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Effect of fermentation on antibacterial activity of milk from Vechur and Kasargod Dwarf cattle

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Abstract

The search for alternative therapeutics is on the rise on account of widespread bacterial resistance to numerous conventional antibiotics. Bioactive peptides produced by lactic acid bacteria found in natural sources such as fermented dairy products have attracted attention as a possible source of biotherapeutic peptides. Bioactive peptides released in fermented milk, on the other hand, is dependent on the proteolytic activities of the cultures used. As a result, this study was carried out to evaluate the effect of fermentation on antibacterial activity of milk from Vechur and Kasargod Dwarf cattle against pathogenic bacteria during 21-days storage period at 5°C. High-speed centrifugation was used to create water-soluble crude peptide extracts. The water-soluble peptides from Kasargod Dwarf and Vechur cattle by fermentation showed maximum inhibition against Escherichia coli NCIM2685 and Salmonella enterica ATCC 6017. The zone of inhibition measured ranges from 5mm to 11mm throughout the storage period. However, both did not show zone of inhibition against Bacillus cereus ATCC 10876 and Staphylococcus aureus ATCC 25923 during 21 days of storage at pH 7.4.

Keywords: *Vechur cattle, Kasargod Dwarf, antibacterial activity, bioactive peptides*

India has the world's largest cattle population, and Vechur (*Bos indicus*) is one of Kerala's indigenous cattle breeds. Regarded as the world's smallest cow (nearly 90cm tall), it has low feed requirements and a high level of disease resistance. The milk of the Vechur cow is high in small

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fat globules and saturated fatty acids, making it suitable for infants and the sick, besides reported to possess medicinal properties (Ravi *et al.,* 2006). The Kasargod Dwarf is another dwarf cattle in Kerala, well known for its superior milking ability and mineral-rich milk. Moreover, the milk of Kasargod Dwarf contains high amounts of α-s2 casein which makes it a beneficial for diabetes and hypertensive patients (Anu *et al.,* 2018).

Fermented dairy products are high in bioactive peptides and provide both energy and nutrients (Mills *et al.*, 2011). Bioactive peptides are protein fragments that have a favourable effect on physiological processes or circumstances, and may influence health. These peptides with certain amino acid sequences are generated by casein degradation by extracellular proteases like tripeptidases, endopeptidases, amino-peptidases and di-peptidases (Fitzgeraldy and Murray, 2006) from microbial cells. Antibacterial properties (Hoskin and Ramamoorthy, 2008), antioxidant activities (Pihlanto-Leppala, 2000), mineral binding (Lorenzen and Meisel, 2005), and ACE inhibitory activities are some of the health benefits of these peptides (Yamamoto *et al* ., 1999; Gobbetti *et al.*, 2004). Antimicrobial peptides can either eradicate or suppress the growth of microorganisms. They have been derived from a variety of milk proteins including β-lactoglobulin, Lactoferrin, α-s1 casein, α-lactalbumin and κ-casein. They can inhibit Gram-positive and Gram-negative microorganisms. These peptides are rich in amino acids like arginine, lysine, etc.

Positively charged antimicrobial peptides (AMP) can bind with negatively charged lipopolysaccharides of bacterial cells electrostatically, which may play a critical role for the disruption of bacterial cell membranes (Yeaman and Yount, 2003). The widespread increase in bacterial resistance to various conventional antibiotics has prompted scientists to focus on developing new antibiotic classes with novel target locations and action modalities. The present work aims to study the effect of fermentation on antibacterial activity of milk from Vechur and Kasargod Dwarf cattle on refrigerated storage.

Materials and methods

Preparation of milk sample

Fresh, pooled, whole milk from Vechur and Kasargod dwarf cattle were collected separately from the animals maintained at Vechur Conservation unit, KVASU, Mannuthy. The milk samples from both the cattle were heated separately at 80°C for 5 min to kill the microorganisms and to denature the indigenous enzymes of milk. The heated samples were cooled immediately to 42°C and then inoculated with yoghurt culture (*Streptococcus thermophilus and Lactobacillus bulgaricus* 1:1). The inoculated milk was packed in sterilized plastic cups and incubated at 42°C for 4-5 hours. The samples were then stored at 5°C±2º C for storage studies for 21 days of storage. The antibacterial analysis was carried out at 0^{th} day (after 4 to 5 hours), 7^{th} day, 14^{th} day and 21^{st} day.

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 16,099 × g using a Remi High-performance centrifuge at 4°C for 30 min. The supernatant was collected, the pH adjusted to 7.4, then freeze-dried (ANN-FD 808 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. The culture was prepared by activating the test organism through inoculation into the nutrient broth, incubating it at 37ºC overnight and then confirmed by staining. Later, the test organism was inoculated to nutrient broth and incubated overnight at 37ºC for inoculation over the agar plate for antibacterial study. Briefly, 100 µL of an overnight grown

culture broth of the test organism was spread on nutrient agar plates. Wells (6mm in diameter) were made in agar using a sterilised stainless-steel borer. Each well was filled with 100 µL of sterilised WSPE in 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 for overnight. Subsequently, the diameter of inhibition zones in mm 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 and *S. enterica* ATCC 6017 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. The WSPE was prepared from fermented milk samples on 0th day and samples stored at 5°C for 14 days. 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, dehydrated rapidly with ascending concentrations of aqueous ethanol series (25, 50, 75, and 90%, and 3 times with 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 SC7620). Fields of the specimen were examined under a high-vacuum TESCAN VEGA-3-LMU benchtop SEM (TESCAN, Czech Republic) and scanning electron micrographs were recorded.

Result and discussion

The antibacterial activity of fermented milk supernatant (WSPF) was determined against four indicated pathogens (*B. cereus* ATCC 10876*, E. coli* NCIM 2685*, S. enterica* ATCC 6017 and *S. aureus* ATCC 25923). The WSPF obtained from the fermented milk of Kasargod Dwarf and Vechur cattle showed antimicrobial activity against *E. coli* NCIM 2685 and *S. enterica* ATCC 6017 pathogens (Table 1, Fig. 1 and 2). Vechur cattle samples showed lower antimicrobial activity than Kasargod Dwarf samples, which may be explained by their slow release of peptides throughout storage period.

Because there is no apparent pattern for samples containing a specific peptide and antibacterial activity, we can assume that the peptides can operate against only some of the bacterial strain and have a synergistic impact. The majority of antimicrobial active peptides can function by penetrating and compromising the integrity of microbial membranes or by translocating across the bacterial membrane and acting on interior targets of bacteria (Sah *et al.,* 2016).

According to Quiros *et al.* (2005) and Galia *et al*. (2009), *S. thermophilus* strains

Table 1. Antibacterial activity of the WSPE of fermented milk during storage (inhibition zone in mm). A-fermented milk made from Kasargod Dwarf cattle milk; B-fermented milk made from Vechur cattle milk.

Pathogens	Samples	Storage period (days)			
		0th day	7th day	14th day	21stday
Bacillus cereus	A	Absent	Absent	Absent	Absent
	B	Absent	Absent	Absent	Absent
Escherichia coli	A	10	10	11	Absent
	B	Absent	Absent	5	10
Staphylococcus aureus	A	Absent	Absent	Absent	Absent
	B	Absent	Absent	Absent	Absent
Salmonella enterica	A	11	Absent	Absent	Absent
	B	7	10	Absent	Absent

**Zone of inhibition excluding 6mm well diameter as well, Figures are mean values of the triplicate analysis*

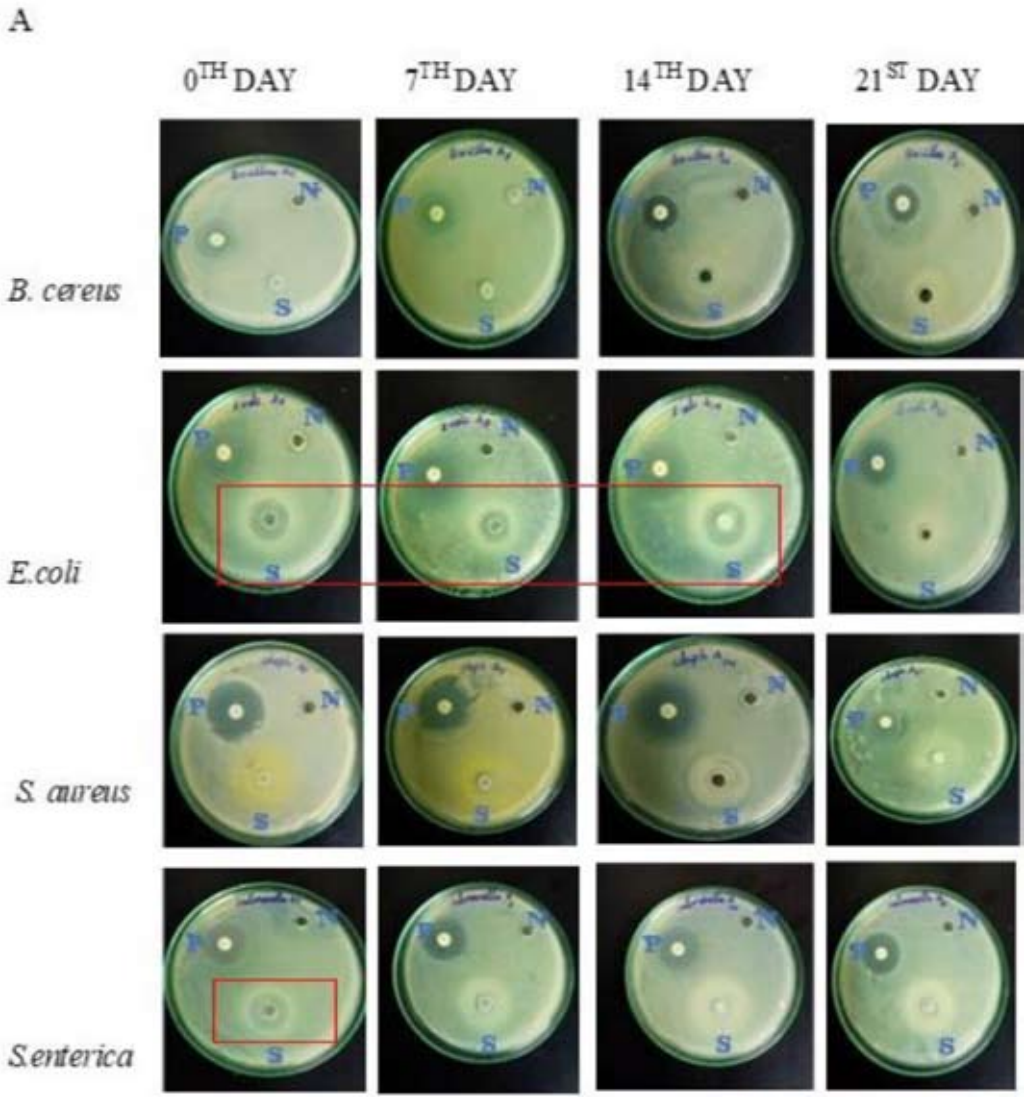

Fig. 1. Antibacterial activity of the WSPE of fermented milk made from Kasargod Dwarf milk(A) against different pathogens during cold storage (inhibition zone in mm). P-positive control, N-negative control, S-sample

were able to liberate peptides from casein based on their proteolytic activity, and some of these peptides were classified as antimicrobial peptides. A large number of peptides were released when β-casein and α-s1-casein were hydrolysed with *L. bulgaricus* (Miclo *et al.*, 2012). Anisha *et al.* (2012) reported that single nucleotide polymorphisms in the Lf gene resulted in a larger level of arginine.

Bacterial morphology of *E. coli* NCIM 2685 and *S. enterica* ATCC 6017 were observed after treatment with WSPE using scanning electron microscopy. The observations demonstrated that the peptides possessed membrane-lytic activities against microbial cells (Fig. 3). As can also be seen in Fig. 3, fibrous material, which is likely the result of cell leakage, and cell debris appear scattered around the cells. This may occur as a result of cationic peptides binding with anionic lipopolysaccharides in gram-negative bacteria's cell membrane (Yeaman and Yount, 2003; Hoskin and Ramamoorthy, 2008). Moreover, these peptides can also displace divalent cations such as Ca2+ and Mg2+, causing

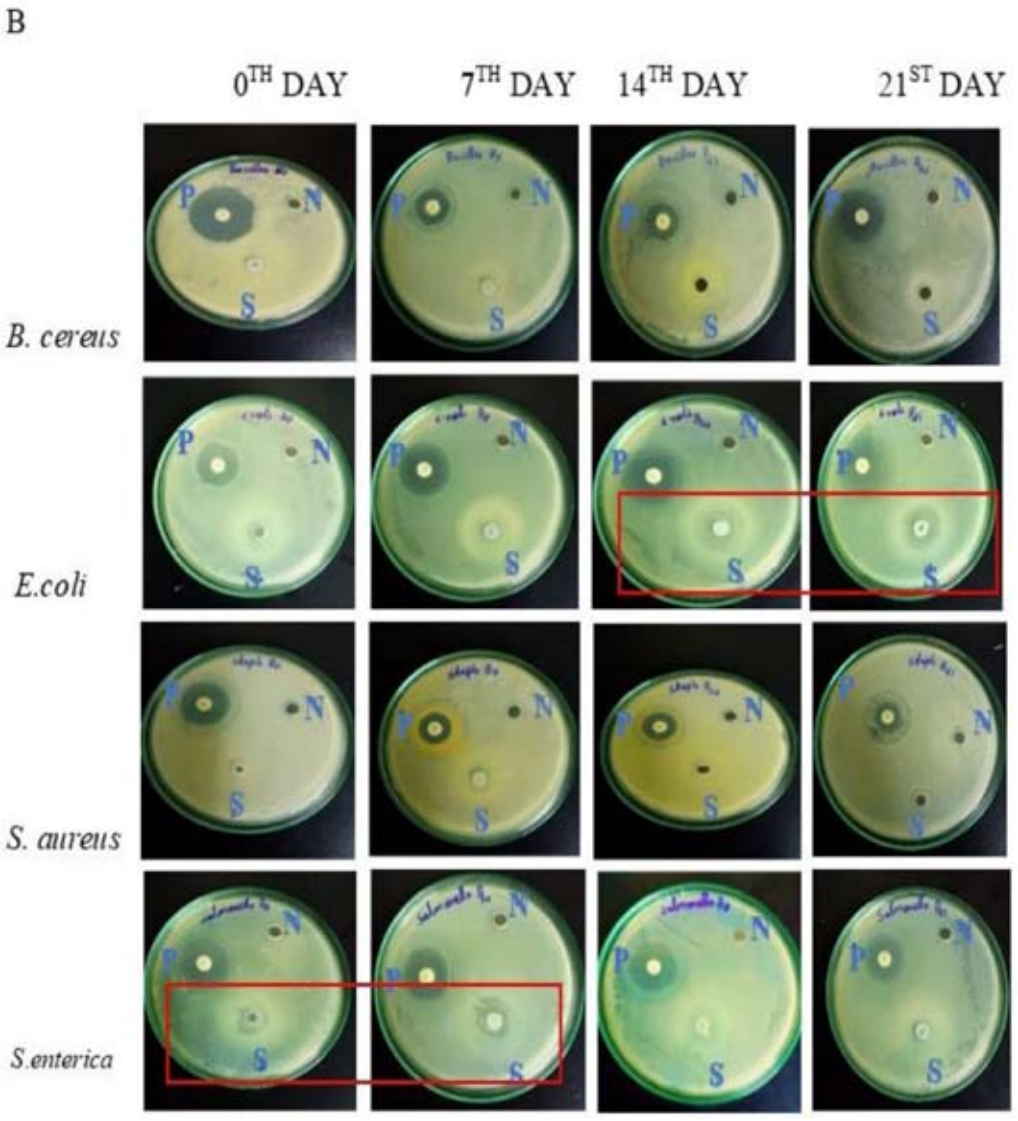

Fig. 2. Antibacterial activity of the WSPE of fermented milk made from Vechur cattle milk (B) against different pathogens during cold storage (inhibition zone in mm). P-positive control, N-negative control, S-sample

distortion of the outer membrane bilayer because these ions are vital to the integrity of the outer membrane. Therefore, the membrane ruptures, causing cell death.

Conclusion

According to the findings of this study, fermentation of milk from Kasargod Dwarf and Vechur cattle by yoghurt starter culture produces a considerable quantity of bioactive peptides and amino acids. These bioactive peptides showed antibacterial activity against different pathogenic bacteria during storage at 5°C for 21 days. The findings show that fermented milk made from native cattle breeds has a higher medicinal potential. More research is needed to isolate the antibacterial components found in Vechur cattle and Kasargod Dwarf fermented milk and use their medicinal potential in pharmaceutical formulations.

Untreated cells Treated cells

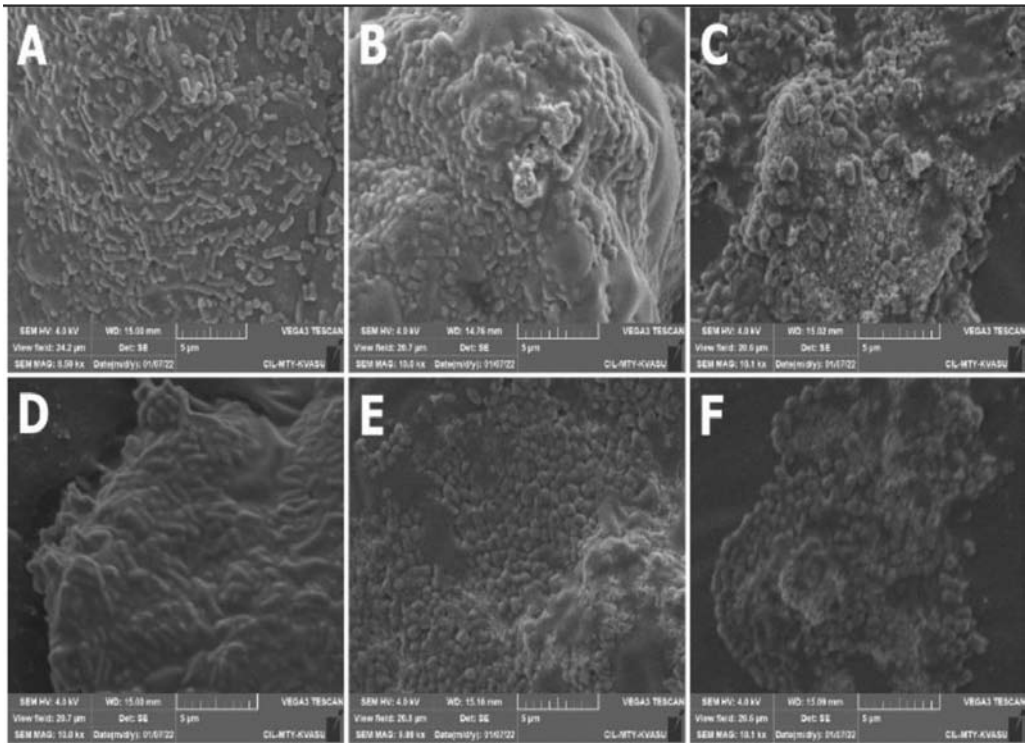

Fig. 3. Scanning electron micrographs showing morphological changes of *Escherichia coli* (A-untreated control cells; B- cells treated with WSPE of 14th day fermented milk made from Vechur cattle milk; C- cells treated with WSPE of 14th day fermented milk made from Kasargod Dwarf cattle milk), and *Salmonella enterica* (D- untreated control cells; E- cells treated with WSPE of 0th day fermented milk made from Vechur cattle milk; F- cells treated with WSPE of 0th day fermented milk made from Kasargod Dwarf cattle milk) induced by treating for 6 hours at 37 with WSPEs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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