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Normalization of Deviant Behavior in *Muc2*$^{+/+}$ Mice through Dietary Incorporation of *Bacillus subtilis* Spores

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Abstract: It is well-known that the intestinal microbiota influences the host's health, including the activities of the central nervous system. This phenomenon is known as the brain-gut axis. Mental and emotional disorders are now fairly widespread. Many of them are lifelong, and medications frequently cause side effects. Not surprisingly, researchers have been drawn to the study of probiotic bacterial cultures as an adjunct or primary therapy for psycho-emotional illnesses. There is a lot of evidence that bacterial strains from the genera *Bifidobacterium* and *Lactobacillus* have a favorable influence on the activities of the central nervous system. However, the effect of *Bacillus* spp. on the behavior is not clearly known. In contrast to *Muc2*$^{-/-}$, the wild type mice (*Muc2*$^{+/+}$) have normal gut barrier function, but the intestinal microbiota and behavior are similar to their mutant siblings *Muc2*$^{-/-}$ mice. Similar to the mutant sibling, *Muc2*$^{+/+}$mice exhibit greater locomotor activity in the open field and light-dark tests, as well as decreased anxiety in the open field test, light-dark test, and marble burying tests. The addition of kanamycin-resistant strain *Bacillus subtilis* BS20 spores to the diet of *Muc2*$^{+/+}$ mice for 3 weeks at 10^7 CFU/g led to a change in behavior and cytokine levels in the gut compared to control C57BL/6 mice. Furthermore, a drop in serotonin and a rise in tyrosine were identified in the blood serum. Changes in cytokines, serotonin, and tyrosine levels may mediate the normalizing impact of *B. subtilis* spores on *Muc2*$^{+/+}$ mice behavior.

Keywords: *Bacillus subtilis*; mice; behavior; cytokines; serotonin

1. Introduction

Currently, a lot of study has been done on the impact of intestinal microbiota on the host organism, including psycho-emotional health [1-3]. It is well known that the presence of some microorganisms in the intestines can cause or predispose to certain diseases [4-7]. Therefore, one of the therapeutic strategies is to modulate the intestinal microbiota in such a way that it contributes to the host's health [8, 9]. Microbiota can be modulated by fecal transplantation, antibiotics, probiotics, prebiotics, and symbiotics [10–17]. It is also interesting to explore the impact of the gut microbiota, both commensal and allochthonous, that transit from the environment. It is well known that probiotics have no side effects, and are quite effective. There are studies of both classical strains of lacto- and bifidobacterium that have wide presence in the pharmacy, and are looking for new ones [18]. Among the new probiotics, spore-forming bacteria *Bacillus* spp. are interesting. Most used probiotic cultures are *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus licheniformis*, *Enterococcus faecium*, *Enterococcus faecalis* [19–21]. Initially, these microorganisms were developed as an alternative to antibiotics and utilized in agriculture [22–25]. This experience was so successful that probiotics based on *Bacillus* spp. are currently used in human therapy. This is accompanied by a greater interest in understanding the mechanism of action of *Bacillus* spp. on humans and animals. *Bacillus* spp. are producers of a large number of bioactive substances [26]. *Bacillus* spp. is known to improve farm animal productivity as well as their resistance to diseases, particularly diarrhea [25, 27–29]. In humans, *B. subtilis* has also been proven to help to relieve abdominal discomfort, gas, and bloating, inflammatory bowel disease, obesity and diabetes, and other conditions [30–33].

Since the bacteria *Bacillus* spp. are new probiotics for humans, they are less studied than lacto- and bifidobacterium. In particular, there is not enough data on the effect of *Bacillus* spp. on the behavior. At the same time, the effect of classical probiotic strains is studied and shown integration into the brain-gut axis. Bacteria are able to produce important neurotransmitters in the gut [34]. Probiotic strains from the genera *Bifidobacterium* and *Lactobacillus* have a neuroprotective impact and have a beneficial impact on anxiety and depression-like behavior via their effects on the serotonergic and immunological systems [34, 35]. Intestinal colonization of mice with *Bifidobacterium longum* reduced mice anxiety and restored BDNF levels [36]. The *Bifidobacterium infantis*-based probiotic also normalized rat behavior, as shown to reduce the time of immobility in the forced swim test [37]. It is crucial to point out that the *Bacillus* spp. have the capacity to impact psycho-emotional health, since *B. subtilis*, for example, is a reasonably good generator of tryptophan, a precursor of serotonin [38]. *B. subtilis* has also been shown to affect serotonergic, noradrenergic, and dopaminergic systems in broiler chicken brains [39].

Previously shown that *Muc2*$^{-/-}$ mice with impaired gut barrier function behave differently than the parent strain C57BL/6 mice [40]. These behavioral impairments were also observed in *Muc2*$^{+/+}$ siblings with normal gut and gut microbiota like *Muc2*$^{-/-}$ mice. As a result, we conclude that *Muc2*$^{-/-}$ behavioral impairments are driven by alterations in the intestinal microbiota rather than an inflammatory process.

The present study assesses the effect of feeding spores of *B. subtilis* BS20 on the behavior of *Muc2*$^{+/+}$ males. These mice have normal gut barrier function, but an altered gut microbiota that is passed on to them from their *Muc2*$^{-/-}$ mothers. Since the bacteria *Bacillus* spp. are allochthonous, they are constantly present in small amounts in the diet of laboratory mice [41, 42]. Since *Bacillus* spp. are present in mouse diet, it was decided to determine if there is enough to change behavior. To address this, two groups of mice fed autoclaved and non-autoclaved diets were compared. To increase the bacterial titer in the diet, spores of the laboratory strain *B. subtilis* BS20 were added. Since *Bacillus* spp. in the diet did not have kanamycin resistance, the strain *B. subtilis* BS20 with resistance to kanamycin was chosen. This allowed for the count of bacteria in the feces that have progressed past the vegetative phase. *B. subtilis* BS20 were added to both autoclaved and non-autoclaved diets of *Muc2*$^{+/+}$ mice. And the CFU of the diet supplemented strain in the feces of mice was measured, also the effect of the presence of *B. subtilis* on immune parameters as an example of cytokines, blood levels of serotonin, tryptophan, and tyrosine, and mice behavior was determined in this study.

2. Materials and Methods

2.1. Animals

The study was carried out at the SRINM (Scientific Research Institute of Neurosciences and Medicine). We used two-month-old male *Muc2*$^{+/+}$ mice fed both an autoclaved (16 mice) and non-autoclaved (9 mice) diet. *Muc2*$^{+/+}$ mice were fed a diet of chow soaked in a suspension of *B. subtilis* BS20 spores at 10^9 spores/g for three weeks after the first series of behavioral testing. The mice ate about 4 g of food per day. After three weeks of feeding on the diet with *B. subtilis* BS20 spores, these mice were tested again. And as a behavioral control, 2-month-old male C57BL/6JNskrc mice (sub-colony of C57BL/6J) were fed a non-autoclaved diet.

Muc2$^{-/-}$ mice were obtained after rederivation of *Muc2*^{tm1Avel}/*Muc2*^{tm1Avel} mice that had a genetic background of C57BL/6J mice [43] used CD1 mice with specific-pathogen free (SPF) status as recipient and next back-crossing to C57BL/6JNskrc [37]. *Muc2*$^{+/-}$ males and females are currently used in breeding because *Muc2*$^{-/-}$ mice exhibit early rectal prolapse, which makes them unsuitable for breeding. The genotypes of the offspring were determined.

To investigate the immunological profile of *Muc2*$^{+/+}$ mice fed with *B. subtilis* BS20 as control animals, intact male *Muc2*$^{+/+}$ mice of the same age (4 months at the time of slaughter) were utilized and housed under the same circumstances as the experimental mice.

The mice were kept in groups of 4–5 animals in individually ventilated cages at a temperature of 22–24°C. Photoperiod was 12h light and 12 h dark (turn off at 12:00 h local time). Water and diet were available *ad libitum*. Cages with bottles, birch sawdust as bedding, diet was autoclaved with 2 atm, 121 °C for 30 min using GE 91415 AR-2 (Getinge Sterilization AB, Getinge, Sweden).

Manipulations with animals were carried out under institutional ethical committee guidelines, Russian legislation according to the standards of Good Laboratory Practice (directive # 267 from 19.06.2003 of the Ministry of Health of the Russian Federation) and the European Convention for the protection of vertebrate animals. Study approved by the Bioethical committee at SRINM, protocol #3 dated 19.05.2022. SPF status of mice validated quarterly as per Federation of European laboratory animal science association's (FELASA) recommendations [44]. Results are presented as recommended ARRIVE guidelines [45].

2.2. Growth of Bacillus subtilis

The laboratory strain *B. subtilis* BS20, which is resistant to kanamycin, was used. The strain was determined by the need to determine the number of bacteria that have passed through the gastrointestinal tract. *B. subtilis* was confirmed by mass spectrometry on the Microflex LT MALDI Biotyper Bruker Daltonik (Germany) and using the VITEK2 Compact Biomerieux (France) with related identification cards BCL. Previously, this strain of bacteria was not assessed for physiological parameters of the host organism. To enable the bacterium to undergo a vegetation cycle from the same stage, a spore suspension was used. Spores of *B. subtilis* BS20 were added to the diet of mice. Strain of bacteria was able to grow on a selective medium with kanamycin. It allows for the quantification of CFU of *B. subtilis* BS20 that was eaten by mice. Growth media for *B. subtilis* BS20 was Dextrose Casein–peptone agar (Merck, Darmstadt, Germany). The bacteria were allowed to grow at + 37 ◦C for 72 to 96 hours till spore formation. By heating the suspension to 85 ◦C for 15 minutes, vegetative forms were eliminated. Spores of *B. subtilis* BS20 were diluted in sterile phosphate-buffered saline (PBS) and were added to the mice's diet [46].

2.3. Count CFU of Bacillus subtilis BS20

The diet and feces were used to count the CFU of *B. subtilis* BS20. Ten g of diet pellet homogenized in 9 mL of PBS (sterile). One hundred mg of feces were resuspended in 0.9 mL of PBS (sterile). Serial dilutions were conducted to measure the titer of CFU. All dilutions were grown on Dextrose Casein-peptone agar (Merck, Darmstadt, Germany) with 0.001% kanamycin (AO Biochimik, Russia) under aerobic condition of 48 h at 36 ◦C.

2.4. Marble burying test

Twenty glass marbles (1 cm diameter) were used in the marble burying tests. Marbles were laid on the top of sawdust with 4 cm thickness. Chamber for the test was a plastic box with 37 x 21 x 15 cm in dimension (width x length x height). Mouse was placed in a plastic box for 30 minutes. During this time, the mouse explored cages and burned marbles. The number of marbles buried at least two-thirds deep were counted.

2.5. Light–dark test

To conduct the light–dark test, a box (42 × 21 × 25 cm) divided into a dark compartment (one-third of the total space) and a light compartment under illumination (two-

thirds of the total space) was used. The compartments were connected to each other via a pass (3 cm × 4 cm). At the start of the test, the mouse was placed in the middle part of the dark compartment. All of the movements of each mouse were recorded by video during the first 5 min of the test. The distance, and time in the light compartment were analyzed using EthoVision XT10 software (Noldus, Wageningen, the Netherlands).

2.6. Open field test

Open field tests with mice were conducted in a square plastic setting sized 40 cm × 40 cm with 15 cm walls and a transparent bottom that lights up in red. Mouse was placed in the central square (20 cm × 20 cm). The movements of each mouse were recorded by video during the first 6 min. Distance and time in the center were analyzed using the EthoVision XT10 software (Noldus, Wageningen, the Netherlands).

2.7. Cytokine level assay

Cytokine levels of colon samples were assayed by multiplex assay according to the manufacturer's instructions. Briefly, samples were homogenized in liquid nitrogen, then resuspended in PBS (100μL). Samples were centrifuged for 15 min at 12,000 rpm 4 °C. Supernatant were collected and used for assay cytokine by Magnetic Luminex assay Kit for Mouse Cytokine/Chemokine Magnetic Bead Panel (Cloud-Clone, China). Cytokine level was calculated by Luminex 200 System (Merck, Germany) with xPONENT 3.1. software. Concentration of cytokines (pg/mg of total protein) normalized by protein that assayed by Bradford method [47].

2.8. HPLC-FLD analysis

The frozen plasma was thawed at room temperature, then mixed with 0.4M perchloric acid solution (1:1, v/v), containing 4 μg/mL of 3,4-dihydroxybenzylamine hydrobromide as internal standard (IS) and vortexed for 30 sec for protein precipitation. The sample was centrifuged at 20000g at 4°C for 15 min, and the resulting supernatant was analyzed by HPLC-FLD.

For the chromatographic analyses, an Agilent 1260 Infinity HPLC system (Agilent Technologies, Singapore) was used with G1329B autosampler, G1311C quaternary pump, G1315D DAD and G1321B fluorescence detector. Agilent ChemStation for LC systems B04.03 software was used to process HPLC data.

To determine the optimal lengths for FLD, spectral data were collected in the range of 220–380 nm (excitation) and 300–500 nm (emission) for each fluorescent compound, i.e., L-DOPA, L-dopamine, 3,4-dihydroxybenzylamine hydrobromide (internal standard – IS), tryptophan, 5-HT and tyrosine. Peaks were identified by comparing their retention time, UV and fluorescence emission spectra in the sample with that of standard solutions.

Isocratic separations were run on a reversed-phase C18 column (Zorbax-SB, 250×4.6mm I.D., 5 μm particle size, Agilent, CA, USA) with a guard column (Zorbax-SB, 12.5×4.6mm). The temperature of column was 35°C. Mobile phase consisted of 13% acetonitrile/methanol (1:1 v/v) and 87% of buffer (25 mM of potassium dihydrogen phosphate, 1.85 mM of octane-1-sulfonic acid sodium salt as ion-pair reagent, pH 3.0 adjusted with ortho-phosphoric acid). Flow rate was 1 mL/min, injection volume was 5 μL.

Elution control was performed using a PDA detector (collects UV-Vis spectra from 190 to 500 nm) and a fluorescence detector. The fluorescence detector has 2 channels. The first channel (excitation at 279 nm and emission at 315 nm for detection) was used to measure L-DOPA, tyrosine, IS and L-dopamine. The second channel (excitation at 279 nm and emission at 340 nm) was used for the detection of L-tryptophan and 5-HT.

2.9. Statistical Analysis

The data are shown as the mean ± standard deviation. Statistical analysis of the data was carried out using the Statistica 10.0 software. Distribution was assessed using the Kolmogorov–Smirnov test. Mann–Whitney U test for independent groups were applied for non-normally distributed values. Analyzing the results of behavioral tests, we used *t*-test for dependent samples when comparing the results of the same mice before and after feeding *B. subtilis* BS20, and *t*-test for independent samples when comparing independent groups.

3. Results

3.1. Analysis of CFU Count in diet and feces

Before undertaking behavioral testing, it was confirmed that viable *B. subtilis* spores were present in the mice's diet and feces. Fecal suspensions were plated and growth on Dextrose Casein-peptone agar with kanamycin. The number of *B. subtilis* BS20 in the autoclaved diet was 1.25±0.05 x 10^9 CFU/g, whereas the non-autoclaved diet was 2.3±0.7 x 10^9 CFU/g. The number of *B. subtilis* BS20 spores in the feces of mice fed autoclaved diet with *B. subtilis* was 1.8±0.23 x 10^7 CFU/g, while the number of spores in the feces of mice fed non-autoclaved diet with *B. subtilis* was 2.97 ±0.15 x 10^7 CFU/g. Spores of *B. subtilis* BS20 pass through the acidic environment of the stomach with little loss.

3.2. Mouse behavior study

Previously shown that *Muc2*$^{+/+}$ males vary in behavior from the C57BL/6 males despite both having mucin 2 and a healthy gut [40]. *Muc2*$^{+/+}$ mice exhibit higher locomotor activity in the open field and light-dark tests, as well as decreased anxiety in the open field, light-dark box, and marble burying tests.

In the open field test, *Muc2*$^{+/+}$ males on non-autoclaved diet had higher locomotor activity than C57BL/6 males (n=9) ($p< 0.05$, t=-2.13, Student's *t*-test, Fig. 1 A). Locomotor activity was considerably reduced after feeding diet with *B. subtilis* BS20 in both mice on

non-autoclaved diet (*Muc2*$^{+/+}$, n=9) (p<0.001, t=6.66 paired Student's *t*-test) and on autoclaved diet (*AD-Muc2*$^{+/+}$, n=16) (p< 0.001, t=4.26, paired Student's *t*-test).

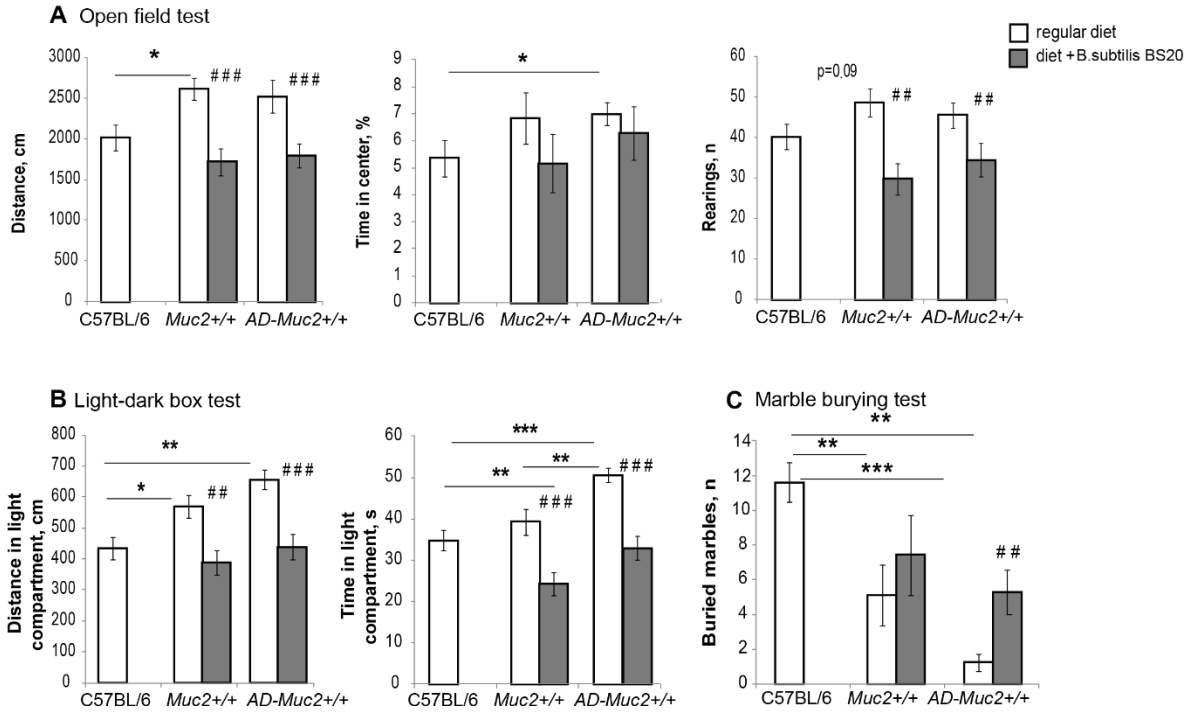

Figure 1. Behavior of C57BL/6 mice (n=9) and *Muc2*$^{+/+}$ fed a non-autoclaved diet (n=9) and autoclaved diet (n=9) with *B. subtilis* BS20 spores. (A) Open field test: distance in open field, cm; time in center of open field, %; number of rearing. (B) Light-dark box test: distance in light compartment, cm; time in light compartment. (C) Marble burying test: number of buried marbles.
* p < 0.05, ** p < 0.01, *** p < 0.001 *t*-test for independent samples (Open field test, Light-dark box test)
# p < 0.05, ## p < 0.01 , ### p < 0.001 *t*-test for dependent samples (Open field test, Light-dark box test)
* p < 0.05, ** p < 0.01 Mann–Whitney *U*-test (Marble burying test)

In the center of the open field, *Muc2*$^{+/+}$ males only on autoclaved diet stayed significantly longer than C57BL/6 males (p<0.05, t=-2.18, Student's *t*-test, Fig. 1 A). Feeding *B. subtilis* BS20 did not significantly affect the time stayed in the center for *Muc2*$^{+/+}$ mice on non-autoclaved diet (p>0.05, t=1.72 paired Student's *t*-test) and on autoclaved diet (p>0.05, t=0.67 paired Student's *t*-test).

In the light-dark box test, *Muc2*$^{+/+}$ mice had increased locomotor activity in the light compartment compared to C57BL/6 mice, both when fed autoclaved diet (p<0.001, t=5.37, Student's *t*-test, Fig. 1 B) and non-autoclaved (p<0.05, t=2.69, Student's *t*-test). After the addition of *B. subtilis* BS20 spores to the diet, the locomotor activity in *Muc2*$^{+/+}$ mice of both groups significantly decreased on non-autoclaved (p<0.01, t= -4.78, paired Student's *t*-test) and on autoclaved diet (p<0.001, t=-6.79, paired Student's *t*-test).

Only the *Muc2*$^{+/+}$ mice on autoclaved diet significantly differed from C57BL/6 in spending longer time in the light compartment (p< 0.001, t=-5.3, Student's *t*-test, Fig. 1 B). *Muc2*$^{+/+}$ mice on autoclaved diet were also significantly longer in the light compartment

compared to *Muc2*$^{+/+}$ mice on non-autoclaved diet (p<0.01, t=-3,36, Student's *t*-test). After feeding *B. subtilis* BS20, the stay in the light compartment decreased in both groups of the *Muc2*$^{+/+}$mice with non-autoclaved diet (p<0.001, t=6.65, paired Student's *t*-test) and with autoclaved diet (*AD-Muc2*$^{+/+}$) (p<0.001, t=5.49, paired Student's *t*-test). In mice of the *Muc2*$^{+/+}$ feeding non-autoclaved diet with *B. subtilis* BS20 time in the light compartment was lower than C57BL/6 (p<0.01, t=2.9, Student's *t*-test).

In the marble burying test, *Muc2*$^{+/+}$males of both groups without *B. subtilis* BS20 buried significantly fewer marbles compared to C57BL/6 (p<0.01, t=3.18, Student's *t*-test and p< 0.001, t=9.65, Student's *t*-test, corresponding, Fig. 1C). After adding *B. subtilis* BS20 to the diet, the *Muc2*$^{+/+}$ group with autoclaved diet buried significantly more marbles to compare with mice before adding *B. subtilis* BS20 to the diet (p<0.01, t=-3.34, paired Student's *t*-test). However, the level of buried marbles by C57BL/6 mice was not reached (p< 0.01, t=3.27, Student's *t*-test).

It can be concluded that *Muc2*$^{+/+}$ mice differed from C57BL/6, as was previously demonstrated [40]. *Muc2*$^{+/+}$ mice increased motor activity in the open field test and decreased anxiety, as evidenced by spending a lot of time in the center of the open field and buried fewer marbles. Such behavior was typical for *Muc2*$^{+/+}$ mice that fed an autoclaved diet compared to those fed non-autoclaved diet. It is crucial to highlight, however, that comparing C57BL/6 with a *Muc2*$^{+/+}$ mice, both provided a non-autoclaved diet is sufficient to get a difference in behavior. It was interesting to know whether the behavior of *Muc2*$^{+/+}$ mice that fed autoclaved diet would be different from that of those fed non-autoclaved one. We can observe that an autoclaved diet had no significant effect on behavior. The exception is time spent in the light compartment, where *Muc2*$^{+/+}$ mice fed an autoclaved diet stayed in the light compartment substantially longer than mice fed non-autoclaved diet. The addition of *B. subtilis* BS20 spores to the diet normalized the behavior of both groups of *Muc2*$^{+/+}$ mice, making them closer to the C57BL/6. In both groups of *Muc2*$^{+/+}$ mice reduced motor activity in the open field and light-dark tests, the time in the light compartment and the number of buried marbles. Commonly, laboratory animals are fed on non-autoclaved diet. Given the behavior of the *Muc2*$^{+/+}$ mice on autoclaved and non-autoclaved diets did not differ significantly, all experiments were done with mice fed a non-autoclaved diet.

3.3. Immunological multiplex analysis of the distal colon of mice

There are various processes by which the gut microbiota impacts the central nervous system, one of which is immune system activation. Thus, we measured the level of cytokines in the colon, an organ where the immune system is abundant and is in direct contact with the microbiota. *Muc2*$^{+/+}$ mice with healthy gut do not differ in the level of colon cytokines from C57BL/6 (Fig. 2). Significant difference was found only in the level of IL13 (Z = 1.96, p=0.049; Mann–Whitney U test, Fig.2 B). There were no changes in the cytokine profile after adding *B. subtilis* BS20 since *Muc2*$^{+/+}$ did not differ from the C57BL/6. However, there was a decrease in the IL10 level at the trend level (Z = 1.77, p = 0.08; Mann–Whitney U test),

and a significant decrease in IL13 (Z = 2.12, p = 0.03; Mann–Whitney U test) and IL17 (Z = 2.12, p = 0.03; Mann–Whitney U test) to a level close to that of C57BL/6.

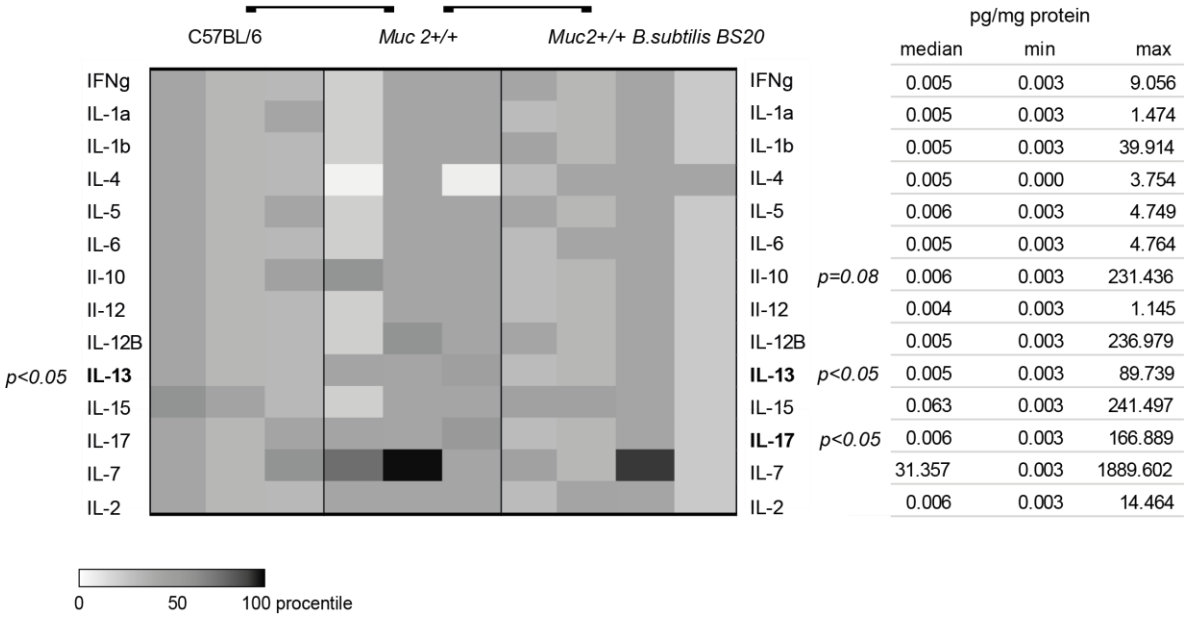

Figure 2. Cytokine profile of the distal colon of male mice C57BL/6 (n=3), *Muc2*$^{+/+}$ (n=3) fed diet with and without *B. subtilis* BS20 spores (*Muc2*$^{+/+}$ *B. subtilis* BS20, n=4). Heat map of cytokine levels in the distal colon, pg/mg.
* p < 0.05, Mann–Whitney *U*-test

Therefore, adding *B. subtilis* BS20 to the diet normalizes even minor aberrations in cytokine levels of *Muc2*$^{+/+}$ mice.

3.4. HPLC-FLD analysis of blood serum of mice

Bacterial metabolites also affect the central nervous system. The levels of serotonin and its precursor tryptophan, as well as tyrosine, were measured in mice blood serum using the HPLC method.

Muc2$^{+/+}$ mice fed with *B. subtilis* BS20 had a drop in serum serotonin (Z=2.12, p=0.034 Mann–Whitney *U*-test, Fig. 3 A), a rise in serum tyrosine (Z=2.12, p=0.034 Mann–Whitney *U*-test, Fig. 3 B), and no differ in tryptophan (Z=0.35, p=0.72 Mann–Whitney *U*-test, Fig. 3 C).

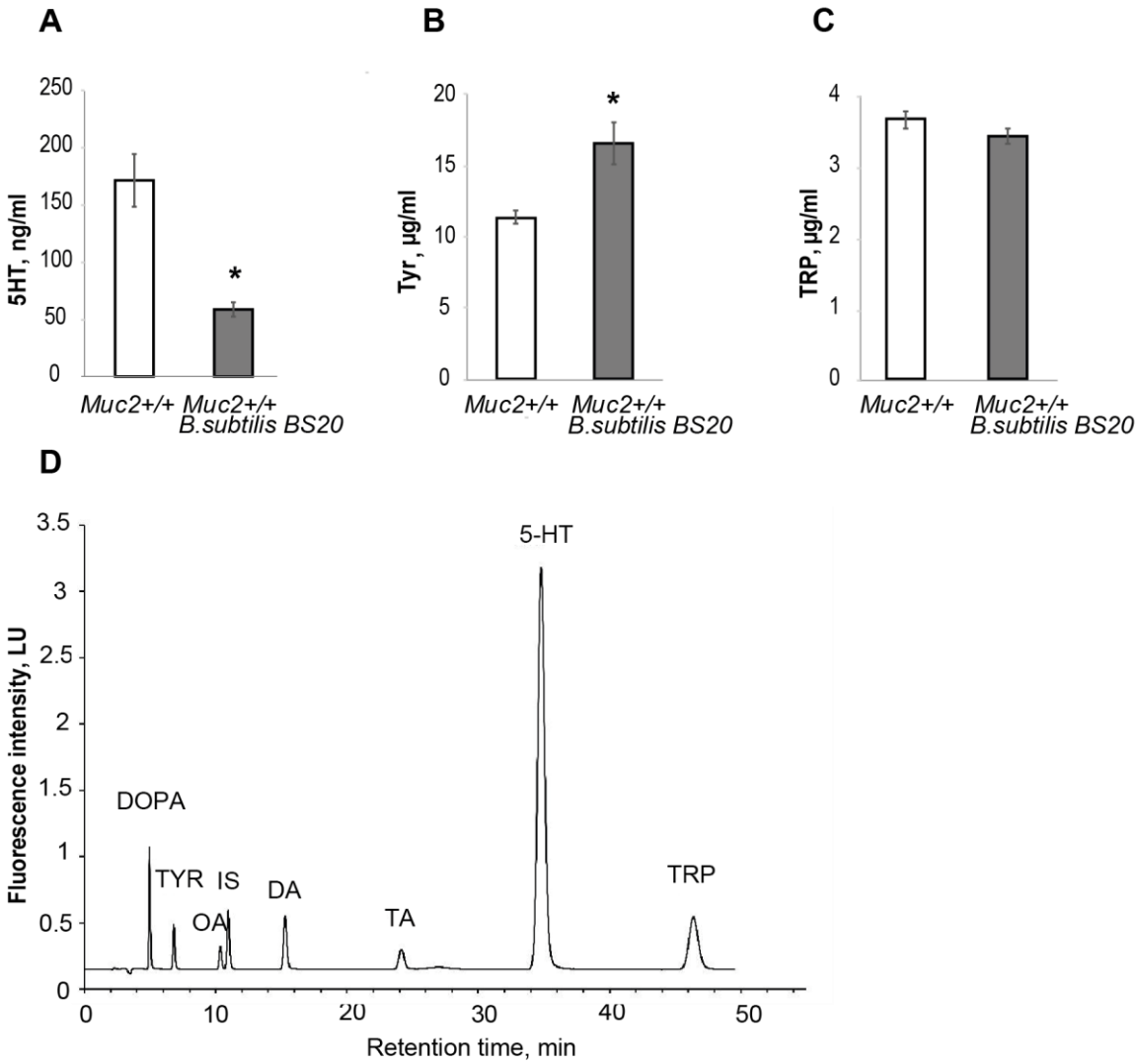

Figure 3. HPLC-FLD analysis of blood serum from *Muc2*$^{+/+}$ mice fed diet without (n=4) and with *B. subtilis* BS20 spores (*Muc2*$^{+/+}$ *B. subtilis* BS20, n=3). A) Serum 5-hydroxytryptamine (5HT), ng/ml. B) Serum tyrosine (Tyr), µg/ml. C) Serum tryptophan (TRP), µg/ml. D) HPLC-FLD chromatogram of 1 µg/ml standards (DOPA – L-3,4-dihydroxyphenylalanine, TYR – L-tyrosine, OA – p-octopamine, IS – internal standard 3,4-dihydroxybenzylamine, DA-dopamine, TA – p-tyramine, 5-HT – serotonin, TRP – L-tryptophan). Native fluorescence using excitation at 279 nm and emission at 340 nm.
* $p < 0.05$, ** $p < 0.01$ Mann–Whitney *U*-test

As a result of including *B. subtilis* BS20 spores in the diet of *Muc2*$^{+/+}$ mice, the metabolic profile of their blood serum changed. To be more specific, it led to increased tyrosine levels while decreasing serotonin levels.

It can be concluded that *Muc2*$^{+/+}$ males differ in behavior from C57BL/6 males, exhibiting increased motor activity and reduced anxiety. It is important to note that the complete exclusion from the diet of viable spores of *Bacillus* spp. caused a greater deviation of behavior from the norm. The addition of viable *B. subtilis* BS20 spores to the *Muc2*$^{+/+}$ diet

normalized behavior, bring closer to the C57BL/6. The study of the colon cytokines showed differences between C57BL/6 and *Muc2*$^{+/+}$ mice only in the level of IL13, which was increased in *Muc2*$^{+/+}$. The addition of *B. subtilis* BS20 spores to the *Muc2*$^{+/+}$ diet reduced the levels of IL10, L13, and IL17 to the levels of those found in C57BL/6 mice. Also, *B. subtilis* BS20 spores in the diet increased *Muc2*$^{+/+}$ serum tyrosine levels and decreased serotonin levels.

4. Discussion

The obtained behavioral data demonstrate that the addition of *B. subtilis* BS20 to the diet normalizes the behavior of *Muc2*$^{+/+}$ mice. Traditionally, it is customary to look for new anxiolytics, i.e., substances that reduce anxiety. The anxiety was increased in our study with *B. subtilis* BS20, but this is a favorable result in our animal model, because the behavior of healthy C57BL/6 should be regarded as the norm. Fear and anxiety are evolutionary formed mechanisms of self-preservation. Reduced anxiety among wild mice will certainly result in greater mortality [48]. As a result, reduced anxiety in open field, light-dark, and marble burying tests in *Muc2*$^{+/+}$ mice is a pathology. A reduction in motor activity in the open field and light-dark tests following a *B. subtilis* BS20 diet to the levels of those of C57BL/6 should also be considered a normalization of behavior, because motor activity in totally healthy C57BL/6 mice should be considered the norm.

Bacillus spp. are present in laboratory diets for animals and can influence reproductive rates [41-42]. It was found that adding spores *B. subtilis* BS20 to the non-autoclaved diet had a higher effect on restoring normal behavior in *Muc2*$^{+/+}$ mice. But the presence of *Bacillus* spp. in the non-autoclaved diet was insufficient to regulate behavior of *Muc2*$^{+/+}$ mice. There are studies on the influence of probiotic strains of *B. subtilis* on chicken behavior. For example, *B. subtilis* reduces aggressive behavior in laying hens [49] and heat stress-related behaviors in chickens [50]. There have been no previous studies showing that a probiotic strain of *B. subtilis* can normalize behavior caused by inherited microbiomes from mice with inflammation.

It is difficult to determine the process by which behavior is normalized in *Muc2*$^{+/+}$ mice. *Muc2*$^{+/+}$ mice are physiologically healthy and share many physiological parameters with the C57BL/6 mice. Intestine microbiota has been shown to regulate the functioning of the central nervous system. This phenomenon is called the gut-brain axis. The immune system is one of the well-established pathways through which the gut microbiota influences the brain. The gut is colonized by about 70-80% of the bacteria of the host. Bacteria are in direct contact with the immune system of the host. It is assumed that if the immune system reacts to the addition of *B. subtilis* BS20 to the diet, then this will affect the intestinal immunity. It was found that the only level of IL13 in *Muc2*$^{+/+}$ mice differed from C57BL/6, and feeding with *B. subtilis* BS20 reduced the level of this cytokine to the level of C57BL/6 mice. Despite having a healthy gut barrier and phenotype, the gut microbiota of *Muc2*$^{+/+}$ mice is inherited from *Muc2*$^{-/-}$ mutant mothers with signs of colitis [40]. This microbiota is expected to increase the pro-inflammatory cytokine IL13, and feeding *B. subtilis* BS20

restores the cytokine profile to a healthy level. Furthermore, *B. subtilis* BS20 lowered the levels of Il10 and IL17 to the levels of those of C57BL/6 mice. We believe that in this model, *Muc2*$^{+/+}$ mice, as well as with behavior, it is worthwhile to analyze changes in cytokine levels not according to their recognized biological function, but according to the variant of the norm for animals, which in this case the standard is C57BL/6 mice. This finding is consistent with earlier studies on *B. subtilis'* potential influence on immunological parameters. For example, consumption of *B. subtilis* reduces the expression of cytokines (IL-6, IL-17, IL-23 and TNF) [51]. Other studies have revealed that *B. subtilis* stimulates the development of immune responses through the synthesis of pro-inflammatory cytokines (IL-1β, IL-6, and IL-8) [52].

In the present study a laboratory strain of *B. subtilis* BS20 was used as a probiotic strain. Identification of *B. subtilis* BS20 was confirmed by mass spectrometry. The strain was selected due to its resistance to kanamycin. Strains of the *Bacillus* spp. isolated from the non-autoclaved diet and mice feces were not resistant to kanamycin [41-42]. In this regard, it was possible to count the titer of *B. subtilis* BS20 that the mice received with diet [46]. This strain of *B. subtilis* BS20 can potentially be considered a probiotic. However, to use it as a food additive, it is necessary to perform studies confirming its safety. It is also possible to test commercially available probiotic strains of *B. subtilis* for the effectiveness of restoring behavior and immune parameters in *Muc2*$^{+/+}$ mice. If all strains have the same effect, future research is warranted to investigate the universal effects of *B. subtilis* on the behavior and immunity of mice.

Serotonin has been shown to have an essential role in the pathophysiology of inflammatory bowel disease [53–55]. Also, serotonin is regarded as an important neurotransmitter in the gut-brain axis since it is required for both normal gastrointestinal tract function and brain physiology, as well as their interaction (i.e. the gut-brain axis) [56]. HPLC-FLD analysis demonstrated that *Muc2*$^{+/+}$ mice ingesting *B. subtilis* BS20 had lower serum serotonin and higher tyrosine levels than control *Muc2*$^{+/+}$ mice. IL13 may affect serotonin levels and the number of enterochromaffin cells in the mucosal lining of the gastrointestinal tract, increasing their number [53]. As a result, we may presume that the drop in serotonin after feeding *B. subtilis* BS20 is mediated by a decrease in pro-inflammatory IL13. Other cytokines also are known to have an effect on the serotonergic system [57]. In turn, serotonin also influences the production and release of cytokines [55]. The gut microbiota may be directly involved in the production of 5-HT, and may also influence SERT and regulate 5-HT levels through short-chain fatty acids (SCFAs) [58]. There is evidence identifying strains of *B. subtilis* that can produce tryptophan, a precursor of serotonin [59]. Basically, these mutant strains [38, 60] of *B. subtilis*, *E. faecium* and *E. faecalis* can increase the expression of SERT in intestinal epithelial cells and tissues [19]. There were no changes in tryptophan levels in the blood serum of *Muc2*$^{+/+}$ mice after adding *B. subtilis* BS20. We can conclude that *B. subtilis* BS20 does not produce tryptophan. Furthermore, feeding *B. subtilis* BS20 resulted in a drop in serotonin levels in serum. The levels of neurotransmitters and their precursors in the brain influence the behavior. However, only the blood serum level of serotonin was assayed because we hypothesized that the greatest changes in tryptophan and serotonin would be in serum if caused by the gut microbiota. Blood serotonin is mostly stored in platelets,

whereas plasma serotonin is synthesized either by gastrointestinal synthesis of dietary tryptophan or through the destruction of platelets [61]. There is an opinion that the level of peripheral serotonin does not directly reflect its level in the brain [62]. However, both peripheral and central serotonin react in the same direction to medications that regulate their levels [61]. It is assumed that a drop in serotonin in the periphery after feeding *B. subtilis* BS20 leads to a decrease in serotonin in the brain. The reduction in postnatal serotonin levels by subcutaneous administration of para-chlorophenylalanine is associated with an increase in anxiety and a decrease in motor activity, but the latter only in females [63]. Another study found that serotonin deficiency in both the brain and the periphery did not cause changes in locomotor activity, but did result in an increase in marbles burying [64]. Our data in the marble burying and open field tests are generally consistent with these studies. As for the decrease in motor activity in mice treated with *B. subtilis* BS20, this effect may be associated with the action of tyrosine, which was increased. In the study by Shipelin et al. [65], when an increased amount of tyrosine was introduced into the diet, the motor activity of animals decreased with age.

5. Conclusion

In sum, the spores of *B. subtilis* BS20 in the diet normalize the behavior of male *Muc2*$^{+/+}$ mice, as evidenced by a change in locomotor activity and anxiety normalized to C57BL/6 mice. These alterations can be mediated both by the immune system as well as by the levels of individual neurotransmitters and their precursors. *B. subtilis* BS20 has been shown to normalize gut cytokine levels to levels of those of control C57BL/6. In mice fed a diet containing *B. subtilis* BS20, blood serum analysis revealed a drop in serotonin levels and an increase in tyrosine levels. The drop in serotonin levels might be mediated by a decrease in IL13. It can be concluded that these changes in the metabolomic and immune profiles are apparently interrelated and may explain the normalization of behavior.

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