

**Yeast Communities in *Dahi* and *Chhurpi*, Ethnic
Fermented Milk Products of Sikkim: Molecular
Characterization and Probiotic Properties**

A Thesis Submitted
To
Sikkim University



In Partial Fulfilment of the Requirement for the
Degree of Doctor of Philosophy

By
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JULY 2025



*In loving memory of our beloved
Sir Sr. Prof. Dr. Jyoti Prakash Tamang
16th November 1961 – 29th April 2025
Your light lives on.
Forever in our hearts.*



Dedicated to my family:

Dhanbir Lama (Father)

Indira Lama (Mother)

Saila Lama (Grandfather)

Saili Lama (Grandmother)

Sherab Lama (Uncle)

Soni Lama (Sister)

Chhewang Lama (Brother)



तुम यसक काह को डर ना हो-१३

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Submitted by **Shri Sonam Lama** under the supervision of **Dr. Vijay Shankar Singh**, Assistant Professor, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok.

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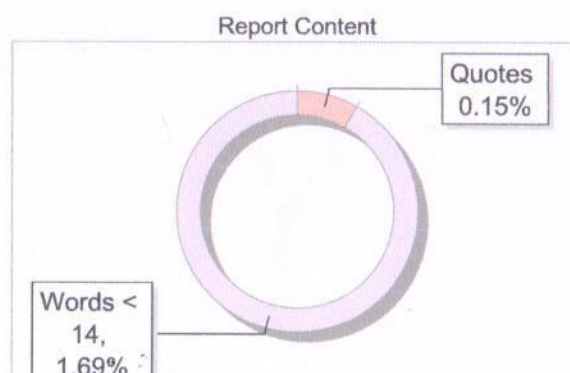
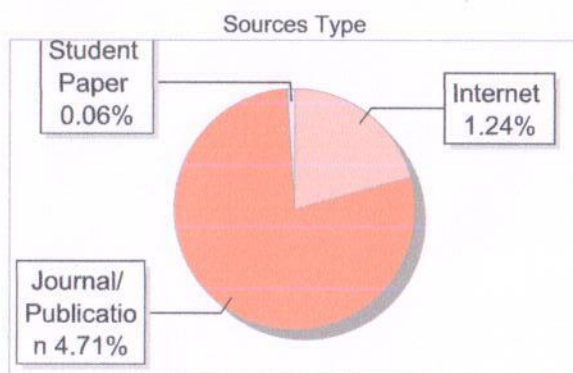
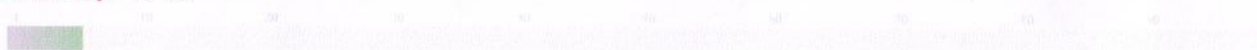
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DECLARATION

I declare that the present Ph.D thesis entitled “**Yeast Communities in *Dahi* and *Chhurpi*, Ethnic Fermented Milk Products of Sikkim: Molecular Characterization and Probiotic Properties**” submitted by me for the award of the degree of **Doctor of Philosophy** in Microbiology in Sikkim University, under the supervision of **Late Sr. Prof. Dr. Jyoti Prakash Tamang**, Senior Professor, Department of Microbiology and **Dr. Vijay Shankar Singh**, Assistant Professor, Department of Microbiology, School of Life Sciences, Sikkim University, is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. No part thereof has been submitted for any degree or diploma in any University/Institution.


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INTRODUCTION

1. INTRODUCTION

Traditionally prepared fermented foods have achieved an important place in human society as fermentation improves the durability, shelf life, taste, texture, aroma, and nutritional content of food (Tamang et al., 2020). Additionally, they harbour beneficial microorganisms which contribute to gut health and overall well-being (Tamang et al., 2016). Around 3500 fermented foods and alcoholic beverages from all over the world have been documented and reported by Campbell-Platt (1994), who also classified them into around 250 groups. Today, billions of people throughout the world consuming over 5000 distinct variety of fermented foods and alcoholic beverages as staples and other culinary ingredients (Tamang, 2010a; Ray and Joshi, 2014; Tamang et al., 2020; Ray et al., 2024). Based on the substrates or raw ingredients derived from animal or plant origin, fermented foods are grouped or categorized into nine major categories as fermented (1) milk products, (2) cereals, (3) legumes, (4) alcoholic beverages, (5) roots and tubers, (6) vegetables and bamboo shoots, (7) fish products (smoked and dried), (8) preserved meat products, and (9) miscellaneous fermented products (Steinkraus, 1997; Tamang, 2010b; Tamang and Samuel, 2010). The traditionally fermented milk products have gained global popularity owing to their enhanced nutritional value and influence of modern technological advancement (Shiby and Mishra, 2013; Bintsis, 2023). The Food and Agriculture Organization (FAO) described fermented milk as a product made by fermenting milk with the assistance of appropriate microorganisms, which must be active, viable, and prevalent in the product until its minimal durability and pH is lowered (Alimentarius, 2018). Based on microorganisms, fermented milk products are divided into two main categories: (a) lactic acid fermentation, fermented by Lactic Acid Bacteria (LAB) species, including probiotic type (e.g., acidophilus milk, bifidus milk), mesophilic (e.g., natural fermented milk, cultured milk, cultured buttermilk, cultured

cream), and the “thermophilic” type (e.g., Bulgarian buttermilk, yogurt); and (b) lactic-fungal fermentations, where the interaction of Yeasts and LAB produces the end result, which includes alcoholic milks (e.g., *kefir*, *koumiss*, acidophilus-yeast milk), and moldy milks (e.g., *viili*) (Tamang et al., 2016).

Naturally fermented milk (NFM) products are usually prepared utilizing one of the oldest approaches of milk fermentation known as “back-slopping” approach, where the previous batch of the fermented product is employed to re-inoculate the fresh batch (Tamang et al., 2016; Tamang et al., 2020). In the Himalayan regions of India, Nepal, and Bhutan, ethnic communities consume a variety of fermented milk products made from yak and cow milk (Tamang et al., 2016; Rai et al., 2016). The Northeastern part of India can be recognized for its diverse ethnicity, rich cultural legacy, and linguistic variations. It is inhabited by diverse ethnic groups, tribes, and castes from various linguistic groups, racial stocks, and socio-cultural heritage (Baruah, 2007; Gupta, 2018; Thapa, 2022). In 2002-2003, Sikkim became the eighth member state to join the North Eastern Region (Bhattacharyya, 2018). Sikkim is a multi-ethnic society which embodies mixed cultural heritage and linguistic diversity consisting of various communities (Government of Sikkim, 2006), which are allocated into three predominant ethnic groups- *Lepcha*, *Bhutia* and *Nepali* (Thapa, 2022). The term ‘*Nepali*’ is generic encompassing various hill tribes and the Hindu castes which incorporates various communities within this broad category (Sinha, 1975; Government of Sikkim, 2006). Various communities are encompassed under the *Nepali* constellation, each embodying unique sociocultural structures, which includes *Bahun*, *Chettri*, *Sanyasi/Jogi*, *Kami*, *Damai*, *Sarki*, *Sunwar*, *Sherpa*, *Mukhiya*, *Giri*, *Bhujel*, *Yakha Dewan*, *Magar*, *Gurung*, *Sunuwar*, *Majhi*, *Thami*, *Limbu*, *Tamang*, *Rai*, and *Newar/Pradhan* (Thapa and Tamang, 2020). Ethnic communities from Sikkim prepare and consume a range of traditionally

prepared fermented milk products prepared from cow milk as well as yak milk, such as *dahi*, *chhurpi* (hard and soft variety), *mohi*, *gheu*, *chhu*, *somar*, *maa*, *philu*, and *shyow*. Some of the milk products, notably *dahi* (curd), *mohi* (buttermilk), *gheu* (butter), and *chhurpi* (hard and soft variety) are acquainted in all locality of the Sikkim Himalayas (Tamang, 2005; Tamang, 2010c; Rai et al., 2016).

The traditionally fermented dairy products are among of the most frequently utilized methods to deliver probiotic culture (Sanchez et al., 2009; Ali et al., 2022; Jang et al., 2024). The term “probiotic” originated from the Greek language which means “for life” (Zendeboodi et al., 2020; Georgieva et al., 2022; Dimitrellou et al., 2025). Probiotics are defined as “*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*” (Hill et al., 2014). Numerous investigations have validated the health advantages pertaining to probiotics in relation to gastrointestinal infections, enhancement of the lactose metabolism, modulation of the GI barrier function, immune system stimulation and modification, anti-carcinogenic, anti-mutagenic, anti-diarrheal properties, antimicrobial activity, enhanced activity of certain beneficial enzymes, improvement of inflammatory bowel syndrome, establishing brain-gut axis communication, inhibition of pathogenic microbes, and reduction in serum cholesterol level (Ritchie and Romanuk, 2012; Kechagia et al., 2013; Alakeji et al., 2015; Tomićić et al., 2016; Brasiel et al., 2020; de Andrade Calaça et al., 2017; Rondanelli et al., 2017; Liu et al., 2018; Skonieczna-Żydecka et al., 2018; Nadeem et al., 2019; Sen and Mansell, 2020; Dai et al., 2022; Moonsamy et al., 2024; Kazemi et al., 2025). The International Scientific Association for Probiotics and Prebiotics (ISAPP) has recognized that consuming foods with live microorganisms, particularly fermented dairy products, is linked to a reduced risk of certain health problems (Hill et al., 2014). In contrast to bacteria, the potentiality of yeast as probiotics is currently less frequently investigated

and recognized (Didari et al., 2014). In terms of selecting probiotics, there have been a numerous test criteria offered, but none of them have gained widespread acceptance as gold standards (Binda et al., 2020). FAO/WHO guidelines require that probiotic microbes survive and proliferate in the human Gastrointestinal tract and also be safe concerning human consumption (FAO/WHO, 2002). The European Food Safety Authority (EFSA) does not establish selection criteria for probiotics; rather, they have particular standard guidelines for microorganisms, including yeasts, that can be used as supplements in animal feed. According to EFSA, these microbes must be identified at the strain level, followed by scientific assessments for antimicrobial production, toxigenicity, and pathogenicity (EFSA FEEDAP Panel, 2018). The ICMR-DBT guidelines has specified certain *in vitro* tests, including resistance to gastric acidity, bile, and antimicrobial activity, along with preclinical and clinical safety and efficacy studies of probiotics (Ganguly et al., 2011). Therefore, lack of standard guidelines and selection criteria for evaluating efficacy and safety is a major challenge in probiotic research and their potential commercial use in the future (Fernández-Pacheco et al., 2021a). Safety studies are another important criterion in the selection of probiotic yeasts (FAO/WHO, 2002). The lack of safety studies, such as pathogenic potential, genomic structure specifically for potential virulence genes, and its general stability over time, are other hurdles in its application in food and therapeutics (Kunyeit et al., 2024). The majority of probiotics are LAB and belong to the genera *Enterococcus*, *Lactobacillus*, and *Bifidobacterium* (Alvarez et al., 2023). Compared to bacteria, a lower number of commercial preparations are available in the market (Alvarez et al., 2023). One of the advantages of yeasts is their natural resistance to antibiotics, whereas the possible transfer of antibiotic resistance genes between lactic acid bacteria and pathogenic bacteria constitutes a threat (Galli et al., 2022). *Saccharomyces cerevisiae* var. *boulardii*

is the most common probiotic yeast distributed in the market presently (Galli et al., 2022). Indeed, hardly any studies have explored the effectiveness of probiotic yeasts, like *S. cerevisiae* var. *boulardii* and *Kluyveromyces marxianus*, which are the only two commercialized yeasts available until now (Homayouni-Rad et al., 2020; Czerucka et al., 2007). *S. cerevisiae* is widely regarded as the principal yeast species in the formulation of various foods and food additives/supplements, which has been granted the “Qualified Presumption of Safety” (QPS) status by the EFSA (Siesto et al., 2022). A number of non-Saccharomyces species, including *Kl. lactis*, *Kl. fragilis*, and *Debaryomyces hansenii* were certified by the EFSA and added to the list of QPS species (EFSA BIOHAZ Panel, 2012; Fernández-Pacheco et al., 2021b; Kazemi et al., 2025).

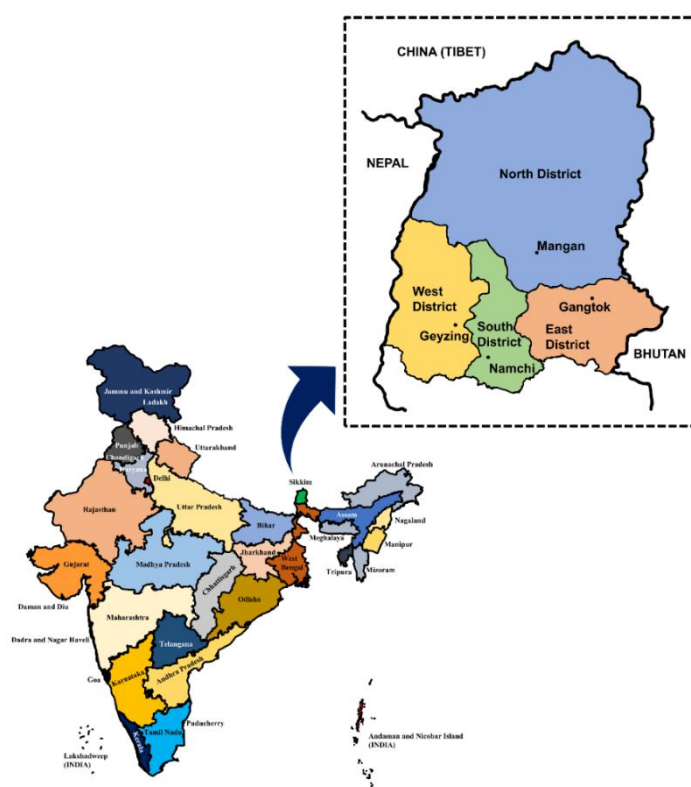


Figure A: Map of Sikkim.

1.1 Research Gap

Numerous studies have documented the presence of diverse lactic acid bacteria (LAB) in traditionally prepared fermented milk (NFM) products of Sikkim, highlighting their probiotic potential. Although various species of yeast have also been identified and reported in these traditional dairy products, research specifically focused on their probiotic and functional properties remains limited. Given the unique microbial ecology and traditional fermentation practices in the region, it is essential to systematically profile the yeast communities present in NFM products of Sikkim and evaluate their probiotic attributes and other functional characteristics. Such investigations can be a factor to the discovery of novel probiotic yeasts and promote the growth and development of region-specific functional foods and biotherapeutic applications.

1.2 Approved Objectives:

- ❖ To profile the yeast communities in *dahi* and *chhurpi* of Sikkim using phenotypic and molecular methods.
- ❖ To screen the probable probiotic yeasts isolated from *dahi* and *chhurpi*.
- ❖ To determine the functional properties of probiotic yeasts.

Therefore, the current study aims to isolate and identify yeast species from *dahi* and *chhurpi* samples of Sikkim using phenotypic and molecular methods. Additionally, it also aims to screen the isolates for probiotic and other functional properties.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

For thousands of years, the fermented milk/dairy products have been an essential component of human diets across the entire globe, with profound cultural, nutritional, and economic significance (Rosenstock et al., 2021; Tamang, 2022b; Ray et al., 2024). Milk fermentation can be traced back to the Neolithic era, around 10,000 years ago, when humans started domesticating animals and preserving milk in animal stomach receptacles, unintentionally allowing lactic acid bacteria to ferment the milk (Rosenstock et al., 2021). Over time, these spontaneous fermentations produced a range of fermented milk products, including yogurt, kefir, koumiss, and dahi, which became culturally distinctive meals (Ray et al., 2024). In ancient India, *Dahi*, a yoghurt-like fermented milk product of India, was mentioned in about 6000 to 4000 BCE in the *Rig Veda* and *Upanishad*, ancient sacred books of the Hindus (Yegna Narayan Aiyar, 1953; Tamang, 2020; Tamang, 2022a). It is also mentioned in the Ayurvedic texts like the *Charaka Samhita* and *Sushruta Samhita*, where products like “*dadhi*” (curd or yogurt) and “*takra*” (buttermilk) were valued for their therapeutic and digestive properties (Tamang et al., 2020; Mallappa et al., 2021). Similarly, the fermented milk/dairy products have been documented over the centuries. For example, Egypt’s *Laban Zeer*, which dates back to 5000-3000 BCE, cultured cream in Mesopotamia (1300 BCE), *Feta cheese* from Greece (1184 BCE), and *Kumis* in Mongolia and Russia (400 BCE) were well known in ancient era (Prajapati and Nair, 2003). Fermented milk products vary significantly throughout the world depending on regional and native milk sources, microbial flora, and traditions (Tamang et al., 2020; Tamang, 2022a). As a result, similar products with various local vernacular languages and dialects (Zhong et al., 2016) are produced throughout the world. The following table elucidates the most well-known and widespread traditional fermented milk/dairy products worldwide (Table A):

Table A: Traditional Fermented Milk Products Across the Globe.

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Amasi</i>	Cow milk	Yoghurt-like product	South Africa and Zimbabwe	<i>Lactococcus lactis</i> , <i>Lactobacillus</i> sp., <i>Leuconostoc</i> sp., <i>Enterococcus</i> sp., <i>Saccharomyces cerevisiae</i> , <i>S. dairenensis</i> , <i>Candida lusitanae</i> , <i>C. tropicalis</i> , <i>C. colliculosa</i> , <i>Dekera bruxillensis</i> , <i>C. lipolytica</i> , and <i>C. kefir</i>	Gadaga et al., 2000; Osvik et al., 2013
<i>Amabere amaruranu</i>	Cow milk	Yoghurt-like product	Kenya	<i>Lactiplantibacillus plantarum</i> subsp. <i>plantarum</i> , <i>Staphylococcus warneri</i> , and <i>Epicoccum</i> spp.	Sichangi et al., 2020
<i>Arrera/ Arera</i>	Cow, goat and camel milk	Buttermilks	Ethiopia	<i>Escherichia coli</i> , <i>Cronobacter sakazakii</i> , <i>Enterobacter cloacae</i> , <i>Klebsiella pneumoniae</i> , and <i>K. oxytoca</i>	Yilma et al., 2005
<i>Ayib</i>	Cow and goat milk	Soft curd cheese	Ethiopia	<i>Lacticaseibacillus paracasei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lcb. rhamnosus</i> , <i>Streptococcus thermophilus</i> , <i>Leuconostoc mesenteroides</i> , <i>Lactococcus lactis</i> , and <i>Lactobacillus acidophilus</i>	Girma and Aemiro, 2021
<i>Airag</i>	Cow, ewe, yak, camel, or goat milk	Yoghurt-like	Kazakhstan, Mongolia, Kyrgyzstan, and some Central Asian regions of Russia	<i>Lactobacillus kefiranofaciens</i> , <i>Lb. helveticus</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lacticaseibacillus paracasei</i> , <i>Companilactobacillus farciminis</i> , <i>Lentilactobacillus kefir</i> , <i>Saccharomyces cerevisiae</i> , <i>S. dairensis</i> , <i>Kluyveromyces wickerhamii</i> , <i>Kl. marxianus</i> , <i>Issachenkia orientalis</i> , <i>Lb. fermentum</i> , <i>Lb. acetotolerans</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Streptococcus thermophilus</i> , <i>Lcb. paracasei</i> , <i>Len. kefir</i> , <i>Kazachstania unispora</i> , <i>Len. hilgardii</i> , <i>Len. parakefiri</i> , <i>Lactiplantibacillus pentosus</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Enterococcus hirae</i> , <i>Strep. thermophilus</i> , <i>Bacillus safensis</i> , <i>Bacillus pumilus</i> , <i>Exiguobacterium profundum</i> , <i>Acinetobacter calcoaceticus</i> , <i>Kocuria rhizophila</i> , <i>Acetobacter pasteurianus</i> , <i>Gluconobacter cerinus</i> , <i>Psychrobacter</i> sp., and <i>Paenibacillus</i> sp.	Uchida et al., 2007; Watanabe et al., 2008; Ringø et al., 2014; Su et al., 2025
<i>Ayran</i>	Camel milk	Yoghurt-like	Kazakhstan, Turkey and in Russia	<i>Lactobacillus helveticus</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lacticaseibacillus paracasei</i> , <i>Streptococcus thermophilus</i> , and <i>Limosilactobacillus fermentum</i>	Baruzzi et al., 2016; Zhadyra et al., 2021
<i>Bongo</i>	Cow milk	Yoghurt-like product	Uganda	<i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>Lc. lactis</i> , <i>Lc. garvieae</i> , <i>Lactobacillus casei</i> , <i>Lb. plantarum</i> , <i>Lb. rhamnosus</i> , <i>Lc. lactis</i> ssp. <i>cremoris</i> , <i>Leuconostoc pseudomesenteroides</i> , <i>Leuconostoc mesenteroides</i> , <i>Streptococcus lutetiensis</i> , <i>Strep. equinus</i> , <i>Enterococcus italicus</i> , <i>Staphylococcus saprophyticus</i> , <i>Saccharomyces cerevisiae</i> , <i>Yarrowia lipolytica</i> , <i>Pichia kudriavzevii</i> , <i>Candida tropicalis</i> , <i>C. glabrata</i> , <i>C. albicans</i> , <i>Kluyveromyces marxianus</i> , and <i>Kazachstania bulderi</i>	Byakika and Mukisa, 2024

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Chhurpi</i> (hard and soft)	Cow and Yak Milk	Cottage cheese	India, Nepal, and Bhutan	<i>Lacticaseibacillus paracasei</i> subsp. <i>paracasei</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lc. lactis</i> subsp. <i>hordniae</i> , <i>Enterococcus faecium</i> , <i>E. pseudoavium</i> , <i>E. faecalis</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>jonggajibkimchii</i> , <i>Lactiplantibacillus plantarum</i> , <i>Limosilactobacillus fermentum</i> , <i>Latilactobacillus curvatus</i> , <i>Lentilactobacillus kefir</i> , <i>Len. hilgardii</i> , <i>Companilactobacillus alimentarius</i> , <i>Lb. helveticus</i> , <i>Lb. delbrueckii</i> , <i>Leu. pseudomesenteroides</i> , <i>Acetobacter pasteurianus</i> , <i>Acetobacter lovaniensis</i> , <i>Gluconobacter oxydans</i> , <i>Hafnia alvei</i> , <i>Pseudomonas fluorescens</i> , <i>Saccharomyces cerevisiae</i> , <i>Kluyveromyces marxianus</i> , <i>Issatchenkia orientalis</i> , and <i>Candida parapsilosis</i>	Tamang et al., 2000; Dewan and Tamang, 2007; Rai et al., 2016; Rai, 2020
<i>Dhanaan</i>	Camel milk	Yoghurt-like product	Somalia	<i>Lactococcus</i> , <i>Streptococcus</i> , <i>Weissella</i> , <i>Enterobacter</i> , <i>Cronobacter</i> , <i>Acinetobacter</i> , <i>Buttiauxella</i> , <i>Clostridium</i> , <i>Brenneria</i> , <i>Salmonella</i> , <i>Erwinia</i> , <i>Shimwellia</i> , <i>Tatumella</i> , <i>Hafnia</i> , <i>Shigella</i> , <i>Kluyvera</i> , <i>Pectobacterium</i> , <i>Escherichia</i> , <i>Lelliottia</i> , and <i>Klebsiella</i>	Berhe et al., 2019
<i>Dadih/dadiyah</i>	Buffalo milk	Yoghurt-like	Indonesia	<i>Lactiplantibacillus plantarum</i> , <i>Enterococcus fecium</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus</i> sp., <i>Lactococcus</i> sp., <i>Acetobacter</i> sp., <i>Bifidobacterium</i> sp., <i>Enterococcus</i> sp., <i>Leuconostoc</i> sp., <i>Staphylococcus</i> sp., <i>Klebsiella</i> sp., <i>Corynebacterium</i> sp., <i>Acinetobacter</i> sp., <i>Serratia</i> sp., <i>Frateuria</i> sp., <i>Chryseobacterium</i> sp., and <i>Raoultella</i> sp.	Collado et al., 2007; Sukma et al., 2018; Amelia et al., 2020; Fatdillah et al., 2021
<i>Dahi</i>	Cow and buffalo milk	Yoghurt-like	Bangladesh, Nepal, Bhutan, India, and Pakistan	<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> , <i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Lactococcus raffinolactis</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , <i>Pediococcus pentosaceus</i> , <i>Strep. bovis</i> , <i>Streptococcus thermophilus</i> , <i>Enterococcus faecium</i> , <i>Limosilactobacillus fermentum</i> , <i>Kodamaea ohmeri</i> , <i>Candida parapsilosis</i> , <i>Clavispora lusitaniae</i> , <i>Trichosporon</i> sp., <i>C. tropicalis</i> , <i>Lichtheimia</i> , <i>Syncephalastrum</i> , <i>Lactobacillus acidophilus</i> , <i>Lacticaseibacillus casei</i> , <i>Lb. helveticus</i> , <i>Enterococcus mundtii</i> , <i>E. faecium</i> , <i>Lim. fermentum</i> , <i>Lcb. rhamnosus</i> , <i>Strep. thermophilus</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Companilactobacillus alimentarius</i> , <i>Ligilactobacillus bifermentans</i> , <i>Lcb. paracasei</i> subsp. <i>paracasei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactiplantibacillus paraplantarum</i> , <i>Lactiplantibacillus pentosus</i> , <i>Lc. lactis</i> subsp. <i>tractae</i> , <i>E. italicus</i> , <i>Lc. raffinolactis</i> , <i>Lb. gasseri</i> , <i>Leu. pseudomesenteroides</i> , <i>Acetobacter pasteurianus</i> , <i>Acetobacter lovaniensis</i> , <i>Acetobacter syzygii</i> , and <i>Pseudomonas fluorescens</i>	Dewan and Tamang, 2007; Soomro and Masud, 2007; Harun-ur-Rashid et al., 2007; Mahmood et al., 2013; Koirala et al., 2014; Bhattarai et al., 2016; Ghatani and Tamang, 2017; Shangpliang et al., 2017; Shangpliang et al., 2018; Nahidul-Islam et al., 2018; Nawaz et al., 2019; Rai, 2020; Tirwa et al., 2021

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Ergo/Ititu</i>	Cow, goat or camel milk	Yoghurt-like product	Ethiopia	<i>Lacticaseibacillus paracasei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Streptococcus thermophilus</i> , <i>Strep. acidominimus</i> , <i>Enterococcus faecalis</i> var. <i>liquefaciens</i> , <i>Strep. bovis</i> , <i>Strep. mitis</i> , <i>Strep. agalactiae</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> , <i>Leu. lactis</i> , and <i>Lc. lactis</i> subsp. <i>lactis</i>	Gonfa et al., 1999; Gonfa et al., 2001; Mulaw et al., 2019
<i>Emasi</i>	Cow milk	Yoghurt-like product	Kingdom of Eswatini	<i>Lactococcus lactis</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lacticaseibacillus paracasei</i> , <i>Levilactobacillus brevis</i> , and <i>Leuconostoc mesenteroides</i>	Simatende et al., 2019
<i>Garris</i>	Camel milk	Yoghurt-like product	Somalia and Sudan	<i>Lactobacillus acidophilus</i> , <i>Ligilactobacillus animalis</i> , <i>Enterococcus faecium</i> , <i>Companilactobacillus alimentarius</i> , <i>Carnobacterium divergens</i> , <i>Lactiplantibacillus plantarum</i> , <i>Levilactobacillus brevis</i> , <i>Lacticaseibacillus casei</i> , <i>Lb. helveticus</i> , <i>Lb. delbrueckii</i> subsp. <i>lactis</i> , <i>Lb. gasseri</i> , <i>Lcb. rhamnosus</i> , <i>Limosilactobacillus fermentum</i> , <i>Lcb. paracasei</i> , <i>Lc. raffinolactis</i> , <i>Streptococcus infantarius</i> subsp. <i>infantarius</i> , <i>Strep. lactis</i> , <i>Strep. lactis</i> subsp. <i>diactylactis</i> , <i>Strep. thermophilus</i> , <i>Strep. acidominimus</i> , <i>Kluyveromyces marxianus</i> , and <i>Issatchenkia</i> sp.	Abdelgadir et al., 2008; Hassan et al., 2008; Ashmaig et al., 2009; Ahmed, 2022; Hafiz and Ibtisam, 2024
<i>Gioddu</i>	Ovine or goat milk	Acid-alcoholic fermented milk	Italy	<i>Lactobacillus delbrueckii</i> , <i>Streptococcus thermophilus</i> , <i>Lactococcus lactis</i> , <i>Lb. kefir</i> , <i>Kluyveromyces marxianus</i> , <i>Saccharomyces cerevisiae</i> , <i>Pichia cactophila</i> , <i>Glomus hyderabadensis</i> , <i>Galactomyces</i> , <i>Geotrichum</i> , <i>Galactomyces candidum</i> , <i>Cladosporium</i> , <i>Alternaria</i> , and <i>Aerobasidium</i>	Maoloni et al., 2020
<i>Gwell</i>	Cow milk	Fermented milk	France	<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacetyllactis</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Lactobacillus acidophilus</i> , <i>Lb. helveticus</i> , <i>Staphylococcus warnerii</i> , <i>Streptococcus thermophilus</i> , <i>Kazachstania servazii</i> , <i>Yarrowia lipolytica</i> , and <i>Geotrichum candidum</i>	von Gastrow et al., 2020
<i>Gheu</i>	Cow or yak milk	Artisanal butter	India and Nepal	<i>Lactococcus piscium</i> , <i>Pseudomonas fluorescens</i> , <i>Lactobacillus helveticus</i> , <i>Lc. lactis</i> , <i>Lc. raffinolactis</i> , <i>Leuconostoc mesenteroides</i> , <i>Leu. lactis</i> , and <i>Lb. gasseri</i>	Rai, 2020
<i>Hurunge</i>	Cow, camel, sheep, horse, and goat milk	Fermented milk	Mongolia	<i>Lactococcus raffinolactis</i> , <i>Lc. lactis</i> ssp. <i>cremoris</i> , <i>Lc. lactis</i> ssp. <i>lactis</i> , <i>Lacticaseibacillus casei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactobacillus homohiochii</i> , <i>Lb. kefir</i> , <i>anofaciens</i> , <i>Lb. acetotolerans</i> , <i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i> , <i>Saccharomyces cerevisiae</i> , <i>Candida valida</i> , <i>C. kefir</i> , <i>C. krusei</i> , and <i>Kluyveromyces marxianus</i> var. <i>lactis</i>	Shuangquan et al., 2006

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Jben</i>	Goat, sheep and cow milk	Cheese-like	Libya, Morocco, Algeria, Tunisia, and Mauritania	<i>Lacticaseibacillus casei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lcb. paracasei</i> , <i>Lcb. rhamnosus</i> , <i>Lactococcus raffinolactis</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , <i>Lc. garvieae</i> , <i>Leuconostoc mesenteroides</i> , <i>Leu. lactis</i> , <i>Leu. citreum</i> , <i>Leu. pseudomesenteroides</i> , <i>Enterococcus faecium</i> , <i>E. durans</i> , <i>E. saccharominimus</i> , <i>E. faecalis</i> , <i>Lentilactobacillus buchneri</i> , <i>Levilactobacillus brevis</i> , and <i>Streptococcus</i> sp.	Hamama, 1992; Ouadghiri et al., 2006
<i>Jiaoke</i>	Cow, yak, goat, and sheep	Fermented cream	Mongolia, China	<i>Lacticaseibacillus casei</i> , <i>Levilactobacillus brevis</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactococcus garvieae</i> , <i>Lactobacillus helveticus</i> , <i>Lc. lactis</i> , <i>Leuconostoc lactis</i> , <i>Streptococcus gallolyticus</i> , <i>Strep. thermophilus</i> , <i>Enterococcus faecium</i> , <i>E. gilvus</i> , <i>E. italicus</i> , <i>E. faecalis</i> , and <i>E. gallinarum</i>	Fan et al., 2020
<i>Kefir</i>	Cow, goat and Sheep milk	Fermented milk	Tibet (China), Taiwan (China), Turkey, South Africa, Russia, Bosnia, Herzegovina, Czech Republic/Czechia, Belgium, Estonia, Ireland, Greece, Romania, Hungary, Norway, Italy, Slovakia, Latvia, Poland, Ukraine, Brazil and Argentina	<i>Lentilactobacillus buchneri</i> , <i>Len. kefir</i> , <i>Len. parabuchneri</i> , <i>Len. diolivorans</i> , <i>Len. rapi</i> , <i>Len. otakiensis</i> , <i>Limosilactobacillus reuteri</i> , <i>Lim. fermentum</i> , <i>Liquorilactobacillus satsumensis</i> , <i>Ligilactobacillus salivarius</i> , <i>Furfurilactobacillus rossiae</i> , <i>Levilactobacillus brevis</i> , <i>Latilactobacillus sakei</i> , <i>Latilactobacillus curvatus</i> , <i>Lacticaseibacillus paracasei</i> , <i>Lcb. rhamnosus</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lcb. casei</i> , <i>Lactiplantibacillus pentosus</i> , <i>Lactobacillus delbrueckii</i> , <i>Lb. helveticus</i> , <i>Lb. crispatus</i> , <i>L. garvieae</i> , <i>L. instestinalis</i> , <i>L. kefiranofaciens</i> , <i>Lb. kefirgranum</i> , <i>Lb. johnsonii</i> , <i>Lb. amylovorus</i> , <i>Lb. gasseri</i> , <i>Lb. acidophilus</i> , <i>Lb. kalixensis</i> , <i>Lb. gallinarum</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Lc. garvieae</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Leu. pseudomesenteroides</i> , <i>Pediococcus claussenii</i> , <i>Pediococcus pentosaceus</i> , <i>Pediococcus lolii</i> , <i>Pediococcus halophilus</i> , <i>Streptococcus durans</i> , <i>Strep. thermophilus</i> , <i>Weissella viridescens</i> , <i>Acetobacter lovaniensis</i> , <i>Acetobacter orientalis</i> , <i>Acetobacter sicerae</i> , <i>Bifidobacterium pseudolongum</i> , <i>Bifidobacterium breve</i> , <i>Bifidobacterium choerinum</i> , <i>Bifidobacterium longum</i> , , , <i>Candida kefir</i> , <i>C. holmii</i> , <i>C. lipolytica</i> , <i>C. maris</i> , <i>C. friedrichii</i> , <i>C. inconspicua</i> , <i>C. sake</i> , <i>Cyberlindnera jadinii</i> , <i>Cryptococcus humicolus</i> , <i>Issatchenkia orientalis</i> , <i>Geotrichum candidum</i> , <i>Saccharomyces cariocanus</i> , <i>S. turicensis</i> , <i>S. cerevisiae</i> , <i>S. unisporus</i> , <i>S. humaticus</i> , <i>Pichia fermentans</i> , <i>Yarrowia lipolytica</i> , <i>Zygosaccharomyces lentus</i> , <i>Torulaspora delbrueckii</i> , <i>Kazachstania barnettii</i> , <i>Kazachstania servazzii</i> , <i>Kazachstania aerobia</i> , <i>Kazachstania solicola</i> , <i>Kazachstania exigua</i> , <i>Kluyveromyces marxianus</i> var. <i>lactis</i> , and <i>Kl. lactis</i>	Bourrie et al., 2016

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Kashk zard</i>	Cow and Sheep milk	Yellow curd	Iran	<i>Limosilactobacillus fermentum</i> , <i>Lim. reuteri</i> , <i>Lim. vaginalis</i> , <i>Lim. pontis</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lacticaseibacillus zeae</i> , <i>Levilactobacillus brevis</i> , <i>Streptococcus thermophilus</i> , <i>Pediococcus acidilactici</i> , and <i>Lactobacillus delbrueckii</i>	Pakroo et al., 2020
<i>Krokmach</i>	Ewe milk	Smear cheese than yoghurt	Bulgaria	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Kluyvera georgiana</i> , <i>Exiguobacterium</i> sp., <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lc. garvieae</i> subsp. <i>garvieae</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i> , <i>Apilactobacillus kunkeei</i> , <i>Enterococcus faecalis</i> , <i>Aeromonas</i> sp., <i>Klebsiella</i> sp., and <i>Escherichia-Shigella</i>	Dimov, 2022
<i>Kurut</i>	Yak milk	yoghurt-like product	Tibet, China,	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lb. helveticus</i> , <i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>Limosilactobacillus fermentum</i> , <i>Lacticaseibacillus casei</i> , <i>Streptococcus thermophilus</i> , and <i>Acetobacter</i> sp.	Chen et al., 2010; Liu et al., 2012
<i>Kivuguto</i>	Cow milk	Yoghurt-like product	Rwanda	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Leuconostoc pseudomesenteroides</i> , and <i>Lactococcus lactis</i>	Karenzi et al., 2012
<i>Kindirmou and pendidam</i>	Cow milk	Yoghurt-like product	Cameroon	<i>Limosilactobacillus fermentum</i> , <i>Pediococcus acidilactici</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactococcus lactis</i> , <i>Lacticaseibacillus casei</i> , <i>Saccharomyces cerevisiae/paradoxus</i> , <i>Galactomyces candidum</i> , <i>Torulaspora delbrueckii</i> , <i>Kluyveromyces marxianus</i> , and <i>Candida parapsilosis</i>	Maïworé et al., 2019; Sohanang et al., 2021; Steve et al., 2024
<i>Lait caillé</i>	Cow milk	Yoghurt-like product	Senegal and Burkina Faso	<i>Pediococcus pentosaceus</i> , <i>Lactococcus lactis</i> , <i>Leuconostoc mesenteroides</i> , <i>Weissella paramesenteroides</i> , <i>Streptococcus</i> spp., <i>Lactobacillus</i> spp., <i>Enterococcus</i> spp., <i>Acetobacter</i> spp., <i>Leuconostoc</i> spp., <i>Saccharomyces cerevisiae</i> , and <i>Candida parapsilosis</i>	Parker et al., 2018; Bayili et al., 2019; Compaore et al., 2021
<i>Lben/leben</i>	Cow, goat, sheep, or camel milk	buttermilk	Morocco	<i>Enterococcus gilvus</i> , <i>E. faecalis</i> , <i>E. durans</i> , <i>E. hirae</i> , <i>E. faecium</i> , <i>Lactobacillus plantarum</i> , <i>Lb. brevis</i> , <i>Lactococcus garvieae</i> , <i>Lb. rhamnosus</i> , <i>Lb. paracasei</i> , <i>Lc. lactis</i> , <i>Leu. kimchi</i> , <i>Leu. pseudomesenteroides</i> , <i>Leu. citreum</i> , <i>Leu. mesenteroides</i> , <i>Weissella viridescens</i> , <i>Weissella confuse</i> , <i>Weissella cibaria</i> , <i>Weissella paramesenteroides</i> , <i>Pediococcus pentosaceus</i> , <i>Kluyveromyces marxianus</i> var. <i>marxianus</i> , <i>Kl. lactis</i> , and <i>Saccharomyces cerevisiae</i>	Hamama, 1992; Benkerroum and Tamime, 2004; Ouadghiri et al., 2009; Mangia et al., 2014
<i>Långfil and Filmjök</i>	Cow milk	Fermented yoghurt	Sweden	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , and <i>Leuconostoc mesenteroides</i>	Hati et al., 2019

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Meekiri/mee-deekiri</i>	Water buffalo	Yoghurt-like product	Sri Lanka	<i>Latilactobacillus curvatus</i> , <i>Lactobacillus acidophilus</i> , <i>Lb. delbrueckii</i> subsp. <i>lactis</i> , <i>Lb. helveticus</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lacticaseibacillus casei</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactococcus lactis</i> , <i>Streptococcus thermophilus</i> , <i>Limosilactobacillus fermentum</i> , <i>Bacillus</i> spp., <i>Micrococcus</i> spp., and <i>Saccharomyces cerevisiae</i>	Dekumpitiya et al., 2016; Adikari et al., 2021
<i>Mursik</i>	cow and goat milk	Yoghurt-like product	Kenya	<i>Lacticaseibacillus casei</i> , <i>Lentilactobacillus kefir</i> , <i>Lcb. rhamnosus</i> , <i>Lcb. paracasei</i> , <i>Levilactobacillus brevis</i> , <i>Limosilactobacillus fermentum</i> , <i>Lactiplantibacillus plantarum</i> , <i>Candida sphaerica</i> , <i>C. kefir</i> , <i>C. krusei</i> , and <i>Saccharomyces fermentati</i>	Nieminen et al., 2013; Khayeka-Wandabwa et al., 2024
<i>Madila</i>	Cow and goat milk	Yoghurt-like product	Botswana	<i>Lactobacillus acidophilus</i> , <i>Lactiplantibacillus plantarum</i> , <i>Levilactobacillus brevis</i> , <i>Lb. delbrueckii</i> , <i>Lactococcus lactis</i> , <i>Limosilactobacillus fermentum</i> , <i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , and <i>Bacillus cereus</i>	Ohenhen et al., 2013
<i>Mohi</i>	Cow milk	Buttermilk	India and Nepal	<i>Lacticaseibacillus rhamnosus</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , <i>Leuconostoc mesenteroides</i> , <i>Companilactobacillus alimentarius</i> , <i>Lactobacillus delbrueckii</i> , <i>Streptococcus thermophilus</i> , <i>Macrococcus caseolyticus</i> , <i>Ralstonia solanacearum</i> , <i>Aeromonas salmonicida</i> , <i>Aeromonas hydrophila</i> , <i>Psychrobacter arcticus</i> , <i>Enterococcus faecalis</i> , <i>Dictyoglomus turgidum</i> , <i>Methylobacterium radiotolerans</i> , <i>Bartonella quintana</i> , <i>Methylobacterium populi</i> , <i>Synechocystis</i> sp., and <i>Thermoanaerobacter</i> sp.	Dewan and Tamang, 2007; Jayashree et al., 2013; Ansari et al., 2019; Rai, 2020
<i>Nunu</i>	Cow milk	Yoghurt-like product	Ghana	<i>Limosilactobacillus fermentum</i> , <i>Lactiplantibacillus plantarum</i> , <i>Leuconostoc mesenteroides</i> , <i>Lactobacillus helveticus</i> , <i>Enterococcus faecium</i> , <i>E. italicus</i> , <i>Weissella confuse</i> , <i>Saccharomyces cerevisiae</i> , <i>Pichia kudriavzevii</i> , <i>S. pastorianus</i> , <i>Khuyveromyce maxianus</i> , <i>Zygosaccharomyces bisporus</i> , <i>Candida stellata</i> , <i>Zygosaccharomyces rouxii</i> , <i>C. parapsilosis</i> , <i>Diutina rugosa</i> , <i>C. tropicalis</i> , <i>Geotrichum candidum</i> , <i>Listeria monocytogenes</i> , <i>Escherichia</i> , <i>Klebsiella</i> , <i>Proteus vulgaris</i> , <i>Enterobacter</i> , and <i>Shigella</i>	Akabanda et al., 2010; Akabanda et al., 2013; Owusu-Kwarteng et al., 2018

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Nono</i>	Cow and goat milk	Yoghurt-like product	Nigeria	<i>Limosilactobacillus fermentum</i> , <i>Lactobacillus plantarum</i> , <i>Lb. helveticus</i> , <i>Lb. casei</i> , <i>Lb. delbrueckii</i> , <i>Lactococcus lactis</i> , <i>Lb. brevis</i> , <i>Lb. bulgaricus</i> , <i>Leuconostoc pseudomesenteroides</i> , <i>Lb. senioris</i> , <i>Lb. cremoris</i> , <i>Streptococcus thermophilus</i> , <i>Weissella</i> spp., <i>Saccharomyces cerevisiae</i> , <i>Candida glabrata</i> , and <i>Cyberlindnera</i>	Okiki et al., 2018; Diaz et al., 2019; Ayanniran et al., 2020; Obioha et al., 2023; Ikele et al., 2024
<i>Omaere/omashikwa</i>	Cow and goat milk	Buttermilk	Namibia	<i>Lacticaseibacillus paracasei</i> subsp. <i>paracasei</i> , <i>Lcb. casei</i> , <i>Lcb. rhamnosus</i> , <i>Lentilactobacillus kefir</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactococcus lactis</i> , <i>Saccharomyces cerevisiae</i> , <i>Kazachstania unispora</i> , and <i>Candida pararugosa</i>	Heita, 2014; Kutaa, 2017
<i>Philu</i> (or <i>philuk</i>)	Cow or yak milk	Cream-like milk product	India	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lacticaseibacillus paracasei</i> subsp. <i>paracasei</i> , <i>Enterococcus faecium</i> , and <i>Loigolactobacillus bifermantans</i>	Dewan and Tamang, 2007; Rai, 2020
<i>Rob/Roub</i>	Camel milk	Yoghurt-like product	Sudan	<i>Lactobacillus delbrueckii</i> , <i>Lb. acidophilus</i> , <i>Lactococcus lactis</i> , <i>Limosilactobacillus fermentum</i> , <i>Streptococcus salivarius</i> , <i>Saccharomyces cerevisiae</i> , and <i>Candida kefir</i>	Abdelgadir et al., 2001; Abdullah and Osman, 2010
<i>Raib</i>	Cow, sheep, goats and camel milk	Cheese-like	Morocco	<i>Enterococcus faecalis</i> , <i>E. durans</i> , <i>E. faecium</i> , <i>E. hirae</i> , <i>Lactococcus garvieae</i> , <i>Lc. lactis</i> , <i>Lactiplantibacillus plantarum</i> , <i>Leu. mesenteroides</i> subsp. <i>dextranicum</i> , and <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	Hamama, 1992; Elotmani et al., 2002; Bendimerad et al., 2012; Moumene et al., 2016
<i>Rayeb</i>	Buffalo milk	Cheese-like	Libya and Egypt	<i>Lactobacillus helveticus</i> , <i>Lactococcus acidophilus</i> , <i>Lb. bulgaricus</i> , <i>Lb. delbrueckii</i> , <i>Streptococcus thermophilus</i> , <i>Strep. acidominimus</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> , <i>Aerococcus viridans</i> , <i>Enterococcus durans</i> , and <i>E. faecium</i>	Abd El Gawad et al., 2010
<i>Somar</i>	Yak milk	Cottage cheese	India	<i>Lacticaseibacillus paracasei</i> subsp. <i>paracasei</i> and <i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Dewan and Tamang, 2007
<i>Shubat</i>	Camel milk	Fermented camel milk, is less thick than yoghurt	China (Xinjiang) and Kazakhstan	<i>Levilactobacillus brevis</i> , <i>Latilactobacillus sakei</i> , <i>Lactobacillus delbrueckii</i> , <i>Lb. helveticus</i> , <i>Lb. kefir</i> , <i>Enterococcus faecium</i> , <i>E. faecalis</i> , <i>Leuconostoc mesenteroides</i> , <i>Leu. lactis</i> , <i>Streptococcus thermophilus</i> , <i>Weissella hellenica</i> , <i>Kazachstania unispora</i> , <i>Kluyveromyces marxianus</i> , and <i>Candida ethanolica</i>	Rahman et al., 2009; Zhadyra et al., 2021

Table A: Traditional Fermented Milk Products Across the Globe (contd.).

Product	Milk Source	Nature of the product	Country	Microorganisms reported	References
<i>Shmen</i>	Camel milk	Butter	Algeria	<i>Lactobacillus delbrueckii</i> ssp. <i>Bulgaricus</i> , <i>Lactococcus lactis</i> ssp. <i>cremoris</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lacticaseibacillus paracasei</i> subsp. <i>paracasei</i> , <i>Enterococcus faecium</i> , <i>Leuconostoc gelidum</i> , <i>Leu. pseudomesenteroides</i> , and <i>Saccharomyces cerevisiae</i>	Mourad and Nour-Eddine, 2006
<i>Suusac</i>	Camel milk	Yoghurt-like product	Somalia and Kenya	<i>Lactiplantibacillus plantarum</i> , <i>Latilactobacillus curvatus</i> , <i>Lactococcus raffinolactis</i> , <i>Ligilactobacillus salivarius</i> , <i>Lc. lactis</i> subsp. <i>lactis</i> , <i>Streptococcus thermophilus</i> , <i>Strep. infantarius</i> subsp. <i>infantarius</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ; <i>Saccharomyces cerevisiae</i> , <i>Candida guilliermondii</i> , <i>C. krusei</i> , <i>C. famata</i> , <i>C. lusitaniae</i> , <i>C. inconspicua</i> , <i>Cryptococcus laurentii</i> , <i>Cryptococcus albidus</i> , <i>Geotrichum penicillatum</i> , <i>Rhodotorula mucilaginosa</i> , <i>Trichosporon mucoides</i> , and <i>Trichosporon cutaneum</i>	Holzapfel, 2002; Lore et al., 2005; Njage et al., 2011; Jans et al., 2012
<i>Vili</i>	Cow milk	Mesophilic yoghurt	Finland and Taiwan	<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Lc. lactis</i> subsp. <i>cremoris</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> , <i>Saccharomyces unisporus</i> , <i>Pichia fermentans</i> , and <i>Kluyveromyces marxianus</i>	Wang et al., 2008; Kahala et al., 2008
<i>Vrum/wrum</i>	Yak milk	Cottage cheese	Mongolia (China)	<i>Lentilactobacillus kefir</i> , <i>Lactobacillus kefiranoformis</i> , <i>Lactococcus lactis</i> , <i>Lb. helveticus</i> , <i>Streptococcus salivarius</i> , <i>Len. diolivorans</i> , <i>Acetobacter orientalis</i> , <i>Acetobacter malorum</i> , <i>Saccharomyces cerevisiae</i> , <i>Kluyveromyces lactis</i> , <i>Kl. marxianus</i> , <i>Kazachstania unispora</i> , <i>Pichia fermentans</i> , <i>Trichosporon asahii</i> , <i>Mucor circinelloides</i> , <i>Mucor racemosus</i> , <i>Levilactobacillus brevis</i> , <i>Lactiplantibacillus plantarum</i> , <i>Lactococcus raffinolactis</i> , <i>Pseudomonas brenneri</i> , <i>Leuconostoc mesenteroides</i> , and <i>Pediococcus acidilactici</i>	Yamei et al., 2019; Liang et al., 2021
<i>Wagashi</i> (or <i>gassire</i>)	Cow milk	Cheese-like	Benin and Nigeria	<i>Saccharomyces cerevisiae</i> , <i>Kluyveromyces marxianus</i> , <i>Sagenomella keratitidis</i> , and <i>Candida parapsilosis</i>	Sessou et al., 2019
<i>Zabady</i>	Buffalo and cow milk	Yoghurt-like product	Egypt	<i>Lactococcus raffinolactis</i> , <i>Lc. lactis</i> , <i>Lc. garvieae</i> , <i>Lactobacillus johnsonii</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Streptococcus thermophilus</i> , and <i>Leuconostoc citreum</i>	El-Baradei et al., 2008

2.1 NFM products from Africa and their microbiological diversity

2.1.1 Yoghurt-like NFM products

2.1.1.1 *Amasi*

It is a yoghurt-like product prepared by fermenting cow's milk in a calabash by Xhosa and Zulu speaking people in the eastern coastal regions of South Africa (KwaZulu-Natal) and Zimbabwe (Gwanda) (Todorov et al., 2007; Osvik et al., 2013; Agyei et al., 2020). It is also referred to as *mukaka wakakora/zifa* by the *Shona (Bantu)* ethnic group in Zimbabwe, while it is called *amasi* by the *Ndebele* ethnic group (Gadaga et al., 1999). *Lactococcus lactis* was the main LAB species observed in this product in results of Denaturing Gradient Gel Electrophoresis (DGGE, culture-independent method); other minor LAB species reported were belonging to *Lactobacillus*, *Leuconostoc*, and *Enterococcus* (Osvik et al., 2013). Major yeast species identified as *Saccharomyces cerevisiae*, *Candida lusitanae*, *C. colliculosa* and *Saccharomyces dairenensis*; followed by minor species including *Dekera bruxillensis*, *C. tropicalis* and *C. lipolytica*, and *C. kefir* (Gadaga et al., 2000).

2.1.1.2 *Amabere amaruranu*

It is a spontaneously fermented Kenyan milk product which is commonly prepared by the neolithic people who practiced agropastoral also known as *abagusii* (Nyambane et al., 2014). In the Kisii region, southwest of the country, it is typically prepared with cow's milk. Nyambane et al. (2014) has reported many LAB and yeast species using culture dependent technique. *Lactiplantibacillus plantarum* subsp. *plantarum* has also been identified and reported from *Amabere amaruranu* as the potential probiotic bacteria (Sichangi et al., 2020). Additionally, the non-fermenter *Staphylococcus warneri* and the fungus *Epicoccum* spp. have been reported (Sichangi et al., 2020).

2.1.1.3 Bongo

In the central and western regions of Uganda, it is among the most widely consumed fermented milk products. It is made similarly to other dairy products that spontaneously ferment, mostly from cow's milk (Mukisa et al., 2020). According to Mukisa et al. (2020), yeast and the lactic acid bacteria constitute the majority of the microbiota. *Lactococcus lactis* ssp. *lactis*, *Lc. lactis*, *Lc. garvieae*, *Lactobacillus casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lc. Lactis* ssp. *cremoris*, *Leuconostoc pseudomesenteroides*, *Leu. mesenteroides*, *Streptococcus lutetiensis*, *Strep. equinus*, *Enterococcus italicus*, *Staphylococcus saprophyticus*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Pichia kudriavzevii*, *Candida tropicalis*, *C. glabrata*, *C. albicans*, *Kluyveromyces marxianus*, and *Kazachstania bulderi* were reported to be the dominant bacteria and yeast species by sequencing 16s rRNA and ITS1-5.8S -ITS2 region, respectively (Byakika and Mukisa, 2024).

2.1.1.4 Dhanaan

Dhanaan is widely consumed fermented camel milk product in rural and urban settlements of Somalia region (Biratu and Seifu, 2016; Berhe et al., 2017; Bekele et al., 2021). In the Somalia region, pastoralists prepare *dhanaan* by spontaneously fermenting camel milk in a clean and smoked containers with *Olea africana* splinters wrapped in a piece of cloth for 12 to 24 hours at room temperature (25-35 °C) (Berhe et al., 2017). Herders produce *dhanaan* because of its high nutritional value, better taste and extended shelf-life of up to five months (Seifu, 2007; Karssa et al., 2024). Berhe et al. (2019) reported *Lactococcus*, *Streptococcus*, and *Weissella* as predominant LAB genera by Next generation sequencing analysis using Ion Personal Genome Machine (PGM); other bacterial genera include *Enterobacter*, *Cronobacter*, *Acinetobacter*, *Buttiauxella*, *Clostridium*, *Brenneria*, *Obesumbacterium*, *Salmonella*, *Erwinia*, *Shimwellia*,

Tatumella, *Hafnia*, *Shigella*, *Kluyvera*, *Pectobacterium*, *Leclercia*, *Escherichia*, *Lelliottia* and *Klebsiella*.

2.1.1.5 Emasi

Another naturally fermented milk product identical to yogurt that is rarely manufactured in the Hhohho province of the Kingdom of Eswatini (formerly known as Swaziland) (Nyambane et al., 2014; Simatende et al., 2019). Predominant LAB species were reported as *Lactococcus lactis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis*, and *Leuconostoc mesenteroides* (Simatende et al., 2019).

2.1.1.6 Ergo/Ititu

Ergo is a spontaneously fermented yoghurt-like product commonly prepared at the household level in Ethiopia (Gonfa et al., 2001; Berhe et al., 2017). It is smooth, thick and semi-solid in nature and has a pleasant odour and taste (Gonfa et al., 2001). It is usually prepared from raw cattle milk. However, in the lowland regions of Ethiopia goat or camel milk is also used (Gonfa et al., 2001). For its production, Raw milk is allowed to ferment for 24 to 72 hours at 16-18 °C temperature (Frew and Abebe, 2020). Herders usually use well-smoked clay pots called *Ensira* for spontaneous fermentation and at the room temperature of 25-35 °C, the product has a shelf-life of about 15-20 days (Andualemm and Geremew, 2014; Karssa et al., 2024). Reported LAB strain from this product were identified as *Lactococcus lactis* subsp. *cremoris*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Streptococcus thermophilus*, *Strep. acidominimus*, *Enterococcus faecalis* var. *liquefaciens*, *Strep. bovis*, *Strep. mitis*, *Strep. agalactiae*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leu. lactis*, and *Lc. lactis* subsp. *lactis* (Gonfa et al., 1999; Gonfa et al., 2001; Mulaw et al., 2019). The increase

in yeast population was also recorded from 1×10^1 CFU mL⁻¹ to 4×10^9 CFU mL⁻¹ during 24 h of *ergo* fermentation (Gonfa et al., 1999).

2.1.1.7 *Garris*

Garris is one more type under the fermented milk/dairy product naturally prepared from camel's milk using semi-continuous or fed-batch fermentation and it is typically made in nations like Somalia and Sudan (Sulieman et al., 2006; Osman et al., 2023). In areas like Sudan, it is ordinarily prepared in fields while the camels are being driven to pastures in distant locations (Dirar, 1993). *Gariss* is kept in two leather bags made of tanned goat skin that are pulled on the back of a camel and are constantly shaken by the jerky gait that camels have (Mirghani, 1994) and is also widely referred as humadah or *hameedh* which means “sour” (Shori, 2012). Several Lactic acid bacterial species were reported in *gariss* such as *Lactobacillus acidophilus*, *Ligilactobacillus animalis*, *Enterococcus faecium*, *Companilactobacillus alimentarius*, *Carnobacterium divergens*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. gasseri*, *Limosilactobacillus fermentum*, *Lcb. rhamnosus*, *Lcb. paracasei*, *Lc. raffinolactis*, *Streptococcus infantarius* subsp. *infantarius*, *Strep. lactis*, *Strep. lactis* subsp. *diactylactis*, *Strep. thermophilus* and *Strep. acidominimus* (Abdelgadir et al., 2008; Hassan et al., 2008; Ashmaig et al., 2009; Ahmed, 2022; Hafiz and Ibtisam, 2024). *Issatchenkia orientalis* and *Kluyveromyces marxianus* are known to be the yeast species preliminary identified in *gariss* (Abdelgadir et al., 2008).

2.1.1.8 *Kindirmou* and *pendidam*

The other naturally prepared fermented milk/dairy products resembling yoghurt are *Pendidam* and *Kindirmou*, normally prepared in areas of Adamawa in Cameroon

(Maïworé et al., 2019; Sohanang et al., 2021). *Pendidam*, the other fermented milk/dairy product made in Cameroon, are acidifying porridges containing reduced milk fat and typically fermented for 48 to 72 hours. On the contrary, *kindirmou* is typically made with cow milk (freshly boiled) that remains under incubation for 12 hours (referred as back-slopping process) and is consumed after adding sugar (Maïworé et al., 2019). Using the culture dependent and independent methods, the studies report the availability of lactic acid bacteria- *Limosilactobacillus fermentum*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus casei*; and yeast species- *Galactomyces candidum*, *Saccharomyces cerevisiae/paradoxus*, *Torulaspora delbrueckii*, *Candida parapsilosis*, and *Kluyveromyces marxianus* (Maïworé et al., 2019; Sohanang et al., 2021; Steve et al., 2024).

2.1.1.9 Kivuguto

Similar to yoghurt-like cow milk product widely made in Rwanda, the identified LAB species in naturally fermented milk product *Kivuguto* include *Lactococcus lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, and *Leu. Pseudomesenteroides* (Karenzi et al., 2012; Karenzi et al., 2013).

2.1.1.10 Lait caillé

Lait caillé, a spontaneously fermented milk/dairy product prepared with unpasteurized milk is the regional product of Northern Senegal (Parker et al., 2018) and Burkina Faso (Bayili et al., 2019). Predominantly reported LAB includes *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Streptococcus* spp., *Lactobacillus* spp., *Enterococcus* spp., *Leuconostoc* spp., *Acetobacter* spp., and *Weissella paramesenteroides*, including the yeast species *Saccharomyces cerevisiae* and *Candida parapsilosis* (Parker et al., 2018; Bayili et al., 2019; Compaore et al., 2021).

2.1.1.11 *Mursik*

Mursik is another naturally fermented milk/dairy product in Kenya, mainly prepared from goat and cow milk by the *Kalenjin* tribe (Riang'a et al., 2017; Nduko et al., 2017). The *Kalenjin* tribe, which has eight separate subtribes, first appeared before the seventeenth century. It is known for a major diet type that makes up over half of Kenyan's daily food intake. The milk is fermented in calabash/gourd, which is locally known as *sotets* for 3-5 days or longer, in accordance with consumer's sensory preferences. Blood is often used in the manufacture of milk locally (Nduko et al., 2017; Kanetkar et al., 2023). When *mursik* is suitable for consumption, a blue or crimson layer form on its surface (Network, 2000). The Lactic acid bacteria reported from *mursik* includes *Lacticaseibacillus casei*, *Lcb. rhamnosus*, and *Lcb. paracasei*. Several other species viz., *Lentilactobacillus kefir*, *Limosilactobacillus fermentum*, *Levilactobacillus brevis*, and *Lactiplantibacillus plantarum* are also found to be reported in *Mursik* (Nieminen et al., 2013; Digo et al., 2017; Khayeka-Wandabwa et al., 2024). The yeast species like *Candida sphaerica*, *C. kefir*, *C. krusei*, and *Saccharomyces fermentati* are also detected in this product (Nieminen et al., 2013; Khayeka-Wandabwa et al., 2024).

2.1.1.12 *Madila*

Madila is naturally fermented cow and goat milk in southern Africa, particularly Botswana. Milk is fermented for 3-4 days and it is collected by removing whey by hanging in a cloth. It is mixed with previously fermented milk to make a liquid or semi-solid curdled product. It is often flavoured with fruit juice or artificial colouring agents (Ohenhen et al., 2013; Mattiello et al., 2018). Most common bacteria isolated and reported in *Madila* includes *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Lb. delbrueckii*, *Lactococcus lactis*, *Limosilactobacillus*

fermentum. Other minor contaminants/ non fermenters viz., *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* are also reported (Ohenhen et al., 2013).

2.1.1.13 Mafi

Another spontaneously fermented milk product that is frequently produced in Lesotho is *Mafi* (Gadaga et al., 2013; Maleke et al., 2021), however, there have been no microbiological reports on the major fermenters so far.

2.1.1.14 Nono

A naturally fermented yogurt-like product attributed to the Nigerian ethnic group, called *Fulani* (Bankole and Okagbue, 1992), is quite similar to yogurt but typically thinner in consistency. It is typically prepared with cow's milk with occasional use of goat's milk (Obadina et al., 2013). Several prominent lactic acid bacterial species are reported in this product: *Limosilactobacillus fermentum*, *Lactobacillus plantarum*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii*, *Lactococcus lactis*, *Lb. brevis*, *Lb. bulgaricus*, *Leuconostoc pseudomesenteroides*, *Lb. senioris*, *Lb. cremoris*, *Weissella* spp., and *Streptococcus thermophilus*. The yeast genera *Saccharomyces*, *Meyerozyma*, *Trichosporon*, *Galactomyces*, *Candida*, and *Cyberlindnera* are found, where *Saccharomyces cerevisiae* remains as the dominant species, followed by *C. glabrata* (Okiki et al., 2018; Diaz et al., 2019; Ayanniran et al., 2020; Uzoaga et al., 2020; Fagbemigun et al., 2021; Obioha et al., 2023; Ikele et al., 2024).

2.1.1.15 Nonnonkoumou

Derived from cow milk, *Nonnonkoumou* is a traditionally fermented dairy product widely consumed in Côte d'Ivoire, particularly in the city of Daloa, situated in the region of Haut-Sassandra (Christelle et al., 2021). Not enough research has been done on this product's microbiological content. To date, research has been limited to the product's

physiological traits and microbiological enumeration (Christelle et al., 2021). Despite the wide prevalence of lactic acid bacteria, yeasts and population of fungi have also been reported beside other pathogenic contaminants (Assohoun-Djeni et al., 2020).

2.1.1.16 *Nunu*

Nunu is a traditional fermented yoghurt-like product in west Africa, particularly among the *Fulani* people in Ghana. Production of *nunu* in northern Ghana does not involve the starter cultures. It is prepared by spontaneously fermenting fresh cow milk in a calabash or in a rubber bucket for a day or two. It is yogurt-like in taste and can be consumed alone or with sugar and *fura* (Owusu-Kwarteng et al., 2010; Akabanda, 2025). As per Owusu-Kwarteng et al. (2010), the latter is made from millet dough mixed with spices, compacted into balls, and cooked for around half an hour. *Fura de nunu* is made by crumbling the cooked *fura* in a bowl of *nunu*. It is often consumed during festivals and community gatherings. However, because of its unhygienic preparation methods and its short shelf-life, it is not appealing to the majority of people (Yahuza, 2001). *Limosilactobacillus fermentum* was the dominant LAB strain *Nunu*, followed by *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *Enterococcus faecium*, *E. italicus* and *Weissella confusa*. The predominant yeast species involved were reported as *Saccharomyces cerevisiae* and *Pichia kudriavzevii* followed by *S. pastorianus*, *Kluyveromyces marxianus*, *Zygosaccharomyces bisporus*, *Candida stellata*, *Zygosaccharomyces rouxii*, *C. parapsilosis*, *Diutina rugosa*, *C. tropicalis* and *Geotrichum candidum* (Akabanda et al., 2013). Many members of Enterobacteriaceae family such as *Escherichia*, *Klebsiella*, *Proteus vulgaris*, *Enterobacter* and *Shigella* were reported in the early stage of fermentation which were eliminated as fermentation progressed (Akabanda et al., 2010). *Listeria monocytogenes* was also observed during

fermentation which may contribute to the post-processing contamination (Owusu-Kwarteng et al., 2018).

2.1.1.17 Rob/Roub

It is a yogurt-like fermented dairy product regional to Sudan (Abdelgadir et al., 2001). Reported LAB were identified as *Lactobacillus delbrueckii*, *Lb. acidophilus*, *Lactococcus lactis*, *Limosilactobacillus fermentum* and *Streptococcus salivarius*, with yeasts identified as *Candida kefy*, and *Saccharomyces cerevisiae* (Abdelgadir et al., 2001; Abdullah and Osman, 2010).

2.1.1.18 Suusac

Derived from camel milk, *Suusac* is a naturally fermented milk/dairy product prevalent in Somalia and Kenya (Lore et al., 2005). Several LAB species found are identified as *Lactiplantibacillus plantarum*, *Latilactobacillus curvatus*, *Lactococcus raffinolactis*, *Ligilactobacillus salivarius*, *Lc. lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Streptococcus thermophilus* and *Strep. infantarius* subsp. *infantarius* (Holzapfel, 2002; Lore et al., 2005; Jans et al., 2012). Yeast species were identified as *Saccharomyces cerevisiae*, *Cryptococcus laurentii*, *Cryptococcus albidus*, *Candida guilliermondii*, *C. krusei*, *C. famata*, *C. lusitaniae*, *C. inconspicua*, *Geotrichum penicillatum*, *Rhodotorula mucilaginosa*, *Trichosporon mucoides* and *Trichosporon cutaneum* (Lore et al., 2005, Njage et al., 2011).

2.1.1.19 Shubat

Shubat, another traditional fermented product derived from camel milk with less viscosity than yogurt, is known as *shubat* in Kazakhstan and China (Xinjiang), and *chal* in Turkey (Shori, 2012). With the advent of culture independent and culture dependent technique, the predominant detected LAB include *Lactobacillus delbrueckii*, *Lb.*

helveticus, *Latilactobacillus sakei*, *Lb. kefir*, *Levilactobacillus brevis*, *Leuconostoc mesenteroides*, *Leu. lactis*, *Weissella hellenica*, *Enterococcus faecalis*, *Enterococcus faecium* and *Streptococcus thermophilus* (Rahman et al., 2009; Zhadyra et al., 2021). The major yeast species reported are *Kluyveromyces marxianus*, *Candida ethanolica* and *Kazachstania unispora* (Rahman et al., 2009).

2.1.1.20 Zabady

It is considered as world's oldest fermented yoghurt-like product, notably in Middle-Eastern countries (especially Egypt) (El-Baradei et al., 2008). The major LAB species reported in *Zabady* are *Lactococcus raffinolactis*, *Lc. lactis*, *Lc. garvieae*, *Lc. garvieae*, *Lactobacillus johnsonii*, *Lb. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc citreum* (El-Baradei et al., 2008).

2.1.2 Artisanal butter

2.1.2.1 Amavuta

This naturally fermented product locally available in Burundi, is an artisanal butter traditionally obtained by churning cow's milk (Aloys and Angeline, 2009; Mattiello et al., 2018). So far, the product reports no microbiological evidences.

2.1.2.2 Qibe/Kibe

Another artisanal butter normally prepared by churning an *ergo*, is *Qibe/Kibe* regional to Ethiopia (Getahun et al., 2019; Karssa et al., 2024). Once *ergo* (fermented yoghurt-like product) has sufficiently fermented, it is churned in a conventional clay pot churner and pseudo-stem of *enset* (*Ensete ventricosum*) is used to cover the churner's mouth (Berhe et al., 2017). Small granules are removed after churning and kneaded in cold water to remove any residual buttermilk (Kuyu and Bereka, 2020). In addition to human consumption, it is also used as a traditional skin and hair cosmetic (Andualemm and

Geremew, 2014). By adding herbs and spices, fresh butter can also be further processed into *nitir qibe*, a traditional *ghee*, which is then melted by heating before consumption. Microorganisms were not reported for this product (Andualemm and Geremew, 2014). No microorganisms were reported till date.

2.1.2.3 Shmen

Camel milk is also utilized to prepare fermented items in North Africa's Saharan regions. *Shmen* is a traditionally significant butter prepared in Algeria that is commonly linked to nomadic ethnic population *Tuareg* (or *Touareg*) (Mourad and Nour-Eddine, 2006). The substance is deemed unusual because no further microbiological studies exist except from documented in study of Mourad and Nour-Eddine, (2006). The bioMérieux analytical profile index (API) identification system report certain species of yeasts and lactic acid bacteria. LAB includes: *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *cremoris*, *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Enterococcus faecium*, *Leuconostoc gelidum*, and *Leu. pseudomesenteroides*, with *Saccharomyces cerevisiae* being the only identified yeast (Mourad and Nour-Eddine, 2006).

2.1.3 Buttermilks

2.1.3.1 Amateregwa

The local term for buttermilk is *amateregwa*, regional to Burundi (Aloys and Angeline, 2009; Mattiello et al., 2018). There have been no reports of microbiological studies using this product.

2.1.3.2 Arrera/Arera

Arrera, a semi-liquid byproduct of butter production, is typically found in Ethiopia (Berhe et al., 2017). It comprises a thin and smooth consistency containing whey and casein (Bereda et al., 2014). With close resemblance to *ergo* in flavour, aroma and colour, it can be heated to further processed into *ayib* or consumed fresh (Karssa et al., 2024). Compared to other conventional milk products, *Arrera* is known to have fewer calories and a shorter shelf life of 24-48 hour (Goa et al., 2022). Yilma et al. (2005) reported *Escherichia coli*, *Cronobacter sakazakii*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* during sample collection in wet and dry seasons.

2.1.3.3 Hazo

Hazo is another traditionally prepared buttermilk from *arrera* (defatted buttermilk) in Tigray Regional State of northern Ethiopia (Awulachew, 2021). The buttermilk is normally prepared with the addition of cereal grain flour, pulses and spices, such as *Alium sativum* (Garlic), *Curcuma domestica* (turmeric), and *Cuminum cyminum* (cumin). Other spices: *Lepidium sativum* (garden cress), *Ocimum basilicum* (basil), *Nigella sativa* (nigella), *Trachyspermum ammi* (ajwain seed), *Ruta chalepensis* (rue), *Trigonella foenum-graecum* (fenugreek), *Zingiber officinale* (ginger), *Piper igrum* (black pepper), and *Aframomum corrorima* (Ethiopian cardamom) are also used (Berhe et al., 2017). Addition of spices is mainly done to preserve the product's quality and enhance its nutritional content (Gebreselassie et al., 2012). With weekly supplementation of a freshly fermented *hazo*, its shelf life can be extended from one to two weeks to up to one month and it is served to visitors and honoured relatives (Gebreselassie et al., 2012; Berhe et al., 2017; Karssa et al., 2024). Microbial composition has not been documented for this product.

2.1.3.4 Lben/leben

It is a fermented buttermilk-like product indigenous to Morocco, and is simply prepared by allowing milk to ferment at ambient room temperature, followed by the coagulated milk extraction (Hamama, 1992). Similar to this product is *laban rayeb* of the Egypt (El-Gendy, 1983). Predominant species reported in *Lben* includes *Leuconostoc*, *Enterococcus*, and *Lactococcus*, with *Lactobacillus* species observed at low numbers. Bacterial species consists of *Enterococcus gilvus*, *E. faecalis*, *E. durans*, *E. hirae*, *E. faecium*, *Lactobacillus plantarum*, *Lb. brevis*, *Lactococcus garvieae*, *Lb. rhamnosus*, *Lb. paracasei*, *Lactococcus lactis* (*Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and *Lc. lactis* subsp. *lactis*), *Leuconostoc kimchi*, *Leu. pseudomesenteroides*, *Leu. citreum*, *Leu. mesenteroides*, *Weissella viridescens*, *Weissella confuse*, *Weissella cibaria*, *Weissella paramesenteroides*, and *Pediococcus pentosaceus*. Certain predominant yeast species are *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* var. *marxianus*, and *Kl. Lactis* (Hamama, 1992; Benkerroum and Tamime, 2004; Ouadghiri et al., 2009; Mangia et al., 2014).

2.1.3.5 Omaere/omashikwa/mpofu

Widely accessible in Namibia's Oshikoto, Oshana, Omusati, Omaheke, and Ohangwena (Misihairabgwi and Cheikhyoussef, 2017), this dairy product is frequently prepared collecting fresh milk into a calabash, followed by adding *omukwa* (*Adansonia digitata*) or *omunkuzi* (*Boscia albitrunca*) roots, and back-slopping with old starter culture (Misihairabgwi and Cheikhyoussef, 2017). Also produced in Angola region and by Botswana's *Herero* (*Bantu*) ethnic communities (Plaatjie, 2018), *Omashikwa* is distinguished by its bitter and rotten flavor, strong acidity, root-like taste, low pH, and viscous (Bille et al., 2007). Culture-dependent investigation identified LAB species viz., *Lacticaseibacillus casei*, *Lcb. paracasei* subsp. *paracasei*, *Lcb. rhamnosus*, *Lactiplantibacillus plantarum*, *Lentilactobacillus kefir*, and *Lactococcus lactis*.

Saccharomyces cerevisiae, *Kazachstania unispora*, and certain dominant yeast species like *Candida pararugosa* (Heita, 2014; Kutaa, 2017).

2.1.4 Artisanal cheese-like products

2.1.4.1 Ayib

Ayib, a popular fermented milk product is typically prepared in Ethiopia. It is essentially an artisanal cottage cheese prepared by heating it in a clay-made pot between 40 and 70 °C (Berhe et al., 2017), and is also known as *metata ayib* when spice is added (Geremew et al., 2015). Reported LAB species from this product includes *Lacticaseibacillus paracasei*, *Lcb. rhamnosus*, *Streptococcus thermophilus*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Lactobacillus acidophilus* (Girma and Aemiro, 2021). *Aguat* is a term devised for the acid whey that remains over after making *ayib* in Ethiopia (Berhe et al., 2017). There is no microbiological report for this product.

2.1.4.2 Jben

It resembles a cheese-like fermented milk product usually made by fermenting *raib* for an extended period of time after draining the whey. Sometimes, the product is also blended with salt (Hamama, 1992). Prepared from ewe or goat milk, it is typically found in Maghreb nations such as Libya, Morocco, Algeria, Tunisia, and Mauritania (Leksir et al., 2019). Certain reported LAB species includes- *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Lcb. paracasei*, *Lcb. rhamnosus*, *Lactococcus raffinolactis*, *Lc. lactis* subsp. *lactis*, *Lc. garvieae*, *Leuconostoc mesenteroides*, *Leu. lactis*, *Leu. citreum*, *Leu. pseudomesenteroides*, *Enterococcus faecium*, *E. durans*, *E. saccharominimus*, *E. faecalis*, *Lentilactobacillus buchneri*, *Streptococcus* sp., and *Levilactobacillus brevis* (Hamama, 1992; Ouadghiri et al., 2006).

2.1.4.3 *Raib*

Seasonally, raw milk in Morocco undergoes natural fermentation at ambient room temperature for one to three days. Following fermentation, *raib* (coagulated milk) is collected and eaten as it is (Hamama, 1992). The LAB species reported are *Enterococcus faecalis*, *E. durans*, *E. faecium*, *E. hirae*, *Lactococcus garvieae*, *Lc. lactis* (*Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and *Lc. lactis* subsp. *lactis*), *Lactiplantibacillus plantarum*, *Leu. mesenteroides* subsp. *mesenteroides*, and *Leuconostoc mesenteroides* subsp. *dextranicum* (Hamama, 1992; Elotmani et al., 2002; Bendimerad et al., 2012; Moumene et al., 2016).

2.1.4.4 *Rayeb*

In Egypt's rural areas, the buffalo's raw milk is used for the production of this traditional fermented milk product (Abd El Gawad et al., 2010). The identified LAB species were reported as *Lactobacillus helveticus*, *Lactococcus acidophilus*, *Lb. bulgaricus*, *Lb. delbrueckii*, *Streptococcus thermophilus*, *Strep. acidominimus*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Aerococcus viridans*, *Enterococcus durans*, and *E. faecium* (Abd El Gawad et al., 2010).

2.1.4.5 *Wagashi* (or *gassire*)

In traditional dairy practices of Benin and Niger, milk is typically fermented to make a cheese-like product referred to as *Wagashi* (or *gassire*) (Sessou et al., 2013; Akabanda, 2025). High-throughput Illumina MiSeq amplicon sequencing reports the studies of yeasts communities only, with the dominant yeast species reported as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Sagenomella keratitidis*, and *Candida parapsilosis* (Sessou et al., 2019).

2.2 Microbial diversity of NFM products from Europe, South America, and Asia (including Russia and the Middle East)

2.2.1 Yoghurt-like NFM products

2.2.1.1 *Airag*

It (also known as *koumiss*) refers to a traditionally fermented milk/dairy beverage prepared from mare's milk in Kazakhstan, Mongolia, Kyrgyzstan, and some parts of Southern Russian Steppe Regions (Watanabe et al., 2008). When prepared from cow, ewe, yak, camel, or goat milk, it is referred as *tarag* or *isgelen tarag* (Uchida et al., 2007; Watanabe et al., 2008). Studies on *airag* report LAB species viz., *Lactobacillus kefiranofaciens*, *Lb. helveticus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Companilactobacillus farciminis*, and *Lentilactobacillus kefiri*; with yeast species *Saccharomyces cerevisiae*, *S. dairensis*, *Kluyveromyces wickerhamii*, *Kl. marxianus*, and *Issachenkia orientalis* identified (Uchida et al., 2007; Watanabe et al., 2008). *Tarag* report LAB species identified as *Lactobacillus fermentum*, *Lb. acetotolerans*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Streptococcus thermophilus*, *Lcb. paracasei*, *Len. kefiri*, with yeast species identified as *S. cerevisiae*, *Issatchenkia orientalis*, and *Kazachstania unispora* (Uchida et al., 2007; Watanabe et al., 2008; Su et al., 2025). The 16S rRNA clone library construction and denaturing gradient gel electrophoresis (DGGE), diverse microbial prevalence revealed an abundance of *Len. kefiri*, *Len. hilgardii*, *Lactobacillus kefiranofaciens*, *Lcb. paracasei*, *Len. parakefiri*, *Lactiplantibacillus pentosus*, *Lactococcus lactis* subsp. *lactis*, *Lb. helveticus*, *Enterococcus hirae*, *Strep. thermophilus*, *Bacillus safensis*, *Bacillus pumilus*, *Exiguobacterium profundum*, *Acinetobacter calcoaceticus*, *Kocuria rhizophila*,

Acetobacter pasteurianus, *Gluconobacter cerinus*, *Psychrobacter* sp., and *Paenibacillus* sp. (Ringø et al., 2014; Su et al., 2025).

2.2.1.2 Ayrān

Resembling to yoghurt-like fermented milk, this product is widely prepared in Russia, Kazakhstan, and Turkey (Baruzzi et al., 2016; Zhadyra et al., 2021; Kaledina et al., 2021). The product is denoted by several names depending on the country origin- *dough/doogh* (Iran), *ayrani* (Cyprus), *shenina* (Jordan), *laban arbil* (Iraq), *tan* (Armenia), and *laban ayrān* (Syria) and *laban ayrān* (Lebanon) (Baruzzi et al., 2016). The culture-dependent and independent research report certain LAB species- *Lactobacillus helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lactocaseibacillus paracasei*, *Streptococcus thermophilus*, and *Limosilactobacillus fermentum* (Baruzzi et al., 2016; Zhadyra et al., 2021).

2.2.1.3 Dadih/dadiāh

The Indonesian ethnic minority called *Minangkabau*, or *Minang*, prepares this fermented milk from buffalo (Arnold et al., 2021). Based on culture-independent analysis, the LAB genera *Lactobacillus*, *Lactococcus*, *Acetobacter*, *Bifidobacterium*, *Enterococcus*, and *Leuconostoc* are predominant. Unwanted or contaminating genera, such as *Staphylococcus*, *Klebsiella*, *Corynebacterium*, *Acinetobacter*, *Serratia*, *Frateuria*, *Stenotrophomonas*, *Chryseobacterium*, and *Raoultella* are also detected (Sukma et al., 2018; Fatdillah et al., 2021). Potential probiotic bacteria, such as *Lactiplantibacillus plantarum*, *Enterococcus fecium*, and *Lactobacillus fermentum* have also been found in culture-dependent studies (Collado et al., 2007; Amelia et al., 2020).

2.2.1.4 Dahi

A major and widely consumed fermented yoghurt-like products in Bangladesh, Bhutan, Nepal, India, and Pakistan is *Dahi* (Tamang et al., 2020).

2.2.1.4.1 *Dahi* (Bangladesh)

The majority of LAB species have been reported by culture-dependent study of *dahi*, including *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus raffinolactis*, *Lc. lactis* subsp. *lactis*, *Pediococcus pentosaceus*, *Enterococcus faecium*, *Streptococcus bovis*, *Strep. thermophilus*, and *Limosilactobacillus fermentum* (Harun-ur-Rashid et al., 2007). Additionally, culture-independent analysis has demonstrated that *Streptococcus* and Firmicutes-associated *Lactobacillus* are the most prevalent LAB species, along with less abundant genera *Acinetobacter*, *Pseudomonas*, and *Micrococcaceae* associated with Proteobacteria and Enterobacteriaceae (Nahidul-Islam et al., 2018). However, the most prevalent species of yeast were *Kodamaea ohmeri*, following *Candida parapsilosis*, *Clavispora lusitaniae*, *Trichosporon* sp., and *C. tropicalis*, while minor genera included *Lichtheimia*, *Syncephalastrum*, and *Moniliera* (Nahidul-Islam et al., 2018).

2.2.1.4.2 *Dahi* (Pakistan)

Culture-dependent study of *dahi* regional to Pakistan has revealed that *Lactobacillus delbrueckii* subsp. *delbrueckii* remains as predominant LAB species, followed by *Lb. delbrueckii* subsp. *lactis*, *Lb. acidophilus*, *Lacticaseibacillus casei*, *Streptococcus thermophilus*, *Lb. helveticus*, *Enterococcus mundtii*, and *Lactococcus lactis* (Soomro and Masud, 2007; Mahmood et al., 2013; Nawaz et al., 2019).

2.2.1.4.3 *Dahi* (Bhutan)

In Bhutan, *dahi* has gained significant popularity and is prepared regularly using a back-slopping technique and spontaneous fermentation (Shangpliang et al., 2017). A culture-dependent investigation reported by Shangpliang et al. (2017), revealed the existence of a possible probiotic strain, *Enterococcus faecium*.

2.2.1.4.4 *Dahi* (Nepal)

Nepali communities in Nepal also prepare *dahi* on a daily basis. Fermentative LAB members reported based on culture dependent study includes *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Lacticaseibacillus rhamnosus*, *Lcb. paracasei* (Koirala et al., 2014; Bhattarai et al., 2016).

2.2.1.4.5 *Dahi* (India)

Dahi, a significant traditionally prepared fermented milk/dairy product primarily prepared using cow or buffalo milk, is a staple in most Indian homes. Also termed as *shyow* when made from yak's milk, it is a popular and common delicacy for the Tibetans (Dewan and Tamang, 2007). With the advent of culture dependent study, the microbes identified and reported are *Companilactobacillus alimentarius*, *Ligilactobacillus bifermentans*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus paraplantarum*, *Lactiplantibacillus pentosus*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *tructae*, *Enterococcus italicus*, and *Leuconostoc mesenteroides* (Dewan and Tamang, 2007; Ghatani and Tamang, 2017; Rai, 2020; Tirwa et al., 2021). Furthermore, culture-independent analysis reported the abundance of *Lc. lactis*, *Lc. raffinolactis*, *Lactobacillus helveticus*, *Lb. gasseri*, *Leuconostoc mesenteroides*, *Leu.*

pseudomesenteroides, *Acetobacter pasteurianus*, *Acetobacter lovaniensis*, *Pseudomonas fluorescens*, *Gluconobacter oxydans*, and *Acetobacter syzygii* (Shangpliang et al., 2018; Rai, 2020).

2.2.1.5 Gioddu

Goat or Ovine milk is used to prepare this Italian acidulous milk, also called acid-alcoholic fermented milk (Maoloni et al., 2020). It has various other names as well viz., *mezzoraddu*, *latte ischidu*, or *miciuratu*. Culture-independent study identified *Lactobacillus delbrueckii* as the predominant LAB species, following prevalence of *Streptococcus thermophilus*, *Lactococcus lactis*, and *Lactobacillus kefir*. *Kluyveromyces marxianus* were detected as a predominant yeast species, followed by *Saccharomyces cerevisiae*, *Pichia cactophila*, *Glomus hyderabadensis*, *Galactomyces*, *Geotrichum*, *Galactomyces candidum*; with reports of certain low-abundant species of *Cladosporium*, *Alternaria*, and *Aerobasidium* (Maoloni et al., 2020).

2.2.1.6 Gwell

Back-slopping serves as a typical method of preparing this traditional fermented milk/dairy product in the Brittany region of France (von Gastrow et al., 2020). The outcomes of both culture-dependent and culture-independent analyses showed that *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Lc. lactis* subsp. *cremoris* were more prevalent than *Lactobacillus acidophilus*, *Lb. helveticus*, *Streptococcus thermophilus* and *Staphylococcus warnerii*, along with a few eukaryotic species, including the yeasts *Kazachstania servazii*, *Yarrowia lipolytica*, and the fungi *Geotrichum candidum* (von Gastrow et al., 2020).

2.2.1.7 Hurunge

Regional to Mongolia, it is often prepared from the milk of cows, camels, sheep, horses, and goats (Shuangquan et al., 2006). The most prevalent LAB species reported were *Lactococcus raffinolactis*, followed by *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Lactobacillus homohiochii*, *Lb. kefiranofaciens*, *Lb. acetotolerans*, and *Leuconostoc mesenteroides* ssp. *Cremoris* (Shuangquan et al., 2006). Certain yeast species like *Saccharomyces cerevisiae*, *Candida valida*, *C. kefir*, *C. krusei*, and *Kluyveromyces marxianus* var. *lactis* were also prevalent in accordance to the study made by Shuangquan et al. (2006).

2.2.1.8 Kashk zard

This well-known fermented milk/dairy product in Persia, is also referred to as yellow curd (Pakroo et al., 2020). The major Firmicutes LAB species reported by Culture-independent study, includes *Limosilactobacillus fermentum*, *Lim. reuteri*, *Lim. vaginalis*, *Lim. pontis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus zae*, *Levilactobacillus brevis*, *Streptococcus thermophilus*, *Pediococcus acidilactici*, and *Lactobacillus delbrueckii* (Pakroo et al., 2020).

2.2.1.9 Kefir

This symbiotic fermentation involving yeasts and lactic acid bacteria embedded in the *kefir* grain structure makes it among the most extensively and globally consumed fermented milk drinks with both acidic and alcoholic characteristics (Prado et al., 2015; Bourrie et al., 2016). Kefir is a favoured fermented milk product that has been the subject of extensive research on microbial diversity. It is reported from many countries, including those in Asian regions such as in Tibet (China) and Taiwan (China), Turkey; in Africa (majorly South Africa); across Asia and Europe including Russia; throughout Europe in countries like Bosnia and Herzegovina, Czech Republic/Czechia, Belgium,

Estonia, Ireland, Greece, Romania, Hungary, Norway, Italy, Slovakia, Latvia, Poland, and Ukraine; in South American countries like Brazil and Argentina (Bengoa et al., 2019). Several studies have reported diverse lactobacilli associated with *kefir* fermentation, particularly those belonging to *Lentilactobacillus* genera. Notable species include *Len. buchneri*, *Len. kefir*, *Len. parabuchneri*, *Len. diolivorans*, *Len. rapi*, *Len. otakiensis*, *Len. sunkii*, *Len. parakefiri*, and *Len. parafarraginis*. Additional genera like *Limosilactobacillus* and *Liquorilactobacillus* are also reported, including *Lim. reuteri*, *Lim. fermentum*, and *Liquorilactobacillus satsumensis*. Other identified species include *Ligilactobacillus salivarius*, *Furfurilactobacillus rossiae*, *Levilactobacillus brevis*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus*. Members of *Lacticaseibacillus* genus- *Lcb. paracasei*, *Lcb. rhamnosus*, along with *Lactiplantibacillus plantarum*, *Lactiplantibacillus casei*, *Lactiplantibacillus pentosus* are also found. Classic *Lactobacillus* species: *Lb. delbrueckii*, *Lb. helveticus*, *Lb. crispatus*, *Lb. garvieae*, *Lb. intestinalis*, *Lb. kefiranoferiens*, *Lb. kefirgranum*, *Lb. johnsonii*, *Lb. amylovorus*, *Lb. gasseri*, *Lb. acidophilus*, *Lb. kalixensis*, and *Lb. gallinarum* have also been documented (Bourrie et al., 2016). The other species of LAB includes Lactococci- *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Lc. lactis* subsp. *cremoris*, and *Lc. garvieae* were reported. This followed the prevalence of *Leuconostoc* members like *Leu. mesenteroides* subsp. *mesenteroides*, *Leu. pseudomesenteroides*, and *Leu. mesenteroides* subsp. *cremoris*. Certain *Pediococcus* species include *Pediococcus claussenii*, *Pediococcus pentosaceus*, *Pediococcus lolii*, *Pediococcus halophilus*, *Pediococcus damnosus* have also been reported; with prevalence of *Oenococcus oeni*, *Streptococcus salivarius* subsp. *thermophilus*, *Strep. durans*, *Strep. thermophilus*, and *Weissella viridescens* (Bourrie et al., 2016). The other reported fermenters from kefir include- acetic acid bacteria- *Acetobacter lovaniensis*, *Acetobacter orientalis*, *Acetobacter sicerae*, and non-

Firmicutes LAB species- *Bifidobacterium pseudolongum*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium choerinum* (Bourrie et al., 2016). The following yeast and fungus species have been associated to kefir: *Candida kefir*, *C. holmii*, *C. lipolytica*, *C. maris*, *C. friedrichii*, *C. inconspicua*, *C. sake*, *Cyberlindnera jadinii*, *Cryptococcus humicolus*, *Eurotium amstelami*, *Dipodascus capitatus*, *Ganoderma lucidum*, *Dioszegia hungarica*, *Issatchenkia orientalis*, *Heterbasidion annosum*, *Dekkera anomala*, *Davidiella bruxellensis*, *Davidiella tassiana*, *Geotrichum candidum*, *Saccharomyces cariocanus*, *S. turicensis*, *S. cerevisiae*, *S. unisporus*, *S. humaticus*, *Microdochium nivale*, *Malassezia pachydermatis*, *Naumovozyma castelli*, *Pichia fermentans*, *Yarrowia lipolytica*, *Peziza campestris*, *Zygosaccharomyces lentus*, *Torulaspora delbrueckii*, *Wallemia sebi*, *Trichosporon coremiiforme*, *Kazachstania barnettii*, *Kazachstania servazzii*, *Kazachstania aerobia*, *Kazachstania solicola*, *Kazachstania exigua*, *Khuyveromyces marxianus* var. *lactis*, *Kl. marxianus*, *Kl. lactis*, and *Teratosphaeria knoxdavisii* (Bourrie et al., 2016).

2.2.1.10 Krokmath

Traditionally prepared fermented dairy product in Bulgaria, it is soft and creamy, and has enhanced fat and salt content, and appears as soft smear cheese as compared to yoghurt (Dimov, 2022). Predominating LAB species were identified and reported as *Lactococcus lactis* subsp. *lactis*, followed by *Khuyvera georgiana*, *Exiguobacterium* sp.; while other less prevalent bacteria such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus garvieae* subsp. *garvieae*, *Streptococcus salivarius* subsp. *thermophilus*, *Apilactobacillus kunkeei*, *Enterococcus faecalis*, *Aeromonas* sp., *Klebsiella* sp., and *Escherichia-Shigella* (Dimov, 2022).

2.2.1.11 Kurut

Yak's milk is used to prepare *Kurut*, a naturally fermented yoghurt-like product in Tibet, China, that can also be dried (Liu et al., 2012). LAB species commonly found include: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactococcus lactis* ssp. *Lactis*, *Limosilactobacillus fermentum*, *Lacticaseibacillus casei*, *Streptococcus thermophilus*, and *Acetobacter* sp. (Chen et al., 2010; Liu et al., 2012).

2.2.1.12 *Långfil* and *Filmjöl*

Långfil represents a fermented yoghurt-like product from Sweden, with ropy and slimy qualities (Widyastuti et al., 2021), prominently due to the exopolysaccharides produced by LAB species. LAB species includes: *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. However, *Filmjöl* is somewhat less sour than yoghurt, and is typically made by *Leuconostoc mesenteroides* and *Lc. lactis*, but still shows resemblance to *långfil* (Hati et al., 2019).

2.2.1.13 *Meekiri/mee-deekiri*

A naturally fermented milk/dairy product resembling curd, this product is prepared exclusively from the milk of water buffalo in Sri Lanka (Priyashantha et al., 2021). Based on Culture-dependent study, identified and reported LAB species comprises of *Latilactobacillus curvatus*, *Lactobacillus acidophilus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Streptococcus thermophilus*, and *Limosilactobacillus fermentum*. *Saccharomyces cerevisiae* is the only reported yeast species (Dekumpitiya et al., 2016; Adikari et al., 2021).

2.2.1.14 *Vili*

This fermented milk/dairy product, which resembles mesophilic yoghurt, is frequently reported in Taiwan and Finland (Wang et al., 2008; Kahala et al., 2008). The majority

of LAB species are *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Leuconostoc mesenteroides* subsp. *cremoris*. *Saccharomyces unisporus*, *Kluyveromyces marxianus* and *Pichia fermentans* are the predominant species of yeasts found (Wang et al., 2008; Kahala et al., 2008).

2.2.2 Fermented creams

2.2.2.1 Jiaoke

In Inner Mongolia, China, this product is traditionally prepared fermented cream prepared from the fat extracted from the fermented milk (Gong et al., 2010; Fan et al., 2020). Microorganisms reported from *Jiaoke* includes *Lacticaseibacillus casei*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lactococcus garvieae*, *Lactobacillus helveticus*, and *Lc. Lactis*. Several other strains viz., *Leuconostoc lactis*, *Streptococcus gallolyticus*, *Strep. thermophilus*, *Enterococcus faecium*, *E. gilvus*, *E. italicus*, *E. faecalis*, and *E. gallinarum* are also reported (Fan et al., 2020).

2.2.2.2 Philu (or philuk)

It is also a traditionally prepared milk product with creamy consistency, prepared using either cow or yak milk, and is usually prepared and consumed by the Sikkimese *Bhutia* and *Sherpa* ethnic groups (Dewan and Tamang, 2007). LAB species reported by culture-dependent investigation includes *Lactococcus lactis* subsp. *cremoris*, *Lacticaseibacillus paracasei* subsp. *paracasei*, *Enterococcus faecium*, and *Loigolactobacillus bifermentans* (Dewan and Tamang, 2007; Rai, 2020).

2.2.3 Artisanal butter products

2.2.3.1 Gheu

In Nepal, Sikkim, and the Darjeeling Hills, this artisanal butter is often prepared using cow or yak's milk by churning raw or cooked milk utilising a traditional tool, referred as *theke* (Tamang, 2010c). Various dialects have different names for it, such as *ghee* or *makhan* in Hindi, *mor* in *Lepcha*, and *maa* in Tibetan (Tamang, 2010c). The most dominant species, according to culture-independent analysis, was *Lactococcus piscium*, followed by *Pseudomonas fluorescens*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Lc. raffinolactis*, *Leuconostoc mesenteroides*, *Leu. lactis*, *Lb. gasseri*, and uncultured bacteria (Rai, 2020). Till now, no reports of culture-dependent study have been examined.

2.2.4 Buttermilks

2.2.4.1 Mohi

Mohi is a well-known traditionally fermented milk/dairy product frequently prepared in some regions of India, which is commonly referred in various regions of the country as "*chaas*" (Mallappa et al., 2020). The culture-based analysis reported the presence of *Lactocaseibacillus rhamnosus*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, and *Companilactobacillus alimentarius* (Dewan and Tamang, 2007; Ansari et al., 2019; Rai, 2020). According to a next-generation sequencing study based on pyrosequencing, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were reported as the most prevalent species. Other minor species included *Macroccoccus caseolyticus*, *Ralstonia solanacearum*, *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Psychrobacter arcticus*, *Enterococcus faecalis*, *Dictyoglomus turgidum*, *Methylobacterium radiotolerans*, *Bartonella quintana*, *Methylobacterium populi*, *Synechocystis* sp., and *Thermoanaerobacter* sp. (Jayashree et al., 2013).

2.2.5 Artisanal cheese-like products

2.2.5.1 *Chhurpi* (hard and soft)

Chhurpi, either prepared as soft or firm type of artisanal or cottage cheese, is widely known in the Himalayan regions that include Sikkim (called "*sherkam*" by the *Sherpas*), Darjeeling, Ladakh, Arunachal Pradesh, Nepal, and Bhutan (Tamang, 2022b). In Bhutan, the hard-variety *chhurpi* is referred to as *chugo/churkam* and the soft-variety is identified as *datshi* (Shangpliang et al., 2017). Culture-based investigations have documented the prevalence of *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *hordniae*, *Enterococcus faecium*, *E. pseudoavium*, and *E. faecalis*. Other reported bacteria include *Leuconostoc mesenteroides* subsp. *jonggajibkimchii*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Latilactobacillus curvatus*, *Lentilactobacillus kefir*, *Len. hilgardii*, and *Companilactobacillus alimentarius* (Tamang et al., 2000; Dewan and Tamang, 2007; Rai, 2020). According to culture-independent analysis, the most prevalent bacteria are *Lc. lactis* and *Lactobacillus helveticus*, followed by *Lb. delbrueckii*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, *Acetobacter pasteurianus*, *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Gluconobacter oxydans*, *Hafnia alvei*, *Pseudomonas fluorescens*, and *Staphylococcus cohnii* (Rai, 2020). *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Issatchenkia orientalis*, and *Candida parapsilosis* are among the yeast species found in soft *chhurpi* (Rai et al., 2016).

2.2.5.2 *Somar*

It is a different type of *chhurpi* which is frequently made in Sikkim and is typically fermented for roughly 15 days in an anaerobic environment (Dewan and Tamang, 2007). Additionally, it is a rare product that is naturally fermented, with only two LAB species

(*Lacticaseibacillus paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *cremoris*) identified by culture-dependent study (Dewan and Tamang, 2007).

2.2.5.3 *Vrum/wrum*

This particular kind of artisanal cottage cheese refers as a typically fermented product in Inner Mongolia, China (Yamei et al., 2019). The majority of LAB includes: *Lentilactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactococcus lactis*, *Lb. helveticus*, *Streptococcus salivarius*, *Len. diolivorans*, and *Acetobacter orientalis*. Reported yeast species comprising of *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Kl. marxianus*, *Kazachstania unispora*, *Pichia fermentans*, *Trichosporon asahii*, *Mucor circinelloides*, and *Mucor racemosus* (Yamei et al., 2019). With the culture-independent Illumina MiSeq amplicon sequencing, majority of species, including *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lc. raffinolactis*, *Pseudomonas brenneri*, *Leuconostoc mesenteroides*, and *Pediococcus acidilactici* were recently discovered (Liang et al., 2021).

2.2.6 Artisanal cheeses

Cheese, a yet another well-liked fermented milk/dairy product, is widely prepared on several countries worldwide. *Lactobacillus lactis* and *Streptococcus thermophilus* are the two most common species found in the core and rind of most cheese varieties. The most prevalent fermenting microbes in cheese belong to the *Lactobacillaceae*, *Streptococcaceae*, *Leuconostocaceae*, and *Enterococcaceae* families (Dugat-Bony et al., 2016; Choi et al., 2020a; Choi et al., 2020b). *Surk* is another traditional cheese commonly prepared in the East Mediterranean region of Lebanon, Turkey, Syria, and other parts of Middle East. Predominant LAB species common in *Surk* are *Lactiplantibacillus plantarum*, *Ligilactobacillus acidipiscis*, and *Companilactobacillus*

alimentarius; non-LAB species *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Staphylococcus sciuri*, *Staphylococcus lentus*, and *Bacillus* sp. (Esen and Çetin, 2021).

Portugal origin, the traditional cheese also known as *Queijo de Azeitão* PDO cheese revealed *Lentilactobacillus kefir*, *Lacticaseibacillus zae*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* as the predominant members (Cardinali et al., 2021). With similar reports made in cheddar cheese (Afshari et al., 2020; Camargo et al., 2021), the majority of the core bacterial genera identified in Historic Rebel (HR) cheese (99%) consisted Firmicutes-associated genera, such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Turri et al., 2021). The predominant fermenting bacteria in *Kazak* artisanal cheese were identified to be *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter*, *Anoxybacillus*, *Kurthia*, *Macroccoccus*, *Lelliottia*, and *Acinetobacter* (Zheng et al., 2021). Several other *Lactococcus* and *Enterococcus* (*E. faecium*) were in association to bacteriogenic potential of the cheese microbiome, according to a shotgun metagenomics study conducted in artisanal cheese from Argentina (Suárez et al., 2020). Furthermore, *Lactococcus lactis*, *Strep. thermophilus*, *Acinetobacter johnsonii*, and *Strep. salivarius* are among the core microbiota identified in *Minas* artisanal cheese of Brazil (Nero et al., 2021). Prevalent microbiota based on culture-based and PCR-DGGE based culture-independent techniques in traditional Turkish *goatskin Tulum* cheese are *Lb. delbrueckii* subsp. *bulgaricus*, *Strep. lutetiensis*, *Strep. gallolyticus*, *Enterococcus hirae*, and *Streptococcus* spp.; with *Enterococcus* and *Lactococcus* spp. as predominant genera as reported in the culture-based study of the cheese ripening (Demirci et al., 2021). *Leu. mesenteroides*, *Limosilactobacillus fermentum*, *Lcb. casei*, and *Enterococcus* sp. are the most commonly found microbes found during the entire fermentation process of Brazilian buffalo *mozzarella* cheese (Silva et al., 2021). Yeasts are another type of fermenting

microorganism that is frequently present in handmade cheeses. According to Yildiz et al. (2021), the traditional moldy civil cheese of Turkey was found to contain *Kl. marxianus*, *Debaryomyces hansenii*, *Pichia membranifaciens*, *Pichia fermentans*, *Yarrowia lipolytica*, and *Candida zeylanoides*. *Skyr* also showed the predominance of *Kl. lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, and *C. Zeylanoides* (Esen and Çetin, 2021). *Kl. lactis*, *Kl. marxianus*, and *Pichia kudriavzevii* were also found to be prevalent in *Kazak* artisanal cheese as a predominant yeast species (Zheng et al., 2018). The identification of *Kl. marxianus*, a putative probiotic yeast, which was isolated from *Fiore Sardo* cheese in Sardinia, Italy, is especially crucial because yeasts are necessary during cheese fermentation (Fadda et al., 2017). The salted "*Pecorino di Filiano*" cheese consisted mainly of the halotolerant yeast species *Debaryomyces hansenii*, with less prevalent species such as *Kl. lactis* and *Dekkera anomala* (Capece and Romano, 2009). According to reports, the two most common fungi found in traditional Turkish cheese are *Penicillium roqueforti* and *Debaryomyces hansenii* (Onmaz et al., 2021). "*Tomme d'Orchies*", a French cheese was also found to have two uncommon fungus species: *Cladosporium cladosporioides* and *Geotrichum candidum* (Ceugniez et al., 2017).

2.3 Rare traditional milk products of Europe and Middle Eastern Asia

Fermented milk is rare in various parts concerning Europe, and there is a dearth of literature on its microbial diversity. *Blaand*, a unique NFM product with a low alcohol concentration and an acidic flavour, is traditionally made in Iceland, Scotland, and Norway (Kaur et al., 2019). Another traditional Scandinavian yoghurt-like product, *tätmjölk*, is prepared by the EPS-producing *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* (Fondén et al., 2006). Similar to *tätmjölk*, *surmjölk* and *filbunke* are typically made without the bacteria that produce EPS. On the contrary, *skyr* is fermented milk that is made using thermophilic microbes like *Lactobacillus* species,

Streptococcus thermophilus, yeasts, and molds, (Fondén et al., 2006). Besides these, the other traditional fermented milk/dairy products are the yoghurt-like *tettemelk*, buttermilk (*kjernemelk*, *kaernemælk*, *kirnupiimä*, and *kärnmjölk*), concentrated fermented milk (*lactofil*, *kokkeli*, and *ymer*), cultured cream (*crème*, *kermapiimä*, *gräddfil*, *fraîche*, and *rømme*), cultured milk (*kulturmælk*, *lättfil*, *kulturmælk*, *skummet*, *rasvatonpiimä*, *tykmælk*, and *talouspiimä*), and yoghurt-like. The fermented milk product known as *zivda* that includes *Lactococcus lactis* as a major fermenter is typically made in Israel (Keller et al., 1974; Karenzi et al., 2013).

2.4 Global market of probiotics

The global probiotics market has experienced prompt expansion due to growing awareness of gut health, immune support, and the overall benefits of microbiome-targeted interventions (Hill et al., 2014). The global market of probiotics valued at USD 32.06 billion in 2013, was later estimated at USD 33.19 billion in 2015 (Byakika et al., 2019). As of 2023, the global market for probiotics valued at approximately USD 61.1 billion, is projected to grow to between USD 85 and 100 billion by 2030, with an expected compound annual growth rate (CAGR) of 7-9% (Grand View Research, 2023). This growth is primarily driven by rising consumer interest in preventive health, functional foods, and naturally sourced alternatives to pharmaceuticals (Mukisa, 2016; Das et al., 2022; Anwer and Wei, 2024). Technological advancements in microbial genomics and the human microbiome are also facilitating the development of targeted probiotic formulations (Speckmann et al., 2024; Chakravarty et al., 2025). The Asia-Pacific region continues to dominate the probiotics market, particularly countries such as China, India, Japan, and South Korea, where there is a historical reliance on fermented foods and increasing acceptance of probiotic-based products (Cosme et al., 2022; Murali et al., 2024; Caetano et al., 2024). In North America and Europe, the growth of the

probiotics market is shaped by trends in functional beverages, personalized nutrition, and gut-brain axis health (Spacova et al., 2023; Speckmann et al., 2024). Simultaneously, developing regions in Latin America, Africa, and the Middle East are witnessing growing demand, supported by urbanization, improved healthcare infrastructure, and global marketing campaigns (Meybodi et al., 2021; LeBlanc et al., 2024; Lasso García et al., 2024). A key factor contributing to market expansion is the innovation in functional foods and beverages enriched with probiotics (Murali et al., 2024; Caetano et al., 2024). New product categories include non-dairy fermented drinks, cereals, plant-based yogurts, and infant formulas fortified with beneficial isolates (Shori and Al Zahrani, 2021; Palanivelu et al., 2022; Dahiya and Nigam, 2022). Consumers are particularly attracted to probiotics for their documented roles in digestive health, immune modulation, and mental well-being, including the mitigation of anxiety and depression symptoms (Hill et al., 2014; Anwer and Wei, 2024). The COVID-19 pandemic further catalyzed interest in immune-supportive probiotics, with increased global sales of dietary supplements and fortified foods (Hu et al., 2021; Xavier-Santos et al., 2022). With the integration of omics technologies such as metagenomics, transcriptomics, and metabolomics researchers are developing next-generation probiotics and synbiotic formulations tailored to individual microbiomes (Murali and Mansell, 2024; Abouelela and Helmy, 2024). These innovations are not only advancing human healthcare but also revolutionizing agricultural biotechnology and animal nutrition (Sarsan et al., 2024; Akutko and Stawarski, 2021). Hence, the global probiotics market represents a rapidly evolving field with far-reaching implications for health, food security, and environmental sustainability.

2.5 Growth of yeast as a probiotic agent

The application of yeast as a probiotic agent has expanded rapidly in recent years, driven by its resilience, therapeutic potential, and suitability across diverse sectors including human health, animal nutrition, and agriculture (Staniszewski and Kordowska-Wiater, 2021; Sadeghi et al., 2022). It is not surprising that *Saccharomyces* isolates are so prevalent in the human gastrointestinal tract since humans have been purposely consuming live *S. cerevisiae* and related species in beer, bread, and other fermented products for thousands of years. Few isolates of *S. cerevisiae*, nevertheless, have been shown health promoting traits to the host (Fernandez-Pacheco et al., 2018; Sen and Mansell, 2020). Concerning probiotics, research pertaining to yeasts has not gained significant attention as the bacterial population (Didari et al., 2014). While probiotic research was initially dominated by bacterial isolates like *Lactobacillus* and *Bifidobacterium*, yeast, particularly *Saccharomyces cerevisiae* var. *boulardii*, has surfaced as a powerful eukaryotic alternative with significant advantages (Czerucka et al., 2007; Kelesidis and Pothoulakis, 2012; Sen and Mansell, 2020; Staniszewski and Kordowska-Wiater, 2021; Fu et al., 2023). Unlike bacteria, yeasts are naturally resistant to antibiotics and has ability to survive harsh gastrointestinal conditions, making them especially effective during antibiotic-associated disturbances (Staniszewski and Kordowska-Wiater, 2021). The prominent mechanism includes competitive inhibition of pathogens, secretion of antimicrobial substances, degradation of bacterial toxins, reinforcement of the intestinal barrier, and modulation of host immune responses (Bordet et al., 2020; Sen and Mansell, 2020; Pereyra et al., 2024; Moonsamy et al., 2024). Numerous studies conducted on non-*Saccharomyces* yeasts revealed the presence potential probiotic traits (Staniszewski and Kordowska-Wiater, 2021). Yeasts with probiotic potential include numerous species such as *S. cerevisiae* and *S. boulardii* (Song

et al., 2014; Diosma et al., 2014; Pienaar et al., 2015; Gil-Rodríguez et al., 2015; Motey et al., 2020; Sen and Mansell, 2020; Pereira et al., 2021; Wang et al., 2024a; Helmy et al., 2024), *Kluyveromyces lactis* (Binetti et al., 2013; Oliveira et al., 2019; Gut et al., 2019; Hugot et al., 2023; Hong et al., 2024), *Kl. marxianus* (Binetti et al., 2013; Diosma et al., 2014; Smith et al., 2015; Motey et al., 2020; González-Orozco et al., 2023; Buonanno et al., 2025), *Debaryomyces hansenii* (Ochangco et al., 2016; Jeong et al., 2022; Lee et al., 2023; Angulo and Angulo, 2025), *Yarrowia lipolytica* (Reyes-Becerril et al., 2021; Keskin et al., 2024), *Pichia kudriavzevii* (Ogunremi et al., 2015a; Lata et al., 2022; Wang et al., 2024a; Chihombori et al., 2025), *Pichia kluyveri* (Ogunremi et al., 2015a; Piraine et al., 2023), *Pichia jadinii* (Buerth et al., 2016), *Pichia guilliermondii* (Bonatsou et al., 2015), *Pichia pastoris* (França et al., 2015), *Meyerozyma caribbica* (Amorim et al., 2018), *Metschnikowia gruessii* (Smith et al., 2015), *Issatchenkia orientalis* (Ogunremi et al., 2015a), *Candida tropicalis* (Ogunremi et al., 2015a), and *C. famata* (Al-Seraih et al., 2015). This investigation broadens the range of potential probiotic yeasts beyond the extensively studied *Saccharomyces boulardii* (Pais et al., 2020). In accordance to studies, certain yeast isolates possess attributes that might assist with treating conditions such as Inflammatory Bowel Disease (IBD), irritable bowel syndrome (IBS), allergies, and skin disorders (Huang et al., 2022; Tullio, 2024). Furthermore, probiotic yeasts can compete with pathogenic enteric bacteria for adhesion sites and nutrition, restricting their growth and colonization (Xu et al., 2018; Rosseto et al., 2022). They can additionally synthesize antimicrobial compounds that directly eliminate harmful bacteria (Nascimento et al., 2020; Junges et al., 2020). The significance of probiotic yeasts in promoting animal nutrition and health has also increased (Pang et al., 2022; Elghandour et al., 2024). Yeast used to be utilized as a feed additive due to its high fiber, protein, vitamin B, mineral, and organic acid content

(Alkalbani et al., 2022a). Adding both viable and nonviable yeast cells to the diet of livestock enhances health and growth (Roto et al., 2015). *S. cerevisiae* found to improve poultry health by increasing egg-laying capacity, enhancing feed intake, and lowering plasma cholesterol levels (Bilal et al., 2020; Krysiak et al., 2021; Dixon et al., 2022). Furthermore, *S. cerevisiae* has been employed in ruminant feed to decrease accumulation of the lactate (Zhang et al., 2021; Zhou et al., 2025). Probiotic yeast has also gained significant attention as a nutritional additive in aquaculture to stimulate the enzymatic antioxidative response in farmed fish (Diguță et al., 2023; del Valle et al., 2023). Recent studies have increasingly focused on isolating novel probiotic yeast isolates from traditional fermented foods, such as fermented milk, sourdough, kimchi, tempeh, kefir, and indigenous fermented beverages (Menezes et al., 2020; Pereira et al., 2021; De Guidi et al., 2023; Shruthi et al., 2024; Kunyeit et al., 2024). These foods serve as rich microbial reservoirs, offering yeast species adapted to dynamic microbial ecosystems and gastrointestinal-like conditions (Cichońska et al., 2023; Mukherjee et al., 2024; Padhi et al., 2025).

MATERIALS AND METHODS

3.1 Materials

3.1.1 Media used

Commercial media	Manufacturing details
Yeast Malt Agar	M424, HiMedia, Mumbai, India
Yeast Malt Broth	M425, HiMedia, Mumbai, India
Potato Dextrose Agar	MH096, HiMedia, Mumbai, India
Nutrient Agar	M001, HiMedia, Mumbai, India
Mueller Hinton Agar	M1084, HiMedia, Mumbai, India
Sheep Blood Agar Plate	MP1301, HiMedia, Mumbai, India
Nitrate Broth	M439, HiMedia, Mumbai, India
Gelatin Agar	M920, HiMedia, Mumbai, India
Yeast Fermentation Broth Base	M676, HiMedia, Mumbai, India
DNase Test Agar w/ Methyl Green	M1419, HiMedia, Mumbai, India
B12 Assay Medium	MM036, HiMedia, Mumbai, India
Tributyrin Agar Base w/o Tributyrin	M157, HiMedia, Mumbai, India
Yeast Nitrogen Base	M139, HiMedia, Mumbai, India
Urea Agar Base (Christensen)	M112S, HiMedia, Mumbai, India
Malt Extract Agar (2%)	M1964, HiMedia, Mumbai, India
Sabouraud Dextrose Agar	MH063, HiMedia, Mumbai, India

Modified Czapek Dox medium (Saxena and Sinha, 1981)

Ingredients	Gram/Litre
Glucose	2.000
L-asparagine	10.000
Monopotassium phosphate	1.520
Potassium chloride	0.520
Copper (II) nitrate trihydrate	0.001
Zinc sulfate heptahydrate	0.001
Ferrous sulfate heptahydrate	0.010
Agar	20.000
Final pH (at 25 °C)	6.2

3.1.2 Amino acids used	Manufacturing details
L-tyrosine	RM069, HiMedia, Mumbai, India
L-Histidine monohydrochloride monohydrate	TC077M, HiMedia, Mumbai, India
D-phenylalanine	RM1738, HiMedia, Mumbai, India
D-leucine	TC078, HiMedia, Mumbai, India
D-tryptophan	RM1761, HiMedia, Mumbai, India
L-arginine	GRM038, HiMedia, Mumbai, India
L-lysine	TC207, HiMedia, Mumbai, India

3.1.3 Carbohydrates used	Manufacturing details
Lactose monohydrate	RM565G, HiMedia, Mumbai, India
Sucrose	GRM601, HiMedia, Mumbai, India
D-(+)-Maltose monohydrate	GRM3050, HiMedia, Mumbai, India
D-(+)-Glucose monohydrate	TC208, HiMedia, Mumbai, India
D-(+) Raffinose pentahydrate	RM107, HiMedia, Mumbai, India
L(+) Rhamnose monohydrate	RM062, HiMedia, Mumbai, India
D-(+)-Mannose	RM104, HiMedia, Mumbai, India
D-(-)-Ribose	GRM197, HiMedia, Mumbai, India
D-(-)-Fructose	GRM196, HiMedia, Mumbai, India
D-Xylose	GRM111, HiMedia, Mumbai, India
D(+)-Trehalose dihydrate	GRM110, HiMedia, Mumbai, India
L(+) Arabinose	GRM037, HiMedia, Mumbai, India

3.1.4 Antibiotics and Antifungal agents used	Manufacturing details
Erythromycin (E) 15 mcg/disc	SD013, HiMedia, Mumbai, India
Gentamicin (GEN) 10 mcg/disc	SD016, HiMedia, Mumbai, India
Penicillin G (P) units/disc	SD028, HiMedia, Mumbai, India
Tetracycline (TE) 30 mcg/disc	SD037, HiMedia, Mumbai, India
Co-trimoxazole (COT) 25 mcg/disc	SD010, HiMedia, Mumbai, India
Vancomycin (VA) 30 mcg/disc	SD045, HiMedia, Mumbai, India
Clindamycin (CD) 2 mcg/disc	SD051, HiMedia, Mumbai, India
Ampicillin (AMP) 10 mcg/disc	SD002, HiMedia, Mumbai, India
Amphotericin B (AP) 20 mcg/disc	SD233, HiMedia, Mumbai, India
Clotrimazole (CC) 10 mcg/disc	SD115, HiMedia, Mumbai, India
Itraconazole (IT) 10 mcg/disc	SD221, HiMedia, Mumbai, India
Ketoconazole (KT) 10 mcg/disc	SD224, HiMedia, Mumbai, India
Nystatin (NS) 50 mcg/disc	SD271, HiMedia, Mumbai, India
Voriconazole (VRC) 1 mcg/disc	SD277, HiMedia, Mumbai, India

3.1.5 Reference type strains	Accession no.
<i>Salmonella enterica</i> subsp. <i>enterica</i> ser. Typhimurium	MTCC 3223
<i>Escherichia coli</i> KL96	MTCC 1583
<i>Bacillus cereus</i>	MTCC 1272
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>	MTCC 740
<i>Aspergillus niger</i>	NCIM 1248
<i>Fusarium oxysporum</i>	NCIM 1350
<i>Aspergillus niger</i>	NCIM 1358
<i>Aspergillus oryzae</i>	NCIM 1212
<i>Aspergillus flavus</i>	NCIM 549

Note:

Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India
 National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India

3.1.6 Software/database used	Links
BLAST (Basic Local Alignment Search Tool)	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ClustVis (Beta)	https://biit.cs.ut.ee/clustvis/
ChromasPro v1.34	http://technelysium.com.au/wp/chromas/
ClustalW	https://www.genome.jp/tools-bin/clustalw
Sequence Scanner v2.0	https://www.thermofisher.com/in/en/home/lifescience/sequencing/sanger-sequencing/sanger-dnasequencing/sanger-sequencing-data-analysis.html
Microsoft office	https://www.microsoft.com/en-in/
Molecular Evolutionary Genetics Analysis version 7 (MEGA v11.0.13)	https://www.megasoftware.net/
OriginPro software v.2024	https://www.originlab.com/index.aspx?go=Products/Origin
PAST v4.03 (Paleontological Statistics Software Package for Education and Data Analysis)	https://past.en.lo4d.com/windows

3.1.7 Chemicals/reagents used	Manufacturing details
2-Propanol	1945240521, EMPLURA®, Supelco, Sigma-Aldrich
GoTaq® Green Master Mix	M7122, Promega, Madison, WI, USA
Proteinase K, From <i>Pichia pastoris</i>	RM2957, HiMedia, Mumbai, India
RNase A (20 mg/mL)	DS0003, HiMedia, Mumbai, India
Phenol:Chloroform:Isoamyl alcohol mixture	MB078, HiMedia, Mumbai, India
Ethanol	1.00983, Merck, Germany
1X TE buffer, pH 8.0	ML060, HiMedia, India
50X TAE	ML016, HiMedia, Mumbai, India
Nuclease-free Water	MC1191, Promega, Madison, WI, USA
Agarose special, Low EEO	MB002, HiMedia, Mumbai, India
Polyethylene glycol (PEG)	TC740, HiMedia, Mumbai, India
1kb DNA Ladder	MBT051, HiMedia, Mumbai, India
100bp DNA Ladder	MBT049, HiMedia, Mumbai, India
50bp DNA Ladder	MBT084, HiMedia, Mumbai, India
Ethidium bromide	RM813, HiMedia, Mumbai, India
Sulphanilic Acid, 0.8%	R015, HiMedia, Mumbai, India
α -Naphthylamine Solution	R009, HiMedia, Mumbai, India
Tribut (Tributyrin)	FD081, HiMedia, Mumbai, India
Coagulase Plasma (from rabbit)	FD248, HiMedia, Mumbai, India
Egg Yolk Emulsion	FD045L, HiMedia, Mumbai, India
SM Powder	GRM1254, HiMedia, Mumbai, India
1N Hydrochloric acid Solution	TCL003, HiMedia, Mumbai, India
1N Sodium hydroxide	TCL002, HiMedia, Mumbai, India
Bile, Dried	CR010, HiMedia, Mumbai, India
Toluene	1.08323, Merck, Germany
n-Hexadecane	RM2238, HiMedia, Mumbai, India
Xylene	1086342500, Merck, Germany
Diethyl ether	1009231000, Merck, Germany
Sodium cholate	RM202, HiMedia, Mumbai, India
Sodium Taurocholate	RM011, HiMedia, Mumbai, India
Sodium glycocholate hydrate	GRM8907, HiMedia, Mumbai, India
Lysozyme	RM074, HiMedia, Mumbai, India
Sodium chloride, Hi-AR™	GRM853, HiMedia, Mumbai, India
Potassium chloride, Hi-AR™/ACS	GRM3934, HiMedia, Mumbai, India
Disodium phosphate (1-Naphthyl phosphate disodium salt)	RM10598, HiMedia, Mumbai, India
Monopotassium phosphate (Potassium dihydrogen phosphate)	GRM2951, HiMedia, Mumbai, India

3.1.7 Chemicals/reagents used	Manufacturing details (contd.)
Phenolphthalein, Hi-Cert™/ACS	GRM076, HiMedia, Mumbai, India
Streptomycin sulphate	TC035, HiMedia, Mumbai, India
Phosphate Buffered Saline, pH 7.2	TS1099, HiMedia, Mumbai, India
Sodium dodecyl sulphate, Ultrapure	GRM6218, HiMedia, Mumbai, India
Tris hydrochloride, Hi-AR™	GRM613, HiMedia, Mumbai, India
EDTA disodium salt dihydrate, Hi-AR™	GRM1370, HiMedia, Mumbai, India
Calcium chloride anhydrous, Hi-AR™	GRM710, HiMedia, Mumbai, India
Pepsin (1:3000), Hi-LR™	GRM1250, HiMedia, Mumbai, India
Pancreatin 3X (3NF/USP)	RM7348, HiMedia, Mumbai, India
Mucin from porcine stomach	M1778, Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA
Acetic acid, glacial	A6283, Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA
Bromocresol purple	PCT1515, HiMedia, Mumbai, India
Potassium hydroxide	PCT1560, HiMedia, Mumbai, India
Cholesterol-Water Soluble	C4951, Sigma-Aldrich, USA
Glycerol	MB060, HiMedia, Mumbai, India
Hexane	HX0293, Merck, Germany
o-Phthalaldehyde, Hi-LR™	RM1143, HiMedia, Mumbai, India
Sulfuric acid 95-97%	100731, Merck, Germany
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	D9132, Sigma-Aldrich, Merck, Germany
Bovine serum albumin	MB083, HiMedia, Mumbai, India
3,5-Dinitrosalicylic acid (DNSA), Hi-AR™	GRM1582, HiMedia, Mumbai, India
α-amylase	86250, Sigma-Aldrich, Merck, Germany
Starch, soluble	S9765, Sigma-Aldrich, Merck, Germany
Ruthenium red tetrahydrate	GRM7463, HiMedia, Mumbai, India
Sodium phytate, From Rice	GRM6226, HiMedia, Mumbai, India
Ammonium sulphate, Hi-LR™	GRM1192, HiMedia, Mumbai, India
Magnesium sulphate heptahydrate, Hi-AR™/ACS	GRM684, HiMedia, Mumbai, India
Ferrous sulphate heptahydrate, Hi-LR™	GRM372, HiMedia, Mumbai, India
Manganese (II) sulphate monohydrate	GRM687, HiMedia, Mumbai, India
Potassium dihydrogen phosphate	PCT0009, HiMedia, Mumbai, India
Ammonium chloride, Hi-LR™	GRM730, HiMedia, Mumbai, India
Ferric chloride hexahydrate	PCT0108, HiMedia, Mumbai, India
Copper (II) nitrate trihydrate	1.02753, Merck KGaA, Darmstadt, Germany

3.1.7 Chemicals/reagents used	Manufacturing details (contd.)
Zinc sulphate heptahydrate, Hi-LR™	GRM1196, HiMedia, Mumbai, India
Phenol red	I010, HiMedia, Mumbai, India
Agar Agar, Type I	GRM666, HiMedia, Mumbai, India
X-gal	MB069, HiMedia, Mumbai, India
U40 Urea Supplement	FD048, HiMedia, Mumbai, India
Dimethyl sulphoxide (DMSO)	GRM5856, HiMedia, Mumbai, India
Isopropyl-β-D-thiogalactopyranoside (IPTG)	MB072, HiMedia, Mumbai, India
Arbutin (p-hydroxyphenyl-β-D-glucopyranoside)	13492, Sisco Research Laboratories Pvt. Ltd., Mumbai, India
Ammonium ferric citrate	GRM475, HiMedia, Mumbai, India
L-asparagine	PCT0303, HiMedia, Mumbai, India
CAS (Eriochrome® azurol S)	RM336, Himedia, Mumbai, India
CTAB	MB101, HiMedia, Mumbai, India
Casamino acid	68806, Sisco Research Laboratories Pvt. Ltd., Mumbai, India
8-hydroxyquinoline	GRM1061, HiMedia, Mumbai, India
Chloroform	AS114, HiMedia, Mumbai, India
PIPES	MB022, HiMedia, Mumbai, India
Bile salts	RM008, HiMedia, Mumbai, India

3.1.8 Machines/Instruments	Manufacturing details
Analytical Weighing Balance	AX 204, Mettler, USA
Stomacher	400, Seward, London, UK
Cyclomixer	CM-101, Remi, India
Biological Incubator	Accumax, CIS-24BL, India
Orbital Shaker Incubator	RSB-12, Remi, Mumbai, India
Autoclave	Instrumentation India, Mumbai, India
Laminar Air Flow	1386, Thermo Scientific, USA
Digital pH meter	GeNei™, Bangalore, India
Hot Air Oven	Instrumentation India, Kolkata
Water Bath	RIME-1322, Remi, Mumbai, India
Water Distillation unit	72240020, Riviera, Mumbai, India
Freezer (-20 °C)	ROFV-170, Remi, Mumbai, India
Freezer (-80 °C)	TSE240A, Thermo Fisher, USA
Compound Microscope	EX1000, Olympus, Japan
Phase contrast microscope	ECLIPSE Si, Nikon, Tokyo, Japan
Viscometer	DV1MRVTJ0, Brookfield AMETEK, MA, USA
Eppendorf Bio-Spectrometer	Model 6135000009, Hamburg, Germany
Thermal Cycler	2720, Applied Biosystems, USA
Gel Doc™ EZ Imager	Model No. EZ735BR06435, BioRad, Hercules, CA, USA
Automated DNA sequencer	3500 Genetic Analyzer, Applied Biosystems, Waltham, MA, USA
Centrifuge	CL21, Thermo Scientific, USA
Microcentrifuge	MicroCL 21R, ThermoFisher Scientific, Carlsbad, CA, USA
Microwave Oven	28L, Samsung, India
Electrophoresis Unit	Bio Rad, USA
UV-Transilluminator	MD-25/HD-25, Wealtech, USA
Eppendorf BioSpectrometer	Eppendorf, Hamburg, Germany
Qubit 3.0 fluorometer	Invitrogen, Carlsbad, CA, USA
Microplate photometer	Multiskan FC, Thermo Scientific, USA

3.2 METHODS

3.2.1 Field survey and documentation

A survey was conducted to document the naturally fermented milk (NFM) products of various districts of Sikkim. A systematic questionnaire (Table A) was developed to record indigenous knowledge of traditional NFM product preparation through interactions in person with tribal communities. We identified households from ethnic groups where milk fermentation is prominent. During the survey, various variables were considered, including milk origin, preparation method, specialization of preparation, associated ceremonies, sensory characteristics, culinary practices, mode of consumption, and socioeconomic status.

Table B: Questionnaire on Consumption of Naturally Fermented Milk Products in Sikkim.

I. General Information

- [1] Identification Number:
- [2] Name of the Informant:
- [3] Ethnic Group:
- [4] Name of

(i) Village/Revenue Block:

(ii) Sub-division:

(iii) District:
- [5] Approximate number of

(i) Households in the village:

(ii) Population of the village:
- [6] Distance of the village from

(i) Nearest Market (km):

(ii) Nearest Town (km):

II. Information on Naturally Fermented Milk Products

- [1] Name of the product in local dialect
- [2] Indigenous method of preparation
- [3] Flow chart of the traditional method of preparation
- [4] Are there any taboos associated with making or consumption of the NFM products you mentioned? Yes/No. If yes, provide details:
- [5] Do you have any preferred times of year or social/religious rituals for preparing or consuming NFM products? Yes/No. If yes, provide details:
- [6] Does your consumption of fermented products include rituals or worship of any particular god or goddesses? Yes/No. If yes, provide details:
- [7] Do you think the NFM products you mentioned have therapeutic benefits or contribute to better health? Yes/No. If yes, provide details:
- [8] Does your livelihood depend on any NFM products? Yes/No.
- [9] Do you prepare the NFM products that you eat at home? If so, how did you know the traditional method of preparation?
- [10] What is the estimated amount of NFM products produced each month or year?
- [11] What is the expected cost of production?
- [12] What is the estimated revenue generated by selling this type of products?

Remarks:

Name and Signature (Investigator):

Date:

3.2.2 Collection of samples

Traditionally prepared NFM products (40 cow *dahi*, 40 cow *chhurpi*, 9 yak *dahi*, and 9 yak *chhurpi*) were collected aseptically in a labelled pre-sterile container from various locations of Sikkim, India. Sample collection site, geographical coordinates, altitude and number of samples collected were recorded. Samples were immediately transported to the laboratory maintaining minimum temperature in an ice box and stored at 4 °C for further analysis.

3.2.3 Physicochemical analysis

3.2.3.1 Analysis of pH

One gram of sample was dissolved in 10 mL of pre-sterilised physiological saline (0.85% NaCl) and the pH was measured in a pH meter (GeNei™, Bangalore, India) calibrated using standard buffers (Shangpliang et al., 2017). The pH readings were reported as mean ± SD for triplicate sets.

3.2.3.2 Moisture Content

The moisture content of the sample was determined using the basic weight difference method (Pratap et al., 2018). The sample was kept in the hot air oven at 105 °C for 5 hours, and the moisture content was calculated on the basis of the final and initial weight differences.

Using the following formula, the percentage of moisture content was calculated:

$$\text{Moisture content (\%)} = [(W1 - W2 / W1) \times 100]$$

where, W1= Fresh weight; W2 = Dried weight

3.2.3.3 Titratable acidity

20 gm of the sample were measured in a suitable vessel, and it was diluted with twice the volume of double distilled water. After adding 2 mL of phenolphthalein (GRM076, HiMedia, Mumbai, India), 0.1 M NaOH was used to titrate until the colour turned pink (AOAC, 2016).

3.2.3.4 Apparent viscosity

On a beaker at 20 °C, the apparent viscosity was measured using a viscometer (DV1MRVTJ0, Brookfield AMETEK, MA, USA) in triplicates. In 150 mL of sample, spindle (LV-SC4-34 spindle, 4 rpm) was allowed to rotate at 20 °C for 1 minute (Ali et al., 2019). Once the reading had stabilized, the viscosity was measured in centipoise (cP).

3.2.4 Microbiological Analysis

The samples were dissolved in a physiological saline (0.85% NaCl) in a 1:10 w/v ratio and homogenised using a stomacher (400, Seward, London, UK) for 2-3 minutes at 250 rpm (Girma and Aemiro, 2021). In order to determine the microbial load (CFU g⁻¹), each homogenized sample was diluted ten times, and 1 mL of each dilution was then transferred to a yeast malt (YM) agar (M424, HiMedia, Mumbai, India) plate supplemented with an antibiotic, streptomycin sulphate (TC035, HiMedia, Mumbai, India). The plates were then incubated at 28 °C for 48 to 72 hours. The isolation was performed randomly, and the pure culture was obtained by streaking on fresh agar plates. Purified colonies were observed using a phase-contrast microscope (ECLIPSE Si, Nikon, Tokyo, Japan) at 400X magnification and stored in 20% glycerol at -20 °C.

3.2.5 Preliminary screening of potential probiotic yeast

3.2.5.1 Low pH tolerance

Low pH tolerance of the yeast isolates was examined according to Greppi et al. (2017), with slight modifications. Yeast Malt (YM) broth (M425, HiMedia, Mumbai, India) were inoculated with yeast isolates and incubated at 28 °C for 24 hours, after which 1% (v/v) of the fresh cultures were inoculated in acidified (pH 2.0) YM broth and kept for incubation at 37 °C. The Optical density of the inoculated broth was measured at 600 nm wavelength at 0 and 24 hours. YM broth without inoculation was used as negative control. $\Delta OD_{600} \geq 0.500$ was considered as a threshold for significant growth after 24 hours of incubation (Bao et al., 2010).

3.2.5.2 Bile tolerance

The ability of yeast isolates to tolerate the bile was tested using the same method as for the Low pH tolerance test (Greppi et al., 2017). After incubating the yeast inoculated YM broth for 24 hours at 28 °C, 1% (v/v) of the fresh culture were inoculated in YM broth containing 0.3% bile (w/v) (CR010, HiMedia, Mumbai, India) and kept for incubation at 37 °C for 24 hours. Optical density of the inoculated broth was measured at 600 nm wavelength at 0 and 24 hours. YM broth without inoculation was used as negative control. $\Delta OD_{600} \geq 0.500$ was considered as a threshold for significant growth after 24 hours of incubation (Bao et al., 2010).

3.2.5.3 Microbial attachment to hydrocarbons (MATHs)

The isolates demonstrating both low pH tolerance and bile tolerance were subjected to MATHs assay, following the methodology described by Shen et al. (2022). In the experiment, hydrocarbons such as n-hexadecane (RM2238, HiMedia, Mumbai, India), toluene (1.08323, Merck, Germany), xylene (61772105001730, Merck, Germany), and diethyl ether (1009231000, Merck, Germany) were used as solvents. Yeast malt (YM) broth was inoculated with the screened isolates and incubated at 28 °C for 24 hours.

Following 5 minutes of centrifugation at $5000 \times g$ at 5°C , the pellets were washed twice with phosphate-buffered saline (PBS, pH 7.2). At 600 nm wavelength, the cell suspension was adjusted to an optical density (OD) of 1.0, designated as $\text{OD}_{\text{initial}}$. Subsequently, 1 mL of each hydrocarbon was mixed with 3 mL of the cell suspension and vortexed vigorously. To allow the two phases to separate, the mixture was left undisturbed at 37°C for 3 hours. After incubation, 1 mL of the upper aqueous layer was collected carefully, and its absorbance (OD) was measured at a 600 nm wavelength and recorded as OD_{final} .

The percentage of microbial attachment to hydrocarbons was calculated using the formula below:

$$\text{MATHs (\%)} = (\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}) / \text{OD}_{\text{initial}} \times 100$$

The yeast isolates were selected for further analysis when their MATHs value exceeded 80%, which was regarded as the threshold value for high hydrophobicity (Alkalbani et al., 2022b).

3.2.6 Identification of the isolates

3.2.6.1 Phenotypic and Biochemical Characterization

Preliminarily selected isolates were tentatively identified based on phenotypic and biochemical characterization. All isolates were tested for colony morphology, cell morphology, flocculation (Umeh et al., 2017), nitrate reduction test (Kotzekidou, 1997), H_2S production (Oliveira et al., 2008), and ability to ferment lactose, sucrose, maltose, glucose, raffinose, rhamnose, mannose, ribose, fructose, xylose, trehalose, and L-arabinose (Umeh et al., 2017).

3.2.6.2 Molecular identification of the selected isolates

3.2.6.2.1 DNA extraction

Genomic DNA of the selected yeast isolates was extracted using the method followed by Renshaw et al. (2015), with some modifications. 2 mL of overnight culture was centrifuged at $12000 \times g$ for 10 minutes, the supernatant was discarded, and the pellet was washed twice with sterile 0.5M NaCl. 400 μ L lysis buffer (10% SDS, Tris-HCl pH 8.0, 5 M NaCl, and 0.5 M EDTA, pH 8.0) was used to lyse the cell, followed by incubation at 60 °C for 30 minutes after the addition of each of 5 μ L of Proteinase K (RM2957, HiMedia, Mumbai, India) and 2 μ L RNase A (20 mg/mL) (DS0003, HiMedia, Mumbai, India). Following the addition of 100 μ L of 5 M NaCl, the mixture was incubated for 10 minutes at -20 °C. To separate the genomic DNA from the proteins and cellular debris, an equal volume of phenol:chloroform:isoamyl-alcohol mixture (25:24:1 v/v) (MB078, HiMedia, Mumbai, India) was mixed with the supernatant in a sterile tube and centrifuged at $12000 \times g$ for 10 minutes. After carefully transferring the upper aqueous layer to a fresh tube, double the volume of the chilled 2-propanol (1945240521, EMPLURA®, Supelco, Sigma-Aldrich) was added and stored at -20 °C overnight. The suspension was centrifuged at $14,000 \times g$ for 10 minutes, and the supernatant was disposed of. After washing the pellet with 100 μ L of chilled 70% ethanol (1.00983, Merck, Germany), it was centrifuged for 5 minutes at $8000 \times g$. The supernatant was disposed of, and the pellet was allowed to air dry at room temperature. Lastly, 50 μ L of nuclease-free water (MC1191, Promega, Madison, WI, USA) was used to dissolve the pellet. The DNA quality was checked with the Eppendorf Bio-Spectrometer (Model 6135000009, Hamburg, Germany), and purity of the DNA was observed at A_{260}/A_{280} ranging from 1.8 to 2.2 to perform PCR reaction.

3.2.6.2.2 PCR amplification

Following the procedure outlined by Palla et al. (2020), the D1/D2 region of the isolated genomic DNA of the screened isolates was amplified using NL1 and NL4 primers. A 50 µL PCR reaction mixture was prepared containing GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA), primers NL1 (5'-GCA TATCAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'), DNA template, and nuclease-free water. Under the following conditions, PCR amplification was carried out using the Thermal Cycler (2720, Applied Biosystems, USA): initial denaturation at 94 °C for 1 minute; 35 amplification cycles of 30 seconds each at 94, 58, and 72 °C; and final extension at 72 °C for 5 minutes. Agarose gel electrophoresis (1%) was conducted to examine the presence of amplicons, and visualization was carried out using a Gel Doc™ EZ Imager (Model No. EZ735BR06435, BioRad, Hercules, CA, USA).

3.2.6.2.3 Purification of PCR Amplicons

Purification of the PCR products was accomplished by following the protocol described by Pariyar et al. (2022), with minor modifications using PEG (polyethylene glycol)-NaCl and 20% (w/v) PEG (TC740, HiMedia, Mumbai, India) and 2.5 M NaCl. The PCR amplicons were mixed with 0.6 volumes of PEG-NaCl and then incubated at 37 °C for 30 minutes. After that, the mixture was centrifuged at $12,000 \times g$ at 4 °C for 30 minutes. Following the careful disposal of the aqueous supernatant without disturbing the pellet, it was washed with 70% ethanol and left to air-dry at room temperature. Finally, the purified DNA was mixed with 20 µL of nuclease-free water, and the Gel Doc™ EZ Imager (Model No. EZ735BR06435, BioRad, Hercules, CA, USA) was used to visualise the DNA, and the quality was evaluated using 1% agarose gel (MB002, HiMedia, Mumbai, India).

3.2.6.2.4 DNA sequencing and bioinformatics analysis

To perform the sequencing, an automated DNA sequencer (3500 Genetic Analyzer, Applied Biosystems, Waltham, MA, USA) was used. Prior to sequencing, sample preparation protocols were strictly followed as per the instruction of Applied Biosystems. Raw sequence analysis was carried out as per the method described by Palla et al. (2020). Sequence scanner software v2.0 (Applied Biosystems, U.S.A) was used to perform initial quality checking and primary editing of the raw sequences. ChromasPro v1.34 (Technelysium Ltd., South Brisbane, Australia) was adopted for contig formation and sequence alignment. Finally, assembled sequences were identified using the NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) (Nutaratat et al., 2022). A phylogenetic tree was constructed to verify the evolutionary relationship between the isolates, using the Maximum Likelihood method based on the Kimura 2-parameter (Kimura, 1980) by Molecular Evolutionary Genetics Analysis software, version 11.0.13 (MEGA v11.0.13) (Tamura et al., 2021), and ClustalW was used to align the identified sequences. In order to obtain accession numbers, the sequences were submitted to the NCBI GenBank.

3.2.7 *In Vitro* Screening of Probiotic Traits

3.2.7.1 Auto-aggregation and Co-aggregation

The auto-aggregation and co-aggregation traits of the screened yeast isolates were determined using the procedure described by Ogunremi et al. (2015a), with slight modifications. The overnight culture of the screened isolates was centrifuged at $5000 \times g$ at 5°C for 10 minutes. The pellets were washed twice with 10 mL of PBS (pH 7.2) and resuspended in 3 mL of the same solution and vortexed for ten seconds. After

carefully removing 1 mL of the suspension from the upper zone, the OD₆₀₀ was obtained and annotated as 'A_{initial}'. Again, the suspension was vortexed and incubated at 37 °C for 3 hours without agitation, followed by measuring the OD₆₀₀ and marking it as 'A_{time}'. The following formula was used to determine the percentage:

$$\text{Auto-aggregation (\%)} = [1 - (A_{\text{time}}/A_{\text{initial}}) \times 100]$$

For the co-aggregation, overnight yeast cultures were tested for their ability to adhere to the tested potential pathogens that included *Salmonella enterica* subsp. *enterica* ser. Typhimurium MTCC (Microbial Type Culture Collection, Chandigarh, India) 3223, *Escherichia coli* KL96 MTCC 1583, *Bacillus cereus* MTCC 1272, and *Staphylococcus aureus* subsp. *aureus* MTCC 740. Both the tested pathogen and overnight yeast isolates were harvested by centrifugation at 5 °C at 5000 × g for 10 minutes, washed twice using 10 mL of PBS (pH 7.2), resuspended in 10 mL of PBS buffer, and optical density was adjusted to 0.1 at 600 nm wavelength, denoted as A_{Yeast} and A_{Pathogen}, respectively. The yeast suspensions and pathogen were mixed in equal volume (2 mL each). After that, the mixture was vortexed and left undisturbed for 3 hours at 37 °C. At a wavelength of 600 nm, absorbance (A_{Mix}) of the mixture was measured, and the percentage of the co-aggregation was calculated as follows:

$$\text{Co-aggregation (\%)} = ((A_{\text{Yeast}} + A_{\text{Pathogen}}/2) - A_{\text{Mix}} / (A_{\text{Yeast}} + A_{\text{Pathogen}}/2) \times 100$$

3.2.7.2 Antimicrobial activity

The antimicrobial activity was carried out following the protocol described by Fernandez-Pacheco et al. (2018), with few modifications. The agar well diffusion method was used where the target pathogens used were *Staphylococcus aureus* subsp. *aureus* MTCC 740, *Escherichia coli* KL96 MTCC 1583, *Bacillus cereus* MTCC 1272, and *Salmonella enteric* subsp. *enteric* ser. Typhimurium MTCC 3223. Pathogenic

isolates were cultured on nutrient broth for 24 hours, and then freshly prepared suspensions (1.5×10^8 CFU mL⁻¹, OD₆₀₀ 0.08-0.1) of the pathogenic isolates (100 µL) were seeded on a plate of Muller Hinton agar (M173, HiMedia, Bombay, India). Using a cork borer, wells were prepared and filled with 100 µL of active yeast isolates (1.5×10^8 CFU mL⁻¹, OD₆₀₀ 0.08-0.1). The plates were then incubated at 37 °C for 48-72 hours. The zone of inhibition that developed following the incubation period was used to determine the antimicrobial activity.

3.2.7.3 Deconjugation of bile salts (BSH activity)

BSH (bile salt hydrolase) activity was assessed using the methodology described by Coutinho et al. (2021), with some modifications. For the screening of BSH activity, cultures were grown overnight in yeast malt (YM) broth, and point inoculated on the surface of YM agar plates supplemented with 0.2% (w/v) sodium glycocholate (GRM8907, HiMedia, India), 0.5% (w/v) sodium taurocholate (RM011, HiMedia, Mumbai, India), and sodium cholate (RM202, HiMedia, Mumbai, India); and 0.37 gL⁻¹ of CaCl₂ (TC097, HiMedia, Mumbai, India), respectively. For the next 72 hours, the plates were incubated at 30 °C. BSH activity is indicated by the presence of white opaque colonies and halos surrounding colonies.

3.2.7.4 Lysozyme tolerance assay

The resistance of the isolates to lysozyme was tested as described by Vera-Pingitore et al. (2016), with some modifications. After being centrifuged for 10 minutes at 5000 × g, the overnight-grown yeast cultures were washed twice with 10 mL of PBS (pH 7.2) and then resuspended in 10 mL of PBS (pH 7.2). Following the vortexing of the suspended cells, 1 mL of the suspension was taken carefully for OD measurement at 600 nm wavelength as 'A_{initial}'. Following another vortexing of the suspended cells, 100 µg mL⁻¹

¹ of lysozyme (RM074, HiMedia, Mumbai, India) was added, and the mixture was incubated for 1 hour at 37 °C without agitation. After this incubation, OD₆₀₀ was measured again as 'A_{final}', and the tolerance was calculated in percentage using the following formula:

$$\text{Lysozyme tolerance (\%)} = (A_{\text{final}}/A_{\text{initial}} \times 100)$$

3.2.7.5 Growth at different temperatures

The growth of the yeast isolates at different temperatures was evaluated by following the protocol described by Muche et al. (2023), with some modifications. About 1 mL of fresh yeast culture was standardized to 10⁸ cells mL⁻¹ (OD₆₀₀ 0.08-0.1) and inoculated separately into 40 mL of YM broth and adjusted to pH 5 before autoclaving. The inoculated test tubes were incubated at 20, 25, 28, 30, 35, 37, 40, and 45 °C temperatures. The growth of each yeast isolate was measured using a spectrophotometer at a 600 nm wavelength at 0 and 24 hours. Non-inoculated YM broth was used as a blank.

3.2.7.6 Growth at different salt concentrations

The growth of yeast isolates at different NaCl concentrations was evaluated (Shruthi et al., 2024). As previously stated, the cell suspension was prepared and resuspended in freshly prepared YMB supplemented with 2%, 4%, 6%, 8%, and 10% NaCl and incubated at 28 °C for 24 hours. OD₆₀₀ was measured at 0 and 24 hours, and non-inoculated YM broth was used as a blank.

3.2.7.7 Tolerance to *in vitro* digestive conditions

Tolerance to *in vitro* digestion conditions was evaluated by following the protocol described by Alkalbani et al. (2022b), with some modifications. A 2 mL aliquot of the yeast pellet suspension was subjected to three different types of *in vitro* digestion fluids, viz. simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated

intestinal fluid (SIF), consisting of amylase (86250, Sigma-Aldrich, Merck, Germany) 75 U mL⁻¹ (pH 7.0), pepsin (GRM1250, HiMedia, Mumbai, India) 2000 U mL⁻¹ (pH 3.0), and pancreatin (RM7348, HiMedia, Mumbai, India) 100 U mL⁻¹ (pH 7.0), respectively. A constant shaking was maintained at 120 rpm during the *in-vitro* digestion process. Serial dilution was performed before and after the *in vitro* digestion to determine the survivability of the yeast isolates and CFU log reduction.

3.2.8 Genetic Screening for Probiotic Traits

The potential probiotic genes (Table 1-4) responsible for various probiotic properties were detected in yeast. A PCR reaction mixture was prepared in order to carry out the PCR amplification of the probiotic genes. Each PCR reaction mixture contained 6 µL GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA), 0.6 µL forward primer, 0.6 µL reverse primer, and 1 µL of template DNA, finally making a volume of 12 µL. The PCR products were run in 1% agarose gel (MB002, HiMedia, Mumbai, India) with a 1 kb DNA ladder (MBT051, HiMedia, Mumbai, India), and a 100 bp (MBT049, HiMedia, Mumbai, India) DNA ladder for more than 500 bp amplicons and 2% agarose gel for less than 100 bp amplicons with a 50 bp (MBT084, HiMedia, Mumbai, India) DNA ladder, followed by ethidium bromide (RM813, HiMedia, Mumbai, India) staining. The PCR configurations were as follows: 1 cycle at 95 °C for 5 minutes; 40 cycles at 95 °C for 30 seconds, an annealing temperature for 10 seconds (depending on the primers specified in Table 1), and at 72 °C for 15 seconds; and 1 cycle at 72 °C for 5 minutes.

Table 1: List of the primers used for the detection of possible low pH tolerance genes.

Sl. no.	Target genes	Primers	Amplified product (bp)	Annealing temperatures (°C)	References
1	<i>TPS1</i>	F 5'-ATGACTACGGATAACG-3' R 5'-TCAGTTTTTGGTGGCAGAGG-3'	1600	65	Wilson et al., 2007
2	<i>HSP150</i>	F 5'-CACTTTGACTCCAACAGCCACTTACA-3' R 5'-TACCGGACAAACATTGGTAGAAGACA-3'	781	65	Marinangeli et al., 2004
3	<i>SED1</i>	F 5'-ATGAAATTATCAACTGTCCTATTATCTGCCGG-3' R 5'-TTATAAGAATAACATAGCAACACCAGCCAAACC-3'	950-1300	64	Mannazzu et al., 2002
4	<i>CYC1</i>	F 5'-ATGACTGAATTCAAGGC-3' R 5'-TTACTCACAGGCTTTTTTCAAGTAG-3'	330	55	Rai et al., 2024
5	<i>VM42</i>	F 5'-ATGGTTTTGTCTGATAAGG-3' R 5'-TTAGATTAGAGATTCTTCTTGGC-3'	1554	58	
6	<i>VM46</i>	F 5'-ATGGAAGGCGTGTATTTC-3' R 5'-TCAATAAACGGAAATATAATTGTTG-3'	1038	58	

Table 2: List of the primers used for the detection of possible bile tolerance genes.

Sl. no.	Target genes	Primers	Amplified product (bp)	Annealing temperatures (°C)	References
1	<i>YIMI</i>	F 5'-CAAGAAATGGACCCCCGAGT-3' R 5'-TGCGTGGAAGCACCATATAC-3'	51	64	Caudle et al., 2011
2	<i>PDR1</i>	F 5'-TTTGACTCTGTTATGAGCGATTACG-3' R 5'-TTCGGATTTTCTGTGACAATGG-3'	51	64	
3	<i>YORI</i>	F 5'-CCATCGGTGCTTGTGTAATGTTA-3' R 5'-TTGAGAGGCGTGGAATAAATG-3'	68	64	
4	<i>FLUI</i>	F 5'-ATGAATAACAATACCAACAGC-3' R 5'-CTATTCCAAAACAAAGGC-3'	1833	55	Rai et al., 2024

Table 3: List of the primers used for the detection of possible genes responsible for antimicrobial activity.

Sl. no.	Target genes	Primers	Amplified product (bp)	Annealing temperatures (°C)	References
1	<i>khs</i>	F 5'-AAGCATCCGAAACAGTACT-3' R 5'-TCAAGGATGCTGCTAAGCTG-3'	919	53	Younis et al., 2017
2	<i>pelA</i>	F 5'-ATCGAATTCATGAAGTTCAGTCTGCTTTC-3' R 5'-ACGGAATTCGCAGCTCGTGGTGGAGCCAGT-3'	727	55	

Table 4: List of the primers used for the detection of possible adhesion genes.

Sl. no.	Target genes	Primers	Amplified product (bp)	Annealing temperatures (°C)	References
1	<i>FLO1</i>	F 5'-ATGACAATGCCTCATCGC-3' R 5'-CTTCCACCCCATGGCTTGATACCGTC-3'	596	52	Damas-Buenrostro et al., 2008
2	<i>FLO5</i>	F 5'-GACAATTGCACACCACTGC-3' R 5'-CCTGTCATTTCTAGGGTTACG-3'	423	52	
3	<i>FLO10</i>	F 5'-AACTGGTACCTACACATTTGGC-3' R 5'-GGCAATACCACACTAACAGG-3'	270	52	
4	<i>FLO11</i>	F 5'-CCTTGTTCAACTGGTACTGGCG-3' R 5'-TAGAATACAACCTGGAAGAGCG-3'	749	52	
5	<i>AGA1</i>	F 5'-GTGACGATAACCAAGACAAACGATGCAA-3' R 5'-CCGTTTCATGCATACTGGTTAATGTGCT-3'	1198	64	Marinangeli et al., 2004
6	<i>EPA1</i>	F 5'-AACCGCAAGAAAATCCTCCT-3' R 5'-GGACTGGAAGTGGGGTATGA-3'	60	64	Caudle et al., 2011
7	<i>CCW12</i>	F 5'-ATGCAATTTTCTACTGTTCGC-3' R 5'-TTACAACAACAAAGCAGCG-3'	402	60	Rai et al., 2024
8	<i>PST1</i>	F 5'-ATGCAATTACATTCACTTATCGC-3' R 5'-TTACATTATAGACATGATGATTGCC-3'	1335	60	

3.2.9 Safety evaluation of the isolates

3.2.9.1 Hemolytic activity

Hemolytic activity of the isolates was determined by following the protocol described by Wang et al. (2024a), with some modifications. Isolates were inoculated in Sheep Blood Agar Plate (MP1301, HiMedia, Mumbai, India) and kept for incubation at 28 °C for 48-72 hours. The presence of clear zones around the colonies was confirmed for β -hemolysis.

3.2.9.2 Gelatinase Production

The yeast isolates' potential to produce gelatinase was investigated using the methodology outlined by Pereira et al. (2021). After being stab-inoculated into butts of gelatin agar (M920, HiMedia, Mumbai, India), the yeast culture was incubated for 5 to 7 days at 37 °C. The tubes were refrigerated for one hour after culture growth to check for gelatin liquefaction.

3.2.9.3 DNase Activity

DNase activity of the isolates was determined as described by Pereira et al. (2021). The isolates were streaked on DNase Test Agar w/ Methyl Green medium (M1419, HiMedia, Mumbai, India) plates and then incubated for five to seven days at 37 °C. A clear or pinkish area surrounding the colony suggested the activity and production of the DNase enzyme.

3.2.9.4 Mucin Degradation Test

The degradation of mucin will be confirmed in Yeast Nitrogen Base (M139, HiMedia, Mumbai, India) agar medium supplemented with 0.5% mucin (M1778, Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) in a Petri dish as described by Coutinho et al.

(2021), with some modifications. The isolates were cultured into YM broth, and a 5 μ L aliquot of each culture was spotted onto the surface of YNB agar supplemented with 0.5% mucin. Plates were incubated at 28 °C for 48 hours and then stained with 0.1% black starch (S9765, Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) in acetic acid (3.5 M) (A6283, Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) for 30 minutes. After that, plates were cleaned with acetic acid (1.2 M) until any potential halos surrounding the spots were discoloured.

3.2.9.5 Biogenic amine production

To assess the capacity of isolates to produce biogenic amines, protocol described by Staniszewski and Kordowska-Wiater, (2023) were used. Isolates were streaked onto YM agar plates supplemented with 0.006% bromocresol purple (PCT1515, HiMedia, Mumbai, India) and a mixture of amino acids: L-tyrosine (RM069, HiMedia, Mumbai, India), L-histidine monohydrochloride monohydrate (TC077M, HiMedia, Mumbai, India), D-phenylalanine (RM1738, HiMedia, Mumbai, India), D-leucine (TC078, HiMedia, Mumbai, India), D-tryptophan (RM1761, HiMedia, Mumbai, India), L-arginine (GRM038, HiMedia, Mumbai, India), and L-lysine (TC207, HiMedia, Mumbai, India) in equal ratios, with a total mass concentration of 1%. By incubating the plates for seven days at 30 °C, the appearance of the biogenic amines (BAs) was checked. The presence of biogenic amine production was monitored by detecting the changes in the medium's colour.

3.2.9.6 Pseudohyphae formation

Pseudohyphae formation was observed by following the protocol described by Youn et al. (2023). The formation of pseudohyphae will be observed by smearing a single colony of each strain of yeast on a glass slide and covering it for ten minutes with 10 μ L of 10%

potassium hydroxide (PCT1560, HiMedia, Mumbai, India). Slides will then be examined at 400x magnification using a light microscope after being covered with a coverslip.

3.2.9.7 Antibiotic and antifungal susceptibility testing

Antibiotic and antifungal susceptibility testing of the isolates was determined by the disc diffusion method by following the protocol described by Diguță et al. (2023), Boranbayeva et al. (2020), Piame et al. (2022), and Alkalbani et al. (2022b), with some modifications. Isolates were inoculated in YM broth and kept for incubation at 28 °C. The concentrations of these cultures were adjusted to McFarland 0.5. Lawn culture was prepared on Mueller-Hinton agar (M173, HiMedia, Mumbai, India). Antibiotics (erythromycin 15 mcg/disc (SD013, HiMedia, Mumbai, India), gentamicin 10 mcg/disc (SD016, HiMedia, Mumbai, India), penicillin G units/disc (SD028, HiMedia, Mumbai, India), tetracycline 30 mcg/disc (SD037, HiMedia, Mumbai, India), Co-trimoxazole 25 mcg/disc (SD010, HiMedia, Mumbai, India), vancomycin 30 mcg/disc (SD045, HiMedia, Mumbai, India), clindamycin 2 mcg/disc (SD051, HiMedia, Mumbai, India), and ampicillin 10 mcg/disc (SD002, HiMedia, Mumbai, India) and antifungal agents (amphotericin B 20 mcg/disc (SD233, HiMedia, Mumbai, India), clotrimazole 10 mcg/disc (SD115, HiMedia, Mumbai, India), itraconazole 10 mcg/disc (SD221, HiMedia, Mumbai, India), ketoconazole 10 mcg/disc (SD224, HiMedia, Mumbai, India), nystatin 50 mcg/disc (SD271, HiMedia, Mumbai, India) and voriconazole 1 mcg/disc (SD277, HiMedia, Mumbai, India) were placed on the lawn culture. Plates were kept for incubation for 24-48 hours at 28 °C, and zones of inhibition were observed. In order to check the antifungal susceptibility of yeasts, CLSI and NCCLS guidelines (NCCLS, 2004; CLSI, 2016) were used.

3.2.9.8 Lipase activity

Lipase activity of yeast isolates was tested on Tributyrin Agar Base w/o Tributyrin media (M157, HiMedia, Mumbai, India), and tributyrin (FD081, HiMedia, Mumbai, India) was used as a supplement (Zheng et al., 2018). Plates were inoculated and incubated for 24-48 hours at 28 °C. A positive reaction was indicated by the clear halo zone surrounding the colonies.

3.2.9.9 Urease activity

Urease activity was determined by following the protocol described by Alvarez et al. (2023), with some modifications. Urea Agar Base (Christensen) (M112, HiMedia, Mumbai, India) were sterilized, and U40 urea supplement (FD048, HiMedia, Mumbai, India) was added and mixed well. The contents were allowed to solidify with the tubes held in a slanting position. After solidification, the tubes were inoculated and incubated at 28 °C for 24-48 hours. When urea is hydrolysed, the medium was found to exhibit a noticeable colour shift.

3.2.9.10 Coagulase activity

The coagulase activity was evaluated using the classical tube test assay described by Tran et al. (2024). 1 mL of yeast strain suspension in YM broth (10^6 CFU mL⁻¹) was added to a tube containing 0.5 mL of coagulase plasma (FD248, HiMedia, Mumbai, India). By inverting the tube, clot development was observed after 2, 4, 6, and 24 hours of incubation. Any degree of clot formation (ranging from a loose, thread-like structure to one that is indicated by a solid clot) was recorded as positive.

3.2.9.11 Phospholipase activity

The phospholipase activity was assessed by following the procedure described by Tran et al. (2024). 10 µL of the yeast strain suspension in PBS (10^6 CFU mL⁻¹) were streaked

onto Sabouraud Egg Agar (SEA): Sabouraud Dextrose Agar (MH063, HiMedia, Mumbai, India) 65 g L⁻¹, NaCl 5.85 g L⁻¹, CaCl₂ 0.06 g L⁻¹, with 10% (v/v) sterile egg yolk emulsion (FD045L, HiMedia, Mumbai, India); Malt Egg Agar (MEA): Malt Extract Agar (M1964, HiMedia, Mumbai, India) 48 g L⁻¹, glucose 20 g L⁻¹, NaCl 5.85 g L⁻¹, CaCl₂ 0.005 g L⁻¹, with 10% (v/v) egg yolk emulsion. Isolates were incubated at 28 °C for 48-72 hours. The development of discrete halos around the colonies indicates phospholipase activity.

3.2.10 Functional properties

3.2.10.1 Cholesterol-lowering capacity

Cholesterol-lowering capacity was determined by following the protocol described by Aswani et al. (2021) and Alameri et al. (2022), with some modifications. Freshly prepared YM broth supplemented with 0.3% of bile salts and filter-sterilized water-soluble cholesterol (1 mg mL^{-1}) (C4951, Sigma-Aldrich, USA) was inoculated and incubated for 48 hours at 37°C . After incubation, cells were harvested by centrifugation at $6000 \times g$ for 15 minutes, and cell-free supernatant (1 mL) was mixed with 3 mL of potassium hydroxide (PCT1560, HiMedia, Mumbai, India) (50% w/v) and 3 mL of 95% ethanol. It was vortexed for 1 minute and incubated at 65°C for 15 minutes in a water bath and subsequently cooled. After removal of the ethanol layer, hexane (5 mL) (HX0293, Merck, Germany) and distilled water (3 mL) were thoroughly mixed for 1 minute. To separate the phases, the tubes were left to stand at room temperature for fifteen minutes. Following phase separation, 2.5 mL of the upper hexane layer were extracted and placed in a clean, dry test tube and allowed to dry completely in a water bath at 65°C . 4 mL of o-phthaldehyde reagent (RM1143, HiMedia, Mumbai, India) were added and allowed to stand at room temperature for 10 minutes. To each tube, 2 mL of concentrated sulfuric acid (100731, Merck, Germany) was added slowly to the side of the tube, mixed, and kept for incubation at room temperature. Absorbance was measured at 550 nm following a 30-minute dark incubation period. The cholesterol-lowering ability was calculated as follows:

$$\text{Cholesterol-lowering activity (\%)} = [\{ 100 - (B / C) \} \times 100]$$

Where, B is absorbance of the culture supernatant mixture; and C is absorbance of the control.

3.2.10.2 Antioxidant activity

The reduction percentage of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D9132, Sigma-Aldrich, Merck, Germany) radical was determined to evaluate the antioxidant activity of the yeast isolates by following the protocol described by Merchán et al. (2020), with some modifications. Eight hundred microliters of each isolate suspension and 1 mL of 0.2 mmol L⁻¹ 2,2-Diphenyl-1-picrylhydrazyl solution in methanol were mixed and incubated for 30 minutes at room temperature in darkness. The reaction tubes were centrifuged for 2 minutes at 2000 x g in order to collect the supernatant. To measure the antioxidant capacity percentage, the absorbance was measured at 517 nm, using sterile PBS solution as the blank.

The scavenging ability was defined as follows:

$$\text{Antioxidant activity (\%)} = \{[1 - A_{517}(\text{sample})/A_{517}(\text{blank})] \times 100\}$$

3.2.10.3 Anti-inflammatory Activity by Inhibition of Albumin Denaturation

The anti-inflammatory activity of the yeast isolates was investigated by the protein denaturation inhibition assay as explained by Pereira et al. (2021). Overnight grown cultures in YM broth were centrifuged at 5000 × g for 10 minutes, and the supernatant was examined for anti-inflammatory activity. 100 µL of the supernatant was mixed with 400 µL of 5% bovine serum albumin (BSA) (w/v aqueous solution) (MB083, HiMedia, Mumbai, India), and this reaction mixture was considered as the test (T). The control reaction mixture (C) was prepared by adding 100 µL of distilled water to 400 µL of BSA. The pH of the reaction mixtures was adjusted to 6.5 using 1 N HCl. The reaction mixtures were incubated at for 20 minutes 37 °C, followed by heating for 20 minutes at 51 °C. The mixtures were allowed to cooled down, 2.5 mL of PBS was added to the

mixtures, and absorbance was measured at 416 nm of wavelength. The percentage inhibition of protein denaturation was determined using the following formula:

$$\text{Inhibition (\%)} = [\{ 1 - (\text{Abs}_T / \text{Abs}_C) \} \times 100]$$

3.2.10.4 Antidiabetic Activity by Inhibition of α -Amylase Activity

Antidiabetic activity was examined by the inhibition of α -amylase activity by using the 3,5-dinitrosalicylic acid (DNSA) (GRM1582, HiMedia, Mumbai, India) method of Pereira et al. (2021). The overnight culture of yeast was centrifuged at $5000 \times g$ for 10 minutes, and 1 mL of the supernatant was pre-incubated for 30 minutes with 0.5 mL of 0.1% α -amylase (in 20 mM sodium phosphate buffer, pH 6.9) (86250, Sigma-Aldrich, Merck, Germany). Then, 1 mL of 0.1% starch (S9765, Sigma-Aldrich, Merck, Germany) was added to the mixture and incubated for 10 minutes at 37 °C. After that, 1 mL of DNSA reagent was added to stop the reaction. For five minutes, each sample's test and control tubes were submerged in a boiling water bath. 10 mL distilled water was added to the reaction mixture after it cooled down, and OD was observed at 540 nm. In the control tubes, all steps performed were similar to those test tubes, except that the culture supernatant was substituted by distilled water. The enzyme activity in the control tubes was 100%.

The following formula was used to calculate the percentage inhibition of α -amylase activity:

$$\text{Inhibition (\%)} = [\{ 1 - (B/A) \} \times 100]$$

where A = absorbance of the control (reaction mixture, with the exclusion of test samples) and B = absorbance of the test (reaction mixture, with the inclusion of test samples).

3.2.10.5 Exopolysaccharide production

The production of exopolysaccharide by the isolates was ascertained by following the protocol described by Alameri et al. (2022). Overnight grown yeast cultures were streaked on the plates containing ruthenium red milk (g L⁻¹: skim milk powder (GRM1254, HiMedia, Mumbai, India) 10.0, ruthenium red (GRM7463, HiMedia, Mumbai, India) 0.08 and agar 20.0), and incubated at 37 °C for 24 hours. Yeast isolates producing exopolysaccharides were observed as white ropy colonies, while non-ropy isolates appeared as red colonies due to the staining of the microbial cell wall.

3.2.10.6 Phytate hydrolyzing activity

The phytate hydrolyzing capacity was determined according to Menezes et al. (2020), with some modifications. The ability of the yeast to produce phytase was examined in solid media. The phytase screening medium agar was composed as follows: glucose 15.0 g L⁻¹; sodium phytate (GRM6226, HiMedia, Mumbai, India) 1.0 g L⁻¹; (NH₄)₂SO₄ 5.0 g L⁻¹; MgSO₄·7H₂O 0.5 g L⁻¹; KCl 0.5 g L⁻¹; FeSO₄·7H₂O 0.01 g L⁻¹; MnSO₄·1H₂O 0.1 g L⁻¹; and agar 15.0 g L⁻¹. With the use of 0.1 N NaOH, the pH was adjusted to 5.5. The inoculated plates were incubated at 30 °C for 24 hours. Phytase production was observed in the plates by the formation of a clear zone around the colonies.

3.2.10.7 β-galactosidase activity

In order to determine the positive activity for β-galactosidase enzymes, freshly grown yeast isolates were streaked on YM agar supplemented with 100 μL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (MB069, HiMedia, Mumbai, India) (20 mg/mL in DMSO) and 20 μL of IPTG (iso-propyl-thio-β-D galactopyranoside) (MB072, HiMedia, Mumbai, India) solution as inducer (Angmo et al., 2016). Positive isolates were shown as greenish to bluish colonies after 48 to 72 hours of incubation at 30 °C.

3.2.10.8 β -D-Glucosidase activity

β -D-glucosidase activity was examined by the arbutin method explained by Fiore et al. (2005). The medium contained Yeast Nitrogen Base (M139, HiMedia, Mumbai, India) 0.67 g L^{-1} , arbutin (p-hydroxyphenyl-b-D-glucopyranoside) (13492, Sisco Research Laboratories Pvt. Ltd., Mumbai, India) 0.5 g L^{-1} , and agar 2 g L^{-1} were prepared, and the final pH of the medium was modified to pH 5.0 before autoclaving. Before pouring the plates, ammonium ferric citrate (filter-sterilized) (GRM475, HiMedia, Mumbai, India) (2 g L^{-1}) was added to the medium. The plates were incubated at 26°C for 5 days, and the colonies showing activity were identified by the brown colours around the colony.

3.2.10.9 Vitamin B12 Assay

Vitamin B12 Assays were performed by following the protocol described by Romero-Luna et al. (2019). To determine vitamin B12 production, isolates were streaked on vitamin B12 assay medium (MM036, HiMedia, Mumbai, India), containing all the vitamins except vitamin B12, followed by incubation at 37°C for 48-72 hours. The growth of the isolates on the assay plate was considered positive for the production of vitamin B12.

3.2.10.10 L-asparaginase activity

L-asparaginase was detected by following the protocol described by Vimal and Kumar, (2017). A modified Czapek Dox medium (pH 6.2) (g L^{-1} : glucose 2.0, L-asparagine (PCT0303, HiMedia, Mumbai, India) 10.0, KH_2PO_4 1.52, KCl 0.52, $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$ 0.001, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001, $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, Agar 20.0) was used for plate assay. 2.5% stock solution of phenol red (I010, HiMedia, Mumbai, India) was prepared in ethanol (pH 6.2), and 3 mL of this was added to 1 L of Czapek Dox medium. Following point inoculation of plates containing this medium, L-asparaginase activity was detected

when a pink zone surrounding the colony was developed after 48 hours of incubation at 30 °C.

3.2.10.11 Antifungal activity

The antifungal activity of the isolates was investigated using the method described by Abouloifa et al. (2020), with minor modifications. The isolates will be seeded in two streaks, 2 cm each, on a YM agar plate and then incubated at 28 °C for 48 hours. The resulting colonies will be overlaid with 10 mL of soft (0.75% agar) PDA medium containing tested molds viz., *Aspergillus niger* NCIM 1248, *Fusarium oxysporum* NCIM 1350, *Aspergillus niger* NCIM 1358, *Aspergillus oryzae* NCIM 1212, and *Aspergillus flavus* NCIM 549. After 72 hours of incubation at 28 °C, results were interpreted as zone formation. Results were categorized into 4 categories: “-” for no zone of inhibition, “+” for zone of a more than 5 mm, “++” for a zone of more than 10 mm, and “+++” for a zone of more than 15 mm.

3.2.10.12 Production of siderophore

Screening for siderophore production was performed by the method of Sourabh et al. (2011). After being acid rinsed, the glassware used in this experiment was rinsed three times with double-distilled water. To prepare CAS agar for siderophore detection, the blue dye solution was first prepared. A total of 0.06 g of CAS (Eriochrome® azurol S) (RM336, HiMedia, Mumbai, India) was dissolved in 50 mL of ddH₂O (Solution 1). Solution 2 was prepared by dissolving 0.0027 g of FeCl₃.6H₂O in 10 mL of 10 mM HCl. For Solution 3, 0.073 g of CTAB (MB101, HiMedia, Mumbai, India) was dissolved in 40 mL of ddH₂O. Solution 1 was mixed with 9 mL of Solution 2, and the resulting mixture was then combined with Solution 3 to form a blue-coloured solution. This final dye solution was autoclaved and stored in a plastic container. For the mixture solution,

MM9 salt stock was prepared by dissolving 15 g of KH_2PO_4 , 25 g of NaCl , and 50 g of NH_4Cl in 500 mL of ddH_2O . 20 g of glucose were dissolved in 100 mL of ddH_2O to create a 20% glucose stock. To make the NaOH stock, 25 g of NaOH was dissolved in 150 mL of ddH_2O , and the pH was adjusted to 12. To prepare the Casamino acid solution, 3 g of Casamino acid (68806, Sisco Research Laboratories Pvt. Ltd., Mumbai, India) was dissolved in 27 mL of ddH_2O , extracted with 3% 8-hydroxyquinoline (GRM1061, HiMedia, Mumbai, India) in chloroform to remove trace iron, and then filter sterilized the solution. For CAS agar preparation, 100 mL of MM9 salt solution was mixed with 750 mL of ddH_2O , and 32.24 g of PIPES (MB022, HiMedia, Mumbai, India) buffer was dissolved into the solution. Since PIPES does not dissolve below pH 5, the pH was first raised to 6 before gradually adding PIPES with continuous stirring. The pH was carefully adjusted to 6.8 during the process, ensuring it did not exceed this value to prevent the solution from turning green. Then, 15 g of agar was added, and the mixture was autoclaved and cooled to 50 °C. After cooling, 10 mL of sterile 20% glucose solution and 30 mL of sterile Casamino acid solution were added to the mixture of PIPES/MM9. Finally, 100 mL of the sterile Blue Dye solution was slowly added along the inner wall of the container with gentle agitation to ensure thorough mixing. The final mixture was aseptically poured into plates and allowed to solidify. The plates prepared were spot inoculated with the test yeast cultures on CAS agar medium and incubated at 28 °C for 48 hours. The development of a yellow-orange zone around the colony on blue-coloured medium plates was interpreted as a positive result.

3.2.11 Statistical analysis

All the experiments were conducted in triplicate, and results were expressed as the mean $\pm$ SD ($n = 3$) using Microsoft Office 2019. Diversity indices [Simpson's diversity index (1-D), Shannon's diversity index (H)] were calculated using PAST v4.03

(Paleontological Statistics Software Package for Education and Data Analysis) (Hammer et al., 2001).

Shannon's diversity index was given as follows:

$$H = - \sum_{i=1}^s p_i \ln(p_i)$$

Simpson's diversity index was given as follows:

$$1 - D = \sum_{i=1}^s p_i^2$$

Where S is the number of species, p_i is the number of the given species divided by the total number of isolates observed, and $1-D$ is Simpson's index (Daly et al., 2018).

Significant statistical differences ($p < 0.05$) between yeast isolates were performed according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA), and Pearson pairwise correlation was also performed with a 2-tailed test of significance where '*' signifies the significant correlation at the 0.05 level using statistical software OriginPro v2024 (OriginLab Corporation, Northampton, United States). Normalization of data generated from qualitative experiments was carried out using $\log(x + 1)$ transformation. Additionally, ClustVis (Beta) was used to plot the heatmap analysis (Metsalu and Vilo, 2015). Differences and similarities amongst the isolates were evaluated, and potential isolates were selected based on the score value by PCA (principal component analysis) biplot using PAST v4.03 (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer et al., 2001).

3.2.12 Data availability

The deposited sequences from various samples are accessible on the NCBI (National Center for Biotechnology Information, USA) database. From *dahi* samples from cow milk, the sequences were submitted with GenBank accession number are: OP071251 (*Saccharomyces cerevisiae* DAO-17), OP071252 (*Saccharomyces cerevisiae* DJT-2), OP071253 (*Saccharomyces cerevisiae* DKL-13), OP071254 (*Saccharomyces cerevisiae* DMS-15), OP071255 (*Kluyveromyces marxianus* DPA-41), OP071256 (*Saccharomyces cerevisiae* DRC-41), OP071257 (*Saccharomyces cerevisiae* DRC-42), and OP071258 (*Kluyveromyces lactis* DTL-6). From *chhurpi* from cow milk, the sequences were submitted with GenBank accession number are: OP479882 (*Debaryomyces prosopidis* CPA-55), OP479875 (*Debaryomyces prosopidis* CCG-4), OP479876 (*Saccharomyces cerevisiae* CGI-29), OP479877 (*Saccharomyces cerevisiae* CKL-10), OP479878 (*Kazachstania exigua* CMB-19), OP479879 (*Meyerozyma guilliermondii* CMN-3), OP479880 (*Kazachstania exigua* CMNB-4), OP479881 (*Pichia bruneiensis* CMS-3), OP479883 (*Pichia bruneiensis* CPAL-16), OP479884 (*Saccharomyces cerevisiae* CRC-35), OP479874 (*Yarrowia lipolytica* CBG-9), and OP561448 (*Pichia kudriavzevii* CNT-3). From *dahi* from yak milk, the sequences were submitted with GenBank accession number are: OR681920 (*Saccharomyces cerevisiae* DKY-22), OR681921 (*Saccharomyces cerevisiae* DCY-27), OR681922 (*Yarrowia lipolytica* DUAY-11), OR681923 (*Saccharomyces cerevisiae* DUAY-53), OR682643 (*Saccharomyces cerevisiae* DUBY-47), OR681924 (*Yarrowia lipolytica* DUCY-6), OR681925 (*Wickerhamomyces anomalus* DUCY-48), OR681926 (*Candida parapsilosis* DLAY-48), and OR681927 (*Yarrowia lipolytica* DZBY-37). From *chhurpi* from yak milk, the sequences were submitted with GenBank accession number are: OR681928 (*Candida metapsilosis* CKY-12), OR682644 (*Yarrowia lipolytica* CLAY-2), OR681929

(*Kluyveromyces lactis* CLBY-16), OR681930 (*Kluyveromyces marxianus* CZAY-19), OR681931 (*Yarrowia lipolytica* CZBY-49), and OR681932 (*Kluyveromyces lactis* CUBY-19).

RESULTS

Objective 1:

To profile the yeast communities in *dahi* and *chhurpi* of Sikkim using phenotypic and molecular methods.

4.1 DOCUMENTATION OF THE ETHNIC FERMENTED MILK PRODUCTS FROM SIKKIM

4.1.1 Survey on ethnic fermented milk products

A survey was accomplished using a structured questionnaire to study artisanal, rare, and easily accessible traditionally fermented milk (NFM) products in various regions of Sikkim, India (Table B). In Sikkim, the following survey data was gathered and recorded about socioeconomic practices, ecological diversity, commercial production, and traditional ways of preparing, cooking, and consumption of the milk products.

4.1.2 Ethnic NFM products of Sikkim

In all four districts of Sikkim, it was found that NFM products are widely prepared. These NFM products are generally prepared from cow and yak milk. Cows are often found in the lower altitudes, while yaks are found exclusively in the higher altitudes. Common NFM products from Sikkim include *dahi*, *mohi*, *chhurpi* (soft variety), *chhurpi* (hard variety), *gheu*, and *mar* (Figure 1).

4.1.3 Indigenous method of preparation

The traditionally prepared fermented cow and yak milk products of Sikkim include *dahi*, *chhurpi* (both soft and hard varieties), *chhu* or *sheden*, *gheu*, *mar*, *mohi*, *philu*, *shyou*, and *somar*. Among these, *Dahi* and *Chhurpi* are the most important naturally fermented milk products from Sikkim, which are usually prepared from cow and yak milk. The process for making *dahi* and *chhurpi* from both types of milk is similar (Figure 2). To make *dahi*, fresh milk is boiled, cooled to room temperature, and mixed with a small amount of old *dahi*. This mixture is then allowed to ferment for 48-72 hours. It uses the “back-slopping” method, which uses the previously fermented batch to start the new batch. The traditional container used for *dahi*-making is known as the *theki*. After *dahi*

is ready, it can also be churned in a wooden vessel called "*tholung*" to separate *gheu* (butter) and *mohi* (buttermilk). The *mohi* is cooked for 20-25 minutes, strained through a cloth or net to remove whey, and then hung to drain. Finally, the soft variety of *chhurpi* is prepared.

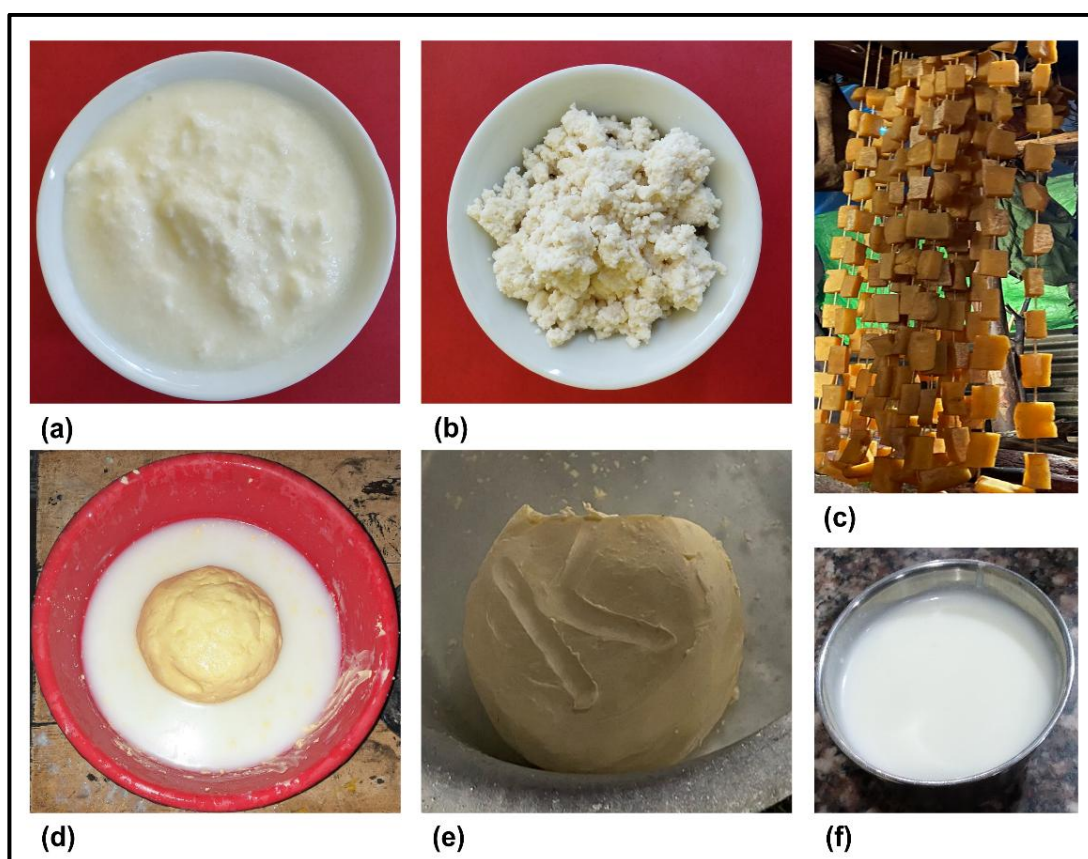


Figure 1: Traditionally prepared naturally fermented milk products from Sikkim. (a) *Dahi*, (b) *Chhurpi* (soft variety), (c) *Chhurpi* (hard variety), (d) *Gheu* (butter from cow's milk), (e) *Mar* (butter from yak's milk), and (f) *Mohi* (Buttermilk from cow milk).

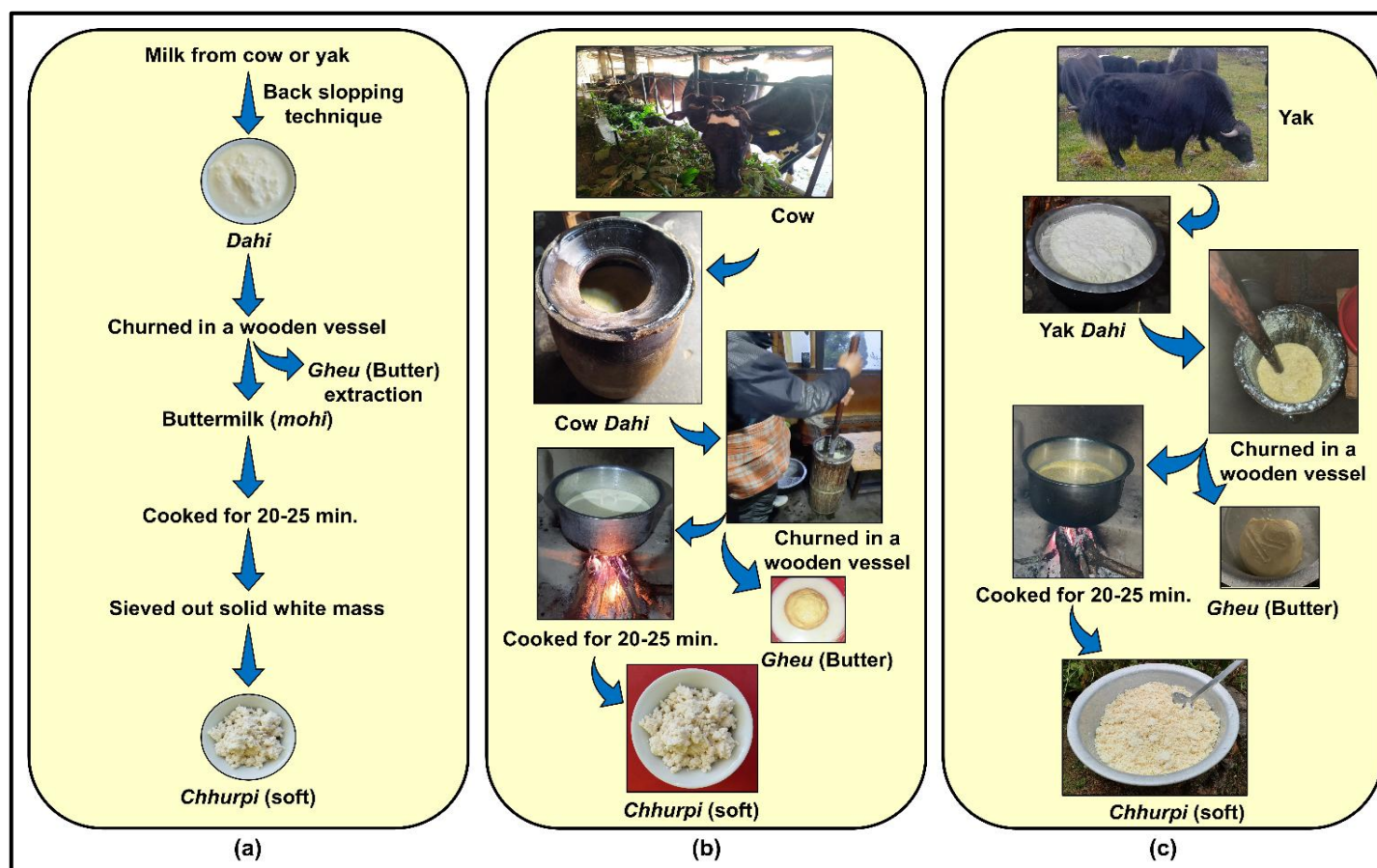


Figure 2: Indigenous methods of *dahi* and *chhurpi* preparation from both cow and yak milk in Sikkim. (a) Common methods for *dahi* and *chhurpi* preparation, (b) *dahi* and *chhurpi* preparation from cow milk, and (c) yak milk.

4.1.4 Ethnic values and consumption patterns

NFM product of Sikkim plays a vital role in culinary traditions, representing the state's rich cultural heritage and dietary patterns of its diverse ethnic communities. NFM products are widely prepared by the ethnic community of Sikkim essentially for daily consumption and as a source of income. NFM products from cow are widely prepared by *Chhetri, Bahun, Rai, Limbo, Gurung, Sherpa, Tamang, Bhutia*, and *Lepcha* communities in low altitudes; however, NFM products from yak are mainly produced by *Bhutia, Lepcha, Sherpa, Lachungpas, Lachenpas*, and *Dokpa* herders living in high altitudes of the North, East, and West districts. NFM products hold immense nutritional, traditional, and ethnic value, often playing important roles in cuisine, rituals, and daily life.

Dahi is a savoury, non-alcoholic beverage which is found in a semi-solid state. People often tend to add sugar before eating. Most likely, people mix it with *cheura* (beaten rice). During the celebration of “*Aashar pandhra*” rice sowing, banana, *cheura* and *dahi* are regarded as the staple foods during the monsoon season. In *Nepali* culture, *dahi* is mixed with raw rice, and the head of the family applies the mixture to each member's forehead during *Dussehra*. *Dahi* is also used to prepare other dairy products like *mohi* (buttermilk), *gheu* (butter) and *chhurpi* (cottage cheese). A wooden vessel called *theki* is considered sacred in *Nepali* culture, it is not washed with other utensils and generally do not get touched by everyone. The reason is that once the butter is extracted, it is offered to their deity or God over a burning coal and buttery aroma is used to purify the house. *Chhurpi* has two varieties- soft and hard. Hard *chhurpi* is a popular chewing snack, especially when traveling. Soft *chhurpi* is consumed fresh and often used in soups, stews and side dishes. Curry and pickle are also prepared with onion, chillies and edible ferns locally called *ningro* (*Diplazium sculentum*). *Dahi* and *chhurpi* are the

main NFM products of Sikkim and it is a vital source of income for farmers and pastoral communities at high altitudes supporting sustainable agriculture and dairy farming in remote areas.

4.1.5 Ecology and diversity

NFM products made from cows are primarily produced by indigenous communities in all four districts of Sikkim. On the other hand, NFM products prepared from yaks (*Bos grunniens* L.) are predominantly produced by nomadic *Lachungpas*, *Lachenpas*, and *Dokpa* herders living in high altitudes of the North, East, and West districts of Sikkim. These yak herders also benefit from herding crossbreeds of cows and yaks known as *urang* or *dzomo* females, and *dzo* males. Yaks in Sikkim can be found in various locations such as Lachen, Gurudongmar Lake, Chopta valley, Lachung valley, Yumthang, Tsomgu Lake, Kupup, Thegu, Nathang, and Yuksom-Dzongri. *Siri* cow (*Bos indicus*) is mostly domesticated small sized draught purpose local cattle breed. The indigenous cattle in urban and nearby regions are usually crossbred with Jersey and Holstein Friesian breeds to increase milk production. According to the 20th livestock census data, Sikkim has 148,010 cattle and 5,219 yaks, accounting for approximately 5.26% of the total yak population in India (Table 5) (20th Livestock Census-2019). The East District reported the largest number of cattle among the districts, with 46,864 cattle, followed by the South District with 46,558 cattle, and the West District had 42,670 cattle. With 11,918 cattle, the North District had the smallest population, but with 3,616 yaks, the largest, since yaks are primarily found in high-altitude areas. Yaks were not reported in the South districts, but comparatively smaller yak populations of 1,509 and 94 were reported in the East and West Districts, respectively. Yaks in Sikkim are informally classified into two breeds: *Bho* and *Aho*, based on the communities that raise them. *Bho* yaks are predominantly found in the western part of Sikkim, while *Aho* yaks

are more common in the eastern region, being smaller and less bulky than the *Bho* variety.

Table 5: District wise distribution of cattle and yak in Sikkim (Source: 20th Livestock Census- 2019).

District	Cattle	Yak
East	46,864	1,509
North	11,918	3,616
South	46,558	0
West	42,670	94
Total	1,48,010	5,219

4.1.6 Milk production and composition

Cow milk production in Sikkim increased from 54,350 Metric Tons to 86,530 Metric Tons between 2016 and 2023 (Basic Animal Husbandry Statistics-2023). The milk production and yield from both native and crossbred cattle in Sikkim during 2022-2023 are highlighted in the Basic Animal Husbandry Statistics-2023 (Table 6).

Table 6: Total number of Cattle, Milk Production and Average Yield per In-Milk Animal in 2022-23 in Sikkim (Source: Basic Animal Husbandry Statistics- 2023, Govt. of India).

Cattle	Number of Animals (figures in '000 Nos.)	Milk production in 2022-23 (figures in '000 Tonnes)	Average Yield per In-Milk Animal (figures in kg/day/Animal)
Crossbred	43.20	82.94	5.26
Indigenous	2.12	3.32	4.3

According to the data, crossbred cattle account for 82.94 thousand tonnes of milk production, while native cattle only contribute 3.32 thousand tonnes. Due to their superior milk yield, which averaged 5.26 kg per animal per day, crossbred cattle contributed more than native cattle, which produced 4.3 kg per animal per day. The widespread adoption of crossbred cattle, particularly in low and mid-altitude regions such as the South and certain areas of the East Districts, is primarily due to their increased productivity and adaptability to enhanced feeding and breeding practices. Despite the fact that indigenous cattle have a lower productivity, they are crucial in remote areas due to their ability to acclimate to adverse environmental conditions and their minimal maintenance requirements. In addition to the rising demand for cow milk, the demand for buffalo milk has also increased from 30 Metric Tons to 270 Metric Tons between 2021-22 and 2022-23 (Basic Animal Husbandry Statistics-2023). Indigenous yaks yield 250-500 kg of milk per lactation over 260-300 days, while cattle-yak hybrids produce about 400 kg of milk per lactation within about 200 days, with a daily yield of 1.4 (ranging from 0.8 to 4.4) liters compared to the 1.1 (ranging from 0.4 to 3.8) liters of yak milk (Behl et al., 2020). Yak milk was found to have higher total solids, fat, protein, lactose, and ash percentage compared to cow milk (Table 7). Although the production of yak milk is relatively low, it is of great cultural and economic importance in the high-altitude areas of north and west Sikkim. However, the limited population and harsh climate limit the overall contribution of Yak milk to the milk production in the State. The Animal Husbandry & Veterinary Services Department of Sikkim does not publish specific data on yak milk production. This could be due to various factors such as geographical constraints, small-scale farming, seasonal and low yield, a lack of institutional focus, and an informal market system. Nevertheless, the Department has been proactively assisting yak herders by offering veterinary services, conservation

initiatives, and market connections to enhance the productivity and sustainability of yak farming.

Table 7: Gross composition of cow and yak milk (**Source:** Singh et al., 2023).

Composition	Cow	Yak
Total solids (%)	11-13	15-19
Fat (%)	3.5-4.0	5-9
Protein (%)	3.0-3.5	4.0-6.5
Lactose (%)	4.5-5.0	4.0-6.0
Ash (%)	0.7-0.9	0.4-0.9
Energy (Kcal 100 mL ⁻¹)	59-70	87-91
Oligosaccharide (mg L ⁻¹)	30-90	-

4.1.7 Commercial production of milk products in Sikkim

In addition to traditional Indigenous methods of milk preparation at the local level, there are many milk products that can be purchased commercially through the Sikkim Co-operative Milk Producers Union Limited. Since more small and marginal farmers are raising dairy animals in the state, dairy farming has become popular among livestock farmers. Dairy farming and milk supply in organized sectors such as the Sikkim Milk Producer's Union are popular primarily because of the Rs.8 per litre incentive offered to farmers. The State Government made the ground-breaking decision to offer milk production incentives of ₹ 8 per litre of milk to dairy farmers under organized sector" during the financial year 2020-2021. The State Government had planned the scheme in order to encourage milk producers and dairy farmers in the State to take up dairy farming and thus to maximise their income by producing milk, with farmers benefiting from an incentive of 8 rupees per litre of milk produced from their cows. It is also evident that the milk producers in the State have been enjoying the scheme and more and more

farmers are participating in the sale of their milk to organised sectors such as the Sikkim Milk Union cooperative Society and North District Dairy Cooperative Union. In the 2023-24 period, the Union procured approximately 12,266.60 metric tons of milk from local level and produced various products as shown in Table 8.

Table 8: Annual production of commercially available milk products in Sikkim Co-operative Milk Producers Union Limited (2023-24).

Sl. No.	Products	Annual production (KGS)
1	Butter (1 kg)	4238
2	Chhurpi (500 gm)	9008.5
3	Chhurpi (200 gm)	1224.4
4	Paneer (200 gm)	9390.8
5	Paneer (1 kg)	10428.8
6	Dahi (500 gm)	602661
7	Ice cream	1926.412
8	Pasteurized High Cream Milk (1000 ml)	11984394
9	Pasteurized High Cream Milk (500 ml)	2072871.5
10	Pasteurized Toned Milk (500 ml)	296884.5
11	Pasteurized Loose Milk	171045.5

4.2 SAMPLE COLLECTION, PHYSICOCHEMICAL AND MICROBIOLOGICAL ANALYSIS OF THE SAMPLE AND PHENOTYPIC AND GENOTYPIC IDENTIFICATION

4.2.1 Sample collection

Traditionally prepared NFM products (40 cow *dahi*, 40 cow *chhurpi*, 9 yak *dahi*, and 9 yak *chhurpi*) were collected from various locations in Sikkim, and the date, sample collection site, nature of the sample, altitude, and geographical coordinates were recorded (Table 9-12). Samples were collected in a sterilized container and transported to the laboratory, maintaining the lowest temperature in an icebox, and transferred to a refrigerator at 4 °C for further analysis.

Table 9: Sample collection details for *dahi* from cow milk of Sikkim.

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude	Number of samples collected
Cow <i>dahi</i>	Dugalakha, Pakyong, East Sikkim	22-11-2020	1118	27°14'23.6"N, 88°36'09.4"E	3
	Sorung, Gangtok, East Sikkim	27-01-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Below ICAR, Gangtok	16-02-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Adampul, Gangtok	16-02-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Lingdum, Gangtok	19-02-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Ranka, Gangtok	19-02-2021	1500	27°20'29.5"N, 88°35'08.9"E	1
	Central Pendam, Singtam	03-07-2021	850	27°12'19.0"N, 88°33'48.0"E	1
	Chanatar, Rangpo	03-07-2021	400	27°10'35.8"N, 88°31'48.5"E	1
	Mangan, North Sikkim	13-11-2021	1750	27°30'01.7"N, 88°32'18.8"E	2
	Kabi, North Sikkim	06-01-2022	1600	27°24'20.6"N, 88°37'06.9"E	2
	Phodong, North Sikkim	06-01-2022	1700	27°24'30"N, 88°34'14"E	2
	Tumlong, North Sikkim	06-01-2022	1700	27°24'47.3"N, 88°35'07.2"E	2
	Ramthang, North Sikkim	06-01-2022	1900	27°25'05.9"N, 88°32'35.0"E	2
	Nesha, Tashiding, West Sikkim	13-02-2022	1050	27°18'31.7"N, 88°17'21.3"E	1
	Barthang, Geyzing, West Sikkim	13-02-2022	600	27°16'47.4"N, 88°16'11.9"E	1
	Chongpung, Geyzing, West Sikkim	13-02-2022	600	27°16'42.0"N, 88°15'52.6"E	1

Table 9: Sample collection details for *dahi* from cow milk of Sikkim (contd.).

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude
Sombaria, West Sikkim	13-02-2022	1500	27°08'30.8"N, 88°08'28.6"E	3
Lidung, Dentam, West Sikkim	13-02-2022	1600	27°15'08.8"N, 88°08'02.4"E	2
Daragaon, Soreng, West Sikkim	13-02-2022	1400	27°10'06.9"N, 88°11'45.5"E	2
Dong, Namchi, South Sikkim	13-02-2022	700	27°10'26.5"N, 88°18'57.4"E	1
Jorethang, South Sikkim	13-02-2022	700	27°08'11.0"N, 88°17'32.5"E	2
Bhanjyang, Namchi, South Sikkim	13-02-2022	1600	27°09'37.6"N, 88°24'21.7"E	2
Ravangla, South Sikkim	13-02-2022	1800	27°17'46.8"N, 88°22'27.0"E	2
Yangang, South Sikkim	03-03-2022	1300	27°17'22.4"N, 88°25'10.4"E	2
Maniram, Namchi, South Sikkim	13-02-2022	1700	27°09'09.1"N, 88°24'36.8"E	1
Total <i>dahi</i> samples from cow:				40

m=meter

Table 10: Samples collection details for *chhurpi* from cow milk of Sikkim.

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude	Number of samples collected
Cow <i>chhurpi</i>	Dugalakha, Pakyong, East Sikkim	22-11-2020	1118	27°14'23.6"N, 88°36'09.4"E	2
	Chanatar, Rangpo	03-07-2021	400	27°10'35.8"N 88°31'48.5"E	1
	Lingzey, Singtam	03-07-2021	1400	27°18'06.4"N 88°28'24.6"E	2
	Khamdong, Singtam	25-03-2021	1100	27°15'54.1"N 88°28'59.7"E	2
	Adampul, Gangtok	14-03-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Makha, Singtam	25-03-2021	1200	27°17'39.9"N 88°27'36.6"E	1
	Chamile, Gangtok	25-03-2021	1600	27°32'36.9"N, 88°59'06.1"E	1
	Mangan, North Sikkim	13-11-2021	1750	27°30'01.7"N, 88°32'18.8"E	4
	Kabi, North Sikkim	06-01-2022	1600	27°24'20.6"N, 88°37'06.9"E	2
	Phodong, North Sikkim	06-01-2022	1700	27°24'30"N 88°34'14"E	2
	Tumlong, North Sikkim	06-01-2022	1700	27°24'47.3"N 88°35'07.2"E	2
	Darap, Geyzing, West Sikkim	13-02-2022	1600	27°18'07.2"N 88°11'20.4"E	1
	Sombaria, West Sikkim	13-02-2022	1500	27°08'30.8"N 88°08'28.6"E	1
	Barthang, Geyzing, West Sikkim	13-02-2022	600	27°16'47.4"N 88°16'11.9"E	1
	Chongpung, Geyzing, West Sikkim	13-02-2022	600	27°16'42.0"N 88°15'52.6"E	1
	Karmatar, Geyzing, West Sikkim	13-02-2022	600	27°16'51.9"N 88°16'31.6"E	1

Table 10: Samples collection details for *chhurpi* from cow milk of Sikkim (contd.)

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude
Liching, Geyzing, West Sikkim	13-02-2022	700	27°16'48.1"N 88°16'13.6"E	1
Narkhola, Geyzing, West Sikkim	13-02-2022	700	27°17'31.5"N 88°17'30.8"E	1
Lidung, Dentam, West Sikkim	13-02-2022	1600	27°15'08.8"N 88°08'02.4"E	2
Daragaon, Soreng, West Sikkim	13-02-2022	1400	27°10'06.9"N 88°11'45.5"E	1
Kholaghari, Namchi, South Sikkim	13-02-2022	1200	27°09'23.9"N 88°22'08.6"E	1
Bhanjyang, Namchi, South Sikkim	13-02-2022	1600	27°09'32.9"N 88°24'37.7"E	1
Ravangla, South Sikkim	13-02-2022	1800	27°18'24.6"N 88°21'17.6"E	1
Yangang, South Sikkim	03-03-2022	1300	27°17'30.6"N 88°25'10.2"E	1
Jorethang, South Sikkim	13-02-2022	700	27°08'11.0"N 88°17'32.5"E	3
Maniram, Namchi, South Sikkim	13-02-2022	1700	27°09'09.1"N 88°24'36.8"E	3
Total <i>chhurpi</i> samples from cow:				40

m=meter

Table 11: Sample collection details for *dahi* from yak milk of Sikkim.

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude	Number of samples collected
Yak <i>dahi</i>	Kupup, East Sikkim	21-06-2022	4000	27.3423° N, 88.8413° E	1
	Chungthang, North Sikkim	04-08-2022	3400	27.6039° N, 88.6464° E	1
	Uttarey, West Sikkim	07-11-2022	3600	27.2606° N, 88.0950° E	3
	Lachung, North Sikkim	05-12-2022	4200	27.6891° N, 88.7430° E	2
	Zuluk, East Sikkim	06-12-2022	3200	27.2518° N, 88.7775° E	2
Total <i>dahi</i> samples from yak:					09

m=meter

Table 12: Sample collection details for *chhurpi* from yak milk of Sikkim.

Sample	Sample collection site	Date	Altitude (m)	Latitude and Longitude	Number of samples collected
Yak <i>chhurpi</i>	Kupup, East Sikkim	21-06-2022	4000	27.3423° N, 88.8413° E	1
	Chungthang, North Sikkim	04-08-2022	3400	27.6039° N, 88.6464° E	1
	Uttarey, West Sikkim	07-11-2022	3600	27.2606° N, 88.0950° E	3
	Lachung, North Sikkim	05-12-2022	4200	27.6891° N, 88.7430° E	2
	Zuluk, East Sikkim	06-12-2022	3200	27.2518° N, 88.7775° E	2
Total <i>chhurpi</i> samples from yak:					09

m=meter

4.2.2 Physicochemical analysis

Physicochemical analysis such as pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) for cow *dahi* ranged from 3.88 ± 0.01 to 4.76 ± 0.03 ; 242 ± 2 to 492.67 ± 2.52 ; 86.54 ± 0.24 to 98.11 ± 0.18 ; and 0.80 ± 0.01 to 0.95 ± 0.03 (Table 13). Similarly, the pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) for cow *chhurpi* were also recorded as 3.93 ± 0.03 to 5.13 ± 0.02 ; 130.33 ± 1.53 to 291.33 ± 2.87 ; 55.79 ± 0.59 to 74.67 ± 1.37 ; and 0.63 ± 0.04 to 0.92 ± 0.01 (Table 14). Yak *dahi* showed a pH value, apparent viscosity (cP), moisture content (%), and titratable acidity (%) ranging from 4.59 ± 0.04 to 4.72 ± 0.04 ; 365.67 ± 3.4 to 462 ± 3.74 ; 86.46 ± 0.25 to 94.38 ± 0.82 ; and 0.83 ± 0.03 to 0.95 ± 0.02 (Table 15). For yak *chhurpi*, the results for pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) are between 4.65 ± 0.04 and 5.72 ± 0.05 ; 128.33 ± 2.49 to 150 ± 4.55 ; 42.49 ± 25.64 to 68.73 ± 0.83 ; and 0.75 ± 0.03 to 0.91 ± 0.01 (Table 16). The physicochemical parameters of *dahi* and *chhurpi* from cow and yak were analysed in the Tukey's boxplot based on the properties such as pH (Figure 3), apparent viscosity (cP) (Figure 4), moisture content (%) (Figure 5), and titratable acidity (%) (Figure 6).

Table 13: Physicochemical properties of *dahi* samples prepared from cow milk of Sikkim.

Sl.no.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
1	PKD-1a	4.76±0.03 ^a	399.6±5.24 ^{lmn}	86.54±0.24 ^k	0.88±0.01 ^{abcdef}
2	PKD-2a	4.36±0.02 ^{hij}	484.33±1.53 ^{ab}	91.6±0.33 ^{hi}	0.91±0.01 ^{abcd}
3	PKD-3a	4.84±0.01 ^a	492.67±2.52 ^a	89.37±0.03 ^j	0.88±0.02 ^{abcdef}
4	GSD-1a	4.31±0.01 ^{jk}	408±2.83 ^{kl}	91.08±0.04 ^{hij}	0.86±0.02 ^{abcdef}
5	GID-1a	4.03±0.02 ^{no}	390.67±5.51 ⁿ	90.92±0.77 ^{ij}	0.82±0.02 ^{ef}
6	GAD-1a	4.08±0.01 ^{mno}	415±3 ^k	93.8±1.49 ^{fg}	0.86±0.001 ^{bcdef}
7	GLD-1a	4.01±0.00 ^o	300±5 ^q	95.07±0.21 ^{cdefg}	0.83±0.02 ^{cdef}
8	GRD-1a	4.33±0.02 ^{ijk}	310±5 ^q	95.54±1.47 ^{bcdef}	0.80±0.01 ^f
9	CPD-1a	3.98±0.01 ^{op}	418.67±3.51 ^{jk}	96.08±0.59 ^{abcde}	0.91±0.01 ^{abcd}
10	RCD-1a	3.88±0.01 ^p	360.33±5.03 ^o	96.95±0.58 ^{abc}	0.87±0.01 ^{abcdef}
11	MNBD-1	4.13±0.01 ^{lmn}	230±1.53 ^s	86.54±0.24 ^k	0.85±0.01 ^{bcdef}
12	MNBD-2	4.16±0.01 ^{lm}	242±2 ^{rs}	93.8±1.49 ^{fg}	0.85±0.02 ^{bcdef}
13	KLD-1	4.34±0.2 ^{ij}	243.33±12.58 ^r	93.09±1.65 ^{gh}	0.87±0.02 ^{abcdef}
14	KLD-2	4.45±0.01 ^{bcdefgh}	451±1 ^{cde}	96.47±0.02 ^{abcde}	0.91±0.01 ^{abcd}
15	POD-1	4.33±0.01 ^{ijk}	446.66±1.52 ^{defg}	97.2±0.07 ^{ab}	0.91±0.01 ^{abcd}
16	POD-2	4.33±0.01 ^{ijk}	440±0.57 ^{efgh}	94.61±0.59 ^{efg}	0.87±0.01 ^{abcdef}
17	TLD-1	4.47±0.01 ^{bcdef}	449±1 ^{def}	95.29±0.02 ^{bcdef}	0.83±0.02 ^{cdef}
18	TLD-2	4.48±0.01 ^{bcdef}	432±2 ^{hi}	94.87±0.02 ^{defg}	0.85±0.02 ^{bcdef}
19	RTD-1	4.38±0.01 ^{efghij}	427.33±2.08 ^{ij}	93.87±0.02 ^{fg}	0.91±0.01 ^{abcd}
20	RTD-2	4.36±0.01 ^{ghij}	485±2 ^{ab}	96.79±0.01 ^{abcd}	0.87±0.02 ^{abcdef}

Table 13: Physicochemical properties of *dahi* samples prepared from cow milk of Sikkim (contd.).

Sl.no.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
21	NTD-1	4.32±0.02 ^{ijk}	432.67±1.15 ^{hi}	94.86±0.01 ^{defg}	0.83±0.02 ^{cdef}
22	BGD-1	4.47±0.01 ^{bcdefg}	428±1 ^{ij}	93.86±0.02 ^{fg}	0.91±0.02 ^{abc}
23	CGD-1	4.49±0.01 ^{bed}	485±2 ^{ab}	96.78±0.02 ^{abcd}	0.86±0.02 ^{bcdef}
24	SBD-1	4.48±0.03 ^{bcdef}	343±1.63 ^p	95.39±0.82 ^{bcdef}	0.93±0.03 ^{ab}
25	SBD-2	4.38±0.06 ^{fghij}	397±1.63 ^{lmn}	96.23±0.59 ^{abcde}	0.86±0.03 ^{bcdef}
26	SBD-3	4.38±0.03 ^{fghij}	478.67±0.94 ^b	98.11±0.18 ^a	0.85±0.04 ^{bcdef}
27	LDD-1	4.35±0.06 ^{hij}	407.33±1.7 ^{klm}	96.44±0.27 ^{abcde}	0.84±0.03 ^{cdef}
28	LDD-2	4.16±0.04 ^{lm}	408.33±0.94 ^{kl}	96.37±0.14 ^{abcde}	0.95±0.03 ^a
29	DSD-1	4.23±0.04 ^{kl}	396±2.16 ^{mn}	97.11±0.27 ^{abc}	0.87±0.05 ^{abcdef}
30	DSD-2	4.38±0.06 ^{efghij}	418.67±1.7 ^{jk}	96.15±0.28 ^{abcde}	0.88±0.04 ^{abcdef}
31	DND-1	4.5±0.02 ^{bc}	487.33±2.52 ^{ab}	96.8±0.03 ^{abcd}	0.88±0.03 ^{abcdef}
32	JRD-1	4.52±0.02 ^b	486.33±3.21 ^{ab}	96.8±0.03 ^{abcd}	0.85±0.04 ^{bcdef}
33	JRD-2	4.49±0.04 ^{bcde}	485.33±1.52 ^{ab}	96.78±0.02 ^{abcd}	0.86±0.03 ^{bcdef}
34	BND-1	4.39±0.04 ^{defghij}	448.67±2.05 ^{defg}	96.95±0.55 ^{abc}	0.89±0.03 ^{abcde}
35	BND-2	4.42±0.03 ^{bcdefghi}	461.67±4.11 ^c	98.01±0.68 ^a	0.85±0.04 ^{bcdef}
36	RVD-1	4.41±0.04 ^{cdefghij}	429.67±1.7 ^{hij}	97.14±0.40 ^{ab}	0.83±0.03 ^{cdef}
37	RVD-2	4.37±0.04 ^{fghij}	432±3.27 ^{hi}	97.71±0.39 ^a	0.86±0.04 ^{bcdef}
38	YGD-1	4.36±0.03 ^{hij}	437±3.74 ^{ghi}	97.15±0.39 ^{ab}	0.86±0.04 ^{bcdef}
39	YGD-2	4.42±0.03 ^{bcdefghij}	454.67±2.62 ^{cd}	97.71±0.56 ^a	0.86±0.04 ^{bcdef}
40	MND-1	4.39±0.03 ^{defghij}	439±1.63 ^{fghi}	96.85±0.78 ^{abcd}	0.82±0.04 ^{def}

Note: All the experiments were conducted in triplicate. Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between *dahi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 14: Physicochemical properties of the *chhurpi* samples prepared from cow milk of Sikkim.

Sl. no.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
1	PKC-1a	5.13±0.02 ^a	165.16±10.12 ^e	67.42±2.2 ^c	0.78±0.03 ^{defghij}
2	PKC-3a	4.96±0.01 ^b	147.85±2.38 ^f	66.29±1.02 ^c	0.63±0.04 ^l
3	RCC-1a	4.05±0.01 ^o	142.33±2.08 ^{fgh}	57.14±0.57 ^{ghi}	0.86±0.01 ^{abcd}
4	SLC-1a	4.17±0.01 ^m	132.67±1.53 ^{ijklm}	56.94±0.03 ^{ghi}	0.83±0.01 ^{bcdefg}
5	GAC-1a	4.29±0.02 ^l	134.67±1.53 ^{ghijklm}	58.55±0.04 ^{fg}	0.85±0.02 ^{abcde}
6	SKC-1a	4.05±0.02 ^o	130.33±1.53 ^{lm}	59.58±0.03 ^{ef}	0.84±0.02 ^{abcdef}
7	GRC-1a	4.77±0.01 ^c	125.67±1.15 ^m	61.68±0.03 ^{de}	0.85±0.02 ^{abcde}
8	SMC-1a	3.93±0.03 ^p	134±1 ^{hijklm}	62.58±0.02 ^d	0.92±0.01 ^a
9	PAC-1a	4.28±0.01 ^l	146.33±1.53 ^f	57.14±0.57 ^{ghi}	0.84±0.01 ^{bcdefg}
10	GCC-1a	4.64±0.02 ^d	143.67±0.58 ^{fg}	55.79±0.59 ⁱ	0.87±0.01 ^{abc}
11	MNDC-1	4.55±0.01 ^{efgh}	175.50±1.41 ^d	55.94±0.35 ^{hi}	0.81±0.02 ^{bcdefghi}
12	MNDC-2	4.54±0.01 ^{fghi}	158.50±2.06 ^e	58.58±0.57 ^{fg}	0.81±0.01 ^{bcdefgh}
13	MNBC-1	4.16±0.01 ^m	135.67±0.53 ^{ghijkl}	57.68±0.81 ^{fghi}	0.88±0.01 ^{ab}
14	MNBC-2	4.15±0.01 ^m	140±1 ^{fghij}	58.58±0.57 ^{fg}	0.85±0.01 ^{abcde}
15	KLC-1	4.96±0.01 ^b	132.67±1.53 ^{ijklm}	58.38±1.07 ^{fg}	0.78±0.03 ^{defghij}
16	KLC-2	4.44±0.01 ^{jk}	134.67±1.53 ^{ghijklm}	55.94±0.35 ^{hi}	0.73±0.02 ^{ijk}
17	POC-1	5.13±0.02 ^a	130.33±1.53 ^{lm}	58.58±0.57 ^{fg}	0.66±0.02 ^{kl}
18	POC-2	4.66±0.02 ^d	135.67±0.53 ^{ghijkl}	57.68±0.81 ^{fghi}	0.76±0.02 ^{fghij}
19	TLC-1	4.49±0.01 ^{ghijk}	140±1 ^{fghij}	58.58±0.57 ^{fg}	0.78±0.02 ^{defghij}
20	TLC-2	4.66±0.01 ^d	132±1 ^{ijklm}	60.94±0.02 ^{de}	0.71±0.03 ^{ijk}

Table 14: Physicochemical properties of the *chhurpi* samples prepared from cow milk of Sikkim (contd.)

Sl. no.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
21	GDC-1	4.95±0.02 ^b	135.33±2.08 ^{ghijkl}	58.01±0.66 ^{fgh}	0.67±0.03 ^{kl}
22	SBC-1	4.43±0.02 ^k	138.85±1.77 ^{fghijkl}	58.24±0.15 ^{fg}	0.76±0.03 ^{ghij}
23	BGC-1	5.12±0.02 ^a	133±2 ^{ijklm}	61.04±0.18 ^{de}	0.78±0.04 ^{efghij}
24	CGC-1	4.64±0.02 ^d	134±3 ^{hijklm}	57.68±0.4 ^{fghi}	0.75±0.04 ^{hijk}
25	KGC-1	4.47±0.02 ^{hijk}	139.58±1.23 ^{fghijk}	58.47±0.29 ^{fg}	0.78±0.02 ^{efghij}
26	LGC-1	4.65±0.03 ^d	133.67±3.05 ^{hijklm}	61.37±0.49 ^{de}	0.79±0.02 ^{cdefghij}
27	NGC-1	4.62±0.02 ^{de}	131.85±1.03 ^{ijklm}	61.04±0.84 ^{de}	0.78±0.03 ^{efghij}
28	LDC-1	4.60±0.03 ^{def}	222.33±3.3 ^b	74.67±1.37 ^a	0.85±0.03 ^{abcdef}
29	LDC-2	4.67±0.04 ^d	291.33±2.87 ^a	68.01±0.73 ^c	0.81±0.03 ^{bcdefghi}
30	DSC-1	4.61±0.03 ^{def}	192.67±3.68 ^c	70.93±1.04 ^b	0.76±0.02 ^{ghij}
31	NKC-1	4.06±0.03 ^{no}	140.67±2.08 ^{fghi}	57.29±0.56 ^{ghi}	0.84±0.03 ^{abcdefg}
32	NBC-1	4.16±0.02 ^m	130.67±2.53 ^{klm}	56.91±0.04 ^{ghi}	0.83±0.01 ^{bcdefg}
33	RVC-1	4.32±0.03 ^l	136.33±1.53 ^{ghijkl}	58.51±0.04 ^{fg}	0.86±0.02 ^{abcde}
34	YGC-1	4.03±0.02 ^o	133.33±2.52 ^{ijklm}	59.58±0.04 ^{ef}	0.85±0.02 ^{abcdef}
35	JRC-1	4.14±0.02 ^{mn}	133±2 ^{ijklm}	56.95±0.04 ^{ghi}	0.85±0.02 ^{abcdef}
36	JRC-2	4.29±0.02 ^l	134.67±1.53 ^{ghijklm}	58.53±0.02 ^{fg}	0.84±0.03 ^{abcdefg}
37	JRC-3	4.47±0.01 ^{ijk}	135±3 ^{ghijkl}	59.56±0.02 ^{ef}	0.83±0.02 ^{bcdefgh}
38	MNC-1	4.62±0.04 ^{de}	131.33±2.05 ^{jklm}	57.05±0.32 ^{ghi}	0.84±0.04 ^{abcdefg}
39	MNC-2	4.56±0.04 ^{efg}	133±3.27 ^{ijklm}	57.99±0.51 ^{fgh}	0.84±0.05 ^{abcdefg}
40	MNC-3	4.52±0.03 ^{ghij}	198±3.56 ^c	57.48±0.55 ^{fghi}	0.84±0.04 ^{abcdefg}

Note: All the experiments were conducted in triplicate. Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between *chhurpi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 15: Physicochemical properties of the *dahi* samples prepared from yak milk of Sikkim.

Sl. No.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
1	KYD-1	4.7±0.02 ^{ab}	392±7.26 ^c	91.97±0.26 ^b	0.91±0.03 ^a
2	CYD-1	4.57±0.04 ^{cd}	450±3.74 ^a	90.87±0.61 ^b	0.89±0.03 ^a
3	UYD-1	4.57±0.05 ^{cd}	416±4.55 ^b	91.20±0.25 ^b	0.92±0.04 ^a
4	UYD-2	4.51±0.02 ^d	420.33±4.11 ^b	91.16±0.12 ^b	0.95±0.02 ^a
5	UYD-3	4.72±0.04 ^a	416.33±4.03 ^b	91.01±0.64 ^b	0.87±0.05 ^a
6	LYD-1	4.60±0.03 ^{bcd}	462±3.74 ^a	94.38±0.82 ^a	0.93±0.02 ^a
7	LYD-2	4.68±0.02 ^{abc}	418.33±2.05 ^b	86.46±0.25 ^c	0.87±0.04 ^a
8	ZYD-1	4.66±0.04 ^{abc}	365.67±3.4 ^d	92.07±0.18 ^b	0.89±0.04 ^a
9	ZYD-2	4.59±0.04 ^{bcd}	428.33±4.92 ^b	92.15±0.69 ^b	0.83±0.03 ^a

Note: All the experiments were conducted in triplicate. Results are expressed as the mean ± SD (n = 3). Significant statistical differences (p < 0.05) between *dahi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 16: Physicochemical properties of the *chhurpi* samples prepared from yak milk of Sikkim.

Sl. No.	Sample Code	Physicochemical properties			
		pH	Apparent Viscosity (cP)	Moisture content (%)	Titrateable Acidity (%)
1	KYC-1	4.9±0.23 bc	136.33±3.68 ^b	68.23±1.34 ^a	0.82±0.03 ^{ab}
2	CYC-1	4.95±0.06 bc	137.33±1.25 ^b	66.52±0.87 ^a	0.83±0.04 ^{ab}
3	UYC-1	4.94±0.07 bc	138±2.16 ^{ab}	58.55±0.42 ^a	0.83±0.03 ^{ab}
4	UYC-2	4.99±0.07 b	132±5.35 ^b	67.60±1.77 ^a	0.77±0.03 ^b
5	UYC-3	4.83±0.05 bc	128.33±2.49 ^b	42.49±25.64 ^a	0.77±0.05 ^b
6	LYC-1	4.66±0.04 c	135±2.94 ^b	66.61±1.08 ^a	0.87±0.04 ^{ab}
7	LYC-2	4.65±0.04 c	137±3.74 ^b	66.48±0.76 ^a	0.91±0.01 ^a
8	ZYC-1	5.62±0.04 a	150±4.55 ^a	66.29±0.78 ^a	0.78±0.03 ^b
9	ZYC-2	5.72±0.05 a	138±3.27 ^{ab}	68.73±0.83 ^a	0.75±0.03 ^b

Note: All the experiments were conducted in triplicate. Results are expressed as the mean ± SD (n = 3). Significant statistical differences (p < 0.05) between *chhurpi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

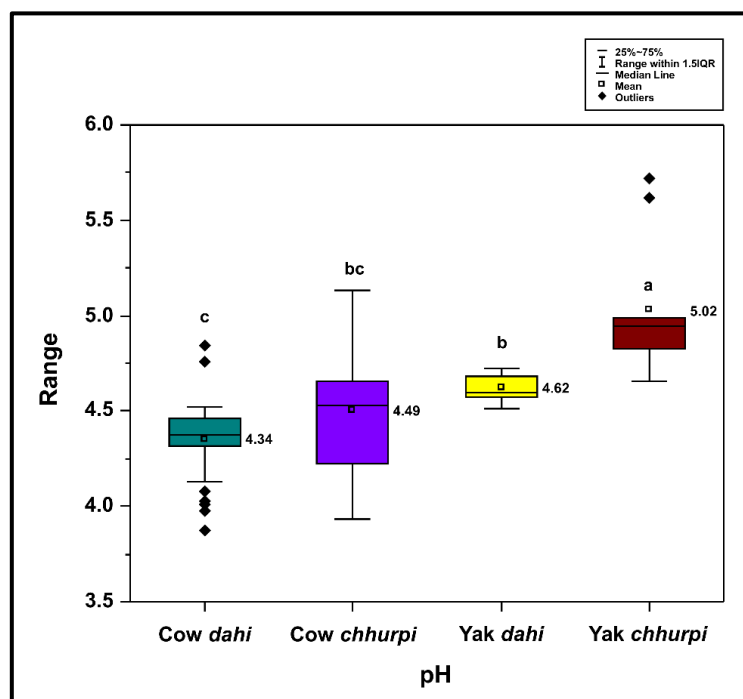


Figure 3: pH of *dahi* and *chhurpi* samples traditionally prepared from cow and yak milk of Sikkim.

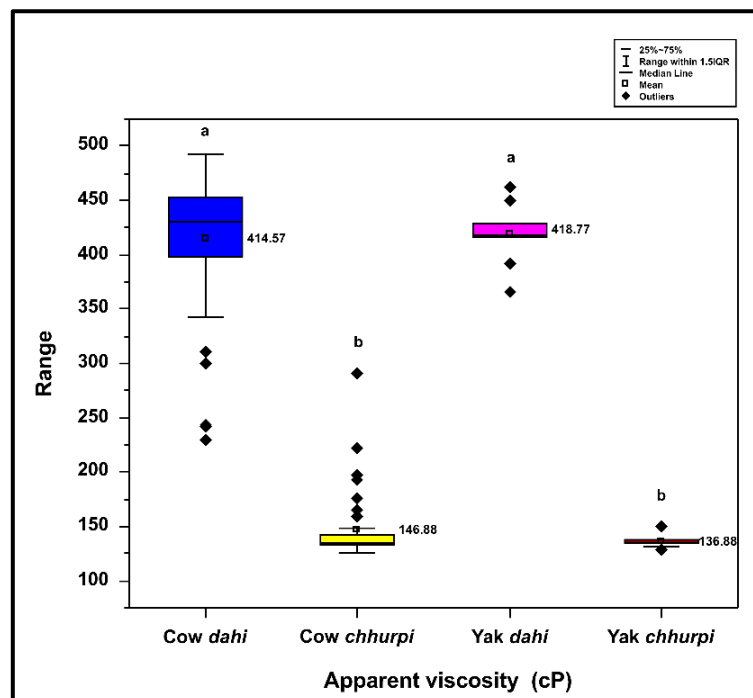


Figure 4: Apparent viscosity of *dahi* and *chhurpi* samples traditionally prepared from cow and yak milk of Sikkim.

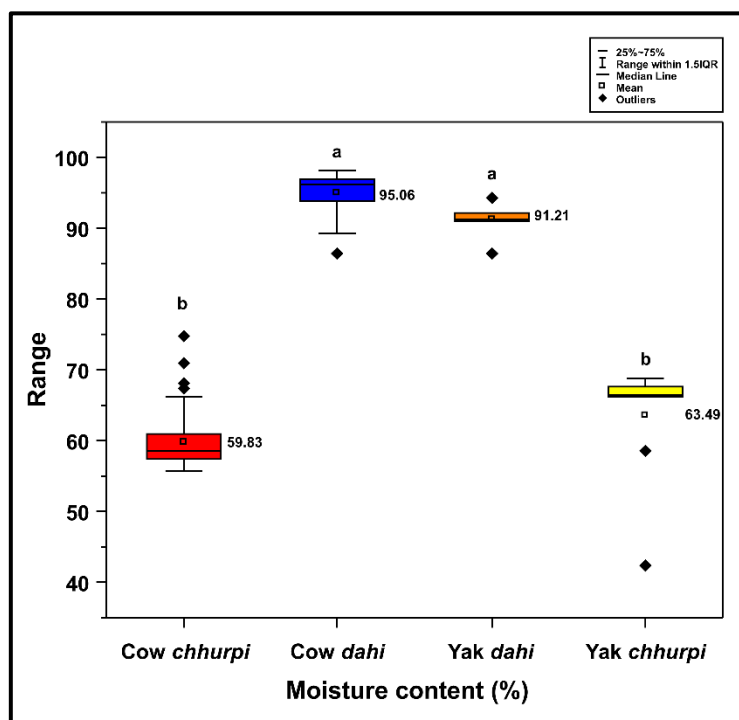


Figure 5: Moisture content of *dahi* and *chhurpi* samples traditionally prepared from cow and yak milk of Sikkim.

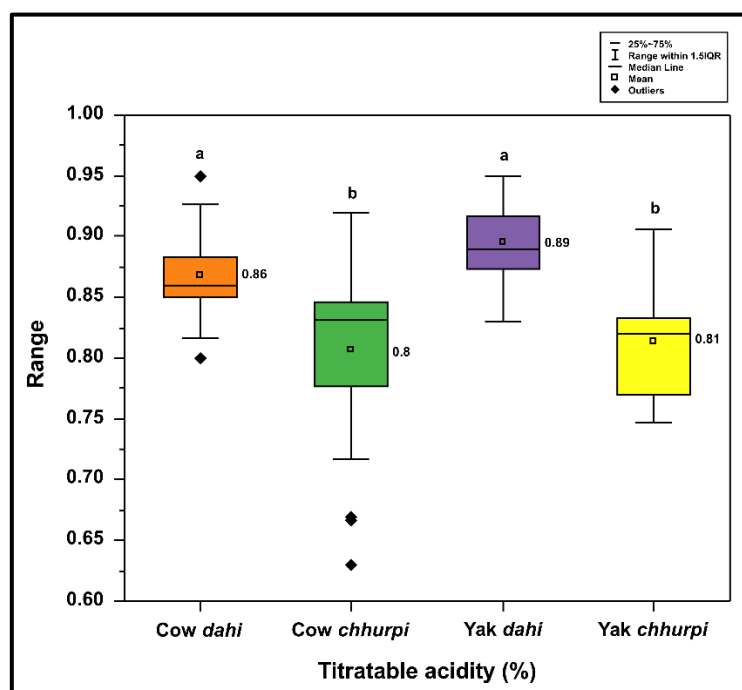


Figure 6: Titratable acidity of *dahi* and *chhurpi* samples traditionally prepared from cow and yak milk of Sikkim.

4.2.3 Microbiological analysis

The microbial load of an NFM product was reported in various culture media such as Yeast Malt Agar (YMA), Potato Dextrose Agar (PDA), Plate Count Agar (PCA), and Nutrient Agar (NA). The Microbial load ($\log \text{CFU g}^{-1}$) reported in YMA, PDA, PCA and NA from cow *dahi* samples ranges from 4.73 ± 0.1 to 7.46 ± 0.02 ; 4.82 ± 0.02 to 7.45 ± 0.05 ; 7.28 ± 0.01 to 9.64 ± 0.02 ; and 5.44 ± 0.04 to 9.64 ± 0.02 , respectively (Table 17). The Microbial load ($\log \text{CFU g}^{-1}$) reported in YMA, PDA, PCA and NA from cow *chhurpi* samples ranges from 5.17 ± 0.02 to 7.48 ± 0.02 ; 4.68 ± 0.07 to 7.37 ± 0.04 ; 6.51 ± 0.03 to 9.22 ± 0.04 ; and 6.78 ± 0.25 to 9.25 ± 0.02 , respectively (Table 18). In yak *dahi*, microbial load ($\log \text{CFU g}^{-1}$) in YMA, PDA, PCA and NA were reported from 4.96 ± 0.08 to 5.82 ± 0.11 ; 4.22 ± 0.09 to 5.51 ± 0.07 ; 5.65 ± 0.11 to 7.45 ± 0.08 ; and 5.06 ± 0.11 to 6.99 ± 0.09 , respectively (Table 19). The Microbial load ($\log \text{CFU g}^{-1}$) reported in YMA, PDA, PCA and NA from yak *chhurpi* samples ranges from 5.06 ± 0.11 to 5.83 ± 0.14 ; 4.69 ± 0.07 to 5.08 ± 0.11 ; 5.65 ± 0.06 to 7.02 ± 0.1 ; and 5.27 ± 0.05 to 6.41 ± 0.11 , respectively (Table 20). Microbial load in *dahi* and *chhurpi* from cow and yak were analysed in Tukey's boxplot analysis (Figure 7).

Table 17: Microbial load of the *dahi* samples prepared from cow milk of Sikkim.

Sl.no.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
1	PKD-1a	5.1±0.05 ^{lm}	6.48±0.02 ^{cd}	8.52±0.03 ^{cdefg}	8.87±0.02 ^{bc}
2	PKD-2a	7.46±0.02 ^a	7.45±0.05 ^a	8.34±0.05 ^{efghi}	8.13±0.02 ^{def}
3	PKD-3a	5.49±0.04 ^{jkl}	5.59±0.01 ^{ijkl}	7.89±0.09 ^{ijklm}	5.44±0.04 ^{efg}
4	GSD-1a	5.74±0.0173 ^{ijk}	6.49±0.05 ^{cd}	8.95±0.026 ^{bc}	8.82±0.11 ^{bc}
5	GID-1a	6.18±0.08 ^{fghi}	6.29±0.17 ^{cdefg}	8.84±0.1 ^{bcd}	8.53±0.03 ^{cde}
6	GAD-1a	4.73±0.1 ^m	4.82±0.02 ⁿ	8.89±0.06 ^{bc}	8.6±0.01 ^{cd}
7	GLD-1a	6.63±0.05 ^{cdefg}	6.77±0.06 ^{bc}	9.64±0.02 ^a	9.64±0.02 ^a
8	GRD-1a	5.4±0.06 ^{kl}	6.28±0.03 ^{cdefg}	8.58±0.02 ^{cdefg}	8.65±0.04 ^c
9	CPD-1a	6.27±0.05 ^{efghi}	6.33±0.12 ^{cdef}	8.17±0.02 ^{fghijk}	8.16±0.01 ^{def}
10	RCD-1a	7.32±0.02 ^{ab}	7.28±0.01 ^{ab}	9.16±0.03 ^b	9.22±0.03 ^{ab}
11	MNBD-1	7.19±0.02 ^{abc}	6.27±0.02 ^{cdefg}	8.58±0.02 ^{cdefg}	8.84±0.1 ^{bc}
12	MNBD-2	6.29±0.04 ^{efghi}	6.81±0.02 ^{bc}	8.17±0.02 ^{fghijk}	8.89±0.06 ^{bc}
13	KLD-1	5.1±0.05 ^{lm}	6.29±0.17 ^{cdefg}	7.28±0.01 ⁿ	8.95±0.026 ^{bc}
14	KLD-2	4.73±0.1 ^m	5.76±0.02 ^{ghijkl}	7.46±0.02 ^{mn}	8.84±0.1 ^{bc}
15	POD-1	6.18±0.08 ^{fghi}	6.18±0.08 ^{defgh}	8.16±0.02 ^{fghijk}	8.52±0.03 ^{cde}
16	POD-2	5.4±0.06 ^{kl}	6.33±0.12 ^{cdef}	7.89±0.09 ^{ijklm}	9.64±0.02 ^a
17	TLD-1	7.46±0.02 ^a	6.52±0.05 ^{cd}	8.94±0.01 ^{bc}	8.87±0.02 ^{bc}
18	TLD-2	7.32±0.02 ^{ab}	6.37±0.08 ^{cde}	8.83±0.01 ^{bcd}	8.77±0.02 ^{bc}
19	RTD-1	7.19±0.02 ^{abc}	4.82±0.02 ⁿ	8.82±0.04 ^{bcd}	7.94±0.02 ^{fgh}
20	RTD-2	6.29±0.04 ^{efghi}	5.87±0.02 ^{efghij}	8.78±0.03 ^{bcde}	7.92±0.03 ^{fgh}

Table 17: Microbial load of the *dahi* samples prepared from cow milk of Sikkim (contd.).

Sl.no.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
21	NTD-1	7.31±0.02 ^{ab}	6.34±0.04 ^{cdef}	8.83±0.02 ^{bcd}	8.76±0.02 ^{bc}
22	BGD-1	7.2±0.02 ^{abc}	4.83±0.02 ⁿ	8.84±0.04 ^{bcd}	7.96±0.01 ^{fgh}
23	CGD-1	6.29±0.05 ^{efghi}	5.84±0.02 ^{efghij}	8.77±0.04 ^{bcde}	7.91±0.02 ^{fgh}
24	SBD-1	6.09±0.19 ^{ghij}	5.52±0.36 ^{ijklm}	7.63±0.38 ^{lmn}	7.27±0.17 ^{jkl}
25	SBD-2	5.9±0.79 ^{hijk}	5±0.41 ^{mn}	7.85±0.28 ^{klmn}	7.08±0.17 ^{kl}
26	SBD-3	6.53±0.39 ^{defgh}	5.89±0.22 ^{efghi}	7.69±0.29 ^{klmn}	7.03±0.28 ^l
27	LDD-1	5.96±0.17 ^{hijk}	5.02±0.12 ^{mn}	8.03±0.18 ^{hijkl}	7.37±0.28 ^{jkl}
28	LDD-2	6.75±0.5 ^{bcdef}	5.3±0.39 ^{klmn}	8.17±0.23 ^{fghij}	7.42±0.38 ^{ijkl}
29	DSD-1	6.82±0.28 ^{bcde}	5.28±0.26 ^{lmn}	8.4±0.33 ^{defgh}	7.74±0.27 ^{fghij}
30	DSD-2	7.02±0.11 ^{abcd}	6.37±0.4 ^{cde}	8.11±0.13 ^{ghijkl}	7.69±0.19 ^{fghij}
31	DND-1	6.33±0.04 ^{efghi}	5.83±0.02 ^{efghijkl}	8.73±0.02 ^{bcde}	7.93±0.02 ^{fgh}
32	JRD-1	6.34±0.02 ^{efghi}	5.8±0.02 ^{fghijkl}	8.76±0.02 ^{bcde}	7.9±0.02 ^{fghi}
33	JRD-2	6.36±0.01 ^{efghi}	5.85±0.03 ^{efghijk}	8.71±0.03 ^{bcde}	7.95±0.03 ^{fgh}
34	BND-1	6.4±0.14 ^{defgh}	5.46±0.2 ^{ijklm}	8.54±0.23 ^{cdefg}	7.33±0.23 ^{jkl}
35	BND-2	6.17±0.06 ^{fghi}	5.42±0.25 ^{ijklm}	8.56±0.16 ^{cdefg}	7.41±0.23 ^{jkl}
36	RVD-1	6.46±0.09 ^{defgh}	5.52±0.13 ^{ijklm}	8.34±0.23 ^{efghi}	7.49±0.23 ^{hijkl}
37	RVD-2	6.46±0.2 ^{defgh}	5.45±0.22 ^{ijklm}	8.63±0.17 ^{cdef}	7.49±0.25 ^{hijkl}
38	YGD-1	6.41±0.28 ^{defgh}	5.64±0.22 ^{hijkl}	8.49±0.18 ^{cdefgh}	7.62±0.21 ^{ghij}
39	YGD-2	6.67±0.11 ^{cdefg}	5.47±0.21 ^{ijklm}	8.52±0.27 ^{cdefg}	7.54±0.19 ^{hijk}
40	MND-1	6.49±0.23 ^{defgh}	5.32±0.19 ^{jklmn}	8.58±0.18 ^{cdefg}	7.48±0.23 ^{hijkl}

Note: All the experiments were conducted in triplicate. Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between *dahi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 18: Microbial load of the *chhurpi* samples prepared from cow milk of Sikkim.

Sl. No.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
1	PKC-1a	5.90±0.01 ^{lmn}	6.53±0.02 ^b	7.29±0.02 ^g	8.54±0.02 ^e
2	PKC-3a	6.27±0.07 ^{ijk}	6.14±0.02 ^c	8.14±0.03 ^{cd}	8.01±0.08 ^{gh}
3	RCC-1a	6.65±0.03 ^{fg}	6.70±0.07 ^b	8.71±0.03 ^b	8.74±0.03 ^{bcde}
4	SLC-1a	7.48±0.02 ^a	7.37±0.04 ^a	9.19±0.03 ^a	9.22±0.02 ^a
5	GAC-1a	7.36±0.02 ^{ab}	7.22±0.03 ^a	8.15±0.03 ^{cd}	8.24±0.02 ^{fg}
6	SKC-1a	6.39±0.01 ^{hi}	6.16±0.02 ^c	8.86±0.02 ^b	8.74±0.03 ^{bcde}
7	GRC-1a	5.56±0.01 ^{opq}	5.67±0.02 ^{de}	7.16±0.01 ^g	9.22±0.02 ^a
8	SMC-1a	6.14±0.03 ^{jkl}	6.17±0.01 ^c	8.15±0.03 ^{cd}	8.24±0.02 ^{fg}
9	PAC-1a	5.17±0.02 ^s	5.17±0.02 ^f	8.14±0.03 ^{cd}	7.9±0.01 ^{hi}
10	GCC-1a	7.18±0.01 ^{bc}	5.58±0.03 ^{de}	7.91±0.09 ^{ef}	7.83±0.01 ^{hij}
11	MNDC-1	5.57±0.02 ^{opq}	5.61±0.02 ^{de}	8.71±0.03 ^b	8.71±0.03 ^{cde}
12	MNDC-2	5.69±0.17 ^{nop}	5.58±0.02 ^{de}	9.19±0.03 ^a	9.19±0.03 ^a
13	MNBC-1	6.11±0.02 ^{kl}	5.71±0.02 ^{de}	8.15±0.03 ^{cd}	8.15±0.03 ^{fg}
14	MNBC-2	5.27±0.02 ^{rs}	5.47±0.02 ^e	8.86±0.02 ^b	8.86±0.02 ^{bcd}
15	KLC-1	6.48±0.02 ^{ghi}	5.74±0.0173 ^d	8.01±0.08 ^{de}	7.29±0.02 ^k
16	KLC-2	5.58±0.02 ^{opq}	6.18±0.08 ^c	8.71±0.03 ^b	8.71±0.03 ^{cde}
17	POC-1	5.71±0.02 ^{nop}	4.73±0.1 ^{gh}	9.19±0.03 ^a	9.19±0.03 ^a
18	POC-2	5.47±0.02 ^{pqr}	6.63±0.05 ^b	8.15±0.03 ^{cd}	7.89±0.09 ^{hi}
19	TLC-1	6.27±0.05 ^{ijk}	5.58±0.02 ^{de}	8.71±0.03 ^b	8.94±0.01 ^b
20	TLC-2	6.65±0.01 ^{fg}	6.16±0.02 ^c	6.48±0.02 ^h	8.71±0.03 ^{cde}

Table 18: Microbial load of the *chhurpi* samples prepared from cow milk of Sikkim (contd.).

Sl. No.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
21	GDC-1	5.74±0.04 ^{mno}	4.72±0.1 ^h	7.97±0.02 ^{de}	7.18±0.03 ^k
22	SBC-1	5.45±0.02 ^{qr}	6.65±0.04 ^b	8.74±0.03 ^b	8.68±0.03 ^{de}
23	BGC-1	6.32±0.05 ^{ijk}	5.57±0.03 ^{de}	9.16±0.02 ^a	8.82±0.04 ^{bcd}
24	CGC-1	6.66±0.02 ^{efg}	6.19±0.02 ^c	8.13±0.02 ^{cde}	7.83±0.02 ^{hij}
25	KGC-1	5.57±0.02 ^{qr}	4.68±0.07 ^h	8.68±0.03 ^b	8.92±0.03 ^{bc}
26	LGC-1	5.44±0.03 ^{qr}	5.04±0.17 ^f	6.51±0.03 ^h	6.78±0.25 ^l
27	NGC-1	6.36±0.03 ^{hij}	5.54±0.03 ^{de}	8.65±0.04 ^b	7.74±0.04 ^{ij}
28	LDC-1	6.36±0.2 ^{ij}	5.58±0.18 ^{de}	8.15±0.28 ^{cd}	7.9±0.13 ^{hi}
29	LDC-2	6.6±0.23 ^{fgh}	5.04±0.23 ^f	7.98±0.13 ^{de}	7.66±0.21 ^j
30	DSC-1	5.98±0.17 ^{lm}	4.99±0.21 ^{fg}	7.71±0.17 ^f	7.23±0.12 ^k
31	NKC-1	6.68±0.02 ^{efg}	6.65±0.04 ^b	8.73±0.02 ^b	8.71±0.03 ^{cde}
32	NBC-1	7.46±0.03 ^a	7.34±0.03 ^a	9.22±0.04 ^a	9.24±0.02 ^a
33	RVC-1	7.37±0.02 ^{ab}	7.19±0.04 ^a	8.13±0.02 ^{cde}	8.27±0.02 ^f
34	YGC-1	6.36±0.04 ^{hij}	6.14±0.3 ^c	8.83±0.02 ^b	8.71±0.03 ^{cde}
35	JRC-1	7.43±0.03 ^a	7.36±0.02 ^a	9.22±0.05 ^a	9.25±0.02 ^a
36	JRC-2	7.36±0.03 ^{ab}	7.21±0.02 ^a	8.14±0.02 ^{cd}	8.24±0.03 ^{fg}
37	JRC-3	6.36±0.01 ^{hi}	6.12±0.2 ^c	8.86±0.03 ^b	8.74±0.04 ^{bcde}
38	MNC-1	6.89±0.12 ^{de}	5.52±0.1 ^{de}	8.25±0.08 ^c	7.76±0.1 ^{ij}
39	MNC-2	7.07±0.13 ^{cd}	5.75±0.07 ^d	7.92±0.04 ^{ef}	7.77±0.1 ^{ij}
40	MNC-3	6.83±0.06 ^{ef}	5.61±0.13 ^{de}	7.95±0.07 ^{de}	7.12±0.1 ^k

Note: All the experiments were conducted in triplicate. Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between *chhurpi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 19: Microbial load of the *dahi* samples prepared from yak milk of Sikkim.

Sl. No.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
1	KYD-1	5.21±0.09 ^b	5.31±0.15 ^{ab}	6.83±0.15 ^b	5.92±0.05 ^{cd}
2	CYD-1	5.80±0.12 ^a	5.51±0.07 ^a	7.45±0.08 ^a	6.99±0.09 ^a
3	UYD-1	5.70±0.15 ^a	4.99±0.09 ^{bc}	7.09±0.09 ^{ab}	6.26±0.17 ^b
4	UYD-2	4.96±0.08 ^b	4.22±0.09 ^d	5.92±0.13 ^{cd}	5.47±0.14 ^d
5	UYD-3	5.08±0.13 ^b	4.89±0.09 ^c	5.65±0.11 ^d	5.06±0.11 ^e
6	LYD-1	5.67±0.16 ^a	5.01±0.08 ^{bc}	5.9±0.1 ^{cd}	5.66±0.04 ^{cd}
7	LYD-2	5.82±0.11 ^a	5.1±0.09 ^{abc}	5.92±0.1 ^{cd}	5.72±0.05 ^{cd}
8	ZYD-1	5.69±0.06 ^a	5.16±0.04 ^{abc}	6.12±0.06 ^c	5.83±0.05 ^c
9	ZYD-2	5.68±0.08 ^a	5.09±0.1 ^{abc}	6.08±0.09 ^c	5.76±0.09 ^{cd}

Note: All the experiments were conducted in triplicate. Results are expressed as the mean ± SD (n = 3). Significant statistical differences (p < 0.05) between *dahi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

Table 20: Microbial load of the *chhurpi* samples prepared from yak milk of Sikkim.

Sl. No.	Sample Code	Microbial load			
		Yeast Malt Agar (Log CFU g ⁻¹)	Potato Dextrose Agar (Log CFU g ⁻¹)	Plate Count Agar (Log CFU g ⁻¹)	Nutrient Agar (Log CFU g ⁻¹)
1	KYC-1	5.66±0.11 ^{ab}	5.08±0.11 ^a	7.02±0.1 ^a	6.41±0.12 ^{ab}
2	CYC-1	5.65±0.11 ^{ab}	5.08±0.14 ^a	6.88±0.08 ^a	6.41±0.11 ^a
3	UYC-1	5.76±0.1 ^a	4.69±0.07 ^a	6.18±0.13 ^b	5.8±0.1 ^c
4	UYC-2	5.06±0.11 ^c	4.73±0.04 ^a	5.76±0.07 ^{cd}	5.27±0.05 ^c
5	UYC-3	5.61±0.11 ^{ab}	5.07±0.08 ^b	6.01±0.09 ^{bc}	5.96±0.05 ^b
6	LYC-1	5.83±0.14 ^a	4.70±0.13 ^a	5.89±0.09 ^{bcd}	5.78±0.09 ^b
7	LYC-2	5.28±0.14 ^{bc}	4.75±0.06 ^a	5.84±0.05 ^{cd}	5.64±0.07 ^b
8	ZYC-1	5.64±0.07 ^{ab}	4.93±0.04 ^a	5.95±0.06 ^{bc}	5.72±0.05 ^b
9	ZYC-2	5.28±0.1 ^{bc}	4.76±0.09 ^a	5.65±0.06 ^d	5.71±0.15 ^b

Note: All the experiments were conducted in triplicate. Results are expressed as the mean ± SD (n = 3). Significant statistical differences (p < 0.05) between *chhurpi* samples according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

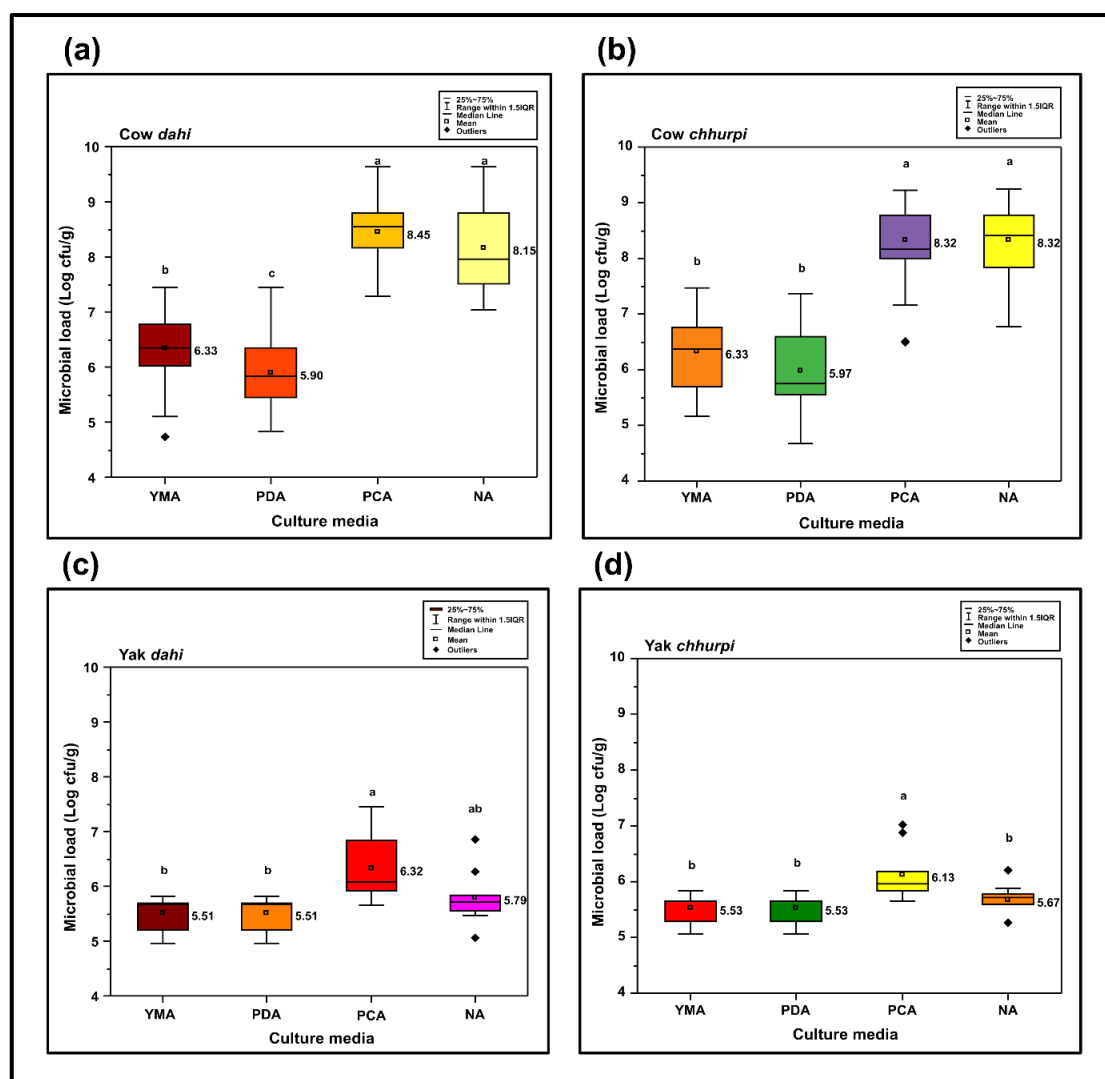


Figure 7: Microbial load of *dahi* and *chhurpi* samples traditionally prepared from cow and yak milk of Sikkim.

4.2.4 Yeast isolation

A total of 3438 yeast from cow milk products (cow *dahi*- 1779 and cow *chhurpi*- 1659) and 850 yeast from yak milk products (yak *dahi*- 460 and yak *chhurpi*- 390) were isolated (Table 21-22) and confirmed in phase contrast microscopy.

4.2.5 Preliminary screening for probiotic potential

Isolated yeast was processed for preliminary screening based on low pH tolerance (pH 2.0), bile tolerance (0.3% w/v), and Microbial attachment to hydrocarbons (MATHs) ($\geq$

80%). Growth at pH 2.0 and 0.3% of bile was analysed by measuring the difference in optical density of $\Delta OD_{600} \geq 0.5$ at 0 h and 24 h. Initially, 20 yeast from cow milk products (cow *dahi*-8 and cow *chhurpi*-12) (Table 21) and 15 yeast from yak milk products (yak *dahi*- 9, yak *chhurpi*- 6) were screened (Table 22) for identification and further analysis.

Table 21: Isolation details and selection of the potential yeast isolates from cow *dahi* and *chhurpi* samples of Sikkim.

Sample	No. of Samples	No. of Isolates	Low pH (pH 2.0) and Bile tolerance (0.3% w/v)	Microbial attachment to hydrocarbons ($\geq 80\%$)
Cow <i>Dahi</i>	40	1779	54	8
Cow <i>Chhurpi</i>	40	1659	61	12
Total	80	3438	115	20

Table 22: Isolation details and selection of the potential yeast isolates from yak *dahi* and *chhurpi* samples of Sikkim.

Sample	No. of Samples	No. of Isolates	Low pH (pH 2.0) and Bile tolerance (0.3% w/v)	Microbial attachment to hydrocarbons ($\geq 80\%$)
Yak <i>Dahi</i>	9	460	11	9
Yak <i>Chhurpi</i>	9	390	14	6
Total	18	850	25	15

4.2.6 Identification of the isolates

4.2.6.1 Phenotypic and biochemical characterization

Screened isolates were processed for phenotypic and biochemical characterization and isolates were tentatively identified and grouped up to genus level as *Saccharomyces* (Cow *dahi*- 6, cow *chhurpi*- 3, yak *dahi*- 4 and yak *chhurpi*- 0); *Yarrowia* (Cow *dahi*- 0,

cow *chhurpi*- 1, yak *dahi*- 3 and yak *chhurpi*- 2); *Kluyveromyces* (Cow *dahi*- 2, cow *chhurpi*- 0, yak *dahi*- 0 and yak *chhurpi*- 3); *Pichia* (Cow *dahi*- 0, cow *chhurpi*- 3, yak *dahi*- 0 and yak *chhurpi*- 0); *Candida* (Cow *dahi*- 0, cow *chhurpi*- 1, yak *dahi*- 1 and yak *chhurpi*- 1); *Debaryomyces* (Cow *dahi*- 0, cow *chhurpi*- 2, yak *dahi*- 0 and yak *chhurpi*- 0); *Kazachstania* (Cow *dahi*- 0, cow *chhurpi*- 2, yak *dahi*- 0 and yak *chhurpi*- 0); and *Wickerhamomyces* (Cow *dahi*- 0, cow *chhurpi*- 0, yak *dahi*- 1 and yak *chhurpi*- 0) (Table 23).

Table 23: Tentative identification and grouping of the isolates based on phenotypic and biochemical characterizations.

Characteristics		<i>Saccharomyces</i> sp.	<i>Pichia</i> sp.	<i>Kluyveromyces</i> sp.	<i>Debaryomyces</i> sp.	<i>Kazachstania</i> sp.	<i>Yarrowia</i> sp.	<i>Wickerhamomyces</i> sp.	<i>Candida</i> sp.
Colony morphology	Size	Large	Large	Large	Moderate	Small	Moderate	Moderate	Moderate
	Form	Round	Round	Round	Round	Round	Round	Round	Round
	Margin	Entire	Entire	Entire	Entire	Lobate	Undulate	Entire	Entire
	Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised
	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Appearance	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white	Off-white
	Optical activity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Cell morphology		Oval, budding	Oval to cylindrical, budding	Ovoid, budding	Ovoid or short ellipsoidal, budding	Oval, budding	Ellipsoidal, budding	Ellipsoidal, budding	Oval, budding
Flocculation		+	+	+	+	+	+	+	+
Nitrate reduction test		-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve
H ₂ S production test		+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve
Carbohydrate fermentation	Lactose	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
	Sucrose	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
	Maltose	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
	Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	Raffinose	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve
	Rhamnose	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
	Mannose	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve

	Ribose	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
	Fructose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	Xylose	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
	Trehalose	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve
	L-arabinose	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve
Representatives	Cow <i>dahi</i>	6	0	2	0	0	0	0	0
	Cow <i>chhurpi</i>	3	3	0	2	2	1	0	1
	Yak <i>dahi</i>	4	0	0	0	0	3	1	1
	Yak <i>chhurpi</i>	0	0	3	0	0	2	0	1

4.2.6.2 Molecular identification based on sequencing D1/D2 domain of large ribosomal subunit

Preliminarily identified 35 yeast isolates comprising of major genera *Saccharomyces*, *Yarrowia*, *Kluyveromyces*, *Pichia*, *Candida*, *Debaryomyces*, *Kazachstania*, and *Wickerhamomyces* were further identified by genotypic methods. Yeast was identified genotypically by sequencing the D1/D2 domain of a large ribosomal subunit using NL1 and NL4 primers and validated in 1% agarose gel electrophoresis (Figure 8). For identification, sequences were aligned using BLAST against the sequences available in the NCBI GenBank database. Sequences were submitted to NCBI GenBank for accession number (Table 24-25) and phylogenetic tree were constructed using the Neighbor-joining method based on Kimura-2 parameter with a bootstrap of 1000 replicates using MEGA v11.0.13 (Figure 9).

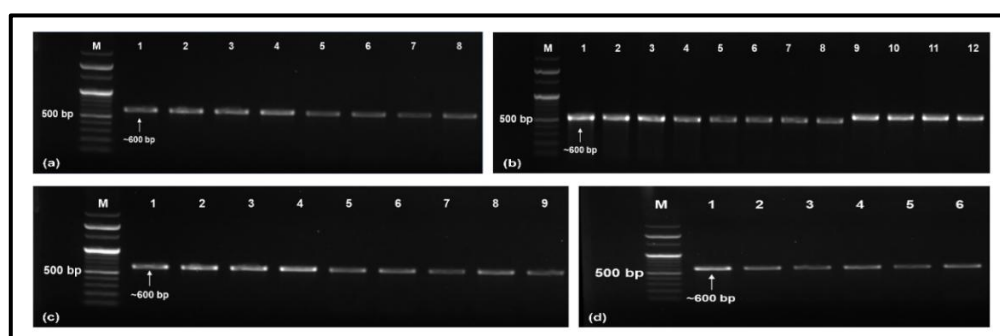


Figure 8: Confirmation of the isolates by sequencing the D1/D2 domain of the large ribosomal subunit (~600 bp) from (a) cow *dahi* (8 isolates), (b) cow *chhurpi* (12 isolates), (c) yak *dahi* (9 isolates), and (d) yak *chhurpi* (6 isolates). The size of the amplicon was confirmed by 1% agarose gel electrophoresis using a 100 bp DNA ladder (MBT049, HiMedia, India), and the gel was visualized in a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

Table 24: Molecular identification of yeasts isolates isolated from cow *dahi* and *chhurpi* of Sikkim, based on sequencing of the D1/D2 domain of a large ribosomal subunit.

Product	Isolate Code	Identification	Type species	Sequence length (bp)	Similarity (%)	GenBank Accession number
Cow <i>dahi</i>	DAO-17	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	535	99.07	OP071251
	DJT-2	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	604	99.83	OP071252
	DKL-13	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	604	99.83	OP071253
	DMS-15	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	578	100	OP071254
	DPA-41	<i>Kluyveromyces marxianus</i>	NRRL Y-8281	556	99.82	OP071255
	DRC-41	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	584	99.64	OP071256
	DRC-42	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	500	99.80	OP071257
	DTL-6	<i>Kluyveromyces lactis</i>	NRRL Y-8279	511	100	OP071258
Cow <i>chhurpi</i>	CPA-55	<i>Debaryomyces prosopidis</i>	JCM 9913	550	100	OP479882
	CCG-4	<i>Debaryomyces prosopidis</i>	JCM 9913	568	100	OP479875
	CGI-29	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	530	99.43	OP479876
	CKL-10	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	495	99.21	OP479877
	CMB-19	<i>Kazachstania exigua</i>	CBS 379	519	99.81	OP479878
	CMN-3	<i>Meyerozyma guilliermondii</i>	NRRL Y-2075	442	99.73	OP479879
	CMNB-4	<i>Kazachstania exigua</i>	CBS 379	558	99.64	OP479880

CMS-3	<i>Pichia bruneiensis</i>	CBS 12611	527	99.05	OP479881
CPAL-16	<i>Pichia bruneiensis</i>	CBS 12611	567	99.01	OP479883
CRC-35	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	557	99.12	OP479884
CBG-9	<i>Yarrowia lipolytica</i>	NRRL YB-423	507	99.06	OP479874
CNT-3	<i>Pichia kudriavzevii</i>	NRRL Y-5396	529	100	OP561448

bp- base pair

Table 25: Molecular identification of yeasts isolates isolated from yak *dahi* and *chhurpi* of Sikkim, based on sequencing of the D1/D2 domain of a large ribosomal subunit.

Product	Isolate code	Identification	Type species	Sequence length (bp)	Similarity (%)	GenBank Accession Number
Yak <i>dahi</i>	DKY-22	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	573	98.60	OR681920
	DCY-27	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	563	98.05	OR681921
	DUAY-11	<i>Yarrowia lipolytica</i>	NRRL YB-423	550	99.43	OR681922
	DUAY-53	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	446	99.10	OR681923
	DUBY-47	<i>Saccharomyces cerevisiae</i>	NRRL Y-12632	432	98	OR682643
	DUCY-6	<i>Yarrowia lipolytica</i>	NRRL YB-423	479	99.38	OR681924
	DUCY-48	<i>Wickerhamomyces anomalus</i>	NRRL Y-366	584	98.64	OR681925
	DLAY-48	<i>Candida parapsilosis</i>	ATCC 22019	520	98.46	OR681926
Yak <i>chhurpi</i>	DZBY-37	<i>Yarrowia lipolytica</i>	NRRL YB-423	494	99.19	OR681927
	CKY-12	<i>Candida metapsilosis</i>	ATCC 96144	502	100	OR681928
	CLAY-2	<i>Yarrowia lipolytica</i>	NRRL YB-423	492	98.58	OR682644
	CLBY-16	<i>Kluyveromyces lactis</i>	NRRL Y-8279	549	100	OR681929
	CZAY-19	<i>Kluyveromyces marxianus</i>	NRRL Y-8281	547	99.63	OR681930
	CZBY-49	<i>Yarrowia lipolytica</i>	NRRL YB-423	452	98.64	OR681931
	CUBY-19	<i>Kluyveromyces lactis</i>	NRRL Y-8279	573	98.90	OR681932

bp- base pair

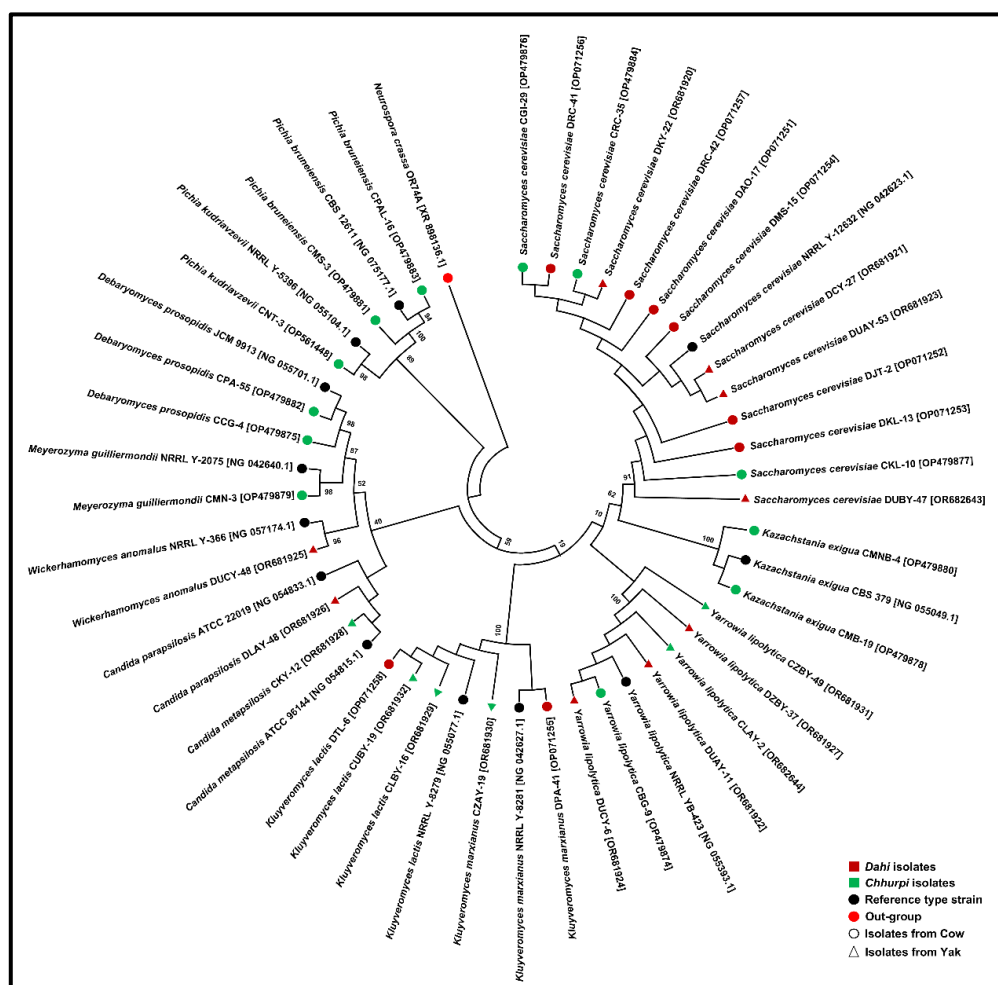


Figure 9: Molecular phylogenetic analysis of 35 yeast isolates from NFM products of Sikkim by sequencing the D1/D2 domain of the large ribosomal subunit. The evolutionary history was derived using the Neighbor-Joining method and the Kimura-2 parameter with 1000 replicates of the bootstrap value. Molecular Evolutionary Genetics Analysis (MEGA) version 11.0.13 was used for the evolutionary studies. Isolates depicted in maroon are *dahi* isolates, in green they are *chhurpi* isolates, in black they represent reference-type isolates, and in red are an out-group. The circle and triangle indicate that the isolates are from cow and yak milk products, respectively. *Neurospora crassa* OR74A [XR 898136.1] served as an outgroup for molecular phylogenetic tree construction.

4.2.7 Relative abundances of the identified yeast isolates

Saccharomyces cerevisiae (75%) was the dominant species in cow *dahi* followed by *Kluyveromyces marxianus* (13%) and *Kluyveromyces lactis* (12%) (Figure 10). Most diverse yeast species were identified from cow *chhurpi*; *Saccharomyces cerevisiae* (25%) was the dominant species followed by *Debaryomyces prosopidis* (17%), *Kazachstania exigua* (17%), *Pichia bruneiensis* (17%), *Meyerozyma guilliermondii* (8%), *Yarrowia lipolytica* (8%), and *Pichia kudriavzevii* (8%) (Figure 11). In yak *dahi*, *Saccharomyces cerevisiae* (45%) was identified as dominant species followed by *Yarrowia lipolytica* (33%), *Wickerhamomyces anomalus* (11%), and *Candida parapsilosis* (11%) (Figure 12). In yak *chhurpi*, *Yarrowia lipolytica* (33%) was the dominant species followed by *Kluyveromyces lactis* (33%), *Kluyveromyces marxianus* (17%), and *Candida metapsilosis* (17%) (Figure 13). iGraph-R-package were used to prepare a simple network analysis presenting distribution of the identified yeast species in *dahi* and *chhurpi* samples from cow and yak (Figure 14).

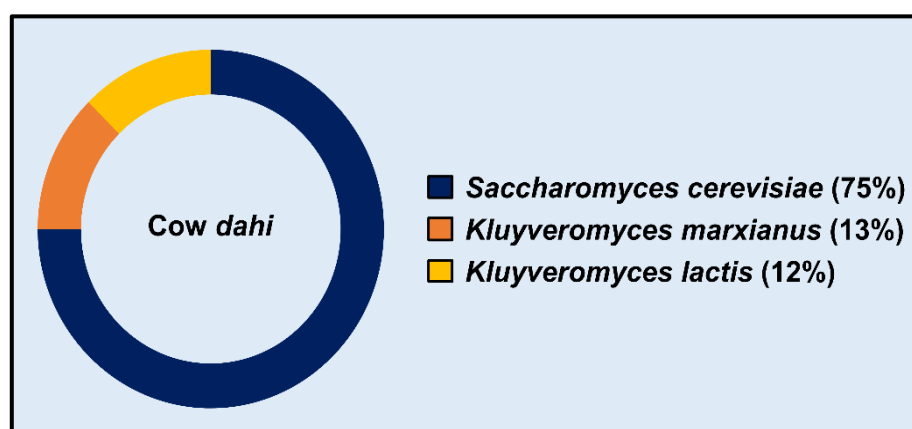


Figure 10: Doughnut-chart represents the yeast diversity identified in cow *dahi* by sequencing the D1/D2 domain of a large ribosomal subunit.

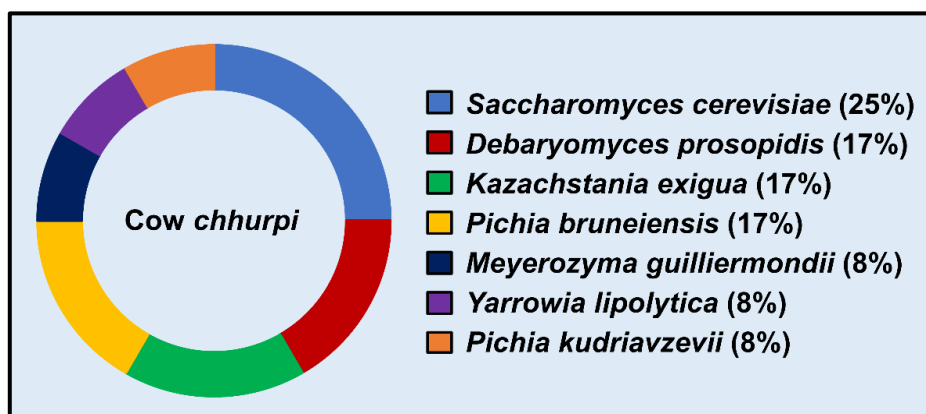


Figure 11: Doughnut-chart represents the yeast diversity identified in cow *chhurpi* by sequencing the D1/D2 domain of a large ribosomal subunit.

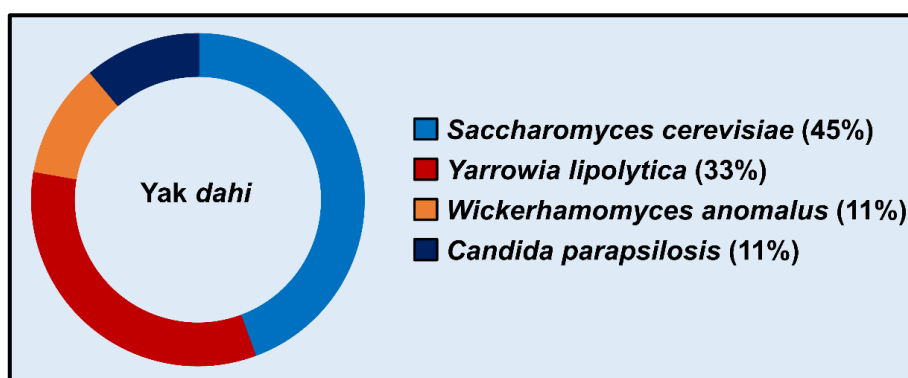


Figure 12: Doughnut-chart represents the yeast diversity identified in yak *dahi* by sequencing the D1/D2 domain of a large ribosomal subunit.

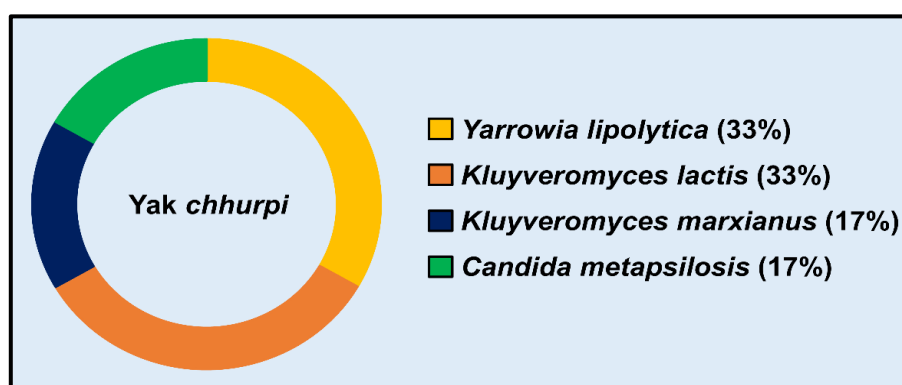


Figure 13: Doughnut-chart represents the yeast diversity identified in yak *chhurpi* by sequencing the D1/D2 domain of a large ribosomal subunit.

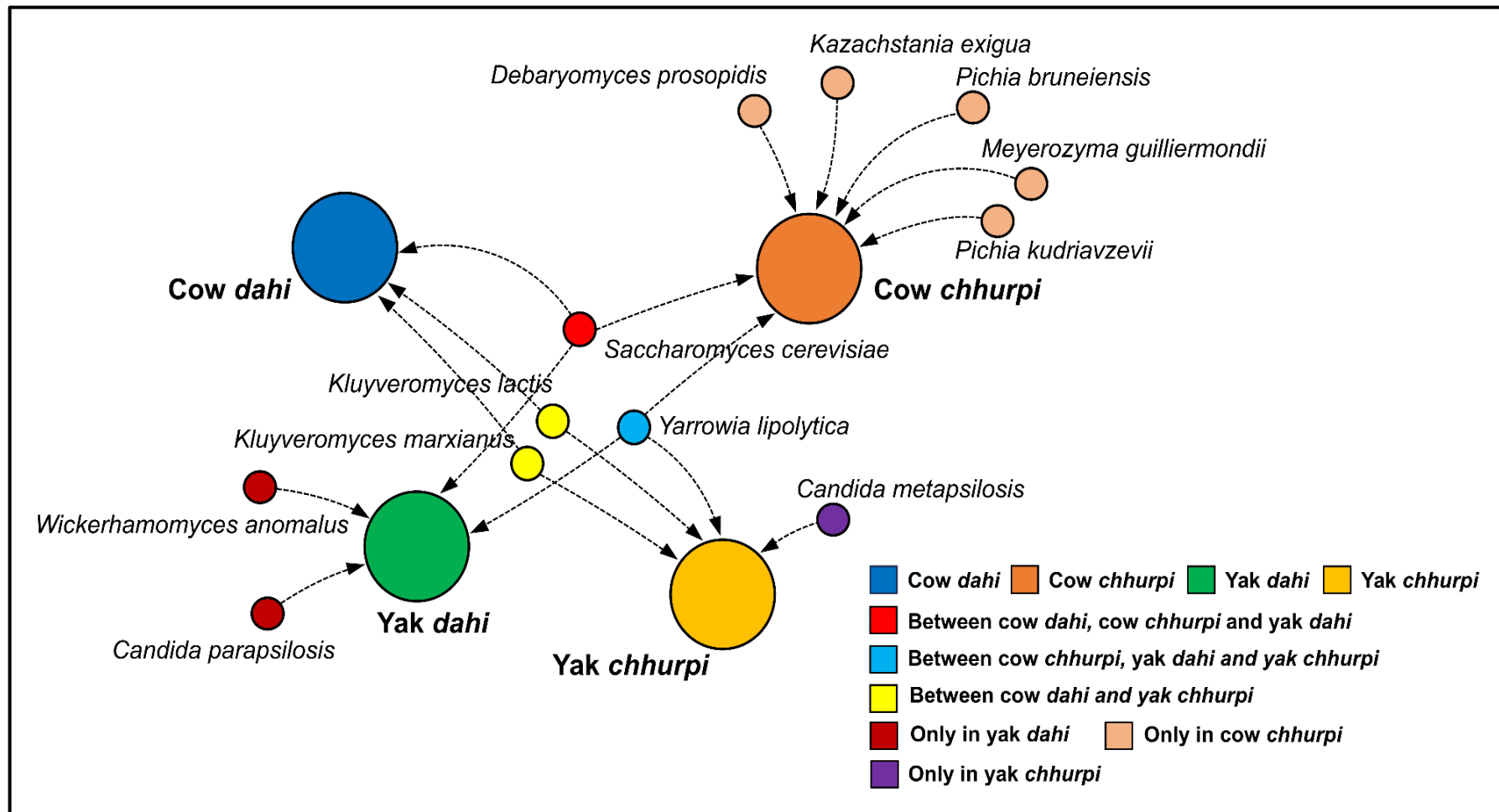


Figure 14: Simple network analysis illustrates the common and distinct yeast isolates among NFM products using the iGraph-R-package.

4.2.7.1 Alpha diversities

Cow *chhurpi* showed the highest diversity indices, 1.85 for Shannon's diversity index (H) and 0.83 for Simpson's diversity index (1-D); followed by yak *chhurpi* (Shannon's diversity index (H)- 1.33 and Simpson's diversity index (1-D)- 0.72), yak *dahi* (Shannon's diversity index (H)- 1.21 and Simpson's diversity index (1-D)- 0.66) and cow *dahi* (Shannon's diversity index (H)- 0.73 and Simpson's diversity index (1-D)- 0.40) (Table 26).

Table 26: Frequency and species diversity indices of yeast species isolated from the exotic NFM products of Sikkim.

Yeast	Cow		Yak	
	<i>Dahi</i> (%)	<i>Chhurpi</i> (%)	<i>Dahi</i> (%)	<i>Chhurpi</i> (%)
<i>Saccharomyces cerevisiae</i>	75	25	45	0
<i>Kluyveromyces marxianus</i>	12	0	0	17
<i>Kluyveromyces lactis</i>	13	0	0	33
<i>Debaryomyces prosopidis</i>	0	17	0	0
<i>Kazachstania exigua</i>	0	17	0	0
<i>Pichia bruneiensis</i>	0	17	0	0
<i>Meyerozyma guilliermondii</i>	0	8	0	0
<i>Yarrowia lipolytica</i>	0	8	33	33
<i>Pichia kudriavzevii</i>	0	8	0	0
<i>Wickerhamomyces anomalus</i>	0	0	11	0
<i>Candida parapsilosis</i>	0	0	11	0
<i>Candida metapsilosis</i>	0	0	0	17
Species diversity indices				
Simpson's diversity index (1-D)	0.40	0.83	0.66	0.72
Shannon's diversity index (H)	0.73	1.85	1.21	1.33

Objective 2:

To screen the probable probiotic yeasts isolated from *dahi* and *chhurpi*.

4.3 SCREENING FOR POTENTIAL PROBIOTIC CANDIDATE BY *IN VITRO* METHODS

Preliminarily screened isolates from NFM products were further screened for potential probiotic candidate on the basis of standard protocol and analysis.

4.3.1 Low pH tolerance

Tolerance to low pH (pH 2.0) is an essential criterion for a yeast to be screened as a potential probiotic candidate, as it determines their ability to survive passage through the highly acidic environment of the stomach. In this study, isolates were subjected to pH 2.0, and their survival was assessed by measuring the change in optical density (ΔOD_{600}), and only those isolates were selected that showed $\Delta OD_{600} \geq 0.5$ at 0 h and 24 h. *Saccharomyces cerevisiae* DAO-17 from cow *dahi* and *Pichia kudriavzevii* CNT-3 from cow *chhurpi* showed the highest tolerance with ΔOD_{600} of 0.79 ± 0.012 and 0.62 ± 0.02 , respectively (Figure 15). From yak *dahi*, *S. cerevisiae* DCY-27 (0.59 ± 0.012) and *Yarrowia lipolytica* CZBY-49 (0.57 ± 0.01) from yak *chhurpi* showed the highest tolerance to pH 2.0 (Figure 16).

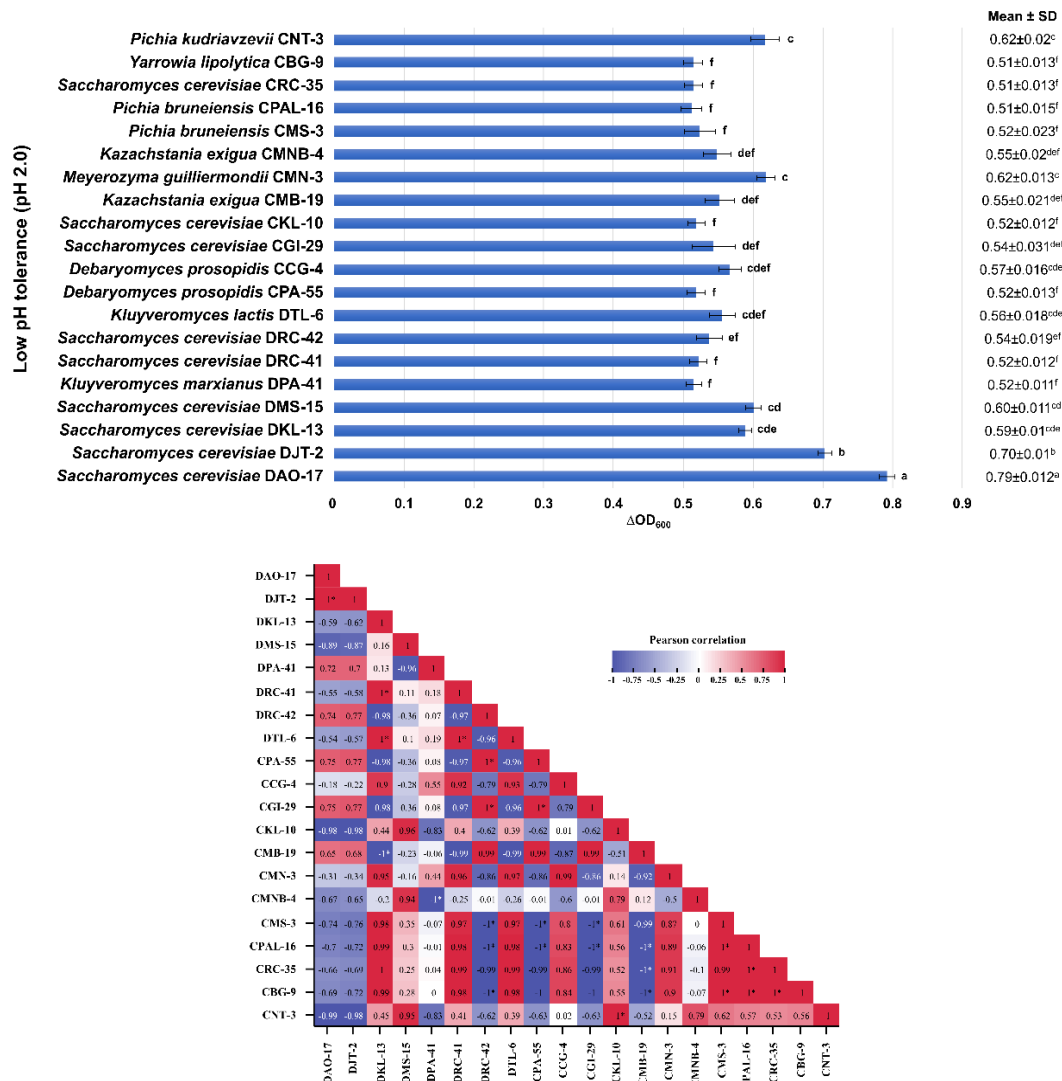


Figure 15: Low pH tolerance (pH 2.0) of the potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for low pH tolerance (pH 2.0) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and "*" denotes the significant correlation at the 0.05 level.

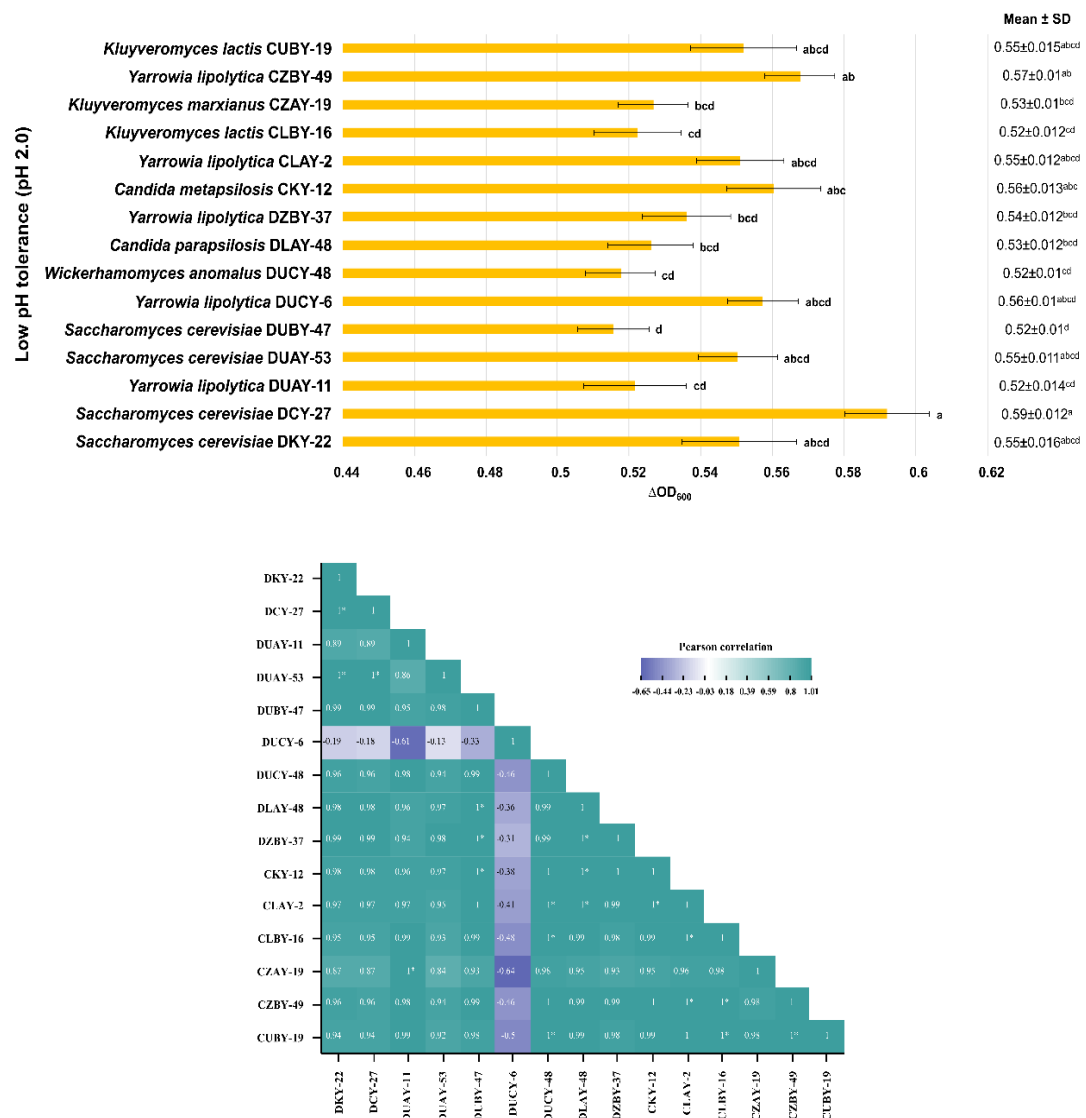


Figure 16: Low pH tolerance (pH 2.0) of the potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for low pH tolerance (pH 2.0) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.3.2 Bile tolerance

Bile tolerance is an important factor to consider when screening probiotics since it shows if a microorganism can survive and endure metabolically active in the small intestine, where bile salts are secreted. The method used for evaluating bile tolerance was similar to the method used to assess low pH tolerance. From cow *dahi*, *Saccharomyces cerevisiae* DRC-42 and from cow *chhurpi*, *Kazachstania exigua* CMNB-4 showed the highest bile tolerance, with ΔOD_{600} of 0.91 ± 0.012 and 0.95 ± 0.022 , respectively (Figure 17). *S. cerevisiae* DCY-27 from yak *dahi* and *Yarrowia lipolytica* CLAY-2 from yak *chhurpi* showed the highest tolerance to bile, with ΔOD_{600} of 0.71 ± 0.10 and 0.71 ± 0.012 , respectively (Figure 18).

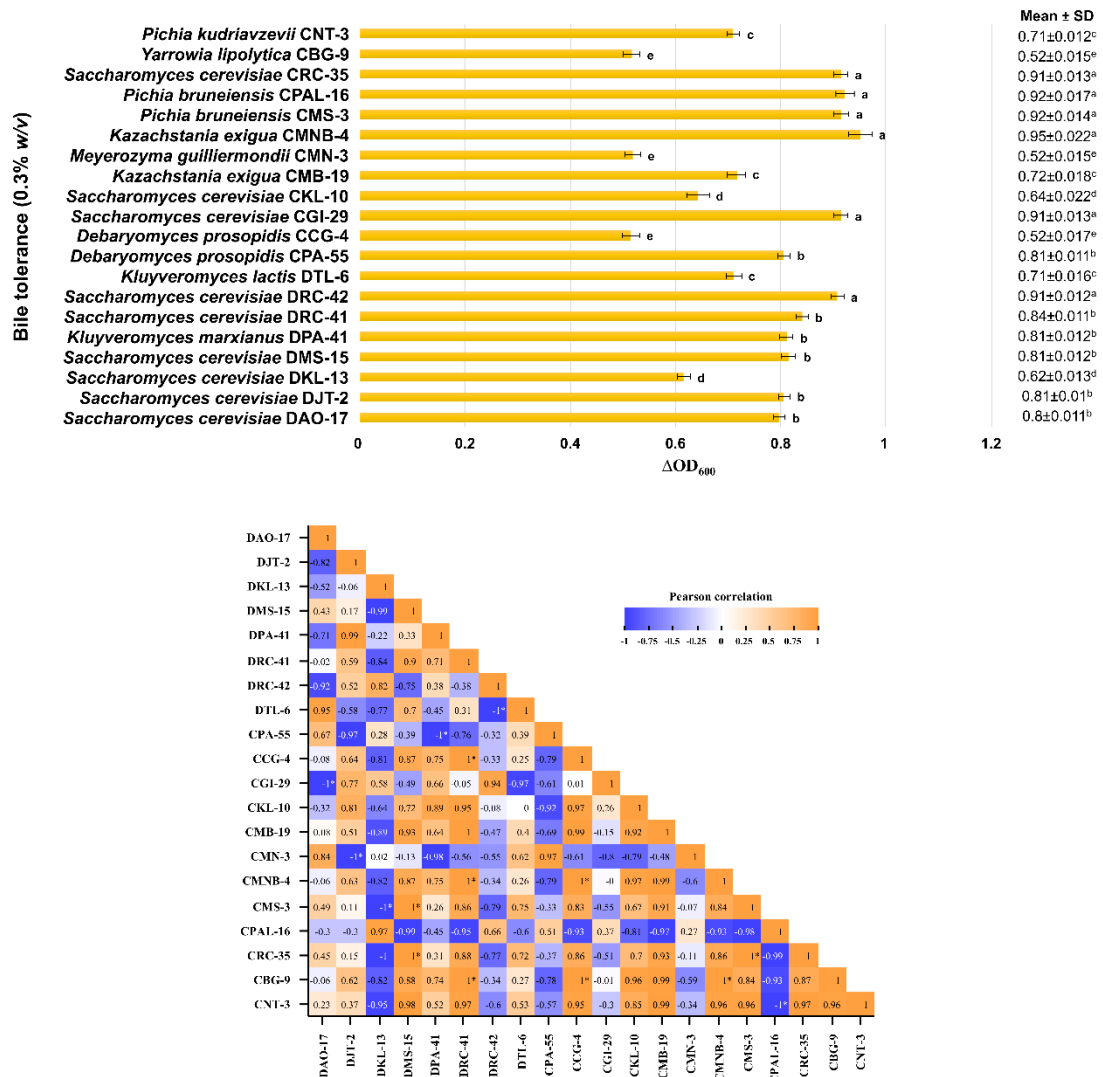


Figure 17: Bile tolerance (0.3% w/v) of the potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences (p < 0.05) between yeast isolates according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for bile tolerance (0.3% w/v) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

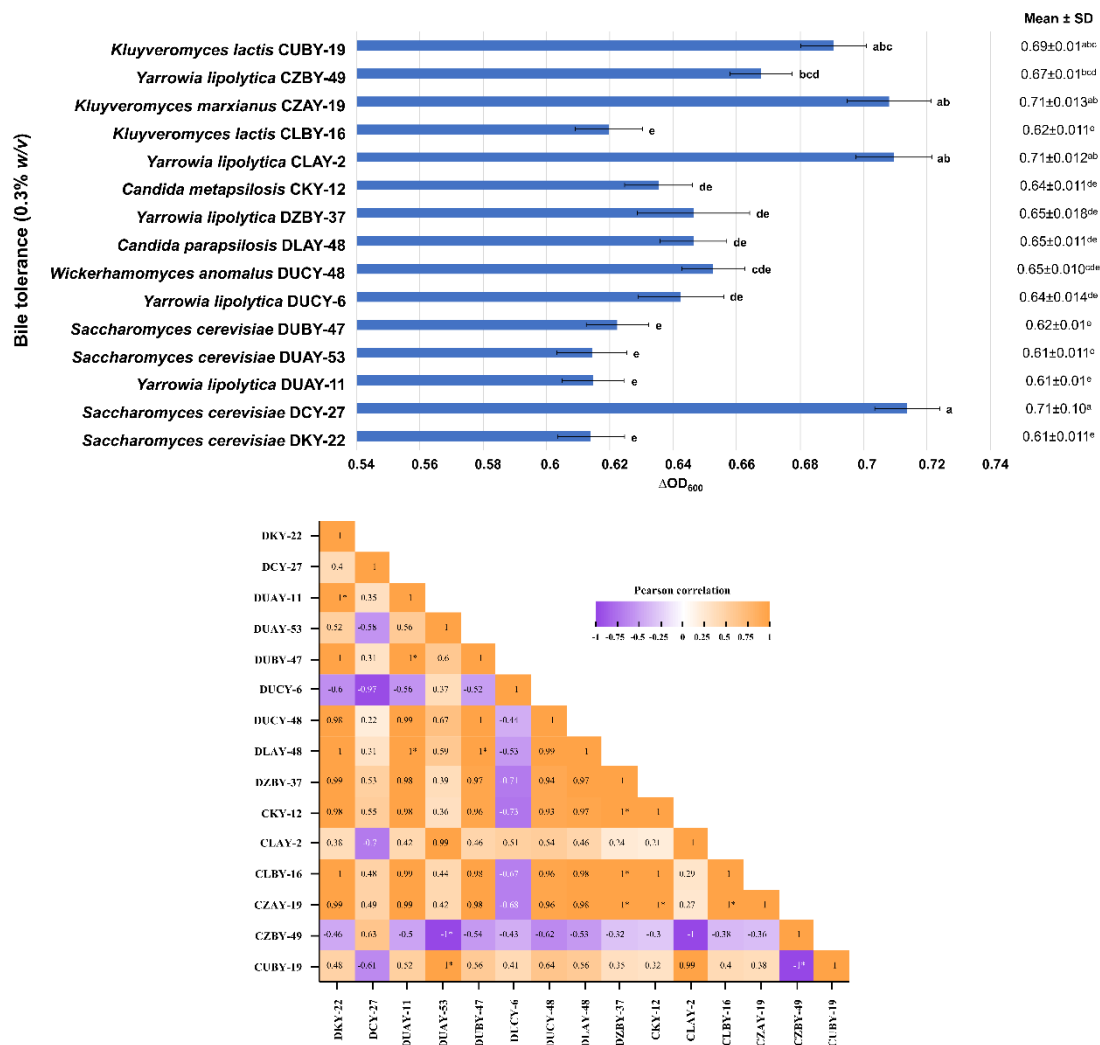


Figure 18: Bile tolerance (0.3% w/v) of the potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for bile tolerance (0.3% w/v) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.3.3 Microbial attachment to hydrocarbons (MATHs)

After low pH tolerance and bile tolerance, microbial attachment to hydrocarbons is one of the essential traits on the basis of which preliminary screening of the isolates was carried out. Cell surface hydrophobicity of the isolates was checked by measuring the adherence property of the isolates with hydrocarbons such as n-hexadecane, toluene, xylene, and diethyl ether. On preliminary screening, only those isolates were selected that showed $\geq 80\%$ cell surface hydrophobicity. Isolate *Saccharomyces cerevisiae* DJT-2 from cow *dahi*, *Pichia bruneiensis* CMS-3 from cow *chhurpi*, *Yarrowia lipolytica* DUCY-6 from yak *dahi*, and *Y. lipolytica* CZBY-49 from yak *chhurpi* showed the highest hydrophobicity for all four hydrocarbons (Table 27-28).

Table 27: Microbial attachment to hydrocarbons (MATHs) of the potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Microbial attachment to hydrocarbons (%)			
		n-hexadecane	Toluene	Xylene	Diethyl ether
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DAO-17	80.53±0.75 ⁱ	87.56±0.46 ^c	88.04±0.48 ^c	88.99±0.64 ^{ef}
	<i>Saccharomyces cerevisiae</i> DJT-2	96.99±0.19 ^a	96.87±0.32 ^a	96.91±0.31 ^a	96.78±0.29 ^a
	<i>Saccharomyces cerevisiae</i> DKL-13	83.97±0.05 ^g	84.36±0.24 ^f	83.71±0.57 ^f	84.17±0.31 ^g
	<i>Saccharomyces cerevisiae</i> DMS-15	80.53±0.12 ⁱ	80.15±0.36 ⁱ	79.94±0.32 ⁱ	80.23±0.29 ⁱ
	<i>Kluyveromyces marxianus</i> DPA-41	87.86±0.07 ^f	87.96±0.24 ^c	88.18±0.27 ^c	88.23±0.36 ^f
	<i>Saccharomyces cerevisiae</i> DRC-41	81.92±0.14 ^h	82.03±0.34 ^g	82.00±0.25 ^g	81.86±0.41 ^{hi}
	<i>Saccharomyces cerevisiae</i> DRC-42	91.62±0.16 ^d	91.93±0.44 ^c	92.10±0.21 ^c	91.58±0.56 ^{cd}
	<i>Kluyveromyces lactis</i> DTL-6	94.42±0.23 ^c	94.31±0.26 ^b	94.31±0.26 ^b	93.96±0.34 ^b
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	91.62±0.16 ^d	91.75±0.44 ^c	92.27±0.53 ^c	91.84±0.76 ^c
	<i>Debaryomyces prosopidis</i> CCG-4	81.92±0.14 ^h	82.08±0.24 ^g	82.03±0.28 ^g	81.92±0.36 ^h
	<i>Saccharomyces cerevisiae</i> CGI-29	80.53±0.12 ⁱ	80.61±0.21 ^{hi}	80.85±0.18 ^{ghi}	80.56±0.29 ^{hi}
	<i>Saccharomyces cerevisiae</i> CKL-10	82.00±0.29 ^h	81.70±0.37 ^{gh}	81.53±0.51 ^{gh}	80.91±0.43 ^{hi}
	<i>Kazachstania exigua</i> CMB-19	80.53±0.12 ⁱ	80.56±0.29 ^{hi}	80.85±0.49 ^{ghi}	80.38±0.49 ^{hi}
	<i>Meyerozyma guilliermondii</i> CMN-3	80.53±0.12 ⁱ	80.35±0.21 ⁱ	80.50±0.44 ^{hi}	80.23±0.37 ⁱ
	<i>Kazachstania exigua</i> CMNB-4	80.53±0.12 ⁱ	80.61±0.21 ^{hi}	80.29±0.37 ^{hi}	80.29±0.37 ^{hi}

<i>Pichia bruneiensis</i> CMS-3	95.84±0.19 ^b	96.05±0.26 ^a	96.34±0.32 ^a	96.32±0.36 ^a
<i>Pichia bruneiensis</i> CPAL-16	92.18±0.12 ^d	91.84±0.42 ^c	91.93±0.44 ^c	92.01±0.53 ^c
<i>Saccharomyces cerevisiae</i> CRC-35	81.92±0.14 ^h	81.92±0.25 ^g	81.94±0.27 ^g	81.92±0.31 ^h
<i>Yarrowia lipolytica</i> CBG-9	88.20±0.08 ^f	88.15±0.13 ^e	88.12±0.31 ^e	88.01±0.43 ^f
<i>Pichia kudriavzevii</i> CNT-3	90.62±0.12 ^e	90.48±0.27 ^d	90.37±0.34 ^d	89.99±0.37 ^{de}

Note: Results are expressed as the mean ± SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters.

Table 28: Microbial attachment to hydrocarbons (MATHs) of the potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Microbial attachment to hydrocarbons (%)			
		n-hexadecane	Toluene	Xylene	Diethyl ether
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	90.62±0.12 ^d	90.62±0.2 ^b	90.07±0.08 ^d	90.65±0.16 ^c
	<i>Saccharomyces cerevisiae</i> DCY-27	90.68±0.08 ^d	90.35±0.12 ^{bc}	89.89±0.08 ^d	90.44±0.14 ^c
	<i>Yarrowia lipolytica</i> DUAY-11	91.62±0.16 ^c	90.76±0.32 ^b	92.18±0.12 ^c	91.19±0.11 ^b
	<i>Saccharomyces cerevisiae</i> DUAY-53	87.86±0.07 ^f	87.86±0.09 ^d	88.25±0.17 ^c	87.84±0.10 ^c
	<i>Saccharomyces cerevisiae</i> DUBY-47	84.58±0.11 ^g	84.38±0.14 ^e	85.22±0.11 ^f	84.41±0.11 ^f
	<i>Yarrowia lipolytica</i> DUCY-6	95.34±0.07 ^b	95.39±0.14 ^a	94.91±0.15 ^b	95.36±0.17 ^a
	<i>Wickerhamomyces anomalus</i> DUCY-48	81.34±0.05 ^j	81.58±0.12 ^h	81.57±0.10 ⁱ	81.22±0.07 ⁱ
	<i>Candida parapsilosis</i> DLAY-48	82.72±0.09 ⁱ	82.12±0.1 ^{gh}	82.37±0.18 ^h	82.33±0.1 ^h
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> DZBY-37	80.67±0.07 ^k	80.04±0.13 ⁱ	80.28±0.11 ^j	80.34±0.15 ^j
	<i>Candida metapsilosis</i> CKY-12	80.21±0.10 ^l	80.13±0.1 ⁱ	80.11±0.13 ^j	80.05±0.1 ^j
	<i>Yarrowia lipolytica</i> CLAY-2	82.99±0.10 ⁱ	82.38±0.13 ^g	82.81±0.25 ^h	82.58±0.14 ^h
	<i>Kluyveromyces lactis</i> CLBY-16	89.77±0.06 ^e	89.85±0.09 ^c	89.75±0.12 ^d	89.66±0.05 ^d
	<i>Kluyveromyces marxianus</i> CZAY-19	88.20±0.08 ^f	87.72±0.19 ^d	87.93±0.08 ^c	87.66±0.11 ^c
	<i>Yarrowia lipolytica</i> CZBY-49	96.45±0.16 ^a	95.84±0.19 ^a	95.84±0.1 ^a	95.63±0.17 ^a
	<i>Kluyveromyces lactis</i> CUBY-19	83.97±0.05 ^h	83.71±0.07 ^f	83.69±0.12 ^g	83.73±0.10 ^g

Note: Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters.

4.3.4 Auto-aggregation and Co-aggregation

The ability of yeast isolates to auto-aggregate with one another was evaluated in order to determine their affinity to colonize intestinal epithelial cells. Out of 20 isolates from cow milk products, *Saccharomyces cerevisiae* DRC-42 ($93.13 \pm 0.18\%$) from cow *dahi* and *S. cerevisiae* CGI-29 ($91.67 \pm 0.26\%$) from cow *chhurpi* showed the highest percentage of auto-aggregation (Figure 19). *S. cerevisiae* DKY-22 ($96.24 \pm 0.24\%$) from yak *dahi* and *Candida metapsilosis* CKY-12 ($88.96 \pm 0.08\%$) from yak *chhurpi* showed maximum auto-aggregation among 15 isolates from yak milk products (Figure 20). Co-aggregation is another aggregation property of isolates to determine their ability to aggregate with MTCC reference isolates, namely *Escherichia coli* KL96 MTCC 1583, *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740, and *Bacillus cereus* MTCC 1272. *Kluyveromyces marxianus* DPA-41 (MTCC 740- $53.19 \pm 1.45\%$; MTCC 1583- $59.96 \pm 0.45\%$; MTCC 3223- $61.16 \pm 0.40\%$; and MTCC 1272- $50.71 \pm 0.55\%$) from cow *dahi* and *S. cerevisiae* CRC-35 (MTCC 740- $58.82 \pm 1.32\%$; MTCC 1583- $57.25 \pm 0.45\%$; MTCC 3223 $59.35 \pm 1.7\%$; and MTCC 1272- $45.81 \pm 0.42\%$) from cow *chhurpi* showed the highest co-aggregation against all four MTCC reference isolates (Table 29). From yak *dahi*, *S. cerevisiae* DKY-22 showed the maximum percentage of co-aggregation against MTCC 740 ($62.96 \pm 0.74\%$); *S. cerevisiae* DUAY-53 showed highest co-aggregation against MTCC 1583 ($19.56 \pm 0.04\%$), MTCC 3223 ($18.89 \pm 0.05\%$), and MTCC 1272 ($20.99 \pm 0.03\%$). From yak *chhurpi*, *Kl. lactis* CUBY-19 demonstrated the highest co-aggregation against MTCC 740 ($58.88 \pm 0.52\%$), MTCC 1583 ($17.81 \pm 0.26\%$), and MTCC 1272 ($18.6 \pm 0.04\%$); and *Kl. marxianus* CZAY-19 showed the highest co-aggregation against MTCC 3223 ($18.87 \pm 0.05\%$) (Table 30).

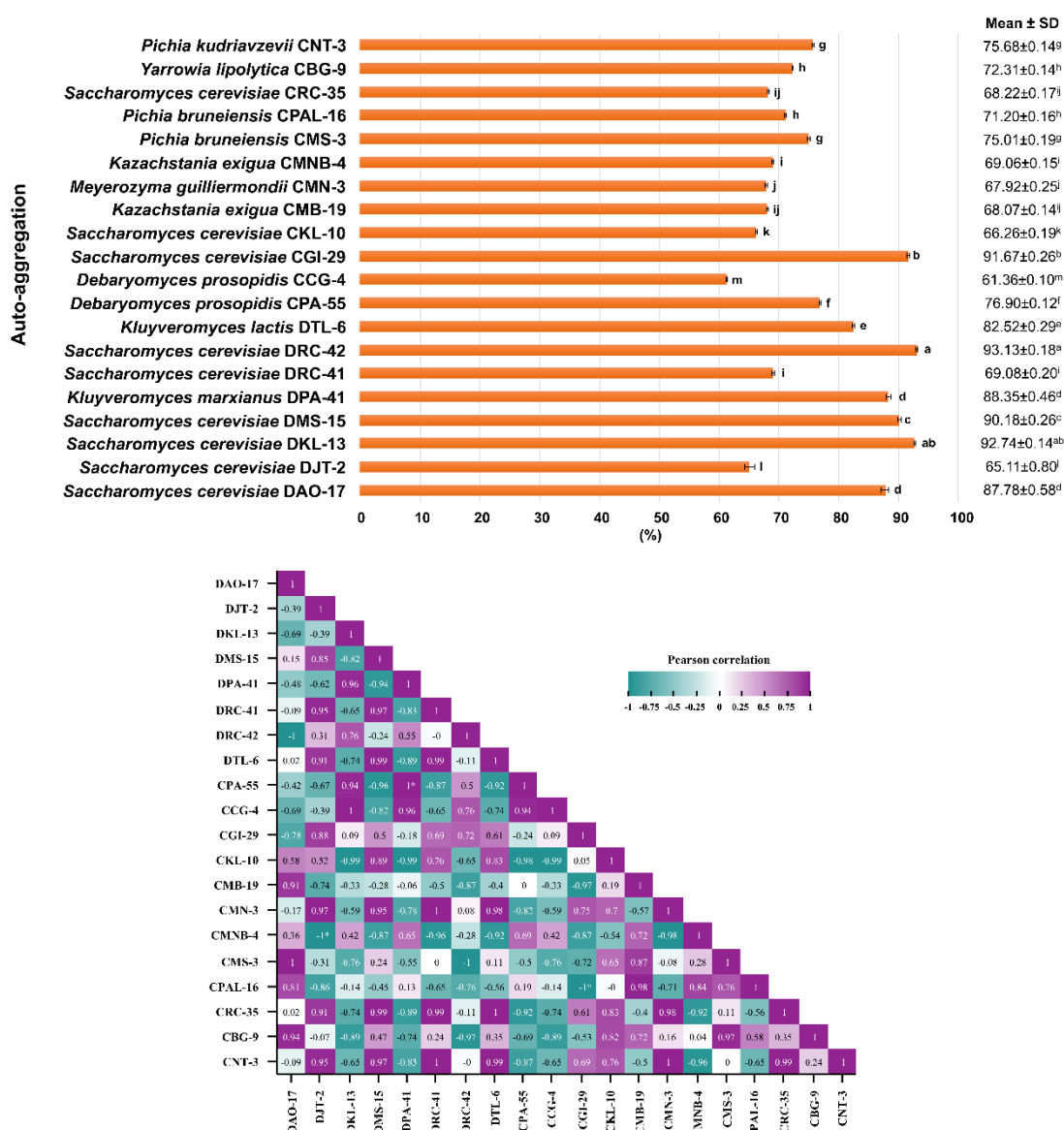


Figure 19: Auto-aggregation (%) of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for auto-aggregation (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

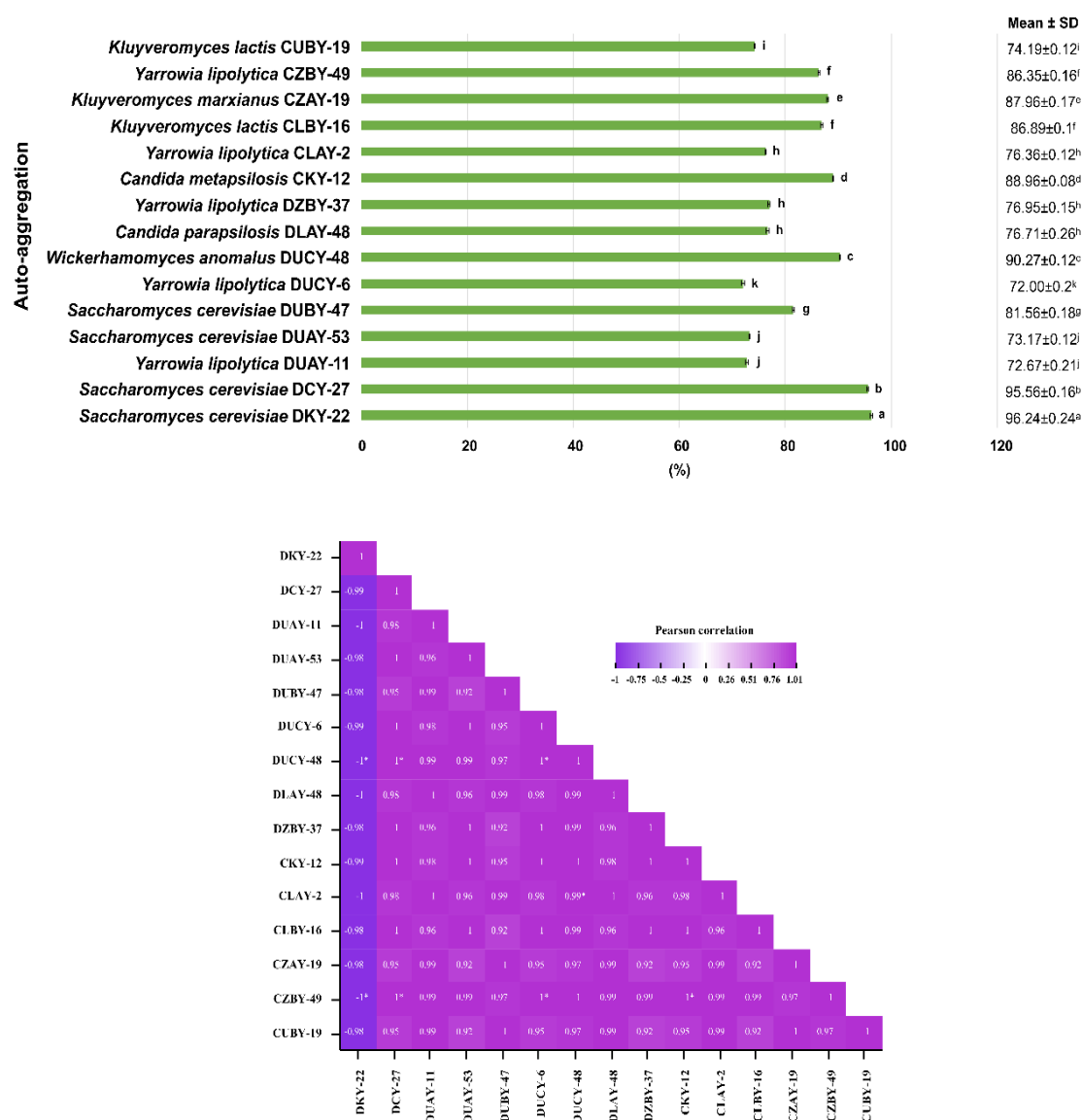


Figure 20: Auto-aggregation (%) of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for auto-aggregation (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

Table 29: Co-aggregation of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Co-aggregation (%)			
		MTCC 740	MTCC 1583	MTCC 3223	MTCC 1272
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DAO-17	43.63±1.8 ^{cd}	31.79±0.11 ^{ef}	48.83±0.36 ^{ef}	39.07±1.5 ^{ef}
	<i>Saccharomyces cerevisiae</i> DJT-2	38.28±1.92 ^d	28.80±1.02 ^{fg}	31.15±0.31 ^{fg}	12.61±1.44 ^{fg}
	<i>Saccharomyces cerevisiae</i> DKL-13	27.47±1.57 ^e	28.85±1.44 ^{fg}	41.68±1.68 ^{fg}	27.76±1.57 ^{fg}
	<i>Saccharomyces cerevisiae</i> DMS-15	45.28±1.06 ^c	32.70±0.9 ^e	38.92±0.41 ^c	28.99±0.55 ^c
	<i>Kluyveromyces marxianus</i> DPA-41	53.19±1.45 ^b	59.96±0.45 ^a	61.16±0.40 ^a	50.71±0.55 ^a
	<i>Saccharomyces cerevisiae</i> DRC-41	20.82±1.34 ^{fg}	12.36±0.44 ^j	22.81±1.68 ^j	29.55±1.37 ^j
	<i>Saccharomyces cerevisiae</i> DRC-42	27.7±1.67 ^e	28.26±1.71 ^g	47.39±0.52 ^g	5.6±0.55 ^g
	<i>Kluyveromyces lactis</i> DTL-6	41.58±1.89 ^{cd}	48.78±1.17 ^b	19.03±0.55 ^b	17.24±0.89 ^b
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	10.64±1.81 ⁱ	16.39±0.45 ⁱ	39.91±1.01 ⁱ	29.55±1.37 ⁱ
	<i>Debaryomyces prosopidis</i> CCG-4	18.54±1.46 ^{fg}	19.16±0.44 ^{hi}	8.67±0.41 ^{hi}	15.62±0.55 ^{hi}
	<i>Saccharomyces cerevisiae</i> CGI-29	27.59±1.67 ^e	43.48±0.62 ^c	6.82±0.61 ^c	17.74±0.7 ^c
	<i>Saccharomyces cerevisiae</i> CKL-10	23.55±2.47 ^{ef}	47.69±0.44 ^b	15.62±0.41 ^b	20.75±0.42 ^b
	<i>Kazachstania exigua</i> CMB-19	4.72±0.43 ^j	47.60±0.56 ^b	31.61±0.41 ^b	34.06±0.55 ^b
	<i>Meyerozyma guilliermondii</i> CMN-3	3.64±0.35 ^j	28.94±1.39 ^{fg}	11.84±0.40 ^{fg}	30.66±0.55 ^{fg}
	<i>Kazachstania exigua</i> CMNB-4	10.75±1.53 ^{hi}	39.99±0.56 ^d	34.03±0.4 ^d	32.50±0.41 ^d

<i>Pichia bruneiensis</i> CMS-3	16.10±0.56 ^{gh}	21.60±1.17 ^h	21.70±0.4 ^h	7.32±0.55 ^h
<i>Pichia bruneiensis</i> CPAL-16	42.66±0.61 ^{cd}	20.20±1.22 ^h	36.50±0.4 ^h	59.06±0.55 ^h
<i>Saccharomyces cerevisiae</i> CRC-35	58.82±1.32 ^a	57.25±0.45 ^a	59.35±1.7 ^a	45.81±0.42 ^a
<i>Yarrowia lipolytica</i> CBG-9	8.65±0.56 ^{ij}	48.14±0.34 ^b	23.35±1.01 ^b	24.03±0.55 ^b
<i>Pichia kudriavzevii</i> CNT-3	46.25±0.56 ^c	37.50±0.59 ^d	14.47±0.31 ^d	37.45±0.42 ^d

Note: Results are expressed as the mean ± SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters. MTCC 740- *Staphylococcus aureus* subsp. *aureus* MTCC 740; MTCC 1583- *Escherichia coli* KL96 MTCC 1583; MTCC 3223- *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223; and MTCC 1272- *Bacillus cereus* MTCC 1272.

Table 30: Co-aggregation of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim.

Sl. no.	Isolates	Co-aggregation (%)			
		MTCC 740	MTCC 1583	MTCC 3223	MTCC 1272
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	62.96±0.74 ^a	4.19±0.04 ^m	17.81±0.03 ^d	18.51±0.04 ^b
	<i>Saccharomyces cerevisiae</i> DCY-27	60.46±0.28 ^b	14.68±0.06 ^d	17.86±0.06 ^d	14.25±0.06 ^e
	<i>Yarrowia lipolytica</i> DUAY-11	52.22±0.36 ^d	13.51±0.07 ^e	11.88±0.11 ^j	13.22±0.04 ^f
	<i>Saccharomyces cerevisiae</i> DUAY-53	39.57±0.21 ^h	19.56±0.04 ^a	18.89±0.05 ^a	20.99±0.03 ^a
	<i>Saccharomyces cerevisiae</i> DUBY-47	50.43±0.34 ^e	13.35±0.07 ^e	18.09±0.04 ^c	7.72±0.06 ^j
	<i>Yarrowia lipolytica</i> DUCY-6	8.37±0.29 ^l	10.07±0.04 ^h	8.90±0.06 ^k	16.31±0.03 ^c
	<i>Wickerhamomyces anomalus</i> DUCY-48	37.76±0.30 ⁱ	6.83±0.05 ^j	17.55±0.04 ^e	13.17±0.04 ^f
	<i>Candida parapsilosis</i> DLAY-48	44.89±0.24 ^g	8.65±0.04 ⁱ	15.92±0.03 ^g	7.53±0.03 ^j
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> DZBY-37	44.67±0.18 ^g	11.57±0.05 ^g	12.08±0.06 ^j	12.13±0.07 ^h
	<i>Candida metapsilosis</i> CKY-12	26.51±0.24 ^j	10.28±0.06 ^h	18.57±0.04 ^b	12.72±0.13 ^g
	<i>Yarrowia lipolytica</i> CLAY-2	20.63±0.21 ^k	6.21±0.05 ^k	16.53±0.06 ^f	5.57±0.04 ^k
	<i>Kluyveromyces lactis</i> CLBY-16	21.89±0.31 ^k	5.61±0.06 ^l	15.11±0.07 ^h	12.79±0.06 ^g
	<i>Kluyveromyces marxianus</i> CZAY-19	50.52±0.28 ^e	17.04±0.06 ^c	18.87±0.05 ^a	11.64±0.06 ⁱ
	<i>Yarrowia lipolytica</i> CZBY-49	48.00±0.54 ^f	11.94±0.1 ^f	13.15±0.05 ⁱ	14.93±0.06 ^d
	<i>Kluyveromyces lactis</i> CUBY-19	58.88±0.52 ^c	17.81±0.26 ^b	18.11±0.06 ^c	18.6±0.04 ^b

Note: Results are expressed as the mean $\pm$ SD ($n = 3$). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters. MTCC 740- *Staphylococcus aureus* subsp. *aureus* MTCC 740; MTCC 1583- *Escherichia coli* KL96 MTCC 1583; MTCC 3223- *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223; and MTCC 1272- *Bacillus cereus* MTCC 1272.

4.3.5 Bile salt hydrolase (BSH) activity

Bile salt hydrolase (BSH) activity is another key functional characteristic in probiotic selection. It facilitates the detoxification of bile salts by hydrolyzing conjugated bile acids, assisting probiotics to thrive in the adverse conditions of the gastrointestinal tract. Isolates were checked to hydrolyse sodium cholate, sodium taurocholate, and sodium glycocholate. *Kluyveromyces marxianus* DPA-41 from cow *dahi* and *Meyerozyma guilliermondii* CMN-3 and *Pichia kudriavzevii* CNT-3 from cow *chhurpi* were found to hydrolyse all three sodium salts (Table 31). From yak *dahi*, *Saccharomyces cerevisiae* DCY-27, *S. cerevisiae* DUAY-53, *Yarrowia lipolytica* DUCY-6, *Candida parapsilosis* DLAY-48, and *Y. lipolytica* DZBY-37; from yak *chhurpi*, *Kl. lactis* CLBY-16, *Kl. marxianus* CZAY-19, and *Kl. lactis* CUBY-19 were found to hydrolyse three different sodium salts with different intensities (Table 32).

Table 31: BSH activity of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Sodium cholate	Sodium Taurocholate	Sodium Glycocholate
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DAO-17	-	-	+
	<i>Saccharomyces cerevisiae</i> DJT-2	-	-	+
	<i>Saccharomyces cerevisiae</i> DKL-13	-	-	-
	<i>Saccharomyces cerevisiae</i> DMS-15	-	-	-
	<i>Kluyveromyces marxianus</i> DPA-41	++	++	++
	<i>Saccharomyces cerevisiae</i> DRC-41	-	-	-
	<i>Saccharomyces cerevisiae</i> DRC-42	-	-	-
	<i>Kluyveromyces lactis</i> DTL-6	-	-	-
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	-	-	+
	<i>Debaryomyces prosopidis</i> CCG-4	-	-	-
	<i>Saccharomyces cerevisiae</i> CGI-29	-	-	+
	<i>Saccharomyces cerevisiae</i> CKL-10	-	-	+
	<i>Kazachstania exigua</i> CMB-19	-	-	-
	<i>Meyerozyma guilliermondii</i> CMN-3	+	++	++
	<i>Kazachstania exigua</i> CMNB-4	-	-	-
	<i>Pichia bruneiensis</i> CMS-3	-	-	-
	<i>Pichia bruneiensis</i> CPAL-16	-	-	-
	<i>Saccharomyces cerevisiae</i> CRC-35	+	-	+++
	<i>Yarrowia lipolytica</i> CBG-9	-	-	-
	<i>Pichia kudriavzevii</i> CNT-3	+	+	+

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone.

Table 32: BSH activity of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Sodium cholate	Sodium Taurocholate	Sodium Glycocholate
Yak dahi	<i>Saccharomyces cerevisiae</i> DKY-22	+	+++	-
	<i>Saccharomyces cerevisiae</i> DCY-27	+	++	++
	<i>Yarrowia lipolytica</i> DUAY-11	++	-	-
	<i>Saccharomyces cerevisiae</i> DUAY-53	+	+++	++
	<i>Saccharomyces cerevisiae</i> DUBY-47	-	++	+
	<i>Yarrowia lipolytica</i> DUCY-6	+	+	+
	<i>Wickerhamomyces anomalus</i> DUCY-48	-	+++	+
	<i>Candida parapsilosis</i> DLAY-48	+	+	+
Yak chhurpi	<i>Yarrowia lipolytica</i> DZBY-37	+	+	-
	<i>Candida metapsilosis</i> CKY-12	+	-	+
	<i>Yarrowia lipolytica</i> CLAY-2	++	++	-
	<i>Kluyveromyces lactis</i> CLBY-16	++	++	++
	<i>Kluyveromyces marxianus</i> CZAY-19	+	+	+
	<i>Yarrowia lipolytica</i> CZBY-49	-	++	+
	<i>Kluyveromyces lactis</i> CUBY-19	+++	+++	+

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone.

4.3.6 Antimicrobial activity

The ability of probiotic isolates to demonstrate antimicrobial properties is considered an essential factor in their selection, since it improves colonization resistance by inhibiting pathogenic microbes through the synthesis of various organic compounds. Antimicrobial activity of the isolates was determined against four different MTCC reference isolates, namely *Escherichia coli* KL96 MTCC 1583, *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus*

MTCC 740, and *Bacillus cereus* MTCC 1272. *Saccharomyces cerevisiae* DKL-13 from cow *dahi* showed positive antimicrobial activity against MTCC 740, MTCC 1583, and MTCC 1272; and from cow *chhurpi*, *S. cerevisiae* CKL-10, *Kazachstania exigua* CMB-19, and *K. exigua* CMNB-4 showed positive antimicrobial activity against all four MTCC reference isolates (namely MTCC 740, MTCC 1583, MTCC 3223, and MTCC 1272) (Table 33). From yak *dahi*, *Yarrowia lipolytica* DUAY-11 and *S. cerevisiae* DUBY-47 showed positive antimicrobial activity against MTCC 1583, MTCC 3223, and MTCC 1272, and from yak *chhurpi*, *Kluyveromyces lactis* CUBY-19 showed antimicrobial activity against MTCC 740, MTCC 3223, and MTCC 1272 (Table 34).

Table 33: Antimicrobial activity of the screened yeast isolates from cow *dahi* and *chhurpi* against standard MTCC reference isolates.

NFM products	Isolates	Antimicrobial activity against MTCC isolates			
		MTCC 740	MTCC 1583	MTCC 3223	MTCC 1272
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DAO-17	++	-	-	-
	<i>Saccharomyces cerevisiae</i> DJT-2	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DKL-13	+	+	-	++
	<i>Saccharomyces cerevisiae</i> DMS-15	-	-	-	-
	<i>Kluyveromyces marxianus</i> DPA-41	+	-	-	-
	<i>Saccharomyces cerevisiae</i> DRC-41	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DRC-42	-	-	-	-
	<i>Kluyveromyces lactis</i> DTL-6	-	-	-	-
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	-	-	-	-
	<i>Debaryomyces prosopidis</i> CCG-4	-	-	++	-
	<i>Saccharomyces cerevisiae</i> CGI-29	-	-	-	-
	<i>Saccharomyces cerevisiae</i> CKL-10	+	+	+	+
	<i>Kazachstania exigua</i> CMB-19	+	+	+	+
	<i>Meyerozyma guilliermondii</i> CMN-3	-	-	-	-
	<i>Kazachstania exigua</i> CMNB-4	+	+	+	+

<i>Pichia bruneiensis</i> CMS-3	-	-	-	-
<i>Pichia bruneiensis</i> CPAL-16	+	-	-	++
<i>Saccharomyces cerevisiae</i> CRC-35	+	-	-	-
<i>Yarrowia lipolytica</i> CBG-9	-	-	+	-
<i>Pichia kudriavzevii</i> CNT-3	-	+	+	-

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone. MTCC 740- *Staphylococcus aureus* subsp. *aureus* MTCC 740; MTCC 1583- *Escherichia coli* KL96 MTCC 1583; MTCC 3223- *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223; and MTCC 1272- *Bacillus cereus* MTCC 1272.

Table 34: Antimicrobial activity of the screened yeast isolates from yak *dahi* and *chhurpi* against standard MTCC reference isolates.

NFM products	Isolates	Antimicrobial activity against MTCC isolates			
		MTCC 740	MTCC 1583	MTCC 3223	MTCC 1272
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	-	+	+++	-
	<i>Saccharomyces cerevisiae</i> DCY-27	-	+	++	-
	<i>Yarrowia lipolytica</i> DUAY-11	-	+	++	++
	<i>Saccharomyces cerevisiae</i> DUAY-53	-	-	++	-
	<i>Saccharomyces cerevisiae</i> DUBY-47	-	+	++	++
	<i>Yarrowia lipolytica</i> DUCY-6	-	-	++	-
	<i>Wickerhamomyces anomalus</i> DUCY-48	-	-	-	-
	<i>Candida parapsilosis</i> DLAY-48	-	-	+++	-
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> DZBY-37	++	-	-	-
	<i>Candida metapsilosis</i> CKY-12	-	-	++	-
	<i>Yarrowia lipolytica</i> CLAY-2	+++	-	++	-
	<i>Kluyveromyces lactis</i> CLBY-16	-	-	++	-
	<i>Kluyveromyces marxianus</i> CZAY-19	-	-	++	-
	<i>Yarrowia lipolytica</i> CZBY-49	+	-	++	-
	<i>Kluyveromyces lactis</i> CUBY-19	++	-	+++	+

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone. MTCC 740- *Staphylococcus aureus* subsp. *aureus* MTCC 740; MTCC 1583- *Escherichia coli* KL96 MTCC 1583; MTCC 3223- *Salmonella enteric* subsp. *enterica* ser. *typhimurium* MTCC 3223; and MTCC 1272- *Bacillus cereus* MTCC 1272.

4.3.7 Lysozyme tolerance

Tolerance to lysozyme is one of the essential *in vitro* traits of potential probiotics to tolerate the enzymatic activity of the mouth cavity. Isolates from cow and yak milk products exhibited more than 70% tolerance to lysozyme. From cow *dahi*, *Saccharomyces cerevisiae* DJT-2 ($91.63 \pm 0.11\%$) and from cow *chhurpi*, *S. cerevisiae* CGI-29 ($94.42 \pm 0.23\%$) showed highest tolerance to lysozyme (Figure 21). From yak *dahi*, *Wickerhamomyces anomalus* DUCY-48 ($87.97 \pm 0.28\%$) and from yak *chhurpi*, *Kluyveromyces lactis* CUBY-19 ($90.31 \pm 0.16\%$) showed highest lysozyme tolerance (Figure 22).

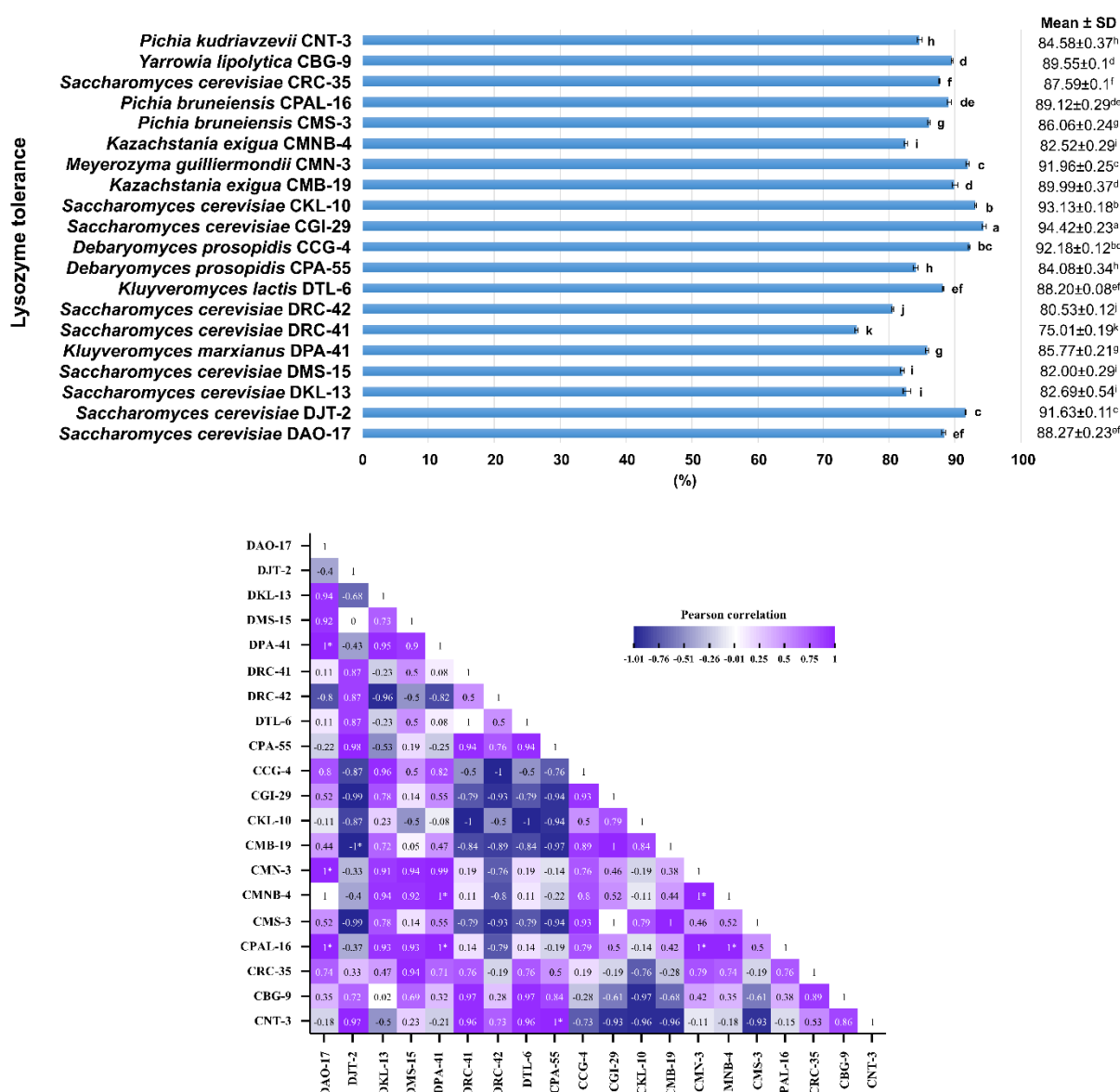


Figure 21: Lysozyme tolerance (%) of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap demonstrates the Pearson paired-wise correlation for lysozyme tolerance (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

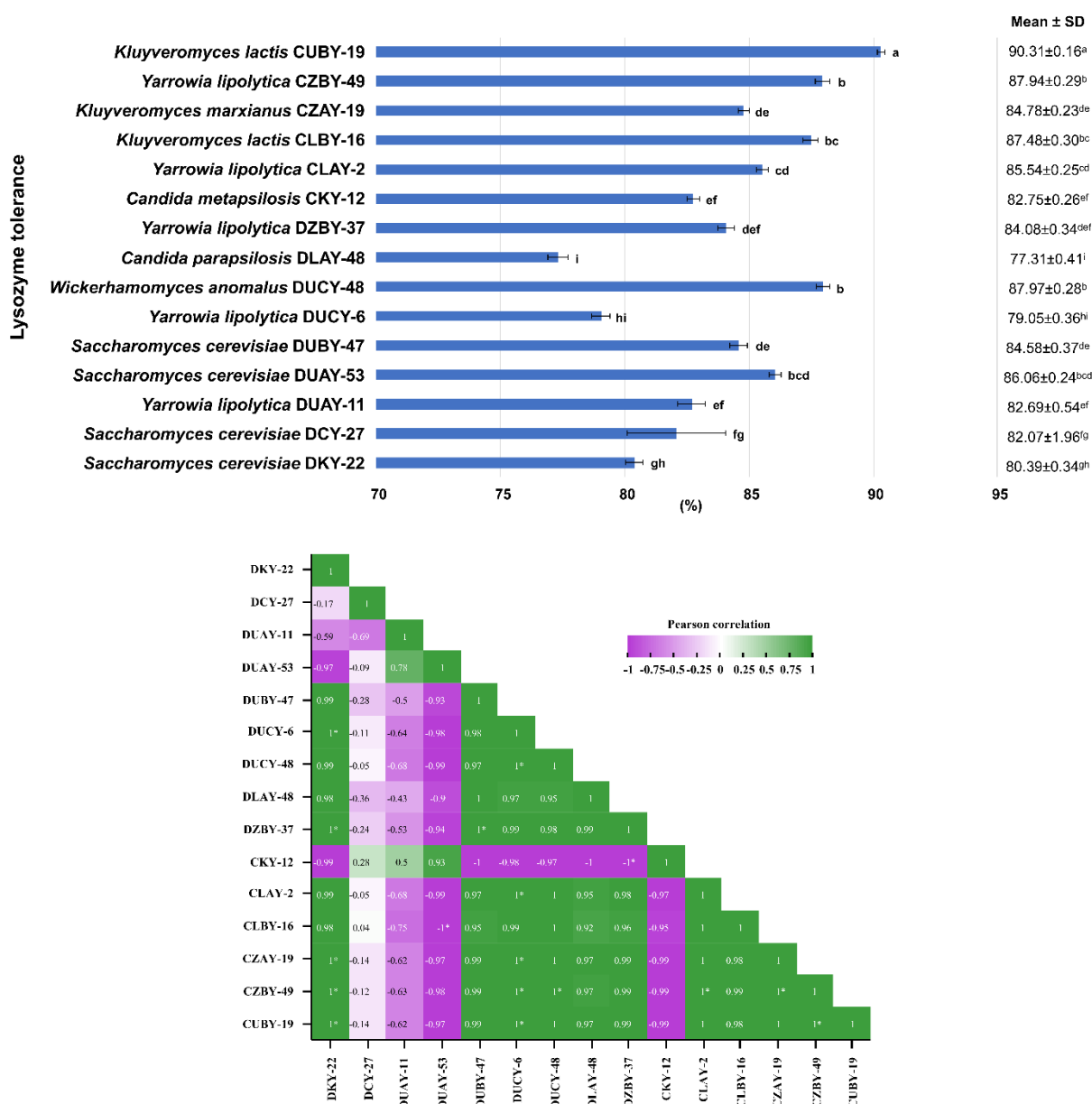


Figure 22: Lysozyme tolerance (%) of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences (p < 0.05) between yeast isolates according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been illustrated by different letters. The triangle heatmap shows the Pearson paired wise correlation for lysozyme tolerance (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.3.8 Growth at different temperatures

The potential yeast isolates were examined for their growth at different temperatures (20, 25, 28, 30, 35, 37, 40 and 45 °C), and their growth was assessed. It was found that the temperature of 28 °C is the optimal growth temperature for yeast. At 37 °C temperature, *Kluyveromyces marxianus* DPA-41 ($89.29 \pm 0.37\%$) from cow *dahi* and *Saccharomyces cerevisiae* CRC-35 ($87.78 \pm 0.37\%$) from cow *chhurpi* showed maximum growth (Figure 23). *S. cerevisiae* DUBY-47 ($82.86 \pm 0.65\%$) from yak *dahi* and *Kl. lactis* CUBY-19 ($77.68 \pm 0.58\%$) from yak *chhurpi* showed highest growth at 37 °C (Figure 24). Growth at a temperature of 37 °C is an essential characteristic of a probiotic microorganism, as it must tolerate human body temperature. After 37 °C temperature, growth of the yeast was found to be reduced drastically at 40 and 45 °C.

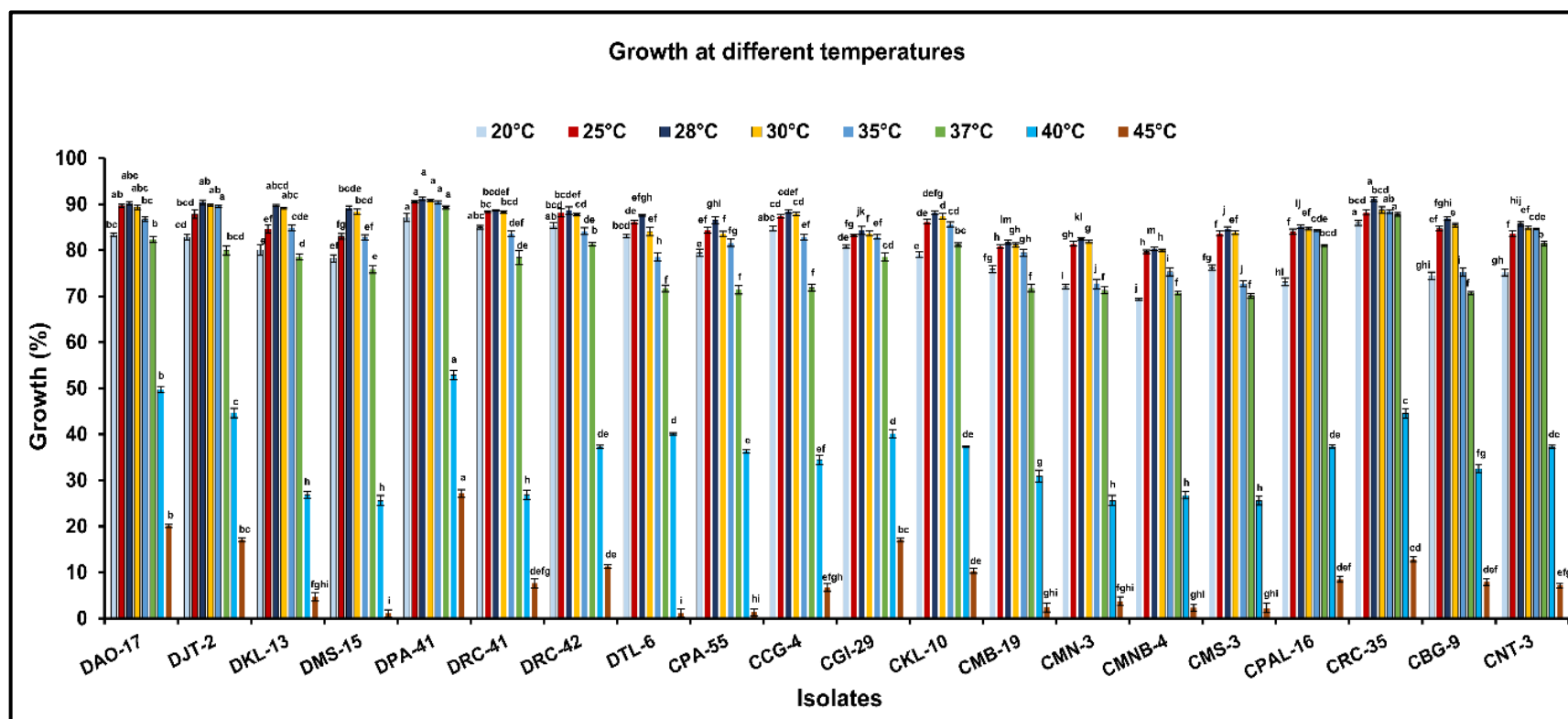


Figure 23: Growth at different temperatures (%) of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Different letters have been utilized to indicate significant statistical differences ($p < 0.05$) between yeast isolates based on the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA).

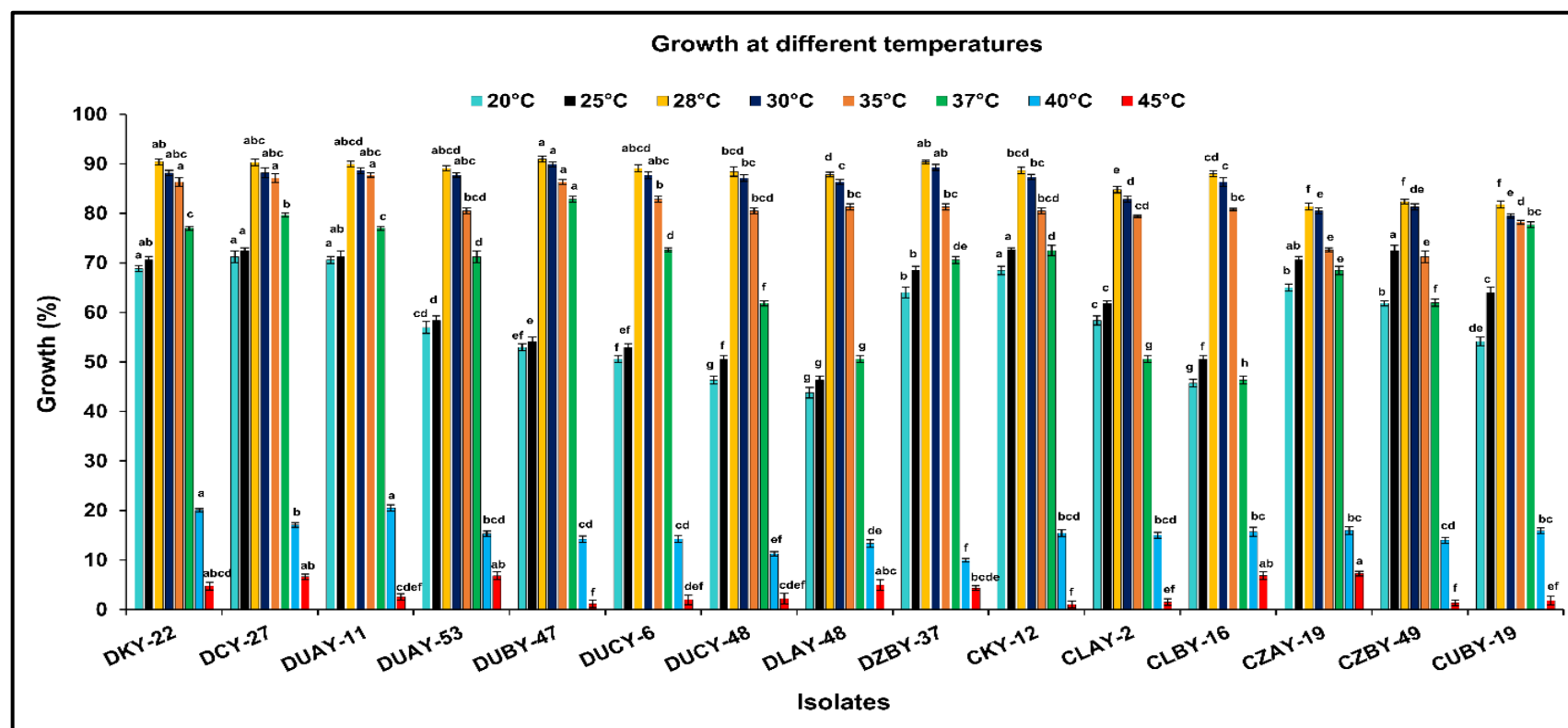


Figure 24: Growth at different temperatures (%) of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Different letters have been utilized to indicate significant statistical differences ($p < 0.05$) between yeast isolates based on the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA).

4.3.9 Growth at Different NaCl Concentrations

Potential isolates were subjected to different NaCl concentrations (2, 4, 6, 8, and 10%), and their growth was assessed. Isolates were found to tolerate up to 4% NaCl, however growth was significantly reduced at 6, 8, and 10% NaCl. *Kluyveromyces marxianus* DPA-41 ($90.54 \pm 0.28\%$) from cow *dahi* and *Debaryomyces prosopidis* CPA-55 ($88.51 \pm 0.48\%$) from cow *chhurpi* showed maximum growth at 4% NaCl concentration (Figure 25). From yak *dahi*, *Saccharomyces cerevisiae* DUAY-53 ($80.79 \pm 0.53\%$) and from yak *chhurpi*, *Candida metapsilosis* CKY-12 ($77.42 \pm 0.85\%$) found to showed highest growth at 4% NaCl concentration (Figure 26).

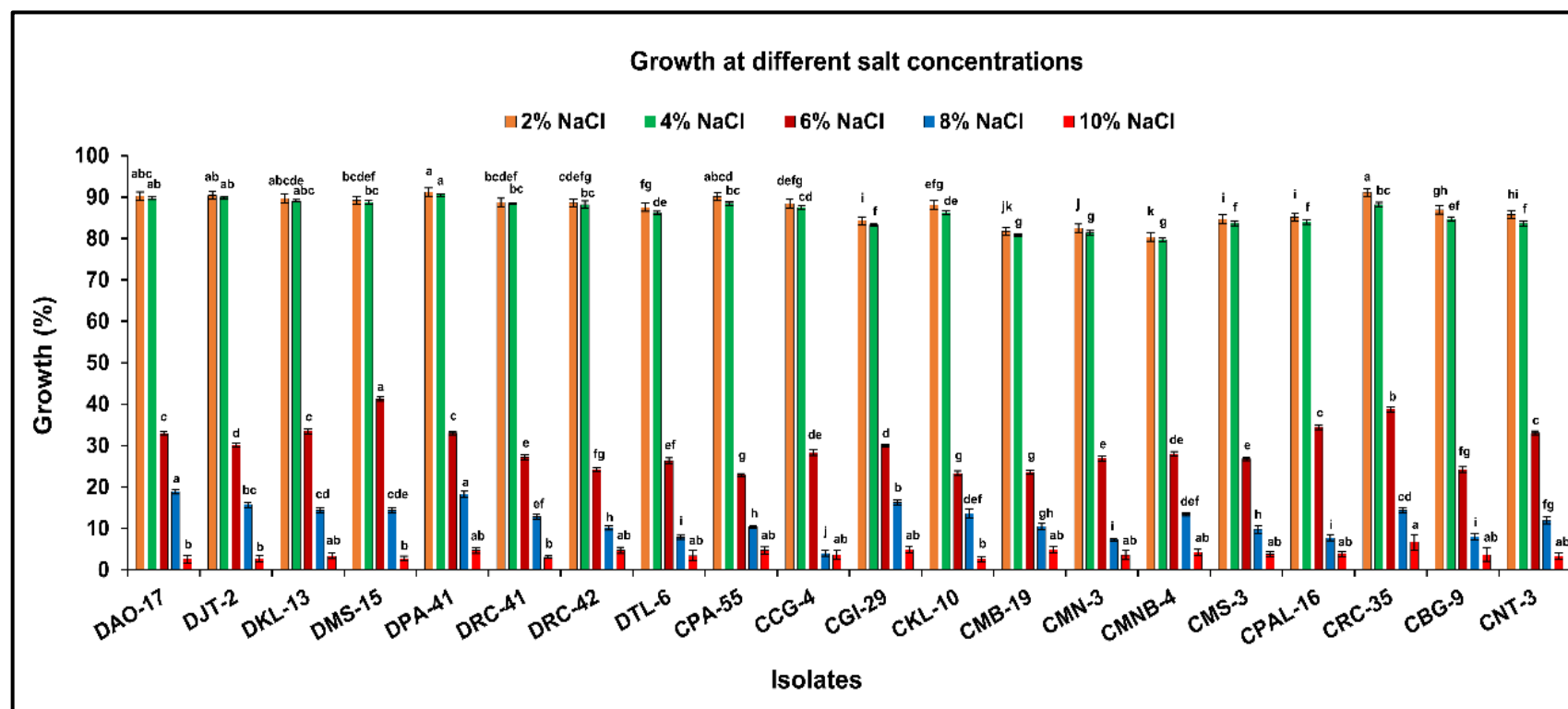


Figure 25: Growth at different salt concentrations (%) of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

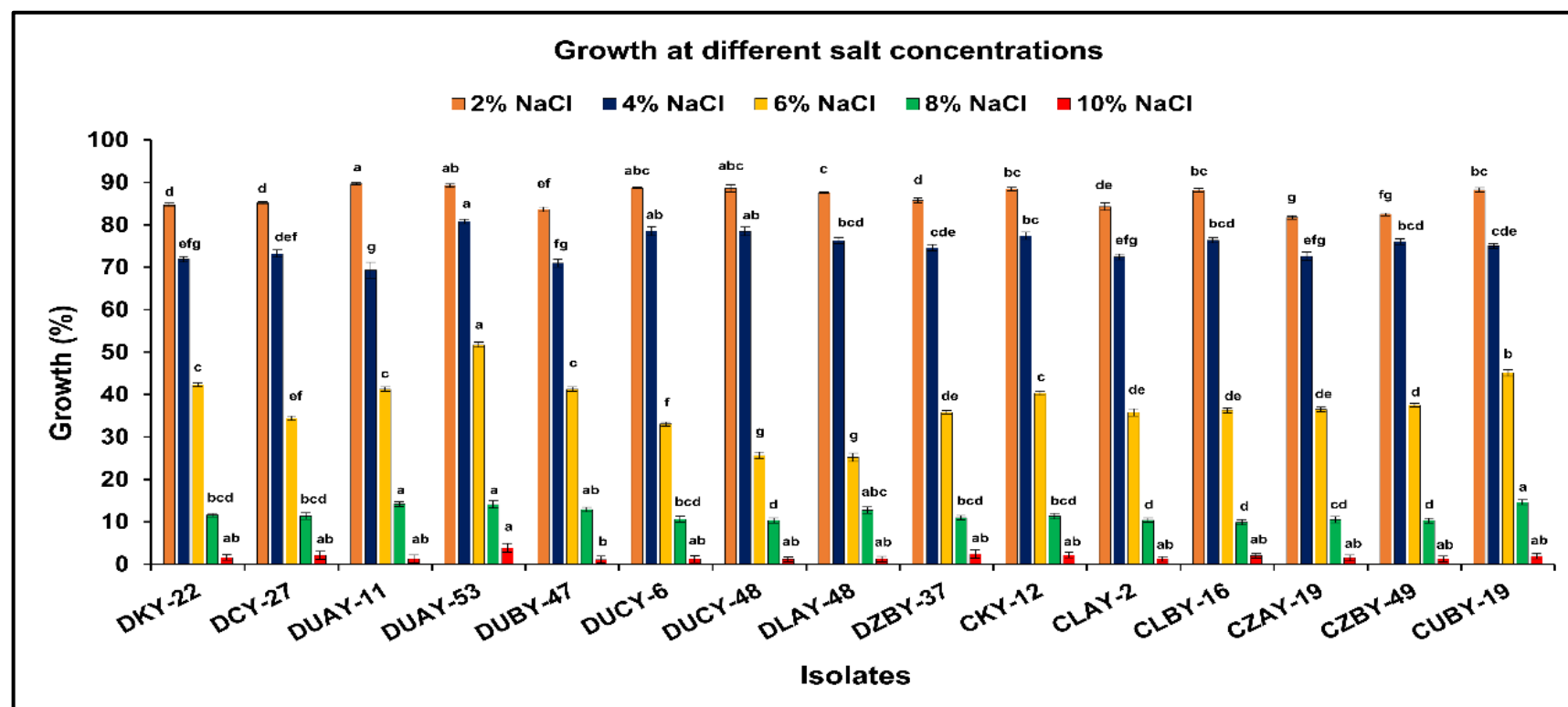


Figure 26: Growth at different salt concentrations (%) of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters.

4.3.10 Tolerance to *in vitro* digestive conditions

Probiotic candidate must demonstrate high survivability in stressful condition of digestive system which includes inhibitory condition of salivary fluid, gastric fluid and intestinal fluid. *Kluyveromyces marxianus* DPA-41 from cow *dahi* and *Saccharomyces cerevisiae* CRC-35 from cow *chhurpi* showed highest survivability and lowest CFU log reduction in Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) (Table 35). Similarly, *Wickerhamomyces anomalus* DUCY-48 from yak *dahi* and *Kl. lactis* CUBY-19 from yak *chhurpi* showed highest survivability and lowest CFU log reduction in SSF, SGF and SIF (Table 36).

Table 35: Tolerance to *in vitro* digestive conditions of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim.

Sl. no.	Isolates	Tolerance to <i>in vitro</i> digestive conditions					
		Simulated Salivary Fluid (SSF)		Simulated Gastric Fluid (SGF)		Simulated Intestinal Fluid (SIF)	
		CFU Log reduction	Survivability (%)	CFU Log reduction	Survivability (%)	CFU Log reduction	Survivability (%)
Cow dahi	<i>Saccharomyces cerevisiae</i> DAO-17	1.43±0.12 ^{abcd}	76.71±0.11 ^{bcde}	2.6±0.12 ^{ab}	60±0.11 ^{fg}	2.11±0.08 ^{ab}	67.23±0.08 ^{de}
	<i>Saccharomyces cerevisiae</i> DJT-2	0.69±0.22 ^{cd}	89.76±0.21 ^{abc}	1.45±0.34 ^{efgh}	77.34±0.33 ^{abcd}	1.59±0.15 ^{bcd}	75.68±0.15 ^{bcd}
	<i>Saccharomyces cerevisiae</i> DKL-13	1.22±0.14 ^{bcd}	82.47±0.13 ^{abcd}	2.09±0.18 ^{abcdef}	71.87±0.18 ^{bcde}	1.97±0.19 ^{abc}	72.97±0.19 ^{bcde}
	<i>Saccharomyces cerevisiae</i> DMS-15	1.35±0.19 ^{abcd}	76.06±0.18 ^{cde}	1.74±0.35 ^{cdefgh}	68.47±0.35 ^{bcdefg}	1.86±0.35 ^{abcd}	67.82±0.34 ^{de}
	<i>Kluyveromyces marxianus</i> DPA-41	0.56±0.19 ^d	90.68±0.18 ^{ab}	1.17±0.12 ^{gh}	79.21±0.12 ^{ab}	1.38±0.12 ^{cde}	75.48±0.11 ^{bcd}
	<i>Saccharomyces cerevisiae</i> DRC-41	2.28±0.29 ^a	64.65±0.29 ^e	2±0.37 ^{abcdefg}	62.26±0.37 ^{efg}	2.14±0.08 ^{ab}	66.51±0.07 ^e
	<i>Saccharomyces cerevisiae</i> DRC-42	1.37±0.42 ^{abcd}	81.83±0.41 ^{abcd}	2.3±0.19 ^{abcd}	69.21±0.19 ^{bcdef}	2.25±0.15 ^{ab}	69.97±0.14 ^{cde}
	<i>Kluyveromyces lactis</i> DTL-6	1.35±0.13 ^{abcd}	80.11±0.12 ^{abcd}	1.97±0.09 ^{abcdefg}	70.28±0.09 ^{bcdef}	1.96±0.21 ^{abc}	69.93±0.20 ^{cde}
Cow chhurpi	<i>Debaryomyces prosopidis</i> CPA-55	0.61±0.14 ^d	89.73±0.13 ^{abc}	1.28±0.12 ^{fgh}	79.42±0.12 ^{ab}	1.28±0.15 ^{de}	79.84±0.14 ^{ab}
	<i>Debaryomyces prosopidis</i> CCG-4	1.45±0.26 ^{abcd}	79.91±0.25 ^{abcd}	2.39±0.12 ^{abc}	67.17±0.12 ^{cdefg}	2.25±0.13 ^{ab}	69.91±0.13 ^{cde}
	<i>Saccharomyces cerevisiae</i> CGI-29	1.65±0.07 ^{abc}	76.05±0.06 ^{cde}	2.02±0.08 ^{abcdef}	70.38±0.08 ^{bcdef}	1.92±0.08 ^{abcd}	71.84±0.08 ^{bcde}

<i>Saccharomyces cerevisiae</i> CKL-10	1.96±0.12 ^{ab}	70.39±0.11 ^{de}	2.23±0.09 ^{abcde}	66.46±0.08 ^{defg}	2.15±0.17 ^{ab}	67.86±0.17 ^{de}
<i>Kazachstania exigua</i> CMB-19	1.22±0.06 ^{bcd}	81.4±0.05 ^{abcd}	2.64±0.37 ^{abcdef}	69.27±0.37 ^{bcdef}	1.88±0.12 ^{abcd}	71.81±0.12 ^{bcde}
<i>Meyerozyma guilliermondii</i> CMN-3	1.34±0.054 ^{abcd}	79.28±0.53 ^{abcd}	2.58±0.18 ^{ab}	60.84±0.18 ^{efg}	2.36±0.11 ^a	64.29±0.11 ^e
<i>Kazachstania exigua</i> CMNB-4	1.26±0.18 ^{bcd}	77.85±0.19 ^{bcde}	1.82±0.08 ^{bcdefgh}	68.23±0.08 ^{bcdefg}	1.68±0.09 ^{bcd}	71.03±0.09 ^{bcde}
<i>Pichia bruneiensis</i> CMS-3	1.21±0.18 ^{bcd}	82.68±0.18 ^{abcd}	2.01±0.06 ^{abcdef}	72.46±0.06 ^{bcde}	2.07±0.15 ^{ab}	71.36±0.15 ^{bcde}
<i>Pichia bruneiensis</i> CPAL-16	1.24±0.13 ^{bcd}	82.46±0.12 ^{abcd}	1.55±0.09 ^{defgh}	78.23±0.09 ^{abc}	1.6±0.13 ^{bcd}	77.33±0.13 ^{bc}
<i>Saccharomyces cerevisiae</i> CRC-35	0.55±0.38 ^d	92.16±0.37 ^a	1.06±0.11 ^h	85.67±0.1 ^a	0.88±0.08 ^e	88.34±0.08 ^a
<i>Yarrowia lipolytica</i> CBG-9	1.42±0.11 ^{abcd}	76.49±0.10 ^{bcde}	2.66±0.28 ^a	57.57±0.27 ^g	2.19±0.15 ^{ab}	65.29±0.15 ^e
<i>Pichia kudriavzevii</i> CNT-3	0.92±0.29 ^{cd}	86.56±0.29 ^{abc}	1.51±0.15 ^{defgh}	77.79±0.14 ^{abcd}	1.85±0.13 ^{abcd}	72.51±0.13 ^{bcde}

Note: Results are expressed as the mean ± SD (n = 3). Significant statistical differences (p < 0.05) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters.

Table 36: Tolerance to *in vitro* digestive conditions of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Tolerance to <i>in vitro</i> digestive conditions					
		Simulated Salivary Fluid (SSF)		Simulated Gastric Fluid (SGF)		Simulated Intestinal Fluid (SIF)	
		CFU Log reduction	Survivability (%)	CFU Log reduction	Survivability (%)	CFU Log reduction	Survivability (%)
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	1.76±0.24 ^{ab}	73.17±0.24 ^{de}	2.58±0.18 ^a	60.84±0.18 ^{efg}	2.24±0.13 ^a	66.11±0.13 ^{de}
	<i>Saccharomyces cerevisiae</i> DCY-27	1.45±0.25 ^{abcd}	79.91±0.25 ^{abcd}	2.27±0.22 ^a	68.81±0.22 ^{def}	2.25±0.09 ^a	69.91±0.09 ^{cde}
	<i>Yarrowia lipolytica</i> DUAY-11	1.42±0.1 ^{abcd}	76.49±0.1 ^{bcd}	2.66±0.12 ^a	57.57±0.12 ^{fg}	2.19±0.09 ^a	65.29±0.09 ^e
	<i>Saccharomyces cerevisiae</i> DUAY-53	1.26±0.18 ^{abcd}	82.1±0.18 ^{abcd}	1.92±0.23 ^{abcd}	73.69±0.23 ^{cd}	2.07±0.14 ^{ab}	71.36±0.14 ^{cde}
	<i>Saccharomyces cerevisiae</i> DUBY-47	1.26±0.21 ^{abcd}	81.71±0.21 ^{abcd}	2.03±0.17 ^{abc}	70.14±0.17 ^{cde}	1.98±0.09 ^{ab}	70.71±0.09 ^{cde}
	<i>Yarrowia lipolytica</i> DUCY-6	2.09±0.64 ^a	63.26±0.64 ^e	2.67±0.22 ^a	53.32±0.22 ^g	2.22±0.43 ^a	61.39±0.43 ^e
	<i>Wickerhamomyces anomalus</i> DUCY-48	0.72±0.17 ^{cd}	89.02±0.17 ^{abc}	0.93±0.16 ^c	85.99±0.16 ^{ab}	1.17±0.19 ^{cde}	82.43±0.19 ^{ab}
	<i>Candida parapsilosis</i> DLAY-48	0.93±0.13 ^{bcd}	83.59±0.13 ^{abcd}	1.36±0.31 ^{bcd}	76.26±0.31 ^{bcd}	1.36±0.06 ^{bcd}	76.01±0.06 ^{bcd}
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> DZBY-37	0.49±0.18 ^d	91.98±0.18 ^{ab}	1.13±0.05 ^{de}	82.12±0.05 ^{abc}	1.11±0.02 ^{de}	82.87±0.02 ^{ab}
	<i>Candida metapsilosis</i> CKY-12	1.13±0.09 ^{abcd}	83.94±0.09 ^{abcd}	1.3±0.1 ^{cde}	81.71±0.1 ^{abc}	1.6±0.13 ^{abcd}	77.74±0.13 ^{bc}
	<i>Yarrowia lipolytica</i> CLAY-2	1.65±0.02 ^{abc}	76.05±0.02 ^{cde}	2.02±0.05 ^{abc}	70.38±0.05 ^{cde}	1.92±0.05 ^{ab}	71.84±0.05 ^{cde}
	<i>Kluyveromyces lactis</i> CLBY-16	2.04±0.11 ^a	69.55±0.11 ^{de}	2.23±0.08 ^{ab}	66.46±0.08 ^{def}	2.15±0.17 ^a	67.86±0.17 ^{cde}

<i>Kluyveromyces marxianus</i> CZAY-19	1.26±0.19 ^{abcd}	77.85±0.19 ^{bcde}	1.82±0.08 ^{abcd}	68.23±0.08 ^{def}	1.68±0.02 ^{abcd}	71.03±0.02 ^{cde}
<i>Yarrowia lipolytica</i> CZBY-49	1.22±0.04 ^{abcd}	81.4±0.04 ^{abcd}	2.64±0.37 ^{abc}	69.27±0.37 ^{def}	1.88±0.12 ^{abc}	71.81±0.12 ^{cde}
<i>Kluyveromyces lactis</i> CUBY-19	0.46±0.09 ^d	93.72±0.09 ^a	0.85±0.15 ^e	88.51±0.15 ^a	0.78±0.08 ^e	89.66±0.08 ^a

Note: Results are expressed as the mean ± SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to the Tukey's HSD test from one-way ANOVA have been indicated by different letters.

4.3.11 Detection of genes responsible for major probiotic traits

Based on *in vitro* probiotic traits, genes responsible for low pH tolerance, bile tolerance, adhesion capacity and antimicrobial activity were detected for yeast isolates from *dahi* and *chhurpi* from cow (Table 37) and yak (Table 38).

Table 37: Probiotic gene detection in the screened yeast isolates from cow *dahi* and *chhurpi* samples of Sikkim.

NFM products	Yeast isolates	Low pH tolerance	Bile tolerance	Adhesion	Antimicrobial activity
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DAO-17	<i>TPS1, HSP150, SED1</i>	<i>YOR1</i>	<i>FLO1, FLO5, FLO10, FLO11</i>	<i>khs</i>
	<i>Saccharomyces cerevisiae</i> DJT-2	<i>TPS1, HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO1, FLO5, FLO10, FLO11, EPA1</i>	-
	<i>Saccharomyces cerevisiae</i> DKL-13	<i>TPS1, HSP150</i>	<i>PDR1, YOR1, FLU1</i>	<i>FLO10</i>	-
	<i>Saccharomyces cerevisiae</i> DMS-15	<i>TPS1, HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO1, FLO5, FLO11</i>	-
	<i>Kluyveromyces marxianus</i> DPA-41	<i>TPS1, HSP150, SED1, VMA2</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO10, CCW12, EPA1</i>	<i>khs</i>
	<i>Saccharomyces cerevisiae</i> DRC-41	<i>TPS1, HSP150, SED1</i>	<i>YIM1, YOR1, FLU1</i>	<i>FLO1, FLO5, FLO10, EPA1</i>	-
	<i>Saccharomyces cerevisiae</i> DRC-42	<i>TPS1, HSP150, SED1</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO1, FLO5, FLO10</i>	-
	<i>Kluyveromyces lactis</i> DTL-6	<i>TPS1, SED1</i>	<i>YIM1</i>	<i>FLO10, CCW12, EPA1</i>	-
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	<i>TPS1, HSP150, SED1, VMA2</i>	<i>YIM1, PDR1, YOR1</i>	<i>EPA1</i>	-
	<i>Debaryomyces prosopidis</i> CCG-4	<i>TPS1, HSP150, SED1</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO10, EPA1</i>	-
	<i>Saccharomyces cerevisiae</i> CGI-29	<i>TPS1, HSP150, SED1</i>	<i>YOR1</i>	<i>FLO1, FLO5, FLO10, EPA1</i>	-
	<i>Saccharomyces cerevisiae</i> CKL-10	<i>TPS1, SED1</i>	<i>PDR1</i>	<i>FLO1, FLO5, FLO10, FLO11, EPA1</i>	-

<i>Kazachstania exigua</i> CMB-19	<i>TPS1, HSP150</i>	<i>YIM1, YOR1</i>	<i>CCW12</i>	-
<i>Meyerozyma guilliermondii</i> CMN-3	<i>TPS1, HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO10, EPA1</i>	-
<i>Kazachstania exigua</i> CMNB-4	<i>TPS1, HSP150</i>	<i>YIM1, PDR1</i>	<i>FLO5, FLO10, EPA1</i>	-
<i>Pichia bruneiensis</i> CMS-3	<i>HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>CCW12, EPA1</i>	-
<i>Pichia bruneiensis</i> CPAL-16	<i>TPS1, HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO10, CCW12, EPA1</i>	-
<i>Saccharomyces cerevisiae</i> CRC-35	<i>TPS1, HSP150, SED1</i>	<i>YIM1, PDR1, YOR1</i>	<i>FLO1, FLO5, FLO11, EPA1</i>	<i>khs</i>
<i>Yarrowia lipolytica</i> CBG-9	<i>TPS1, HSP150</i>	<i>YIM1, PDR1, YOR1</i>	<i>CCW12, EPA1</i>	-
<i>Pichia kudriavzevii</i> CNT-3	<i>TPS1, HSP150, VMA2</i>	<i>YOR1</i>	<i>FLO1, FLO5, FLO10, FLO11, EPA1</i>	<i>pelA</i>

Table 38: Probiotic gene detection in the screened yeast isolates from yak *dahi* and *chhurpi* samples of Sikkim.

NFM products	Yeast isolates	Low pH tolerance	Bile tolerance	Adhesion	Antimicrobial activity
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	<i>TPS1, HSP150</i>	<i>YOR1, FLU1</i>	<i>FLO5, FLO10</i>	-
	<i>Saccharomyces cerevisiae</i> DCY-27	<i>TPS1, HSP150, SEDI</i>	<i>YOR1, FLU1</i>	<i>FLO1, FLO11</i>	-
	<i>Yarrowia lipolytica</i> DUAY-11	<i>HSP150, SEDI</i>	<i>YOR1</i>	<i>FLO10</i>	-
	<i>Saccharomyces cerevisiae</i> DUAY-53	<i>TPS1, HSP150, SEDI</i>	<i>PDR1, FLU1</i>	<i>FLO1, FLO5</i>	-
	<i>Saccharomyces cerevisiae</i> DUBY-47	<i>TPS1, HSP150</i>	<i>YIM1</i>	<i>FLO5, FLO11, EPA1</i>	-
	<i>Yarrowia lipolytica</i> DUCY-6	<i>TPS1</i>	<i>FLU1</i>	<i>FLO1, FLO5</i>	-
	<i>Wickerhamomyces anomalus</i> DUCY-48	<i>TPS1, HSP150, VMA2</i>	<i>PDR1</i>	<i>FLO5</i>	-
	<i>Candida parapsilosis</i> DLAY-48	<i>HSP150, VMA2</i>	<i>YIM1, YOR1</i>	<i>FLO5</i>	-
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> DZBY-37	<i>TPS1, HSP150</i>	<i>YIM1</i>	-	-
	<i>Candida metapsilosis</i> CKY-12	<i>TPS1, HSP150, SEDI, VMA2</i>	<i>PDR1</i>	<i>FLO10</i>	-
	<i>Yarrowia lipolytica</i> CLAY-2	<i>TPS1, HSP150</i>	<i>FLU1</i>	<i>FLO5, CCW12</i>	-
	<i>Kluyveromyces lactis</i> CLBY-16	<i>TPS1, HSP150</i>	<i>YIM1, YOR1</i>	<i>FLO5, FLO11, EPA1</i>	-
	<i>Kluyveromyces marxianus</i> CZAY-19	<i>HSP150, SEDI</i>	<i>YOR1</i>	<i>FLO5, CCW12</i>	-
	<i>Yarrowia lipolytica</i> CZBY-49	<i>TPS1</i>	<i>YIM1</i>	-	-
	<i>Kluyveromyces lactis</i> CUBY-19	<i>TPS1, HSP150</i>	<i>PDR1, YOR1, FLU1</i>	<i>FLO10</i>	<i>pela</i>

4.3.11.1 Detection of Low pH tolerance genes

There are many probable mechanisms to tolerate low pH levels. However, in this study, we sought to detect genes that contribute to withstand low pH environments. Among *TPS1*, *HSP150*, *SEDI*, *CYC1*, *VMA2*, and *VMA6*, the *CYC1*, and *VMA6* genes were not detected in any of the isolates. All isolates from cow *dahi*, 91.67% of the isolates from cow *chhurpi*, 77.78% of the isolates from yak *dahi*, and 83.33 % of the isolates from yak *chhurpi* were found to be positive for the *TPS1* gene (Figure 27a). About 87.5% of the isolates from cow *dahi*, 91.67% of the isolates from cow *chhurpi*, 88.89% of the isolates from yak *dahi*, and 83.33% of the isolates from yak *chhurpi* were detected for *HSP150* (Figure 27b). About 62.5% of isolates from cow *dahi*, 41.67% isolates from cow *chhurpi*, 33.33% of isolates from yak *dahi*, and 33.33% of the isolates from yak *chhurpi* were found to be positive for gene *SEDI* (Figure 27c). For gene *VMA2*, 12.5% stains from cow *dahi*, 16.67% isolates from cow *chhurpi*, 22.22% isolates from yak *dahi*, and 16.67% isolates from yak *chhurpi* were detected positive (Figure 27d).

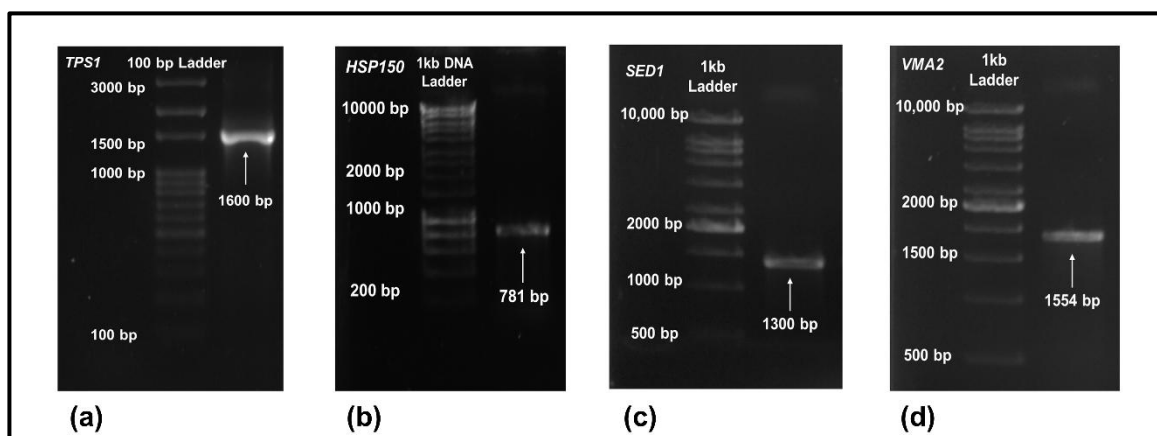


Figure 27: Agarose gel electrophoresis showing PCR amplification of low pH tolerance genes, namely (a) *TPS1*, (b) *HSP150*, (c) *SED1*, and (d) *VMA2*. Amplicon size was confirmed using a 100 bp (MBT049, HiMedia, India) and 1 kb DNA ladder (MBT051, HiMedia, India) in a 1% agarose gel, and the gel was visualized in a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

4.3.11.2 Detection of bile tolerance genes

Possible genes responsible for bile tolerance include *YIMI*, *PDR1*, *YORI*, and *FLU1*. About 75% of the isolates from cow *dahi*, 75% of the isolates from cow *chhurpi*, 33.33% of the isolates from yak *dahi*, and 33.33% of the isolates from yak *chhurpi* detected the gene *YIMI* (Figure 28a). About 62.5% of the isolates from cow *dahi*, 75% of the isolates from cow *chhurpi*, 22.22% of the isolates from yak *dahi*, and 33.33% of the isolates from yak *chhurpi* detected the gene *PDR1* (Figure 28b). Around 87.5% of the isolates from cow *dahi*, 83.33% of the isolates from cow *chhurpi*, 44.44% of the isolates from yak *dahi*, and 50% of the isolates from yak *chhurpi* were found to be positive for gene *YORI* (Figure 28c). About 25% of the isolates from cow *dahi*, 44.44% of the isolates from yak *dahi*, and 33.33% of the isolates from yak *chhurpi* detected the gene *FLU1* (Figure 28d), however, it was not detected in the isolates from cow *chhurpi*.

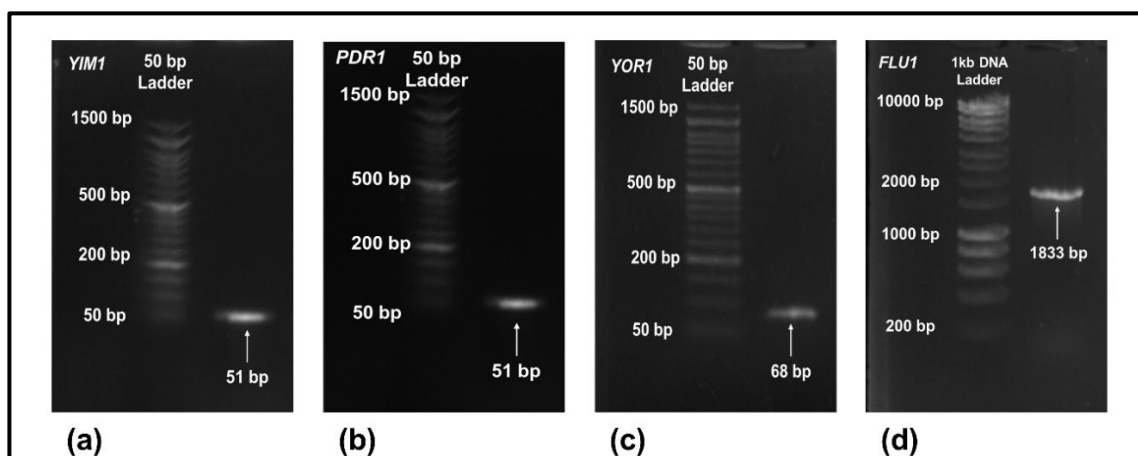


Figure 28: Agarose gel electrophoresis showing PCR amplification of bile tolerance genes, namely (a) *YIM1*, (b) *PDR1*, (c) *YOR1*, and (d) *FLU1*. Amplicon size was confirmed using a 50 bp (MBT084, HiMedia, India) and 1 kb DNA ladder (MBT051, HiMedia, India) in 2% and 1% agarose gel, respectively, and the gel was visualized in a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

4.3.11.3 Detection of adhesion genes

Possible genes responsible for adhesion to the mucous layer of epithelial cells, namely *FLO1*, *FLO5*, *FLO10*, *FLO11*, *AGA1*, *CCW12*, and *PST1*, were examined for yeast isolates. Of the total isolates, *FLO1* gene was detected in 62.5% of cow *dahi*, 33.33% of cow *chhurpi*, and 33.33% of yak *dahi* (Figure 29a), but was absent in yak *chhurpi*. The *FLO5* gene was detected in 62.5% of cow *dahi* isolates, 41.67% of cow *chhurpi* isolates, 66.67% of yak *dahi* isolates, and 50% of yak *chhurpi* isolates (Figure 29b). About 87.5% of the isolates from cow *dahi*, 58.33% of the isolates from cow *chhurpi*, 22.22% of the isolates from yak *dahi*, and 33.33% of the isolates from yak *chhurpi* found positive for the gene *FLO10* (Figure 29c). The gene *FLO11* was detected in 37.5% of cow *dahi* isolates, 25% of cow *chhurpi* isolates, 22.22% of yak *dahi* isolates, and 16.67% of yak *chhurpi* isolates (Figure 29d). *CCW12* gene positivity rates were about 25% of isolates in cow *dahi*, and 33.33% of isolates from both cow and yak *chhurpi* (Figure 29e).

Around 50% of the isolates from cow *dahi*, 91.67% of the isolates from cow *chhurpi*, 11.11% of the isolates from yak *dahi*, and 16.67% of the isolates from yak *chhurpi* detected the gene *EPA1* (Figure 29f). None of the isolates showed the presence of *AGA1* and *PST1* genes.

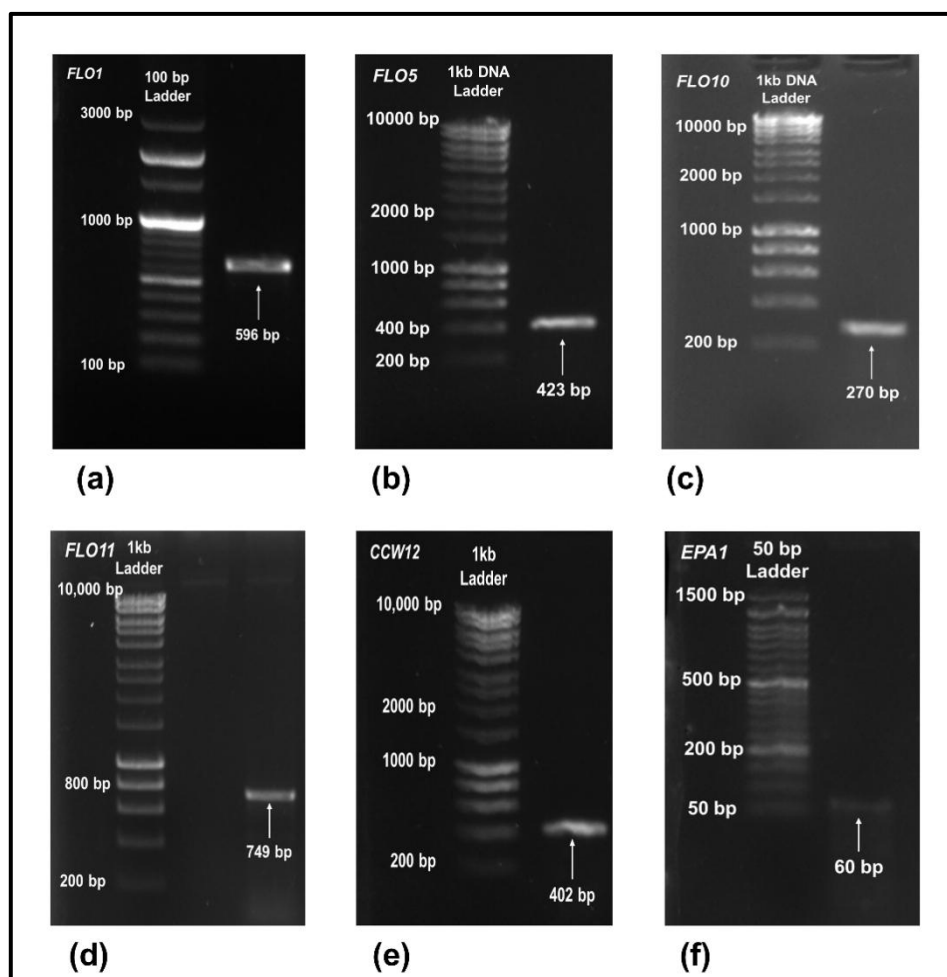


Figure 29: Agarose gel electrophoresis showing PCR amplification of adhesion genes, namely (a) *FLO1*, (b) *FLO5*, (c) *FLO10*, (d) *FLO11*, (e) *CCW12*, and (f) *EPA1*. Amplicon size was confirmed using a 50 bp (MBT084, HiMedia, India), 100 bp (MBT049, HiMedia, India) and 1 kb DNA ladder (MBT051, HiMedia, India) in a 1% agarose gel, and the gel was visualized in a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

4.3.11.4 Detection of antimicrobial genes

Yeast isolates were also examined for the genes *khs* and *pelA*, which are thought to be involved in antimicrobial activity. About 25% of the isolates from cow *dahi* and 8.33% of the isolates from cow *chhurpi* detected the *khs* gene (Figure 30a), but it was not detected in the isolates from yak *dahi* and *chhurpi*. The *pelA* gene was detected in 8.33% of cow *chhurpi* isolates and 16.67% of the isolates from yak *chhurpi* (Figure 30b), and not detected in isolates from cow *dahi* and yak *dahi*.

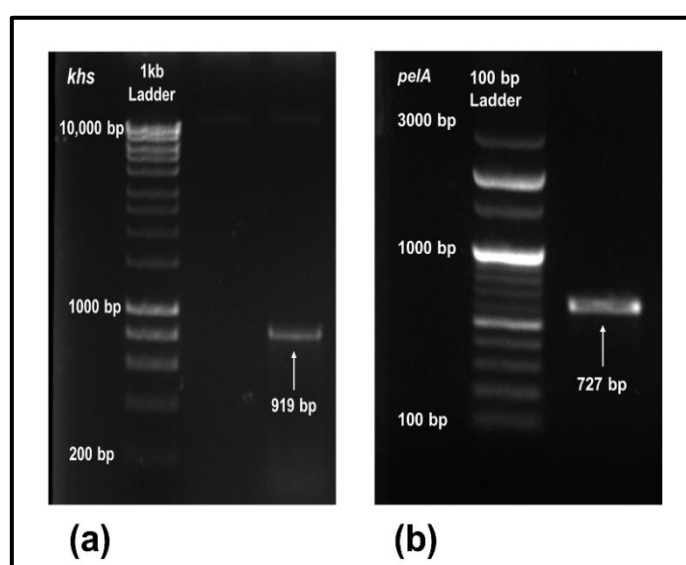


Figure 30: Agarose gel electrophoresis showing PCR amplification of antimicrobial genes, namely (a) *khs* and (b) *pelA*. Amplicon size was confirmed using a 100 bp (MBT049, HiMedia, India) and 1 kb DNA ladder (MBT051, HiMedia, India) in a 1% agarose gel, and the gel was visualized in a Gel Doc™ EZ Imager (BioRad, Hercules, CA, USA).

4.4 Safety evaluation of the yeast isolates

After an *in vitro* screening of the probiotic properties, the yeast isolates were subjected to a safety evaluation by performing Hemolytic activity, DNase activity, gelatinase activity, biogenic amine production, coagulase activity, urease activity, pseudohyphae

formation, lipase activity, phospholipase activity, mucin degradation, and antibiotic and antifungal susceptibility testing. The yeast isolates were tested negative for hemolysis, which indicates an inability to lyse red blood cells. The yeast isolates were unable to produce gelatinase and DNase, which are able to break down gelatin and DNA, respectively. High levels of biogenic amines in the food have potential to cause food poisoning and show allergic reactions such as headache, nausea, respiratory distress, hypertension, and histamine poisoning. Mucins act as a barrier which keep pathogenic microbes from adhering to the lining of the epithelium. In our study, it was found that the yeast isolates did not produce biogenic amines or break down mucin. Phospholipase and urease activities were absent in all yeast isolates. Both phospholipase and urease activity are critical parameters for evaluating the safety of potential probiotic yeast isolates. Since these are key factors in microbial virulence, yeast isolates must demonstrate negative phospholipase and urease activity. However, lipase activity and the formation of pseudohyphae were demonstrated by *Meyerozyma guilliermondii* CMN-3 and *Yarrowia lipolytica* CBG-9 from cow *chhurpi*, *Candida metapsilosis* CKY-12 and *Y. lipolytica* CZBY-49 from yak *chhurpi*, and *C. parapsilosis* DLAY-48 and *Y. lipolytica* DUAY-11 from yak *dahi*. *Y. lipolytica* CLAY-2 was found to exhibit lipase activity. Lipase is an enzyme that hydrolyses triglycerides to produce free fatty acids and glycerol. In addition to being necessary for lipid metabolism, it is thought to be a virulence factor for certain harmful microbes. By dissolving host lipid membranes, lipase activity aids in tissue invasion by allowing microorganisms to enter host cells. Thus, it is anticipated that the probiotic isolates will not exhibit lipase activity or the formation of pseudohyphae in order to protect host tissue. With the exception of *C. parapsilosis* DLAY-48 from yak *dahi*, none of the yeast isolates exhibited coagulase activity. For antibiotic and antifungal activity, all isolates from cow *dahi* and *chhurpi*

showed susceptibility to antifungal agents tested. *Saccharomyces cerevisiae* DMS-15 from cow *dahi* and *M. guilliermondii* CMN-3 from cow *chhurpi* showed intermediate results for gentamicin and penicillin G, respectively; other isolates from cow *dahi* and *chhurpi* were found to be resistant to other antibiotics namely ampicillin, clindamycin, vancomycin, co-trimoxazole, tetracycline, and erythromycin. From yak *dahi*, *Y. lipolytica* DZBY-37 showed intermediate results for voriconazole and Itraconazole; *Y. lipolytica* CLAY-2 from yak *chhurpi* showed intermediate results for itraconazole. From yak *dahi*, *C. parapsilosis* DLAY-48 showed intermediate results for gentamicin and vancomycin, and *Y. lipolytica* DUAY-11 from yak *dahi* showed intermediate results for erythromycin. *C. metapsilosis* CKY-12 from yak *chhurpi* showed intermediate results for vancomycin (Figure 31-32). When assessing the safety of probiotic yeast isolates, testing for antibiotic resistance is an essential component. During antibiotic treatments, probiotic yeast isolates should naturally be resistant to antibacterial antibiotics in order to survive in the human gut. Yeast isolates, however, must not harbour or transmit antibiotic resistance genes that might be horizontally transferred to other microbes. Probiotic yeast isolates, on the other hand, need to be sensitive to antifungal medications in order to shield immunocompromised patients from drug-resistant yeast infections and prevent the spread of genes that may develop the resistance.

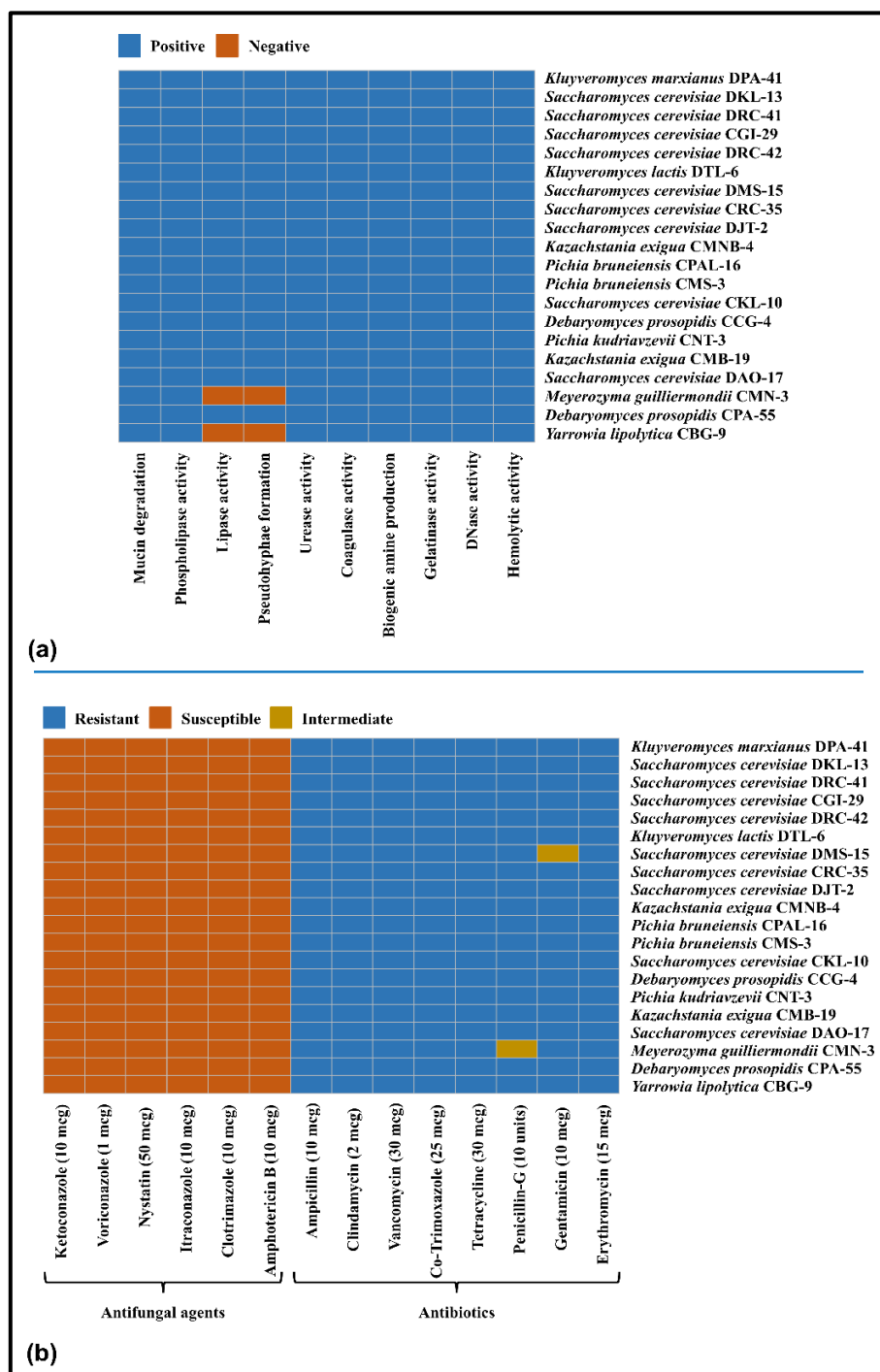


Figure 31: Safety evaluation of the screened yeast isolates from cow *dahi* and *chhurpi*.

(a) Hemolytic activity, DNase activity, gelatinase activity, biogenic amine production, pseudohyphae formation, urease activity, lipase activity, phospholipase activity, coagulase activity, and mucin degradation. (b) Antibiotics and antifungal susceptibility testing.

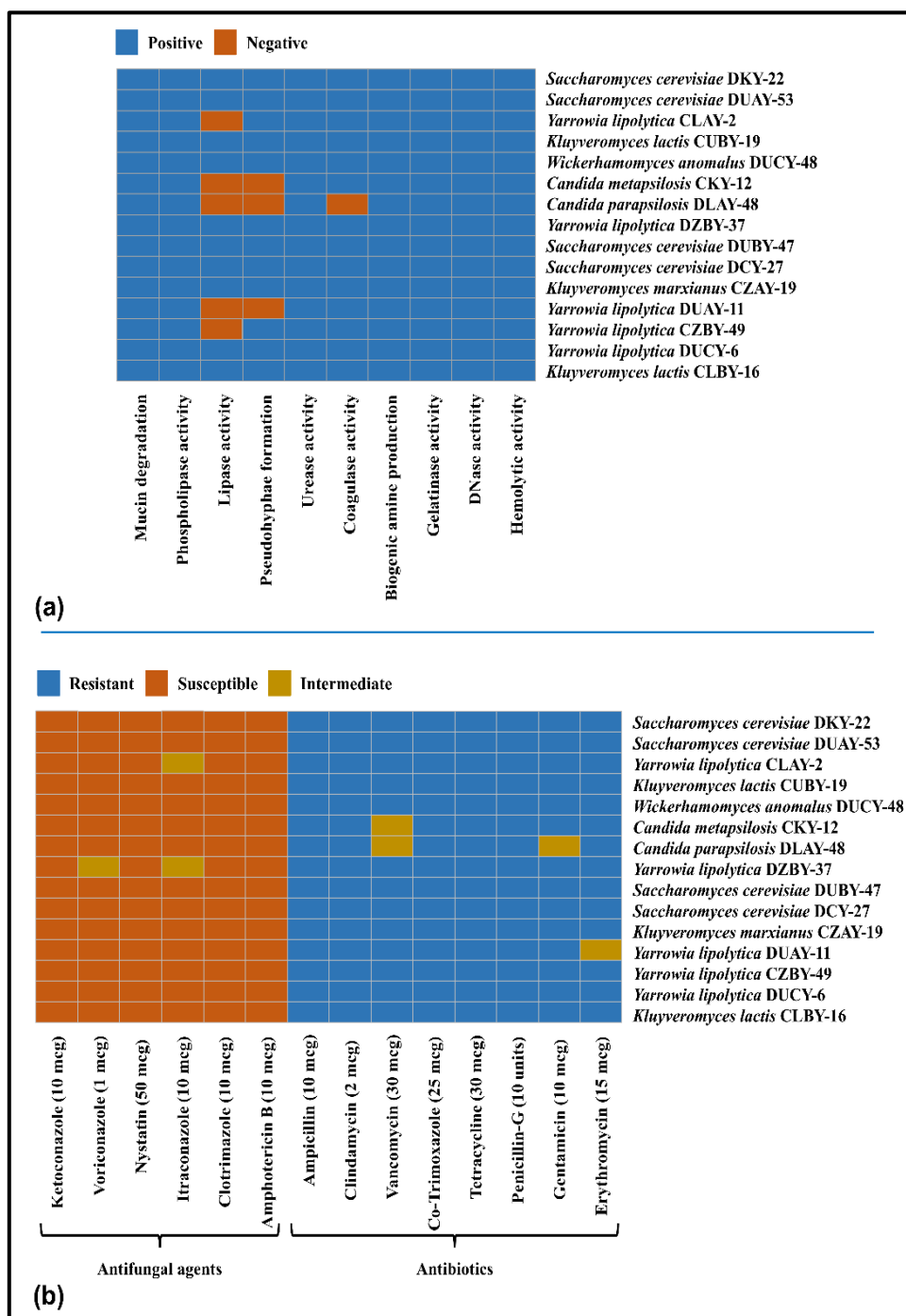


Figure 32: Safety evaluation of the screened yeast isolates from yak *dahi* and *chhurpi*.

(a) Hemolytic activity, DNase activity, gelatinase activity, biogenic amine production, pseudohyphae formation, urease activity, lipase activity, phospholipase activity, coagulase activity, and mucin degradation. (b) Antibiotics and antifungal susceptibility testing.

4.5 Grouping and selection of the most potent probiotic yeast isolates from NFM products of Sikkim

Based on the *in vitro* probiotic test performed, yeast isolates were grouped on the basis of similarities and dissimilarities of the results using a heatmap, and most potential probiotic isolates were selected using the principal component analysis (PCA) biplot score value. For qualitative tests, "1" or "0" indicated for presence and absence, respectively, and all quantitative values were normalized using a $\log(x+1)$ transformation. Analysis of the heatmap visualization revealed a significant clustering that formed two significant clusters (Cluster A and B) by the yeast isolates from cow (Figure 33a) and yak (Figure 33b) milk products. For isolates from cow *dahi* and *chhurpi*, *Saccharomyces cerevisiae* DAO-17, *S. cerevisiae* DKL-13, *S. cerevisiae* DMS-15, *Kluyveromyces marxianus* DPA-41 from cow *dahi*, and *S. cerevisiae* CRC-35 and *Pichia bruneiensis* CPAL-16 from cow *chhurpi* grouped in cluster A, and *S. cerevisiae* DJT-2, *Kl. lactis* DTL-6, *S. cerevisiae* DRC-41, and *S. cerevisiae* DRC-42 from cow *dahi*, and *D. prosopidis* CPA-55, *P. bruneiensis* CMS-3, *Kazachstania exigua* CMB-19, *K. exigua* CMNB-4, *Meyerozyma guilliermondii* CMN-3, *P. kudriavzevii* CNT-3, *S. cerevisiae* CGI-29, *S. cerevisiae* CKL-10, *D. prosopidis* CCG-4, and *Y. lipolytica* CBG-9 from cow *chhurpi* falls in cluster B (Figure 33a). For isolates from yak *dahi* and *chhurpi*, *Y. lipolytica* DUCY-6, *Wickerhamomyces anomalus* DUCY-48, and *Candida parapsilosis* DLAY-48 from yak *dahi*, and *Y. lipolytica* CLAY-2, and *Kl. lactis* CLBY-16 from yak *chhurpi* grouped in cluster A, and *S. cerevisiae* DKY-22, *S. cerevisiae* DCY-27, *Y. lipolytica* DUAY-11, *S. cerevisiae* DUAY-53, *S. cerevisiae* DUBY-47, and *Y. lipolytica* DZBY-37 from yak *dahi*, and *Kl. marxianus* CZAY-19, *Y. lipolytica* CZBY-49, *C. metapsilosis* CKY-12, and *Kl. lactis* CUBY-19 from yak *chhurpi* grouped in cluster B (Figure 33b).

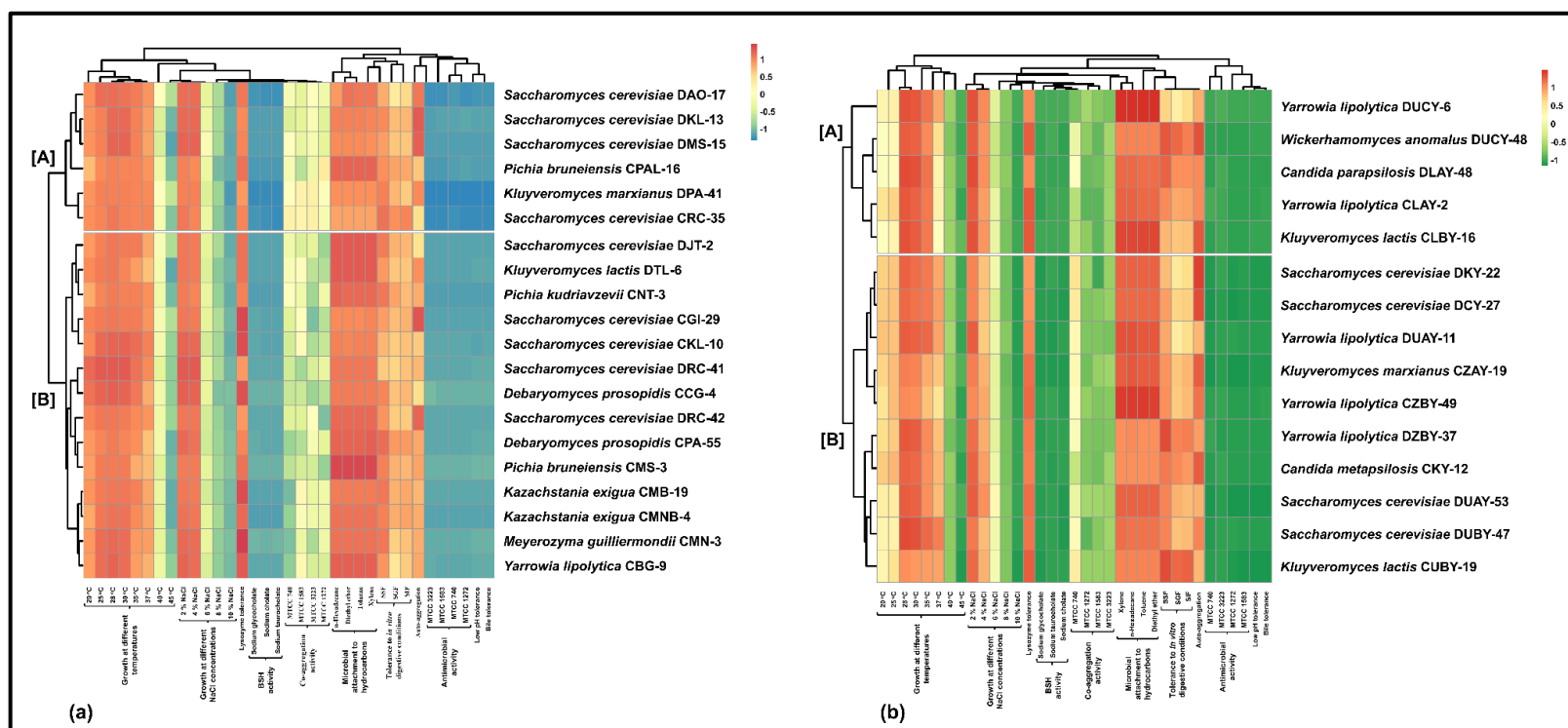


Figure 33: Heatmap visualization of the potential probiotic yeast isolates from *dahi* and *chhurpi* from (a) cow and (b) yak based on low pH tolerance, bile tolerance, microbial attachment to hydrocarbons (MATHs), auto-aggregation, co-aggregation, BSH activity, antimicrobial activity, growth at different temperatures, growth at different salt concentrations, lysozyme tolerance, and tolerance to in vitro digestive conditions. Based on the similarity and dissimilarity of the results, isolates were grouped into two clusters, cluster A and B.

For isolates from cow *dahi* and *chhurpi*, PC1 and PC2 component of PCA biplot shows the variance of 46.64% and 24.62%, respectively (Figure 34a). Three clusters (Cluster A, B and C) were formed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Euclidean similarity index in a hierarchical clustering system (Figure 34b). Cluster A comprises of *S. cerevisiae* DRC-41, *D. prosopidis* CCG-4, *S. cerevisiae* CKL-10, *K. exigua* CMB-19, *K. exigua* CMNB-4, *M. guilliermondii* CMN-3, *Y. lipolytica* CBG-9, *P. bruneiensis* CMS-3, *S. cerevisiae* DJT-2, *D. prosopidis* CPA-55, *P. bruneiensis* CPAL-16, and *P. kudriavzevii* CNT-3, while cluster B consist of *S. cerevisiae* DKL-13, *S. cerevisiae* DRC-42, *S. cerevisiae* DMS-15, *Kl. lactis* DTL-6, *S. cerevisiae* CGI-29, and *S. cerevisiae* DAO-17, and cluster C comprises of *Kl. marxianus* DPA-41 and *S. cerevisiae* CRC-35. Based on score value obtained by the isolates on variance-covariance matrix at 10K bootstrap value, *Kl. marxianus* DPA-41 and *S. cerevisiae* CRC-35 from cow *dahi* and *chhurpi*, were found to have highest potential as probiotic yeast.

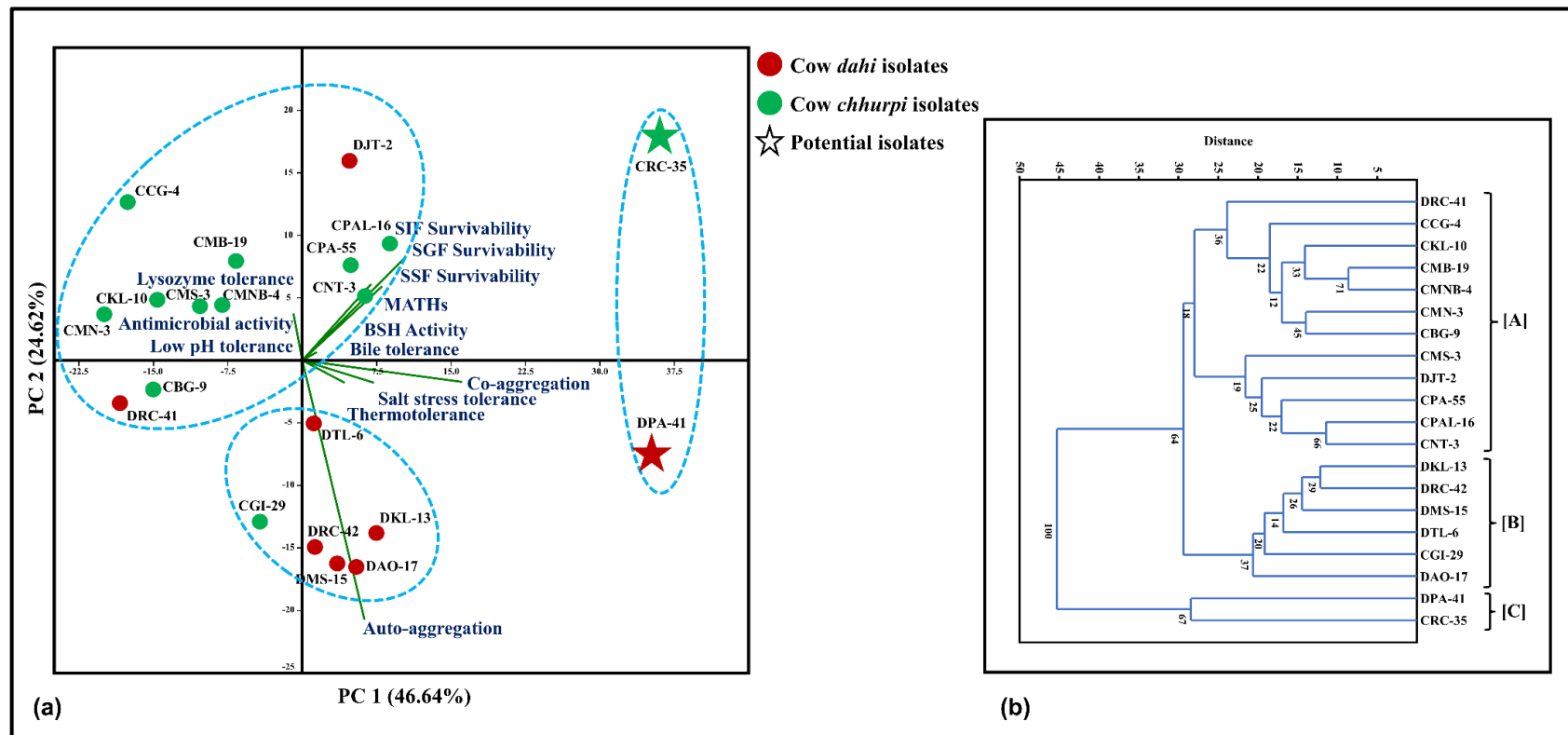


Figure 34: Selection of the most potential probiotic yeast isolates from cow *dahi* and *chhurpi* using (a) PCA bi-plot analysis and (b) a hierarchical clustering system illustrates the formation of the clusters.

Similarly, A PCA biplot was designed for isolates from yak *dahi* and *chhurpi* (Figure 35a). The PC1 component shows 52.61% variance, while the PC2 component shows 22.45% variance based on the variables. Three clusters (Cluster A, B and C) were formed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Euclidean similarity index in a hierarchical clustering system (Figure 35b) and isolates *W. anomalus* DUCY-48 and *Y. lipolytica* DUAY-11 was found to be out-grouped. Cluster A comprises of *Y. lipolytica* DUCY-6, *C. metapsilosis* CKY-12, *Y. lipolytica* DZBY-37, *C. parapsilosis* DLAY-48, and *Kl. marxianus* CZAY-19, while cluster B comprises of *Y. lipolytica* CLAY-2, *S. cerevisiae* DUBY-47, *Y. lipolytica* CZBY-49, *S. cerevisiae* DKY-22, and *Kl. lactis* CLBY-16, and cluster C comprises of *Kl. lactis* CUBY-19, *S. cerevisiae* DUAY-53, and *S. cerevisiae* DCY-27. Hence, based on score value obtained by the isolates on variance-covariance matrix at 10k bootstrap value, *S. cerevisiae* DUAY-53 and *Kl. lactis* CUBY-19 from yak *dahi* and *chhurpi*, were found to have highest potential as probiotic yeast.

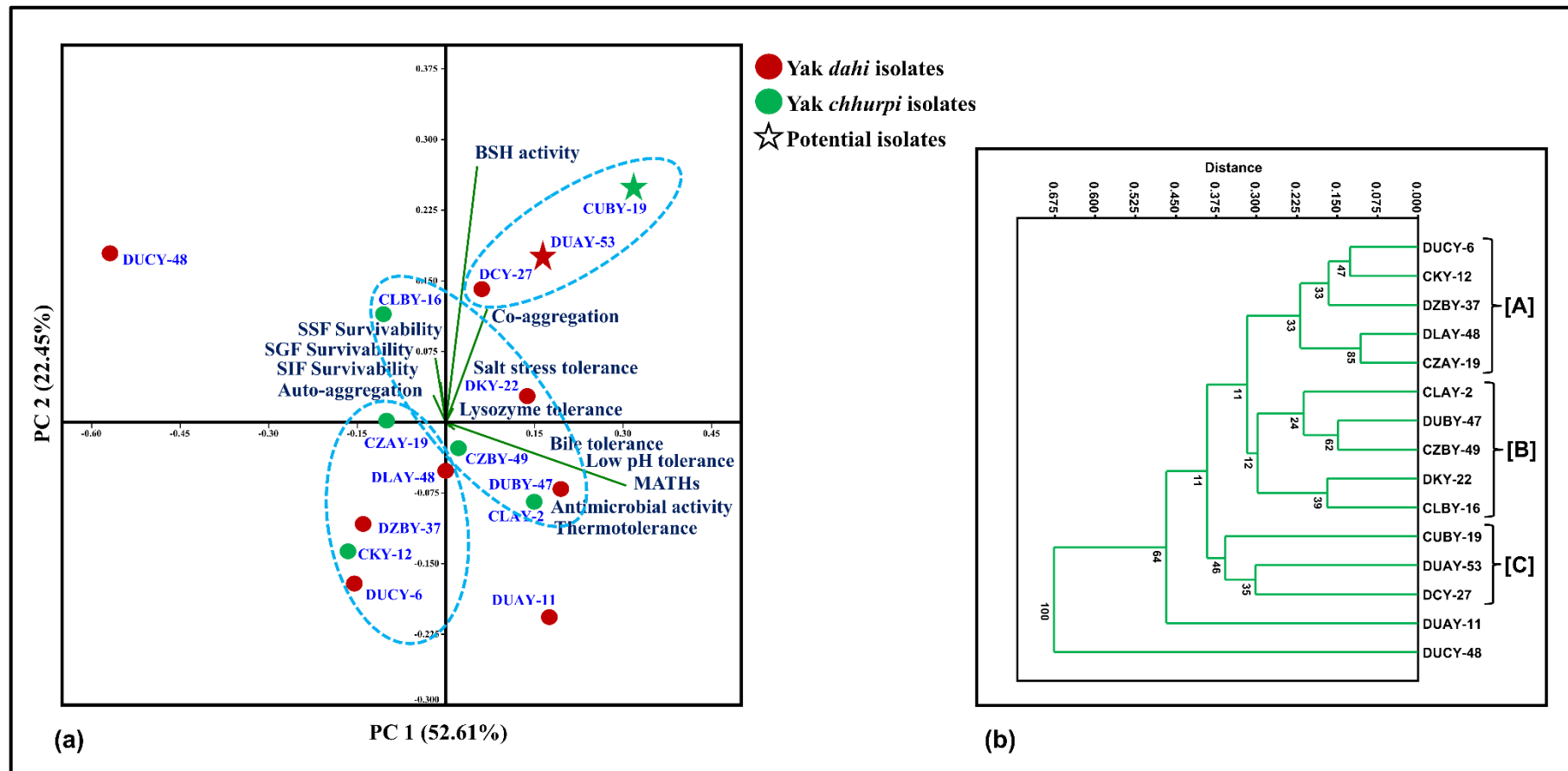


Figure 35: Selection of the most potential probiotic yeast isolates from yak *dahi* and *chhurpi* using (a) PCA bi-plot analysis and (b) a hierarchical clustering system illustrates the formation of the clusters.

Objective 3:

To determine the functional properties of probiotic yeast.

4.6 SCREENING FOR FUNCTIONAL PROPERTIES OF THE POTENTIAL YEAST ISOLATES FROM NFM PRODUCTS OF SIKKIM

4.6.1 Cholesterol lowering activity

Potential probiotic yeast isolates illustrate cholesterol-lowering properties, seen as a beneficial functional characteristic for health-promoting microorganisms. The ability of isolates from cow *dahi* and *chhurpi* to lower cholesterol ranges from 9.97 ± 0.8 to $54.48 \pm 0.7\%$ (Figure 36), whereas isolates from yak *dahi* and *chhurpi* range from 7.00 ± 0.7 to $37.37 \pm 0.8\%$ (Figure 37). The highest capacity to decrease cholesterol was demonstrated by *Saccharomyces cerevisiae* DRC-41 ($54.48 \pm 0.7\%$) from cow *dahi*, *S. cerevisiae* CRC-35 ($36.23 \pm 0.6\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($30.51 \pm 0.6\%$) from yak *dahi*, and *Yarrowia lipolytica* CZBY-49 ($37.37 \pm 0.8\%$) from yak *chhurpi*.

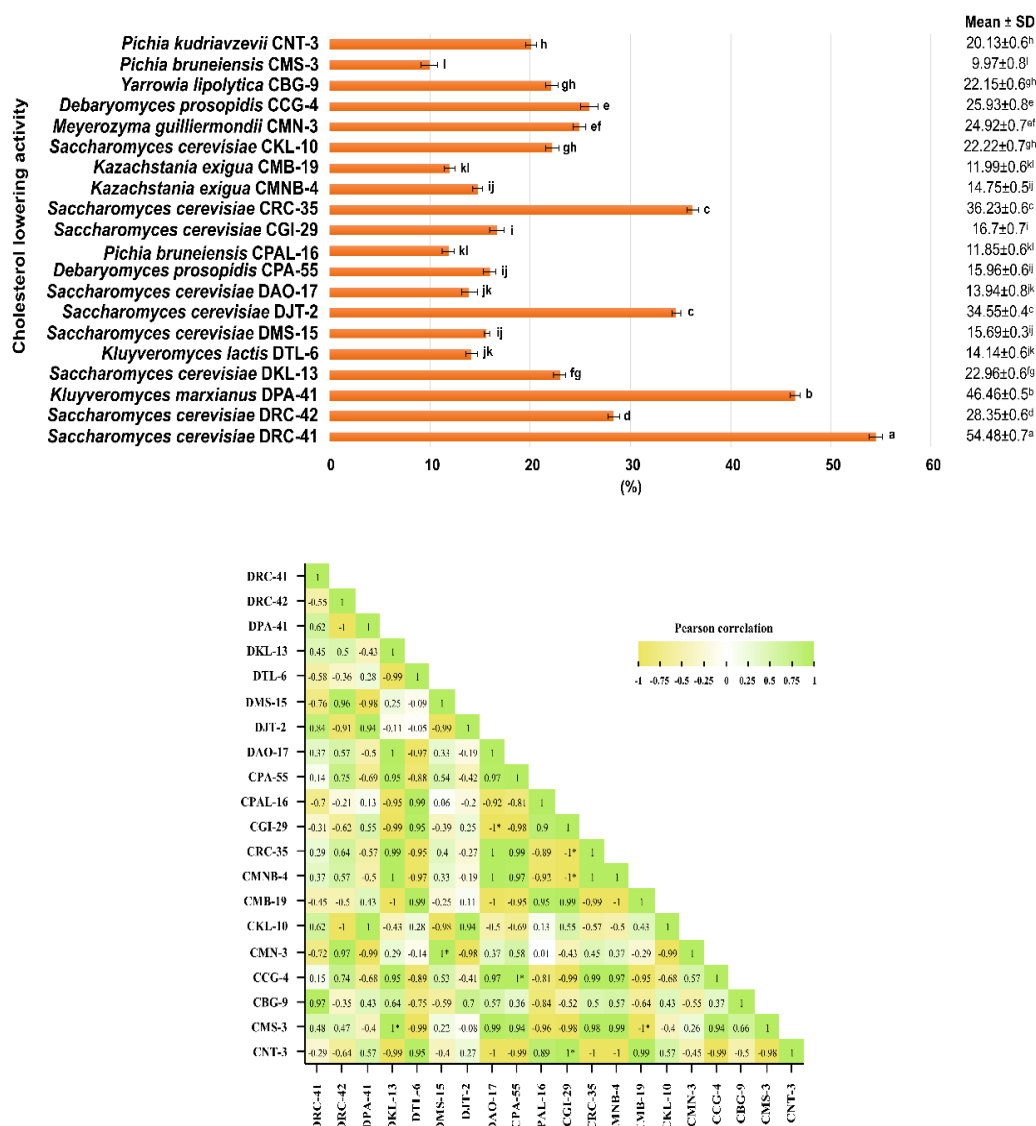


Figure 36: Cholesterol lowering activity (%) of potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for cholesterol lowering activity (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.



Figure 37: Cholesterol lowering activity (%) of potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for cholesterol lowering activity (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.6.2 Anti-oxidant activity

Antioxidant activity is a vital functional trait of potential probiotic yeast isolates, contributing to their potential health benefits. The antioxidant activity of the yeast isolates was determined based on the percentage reduction of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Antioxidant activity varies between 10.84 ± 0.9 and $82.22 \pm 0.3\%$ for yeast isolates from cow *dahi* and *chhurpi* (Figure 38) and between 24.24 ± 0.6 and $63.77 \pm 0.3\%$ for yeast isolates from yak *dahi* and *chhurpi* (Figure 39). The maximum antioxidant activity was detected in *Candida parapsilosis* DLAY-48 ($62.63 \pm 0.3\%$) from yak *dahi*, *Kluyveromyces lactis* CUBY-19 ($63.77 \pm 0.3\%$) from yak *chhurpi*, and *Kl. marxianus* DPA-41 ($82.22 \pm 0.3\%$) from cow *dahi* and *Saccharomyces cerevisiae* CRC-35 ($66.13 \pm 0.7\%$) from cow *chhurpi*.

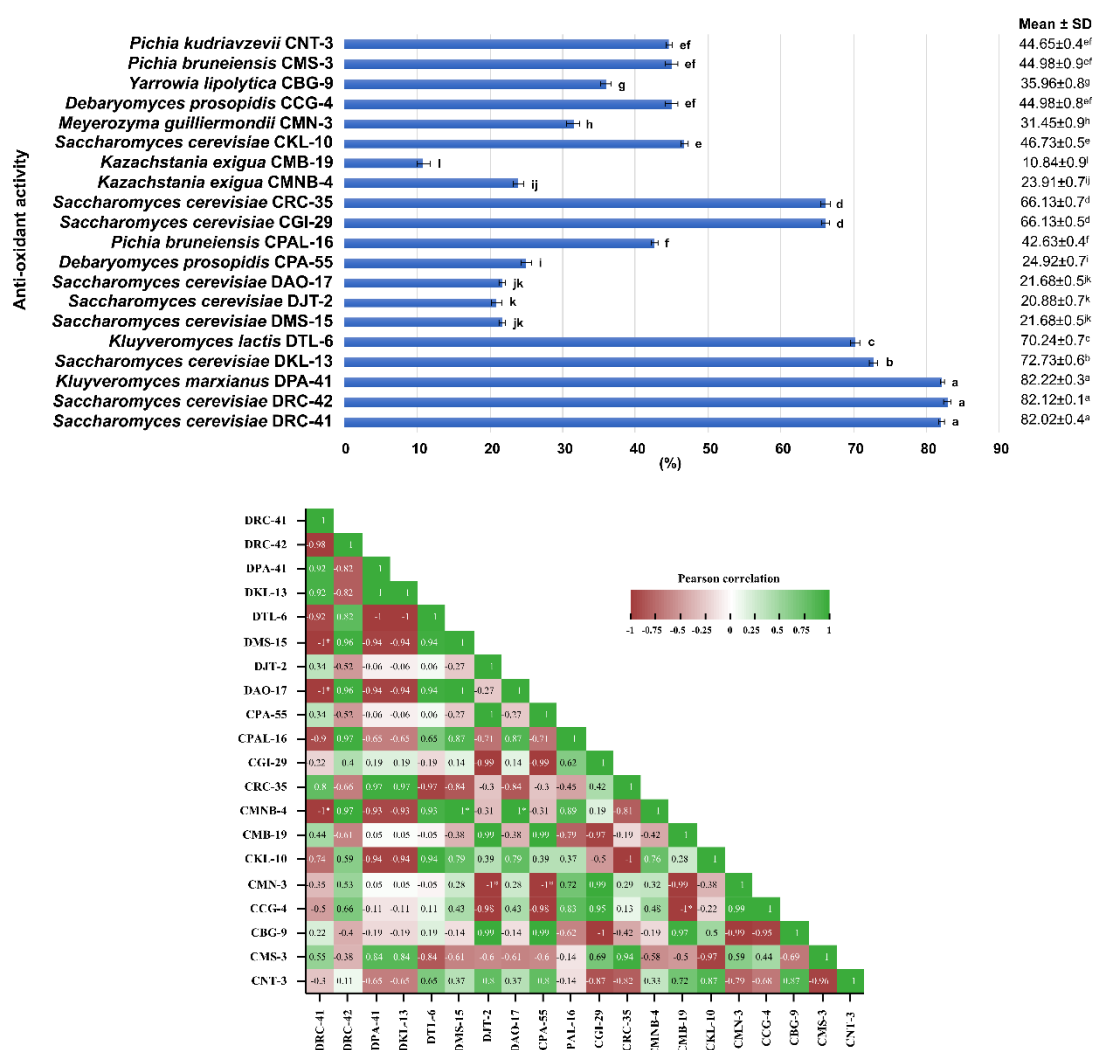


Figure 38: Anti-oxidant activity (%) of potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-oxidant activity (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

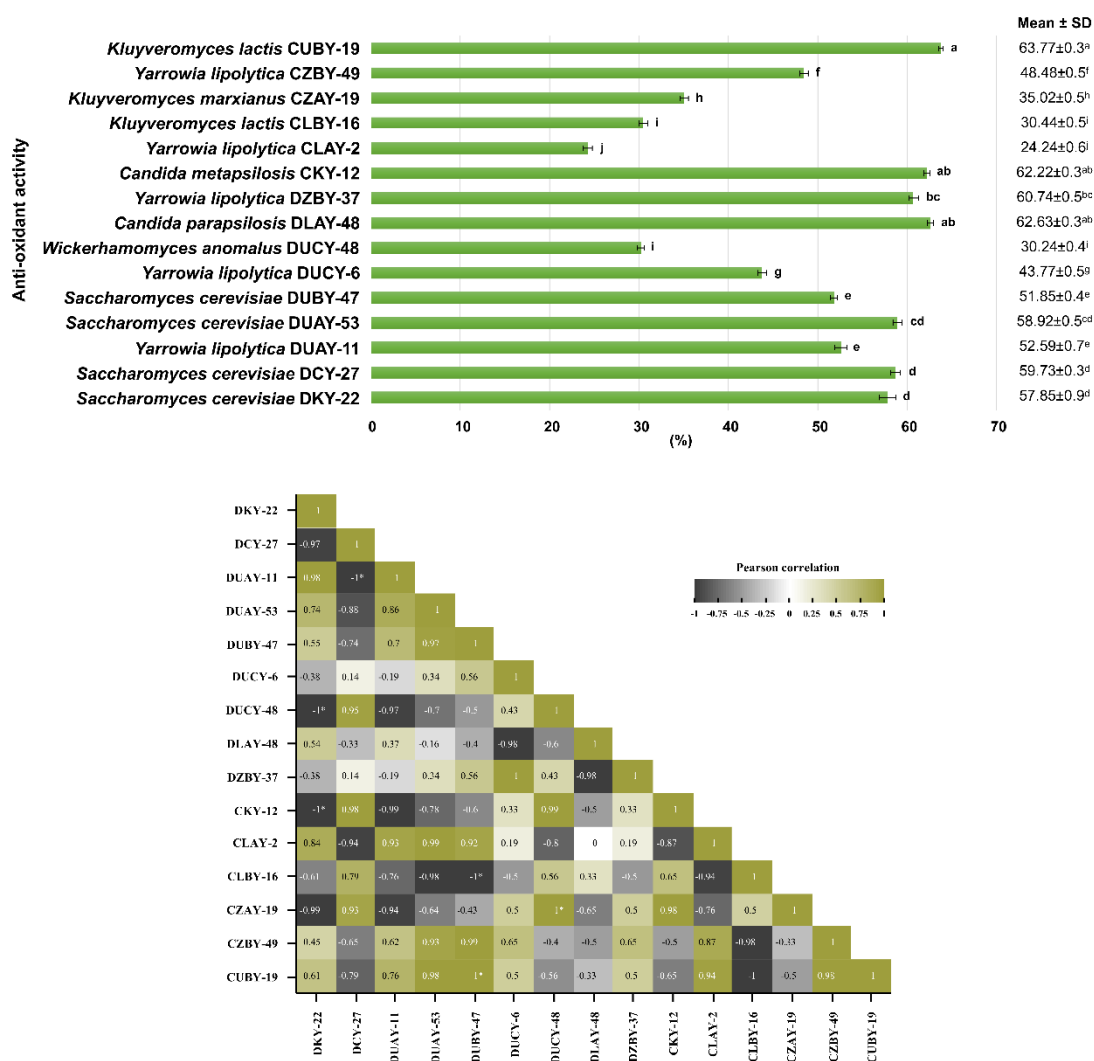


Figure 39: Anti-oxidant activity (%) of potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-oxidant activity (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.6.3 Anti-inflammatory activity

Anti-inflammatory activity is an emerging functional attribute of potential probiotic yeast, enhancing their potential as therapeutic agents. The anti-inflammatory activity of yeast isolates was investigated using the protein denaturation inhibition assay. Yeast isolates from cow *dahi* and *chhurpi* had anti-inflammatory activity ranging from 24.08 ± 0.72 to $59.74 \pm 0.33\%$ (Figure 40), whereas isolates from yak *dahi* and *chhurpi* exhibit anti-inflammatory activity ranging from 47.84 ± 0.81 to $76.84 \pm 0.15\%$ (Figure 41). The highest levels of anti-inflammatory activity were demonstrated by *Kluyveromyces marxianus* DPA-41 ($59.74 \pm 0.33\%$) from cow *dahi*, *Saccharomyces cerevisiae* CRC-35 ($58.03 \pm 0.7\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($76.84 \pm 0.15\%$) from yak *dahi*, and *Kl. lactis* CUBY-19 ($67.33 \pm 0.56\%$) from yak *chhurpi*.

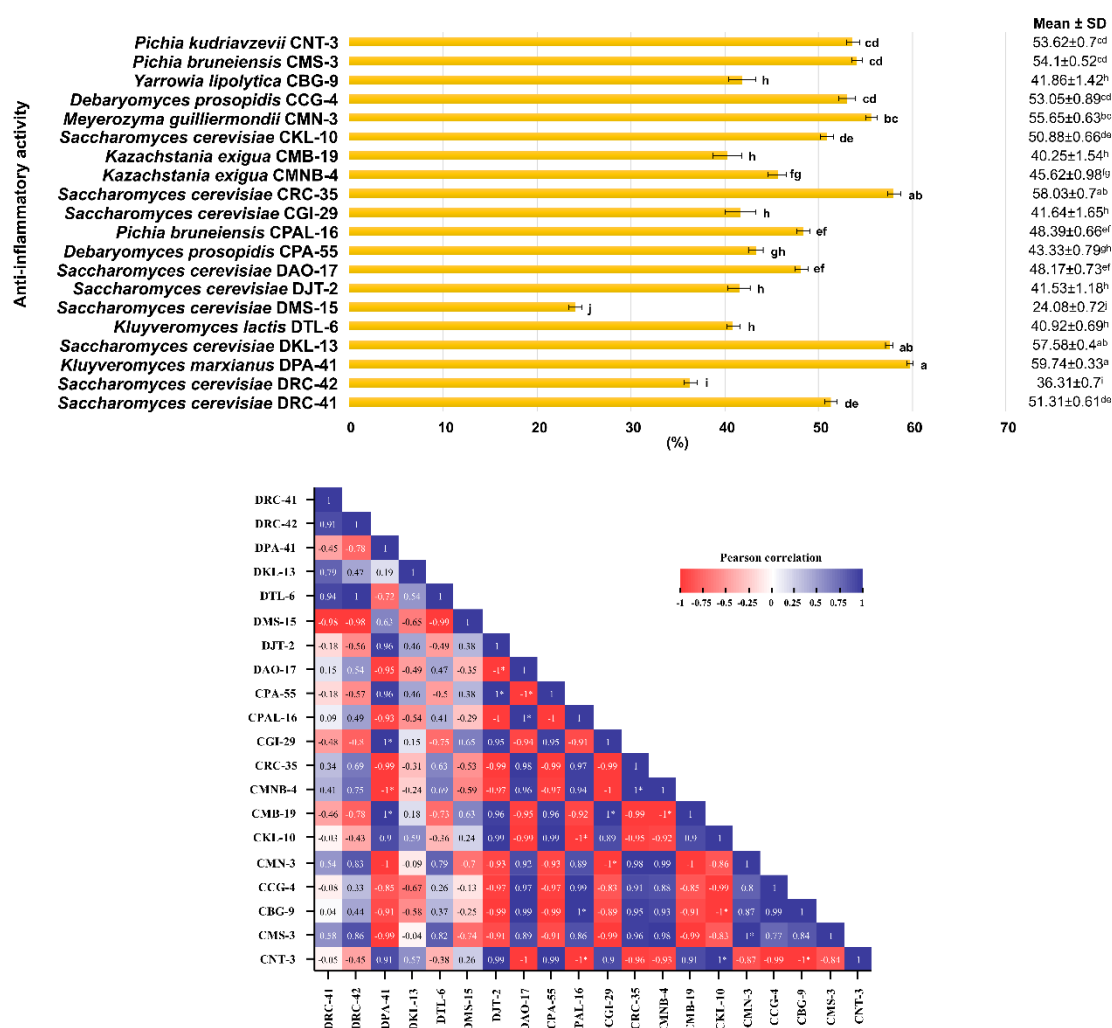


Figure 40: Anti-inflammatory activity (%) of potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-inflammatory activity (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

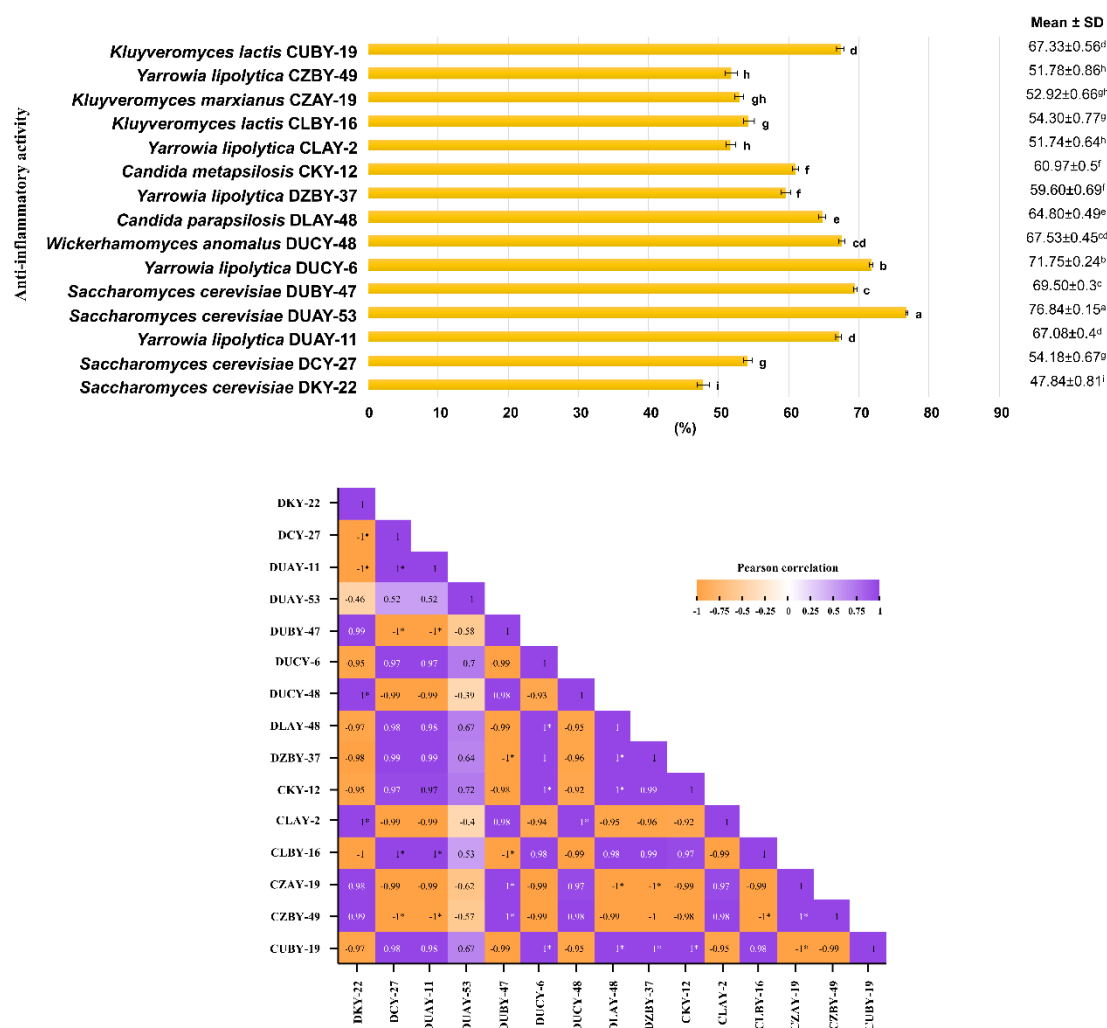


Figure 41: Anti-inflammatory activity (%) of potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-inflammatory activity (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.6.4 Anti-diabetic activity

The anti-diabetic activity of the yeast isolates from cow *dahi* and *chhurpi* ranges from 0.11 ± 0.02 to $12.1 \pm 0.86\%$ (Figure 42), while that of the isolates from yak *dahi* and *chhurpi* ranges from 0.22 ± 0.09 to $5.6 \pm 0.58\%$ (Figure 43). *Kluyveromyces marxianus* DPA-41 ($12.1 \pm 0.86\%$) from cow *dahi*, *Saccharomyces cerevisiae* CRC-35 ($11.49 \pm 0.45\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($5.6 \pm 0.58\%$) from yak *dahi*, and *Kl. lactis* CUBY-19 ($1.27 \pm 0.93\%$) from yak *chhurpi* demonstrated the highest degree of anti-diabetic activity.

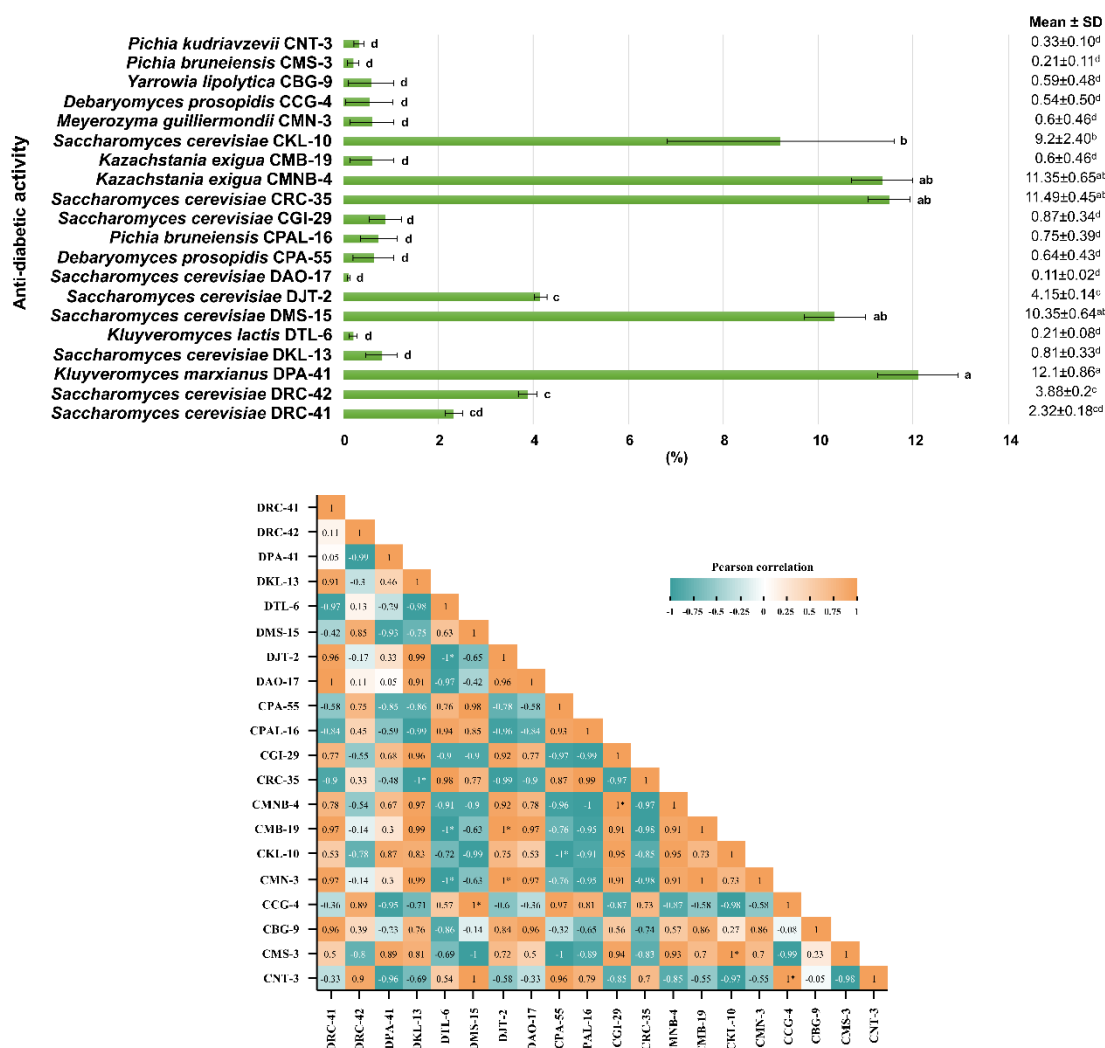


Figure 42: Anti-diabetic activity (%) of potential yeast isolates from cow *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean ± SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-diabetic activity (%) shown by yeast isolates from cow *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

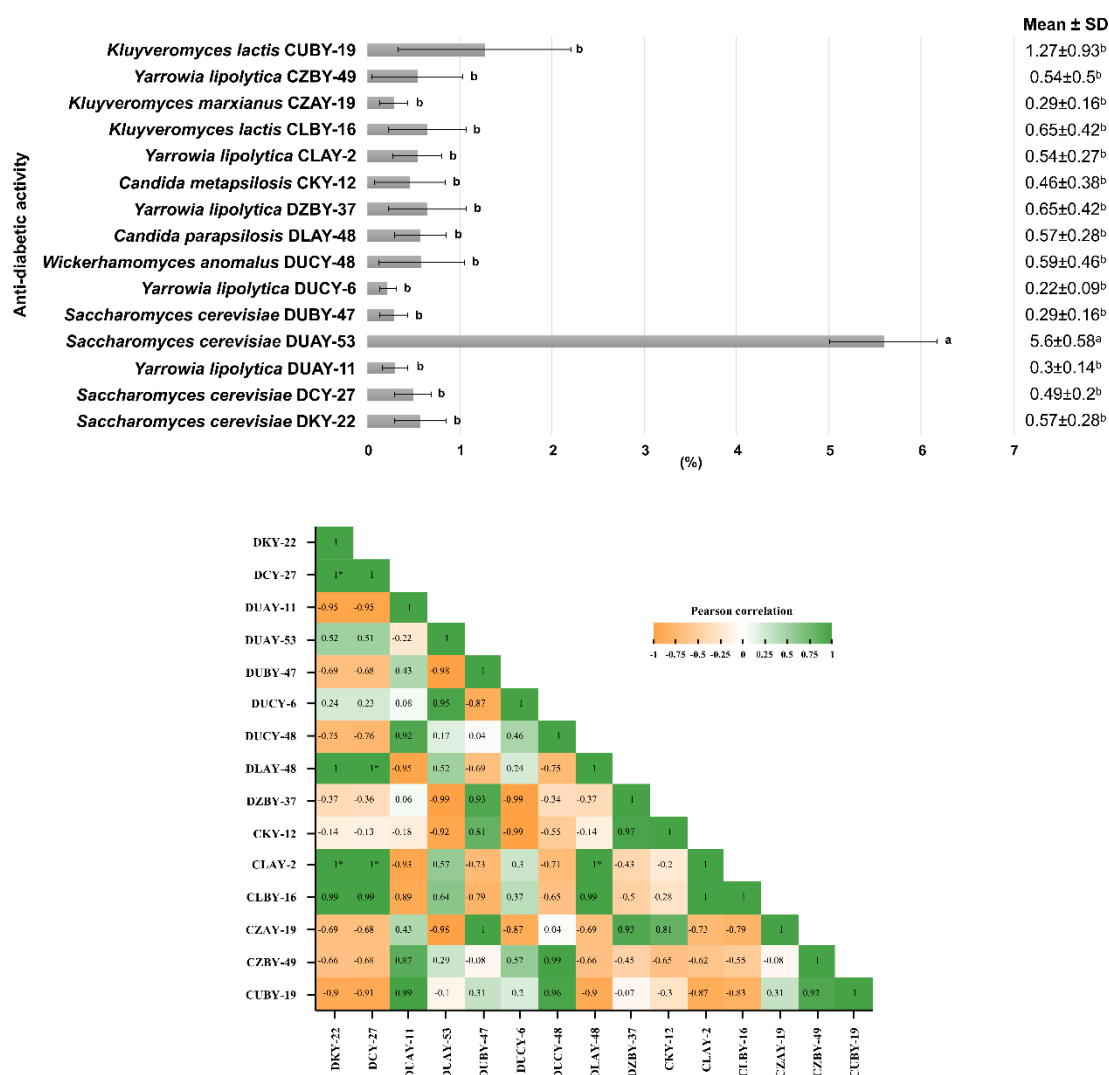


Figure 43: Anti-diabetic activity (%) of potential yeast isolates from yak *dahi* and *chhurpi* of Sikkim. Results are expressed as the mean $\pm$ SD (n = 3). Significant statistical differences ($p < 0.05$) between yeast isolates according to Tukey's honestly significant difference (HSD) test from one-way analysis of variance (ANOVA) have been indicated by different letters. The triangle heatmap shows the Pearson paired-wise correlation for anti-diabetic activity (%) shown by yeast isolates from yak *dahi* and *chhurpi*. A 2-tailed test of significance is used, and '*' signifies the significant correlation at the 0.05 level.

4.6.5 Exopolysaccharide production

On plate-based assay, *Kluyveromyces marxianus* DPA-41 and *Kl. lactis* DTL-6 from cow *dahi*, *Debaryomyces prosopidis* CPA-55, *Meyerozyma guilliermondii* CMN-3 and *D. prosopidis* CCG-4 from cow *chhurpi*, *Saccharomyces cerevisiae* DKY-22, *S. cerevisiae* DCY-27, *Yarrowia lipolytica* DUCY-6 and *Y. lipolytica* DUAY-11 from yak *dahi*, and *Y. lipolytica* CLAY-2, *Y. lipolytica* CZBY-49 and *Kl. lactis* CUBY-19 from yak *chhurpi* showed positive result for exopolysaccharide production.

4.6.6 Phytate hydrolyzing activity

The majority of the isolates were found to hydrolyse phytate, except *Saccharomyces cerevisiae* DRC-42 and *S. cerevisiae* DJT-2 from cow *dahi*, *S. cerevisiae* CGI-29, *S. cerevisiae* CRC-35, and *Yarrowia lipolytica* CBG-9 from cow *chhurpi*, and *Y. lipolytica* DZBY-37 and *Candida metapsilosis* CKY-12 from yak *chhurpi*. All yeast isolates from yak *dahi* were found to hydrolyse phytate.

4.6.7 Beta-D-glucosidase activity

Beta-D-glucosidase activity was observed in *Saccharomyces cerevisiae* DRC-41, *Kluyveromyces marxianus* DPA-41, *S. cerevisiae* DKL-13 and DAO-17 from cow *dahi*, *Pichia bruneiensis* CPAL-16, *S. cerevisiae* CRC-35, *Kazachstania exigua* CMNB-4, *K. exigua* CMB-19, and *Yarrowia lipolytica* CBG-9 from cow *chhurpi*, *Y. lipolytica* DUAY-11, *Y. lipolytica* DUCY-6, *Y. lipolytica* DZBY-37 from yak *dahi*, and *Y. lipolytica* CZBY-49 from yak *chhurpi*.

4.6.8 Beta-galactosidase activity

Beta-galactosidase activity was detected in *Kluyveromyces marxianus* DPA-41 and *Kl. lactis* DTL-6 from cow *dahi*, *Yarrowia lipolytica* CLAY-2, *K. lactis* CLBY-16, *Kl. marxianus* CZAY-19, and *Kl. lactis* CUBY-19 from yak *chhurpi*. It became apparent

that the yeast isolates from yak *dahi* and cow *chhurpi* failed to exhibit beta-galactosidase activity.

4.6.9 Antifungal activity

Antifungal activity of the isolates was evaluated against the reference isolates, namely *Aspergillus niger* NCIM 1248, *Fusarium oxysporum* NCIM 1350, *A. niger* NCIM 1358, *A. oryzae* NCIM 1212, and *A. flavus* NCIM 549. Based on the zone formation, results were categorized into 4 categories: “-” for no zone of inhibition, “+” for zone of more than 5 mm, “++” for zone of more than 10 mm, and “+++” for zone of more than 15 mm. *Saccharomyces cerevisiae* DKL-13 from cow *dahi*; *Pichia bruneiensis* CPAL-16 and *S. cerevisiae* CRC-35 from cow *chhurpi*, and *Yarrowia lipolytica* DUAY-11 from yak *dahi* exhibited highest degree of antifungal activity against tested isolates as shown in table 39 and 40.

Table 39: Antifungal activity of the screened yeast isolates from cow *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Antifungal activity				
		NCIM 1248	NCIM 1350	NCIM 1358	NCIM 1212	NCIM 549
Cow <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DRC-41	-	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DRC-42	+	-	-	-	-
	<i>Kluyveromyces marxianus</i> DPA-41	+	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DKL-13	-	+	+	++	++
	<i>Kluyveromyces lactis</i> DTL-6	+	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DMS-15	-	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DJT-2	-	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DAO-17	-	-	-	+	-
Cow <i>chhurpi</i>	<i>Debaryomyces prosopidis</i> CPA-55	-	-	-	-	-
	<i>Pichia bruneiensis</i> CPAL-16	+	+	-	++	+
	<i>Saccharomyces cerevisiae</i> CGI-29	+	+	-	-	+
	<i>Saccharomyces cerevisiae</i> CRC-35	++	+	+	-	-
	<i>Kazachstania exigua</i> CMNB-4	-	-	-	-	-
	<i>Kazachstania exigua</i> CMB-19	-	-	-	-	-
	<i>Saccharomyces cerevisiae</i> CKL-10	-	-	-	-	-

<i>Meyerozyma guilliermondii</i> CMN-3	-	-	-	+	+
<i>Debaryomyces prosopidis</i> CCG-4	-	-	-	-	-
<i>Yarrowia lipolytica</i> CBG-9	-	-	-	-	-
<i>Pichia bruneiensis</i> CMS-3	+	+	-	-	-
<i>Pichia kudriavzevii</i> CNT-3	-	-	-	+	-

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone of inhibition. NCIM 1248: *Aspergillus niger* NCIM 1248; NCIM 1350: *Fusarium oxysporum* NCIM 1350; NCIM 1358: *Aspergillus niger* NCIM 1358; NCIM 1212: *Aspergillus oryzae* NCIM 1212; and NCIM 549: *Aspergillus flavus* NCIM 549.

Table 40: Antifungal activity of the screened yeast isolates from yak *dahi* and *chhurpi* of Sikkim.

NFM products	Isolates	Antifungal activity				
		NCIM 1248	NCIM 1350	NCIM 1358	NCIM 1212	NCIM 549
Yak <i>dahi</i>	<i>Saccharomyces cerevisiae</i> DKY-22	+	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DCY-27	+	+	-	-	-
	<i>Yarrowia lipolytica</i> DUAY-11	+	+	-	++	++
	<i>Saccharomyces cerevisiae</i> DUAY-53	+	-	-	-	-
	<i>Saccharomyces cerevisiae</i> DUBY-47	-	-	-	-	-
	<i>Yarrowia lipolytica</i> DUCY-6	+	-	-	-	++
	<i>Wickerhamomyces anomalus</i> DUCY-48	+	-	-	-	-
	<i>Candida parapsilosis</i> DLAY-48	-	+	-	-	-
	<i>Yarrowia lipolytica</i> DZBY-37	+	+	-	-	-
	<i>Candida metapsilosis</i> CKY-12	-	-	-	-	-
Yak <i>chhurpi</i>	<i>Yarrowia lipolytica</i> CLAY-2	+	-	-	-	-
	<i>Kluyveromyces lactis</i> CLBY-16	+	-	-	-	-
	<i>Kluyveromyces marxianus</i> CZAY-19	+	-	-	-	-
	<i>Yarrowia lipolytica</i> CZBY-49	+	+	-	-	-
	<i>Kluyveromyces lactis</i> CUBY-19	++	-	+	-	-

Note: Experiment were conducted in triplicate. “+++” > 15mm, “++” > 10mm, and “+” > 5mm, and “-” denotes no activity or no zone of inhibition. NCIM 1248: *Aspergillus niger* NCIM 1248; NCIM 1350: *Fusarium oxysporum* NCIM 1350; NCIM 1358: *Aspergillus niger* NCIM 1358; NCIM 1212: *Aspergillus oryzae* NCIM 1212; and NCIM 549: *Aspergillus flavus* NCIM 549.

4.6.10 Siderophore production

A plate-based assay demonstrated that *Saccharomyces cerevisiae* DKL-13 from cow *dahi*, *Meyerozyma guilliermondii* CMN-3 and *Pichia bruneiensis* CMS-3 from cow *chhurpi*, *S. cerevisiae* DCY-27, *Yarrowia lipolytica* DUAY-11, *S. cerevisiae* DUAY-53, *S. cerevisiae* DUBY-47, *Y. lipolytica* DUCY-6, and *Wickerhamomyces anomalus* DUCY-48 from yak *dahi*, and *Y. lipolytica* CLAY-2 and *Y. lipolytica* CZBY-49 from yak *chhurpi* produce siderophore.

4.6.11 Vitamin B12 production

Vitamin B12 was found to be produced by *Saccharomyces cerevisiae* DRC-42, *Kluyveromyces marxianus* DPA-41, *S. cerevisiae* DKL-13, *Kl. lactis* DTL-6, and *S. cerevisiae* DJT-2 from cow *dahi*, *S. cerevisiae* CGI-29, *S. cerevisiae* CRC-35, *Debaryomyces prosopidis* CCG-4, *Yarrowia lipolytica* CBG-9, *Pichia bruneiensis* CMS-3, and *P. kudriavzevii* CNT-3 from cow *chhurpi*, *Y. lipolytica* DUAY-11, *S. cerevisiae* DUAY-53, *S. cerevisiae* DUBY-47, *Y. lipolytica* DUCY-6, and *Candida parapsilosis* DLAY-48 from yak *dahi*, and *Y. lipolytica* CLAY-2, *Y. lipolytica* CZBY-49, and *Kl. lactis* CUBY-19 from yak *chhurpi*.

4.6.12 L-asparaginase activity

Except for *Debaryomyces prosopidis* CCG-4 and *Yarrowia lipolytica* CBG-9 from cow *chhurpi*, the majority of the isolates lacked L-asparaginase activity.

4.7 Grouping and selection of the yeast isolates based on functional properties

Similarly, in addition to the probiotic properties, the functional traits viz. cholesterol lowering activity, anti-diabetic activity, antioxidant activity, anti-inflammatory activity, phytate hydrolyzing activity, beta-D-glucosidase activity, antifungal activity, siderophore production, beta-galactosidase activity, exopolysaccharide production,

vitamin B12 production, and L-asparaginase activity, of the stains were used to group and select the representative isolates utilizing heatmap visualization and PCA biplot analysis. A $\log(x+1)$ transformation was used to standardize all quantitative values, while "1" or "0" denoted presence and absence, respectively, for qualitative tests. Based on the results obtained by the yeast isolates for functional properties, two significant clusters (Cluster A and B) were formed by the yeast isolates from cow (Figure 44a) and yak (Figure 44b) milk products in a heatmap visualization analysis. For isolates from cow *dahi* and *chhurpi*, *Saccharomyces cerevisiae* DRC-41, *Kluyveromyces marxianus* DPA-41, *S. cerevisiae* DRC-42, *S. cerevisiae* DKL-13, and *Kl. lactis* DTL-6 from cow *dahi*, and *S. cerevisiae* CRC-35, and *S. cerevisiae* CGI-29 from cow *chhurpi* grouped in cluster A, while cluster B comprises of *S. cerevisiae* DJT-2, *S. cerevisiae* DMS-15, and *S. cerevisiae* DAO-17 from cow *dahi*, and *Pichia bruneiensis* CPAL-16, *P. bruneiensis* CMS-3, *S. cerevisiae* CKL-10, *P. kudriavzevii* CNT-3, *Debaryomyces prosopidis* CCG-4, *Yarrowia lipolytica* CBG-9, *Kazachstania exigua* CMNB-4, *K. exigua* CMB-19, *D. prosopidis* CPA-55, and *Meyerozyma guilliermondii* CMN-3 from cow *chhurpi*. For isolates from yak *dahi* and *chhurpi*, *S. cerevisiae* DUAY-53, *S. cerevisiae* DUBY-47, *Candida parapsilosis* DLAY-48, *Wickerhamomyces anomalus* DUCY-48, *S. cerevisiae* DKY-22, and *S. cerevisiae* DCY-27 from yak *dahi*, and *Y. lipolytica* CLAY-2, *Kl. lactis* CUBY-19, *Kl. lactis* CLBY-16, and *Kl. marxianus* CZAY-19 from yak *chhurpi* comprises of cluster A, while *Y. lipolytica* DZBY-37, *Y. lipolytica* DUAY-11, and *Y. lipolytica* DUCY-6 from yak *dahi*, and *Y. lipolytica* CZBY-49, and *C. metapsilosis* CKY-12 from yak *chhurpi* grouped in cluster B.

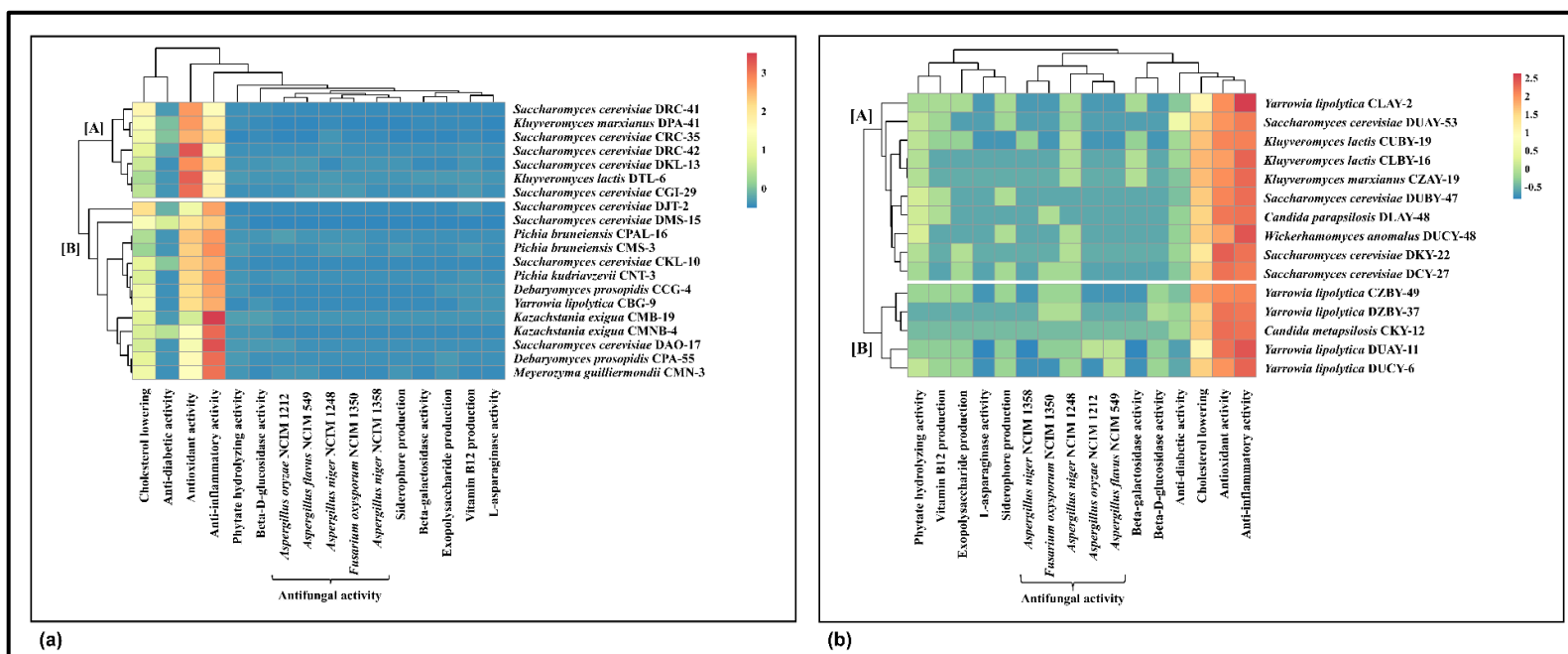


Figure 44: Heatmap visualization of the functional properties of the yeast isolates from *dahi* and *chhurpi* from (a) cow and (b) yak based on cholesterol lowering activity, anti-oxidant activity, anti-inflammatory activity, anti-diabetic activity, phytate hydrolyzing activity, beta-D-glucosidase activity, siderophore production, beta-galactosidase activity, exopolysaccharide production, vitamin B12 production, L-asparaginase activity, and antifungal activity. Based on the similarity and dissimilarity of the results, isolates were grouped into two clusters, cluster A and B.

A PCA biplot was designed to evaluate the differences and similarities among the isolates from cow *dahi* and *chhurpi* (Figure 45a). The PC1 component shows 38.60% variance, while the PC2 component shows 25.67% variance. Two clusters were formed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Euclidean similarity index in a hierarchical clustering system (Figure 45b). Cluster A comprises of *S. cerevisiae* DMS-15, *S. cerevisiae* DRC-42, *S. cerevisiae* DJT-2, *S. cerevisiae* DRC-41, and *Kl. marxianus* DPA-41 from cow *dahi*, and *K. exigua* CMNB-4, *S. cerevisiae* CKL-10, and *S. cerevisiae* CRC-35 from cow *chhurpi*, and cluster B comprises of *S. cerevisiae* DKL-13, *Kl. lactis* DTL-6, and *S. cerevisiae* DAO-17 from cow *dahi*, and *K. exigua* CMB-19, *P. bruneiensis* CPAL-16, *S. cerevisiae* CGI-29, *P. bruneiensis* CMS-3, *P. kudriavzevii* CNT-3, *M. guilliermondii* CMN-3, *D. prosopidis* CPA-55, *D. prosopidis* CCG-4, and *Y. lipolytica* CBG-9 from cow *chhurpi*. Hence, based on score value obtained by the isolates on variance-covariance matrix at 10k bootstrap value, *Kl. marxianus* DPA-41 and *S. cerevisiae* CRC-35 from cow *dahi* and *chhurpi*, were selected as functional isolates with highest potential amongst the other isolates.

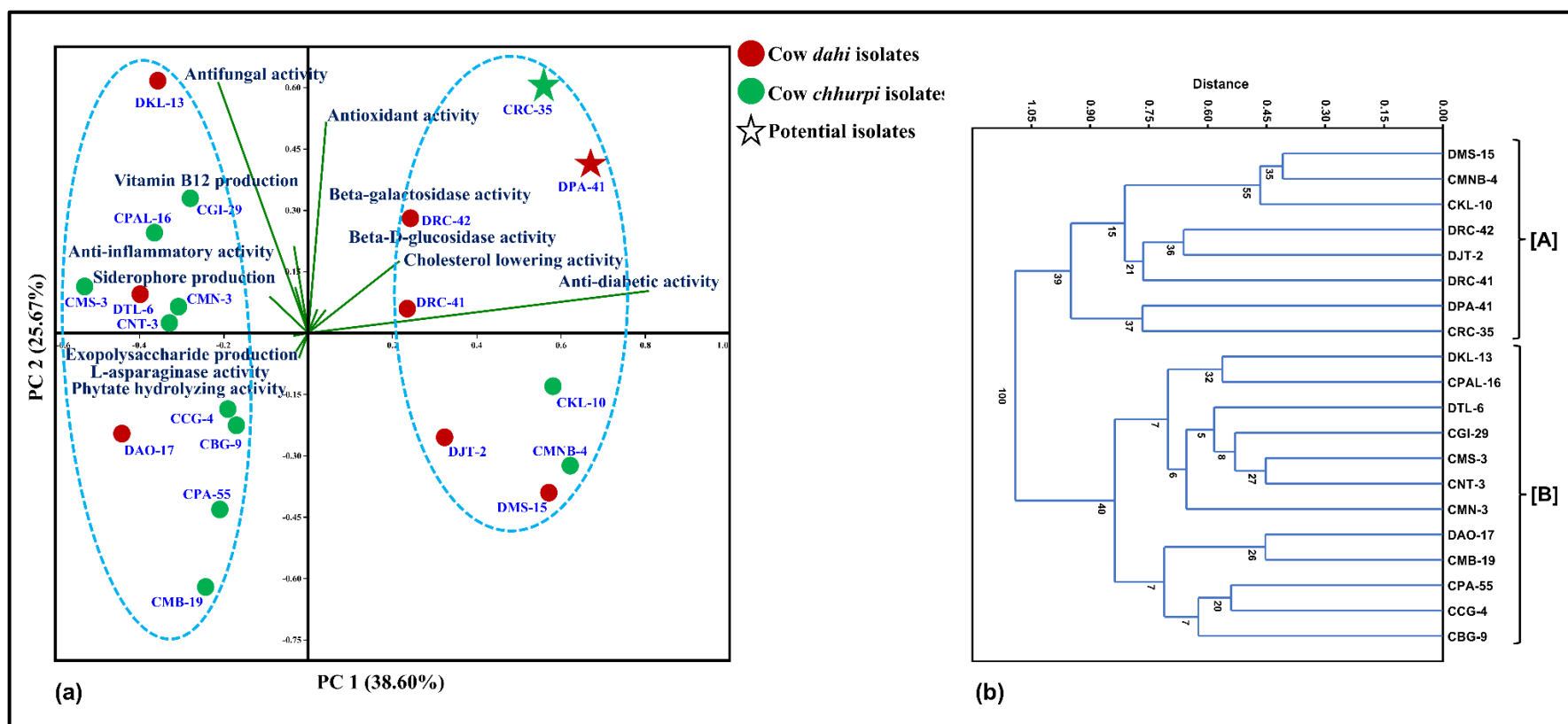


Figure 45: Selection of the most potentially functional yeast isolates from cow *dahi* and *chhurpi* using (a) PCA bi-plot analysis and (b) a hierarchical clustering system illustrates the formation of the clusters.

Similarly, a PCA biplot was designed for the isolates from yak *dahi* and *chhurpi* (Figure 46a). The PC1 component shows 29.49% variance, while the PC2 component shows 20.77% variance. Two clusters were formed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Euclidean similarity index in a hierarchical clustering system (Figure 46b). *S. cerevisiae* DUAY-53 and *Y. lipolytica* CLAY-2 were out-grouped. Cluster A comprises of *S. cerevisiae* DUBY-47, *C. parapsilosis* DLAY-48, *S. cerevisiae* DCY-27, *S. cerevisiae* DKY-22, *Y. lipolytica* DZBY-37, and *W. anomalus* DUCY-48 from yak *dahi*, and *Kl. lactis* CLBY-16, *C. metapsilosis* CKY-12, and *Kl. marxianus* CZAY-19 from yak *chhurpi*, while cluster B was grouped with *Y. lipolytica* DUCY-6, and *Y. lipolytica* DUAY-11 from yak *dahi*, and *Kl. lactis* CUBY-19, and *Y. lipolytica* CZBY-49 from yak *chhurpi*. Hence, based on score value obtained by the isolates on variance-covariance matrix at 10k bootstrap value, *S. cerevisiae* DUAY-53 and *Kl. lactis* CUBY-19 from yak *dahi* and *chhurpi*, were selected as functional isolates with highest potential amongst the other isolates.

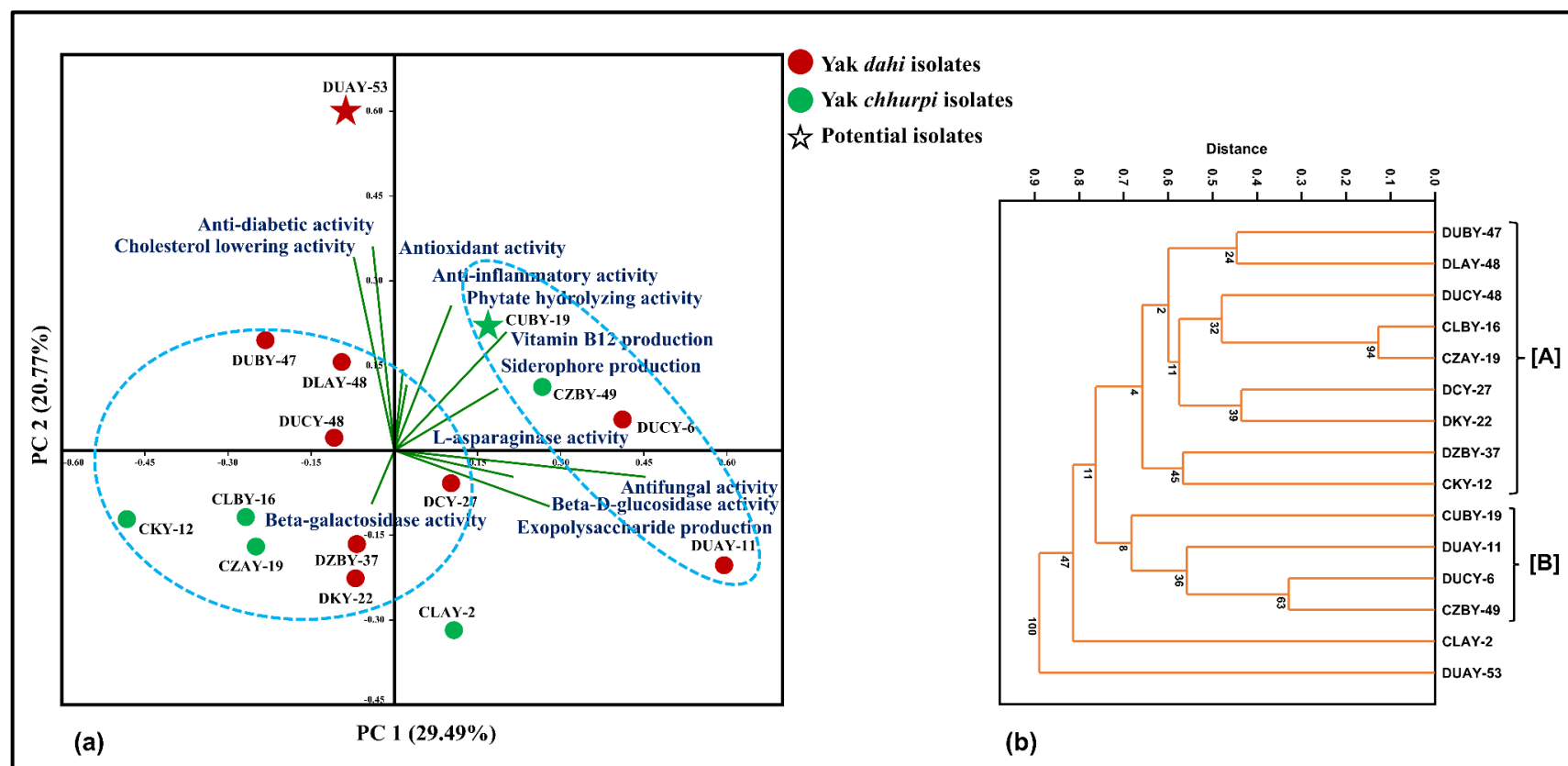


Figure 46: Selection of the most potentially functional yeast isolates from yak *dahi* and *chhurpi* using (a) PCA bi-plot analysis and (b) a hierarchical clustering system illustrates the formation of the clusters.

DISCUSSION

5. DISCUSSION

5.1 Indigenous knowledge of NFM products

The ethnic groups of the Eastern Himalaya share many characteristics, and their traditional knowledge of the techniques used to prepare NFM products reflects their traditions, common culture, religions and belief systems (Dewan and Tamang, 2006; Tamang and Samuel, 2010). In the dietary cultures of ethnic groups residing in the various elevations and climatic zones of the Himalayas, where pastoral and agrarian systems coexist, milk and milk products are the main food components (Tamang, 2010c). The Himalayan people prepare and consume various ethnic fermented milk products prepared from cow milk and yak milk, such as *dahi*, *mohi*, *gheu*, soft *chhurpi*, hard *chhurpi*, *chhu*, *somar*, *maa*, *philu*, and *shyow*. Some of the milk products, notably *dahi* (curd), *chhurpi* (cottage cheese), *mohi* (buttermilk), and *gheu* (butter), are familiar in all regions of the Sikkim Himalayas (Tamang, 2010c).

Dahi is the earliest fermentation product derived from milk. It is the most popular fermented milk product in Sikkim, widely consumed as a refreshing, non-alcoholic, and savoury beverage. Beyond its dietary use, it holds significant cultural and religious importance among various communities, including Hindus, Buddhists, Tibetans, Bhutias, and Lepchas, being used in several rituals, marriages, funerals, and festivals like *Dashai* and *Shoton*. It is also mixed with beaten rice for traditional celebrations such as *Ashar ko Pandra*, symbolizing agricultural beginnings and good fortune (Tamang, 2005; Tamang, 2010c). Other fermented milk products prepared from *dahi* are *mohi*, *chhurpi*, *churkam*, *gheu*, etc. In Sikkim, *Mohi* (buttermilk) is commonly served as a refreshing drink at social occasions and religious ceremonies, while *gheu*, or *ghee*, has a sacred meaning in Hindu and Buddhist ceremonies, used on various occasions from

birth to death and for lighting prayer lamps in temples and monasteries. Soft *chhurpi* is one of the most popular dishes served as curry or pickle during various festivals, whereas hard *chhurpi* is chewed like gum by Bhutia and Sherpa communities in high altitudes for energy. According to a study conducted by Choudhary et al. (2024), 100 g of *chhurpi* may provide more than the entire recommended dietary allowance (RDA) of proteins for the Indian population (adults and children) as per ICMR-NIN Expert Group (2020). *Chhu* is an important local food consumed as soup with rice, especially by the Bhutia community when other foods are scarce, and *somar* is mainly used by *Sherpas* to boost appetite and aid digestion. The indigenous knowledge of Sikkim's ethnic groups in preparing naturally fermented milk products from cow and yak milk is unique in India, celebrated for its distinctive taste, flavour, and traditional culinary practices (Dewan and Tamang, 2006; Rai et al., 2016; Rawat et al., 2018; Tamang, 2022b).

In Sikkim, both cow and yak milk are used to produce NFM products. The Siri cow (*Bos indicus*) is a small, draught-purpose local cattle breed that was primarily domesticated in Sikkim and the Darjeeling Hills (Pundir et al., 2016). The indigenous cattle in urban and nearby regions are usually crossbred with Jersey and Holstein Friesian breeds to increase milk production. Siri cattle have been reported to have drastically decreased in number from 79,000 to 13,948 between 2003 and 2012; as a result, the breed is considered endangered. In 1982, 140,036 indigenous cattle were reported (Tantia et al., 1996). The milk yield varied from 2.0 to 6.5 kg per day. The first calving age, lactation duration, and calving interval were 40-60 months, 200-240 days, and 450-600 days, respectively (Pundir et al., 2016). Despite making up a very small percentage of our nation's total livestock population, yaks are important to the local communities that live in high-altitude areas of the Himalayas from 3,000 to 5,000 meters (Niranjan and Biswas, 2013). With roughly 71% of the nation's total yak population, Jammu and

Kashmir has the largest number (0.54 lakh), followed by Arunachal Pradesh (18.34%) and Sikkim (5.26%). Sikkim has the highest population density of yak (0.569 yak per square km), followed by Arunachal Pradesh (0.169) and Jammu and Kashmir (0.245).

5.2 Yeast in NFM products

Culture-dependent and culture-independent techniques can be used to identify both culturable and unculturable microbes from many fermented milk products; the latter techniques have been shown to reveal inter- and intra-species diversity within or among the genera (Cocolin et al., 2002; Ramos et al., 2010). Profiling yeast population in fermented foods using PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is the most widely used culture-independent method for identifying microorganisms from fermented foods (Cocolin et al., 2002; Geronikou et al., 2020). Using metagenomic analysis along with the DGGE technology, Ding et al. (2022) reported *Saccharomyces cerevisiae* as the prevalent species in traditional fermented yak milk besides other yeast species such as *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Candida zeylanoies*, *Mrakia blollopis*, *Pichia fermentans*, *Pichia kudriavvii*, *Pichia norvegensis*, and *Pichia cactophila*. In our study, *Saccharomyces cerevisiae* (6), *Kluyveromyces marxianus* (1), and *Kluyveromyces lactis* (1) from cow *dahi* samples; *Saccharomyces cerevisiae* (3), *Debaryomyces prosopidis* (2), *Pichia bruneiensis* (2), *Kazachstania exigua* (2), *Pichia kudriavzevii* (1), *Yarrowia lipolytica* (1), and *Meyerozyma guilliermondii* (1) from cow *chhurpi* samples; *Saccharomyces cerevisiae* (4), *Yarrowia lipolytica* (3), *Yarrowia lipolytica* (1), and *Wickerhamomyces anomalus* (1) from yak *dahi* samples; and *Yarrowia lipolytica* (2), *Kluyveromyces lactis* (2), *Kluyveromyces marxianus* (1), and *Candida metapsilosis* (1) from yak *chhurpi* samples were identified by sequencing D1/D2 domain of large ribosomal subunits. In a study conducted by Liu et al. (2015), *Galactomyces* and *Saccharomyces cerevisiae* were revealed as a dominant

fungal genus by High-throughput sequencing analysis in the Tibetan naturally fermented yak milk after predominant bacterial genera. The yeast isolates isolated from *kurut*, naturally fermented yak milk, were identified as belonging to 7 species: *Kl. marxianus*, *S. cerevisiae*, *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Issatchenkia orientalis*, *Kazachstania unisporus*, and *P. fermentans* (Bai et al., 2010), similar to the species identified in the study of Wu et al. (2009). *S. cerevisiae* is the dominant yeast species found in the fermented yak milk products (Wu et al., 2009; Bai et al., 2010; Bao et al., 2012; Liu et al., 2012; Liu et al., 2015). In the case of yak milk-*chhurpi*, a combination of yeast, AAB, and LAB has co-evolved during natural fermentation (Tamang et al., 2012; Tamang et al., 2021). Dewan and Tamang, (2006) reported *Saccharomycopsis crataegensis* and *C. castelli* as the dominant yeast species from the naturally fermented yak milk product, *chhu*. Based on 18s rRNA sequencing, *C. humilis*, *C. parapsilosis*, *Clavispora lusitaniae*, *Ogataea siamensis*, and *Ogataea polymorpha*/*Ogataea parapolyomorpha* were identified in Sumbawa horse milk in Sumbawa Island, West Nusa Tenggara (Ekawati et al., 2024).

5.3 Probiotic attributes

In contrast to probiotic bacteria, which have a well-established history and widespread use, research on probiotic yeast has been limited (Huang et al., 2024). Nevertheless, yeast exhibits specific probiotic characteristics distinct from bacteria, such as enhanced tolerance to gastric acid, immunomodulatory effects, and antimicrobial activity (Staniszewski and Kordowska-Wiater, 2021; Kazemi et al., 2025). Compared to bacterial probiotic potential, yeast species exhibit higher rates of survival in lower pH (less than 1.5), making them feasible probiotic candidates (Hsiung et al., 2021; Zahoor et al., 2021). Although many test conditions have been proposed, none of them have been officially recognized as standard screening techniques for the selection of

probiotics. According to FAO/WHO guidelines, probiotic microbes must be safe for human consumption and be able to survive and multiply in the GI tract (FAO/WHO 2002). The European Food Safety Authority (EFSA) has particular guidelines for the microbes, including yeasts, that may be added to animal feed, but it does not specify selection criteria for probiotics. The EFSA Panel on Additives and Products or Substances Used in Animal Feed (EFSA FEEDAP Panel, 2018) states that these microorganisms first must be identified at the strain level, and then they must undergo scientific evaluations for pathogenicity, toxigenicity, and antimicrobial production. In addition to preclinical and clinical safety and efficacy studies of probiotics, the Indian Council of Medical Research/Department of Biotechnology (ICMR/DBT) has established specific *in vitro* tests, such as resistance to gastric acidity and bile and antimicrobial activity, as well as preclinical and clinical safety and efficacy investigations of probiotic candidates (Ganguly et al., 2011).

To sustain survivability throughout the GI system, suitable probiotic yeast isolates must be tolerant of low pH (pH 1.5-3.0) and bile (0.3%, w/v) (Kazemi et al., 2025). The ability to grow at a pH as low as 2.0 has been reported by several genera, including *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Kazachstania*, *Meyerozyma*, *Pichia*, *Candida*, *Yarrowia*, and *Wickerhamomyces* (Pedersen et al., 2012; Qvirist et al., 2016; Oliveira et al., 2017; Ragavan and Das, 2017; Menezes et al., 2020; Gürkan Özlü et al., 2022). In our study, only 3.34% of the total isolates from cow *dahi* and *chhurpi* and 2.94% from yak *dahi* and *chhurpi* were found to tolerate low pH (pH 2.0) and bile (0.3%). Wang et al. (2024a) reported that 73.33% of the total isolates were found to have higher tolerance at pH 3.0 than pH 2.0, and the same kind of result was reported by Alvarez et al. (2023) and Maione et al. (2024). Probiotic yeasts are generally able to withstand strong inorganic acid by adjusting their cell walls, activating cell wall

integrity, and activating general stress response pathways (Chihombori et al., 2025). Bile salts, which are amphipathic compounds, are crucial for emulsifying lipids in the gastrointestinal system. Their tolerance is essential for the survivability of the probiotic isolates in the gut, as bile salts can disrupt cell membranes and directly impact their viability, particularly in the small intestine (Makzum et al., 2023). The optimum bile concentration for the human gut environment ranges from 0.2% to 0.6% (Helmy et al., 2019; Staniszewski and Kordowska-Wiater, 2021; Galli et al., 2022; Shivani and Sathiavelu, 2024).

Cell surface hydrophobicity influences the isolate's ability to adhere to hydrocarbons, which subsequently affects the tendency of the isolates to adhere to the intestinal epithelium (Alkalbani et al., 2022b; López-Palestino et al., 2025). Hydrophobicity alone is not sufficient for epithelial adhesion; it should be evaluated along with aggregation property (Kanak and Öztürk Yılmaz, 2025). In our study, microbial attachment to hydrocarbons (MATHs) is a significant variable in the preliminary selection of isolates. Cell surface hydrophobicity was checked by measuring the adherence property of the isolates with hydrocarbons such as n-hexadecane, toluene, xylene, and diethyl ether, and only those isolates that showed more than 80% MATHs were selected. As a result, a total of 35 yeasts were selected and processed for further investigation. According to Marina et al. (2005), hydrophobicity is an assessment of a substance's relative tendency toward a non-aqueous as opposed to an aqueous environment. Nonpolar groups tend to be excluded by water molecules in an aquatic environment. This results in the nonpolar groups associating only among themselves. By interacting with nonpolar groups on the cell surface, hydrophobicity allows an organism to make initial contact with its host cells (Shobharani and Agrawal, 2011). The hydrophobicity values $\geq 70\%$ were considered high hydrophobicity, between 40 and 70% intermediary, and $< 40\%$ low (Zupančič et

al., 2019). *S. bacillaris* CC-ZZ5 and *S. bacillaris* CC-N1 had high hydrophobicity, *S. bacillaris* CC-ZZ3, *S. bacillaris* CC-PT4, and *S. bacillaris* CC-PT10 had average hydrophobicity, and the remaining isolates, including the control strain *S. boulardii* CNCM I-745 (15.50%), had lower hydrophobicity (Shen et al., 2022). Gut et al. (2019) measured the hydrophobicity of *S. boulardii* SB 48 and SB 49 to be 15.58% and 21.18%, respectively. It is desired that a probiotic organism has high cell surface hydrophobicity (Amorim et al., 2018; Byakika et al., 2019). Auto-aggregation capacity reflects probiotic's ability to cluster together and form cell aggregates, whereas robust cell hydrophobicity enhances the attachment between microorganisms and epithelial cells (Huang et al., 2024). Therefore, these two parameters are important indicators of the adhesion characteristics of probiotic isolates (Krausova et al., 2019). Auto-aggregation values exceeding 60% indicate strong capability, whereas values below 30% are considered low (Menezes et al., 2020). The higher percentage of cell surface hydrophobicity of the yeast isolates contributes to greater aggregation and adhesion capacity to epithelial mucosa (Bonatsou et al., 2018; Simões et al., 2021; Tran et al., 2024). Yeasts aggregate through their cell wall mannose; a high aggregation propensity indicates an abundance of mannose (Kil et al., 2023). The ability of yeast isolates to auto-aggregate with one another was evaluated in order to determine their affinity to colonize intestinal epithelial cells. Out of 20 isolates from cow milk products, *S. cerevisiae* DRC-42 ($93.13 \pm 0.18\%$) from cow *dahi* and *S. cerevisiae* CGI-29 ($91.67 \pm 0.26\%$) from cow *chhurpi* showed the highest percentage of auto-aggregation. *S. cerevisiae* DKY-22 ($96.24 \pm 0.24\%$) from yak *dahi* and *Candida metapsilosis* CKY-12 ($88.96 \pm 0.08\%$) from yak *chhurpi* showed the maximum auto-aggregation among 15 isolates from yak milk products. Tran et al. (2024) showed the hydrophobicity and auto-aggregation of *Pichia kudriavzevii* YGM091 strain isolated from fermented goat milk

as $>60\%$ and $66.56 \pm 1.45\%$ after 45 min of incubation, respectively. While the hydrophobicity and auto-aggregation of *P. kudriavzevii* MYSSBYPS10 were reported as 91.30% and 97.2% after 24 h of incubation at 37 °C, respectively (Shruthi et al., 2025), while Rai et al. (2024) reported the auto-aggregation ability of *P. kudriavzevii* and *K. ohmeri* as 95.09% and 88.71%, respectively. The auto-aggregation ability of the yeast isolates was found to increase with longer incubation time. Shruthi et al. (2024) reported the auto-aggregation capability of *Meyerozyma guilliermondii* MYSY23 as 74.07% and 97.88% at 5 h and 24 h of incubation time. While the auto-aggregation percentages of *S. cerevisiae* MYSY17, *S. cerevisiae* MYSY8, *S. cerevisiae* MYSY24, and *S. cerevisiae* MYSY22 were reported as 93.65%, 94.67%, 93.01%, and 94.94% respectively, after 24 h of incubation (Shruthi et al., 2024). Additionally, flocculation is one of the important characteristics of potential probiotic yeasts that contributes to adhesion characteristics, aiding in colonization, biofilm formation, and pathogen exclusion (Alkalbani et al., 2022b; Saguez et al., 2022; Chantanawilas et al., 2024).

The ability of a putative probiotic to co-aggregate with pathogenic bacteria is a pertinently advantageous trait, since it provides a defense mechanism against intestinal invasion by trapping pathogen cells (Tiago et al., 2012; Chelliah et al., 2016; Bonatsoua et al., 2018). In this study, *Escherichia coli* KL96 MTCC 1583, *Staphylococcus aureus* subsp. *aureus* MTCC 740, *Salmonella enteric* subsp. *enterica* ser. Typhimurium MTCC 3223, and *Bacillus cereus* MTCC 1272 were used as reference isolates for co-aggregation and antimicrobial activity. From cow *dahi*, *Kluyveromyces marxianus* DPA-41 (MTCC 740- $53.19 \pm 1.45\%$; MTCC 1583- $59.96 \pm 0.45\%$; MTCC 3223- $61.16 \pm 0.40\%$; and MTCC 1272- $50.71 \pm 0.55\%$) and *S. cerevisiae* CRC-35 (MTCC 740- $58.82 \pm 1.32\%$; MTCC 1583- $57.25 \pm 0.45\%$; MTCC 3223- $59.35 \pm 1.7\%$; and MTCC 1272- $45.81 \pm 0.42\%$) from cow *chhurpi* showed the highest co-aggregation against all

four MTCC reference isolates. From yak *dahi*, *S. cerevisiae* DKY-22 showed the maximum percentage of co-aggregation against MTCC 740 ($62.96 \pm 0.74\%$); *S. cerevisiae* DUAY-53 showed the highest co-aggregation against MTCC 1583 ($19.56 \pm 0.04\%$), MTCC 3223 ($18.89 \pm 0.05\%$), and MTCC 1272 ($20.99 \pm 0.03\%$). From yak *chhurpi*, *Kl. lactis* CUBY-19 demonstrated the highest co-aggregation against MTCC 740 ($58.88 \pm 0.52\%$), MTCC 1583 ($17.81 \pm 0.26\%$), and MTCC 1272 ($18.6 \pm 0.04\%$), and *Kl. marxianus* CZAY-19 showed the highest co-aggregation against MTCC 3223 ($18.87 \pm 0.05\%$). Coutinho et al. (2021) found that two yeast isolates (*S. cerevisiae* UFMGCB 11120 and *Rhodotorula mucilaginosa* UFMGCB 18377) were assessed for auto-aggregation and co-aggregation, where *Salmonella typhimurium* and *Shigella sonnei* were used as reference isolates. Since it has a high growth yield and co-aggregation capability with *S. Typhimurium*, these two yeast isolates were selected to perform *in vivo* tests to determine their protective effect against salmonellosis (Coutinho et al., 2021), one of the main reasons of diarrhoea and inflammation of the intestinal mucosa, which annually infects around 180 million people (Besser, 2018). Tran et al. (2024) reported that *P. kudriavzevii* YGM091 exhibited high levels of co-aggregation with *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Salmonella enterica* serovar Typhimurium (ATCC 14028). Apart from the co-aggregation property, antimicrobial activity is another antagonistic property of the isolates. It serves to extend the shelf life of foods (Kanak and Öztürk Yılmaz, 2025). Additionally, killer toxins (K1, K2, and K28), volatile organic compounds (VOCs; alcohols, hydrocarbons, and benzene derivatives), and cell wall-degrading enzymes (chitinases, glucanases, and proteases) released by yeast can also influence the efficacy of antimicrobial activity (Ma et al., 2023; Sun et al., 2023). Competition for nutrients is potentially one of the most prominent antagonistic traits of yeasts (Vergara et al., 2023).

S. cerevisiae DKL-13 from cow *dahi* and *S. cerevisiae* CKL-10, *K. exigua* CMB-19, and *K. exigua* CMNB-4 from cow *chhurpi* showed positive antimicrobial activity against all four MTCC reference strains. From yak *dahi*, *Y. lipolytica* DUAY-11 and *S. cerevisiae* DUBY-47 showed positive antimicrobial activity against MTCC 1583, MTCC 3223, and MTCC 1272; and from yak *chhurpi*, *Kl. lactis* CUBY-19 showed antimicrobial activity against MTCC 740, MTCC 3223, and MTCC 1272. During the growth of yeast isolates, various compounds, such as organic acids, bacteriocins, volatile acids, and antibiotic factors, are produced, exerting antagonistic effects against pathogenic bacteria (Huang et al., 2024). The *Pichia* genus is renowned for its capacity to generate killer toxins that can inhibit specific pathogens (Sun et al., 2023). The antibacterial effects of *S. cerevisiae* var. *boulardii* against various Gram-positive and negative bacterial and viral pathogens, including *Bacillus anthracis*, *Shigella*, *Escherichia coli*, *Vibrio cholerae*, *Helicobacter pylori*, *Clostridium difficile*, *Salmonella*, and *Rotavirus*, have been previously reported (Kaźmierczak-Siedlecka et al., 2020; Abid et al., 2022). Additionally, some yeast may synthesize antimicrobial compounds that may inhibit the growth of pathogenic bacteria and other fungi (Al-Qaysi et al., 2017; Sampaolesi et al., 2022; Piraine et al., 2023). Oliveira et al. (2017) reported the ability of a *C. norvegica* strain to inhibit *Cryptococcus neoformans* with an inhibition zone equivalent to 1.5 cm. The growth of *Salmonella* sp., *Vibrio cholerae*, *Pseudomonas* sp., and *Escherichia coli* was found to be inhibited by *Pichia* and *Candida* species, according to Syal and Vohra (2013). The clear zone around the yeast colonies ranged in diameter from 16 to 36 mm. Gürkan Özlü et al. (2022) has recorded the highest antimicrobial activity for *P. kudriavzevii* TEM-AP6 and *W. anomalus* TEM-KMZ3 against *Listeria monocytogenes* ATCC 19115 and *Escherichia coli* O157:H7 RSSK 234 with an inhibition zone of 26 and 14 mm, respectively. The results showed that the production of various organic acids

such as capric acid, caprylic acid, and caproic acid by *S. boulardii* was the reason for its antimicrobial activity (Ansari et al., 2023).

The capacity to exhibit BSH activity is a crucial factor when choosing probiotic isolates because BSH breaks down bile salts that the liver produces to aid in the digestion and absorption of dietary fats (Gebre et al., 2023). Probiotic isolates with BSH activity are more bile salt tolerant, aiding their gut survival and colonization (Tsigkrmani et al., 2022). *Kl. marxianus* DPA-41 from cow *dahi*, *M. guilliermondii* CMN-3 and *P. kudriavzevii* CNT-3 from cow *chhurpi*, from yak *dahi*, *S. cerevisiae* DCY-27, *S. cerevisiae* DUAY-53, *Y. lipolytica* DUCY-6, *C. parapsilosis* DLAY-48, and *Y. lipolytica* DZBY-37; and from yak *chhurpi*, *Kl. lactis* CLBY-16, *Kl. marxianus* CZAY-19, and *Kl. lactis* CUBY-19 were found to hydrolyse three different sodium salts with different intensities.

The optimum temperature range for yeast growth is 20-30 °C (Zhuang et al., 2024). In order to ensure that the probiotic yeast will thrive and have its beneficial effects on the human body, it is essential to select probiotic yeast that can tolerate and grow at 37 °C (Huang et al., 2024; Piraine et al., 2023; Oliveira et al., 2017; Gut et al., 2019). Numerous yeast isolates from Ethiopian fermented sourdough (Muche et al., 2023; Huang et al., 2024), fermented cheese in Brazil (De Miranda et al., 2023), and Turkish dairy products (Gürkan Özlü et al., 2022) were found to show robust growth at 37 °C temperature. In our study, isolates were found to tolerate up to 4% of NaCl concentrations, and the growth of the yeast was reduced drastically at 6, 8, and 10%. Wang et al. (2024a) reported the good tolerance of yeast in 2.5-4% NaCl (w/v). *Schizosaccharomyces pombe* LBY5 was the only strain that showed weak growth at 6.5% NaCl (w/v) (Wang et al., 2024a). In a study conducted by Diguță et al. (2023), it was shown that the majority of yeast isolates (86.67%) were observed to thrive at NaCl

concentrations ranging from 2 to 6.5% w/v. A rapid decline in growth was observed after a concentration of 3.0% NaCl, while some isolates of yeast were also observed to grow at 8.0% NaCl (Hossain et al., 2020). Salt is added to some fermented food products as a preservative that inhibits the growth of harmful microbes while also contributing to the aromatic profile (Zeng et al., 2019; Szutowska and Gwiazdowska, 2021; Alkalbani et al., 2022a, 2022b). Yeasts adopt hyperosmotic stress tolerance through the high osmolarity glycerol (HOG) pathway, which uses two cell-membrane-bound receptors to activate mitogen-induced protein kinase (Pascual-Ahuir et al., 2018; Brewster and Gustin, 2014). The HOG pathway genes are stimulated as a result of osmotic changes revealed by *sho1p* and *sln1p* (Skoneczny and Skoneczna, 2018; Rodrigues-Pousada et al., 2004), causing genes linked to resistance to salt stress to be triggered downstream (Dhar et al., 2011).

The main factors preventing yeast from surviving in the GI system are high levels of bile salts and organic acids (Pais et al., 2020). The human digestive system was modelled using three distinct simulated fluids: simulated intestinal fluid (SIF) contains pancreatin and bile salt, simulated gastric fluid (SGF) contains pepsin and has a pH of 2.0, and simulated salivary fluid (SSF) contains amylase as its primary component. After treatment with different simulated fluids, CFU log reduction and survivability of the tested isolates were determined. *Kl. marxianus* DPA-41 from cow *dahi* and *S. cerevisiae* CRC-35 from cow *chhurpi*, *Wickerhamomyces anomalus* DUCY-48 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi* showed the highest survivability and lowest CFU log reduction in SSF, SGF, and SIF. When a yeast cell is placed in a low pH environment, the mechanism for the cell wall integrity pathway gets activated (Chen et al., 2009; de Lucena et al., 2015). It is dependent on Rho1 GTPase receiving signals from stressed walls, which forms different carbohydrate polymers that are used to

remodel the cell wall (Levin, 2011). A general stress response pathway is another way to withstand the strong inorganic acid. This pathway is crucial for any conditional change, and the conductance of the protein kinase C pathway is essential for this response (Lucena et al., 2020). In keeping with earlier research, it is advised to use pepsin and a low pH (2.0) to simulate human stomach conditions in order to accurately assess novel isolates in gastric juice (Alkalbani et al., 2022b). Additionally, Pais et al. (2020) pointed out that the gut microbiome's secondary metabolic products, pancreatic and hydrolytic enzymes, and the small intestine's epithelial brush border may neutralize microbes, including yeasts. Yeast isolates in the intestinal tract are also unavoidably exposed to alkaline stress. The active transporters in the plasma membrane are activated by a persistent proton gradient between extracellular and intracellular media. This gradient is shielded by the corresponding fungal orthologs, such as Pma1p H⁺-ATPase in *S. cerevisiae* (Skoneczny and Skoneczna, 2018). This gradient is significant because it functionally absorbs a number of essential compounds (Guan and Liu, 2020). Numerous processes have been brought forward to explain yeast alkaline tolerance. The plasma membrane of *S. cerevisiae* has Ena1 P-type ATPase, which operates on the cell's efflux of K⁺ and Na⁺ cations to counterbalance the internal alkaline medium (Skoneczny and Skoneczna, 2018; Nakayama et al., 2004). Under alkaline stress, the protein kinase A pathway, another mechanism in *S. cerevisiae*, is inhibited, causing environmental alkalization, which is counteracted by remodelling the expression of the *Msn2* and *Msn4* genes of stress-responsive transcriptional activators (Casado et al., 2011). Additionally, a yeast cell's intracellular pH must be regulated by the vacuolar H⁺-ATPase enzyme (Wilms et al., 2017; Diab and Kane, 2013). *Y. lipolytica* uses polyphosphate storage molecules to deal with alkaline stress (Zvyagilskaya et al., 2001).

5.4 Detection of potential probiotic genes in yeast isolates

Probiotic yeasts are generally able to withstand strong inorganic acid by adjusting their cell walls, activating cell wall integrity, and activating general stress response pathways (Chihombori et al., 2025). Various potential genes were observed to contribute in low pH environments. The cell architecture is found to be depleted under acidic conditions, and the acid shock response involves a number of protein complexes and mechanisms (Kawahata et al., 2006; Sun et al., 2020). Increasing the activity of plasma membrane H^+ -ATPase and vacuolar H^+ -ATPase is necessary for adaptation to low pH environments in order to counteract the acidification of cytosolic pH and the dissipation of the plasma membrane H^+ gradient (Henriques et al., 2017). The presence of the genes responsible for low pH tolerance (*TPS1*, *HSP150*, *SED1*, and *VMA2*) was found to be present in most of the isolates. The *TPS1* gene was detected in most of the isolates except *Pichia bruneiensis* CMS-3, *Yarrowia lipolytica* DUAY-11, *Candida parapsilosis* DLAY-48, and *Kluyveromyces marxianus* CZAY-19. In the acid shock response, it was further found that metal resistance genes such as *FIT2*, *ARN1*, and *ARN2* were upregulated (Kawahata et al., 2006; Sun et al., 2020). *VMA2*, *VMA4*, and *VMA6* genes encode the hydrogen translocating V-ATPase complex proteins that surround the vacuoles, and their depletion results in sensitivity to all acids (Kawahata et al., 2006). Additionally, it implies that the vacuole is necessary for growth at low pH and on organic acid media (Mollapour et al., 2004). Loss of the V-ATPase and HOG MAPK proteins can also cause acid sensitivity (Chen et al., 2009; Brandão et al., 2014). Numerous protein subunits are found in the vacuolar membrane, inner mitochondrial membrane, and plasma membrane, which are home to ATP binding cassette (ABC) transporters and other efflux pumps involved in bile stress resistance (Kumari et al., 2021). The discovery of the bile tolerance-related genes *YIMI*, *PDRI*, and *FLUI* (Rai et al., 2024; Das et al.,

2024) supports the isolate's ability to survive in environments with low bile salt levels (Diguță et al., 2023). Genes like *PDR1* and *YOR1* were also found to cope with the aldehyde stress with functions such as multidrug efflux pumps, drug/toxin transporters, and ATP-binding cassette transporters (Katzmann et al., 1999; Alenquer et al., 2006; Ma and Liu, 2010; Zheng et al., 2024). Transcripts of *YOR1* and *PDR5* were also found to induce in the presence of organic acids, contributing to cell survival under low pH conditions (Teixeira and Sá-Correia, 2002). The expression of *PDR5* is regulated by *PDR1*, and deletion of *PDR1* leads to a significant reduction in *PDR5* expression (Wolfger et al., 2004; Kumari et al., 2021). Genes from the *FLO* family were found to encode cell wall associated surface proteins and exhibit adhesion (Collins et al., 2023). Apart from adhesion, higher expression of *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast was also found to enhance thermotolerance and viability (Vergara-Álvarez et al., 2019). The adhesion-related genes *FLO1*, *FLO10*, *AGA1*, *CCW12*, *EPA1*, and *PST1* were detected in some of the yeast isolates, indicating their involvement in cell-substrate adhesion and flocculation (Guo et al., 2000; Vergara-Álvarez et al., 2019; Rai et al., 2024; Das et al., 2024). Additionally, flocculation in probiotic yeasts is integral to their adhesion characteristics, aiding in colonization, pathogen exclusion, and biofilm formation, all of which are essential for their probiotic functionality (Alkalbani et al., 2022b; Saguez et al., 2022; Chantanawilas et al., 2024). Antimicrobial activity shown by the isolates was validated by detection of the genes potentially responsible for antimicrobial activity. The *khs* gene was detected in *S. cerevisiae* DAO-17, *Kl. marxianus* DPA-41, and *S. cerevisiae* CRC-35, while the *pelA* gene was detected in *P. kudriavzevii* CNT-3 and *Kl. lactis* CUBY-19. These genes were known for inhibiting protein synthesis (Florin et al., 2017; Rai et al., 2024). Other possible mechanisms of antimicrobial activity are production of killer toxins,

competition for nutrients and space, cell wall and membrane barriers, biofilm and adhesion-mediated competition, and production of volatile organic compounds (Oliveira et al., 2017; Sampaolesi et al., 2022; Gürkan Özlü et al., 2022; Piraine et al., 2023; Ansari et al., 2023).

5.5 Safety evaluation of the screened yeast isolates

Yeast isolates were also evaluated for hemolytic activity, DNase activity, gelatinase activity, biogenic amine production, coagulase activity, urease activity, pseudohyphae formation, lipase activity, phospholipase activity, mucin degradation, and antibiotic and antifungal susceptibility testing. The yeast isolates did not demonstrate hemolysis, which indicates an inability to lyse red blood cells (Romero-Luna et al., 2019; Muche et al., 2023; Tran et al., 2024; Kaewda et al., 2025). The yeast isolates were unable to produce gelatinase and DNase, which are able to break down gelatin and DNA, respectively (Lara-Hidalgo et al., 2019; Fernández-Pacheco et al., 2021b; Wang et al., 2024a). High levels of biogenic amines in the food may cause food poisoning symptoms and allergic reactions such as headache, nausea, respiratory distress, hypertension, and histamine poisoning (Fernández-Pacheco et al., 2021b). Mucins act as a barrier that keeps pathogenic microbes from adhering to the lining of the epithelium (Coutinho et al., 2021). In our study, it was found that the yeast isolates did not produce biogenic amines or break down mucin. Phospholipase and urease activities were absent in all yeast isolates. Both phospholipase and urease activity are critical parameters for determining the safety of potential probiotic yeast isolates (Lara-Hidalgo et al., 2019; Pérez-Través et al., 2021). Since these are key factors in microbial virulence, yeast isolates must demonstrate negative phospholipase and urease activity (Fernández-Pacheco et al., 2021b). However, lipase activity and the formation of pseudohyphae were demonstrated by *Meyerozyma guilliermondii* CMN-3 and *Yarrowia lipolytica*

CBG-9 from cow *chhurpi*, *Candida metapsilosis* CKY-12 and *Y. lipolytica* CZBY-49 from yak *chhurpi*, and *C. parapsilosis* DLAY-48 and *Y. lipolytica* DUAY-11 from yak *dahi*. *Y. lipolytica* CLAY-2 was found to exhibit lipase activity. The hydrolysis of triglycerides by the enzyme lipase produces free fatty acids and glycerol. In addition to being necessary for lipid metabolism, it is thought to be a virulence factor for certain harmful microbes. By dissolving host lipid membranes, lipase activity aids in tissue invasion by allowing microorganisms to enter host cells (Tran et al., 2024; Muche et al., 2023). Thus, it is anticipated that the probiotic isolates should not exhibit lipase activity or the formation of pseudohyphae in order to protect host tissue. Pseudohyphae formation is another necessary virulence factor that has the ability to invade epithelial lining, causing damage to tissues and allowing access to the bloodstream (Sudbery, 2011). With the exception of *C. parapsilosis* DLAY-48 from yak *dahi*, none of the yeast isolates exhibited coagulase activity. Another potential virulence factor is coagulase activity, which may increase the risk of blood clotting by binding to plasma fibrinogen and converting it to fibrin (Jafari et al., 2017; da Silva et al., 2019). When assessing the safety of probiotic yeast isolates, testing for antibiotic resistance is a vital component. It is recognized that administering antibiotics to treat infectious diseases might change a patient's gut microbiota, leading to gut dysbiosis (Chattopadhyay et al., 2023). During susceptibility testing of the isolates, most of the isolates demonstrated resistance against antibiotics and susceptible to antifungal agents. Our findings are nearly consistent with those of Amorim et al. (2018), Hossain et al. (2020), and Diguță et al. (2023). During antibiotic treatments, probiotic yeast isolates should naturally be resistant to antibacterial antibiotics in order to survive in the human gut (Czerucka et al., 2007; Pereira et al., 2021; Fernández-Pacheco et al., 2021b; Muche et al., 2023). Yeast isolates, however, must not harbour or transmit antibiotic resistance genes that might be horizontally

transferred to other microbes (de Melo Pereira et al., 2018). However, in order to protect immunocompromised individuals from drug-resistant yeast infections and stop the propagation of genes that would contribute to the drug resistance, probiotic yeast isolates must be susceptible to antifungal agents (Moradi et al., 2018; Lara-Hidalgo et al., 2019; Tran et al., 2024; Ekawati et al., 2024).

5.6 Functional attributes of the yeast isolates

Yeasts are globally known mainly for their application in the food industry, but their application in diverse markets has increased in recent years. Yeasts are currently among the most common organisms used in biomedical research (Gasser et al., 2013), and they have a great impact on the biofuel production industry (Kwak et al., 2019; Pattanakittivorakul et al., 2019). In the fields of food science, nutrition, cosmetics, and medicine, antioxidant activity is a crucial functional characteristic (Chen et al., 2024; Tumilaar et al., 2024; Wang et al., 2024b; Yusof et al., 2025). Antioxidant activity is one of the most crucial traits, as prior research has shown that diseases like cardiovascular disease, arthritis, and certain infections are caused by reactive oxygen species (ROS) (Romero-Luna et al., 2019; Barache et al., 2024). The biological system's ability to detoxify reactive oxygen species (ROS) is not in equilibrium due to the subsequent generation and accumulation of ROS in cells and tissues, resulting in oxidative stress (Pizzino et al., 2017). Cellular antioxidants, both enzymatic and non-enzymatic, can prevent oxidative stress by preserving the equilibrium between the generation and breakdown of ROS. SOD, CAT, and the glutathione and POD systems are well-known enzyme antioxidants that are employed to scavenge excess ROS and reactive nitrogen species (Staerck et al., 2017). The antioxidant capacities of yeast could be caused by glutathione peroxidase, superoxide dismutase, and catalase, as well as lipids and exopolysaccharides (Merchán et al., 2020; Wang et al., 2024a; Wang et al.,

2024b). In this investigation, the scavenging of DPPH radicals was used to assess the antioxidant capacity of the yeast isolates. *Candida parapsilosis* DLAY-48 ($62.63 \pm 0.3\%$) from yak *dahi*, *Kluyveromyces lactis* CUBY-19 ($63.77 \pm 0.3\%$) from yak *chhurpi*, *Kl. marxianus* DPA-41 ($82.22 \pm 0.3\%$) from cow *dahi*, and *Saccharomyces cerevisiae* CRC-35 ($66.13 \pm 0.7\%$) from cow *chhurpi* were found to have maximum antioxidant activity. Four isolates of *S. cerevisiae* were tested for their ability to neutralize DPPH radicals by Siesto et al. (2022). In a study by Abduxukur et al. (2023), their effectiveness ranged from 36% to 49%, with no significant difference ($p < 0.05$) observed when compared to the results for 6TY-2 (*S. cerevisiae*) and other yeast. In a study conducted by Menezes et al. (2020), *S. cerevisiae* CCMA 0731 (61.6 ± 1.8) was selected as a potential probiotic with the ability to exhibit the highest percentage of DPPH reduction out of 15 isolates studied. The risk of cardiovascular diseases can be reduced with even a small decrease in cholesterol level (Manson et al., 1993). Excess cholesterol in the bloodstream is recognized as a risk factor for coronary heart disease and causes hypercholesterolemia (Aloğlu et al., 2015). *In vitro* and *in vivo* studies have shown that only a few yeast species have the ability to reduce cholesterol levels (Ogunremi et al., 2015b). The *in vitro* cholesterol-lowering capacity of the test isolates in this investigation demonstrated that each strain had a different capacity to remove cholesterol, and *S. cerevisiae* DRC-41 ($54.48 \pm 0.7\%$) from cow *dahi*, *S. cerevisiae* CRC-35 ($36.23 \pm 0.6\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($30.51 \pm 0.6\%$) from yak *dahi*, and *Yarrowia lipolytica* CZBY-49 ($37.37 \pm 0.8\%$) from yak *chhurpi* showed the highest capacity to reduce cholesterol. Numerous studies have reported the ability of yeast to lower cholesterol (Ogunremi et al., 2015b; Aloğlu et al., 2015; Fernández-Pacheco et al., 2021a; Alkalbani et al., 2022b; Lata et al., 2022; Gebre et al., 2023; Barache et al., 2024). The probiotic microbes involved in the modulation of immune responses have anti-inflammatory properties

(Menezes et al., 2020). Crohn's disease, ulcerative colitis, and pouchitis are three conditions that are part of the long-term degenerative disease known as irritable bowel disorder (IBD), which causes extreme irritation of the gastrointestinal tract. It may further lead to breast and colon cancer. Probiotics have been found to treat IBD (Pereira et al., 2021). Jawhara and Poulain, (2007) and Jawhara et al. (2012) suggested that the probiotic yeast *S. cerevisiae* var. *boulardii* reduces intestinal inflammation. Foligné et al. (2010) also demonstrated the anti-inflammatory potential of probiotic yeasts and their application in treating IBD. In our study, the highest levels of anti-inflammatory activity were demonstrated by *Kl. marxianus* DPA-41 ($59.74 \pm 0.33\%$) from cow *dahi*, *S. cerevisiae* CRC-35 ($58.03 \pm 0.7\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($76.84 \pm 0.15\%$) from yak *dahi*, and *Kl. lactis* CUBY-19 ($67.33 \pm 0.56\%$) from yak *chhurpi*. The anti-inflammatory potential of the isolates *S. cerevisiae* DABRP5 and *S. boulardii* was studied through the inhibition of protein denaturation assay *in vitro* (Pereira et al., 2021). *S. cerevisiae* DABRP5 showed a slightly lower percent inhibition ($60.39 \pm 0.34\%$) than *S. boulardii* ($62.95 \pm 0.96\%$) (Pereira et al., 2021). According to Tilg and Moschen (2014), using human and animal models of type 2 diabetes mellitus (T2DM) and obesity, T2DM is linked to gut microbial dysbiosis, meaning that gut microbiota is important in determining a person's susceptibility to obesity and related metabolic diseases. Inhibition of the α -amylase by disruption of carbohydrate is one of the strategies to manage diabetes (Ayyash et al., 2018). Similar to *S. cerevisiae* DABRP5 (Pereira et al., 2021), *Kl. marxianus* DPA-41 ($12.1 \pm 0.86\%$) from cow *dahi*, *S. cerevisiae* CRC-35 ($11.49 \pm 0.45\%$) from cow *chhurpi*, *S. cerevisiae* DUAY-53 ($5.6 \pm 0.58\%$) from yak *dahi*, and *Kl. lactis* CUBY-19 ($1.27 \pm 0.93\%$) from yak *chhurpi* have also inhibited α -amylase activity, proving the antidiabetic trait of potential probiotic yeast isolates. Ekawati et al. (2024) reported the highest antidiabetic activity of *Ogataea siamensis* K_1

(60.80%), followed by *Ogataea polymorpha* K_6 (60.44%) and *Ogataea polymorpha* K_8 (54.51%). Exopolysaccharides are polymeric molecules that assist probiotics in colonizing the gut mucosa gradually (Lata et al., 2022). Yeast EPS has antioxidant, metal adsorption, antitumour, immune regulation, and other biochemical functions and is widely used in the fields of biomedicine and food industries (Rahbar Saadat et al., 2021; Liu et al., 2022). In our study, *Kl. marxianus* DPA-41 and *Kl. lactis* DTL-6 from cow *dahi*, *Debaryomyces prosopidis* CPA-55, *Meyerozyma guilliermondii* CMN-3, and *D. prosopidis* CCG-4 from cow *chhurpi*, *S. cerevisiae* DKY-22, *S. cerevisiae* DCY-27, *Yarrowia lipolytica* DUCY-6, and *Y. lipolytica* DUAY-11 from yak *dahi*, and *Y. lipolytica* CLAY-2, *Y. lipolytica* CZBY-49, and *Kl. lactis* CUBY-19 from yak *chhurpi* showed positive results for exopolysaccharide production. Until now, it has been reported that yeasts belonging to different genera of *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Kazachstania*, *Candida*, *Rhodotorula*, *Hanseniaspora*, *Sporobolomyces*, *Trichosporon*, *Cryptococcus*, *Metschnikowia*, and *Clavispora* can produce EPS (Yadav et al., 2014; Gientka et al., 2015; Yildiran et al., 2019; Fekri et al., 2020; Rahbar Saadat et al., 2021). The nutritional value and functioning of proteins may be adversely affected by phytate (Andrabi et al., 2016; Puppala et al., 2019). In our study, the majority of yeast isolates were found to hydrolyse phytate. Enzymatic phosphatases, like phytase, catalyse the phosphate hydrolysis from phytate, making it possible for the human gut to absorb phytate complexes (Menezes et al., 2020; Fekri et al., 2020) and make it bioavailable to increase nutritional food value (Puppala et al., 2019). The majority of the isolates were found to hydrolyse phytate, except *S. cerevisiae* DRC-42 and DJT-2 from cow *dahi*, *S. cerevisiae* CGI-29, CRC-35, and *Yarrowia lipolytica* CBG-9 from cow *chhurpi*, and *Y. lipolytica* DZBY-37 and *Candida metapsilosis* CKY-12 from yak *chhurpi*. β -galactosidases are widely used to hydrolyse lactose and improve the digestibility and

sensory aspects of dairy products, especially in the dairy industry (Saqib et al., 2017). Lactose intolerance is a clinical syndrome often characterized by gastrointestinal symptoms and is mainly caused by a deficiency of β -galactosidase in humans. Additionally, lactose intolerance can be alleviated by introducing β -galactosidase-producing probiotic isolates in the gut microbiota (Gingold-Belfer et al., 2020). The yeast *Kl. lactis* is one of the well-known yeasts in nature able to produce β -galactosidase (de Lima et al., 2017; Hsiung et al., 2021). In our study, beta-galactosidase activity was detected in *Kl. marxianus* DPA-41 and *Kl. lactis* DTL-6 from cow *dahi*, *Yarrowia lipolytica* CLAY-2, *Kl. lactis* CLBY-16, *Kl. marxianus* CZAY-19, and *Kl. lactis* CUBY-19 from yak *chhurpi*. Siderophore production is another important feature to tolerate heavy metals (Fernandez-San Millan et al., 2020). El-Maraghy et al. (2020) have also reported the production of siderophore by many yeast species. Flavouring substances such as linalool, nerol, benzyl, geraniol and phenylethyl alcohols are released by beta-glucosidase activity (Sirilun et al., 2016). Vitamin B12 is one of the most significant vitamins in the B complex group. This vitamin is soluble in water and is also known as cobalamin (Jach et al., 2020). In our study, few yeast species were found to produce vitamin B12 on plate-based assay. In our study, isolates were also examined for antifungal activity against *Aspergillus niger* NCIM 1248, *Fusarium oxysporum* NCIM 1350, *Aspergillus niger* NCIM 1358, *Aspergillus oryzae* NCIM 1212, and *Aspergillus flavus* NCIM 549. Alasmar et al. (2020) showed the significant drop in the growth of *Penicillium*, *Aspergillus*, and *Fusarium* genera by *Kl. marxianus* QKM-4. *S. cerevisiae* and *S. boulardii* also have the ability to inhibit the growth of *M. phaseolina* (Hantoosh et al., 2021). In our study, *S. cerevisiae* DKL-13 from cow *dahi*, *Pichia bruneiensis* CPAL-16 and *S. cerevisiae* CRC-35 from cow *chhurpi*, and *Y. lipolytica* DUAY-11 from yak *dahi* exhibited the highest degree of antifungal activity against tested isolates. The

antagonistic characteristics of yeasts are associated with a variety of mechanisms, including the production of toxins, the secretion of enzymes, competition for nutrients and space, the release of volatile organic compounds (VOCs), and the development of plant resistance (Bleve et al., 2006; Choińska et al., 2020; Delali et al., 2021).

CONCLUSION

6. CONCLUSION

This study focused on the diversity of yeast species in naturally fermented milk products of Sikkim by culture-dependent methods, as well as their probiotic attributes and other functional properties. The physicochemical properties and microbial load of the samples were investigated. *Saccharomyces cerevisiae* was the predominant yeast species found in cow *dahi*, cow *chhurpi*, and yak *dahi*, while yak *chhurpi* was found to be dominated by *Yarrowia lipolytica*. Isolates were preliminarily screened based on low pH tolerance (pH 2.0), bile tolerance (0.3% w/v) and MATHs ($\geq 80\%$). The screened isolates were identified based on phenotypic and genotypic methods, and the isolates were then processed for probiotic and functional assessments. Based on the *in vitro* probiotic attributes, *Kluyveromyces marxianus* DPA-41 from cow *dahi*, *S. cerevisiae* CRC-35 from cow *chhurpi*, *S. cerevisiae* DUAY-53 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi* were selected as the potential probiotic candidates. In addition to being safe according to the safety evaluation test, these isolates were shown to have additional functional properties such as cholesterol-lowering, antioxidant, anti-inflammatory, antidiabetic, and antifungal activity, etc. These findings demonstrate that traditional fermented milk products from Sikkim harbour a diverse range of yeast species with notable probiotic potential and various health-enhancing properties. Both the fermentation process and the development of distinctive flavours in these products are greatly aided by the presence of these yeast populations. The promising functional attributes exhibited by the selected isolates suggest their potential for use in functional foods and nutraceuticals. However, further *in vivo* studies and clinical evaluations are essential to validate their efficacy and safety, ultimately paving the way for the development of novel probiotic formulations based on these indigenous yeast isolates.

SUMMARY

Ethnic people of Sikkim Himalaya consume a variety of traditionally prepared fermented cow and yak milk products. Among the various NFM products of the region, *dahi* (curd) and *chhurpi* (hard and soft variety) are familiar and widely consumed by all ethnic groups. Therefore, the prime objective of this study was to explore the traditional practices of milk fermentation by the ethnic communities and the study of probiotic and other functional attributes of yeast isolates native to NFM products. Traditionally prepared *dahi* and *chhurpi* samples prepared from cow and yak milk were collected in a sterilized container from various locations of Sikkim and transported to the laboratory maintaining the lowest temperature in an icebox, and kept in a refrigerator at 4 °C for further analysis. Physicochemical analysis such as pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) were analysed of all the samples collected. Physicochemical analysis such as pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) for cow *dahi* ranged from 3.88 ± 0.01 to 4.76 ± 0.03 ; 242 ± 2 to 492.67 ± 2.52 ; 86.54 ± 0.24 to 98.11 ± 0.18 ; and 0.80 ± 0.01 to 0.95 ± 0.03 . Similarly, the pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) for cow *chhurpi* were also recorded as 3.93 ± 0.03 to 5.13 ± 0.02 ; 130.33 ± 1.53 to 291.33 ± 2.87 ; 55.79 ± 0.59 to 74.67 ± 1.37 ; and 0.63 ± 0.04 to 0.92 ± 0.01 . Yak *dahi* showed a pH value, apparent viscosity (cP), moisture content (%), and titratable acidity (%) ranging from 4.59 ± 0.04 to 4.72 ± 0.04 ; 365.67 ± 3.4 to 462 ± 3.74 ; 86.46 ± 0.25 to 94.38 ± 0.82 ; and 0.83 ± 0.03 to 0.95 ± 0.02 . For yak *chhurpi*, the results for pH, apparent viscosity (cP), moisture content (%), and titratable acidity (%) are between 4.65 ± 0.04 and 5.72 ± 0.05 ; 128.33 ± 2.49 to 150 ± 4.55 ; 42.49 ± 25.64 to 68.73 ± 0.83 ; and 0.75 ± 0.03 to 0.91 ± 0.01 . The Microbial load (Log CFU g⁻¹) reported in YMA, PDA, PCA and NA from cow *dahi* samples ranges from 4.73 ± 0.1 to 7.46 ± 0.02 ; 4.82 ± 0.02 to 7.45 ± 0.05 ; 7.28 ± 0.01 to 9.64 ± 0.02 ; and 5.44 ± 0.04 to 9.64 ± 0.02 , respectively. The Microbial load (Log CFU g⁻¹) reported in

YMA, PDA, PCA and NA from cow *chhurpi* samples ranges from 5.17 ± 0.02 to 7.48 ± 0.02 ; 4.68 ± 0.07 to 7.37 ± 0.04 ; 6.51 ± 0.03 to 9.22 ± 0.04 ; and 6.78 ± 0.25 to 9.25 ± 0.02 , respectively. In yak *dahi*, microbial load (Log CFU g⁻¹) in YMA, PDA, PCA and NA were reported from 4.96 ± 0.08 to 5.82 ± 0.11 ; 4.22 ± 0.09 to 5.51 ± 0.07 ; 5.65 ± 0.11 to 7.45 ± 0.08 ; and 5.06 ± 0.11 to 6.99 ± 0.09 , respectively. The Microbial load (Log CFU g⁻¹) reported in YMA, PDA, PCA and NA from yak *chhurpi* samples ranges from 5.06 ± 0.11 to 5.83 ± 0.14 ; 4.69 ± 0.07 to 5.08 ± 0.11 ; 5.65 ± 0.06 to 7.02 ± 0.1 ; and 5.27 ± 0.05 to 6.41 ± 0.11 , respectively. After physicochemical analysis of the sample, total of 3438 yeast from cow milk products (cow *dahi*- 1779 and cow *chhurpi*- 1659) and 850 yeast from yak milk products (yak *dahi*- 460 and yak *chhurpi*- 390) were isolated and confirmed by phase contrast microscopy. Based on preliminary screening such as low pH tolerance (pH 2.0), bile tolerance (0.3% w/v), and Microbial attachment to hydrocarbons (MATHs) ($\geq 80\%$) test, 20 yeast from cow milk products (cow *dahi*-8 and cow *chhurpi*-12) and 15 yeast from yak milk products (yak *dahi*- 9, yak *chhurpi*- 6) were screened for identification and further analysis. Based on phenotypic and biochemical characterization, isolates were tentatively identified and grouped up to genus level as *Saccharomyces*, *Yarrowia*, *Kluyveromyces*, *Pichia*, *Candida*, *Debaryomyces*, *Kazachstania*, and *Wickerhamomyces*. Identification of the isolates was further confirmed by sequencing D1/D2 domain of large ribosomal subunit and the sequences were submitted to NCBI GenBank for accession number. *Saccharomyces cerevisiae* was found to be the dominant species in *dahi* and *chhurpi* from cow milk, and *dahi* from yak milk. However, *Yarrowia lipolytica* (33%) was found to be the dominant species in yak *chhurpi*.

Likewise, yeast isolates were further tested for other probiotic attributes based on *in vitro* methods. *S. cerevisiae* DAO-17 from cow *dahi*, *Pichia kudriavzevii* CNT-3 from

cow *chhurpi*, *S. cerevisiae* DCY-27 from yak *dahi* and *Y. lipolytica* CZBY-49 from yak *chhurpi* showed highest tolerance to pH 2.0. Similarly, *S. cerevisiae* DRC-42 from cow *dahi*, *Kazachstania exigua* CMNB-4 cow *chhurpi*, *S. cerevisiae* DCY-27 from yak *dahi*, and *Y. lipolytica* CLAY-2 from yak *chhurpi* showed highest tolerance to bile (0.3% w/v). For the Microbial attachment to hydrocarbons (MATHs) test, *S. cerevisiae* DJT-2 from cow *dahi*, *P. bruneiensis* CMS-3 from cow *chhurpi*, *Y. lipolytica* DUCY-6 from yak *dahi* and *Y. lipolytica* CZBY-49 from yak *chhurpi* showed highest hydrophobicity for all four hydrocarbons tested. Furthermore, *S. cerevisiae* DRC-42 from cow *dahi*, *S. cerevisiae* CGI-29 from cow *chhurpi*, *S. cerevisiae* DKY-22 from yak *dahi*, and *Candida metapsilosis* CKY-12 from yak *chhurpi* showed maximum auto-aggregation potential. For co-aggregation assay, *Kluyveromyces marxianus* DPA-41 from cow *dahi* showed highest co-aggregation against MTCC 3223 ($61.16 \pm 0.40\%$), *S. cerevisiae* CRC-35 from cow *chhurpi* showed highest co-aggregation against MTCC 3223 ($59.35 \pm 1.7\%$), from yak *dahi*, *S. cerevisiae* DKY-22 showed maximum percentage of co-aggregation against MTCC 740 ($62.96 \pm 0.74\%$), and from yak *chhurpi*, *K. lactis* CUBY-19 demonstrated highest co-aggregation against MTCC 740 ($58.88 \pm 0.52\%$). Additionally, *K. marxianus* DPA-41 from cow *dahi*, and *Meyerozyma guilliermondii* CMN-3 and *Pichia kudriavzevii* CNT-3 from cow *chhurpi* were found to hydrolyse all three sodium salts, and, *S. cerevisiae* DCY-27, *S. cerevisiae* DUAY-53, *Yarrowia lipolytica* DUCY-6, *C. parapsilosis* DLAY-48, and *Y. lipolytica* DZBY-37 from yak *dahi*, and *Kl. lactis* CLBY-16, *Kl. marxianus* CZAY-19, and *Kl. lactis* CUBY-19 from yak *chhurpi* found to hydrolyse three different sodium salts with different intensities. *Saccharomyces cerevisiae* DKL-13 from cow *dahi*, *S. cerevisiae* CKL-10, *Kazachstania exigua* CMB-19, and *K. exigua* CMNB-4 from cow *chhurpi*, *Yarrowia lipolytica* DUAY-11 and *S. cerevisiae* DUBY-47 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi* showed

antimicrobial activity against MTCC 740, MTCC 3223, and MTCC 1272, and MTCC 740. For lysozyme tolerance test, *S. cerevisiae* DJT-2 from cow *dahi*, *S. cerevisiae* CGI-29 from cow *chhurpi*, *Wickerhamomyces anomalus* DUCY-48 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi* showed highest tolerance. It was found that the temperature of 28 °C is the optimal growth temperature for yeast and growth was also observed at 37 °C temperature. After 37 °C, growth of the yeast was found to be reduced drastically at 40 and 45 °C. For NaCl tolerance, isolates were found to tolerate up to 4% NaCl concentration, however growth was significantly reduced at 6, 8, and 10% NaCl. Probiotic candidate must demonstrate high survivability in stressful condition of the digestive system. *Kl. marxianus* DPA-41 from cow *dahi*, *S. cerevisiae* CRC-35 from cow *chhurpi*, *Wickerhamomyces anomalus* DUCY-48 from yak *dahi* and *Kl. lactis* CUBY-19 from yak *chhurpi* showed highest survivability and lowest CFU log reduction in all three simulated fluids.

Genetic screening of potential probiotic traits by gene detection demonstrated that yeast isolates contain the genes associated with low pH tolerance, bile tolerance, adhesion, and antimicrobial attributes. Most of the isolates showed presence of genes responsible for low pH tolerance, bile tolerance, and adhesion. However, *Kl. marxianus* DPA-41 from cow *dahi*, *S. cerevisiae* CRC-35 from cow *chhurpi*, *S. cerevisiae* DCY-27 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi* were found to show highest number of potential probiotic genes. After genetic screening, isolates were further processed for safety evaluation test. Most of the isolates found to be safe based on the experiments performed. However, lipase activity and the formation of pseudohyphae were demonstrated by *M. guilliermondii* CMN-3 and *Y. lipolytica* CBG-9 from cow *chhurpi*, *C. metapsilosis* CKY-12 and *Y. lipolytica* CZBY-49 from yak *chhurpi*, and *C. parapsilosis* DLAY-48 and *Y. lipolytica* DUAY-11 from yak *dahi*. *Y. lipolytica* CLAY-

2 was found to exhibit lipase activity. Additionally, *C. parapsilosis* DLAY-48 from yak *dahi* were found to exhibited coagulase activity. In addition, isolates were further tested for other functional properties such as cholesterol lowering activity, antioxidant activity, anti-inflammatory activity, anti-diabetic activity, exopolysaccharide production, phytate hydrolyzing activity, beta-D-glucosidase activity, beta-galactosidase activity, antifungal activity, siderophore production, vitamin B12 production, and L-asparaginase activity. After performing all the experiments, heatmap visualization and PCA biplot analysis was performed for both probiotic and other functional attributes. Based on the parameters mentioned above, *Kl. marxianus* DPA-41 from cow *dahi*, *S. cerevisiae* CRC-35 from *chhurpi*, *S. cerevisiae* DUAY-53 from yak *dahi*, and *Kl. lactis* CUBY-19 from yak *chhurpi*, were selected as best potential probiotic yeast with other functional traits.

In conclusion, this study comprehensively explored the traditionally fermented milk products of the Sikkim Himalaya, focusing on *dahi* and *chhurpi* prepared from cow and yak milk. Detailed physicochemical and microbiological analyses of these indigenous products revealed diverse and abundant yeast communities. Through systematic isolation and rigorous screening, several yeast strains were identified with significant probiotic potential. Selected isolates demonstrated key probiotic attributes based on *in vitro* probiotic attributes. Genetic screening showed that these yeast isolates have genes related to probiotic traits, and safety checks revealed that most of them are not harmful and can be used safely. Moreover, functional assessments highlighted additional health-promoting properties, including antioxidant, anti-inflammatory, anti-diabetic, and cholesterol-lowering activities, among others. The findings of this research underscore the immense potential of native yeast strains from traditional fermented milk products of Sikkim as potential probiotic yeast candidates, paving the way for their future application in functional foods and nutraceuticals.

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CURRICULUM VITAE (CV)

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Award/Fellowships:

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3. Best Oral Presentation (Student) award at the **7th International Conference of Indian Network for Soil Contamination Research (INSCR)** organized by Phixgen Pvt. Ltd, Gurugram in association with Delhi University from 07th -11th November 2022.
4. Young Investigator Award at **13th India Probiotic Symposium**, “Science Based Evidence on the Benefits of Probiotics for Human Health” organized by the Gut Microbiota and Probiotic Science Foundation (India) and the Institute of Liver and Biliary Sciences (ILBS), Delhi, on 25th & 26th March 2023.

5. Young Investigator Award at the **15th India Probiotic Symposium**, "Celebrating the Science of Gut Microbiota and Probiotics" organized by the Gut Microbiota and Probiotic Science Foundation (India) and the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, on 22nd & 23rd February 2025.
6. Best speaker award (3rd position) in the **Young Scientist Flash Talk at the First National Symposium on Traditional Knowledge in Food, Beverages & Pharmaceuticals: Tradition to Translation (IndiGen-Symp-2025)** Organised by Institute of Advanced Study in Science and Technology (IASST), Guwahati, India on 8-9 May 2025.
7. **National Fellowship for Higher Education of ST Students (NFST)** by the Ministry of Tribal Affairs, Govt. of India.
8. Currently working as a **Junior Research Fellow (JRF)** in a Department of Biotechnology, Government of India sponsored National Himalayan Bioresource Project in the Department of Microbiology, Sikkim University, since December 2024.

Oral/Poster presentation:

1. Presented research paper (oral) entitled "*Screening of Probiotic Yeasts from some Naturally Fermented Milk products of Sikkim*" at **7th International Conference of Indian Network for Soil Contamination Research (INSCR)** organized by Phixgen Pvt. Ltd, Gurugram in association with Delhi University (7th -11th November 2022).
2. Presented research paper (oral) entitled "*Potential Probiotic Yeasts from Fermented Cow-milk and Yak-milk Products of Sikkim*" at **National Conference on "Contribution of Microbial Science for Societal Development"** Organized by Department of Microbiology, Sikkim University Sponsored by SERB, Department of Science and Technology, New Delhi (15th December 2023).
3. Presented research paper (poster) entitled "*In vitro assessment of the probiotic and functional traits of yeast from naturally fermented yak milk products of Sikkim, India*" at the **15th India Probiotic Symposium organized by the Gut Microbiota and Probiotic Science Foundation (India)** in collaboration with the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, on 22nd & 23rd February 2025.
4. Presented research paper (oral) entitled "Probiotic yeasts from fermented yak-milk" at the **First National Symposium on Traditional Knowledge in Food, Beverages & Pharmaceuticals: Tradition to Translation (IndiGen-Symp-2025)** Organised by Institute of Advanced Study in Science and Technology (IASST) (An autonomous R&D Institute Under DST, Govt. of India), Guwahati, India on 8 and 9th May 2025.

Workshop/Training:

1. Attended two-day National Workshop on "Bifidobacterial Probiotics: Supplementation through fermented foods" sponsored by Probiotic Association of India (PAI) and organized by Microbiology & Fermentation Technology Department, CSIR- Central Food Technological Research Institute, Mysuru- 570020 on 24th & 25th March 2022.

2. Attended “Hands on Training on Basic Molecular Biology & Bioinformatics” held at the Institute for Development of Advanced Computing, ONGC Centre of Advanced Studies, University of Lucknow, Lucknow in collaboration with Centre of Excellence in Computational Biology & Bioinformatics Infrastructure Facility, Lucknow University, Lucknow from 20th to 22nd August, 2019.
3. Completed internship at the Bacteriology laboratory, Central Referral Hospital (CRH), Gangtok from 10th December 2017 to 31st January 2018.

Publications:

1. **Lama, S.** and Tamang, J.P. (2022). Isolation of yeasts from some homemade fermented cow-milk products of Sikkim and their probiotic characteristics. *Fermentation* 8: 664. doi: 10.3390/fermentation8120664 (**Impact Factor: 3.3**) (**Research article**)
2. Tamang, J.P. and **Lama, S.** (2022). Probiotic properties of yeasts in traditional fermented foods and beverages. *Journal of Applied Microbiology* 132: 3533-3542. doi: 10.1111/jam.15467 (**Impact Factor: 3.2**) (**Review**)
3. Tamang, J.P. and **Lama, S.** (2023). Diversity of yeasts in Indian fermented foods and alcoholic beverages. *FEMS Yeast Research* 23:11. doi: 10.1093/femsyr/foad011 (**Impact Factor: 2.4**) (**Review**)
4. Rai, A., **Lama, S.**, Das, S., Thatal, B. and Tamang, J.P. (2024). Potential probiotic yeasts isolated from idli and selroti, Indian ethnic fermented cereal-based foods. *Current Probiotics* 1, e26666499321746, 47-61. doi: 10.2174/0126666499321746240809073632. Bentham Science. (**Research article**)
5. Tamang, J.P., Das, S., **Lama, S.**, Utama, G.L., Safitri, R., Balia, R.L. and Thapa, N. (2025). Analysis of meta-transcriptomics and identification of genes linked to bioactive peptides and vitamins in Indonesian tempe. *Food Research International* 202, 115757. doi: 10.1016/j.foodres.2025.115757 (**Impact Factor: 8.0**) (**Research article**)

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Sonam Lama

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LIST OF PUBLICATIONS



Article

Isolation of Yeasts from Some Homemade Fermented Cow-Milk Products of Sikkim and Their Probiotic Characteristics

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Abstract: *Dahi* and *chhurpi* are the homemade, mildly acidic and mouthfeel fermented dairy products of Sikkim in India. Since yeasts co-exist among traditional fermented dairy foods, we believe that some species of yeasts may have some probiotic properties. Hence, the present study is aimed at screening some probiotic yeasts from *dahi* and *chhurpi*. A total of 3438 yeasts were isolated from 40 samples of *dahi* (1779 isolates) and 40 *chhurpi* (1659 isolates) and were preliminarily screened for probiotic properties on the basis of survival in low pH, resistance to bile salts and the percentage of hydrophobicity, out of which only 20 yeasts were selected for in vitro and genetic screening of probiotic properties. *Saccharomyces cerevisiae* DJT-2 and *Debaryomyces prosopidis* CPA-55 showed the highest hydrophobicity of 97.54% and 98.33%, respectively. *S. cerevisiae* DRC-42 and *S. cerevisiae* CGI-29 showed 93.88% and 91.69% auto-aggregation, respectively. All yeasts showed co-aggregation properties against pathogenic bacteria. *Kluyveromyces marxianus* DPA-41 and *Pichia kudriavzevii* CNT-3 showed excellent deconjugation activities. Probiotic genes for acid tolerance, bile tolerance, adhesion and antimicrobial activity were detected in *S. cerevisiae* DAO-17, *K. marxianus* DPA-41, *S. cerevisiae* CKL-10 and *P. kudriavzevii* CNT-3. Based on the results of in vitro and genetic screening of probiotic yeasts strains, *S. cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *P. kudriavzevii* CNT-3 (*chhurpi*) and *K. marxianus* DPA-41 (*dahi*) were selected as the potential probiotic yeasts.

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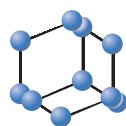
Keywords: fermented dairy products; probiotics; yeasts; *dahi*; *chhurpi*; *Saccharomyces*; *Kluyveromyces*

1. Introduction

The souring of animal milk, by natural or back-sloping processes, is one of the oldest inventions of humans for prolonging the shelf life of perishable animal milk. Among the fermented dairy products, cheese and yogurt are the most popular products around the world with several health claims including having probiotic properties [1–4], followed by *kefir*, a viscous and slightly fizzy product obtained by the fermentation of milk and *kefir* grains [5], as a probiotic milk product [6,7]. The majority of populaces in the world cannot afford to buy the commercial probiotic milk products; however, they traditionally prepare various artisanal, naturally fermented dairy products from domesticated animals such as cow, buffalo, yak, camel, mare, sheep and donkey at household levels in different regions of the world [8–14], which may or may not have probiotics properties.

The probiotic properties of some region-specific and artisan-fermented dairy products of a few countries have also been reported, such as the *lait caillé* of Senegal [15], *tarag* and *airag* of Mongolia [16], *amasi* of South Africa [17], *dadih* of Indonesia [18], *dahi* of India [19,20] and *nunu* and *wara* of West Africa [21]. However, most of these probiotics properties in fermented dairy products have been shown by species of lactic acid bacteria (LAB) of *Lactocaseibacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Limosilactobacillus* and *Lactobacillus* [22–24], and non-lactic acid bacteria such as *Propionibacterium* [25] and *Bifidobacterium* [26]. The probiotic properties of yeasts in fermented dairy products are barely reported [27–29]. *Saccharomyces cerevisiae* var. *boulardii* is the only clinically claimed probiotic yeast that is commercially available for human use [30], and it has also been reported from few fermented dairy products [31–33].

RESEARCH ARTICLE

BENTHAM
SCIENCE

Potential Probiotic Yeasts Isolated from Idli and Selroti, Indian Ethnic Fermented Cereal-based Foods

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Abstract: Introduction: In the present study, we focused on the screening of the potential probiotic yeasts isolated from two Indian fermented cereal-based foods, viz., Idli and Selroti. A total of 260 yeast isolates were isolated from the batters of Idli (140 isolates) and Selroti (120 isolates).

Methods: Preliminary screening of basic probiotic traits such as tolerance to low pH, bile, and cell surface attachment was checked for the selection of potential probiotic yeasts from total isolates. Finally, 8 yeast isolates were selected for further in-depth assessment by *in vitro* and genetic screening, which included *Clavispora lusitaniae* AIY-4, *Wickerhamomyces anomalus* MIY-30, *Pichia kudriavzevii* BIY-8 (from idli), *Yarrowia lipolytica* SGLY-15, *Y. lipolytica* SGLY-21, *Candida parapsilosis* SPRY-17, *C. parapsilosis* SBRY-12, and *Kodamaea ohmeri* SBRY-25 (from selroti).

Results: A principal component analysis (PCA) biplot was designed to evaluate the differences and similarities amongst the yeast strains, and two clusters were formed using the paired group (UP-GMA) algorithm and Euclidean similarity index. Cluster one was comprised of *Cl. lusitaniae* AIY-4, *W. anomalus* MIY-30, *C. parapsilosis* SBRY-12, and *P. kudriavzevii* BIY-8, and another cluster included *C. parapsilosis* SBRY-12 and *Y. lipolytica* SGLY-21.

Conclusion: Hence, based on statistical analysis for probiotic *in vitro* and genetic screening, *Wickerhamomyces anomalus* MIY-30 (Idli) and *Kodamaea ohmeri* SBRY-25 (selroti) were selected as the most potential probiotic strains.

Keywords: Probiotic yeasts, idli, selroti, *wickerhamomyces*, *kodamaea*, *candida*, *pichia*.

1. INTRODUCTION

Millions of individuals globally depend on cereals as a staple in their diets due to their readily available calorie content, distinctive qualitative attributes, and widespread acceptance. Some cereals, such as rice, maize, and wheat, are traditionally fermented either naturally or by introducing starter cultures to various ethnic fermented cereal dishes and alcoholic beverages [1, 2]. In India, there is a wide range of fermented foods made from various agricultural products, including cereals [3, 4]. One of the most popular fermented cereal-based foods in South India is Idli, while Selroti is a favoured fermented cereal-based confectionery in North East India. Idli (Supplementary Fig. 1a) is a soft, white, round, and porous steamed cake from South India made from de-hulled black gram (*Vigna mungo*) and rice (*Oryza sativa*) batter through spontaneous fermentation, commonly consumed for breakfast [5]. Selroti (Supplementary Fig. 1b), on the other hand, is a unique fermented cereal confectionery

resembling a fritter shaped like a donut and is a common snack in the diets of North East Indian people [6, 7]. Species of lactic acid bacteria (LAB) viz., *Leuconostoc*, *Lactococcus*, *Weissella*, *Lactobacillus*, *Enterococcus*, *Bacillus*, and *Macrorococcus* [7-12]; and certain yeast species like *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Hansenula anomala*, and *Candida versatilis* [13, 14] are the primary microorganisms involved in fermenting idli. Similarly, a small number of LAB and yeasts, including *Saccharomyces cerevisiae*, *S. kluyveri*, *Debaryomyces hansenii*, *Pichia burtonii*, and *Zygosaccharomyces rouxii*, have been identified in Selroti based on phenotypic analysis [15, 16].

Probiotics are live microorganisms that offer various health benefits to consumers, such as treating dysbiosis in the human gut microbiome [17-19]. Some microorganisms have probiotic traits and are considered potential probiotic cultures [20, 21]. While there are reports on the probiotic properties of LAB in fermented cereal foods, the probiotic potential of yeasts in fermented cereal foods is not widely recognized [22-26]. Probiotic yeast may have an advantage over probiotic bacteria as it is believed to have a better chance of surviving in the stomach due to the presence of

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Analysis of meta-transcriptomics and identification of genes linked to bioactive peptides and vitamins in Indonesian *tempe*

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ARTICLE INFO

Keywords:

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RNA-seq
Meta-transcriptome
Gene expression

ABSTRACT

Tempe, a fermented soybean product, plays a significant role in Indonesian dietary culture. Currently, there have been no studies that classify *tempe* as a functional food using up-to-date genomic data. This research used an Illumina-based meta-transcriptomic method to explore the microbial communities that mainly contribute to *tempe* fermentation and their functions related to its health benefits. The analysis revealed 8 phyla, 19 families, 20 genera, and 28 species of both bacteria and fungi. The dominant species found were *Klebsiella africana*, followed by *Rhizopus microsporus*, *Klebsiella pneumoniae*, *Rhizopus oligosporus*, *Propionibacterium humerusii*, and *Lactobacillus brevis*. *Klebsiella* and *Propionibacterium* are involved in vitamin B12 production, while *Rhizopus* is associated with GABA production and isoflavones like genistein and daidzein, along with spermidine, caffeic acid, and chlorogenic acid. *Lactobacillus* contributes proteases, GABA, and beta-glucosidase. The study also strengthened the link between microbes and health benefits by identifying certain microbial genes and their expression levels in the samples. Changes in gene expression reveal how fermentation environments affect diversity and functionality. Overall, finding a variety of bioactive compounds and their genetic backgrounds supports *tempe*'s position as a functional superfood that could gain global popularity through further research and development.

1. Introduction

The ancient inhabitants of Southeast Asia had an early proficiency in utilizing beneficial microorganisms for food production (Tamang et al., 2020). A notable example is *tempe*, an ethnic fermented soybean product covered in fungal mycelium, resembling dense bacon with a chewy texture and a meaty flavour, originating from Indonesia. The conventional method includes wrapping cooked soybeans in leaves containing *Rhizopus* spp. for fermentation to produce *tempe* (Tamang et al., 2022). The primary culture used is *Rhizopus oligosporus*, though yeasts and bacteria can also affect the microbial makeup during the fermentation process (Nurdini et al., 2015). The traditional *tempe*-making process involves soaking, dehulling, and boiling soybeans before wrapping and fermenting them for 24–48 h. The final product is a compact cake of

soybeans encased in *Rhizopus* mycelium, which can be fried or served alongside rice. Variations in traditional preparation techniques may alter the microbial ecology during fermentation, resulting in differences based on location and individual producers.

Tempe fermentation has been an interesting area of research in food microbiology for a long time due to its nutritional value, health benefits, and the factors that affect it. Various species of the *Rhizopus* have been found in *tempe* through earlier studies that used traditional methods, genetic sequencing, and modern sequencing techniques. These include *Rhizopus microsporus* variety *oligosporus*, *R. oryzae*, *R. delemar*, *R. arrhizus*, *R. stolonifer*, and *R. chinensis*, as well as *Mucor* species, other fungi, and yeasts (Sjamsuridzal et al., 2021; Vebliza et al., 2023). Various types of *Rhizopus* have been reported in *tempe* for their ability to change isoflavone glycosides into isoflavone aglycones, which offer several health

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REVIEW ARTICLE

Probiotic properties of yeasts in traditional fermented foods and beverages

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Abstract

The interest in potentiality and functionality of probiotic yeasts from fermented foods has increased drastically over the years. In many fermented foods and beverages, lactic acid bacteria and yeasts exist synergistically by stimulating their growth and survival. Probiotic strains of lactic acid bacteria are more widely studied than potential probiotic yeasts. *Saccharomyces cerevisiae* variety *boulardii* is the only commercialized probiotic yeast, which are extensively studied. This review article provides information on the presence of potential probiotic yeasts in some traditional fermented foods and beverages.

KEYWORDS

fermented beverages, fermented foods, probiotics, *Saccharomyces boulardii*, yeasts

INTRODUCTION

Traditional food fermentation has always been considered as affordable and convenient practice to store perishable food bio-resources by preserving and enhancing the shelf-life, organoleptic and functional properties of the edible products (Rezac et al., 2018). Fermentation-associated microorganisms generally out-compete with potential pathogenic microorganisms, further intensify food safety and stability (Marco et al., 2021). Diverse groups of culturable and unculturable bacteria, yeasts, filamentous moulds, viruses and archaea appear and coexist during the natural fermentation of foods (Tamang, 2021; Tamang et al., 2020; Voidarou et al., 2021), contributing a huge microbial diversity in the food ecosystem (Rezac et al., 2018; Verardo et al., 2020). World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have defined probiotics as “viable microorganisms which, when administered in adequate amount, provide health benefits to the host” (Hill et al., 2014) and are considered as dietary supplements (Binda et al., 2020) that stabilize the gut microbiome (Ma et al., 2020). Probiotic strains should exhibit the subsequent characteristics to be observed as functional food ingredients such as acid and bile-stability, tolerance to

digestive enzymes, adhesion to intestinal wall, antagonistic activity, anti-carcinogenic and anti-mutagenic activity, cholesterol-reducing effects, stimulation of the immune system without inflammatory effects, improvement of bowel motility, conservation of mucosal stability, antioxidant, auto-aggregation, development of bioavailability of food ingredients and production of vitamins and enzymes (Krausova et al., 2019; Kwofie et al., 2020; Menezes et al., 2020; Staniszewski & Kordowska-Wiater, 2021). Probiotics have diverse mechanisms to operate such as production of antimicrobial compounds (Silva et al., 2020), regulation of the immune response through the secretion of IgA against potential pathogens (Ahiwe et al., 2021), improvement of function of the intestinal mucosal barrier (Roohvand et al., 2020) and modulation of expression of host genes in gut (Plaza-Diaz et al., 2019). Probiotics which is administered orally must pass through stressful gastric environment of the host's upper gastrointestinal tract (GIT) (Shokryazdan et al., 2017). Probiotics must have acid and bile tolerance or exclusion mechanisms in order to survive in the gut (Suvarna et al., 2018). As compared to bacterial candidate for probiotics, yeast species have higher survivability in low pH (1.5), and thus are promising candidates as probiotics (Hsiung et al., 2021). Tolerance

Diversity of yeasts in Indian fermented foods and alcoholic beverages

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Abstract

Ethnic Indian people have been domesticating beneficial microorganisms (bacteria, yeasts, and moulds) by their wisdom of ethno-microbiological knowledge for production of flavoured and socio-culturally preferred fermented foods and alcoholic beverages for more than 8000 years. The purpose of this review is to collate the available literatures of diversity of *Saccharomyces* and non-*Saccharomyces* species associated with Indian fermented foods and alcoholic beverages. A colossal diversity of enzyme- and alcohol-producing yeasts under the phylum *Ascomycota* has been reported from Indian fermented foods and alcoholic beverages. The distributions of yeast species show 13.5% of *Saccharomyces cerevisiae* and 86.5% of some non-*Saccharomyces* spp. in Indian fermented foods and alcoholic beverages, based on the reported literatures available till date. There is a research gap on prospect of yeasts research in India. Hence, we suggest that validation of traditional knowledge of domestication of functional yeasts needs to be studied to develop the functional genomics platforms for *Saccharomyces* and non-*Saccharomyces* spp. in Indian fermented foods and alcoholic beverages.

Keywords: yeasts, Indian fermented foods, alcoholic beverages, *Saccharomyces*, non-*Saccharomyces*

Introduction

Glimpse on Indian gastronomy

The age-old wholesome staple meal called *Ayurveda ahar* was the ancient Indian wholesome diet comprising of rice, legumes, vegetables, and milk products as a healthy vegetarian meal since Vedic period around 1500 BCE (Payyappallimana and Venkatasubramanian 2016, Tamang 2016). Indian gastronomy is a mixture of multicuisines and recipes due to vast multiracial communities, diverse agro-climatic conditions, and variable geographical locations. Cooked rice is a staple diet, followed by baked-wheat and other minor cereals, which is eaten with legumes, varieties of seasonal vegetables, milk and milk products, meat, and also fish mostly in the coastal regions. Every Indian meal has the recipes of fermented foods as staples or side-dish or taste maker and alcoholic/nonalcoholic beverages. More than 1000 types of common, uncommon, artisan, exotic fermented foods, and alcoholic beverages and drinks are consumed in India (Tamang 2022a), mostly based on accessibility of edible plant/animal resources, geographical locations, and divergent food preferences. Ethnic Indian people have been domesticating functional microorganisms (bacteria, yeasts, and moulds) through their ethno-microbiological knowledge for production of flavoured and socio-culturally preferred fermented foods and alcoholic beverages for more than 8000 years (Tamang 2022a). Most of the Indian fermented foods are traditionally produced by spontaneous fermentation, which may facilitate the growth of bacteria, yeasts, moulds along with viruses, and archaea. Substrate-based fermented foods of India are fermented cereals (mostly fermented by bacteria and yeasts), fermented legumes (fermented by yeasts), fermented cereal (fermented by yeasts and bacteria), fermented mixture of legume and cereals (fermented by bacteria and yeasts), fermented dairy prod-

ucts (fermented by bacteria, and followed by yeasts), fermented soybeans (fermented by bacteria predominantly by *Bacillus* spp.), fermented vegetables and bamboo shoots products (fermented by bacteria mostly lactic acid bacteria), fermented meat and fish products (fermented by bacteria), and fermented alcoholic beverages (moulds, yeasts, and bacteria) (Tamang 2022b).

Few researchers have reviewed the bacterial-fermented foods of India and their health benefits (Satish Kumar et al. 2013, Das et al. 2016, Rasane et al. 2017, Tamang et al. 2021, Tamang 2022a, b). However, very sporadic publications are available exclusively on yeasts-fermented Indian foods (Table 1) (Syal and Vohra 2013, Bardhan et al. 2020, Aggarwal et al. 2021, Nath et al. 2021, Lama and Tamang 2022, Tamang and Lama 2022). The objective of this review is to collate the available literatures on yeasts-fermented and alcoholic beverages of foods.

Yeasts in Indian fermented cereal foods

Cereals and legumes are the lead edge prime foodstuffs consumed by majority of Indians in diversified forms including fermented cereals. Although most of these traditional foods are homemade, few are commercially feasible in restaurants and industries as well. *Jalebi* is an Indian sweetened fermented confectionary product, prepared from refined wheat flour, mixed with *dahi* (curd), sugar syrup, and water, and is eaten as deep-fried pretzel-like sweet confectionary in all over India (Table 1). *Saccharomyces cerevisiae*, *S. bayanus*, *Candida tropicalis*, and *Pichia manshurica* (Fig. 1) are the major yeast species found in the fermented batter of *jalebi* (Batra and Millner 1974, Syal and Vohra 2013). Recently, yeasts community in fermented batter of *jalebi* was profiled by shotgun metagenomic tool and detected *S. cerevisiae*, *Wickerhamomyces anomalus*, *Debaryomyces hansenii* (= *Candida famata*), and *Pichia ku-*

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