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Microbial consortia in a household Dahi sample: An assessment

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Abstract
This study intended to disclose the microbial tapestry of a randomly collected household dahi sample that exhibited ropiness. Pour plating in Nutrient agar and MRS agar revealed the prevalence of four types of colonies with distinct colony morphology. Preliminary characterisation indicated that none of them belonged to lactic acid bacteria. Molecular level identification confirmed the isolates as *Pichia kudriavzevii* strain DMA06, *Saccharomyces cerevisiae* strain DMA05, *Staphylococcus warneri* strain DMA04 and *Staphylococcus saprophyticus* strain DMA03. Both Staphylococcal strains were lipolytic and hemolytic but coagulase negative and failed to liquify gelatin. Remarkable proteolytic activity was evident for *Saccharomyces cerevisiae* and *Staphylococcus saprophyticus*. Isolates except *Pichia kudriavzevii* DMA06 showed growth both at 70 °C and 450 °C. It was also observed that none of the isolates were completely inhibited by 6.5 % NaCl. Prevalence of resistance to B-lactams in staphylococci can be a safety concern.

Keywords: Dahi, yeast, pathogens, backslopping

Introduction
In recent years, the exploration of microbial communities inhabiting various ecosystems has garnered significant attention due to its implications for human health, food safety, and environmental sustainability. Among these ecosystems, fermented foods represent unique niches where diverse microbial consortia thrive, contributing to both the sensory attributes and nutritional qualities of these products. Household dahi, a traditional fermented milk product popular in many cultures, serves as an excellent model system for studying microbial consortia dynamics [1, 2]. The process of preparing dahi typically involves inoculating fresh milk with a small portion of previously fermented dahi, a practice commonly known as backslopping. This traditional method not only serves as a means of inoculating desirable microorganisms but also facilitates the establishment of a stable microbial community. While the presence of lactic acid bacteria (LAB) such as Lactobacillus and Streptococcus species is traditionally associated with dahi fermentation, recent studies have highlighted the presence of other microbial taxa, including opportunistic pathogens and yeast species, in these consortia [3, 4].
Of particular interest is the prevalence of opportunistic pathogens like Staphylococcus species within household dahi. Despite the potential health risks associated with these microorganisms, their presence underscores the complexity of microbial communities in fermented foods and the need for comprehensive investigations into their dynamics. Additionally, the occurrence of yeast species, notably Pichia, further enriches our understanding of the microbial diversity within dahi consortia [5]. Remarkably, some studies have reported instances of dahi fermentation occurring in the absence of typical LAB populations, challenging conventional notions regarding the essential microbial players in this fermentation process. This phenomenon underscores the resilience and adaptability of microbial communities in fermented foods, prompting further exploration into the ecological factors shaping their composition and function [1, 6].
In this article, we aim to provide a comprehensive overview of microbial consortia inhabiting household dahi prepared by backslopping, with a specific focus on the prevalence of opportunistic pathogens such as Staphylococcus species and yeast species like Pichia. By elucidating the dynamics of these microbial communities, we can gain valuable insights into

the factors influencing dahi fermentation, as well as potential strategies for enhancing the safety and quality of this traditional fermented product.

Materials and Methods

Study situation

As a part of isolation of lactic acid acid bacteria, a random house hold dahi sample was collected from Thrissur district, Kerala. As per the literatures the main flora that contributing to dahi fermentation is lactic acid bacteria. The sample was Appropriately diluted and pour plated in MRS agar as well as nutrient agar and incubated at 37 ℃ for 48h. After incubation four types of colonies was observed in the plates but an interesting fact was noted that none of these isolates were catalase negative (lactic acid bacteria belongs to catalase negative group). So, we started to study four types of the flora present in the sample.

Preliminary Characterization of isolates

Primary identification tests such as Grams reaction, catalase test and oxidase test were conducted as per the procedures described by Barrow and Feltham [7].

Molecular Level Identification

16S rRNA sequencing was done by outsourcing for molecular level confirmation of the isolates. The isolates were sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for identification.

Growth at Different Temperature

Sterile nutrient broth tubes were inoculated with two percent of freshly activated culture and incubated at different temperatures: 7 ℃, 15 ℃, 37 ℃ and 45 ℃. After 24h, the growth was qualitatively evaluated by comparing the turbidity developed with that of control. Uninoculated broth tubes served as control.

Growth at Different Salt Concentrations

Freshly activated culture was inoculated at a level of two percent in sterile nutrient broth tubes containing Sodium chloride at a level of 4 and 6.5 per cent. After incubation at 37 ℃ for 24 h, the turbidity developed was compared with control. Uninoculated broth tubes with salt served as control.

Carbohydrate Fermentation Test

The potential of the isolates to ferment carbohydrates was determined, using andrade petone water. The color change from light pink to dark pink was indicative of acid production [8].

Proteolysis and lipolysis

The enzymatic activity of the isolates are determined in terms of proteolytic and lipolytic activity. for the evaluation of proteolytic and lipolytic activity. The isolates were streaked on skim milk agar and Tributirin agar respectively, and incubated at 37 ºC for 24h [9].

Hemolytic property and Gelatin liquefaction

All the four isolates were streaked on blood agar and incubated at 37 ℃ for 24 h. Plates were examined for the presence of zones around the growth. Those producing zones of blood lyses around the colonies (Beta-hemolysis) were classified as hemolytic [10]. For checking the gelatin liquifying ability, the isolates were streaked on gelatin agar (HiMedia) slant and incubated at 37 ºC for 24 h. Tubes were examined for gelatin liquefaction after 3h of refrigeration.

Antibiogram of isolates

The antibiotic susceptibility of isolates was done by using antibiotic disc diffusion assay. MRS agar plates were spread with 100 µl of an overnight LAB culture and antibiotic discs were placed under sterile conditions. After incubation for 24 h at 37 °C, the diameter (mm) of inhibition zone was measured and results were expressed as resistant and susceptible [11].

Biofilm forming ability

Freshly activated cultures were streaked on congored agar for the detection of biofilm formation [12].

Coagulase test for *staphylococcus* strains

Bacterial cultures were incubated at 37 °C for 16 hours in tryptic soy broth. One tenth milliliter of each culture was placed in a glass tube containing 0.4 ml of plasma (Himedia). The coagulase-positive organism caused the plasma to form a clot in the tube. Whereas, the coagulase-negative organism.

Staphylococcus isolates reaction on MSA

Bacterial cultures were streaked on mannitol salt agar and incubated at 37 °C for 24 hours.

Results and Discussion

Microscopic examination of curd sample revealed the presence gram positive cocci and yeast in the sample. A total of four types colonies found on the nutrient agar plated with different dilutions of dahi sample. The preliminary characteristics of the isolates are plotted on the table Sample dahi with a typical ropy nature is depicted in the Fig 1.

Table I: The preliminary characteristics of the isolates

Isolates	Grams reaction	Catalase	Oxidase
DMA03	Gram positive cocci	Positive	Positive
DMA04	Gram positive cocci	Positive	Positive
DMA05	Gram positive (Yeast cells)	Positive	Negative
DMA06	Gram positive (yeast cells)	Positive	Negative

Molecular level identification of the isolate revealed that none of the isolate belong to the lactic acid bacteria (Commonly use starter culture for the dahi preparation). The isolates one, two, three and four being identified as *Pichia kudriavzevii* strain DMA06, *Saccharomyces cerevisiae* strain DMA05, *Staphylococcus warneri* strain DMA04, *Staphylococcus saprophyticus* strain DMA03 (Fig 2) and deposited in NCBI with accession number ON506052, ON506046, ON506040 and ON506028 respectively.

Fig 1: Dahi sample exhibiting ropiness

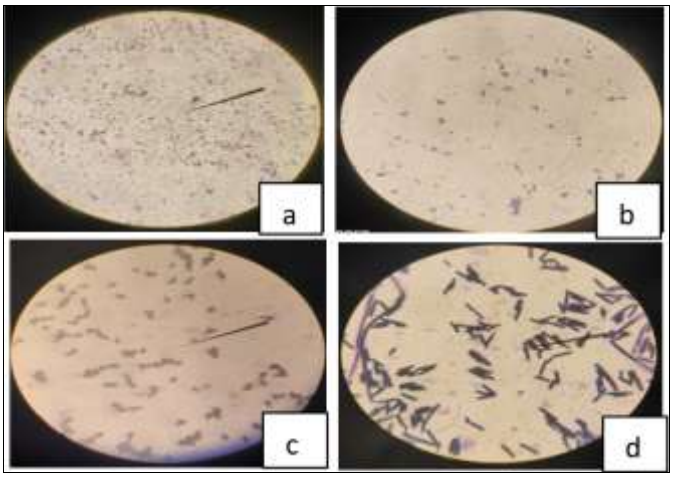

Fig 2: Gram staining reaction: *S. warneri* DMA04 (a), *S. saprophyticus* DMA03 (b), *S. cerevisiae* DMA05(c) and *P. kudriavzevii* DMA06 (d)

Dairy products like yoghurt and sour milk, are prone to contamination by yeast species, which ultimately lead to deterioration. This is because these items low pH provides an environment that is conducive to their growth [13]. staphylococcal food poisoning is one of the most prevalent types of foodborne illness and is likely to occur in every country in the world. Inadequate inquiry or laboratory examination often inhibits correct diagnosis, even in cases where the symptoms are recorded. Because enterotoxigenic staphylococci grow in food, enterotoxins found in the food are what cause the sickness. Despite large variations in kitchen practices, cleaning regimes and environmental quality core kitchen microbiota comprised of eight genera including staphylococci as per Moen *et al.* [14] from a study in household across five countries. Microbes colonising refrigerators in kitchen could pose a risk of indirect long-term contamination of subsequent food preparation activities [15].

All the isolates grew optimally at 37 °C after 24 h incubation (Table II). Nevertheless, at 7 °C growths were drastically reduced for all the isolates examined. Growth of staphylococci and production of toxin are greatest most commonly reported to be at 20–37 °C, but growth can occur between 8 and 48 °C [16]. Furthermore, all the isolates tolerated increasing NaCl concentration to 6.5%. Members of the Staphylococcus genus have been isolated from diverse environments and characterized as having salt-tolerance potential [17]. Adaptation of yeast cells and staphylococcal sp in high level saline conditions are reported by several authors in their studies validating our results [18, 19].

Table II: Growth characteristics of isolates

Isolates	Growth at different temperature (0C)				Concentration of NaCl (%)	
	7^0C	15^0C	37^0C	45^0C	4%	6.5%
DMA03	+	++	++++	+++	++++	+++
DMA04	+	+	++++	+++	++++	+++
DMA05	+	++	+++	++	+++	+
DMA06	-	+	+++	++	+++	+

--No growth, +-less growth, ++- Moderate growth, +++-Good growth, ++++-Heavy growth

Both the Staphylococcus isolates were found to be alpha hemolytic in nature but none of the isolates were showed gelatin liquifying nature. When the hemoglobin in red blood cells is converted to methemoglobin, an α-hemolytic reaction takes place, which results in a greenish tint on the agar surrounding the colonies. The bacteria that are most commonly isolated from human illnesses and bovine mastitis are Staphylococci. The many hemolysins, which are crucial for the virulence of Staphylococci, are one of the virulence factors that combine to cause diseases caused by this genus [20].

Table III: Carbohydrate test for isolates

Isolates / Carbohydrates	Staphylococcus	Staphylococcus	*Saccharomyces cerevisiae*	*Pichia kudriavzevii*
Lactose	-	-	-	+
Sucrose	-	+	+	-
Maltose	+	+	+	+
Mannitol	+	-	-	-
Arabinose	-	-	-	_

+- Positive - -negative

In carbohydrate fermentation, an interesting observation was found that the Pichia was lactose positive and that was it can be assumed the responsible microbe for the milk fermentation, in place of lactic acid bacteria. The yeasts that assimilate lactose aerobically are widespread, but those that ferment lactose and producing lactic acid are rather rare, including *Kluyveromyces lactis, K. marxianus* and *Candida pseudotropicalis* are some of the yeasts found to lactose fermenters according to the literatures. Because of its potential use in the food and biotechnology industries, *Pichia kudriavzevii* is a newly discovered non-conventional yeast. widespread in many different environments, it frequently happens during the natural fermentation of traditional fermented foods and drinks. *P. kudriavzevii* is a good starter culture for the food and feed industries because of its abilities to break down organic acid, release different hydrolases and taste compounds, and exhibit probiotic qualities. Its natural qualities also provide it the ability to overcome technical difficulties in industrial applications because to its strong tolerance to severe pH, high temperature, hyperosmotic stress, and fermentation inhibitors. *P. kudriavzevii* has been isolated from several indigenous fermented milk products from different areas [21]. Several studies highlighted the spoilage potential of *P. kudriavzevii* because the release of alcohols and esters thought to be associated with off-flavors of fermented milk beverages [22].

Table IV: Antibiogram study of isolates

Isolates / Antibiotics	*S. warneri*	*S. saprophyticus*	*Pichia kudriavzevii*	*Saccharomyces cerevisiae*
Pencillin	R	R	S	I
Amoxyllin	I	R	I	I
Streptomycin	S	S	S	S
vancomicin	S	S	R	S
Azythromicin	S	S	S	S
Tetracycline	R	R	S	S

R-resistant S-susceptible I-Intermediate resistant (CLSI Guide lines)

All the isolates showed some extent of resistance against beta lactam group of antibiotics. It is major concern in our society. Beta lactam group of antibiotics are them. There is a risk associated with the ability of these resistant strains to transmit the resistance gene to pathogenic bacteria [23]. This may result to highly antibiotic resistant enteropathogenic bacteria. *Staphylococcus* strains Have a notorious ability to become resistant to antibiotics. These organisms have the potential to spread throughout our biosystem, so it's important to pay strict attention to infection control measures and medication stewardship on drug resistance of pathogen to prevent the development of even more harmful diseases.

In congored agar all the isolates gaves a negative reaction i.e, none of the isolates gave a black colony on the congored agar indicates the inability of the isolates to produce biofilms [24]. The proteolytic and lipolytic activity of the isolates were tested. All the isolates were found to be proteolytic in nature but only staphylococcus strains showed the lipolytic activity (Fig. 3)

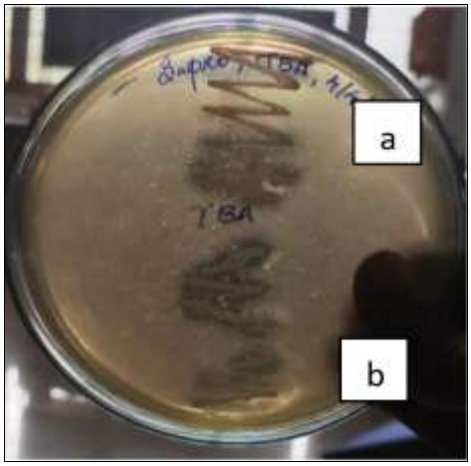

Fig 3: Lipolysis in TBA a) *Staphylococcus saprophyticus* b) *Staphylococcus warnerii*

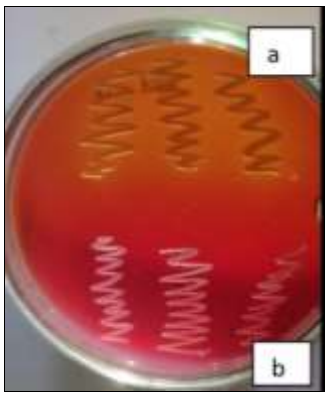

Fig 4: Mannitol salt agar plates a) *Staphylococcus saprophyticus* b) *Staphylococcus warnerii*

Pathogen test for staphylococcus were done using coagulase test, gave no reaction for both. *Staphylococcus saprophyticus* gave a positive reaction in MSA reaction, the agar become red to yellow (Fig. 4). Mannitol Salt Agar (MSA) is a selective medium used for isolation, enumeration, and differentiation of pathogenic staphylococci. Several studies have also reported the presence of coagulase-negative. Staphylococci, are usually resistant to β-lactam antibiotics and other antibiotic classes and exhibit Multiple drug resistance. This report aligns with the findings of our study [25, 26]. *Staphylococcus* spp. have been implicated in many nosocomial infections. They are considered a potential pathogen that can cause various inflammatory diseases in immunosuppressed patients. After *Escherichia coli*, *Staphylococcus saprophyticus* is the second most common acute agent of community-acquired urinary tract infections (UTI). It is noteworthy to highlight the importance of detecting not only the pathogenic microbiota but also their toxic metabolites so that putative outbreaks can thereby be prevented or detected even before they cause harmful effects to human health. The major cause of subclinical mastitis is coagulase-negative staphylococci is mainly involved in clinical mastitis of dairy herds [27]. Multiple drug resistant is a major public health concern and is reported for major community-associated outbreaks in worldwide. The occurrence and distribution of these organisms have been reported to be from the environment, especially different types of water available for human consumption and/or recreation has also been reported like foods, water, soil etc. Joen *et al*. [28] in their studies house hold bacterial community observed that bacterial communities from refrigerators and toilet shared more species in common with human skin than with human gut. Human skin is an important sources of indoor bacteria that could adhere and survive for long periods on indoor surfaces [29].

Conclusion

Our study encounter on the point that the need of critical evaluation of dahi or other household indigenous products to ensure the consumer safety as well as the wellbeing. It is necessary to develop quick and accurate detection techniques since Staphylococcus species are becoming human pathogens and carriers of antibiotic resistance determinants. If consumers are well- informed about the dangers of consuming contaminated products, they will demand the highest safety standards from the milk distributors and regulatory authorities. The outcome of all these would be a well-functioning food system that not only brings economic incentives, but also protects the health of the consumers.

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