Investigating the Potential for Novel Probiotic Bacteria Isolated in Turkey to Impact on the Microbial Ecology of Gastrointestinal Tract

Submitted to

European Network for Gastrointestinal Health Research (ENGIHR)

Rowett Institute of Nutrition and Health

Izmir Institute of Technology (IzTech)

Exchange Visit

23rd August – 25th September 2011

Rowett Institute of Nutrition and Health
Aberdeen, UK

Performed by:

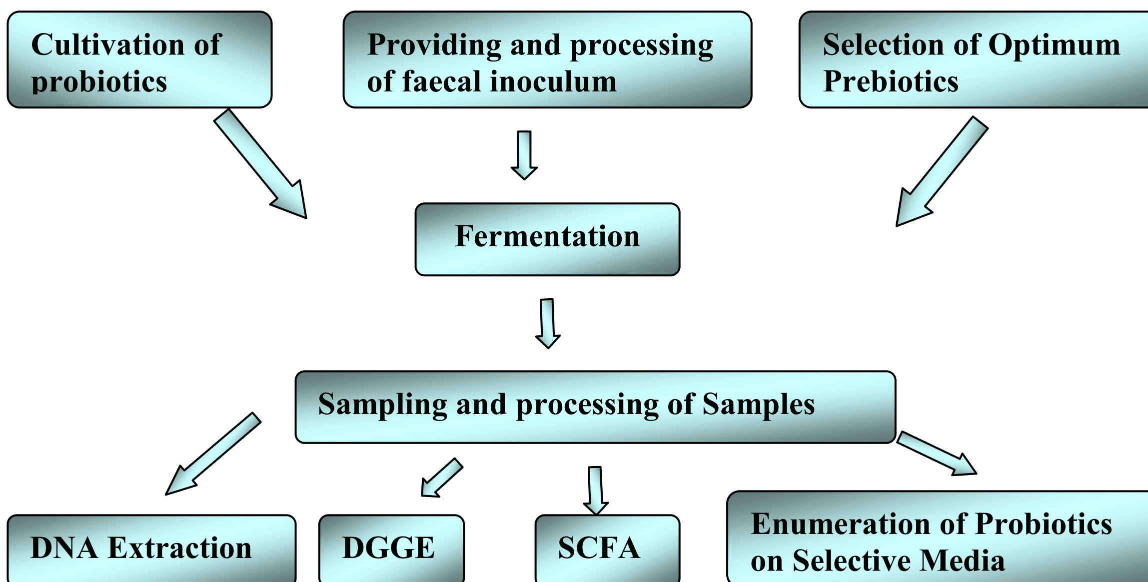
Merve Şamlı

1. Purpose of the visit;

In our project we investigated the potential for novel probiotic bacteria, which is isolated from Turkish dairy products- to impact on the microbial ecology of gastrointestinal tract. We introduced some of these 'novel probiotics' to a simulation of the human colon – a fermentor system – and assess the persistence of the introduced bacterium in the presence or absence of galactooligosaccharides (GOS) as a prebiotic source (a synbiotic approach). At the same time the overall impact of the addition on the total gut microbiota assessed using SCFA and molecular technique; PCR-DGGE. Fermentor systems to assess the impact of dietary changes on the microbial community and all the techniques required has been routinely used in Microbial Ecology Laboratory of Rowett Institute. Studies on isolation of lactobacillus species from Turkish dairy products have been continuing for many years in IzTech.

2. Description of the work carried out during the visit;

Lactobacillus delbrueckii subsp. bulgaricus and *Streptococcus salivarus subsp. thermophilus* species were previously isolated from local Turkish Dairy products in IzTech Food Engineering Department laboratories. These bacteria were carried from Iztech laboratories to Rowett Institute of Nutrition and Health in aseptic conditions by researcher Merve Samli.



Culture of *Lactobacillus* and *Streptococcus* species and preparation of the media

Lactobacillus delbrueckii subsp. *bulgaricus* species were cultivated on MRS broth and *Streptococcus salivarus* subsp. *thermophilus* species on M17 broth under aerobic conditions at 37°C.

Secondary inoculations were applied to MRS agar and M17 agar for *L. delbrueckii* and *S. salivarus* sequentially in order to verify their colony counts.

Monitoring of effect selected prebiotics on probiotic bacteria's growth

Firstly, general media and the basal media with some selected prebiotics were prepared for *L. delbrueckii* and *S. salivarus*. Detailed information was given in Table 1.

Table 1. Monitoring of effect selected prebiotics on probiotic bacteria's growth

Strain	*L. delbrueckii*	*S. salivarus*
Confirmation of Growth	MRS	M17
	Basal MRS	Basal M17
Growth in presence of Prebiotics	Basal MRS + 1% fructose	Basal M17 + 1% fructose
	Basal MRS + 1% FOS	Basal M17 + 1% FOS
	Basal MRS + 1% GOS	Basal M17 + 1% GOS
	Basal MRS + 1% lactulose	Basal M17 + 1% lactulose

Probiotic bacteria were inoculated into Microtiter 96 well plates containing the media described above and incubation were conducted in Tecan Safire[2] Microplate Reader in 37°C / 24h. OD_{650} were measured at each one hour intervals. Each of the samples was inoculated into 6 parallel wells and also OD_{650} of media were monitored as blank in two parallel wells.

Fermentation was done with 3 single vessel fermentors. Three different cases were tested in this fermentation:

- general media + faecal inoculum (F1)
- media without carbon source and with prebiotic (GOS) + faecal inoculum + *L.delbrueckii* + *S. salivarus* (F2)
- general media + faecal inoculum + *L.delbrueckii* + *S. salivarus* (F3)

3 fermentors were inoculated with same faecal inoculum and allowed to establish and stabilise for 4 days. At the 5th day of fermentation *L. delbrueckii* + *S. salivarus* – that are incubated in their enrichment broth media overnight- inoculated into the fermentor containing general media + faecal inoculum and to the fermentor containing media without carbon source and with GOS + faecal inoculum, after the cultures washed from the enrichment media with peptone water. Each day fresh overnight bacteria were inoculated to two of the fermentors after taking samples from each fermentor.

Analysis conducted to fermentation products and the media

Enumeration of probiotic bacteria on selective media

Survival of novel lactic acid bacteria (*L.delbrueckii* and *S. salivarus*) in a fermentor system in the presence and absence of added prebiotics were checked via selective enumeration media on 8th, 9th, 10th and 11th days of fermentation. Plate counts are repeated two times for each condition.

For selective enumeration of *L.delbrueckii* in fermentor samples basal MRS media with 1% fructose were used. Samples were diluted with a ratio of 10^{-4} inoculated with pour plate technique and incubated in 45°C/72h in anaerobic conditions. For control, pure culture of *L.delbrueckii* was inoculated and incubated at the same conditions with the other samples.

For selective enumeration of *S. salivarus* in fermentor samples basal M17 media with 1% lactose were used. Samples were diluted with a ratio of 10^{-4} inoculated with pour plate technique and incubated in 45°C/24h in aerobic conditions. For control, pure culture of *S. salivarus* was inoculated and incubated at the same conditions with the other samples.

Short Chain Fatty Acid Analysis (SCFA)

SCFA analyses were applied to both fermentor samples, faecal inoculum and to anaerobic media just before probiotic inoculation and at the end of the fermentation: in order to examine supernatants for any changes in profiles of usual SCFAs. For the SCFA analyses 2-ethyl butyric acid, HCl and ether were added into the samples. After mixing and spinning the upper ether phase were taken carefully and taken into thicker tube. The samples were re-treated with ether and the ether phase is added again into the thicker tube. The ether phase were transferred into agilent crimp cap GC vials, mixed with N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA), heated in 80°C/20 minutes and injected to the GC for analysis.

Polymerase Chain Reaction- Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

PCR-DGGE analysis for Faecal Microbioata

Total DNA was extracted from fermentor samples and original feacal samples using a Soil Bio101 DNA extraction kit by following the manufacturer’s instructions. Amplification was performed using a DNA Thermal i-Cycler (Biorad, USA) and the specific primers F357-GC and 518R were used. The following PCR program was used: initial denaturation at 95°C for 5 mins, 30 cycles of denaturation at 94°C for 20 secs, annealing at 55°C for 45 secs, extension at 72°C for 1 min and final extension at 72°C for 7 mins, followed by cooling to 25°C.

The forward primer contained a GC clamp (5’-CGCCCGCCGCGCGGCGGGCGGG GCGGGGGCACGGGGG-3’) to facilitate separation of the amplicons in a DGGE gel. PCR products were analyzed on DGGE gels on the basis of the protocol of Rowett Research Institute Gut Health Division. A DGGE-2001: universal mutation detection system (C.B.S. Scientific, USA) was used. 35-60% gradient gels were used for the separation of the bands from amplified DNAs. Before polymerization of the denaturing gel, stacking gel without denaturing chemicals was added and the appropriate comb was subsequently inserted. PCR products were separated by electrophoresis at a constant voltage of 80V in TAE buffer at a constant temperature of 60°C for 16 h. Gels were stained

in SYBR gold for 30 min, allowing digital capture of the DGGE band profiles under UV light. As control faecal samples were loaded into gels parallel to PCR products of fermentor samples.

PCR-DGGE analysis for *Lactobacillus*

Different from the analysis of faecal microbioata, again extracted DNAs were also amplified using specific primers Lac1 and Lac2-GC by following the program; initial denaturation at 94°C for 5 mins, 35 cycles of denaturation at 94°C for 30 secs, annealing at 56°C for 45 secs, extension at 68°C for 1 min and final extension at 61°C for 7 mins, followed by cooling to 25°C. 30-50% gradient gels were used for the separation of the bands from amplified DNAs. PCR products were separated by electrophoresis at a constant voltage of 85V in TAE buffer at a constant temperature of 60°C for 16h. Also Genomic DNA from *L.delbrueckii* isolated using a Promega DNA extraction kit via applying the manufacturer's protocol for Gram Positive and Gram Negative Bacteria. Isolated DNA was amplified using the previously mentioned PCR protocol for *Lactobacillus*. They were also put into the gel with the other PCR products.

Amplification of genomic bacterial DNA and total DNA from original faecal inoculum were verified by agarose gel electrophoresis.

3. Description of the main results obtained;

Colony counts in enrichment media can be given as 172-201*10^{-6} c.f.u./ml for *L. delbrueckii* subsp. *bulgaricus* and 149-176 *10^{-5} c.f.u./ml for *S. salivarus* subsp. *thermophilus*.

Determination of the optimum prebiotic

Observation of growth curves at OD_{650nm} showed that highest amount of bacteria obtained in a shorter time via adding GOS to the basal media of both *L. delbrueckii* and *S.salivarus* see Figure 1 and Figure 2.

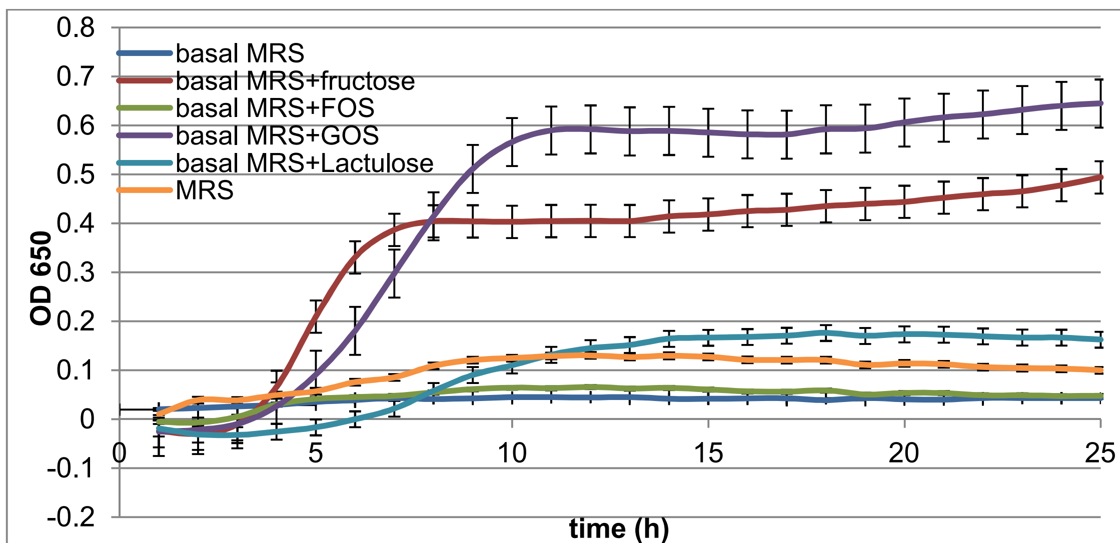


Figure 1. Growth curves of *L. delbrueckii* in presence/absence of carbon sources/prebiotics.

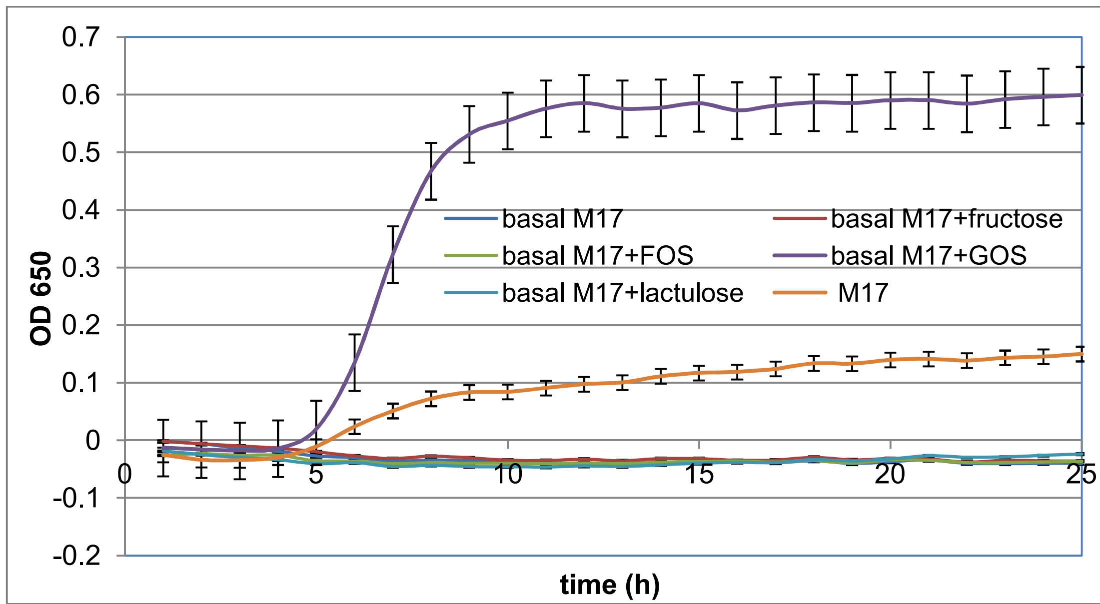


Figure 2. Growth curves of S.salivarus in presence/absence of carbon sources/prebiotics.

Viability of probiotic bacteria during fermentation

Average of colony counts for probiotic bacteria with samples taken from fermentor and probiotic inoculum added into fermentors given in Table 2 and Table 3. Results for Fermentor 1 proving presence of *L. delbrueckii* and *S. salivarus* in original faecal inoculum. Results for Fermentor 2 showing effect of addition of GOS+ probiotic lactic acid bacteria on total growth of probiotic lactic acid bacteria + lactic acid bacteria existing in faecal inoculum. Finally results for Fermentor 3 showing effect of addition of probiotic lactic acid bacteria on total growth of probiotic lactic acid bacteria + lactic acid bacteria existing in faecal inoculum.

Table 2. Enumeration of *L. delbrueckii* in fermentor samples

Day of the fermentation	Pure *L. delbrueckii*	Fermentor 1	Fermentor 2	Fermentor 3
8th day	>503	3	6	>173
9th day	>1000	6	~120	>190
10th day	>1000	21	75	382
11th day	1	219	51	373

Table 3. Enumeration of *S. salivarus* in fermentor samples

Day of the fermentation	Pure *S. salivarus*	Fermentor 1	Fermentor 2	Fermentor 3
8th day	419	183	140	197
9th day	268	142	144	124
10th day	>689	65	2	329
11th day	332	21	4	362

SCFA Analyses

We can observe the change in the SCFA concentration and composition during fermentation process. Abbreviations[1] for Figures 3,4 and 5 are denoted as; **blank:** media at 4th day, **change blank:** media

[1] Some of these are also used in Figure 6.

at 12th day, t_0- t_{11} are fermentor samples; t_0: 1st day (before faecal inoculation), t_1: 2nd day (only faecal inoculum), t_2: 3rd day (only faecal inoculum), t_3: 4th day (only faecal inoculum), t_4: 5th day (only faecal inoculum), t_5: 6th day (faecal inoculum+ probiotics), t_6: 7th day (faecal inoculum+ probiotics), t_7: 8th day (faecal inoculum+ probiotics), t_8: 9th day (faecal inoculum+ probiotics), t_9: 10th day (faecal inoculum+ probiotics), t_{10}: 11th day (faecal inoculum+ probiotics), t_{11}: 12th day (faecal inoculum+ probiotics), **f:** original faecal sample. From figures 3, 4 and 5, we can see a prominent change in acetate, butyrate and propionate concentrations of fermentor samples during fermentation in all of three fermentors.

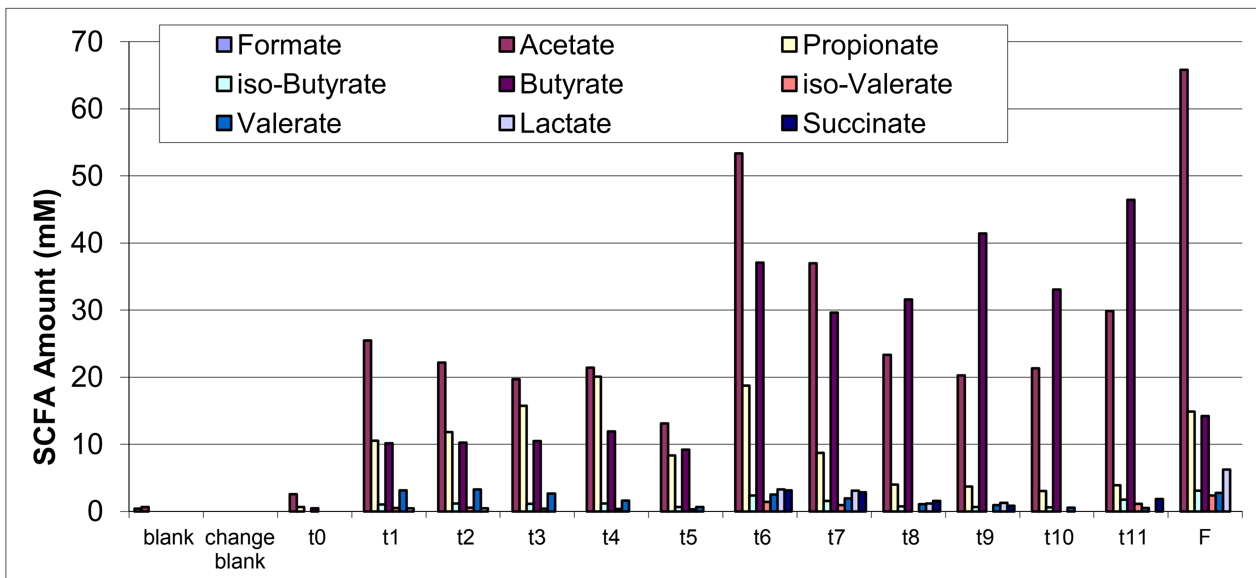


Figure 3. SCFA profile of the faecal inoculum in the general media

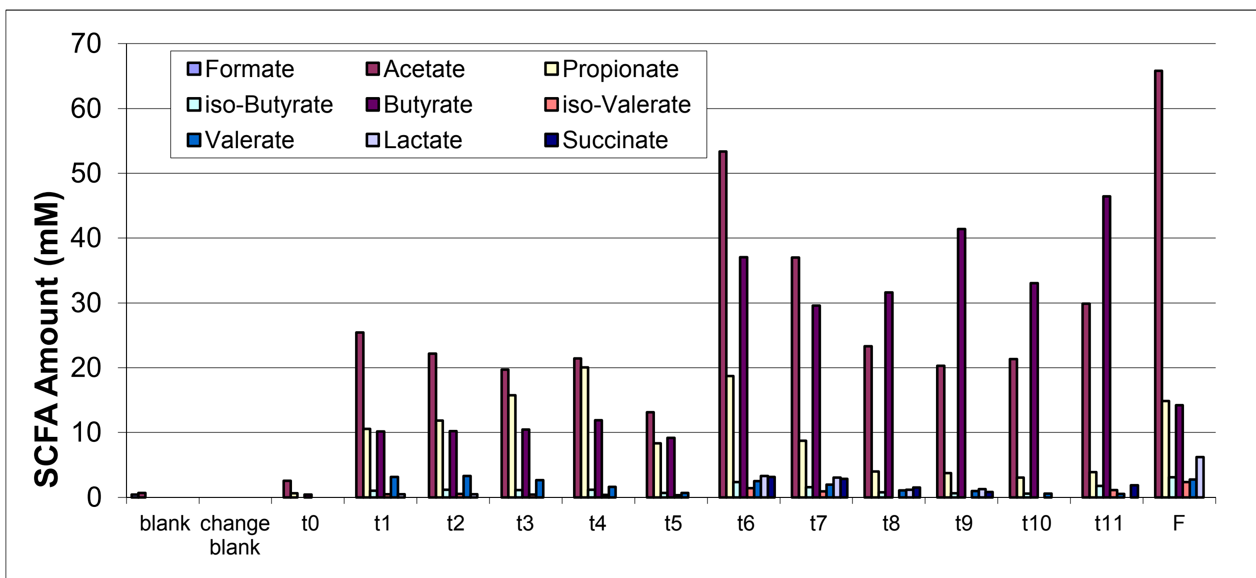


Figure 4. SCFA profile of the faecal inoculum in the media without carbon source and with GOS + (*L.delbrueckii* + *S. salivarus*)

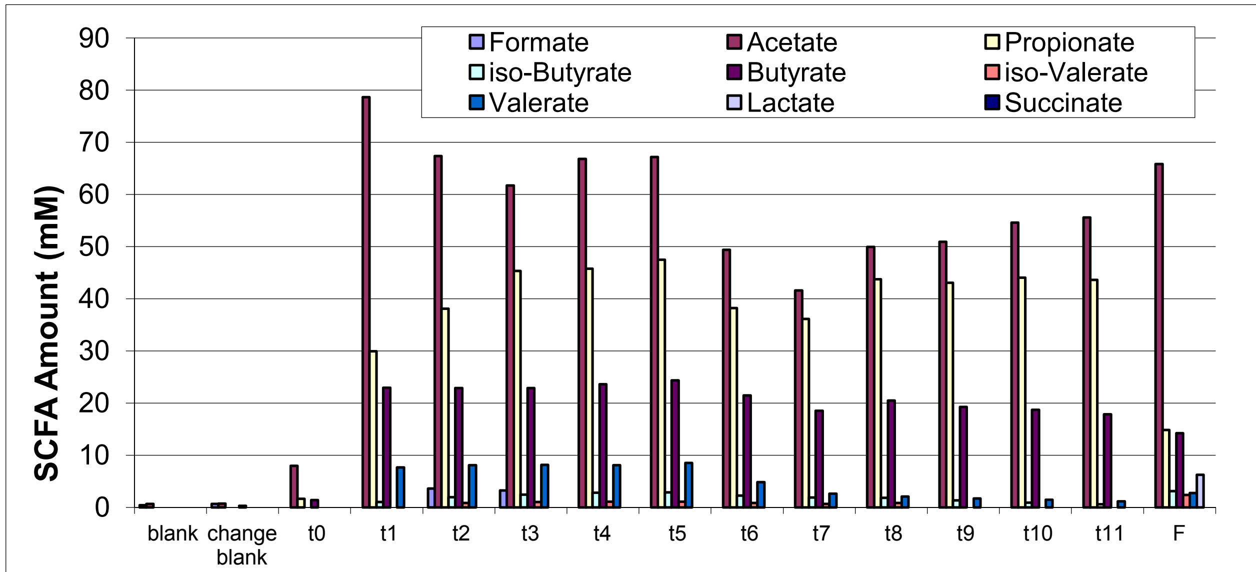


Figure 5. SCFA profile of faecal inoculum in the general media with *L.delbrueckii + S. salivarus*

In fermentor 1 there is an increasing tendency in SCFA amount during fermentation process but not in a direct manner, an increase with fluctuation. In fermentor 3 addition of probiotics have a decreasing effect on SCFA amount at 1st day, but during fermentation again a direct increase pattern can be observed.

DGGE Analyses

At present, DGGE is one of the most frequently applied molecular techniques to analyze gut microbial ecosystems. Results for DGGE analyses of original faecal sample's (from human gut) and fermentor samples' PCR-products (with V3 and Lac) are shown in Figure 6. The mobility of the PCR products obtained in DGGE were compared to faecal ladder, lac ladder (which were previously prepared in Microbial Ecology laboratory of Rowett Institute, UK) and GC tail of known band sizes. Also, the results of DGGE analyses with V3 were evaluated via FPQuest 5.0 software and sought for the ratio of similarity between the samples, see Figure 7. From the results of similarity analyses, we could conclude that, before probiotic inoculation samples taken from fermentor 2 and 3 ran in a similar way through the gel while samples from fermentor 1 ran in a more different way, interestingly, while it should be similar, though.

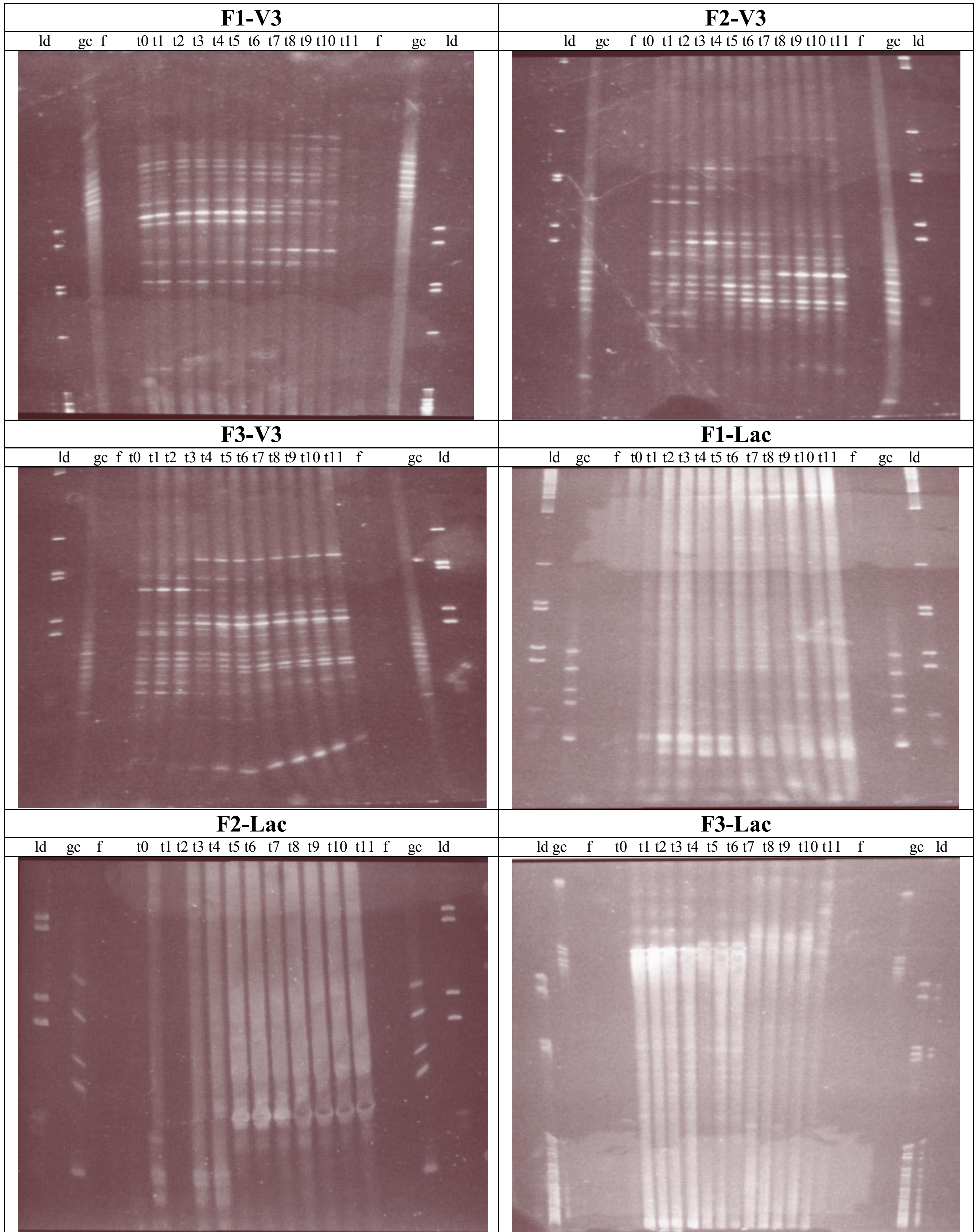


Figure 6[2]. DGGE of faecal and fermentor samples' PCR-products (with V3 and Lac) from human gut

[2] [abbreviations used, ld: Faecal ladder (for V3) ; ld: Lac ladder (for Lac) ; gc: GC tail of known band size]

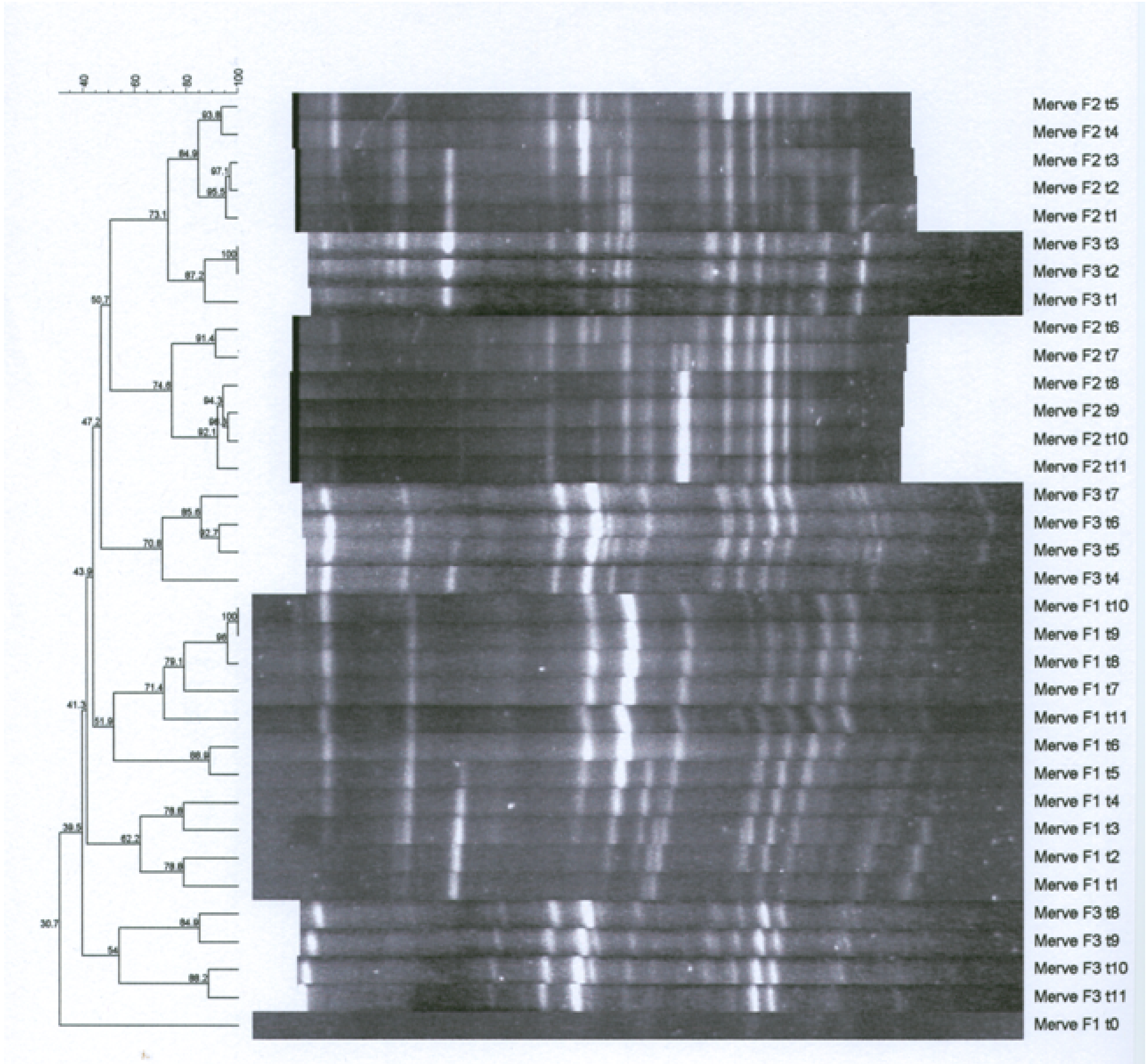


Figure 7. Similarity analysis of fermentor samples that run on DGGE with V3

4. Future collaboration with host institution;

Future collaborations will be sought in between Rowett Health and Nutrition Institute and IZTECH. The framework can be student and researcher exchange and/or joint research projects. In terms of research projects, we have been planning to prepare a project on the area of synbiotics to be submitted to TUBITAK (The Scientific and Technological Research Council of Turkey). We are going to propose to Rowett Health and Nutrition Institute to join this project.

5. Projected publications / articles resulting or to result from the grant (ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant);

No publications exist, for now.

6. Other comments

I would like to thank to ESF-ENGIHR Committee for funding this study and creating such a helpful network for scientists interested in gut health research, thanks to Rowett Health and Nutrition Institute-Gut Health Group for giving permission to use their laboratories and equipments in this study, especially special thanks to Dr. Karen Scott for giving me this opportunity to visit her laboratory and conducting beneficial experimental studies and guiding me with patience anytime I needed, thanks to Jennifer Martin helping me in my experiments whenever I needed and also thanks to IZTECH Food Engineering Department and Biotechnology & Bioengineering Research & Application Center for providing laboratory facilities in the studies conducted in Turkey.