# Serum neurofilament light chain in response to probiotics in bi-center, double-blind, randomized, placebo-controlled clinical trial (CleverAge Biota)

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**Background and Aims.** Neurodegenerative disorders affecting the brain and spinal cord are caused by a large number of factors. More recently, imbalances in gut microbiota are found to be one factor linked directly to neurological dysfunction. Probiotics prevent cognitive decline. For the first time, the effect of probiotics was assessed by monitoring the concentrations of the neurodegeneration biomarker neurofilament light chains (NfL) in a well-defined group of community-dwelling individuals. The aim of this study was to determine whether administration of our new probiotics could reduce NfL concentrations.
**Methods.** The serum NfL concentrations were measured in total of 190 serum samples of 85 older community-dwelling individuals. The participants were randomly divided into two groups: the PROPLA group and the PLAPRO group. Individuals in the PROPLA group started with a three-month use of probiotics and continued with a three-month use of placebo while the order was reversed in the PLAPRO group. The participants underwent detailed examinations at three time points: at baseline, in three and six months. The serum NfL concentrations were determined using ultrasensitive single-molecule array (SIMOA) assay.
**Results.** Longitudinal comparisons of NfL concentrations between samplings at different time points in the PROPLA and PLAPRO groups showed no statistically significant differences. Baseline NfL concentrations at the beginning of the study and in the succeeding samplings were not significantly different for the two groups in cross-sectional comparisons.
**Conclusions.** Serum NfL concentrations were not influenced by the three-month use of probiotics.

**Key words:** serum, neurofilament light chain, probiotics, gastrointestinal microbiome, cognition

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## INTRODUCTION

Probiotics appear to be promising therapeutic agents that can favorably modulate the imbalance in the intestinal microflora in various disorders affecting the gastrointestinal tract and skin and immune system, among others[1,2]. The positive effects of probiotics have been also proven in neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's diseases (PD) or multiple sclerosis[2]. There are assumptions that modification of the intestinal flora by probiotics, prebiotics, and synbiotics could improve cognitive function or show other positive effects[3-5].

Some studies report that the administration of probiotics acts through the amelioration of some general mechanisms involved in the pathogenesis of many diseases, including inflammation or oxidative stress, which can be monitored by appropriate biomarkers. A recent meta-analysis suggested that probiotic supplementation can improve cognition in individuals with AD or mild cognitive impairment and decrease inflammatory and oxidative biomarkers such as high-sensitivity C-reactive protein and malondialdehyde[6], while other meta-analyses[7,8] have not shown a significant improvement in cognition in the elderly or those with AD. However, the current knowledge about the administration of probiotics does not yet allow us to draw clear conclusions about their effects on cognitive function. Another meta-analysis showed reduced serum concentrations of some inflammatory cytokines[9].

Inflammation, oxidative stress with other mechanisms may participate in the development of neurodegeneration, which is the main pathologic feature in many important neurological diseases[10]. Neurodegeneration can be assessed by monitoring the concentrations of neurocytoskeletal components specific to nervous tissue such as neurofilament light chains (NfL) or antibodies against them as were explored by us and others[11-24].

In the present study, we focus on the effect of human probiotics on serum NfL concentrations in older community-dwelling individuals. Previous studies have used cognitive tests or biomarkers of inflammation and oxidative stress to evaluate the effect of probiotics[6,9,25]. To our knowledge, no studies have investigated serum NfL in older community-dwelling individuals before and after probiotic administration. We hypothesized that administration of our new probiotics can reduce NfL concentrations.

## MATERIALS AND METHODS

### Study design

This study presents results of our randomized clinical trial regarding the effect of human probiotics on serum NfL following our study protocol registered at clinical trial registration: clinicaltrials.gov, identifier (NCT05051501). The design and a protocol of the study was recently reported in detail[26]. Here we only briefly mention the key data about the study design, participants, and methods in the following sections.

CleverAge Biota study was a randomized double-blind placebo-controlled trial with a cross-over design. The study was carried out at two centres – the University Hospital Kralovske Vinohrady (UHKV), Charles University, Third Faculty of Medicine, Prague and at the National Institute of Mental Health (NIMH) in Klecany near Prague, Czech Republic from January 2021 to April 2022.

### Participants

We recruited older community-dwelling individuals. The inclusion criteria were age between 55–80 years, Czech as a native language, preserved activities of daily living in a community and good sight and hearing. Participants were excluded from the study if they had any of the following diseases or conditions currently or in the past: a disease of the digestive tract (celiac disease, Crohn's disease etc.), a neurological disease of the brain (epilepsy, major head injury, stroke, brain operation, brain tumor etc.), a psychiatric disease or treatment (schizophrenia, bipolar disorder, drug addiction, alcoholism etc.), organ failure (heart, kidney etc.), an oncological disease in the last five years or are after chemotherapy or radiotherapy, an immune-mediated disease, an operation in general anaesthesia in the last three months, use of cognitive enhancers, and use of antibiotics or another probiotic supplements three months prior to the start of the study. Depression was not an exclusion criterion. Participants were recruited and selected using an online form with all inclusion and exclusion criteria and using a short online memory test named ALBAV to increase chances for identifying those with memory impairment prior to the trial onset. More information about the memory test ALBAV is available in our previous reports of this clinical trial[25-27,29]. Cognitive functions of the participants were normal in the majority (approximately 75%) or were mildly impaired.

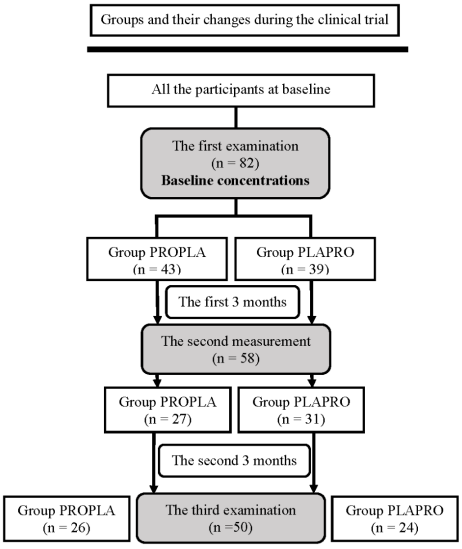


**Fig. 1.** Timeline of serum neurofilament light (NfL) measurements in groups PROPLA and PLAPRO.

A total of 91 persons were included in the study. The participants were randomly divided into two groups: the PROPLA group and the PLAPRO group. Individuals in the PROPLA group started with a three-month use of probiotics (PRO) and continued with a three-month use of placebo (PLA). The order of administration of the probiotic in the PLAPRO group was reversed. At baseline, both groups (PROPLA and PLAPRO groups) did not differ in scores of brief and neuropsychological tests. The participants underwent detailed examinations at three time points: at baseline, in 3 and 6 months[26].

### Probiotic intervention

A single dose of probiotic (one tablet) was composed of 106 colony forming units of human Streptococcus thermophilus GH, Streptococcus salivarius GH NEXARS, Lactobacillus plantarum GH and Pediococcus pentosaceus GH. Probiotics were prepared using human-stemmed lines. Each participant received one tablet of probiotics together with two fibre tablets per day for 3 months either in months 1–3 of the study (PROPLA group) or in months 4–6 of the study (PLAPRO group). The participants received placebo tablets during the period when they did not take the probiotics. The placebo tablets did not visually and tastefully differ from probiotic supplements. Probiotic supplements, prebiotics and placebo tablets were produced in NEXARS (Brno, Czech Republic). Both participants and administrators were blinded to the type and the order of intervention and placebo.

### Blood sampling and NfL measurement

Fasting blood was collected between 7 and 9 in the morning prior to cognitive testing and other evaluations on the same day. Blood was immediately centrifuged at 4,000 rpm for 10 min and supernatant was divided into Eppendorf tubes and stored at −80 °C till the analysis. The serum NfL concentrations were measured in 85 participants in total of 190 serum samples. One to three measurements of serum NfL concentrations were performed in individual participants (1st examination – 82 participants, 2nd examination – 58 participants, 3rd examination – 50 participants). The first examination of NfL represented the baseline NfL levels for both groups (PROPLA group and PLAPRO group), i.e., prior to any intervention meaning natural individual concentrations. The next time points for NfL sampling were after 3 and 6 months of the study. Fig. 1 shows an overview and flowchart of the study.

The serum NfL concentrations were determined using ultrasensitive single molecule array (SIMOA) assay on platform SR-X. We used a Simoa NF-Light Advantage Kits (Quanterix Corporation, Billerica, MA) according to the manufacturer's instructions.

**Ethics Statements**

The study conforms with World Medical Association Declaration of Helsinki. All participants sign the informed consent. The study was approved by the Ethics Committees of NIMH Klecany in 2020 (No.78 and 165/20) and UHKV Prague in 2020 (No EK-VP 17/0 and 1/2020).

**Statistical analyses**

MedCalc® Statistical Software version 20.008 (MedCalc Software Ltd, Ostend, Belgium) was used for statistical analysis.

Data are presented as means with standard deviations, percentages or medians with percentiles. The Chi-squared test was used to compare categorical data. The Mann-Whitney test for independent samples was used to compare differences between two groups and the Wilcoxon test for paired samples for comparisons of NfL concentration in the same participant in longitudinal evaluation. The Spearman rank correlation and multiple regression were used for the evaluation of relationships between variables. A level of $P<0.05$ was considered statistically significant.

## RESULTS

**Participants' characteristics**

Participants' characteristics as the whole group and in separated groups PROPLA and PLAPRO are shown in Table 1. The groups did not differ in age, gender, BMI, and years of education.

**Baseline serum NfL concentrations**

First, we evaluated NfL concentrations in serum samples taken at the beginning of the study prior the administration of probiotic or placebo tablets.

NfL serum concentrations obtained from the total group of participants at the beginning of the study were used to determine baseline concentrations in older community-dwelling individuals (Fig. 2, Table 2). Baseline NfL values in our group did not exceed the 95th percentile of the reference range determined by Vermunt et al.[28] except three values. Comparisons of the upper limits of the baseline concentration (90th and 95th percentiles) with those of the reference range[28] showed minimal differences only 0.7 pg/mL (90th percentile) or 1.7 pg/mL (95th percentile) on average.

In contrast to age ($P=0.0007$), univariate and multivariate regression analyses did not reveal the influence of sex, BMI and years of education on serum NfL concentrations.

**Comparisons of serum NfL concentrations at different time points depending on probiotic administration**

We compared serum NfL concentrations separately in individual groups between baseline concentrations and after three months of administration of probiotics (PROPLA group) or placebo (PLAPRO group) (Fig. 3A). The other comparison focused on changes in NfL concentrations in the next three months between the second sampling and the third one when the administration of probiotics and placebo was opposite in each group (Fig. 3B). Longitudinal comparisons of NfL concentrations between samplings at different time points in the PROPLA and PLAPRO groups did not show statistically significant differences.

We also compared NfL concentrations at individual time points between the two groups. Baseline NfL concentrations at the beginning of the study as well as those in the following samplings were not different between the two groups in cross-sectional comparisons (Fig. 4A, B, C).

## DISCUSSION

Our study evaluated serum NfL concentrations before and after three months of probiotic administration. The results suggest that there were no changes in NfL concentrations after administration of our original probiotics to community-dwelling elderly people. In addition, we did not show statistically significant differences between the probiotic group and the group without intervention in cross-sectional comparisons. This biological report is in accordance with similar outcomes as our previous paper on the same cohort showing no effect on cognitive functions and mood[29].

The baseline concentrations of NfL in our study obtained before probiotic administration closely matched

**Table 1.** Participants' characteristics at baseline and a comparison of two subgroups.

| | Total (n=85) | Group PROPLA (n=45) | Group PLAPRO (n=40) | *P* |
|---|---|---|---|---|
| Gender (%) | | | | n.s. |
| Male | 34 (40) | 17 (20) | 17 (20) | |
| Female | 51 (60) | 28 (33) | 23 (27) | |
| Age (y) | 70 (66 to 75) | 69 (63 to 74) | 72 (67 to 76) | n.s. |
| | 70±7 | 69±1 | 71±6 | |
| Education (years of schooling) (y) | 17 (13 to 18) | 18 (13 to 18) | 17 (14 to 18) | n.s. |
| | 17±3 | 17±3 | 17±3 | |

Unless otherwise indicated, data reported as median and interquartile range and as a mean±mean standard deviations.
Group PROPLA, a group starting with probiotic period followed by placebo one; group PLAPRO, a group starting with placebo period followed by probiotic one; y, year; *P*, statistical significance.

**Table 2.** The age-related serum NfL concentrations in older community-dwelling individuals at baseline (n = 82).

| Age (y) | Percentiles NfL(pg/mL) | | | |
|---|---|---|---|---|
| | 5th | 10th | 90th | 95th |
| 55 | 6.0 | 6.6 | 13.2 | 14.7 |
| 60 | 6.8 | 7.5 | 15.5 | 17.4 |
| 65 | 7.7 | 8.5 | 18.2 | 20.6 |
| 70 | 8.9 | 9.8 | 21.7 | 24.7 |
| 75 | 10.2 | 11.3 | 26.0 | 29.8 |
| 80 | 11.8 | 13.1 | 31.6 | 36.4 |

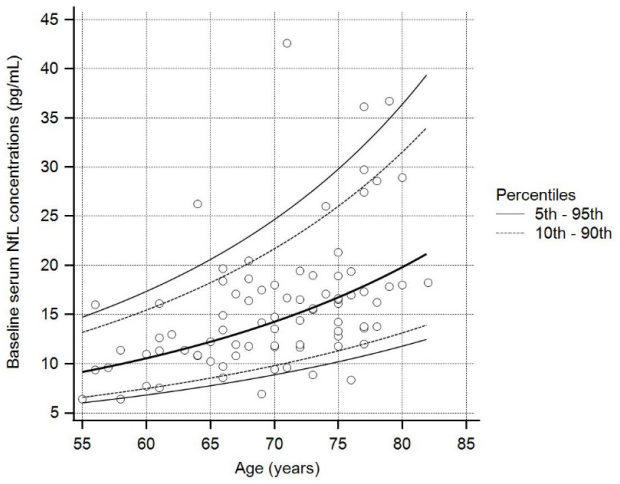


**Fig. 2.** The age-related baseline serum NfL concentrations in older community-dwelling individuals.

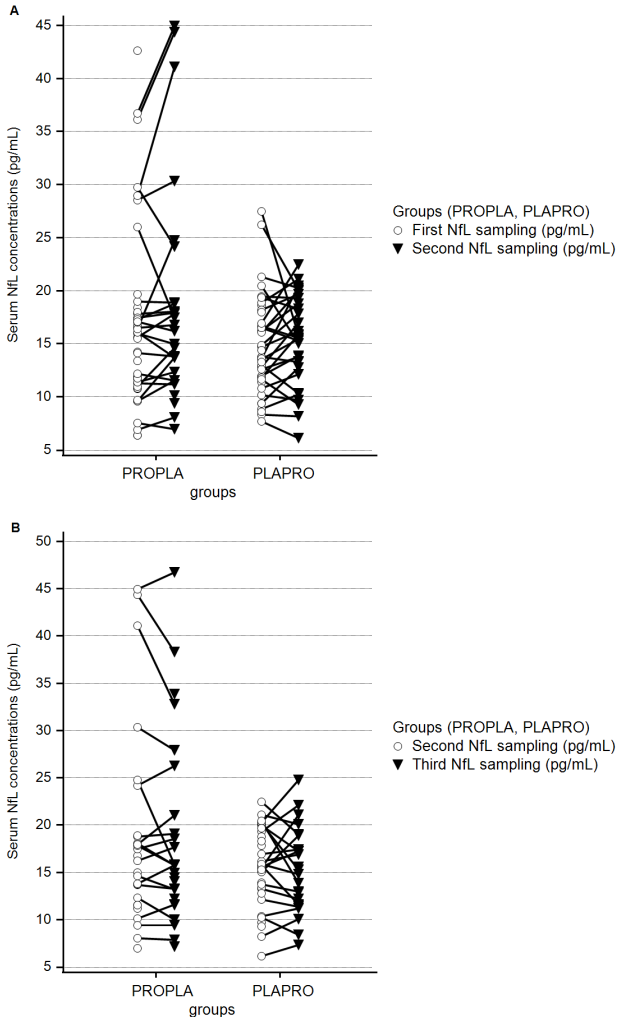


**Fig. 3.** Longitudinal comparisons of serum NfL concentrations between different time points in the groups PROPLA and PLAPRO: **A.** Comparisons of serum NfL concentrations between the beginning of the study (1st sampling) and after three months (2nd sampling) in the group PROPLA and the group PLAPRO; **B.** Comparisons of serum NfL concentrations between sampling after the three months (2nd sampling) and after six months of the trial (3rd sampling) in the group PROPLA and the group PLAPRO
Group PROPLA, a group starting with probiotic period followed by placebo one; group PLAPRO, a group starting with placebo period followed by probiotic one; NfL, neurofilament light chains.

the recently published reference range for controls using the same methodology for NfL analyses. Age-dependent upper limits of baseline serum NfL concentrations (90th and 95th percentiles) determined from our values differed minimally from those of Vermunt et al.[28].

Increased concentrations of neurofilaments in cerebrospinal fluid and blood (serum, plasma) reflect an injury of the neuronal axons caused by various acute or chronic processes such as inflammation, neurodegeneration, or trauma[13,14,17,20,22,30-40]. However, axonal degeneration also occurs as a consequence of normal aging[34]. This is reflected in the increase in NfL concentrations in biological fluids with increasing age in healthy individuals[34]. However, the molecular basis of the transition to development to neurodegeneration is not fully understood[34]. A more pronounced increase in NfL concentrations is evident, especially in those older than the age of 60 years[33]. We also observed a similar trend with increasing concentrations especially in participants aged 60 years and more in our study. Baseline serum NfL concentrations with only three exceptions corresponded to the recently published reference range[28]. Our participants were independently living individuals without clear manifestation of brain disorders. It may indicate that the degree of neurodegeneration did not exceed an age-appropriate one. In agreement with some studies, we did not show differences in serum NfL concentrations between men and women[30].

Some studies focused on the neuroprotective role of the gut microbiota in different neurodegenerative diseases in models of human or animal diseases reported various positive effects[2,41]. At the beginning of the study NfL concentrations of our participants corresponded to the recently published reference values[28]. Therefore, we did not confirm a reduction in NfL concentrations in the reference range. Furthermore, the three-month period of administration of probiotics may not have been sufficient to show their effects.

The group of persons who started the study with placebo administration allowed us to evaluate the rate of axo-

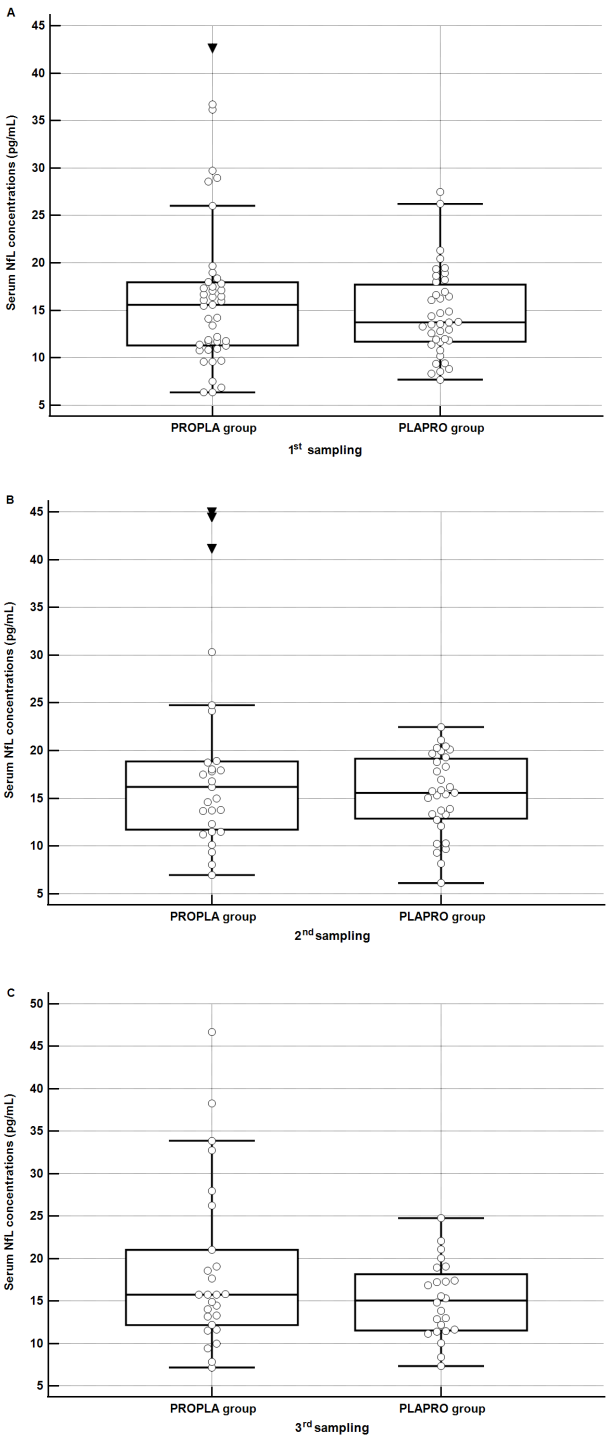


**Fig. 4.** Cross-sectional comparisons of serum NfL concentrations between the groups PROPLA and PLAPRO at different timepoints: **A.** The first baseline serum NfL concentrations in the group PROPLA and the group PLAPRO at the beginning of the study (1st sampling); **B.** The second serum NfL concentrations in the group A (PROPLA) and the group PLAPRO after three months (2nd sampling); **C.** The third serum NfL concentrations in the group PROPLA and the group PLAPRO after six months (3rd sampling). No differences were found.
Group PROPLA, a group starting with probiotic period followed by placebo one; group PLAPRO, a group starting with placebo period followed by probiotic one; NfL, neurofilament light chains.

nal degeneration in community-dwelling older individuals. The unchanged NfL concentrations in the placebo group could indicate that the participants did not experience axonal damage associated with increased NfL concentrations during the three-month follow-up.

The strength of the study is the first attempt to monitor probiotic effects using neuron-specific protein. Our study used an intervention with an original formulation of probiotics. For the first time, the effect of probiotics was assessed by monitoring the concentrations of the neurodegeneration biomarker NfL. We monitored the process of age-related damage to axons in a well-defined group of community-dwelling individuals.

A limitation of the study is the lack of brain neuroimaging. Longitudinal and cross-sectional effects of probiotics were not studied in patients with different degrees of cognitive deficits because our main aim was to restore and maintain functioning of the common older people without apparent mental disorders.

## CONCLUSION

We found that a three-month intervention with original probiotics was not reflected in a reduction of serum NfL concentrations. Monitoring serum NfL concentrations did not show significant axonal degeneration at a three-month interval in community-dwelling elderly without probiotic intervention.

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**Conflict of interest statement:** The authors state that there are no conflicts of interest regarding the publication of this article.

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