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Research Article
Dose-Dependent Variation of Typhi Vaccine-Induced Immunity by Bifidobacteria in a Murine Model: Implications for Probiotic Adjuvant Use

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Abstract

Background and Objective: The potential of probiotics as adjuvants in vaccination strategies is a promising area of research, particularly with concerns for immunocompromised populations. This study investigates how different doses of bifidobacteria affect the immune response to a *Salmonella typhi* (*S. typhi*) vaccine in mice. **Materials and Methods:** Forty female Swiss mice, aged six to eight weeks, were divided into four groups. These groups included a control group, a vaccine-only group and two groups that received the vaccine with either a low (10^8 CFU) or high (10^{12} CFU) dose of bifidobacteria. The immune response was measured on days 28 and 42 post-vaccination, focusing on mucosal gene expression and immunoglobulin levels. Statistical tests, including One-way ANOVA, were performed on all groups, with statistical significance determined by $p<0.05$. **Results:** The study found that bifidobacteria modulate immunity dose-dependently, reducing mucosal TLR2 expression and suppressing cytokines IFN-γ, IL-4 and IL-10, with higher doses enhancing vaccine efficacy. Serum antibody analysis showed increased IgG, IgM and IgA, with a dose-response effect for IgA but not IgG. By day 42, bifidobacteria sustained IgG and IgM levels but did not further elevate IgA beyond vaccine-induced levels. **Conclusion:** This study underscores the efficacy of lower *Bifidobacterium* doses in boosting immune regulation, highlighting the crucial need for stringent dose control when administering probiotics alongside vaccines.

Key words: Probiotics, mucosal immunity, bifidobacteria, Typhi vaccine, Polysaccharide vaccine

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Typhoid fever, predominantly caused by the bacterium (*Salmonella enterica*) serotype Typhi (*S. typhi*), poses a significant health threat globally, particularly in areas with inadequate sanitation[1,2]. Two main types of vaccines are available to combat this disease: The live oral Ty21a vaccine and the injectable polysaccharide vaccine that targets the *S. typhi* Vi antigen[3]. Despite the success of these vaccines in diminishing typhoid incidences, the emergence of antibiotic-resistant strains underscores their critical role[4,5]. The Ty21a vaccine, when taken orally over several days, can trigger both systemic and mucosal immune responses[6]. In contrast, the injectable polysaccharide Typhi vaccine primarily induces a systemic immune response[7,8]. This injectable vaccine utilizes purified antigens extracted from the *S. typhi* Vi capsular polysaccharide[9,10]. Once injected, these antigens stimulate the production of specific antibodies, predominantly immunoglobulin G (IgG). These antibodies circulate in the bloodstream, ready to detect and neutralize the *S. typhi* bacteria upon infection, thus offering widespread protection[11].

Bifidobacteria are essential gram-positive probiotics that promote gut health, particularly in infants. These bacteria provide several benefits, including defending against pathogens, aiding digestion, modulating the immune system and preventing diarrhea[12]. Strains like (*B. bifidum*, *B. longum*, *B. breve*, *B. lactis*, *B. infantis* and *B. adolescentis*) provide several specific advantages like enhanced digestion, stress relief and immune support[13]. The function of bifidobacteria can be impacted by factors such as diet, age and environment[14] and it's advisable to consult a healthcare provider before starting probiotics[15]. Bifidobacteria play a critical role in immune system balance by modulating inflammatory responses and maintaining the integrity of the gut barrier, which prevents harmful substances from entering the body[16]. They also improve immune cell functions, enhancing overall immune surveillance and tolerance[16,17]. Certain strains can enhance vaccine effectiveness and may also assist in reducing the risk of allergies[18].

Probiotics, including bifidobacteria, are generally safe and beneficial for most people, enhancing gut health and immune function[12]. However, they can pose risks, particularly for immunocompromised individuals, such as those with conditions like HIV/AIDS, those undergoing chemotherapy or organ transplant recipients, who may face a higher risk of infections[19]. There is also potential for immune system overstimulation, which could exacerbate autoimmune or chronic inflammatory conditions and rare allergic reactions could occur[20]. Additionally, in specific cases, probiotics might produce D-lactic acid, leading to D-lactic acidosis, especially in individuals with conditions like short bowel syndrome[21]. There is also a theoretical risk of gene transfer, where probiotics could pass antibiotic resistance genes to harmful gut bacteria[22]. While the benefits of probiotics usually outweigh these risks, those with underlying health conditions should consult a healthcare provider before starting probiotic supplements to ensure safety[23]. This study examined the impact of two different doses of bifidobacteria on mucosal and humoral immune responses in immunized mice (injected with the Vi *Salmonella typhi* vaccine). The primary objectives were to analyze changes in some inflammatory gene expression relevant to mucosal immunity and the levels of total serum immunoglobulins. This investigation aims to clarify how probiotics might influence the effectiveness of the polysaccharide typhoid vaccine, which primarily induces a systemic immune response.

MATERIALS AND METHODS

Study area: The study was conducted at the Faculty of Science, King Abdulaziz University in Jeddah, Saudi Arabia, from January, 2019 to April, 2024.

Supplies of bifidobacteria and Typhoid Vi vaccine: This study used a probiotic powder (from Custom Probiotics Inc.) containing five types of bifidobacteria (*B. lactis*, *B. longum*, *B. bifidum*, *B. infantis* and *B. breve*) at concentrations of 10^{12} and 10^{8} CFU per 0.5 mL dose. Typhoid fever was addressed with the injectable Typhim Vi® vaccine (from Sanofi Pasteur SA), a conjugated vaccine derived from the Ty2 strain of *Salmonella typhi* (*S. typhi*).

Experimental design and grouping in Typhoid vaccine and probiotic interaction study: The study involved 40 female Swiss mice, each aged six to eight weeks and with a body weight ranging from 25 to 30 g. These mice were acquired and housed under rigorously controlled conditions at the Faculty of Science, King Abdulaziz University in Jeddah. The facility was carefully regulated to maintain a constant temperature of approximately 22°C, with possible variations of ±2 degrees. During the study, the mice had uninterrupted water access, ensuring their consistent care and welfare.

The mice were divided into four groups through a random selection process: One control group received no treatment, while the other three experimental groups each underwent distinct treatments. Specifically, on the 14th and 28th days, a subcutaneous injection of 0.25 mL of the Typhoid Vi vaccine was administered to these three groups.

The first group, known as STV, received only the Typhi vaccine with no additional treatments. The second group, labeled STV+B 10^8, was given an oral dose of *Bifidobacterium*, at a concentration of 10^8 CFU in 0.5 mL, administered three times a week from the start of the study. The third group, STV+B 10^{12}, received the same *Bifidobacterium* treatment but at a higher concentration of 10^{12} CFU.

On days 28 and 42, the mice from each group were humanely euthanized to collect and store their intestinal tissue and blood serum at -80°C for detailed analysis.

Ethical consideration: The entire study was conducted with the highest ethical standards, having received approval from the Scientific Research Ethical Committee of the Faculty of Science at King Abdulaziz University and it was also sanctioned by the King Abdulaziz City for Science and Technology (KACST). This careful and thorough approach ensures the reliability and ethical integrity of the findings.

RNA preservation and quantitative analysis in intestinal tissue of Typhoid-vaccinated mice: Mouse intestinal tissue samples were preserved using RNAlater (QIAGEN, Cat No. 76106) to ensure RNA integrity. Following the manufacturer's instructions, RNA was extracted with the RNeasy Maxi kit (QIAGEN, Cat No. 75162). The extracted RNA was then diluted in RNase-free water, divided into aliquots and stored at -80°C for later analysis. After measuring the RNA concentration, qRT-PCR amplification was performed using the Verso SYBR Green 1-Step qRT-PCR Kit (Thermo Scientific, Cat. No. AB-4108/C) and specific primers[20]. The mRNA levels of TLR2, IFN-γ, IL-4 and IL-10 were quantified employing the $2^{-\Delta\Delta Ct}$ method, normalizing to a reference sample[24].

Serum immunoglobulin quantification via capture ELISA: The IgG, IgM and IgA serum levels were measured using ELISA kits supplied by Abcam (Cat. No. ab157717, ab133047, ab157719), strictly adhering to the provided protocols. Absorbance readings were recorded at 450 nm and determined the concentrations of these immunoglobulins were by comparing the readings against established standards. To calculate the total immunoglobulin content in the serum, we summed the values of IgA, IgM and IgG[20].

Data analysis

Comparing control and treated groups using ANOVA: Statistical comparisons were conducted between the control group and two groups that received treatment using the MegaStat Software, version 10.1. A One-way ANOVA test was implemented for parametric analysis, with a statistical significance threshold set at a $p<0.05$.

RESULTS

Effects of *Bifidobacterium* dosage on intestine mucosal gene expression

TLR2 regulation: By the 28th day of the experiment, the level of TLR2 gene expression in the intestinal biopsies of mice injected with the *Salmonella typhi* vaccine was comparable to that of the untreated group, with a non-significant difference ($p = 0.27$). Meanwhile, mice that received a low dose of *Bifidobacterium* (10^8 CFU) in combination with the *Salmonella typhi* vaccine showed a significant decrease in TLR2 gene expression in the intestinal mucosa. This reduction was more substantial than that observed in the untreated mice ($p = 0.0008$), those vaccinated without *Bifidobacterium* ($p = 0.001$) and those administered a high dose of *Bifidobacterium* (10^{12} CFU), with the latter group showing highly significant differences ($p = 0.000$). Conversely, the high dose of *Bifidobacterium* led to an increase in TLR2 expression compared to the control group ($p = 0.0103$), however, this increase was not significantly different from the vaccine-only group ($p = 0.081$), as detailed in Fig. 1a.

By the 42nd day of the study, the level of TLR2 gene expression still showed no significant difference in the *Salmonella typhi* vaccine mice group compared to the untreated group ($p = 0.43$). Both low and high doses of *Bifidobacterium* combined with the *Salmonella typhi* vaccine significantly reduced TLR2 expression in mice compared to controls and those receiving only the vaccine. This trend was documented with highly significant $p = 0.000$ each comparable group, as shown in Fig. 1b.

Th1/IFN-γ regulation: After a 28 days regimen, mice administered the *Salmonella typhi* vaccine exhibited a significant boost in IFN-γ RNA expression within their intestinal mucosa, compared to the untreated control group ($p = 0.0001$). Moreover, the combination of low (10^8 CFU) and high (10^{12} CFU) doses of *Bifidobacterium* to the vaccine notably reduced IFN-γ expression relative to the control and the vaccine-only groups. The higher dose of *Bifidobacterium* combined with the Typhi vaccine was more potent, significantly suppressing IFN-γ levels, with each comparison group showing a p-value of 0.000 (Fig. 2a).

Progressing to day 42, the vaccine-only group displayed a dramatic decrease in IFN-γ gene expression compared to the untreated control, with a highly significant p-value of 0.0000.

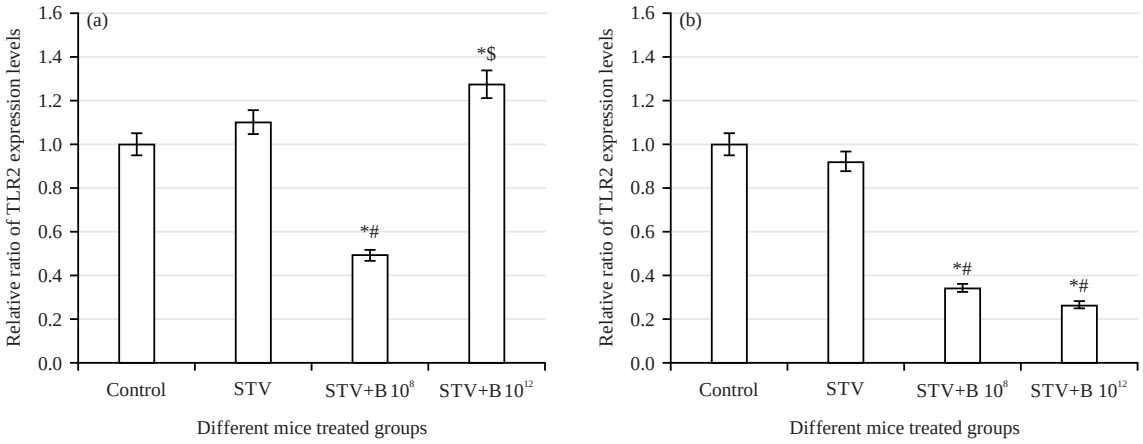

Fig. 1(a-b): Comparison of TLR2 gene expression in the intestinal mucosa of mice over two time frames, (a) Presents the changes after 28 days and (b) Extends this observation to 42 days

Control group was not treated, the STV group only received the *Salmonella typhi* vaccine, the STV+B 10^8 group was given a low dose of bifidobacteria with the vaccine and the STV+B 10^{12} group received a high dose alongside the vaccine, statistically significant differences, marked by asterisks, *Comparisons to the untreated group, #Comparisons to the STV group and $Comparisons to the 10^8 CFU group

Additionally, the mice receiving a low dose of *Bifidobacterium* with the Typhi vaccine experienced a substantial reduction in IFN-γ levels compared to the vaccine-only group (p = 0.028) and the untreated control (p = 0.0000). On the other hand, mice treated with a high dose of *Bifidobacterium* showed an elevation in IFN-γ levels when compared to those receiving the low dose or the vaccine alone (p = -0.000 for each group). However, this increase did not significantly differ from the untreated control group (p = 0.86) (Fig. 2b).

Th2/IL-4 regulations: After 28 days of treatment, all vaccinated groups, including those receiving low and high doses of *Bifidobacterium*, demonstrated significantly reduced IL-4 gene expression compared to the untreated control group, with each registering a significant result (p = 0.0000). The group receiving a low dose of *Bifidobacterium* along with the vaccine showed a minor increase in IL-4 expression compared to the vaccine-only group, though this was not statistically significant (p = 0.38). Conversely, the group given a higher dose of *Bifidobacterium* achieved a more pronounced reduction in IL-4 levels than the low-dose and vaccine-only groups, with significant differences noted (p-values of 0.001 and 0.0002, respectively), as shown in Fig. 2c.

By day 42, IL-4 levels remained significantly lower in all vaccinated groups, including those treated with bifidobacteria, compared to the untreated control (p = 0.0000 for each group). Moreover, IL-4 expression significantly increased in the low and high bifidobacteria dose groups compared to the vaccine-only group (p = 0.0000 for each). Yet there was no significant difference between the low and high doses of bifidobacteria when combined with the Typhi vaccine (p = 0.82), as detailed in Fig. 2d.

Treg/IL-10 regulations: At day 28 post-vaccination, the study revealed that both low and high doses of *Bifidobacterium* combined with the Typhi vaccine considerably lowered IL-10 levels in the intestinal mucosa compared to both the untreated control and the vaccine-only groups, with each comparison showing notable significance (p-value of 0.0000). This consistent reduction in IL-10 was observed across both dosages of *Bifidobacterium*, indicating similar efficacy between the two (p = 0.932). Mice administered only the Typhi vaccine also showed a significant decrease in IL-10 expression relative to the untreated control group (p = 0.0000), as illustrated in Fig. 2e.

By the 42 days mark, the two groups treated with *Bifidobacterium* and the Typhi vaccine continued to exhibit significantly suppressed IL-10 levels compared to the untreated control group, with highly significant results (p<0.000). While the differences in IL-10 levels between the high and low doses of *Bifidobacterium* combined with the Typhi vaccine weren't statistically significant when compared to the vaccine-only group (p = 0.059 and 0.132, respectively), the high-dose group did show a notable reduction in IL-10 expression than the low-dose group (p = 0.0003). Additionally, mice administered only the Typhi vaccine displayed a significant decrease in IL-10 expression relative to the untreated controls (p = 0.0000), as highlighted in Fig. 2f.

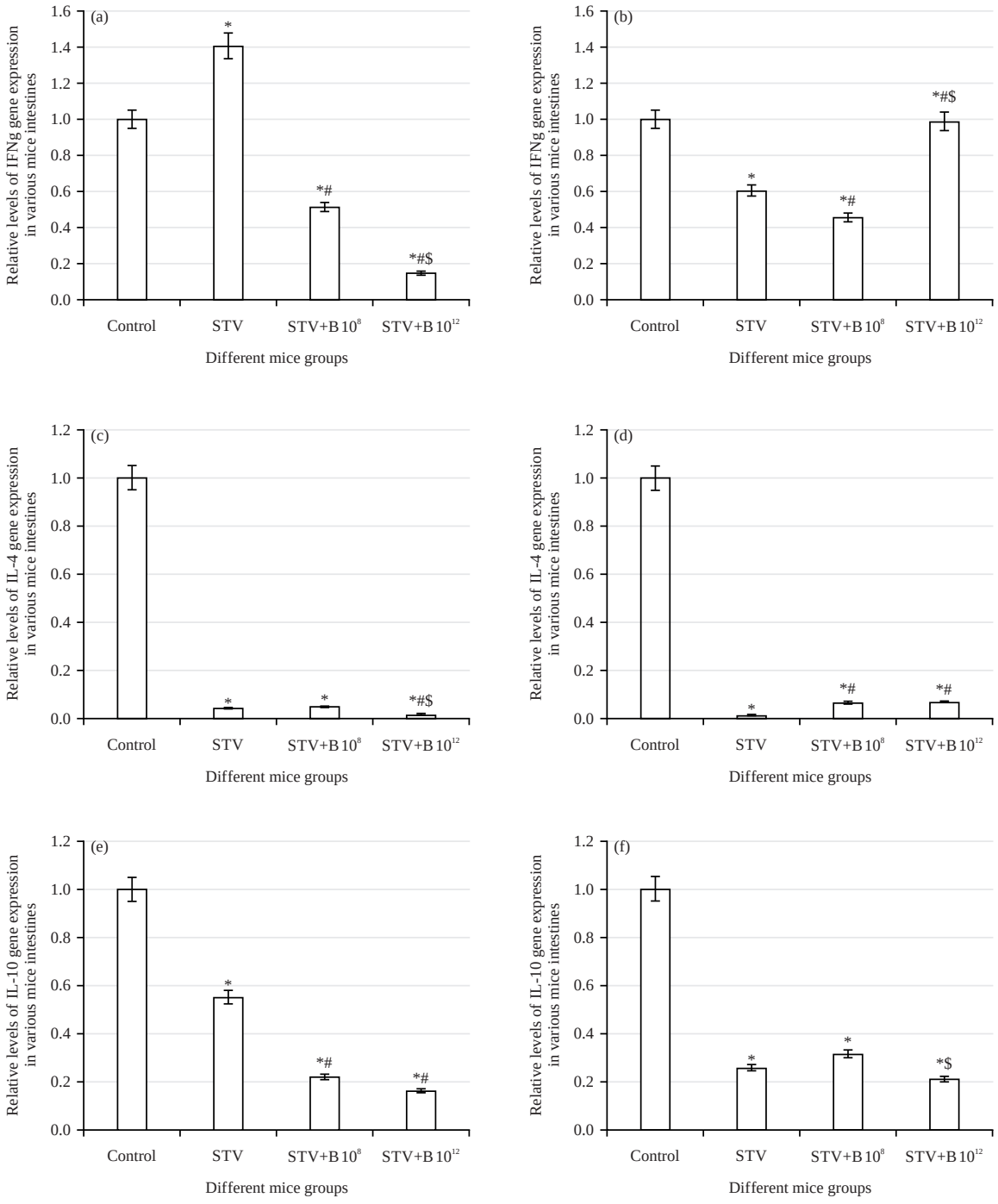

Fig. 2(a-f): Change in the intestinal mucosa of mice treated with bifidobacteria and the Typhi vaccine, (a) Captures the pro-inflammatory IFN-γ gene expression changes after 28 days, (b) Continues to monitor these changes for 42 days, (c) Anti-inflammatory IL-4 gene expression shifts at 28 days, (d) Extends the observation of IL-4 expression to 42 days and (e-f) Trace the alterations in IL-10 gene expression at 28 and 42 days, respectively

Study involved four groups: A control group, an STV group (vaccine only), a STV+B 10^8 group (low dose of bifidobacteria and vaccine) and a STV+B 10^{12} group (high dose of bifidobacteria and vaccine), with statistically significant differences marked by *Control comparisons, #Comparisons with the STV group and $Comparisons with the 10^8 CFU group, analyzed using variance analysis and One-way ANOVA where $p<0.05$ were deemed significant and this structured visualization helps delineate the impact of varying treatments on both inflammatory and anti-inflammatory responses over time

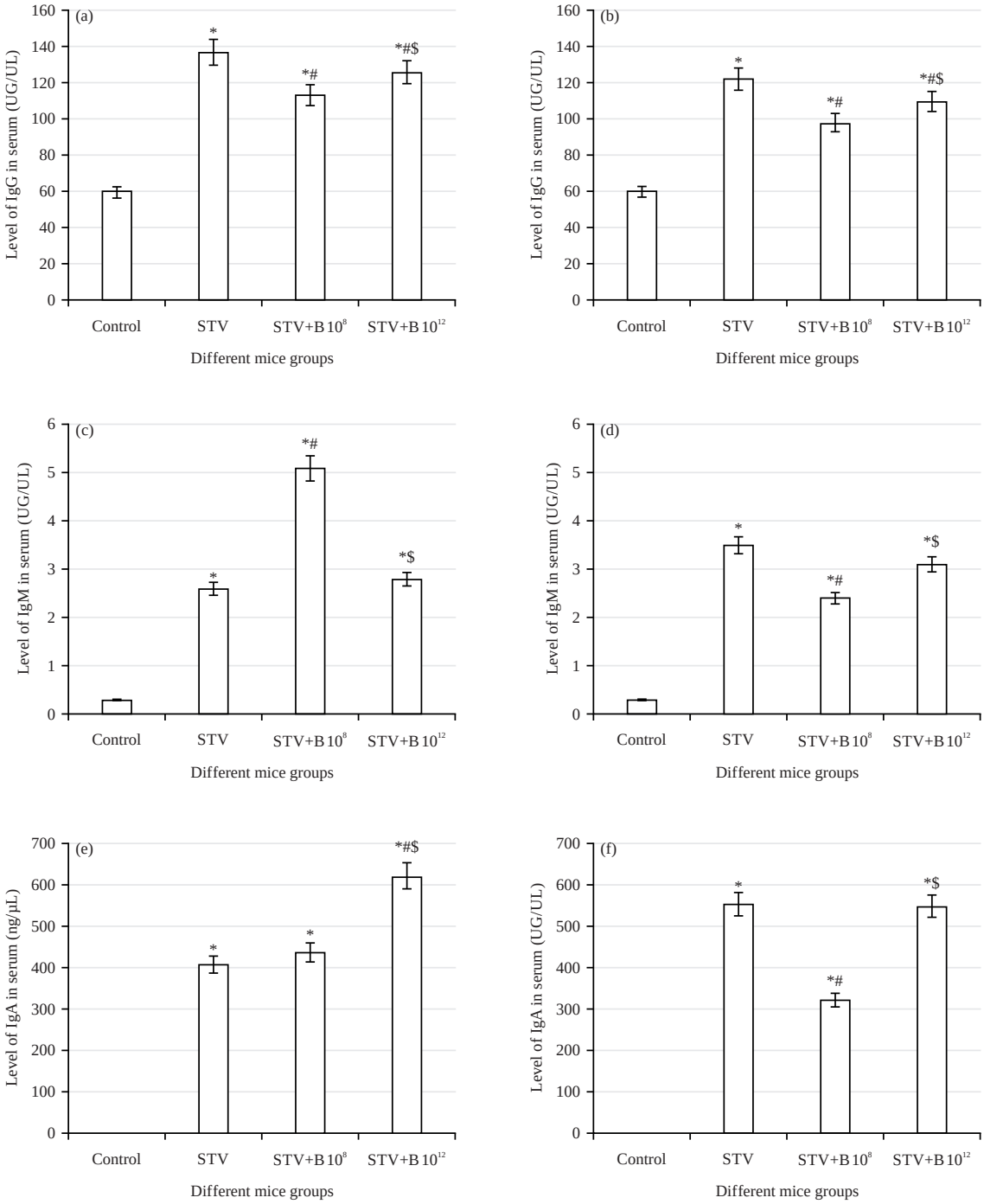

Fig. 3(a-f): Changes in polyclonal immunoglobulins in the serum of mice treated with bifidobacteria and the Typhi vaccine, (a) Details the change in total IgG levels at 28 days post-vaccination, (b) Extends the IgG observations to 42 days, (c) Presents shifts in polyclonal IgM levels at 28 days, (d) Continues the IgM data up to 42 days and (e-f) Changes in IgA levels at 28 and 42 days, respectively

Groups studied include an untreated control group, an STV group that received only the *Salmonella typhi* vaccine, a STV+B 10^8 group treated with a low dose of bifidobacteria combined with the vaccine and a STV+B 10^{12} group that received a high dose of bifidobacteria in conjunction with the vaccine, statistically significant differences between the groups are indicated using different symbols: *Comparisons with the control group, $^{\#}$Comparisons with the STV group and $^{\$}$Comparisons with the 10^8 CFU group and these differences were evaluated using variance analysis and one-way ANOVA, with significance thresholds set at $p<0.05$

Effects of *Bifidobacterium* dosage on total immunoglobulin

Immunoglobulin G isotype: Twenty-eight days following vaccination, all groups that received the vaccine, including those treated with varying doses of bifidobacteria, exhibited significantly higher total IgG levels than the untreated controls, with each group showing a notable increase ($p = 0.0000$ for each). Interestingly, both the low and high doses of *Bifidobacterium* combined with the vaccine led to lower polyclonal IgG levels than the vaccine-only group ($p = 0.000$ for each comparison). Moreover, the group receiving the high dose of *Bifidobacterium* showed significantly higher total IgG levels than those given the low dose ($p = 0.0002$), as detailed in Fig. 3a.

By the 42nd day post-vaccination, the pattern in IgG levels observed at day 28 persisted. Both groups treated with *Bifidobacterium* combined with the vaccine still demonstrated a significant increase in total IgG levels over the control group ($p = 0.0000$). Yet they still maintained lower total IgG levels than the vaccine-only group ($p = 0.0000$ for each). Additionally, the high-dose group displayed higher polyclonal IgG levels than the low-dose group ($p = 0.0003$), indicating a dose-response effect in enhancing the immune response, as shown in Fig. 3b.

Immunoglobulin M isotype: Twenty-eight days after vaccination, both the low and high doses of *Bifidobacterium* combined with the Typhi vaccine, as well as the vaccine-only group, showed a significant increase in serum IgM levels compared to the untreated control group (p-value of 0.0004, 0.0041 and 0.0061, respectively). Notably, the group receiving the low dose of *Bifidobacterium* with the vaccine exhibited the most substantial rise in IgM levels, significantly exceeding those observed in both the vaccine-only group and the high-dose *Bifidobacterium* group (p-value of 0.0048 and 0.0073, respectively). No significant difference was found between the vaccine-only group and the high-dose *Bifidobacterium* combined with the vaccine group regarding IgM levels ($p = 0.57$), as illustrated in Fig. 3c.

By day 42 post-vaccination, the trends in IgM levels remained consistent with those observed at day 28. The groups treated with both low and high doses of *Bifidobacterium* combined with the Typhi vaccine and the vaccine-only group continued to show significantly elevated IgM levels compared to the untreated control group, with p-values of 0.0009, 0.0003 and 0.002, respectively. However, the low-dose *Bifidobacterium* group saw a notable drop in IgM levels compared to the vaccine-only and the high-dose *Bifidobacterium* groups, with p-values of 0.0098 and 0.0286, respectively. There remained no significant difference in IgM levels between the high-dose *Bifidobacterium* combined with vaccine and the vaccine-only groups ($p = 0.267$), as depicted in Fig. 3d.

Immunoglobulin A isotype: By day 28 post-vaccination, all groups that received the vaccine-alone or combined with various doses of *Bifidobacterium*-demonstrated significant increases in serum IgA levels compared to the untreated control group, achieving a notable p-value of 0.0000 for each. The groups treated with both low and high doses of *Bifidobacterium* alongside the Typhi vaccine showed enhanced IgA levels beyond those seen in the vaccine-only group, with respective p-values of 0.0277 and 0.0000. Particularly, the high-dose *Bifidobacterium* group exhibited a markedly greater increase in IgA levels than those belonging to the low-dose group, with a p-value of 0.0000, as highlighted in Fig. 3e.

By day 42 post-vaccination, the trend continued with all vaccinated groups-whether administered alone or with *Bifidobacterium*-maintaining significantly higher IgA levels than the control group, each registering a p-value of 0.0000. The high-dose *Bifidobacterium* group not only sustained higher IgA levels than the low-dose group ($p = 0.0000$) but also achieved IgA levels than those of the vaccine-only group, showing no significant difference ($p = 0.683$). Conversely, the low-dose *Bifidobacterium* and vaccine combination resulted in significantly lower IgA levels than the vaccine-only group ($p = 0.0000$), as depicted in Fig. 3f.

DISCUSSION

This study explores how different levels of bifidobacteria affect immune responses in mice immunized against *Salmonella typhi*, focusing on changing vaccine efficacy by interacting these probiotics with the immune system.

This current study has shown that the level of *Bifidobacterium* administered to mice following their immunization with a *Salmonella typhi* vaccine can significantly influence the expression of TLR2, a receptor critical for recognizing gram-positive bacteria and modulating immune responses[25-28]. In this study, on day 28, a low dose of *Bifidobacterium* (10^8 CFU) administered with the *Salmonella typhi* vaccine significantly reduced TLR2 gene expression in the intestinal mucosa of mice, outperforming both untreated and vaccine-only groups, as well as those given a high dose of *Bifidobacterium* (10^{12} CFU). This lower TLR2 expression was maintained through day 42. In contrast, the high dose initially increased TLR2 expression compared to controls and did not differ from the vaccine-only group. By day 42, however, it

reduced TLR2 expression to levels comparable to the low dose. These results suggested that while both doses eventually modulate TLR2 expression by day 42, the high dose may provoke an initial, less targeted immune response. These results indicate that when combined with the Typhi vaccine, high doses of *Bifidobacterium* might excessively activate TLR2 pathways. This overactivation increases TLR2 expression as the immune system responds to what it interprets as an overwhelming bacterial presence[29], thereby surpassing the microbial tolerance threshold and prompting a strong immune reaction[29,30]. In contrast, lower doses of *Bifidobacterium* with the Typhi vaccine gently steer the immune response, avoiding an overly aggressive reaction and reducing TLR2 expression. This reduction is facilitated by a process known as microbial tolerance, in which the immune system considers the bacteria benign[31].

In this study, combining low doses of *Bifidobacterium* with the *Salmonella typhi* vaccine enhanced regulatory T cell induction, suppressing inflammation and modulating immune responses, possibly reducing TLR2 expression[32]. This treatment significantly lowered c levels by days 28 and 42, suggesting low doses promote sustained, balanced immune regulation and prevent inflammatory spikes seen with higher doses. Conversely, high doses initially suppressed inflammation more effectively, achieving the lowest IFN-γ levels by day 28. However, this did not foster Treg-mediated control, potentially increasing TLR2 expression[33] and leading to an uncontrolled inflammatory response[34]. By day 42, a rebound in IFN-γ levels was observed, linked to higher TLR2 levels, suggesting that high doses might promote a pro-inflammatory environment. The modulation of the Th1 response by *Bifidobacterium*, particularly the initial suppression followed by a later increase in IFN-γ levels in the high-dose group, indicates a more dynamically regulated immune response[17,27] that could prevent overactivation and tissue damage while still combating *Salmonella* effectively[16].

At day 28 of this study, administering either low or high doses of *Bifidobacterium* with the *Salmonella typhi* vaccine significantly lowered the IL-10 expression levels in the intestinal mucosa of both groups compared to those either untreated or only receiving the vaccine. Normally, probiotics like *Bifidobacterium* would boost IL-10 to foster an anti-inflammatory state[35]. Yet, the decrease in mucosal IL-10 expression corresponded with the suppression of mucosal IFN-γ, a key inflammatory cytokine vital for stimulating immune responses[36] against the *Salmonella typhi* vaccine. This suppression could potentially sharpen and strengthen the vaccine's immune response[37]. Notably, the high-dose group experienced a more pronounced reduction in IL-10 and a significantly delayed surge in IFN-γ levels by day 42, indicating a late adjustment in immune strategy against the vaccine[38]. In contrast, the low-dose group maintained reduced levels of both IL-10 and IFN-γ throughout the study, highlighting a dose-dependent effect on how the immune system modulates its response to the vaccine.

Conjugate vaccines like the Vi conjugate vaccines boost immune responses by attaching polysaccharides to protein carriers, converting the antigens from T-cell-independent to T-cell-dependent[39]. This innovation in vaccine technology enhances both immunogenicity and effectiveness by involving critical cytokines such as IL-4, which support a strong and sustained immune defense[34,39,40]. In disagreeing with this theory, from the beginning to day 42 of this experiment, all vaccinated groups-whether with just the vaccine or even with added low or high doses of *Bifidobacterium*-demonstrated lower mucosal IL-4 levels compared to the untreated group. This decrease indicates a move towards T-independent immune responses, characteristic of the polysaccharide vaccine, which promotes direct B cell activation with minimal dependence on T-helper cells[41]. This suggests that the *Salmonella typhi* vaccine, particularly when used alongside *Bifidobacterium*, may reduce the Th2 immune response, favoring a more direct and potent immune defense mechanism[17,42]. Twenty-eight days following vaccination, both low and high doses of *Bifidobacterium* administered with the vaccine significantly lowered mucosal IL-4 gene expression compared to the vaccine-only group. The high dose was particularly effective, showing a more substantial reduction than both the low dose and the vaccine-only group, indicating a dose-dependent enhancement in suppressing the Th2 immune response-a response typically marked by IL-4. By day 42, IL-4 levels in both the low and high bifidobacteria groups remained considerably lower than in the untreated control, with no significant differences between the two doses, suggesting that the effectiveness of both doses eventually converged. Despite this convergence, both bifidobacteria doses continued to maintain higher IL-4 levels compared to the vaccine-only group. This ongoing presence of higher IL-4 levels suggests that while *Bifidobacterium* markedly reduces IL-4, it also moderates its decline, potentially preserving a balanced level of the Th2 response[13]. This could be advantageous for maintaining a well-regulated immune system[43]. The initial stronger suppression seen with the high dose appears to hit a plateau, after which the effects of both doses level out, becoming equally effective. This nuanced interaction between *Bifidobacterium* dosage and immune response modulation provides intriguing insights into the potential of probiotics to enhance vaccine efficacy while ensuring immune balance[44,45].

An ongoing analysis of the dosage of bifidobacteria on humoral immune response post-vaccination has been recorded. By day 28 post-vaccination, both low-dose and high-dose *Bifidobacterium* groups, when paired with a vaccine, significantly increased total IgG levels over the control group, demonstrating *Bifidobacterium's* ability to boost overall IgG antibody responses[20,46]. However, polyclonal IgG levels in these groups were still lower than in the vaccine-only group, indicating that *Bifidobacterium* might affect antibody diversity differently[14,16]. Conversely, the low-dose group showed a marked increase in IgM levels, the highest among all groups, hinting at an optimal threshold for maximum early immune response that may not benefit from higher doses. These results underscore a complex interaction between *Bifidobacterium* dosage and immune response, with varying impacts on IgG, IgM and IgA antibody types[47]. By day 42 after vaccination, both low-dose and high-dose *Bifidobacterium* groups maintained higher total IgG levels than the control but were still lower than the vaccine-only group, with the high-dose group showing more specific immune responses, indicating a dose-dependent effect[48]. While IgM levels remained elevated compared to the control, they declined in the low-dose group, suggesting that higher doses might sustain IgM levels better over time[49]. The IgA levels were significantly higher in both *Bifidobacterium* groups than in the control, with the high-dose group achieving levels comparable to the vaccine-only group, further supporting the dose-dependency in enhancing mucosal immunity[48,49]. The high-dose group showed increased total IgG and IgA levels, indicating a dose-dependent enhancement of these antibodies. This suggests *Bifidobacterium* may promote a balance of Th1 and possibly Th17 cytokines without a strong Th2 bias[50]. These could be particularly effective in enhancing cellular immunity and mucosal defenses[17,42,51].

CONCLUSION

This research highlights how varying doses of *Bifidobacterium* can effectively modulate immune responses, offering a beneficial approach to boost mucosal immunity against the *Salmonella typhi* vaccine. Administering a low dose (10^8 CFU) consistently suppressed TLR2 expression and kept inflammatory cytokines IFN-γ and IL-10 low up to day 42, indicating a more regulated immune modulation. On the other hand, a high dose (10^{12} CFU) initially caused a spike in TLR2 expression and inflammation, which then normalized by day 42, marking an initially broad but subsequently refined immune response. Both doses significantly cut down IL-4 expression, tailoring the immune reaction to the vaccine, with a more marked reduction seen at the high dose. Moreover, while both doses boosted total IgG levels, the low dose particularly increased IgM levels and the high dose raised IgA levels, showing a dose-dependent effect on different types of antibodies. Overall, the findings suggested that dosage levels of *Bifidobacterium* can markedly affect vaccine effectiveness and immune regulation, with lower doses promoting a consistent and controlled response and higher doses inducing a vigorous but eventually balanced response.

SIGNIFICANCE STATEMENT

This study indicates that smaller doses of *Bifidobacterium* could be more beneficial for immune regulation than larger doses when combined with vaccines, emphasizing the importance of accurate dosage control to maximize the immunomodulatory effects of probiotics. This insight reveals the intricate relationship between probiotic dosage and immune response, suggesting that optimizing vaccine effectiveness could involve a meticulously balanced approach to probiotic supplementation.

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