



Original Research

Ameliorative effect of probiotics (*Lactobacillus paracasei* and Protexin®) and prebiotics (propolis and bee pollen) on clindamycin and propionic acid-induced oxidative stress and altered gut microbiota in a rodent model of autism

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Abstract: Colonization by toxin-producing bacteria in the gut plays a major role in bowel problems in autistic patients. Prebiotics can inhibit the growth of these pathogenic microbes by nourishing beneficial bacteria, while probiotics--live microorganisms--can balance the gut bacteria; thus, both together can maintain healthy bacteria in the gut. The present study was conducted to find the effect of probiotics and prebiotics in balancing the gut flora in a rodent model of autism linked with a clindamycin-induced altered gut. The effects of probiotics and prebiotics on oxidative stress markers in the brain were also evaluated. Eight groups of hamsters were assigned, with Group I serving as the control; Group II, as the autistic model, was treated with 250 mg propionic acid/kg BW/day for 3 days; Group III was treated with clindamycin 30 mg/kg BW for 3 days; Groups IV and V were treated with bee pollen and propolis (supposed prebiotics) at a dose of 250 mg/kg BW/day for 28 days; Group VI and Group VII were treated with *Lactobacillus paracasei* and Protexin® (supposed probiotics) for 28 days; and finally, Group VIII was anorectally transplanted with stool from normal animals for 5 days. Remarkable changes were measured in oxidative stress markers, primarily glutathione and vitamin C, in the brains of hamsters in the propionic acid- and clindamycin-treated group. All probiotic/prebiotic treatments showed ameliorative effects; however, lactobacillus had the strongest effect. We conclude that pro- and prebiotic supplements may be effective to revive healthy digestive system function in autistic patients. The disappointing results of the fecal transplants suggest that further study is needed to develop an appropriate technique.

Key words: Probiotic; Prebiotic; Autism; Lactobacillus; Fecal transplant; Propolis; Bee pollen; Propionic acid; Clindamycin.

Introduction

Autism is one of the largest medical challenges being faced worldwide, and in recent decades, this condition has exhibited an unexplained increase in prevalence. This neurodevelopmental disorder is characterized by stereotypical behavior, loss of social interaction, and impaired communication. Because of the exquisite complexity of the brain, it is almost impossible to completely understand the etiological mechanisms responsible for the behavioral phenotypes in the patients with autism. Multiple rodent models of autism have been developed, each of which may reveal one or two of the mechanisms involved in the pathogenesis of this disorder and hence might help to develop an effective treatment strategy.

Exposure to neurotoxic doses of propionic acid (PPA) is related to the pathology of autism. Intraventricular infusions or oral administration of PPA to rat pups have been used previously as a valid rodent model of autism (2, 3, 4). Recently, Xiong et al. (5) reported that the introduction of propionibacteria capable of producing PPA, such as Clostridia species, could be a contributing etiology to the PPA rodent model of autism.

The therapeutic effects of probiotics are directly or indirectly realized through their action on the gastrointestinal tract (GIT). Because probiotics are usually taken orally, these supplements have the ability to interact with the host microbiota (6). In spite of the well-documented beneficial effects of probiotics, the exact mechanism of their action has not been thoroughly elucidated to date (7). Certain of these bacteria, such as *Bifidobacterium* and *Lactobacillus*, also produce metabolites, such as lactic, propionic and acetic acids, which lower the pH of the intestine, prevent the growth of pathogenic bacteria, and assist the ecosystem of the gut through the modification of the activities of other bacteria (7, 8).

Treatment of clindamycin-associated diarrhea with probiotics is used to test the effectiveness of probiotics and to validate their health benefits (9). A remarkable reduction of diarrheal episodes associated with *C. difficile* through the use of *Lactobacillus* and *Streptococcus* probiotic species has been reported (10-12).

The recently reported apitherapeutic effect of propolis and bee pollen was attributed to antimicrobial effects (13). Bee pollen has well-documented antibiotic activity against Gram-positive pathogenic bacteria, including

propionobacteria. Additionally, the effects of propolis and bee pollen can be attributed to their high content of flavonoids, which are potent antioxidants (14, 15).

Based on these data, we decided to test the therapeutic effects of propolis, bee pollen, *Lactobacillus paracasei*, Protexin® (a commercially available mixture of probiotics), and fecal transplantation on selected oxidative stress biomarkers and the overgrowth of pathogenic bacteria that are known to be clinically presented in autistic patients and in animal models of autism.

Materials and Methods

Animals

Eighty male golden Syrian hamsters weighing approximately 100 g purchased from a live safari store in Riyadh, Saudi Arabia were obtained for in this study. The hamsters were given tap water and were raised under standard laboratory animal feeding conditions (standard pellets).

Bee pollen and propolis

100% natural bee pollen, and propolis imported from Wadi Al-Nahil, a marketing company in Saudi Arabia.

Experimental design

The hamsters were randomly divided into eight groups with ten animals per group, as follows: Group I was the control group; Group II was an autistic model treated with 250 mg PPA/kg BW/day for three days; Group III received a single dose of 30 mg clindamycin/kg BW for 3 days. After PPA intoxication, Group IV was given 250 mg/kg BW of bee pollen for 4 weeks; Group V was treated with 250 mg/kg of propolis for 4 weeks; Group VI was given *Lactobacillus paracasei* (109 CFU/mL) suspended in PBS (phosphate buffered solution pH 7.2) for 4 weeks; Group VII was given Protexin®, a mixture of probiotics, for 4 weeks; and Group VIII was transplanted with stool from normal animals. The transplants were performed by suspending the stool samples in PBS solution pH 7.2 and transplanting it anorectally into the study group daily for 5 days (16).

Ethics approval and consent to participate

All methods were performed in conformity with the National Institutes of Health Guide for the Care and treatment of Laboratory Animals in experimental investigations and approved by the Ethics of Animal use in Research committee of Princess Nourah Bint Abdulrahman University, Riyadh (approval number IBR-16-0031).

Sample collection

Brain homogenate preparation

Brain tissue was collected and washed with cold normal saline and then homogenized in ten volumes/weight of distilled water. The homogenate was then centrifuged at 3000 rpm for 10 min. The supernatant obtained was used for various biochemical assays.

Fecal pellets

The fecal pellets were collected from all the groups at different time intervals and stored at -80°C for micro-

biological analysis.

Biochemical analyses

The method described by Ruiz-Larrea *et al.* (17) was used to measure lipid oxidation by the formation of thiobarbituric acid reactive substances (TBARS). Vitamin C levels were estimated according to the method described by Jagota and Dani (18). The method of Beutle *et al.* (19) was used to measure glutathione by using 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and sulfhydryl compounds. An assay kit from Biovision, USA using the GST-catalyzed reaction between glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) was used to measure Glutathione -S-transferase activity (20).

Microbiological analysis

Fecal sample suspension preparation

Approximately 1 g of each of the fecal samples collected was dissolved in 10 ml phosphate buffer solution PBS (pH 7.2). Feces were homogenized using beads and vortexing and were centrifuged at 4500 rpm for 3 min at 4°C. Following centrifugation, 1 ml of each fecal suspension was serially diluted to 9 ml with PBS until dilution 4. 100 µl of the 4 dilutions was inoculated on different culture media: Nutrient agar (NA) (Oxoid, USA); MacConkey agar (MAC); blood agar (BLD); cefoxitine cycloserine fructose agar (CCFA), which is a selective for *Clostridium difficile* and *Bacteroides*; and Bile Esculin agar (BBE), which is selective for *Bacteroides* sp. Plates were then incubated at 37°C aerobically for 18-24 h, except for the CCFA and BBE plates, which were incubated in an anaerobic jar supplied with 5% CO₂ for 3 d.

Bacterial enumeration

Bacterial strains were counted from the different culture media listed above as colonies per plate and were tabulated. Plates with a bacterial count higher than 300 colonies per plate were not taken into consideration. Plates with colonies per plate ranging from 30-100 were considered. Susceptible colonies giving yellow color on CCFA medium were preliminary identified as *Clostridium difficile*.

Bacterial identification

All colonies were chosen from each culture plate according to their morphological features. All isolates were purified and later stored in nutrient broth with 30% glycerol in sterile microcentrifuge tubes at -80°C until use. Bacterial colonies were identified to the genus level on the basis of their microscopic appearance, morphological features, and biochemical analyses (21, 22).

Results

The obtained data are presented as means ± S.D. for the three measured variables (Table 1). While PPA and clindamycin induced remarkable changes in GSH and vitamin C, all of the tested treatments demonstrated ameliorative effects, among which *Lactobacillus* treatment was determined to be outstanding. Table 2

Table 1. Mean \pm S.D and percentage changes of the three measured parameters in brain homogenate.

Parameters	Groups	Min.	Max.	Mean \pm S.D.	Percent Change	P value ^a	P value ^b
Glutathione	Control	32.46	53.14	39.05 \pm 6.22	100.00		
	PPA	31.40	38.64	35.07 \pm 2.83	89.82	0.627	
	Clindamycin	31.88	48.30	40.98 \pm 5.93	104.94	0.976	
	Be pollen	37.68	45.61	42.40 \pm 2.96	108.59	0.713	0.001
	Propolis	38.16	50.24	46.37 \pm 3.94	118.76	0.045	
	Lactobacillus	40.09	62.80	49.87 \pm 7.27	127.72	0.001	
	Mixture of probiotic	39.13	62.00	45.91 \pm 7.34	117.59	0.067	
	Fecal transplant	40.57	52.65	45.69 \pm 3.94	117.02	0.096	
Vitamin C	Control	17.89	31.57	22.60 \pm 4.90	100.00		
	PPA	10.00	16.31	12.54 \pm 1.92	55.49	0.001	
	Clindamycin	11.05	24.73	14.73 \pm 4.29	65.20	0.003	
	Be pollen	14.21	30.00	20.98 \pm 4.99	92.83	0.957	0.001
	Propolis	11.05	23.15	15.49 \pm 4.13	68.54	0.009	
	<i>Lactobacillus</i>	12.63	20.00	16.64 \pm 3.16	73.66	0.039	
	Mixture of probiotic	13.68	26.84	19.35 \pm 5.22	85.65	0.466	
	Fecal transplant	11.57	25.26	16.61 \pm 4.35	73.52	0.038	
Lipid peroxidase	Control	0.33	0.37	0.35 \pm 0.01	100.00		
	PPA	0.36	0.40	0.38 \pm 0.01	109.70	0.339	
	Clindamycin	0.33	0.48	0.38 \pm 0.06	108.26	0.506	
	Be pollen	0.34	0.38	0.36 \pm 0.01	103.60	0.980	0.001
	Propolis	0.27	0.33	0.31 \pm 0.03	89.21	0.271	
	<i>Lactobacillus</i>	0.32	0.37	0.35 \pm 0.02	99.28	1.000	
	Mixture of probiotic	0.29	0.43	0.37 \pm 0.06	106.82	0.695	
	Fecal transplant	0.40	0.56	0.44 \pm 0.05	125.90	0.001	

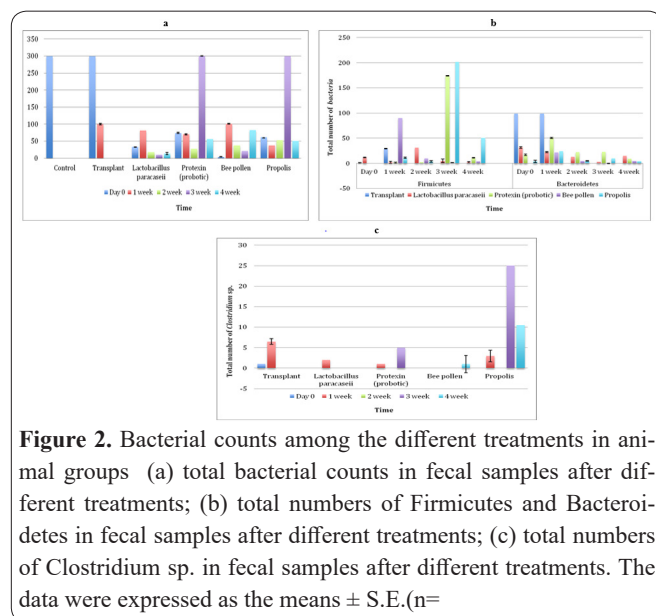
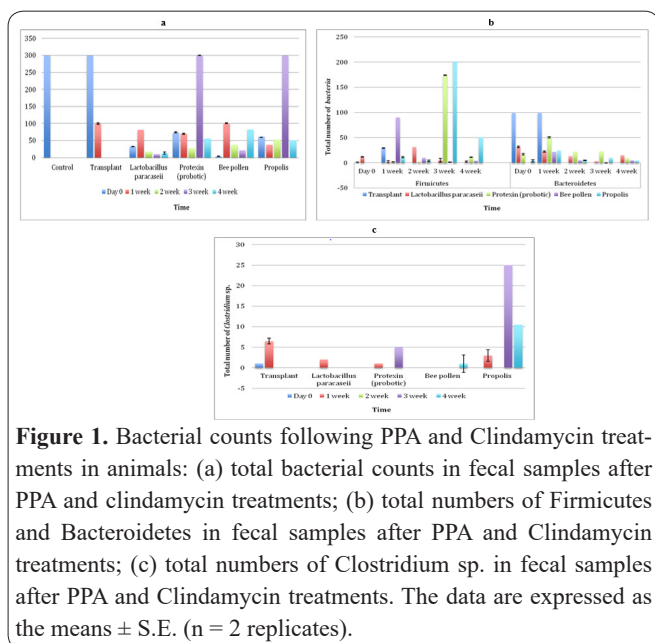
^a P value between each group and the control group. ^b P value between all groups.

Table 2. ROC-Curve of all parameters in all groups.

Parameters	Groups	AUC	Cut-off value	Sensitivity %	Specificity %	P value	95% CI
Glutathione	PPA	0.750	37.410	83.3 %	62.5 %	0.121	0.487 - 1.013
	Clindamycin	0.643	40.770	71.4 %	87.5 %	0.355	0.317 - 0.969
	Be pollen	0.789	39.325	87.5 %	75.0 %	0.052	0.541 - 1.038
	Propolis	0.833	41.540	88.9 %	87.5 %	0.021	0.597 - 1.070
	<i>Lactobacillus</i>	0.881	39.565	100.0 %	75.0 %	0.007	0.690 - 1.072
	Mixture of probiotic	0.861	39.085	100.0 %	75.0 %	0.012	0.649 - 1.073
	Fecal transplant	0.867	39.805	100.0 %	75.0 %	0.014	0.638 - 1.096
	Vitamin C	PPA	1.000	17.100	100.0 %	100.0 %	0.001
Clindamycin		0.906	16.315	87.5 %	100.0 %	0.006	0.727 - 1.086
Be pollen		0.562	16.575	25.0 %	100.0 %	0.674	0.268 - 0.857
Propolis		0.875	17.758	75.0 %	100.0 %	0.012	0.685 - 1.065
<i>Lactobacillus</i>		0.836	17.365	62.5 %	100.0 %	0.024	0.634 - 1.037
Mixture of probiotic		0.722	18.680	66.7 %	87.5 %	0.124	0.465 - 0.980
Fecal transplant		0.875	17.625	75.0 %	100.0 %	0.012	0.685 - 1.065
Lipid peroxidase	PPA	0.969	0.365	87.5 %	87.5 %	0.002	0.895 - 1.042
	Clindamycin	0.523	0.425	25.0 %	100.0 %	0.875	0.222 - 0.825
	Be pollen	0.758	0.355	75.0 %	75.0 %	0.083	0.512 - 1.003
	Propolis	0.946	0.335	100.0 %	75.0 %	0.004	0.839 - 1.054
	<i>Lactobacillus</i>	0.547	0.355	87.5 %	25.0 %	0.753	0.256 - 0.838
	Mixture of probiotic	0.656	0.365	62.5 %	87.5 %	0.294	0.347 - 0.966
	Fecal transplant	1.000	0.385	100.0 %	100.0 %	0.001	1.000 - 1.000

demonstrates the Receiver Operating Characteristics (ROC) analysis for the measured parameters. Out of the three measured variables, while GSH demonstrates the predictive value of PPA and clindamycin, vitamin C shows excellent predictive value for both neurotoxins, while lipid peroxides reflect the neurotoxic effect

of PPA but not clindamycin. All the ROC results of the treatments show ranges of good-excellent AUC, sensitivity, and specificity, except for vitamin C for the bee pollen treatment and lipid peroxides for the *Lactobacillus* treatment.



Effect of PPA and clindamycin on the gut microbiota

The group of hamsters treated with PPA and clindamycin correspondingly showed an increase in the total number of bacterial gut microbiota at day 0 (300 ± 0 , 300 ± 0 , respectively) compared to the control group (120.5 ± 0.5) (Fig. 1a). PPA, however, showed a decrease in the total number of intestinal bacteria at day 3 (95 ± 5) (Fig. 1a). Fig. 1b demonstrates a reduction in *Firmicutes* (4.1%) and an increase in *Bacteroidetes* (66.5%) at day 0; conversely, no significant difference in *Firmicutes* and *Bacteroidetes* ratios was observed (52.7% and 43.2%) at day 3. In contrast, clindamycin, on day 0, increased the *Firmicutes* (27.7%) and decreased *Bacteroidetes* numbers (1.5%). Additionally, clindamycin induced an increase in both *Firmicutes* and *Bacteroidetes* numbers (100% and 40.8%) at day 3. Results for *Clostridium* sp. are shown in Fig. 1c. Increased *Clostridium* sp. were observed in both the PPA- and clindamycin-treated groups (3.5 ± 0.7 , 19.5 ± 0.7 , respectively) compared to the control group (0) at day 0.

Effects of fecal transplant, *Lactobacillus paracasei*, Protexin® (probiotic), bee pollen, and propolis on the gut microbiota

Hamsters treated with *Lactobacillus paracasei*, Protexin®, bee pollen, and propolis showed a significant decrease in the total number of gut microbiota at day 0 (32.5 ± 0.7 , 74 ± 1.4 , 3 ± 0 , 61 ± 1.4 , respectively) compared with the control group (Fig. 2a), while the transplant group showed an increase in the total number of gut microbes (300 ± 0). All groups revealed a decrease in the total number of gut microbiota until the end of the experiment (Fig. 1a), except in week 3, when Protexin® and propolis showed an increase in the total intestinal bacteria. Fig. 2b demonstrated a reduction in *Firmicutes* (0 %) and an increase in *Bacteroidetes* (33 %) at day 0 and week 1 in the transplant group; however, the rest of the groups in the study showed no significant difference between *Firmicutes* and *Bacteroidetes*. Conversely, the Protexin® (probiotic) and propolis week 3 data indicated an increase in *Firmicutes* (58% and 67.2%, respectively). The results for *Clostridium* sp. are presented in Fig. 2c. Similarly, an increase in *Clostridium* sp. num-

bers was observed in the transplant group at week 1 (99 ± 1.4) and propolis group at weeks 3 and 4 (25 ± 0 , 10.5 ± 2.1) compared to the other groups.

Discussion

The results of the present study demonstrate the neurotoxic effects of both PPA and clindamycin through the development of oxidative stress and impairment of gut microbiota together with the ameliorative effects of prebiotic (propolis and bee pollen), probiotic (pure *Lactobacillus paracasei* and the mixture of probiotic bacteria), and fecal transplant treatments.

Glutathione, as the major intracellular defense against oxidative stress, protects against PPA-induced cell damage and apoptosis (23). Neurotoxic agents that deplete brain glutathione indirectly increase lipid peroxide levels and induce cell death in a variety of cell types (24). The remarkable but still non-significant depletion of GSH reported in the present study is consistent with the previous work of MacFabe et al. (3) and El-Ansary et al. (4), who hypothesized oxidative stress to be among the neurotoxic effects of intraventricular or orally-administered PPA. These researchers' hypothesis is supported by the present study, in which PPA induced a remarkable decrease of GSH together with increased lipid peroxides in PPA-treated animals but not clindamycin-treated ones. The ameliorating effects of the bee pollen, propolis, and probiotics are clearly demonstrated by the remarkable increases in GSH and vitamin C, two antioxidants that were measured. The remarkable increase of vitamin C in the bee pollen-treated group is consistent with multiple studies that have reported the presence of vitamin C among the active ingredients of bee pollen (25).

Moreover, the significant increase of GSH and decrease of lipid peroxidation in propolis-treated animals agrees with a recent report from Oryan et al. (26), in which propolis displayed a notable antioxidant effect due to the presence of caffeic acid phenethyl ester (CAPE) as major component. Propolis reduces lipid peroxide levels and replenishes GSH levels through inhibiting xanthine oxidase enzyme, reducing ROS generation, scavenging H_2O_2 and restoring normal

GSH/GSSG status in human burn patients, rats with thermal injury, and acute diazinon-intoxicated rats (26, 27). The antioxidant properties of propolis may be due to its chemo-preventive effect and may relate to its high content of flavonoids (28). Additionally, the neuro-protective effects of propolis presented in Table 1 are confirmed by the recent work of Nanaware *et al.* (29), in which these researchers reported that an amyloid beta-induced decrease in hippocampus GSH levels was ameliorated with macerated ethanolic extract of Indian propolis (MEEP).

The ameliorative effects of probiotic treatments presented in Table 1 are well-supported by the multiple studies that have reported that *Lactobacillus* strains have antioxidative effects, measured not only by reduced lipid peroxides but also by enhanced antioxidant production, including GSH (30-35).

Receiver operating characteristics analysis as a statistical tool usually used to measure the effectiveness of biomarkers are presented in Table 2. The neurotoxic effect of PPA is demonstrated by the oxidative stress-related markers seen; the high AUCs for all the measured variables with vitamin C and lipid peroxides demonstrating the most affected toxicity markers (AUC of 1.0 and 0.969, respectively). It is reasonable to observe that clindamycin demonstrates less toxicity as antibiotic induces propionic acid-producing bacteria compared to the direct oral administration of PPA. The ameliorative effects of most of the treatments are verified by AUC together with satisfactory specificity and sensitivity with minor exceptions.

The impairment of gut microbiota has been associated with autism and is usually seen as a high frequency of *Clostridium* clusters in autistic patients compared to control subjects. Important changes can be readily observed at the phylum level in *Firmicutes* and *Bacteroidetes*, leading to a general microbiota imbalance of the gut referred to as dysbiosis (36,37). On the other hand, intestinal homeostasis can be restored by probiotic intake and fecal transplantation, both of which are promising therapies for *Clostridium difficile* infection (38,39).

Different studies on humans (40) and mice (41,42) have shown that the diversity of the intestinal microbiota is initially reduced by antibiotic therapies before the normal intestinal flora re-establish. However, the recovery of the variable gut microbiota composition is time- and dose-dependent in relation to the antibiotic treatment used and depends on the types of bacteria present initially in the intestinal environment that are able to re-colonize the intestinal tract (41). This dependence is apparent the presented data, where fluctuations in the number and type of bacteria were recorded and were significant at week 3 of the treatment period. (Figure 2.B). Notably, PPA treatment altered the gut bacterial composition in hamsters and induced increased number of *Clostridium* sp. In relation to the effect of antibiotic treatment on gut microbiota, the present study demonstrates the effect of clindamycin on the bacterial gut composition. Clindamycin treatment led to an increase in *Firmicutes*, particularly *Clostridium* sp., but a decrease in *Bacteroidetes*. This observation may be related to a previous report from Finegold (42), which explained that the return of autistic disease after the

cessation of oral vancomycin was due to the presence of spores (which are not destroyed by antibiotics), which then developed to reflect the disease. The present study demonstrate that bee pollen and propolis can be beneficial to rebalance the microbial gut composition.

Fecal transplantation has lately been considered as a promising alternative treatment for *Clostridium difficile* disease to restore the normal gut microbiota; however, it is not widely used due to the difficulty of finding an appropriate healthy donor and to the risk of introducing a pathogenic bacterium during transplantation (43). Thus, developing a standardized treatment mixture of probiotic bacteria is necessary. In this study, various bacteria were used, including the probiotic bacterium *Lactobacillus paracasei* and Protexin®, a mixture of probiotic bacteria, and a fecal transplant from healthy donor hamsters. All of the applied protocols were able to decrease *Clostridium* growth and recover the suppressed microbiota. The data showed higher microbiota recovery ratios from fecal transplant and *Lactobacillus paracasei* (day 0 and week 1) followed by Protexin® and propolis week 3 (Fig. 2B and 2C); as a consequence, *Clostridium* number is reduced, providing a potential therapeutic efficacy for the treatment of *Clostridium* infections and also decreasing the autistic features. These findings may open new way in the health associated microbial therapeutic protocols and potentially decrease the risk of developing autism (42,43).

The use of commercial probiotics which contain only a minute fraction of the total bacteria in the gut and the bacteria they do enclose are restricted in diversity compared to the broad number and types of bacteria in the human gut. The relationship or crosstalk between intestinal bacteria and the host is so specific, so observations of this study might need more work to be applicable in humans.

Bee pollen and propolis, due to the biological properties of their components (in particular, phenolic compounds), have been determined to exhibit free radical scavenging and antioxidant activity. Moreover, consumption of *Lactobacillus paracasei* independently, either in mixture or through fecal transplantation, had beneficial effects in ameliorating oxidative stress and restoring balanced gut microbiota in PPA or clindamycin-intoxicated hamsters.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KA: Suggested the microbiological idea of the study and co-drafted the manuscript; RS: Acquisition of data and co-drafted the manuscript, NM: Acquisition of data and co-drafted the manuscript; MA: Acquisition of data; MM: Acquisition of data; AA: Acquisition of data; AE: Suggested the study and co-drafted the manuscript.

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