SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

Action number: COST Action FA 1403-POSITIVe

STSM title: Measuring diet: microbiota in the human gut using culture independent molecular methods and metabolite profiling

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PURPOSE OF THE STSM

A centre will be established at University of Health Sciences that aims to develop functional foods, isolate plant-derived bioactive components, investigate their health effects at the molecular level, prevent/delay the formation of chronic diseases by providing effective biological markers, evaluate the mechanism of action of plant bioactives and developed functional food products on health using “omics” approaches, identify new biomarkers for early detection of chronic diseases / metabolic disorders and identify diet signatures by examining the effect of nutrients on the structure and expression of human genome.

Measuring diet:microbe interactions are emerging as key players in human health and disease risk. The gut microbiota is a complex collection of mainly anaerobic microorganisms, many of which are difficult to cultivate in pure culture under laboratory conditions. Culture independent molecular methods and metabolite analysis using analytical chemistry methods are therefore necessary to study both the composition of the gut microbiota and their metabolic output. This short term scientific mission (STSM) is designed to get experience in culture independent microbiota analysis (specifically employing fluorescent in situ hybridization using 16S rRNA targeted oligonucleotide probes and 16S rRNA sequencing) and design of targeted and untargeted metabolomics approaches for quantifying key metabolites produced by the gut microbiota. It is aimed to provide the initial training to enable them to transfer these technologies to our research centre.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

In order to identify and quantify key metabolites produced by the gut microbiota in response to dietary intervention, a training of LC-Orbitrap LTQ-XL and triple quad 5500 ABSciex was given by Dr. Maria M. Ulaszewska for targeted and untargeted metabolomics approaches. Targeted approach focused on bile acid quantification in plasma, while untargeted approach allowed for identification of any metabolites resulting statistically significant against baseline sample. The latter approach allows for identification of both early term biomarkers (Tmax 1-3h) and gut microbiota biomarkers (Tmax 5h and more). The preparation of the sequence and QC samples, calibration and cleaning of the equipment, information on instrument parameters were explained. As wells as a presentation was given by Dr. Ulaszewska about annotation in MS including the topics of elemental composition, molecular ions and adducts, related on-line databases, fragmentation patterns, and an exercise for a metabolite. Furthermore a practical training for Compound Discoverer program was performed by Dr. Ulaszewska using the analysis results of the samples provided by University of Barcelona. As the analysis of the compounds is still under evaluation, no data from that study is provided in this report. Besides metabolomics-related useful links and information for softwares as well as tips were given to be used throughout the metabolomics analysis.

A four week course on metabolomics was given by Dr. Panagiotis Arapitsas (3 hours/week) including a practical application with wine samples. Study design, sampling (ISACreator, MetaMS, Metabolights), MS analysis (randomization, method adaptation, sample preparation), data processing (QC, XCMS), marker detection, markers validation (targeted analysis, TargetLynx statistics, visual control), markers identification (databases such as mzMine, MetaboAnalyst, Metabox) were explained by Dr. Arapitsas. In the practical part of the course, Progenesis QI was introduced for the analysis of the samples.

Culture independent microbiota analysis using FISH (fluorescent in situ hybridization) with 16S rRNA targeted oligonucleotide probes was performed with Dr. Andrea Mancini. The fecal batch culture fermentation system was set up, all parts of the mechanism, critical steps were well-explained and useful tips were given by him. Also processes that need to be handled in care were highlighted. Samples of an ongoing project were used for FISH analysis using probes of *Lactobacillus* 158 and *Bifidobacterium* 164 according to the method of Sanchez-Patan *et al*. (2012). In this study, samples were taken in three different time points (0h, 24h, 48h) from a batch culture fermentation containing 10 treatments. In Table 1 study design was given. Briefly 10µl of sample stored at -20°C in PBS:Ethanol (50:50), was resuspended in 190 µl of PBS. After mixing and centrifugation steps, samples were resuspended in Tris-EDTA buffer and Tris-EDTA buffer containing lysozyme, respectively. After incubation for 10 min at RT, samples were centrifuged and resuspended in 200 µl PBS and 50 µl of hybridization buffer containing probes (5µl of 50

ng/μl), respectively. Following incubation at hybridization temperature (50°C) overnight, samples were centrifuged and resuspended in 200 μl wash buffer following incubation at wash temperature (50°C). Finally samples were resuspended in 50 μl of 1:10000 SYBR Green in PBS and PBS. Then samples were incubated at RT for 10 min and 150 μl PBS was added. Cells were pelleted by centrifugation and resuspended in 100 μl PBS. At the end flow cytometry was used to obtain the number of cells in ml fecal sample.

Table 1: Study design for batch culture fermentation

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Control pH1	Control pH2	Fatty acid pH1	Fatty acid pH2	Fatty acid+ probiotic pH1	Fatty acid+ probiotic pH2	Inulin+ probiotic pH1	Inulin+ probiotic pH2	Inulin pH1	Inulin pH2

DESCRIPTION OF THE MAIN RESULTS OBTAINED

The main outcome of the STSM was a very well training of targeted and untargeted metabolomics techniques applied to biological fluids (plasma and urine). Useful and valuable information given with the training by Dr. Ulaszewska about critical points in untargeted nutritional metabolomics including study design, sample preparation, aliquoting, storage etc. Samples used for showcase of untargeted metabolomics protocol for plasma extraction were taken from EU Project FoodBall, and are still on-going. Additionally urine dataset used for exercise with Compound Discoverer 2.1 Software (ThermoFisher) aiming at data pre-processing and statistical analysis were taken from the same on-going EU Project FoodBall, previously extracted. Dataset consisted of raw Orbitrap files from an acute kinetic study, where samples were taken at time zero (T_0), and several hours postprandial (1h, 2h, 4h, 6h, 12h, 24h, and 48h) after consumption of three different foods A, B and C. From general overview of kinetic curves it can be easily assessed that some metabolites are characteristic for a single Food Item (Figure 1 & 2), and some of them share common metabolism pathway, and are a postprandial reaction of human metabolism (Figure 3). Full statistical analysis of biomarkers resulting statistically significant is under development. Also phase of marker annotation is under development, and names of compounds that were found statistically significant, cannot be given in this report.

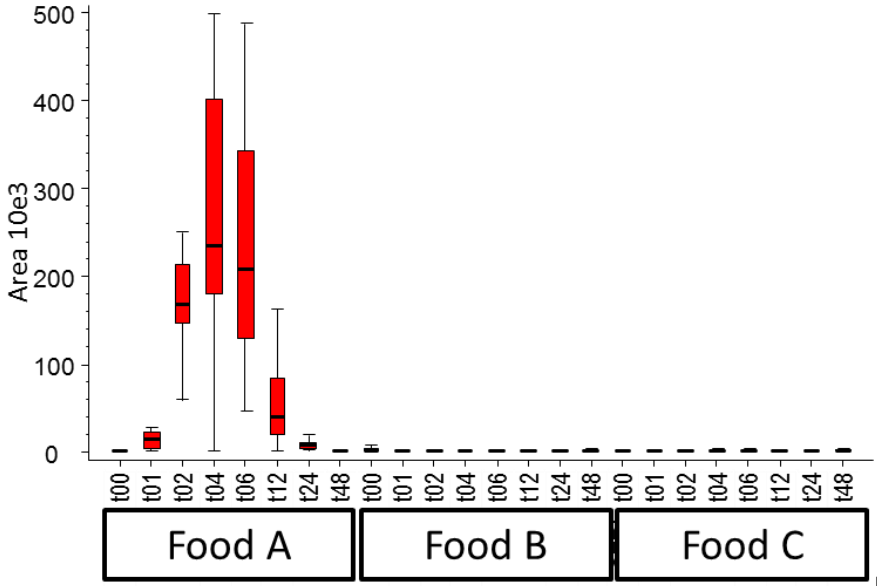

Figure 1

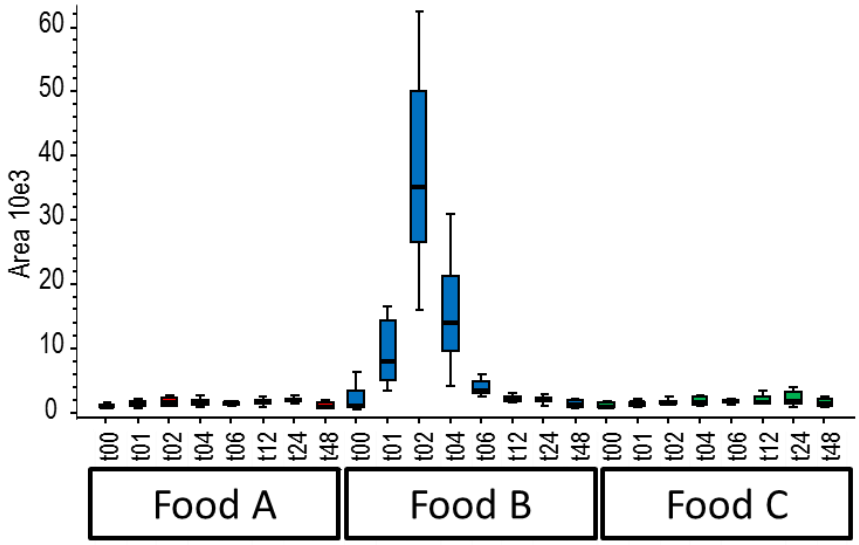

Figure 2

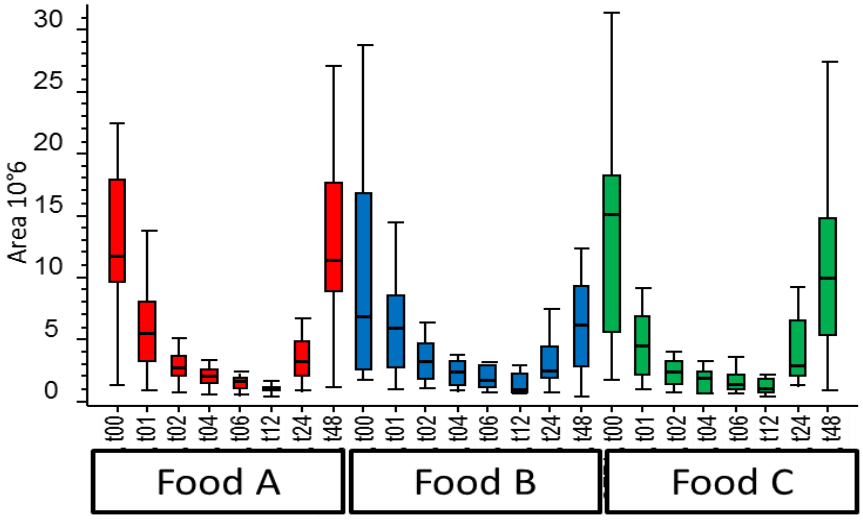

Figure 3

Another part of the STSM was composed of a training for FISH using 16S rRNA targeted oligonucleotide probes. Ready samples from an ongoing project were used for FISH analysis. The amount of *Lactobacillus* and *Bifidobacterium* cells in fecal samples were given in Figure 4&5. As the results of this study has not been published yet, it is not possible to report the details of the study and the type of the specific compounds used. It is obvious to see the effect of the different compounds in the study. In terms of *Bif* 164, a clear increase in the cell number was observed at pH 1 with inulin and inulin+probiotic combination ($4.6x10^7$ cells/ml) after 24h of fermentation.

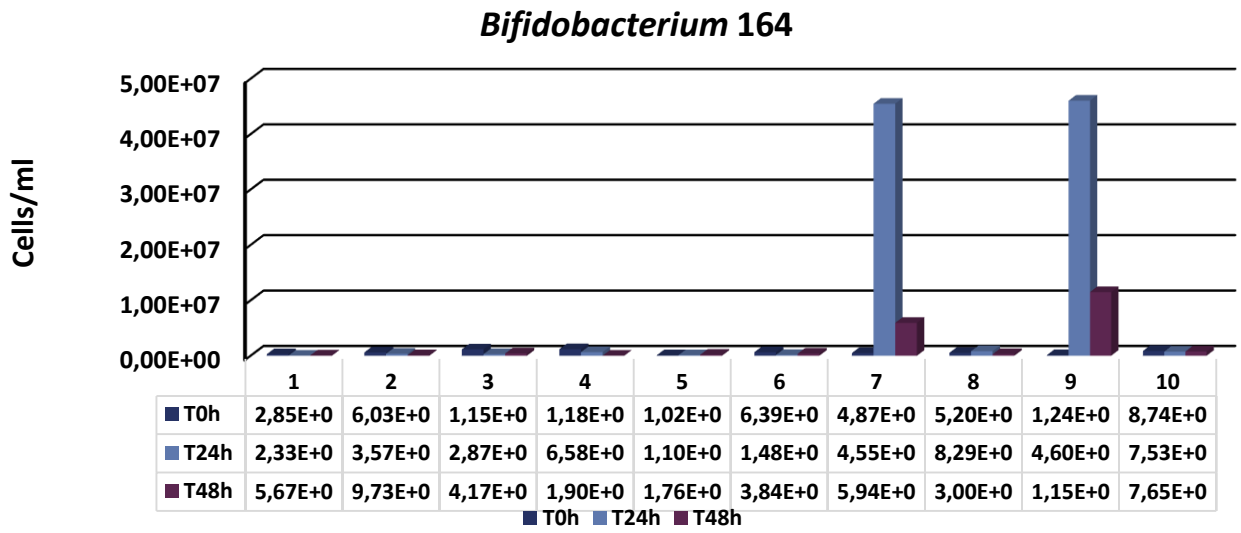

Figure 4 The amount of *Bifidobacterium* cells in fecal samples

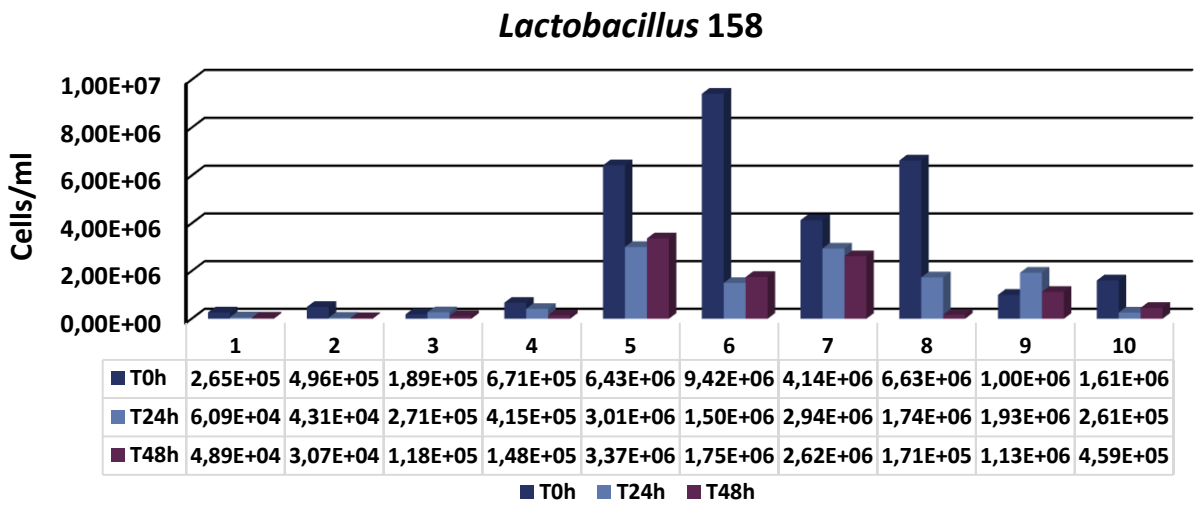

Figure 5 The amount of *Lactobacillus* cells in fecal samples

FUTURE COLLABORATIONS

This STSM allowed to create a new collaboration between Foundazione Edmund Mach and the University of Health Sciences. It is a great pleasure for us that Dr. Tuohy accepted to be the member of the advisory board member of our research centre. It has been of great importance for strengthening the existing collaboration, as well as for reaching their and overall scientific objectives of the COST action POSITIVe including supporting mobility, strengthening the existing networks and fostering collaborations between researchers, learning new techniques, instruments, and methods which are not available in the institutions of the applicants.

Besides this visit enabled a great opportunity to get in touch with researchers in nutrition and nutigenomics area particularly, for prospective EU projects.

Acknowledgements

I would like to thank the COST action POSITIVe for funding this visit and also to Dr. Maria M. Ulaszewska, Dr. Andrea Mancini and Dr. Panagiotis Arapitsas at Fondazione Edmund Mach for their kind help and trainings.

Reference

Sanchez-Patan, F.; Cueva, C.; Monagas, M.; Walton, G.E.; Gibson, G.R.; Quintanilla-Lopez, J.E.; Lebron-Aguilar, R.; Martin-Alvarez, P.J.; Moreno-Arribas, M.V.; Bartolome, B. In vitro fermentation of a red wine extract by human gut microbiota: Changes in microbial groups and formation of phenolic metabolites. J. Agric. Food Chem. 2012, 60, 2136-2147