Thesis

PROVIT-CLOCK - A potential influence of probiotics and Vitamin B7 add-on treatment and metabolites on clock gene expression in major depression

submitted by

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Graz, 04.09.2024

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 04.09.2024 *Kathrin Kreuzer m.p.*

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Zusammenfassung

Immer mehr Publikationen deuten auf einen engen Zusammenhang zwischen Darmgesundheit und psychischem Wohlbefinden hin. In letzter Zeit wurde speziell über die beeinflussende Rolle von Butyrat-produzierenden Bakterien und der Schlafqualität diskutiert. Die PROVIT-Studie, eine randomisierte, doppelblinde, vierwöchige, probiotische Interventionsstudie, zielte auf die Untersuchung potenzieller Verbindungen zwischen dem Metabolom des Darms und der molekularen Uhr bei Personen mit schwerer depressiver Störung (MDD) ab.

Ziel der PROVIT-CLOCK-Studie war es, bei Patienten*innen mit MDD (n = 53) Veränderungen der Genexpression der inneren Uhr während der Behandlung mit Probiotika im Vergleich zu Placebo im Nüchternblut, sowie im Serum- und Stuhl-Metabolom zu analysieren. Zusätzlich zu den klinisch-psychologischen Testungen in der PROVIT-Studie wurden Metabolomanalysen mit ^{1}H-Kernspinresonanz (NMR)-Spektroskopie (Stuhl und Serum) und Genexpressionsanalysen (RT-qPCR) der zentralen CLOCK-Gene *ARNTL*, *PER3*, *CLOCK*, *TIMELESS* und *NR1D1* in peripheren mononukleären Blutzellen (PBMCs) aus Nüchternblut durchgeführt.

Die Genexpressionswerte des eines Taktgebers des Uhrengens *CLOCK* zeigten nur bei Personen, die zusätzlich Probiotika erhielten, eine signifikante Veränderung. Die Genexpression von *TIMELESS* und *ARNTL* veränderte sich während der vierwöchigen Interventionsphase in beiden Gruppen signifikant. Zusätzlich beobachtete man verschiedene positive und negative Korrelationen zwischen Metaboliten im Serum/Stuhl und der Expression von CLOCK-Genen.

Eine Veränderung des Darmmikrobioms mittels Probiotika beeinflusste möglicherweise die CLOCK-Genexpression. Die vorläufigen Ergebnisse der PROVIT-CLOCK-Studie deuten auf einen Zusammenhang zwischen dem Darmmikrobiom und dem zirkadianen Rhythmus hin, der möglicherweise durch Metaboliten gesteuert wird.

Abstract

An increasing body of evidence suggests a strong relationship between gut health and mental state. Lately, a connection between butyrate-producing bacteria and sleep quality has been discussed. The PROVIT study, as a randomized, double-blind, four-week, multispecies probiotic intervention study, aims at elucidating the potential interconnection between the gut's metabolome and the molecular clock in individuals with major depressive disorder (MDD).

The PROVIT-CLOCK study aimed to analyze changes in core clock gene expression during treatment with probiotic intervention versus placebo in fasting blood and the connection with the serum- and stool-metabolome in patients with MDD (n = 53). In addition to clinical assessments in the PROVIT study, metabolomics analyses with ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy (stool and serum) and gene expression (RT-qPCR) analysis of the core clock genes *ARNTL, PER3, CLOCK, TIMELESS, NR1D1* in peripheral blood mononuclear cells (PBMCs) of fasting blood, were performed.

The gene expression levels of the clock gene *CLOCK* were significantly altered only in individuals receiving probiotic add-on treatment. *TIMELESS* and *ARNTL* gene expression changed significantly over the four-week intervention period in both groups. Various positive and negative correlations between metabolites in serum/stool and core clock gene expression levels were observed.

Changing the gut microbiome by probiotic treatment potentially influences *CLOCK* gene expression. The preliminary results of the PROVIT-CLOCK study indicate a possible interconnection between the gut microbiome and circadian rhythm potentially orchestrated by metabolites.

Publication

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Contents

List of Abbreviations

ANOVA	(Analysis of Variance)
ARNTL	(aryl hydrocarbon receptor nuclear translocator-like protein)
BDI-II	(Beck Depression Inventory-II)
BMI	(body mass index)
CNS	(central nervous system)
CPMG	(Carr-Purcell_Meiboom_Gill)
CLOCK	(circadian locomotor output cycles kaput)
D_2O	(Deuterium oxid)
FC	(x-fold change)
GWAS	(genome-wide association study)
GABA	(γ-Aminobutter acid)
GAPDH	(Glyceraldehyde-3-phosphate dehydrogenase
HAMD	(Hamilton Rating Scale for Depression)
IL-6	(Interleukin-6)
ICD-10	(International Statistical Classification of Diseases and Related Health Problems)
MAO-A	(monoamine oxidase A)
MDD	(major depressive disorder)
MINI	(Mini International Neuropsychiatric Interview)
NMR	(nuclear magnetic resonance)
NR1D1	(Nuclear Receptor Subfamily 1 Group D Member 1)
Na_2HPO_4	(dibasic sodium phosphate)
NaN_3	(sodium azide)
n	(number)
PBMCs	(peripheral blood mononuclear cells)
PER3	(period circadian protein homolog 3)
p	(p-value)
RT-qPCR	(real-time quantitative PCR)
SCFAs	(short-chain fatty acids)
SCN	(suprachiasmatic nucleus)
SD	(standard deviation)
SNRIs	(serotonin norepinephrine reuptake inhibitors)
SSRIs	(selective serotonin reuptake inhibitors)

TBP	(TATA box binding protein)
TSP	(3(trimethylsilyl) propionic acid-2,2,3,3-d4 sodium salt)
t_0	(Admission)
t_1	(2-weeks visit)
t_2	(4-weeks visit)

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Einleitung

Depressive Störungen (MDD) gehören zu den weit verbreiteten psychiatrischen Erkrankungen. Vor allem während der COVID-19-Pandemie ist die Zahl der MDD-Fälle mit schätzungsweise 53,2 Millionen zusätzlich Erkrankten weltweit dramatisch gestiegen, was die dringende Notwendigkeit unterstreicht, neue Zusatztherapien zur Behandlung der Depression zu finden [1].

Obwohl es sich bei MDD um eine häufige, verheerende Erkrankung handelt, sind die zugrunde liegenden pathophysiologischen Mechanismen noch nicht zurreichend erforscht. Es ist allgemein bekannt, dass hunderter Genvarianten von Kandidatengenen, zu einer individuellen genetischen Prädisposition für MDD führen [2]. Neben der genetischen Veranlagung, beeinflussen eine Verkettung von chronischem und akutem psychosozialem Stress sowie Gen-Umwelt-Interaktionen, wie das Mikrobiom im Darm und die zirkadianen Rhythmen, die komplexe Pathophysiologie der MDD [3]. In jüngster Zeit sind die Darm-Hirn-Achse und deren Auswirkung auf einen gestörten zirkadianen Rhythmus bei Menschen mit MDD in den Mittelpunkt der Aufmerksamkeit gerückt [4–6]. Mittlerweile ist bekannt, dass psychiatrische Störungen mit Veränderungen in der Zusammensetzung des Darmmikrobioms einhergehen. Personen mit MDD weisen eine Verarmung an Butyrat-produzierenden Bakterien wie *Faecalibacterium*, *Coprococcus spp*., *Dialister* und eine erhöhte Anzahl an entzündungsfördernden Bakterien wie *Eggerthella* auf [7, 8].

Doch wie beeinflussen die Darmbakterien die Depression? Die Zusammensetzung der Darmbakterien können den menschlichen Körper und das Gehirn über mehrere Wege beeinflussen. So können die Bakterien im Darm beispielsweise kleine Stoffwechselprodukte produzieren, die zu einer bidirektionalen Kommunikation, der Darm-Hirn-Achse, zwischen dem Magen-Darm-Trakt und dem zentralen Nervensystem (ZNS) führen. Von Bedeutung ist im speziellen die Produktion kurzkettiger Fettsäuren (SCFAs), die von Darmmikrobiota durch Fermentierung und Metabolisierung von Ballaststoffen entstehen [9, 10]. SCFAs (z. B. Butyrat) können die Blut-Hirn-Schranke passieren und unsere 24-Stunden-Uhr sowie den Schlaf beeinflussen [11, 12]. Die Supplementierung von Butyrat hat in Tiermodellen gezeigt, dass die Dauer des Nicht-REM-Schlafs (Non-Rapid Eye Movement) zunimmt und der Schlaf insgesamt gefördert wird [13]. Seit Jahrzehnten wird ein gestörter Schlaf klinisch mit Depressionen in Verbindung gebracht. Neuere Studien unterstreichen diese Verbindung [14, 15].

Hypothesengeleitete Genassoziations- und Genexpressionsstudien haben bereits in relativ kleinen Kohorten nominelle Assoziationen zwischen Uhrengenvarianten und Stimmungsstörungen (MDD und bipolare affektive Störung) entdeckt [16–25]. Die Ära der genomweiten Assoziationsstudien (GWAS) brachte neue Erkenntnisse und untermauerte Hinweise für den Zusammenhang zwischen gestörten zirkadianen Rhythmen und psychisch affektiven Störungen auf Grundlage einer polygenen Erblichkeit und Stimmungsinstabilität auf genetischer Ebene [26]. Dies ist nicht überraschend, da die molekulare Uhr die Stimmung durch Aktivierung der Transkription von *Monoaminoxidase A* (*MAO-A*) reguliert [27, 28]. Genauer gesagt, kodiert das Uhrengen *ARNTL* für einen Transkriptionsfaktor, der zusammen mit dem Genprodukt von *NPAS2* als Heterodimer die Transkription des Neurotransmitter abbauenden Enzyms MAO-A aktiviert [27, 28]. Die molekulare Uhr und die Stimmung zeigen also eine starke gegenseitige Beeinflussung. Die zirkadiane Uhr im suprachiasmatischen Kern (SCN) des Hypothalamus im ZNS spielt jedoch auch eine entscheidende Rolle in dieser Gleichung, indem sie die verschiedene endokrinologische und vegetative Lebensvorgänge steuert (Cortisol- und Melatoninausschüttung, Körpertemperatur, Herzschlag und Stoffwechsel), da sie über 200 von Uhrengenen gesteuerte Zielgene aktiviert [11]. Demzufolge gibt es eine starke Verbindung zwischen der Darm-Hirn-Achse, dem zirkadianen Rhythmus und der Stimmung, die noch genauer erforscht werden muss .

Die PROVIT-Studie war eine randomisierte, doppelblinde, placebokontrollierte Probiotika-Interventionsstudie, die darauf abzielte, die Auswirkungen einer vierwöchigen Probiotikaeinnahme bei stationären Patienten*innen mit depressiver Störung auf klinischer und molekularer Ebene zu untersuchen. Darüber hinaus war die PROVIT-Studie eine der ersten Studien weltweit, die das Mikrobiom, das Metabolom, die Genexpression und die Kognition nach einer 28-tägigen placebokontrollierten, doppelblinden Einnahme von Multispezies-Probiotika analysiert hat. In einem früheren Ergebnis der PROVIT-Studie zeigte sich eine Verschiebung des Metabolom des Stuhls hin zu höheren normalisierten Konzentrationen von Butyrat bei Personen mit MDD, die 28 Tage lang zusätzlich mit Probiotika behandelt wurden. Um genauere Erkenntnisse über das Zusammenspiel zwischen den Metaboliten der Darmmikrobiota und der molekularen Uhr zu gewinnen wurde die aktuelle PROVIT-CLOCK-Untersuchung als ein weiterer Schritt konzipiert.

Die PROVIT-CLOCK-Studie zielte darauf ab, die Expression der wichtigsten Uhrengene (*ARNTL*, *PER3*, *CLOCK*, *TIMELESS*, *NR1D1*) in mononukleären Zellen des peripheren

Blutes (PBMCs) aus Nüchternblut sowie das Metabolom (NMR) in Serum- und Stuhlproben von stationären Patienten*innen mit MDD zu analysieren. Das primäre Ziel der Studie war es, zu untersuchen, ob die Genexpression der Uhrengene in PBMCs bei Patienten*innen mit MDD, die zusätzlich Probiotika erhielten, verändert ist. Zudem wurde analysiert, wie sich die Uhrengenexpression nach vierwöchiger probiotischer Intervention zeitlich entwickelt (Gruppe-Zeit-Interaktion). Darüber hinaus wurden die Auswirkungen der Zeit auf die molekulare Uhr untersucht. Schließlich wurde erforscht, ob Metaboliten in Stuhl und Serum die Uhrengenexpression in PBMCs beeinflussen, um die Verbindung zwischen dem Metabolom und der molekularen Uhr zu verstehen.

Die folgende Publikation wurde eigenhändig von Kathrin Kreuzer verfasst. Die klinische Abhandlung der PROVIT Studie war bereits vor Beginn der Diplomarbeit abgeschlossen. Zu Beginn war eine Einführung in die Literatur nötig. Darauffolgend stand die Auswertung und Qualitätskontrolle der Genexpressionsdaten. Kathrin Kreuzer, war maßgeblich für die statistische Auswertung der Genexpressionsdaten sowie in Zusammenarbeit mit Univ.-Prof. Tobias Madl für die Auswertung der Metabolomics Daten verantwortlich. Im Anschluss wurde die untenstehende Publikation von Kathrin Kreuzer mit Einverständnis der Betreuer verfasst und beim Journal Neuropsychobiology wie geplant am 11.07.2023 eingereicht. Kathrin Kreuzer war auch für die notwendige Revision verantwortlich, wo erforderliche Änderungen im Manuskript durchgeführt wurden und die Kommentare der Reviewer beantwortet wurden. Die Originalarbeit wurde am 28.03.2024 angenommen und am 22.05.2024 erfolgte schließlich die Publikation.
Das Literaturverzeichnis der Publikation dient gleichzeitig als Literaturverzeichnis der Einleitung, da die Zahlen und Quellen deckungsgleich sind.

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PROVIT-CLOCK: A Potential Influence of Probiotics and Vitamin B7 Add-On Treatment and Metabolites on Clock Gene Expression in Major Depression

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Keywords
Major depressive disorder · Gut-brain axis · Circadian clock genes · Randomized controlled trial

Abstract
Introduction: An increasing body of evidence suggests a strong relationship between gut health and mental state. Lately, a connection between butyrate-producing bacteria and sleep quality has been discussed. The PROVIT study, as a

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randomized, double-blind, 4-week, multispecies probiotic intervention study, aims at elucidating the potential interconnection between the gut's metabolome and the molecular clock in individuals with major depressive disorder (MDD). ***Methods:*** The aim of the PROVIT-CLOCK study was to analyze changes in core clock gene expression during treatment with probiotic intervention versus placebo in fasting blood and the connection with the serum- and stool-metabolome in patients with MDD (n = 53). In addition to clinical assessments in the PROVIT study, metabolomics analyses with ^{1}H nuclear magnetic resonance spectroscopy (stool and serum) and gene expression (RT-qPCR) analysis of the core clock genes *ARNTL, PER3, CLOCK, TIMELESS, NR1D1* in peripheral blood mononuclear cells of fasting blood were performed. ***Results:*** The gene expression levels of the clock gene *CLOCK* were significantly altered only in individuals receiving probiotic add-on treatment. *TIMELESS* and *ARNTL* gene expression changed significantly over the 4-week intervention period in both groups. Various positive and negative correlations between metabolites in serum/stool and core clock gene expression levels were observed. ***Conclusion:*** Changing the gut microbiome by probiotic treatment potentially influences *CLOCK* gene expression. The preliminary results of the PROVIT-CLOCK study indicate a possible interconnection between the gut microbiome and circadian rhythm potentially orchestrated by metabolites.

Introduction

Major depressive disorder (MDD) is a highly prevalent psychiatric disorder. Especially during the COVID-19 pandemic, the numbers increased dramatically with an estimation of 53.2 million additional MDD cases worldwide, which underlines the urgent necessity to explore new add-on therapies to treat the widespread MDD [1].

Even though MDD is a common, devastating disorder, the underlying pathological mechanisms are still not completely uncovered. It is common scientific knowledge that a polygenic orchestra builds up the genetic heritability, including hundreds of gene variants, leading to an individual's genetic predisposition for MDD [2]. In addition to the genetic predisposition, a concatenation of chronic and acute psychosocial stress, gene-environment interactions (e.g., the microbiome in the gut and the circadian rhythms) are influencing the complex pathophysiology of MDD [3]. Recently, the gut-brain axis and their impact on disturbed circadian rhythm in individuals with MDD got in the spotlight of attention [4–6]. Today we know that psychiatric disorders are associated with alterations in the gut microbiome composition. Individuals with MDD exhibit a depletion of butyrate-producing bacteria such as *Faecalibacterium*, *Coprococcus spp.*, as well as *Dialister* and an increased abundance of proinflammatory bacteria like *Eggerthella* [7, 8].

Nevertheless, how do gut bacteria influence depression? The gut bacteria can affect the human body and brain via several pathways. For example, bacteria can release small metabolites, which facilitate bidirectional communication between the gastrointestinal tract and the central nervous system (CNS) via the gut-brain axis. Of importance is the production of short-chain fatty acids by fermenting and metabolizing dietary fiber by gut microbiota, which can have far-reaching effects [9, 10]. Short-chain fatty acids (e.g., butyrate) can pass the blood-brain barrier and can even affect our 24-h clock and sleep [11, 12]. Supplementation of butyrate in animal models demonstrated increases in non-rapid eye movement, sleep duration, and promoting sleep [13]. Gut microbial metabolites influence central and hepatic clock gene expression, sleep duration, and body composition [14]. Disturbed sleep has been clinically associated with depression for decades and recent studies underline this concatenation [15].

Hypothesis-driven gene association and gene expression studies already discovered, in relatively small cohorts, nominal associations between clock gene variants and mood disorders (MDD and bipolar affective disorder) [16–25]. The genome-wide association study (GWAS) era brought new insights and provided evidence for the association between disturbed circadian rhythms based on a polygenic heritability and mood instability on a genetic level [26]. This is not surprising because the molecular clock regulates mood by activating the transcription of *monoamine oxidase A* (*MAO-A*). More precisely, the clock gene *ARNTL* encodes for a transcription factor, which activates together with the gene product of *NPAS2* as a heterodimer, the transcription of the neurotransmitter degrading enzyme *MAO-A* [27, 28]. The molecular clock and mood show therefore a strong concatenation. Nevertheless, the circadian clock in the suprachiasmatic nucleus of the hypothalamus in the CNS also plays a crucial role in this equation by managing various endocrinological and vegetative vital pathways (cortisol and melatonin release, body temperature, heartbeat and metabolism) as it activates over 200 clock gene controlled target genes [11]. As previously mentioned, there is a strong cross-link between the gut-brain

axis, circadian rhythm, and mood, which needs to be further elucidated [11].

The PROVIT study, as a randomized double-blind placebo-controlled probiotic intervention study, aimed at exploring the effects of a 4-week probiotic intake in inpatients with MDD on a clinical and molecular level. In addition, the PROVIT study is one of the first worldwide studies that analyzed microbiome, metabolome, gene expression, and cognition after 28 days of placebo-controlled, double blind, multispecies probiotic intake. One prior finding of the PROVIT study was a shift in the stools' metabolome of individuals with MDD receiving probiotic add-on treatment for 28 days toward higher normalized concentrations of butyrate [29]. As butyrate is suspected to influence the circadian rhythm, the current PROVIT-CLOCK investigation was designed as a further step to gain knowledge about the interplay between gut-microbiota metabolites and the molecular clock [11, 12].

More precisely, PROVIT-CLOCK aimed to analyze the core clock gene expression (*ARNTL*, *PER3*, *CLOCK*, *TIMELESS*, *NR1D1*) in peripheral blood mononuclear cells (PBMCs) of fasting blood and the metabolome (NMR) in serum and stool samples of inpatients with MDD. In detail, the primary aim of the current investigation of the PROVIT-CLOCK study was to analyze if the clock gene expression in PBMCs is altered in the group of individuals with MDD receiving probiotic add-on treatment. Second, we aimed to study a group*time interaction of clock gene expression after 4 weeks of probiotic intervention in inpatients with MDD. Third, we targeted to examine a time effect on the molecular clock. Fourth, we aimed to explore whether metabolites in stool and serum affect the clock gene expression in PBMCs of study participants with MDD to investigate the interconnection between the metabolome and the molecular clock.

Materials and Methods

Study Design of the PROVIT Trial

The detailed PROVIT study protocol was already published by Kreuzer et al. [30], Reininghaus et al. [31], and Reiter et al. [32]. In short, the PROVIT study was designed to investigate the influence of multispecies probiotic add-on treatment in individuals with MDD, in a double-blind, randomized, placebo-controlled study setting (the timeline of the PROVIT project is depicted in Fig. 1). Parameters included microbiome, metabolome, routine blood work markers and targeted gene expression, as well as scores for psychiatric symptomatology and cognition. All patients were recruited at the Department for Psychiatry and Psychotherapeutic Medicine at the Medical University of Graz in Austria. Patients were recruited via our acute outpatient clinic or referred by a psychiatrist for in-hospital psychiatric treatment.

Inclusion criteria demanded a current diagnosis of a depressive episode and age between 18 and 75 years. A psychiatrist according to the International Statistical Classification of Diseases and Related Health Problems (ICD-10) guidelines and Mini International Neuropsychiatric Interview (MINI) confirmed the MDD diagnosis of all individuals recruited. All participants were aged at least 18 years, legally insightful, and able to consent. All study participants provided written and informed consent. The probiotics study was registered at clinicaltrials.com (NCT03300440) and the local Ethics Committee gave their approval (EK 29-235 ex 16/17). In addition to the inpatient standard care, all patients with MDD received the placebo or probiotic drink (OMNi-BiOTiC Stress Repair with the bacterial strains: bifidobacteria (*B. bifidum W23, B. lactis W51, B. lactis W52*) and lactobacilli [*L. acidophilus W22, L. casei W56, L. paracasei W20, L. plantarum W62, L. salivarius W24, L. lactis W19* provided by AllergoSan]). Both groups received biotin additionally in the study medication, due to ethical considerations. All study participants were required to receive a substance that might be beneficial for them. Therefore, both formulas consisted of 125 mg D-biotin (vitamin B7), 30 mg of common horsetail, 30 mg of fish collagen, and 30 mg of keratin in a matrix with maize starch, maltodextrin, inulin, potassium chloride, magnesium sulfate, fructooligosaccharides, enzymes (amylases), and manganese sulfate. The physician on call provided and supervised the intake of the drink every morning at 7:00–7:30 am for 28 days in a double-blind manner. As the ethical board required all patients to receive a beneficial substance, vitamin B7 (biotin) was added to the probiotics and placebo formula. Fasting blood was collected at the beginning of the trial (t_0) and at the end of the intervention after 4 weeks (t_2). At the beginning (t_0), after 2 weeks (t_1), and at the end of the intervention (t_2), stool was collected. Demographic parameters (age, weight [kg], height [m], body mass index, sex, and standard medication), data on cognitive testing, and lifestyle questionnaires were obtained at the intervention start and the end. A psychiatrist according to the International Statistical Classification of Diseases and Related Health Problems (ICD-10) guidelines and Mini International Neuropsychiatric Interview (MINI) confirmed the MDD diagnosis of all individuals recruited. The depression severity was rated with the Beck Depression Inventory-II (BDI-II) and the Hamilton Rating Scale for Depression (HAMD). Further details on exclusion and inclusion criteria and details on the PROVIT study can be obtained by the former publications [29, 30, 35].

Randomization and Double-Blind Design

All participants and the study team were blinded until the end of the study. The placebo drink had the same texture, color, and taste as the probiotic drink. The doctors on call, who provided the drink, did not know whether they handed out a placebo or probiotic drink. The platform www.randomization.com was used to randomize 96 subjects into 24 blocks of 4 to assign the individuals either to the intervention or placebo group. The manufacturer assembled the test product packages. For each study participant, one package, with the relevant participant number, was provided. The person in charge of the randomization, as well as all unblinded employees of the manufacturer, was bound to secrecy toward thirds. The ratio of the probiotic group:placebo group was 1:1.

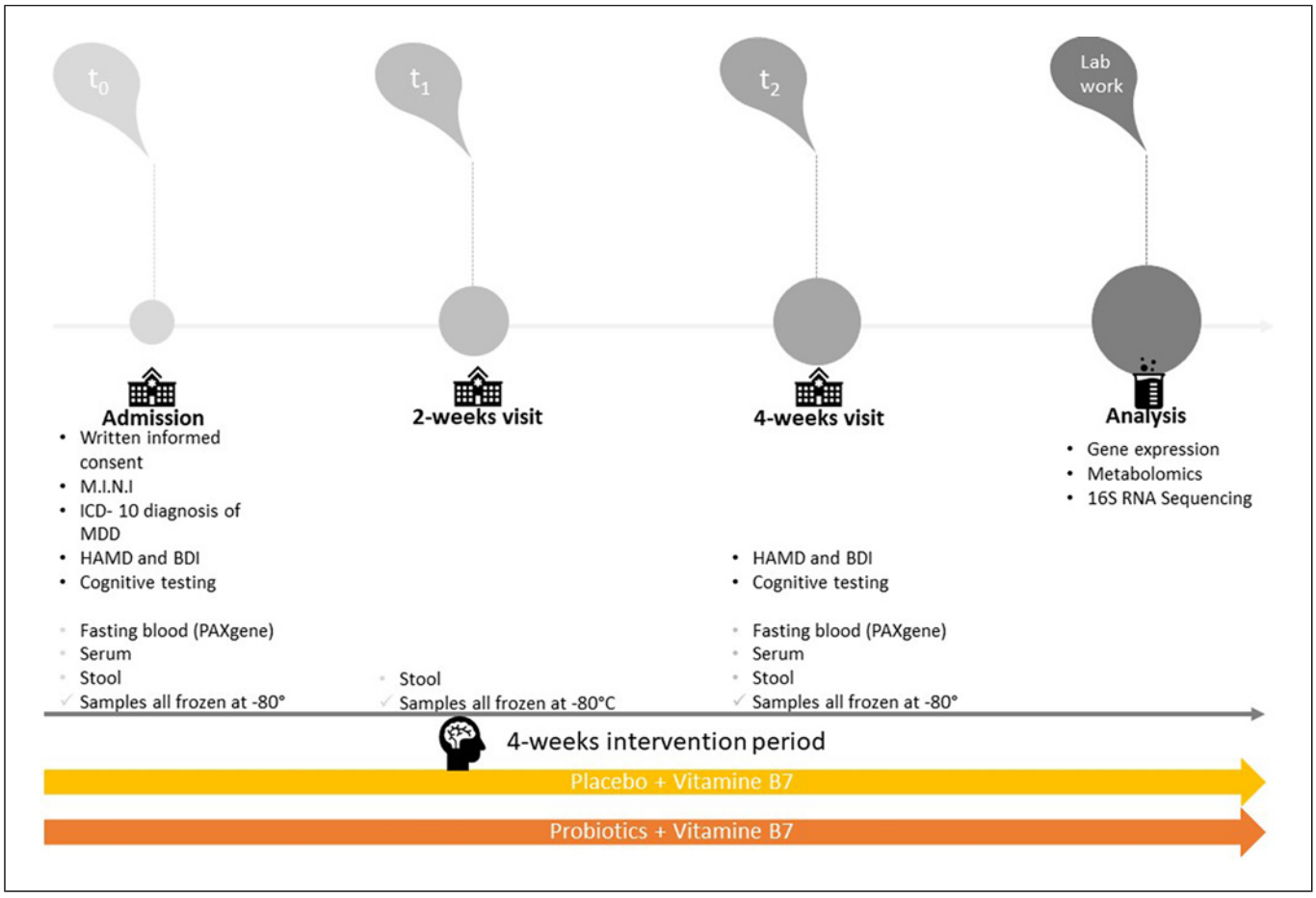

Fig. 1. Timeline of the PROVIT study.

Details of the Probiotic Add-On Treatment

The provider "Institute AllergoSan" with their producer Winclove BV in Amsterdam provided the probiotic and placebo additives. The probiotic group received the commercially available multistrain probiotic "OMNi-BiOTiC® Stress Repair" supplement, which includes nine bacterial strains with ≥2.5 × 109 colony-forming units per gram. A probiotic drink consisted of at least 3 g, with a total number of 7.5 × 109 colony-forming units per bag. More precisely, the probiotic drink included bifidobacteria (*B. bifidum* W23, B. lactis W51, B. lactis W52) and lactobacilli (*L. acidophilus* W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24, *L. lactis* W19). Additional ingredients included in the probiotic and placebo supplement were D-biotin (vitamin B7), common horsetail, fish collagen, and keratin. The matrix of the supplement further compromised maize starch, maltodextrin, inulin, potassium chloride, magnesium sulfate, fructooligosaccharides, enzymes (amylases), and manganese sulfate. The placebo drink had the exact same looking color, consistency, and taste texture, containing the matrix and D-biotin (vitamin B7). The detailed study procedure and ingredients of the add-on treatment were already described before [29, 30, 35].

Gene Expression Analysis of Core Clock Genes Using qPCR

The gene expression analysis of the target clock genes in this current PROVIT investigation was conducted in the same way as previously published by Kreuzer et al. [30]. At the beginning and the end of the 28-day PROVIT intervention, fasting blood was collected in PAXgene blood RNA tubes (Nr 762165, PreAnalytix GmbH, Hombrechtikon, Switzerland) and stored in a freezer at −80°C. In the following step, RNA was isolated from PBMCs in the PAXgene blood RNA tubes according to the manufacturer's instruction with PAXgene Blood RNA Kit (PreAnalytix GmbH, Hombrechtikon, Switzerland). The purity and quantity of the RNA were determined by using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of the RNA to cDNA was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) containing RNAse inhibitor (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed on the QuantStudioTM 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA). Per reaction, 2.5 ng of the cDNA and 200 nM of the primers

were used. For the gene expression analysis of the targeted clock genes *ARNTL*, *PER3*, *CLOCK*, *TIMELESS*, *NR1D1*, QuantiTect Primer Assay (QT00000721, Qiagen, Hilden, Germany) were used. The cycling conditions included the initial denaturation at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and an extension period at 60°C for 60 s. The sample's melting curves were measured to confirm the specificity of amplified products. In general, all samples were measured as technical triplicates. For determining the transcript abundance, we used the $2^{-\Delta\Delta Ct}$ method, normalized to the Ct values of housekeeping genes *GAPDH* and *TBP*. The gene expression levels of the housekeeping genes were stable over the time course of intervention and between the verum and the placebo group. Detailed information about primer sequences (online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000538781), gene expression levels of housekeeping genes (online suppl. Fig. 1–2), and melting curves (online suppl. Fig. 3) are given in the supplementary material. The gene expression values represent the x-fold change (FC).

Metabolomics

Reagents

From VWR International (Darmstadt, Germany), dibasic sodium phosphate (Na_2HPO_4), sodium hydroxide, hydrochloric acid (32% m/v), and sodium azide (NaN_3), were purchased. 3(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt (TSP) was obtained from Alfa Aesar (Karlsruhe, Germany). Deuterium oxide (D_2O) was ordered from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). The in-house Milli-Q Advantage Water Purification System from Millipore (Schwalbach, Germany) was used to purify deionized water. All other chemicals were used for no further purification. The phosphate NMR buffer solution was prepared by dissolving 5.56 g of anhydrous Na_2HPO_4, 0.4 g of TSP, and 0.2 g NaN_3, in 400 mL of D_2O and adjusted to pH 7.4 with 1 M NaOH and HCl. Upon addition of D_2O to a final volume of 500 mL, the pH was re-adjusted to pH 7.4 with 1 M NaOH and HCl.

Metabolite Quantification Using NMR

The detailed NMR protocol was already published by Kreuzer et al. [30]. In summary, in the first step proteins were removed and the ongoing enzymatic reactions were quenched by adding a methanol-water solution in a 2:1 ratio to stool and serum samples (200 µL serum plus 400 µL methanol; 1 g stool plus 2 mL methanol). In the following step, the Precellys homogenizer was used to lyse the samples. Until further processing, the samples were stored at −20°C. Then, the samples were spun at 17,949 rcf at 4°C for 30 min. Before transferring the samples to 5 mm NMR tubes, the supernatants were lyophilized, and 500 µL of NMR buffer in D_2O was added to the samples. The NMR measurements were conducted at 310 K on an AVANCE™ Neo Bruker Ultrashield 600 MHz spectrometer, equipped with a TXI probe head and processed as described previously [31]. Specifically, for the ^1H 1D NMR experiments, the 1D CPMG (Carr-Purcell_Meiboom_Gill) pulse sequence (cpmgpr1d, 128 scans, 73,728 points in F1, 11,904.76 HZ spectral width, recycle delays 4 s) with water suppression by presaturation was used. For data acquisition, the Bruker Topspin version 4.0.2 was used. The spectra of all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced using TSP at 0.0 ppm, using the Bruker Topspin 4.0.2 software (Bruker GmbH, Rheinstetten, Germany). Next, the spectra were imported to Matlab2014b; the regions around the water, TSP and remaining methanol signals were excluded. For correcting the sample metabolite dilution, a probabilistic quotient normalization was performed [32–34]. Data analysis and preparation of figures was carried out in MetaboAnalyst 5.0 (vide infra).

Statistical Analysis

Descriptive Statistics of the PROVIT Gene Expression Sample

According to per-protocol analysis, only participants providing a full set of serum samples (t_0 and t_2) were included in the PROVIT-CLOCK sample. First, the normal distribution of the variables was measured with the Kolmogorov-Smirnov test, Shapiro-Wilk test, and scale level. Depending on the normal distribution, baseline differences between the groups were analyzed with a two-tailed Students' *t* test, Mann-Whitney U test, or χ^2 test. Descriptive data were assessed by using means, mean rank, standard deviations and percentages of the respective variables. The description of the sample concerning clinical and sociodemographic data is shown in Table 1. Table 2 shows the medication. The level of significance was set at $p < 0.05$. All analyses were carried out with SPSS version 27 (SPSS Inc., Chicago, IL, USA). Visualization was performed with SPSS version 27, MetaboAnalyst 5.0, and Figure 1 with the online tool Visme.

Depression Scores and Gene Expression Analyses

The mean Ct value of *GAPDH* and *TBP* was used for normalization of transcript abundance. The expression values represent the x-FC. Group (probiotics vs. placebo), time effects (before vs. after the 4-week PROVIT intervention) and group-time interaction of clock gene expression and depression scores HAMD and BDI-II were analyzed with two repeated-measures two-way analysis of variances with the independent factors time point (t_0 vs. t_2) and group (intervention vs. placebo). The dependent variables in the model were the FC values of *ARNTL*, *PER3*, *CLOCK*, *TIMELESS*, and *NR1D1*. Sphericity was checked and the homogeneity of the sample was measured with the Levene test. The Greenhouse-Geisser adjustment was used to correct for violations of sphericity for the variables *TIMELESS* and *ARNTL*. Diagrams were illustrated with SPSS 27 (SPSS Inc., Chicago, IL, USA).

Correlation Analyses between Serum Metabolome and Clock Gene Expression

Based on the PROVIT-metabolomics data, published by Kreuzer et al. [30], the association between serum metabolites and clock gene expression (*ARNTL*, *PER3*, *CLOCK*, *TIMELESS*, *NR1D1*) was measured in the total sample at time point t_2 using Pearson correlation coefficient.

Results

Description of the Patient Population of the PROVIT-CLOCK Cohort

Forty-two patients with MDD (32 women and 10 men) were allocated to the intervention group. Forty patients with MDD (32 women and 8 men) were allocated to the

Table 1. Description of the PROVIT gene expression sample at baseline

Description	Intervention group (n = 24), n (%)	Placebo group (n = 29), n (%)	Statistics	
			χ^2	Sig (p value)
Sex (female)	18 (75.0)	23 (79.3)	0.139	0.709
Smoking (yes)	8 (33.3)	16 (55.2)	2.528	0.112
	Mean (SD)	Mean (SD)	t	p value
Age, years	44.15 (14.48)	40.36 (10.87)	−1.088	0.162
Waist-to-hip ratio	0.85	0.86	−0.160	0.257
	Median (mean rank)	Median (mean rank)	U	p value
Education, years	9.00 (26.33)	9.00 (27.55)	332	0.753
Illness duration, years	6.00 (25.32)	10.00 (26.52)	304	0.775
BMI, kg/m^2	23.86 (27.83)	27.18 (26.31)	328	0.721

MDD, major depressive disorder; SD, standard deviation; BMI, body mass index.

Table 2. Medication of PROVIT-CLOCK gene expression sample at baseline (in alphabetical order)

	Intervention group (N = 24), n (%)	Placebo group (N = 29), n (%)	Statistics	
			χ^2	p value
Anticonvulsants	3 (12.5)	2 (6.9)	0.483	0.487
Benzodiazepines/hypnotics	4 (16.7)	5 (17.2)	0.003	0.956
Atypical antipsychotics	8 (33.3)	9 (31.0)	0.032	0.858
Other antidepressants (SNRIs, etc.)	17 (70.8)	21 (72.4)	0.016	0.899
Selective serotonin reuptake inhibitors (SSRIs)	8 (33.3)	13 (44.8)	0.725	0.394

N, number of study participants with MDD; MDD, major depressive disorder; SNRIs, serotonin norepinephrine reuptake inhibitors; SSRIs, selective serotonin reuptake inhibitors.

placebo group. In the intervention group, 5 women and 1 man were lost to follow-up and 7 women and 1 man discontinued intervention because of non-compliance or antibiotic intake. In the placebo group, three women and two men were lost to follow-up and two women discontinued intervention. The consort flowchart was published by Reininghaus et al. [31]. In the PROVIT-CLOCK sample, 4 patients in the probiotics group and 4 patients in the placebo group were excluded from the whole PROVIT sample because of lacking serum samples or not passing the quality control of the processing steps during gene expression analysis. Study participants with lacking data were completely excluded from analysis. There was no significant difference in sex, smoking, age, waist-to-hip ratio, education in years, illness duration, and body mass index between participants subjected to the intervention (n = 24) or placebo group (n = 29) (see Table 1).

The psychopharmacological medication at admission is presented in Table 2. No significant differences between the intervention and the placebo group, in assessed depression scores HAMD and BDI, occurred in the PROVIT-CLOCK cohort. Further details on the psychopharmacological medication were already published in previous PROVIT publications [29, 30, 35].

All Individuals of PROVIT-CLOCK Cohort Improved in Depression Scores

The mean values, standard deviation, and results of repeated-measures two-way analysis of variances are depicted in Table 3. Both groups significantly improved over the time course of the intervention period of 28 days

Table 3. Mean values, SD, and results of repeated-measures ANOVA of BDI-II and HAMD of the PROVIT-CLOCK cohort

	Intervention group (N = 24)		Placebo group (N = 29)		Time		Group		Interaction	
	mean	SD	mean	SD	F	p value	F	p value	F	p value
BDI-II t_0	30.25	8.38	31.19	10.89	91.71	<**0.001**	0.636	0.429	0.351	0.556
BDI-II t_2	14.71	8.28	17.46	11.09						
HAMD t_0	14.75	5.56	14.55	4.7	61.85	<**0.001**	0.192	0.663	0.155	0.695
HAMD t_2	8.79	4.97	7.97	5.25						

N, number of study participants with MDD; MDD, major depressive disorder; SD, standard deviation.

(BDI: $F_{(1,\ 48)}$ = 91.71, p < 0.001, η^2 = 0.656; HAMD: $F_{(1,51)}$ = 61.85, p < 0.001, η^2 = 0.548). There was no significant difference in the change of depression scores between the placebo or probiotic group. No significant time*group interaction occurred (see Table 3). The results did not change when adjusted for smoking.

CLOCK Gene Expression Is Altered in Individuals Receiving Probiotic Treatment

The gene expression level of *CLOCK* ($F_{(1,\ 48)}$ = 981.96, p = < 0.*018*, η^2 = 0.953) was significantly upregulated in the intervention group after 28 days (see Fig. 2). *ARNTL* ($F_{(1,\ 51)}$ = 4.579, p = 0.037, η^2 = 0.082; Fig. 3) and *TIMELESS* ($F_{(1,\ 47)}$ = 5.558, p = 0.023, η^2 = 0.106; see Fig. 4) were significantly upregulated over the course of the intervention period in both groups (see Table 4). The gene expression of ARNTL did not differ significantly at the beginning of the study. The results did not change when adjusted for smoking.

Correlation between Metabolome and Clock Gene Expression

The comprehensive results of the correlation analyses between serum and stool metabolites (49 and 50 metabolites, respectively, were analyzed) and clock gene expression markers are depicted in Figures 5–7. *ARNTL* gene expression levels in serum positively correlated with lysine (r = 0.28; p_{raw} = 0.0048), valine (r = 0.24; p_{raw} 0.019), isoleucine (r = 0.24; p_{raw} 0.021), and alanine (r = 0.23; p_{raw} 0.023) in stool and with betaine (r = 0.28; p_{raw} 0.0046) and glucose (r = 0.27; p_{raw} 0.074) in serum. CLOCK gene expression levels in serum significantly correlated with formic acid (r = −0.27; p_{raw} 0.0090), fumaric acid (r = 0.26; p_{raw} 0.012), and glycerol (r = −0.23; p_{raw} 0.028) in stool. *TIMELESS* and *NR1D1* gene expression levels in serum positively correlated with propylene glycol (r = 0.28; p_{raw} 0.0064 and r = 0.27; p_{raw} 0.011, respectively) in stool. *NR1D1* gene expression levels in serum positively correlated with valeric acid (r = 0.26; p_{raw} 0.013) in stool and glycerophosphocholine (r = 0.37; p_{raw} 0.00017) and carnitine (r = 0.36; p_{raw} 0.00032) in serum. *PER3* gene expression levels in serum positively correlated with butyric acid (r = 0.21; p_{raw} 0.042) in stool and levels of tryptophan (r = 0.44; p_{raw} 5 × 10^{-6}), trimethylamine-N-oxide (r = 0.30; 0.0026), leucine (r = 0.27; p_{raw} 0.0082), and arginine (r = 0.28; p_{raw} 0.0058) in serum.

Discussion

The current analyses of the PROVIT sample, which was recruited as a randomized, placebo-controlled, multispecies probiotics study in individuals with MDD, showed significant changes in the circadian clock gene expression levels measured with RT-qPCR. The transcription of the core clock gene *CLOCK* changed significantly only in participants with MDD receiving the probiotics add-on therapy for 28 days and did not change in the placebo group. Gene expression levels of the other core clock genes *ARNTL* and *TIMELESS* changed significantly over the course of the intervention period in both groups. Comprehensive weak correlations between stool and serum metabolites in different pathways, e.g., amino acids, microbial derived metabolites, cofactors for epigenetic regulation, and other signaling pathways (see Fig. 5–7), and core clock gene expression markers may explain the interplay between the gut-brain axis and the molecular 24-h clock. Obviously, an orchestra of metabolites appears to be linked to the molecular 24-h clock aside from other time givers, such as light, day structure, and food intake, which in turn influence the microbiome.

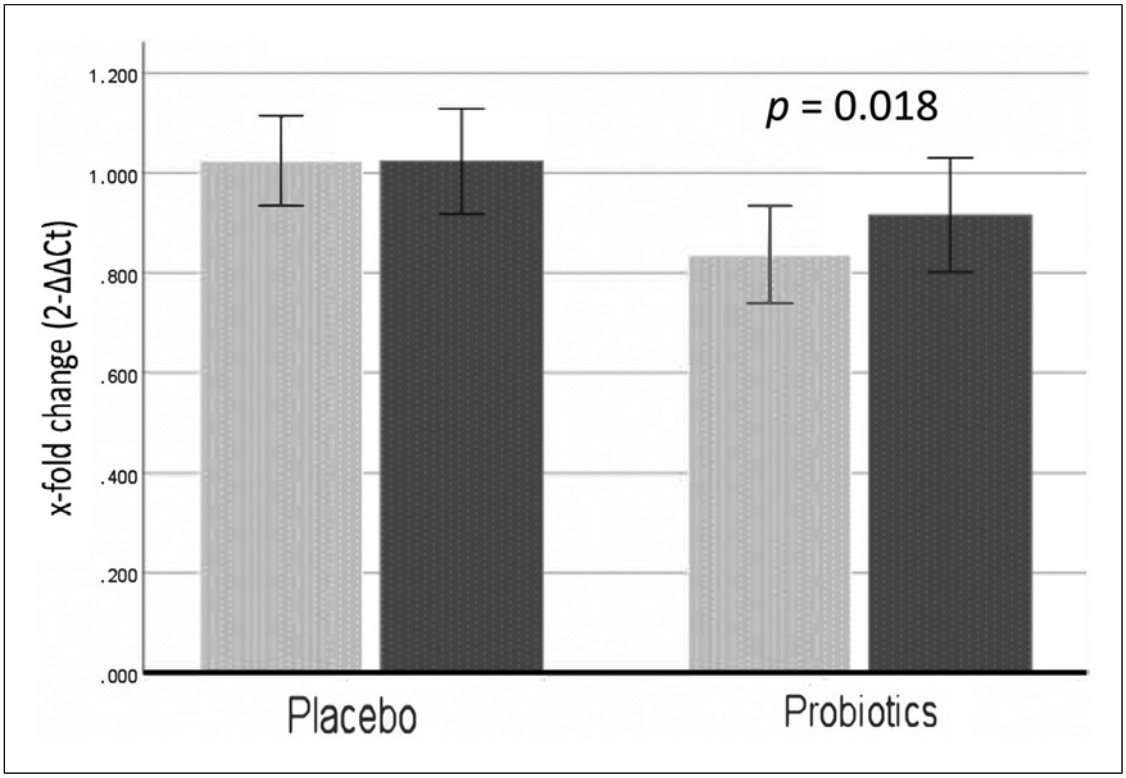

Fig. 2. Group effects of CLOCK expression: qPCR expression changes (FC) of the clock gene *CLOCK* in fasting blood samples of study participants with MDD over time (t_0 at admission vs. t_2 after 4 weeks of intervention). The black boxes illustrate probiotics; the gray boxes illustrate placebo.

From a molecular perspective, the circadian clock gene expression is regulated by a transcriptional/translational feedback loop of four key clock proteins, namely, CLOCK and ARNTL (two activators) and PER and CRY (two repressors). CLOCK and ARNTL form a heterodimeric complex; thereby, the CLOCK:ARNTL complex activates the transcription of *PER* and *CRY*. After translation, PER and CRY heterodimerize and are phosphorylated in the cytoplasm. Next, the phosphorylated PER:CRY complex translocates to the nucleus, where PER:CRY acts as an inhibitor of the transcriptional activator CLOCK:ARNTL. This complex circle generates a 24-h rhythm in the human organism, which manages crucial body functions such as heartbeat, body temperature, metabolic mechanisms, sleep, and hormone release [27, 28]. In our current PROVIT study, the gene expression levels of the core clock gene *CLOCK* changed significantly and the gene expression levels of *ARNTL* showed a trend toward significance. Linking these findings further substantiates an influence of the gut microbiome on the molecular 24-h clock. Hence, changing the gut microbiome could have favorable effects on the molecular clock and mood as the 24-h clock is directly linked to the regulation of neurotransmitter breakdown via *MAO-A* as described in detail in the introduction [27, 28].

However, the hospital inpatient treatment with regular day structure (regular get up and sleeping times, regular meals, and sport sessions) and the added vitamin B7 to the placebo and verum supplement could be an additional influencing factor for regulating the inner clock and the change of *ARNTL* and *TIMELESS* gene expression in both groups over time. It is known from previous literature that short-chain fatty acids, such as butyrate, released from gut microbiota have significant effects on the 24-h clock in the CNS, which might be one relevant factor for changes in clock gene expression in our current investigation [11, 12]. Results from a mechanistic murine study suggest that the sleep-inducing effects of butyrate are mediated by a sensory mechanism located in the liver and/or in the portal vein wall. Hepatoportal butyrate-sensitive mechanisms may play a role in sleep modulation by the intestinal microbiota [29]. Furthermore, butyrate may also lead to enhanced GABA production, by alleviating hypothalamic inflammation [35].

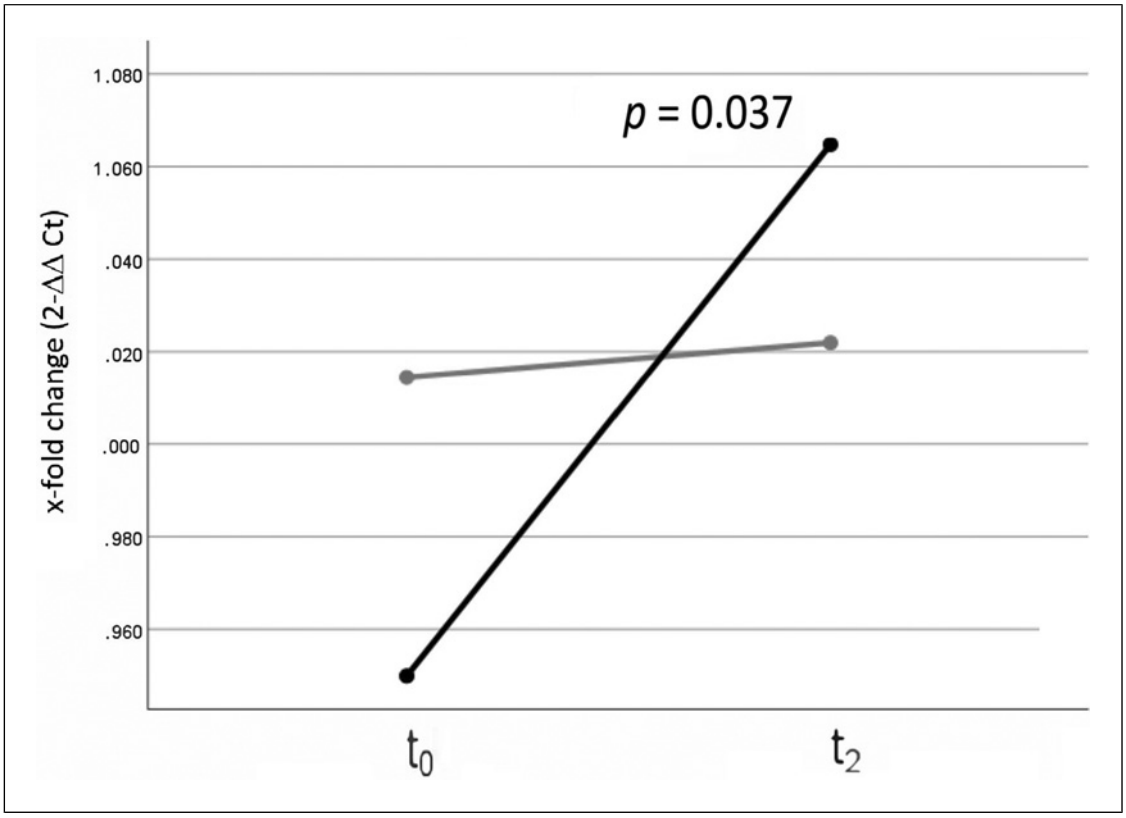

Fig. 3. Time effects of *ARNTL* expression: RT-qPCR expression changes (FC) of the clock gene *ARNTL* in fasting blood samples of study participants with MDD over time (t_0 at admission vs. t_2 after 4 weeks of intervention). The black line illustrates probiotics; the gray line illustrates placebo.

Interconnecting these findings with our PROVIT study, participants with MDD receiving probiotic add-on treatment elucidated a higher relative abundance of two butyrate-producing bacteria, namely, *Coprococcus 3* and *Ruminococcus gauvreauii*, analyzed with 16S rRNA sequencing. This change in the gut microbiome is supported by the findings of the PROVIT-metabolomics project. The stools metabolome of depressed individuals in the probiotic group shifted toward higher normalized concentrations of butyrate, in comparison to the placebo group [30–32]. Interestingly, Grosicki et al. [36] observed an association between the relative abundance of butyrate-producing bacteria *Ruminococcus* and *Blautia* and self-reported quality of sleep in a pilot study. Correspondingly, *Ruminococcus* was relatively more abundant after 28 days of probiotic add-on treatment in the PROVIT study [30–32], which further suggests a connection between gut microbiome and sleep physiology [36].

In the literature, inflammation has been associated with the circadian rhythm [37]. In the PROVIT study, we additionally observed decreasing *IL-6* proinflammatory cytokine levels, in individuals subjected to the probiotic group, whereas *IL-6* gene expression levels increased in the placebo group. Linking these findings, a pathophysiological relationship between the proinflammatory cytokine IL-6 and a disruption in the ultradian activity rhythms in a mice model has been discussed lately [38].

However, changing the gut microbiome by probiotic supplementation could help to balance the homeostasis of the metabolome, which had clear alterations between patients with depression and healthy controls in one of the largest metabolomics MDD study [39]. Interconnecting our PROVIT-metabolomics data with the clock gene expression data, we discovered weak correlations between gene expression levels in PBMCs and various metabolites in stool and serum. The gene expression levels of *ARNTL* correlated with the branched-chain amino acids isoleucine, valine, the essential amino acid lysine, and the nonessential amino acid alanine in stool. Linking these results with the PROVIT-metabolomics analysis, interestingly, the amino acids

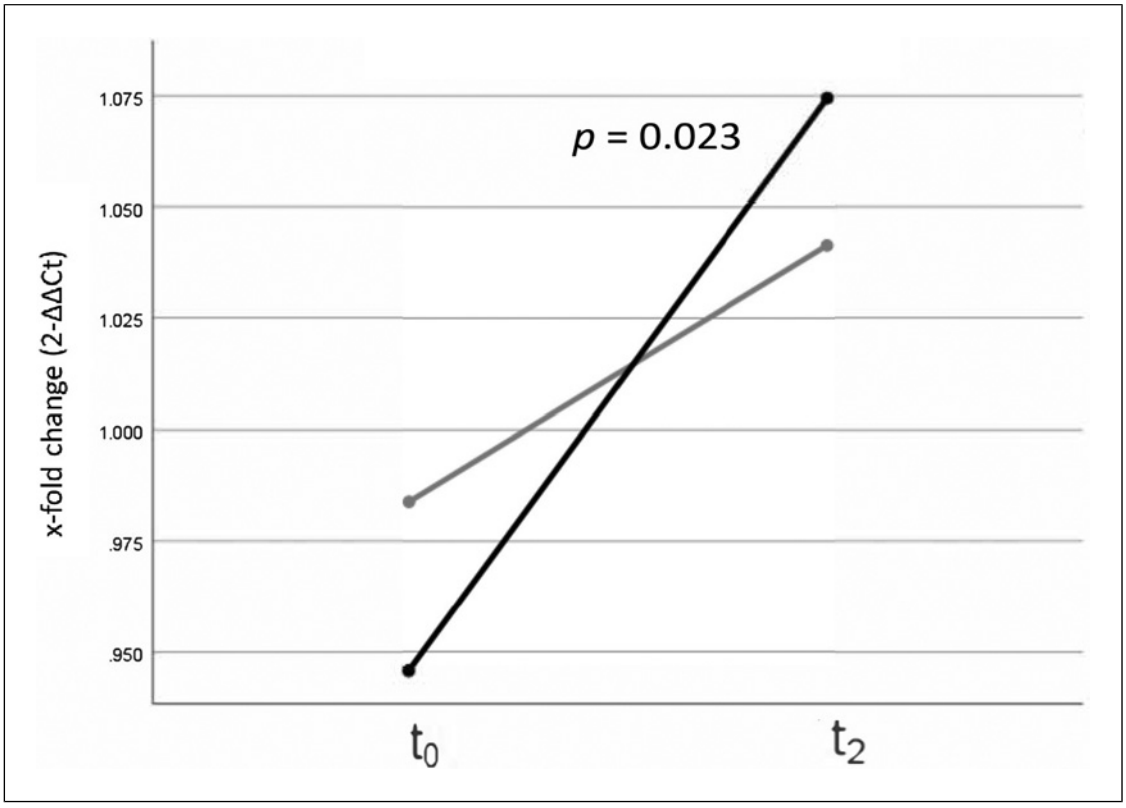

Fig. 4. Time effects of TIMELESS expression: qPCR expression changes (FC) of the clock gene *TIMELESS* in fasting blood samples of study participants with MDD over time in the Austrian PROVIT study (t_0 at admission vs. t_2 after 4 weeks of intervention). The black line illustrates probiotics; the gray line illustrates placebo.

Table 4. Results in core clock gene expression after 4 weeks of placebo-controlled probiotic intervention

	Intervention group (N = 24), mean (SD)	Placebo group (N = 29), mean (SD)	Time		Group		Interaction	
			F	p value	F	p value	F	p value
ARNTL t_0	0.95 (0.20)	1.01 (0.18)	4.58	0.037	0.048	0.827	3.523	0.065
ARNTL t_2	1.06 (0.22)	1.02 (0.23)						
CLOCK t_0	0.84 (0.24)	1.02 (0.22)	1.00	0.321	5.97	0.018	1.04	0.314
CLOCK t_2	0.92 (0.27)	1.02 (0.27)						
PER3 t_0	0.96 (0.20)	1.02 (0.23)	0.230	0.634	0.027	0.870	2.49	0.121
PER3 t_2	1.02 (0.28)	0.98 (0.22)						
TIMELESS t_0	0.95 (019)	0.98 (0.30)	5.56	0.023	0.001	0.973	0.813	0.372
TIMELESS t_2	1.07 (0.30)	1.04 (0.34)						
NR1D1 t_0	1.07 (0.29)	1.03 (0.34)	3.74	0.059	0.209	0.650	3.073	0.086
NR1D1 t_2	0.91 (0.25)	1.02 (0.32)						

Mean values, SD, and results of the repeated-measures ANOVA. ARNTL, aryl hydrocarbon receptor nuclear translocator-like protein; CLOCK, circadian locomotor output cycles kaput protein; PER3, period circadian protein homolog 3; NR1D1, nuclear receptor subfamily 1, group *D* member 1.

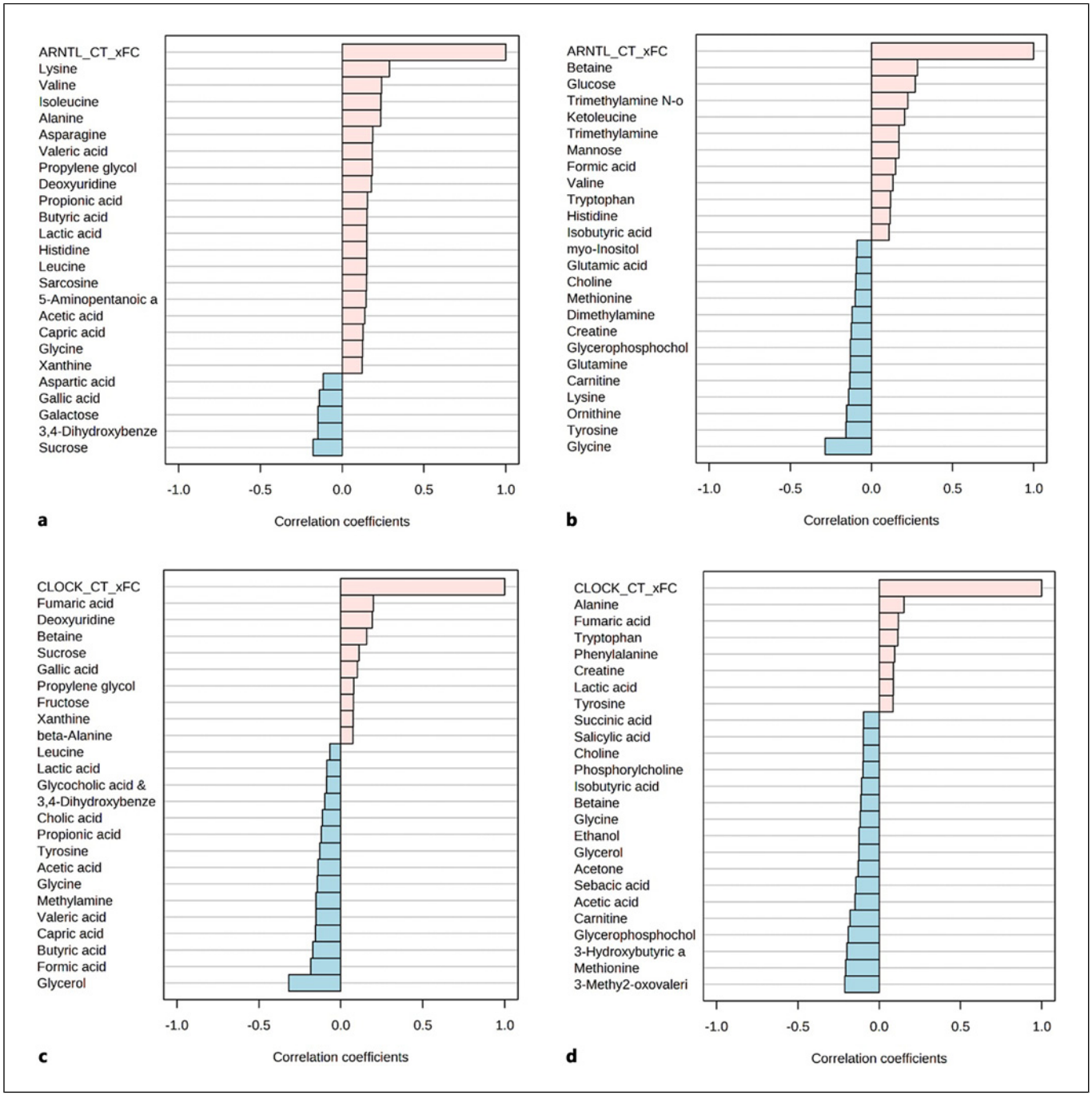

Fig. 5. Top 25 correlations with gene expression levels of *ARNTL* in stool (**a**), *ARNTL* in serum (**b**), *CLOCK* in stool (**c**), *CLOCK* in serum (**d**) and with the quantitatively assessed metabolites by NMR metabolomics. *x*-axis correlation coefficients (raw values, irrespective of *p* value) and on the y-axis the top 24 metabolites analyzed by NMR spectroscopy correlating with the upper most feature ($r = 1$).

lysine, alanine, isoleucine, and valine were altered in the stool's metabolome of patients with MDD receiving the probiotic add-on treatment [30]. The transcriptional level of the core clock gene *ARNTL* correlated with the important methyl donor betaine in serum. Betaine is an essential metabolite in various biochemical processes,

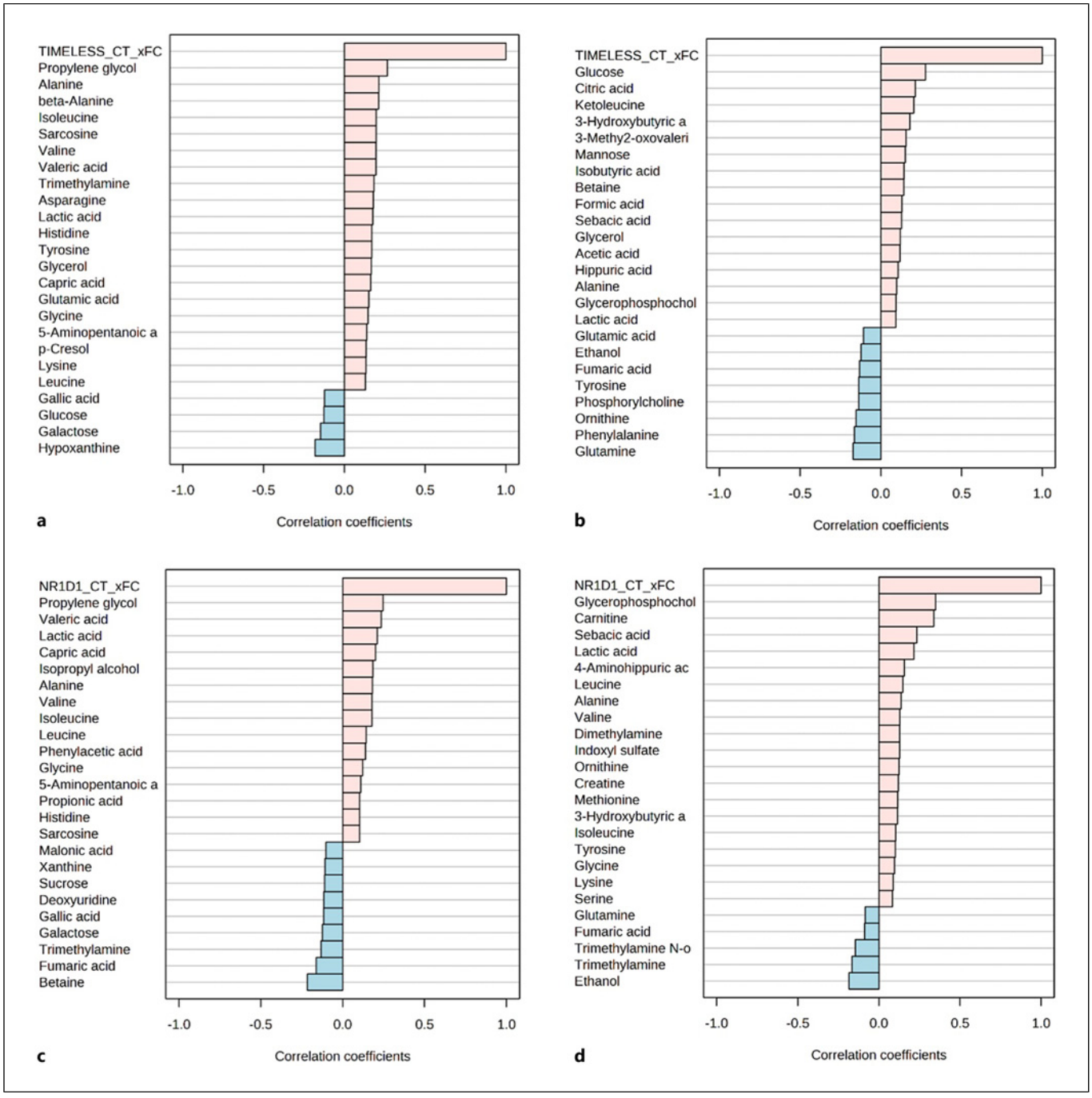

Fig. 6. Top 25 correlations with gene expression levels of *TIMELESS* in stool (**a**), *TIMELESS* in serum (**b**), *NR1D1* in stool (**c**), *NR1D1* in serum (**d**) and with the quantitatively assessed metabolites by NMR metabolomics. *x*-axis correlation coefficients (raw values, irrespective of *p* value) and on the y-axis the top 24 metabolites analyzed by NMR spectroscopy correlating with the upper most feature ($r = 1$).

such as cellular replication, detoxification, and epigenetics [40]. In the current analysis, we observed a weak negative correlation between glycine levels and the gene expression levels of *ARNTL* in serum. Various studies suggest that the simplest nonessential amino acid glycine appears to be associated with sleep physiology [41–44].

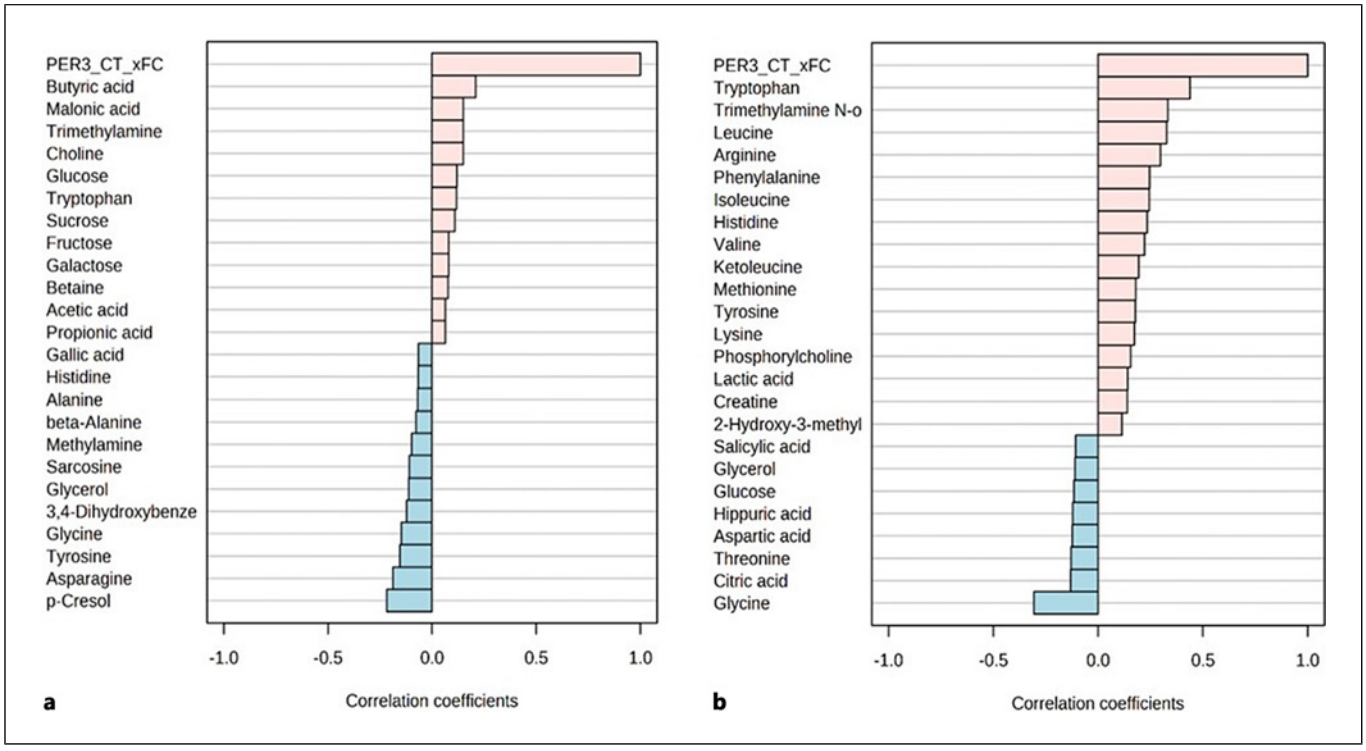

Fig. 7. Top 25 correlations with gene expression levels of PER3 in stool (**a**), PER3 in serum (**b**) and with the quantitatively assessed metabolites by NMR metabolomics. *x*-axis correlation coefficients (raw values, irrespective of *p* value) and on the y-axis the top 24 metabolites analyzed by NMR spectroscopy correlating with the upper most feature ($r = 1$).

The gene expression level of the core clock gene *CLOCK* negatively correlated with the metabolite glycerol in the stool. Glycerol is an end product of lipolysis and is also potentially controlled by the circadian rhythm [44]. *TIMELESS* and *NR1D1* gene expression levels correlate with propylene levels in serum. These results should be interpreted with caution as propylene glycol is a solvent of many medications [45].

The gene expression of *NR1D1* shows a weak correlation with the MCFAs valeric acid in stool. Valeric acid is a microbiome-derived carboxylic acid, with protective effects on gut health. Like butyrate, valeric acid acts as a histone deacetylase inhibitor, which is a main enzyme in regulating epigenetic processes [40]. In serum, *NR1D1* gene expression levels correlate with the choline derivate glycerophosphocholine and carnitine.

The more surprising correlation of the current investigation is the gene expression level of cycle length controlling *PER3* with the essential amino acid tryptophan – the precursor of the sleep-regulating hormone melatonin. This result is of indispensable interest because tryptophan metabolism imbalance is a key player in the pathophysiology of neuropsychiatric disorders [46]. In serum, the metabolite trimethyl-N-oxide and the amino acids leucine and arginine correlate with the clock gene expression of *PER3* in PBMCs.

As our PROVIT study showed that multispecies probiotics add-on therapy could change with the microbiome and metabolite pattern in the gut and in consequence the gene expression of important pathways (circadian rhythms, inflammation) [30–32], we hope that in the future we will be able to improve the treatment of depression and sleeping disorders in a multi-factorial, biopsychosocial way including treatment of the gut-brain axis with probiotics. For example, probiotic treatment with strains of *Lactobacillus* and *Bifidobacterium* was associated with increased subjective sleep quality in healthy individuals, which was revealed in a recent meta-analysis [15]. Additionally, probiotic supplementation in MDD patients increased subjective sleep quality in a small pilot study after 8 weeks [47]. The molecular perspective of our probiotic intervention study underpins the findings of Wallace et al. [47]

and Geoffroy et al. [15], by adding a potential interconnection of the clock gene expression in PBMCs and associated metabolites.

Nevertheless, further research is necessary to investigate the interplay between probiotics, microbiome, metabolome, and the molecular clock in MDD with new techniques and trials. For in-depth characterization of the influence of probiotics on the gut microbiome in individuals with MDD, shot gut sequencing will be necessary and has the potential to elucidate further mechanisms of the gut-brain axis, sleep, and depression. If the interplay between the gut-brain axis, the molecular clock, and mood can be replicated with other advanced techniques in the future, this may lead to the availability of even more sophisticated treatment options for MDD. Psychobiotics – tailored probiotic strains with mental health benefit – could be a promising treatment for MDD [48–50]. Some studies already analyzed fecal microbiota transplantation and its utility in the treatment of MDD. As MDD is clearly associated with microbiome changes, this intervention becomes more and more realistic as a treatment with fewer side effects in the future [12, 51]. Probiotics are a milder form of influencing the gut-brain axis and can be easily applied as add-on therapy in clinical practice. In general, probiotics are considered safe in psychiatric settings. They are now even recommended with grade A evidence in the guidelines of the World Federation of Societies of Biological Psychiatry (WFSBP) for patients with major depression as add-on therapy [13]. Only few case studies describe a worsening of outcomes in critically and severely immunocompromised patients after probiotic interventions, mainly when *Sacharomyces boulardii*, a yeast, was administered to critically ill patients with weak immune status [52]. Overall, our results strengthen the idea that tailored probiotics with mental health benefits are beneficial add-on therapies in MDD with effects on the gut-brain axis and circadian rhythms. Nevertheless, further research is necessary to investigate the complex interplay between probiotics, the gut-brain axis, and the molecular clock in MDD.

Limitations

The PROVIT sample is relatively small and gene expression is measured in peripheral blood in PBMCs. Other samples, such as brain tissue, are difficult to obtain from study participants. In the field of psychiatry, we are always limited to the fact that we cannot obtain brain tissue to study gene expression. To date, it remains elusive if the gene expression in the periphery exactly reflects the gene expression in the brain. However, the clock genes are oscillating in every cell in our body; therefore, it is perceivable that a change in the periphery may also occur in the brain. Additionally, it cannot be ruled out that the higher number of smokers in the placebo group had a confounding influence on the results. However, the results did not change when adjusted for smoking. Medication intake across groups did not differ significantly, yet it might be a confounding factor via interactions in pharmacodynamics and pharmacokinetic pathways of medication and probiotic supplementation. Therefore, a replication of the demonstrated results in drug-free patients with MDD would be ideal. Moreover, vitamin B7 was added to the multispecies probiotic formula due to the requirement of the Ethical Committee that all participants get a beneficial substance. Vitamin B7 might be an additional confounding factor. In future study designs, subjective or objective sleep quality assessments via self-rated questionnaires or polysomnography should be utilized to measure the potential therapeutic effect of clock gene expression, probiotic treatment, and gut microbiome metabolites on sleep. In order to get a comprehensive overview of the alteration of the whole transcriptome influenced by probiotic add-on treatment, RNA sequencing or gene expression arrays could be used in the future to increase the knowledge. Nevertheless, larger sample sizes are warranted. Based on the rather small sample, we approached the targeted design with qPCR.

In the 4 weeks study period, we could not observe a significant difference in the clinical depression scores HAMD and BDI-II between the probiotics and intervention group. This is probable due to our limited study duration of 4 weeks, which was limited to the inpatient stay in our acute hospital setting. Further studies with an expanded study duration are necessary. An influence of probiotic add-on treatment, over a longer time course than 4 weeks, on depression parameters is likely as the findings of the PROVIT study are strongly pointing in that direction.

Conclusion

The gene expression of the core clock gene *CLOCK* differed significantly only in individuals with MDD receiving multispecies add-on treatment for 28 days. In addition, we observed an orchestra of metabolites in serum and stool correlating with clock gene expression in PBMCs, which strengthens the idea of a potential

interplay between the gut-brain axis and circadian rhythm. Hence, probiotics might be a well-tolerated add-on therapy option, in individuals with MDD.

Although our results are pioneering, they should be interpreted with caution as the intervention period was limited and the size of the study group was relatively small. Future studies are necessary to get a more precise picture of how probiotics potentially influence the pathophysiological mechanisms, such as impaired circadian rhythms and inflammation processes in patients with MDD.

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Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Medical University of Graz, Austria (EK 29-235 ex 16/17). Written informed consent was obtained from all subjects involved in the study.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Kathrin Kreuzer: formal analysis, resources, data curation, writing – original draft, and visualization; Anna Maria Birkl-Toeglhofer and Johannes Haybaeck: sample management, gene expression analysis, and writing – review and editing; Alexandra Reiter, Nina Dalkner, Jolana Wagner-Skacel, Eva Reininghaus, and Susanne Astrid Bengesser: conceptualization, formal analysis, resources, data curation, writing – original draft, visualization, funding acquisition, and clinical study management; Frederike T. Fellendorf, Armin Birner, Robert Queissner, Alexandra Kohlhammer-Dohr, Annamaria Painold, and Theresa Lahousen-Luxenberger: recruitment, resources, data curation, and writing – review and editing; Alexander Maget and Martina Platzer: resources, data curation, writing – review and editing, and investigation; Matthias Seidl, Lilli-Marie Mendel, Melanie Lenger, Marco Mairinger, Anna Obermayer, and Tanja Färber: resources, data curation, and writing – review and editing; Tatjana Maria Stross: data curation and writing – review and editing; Alfred Häussl: writing – review and editing; Helmut Schöggl, Carlo Hamm, Daniela Amberger-Otti, Birgitta Leitner-Afschar, Nathalie Meier-Allard, Sandra Holasek, and Sonja Lackner: resources and writing – review and editing; Sabrina Mörkl: conceptualization, formal analysis, resources, data curation, writing – original draft, visualization, and clinical study management; and Hansjörg Habisch and Tobias Madl: metabolomics analyses, visualization, formal analysis, data curation, and writing – review and editing.

Data Availability Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the lack of a suited deposition platform.

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