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Research Article

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Focus On Prebiotic and Probiotic Preventive and Therapeutic Diet Approaches to Restore Gut Homeostasis in Rodent Models of Autism

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Abstract: Although variable diet approaches shaped the gut bacterial abundance differently, they are considered the core factors in modulating gut microbiota through different mechanisms. Our aim in this study is to focus on the dietary candidates that could maintain a healthy gut microbiota by promoting commensal bacteria and reducing or inhibiting pathogens. In the current study, our objective is to demonstrate that a novel combined dietary approach of pre-and probiotic mixtures could be more effective in inducing significant improvements in gut bacterial composition as a target to treat ASD. Luteolin complex veggie capsules (Swanson, USA), artichoke extract, as well as yogurt and *Lactobacillus rhamnosus* veggie capsules (5 billion CFU) (Swanson, USA), recently known as *Lacticaseibacillus rhamnosus* were selected as prebiotics and probiotics respectively and were screened for their protective and therapeutic effects on the gut microbiota. They were all administered orally over 5 weeks to propionic acid PPA-treated rats as rodent models of Autism (groups 4-10). Their results were compared to the control animal group undertaking a standard diet (group 1) and to post and pre-PPA-treated groups (groups 2 and 3). *Lactobacillus* and *Bifidobacterium* were noted as the most dominant, non-fluctuating strains in response to all diet intakes, and they negatively influenced the presence of Proteobacteria (*Enterobacteriaceae*), which decreased in almost all treated groups during weeks 2 and 3, and 4.Interestingly, *Clostridium* sp. was observed to be high in number at week 1, mainly in the control group (1) and pre and post-PPA groups (2 and 3), but were inhibited in all treated groups over the extended treatment period. *Bacteroidetes* were positively increased with the high presence of *Lactobacillus* and *Bifidobacterium* spp. Levels throughout the experiment in almost all animal groups. Thus, both prebiotics and probiotics study designed candidates (Luteolin complex capsules, artichoke extract, yogurt, and *L. rhamnosus* capsules) inhibited *Clostridium* sp., promoted commensal bacteria, specifically *Lactobacillus* and *Bifidobacterium* spp., restored the normal gut bacterial niches and therefore could be considered as potential promising dietary approaches for maintaining healthy physical and microbial gut strategies.

Keywords: Gut Microbiota; Homeostasis; *Lacticasei Bacillus*(*Lactobacillus) Rhamnosus* Veggie Capsules; Luteolin; Prebiotics; Probiotics

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1. INTRODUCTION

A host's homeostatic physiology is crucially dependent on the gut microbiota, described as an intimate symbiotic relationship. In this scenario, apparently a healthy gut microbiota is defined as a set of bacterial taxa[1] belonging mainly to Firmicutes (90%) and Bacteroidetes phyla followed by lesser extents to *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*[2], where each individual harbors a unique gut microbiota, determining as such the highly variable gut bacterial composition among healthy individuals[3,4]. In addition, a healthy gut microbiota is defined by the ability of the commensal existing bacterial groups to resist external perturbation and to maintain a balanced environment; therefore, the gut microbiota should have a high plasticity towards the external factors[5]. An unbalanced diet, lack of sleep, long-term intake of drugs, and a poor lifestyle all contribute to disease development or "dysbiosis," which should be taken into consideration during any health disorders[6]. Indeed, this interconnection between the host and the gut microbiota is very complicated and is multidirectional, affecting, for example, the gut-brain axis, the gut-liver axis, and the gut-muscle axis. Recent research has revealed that the gut microbiota highly influences the human brain function via gut–brain interaction and that pre and probiotics intake helps maintain and improve mental health[7]. Hence, the gut microbiota is now considered the core therapeutic target for many chronic social-related diseases, specifically autism spectrum disorder (ASD). Multiple studies showed that the abundance of intestinal flora was significantly altered in children with ASD and that reduced abundance of probiotics was associated with the severity of the disorder [8, 9]. Most recently, probiotics demonstrated effective treatment for children with ASD in daily clinical practice [10]. In the current study, the use of animal modeling of ASD could help to clarify the changes in the gut bacteria of the ASD rodent model, setting a basis for understanding its pathogenesis. Additionally, prebiotics and probiotics are much more risk-free than drugs [10]. Our recent work ascertained the effectiveness of probiotics in alleviating behavioral deficits and related neural biochemistry and indicated the potential of gut microbiota-targeted therapeutic strategy in ASD [11]. As mentioned earlier, among the multifactors affecting the gut bacterial composition, diet is considered the main geographical factor that promotes differences in gut compositions [12], not only by variable diet patterns but also by long-term dietary habits, meal times, and consummatory behaviors. In particular, long-term dietary habits account for deeper and chronic gut bacterial alterations than short dietary intakes [13, 14]. Diet is noted to modulate the intestinal bacterial composition by providing end byproducts of dual effects utilized by both the host and the gut microbiota. Prebiotics, for instance, were defined as the main dietary substrates that contribute to a balanced host gut microbiota until recently, when polyphenols were considered prebiotics by promoting both beneficial bacterial growth and function, reducing disease occurrence [15]. Their great impact on the gut intestinal composition relies on both direct stimulation of bacterial growth and on their known direct antimicrobial effect [16]. Another example of a diet promoting gut homeostasis is yogurt, which is defined as a complex food composed of high amounts of calcium, phosphorus, vitamin B, essential fatty acids, and lactic acid bacteria; hence, it has a high nutritional value and has healthy effects on the gut [17]. Yogurt improves intestinal disturbances such as constipation and diarrhea due to the probiotic cultures present and consequently restores the healthy gut environment [18]. Probably by either producing bioactive peptides, which in turn enhance the intestinal barrier and prevent pathogens [19], or by the direct interaction of live yogurt bacterial strains with the gastrointestinal (GI) existing bacteria, restoring any gut bacterial disturbance as such. Similarly, probiotics are referred to as "live microorganisms that, when given in adequate amounts, confer a health benefit on the host" [20] by mainly maintaining gut microbiota balance, interacting with the host gastrointestinal cell, immune, nerve, and endocrine cells, and producing short-chain fatty acid (SCFA) for this, they are also called "psychobiotic"[7]. Among the broad range of microbes and applications, while capturing the essence of probiotics and in the context of positively modulating the gut microbiota in terms of alleviating the gut physiology, restoring homeostasis together with its bacterial composition, pre and probiotics including Luteolin complex capsules (Swanson, USA), artichoke extract (polyphenol-rich food), yogurt and *Lacticaseibacillus rhamnosus,* formerly known as *Lactobacillus rhamnosus*[21], capsules (5 billion CFU/capsule)(Swanson, USA) were administered orally for 5 consecutive weeks to the 7 dietary- protected or treated groups each of 6 animals. Fecal samples from each animal in each of the tested groups were processed for gut bacterial alteration in comparison to control groups. The molecular technique was applied to identify further some selected bacterial isolates representative of normal gut microbiota to reveal the gut bacterial composition changes pre and post-d dietary intake, particularly before and post-PPA administration, as an example of induced autism. In the current study, a novel combined dietary approach of pre-and probiotic mixtures could be more effective in inducing significant improvements in gut bacterial composition as a target to treat ASD. It is well accepted that combined interventions might be more appropriate for the improvement of altered gut microbiota as well as social behavioral deficit in ASD [22, 23].

2. MATERIALS AND METHODS

2.1. Food pattern collection, extraction, and administration

Fresh commercially available artichoke (*Cynara scolymus* L.), imported from the Netherlands, was purchased from local supermarkets in Riyadh, Saudi Arabia, in March 2019. *Cynara scolymus* leaf samples were cleaned, chopped, shade-dried at room temperature, and ground into powder. 10g of the dried powder was then extracted separately with methanol/water (80/20, V/V) for 72 h on an orbital shaker adjusted to 150 rpm. The extract was then filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK) in a Buchner funnel. The filtrate was allowed to evaporate in a vacuum rotary evaporator (EYELA, Tokyo, Japan) at 40°C until the methanol was completely evaporated. The dry extract was stored at 4°C until further use. Yogurt, as a probiotic source, was purchased from the local market and stored at 4°C until use. Both *Lacticaseibacillus rhamnosus,* previously known as *Lactobacillus rhamnosus* [21], veggie capsules (5 billion CFU/capsule), and Luteolin complex as probiotic and prebiotic supplements, respectively, were purchased from Swanson Health Products, Fargo, North Dakota, USA.

2.2. Ethics approval

This work was approved by the ethical committee of the Science College, based on the recommendation of the

Research Ethics Sub-committee on Animals number (SE-19-142), King Saud University.

2.3. *Animal house, groups, and dosage*

Sixty males, three Wistar albino rats, each weighing 70±20g, were purchased from the Center for Laboratory Animals and Experimental Surgery (CLAES), Prince Naif Health Research Center (PNHRC), King Khaled University Hospital (KKUH), Riyadh, SA. All animals were hosted in polypropylene cages in an environmentally controlled clean air room at 25°C ±1, a 12 h light/12 h dark cycle, and a relative humidity of 50 ±5%. Rats were divided into 10 groups of six animals each (labeled as A and B) as follows: Group 1 was the control group with rats of regular weight following a standard food diet (laboratory animal feed pellets) with water as described in the Association of Official Agricultural Chemists (AOAC) [24] for 30 days. Group 2 (pre-PPA) (autism rodent model): six rats of regular weights were given orally a PPA neurotoxic dose (250 mg/kg body weight) for three days [25], followed by water and standard diet intake for 27 days. In group 3 (post-PPA), six rats received the first standard diet with water for 27 days, followed by oral intake of PPA (250 mg/kg body weight) for three days, and served as a control-post-treated autism model. Animal groups 4-7 (protected groups) were given the dietary interventions followed by PPA neurotoxic dose (250 mg/kg body weight) [25] for 3 days as such: Group 4 (yogurt-protected group) had a standard diet, water and yogurt (3 mL/kg body weight) were first given orally for 27 days, followed by PPA for three days. Group 5 (Artichoke-protected group) of standard diet, water, and Artichoke (400 mL/kg body weight) were first administered for 27 days, followed by PPA for three days. Group 6 (*L. rhamnosus*-protected group) standard diet, water, and *L. rhamnosus* (1 mg/mL/day) were first given for 27 days, and then PPA, Group 7 (Luteolin-protected) standard diet, water, and Luteolin (50 mg/kg body weight) for 27 days followed by PPA. Groups 8-10, however, will receive the first PPA followed by different dietary interventions as follows: Group 8 (Yogurt-treated group) where PPA neurotoxic dose was given first for three days and then treated with a standard diet, water and yogurt (3 mL/kg body weight). Group 9 (Artichoke-treated group) again PPA was received first for three days, followed by standard diet, water, and orally administered Artichoke (400 mL/kg body weight) for 27 days. Group 10 (*L. rhamnosus* + Luteolin –treated) PPA was administered first and then treated with standard diet, water Luteolin (50 mg/kg body weight) and *L. rhamnosus* (1 mg/mL/day) for 27 days. A summary of both dietary interventions, groups, and dosage is given in Tables 1 and 2.

Table 1. Dietary intake and dosage.

Dosage	Control	PPA	Yogurt	Artichoke	Luteolin	*L. rhamnosus*
		250 mg/kg body weight	3 mL/kg	400 mL/kg body weight	50 mg/kg/day	1 mg/mL/day

Table 2. Study-designed animal groups.

Animal groups	Food intake				
	Yogurt+PPA/ PPA+Yogurt^	Artichoke+PPA/ PPA+Artichoke^	*L. rhamnosus*+PPA/ PPA+ L. rhamnosus^	Luteolin+ PPA/ PPA+Luteolin^	
Group 1					Control
Group 2					PPA+normal diet
Group 3					Normal diet +PPA
Group 4	*				
Group 5		*			
Group 6			*		
Groups 7				*	
Group 8	^				
Group 9		^			
Group 10			^	^	

2.4. *Isolation of bacterial strains*

In brief, 1 g of fresh stool was homogenized in 10 mL phosphate buffered saline (pH 7.2) for 30 seconds, centrifuged for 3min at 4500rpm at 4ºC, and then 4 serial dilutions were performed. 0.1mL from the last 2 dilutions was plated on MacConkey agar (Mac - Oxoid, USA) for *Enterobacteriaceae*, Man Rogosa Sharpe agar (MRS- Oxoid, USA) for *Lactobacillus* spp. and *Bifidobacterium* spp. Bacteroides Bile Esculin (BBE-Oxoid, USA) agar for *Bacteroides* spp. and CCFA (Oxoid, USA) for *Clostridium* spp. Growth respectively. BBE, CCFA, and MRS plates were incubated under anaerobic conditions at 37℃ for 72 h, whereas Mac was incubated under aerobic conditions at 37℃ for 18-21 h. Similar bacterial strains were observed on each of the selective media among the animal groups and throughout the experiment. Selection was made in an attempt to reveal the mostly known commensal gut bacterial strains in response to the presence of the representative bacterial species *Clostridium* spp. in autism following the pre and probiotic intake. A few colonies with distinct morphologies were selected from each selective media used and from selected samples, too; for purification and preliminary identification with the Gram staining technique, they were divided into Gram-positive or Gram-negative according to their microscopical description (color and shape). These same colonies were further identified using molecular technique, precisely conventional PCR amplification reaction.

2.5. *Molecular identification*

All selected presumptive isolated strains were further identified with PCR reaction following the genomic DNA

extraction kit instructions (Invitrogen, USA) to confirm the target genera, with the use of specific sets of primers: LAC F and Lac R, Bifido F and Bifido R, TEcol 553F TEcol 754, hha gene F hha R, AllBac R, AllBac R, tcdA F tcd A R, tcdB F and tcdB R as indicacted in Table 3 for the identification of *Lactobacillus* spp. *Bifidobacterium* spp., *E.coli*, *Bacteroides,* and *Clostridium* spp. Correspondingly. A total volume of 25 µL PCR reaction was carried in Genepro thermal cycler (Bioer, China); each reaction mixture contained 2 µL of each of the DNA samples, 12.5 µL of the GoTaq Green Master Mix (Promega, USA), 0.125 µL of each of the primers forward and reverse (Invitrogen, USA), 10.25 µL of RNAse DNAse free water. The cycling parameters were as follows: initial denaturation for 2 min at 94°C followed by 35 cycles of 94 °C for 15 sec, 63 °C for 1min as for the annealing of each primer, 72 °C for 2 min, and a final elongation step at 72 °C for 5 min[26]. DNA concentration and quality were determined using the genova nanodrop (Italy) and 1.5% agarose gel electrophoresis; positive samples showed bands ranging from 200-600bp for the different sets of primers used.

Table 3. Sets of primers for bacterial identification

Primers	Sequence (5'-3')	Bands (bp)
Lac F	5'- AGCAGTAGGGAATCTTCCA -3'	600
Lac R	5' -CACCGCTACACATGGAG -3'	
Bifido F	5'- GATTCTGGCTCAGGATGAACGC -3'	400
Bifido R	5'- CTGATAGGACGCGACCCCAT-3'	
tuf gene TEcol553	5'-TGGGAAGCGAAAATCCTG -3'	200
TEcol754	5'-CAGTACAGGTAGACTTCTG -3'	
hha R gene	5' - GTTTACGTCGTTGCCAGACA-3'	166
hha F	5'- TTCCATACTGAGGAAGGGATCT -3'	
AllBac 296F	5'-GAGAGGAAGGTCCCCCAC -3'	600
AllBac 412R	5'-CGCTACTTGGCTGGTTCAG -3'	
tcdA F	5'- GGTAATAATTCAAAAGCGGCT -3'	600
tcdA R	5'- AGCATCCGTATTAGCAGGTG -3'	
tcdB F	5'- GAAAGTCCAAGTTTACGCTCAAT -3'	400
tcdB R	5'- GCTGCACCTAAACTTACACCA -3'	

3. STATISTICAL ANALYSIS

Two-way analysis of variance (ANOVA) was performed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) or IBM SPSS for Windows, version 27.0.1.0 (IBM Corp., Armonk, NY). Multivariate ANOVA (MANOVA), Mauchly's test of sphericity, and discriminant analysis (DA) were performed using IBM SPSS for Windows, version 27.0.1.0. Mauchly's test of sphericity was performed in association with repeated measures ANOVA to validate the sphericity assumption. Whenever the assumption of sphericity was violated ($p<0.05$), p values were corrected using the Greenhouse-Geisser method.

4. RESULTS

4.1. Morphology and molecular identification

Based on the macroscopical and microscopical observation, all selected colonies displayed specific characteristics on the selected media, confirming their preliminary identification. As *Escherichia coli* with distinct pink colonies on Mac, *Lactobacillus and Bifidobacterium* on MRS identified according to their difference in color, *Bacteroides* on BBE appeared as brown colonies on BBE plates, and last *Clostridium* spp. were identified as yellow colonies on CCFA.

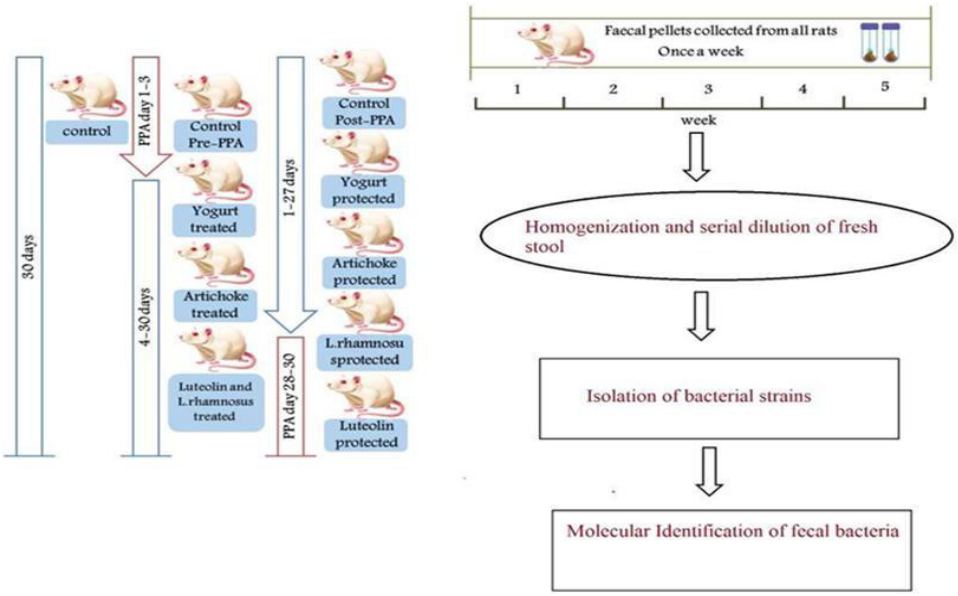
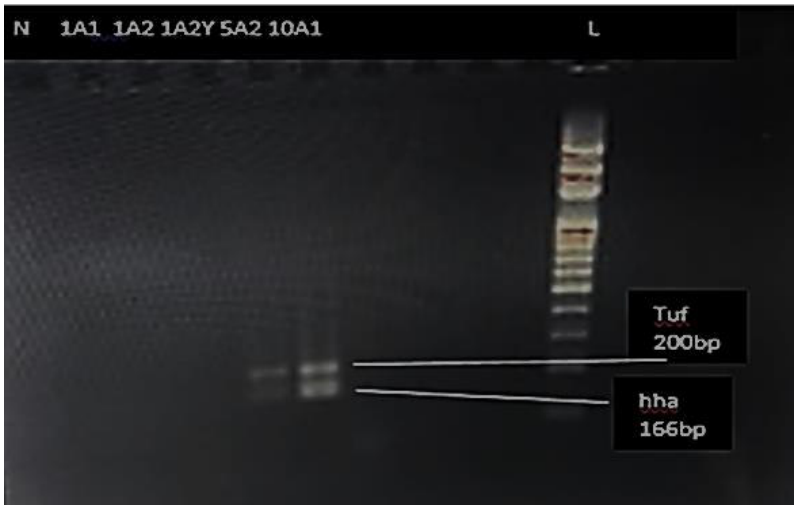


Fig 1: Microbial analysis experimental design showing stool collection followed by fecal dilutions of each of the animal groups for bacterial isolation and molecular identification

The PCR product of the selected isolated strains from specific animal groups revealed different band profiles. *E. coli* were revealed at 166bp and 200 bp with hha and tuf primers, respectively, from groups 5 and 10 only (Figure 2). *Lactobacillus* and *Bifidobacterium* spp. appeared mostly together, almost in all selected animal groups at 600 and 400bp, with LacR and LacF, BifidoR, and BifidoF, respectively, revealing their inbred-cohabitation nature. Animal groups 1, 2, 3, 4, 5, 8, 9, and 10 were all positive for the co-presence of *Lactobacillus* and *Bifidobacterium* spp. (Figure 3). On the other hand, all selected samples were negative for the presence of *Bacteroides* sp. All samples failed to show bands at 600bp with allBac primer; as for *Clostridium* sp., animal groups 1,2,3,4,5 and 8 were positive for the toxin A and toxin B genes at 600 and 400 bp, respectively, whereas group 1rat 3 and group 2 rat 1 showed bands for only tcdA (Figure 4).



Both groups, Artichoke treated 5, and L. rhamonosus + Luteolin treated 10 were positive for the hha gene at 166bp and 200bp, respectively. N: negative, L: DNA Ladder (100-3000bp) (Solis, USA), A: rat numbers per animal group.

Fig 2: PCR products for *E. coli* identified strains with hha gene and tuf gene from the selected animal groups.

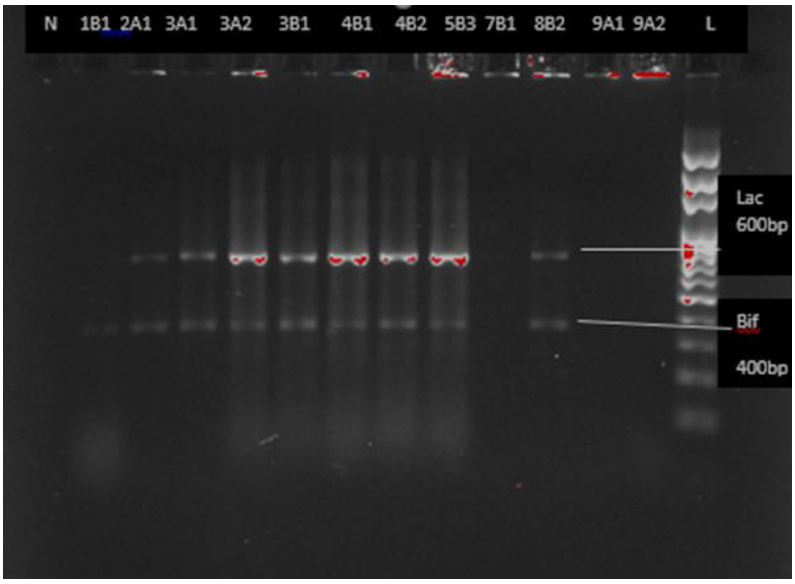


Fig 3: Amplification gel image for the identification of *Lactobacillus* and *Bifidobacterium* sp

In selected animal groups (1 lean control, pre-PPA 2, post-PPA 3, yogurt protected 4, Artichoke protected 5, and yogurt treated 8) showed positive bands at 400 and 600bp for the Bif gene and Lac gene, respectively. Only one sample from group 1 was solely positive for *Bifidobacterium* sp. presence. with an observed band at 400bp. At the same time, Luteolin-protected 7 animal groups failed to show bands for both strains in the study. N: negative, L: DNA Ladder, A and B: rat numbers per each animal group.

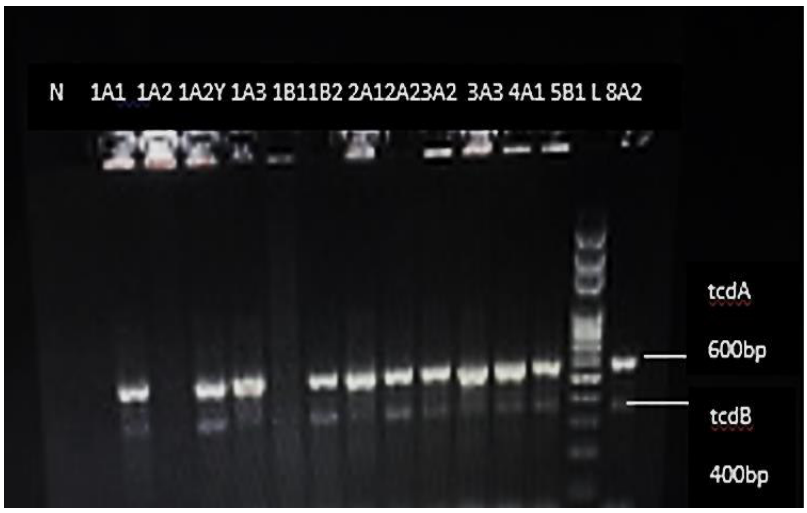


Fig 4: Amplified PCR products on 1.5% agarose gel showing bands at 400bp and 600bp for tcdA and tcdB (toxin A and toxin B genes) for the identification of *Clostridium* sp. when compared to the DNA ladder.

Animal groups 1 (lean control), 2 (pre-PPA),3 (post-PPA),4 (Yogurt protected), 5 (Artichoke protected), and 8 (Yogurt treated) were positive for toxin A and toxin B genes, whereas 1A3, 2A1 showed bands for only tcdA. G1A2 failed to show any band. N: negative, L: DNA Ladder, A and B: number of rats per each animal group.

4.2. Microbiome dynamics in dietary-intake groups

One-way repeated measures MANOVA showed significant temporal changes in the gut microbiome – represented in four bacterial groups, *Enterobacteriaceae*, *Lactobacillus*/*Bifidobacterium*, *Bacteroidetes*, and *Clostridia* – of PPA-post ($p<0.001$), Artichoke-protected ($p=0.032$), Yogurt-treated ($p=0.013$), Artichoke-treated ($p<0.001$), and *L. rhamnosus*-protected groups ($p<0.001$). No such changes were observed in the remaining groups, including the control group ($p>0.05$) (Table S1). One-way repeated measures ANOVA was used to examine each of the individual bacterial groups, which revealed significant temporal changes in *Lactobacillus*/*Bifidobacterium* and *Clostridia* of the PP-post ($p=0.024$ & 0.006, respectively), the Artichoke-treated ($p=0.049$ & 0.007, respectively), and the *L. rhamnosus-protected* groups ($p=0.018$ & 0.038, respectively). In addition, the *Lactobacillus*/*Bifidobacterium* group changed over time in the artichoke-protected group ($p=0.012$) (Table S1). *Lactobacillus*/*Bifidobacterium* seemed to fluctuate over the course of the experiment in these four groups (PPA-post, Artichoke-treated, *L. rhamnosus-protected*, and Artichoke-protected), while clostridia were highest during the first week, diminishing afterward (Figure 5). To evaluate microbiome distinctiveness among the dietary groups, we performed discriminant analysis on each of the four microbial groups studied. Discriminant analysis failed to effectively separate the groups using *Enterobacteriaceae*, *Lactobacillus*/*Bifidobacterium*, *Bacteroidetes*, and *Clostridia* (Figure 6), indicating that, despite the specific differences described above, the microbiomes of

the dietary groups are not wholly different enough to distinguish between the groups. To individually examine weekly results, we performed two-way ANOVA to determine whether the differences between dietary-intake groups in bacterial counts were significant and whether the influence of dietary intake on bacterial counts significantly differed between microbiome groups. We showed that dietary intake significantly influenced fecal bacteria counts in each of the five weeks, except for weeks 2 and 5 (p values: <0.0001, 0.3557, 0.0002, 0.0239, and 0.2386 for weeks 1 through 5, respectively). This influence significantly differed by microbiome group only in weeks 2 and 3 (p values: 0.2592, 0.0087, <0.0001, 0.3841, and 0.1244 for weeks 1 through 5, respectively). We then used MANOVA whether the influence of dietary intake on fecal bacteria counts differed significantly between microbiome groups throughout the entire study, and we found that it did (p-value of 3.8×10^{5}).

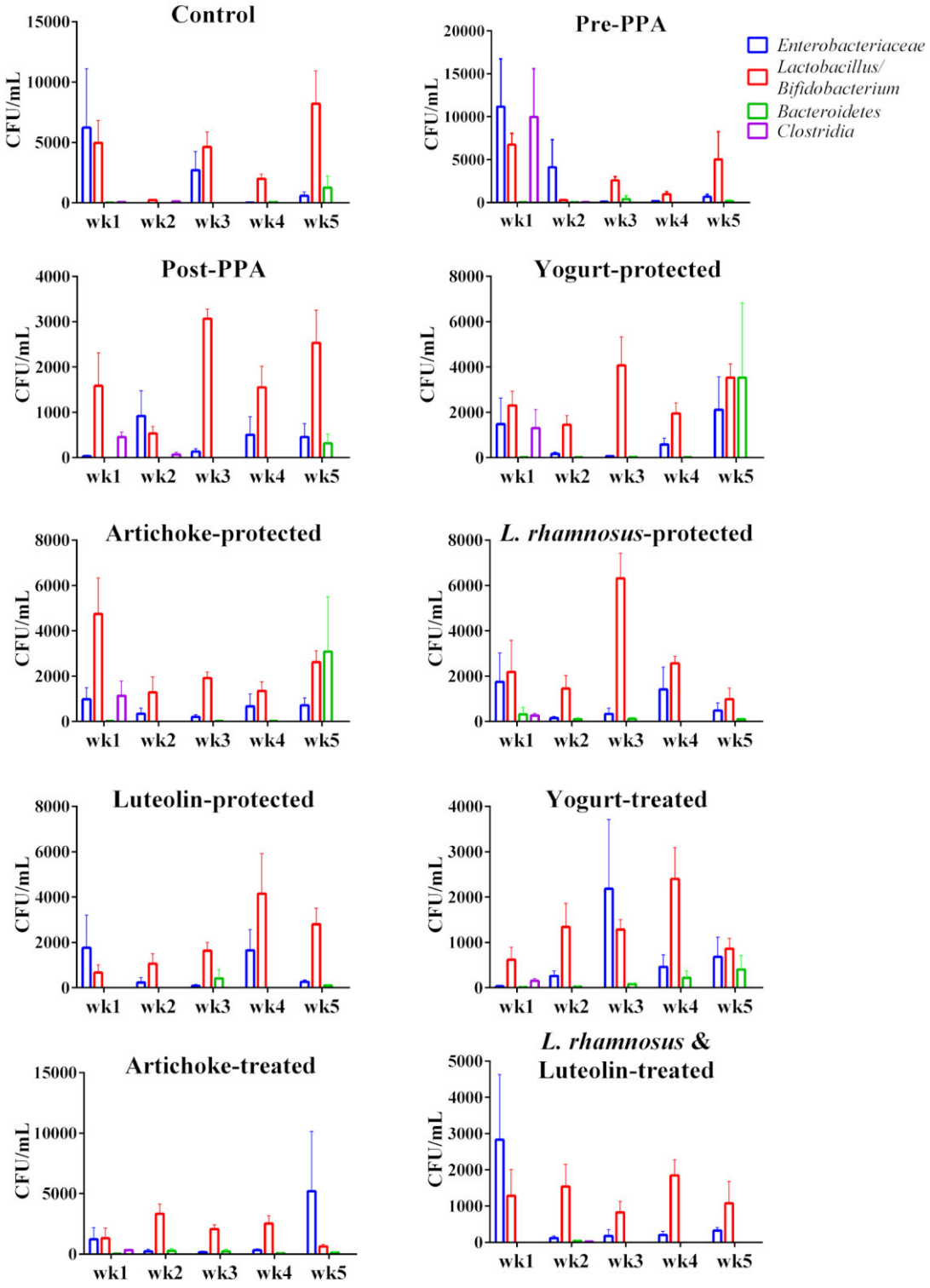


Fig 5: Fecal bacterial counts of rats in different food intake groups over a 5-week period.

Lactobacillus and *Bifidobacterium* sp. were dominant throughout the experiment in all tested animal groups. *Enterobacteriaceae,* on the other hand, appeared to reduce in number, particularly by the end of the experiment, mainly with the prebiotic group (Luteolin and artichoke extract as polyphenol-rich food), probiotics *L. rhamnosus,* yogurt compared to the control groups 1, 2 pre-PPA treated and 3 post-PPA treated. The same pattern was observed for *Clostridium* sp., where high numbers were observed at the beginning of the experiment, particularly with groups 1,2 and 3. Then, it disappeared throughout the experiment with all prebiotics (Luteolin and artichoke extract), probiotics, and yogurt animal groups (4,5,6,7,8,9 and 10). BBE growing strains were variable in their abundance; they decreased in week 1, increased in week 2 and week 3, and then decreased in number in weeks 4 and 5. Thus, a prolonged food intake period greatly affected the presence of the bacterial strains; it was noted that all of the dietary interventions used in the present study had similar effects on the gut microbiota; they all promoted the growth of the commensal beneficial bacteria *Lactobacillus* and *Bifidobacterium* sp. and inhibited *Clostridium* sp.

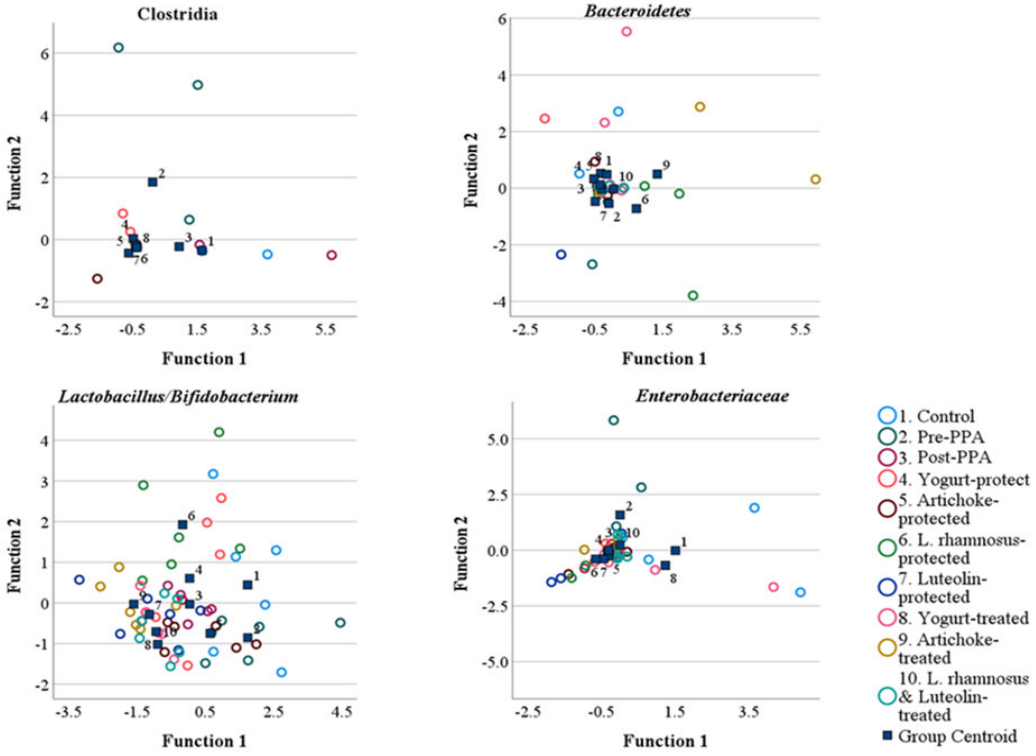


Fig 6: Group separation using discriminant analysis MANOVA (P value of 3.8 x10^{5}) fails to separate dietary intake groups.

Discriminant analysis was used to attempt to separate dietary intake groups based on fecal bacterial counts obtained during each of the five weeks of this study. This was done separately for *Enterobacteriaceae*, *Lactobacillus* /*Bifidobacterium*, *Bacteroidetes*, and *Clostridia*. No clear separation was observed, indicating these variables combined could not discern the 10 experimental groups.

5. DISCUSSION

Different health dietary consequence patterns were pointed out [27- 29]; however, deeper studies are required to ascertain their nutrients' effects on the gut microbiota. Although it has been demonstrated that diet is one of the most critical factors affecting the gut microbiota together with the duration of the intake and the life style, however, still the mechanism is not very clear. A healthy gut is defined as the interconnection between the host and the commensal bacteria present in the gut; any disturbance in this interrelationship will cause gut imbalance interlinked to social and behavioral disturbances revealed by the abundance of pathogenic bacteria in fecal samples, such as the case with autism where it is mainly characterized by the dominance of *Clostridium* sp. in stool samples. In the context of promoting gut homeostasis, the present data reported fluctuation in the bacterial abundance in response to the variable experimented dietary intervention using pre and probiotics [30]. At the bacterial level, *Lactobacillus*/ *Bifidobacterium* was observed as the dominant species throughout the experiment in all animal groups 1-10; they reached their maximal level particularly following the probiotic intake (*Lactobacillus* and Yogurt) in groups 4, 6, 8, and 10. *Enterobacteriaceae* (*E.coli*), on the contrary, were noted high at the beginning of the experiment week 1 in almost all experimented groups 1-10, then decreased to disappear in group 1(control) week 2 and 3, but then increased with protected and treated animal groups (4, 5, 6, 7, 8, 9 and10) during week 4 and 5. *Bacteroidetes* were observed in the control group (group 1) and groups 4, 6, 8, and 10, and with the prolonged diet intake, their number increased specifically at week 3 and 4 in groups to reach their utmost occurrence at week 5 mainly in groups 4, 5, 6, 7, 8, 9 and 10 in correlation with the overgrowth of *Lactobacillus* and *Bifidobacterium*. *Clostridium* sp., oppositely, were noted high at the beginning of the experiment (week 1) in almost all animal groups except for group 7 and group 10, but then were reduced significantly in group 1 control as well as in pre and post-PPA groups (groups 2 and 3) at week 2; to remarkably disappear in all animal groups (1-10) from week 3 until the end of the

experimental period (Figure 5) Now at the dietary intervention level, Luteolin and artichoke extract (polyphenol-rich food) given as prebiotics (groups 5, 7, 9 and 10), revealed similar gut bacterial composition alteration, both triggered the growth of the beneficial bacteria *Lactobacillus* and *Bifidobacterium* sp. and decreased with time the presence of *Enterobacteriaceae*, *Clostridium* sp. and *Bacteroidetes*. Gonzalez-Sarrias et al. [31], Liu et al. [32], and Rodriguez-Daza et al. [33] reported similar findings where polyphenols can reshape the gut microbiota by promoting the growth of probiotic bacteria, mainly *Bifidobacteria* and *Lactobacilli* spp. and shifted the ecological niches either by triggering mucosal pro and anti-inflammatory balance and or inhibiting pathogens by direct polyphenols intake by the gut bacteria. Similar to the prebiotic effect, probiotics administered as yogurt and *L. rhamnosus* in this study (groups 4, 6, 8, and 10) showed identical overgrowth patterns of *Lactobacillus* and *Bifidobacterium* sp.; however, their dominance remarkably stimulated Bacteroidetes presence precisely on week 3 in almost all animal groups (Figure 5). On the other hand, Proteobacteria, including *Enterobacteriaceae* (*E. coli*) and *Clostridium* sp., were negatively affected following the prolonged probiotic intake, in agreement with previous findings, which demonstrated that probiotic intake altered the gut bacterial composition. Ferrario et al. [34], for instance, in their randomized double-blinded study of *Lactobacillus paracasei* DG capsules of daily intake over 4 weeks, reported an increase in Proteobacteria and a decrease in *Clostridial* sp. Nagata et al. [35] also indicated that after 6 months of *Lactobacillus casei* intake, both *Bifidobacterium* and *Lactobacillus* were significantly more abundant compared to other bacterial strains, *Clostridium* sp. in contrast, were observed to be low in number. *L. rhamnosus* GG intake for 4 weeks encouraged the development of healthy microbiota in infants by increasing *Bifidobacterial* diversity, as demonstrated by Lahtinen et al. [36] The Same scenario was revealed with prolonged yogurt intake throughout this study, although research on yogurt is still scarce and controversial; however, 5 weeks of yogurt intake period overgrew *Lactobacillus* and *Bifidobacterium* compared to all other species studied, significantly inhibited *Clostridium* sp. and affected the growth of *E. coli* and *Bacteroidetes*. Gracia-Albiach et al. [37], on the other hand, revealed the difference between fresh and pasteurized yogurt, where a higher number of *Lactobacillus* was observed with the fresh yogurt compared to the pasteurized, whereas *Bacteroidetes* decreased with both intakes. All this gut bacterial alteration could be due to the production of bioactive peptides which enhance the intestinal barrier and prevent pathogens [19], or to the direct interaction of live yoghurt bacterial strains with the GI existing bacteria improving intestinal disturbances such as constipation and diarrhea due to the probiotic cultures present and consequently restoring the healthy gut environment [18]. Last but not least, the molecular identification with conventional PCR, using selective primers for each of the presumptive isolates obtained, revealed bands between 166bp hha gene (*E. coli*), 400bp *Bifidobacterium* and toxin B (*Clostridium* sp.) and 600 bp for *Lactobacillus* and toxin A (*Clostridium* sp.) (Figures 2-4). This technique was performed as a confirmation step of the bacterial genera in an attempt to develop an effective approach of pre and probiotics diet administration for maintaining a healthy gut microbiota.

6. CONCLUSION

Based on the obtained results, it can be concluded that pre and probiotic prolonged ingestion (Luteolin, artichoke, *L. rhamnosus*, and yogurt), independent of the strain used and or fresh or pasteurized yogurt, revealed similar effects in terms of gut bacterial composition and dysbiosis prevention, indicated the existence of an interrelationship between the host and the gut microbiota either through the production of secondary metabolites and or through the known pre and probiotics antimicrobial and anti-inflammatory effects. It also indicated the ability of pre and probiotics to persist in the gut for an extended period; however, still more studies should be elaborated to build a standardized dietary pattern. Pre and probiotics designed candidates in this study were noted to inhibit pathogens and promoted commensal bacteria, specifically *Lactobacillus* and *Bifidobacterium* sp., restored the normal gut bacterial niches and therefore could be considered as potential promising dietary approaches for maintaining a healthy physical and microbial gut strategies.

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9. AUTHOR CONTRIBUTION

S.S provided the resources. H.A.A. supervised, validated, and edited the manuscript. W.H performed the statistical analysis. A. A conceptualized, validated, and edited the manuscript. N.M. conducted the microbiology analysis, validated the data, and wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

10. CONFLICTS OF INTEREST

Conflict of interest declared none.

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