Turk J Pharm Sci 2020;17(3):293-298
DOI: 10.4274/tjps.galenos.2019.94210

ORIGINAL ARTICLE

A Synbiotic Mixture Augmented the Efficacy of Doxepin, Venlafaxine, and Fluvoxamine in a Mouse Model of Depression

Sinbiyotik Karışım, Fare Depresyon Modelinde Doksepin, Venlafaksin ve Fluvoksaminin Etkinliğini Artırdı

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ABSTRACT

Objectives: Currently available antidepressant drugs have notable downsides; in addition to their side effects and slow onset of action their moderate efficacy in some individuals may influence compliance. Previous literature has shown that probiotics may have antidepressant effects. Introducing complementary medicine in order to augment the efficacy of therapeutic doses of antidepressant drugs appears to be very important. Therefore, the effect of adding a synbiotic mixture to drinking water was assessed in a mouse model of depression following the administration of three antidepressant drugs belonging to different classes.

Materials and Methods: The marble burying test (MBT) and forced swimming test (FST) were used as animal models of obsessive behavior and despair. The synbiotic mixture was administered to the mice's drinking water ($6.25x10^6$ CFU) for 14 days and the tests were performed 30 min after the injection of the lowest dose of doxepin (1 mg/kg), venlafaxine (15 mg/kg), and fluvoxamine (15 mg/kg) on days 7 and 14.

Results: After 7 days of ingestion of the synbiotic mixture, immobility time decreased in the FST for doxepin (92±5.5 s) and venlafaxine (17.3±2.5 s) compared to the control group (drinking water), but fluvoxamine decreased immobility time after 14 days of ingestion of the synbiotic mixture (70±7.5 s). Preadministration of the synbiotic mixture improved the MBT test response for venlafaxine, while it did not change the results for the other two drugs.

Conclusion: Adding the synbiotic mixture to drinking water improved the efficacy of discrete antidepressant drugs particularly during the FST. Probiotics could be a useful complementary medicine for drug-resistant depressed individuals.

Key words: Probiotic, synbiotic, depression, antidepressant, forced swimming test, complementary medicine

ÖZ

Amaç: Günümüzde temin edilebilen antidepresan ilaçların belirgin dezavantajları vardır; yan etkilerine ve yavaş etki etmelerine ek olarak, orta derecedeki etkinlikleri bazı kişilerde hasta uyuncunu etkileyebilir. Daha önceki literatür bilgileri probiyotiklerin antidepresan etkilere sahip olabileceğini göstermiştir. Antidepresan ilaçların terapötik dozlarının etkinliğini artırmak için tamamlayıcı tıbbın etkisinin çok önemli olduğu düşünülmektedir. Burdan hareketle, bu çalışmada içme suyuna sinbiyotik bir karışımın eklenmesinin etkisi, farklı sınıflara ait üç antidepresan ilacın uygulanmasını takiben fare depresyon modelinde değerlendirilmiştir.

Gereç ve Yöntemler: Obsesif davranış ve umutsuzluğun hayvan modeli olarak misket gömme testi (MBT) ve zorunlu yüzme testi (FST) kullanılmıştır. Sinbiyotik karışım, farelerin içme suyuna ($6,25x10^6$ CFU) 14 gün boyunca uygulanmış ve testler, 7. ve 14. günlerde en düşük dozda doksepin (1 mg/kg), venlafaksin (15 mg/kg) ve fluvoksamin (15 mg/kg) enjeksiyonundan 30 dakika sonra yapılmıştır.

Bulgular: Sinbiyotik karışımın 7 gün süreyle uygulanmasından sonra, doksepin (92±5,5 s) ve venlafaksin (17,3±2,5 s) için FST'de kontrol grubuna (içme suyu) kıyasla hareketsizlik süresi azalmış, ancak fluvoksamin ile, sinbiyotik karışımın 14 gün süreyle uygulanmasından (70±7,5 s) sonra hareketsizlik süresi azalmıştır. Sinbiyotik karışımın önceden uygulanması venlafaksin için MBT test yanıtını geliştirmiş, diğer iki ilacın sonuçlarını değiştirmemiştir.

Sonuç: Sinbiyotik karışımın içme suyuna eklenmesi, özellikle FST sırasında ayrı antidepresan ilaçların etkinliğini arttırmıştır. Probiyotikler, ilaca dirençli depresif bireyler için yararlı bir tamamlayıcı olabilir.

Anahtar kelimeler: Probiyotik, sinbiyotik, depresyon, antidepresan, zorunlu yüzme testi, tamamlayıcı tıp

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Received: 04.02.2019, **Accepted:** 07.03.2019

INTRODUCTION

Probiotics are live organisms that when chronically ingested in adequate amounts could induce beneficial effects in the host.[1] Single strain probiotics or their combinations are useful for a variety of diseases, including gastrointestinal (GI) disorders such as inflammatory bowel disease, diarrhea, Irritable Bowel syndrome (IBS), and allergies.[2] This is now even being extended to some ailments of the central nervous system (CNS).[3,4] Gut bacterial microorganisms have two dominant genera, *Bacteroidetes* and *Firmicutes*, while other types have lower abundance, for instance *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*.[5] Evidently, the brain-gut axis is a bidirectional homeostatic route and so its aberration may cause important pathophysiological results.[3] The high risk of co-existing psychiatric symptoms such as anxiety with GI disorders, for instance IBS, provides further evidence of the importance of this axis.[6] The brain-gut axis is connected not only by complex systems including the vagus nerve, endocrine, immune, and humoral links but also the gut microbiota in order to maintain GI stability and to connect cognitive and emotional areas of the brain with GI functions.[7]

Major depressive disorder is the most common mood disorder, affecting 5% of the population each year.[8] Several mechanisms are involved in its pathogenesis, such as altered monoaminergic (serotonergic, noradrenergic, and dopaminergic) and glutamatergic systems, increased inflammation, hypothalamic-pituitary-adrenal (HPA) axis aberrations, and decreased neuroplasticity.[9] Probiotics have shown antidepressant effects in animal models of depression. Species of the genus *Lactobacillus* are particularly characterized as antidepressants.[10] A probiotic mixture of *Lactobacillus* strains (*L. rhamnosus* and *L. helveticus*) ameliorated depression by regulating corticosterone levels in rat pups.[11] Likewise, chronic ingestion of a *L. rhamnosus* strain (JB-1) in mice alleviated depressive behavior and caused regional alteration in GABA receptor expression and since these changes were not observed in vagotomized mice the vagus nerve was considered critical for the brain-gut axis.[12] *Bifidobacterium* species also induce physiologic changes in favor of antidepressants effects in animal studies; for instance, *B. infantis* ameliorated depressive behavior in the maternal separation model.[13] In a systematic review regarding the effects of probiotics on depression in humans most of the studies found positive results on all values of depressive symptoms, but there were wide differences in the strain of probiotic, the dosing, and the duration of treatment.[14] The possible mechanisms that were considered for antidepressant effects of *B. infantis* by use in a rat model were reduction of pro-inflammatory cytokines, alteration of tryptophan metabolism, and CNS neurotransmitters.[15] The combination of *Lactobacilli* and *Bifidobacteria* was tested in depression following post-myocardial infarction (MI) in rats. Administration of *L. helveticus* and *B. longum* together had positive effects on post-MI depression through reduction of pro-inflammatory cytokines (for instance IL-4) and intestinal permeability restoration.[16,17] In addition, prebiotics such as oligosaccharides that stimulate the growth of nonpathogenic intestinal microflora such as *Lactobacilli* and *Bifidobacteria* also have neurotropic effects.[18]

Although drug therapy of depression is usually safe and effective, it is still not ideal because the latency time for clinical results is quite long (about 3-5 weeks) and there are still concerns regarding side effects such as loss of libido and weight gain. Additionally, there are a significant number of patients that do not respond well to antidepressant drugs, psychotherapy, and electroconvulsive therapy.[19] Thus the aim of the present study was to first observe the effect of a synbiotic (probiotic + prebiotic) mixture on behavior in an animal model of depression. Since it has been previously shown[11-15] that the probiotics *Lactobacilli* and *Bifidobacteria* have antidepressant effects in animal studies, a manufactured premixed product was chosen that comprised these genus and also a prebiotic, fructooligosaccharides. Second, in order to observe the effect of the synbiotic mixture on the efficacy of antidepressant drugs from different classes, subthreshold doses of doxepin [a tricyclic antidepressant (TCA)], venlafaxine [a serotonin-norepinephrine reuptake inhibitor (SNRI)), and fluvoxamine (a selective serotonin reuptake inhibitor (SSRI)] were chosen.

MATERIALS AND METHODS

Animals

Male Swiss mice weighing 23-26 g were housed at 21±2 ºC in a 12-h light/dark cycle with the lights on in the day time 6 am-6 pm. Tap water or the synbiotic mixture and standard food pellets were available *ad libitum*. For each experiment 6 mice were used and they were housed 3 per cage. Tests were performed in the behavior laboratory 24 h after the mice had become acclimatized to the environment. In order to minimize the influence of circadian rhythm, all experiments were performed between 8 am and 1 pm in the pharmacology laboratory. All animal procedures were approved by the Ethics Committee of Isfahan University of Medical Science and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Ethical no: IR.MUI.REC.1395.3.864).

The synbiotic mixture and drug therapy

The synbiotic mixture comprised 10^9 CFU [*Lactobacillus casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *Bifidobacterium breve*, *B. infantis*, *Streptococcus thermophilus*, fructooligosaccharides (a prebiotic); a product of Zist Takhmir, Iran]. The animals had free access to the synbiotic mixture solution in drinking water that was prepared freshly each day in three concentrations: 6.25, 12.5, and $25x10^6$ CFU (based on pilot studies); the control animals had tap water *ad libitum*. The amounts of the synbiotic mixture solution or normal drinking water ingested by the animals were measured daily. The therapy continued for 14 days and the tests were performed on days 7 and 14.

The antidepressants were first administered intraperitoneally alone 30 min before starting the first test. Two doses were applied for each drug according to previous studies[20] and finally the lowest effective dose (data not shown) was chosen to be

applied following the synbiotic mixture administration. The selected antidepressants were doxepin 1 mg/kg (Razak, Iran), venlafaxine 15 mg/kg (Sigma-Aldrich, India), and fluvoxamine 15 mg/kg (Sigma-Aldrich, India). All the drugs were freshly prepared in normal saline solution and injections were adjusted for a volume of 10 mL/kg mouse body weight. The selected dose of each antidepressant drug was administered on days 7 and 14 following the synbiotic therapy (6.25x10^6 CFU), 30 min before the tests were performed.

Marble burying test (MBT)

The first test was the MBT. This is a method used to evaluate anxiety behavior and obsessive compulsive behavior. With minor modification to the method presented by Njung'e and Handley[21], the mice were separately placed in plastic cages (42x24x12 cm) containing 12 opaque glass marbles (1 cm diameter) that were distributed evenly over 5 cm of deep sawdust without food or water for 30 min. The number of marbles at least two-thirds buried (MB) was counted after 30 min.[22]

Forced swimming test (FST)

After performing the MBT each mouse was subjected to the FST. With some modifications[23] the mice were forced to swim in a glass beaker (diameter 14 cm) containing 25 ºC water for 6 min. The depth was about 15 cm to prevent the mice from escaping or touching the bottom of the glass beaker with their paws or tail. After 2 min of adaptation the total immobility time was measured by a chronometer in the last 4 min of the trial. Immobility is defined as the time the animal was floating and staying still when no additional activity was observed other than that required to keep the animal's head above water. Finally the mice were dried carefully and returned to their home cage.

Data processing and statistical analysis

The results were expressed as group mean ± standard error of the mean. The results were analyzed by One-Way ANOVA, followed by Tukey's multiple comparison tests and p values less than 0.05 were considered significant. The software programs used for data analysis and making graphs were Excel 2010 and the GraphPad Prism 6.

RESULTS

Daily drinking intake

According to Table 1, daily measurements of the synbiotic mixture ingestion showed that approximately a dose of 2.4-9.2x10^6 CFU/g body weight of synbiotic was ingested.

Effect of the synbiotic mixture on the MBT and FST

The number of marbles buried during the MBT showed that consuming 12.5x10^6 CFU synbiotic after 7 days or 14 days reduced obsessive behavior in the mice (Figure 1A). The percentage of marbles buried after 30 min on days 7 and 14 of ingesting the middle concentration of the synbiotic mixture was 25% and 41%, respectively, which was significantly ($p<0.05$) different from the corresponding control group (50% and 58.3% on days 7 and 14, respectively; $p<0.05$). The lowest synbiotic concentration only reduced MB to a significant amount on day 7 compared to the control animals (33.3% vs 50%; $p<0.05$). However, consuming the highest synbiotic concentration showed opposite results on MB behavior, since the animals buried more marbles measured after 7 (87%, $p<0.001$ vs control) and 14 days of therapy. During the FST the animals that ingested the synbiotic showed antidepressant behavior as presented in Figure 1B. After the mice had ingested the synbiotic mixture for

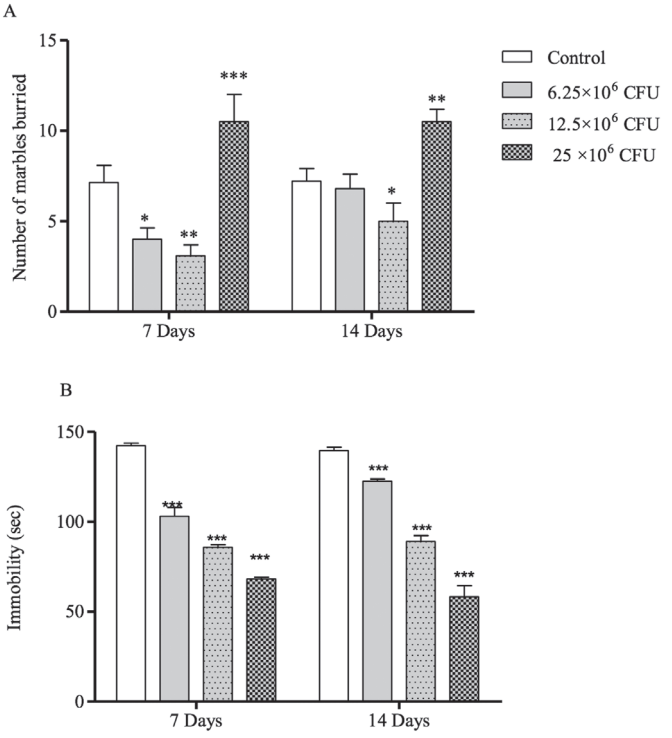

Figure 1. The effect of 3 concentrations of the synbiotic cocktail ingestion for 14 days on mouse behavior. A) The MBT the number of marbles at least two-thirds buried after 30 min, and B) the total immobility time during the last 4 min in the FST. The tests took place on days 7 and 14; each animal was first subjected to the MBT and then the FST. Number of animals in each group was 6; the control animals drank tap water. Results are expressed as group mean ± SEM and analyzed by One-Way ANOVA, followed by Tukey's post-hoc test. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ compared with the control group

MBT: Marble burring test, FST: Forced swimming test, SEM: Standard error of the mean

Table 1. Daily liquid intake

Synbiotic mixture CFU	6.25x10^6	12.5x10^6	25x10^6	0 (Tap water)
Daily intake mL/g body weight	0.38	0.37	0.37	0.39
Daily dose CFU/g body weight	2.375x10^6	4.625x10^6	9.25x10^6	0

The synbiotic mixture comprised *Lactobacillus casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *Bifidobacterium breve*, *B. longum*, *Streptococcus thermophilus*, fructooligosaccharides (a prebiotic)

a week the immobility time measured during the FST decreased in a dose-dependent manner since the highest mixture concentration (25x10^6 CFU) caused the lowest immobility time (68.1±2.5 s vs 142.3±4.3 s, p<0.001). After 14 days of synbiotic ingestion, the animals dose-dependently showed a shorter immobility time compared with the control animals (139.6±5.4 s); the animals that had ingested the highest concentration of the synbiotic mixture had the shortest immobility time (58.3±15 s, p<0.001).

Effect of the synbiotic mixture pretreatment on mouse response to antidepressants during the MBT and FST

The lowest concentration of the synbiotic mixture (6.25x10^6 CFU) was chosen to observe the animals' behavior during the MBT and FST on days 7 and 14 after injection of the lowest dose of each antidepressant drug (Figure 2). The antidepressant drugs all significantly reduced the number of MB compared to the control group (Figure 2A, p<0.05). Synbiotic ingestion significantly influenced the effects of venlafaxine on MB behavior since the animals significantly buried fewer marbles compared to those that had drunk water (percentage of MB decreased from 25% to 7%). However, consumption of the synbiotic mixture did not have any considerable effect on the MB behavior of animals injected with doxepin or fluvoxamine. On the other hand, drinking the synbiotic mixture increased the antidepressant effects of the lowest dose of each of the drugs (Figure 2B). The immobility time decreased dramatically in the animals that had drunk the synbiotic mixture either for 7 days (17.3±2.5 s) or for 14 days (60±16 s) prior to the administration of venlafaxine (p<0.001, vs the venlafaxine alone group 146±1 s). The results for synbiotic drinking prior to doxepin injection followed the same trend; after 7 days the immobility time was 92±5.5 s and after 14 days it was 70±7.4 s, which was decreased significantly compared to the doxepin alone group, 100±19 s (p<0.001). The synbiotic effect on fluvoxamine was slightly different as only after 14 days immobility time decreased significantly compared to the drug administered alone (70±7.5 s vs 100±9 s, p<0.001).

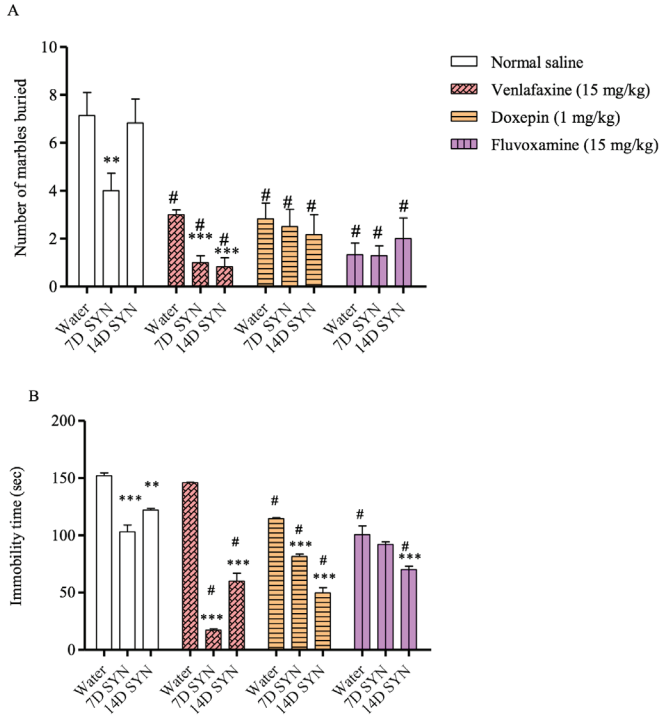

Figure 2. The effect of drinking the synbiotic mixture and the lowest dose of antidepressants on mouse behavior. A) The MBT the number of marbles at least two-thirds buried after 30 min are presented, and B) the total immobility time during the last 4 min in the FST. Number of animals in each of the groups was 6; animals ingested the synbiotic mixture (SYN; 6.25x10^6) for 14 days; the control group ingested tap water. The drugs were injected IP 30 min before testing; each animal was first subjected to the MBT and then the FST. Results are expressed as group mean ± SEM and analyzed by One-Way ANOVA followed by Tukey's post-hoc test. ** p<0.01 and *** p<0.001 compared with the probiotic free group. # p<0.01 compared with the corresponding control groups (the plane bars)

IP: Intraperitoneally, MBT: Marble burying test, FST: Forced swimming test, SYN: Synbiotic, SEM: Standard error of the mean

DISCUSSION

Our results showed that the synbiotic mixture could mitigate the immobility time in the FST, which denotes its possible antidepressant effects. Interestingly, only after a week of ingesting the synbiotic mixture did response to the lowest dose of the antidepressants increase dramatically. However, the MB behavior showed variable results. The MB behavior appears to be just a form of digging, but the behavior in mice has been used as a model of anxiety disorders including obsessive compulsive disorder (OCD).[24] This method is also useful for evaluating the mechanisms of action of drugs.[25] Venlafaxine (SNRI), doxepin (TCA), and fluvoxamine (SSRI) in our set of experiment clearly reduced the number of marbles buried, which was parallel with previous literature.[22,25] The FST is a reliable tool for drug discovery in industrial settings where high quantity screening of new compounds is necessary, as well as in research regarding complementary medicine.[23] By leaving the mouse in the water container it gradually loses hope to escape the stressful environment, and thus the immobility time reflects a measure of "behavioral despair". In the same trend as previous results, all antidepressant drugs of different classes that were tested in our study presented antidepressant effects in mice by reducing the immobility time.[22]

The daily preparation of the synbiotic mixture in the mice's drinking water was based on previous literature that *B. infantis* was administered by dissolving a powder containing 10^{10} bacterial cells in 100 mL of the rats' drinking water every morning.[26] There is also evidence that dead probiotic bacteria or just integral components of the bacterial cell such as peptidoglycan fragments or DNA would also be effective.[27] Therefore, although survivability of the mixture was not assessed before the new batch the next morning the research showed that the content remained effective. The synbiotic mixture dose-dependently reduced the immobility time during the FST, which obviously denotes its antidepressant-like effect in mice. However, a dose-dependent effect on obsessive behavior was not observed during the MBT; although lower doses of the synbiotic mixture reduced the MB behavior, this was not observed with the higher dose. A high dose of the synbiotic mixture had opposite effects

on the MBT. The different effects of the synbiotic mixture on the FST and MBT could be because of the different mechanisms involved in each test. Earlier research has proven that although the MBT and FST are invaluable predictive tests of OCD and antidepressant action, each assay appears to engage completely distinct neurochemical systems.[28] While the FST is more susceptible to compounds that are effective by altering activity in the noradrenergic system, the MBT mostly depends on modulation of the serotonergic system.[28] The downside of our research was that we did not measure the monoamine neurotransmitters, but this has been proven earlier.[29,30] Reports have shown that chronic gavage of *L. plantarum* in mice reduced stress-induced depression-like behaviors, normalized the HPA axis and immune systems, and modulated the changes in the dopamine and serotonin system in the prefrontal cortex.[29] Additionally, probiotics changed behavior and the CNS function in naïve adult animals.[30] The consumption of a fermented milk product containing *B. aimalis*, *S. thermophilus*, *L. bulgaricus*, and *L. lactis* in healthy women without psychiatric symptoms as observed by functional magnetic resonance imaging induced robust alterations in the activity of brain regions that control the central processing of emotions and sensations.[31]

Pretreatment with the synbiotic mixture before the administration of venlafaxine (SNRI) had a synergistic effect on the MBT, but this synergistic effect was not observed with doxepin or fluvoxamine. Previous studies on rat models of depression have reported that the ingestion of probiotics has a role in restoring the monoamine levels in important brain regions.[4,13] Therefore, it is possible that restoring the monoamine level improved the efficacy of venlafaxine in the MBT. On the other hand, the synbiotic mixture had a synergistic antidepressant-like effect on venlafaxine and doxepin (TCA); the antidepressant efficacy of fluvoxamine (SSRI) only increased after 14 days of synbiotic ingestion. Several studies have supported the argument that probiotics can exert psychotropic potential and this effect could be mediated by alterations in the monoamines. For instance, it has been shown that *B. infantis* reverses maternal separation-induced depressive-like behavior in rats during the FST,[13] and at least part of this antidepressant effect is due to elevation of tryptophan.[15] Previous findings suggest that in naïve mice the probiotic *L. plantarum* could modulate both serotonergic and dopaminergic systems,[29] and interestingly the probiotic increased dopamine, 3,4-dihydroxyphenylaceticacid, and homovanillicacid but there was no change in the dopamine turnover rate.[29] Earlier literature advocates that the vagus mediates the behavioral and neurochemical effects of probiotics.[7,12] Nevertheless, the antidepressant effects of the probiotic mixture may arise independently of any changes to the monoamine systems; for instance, it may be caused by attenuation of pro-inflammatory immune responses.[15]

The results of the present research were not only parallel with the previous literature regarding the antidepressant effects of probiotics but also extended it in several ways: first, the synbiotic mixture could decrease MB behavior. Second, the cocktail ingested by animals augmented the efficacy of the SNRI antidepressant venlafaxine during the MBT. Finally, after 7 days of the synbiotic drink it had a synergistic antidepressant-like effect on venlafaxine and doxepin, and it increased fluvoxamine efficacy after 14 days. Previous results proved that fluoxetine and escitalopram have antimicrobial activity *in vitro*, and psychotropic medications differentially influence the composition of gut microbiota *in vivo*.[32] Therefore, adding probiotics may help to improve the gut microbial composition in patient receiving antidepressant therapies.

CONCLUSION

Since extrapolating animal results to humans must be done with caution, further clinical research is warranted regarding adding synbiotics to drug-resistant patients and adding synbiotics in order to reduce antidepressant drug dosage and side effects mainly when SNRI or TCA are administered.

ACKNOWLEDGEMENT

This work was supported by the School of Pharmacy and Pharmaceutical Sciences Research Council (grant number: 395864, 12/05/2016), Isfahan University of Medical Sciences.

Conflict of Interest: Authors confirm that there is no conflict of interest in relation to this article.

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