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Evaluating the Impact of Vitamin D₃ on NF-κB and JAK/STAT Signaling Pathways in *Drosophila melanogaster*

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ABSTRACT: This study delved into the consequences of prolonged administration of vitamin D₃ on innate immune systems, particularly NF-κB and JAK/STAT, in *Drosophila melanogaster*. The outcomes indicated that vitamin D₃ treatment exhibited a notable capacity to improve the survival of adult flies with compromised immune functions, a condition induced by the loss of PGRP-LB, particularly when the flies were exposed to heat-killed *Escherichia coli*. The *PGRP-LB*^Δ mutant line that was treated with heat-killed *E. coli* experienced reduced survival. Treatment of heat-killed *E. coli*-treated *PGRP-LB*^Δ with vitamin D₃ resulted in improved survival, and this phenotypic feature might be due to the downregulation of gene expression in the NF-κB and JAK/STAT pathways. However, a higher concentration of vitamin D₃ was associated with decreased survival, potentially linked to intricate immunological responses. The research also underscored the influence of vitamin D₃ on the expression of antioxidant genes, *sod1* and *sod2*, indicating an augmented resistance to oxidative stress. Further, this study revealed the effect of vitamin D₃ on the reproductive status of the autoinflammatory model, showing an increase in pupae and adult flies with a treatment of 10 mM vitamin D₃, suggesting the potential benefits of vitamin D₃ on the reproductive profile. Overall, this study provides preliminary insights into the complex interactions between vitamin D₃, immune pathways, oxidative responses in the cell, and reproduction in *Drosophila*.
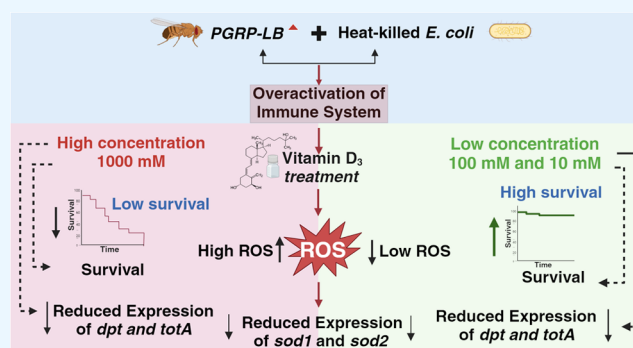
1. INTRODUCTION

The human body relies on its immune system to detect and eliminate harmful microbes and foreign entities.[1,2] In this modern era, immunomodulators are often sought to regulate the immune system's defensive capabilities against pathogens, reflecting a broader interest in proactive health measures. Immunomodulators can regulate the immune system by either stimulating or suppressing its components. Immunostimulation aims to boost immune responses, potentially improving resistance to infections and diseases.[3,4] However, in certain instances, the improper use of immunomodulators (specifically immunostimulants) can paradoxically trigger pathological conditions, emphasizing the need for a nuanced and evidence-based approach to the use of immunomodulatory agents.[5]

Vitamin D₃ has emerged as a versatile immunomodulator, exhibiting antibacterial and antiviral properties. It enhances the production of antimicrobial peptides, aiding in immune balance. Moreover, vitamin D₃ influences T cell activation, regulates antigen-presenting cells, and coordinates dendritic cell functions.[6−9] Vitamin D₃ administration inhibits proin-

flammatory cytokine synthesis and promotes anti-inflammatory cytokine production by macrophages.[6] In addition to regulating cellular immunity, vitamin D₃ has been shown to be important in the mitigation of cytokine storms[10] and mutations within the vitamin D receptor pose potential risk factors for the emergence of immune-related disorders.[11]

In the context of COVID-19, vitamin D₃ has garnered attention, with recommended dosages outlined in the management guidelines National Institute for Health and Care Excellence (NICE).[12] Patients with confirmed COVID-19, regardless of symptom severity, are advised to take vitamin D₃ as an adjuvant therapy.[13] Previous studies suggest that vitamin D₃ plays a role in the regulation of the immune system. Inadequate levels of vitamin D₃ in the body weaken the
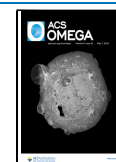
effectiveness of the immune system, making it vulnerable to infection.[10,14,15] However, caution is warranted, as prolonged and high-dose intake of vitamin D_3 supplements may lead to complications such as hypercalcemia and disruptions in kidney function.[16] Nevertheless, while much is known about the pharmacological and side effects of vitamin D_3, as well as its effects on the adaptive immune system, less is known about its impact on the innate immune system, particularly on the NF-κB and JAK/STAT pathways.

To comprehensively understand the enduring impacts of vitamin D_3 on the innate immune system, particularly NF-κB and JAK/STAT, a detailed exploration using the model organism *Drosophila melanogaster* is proposed. Scientifically validated as a model organism with around 70% genetic similarity to humans,[17] *D. melanogaster* offers an array of advantages including cost-effectiveness, low maintenance requirements, accelerated growth, and genetic manipulability,[18] further justifying the use of *D. melanogaster* as a model organism in this study.

D. melanogaster has served as an invaluable model organism for exploring diverse biological and physiological phenomena, offering insights into the emergence and management of human diseases.[17] It has been utilized extensively in the investigation of neurodegenerative disorders[19,20] and the discovery of therapeutic interventions for such conditions.[21] Moreover, *Drosophila* has played a crucial role in advancing our understanding of antioxidant protection in metazoans[22,23] and the toxicological implications of insecticide exposure, including the emergence of insect resistance.[24] Additionally, it has provided a valuable platform for elucidating the mechanisms of vitamin sensing[25] and exploring the pharmacological effects of vitamins[26−30] and vitamin analogs[31] in metazoan species. Furthermore, *Drosophila* has been pivotal in the evaluation of drug candidates aimed at treating a diverse spectrum of human diseases, including cancer,[32] diabetes,[33] inflammatory bowel diseases,[34] among others.

In this study, we utilized *PGRP-LB*$^\Delta$, a mutant line that lacks the function of PGRP-LB. PGRP-LB is a protein involved in the immune response of *Drosophila* by breaking down peptidoglycan (PGN) into nonimmunogenic compounds. This protein specifically targets meso-2,6-diaminopimelic acid (meso-DAP or DAP)-type PGN and regulates the immune deficiency (Imd) pathway by cutting DAP-type PGN.[35,36] This action helps maintain immune tolerance and protects symbiotic bacteria from host immune responses. PGRP-LB is a secreted protein with a typical peptidoglycan binding domain (PGN) and five amino acid residues required for its enzymatic activity. The mechanism of its enzymatic reaction involves a nucleophilic attack by water molecules, stabilized by tyrosine residues (Y78), on the carbonyl group of the amide function in PGN. This is important in avoiding excessive immune reactions and protecting symbiotic bacteria after infection.[36]

PGRP-LB expression is regulated by the Imd (NF-κB) signaling pathway. Previously, it has been suggested that the activation of the Imd pathway can induce the JAK/STAT pathway in *Drosophila*.[37] Upd3 production, triggered by Imd pathway activation, subsequently activates the JAK/STAT pathway via binding to its receptor Domeless, contributing to the systemic immune response.[37] Another study proposed that the JAK/STAT pathway can suppress Imd signaling.[38] Through experiments with cultured cells, Kim and colleagues demonstrated that transcription factors Stat93E, which are downstream of the JAK/STAT pathway, can displace Relish

from a promoter. This action terminates the transcription of antimicrobial peptide (AMP) genes and suppresses the output of the Imd pathway.[38] Overall, the Imd and JAK/STAT pathways, although distinct, are interconnected and able to influence each other.

The choice of *D. melanogaster* as an in vivo model is not only scientifically sound but also beneficial for researchers in resource-constrained nations, such as Indonesia. The model's unique features, including mutant lines expressing heightened AMPs akin to conditions of a cytokine storm, provide an ideal platform for investigating the long-term impacts of vitamin D_3 on the immune system. The results obtained in this study hold promise for advancing our understanding and may lay the groundwork for future considerations in the realm of immunomodulation.

2. MATERIALS AND METHODS

2.1. Materials. Vitamin D_3 (CAS No.: 67-97-0) was obtained from Wellgreen Technology Co. Ltd., Tween 80 (CAS No.: 9005-65-6) was obtained from MERCK, PEG 40 (CAS No.: 61788-85-0) was obtained from Hefei TNJ Chemical Industry Co., Ltd., and Luria−Bertani (LB) Broth medium was obtained from HIMEDIA Lab Chemicals & Biochemicals (India).

2.2. *Drosophila* Stocks. *Drosophila PGPR-LB*$^\Delta$ line was acquired from the Laboratory Host Defense and Responses (Kanazawa University, Japan). The fly line was maintained on standard fly food at a temperature of 25 °C.

2.3. Preparation of Vitamin D_3 Solution. A solution of vitamin D_3 was prepared using 1% Tween 80 and 1% PEG 40 as the solvents. Vitamin D_3 was then diluted with a 1:1 solvent ratio to achieve concentrations of 1000, 100, and 10 mM.

2.4. Preparation of Heat-Killed *Escherichia coli*. The *E. coli* FNCC 0091 strain was cultured in LB broth medium at 37 °C for 24 h with agitation. After incubation, the 24 h *E. coli* culture was autoclaved at 121 °C and 2 atm pressure for 30 min to kill the bacteria.

2.5. Preparation of *Drosophila* Food Containing Heat-Killed *E. coli*. *Drosophila* food was prepared by combining corn, yeast, sugar, and agar into a mixture, which was then supplemented with heat-killed *E. coli*. The entire mixture was heated to 100 °C while being stirred until it reached a thickened consistency. Subsequently, the resulting mixture was carefully transferred into vials to establish a controlled environment for further study.

2.6. Survival Assay. A survival assay was conducted to assess the flies' ability to endure specified treatments. Briefly, second instar larvae of *PGRP-LB*$^\Delta$ were exposed to heat-killed *E. coli* in the presence or absence of vitamin D_3. Daily recording of the number of larvae progressing into pupae and subsequently emerging as adult flies continued until the entire population succumbed.

2.7. Fecundity Assay. Three virgin female and three male *PGRP-LB*$^\Delta$ flies (parental flies) were placed in each vial containing the treatment media. The treatments were categorized into three groups, each receiving heat-killed *E. coli* and vitamin D_3 at different concentrations: 1000, 100, or 10 mM. Additionally, a control group comprising three virgin female and three male *PGRP-LB*$^\Delta$ flies treated with heat-killed *E. coli* only was included. Flies were given a 5 day period to reproduce, after which all parental flies were removed. Analysis was performed to determine the quantity of pupae and adult flies emerging in each experimental group.

Table 1. Primers Used in the RT-qPCR Assay

genes	forward primer	reverse primer
dpt	5′-AGGTGTGGACCAGCGACAA-3′	5′-TGCTGTCCATATCCTCCATTCA-3′
totA	5′-GAATAGCCCATGCATAGAGGAC-3′	5′-CCAAAATGAATTCTTCAACTGCT-3′
sod1	5′-AGGTCAACATCACCGACTCC-3′	5′-GTTGACTTGCTCAGCTCGTG-3′
sod2	5′-TGGCCACATCAACCACAC-3′	5′-TTCCACTGCGACTCGATG-3′
rp49	5′-GACGCTTCAAGGGACAGTATCTG-3′	5′-AAACGCGGTTCTGCATGAG-3′

2.8. Nitroblue Tetrazolium Reduction Assay. To quantify the levels of reactive oxygen species (ROS) in adult flies' hemolymph, we conducted the nitroblue tetrazolium (NBT) reduction assay based on the previously published protocol.[39] In this assay, materials containing ROS were exposed to nitroblue tetrazolium, a yellow dye. Upon incubation, blue superoxide molecules reduced the dye to water-insoluble formazan particles, with absorbance at 595 nm correlating with the ROS concentration. Specifically, 100 adult flies were collected and rinsed in PBS to remove food residues. Hemolymph extraction was conducted on ice to prevent melanization. A total volume of 300 μL was prepared by combining 100 μL of hemolymph with 200 μL of 1× PBS, followed by the addition of an equal volume of the NBT solution. The mixture was incubated at room temperature in the dark for 1 h before the addition of 300 μL of 100% glacial acetic acid to halt the reaction. After centrifugation at full speed for 1 min, absorbance was measured at 595 nm following the addition of 50% acetic acid.[39]

2.9. Gene Expression Analysis. Five adult *PGRP-LB*$^\Delta$ flies, previously subjected to compound treatments, were placed in Treff tubes for total RNA isolation using the SV total RNA Isolation System (Promega) following the manufacturer's protocol. The expression level of target genes was quantitatively assessed using the reverse transcriptase quantitative PCR (RT-qPCR) method with SuperScriptIII Platinum One-Step qRT-PCR Kit (Invitrogen), in accordance with the manufacturer's protocol. The RT-qPCR reaction for the target gene was performed in a 10 μL volume, involving one cycle at 37 °C for 15 min, followed by 95 °C for 10 min, and then 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Standard melt curve analysis was performed in each RT-qPCR run to validate the specific amplification of the expected products. Additionally, the RNA levels of the host ribosomal protein rp49, used as an internal control, were examined using a set of *rp49* primers following a similar protocol applied to the target genes. The primer sequences used in this assay are listed in Table 1.

2.10. Data Analysis. All data were processed using GraphPad Prism 9, with at least three independent biological replicates. Bar graphs were utilized to present most of the data, and a one-way analysis of variance (ANOVA) was subsequently applied. All data were expressed as mean values with standard deviation (mean ± SD), with p values less than 0.05 considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Vitamin D$_3$ Augments the Survival of Heat-Killed *E. coli*-Treated *PGRP-LB*$^\Delta$ Flies. To assess the safety of vitamin D$_3$ in *D. melanogaster* and investigate its potential phenotypical implications in an autoinflammatory fly model, we conducted a survival assay using *PGRP-LB*$^\Delta$ flies. The peptidoglycan recognition protein (PGRP) in *D. melanogaster* functions as a crucial regulator inhibiting the immune

deficiency (IMD) pathway and tightly controlling the production of antimicrobial peptides (AMPs).[40] In the absence of PGRP-LB, there is a substantial increase in the proinflammatory AMPs triggered by the presence of Gram-negative bacterial peptidoglycan, such as that from heat-killed *E. coli*. Consequently, the induction of AMPs may occur through the IMD pathway. Continuous activation of the IMD pathway has been previously demonstrated to lead to reduced survival in *PGRP-LB*$^\Delta$ flies.[18,41]

In the comparison of the 7 day survival rates of adult flies in the heat-killed *E. coli* control group to the untreated group, the *PGRP-LB*$^\Delta$ mutant exhibited a lower survival rate in the presence of heat-killed *E. coli* (Figure 1), confirming our
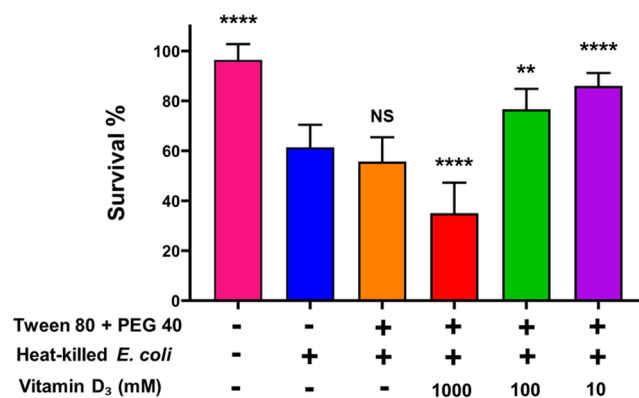

Figure 1. Concentration-dependent effect of vitamin D$_3$ on the survival of heat-killed *E. coli*-treated *PGRP-LB*$^\Delta$ *D. melanogaster*. Daily assessments of the survival of *PGRP-LB*$^\Delta$ flies treated with heat-killed *E. coli* commenced on the first day as adult flies and continued for 7 days. The survival data from each group are compared to that of the heat-killed *E. coli* control group. NS, nonsignificant; ** $p < 0.01$; **** $p < 0.0001$.

previous observation.[18,41] Notably, there was no significant disparity between the heat-killed *E. coli* and the solvent control (Tween 80 + PEG 40), signifying the inert effect of solvent on the survival of *PGRP-LB*$^\Delta$ mutant flies.

As shown in Figure 1, our finding suggests that heat-killed *E. coli* reduces the survival of flies, while the solvent used for dissolving vitamin D$_3$ (Tween 80 + PEG 40) does not have any adverse effects on fly survival. However, it is essential to recognize that a high concentration of vitamin D$_3$ (1000 mM) can reduce fly survival. In contrast, lower concentrations (10 and 100 mM) appear beneficial for *PGRP-LB*$^\Delta$ flies, as they demonstrate improved survival upon treatment with heat-killed *E. coli*. These results underscore the dosage-dependent effects of vitamin D$_3$ on the survival dynamics of *PGRP-LB*$^\Delta$ flies and highlight the importance of dosage considerations in potential interventions.

3.2. Vitamin D$_3$ Inhibits the Expression of NF-κB and JAK/STAT-Related Genes. *Drosophila*, with its robust immune system, exhibits a remarkable ability to combat
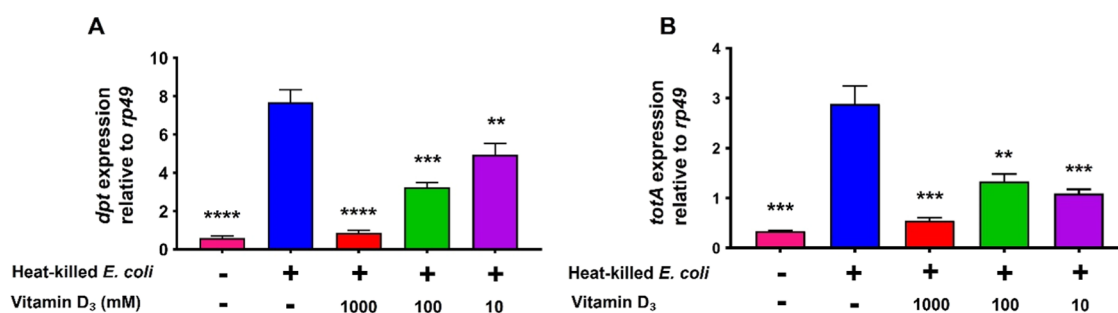
Figure 2. Immunomodulatory effect of vitamin D_3 on the immune response of adult $PGRP-LB^\Delta$ *D. melanogaster*. Downregulated expression of *dpt*, NF-κB-target gene (A) and *totA*, JAK/STAT-target gene (B) is observed in adult $PGRP-LB^\Delta$ flies upon treatment with vitamin D_3 at concentrations of 1000, 100, and 10 mM. The expression data from each group is compared to that of the heat-killed *E. coli* control group. NS, nonsignificant; ** p < 0.01; *** p < 0.001; and **** p < 0.0001.
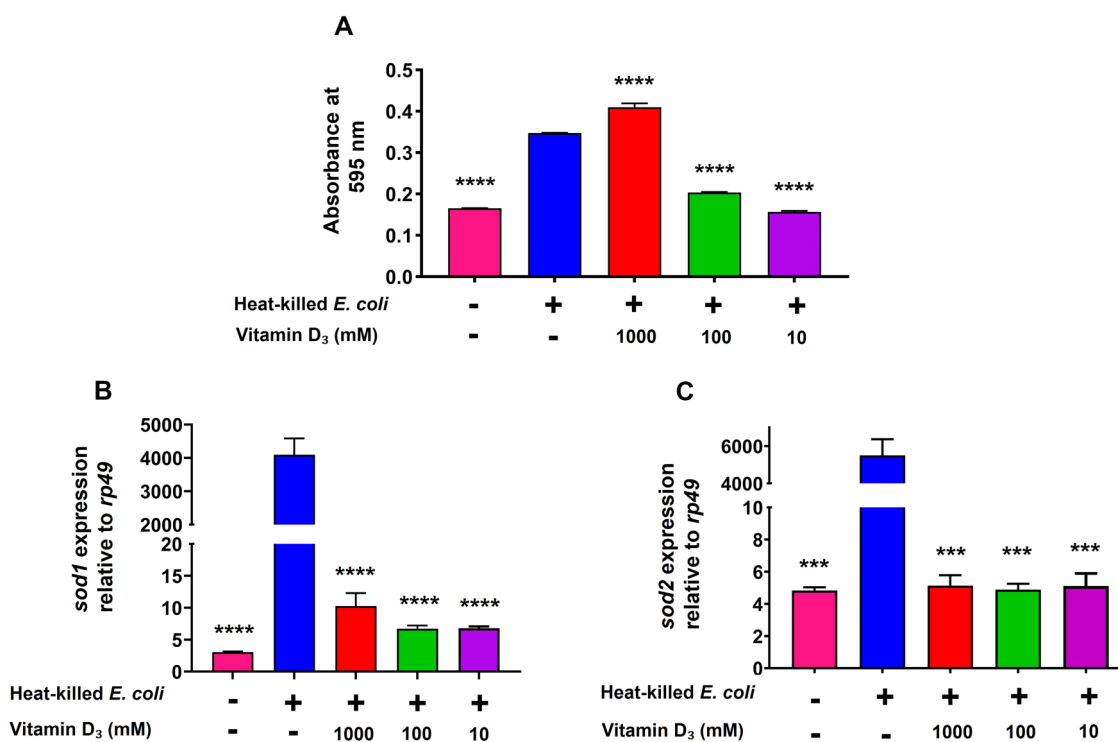
Figure 3. Elevated level of ROS prompts the dynamic expression of superoxide dismutases, which are downregulated by vitamin D_3. (A) Treatment with heat-killed *E. coli* increases ROS levels in $PGRP-LB^\Delta$ adult flies, while supplementation with vitamin D_3 at concentrations of 100 and 10 mM decreases the level of ROS. The level of ROS was assessed using NBT assay coupled with spectrophotometry analysis. Downregulated expression of superoxide dismutase genes *sod1* (B) and *sod2* (C) in response to vitamin D_3 treatments. $PGRP-LB^\Delta$ flies were pretreated with heat-killed *E. coli* before receiving vitamin D_3 treatment and subjected to molecular analysis using RT-qPCR. Data derived from each group is compared to that of the heat-killed *E. coli* control group. NS, nonsignificant; *** p < 0.001; **** p < 0.0001; ROS, reactive oxygen species.

infectious pathogens through humoral and cellular responses. The humoral reaction involves the synthesis of AMPs, while the cellular response engages pathogenic entities, virus-infected cells, and apoptotic cells via phagocytosis.[42−44] However, continuous and aberrant expression of AMPs has been demonstrated to have detrimental effects on the survival of flies.[18,41] Next, we aimed to elucidate whether the enhanced survival of $PGRP-LB^\Delta$ flies in the presence of lower concentrations of vitamin D_3 (10 and 100 mM) could be attributed to a reduction in the expression of AMPs. Among the AMPs expressed under the Imd (NF-κB) signaling pathway is Diptericin, encoded by the *dpt* gene. Our analysis focused on assessing whether vitamin D_3 could modulate the expression of *dpt*.

As depicted in Figure 2A, heat-killed *E. coli* induces the expression of *dpt* in $PGRP-LB^\Delta$ flies, suggesting that heat-killed *E. coli* serves as an inducer of the Imd signaling pathway. Notably, the overactivation of the Imd signaling pathway has been associated with reduced survival. However, when heat-killed *E. coli* is coadministered with vitamin D_3 at concentrations of 10 and 100 mM, there is a decrease in the expressions of NF-κB-related gene (*dpt*, Figure 2A) and JAK/STAT-related gene (*totA*, Figure 2B). This reduction implies an immunosuppressive effect, inhibiting the activation of the immune system, leading to the improvement of the $PGRP-LB^\Delta$ survival rate (Figure 1B). Conversely, when heat-inactivated *E. coli* was combined with a concentration of 1000 mM vitamin D_3, there is a decrease in gene expression (Figure 2B) coupled with reduced survival (Figure 1B). This phenomenon may be
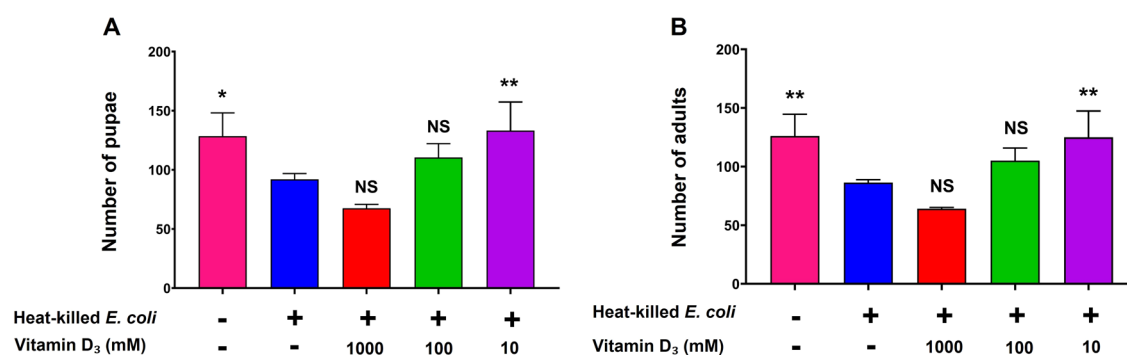
Figure 4. Improved reproductive capacity of *D. melanogaster* in response to vitamin D_3 treatment. A significant increase in the number of offspring produced at the form of pupae (A) and adult flies (B) when *PGRP-LB*$^\Delta$ flies were treated with 10 mM vitamin D_3, compared to the control group and groups treated with vitamin D_3 at 100 and 1000 mM. Data derived from each group is compared to that of the heat-killed *E. coli* control group. NS, nonsignificant; *$p < 0.05$; ** $p < 0.01$.

attributed to an elevated level of cell death, most likely due to vitamin D_3 toxicity at a 1000 mM concentration, consequently leading to a decrease in the expression of immune genes and implicated in the reduced survival rate. It is important to note that the overexpression of the *dpt* gene can be potentially lethal.[18,45] Overall, these findings highlight the intricate interplay between vitamin D_3, immune modulation, and survival outcomes in the context of *Drosophila*'s immune response.

In addition to *dpt*, we also examined the expression of *totA*, one of the genes expressed under the JAK/STAT pathway.[37] The expression of the *totA* undergoes a notable reduction in gene expression when heat-killed *E. coli* is coadministered with vitamin D_3 at concentrations of 1000, 100, and 10 mM, as compared to the treatment with heat-killed *E. coli* alone. This observation suggests that vitamin D_3 may exert an immunomodulatory effect on the immune response in *D. melanogaster*, restricted not only to the Imd pathway but also to other signaling pathways. This broad-reaching effect across multiple signaling pathways indicates the complex and systemic nature of vitamin D_3's influence on the immune response. The reduction in *dpt* expression might be linked to the observed decrease in *totA* expression, suggesting potential crosstalk or interdependence between the IMD and JAK/STAT pathways in the expression of *totA*, as has been suggested previously.[46] In summary, the reduction in *totA* expression in response to the coadministration of heat-killed *E. coli* and vitamin D_3 at various concentrations suggests that the immunomodulatory activity of vitamin D_3 extends beyond the Imd (NF-κB) pathway, influencing other signaling pathways such as JAK/STAT, at least in *D. melanogaster*.

3.3. Downregulated Expression of *sod1* and *sod2* in Response to Vitamin D_3 Treatment.

totA has been implicated as one of the genes involved in the stress response.[46,47] Consequently, the observed reduction in *totA* expression in the presence of vitamin D_3 suggests a potential modulatory effect on the stress response pathway. Stress responses often entail heightened levels of reactive oxygen species (ROS),[48] probably due to the administration of heat-killed *E. coli* and vitamin D_3 at high concentration (1000 mM). To test this, we carried out an NBT assay to examine the level of ROS in the presence or absence of vitamin D_3 treatments following the administration of heat-killed *E. coli*. We found that the level of ROS is increased in the presence of heat-killed *E. coli* and also in the presence of both heat-killed *E. coli* and 1000 mM vitamin D_3 (Figure 3A). However, the level of ROS

is decreased upon the administration of 100 and 10 mM vitamin D_3. Vitamin D_3 might play a crucial role in mitigating the impact of ROS, possibly by upregulating the expression of key antioxidant enzymes such as superoxide dismutases (SODs), thus facilitating the neutralization of ROS and the preservation of cellular redox equilibrium.[49] The decrease in *totA* expression might imply a concurrent reduction in ROS levels. In response to this, endogenous enzymes responsible for ROS mitigation may undergo downregulation. To experimentally validate this hypothesis, we conducted an RT-qPCR assay to examine the expression levels of *sod1* and *sod2*.

As shown in Figure 3, the expression of *sod1* (Figure 3B) and *sod2* (Figure 3C) in *PGRP-LB*$^\Delta$ flies was upregulated in response to the treatment of heat-killed *E. coli*. This upregulation is posited to result from the heightened production of reactive oxygen species (ROS) by host cells, triggered by the overactivation of immune signaling in response to heat-killed *E. coli*. However, following treatment with heat-killed *E. coli* in the presence of vitamin D_3, the expression of both *sod1* and *sod2* was observed to be downregulated. These findings suggest that an excessive immune response may induce elevated levels of reactive oxygen species (ROS), possibly causing oxidative stress, which, in turn, prompts an upregulation in the expression of SODs as a compensatory mechanism. Once the ROS levels are reduced, the expression of *sod1* and *sod2* is then decreased to the basal state.

3.4. Improved Fly Fecundity in Response to Vitamin D_3 Treatment.

The modulation of immune responses may result in a trade-off with reproductive processes.[50,51] In order to investigate this phenomenon, a fecundity assay was carried out to examine the impact of continuous treatment with vitamin D_3 and heat-killed *E. coli* on offspring quantity, serving as a parameter for fecundity, in *Drosophila*. The findings, as shown in Figure 4, revealed a notable increase in the number of pupae (Figure 4A) and adult flies (Figure 4B) when 10 mM vitamin D_3 was administered, while other concentrations of vitamin D_3 did not exhibit a similar effect. Specifically, the *PGRP-LB*$^\Delta$ mutant flies, treated with a 10 mM concentration of vitamin D_3 in the presence of heat-killed *E. coli*, demonstrated an enhanced capability to produce greater quantities of progeny. Contrastingly, an elevation in the vitamin D_3 concentration beyond 10 mM did not result in an improved number of offspring. This observation suggests that the regulation of immune responses through the utilization of higher concentrations of vitamin D_3 may demand more energy,

likely due to the modulation of Imd signaling, consequently leading to a trade-off with reproduction.

4. CONCLUSIONS

This study investigated the impact of vitamin D_3 supplementation on the immune system of *Drosophila*. The results indicated that vitamin D_3 contributed to an increased survival rate, particularly in flies with compromised immune functions. Notably, vitamin D_3 exhibited inhibitory effects on specific immunological responses, leading to an overall improvement in survival. The study also highlighted the influence of vitamin D_3 on key genes involved in immunological pathways, such as NF-κB and JAK/STAT. Furthermore, vitamin D_3 was found to downregulate the activation of genes associated with endogenous antioxidants, suggesting a potential link between immune responses and cell stress. An intriguing observation emerged when treatment of an autoinflammatory model with vitamin D_3 at a concentration of 10 mM resulted in a higher reproductive rate in flies. In summary, the study underscores the potential benefits of vitamin D_3 in the regulation of both immunological responses and reproductive activity in *Drosophila*, two features that are conserved from flies to mammals.

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Author Contributions

W.H., Y.Y.D., and F.N. were responsible for conceptualization, W.H., R.M.A., and F.N. were responsible for methodology, W.H., D.F., M.R.P., T.Z.A.D.P., R.C., N.P.L., and M.M. were responsible for data curation and formal analysis, W.H. and F.N. were responsible for preparing the original draft , W.H., Y.Y.D., R.M.A., and F.N. were responsible for reviewing and editing the original draft, W.H. was responsible for visualization, and Y.Y.D., R.M.A., and F.N. were responsible for supervision. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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