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Isolation and Identification of Complex Microbiota from Milk and Milk Products and Anti-Microbial Activity of Fruit Peels Against Isolated Bacteria

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Abstract
Milk and milk products are highly nutritious food source that can support growth of many complex microbiota. The development of various culture techniques helps us in isolating and culturing such microorganisms from milk and milk products. Gram staining was used as a preliminary approach. Initially, bacterial identification was done by targeting the cell wall composition by gram staining. Further to identify the genus and species of bacteria different biochemical tests were conducted. These microorganisms enter from a variety of sources and can play a number of roles like facilitating dairy fermentations (e.g., *Streptococcus, Lactobacillus*), spoilage (e.g., *Clostridium, Pseudomonas*), causing disease (e.g., *Salmonella spp., E. coli).* The growth of these causative organisms can be inhibited by the implementation of various anti-microbial tests. The microbial inhibition properties inflicted by voluntarily selected fruit peel extracts was studied against the identified disease-causing microbes. Fruit peels that are being left out after the fruit extract is processed out, are generally rich in bioactive compounds like polyphenols and carotenoids, exhibiting antimicrobial, antioxidant, and anti-cancer properties. The anti-oxidants present in the peels of citrus fruits act against the growth of spoilage and disease-causing microbes.

Keywords: Dairy product, polyphenol, carotenoid, antimicrobial activity, *Vibrio, Staphylococcus, Pseudomonas*.

Introduction:

Milk and milk products like curd, ghee, paneer, cheese etc. are primary source of daily nutrition containing essential nutrients and minerals. High nutrient contents include carbohydrate, fats, proteins, vitamins, minerals and amino acids. These are highly nutritious foods that provide an ideal environment for the growth of many microorganisms due to its high nutrient content **[1]**. Pathogenic microorganisms can introduce contamination into raw milk through two primary pathways. The first way involves

endogenous contamination, wherein the milk becomes contaminated through direct transfer from the bloodstream resulting from a systemic infection or via an infection in the udder known as mastitis. The second way involves exogenous contamination, which occurs during or after the milking process when the milk comes into contact with feces, the external surface of the udder and teats, the skin, the surrounding environment **[2]**.

The consumption of raw milk carries the risk of pathogenic microorganisms, which can pose health risks to consumers. The milk samples analyzed are generally contaminated with coliform, Staphylococcus aureus, Enterobacter aerogenes, and Proteus vulgaris. Psychrotrophic populations, including Pseudomonas and Acinetobacter spp., are also present and can impact milk quality and shelf life **[1]**. Common sources of contamination include hands and clothing of food processors and dirty surfaces of equipment. Microorganisms such as Staphylococcus aureus, Salmonella spp., Escherichia coli, and Shigella spp. have been reported as the most common foodborne pathogens present in many foods, including milk and fermented milk products **[3]**. Lactic acid bacteria, particularly the genus Lactobacillus, are important microorganisms used in the dairy industry for the production of fermented dairy products. Lactobacillus species are classified into three major groups based on their ability to ferment sugars and produce different end products. They are non-motile, non-sporulating, and produce lactic acid as a major product of their metabolism **[4]**. The Lactobacillus genus has beneficial characteristics, such as its ability to resist weak acids and produce high yields of lactic acid, making it useful for industrial applications. Lactic acid is commonly used as an acidulent and preservative in the production of cheese and yogurt **[5]**.

Pathogenic bacteria in milk have been a major factor for public health concern since the early days of the industry, with many diseases being transmissible via milk products. Unclean teats and equipment are sources of contamination by microorganisms. Milk can also be contaminated with various bacteria during milking and processing, including *Staphylococcus aureus* and *Escherichia coli*, which can cause foodborne diseases and mastitis in dairy ruminants **[6]**. The presence of these pathogens in milk largely depends on faecal contamination, which can originate from feed contamination. Poor sanitation during collection and processing of milk is a particular concern in developing countries. Identification of pathogenic bacteria from dairy products is crucial to understand their hazardous effects and microbial quality **[7]**. Various biochemical tests further help in identification of these pathogenic bacteria. Along with identification, they provide valuable information about their metabolic capabilities and biochemical reactions. These tests involve the detection and measurement of specific enzymatic activities and other chemical reactions within microbial cells **[8]**.

Fruits play a crucial role in a healthy diet and are associated with a reduced risk of various chronic diseases. They are rich in bioactive compounds that offer numerous health benefits. However, the processing of fruits and vegetables generates substantial amounts of waste and by-products, posing significant environmental challenges **[9]**. The accumulation of these waste materials has harmful consequences on the environment. Therefore, finding sustainable solutions for managing fruit and vegetable waste is essential to minimize their negative impact on environment **[10]**.

Fruit peels obtained during fruit processing are rich in bioactive compounds like polyphenols and carotenoids, exhibiting antimicrobial, antioxidant, and anti-cancer properties. These findings highlight the potential of fruit peels as valuable sources of beneficial compounds with various health benefits **[13]**. Natural antioxidants often extracted from citrus fruits like oranges can be added to any food to increase their shell life **[11]**. As the orange peel is a valuable by-product that can be utilized for the production of β-carotene. It contains volatile oil and has commercial potential **[12]**.

The risk of spoilage in butter manufacturing due to microbial growth, particularly by Psychrotrophic bacteria, can be encountered by use of orange peel as natural oxidant, as a source of oil extract, and antimicrobial agent in ghee and butter **[14]**. The peels of bananas are known to have potential medicinal properties and have been used in various industrial applications. The presence of chemical components such as phenols, tannins, saponins, alkaloids, steroids, flavonoids, and carbohydrates in banana peels may contribute to their antimicrobial activity **[15]**.

Materials and Methods:

Collection of milk samples:

Dairy products were collected from different sources available in Bhubaneswar, Odisha. A total of 4 samples were analyzed including raw milk, ghee, curd and paneer. These samples were then serially diluted to decrease the initial microbial count present.

Processing of the samples:

Diluted milk samples were directly inoculated in the nutrient agar media aseptically, after inoculation samples were incubated at 37°C for 24 hours. After Gram staining procedure according to morphological analysis various selective media were used to isolate the microorganism.

Direct microscopic examination:

Raw milk sample was spread on microscopic slide using a platinum wire loop to apply the milk resulting in a smear. After drying, xylol was flooded on slide then stained with methylene blue solution and examined under light microscope (40×).

Biochemical tests:

Confirmation of these isolated bacteria was conducted with classical biochemical tests for each experiment carried out in duplicate with appropriate positive and negative controls. Catalase test was used to identify organisms that produce the enzyme, catalase and detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. Production of oxygen gas with bubbles indicates positive result.

Voges-Proskauer test was performed by adding alpha-naphthol and potassium hydroxide to the VP broth to detect the presence of acetoin, a precursor of 2, 3 butanediol. A cherry red colour indicates a positive result for acetoin while a yellow-brown colour indicates a negative result. Indole test was conducted for the identification of organisms having tryptophanase enzymes which convert amino acid tryptophan into indole. The organism was inoculated in a test tube containing culture broth with the addition of 0.5 mL of Kovac's reagent and incubated at 37 °C for 24 hours. Development of red-violet ring layer indicates

positive whereas yellow ring layer indicates negative test. Motility test was used to determine the ability of an organism to swim randomly with flagella monitored under microscope. Turbidity in the entire tube against the current of water streaming indicates that bacteria are motile in a wet mount and vice versa.

Antimicrobial activity:

Nutrient Agar (NA) plates were seeded with 24 hours broth culture of different bacteria. In each of these plates, 4 wells (8 mm) were cut out. Using sterilized dropping pipettes, 0.1 ml of extracts (Apple, Banana, Orange) were carefully added into the wells and allowed to diffuse at room temperature for 2 hours. The plates were then incubated at 37°C for 24 hours. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone. The experiment was carried out in triplicate and the mean of the diameter of the inhibition zones was calculated **[16]**.

Results and Discussion:

Not all microorganisms obtained from Diary and dairy products are useful and health promoting, some of them might be disease and spoilage causing. They can be present from the animal's gut or may be contaminated during collection etc. These can be identified using various biochemical tests.

Biochemical Tests	Milk 10^{-5}	Milk 10^{-4}	Ghee 10^{-3}	Ghee 10^{-2}	Curd 10^{-4}	Curd 10^{-2}	Curd 10^{-4}	Paneer 10^{-4}	Paneer 10^{-3}
Catalase	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
Motility	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve
Urease	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve
Carbohydrate Fermentation	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve
Salt Tolerance	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve
Indole	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
Methyl Red	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve
Voges-Proskauer	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve
Citrate Utilization	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve

[Table.1: Biochemical tests of the isolates which are isolated from Milk, Ghee, Curd, and Paneer]

In the present study total 4 samples were collected from different sources located in Bhubaneswar. In total 4 isolates were selected characterized through colony characters on specific media, and gram staining and biochemical tests and it was found that one isolate is gram positive and other two are gram negative bacteria.

The most common bacterial species found are *E. coli*, *Salmonella*, *Clostridium*, *Pseudomonas*. Naturally, fruits are of high nutritional value, it also contains antioxidants which provides resistance towards microbial activity.

Dairy Products	Concentration of Samples	Gram's Staining Result	Microorganism Identified
Milk	10^{-4}	Negative	*Vibrio*
	10^{-5}	Positive	*Staphylococcus*
Ghee	10^{-2}	Negative	*Pseudomonas*
	10^{-3}	Positive	*Staphylococcus*
Curd	10^{-2}	Positive	*Lactobacillus*
	10^{-4}	Positive	*Lactobacillus*
	10^{-4}	Positive	*Streptococcus*
Paneer	10^{-3}	Negative	*Pseudomonas*
	10^{-4}	Positive	*Staphylococcus*

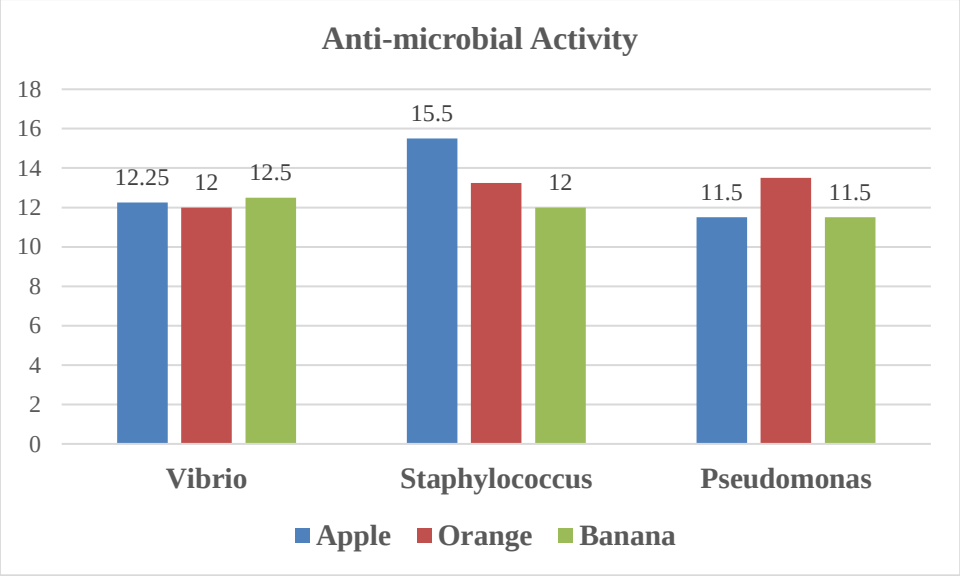
[Table.2: Gram's staining of the isolates and microbial identification]

In this study, the fruit peels were treated with hydro-alcohol to extract the antioxidants present in them. Upon inhibition zone analysis, it was observed that the fruit peels of apple, orange and banana showed a clear zone of inhibition against the disease-causing microorganisms found in the dairy product samples.

Fruit Peel Extract	Zone of Inhibition (mm)		
	Vibrio	Staphylococcus	Pseudomonas
Apple	12.25	15.5	11.5
Orange	12	13.25	13.5
Banana	12.5	12	11.5

[Table.3: Zone of inhibition created by different fruit peel extracts against different bacteria]

Apple had the maximum inhibited zone against *Staphylococcus spp.* of 15.5 mm whereas, orange and banana had the largest zone of inhibition against *Pseudomonas spp.* and *Vibrio spp.* of 13.5 mm and 12.5 mm respectively.



[Fig.1: Graph showing zone of inhibition for different fruit peel extracts against different disease-causing bacteria]

The identified microbial species found in different milk and milk product samples taken are *Vibrio, Staphylococcus, Pseudomonas, Lactobacillus, Streptococcus*. Out of these, *Streptococcus* species and *Lactobacillus* species are used in dairy fermentation processes. *Staphylococcus* species can be harmful if they are present in higher quantities. *Pseudomonas* species take part in spoilage of food items whereas organisms of *Vibrio* species are causative agents in various diseases.

Conclusion:

The milk product samples viz. raw milk, ghee, curd and paneer were serially diluted to minimize the microorganisms present in them. Using culture techniques like mother culture, daughter culture, pure culture and broth culture microbes were isolated and preserved for further use. From the pure culture gram positive and gram-negative bacteria were identified by Gram's staining procedure. Various biochemical tests were conducted further to identify the bacterial genus. These tests included catalase test, motility test, carbohydrate fermentation test, salt tolerance test, indole test, MR-VP test and citrate test. Hence, confirming the presence of *Vibrio, Staphylococcus and Pseudomonas* genus of bacteria. Furthermore, the antimicrobial activity of each identified bacterial samples were tested using antioxidants extracted from peels of apple, orange and Banana by agar well diffusion method. Zone of inhibition was calculated for each peel extract. The following conclusions were made after analyzing the anti-microbial activity against fruit peels:

1. For *Vibrio*, the anti-microbial activity of Banana>Apple>Orange.
2. For *Staphylococcus*, the anti-microbial activity of Apple>Orange>Banana.
3. For, *Pseudomonas*, the anti-microbial activity of Orange>Apple=Banana.

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Conflict of Interest:
Nil

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