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Original Article

Impact of lactobacillus probiotics on vaccine response in diabetic rats: modulation of inflammatory cytokines

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Abstract: Lymph nodes are essential for immune function as they contain immune cells that activate responses and filter pathogens from lymph. This study investigates how diabetes-related metabolic challenges affect immune function, focusing on the impact of Lactobacillus probiotics on lymph node responses to meningococcal vaccines in thirty male Albino rats with Streptozotocin-induced diabetes, established two weeks before vaccination. The diabetic rats were divided equally and randomly into three groups: one untreated (UD group), one receiving two shots of the meningococcal vaccine (DM group), and one receiving the same vaccination regimen alongside oral doses of *Lactobacillus rhamnosus* probiotics (DML group). We monitored the rats' weights and measured the expression levels of inflammatory cytokines (IL-1β, TNF-α, and IL-2) in their lymph nodes as markers of immune activation after vaccination. Diabetic rats vaccinated against meningococcal disease showed increased levels of IL-1β and TNF-α, which showed a significant reduction by *Lactobacillus* supplementation after three weeks. However, following the second vaccination, *Lactobacillus* significantly increased IL-1β and TNF-α levels. Also, *Lactobacillus* appeared to modulate the initial spike in IL-2, with a notable increase observed five weeks after the second vaccine dose. Notably, the vaccination protocol did not affect the body weight of the diabetic rats. These findings suggest that while the vaccine elevates inflammatory cytokine levels in the lymph nodes of diabetic rats, *Lactobacillus* may help mitigate these responses and regulate IL-2 levels, indicating its potential value in enhancing diabetes management, optimizing vaccine effectiveness, and addressing autoimmune issues in diabetic individuals.

Keywords: Probiotics, IL-1β, IL-2, TNF-α, diabetic diseases, autoimmune proinflammatory cytokines, meningococcal vaccine, lymph nodes

Introduction

Lymph nodes play a vital role in immune function by serving as sites for the congregation of B and T lymphocytes, with B cells located in the cortex and T cells in the paracortex [1]. Dendritic cells (DCs) and other antigen-presenting cells (APCs) help initiate immune responses by presenting antigens to T cells, while macrophages filter pathogens from the lymph [2]. Key cytokines, such as TNF and IL-1, regulate the movement of immune cells, and IL-2 is crucial for developing regulatory T cells [3]. TNF-α, primarily produced by macrophages and T cells, is necessary for combating infections and inducing apoptosis in cancer cells [4], whereas IL-1β plays a critical role in defense and inflammation [5]. IL-2 also supports T cell proliferation and helps prevent autoimmune disorders [6]. The association between diabetes and lymph node effectiveness is significant, as diabetes can impair the immune system and the lymph nodes' response to infections, often leading to chronic low-grade inflammation that disrupts lymph node function and immune cell production, increasing the risk of infections [7]. Furthermore, cytokine imbalances in diabetes can negatively impact signaling pathways within lymph nodes, reducing vaccine efficacy [8].

Lactobacillus probiotics boost immune responses by influencing both innate and adaptive immunity. They enhance the function of immune cells, such as macrophages and T lym-

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phocytes, and promote the production of beneficial cytokines like IL-10 while decreasing pro-inflammatory cytokines [9]. This balance fosters a healthy immune system. Additionally, Lactobacillus supports gut-associated lymphoid tissue (GALT), improves antigen presentation, and may enhance vaccine efficacy and antibody production [10]. By out-competing pathogens and producing antimicrobial substances, they help prevent infections, showcasing their therapeutic potential for health enhancement and disease prevention [11]. In diabetic patients, Lactobacillus may improve immune responses by enhancing lymph node function and cytokine profiles, leading to better vaccine efficacy [12].

Meningitis, an inflammation of the meninges surrounding the brain and spinal cord, can be caused by various pathogens, with bacterial meningitis being particularly concerning due to its rapid onset and severe potential consequences [13]. In response to the threat of meningitis, several vaccines have been developed. Among the most common are meningococcal vaccines, such as MenACWY and MenB, designed to protect against Neisseria meningitidis [14]. Generally, these vaccines are recommended for young adolescents, preteens, and individuals at increased risk [15]. Moreover, specific vaccines like MenAfriVac target meningitis A and are crucial for controlling epidemic outbreaks in the sub-Saharan African region known as the "meningitis belt" [16]. The relationship between the frequency of vaccine doses and immunogenicity is vital for vaccine effectiveness [17]. An initial vaccine dose primes the immune system to recognize the antigen, initiating antibody and T-cell responses. Booster's doses enhance the immune response, strengthening the system's memory for future antigen encounters [18]. The number of doses needed for optimal immunogenicity varies by vaccine type: live attenuated vaccines typically require fewer doses than inactivated or subunit vaccines [19]. Dosing schedules can be tailored for specific populations or modified based on emerging pathogen variants. Following the recommended vaccination schedule is crucial for optimal protection against infectious diseases [20].

Reduced vaccine efficacy in diabetic patients has significant public health implications, including increased infection rates, higher morbidity and mortality, and a strain on healthcare resources [21]. This diminished immunity can lead to vaccine hesitancy and localized outbreaks of vaccine-preventable diseases, affecting not only diabetic individuals but also the broader community [22]. Public health strategies may need to adapt by implementing targeted vaccination approaches, educational campaigns, and tailored interventions to enhance immune responses [23]. Previous research shows that diabetic individuals often experience diminished responses to vaccines due to compromised lymph node function [24]. This research explored how Lactobacillus probiotic supplementation affects the immune response in the lymphatic system after administering a two-dose meningococcal polysaccharide vaccine to diabetic rats undergoing diabetes treatment. This study focused on modulating innate cytokines, such as TNF-α and IL-1β, and IL-2 as representative of adaptive immune responses, as markers of immune activation triggered by the vaccine.

Materials and methods

Administration of meningococcal vaccine and lactobacillus rhamnosus in rat studies

The Meningococcal vaccine (Menactra), developed by SANOFI PASTEUR, targets meningococcal serogroups ACWY and is conjugated with diphtheria toxoid protein. In this study, Menactra was administered intraperitoneally to rats in two doses of 0.1 ml each. Additionally, *Lactobacillus rhamnosus* GG (LGG) probiotic powder, containing 200 billion CFU per 30 g, was sourced from Bioanalytical Technologies in KSA. For administration, 0.6 g of LGG was dissolved in 160 ml of water, resulting in a concentration of 1.0×10^9 CFU/ml, with each rat receiving a dose of 1 ml of this solution.

Induction of diabetes and treatment protocols in albino rats

The diabetes model in rats was induced using a 75 mg dose of Streptozotocin (STZ) obtained from Sigma-Aldrich, dissolved in a 0.1 M sodium citrate buffer. Each rat received a 40 mg/kg intraperitoneal injection of STZ two weeks before day 0 of the vaccination administration. Moreover, metformin, a diabetes medication from Merck, was orally administered at a daily

Table 1. Timeline of experimental treatments

Experiment procedures	Experiment durations/Weeks								
	-2	-1	0	1	2	3	4	5	6
STZ Dose	√								
Lactobacillus treatment		√	√	√	√	√	√	√	
Vaccine dose1 injection			√						
Scarification1						√			
Vaccine dose 2 injection						√			
Scarification 2									√

During the study, the rats were kept in wire-bottom cages within a stable environment maintained at 22°C, with a permissible variation of ±2 degrees. The lighting was consistently set to alternate between 12 hours of light and 12 hours of darkness. Note: The table above is a simplified representation of the experimental timeline, and the '√' marks indicate the weeks in which treatments were administered.

Table 2. Primer sets for SYBR green qRT-PCR gene expression quantification

Primers	Primer sequence (5'-3')	GenBank References
GABDH	F "GGTGGTCCAGGGTTTCTTA" R "GTTGTCTCCTGCGACTTCA"	XM_017321385.1
TNF-α	F "ATGAGCACAGAAAGCATGA" R "AGTAGACAGAAGAGCGTGGT"	AB185894.1
IL-1β	F "TCATGGGATGATGATGATAACCTGCT" R "CCCATACTTTAGGAAGACACGGATT"	XM_006498795
IL-2	F "GTCAAATCCAGAACATGCCGCAGA GTGTCA" R "ATGTACAGCATGCAGCTCGCATCCTGGTCCA"	AB185894.1

dose of 150 mg/kg, dissolved in 1 ml of water, to manage elevated blood sugar levels in diabetic rats. A total of thirty adult male albino rats, each weighing between 200-300 g, were used in this study. Diabetes was induced in the rats using streptozotocin (STZ), and they were subsequently divided into three groups (with all treatment timelines detailed in **Table 1**): the UD group, consisting of diabetic rats that received no treatment; the DM group, which included diabetic rats that were injected twice with the Men ACWY-D vaccine; and the DML group, which comprised diabetic rats treated similarly to the DM group but also received biweekly oral doses of *Lactobacillus rhamnosus* GG (LGG) beginning one week before vaccination. At the end of the third week, five rats from each group were humanely euthanized for lymph node collection. The remaining rats continued their treatment until the fifth week, at which point they were also euthanized for lymph node analysis. Ethical approval for the experiment was obtained from the Scientific Research Ethics Committee at the Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

Inflammatory cytokine RNA expression in diabetic rat lymph nodes

In our study, lymph node samples from the three groups were meticulously preserved using RNA*later* (Cat No. AM7022) before being stored at 4°C and then frozen at -80°C. RNA extraction was performed using the RNeasy Mini kit (Cat No: 74104). The expression levels of genes such as GAPDH, TNF-α, IL-1β, and IL-2 were measured using SYBR Green qRT-PCR (Cat No. 11736051) with primers listed in **Table 2**, which included steps for RNA reverse transcription and cDNA amplification. The cDNA was standardized to 200 ng per reaction for qRT-PCR, conducted in quadruplicate in a 96-well plate format, along with negative controls. The plates were analyzed using an Applied Biosystems qRT-PCR system, and gene expression was normalized to GAPDH using the ΔΔ Ct method [25]. This comprehensive approach enabled the assessment of the impact of probiotic treatment on gene expression in diabetic rats.

Statistical analysis of data

The data collected from this study were processed and analyzed with the Mega Stat statistical software, version 10.2 release 2.1. The result was deemed to have statistical significance if the *P*-value was less than 0.05, as determined by the One-way Analysis of Variance (ANOVA) test.

Results

Effects of meningococcal vaccine and lactobacillus probiotics on body weight in diabetic rats

Three weeks into the study, following the initial vaccination, the diabetic rats that received the vaccine exhibited a slight, non-significant

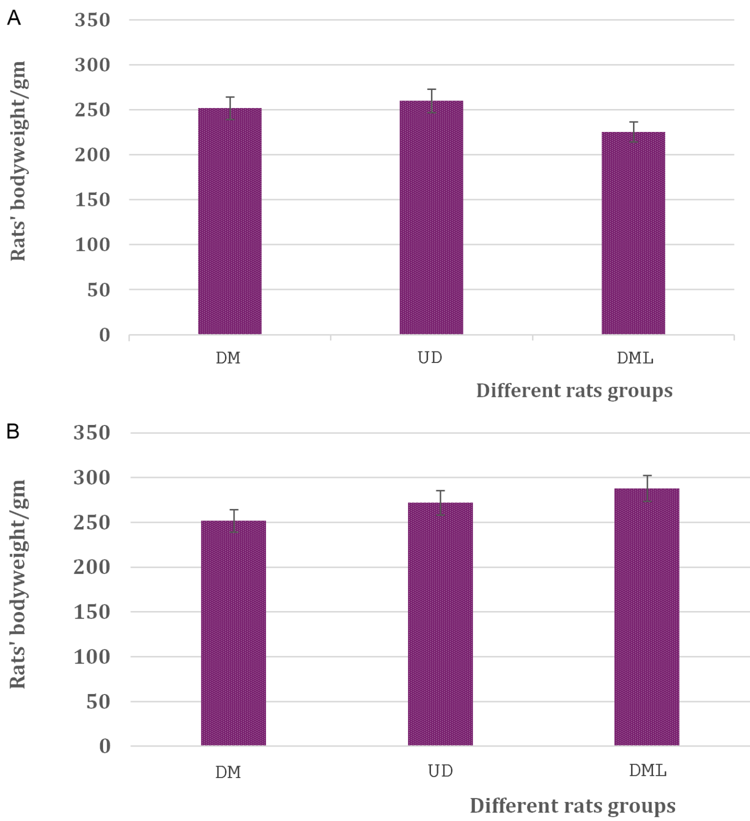

Figure 1. The image depicts the body weight variations in different treated and untreated rat groups after (A) 3 weeks and (B) 5 weeks post-vaccination. A one-way ANOVA was conducted to assess differences among the groups: UD indicates untreated diabetic rats, DM refers to diabetic rats that received the meningitis vaccine, and DML represents diabetic rats that were vaccinated and received oral LGG probiotic bacteria. The vertical bars show a 5% margin around the mean weight.

increase in body weight compared to their unvaccinated diabetic counterparts, as shown in **Figure 1A**. In contrast, diabetic rats that received both the vaccine and Lactobacillus probiotic supplementation experienced a quiet, non-significant decrease in body weight compared to the vaccinated and unvaccinated groups. By the fifth week, after the second round of vaccinations, there were no statistically significant differences in body weight between the vaccinated diabetic rats and those that did not receive the vaccine. However, diabetic rats that received the meningococcal vaccine and Lactobacillus probiotics demonstrated a slight but statistically significant increase in body weight compared to untreated diabetic rats. Additionally, there was no significant difference in body weight between the group that received the meningococcal vaccine and Lactobacillus probiotics and the group that was solely vaccinated, as shown in **Figure 1B**.

Evaluating IL-1β expression in diabetic rats post-vaccination and probiotic treatment

Three weeks after vaccination, lymph nodes from diabetic rats that received the vaccine showed a significant increase in IL-1β expression levels ($P<0.0001$) compared to those from unvaccinated rats, as illustrated in **Figure 2A**. In contrast, lymph nodes from rats treated with both Lactobacillus and the vaccine didn't demonstrate a significant increase in IL-1β expression compared to the unvaccinated group. Notably, there was a substantial decrease in IL-1β expression in the lymph nodes of the Lactobacillus-treated and vaccinated group compared to those vaccinated only ($P<0.0001$), as shown in **Figure 2A**.

By the fifth week, following the second round of vaccinations, the lymph nodes of the group receiving both treatments showed a significant increase in IL-1β expression ($P<0.0001$) compared to both the vaccinated and unvaccinated groups, as indicated in **Figure 2B**. Furthermore, changes in IL-1β expression in the lymph nodes of unvaccinated rats were not statistically significant compared to those of the vaccinated group, as demonstrated in **Figure 2B**.

Evaluating TNF-α expression in diabetic rats post-vaccination and probiotic treatment

Three weeks after the initial vaccination, the lymph nodes of diabetic rats given both the vaccine and Lactobacillus showed a significant drop in TNF-α levels ($P<0.0001$) compared to those receiving only the vaccine. This combined treatment group, however, showed no significant difference in TNF-α levels compared to the unvaccinated group (**Figure 3A**). Conversely,

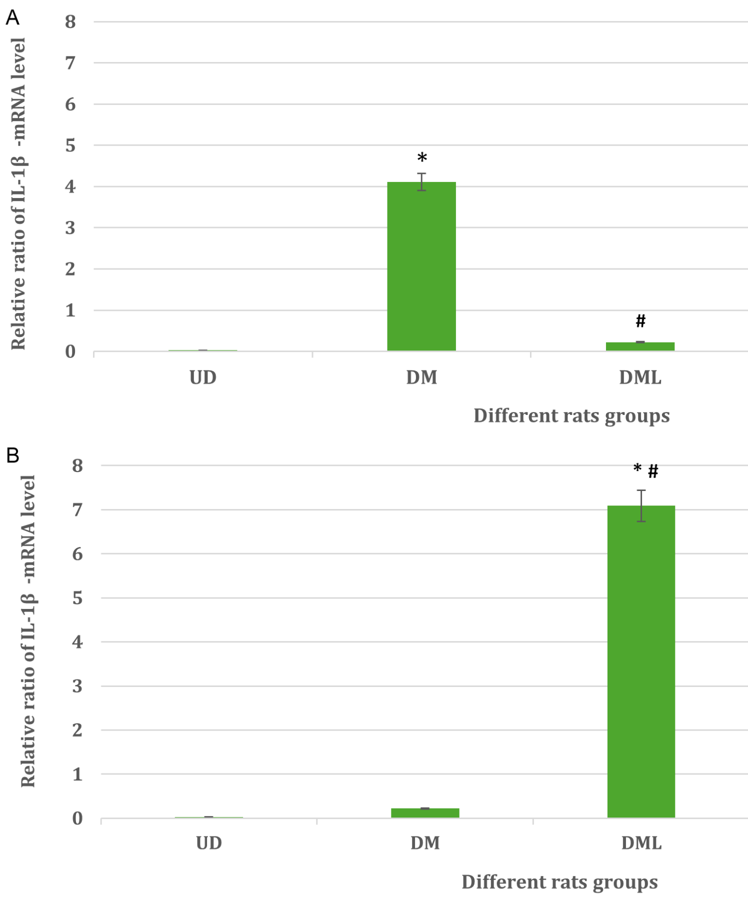

Figure 2. The image presents the mRNA expression levels of IL-1β in the lymph nodes of various treated and untreated rat groups, measured after three weeks (A) and five weeks (B) following vaccination. Statistical analysis was conducted using a one-way ANOVA: 'UD' denotes untreated diabetic rats, 'DM' refers to diabetic rats that received the Meningitis vaccine, and 'DML' signifies diabetic rats vaccinated and administered orally with lactobacillus probiotic bacteria. The data points shown represent average values from eight individual qRT-PCR experiments. A *P* value below 0.05 was considered statistically significant, with (*) indicating substantial differences from the UD group and (#) denoting significant differences from the DM group. Error bars indicate a 5% standard error of the mean.

the vaccine alone led to a substantial increase in TNF-α expression in the lymph nodes of diabetic rats compared to those that were not vaccinated ($P<0.0001$, **Figure 3A**).

By the fifth week, following the second vaccine dose, the diabetic rats treated with both the vaccine and Lactobacillus supplementation showed a significant increase in TNF-α expression ($P<0.0001$) compared to both the solely vaccinated diabetic rats and the unvaccinated group, as indicated in **Figure 3B**. Additionally, TNF-α levels in the diabetic rats that received only the vaccine also significantly increased ($P=0.0001$) compared to the unvaccinated diabetic rats, as depicted in **Figure 3B**.

Evaluating IL-2 expression in diabetic rats post-vaccination and probiotic treatment

Three weeks after the initial dose of the meningococcal vaccine, lymph nodes from diabetic rats that received both the vaccine and Lactobacillus supplementation showed a slight decrease in IL-2 expression compared to the unvaccinated group. This decrease was highly significant ($P=0.0000$) compared to the vaccinated rat's group, as illustrated in **Figure 4A**. In contrast, IL-2 levels in the lymph nodes of vaccinated diabetic rats significantly increased ($P=0.0000$) compared to baseline measurements from the unvaccinated diabetic rats, as shown in **Figure 4A**.

By week five, after the second vaccination, the group that received both Lactobacillus supplementation and the vaccine showed a significant increase in IL-2 expression ($P=0.0000$) compared to the vaccinated or the unvaccinated diabetic groups. Additionally, there was no significant increase in IL-2 levels in the vaccinated diabetic group compared to the unvaccinated diabetic rats, as illustrated in **Figure 4B**.

Discussion

Diabetes affects body weight differently in type 1 and type 2 cases. In type 2 diabetes, obesity or weight gain is often linked to insulin resistance, while unexpected weight loss can indicate poorly managed diabetes [26]. Weight loss happens when the body cannot effectively utilize insulin and starts burning fat and muscle for energy, which may also be influenced by

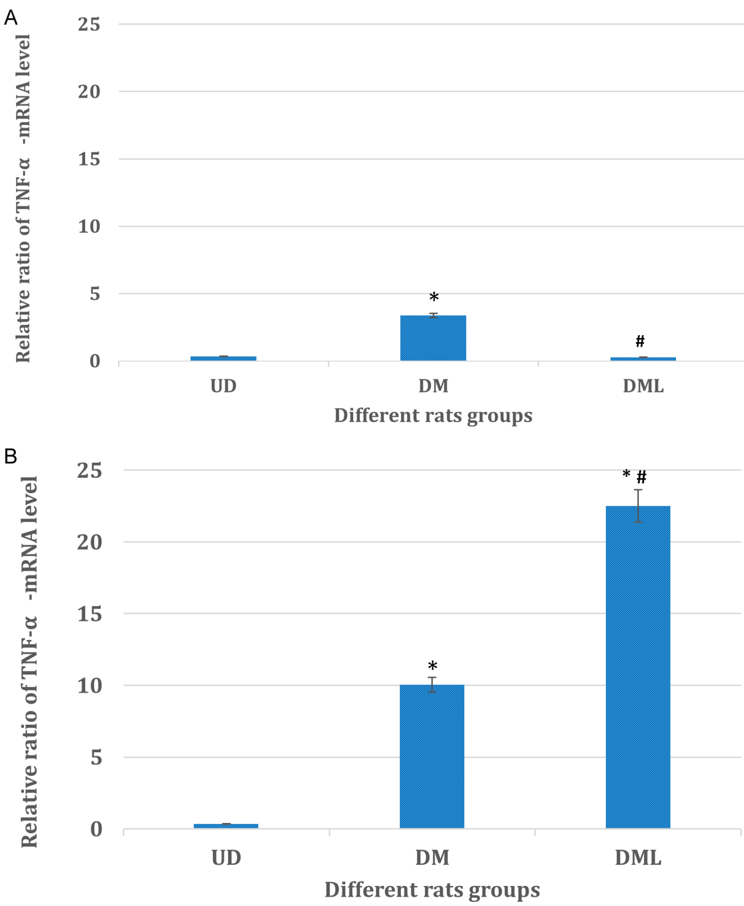

Figure 3. The image illustrates the mRNA expression levels of TNF-α in the lymph nodes of different treated and untreated rat groups, assessed after three weeks (A) and five weeks (B) post-vaccination. Statistical analysis was performed using one-way ANOVA. In this context, 'UD' refers to untreated diabetic rats, 'DM' denotes diabetic rats that received the Meningitis vaccine, and 'DML' indicates diabetic rats that were both vaccinated and given oral lactobacillus probiotic bacteria. The data points represent average values obtained from eight separate qRT-PCR experiments. A *P* value below 0.05 was considered statistically significant, (*) marking substantial differences from the UD group, and (#) indicating significant differences from the DM group. Error bars illustrate a 5% standard error of the mean.

medications or dietary restrictions for blood sugar control, indicating a potential need for treatment reassessment [27]. In this study, involving diabetic rats over five weeks, their weight remained stable, regardless of whether they received a meningitis vaccine or Lactobacillus supplements. This stable weight may be due to Metformin, which can promote slight weight loss by lowering glucose production in the liver, enhancing glucose uptake in muscles, and possibly reducing appetite [28]. The effects of Metformin on weight management, particularly alongside vaccinations, depend on factors like dosage, treatment duration, and the individual metabolic condition of each rat [29].

Lymph nodes play a crucial role in the immune response and inflammation [30], which are complicatedly associated with diabetes [31]. They filter lymphatic fluids and house immune cells that react to pathogens [30]. However, diabetes can hinder lymphatic function, disrupting fluid balance and the movement of immune cells, leading to complications like infections and delayed healing [32]. Additionally, diabetes is commonly associated with chronic low-grade inflammation, characterized by elevated markers like IL-1β and TNF-α in lymph nodes [7]. These agreed with the current findings, which showed that lymph nodes from diabetic rats had lower levels of both cytokines than the other groups.

The present study showed that IL-1β and TNF-α levels elevated slightly after three weeks but further increased five weeks post-vaccination in lymph nodes of diabetic rats compared to their unvaccinated diabetic counterparts. It seems that the ongoing increase in both proinflammatory cytokines levels is due to the immune response to the meningitis vaccine, with the booster dose further boosting immune activity in the lymph nodes [33, 34]. In contrast, lymph nodes from current diabetic rats treated with Lactobacillus alongside vaccination showed lower levels of IL-1β and TNF-α after three weeks compared to the vaccination-only group, resembling those in the untreated diabetic group. These implied that Lactobacillus may help lower the inflammatory cytokines IL-1β and TNF-α over time or that its full potential is not yet fully realized [35]. This finding reinforces the theory of immune suppression in diabetic rats, which appear to primarily

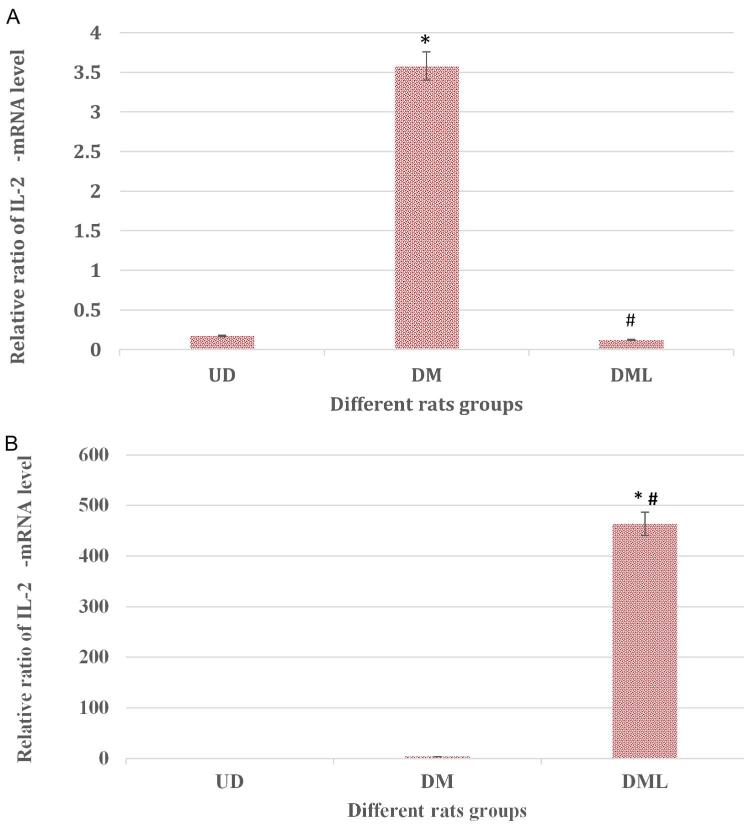

Figure 4. The image displays the mRNA expression levels of IL-2 in the lymph nodes of various treated and untreated rat groups, assessed after three weeks (A) and five weeks (B) post-vaccination. A one-way ANOVA was used for statistical analysis. Here, 'UD' represents untreated diabetic rats, 'DM' refers to diabetic rats that received the Meningitis vaccine, and 'DML' indicates diabetic rats that were both vaccinated and given oral lactobacillus probiotic bacteria. The data points reflect average results from eight separate qRT-PCR assays. The *P* value (<0.05) was considered statistically significant. (*) denotes a significant difference compared to the UD group, while (#) indicates a significant difference relative to the DM group. Error bars represent the standard error, calculated as 5% of the mean value.

respond to the master antigen, represented in this study by the meningitis vaccine. Moreover, five weeks after vaccination, lymph nodes from current diabetic rats that received both the meningitis vaccine and Lactobacillus exhibited significant increases in IL-1β and TNF-α levels compared to other groups. These suggested that Lactobacillus may play a role in modulating inflammatory responses post-vaccination and could enhance vaccine effectiveness, as seen in prior research on influenza [12, 36] and SARS-CoV-2 vaccines [37]. However, it is crucial to recommend proper dosages of probiotics to avoid excessive increases in IL-1β and TNF-α, which could worsen inflammation and insulin resistance [38, 39], thereby exacerbating type 2 diabetes heightening the risk of beta-cell damage, and reducing insulin production [40]. Elevated pro-inflammatory cytokine levels may also trigger autoimmune responses and influence vaccine efficacy, potentially leading to enhanced effects and adverse reactions [5, 41].

IL-2 is essential for modulating immune responses and inflammation in managing diabetes [42]. In this study, three weeks after the first dose of the meningococcal vaccine, lymph nodes from vaccinated diabetic rats exhibited a significant increase in IL-2 expression compared to the unvaccinated group. These findings confirmed that the vaccine effectively stimulated the adaptive immune response in these animals [43], suggesting an enhancement in enhancing the capacity of T cells, and humoral immune responses [44]. Overall, an increase in IL-2 is essential in diabetic rats, as they may experience compromised immune function [45]. Therefore, the vaccine's ability to boost IL-2 levels could enhance their immune readiness and protection against pathogens. In contrast, the present lymph nodes from diabetic rats that received both the vaccine and lactobacillus showed only a non-significant rise in IL-2 levels during the same period, with these levels being significantly lower than those of either the vaccinated or unvaccinated groups. Lower IL-2 levels in the lactobacillus group may indicate reduced T-cell activation, potentially leading to a weaker immune response to pathogens and vaccines [46]. While this decline could help modulate diabetes-related inflammation, it might also compromise the vaccine's efficacy [47]. However, by the fifth week (the second vaccination dose), the rise in IL-2 among the lymph nodes of the vaccinated diabetic rats was no longer significant, when compared to the unvaccinated diabetic group. This decline may be due to immune regulatory mechanisms [48], potential immune exhaustion from repeated stimulation [49], and the compromised immune function typical in diabetic rats, which collectively hinder the sustained elevation of IL-2 [50]. Remarkably, five weeks after the second dose of the meningococcal vaccine, lymph nodes from Lactobacillus-treated rats showed significantly higher IL-2 levels than both unvaccinated and vaccinated-only groups. This suggests that Lactobacillus could be an effective strategy for managing T1D by reducing autoimmune reactions and improving vaccine responses [12]. The late increase in IL-2 levels may be due to the gradual activation of the immune system through probiotics, along with the modulation of gut microbiota and cumulative effects from repeated exposure and vaccination [51].

Conclusion

This study is the first to explore the interactions between inflammatory cytokines (IL-2, IL-1β, and TNF-α), diabetes, and Lactobacillus supplementation following a meningitis vaccine booster. The results revealed no significant weight changes among diabetic rats, regardless of vaccine or probiotic treatment. Initially, the meningitis vaccine increased IL-1β and TNF-α levels in the lymph nodes, potentially enhancing vaccine efficacy, but also raising diabetes-related inflammation, which later declined even after the booster. However, Lactobacillus supplementation appeared to mitigate these inflammatory responses, possibly improving the vaccine's protective effects. After the second booster, IL-1β and TNF-α levels rose again, which could enhance efficacy but also worsen inflammation. Additionally, Lactobacillus moderates the rise in IL-2, an important immune factor that, if unchecked, could exacerbate diabetes. Overall, these findings suggest that Lactobacillus may enhance diabetes management and affect vaccine responses.

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Disclosure of conflict of interest

None.

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