# Examining immune-inflammatory mechanisms of probiotic supplementation in depression: secondary findings from a randomized clinical trial

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# Abstract

We recently indicated that four-week probiotic supplementation significantly reduced depression along with microbial and neural changes in people with depression. Here we further elucidated the biological modes of action underlying the beneficial clinical effects of probiotics by focusing on immune-inflammatory processes.

The analysis included a total of N=39 participants with depression, from which N=18 received the probiotic supplement and N=21 received a placebo over four weeks, in addition to treatment as usual. Blood and saliva were collected at baseline, at post-intervention (week 4) and follow-up (week 8) to assess immune-inflammatory markers (IL-1β, IL-6, CRP, MIF), gut-related hormones (ghrelin, leptin), and a stress marker (cortisol). Furthermore, transcriptomic analyses were conducted to identify differentially expressed genes (DEG). Finally, we analyzed the associations between probiotic-induced clinical and immune-inflammatory changes.

We observed a significant group x time interaction for the gut hormone ghrelin, indicative of an increase in the probiotics group. Additionally, the increase in ghrelin was correlated with the decrease in depressive symptoms in the probiotics group. Transcriptomic analyses identified 51 up- and 57 down-regulated genes, which were involved in functional pathways related to enhanced immune activity. We identified a probiotic-dependent upregulation of the genes ELANE, DEFA4 and OLFM4 associated to immune activation and ghrelin concentration.

These results underscore the potential of probiotic supplementation to produce biological meaningful changes in immune activation in patients with depression. Further large-scale mechanistic trials are warranted to validate and extend our understanding of immune-inflammatory measures as potential biomarkers for stratification and treatment response in depression.

Trial Registration: www.clinicaltrials.gov, identifier: NCT02957591.


# Introduction

With a global lifetime prevalence of approximately 25%, major depressive disorder (MDD), poses significant individual and societal burden [1,2]. Antidepressants bring symptom relief, but over 50% of cases do not respond to initial treatment, with one in three patients showing resistance to antidepressants [3]. The limitations of current treatments, in light of the global impact of MDD, underscore the urgent need for alternative treatment approaches. Probiotic interventions targeting the gut microbiota show promise in relieving depressive symptoms [4]. Multiple randomized controlled trials (RCTs) demonstrated a beneficial effect of probiotic supplementation on depressive symptoms in MDD patients [5–8], including our own trial [9]. However, a significant research gap remains in understanding the specific biological mechanisms underlying the beneficial clinical effect of probiotic supplementation in MDD.

Such mechanistic analyses aim to identify biological targets for patients' stratification and developing more efficient and tailored microbial interventions.

Probiotic supplements are formulations of living microorganisms that provide a health benefit through modulation of the microbiota. They are thought to positively impact mental health, via altering the microbiota gut brain (MGB) axis [10], a set of bi-directional communication pathways between the gut and the brain, including endocrine, immune and neurotransmitters systems [11]. Dysfunctions of the MGB axis, alongside compositional and functional (e.g., metabolomic and transcriptional) changes in gut microbiota, known as dysbiosis, have been detected as important factors in the pathology and treatment of depression [12]. In patients with MDD, gut dysbiosis is associated with a disrupted gut microenvironment, harming the protective functioning of the gut epithelium which leads to intestinal barrier dysfunction [13]. The damaged intestinal barrier (commonly referred to as "leaky gut") allows increased systemic translocation of gut metabolites, microbial cell components, or even the microbiota causing a range of negative consequences that have been implicated in the pathogenesis of depression [14–16].

Psychiatric disorders, including depression, exhibit a transdiagnostic pattern of gut microbial disarray, marked by a distinct pattern of depleted anti-inflammatory and enriched pro-inflammatory bacteria [17]. This pro-inflammatory microbial state is particularly noteworthy for depression, as chronic low-grade inflammation is a known pathologic feature of depression [18]. Approximately 1/3 of patients with depression have elevated immune-inflammatory markers [19], and a pro-inflammatory state is a common feature of no-responders to antidepressant medication [20,21]. In animal models, probiotics were found to counter gut microbiota perturbation by increasing beneficial bacteria and improving overall microbial diversity, causing a reduction in circulating immune-inflammatory markers [22,23]. But whether probiotics can produce similar reparative effects on dysbiosis and inflammatory mechanisms in patients with depression, and whether these changes can have antidepressant effects remains an area of limited exploration. A comprehensive meta-analysis on the impact of probiotic supplementation on immune-inflammatory markers in a clinical population did report reductions in a range of cytokines, including high sensitivity C-reactive protein (hs-CRP), and interleukin 6 (IL-6), while showing no effects for other markers including interleukin 1β (IL-1β) [24]. However, these implications are limited due to the diverse patient groups and their physiological states. In terms of immune-inflammatory mechanism of probiotics in depression only few RCTs assessed cytokines. Some studies found that probiotic supplementation reduces hs-CRP [8], and decreases IL-6 gene expression levels [25], although other studies reported no changes for these cytokines [26–28].

Hyperactivation of the hypothalamus–pituitary–adrenal (HPA) axis is a major factor linked to the pathophysiological development of depression and inflammation-related alterations in the gut microbiota [12]. Animal models of chronic psychosocial stress demonstrated that the composition of microbiota from mice exposed to stress was negatively affected alongside increased circulating levels of IL-6 [29]. Multiple studies in healthy humans corroborate the view that psychosocial stress reduces the overall diversity and

specifically the abundance of beneficial gut microbes [30]. A potential mechanism through which probiotics exert their anti-inflammatory effects involves the modulation of the gut hormone ghrelin. Increasing evidence suggests that changes in the gut microbiota composition alter ghrelin expression, secretion, activation and signaling [31]. Furthermore, ghrelin seems to interact with the HPA axis and immune-inflammatory markers. For instance, exposure to the trier social stress test increases subjective stress ratings and in some it also enhances serum cortisol in association with the secretion of ghrelin [32]. In turn, injections of ghrelin enhanced plasma cortisol in healthy, normal weight adults [33]. Moreover, in animal studies ghrelin was found to influence inflammatory pathways that are disturbed in depression. Specifically, ghrelin injection reduced pro-inflammatory cytokine secretion (i.e., IL-6) in rats [34]. This effect was attenuated after vagotomy, suggesting ghrelin down-regulates pro-inflammatory cytokines by activating the vagus nerve. Similarly, a study in humans found that ghrelin inhibits IL-6, IL-1β, and tumor necrosis factor-α secretion [35]. In sum, there is evidence for a direct influence of gut microbiota on systemic immune-inflammation processes, and bidirectional neuroendocrine regulation on the MGB axis seems to play a key role in this [36].

We recently performed an RCT investigating probiotic supplementation in individuals with depression [9]. Primarily, we found that the intervention alleviated depressive symptoms (d = 0.62) and positively affected the gut microbiota composition. Here, we present additional analyses that further uncover the biological mechanisms underlying the positive impact of probiotic supplementation on depressive symptoms. Specifically, we report serum concentrations of 1) immune-inflammatory cytokines (IL-1β, IL-6, CRP, MIF), and 2) gut-related hormones (ghrelin, leptin), 3) saliva concentrations of cortisol, 4) transcriptional (gene expression) changes, and 5) subjective appetite measures.

# Patients and Methods

This is a secondary analysis of a double-blinded RCT of probiotic supplementation in patients with depression (NCT02957591, www.clinicaltrials.gov). Clinical, microbial, and neural findings have previously been published [9,37,38], indicating beneficial effects of the probiotic intervention on depressive and cognitive symptoms, gut microbiota composition and fronto-limbic brain structure and function. Here we further explored probiotic effects on immune-inflammatory mechanisms.

Participants

Adult inpatients (n = 60; 18–65 years of age) with a current depressive episode (F31.3-F34 according to ICD-10 criteria) were recruited at the University Psychiatry Clinics (UPK) in Basel, Switzerland between March 2017, and January 2020. All participants met the criteria for a mild depressive episode, assessed with the Hamilton Depression Rating Scale (HAMD-17 [39]) score > 7 [40], and received treatment as usual (TAU) for depression (Supplementary Table 1). Exclusion criteria were psychiatric comorbidities (such as addiction, bipolar disorder, and schizophrenia), dietary restrictions, immunosuppressive treatment, or other acute somatic medical conditions. All participants provided written informed consent prior to the

initiation of the study, and the study was approved by the local ethics committee (Ethikkommission Nordwest- und Zentralschweiz).

## Study intervention

Participants received either a placebo or a probiotic supplement in addition to TAU over four weeks. The probiotic supplement (DSFormulation; Vivomixx®; Visbiome®) consisted of eight different bacterial strains: *Streptococcus thermophilus* NCIMB 30438, *Bifidobacterium breve* NCIMB 30441, *B. longum* NCIMB 30435 (Re-classified as *B. lactis*), *B. infantis* NCIMB 30436 (Re-classified as *B. lactis*), *Lactobacillus acidophilus* NCIMB 30442, *L. plantarum* NCIMB 30437, *L. paracasei* NCIMB 30439, and *L. delbrueckii subsp. bulgaricus* NCIMB 30440 (Re-classified as *L. helveticus*). The daily dose consisted of two sachets containing a high dose of 900 billion colony forming units (CFU)/day that could be mixed with any cold, non-carbonated drink. In the control group, participants received a placebo containing maltose and no bacteria which was indistinguishable in color, shape, size, smell, and taste from the probiotic supplement.

## Study design and procedure

Participants were randomly allocated to one of the two study arms and assessed at three time points: Week zero (baseline), week four (post-intervention) and week eight (follow-up). A standardized clinical assessment of depression (HAMD-17, Beck Depression Inventory-II (BDI-II) [41]) was conducted at all three time points. Blood samples and subjective measurements of appetite were obtained at all three time points, and saliva samples at baseline and post-intervention (see Supplementary Fig. 1). During the intervention period, all medication of the participants was registered (see Supplement) and a standardized diet containing stable amounts of fibers, starch and protein was provided. Fidelity to the protocol was assessed by the nursing personnel administering the intervention.

## Blood analysis of immune-inflammatory markers and gut hormones

Blood serum concentrations of immune-inflammatory markers (IL-1β, IL-6, macrophage inhibitory factor (MIF), and CRP) and gut-related hormones (ghrelin and leptin) were obtained. Blood samples were collected at 7 am after overnight fasting according to a standardized laboratory procedure using a serum Monovette® (Sarstedt; Nümbrecht, Germany) per manufacturer's protocol and stored at − 80°C up until further analysis. Analysis of immune-inflammatory markers and gut-related hormones was performed by an external laboratory (Labor Rothen AG, Basel, Switzerland). Quantitative CRP was determined in the laboratory using the CRP Latex reagent system on Beckman Coulter AU Analyzers, while the other immune-inflammatory markers and gut-related hormones were measured by the U-Plex® Metabolic Group 1 (Human) Multiplex Assays by Meso Scale Discovery®.

## Saliva cortisol analysis

The saliva concentration of the stress-hormone cortisol was obtained. Saliva samples were drawn at 9 pm before going to bed, and at the following morning at 7 am immediately upon awakening (S1), and after 10 min (S2), 20 min (S3), and 30 min (S4). A blue cap Salivette® (Sarstedt; Nümbrecht, Germany) with synthetic swab was employed to obtain the cortisol concentrations. Saliva samples were kept frozen at -80°C until analysis. A time-resolved fluorescence immunoassay was used to determine cortisol concentrations by the biochemical laboratory from the Department of Biological and Clinical Psychology at the University of Trier, Germany. The cortisol awakening response (CAR) of participants was computed as the inverted area under the curve (AUCi) of the morning cortisol concentrations S1 to S4 [44].

Analysis of subjective appetite ratings

Subjective measures of appetite-related sensations were assessed using a 10-point Likert Scale in the morning after overnight fasting in addition to the blood and saliva sampling. The Likert scale was anchored by two contrasting descriptors, "not at all" and "extremely" accompanied by four measures of appetite-related sensations (“hunger”, “desire to eat”, “feeling of fullness”, “satiety”).

Transcriptomic analysis

## RNA isolation, sequencing and quantification

Blood samples were collected at 7 am after overnight fasting according to a standardized laboratory procedure into a PAXgene tube (Qiagen; Hilden, Germany) per manufacturer’s protocol and stored at −80°C up until further analysis. RNA isolation (Quantification-OD measurement, Gel electrophoresis-integrity, RNA isolation PaxGene) has been conducted by Qiagen (Qiagen; Hilden, Germany). RNA sequencing and quantification was performed at the Lausanne Genomic Technology Facility. RNA-seq libraries were prepared from 250 ng of total RNA with the Illumina TruSeq Stranded mRNA reagents (Illumina) and the QIAseq FastSelect -Globin reagents (Qiagen; Hilden, Germany) for globin transcript depletion. Library preparation was performed on a Sciclone liquid handling robot (PerkinElmer; Waltham, Massachusetts, USA) with a PerkinElmer-developed automated script. Unique dual indexes were used for barcoding of the libraries. Libraries were quantified by a fluorometric method (QubIT, Life Technologies) and their quality assessed on a Fragment Analyzer (Agilent Technologies). Sequencing was performed on an Illumina NovaSeq 6000 for 300 cycles (paired end 150 nt reads). Sequencing data were demultiplexed using the bcl2fastq2 Conversion Software (version 2.20, Illumina). Low quality sequences and adapters were trimmed using Cutadapt (v. 2.5) [45]. Reads matching to ribosomal sequences were removed with fastq_screen (v. 0.11.1) [46] and low complexity reads were subsequently removed with reaper (v. 15–065) [47]. Reads were aligned against Homo sapiens genome (build GRCh38 and Ensembl version 102) using STAR (v. 2.5.3a) [48] and read counts per gene locus were summarized using htseq-count (v. 0.9.1) [49].

# Differential gene expression (DGE) analysis

For downstream analyses, libraries were filtered for minimal expression retaining only the genes with at least 1 count per million reads (CPM) in at least 10% of the samples.

Library size adjustment and differential gene expression analysis was done using the R package DESeq2 (v1.42.0) [50].

# Weighted gene correlation network analysis

Modules of co-expressed genes were generated using a weighted gene co-expression network analysis (WGCNA) approach [51]. WGCNA was performed on normalized, and variance-stabilizing transformed expression data measured at baseline or post-intervention from the participants who underwent transcriptional profiling. A signed hybrid network was constructed by calculating an adjacency matrix using Pearson correlation with pairwise complete observations and a soft-thresholding power of 6. A topological overlap matrix was computed from the adjacency matrix, converted to distances, and clustered by hierarchical clustering using average linkage clustering. Modules were identified by dynamic tree cut method with a minimum size of 20 and deepSplit value of 2. The expression profiles of the co-expressed genes in each module were summarized by calculating the first principal component of the corresponding expression matrix, referred to as the “module eigengene” (ME). Similar modules were merged using a ME distance of 0.2 as the threshold. MEs were also used to computationally relate modules to clinical traits. For each gene, irrespective of its original module assignment, we also defined a "module membership" (MM) by correlating its expression profile with the module eigengene of a given module.

# Statistical analysis

All analyses were conducted on a modified intention-to-treat (mITT) sample excluding non-compliant participants and drop-outs. The compliance rate cut-off of > 65% resulted in the exclusion of two patients per group from the study sample (for details see [9]). All analyses and visualizations were performed in R (v4.3.1). Unless otherwise specified, the significance level was set at $p < .05$, and multiple comparison adjustment for post-hoc comparison was performed.

# Effect of probiotics on blood/saliva markers and appetite measures

To reach quasi-normal distributions of blood and saliva measures, Tukey transformation with optimized lambda values was performed [52]. Additionally, outlier values were defined at 1.5 times the interquartile range (IQR) below the first quartile or above the third quartile and excluded from the final analysis. Linear mixed-effects models (LMM) were applied to assess the probiotic effect including the following fixed effects: Treatment-group (probiotics, placebo) as between factor, time (baseline, post-intervention, follow-up (if available)) as within factor, a group x time interaction. The LMM included a random effect for participant, to account for individual differences. To avoid confounding, sex, age, and body-mass-index (BMI), were additionally added as fixed effects in the model. An analysis of variance (ANOVA, type III) was

computed, and for significant main effects of group, time, and group x time interactions pairwise post-hoc multiple comparisons using estimated marginal means with t-tests were performed.

## Association between probiotics' effect on blood/saliva markers and depression

To explore the links between significant probiotic-induced changes on blood/saliva markers and depressive symptoms (HAMD-17), a partial correlation analysis was conducted for both treatment groups. Age, sex, and BMI were included as covariates. Fischer's z test was applied to compare correlations between treatment groups. Cook's Distance, with a cutoff of > 4/N, was used for bivariate outlier detection.

# Effect of probiotics on gene expression and functional enrichment analysis

To test the effect of probiotics over time, while controlling for random participant effects, we used DESeq2 to fit a negative binomial generalized linear model with the following design formula: expression ~ group + group:participant + group:time, where group was a two-levelled factor (placebo or probiotics) and time was a two-levelled factors (baseline or post-intervention). Separate models were fit to compare different time-points. Differentially expressed genes (DEGs) were identified using Wald's test $p < .05$ and |fold-change| > 1.5. REACTOME gene set enrichment analysis (GSEA) was performed on the entire lists of expressed genes pre-ranked by signed p-value as determined by Wald's test, using the "GSEA" function of the R package clusterProfiler (v4.10.0) [53]. The enrichment scores were normalized by gene set size, and their statistical significance was assessed by permutation tests (n = 1,000). Testing for over-representation of REACTOME pathways in gene modules was performed using hypergeometric test implemented in the "enricher" function from clusterProfiler. Genes with low module membership ($|MM| \leq 0.6$) were discarded from this analysis. Benjamini-Hochberg corrections were applied to functional enrichment p values to correct for multiple comparisons.

# Linking DEGs to probiotics' effect on biological and clinical measures

To evaluate the link between transcriptional changes and the effect of probiotics on biological and clinical measures, LMMs were applied including gene expression as fixed effect. The LMM was built and analyzed analogue to the description in the previous section: *Effect of probiotics on blood/saliva markers and appetite measures*, with the addition of a three-way interaction group x time x gene expression. The expression level (log transformed and normalized gene counts) of one gene at the time was included and only DEGs identified between probiotics and placebo groups, at post-intervention vs baseline, were analyzed. The same analysis was extended to gene modules by replacing individual gene counts with MEs as fixed effects.

# Results

Participant characteristics

The final study sample included 43 participants (mITT; see [9] for details). Blood samples of N = 40 (93%), saliva samples of N = 38 (88%), transcriptome data of N = 35 (81%), and appetite measures of N = 43 (100%) were available (Supplementary Fig. 1). Baseline characteristics of all participants are presented in Table 1. Group comparisons showed no significant differences in demographic characteristics, medication, and clinical measures at baseline except for HAMD-17 scores, which showed a higher score for the probiotics group ($W = 311$, $p < .05$). Blood, saliva, and appetite measures showed no differences between the study groups at baseline except for MIF, which showed a lower concentration for the probiotics group ($W = 250$, $p < .05$).

Blood analysis

# Immune-inflammatory markers

IL-1β and IL-6 levels did not show a significant main effect of group or time, nor a significant group x time interaction (Supplementary Table 2, Supplementary Fig. 2AB). MIF levels did not show a main effect of group, but a significant main effect of time ($F(2, 96) = 11.67$, $p < .001$), and a significant group x time interaction ($F(2, 96) = 4.66$, $p < .05$) was observed (Supplementary Table 2, Fig. 1A). Post-hoc tests demonstrated a significant increase in MIF levels from baseline to follow-up ($p_{Tukey} < .01$) and from post-intervention to follow-up ($p_{Tukey} < .001$) in the probiotics group, but not from baseline to post-intervention. No change was observed in the placebo group at any time point. CRP levels showed a main effect of group ($F(1, 34.75) = 9.57$, $p < .01$), but no main effect of time or group x time interaction was observed (Supplementary Table 2, Supplementary Fig. 2D). Post-hoc tests demonstrated higher CRP levels across all time points in the probiotics compared to the placebo group ($p_{Tukey} < .01$).

# Association between probiotics' effect on MIF and depression symptoms

The partial correlation between MIF and HAMD-17 change-scores ($x_{Follow\text{-}Up} - x_{Post\text{-}Intervention}$) did not demonstrate associations in the probiotics group ($r = 0.17$, $p = .64$) nor in the placebo group ($r = 0.23$, $p = .55$) (Fig. 1B).

# Gut-related hormones

Ghrelin levels did not show a main effect of group or time, but a significant group x time interaction ($F(2, 64.69) = 4.36$, $p < .05$) was observed (Supplementary Table 3, Supplementary Fig. 2E). Post-hoc tests demonstrated a significant increase of ghrelin levels from baseline to post-intervention ($p_{Tukey} < .05$) in the probiotics group, which was no longer observable at follow-up (Fig. 1C). No change was observed in the placebo group at any time point.

Leptin levels showed a significant main effect of time ($F(2, 58.77) = 4.18$, $p < .05$), but no main effect of group and no group x time interaction was observed (Supplementary Table 3, Supplementary Fig. 2F). Post-hoc tests demonstrated an increase of leptin levels from baseline to follow-up ($p_{Tukey} < .05$) across both groups.

# Association between probiotics' effect on ghrelin and depression symptoms

The partial correlation between ghrelin and HAMD-17 change-scores ($x_{Post\text{-}intervention} - x_{Baseline}$) demonstrated a significant negative correlation in the probiotics group ($r = -0.63$, $p < .05$) but not in the placebo group ($r = 0.35$, $p = .2$) (Fig. 1D). That is the higher the increase in ghrelin levels from baseline to post-intervention the stronger the decrease in HAMD-17 for the probiotics but not the placebo group.

Saliva cortisol

Evening cortisol concentrations (9 pm), waking cortisol concentrations (7 am) and the CAR did not show a significant main effect of group or time, nor a significant group x time interaction (Supplementary Table 4, Supplementary Fig. 3).

Subjective appetite ratings

The appetite sensations, hunger, satiety, fullness, and desire to eat did not show a significant main effect of group or time, nor a significant group x time interaction (Supplementary Table 5, Supplementary Fig. 4).

Transcriptomics analysis

# DGE and functional enrichment

From baseline to post-intervention, DGE analysis revealed the upregulation of 51 genes (fold change > 1.5) and downregulation of 57 genes (fold change < -1.5) (unadjusted $p < .05$) in the probiotics compared to the placebo group (Fig. 2AB). Gene set enrichment analysis (GSEA) of REACTOME pathways demonstrated the DGE to be a coordinated upregulation of genes involved in functional pathways of immune activation. Probiotic supplementation was associated with an upregulation of biological processes of "Neutrophil degranulation", "Antigen processing cross presentation", "Signaling by CSF3 (G-CSF)", "Antimicrobial peptides", "Negative regulation of NOTCH4 signaling", "Inactivation of CSF3 (G-CSF) signaling", all functionally associated to immune mechanisms (Fig. 3A). Analyses of overlapping DEG and enriched pathways identified the DEG (HBB, ELANE, DEFA4, OLFM4, KRT1) in "Neutrophil degranulation", and the DEG (ELANE, DEFA4) in "Antimicrobial peptides" (Fig. 3BC). The GSEA of downregulated genes revealed no functional coordination, as no association to consensus REACTOME pathways was obtained. The DGE from post-intervention to follow-up demonstrated no upregulation of genes functionally associated to immune activity, indicating a transient and immediate effect of probiotic supplementation on DGE related to immune activation (Fig. 3A). Changes in multiple DEGs were

significantly linked to changes in ghrelin levels in the probiotics compared to the placebo groups from baseline to post-intervention (Fig. 4A). For two DEGs (TREML1 and ELANE) the change score ($x_{Post\text{-}intervention} - x_{Baseline}$) demonstrated a significant partial correlation to the ghrelin change score ($x_{Post\text{-}intervention} - x_{Baseline}$) in the probiotics group (Fig. 4B).

Weighted gene correlation network analysis

To capture correlation pattern among genes in response to the probiotic intervention, we grouped co-expressed genes into 37 modules using a network-based approach [51]. We found that the Module 24 (M24) exhibited the highest degree of overlap to DEGs, containing 47 (8.5%) upregulated DEGs (Fig. 5B). The REACTOME over-representation analysis of module genes found M24 to be enriched for functional pathways which we already identified as upregulated in the DGE analysis ("Neutrophil degranulation" and "Antimicrobial peptides") (Fig. 5D). This indicates that the module M24 recapitulates probiotics-specific transcriptional changes on immune activity. Interestingly, we also found that the interaction group x time x ME (module eigengene) of M24 was significantly associated to ghrelin (Fig. 5CE).

# Discussion

We previously reported a beneficial impact of a multi-strain probiotic intervention on both depressive symptoms and gut microbiota composition in individuals with depression receiving TAU [9]. Following this, the current secondary analysis focusing on immune-inflammatory mechanisms of probiotics revealed three key outcomes: Firstly, probiotics exhibited a significant, albeit transient, increase in circulating levels of ghrelin over the intervention period. This effect was associated with the improvement in depressive symptoms during the intervention phase. Secondly, probiotics showed no immediate effect on pro-inflammatory cytokines, cortisol concentrations, and leptin. However, MIF levels showed a significant increase at follow-up in the probiotic group. Thirdly, probiotics induced changes in gene expression patterns functionally associated with the immune system. Similar to the elevation of circulating ghrelin, the transcriptional changes were only evident during the intervention period and no longer evident at follow-up.

The association between the probiotic-induced increase of ghrelin levels and decrease of depressive symptoms adds to the existing literature suggesting antidepressant effects of ghrelin [54,55]. Multiple preclinical studies reported antidepressant-like properties of ghrelin in rodents [56–60]. Clinical data show a more mixed picture of ghrelin's effect on depressive symptoms [61,62]. There is evidence for improved depressive symptoms following ghrelin administration in a study on patients with MDD [63], while another study reported an association between higher severity of depressive symptoms and increased ghrelin concentrations in patients with depression [64]. However, other studies reported no such association in patients with depression [65–67]. In addition, compared to healthy individuals, in patients with depression higher [68–70], lower [71] and comparable [67,72,73] ghrelin concentrations were reported, indicating variability

amongst patients with depression in terms of ghrelin concentrations. In this study, the obtained increase in ghrelin levels was due to the intervention effect of a multi-strain probiotic over four weeks, which returned to baseline concentrations at follow-up four weeks after the intervention was completed. The finding of higher ghrelin concentrations following probiotic supplementation in patients with depression is in line with preclinical results reporting higher ghrelin gene expression in mice treated with the same multi-strain probiotic [74]. Furthermore, mice treated with the probiotic were found to have increased numbers of ghrelin secreting cells in the mouse intestine, alongside higher numbers of cells shielding the gastric epithelium [74]. In addition, probiotics (*Lactobacillus* spp.) were reported to enhance ghrelin gene expression and ghrelin secretion in other animal studies [75,76]. These findings stand in contrast to another animal study reporting reduced ghrelin concentrations after administering the same multi-strain probiotic we used [77]. However, none of these preclinical findings were obtained using an animal model of depression, limiting the translation to a clinical population of patients with depression. Nonetheless, in accordance with our initial finding of increased abundances of the genus *Lactobacillus* following the probiotic supplementations [9], most animal studies demonstrated that probiotics (*Lactobacillus* spp.) have the potential to enhance ghrelin gene expression and secretion.

We found no treatment effect over time of probiotic supplementation on all immune-inflammatory blood markers except for MIF, for which we obtained a significant increase at follow-up. The lack of effects at post-intervention is in line with other clinical trials in patients with depression, which did not find the proposed anti-inflammatory properties of probiotics reported in animal models [78,79]. In accordance with our results, no changes in IL-1β and IL-6 levels, were reported in a human study administering the same multi-strain probiotic supplement we used and in a recent meta-analysis evaluating probiotics trials [80,81]. However, while most results point towards no changes in immune-inflammatory markers, two trials using the identical probiotic supplementation reported decreased hs-CRP levels in patients with depression [8], and reduced IL-6 levels in patients with multiple sclerosis [82]. These mixed findings on probiotic supplement effects on immune-inflammatory markers might be due to the variability in used probiotic strains and targeted clinical populations [83]. Some probiotics may exhibit anti-inflammatory properties, while others might function as immune stimulants, thereby enhancing physiological inflammation [84]. This may explain our probiotic-induced increase in MIF concentrations at follow-up. MIF acts as a regulator of the innate immune activity [85]. Thus, the increase in MIF is likely due to the enhancement of macrophage activation through administration of genus *Lactobacillus* strains in the probiotic supplement [86].

Gene expression analysis results indicated transcriptional changes in the probiotic relative to the placebo group during the intervention. Adding to the growing number of studies assessing the effects of probiotics at the gene expression level [87], we obtained 108 DEG after the multi-strain probiotic intervention. In patients with depression, only one other study assessed gene expression effects after probiotic administration [88]. Using a different multi-strain probiotic, this study reported higher IL-6 gene expression after the intervention [88]. In addition, other clinical studies reported anti-inflammation related

gene expression changes following probiotic supplementation in neurodegenerative disorders [89,90]. Here, we found effects on DGE functionally related to pathways of immune activation rather than anti-inflammatory processes. This difference between probiotic supplements in altering gene expression is in line with the literature, suggesting different biological mechanisms of action for different probiotic strains [91]. Our results indicate that neutrophil-associated immune activation was functionally most clearly associated to the DGE. This finding is in accordance with preclinical research showing that the same multi-strain probiotic that we used activates the epithelial innate immune system [84]. The authors of the study propose that “physiologic inflammation” induced by probiotics is beneficial, both for the defense against adverse gut-bacteria and for supporting the innate immune system [84]. This boost of immune activity induced by probiotic supplementation via immunoregulatory functions is well documented [92].

We identified functionally and biologically relevant probiotic induced changes in gene expression by computing a WGCNA which we overlapped to DEGs. We identified M24, which not only captured the upregulation to immune activity but was also significantly associated to probiotics induced ghrelin changes over time. M24 contains the DEGs Olfactomedin 4 (OLFM4) an intestinal glycoprotein, Elastase (ELANE) a neutrophil serine protease and Defensin Alpha 4 (DEFA4) an antimicrobial peptide. Interestingly, expression of OLFM4 and DEFA4 was previously associated with host defense in the mature intestine [93], and recently found to be increased following probiotic supplementation of the genus *Lactobacillus* in mice [94]. Both OLFM4 and DEFA4 are important contributors to gastrointestinal defense mechanisms. While OLFM4 is part of the gastrointestinal mucosal surface and has a potential role in epithelial defense [95], DEFA4 is involved in killing Gram-negative bacteria, which are associated with gut dysbiosis [96].

This study had several strengths and limitations that necessitate careful consideration. A key strength lies in the systematic and comprehensive examination of diverse biological parameters associated with the mechanisms of the MGB axis involved in depression. By employing a combination of transcriptomics and blood concentration analyses, our methodology facilitated an in-depth exploration of the biological underpinnings relevant to the reported antidepressant mechanisms of probiotics. Notwithstanding these strengths, it should be noted that sample size was small, which limits the generalizability of our findings, particularly in light of the clinical and physiological heterogeneity of depression. Moreover, despite our efforts to record and control for medication effects, we cannot definitively ascertain whether the observed effects are specific to interactions with certain antidepressants.

In conclusion, the biological mechanisms of add-on multi-strain probiotic supplementation in patients with depression were linked to the gut hormone ghrelin and the upregulation of genes of immune activation. Higher ghrelin levels after probiotic supplementation were furthermore related to improved depressive symptoms, hinting at a potential link between ghrelin secretion and antidepressant mechanisms. These findings emphasize probiotics’ biological mechanism of action as promoting immune activation ultimately associated to symptom relief in patients with depression. Moving forward, our results warrant replication in large-scale mechanistic trials of probiotic supplementation to test the

potential of immune-inflammatory measures as stratification and treatment response biomarkers in depression. This could pave the way for more targeted and personalized approaches in the treatment of depression.

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AS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. LM, UEL, and AS designed the study. LS, JPKD, VL, ACS, CK, LM, NS and AS contributed to the acquisition, analysis, or interpretation of data. Statistical analyses of blood/saliva concentration and appetite measures were performed by LS and VL. Transcriptomic analyses and interpretations were performed by FM and MI from the Vital-IT Competence Center in Bioinformatics, SIB Swiss Institute of Bioinformatics, University of Lausanne. Administrative, technical, or material support was provided by LM, AS, and UEL. LS and AS drafted the manuscript. All authors critically reviewed the article and approved the final manuscript.

# Declarations

Acknowledgments and disclosures

The study was supported by the Gertrud Thalmann Foundation of the University Psychiatric Clinics (UPK) Basel (AS, UEL), the Kämpf-Bötschi Foundation (UEL), the research fund junior researchers from University of Basel (Appln 3MS1041, AS), the research fund of the UPK Basel (AS) and the Stiftung zur Förderung der gastroenterologischen und allgemeinen klinischen Forschung sowie der medizinischen Bildauswertung (AS). MENDES S.A., Switzerland, supplied the investigational medicinal product. The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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Swiss Institute of Bioinformatics, University of Lausanne. Administrative, technical, or material support was provided by LM, AS, and UEL. LS and AS drafted the manuscript. All authors critically reviewed the article and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest and no biomedical financial interests.

Availability of data

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary information

Supplementary information is available at TP's website.

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# Tables

**Table 1.** Demographics, clinical characteristics, and secondary measures at baseline.

| | Probiotics group | Placebo group | Group comparison |
|---|---|---|---|
| **Demographic** | **(n=19)** | **(n=24)** | |
| Age | 39.21 (11.53) | 38.04 (10.24) | $\chi^2(1)=1.60$, *p=0.21* |
| Sex, n (%; female) | 14 (74) | 12 (50) | W=238.5, *p=0.81* |
| BMI | 23.83 (3.66) | 25.13 (4.01) | W=177, *p=0.30* |
| Compliance Rate | 87 (8.44) | 88 (8.17) | W=186, *p=0.84* |
| **Depression Severity** | | | |
| HAMD-17 | 19.13 (4.89) | 16.5 (4.18) | W=311, *p=0.04* |
| BDI-II | 21.53 (7.59) | 22.31 (9.94) | W=218.5, *p=0.96* |
| **Medication (DDD)** | | | |
| Antidepressant equivalents | 1.86 (1.30) | 1.82 (1.12) | W=227, *p=0.99* |
| Antipsychotic equivalents | 0.33 (0.71) | 0.24 (0.31) | W=241, *p=0.76* |
| **Clinical Measures** | | | |
| N of Hospitalizations | 2.29 (1.48) | 1.85 (1.23) | W=210.5, *p=0.32* |
| STAI 1 | 49 (14.11) | 51.83 (10.61) | W=191, *p=0.68* |
| GSRS | 28.16 (9.65) | 29.96 (12.79) | W=211.5, *p=0.87* |
| **Blood Measures** | | | |
| **Immune-Inflammatory Markers** | **(n=18)** | **(n=22)** | |
| IL-1b[a] | 0.002 (0.003) | 0.001 (0.001) | *W*=73.5, *p*=0.49 |
| IL-6[a] | 0.098 (0.076) | 0.104 (0.081) | *W*=166, *p*=0.86 |
| MIF[a] | 169.15 (88.84) | 251.19 (106.21) | *W*=250, *p*=0.01 |
| CRP[b] | 1.78 (1.36) | 1.11 (0.82) | *W*=110, *p*=0.08 |
| **Gut-Related Hormones** | | | |
| Ghrelin[a] | 7.92 (6.26) | 8.43 (5.99) | *W*=191, *p*=0.72 |
| Leptin[b] | 0.014 (0.013) | 0.01 (0.011) | *W*=103, *p*=0.24 |
| **Saliva Measures** | | | |
| **Cortisol** | **(n=19)** | **(n=24)** | |
| Evening Cortisol[c] | 1.03 (1.09) | 1 (0.37) | *W*=136, *p*=0.80 |

| | | | |
|---|---|---|---|
| Waking Cortisol[c] | 6.55 (3.72) | 8.05 (4.36) | *W*=120, *p*=0.41 |
| CAR[c] | 7.09 (6.86) | 7.99 (5.71) | *W*=145, *p*=0.99 |
| **Appetite Measures** | **(n=19)** | **(n=24)** | |
| Satiety | 4.76 (2.73) | 4.55 (2.72) | *W*=164, *p*=0.87 |
| Hunger | 4.29 (2.28) | 5 (2.64) | *W*=193.5, *p*=0.48 |
| Feeling of fullness | 2.35 (1.93) | 3.35 (2.62) | *W*=207, *p*=0.23 |
| Desire to eat | 3.82 (2.65) | 5.05 (2.91) | *W*=212.5, *p*=0.20 |

Measures are presented as mean (SD) unless stated otherwise; BMI body mass index, HAMD-17 Hamilton Rating Scale for Depression 17-item, BDI-II Beck Depression Inventory 2nd edition, DDD defined daily dose, STAI 1 State-Trait Anxiety Inventory 1, GSRS Gastrointestinal Symptom Rating Scale, IL Interleukin, MIF Macrophage Inhibitory Factor, CRP C-reactive Protein, CAR Cortisol Awakening Response; [a] in pg/ml, [b] in mg/l, [c] in nmol/l.

## Supplementary Files

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