The impact of breastmilk lactobacilli isolates on immune cell responses

Short term (three months) EAACI Research Fellowship at Stockholm University in Stockholm, Sweden.

What questions were addressed and why? What was the nature of the research? What was the result? How will the findings impact future research? ***

Appropriate initial bacterial colonization of the newborn is critical for adequate intestinal and systemic immune development. Breastmilk, being a direct source of microbes, promotes the initial colonization and maturation of the infant gut microbiome, and acquired disturbances may have an important and timely impact on immune maturation.

Potential health promoting microorganisms detected in breastmilk, including species from the *Lactobacillus, Bacteroides, Bifidobacteria* and *Staphylococcus* genera, have been shown to be important during the neonatal period in activating different immunologic functions such as tolerance and intestinal barrier integrity. Lactobacilli play an important role in the healthy human intestinal microbiota and are considered to be involved in immune priming and the maintenance of other commensals. Various *Lactobacillus* strains have been demonstrated to have probiotic properties, although the mechanisms by which they modulate immune responses in the newborn are not completely understood.

In this study, we aimed to understand the impact of the breastmilk-isolated *Lactobacillus* species and breastmilk pools, of mothers whose children did not or did develop allergies, on cord blood immune cell- responses. We hypothesize that the isolated *Lactobacillus* species may alter immune cell gene and protein expression towards an anti-inflammatory profile. Moreover, we hypothesize that breastmilk might impact the specific immune cell regulation in relation to allergy development. Our preliminary results showed that the *Lactobacillus gasseri* isolate was able to decrease the relative amount of regulatory T cells (here measured by expression of CD25+FOXP3+), when compared to positive control, while *Lactobacillus rhamnosus* appears to have an opposite effect (Fig. 1 and Fig. 2A). We could also observe that the cord-blood cells are dominantly characterized by a naïve phenotype (CD45RA+ CCR7+), even upon bacterial stimulation, and that stimulation with *L. gasseri* resulted in loss of CCR7 expression. However, this needs to be further confirmed by ELISA immunoassay by measuring appropriate cytokines.

Upon stimulating cord blood monocytes (CBMCs) with breastmilk pools (containing a mix of five samples) from mothers whose children did or did not develop allergies, no differences in cellular responses, as observed by fluorescent activated cell sorting (FACS), could be observed (Fig. 2B). Nevertheless, this should be studied further with greater amount of cord blood samples and ideally with breastmilk pools of other mothers.

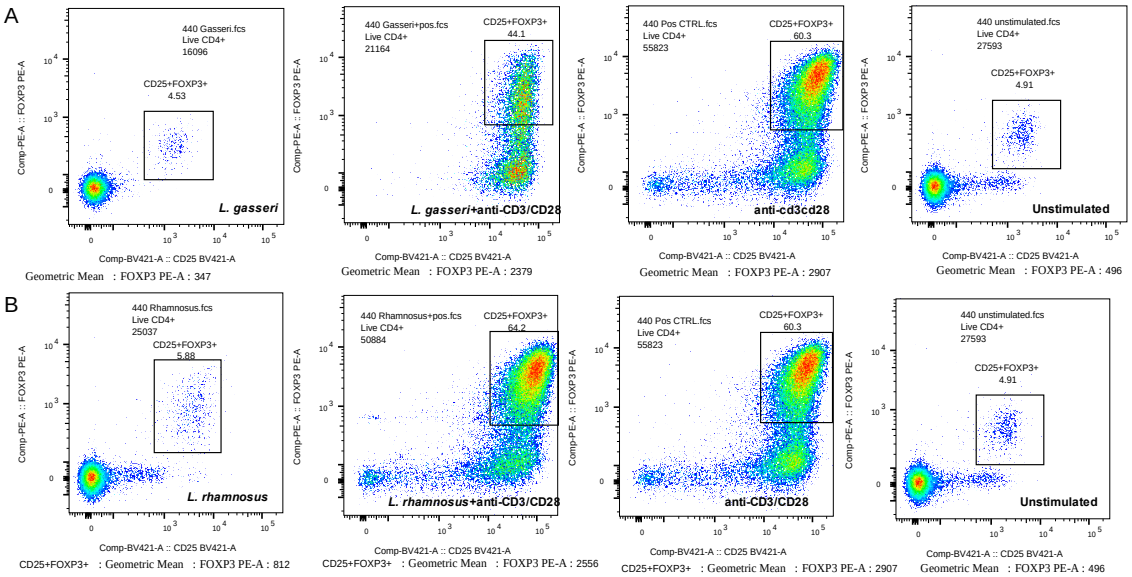

Figure 1. An example of CD25+FOXP3+ expression upon stimulation with *L. gasseri* (A) and *L. rhamnosus* (B). Plots presented were obtained using FlowJo software.

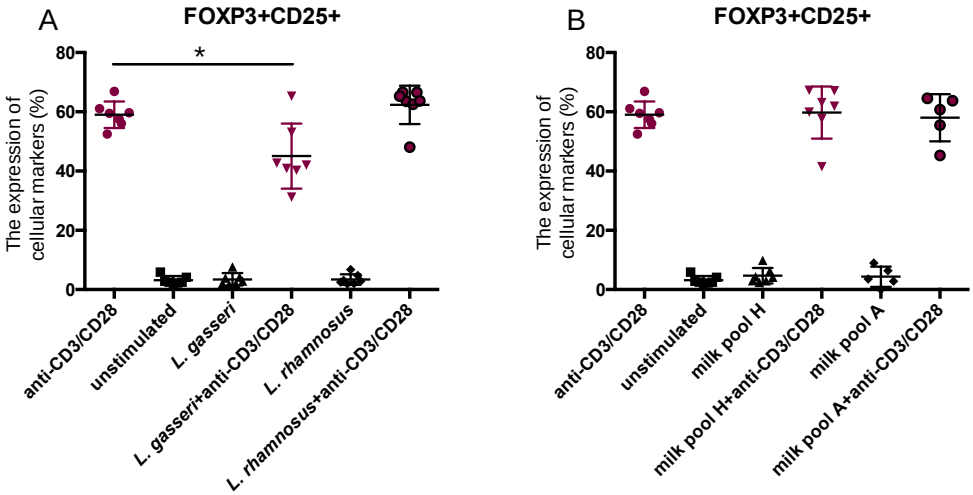

Figure 2. The expression of CD25+FOXP3+ regulatory T cell markers upon CBMCs stimulation. A. The plot represents CD25+FOXP3+ expression upon stimulation with *L. gasseri* and *L. rhamnosus*. B. The plot represents CD25+FOXP3+ expression upon stimulation with milk pools obtained from mothers whose children did (milk pool A) or did not (milk pool H) develop allergies during the first year of age. The figures present data as interquartile ranges with ± SEM. *p < 0.05. Analyses were performed using Prism software (GraphPad).

The majority of microbiota studies that have been published during the last years have been of observational nature and we believe that the research considering the specific biological mechanisms of host-microbiota interactions are lacking and should be further elucidated. Knowing that breastfeeding may shape the intestinal microbiota, and the gut milieu in general, it is of great interest to understand the role of breastmilk bacteria as a potential regulator of different immune cell types. By knowing how *Lactobacillus* isolates can regulate specific immune cells, this understanding could further pave a way for a potential probiotic that may be used in early immune instructions.

The report should include an informative description of the activity during the fellowship period and answers to the previously defined objectives. The outcomes presented should correspond and match with those specified in the plan.

Our previously defined objectives:

(1) To understand the impact of the breastmilk-isolated *Lactobacillus* species on cord blood immune cell- responses.
(2) To understand the significance of breastmilk on the above responses, by using breastmilk from mothers whose children do or do not develop atopic symptoms during the first year of life.

Laboratory work performed before the research stay:
Before starting the stay at Stockholm University, bacterial isolates from breastmilk samples were obtained as followed:

1. Breastmilk samples from mothers whose children do or do not develop atopic manifestations during the first year of life.
2. Bacterial isolation from breastmilk samples using basic microbiology approaches.
3. PCRs and Sanger sequencing of PCR products for identification of lactobacilli isolates.

L. gasseri, L. rhamnosus and *L. fermentum* were isolated, among other bacteria.

Laboratory work performed during the stay:
Upon arrival to the host lab, I needed to optimize the protocol for my experiments since at the host laboratory they mainly worked with adult PBMCs that are behaving in a different way. For instance, it was needed to define the optimal incubation time of 24h, 48h or 72h and compare different an ideal bacterial concentration. Thus, first months I was mainly working with PBMCs isolated from adults where I learned the methodology and explored the cell responses, by measuring different cytokine responses, upon different bacterial stimuli. The colleagues from the research hosting group showed me how to work correctly and sterile with the cell cultures, how to treat the cells, how to examine them microscopically and how to perform a viability staining. Moreover, I tried three different lactobacilli isolates and realized that one of them did not have any influence on the cells, e.g. did not cause any production of cytokines that we were measuring with ELISA, and therefore I decided to not continue using it in further experiments.

After having optimized the method for the specific samples, the following experiments were performed:

1. **Cell culturing of cord blood monocyte cells that were stimulated with different microbial lactobacilli supernatants.** The cells were incubated for 48h in the presence or absence of lactobacilli isolates from breastmilk and in combination with a positive control (anti-CD3/CD28). Also, skimmed milk pools were used (total milk sample without the fat content) for the stimulation of the cord blood monocytes. For every stimulation, at least 250 000 cells were needed and since the CBMCs samples were limited (around 2 million cells/sample), we needed to select carefully which bacterial stimuli could be should use. Moreover, further problems were encountered upon thawing the CBMCs samples since in only 9 of 20 cases, viable cells could be found. Likely, there must have been some problem upon initial CBMCs isolation performed by biobank.
2. **Collection of supernatant**. After 48 hours of incubation with different bacterial stimuli, the cells were centrifuged and the supernatant was collected into plates and stored at -80 degrees for further immune assays. We are planning to perform ELISA immune

assay for measuring the levels of different cytokines, thus reflecting the inflammatory/anti-inflammatory responses that bacteria likely cause, but there was no time for that during this visit.

3. **Phenotyping of the cells by using fluorescence activated cell sorting (FACS).** Beside collecting the supernatants, the stimulated cell pellets were used for antibody staining in order to phenotype the expression of different T cell markers by using flow cytometry. The T cell panel consisted of: CD4, CD25, CCR7, CD45RA, CD127, FOXP3. The data obtained was analysed by a software FlowJo.

My plan from beginning was to compare the responses between children that do and do not develop allergies but since not all the cord-blood samples had viable cells, I could not fulfil the entire experiment as planned. Due to sample unavailability, I modified the analyses in the way that I am comparing the cellular responses according to the different bacterial stimuli, without taking into account if they develop allergies or not later in life. For the future experiments, I would like to increase the number of samples in order to determine if allergy status would have an effect on cellular responses.

Conclude with a personal reflection on what you have learned and how you can improve for the future.

During my research stay I learned many different methodologies, including ELISA immunoassay, fluorescence activated cell sorting, but also how to analyse FACS generated data by using a specific software. Moreover, I have learned how to work with cell cultures and how to handle blood samples. The stay has been a great experience for me, both when it comes to new knowledge but also at the personal level since I learned how to design, plan and conduct a study and adapt to a new lab group. For the future stays, I would like to decide more in detail about the lab work that needs to be performed and also to confirm the viability of the samples before starting with the experiments. Moreover, I would have liked to have more time for this project because it took me nearly two months to optimize everything before I could start with the main experiment. However, I am really happy that I got the possibility to visit this outstanding lab and scientific team and learn from them. I am truly satisfied with this stay and I hope that we will continue collaborating in the future.