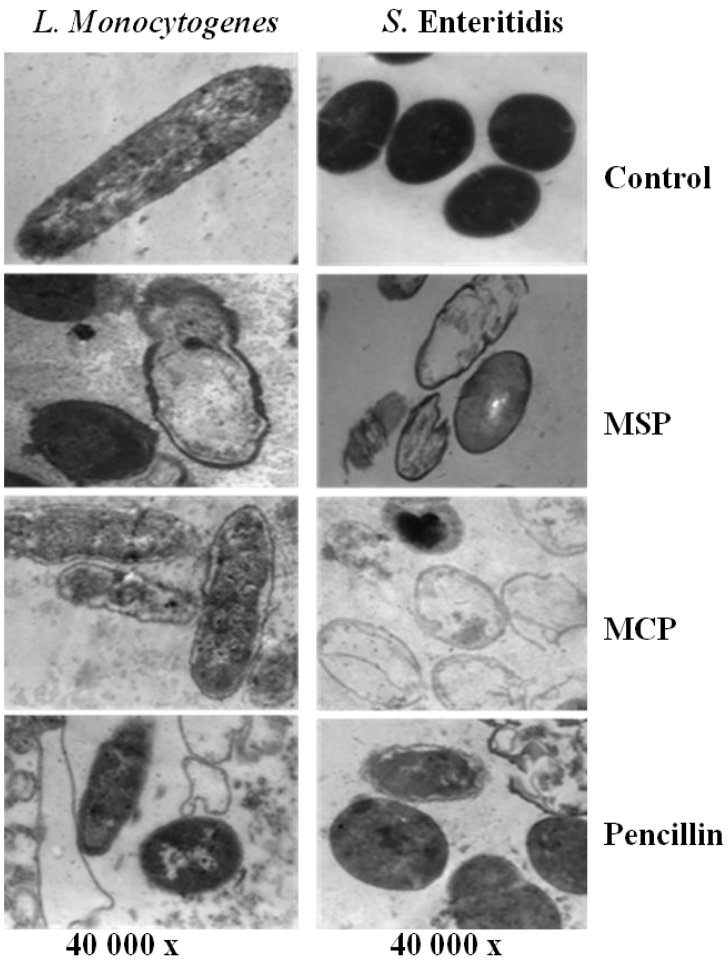

Figure 5. Transmission Electron Microscopy (TEM) of *L. monocytogenes* Scott A and *S.* Enteritidis PT4 as affected by 100 µg/mL of cationic soybean or chickpea protein (MSP and MCP), as compared to penicillin.

Esterification of legume proteins turns them positively charged and hence exhibits outstanding anti-Listeria and anti-Salmonella actions. This action turns the net charge of □-conglycinin from negative into positive while it intensifies the positive charge on glycinin. This modification eliminates the electrostatic interactions between these two subunits, allowing the whole protein to exert antibacterial action. The current biotechnological technique can provide antimicrobially active cationic proteins. These prepared mixtures of cationic proteins can be invested in the antimicrobial applications without the need to use costly and time-consuming procedures for isolating the active protein component (glycinin). The antimicrobial action of the

cationic proteins may be initiated by an electrostatic interaction between their positively charged regions and the negatively charged regions of the cell wall or the cell membrane accompanied by a hydrophobic interaction between like regions of the two reactants.

3.2.3. *In Situ*

Supplementation of raw milk with esterified legume proteins (Methylated Soybean Protein and Methylated Chickpea Protein) has significantly ($p < 0.05$) reduced the levels of TBC, PBC and PSC in raw milk preserved at 4 °C, i.e. it could potentially delay the start of spoilage from the second day to the sixth day. This antimicrobial action against psychrotrophic bacteria primarily originates from the chemical esterification altering the protein net charge to positive an. Thus, being capable of interacting with the negatively charged components of the bacterial cell wall and membrane, it leads to their disintegration and finally bacterial inhibition. Supplementing raw milk with methylated legume proteins could significantly ($p < 0.05$) limit the changes in the pH and the titratable acidity in raw milk stored under cold conditions. This could also protect casein from degradation for a longer period (10 days) of preservation at 4 °C, as compared to 2 days in the case of the control. Collectively, it can be concluded that esterified proteins can generally inhibit the bacterial growth and its associated activities: acid production and proteolysis [12].

Protein isolates from the soybean and chickpea, as well as their methylated esters, were tested for their inhibitory action against the propagation of pathogenic bacteria in raw milk during its storage at room temperature or under refrigeration. Raw milk was inoculated with a mixed culture of *Listeria monocytogenes* Scott A and *Salmonella enterica serovar* Enteritidis strain PT4 at ca. 2 log CFU ml^{-1}. Aerobic plate count, coliform count, and presumptive E. coli in raw milk treated with esterified legume proteins were inhibited by 2 to 3 log relative to a control after 6 to 8 days of storage at 4°C. At room temperature, bacterial populations (aerobic plate count, coliform count, and presumptive *E. coli*) in raw milk, treated with esterified legume proteins, were inhibited by ca. 1.5 to 1.6 log relative to the control after 12 h. Supplementation of raw milk with esterified soybean protein could significantly inhibit the counts of the two inoculated pathogens (*L. monocytogenes* Scott A and *Salmonella* Enteritidis PT4), initially inoculated at ca. 2 log CFU ml^{-1}, by ca. 2.4 log and 1.6 log CFU ml^{-1}, respectively, on day eight of storage under cold conditions. Corresponding reductions amounting to 2.7 and 1.8 log CFU ml^{-1} were observed after 12 h of storage at room temperature. Supplementation of raw milk with esterified soybean protein (0.5%) reduced the maximum level of titratable acidity to 0.21 and maintained the pH level at 6.4, after 8 days of storage under cold conditions as compared with 4 days for untreated raw milk. Similar results were observed when raw milk was stored at room temperature for 10 h [10] (.

Methylated soy protein (MSP) was evaluated as an antimicrobial agent that can counteract the potential post-pasteurization contamination of milk during a 30-day cold storage (4 °C) or 24-h room temperature storage (25 °C)- as compared to its native form (SP) [10] SP and MSP were added to buffalo milk at 0.5% (w/v) after pasteurization and before storage. Microbiological and chemical analyses of the milk were carried out directly to follow the spontaneous microbial contamination or after artificial contamination with three pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes* Scott A, and *Salmonella enterica subsp.*

enterica serovar Enteritidis PT4). Supplementation of milk samples with MSP (0.5%) significantly ($p<0.05$) and considerably inhibited the levels of the spontaneously proliferating bacterial counts, i.e., total viable and Enterobacteriaceae counts were inhibited by about 2.5 log CFU mL^{-1}.However; psychrotrophic and pseudomonads counts were inhibited by 1.8 and 1.6 log CFU mL^{-1}, respectively, after 16 days of preservation at 4 °C. Similar trends of effects were also produced after 12–18 h of milk storage at 25 °C. MSP (0.5%) nearly hindered the proliferation of the three inoculated pathogens in pasteurized milk during 16 day of storage at 4 °C or 12–18-h storage at 25 °C. Based on milk acidity, SDS–PAGE pattern, and microbiological analysis, it is concluded that supplementation with MSP (0.5%) can prolong the shelf life of pasteurized milk from 6 to 16 days when stored under cold conditions and from 8 to 18 h under room temperature conditions. Methylated soy protein (MSP) is a potent antimicrobial agent that can counteract the potential post-pasteurization contamination of milk during cold (4 °C) or room temperature (25 °C) storage. Supplementation of milk samples with MSP (0.5% w/v) significantly ($p<0.05$) and considerably inhibited the levels of the spontaneously proliferating spoilage bacterial counts, i.e., TVC, PBC, PSC, and ENC counts, after 16 days of preservation at 4 °C or after 12–18 h of storage at 25 °C. In parallel, supplementation of pasteurized milk with MSP nearly hindered the proliferation of three inoculated pathogens during 16 days of storage at 4 °C or during 12–18 h of storage at 25 °C.

It is concluded that supplementation with MSP (0.5%) can prolong the shelf life of pasteurized milk from 6 to 16 days when stored under cold conditions and from 8 to 18 h when stored at room temperature based on milk total bacterial count, acidity, and pH measurements- as well as the intact status of milk proteins visualized by SDS electrophoresis [14].

Methylated chickpea protein or a native chickpea protein was supplemented to milk (0.5 %) and combined with a mild thermization treatment (65 ºC/ 5 min) before storing at 4 ºC for 30 days. The influence of these combined treatments was assessed on milk physicochemical, nutritional and sensorial quality during storage. Supplementation of milk samples with MCP (0.5% w/v) significantly ($p < 0.05$) and considerably reduced the levels of the bacterial counts; i.e. total bacterial, psychrotrophic and Pseudomonas spp. counts by about 1.6-1.9 log CFU ml^{-1} ,after 16 days of storage at 4 ºC. Within the same period, it could control the development of titratable acidity, limit lypolysis & proteolysis, maintain most of the vitamin contents, and keep considerable heat stability, oxidative stability, rennetablity and sensorial properties [17].

CONCLUSIONS

Both glycinin and the basic subunit have a more swift antimicrobial action than that of penicillin. The basic subunit exhibited the highest efficiency at killing bacterial cells followed by glycinin, penicillin, and β-conglycinin with the lowest while the bacteria that was most susceptible to the antimicrobial agents was shown to be *L. monocytogenes*, followed by *B. Subtilis* and *S. Enteritidis*, with the lowest susceptibility. The antibacterial action of glycinin against these three groups of contaminating bacteria was similar to the effects exerted by nisin and was much more effective than lysozyme (data not shown). Chemical esterification can turn the originally inactive forms of legume proteins into biologically active forms against pathogenic and spoilage bacteria. Furthermore, esterification can enhance the antibacterial activity of the originally active sub-

units (e.g. glycinin). Both active legume subunits (glycinin and its basic subunits) as well as the esterified legume proteins have general antibacterial properties against both spoilage and pathogenic bacteria in raw milk, preserved under refrigeration or at room temperature, and thus can be used as safe food grade preservatives.

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