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RESEARCH ARTICLE

A Study On Application Of Listeria Monocytogenes Bacteriophage As Biocontrol Agents For Dairy Products.

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Abstract: ***Background***: Listeriosis is epidemiologically linked to the consumption of foods contaminated with Listeria monocytogenes (LM), especially ready-to-eat dairy food foods that are stored for extended periods at refrigeration temperatures and do not require heating for consumption. The use of lytic bacteriophages has been successfully tested for the biocontrol of L. monocytogenes in different food matrices such as artificially contaminated dairy products. Hence, this study was conducted with the main objective to evaluate the application of L. monocytogenes bacteriophage as biocontrol agents for dairy products such as milk, butter milk, and curd. The samples of dairy products viz. milk, butter milk, and curd were collected, and observed for morphological changes like texture, odour and colour before and after two days of incubation. Dairy products underwent treatment with bacteriophage (107PFU/g), while an uninoculated sample served as control. Each treatment was sealed with sterile aluminium foil under various conditions viz. room temperature, 15-18ºC, 4ºC, and deep freezer (-20ºC). Subsequently, the samples were subjected to microbiological examination. Results revealed that there was statistically significant ($p<0.001$) decrease in L. monocytogenes colony count was observed in all the dairy product's samples viz. milk, butter milk, and curd following treatment with L. monocytogenes bacteriophage for two consecutive days. In conclusion, L. monocytogenes bacteriophage treatment of dairy products like milk, butter milk, and curd significantly controls the L. monocytogenes infections. Hence, bacteriophages could be explored as biocontrol agents for the prevention of dairy products from getting infected with pathogenic L. monocytogenes infections.

Keywords: Listeria monocytogenes, Bacteriophage, Biocontrol agents, Milk, Butter milk, Curd.

INTRODUCTION

Dairy farming in India predominantly involves numerous smallholder dairy farmers and entrepreneurs, making it a subsidiary activity. The economic significance of the dairy sector is evident, with the value of milk alone exceeding Rs 35 thousand crores. Dairying plays a key role in Indian agriculture by efficiently utilizing low-valued agricultural by-products and engaging an underemployed labor force for milk production.1

Milk consumption in India is linked to health benefits. The intake of milk can serve as an indicator of overall good nutrition, and dairy products offer essential nutrients such as calcium in milk, that support the growth and development of young children.2 The majority of the milk produced is used domestically in various forms, including liquid milk and dairy-products like butter, clarified butter (ghee), yogurt, curd, cheese, paneer, flavored milk, and ice cream. Consumption patterns exhibit diversity across the country, influenced by regional or community-specific factors, including levels of deprivation/food security and adherence to traditional practices. 3

The composition of milk encompasses water (86-88%), fat (3-5%), protein (3-4%), lactose (4.5-5%), and minerals (0.7%). Additionally, milk contains minor components such as phospholipids, sterols, vitamins, enzymes, and pigments. In dairy products, these components are present in significant amounts, playing crucial roles in maintaining and developing the human body while offering therapeutic and other beneficial effects. Andersen and colleagues emphasize about the interdependence of food and health, asserting that the consumption of nutritious food leads to good health. In line with this understanding, Indian dairy products inherently contain various health-promoting components with therapeutic values, such as antiallergenic qualities, prevention of osteoporosis, reduced risk of heart disease, antimicrobial properties, immune enhancement, anti-diarrheal effects, and alleviation of lactose intolerance.4 In the 1920s, listeriosis was identified as an infection brought on by a Gram-positive bacterium that was subsequently identified as Listeria monocytogenes (LM).5 L.

monocytogenes is extensively distributed in a wide range of agricultural environment such as soil, manure, and water.6 Furthermore, L. monocytogenes is a global occurrence with widespread distribution in the environment, posing a potential threat as a contaminant in various dairy products, including raw milk. While pasteurization is known to eliminate L. monocytogenes, instances of post-pasteurization contamination may arise due to inadequate temperature control and technical errors.7 Microbes, especially bacteria, exhibit varying responses to antibiotics and anti-microbial compounds, due to inherent differences or the progress of resistance through application or genetic exchange. Resistant bacteria will cause severe health effects directly or by transmitting antibiotic resistance.8

One promising strategy to improve food safety is the use of bacteriophages to specifically eliminate unwanted bacteria like Listeria. Phages contribute to the natural microflora present in foods, are extensively dispersed throughout the environment, and provide their hosts with a high degree of specificity.9 Because of this, they are perfect for controlling infections without affecting the resident microbiota or starter culture organisms needed to produce fermented foods.10 Hence, current study was conducted with the main purpose of evaluation of application of Listeria monocytogenes bacteriophage as biocontrol agents for dairy products like milk, butter milk, and curd.

MATERIALS AND METHODS

The samples of dairy products viz. milk, butter milk, and curd were collected KMF outlets located around the Bagalkot district, Karnataka, India and transferred to the laboratory.

Morphological characterization

Dairy products were observed for morphological changes like texture, odour and colour before and after two days of incubation and recorded.

Preparation of bacteriophages

Bacteriophages specific to L. monocytogenes were introduced at a dilution of 1:100 in 200 ml of L. monocytogenes and then subjected to incubation at 37°C in a shaking incubator set at 240 rpm. Once the optical density at 600nm reached 0.1, stock phages were introduced at a concentration of 107 plaque-forming units (PFU/g).

Application of LM bacteriophage as biocontrol agents for dairy products

Dairy products underwent treatment with LM Bacteriophage (107PFU/g), while an uninoculated sample served as control. Each treatment was sealed with sterile aluminium foil under various conditions i.e., room temperature, 15-18ºC, 4ºC, and deep freezer (-20ºC). Subsequently, the samples were subjected to microbiological examination i.e., L. monocytogenes colony count.

Statistical analysis

The data were expressed as the mean. Data were subjected to statistical analysis using unpaired t-test to draw a comparison of the L. monocytogenes colony count between control and treatment groups on both the incubations periods i.e., 0th day and 2nd day. $p<0.05$ was considered as statistically significant. Statistical analyses were conducted using SPSS (Statistical Package for Social Sciences) version 21 software (IBM SPASS statistics [IBM corp. released 2011].

RESULTS AND OBSERVATIONS:

Dairy products *viz.* milk, butter milk, and curd were observed for morphological changes like texture, odour and colour changes before and after two days of incubation. Results revealed that after two days of incubation the normal consistency of dairy products was disturbed and observed odour of microbial infection in the dairy products (Table 1 and Figure 1 and 2).

Table 1. Morphological characterization of dairy products

Days	Appearance/ Colour	Texture	Odour	Taste
Milk				
0^{th} Day	White	Watery Consistency	No smell	Normal
2^{nd} Day	Yellowish	Semisolid Consistency	Smell	Bitter
Butter milk				
0^{th} Day	White	Watery Consistency	No smell	Normal
2^{nd} Day	Yellowish	Semisolid Consistency	Smell	Bitter
Curd				
0^{th} Day	White	Normal consistency	No smell	Normal
2^{nd} Day	Yellowish	Hard consistency	Smell	Bitter

Fig 1. Showing dairy products before being infected with L. *monocytogenes*

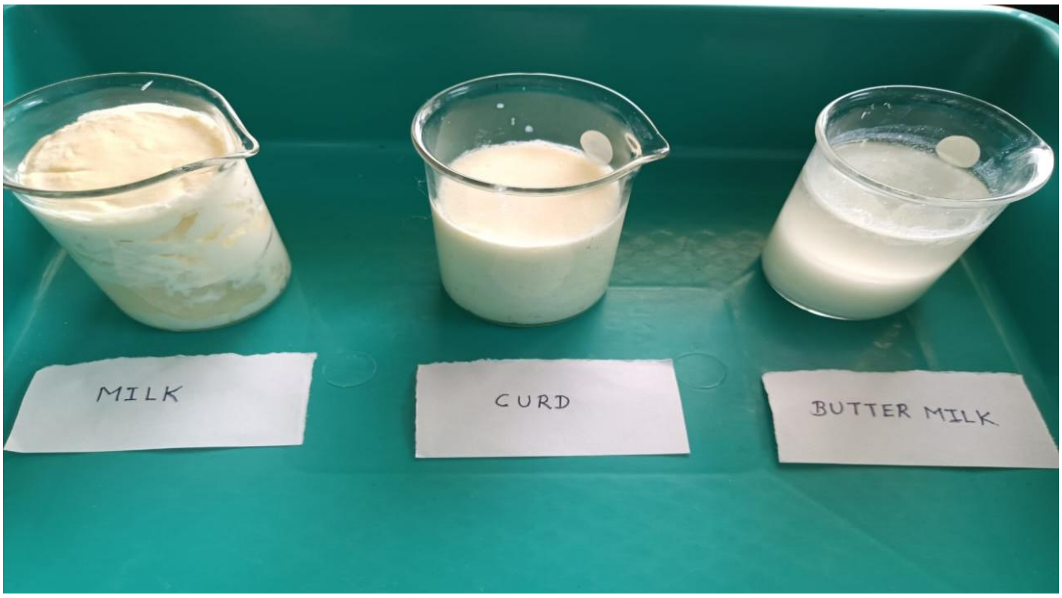

Fig 2. Showing dairy products after being infected with L. *monocytogenes*

Dairy products were treated with bacteriophage (10^7PFU/g) and uninoculated sample served as control. Each treatment was closed with sterile aluminium foil, and incubated at different conditions *viz.* room temperature, at 15-18ºC, at 4ºC and deep freezer (-20ºC) and then were examined by microbiologically for *L. monocytogenes* colony count. Results depicted that on day 2, there was a statistically significant ($p<0.001$) decrease in *L. monocytogenes* colony count was observed in milk samples treated with LM bacteriophage (Table 2).

Table 2. Effect of LM bacteriophage treatment on *L. monocytogenes* colony count in milk sample

Milk Sample			
Incubation Period	***L. monocytogenes* Colony Count**		
	Control	**Milk + LM Phage**	**p-value**
0th Day	360	355	0.062
2nd Day	365	310	<0.0001

Values are expressed as mean; n=3

In butter milk sample also, there was a statistically significant (p<0.001) decrease in *L. monocytogenes* colony count was observed on day 2 (Table 3).

Table 3. Effect of LM bacteriophage treatment on *L. monocytogenes* colony count in butter milk sample

Butter Milk Sample			
Incubation Period	***L. monocytogenes* Colony Count**		
	Control	**Milk + LM Phage**	**p-value**
0^{th} Day	380	365	0.051
2^{nd} Day	390	325	<0.0001

Values are expressed as mean; n=3

Furthermore, there was a statistically significant (p<0.001) decrease in *L. monocytogenes* colony count was observed on day 2 in curd sample also (Table 4).

Table 4. Effect of LM bacteriophage treatment on *L. monocytogenes* colony count in curd sample

Curd Sample			
Incubation Period	***L. monocytogenes* Colony Count**		
	Control	**Milk + LM Phage**	**p-value**
0^{th} Day	428	425	0.1401
2^{nd} Day	410	335	<0.001

Values are expressed as mean; n=3

DISCUSSION

The dairy industry is among the largest food industries in the world, producing a wide range of perishable and semi-perishable products that are widely demanded. It is estimated that, by 2026, the global dairy trade will exceed one billion USD.11 Ensuring the quality and safety of these products is a major concern for dairy companies. However, due to their highly nutritious nature, dairy products are excellent environments for the growth of a wide variety of microorganisms, and undesirable (spoilage-inducing and pathogenic) microbes can be introduced into the dairy chain during any step, from milking to serving.12-14 As a result, many milk products have a relatively short shelf life or are associated with cases of foodborne disease, such as listeriosis.11 L. monocytogenes is the causative agent of listeriosis, a disease that mainly affects specific groups of individuals. It is especially concerning due to the severity of its sequelae and high mortality rate (up to 30%).15,16

Listeriosis is epidemiologically linked to the consumption of foods contaminated with L. monocytogenes, especially ready-to-eat foods that are stored for extended periods at refrigeration temperatures and do not require heating for consumption.16,17 The use of lytic bacteriophages has been successfully tested for the biocontrol of L. monocytogenes in different food matrices such as artificially contaminated dairy products.18,19 With this scenario, present study was conducted with the main purpose of evaluation of application of LM bacteriophage as biocontrol agents for dairy products such as milk, butter milk, and curd.

Our study findings showed deteriorated morphology of dairy products under investigations viz. milk, butter milk, and curd following 2 days incubation due to pathogenic L. monocytogenes infections. However, upon treatment with bacteriophage at 107PFU/g the colony count of L. monocytogenes count significantly decreased. These findings implied that bacteriophages could be used as biocontrol agents for the prevention of dairy products from getting infected with pathogenic L.

monocytogenes infections. In concurrence with out study results, literature reports evidenced that lytic bacteriophages hijack the metabolic mechanisms of their hosts for their growth and multiplication, leading the death of host cells due to lysis, without disturbing the normal microbiota of the food, as there is a high specificity of phage-host interactions.19 Furthermore, Lee et al., showed that different lytic bacteriophages inhibited the growth of L. monocytogenes in a culture medium and milk at various temperatures.20 Moreover, Silva et al., observed that the application of a lytic phage in soft cheeses reduced the initial pathogen counts only at the beginning of the cold storage of the products, indicating that the effectiveness of the phage treatment was dependent on the initial contamination of the product with L. monocytogenes.21

Furthermore, Guenther and colleagues used a lytic bacteriophage to control L. monocytogenes during the production and ripening of soft-ripened cheeses, and observed that a single larger dose of the phage was more effective than repeated smaller applications at reducing the pathogen counts.10 In addition, bacteriophages were able to reduce L. monocytogenes counts below the detection limit when low initial counts of the pathogen were added to cheeses, which was in accordance with Silva et al., observations.21.

CONCLUSION

In conclusion, L. monocytogenes specific bacteriophage treatment of dairy products like milk, butter milk, and curd significantly controls the L. monocytogenes infections. Hence, bacteriophages could be explored as biocontrol agents for the prevention of dairy products from getting infected with pathogenic L. monocytogenes infections. However, although the use of bacteriophages for the biocontrol of L. monocytogenes may offer a safe and ecological approach to reduce the contamination of foods with the pathogen, further studies are warranted to optimize their use in different food products and environments.

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