

Special Article - Probiotics and Functional Foods

Transcriptomic Analysis of PBMCs from Allergic Patients after Probiotic Treatment

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Abstract

Background: Allergic rhinitis is one of the most prevalent manifestation of allergy, affecting over 15% of the population worldwide. Recent published clinical studies have shown that specific probiotics can improve allergic rhinitis clinical symptoms.

Findings: In this study, thirty one adult volunteers suffering from allergic rhinitis were enrolled in a crossover study evaluating the efficacy of the consumption of a product containing either *L. paracasei*-fermented milk or the placebo. Transcriptomic analysis was performed on unstimulated PBMC after each treatment period and analysis was adjusted for the crossover design. No differences were observed between PBMCs from probiotic treated allergen challenged allergic patients and PBMCs from placebo treated allergen challenged allergic patients.

Conclusion: This study shows that, in the blood compartment, PBMCs mRNA levels are too stable to mirror the changes of symptoms and alteration of cytokine expressions observed after a treatment with *L. paracasei*.

Trial Registration: NCT01150253. Registered 23 June 2010.

Keywords: Allergic rhinitis; Probiotic; Grass pollen; PBMC; Transcriptomic

Abbreviations

PBMC: Peripheral Blood Mononuclear Cell; RT-Q-PCR: Reverse Transcription-Quantitative-Polymerase Chain Reaction; IL: Interleukin; NPT: Nasal Provocation Test, B2M: Beta-2-Microglobulin

Introduction

Allergic rhinitis prevalence has dramatically increased over the last past decades reaching up to 15.7% of the populations [1]. Pharmacologic treatments for allergic rhinitis are available but with some side effects that affects the quality of life of the patients. Probiotics have been largely tested in the context of atopic dermatitis, allergic rhinitis and food allergy prevention or treatment [2-7] and have been shown to modulate the immune system dampening the Th2 response and potentiating T regulatory cell population and IL-10 production [8]. *Ex vivo*, Th2 skewed PBMCs stimulated with probiotics such as *L. paracasei* NCC2461 ST11 show an increase in TNF- α , IFN- γ and IL-10 productions and decrease in IL-5 production [9], or *ex vivo* in atopic patients [10,11]. Epigenetic mechanisms involving RNA stability have been described with *L. paracasei* NCC2461-stimulated PBMCs [12]. Two previous clinical trials (CT) have shown that the probiotic *L. paracasei* NCC2461 can improve the nasal congestion or the nasal pruritus after a nasal provocation test [13,14]. Both trials strongly suggested that the consumption of *L. Paracasei* NCC2461 may benefit grass pollen induced allergic reaction. In the second trial [14], designed as a randomized double-blind placebo controlled crossover study, 31 adult volunteers, aged between 18 and 35 years old, suffering from allergic rhinitis were enrolled. The crossover study was based on two-times 4-week periods of product consumption either

with *L. paracasei*-fermented milk or with the placebo, separated by a wash-out period of 6 weeks (Figure 1). This clinical trial demonstrated a significant reduction in nasal congestion in subjects receiving fermented *L. paracasei* milk compared to placebo treated subjects. Additionally, lower IL-5 expression level in grass pollen restimulated peripheral blood mononuclear cells (PBMCs) was found associated with the consumption of *L. paracasei*-fermented milk and with the decreased in nasal congestion. Similar decrease was observed for allergen specific immunoglobulin G4 (IgG4) level in the *L. paracasei*-fermented milk treated group. Nasal pruritus was improved in the group treated with *L. paracasei*-fermented milk but without reaching significant difference.

In order to test whether PBMCs could be used as a predictor of symptom response, we sought to identify whether the *L. paracasei*-fermented milk treatment was able to modify the gene expression profile of unstimulated PBMCs compared to the placebo, and to correlate these gene expression levels with the cell counts in nasal fluids, immunoglobulin levels and cytokine expressions in grass

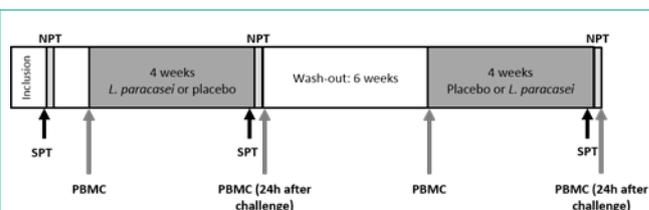


Figure 1: Schematic design of the crossover, placebo-controlled study. Double blind and cross over study for comparison of two interventions (with or without *L. paracasei*). SPT: Skin Prick Test; PBMC: Peripheral Blood Mononuclear Cells; NPT: Nasal Provocation Test.

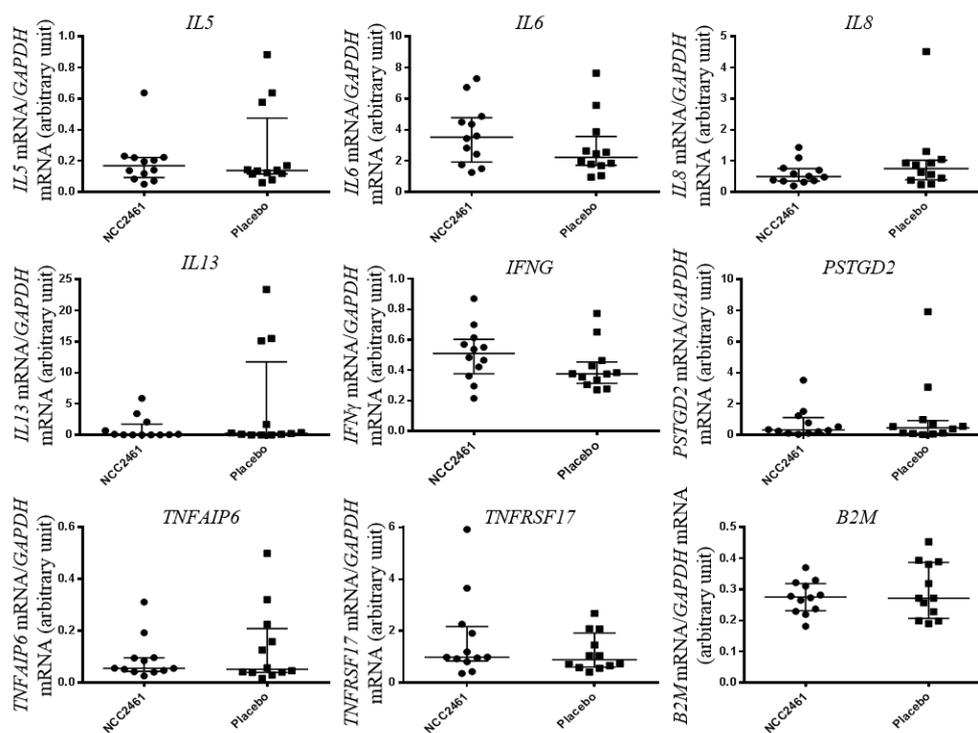


Figure 2: Relative mRNA expression of candidate genes. Effect of *L. paracasei* NCC2461 and placebo treatment of inflammatory cytokine gene expression in PBMC. *IL5*, *IL6*, *IL8*, *IL13*, *IFNG*, *TNFAIP6* and *TNFRSF17* mRNA expression levels were measured by RT-Q-PCR. Results are expressed as ratio relative to housekeeping gene GAPDH.

pollen re-stimulated PBMCs.

Materials and Methods

RNA extraction and transcriptomics analysis

RNA was prepared from PBMC (10^7 cells) using RN Easy midi kits (Qiagen, Venlo, Netherlands), incorporating a DNase1 digestion step, according to the manufacturer's instructions. Total RNA yield was determined using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, United States) and purity assessed by the ratios of absorbance at 260:280 and 260:230 nm. RNA integrity was assessed using Agilent RNA6000nano LabChips[®] (Agilent Technology 2100 Bioanalyzer Version A.01.20 SI211, Santa Clara, California, USA). The RNA samples were aliquoted (10 μ g) and stored at -80°C . RNA were prepared according to manufacturer's instruction for the hybridization to Affymetrix human U133 Plus 2 arrays (Santa Clara, California, USA). The raw data for all the 48 arrays (.CEL files) were uploaded into GeneSpring 9.0.6 (Agilent Technologies, Santa Clara, California, USA) and analyzed. Statistical analyses (paired t-tests incorporating Benjamini and Hochberg multiple test correction) were then used to perform a number of specific group comparisons.

Real time PCR-PCT

Reverse transcription was performed using qScript reverse transcriptase (Invitrogen, Carlsbad, California, United States) and quantitative real-time polymerase chain reaction (RT-Q-PCR) was carried out using Platinum SYBR Green qPCR Supermix-UDG with ROX (Invitrogen) on an ABI Prism 7900HT Fast Real-Time PCR

Table 1: Human primers pairs used for real-time PCR.

Gene	Forward primer (3'-5')	Reverse primer (5'-3')
<i>B2M</i>	TTCATCCATCCGACATTGAA	CCTCCATGATGCTGCTTACA
<i>IL5</i>	GAGACCTTGGCACTGCTTTC	CAGTACCCCTTGCACAGTT
<i>IL6</i>	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT
<i>IL8</i>	TAGCAAATTGAGGCCAAGG	AAACCAAGGCACAGTGGAAAC
<i>IFNg</i>	TCCCATGGGTTGTGTGTTA	AAGCACCAGGCATGAAATCT
<i>TNFAIP6</i>	TCACATTTTCAGCCACTGCTC	AGACCGTGCTTCTCTGTGGT
<i>TNFRSF17</i>	GCAGTGCTCCCAAATGAAT	GTCCCAAACAGGTCCAGAGA
<i>PTGS2</i>	TGAGCATCTACGGTTTGCTG	TGCTTGCTGGAACAACCTGC
<i>GAPDH</i>	GAGTCAACGGATTTGGTCTG	TTGATTTTGGAGGGATCTCG

system (Applied Biosystems, Foster City, California, United States). The PCR was performed with 40 cycles of the following conditions: denaturation for 15s at 95°C , annealing for 15s at 60°C and extension for 30s at 72°C . The primer sets used to perform real-time PCR are listed in Table 1. The mRNA sequences were used in the Primers3 software (<http://simgene.com/Primer3>) to select pairs of matching primers. Synthetic primers were obtained by Microsynth AG (Balgach, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene and the results were calculated using a standard curve for each gene. Results are then expressed as a ratio compared to the house keeping gene.

Cytokines expression

Cytokines (IL-4, IL-5, IL-8, IL-10 and IFN-g) were measured

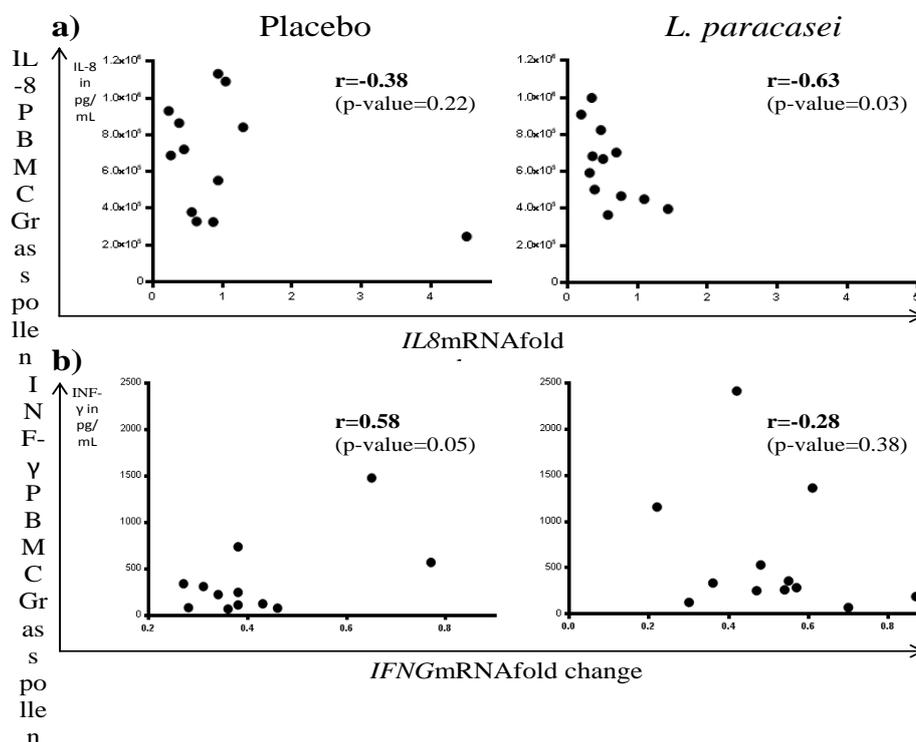


Figure 3: Correlation between mRNA and protein expression level in PBMCs. Comparison of cytokine expression in restimulated PBMC with grass pollen and mRNA expression level by quantitative PCR in unstimulated PBMCs. Correlation of IL-8 (a) and IFN- γ (b) for both the placebo and *L. paracasei* treated group are shown.

on grass pollen re-stimulated PBMCs as previously described [14]. Detection limits of the multiplexed bead-based flow cytometry assay were 0.6, 0.2, 0.55, 1.19 and 0.87 pg/L, respectively.

Statistical analysis

PCR cytokines expression levels and correlations were performed using R version 3.1.2 (<https://cran.r-project.org/bin/windows/base/old/3.1.2/>) and graphs generated with GraphPad Prism 7 (GraphPad Software, Inc. La Jolla, California, USA).

Results

Transcriptomic analysis using Affymetrix human U133 Plus 2 arrays was performed and adjusted for the crossover design (Figure 1). Comparison of the ~47,000 transcripts expression profiles for samples collected immediately prior to both interventions identified no significant differences between the groups when a false discovery rate was applied ($FDR < 0.05$) in the statistical analysis. Due to the challenge of detecting the often small changes in gene transcription resulting from nutritional interventions, such comparisons are often reported without corrections for multiple tests. However, even with the false discovery rate omitted from the analysis, the number of significant genes ($p < 0.05$) was lower than the expected number of false positive tests for the number of comparisons performed before and after the intervention with placebo or *L. paracasei*. Similarly, no genes were identified as changing in expression as a result of the *L. paracasei*-fermented milk intervention compared to placebo when the false discovery rate was applied. However, in this case, when the false discovery rate was omitted, the number of genes for which the uncorrected p values was < 0.05 was substantially higher

(approximately 3-fold) than the expected number of false positive tests for the number of comparisons performed.

Because some cytokine transcripts are not always detected using microarray techniques, we used a candidate approach. To address this issue, a subset of candidate genes was selected based on the transcriptomic results and the known Th2 cytokines responding to probiotics intervention (IL-8; IFN γ). No significant differences were observed between the placebo group and the *L. paracasei*-fermented milk treated group for the different molecular markers analyzed by RT-Q-PCR in PBMCs (Figure 2) thus confirming the null findings obtained by transcriptomics. Interestingly, a negative correlation (Spearman's $r = -0.63$, $p = 0.03$) between interleukin-8 (*IL8*) mRNA expression and IL-8 secretion in the supernatant of grass pollen re-stimulated PBMC was observed in the *L. paracasei*-fermented milk treated group, while no statistical association was found for the placebo treatment (Spearman's $r = 0.58$, $p = 0.05$, Figure 3A). A significant positive correlation was also observed with *IFNG* mRNA level in PBMC and interferon- γ (IFN- γ) expression level in grass pollen re-stimulated PBMC in the placebo group (Figure 3B).

Correlations between interleukin-13 (*IL13*), interleukin-6 (*IL6*) and tumor necrosis factor alpha induced protein 6 (*TNFAIP6*) expression and neutrophil, leucocyte and eosinophil cell counts in nasal fluids, IgE in serum, and cytokine expressions in grass pollen restimulated PMBCs were all non-significant (data not shown).

Discussion

Transcriptomics analyses have been widely used in human

tissues including some studies with PBMCs [15,16]. Persson H, et al. have recently demonstrated differential expression levels in therapy sensitive asthma patients versus treatment refractory asthma patients using peripheral blood leucocytes transcriptomic [17]. However blood PBMC is a mix of different cells that seems to be very well controlled for its overall gene expression [18]. Interestingly, a more recent study has shown that even when cells were isolated, proteomics emphasized only mild differences between allergic and non-allergic individuals [19]. It is thus not surprising retrospectively, that no differences were observed between PBMCs from probiotic treated allergen challenged allergic patients and PBMCs from placebo treated allergen challenged allergic patients. The intervention with *L. paracasei*-fermented milk may have influenced gene expression profiles but the effect was subtle resulting in a difficulty to separate real effects on the level of individual genes from random effects due to biological noise. It is possible that only a small subset of cells did respond to the probiotic treatment but the response may be diluted in the whole PBMC leading to an undetectable signal when looking at transcriptomic analysis of non-stimulated PBMCs. All together, these results suggest that the gene expression levels were highly stable in all individuals no matter the timing in the protocol or treatment at the time of the sampling. The negative correlation of *IL8* mRNA and IL-8 secretion level in the supernatant of grass pollen re-stimulated PBMCs suggests that PBMC mRNA level for *IL8* may inversely predict the responses in re-stimulated PBMCs in *L. paracasei*-fermented milk treated group. The discrepancy between the observed IL-8 results could be explained by the nature of the probiotics used. Depending on the physiological state of a given probiotic, specific mRNA may be specifically stabilized hence creating variations in effects after a probiotic treatment [12].

One should keep in mind that the results of the clinical trial have shown significant but mild decrease of clinical symptoms possibly explaining the lack of differences in our transcriptomic analysis. Another limitation of the study is the absence of results before the nasal provocation test (NPT) as the clinical trial was designed to assess clinical and molecular parameters (including gene expression in PBMCs) before and after probiotic treatment. As such the lack of PBMCs analysis before the NPT prevent analysis of the probiotic treatment efficacy on gene expression in PBMCs before challenge. Additionally the PBMCs were not stimulated *ex vivo* with the allergens, while this could have led to results better correlated to the cytokine expressions quantified after restimulation. Whalen K, et al. have shown that restimulated PBMC transcriptomic could separate healthy from allergic patients [20]. However the goal here was to assess baseline unstimulated gene expression changes that could be responsible for the differences observed *ex vivo* on cytokines secretion levels following *ex vivo* re-stimulation with the grass pollen extract.

Taken altogether, these results suggest that in the context of this probiotic intervention, the blood compartment and more specifically PBMC mRNA level is too stable to reflect the subtle changes of symptoms and alteration of cytokine expression triggered by a treatment with *L. paracasei*. The effect of a probiotic treatment on the systemic compartment may be too tenuous to reflect the clinical symptoms alterations.

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Declarations

Ethics approval and consent to participate

Ethical approval for the study was obtained from the Centre Hospitalier Universitaire Vaudois Ethics Committee. NCT01150253.

Consent for publication

All patients consented to participate to the study and gave their written approval and the study was performed in accordance with the principle of the Declaration of Helsinki.

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Authors' contributions

All authors approved the final manuscript. SN, RE, RA and AM participated in the study design or the conduct of the clinical trial. FS was principal investigator for the study. RE, CB and FR analyzed the transcriptomics profiles. TB analyzed the gene expression levels relevant to this publication and drafted the text. LG performed the correlation and the statistical analysis. CB led the writing of this manuscript.

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