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The effect of lactic acid bacteria as probiotics isolated from different sources on some parameters of the immune system of Wistar albino rats

Sara Imad Hajwal¹, Laith Muslih Najeeb ^{1*}

¹Biology Department, College of Science, University of Anbar

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Corresponding author Laith Muslih Najeeb drlaith@uoanbar.edu.iq

ABSTRACT

The current study aimed to determine the altered immune indicators resulting from the use of lactic acid bacteria as probiotics, isolated from various sources. In this experiment, a sample of twelve-month-old male Wistar albino rats was taken with an average weight of 204.67 grams and a standard deviation of 8.04 grams. Three mice were randomly assigned to each of the four experimental groups for a period of one month. The groups were placed in different types of water, with the first group receiving plain water (control group) and the second and third groups receiving plain water mixed with sedimentary cells of lactic acid bacteria (LAB) isolated from the excreta of breastfed children. The fourth is on plain water mixed with lactic acid bacteria cells isolated from the excreta of bottle-fed infants. One month after the experiment began, blood samples were collected and subjected to six different immunological tests (IL-2, IL-8, TNF- α , TNF- κ , ADP, and LEP) using ELISA kits. Both groups of natural feeders respond better than the other two groups, control and formula-fed. The mean optical density for the natural feeding groups is significantly different between the two natural-fed groups as well as between them and the other two groups of formula-fed and control. LEP and IL-8 recorded the highest optical densities among all others, which indicates that these factors mostly respond to the lactic acid bacteria (LAB) isolated from the excreta of breastfed children.

Keywords: Immune system, Interleukin, Optical density, Probiotics, Wistar Albino rats

1 INTRODUCTION

A wide variety of Gram-positive bacteria, including Lactobacillus, Lactococcus, Streptococcus, Enterococcus, Leuconostoc, Carnobacterium, Oenococcus, Pediococcus, Tetragenococcus, Vagococcus, and Weissella, make up the lactic acid bacteria (LAB) group [1]. Intestinal and gut mammalian systems are home to trillions of microorganisms, "microbiota/microflora" that play an essential role in human health. The most commonly isolated organisms from fermented foods, beverages, and human and animal GUT are Lactobacillus, Pediococcus, Bifidobacterium, Lactococcus, Streptococcus, and Leuconostoc [2,3]. The potentially beneficial effects of LABs on human health include lowering blood cholesterol [4], alleviating lactose intolerance [5,6], inhibiting tumor

growth [5,7-9], and enhancing the immune system [10]. Due to these positive effects, there has been a surge in studies investigating how to isolate and characterize LAB from various food sources for use in the food industry. Bifidobacterium spp., Lactococcus spp., and Lactobacillus spp. are only a few examples of health-promoting LAB strains that have been discovered [11]. There are several benefits to human from consuming probiotic LAB, including improve immune responses by increasing serum antibody release (IgM, IgG, and IgA), balancing proinflammatory and anti-inflammatory cytokines [6,12–14], shorten the duration and prevent intestinal diseases like inflammatory bowel disease, diarrhea and constipation by colonizing and modulating the gut microbiota [15, 16], improves gut microbiota, revives the antioxidant system, and reduces insulin resistance and inflammation. All of

which aid in warding off metabolic disorders like diabetes, obesity, cardiovascular disease [14], prevent intestinal infection from Candida and Helicobacter pylori by fighting for adhesion sites on the mucosal surface and boosting immune responses [12], anti-mutagenic effect [17, 18], detoxification of chemical carcinogens decreased release of toxic metabolites improved antioxidant system modulation of immune response to inhibit self-proliferation of cancer [14, 17, 18], generation of metabolites like butyrate [19–21], provides the -galactosidase (Lactase) enzyme, which breaks down lactose into its component parts, aiding in the maintenance and alleviation of lactose intolerance [14], benefits in lowering the cholesterol level by precipitating cholesterol with free bile salts into bile acids, reducing cholesterol absorption [22], and improves mineral absorption, particularly calcium, to prevent mineral deficiency.

One of the most microbiologically active ecosystems, the gastrointestinal tract (GT) houses many bacteria essential to immune cell development. An abundance of microbiota and food-borne bacteria live in harmony with each other and with the immune cells that are embedded in the villous lamina propria. Although the intestinal microbiota has no direct interaction with the epithelial cells, their metabolites promote the development and function of immune cells [23]. The original definition for probiotics was "Live microbial feed supplements which beneficially affect the host, improving the intestinal microbial balance of the host." [24]. An updated definition of probiotics is that they are "Live microorganisms that when being administered in appropriate doses, confer a benefit to the health of the host" [25] Some of the many probiotic bacteria that make up the intestinal microbiota have been increasingly incorporated into foods in an effort to boost gut health through the promotion of a healthy microbial balance in the digestive tract. For example, *Lactobacilli*, Streptococcus, Pediococcus, Enterococcus, Bifidobacteria, and even yeasts like Saccharomyces boulardii are all commonly utilized as probiotics [26]. However, only some strains of bacteria may serve as probiotics. In animal models, the positive effects of probiotics have been widely exploited to enhance host health and treat a variety of viral and non-infectious diseases. This includes defense against infections [27], alleviation of IBS symptoms [28], suppression of Helicobacter pylori growth, attenuation of the inflammatory response in the gut, and protection against allergic reactions. Although probiotics have shown promising results in a number of human health conditions, including diabetes, MDR- pathogens, and irritable bowel syndrome, much more research is needed before probiotics can be routinely incorporated into human health, nutrition, and the regulation of various abnormalities. Probiotic bacteria must be able to endure the acidic and alkaline environments of the human stomach and gastrointestinal tract in order to have a positive effect on human health. That probiotics can survive gastric juice and bile salt, make it through the upper GI tract intact, proliferate, colonize, and perform their function in the GI tract are all factors that could contribute to this claim [29].

Probiotics such as *Lactobacillus rhamnosus* strain GG and *L. plantarum* have been shown in multiple studies to prevent enteropathogenic *Escherichia coli* from attaching to the gastrointestinal tract [30]. Furthermore, the ability to adhere to epithelial cells is one of the most critical features required for a potential probiotic strain. For example, [31] used electronic microscopy to show that the probiotic bacteria *L. casei* CRL 431 and *L. paracasei* CNCM I-1518 bind to and stimulate the immune system by adhering to intestinal epithelial cells (IECs) via Toll-like receptors (TLRs).

There was no change to the intestinal barrier or the invasion of mononuclear cells into the small intestine after this interaction, but IECs increased their production of cytokines such as interleukin-6 and macrophage chemoattractant protein 1. The scientists also showed that only bacterial pieces, not complete germs, were taken up by the IECs. Therefore, the IECs set in motion a complex network of signals that excite the lamina propria-associated immune cells, primarily activating the innate response and the cytokines released by T cells [31].

The intestinal epithelium displays a wide variety of morphological adjustments to protect the host's connective tissue against environmental assault. There is one layer of epithelial cells, which form tight connections with one another, and mucus that coats the epithelial surface, all of which contribute to this physical barrier [32].

Goblet cells secrete a strongly glycosylated mucinrich layer, known as the glycol calyx, and bind it to the epithelium at the epithelial cell surface. Collectively, these make up the epithelium's apical surface, where they form a thick and largely permeable layer [33]. This is because probiotics boost the amount of Goblet cells, which reinforce the mucus layer, in the intestines [34]. A variety of antimicrobial peptides, including lysozyme, secretory phospholipase A2, defensins, defensin-like peptides (elafin and SLPI), and cathelicidins, are secreted by paneth cells, which are epithelial cells unique to

the small intestine and found at the base of intestinal crypts [35].

Plasma cells in the intestinal lamina propria produce the antibodies primarily as dimers joined by the connecting chain. Transport through IECs and secretion into the intestinal lumen of dimeric IgA is aided by its binding to the polymeric immunoglobulin receptor. The mucosal immune system is able to exclude mucosal antigens because sIgA binds to the mucus layer [36].

When food consumption falls short of energy needs, a condition known as malnutrition occurs. Because of its effect on the immunological response, the host's defenses are significantly weakened, increasing the likelihood of infection. As a result, malnutrition can serve as a model for investigating how probiotics influence host health. Re-feeding mice with PFM restored normal intestinal mucosa architecture and boosted both local and systemic immunity after they had been undernourished [37]. Given that starvation significantly impairs the immune system, with the thymus being one of the most afflicted organs, restoring thymus histology by probiotic ingestion becomes important.

Additionally, in vivo investigations demonstrated that probiotics are efficient in lowering adipose tissue, serum/plasma total cholesterol, LDL-cholesterol, and triglycerides while simultaneously raising HDL-cholesterol [38–40]. Probiotics have been shown to lower glucose and insulin levels in diabetic patients in clinical trials. Hb1Ac and insulin resistance can also be lowered with their help. These consequences of obesity can be explained by a variety of mechanisms, such as the enhancement of intestinal barrier function, the modulation of immunological differentiation, and the prevention of harmful microorganisms from adhering to and migrating to adipose tissue. Multiple factors appear to contribute to probiotics' undeniable effectiveness as anti-cancer medicines.

2 MATERIALS AND METHODS

2.1 Animal preparation

Wistar albino male rats were considered in this experiment. Twelve rats, four months old, with an average weight of 204.67 g and a standard deviation of 80.04 g, and a weight range between 192-219 g, were prepared in advance for this experiment. All of them were supplied by the Cancer Research Center/Al-Mustansiriya University. The animals were housed in plastic cages and maintained on a 12:12 light-dark cycle at a controlled temperature

of 25 °C \pm 1°C; their water was changed daily, and they were fed a commercial rat diet (pellets) throughout the experiment.

Out of the 12 rats utilized in this experiment, three rats were randomly assigned to each of the four experimental groups for one month. The groups were put on different types of water such that the first group was put on water (control group), the second and third groups were offered the water mixed with sediment' cells of breastfed LAB, and the fourth group was offered the water mixed with sediment' cells of formula-fed LAB (Figure 1).

In light of preparing the water for the groups of rats, the three groups of the experiment received drinking water by adding 5 ml of sediment cells from the breast and formula to 500 ml of drinking water.

2.2 Blood collection and serum preparation

After 30 days on the assigned water regimens, rats were deeply anesthetized with isoflurane and terminally exsanguinated by cardiac puncture. Whole blood was collected into plain tubes, allowed to clot for 20-30 min, and centrifuged at 1,500-2,000 \times g for 15 min at 4 °C. Serum was aliquoted, checked for hemolysis, and stored at -80 °C. ELISAs measured IL-2, IL-8, TNF- α , TNF- γ , ADP, and LEP. All procedures were conducted under institutional animal ethics approval.

3 RESULT

3.1 Effects of probiotics on the immune system

Each group received a specific regimen as presented in Table 1. After one month of starting the experiment, samples of blood were taken and exposed to six different immune tests (IL-2, IL-8, TNF- α , TNF- γ , ADP, and LEP) using ELISA kits. Each of the previously mentioned tests investigated the response of the samples (optical density) at each titer. According to the user manual of the performed tests, the theoretical relationship between optical density and concentration should give a curvature shape. Actually, this is not always adequate and therefore variation is accepted. The plot of the scatter diagram should be applied on a log scale for both the x-axis and the y-axis. The fitted equation of the shape is very important to estimate the concentration when the optical density is known. The scatter diagram shown in Figure 2 indicates that both groups of natural feeding respond better than the other two groups of control and formulafed. Mean optical density for the natural feeding groups is significantly different between the two natural fed groups, as well as between them and the other two groups of formula-fed and control.

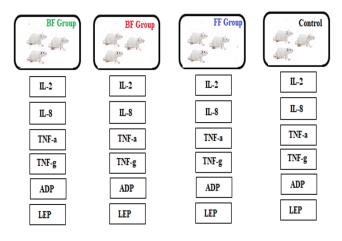


Fig. 1 Experiment layout. Red = 207Breastfeeding isolate, Green=205 Breastfeeding isolate, Blue=208 Artificial feeding isolate

Table 1 Feeding regime of the experimental rats with respect to their groups

Group	Feeding			
Control	Common daily food regime and drinking water (Gray)			
	Common daily food regime plus 5 ml culture of bacteria			
Green	isolated from breast fed sample added to 500 ml of			
	drinking water. Green=205 Breastfeeding isolate			
	Common daily food regime plus 5 ml of culture of bacteria			
Red	isolated from breast fed added to 500 ml of drinking water (Red)			
	Red = 207 Breastfeeding isolate			
	Common daily food regime plus 5 ml culture of bacteria			
Blue	isolated from Artificia fed added to 500 ml of			
Diuc	drinking water (Blue)			
	Blue=208 Artificial feeding isolate			
Group	Feeding			
Control	Common daily food regime and drinking water (Gray)			
Green	Common daily food regime plus 5 ml of crude extract			
Green	from breastfed sample added to 500 ml of drinking water (40.)			
Red	Common daily food regime plus 5 ml of crude extract from			
Keu	breastfed sample added to 500 ml of drinking water (Red)			
Blue	Common daily food regime plus 5 ml of crude extract from			
Diuc	formula fed sample added to 500 ml of drinking water (Blue)			

The mean optical density of the formula-fed group was not significantly different from that of the control group. There is still a notable difference between the means of the last two groups, and the mean of the formula-fed group is greater than that of the control group (approaching zero), but the difference is not significant. The optical density reflects the effect of the crude extract on the vital functions of these rats. In addition to this comparison, the response of the samples to the investigation parameters will also be studied, and the scatter diagram of optical

density with respect to the experimental kits (immune factors) was obtained and presented in Figure 3.

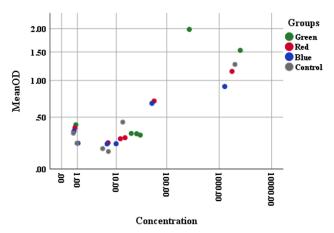


Fig. 2 Scatter diagram of optical density versus concentration with regard to experimental groups

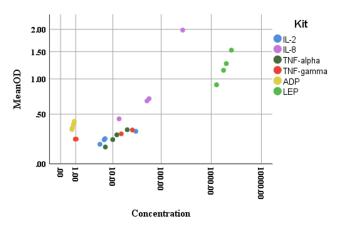


Fig. 3 Scatter diagram of optical density versus concentration with regard to immune factors

It is clear from Figure 3 that LEP and IL-8 recorded the highest optical densities among all others, indicating that these factors respond most strongly to the crude extract. This experiment was carried out six times with six different ELISA kits, such that each kit was responsible for a certain immune factor. The analysis of variance was carried out for the optical density data.

Table 2 shows the components of the analysis of variance for the optical density with group, kit, and replicates factors, as well as the interaction term between the groups and the kits. This table shows that groups and

kits are significantly different, and the interaction term is also significant. The significant interaction indicated that the immune factor is affected by the type of regime of the tested groups. Figure 4 shows the performance of the fitted model through the residual analysis, which reveals a perfect fit. The multiple comparison test by Tukey's 95% confidence intervals was used to detect differences between groups as well as between parameters. Tables 3 and 4 show the differences between each pair of means. With regard to the groups, groups of breastfeeding (Green and Red) are significantly different from other groups. The group of formula feeding is not significantly different from the control group.

Table 2 Feeding regime of the experimental rats with respect to their groups

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Kits	5	11.0705	2.21409	6757.44	0
Groups	3	1.7753	0.59177	1806.09	0
Replicates	2	0.0019	0.00097	2.96	0.062
Kits*Groups	15	3.2666	0.21777	664.64	0
Error	46	0.0151	0.00033		
Total	71	16.1293			

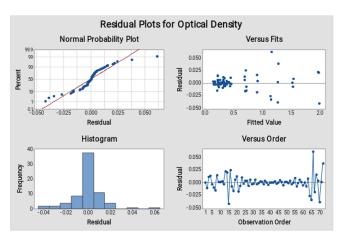


Fig. 4 Residual plot of the fitted model of optical density

Figure 5 shows the Tukey simultaneous 95% confidence interval for the means of optical density for the groups. Differences that are near or otherwise on the zero line are not significant. This graph is very important because it explains the details of the difference, that is, how far apart the means are from each other. Formulafed and control groups revealed a closer response to the test than other groups of breastfeeding. Breastfeeding significantly stimulates the production of LAB more than formula feeding.

Figure 6 shows the confidence intervals for the mean differences of the immune factors as listed on the vertical axis of the plot. Differences that are away from the zero line are significant, and one can observe the sign of the difference on the horizontal line of the plot to judge the type of difference. Optical densities of tumor necrosis factor (alpha), tumor necrosis factor (gamma), and IL-2 showed no significant difference among their means, and they almost had the same response to the feeding regime. Table 4 shows the mean values of the immune factors as well as the letters of the pairwise comparison.

Table 3 Tukey pairwise comparisons for the groups of the experimental rats

Groups	N	Mean		Grouping	
Green	18	0.811722	A		
Red	18	0.502056		В	
Blue	18	0.433611			C
Control	18	0.429944			C

Note. Means that do not share a letter are significantly different. Grouping information using the Tukey method and 95% confidence

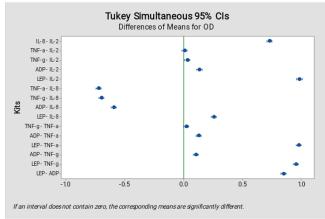


Fig. 5 Tukey's multiple comparisons for the groups of experimental rats with respect to means of optical density

Figure 7 shows the confidence intervals of the mean optical densities for each of the immune factors as obtained by the use of ELISA kits. The high optical density corresponded to the factor LEP, which indicates that this factor is mostly affected by the experimental treatment of the groups. The lowest optical density was recorded for the immune factor IL-2.

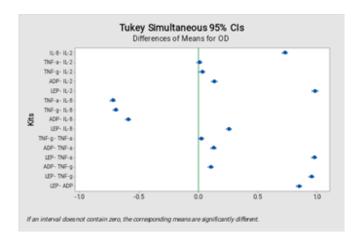
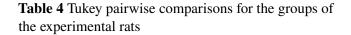


Fig. 6 Tukey's multiple comparisons for means of optical density in regard to the kits of immune factors



Grouping Information Using the Tukey Method and 95% Confidence					
Kits	N	Mean	Grouping		
LEP	12	1.21317 A			
IL-8	12	0.95767	В		
ADP	12	0.365	C		
TNFg	12	0.26092		D	
TNF a	12	0.23858			E
IL-2	12	0.23067			E

Note. Means that do not share a letter are significantly different.

Figure 7 shows the confidence intervals of the mean optical densities for each of the immune factors as obtained by the use of ELISA kits. The high optical density corresponded to the factor LEP, which indicates that this factor is mostly affected by the experimental treatment of the groups. The lowest optical density was recorded for the immune factor IL-2.

Breastfeeding groups were found to have the highest mean optical density compared to other groups, and their variation around the mean is also noted as a wide range, overlapping with the values of optical densities from other groups (Figure 8). Such a variation indicates the potentiality of enhancing the response of the immune factors to the probiotics may need some manipulation of the experimental setting, such as the proportion of crude extract to drinking water, or maybe the duration of the experiment, which might need further studies.

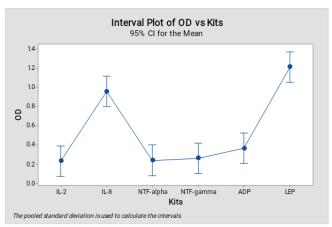


Fig. 7 Means 95% confidence intervals for optical density of immune factors.

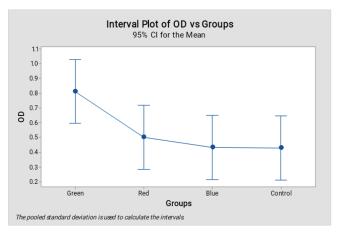


Fig. 8 Means 95% confidence intervals for optical density of experimental groups.

The scatter diagram (Figure 9) shows that there is a curvature trend in the relationship between optical density and concentration. The overlapping of some points happened due to the response of different immune factors to optical density. It is therefore necessary to find a fitting that can be used in similar experiments to predict the concentration of a certain optical density. In this context, the non-linear regression was used. The best-estimated fitted model was found cubic with an adjusted R-square of 94.7%.

Table 5 shows the analysis of the variance table for the regression model, which explains the adequacy of a model in estimating concentrations. Figure 10 shows the plot of the fitted model.

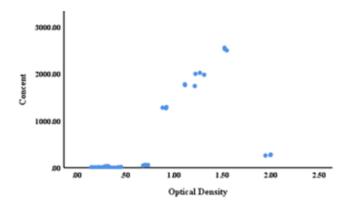


Fig. 9 Scatter diagram of concentration versus optical density readings.

Table 5 Tukey pairwise comparisons for the groups of the experimental rats

	Sum of Squares	df	Mean Square	F	Sig
Regression	35503700.574	3	11834566.858	421.574	0.0
Residual	1908918.458	68	28072.330		
Total	37412619.032	71			

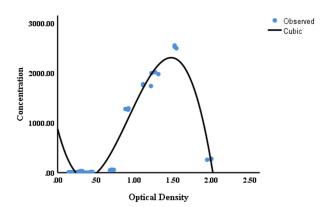


Fig. 10 Cubic fitting for the estimated concentrations versus optical density.

4 DISCUSSION

The health benefits that functional foods deliver and maintain have piqued the public's interest in them [41]. Anti-inflammatory efficacy is provided by probiotics because of their ability to modulate the immune system [42]. These advantages are strain-dependent and may vary even across isolates of the same species [43]. In order for their anti-inflammatory qualities to be certified and used

in therapeutic settings, they must be identified for each new isolate [44]. The immune system of the host is affected by probiotics because they stimulate, modulate, and regulate immunological responses. TNF is crucial for immune modulation as one of the most important pro-inflammatory cytokines. Inhibition of TNF- TNF expression by probiotics may have immune-suppressive effects, while elevation of TNF- TNF expression by probiotics may have immune-stimulating effects [45].

This work focuses on the well-documented finding that probiotics can lower TNF-expression, leading to an immunosuppressive and anti-inflammatory response. For example, [46] found that mice given the probiotic combination VSL#3 had lower levels of TNF. On the other hand, a study conducted by [47] (on VSL#3) implies that it can alleviate colitis symptoms by decreasing TNF-expression. Memory deficits, neuron and synaptic lesions, and glial activation were all significantly improved after administration of a probiotic solution containing *Bifidobacterium* lactis, Lactobacillus casei, Bifidobacterium bifidum, and Lactobacillus acidophilus (ProBiotic-4) to mice with accelerated senescence (SAMP8) [48]. TNF and other pro-inflammatory cytokines are released in response to an imbalance in the microbiota, gut, and brain, as shown by the activation of the TLR4-mediated NF-kB signaling pathway. Pro-inflammatory cytokines (IL-6 and TNF) were considerably reduced in intestinal mRNA levels and plasma protein concentration in SAMP8 mice treated with ProBiotic-4. This medication was able to improve the deficit of the microbiota-gut-brain axis and cognitive function associated with aging, acting as a neuroprotector in the process. Therapeutic potential for the treatment of postmenopausal osteoporosis has also been demonstrated by the probiotics Lactobacillus plantarum A41 and Lactobacillus fermentum SRK414 [49] via the reduced expression of pro-inflammatory cytokines, specifically TNF, and increased osteoprotegerin expression, leading to mitigated bone resorption.

Clinical symptoms and serum IgE levels did not change between the *Lactobacillus* GG-administered group and the placebo group in a study of 230 infants with atopic eczema/dermatitis syndrome [50]. No change in cytokine levels was reported after probiotic administration in any of the other clinical studies. In conclusion, we discovered that probiotics were successful in lowering the SCORAD index, serum IL-5, IL-6, IFN, and total serum IgE levels of AD patients but were ineffective in lowering serum IL-2, IL-4, IL-10, ECP, and TNF levels. Modifying the immunogenicity of probable allergens may mitigate

the effect of probiotics on SCORAD indicators. Restoring intestinal mucosal barrier integrity, decomposing dietary antigens, modulating intestinal microbial makeup and activity, and increasing secretory IgA synthesis are all ways in which probiotics aid in the pathogenesis of AD. They counteract the Th2 immune response by boosting the Th1 response. Clinical symptoms can be lessened thanks to probiotics because they modulate both local and systemic immunity [51].

The leptin levels of the LC, LR, and LP groups were lower than the HFD group (p < 0.05), while the leptin levels of the LF and LA groups were lower than the HFD group. Adiponectin levels also differed significantly (p <0.05) between the HFD group and the LF, LA, LR, and LP groups. There was no statistically significant difference between LC and the control group. Obesity in mice was reduced by five different Lactobacillus strains, according to a study published in 2020 [46]. Serum levels of LEP and ADP were decreased in the intervention groups compared to the HFD group (P 0.05). Serum levels of LEP and ADP were lower in Groups 2 and 3 (BPL1 1.67 x 109 cfu/d.kg and CSE 166.67 mg/d+BPL1 1.67 x 109 cfu/d.kg, respectively) compared to Group 1 (CSE 166.67 mg/d.kg) (P 0.05). Interventions combining CSE and BPL1 were more effective at lowering LEP and ADP than CSE alone (51).

5 CONCLUSION

Probiotics showed selective benefits. They lowered SCORAD and serum IL-5, IL-6, IFN, and total IgE in atopic dermatitis, with no change in IL-2, IL-4, IL-10, ECP, or TNF. In the high-fat-diet model, several Lactobacillus strains reduced leptin (and adiponectin) versus HFD, with the CSE+BPL1 combo outperforming CSE alone; one strain (LC) was comparable to control. Overall, the effects suggest immune rebalancing and gutbarrier support, rather than a blanket anti-inflammatory response.

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DATA AVAILABILITY

N/A

DECLARATIONS

Conflict of interest

The authors declare no conflict of interest.

Consent to publish

N/A

Ethical approval

All animal procedures complied with internationally accepted standards for the care and use of laboratory animals. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Anbar.

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