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Microbiological Investigation of the Effects of Olanzapine with Thymoquinone on the Intestine

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Abstract

The aim of our study is to examine the effect of thymoquinone (TQ) in obese rats induced with the antipsychotic drug olanzapine (OL). Thirty-five female Spraque-Dawley rats were divided into five groups (n = 7): Control, OL (2 mg / kg OL daily), OL + TQ1 (2 mg / kg OL + 20 mg / kg TQ), OL + TQ2 (2 mg / kg OL + 40 mg / kg TQ) and the OL + TQ3 group (2 mg / kg OL + 80 mg / kg TQ). On the 15th day of treatment, intestinal tissue was removed for analysis. It has been found that TQ treatment affects the levels of Firmicutes and Bacteroides at varying rates in the intestinal flora in OL + TQ1, OL + TQ2, and OL + TQ3 groups, and also has a significant role in the apoptotic effect of TQ. In conclusion, with this study, it was determined that the

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treatment of TQ has a protective property against the side effects of OL. TQ can be an effective treatment method to increase therapeutic effectiveness.

Keywords: Olanzapine; Thymoquinone; Obesity; *Firmicutes*; *Bacteroides*.

Olanzapin ile Timokinon'un Bağırsak Üzerindeki Etkilerinin Mikrobiyolojik Olarak Araştırılması

Öz

Çalışmamızın amacı, bir antipsikotik ilaç olanzapin (OL) ile indüklenen obez sıçanlarda timokinonun (TQ) etkisini incelemektir. Otuz beş dişi Spraque-Dawley sıçanı beş gruba ayrıldı (n = 7): Kontrol, OL (günlük 2 mg / kg OL), OL + TQ1 (2 mg / kg OL + 20 mg / kg TQ), OL + TQ2 (2 mg/kg OL + 40 mg/kg TQ) ve OL + TQ3 grubu (2 mg/kg OL + 80 mg/kg TQ). Tedavinin 15. gününde, analiz için bağırsak dokusu çıkarıldı. TQ tedavisinin, OL+TQ1, OL+TQ2 ve OL+TQ3 gruplarında bağırsak florasındaki Firmicutes ve Bacteroides düzeylerini değişen oranlarda etkilediği ve ayrıca TQ'nun apoptotik etkisinde önemli rolü olduğu tespit edilmiştir. Sonuç olarak, bu çalışma ile TQ tedavisinin OL'nin yan etkilerine karşı koruyucu özelliği olduğu belirlendi. TQ, terapötik etkinliği artırmak için etkili bir tedavi yöntemi olabilir.

Anahtar Kelimeler: Olanzapin; Timokinon; Obezite; Firmicutes; Bacteroides.

1. Introduction

Obesity is a disease that has increased in number in recent years and has become a serious problem in the world. In humans; cardiovascular, liver and gallbladder diseases, diabetes, osteoarthritis, hyperlipidemia, cancer, asthma, obstructive sleep apnea syndrome, and may result in death [1]. In 2015, 107.7 million children and 603.7 million adults were reported to be obese worldwide [2]. It is also estimated that obesity will affect 51%[3] and about a quarter of the adult population by 2030 [4]. According to data from the World Health Organization, weight and obesity in Europe cause 80% of diabetes in adults, 35% of heart disease, 55% of hypertension, and deaths of more than one million people per year [5]. Since obesity develops due to changes in adipose tissue, we can say obesity = body mass index (BMI). BMI is calculated as the ratio of body weight to the square of the neck. 32 genes affecting BMI were identified, but the most effective factor was considered to be environmental factors caused by energy-intensive nutrition and reduced [6]. Recent research has shown that microbial changes in the intestine have an impact on obesity. Intestinal microbiota affects human metabolism. The presence of microbial flora and its metabolites are responsible for these effects. The microbiota has important effects on the

production of vitamins, destruction of non-breakable nutrients, metabolites, and immunity [3, 4, 7]. It benefits energy metabolism by producing short-chain fatty acids stimulating substances, affecting the metabolic pathway and insulin resistance in fat cells and peripheral organs. Alcohol, stress, smoking, socioeconomic status, and eating habits are effective on the microbiota. [3, 4, 8]. Dysbiosis with the change of the intestinal flora causes some metabolic disorders [9]. These metabolic disorders include impaired glucose, lipid levels, inflammation, altered intestinal permeability, insulin resistance, high calorie increase, obesity, and physiological balance changes [4, 10]. In the last decade, different results have been obtained on the effect of intestinal microbiota on obesity, leading studies to be in this direction [9]. Various methods of analysis, methods of taking samples, differences in body mass index classifications around the world and the increase in research findings have led to differences in the results of the study at the level of obesity relation of intestinal microbiota. Until the last five years, it has been known that the intestinal microbial flora of obese people is less than the weak ones, today it has been shown that this result is the opposite. As a result of the research findings, changes in the intestinal flora, namely intestinal dysbiosis, have gained importance [4, 7, 11]. In microbiome studies, it has been tried to understand the cause and effect relationships that cause obesity and intestinal microbiota connection, except for the types and rates of bacteria [12]. As is generally known, changes in the intestinal microbiota profile are important in obesity [9]. In healthy humans, the intestinal microbial consists of 6 classes: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia. Bacteroidetes* and *Firmicutes* make up 90% of the intestinal microbiota. *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, Bifidobacterium, Fusobacterium are the most obligatory anaerobes at the class level and the facultative anaerobes are *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Proteus* and *Lactobacillus*. *Bacteroidetes* / *Firmicutes* ratio is thought to be very effective on obesity [1]. It is known that some bacterial species belonging to *Bacteroidetes* and *Firmicutes* branches are dominant in the normal intestinal flora [13]. The main causes of obesity are not thought to be solely due to genetic changes and dietary differences. Genetic and environmental factors increase the tendency to obesity. Even antipsychotic drugs used are closely related to obesity, and antipsychotic drugs are known to cause weight gain in obesity. Olanzapine (OL), one of these antipsychotic drugs, has less side effects than other antipsychotic drugs [14]. However, significant weight gain leads to an increase in serum cholesterol and triglyceride levels. Again, OL has a stronger association with obesity and insulin resistance. Studies have shown that OL causes the most weight gain compared to other antipsychotic drugs. Therefore, patients taking antipsychotic drugs are thought to be at risk. The increase in the use of antipsychotic drugs worldwide and the numerous side effects of these drugs have necessitated the use of natural

products. One of these natural products is thymoquinone (TQ). TQ black seed (*Nigella Sativa*) is the most important bioactive component found in the essential oil of 18.4-24%. TQ has many beneficial effects such as antioxidant, antihyperlipidemic, antidiabetic, anti-inflammatory, gastroprotective and hepatoprotective. Studies have shown that TQ has hypoglycemic, hypolipidemic and hypocholesterolemic effects [15]. In our study, it is thought that atypical antipsychotic drugs can be removed with TQ which is a natural protective product against various metabolic changes such as weight gain induced by side effects.

The aim of our study is to determine to what extent the protective effect of TQ against OL, which is thought to cause obesity, on some *Bacteroides* and *Firmicutes* strains in the intestinal microflora. Recent studies show that more research is needed to determine the effect of intestinal microbiota on obesity. Our research will guide other studies in this field.

2. Materials and Methods

2.1. Chemicals

OL was obtained from Ali Arif Ilac Sanayi (ARIS), Istanbul, Türkiye. TQ (purity > 98 %) was purchased from Sigma. All other chemicals used were of the best analytical grade.

2.2. Animals

In this study, 35 female Sprague Dawley rats (230-280 g and 4 months old) were obtained from Fırat University Laboratory Livestock and Research Center. The experiments were carried out according to the protocol (Protocol # 2015/36) approved by Fırat University Faculty of Medicine Laboratory Animals Ethics Committee. The rats were provided with appropriate nutrition and shelter (rat food and tap water at 21 ± 1 °C for 12 hours without light and light). The drug and preservative application lasted 2 weeks.

2.3. Experimental design

In this study, 35 rats were randomly divided into 5 groups with 5 sherds. Doses of 25 mg, 50 mg, and 100 mg of TQ were administered. 1st group control, 2nd group OL, 3rd group OL + TQ1 (OL + 25 mg TQ), 4th group OL + TQ2 (OL + 50 mg TQ), and 5th group OL + TQ3 (OL + 100 mg TQ). Saline solution was given to the control group by gavage once a day. Apart from the first group, OL was given to all groups 4 mg/kg once a day in the first week and 8 mg/kg in the second week. The TQ was given 25 to the third group, 50 to the fourth group, and 100 mg/kg body weight/day to the third group. In female Sprague Dawley rats, OL and TQ doses and durations were determined according to certain methods, and TQ was administered daily between

08:00 and 09:00 a.m. by gastric tube [16,17]. All compounds were treated with saline and administered by gavage once a day. At the end of the application, which continued for 2 weeks, the rats were euthanized by cardiac puncture. Intestinal tissues and blood samples were stored at -80 °C.

2.4. Bacterial RNA isolation and quantitative real-time PCR (qRT-PCR)

In our study, 4 genus levels of *Lactobacillus* sp. (LAC), *Faecalibacterium* sp. (FAE) from Firmicutes branch, and 2 genera of *Bacteroides* sp. (BAC) and *Prevotella* sp. (PRE) from Bacteridetes branch were determined in intestinal tissues. qRT-PCR was used to detect the mRNA expression of LAC, FAE, BAC and PRE receptors. RNA isolation of intestinal tissues was performed. 30 mg intestinal tissue homogenizer (Bioprep-24, Allsheng) was homogenized. Total RNAs were extracted using an ExiPrepTM Tissue Total RNA isolation kit (Bioneer, K-3325) and quantified by measuring the absorbance at 260/230 nm and 260/280 nm using a NanoDrop spectrophotometer (Denovix DS-11). RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. We also used primer pairs (Bionner S-1001) for qRT-PCR in our study of AccuPower® RT PreMix (Bioneer K-2041) according to the instructions. The RT-PCR was conducted following the instructions of the AccuPower GreenStar qPCR PreMix (Bioneer, Cat No: K-6210). The level of the mRNA expression of the LAC, FAC, PRO and BAC genes, was detected using the ExiCyclerTM96 qRT- PCR system (Bioneer). The PCR conditions were 95 °C for 1 min, followed by 45 cycles at 95 °C for 5 sec, and 55 °C for 40 second. The 2^-ΔΔCt method was used to calculate the results 30-35 (Table 1).

Table 1: Primer sequences used to replicate the gene region

Primer Sets	F.	R.
***Lactobacillus* spp.**	GAGGCAGCAGTAGGGAATCTTC	GGCCAGTTACTACCTCTATCCTTCTTC
***Faecalibacterium* spp.**	GAAGGCGGCCTACTGGGCAC	GTGCAGGCGAGTTGCAGCCT
***Bacteroides* spp.**	GAAGGTCCCCCACATTG	CGCTACTTGGCTGGTTCAG
***Provetella* spp.**	AAGGTCCCCCACATTGG	CCGCGGCKGCTGGCAC

2.5. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

TUNEL test was used to determine the rate of apoptosis in the intestinal samples of all study groups. Intestinal samples were waxed, sectioned, and placed on slides covered with polylysine. Apoptotic cells were identified with the ApopTag Plus Peroxidase In situ Apoptosis Detection Kit (Chemicon, Cat no: S7101, USA). Samples were examined, evaluated, and visualized using an imaging-assisted binocular light microscope (Eclipse Ni–U; Nikon, Tokyo,

Japan). Nuclei stained blue with hematoxylin were evaluated as normal, brown ones as apoptotic cells. 10 randomly selected areas in the sections were scanned and at least 400 cells were examined. Apoptotic index (AI) was calculated as apoptotic cells / total (normal + apoptotic) cells [18,19].

2.6. Statistical analysis

Statistical analyses were performed using a statistical software package (SPSS version 20.0, SPSS, Chicago, IL). For histopathological analysis, results were expressed as means ± standard deviation. The statistically significant difference was determined by ANOVA followed by Tukey's multiple comparison test. Probability values (p) less than 0.05 were considered to be statistically significant.

2.7. qPCR relative assessment

In the study, it was assumed that the reactions work 100% efficiently. Increase or decrease in the number of bacteria compared to control. Control 1 was accepted. The number of bacteria in the groups is how many times it increases and how many times it decreases compared to the control. $2^{(\text{average Control ct - average sample ct})}$. Relative evaluation [20], 2^{nd} calculation [21].

3. Results

3.1. Evaluation of apoptosis in intestine tissue

Examination of TUNEL staining for the determination of apoptotic cells under light microscopy; TUNEL positivity was significantly increased in the OL group (Figure 1B), OL + TQ1 (Figure 1C), OL + TQ2 (Figure 1D) and OL + TQ3 (Figure 1E) compared to control group (Figure 1A) ($p < 0.05$). TUNEL positivity was significantly decreased in OL + TQ1, OL + TQ2 and OL + TQ3 groups compared to OL group ($p < 0.05$). However, no significant change was observed between OL + TQ1, OL + TQ2, and OL + TQ3 (Table 2, Fig. 1).

Table 2: Effects of olanzapine and thymoquinone on apoptotic index (%)

Groups	Apoptotic Index (%) (AI; mean ± SD)
Control	4.66 ± 1.75 [b,c,d,]
OL	26.50 ± 3.27 [a,c,d,e]
OL + TQ-1	11.51 ± 1.51 [a,b]
OL + TQ-2	9.16 ± 1.16 [a,b]
OL + TQ-3	10.16 ± 2.85 [a,b]

The apoptotic index of all the groups. Values are mean ± SD for seven rats in each group. a: Significant from control; b: Significant from OL; c: Significant from OL + TQ-1; d: Significant from OL + TQ-2; e: Significant from OL + TQ-3 ($p \leq 0.05$). Abbreviations: OL, olanzapine; TQ, thymoquinone; OL + TQ-1, OL + 25 mg/kg TQ; OL + TQ-2, OL + 50 mg/kg TQ; OL + TQ-3, OL + 100 mg/kg TQ. 4 mg/kg once a day for the first week, 8 mg/kg once a day for the second week of OL was given to all groups, except control group

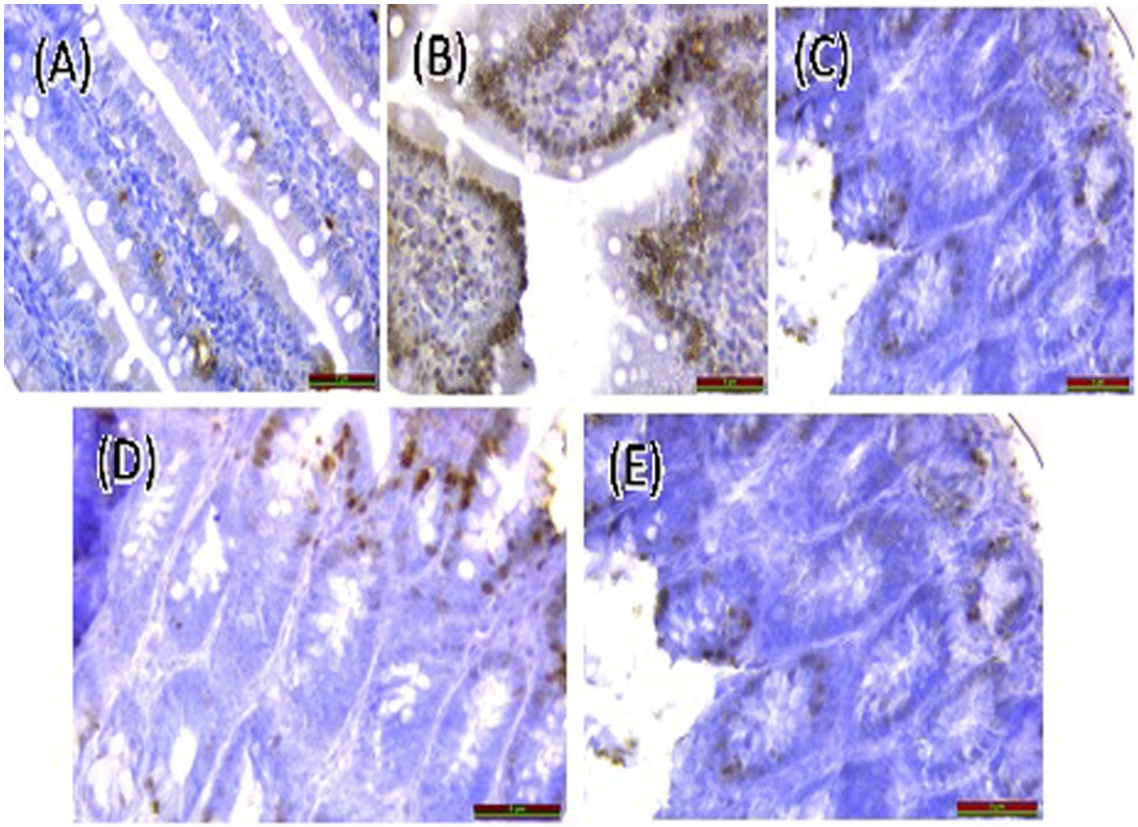

Figure 1: TUNEL staining for the determination of apoptotic cells

3.2. Evaluation of microbiological in intestine tissue

As a result of our study, the number of BAC was 5.8 times decreased in the OL group, OL + TQ1 decreased 3.36 times, OL + TQ2 group decreased 2.53 times, OL + TQ3 group decreased 6.06 times compared to the control (Table 3-4). According to the PRE control group OL group increased 149.08 times, OL + TQ1 12.64 times increased, OL + TQ2 group increased 9.98 times, and OL + TQ3 group decreased 81 times (Table 3-4). The number of LAC compared to the control group OL group 1.74 times, OL + TQ1 decreased 1.75 times, OL + TQ2 group decreased 2.11 times and OL + TQ3 group decreased 1.71 times (Table 3-4). The number of FAE compared to the control group OL group increased 5.89 times, OL + TQ1 decreased by 4.03 times, OL + TQ2 group increased 4.44 times, and OL + TQ3 group increased 3.56 times (Table 3-4).

Table 3: qPCR Relative Evaluation of bacterial levels

	Control Groups Avr Ct	OL Group			OL + TQ1			OL + TQ2			OL + TQ3		
		Avr Ct	ΔCt	Fold fark	AvrCt	ΔCt	Fold fark	AvrCt	ΔCt	Fold fark	AvrCt	ΔCt	Fold fark
Bac.	27.45 ± 2.29	30 ± 1.7	-2.55	**5.8 d (0.17)**	29.2 ± 3.2	-1.75	**3.36 d (0.27)**	28.8 ± 3.1	-1.34	**2.53 d (0.395)**	30.1 ± 3	-2.6	**6.06 d (0.164)**
Pre.	27.61 ± 5	20.39 ±1.16	7.22	**149.08 i**	23.94 ±2.01	3.66	**12.64 i**	24.29 ± 2.8	3.32	**9.98 i**	21.27 ±1.97	6.34	**81 i**
Lac.	19.79 ± 3.1	18.99 ±2.76	0.8	**1.74 i**	20.6 ±4.71	-0.81	**1.75 d (0.57)**	20.87 ±4.03	-1.08	**2.11 d (0.473)**	20.57 ± 6.2	-0.78	**1,71 d (0.582)**
Fae.	32.86 ± 2.12	30.30 ±5.64	2.56	**5.89 i**	34.87 ±1.87	-2.01	**4.03 d (0.248)**	30.71 ±3.08	2.15	**4.44 i**	31.03 ±1.39	1.83	**3.56 i**

(Increasing or decreasing the number of bacteria by control) = $2^{(\text{averaj Control ct - averaj example ct})}$. (d: floor decreased compared to control, i: floor increased compared to control).

Table 4: qPCR Realistic Evaluation (Decrease in floor or multiplication according to control, if we accept control: 1)

Groups	BAC	PRE	LAC	FAE
Control	1	1	1	1
OL	1.17	149.08	1.74	5.89
OL + TQ1	0.279	12.64	0.57	0.248
OL + TQ2	0.395	9.98	0.473	4.44
OL + TQ3	0.164	81	0.582	3.56

4. Discussion

In our study, it was observed that the effect of TQ against the damage caused by OL in cells greatly reduced apoptotic cell damage and death. On the basis of this, TQ is thought to suppress apoptosis. Our results support other studies in terms of the antioxidant activity of TQ [22-27]. According to the microbiological results of our study, the decrease in the number of BAC in the OL group associated with obesity and the increase in the number of LAC and FAE belonging to the Firmicutes branch are similar to other studies [28-29]. The increase in the number of PRE from the Bacteroidetes phylum in the OL group was evaluated as a different result. The taxonomic categories within the phyla Firmicutes and Bacteroidetes cause changes in flora (dysbiosis) [4,11], as a result, the diversity on the basis of genus and species has become very important in different microbiota tables in obesity. *Bacteroides fragilis* and *Lactobacillus* sp. in a

study of microbiome levels in obese and overweight people. It has been reported that species are higher than lean ones and are directly proportional to body mass index [11]. In the OL + TQ1 group of our study, LAC and FAE decreased, the number of PRE increased, TQ was 25 mg *Bacteroides* level in rat intestines with weight reduction was found to be higher than Firmicutes. It has been observed that the level of protection is in PRE and FAE, but not in BAC and LAC. In other studies, at the phylum level in obese; while an increase in the *Firmicutes* level was observed, a decrease in *Bacteroidetes* was reported, and the situation was reversed in people who were thin and dieted. [4, 7, 11, 30]. *Firmicutes* / *Bacteroidetes* ratio was higher in females with increased body mass index compared to males [11] and an increase in *Firmicutes* / *Bacteroidetes* ratio showed that the person was a candidate for obesity. *Firmicutes* bacteria break down non-degradable polysaccharides. Studies have shown that an increase in *Firmicutes* density and a decrease in *Bacteroidetes* take more energy and fat from foods than routine [4]. It has been found that there is an increase in the level of *Bacteroidetes* in people who lose weight with a poor calorie diet [31]. *Lactobacillus* sp. and *Bifidobacterium* sp. levels have been found to decrease in obese patients by reducing fatty food intake [32]. It has been determined that the intestinal microbiota in obese individuals varies according to the amount of calories taken with food, this variability is observed in thin individuals and not observed in obese individuals [33]. It has been reported that the composition of the gut microbiota is in mutual interaction with obesity, the level of *Bacteroidetes* in the microbiota in obese people is higher than *Firmicutes* in weight loss, and when these people have their previous eating habits and weight gain, the number turns in favor of *Firmicutes* [28-29]. In studies conducted with obese people, it was found that the number of Actinobacteria was high in the intestinal flora, the amount of *Firmicutes* was not affected and the number of *Bacteroidetes* decreased [34]. In another study, some *Lactobacillus* sp. numbers were thought to be associated with obesity [35]. In addition, it has been reported that the number of *Firmicutes* decreases with diet application in obese people [36]. It has been reported that the number of some *Firmicutes* species is increased in obese children compared to non-obese children [37]. *Bacteroidetes-Prevotella* sp. species have been found to increase after adolescent children lose weight [38]. In mice, intestinal microflora was observed in 12 obese subjects. The amount of *Bacteroidetes* in the non-obese control group was found to be less than the rate of excess *Firmicutes*. Then, it was observed that the number of *Bacteroidetes* increased and weight loss occurred in people who received food therapy [39]. Again, in a study on mice colon microbiota of obese mice was found to increase *Firmicutes* and decreased *Bacteroidetes* [40]. *Bacteroidetes* have fewer enzyme genes that concern less lipid and carbohydrate metabolism than *Firmicutes* [41]. *Bacteroidetes thetaiotaomicron* species have been found to have a good effect on food absorption in the body [42]. The variable *Firmicutes* / *Bacteroidetes* ratio was determined in the

intestinal flora of obese people. It was found that this ratio increased in some and was not related in others [43-48]. Studies have reported that high levels of *Lactobacillus* sp. species (from the *Firmicutes* family) are reported in obese patients compared to poor controls [43]. *Lactobacillus rhamnosus* probiotic species have been reported to lose weight at the end of a given period of time given to mice [49].

5. Conclusion

In conclusion, the results of our study revealed that there is an interaction between obesity and intestinal microflora. Our findings suggest that OL, an antipsychotic drug that causes obesity, affects the microflora in intestinal tissues at different levels, and the protective effect of TQ, which we use as a preservative, also creates differences in the groups. What type of microorganism is at what level and how on obesity is still unexplained. Our study will guide the comprehensive and well-equipped studies to be conducted regarding the interaction of metabolism and intestinal microbiota, which are still uncertain and need research.

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Declaration of conflicting interests

No conflict of interest is reported by the authors.

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