AAU's Perspectives into Probiotics from Phenotyping to Pyrosequencing

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Microorganisms and hence fermentation precedes human civilization. This explains and emphasizes the role of microbes in the physiological and socio economical development of the human race. Among the roles microbes play, their beneficial activities in food have been the least acknowledged and appreciated. In India, the *Rigveda* (1500BC) and *Sukla Yajurveda* have texts mentioning the preparation of *Soma* (Plant juice) and *Sura* (Wine/beer) prepared by fermentation. Curd finds its mention in *Yajurved and Charaka Samhita.* The brilliant observation by Nobel laureate Elie Metchnikoff on the thereaupetic benefits of gut bacteria on the host ignited the "probiotic" revolution that we witness today. There is more than an ounce of wisdom in the words that "http://www.searchquotes.com/quotes/author/Maria_Montessori/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 andthrough the PAI newsletter would like to showcase our research and developments in this promising area of interest.

The first step

The journey began with the combination of keen observation and sharp acumen of Dr J. M. 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 from thereon. Research was channelized at the department to prepare acidophilus products with increased viability, storage ability and sensory appeal. 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. Isolation of bifidobacteria wasdone from breast fed infant faeces. 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 acid tolerance, phenol and bile tolerance, adhesion and so forth.

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.

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 (pH 3). 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 superioradhesion ability and the product gave complete cure of complaints within nine days of consumption. 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. Selected milk-rice blend was converted into a probiotic food by incorporating freeze-dried cells of *Lactobacillus acidophilus* V3 in it. The product had on an average 10.9% fat, 22.3% protein, 3.2% ash, 61.7% carbohydrate and 10^9 cfu/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.

Polyphasic strain identification

Peer-reviewed papers published worldwide have reported instances of probiotic products being unreliable in content and unproven clinically. The discrepancies in the safety and efficacy of probiotic product are (1) inaccurately reflected on the label; (2) Microbes not always named in accordance with scientifically valid nomenclature (3) Claims of efficacy not always adequately substantiated; and (4) Use of the term 'probiotic' on labels of products with no established record of a physiological (health) benefit in humans. 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. 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 first emphasis is on proper and precise genus, species and strain identification, because the effects of probiotics are strains specific. The point that both phenotypic and genotypic tests should be done using validated standard methodology was emphasized. Nomenclature of the bacteria must conform to the current, scientifically recognized names as per the International Committee on Systematics of Prokaryotes (ICPS). It was recommended that probiotic strains in use in India should be deposited in internationally recognized culture collection/repositories.

Partial Gene sequencing

Following these guidelines our probiotic culture went through the following regime. The partial16S 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.

16 SrRNA sequencing of Lactobacillus strain

No.	Strain	Phenotypic identification	Genotypic identification	GenBank Accession number
1	V3/ MTCC 5463	*Lactobacillus acidophilus*	*Lactobacillus helveticus*	GQ253959
2	I4/ MTCC 5462	*Lactobacillus acidophilus*	*Lactobacillus rhamnosus*	GQ253960

Whole Genome sequencing

The partial 16S rRNA 1053 bp sequence of the *L.acidophilus* V3 showed similarities of above 97%, which is the generally accepted limit, with DNA sequences from other species too. Hence the rational next step was to go for a 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 "nextgeneration" 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. Our results with 454 sequencing are as shown in Table below.

Whole genome sequenced Lactobacillus strain

Run	No. of Reads	Total Bases	Genome Size
L. helveticus MTCC 5463	119526	28319328	2080931
L. rhamnosus MTCC 5462	57784	10906515	2080931

The complete sequence of the *L. helveticus* MTCC 5463 genome can be accessed under the GenBank accession numbers AEYL01000001 to AEYL01000593. 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.

Bioinformatic analysis

The Genome Sequencer FLX System comes with a suite of state - of - the - art analysis tools that integrate seamlessly with the instrument and are optimized for 454 Sequence data analysis. The GS Reference Mapper softwaremaps shotgun reads against a given reference sequence and assembles them into a consensus sequence. The GS De Novo Assembler software generates a consensus sequence by de novo assembly of the shotgun sequencing reads into contigs. The AVA software application computes the alignment of reads from amplicon libraries sequenced using the Genome Sequencer FLX System, and identifies differences between the reads and a reference sequence. We also used the Metagenomics RAST server (MG-RAST), a web - based, open source system that offers a unique suite of tools for analyzing these data sets. After de-replication and quality control, fragments are mapped against a comprehensive nonredundant database (NR). Phylogenetic and metabolic reconstructions are computed from the set of hits against the NR. The resulting data are made available for browsing, download, and most importantly, comparison against a comprehensive collection of public metagenomes.

Synbiotic product profile

Several synbiotic products with probiotic culture *Lactobacillus helveticus* MTCC 5463 and probiotic ingredients like inulin and oligofructose have been developed.

Probiotic and synbiotic products developed at Anand

Sr. No.	Product	Ingredients	Remarks
1	Synbiotic dahi	Milk, Inulin, Sugar	Set coagulated product with 10^8 viable cells of probiotic lactobacilli per gram.
2	Synbiotic raita	Milk, Inulin, Fructooligosachharide, Tomato, Cucumber, Onion, Banana, Sapota, sugar	Stirred yoghurt type products fermented by probiotic lactobacilli and garnished with fruits and vegetables.
3	Synbiotic lassi	Milk, Oat, FOS, Carrot, Mango, sugar	Thick liquid with probiotics and shelf life of 3 weeks at 5°C.
4	Whey drink	Whey, Sugar, Pineapple	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.

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".

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.

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. However, the extent of protection offered, varied with the strain of *Lb. acidophilus*.

MTCC 5463 was 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.

Human clinical trials

Live lactobacilli or their metabolites may lower the blood cholesterol are supposed to be directly effective 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 with the advice of medical doctors. 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. 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.

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 synbiotic lassi 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.

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.

We have recently concluded another human clinical trial in geriatric population (N=72) for study the effect of probiotic intervention on immune responses and management of cholesterol. The data are under analysis. With this we also did metagenome analyses of human gut flora as affected by our probiotic culture intervention. The results are enriching the knowledge of scientist working in this area.

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 Active Ingredients (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/1 tablet of 300 mg as inocula per 100 ml of milk and incubation at 37°C for overnight (12 to 14 h) is recommended.