

Indian *Chilika* curd – A potential dairy product for Geographical Indication registration

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India has a rich heritage of traditional fermented foods, significantly known for their tremendous nutritional and therapeutic properties, and great economic potentials. *Chilika* curd is one of such dairy food traditionally prepared in a cup shaped bamboo basket using milk of *Chilika* Buffalo by ethnic community of *Chilika* (Odisha, India), it is known to have an exceptionally extended shelf life. In the current study, our objective was to explore this unique character of *Chilika* curd. We found it positively showing longer shelf life and could validate it experimentally, a total of 64 microbial isolates were isolated from curd and milk samples collected from *Chilika*. Phenotypically these isolates were found diverse and characterized as *Lactobacillus* (18), *Leuconostoc* (13), *Lactococcus* (12), *Streptococcus* (9) and Yeast (12) which was further confirmed by specific PCR. Surprisingly, 8 *Lactobacillus* isolates out of the above were found to show anti-fungal effect against the test organism *Candida parapsilosis* (NCDC 279), which can be correlated with preservation of curd from fungal spoilage, and enhancing its shelf life. As a conclusion to legally protect the heritage of *Chilika* curd it can be suggested as a potential candidate for getting it registered as Geographical Indication.

Keywords: *Chilika* curd, Long shelf life, Geographical indication, Antifungal, *Lactobacillus*

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Some products of particular regions do have some superior qualities either because of climate, recipes or food processing techniques that are local to a region or indigenous manufacturing skills and are found to be more saleable in the market than comparable products from other region. In order to take advantage of the commercial attractiveness of these local reputations, quality and attributes associated with Geographical origin, some laws were passed by local legislators preventing adulteration of local products and introduction of certification systems for quality and this paved the way to the development of a new branch of Intellectual Property Rights known to be Geographical Indications (GI).

GI indicates that particular goods originate from a country, region, or locality, and have some special

characteristics or qualities, or reputations, that are attributable to its place of origin and are part and parcel of the cultures and traditions of a country. They contribute to the reputation of the products concerned and create goodwill among consumers and are therefore, of considerable importance for both developed and developing countries. Indian case studies on GIs include 172 registered GI's till March 2012¹ including Darjeeling tea², Mysore silk, Solapur chaddar, Kashmir Pashmina shawl, Pokkali rice, Santiniketan leather goods, Banaras sarees and Brocades³, etc.

Indian *Chilika* curd and the need for GI protection

India is popular for its wide and unique collection of cuisine and indigenous traditional food products, which are known for their immense nutritional and health beneficial values and make them a suitable candidate for global market. Indian fermented milk

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products utilize only 7% of total milk produced⁴ and mainly includes three product *dahi* (curd), *shrikhand* (sweetened concentrated curd) and *lassi* (stirred curd), which may be considered the western equivalent to yogurt, quarg and stirred yogurt, respectively. Curd/*Dahi* is considered as the oldest Indian fermented dairy product⁵ and has been used as a regular item of the diet. It was mentioned in the Ayurveda literature from around 600 AD for treatment of diarrhoea. *Dahi* along with a usual diet is known to reduce the number of episodes as well as duration of diarrhoea⁶ and *Chilika* curd is one of the best example. Such fermented dairy products can be produced by employing traditional techniques where in many cases the microorganisms involved have not been characterized⁷.

Chilika is an integral region of coastal Odisha (erstwhile Orissa), Mythologically about 400 yrs ago a saint poet and devotee of Lord Jagannath Purshottam Das, wrote a poem about Lord Krishna dancing with a milkmaid called *Maniki*, who had come to sell curd on the banks of the Chilika. *Chilika* curd is prepared from milk of *Chilika* buffaloes which are unique as they are found only around Chilika lake, in the Khordha, Puri, and Ganjam districts and feeds on sea weed in neck-deep, brackish waters on the shores of Chilika lake. Perhaps because of the high salt content in the animal's diet, the milk is not only very tasty but can also be stored without refrigeration for days. Both the milk and other products such as curd made from milk are attracting attention in the regional market place, and can be suggested for GI registration.

International Jurisprudence and forms of protection available to GIs include Paris Convention for the Protection of Industrial Property, 1883; Madrid Agreement for the Repression of False or Deceptive Indications of Source of Goods, 1891 and Lisbon Agreement for the Protection of Appellations of Origin and their Registration, 1958.

It is widely observed and reported that *Chilika* curd has longer shelf life but no scientific study has been undertaken to document this unique character⁸. In the present study, curd sample prepared from Buffalo milk were collected from Chilika (19°43'N 85°19'E) region of Odisha (Fig. 1), and study was undertaken in the context to explore microbes involved in fermentation of curd, with an aim to know the reasons behind the shelf life of the *Chilika* curd.



Fig. 1—Map of region of *Chilika* curd.



Fig. 2—*Chilika* curd prepared in bamboo basket bowl.

Material and methods

Ten representative random samples each of milk and curd were collected aseptically from farmers of *Chilika* Buffalo Society (Chilika, Odisha, India) covering regions in and around Satapada. Samples were collected in sterile Multipurpose Clinical Sample Collector (Himedia) and were brought to laboratory in refrigerated condition during transportation. The samples were stored at room temperature for 14 days.

Chilika curd was prepared by farmers of Chilika Buffalo Society traditionally in a bowl made out of fresh bamboo mats which are interwoven to make small basket type bowl (Fig. 2). To make the basket impervious it was coated with a layer of inoculum curd and was dried in open sun, this process was repeated with multiple curd layers with intermittent sun treatment. For preparing curd *Chilika* buffalo milk was concentrated by boiling for longer duration. Finally, the concentrated

milk was poured in the pre-treated bamboo container covered with leaves and incubated to prepare curd.

Chemical analysis

Chemical analysis of *Chilika* curd and milk samples was carried out for fat, protein, moisture content and acidity. Gerber method⁹ was used for determination of fat in samples, standard Kjeldahl's method¹⁰ was used for determination of total protein in samples. Moisture content was determined by drying 5 gm of sample in a hot air oven at 70°C to a constant weight. pH was measured with help of electronic pH meter (Thermo Electron, Madison, WI, USA) and titratable acidity of samples was measured by titrating samples with 0.1 N NaOH to an end point indicated by pale pink colour by using phenolphthalein as indicator.

Microbiological analysis

Ten gm of curd sample was homogenized with 90 mL of 2% sodium citrate, serially diluted (10^{-1} to 10^{-8}) in 1% peptone (Himedia, Bombay, India) and one mL of decimal dilutions of the samples were pour plated with 20 mL of the following media: (i) de Man, Rogosa and Sharpe (MRS) agar (Himedia, Bombay, India) incubated for 48 hrs at 37°C and 30°C for enumeration of *Lactobacilli* and *Leuconostoc* (ii) Violet Red Bile Agar (VRBA) (Himedia), incubated at 37°C for 48 hrs for enumeration of the coliform (iii) M17 Agar (Himedia), incubated at 42°C and 30°C for 48 hrs for enumeration of thermophilic microbes and *Lactococcus* (iv) Potato Dextrose Agar (PDA) (Himedia), incubated at 30°C for 48 hrs for enumeration of yeast and moulds.

Isolation and identification of microbes

For isolation of microbes 10 colonies were picked randomly from each plates of MRS, M17 and PDA of highest dilution and were transferred to respective

broth tube and were incubated overnight, next day broth culture were observed microscopically. These isolates were further purified by successive streaking on agar plates of respective medium. These purified isolates were phenotypically identified by catalase test, microscopic observation and biochemical tests. These isolates were stored in glycerol stocks (15%) at - 20°C for further studies.

Molecular characterization of microbes

Genomic DNA of phenotypically identified bacterial isolates was extracted from 2 mL samples of overnight cultures grown in broth as previously described¹¹. Primers for molecular characterization of isolates as in Table 1 were used targeting 16S rRNA gene was used for the confirmation of *Lactobacillus* genus¹² and *Leuconostoc*¹³, *gad B* gene for *Lactococcus* isolates¹⁴, and *lac Z* gene for *Streptococcus thermophilus*¹⁵. PCR was performed in 25µL of reaction volume, containing 50–100 ng of genomic DNA, 1X Taq buffer, 1.5 mmol/L MgCl₂, 10mmol/L of each deoxyribonucleotide tri phosphate (dNTP), 50 ng of each primer and one unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India). PCR amplification was performed on an Eppendorf Mastercycler (Hamburg, Germany) according to earlier published literature¹²⁻¹⁵. Amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 1X TAE buffer using a 100-bp ladder (Bangalore Genei) as a molecular weight marker, and gels were stained with ethidium bromide (1 mg/mL).

Shelf life studies

For shelf life studies representative of curd samples stored at room temperature were analysed from day one of collection and upto 14 days for changes in Physio-chemical properties such as colour and appearance, body and texture. Changes in pH of

Table 1—List of primers used in this study for molecular identification of cultures

Primer	Primer Sequence 5'-3'	Target	Product size (bp)	Reference
<i>Lactobacillus</i> genus specific	LbF-CTCAAACTAAACAAAGTTTC LbR-CTTGACACACCGCCCGTCA	16S r DNA	250	Dubernet <i>et al.</i> 2002 ¹²
<i>Leuconostoc</i>	LEUF-GTGCCTAATACATGCAAGTCG LEUR- AGCTTCAAAGGTAGTCAAGAG	16S r DNA	850	Eom <i>et al.</i> 2007 ¹³
<i>Lactococcus</i>	<i>gadB21</i> -CGTTATGGATTTGATGGATATAAAGC GAD7-ACTCTTCTTAAGAACAAGTTTAACAGC	<i>gadB</i> gene	334	Nomura <i>et al.</i> 2002 ¹⁴
<i>Streptococcus</i>	F -CACTATGCTCAGAATACA R -CGAACAGCATTGATGTTA	<i>lacZ</i> gene	968	Lick <i>et al.</i> 1996 ¹⁵

stored curd sample were measured by using protocol as described previously. Stored samples of curd were also analysed for changes in microbiological count in Plate Count Agar, MRA agar, M17 agar and VRBA.

Anti-fungal studies

All strains of *Lactobacillus* isolated from chilika samples were screened for anti-fungal activity against test organism *Candida parapsilosis* (NCDC 279) by using dual agar plate assay method as described by Yousef *et al.*¹⁶

Results and discussion

Curd/*Dahi* is considered as the oldest Indian fermented dairy product, which is related to our mythology with lord Krishna and has been used as a regular item of our diet from ancient time, curd is been recommended for treatment of various disease in Ayurveda and have also been scientifically proved for control of diarrhoea^{6,17}. It is recommended that regular use of curd will reduce a number of episodes of diarrhoea⁶. *Chilika* curd can be suggested as one of the best example which is prepared by a traditional process and has an enhanced shelf life.

With apparent sensory evaluation of involved research workers it was found that *Chilika* curd has creamy appearance, thick consistency, granular texture, slightly salty flavour and a rich mouth feel, and was overall acceptable. Titrable acidity of curd was found significantly high, *i.e.* 1.09 ± 0.01 % Lactic acid (LA) with *pH* 3.87 ± 0.07 whereas chemical study of milk of Chilika buffalo shows *pH*: 6.59 ± 0.08 , and acidity: 0.09 ± 0.01 % LA, with moisture content 89.2 ± 2.95 % with protein content 3.9%, fat content in milk was found significantly high 8.6 %. Thicker consistency of curd can be correlated with high fat content of the milk where as sour taste can be due to high LA in curd.

Lactic acid bacteria were found to proliferate in curd sample giving microbial count of 1.7 to 2.2×10^5 CFU/gm of curd, a total of 64 microbial isolates were isolated from collected curd and milk samples, out of 64 isolates by phenotypic methods 18 were found to be *Lactobacillus*, 13 were *Leuconostoc*, 12 were *Lactococcus*, 9 were *Streptococcus*, and 12 were Yeast. Biochemical characterization especially sugar fermentation pattern was used as a phenotypic method for identification of isolates. Genotypically the *Lactobacillus* isolates were confirmed by genus specific PCR giving a positive band of 250 bp for all 18 isolates (Fig. 3), *Lactococcus lactis* was confirmed giving a product of 334 bp (Fig. 4) for *gad B* gene,

Leuconostoc isolates were confirmed giving a PCR product of 850 bp, *Streptococcus* isolates were confirmed to be *S. thermophilus* giving a product of 968 bp for *lac Z* gene (Fig. 5).⁷

Shelf life studies reveals good storage stability of *Chilika* curd up to 7 days at room temperature, physical appearance, colour, body and texture of the stored curd samples remained same as fresh sample. There was slight change in acidity of stored curd from 1.044% LA to 1.095% LA. Microbiological analysis of stored curd samples revealed increase in LAB count in MRS from 207×10^4 CFU/mL to 165×10^5 CFU/mL for 7 days followed by gradual fall in count and in M17 from 240×10^5 CFU/mL to 119×10^6 CFU/mL medium which gradually falls, showing stability of curd up to a week.

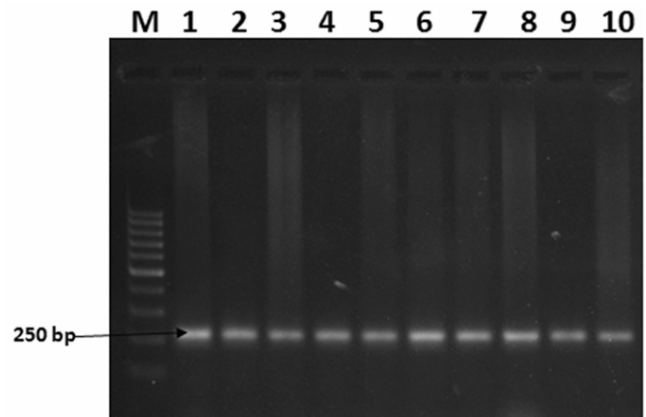


Fig. 3—PCR amplification product of 250bp for *Lactobacillus* genus-specific PCR, lane M = 100bp ladder, lane 1= standard *Lactobacillus* lane 2 to 10 = *Lactobacillus* isolates.

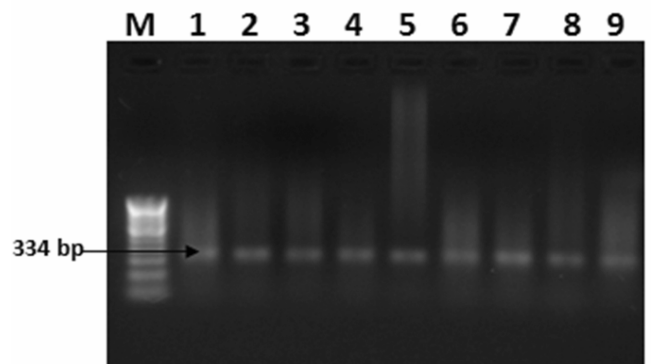


Fig. 4—PCR amplification product for *gadB* gene of 334bp confirming *Lactococcus*, lane M = 100 bp ladder, lane 1= standard culture lane 2 to 9 = *Lactococcus* isolates.

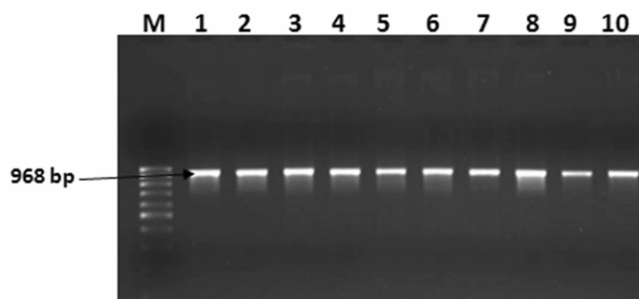


Fig. 5—PCR amplification product for *lacZ* gene of 968 bp confirming *Streptococcus thermophilus*, lane M = 100 bp ladder, lane 1= standard culture lane 2 to 10 = *Streptococcus* isolates

It has been found that about 5 - 10% of the world's food production is lost due to fungal deterioration¹⁸. Yeasts such as *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Kluyveromyces marxianus* and *Debaromyces hansenii* have been reported as common spoilage organisms of yoghurt and other fermented dairy products¹⁹. Keeping in mind *Lactobacillus* isolates were tested for their antifungal properties and surprisingly out of 18 *Lactobacillus* isolates, 6 isolates showed good and 2 low antifungal effect against test organism *Candida parapsilosis* (NCDC 279) (Fig. 6), antifungal properties of these isolates can be correlated with the longer shelf life of the product which protect them from fungal contamination²⁰. Lactic acid bacteria (LAB) producing antifungal compounds such as caproic acid, 3-hydroxy fatty acid, fungicins (proteinaceous compounds), phenyllactic acid, etc. has been reported previously²⁰. Such isolates can be used as starter for making curd with better shelf life. Such products with extended shelf life can be beneficial for country like India where major population reside in villages who can't afford refrigeration facility and there is no need of cold chain during transportation.

Chilika curd as a traditional product has number of beneficial attributes. Curd is prepared with concentrated milk by boiling for longer time, this process makes milk sterile and free for any pathogen which has also been proved by microbiological analysis of processed milk sample collected aseptically from Chilika, besides this it also gives a thick consistency which is comparable with other products. As curd is prepared inside fresh bamboo basket there is less chance of cross contamination. The intermittent sun treatment during processing eliminates most of the contaminants from inoculum and only thermo tolerants get selected thus this is a

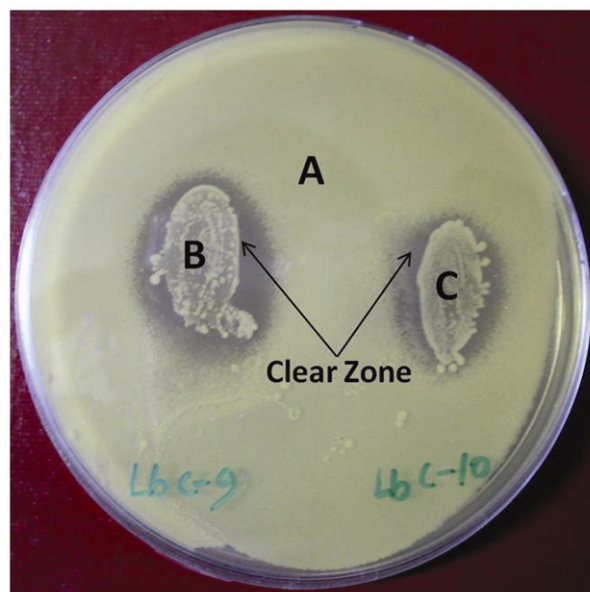


Fig. 6—Antifungal activity of *Lactobacillus* isolates (B and C) towards *Candida parapsilosis* (NCDC-279) (A) showing clear zone around the colony.

traditional process of microbial enrichment. These all attributes are comparable with other curd. This longer shelf life of the Chilika curd may be partly due to bacterial population characterised in the present study, which shows antifungal activity. The reasons for the longer shelf life of *Chilika* curd may also be due to the inherent properties of selectively enriched thermo tolerant *Lactobacilli* inoculum, dendrocin²¹ (An anti-fungal protein isolated from fresh bamboo shoots), or it's because of special diet of Chilika buffaloes which needs further investigations in future.

Products marketed under GIs have been a major source of income for many developing and developed countries even before TRIPS came into force. For instance, producers of Italian Toscano olive oil have managed to earn a premium of 20 % since registration as a Geographical indication in 1998²².

The economies of most developing countries depend heavily on agriculture and many of the much sought after products like Antigua coffee, Jasmine rice, Darjeeling tea, Ceylon tea, etc. come from developing countries. Statistics indicate that more Antigua coffee and Darjeeling tea are sold under the respective names than what is actually produced in Antigua and Darjeeling. This only proves that there is definitely a market for the genuine products indicated by the GI and a section of the consuming public, in their quest for quality, is being deceived. GIs therefore, reflect *inter alia* the traditions, culture,

human efforts, and environment of particular regions. They are also valuable property to producers from particular Geographical regions as it helps them benefit exclusively from the reputation of the product because of its Geographical origin. It should also be noted that GI does not protect the breed or genetic material *per se* but adds commercial value to the animals of breed reared in a particular region that may be used as a value addition mechanism. Prior to the TRIPS Agreement, although GIs were protected in many countries the momentous role that GIs could play in the economy of a country was never discussed at the rate it is being done today.

Significance of study to the researcher/society

Lactobacillus isolates with antifungal activity from this study can be used as starter for making curd with better shelf life, such dairy products with extended shelf life can be an option for country like India where major population resides in villages where refrigeration is unaffordable, it can also be a better option for conditions like war fares where food preservation is a major problem. This study can further be extended to find role of selective enrichment of thermo tolerance microbes, dendrocin or special diet of buffalo in extended shelf life of Chilika curd.

Conclusion

Chilika curd samples collected from Chilika (Odisha) were found to have extended shelf life. A diverse population of microbes were screened from the curd sample consisting of Yeast, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*. Antifungal activity of *Lactobacillus* isolates can be correlated with the increase in shelf life of product. The reasons for the longer shelf life of *Chilika* curd may also be due to inherent properties of selectively enriched thermotolerant *Lactobacilli* inoculum, dendrocin, or because of special diet of these buffaloes which needs further investigations in future. The findings of the present investigation strongly support for the GI registration of the Indian *Chilika* curd.

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