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Microbial Risk Assessment of Psychrophilic and Psychrotrophic Pathogens in Refrigerated Food Items

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Abstract

Refrigeration is a common practice for food preservation, however it can also support the growth of psychrophilic and psychrotrophic microorganisms, some of which may pose health risks. This study aimed to isolate and identify such microorganisms from refrigerated food samples. A total of thirty samples, including ice cream, dairy products, spoiled vegetables, Indian cooked food and processed food, were collected and inoculated onto various selective media. The plates were incubated at low-temperatures with additional cold-shock treatment to enhance the recovery of psychrophiles. Isolates were identified through Gram staining and biochemical characterization, including Carbohydrate fermentation tests and enzyme tests. Furthermore, the physiological growth patterns of the isolates were analyzed under varying temperature, pH and NaCl concentrations. The antimicrobial susceptibility of the identified strains was also assessed. The findings provide valuable insights into the adaptability, biochemical properties and resistance profiles of psychrophiles and psychrotrophs, contributing to a better understanding of their role in food spoilage and safety.

Keywords: Psychrophiles, Psychrotrophs, Food spoilage, Refrigeration, Antimicrobial Susceptibility.

INTRODUCTION

Refrigeration is a widely used method for food preservation to increase the shelf life of food items by minimizing the microbial growth. However, despite storage at low temperatures, certain microorganisms such as psychrophiles and psychrotrophs, are able to grow and survive leading to spoilage of food and potential health risks. Psychrophilic bacteria are the ones which can grow at low temperatures ranging from -20°C to 15°C whereas Psychrotrophic bacteria are the bacteria which are able to grow at low temperatures but exhibit optimal growth at mesophilic conditions. This cold-adapted nature of microorganisms pose a threat in foodborne diseases and contamination of refrigerated food products.

A food is considered spoiled when the food is not acceptable for human consumption. Acceptance for consumption and organoleptic qualities of food (colour, texture, flavor, shape etc.) being subjective varies with

culture and socioeconomic condition, so the parameters to judge spoilage also change (Kondratowicz and Matusevicius, 2002).

Spores of various *Bacillus* species are metabolically dormant and extremely resistant to a variety of harsh treatments. Due to this extreme resistance, Bacilli are involved in spoilage of food in the fridges and refrigerators and emerging food-borne diseases (Ghosh *et. al*., 2008). *E. coli* strains, especially serotype O157: H7. E. coli O157: H7 consider has major concern in dairy industries and to public health because of its ability to cause severe illness, such as haemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Reuben *et. al*., 2013).

The previous study showed that children who consumed ice cream were suffered from several food-borne diseases like cholera, typhoid, bacillary dysentery, salmonellosis, dysentery, acidity, poisoning, vomiting, dysuria, diarrhea, stomach pain, and weakness (Md.Sohel *et. al*., 2022).

The present study aimed to investigate psychrophilic and psychrotrophic microorganisms from refrigerated food samples. A total of thirty samples were collected comprising of ice cream samples, dairy products, spoiled vegetables, Indian cooked food and processed food. These specimens were directly inoculated onto specific media. The plates were then incubated at low-temperatures with an additional cold-shock treatment applied to enhance the growth of psychrophiles and psychrotrophs. Following incubation, the microorganisms were subjected to gram staining and biochemical characteristics for precise identification. Furthermore the growth pattern of isolates were analyzed under varying physiological parameters, including temperature, pH and NaCl concentration to determine their growth preferences and adaptability. Finally, the Antimicrobial Susceptibility Testing (AST) was conducted to assess resistance profiles of the isolates.

MATERIAL AND METHODS

In the present study, thirty refrigerated food samples were collected, including frozen desserts, spoiled vegetables, dairy products, Indian cooked food and processed food.

Isolation:- The samples were streaked onto various solid culture media including Nutrient Agar, Eosin Methylene Blue (EMB) Agar, Mannitol Salt Agar (MSA), Pseudomonas Isolation Agar, Reasoner's 2A Agar (R2A) and Brain Heart Infusion (BHI) Agar. The inoculated plates were incubated at 4°C, 10°C, and 15°C and monitored daily for microbial growth. Additionally a cold-shock treatment was employed to enhance the growth of psychrophiles and psychrotrophs.

Identification:- For identification of the isolates gram staining was performed, followed by further characterization through various biochemical tests including Indole, Methyl red, Voges Proskauer, Citrate test as well as Carbohydrate fermentation test using Glucose, Lactose, Mannitol, Fructose, Sucrose and enzyme tests including Amylase, Coagulase, Catalase, Urease.

Assessment of growth under various physiological conditions:- All bacterial isolates were inoculated into nutrient broth test tubes and incubated under varying temperature ranges (5°C, 10°C and 15°C), pH ranges (pH 5-5.5, pH 7 and pH 8.5-9) and NaCl concentrations (5%, 10% and 15%). The test tubes were monitored daily for bacterial growth.

Antibiotic Susceptibility Testing (AST):- The Kirby-Bauer disk diffusion method was employed to determine the antibiotic resistance and susceptibility profiles of the identified psychrophiles and psychrotrophs. In the present study the antibiotics used to check the susceptibility were; Ampicillin (AMP), Amoxyclav (AMC),

Chloramphenicol (C), Gentamicin (GEN), Tetracycline (TE), Vancomycin (VA), Ciprofloxacin (CIP), Imipenem (IPM), Erythromycin (E). The antibiotic discs were gently pressed onto the agar surface, then the plates were incubated at 37°C for 18–24 hours. After incubation, the zones of inhibition were measured in millimeters (mm) using a ruler or caliper.

RESULTS

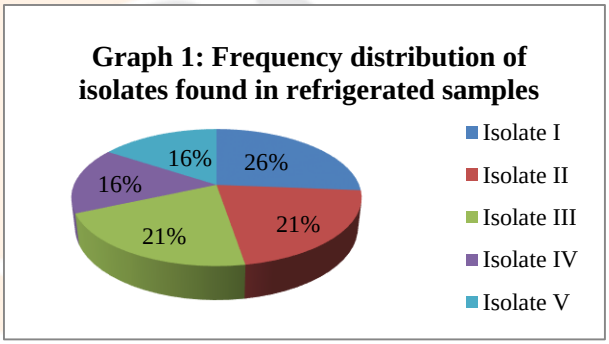
The present study aimed to isolate and identify psychrophilic and psychrotrophic microorganisms from refrigerated food samples, followed by assessment of their growth under varying physiological conditions.

Collection of samples:- A total of thirty samples were collected comprising of ice cream samples, dairy products, spoiled vegetables, Indian cooked food and processed food which are commonly stored under low-temperature conditions.

Isolation:- The isolation of psychrophilic and psychrotrophic microorganisms from the collected refrigerated food samples were carried out by inoculating the samples onto Nutrient Agar, Eosin Methylene Blue (EMB) Agar, Mannitol Salt Agar (MSA), Pseudomonas Isolation Agar, Reasoner's 2A Agar (R2A) and Brain Heart Infusion (BHI) Agar. The inoculated plates were incubated at 4°C, 10°C, and 15°C and additionally a cold-shock treatment was employed.

Table 1: Frequency distribution of isolates in refrigerated samples

Isolates	No. of Isolates	Percentage (%)
Isolate I	5	26.3%
Isolate II	4	21.1%
Isolate III	4	21.1%
Isolate IV	3	15.8%
Isolate V	3	15.8%
Total	19	100%



Identification:-

The purified bacterial isolates isolated from Refrigerated food samples were subjected to a series of morphological and biochemical tests to confirm their identity. Gram staining was initially performed followed by IMViC tests (Indole, Methyl Red, Voges Proakauer and Citrate utilization), Carbohydrate fermentation tests (Glucose, Lactose, Fructose, Sucrose and Mannitol) along with enzyme tests such as catalase, urease, amylase and coagulase tests to further characterize the isolates.

Table 2: Morphological Characteristics of Bacterial Isolates

Isolate	Isolate I	Isolate II	Isolate III	Isolate IV	Isolate V
Size	1-3 mm	0.8-2.5 mm	2-5 mm	0.5-2 mm	3-6 mm
Shape	Rods	Cocci	Rods	Rods	Rods
Color	Green metallic sheen	Golden yellow	Creamy white	White	Off-white
Elevation	Raised	Convex	Flat	Raised	Flat
Margin	Entire	Entire	Irregular	Entire	Irregular

Opacity	Opaque	Opaque	Translucent	Opaque	Opaque
Texture	Smooth	Smooth	Mucoid	Smooth	Dry-Wrinkled
Gram Character	Gram -ve	Gram +ve	Gram -ve	Gram +ve	Gram +ve

Table 3: IMViC and Enzyme Test:-

Isolates	IMViC Test				Enzyme Test			
	Indole	MR	VP	Citrate	Catalase	Amylase	Urease	Coagulase
E. coli	+	+	-	-	+	-	-	-
S. aureus	-	+	+	+	+	+	-	+
P.fluorescens	-	-	-	+	+	+	-	-
L. plantarum	-	+	+	-	-	+	-	-
B. cereus	-	+	+	+	+	+	+	-

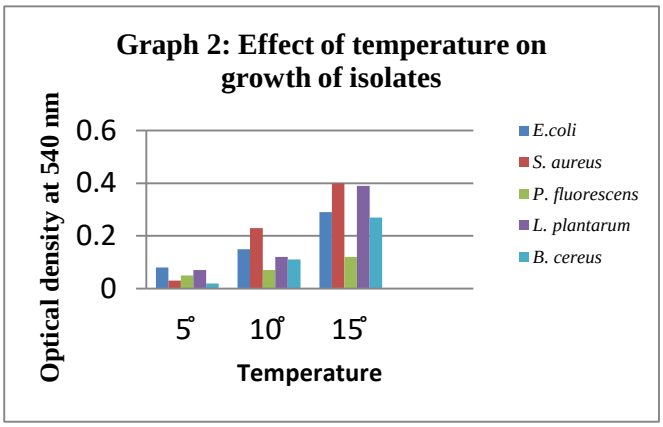
Table 4: Carbohydrate fermentation Test

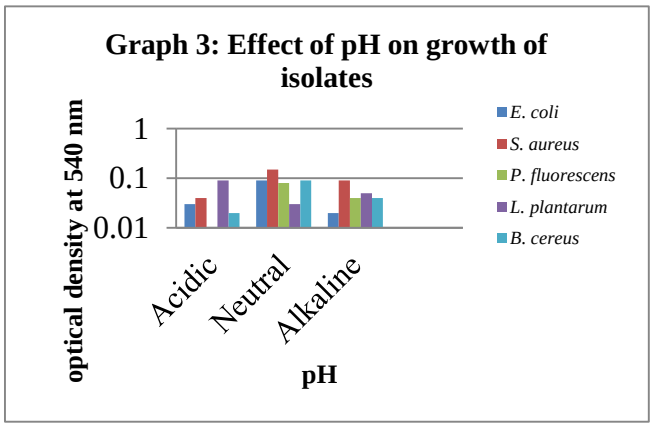
S.No.	Isolates	Lactose		Fructose		Mannitol		Glucose		Sucrose	
		Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
1	*E. coli*	+	+	+	+	+	+	+	+	+	+
2	*S. aureus*	+	-	+	-	+	-	+	-	+	+
3	*P. fluorescens*	-	-	-	-	-	-	+	-	-	-
4	*L. plantarum*	+	+	+	+	+	+	+	+	+	+
5	*B. cereus*	+	-	+	-	+	+	+	+	+	+

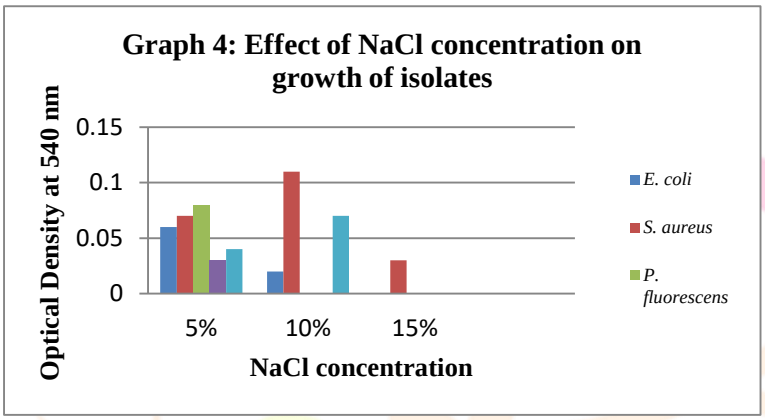
Five bacterial isolates were identified based on their morphological and biochemical characteristics. Isolate I formed green metallic sheen colonies on EMB agar and was identified as ***Escherichia coli***, showing positive results for indole, methyl red, catalase and fermentation of lactose, glucose and mannitol. Isolate II with golden yellow colonies was identified as ***Staphylococcus aureus***, exhibiting catalase, amylase and coagulase positivity and acid production in glucose, fructose, sucrose and mannitol. Isolate III displayed creamy-white colonies and was identified as ***Pseudomonas fluorescens***, positive for Voges-Proskauer, citrate, catalase, and amylase, fermenting only glucose. Isolate IV formed small, white colonies and was identified as ***Lactobacillus plantarum***, showing positive results of glucose, lactose, fructose and sucrose, and amylase. Isolate V with large, irregular colonies was identified as ***Bacillus cereus***, positive for Vages-Proskauer, citrate, catalase, amylase, and urease, fermenting glucose, fructose, sucrose and mannitol.

Table 6: Optimisation of growth under varying physiological condition (OD at 540 nm):-

Parameters		*E. coli*	*S. aureus*	*P.fluorescens*	*L.plantarum*	*B. cereus*
Temperature	5 °C	0.08	0.03	0.05	0.07	0.02
	10 °C	0.15	0.23	0.07	0.12	0.11
	15 °C	0.29	0.40	0.12	0.39	0.27
Std. temp. range		4°- 45°C	7°- 48°C	0°- 35°C	1°- 45°C	4°- 50°C
pH	Acidic	0.03	0.04	0.01	0.09	0.02
	Neutral	0.09	0.15	0.08	0.03	0.09
	Basic	0.02	0.09	0.04	0.04	0.04
Std. pH range		4.4-9.0	4.0-10.0	5.5-8.0	3.0-8.5	4.9-9.3
NaCl conc.	5%	0.06	0.07	0.08	0.03	0.04
	10%	0.02	0.11	0.0	0.0	0.07
	15%	0.0	0.03	0.0	0.0	0.0
Std. NaCl range		5%	15%	Low	7%	7%







Effect of temperature on growth of bacterial isolates:-

The optical density at 540 nm measured at 5°C, 10°C and 15°C revealed the temperature-dependent growth of the isolates. *E. coli* showed increased growth with temperature, with OD values rising from 0.08 (5°C) to 0.29 (15°C). *S. aureus* exhibited a significant OD increase from 0.03 to 0.40, indicating efficient growth at higher temperatures making it cold-tolerant. *P. fluorescens* showed moderate growth (0.05 to 0.12). *L. plantarum* displayed substantial variation with OD peaking at 0.39 (15°C). *B. cereus* exhibited gradual growth, with OD increasing from 0.02 to 0.27. From the results we can categorize *P. fluorescens* as a psychrophile, whereas *E. coli*, *S. aureus*, *L. plantarum* and *B. cereus* can be categorized as psychrotrophs based on their ability to grow at low temperatures with enhanced growth at higher ranges.

Effect of pH on growth of bacterial isolates:-

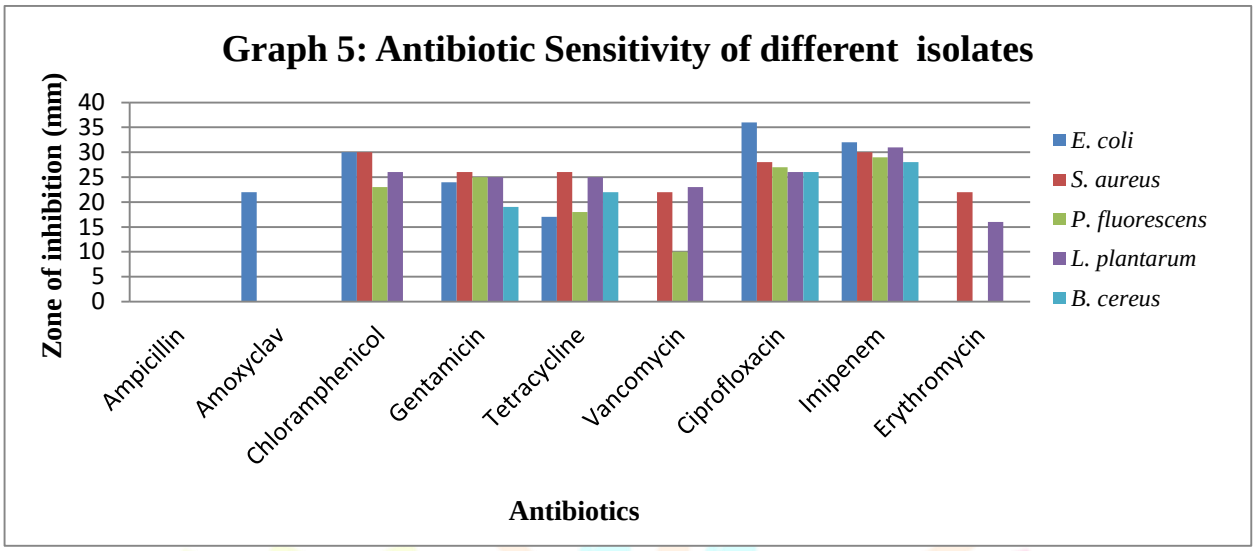
The optical density at 540 nm measured under different pH conditions revealed the pH-dependent growth of the isolates. *E. coli*, *S. aureus*, *P. fluorescens* and *B. cereus* exhibited optimal growth at neutral pH, with reduced growth in acidic and alkaline environments. *L. plantarum* displayed maximum growth under acidic conditions, aligning with its fermentation-based metabolism. The ability of *S. aureus* and *B. cereus* to grow in both neutral and alkaline conditions suggests their adaptability, whereas *P. fluorescens* showed a preference for neutral pH with limited tolerance to alkaline conditions.

Effect of NaCl on growth of bacterial isolates:-

The effect of NaCl concentration on bacterial growth was assessed by measuring OD at 540 nm. *E. coli* and *P. fluorescens* showed optimal growth at 5% NaCl but unable to grow at higher concentrations, indicating salt sensitivity. *S. aureus* exhibited highest growth at 10% NaCl, demonstrating halotolerance. *B. cereus* displayed

moderate growth at 5% and 10% NaCl, while *L. plantarum* showed minimal growth at 5% NaCl and no growth beyond, confirming its salt sensitivity. These findings highlight species specific NaCl tolerance, influencing bacterial survival in saline environments.

Table 7: Antibiotic Susceptibility Testing of isolates:-



Antibiotics	*E.coli*	*S.aureus*	*P.fluorescens*	*L.plantarum*	*B.cereus*
Ampicillin	R	R	R	R	R
Amoxyclav	22 mm	R	R	R	R
Chloramphenicol	30 mm	30 mm	23 mm	26 mm	R
Gentamicin	24 mm	26 mm	25 mm	25 mm	19 mm
Tetracycline	17 mm	26 mm	18 mm	25 mm	22 mm
Vancomycin	R	22 mm	10 mm	23 mm	R
Ciprofloxacin	36 mm	28 mm	27 mm	26 mm	26 mm
Imipenem	32 mm	30 mm	29 mm	31 mm	28 mm
Erythromycin	R	22 mm	R	16 mm	R

Antibiotic Susceptibility Testing of isolates:-

The antibiotic susceptibility of the isolates was determined using Kirby Bauer method. *E. coli* exhibited resistance to ampicillin, vancomycin and erythromycin but was sensitive to ciprofloxacin (36 mm), chloramphenicol (30 mm), gentamicin (24 mm), tetracycline (26 mm), amoxyclav (22 mm) and imipenem (32 mm). *S. aureus* showed resistance to ampicillin and amoxyclav but showed susceptibility to chloramphenicol (30 mm), gentamicin (26 mm), tetracycline (26 mm), imipenem (30 mm), erythromycin (22 mm), vancomycin (22 mm) and ciprofloxacin (28 mm). Similarly the susceptibility patterns of *P. fluorescens*, *L. plantarum* and *B. cereus* to the antibiotics are detailed in Table 7.

Photoplate 1- Enzyme test

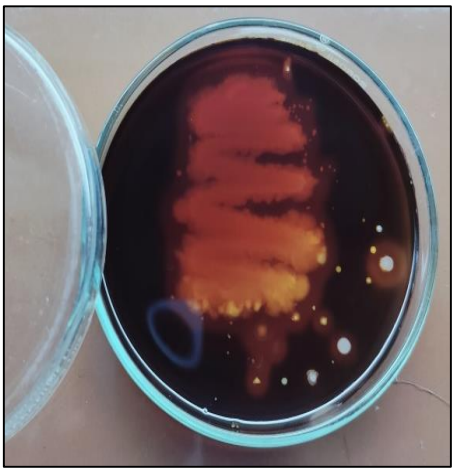


Fig 1- Amylase test

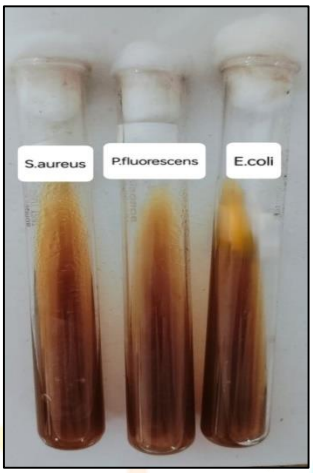


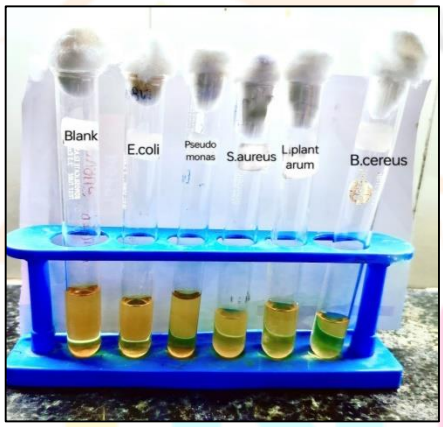
Fig 2- Urease test

Fig 3- Catalase test

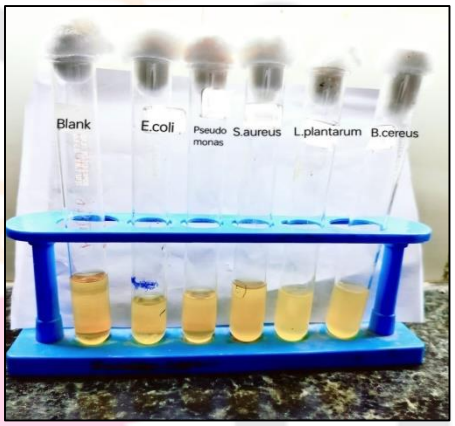
Fig 4- Coagulase test

Photoplate 2- Optimisation of growth under varying physiological conditions

5 °C

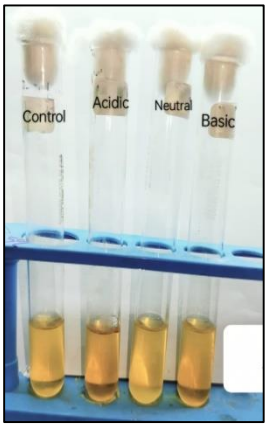


10 °C

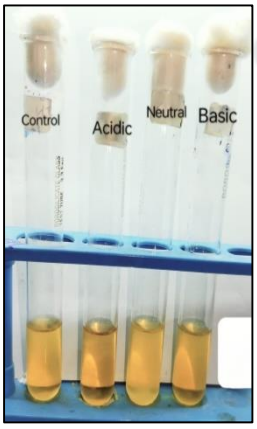


15 °C

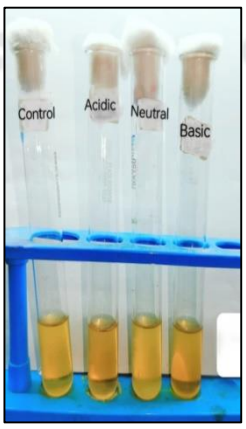
Fig 5- Temperature-Dependent Growth Variations in Bacterial Isolates



E. coli

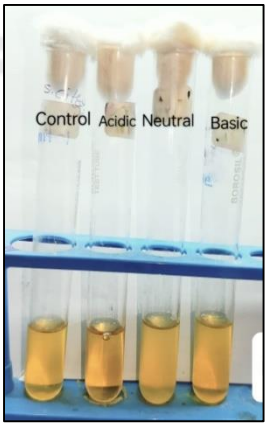


S. aureus



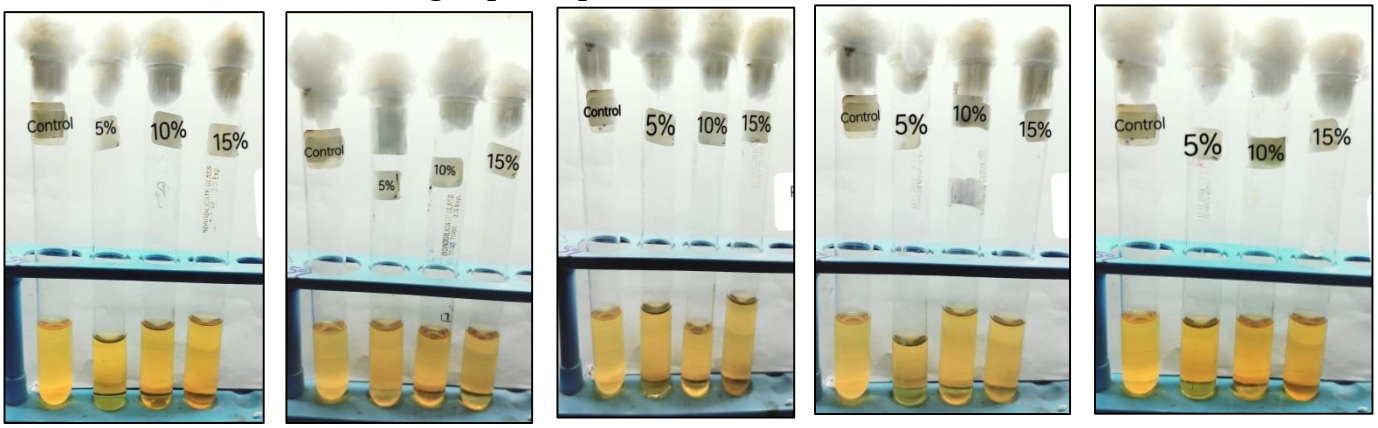
P. fluorescens

L. plantarum



B. cereus

Fig 6- pH-Dependent Growth Variations in Bacterial Isolates



E. coli *S. aureus* *P. fluorescens* *L. plantarum* *B. cereus*

Fig 7- Influence of NaCl on Bacterial growth

Photoplate 3- Antibiotic Susceptibility Testing

Fig 8- Antimicrobial Susceptibility of *E.coli*

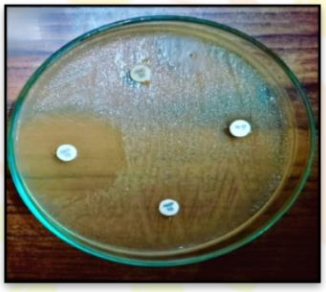


Fig 9- Antimicrobial Susceptibility of *S.aureus*

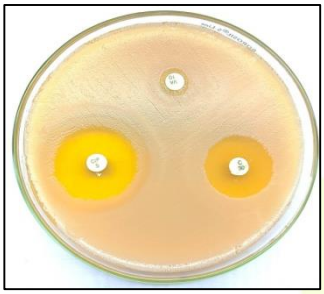
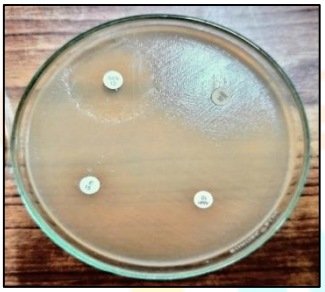


Fig 10- Antimicrobial Susceptibility of *P.fluorescens*



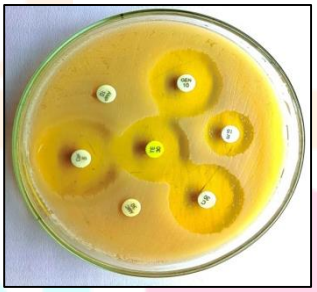


Fig 11- Antimicrobial Susceptibility of *L.plantarum*

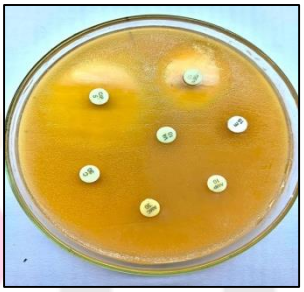


Fig 12- Antimicrobial Susceptibility of *B.cereus*

DISCUSSION

In the present study, out of thirty refrigerated food samples 19 tested positive for bacterial contamination. The isolates include 5 isolates of *Escherichia coli*, 4 isolates of *Staphylococcus aureus*, 4 isolates of *Pseudomonas fluorescens*, 3 isolates of *Lactobacillus plantarum* and 3 isolates of *Bacillus cereus*. These bacteria were identified using selective media. Similar studies by S. Metamoros *et. al.,* (2006), Susmita Das *et. al.,* (2014) and V. B. Hemalata and D. B. M. Virupakshaiah (2016) have reported bacterial contamination in refrigerated foods emphasizing the risk of spoilage and potential health hazards. The growth of isolates was assist under different temperature, pH and Salt concentration. *E. coli* and *P. fluorescens* exhibited moderate growth at 5°C, increasing at 15°C while *S. aureus, L. plantarum* and *B. cereus* thrived at higher temperatures.

Salt tolerance varied, with S. aureus showing the highest resistance at 10% and 15% NaCl while other species demonstrated limited growth at higher concentration.

Antibiotic Susceptibility Testing revealed varying resistance patterns among *E. coli*, *S. aureus*, *P. fluorescens*, *L. planatrum* and *B. cereus*. These findings align with studies by Olopade Bunmi *et. al.*, (2016), Muayad S. Badr (2018) and Abdullah Al Mamun *et. al.*, (2022), indicating an increase in antibiotic resistance strain in food-borne bacteria.

The presence of multi-drug resistance bacteria in refrigerated food samples highlights potential health risks, necessity and strict hygiene measures during storage and handling. Studies by Ali Aydin *et. al.*, (2012) and Susmita Das *et. al.*, (2014) support the importance of maintaining proper food storage conditions to prevent contamination. The results emphasize the adaptability of these bacterial species to refrigeration condition and their potential to cause food-borne illnesses, underscoring the need for regular monitoring and stringent food safety practices.

CONCLUSION

In this study, a comprehensive analysis of refrigerated food samples was conducted to isolate and identify psychrophilic and psychrotrophic bacteria responsible for food spoilage. A total of thirty samples were collected and examined for microbial contamination. The isolation and identification of bacterial species were performed using morphological and biochemical characterization, which led to the identification of *E. coli*, *P. fluorescens*, *S. aureus*, *L. plantarum* and *B. cereus* as the predominant isolates. The presence of these bacteria in refrigerated foods highlights the risk of microbial survival and proliferation under cold storage conditions. Analysis of physiological parameters including temperature, pH and NaCl concentrations, provided insights into the growth preference of the isolates. The results demonstrated that most of the bacteria exhibit optimal growth at 10 °-15 °C, with variations in the pH and salt tolerance, suggesting their adaptability to different storage conditions. The Antibiotic Susceptibility Testing revealed varying resistance patterns among the isolates, with certain bacteria exhibiting resistance to commonly used antibiotics such as ampicillin and vancomycin. This finding underscores the potential risk of antimicrobial resistance bacteria in refrigerated food, which could pose a threat to public health.

The identified bacterial species associated with various food-borne diseases. *E.coli* particularly the pathogenic strains, can cause gastroenteritis and severe food poisoning. *S.aureus* is a well known producer of enterotoxins that can lead to food poisoning with symptoms nausea, vomiting and diarrhea. *P. fluorescens* contributes to food spoilage and can affect immune-compromised individuals. *L. plantarum* is generally considered beneficial but may cause opportunistic infection in susceptible individuals. *B. cereus* is a significant food-borne pathogen capable of producing heat stable toxins, leading to diarrheal and emetic syndromes. The presence of these bacteria in refrigerated foods highlights the importance of proper food handling, storage and hygiene to prevent potential health risks. Despite refrigeration being a common food preservation method, the persistence of these bacteria suggest that prolonged storage may not always ensure food safety. Proper handling, timely consumption, and awareness of microbial contamination risks are essential to minimize food-borne illness and maintain food quality.

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