Psychoneuroendocrinology 100 (2019) 213–222

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Probiotic *Lactobacillus Plantarum 299v* decreases kynurenine concentration and improves cognitive functions in patients with major depression: A double-blind, randomized, placebo controlled study

Leszek Rudzki[a,*], Lucyna Ostrowska[b], Dariusz Pawlak[c], Aleksandra Małus[d], Krystyna Pawlak[e], Napoleon Waszkiewicz[f], Agata Szulc[g]

[a] Department of Psychiatry, Medical University of Bialystok, Poland
[b] Department of Dietetics and Clinical Nutrition, Medical University of Bialystok, Poland
[c] Department of Pharmacodynamics, Medical University of Bialystok, Poland
[d] Department of Psychiatry, Medical University of Bialystok, Poland
[e] Department of Monitored Pharmacotherapy, Medical University of Bialystok, Poland
[f] Department of Psychiatry, Medical University of Bialystok, Poland
[g] Department of Psychiatry, Medical University of Warsaw, Poland

ARTICLE INFO

Keywords:
Probiotic
Psychobiotic
Microbiota
Lactobacillus Plantarum 299v
Microbiota-gut-brain axis
Gut-brain axis
Major depression
Kynurenine
Kynurenine pathway
Placebo controlled trial
Cytokines
Cortisol
IL-1b
TNF-alpha
IL-6

ABSTRACT

Background: Interactions between the digestive system and the brain functions have become in recent years an important field of psychiatric research. These multidirectional interactions take place in the so called microbiota-gut-brain axis and emerging scientific data indicate to the significant role of microbiota in the modulation of the central nervous system (CNS) including affective and cognitive functions.

Objective: An assessment of psychobiotic and immunomodulatory effects of probiotic bacteria *Lactobacillus Plantarum 299v (LP299v)* by measuring affective, cognitive functions and biochemical parameters in patients with MDD undergoing treatment with selective serotonin reuptake inhibitors (SSRI).

Design: Seventy nine patients with MDD were randomized and allocated to a double-blind, placebo-controlled trial. Participants received either a SSRI with the probiotic *LP299v* (n = 40) for a period of 8 weeks or a SSRI with the placebo of the probiotic (n = 39) for the same period. The severity of psychiatric symptoms was assessed using Hamilton Depression Rating Scale (HAM-D 17), Symptom Checklist (SCL-90) and Perceived Stress Scale (PSS-10). Cognitive functions were assessed using the Attention and Perceptivity Test (APT), Stroop Test parts A and B, Ruff Figural Fluency Test (RFFT), Trail Making Test (TMT) Parts A and B and the California Verbal Learning Test (CVLT). Biochemical parameters such as tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxykynurenine (3HKYN), anthranilic acid (AA), 3-hydroxy anthranilic acid (3HAA), tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), interleukin 1-beta (IL-1b) and cortisol plasma concentrations were measured.

Results: Sixty participants finished the study and were analyzed: 30 participants in the *LP299v* group and 30 participants in the placebo group. There was an improvement in APT and in CVLT total recall of trials 1–5 in the *LP299v* group compared with the placebo between baseline and after 8 weeks of intervention. There was a significant decrease in KYN concentration in the *LP299v* group compared to the placebo group. We also observed significant increase in 3HKYN:KYN ratio in the *LP299v* group compared with the placebo group. Additionally, Repeated Measures ANOVA revealed a significant effect of interaction of Treatment x time for AA concentration. However, results of *post hoc* analysis did not reach statistical significance in neither probiotic nor placebo group. There were no significant changes of TNF-α, IL-6 and IL-1b and cortisol concentrations in neither probiotic nor placebo groups.

Conclusions: Augmentation of SSRI treatment with probiotic bacteria *Lactobacillus Plantarum 299v* improved cognitive performance and decreased KYN concentration in MDD patients. Decreased KYN concentration could contribute to the improvement of cognitive functions in the *LP299v* group compared to the placebo group. To our knowledge results of this study are the first evidence of improvement of cognitive functions in MDD patients

* Corresponding author at: Three Towns Resource Centre, Nelson Rd, Saltcoats KA21 5RF, Scotland, UK.
E-mail addresses: rudzki.l@gmail.com (L. Rudzki), lucyna@umb.edu.pl (L. Ostrowska), dariuszpawlak@poczta.onet.pl (D. Pawlak), aleksandrawalukmalus@gmail.com (A. Małus), krystynapawlak@poczta.onet.pl (K. Pawlak), napwas@wp.pl (N. Waszkiewicz), agataszulc@poczta.onet.pl (A. Szulc).

https://doi.org/10.1016/j.psyneuen.2018.10.010
Received 27 March 2018; Received in revised form 13 October 2018; Accepted 14 October 2018

due to probiotic bacteria and this is the first evidence of decreased KYN concentration in MDD patients due to probiotic bacteria.

1. Introduction

In recent years, the role of intestinal microbiota, as a component of the microbiota-gut-brain axis, has become an important player in neuroscience and psychiatric research. Animal and human studies point out the role of those bacteria in maintaining the psycho-neuro-immunological balance through various actions, such as the modulation of the immune and the neuroendocrine systems e.g. hypothalamic-pituitary adrenal axis (HPA), changes of the TRP metabolism in the serotonin (5-HT) and the kynurenine axes, regulation of the neurogenesis and improvement of intestinal permeability. Moreover, those bacteria are able to produce and metabolize various neuroactive compounds, such as the short-chain fatty acids (SCFAs) and neurotransmitters. Additionally, they can influence neurogenesis and the expression of neurotransmitters' receptors in the central nervous system (CNS) (Cryan and Dinan, 2012; Desbonnet et al., 2010; Gareau, 2014; Huang et al., 2016).

Extensive research suggest that activation of inflammatory response, pro-inflammatory cytokines, hypercortisolemia, detrimental kynurenines catabolites (TRYCATs), oxidative and nitrosative stress play essential parts in the pathophysiology of various psychiatric disorders including major depression and cognitive decline (Schwarcz et al., 2012). Consequently, since gastrointestinal (GI) tract with its gut-associated lymphoid tissue (GALT) creates the largest immune organ of the human body its role in psychopathology is drawing a lot of attention in psychiatric research. Multiple factors influence permeability and immunity of this barrier. For example, psychological stress (mediated by corticoliberin – CRH), proinflammatory cytokines, dysbiosis, oxidative and nitrosative stress, the nuclear factor NF-κB can lead to increased intestinal permeability and subsequent activation of the inflammatory response with its consequences in the CNS (Ferrier, 2008). Microbiota, as natural guardians of the intestinal epithelium, modulate intestinal permeability and regulate various functions of GALT (Zareie et al., 2006). Previously, the role of intestinal barrier disruption and gut-derived activation of inflammatory response have been described in various psychiatric disorders (Esnafoglu et al., 2017; Leclercq et al., 2014; Maes and Leunis, 2008; Severance et al., 2012, 2010) including major depression (Maes et al., 2008, 2012).

There is growing evidence of the therapeutic effects of the microbiota on cognitive functions, symptoms of anxiety, low mood, depression and chronic fatigue syndrome (CFS) (Akbari et al., 2016; Akkasheh et al., 2016; Cryan and Dinan, 2012; Huang et al., 2016; Rao et al., 2009) and due to the therapeutic effect of some of the microbiota on CNS, this beneficial group of bacteria have recently earned a general name of psychobiotics.

Kynurenines have neurotoxic and neurodegenerative effects on CNS but in physiological levels they have significance in immunomodulation, neuroprotection and energetic balance of CNS. Pro-inflammatory cytokines e.g. TNF-α, IL-6, IL-1b, bacterial lipopolysaccharides (LPS), glucocorticoids, oxidative and nitrosative stress can activate enzymes of the kynurenine pathway, such as 2,3 indoleamine dioxygenase (IDO) and tryptophan 2,3 dioxygenase (TDO) which initiate conversion of TRP (serotonin precursor) towards detrimental TRYCATs depleting TRP from the conversion to serotonin (5-HT) and melatonin (Fig. 1). As a consequence, TRP is converted to KYN and further various kynurenines which have vast immunological actions and neuronal effects. For instance, the negative effects of QUIN and KYNA on cognitive functions are well established (Stone and Darlington, 2013). Moreover, kynurenines are believed to play a significant role in the pathogenesis of major depressive disorder (Schwarcz et al., 2012; Steiner et al., 2011; Wichers et al., 2005).

Lactobacillus Plantarum 299v (DSM 9843) (*LP299v*) is a probiotic bacteria with characteristics which we found valuable in the context of our research. *LP299v* is a natural habitat of human intestinal mucosa and it is widely distributed in the gastrointestinal tract. It is a safe probiotic and capable of surviving passage and colonizing human gastrointestinal tract (Johansson et al., 1998; Klarin et al., 2005). *LP299v* has a positive impact on the intestinal barrier and was shown to decrease its permeability (Mangell et al., 2002; White et al., 2006). It is also able to reduce intestinal and systemic inflammation (Schultz et al., 2002) and has a positive influence on IBS symptoms, a prototypical gut-brain axis disorder (Ducrotte et al., 2012).

Taking into consideration all of the above, pharmacological and psychobiotical modulation of kynurenine pathway became a subject of interest of psychopharmacological research with particular emphasis on cognitive performance, neuroprotection and symptoms of depression and anxiety. For those reasons we found it valuable to assess the influence of augmentation of probiotic bacteria *Lactobacillus Plantarum 299v* on cognitive, affective and immune parameters of depressed patients undergoing SSRI treatment. In our study we hypothesized the beneficial influence of probiotic *LP299v* on symptoms of major depression and/or cognitive functions. Moreover, we hypothesized that those actions could take place due to probiotic via modulation of proinflammatory cytokines and cortisol (characterizing HPA axis activity) levels and changes in kynurenine pathway. Proinflammatory cytokines, such as TNF-α, IL-6, IL-1b were previously described to be elevated in depressed patients and to play a significant role in the pathogenesis of major depression. Moreover, these cytokines were shown to influence integrity of intestinal barrier, activity of HPA-axis and metabolism of TRP in kynurenine pathway. These are the reasons why we measured those particular cytokines in our study.

2. Material and methods

2.1. Participants

Participants were admitted to Outpatient Clinic of Stanislaw Deresz Psychiatric Hospital (Bialystok, Poland). Patients during SSRI monotherapy or drug free at admission were classified according to the DSM-

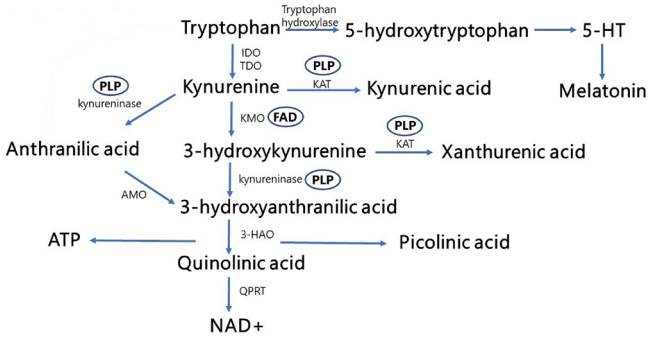

Fig. 1. Overview of the tryptophan metabolism through kynurenine and 5-HT pathways including the key enzymes and their cofactors.
IDO: indoleamine 2,3 dioxygenase; TDO: tryptophan 2,3 dioxygenase; KAT: kynurenine aminotransferases; KMO: kynurenine monooxygenase; AMO: anthranilate 3-monooxygenase; 3-HAO: 3-hydroxyanthranilic acid oxidase; QPRT: quinolinic-acid phosphoribosyl transferase; NAD+: nicotinamide adenine dinucleotide; ATP: adenosine triphosphate; PLP: pyridoxal 5′-phosphate (an active form of pyridoxine - vitamin B6); FAD: flavine adenine dinucleotide (an active form of riboflavin - vitamin B2)

IV-R (Diagnostic and Statistical Manual of Mental Disorders) diagnostic criteria of major depression. Subjects with inflammatory, oncological, autoimmune disorders, diabetes, previously diagnosed other psychiatric diseases other than depression, psychoactive substances abusers, patients with organic brain dysfunctions, smokers, and changes in routine blood biochemical parameters were excluded from the study. Pregnancy, lactation, Body Mass Index (BMI) < 18.5 kg/m^2 and > 30 kg/m^2, treatment with antipsychotic drugs, mood stabilizers, antibiotics, glucocorticosteroids were also criteria of exclusion from the study. All subjects gave written consent after the study protocol was explained. Seventy nine patients with MDD were randomized and allocated to a double-blind, placebo-controlled trial. Sixty of those finished the study and were analyzed: 30 participants, 20 female and 10 males with a mean age of 38.90 years (SD = 12) and a mean BMI of 23.55 (SD = 3.13) in the placebo group, and 30 participants, 23 female and 7 male with a mean age of 39.13 years (SD = 9.96) and a mean BMI of 24.09 (SD = 3.76) in the probiotic group. Placebo and probiotic groups did not significantly differ in sex, age, BMI, depressive episode characteristics or psychometric and biochemical parameters (Table 1 and 2).

In group of 60 patients who finished the study and were analysed, 9 patients in probiotic group and 7 patients in placebo group were treated with SSRI before inclusion to the study and there was no statistical difference in this parameter between groups ($X^2 = 0.34$, $p = 0.559$), and there was no statistical difference in SSRI type before inclusion to the study between placebo and probiotic groups ($X^2 = 0.06$, $p = 0.969$) (Table 1). Furthermore, the rest of patients were started on SSRI treatment on enrolment to the study, simultaneously with probiotic or placebo and there was no statistical difference regarding SSRI type during treatment between placebo and probiotic groups ($X^2 = 2.08$, $p = 0.555$).

The sample size was calculated to achieve statistical power $\beta = 0.80$ and effect size E = 0.30 with statistical significance threshold $\alpha = 0.05$ for repeated measures ANOVA what gave us sample size n = 24 per group and in total 48 subjects in both experimental groups.

2.2. Study design

The study was an 8 week, randomized, double-blinded, parallel, placebo controlled trial. Patients were randomly assigned to placebo or probiotic group using computer generated randomization list and the study was blinded at group allocator, participants and assessor levels. Different members of the research group were responsible for generation of the random allocation sequence, enrollment of participants, and assigning participants to interventions. The study was approved by the local Ethics Committee of Medical University of Bialystok and was registered with ClinicalTrials.gov registry number: NCT02469545 and was performed in the period between June 2014 and March 2016. Participants received an 8 week augmentation of SSRI treatment with probiotic bacteria *Lactobacillus plantarum 299v* or with placebo of the probiotic. In the probiotic group participants were provided with 60 capsules of probiotic for the first 4 weeks of the intervention to be taken according to the information provided by the manufacturer - 1 capsule in the morning and 1 at night. Each capsule contained 10×10^9CFU of probiotic bacteria *Lactobacillus Plantarum 299v* (Sanprobi IBS®- Sanprobi Sp. z o.o., Sp. k., Szczecin, Poland; probiotic capsules manufacturer - Institute Rosell-Lallemand, Montreal, Canada; *Lactobacillus plantarum299v*(*LP299v*) strain owner - Probi AB, Lund, Sweden). After 4 weeks of intervention patients were appointed for the compliance assessment during which compliance with the treatment regime was verified. Patient were asked to return empty capsule strips after probiotic or placebo. During the same appointment patients received a further 60 capsules of probiotic for the subsequent 4 weeks of intervention. In the placebo group, participants were provided with placebo capsules containing crystalline cellulose powder. Placebo capsules were indistinguishable from the probiotic capsules and were given at the same regime as intervention in the probiotic group. The placebo group underwent the same treatment regime compliance verification as the probiotic group.

2.3. Depressive and anxiety symptoms – primary outcome measures

Depressive and anxiety symptoms were assessed at 3 points of the study: pre intervention at baseline, after 4 weeks and post intervention - after 8 weeks of intervention using 17 items Hamilton Depression Rating (HAM-D 17), Symptom Checklist (SCL-90) and Perceived Stress Scale (PSS-10).

HAM-D 17 is the questionnaire rating severity of depression by assessing in 3–5 point scale mood, feelings of guilt, suicide ideation, insomnia, agitation or retardation, anxiety, weight loss, and somatic symptoms.

SCL-90 is a 90-item self-reported multidimensional questionnaire measuring a wide range of psychopathological dimensions, such as somatization, obsessive–compulsive symptoms, interpersonal sensitivity, depression, anxiety, anger–hostility, phobic anxiety, paranoid ideation, psychoticism and global severity index which is the sum of scoring from all of the scale's dimensions. Each item is rated on a five-point scale, ranging from 'not at all' to 'extremely'.

PSS-10 is a ten-item self-reported questionnaire assessing the degree to which recent life situations are appraised as stressful. Respondents indicate on a five-point scale ranging from 0 (never) to 4 (very often) how often they have felt or thought in a certain way during the period

Table 1
Demographic and clinical characteristics of MDD patients at baseline.

Variable	Placebo (n = 30)	*LP299v* (n = 30)	Statistic value	*p*
Age, years mean, (SD)	38.90 (12)	39.13 (9.96)	1.49	0.935[a]
BMI (kg/m^2) mean, (SD)	23.55 (3.13)	24.09 (3.76)	1.24	0.551[a]
Sex n (%)				
Female	20 (66.7)	23 (76.7)	0.74	0.390 [b]
Male	10 (33.3)	7 (23.3)		
Educational status n (%)				
Primary	2 (6.7)	0.0	4.45	0.217 [b]
Vocational	4 (13.3)	9 (30.0)		
Secondary	10 (33.3)	7 (23.3)		
Higher	14 (46.7)	14 (46.7)		
Psychiatric family history n (%)	13 (43.3)	18 (60.0)	1.67	0.196 [b]
Depression characteristics n (%)				
Recurrent	4 (13.3)	5 (16.7)	0.13	0.718 [b]
Chronic (> 2 years)	2 (6.7)	4 (13.3)	0.74	0.389 [b]
Melancholic	12 (40.0)	9 (30.0)	0.66	0.417 [b]
Atypical	4 (13.3)	2 (6.7)	0.74	0.389[b]
Suicidal thoughts	10 (33.3)	5 (16.7)	2.22	0.136 [b]
SSRI treatment before inclusion n (%)	7 (23.3)	9 (30)	0.34	0.559 [b]
SSRI type before inclusion n (%)				
Escitalopram	4 (13.3)	5 (16.6)	0.06	0.969[b]
Sertraline	2 (6.7)	3 (10.0)		
Paroxetine	1 (3.0)	1 (3.0)		
SSRI type during treatment (n)				
Escitalopram	23 (76.7)	25 (83.3)	2.08	0.555 [b]
Sertraline	4 (13.3)	4 (13.3)		
Paroxetine	1 (3.3)	1 (3.3)		
Fluoxetine	2 (6.7)	0.0		

MDD: major depression; *LP299v*: *Lactobacillus plantarum 299v*; SSRI: serotonin selective reuptake inhibitors.
All results are expressed as mean, standard deviation.
n = number of participants.
$p < 0.05$ *.
[a] Statistical analysis by *t*-test.
[b] *chi square* test.

Table 2
Affective, cognitive and biochemical characteristics of MDD patients at baseline.

Variable		Placebo (n = 30)	*LP299v* (n = 30)	Statistic value	*p*
HAM-D 17		22.00 (7.92)	21.53 (6.03)	−0.26	0.797 [a]
PSS-10		29.70 (3.95)	28.27 (4.91)	−1.25	0.218 [a]
SCL 90	Somatization	1.66 (0.9)	1.87 (0.92)	−0.91	0.369 [a]
	Obsessive–compulsive sympt.	2.38 (0.67)	2.11 (0.71)	1.49	0.143 [a]
	Interpersonal sensitivity	2.04 (0.85)	1.77 (0.78)	1.25	0.215 [a]
	Depression	2.67 (0.64)	2.42 (0.63)	1.51	0.137 [a]
	Anxiety	2.20 (0.75)	2.04 (0.79)	0.84	0.406 [a]
	Anger–hostility	2.06 (1.16)	1.70 (0.80)	1.4	0.167 [a]
	Phobic anxiety	1.11 (0.78)	1.11 (0.97)	0	1.000 [a]
	Paranoid ideation	2.10 (0.81)	1.78 (0.87)	1.44	0.155 [a]
	Psychoticism	1.55 (0.65)	1.38 (0.62)	0.99	0.324 [a]
	Global severity index	17.43(5.08)	16.20 (5.40)	0.91	0.366 [a]
APT	Work speed	718.73 (154.8)	693.4 (131.09)	0.684	0.497 [a]
	Omissions	7.90 (8.82)	7.63 (6.47)	0.13	0.894 [a]
Stroop T.	Time A	24.91 (5.45)	24.72 (4.12)	0.154	0.879 [a]
	Errors A	29.57	31.43	422	0.483 [c]
	Time B	62.51 (13.66)	65.47 (28.58)	−0.510	0.612 [a]
	Errors B	30.82	30.18	440.5	0.886[c]
RFFT	Total unique designs	69.67 (24.31)	70.37 (23.46)	−0.11	0.91 [a]
	Total perseverations	12.93 (12.58)	20.6 (18.96)	−1.85	0.07 [a]
	Total enumerative strategies	8.1 (16.76)	5.47 (10.99)	0.72	0.475 [a]
	Total rotational strategies	9.67 (14.82)	7.9 (13.19)	0.49	0.628[a]
	Total enumerative and rotational strategies combined	17.8 (20.8)	13.27 (18.36)	0.89	0.375 [a]
TMT	Part A	30.15 (8.74)	33.38 (16.81)	−0.94	0.353 [a]
	Part B	73.67 (24.7)	81.41 (47.93)	−0.79	0.435 [a]
CVLT	Total recall of trials 1-5	46.67 (12.9)	41.21 (10.74)	1.63	0.110 [a]
	Perseverative errors	2.37 (2.26)	3.04 (1.92)	−1.14	0.261 [a]
	Intrusion errors	0.48 (1.31)	0.67 (1.17)	−0.53	0.599 [a]
	Semantic clustering	7.19 (4.3)	7.33 (5.43)	−0.11	0.914 [a]
BIOCHEMICAL PARAMETERES	TRP (μmol/L)	63.58 (12.94)	59.08 (10.1)	1.49	0.142 [a]
	KYN (μmol/L)	2.17 (0.76)	2.05 (0.5)	0.7	0.489 [a]
	3HKYN (nmol/L)	37.53 (16.02)	31.40 (12.03)	1.66	0.103 [a]
	KYNA (nmol/L)	27.15 (10.33)	29.90 (9.63)	1.07	0.29 [a]
	AA (nmol/L)	32.38 (18.31)	42.80 (35.18)	−1.38	0.173
	3HAA (nmol/L)	5.39 (2.55)	6.04 (3.72)	−0.76	0.449
	KYN:TRP	0.035 (0.01)	0.035 (0.01)	0.00	1[a]
	3HKYN:KYN	17.82 (5.96)	15.89 (6.28)	−1.22	0.229 [a]
	KYNA:KYN	12.96 (4.06)	15.18 (5.38)	1.8	0.076[a]
	TNF-α (pg/ml)	1.29 (1.21)	1.70 (2.36)	−0.83	0.411 [a]
	IL-6 (pg/ml)	1.74 (1.93)	2.05 (2.78)	−0.49	0.623 [a]
	IL-1b (pg/ml)	0.13 (0.22)	0.16 (0.18)	−0.6	0.55 [a]
	Cortisol (ug/ml)	155.51 (52.66)	160.74 (77.21)	0.31	0.76 [a]
	TSH (μIU/ml)	1.69 (0.87)	1.59 (0.92)	−0.43	0.671 [a]
	CRP (mg/L)	1.36 (1.85)	1.14 (1.52)	−0.47	0.642 [a]

MDD: major depression; *LP299v*: *Lactobacillus plantarum 299v*; HAM-D 17: Hamilton depression rating scale; PSS-10: Perceived Stress Scale; SCL90: Symptom Checklist-90; APT: Attention and Perceptivity Test; Stroop T: Stroop Test; RFFT: Ruff Figural Fluency Test; TMT: Trail Marking Test; CVLT: California Verbal Learning Test Total; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; IL-1b: interleukin 1b; TRP: tryptophan; KYN: kynurenine; 3HKYN: 3-hydroxykynurenine; KYNA: kynurenic acid; AA: anthranilic acid; 3HAA: 3-hydroxyanthranilic acid; TSH: thyroid stimulating hormone; CRP: C-reactive protein.
All results are expressed as mean, standard deviation.
n = number of participants.
$p < 0.05$ *.
[a] Statistical analysis by *t*-test.
[c] U Mann-Whitney test.

of the past month. All responses are then summed to measure the level of perceived stress.

2.4. Cognitive functions – secondary outcome measures

Patients' cognitive functions were assessed pre-intervention at baseline and post intervention - after 8 weeks of intervention with Attention and Perceptivity Test (APT), Stroop Test parts A and B, Ruff Figural Fluency Test (RFFT), Trail Making Test (TMT) Parts A and B and California Verbal Learning Test (CVLT).

Attention and Perceptivity Test (APT) (Jaworski, 2014) is commonly used in Poland equivalent of d2 Brickenkamp Test of Attention and The Dot Cancellation or Bourdon-Wiersma test to assesses selective attention, sustained attention, vigilance, visual perception and visual scanning. Test score is based on variables, such as work speed, number of errors, and number of omissions. Its results correspond well with results of WAIS-R (Wechsler Adult Intelligence Scale Revised Version) subscales measuring attention and perceptivity.

Stroop Test is used for evaluation of selective attention, cognitive flexibility, working memory, processing speed and ability to inhibit cognitive interference.

The Ruff Figural Fluency Test (RFFT) is a non-verbal fluency test that measures executive functions, such as planning strategies, divergent thinking and the ability to shift between different cognitive tasks and executive ability to coordinate those processes. Major depression, dementia, Huntington's and Parkinson's diseases and various brain injuries are conditions which negatively influence the test outcomes.

Trail Making Test (TMT) assesses visual attention and psychomotor speed, speed of processing and scanning, mental flexibility, task switching and executive functioning.

California Verbal Learning Test (CVLT) measures episodic verbal learning and memory and demonstrates sensitivity to a range of clinical conditions. In the present study patients were assessed based on the following variables: total recall of trials 1–5 (learning performance), perseverative errors, intrusion errors, semantic clustering (the use of learning strategy of reorganizing listed words into categorical groups).

2.5. Cytokines, kynurenines and cortisol – secondary outcome measures

Fasting blood was collected between 8.00 a.m. and 9:00 a.m. for pro-inflammatory cytokines, kynurenines and cortisol measurements.

Tryptophan (TRP) and its metabolites were determined by high-performance liquid chromatography (HPLC). The chromatographic equipment was an Agilent 1200 series LC system (Agilent Technologies, Germany) composed of G1322 A degasser, G1311 A quaternary pump, G1329 A autosampler and G1330B thermostat for autosampler, HP1050 variable wavelength detector (VWD), HP1046 A fluorescence detector (FLD). Detectors were connected with a Waters Spherisorb S3 µm ODS2 150 x 2.1 mm column.

TRP, kynurenic acid (KYNA) anthranilic acid (AA) and 3-hydroxyanthranilic acid (3HAA) concentrations were determined according to Herve et al. (Herve et al., 1996). The column effluent was monitored by using a programmable FLD detector. The optimized conditions were determined by recording fluorescence spectra with a stop-flow technique. Excitation and emission wavelengths were set at 254/404 nm for TRP and KYNA, 320/420 nm for AA. The mobile phase consisted of 50 mM acetic acid, 0.25 M zinc acetate (pH-4.9), containing 1.2% of acetonitrile was pumped at a flow-rate of 0.2 ml/min.

Kynurenine (KYN) concentration was measured according to Holmes (Holmes, 1988). Using a VWD detector the column effluent was monitored at 365 nm. The mobile phase consisted of 0.1 M acetic acid, 0.1 M ammonium acetate (pH 4.65) containing 2% of acetonitrile and was pumped at a flow–rate of 0.2 ml/min.

Plasma TNF-α, IL-6, IL-1b concentrations were measured with high sensitive ELISA commercial kits according to manufacturer's recommendations (R&D Systems, TNF-α catalog number HSTA00D, IL-6 catalog number HS600B, IL-1b catalog number HSLB00C).

Plasma cortisol concentration was measured with ELISA commercial kits according to manufacturer's recommendations (IBL International, Germany, catalog number RE52061).

We also assessed baseline values and changes over the time of TRP and various kynurenines ratios such as KYN:TRP, KYNA:KYN, 3HKYN:KYN. Those parameters may indirectly reflect extent of conversion of kynurenines via kynurenine pathway and activities of different enzymes of kynurenine pathway namely: KYN:TRP – 2,3 dioxygenase indoleamine (IDO); KYNA:KYN – kynurenine aminotransferase (KAT); 3HKYN:KYN – kynurenine 3-monooxygenase (KMO) activities (Fig. 1).

2.6. Statistical analysis

Per protocol evaluations were carried out for all measured parameters. The repeated measure analysis of variance with Treatment (Placebo vs *LP299v*) as inter-subject factors and within-subject factor time effect (pre- vs post- intervention) were performed for all biochemical and psychometric measurements. Statistically significant ANOVA results for Treatment x time effect were further analyzed for their significance with *post hoc* Bonferroni test. For comparisons in groups' characteristics Student's *t*-test, nonparametric Mann-Whitney *U* test or *chi square* ($\chi 2$) analyses were used depending on measurement scale of variables. To test correlations between variables we used r Pearson's correlation coefficient.

3. Results

3.1. Randomization

One hundred eighteen patients with a diagnosis of major depressive disorder who attended the outpatient clinic of Stanislaw Deresz Psychiatry Hospital (Bialystok, Poland) were assessed for eligibility for the study. Thirty five patients did not meet inclusion criteria and 4 patients declined to participate in the study. Seventy nine patients were randomized and were allocated to the interventions. Of those, thirty

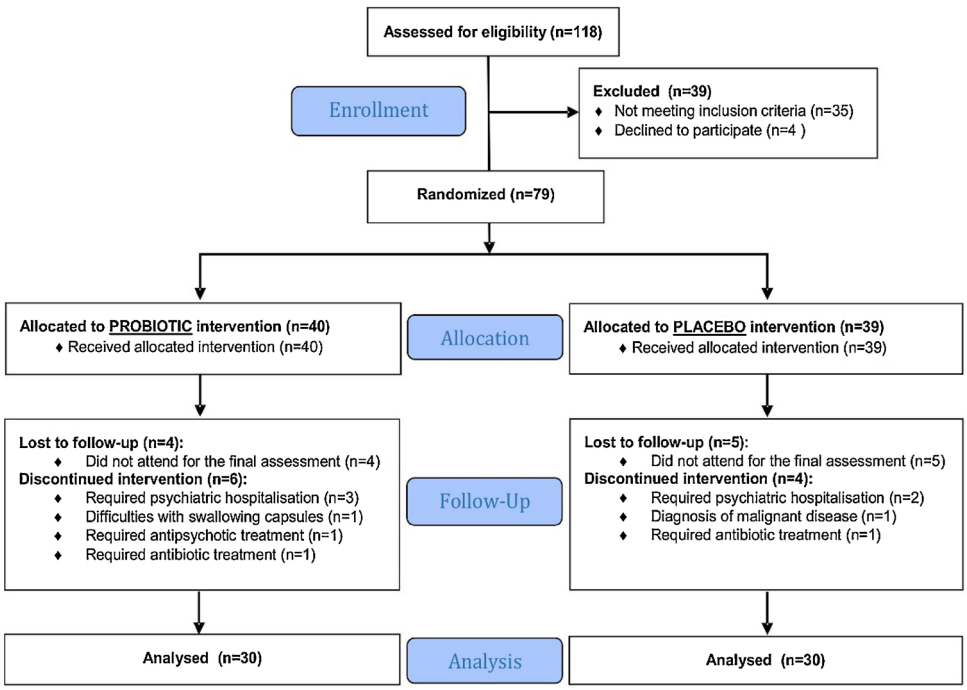

Fig. 2. CONSORT 2010 Flow Diagram of distribution of study participants during the trial.

patients in the probiotic group and 30 patients from the placebo group completed the study up to week 8. Fig. 2 represents the diagram of distribution of study participants during the trial.

3.2. Depressive and anxiety symptoms – primary outcome measures

There were no significant changes in Treatment x time effect (Repeated Measures ANOVA) in primary outcome measures such as Hamilton Depression Rating (HAM-D 17), Symptom Checklist (SCL-90) and Perceived Stress Scale (PSS-10) **(Table 4, supplementary material)**.

3.3. Cognitive functions – secondary outcome measures

Repeated Measures ANOVA revealed significant effect of interaction of Treatment x time in Work Speed in Attention and Perceptivity Test and Bonferroni *post hoc* analysis demonstrated significant improvement of this parameter in probiotic *LP299v* group between baseline and after 8 weeks of intervention when compared with placebo group at baseline and after 8 weeks of intervention (Table 3, Fig. 3).

Repeated Measures ANOVA revealed significant effect of interaction of Treatment x time in CVLT total recall of trials 1–5 and Bonferroni *post hoc* analysis demonstrated a significant improvement of this parameter in probiotic *LP299v* group between baseline and after 8 weeks of intervention when compared with placebo group at baseline and after 8 weeks of intervention **(**Table 3, Fig. 3**).** Due to methodological difficulties with collection of data in CVLT, statistical analyses of this test's results were performed on group of 27 patients in the placebo group and 24 patients in the probiotic *LP299v* group.

There was no significant effect of interaction of Treatment x time in Repeated Measures ANOVA in Stroop Test parts A and B, Ruff Figural Fluency Test (RFFT) and Trail Making Test (TMT) parts A and B in neither the probiotic *LP299v* nor placebo groups **(Table 5, supplementary material).** However, there was a trend ($p = 0.063$) in Treatment x time in Repeated Measures ANOVA towards increase of Total rotational strategies in Ruff Figural Fluency Test (RFFT) in probiotic *LP299v* group compared to the placebo group **(Table 5, supplementary material).**

Since our data for the number of errors in parts A and B of Stroop Test did not meet the criteria for parametric tests (Ordinal scale), we performed U Mann-Whitney Test but we did not find significant differences in errors of part A of Stroop Test (U = 447, $p = 0.932$) between placebo ($M_{rank} = 30.6$) and probiotic *LP299v* ($M_{rank} = 30.4$) and in part B of Stroop Test (U = 397, $p = 0.419$) between placebo ($M_{rank} = 32.27$) and probiotic *LP299v* ($M_{rank} = 28.73$) after intervention.

Since patients from both the probiotic and placebo groups did not have any errors in Attention and Perceptivity Test those results did not met the criteria for any statistical analysis.

3.4. Kynurenines, cytokines and cortisol – secondary outcome measures

In biochemical analyses Repeated Measures ANOVA revealed significant effect of interaction of Treatment x time in KYN concentration and Bonferroni *post hoc* analysis revealed a significant decrease in KYN concentration after 8 weeks of intervention in the probiotic *LP299v* group compared to the placebo group. Moreover, Bonferroni *post hoc* analysis revealed a significant decrease in KYN concentration in the *LP299v* group between baseline, and after 8 weeks of intervention **(**Table 3, Fig. 3**)**.

Repeated Measures ANOVA revealed a significant effect of interaction of Treatment x time for AA concentration. However, results of *post hoc* analysis did not reach statistical significance in neither probiotic nor placebo group (Table 3).

Repeated Measures ANOVA revealed significant effect of interaction of Treatment x time in 3HKYN:KYN ratio and Bonferroni *post hoc*

Table 3
Significant changes in kynurenines and cognitive parameters over time.

Variable	Treatment	Baseline M (SD)	Week 8 M (SD)	Treatment x time effect (Repeated Measures ANOVA)		Bonferroni *post hoc* test (*p*-value)			
				F	*p*	Baseline Placebo – LP299 V	Week 8 Placebo – LP299 V	Baseline – > Week 8 Placebo	Baseline – > Week 8 LP299 V
KYN (μmol/L)	Placebo	2.17 (0.76)	2.32 (0.8)	8.39	0.005[a]*	0.489[b]	0.010[b]*	0.105[b]	0.017[b]*
	LP299v	2.05 (0.5)	1.82 (0.63)						
AA (nmol/L)	Placebo	32.38 (18.31)	43.32 (34.51)	5.09	0.028[a]*	0.173[b]	0.261[b]	0.076[b]	0.176[b]
	LP299v	42.80 (35.18)	34.94 (19.97)						
3HKYN:KYN	Placebo	17.82 (5.96)	15.26 (4.19)	4.69	0.034[a]*	0.489[b]	0.085[b]	0.584[b]	0.015[b]*
	LP299v	15.88 (6.28)	27.68 (4.19)						
AP Test Work Speed	Placebo	718.73 (154.8)	750.13 (140.96)	8.19	0.006[a]*	0.497[b]	0.229[b]	0.078[b]	< 0.001[b]**
	LP299v	693.4 (131.09)	795.6 (148.76)						
CVLT total recall of trials 1-5	Placebo (n = 27)	46.67 (12.9)	44.41 (9.29)	5.51	0.023[a]*	0.110[b]	0.338[b]	0.349[b]	0.024[b]*
	LP299 V (n = 24)	41.21 (10.74)	47.13 (10.76)						

KYN: kynurenine; AA: anthranilic acid; AP Test: Attention and Perceptivity Test; CVLT: Californian Verbal Learning Test; *LP299v: Lactobacillus Plantarum 299v.*
All results are expressed as mean, standard deviation.
$p < 0.05$ *.
$p < 0.001$ **.
[a] Statistical analysis by Repeated Measures ANOVA.
[b] Statistical analysis by Bonferroni *post hoc* test.

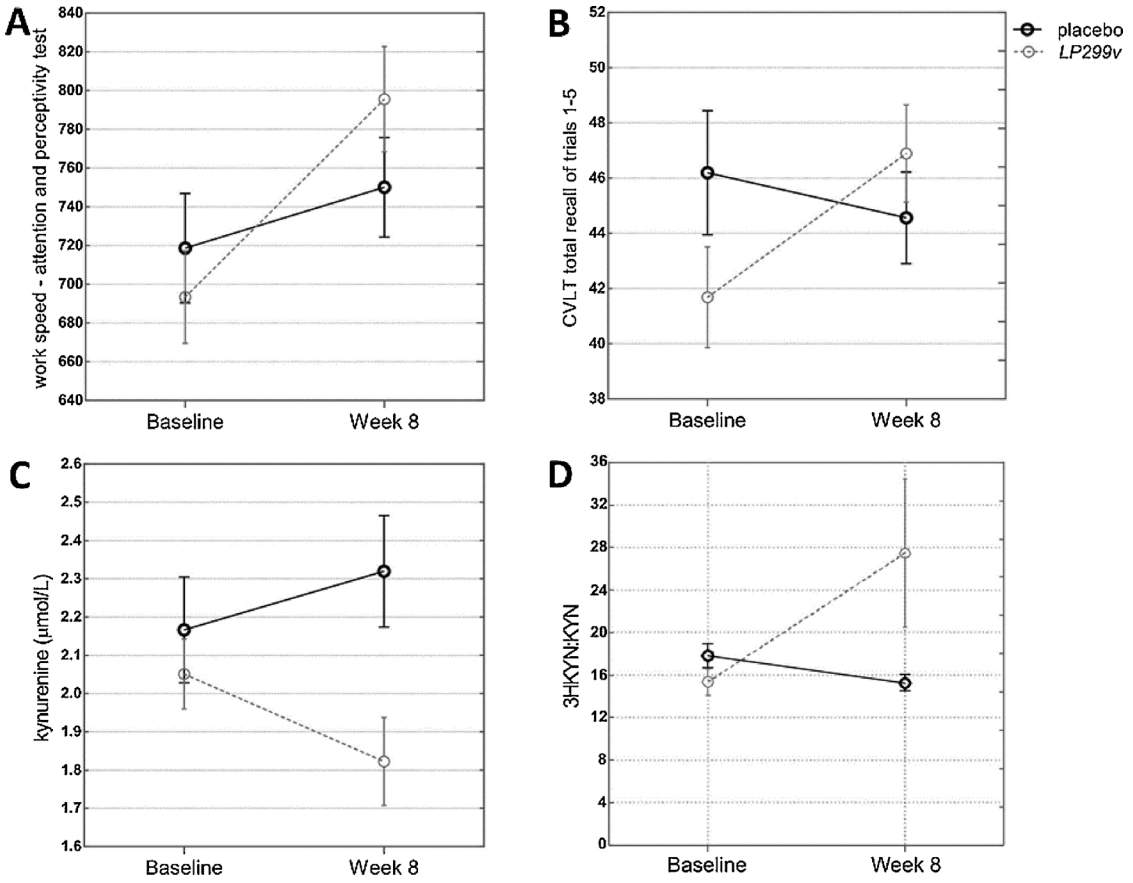

Fig. 3. Significant changes in psychometric and biochemical parameters during 8 weeks of the study. **A:** Improvement of work speed in Attention and Perceptivity test in the *LP299v* group compared to the placebo group. **B:** Improvement of CVLT total recall of trials 1–5 in the *LP299v* group compared to the placebo group. **C:** Decrease of kynurenine concentration in the *LP299v* group compared to the placebo group. **D:** Increase of 3HKYN:KYN ratio in the *LP299v* group compared to the placebo group.

analysis demonstrated significant increase of this parameter in the probiotic *LP299v* group between baseline and after 8 weeks of intervention when compared with the placebo at baseline and after 8 weeks of intervention **(**Table 3, Fig. 3**)**.

There was no significant effect of interaction of Treatment x time in Repeated Measures ANOVA in TRP, 3HKYN, KYNA and 3HAA concentrations in the probiotic and placebo groups. Moreover, there was no significant effect of interaction of Treatment x time in Repeated Measures ANOVA in KYN:TRP and KYNA:KYN ratios neither in the probiotic nor placebo groups **(Table 6, supplementary material)**.

We also did not find any significant effects of interactions of Treatment x time in Repeated Measures ANOVA in pro-inflammatory cytokines TNF-α, IL-6 and IL-1b, and cortisol concentrations neither in the probiotic nor placebo groups **(Table 6, supplementary material)**. Proportion of samples (25 patients) had undetectable levels of IL-1b and in those samples detection limit of the assay was allocated (0.01 pg/ml). For this reason Il-1b plasma results are to be interpreted with caution.

3.5. Adverse effects

During intervention patients did not experience any severe side effects neither in probiotic nor in placebo groups. There were no statistic significant differences in experienced adverse effects between placebo and probiotic groups (Table 4). Interestingly, all patients who experienced gastrointestinal side effects were those subjects who recently were commenced on SSRI (simultaneously with starting probiotic or placebo) and it seems highly likely that SSRI contributed to those side effects.

4. Discussion

To our knowledge this is the first time that administration of probiotic has been shown to decreased kynurenine concentration with subsequent improvement of cognitive functions in depressed patients. There are various hypothetical ways how probiotic bacteria *Lactobacillus Plantarum 299v* could contribute to a decrease of plasma KYN concentration with subsequent improvement of cognitive functions.

Increased intestinal permeability to enteric bacteria due to psychological stress may contribute to low grade inflammation with increased concentration of pro-inflammatory cytokines and increased

Table 4
Adverse effects during intervention.

Reported adverse effects	Placebo (n = 30)	LP299v (n = 30)	X^2	*p*
Neurological n (%)	4 (13.3)	4 (13.3)	0	1[b]
Headache	4 (13.3)	2 (6.6)	0.6	0.439[b]
Vertigo	0	1 (3.3)	1.02	0.313[b]
Tremor	0	1 (3.3)	1.02	0.313[b]
Gastrointestinal (n)%	2 (6.6)	1 (3.3)	0.35	0.554[b]
Looser stool	1 (3.3)	1 (3.3)	0	1[b]
Flatulence	1 (3.3)	0	1.02	0.313[b]
Cardiac (n)%	1 (3.3)	0	1.02	0.313[b]
Palpitations	1 (3.3)	0	1.02	0.313[b]

LP299v: *Lactobacillus plantarum* 299v.
$p < 0.05$ *.
[b] *chi square* test.

levels of detrimental kynurenines which could negatively influence mood and cognition (Maes et al., 2008, 2012; Maes et al., 2011). Bacterial LPS are able to induce IDO and it was demonstrated that peripheral LPS challenge up-regulated CNS expression of pro-inflammatory cytokines along with increased levels of neurotoxic kynurenines. Such a challenge also resulted in deficit in the novel object recognition and a single peripheral injection of KYN induced a deficit in recognition memory in mice. In healthy human LPS challenge had a dose dependent negative impact on cognitive functions. Moreover, microbial translocation and LPS are known to induce monocyte activation and trafficking into the CNS which is believed to be a key mechanisms in the pathogenesis of HIV-associated dementia.

Conversely, it was demonstrated that probiotic bacteria e.g. *Lactobacillus rhamnosus* and *Lactobacillus helveticus* prevented bacterial translocation and improved intestinal barrier function following psychological stress (Gareau et al., 2007; Zareie et al., 2006). Also, positive behavioral changes, attenuation of pro-inflammatory immune responses, changes in TRP and KYNA concertation, and in CNS monoamine activity due to probiotic *Bifidobactrium infantis* were demonstrated in rats (Desbonnet et al., 2010). *Lactobacillus Plantarum 299v* was previously described to have a positive impact on the intestinal barrier and its permeability, it decreased bacterial intestinal translocation, and led to the reduction of intestinal inflammation (Mangell et al., 2002; Schultz et al., 2002; White et al., 2006). Some of the qualities of this bacteria that could contribute to those effects are the ability to adhere to the intestinal mucosa due to expressing mannose-specific adhesins and the capability to enhance mucin production. *LP299v* inhibits growth and epithelial adherence of potentially pathogenic bacteria and fungi, increases number of beneficial intestinal *bifidobacteria* and *lactobacilli*, enhances SCFAs synthesis (Johansson et al., 1998) and positively modulates cytokines status (Schultz et al., 2002). All those mechanisms could contribute to the possible improvement of the gut barrier and subsequent decrease in KYN concentration in patients receiving probiotic *Lactobacillus Plantarum 299v*.

Another potential mechanism how *LP299v* could influence kynurenine concentration is related to modulation of IDO activity by hydrogen peroxide (H_2O_2). It was demonstrated that *Lactobacillus plantarum* is able to accumulate H_2O_2 (Murphy and Condon, 1984) and that H_2O_2 inhibits IDO activity (Freewan et al., 2013). Valladares et al. demonstrated reduced IDO activity in vitro, using HT-29 intestinal epithelial cells due to probiotic *Lactobacillus johnsonii.* Administration of this probiotic to rats resulted in increased H_2O_2 concertation along with decreased KYN synthesis (Valladares et al., 2013). In our study we did not find statistical difference in KYN:TRP ratio between the placebo and probiotic groups. KYN:TRP ratio is believed to reflect IDO activity, however it is rather a “sign post” in this context and it is not an objective assessment of IDO activity, and KYN:TRP ratio is also determined by tryptophan availability of IDO substrate – tryptophan. For those reasons, despite the lack of statistically significant changes in KYN:TRP ratio in our results, the changes in IDO activity due to probiotic *LP299v* should not be ruled out.

Synthesis of 5-HT by various bacterial strains including *Lactobacillus plantarum* is another way how those bacteria could modulate metabolism of TRP and kynurenines (O’Mahony et al., 2015). Increased 5-HT synthesis by probiotic bacteria could lead to decreased TRP availability for kynurenine pathway with subsequent reduction of KYN concentration. Interestingly, *LP299v* is known for its therapeutic effects in IBS (Ducrotte et al., 2012) and alterations in 5-HT biosynthesis, release, reuptake and intestinal content are believed to be a major factor of IBS symptomatology. It is likely that the beneficial effect of *LP299v* in IBS could be related to its modulation of TRP metabolism in serotoninergic and kynurenine pathways and those mechanisms could have contributed to decreased KYN concentration in *LP299v* group in our study.

Finally, another exciting field of interaction between microbiota and the kynurenine pathway is the ability of synthesis of various enzymatic cofactors by those beneficial bacteria. It was previously described that human intestinal microbiota are able to produce multiple vitamins, such as K, various vitamins of group B including folates, pyridoxine, biotin, nicotinic acid, riboflavin, thiamine, pantothenic acid and cobalamin (Gu and Li, 2016; Hill, 1997). Some of those vitamins e.g. vitamin B2, B6 are cofactors in various steps of kynurenines synthesis (Ueland et al., 2017). For instance, pyridoxal 5′-phosphate (PLP), an active form of pyridoxine (vitamin B6), is a cofactor for kynurenine aminotransferase (KAT) and kynureninase. Furthermore, flavine adenine dinucleotide (FAD), an active form of riboflavin (vitamin B2), is a cofactor for kynurenine 3-monooxygenase (KMO) (Oxenkrug et al., 2013; Ueland et al., 2017) **(**Fig. 1**)**. Moreover, folate and pyridoxine need riboflavin for conversion to their bioavailable forms. Previously, it was demonstrated that vitamin B6 administration increased kynurenine pathway enzymes activities with subsequent decrease of KYN levels (Hankes et al., 1971; Leklem, 1971). Interestingly, *Lactobacillus plantarum* strains were shown to synthesize vitamin B2 (Thakur et al., 2016) and administration of *Lactobacillus Plantarum 299v* to healthy volunteers significantly increased numbers of faecal *lactobacilli* and *bifidobacteria* (Johansson et al., 1998) and the latter are well known producers of vitamin B6 (Deguchi et al., 2014). In our study we observed increased 3HKYN:KYN ratio, reflecting KMO activity, in the *LP299v* group but without significant changes in plasma concentrations of 3HKYN nor 3HAA. This could indicate that there was activation of KMO in the *LP299v* group with increased metabolism of KYN towards nicotinamide adenine dinucleotide (NAD+) and ATP synthesis but without accumulation of detrimental kynurenines **(**Fig. 1**)**. This activation could be related to increased synthesis of enzymatic cofactors of kynurenine pathway, such as vitamins B2 and B6 by probiotic bacteria. It is also worth mentioning that psychological stress and inflammation may result in increased utilization and decreased concentrations of abovementioned cofactors of kynurenine pathway with subsequent accumulation of detrimental kynurenines (Paul et al., 2013; Theofylaktopoulou et al., 2014). Consequently, bacterial influence on vitamins B status seems to be another mechanism of gut-brain axis regulation and further research of this potential therapeutic direction is desirable.

On the other hand, increase of 3OHKYN:KYN ratio observed in our study could be solely related to decreased KYN availability in *LP299v* group.

Another aspect is how decreased KYN concentration could contribute to the improvement of cognitive functions in our study. The majority, about 60% of CNS KYN is derived from the periphery after crossing the blood brain barrier (BBB) and changes in plasma KYN concentrations are believed to have a significant influence on CNS kynurenines metabolism (O’Mahony et al., 2015). Increased brain KYNA levels are known to cause cognitive deficits in animals and humans, and increase of KYN and KYNA levels (e.g. in pre frontal cortex - PFC) have been previously described in schizophrenia, bipolar disorder, Alzheimer’s and Parkinson’s diseases. It is believed that elevated KYNA levels could be a major contributor of cognitive deficits due to inhibition of glutamatergic and acetylocholinergic neurotransmissions. Decreased KMO activity with subsequent increased brain KYNA levels along with neurocognitive deficits where described and increased kynurenine levels in the PFC of schizophrenic patients’ postmortem brain tissue correlated with KYNA brain tissue levels (Schwarcz et al., 2001; Wonodi et al., 2011). Besides various examples where elevated kynurenines levels had negative consequences on cognitive functions, there are also scientific data confirming that physiological levels of KYN are involved in baseline cognitive functions (Stone and Darlington, 2013). The deletion of KAT II, the major enzyme responsible for synthesis of KYNA resulted with 71% decrease of hippocampal KYNA concentration and increase of extracellular glutamate in the rats’ CNS. This was accompanied by improvement in synaptic plasticity along with improvement of cognitive functions namely object exploration and recognition, passive avoidance, and spatial discrimination. QUIN is another KYN metabolite detrimental to cognitive functions. It was

demonstrated that patients with HIV-associated dementia had increased concentration of this compound in cerebrospinal fluid. In Alzheimer's disease increased activity of IDO and increased QUIN concentration were detected in neurofibrylary tangles and in the perimeter of senile plaques suggesting the role of kynurenine pathway in pathogenesis of this disorder. In animal studies sub chronic intraventricular infusion of QUIN produced impairment in working memory. Furthermore, due to a significant body of evidence of kynurenine pathway involvement in cognitive functions, this field became an intriguing topic in drug development and various studies were undertaken on the improvement of cognitive functions through modulation of kynurenine pathway (Stone and Darlington, 2013).

Despite changes in kynurenine pathway and improvements of cognitive functions in probiotic group, there were no observed differences in affective parameters between groups. We hypothesize that due to the lack of influence of *LP299v* on plasma TRP concentration, a precursor of central 5-HT, there was no change of TRP brain availability and subsequently no change in the central 5-HT synthesis. This could contribute to the lack of differences in affective parameters of major depression between the probiotic and placebo groups during the treatment. In contrast, decreased peripheral KYN concentration due to *LP299v* observed by us could influence CNS kynurenines levels with consequent improvement in some of the cognitive parameters.

We did not observe more conclusive correlations between psychometric parameters and biochemical measurements, such as KYN concentration and improvement of psychometric parameters (data not shown). However, what should be taken into account is that despite the fact that peripheral KYN concertation influences further synthesis of CNS kynurenines, we did not measure cerebrospinal fluid kynurenines levels which would be obviously much more precise assessment of CNS kynurenines metabolism. Moreover, CNS regional distribution of kynurenines and their region specific functions require far more research involving imaging studies along with cognitive studies to determine more precisely involvement of kynurenine pathway in neurophysiology. Taking this into account we see results of our study as the first steps in building up understanding of complex interactions between microbiota, kynurenines and CNS functions in the perspective of major depressive disorder and discussed above hypothetical models point to exciting directions for future studies.

4.1. Limitations and future directions

There are few limitations in our research which could be also seen as directions for future research. Firstly, due to methodological difficulties with collecting of data in CVLT we performed our analyses on smaller groups of patients - 27 patients in the placebo group and 24 patients in the probiotic *LP299v* group. Second, it could be valuable to perform measurements of intestinal permeability e.g. lactulose/mannitol ratio test to assess whether probiotic could influence this parameter and if changes of intestinal permeability could influence parameters measured by us. Thirdly, we were not able to measure levels of QUIN and this parameter would be valuable in more precise assessment of dynamics of kynurenine pathway. Also, due to logistic and financial limitations, despite extensively discussed potential role of vitamins B in our study, we were not able to assess those parameters. However, measurements of vitamins B levels e.g. vitamin B2 and B6 seems to be an exciting, valuable and recommended direction for the future research in context of microbiota-gut-brain axis. Fourthly, due to logistic limitations we were able to perform cortisol analysis in a single time point. We did not observe significant differences in cortisol concentration between placebo and probiotic groups during the treatment. However, due to restrictions in quality of single time point cortisol analysis, the impact of HPA axis in mediating reduction in kynurenine concentration should not be excluded and those results are to be interpreted with caution.

Furthermore, patients who did not complete the study were, from practical and financial reasons, excluded from biochemical analyses of cytokines, kynurenines and cortisol. For those reasons we were not able to include them in our statistical analyses and for the homogeneity, and consistency of our results we performed per protocol analysis. Moreover, some of our results e.g. number of total rotational strategies in RFFT revealed a tendency towards statistically significant results and possibly research on a larger group of participants could be more conclusive. Finally, another important aspect is that we excluded patients with inflammatory diseases, such as intestinal and autoimmunological disorders. It seemed beneficial in the context of group homogeneity, however, individuals with gastrointestinal and extra intestinal autoimmune diseases, who often suffer from comorbid depression and who seem to be more prone to increased intestinal permeability, possibly would be a group of patients where we could expect more dynamic, and more significant changes in kynurenines, cytokines and cortisol levels due to probiotic intervention. Also, in our study we focused on one particular bacterial strain. However, due to various specific effects and mechanisms of action of different bacterial strains, it could be beneficial to explore if multispecies probiotic formulas could have positive influence on psychometric and biochemical parameters in various psychiatric disorders.

5. Conclusions

Augmentation of SSRI treatment with probiotic bacteria *Lactobacillus Plantarum 299v* improved cognitive performance and decreased kynurenine (KYN) concentration in depressed patients compared to placebo. Decreased kynurenine concentration could contribute to the improvement of cognitive functions in a group of patients receiving *LP299v* compared to placebo.

Declaration of interests

None.

Appendices

None.

Funding

This work was supported with grants from Medical University of Bialystok, Poland.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.10.010.

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