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Research Article

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Impact of pre-enrichment broth on recovery of *S. typhimurium* and reformed water activity on dominance and endurance of *Salmonella* in Indian sweetmeat milk (doodh) peda

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Abstract

Food-borne outbreaks associated with low water activity (a_w) foods involve Salmonella contamination, and its control is a significant challenge. In India, milk (doodh) peda is a low a_w traditional and popular food. Accordingly, it is essential to determine the prevalence and survival of Salmonella spp. in artificially spiked milk peda stored for an extended period at different a_w. Salmonella spp. was not detected in any of the 25 indigenous peda samples. Salmonella did not grow in low a_w. However, S. typhimurium ATCC 25241, which was inoculated artificially at a level of $1.5x10^4$ CFU/g, survived in the spiked peda sample at 0.75, 0.56, and 0.32 a_w. Survival of S. typhimurium ATCC 25241 was observed for a more extended period (19 days) at lower a_w than higher a_w. These results confirmed that even though Salmonella spp. was not detected in milk peda samples, but can survive for a long time in contaminated samples. Salmonella survived for a long time by the osmoadaptation mechanism. These results revealed that the survival of S. typhimurium is influenced by a_w, and the prevalence of Salmonella in the peda sample was inversely proportional to a_w.

Keywords: *Salmonella,* incidence, existence, low water activity, dairy confectionary, peda.

Introduction

Traditional dairy products and confectioneries are an integral part of the Indian food ethos with significant social, religious, cultural, medicinal, and economic significance. Annual milk production in India accounts for 155.5 million tons, and 50-55% of produced milk is converted into a variety of traditional Indian dairy products [1]. Amongst the various milk confectionaries, value-added *Khoa-based* products such as burfi, peda, Gulab Jamun, milk cake, kalakand, and Kunda dwell in more commercial significance than other sweets [2]. These milk products were prepared to aim at value addition and economic development of the food manufacturers. Generally, about 6.5% of milk is used by the private and unorganized sectors to manufacture khoa [3, 4]. Besides small khoa-based dairy confectioners (halwais), many organized dairy companies and large milk business companies have entered this productive venture.

In food processing and preservation, water activity (a_w) measurement is one of the most critical parameters consequent to a product's hygienic quality. The growth of microorganisms can be prohibited by adjusting storage temperature, reducing water activity, lowering pH, using preservatives, and proper packaging [5]. In the past, low a_w foods were suspected to be harmless from a microbiological food safety point of view that, including Indian dairy confectionary milk peda because of low a_w (0.85 or below) [6]. Moreover, peda is offered as 'Prasad' in India during religious worship and festive celebrations. In recent years there has been a rise in the load of food-borne illnesses due to microbial contamination of low a_w foods by food-borne pathogens including *Salmonella enterica, Shigella, Escherichia coli, Yersinia enterocolitica, Vibrios, and Staphylococcus aureus* [7, 8]. Most notably and frequently, *Salmonella* spp. is responsible for most recurrent food-borne illness outbreaks [9]. The first *Salmonella* outbreak related to low-moisture products was registered in the 1970s. However, recent outbreaks of salmonellosis linked to low-moisture foods, such as spices, whole raw almonds, peanut butter, baby formula, wheat flour, puffed cereals, and cookie dough, have proven their survival for significant periods [10, 11]. In the United States of America, 48 million infections of food-borne illnesses account for every year because of these bacteria [12]. Since *Salmonella* has

been confirmed as the leading pathogen of concern for low-moisture foods worldwide about 90 million cases of gastroenteritis and 155,000 deaths were estimated every year [13, 14]. *Salmonella* infections can cause severe illness and even mortality in young, elderly, and immunocompromised patients, even though the diseases are self-limiting in healthy people [15]. Improper processing or storage of dairy products can represent a transmission hazard for many pathogens. It can be responsible for outbreaks of brucellosis, listeriosis and tuberculosis [16, 17].

Salmonella can survive for long periods in low a_w products like halva and peanut butter chocolate [18-20]. There is a growing concern for *Salmonella*'s potential occurrence and survival in low a_w products. Moreover, controlling *Salmonella* in low a_w foods and their production environments represents a significant challenge for all food manufacturers. Thus from a public health standpoint, it is essential to evaluate the prevalence and survival of *Salmonella* in the low a_w peda samples.

Materials and Methods

Bacteriological Media

Lactose broth (LB), peptone, Rappaport Vassiliadis (RV) *Salmonella* enrichment broth, tetrathionate broth (TT), standard plate count (SPC) agar medium, hektoen enteric agar (HEA) medium, xylose-lysine deoxycholate (XLD) agar, bismuth sulphite agar (BSA), violet red bile glucose (VRBG) agar, triple sugar-iron (TSI) agar medium and lysine iron agar (LIA) were purchased from Hi-media Laboratories Pvt. Ltd, Mumbai, India.

Bacterial Strain and Inoculum Preparation

Standard lyophilized culture of *Salmonella typhimurium* ATCC 25241 was obtained from the American Type Culture Collection and stored at -80° C in brain heart infusion (BHI) broth with 10% glycerol. Cultures were revived from -80° C storage and grown in nutrient broth incubated at 37° C. Overnight broth cultures were spread onto nutrient agar (NA) plates, incubated overnight (16-24 h) at 35° C, and used for further spiking study. To determine the viable colony count, *S. typhimurium* ATCC 25241 suspensions were adjusted to McFarland standard one turbidity units (corresponding to approximately 3×10^8 CFU/ml). The cell suspension from the agar plate was prepared, and one ml of ten-fold serial dilutions in sterile physiological saline (0.85%) was made. Subsequently, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} dilutions were enumerated by pour plate technique on plate count agar after 18-24 h of incubation at 37° C.

Sampling

Peda samples were randomly obtained from different local vendors, private manufacturers, and organized dairies (Mysore Milk Dairy, Mysore and Chamarajanagar District Co-operative Milk Producers Societies Union Ltd., Siddartha Nagar, T. Narsipura road, Mysuru- 570011) from different places of Mysore city. The declared ingredients of the samples were milk solids and cane sugar. A total of 25 peda samples randomly collected from various shops were screened for the occurrence of *Salmonella spp*.. Enumeration of *Salmonella* spp. was carried out in all peda samples. A peda sample from Mysore Milk Dairy was used for the survival studies on artificially spiked *Salmonella* spp. with different water activity. NFDM (nonfat dry milk, skim milk) was purchased from wholesale suppliers.

The efficiency of pre-enrichment broth in the recovery of *S. typhimurium* atcc 25241 for spiking study

Pre-enrichment broths like LB and NFDM (100 g/l of distilled water) with 7.5g of brilliant green 2% were used. Sterile distilled water containing 0.002% brilliant green was added to NFDM. The pH of LB was adjusted to 6.8±0.2 with 1 N NaOH, as described in the BAM *Salmonella* culture method (FAO, 2012). The two pre-enrichment broths were sterilized. Simultaneously, 1 ml of 3×10^4 CFU/ml dilutions of *Salmonella* typhimurium ATCC 25241 were inoculated to 225 ml of LB and NFDM media. From that, 1ml of aliquots were taken periodically every half an hour, plated on XLD selective enrichment media for 8 h, and incubated at 35° C for 24 h. Growth was characterized by CFU/ml from XLD plates and optical density from absorbance readings at 600nm (OD600).

Prevalence of *Salmonella* spp. in indigenous milk peda

The pre-enrichment LB was added (225 ml) to a sterilized stomacher bag (Nasco) containing a peda sample (25 g) by maintaining aseptic conditions. This was homogenized in a laboratory stomacher for 1 min. The homogenate was then incubated at 35° C for six h. After incubation, 1 ml and 0.1 ml of culture broth were transferred to the selective enrichment broths RV and TT and incubated at 35° C and 43° C, respectively, for 18-24 h. After incubation, a loop full of inoculum was streaked on the selective agar plates (XLD, BSA, and HEA). Presumptive *Salmonella* colonies grew on XLD (big black centered colonies), BSA (small black colonies with a metallic sheen), and HEA (small to medium pink colonies) were inoculated on TSI and LIA agar.

Retrieval and enumeration of spiked *S. typhimurium* ATCC25241 at different a_w

Equilibration of a_w of peda Sample

The water activity of the peda sample was recorded using a bench top water activity meter (LabMaster- a_w (Novasina AG, Neuheinstrase 12, 8853 lanchen, Switzerland) with an accuracy of +/- 0.003 a_w. The a_w values of the peda sample were adjusted to 0.75, 0.56, and 0.32 a_w in vacuum desiccators containing saturated salt solutions of sodium chloride, sodium bromide, and magnesium chloride, respectively [21]. A homogeneous mass (600 g) of peda was prepared by pooling individual peda (20 g) samples. To attain the set water activity, about 150-200 g of sample was distributed into three different sterile desiccators containing saturated salt solutions. Equilibrium was assumed based on the constant weight of the triplicate sample.

S. typhimurium challenged (artificially spiked) peda sample

Peda sample confirmed to be free from *Salmonella* by conventional culture method was used for the spiking experiment. The standard *S. typhimurium* ATCC 25241 cultures were subcultured on a nutrient agar slant and incubated at 35° C for 24h. After incuba-

tion, a suspension of $3x10^8$ cell/ml was prepared in 0.1% peptone water and adjusted to Mac Farland standard No.1. Two-fold serial dilutions were made from overnight culture to obtain desired cell concentration ($3x10^6$) for an artificially spiked sample. The precise number of CFU was confirmed using the plate count method onto nutritive agar. Twenty gram ($1.5x10^5$ CFU/g) of dry peda sample with different water activity was artificially inoculated with 1 ml of the appropriately diluted suspension ($3x10^6$ dilutions) of *S. typhimurium* ATCC 25241. Colonies were enumerated after 18-24 h incubation at 37° C. The CFU was determined by serial dilution and plating on XLD plates. Serial dilutions and plating determined the viable colony count on XLD.

Survival of *S. typhimurium* in peda at different a_w

Five gram of challenged peda sample ($1.5x10^5$ CFU/g) was mixed with 45g of unchallenged peda sample of different a_w (0.75, 0.56, and 0.32). To evaluate the survival of *S. typhimurium*, the detection and enumeration were performed every day using 1±0.01g of sample aliquot from each a_w level. The 10X diluted peda samples were serially diluted. The enumeration was performed using the 3-tube Most Probable Number (MPN) technique with buffered peptone water (BPW). The aliquots samples (0.1, 0.01, and 0.001 g) were transferred into 10 ml of tetrathionate broth with subsequent plating on XLD agar.

Statistical Analysis

All data were expressed as the mean ± standard deviation (SD, n=3).

Results

Prevalence of *S. typhimurium* in peda samples

Milk peda, a popular traditional sweet product, is consumed by all sections of society. It was presumed that low a_w of peda would be safe and preserved for a longer duration. A total of 25 peda samples collected from different shops were analyzed for the prevalence of *Salmonella*. Our results revealed that none of the samples were positive for *Salmonella*. Because peda is a heat-desiccated product of low a_w, it was free from gram-negative fermentative heat-sensitive bacteria, including hygiene-indicating bacteria, E. coli. The pour plate results of the peda sample on XLD medium revealed the presence of >300, 149, and 12 colonies at 10^{-5}, 10^{-6}, and 10^{-7} dilution, respectively. However, the typical characteristics of *Salmonella* colonies were not observed.

Effectiveness of pre-enrichment broth for recovery of *S. typhimurium* atcc 25241

Since there are few reports on survival studies of *Salmonella* in peda samples, we focused on determining the survival of *S. typhimurium* in different a_w. For Survival studies, the probability of *Salmonella* should be increased by methods like pre-enrichment, selective enrichment, and selective plating to avoid false-negative results and increase the numbers of pertinent organisms [22].

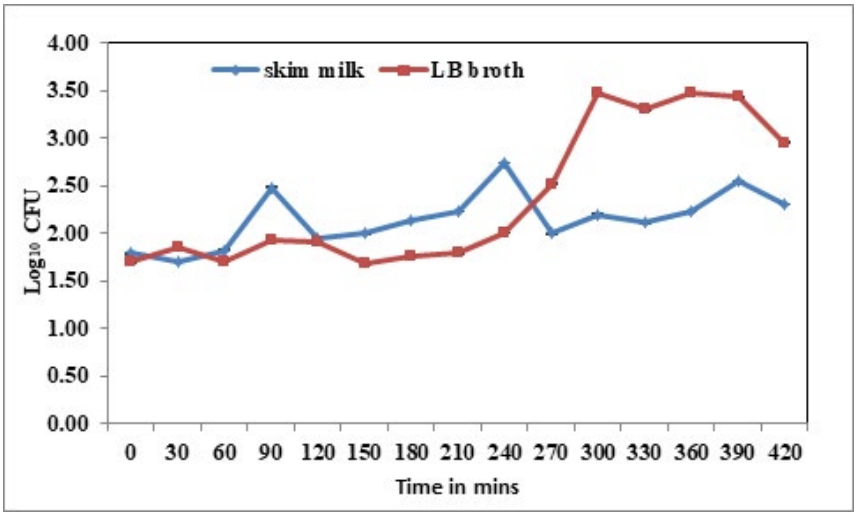

Figure 1: Recovery of *Salmonella* in different pre-enrichment media. Values were obtained from three independent trials with standard deviation.

Figure 1 shows the recovery (every 30 min interval) of *S. typhimurium* from NFDM with BGW and LB as pre-enrichment media for up to 7h. Growth characteristics of *S. typhimurium* varied significantly in the enrichment broths examined. In NFDM as pre-enrichment media, a significant variation in the growth rate was observed, which ranges from 1.70±0.01 to 2.74±0.01 CFU from 30 mins up to 4 h. After four h, a decrease in growth rate was observed. However, the growth rate in LB media ranges from 1.68±0.02 to 3.48±0.00 CFU. The growth rate was maintained at 3.47±0.01 CFU for six h. After six h decline in growth rate was observed. The LB was significantly more productive than NFDM with BGW in recovering *S. typhimurium*. These results suggested that *S. typhimurium* can multiply in the presence of LB as pre-enrichment media.

Survival of *S. typhimurium* at different a_w

The average a_w of the peda samples ranged from 0.732 to 0.784. However, to study the significance of a_w on the survival count of *S. typhimurium* in the spiked peda sample, peda with a different water activity (0.75, 0.56, and 0.32) was prepared. The observed survivability of *Salmonella* in different a_w is presented in Figure 2.

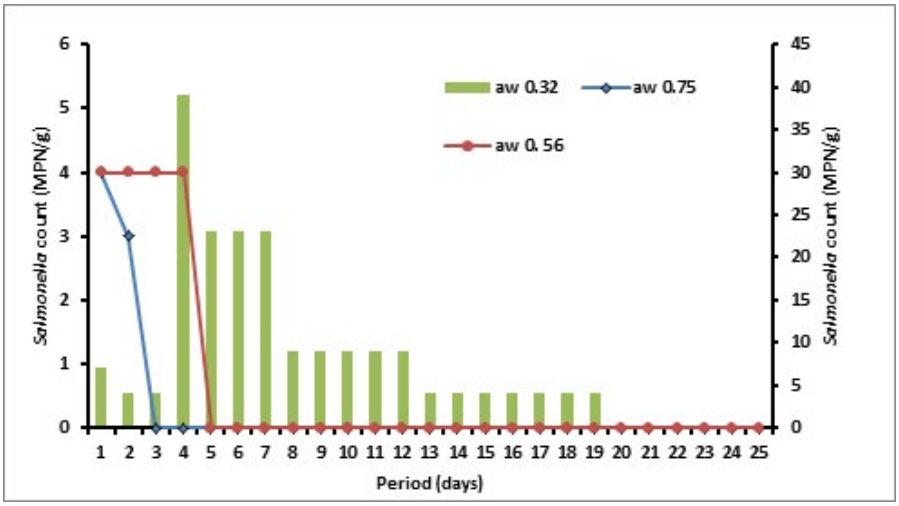

Figure 2: Survival of artificially spiked *Salmonella* in peda sample at different water activity
Values are mean±standard deviation (SD, n = 3). No significant difference between the readings ($p < 0.05$).

A reduction in a count of 150 CFU per g of the sample is observed from the first day. At the highest a_w of 0.75, after the 3rd day, the bacterial numbers were below detectable limits. *Salmonella* becomes undetectable after five days at a_w of 0.56. While at the lowest a_w of 0.32, it was measurable even up to 19 days. The results showed that lower a_w is favorable for the survival of *Salmonella.* However, vegetative cells and spores may continue viable for several months or even years in a dehydrated state even though the metabolism is significantly reduced. Water activity (a_w) 0.75 (Sodium chloride), a_w 0.56 (Sodium bromide), and a_w 0.32 (Magnesium chloride), Initial count is 150 cfu/g.

Discussion

Microbial spoilage of foods can be prevented by lowering water activity. Milk peda, a popular traditional sweet product, is consumed by all sections of society. Low a_w of this product is presumed safe and can be preserved for a longer duration. However, the prevalence of *Salmonella* and other pathogens, such as *E. coli, Shigella*, hemolytic *Streptococci*, and *Pseudomonas aeruginosa*, were reported in peda samples [23]. Similarly, the presence of S. enteritis was reported in peda and khoa samples collected from local markets in Mumbai [24]. 16.3 % of peda is contaminated with *S. Typhi.* These bacteria are present due to improper maintenance and contamination of products during subsequent handling [23, 25].

The pre-enrichment step is imperative and increases the number of pertinent organisms. The first six h of pre-enrichment is a critical period in the recovery of *Salmonella* [22]. In this study, two different pre-enrichment mediums were tested for the growth study of *Salmonella*. It was observed that LB provided a favorable environment for the recovery of damaged *Salmonella* and favored the growth of *Salmonella* over other species. A pre-enrichment medium offers a higher ratio of *Salmonella* to non-*Salmonella* bacteria after incubation. Conversely, a decline in the number of viable *Salmonella* in NFDM media suggested that LB may be more useful in detecting low *Salmonella* levels than NFDM.

Salmonella survived long-term in low a_w peda due to the osmoprotectant metabolites/molecules. The first metabolite trehalose biosynthesis by glucose as the sugar was added during the preparation of peda. The glucose is diverted toward trehalose production, and cells must acquire energy for cellular processes (such as the import of osmoprotectants) solute that is also important for osmoadaptation in *Salmonella* [26-29]. An up-regulation in the trehalose biosynthetic genes has also been observed after the desiccation of *Salmonella* on paper disks and stainless steel [30, 31].

Another mechanism by which *Salmonella* survived under desiccation stress is the catabolism of fatty acid. Since the peda is rich in fat content, the production of more ATP per carbon atom from fatty acids is in comparison to glucose [30-32]. Besides, *Salmonella* appears to become quite heat resistant under low-water activity stress. It was observed that the survival of rate of *Salmonella* in the peda sample was inversely proportional to a_w. These results revealed that water activity significantly influenced the survival of *Salmonella* in low-a_w foods ($a_w < 0.32$). Thus from a public health standpoint, it is essential to implement GHP (good handling practice), GMP (good manufacturing practice), and HACCP (hazard analysis critical control point) during the preparation, retailing, and storage of this product.

Conclusions

Outbreaks of *S. typhimurium*-caused infections due to food con-

tamination are frequent and affect humans worldwide. The study reveals the nonappearance of the top food-borne pathogens, such as *E. coli* and *Salmonella*, from peda samples. Although *Salmonella* was not detected in any peda samples by surveillance study, its survival was confirmed by spiking study at the different a_w activity. This is the first study to show that *S. typhimurium* survived for more extended periods at low a_w (0.32). Thus, focusing attention on food processing safety, low a_w of peda samples revealed the presence of *Salmonella* of clinical significance.

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