Characterization of PsychrotrophicMolds in IndigenousFermented DairyProducts

ABSTRACT

The research work was conducted in the Department of Dairy Microbiology, Dairy Science College, KVAFSU,Hebbal,Bengaluru,560024,Karnataka,IndiabetweenJune2014toJune2015 as part of post graduate research work. The indigenous fermented dairy product samples unbranded (n=6) and branded (n=15) used in the study were Mishtidoi, Shrikhand, Lassi and Buttermilk which are collected from Bengaluru markets. The market samples of indigenous fermented dairy product samples were subjected for isolation on poured sterile Malt Extract Agar of pH 3.5. The study used the milk product samples collected from different shops in Bengaluru and analyzed in Dairy Microbiology Laboratory at Dairy Science College.~~The isolateswere obtained by Pp~~our-plate, staining techniquewith biochemical tests were used to study the Psychotrophic molds cultured on Malt Extract Agar.~~ The mold isolates were identified by colony morphology, microscopic examination for cell morphology and specific biochemical tests.Outoftotalof21psychrotrophicmoldisolates,~~The results revealed that6and15isolates~~wereobtained~~from unbranded and branded samples were obtained, respectively. Based on the preliminary identification, the isolates were characterized as *Penicillium* spp. (n=3), *Mucor* spp. (n=2) and *Cladosporium* spp. (n=1) from unbranded samples while branded indigenous fermented dairy product samples showed *Penicillium* spp. (n=9 and *Alternaria spp*. (n=6) were isolated from branded samples. According to pheno and genotypic characterization, the isolateswereidentifiedas*P.chrysogenum*(57.12%)followedby*A.alternata*(28.56%),*C.cladosporioides*(4.76%)a nd*M.mehei*(9.52%)atthespecieslevel.Except unbranded mishitdoi, all types of indigenous fermented dairy product samples were found to contain psychrotrophicmolds.*P.chrysogenum*wasthe mostabundantspeciesespeciallyin branded butter milk indicating longer refrigeration may lead to selection of psychrotrophic molds due to low temperature storage and favourable pH of the indigenous fermented dairy product samples.

Keywords: Psychrotrophicmold, Morphology, Unbranded, Mishtidoi, Refrigeration

1. INTRODUCTION

India ranks first in the world milk production with 230.58 million tonnes during 2022-23 [1]. Produce ~~M~~milk ~~produced~~is ~~utilized~~ oftenly used to manufacture a variety of milk products such as heat desiccated, acid coagulated products and fermented dairy products to increase the shelf life. Simple processes are usedtopreservemilk'snutritionqualitytoprotect and promote health. Indigenous dairy products (IDPs) may be defined as~~those~~milk products that are native to India, evolved over ages and are prepared ~~utilizing~~ usinglocally available cooking wares and fuels. Out of the total milk production, 50-55% is utilizedtomanufactureIDPsin which 7% is utilized to manufacture fermented dairy products such as Mishtidoi, Shrikhand, Lassi and Butter milk[2].Market for Indigenous Fermented Dairy Products (IFDPs)inBangalore is covered by varieties of brands like AMUL, Danone, MilkyMist, Mother Dairy, Britannia, Nilgiris, K. C. DasanditsregionalbrandssuchasNandini.TheIFDPs need to be stored at low temperature to avoid spoilage by molds. The presence of *Aspergillus*, *Fusarium*, *Mucor* and *Penicillium* spp.,were noticedin yogurt stored at low temperature.Low temperature storage extends the shelf life of IFDPs,~~but significance of psychrotrophic molds and their metabolites need to be considered.~~however, Psychrotrophic molds (*Penicillium, Rhizpous* and *Aspergilllus*) produce proteolytic and lipolytic enzymes ~~even at low~~aat that temperature ~~that~~ whicheventually

Comment [T1]: This must go to the methodology section

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Comment [T4]: After the presenting the key finding of the study, end with the general conclusion, and recommendation. Do not wordy the abstract.

Comment [T5]: Kindly provide the examples of such product, avoid being too technical

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alters the product's composition leading to defects [3].In the fall of 2013, a large spoilage incident resulted in a national recall of Greek yogurt contaminated with the mold *Mucor circinelloides*. Approximately one month's production was recalled for potential contamination, and the event received national media attention. The company initially implemented a product withdrawal, but after several hundred consumers reported symptoms of gastroenteritis, the withdrawal was changed to a voluntary recall [4, 5]. Psychrotrophicmoldcountof 1.8 $\log_{10}$cfu/g was noticed during storage of shrikhand, an IFDP atrefrigerationtemperaturefor10 days[6]. In some cases, contamination of yogurt products with dimorphic mold (e.g., Mucor), or those that can grow as yeast or mold depending on environmental conditions, have also been shown to cause yogurt spoilage[5]. A total of 21 psychrotrophic mold isolates were obtained from the Indigenous Fermented Dairy Products such as Mishti Doi, Shrikhand, Lassi and Buttermilk collected from Bengaluru market, ~~Karnataka, India, that~~ revealed predominance of *Penicillium chrysogenum* (12 nos.) followed by *Alternaria alternata* (6 nos.), *Mucor mehei* (2 nos.) and *Cladosporium cladosporioides* (1 no.) [7]

In a mycological study, a total of 125 random samples from raw milk, locally manufactured kareish cheese, hard cheese (Rass cheese), plain yoghurt and flavored yoghurt (25 of each) collected from dairy shops and super markets in Beni Suef city, Egypt revealed an average log viable counts of molds as 2.34, 0.70, 3.63 and 0.40in the examined milk, Rass, locally manufactured kareish cheese and flavoured yoghurt samples respectively, while molds couldn't be detected in the examined plain yogurt samples. The most predominant species of *Mucor, Absidia, Penicillium, Geotrachum candidum, Cladosporium, Phoma, Fusarium*, *Asperigellus flavus, Asperigellus niger* and *Asperigellus fumigatus* but samples were free ofaflatoxin M1 [8]. The primary mold found in yogurt spoilage ~~incidents~~was *Penicillium*, due to its acidic pH. However, a number of other genera have been reported in yogurt including species of *Mucor, Alternaria, Aspergillus, Monilia* and *Rhizopus*[9].*M. circinelloides* 01180023 required around 14 days to reach a specific colony size of 1.5×10^5 (in pixels) at 5°C with sporulation was observed after incubation ranging from 9 to 11 days. Growth of *P. roqueforti* ISI4 started out slowly withthe highest growth recorded after day 20 at 5°C[10]. Fermented milk products such as yoghurt and sour milk from Western Kenya were contaminated with fungal species that cause spoilage, as the low pH (2.9–3.6) of these products favour their growth. The common molds observed were species of *Penicillium* and *Aspergillus*[11]. Fungal count on an average of 4.99 (77 samples), 5.29 (111 samples) and 4.23(111 samples) were found in raw milk cheese, pasteurized milk cheese and Kajmak samples (a thick, tangy clotted cream from the Balkans usually made with sheep's or cow's milk) collected from small scale producers of Serbia, respectively. The present study showed mostly satisfactory levels of yeasts and molds in the cheese and Kajmak samples, exceeding 8 log CFU/g, indicated potential spoilage in Serbia[12].

In addition to organoleptic properties' deterioration, certain molds like *Penicillium* spp., release the mycotoxins such as citrinin, patulin, penicillic acid, ochratoxin A and sterigmocystinincheeseandotherfermented dairy products which are difficultto destroy duringmilkprocessing~~toproduceFDPs~~[3]. The mycotoxins (low weight metabolites which cause harm known as mycotoxicoses) are the secondary metabolites produced by certain molds which have been found to be present in most food substances like in livestock and their by products therefore of public health significance[13].The *Penicillium* species are highly adaptive to low temperatures and several of them are considered xerophilic species, therefore, their occurrence in spoiled dairy products like yoghurt, soft, semisoft cheeses is not surprising. Citrinin production mainly by *P. citrinicum* and also by *P. chrysogenum* found to be involved in nephrotoxicosis issues by disfunctioning of renal veins of kidney and also caused Balkan nephropathyandyellowricefeverwhenthefood contaminated with the mold ~~was~~ isconsumed. Various cytotoxic and genotoxic effects were offered by patulin like damage to the immune system; pancreas; liver and gastrointestinaltract[14,15]. *Mucor* spp. are common spoilage agents of yogurt and cheese. Apart from the negative impacts on food quality, such as visible growth and off-flavors, some fungal genera possess the ability to produce mycotoxins, which constitute a threat to human health. For instance, *Mucor circinelloides* (*M. circinelloides*) is recognized as an opportunistic pathogen, which can produce the mycotoxin 3-nitropropionic acid[10]. The spoilage mold*Aspergillus*spp. can also produce aflatoxin. The occurrence of AFM1 in milk results from the conversion by dairy animals, fed on aflatoxin B1 (AFB1)-contaminated feedstuffs, of AFB1 to AFM1, which then pass to their urine and milk. Large doses of aflatoxins can lead to acute poisoning (aflatoxicosis) and can be life threatening, usually through damage to the liver. Aflatoxins have also been shown to be genotoxic, meaning they can damage DNA and cause cancer in animal species. There is also evidence that they can cause liver cancer in humans [13, 16].

The present study help to ~~know the number and types of~~ccharacterize thePpsychrotrophic molds occuring in

Comment [T12]: Is this a brand? Is so, try to use pronoun (some, etc…)

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IFDPs and ~~gives an idea regarding their metabolites (enzymes and toxins) that~~establish measures to curb the~~cause~~ the spoilage of the end products~~and health hazards when such products are ingested~~.

2. MATERIALSANDMETHODS

2.1 CollectionofIFDPs: Different varieties of mishtidoi, shrikhand, lassi and buttermilk samples were collected in an ice box from many sweetmeat sellers as well as from retailbrandedsellersofBanashankari,Hebbal,R.T. Nagar, Majestic areas of Bengaluru city. The samples~~which~~ were considered as unbranded ~~samples~~ and branded ~~samples~~ respectively. The samples were brought to the laboratory for the isolation of psychrotrophic molds using Malt Extract Agar (MEA) adjusted to pH 3.5[17] followed by incubation at 7°C for 20-25 days.

2.2 Isolationof Psychrotrophic Molds from IFDPs: Plates, showing cottony colonies were selected and streaked thrice onto MEA plates in order to purify the isolates [18].The purified isolates were grown on MEA slants, subcultured every month and maintained at 2°C till required.

2.3 PhenotypicCharacterizationof Psychrotrophic Mold Isolates

2.3.1 Macroscopic and Microscopic characteristicsofthe isolates: The psychrotrophic mold isolates obtained were characterized macroscopically through thecolony morphology, such as colour of colony, front view, reverse view, surface of the colony and any exudate production over the colony.The isolates were microscopically identified by Wet Mount Staining technique[19].

2.3.2 Specifictestsforselectivemoldisolates:

2.3.2.1 Ehrlichtest:All the mold isolates were examined for production of cyclopiazonic acid and other alkaloidsreactingwithEhrlichusingafilter paper method.The appearance of a violet ring for 2- 6 min indicates the presence of cyclopiazonic acid or related alkaloids[20].

2.3.2.2 ScreeningofPenicilliumisolatefor penicillin production*: Penicillium* isolate was tested for penicillin production. After extraction they are tested byβ-Lactamasetestagainst*Staphylococcus aureus*. The production of yellow color confirms the presence of penicillin [21].After the preliminary test, the penicillin extracted was tested for its antibacterial activity against *Staphylococcus aureus* [22].A clear ~~Z~~zone of inhibition was noted and the activity of the antibiotic was confirmed.

2.3.2.3 ScreeningofthePenicilliumisolatefor citrinin production*:* Citrinin production form penicillium isolates were tested against *S. aureus* and *Escherichia.* Citrininwasfoundtoinhibit*Staphylococcus aureus*butnotthe*Escherichiacoli*.Theinhibition zonesaroundwellsofcitrinininoculatedwiththe*S. aureus* culture confirms the presence ofcitrinin toxin[23].

2.3.2.4 Genotypicidentificationofmoldisolates: The gene sequencing of mold isolates was outsourced for Serene Biosciences, Molecular Diagnostics Research, Custom services & Training. No. 30, II cross, Marappa Garden, Benson Town Post, Bangalore - 560 046 to determine the genotypic identity[23].

3. RESULTSANDDISCUSSION

3.1 Psychrotrophic Mold Isolated from IFDPs

Out of 21 isolates, 6 and 15 numbers of isolates were obtained from unbranded and branded samples, respectively (Table 1). Four different colony morphologies were obtained among the mold isolates.Theseisolateswerefurthersubjectedto preliminary, specific tests and characterized by genotyping methods.

Comment [T23]: What study design did you implore? Was the sample size enough? And how did you arrive to the sampling site or what criteria did you use for study area?

Comment [T24]: Provide a table to show the samples under branded and unbranded

A study design will help to show when and time interval of laboratory data collection

Comment [T25]: Why this media? Does it have the ability to suppress the culturing of other psychrotrophic microorganisms such as lactic acid bacteria?

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20-25 days?

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Your data presentation either in tables, figures must be well organised and articulated. Follow my comments when revising.

Table1.PsychrotrophicmoldsisolatedfromIndigenousfermenteddairyproducts

Sl. no.	Samples	Numberofisolates	
		Unbranded	Branded
1	Mishtidoi	-	4
2	Shrikhand	3	4
3	Lassi	2	2
4	Buttermilk	1	5
Totalisolatesobtained		615	

3.2 PhenotypicIdentificationof Psychrotrophic Molds from Selected IFDPs

The preliminary identification of psychrotrophic molds isolates (n=4) were based on the colony characteristics, cell morphology while specific tests included Ehrlich reaction for screening of toxigenic mold species and antibiotic production by the species (Table 2).Mold isolate -1 formed colony with greenish on the surface, yellow exudate while citrine yellow colouratthebottom.Microscopicexaminationof the same revealed septate hyphae bearing conidiospores resembling the characteristics of *Penicillium* spp., with production of penicillin and citrinin with absence of alkaloid (Fig.1).

Comment [T29]: This information belongs to methodology. Here you just show up the results by a leading statement

Table2.Colonymorphologyoftheisolatedpsychrotrophicmoldisolatesfromindigenous fermented dairy products

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Characteristics	Isolate-1	Isolate–2	Isolates–3	Isolate-4
Colonymorphology				
DiameteronMEA	18mmX 17mm	22mmX 25 mm	9mmX 10mm	25mmX26 mm
ColouronMEA	Greenishwith whitishedges	Brownishred	Blackishwith aerialspores	Dark green
Reverseside	Citrineyellow	Brownishblack	Darkmuddy White	Black
AspectonMEA	Radially grooved	Flatwithraised atthe center	Highlyraised	Flatspread
Textureofthe colony	Grainy,non dissociable spores	Cottonyspread	Grainyof aerial Spores	Velvetyhard, leathery
Cellmorphology				
Sporeshape	Ovalround conidiospores	Beakshaped conidiospores	Round Sporangiospores	Round conidiospores
Typeofhyphae	Septate	Septate	Non septate	Septate
Typeof mycelia	Septate	Septate	Non septate	Septate
Specieswisespecialcharacteristics				
Exudateproduction	Typicalyellow	-	-	-
Ehrlichreaction	-	+(reddishpink) indicates alkaloids	-	-
Antibiotic production:				
Penicillin(β-Lactamasetest)	+	-	-	-
Citrinin(MHAgar)	+	-	-	-
Identityoftheisolate	*Penicillium*sp.	*Alternaria*sp.	*Mucor*sp.	*Cladosporium* sp.

a)Frontview b)Reverseview

c) Exudateproduction d)Cellmorphology

Negative Positive

e) β-Lactamasetest

e) Penicillinproductiong)Citrininproduction

Fig.1.Colonyandcellmorphologyofpsychrotrophic*Penicillium*spp.

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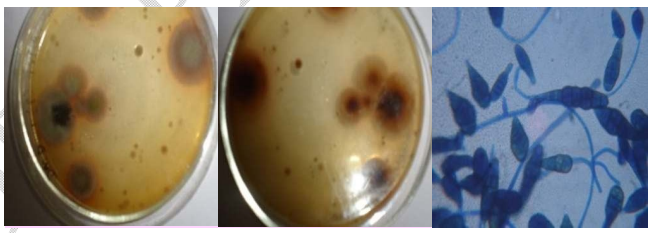
Among the mold isolates, only *Penicillium spp*., produced typical citrine yellow exudate confirming the species and negative to Ehrlich reaction which are in conformationwith the other two studies [20, 24]. Isolate was a producer of antibiotic penicillin which showed antibacterial activity against *Staphylococcus aureus* and citrinin showed antibacterial activity against *S. aureus* but not on *E. coli* in addition to positive results of β-lactamase test. Similarcharacteristicswerenoticedinoneofthestudy[21]withrespectto*Penicillium*spp.Citrininand penicillin production by *P. chrysogenum* was determined in the present study while the same thingswereconfirmedinotherstudies[24, 25].In a study[26], fruity odour from the mold colony showed ter means 3 or quart means 4 branched smooth phialides under microscope. The same was observed in the present isolate hence confirmedas*Penicillium*sp.andthesamewasin relevance to the features of mold by one more study [27].*Penicillium* spp., release the mycotoxins such as citrinin, patulin, penicillic acid, ochratoxin A and sterigmocystinincheeseandotherfermented dairy products which are difficultto destroy duringmilkprocessingtoproduceFDPs[3]. Citrinin production mainly by *P. citrinicum* and also by *P. chrysogenum* found to be involved in nephrotoxicosis issues by disfunctioning of renal veins of kidney and also caused Balkan nephropathyandYellowricefeverwhenthefood contaminated with the mold was consumed. Various cytotoxic effects were offered by patulin like damage to the immune system; pancreas; liver and gastrointestinaltract [14, 15].

The colony of mold isolate – 2 was brownish red at the surface and brownish black colour at the bottom with raise at the center. Microscopic examinationofmoldshowedpearorclubshaped spores with both longitudinal septa, cross walls with septate hyphae and mycelium. The mold produced alkaloids determined by positiveEhrlich test. All the features. The colony and cell morphology revealed the mold isolate as *Alternaria* sp.,(Fig. 2).The mold was identified based on the asexual spores conidia over conidiophore and confirmed itas*Alternaria*byastudy basedoncellstructure [28], while another study [21] identified the mold based on alkaloid production.



a)ColonyFrontview b)ColonyReverseview c)Cell morphology

Negative Positive

d) **Ehrlichtest**

Fig.2.Colonyandcellmorphologyof*Alternaria*spp.

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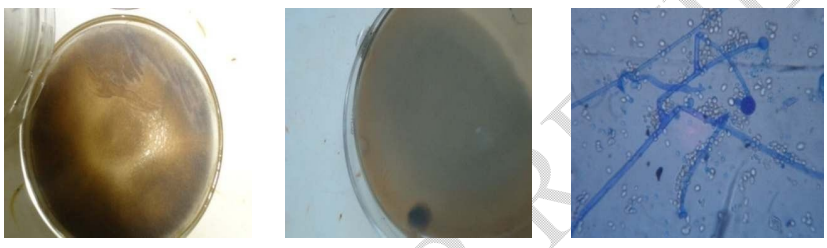
Psychrotrophic mold isolate - 3 was black at surface bearing pin heads of the aerial spores, with non-septate hyphae bearing round sporangiophore and phenotyped as *Mucor* spp., (Fig. 3).A similar results as non-septate hyaline hyphal elements bearing globose sporangia above the sporangiophore confirming*Mucor* spp., was found [29].A mucor species, *Mucor circinelloides*is recognized as an opportunistic pathogen, which can produce the mycotoxin 3-nitropropionic acid. It has been suspected of causing nausea, vomiting and diarrhea, following ingestion of contaminated yogurt stored at 5°C[16].

The colony with dark green on surface and back at the bottom with aseptate hyphae ie., conidiophores bearing 1 to 2 celled conidia in branched chainswasobservedformold isolate – 4undermicroscopeandidentifiedas*Cladosporium*spp.,(Fig. 4).The mold isolate - 4 was identified as *Cladosporium* spp., based on colony and cell morphology[30].Preliminaryandspecifictestsrevealed the identity of psychrotrophic molds as *Penicillium* spp. (12 nos.), *Alternaria* spp. (6 nos.), *Mucor* spp. (2 nos.) and *Cladosporium* spp., (1 no.).



(a) Colony Front view (b) Colony Reverse view c)Cellmorphology

Fig. 3. Colony and cell morphology of *Mucor* spp.

(a) Colony Front view (b)Colony Reverse view c)Cellmorphology

Fig. 4. Colony and cell morphology of *Cladosporium* spp.

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3.3 GenotypicIdentityoftheIsolates: Predominatingphenotypedpsychrotrophicmolds 1,2,3and4isolatedfromIndigenousFermented Milk Products were identified based on 18SrRNA sequencing as *Penicillium chrysogenum*,*Alternariaalternata*,*Cladosporium cladosporioides* and *Mucor meheii,*respectively(Table 3).

Table3.Genotypicidentityofthepredominatingpsychrotrophicmoldisolateswiththe accession number

Isolate	Accessionnumber	Identity
Isolate–1	JQ015265.1	*Penicilliumchrysogenum*
Isolate–2	JN210895.1	*Alternariaalternata*
Isolate–3	KF983515.1	*Cladosporiumcladosporioides*
Isolate–4	KG983517.1	*Mucormehei*

The same identification tool of 18s rRNA sequencing was used in the mold classification by other studies [31, 32, 33].

Outof the psychrotrophic moldisolated (21 nos.) from refrigerated IFDPs, *Penicillium* spp. (12 nos.) predominated followed by *Alternaria* spp., (6 nos.), *Mucor* spp., (2 nos.) and *Cladosporium*spp., (1 no.) in the present study. These molds may enter from the air, utensils used, sugar added for preparationand unhygienic handler as mentioned in one of the study. Post processing contamination especially by local producers may introduce the spores of moldsinto product, low pH and low temperature help in favouring their growth [9].Onpar withthe presentstudy,psychrotrophicmoldcountwas noticed during storage of shrikhand, an IFDP atrefrigerationtemperaturefor10 days [6]. The predominance of *Penicillium spp.*was observedin yoghurt and cheese samples stored at refrigeration condition followed by *Alternaria spp*[34, 9]. Onthecontraryto the present study, predominance of psychrotrophic*Aspergillus* spp., followed by *Fusarium*, *Mucor* and *Penicillium* in case of low temperature stored yoghurt samples while inmilk samples occurrence of the *Alterneria* spp., was observed [3].In some cases, contamination of yogurt products with dimorphic mold e.g., Mucor has been shown to cause yogurt spoilage [5].The most predominant species of *Mucor, Absidia, Penicillium, Geotrachum candidum, Cladosporium, Phoma, Fusarium*, *Asperigellus flavus, Asperigellus niger* and *Asperigellus fumigatus* were identified from used in the study., a total of 125 random samples from raw milk, locally manufactured kareish cheese, hard cheese (Rass cheese), plain yoghurt and flavored yoghurt (25 of each) collected from dairy shops and super markets in Beni Suef city, Egypt but samples were free of aflatoxin M1 [8]. FMPs such as yoghurt and sour milk with low pH (2.9–3.6) from Western Kenya were contaminated with the common molds such as species of *Penicillium* and *Aspergillus* that cause spoilage, as the of these products favour their growth [11]. Fungal count on an average of 4.99 (77 samples), 5.29 (111 samples) and 4.23(111 samples) were found in raw milk cheese, pasteurized milk cheese and Kajmak samples (a thick, tangy clotted cream from the Balkans usually made with sheep's or cow's milk) collected from small scale producers of Serbia, respectively. The study was conducted for only enumeration and isolation as wellas identification of molds was not carried out[12]. The molds in IFDPs need to be considered for their mycotoxin production having public health importance.The mycotoxins that are the secondary metabolites produced by certain molds like species of *Penicilium, Aspergillus, Mucor* which have been found to be present in most food substances like in livestock and their byproducts therefore of public health significance [13].

CONCLUSION: A total of 21 psychrotrophic mold isolates were obtained from the Indigenous Fermented Dairy Products such as Mishti Doi, Shrikhand, Lassi and Buttermilk collected from Bengaluru market, Karnataka, India, out of which 6 isolates were from unbranded samples while 15 from branded samples. Pheno and genotypic characterization of the isolated psychrotrophic molds from indigenous fermented milk products revealed predominance of *Penicillium chrysogenum* (12 nos.) followed by *Alternaria alternata* (6 nos.), *Mucor mehei* (2 nos.) and *Cladosporium cladosporioides* (1 no.) amongisolated psychrotrophic molds.

REFERENCES

Comment [T34]: Be exact by providing the figures or ranges

Comment [T35]: The discussion must be simple and plane. Do not wilder it with words that put readers mind and interests off. Read as many published papers and see how the discussion section interestingly flows.

Comment [T36]: No need to write all of this

Comment [T37]: This is unnecessary and what is it doing here?

Comment [T38]: This part provides the take home massages: what is your general conclusion from the findings,

What is the massage to the milk product manufacturers?

What are the recommendations that people in the dairy industry can comply in as far as food safety is concerned?

What should the future researchers do or what gap has your research left?

Comment [T39]: Some reference are too old

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Comment [T40]: This one is too old

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