Assessment of the Stability and Efficacy of a Newly Developed Probiotic Blend in the Context of IBS through a Pilot Multicentre Study

Laura Brunelli (Laura.Brunelli@unimi.it)

Dept. of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy

Tutor: Prof. Simone Guglielmetti

The first two activities of the PhD thesis project are described. Firstly, the adhesion and antioxidant abilities of bacteria that composed the probiotic blend were assessed. These are two of the main probiotic properties evaluated tocharacterize the bacterial strains from the functional point of view *in vitro*. Secondly, the effect of the probiotic multi-strains supplement on non-constipated IBS patients was assessed, and the results obtained from the microbiomic and metabolite analysis of the faecal samples collected during the clinical study are described.Bottom of Form

**Valutazione della stabilità e dell’efficacia di un nuovo prodotto probiotico multi-ceppo nel contesto dell’IBS tramite uno studio pilota multicentrico**

Sono descritte le prime due attività del progetto di tesi di dottorato. Sono state determinate la capacità adesiva e antiossidante dei batteri contenuti nel blend probiotico, le quali rappresentano due delle principali proprietà utilizzate per caratterizzare *in vitro* la funzionalità dei ceppi. Si è poi valutato l’effetto del probiotico multi-ceppo in soggetti con IBS non costipati, descrivendo i risultati ottenuti dall’analisi microbiomica e dei metaboliti a partire dai campioni fecali raccolti nello studio clinico.

# **1. Introduction**

In accordance with the PhD project, this poster reports the main results of the first 2 activities, which are as follows:

(A1) *in vitro* characterization of strains’ probiotic properties from the functional perspective, particularly by evaluating adhesion and antioxidant abilities of bacterial strains;

(A2) assessment of the effect of the probiotic multi-strain supplement in non-constipated IBS patients, with the primary endpoint being the modulation of the faecal bacterial community structure and metabolites.

# **2. Materials and Methods**

Bacterial adhesion to Caco-2 cell line: 2.0×108 bacteria for each strain were incubated for 1 h at 37°C with a fully differentiated monolayer, then washed three times with PBS and incubated with 3 ml of methanol for 8 min. Afterwards, cells were stained with 3 mL of Giemsa solution (1:20) and left 30 min at room temperature in the dark. Finally, monolayers were washed and examined microscopically.

Evaluation of antioxidant activity of probiotic bacteria: free radical scavenging activity of strains was measured by mixing 500 μl of each bacterial cell concentration (1.0×1010, 5.0×109, 2.5×109, 1.0×109 cells/mL) with 500 μl of 0.4 mM DPPH-ethanol solution. The control group included 0.1 M phosphate buffer and DPPH-ethanol solution, while blank group contained sample and ethanol. The mixtures were incubated at 37°C in the dark for 30 min, then the optical absorbance was measured at 517 nm after samples centrifugation at 10000 × *g* for 3 min.

Assessment of the cellular antioxidant activity in Caco-2 cells: after 15 days of growth, Caco-2 seeded at 1.0×104 cells/well on a black 96-well microplate were treated with 100 μl of 10 μM DCFH-DA up to 30 min at 37°C. Subsequently, the cells were washed with PBS and treated with 100 μl of 0.6 mM ABAP together with bacterial suspensions at MOI of 50, 100, 200 and fluorescence was measured for 13 cycles at 5-min intervals (λ excitation = 485 nm and λ emission = 538 nm). N-acetyl cysteine was used as positive control.

Faecal microbiome analysis: the bacterial community structure of faecal samples was studied by 16S rRNA gene profiling with Illumina HiSeq technology. After the extraction of the total DNA from 150 mg of faeces, the 16S rRNA gene amplicons encompassing the V3 and V4 variable regions were sequenced. Moreover, the concentration of short-chain fatty acids (SCFAs; acetate, butyrate, propionate, valerate, isovalerate) and organic acids (lactate and succinate) was quantified in faecal samples by UPLC-MS.

# **3. Results and Discussion**

## **3.1 Definition and microbiological characterization of the probiotic blend**

The differentiated Caco-2 epithelial cell layer was used to test the potential ability of the bacterial cells to adhere on human enterocytes. As expected, since *Bifidobacterium bifidum* MIMBb23sg has been previously demonstrated to be strongly adhesive, it resulted with the higher adhesion index (i.e., bacterial cells per 100 Caco-2 cells) compared to the other strains (Table 1), and this may play a pivotal role in increasing the intestinal barrier with a concurrent beneficial effect in IBS patients (Guglielmetti et al., 2011).

***Table 1*** *Adhesion properties of the probiotic bacterial strains to a Caco-2 cell monolayer. Data are reported as adhesion index.*

|  |  |
| --- | --- |
| **Enterolactis® Ultra *strains*** | ***Adhesion index*** |
| *Bifidobacterium bifidum* MIMBb23sg (*Bifidobacterium bifidum* BbfIBS01, DSM 32708) | >2000 |
| *Lacticaseibacillus paracasei* DG I1572; L. casei DG**®** (DSM 34154) | >50 |
| *Bifidobacterium breve* BbIBS01 (DSM 33231) | >60 |
| *Bifidobacterium breve* BbIBS02 (DSM 33232) | >60 |
| *Lactiplantibacillus plantarum* LpIBS01 (DSM 33234) | >100 |
| *Bifidobacterium animalis* subsp. *lactis* BlIBS01 (DSM 33233) | >100 |

The DPPH assay was used as a screening for assessing strains’ antioxidant abilities and was defined as scavenging activity, while the cellular antioxidant assays was used to reveal the total antioxidative capacity of the strains by measuring ROS accumulation in Caco-2 cells. The ROS scavenging abilities were consistent with the CAA results, showing that both MIMBb23sg and DG exert a statistically significant ability to lower ROS in a dose-dependent manner. The free radical scavenging ability is plausibly due to the properties of the molecules on the bacterial cell outer surface (e.g., the exopolysaccharide of strain DG; Balzaretti et al., 2017). Reportedly, some probiotic strains are reported to scavenge oxygen free radicals protecting Caco-2 cells against damage, maintaining Caco-2 cell integrity by enhancing the expression of tight junction proteins to protect the host against ROS injury (Mu et al., 2019).

## **3.2 *In vivo* clinical trial**

Regarding the effect of the probiotic treatment on faecal taxonomic diversity, α-diversity was not significantly changed. Furthermore, the analysis of inter-sample biodiversity (β-diversity) indicated that the treatments did not induce a significant alteration in the overall bacterial community structure of faecal samples. However, concerning the impact of the probiotic intervention on specific faecal bacterial taxa, the administration of probiotics resulted in a significant decrease of the phylum *Actinobacteria* and a significant increase of the phylum *Bacteroidetes*. In addition, several taxa of the phylum *Proteobacteria*, such as the genera *Paracoccus*, *Ralstonia*, *Halomonas* and *Vibrio*, were significantly reduced after probiotic treatment compared to the placebo.

Therefore, the daily intake of a sachet of Enterolactis® Ultra modifies the intestinal microbial ecosystem of non-constipated IBS patients. It also led to a significant reduction in the ratio between the faecal levels of the SCFAs propionate and butyrate, as reported in Table 2. Notably, the propionate:butyrate ratio has already been proposed as a potential biomarker for IBS in previous studies, showing that the difference between propionic acid and butyric acid (mmol/l) was significantly higher in diarrhoea-predominant IBS patients (Farup et al., 2016).

Finally, correlation analysis revealed significant positive associations of propionate and the propionate/butyrate ratio with several bacterial taxa that resulted significantly reduced by the probiotic intervention, including the genus *Collinsella*, the family *Leuconostocaceae* and the genus *Coprobacillus*, which resulted also showed negative correlation with butyrate levels.

***Table 2*** *Faecal concentration of organic acids in patients participating to the clinical trial. Data are presented as median values and expressed as mmol/100 g of faeces. \*, P<0.05* *based on non-parametric ANOVA.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|   |   | ***P*** | **Before Probiotic** | **After Probiotic** | **Before Placebo** | **After Placebo** |
| Acetate |   | 0.537 | 3.66 | 4.21 | 4.03 | 3.93 |
| Butyrate |  | 0.152 | 2.23 | 2.95 | 3.98 | 2.55 |
| Propionate |   | 0.100 | 1.08 | 0.97 | 0.92 | 1.15 |
| Valerate |   | 0.140 | 1.24 | 1.13 | 1.21 | 1.42 |
| Succinate |  | 0.555 | 0.09 | 0.15 | 0.10 | 0.09 |
| Isovalerate |   | 0.478 | 0.56 | 0.59 | 0.57 | 0.53 |
| Lactate |   | 0.355 | 0.04 | 0.05 | 0.03 | 0.03 |
| Acetate/Butyrate |   | 0.468 | 1.23 | 1.25 | 1.21 | 1.29 |
| Propionate/Butyrate | **\*** | **0.013** | **0.42** | **0.22** | **0.26** | **0.38** |

# **4. References**

Guglielmetti, S., Mora, D., Gschwender, M., & Popp, K. (2011). Randomised clinical trial: *Bifidobacterium bifidum* MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. Alimentary pharmacology & therapeutics, 33(10), 1123–1132.

Balzaretti, S., Taverniti, V., Guglielmetti, S., Fiore, W., Minuzzo, M., Ngo, H. N., Ngere, J. B., Sadiq, S., Humphreys, P. N., & Laws, A. P. (2017). A Novel Rhamnose-Rich Hetero-exopolysaccharide Isolated from *Lactobacillus paracasei* DG Activates THP-1 Human Monocytic Cells. *Applied and environmental microbiology*, *83*(3), e02702-16.

Mu, G., Li, H., Tuo, Y., Gao, Y., & Zhang, Y. (2019). Antioxidative effect of *Lactobacillus plantarum* Y44 on 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP)-damaged Caco-2 cells. *Journal of dairy science*, *102*(8), 6863–6875.

Farup, P. G., Rudi, K., & Hestad, K. (2016). Faecal short-chain fatty acids - a diagnostic biomarker for irritable bowel syndrome? *BMC gastroenterology*, *16*(1), 51.