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Research Article

Study of Antagonistic Properties of Bacteria from Cow's Milk by Real-Time Surface Plasma Resonance Biosensor (BIAcore)

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ABSTRACT
Probiotics are functional food natural or processed contains known live microorganism or biologically active compounds which confer multiple health benefits conferring in prevention, management and treatment of chronic diseases on the host. Foodborne bacteria confer probiotics that produce antimicrobial substances which shows narrow spectrum antagonistic property against food spoilage bacteria like *Escherichia coli, Listeria monocytogenes* and *Salmonella enterica.* The emergence of multidrug-resistant pathogens and the restriction on the use of antibiotics as growth inhibitors of the pathogens in the processed foods and feeds have drawn attention to the search for possible alternatives. Present work begins with the isolation of probiotics bacteria from raw cow's milk and screening for bacteriocin production by antagonistic assay. The culture's cell free supernatants were isolated from the bacteria for the comparative antimicrobial screening by conventional Kirby-Bauer disc diffusion method and advanced real-time surface plasma resonance biosensor (BIAcore).

Keyword: Antimicrobial activity, Bacteriocins, Kirby-Bauer disc diffusion, Surface Plasma Resonance Biosensor, BIAcore T200

INTRODUCTION

Probiotics are the "live microbial culture or living microorganisms which beneficially influence the health and nutrition when ingested in appropriate concentration exert health benefits beyond inherent basic nutrition for the host" [1]. Probiotics exert their beneficial effects on the host through four main mechanisms: interference with important pathogens, improvement of gut barrier functioning, immunomodulation and production of appropriate neurotransmitters, and their host targets varies from the resident microbiota to the cellular components of the gut-brain axis. The beneficial effect of the probiotics is mainly through their interactions with the intestinal microbiota and with the intestinal mucosa of the host. During fermentation, lactic acid bacteria can produce a number of bioactive peptides also known as metabolites such as bacteriocins, bio-genic amines, exopolysaccharides and proteolytically released peptides [2] . Food products containing probiotics and prebiotics are considered as an important development in

health foods, which enhance intestinal health promoting microbial flora. Food, particularly dairy products are considered as an ideal vehicle for delivering probiotic bacteria to the human gut or gastrointestinal tract. Milk is a complex fluid containing different molecules in several states of dispersion. Cow's milk provides a sole and primary source for nourishment and immunological protection. The components of milk includes water, fat, lactose, protein, small quantities of minerals, vitamins, enzymes, specific blood proteins and somatic cells. The types of microorganisms present in raw milk are *Micrococcus, Staphylococcus, Bacillus, Pseudomonas, Enterobacter, Klebsiella* and *Serratia* [3].

MATERIALS AND METHODS

Screening of non-lactic acid bacteria from different food samples

It is common knowledge that microorganisms, bacteria have capabilities to produce a huge amount of bioactive antimicrobial compounds. Since their elective habitat is food, especially dairy becomes the important source for isolating the bacteria. The isolation bacteria from dairy products begun with collecting the milk aseptically in a sterile bottle used immediately within 2hrs or stored at 4ºC. Raw milk and pasteurized milk were used for isolation of bacteria of which 1ml of cow's milk was serially diluted in the range from 10^{-1} to 10^{-5} in sterile distilled water. Isolation of bacteria was carried out for the dilutions 10^{-3} to 10^{-5} by pour plate method on nutrient agar, incubated at 37°C for 24-48hrs [4].

Procurement of indicator organisms

Pure cultures of food spoilage bacteria were procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh, India and maintained in the table with their respective MTCC numbers in their respective media as instructed and tabulated in Table 01.

Table 01: MTCC organisms with their number

Sl No	Organism	MTCC Number
1.	*Escherichia coli*	10312
2.	*Listeria monocytogenes*	1143
3.	*Salmonella enterica*	1164

All the organisms were revived and tested in nutrient broth and nutrient agar. The organisms are maintained as per the MTCC standard instructions.

Screening

Preparation of cell free supernatants

The isolated bacterial strains were grown in Nutrient Broth (NB), incubated at 30°C for 48 hrs in shaking condition at 120 rpm. The cultures were centrifuged at 10000 g for 15 min. The supernatant collected was filtered with 0.45µM Whatman No.1 filter membrane to get cell free supernatant [5]. Through the statistical tools studied, the maintenance of aerobic condition with constant supply of oxygen plays a crucial role in the growth of non-lactic acid bacteria and in the production of bacteriocin [6].

Preparation of inoculum

The evaluation of antimicrobial activity reckoned by preparing 24hrs fresh culture of indicator bacteria by adjusting the turbidity of cell density with the spectrophotometer to the transmittance to produce absorbance of 0.08-0.1 that correlates to 0.5 McFarland standard at 625 nm wavelength. This procedure yielded the cell stock suspension of 1 x 10^7 to 10^8 colony forming units (CFU) per mL [7].

In vitro antimicrobial assay by Kirby-Bauer disc diffusion method

A volume of 100 µl of inoculum suspension previously adjusted to 0.5 McFarland standard of each indicator bacteria was swabbed evenly on prepoured sterilized nutrient agar plates set for the disc diffusion assay. The lid was left ajar to allow the absorption of excess surface moisture for 10mins before placing the sterile discs. Sterile 5 mm diameter discs were prepared using Whatmann No. 1 filter paper and placed on bacteria swabbed nutrient agar plates equidistantly round the margin of petridish. 10 µl of cell free supernatant from nutrient broth were dispensed on the disc. The plates were incubated at 37°C for 24 hrs. At the end of the incubation period, the antagonistic activity was

recorded as the width of the clear inhibition zone formed around the disc (diameter of inhibition zone plus diameter of the disc). The values are the means of three independent experiment performed in triplicate [8].

Statistical analysis

Statistical analysis was performed using SPSS software: Version 20.0. The results were expressed as mean ± SD (n=3). Experimental data were analysed using multivariate ANOVA depending on the nature of data set. Means were separated by post hoc analysis – Tukey HSD with the level of significance at $P<0.05$.

Advanced automated real time Biacore technique

Real-time, label-free detection of functionalization of antimicrobial peptide present in cell free supernatant with high selectivity and sensitivity for the indicator organism is demonstrated using an interdigitated impedimetric array called the Biacore technique. Biomolecular discrimination is one of the most important ways to discriminate closely related species which can be made easy, versatile and reliable by using different sensing systems and one among is the Biacore-carboxymethylated dextran coated sensor chips.

Surface Plasmon resonance (SPR)

The surface plasmon resonance (SPR) biosensor is a useful tool to analyze numerically the interaction of certain molecules. The most important advantage of the SPR assay as compared with other protein-protein binding affinities and association/dissociation kinetics of complexes in real time, in a label-free environment, and using relatively small quantities of materials and can calculate the affinity between protein and its binding partner. The method is based on the immobilization of one of the binding partner- indicator organism at 0.5 McFarland standard, called the ligand, on a dedicated sensor surface. Immobilization is followed by the injection of the other partner-cell free supernatant, called the analyte, over the surface containing the ligand. The binding is monitored by subsequent changes in the refractive index of the medium close to the sensor surface upon injection of the analyte and a sensogram is produced as a representation of its binding kinetics [9,10].

SPR interaction analyses were performed using a Biacore T200 optical biosensor (GE Healthcare Life Sciences, Bangalore, India). Biacore T200 is a molecular interaction analysis system that delivers the highest quality data for every interaction parameter.

The detailed protocol includes;

The immobilization of indicator organism

The indicator organism is immobilized onto the CM5 (CarboxyMethylated) gold microelectrode chip using amine coupling. Amine coupling is the most applicable covalent coupling chemistry used to immobilize protein ligands. The dextran matrix is activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) to create reactive succinimide esters. For immobilization, the free carboxyl groups of the amino acid residues were activated with EDC/NHS, then reacted with the ethylenediamine-derivatized carboxymethyldextran sensor chip to obtain the desired ligand concentrations. The standard activation time required to reach the immobilization level is 7mins [11]. The ligand is then injected in low salt buffer lacking primary amines at a low pH below the isoelectric point of the protein. Under these conditions, the ligand acquires a positive charge and effectively preconcentrated into the negatively charged carboxymethyl dextran matrix. The unreacted esters are blocked with ethanolamine. This acts as a Baseline which monitors the immobilization level.

Analyte- ligand binding analysis

SPR measurements were carried out in phosphate buffer saline (0.1 M phosphate buffer with 27 mM KCl and 1.37 M NaCl 0.005% polysorbate 20 pH 7.4). Onto the immobilized ligand on the dextran matrix that acts as a substrate film to which the analyte, cell free supernatant to be detected attaches through hydrophilic binding to form a positive interaction, else a negative interaction. SPR causes reduction in the intensity/ density of light at a specific angle from the glass slide of the sensor chip CM5. Nisin at the concentration of 1ppm (1µg/mL) is used as a positive control and nutrient broth as negative control.

Data analysis

Data was collected with the Biacore control software version 3.0. Experiments were performed by monitoring the refractive index changes as a function of time under a constant flow rate of 45µl/min. The relative amount of active compound bound to the indicator organism was determined by measuring the net increase in refractive index over time compared to control running buffer. There is an inline subtraction of reference surface during the run. The result from the detection of the change in RI is displayed as a sensogram plotted with the binding response (RU) on Y-axis against time on X-axis. The representation of sensogram can be either curve graph or a bar chart graph.

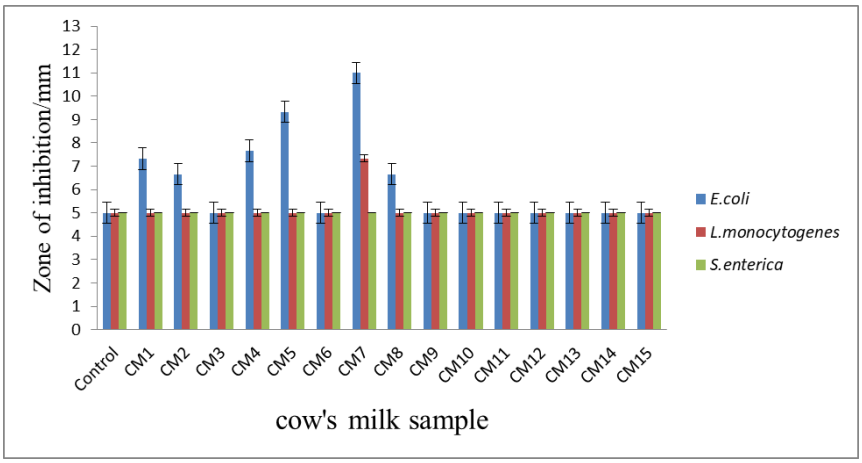
RESULTS AND DISCUSSION

Isolation of colonies

The colonies which are opaque, moderately larger in size, cream/white coloured, prominent surface texture and shape were isolated and dispensed into nutrient broth. 15 colonies with different morphological features were isolated which was considered primarily as a single isolate from raw cow's milk and pasteurized milk did not show any growth of bacterial colonies.

***In vitro* antimicrobial assay by Kirby-Bauer disc diffusion method**

The primary screening of cell free supernatants by Kirby-Bauer disc diffusion method revealed the positive antagonistic activity. Among 15 isolates from cow's milk, 6 isolates (40%), CM7, CM5, CM4, CM1, CM2 and CM8 exhibited antimicrobial activity of 11.66, 9.33, 8.66, 8.33, 7.33 and 6.66 mm against *E.coli* respectively. Only 1 isolates (6.66%), CM7 exhibited antimicrobial activity of 7.66 mm against *L.monocytogenes*. During the conventional procedure of antimicrobial assay none of the isolates showed any antagonistic activity against *S.enterica*. The results are plotted through bar graph (Graph 01) and the antimicrobial assay plates are observed in the figure 01.



Graph 01: Antagonistic activity of cell free supernatants from the isolates of raw cow's milk by Kirby-Bauer disc diffusion method

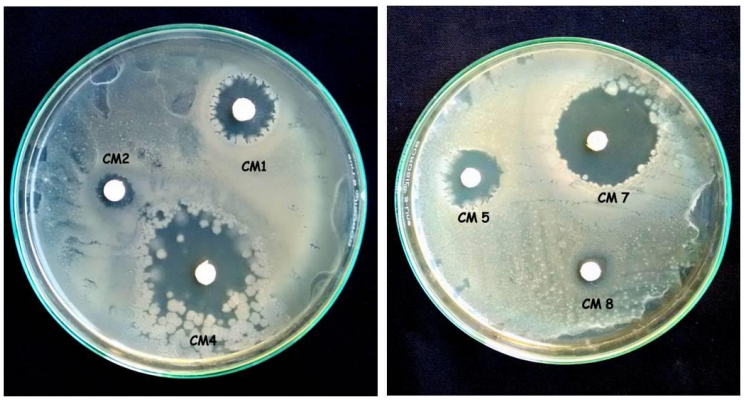
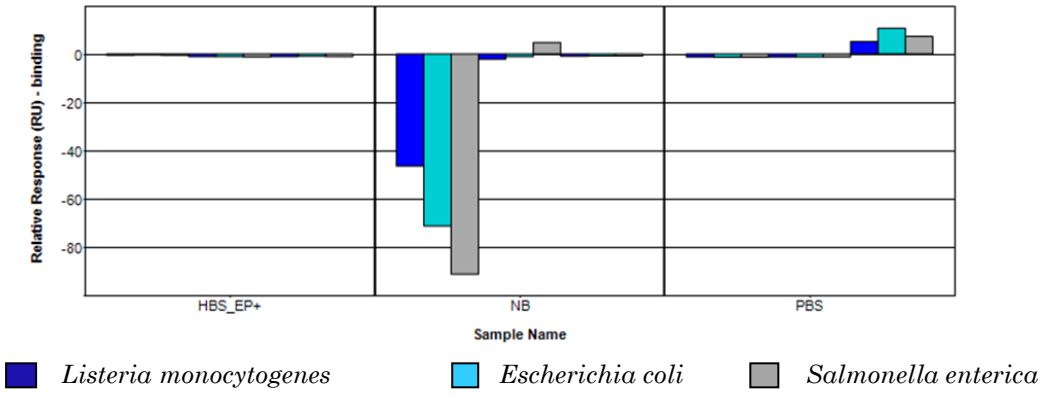


Fig. 01: Antagonistic activity of cell free supernatants from the isolates of raw cow's milk by Kirby-Bauer disc diffusion method

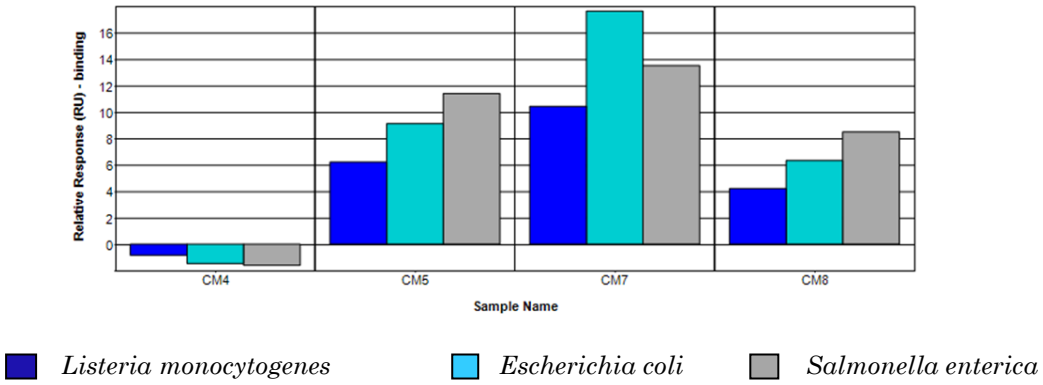
Advanced automated real time Biacore technique

The potential isolates CM4, CM5, CM7 and CM8 that are exhibiting significant antimicrobial activity were selected which resulted in the antagonistic activity against all the three indicator organism viz., *E.coli*, *L.monocytogenes* and *S.enterica*. SPR Biacore T200 showed positive sensogram were plotted for the activity of cell free supernatant that ranged from 0.5 to 18.

The binding response of CM5 is 6.1, 9, 11.4, CM7 is 10.1, 16.5, 13.6 and CM8 is 4.2, 6.3, 8.2 against *L.monocytogenes, E.coli* and *S.enterica* respectively. The buffers, positive and the negative control did not exhibit any activity. The details are represented in the bar graphs and displayed in **Graph 02** and **Graph 03**.



Graph 02: Binding response of the cell free supernatants for buffers and nutrient broth



Graph 03: Binding response of the cell free supernatants against indicator organisms

CONCLUSION

The study clearly showed the antibacterial activity conducted through SPR biosensor Biacore technique revealed a significant activity against all the three indicator organisms even in small quantities. The antibacterial activity conducted through conventional disc diffusion method could not show any sensitivity towards the all the three indicator organisms. The study concludes the better utilization of the advance technique when compared with the conventional method.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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