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Perspectives into probiotics- from phenotyping to pyrosequencing

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ABSTRACT

Global scientific pursuits are percolating towards a common goal of finding a solution for efficient utilization of existing resources, developing low cost effective technologies for making the foods more nutritional and preventing losses due to biological, microbiological, chemical, biochemical, mechanical, physical and physiological factors. One of the possible approaches in finding solutions to these problems is exploiting the full potential of fermentation technology. Fermentation is one of the oldest methods of food preservation known to mankind (Prajapati and Nair, 2003). In India, the *Rigveda* (1500BC) and *Sukla Yajurveda* have texts mentioning the preparation of *Soma* (Plant juice) and *Sura*(Wine/beer) while curd finds its mention in *Yajurved and Charaka Samhita.* Traditional sour milk products were primarily produced to prolong the shelf-life of milk and for pleasant sensory properties. Fermented milk products got a whole new reputation when Elie Metchnikoff in 1907 hypothesized that replacing or diminishing the number of 'putrefactive' bacteria in the gut with lactic acid bacteria could normalize bowel health and prolong life. After more than half a century the term probiotics was coined to reflect Metchnikoff's idea, much progress has been made since in terms of the fermentation of dairy products. Probiotics are proven effective in gastrointestinal diseases along with successful reports in treating allergy, cancer, diabetes to depression.Sales in Western Europe grew to 8 billion U.S. dollars by 2014. The most developed markets for probiotic foods were Europe and Japan, accounting for more than half of total sales.The Indian probiotic scenario is expected to to grow at a CAGR of 25% during 2014-2019 as reported by the Dairy Industry in India. At this point it is imperative to develop and promote indigenous probiotic strains and make serious efforts to explore the novel functional physiological properties of the enormous Indian microbial diversity.

Keywords: bacteria, fermentation, indigenous, strains.

The first step

There is more than an ounce of wisdom in the words that "Ideas can no more flow backward than can a river". We, at the Department of Dairy Microbiology are riding the waves of this vast river of functional probiotic dairy based foods since the last 25 years and through this communication would like to showcase our research and developments in this promising area of interest.

The journey began with the combination of keen observation and sharp acumen of Dr JM Dave, Former Principal of the college and a renowned microbiologist in 1980's. He was inspired by the work of Dr Marvin Speck who proclaimed that the genus *Lactobacillus* are indigenous to the intestinal tract of men and animals to establish and stabilize the intestinal microflora of healthy individuals. The "acidophilus technology" gained momentum thereon. Research was channelized at the department to prepare acidophilus products with increased viability, storage ability and sensory appeal (Prajapati *et al.,* 1983, 1986). Acknowledging the fact that probiotic effects were strain specific, further efforts were put in the isolation of novel strains of lactobacilli from milk and milk products as well as body niches of healthy individuals like intestines and vagina. Preliminary identification of the isolates was done by morphological and biochemical tests. Their usefulness in fermenting milk was also studied by checking their rate of acid production and growth curve in milk. The promising strains were also tested for their antimicrobial activity against human pathogens and for their ability to pass through and reside in the intestinal tract by tests such as adhesion, tolerance to acid, phenol, bile and so forth.

Isolation of indigenous probiotics

It was in 1988 that two strains LBKV3 and LBKI4 were isolated from vaginal mucosa of adult healthy human female and faecal matter of bottle fed healthy child respectively. It was identified as *L.acidophilus* using morphological, physiological and biochemical characteristics. Both isolates showed resistance to phenol (0.4%), bile salt (4%) and low pH (pH3). Acidophilus milk prepared by using both these strains was fed to two groups of subjects to check their curative ability against gastrointestinal complaints and implantation ability. Surprisingly, the vaginal isolate had superior adhesion ability and the product gave complete cure of complaints within nine days of consumption (Khedkar *et al.,* 1990a, 1990b). It is worthy of mention that the coliform counts in faeces decreased significantly in

all the human subjects at the end of the feeding period as compared to initial counts irrespective of the strain of lactobacilli used. To deliver the probiotic effects studies were conducted to formulate food matrix and we standardized the technology for manufacture of acidophilus lassi containing *Lb. acidophilus* V3 and *Streptococcus thermophilus*. The product was highly acceptable, had viable count of 20 x 10^7 lactobacilli/g and shelf life of 27 days in refrigerator (Patidar and Prajapati, 1998). Selected milk-rice blend was converted into a probiotic food by incorporating freeze-dried cells of *Lactobacillus acidophilus* V3. The product had an average 10.9% fat, 22.3% protein, 3.2% ash, 61.7% carbohydrate and 10^9cfu/g of live lactobacilli. The count of lactobacilli reduced to 10^7 cfu/g after four months of refrigerated storage, which still maintained the therapeutic minimum.

Polyphasicstrain identification

Most probiotic microorganisms belong to lactic acid bacteria (LAB). However, not all probiotics display the same properties, and careful selection of specific strains based on their claimed beneficial effects is needed. Moreover, there are reports of probiotic products being unreliable in content and unproven clinically. The discrepancies in the safety and efficacy of probiotic product are mainly due to (1) inaccurate identification and labelling (2) not named in accordance with scientifically valid nomenclature (3) Claims of efficacy not adequately substantiated; and (4) no established record of a physiological (health) benefit in humans (Reid, 2005). FAO (Food and Agricultural Organization**)** and WHO (World Health Organization) initiated work to examine the scientific evidence on the functional and safety aspects and develop guidelines for the evaluation of probiotics in food in 2001. The challenge lies in maintaining a balance between accurate representations of the strain and preserving the consumer familiarity with certain names. Mislabelling closely related species of lactobacilli poses no safety risk, but may raise concerns about a company's credibility, both in the eyes of the consumer and regulatory agencies.

Recently in 2011, Indian counterparts framed the ICMR-DBT guidelines that comprehensively addressed the various concerns regarding safety, efficacy and reliability as well as labelling of probiotic products being sold in India. The emphasis was laid on proper and precise genus, species and strain identification, using both phenotypic and genotypic tests. It was recommended that probiotic strains in use in India should be deposited in internationally recognized culture collection/repositories. We followed the

instructed path and results are delineated below.

Partial Gene sequencing

A valid scheme for the identification of a probiotic strain should be currently based on its morphological, physiological, and biochemical features as well as on aspects of its genetic profile. The 16S rRNA presents the most common target region for phylogenetic analysis at the species level, because sequence data of this region can be used for taxonomic classification. The partial 16S rRNA gene sequencing was outsourced to MTCC, Institute of Medical Technology, Chandigarh, India. The sequence was analyzed using the Basic Local Alignment Search Tool. After which the gene sequences were submitted to GenBank and the accession numbers were duly received and strains were submitted at the International Depository, MTCC, Chandigarh. Genotyping revealed that the strains were *Lactobacillus helveticus* and not previously assumed *Lactobacillus acidophilus*. This is not surprising as the'*L. acidophilus* complex' is a group of several species including *L.acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri*, *L.helveticus and L. johnsonii* that were initially identified as *L. acidophilus*strains.

Whole Genome sequencing:

The rational next step was to go for whole genome sequencing of the strain. Genome sequencing of a probiotic strain also constitutes an essential step to generate primary information for the functional analysis of gene and protein expression, reconstruction of metabolic pathways, cellular transport etc. Genomics-based approaches and post genomic tools will also facilitate the detailed investigation of the interactions between the bacterium and its host organism by gaining an insight into the genes that contribute to this gut functionality, and exploitation of this information will lead to a more complete understanding of these beneficial microbes, their behaviour in the human gut and their effects on human health and will provide a major impetus for the development of probiotic foods by supporting claims concerning their health benefits. Demand for faster, affordable DNA sequencing has led to the development of so-called "next generation" sequencing technologies like 454 Genome Sequencer (GS) FLX instrument from Roche Applied Sciences. It has a significant advantage over Sanger sequencing that it requires no gels or capillaries to separate extension products by size, and that base incorporation can be detected in real time. The complete sequence of the *L. rhamnosus* MTCC 5462 genome

can be accessed under the GenBank accession number AEYM01000001-AEYM01002543. We are proud to announce that we are the first in the country to submit the whole genome sequence of an indigenous novel probiotic strain (Prajapati *et al.,* 2011 and 2012)

Clinical effects of our strains

In animal models

To directly observe the benefits of our cultures in chicks and to extrapolate in humans, we carried out experiments on feeding acidophilus milk to chicks for 8 days and monitored HI- antibody titre as a measure of immune response for 5 weeks. All the fermented milk fed chicks showed higher antibody titre than control, which indicated the immune stimulating ability of the fermented milk (Patidar and Prajapati, 1999). Acidophilus milk prepared using two different strains of *Lactobacillus acidophilus* was fed to one week old chicks for 16 consecutive days. All the chicks were then challenged with pathogenic *E. coli* . Within 72 h post-challenge, 93% birds in the control group died. However, in chicks fed acidophilus milk fermented by *Lb. acidophilus*, the mortality was only 27%. This indicated that the feeding of acidophilus milk offer protective effect to the chicks against *E. coli* infection. MTCC 5463 was again able to completely stop proliferation of tumor *in vitro* on human cell lines. 63% adhesion in *in vitro* adhesion studies on carcinoma HT-29 cells monitoring using flow cytometry studies were established (Vishwananth *et al.,* 2012).

***In silico* models**

Whole-genome based validation of the adaptive properties L. helveticus MTCC 5463

For a polyphasic approach to validate the functionality of a potential probiotic strain we focused on the *in silico* analysis of the strain's genetic basis of robustness in gut and transit, adhesion, autoaggregation, colonization, and antibacterial, hypocholesterolemic and immunomodulatory properties. The strain had excellent adaptive features like the multi subunit F0F1 ATPase, conjugated bile salt hyrolase, chaperones like hsp33, HtrA, GroEL, GroES, dnaK, grpE, starvation-inducible proteins and heavy-metal transporting ATPases. The genome revealed genes for adhesion and aggregation including exopolysaccharides, capsular polysaccharides sortase, elongation factor Tu, aggregation promoting proteins, fibronectin-binding proteins, S-layer and mucus-binding proteins. We could identify

genes conferring physiological benefits like immunostimulation, cholesterol reduction, and folate production. Thus, through trait and gene matching, the study established that the strain possessed the genetic arsenal required to adapt to the gut milieu. (Senan *et al.*, 2014a).

Genome-scale analysis of niche-based stress responsive genes in Lactobacillus helveticusstrains

Next generation sequencing technologies with advanced bioinformatic tools present a unique opportunity to compare genomes from diverse niches. We attempted to compare the stress-responsive genes of a potential probiotic strain, *Lactobacillus helveticus* MTCC 5463, and a cheese starter strain, *Lactobacillus helveticus* DPC 4571, from a gut and dairy niche, respectively. The MTCC 5463 genome carried multiple orthologs of genes governing stress responses, whereas the DPC 4571 genome lacked in the number of major stress response proteins. The absence of the bile salt hydrolase gene in DPC 4571 and its presence in MTCC 5463 clearly indicated niche adaptation. Further, MTCC 5463 carried higher copy numbers of genes contributing towards heat, cold, osmotic, and oxidative stress resistance as compared with DPC 4571. Through comparative genomics, we could thus identify stress-responsive gene sets required to adapt to gut and dairy niches (Senan *et al.*, 2014b).

Human clinical trials:

Live lactobacilli or their metabolites have known to lower the blood cholesterol through assimilation of dietary cholesterol and indirectly via deconjugation of bile salts. After *in vitro* testing, we conducted feeding trial of acidophilus milk on 27 human volunteers of normal as well as hyperlipemia groups. Feeding of acidophilus milk resulted in reduction of total cholesterol by 11.7, 21.0, 12.4 and 16.4 % in volunteer group A1 (40-60 years), C2 (200-220 mg/dl initial cholesterol), C3 (220-250 mg/dl initial cholesterol) and H1 (normal health), respectively (Ashar and Prajapati, 1999). The feeding favourably affected total serum cholesterol and LDL/HDL or Total/HDL ratios. Overall, the feeding was most beneficial to the volunteers of 40-60 years, which have the highest risk of heart attack.

Encouraged by the results of the above mentioned investigation we conducted a clinical trial to study the effect of consumption of milk based synbiotic product on the intestinal well being and humoral immune response in health human subjects. The intervention study could not confirm a beneficial effect of probiotics on health adults in matters of

significant alterations in parameters but confirms its acceptance to a healthy body's microbiota which proclaims its use as a potent prophylactic agent against intestinal disorders (Prajapati *et al.,* 2012). To further prove the therapeutic effects of the strains on patients suffering from elevated levels of cholesterol, another clinical study on "Effect of consumption of synbioticlassi fermented with *Lactobacillus helveticus*MTCC 5463 on serum lipid levels in *hypercholesterolemic* humans" was carried out in 2011. We had 9 subjects showing upto 10% reduction in total cholesterol; 5 showing upto 20% and one showing upto 30% reduction after consuming the synbiotic drink. In 2013, we conducted a human clinical trial with a probiotic oat based lassi developed under our Swedish collaboration and found significant on gut microflora in the subjects (n=32). However, the effect on cholesterol was non-significant. In the same time we targeted the niche geriatric population to study the effect of probiotic intervention on immune responses and management of cholesterol. The data is under statistical analysis. With this we also did metagenome analyses of human gut flora as affected by our probiotic culture intervention.

Bacterial shifts after a probiotic intervention revealed by Metagenomics

Recent explorations of the human gut microbiota suggest that perturbations of microbial communities may increase predisposition to different disease phenotypes. Probiotics may restore the composition of the gut microbiome and introduce beneficial functions to gut microbial communities, resulting in amelioration or prevention of gut inflammation and other intestinal or systemic disease phenotypes. The metagenomic study revealed that the faecal samples were dominated by Firmicutes (50%), Acintobacteria (20%) and Proteobacteria (10%). Changes in the phylum composition after probiotic feeding included a 7% increase in Firmicutes, 1.5 % rise in Actinobacteria and 1.9% increase in Proteobacteria. Proteobacteria were higher in non-responders than respondents. The STAMP analysis revealed that among responders and non-respondents the chief genera of Firmicutes that showed significant difference were *Lactobacillus, Clostridium, Eubacterium*, and *Blautia* ($q < 0.002$) while the genera of Proteobacteria included *Shigella, Escherichia, Burkholderia* and *Camphylobacter* (q-value<0.002). The results are enriching the knowledge of scientist working in this area.

Synbiotic product profile:

A synbiotic has been defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation

of live microbial dietary supplements in the gastrointestinal tract'. The concept of synbiotics was also known by Indian sages as '*Panchamrut*' is an article of every ritual in Hindu culture since *Vedic* times. *Panchamrut* was probably the first synbiotic, which contained dahi with probiotic bacteria and honey as the source of probiotics.

Table 1. Probiotic and synbiotic products developed at Anand

Sr. No.	Product	Ingredients	Remarks
1	Synbioticdahi	Milk, Inulin, Sugar	Set coagulated product with 10^8 viable cellsof probiotic lactobacilli per gram.
2	Synbioticraita	Milk, Inulin, Fructooligosaccharide, Tomato, Cucumber, Onion, Banana, Sapota, sugar	Stirred yoghurt type products fermented by probiotic lactobacilli and garnished with fruits and vegetables.
3	Synbioticlassi	Milk, Oat, FOS, Carrot, Mango, sugar	Thick liquid with probiotics and shelf life of 3 weeks at 5C.
4	Whey drink	Whey, Sugar, Pine-apple	Beverage with fruit pieces and 10^8 cells/ml of probiotic lactobacilli.
5	Herbal probiotic lassi	Milk, Safed musli, sugar, honey	Milk fermented by probiotic lactobacilli and supplemented with herbs.
6	Protein rich lassi	Milk, Spirulina, sugar	Fermented milk enriched in protein by spirulina.
7	Acidophilus banana powder	Acidophilus milk, banana, sugar, elachi	Dried product with 10 million/g viable cells of *Lb. acidophilus*.
8	Acidophilus wheat malt powder	Acidophilus milk, wheat malt, sugar, cocoa powder	Dried product with 10 million/g viable cells of *Lb. acidophilus*.
9	Milk-Rice probiotic food	Milk, Rice, Freeze dried probiotic culture	Milk and rice were fermented and spray dried and blended with freeze dried probiotic lactobacillus cells.

Recognizing the potential for functional dairy products above and beyond traditional dairy products, we developed a number of products into the market. Several synbiotic products with probiotic culture *Lactobacillus*

helveticus MTCC 5463 and probiotic ingredients like inulin and oligofructose have been developed.

Carbonated probiotic whey drink with artificial and natural carbonation have been developed in addition to the above mentioned product overview.

It is further added that a Patent (Application No. 620/MUM/2011) has been filed for invention entitled "process for manufacture of herbal probiotic fermented milk product".

Probiotics in a pouch

Selected strains fulfilling most characteristics were preserved and used in different probiotic food preparations. It was thought appropriate to make them available as regular ready to use food supplements. Hence, the search for suitable carrier media started. As a partial replacement of milk solids, gram, moong and wheat flours were tried. The cultures were blended in different media and dried under vacuum and spray dried for direct commercial practice. However, maintaining viability was the bottleneck. Investigations into reducing processing stress led to the standardization of a spray drying process incorporating tomato and banana in the fermented milk which gave highest possible survival of about 14% in the dried preparation. Similar studies were conducted on spray drying of standard strain of *Lb. acidophilus* in a mix containing wheat malt, fermented milk, sugar and cocoa. The next venture was towards development of milk-cereal blends. The cost of the product was reduced by replacing a part of costly milk solids with cereal solids and secondly, the nutritive value was enhanced due to the complimentary action of milk and cereal nutrients. The modes of packaging the material was also delved upon and spray dried acidophilus-malt preparation was packed in pouches of three different flexible packaging materials. Atmospheric storage upto two months with satisfactory viability and moisture control could be guaranteed using polyethylene-aluminium foil laminate with a logical cost benefit ratio.

Our Probiotic in pharma dosage forms

The increasing application of starter cultures and probiotics demand them to be made available to the consumers in different dosage forms, which can provide ease of handling, ease of addition to food, precise dosage and functionality for specific application and long term preservation. In the current study freeze dried cultures of *Streptococcus thermophilus* MTCC 5460, *Lactobacillus helveticus*MTCC 5463, *Lactobacillus rhamnosus* MTCC

5462 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 were used for preparation of dosage forms viz., sachets, capsules and tablets for use in the household and industrial level either as inocula for product preparation or as food ingredient or dietary supplement.

The dosage forms at refrigerated storage exhibited good viability, activity and physical characteristics for a period of six months. The viable counts of individual cultures in sachets and capsules made of all three culture combinations were found to be more than 9 log cfu/dosage form even after six months of storage at refrigeration temperature. Tablets of all AIs also showed a viable count of 8 log units after 6 months of storage indicating the suitability of tablets for use as inocula for product preparation provided that the tablets are manufactured under good hygienic conditions The physical parameters of the developed tablets satisfied the technological requirements needed for their easy adaptations to the industrial manufacturing. For making dahi at household level, one unit of dosage form, i.e., 1 sachet/1 capsule/1tablet of 300 mg as inocula per 100 ml of milk and incubation at 37^0C for overnight (12 to 14 h) is recommended.

Taking the rubber to the road: Commercialization

A probiotic lassi containing our probiotic and dairy starter culture was optimized for commercial production at Malabar Milk Union, Calicut and the technology for the same has been transferred. The culture *L. helveticus* MTCC 5463 has been licensed to Dr Baboo's Food Science and Biotechnology Pvt. Ltd, Thiruvanantapuram for commercial use. The company will use and market the strain in different products and formats. One of the first formulation is oat based probiotic lassi, which is rich in β-glucan, was developed under collaborative project with Lund University, Sweden. This product was launched on 6th December 2013 in an International conference by the State Agriculture Minister. We are hopeful to provide health claims for this product in near future. In 2014 we were awarded Hari Ohm Ashram sponsored Prof. JP Trivedi award for "Development of first Indian probiotic culture till commercial application" conferred by Gujarat Association of Agricultural Scientists (GAAS). Our latest work on metagenomics of human gut flora as affected by probiotic intervention in geriatric population got special attention at American Dairy Science Association, Kansas and Harvard Medical School recently. This has improved visibility of Anand on the world map.

Epilogue

Department of Dairy Microbiology is the first in India to have deposited Indigenous probiotic culture in an International culture repository, carry out the whole genome sequencing of the culture and license the culture for commercial application in Indian market. We have backed the claims of our indigenous strain with clinical and metagenomics studies. Understanding the role of indigenous intestinal bacteria and their ecological interactions play in human health and disease based on epidemiological, intervention and mechanistic studies will facilitate the optimization of integrated dietary strategies to efficiently modulate the human gut microbiome, leading to improvements in nutrition and clinical practice. Additionally, these studies need to be conceptualized with 'Indian' as the focal point. Although such studies will be challenging they will bring understanding that is relevant to the health of Indians. Our ongoing efforts are directed to assess host gene-diet interactions within the context of the structure and operations of gut microbial communities. The department fosters a vision to play a pioneering role in tailored probiotic therapy.

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