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Effect of differential heat treatments on antibacterial activity of fermented goat milk#

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Abstract

The present study was conducted to evaluate the effect of three different temperatures on the antibacterial potential of fermented goat milk.Goat milk is one of the naturalfoods to complete perfection.Bioactive peptides, the specific protein fragments that have a positive impact on body functions, can be produced during milk fermentation. Thanks to the starter cultures' proteolytic activity.Fresh pooled goat milk sample collected was divided into four lots. One set was used for raw milk analysis and the remaining three were subjected to heat treatment at three different time-temperature combinations namely;72°C for 15 sec, 85°C for 10 min, and 121° C for 15 min. These samples were cooled immediately to 42ºC, inoculated with 0.04 % DVS yogurt culture,incubated at 42º C for 4 hrs, and then stored at 5±2°C for 21 days for storage studies. The analysis was carried out at 7-day intervals. An agar well diffusion assay was performed to assess the inhibitory activity ofWater-soluble peptide extracts (WSPE) of these samples against Bacillus cereus ATCC 10876, Escherichia coli NCIM 2685, Salmonella enterica ATCC 6017, and Staphylococcus aureus ATCC 25923. The milk that was heated to 85°C for 10 min before fermentation showed the highest antibacterial activity against E.coli during the 7th and 14th day of storage, withthe zone of inhibition measuredat 17±0.433mm and 21±0.55mm respectively.Thus, it can be concluded that milk heated at 85°C for 10 min had better antibacterial activity than the other two treatments.

Keywords: *Goat milk, heat treatment, antibacterial, bioactive peptides*

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The conventional view of milk's function has significantly broadened beyond justmeetingthe nutritional needs of babies. Today, milk proteins are regarded as the most significant source of bioactive peptides, and a significant number of bioactive peptides have been found in fermented dairy products and milk protein hydrolysates. Due to consumers' growing knowledge of the beneficial relationship between functional foods and health, the market for bioactive peptides has been growing rapidly.

Bioactive peptides (BAPs) are described as specific protein fragments that positively affect bodily processes or conditions and may ultimately have an impact on health (Kitts and Weiler,2003). These peptides are inactive inside the parent protein's sequence. Usually consisting of 2 to 20 amino acids, milk-derived bioactive peptides become active once they are released from the precursor protein. They can be released in the following ways: (a) proteolysis by enzymes derived from microorganisms or plants, (b) fermentation of milk with proteolytic starter cultures, and (c) enzymatic hydrolysis by digestive enzymes (Korhonen and Pihlanto, 2007).The cost-effective approach for producing BAP is microbial fermentation (Dalirie*t al.,* 2017), which is currently widely used in the dairy industry for the functionalization of milk products and byproducts(Hafeez *et al.,* 2014).

Fermented goat milk products have been demonstrated to have significant health benefits, including being anti-allergenic, probiotic, and anti-carcinogenic. Fermentation is known to enhance the therapeutic characteristics of dairy products. Additionally, it had been demonstrated that the nutritional content of goat milk rose during fermentation and that it lost its distinctively "goaty" flavour, which many consumers found to be unappealing (Slacanac*et al.,* 2010). By utilising the development of specific microbes in food as a benefit, fermentation was also early shown to be an excellent technique of preservation. It had been demonstrated that the fermenting procedure increased dairy products' nutritional properties (Slacanac*et al.,* 2004).

One of the most popular processing techniques used in the dairy sector is heat treatment, which can eliminate bacteria from raw milk and increase product shelf life and food safety (McKinnon *et al.,* 2009; Claeys *et al.,* 2013). When milk is heated, whey proteins get denatured and associate with casein micelles by intermolecular -SH/S-S interchange reactions (Considine *et al.,* 2007). The amount of denatured whey proteins that interact with the casein micelle during heating from 75 to 100 °C for up to 60 min causes the micelle to enlarge (Anema and Li, 2003); however, the rate of whey protein denaturation is faster than the rate of interaction of the denatured whey protein with the κ-CN on the micelles. In addition, the rate of whey proteins interacting with the micelles gradually increases over the course of heating at temperatures between 75 and 85°C.The rate of interactions between whey proteins and casein micelles is rapid between 90 and 100 °C but slows down after prolonged heating to >100 °C (Anema and Li, 2003). Once β-Lg is denatured, revealing its free -SH group, β-Lg interacts with κ-CN. In the initial stages of their contact, β-Lg and κ-CN engage in both hydrophobic and S-S interactions; however, the mechanism of the interaction is heavily reliant on the protein system and heating conditions (Cho *et al.,*2003). Denatured β-Lg initially links with κ-CN through hydrophobic bonding but eventually forms bonds through -SH/S-S interchange reaction.Furthermore, interactions between α-La and κ-CN are possible if a free reactive -SH group is available, following the formation of β-Lg–α-La (Anema and Li, 2003).

Lactoferrin, a minor whey protein, is primarily linked to milk's antibacterial properties. Due to its capacity to chelate iron or attach to bacterial surfaces, this protein exhibited bacteriostatic and bactericidal characteristics. According to Tomita *et al.* (1994), pepsin digestion of bovine lactoferrin yields a powerful bactericidal peptide, and the hydrolysate's antimicrobial effectiveness is greater than that of lactoferrin which has not been digested. Goat's milk contains milk proteins with antibacterial properties, including lactoferrin, immunoglobulins, and lactoperoxidase. Milk's quality would be enhanced by high amounts of lactoferrin, especially in terms of microbiological

quality and milk's usefulness as a functional food. Goat milk is broken down by the gastric enzyme pepsin to produce peptides that are resistant to gram-negative bacteria (Slacanac*et al.,* 2004; Park *et al.,* 2007). Compared to fermented cow milk, fermented goat milk also displayed antibacterial action against *Serratiamarcesens* (Slacanac*et al.,* 2004).Antimicrobial peptides α-S2-CN f183-207 and f164-179 that inhibited both Gram-positive and Gram-negative bacteria were discovered in goat milk by Atanasova and Ivanova in 2010.

Even though the bioactivity of goat milk proteins attracted the attention of many scientists, there is no information in the literature about how thermal processing affects the bioactivity of peptides produced during the fermentation of goat milk, despite the fact that goat milk proteins are more sensitive to heat treatment. Therefore, this project is aimed to study the effect of differential heat treatments on the antimicrobial properties of biopeptides in fermented goat milk.

Materials and methods

Collection of milk

Fresh pooled goat milk sample was collected from Government Goat Farm, Parassala, Trivandrum. It was immediately transported to the laboratory and was divided into four lots. One set was used for raw milk analysis. The remaining three were subjected to three different time-temperature combinations: Treatment A: 72°C for 15 sec, Treatment B: 85°C for 10 min, and Treatment C: 121°C for 15 min.

Preparation of sample

The heated samples were cooled immediately to 42°C and then inoculated with 0.04 % yogurt culture (YO MIX 883 LYO 50 DCU, Danisco). The inoculated milk was then transferred to sterilized plastic cups and incubated at 42°C until pH reached 4.6. The samples were then stored at 5±2ºC for storage studies for 21 days. The antibacterial analysis was carried out on the 0th day, the 7th day, the 14th day, and the 21st day of storage.

Preparation of water-soluble peptide extracts

Water-soluble peptide extracts (WSPE) were prepared by high-speed centrifugation of fermented samples as described by Sah *et al.* (2016). The samples were centrifuged at 16,099g using a REMI C-24 plus high-performance refrigerated centrifuge at 4°C for 30 min. The supernatant was collected and freeze-dried using ANM-FD 80 Freeze dryer and stored at -20°C until further analysis.

Determination of antibacterial activity

An agar well diffusion assay was performed to assess the inhibitory activity of WSPE against target strains (*Bacillus cereus* ATCC 10876*, Escherichia coli* NCIM 2685*, Salmonella enterica* ATCC 6017 and *Staphylococcus aureus* ATCC 25923) as described by Vieira *et al.* (2014) with some modifications. Briefly, 100 µL of an overnight culture of the test organism was spread on nutrient agar plates. Wells (6mm in diameter) were made in agar using a sterilized stainless-steel borer. Each well was filled with 100 µL of sterilized WSPE in Phosphate Buffer Solution (PBS) (NaCl =8.475 g/L, Na_2HPO_4 =1.093g/L, and NaH_2PO_4 = 0.276g/L; pH 7.4; 500µg of protein /mL). The plates were left at 4˚C for 4h to allow peptide diffusion in the medium and then incubated aerobically at 37˚C overnight. Subsequently, the diameter of inhibition zones in mm (including the well) was measured. Streptomycin disc was used as a positive control and PBS was used as a negative control.

The morphological changes induced by the WSPE on *E. coli* ATCC 8739 were studied using scanning electron microscopy as described by Zhao*et al.* (2015) with some modifications. Briefly, 300 µL of suspension of log-phase tested bacteria in Nutrient broth (optical density at 600 nm of ~0.1) was treated with 600 µL of sterilized WSPE sample (at 500 µg of protein/mL in PBS) in a sterile 1.5mL Eppendorf tube and incubated for 6 h at 37°C. After incubation, cells were washed twice with sterile PBS and pelleted by centrifugation (16,099 × g, 2 min). The pellet was washed thrice with sterile Milli-Q water and followed by an ethanolic wash with ascending concentrations

of aqueous ethanol solution (25%, 50%, 75%, 90%, and 100% for 10 min each), and dried. Finally, the cell pellet was directly mounted on aluminium scanning electron microscopy stubs, air-dried overnight at room temperature in a bio-safety cabinet, and sputtered with gold (~18 nm) using a Quorum sputter coater (model SC-7620). Scanning electron micrographs of the specimen were recorded using Tescan Vega 3 LMU scanning electron microscope.

Result and discussion

Skim milk of unfermented milk and fermented milk supernatant (WSPF) were tested for antibacterial activity against four identified pathogens (*B. Cereus* ATCC 10876*, E. coli* NCIM 2685, *S. enterica*ATCC 6017, and *S. aureus* ATCC 25923).From Table 1, Fig. 1, Fig.2, Fig.3 and Fig.4, it is clear that skim milk from goat milk heated at 85°C /10 min and WSPF obtained from the fermented milk heated at 85°C /10 min showed antimicrobial activity against *E. coli* NCIM 2685.

Depending on the temperature and time, heat treatment may alter the structure of milk proteins. Caseins and whey proteins can interact with one another. Whey protein denaturation occurs during heat treatment at temperatures above 60°C, and this is followed by the production of whey protein polymers, either alone or in combination with casein. When milk was heated at 95°C for 10 minutes, nearly all of the whey protein was denatured (Qian *et al.,*2017).The whey protein denaturation ranges from 81.2% under treatment at 85°C for 2 min (Joyce *et al.,* 2017) and complete denaturation occurred under treatment at 85°C for 30 min (Zhao *et al.,*2020). The complex reaction occurs around 121˚C.When heated to 121 °C, the lysine content was reduced by 21% and there was noticeable browning. Similar amounts of lysine are present in both human and bovine milk proteins; hence this reduction may noticeably degrade the milk's protein nutritional quality (Kilshaw*et al.,* 1982). In this study, the maximum antibacterial activity was observed in the case of 85°C/10 min, rather than 72°C/15sec and 121°C/15min. The above-said reasons regarding whey protein denaturation could explain this fact. Arun (2016) studied the antibacterial activity of goatmilk fermented with *Lactobacillus* strains and reported antimicrobial activity against *E.coli, B.cereus, S.typhi, and E.faecalis.* Contrary to this the activity was observed only in the case of *E.coli* in this study. This may be because of differences in goat breed, stage of lactation, anddifference in the strain of pathogen used for the study.

Table 1. Antibacterial activity of the skim milk of unfermented milk and WSPE of fermented milk duringstorage (inhibition zone in mm).

Pathogens	Samples	Unfermented milk*	Fermented goat milk during the storage period (days)*			
			0th day	7th day	14th day	21st day
Bacillus cereus	72°C/15c	Abs.	Abs.	Abs.	Abs.	Abs.
	85°C /10min	Abs.	Abs.	Abs.	Abs.	Abs.
	121°C /15min	Abs.	Abs.	Abs.	Abs.	Abs.
Escherichia coli	72°C/15sec	Abs.	Abs.	Abs.	Abs.	Abs.
	85°C /10min	**12 ± 2.00mm**	**14 ± 0.55mm**	**17±0.433**	**21 ± 0.55 mm**	**15 ± 0.5mm**
	121°C /15min	Abs.	Abs.	Abs.	Abs.	Abs.
Staphylococcus aureus	72°C/15sec	Abs.	Abs.	Abs.	Abs.	Abs.
	85°C /10min	Abs.	Abs.	Abs.	Abs.	Abs.
	121°C /15min	Abs.	Abs.	Abs.	Abs.	Abs.
Salmonella enterica	72°C/15sec	Abs.	Abs.	Abs.	Abs.	Abs.
	85°C /10min	Abs.	Abs.	Abs.	Abs.	Abs.
	121°C /15min	Abs.	Abs.	Abs.	Abs.	Abs.

*Zone of inhibition includes 6mm well diameter as well, Figures are mean ± standard deviation of triplicate analyses.

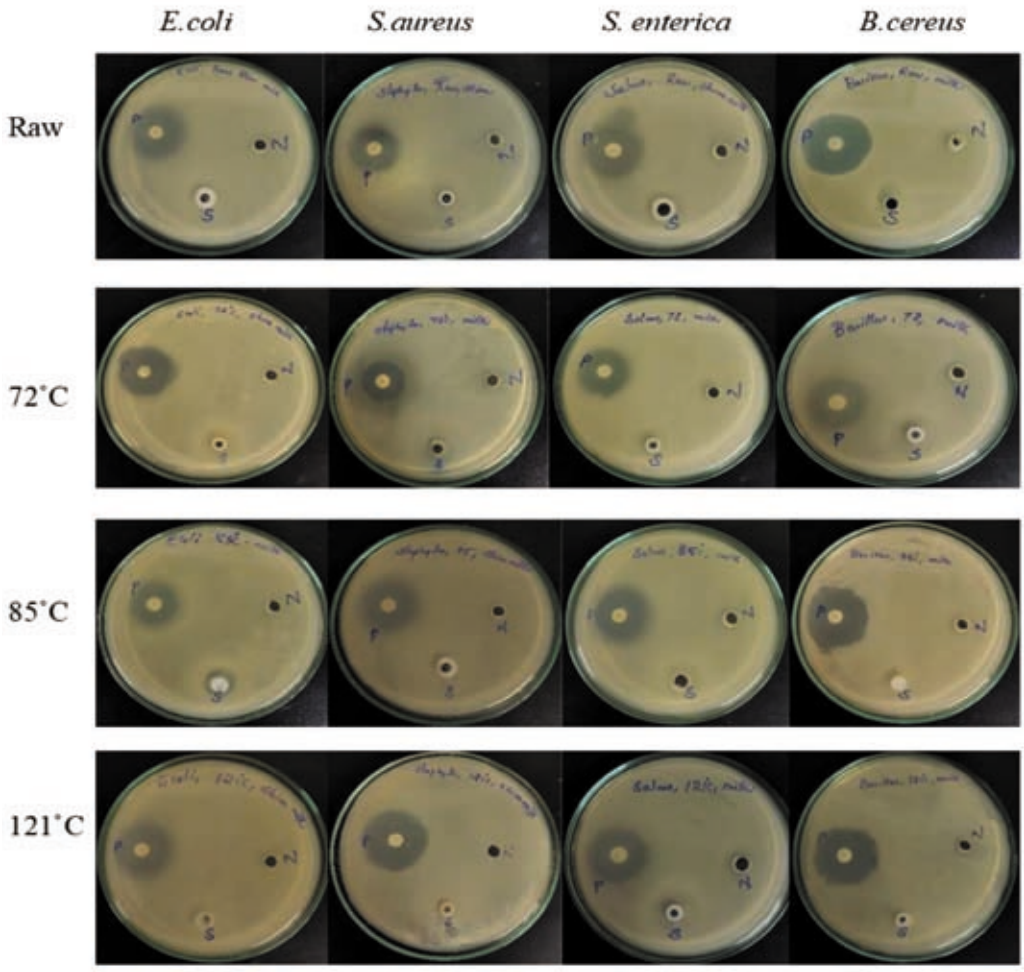

Fig.1. Antibacterial activity of the skim milk made from goat milk (raw, 72°C/15sec, 85°C/10min, 121°C /15min.) P-positive control, N-negative control, S-sample.

Since there is no obvious pattern for the samples with the presence of a particular peptide and antimicrobial activity, the different behaviours of the samples against different strains allow us to speculate that the peptides can act against only some of the bacteria and that they can also have some synergistic effect. The majority of antimicrobial active peptides can either penetrate microbial membrane integrity and damage it or translocate across the membrane and operate on interior targets Sah *et al.* (2016). The strongest inhibitory effect is shown in low molecular mass peptides that are generated during fermentation. According to their proteolytic activity, *S. thermophilus* strains were able to release peptides from the casein, some of which had been categorized as antimicrobial peptides, according to Quiros*et al.* (2005) and Galia*et al.* (2009). Similar to this, a lot of peptides were produced when *L. bulgaricus* degraded β-casein and αS1-casein (Miclo*et al.,* 2012).

SEM (Scanning electron microscopy) was used to examine the bacterial morphology of *E. coli* NCIM 2685 following treatment with WSPE. The findings showed that the peptides had anti-microbial cell membrane-lytic properties (Fig. 5).

There were no bacterial cells, and further, there is the appearance of fibrous material, which is probably the product of

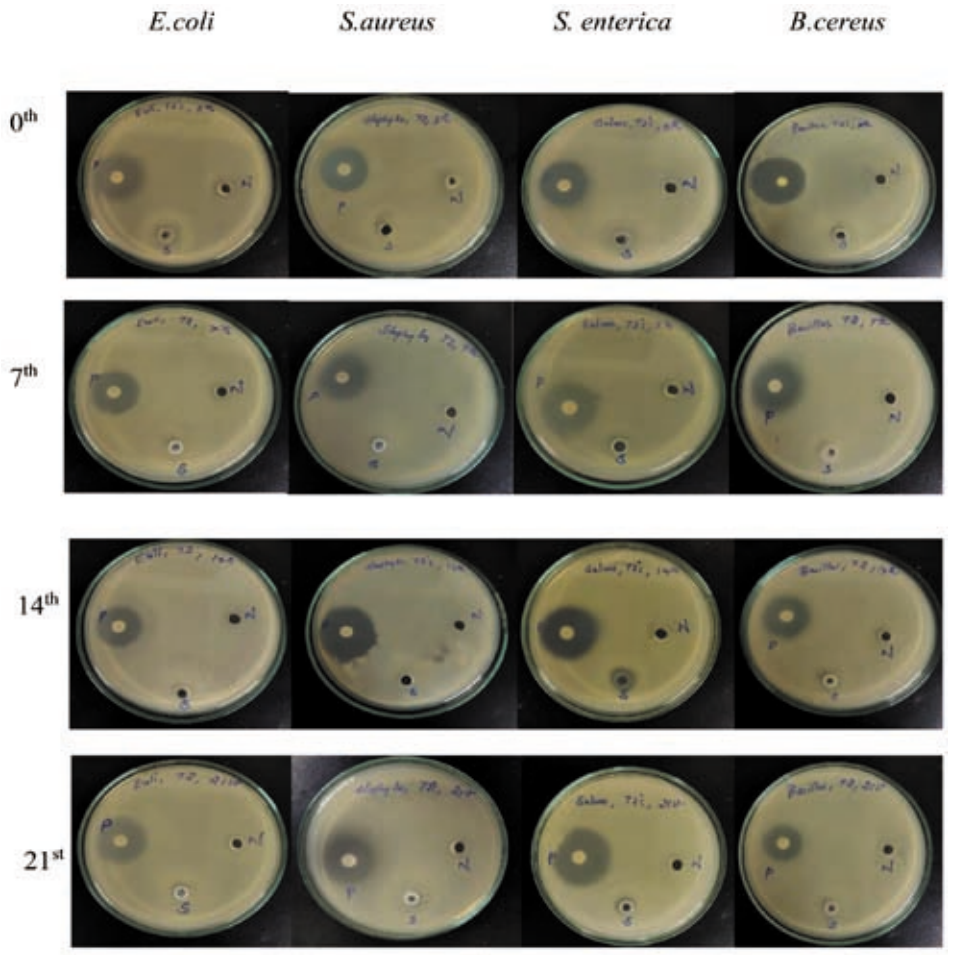

Fig.2. Antibacterial activity of the WSPE of fermented milk made from goat milk heated to **72°C/15sec** against different pathogens during cold storage (inhibition zone in mm). P-positive control, N-negative control, S-sample

cell lysis, cell leakage, and cell detritus, as shown in Fig. 5. This could happen as a result of cationic peptides interacting with anionic lipopolysaccharides in the cell membrane of gram-negative bacteria (Yeaman and Yount, 2003; Hoskin and Rama Moorthy, 2008). Furthermore, because these ions are essential to maintaining the integrity of the outer membrane, these peptides can also displace divalent cations like Ca^{2+} and Mg^{2+}, leading to the deformation of the outer membrane bilayer. Consequently, the membrane shatters, killing the cell.

Conclusion

From the study, it can be concluded that the fermentation of goat milk heated to 72°C for 15 sec, 85°C for 10 min, and 121° C for 15 min releases several bioactive peptides and amino acids. Among them, bioactive peptides from goat milk heated to 85°C for 10 minshowed antibacterial activity against *E. coli* NCIM 2685. Further studies are needed to isolate the antibacterial components in fermented goat milk and to utilize its therapeutic potential in pharmaceutical formulations.

Acknowledgement

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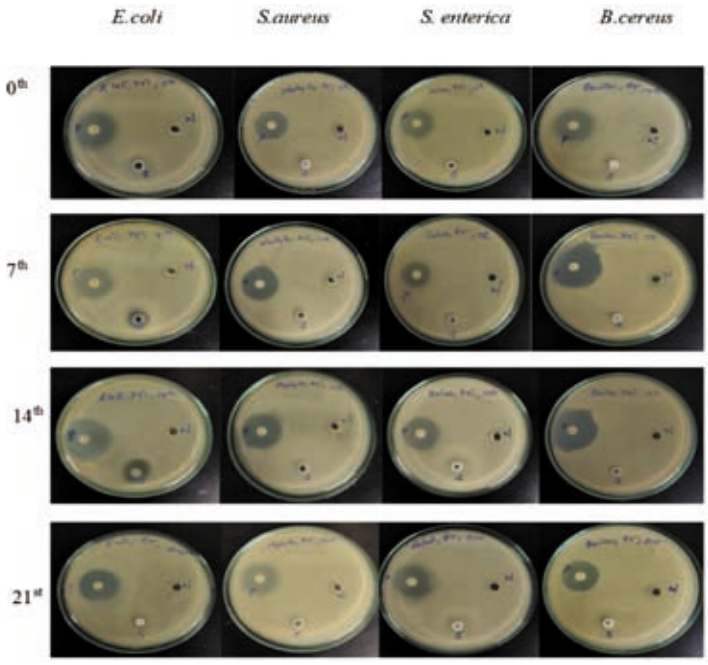

Fig. 3. Antibacterial activity of the WSPE of fermented milk made from goat milk heated to **85°C/10min** against different pathogens during cold storage (inhibition zone in mm). P-positive control, N-negative control, S-sample

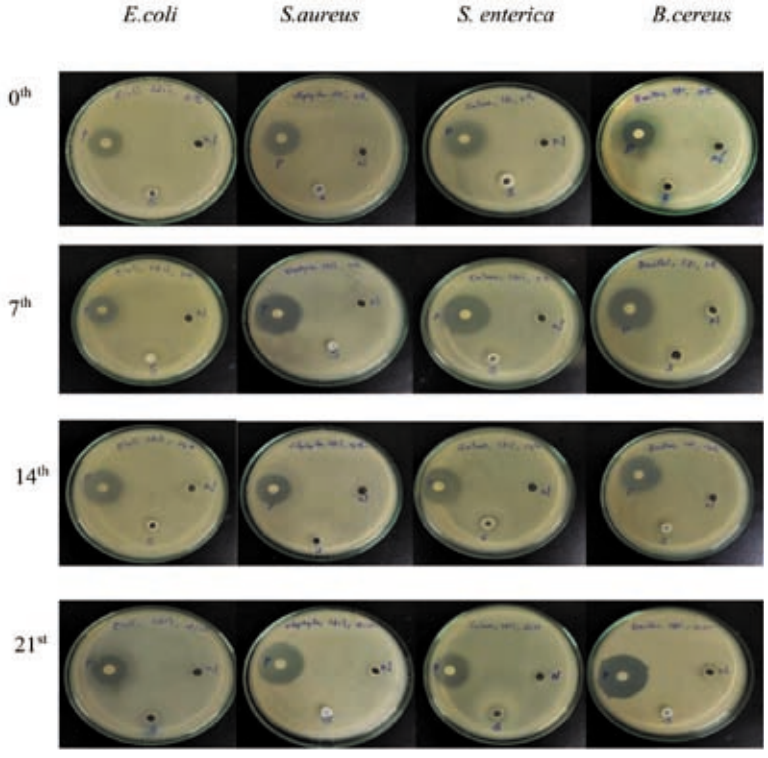

Fig.4. Antibacterial activity of the WSPE of fermented milk made from goat milk heated to **121°C/15sec** against different pathogens during cold storage (inhibition zone in mm). P-positive control, N-negative control, S-sample

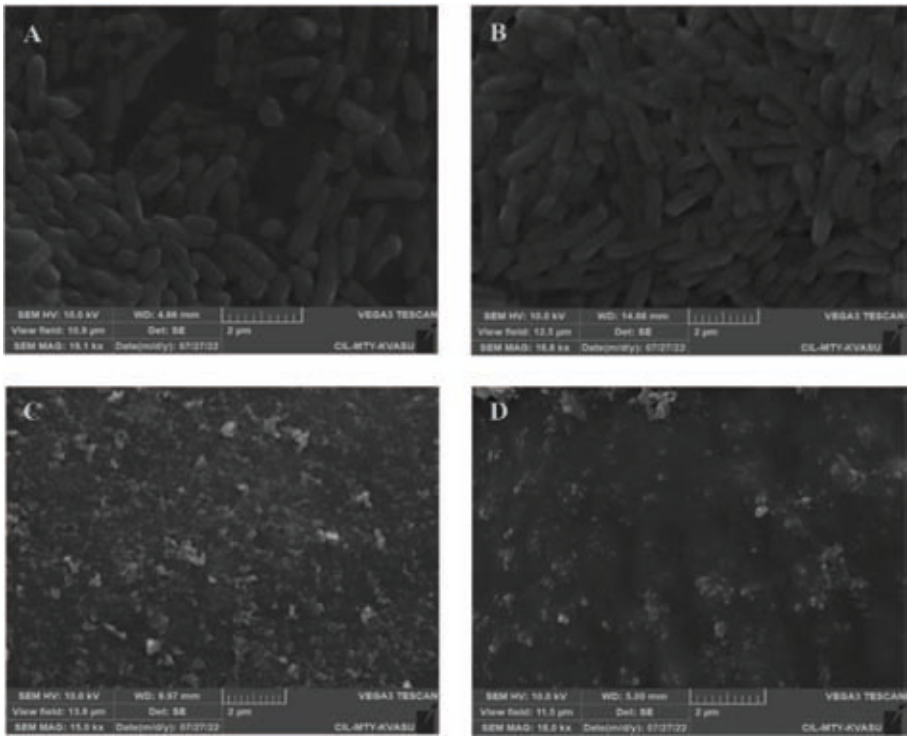

Fig. 5. Scanning electron micrographs showing morphological changes of Escherichia coli (A, B- untreated control cells; C- cells treated with WSPE of 7th day fermented milk made from goat milk heated to 85°C /10min; D- cells treated with WSPE of 14th day fermented milk made from goat milk heated to 85°C /10min) induced by treating for 6 hours at 37°C with WSPEs.

Conflict of interest

The authors declare that they have no conflict of interest.

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