Short Term Scientific Mission (STSM) Scientific Report

Action: FA 1403-POSITIVe: Interindividual variation in response to consumption of plant food bioactives and determinants involved

Chair of the COST Action: Dr Christine MORAND

Vice Chair of the COST Action: Prof Francisco TOMAS-BARBERAN

Date of the visit: 30 January, 2017- 30 March, 2017

STSM Title: "Studying the relationship between microbiome genetic variation and polyphenol metabolic conversions"

STSM Applicant: Ditta Kolimár, Szent István University, Faculty of Food Science, Department of Applied Chemistry

Host: Assoc. Prof David Berry, Department of Microbiology and Ecosystem Science, University of Vienna

1. Purpose of the visit

The goal of this research is to investigate the effect of inter-individual variation of human gut microbiome on the metabolism of rutin, one of the most abundant flavonol conjugates present in widely consumed plant foods. Within the framework of this STSM, the first part of the collaborative research was carried out, namely the quantitative estimation of the proportion of microbial cells using single-cell activity measurements to show physiological activity during rutin intervention. Rutin-related metabolites will be analysed by LC-HRMS in the samples obtained in the framework of this STSM study at the Szent István University, Budapest.

Gut microbiota had been challenged first with "rutin" (encoded as STSM05 in the attached table). We planned four arms in the experimental setup: a) "pre-washed" faecal samples treated with rutin b) regular faecal samples treated with rutin c) "no amendment control" without rutin appropriate amount of DMSO vehicle d) abiotic control (to monitor the chemical degradation of rutin) 3 time points for each treatment (T0, T6, T24). Stool samples

had been collected from 10 volunteers (10 biological replicates) with 1 technical "replicate". In total we practised 31 fermentations (expect arm "d", that we applied it only once), ultimately 93 samples.

At the final plan of the experiment we missed out the first pre-wash step. The reason of this was, we had not seen significant difference between pre-wahsed and regular samples at the stage of microscopical evaluation.

2. Description of the work carried out during the visit

In vitro intestinal microbial fermentation of rutin (Fig 1) was carried out using faecal inoculum obtained from 10 healthy individuals. After some preliminary experiments and method adjustments, the anaerobic batch incubations were performed in autoclaved hungate tubes in the presence (1 mM) of non-canonical amino acid *L*-azidohomoalanine (AHA) (baseclick GmbH, Germany) and with rutin with a final concentration of 500 µM. A negative control containing dimethyl sulfoxide (DMSO) and a positive control with 5mg/ml of glucose were used for each experiment. Abiotic controls for each time point were collected to further study the biochemical degradation of the compound. Samples were incubated under anaerobic condition with a final volume of 5 ml at 3 different time points: T0, T6 and T24 hours. Subsequently, samples were centrifuged at 14000 rpm for 10 minutes, the supernatant was collected and stored at -80°C for further HPLC analysis. Finally, samples were washed twice in PBS and then fixed in ethanol: PBS (1:1).

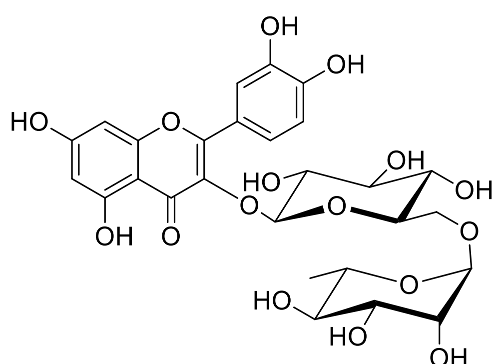

Fig 1 Rutin (quercetin-3-*O*-β-D-glucosyl (1 □6) α-L-rhamnoside) is a disaccharide conjugate of the flavonol quercetin. In this molecule, an ether bond is formed between the 1-OH group of a β-D-glucose and the 3-OH group of a quercetin. In addition, the 1-OH group of an α-L-

rhamnose is attached to the 6-OH group of the glucose. This disaccharide residue is referred to as rutinose, thus this quercetin conjugate is called quercetin-rutinoside or simply rutin.

Physiological activity of microbial cells were visualised by using fluorescence in situ hybridization (FISH) techniques. The total biomass was assessed by staining fixed cells with 4',6-diamidino-2-phenylindole (DAPI). This staining technique visualizes nuclear DNA in fixed cells, thus enables to assess the gross biovolume of the total biomass. In addition to the estimation of total biomass, the physiologically active cells were detected by fluorescent tracking of protein synthesis in individual microorganisms. The technique used, termed bioorthogonal non-canonical amino acid tagging (BONCAT), is based on the *in vivo* incorporation of the non-canonical amino acid L-azidohomoalanine (AHA), a surrogate for L-methionine, followed by fluorescent labelling of AHA-containing cellular proteins by azide-alkyne click chemistry [1]

20 images were collected for each samples and each time point (T6 and T24 hours) with an epifluoresce microscope (Zeiss-Axio-imager, Germany). Images analysis was performed using the software digital image analysis in microbial ecology (Daime) and biovolume fraction was calculated. The biovolume fraction is the measure of the specifically labelled target population relative to the biovolume of the total biomass.

Description of the main results obtained

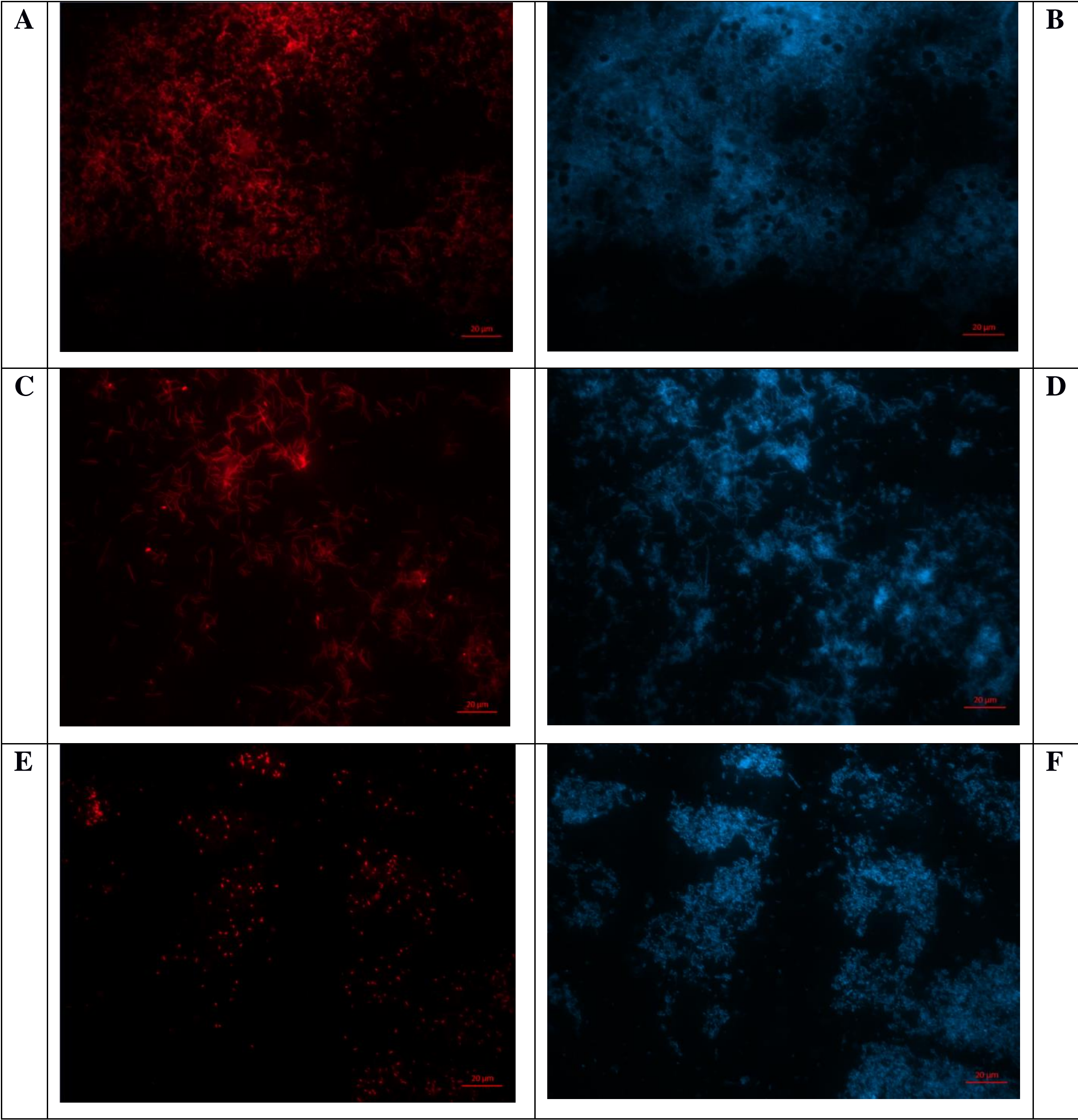

Fig.1: Inter-individual variability between participants. Panels A and B represent participant 1, panel C and D participant 2, panel D and E participant 3. BONCAT signal is represent in red (Cy5 signal) and DAPI staining in blue.

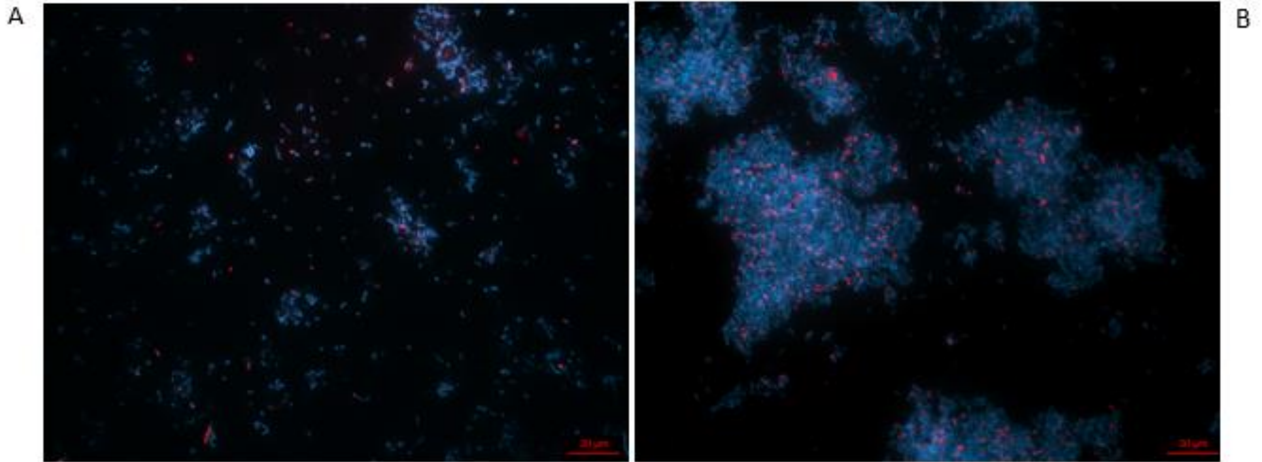

Fig.2: Differences in percentage of labelled cells between time 6 hours (A) and time 24 hours (B).

In the pictures above it is obviously, more cells are activated at the T24 time point, than in case of T6. Based on literature data the maximal absorption rate of rutin is reached after 6-9 hours, but it can be different in each person.

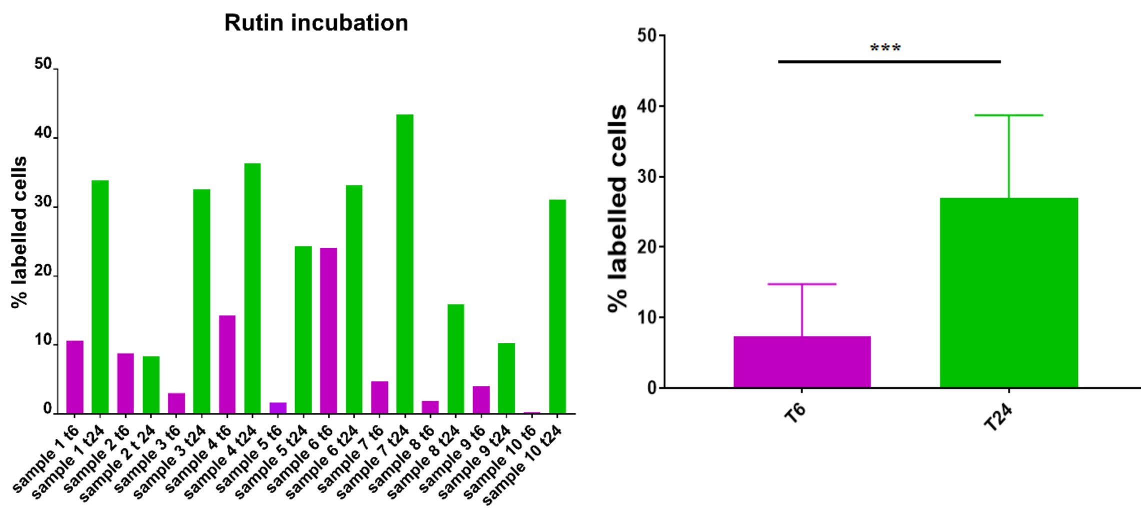

Fig.3: Rutin incubation revealed an inter-individual variability and a difference percentage of active cells between time 6 and time 24. (A) percentage of labelled cells shown for each participant. (B) percentage of labelled cells comparing time 6 and time 24 hours (t-student test p=0.0003).

Projected publications resulting or to result from the STSM

The following oral presentation is accepted at the XIX Eur Food Chem conference 4-6 October, 2017, Budapest:

Ditta Kolimár, Alessanda Riva, David Berry, László Abrankó, Studying the relationship between gut microbiome variation and polyphenol metabolic conversions.

Acknowledgment

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1. Hatzenpichler, R., et al., *In situ visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry.* Environmental Microbiology, 2014. **16**(8): p. 2568-2590.